INVESTIGATING THE IMMUNOREGULATORY ROLE OF BETAGLYCAN

By

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Abstract

Betaglycan, also known as TGFβRIII, is a component of the TGFβ receptor, being thought to act as a co-factor in signal transduction. It has proven roles in TGFβ-mediated embryogenesis and carcinogenesis, and facilitates signalling by activins and inhibins within the endocrine system. Despite the recognised importance of TGFβ in immunity, little is known about betaglycan in immune regulation.

In mice, absolute deficiency of any of the three isoforms of TGFβ, or any of the individual TGFβ receptor components results in embryonic or perinatal lethality, limiting the ability to study peripheral immune responses in these models. We developed a chimeric model in which betaglycan-deficient stem cells were transferred to immunodeficient hosts, allowing study of mature animals with betaglycan deletion restricted to T and B lymphocytes. We were thus able to investigate peripheral immune responses *in vivo*, and assess TGFβ signalling to T lymphocytes *in vitro,* in the presence and absence of betaglycan.

Betaglycan deficiency resulted in upregulated CD4+ and CD8+ T lymphocyte activation, with increased Th1 polarisation in peripheral lymphocyte compartments in both naïve and antigen-experienced animals. These observations confirm the involvement of betaglycan in T lymphocyte biology, providing the first evidence for its role in regulation of peripheral immune responses.

Dedication

To those who ask "why?"

If you should find yourself sat before me in an outpatient clinic with potentially blinding ocular inflammatory disease, I promise that I will manage your inflammation to the absolute best of my ability. I have access to the most advanced diagnostic aids and therapeutic interventions, can call on world-renowned experts for help and advice, and can provide the best available treatment to control your disease. I will do everything within my power to help you.

As a patient in my clinic, I know that your only concern is protecting your eyesight. I know that your livelihood and family are dependent on you maintaining good vision. I know that you will listen to what I say, and use the treatment I suggest, even if that treatment makes you unwell, because your eyes are important to you.

What you probably don't realise, is that despite trying to act calm, confident and reassuring, I don't know *why* you are sat before me. I don't know why you are suffering this condition when others will live a lifetime without it. I don't know why some of the treatment that I use will work for you whilst other treatments won't. Ultimately, I don't know if I am going to be able to completely prevent you from going blind, or just postpone an inevitable loss of your visual function.

I realise that these are the exact questions to which you need answers, but you will be too polite to ask because you think it will take up more of my time, and because you assume that I must know what I'm doing.

Please allow me to reassure you that I ask these questions too. These are the questions that motivate me. I hope that through this thesis, I have taken the first steps towards giving us both the answers that we need. Whilst there is still much to learn, I promise to build on my experience so far, and will continue to ask "*why?*" until we are both satisfied with the answers.

Robert Barry, Birmingham, 2015

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Finally, I must acknowledge the mice. However it is phrased, and whatever scientific justification is given, I will never feel comfortable 'sacrificing' animals in the name of scientific research, and over the three years spent working in the laboratory, the process never became 'easy' or 'routine'. I hope that the animals did not live and die in vain.

Robert Barry, Fight for Sight Clinical Fellow, Speciality Trainee Ophthalmology and occasional husband and father, August 2015.

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List of abbreviations A-E

AF	AlexaFluor
AICD	Activation Induced Cell Death
AIRE	Autoimmune Regulator Protein
AS	Ankylosing Spondylitis
APC	Antigen Presenting Cell
ARN	Acute Retinal Necrosis
BAEC	Bovine Aortic Endothelial Cell
BCR	B Cell Receptor
BD	Behcet's Disease
BMP	Bone Morphogenic Protein
BMSU	Biomedical Support Unit
Breg	Regulatory B Lymphocyte
CD	Cluster of Differentiation
CFA	Complete Freund's Adjuvant
CMV	Cytomegalovirus
CNS	Central Nervous System
COPD	Chronic Obstructive Pulmonary Disease
cTEC	Cortical Thymic Epithelial Cell
CTLA-4	Cytotoxic T Lymphocyte Associated Antigen 4
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic Cell
Dkk3	Dickkopf-3
DMSO	Dimethyl Sulfoxide
dn	Double Negative
dp	Double Positive
EAE	Experimental Autoimmune Encephalomyelitis
EAU	Experimental Autoimmune Uveoretinitis
EBV	Epstein-Barr Virus
ECM	Extra-Cellular Matrix
EDTA	Ethylebediaminetetraacetic Acid
eF	eFluor
E(n)	Embryogenesis (number corresponding to number of days post implantation)

List of abbreviations F-I

FACS	Fluorescence Activated Cell Sorting
FBHE	Foetal Bovine Heart Endothelial
FHC	Fuch's Heterochromic Iridocyclitis
FITC	Fluorescein Isothiocyanate
FoxP3	Forkhead Box Protein 3
FSc	Forward Scatter
FTOC	Foetal Thymic Organ Culture
GDF	Growth and Differentiation Factor
GPS	L-glutamine, benzylpenecillin, streptomycin
GRH	Gonadotrophin Releasing Hormone
HAART	Highly Active Anti-Retroviral Therapy
HBV	Hepatitis B Virus
HCC	Hepatocellular Carcinoma
Het	Heterozygote
HEV	High Endothelial Venule
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HTLV-1	Human T-cell Lymphotropic Virus Type 1
HWE	Hardy-Weinberg Equilibrium
IC_{50}	Inhibitory Concentration (50%)
ICAM	Intercellular Adhesion Molecule
IFA	Incomplete Freund's Adjuvant
IFN	Interferon
Ig	Immunoglobulin
IIU	Idiopathic Intermediate Uveitis
IL	Interleukin
IMT	Immuno-modulatory Therapeutic Agent
IP	Intraperitoneal
IRBP	Interphotoreceptor Retinoid Binding Protein
iTreg	Inducible Regulatory T Lymphocyte
IV	Intravenous

List of abbreviations J-S

JAM	Junctional Adhesion Molecules
JIA	Juvenile Idiopathic Arthritis
KO	Knock-Out
LAP	Latency Associated Peptide
LFA	Lymphocyte Function-Associated Antigen
LH	Luteinizing Hormone
Mac-1	Macrophage-1 Antigen
MACS	Magnetic Activated Cell Sorting
MBP	Myelin Basic Protein
MHC	Major Histocompatibility Complex
MMP	Matrix Metalloproteinase
MOG	Myelin Oligodendrocyte Protein
mTEC	Medullary Thymic Epithelial Cell
NOD	Non-Obese Diabetic
nTreg	Naturally-Occurring Regulatory T Lymphocyte
OCT	Optimal Cutting Temperature
PAI-1	Plasminogen Activator Inhibitor-1
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PECAM	Platelet Endothelial Cell Adhesion Molecule
PNA	Peanut Agglutinin
RAG	Recombinase Activating Gene
RAGE	Receptor for Advanced Glycoprotein Endpoints
RORγ	Retinoid-Related Orphan Receptor Gamma
SC	Subcutaneous
SCID	Severe Combined Immunodeficiency
SMAD	Sma (Small) and Mad (Mothers Against Decapentaplegic) related protein
SNP	Single Nucleotide Polymorphism
S1PR1	Sphingosine-1 Phosphate Receptor 1
SSc	Side Scatter
sTGFβRIII	Soluble domain of TGFβRIII

List of abbreviations T-Z

Tcm	Central Memory T Lymphocyte
TCR	T Cell Receptor
Tem	Effector Memory T Lymphocyte
TGFβ	Transforming Growth Factor β
TGFβRI/II/III	Transforming Growth Factor β Receptor I/II/III
Th	Helper T Lymphocyte
TNF	Tumour Necrosis Factor
Treg	Regulatory T Lymphocyte
Trm	Resident Memory T Lymphocyte
VKH	Vogt Koyanagi Harada
VLA	Very Late Antigen
WT	Wild-Type

1 GENERAL INTRODUCTION

1.1 Key concepts in immunity

1.1.1 Innate and adaptive immunity

Broadly speaking, the human immune system is divided into two components comprising an innate and adaptive response. The activity of these components is intrinsically linked, such that neither in isolation is sufficient to provide comprehensive immunological protection to the host.

The innate immune response provides a first line of defence through the action of macrophages and neutrophils. These cells are important in quickly recognising and destroying pathogens, however their activity is restricted by the limited range of non-varying receptors through which they are able to recognise invading microorganisms. The innate response exists primarily to limit damage whilst activating a more targeted adaptive immune response.

The adaptive immune response has evolved to eradicate a broad range of pathogens, and is activated on recognition of foreign, or 'non-self' antigens. Lymphocytes are the effector cells of the adaptive immune system, and are broadly divided into two groups, each with distinct functions, named according to the circumstances of their discovery. T lymphocytes are involved in cellular immunity, and were named following their discovery in the human thymus (Miller 1961; Miller 1962). B lymphocytes are involved in antibody production, and

whilst they are commonly considered to be named after their main site of production in humans in the bone marrow, the name is actually derived from their initial discovery in the bursa of Fabricius in birds (Glick et al. 1956). The innate immune system utilizes antigen-presenting cells (APC) such as dendritic cells (DC) to present pathogens to the adaptive immune system (specifically T lymphocytes), providing an essential bridge between the two systems and allowing recruitment of antigen-specific effector cells to the site of infection.

1.1.2 T lymphocytes and MHC restriction

T lymphocytes are the main effector cells of the adaptive immune response. T lymphocytes have evolved to react to invading pathogens through a very tightly regulated mechanism involving the T cell receptor (TCR), and its interaction with foreign antigen displayed on the surface of cells of the host organism. The T lymphocyte is able to detect this foreign antigen because infected cells display peptide fragments derived from the invading pathogen in specialized cell-surface glycoproteins known as major histocompatibility complex (MHC) molecules. This interaction between the TCR and MHC molecule is referred to as "MHC restriction" (Zinkernagel & Doherty 1974; Unanue 1984; Townsend & Bodmer 1989).

A diverse array of TCR is produced through rearrangement of germ line-encoded gene segments in developing T lymphocytes in the thymus. Two types of TCR have been described, each with distinct functions and distribution. TCR which engage the MHC / peptide complex are heterodimers comprising an α and β chain (α:β-T lymphocytes); in mice, each α chain is constructed from one of 50-100 Vα, and one of 50 Jα, and each β chain from one of 20-50 Vβ, one of 12 Jβ, and one of two Dβ. The exact number of gene segments varies

between different species (Huseby et al. 2005). Random rearrangement of these genes allows generation of a diverse array of different TCR, able to recognise a multitude of MHC / peptide combinations. Whilst such random gene rearrangement generates a useful repertoire of TCR against foreign peptides, it also allows development of TCR recognising host peptides. This process thus necessitates strict regulation to protect against uncontrolled self-reactivity.

T lymphocytes develop from haemopoietic stem cell precursors which migrate to the thymus, initially entering at the cortico-medullary junction and migrating through the thymic cortex. During T lymphocyte maturation, a variety of self-peptides are initially presented in MHC by cortical thymic epithelial cells (cTEC); low affinity interactions result in positive selection of T lymphocytes enabling further maturation, whilst cells unable to recognise self-peptide / MHC present on cTEC die by apoptosis. Positively selected T lymphocytes next migrate to the cortico-medullary junction, where a process of negative selection results in removal of those T lymphocytes displaying high affinity interaction between self-peptide / MHC and TCR, thereby eliminating potentially harmful self-reactive T lymphocytes. Negative selection is mediated by bone-marrow derived dendritic cells, macrophages and medullary thymic epithelial cells (mTEC) expressing tissue-specific self-antigens under the control of the autoimmune regulator (AIRE) protein (Anderson et al. 2002; Guerau-de-Arellano et al. 2009). Selection of T lymphocytes is therefore determined by their relative affinity for self-peptide / MHC in the thymus (Starr et al. 2003; Kyewski & Klein 2006).

T lymphocytes which fail to react to appropriate MHC stimulation, or those which react to host-derived peptides are deleted to ensure that the population of peripheral T lymphocytes is

sensitive to invading pathogen, without being damaging to host tissue. Failure of this selection process is thought to be at least partially responsible for autoimmune disease and loss of self tolerance (Marrack et al. 2011; Parham 2013) (discussed further in section 1.1.8).

A second subset of T lymphocytes exists with a receptor comprising a γ chains and a δ chain (γ:δ-T lymphocytes). These cells are mainly distributed in mucosal and epithelial tissues and comprise less than 5% of the total T lymphocyte population in peripheral blood. They are thought to be able to act as antigen presenting cells, and have both pro-inflammatory and regulatory functions in different disease states (Su et al. 2013). They do not require antigen presentation by the MHC molecule, and their exact role remains a topic of investigation (Janeway et al. 2001). They will not be discussed further in this review, and the term 'T lymphocyte' will refer to α:β-T lymphocytes unless otherwise specified.

There are two classes of MHC molecule; class I molecules can be expressed by any nucleated cell, and predominantly present peptide fragments of intracellular pathogens (most commonly viruses) to cytotoxic CD8+ T lymphocytes, which respond by killing a cell they recognise. Class II molecules are expressed only by APC which participate in the immune response, such as CD4+ T lymphocytes, B lymphocytes, DC and macrophages (discussed further in section 1.1.3). Cells expressing class II molecules are involved in activation of other effector cells of the immune system, and have thus generally been considered to have a "helper" function. Direct class II-dependent cell lysis has however also been observed both *in vivo* and *in vitro*, with some CD4+ T lymphocytes demonstrating cytotoxicity against influenza virus in the mouse, and measles virus, human immunodeficiency virus (HIV) and Epstein-Barr virus (EBV) in humans (Jellison et al. 2005).

In the mouse, MHC molecules are encoded by clusters of genes on chromosome 17. These are referred to as H2 genes; there are three main MHC class I H2 genes called H2-K, -D and -L, and two main MHC class II genes called H2-A and -E. In humans, a similar arrangement of Human Leukocyte Antigen (HLA) genes exists on chromosome 6 referred to as HLA-A, -B, and -C corresponding to MHC class I molecules, and HLA-DP, -DQ and –DR corresponding to MHC class II. The polygenic nature of the MHC results in generation of a set of MHC molecules with different ranges of peptide affinities, each able to bind a different range of peptides, thus allowing host cells to present peptide from a wide range of potential pathogens. The MHC repertoire is further increased by the polymorphic nature and co-dominant expression of MHC genes, which generates a greater range of specificities than would be permitted simply by different gene combinations alone (Janeway et al. 2001).

The diversity of both the TCR and MHC ensures the host is able to respond to a vast range of invading pathogens, whilst thymic selection restricts TCR stimulation to foreign peptide / MHC-only activation and in theory, prevents the initiation of an immune response against host tissues.

1.1.3 T lymphocyte subsets

As inferred above, T lymphocytes can be broadly classified as being either directly "cytotoxic", acting to kill cells infected with invading bacteria or viruses, or have a "helper" function, signalling to other cells to assist in the immune response. These subsets are determined by the expression of cell-surface co-receptors, which are crucial to the function of the TCR: Cytotoxic T lymphocytes express the cell-surface protein CD8, which binds to the

class I MHC molecule, whereas helper T lymphocytes express CD4, which binds to the class II MHC molecule. Although this cytotoxic / helper distinction is often referenced, it should be acknowledged that this categorisation is an over-simplified version of the true situation, as highlighted by the presence of cytotoxic MHC class II-expressing CD4+ T lymphocytes as previously discussed.

There is step-wise expression of TCR components during intra-thymic development; initially, T lymphocytes express neither CD4 nor CD8 and are referred to as "double negative" (dn). These cells give rise to both γ:δ and α:β-T lymphocytes; γ:δ-T lymphocytes express neither CD4 nor CD8 even as mature cells. Conversely, α:β-T lymphocytes first express both CD4 and CD8 becoming "double positive" (dp), initially expressing the pre-TCR (pTα:β), at which point cells enlarge and divide. They next become smaller resting double positive cells expressing the α:β TCR, at which point most cells undergo apoptosis. Only cells with the ability to recognise self MHC molecules survive, down-regulating either CD4 or CD8 to become "single positive" cells with a mature TCR (Janeway et al. 2001) (Figure 1.1).

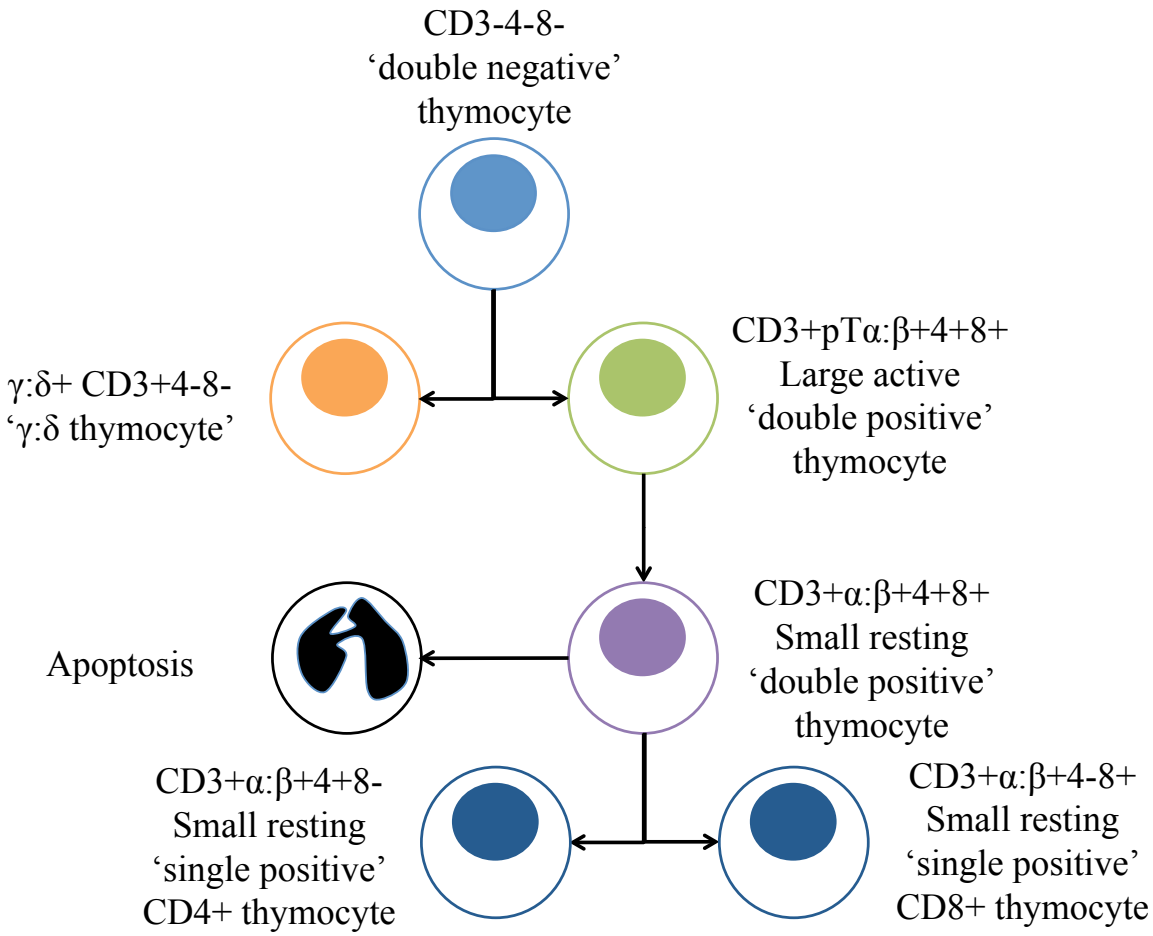


Figure 1.1 T lymphocyte development in the thymus. Mature γ:δ and α:β-T lymphocytes develop from a common precursor which does not express CD3, CD4 or CD8. Mature γ:δ-T lymphocytes express only CD3 and remain both CD4 and CD8 negative. α:β-T lymphocytes follow a sequence of upregulation of CD3, CD4 and CD8, before down regulating either CD4 or CD8 to become mature CD4+ or CD8+ α:β-T lymphocytes.

CD4+ T-helper (Th) lymphocytes can be divided into at least four main lineages; Th1, Th2, Th17 and regulatory (Treg) T cells (Valerie Dardalhon et al. 2008). More recently, additional subsets have been identified (Th9, Th22), and this classification has been further complicated by the discovery of overlap or 'plasticity' between different T lymphocyte subsets; whereas T helper lymphocytes were once considered terminally differentiated, it is now recognised that the mature T lymphocyte phenotype can change (Nakayamada et al. 2012).

Subsets of CD4+ T lymphocytes can be identified by the particular combination of cytokines required for differentiation from naïve precursors, expression of nuclear transcription factors, expression of surface receptors and cytokines secreted. This is illustrated in figure 1.2, and discussed over the forthcoming pages.

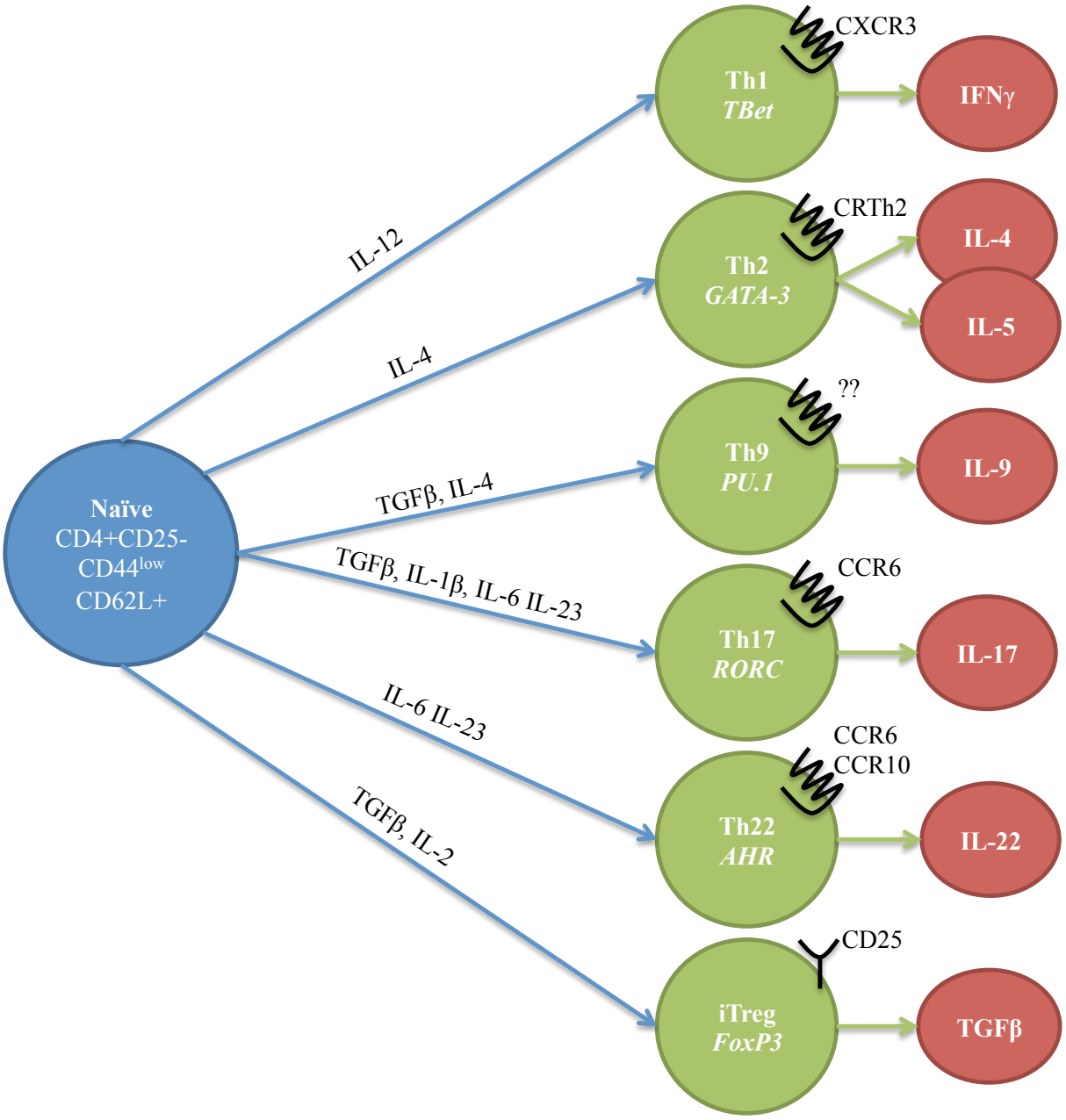


Figure 1.2 T lymphocyte differentiation from naïve progenitor cells. Diagram illustrating cytokines required for murine T lymphocyte lineage differentiation, transcription factors (italic), surface markers and cytokines secreted.

Th1 lymphocytes are important in supporting cellular immunity and are involved in clearance of intra-cellular pathogens (Fietta & Delsante 2009). They differentiate from naïve T lymphocytes in the presence of interleukin-12 (IL-12) and express the transcription factor T-Bet, which in turn induces IFNγ and defines the prototypical Th1 response (Szabo et al. 2000). IFNγ activates macrophages and promotes IL-12 secretion by DC, acting as a feed-forward mechanism to potentiate the Th1 response. T-Bet also induces CXCR3, allowing Th1 lymphocytes to enter peripheral tissues (Lord et al. 2005).

Th2 lymphocytes are critical for clearance of extra-cellular parasites and are involved in allergic responses (Romagnani 1994). They differentiate from naïve T lymphocytes in the presence of IL-4 and express the transcription factor GATA-3, which in turn induces the Th2 cytokines IL-4, IL-5 and IL-13 (Zheng & Flavell 1997; Lantelme et al. 2001). It also induces the chemokine receptor CCR4 and the prostaglandin D2 receptor CRTh2 in humans (De Fanis et al. 2007). The Th2 cytokines potentiate the allergic response; IL-4 inhibits Th1 and Th17 lymphocyte responses and promotes IgE production by B lymphocytes, whilst IL-5 activates eosinophils.

Th17 lymphocytes are critical for immune responses against extracellular bacteria and fungi, and are thought to give rise to pathogenic cells that drive chronic inflammation in organ-specific autoimmune disease (Korn, Oukka, et al. 2007). They express the transcription factor RORC, which drives IL-17 production and expression of the IL-23 receptor (Acosta-Rodriguez et al. 2007; Annunziato et al. 2007; Wilson et al. 2007). In turn, IL-17 acts via epithelial cells to attract neutrophils, and CCR6 is important in recruiting Th17 lymphocytes to inflamed gut or skin (Schutyser et al. 2003). Th17 cells are induced in murine models by

TGFβ and IL-6 (Veldhoen et al. 2006), with IL-1β, IL-21 and IL-23 also contributing to their development (Shainheit et al. 2008; Korn, Bettelli, et al. 2007; Zhou et al. 2007; Nurieva et al. 2007).

Th9 lymphocytes are defined by IL-9 secretion, and express the transcription factors PU1 (Ramming et al. 2012; Chang et al. 2010) and IRF4 (Staudt et al. 2010). They are generated in response to IL-4 and TGFβ (Putheti et al. 2010; Veldhoen et al. 2008) and are involved in allergic asthma and autoimmunity (Valérie Dardalhon et al. 2008).

Th22 lymphocytes are involved in immune responses in the skin, and are identified by secretion of IL-22 and expression of the transcription factor AHR (Geginat et al. 2013). IL-22 induces proliferation of epithelial cells and is involved in psoriasis (Ma et al. 2008; Zheng et al. 2007).

Treg are a subset which suppress otherwise pathogenic immunity and can also induce self-tolerance (Corthay 2009). Treg can be produced in the thymus ("naturally occurring" nTreg) or induced in the periphery ("induced" iTreg) and are characterised by expression of the Forkhead Box P3 (FoxP3) gene, IL-2 receptor α chain (CD25) and low expression of CD127 (Vignali et al. 2009). Treg suppress pathogenic T lymphocyte function through a range of mechanisms including the production of immunosuppressive cytokines such as IL-10 and TGFβ, release of protease enzymes causing destruction of effector cells, and prevention of antigen-presenting cell maturation (Vignali et al. 2009; Corthay 2009). Treg are also known to constitutively express Cytotoxic T Lymphocyte Associated Antigen 4 (CTLA-4), which is considered important in immune homeostasis. CTLA-4 binds CD80 and CD86; these ligands

are mainly expressed by APC, and are also bound by CD28 on T lymphocytes in the process of activation. CTLA-4 binds these ligands with higher affinity than CD28 and is thus able to act as an antagonist of CD28-ligand binding, inhibiting T lymphocyte-mediated immune responses (Walker & Sansom 2011).

1.1.4 T lymphocyte activation and memory subsets

All T lymphocyte subsets originate from naïve cells that have not yet encountered specific antigen. On exposure to antigen, naïve cells undergo clonal expansion targeted towards eradicating the invading pathogen, followed by contraction of the effector cell population. The eventual effector phenotype is determined by the nature of co-stimulation from the innate immune system as described above. A small population of 'memory' cells specific to the invading pathogen persists after cessation of the immune response. This population of already-differentiated memory cells retain their effector function, and are able to proliferate on repeat exposure to the same pathogen, thus enabling a more rapid adaptive immune response to future infection (Mitchell & Williams 2010).

Naïve T lymphocytes circulate in peripheral blood, entering lymph nodes via high endothelial venules (HEV), where they encounter antigen and undergo differentiation to an effector phenotype (Geginat et al. 2013). To be able to enter HEV, naïve cells require L-selectin (CD62L) and the chemokine receptor CCR7 (Bradley et al. 1994; Förster et al. 1999); CD62L binds to vascular addressins present on HEV and is responsible for the initial binding of blood-borne T lymphocytes (Berg et al. 1991), whilst CCR7 regulates responsiveness to chemokines present within the HEV (Gunn et al. 1998). In humans, these naïve cells are

identified by expression of both CD62L and the longest isoform of the common lymphocyte antigen CD45 (CD45RA) in the absence of the short isoform (CD45RO) (Clement 1992). CD45 is a tyrosine phosphatase which regulates the activation threshold of T lymphocytes (Hermiston et al. 2003). On activation, naïve T lymphocytes lose CD62L and express CD45RO in the absence of CD45RA.

On contraction of the effector lymphocyte population, two distinct subsets of memory lymphocytes are retained. 'Effector Memory' lymphocytes (Tem) circulate in the blood and are able to rapidly proliferate in response to repeat antigenic challenge, retaining their effector function without the requirement for trafficking to the lymph node. 'Central Memory' lymphocytes (Tcm) similarly retain their antigen-specificity, but on repeat antigenic challenge retain the ability to migrate to lymph nodes and mount secondary proliferative responses. In humans, memory populations retain CD45RO, and are distinguished by their relative expression of CCR7; Tcm retain CCR7 whilst Tem lose CCR7 (Sallusto et al. 1999).

An analogous situation exists in mice, where the surface marker CD44 is widely used to identify both activated and memory T lymphocyte populations; CD44 is an adhesion molecule expressed by most cells which mediates binding to the extracellular matrix (ECM) via hyaluronic acid. It is up-regulated on active T lymphocytes, and high expression is maintained indefinitely on memory cells (Baaten et al. 2010). Naïve cells are characterised by high expression of CD62L and low expression of CD44, In this state, naïve cells retain the ability to enter HEV (CD62L+) but remain undifferentiated (CD44 low). As described above, memory cells are identified by high expression of CD44, but can be further sub-divided into

Tem and Tcm populations by the presence of low or high CD62L expression respectively (Pepper & Jenkins 2011).

A third population of memory T lymphocyte has been described; tissue-resident T lymphocytes (Trm) are a subset of Tem which remain positioned within non-lymphoid tissues following resolution of antigenic challenge without recirculating through blood. Similar to Tem, Trm are defined by absent CD62L expression, however they also express a number of markers not shared by other memory T lymphocytes, including CD69 and CD103. Whilst the precise functional significance of these markers remains unknown, CD69 is thought to antagonise sphingosine-1 phosphate receptor 1 (S1PR1), which is an important mediator of T lymphocyte egress from lymph nodes, and CD103 is thought to be important in maintaining survival of CD8+ cells and may promote adherence to epithelial cells. Trm are thus thought to reside at epithelial surfaces and provide a first response to invading pathogens (Schenkel & Masopust 2014).

1.1.5 T lymphocyte recruitment to tissue

Once activated, T lymphocytes must enter the circulation and travel to their target site, where they must then localize to tissue. Tissue recruitment is a complex process dependent on integrin binding, adhesion molecule expression and cytokine secretion. These mechanisms are known to be both site and cell-specific (Lukacs 2000).

Leukocytes must first adhere to the vascular endothelium. Contact is initially mediated by tethering and rolling of leukocytes on the endothelial cell surface, dependent on the binding of

P- or E-selectin on the endothelial cell surface to glycoprotein ligands on the leukocyte (Ley et al. 2007; McEver & Zhu 2010). This interaction slows the velocity of the rolling leucocyte, allowing firm adhesion to the endothelium, mediated by leukocyte integrins, such as very late antigen (VLA)-4 , lymphocyte function-associated antigen (LFA)-1 or macrophage-1 antigen (Mac-1). These interact with endothelial ligands, including vascular cell adhesion molecule (VCAM)-1, intercellular adhesion molecule (ICAM)-1, ICAM-2, or receptor for advanced glycation end products (RAGE) (Chavakis 2012; Vestweber 2007; Ley et al. 2007). Finally, transmigration of leukocytes through the endothelium generally occurs by passing through endothelial junctions and involves several adhesion receptors, such as integrins, junctional adhesion molecules (JAM), platelet endothelial cell adhesion molecule-1 (PECAM-1) or CD99 (Chavakis 2012; Vestweber 2007; Ley et al. 2007; Woodfin et al. 2011).

This process can be modulated by cytokines or chemokines; tissue-derived cytokines can upregulate endothelial adhesion molecule expression, whilst tissue-derived chemokines can induce conformational changes on leukocyte integrins, leading to their activation (Vestweber 2007; Ley et al. 2007; Shamri et al. 2005).

1.1.6 B lymphocytes and immunoglobulin

B lymphocytes develop in the bone marrow from haemopoietic precursors. The antigen recognition molecules of B lymphocytes are immunoglobulins (Ig), which when bound to the cell membrane form the B cell receptor (BCR). Similar to the TCR, Ig are produced with a wide range of specificities, with each B lymphocyte producing Ig of a single specificity. Antibodies are the secreted form of the BCR, produced by terminally differentiated B lymphocytes, which are known as plasma cells. Antibody is able to bind pathogens or their toxic products in extra-cellular spaces; this is the main effector function of B lymphocytes in immunity (Janeway et al. 2001).

Antibody molecules are approximately Y-shaped consisting of three portions, loosely connected by a flexible tether. All antibodies consist of two heavy ('H') chains and two light ('L') chains; in any antibody the two H chains and two L chains are identical, giving the antibody two identical antigen-binding sites. Two types of L chain, termed lambda (λ) and kappa (κ) are found in antibodies; a given antibody has either lambda or kappa chains but never both. Five classes of antibody exist, termed IgM, IgD, IgG, IgA and IgE; the class, and therefore effector function of antibodies is determined by the H chain structure, conferred by the carboxyl-terminal part of the H chain (C region). H chains of each class are denoted by the corresponding lower-case Greek letter μ, δ, γ, α, or ε. Antibody is identical to the BCR of the cell that secretes it with the exception of a small portion of the C-terminus of the heavy chain constant region (Janeway et al. 2001).

Generation of Ig begins with functional rearrangement of Ig gene segments. V, D and J segments in the heavy ('H') chain locus, and V and J segments in the light ('L') chain locus combine to generate a repertoire of antibodies recognising greater than 5 x 10^{13} distinct antigens. Three developmental stages are described; pro-B lymphocytes lack functional Ig, and are generated by rearrangement of D and J segments of the H chain, followed by a second rearrangement joining a V region to the DJ segment. Pro-B lymphocytes undergo cell division and further rearrangement of L chain segments to become pre-B lymphocytes, and finally an IgM molecule is formed by functional rearrangement of the μ-H chain gene segments to define the immature B lymphocyte. IgM is expressed at the cell surface. These cells leave the bone marrow and migrate to B cell zones in secondary lymphoid organs, where they differentiate to an effector phenotype, undergoing Ig class-switching on interaction with antigen (Pieper et al. 2013).

1.1.7 B lymphocyte function

The importance of the B lymphocyte in immune responses was initially attributed to the ability to produce antibody. Antibodies provide protection against antigens by several mechanisms, including 'neutralisation' whereby they bind to pathogens or their toxic products and block their access to target cells, 'opsonisation' whereby they bind to pathogens and foreign particles enabling phagocytosis by other immune cells, and finally by activation of the compliment cascade (Janeway et al. 2001).

It is now known that the ability to eradicate invading pathogens is dependent on the interplay between both the T and B lymphocyte response. B lymphocytes are able to internalise

antigens bound by their cell-surface BCR, and present these as peptide:MHC class II complexes. B lymphocytes thus play a crucial role in antigen presentation to CD4+ T lymphocytes, enabling activation and clonal proliferation of antigen-specific T lymphocytes (Lebien & Tedder 2008).

Furthermore, B lymphocytes have been shown to secrete a range of cytokines which modulate the local immune response, including regulation of local T lymphocytes. For example, B lymphocytes secreting IL-10 have an immunosuppressive function and have been termed 'regulatory B lymphocytes' (Breg), (Mauri & Bosma 2012). 'Effector' B lymphocytes have also been identified which are able to regulate differentiation of naïve CD4+ T lymphocytes through secretion of IL-4 and IFNγ (Harris et al. 2000).

1.1.8 Immunological tolerance

The human immune system must achieve a balance between effective elimination of pathogens, whilst maintaining tolerance to self-antigens and achieving homeostasis. Immunological tolerance refers to the reduction or inhibition of the ability to mount an immune response on exposure to antigen. Tolerance can be naturally acquired against host antigens, or induced against exogenous antigens.

There are many determinants of the immune response, including the antigen itself, its dose and route of administration, and the genetic background of the host. In turn, there are many points at which this response is regulated, being further influenced by the effects of

responding immune cells, the nature of associated cytokine stimulation, and the activity of other biological systems in the host.

1.1.8.1 Factors determining the extent, induction and duration of tolerance

The size of a potentially antigenic molecule can determine the level of immune response against that antigen; molecules which exist as aggregates are often more antigenic than those which exist as monomers (Ratanji et al. 2014). For example, serum proteins such as γ-globulin and albumin are strongly antigenic when present as aggregates, but induce tolerance when present as monomers (Ellis & Henney 1969). This is attributed to the rapid ingestion of larger, aggregated molecules by professional APC resulting in efficient presentation to T lymphocytes and potentiation of an immune response, whereas monomers may escape ingestion by APC, remaining in the circulation for prolonged periods and being processed more slowly (Zouali 2014).

Initial induction of an immune response depends on the availability of antigen in secondary lymphoid organs in a dose and time-dependent manner; if antigen is present at very low concentration or does not reach secondary lymphoid tissues, it may be ignored by immune cells and no immune response will be mounted. Conversely, if present at excessively high concentrations in multiple sites, antigen is likely to induce a state of high activity, and may even deplete the immune system of all antigen-specific cells resulting in a state of tolerance or immune deficiency (Zinkernagel et al. 1997).

Route of administration is thought to determine relative availability of an antigen to APC. In general, subcutaneous administration of antigen is thought to result in uptake by Langerhans cells and induction of an immune response, whereas intra-venous immunisation results in presentation by resting B lymphocytes and induction of tolerance. Oral administration is also thought to promote antigenic tolerance (Cuppari et al. 2014).

Genetic influences have been demonstrated between strains of laboratory mouse, with some strains being relatively resistant to tolerance induction. For example BALB/c mice are relatively resistant to tolerance induction by xenogeneic γ-globulin, and this resistance segregates amongst offspring (Lukić et al. 1975). In humans, genetic variation in MHC genes has been shown to influence the level of response to antigen, susceptibility to infection and autoimmune disease. This is thought to be explained by alteration of reporter gene activity and allele-specific expression, and variation in DNA binding-sites for regulatory factors (Handunnetthi et al. 2010).

Interaction between components of the immune system and antigen involves several clones specific for different epitopes, each with different affinities for the invading antigen. The affinity of this interaction with antigen determines whether a clone may be tolerised, with the completeness of resulting tolerance dependent on the proportion of tolerant and non-tolerant clones (Zouali 2014).

Tolerance may be induced by a lack of appropriate co-stimulation of T and B lymphocytes. T lymphocyte activation is dependent on two signals: in addition to the interaction with the processed peptide on APC, co-stimulation by accessory molecules expressed on APC is also

necessary. Well-characterised molecules include CD80 and CD86 ligands on APC interacting with CD28 and CTLA-4 receptors on T lymphocytes, and the CD40 molecule on macrophages, dendritic cells and B lymphocytes interacting with the CD40L receptor on activated T lymphocytes. In the absence of appropriate co-stimulation, T lymphocytes may become apoptotic or unresponsive, abrogating initiation of an adaptive immune response against the antigen (Chen & Flies 2013). Similar co-stimulation is necessary for activation of B lymphocytes following BCR / antigen interaction, with interaction between CD40 and CD40L being necessary to induce germinal center (GC) formation, Ig isotype switching, Ig somatic hypermutation and formation of long-lived plasma cells and memory B lymphocytes. (Clark & Ledbetter 1986; Elgueta et al. 2009; Mauri & Bosma 2012).

1.1.8.2 Mechanisms of central tolerance

Central tolerance describes the multitude of processes which exist to remove immature lymphocytes with the potential to mount immune responses against host tissues from primary lymphoid organs.

As previously discussed, potentially auto-reactive T and B lymphocytes are generated in the thymus and bone marrow respectively, which must be identified and eliminated. T lymphocytes must first undergo a process of positive selection, during which only those recognising peptide in self-MHC molecules are permitted to continue development; T lymphocytes failing to recognise self-MHC undergo apoptosis in the thymus. Positively selected T lymphocytes with high affinity for self-peptide bound to MHC molecules are then eliminated by clonal deletion, in a process termed negative selection (Starr et al. 2003;

Kyewski & Klein 2006; Anderson et al. 2007) (see section 1.1.2). Similarly, auto-reactive B lymphocytes are induced to die by apoptosis at the transition between pre-BCR to mature-BCR in the bone marrow. These process ensure that T and B lymphocytes with the potential to recognise and mount immune responses against self-peptides are deleted before entering the circulation, serving as a mechanism of central tolerance induction (Nemazee & Buerki 1989; Chen et al. 1995).

Whilst tolerance was initially considered to result only from deletion of auto-reactive lymphocytes in primary lymphoid organs, it was later demonstrated that populations of inactive lymphocytes exhibiting poor responsiveness to strong antigenic challenge can be detected in tolerant animals. The term anergy was first used to describe this functionally silent state observed in B lymphocytes (Nossal & Pike 1980). *In vitro*, B lymphocyte stimulation in the absence of T lymphocyte help results in a state of anergy, thus preventing aberrant activation of B lymphocytes (Healy et al. 1997). Similarly, *in vitro* activation of T lymphocytes in the absence of appropriate co-stimulation results in development of a functionally inactive state in the activated T lymphocyte. Such inactive T lymphocytes are incapable of producing IL2, and are unable to proliferate if later exposed to antigen and appropriate co-stimulatory ligands (Fields et al. 1996; L. Li et al. 2006).

B lymphocytes have also been shown to undergo a process of receptor editing, in which variable region genes expressed by B lymphocytes are altered resulting in changes to the specificity of the BCR (Gay et al. 1993; Tiegs et al. 1993; Radic & Zouali 1996). In contrast to the process of clonal deletion, where autoreactive B lymphocytes are eliminated, receptor editing induces gene rearrangement in autoreactive lymphocytes. This has the dual benefit of

neutralising potentially auto-reactive B lymphocytes, whilst expanding the primary B lymphocyte repertoire (Zouali 2008).

1.1.8.3 Peripheral tolerance

Central tolerance enables elimination of lymphocytes sensitive to self antigens present in primary lymphoid organs. Antigens produced in peripheral non-lymphoid tissues do not circulate in sufficient amounts in primary lymphoid organs, and mechanisms of peripheral tolerance are thus necessary to restrict auto-reactive lymphocytes that have not been silenced in primary organs.

There is significant regulation of the immune response by activated T lymphocytes, with the type of response determined by the nature of cytokines secreted. For example, Th1 cells secrete IFNγ, which stimulates macrophages and increases Th1 cell numbers by positive feedback (Szabo et al. 2000), whereas Treg secrete cytokines such as IL-10 and TGFβ, suppressing immune responses and promoting tolerance (Corthay 2009). Peripheral tolerance can thus be induced through preferential activation of specific T lymphocyte subsets.

Activation-induced cell death (AICD) is a process whereby repeated stimulation of T lymphocytes via the TCR results in programmed cell death by apoptosis, and is thus a mechanism of negative regulation of activated T lymphocytes which helps maintain peripheral immune tolerance (Green et al. 2003). Apoptosis is induced through interaction of Fas receptors (CD95) on lymphocytes with Fas receptor ligand (CD95L) expressed by a wide range of effector cells (Nguyen & Russell 2001). Interestingly, CD95L is expressed on

activated T lymphocytes, and can be secreted in a soluble form, thereby enabling autocrine T lymphocyte 'suicide'. This is thought to be an important mechanism through which the pathogenic potential of activated T lymphocytes can be limited (Dhein et al. 1995).

As previously discussed, two distinct groups of Treg exist, including nTreg which are generated in the thymus, and iTreg which develop from naïve T lymphocytes in the periphery (see section 1.1.3). During the process of negative selection of T lymphocytes in the thymus, relatively higher-affinity interactions between self-peptide / MHC and the developing TCR which remain below the threshold for initiating death by apoptosis result in FoxP3 induction and development of nTreg (Ohkura et al. 2013). Development of peripheral iTreg occurs in response to interaction between TCR on naïve T lymphocytes and foreign peptide / MHC in the absence of pro-inflammatory co-stimulation (Li & Zheng 2015). TGFβ is implicated in both processes, by exerting an anti-apoptotic effect on nTreg (Ouyang et al. 2010), and by inducing Smad binding to the FoxP3 locus and directly promoting FoxP3 expression in iTreg (Schlenner et al. 2012) (discussed further in section 1.2.4). Treg induce immunological tolerance through a variety of mechanisms, including secretion of immunosuppressive cytokines such as IL-10 and TGFβ, killing or functional modulation of APC or T lymphocytes by release of perforin, granzymes, or by a CTLA- 4–dependent mechanism (see section 1.1.3) (Walker & Sansom 2011), deprivation of cytokines such as IL- 2, and degradation of ATP through ectonucleotidases (Shevach 2009).

Similarly, Breg are able to negatively regulate inflammation and autoimmune disease through production of IL-10 (Mauri & Bosma 2012)**.** Furthermore, immunoglobulins secreted by B lymphocytes are able both to positively regulate immune responses through processes

including immune complex formation and increased B lymphocyte activation, or negatively regulate antigen presentation through feedback inhibition of B lymphocyte function by mechanisms including reduction of antibody avidity following Ig class switching (Hjelm et al. 2006).

Certain sites appear to be almost exempt from localised immune responses, including the brain, eye and testis. Whilst immune responses are observed in these locations, they appear to be more tightly regulated than those at other sites, usually displaying a far higher threshold for immune activation. Such tissues are considered sites of 'immune privilege', and this characteristic was initially attributed to the lack of lymphatic drainage and natural blood-tissue barriers observed in these areas. It was thought that physical barriers prevented access of effector immune cells to these tissues (Forrester et al. 2008). It is however now accepted that immune privilege is a consequence of interactions between the immune system and specialized tissues (Van Parijs & Abbas 1998). For example, CD95L, the ligand for the Fas receptor CD95, is expressed constitutively in the eye and testis, and activated CD95+ T lymphocytes entering these sites are thus induced to undergo apoptosis by AICD (Griffith & Ferguson 2011).

Finally, there is interaction between the neuro-endocrine and immune system via the hypothalamo-pituitary-adrenal axis, with secretion of endogenous corticosteroids observed to negatively regulate peripheral immune activation (Haddad et al. 2002). The immune response is thus regulated at many points by a wide variety of intrinsic and extrinsic mechanisms.

1.2 Transforming Growth Factor β (TGFβ)

1.2.1 Function

The Transforming Growth Factor Beta (TGFβ) superfamily includes the isoforms of TGFβ, activins and inhibins, mullerian inhibitory substance, growth and differentiation factors (GDF) and bone morphogenic peptides (BMP). These proteins have a wide range of roles including proliferation, differentiation, apoptosis, chemotaxis, tumour suppression and immune regulation (Dallas et al. 2008). TGFβ is also important in embryogenesis, and alterations in TGFβ signalling have been implicated in many human disease states (Santibañez et al. 2011).

1.2.2 Isoforms

Three TGFβ isoforms have been identified in mammals, encoded by different genes (Massagué & Chen 2000). Significant sequence homology is observed between isoforms with 71% similarity between TGFβ1 and TGFβ2, and 76% similarity between TGFβ2 and TGFβ3 (Kondaiah et al. 1990). Interestingly, there is greater than 76% sequence homology between all isoforms of mature TGFβ between most mammalian and avian species, suggesting significant evolutionary conservation of TGFβ molecules (Massagué 1990).
There is differential expression of the isoforms during embryogenesis (Pelton et al. 1989; Schmid et al. 1991; Millan et al. 1991), which is thought to explain why mice with targeted deletions of each isoform show different phenotypes:

TGFβ1 deletions result in two distinct phenotypes; approximately 50% of embryos die around mid-gestation due to defects in yolk sac vasculogenesis and haematopoiesis (Dickson et al. 1995), whilst the remainder survive beyond birth, possibly due to transfer of maternal TGFβ1 across the placenta. Mice surviving to birth proceed to experience spontaneous activation of a self targeted immune responses leading to death between three and four weeks of age (Kulkarni et al. 1993; Shull et al. 1992).

TGFβ2 deletions also result in perinatal death, although this is due to extensive developmental defects in the cardiac, pulmonary, musculoskeletal, craniofacial and urogenital systems (Sanford et al. 1997). Loss of TGFβ3 results in cleft palate and abnormal pulmonary development with death within a few hours of birth (Kaartinen et al. 1995; Proetzel et al. 1995).

These observations suggest that each isoform must have a distinct function during embryogenesis, and thus differential responses to each isoform may persist in mature tissues. Indeed, there is significant variation in the predominant TGFβ isoform at different sites in the human body. TGFβ1 is thought to be the predominant isoform acting in the immune system (Wan & Flavell 2007), and accounts for 80-90% of the TGFβ found in bone (Bonewald 1999; Dallas et al. 2008). TGFβ1 also appears to prevail in the intra-ocular environment during active uveitis, whilst TGFβ2 is the predominant isoform in the un-inflamed eye (Denniston et al. 2011; Stein-Streilein 2008). TGFβ2 is the predominant isoform in the prostate gland (Dallas et al. 2005) and kidney mesangial cells (Marra et al. 1996). TGFβ3 is the only isoform constitutively expressed in the intact epidermis and as a result is specifically implicated in wound healing (Schmid et al. 1993).

1.2.3 Structure

TGFβ molecules encoded as large precursor proteins of 390-412 amino acids, which undergo proteolytic cleavage by endopeptidase furin to yield two products which assemble into homodimers. The 65-75kDa dimer from the N-terminal region is known as the latency-associated peptide (LAP), whilst the 25kDa dimer from the C-terminal portion is the mature TGFβ molecule (Khalil 1999).

Each mature TGFβ monomer comprises nine cysteine residues, of which eight form disulphide bonds comprising the 'cysteine knot' which is common to all TGFβ superfamily members. The ninth cysteine residue forms a bond with the ninth residue of another monomer to form the dimeric structure. The area between the fifth and sixth cysteine residues is exposed at the surface of the molecule and is thought to be involved in receptor binding (Daopin et al. 1992) (Figure 1.3).

All TGFβ molecules are described as having a common structure described as a 'TGFβ-fold', comprising a cysteine knot with two pairs of β-strands extending from an α-helix (Lin et al. 2006) (Figure 1.3). The monomers are arranged such that the central helix of one monomer is oriented in the concave surface formed by the β-strands of the other, with stabilising inter-chain disulphide bonds present in most examples (Hinck 2012).

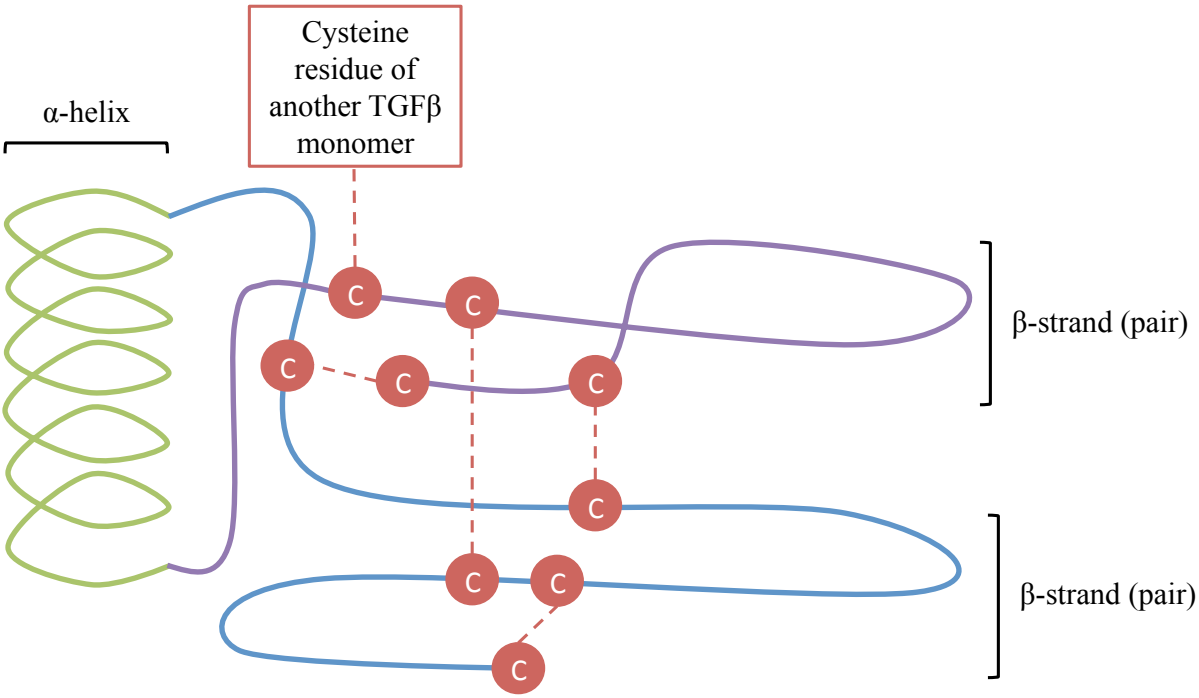


Figure 1.3 Schematic representation of TGFβ monomer structure. Each monomer consists of nine cysteine residues (red circles), of which eight form disulphide bonds (dashed lines) to create a 'cysteine knot', and the ninth forms a bond with the ninth residue of another TGFβ monomer (dashed arrow). The monomeric structure is described as a TGFβ fold, comprising an α-helix (green) with two pairs of β-strands (one pair of strands illustrated by purple line, one pair of strands illustrated by blue line).

1.2.4 TGFβ in immune regulation

In the context of immune regulation, TGFβ has traditionally been considered a potent suppressor of immune activation (Yoshimura et al. 2010). *In-vitro* assays have demonstrated TGFβ to act as a negative regulator of cell proliferation and induce iTreg differentiation of naïve T lymphocytes. TGFβ was first shown to suppress T lymphocyte proliferation through inhibition of IL-2 receptor expression (Kehrl et al. 1986), and later to induce differentiation of naïve T lymphocytes to a regulatory FoxP3+ phenotype (iTreg) (Li & Flavell 2008; Wan & Flavell 2007; Ming O Li et al. 2006; Chen et al. 2003), seemingly justifying its label as an immunosuppressive molecule. More recently, it has been suggested that TGFβ dampens the effect of CD28 signalling to T lymphocytes, inhibiting growth and proliferation of CD4+ T lymphocytes (Delisle et al. 2013).

Such *in-vitro* assays have however demonstrated that the response to TGFβ is highly context-dependent. Paradoxically, some groups demonstrated the ability to induce proliferation of effector T lymphocytes in response to TGFβ (Bode et al. 2004), and others suggested TGFβ to confer protection against T lymphocyte apoptosis in some situations (Chen et al. 2001; Sanjabi et al. 2009) thus providing evidence of a pro-inflammatory effect. In addition, TGFβ has also emerged as an important cytokine in the generation of a pathogenic effector Th17 lymphocyte response. When present at lower levels than those necessary for FoxP3 induction, and with co-stimulation by IL-6, TGFβ will induce differentiation of naïve T lymphocytes to a potentially destructive Th17 phenotype (Korn, Oukka, et al. 2007; Rubtsov & Rudensky 2007; Mangan et al. 2006; Bettelli et al. 2006; Veldhoen et al. 2006) (Figure 1.4).

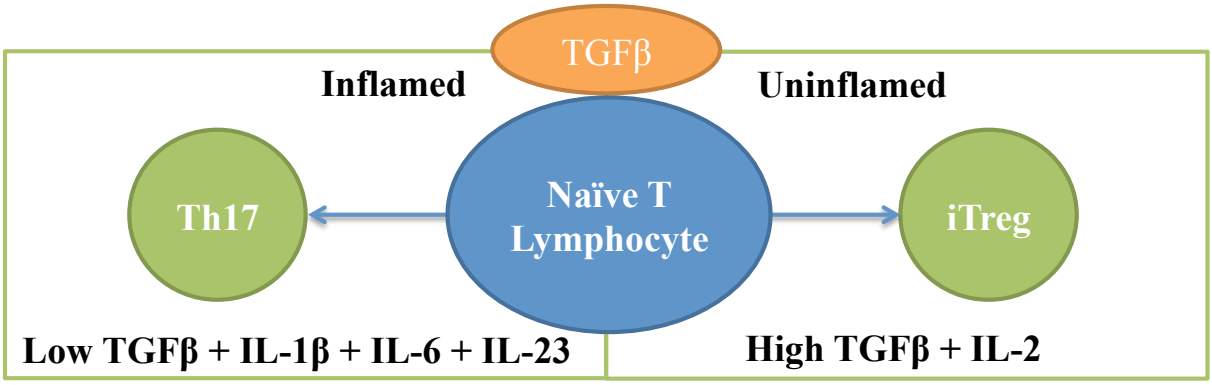


Figure 1.4 The dual role of TGFβ. TGFβ is able to induce differentiation of naïve T lymphocytes to either an immunosuppressive iTreg phenotype or pro-inflammatory Th17 phenotype; TGFβ-dependent immune responses are thus dependent on the co-stimulatory cytokines present at the time of signal transduction.

Perhaps most interestingly, there is evidence that TGFβ3 is able to differentiate naïve CD4+ T lymphocytes to a more pathogenic phenotype than TGFβ1; Lee et al differentiated Th17 cells from naïve CD4+ T lymphocytes using either TGFβ1 or TGFβ3, using the resulting cells to induce a murine model of experimental autoimmune encephalomyelitis (EAE) (Lee et al. 2012). Th17 lymphocytes differentiated with TGFβ3 and IL-6 resulted in EAE induction, whereas lymphocytes induced by TGFβ1 and IL-6 did not readily induce autoimmune disease without further exposure to IL-23. This suggests that not only can TGFβ have a pro-inflammatory function, but that there may also be a differential response between different isoforms.

In-vivo studies of TGFβ in immune regulation have been limited partly by the embryonic lethality of murine knock-out models, and also due to the widespread expression of both TGFβ and its receptors. It is thus difficult to separate the effects of TGFβ on T lymphocytes from its effects on antigen presenting cells or non-lymphoid cells.

To overcome some of these difficulties, Gorelik and Flavell adopted a transgenic approach, whereby they were able to breed mice expressing a dominant negative form of the type-II TGFβ receptor (dnTGFβRII) under the control of a T lymphocyte specific promoter (Gorelik & Flavell 2000). Whilst dnTGFβRII retains the ability to bind all isoforms of TGFβ, it is unable to phosphorylate TGFβRI and mediate signal transduction (Wieser et al. 1993; Wrana et al. 1994). This allowed for specific blockade of TGFβ signalling to T lymphocytes, allowing targeted investigation of TGFβ-dependent T lymphocyte responses *in-vivo*.

Mice expressing dnTGFβRII on T lymphocytes developed autoimmune disease characterised by inflammatory infiltration in multiple organ systems and presence of circulating autoantibodies. In addition, most T lymphocytes appeared to differentiate to an effector phenotype with increased IFNγ and IL-4 secretion. The immunosuppressive activity of TGFβ thus appears to dominate *in vivo*.

1.2.5 TGFβ in disease

1.2.5.1 Uveitis and EAU

An example of the contrasting effects of TGFβ can be found in the eye when considering the disease state of uveitis – a condition characterised by inflammation involving the structures of the uveal tract (the iris, ciliary body and choroid). Visual impairment is common (Darrell et al. 1962; Suttorp-Schulten & Rothova 1996; Goldstein 1980), and may result from direct damage to uveal tract structures, or from involvement of adjacent tissues such as the retina and optic nerve (Forrester 1991). Whilst uveitis can have an infectious aetiology, most disease in the West is non-infectious (Barry et al. 2014); such non-infectious disease is believed to result from influx of effector cells and inappropriate activation of the intra-ocular immune system:

Changes in the ocular microenvironment are thought to result in a switch between an un-inflamed regulatory state, and an inflamed pathogenic state; studies in humans have demonstrated high levels of TGFβ (particularly TGFβ2) in the aqueous humour of un-inflamed eyes (Denniston et al. 2011), whereas TGFβ levels have been shown to decrease in active uveitis (Curnow et al. 2005).

Experimental models also support the role of TGFβ in T lymphocyte driven ocular inflammation; experimental autoimmune uveoretinitis (EAU) is perhaps the most commonly used model of autoimmune ocular inflammation. It is a T lymphocyte mediated disease that targets the neural retina and related tissues, and is thought to be a useful model of posterior

segment uveitis (inflammation of the choroid and retina) in humans (Caspi 2003). It is induced by the subcutaneous injection of a soluble retinal antigen – usually s-antigen or interphotoreceptor retinoid binding protein (IRBP) – in a complete Freund's adjuvant (CFA) vehicle, and results in a predominantly CD4+ T lymphocyte mediated response in the retina (Gasparin et al. 2012). Using EAU as a model for investigation, it has been shown that lower levels of TGFβ, in the presence of other pro-inflammatory cytokines such as IL-6 and IL-23 actually promote a pathogenic Th17-driven uveitis (Zhou et al. 2012; Zhou et al. 2011; Bettelli et al. 2006).

Based on these observations, it thus appears TGFβ is implicated in both the un-inflamed and inflamed ocular microenvironment, and therefore has a crucial function in ocular immune regulation.

1.2.5.2 Multiple sclerosis and EAE

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system (CNS) involving demyelination and neuronal injury (Hauser & Oksenberg 2006; Steinman & Zamvil 2006). The aetiology of MS is currently unknown, although environmental and genetic factors are thought to be involved in disease pathogenesis. Th17 cells have been detected in the CNS of patients with MS and are thought to be the main pathogenic effector cell in this condition (Axtell et al. 2010).

Experimental autoimmune encephalomyelitis (EAE) is a murine model of MS. It is classically induced through the subcutaneous immunisation of susceptible mice with encephalitogenic

antigens derived from CNS proteins, such as myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG). This results in the induction of autoreactive T lymphocytes in the CNS and is considered a useful tool for modelling human disease (Rangachari & Kuchroo 2013).

TGFβ has been found at elevated levels in the CNS of mice in the recovery phase of EAE (Issazadeh et al. 1996), which appears to correlate with an expansion of resident FoxP3+ Treg populations (O'Connor et al. 2007; Zorzella-Pezavento et al. 2013). Activated TGFβ has also been shown to be necessary for differentiation of naïve T lymphocytes to pathogenic Th17 cells in the active phase of EAE, again demonstrating TGFβ to be both an activator and suppressor of localised immune responses (Yoshida et al. 2014).

1.2.5.3 Inflammatory bowel disease and experimental colitis

TGFβ is also known to be a mediator of intestinal epithelial restitution and defence (Dignass & Podolsky 1993), and is strongly implicated in the pathogenesis of inflammatory bowel disease in humans. It is found at elevated levels in active lesions of Crohn's disease and ulcerative colitis, and normalises in quiescent disease (Babyatsky et al. 1996). Whilst traditionally considered to be Th1 /Th2-mediated, Th17 cells have more recently been identified in active disease (Kobayashi et al. 2008; Olsen et al. 2011; Siakavellas & Bamias 2012).

Several models of experimental murine colitis have been developed; the earliest and perhaps best characterised of these involves transfer of either CD4+CD62L+ (central memory) or

CD4+CD25- (non-regulatory) T lymphocytes to a severe combined immunodeficiency (SCID) mouse host (Powrie et al. 1994; Blumberg et al. 1999). Th17 cells have been identified and observed to correlate with disease progression in these mice, with IL-17 blockade shown to prevent development of inflammation (Wedebye Schmidt et al. 2013). Likewise, transfer of TGFβ-induced Treg cells have been demonstrated to suppress active experimental colitis in this model (Mottet et al. 2003; Fantini et al. 2006).

In each of these examples, both active inflammation and resolution of inflammation appear dependent on TGFβ. Differential signalling by TGFβ thus appears important in immune regulation.

1.3 TGFβ signalling

1.3.1 Ligand activation

TGFβ is synthesized as a precursor and secreted in a latent form by most cell types; the mature 25kDa homodimer is released by post-translational processing at the site of activity (Dallas et al. 2008).

Briefly, the pre region of TGFβ precursor contains a signal peptide, and pro-TGFβ is processed in the Golgi apparatus, removing the N terminus of the immature protein. This new protein forms a homodimer known as the latency-associated peptide (LAP), which forms a non-covalent bond to a homodimer of mature TGFβ. In order to bind to, and signal through its receptor, TGFβ must be released from the LAP by the action of a TGFβ activator, either by

proteolysis or a conformational change. Several molecules have been described as latent TGFβ activators, including proteases such as plasmin, matrix meyalloproteinase-1 and 2 (MMP-1 and MMP-2), thrombospondin-1, integrins and reactive oxygen species. Cells producing these TGFβ activators may differ from those secreting TGFβ. This is thought to be an important early regulator of TGFβ signalling (Annes et al. 2003).

1.3.2 Receptor structure

TGFβ (and members of the TGFβ superfamily) signal through a complex of cell surface receptors consisting of two type I (TGFβRI) and two type II (TGFβRII) transmembrane receptors. Both TGFβRI and TGFβRII are serine / threonine kinases, of approximately 55kDa and 75kDa respectively (Chang 2002). Both have short extra-cellular domains with a long cytoplasmic region containing the serine / threonine kinase (Dallas et al. 2008) (Figure 1.5).

TGFβRI and TGFβRII have been shown to have higher affinity for TGFβ1 and TGFβ3 isoforms than for TGFβ2 (Wrana et al. 1992) although subsets of receptors with higher affinity for TGFβ2 have been identified in some cell lines (Cheifetz et al. 1990), suggesting variation in the binding properties of receptors on different cell types at different sites, thus implicating other receptor isoforms in the signalling cascade.

Absolute deficiency of any either of the two main TGFβ receptor subunits is embryonic lethal, resulting in death around mid-gestation. Murine models of TGFβRI or II gene knock-out are characterised by severe defects in vascular development in the placenta and yolk sac (Larsson et al. 2001; Dickson et al. 1995; Oshima et al. 1996).

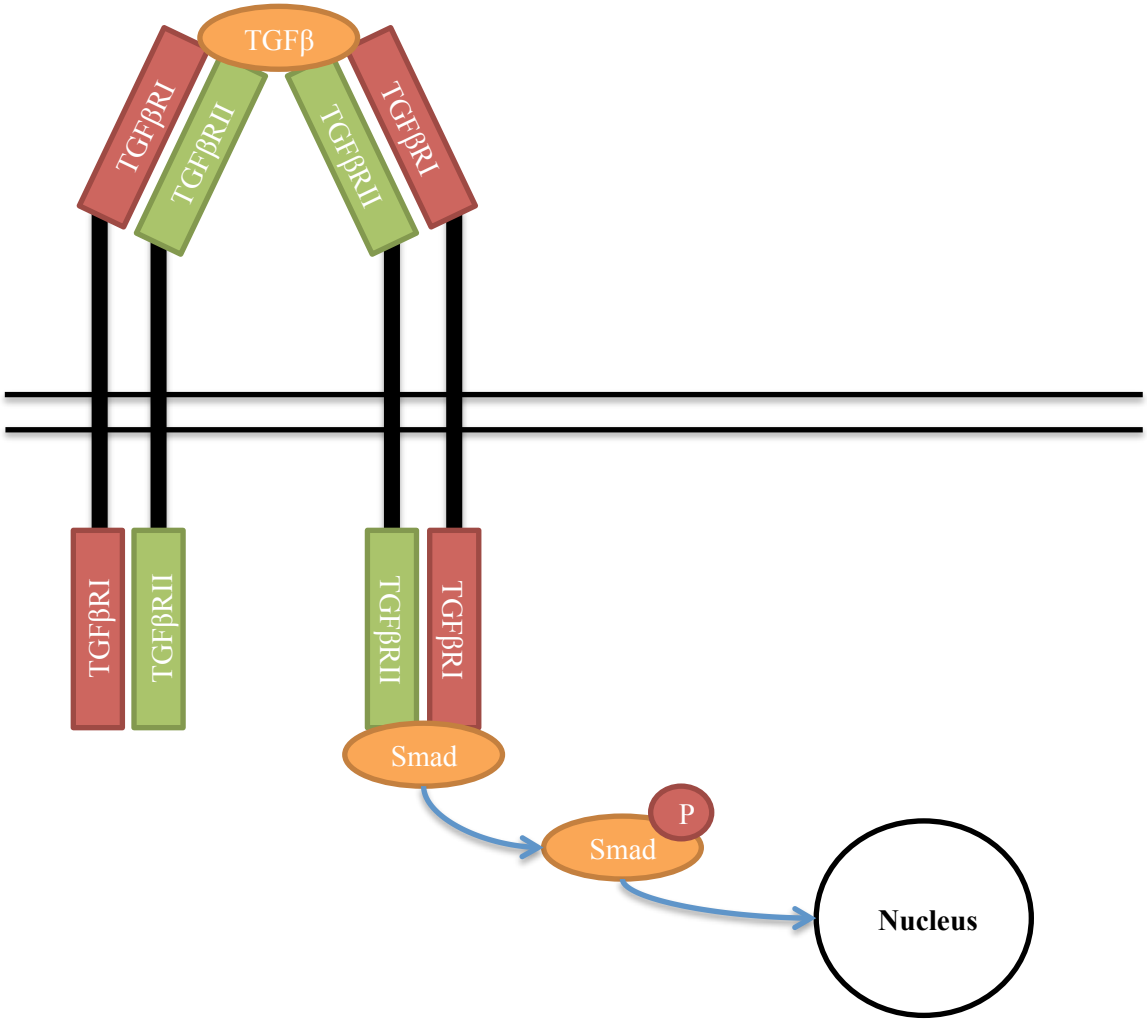


Figure 1.5 Schematic representation of TGFβ receptor and signalling pathway. TGFβ binds to TGFβRI, transphosphorylating TGFβRII, which in turn transphosphorylates intracellular Smad proteins. Phosphorylated Smad proteins are released from the receptor complex and migrate to the cell nucleus where they influence gene expression.

1.3.3 Signalling cascade

All members of the TGFβ superfamily exert their function through binding the aforementioned cell-surface serine/threonine kinase receptors (TGFβRI and TGFβRII) (Santibañez et al. 2011; Massague 1992). Ligand binding forms a stable complex consisting of two of each type of receptor (Feng & Derynck 2005). Ligand initially binds to the TGFβRII component allowing it to phosphorylate the kinase domain of the TGFβRI receptor (Souchelnytskyi et al. 1996; Kang et al. 2009). This in turn initiates the intracellular signalling cascade through the phosphorylation of Smad proteins (Santibañez et al. 2011); TGFβRI initially phosphorylates Smad 2 and Smad 3 which in turn form a complex with Smad 4 (Massagué 1998). These phosphorylated Smad proteins dissociate from the receptor and translocate to the nucleus, acting as transcription factors controlling gene expression within the cell nucleus (Shi & Massagué 2003).

Whilst full details of the signalling cascade are beyond the scope of this thesis, it is important to note that there is significant signalling versatility in TGFβ gene responses, determined by differential Smad-receptor interactions, Smad complex formation, interaction with accessory proteins and involvement of non-Smad signalling pathways. The outcome of TGFβ / receptor interaction is thus ultimately determined by the exact nature of the resulting signalling cascade, allowing TGFβ to induce cell-specific responses and have site-specific effects (Feng & Derynck 2005).

1.4 Betaglycan

1.4.1 Structure

There is a third component of the receptor for TGFβ; TGFβRIII, which is also known as betaglycan. Betaglycan is a membrane-anchored proteoglycan, which binds TGFβ via its core protein. It consists of 853 amino acids, with two large extra-cellular cysteine-containing domains separated by a short spacer region, a single pass transmembrane and short 42 amino-acid cytoplasmic domain (Massague 1992; Bilandzic & Stenvers 2011). It can also exist in a soluble form through proteolytic cleavage of the extra-cellular domains close to the cell membrane (Velasco-Loyden et al. 2004) being found in serum and ECM (Andres et al. 1989).

1.4.2 Current understanding of function

Betaglycan is able to bind all three isoforms of TGFβ, and also binds inhibin A, inhibin B and certain BMP (Bilandzic & Stenvers 2011). Betaglycan is present on a wide range of cell types, including mesenchymal, neuronal, and epithelial cells, being most strongly expressed on CD4+ T lymphocytes (Figure 1.6) and in the placenta (Pakula et al. 2007). It is however absent from certain myoblasts, epithelial, endothelial, and haematopoietic cells which still respond to TGFβ (López-Casillas et al. 1991). This early observation led to the suggestion that betaglycan itself has no intrinsic enzyme activity, but acts as a co-factor in TGFβ signalling, concentrating ligand at the cell surface to potentiate signalling through the TGFβRI/RII complex (Wang et al. 1991). It is thus considered an accessory molecule in TGFβ signalling.

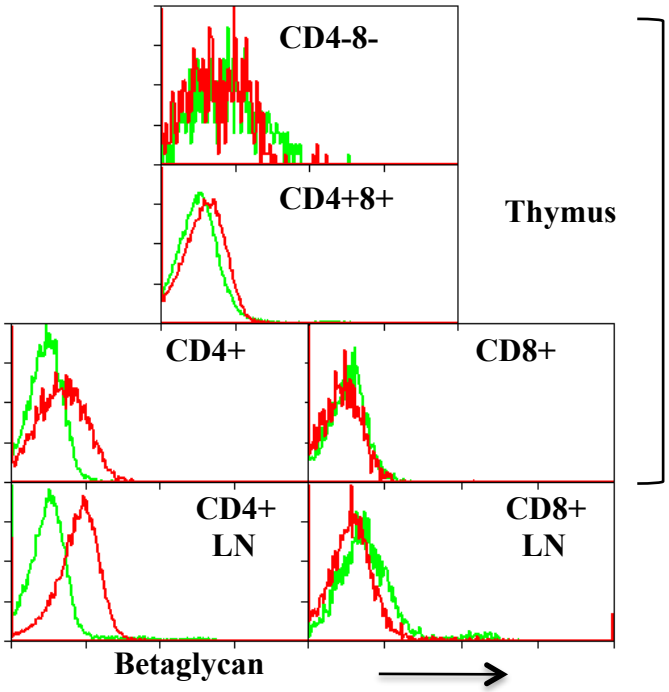


Figure 1.6 Betaglycan expression on T lymphocytes. Murine thymocytes and lymph node (LN) cells were stained with antibodies for CD4, CD8 and betaglycan (red line) or isotype control (green line). Highest expression was observed on CD4+ T lymphocytes.

Betaglycan is thought to have a dual role in regulation of TGFβ signalling: In the membrane bound form, betaglycan is thought to potentiate binding of ligand to the receptor complex, by binding TGFβ and presenting it to TGFβRII as an early regulatory step in initiation of the signalling cascade (Akhurst et al. 1990; López-Casillas et al. 1993; López-Casillas et al. 1994; Wang et al. 1991). Conversely, soluble betaglycan generated through proteolytic cleavage at the cell membrane is thought to have an opposite effect, by sequestering TGFβ away from the cell surface receptor complex (Bandyopadhyay et al. 1999; López-Casillas et al. 1994; Sun & Chen 1997). It therefore appears that betaglycan may have a dual role in regulation of TGFβ binding to the receptor complex.

The effect of betaglycan on TGFβ-TGFβRI/II affinity and subsequent TGFβ potency is complicated by the wide variety of *in vitro* assays reported, and differences in cell lines on which results are based:

Competition assays using tracer radioligands have previously shown that whilst all TGFβ isoforms have a similar affinity for betaglycan, TGFβ2 is of significantly lower affinity for TGFβRI and TGFβRII than TGFβ1 or TGFβ3. Furthermore, in cell lines lacking betaglycan, TGFβ2 has reduced potency in growth inhibition assays. The foetal bovine heart endothelial (FBHE) cell line is naturally betaglycan deficient; TGFβ2 is at least 50 times less potent as a growth inhibitor of FBHE cells than TGFβ1 or TGFβ3 (Cheifetz et al. 1990).

It was subsequently shown that TGFβRII is of intrinsically low affinity for TGFβ; rat L6E9 skeletal muscle myoblast cell lines express TGFβRI and TGFβRII, but lack betaglycan. Lopez-Casillas et al transfected these cells with betaglycan in order to characterise its effect

on TGFβ affinity and cell responsiveness (López-Casillas et al. 1993). In the absence of betaglycan up to 90% of TGFβ1 bound to TGFβRII with low affinity, and TGFβ2 binding was almost undetectable. Transfection with betaglycan resulted in a 50-fold increase in affinity for both TGFβ isoforms. This increased affinity for TGFβRII resulted in a similar increase in TGFβ2 binding to TGFβRI, whilst there was only a limited increase in TGFβ1 binding to TGFβRI. These observations correlated with the growth inhibitory response of both isoforms, with a 10-fold reduction in TGFβ2 potency observed in L6E9 cells when compared to assays using TGFβ1, and a corresponding 10-fold increase in TGFβ2 potency following transfection with betaglycan.

Sankar et al later similarly transfected bovine aortic endothelial cells (BAEC - another betaglycan-deficient cell line) with betaglycan, demonstrating increased potency of TGFβ2 assessed by a number of criteria including inhibition of migration and proliferation (Sankar et al. 1995).

These experiments suggest that betaglycan promotes presentation of all isoforms of TGFβ to TGFβRII, increasing affinity of both TGFβ1 and TGFβ2 for the receptor complex. Whilst this has a limited effect on TGFβ1 signalling, it allows a 10-fold increase in the potency of TGFβ2 signalling.

To examine the function of betaglycan *in vivo*, several groups have developed betaglycan knock-out (TGFβRIII-/-) mice. Similar to other TGFβ receptor knock-out models, these mice display embryonic lethality (discussed later), however they permit investigation of embryonic-derived cell populations. Stenvers el al derived fibroblasts from mutant and wild-

type embryos, and cultured them in the presence of either TGFβ1 or TGFβ2. Cellular proliferation was measured by thymidine incorporation. Whilst both isoforms resulted in dose-dependent inhibition of proliferation, significantly higher doses of TGFβ2 were needed to induce inhibition in cells derived from betaglycan deficient embryos compared to TGFβ1, with calculations of IC_{50} suggesting that TGFβ2 is 10-fold less potent than TGFβ1 in mutant than wild type cell lines (Stenvers et al. 2003). Interestingly, no significant differences in Smad 2 phosphorylation were observed when comparing the response to TGFβ1 or TGFβ2 between mutant and wild type cells. TGFβ3-dependent responses were not investigated. Betaglycan has thus emerged as an important co-factor in TGFβ signalling, with particular significance for the TGFβ2 isoform (Figure 1.7).

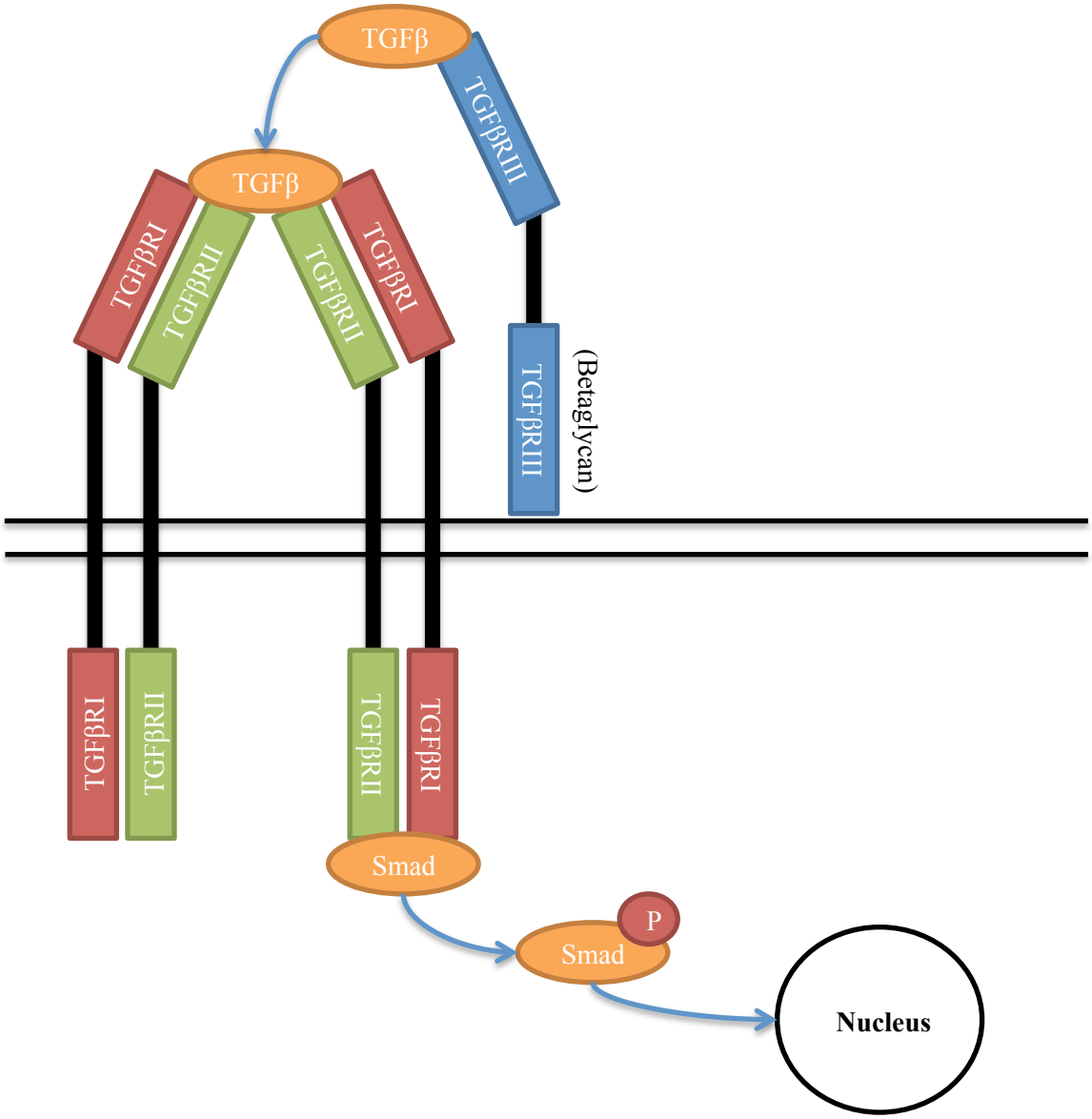


Figure 1.7 Schematic representation of TGFβ receptor and signalling cascade illustrating role of membrane-bound betaglycan. Membrane-bound betaglycan is a non-signalling component of the TGFβ receptor which is thought to present TGFβ to the signalling components. It is also able to exist in a soluble form through cleavage close to the cell membrane; in this form it is believed to sequester TGFβ away from the signalling complex.

1.4.3 Role of betaglycan in embryogenesis

Early observations of betaglycan showed higher expression in foetal than adult tissues, suggesting an important role in embryogenesis (Andres et al. 1989). This was confirmed in studies of betaglycan knock-out (TGFβRIII-/-) mice, which displayed embryonic-lethal cardiac and hepatic defects arising around mid-gestation, which were not observed in heterozygote (TGFβRIII+/-) or wild-type (TGFβRIII+/+) mice (Stenvers et al. 2003). Embryos generated through timed pregnancies between heterozygotes were obtained by caesarean delivery at time-points between E12.5 and E18.5. The resulting embryos followed Mendelian inheritance patterns, with a betaglycan knock-out genotype in 25%. In these knock-out embryos, development was largely unaffected at time points up to E15.5 with less than 10% dying. This death rate increased to over 55% by E18.5, with any remaining knock-outs showing significant anatomical abnormalities incompatible with life. Knock-out embryos were observed to die due to a combination of severe haemorrhagic liver destruction, disrupted erythropoiesis, and defects in the walls of cardiac ventricles.

It has since been shown that the cytoplasmic domain of betaglycan is necessary for TGFβ2-induced transformation of epithelial cells to endocardial cells in heart valve development (Townsend et al. 2012), and betaglycan is also necessary for development of normal coronary vasculature (Compton et al. 2007).

As well as these defects in cardiac development, betaglycan has been linked to other aspects of embryogenesis. Betaglycan deficient mice have been shown to display abnormal kidney development, with knock-out embryos demonstrating evidence of renal hypoplasia at E13.5,

and heterozygote adults exhibiting reduced nephron number when compared to wild-type adults (Walker et al. 2011). Knock-out mice also show impaired seminiferous cord and leydig cell development in the mouse testis with abnormalities of embryogenesis occurring between E11.5 and E14.5 (Sarraj et al. 2013).

1.4.4 Endocrine signalling

Betaglycan also has a role in the activin / inhibin pathway through the ability to bind inhibin, and thereby antagonise activin signalling (Lewis et al. 2000; Looyenga et al. 2010); it is thus important in regulation of gonadal development and functioning at the level of the pituitary. Loss of the inhibin-binding site on betaglycan has been shown to inhibit inhibin-mediated antagonism of activin-induced follicle stimulating hormone (FSH) and gonadotrophin-releasing hormone (GRH) production in animal studies (Wiater et al. 2006; Escalona et al. 2009), which has been linked to a wide range of reproductive disorders in both animal and human investigations (Stenvers et al. 2003; Stenvers & Findlay 2010; Zhu et al. 2010).

In addition to these effects on the reproductive axis at the level of the pituitary, betaglycan and inhibin are thought to have direct effects on somatic cell populations at the gonads (Stenvers & Findlay 2010). Inhibin has been shown to antagonise BMP-mediated down-regulation of luteinising hormone (LH)-induced steroidogenesis on bovine thecal cells (Glister et al. 2010), and loss of betaglycan expression on follicular cells has been linked to several reproductive diseases in humans (Bilandzic et al. 2009; Zhu et al. 2010).

Activins and inhibins are also produced at the adrenal cortex, and perhaps unsurprisingly there is high expression of betaglycan at this site (Vänttinen et al. 2003). Again, reduced betaglycan expression is thought to disrupt inhibin-mediated antagonism of activin signalling, resulting in significant derangements of steroidogenesis and apoptosis. This has been implicated in many diseases of the renal cortex, most notably cancer (Looyenga et al. 2010).

1.4.5 Carcinogenesis

Most of the research on betaglycan function to date has been in the context of carcinogenesis. Betaglycan deletions have been associated with haematological (Lambert et al. 2011), colonic (Gatza, Holtzhausen, et al. 2011), ovarian (Bilandzic et al. 2009), pancreatic (Gordon et al. 2008), prostate (Sharifi et al. 2007), renal (Bilandzic et al. 2013; Cooper et al. 2010), adrenal (Vänttinen et al. 2003), lung (Finger et al. 2008), and breast cancers (Pal et al. 2012; Lee et al. 2010).

TGFβ has a paradoxical role in carcinogenesis; early in disease it is generally considered a tumour suppressor through the ability to inhibit cell growth and promote apoptosis or differentiation, yet it is thought to have tumour-promoting effects later in the disease process by inhibiting the tumour-eradicating effects of the local immune system (Massagué 2008). Betaglycan deletions are thought to display a similar dichotomy, being tumour-promoting if occurring early in the course of disease, but tumour-suppressive if occurring later in the disease (Gatza, Oh, et al. 2011).

This has recently been demonstrated using the example of bladder urothelial cancer; samples either of fresh tumour or metastatic paracarcinoma tissue specimens were retrieved from 56 patients and betaglycan expression determined by western blotting (Liu et al. 2013). Expression was reduced compared to corresponding normal tissue in 18/30 fresh tumour specimens, and increased in 19/26 metastatic samples. These findings correlated with analysis of cell lines; the superficial urothelial bladder cancer cell line 5637 displayed decreased betaglycan expression, whereas the invasive cancer line T24 displayed increased expression when compared with the SV-HUC-1 normal urothelial cell line. Furthermore, knock-down of betaglycan gene expression in the T24 cell line resulted in reduced cell growth, motility and invasion. The authors conclude that loss of betaglycan correlates with initial tumour development, whereas upregulation of betaglycan enhances metastasis.

It should be noted that this observation applies to the membrane-bound form of betaglycan. As previously discussed, soluble betaglycan is believed to have an opposing effect (Velasco-Loyden et al. 2004; López-Casillas et al. 1994), and thus it is considered beneficial to down-regulate soluble betaglycan early in disease, however the ability to sequester TGFβ away from metastatic cells may have an anti-cancer effect in advanced disease. Through this mechanism soluble betaglycan has been identified as a potential therapeutic agent in metastatic cancer, with inhibitory effects on tumour progression demonstrated in breast cancer cell lines (Bandyopadhyay et al. 1999):

The human breast cancer cell line MDA-MB-231 was cultured with and without expression of a truncated soluble extracellular domain of betaglycan (sTGFβRIII). Soluble betaglycan was able to antagonise the tumour promoting activity of TGFβ by sequestering that produced by

proliferating cancer cells, as demonstrated by a reduction in the amount of active TGFβ1 and TGFβ2 in the growth media. Mink lung epithelial CCL64 cells cultured in the resulting media showed reduced growth inhibition in the presence of soluble betaglycan compared to control. To assess the effect of soluble betaglycan *in vivo*, athymic nude mice were inoculated with either control MDA-MB-231 cells, or cells with induced expression of sTGFβRIII. Four of five mice receiving control cells developed lung metastases. None of the ten mice receiving cells expressing sTGFβRIII were observed to develop metastatic disease.

1.4.6 Immune regulation

Despite this wealth of data highlighting the importance of betaglycan as a co-factor in TGFβ signalling, it is surprising that no literature exists describing the role of betaglycan in the context of TGFβ function in immune regulation in the mature immune system.

1.4.6.1 Genetic polymorphism in inflammatory disease

Studies of single nucleotide polymorphisms (SNP) in the betaglycan gene provide indirect evidence of a role in immune regulation. SNP have been identified in a number of diseases with an inflammatory or infective aetiology, including Crohn's disease (The Wellcome Trust Case Control Consortium 2007), chronic obstructive pulmonary disease (COPD) (Hersh et al. 2009) and Behcet's disease (BD) (Chen et al. 2012). SNP have also been implicated in the clearance of hepatitis B virus (HBV) and the subsequent development of hepatocellular carcinoma (HCC) (Kim et al. 2011).

The association of betaglycan SNP with BD appears particularly significant; BD may be considered at least partially TGFβ dependent, as Treg and Th17 cells have been implicated in its pathogenesis (Na et al. 2013). Furthermore, BD is often characterised by frequent episodes of severe uveitis (Wechsler et al. 1990), which as previously discussed appears highly dependent on TGFβ2 signalling.

A particular SNP (RS1805110) in the betaglycan gene (TGFBR3) has previously been identified in Han Chinese patients with BD, with the CC genotype suggested to confer a protective effect against developing disease (Chen et al. 2012). This SNP is a non-synonymous mutation located in the signal sequence that may affect secretion or expression of betaglycan, although this has not been directly addressed. No association was observed with a disease control group of Voygt Kayanagi Harada (VKH) disease – another common cause of uveitis in the Han Chinese population.

1.4.6.2 Betaglycan in thymocyte development

One recent paper linked betaglycan to survival of thymocytes in foetal thymic organ culture (FTOC) from betaglycan-deficient mouse embryos (Aleman-Muench et al. 2012):

In this study, embryos were harvested from pregnant females following timed mating of betaglycan heterozygote mice at time-points E14 onwards and grown under culture conditions. Embryos were genotyped by polymerase chain reaction (PCR) to allow comparison between betaglycan-null and wild-type embryos. Real-time PCR was utilised to quantify betaglycan expression in different populations of developing T lymphocytes (double

negative CD4-CD8-, double positive CD4+CD8+, single positive CD4+ and CD8+) at a range of gestational time-points; FTOC were set up with and without betaglycan blocking antibodies to examine T lymphocyte survival and apoptosis.

Betaglycan was found to be expressed in the thymus throughout development, and was most highly expressed on CD4+ T lymphocytes and in the thymic stroma. Expression increased in the early stages of T lymphocyte maturation, suggesting a possible role for betaglycan in the pre-TCR phase of development.

Wild-type FTOC blocking assays using antisera against the extracellular domain of betaglycan resulted in a reduced proportion of double positive thymocytes and a corresponding increase in double negative subsets by day 7 of culture. A more pronounced reduction in double positive thymocyte proportions was observed in betaglycan-null E14 FTOC compared to wild-type controls, with a statistically significant difference in cellularity observed under conditions of absolute betaglycan deficiency. The difference between cell numbers under conditions of betaglycan blockade and absolute deficiency suggests that the antisera against betaglycan resulted in incomplete blockade, perhaps due to an inability to block soluble betaglycan.

Finally, thymocyte apoptosis was measured by expression of active caspase 3 in FTOC of either wild-type or betaglycan-null E14 embryos. Caspase 3 was increased in both double positive and double negative subsets in betaglycan-null cultures, however this only achieved statistical significance for the more mature double positive population.

The authors conclude that betaglycan is crucial to T lymphocyte development, regulating the transition from more immature double negative to relatively mature double positive cells by protecting double positive cells from apoptosis.

Whilst this paper once again adds to the body of evidence supporting the role of betaglycan in embryological development, and provides an interesting link to T lymphocyte biology, the effect of betaglycan in peripheral T lymphocytes remains unknown.

1.4.7 Clinical significance

As previously discussed, uveitis is thought to result from aberrant trafficking and activation of CD4+ T lymphocytes in the intra-ocular microenvironment (Zhou et al. 2011; Zhou et al. 2012; Gasparin et al. 2012). TGFβ2 is present at high levels in the aqueous humour of un-inflamed eyes (Curnow et al. 2005; Denniston et al. 2011), and thus the inflammation observed in episodes of uveitis is thought to result from disruption of TGFβ2-mediated immunoregulation of intra-ocular T lymphocytes. Uveitis is therefore of particular clinical significance when considering the role of betaglycan in TGFβ-mediated T lymphocyte responses.

The literature review presented throughout sections 1.4.7.1 to 1.4.7.5 has previously been published in a peer-reviewed scientific journal (Barry et al. 2014), comprising the introduction to the paper. See section 8.1 for the full citation and a reproduction of the published version.

1.4.7.1 Introduction to uveitis

Uveitis, a significant cause of blindness worldwide, is a term applied to a wide range of conditions that are characterized by intraocular inflammation. Many cases of 'uveitis' do indeed involve inflammation of the uvea (which comprises the iris, ciliary body and choroid), but may also involve adjacent structures such as the retina or vitreous. Uveitis is highly heterogeneous, varying in aetiology, pattern, tissue involved and extent. The uveitis specialist may be confronted by a small, localized area of inflammation in a single tissue in a non-sight-threatening location, or widespread blinding inflammation involving almost all ocular tissues. Visual impairment is common, affecting between 2.8 and 10% of patients (Darrell et al. 1962; Suttorp-Schulten & Rothova 1996; Goldstein 1980), and may result directly from damage to uveal tract structures, or may occur due to secondary effects on neighbouring tissues, for example accelerated cataract formation, glaucoma, and macular oedema (Forrester 1991).

The Standardization of Uveitis Nomenclature (SUN) working group classifies uveitis according to the site of primary inflammation (Jabbs et al. 2005). Anterior chamber inflammation is categorized as 'anterior uveitis', and includes iritis, iridocyclitis and anterior cyclitis. Inflammation primarily affecting the vitreous is referred to as 'intermediate uveitis', and includes pars planitis, posterior cyclitis and hyalitis. 'Posterior uveitis' describes inflammation of the retina or choroid. Finally, 'pan-uveitis' describes the situation where inflammation is seen throughout the anterior chamber, vitreous and retina or choroid. According to the SUN criteria, disease is further classified according to onset (sudden or insidious), duration (limited or persistent), and course (acute, recurrent or chronic).

1.4.7.2 Pathophysiology of uveitis

Uveitis can be either infectious or non-infectious; whilst both may present with similar clinical features, they are best considered as distinct disease entities since the underlying pathophysiology and treatment strategies are very different.

Whilst common in the developing world, infective causes account for the minority of uveitis cases presenting to tertiary referral centres in the West. Infectious causes include organisms such as toxoplasma, cytomegalovirus, syphilis and herpes viruses (Miserocchi et al. 2013; Cimino et al. 2010). Local infection results in foreign antigen presentation to ocular immune cells, with appropriate immune activation aimed at clearing the invading organism. Uveitis occurs as a secondary effect of this immune activation.

Non-infective uveitis is thought to result from inappropriate activation of the immune system (Curnow et al. 2004) and it is therefore not surprising that it is often associated with systemic autoimmune or auto-inflammatory diseases such as ankylosing spondylitis (AS), sarcoidosis or BD. In the remainder, however, no such systemic association is identified; these cases are generally labelled as 'idiopathic' in recognition of the fact that the autoimmune/autoinflammatory origin of most of these cases is presumed rather than proven. Human data and experimental models indicate parallel changes in the inflammatory milieu of the intra-ocular microenvironment. Uveitis may be induced in animal models by a range of mechanisms which cause differentiation of naïve $CD4^+$ T-cells to pathogenic effector cells, resulting in tissue damage (Denniston et al. 2011; Takeuchi et al. 1998; Takeuchi et al. 1997; Taylor et al. 2000; Taylor et al. 1992). Although similar pathogenic effector cells have been

recovered from ocular fluids and tissue in human uveitis, the evidence for autoreactive T-cells (such as seen in the animal models) is much more limited (Gocho et al. 2001; de Smet et al. 2001).

Such idiopathic cases account for the largest cohort of patients seen in most clinical practices in the West. In a retrospective study of all uveitis cases presenting to a tertiary centre, Rodriguez reported 34% to be idiopathic, 10.4% to be associated with seronegative spondyloarthropathies and 9.6% to be associated with sarcoidosis (Rodriguez et al. 1996), although it should be noted that certain conditions are typically associated with particular anatomical groups such as anterior uveitis with seronegative spondyloarthropathies. It is also of interest to note that in a report from the SITE retrospective study of US tertiary uveitis services, the leading systemic associations in 4911 patients with uveitis were sarcoidosis (7%), seronegative spondyloarthropathy (5%), juvenile idiopathic arthritis (JIA, 5%) and BD (3%) (Zaidi et al. 2010).

1.4.7.3 Overview of treatment strategies

For infective causes, treatment is aimed at eradicating the pathogenic organism with appropriately targeted anti-microbial therapy. In severe cases, such agents may be delivered directly to the eye by intravitreal injection, or are more frequently administered systemically by an oral or intravenous route. Once the infective agent is considered to be under control, immunosuppressive agents such as corticosteroids may be used judiciously to limit tissue damage.

For non-infective causes, treatment involves suppression of the local immune response. It is useful to consider the concept of disease activity versus damage when treating inflammatory disease (Barry et al. 2008; Skopouli et al. 2000). 'Activity' refers to the on-going immune response, which may be acute or chronic, but is usually reversible. 'Damage' refers to the effect of active inflammation on native tissues; it is usually irreversible. In simple terms, persistent activity will lead to accumulation of damage. In theory, effective therapy should suppress all activity and prevent or halt accumulation of damage.

Therapy in non-infectious uveitis is aimed at suppressing the immune system, and ranges from topical therapy (commonly corticosteroid eye drops) to systemic immunosuppression with either high-dose corticosteroids (oral, intravenous, intramuscular or subcutaneous) or a wide range of corticosteroid-sparing immunomodulatory therapeutic (IMT) agents (Tempest-Roe et al. 2013; Durrani et al. 2011). Ideally, treatment should be targeted to the mechanism and localized to the tissue to maximize the efficacy/side-effect profile. However, this is often not achieved and new therapies should therefore aim to suppress disease activity, prevent accumulation of damage, and preserve visual function for patients with the minimum possible adverse events (Denniston & Dick 2013; Gallego-Pinazo et al. 2013; Larson et al. 2012).

1.4.7.4 Epidemiology of uveitis

Uveitis is considered a rare disease (Barisani-Asenbauer et al. 2012), with an estimated incidence between 17 and 52 people per 100,000 population in Europe and the USA (Cimino et al. 2010; Gritz & Wong 2004; Suhler et al. 2008; Darrell et al. 1962), although a higher incidence of disease may be observed in Chinese and Japanese populations (Hwang et al.

2012). Despite this rarity, it is a disproportionately common cause of legally-recognized visual impairment, and is the fourth most common cause of blindness in the working age population in the developed world (Read et al. 2001; Bodaghi et al. 2001; Suttorp-Schulten & Rothova 1996). Uveitis can occur in any age group, however it is particularly prevalent in younger people, with a mean age at onset of less than 40 years (Bodaghi et al. 2001).

Epidemiological studies in uveitis are particularly prone to bias. Most notably, a range of criteria exist for diagnosis and categorization of different uveitis entities; the relatively recent introduction of SUN working group anatomical classification has helped standardize practice, however clinical, etiological and pathological classification criteria have been used variably throughout the literature, and it is often difficult to compare published data from multiple sources (Bloch-Michel & Nussenblatt 1987; Holland 1994; Engstrom et al. 1994; Read et al. 2001; Mandeville et al.). In addition, since the majority of research in uveitis is generated from tertiary referral centres with relatively little data from community-based practice, there is bias towards severe disease and a relative under-representation of more straightforward cases (McCannel et al. 1996). Furthermore, given the heterogeneity of uveitis entities and the wide geographic variation in both clinical features and disease aetiology, comparison between different regions is difficult (Miserocchi et al. 2013).

Disease aetiology shows significant variation with age, with some forms of uveitis affecting specific groups. For example, in European populations, uveitis due to JIA occurs almost exclusively in children (Heiligenhaus et al. 2013; Smith et al. 2009), HLA-B27 positive disease is seen in young adults (Rothova et al. 1987), and masquerade syndromes and lymphoma are more commonly seen in the elderly (Barisani-Asenbauer et al. 2012).

Infectious uveitis is relatively rare in developed countries, accounting for 13-21% of cases, and is thought to be mostly due to infection by herpes viruses (Miserocchi et al. 2013; Barisani-Asenbauer et al. 2012). This is in stark contrast with developing countries, where up to 50% of uveitis is thought to have an infectious aetiology. The most common infectious causes in these populations are toxoplasmosis, tuberculosis (TB), onchocerciasis, cysticerosis, leprosy and leptospirosis (London et al. 2010).

In Western countries, anterior uveitis accounts for at least half of all cases (Wakefiled & Chang 2005), of which idiopathic disease accounts for approximately 50% (Suhler et al. 2008). The most common clinical associations in those countries are HLA-B27 positive disease, AS and Fuchs heterochromic iridocyclitis (FHC) (Miserocchi et al. 2013). It is estimated that up to 55% of Caucasian patients with acute anterior uveitis are HLA-B27 positive, compared to only 5-10% in the general population (Brewerton et al. 1973). In contrast, the prevalence of anterior uveitis is much lower in Asian populations, which is thought to be due to the lower frequency of HLA-B27 positivity and AS (Chang & Wakefield 2002).

Intermediate uveitis is the least common form of disease across all geographic regions, with an estimated incidence of 1.5-2.08 per 100,000 population in Western populations (Miserocchi et al. 2013). There is thought to be a strong association with Human T-cell Lymphotropic Virus Type 1 (HTLV-1) (Ohguro et al. 2012), and several authors have noted an association between pars planitis and MS, although this has yet to be fully characterized (Zein et al. 2004; Zierhut & Foster 1992).

Posterior uveitis accounts for 15-30% of diagnoses. The most common cause worldwide is toxoplasmosis, followed by idiopathic disease (Miserocchi et al. 2013; Wakefiled & Chang 2005; Chang & Wakefield 2002). It follows that posterior uveitis is more common in developing countries, owing to the higher prevalence of infectious diseases in these populations.(London et al. 2010) BD and VKH are two rare but important non-infectious causes of posterior uveitis (Yang et al. 2005; Rathinam & Namperumalsamy). Cytomegalovirus (CMV) retinitis is associated with HIV infection, but its incidence is decreasing with the use of modern Highly Active Anti-Retroviral Therapy (HAART) (Miserocchi et al. 2013).

It is difficult to accurately estimate the prevalence of panuveitis; in one recent large-scale review of epidemiological studies, the prevalence varied from 1% to 69% depending on geographic location (Miserocchi et al. 2013). It is probable that panuveitis is more heavily affected by the biases described above than other types of uveitis, particularly referral bias skewing patient populations towards more severe disease in tertiary referral centres, and as a result, these figures are unlikely to be representative of the general population. Idiopathic disease is most common in Europe, USA, Australia and India, whilst infective causes are again more common in most developing countries, and BD is an important cause in Asia and countries along the historic ‘silk route’ (Middle East and Mediterranean regions) (Bodaghi et al. 2001; Chung et al. 1988; Kotake et al. 1996; Wakabayashi et al. 2003; Tugal-Tutkun 2010).

1.4.7.5 Current treatment options

Any treatment strategy for uveitis needs to consider a number of factors (Jabs & Busingye 2013). First: the aetiology (i.e. infectious vs. non-infectious) which will define the type of treatment required (antimicrobial vs. anti-inflammatory/immunosuppressive). Second: extent of uveitis and associated inflammation. This includes (a) anatomical location within the eye (as per SUN classification) (Jabbs et al. 2005), (b) unilaterality or bilaterality and (c) presence of systemic disease. These factors are important considerations when deciding whether topical, local or systemic treatments are likely to be required. Third: severity of disease which may necessitate 'rescue' therapy or additional treatment. Fourth: potential complications of either the disease itself or of treatments of the disease.

1.4.7.5.1 Infectious vs. Non-infectious Uveitis

One of the most important (and sometimes difficult) challenges to confront the uveitis specialist is whether the inflammatory process is the result of an infectious agent. Establishing or excluding this may sometimes be possible on clinical appearance alone (Mandelcorn 2013), but is often supplemented by investigations on the peripheral blood (commonly serology, interferon gamma release assays for *Mycobacterium tuberculosis*, haematological and biochemical markers) or ocular fluids (PCR for suspected microbes; less commonly Goldmann-Witmer coefficient); imaging is also commonly used to characterize associated systemic disease (Guly & Forrester 2010; De Groot-Mijnes et al. 2006).

In some cases treatment may need to be started before the results of such investigations are available. For example, in the presence of suspected acute retinal necrosis (ARN), samples of aqueous humour and vitreous humour should be taken and sent for PCR concurrently with starting treatment (commonly intravitreal foscarnet and either oral valacyclovir or intravenous acyclovir) (Wong et al. 2013). This case also highlights another issue: that some cases of infectious uveitis may provoke a strong inflammatory reaction both at the time and sometimes beyond the infectious component of disease; this may in turn require cautious immunosuppression to limit tissue damage and maximize visual potential following treatment.

1.4.7.5.2 Extent and severity of uveitis and associated inflammation

In general, isolated uncomplicated anterior uveitis (whether unilateral or bilateral) can often be managed by topical therapy alone; frequent topical corticosteroids are used for rescue treatment, and then titrated down to complete cessation (in acute disease) or to a low frequency maintenance regimen (chronic disease or frequent recurrences); a mydriatic is commonly prescribed to reduce the risk of posterior synechiae (Guly & Forrester 2010).

In cases of posterior segment uveitis (intermediate, posterior or panuveitis), the topical route provides inadequate penetration to the inflamed tissue, although topical therapies may still have an adjunctive role. For these cases the choice of treatment will depend on whether the disease is unilateral or bilateral, and whether it is isolated or is a manifestation of a systemic inflammatory process. Unilateral and increasingly bilateral disease may be treated by local therapies (peribulbar, sub-Tenon's or intravitreal routes); corticosteroids are the most common drugs given by these routes although the role of other agents is also being assessed.

Local therapy may be sufficient to control the disease, but in more severe inflammation, or in the presence of systemic disease, systemic therapy is likely to be necessary. In addition, there may be contraindications to the use of local therapies in some cases (Jabs et al. 2000).

Recognition and treatment of any associated systemic disease is a priority. Therapy initiated for co-existent systemic inflammatory disease may ameliorate any active uveitis, reducing the need for direct ophthalmic intervention. Once again, rescue therapy is traditionally with corticosteroids (either intravenous or oral) with maintenance therapy comprising either lower dose of corticosteroid or a steroid-sparing IMT agent (Jabs et al. 2000). The range of steroid-sparing IMT agents available (including biologics) is steadily increasing, although the evidence of their efficacy and safety in uveitis is often lacking (Denniston & Dick 2013). Current options include anti-metabolites (such as methotrexate and mycophenolate mofetil), T-cell inhibitors (such as cyclosporin), and alkylating agents (such as cyclophosphamide). More recently, biological therapies (such as infliximab) have been utilized in the management of severe and refractory uveitis (Durrani et al. 2011; Gallego-Pinazo et al. 2013; Pato et al. 2011; Cordero-Coma et al. 2013; Saadoun et al. 2013).

1.4.7.6 Betaglycan as a potential therapeutic target in uveitis

Episodes of severe uveitis, in which sight-threatening inflammation is localised to the eye, often necessitate systemic treatment resulting in a state of immunosuppression throughout all organ systems. This has the potential to cause significant additional morbidity and mortality to patients through the occurrence of severe side effects. It is thus desirable to develop

treatment strategies in which a therapeutic immunosuppressive effect can be localised to the intra-ocular microenvironment.

As previously discussed, TGFβ2 is considered an important immunoregulatory molecule in the eye, being thought to regulate the activity of local T lymphocytes and determine the balance between immunosuppressive iTreg and pathogenic Th17-driven processes. The predominance of TGFβ2 is unusual, with TGFβ1 being the most widely expressed isoform in the human immune system. The ability to specifically modulate TGFβ2-driven immune responses thus confers the potential to influence pathways of intra-ocular immune regulation, with little effect in other organ systems. Since betaglycan is strongly implicated in signal transduction by TGFβ2 but appears redundant in signal transduction by TGFβ1 and TGFβ3, it is possible that modulation of betaglycan activity may allow manipulation of only TGFβ2-dependent processes, and thus localise an immunosuppressive effect to the eye.

Such modulation of betaglycan-dependent responses has already been exploited in control of metastatic disease processes through the systemic administration of soluble betaglycan (Section 1.4.5), and it is possible that similar modulation may be useful in controlling inflammatory diseases such as uveitis.

1.5 Hypotheses

Betaglycan is necessary for TGFβ2 signalling to lymphocytes via the cell-surface TGFβ receptor and is therefore an absolute requirement for TGFβ2-mediated immune responses; TGFβ2-mediated T lymphocyte immune responses will be completely abrogated in the absence of betaglycan.

Betaglycan increases the affinity of both TGFβ1 and TGFβ3 for the cell-surface TGFβ receptor on lymphocytes, but is not an absolute requirement for TGFβ1 or TGFβ3-mediated immune responses; TGFβ1 and TGFβ3-mediated T lymphocyte immune responses will be reduced in the absence of betaglycan.

No spontaneous immune dysregulation will be observed *in vivo* under conditions of betaglycan deficiency in environments where TGFβ1 or TGFβ3 signalling predominates, however disordered immune responses will be observed under conditions of antigenic challenge. Since TGFβ is primarily involved in immunosuppressive responses, this will be observed by increases in T lymphocyte activation levels, with alteration in the relative proportions of Th17 and Treg cells involved in immune responses.

1.6 Objectives

In this thesis, I set out to achieve the following objectives:

1. To develop a novel experimental animal model of betaglycan deficiency in which to study peripheral immune responses *in vivo*, and mature immune cells *in vitro*, focusing on T and B lymphocyte responses.

2. To characterise these animals, observing for evidence of spontaneous immune dysregulation or autoimmune disease. Investigation will focus on peripheral T and B lymphocyte populations, with assessment of both circulating (blood) populations, and those resident in secondary lymphoid organs. Structure of secondary lymphoid organs will also be assessed.

3. To assess for evidence of immune dysregulation in response to controlled, traceable antigenic challenge. Given the role of betaglycan in the TGFβ receptor complex, particular attention will be paid to TGFβ-dependent Treg and Th17 responses, determined by levels of FoxP3 expression and IL-17 secretion respectively.

4. To investigate TGFβ signalling to T lymphocytes in the presence or absence of betaglycan through *in vitro* iTreg polarization assays. Naïve CD4+ T lymphocytes will be exposed to all three isoforms of TGFβ, and FoxP3 induction compared in conditions of betaglycan sufficiency and deficiency.

5. Whilst an association has been observed between betaglycan SNP and the development of BD in a Han Chinese population, no equivalent analysis exists for Caucasian patients. This will be addressed through association analysis of betaglycan SNP RS1805110 with BD in a population of Caucasian patients, with comparison to other forms of ocular inflammatory disease and healthy controls.

2 MATERIALS AND METHODS

2.1 Materials

See tables 2.1 to 2.4 for a comprehensive list of all media, reagents, cytokines and antibodies used for experimental procedures described in the following methods (Section 2.2).

Description	Details	Source
Culture media	RPMI (Roswell Park Memorial Institute) 1640	Sigma Aldrich, Dorset, UK
	1% GPS (2mM l-glutamine, 100 U/ml penicillin, 100ug/ml streptomycin)	HyClone, Northumberland, UK
	1% HEPES (1M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)	Sigma Aldrich, Dorset, UK
	10% HIFCS (Heat Inactivated Foetal Calf Serum)	Biosera, Ringmer, UK
0.5% Flow Cytometry Buffer	PBS (see below)	Oxoid, Basingstoke, UK
	0.5% BSA (Bovine Serum Albumin)	Sigma Aldrich, Dorset, UK
2.0% Flow Cytometry Buffer	PBS (see below)	Oxoid, Basingstoke, UK
	2% BSA (Bovine Serum Albumin)	Sigma Aldrich, Dorset, UK

Table 2.1 Commonly used media. (1 of 2).

Description	Details	Source
Gey's Solution	(1) 20ml Solution A; in 1.0l dH2O NH_4Cl 35.0g, KCl 1.85g, $Na_2HPO_4.12$ H_2O 0.119g, Glucose 5g, Gelatin 25g, 1% Phenol Red 1.5ml (2) 5ml Solution B; in 1.0l dH2O $MgCL_2.6H_20$ 4.2g, $MgSO_4.7H_2O$ 1.4g, $CaCl_2$ 3.4g (3) 5ml Solution C; in 1.0l dH2O $NaHCO_3$ 22.5g (4) 70ml dH20	Sigma Aldrich, Dorset, UK
MACS Buffer	PBS (See below) 0.5% BSA (Bovine Serum Albumin) 2mM EDTA (Ethylenediamine tetra-acetic acid)	Oxoid, Basingstoke, UK Sigma Aldrich, Dorset, UK Sigma Aldrich, Dorset, UK
Phosphate Buffered Saline (PBS)	8g/l NaCL, 0.26g/l KCl, 1.15g/l Na_2HPO_4, 0.2 g/ml KH_2PO_4 in dH2O Prepared as 1 PBS tablet per 100ml dH2O	Sigma Aldrich, Dorset, UK

Table 2.1 Commonly used media. (2 of 2).

Specificity	Isotype	Clone	Fluorochrome	Dilution		Source
				CyAn	Fortessa	
Surface stains						
B220	Rat IgG2a	RA3-6B2	FITC	-	1/300	eBioscience
			PE-Cy7	-	1/500	eBioscience
CD3	Rat IgG2b	17A2	APC-eFluor780	1/50	1/100	eBioscience
	Hamster IgG	145-2C11	PE-Cy7	1/50	1/100	eBioscience
CD4	Rat IgG2b	GK1.5	APC	-	1/500	eBioscience
	Rat IgG2a	RM4-5	PerCP-Cy5.5	1/200	-	eBioscience
CD8	Rat IgG2a	53-6.7	V500	1/100	1/200	BD Biosciences
CD11b	Rat IgG2b	M1/70	FITC	-	1/300	eBioscience
			PE	1/160	-	eBioscience
			PE-Cy7	-	1/500	eBioscience
CD11c	Hamster IgG	N418	eFluor450	1/50	-	eBioscience
			FITC	-	1/300	eBioscience
			PECy7	-	1/500	eBioscience
CD19	Rat IgG2a	eBio1D3	eFluor450	1/100	-	eBioscience
			PE	-	1/200	eBioscience
CD25	Rat IgG1	PC61.5	APC	1/160	-	eBioscience

Table 2.2 Antibodies used for flow cytometry analyses. Fluorochromes and dilutions shown for analysis on both Dako-CyAn flow cytometer (CyAn) and BD LSRFortessa (Fortessa) where appropriate. Source details: eBioscience, Hatfield UK; BD Biosciences, Oxford, UK; Biolegend, London, UK (1 of 2).

Specificity	Isotype	Clone	Fluorochrome	Dilution		Source
				CyAn	Fortessa	
Surface stains (continued)						
CD44	Rat IgG2b	IM7	APC-eFluor780	1/160	-	eBioscience
			BV785	-	1/200	Biolegend
			FITC	1/300	-	eBioscience
CD45.2	Mouse	104	FITC	1/200	-	eBioscience
	IgG2a		PerCP-Cy5.5	1/200	-	eBioscience
CD62L	Rat IgG2a	MEL-14	PE	1/400	-	eBioscience
GR1	Rat IgG2b	RB6-8C5	APC	-	1/160	eBioscience
NK1.1	Mouse	PK136	PE-Cy7	-	1/500	eBioscience
	IgG2a					
Transcription factor stains						
FoxP3	Rat IgG2a	FJK-16S	FITC	1/50	1/50	eBioscience
RORγ	Rat IgG2a	AFKJS-9	PE	-	1/50	eBioscience
Cytokine stains						
IFNγ	Rat IgG1	XMG1.2	eFluor450	1/50	-	eBioscience
			PE-Cy7	-	1/200	eBioscience
IL17	Rat IgG2a	eBio17B7	APC	1/100	-	eBioscience
			PE	-	1/100	eBioscience

Table 2.2 Antibodies used for flow cytometry analyses. Fluorochromes and dilutions shown for analysis on both Dako-CyAn flow cytometer (CyAn) and BD LSRFortessa (Fortessa) where appropriate. Source details: eBioscience, Hatfield UK; BD Biosciences, Oxford, UK; Biolegend, London, UK (2 of 2).

Specificity	Isotype	Clone	Fluorochrome	Dilution	Source
Primary stains					
B220	Rat IgG2a	RA3-6B2	Biotin	1/200	eBioscience
CD3	Hamster IgG	eBio500A2	FITC	1/200	eBioscience
CD4	Rat IgG2b	GK1.5	AF647	1/100	eBioscience
IgD	Rat IgG2a	11-26c	FITC	1/200	eBioscience
IgM	Donkey IgM	Polyclonal	Rhodamine Red	1/200	Stratech Scientific
PNA	Rat	-	Biotin	1/200	Vector Laboratories
Secondary / amplification stains					
FITC	Rabbit-anti-FITC	-	AF488	1/200	Invitrogen
Rabbit	Donkey-anti-rabbit	-	AF488	1/200	Invitrogen
Biotin	Streptavidin	-	AF555	1/500	Invitrogen
Biotin	Streptavidin	-	AF647	1/400	Invitrogen

Table 2.3 Antibodies and stains used for immunohistochemical analyses. Dilutions presented for analysis by Zeiss LSM 510 Meta Confocal microscope. Source details: eBioscience, Hatfield, UK; Stratech Scientific, Suffolk, UK; Vector Laboratories, Peterborough, UK; Invitrogen, Paisley, UK.

Description	Isotype	Clone / source	Concentration	Source
AccuCheck Counting Beads	-	-	-	ThermoFisher Scientific
Anti-CD16/CD32	Rat IgG2a	93	0.25µg/well	eBioscience
Anti-CD3	Rat IgG2b	17A2	5µg/ml	eBioscience
Anti-CD28	Hamster IgG	37.51	1µg/ml	eBioscience
Anti-CD3 / Anti-CD28 DynaBeads	Hamster IgG	-	Variable	Life Technologies
PMA	-	-	50ng/ml	Sigma-Aldrich
Ionomycin	-	-	500ng/ml	Sigma-Aldrich
Brefeldin A	-	-	3 µg/ml	Sigma-Aldrich
IL2	-	*E. Coli*	50U/ml	Immunotools
TGFβ1	-	Hek293	0-10ng/ml	Peprotech
TGFβ2	-	Hek293	0-10ng/ml	Peprotech
TGFβ3	-	*E. Coli*	0-10ng/ml	Peprotech
Reddymix PCR Master-mix	-	-	1.1x (2.0mM $MgCl_2$)	ThermoFisher Scientific
Qiagen DNEasy Blood & Tissue Kit	-	-	-	Qiagen

Table 2.4 Miscellaneous cytokines and reagents. Source details: eBioscience, Hatfield, UK; Immunotools, Friesoythe, Germany; Life Technologies, Paisley, UK; Peprotech, London, UK; Qiagen Limburg, Netherlands; Sigma-Aldrich, Dorset, UK; ThermoFisher Scientific, Massachusetts, USA.

2.2 Methods

All experiments involving animals were performed in accordance with UK Home Office regulations under project licences 40/3072 (Professor P.J.L. Lane) and 30/2961 (Dr D.W. Withers). All experiments were performed under the personal licence 30/9567 (Dr R.J. Barry).

2.2.1 Observation of mouse colonies

Animal colonies were housed in the Biomedical Support Unit of the University of Birmingham. All animals were observed daily and assessed for signs of illness according to UK Home Office criteria, including greater than 10% weight loss from baseline, display of stereotypical behaviour, development of starry coat or external evidence of systemic disease (Bleby 1986). Animals displaying signs of illness were culled by an approved schedule 1 procedure.

Survival curves were created for mouse colonies. ‘Survival’ was defined as animals displaying no evidence of illness according to the above criteria by the time of schedule 1 cull for use in planned experiments (at which point survival data was censored). ‘Death’ was defined as the cull of an animal due to evidence of illness according to the above criteria.

2.2.2 Genotyping of mouse colonies

Animal genotype was determined by PCR according to the following method.

2.2.2.1 DNA extraction

Ear clippings of mature mice, or tail samples of murine embryos were frozen at -20°C for later genotyping. DNA was extracted using the Qiagen DNeasy Blood & Tissue Kit (Qiagen Limburg, Netherlands) according to the protocol 'Purification of Total DNA from Animal Tissues (Spin-Column Protocol)'.

Frozen tissue was defrosted at room temperature, then incubated at 56°C in 180µl tissue lysis buffer (ATL) and 20µl proteinase K for three hours or until completely lysed, vortexing occasionally. 200µl lysis buffer (AL) and 200µl ethanol (100%) were added and the mixture pipetted into a DNeasy Mini Spin Column placed in a 2ml collection tube. This was centrifuged at 6,000g for one minute before the flow through and collection tube are discarded. The spin column was placed in a fresh 2ml collection tube, 500µl wash buffer (AW1) added, again centrifuged at 6,000g for one minute before discarding the flow through and collection tube. The spin column was placed in another 2ml collection tube and 500µl of wash buffer (AW2) added. This was centrifuged for three minutes at 16,000g before the flow through and collection tube were discarded. Finally, the spin column was placed in a 1.5ml Eppendorf, 200µl elution buffer (AE) added and incubated at room temperature for one minute before centrifuging at 6,000g for one minute. The elute of this final step contained DNA ready for PCR analysis. DNA samples were stored at 2-8°C until required.

2.2.2.2 PCR preparation

Forward and reverse primers (Invitrogen, Paisley, UK) were diluted 1:2 in sterile water from a stock solution at 100ρmol/µl. 2µl DNA was added to 0.5µl of each primer, 18µl pre-mixed PCR master-mix buffer (ThermoFisher Scientific, Massachusetts, USA) and 2µl nuclease-free water for each sample.

2.2.2.3 PCR protocol

PCR followed the following protocol: Initial denaturing at 95°C for five minutes, followed by 32 cycles of (1) denaturing at 95°C for 30 seconds (2) annealing at 58°C for 30 seconds (3) DNA synthesis at 72°C for 40 seconds, followed by a final cycle at 72°C for four minutes. Processed samples were stored at 8°C until use, at which point they were run on a 1.4% aragose gel at 75V for 20 minutes.

2.2.3 Tissue sample preparation

For all experiments involving the use of murine tissues, experimental animals were first culled by an approved schedule 1 method (either cervical dislocation or rising concentration of inhaled CO_2) in accordance with UK Home Office regulations (Bleby 1986). Where serum samples were required, these were obtained through cardiac puncture under terminal anaesthesia. Lymph nodes (cervical, brachial, axillary, inguinal and mesenteric) and spleen were harvested via a midline incision and transferred to RPMI prior to further processing. Excess adipose tissue was removed from tissues prior to further processing.

2.2.3.1 Generation of cell suspensions

2.2.3.1.1 Blood

Blood was collected either by cardiac puncture under terminal anaesthesia (if an animal was to be sacrificed for experimental analysis) or by tail bleed of live animals; to prevent clotting, blood was collected in 1.5ml Eppendorfs containing 50µl 0.1mM ethylebediaminetetraacetic acid (EDTA) and was mixed thoroughly. 1ml of red cell lysis buffer (Gey's solution; Table 2.1) was added and the sample incubated at 2-8°C in the dark for five minutes. Next, the sample was centrifuged at 400g for five minutes and the supernatant removed by pipetting. The lysis step was repeated if significant red cell contamination was noted after the first cycle. The resulting pellet was re-suspended in RPMI and stored at 2-8°C until required.

2.2.3.1.2 Serum

Blood was collected by cardiac puncture under terminal anaesthesia in a 1.5ml Eppendorf in the absence of EDTA and stored at room temperature for 30 minutes until completely clotted. The sample was centrifuged at 16,000g for 10 minutes; the resulting supernatant comprised the serum, which was removed by pipette and stored at -20°C until required.

2.2.3.1.3 Spleen

Splenic tissue was first cut into small fragments before being transferred to a 70μm cell filter placed in a 50ml Falcon tube, and crushed using a sterile plunger from a 2ml syringe. The filter was rinsed twice with 10ml sterile RPMI to maximise cell yield.

The resulting suspension was centrifuged at 400g at 4°C for six minutes to pellet the splenocytes. The supernatant was removed, and the pellet re-suspended in 5ml sterile Gey's solution to lyse the red blood cells, then incubated at 2-8°C in the dark. After five minutes, 10ml sterile RPMI was added, and the suspension centrifuged at 400g at 4°C for a further six minutes. The lysis step was repeated if significant red cell contamination was noted after the first cycle, and the pellet re-suspended in 5ml sterile RPMI. This was passed through a fresh 70μm filter into a new 50ml Falcon tube, and the filter rinsed with a further 5ml sterile RPMI. Cells were stored at 2-8°C until required. Cell counts were performed with a haemocytometer where appropriate.

2.2.3.1.4 Lymph node

Lymph nodes were prepared according to the above method for splenic tissue, however since lymph nodes contain almost entirely lymphocytes with no significant red blood cells, the red blood cell lysis steps were not necessary, and cell count could be performed as soon as the tissue has been crushed through the 70μm filter.

For experiments requiring maximal cell yield, lymph nodes were manually disaggregated in RPMI using micro-forceps under a microscope, then underwent enzymatic lysis by collagen dispase and DNAse at 36°C for 20 minutes before being transferred to the 70μm filter.

Cell counts were performed with a haemocytometer where appropriate.

2.2.3.2 Generation of tissue sections

2.2.3.2.1 Spleen

Whole spleen was cut into two segments along its short axis, snap-frozen in liquid nitrogen and stored at -80°C until required. Tissue sections were cut to a thickness of 7μm using a cryostat maintained between -15 and -20°C, mounted on glass slides and fixed in acetone for 20 minutes at 4°C. Slides were stored at -20°C until required.

2.2.3.2.2 Lymph node

Lymph nodes were mounted in OCT compound (Agar Scientific, Stansted, UK), frozen in liquid nitrogen and stored at -80°C until required. Tissue sections were cut to a thickness of 7μm using a cryostat maintained between -15 and -20°C, mounted on glass slides and fixed in acetone for 20 minutes at 4°C. Slides were stored at -20°C until use.

2.2.4 Flow cytometry

Where cell numbers permitted, 1-2 x10^6 cells were suspended in 2% flow cytometry buffer, and stained in a volume of 50µl in round-bottom 96-well plates. All antibody dilutions, rinse cycles and cell suspensions were performed using 2% flow cytometry buffer unless otherwise stated. Cell rinse cycles were performed with 150µl buffer followed by centrifugation. All centrifugation was performed at 300g at 4°C for 4 minutes. Cell suspensions were maintained at 2-8°C and shielded from light unless otherwise stated. Stained cells were re-suspended in 300µl buffer prior to analysis on the flow cytometer. Counting beads were added to samples prior to analysis where appropriate (AccuCheck Counting Beads, ThermoFisher Scientific, Massachusetts, USA). See table 2.2 for a list of antibodies used.

For all non-purified cell samples, anti-mouse CD16/CD32 Fc receptor block (eBioscience, Hatfield, UK) was first added to prevent non-specific binding of antibodies to the Fc gamma III (CD16) and Fc gamma II (CD32) receptors expressed by any resident B lymphocytes, monocytes, macrophages, NK cells and neutrophils. These receptors will bind mouse IgG via the Fc portion, resulting in false positive staining for any antibody of this isotype. 10µl of Fc receptor block was added to all samples before incubating for ten minutes at 2-8°C prior to adding further antibodies.

Multi-colour cytometry compensation was performed using either wild-type murine T lymphocytes (derived from either lymph node or spleen as described above), or anti-mouse or anti-rat staining beads (AbC kits, Invitrogen, Paisley, UK) as appropriate, individually stained with each fluorochrome-conjugated antibody. This allowed for compensation of spectral

overlap by adjusting for false positive staining from other fluorochromes. The number of events analysed per sample was between 10,000 and 500,000.

Samples were analysed using the Dako-CyAn flow cytometer (Dako, Colorado, USA) and Summit 4.3 analysis software for Microsoft Windows (Dako, Colorado, USA, 2007), or BD LSRFortessa flow cytometer (BD Biosciences, San Jose, California, USA) and FlowJo 8.8.6 analysis software for Mac (FlowJo LLC, Oregon, USA). Calibration was checked on a daily basis with Flowcheck Fluorospheres (Beckman Coulter Inc.). Isotype controls were used to determine the level of background non-specific binding where appropriate.

2.2.4.1 Surface staining

Cells and beads were centrifuged, the supernatant removed and the plate gently vortexed to re-suspend the resulting pellet. Cells and beads were stained with surface marker antibodies as indicated in table 2.5 and incubated for 20 minutes before performing two cell rinse cycles and analysing on the flow cytometer.

Description	Flow Cytometer	Antibodies
Lymphocyte phenotype	CyAn	*Surface antibodies:* CD3, CD4, CD8, CD19, CD25, CD44, CD45.2, CD62L
Treg	CyAn	*Surface antibodies:* CD3, CD4, CD25, CD44, CD62L *Transcription factor:* FoxP3
	Fortessa	*Surface antibodies:* B220, CD3, CD4, CD8, CD11b, CD11c, CD44 *Transcription factor:* FoxP3, RORγ
Th17	CyAn	*Surface antibodies:* CD3, CD4, CD8, CD44, CD62L *Cytokine antibodies:* IFNγ, IL17
	Fortessa	*Surface antibodies:* B220, CD3, CD4, CD8, CD11b, CD11c, CD44 *Cytokine antibodies:* IFNγ, IL17
Non-T / Non-B populations	CyAn or Fortessa	*Surface antibodies:* CD3, CD19, CD11b, CD11c, CD45.2, GR1, NK1.1
Naïve CD4+ T cell sort	CyAn	*Surface antibodies:* CD4, CD25, CD44, CD62L

Table 2.5 Antibody combinations used in flow cytometry analyses. Combinations shown for analysis on both Dako-CyAn flow cytometer (CyAn) and BD LSRFortessa (Fortessa) where appropriate.

2.2.4.2 Transcription factor staining

Cells were initially stained for surface marker antibodies as indicated in table 2.5 according to the method described above.

Cells were fixed in 200µl 1x fixation/permeabilisation solution (1:3 dilution of FoxP3 fixation/permeabilisation 4x concentrate and diluent, eBioscience, Hatfield, UK) and incubated for 30 minutes. The cells were next centrifuged and rinsed once, then permeabilised by two rinse cycles in 150µl 1x permeabilisation buffer (10x permeabilisation buffer, eBioscience, Hatfield, UK diluted 1:9 in distilled water).

Intracellular transcription factor antibodies were diluted in 1x permeabilisation solution and added both to the cells for analysis and also to the appropriate compensation well. 50µl 1x permeabilisation solution was added to all other compensation wells. Cells were incubated for 30 minutes, before two further rinse cycles in 150µl 1x permeabilisation fluid. Samples were finally re-suspended in flow cytometry buffer for analysis.

2.2.4.3 Cytokine staining

Cells were re-suspended in 200µl complete medium and transferred to a round bottom 96-well plate. Cells were re-stimulated using PMA (Sigma-Aldrich, Dorset, UK) at a concentration of 50ng/ml and ionomycin (Sigma-Aldrich, Dorset, UK) at 500ng/ml. To prevent cytokine release, Brefeldin A was also added at a concentration of 3µg/ml (Sigma-

Aldrich, Dorset, UK). The plate was sealed and transferred to an incubator at 37°C 5% CO_2 for 3 hours.

The plate was centrifuged and the supernatant discarded. Cells were stained according to the protocols detailed in table 2.5. Cells were initially stained for surface marker antibodies according to the method described above.

Cells were fixed in 50µl of fixation reagent A (Fix / Perm reagents, Invitrogen, Camarillo, California, USA) and incubated at room temperature or 15 minutes, shielded from light. A single rinse was performed in flow cytometry buffer and centrifuged as before.

Antibodies for intracellular staining were diluted in 50µl permeabilisation reagent B (Fix / Perm reagents, Invitrogen, Camarillo, California, USA) and added to the cells and appropriate compensation wells. 50µl flow cytometry buffer was added to surface antibody compensation wells. This was incubated at room temperature for 15 minutes shielded from light, before a final rinse cycle in flow cytometry buffer.

Samples were finally resuspended in flow cytometry buffer for analysis.

2.2.5 Immunohistochemistry

2.2.5.1 Autoantibody screen

Serum from experimental animals was screened for autoantibodies using a commercial rat liver, kidney, and stomach indirect immunofluorescence kit and slides (Cambridge Life Sciences, Ely, Cambridgeshire, UK) according to the manufacturers instructions. This kit contains slides which contain aggregate sections of rat stomach, liver and kidney to show anti-nuclear, anti-mitochondrial, anti-smooth muscle and anti-gastric parietal cell antibodies, which can easily be seen through comparison of the different tissues displayed. Serum from a roquin knock-out mouse was used as positive control.

Serum samples were collected as described in section 2.2.3.1.2. After defrosting the serum at room temperature, serial dilutions were made (1/10, 1/40, 1/160) in PBS containing 10% goat serum to block cross-reactivity through non-specific Fc receptor binding. 50µl 10% goat serum was added to each test specimen and incubated at room temperature in a dark chamber for 10 minutes. Next, 50µl of each serum dilution was added to each test specimen and incubated at room temperature in a dark chamber for 20 minutes. Slides were rinsed in PBS, then 50µl of 1:100 FITC-conjugated goat-anti-mouse IgG (Southern Biotech, Alabama, USA) applied to each specimen, and the slide was again incubated in darkness at room temperature for 20 minutes. Slides were again washed in PBS, a coverslip applied, then examined under an immunofluorescent microscope.

2.2.5.2 Immunofluorescent staining

Slides from betaglycan knock-out, heterozygous and wild-type chimeras were stained according to the protocol illustrated in table 2.6. Specimens from a RAG/BoyJ mouse were used as negative control.

Before staining, slides were defrosted at room temperature and hydrated for 10 minutes in PBS. To prevent non-specific binding of antibodies, a block of 75µl of 10% horse serum in PBS / 1% BSA was applied to each spot, and the slides were then incubated for 15 minutes in a dark, humidified environment at room temperature.

Excess horse serum was aspirated from each spot, then 75µl of the primary antibody solutions were added to their relevant spots on the slides. The slides were incubated for a further 40 minutes, then washed in PBS. Next, 75µl of the secondary antibody solution was added to each spot. The slides were again incubated for 30 minutes before being removed and washed once more in PBS. 75µl of the tertiary antibodies added to the relevant spots. The slides were incubated for 30 minutes then washed in PBS.

Finally, each slide was placed in DAPI stain for 30 seconds before three consecutive PBS washes. Slides were allowed to partially air dry then fixed with ProLong Gold (ThermoFisher Scientific, Massachusetts, USA) to preserve fluorescence and cover slips applied. Slides were stored in darkness at -20^{0}C until required for analysis.

Spot 1	Spot 2
Step 1	
Block all spots with 10% horse serum in autoclaved PBS with 1% BSA	
Step 2	
Hamster-anti-mouse CD3 FITC	Rat-anti-mouse IgD FITC
Rat-anti-mouse B220 biotin	Rat-anti-mouse PNA biotin
Rat-anti-mouse CD4 AF647	Donkey-anti-mouse IgM Rhodamine Red
Step 3	
Block with 10% mouse serum in autoclaved PBS with 1% BSA.	
Rabbit-anti-FITC AF488	
Step 4	
Block with 10% mouse serum in autoclaved PBS with 1% BSA.	
Donkey-anti-rabbit AF488	Donkey-anti-rabbit AF488
Streptavidin, AF555 Conjugate	Streptavidin, AF647 Conjugate
Step 5	
DAPI (4', 6-diamidino-2-phenylindole) stain 5mg/ml, Invitrogen, Paisley, UK	

Table 2.6 Protocol for immunofluorescent staining.

2.2.5.3 Image analysis

Imaging of frozen sections was performed using a Zeiss LSM 510 confocal microscope under standardised conditions of 10x magnification and 0.7x zoom. Laser gain was adjusted to minimise background staining. Three representative white-pulp areas were imaged for each spleen section, and a single view of each lymph node was captured.

Images were analysed using ImageJ software (nih.gov); for splenic sections, white pulp area, T-zone area, and number and area of germinal centres were calculated for each of the three white-pulp zones captured for each section. Images were assessed by two masked observers, and results compared to ensure measurements were within 10%. If a discrepancy of greater than 10% was encountered, both observers reassessed the image until agreement was reached. The mean of the two measurements was then taken as the final value.

IgM and IgD co-expression was calculated using the ImageJ software 'co-localisation plug-in'. This enabled separation of each image to its component channels, and counting of the number of pixels which were positive for the immunofluorescent stain in question. Imaging intensity was first standardised to control for any background staining; mean pixel intensity was calculated for each image, then a gate applied to each image from 0 to 1.5x this mean intensity, and the percentage of positive pixels in each resulting image calculated. Co-localization was determined by combining the resulting IgM and IgD images and calculating the percentage of bright pixels appearing in both channels.

2.2.6 *In-vivo* antigenic challenge

Mice underwent controlled antigenic challenge at day 0 and were sacrificed for analysis at day 7; secondary lymphoid tissues were harvested and processed to cell suspensions as described in section 2.2.3.1. Animals were immunised a peptide antigen and either attenuated *Listeria monocytogenes (L. monocytogenes)*, incomplete Freund's adjuvant (IFA) or *Salmonella typhi (S. typhi)* outer membrane porins as adjuvants. For all experiments, a maximum of three non-chimeric mice were immunised as antigen controls, and a maximum of three chimeric mice immunised with PBS as negative controls.

For all experiments, 2W1S peptide was used as an antigen. TCR binding to antigen-derived peptide-MHC (pMHC) ligands induces naïve T lymphocytes to proliferate and differentiate to an effector phenotype (Jenkins et al. 2001). 2W1S peptide consists of amino acids 52-68 from the I-E alpha chain (Rees et al. 1999), and is highly immunogenic in C57BL/6 mice due to the presence of a relatively large population of naïve T lymphocytes capable of recognising 2W1S:I-A^b (Moon et al. 2007). Immunisation by 2W1S peptide thus allows assessment of a peptide-specific T lymphocyte response, by staining cells with a fluorochrome-labelled 2W1S:I-A^b tetramer.

2W1S:I-A^b tetramer staining of cell suspensions was performed prior to further surface, cytokine and/or transcription factor staining according to the methods as described in section 2.2.4. For tetramer staining, cells were incubated at room temperature for 1 hour shielded from light in BV421-conjugated 2W1S:I-A^b at a dilution of 1/40.

2.2.6.1 Attenuated *Listeria monocytogenes*

Animals were either immunised with 2W1S peptide and attenuated *L. monocytogenes* adjuvant engineered to secrete a fusion protein containing a portion of chicken ovalbumin and the 2W1S peptide (Pepper et al. 2010) or PBS alone. Bacteria were grown at 37°C in a shaking incubator to concentration OD600 = 0.1 in Luria-Bertani broth supplemented with 20 µg/mL chloramphenicol. Either 10^7 bacteria in 200µl or PBS alone was injected via a tail vein at day 0, and spleen harvested at day 7.

2.2.6.2 IFA

Animals were immunised with either a 1:1 mix of 20µl IFA (Sigma-Aldrich, Dorset, UK) and 20µl 2W1S peptide in a total volume of 50µl or PBS alone, administered via subcutaneous injection to the right and left flank at day 0. Draining inguinal lymph nodes were harvested at day 7.

2.2.6.3 *Salmonella typhi* porins

Animals were immunised with either a 1:1 mix of 20µl *S. typhi* outer-membrane protein C– and F–based subunit vaccine (porins) and 20µl 2W1S peptide in a total volume of 25µl or PBS alone, administered via subcutaneous injection to right and left front paw pads at day 0. Draining inguinal axillary and brachial lymph nodes were harvested at day 7.

2.2.7 *In-vitro* assays of T lymphocyte differentiation

2.2.7.1 Cell purification

Cell suspensions were first generated from secondary lymphoid tissues under sterile conditions as described above. Naïve T lymphocytes were purified through either magnetic bead isolation (magnetic activated cell sorting - MACS) using the CD4+CD62L+ T cell Isolation Kit II (Miltenti Biotech Inc, California, USA), or by fluorescence activated cell sorting (FACS), using the 'lymphocyte phenotype' panel as illustrated in table 2.5 but staining only for purification of CD4+CD25-CD44lowCD62L+ (naïve CD4+) T lymphocytes. Samples of 2 x 10^5 cells were taken before and after sorting to confirm purity, again being analysed according to the 'lymphocyte phenotype' flow cytometry protocol as described in table 2.5.

MACS purification followed a two-step process. Firstly, non-CD4+ cells were removed through magnetic bead labelling with a cocktail of biotin-conjugated antibodies against CD8a, CD45R, CD11b, CD23, CD49b, TCRγ/δ and Ter-119. These labelled cells were depleted by separation over a MACS column to remove any non-CD4+ cells from the sample. Secondly, the remaining CD4+ cells were directly labelled using monoclonal anti-mouse CD62L microbeads, and labelled cells isolated by positive selection from the pre-enriched fraction to generate a purified CD4+CD62L+ sample.

This process aims to purify a naïve T lymphocyte phenotype by assuming a low population of "central memory" T cells expressing both CD62L and CD44, and also that all CD62L+ cells

will be CD25-. FACS enables positive selection of CD4+CD25- cells, allowing the central memory component to be excluded by positively selecting the CD44lowCD62L+ from this population.

FACS therefore results in a sample of considerably greater purity, however there is a greater risk of microbial contamination as the FACS sorter is not maintained in a sterile environment, and cell recovery and viability is reduced compared to MACS. There is thus a trade-off between the risk of sample infection, and the degree of sample purity, and the cell yield between the two techniques.

2.2.7.2 Culture conditions

Where indicated, purified naïve T lymphocytes were first labelled with an eFluor 450 proliferation dye (eBioscience, Hatfield, UK) according to the following protocol:

The proliferation dye was first reconstituted with 165µl anhydrous dimethyl sulfoxide (DMSO) to give a 10mM stock solution. Cells were washed twice in PBS (300g, 8 minutes per wash) and resuspended in 0.5ml sterile PBS per 5 million cells. A 1/500 dilution of stock solution was then added to the cell suspension at a volume ratio of 1:1, and the mixture incubated in darkness at room temperature for 10 minutes. Cold complete media was then added to stop the labelling, and incubated on ice in darkness for 5 minutes. Cells were finally washed three times in complete media as above.

Following labelling, cells were resuspended in complete media and transferred to sterile 96-well plates. Cells were stimulated with either plate-bound anti-CD3 5μg/ml (eBioscience, Hatfield, UK) and soluble anti-CD28 1μg/ml (eBioscience, Hatfield, UK), or anti-CD3 / anti-CD28 coated activation beads at a ratio between 2:1 and 1:4 beads:cells (Dynabeads Mouse T-activator CD3/CD28, Life Technologies, Paisley, UK).

Flat-bottomed 96-well plates were coated with anti-CD3 (200μl 1/200 anti-CD3 in sterile PBS) for a minimum of 6 hours at 2-6°C; PBS was removed by pipetting and excess un-bound anti-CD3 removed by two washes with 1ml sterile PBS. Care was taken at each pipetting step not to disturb the bound anti-CD3. Round-bottomed 96-well plates were used for cultures involving anti-CD3 / anti-CD28 coated activation beads.

For all experiments cells were cultured at a concentration of 2 x 10^5 cells per 200μl. Cells were incubated at 37°C in an environment of 5% CO_2 for four days and cultured either under iTreg polarizing conditions (IL2 50 U/ml, TGFβ 3.0-0.1ng/ml in Log3 dilutions) or control (Th0) conditions (IL2 50U/ml). Further control conditions were tested in the absence of stimulation. Recombinant TGFβ1, 2 or 3 was tested in appropriate assays.

2.2.8 Single nucleotide polymorphism screening

2.2.8.1 Sample preparation

After informed consent, blood samples were collected by venepuncture by research nurses and doctors working in outpatient clinics at the Birmingham & Midland Eye Centre and

Medical Eye Unit at St Thomas' Hospital London. DNA was prepared by proteinase K digestion, and salt extraction and stored at -70˚C until use.

2.2.8.2 Real-time genotyping and analysis

The SNP RS1805110 (Applied Biosystems, Warrington, UK) in TGFBR3 was genotyped in all subjects using a PCR restriction length polymorphism assay (Table 2.7). PCR was performed in 384-well plates with a 10μl total reaction volume containing 20pg/μl DNA (samples) or water (controls) and 2x LC480 probe master (Roche, West Sussex, UK). Analysis was conducted using the Roche LC480 system (Roche, West Sussex, UK) according to the 'endpoint genotyping PCR read' protocol, comprising pre-incubation of 1 cycle at 95° for 10 minutes, followed by 40 amplification cycles at 95° for 10 seconds, 60° for 1 minute and 72° for 1 second, and finally 1 cooling cycle at 40° for 30 seconds. Genotypes were determined as either homozygous (TT or CC) or heterozygous (CT) according to the presence or absence of fluorescence for each genotype.

RS number	Context sequence
RS1805110	CAACTTACCTGCAGTGGCTAAACAG [A/G] AGCTCATCAGGGCAAAGATGGCAAT

Table 2.7 SNP RS1805110 probe used for analysis. Substitution in context sequence shown in square brackets; Applied Biosystems, Warrington, UK.

2.3 Statistical analysis

All data was entered into a Microsoft Excel spread sheet for initial processing. Statistical analysis and graphical representations were then generated using Graphpad Prism 5 (Graphpad, California, USA).

Descriptive statistics comprising median, mean, standard error, and 95% confidence intervals were calculated where appropriate. Illness and survival amongst experimental animals was assessed using a Kaplan-Meier survival analysis with a Mantel-Cox test for curve comparison.

Non-parametric tests were employed to compare cell populations between experimental groups of animals, using the Mann-Whitney U test for comparisons of two groups, and Kruskal-Wallis test with Dunn's multiple comparison post-test for analyses involving three or more groups. Non-parametric tests were favoured as data could not be considered normally distributed, and therefore non-parametric tests were considered more rigorous (Altman 1991).

For association analysis of SNP with human disease groups, Hardy-Weinberg equilibrium (HWE) was assessed using the Chi-square test. Allele frequencies were estimated by direct counting, and distribution of alleles and genotypes between patients and controls compared. Odds ratio (OR) and 95% confidence intervals were calculated, and the association of this SNP with disease phenotype analysed using Fisher's exact test.

Statistical significance was accepted for $p<0.05$ throughout all analyses.

3 DEVELOPING AN EXPERIMENTAL MODEL TO ENABLE INVESTIGATION OF THE ROLE OF BETAGLYCAN IN PERIPHERAL LYMPHOCYTE RESPONSES

3.1 Introduction

Absolute deficiency of any of the three isoforms of TGFβ, or any components of the TGFβ receptor is incompatible with life. This is due to the wide distribution of TGFβ and its involvement in a multitude of life-essential signalling pathways across all organ systems, and the importance of TGFβ to the developing embryo. In mice, TGFβ1 deletions result in two distinct phenotypes; approximately 50% of embryos die around mid-gestation due to defects in yolk sac vasculogenesis and haematopoiesis (Dickson et al. 1995), whilst the remainder survive beyond birth, possibly due to transfer of maternal TGFβ1 across the placenta. Mice surviving to birth proceed to experience spontaneous activation of a self targeted immune responses leading to death between three and four weeks of age (Kulkarni et al. 1993; Shull et al. 1992). TGFβ2 deletions also result in perinatal death, although this is due to extensive developmental defects in the cardiac, pulmonary, musculoskeletal, craniofacial and urogenital systems (Sanford et al. 1997). Loss of TGFβ3 results in cleft palate and abnormal pulmonary development with death within a few hours of birth (Kaartinen et al. 1995; Proetzel et al. 1995).

Similarly, murine models of complete TGFβRI or II gene knock-out are characterised by embryonic lethal defects in vascular development in the placenta and yolk sac (Larsson et al. 2001; Dickson et al. 1995; Oshima et al. 1996), whilst betaglycan knock-out (TGFβRIII-/-) mice display embryonic-lethal cardiac and hepatic defects arising around mid-gestation (Stenvers et al. 2003).

Whilst absolute betaglycan deficiency is thus incompatible with life, animals with targeted deletions of betaglycan restricted to specific cells and organ systems have the potential to be viable and offer opportunities for studying the role of betaglycan in mature organ systems *in vivo*. Wild-type mice possess two copies of the betaglycan gene; heterozygotes with a single copy of the betaglycan gene have previously been generated, proving to be both viable and fertile, allowing a colony of betaglycan heterozygotic mice to be established (Stenvers et al. 2003). Embryos generated through mating between two betaglycan heterozygotic mice follow a Mendelian pattern of inheritance, with a 1:2:1 ratio of wild-type (TGFβRIII +/+) : heterozygote (TGFβRIII +/-) : knock-out (TGFβRIII -/-) progeny. As previously discussed, absolute deficiency of betaglycan as observed in TGFβRIII -/- progeny results in death of mice *in utero* due to cardiovascular developmental defects; embryogenesis proceeds unaffected until day 12 gestation, after which gradual accumulation of cardiovascular developmental defects results in death of all TGFβRIII -/- embryos. Survival is rarely observed beyond day 18 gestation (E18).

The survival of TGFβRIII -/- embryos to mid gestation has previously enabled observation of betaglycan-deficient foetal thymic organ cultures, which have shown deficiencies in thymic T lymphocyte development (Aleman-Muench et al. 2012). Due to the difficulties in developing

suitable experimental models, there have been no previous studies of betaglycan deficiency in mature animals.

In the previously described foetal thymic organ cultures, developing TGFβRIII -/- embryos were harvested at a time point prior to embryonic death, and grown in culture to allow the resident population of embryonic stem cells to mature. This enabled observation of mature betaglycan-deficient T lymphocytes in an *ex vivo* system. We observed that such populations of embryonic stem cells could be transferred to a host animal in which they could proliferate and differentiate *in vivo*, allowing investigation of mature, peripheral TGFβRIII -/- T lymphocytes in a physiological system.

We subsequently adopted this approach to develop a novel experimental model of betaglycan deficiency, through generation of mixed foetal liver chimeric mice.

3.2 Experimental design

3.2.1 Generation of betaglycan-deficient foetal liver chimeric mice

Our requirements were to develop an experimental model in which the role of betaglycan could be investigated in peripheral lymphocyte responses, whilst avoiding the embryonic lethality observed in conditions of absolute betaglycan deficiency. We therefore employed a technique of generating animals with targeted deletion of betaglycan, restricted primarily to the lymphocyte populations of interest.

Haemopoietic chimeras are created when embryonic stem cells are harvested from a 'donor' source and transferred to a 'host' mouse. Haematopoietic stem cells populate the embryonic liver, which is thus a rich source of lymphocyte precursors. Provided both the donor and host animal are of a similar genetic background, graft versus host disease is avoided, and the donor lymphocyte precursor cell populations will mature and persist in the host mouse. We utilised this concept to generate a model of targeted betaglycan deficiency, where TGFβRIII -/- foetal liver stem cells were transferred to a sub-lethally irradiated RAG -/- host mouse (Figure 3.1).

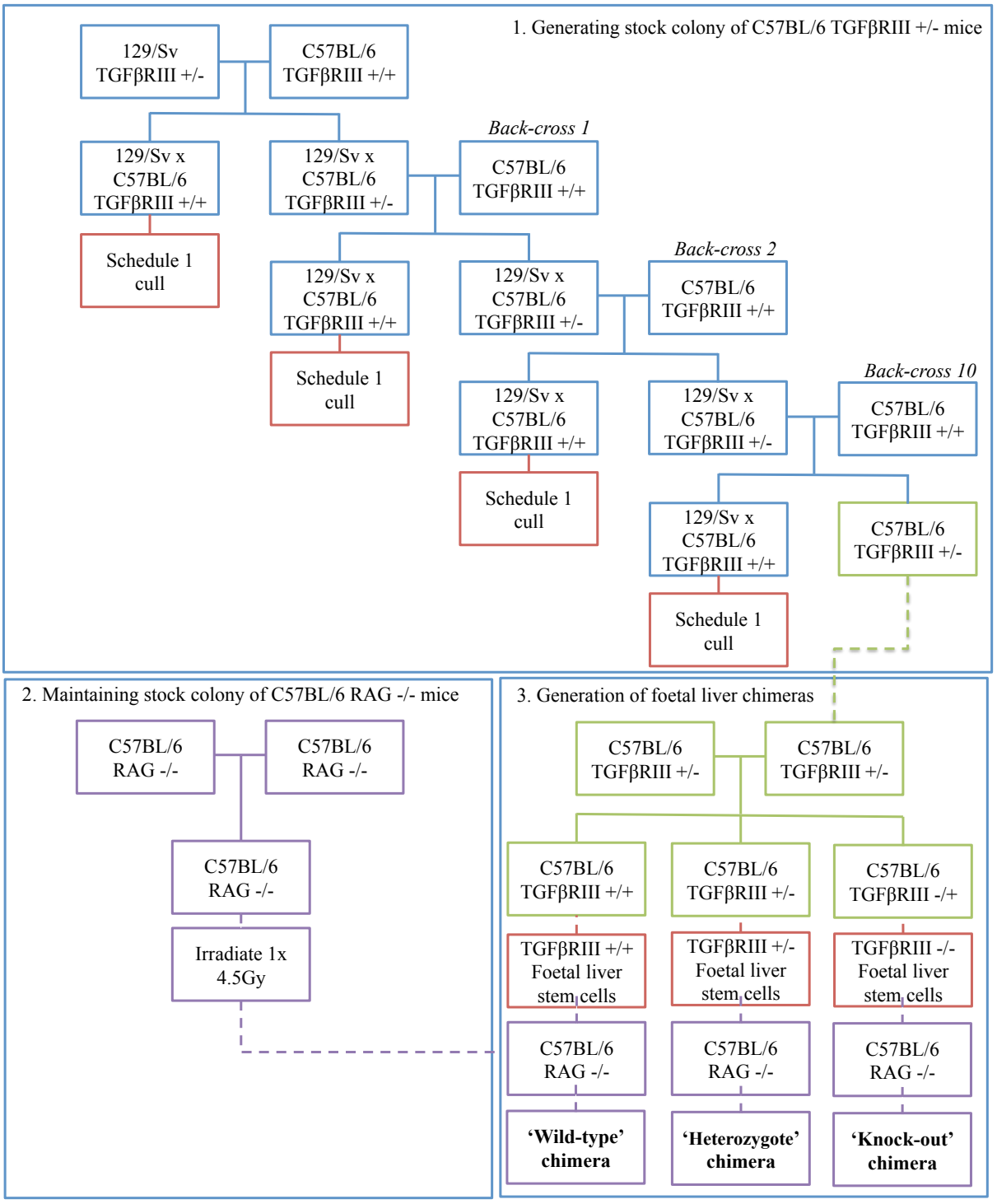


Figure 3.1 Generation of foetal liver chimeras. Flowcharts illustrating maintenance of stock colonies and co-ordination of foetal liver chimera generation. Experimental animals were created following of transfer of embryonic liver stem cells from the products of timed mating between betaglycan heterozygote mice to sub-lethally irradiated RAG/BoyJ hosts between day 12 and 14 gestation. All animals were maintained on a C57BL/6 background.

3.2.2 Animal selection and generation of stock colonies

A population of RAG/BoyJ mice was maintained as hosts for cell transfer; these mice lack the recombinase activating genes which are required for V(D)J gene rearrangement during generation of Ig and the TCR (Schatz et al. 1989). In the absence of V(D)J rearrangement, lymphocyte development is arrested and RAG -/- mice have no circulating T or B lymphocytes. Since the bone marrow of these knock-outs is otherwise functionally normal, lymphocytes will develop if embryonic stem cells are transferred from mice with functioning RAG genes (Chen et al. 1993).

TGFβRIII +/- mice (129/Sv background) were kindly donated by Kaye Stenvers (Head of Reproductive Development and Cancer, Prince Henry's Institute of Medical Research, Clayton, Victoria, Australia). A colony of betaglycan heterozygote mice was maintained for use as donors for cell transfer through mating with wild-type C57BL/6 mice. The offspring of each mating were genotyped according to the PCR method as described in section 2.2.2.

In creating the original heterozygotic mouse, a 2.55-kb phosphoglycerokinase-neomycin (PGK-Neo) expression cassette was inserted in place of the betaglycan gene. Mice or embryos could be identified as TGFβRIII +/+ if only the betaglycan gene was detected by PCR, TGFβRIII -/- if only the neomycin gene was identified, and TGFβRIII +/- if both genes were identified by PCR (Figure 3.2).

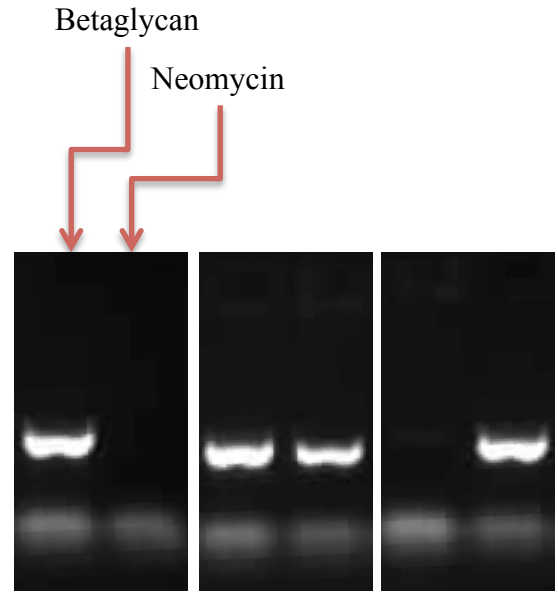


Figure 3.2 Identification of donor cell genotype by PCR. Tissue samples were retained from all donor embryos to enable accurate genotyping following cell transfer. Examples of PCR results are shown for TGFβRIII wild-type (+/+), TGFβRIII heterozygote (+/-) and TGFβRIII knock-out (-/-) tissue samples. In each example, betaglycan probes are loaded in the first column, and neomycin probes loaded in the second column; animals were thus identified as either TGFβRIII wild-type (+/+) if only the betaglycan gene was identified, heterozygote (+/-) if both betaglycan and neomycin genes were identified and knock-out (-/-) if only the neomycin gene was identified.

Since all TGFβRIII -/- embryos die *in utero*, this breeding achieved a TGFβRIII +/+ to TGFβRIII +/- ratio of 1:2 in live births. All +/+ mice were sacrificed, and breeding pairs created between the remaining TGFβRIII +/- mice and C57BL/6 mates: The original betaglycan heterozygote mouse was created on a 129/Sv background, however the host RAG/BoyJ mouse had a C57BL/6 background. To avoid graft-versus-host disease in the resulting chimeras it was important to back-cross the betaglycan heterozygotes to a genetically similar C57BL/6 background. After a minimum of five back-crosses the mice were considered sufficiently close to a C57BL/6 genotype for further experiments to commence. In addition to ensuring genetic compatibility, the back-crossing of betaglycan heterozygotes to a C57BL/6 background also enabled discrimination of donor and host cells in chimeric mice; C57BL/6 mice express the CD45.2 congenic marker, whereas the host RAG/BoyJ mice express CD45.1. By ensuring that donor and host cells express different isoforms of CD45, the two cell populations could be distinguished by flow cytometric analysis at a later stage.

3.2.3 Generation of experimental mice

Embryos were generated through timed mating between TGFβRIII +/- animals. Pregnant females were sacrificed by a UK Home Office-approved schedule 1 method between E12 and E14 and the embryos extracted by caesarean section. Each embryo was transferred to a separate well of a sterile six-well plate in 2ml sterile RPMI, and the liver removed and transferred to a separate well of a sterile 24 well plate in 1ml sterile RPMI. A sample of tissue was retained from the tail of each embryo and immediately frozen at -20°C for later genotyping according to the PCR method described in section 0.

Foetal liver specimens were disaggregated to a cell suspension by repeated pipetting, then transferred to a flow cytometry tube via a 70μm filter. Cells were centrifuged to a pellet, the supernatant removed and cells resuspended in 200μl of sterile PBS in preparation for transfer to a host mice, with cells from each embryo being transferred to a separate host. To maximise the chance of successful reconstitution in chimeric mice, all available cells from each foetal liver sample were transferred to host mice.

To reduce the potential risk of bacterial infection and reduce the risk of death from overwhelming sepsis following sub-lethal irradiation and subsequent bone marrow suppression, RAG/BoyJ drinking water was supplemented with Baytril (active ingredient enrofloxacin, Bayer, Newbury, UK) for seven days prior to the planned procedure. Mice were irradiated at a dose of 4.5Gy up to four hours prior to cell transfer. Hosts were weighed to give a baseline measurement, earmarked for traceability, then donor cells were transferred via injection to a tail vein. Baytril supplementation continued for 1 week following cell transfer. Host animals were observed daily for signs of distress following cell transfer (weight loss, starry coat, stereotypical behaviour, orbital tightening (Bleby 1986)); animals deemed 'distressed' were culled by a UK Home Office-approved schedule 1 procedure.

In the resulting chimeric mice, all mature T and B lymphocytes had differentiated from the donor stem cells, and thus in this model, absolute deficiency of betaglycan deficiency was achieved for all T and B lymphocytes. For all other haemopoietic cell populations, a mixed population of mature donor and host cells was observed. All non-haemopoietic cells of the host mouse also expressed betaglycan within the cell surface TGFβ receptor complex.

3.3 Results

3.3.1 Betaglycan-deficient T and B lymphocytes populate circulating compartments within 6 weeks of donor cell transfer

In order to allow assessment of the effect of betaglycan deficiency on mature lymphocyte populations, it was first necessary to confirm that donor cell populations were able to both persist and populate peripheral compartments in host mice. To confirm this, blood samples were collected via tail bleed of all chimeric mice 6 weeks post cell transfer according to the method described in section 2.2.3. Blood samples were processed for flow cytometric analysis according to the 'lymphocyte phenotype' protocol as described previously (Table 2.5).

Reconstitution of donor cell populations was confirmed by the presence of circulating CD3+CD45.2+ (T lymphocyte) and CD19+CD45.2+ (B lymphocyte) populations, with these populations absent in un-reconstituted RAG/BoyJ mice. Un-reconstituted mice were culled according to a UK Home Office-approved schedule 1 technique at 6 weeks post cell transfer (Figure 3.3). Failure of reconstitution was attributed to transfer of insufficient numbers of donor cells.

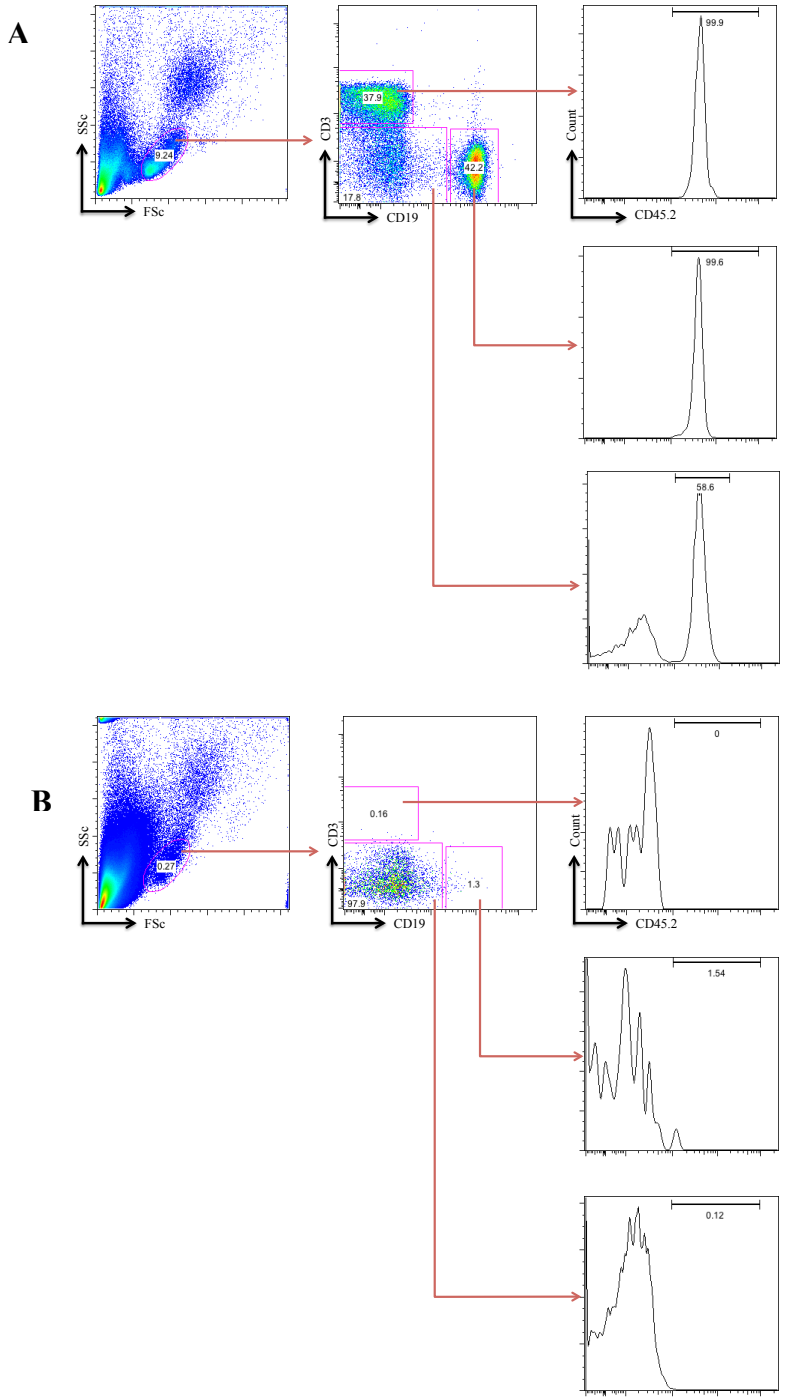


Figure 3.3 Successful reconstitution of foetal liver chimeras is confirmed by the persistence of circulating CD45.2+ cell populations. Example flow cytometric analysis of circulating blood cell populations in RAG/BoyJ host mice 6 weeks after irradiation and cell transfer showing **A** successful reconstitution and **B** unsuccessful reconstitution. Lymphocytes were first identified by forward scatter (FSc) / side scatter (SSc) profile, then T lymphocytes identified by staining for CD3 and B-lymphocytes identified by staining for CD19. Where T and B lymphocytes were successfully identified, the origin of these cells was confirmed by staining for CD45.2, with >99.9% of CD3+ and CD19+ cells displaying CD45.2 positivity. CD3-CD19- cell populations in successfully reconstituted mice displayed both CD45.2 positivity and negativity. Numbers in gates represent percentages for displayed sample.

Based on our observations, we conclude that betaglycan-deficient cells of donor origin are able to persist and circulate in the blood of our chimeric mice, enabling further investigation of peripheral lymphocyte populations. Overall, 289 chimeric mice were successfully generated, with chimera genotype closely following the expected ratio of Mendelian inheritance (1 : 1.94 : 1.19 TGFβRIII -/- : TGFβRIII +/- : TGFβRIII +/+ respectively).

3.3.2 Reconstitution of host bone-marrow is not impaired in betaglycan deficient foetal liver chimeras

To confirm that targeted betaglycan deficiency did not adversely affect reconstitution of host cell populations, an assessment was also made of resident non-T lymphocyte / non-B lymphocyte populations following irradiation and cell transfer. Whilst all lymphocyte populations in reconstituted chimeras must have originated from CD45.2+ donor cell populations, all other bone-marrow derived cells may have originated either from donor CD45.2+ cell populations, or host CD45.2- populations which repopulated bone-marrow compartments following sub-lethal irradiation of host mice. Thus, the presence of lymphocyte populations confirmed persistence of donor cell populations, but gave no indication of host bone-marrow reconstitution.

Flow cytometric analysis was performed to compare proportions of resident splenic non-lymphocyte populations of donor and host origin according to the 'Non-T / Non-B lymphocyte' staining protocol as previously described (Table 2.5).

All T and B lymphocytes were first excluded from analysis by gating on CD3-CD19-CD4-CD8- populations, then macrophages, dendritic cells and neutrophils identified through selection of CD11b+, CD11c+ and GR1+ populations respectively (Figure 3.4). Median ratio CD45.2+:CD45.2- populations ranged from 0.68 to 2.05. No statistically significant difference in ratio was observed for any cell type between genotypes, although a non-statistically significant trend towards impaired reconstitution of donor cell populations in knock-out chimeras was seen.

Based on these observations, we conclude that our experimental conditions of targeted betaglycan deficiency result in no impairment of bone-marrow haematopoiesis in host mice, and reconstitution of non-T lymphocyte and non-B lymphocyte populations is similar between mice of different genotypes. We find no evidence for a role of betaglycan in establishment of cell populations in the bone marrow.

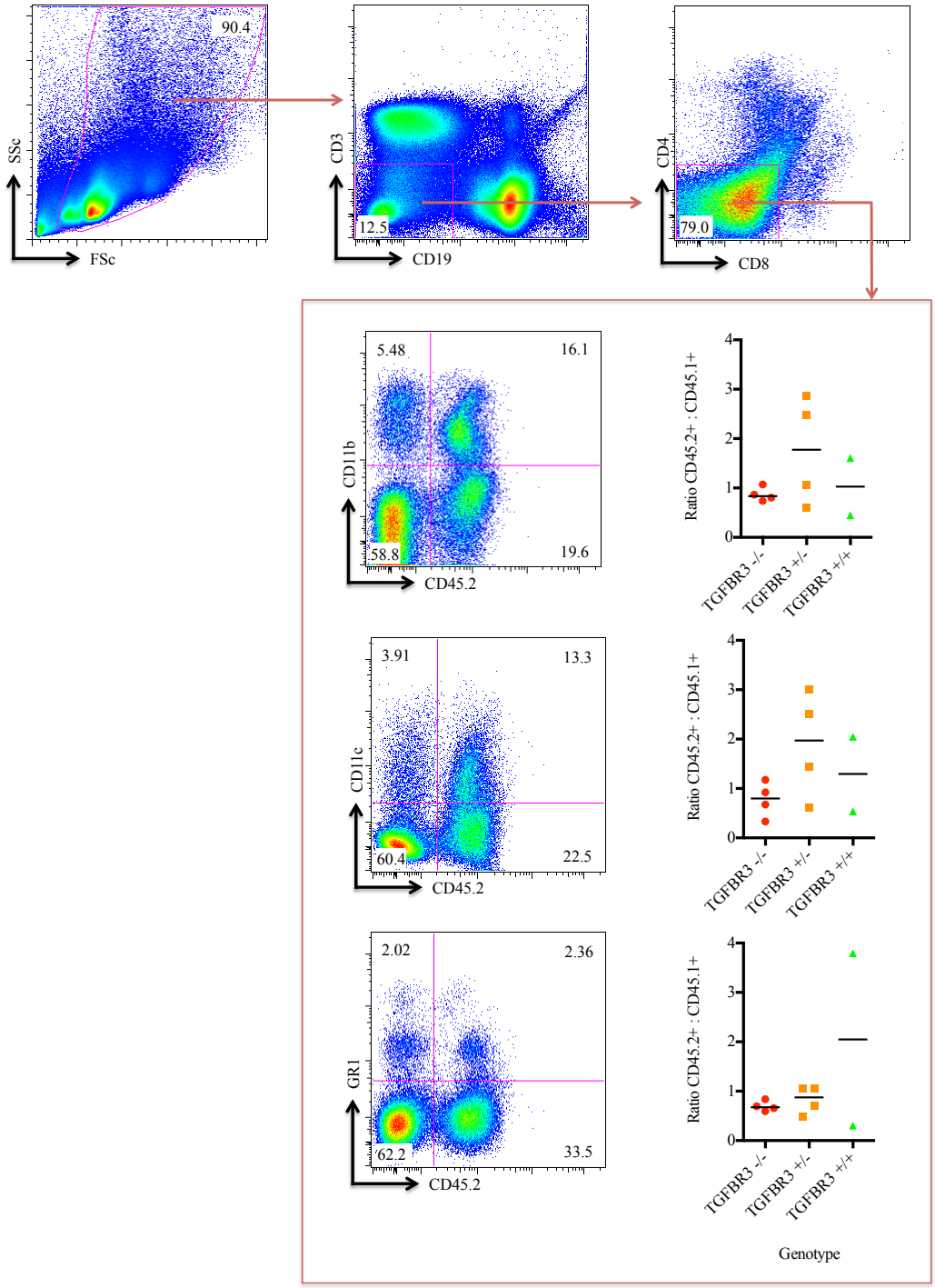


Figure 3.4 Reconstitution of non-T lymphocyte and non-B lymphocyte cell populations is not affected by chimera genotype. Example flow cytometry data plots showing gating strategy and associated column charts illustrating pooled results of analysis (flow cytometry data shown for heterozygote mouse; pooled data n=4 knock-out, n=4 heterozygote, n=2 wild-type; all animals assessed at 6 weeks post-transfer). Live cells were identified by forward scatter (FSc) / side scatter (SSc) profile, T and B lymphocytes excluded by gating on CD3-CD19-CD4-CD8- populations, then macrophages, dendritic cells and neutrophils identified through selection of CD11b+, CD11c+ and GR1+ populations respectively. Numbers in gates represent percentages for displayed sample. Line indicates median value; all comparisons non-significant ($p>0.05$) using Kruskal-Wallis test.

3.3.3 Animals with betaglycan deficient T and B lymphocytes are viable and display no external evidence of systemic disease

Having confirmed reconstitution of cell populations in host mice, it was also necessary to confirm viability of these animals for use in further experiments. Viability was confirmed by gross observations of mouse colonies, with assessment of general health according to recognised UK Home Office criteria (Bleby 1986).

No significant difference in percentage weight gain was observed at either 4 or 6 weeks post cell transfer, or at time of schedule 1 cull (Figure 3.5). This suggests that neither the process of irradiation or transfer of betaglycan-deficient cell populations results in development of significant gastrointestinal disease.

A slow rate of spontaneous illness as defined by Home Office criteria and described in section 2.2.1 was observed in chimeric mice (Prevalence 6.92% overall); whilst the survival curves for knock-out, heterozygote and wild-type mice are statistically significantly different according to Mantel-Cox curve analysis ($p<0.05$), the overall prevalence of spontaneous illness in each group were similar when comparing all animals. Experimental mice were observed for up to 24 weeks post cell transfer, allowing sufficient survival to allow for further experimental investigation (Figure 3.6).

We therefore conclude that restriction of betaglycan deficiency to T and B lymphocytes results in no gross external disease phenotype, and this is a suitable model on which to base further investigation of peripheral lymphocyte responses.

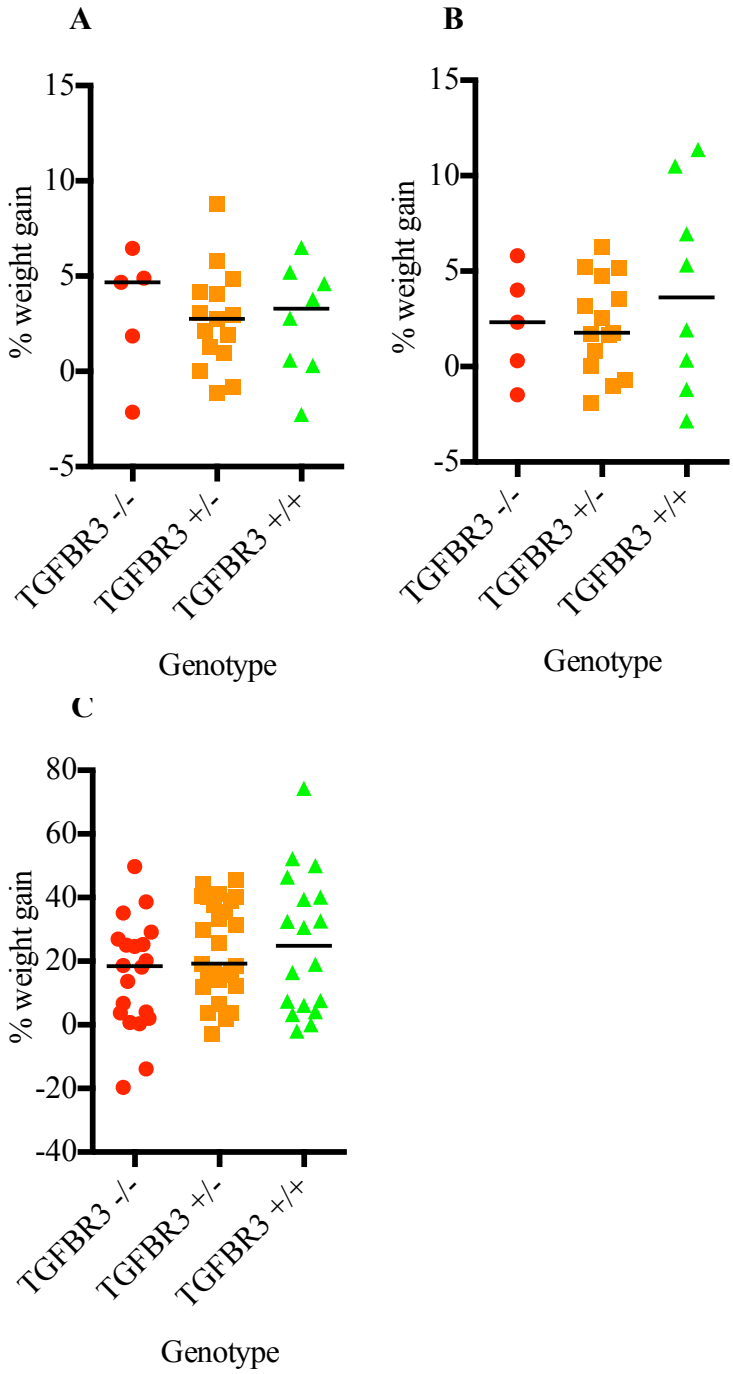
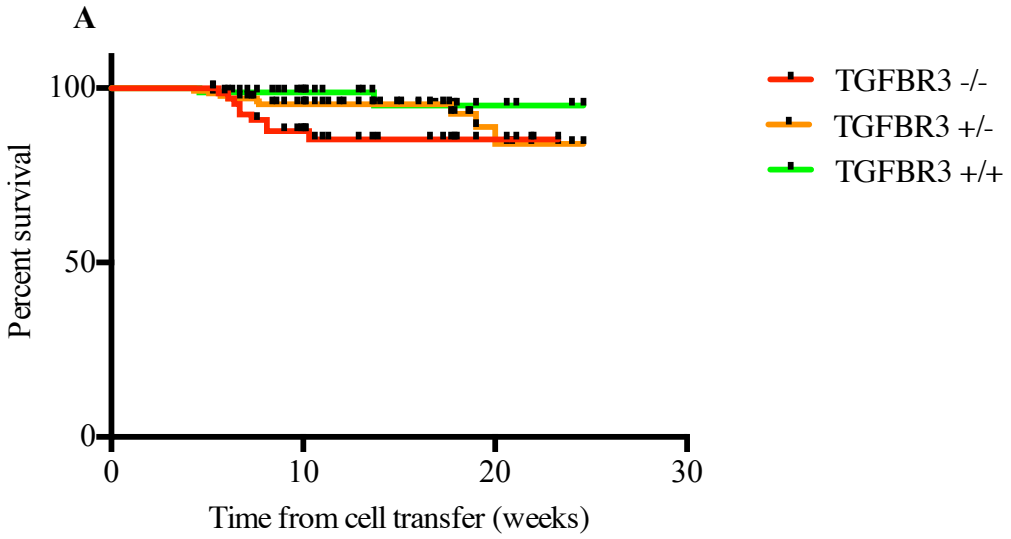


Figure 3.5 Chimera weight gain following irradiation and cell transfer is not affected by betaglycan deficiency. Percentage weight gain shown for **A** 4 weeks post irradiation and cell transfer and **B** 6 weeks post irradiation and cell transfer (data for one experiment involving 3x timed matings shown; n=5 knock out, n=14 heterozygote, n=8 wild type mice), and **C** at schedule 1 cull (all available data pooled). Data points represent individual animals. Line indicates median value; all comparisons non-significant ($p>0.05$) using Kruskal-Wallis test.

A



B

Chimera genotype	*Total*	*%[a]*	*M*	*%[b]*	*F*	*%[b]*
TGFβRIII -/-	70	24.22	39	55.71	31	44.29
TGFβRIII +/-	136	47.06	70	51.47	66	48.53
TGFβRIII +/+	83	28.72	54	65.06	29	34.94
Overall	289	100.00	163	56.40	126	43.60

C

Chimera genotype	*Total*	*%[b]*	*M*	*%[c]*	*F*	*%[c]*
TGFβRIII -/-	9	12.86	3	33.33	6	66.67
TGFβRIII +/-	9	6.62	2	22.22	7	77.78
TGFβRIII +/+	2	2.41	1	50.00	1	50.00
Overall	20	6.92	6	30.00	14	70.00

%[a]: expressed as a percentage of total number of animals
%[b]: expressed as a percentage of total number of animals stratified by genotype
%[c]: expressed as a percentage of total number of animals developing spontaneous illness stratified by genotype

Figure 3.6 No difference in survival is observed between chimeras of different genotype. **A** Survival curves for chimeric mice where survival is defined as absence of spontaneous illness by time of schedule 1 cull for use in an experiment, and summary statistics showing overall prevalence of spontaneous illness stratified by gender and genotype for **B** all animals and **C** only those showing signs of spontaneous illness.

3.4 Discussion

We successfully generated an experimental mouse model in which absolute betaglycan deficiency was observed in all T and B lymphocytes. Experimental animals were viable, with no obvious evidence of immune-mediated disease detected on observation of resulting animal colonies. We were able to demonstrate successful reconstitution of bone marrow haematopoiesis following irradiation and cell transfer, with persistence of donor lymphocyte populations and population of circulating compartments by T and B lymphocytes (Figure 3.3). We can conclude that betaglycan is not an absolute requirement for successful bone-marrow T or B lymphocyte lymphopoiesis, or for the transit of immune cells from the bone marrow to the blood compartment.

We were also able to confirm the persistence of non-T / non-B lymphocyte cell populations of both donor and host origin, confirming the absence of gross defects in bone marrow reconstitution following irradiation (Figure 3.4). Whilst we observed no statistically significant differences in donor:host ratios, we recognised a subtle trend towards impaired reconstitution of donor cells in betaglycan knock-out chimeras, as illustrated by a median ratio of CD45.2:CD45.1 populations of less than 1 for all non-T / non-B lymphocyte populations in knock-out chimeras, compared to a median ratio of greater than 1 for all similar populations in wild-type animals. We speculate that this difference may have become statistically significant with larger sample sizes, raising the possibility that betaglycan may be involved in regulation of these cell populations in the bone marrow.

In experimental models of disease, TGFβ has been implicated in a range of conditions including uveitis (EAU) (Caspi 2003; Caspi et al. 2008), inflammatory bowel disease (experimental colitis) (Fantini et al. 2006) and multiple sclerosis (EAE) (Rangachari & Kuchroo 2013). Whilst we made no specific examination of ocular, gastrointestinal or neural tissues, we observed no external evidence of any of these disease processes. Spontaneous ocular inflammation would be expected to present with ocular surface disturbance and eventual globe perforation, colitis would present with gastrointestinal disturbance and weight loss, and animals suffering encephalomyelitis display well recognised abnormalities of movement and locomotion. No such features were observed in our experimental animals.

This contrasts with other animal models of altered TGFβ signalling. For example, complete knock-out of TGFβ1 through disruption of the TGFβ1 gene results in development of a wasting syndrome accompanied by tissue necrosis around three weeks of age, characterised by a gross phenotypic observations of "dishevelled appearance, hunched posture and skin irritation (Shull et al. 1992)." Death occurs within a few days of developing this phenotype (Kulkarni et al. 1993). Mice deficient in TGFβ2 or TGFβ3 show obvious developmental defects of multiple organ systems, resulting in death in the immediate peri-natal period (Kaartinen et al. 1995; Proetzel et al. 1995; Sanford et al. 1997).

In each of these models, there is complete deficiency of signalling by TGFβ1, 2 or 3; given the widespread functions of TGFβ across multiple organ systems, the severe nature of such deletions is unsurprising, and would not be expected in our model. Gorelik and Flavell developed a murine model with great similarity to ours, in which a dominant-negative TGFβRII was expressed under a T lymphocyte-specific promoter. In this model, the TGFβRII

component of the TGFβ receptor complex was absent on all T lymphocytes, but present on all other cell types (Gorelik & Flavell 2000). Mice expressing the dominant-negative form of TGFβRII were observed to develop normally to 3-4 months of age, at which point they began to develop a spontaneous phenotype of "sickness, wasting and diarrhoea." As expected, targeted disruption of TGFβ signalling to T lymphocytes led to a much milder external phenotype than complete deletion of TGFβ.

Since our betaglycan-deficient chimeras appeared grossly healthy to a maximum age of 6 months post-cell transfer, with no statistically significant difference in percentage weight gain either immediately following cell transfer or at the point of schedule 1 cull (Figure 3.5), we conclude that targeted betaglycan deficiency on T and B lymphocytes does not induce obvious systemic TGFβ-mediated immune disease. This is unsurprising, as betaglycan has primarily been implicated in TGFβ2 signalling (Cheifetz et al. 1990; Sankar et al. 1995; Sarraj et al. 2013), whilst TGFβ1 is the predominant isoform acting in the murine immune system (Rubtsov & Rudensky 2007). The effect of betaglycan deficiency on TGFβ1-dependent T lymphocyte responses was therefore expected to be small, and was not expected to result in an obvious phenotype of spontaneous immune disease.

A slow rate of spontaneous attrition of experimental animals was however observed across our colonies; experimental animals were observed on a daily basis, and recognised evidence of illness or distress including loss of weight, hunched appearance and stereotypical behaviour recorded by trained staff. Animals considered 'unwell' were sacrificed by a UK Home Office-approved schedule 1 method (Bleby 1986). These losses were considered most likely to have resulted from infection by commensal pathogens present in the animal facility.

Experimental colonies were maintained in a specific pathogen free (SPF) environment; this implies that the environment is free of a selection of specified pathogens, but not completely sterile, and thus animals may still be infected by unspecified pathogens.

A statistically significant difference in the rate of spontaneous attrition was observed between animals of different genotype, with increased early attrition of knock-out chimeras compared to wild-type animals. Despite this, the overall prevalence of spontaneous attrition was similar between genotypes by the end of our maximum period of observation. This suggests that knock-out mice displayed an increased susceptibility to commensal pathogens compared to their heterozygote and wild-type litter-mates in the immediate period following irradiation and cell transfer, but overall were no more likely to succumb to disease than heterozygote or wild-type littermates (Figure 3.6).

Our data confirms the presence of CD3+ cell populations in the blood within 6 weeks of cell transfer in all animals, suggesting no gross deficit in immune reconstitution in knock-out chimeras. It is however possible that there was a delay in development of mature effector cells, or a functional defect in the resulting lymphocyte populations which may explain the early attrition of knock-out animals (further investigated in chapter 4).

On closer inspection, we observed that episodes of disease clustered around two time points between November 2011 – February 2012, and August 2012 (data not shown). This suggests there may have been some variation in the level of background commensal pathogens in the animal facility at these times, which may have been sufficient to induce disease in our chimeras. The impact of commensal pathogens on disease development has previously been

reported: In a paper by Goverman et al. a T cell transgenic mouse was created in which to study experimental autoimmune encephalitis (EAE). Two populations of this T cell transgenic mouse model were maintained in two separate animal facilities, yet spontaneous disease was only observed in knock-out mice from one facility. The authors attributed this to differences in environment and the level of local bacterial colonisation, and acknowledged that commensal bacteria may be sufficient to trigger a spontaneous immune response in certain strains of T cell transgenic mice (Goverman et al. 1993).

Based on these results, we concluded that betaglycan deficient mice were viable and showed no obvious external manifestations of immune disease. To our knowledge, this animal model provided the first opportunity to study mature animals with absolute betaglycan deficiency restricted to T and B lymphocytes, and provided a useful basis for further investigation of betaglycan in immune regulation.

4 INVESTIGATING THE ROLE OF BETAGLYCAN IN REGULATION OF RESTING T LYMPHOCYTE POPULATIONS

4.1 Introduction

Whilst betaglycan has a widely reported role in TGFβ2-dependent responses (Bilandzic & Stenvers 2011; Chu et al. 2011; Hanks & Holtzhausen 2013; López-Casillas et al. 1994; López-Casillas et al. 1993), there is a paucity of evidence for its role in TGFβ2 signalling to T lymphocytes. Existing immunological data is limited to the study of foetal thymic organ cultures, demonstrating a role for betaglycan in regulation of lymphocyte maturation and protection of developing lymphocytes from apoptosis (Aleman-Muench et al. 2012). Assessment of the role of betaglycan in mature lymphocyte populations have been limited due to the absence of a suitable model in which to study peripheral immune populations.

In our animal model, betaglycan deficiency is restricted to bone-marrow-derived cell populations of donor origin, whilst all host tissues are betaglycan sufficient. Since our host RAG/BoyJ animals are unable to generate T and B lymphocytes under normal conditions, the resulting chimeras display absolute deficiency of betaglycan in all T and B lymphocytes (since these are derived solely from donor cell populations), and partial betaglycan deficiency in all other bone-marrow-derived cell populations (since these may originate from either donor or host cell populations). This model therefore permits assessment of the role of betaglycan in mature lymphocyte populations.

In order to circumvent the lethality of complete TGFβ receptor component knock-outs and enable investigation of peripheral lymphocyte responses, models of targeted deficiency of TGFβ receptor components have previously been developed and studied. For example, when TGFβRII deletion is restricted to only CD4+ and CD8+ T lymphocytes, a dramatic increase in T lymphocyte activation is observed under resting conditions (Gorelik & Flavell 2000). This is attributed to impairment of the ability of TGFβ to signal to lymphocytes under conditions of TGFβRII deficiency; since TGFβ has a predominantly immunosuppressive effect in the periphery through the induction of iTreg from naïve T lymphocytes (Yoshimura et al. 2010; Li & Flavell 2008; Wan & Flavell 2007; Ming O Li et al. 2006; Chen et al. 2003), an impairment of TGFβ signalling thus appears to have a pro-inflammatory effect.

TGFβRII is required for signal transduction across the cell membrane, being necessary to phosphorylate the kinase domain of TGFβRI (Souchelnytskyi et al. 1996; Kang et al. 2009) which in turn initiates the intracellular signalling cascade through the phosphorylation of Smad proteins (Santibañez et al. 2011). The absence of TGFβRII from T lymphocytes thus completely abrogates TGFβ signalling, and the consequential severity of the auto-inflammatory phenotype is therefore unsurprising. In contrast, betaglycan has no intrinsic enzyme activity, and is considered a co-factor in signal transduction across the TGFβRI / TGFβRII receptor complex. Whilst betaglycan is strongly expressed on CD4+ T lymphocytes and in the placenta (Pakula et al. 2007), it is absent from certain myoblasts, epithelial, endothelial, and haematopoietic cells which still respond to TGFβ suggesting that betaglycan is not an absolute requirement for TGFβ signalling (López-Casillas et al. 1991). Furthermore, betaglycan has been primarily implicated in TGFβ2-mediated signalling, with a seemingly redundant role in TGFβ1 and TGFβ3-mediated processes (Bilandzic & Stenvers 2011; Chu et

al. 2011; Hanks & Holtzhausen 2013; López-Casillas et al. 1994; López-Casillas et al. 1993). Since TGFβ1 is the predominant isoform acting in the immune system (Wan & Flavell 2007), targeted deletion of betaglycan on T and B lymphocytes may thus be expected to result in a far more subtle phenotype of immune dysregulation than that of similarly-targeted TGFβRII deletion.

4.2 Experimental design

We wished to determine whether the absence of betaglycan from the cell-surface TGFβ receptor on T and B lymphocytes resulted in any deficit in the establishment or regulation of the peripheral immune system in our experimental model of targeted betaglycan deficiency. Having proven that donor cells were able to populate circulating compartments with CD3+ T lymphocytes and CD19+ B lymphocytes, we now wished to further phenotype these cells, comparing levels of spontaneous activation between betaglycan deficient and betaglycan sufficient lymphocytes, and comparing relative proportions of T lymphocyte subsets between betaglycan knock-out and wild-type chimeras under resting conditions. Lymphocyte populations were assessed in circulating (blood), splenic and lymph node compartments. For analysis of lymph node populations, cells were pooled from all available nodes (including cervical, brachial, axillary, inguinal and peritoneal lymph nodes).

We also wished to assess whether betaglycan was necessary for the establishment of secondary lymphoid tissues, which would be otherwise absent in RAG/BoyJ hosts, and determine whether betaglycan deficiency in T and B lymphocyte compartments resulted in autoimmunity in chimeric animals.

Experimental animals were culled by a UK Home Office-approved schedule 1 procedure after confirming successful reconstitution, between 6 and 18 weeks following cell transfer. To maximise data collection and avoid wastage of experimental animals, phenotypic analysis of peripheral lymphocyte populations was performed on an opportunistic basis as experimental animals were culled for use in other experiments, with all available results pooled. All experimental animals were included in analysis where cell number permitted. Animals subject to controlled antigenic challenge were not included in this analysis, but were analysed separately (Section 5.3.3).

4.3 Results

4.3.1 Wide variation is observed in the activation level of resting lymphocyte populations in experimental animals

In order to assess the role of betaglycan in the regulation of activation levels of resting lymphocyte populations, cell suspensions were generated from blood, lymph node and spleen according to the method described previously (Section 2.2.3.1) and comparison made between cells of betaglycan knock-out and wild-type origin. Example flow cytometric data is presented in figure 4.1.

To first determine whether there was any deficit in establishment of overall T and B lymphocyte compartments, ratios of CD3+:CD19+ and CD4+:CD8+ lymphocytes were compared between experimental mice of different genotypes. No statistically significant differences or obvious trends were observed, suggesting that there was no underlying deficit in resting populations of either T lymphocytes or B lymphocytes in the absence of betaglycan, and furthermore, betaglycan deficiency resulted in no alteration of gross T lymphocyte subsets at rest (Figure 4.2).

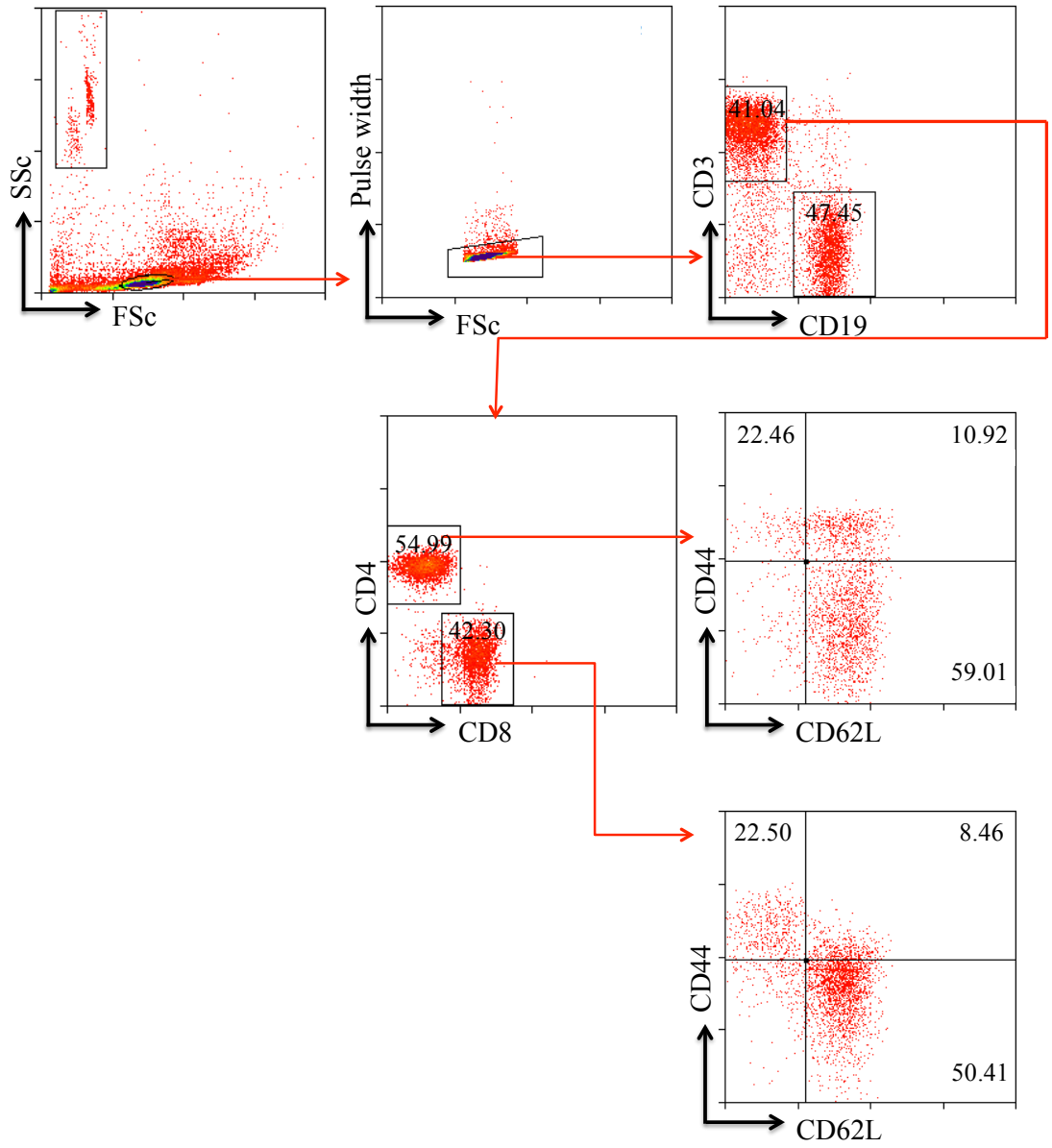


Figure 4.1 Identification of resting lymphocyte activation levels by flow cytometry. Lymphocytes were first identified by their forward scatter (FSc) and side scatter (SSc) profile, doublets excluded by gating on pulse width, T and B lymphocytes identified by gating on CD3+ and CD19+ respectively, T lymphocytes separated to CD4+ and CD8+ lymphocytes and naïve (CD44lowCD62L+) and effector memory (CD44highCD62L-) populations identified for each. Data shown for splenocytes of wild-type chimeric mice at 8 weeks post cell transfer. Numbers in gates represent percentages for displayed sample. FSc / SSc presented on linear axes. All other plots presented on logarithmic axes.

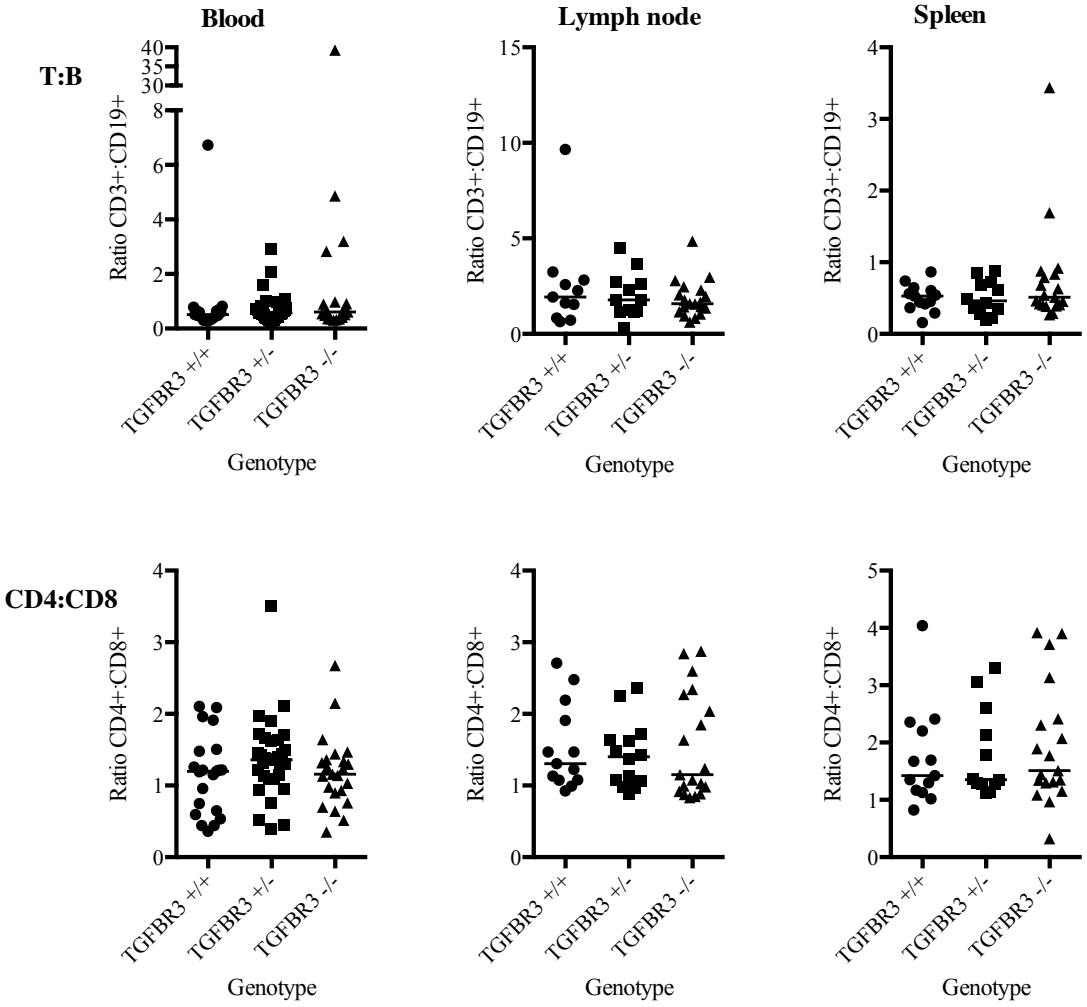


Figure 4.2 Betaglycan deficiency results in no significant alteration in the phenotype of resting lymphocyte populations. Ratios of T lymphocytes (CD3+): B lymphocytes (CD19+) and CD4+:CD8+ T lymphocytes shown for blood, lymph node and splenic populations. Lymph node samples were pooled from all available nodes. Results of flow cytometric analysis by surface staining shown. Pooled data displayed for all animals culled at time points 6-18 weeks post-reconstitution; n=11-21 TGFβRIII+/+, n=11-36 TGFβRIII +/-, n=15-23 TGFβRIII-/-. Data points represent individual animals; line indicates median value. $p>0.05$ for all comparisons using Kruskal-Wallis test with Dunn's multiple comparison post test.

Ratios of cell populations were favoured over percentages, since variable quantities of non-CD3+ / non-CD19+ and non-CD4+ / non-CD8+ populations were demonstrated in cell suspensions by flow cytometry, reducing the calculated percentages of events in CD3+ or CD19+ and CD4+ or CD8+ gates respectively. Presentation of data as ratios of events CD3+:CD19+ and CD4+:CD8+ eliminated the effect of these non-CD3+ / non-CD19+ and non-CD4+ / non-CD8+ populations, and were felt to be more directly comparable between samples than raw percentages.

To next determine whether betaglycan was involved in regulation of activity levels in these resting T lymphocyte populations, proportions of naïve (CD44lowCD62L+), central memory (CD44highCD62L+) and effector memory (CD44highCD62L-) CD4+ and CD8+ T lymphocytes were compared between experimental mice of different genotypes.

When expressed as a percentage of the total T lymphocyte population, a statistically significant increase in the percentage of resident splenic effector memory CD8+ T lymphocytes was observed in TGFβRIII -/- chimeras compared to TGFβRIII +/+ chimeras (median 23.76% and 12.50% respectively, $p<0.05$). Similarly, a non-statistically significant trend towards increased percentages of effector memory CD4+ and CD8+ T lymphocytes and reduced percentages of naïve CD4+ and CD8+ T lymphocytes was also observed in TGFβRIII -/- chimeras compared to TGFβRIII +/+ chimeras across all tissues (Figures 4.3 to 4.4).

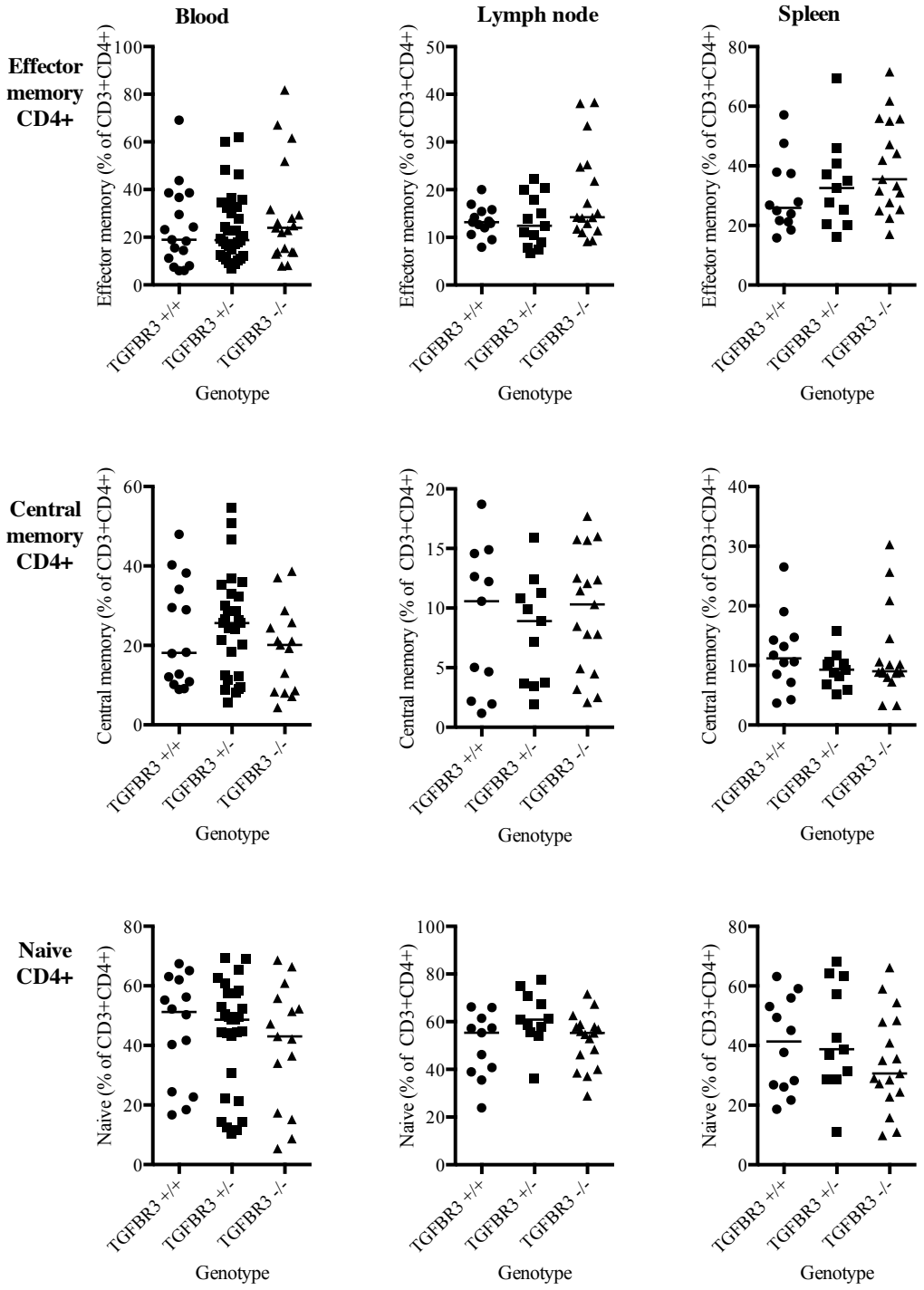


Figure 4.3 Betaglycan deficiency results in no statistically significant difference in CD4+ T lymphocyte activation levels at rest. Relative proportions of effector memory (CD44highCD62L-), central memory (CD44highCD62L+) and naïve (CD44lowCD62L+) CD4+ T lymphocytes shown for blood, lymph node and splenic populations. Lymph node samples were pooled from all available nodes. Results of flow cytometric analysis by surface staining shown. Pooled data displayed for all animals culled at time points 6-18 weeks post-reconstitution; n=11-21 TGFβRIII+/+, n=11-36 TGFβRIII +/-, n=15-23 TGFβRIII-/-. Data points represent individual animals; line indicates median value. $p>0.05$ for all comparisons using Kruskal-Wallis test with Dunn's multiple comparison post test.

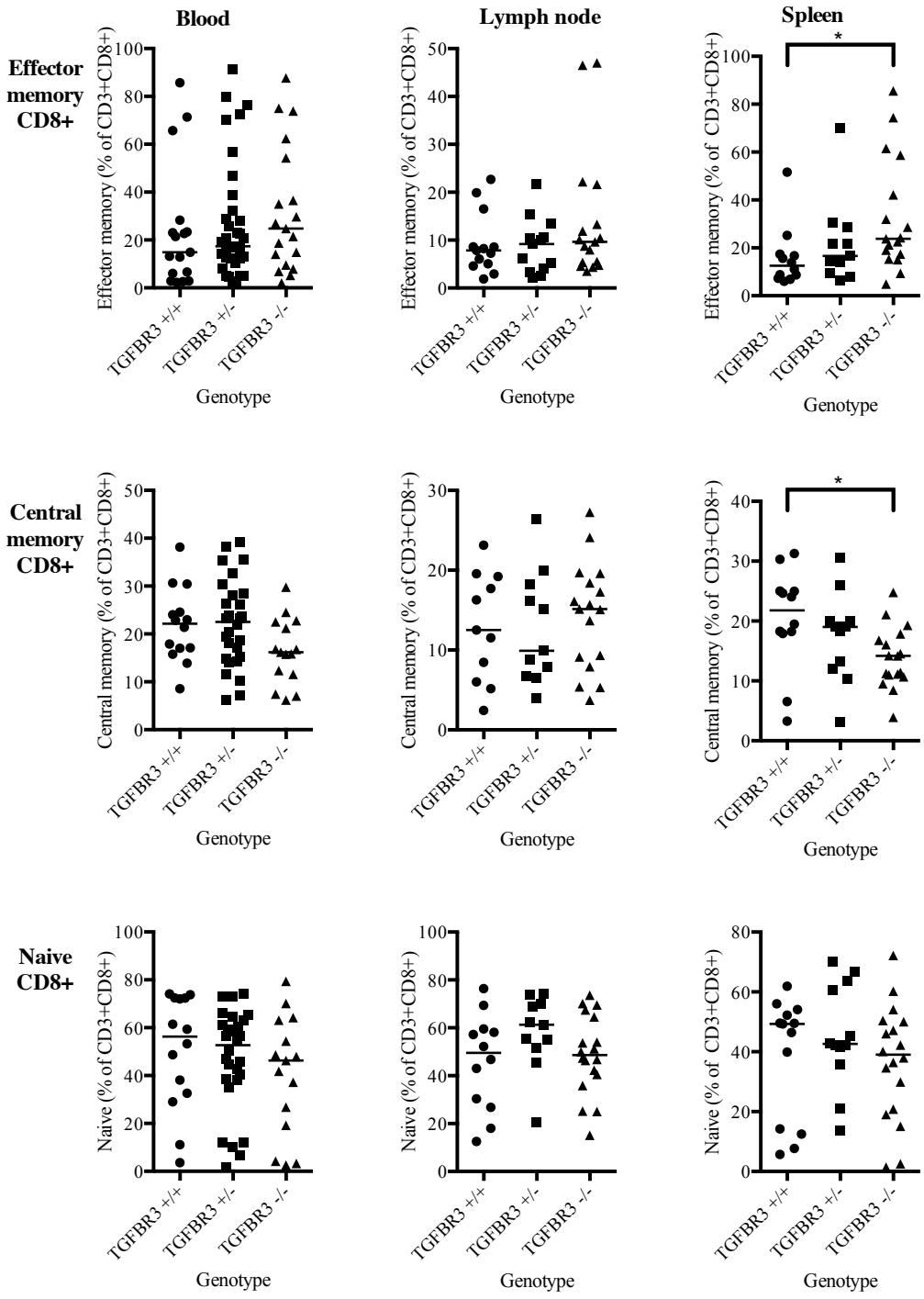


Figure 4.4 Betaglycan deficiency results in a statistically significant increase in splenic CD8+ effector memory populations at rest. Relative proportions of effector memory (CD44highCD62L-), central memory (CD44highCD62L+) and naïve (CD44lowCD62L+) CD8+ T lymphocytes shown for blood, lymph node and splenic populations. Lymph node samples were pooled from all available nodes. Results of flow cytometric analysis by surface staining shown. Pooled data displayed for all animals culled at time points 6-18 weeks post-reconstitution; n=11-21 TGFβRIII+/+, n=11-36 TGFβRIII +/-, n=15-23 TGFβRIII-/-. Data points represent individual animals; line indicates median value. *$p<0.05$ comparisons using Kruskal-Wallis test with Dunn's multiple comparison post test.

Our phenotypic analysis of resident lymphocyte populations revealed the presence of a small number of significant outliers in our data; some experimental animals displayed significantly elevated T:B lymphocyte ratios, elevated CD4:CD8 ratio, increased effector memory CD4+ and CD8+ populations and reduced naïve CD4+ and CD8+ populations. Following calculation of Pearson's product-moment correlation coefficient (Pearson's r), we observed positive correlation between several of these parameters (Table 4.1). In particular, we observed a statistically significant positive correlation between blood T:B lymphocyte ratio and percentage effector memory CD4+ and CD8+ populations in blood (CD4+ r=0.50, CD8+ r=0.47), lymph node (CD4+ r=0.58, CD8+ r=0.70) and spleen (CD4+ r=0.44, CD8+ r=0.69), and strong positive correlation between percentage effector memory CD4+ and CD8+ T lymphocytes throughout all tissues (Pearson r values between 0.59 and 0.94).

Of the seven chimeras with a blood T:B lymphocyte ratio greater than 2.0, four had been identified as showing signs of spontaneous illness and were culled according to UK Home Office regulations (Figure 3.6, discussed in sections 2.2.1 and 3.3.3).

Based on these observations, we conclude that deletion of betaglycan from T lymphocytes results in no significant deficit in resting lymphocyte populations, but may induce a variable state of increased lymphocyte activation at rest.

		Blood				Lymph node					Spleen				
		CD3:CD19		Effector memory (% of CD3+CD4+)		Effector memory (% of CD3+CD8+)		CD3:CD19		Effector memory (% of CD3+CD4+)		Effector memory (% of CD3+CD8+)		CD3:CD19		Effector memory (% of CD3+CD4+)		Effector memory (% of CD3+CD8+)	
		r	p	r	p	r	p	r	p	r	p	r	p	r	p	r	p	r	p
Blood	CD3:CD19			0.50	**	0.47	*	-0.01	NS	0.58	**	0.70	**	0.88	**	0.44	*	0.69	**
	Effector memory (% of CD3+CD4+)					0.91	**	-0.36	*	0.79	**	0.77	**	0.44	*	0.70	**	0.78	**
	Effector memory (% of CD3+CD8+)							-0.41	*	0.71	**	0.66	**	0.37	*	0.59	**	0.71	**
Lymph node	CD3:CD19									-0.31	NS	-0.20	NS	0.08	NS	-0.42	*	-0.33	NS
	Effector memory (% of CD3+CD4+)											0.89	**	0.51	**	0.81	**	0.85	**
	Effector memory (% of CD3+CD8+)													0.72	**	0.81	**	0.94	**
Spleen	CD3:CD19															0.33	NS	0.63	**
	Effector memory (% of CD3+CD4+)																	0.86	**
	Effector memory (% of CD3+CD8+)																		

Table 4.1 Correlation matrix for pooled phenotypic data. Pearson r value (r) and p-value for correlation between T;B (CD3:CD19) ratio, percentage effector memory (CD44highCD62L-) CD4+ and CD8+. NS $p>0.05$ * $p<0.05$, ** $p<0.01$.

4.3.2 Betaglycan deficiency has a variable effect on Th1 polarisation in resting T lymphocyte populations but has no influence on Treg or Th17 proportions

Having demonstrated a variable role for betaglycan in regulation of T lymphocyte activation levels at rest, we next wished to determine whether betaglycan had a role in maintenance of CD4+ T lymphocyte subsets at rest. Cell suspensions were generated from lymph node and spleen according to the method described previously (Section 2.2.3.1) and comparison made between cells of betaglycan knock-out and wild-type origin.

Proportions of Treg (CD3+CD4+FoxP3+) were determined in both naïve and effector memory populations, and proportions of Th1 (CD3+CD4+IL17-IFNγ+) and Th17 (CD3+CD4+IL17+IFNγ-) determined in both naïve (CD44low) and memory (CD44high) populations according to the flow cytometry protocols as previously described (Table 2.5). Since it was necessary to re-stimulate cells prior to staining for secreted cytokines (section 2.2.4.3), CD62L was down regulated for all samples and it was therefore not possible to distinguish between effector memory and central memory populations in these analyses. Example flow cytometry gating strategies are shown in figures 4.5 to 4.6.

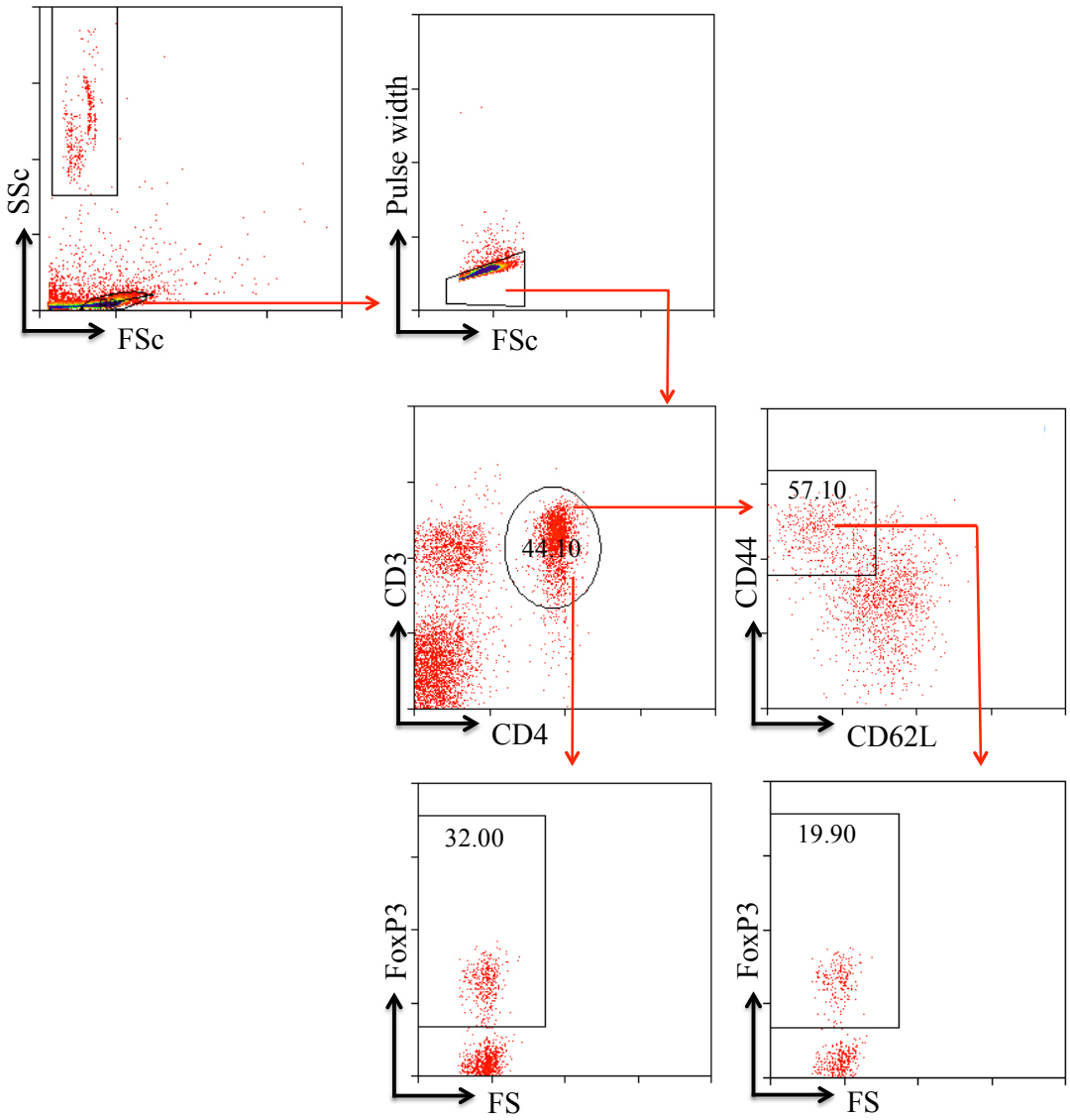


Figure 4.5 Identification of FoxP3+ Treg lymphocyte populations by flow cytometry. Lymphocytes were first identified by their forward scatter (FSc) and side scatter (SSc) profile, doublets excluded by gating on pulse width, CD4+ T lymphocytes identified by gating on CD3+ and CD4+, and FoxP3+ Treg populations identified for both the total CD4+ T lymphocyte population and the effector memory (CD44highCD62L-) populations. Data shown for splenocytes of wild-type chimeric mice at 8 weeks post cell transfer. Numbers in gates represent percentages for displayed sample. FSc / SSc presented on linear axes. All other plots presented on logarithmic axes.

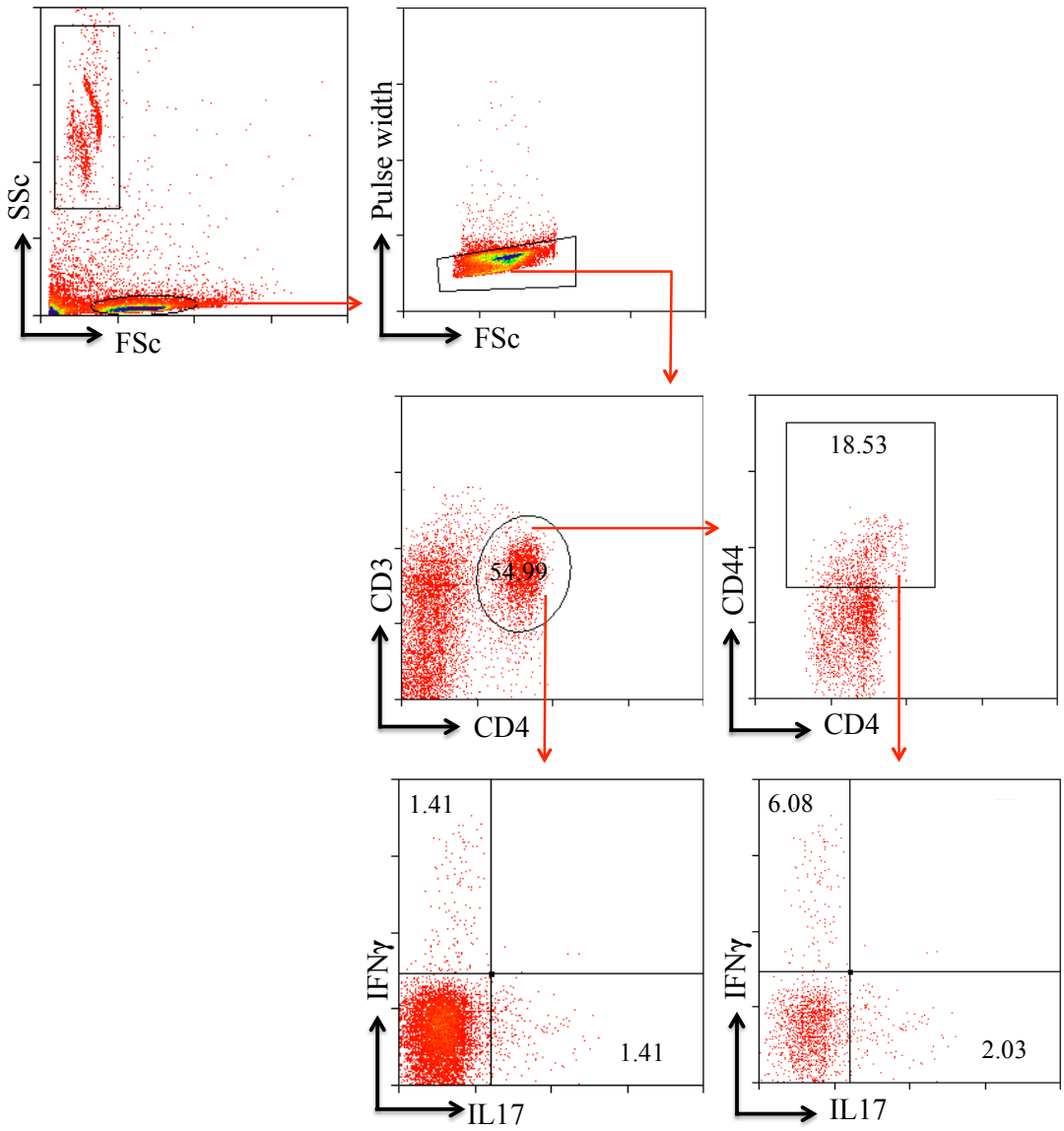


Figure 4.6 Identification of IFNγ+ Th1 and IL17+ Th17 lymphocyte populations by flow cytometry. Lymphocytes were first identified by their forward scatter (FSc) and side scatter (SSc) profile, doublets excluded by gating on pulse width, CD4+ T lymphocytes identified by gating on CD3+ and CD4+, and IFNγ+ Th1 populations and IL17+ Th17 populations identified for both the total CD4+ T lymphocyte population and memory (CD44high populations). Data shown for splenocytes of wild-type chimeric mice at 8 weeks post cell transfer. Numbers in gates represent percentages for displayed sample. FSc / SSc presented on linear axes. All other plots presented on logarithmic axes.

A statistically significant increase in the percentage of resident lymph node IFNγ+ Th1 lymphocytes was observed between TGFβRIII -/- and TGFβRIII +/+ chimeras when expressed both as a percentage of total CD3+CD4+ T lymphocytes (median 2.65% and 1.15% respectively, $p<0.05$) (Figure 4.7), and when expressed as a percentage of only the memory CD3+CD4+CD44+ T lymphocyte population (median 7.10% and 3.00% respectively, $p<0.05$) (Figure 4.8). A similar trend was observed for resident splenic Th1 lymphocyte populations in these analyses, although this did not achieve statistical significance. We observed no statistically significant difference in percentages of FoxP3+ Treg or IL17+ Th17 lymphocytes between genotypes in either spleen or lymph node cell suspensions.

Based on these observations, we conclude that betaglycan has no role in regulation of Treg or Th17 lymphocyte populations at rest, but may have a subtle role in regulation of Th1 lymphocyte subsets, perhaps acting to limit both Th1 polarisation and activation to an effector phenotype under resting conditions.

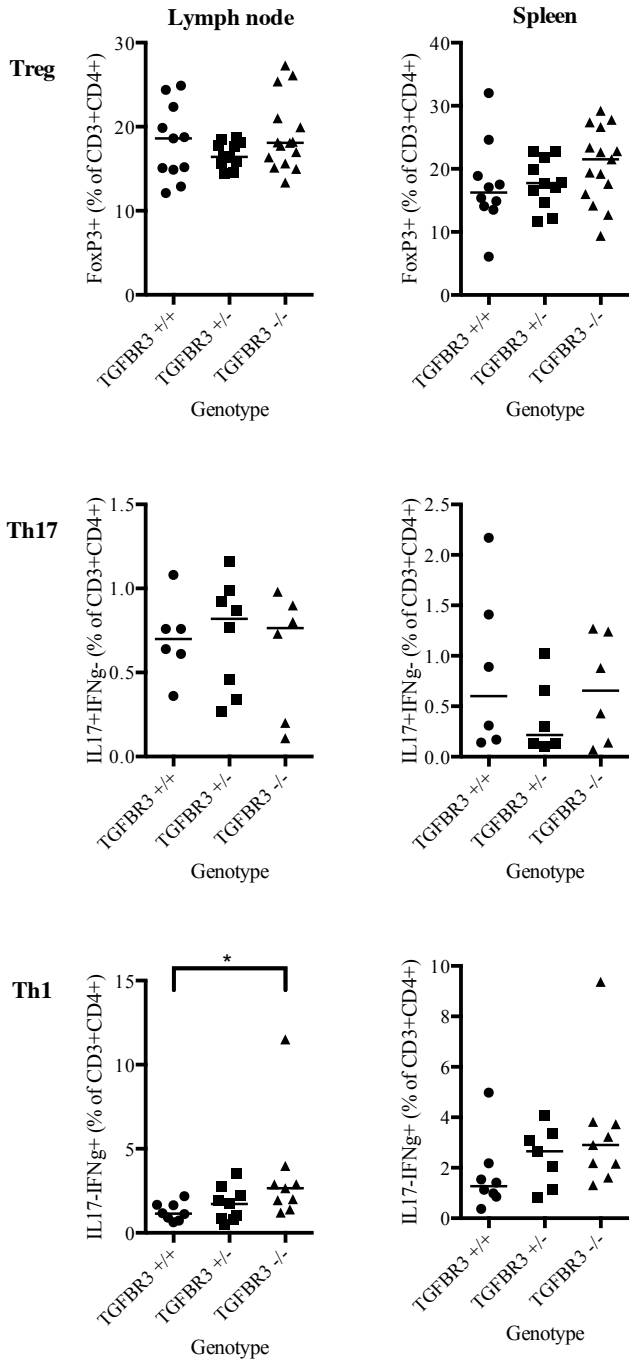


Figure 4.7 Betaglycan deficiency results in a statistically significant increase in lymph node Th1 populations at rest. Relative proportions of Treg (FoxP3+), Th17 (IL17+IFNγ-) and Th1 (IL17-IFNγ+) T lymphocytes shown for blood, lymph node and splenic populations expressed as percentage of total CD3+CD4+ population. Lymph node samples were pooled from all available nodes. Results of flow cytometric analysis by transcription factor (FoxP3) and cytokine (IL17 and IFNγ) staining shown. Pooled data displayed for all animals culled at time points 6-18 weeks post-reconstitution; n=6-11 TGFβRIII+/+, n=6-11 TGFβRIII +/-, n=6-15 TGFβRIII-/-. Data points represent individual animals; line indicates median value. *$p<0.05$ comparisons using Kruskal-Wallis test with Dunn's multiple comparison post test.

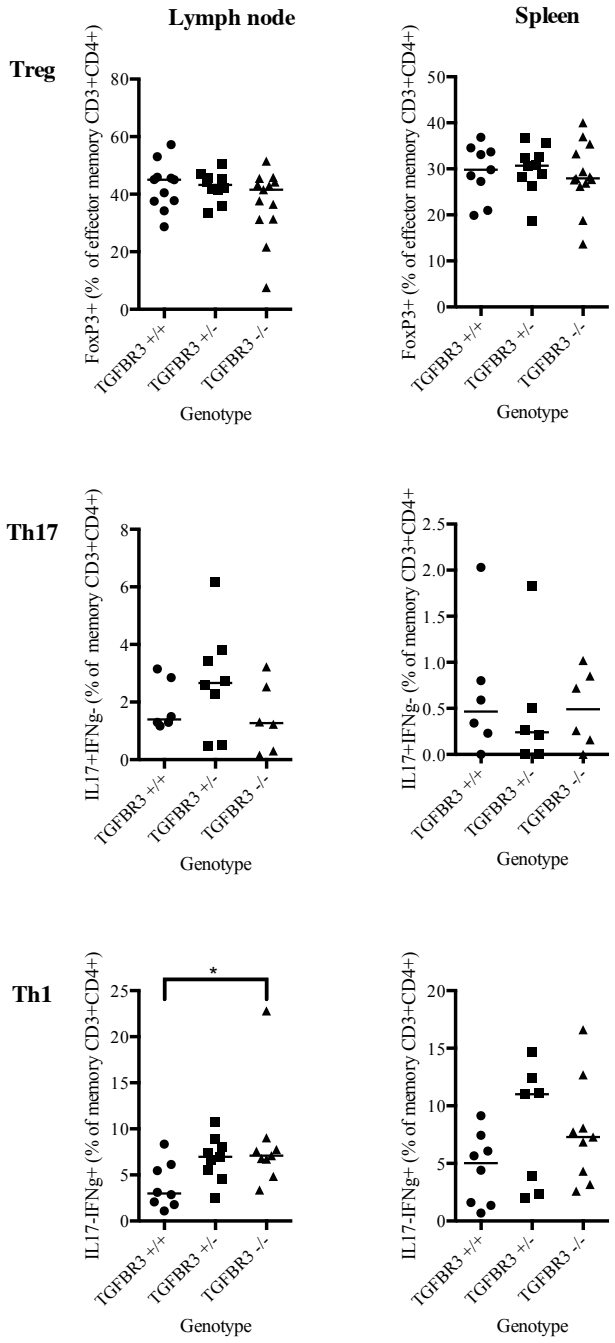


Figure 4.8 Betaglycan deficiency results in a statistically significant increase in lymph node memory Th1 populations at rest. Relative proportions of Treg (FoxP3+) expressed as percentage of the effector memory T lymphocyte population (CD3+CD4+CD44+CD62L-), and Th17 (IL17+IFNγ-) and Th1 (IL17-IFNγ+) expressed as percentage of memory T lymphocyte population (CD3+CD4+CD44+) shown for blood, lymph node and splenic populations. Lymph node samples were pooled from all available nodes. Results of flow cytometric analysis by transcription factor (FoxP3) and cytokine (IL17 and IFNγ) staining shown. Pooled data displayed for all animals culled at time points 6-18 weeks post-reconstitution; n=6-11 TGFβRIII+/+, n=6-11 TGFβRIII +/-, n=6-15 TGFβRIII-/-. Data points represent individual animals; line indicates median value. *$p<0.05$ comparisons using Kruskal-Wallis test with Dunn's multiple comparison post test.

4.3.3 Loss of betaglycan does not result in an autoimmune phenotype

In order to determine whether betaglycan was involved in maintenance of immune tolerance and protection from autoimmune pathology, we wished to assess the level of circulating autoantibodies in our experimental animals, comparing antibody titres between betaglycan knock-out and control animals. Serum from chimeric animals was extracted according to the method described previously (Section 2.2.3.1.2) and tested for the presence of circulating autoantibodies according to the previously described protocol (Section 2.2.5.1). Comparison was made between knock-out, heterozygous and wild-type chimeras (n=4 per genotype), using serum collected from animal 8 weeks post-reconstitution.

No autoantibody staining was observed with serum of TGFβRIII -/-, TGFβRIII +/- or TGFβRIII +/+ chimeras at any of the tested dilutions (Figure 4.9). No differences were observed between serum samples of any two of the tested samples.

These observations suggest that there is no evidence of autoimmune mediated pathology in our betaglycan-deficient chimeras.

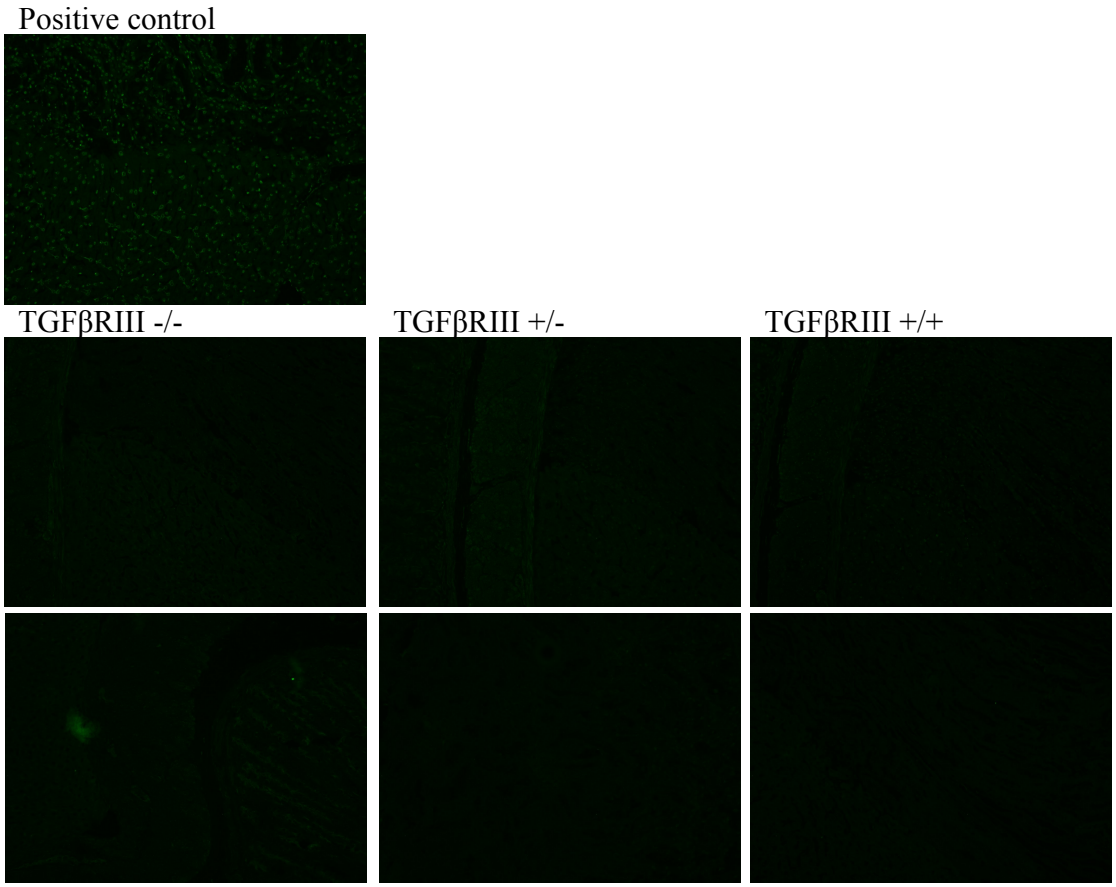


Figure 4.9 Circulating autoantibodies were not demonstrated in the serum of experimental mice. Commercial rat liver, kidney and stomach slides and staining kit (Cambridge Life Sciences, Ely, Cambridgeshire, UK) were used to assess for anti-nuclear, anti-mitochondrial, anti-smooth muscle and anti-gastric parietal cell antibodies in serum from betaglycan chimeric mice. Example tissue sections showing positive control staining with serum from a roquin knock-out mouse and no discernible staining using serum from TGFβRIII -/-, TGFβRIII +/- or TGFβRIII +/+ chimeras at 1/10 dilution (n=4 per genotype, serum extracted at 8 weeks post-reconstitution). For each chimeric sample, two areas of the slide are shown to include stomach (top row), liver (top row) and kidney tissue (bottom row) confirming no staining in any area at 20x magnification.

4.3.4 Secondary lymphoid tissue anatomy is preserved under conditions of betaglycan deficiency

To determine whether betaglycan was necessary for establishment of secondary lymphoid tissues, we investigated splenic and lymph node anatomy in our experimental mice, comparing tissues derived from animals of different genotype.

Our first objective was to determine the cellularity of secondary lymphoid tissues. The size and cellularity of a lymph node varies with the level of immune activation, whilst the size of the spleen remains relatively constant under normal resting conditions. We therefore chose to assess only splenic cellularity in our experimental mice. Cell counts were performed following generation of splenic lymphocyte suspensions according to the method as previously described (section 2.2.3.1).

Splenic cell count was assessed at a range of time points from 6 to 18 weeks and data pooled, demonstrating no statistically significant difference in spleen cell count between knock-out, heterozygous or wild-type chimeras (Figure 4.10). This suggests that betaglycan is not required for establishment or maintenance of splenic cellularity at rest.

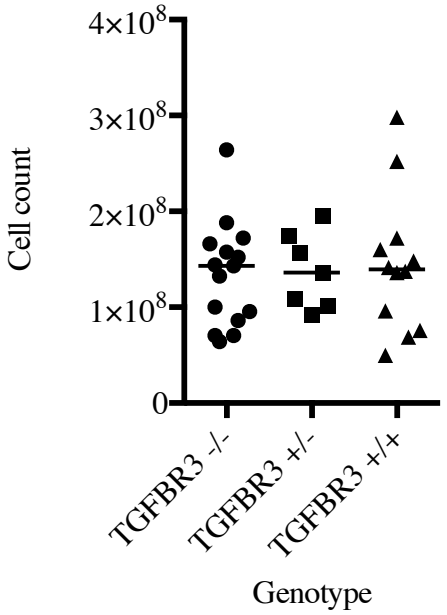


Figure 4.10 Betaglycan is not involved in regulation of splenic cellularity. Column chart illustrating pooled splenic cell count stratified by genotype. Data points represent individual animals; line indicates median value. P>0.05 for all comparisons using Kruskal-Wallis test with Dunn's multiple comparison post test. n=12 TGFβRIII -/-, n=7 TGFβRIII+/-, n=15 TGFβRIII+/+.

Having demonstrated that there was no difference in splenic cellularity between genotype, we next wished to determine whether betaglycan was necessary for establishment of normal tissue micro-anatomy in secondary lymphoid organs.

Spleen and lymph node tissue sections were generated according to the method described previously (Section 2.2.3.2) and stained with a selection of T lymphocyte markers (antibodies to CD3 and CD4), B lymphocyte markers (antibodies to B220, IgM, and IgD) and germinal centres (PNA) according to the previously described method (Section 2.2.5.2). Tissue sections from 4 TGFβRIII -/- chimeras were compared with sections from 4 age-matched control animals (age 12 weeks post-cell transfer, n=2 TGFβRIII +/- chimeras, n=2 TGFβRIII +/+ chimeras). Spleen sections were quantitatively analysed to calculate white pulp, T zone, B cell and germinal centre areas, and co-expression of IgD and IgM as previously described (Section 2.2.5.3)

Chimeric mice developed secondary lymphoid tissues with no obvious architectural defects. T and B-zones were observed in both spleen (Figure 4.11) and lymph node sections (Figure 4.12). On qualitative review, both spleen and lymph node sections appeared anatomically 'normal' across all genotypes, with spleen sections demonstrating identifiable red pulp areas, white-pulp areas, marginal zones and germinal centres, and lymph nodes demonstrating identifiable B lymphocyte (primary lymphoid follicles) and T lymphocyte (paracortical) areas.

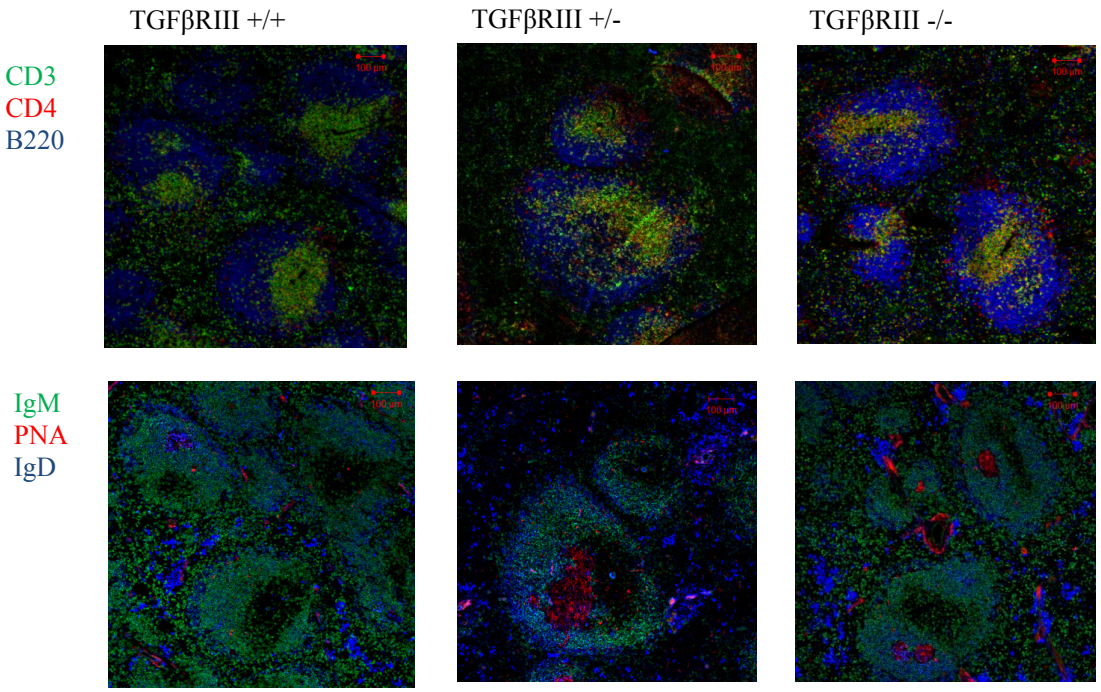


Figure 4.11 Betaglycan is not required for development of normal splenic micro-anatomy. Representative splenic tissue sections from TGFβRIII -/- (n=4), TGFβRIII +/- (n=2) and TGFβRIII +/+ (n=2) chimeras assessed at 12 weeks post cell transfer. Example immunofluorescent staining for CD3 (green) / CD4 (red) / B220 (blue) (top row) and IgM (green) / PNA (red) / IgD (blue) (bottom row). Images acquired at 10x magnification.

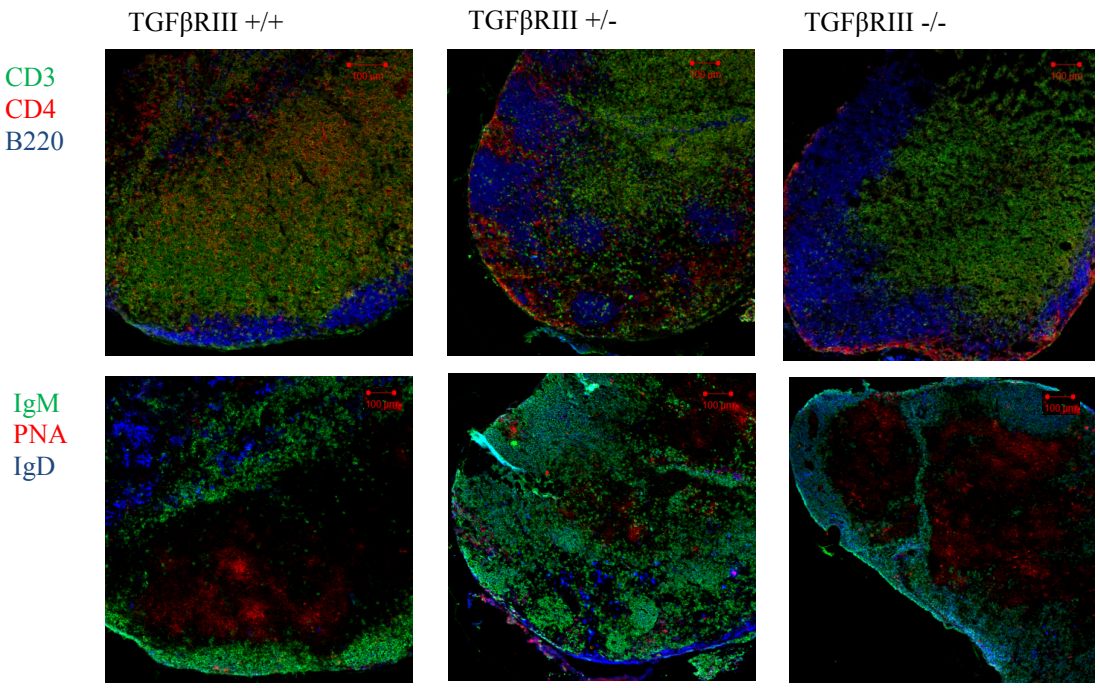


Figure 4.12 Betaglycan is not required for development of normal lymph node micro-anatomy. Representative lymph node tissue sections from TGFβRIII -/- (n=4), TGFβRIII +/- (n=2) and TGFβRIII +/+ (n=2) chimeras assessed at 12 weeks post cell transfer. Example immunofluorescent staining for CD3 (green) / CD4 (red) / B220 (blue) (top row) and IgM (green) / PNA (red) / IgD (blue) (bottom row). Images acquired at 10x magnification.

No statistically significant differences were observed in T zone, B zone or germinal centre area (measured in um^2) between splenic sections from TGFβRIII -/-, TGFβRIII +/- or TGFβRIII +/+ chimeras. Furthermore, no statistically significant difference was observed in co-expression of IgM and IgD between splenic sections from chimeras of different genotype (Figure 4.13).

These observations suggest that betaglycan is not required for development of secondary lymphoid tissues in our experimental mice.

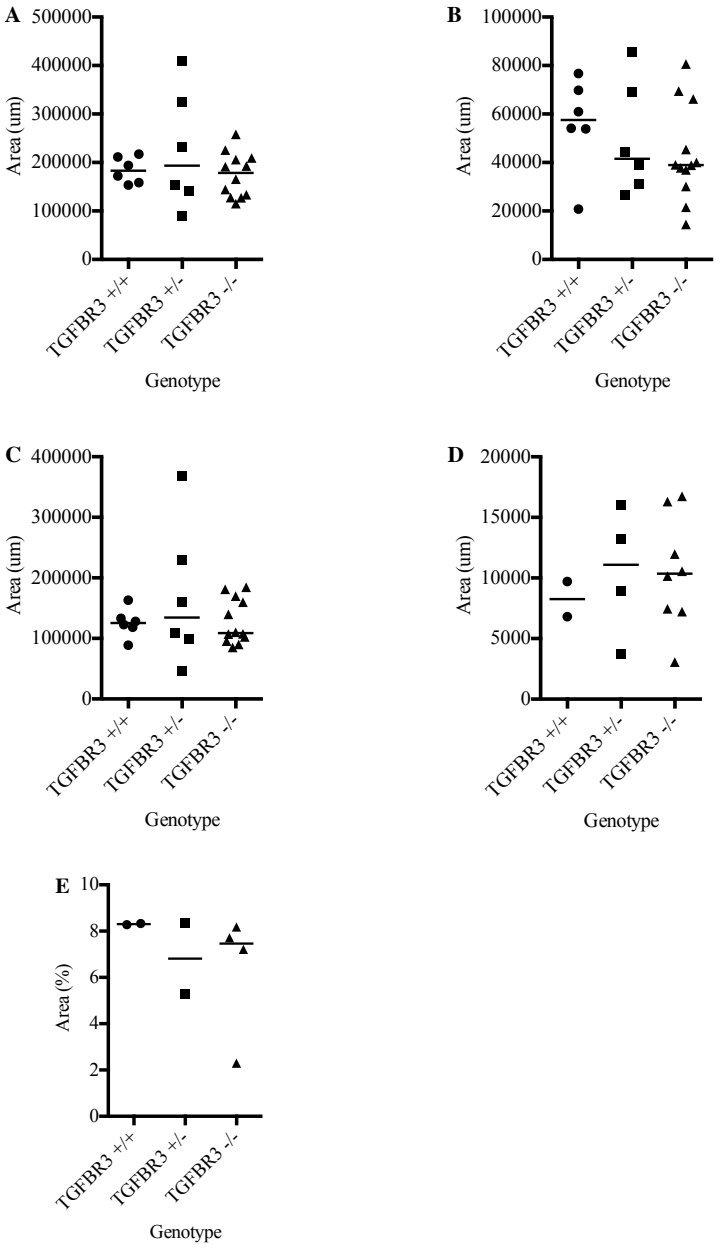


Figure 4.13 No statistically significant differences were observed in quantitative assessment of splenic anatomy between animals of different genotype. Tissue sections from 4 TGFβRIII -/- chimeras, 2 TGFβRIII +/- chimeras and 2 TGFβRIII +/+ chimeras. Two independent assessors each reviewed three pre-determined representative white pulp areas on each slide. Assessors were masked to the genotype of the animals. Measurements of area were accepted if the difference between independent assessors was within 10% of the smaller measurement. For differences greater than 10%, tissue sections were reassessed until agreement was reached. Data points represent the mean average measurement of both assessors; line indicates median value. Data shown for **A** white pulp area **B** T-zone area **C** B-zone area and **D** germinal centre area. **E** Data points represent % total slide area for IgD and IgM co-expression. $p>0.05$ for all comparisons using Kruskal-Wallis test with Dunn's multiple comparison post test.

4.4 Discussion

We present the first data describing the role of betaglycan in lymphocyte responses in an experimental animal model. Under resting conditions we have been able to characterise T lymphocyte activation levels, relative proportions of T lymphocyte subsets and secondary lymphoid organ structure in the presence and absence of betaglycan. We have also been able to assess for the role of betaglycan in development of autoimmune disease.

We observed no statistically significant difference in T:B lymphocyte or CD4:CD8 T lymphocyte ratios across our experimental groups (Figure 4.2). We can therefore conclude that naïve betaglycan-deficient foetal liver chimeras show no obvious deficit in resident proportions of either T or B lymphocytes, or CD4+ or CD8+ T lymphocytes, being phenotypically similar to heterozygous and wild-type chimeras. Betaglycan thus appears to play no role in the establishment of resting lymphocyte populations in naïve animals. We did however observe a statistically significant increase in the percentage of resident splenic effector memory CD8+ T lymphocytes in betaglycan-deficient animals (Figure 4.4), with a trend towards increased percentages of effector memory CD4+ and CD8+ T lymphocytes accompanied by a reciprocal reduction in naïve T lymphocyte percentages across all tissues, although this did not achieve statistical significance (Figures 4.3 to 4.4). This suggests that betaglycan may be involved in regulation of activity levels of resident T lymphocytes, although any potential role appears highly variable, and subtle at best.

This consequences of betaglycan deficiency appear similar to those of targeted TGFβRII deletion on CD4+ and CD8+ T lymphocytes as previously reported (Gorelik & Flavell 2000);

mice expressing dnTGFβRII under the control of a CD4 promoter demonstrated almost complete absence of naïve CD44lowCD62L+ CD4+ and CD8+ T lymphocytes in secondary lymphoid tissues with a dramatic increase in effector memory CD44highCD62L- populations. Over 90% of T lymphocytes displayed a memory phenotype (CD44high) in this model. Similar, less dramatic alteration of activation levels were observed under conditions of betaglycan deficiency, suggesting that betaglycan is involved in similar TGFβ-dependent lymphocyte responses, but has a more minor role in the signalling process.

Significant populations of outliers emerged in our data; these outliers could be observed across all genotypes, and were characterised by increased percentages of CD3+ T lymphocytes, increased percentages of CD4+ lymphocytes with a reciprocal reduction in CD8+ proportions, and increased effector memory CD4+ and CD8+ T lymphocytes with a reciprocal reduction in naïve T lymphocyte populations (Table 4.1). These outliers can thus be described as displaying a state of high immune activation, suggesting the occurrence of sporadic disease within our experimental colonies. As previously discussed (Section 3.4), animals were housed in a 'specific pathogen free' facility, which whilst free of a specified list of potential pathogens was not completely sterile, and animals remained susceptible to infection by other non-specified pathogens. The occurrence of spontaneous immune activation correlates with the observation of a rate of spontaneous attrition across experimental colonies as previously described (Figure 3.6). Furthermore, of the seven animals identified with blood T:B lymphocyte fraction greater than 2.0, four had been culled after being identified as showing external evidence of illness according to Home Office criteria. We therefore conclude that these outliers represent animals succumbing to disease caused by

resident pathogens in the animal facility, and cannot attribute this to the presence or absence of betaglycan on resident T and B lymphocytes.

Even after accounting for these extreme outliers, significant variation was still observed within similar genotypic groups of experimental animals across all experiments, which reduced our ability to draw statistically significant conclusions from our data. This is likely to represent variable reconstitution of our chimeric mice following irradiation and cell transfer. As previously discussed, chimeras were generated through transfer of foetal liver cell suspensions, derived from embryos produced through timed mating between betaglycan heterozygote mice, with donor embryos harvested between day 12 and 14 gestation. The gestational age of the embryos at the point of maternal sacrifice determined the size of the developing liver and the number of cells available for transfer to a RAG/BoyJ host, with animals sacrificed at day 12 having smaller embryonic livers than those sacrificed at day 14.

The process whereby start-dates of successful pregnancies were determined introduced further variation in the number of cells available for transfer. Timed matings were created by technicians working within [redacted]; animals identified as mates were housed together until a vaginal plug was observed in the female, at which point mating was deemed to have taken place. The number of days of gestation were counted from this point, with the day of first observation of the vaginal plug being counted as day zero. Since animals were only usually observed once daily, there was potential for up to a 24 hour delay in noticing the vaginal plug. Furthermore, whilst the presence of a vaginal plug is a reliable indicator of mating, this may not correspond to the day of implantation of a fertilised embryo. This again introduced variation in the size of the embryonic liver by the time of sacrifice.

To maximise the chance of successful reconstitution, the entire cell suspension generated from the foetal liver was transferred to the host RAG/BoyJ mouse; there was no standardisation of the number of cells transferred, with some hosts potentially receiving significantly larger numbers of embryonic stem cells than others. This is likely to have affected the speed and extent of reconstitution of lymphocyte populations following cell transfer, and may further explain the variability in our experimental groups.

A statistically significant increase in the percentage of resident lymph node Th1 lymphocytes was observed in betaglycan deficient chimeras compared to wild-type chimeras, with a similar non-statistically significant trend observed in splenic populations (Figures 4.7 to 4.8). This is consistent with observations of previous murine studies. Experimental autoimmune uveitis (EAU), is characterised by a predominant Th1-driven immune response within the eye (Xu et al. 1997); TGFβ has been shown to suppress both IFNγ production and proliferation of Th1 cells derived from lymph nodes of recently immunised mice with EAU (Xu et al. 2003). Furthermore, TGFβ1 from local T lymphocytes has been shown to down-regulate Th1 differentiation in experimental autoimmune encephalomyelitis (EAE) and experimental colitis (Li et al. 2007; Gutcher et al. 2011). Our data suggests that betaglycan deficiency impairs this immunosuppressive function of TGFβ on Th1 lymphocytes, and provides evidence that betaglycan is therefore involved in TGFβ signalling to lymphocytes.

No statistically significant differences were observed in the percentage of Th17 or Treg lymphocytes in either spleen or lymph node, suggesting that betaglycan is not involved in Th17 or Treg responses in naïve animals (Figure 4.7 to 4.8). These results are surprising, since TGFβ has been implicated in differentiation of naïve T lymphocytes *in vitro* to both

Th17 (Korn, Oukka, et al. 2007; Rubtsov & Rudensky 2007; Mangan et al. 2006; Bettelli et al. 2006; Veldhoen et al. 2006) and Treg (Li & Flavell 2008; Wan & Flavell 2007; Ming O Li et al. 2006; Chen et al. 2003). It is possible that no difference was observed in these subsets as a result of our experimental animals being naïve; in the absence of antigenic challenge it is unlikely that animals would display a strong polarisation of either a pathogenic Th17 or immunosuppressive Treg response, and thus differences between genotypes may exist but not be apparent. This theory is investigated in more detail through controlled antigenic challenge of our animals in section 5.3.3.

The observed differences in lymphocyte subsets do not appear to be a manifestation of autoimmunity, since we were unable to demonstrate the presence of anti-nuclear, anti-mitochondrial, anti-smooth muscle or anti-gastric parietal cell antibodies in the serum of our chimeras at a range of dilutions from 1/10 to 1/160 (Figure 4.9). Furthermore, no significant differences were observed in size or functional anatomy of spleens between animals of different genotypes (Figures 4.10 to 4.11, figure 4.13). Quantitative assessment of lymph node anatomy was not possible due to wide variation in the quality of tissue sections, however again, no obvious gross differences in T or B lymphocyte areas were observed between animals of different genotype (Figure 4.12). The subtle phenotypic differences observed in our betaglycan-deficient chimeras and lack of obvious secondary tissue damage is also in stark contrast to previously published models of autoimmunity, such as those resulting from knock-out of FoxP3 (Brunkow et al. 2001), TGFβ1 (Wahl et al. 2000; Kulkarni et al. 1993; Shull et al. 1992) and CTLA-4 (Waterhouse et al. 1995), each of which results in lethal lymphoproliferative disease in all knock-out offspring. If betaglycan deficiency did induce

autoimmune lymphoproliferative disease, it would be expected in all betaglycan knock-outs, with similar severity in every example.

Collectively, these observations suggest that betaglycan may have a role in immune regulation, potentially facilitating TGFβ signalling in regulation of CD4+ and CD8+ T lymphocyte activation levels, and contributing to the control of Th1 immune responses. The degree of immune dysregulation induced in our betaglycan deficient chimeras was however considered mild, with no gross external evidence of inflammatory disease, and no evidence of inflammatory infiltrates in secondary lymphoid tissues (see also section 3.3.3). The absence of widespread systemic inflammatory disease in betaglycan-deficient chimeras is consistent with our current understanding of the role of betaglycan in TGFβ-mediated processes; since betaglycan has been shown to be predominantly required for signal transduction by TGFβ2 (Cheifetz et al. 1990; Sankar et al. 1995; Sarraj et al. 2013), we would expect betaglycan deficiency to cause significant immune dysregulation in environments where TGFβ2 is the predominant isoform, but not in those where TGFβ1 or TGFβ3 predominates. Since TGFβ1 is thought to be the predominant isoform acting in the murine immune system (Rubtsov & Rudensky 2007), any potential effect of betaglycan deficiency on TGFβ2 signalling is likely to be masked when assessing systemic immune responses.

The interpretation of our experimental findings is limited by the predominantly negative data, and wide variability between genotypically similar experimental animals. We therefore conclude that betaglycan may have a role in regulation of T lymphocyte activation and polarisation, but acknowledge that any potential role for betaglycan in the regulation of resting lymphocyte populations is likely to be subtle.

5 INVESTIGATING THE ROLE OF BETAGLYCAN IN REGULATION OF ACTIVATED T LYMPHOCYTE POPULATIONS

5.1 Introduction

In-vitro assays of the role of TGFβ in lymphocyte biology have demonstrated it to act as a negative regulator of cell proliferation with the ability to induce iTreg differentiation of naïve T lymphocytes; TGFβ was first shown to suppress T lymphocyte proliferation through inhibition of IL-2 receptor expression (Kehrl et al. 1986), and later to induce FoxP3 expression and differentiation of naïve T lymphocytes to a regulatory phenotype (iTreg) (Li & Flavell 2008; Wan & Flavell 2007; Ming O Li et al. 2006; Chen et al. 2003). More recently, it has been suggested that TGFβ dampens the effect of CD28 signalling to T lymphocytes, inhibiting growth and proliferation of CD4+ T lymphocytes (Delisle et al. 2013). Paradoxically, TGFβ is also able to mediate pro-inflammatory lymphocyte responses; when present at lower levels than those necessary for FoxP3 induction, and with co-stimulation by IL-6, TGFβ will induce differentiation of naïve T lymphocytes to a potentially destructive Th17 phenotype (Korn, Oukka, et al. 2007; Rubtsov & Rudensky 2007; Mangan et al. 2006; Bettelli et al. 2006; Veldhoen et al. 2006).

Extrapolation of these *in vitro* observations suggests that lymphocyte responses to TGFβ signalling *in vivo* are highly dependent on the nature of co-stimulation at the time of ligand

binding, enabling both disease and site-specific effects. Indeed, TGFβ is considered a crucial cytokine in diverse disease processes such as uveitis and EAE (Zhou et al. 2012; Zhou et al. 2011; Denniston et al. 2011; Curnow et al. 2005), MS and EAE (Axtell et al. 2010; Issazadeh et al. 1996; O'Connor et al. 2007; Zorzella-Pezavento et al. 2013; Yoshida et al. 2014), and inflammatory bowel disease and murine colitis (Dignass & Podolsky 1993; Babyatsky et al. 1996; Kobayashi et al. 2008; Olsen et al. 2011; Siakavellas & Bamias 2012; Wedebye Schmidt et al. 2013; Mottet et al. 2003; Fantini et al. 2006), with variable involvement of Th17 and Treg CD4+ lymphocytes in all examples. In each of these disease processes, TGFβ mediates its varying effects by signalling through a common cell surface receptor, where intracellular signalling is initiated after phosphorylation of TGFβRI and TGFβRII by TGFβ (Souchelnytskyi et al. 1996; Kang et al. 2009; Santibañez et al. 2011).

The role of betaglycan has not yet been investigated in these processes, either *in vitro* or *in vivo*. It has previously been shown that whilst all isoforms of TGFβ have a similar affinity for betaglycan, TGFβ2 is of significantly lower affinity for TGFβRI and TGFβRII than TGFβ1 or TGFβ3. Furthermore, in cell lines lacking betaglycan, TGFβ2 has reduced potency in growth inhibition assays whilst the relative potency of TGFβ1 and TGFβ3 is unaffected (Cheifetz et al. 1990). It has thus been suggested that betaglycan is necessary for TGFβ2 signalling via the cell surface receptor, acting to concentrate ligand in a favourable conformation at the cell surface, but may be considered unnecessary for signalling by TGFβ1 and TGFβ3. Existing data is however limited to investigation of non-lymphocyte cell lines (López-Casillas et al. 1993; Cheifetz et al. 1990) and the role of betaglycan in TGFβ signalling has not previously been investigated in T lymphocyte-specific assays. Furthermore, due to the lack of a suitable

experimental system, there have been no *in vivo* studies of betaglycan in either site or disease-specific models.

We wished to address these shortcomings; using our novel experimental model of targeted betaglycan deficiency, we first created *in vitro* iTreg polarisation assays using purified lymphocyte populations derived from lymph node and spleen of betaglycan-deficient and betaglycan-sufficient mice. Induction of iTreg from naïve T lymphocytes is accurately quantified by assessment of FoxP3 expression (Vignali et al. 2009); FoxP3 induction in naïve T lymphocytes can thus be considered a surrogate marker of successful TGFβ signalling. We therefore compared FoxP3 induction in naïve T lymphocytes by all isoforms of TGFβ in the presence and absence of betaglycan.

We next wished to assess the role of betaglycan in TGFβ-dependent T lymphocyte responses *in vivo*, using an experimental model of controlled antigenic challenge in which Treg and Th17 polarisation could be compared between betaglycan-deficient and betaglycan-sufficient animals. To determine the most appropriate model in which to assess Treg and Th17 responses, a series of pilot experiments were initially performed assessing T lymphocyte induction following antigenic challenge by 2W1S peptide and adjuvants of either attenuated *L. monocytogenes*, IFA or *S. typhi* porins according to the methods described in section 2.2.6.

L. monocytogenes is a gram-positive bacterium which is widely used as an adjuvant in antigenic challenge in mammals, allowing detailed study of the immune response to bacterial infection. It is known to induce a strong CD8+ T lymphocyte response, with a relatively small CD4+ T lymphocyte component (Pamer 2004). This model was not expected to induce

a strong Th17 or Treg response; it was however included in pilot studies as a baseline assessment with which to compare more targeted antigenic challenges.

IFA has been widely used as an adjuvant in experimental protocols for induction of immune disease in laboratory animals; it is an emulsion of paraffin oil containing mannide mono-oleate as a surfactant (Billiau & Matthys 2001). It is known to induce a strong CD4+ Th1 / Th2 response (Chang et al. 1998), and has been shown to induce Th17 cells in C57BL/6 mice (Nian et al. 2012).

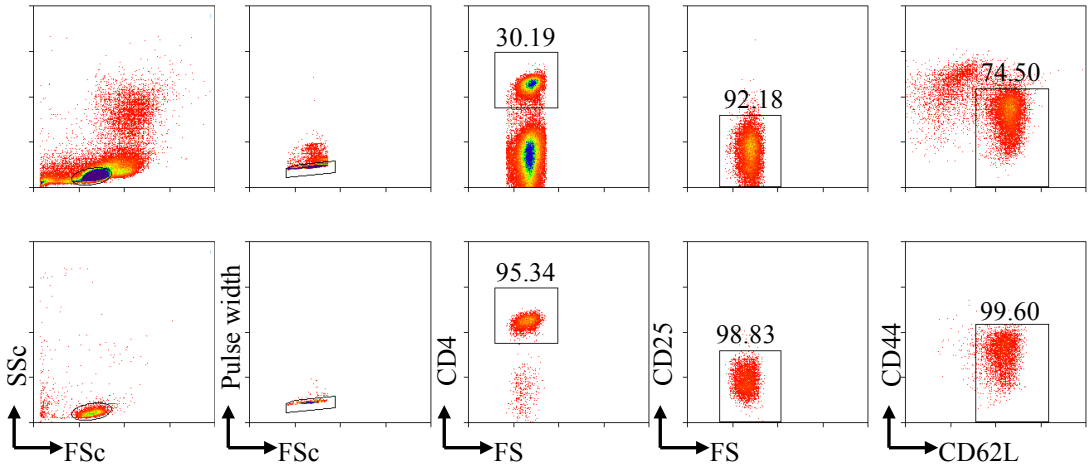
Many gram-negative bacteria express outer membrane proteins, which are highly immunogenic and able to elicit strong immune responses in mice (Singh et al. 1999; Isibasi et al. 1992; Secundino et al. 2006; Cervantes-Barragán et al. 2009). In humans, antibodies against pore forming outer membrane proteins (porins) can be found in patients recovering from *S. typhi* infection, which can be purified and produced on a large scale (Salazar-González et al. 2004). Such porins have been demonstrated to promote CD4+ T lymphocyte responses when used as an adjuvant in antigenic challenge of C57BL/6 mice, characterised by production of both IFNγ and IL-17 (Perez-Shibayama et al. 2014).

5.2 Experimental design

For all *in vitro* polarisation assays, cell suspensions were generated from lymph node and spleen according to the method described in section 2.2.3.1. For all experiments, betaglycan knock-out (KO) samples were pooled from between 3-6 TGFβRIII -/- chimeras, and betaglycan wild-type (WT) samples pooled from between 3-6 TGFβRIII +/+ chimeras, enabling between 2-6 replicates for each culture condition depending on total cell number available. All experimental animals were matched for age and gender. Naïve T lymphocytes were stimulated and grown under the iTreg polarising conditions as described in section 2.2.7.2 and FoxP3 assessed at day 4. Cells were cultured with individual isoforms of TGFβ (1,2 or 3) at physiological dilutions (3.0ng/ml to 0.1ng/ml at Log3 dilutions). Control conditions of un-stimulated cells and cultures lacking TGFβ were also assessed.

Naïve CD4+ T lymphocytes were initially purified using the MACS CD4+CD62L+ T Lymphocyte II kit according to the previously described method (Section 2.2.7.1) however the resulting purity of cell samples was deemed to be unacceptably low, with a mean post-sort purity of naïve CD4+ T lymphocytes of 59.15%, and a lowest recorded purity of 20.87%. As a result, purification was subsequently performed by FACS according to the method described previously (Section 2.2.7.1) resulting in a mean purity of naïve CD4+ T lymphocytes of 89.14%, and a minimum purity of 77.19% (Figure 5.1).

A



B

Sort type		n	% CD4+	% CD25-	% naïve CD4+ CD25-
MACS	*Pre*	5	21.93 (8.81 – 46.67)	21.05 (7.31 – 46.64)	15.56 (3.74 – 34.21)
FACS	*Pre*	11	17.55 (3.41 – 28.39)	24.59 (2.09 – 78.53)	19.04 (1.68 -52.32)
MACS	*Post*	5	66.27 (24.49 – 91.62)	65.55 (22.87 – 91.58)	59.15 (20.87 – 90.71)
FACS	*Post*	11	93.77 (84.04 – 97.77)	89.57 (77.52 – 96.14)	89.41 (77.19 – 96.35)

Figure 5.1 Identification of naïve CD4+ T lymphocytes by flow cytometry and purity of sorted cells. A Example flow cytometry data shown for pooled cell sample pre-sort (top row) and post-sort (bottom row) for FACS-sorted wild-type cells. Cell suspension generated from lymph node and spleen as previously described (section 2.2.3.1). Live lymphocytes were first identified according to their forward-scatter (FSs) / side-scatter (SSs) profile, doublets excluded according to pulse-width, then CD4+ populations identified by positive selection. Naïve cells were identified first by lack of CD25 expression (CD25-), and further defined by positive selection of L-selectin positive CD44 low populations (CD44lowCD62L+). Numbers in gates represent percentages as a proportion of events on each individual plot for displayed sample. FSc / SSc presented on linear axes. All other plots presented on logarithmic axes. **B** Mean purity (range shown in brackets) of CD4+, CD25-, and naïve (CD44lowCD62L+) CD4+CD25- cell populations pre- and post-sort, comparing MACS and FACS sorted populations. Percentages expressed as a proportion of total cell population. n=number of samples analysed.

T lymphocyte responses in chimeric mice were assessed following controlled immune challenge according to the methods described previously (Section 2.2.6). For all immunisations, a known quantity of adjuvant was administered with a known quantity of 2W1S peptide antigen; this allowed assessment of both the peptide-specific T lymphocyte response (quantifying T lymphocyte responses specific for the 2W1S peptide, by staining cells with a fluorochrome-labelled 2W1S:I-A^b tetramer), and the non-specific T lymphocyte response.

To avoid wastage of experimental animals, models of antigenic challenge were first tested in non-chimeric C57BL/6 mice to assess the efficacy of each adjuvant in induction of TGFβ-dependent T lymphocyte responses. For these experiments 3-4 mice were immunised with antigen, and 3-4 age-matched mice immunised with PBS as control. Experiments were performed on two separate occasions and results pooled. All immune responses were assessed at day 7 following immunisation.

For experiments involving chimeric mice, comparison was made between age-matched TGFβRIII +/+ (WT) and TGFβRIII -/- (KO) chimeras. For all experiments, 3 KO and 3 WT chimeric mice were immunised with antigen and adjuvant. In addition, 1-2 WT chimeras were immunised with PBS as negative controls, and 1-2 non-chimeric C57BL/6 mice were immunised with antigen and adjuvant as positive controls. All experiments were performed on two separate occasions and results pooled. All immune responses were assessed at day 7 following immunisation.

5.3 Results

5.3.1 Betaglycan is not required for TGFβ-mediated iTreg induction in naïve T lymphocytes

To first confirm viability of the assay and assess the effect of varying levels of stimulation, wild-type cell proliferation assays were created under iTreg polarising conditions as described in section 2.2.7.2 with anti-CD3 / anti-CD28 coated activation beads at a range of ratios from 2:1 to 1:4 beads:cells, and TGFβ1 added across a range of physiological dilutions (Log3 dilutions from a maximum of 3.0ng/ml to a minimum of 0.1ng/ml). Results were compared to control conditions in the absence of both TGFβ and activation beads. Cells were stained with an eF450 proliferation dye and proliferation assessed at day 4 (Figure 5.2).

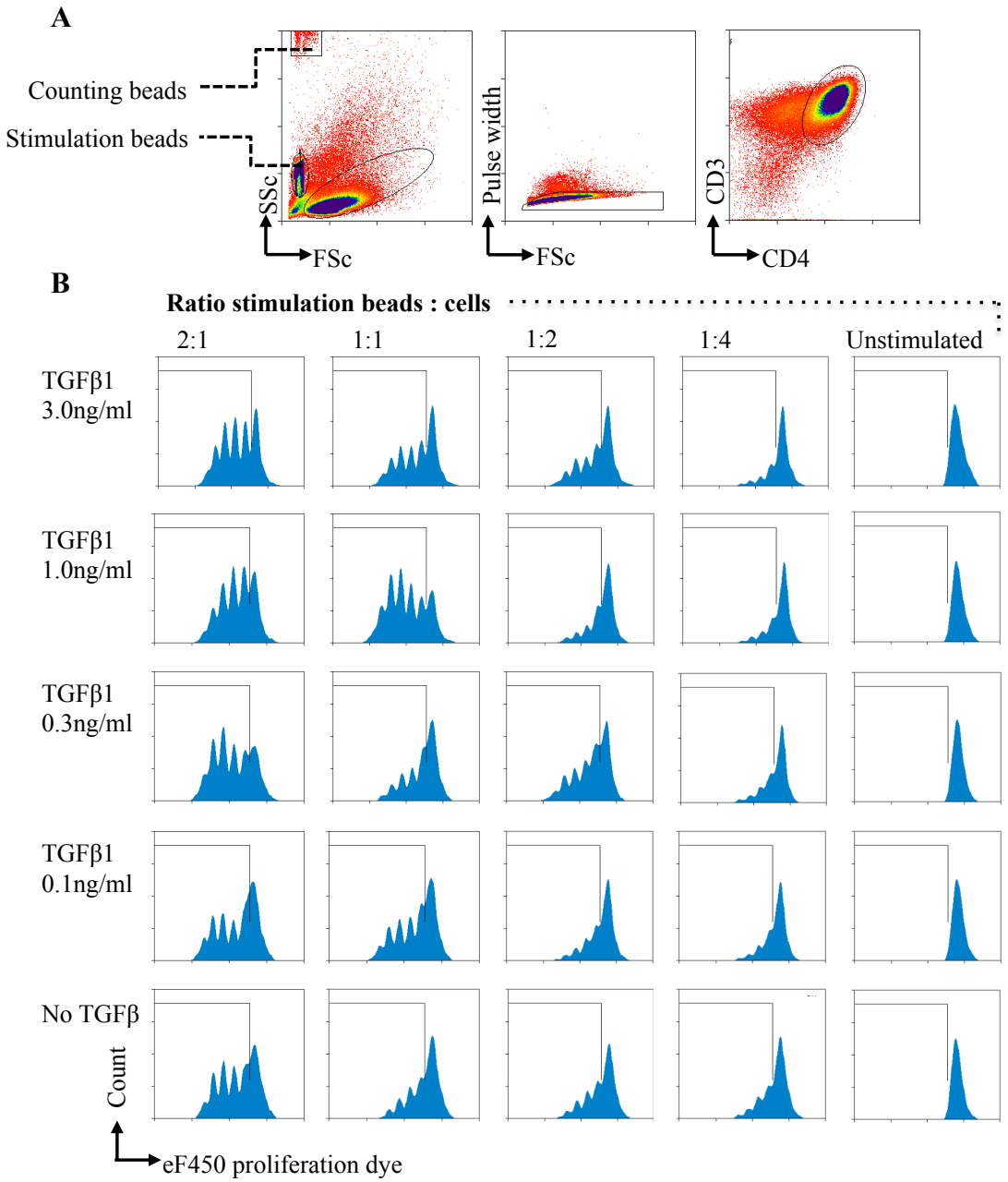


Figure 5.2 Proliferation of wild-type naïve CD4+ T lymphocytes under varying levels of stimulation with Log3 dilutions of TGFβ1. Wild-type lymph node cell suspensions derived from C57BL/6 mice and FACS sorted to naïve CD4+CD25-CD44lowCD62L+ population stimulated with anti-CD3 / anti-CD28 coated activation beads at range of ratios shown. IL2 added to all cultures at concentration of 50U/ml. TGFβ added at Log3 dilutions from 3.0mg/ml to 0.1ng/ml. Control conditions in absence of activation beads and TGFβ also shown. Proliferation assessed at day 4. **A** Flow cytometry gating strategy used for identification of proliferating lymphocytes in all *in vitro* assays: Live lymphocytes were first identified according to their forward-scatter (FSc) / side-scatter (SSc) profile, doublets excluded according to pulse-width, then CD4+ T lymphocytes identified by positive selection of CD3+CD4+ cells. FSc / SSc presented on linear axes. All other plots presented on logarithmic axes. **B** Cell proliferation was assessed at day 4 by reducing intensity of eF450 proliferation dye staining. Proliferation presented on linear scale.

Level of stimulation was determined by the percentage of proliferating cells, and differential response to varying concentrations of TGFβ assessed by calculating the range of percentage proliferating cells for each level of stimulation (maximum – minimum) (Table 5.1).

Whilst a ratio of 2:1 beads:cells achieved the highest levels of stimulation (65.41% – 71.73%), the differential response to varying concentrations of TGFβ was small (9.62%), suggesting that this level of stimulation was sufficient to overcome any subtle influence of additional TGFβ. The greatest differential response was observed with a ratio of 1:1 beads:cells (34.55%), with stimulation ranging from 44.40% - 59.32%%. Both level of stimulation and differential response decreased with lower levels ratios of beads:cells.

Interestingly, the number of proliferating cells increased with the addition of TGFβ in most assays, with the greatest number of proliferating cells observed on the addition of TGFβ at a concentration of 1.0ng/ml and a 1:1 ratio of beads:cells. This is perhaps surprising since TGFβ is usually considered a potent suppressor of lymphocyte proliferation (Kehrl et al. 1986), and most likely represents an effect of TGFβ-restriction of activation-induced cell death (Chen et al. 2001; Sanjabi et al. 2009).

A ratio of 1:1 stimulation beads:cells was therefore adopted for all subsequent assays, with cells grown under iTreg polarising conditions as described previously (Section 2.2.7.2), and FoxP3 induction assessed at day 4.

Conc. TGFb1 (ng/ml)	Ratio stimulation beads : cells									
	2:1		1:1		1:2		1:4		0	
	%	*n*	*%*	*n*	*%*	*n*	*%*	*n*	*%*	*n*
3.00	71.73	81657	59.32	57438	51.29	44552	29.00	19862	3.67	2151
1.00	72.75	72026	78.95	111238	39.42	24834	31.26	19841	1.89	1030
0.30	75.03	76393	52.21	42171	61.33	54220	36.77	23939	2.04	928
0.10	65.72	45336	58.48	41894	40.70	25917	34.22	18642	1.83	829
0.00	65.41	41328	44.40	27922	44.01	30480	36.13	26829	2.00	1529
Range	**9.62**	**35065**	**34.55**	**83316**	**21.91**	**29386**	**7.77**	**4077**	**1.84**	**1322**

Table 5.1 Cell proliferation increases with the concentration of activation beads whilst increasing concentration of TGFβ1 has variable effects. Table illustrating percentage and absolute number of proliferating cells in iTreg cultures used for assay optimisation. Wild-type lymphocytes derived from C57BL/6 mice and FACS sorted to naïve CD4+CD25-CD44lowCD62L+ population stimulated with anti-CD3 / anti-CD28 coated activation beads at range of ratios shown. IL2 added to all cultures at concentration of 50U/ml. TGFβ added at Log3 dilutions from 3.0mg/ml to 0.1ng/ml. Control conditions in absence of activation beads and TGFβ also shown. Proliferation assessed at day 4. Range of values for percentage and absolute cell number shown for each level of stimulation (n=1 for each culture condition).

In order to determine whether betaglycan was necessary for TGFβ signalling to in naïve T lymphocytes *in vitro*, FoxP3 induction was first assessed by all isoforms of TGFβ at a concentration of 1.0ng/ml TGFβ. Sample flow cytometry data is shown in figure 5.3 (gating strategy as shown in figure 5.2). Data for three individual experiments is presented in figure 5.4, in addition to graphical representation of pooled data for percentage FoxP3+ cells in cultures from all three experiments combined. Data for cell number could not be pooled due to the significant variability between experiments performed on different days.

FoxP3 induction was demonstrated with all isoforms of TGFβ in both WT and KO cell samples for TGFβ concentrations of 1.0ng/ml. Whilst there was a general trend towards TGFβ2 being less potent than either TGFβ1 or TGFβ3, no obvious trends were observed in the difference between the response of KO and WT cells to any isoform of TGFβ. Furthermore, no statistically significant difference was observed in either percentage or absolute number of FoxP3+ T lymphocytes between KO and WT cultures in response to any isoform of TGFβ using the Mann-Witney U-test (Figure 5.4).

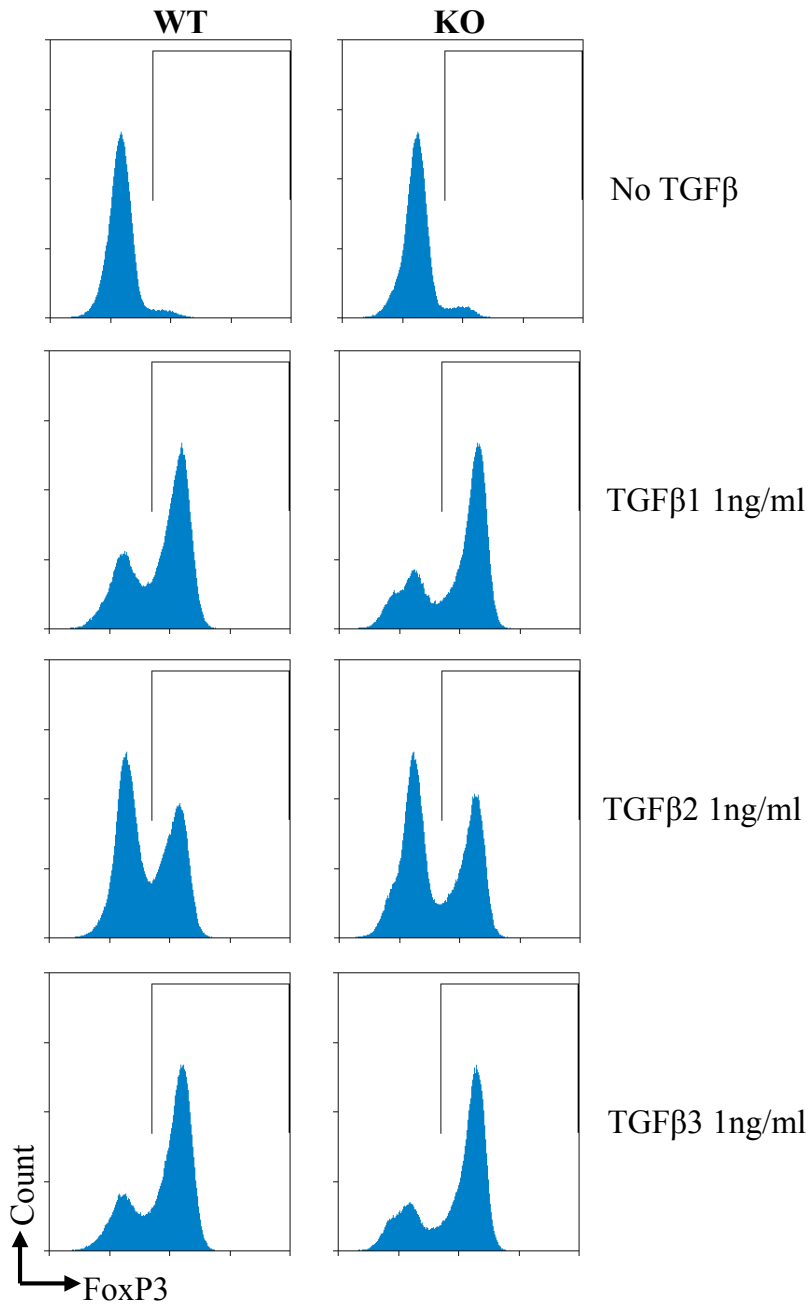


Figure 5.3 FoxP3 induction by TGFβ in naïve CD4+ T lymphocytes. Representative plots illustrating frequency of FoxP3+ iTreg lymphocytes in polarisation assays. Lymphocytes identified according to gating strategy shown in figure 5.2. **WT** Wild-type lymphocytes derived from lymph nodes of TGFβRIII +/+ chimeras **KO** Knock-out lymphocytes derived from lymph nodes of TGFβRIII -/- chimeras. Cells FACS sorted to naïve CD4+CD25-CD44lowCD62L+ population and stimulated with anti-CD3 / anti-CD28 coated activation beads at a ratio of 1:1 beads:cells. IL2 added to all cultures at concentration of 50U/ml. TGFβ1, 2 and 3 added at concentration of 1.0ng/ml. Control conditions in absence of TGFβ also shown. Proliferation assessed at day 4. Range of values for percentage and absolute cell number shown for each level of stimulation (n=2-6 for each culture condition).

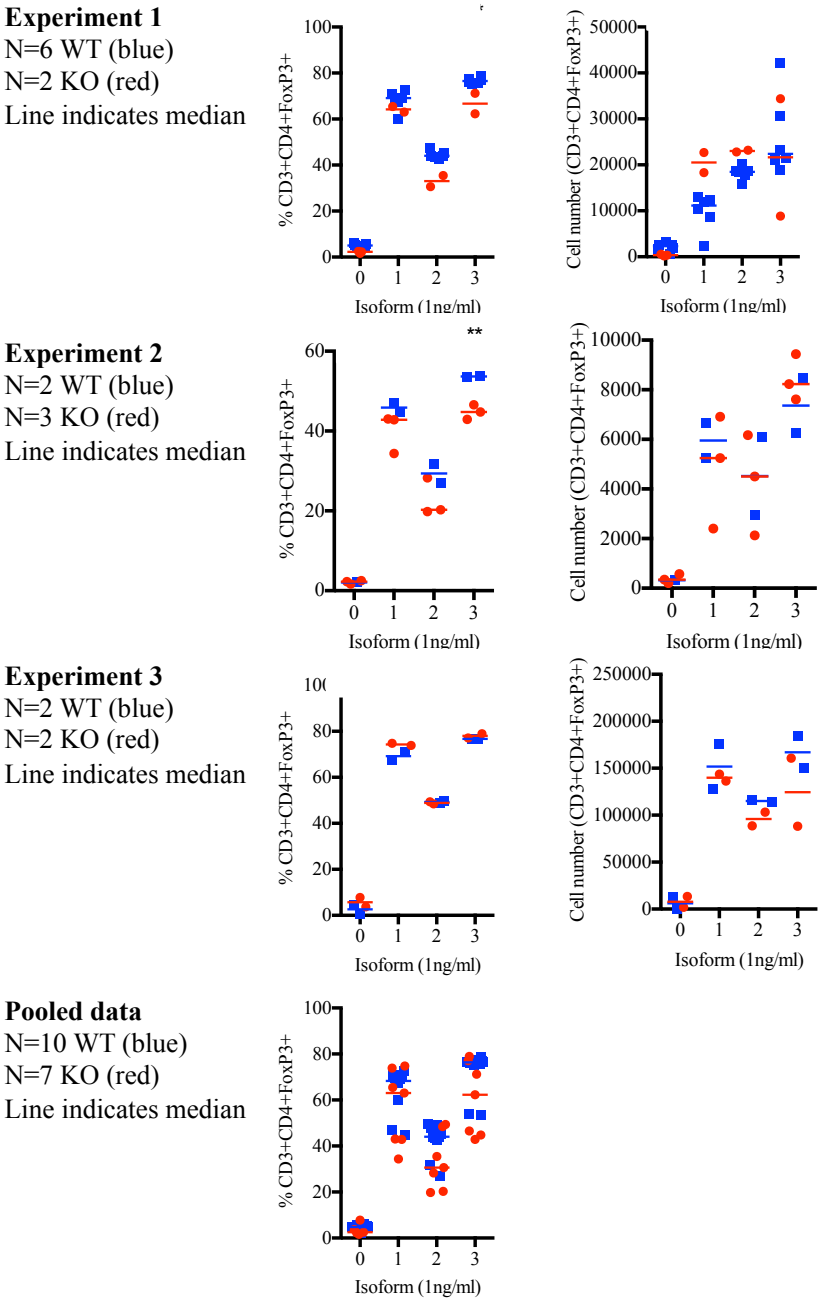


Figure 5.4 Betaglycan is not required for TGFβ-mediated FoxP3 induction in naïve CD4+ T lymphocytes *in vitro* (1). Column charts illustrating percentage FoxP3 induction (left) and absolute number of FoxP3+ CD4+ T lymphocytes (right). Data shown for 3 separate experiments (top 3 rows) and pooled data presented (bottom row). Lymphocytes identified according to gating strategy shown in figure 5.2. WT lymphocytes derived from lymph nodes of TGFβRIII +/+ chimeras KO lymphocytes derived from lymph nodes of TGFβRIII -/- chimeras. Cells FACS sorted to naïve CD4+CD25-CD44lowCD62L+ population and stimulated with anti-CD3 / anti-CD28 coated activation beads at a ratio of 1:1 beads:cells. IL2 added to all cultures at concentration of 50U/ml. TGFβ1, 2 and 3 added at concentration of 1.0ng/ml. Control conditions in absence of TGFβ also shown. Proliferation assessed at day 4. Data points represent individual cultures; line represents median value; $p>0.05$ for all comparisons between KO and WT samples using Mann-Witney U-test.

To determine whether these results were masking a more subtle dose-response effect, FoxP3 induction was next assessed by culturing naïve T lymphocytes with all isoforms of TGFβ at a range of physiological dilutions of TGFβ from 3.0ng/ml to 0.1ng/ml (Log3 dilutions) and compared with cells cultured in the absence of TGFβ. Data for two individual experiments is presented in Figures 5.5 and 5.6.

FoxP3 induction was demonstrated as a dose-dependent response to TGFβ, with all isoforms of TGFβ able to induce FoxP3 in both WT and KO samples. Again, we observed no obvious trend between WT and KO samples, and no statistically significant difference in either percentage FoxP3 induction or total FoxP3+ cell number between KO or WT cells across the range of physiological dilutions of TGFβ tested. We therefore conclude that betaglycan is not necessary for TGFβ-mediated iTreg induction in our assays.

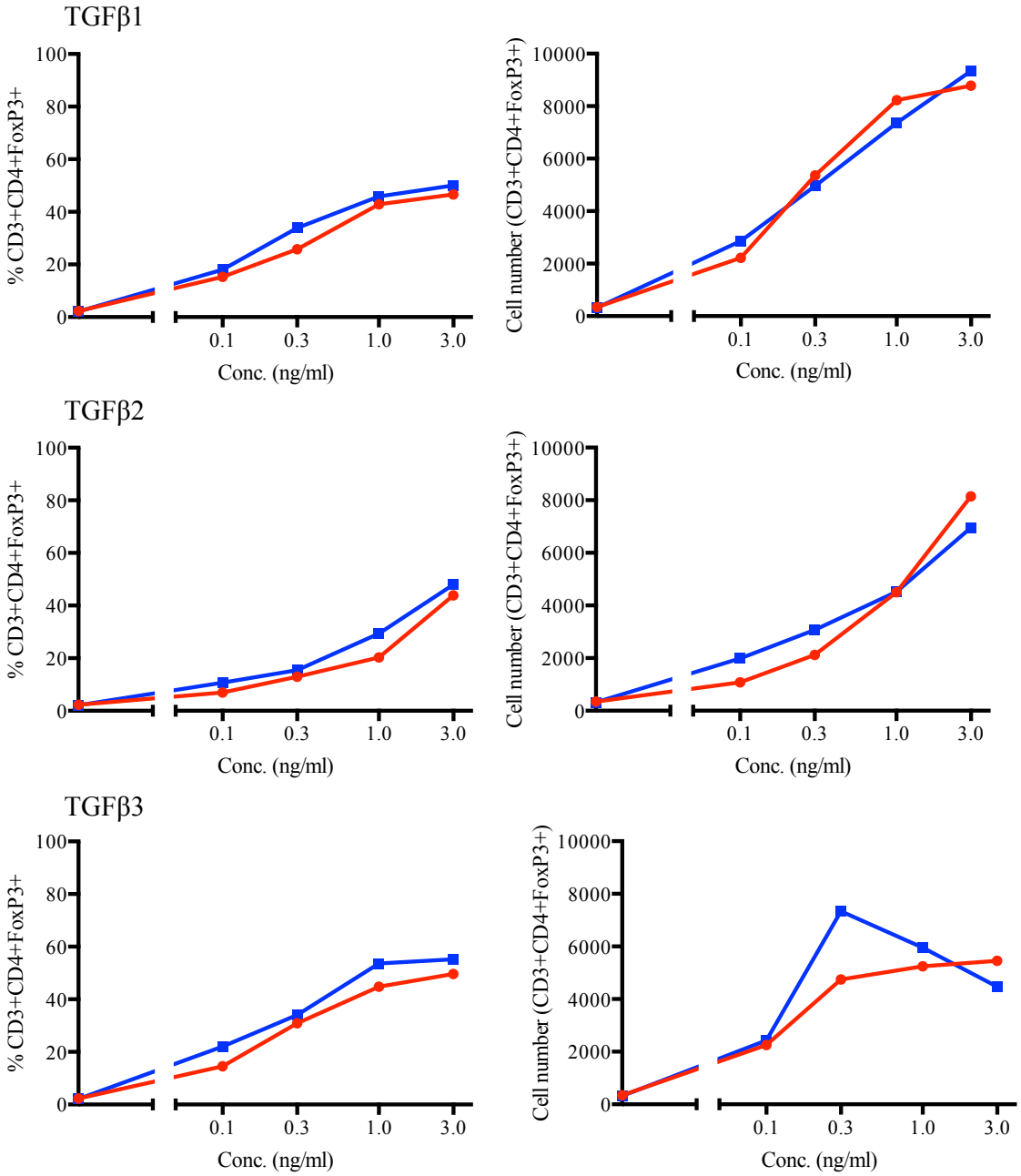


Figure 5.5 Betaglycan is not required for TGFβ-mediated FoxP3 induction in naïve CD4+ T lymphocytes *in vitro* (2). Line graphs illustrating percentage FoxP3 induction (left) and absolute number of FoxP3+ CD4+ T lymphocytes (right). Data presented for experiment 1 of 2. Lymphocytes identified according to gating strategy shown in figure 5.2. WT lymphocytes derived from lymph nodes of TGFβRIII +/+ chimeras (blue line, n=3) KO lymphocytes derived from lymph nodes of TGFβRIII -/- chimeras (red line, n=2). Cells FACS sorted to naïve CD4+CD25-CD44lowCD62L+ population and stimulated with anti-CD3 / anti-CD28 coated activation beads at a ratio of 1:1 beads:cells. IL2 added to all cultures at concentration of 50U/ml. TGFβ1, 2 and 3 added at Log3 dilutions from 3.0.0ng/ml to 0.1ng/ml. Control conditions in absence of TGFβ also shown. Proliferation assessed at day 4. Data points represent median values for replicate cultures; $p>0.05$ for all comparisons between KO and WT samples using Mann-Witney U-test.

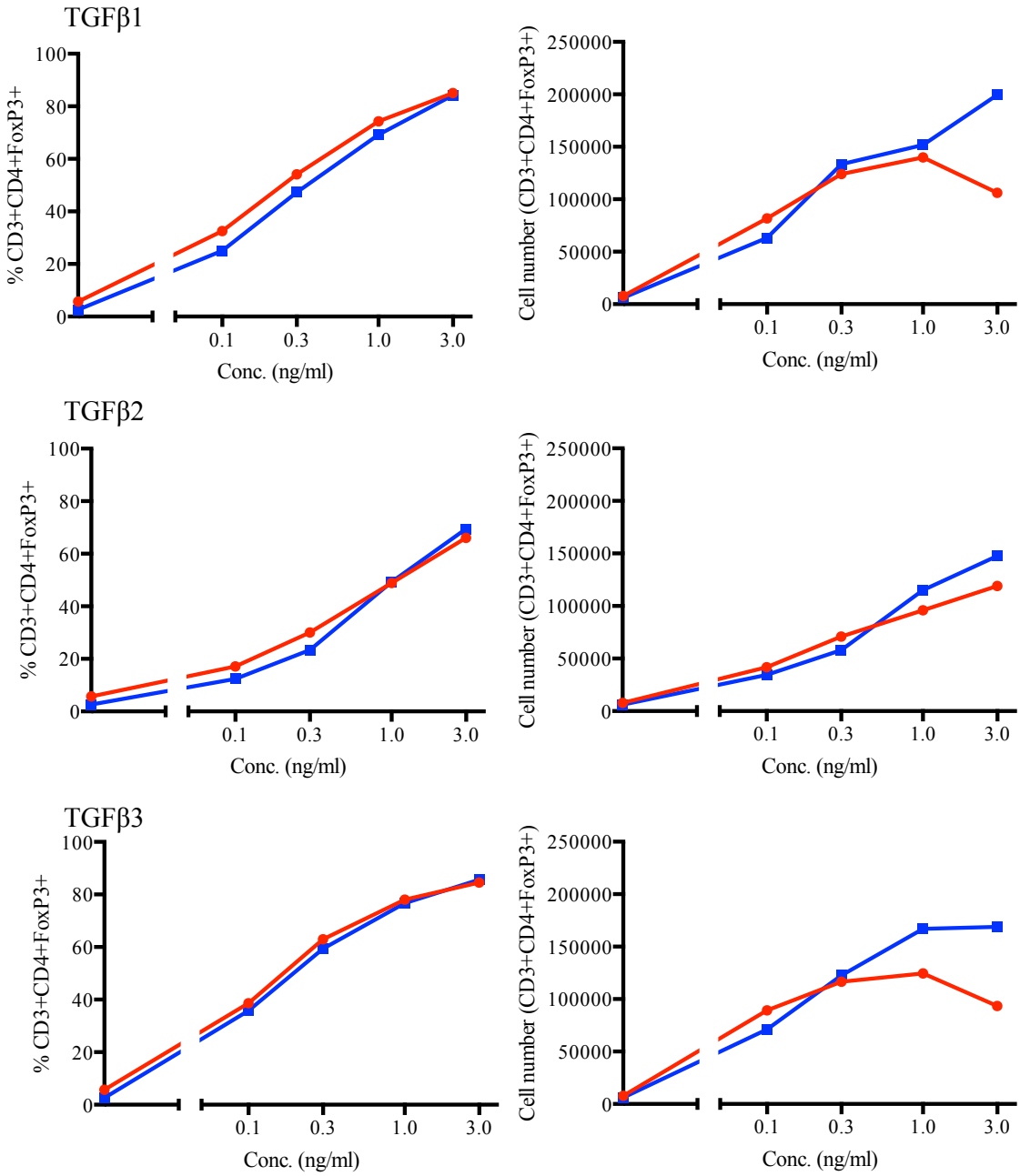


Figure 5.6 Betaglycan is not required for TGFβ-mediated FoxP3 induction in naïve CD4+ T lymphocytes *in vitro* (3). Line graphs illustrating percentage FoxP3 induction (left) and absolute number of FoxP3+ CD4+ T lymphocytes (right). Data presented for experiment 2 of 2. Lymphocytes identified according to gating strategy shown in figure 5.2. WT lymphocytes derived from lymph nodes of TGFβRIII +/+ chimeras (blue line, n=2) KO lymphocytes derived from lymph nodes of TGFβRIII -/- chimeras (red line, n=2). Cells FACS sorted to naïve CD4+CD25-CD44lowCD62L+ population and stimulated with anti-CD3 / anti-CD28 coated activation beads at a ratio of 1:1 beads:cells. IL2 added to all cultures at concentration of 50U/ml. TGFβ1, 2 and 3 added at Log3 dilutions from 3.0.0ng/ml to 0.1ng/ml. Control conditions in absence of TGFβ also shown. Proliferation assessed at day 4. Data points represent median values for replicate cultures; $p>0.05$ for all comparisons between KO and WT samples using Mann-Witney U-test.

5.3.2 The extent of Treg and Th17 polarisation of lymphocyte populations in C57BL/6 mice is dependent on the method of antigenic challenge

To determine an appropriate model for assessment of Treg and Th17 lymphocyte responses in our experimental mice, control experiments were performed in C57BL/6 mice using 2W1S peptide antigen and attenuated *L. monocytogenes*, IFA or *S. typhi* porins as adjuvants according to the methods described in section 2.2.6. Response to antigenic challenge was assessed by observation of T lymphocyte populations in draining lymphoid tissues, calculated as absolute number of cells per draining lymph node for challenge by 2W1S peptide with *S. typhi* porin and IFA (mean cell number per lymph node), or absolute number per whole spleen for challenge by 2W1S peptide with attenuated *L. monocytogenes*.

For each method of antigenic challenge, we first determined the number of activated, peptide-specific CD4+ T lymphocytes present (CD3+CD4+CD44+2W+), to give an overall indication of the success of antigenic induction. Within this population, we then determined the number of FoxP3+ and RORγ+ lymphocytes present. As previously discussed, both iTreg and nTreg can be identified by their expression of the transcription factor FoxP3 (Vignali et al. 2009), which is easily assessed through transcription factor staining by flow cytometry. Th17 cells can also be identified by transcription factor staining for RORγ, however this is considered less specific than staining for the cytokine IL-17 (Dong 2008). RORγ was accepted as a useful indicator of Th17 induction for the purpose of our pilot experiments. Example flow cytometry plots are shown for pilot experiments in Figures 5.7 to 5.9 with corresponding column charts illustrating pooled data for all experiments.

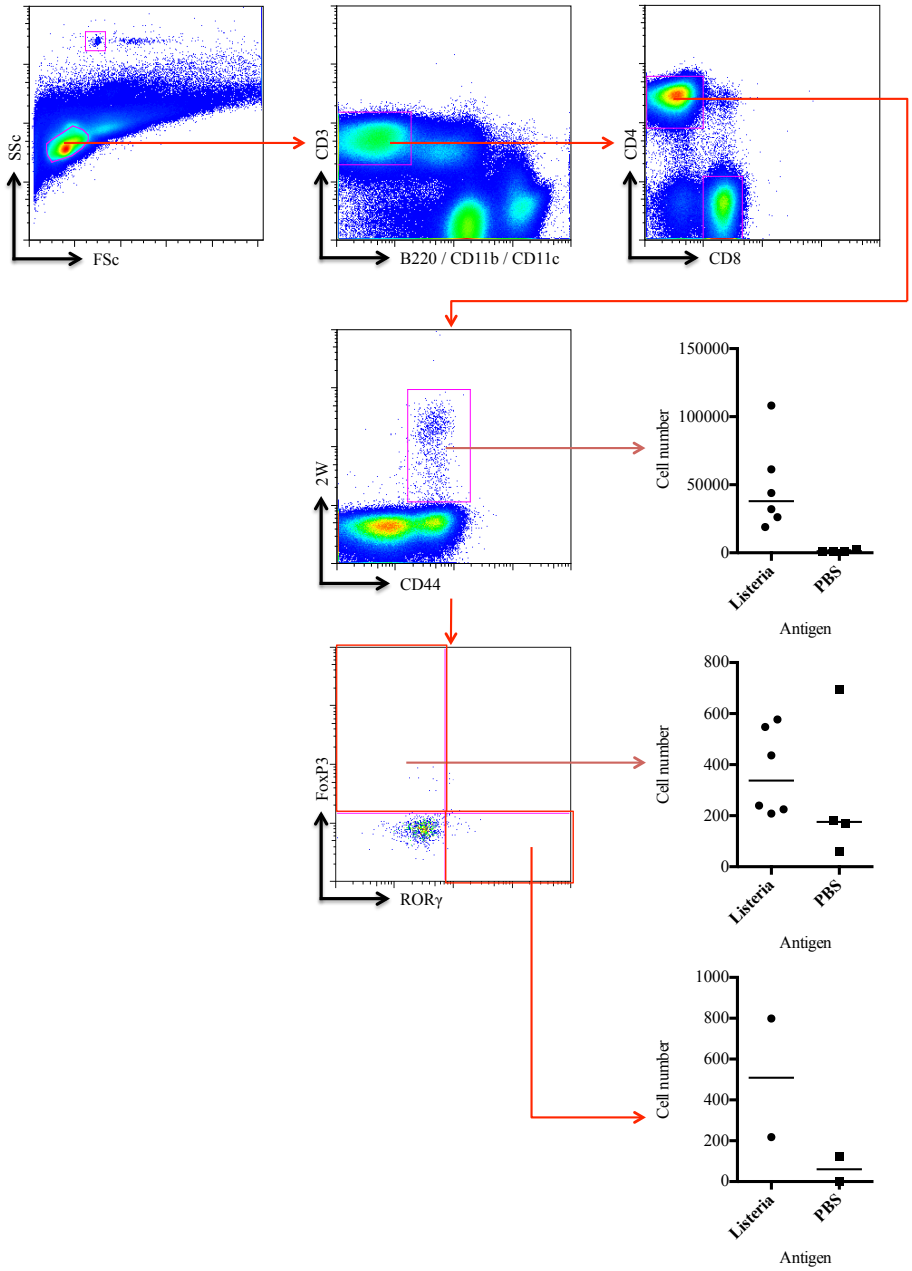


Figure 5.7 Polarisation of FoxP3+ and RORγ+ CD4+ T lymphocyte responses in C57BL/6 mice by *L. monocytogenes* and 2W1S peptide. Example flow cytometry gating strategy for identification of splenic peptide-specific T lymphocytes. Live lymphocytes were first identified according to their forward scatter (FSc) / side-scatter (SSc) profile, doublets excluded according to pulse-width (not shown), then CD4+ T lymphocytes identified by positive selection of CD3+CD4+ cells. Activated cells were identified by staining for CD44 (CD44+) and peptide-specific cells identified by staining with 2W tetramer (2W+). Th17 (FoxP3-RORγ+) and Treg (FoxP3+RORγ-) cells were identified from this population. FSc presented on linear axis. All other plots presented on logarithmic axes. Pooled pilot data shown for analysis at day 7 following antigenic challenge by attenuated *Listeria monocytogenes* and 2W1s peptide compared to PBS control (data for CD44+2W+ and FoxP3+ populations pooled from 2 experiments; n=6 per group. Data for RORγ+ populations from single experiment; n=2 per group). Cell number represents absolute number of cells per whole spleen. Data points represent individual animals. Line indicates median value.

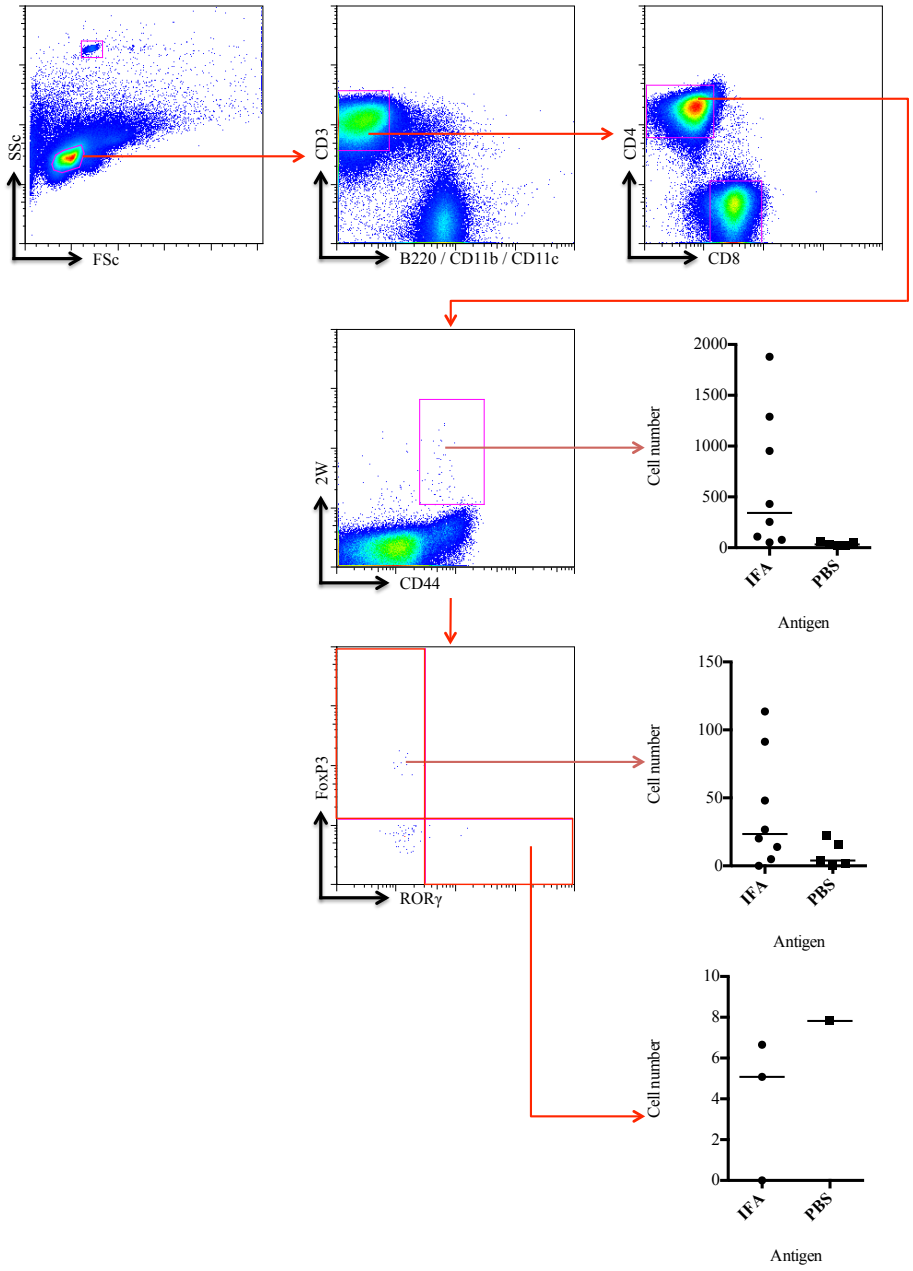


Figure 5.8 Polarisation of FoxP3+ and RORγ+ CD4+ T lymphocyte responses in C57BL/6 mice by IFA and 2W1S peptide. Example flow cytometry gating strategy for identification of peptide-specific T lymphocytes in draining inguinal lymph nodes. Live lymphocytes were first identified according to their forward scatter (FSc) / side-scatter (SSc) profile, doublets excluded according to pulse-width (not shown), then CD4+ T lymphocytes identified by positive selection of CD3+CD4+ cells. Activated cells were identified by staining for CD44 (CD44+) and peptide-specific cells identified by staining with 2W tetramer (2W+). Th17 (FoxP3-RORγ+) and Treg (FoxP3+RORγ-) cells were identified from this population. FSc presented on linear axis. All other plots presented on logarithmic axes. Pooled pilot data shown for analysis at day 7 following antigenic challenge by IFA and 2W1s peptide compared to PBS control (data for CD44+2W+ and FoxP3+ populations pooled from 2 experiments; n=8 per group. Data for RORγ+ populations from single experiment; n=3 per group). Cell number represents absolute number of cells per lymph node. Data points represent individual animals. Line indicates median value.

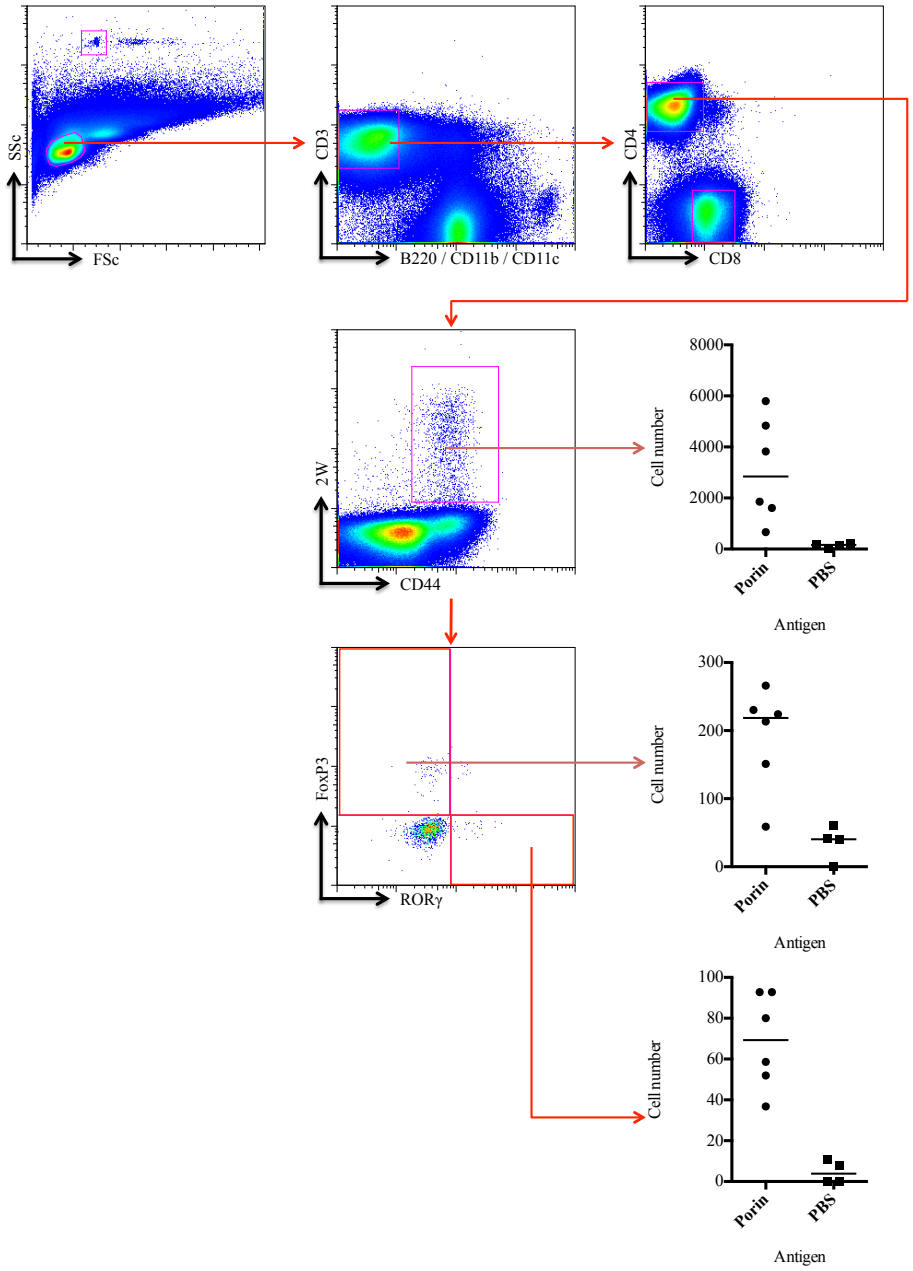


Figure 5.9 Polarisation of FoxP3+ and RORγ+ CD4+ T lymphocyte responses in C57BL/6 mice by *S. typhi* outer membrane porins and 2W1S peptide. Example flow cytometry gating strategy for identification of peptide-specific T lymphocytes in draining axillary and brachial lymph nodes. Live lymphocytes were first identified according to their forward scatter (FSc) / side-scatter (SSc) profile, doublets excluded according to pulse-width (not shown), then CD4+ T lymphocytes identified by positive selection of CD3+CD4+ cells. Activated cells were identified by staining for CD44 (CD44+) and peptide-specific cells identified by staining with 2W tetramer (2W+). Th17 (FoxP3-RORγ+) and Treg (FoxP3+RORγ-) cells were identified from this population. FSc presented on linear axis. All other plots presented on logarithmic axes. Pooled pilot data shown for analysis at day 7 following antigenic challenge by *Salmonella typhi* outer membrane porins and 2W1S peptide compared to PBS control (data for CD44+2W+ and FoxP3+ populations pooled from 2 experiments; n=6 per group). Cell number represents absolute number of cells per lymph node. Data points represent individual animals. Line indicates median value.

Immunisation with attenuated 2W1S peptide and *L. monocytogenes* adjuvant generated a strong immune response as demonstrated by high numbers of peptide-specific activated CD4+ T lymphocytes in splenic populations (median 37988 cells, n=6), however of these, there were very few Treg cells (median 338 cells, n=6), and similar levels of Th17 cells (median 509 cells, n=2) (Figures 5.7 and 5.10).

IFA and 2W1S peptide immunisation was poorly antigenic, with very few peptide-specific activated CD4+ T lymphocytes in draining lymph nodes (median 343 cells, n=8). Such small numbers of activated cells made further assessment of Treg and Th17 populations highly unreliable (Figures 5.8 and 5.10).

Antigenic challenge by 2W1S peptide and *S. typhi* porin adjuvant resulted in a stronger immune response than that achieved with IFA and 2W1S peptide, as demonstrated by the greater numbers of peptide-specific activated CD4+ T lymphocytes in draining lymph nodes (median 2841 cells, n=6). Similar numbers of both Treg (median 219 cells, n=6) and Th17 lymphocytes (median 69 cells, n=6) were induced compared to those observed in response to immunization by attenuated 2W1S peptide and *L. monocytogenes* in this population (Figures 5.9 to 5.10).

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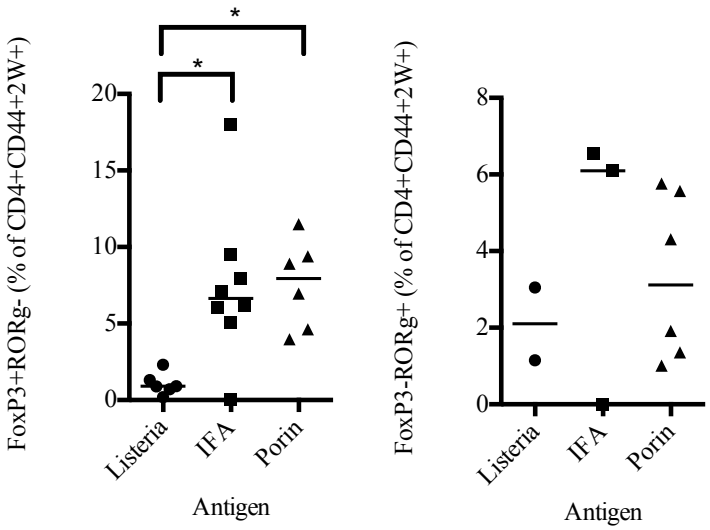
A



B

Population	Statistic	Listeria n	Listeria %	IFA n	IFA %	Porin n	Porin %
CD4+CD44+2W+	Median	37988	0.47	343	0.13	2841	0.24
	Mean	48430	0.46	631	0.14	3098	0.23
	95% CI lower	13965	0.28	66	0.05	971	0.12
	95% CI upper	82896	0.64	1195	0.24	5225	0.34
CD4+CD44+2W+	Median	338	0.90	23	6.64	219	7.94
FoxP3+RORγ-	Mean	372	1.05	40	7.48	191	7.56
	95% CI lower	195	0.31	5	3.23	112	4.50
	95% CI upper	550	1.80	75	11.74	269	10.62
CD4+CD44+2W+	Median	509	2.10	5	6.10	69	3.11
FoxP3-RORg+	Mean	509	2.10	4	4.22	69	3.32
	95% CI lower	-3182	-9.96	-5	-4.88	45	1.06
	95% CI upper	4199	14.16	13	13.31	93	5.58

Figure 5.10 Antigenic challenge by *S. typhi* porins and 2W1S peptide is the most effective method of generating peptide-specific FoxP3+ and RORγ+ CD4+ T lymphocytes. A Column charts showing percentage FoxP3+ and RORγ+ lymphocytes as a proportion of activated (CD44+) peptide-specific (2W+) CD4+ T lymphocytes induced by Listeria (*L. monocytogenes* and 2W1S peptide injected via tail vein, lymphocyte response in spleen assessed, n=6 FoxP3+RORγ-, n= 2 FoxP3-RORγ+), IFA (IFA and 2W1S peptide administered via subcutaneous injection to flank, lymphocyte response in draining inguinal lymph nodes assessed, n=6 FoxP3+RORγ-, n= 2 FoxP3-RORγ+), porin (*S. typhi* outer membrane porins and 2W1S peptide administered via subcutaneous injection to paw pad, lymphocyte response in draining axillary and brachial lymph nodes assessed, n=6 FoxP3+RORγ-, n= 6 FoxP3-RORγ+). All animals assessed at day 7 following challenge. Data points represent individual animals. Line indicates median value. * p,<0.05 using Kruskal-Wallis test. **B** Table illustrating median, mean and 95% confidence intervals of mean number and percentage of cells induced by each method of antigenic challenge. n = absolute number of cells per lymph node for IFA / Porin columns (calculated as mean cell number per lymph node) and absolute number of cells per whole spleen for Listeria column.

In conclusion, IFA and 2W1S peptide was excluded as a suitable method of further antigenic challenge based on the very weak peptide-specific immune response. The administration of 2W1S peptide and either attenuated *L. monocytogenes* and *S. typhi* porin adjuvants achieved satisfactory levels of immune activation, with immunisation by 2W1S peptide and attenuated *L. monocytogenes* generating a 10-fold greater number of activated peptide-specific CD4+ T lymphocytes than that achieved following immunisation with 2W1S peptide and *S. typhi* porin adjuvant. This was expected given the greater number of T lymphocytes in spleen compared to a single lymph node. However, of these cells, percentage FoxP3 expression was significantly greater following immunisation by 2W1S peptide and *S. typhi* porins (median expression 7.94% *S. typhi* porin / 2W1S vs. 0.90% *l. monocytogenes* / 2W1S, $p<0.05$); percentage RORγ expression was similar between the two methods (3.11% vs. 2.10%, $p>0.05$) (Figure 5.10).

Controlled antigenic challenge with 2W1S peptide and *S. typhi* porin was therefore adopted for all subsequent experiments in chimeric mice.

5.3.3 Betaglycan is not required for Treg or Th17 polarisation following controlled antigenic challenge *in vivo* but may have a subtle role in Th1 polarisation

Having identified a suitable model of controlled antigenic challenge in which to assess Treg and Th17 lymphocyte responses in secondary lymphoid organs, we now wished to assess the role of betaglycan in polarisation of the resulting immune response. Both peptide-specific and non-peptide-specific T lymphocyte responses in draining lymph nodes were assessed following immunisation by 2W1S peptide and *S. typhi* outer membrane porin adjuvant according to the method as previously described (Section 2.2.6).

Pooled lymph node cell suspensions from each experimental animal were divided into two equal samples; one was analysed according to the transcription factor staining protocol, and the other according to the cytokine staining protocol (Table 2.5). For all results, absolute cell numbers were expressed per lymph node assuming four equal sized lymph nodes were extracted from each animal (cells per lymph node = total cell number / 4).

In order to assess the strength of antigenic induction following controlled challenge, we first determined the number of activated (CD44+) CD4+ T lymphocytes present in both peptide-specific and non-peptide-specific populations, and determined the percentage of peptide-specific and non-peptide-specific cells as a proportion of the total CD44+ population. We next examined the phenotype of responding cells within each population; Treg were identified by FoxP3 expression (which identified both iTreg and nTreg in draining lymph nodes), Th17 cells were identified by both RORγ expression and IL17 secretion (in transcription factor and

cytokine analyses respectively), and Th1 identified by IFNγ secretion. In each analysis, assessment was also made of cells positive for both FoxP3 and RORγ (following transcription factor staining) and both IFNγ and IL17 (following cytokine staining). Example flow cytometry data with explanation of the gating strategy used is shown in Figure 5.11.

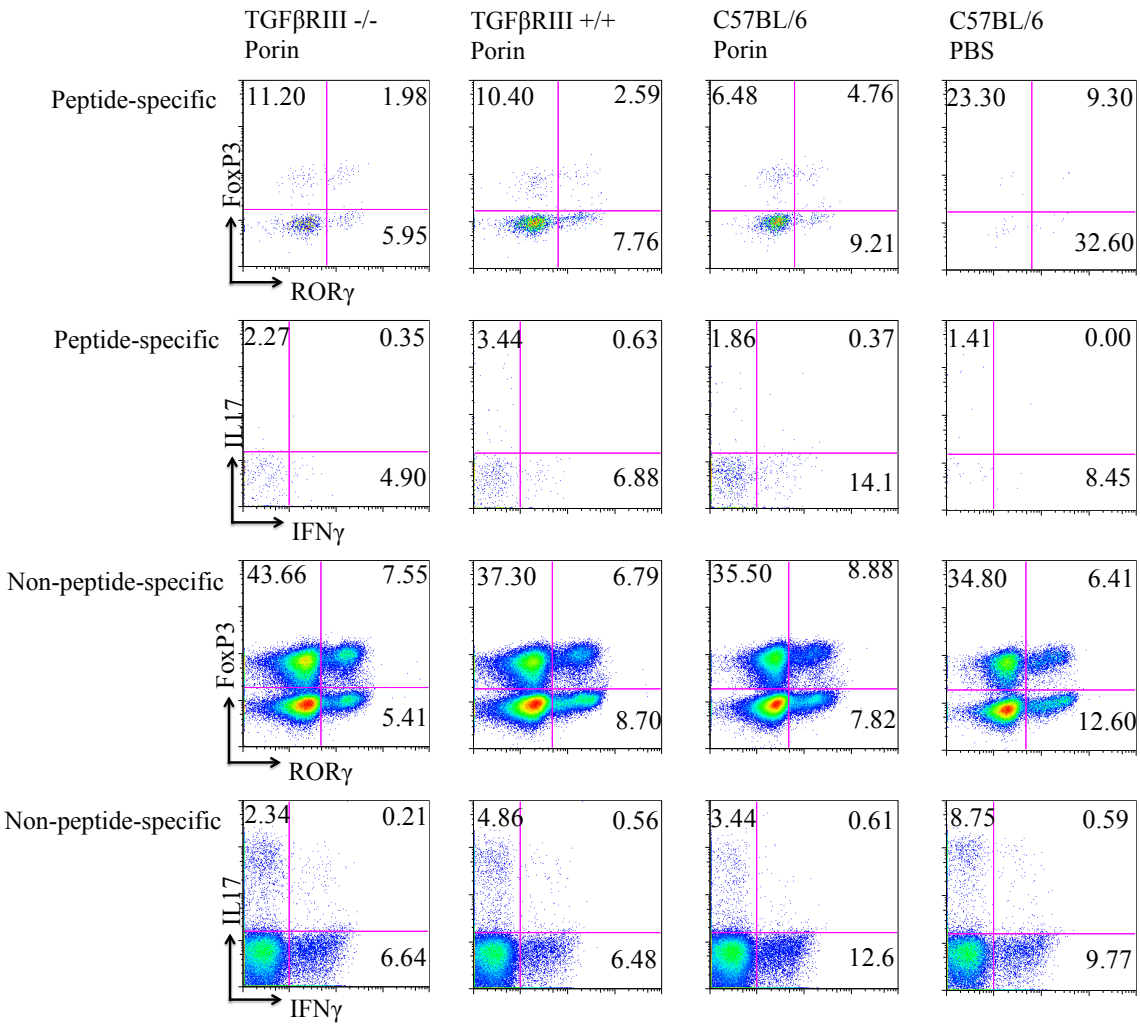


Figure 5.11 Polarisation of Treg and Th17 lymphocyte responses in betaglycan chimeric mice by *S. typhi* outer membrane porins and 2W1S peptide. Example flow cytometry data illustrating activated (CD44+) T lymphocyte populations in draining axillary and brachial lymph nodes of both betaglycan chimeric mice and C57BL/6 controls. Data analysed at day 7 following antigenic challenge by *Salmonella typhi* outer membrane porins and 2W1S peptide compared to PBS control. Activated cells were identified as illustrated in figures 5.7 to 5.9; peptide-specific and non-peptide-specific cells were separated by the presence or absence of staining with 2W tetramer (2W+ / 2W-). Th17 (FoxP3-RORγ+ and IL17+IFNγ-), Treg (FoxP3+RORγ-), Th1 (IL17-IFNγ+) and double positive (FoxP3+RORγ+ and IL17+IFNγ+) cells were identified from these populations. Numbers in gates represent percentages for displayed sample. All plots presented on logarithmic axes.

The peptide-specific immune response was considered adequate for experimental mice, with similar numbers of active CD4+ cells observed in draining lymph nodes of TGFβRIII -/- and TGFβRIII +/+ chimeras compared to C57BL/6 controls (median values 1628, 1366 and 1980 cells respectively), with very few peptide-specific active CD4+ cells observed in PBS controls (median 176 cells) (Figure 5.12).

As with previous observation of resting lymphocyte populations, there was significant variation within genotypic groupings. This limited our ability to detect significant associations within our data, however no differences were observed in expression of transcription factors FoxP3 or RORγ either in absolute number or percentage of either peptide-specific (Figure 5.12) or non-peptide-specific populations (Figure 5.13).

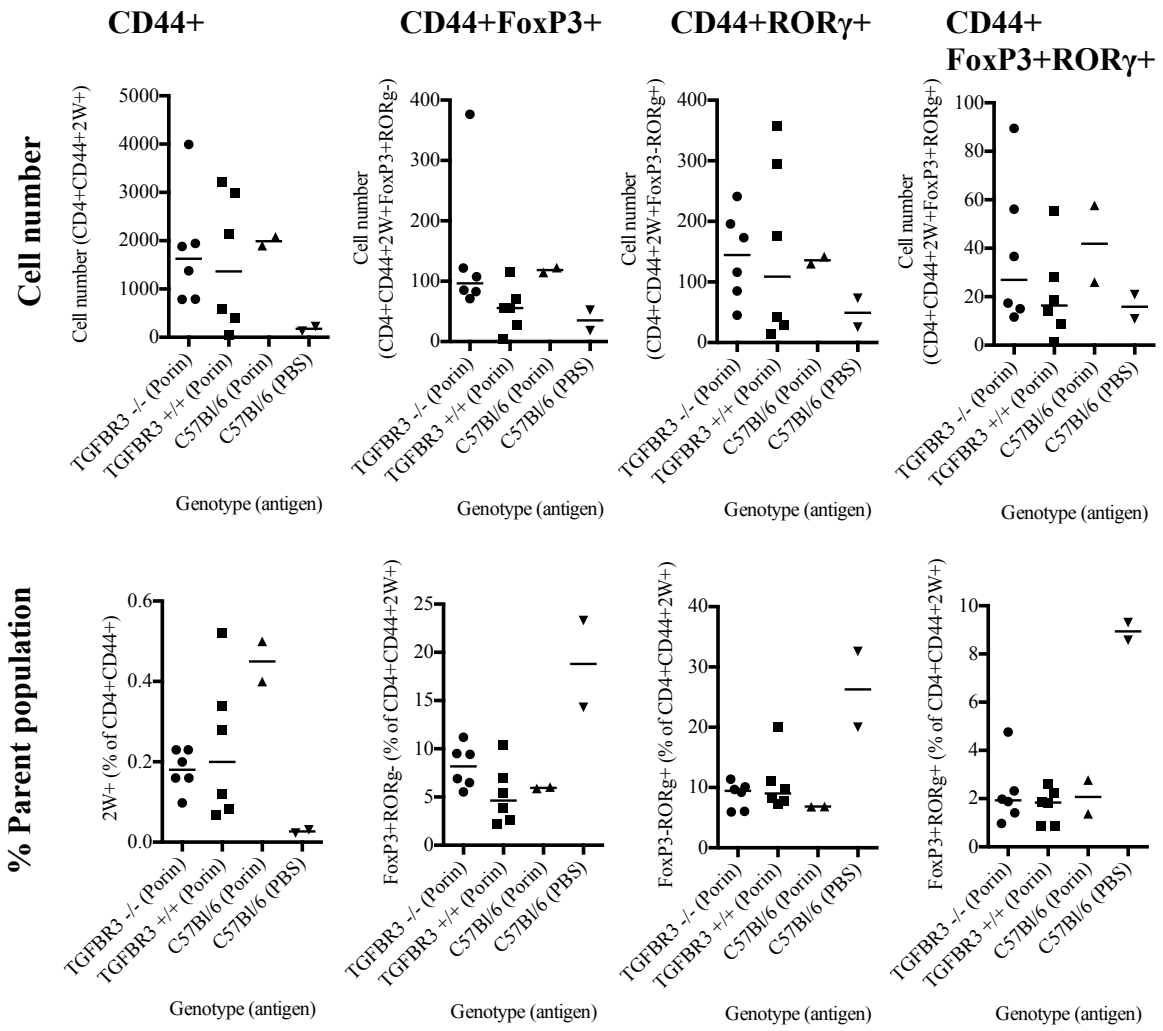


Figure 5.12 Betaglycan is not necessary for polarisation of peptide-specific FoxP3+ Treg or RORγ+ Th17 lymphocyte responses following antigenic challenge with *S. typhi* outer membrane porins and 2W1S peptide. Column charts showing pooled peptide-specific (2W+) experimental data following controlled antigenic challenge of betaglycan chimeras and non-chimeric C57BL/6 controls with *Salmonella typhi* outer membrane porins and 2W1S peptide compared to PBS control. Results of FoxP3 and RORγ staining shown. Data pooled from 2 separate experiments; n=6 per group for chimeric mice; n=2 per group for C57BL/6 mice. Data analysed at day 7 post challenge. Absolute number of cells per draining lymph node (top row) and percentages (bottom row) shown. Data points represent individual animals; line indicates median value. $p>0.05$ for all comparisons using Mann-Witney U-test.

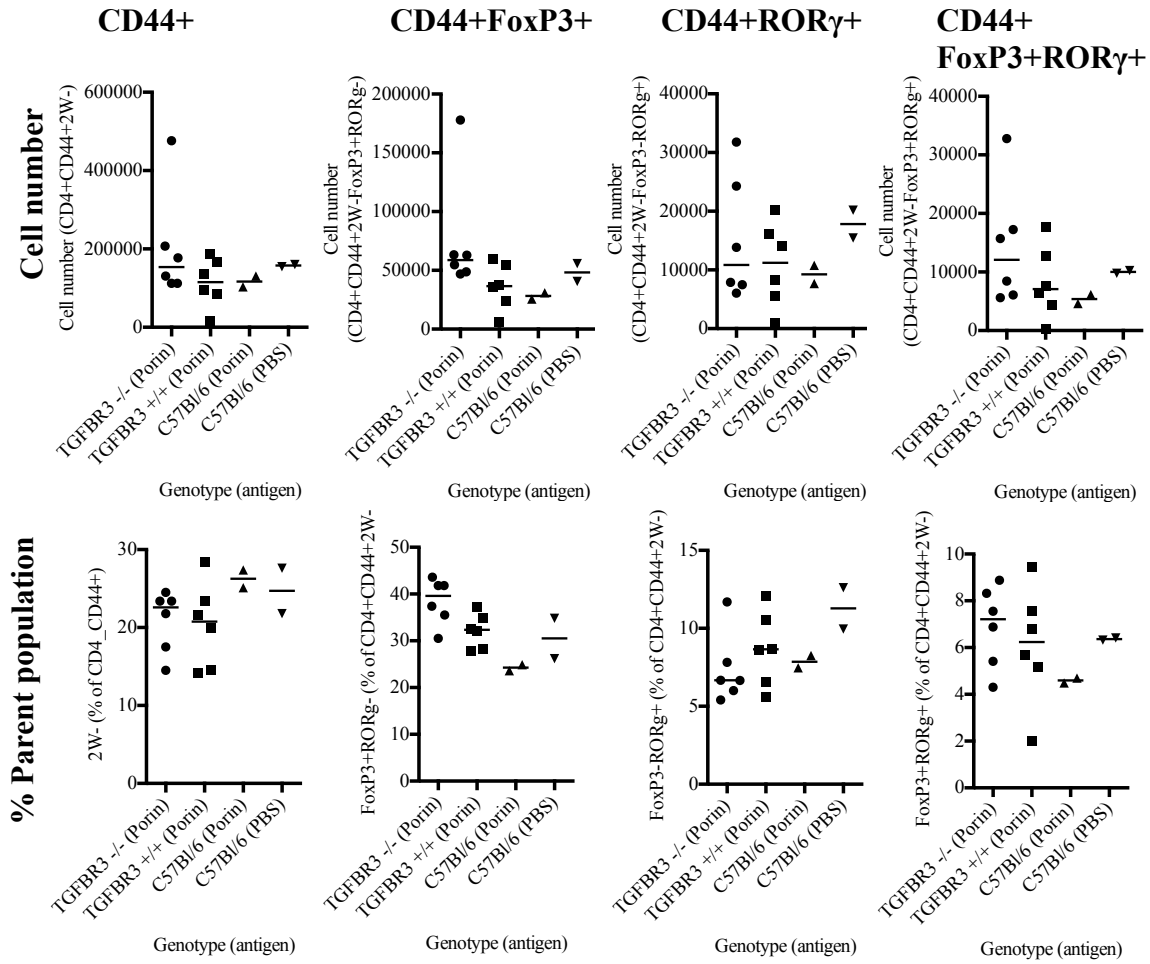


Figure 5.13 Betaglycan is not necessary for polarisation of non-peptide-specific FoxP3+ Treg or RORγ+ Th17 lymphocyte responses following antigenic challenge with *S. typhi* outer membrane porins and 2W1S peptide. Column charts showing pooled non-peptide-specific (2W-) experimental data following controlled antigenic challenge of betaglycan chimeras and non-chimeric C57BL/6 controls with *Salmonella typhi* outer membrane porins and 2W1S peptide compared to PBS control. Results of FoxP3 and RORγ staining shown. Data pooled from 2 separate experiments; n=6 per group for chimeric mice; n=2 per group for C57BL/6 mice. Data analysed at day 7 post challenge. Absolute number of cells per draining lymph node (top row) and percentages (bottom row) shown. Data points represent individual mice; line indicates median value. $p>0.05$ for all comparisons using Mann-Witney U-test.

No statistically significant differences in absolute number of IL17 or IFNγ secreting cells were observed between TGFβRIII -/- or TGFβRIII +/+ chimeras, or C57BL/6 controls either for peptide-specific or non-peptide-specific populations (Figures 5.14 to 5.15).

Statistically significant differences were however observed between betaglycan deficient and wild-type C57BL/6 non-chimeric control animals in the proportion of non-peptide-specific populations. Statistically significant increases were detected in IFNγ+ Th1 lymphocytes in TGFβRIII-/- chimeras compared to TGFβRIII +/+ chimeras, and double positive IFNγ+IL17+ CD4+ T lymphocytes in TGFβRIII -/- chimeras compared to C57BL/6 non-chimeric controls (Figure 5.15).

We also observed a statistically significant reduction in the number of peptide-specific IFNγ-secreting lymphocytes in TGFβRIII +/+ chimeras compared to C57BL/6 controls (Figure 5.14), and a statistically significant increase in non-peptide-specific IL17-secreting CD4+ T lymphocytes in TGFβRIII +/+ chimeras compared to C57BL/6 control animals (Figure 5.15). Since these differences were observed between populations of 'control' animals, they can not be attributed to a deficiency of betaglycan, and the functional significance is therefore questionable.

These *in vivo* experiments provide further evidence that betaglycan is involved in regulation of Th1 lymphocyte responses. We must however acknowledge that the response to controlled antigenic challenge was again variable in our experimental animals, and any consequence of betaglycan deficiency is subtle.

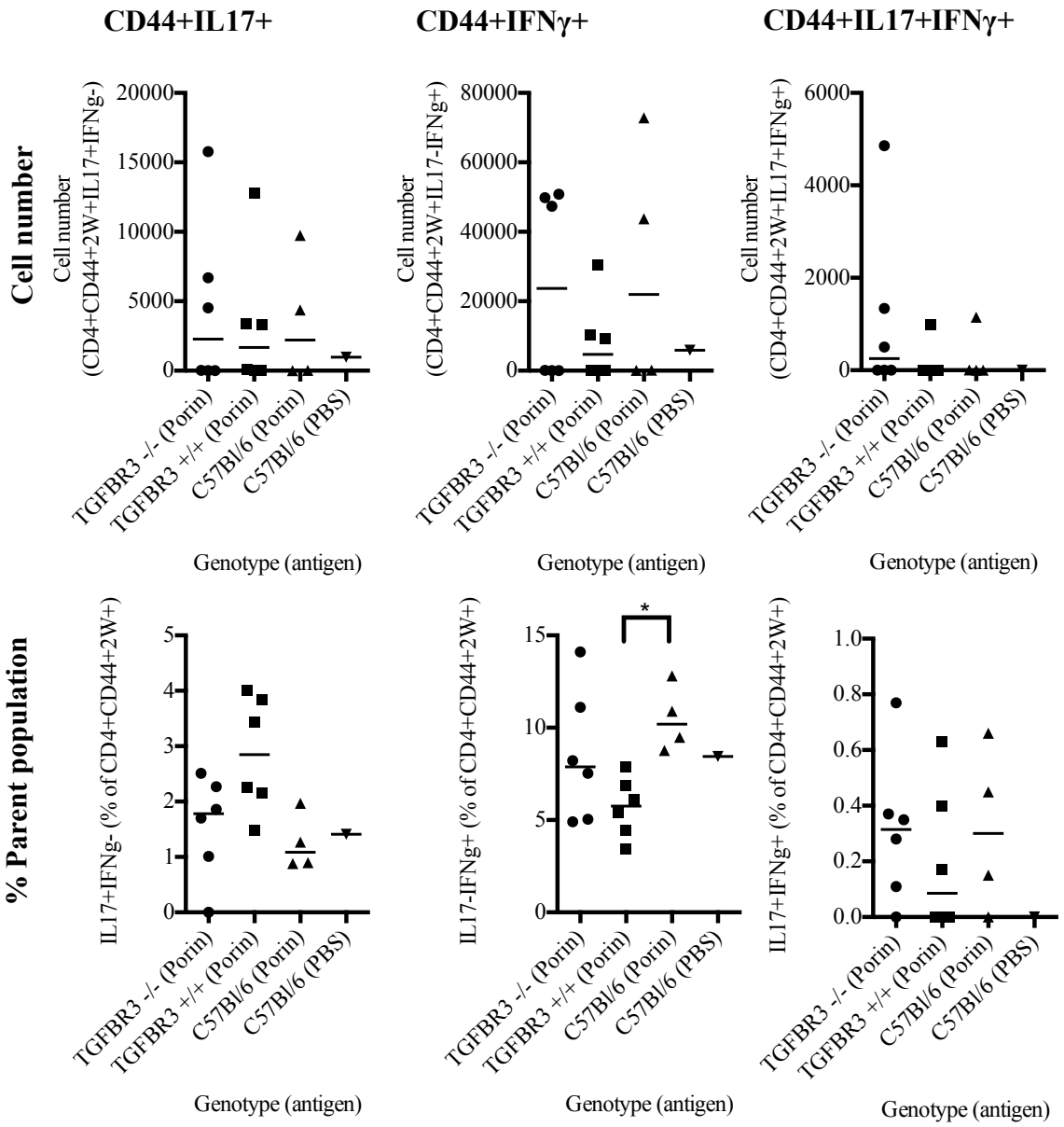


Figure 5.14 Betaglycan is not necessary for polarisation of peptide-specific IFNγ+ Th1 or IL17+ Th17 lymphocyte responses following antigenic challenge with *S. typhi* outer membrane porins and 2W1S peptide. Column charts showing pooled peptide-specific (2W+) experimental data following controlled antigenic challenge of betaglycan chimeras and non-chimeric C57BL/6 controls with *Salmonella typhi* outer membrane porins and 2W1S peptide compared to PBS control. Results of IFNγ and IL17 staining shown. Data pooled from 2 separate experiments; n=6 per group for chimeric mice; n=4 per group for C57BL/6 mice. Data analysed at day 7 post challenge. Absolute number of cells per draining lymph node (top row) and percentages (bottom row) shown. Data points represent individual animals; line indicates median value. * $p<0.05$ using Mann-Witney U-test.

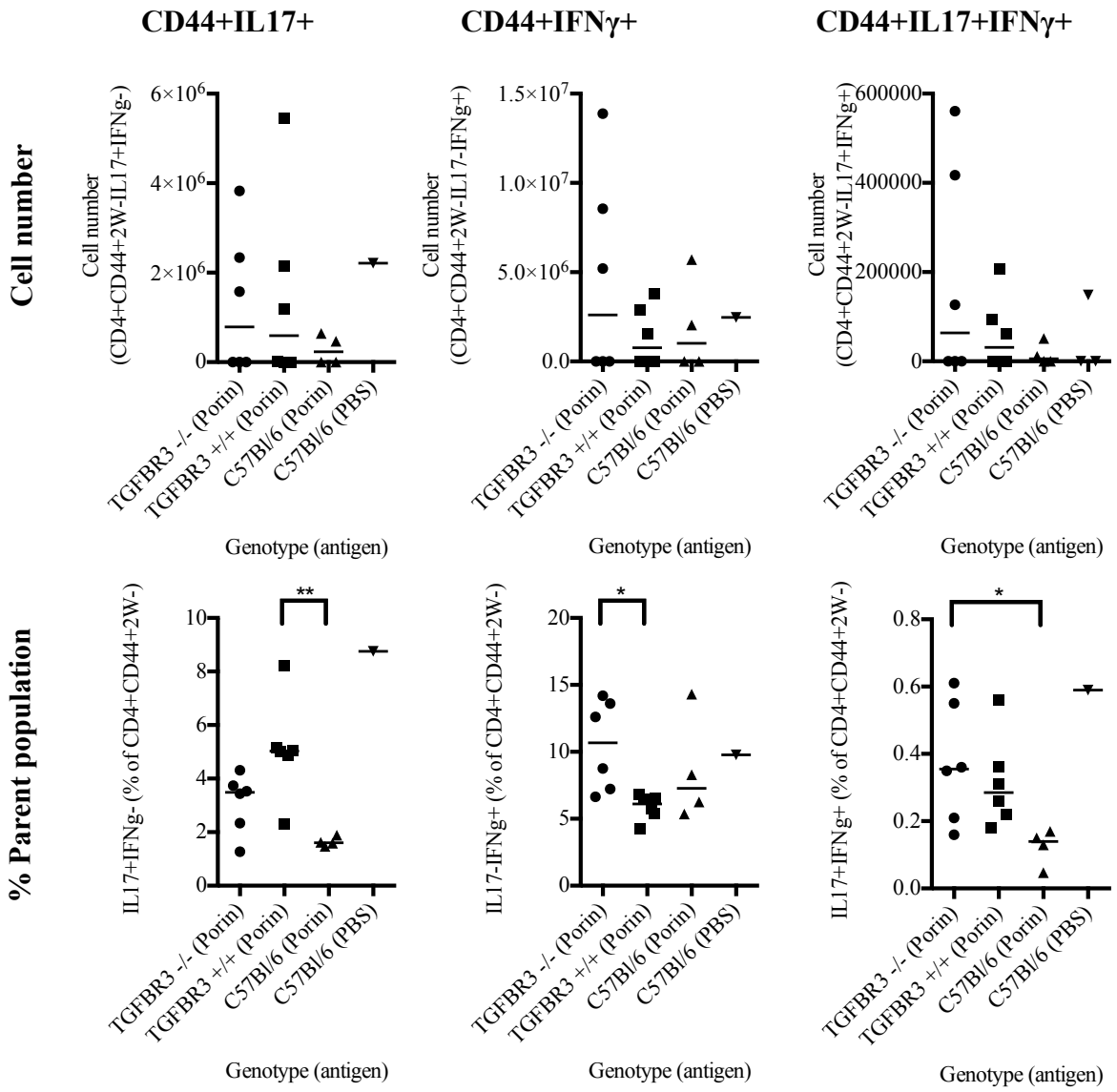


Figure 5.15 Betaglycan has variable effects on polarisation of non-peptide-specific IFNγ+ Th1 and IL17+ Th17 lymphocyte responses following antigenic challenge with *S. typhi* outer membrane porins and 2W1S peptide. Column charts showing pooled peptide-specific (2W+) experimental data following controlled antigenic challenge of betaglycan chimeras and non-chimeric C57BL/6 controls with *Salmonella typhi* outer membrane porins and 2W1S peptide compared to PBS control. Results of IFNγ and IL17 staining shown. Data pooled from 2 separate experiments; n=6 per group for chimeric mice; n=4 per group for C57BL/6 mice. Data analysed at day 7 post challenge. Absolute number of cells per draining lymph node (top row) and percentages (bottom row) shown. Data points represent individual animals; line indicates median value. * $p<0.05$, ** $p<0.01$ using Mann-Witney U-test.

5.4 Discussion

We were able to successfully extract T lymphocytes from the tissues of our betaglycan foetal liver chimeric mice, sort the resulting cell suspensions to a highly purified naïve CD4+ T lymphocyte population, and grow these *in vitro* under iTreg polarising conditions achieving up to 85% FoxP3+ induction in the resulting cell cultures (Figures 5.4 to 5.6). We observed no statistically significant difference in TGFβ-dependent T lymphocyte differentiation and proliferation between wild-type or betaglycan knock-out cell populations across the range of TGFβ conditions tested. We have demonstrated that all isoforms of TGFβ are able to signal to T lymphocytes independently of betaglycan, and have found no evidence of a role for betaglycan in TGFβ signalling to T lymphocytes.

This appears to contrast with published data for the role of betaglycan in TGFβ signalling; assays of heart endothelial and skeletal muscle cell lines in the presence and absence of betaglycan have previously suggested that whilst betaglycan is unnecessary for TGFβ1 and TGFβ3 mediated responses, betaglycan is necessary for TGFβ2 signalling (López-Casillas et al. 1993; Cheifetz et al. 1990). Our data demonstrates that TGFβ2 is able to signal to T lymphocytes in conditions of complete betaglycan insufficiency, with no differences observed between betaglycan-sufficient and betaglycan-deficient cells.

This discrepancy is likely to be explained by the differences in each of the assays used. The previously cited research groups published findings based on data derived only from cell lines; it is often argued that cell lines are unrepresentative of the cells from which they are derived, at high risk of both cross-contamination from other cell lines, and microbial

contamination by resident pathogens (Masters 2000). Our data is derived from primary cell cultures, and we therefore suggest that our assays are likely to be more representative of the true situation *in vivo*.

Furthermore, different end-points were assessed in our assays compared to those in previously published literature; both Cheifetz et al. and López-Casillas et al. based their conclusions on growth inhibition assays, demonstrating a 50-fold reduced potency of TGFβ2 compared to TGFβ1 or TGFβ3 in conditions of betaglycan deficiency (López-Casillas et al. 1993; Cheifetz et al. 1990). In contrast, we base our conclusions on FoxP3 induction in live cells after four days in cell culture, and did not directly assess growth inhibition. It is possible that cells require a stronger TGFβ signal for growth inhibition than for differentiation to an effector phenotype. In principle this appears logical, since at times of immunological stress the TGFβ-dependent signal to differentiate is thus likely to be stronger (and more important) than the TGFβ-dependent signal to down-regulate lymphocyte cell populations. Both conclusions may therefore be equally valid.

Variation in biological potency of TGFβ2 in the absence of betaglycan has also previously been observed in FHBE cell lines. FHBE cells are naturally deficient in betaglycan, and whilst they have been shown to display reduced TGFβ2-mediated growth inhibition compared to wild-type cells (Cheifetz et al. 1990), they display similar TGFβ2-mediated plasminogen-activator inhibitor 1 (PAI-1) inhibition (ten Dijke et al. 1988). The difference between 'receptor affinity' and 'biological potency' may also be important; whilst TGFβRI and TGFβRII have previously been shown to have higher affinity for TGFβ1 and TGFβ3 isoforms than for TGFβ2 (Wrana et al. 1992), it may be possible for low affinity binding to induce a

strong biological effect (Cheifetz et al. 1990) inducing similar signal transduction across the cell surface receptor.

Finally, whilst differences in TGFβ-mediated growth inhibition have been observed in betaglycan-deficient fibroblasts, TGFβ-mediated Smad phosphorylation has been demonstrated to be similar in knock-out and wild-type cell populations (Stenvers et al. 2003). TGFβ-mediated growth inhibition thus appears to proceed independent of Smad signalling pathways, and therefore may not directly correlate with the availability of betaglycan at the cell surface receptor, or reflect the role of betaglycan in signal transduction.

Collectively, these observations illustrate that *in vitro* responses to TGFβ2 vary depending on the assay used. The impact of betaglycan deficiency on TGFβ signal transduction *in vivo* may therefore be highly context specific, dependent both on the cell type and biological process. Ultimately, we conclude that betaglycan is not an absolute requirement for TGFβ1,2 or 3-mediated FoxP3 induction by naive T lymphocytes. We cannot however exclude the possibility that betaglycan is necessary for other TGFβ-mediated processes.

We were able to successfully immunise our betaglycan foetal liver chimeras, using 2W1S peptide antigen and *S. typhi* outer membrane porin adjuvant to induce Th1, Th17, and Treg lymphocyte responses in draining lymph nodes (Figure 5.11). Furthermore, we were able to identify activated 2W1S peptide-specific CD4+ T lymphocytes, and were thus able to track a highly challenge-specific immune response. We observed no statistically significant difference in the absolute number of 2W1S peptide-specific or non-specific CD4+ T lymphocytes recruited to the site of antigenic challenge between TGFβRIII +/+ or TGFβRIII -

/- chimeras, or C57BL/6 controls (Figures 5.12 to 5.15). We did however observe statistically significant differences in relative proportions of Th1 and Th17 cells across our experimental groups.

A statistically significant reduction in the proportion of peptide-specific Th1 lymphocytes was observed in TGFβRIII +/+ chimeras compared to C57BL/6 controls (Figure 5.14), with a similar, non-statistically significant trend observed in the non-peptide specific response in these animals (Figure 5.15). In addition, a statistically significant increase in the proportion of non-peptide-specific Th17 lymphocytes was observed in TGFβRIII +/+ chimeras compared to C57BL/6 controls (Figure 5.15), with a similar, non-statistically significant trend observed in the peptide-specific response in these animals (Figure 5.14). Whilst not statistically significant, a similar trend was observed in absolute cell number of Th1 and Th17 cells in these experimental groups.

These observations are surprising, since our TGFβRIII +/+ chimeras were considered genetically identical to wild-type C57BL/6 mice, and we would therefore expect a similar response to antigenic challenge. Our data suggests that the Th1 response is impaired in 'wild-type', betaglycan-sufficient foetal liver chimeric animals compared to wild-type non-chimeras, with a relative shift towards the Th17 response. This raises the possibility of differences in our experimental mice which arise due to the process of generating chimeras; perhaps suggesting that the process of sub-lethal irradiation leads to disruption of either the bone marrow or thymus which persists following reconstitution, resulting in altered function and disruption to the subsequent development of lymphocyte populations.

Previous studies of mice subject to sub-lethal irradiation have demonstrated differential radio-sensitivities of mature T lymphocyte populations, with relative sparing of Treg subsets compared to other effector populations in response to any given dose of gamma radiation (Qu et al. 2010); since our chimeras were generated in RAG deficient mice which lacked mature lymphocyte populations at the time of irradiation, this is unlikely to explain differences in our model. More significantly, proliferation of bone marrow stroma *in vitro* has been shown to be significantly suppressed following irradiation (Zhang et al. 2010); it is thus more likely that the supportive functions of stromal cells were impaired in our irradiated chimeric mice compared to non-irradiated C57BL/6 controls, and this may explain any subsequent differences in lymphocyte development between otherwise genetically-identical animals.

In contrast, no significant differences were observed between TGFβRIII -/- chimeras or C57BL/6 controls in these comparisons. This could be interpreted in a variety of ways. Firstly, betaglycan deficiency may somehow compensate for the detrimental effects of irradiation, allowing restoration of more physiologically normal bone marrow and thymic lymphocyte compartments, with the resulting chimeras appearing more similar to non-chimeric C57BL/6 animals than wild-type chimeras. Alternatively, both TGFβRIII -/- and TGFβRIII +/+ chimeras may sustain similar disruption of lymphocyte pools following irradiation, but there may be polarisation towards a stronger Th1 (and therefore weaker Th17) response following antigenic challenge in the absence of betaglycan. Finally, it is possible that a significant difference does indeed exist, but our *in vivo* system was too variable for us to demonstrate this.

We observed a statistically significant increase in non-peptide-specific Th1 cell proportions in TGFβRIII-/- chimeras compared TGFβRIII +/+ chimeras (Figure 5.15), again, with non-statistically significant trends towards increased Th1 and reduced Th17 proportions across both the peptide-specific and non-peptide-specific data (Figure 5.14 to 5.15). This suggests that a preferential Th1-driven response may predominate under conditions of betaglycan deficiency, with a possible reduction in the Th17 response. This is consistent with published data in EAE and experimental colitis, in which TGFβ has been shown to modulate the balance between Th1 and Th17 responses, being observed to suppress Th1 mediated inflammation whilst promoting Th17-mediated disease (Li et al. 2007).

Indeed, excessive Th1 responses have been widely reported in response to disruption of TGFβ signalling. This has largely been considered a secondary effect of reduced Treg differentiation in the absence of TGFβ, for example in a murine transfer model, TGFβ from FoxP3+ Treg cells was shown to be necessary to suppress Th1 cell differentiation and prevent development of experimental colitis (Ishigame et al. 2013). Several studies have demonstrated an association between reduced proportions of peripheral Treg lymphocytes and development of immune-mediated disease in TGFβ-deficient mice (Ouyang et al. 2010; Gu et al. 2012; Takimoto et al. 2010), however these observations do not correlate with our data, since we observed no disruption of Treg populations in either our naïve or antigen-experienced mice.

In a mouse model of Th1-mediated type-1 diabetes, Ishigame et al. demonstrated that inhibition of TGFβ signalling to CD4+ T lymphocytes through conditional TGFβRII knock-out resulted in increased Th1 polarisation and development of diabetes, in the presence of normal Treg development, maintenance and function (Ishigame et al. 2013). Peripheral Th1

and Treg lymphocytes are thought to exist in a state of balance, whereby local Th1 numbers are regulated by reciprocal changes in levels of local Treg (Caretto et al. 2010). The observations of Ishigame et. al. suggest that Treg-independent mechanisms of Th1 regulation exist under conditions of deficient TGFβ signalling, and this may explain the trends in our data. It is thus possible that betaglycan deficiency results in direct up-regulation of Th1 polarisation, in a similar, Treg-independent manner.

Finally, a statistically significant increase in the proportion of non-peptide-specific double-positive IL17+IFN+ CD4+ T lymphocytes was observed in TGFβRIII -/- chimeras compared to C57BL/6 controls (Figure 5.15); since these cells constitute less than 1% of the total effector lymphocyte population in each of our comparisons, this is likely to be of minimal functional significance, but is further evidence of an exaggerated immune response in betaglycan deficient chimeras.

No significant differences were observed in either absolute number or relative proportion of FoxP3+ CD4+ lymphocyte populations; this is consistent with our *in vitro* data (Section 5.3.1), and provides further evidence that betaglycan is not involved in Treg polarisation.

We conclude that betaglycan may be implicated in Th1-driven responses to antigenic challenge, perhaps acting to suppress Th1-mediated immune responses under normal conditions, but is not involved in Th17 or Treg responses.

6 ASSOCIATION ANALYSIS OF TGFBR3 GENE WITH BEHÇET'S DISEASE AND IDIOPATHIC INTERMEDIATE UVEITIS IN A CAUCASIAN POPULATION

The introduction, methods, results and discussion presented within this chapter have previously been published in a peer-reviewed scientific journal (Barry et al. 2015); see section 8.2 for the full citation and a reproduction of the published version.

6.1 Introduction

Behçet's disease (BD) and idiopathic intermediate uveitis (IIU) are two diseases characterised by sight-threatening intra-ocular inflammation: BD is a multisystem vasculitis characterised by inflammatory lesions of the oro-genital mucosa, eyes, skin, central nervous system and joints. The underlying cause is unknown, but it is believed to be an autoinflammatory process triggered by an infectious or environmental agent. There is significant geographical clustering of BD, being most prevalent in countries along the historic 'Silk Route' (Mendes et al. 2009). IIU is a localized ocular inflammatory disease characterized by breakdown of the blood–retinal barrier and leucocyte infiltration of the retina with photoreceptor cell loss, often leading to blindness. It is usually considered to be an autoimmune, cell mediated, organ specific disease based on the findings of autoreactive T lymphocytes and antibodies in patients and its response to immunosuppression (Walton & Ashmore 2003). Both conditions

have also been associated with HLA and cytokine gene polymorphisms (Verity et al. 1999; Stanford et al. 2005).

BD and IIU may be considered to be at least partially TGFβ dependent, as Treg and Th17 cells have been implicated in the pathogenesis of each (Na et al. 2013; Amadi-Obi et al. 2007). Furthermore, since TGFβ2 is the predominant intra-ocular isoform, betaglycan may be particularly important in the ocular microenvironment, and may be implicated in the pathogenesis of both diseases (Curnow et al. 2005).

Single nucleotide polymorphisms (SNP) in the gene controlling TGFβRIII (TGFBR3) have been identified in both systemic and ophthalmic disease (Xiong et al. 2009; Khor et al. 2011; Ramdas et al. 2011). Several studies in ophthalmology have identified SNP in TGFBR3 in patients with glaucoma, demonstrating an association with optic disc area in both Caucasian and Asian cohorts, suggesting a role in the onset and progression of glaucomatous optic disc damage (Axenovich et al. 2011; Khor et al. 2011; Ramdas et al. 2011). A particular SNP (RS1805110) has previously been identified in a Han Chinese population with BD, with the CC genotype suggested to confer a protective effect against developing disease. This SNP is a non-synonymous mutation located in the signal sequence that may affect secretion or expression of TGFBR3, although this has not addressed specifically (Chen et al. 2012).

This finding is of particular interest given the possible importance of the TGFBR3 gene and TGFβRIII receptor to ocular immunoregulation. Having investigated the role of betaglycan in a series of tightly controlled experimental animal models, we wished to extend our investigation to human subjects, and attempt to replicate the findings of Chen et. al. by testing

for this association in a population of Caucasian patients with BD, making comparison to disease-free controls and patients with IIU.

6.2 Experimental design

Blood samples were collected from 167 consecutive Caucasian patients (75 with BD, 92 disease control with IIU) attending the Birmingham and Midland Eye Centre, and Medical Eye Unit at St. Thomas' Hospital, London. All patients with BD fulfilled the International Study Group criteria for diagnosis (Wechsler et al. 1990) and underwent a prospective clinical and ophthalmological examination. IIU was diagnosed clinically by expert ophthalmological review. Blood samples were collected from 85 age- and ethnicity-matched healthy controls without BD or IIU for comparison.

This study received the approval of the St Thomas' Hospital and Sandwell Local Ethics Research Committees.

6.3 Results

6.3.1 TGFBR3 SNP RS1805110 is not associated with Behcet's Disease in a Caucasian population

Representative data plots of the endpoint genotype analysis are shown in figure 6.1 and summary statistics are shown in table 6.1. Across all diagnostic groups, the CC genotype was most prevalent, ranging from a frequency of 79.35% in IIU samples to 89.41% in control samples. A considerably lower frequency of the CT genotype was observed, ranging from 9.41% in healthy control to 20.65% in IIU samples. A very low frequency of the RS1805110 T allele was observed across all diagnostic groups (5.88% control, 9.33% BD, 10.33% IIU), with complete absence of the homozygous RS1805110 TT genotype in patients with BD and IIU in this sample (1.18% control, 0% BD, 0% IIU) (Table 6.1).

No significant difference in RS1805110 allele or genotype frequency was observed between either BD or IIU and healthy controls ($p>0.05$ for all comparisons). Distribution of genotype in each group did not deviate from HWE.

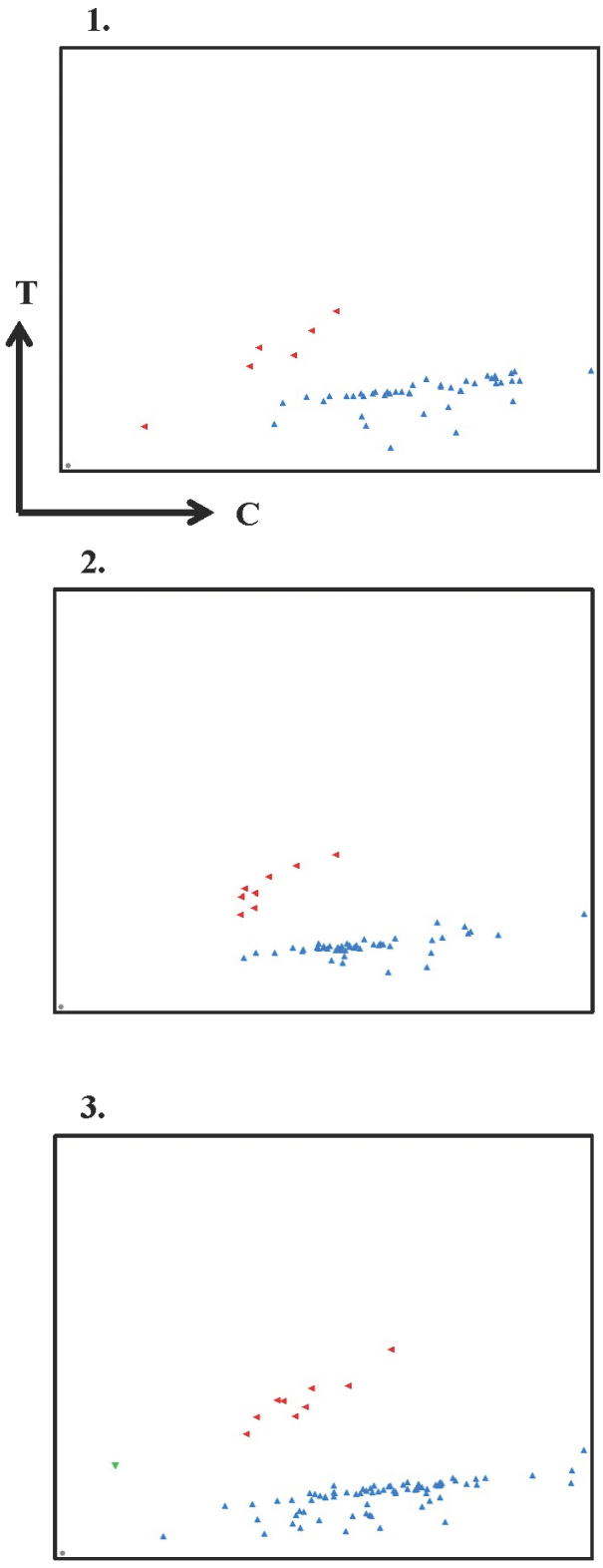


Figure 6.1 Representative plots of endpoint genotyping analysis. Data shown for BD (1), IIU (2) and healthy controls (3). Blue triangles CC, red triangles CT, green triangles TT, grey circles negative control.

Genotype / allele	**BD (n=75)**		**IIU (n=92)**		**Control (n=85)**		**BD vs. Control**				**IIU vs. Control**			
	n	*%*	*n*	*%*	*n*	*%*	*OR*	*95% CI*	*p*	*OR*	*95% CI*	*p*
CC	61	81.33	73	79.35	76	89.41	0.52	0.21 - 1.27	0.178	0.46	0.19 - 1.07	0.098
TT	0	0.00	0	0.00	1	1.18	*	*	1.000	*	*	0.480
CT	14	18.67	19	20.65	8	9.41	2.21	0.87 - 5.61	0.109	2.51	1.03 - 7.58	0.058
C	136	90.67	165	89.67	160	95.12	0.61	0.26 - 1.41	0.290	0.54	0.24 - 1.20	0.174
T	14	9.33	19	10.33	10	5.88	1.65	0.71 - 3.83	0.290	1.84	0.83 - 4.09	0.174

Table 6.1 SNP RS1805110 genotype and allele frequency. Data stratified by diagnostic group. n = number of samples; OR = Odds Ratio; 95% CI = 95% Confidence Interval of Odds Ratio. p-value calculated by Fisher's exact test. * Unable to calculate statistics with n=0 in one comparison group.

6.3.2 The RS1805110 minor allele frequency is low in Caucasian populations

The T allele frequency was very low amongst Caucasian patients within our sample, with a frequency of only 5.88% in our healthy controls (Table 6.1). Our observed allele frequencies differ significantly from those previously published in Han Chinese patients with BD ($p<0.0001$), but are consistent with those observed in Caucasian populations as presented in the HapMap database (The International HapMap Consortium 2015) ($p=0.149$) (Table 6.2).

Stratification analysis according to gender or clinical features of BD did not associate with the tested SNP ($p>0.05$).

Data source	Diagnostic group	n	C	T	OR	95% Confidence Interval	p
Present study	BD	150	136	14	-	-	-
Chen et al. 2012	BD	634	330	304	8.95	5.05 - 15.85	<0.0001
HapMap CEU[a]	Control	226	214	12	0.52	0.23 - 1.16	0.149

Table 6.2 SNP RS1805110 allele frequencies in published literature. Comparison of allele frequency observed in patients with BD between present study and Chen 2012 (Chen et al. 2012), and with published background allele frequency in Caucasian patients presented in HapMap database (The International HapMap Consortium 2015). n = total number of alleles in sample; OR = Odds Ratio; p-value calculated using Fisher's exact test. a = CEU: Utah residents with Northern and Western European ancestry from the CEPH collection.

6.4 Discussion

We found no statistically significant association between TGFBR3 RS1805110 and either BD or IIU. This was probably due to the low prevalence of the RS1805110 T allele observed throughout our study populations (Table 6.1). These observations contrast with the results of Chen et al. who reported a statistically significant reduction in RS1805110 CC genotype frequency in patients with BD compared to control (19.9% vs. 28.8%) and an overall T allele frequency of 46.53% in their study sample. It was postulated that the CC genotype conferred a potential protective effect against developing BD (Chen et al. 2012). Whilst we also observe a lower frequency of the CC genotype in BD patients compared to control in our study (81.33% vs. 89.41%), this difference was not statistically significant. As a result, the possibility that RS1805110 may influence BD in Caucasian cannot be fully excluded, however due to the low frequency of the C allele we conclude that any influence would be very weak.

The observed T allele frequency in our study is consistent with that reported for Caucasian populations on the HapMap database (The International HapMap Consortium 2015) (5.6% for Utah residents with Northern and Western European ancestry from the CEPH collection), whilst the results of Chen et al. are consistent with the T allele frequency reported for Chinese populations (39.0% for Chinese in Metropolitan Denver and 42.7% for Han Chinese in Beijing) and suggests that there is significant genetic variation between patients with BD depending on geographic region (Table 6.2). Similar findings have been reported in studies of other candidate genes in BD. Recent genome-wide association studies have identified several BD associated genes such as HLA-B*51, IL23R, and IL10, in patients cohorts across the

geographical range of BD. By comparison, other genes identified in these and candidate gene studies are specific to particular regions (Remmers et al. 2010; Mizuki et al. 2010). For example, an inverse correlation has been observed between the PTPN22 620W polymorphism and BD in the UK population, but this association was not seen in Middle Eastern populations where the prevalence of the 620W polymorphism is very low (Baranathan et al. 2007). Similarly, no association has been demonstrated between PTPN22 SNP and BD in Han Chinese patients (Zhang et al. 2012). This suggests that while HLA-B*51 is the strongest association in all BD patient cohorts, different polymorphisms are linked with the condition in different geographical areas.

Similar geographic variations in TGFBR3 SNP have been reported in a number of other contexts. Studies in primary open angle glaucoma indicate that TGFBR3 SNP RS1192415 is a common determinant of optic disc area in patients of Caucasian (European) and Asian (Indian and Malay populations in Singapore) backgrounds (Khor et al. 2011; Ramdas et al. 2011), however this association is absent in Afro-Caribbean patients where other SNP appear more significant (Cao et al. 2012). Studies in osteoporosis show comparable variation, with the SNP RS17131547 showing a statistically significant association with reduced bone mineral density in Afro-Caribbean and White US populations, but being monomorphic in Chinese populations (Xiong et al. 2009).

Most existing research of TGFBR3 function has been performed in the context of cancer studies where TGFBR3 deletions are thought to have a paradoxical role, being tumour-promoting if occurring early in disease through presumed impairment of immune suppression by TGFβ, but tumour-suppressive if occurring later in disease through probable enhancement

of the anti-tumour effect of the local immune system (Gatza, Oh, et al. 2011). In each circumstance, loss of TGFBR3 is thought to impair local immune responses through the reduction in potency of TGFβ at sites of antigenic challenge.

The functional relevance of RS1805110 has not been studied to date. This polymorphism is a non-synonymous SNP (Ser/Phe), which changes the amino acid sequence, predicted to be located in the signal sequence region, where it might affect secretion or expression of the protein. A protective effect postulated by Chen et al. would suggest either a "gain of function" in relation to the anti-inflammatory effect of TGFβ, or a "loss of function" in relation to induction of Th17 cells which have been implicated in BD (Na et al. 2013). Future studies will be needed to address this.

Our investigation is subject to a number of limitations. Firstly, given the very low frequency of the T allele in Caucasian populations, our sample size may have been too small to detect statistically significant associations between this SNP and disease groups. Second, since patients were recruited from ophthalmology clinics we are unable to comment on any association in patients with non-ocular BD. Finally, we have screened for a single polymorphism; significant associations may exist with other TGFBR3 SNPs in the Caucasian population.

In conclusion, whilst TGFBR3 RS1805110 has been proven to be associated with BD in a Han Chinese population, it is not associated with BD or IIU in Caucasian a population. This study highlights the complexity in genetic determinants of BD, and reveals further geographical genetic variation in patients with BD.

7 FINAL DISCUSSION

7.1 Introduction

We were able to successfully develop a novel experimental model in which all resident T and B lymphocytes were betaglycan-deficient. To our knowledge, this is the first example of an *in vivo* system in which to study betaglycan-deficient T and B lymphocytes, and we are the first group to investigate the role of betaglycan in peripheral immune responses. We observed population of peripheral lymphocyte compartments (Chapter 3) and the development of secondary lymphoid tissues in our chimeric animals (Chapter 4), and were able to use our chimeras for both *in vivo* and *in vitro* assays of TGFβ-dependent T lymphocyte responses (Chapter 5).

In addition, we developed a model of antigenic challenge comprising subcutaneous injection of 2W1S peptide antigen in conjunction with an adjuvant of *Salmonella typhi* outer membrane porins, in which we were able to induce polarisation of Th1, Treg and Th17 lymphocytes in draining lymph nodes, and identify a population of responding 2W1S-specific CD4+ T cells (Figure 5.11). To our knowledge, this model has never previously been reported.

7.2 Refuting the hypothesis

Our initial hypothesis consisted of three broad statements; through the experiments described we have been able to refute each of these.

We hypothesized that betaglycan would be necessary for TGFβ2 signalling to T lymphocytes via the cell-surface TGFβ receptor, being an absolute requirement for TGFβ2-mediated immune responses. Our *in vitro* assays demonstrated FoxP3 induction in naïve T lymphocytes following exposure to all isoforms of TGFβ in both the presence and absence of betaglycan, with no statistically significant difference in the number of FoxP3+ cells induced by TGFβ2 between betaglycan-deficient and betaglycan-sufficient cell populations at any of the tested concentrations of TGFβ (Figures 5.4 to 5.6). We therefore conclude that betaglycan is not an absolute requirement for TGFβ2 signalling to T lymphocytes in our assays.

We hypothesized that betaglycan would increase the affinity of both TGFβ1 and TGFβ3 for the cell-surface TGFβ receptor on lymphocytes, resulting in impairment of TGFβ1 and TGFβ3-mediated T lymphocyte immune responses in the absence of betaglycan. Our *in vitro* assays demonstrated no significant difference in the number of FoxP3+ cells induced by TGFβ1 or TGFβ3 when comparing betaglycan-sufficient and betaglycan-deficient cell populations (Figures 5.4 to 5.6). We therefore conclude that betaglycan has no influence on the relative potency of either TGFβ1 or TGFβ3 for the cell surface receptor on T lymphocytes, and does not increase influence the affinity of TGFβ1 or TGFβ3 for the receptor in our assays.

Finally, we hypothesized that no spontaneous immune dysregulation would be observed *in vivo* under conditions of betaglycan deficiency in environments where TGFβ1 or TGFβ3 signalling predominates, however disordered Th17 and Treg lymphocyte responses would be observed under conditions of antigenic challenge. Observation of naïve chimeras revealed a subtle phenotype of increased T lymphocyte activation in betaglycan-deficient chimeras (Figure 4.4), with an exaggerated Th1 response, but with no differences in Th17 or Treg lymphocyte populations (Figures 4.7 to 4.8). These observations correlated with the results of our controlled antigenic challenge experiments, which again showed no alteration of Treg or Th17 responses, but demonstrated an exaggerated Th1 response in betaglycan-deficient chimeras (Figure 5.12, figures 5.14 to 5.15). We therefore conclude that betaglycan deficiency may result in spontaneous (but subclinical) immune dysregulation, with particular influence on Th1-mediated immune responses.

7.3 Current understanding of the immunoregulatory role of betaglycan

Based on our observations, we conclude that betaglycan is involved in the TGFβ signalling process at the T lymphocyte cell surface. We speculate that it mediates TGFβ signalling in T lymphocyte homeostasis, contributing to regulation of T lymphocyte activation levels. Targeted betaglycan deficiency restricted to T and B lymphocytes appears to cause spontaneous CD4+ and CD8+ T lymphocyte activation in naïve mice. Betaglycan is also implicated in T lymphocyte polarisation, with targeted deficiency on T and B lymphocytes

resulting in increased proportions of Th1 CD4+ T lymphocytes in both naïve and antigen-experienced mice.

Betaglycan is not implicated in Treg or Th17 polarisation. Targeted betaglycan deficiency restricted to T and B lymphocytes results in no gross phenotype of immune mediated disease.

7.3.1 Betaglycan as a regulator of T lymphocyte activation and polarisation

Our data suggests that betaglycan deficiency results in spontaneous CD4+ and CD8+ T lymphocyte activation in naïve mice (Figure 4.4). This suggests that betaglycan is involved in TGFβ signalling across the cell membrane in T and B lymphocytes, with deletion resulting in impairment of the immunosuppressive effect of TGFβ on these cell populations. The exact mechanism by which this occurs remains unclear; TGFβ may have a direct influence on antigen presentation, since it is known to play an important role in regulation of MHC levels (Lee et al. 1997; Panek et al. 1995).

In studies of the TGFβ1 knock-out mouse, increased expression of MHC class I and II has been observed on antigen presenting cells in the absence of TGFβ (Geiser et al. 1993). Impaired TGFβ signalling may thus cause up-regulation of MHC molecules, resulting in abnormal antigen presentation to T lymphocytes and initiation of immune activation in apparently naïve animals. This mechanism is however unlikely to explain the variation in lymphocyte activation observed in our model, since betaglycan was absent only from T and B lymphocytes, whilst all other MHC-expressing cells retained normal levels of betaglycan.

Others have suggested that TGFβ may have an indirect role in lymphocyte activation through regulation of Treg numbers (Li et al. 2007; Ishigame et al. 2013). Naïve T lymphocytes are induced to differentiate to FoxP3+ Treg under the influence of TGFβ (Ming O. Li et al. 2006), which subsequently act to suppress local immune responses. Abrogation of TGFβ signalling is thus assumed to prevent Treg polarisation, resulting in a shift towards a pro-inflammatory environment with disordered activation of local T lymphocytes. Whilst this may contribute to the process of T lymphocyte activation in some situations, we observed no significant reduction in Treg proportions either in our naïve (Figures 4.7 to 4.8) or antigen-experienced animals (Figures 5.12 to 5.15), and furthermore, were unable to demonstrate any role for betaglycan in *in vitro* assays of FoxP3 induction in naïve T lymphocytes (Figures 5.4 to 5.6).

Similar increases in lymphocyte activation have been observed following targeted deletion of TGFβRII, although the resulting phenotype was considerably more severe, with over 90% of CD4+ and CD8+ T lymphocytes are observed to display a memory phenotype (Gorelik & Flavell 2000). This suggests that betaglycan functions in a similar manner to TGFβRII, but has less overall influence on TGFβ signalling across the cell surface receptor complex, and is consistent with previous observations of betaglycan acting as a co-factor in the signalling process rather than being an absolute requirement for TGFβ signal transduction (Cheifetz et al. 1990; López-Casillas et al. 1991; Sankar et al. 1995; Stenvers et al. 2003).

In addition to its role in T lymphocyte activation, betaglycan also appears to regulate T lymphocyte polarisation, being necessary for regulation of Th1-mediated immune responses both at rest (Figures 4.7 to 4.8) and following antigenic challenge (Figures 5.14 to 5.15). Excessive Th1 responses have been widely reported in response to the absence of TGFβ

signalling. This has largely been considered a secondary effect of reduced Treg differentiation in the absence of TGFβ, for example in a murine transfer model, TGFβ from FoxP3+ Treg cells was shown to be necessary to suppress Th1 cell differentiation and prevent development of experimental colitis (Fantini et al. 2006). Indeed, several studies have demonstrated an association between reduced proportions of peripheral Treg lymphocytes and development of immune-mediated disease in TGFβ-deficient mice (Ouyang et al. 2010; Gu et al. 2012; Takimoto et al. 2010), however these observations do not correlate with our data, since we observed no disruption of Treg populations in either our naïve (Figures 4.7 to 4.8) or antigen-experienced mice (Figures 5.12 to 5.15).

In a mouse model of Th1-mediated type-1 diabetes, Ishigame et al. demonstrated that inhibition of TGFβ signalling to CD4+ T lymphocytes through conditional TGFβRII knock-out resulted in increased Th1 polarisation and development of diabetes, in the presence of normal Treg development, maintenance and function (Ishigame et al. 2013). This suggests that Treg-independent mechanisms of Th1 polarisation exist under conditions of deficient TGFβ signalling, and this may explain the trends in our data. It is thus possible that betaglycan deficiency results in direct up-regulation of Th1 polarisation, in a similar, Treg-independent manner.

Mouse models of targeted TGFβRII deficiency on T lymphocytes result in lethal autoimmune disease with marked T lymphocyte activation and inflammatory infiltration of multiple organ systems (Gorelik & Flavell 2000; Ming O. Li et al. 2006). In contrast, we observed relatively mild T lymphocyte activation, with no evidence of autoimmunity (Figure 4.9) or tissue-specific inflammatory disease (Figures 4.10 to 4.13) in response to targeted betaglycan

deficiency. Betaglycan is ubiquitously expressed by almost all cell types (Pakula et al. 2007), and expression of betaglycan by non-T non-B lymphocyte cell populations was unaffected in our betaglycan-deficient chimeras (Figure 3.4); it is thus possible that betaglycan-deficient T lymphocytes were able to utilise these external sources of betaglycan in TGFβ signal transduction.

This may have been achieved in two ways; firstly, it is known that betaglycan can exist in a soluble form following cleavage of the receptor at the cell membrane (Velasco-Loyden et al. 2004). Soluble betaglycan released from resident non-lymphocyte cells in this way may be able to interact with the cell-surface TGFβ receptor on local lymphocytes, being able to function in a comparable way to membrane-bound betaglycan. Secondly, it has recently been shown that IL15 is able to regulate CD4+ T lymphocyte homeostasis via trans-presentation by non-lymphocyte cells (Chen et al. 2014); an analogous mechanism may allow TGFβ to be trans-presented to the lymphocyte by betaglycan at the cell surface of resident non-lymphocyte cells. If either of these mechanisms are indeed present in the murine immune system, lymphocytes may be able to compensate for betaglycan deficiency at the cell surface, and the lymphocytes could not be considered truly betaglycan-deficient in our experimental system. In future work this could be addressed with the use of betaglycan blocking antibodies administered at the time of antigenic challenge.

Furthermore, the possibility of Smad-independent, and therefore TGFβ-independent FoxP3+ induction has been postulated by some authors, who have identified other transcription factors such as NFAT, which are able to bind the FoxP3 promoter and induce Treg cells (Tone et al. 2008; von Boehmer & Nolting 2008). This raises the possibility that in the absence of a fully

functioning TGFβ receptor, there may be up-regulation of TGFβ-independent signalling pathways and maintenance of immune homeostasis.

Our in vitro assays of TGFβ-dependent T lymphocyte responses were designed to address some of these issues. We sorted pooled lymph node and spleen cell suspensions to highly purified populations of naïve T lymphocytes, and thus removed all non-lymphocyte cells from these assays (Figure 5.1). In the absence of non-T lymphocyte cells, trans-presentation of betaglycan was not possible, avoiding this potential source of betaglycan in our knock-out cells. It is however possible that our naïve cells may have been 'contaminated' with soluble betaglycan prior to being sorted; if lymphocytes are indeed able to retain soluble betaglycan at the cell surface the effects of our knock-out may again have been masked.

7.3.2 Betaglycan is redundant in Treg and Th17-mediated immune responses and is not involved in differential responses to TGFβ

TGFβ is required for *in vitro* differentiation of both Treg and Th17 from naïve T lymphocytes (Yamagiwa et al. 2001; Chen et al. 2003; Ming O. Li et al. 2006; McGeachy & Cua 2008; Bettelli et al. 2006). Despite this, we observed no evidence of dysregulated Treg or Th17-mediated immune responses in our *in vivo* experiments (Figures 4.7 to 4.8, Figures 5.12 to 5.15), and furthermore, observed no difference in TGFβ-induced Treg differentiation between betaglycan-deficient and betaglycan-sufficient cell populations in our *in* vitro assays (Figures 5.4 to 5.6). This was an unexpected observation, having already demonstrated a potential role for betaglycan in the regulation of T lymphocyte responses at rest (Chapter 4).

As with all *in vitro* assays, this is a highly artificial scenario, which over-simplifies the true biological process; *in vivo*, TGFβ would first be released in latent form, being activated at its target destination (Dallas et al. 2008). We cannot rule out the possibility that betaglycan is in fact involved in this activation of TGFβ at the T lymphocyte cell surface. In addition, the interaction of recombinant TGFβ with the TGFβ receptor may differ from the interaction with native TGFβ, with the role of betaglycan being specific to interaction with the naïve form. This could be addressed by the addition of latent, native (human) TGFβ to our assays; aqueous humour from un-inflamed eyes would be one possible source, with the additional advantage that this is known to be a rich source of TGFβ2 (Denniston et al. 2011).
It should also be noted that published data suggesting a role for betaglycan in TGFβ2-dependent cell proliferation is based on experiments using *in vitro* assays of cell lines (López-Casillas et al. 1993; Sankar et al. 1995). Whilst cell lines are useful in generating *in vitro* data, they are a highly manipulated and artificial system. It may not be possible to extrapolate such data to resident cell populations from *in vivo* sources.

We must also acknowledge that our *in vitro* assays do not directly address signalling by TGFβ across the cell surface receptor; we are able to draw conclusions concerning the ability of TGFβ to induce nuclear transcription factors, but are unable to describe how TGFβ achieves this, and cannot comment on the signalling process. Gu et al. investigated TGFβ-dependent Treg responses in a transgenic mouse model in which all Treg lacked Smad2 and Smad3 (Gu et al. 2012). The resulting animals displayed normal thymic development and peripheral maintenance of Treg, suggesting that Smad-independent pathways are important for Treg function. Although unlikely, it is possible that the TGFβ-dependent responses observed in our assays occurred independently of the TGFβ receptor. Future work is required to measure

products of the signalling cascade, such as Smad protein phosphorylation, to confirm that receptor signalling is involved in these assays.

It is also possible that betaglycan is not primarily involved in TGFβ signalling, but is involved in signal transduction by other ligands, and the previously observed effects of betaglycan deficiency on T lymphocyte homeostasis were independent of TGFβ signalling. In addition to binding all isoforms of TGFβ, betaglycan is also able to bind a range of other molecules, including all members of the TGFβ superfamily including inhibin A, inhibin B and certain BMPs (Bilandzic & Stenvers 2011). It is therefore possible that other non-TGFβ dependent pathways are involved in signalling to T lymphocytes *in vivo*.

This is supported by a recent publication investigating role of betaglycan in foetal thymic organ cultures, in which betaglycan was found to be necessary for T lymphocyte maturation, primarily through its ability to bind inhibins (Aleman-Muench et al. 2012). Similarly, BMP have previously been implicated in T lymphocyte maturation in the thymus (Cejalvo et al. 2007); it is possible that this effect is also mediated by betaglycan and may be important in other resident lymphocyte populations.

Again, our *in vitro* assays were designed to address this, since we were able to test specific isoforms of TGFβ, and in particular were able to assess TGFβ2-dependent responses in the complete absence of TGFβ1. Despite this, we remained unable to demonstrate a clear role for betaglycan in TGFβ2-dependent T lymphocyte responses (Figures 5.4 to 5.6).

Finally, we must acknowledge that we may have chosen an inappropriate assay to assess TGFβ signalling via betaglycan, since TGFβ potency is known to vary in different biological processes in the same cell. As previously discussed, FHBE cells are naturally deficient in betaglycan, and whilst they have been shown to display reduced TGFβ2-mediated growth inhibition compared to wild-type cells (Cheifetz et al. 1990), they display similar TGFβ2-mediated PAI-1 inhibition (ten Dijke et al. 1988). It is thus possible that we may have observed a difference in our betaglycan-deficient cell cultures by assessing an alternative end-point. Given the observation of the potential involvement of betaglycan in Th1 responses, we speculate that *in vitro* assays of Th1 polarisation of naïve T lymphocytes (as discussed in Kato & Nariuchi 2000) may reveal differences between betaglycan knock out and wild type cells.

7.3.3 Betaglycan as a regulator of tissue-specific immune responses

Overall, we have observed a subtle role for betaglycan in immune responses; whilst we have evidence for increases in T lymphocyte activation and Th1 polarisation, this resulted in no obvious external phenotype (Chapter 3).

It is perhaps surprising that our naïve betaglycan-deficient chimeras displayed any evidence of immune dysregulation, since previously published literature suggests that betaglycan is involved in TGFβ2-dependent responses, being necessary for high affinity interactions between the ligand and cell-surface receptor complex, but is not required for TGFβ1 or TGFβ3 driven processes (López-Casillas et al. 1994; López-Casillas et al. 1993; Sarraj et al. 2013; Cheifetz et al. 1990). Since the predominant isoform of TGFβ acting in the peripheral

murine immune system is TGFβ1 (Rubtsov & Rudensky 2007), any potential influence of betaglycan deficiency on TGFβ2 signalling was expected to be masked. We therefore speculate that the role of betaglycan may still be more significant in environments where TGFβ2 predominates, and thus tissue-specific phenotypes may exist.

Although speculative, there are notable similarities between this observed / predicted action of betaglycan and that of another molecule known as Dickkopf-3 (Dkk3). Dkk3 is a secreted protein with high expression in immune privileged organs such as the embryo, placenta, brain and eye (Niehrs 2006; Nakamura et al. 2007). Animals deficient in Dkk3 show no external evidence of immune-mediated disease, and display normal resting T lymphocyte populations and normal T lymphocyte activation levels (Meister et al. 2015). Differences in Dkk3-deficient mice become apparent on induction of EAE, with increased IFNγ production and T lymphocyte activation within the CNS, whilst levels in lymph node and spleen are unaffected. Dkk3 has thus been suggested as an important tissue-specific modulator of T lymphocyte activity, usually functioning to limit T lymphocyte activation and Th1 polarization.

We speculate that betaglycan-deficient animals may display similar exacerbation of EAE and EAU, associated with an exaggeration of the previously observed changes in T lymphocyte populations.

7.4 The role of betaglycan in inflammatory disease in humans

The only published association between betaglycan and human inflammatory disease exists for BD, specifically in a Han Chinese population (Chen et al. 2012). This is an interesting

association, since BD is characterised by severe intra-ocular inflammation (uveitis), which is thought to be mediated by CD4+ T lymphocytes under the control of TGFβ. Furthermore, TGFβ2 is the predominant intra-ocular isoform (Denniston et al. 2011; Stein-Streilein 2008), and despite the lack of supportive evidence in our experiments documented herewith, betaglycan has previously been considered particularly important in TGFβ2 signalling (Cheifetz et al. 1990; López-Casillas et al. 1991; Sankar et al. 1995).

In uveitis, inflammation is often limited solely to the eye. Despite this, current therapeutic strategies in uveitis usually involve systemic immunosuppression with significant unwanted side effects in multiple organ systems (Barry et al. 2014). The ideal pharmacological agent in uveitis should enable potent suppression of intra-ocular CD4+ T lymphocyte activity, without modulation of T lymphocyte activity at other, non-inflamed sites. Whilst TGFβ2 predominates in the ocular microenvironment (Denniston et al. 2011; Stein-Streilein 2008), TGFβ1 is the predominant isoform acting in the peripheral immune system (Rubtsov & Rudensky 2007). Modulation of betaglycan by a therapeutic agent may allow targeted manipulation TGFβ2 signalling and thus confer the ability to localise the resulting immunosuppressive effect to the eye, avoiding unnecessary systemic side effects.

We have observed a variable role for betaglycan in regulating activation and polarisation of CD4+ T lymphocytes with increases in lymphocyte activation and Th1 polarisation observed in its absence; potentiation of signalling either through up-regulation of betaglycan receptors on T lymphocytes or addition of soluble betaglycan may therefore emerge as a useful therapeutic strategy in uveitis.

Whilst we were unable to demonstrate a statistically significant association between betaglycan and BD in a Caucasian population (Barry et al. 2015), we did observe a non-significant trend towards reduced prevalence of the RS1805110 CC genotype (Table 6.1). It is thus possible that a similar association exists in Caucasian patients compared to that observed in Han Chinese patients, which may have become statistically significant with a larger sample size. Whilst our study is therefore inconclusive, it is supportive of a wider role for betaglycan in ocular inflammatory disease.

7.5 Limitations of current investigation and future work

All investigation presented herewith is based on the study of mixed foetal liver chimeric mice; whilst this enabled us to generate a novel model of targeted betaglycan deficiency on T and B lymphocytes, the relative complexity of the breeding programme (Figure 3.1) resulted in unavoidable time constraints in the generation of experimental mice. Furthermore, chimeras could only be genotyped after cell transfer, resulting in significant surplus of heterozygote mice, with relatively few wild-type and knock-out chimeras. As previously documented, this necessitated a more 'opportunistic' approach to phenotypic data collection than would be ideal, and limited the number of available animals for use in our *in vivo* and *in vitro* experiments. Future experiments may benefit from the development of models of adoptive transfer or conditional betaglycan knock-out mice.

Our investigation was largely restricted to CD4+ T lymphocyte responses with more limited assessment of CD8+ responses, and very limited assessment of B lymphocyte and other immune cell populations. Given the paucity of literature to inform our investigation, we felt

that we were most likely to find a role for betaglycan in known TGFβ-dependent T lymphocyte responses, and therefore made this the focus of our investigation. Whilst we observed no obvious difference in ratios of T:B lymphocytes (Figure 4.2) or host:donor non-T / non-B lymphocyte populations (Figure 3.4) in our chimeras, we cannot exclude the possibility that betaglycan is involved in TGFβ signalling to other cell types, or that deficiency on T lymphocyte has indirect effects on other cell populations. In the future, we would thus be interested to study proportions of B lymphocyte subsets both in naïve animals and in response to controlled antigenic challenge, and also investigate APC subsets in greater detail, which may be involved in trans-presentation of betaglycan to the T lymphocyte.

Similarly, histological investigation was restricted to the study of secondary lymphoid organs (Figures 4.10 to 4.13), with no assessment of other tissues. Again, we felt that betaglycan deficiency was most likely to manifest as disruption of lymphocyte populations and micro-anatomy in these tissues, and whilst we observed no external evidence of disease in other sites (for example diarrhoea to suggest bowel disease, weakness and paralysis to suggest disease of the central nervous system), we cannot exclude the possibility of undetected subclinical disease in other sites. We would recommend detailed histological examination of brain, eye, thymus, liver, kidney and bowel in future work.

As discussed above, betaglycan is unlike other components of the TGFβ receptor complex due to the observation that it can also exist in a soluble form. Whilst we are confident that we were able to knock-out the cell-surface betaglycan receptor on T and B lymphocytes, it remained present on all other cell types. We made no attempt to block the action of soluble betaglycan in our *in vivo* experiments, and it is thus possible that T and B lymphocytes in our

betaglycan deficient chimeras were able to compensate for betaglycan deficiency at the cell surface by utilising soluble betaglycan from other sources. Whilst our *in vitro* assays addressed this concern to a certain extent, it may be necessary to consider the use of blocking-antibodies in future experiments.

Considering existing literature, we were surprised to find no evidence of a role for betaglycan in TGFβ2 dependent responses in our *in vitro* assays (Figures 5.4 to 5.6); we suspect this is attributable either to the use of activated rather than latent TGFβ in our assays, or to the use of TGFβ receptor-independent signalling pathways. We would recommend the use of latent TGFβ in future assays, and would also recommend the measurement of Smad phosphorylation in these assays to confirm signalling via the TGFβ receptor. We would also like to replicate the previously-published growth inhibition assays using cells derived from our chimeras.

We were unable to investigate tissue-specific immune responses in our *in vivo* experiments of controlled antigenic challenge. As previously discussed, we suspect betaglycan may act as a tissue-specific regulator of T lymphocyte function, being more likely to be important in environments where TGFβ2 signalling predominates; our preferred experimental model of such tissue-specific responses is EAU, and we would be keen to induce this process in our chimeras in the future.

We would also be interested to investigate other polymorphisms in the betaglycan gene across a range of ocular inflammatory diseases, and to investigate betaglycan expression in aqueous

humour samples of patients with uveitis and controls, to further elucidate whether betaglycan is an important mediator of intra-ocular inflammation in humans.

7.6 Conclusion

Our aim was to determine the immunoregulatory role of betaglycan; based on the experimental research described within this report, we conclude that betaglycan does indeed have a role in immunoregulation, being implicated in activation and polarisation of T lymphocytes. Based on our observations, we speculate that betaglycan may act to limit T lymphocyte activation and inhibit Th1-mediated immune responses. Betaglycan is not an absolute requirement for TGFβ signalling, and appears to have less influence over TGFβ-mediated signalling processes than the other components of the cell surface receptor. In contrast to most existing literature, we have found no clear evidence for a predominant role in TGFβ2 signalling, however we suspect this may be due to the endpoints assessed in our experiments, and remain open to the possibility that a differential response may become more obvious in other assays.

Our understanding of betaglycan remains incomplete, and we acknowledge that we have generated far more questions than answers. We have however developed and characterised a novel animal model of targeted betaglycan deficiency, which hope provides a strong foundation on which to base future work.

8 APPENDIX

8.1 Pharmacotherapy for uveitis: current management and emerging therapy (published data)

The introduction to uveitis detailed in section 1.4.7 was written as a review article and accepted for publication in Clinical Ophthalmology.

Full citation:

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REVIEW

Pharmacotherapy for uveitis: current management and emerging therapy

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Abstract: Uveitis, a group of conditions characterized by intraocular inflammation, is a major cause of sight loss in the working population. Most uveitis seen in Western countries is non-infectious and appears to be autoimmune or autoinflammatory in nature, requiring treatment with immunosuppressive and/or anti-inflammatory drugs. In this educational review, we outline the ideal characteristics of drugs for uveitis and review the data to support the use of current and emerging therapies in this context. It is crucial that we continue to develop new therapies for use in uveitis that aim to suppress disease activity, prevent accumulation of damage, and preserve visual function for patients with the minimum possible side effects.

Keywords: clinical trials, immunomodulatory therapeutic agents, immunosuppression, inflammation, uvea

Background

Uveitis, a significant cause of blindness worldwide, is a term applied to a wide range of conditions that are characterized by intraocular inflammation. Many cases of "uveitis" do indeed involve inflammation of the uvea (which comprises the iris, ciliary body, and choroid), but may also involve adjacent structures such as the retina or vitreous. Uveitis is highly heterogeneous, varying in etiology, pattern, tissue involved, and extent. The uveitis specialist may be confronted by a small, localized area of inflammation in a single tissue in a non-sight-threatening location, or widespread blinding inflammation involving almost all ocular tissues. Visual impairment is common, affecting between 2.8% and 10% of patients,[1–3] and may result directly from damage to uveal tract structures, or may occur due to secondary effects on neighboring tissues: for example, accelerated cataract formation, glaucoma, and macular edema.[4]

The Standardization of Uveitis Nomenclature (SUN) working group classifies uveitis according to the site of primary inflammation.[5] Anterior chamber inflammation is categorized as "anterior uveitis", and includes iritis, iridocyclitis, and anterior cyclitis. Inflammation primarily affecting the vitreous is referred to as "intermediate uveitis", and includes pars planitis, posterior cyclitis, and hyalitis. "Posterior uveitis" describes inflammation of the retina or choroid. Finally, "pan-uveitis" describes the situation where inflammation is seen throughout the anterior chamber, vitreous, and retina or choroid. According to the SUN criteria, disease is further classified according to onset (sudden or insidious), duration (limited or persistent), and course (acute, recurrent, or chronic).

Pathophysiology

Uveitis can be either infectious or noninfectious; whilst both may present with similar clinical features, they are best considered as distinct disease entities since the underlying pathophysiology and treatment strategies are very different.

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Whilst common in the developing world, infectious causes account for the minority of uveitis cases presenting to tertiary referral centers in the West. Infectious causes include organisms such as toxoplasma, cytomegalovirus, syphilis, and herpes viruses.[6,7] Local infection results in foreign antigen presentation to ocular immune cells, with appropriate immune activation aimed at clearing the invading organism. Uveitis occurs as a secondary effect of this immune activation.

Noninfectious uveitis is thought to result from inappropriate activation of the immune system[8] and it is therefore not surprising that it is often associated with systemic autoimmune or autoinflammatory diseases such as ankylosing spondylitis (AS), sarcoidosis, or Behçet's Disease (BD). In the remainder, however, no such systemic association is identified; these cases are generally labeled as "idiopathic" in recognition of the fact that the autoimmune/autoinflammatory origin of most of these cases is presumed rather than proven. Human data and experimental models indicate parallel changes in the inflammatory milieu of the intraocular microenvironment. Uveitis may be induced in animal models by a range of mechanisms that cause differentiation of naïve CD4+ T-cells to pathogenic effector cells, resulting in tissue damage.[9–13] Although similar pathogenic effector cells have been recovered from ocular fluids and tissue in human uveitis, the evidence for autoreactive T-cells (such as seen in the animal models) is much more limited.[14,15]

Such idiopathic cases account for the largest cohort of patients seen in most clinical practices in the West. In a retrospective study of all uveitis cases presenting to a tertiary center, Rodriguez et al reported 34% to be idiopathic, 10.4% to be associated with seronegative spondyloarthropathies, and 9.6% to be associated with sarcoidosis,[16] although it should be noted that certain conditions are typically associated with particular anatomical groups, such as anterior uveitis with seronegative spondyloarthropathies. It is also of interest to note that, in a report from the Systemic Immunosuppressive Therapy for Eye Diseases (SITE) retrospective study of US tertiary uveitis services, the leading systemic associations in 4,911 patients with uveitis were sarcoidosis (7%), seronegative spondyloarthropathy (5%), juvenile idiopathic arthritis (JIA) (5%), and BD (3%).[17]

Brief overview of treatment strategies

For infectious causes, treatment is aimed at eradicating the pathogenic organism with appropriately targeted antimicrobial therapy. In severe cases, such agents may be delivered directly to the eye by intravitreal injection, or are more frequently administered systemically by an oral or intravenous route. Once the infectious agent is considered to be under control, immunosuppressive agents such as corticosteroids may be used judiciously to limit tissue damage.

For noninfectious causes, treatment involves suppression of the local immune response. It is useful to consider the concept of disease activity versus damage when treating inflammatory disease.[18,19] "Activity" refers to the ongoing immune response, which may be acute or chronic, but is usually reversible. "Damage" refers to the effect of active inflammation on native tissues; it is usually irreversible. In simple terms, persistent activity will lead to accumulation of damage. In theory, effective therapy should suppress all activity and prevent or halt accumulation of damage.

Therapy in noninfectious uveitis is aimed at suppressing the immune system, and ranges from topical therapy (commonly corticosteroid eye drops) to systemic immunosuppression with either high-dose corticosteroids (oral, intravenous, intramuscular, or subcutaneous) or a wide range of corticosteroid-sparing immunomodulatory therapeutic (IMT) agents.[20,21] Ideally, treatment should be targeted to the mechanism and localized to the tissue to maximize the efficacy/side-effect profile. However, this is often not achieved and new therapies should therefore aim to suppress disease activity, prevent accumulation of damage, and preserve visual function for patients with the minimum number of possible adverse events.[22–24]

Introduction to the epidemiology of uveitis

Uveitis is considered a rare disease,[25] with an estimated incidence between 17 and 52 people per 100,000 population in Europe and the USA,[1,7,26,27] although a higher incidence of disease may be observed in Chinese and Japanese populations.[28] Despite this rarity, it is a disproportionately common cause of legally-recognized visual impairment, and is the fourth most common cause of blindness in the working-age population in the developed world.[2,29,30] Uveitis can occur in any age group; however, it is particularly prevalent in younger people, with a mean age at onset of less than 40 years.[30]

Epidemiological studies in uveitis are particularly prone to bias. Most notably, a range of criteria exist for diagnosis and categorization of different uveitis entities; the relatively recent introduction of SUN working group anatomical classification has helped standardize practice; however, clinical, etiological, and pathological classification criteria have been used variably throughout the literature, and it is often difficult to compare published data from multiple sources.[29,31–34] In addition, since the majority of research in uveitis is

generated from tertiary referral centers with relatively little data from community-based practice, there is bias towards severe disease and a relative under-representation of more straightforward cases.[35] Furthermore, given the heterogeneity of uveitis entities and the wide geographic variation in both clinical features and disease etiology, comparison between different regions is difficult.[6]

Disease etiology shows significant variation with age, with some forms of uveitis affecting specific groups. For example, in European populations, uveitis due to JIA occurs almost exclusively in children;[36,37] human leucocyte antigen (HLA)-B27-positive disease is seen in young adults;[38] and masquerade syndromes and lymphoma are more commonly seen in the elderly.[25]

Infectious uveitis is relatively rare in developed countries, accounting for 13%–21% of cases, and is thought to be mostly due to infection by herpes viruses.[6,25] This is in stark contrast with developing countries, where up to 50% of uveitis is thought to have an infectious etiology. The most common infectious causes in these populations are toxoplasmosis, tuberculosis, onchocerciasis, cysticercosis, leprosy, and leptospirosis.[39]

In Western countries, anterior uveitis accounts for at least half of all cases,[40] of which idiopathic disease accounts for approximately 50%.[27] The most common clinical associations in those countries are HLA-B27-positive disease, AS, and Fuchs heterochromic iridocyclitis.[6] It is estimated that up to 55% of Caucasian patients with acute anterior uveitis are HLA-B27 positive, compared to only 5%–10% in the general population.[41] In contrast, the prevalence of anterior uveitis is much lower in Asian populations, which is thought to be due to the lower frequency of HLA-B27 positivity and AS.[42] Despite this, it should be noted that HLA-B27 positivity remains the most common association with acute anterior uveitis in most Asian populations.[40,42]

Intermediate uveitis is the least common form of disease across all geographic regions, with an estimated incidence of 1.5–2.08 per 100,000 population in Western populations.[6] There is thought to be a strong association with human T-cell lymphotropic virus type 1,[43] and several authors have noted an association between pars planitis and multiple sclerosis (MS), although this has yet to be fully characterized.[44,45]

Posterior uveitis accounts for 15%–30% of diagnoses. The most common cause worldwide is toxoplasmosis, followed by idiopathic disease.[6,40,42] It follows that posterior uveitis is more common in developing countries, owing to the higher prevalence of infectious diseases in these populations.[39] BD and Vogt–Koyanagi–Harada (VKH) disease are two rare but important noninfectious causes of posterior uveitis.[46,47] Cytomegalovirus retinitis is associated with human immunodeficiency virus infection, but its incidence is decreasing with the use of modern highly active anti-retroviral therapy.[6]

It is difficult to accurately estimate the prevalence of panuveitis; in one recent large-scale review of epidemiological studies, the prevalence varied from 1% to 69% depending on geographic location.[6] It is probable that panuveitis is more heavily affected by the biases described above than other types of uveitis, particularly referral bias skewing patient populations towards more severe disease in tertiary referral centers, and, as a result, these figures are unlikely to be representative of the general population. Idiopathic disease is most common in Europe, USA, Australia, and India, whilst infectious causes are again more common in most developing countries, and BD is an important cause in Asia and countries along the historic "silk route" (Middle East and Mediterranean regions).[30,48–51]

Current treatment options

Any treatment strategy for uveitis needs to consider a number of factors.[52] First, the etiology (ie, infectious versus noninfectious), which will define the type of treatment required (antimicrobial versus anti-inflammatory/immunosuppressive). Second, the extent of uveitis and associated inflammation – this includes: 1) anatomical location within the eye (as per SUN classification);[5] 2) unilaterality or bilaterality; and 3) presence of systemic disease. These factors are important considerations when deciding whether topical, local, or systemic treatments are likely to be required. Third, the severity of disease, which may necessitate "rescue" therapy or additional treatment. Fourth, potential complications of either the disease itself or of treatments of the disease.

Infectious versus noninfectious uveitis

One of the most important (and sometimes difficult) challenges to confront the uveitis specialist is whether the inflammatory process is the result of an infectious agent. Establishing or excluding this may sometimes be possible on clinical appearance alone,[53] but is often supplemented by investigations on the peripheral blood (commonly serology, interferon gamma release assays for *Mycobacterium tuberculosis*, and hematological and biochemical markers) or ocular fluids (polymerase chain reaction for suspected microbes; less commonly Goldmann–Witmer coefficient); imaging is also commonly used to characterize associated systemic disease.[54,55]

In some cases, treatment may need to be started before the results of such investigations are available. For example, in the presence of suspected acute retinal necrosis, samples of aqueous humor and vitreous humor should be taken and sent for polymerase chain reaction concurrently with starting treatment (commonly intravitreal foscarnet and either oral valacyclovir or intravenous acyclovir).[56] This situation also highlights another issue: that some cases of infectious uveitis may provoke a strong inflammatory reaction both at the time and sometimes beyond the infectious component of disease; this may in turn require cautious immunosuppression to limit tissue damage and maximize visual potential following treatment. The focus of the remainder of this review will be on noninfectious uveitis. Although this noninfectious group is markedly heterogeneous, the apparent overlap in etiology (autoimmune or autoinflammatory) leads to an overlap in the strategies for treatment and in the types of drugs used.

Extent and severity of uveitis and associated inflammation

In general, isolated uncomplicated anterior uveitis (whether unilateral or bilateral) can often be managed by topical therapy alone; frequent topical corticosteroids are used for rescue treatment, and then titrated down to complete cessation (in acute disease) or to a low frequency maintenance regimen (chronic disease or frequent recurrences); a mydriatic is commonly prescribed to reduce the risk of posterior synechiae.[54]

In cases of posterior segment uveitis (intermediate, posterior, or panuveitis), the topical route provides inadequate penetration to the inflamed tissue, although topical therapies may still have an adjunctive role. For these cases, the choice of treatment will depend on whether the disease is unilateral or bilateral, and whether it is isolated or is a manifestation of a systemic inflammatory process. Unilateral and, increasingly, bilateral disease may be treated by local therapies (peribulbar, sub-Tenon's, or intravitreal routes); corticosteroids are the most common drugs given by these routes although the role of other agents (see "Update on drugs currently in development stages for uveitis treatment" section for further details) are also being assessed. Local therapy may be sufficient to control the disease, but in more severe inflammation, or in the presence of systemic disease, systemic therapy is likely to be necessary. In addition, there may be contraindications to the use of local therapies in some cases.[57]

Recognition and treatment of any associated systemic disease is a priority. Therapy initiated for coexistent systemic inflammatory disease may ameliorate any active uveitis, reducing the need for direct ophthalmic intervention. Once again, rescue therapy is traditionally performed with corticosteroids (either intravenous or oral), with maintenance therapy comprising either a lower dose of corticosteroid or a steroid-sparing IMT agent.[57] The range of steroid-sparing IMT agents available (including biologics) is steadily increasing, although the evidence of their efficacy and safety in uveitis is often lacking.[22] Current options include antimetabolites (such as methotrexate and mycophenolate mofetil), T-cell inhibitors (such as cyclosporine), and alkylating agents (such as cyclophosphamide). More recently, biological therapies (such as infliximab) have been utilized in the management of severe and refractory uveitis.[21,23,58–60] A brief discussion of such agents is included in the next section.

Comparative safety, efficacy, and tolerability of the drugs available for uveitis treatment

When considering the drugs currently available for use in the treatment of ocular inflammation, it is important to recognize the limitations in the evidence that supports their role in uveitis.[22] For many agents there are no randomized controlled trials in uveitis and, thus, justification of their use is based on open-label cohort studies, uncontrolled case-series, extrapolation from their use in other inflammatory conditions, and expert opinion. As a result, it is not yet possible to provide a definitive account of which treatment is "best" for any particular variant of uveitis. Similarly, given the acknowledged limitations in the evidence base, there is no clear line between those drugs that are "established" and those that are still "in development". For the purposes of this review, we have included within the "established" group those agents that are commonly used by specialists in uveitis services (this includes many second-line agents and the older anti-tumor necrosis factor [TNF] agents). Within the "in development" group, we consider both those agents that are in early phase of development and those agents that have established efficacy in other diseases, but have as yet limited evidence for their role in uveitis.

Please refer to Table 1 for a summary of the therapeutic agents discussed in this review. Further guidance for the monitoring of these agents may be found from a number of external resources;[61,62] however, it should be noted that guidance varies between institutions and, thus, all such advice should be checked against local protocols.

Corticosteroids

Corticosteroids are well known for their potent anti-inflammatory effects and have been used to treat uveitis

Table 1 List of therapeutic agents discussed in review

Category	Class	Subtypes	Examples
Corticosteroids	Corticosteroids	–	Prednisolone
			Methylprednisolone
			Iluvien
			Ozurdex
			Retisert
Second-line agents	T-cell inhibitors	Calcineurin inhibitor	Cyclosporine
			Tacrolimus
			Voclosporin
		mTOR inhibitor	Everolimus
			Sirolimus
	Antimetabolites	Purine antagonist	Azathioprine
		DHFR inhibitor	Methotrexate
		IMPDH inhibitor	Mycophenolate mofetil
	Alkylating agents	–	Chlorambucil
			Cyclophosphamide
Biologics	Anti-TNF	Anti-TNFα	Adalimumab
			Certolizumab
			Golimumab
			Infliximab
		Anti-TNFα and β	Etanercept
	Other biologic agents	Anti-CD11a	Efalizumab
		Anti-CD20	Rituximab
		Anti-CD28	Abatacept
		Anti-CD52	Alemtuzumab
		Anti-IL1	Anakinra
		Anti-IL1β	Canakinumab
			Gevokizumab
		Anti-IL2	Daclizumab
		Anti-IL6	Tocilizumab
		Anti-IL17A	Secukinumab
		Interferons	Interferon α/β
		S1P inhibitor	Fingolimod
		VEGF inhibitors	Aflibercept
			Bevacizumab
			Ranibizumab
Other agents	Other	–	IVIG

Abbreviations: CD, cluster of differentiation; DHFR, dihydrofolate reductase; IL, interleukin; IMPDH, inosine monophosphate dehydrogenase; IVIG, intravenous immunoglobulin; mTOR, mammalian target of rapamycin; S1P, sphingosine-1-phosphate; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

since 1950.[63,64] Their anti-inflammatory mechanism is complex, and only partly understood:[65] key components appear to include: direct binding of the glucocorticoid/receptor complex to genes involved in the inflammatory cascade; indirect effects on other transcription factors such as activating protein-1 or nuclear factor-kappa B; inhibitory effects on many inflammation-associated molecules such as cytokines, chemokines, arachidonic acid metabolites, and adhesion molecules; and upregulation of many anti-inflammatory mediators.

Whilst this diverse mechanism of action drives an excellent anti-inflammatory response, it also causes significant unwanted side effects.[23] Ocular side effects (more common with topical or local administration) include accelerated cataract formation and increase in intraocular pressure.[66,67] Systemic side effects (more common with systemic administration) include hypertension, diabetes, Cushing's syndrome, osteoporosis, and disorders of sleep, mood, and appetite.[57]

Corticosteroids continue to have a vital role in terms of "rescue" therapy, but their use as a maintenance therapy is limited by their associated side effects. The American Uveitis Society expert consensus recommendations suggest a maintenance dose of no more than 10 mg oral prednisolone equivalent per day, and this is broadly in line with guidelines from other inflammatory diseases.[57] However, in a survey study among physicians who manage patients with uveitis, it was learned that such guidelines are not always followed.[68]

There is thus a desire to develop treatments with similar anti-inflammatory effects to corticosteroids, but with fewer side effects; this concept of "steroid-sparing agents" describes any anti-inflammatory medication/IMT agent which may be used in place of corticosteroids, or which may allow reduction of corticosteroid dose and attenuation of steroid-induced side effects.

T-cell inhibitors

Cyclosporine was initially developed for use in solid organ transplantation and is known to reversibly bind to and inhibit calcineurin, which in turn inhibits the activity of circulating T-cells.[69,70] It is fast acting, reaching peak efficacy within 7–15 days of initiation of therapy. It is administered orally and in uveitis is commonly given at 2.5–5 mg/kg twice daily, although it should be noted that, within the retrospective multicenter SITE study, it appeared that the dose range of 150–250 mg/day appeared to have equal effect, but with lower rates of side effects, than doses of greater than 250 mg/day.[71] Cyclosporine has demonstrated efficacy in various forms of posterior uveitis. In uveitis secondary to BD, Masuda et al conducted a randomized controlled trial of cyclosporine versus colchicine in 96 patients, demonstrating that both frequency and severity of disease flares were reduced in the cyclosporine group.[72] Smaller noncontrolled studies by Nussenblatt et al[73] and Graham et al[74] reported apparent benefit in mixed cohorts of patients with severe refractory posterior uveitis that included sarcoidosis, idiopathic retinal vasculitis, and BD. Side effects include hypertension, renal impairment, gingivitis, and hirsutism. In the retrospective multicenter SITE study, there were 373 patients who received cyclosporine for uveitis, of whom 11% had to cease the drug within 1 year due to side effects.[71]

Tacrolimus (FK-506) is a macrolide antibiotic with a similar mechanism of action to cyclosporine.[69,70] It is orally administered and, in uveitis, is commonly given at 0.03–0.08 mg/kg/day, but requires trough-level monitoring (aiming for 8–12 ng/L). Like cyclosporine, it has been used extensively in solid organ transplantation. It appears to have equivalent efficacy and better tolerability than cyclosporine. In a randomized but nonmasked controlled trial of 37 patients with refractory posterior segment uveitis (including idiopathic, BD, and sarcoidosis), Murphy et al reported equivalent "success" in the two groups, but significantly lower rates of hypertension and hypercholesterolemia in the tacrolimus group; there was a 5% discontinuation rate for renal impairment in both groups.[75] Other side effects include neurological symptoms, gastrointestinal symptoms, and hyperglycemia.

Antimetabolites

Azathioprine interferes with purine incorporation into DNA, resulting in synthesis of nonfunctional DNA sequences and blocking protein synthesis in lymphoid cells. It is also known to selectively inhibit T-cell function, suppress homing in circulating T-cells, decrease development of monocyte precursors, and suppress the role of natural killer cells in antibody-dependent cytotoxic reactions.[76,77] It is orally administered at a starting dose of 2–3 mg/kg/day, before being titrated according to response and side effects,[21] achieving efficacy within 4–12 weeks. It has been suggested to be effective in management of a wide range of ocular inflammatory conditions including: scleritis secondary to relapsing polychondritis;[78] ocular cicatricial pemphigoid;[79] JIA-associated iridocyclitis unresponsive to corticosteroids;[80] intermediate uveitis;[81] and sympathetic ophthalmia.[82] It has also been shown to be useful in controlling ocular inflammation in the context of BD.[83] The SITE retrospective review reported control of inflammation in 62% and steroid-sparing effect in 47% of patients using azathioprine for ocular inflammatory disease at 1 year.[84] Side effects include gastrointestinal upset, affecting up to 12% of patients,[85] and myelosuppression for which regular monitoring of blood count is required.[86] In the aforementioned SITE retrospective review, discontinuation due to side effects occurred at a rate of 0.16 per person year.[84] Despite numerous reports suggesting an increased risk of neoplasia with long-term use of azathioprine,[87,88] a recent critical appraisal of the literature concluded there was no significant increase in the incidence of malignancy associated with its use for ocular inflammation.[89]

Methotrexate is a folic acid analogue and inhibitor of dihydrofolate reductase, and therefore inhibits DNA reproduction.[77] It is administered weekly either orally or by intramuscular injection at a starting dose of 2.5–10 mg/week and is titrated to effect with a maximum dose of 50 mg/week,[21] with one study demonstrating control of ocular inflammation in 76% of patients at a mean dose of 12.3 mg/week.[90] It has a lag time of 3–6 weeks from initiation of treatment to full therapeutic effect. Its efficacy has been demonstrated in a variety of uveitis entities in several retrospective reviews,[91–94] and, in the SITE retrospective cohort study of 384 patients, ocular inflammatory disease was suppressed in 66% of patients at 1 year, allowing for tapering of steroids to 10 mg/day in 58%.[95] Successful outcomes

were again observed in a variety of conditions, with highest rates observed in anterior uveitis and scleritis. Side effects include gastrointestinal symptoms, cytopenia, and hepatotoxicity, with abnormal liver function observed in 15% of patients.[96] No increased risk of neoplasia has been demonstrated with long-term use.[89]

Mycophenolate mofetil is an inosine monophosphate dehydrogenase inhibitor that disrupts purine synthesis, and preferentially inhibits DNA synthesis by B- and T-cells.[97] It is administered orally at a starting dose of 500 mg twice daily, and is increased to 1 g twice daily after 2 weeks provided that side effects are acceptable[21] with regular monitoring of blood count and liver-function tests.[57] Efficacy is achieved within 2–12 weeks after commencing treatment. In one study, 65% of patients achieved control of ocular inflammation on monotherapy, with steroid-sparing effects in 54% of patients.[98] In another case series of patients with uveitis and scleritis intolerant to methotrexate, 47/85 patients achieved control of inflammation on mycophenolate, with five patients achieving complete remission and being able to discontinue all immunosuppression.[99] These findings are concordant with the retrospective SITE review, which demonstrated control of ocular inflammation in 73%, and steroid-sparing effects in 55% of patients.[100] The most frequent side effects are those of gastrointestinal disturbance. Elevation of liver enzymes, leukopenia, and thrombocytopenia are less commonly reported.[77]

Alkylating agents

Cyclophosphamide exerts a cytotoxic effect on rapidly proliferating cells by alkylating nucleophilic groups on DNA bases. This results in cross-linking of DNA bases, abnormal base pairing, and strand breakage. Immunosuppressive effects are thought to be mediated by these cytotoxic effects on immunocompetent lymphocytes.[101] It may be administered either orally or as intravenous pulses. Some authors have demonstrated greater efficacy with oral administration; however, pulsed intravenous dosing is widely regarded to have a more preferable side-effect profile than oral dosing.[102,103] A loading course of infusions is usually commenced at 2-weekly intervals; since the degree of immunosuppression is reflected by the degree of lymphopenia, dosage is titrated to a leukocyte count of 3,500–4,500 cells/μL. Therapeutic efficacy is usually achieved within 2–8 weeks, and frequency of infusions is reduced as the inflammatory disease stabilizes.[57,104] In one widely-used protocol in the UK, cyclophosphamide is administered by intravenous infusion at an initial dose of 15 mg/kg at 2, 4, 7, 10, and 13 weeks, with monthly infusions thereafter to a maximum of nine pulses.[105] This protocol has been reported to successfully treat both refractory sclerokeratitis and uveitis, achieving control of inflammation in 71% and 41% of patients, respectively.[106] In the SITE retrospective review of 215 patients, 76% achieved control of ocular inflammation, and a steroid-sparing effect was demonstrated in 61%.[107] There are a number of potentially serious side effects, including leukopenia, hemorrhagic cystitis, secondary malignancy, and sterility; however, treatment benefits are thought to outweigh risks when used as short-term intravenous therapy.[108–110]

Chlorambucil has a similar action to cyclophosphamide, replacing hydrogen ions with alkyl groups on DNA bases, resulting in disruption of DNA synthesis in rapidly dividing cells.[111] It is orally administered at a starting dose of 0.1 mg/kg/day, with dosage titrated to response and side effects to a maximum dose of 6–12 mg/day.[21] Therapeutic efficacy is usually achieved within 4–12 weeks of onset, but it is less predictable than that of cyclophosphamide. In a review of 44 patients with refractory uveitis secondary to BD, Mudun et al demonstrated resolution of inflammation in 66% of patients with short-term chlorambucil therapy.[112] In another review of 53 patients with refractory uveitis due to a range of etiologies, 70% achieved remission with an average dose of 20 mg chlorambucil daily for an average of 16 weeks' duration.[113] Furthermore, in a review of 28 patients with refractory uveitis secondary to BD, JIA, pars planitis, sympathetic ophthalmia, idiopathic uveitis, Crohn's disease, and HLA-B27-positive disease, a positive response was observed in 68% with a median daily dose of 8 mg, over a median duration of 12 months.[114] The main side effect is bone marrow suppression, which, whilst usually reversible, may progress to irreversible aplastic anemia. It is also associated with an increased incidence of cutaneous malignancy, and in patients with preexisting neoplastic disease may lead to an increase in secondary hematological malignancy.[57] Chlorambucil is not used as often as cyclophosphamide by uveitis specialists who wish to employ alkylating agents to control the inflammation.

Anti-TNFα

Infliximab (Remicade; Janssen Biotech, Inc., Horsham, PA, USA) is a chimeric immunoglobulin (Ig)G monoclonal antibody that binds to TNFα and inhibits its biological function. It is composed of a human constant region, and murine variable region.[115–117] As is common to all anti-TNFα therapy, patients with evidence of acute infection are excluded from treatment, and, prior to commencing treatment, all patients should be screened for latent tuberculosis infection, in addition to hepatitis B virus if there are any known risk factors. Patients

with moderate to severe heart failure are also excluded, and those with mild cardiac disease should be closely monitored. Blood count and liver function are checked at baseline and monitored monthly for the duration of therapy.[118] Infliximab is administered intravenously, usually with a short "loading course" of three doses at 2-weekly intervals at a dose of 3–5 mg/kg, then maintenance doses are administered every 4–8 weeks at 5–10 mg/kg.[57,119,120] Treatment is usually continued for up to 2 years after disease quiescence is achieved.[121]

Infliximab has been used successfully to treat uveitis associated with BD unresponsive to other therapies,[122–124] and also in uveitis secondary to birdshot chorioretinopathy (BSCR),[125] JIA,[126,127] AS, sarcoidosis, and Crohn's disease.[128,129] In a 2-year prospective study by Suhler et al of 31 patients with refractory uveitis, 77% were deemed to have achieved clinical success within 10 weeks of commencing treatment with infliximab.[130,131] In another review of 25 patients with BD-related uveitis, Sfikakis et al demonstrated resolution of vitritis, macular edema, retinitis, and retinal vasculitis in over 90% of patients within 4 weeks of commencing treatment.[124]

Reactions during infusion are common and are thought to be largely due to the presence of the murine variable region, but can usually be managed successfully with antihistamines and analgesics. Potentially, fatal opportunistic infections have been observed in patients treated with infliximab, and, as a result, infliximab is contraindicated for patients with active, clinically significant infections, and should be used with caution in those with a history of chronic or recurrent infections. Caution is also required in patients with preexisting heart disease and neurological disease.[119] Studies of infliximab in ocular inflammatory disease have shown increased incidence of thromboembolism, drug-induced lupus-type reactions, and possibly solid malignancy.[131]

Adalimumab (Humira; AbbVie, Inc., North Chicago, IL, USA) is a humanized monoclonal IgG antibody to TNFα,[132] which is administered subcutaneously 2-weekly at a typical dose of 40 mg[133] and has been shown to be effective in the management of refractory uveitis due to a range of etiologies.[133–137] In a recent retrospective review of 60 patients with noninfectious uveitis across three centers, adalimumab was administered at a dose of 40 mg every other week for a mean duration of 87.9 weeks. Overall, 81.7% of patients were deemed to have shown improvement in their clinical condition, as judged by reduction in macular edema, increase in visual acuity, reduction in anterior chamber cells, reduced frequency of disease flares, or by steroid-sparing effect.[134] In another open-label, uncontrolled review of 274 patients with anterior uveitis secondary to AS, disease flare frequency was reduced by 51%, and when they did occur, disease flares were deemed to be less severe than prior to commencing adalimumab therapy.[133] It has a similar mechanism of action and side effect profile to infliximab, with the advantage of fewer injection site reactions, owing to the fact that it is completely humanized.[23] Currently, the VISUAL studies are being conducted worldwide to evaluate the potential role of adalimumab in the management of noninfectious uveitis (VISUAL I: NCT 01138657; VISUAL II: NCT 01124838; VISUAL III: NCT 01148225).

Etanercept (Enbrel; Immunex Corporation, Thousand Oaks, CA, USA) comprises a soluble TNF receptor and a human IgG Fc fragment, which is able to block the activity of both TNFα and TNFβ.[115,116,138] It is administered subcutaneously twice per week at a typical dose of 25 mg.[57] Whilst early studies showed favorable outcomes of etanercept therapy,[139–141] subsequent studies were generally disappointing. Quartier et al demonstrated that whilst there was an initial improvement in uveitis secondary to JIA, this was maintained in only 50% of patients at 1 year.[142] In a randomized controlled trial of the use of etanercept in JIA-associated uveitis, Smith et al found no benefit of etanercept over placebo.[143] Similarly Foster et al found that etanercept was no better than placebo in preventing uveitis relapses in previously controlled patients when tapering methotrexate therapy.[144] When compared to infliximab, etanercept has been shown to be inferior in controlling and preventing recurrence of ocular inflammation.[145] Side effects are similar to infliximab and adalimumab.[21] Because of low efficacy, etanercept is rarely used to manage uveitis.

Other biologic agents

Rituximab (Rituxan; Genentech, Inc., South San Francisco, CA, USA) is a human/murine chimeric monoclonal IgG antibody directed against cluster of differentiation (CD)20 on the surface of B-cells. CD20 regulates the early differentiation and maturation of B-cells, and inhibition results in cell death by apoptosis.[146] Rituximab therapy results in depletion of B-cells and reduction of IgG and IgM levels for 6–12 months following therapy.[147] It is commonly administered as two infusions, 2-weeks apart, at a quoted dose of either 375 mg/m^2 body area,[21,148] or 1,000 mg per infusion.[149,150] It has been demonstrated to be effective in the management of refractory uveitis secondary to BD,[149,150] JIA,[148,151] and BSCR.[152] Side effects are potentially fatal, and include severe sepsis, infusion reactions with severe adult respiratory distress syndrome, bronchospasm, pulmonary edema,

angioedema, Steven–Johnson syndrome, and toxic epidermal necrolysis.[153]

Abatacept (Orencia; Bristol-Myers Squibb, New York, NY, USA) is a fusion protein that inhibits costimulation of T-cells through blockage of the interaction between CD28 and CD80/86.[154] It is administered intravenously at monthly intervals.[155] Current data in uveitis is limited to case reports and case series; however, it has been repeatedly demonstrated to successfully treat uveitis in JIA.[156–158] Side effects include pneumonia and malignancy.[23]

Daclizumab (Zenapax; Hoffman-La Roche Ltd., Basel, Switzerland) is a humanized monoclonal antibody to the alpha subunit of the IL-2 receptor on the surface of T-cells. It binds CD25 on the IL-2 receptor of activated human lymphocytes, thus blocking the activity of autoreactive T-cells without suppressing the function of the immune system.[159] It is administered intravenously, usually at a starting dose of 1 mg/kg once every 2 weeks, with dose and frequency titrated to response and side effects to a maximum dose of 200 mg.[160] It has been shown to be well tolerated by patients in the management of uveitis,[161,162] with side effects including lymphadenopathy, psoriasiform rashes, mild peripheral edema, and infections. There appears to be no increased risk of malignancy associated with its use.[163] In one open label trial of daclizumab for various etiologies of intermediate and posterior uveitis, 4-weekly infusions resulted in improved inflammation and increased visual acuity in 80% of patients at 1 year,[164] which was maintained at 4 years.[165] Other reviews have shown daclizumab to be effective in uveitis secondary to BSCR, JIA, VKH, and idiopathic intermediate and posterior uveitis.[162,166,167] Data for BD has been equivocal, with one double-masked, randomized controlled trial finding daclizumab less effective than placebo.[168] Despite showing promise in the treatment of uveitis, daclizumab was discontinued by the manufacturer in 2009 due to decreasing market demand.[155]

Interferons (IFNs) are a group of cytokines synthesized by a variety of cell types with immunomodulatory, antiproliferative, and antiviral properties. The type I interferons (IFNα and IFNβ) act to increase expression of major histocompatibility complex class 1 molecules, and activate macrophages and natural killer cells.[60,169] They are typically administered subcutaneously at a dose of 3–6 million IU/day, at a frequency between three times weekly and once daily.[119] Interferons have been used to treat ocular inflammation since 1994, with strong evidence of efficacy in the management of uveitis secondary to BD[170,171] and MS.[172,173] Kötter et al performed a large scale systematic review of 36 studies assessing the use of interferons for uveitis secondary to BD; 182 of 338 patients were commenced on interferon therapy, of which 94% showed partial or complete resolution of inflammation within 2–4 weeks.[171] Common side effects include a flu-like illness and mild leukopenia, whilst mood disturbance such as depression and suicidal ideation have been less frequently reported.[169,171]

Intravenous Ig (IVIg)

IVIg contains 97%–98% intact human IgG with subclass distribution similar to normal plasma. Donations originate from a large number of donors (7,000–10,000) and therefore provide a broad spectrum of antibodies against pathogens.[174] IVIg is thought to have a multifactorial mechanism of action, including: blockage of IgG Fc fragment receptors on macrophages; modulation of cytokine synthesis and release; modulation of compliment; selection of B- and T-cell repertoires; neutralization of circulating autoantibodies; and interaction with other B- and T-cell surface receptors.[175] IVIg is administered intravenously at a dose of 1–2.5 g/kg/cycle of treatment divided over 3 days. Infusions are repeated at 2- to 4-weekly intervals until inflammation is controlled, after which infusions are repeated every 5–6 weeks for at least 2 years after remission has been achieved.[176] Side effects include aseptic meningitis, thromboembolism, and risk of transmission of blood-borne disorders.[147] It has been shown to be effective in treatment of cases of uveitis secondary to BSCR,[177] BD,[178] and VKH.[179]

Vascular endothelial growth factor (VEGF) inhibitors

VEGF has been implicated in the induction of inflammation in uveitic eyes,[180,181] and, as a result, intravitreal VEGF inhibitors have been used in the management of persistent macular edema secondary to inflammatory disorders. Currently available VEGF inhibitors include ranibizumab (Lucentis; Novartis International AG, Basel, Switzerland), bevacizumab (Avastin; Hoffman-La Roche Ltd./Genentech, Inc.), and aflibercept (Eylea; Regeneron, Tarrytown, NY, USA, and Bayer AG, Leverkusen, Germany). Treatment usually involves a course of three intravitreal injections at monthly intervals, followed by further injections depending on treatment response. Most data suggests that whilst such agents may successfully reduce macular thickness, the reduction in edema does not correspond to an increase in visual acuity.[180,182–184] When direct comparisons have been made, macular thickness and visual outcomes in uveitic macular edema are in fact better with intravitreal triamcinolone injections than with anti-VEGF agents.[185–187]

This is likely to be due to the increased anti-inflammatory effect of steroids compared to VEGF inhibitors.[188] There are potential risks associated with the injection procedure itself: intravitreal injection may cause increased intraocular pressure, and also carries the infrequent but serious risk of infectious endophthalmitis. Whilst systemic side effects are rare, the risk of serious cardiovascular events remains a topic of debate.[189,190]

Update on drugs currently in development stages for uveitis treatment

Therapeutic agents are very rarely developed for the primary purpose of treating ocular inflammatory disease; instead, most advances in treatment occur when drugs previously used to successfully manage systemic inflammatory diseases are adapted for use in ophthalmology. Most immunomodulatory therapy is thus first used in conditions such as transplant medicine, rheumatoid arthritis (RA), psoriasis, or MS before being trialed in patients with uveitis, and is then usually used off-label, being documented as case reports or case series in the medical literature.

Corticosteroids

Intravitreal injection of triamcinolone acetonide is commonly used in control of uveitic macular edema, since it allows higher concentrations of corticosteroid to be delivered directly to the posterior pole than can be achieved with topical or systemic therapy.[191] Duration of action is, however, limited to 3–4 months at best, necessitating frequent repeat therapy and carrying risks of complications for the patient.[192] As a result, there has been a drive to develop longer-acting corticosteroid implants, which provide similar anti-inflammatory activity over a prolonged period of time.

Ozurdex (Allergan, Inc., Irvine, CA, USA) is a "bioerodible" dexamethasone implant using a solid polymer delivery system, in which dexamethasone is combined with biodegradable material in the form of a small rod, which is injected into the vitreous cavity using a specific injector.[20] Dexamethasone is released in a biphasic manner over 6 months, with higher concentrations released for the first 6 weeks, followed by lower concentrations for the following months.[193] After this time, the implant dissolves to CO_2 and H_2O leaving no residue within the eye.[194] It is licensed for use in uveitis in USA and Europe.

The license for Ozurdex is primarily based on HURON, a Phase III posterior segment uveitis study. This study, published in 2011, was a double-masked, randomized, controlled trial that compared the effect of two implant doses (0.7 mg and 0.35 mg) with sham injection. Both implant doses proved effective in controlling vitreous inflammation and improving visual acuity with reduction in cystoid macular edema, but the higher-dose implant had a longer duration of action without a significant increase in side effects, and is the implant now available. A total of 47% of patients treated with the 0.7 mg implant had a vitreous haze score of zero at 8 weeks (versus 12% for the sham injection group). The implant was still effective at the 6-month time point. Ozurdex was found to be safe with a low incidence of cataract reported over the 26-week period (15% of patients developed clinically evident lens opacity, but none sufficient to require surgery). The key potential side effect of raised intraocular pressure (IOP) was also low with IOP >25 mmHg occurring in less than 10% of patients.[195] Ozurdex 0.7 mg implants have also been shown to be effective in reducing macular thickness and increasing visual acuity in uveitic macular edema in vitrectomized eyes,[196] and have been shown to be effective in cases of pediatric uveitis.[197]

Given the success of these implantable therapies, there has been a desire to develop similar implants with an extended half-life. Fluocinolone acetate (FA) is significantly less soluble than dexamethasone in aqueous humor, and thus has potential to persist for far longer within the eye.[198] Retisert (Bausch & Lomb Incorporated, Bridgewater, NJ, USA) contains 0.59 mg FA, which is surgically placed into the vitreous cavity, where it persists for 30 months.[199] It was approved for use in noninfectious uveitis by the US Food and Drug Administration (FDA) in 2005, and several large-scale studies have documented its efficacy.[200–202] It is, however, associated with significant side effects. In one large scale review, all phakic patients were observed to develop cataract within 3 years of implantation, and significant IOP increases requiring trabeculectomy were observed in 40%.[200]

Iluvien (Alimera Sciences, Alpharetta, GA, USA; and pSivida Corp, Watertown, MA, USA) is an alternative FA insert that has been licensed for usage in diabetic macular edema[203–205] and is currently under evaluation in uveitis (Phase III trial ongoing, NCT 01694186). It has advantages over Retisert of being injectable via a 25-gauge injector system, and is thought to cause fewer corticosteroid side effects due to the release of a lower dose of the drug (0.2 or 0.5 µg/day compared to 0.59 µg/day with Retisert), although there still appears to be significant cataract progression following implantation.[204,205] Phase III clinical trials of FA inserts in eyes with noninfectious uveitis are currently underway, being led by pSivida Corp.[206]

T-cell inhibitors

Everolimus (Zortress [USA]/Certican [Europe and other countries]; Novartis International AG) is a mammalian target of rapamycin (mTOR) inhibitor, which inhibits T-cell proliferation and differentiation by blocking IL-2 signal transduction. mTOR inhibitors act at a later stage in the IL-2 signaling pathway than the commonly used calcineurin antagonists, and as a result are thought to have differential effects on different classes of CD4 T-cells, preserving regulatory T-cell responses whilst blocking development of other more pathogenic $CD4^+$ T-cells.[207–209] For this reason, mTOR inhibitors are thought to be particularly useful in autoimmune disease where a regulatory T-cell effect may be beneficial, and as such have been used in the treatment of RA.[210] Everolimus is thought to have a preferable side effect profile to other mTOR inhibitors,[211,212] with higher bioavailability and shorter half-life than other agents.[213]

Given these qualities, there has been interest in Everolimus as an alternative therapy in uveitis refractory to corticosteroids and other calcineurin inhibitors. Heiligenhaus et al performed a nonrandom, open-label, prospective pilot study of 12 patients with endogenous uveitis refractory to corticosteroids and cyclosporine (nine patients with anterior and intermediate uveitis, three patients with panuveitis).[214] Everolimus was initiated at an oral dose of 0.75 mg twice daily and adjusted from week 1 to obtain trough serum levels in the range of 3–8 ng/mL, with a maximum daily dose of 2.5 mg. On review at 3 months, uveitis was deemed inactive in all patients; however, by 12 months, inflammation had returned in four patients in whom therapy had been tapered or withdrawn. Visual acuity was stable throughout the period of observation, macular thickness was reduced, and a 50% reduction in dose of steroids or cyclosporine was achieved for all patients. Disease recurrence was observed in 50% of patients within 1 month of terminating everolimus therapy. No significant adverse events were documented.

Everolimus therefore appears as an attractive option in the management of disease where other calcineurin inhibitors have failed, or where there is thought to be an autoimmune etiology. Further studies are required in this area.

Sirolimus (Rapamune; Pfizer, Inc., New York, NY, USA) is another mTOR inhibitor with a proven track record in controlling inflammation.[215] Systemic administration of sirolimus has been approved in multiple, diverse clinical applications including prevention of organ rejection,[216] prevention of coronary restenosis,[217] and treatment of advanced renal cell carcinoma[218] in the US, Japan, and European Union. Systemically delivered sirolimus has also been used in cases of refractory uveitis.[219,220] A proprietary formulation of sirolimus for local (intravitreal or subconjunctival) injection has been developed.[221] The SAVE (Sirolimus as a Therapeutic Approach for UVEitis) study has reported the safety and bioactivity of subconjunctival and intravitreal sirolimus in noninfectious uveitis.[222] The SAVE-2 Phase II study is being conducted to evaluate two different doses of intravitreal sirolimus for uveitis (NCT 01280669). In addition, SAKURA studies are two Phase III multicenter, randomized, international clinical trials that are being performed at this time to seek indication for intravitreal sirolimus in the management of noninfectious uveitis of the posterior segment.[223]

Voclosporin (Luveniq; Lux Biosciences, Inc., New Jersey City, NJ, USA) is a calcineurin inhibitor with a similar structure to cyclosporine, with the exception of a functional group in an amino acid.[224] It has been associated with less nephrotoxicity than cyclosporine[225] and used successfully in the management of plaque psoriasis.[226,227] The LUMINATE (Lux Uveitis Multicenter Investigation of a New Approach to Treatment) trials sought to assess the safety and efficacy of voclosporin for treatment, maintenance, and control of all forms of noninfectious uveitis, and took the form of three randomized, double-blind, placebo-controlled Phase II/III trials in patients with severe, sight-threatening disease.[228] Initial results were promising, with a 50% reduction in inflammation in patients with active uveitis compared to 29% in placebo at 16 and 24 weeks, a reduction in recurrence of inflammation in quiescent disease of 50%, and reduction of oral prednisolone to 5 mg/day or less in 96%–98% of patients.[229,230] Unfortunately, subsequent Phase III trials did not meet the primary end point of change from baseline in vitreous haze at 12 weeks or at the time of treatment failure, if earlier, and the manufacturers did not seek regulatory approval for use of voclosporin in uveitis in USA or Europe.[231]

Anti-TNFα

Golimumab (Simponi; Janssen Biotech, Inc.) is a human monoclonal antibody to TNFα with the advantage of requiring only monthly subcutaneous injection; it is administered at a dosage of 50 mg. Published case reports have demonstrated treatment success in refractory uveitis secondary to BD,[232] JIA,[233,234] idiopathic retinal vasculitis,[235] seronegative spondyloarthropathy-associated disease,[236] and HLA-B27-positive disease.[234] In one series including 34 eyes of 17 patients (13 patients with JIA-associated disease and four with HLA-B27-positive disease), an initial response was observed in 14 patients, with 12 patients achieving

cessation of active inflammation at their most recent clinic visit. Mean follow-up time was 21.9 months, during which time visual acuity remained stable in 26 eyes, improved in seven eyes, and worsened in one eye. Mean oral prednisolone dose was 12.5 mg/day prior to commencing treatment, and had reduced to 3.5 mg/day at the end of the period of observation.[234] In another recent series of three patients with JIA-associated disease, one patient achieved long-term quiescence with golimumab therapy, one showed an initial improvement but developed recurrent disease, and the final patient showed significant improvement without achieving complete quiescence.[233] No controlled trials have been published, and no direct comparisons against cyclosporine have yet been made.

Certolizumab (Cimzia; UCB, Inc., Smyrna, GA, USA) consists only of the pegylated humanized Fab fragment of a monoclonal antibody directed against TNFα. It is administered subcutaneously at a dose of 400 mg once every 2 weeks.[237] There is a single case report documenting successful treatment of RA-associated scleritis, in which quiescence was achieved at 6 months;[238] however, there are currently no studies reporting outcomes in noninfectious uveitis.

Other immune modulators

Anakinra (Kineret; Swedish Orphan Biovitrum AB [publ], Stockholm, Sweden) is a recombinant interleukin (IL)-1 receptor antagonist that has been approved for use in RA.[239] There is experimental evidence to suggest that IL-1 is involved in the pathogenesis of uveitis,[240] and anakinra has thus been suggested as a potential agent for management of refractory inflammation.[241] Interestingly, the only clinical data for use in uveitis is a case report of a patient with chronic infantile neurological cutaneous articular (CINCA) syndrome, which is thought to be an IL-1 mediated condition. In this report, a 4-year old boy with bilateral CINCA-associated panuveitis unresponsive to corticosteroids, methotrexate, and etanercept achieved quiescent anterior uveitis, and resolution of vitritis and papillitis, at a dose of 1 mg/kg/day.[240]

Gevokizumab (XOMA 052; XOMA Corporation, Berkeley, CA, USA) is a humanized monoclonal antibody to IL-1β, which is a proinflammatory cytokine involved in a wide range of pathological processes.[242] In an open-label pilot study by Gül et al seven patients with BD-associated acute posterior or panuveitis resistant to azathioprine and/or cyclosporine and receiving oral prednisolone at a dose of 10 mg/day or less were treated with a single infusion of 0.3 mg/kg gevokizumab. All immunosuppressive medication was stopped at baseline, and safety, pharmacokinetics, and uveitis status was evaluated. Complete resolution of intraocular inflammation was observed in all patients within 4–21 days (median 14 days), and response was maintained for a median duration of 49 days.[243] Phase III, double-masked, placebo-controlled trials are currently underway for patients with BD-associated uveitis (EYEGUARD-B), and for patients with active noninfectious and controlled noninfectious intermediate, posterior, or panuveitis (EYEGUARD-A and EYEGUARD-C).[244]

Canakinumab (Ilaris; Novartis International AG) is a humanized monoclonal antibody to IL-1β licensed for use in systemic JIA and cryopyrin-associated periodic syndromes in adults and children 4 years of age or older. It is usually administered by subcutaneous injection every 4–8 weeks, but can also be given intravenously.[245] It has been used to successfully treat uveitis associated with BD[246] and Blau syndrome,[247] but data is currently limited to case reports only.

Alemtuzumab (Campath; Genzyme Corporation, Cambridge, MA, USA) is a humanized monoclonal antibody to CD52 primarily used in the management of B-cell chronic lymphocytic leukemia, which has also shown benefit in conditions such as RA and MS.[248,249] Studies of its use in ophthalmology are limited. An early report by Isaacs et al described a 45-year-old male with severe panuveitis, with choroiditis and retinal vasculitis, that was resistant to therapy with corticosteroids, cyclophosphamide, and IVIg. Alemtuzumab was administered intravenously at a dose of 12 mg/day for 5 days with an initial improvement in inflammation. However, the patient suffered a fatal myocardial infarction whilst under follow-up.[250] In a later, uncontrolled series of ten patients, Dick et al reported good initial responses and long-lasting remission in six patients with uveitis (four with retinal vasculitis, one with BD, and one with sympathetic ophthalmia).[251] Side effects include flu-like illness and cytopenias.

Efalizumab (Raptiva; Genentech, Inc.) is a humanized monoclonal antibody to CD11a, which is a subunit of lymphocyte function-associated antigen-1 that is involved in antigen presentation and T-cell adhesion to the vascular endothelium. It is administered subcutaneously on a weekly basis and was originally approved by the FDA for use in psoriasis. Initial ophthalmic data was promising, with one case report and one prospective noncomparative Phase I/II trial both reporting resolution of uveitic macular edema with efalizumab therapy.[252,253] However, it was later shown to increase the risk of progressive multifocal leukoencephalopathy, and, thus, the drug was withdrawn from the market.[254]

Tocilizumab (Actemr; Genentech, Inc.) is a humanized monoclonal IL-6 receptor antibody that inhibits downstream signaling;[255] IL-6 is a cytokine secreted by both B- and T-cells, and is known to participate in T-cell activation,

Ig secretion, leukocyte recruitment, and the differentiation and proliferation of hematopoietic precursor cells.[256] IL-6 has been shown to be an important inflammatory cytokine in the aqueous humor, and is thus an interesting target for therapeutic intervention.[8,257,258] Tocilizumab has previously been used in the treatment of RA, and is usually administered intravenously at a dose range of 4–12 mg/kg at 2- to 4-weekly intervals.[155] It has been demonstrated to successfully control refractory uveitis of various etiologies, including JIA, BD, BSCR, and Castleman disease.[259–263] There is also an evolving body of literature to suggest that tocilizumab is effective at reducing macular thickness and increasing visual acuity in uveitic macular edema.[262,264,265] A Phase I/II clinical trial of tocilizumab in JIA-associated uveitis has been launched (NCT 01717170). In addition, the multicenter, randomized STOP-UVEITIS study on the safety, tolerability, and bioactivity of two different doses of tocilizumab in intermediate, posterior, and panuveitis is currently underway in the United States.[155]

Interestingly, episodes of paradoxical ocular inflammation have been reported in patients receiving tocilizumab therapy for systemic disease; Wendling et al reported a first episode of uveitis in a 45-year-old male being treated for HLA-B27-positive AS, and a first episode of peripheral ulcerative keratitis in another 65-year-old male being treated for RA.[266] Sato et al also published a case report of a 72-year-old female with RA who, after discontinuing tocilizumab, developed severe uveitis associated with hypopyon, requiring rescue therapy with corticosteroids.[267] Careful observation for similar paradoxical effects is required as clinical trials progress.

In addition, the SATURN Study, a multicenter, randomized clinical trial of sarilumab (another IL-6 inhibitor) in intermediate, posterior, and panuveitis, is being conducted in Europe and the US.[268]

Secukinumab (AIN457; Novartis International AG) is a selective, high-affinity, fully human monoclonal antibody that binds and neutralizes human IL-17A,[269,270] which is an inflammatory cytokine secreted by Th17 $CD4^+$ T-cells.[271–273] It has been evaluated in a range of inflammatory conditions, including AS, RA, and psoriasis.[274] Th17 cells have been implicated in the pathogenesis of uveitis in experimental models,[275,276] and circulating levels of IL-17A have been shown to be increased in patients with VKH, BD, and other forms of uveitis.[277–279]

Dick et al recently published the results of three clinical trials assessing the effect of secukinumab versus placebo in BD-associated posterior and panuveitis (SHIELD study, 118 patients randomized), non-BD-associated active noninfectious uveitis (INSURE study, 31 patients randomized), and non-BD-associated quiescent noninfectious uveitis (ENDURE study, 125 patients randomized). After a loading phase, secukinumab was administered subcutaneously at a dose of 300 mg 2- or 4-weekly in the SHIELD study, and 300 mg 2- or 4-weekly or 150 mg 4-weekly in both the INSURE and ENDURE studies. Primary outcome measures were reduction of uveitis recurrence or vitreous haze score during withdrawal of concomitant immunosuppressive medication. Unfortunately, on completion of the SHIELD study, there was insufficient evidence of efficacy, resulting in the early termination of the remaining studies.[280]

Fingolimod (FTY720, Gilenya; Novartis International AG) prevents T-cell migration to inflammatory sites by reducing expression of the sphingosine-1 phosphate receptor normally required for egress from secondary lymphoid tissues. It is orally administered with once-daily dosing of 0.5–1.25 mg, and has been shown to be effective in Phase III trials in relapsing MS, for which it has been FDA-approved.[281] In vivo data suggested a beneficial effect in experimental models of uveitis,[282–284] leading to considerable interest in its use for ophthalmic inflammation. Further development has been held back by concerns over a potential increased risk of developing macular edema. In a pooled analysis of 2,615 patients enrolled in three clinical trials in MS, 19 patients with no previous history of uveitis were observed to develop macular edema.[285,286] This was considered more likely with higher doses of treatment, occurring in 15 patients (1.2%) receiving 1.25 mg/day versus four patients (0.3%) receiving 0.5 mg/day. A Phase II trial was commenced evaluating fingolimod in noninfectious posterior segment uveitis, but was terminated early.

Ultimately, future therapeutic strategies must be based upon rigorous scientific research; as our understanding of the pathogenesis of uveitis increases, it is likely that more mechanism-focused therapies will be developed, for example, targeting of IL-15, IL-22, and IL-23.[287] It is also important to note that considerable in vivo data exists for the use of both gene therapy[288–290] and RNA interference[291–293] in models of ocular inflammation. Both modalities offer the potential of being able to suppress specific genes and proteins in the inflammatory cascade, and may provide the most targeted treatment, and therefore the fewest side effects in the management of refractory ocular inflammation in the future.[294]

Patient-specific considerations

Uveitis specialists should seek to treat the patient, rather than simply suppress the uveitis. As such, it is important to take a holistic approach to treatment, and be aware that issues of compliance and confidence in the doctor–patient

relationship are equally as important as the efficacy of a therapeutic intervention.

Past medical and past ocular history will dictate treatment choice; severe allergies and coexistent disease may be absolute contraindications to some medications, for example, chronic liver disease and methotrexate.[96] Similarly, high-dose steroids may be avoided in patients known to respond with increased intraocular pressure.[67] Patients may also have uveitis secondary to systemic disease for which they may already be receiving IMT agents; it may be necessary to consider switching treatment, or deciding on an additional therapy, for example, adding oral steroids or methotrexate to an anti-TNFα agent.

Method and frequency of administration may also guide treatment choice. For example, children may not tolerate frequent topical application of eye drops and may be more compliant with daily oral medication, and patients of working age may find it difficult to attend regular clinic appointments for intravenous infusions of therapy.

There is also the issue of extrapolation of trial data to "real-world" settings. Phase I clinical trials usually recruit young, healthy patients to assess safety and bioavailability of a novel therapeutic agent, whilst Phase II and III trials will usually exclude children and pregnant females, and may avoid women of child-bearing age. Caution is thus required before any medication is used off-label in these patient groups.

Women of child-bearing age require particular caution; medications such as methotrexate are teratogenic, whilst others such as chlorambucil and cyclophosphamide may cause sterility and early menopause.[24,57]

Patients should be given sufficient information regarding the potential risks and benefits of any therapeutic agent, so that they can give proper informed consent, and feel confident in the proposed course of treatment.

Conclusion

The increasing choices of immunosuppressives/immunomodulatory therapy and the advent of more targeted therapies are both an opportunity and a challenge. The clinicians should always consider the patient first and foremost. It is vital that one establishes the efficacy and safety profile of both established and emerging therapies so that treatment decisions can be informed and appropriate to the individual. In addition, it is likely that stricter regulatory frameworks and tighter budgetary constraints may make it even more difficult to navigate the latest hurdles in the drug-development pathway of licensing and funding approval. With such considerations in mind, we need to recognize that to some extent the whole uveitis pharmacopoeia is "in development".

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Disclosure

The authors report no conflicts of interest in this work.

References

1. Darrell RW, Wagener HP, Kurland LT. Epidemiology of uveitis. Incidence and prevalence in a small urban community. *Arch Ophthalmol*. 1962;68:502–514.
2. Suttorp-Schulten MS, Rothova A. The possible impact of uveitis in blindness: a literature survey. *Br J Ophthalmol*. 1996;80(9):844–848.
3. Goldstein H. The reported demography and causes of blindness throughout the world. *Adv Ophthalmol*. 1980;40:1–99.
4. Forrester JV. Uveitis: pathogenesis. *Lancet*. 1991;338(8781):1498–1501.
5. Jabbs D, Nussenblatt RB, Rosenbaum JT; Standardization of Uveitis Nomenclature (SUN) Working Group. Standardization of uveitis nomenclature for reporting clinical data. Results of the First International Workshop. *Am J Ophthalmol*. 2005;140:509–516.
6. Miserocchi E, Fogliato G, Modorati G, Bandello F. Review on the worldwide epidemiology of uveitis. *Eur J Ophthalmol*. 2013;23(5):705–717.
7. Cimino L, Aldigeri R, Salvarani C, et al. The causes of uveitis in a referral centre of Northern Italy. *Int Ophthalmol*. 2010;30(5):521–529.
8. Curnow SJ, Scheel-Toellner D, Jenkinson W, et al. Inhibition of T-cell apoptosis in the aqueous humor of patients with uveitis by IL-6/soluble IL-6 receptor trans-signaling. *J Immunol*. 2004;173(8):5290–5297.
9. Denniston AK, Kottoor SH, Khan I, et al. Endogenous cortisol and TGF-beta in human aqueous humor contribute to ocular immune privilege by regulating dendritic cell function. *J Immunol*. 2011;186(1):305–311.
10. Takeuchi M, Alard P, Streilein JW. TGF-beta promotes immune deviation by altering accessory signals of antigen-presenting cells. *J Immunol*. 1998;160(4):1589–1597.
11. Takeuchi M, Kosiewicz MM, Alard P, Streilein JW. On the mechanisms by which transforming growth factor-beta 2 alters antigen-presenting abilities of macrophages on T-cell activation. *Eur J Immunol*. 1997;27(7):1648–1656.
12. Taylor AW, Yee DG, Nishida T, Namba K. Neuropeptide regulation of immunity. The immunosuppressive activity of alpha-melanocyte-stimulating hormone (alpha-MSH). *Ann N Y Acad Sci*. 2000;917:239–247.
13. Taylor AW, Streilein JW, Cousins SW. Identification of alpha-melanocyte stimulating hormone as a potential immunosuppressive factor in aqueous humor. *Curr Eye Res*. 1992;11(12):1199–1206.
14. Gocho K, Kondo I, Yamaki K. Identification of Autoreactive T-Cells in Vogt-Koyanagi-Harada Disease. *Invest Ophthalmol Vis Sci*. 2001;42(9):2004–2009.
15. De Smet MD, Bitar G, Mainigi S, Nussenblatt RB. Human S-antigen determinant recognition in uveitis. *Invest Ophthalmol Vis Sci*. 2001;42(13):3233–3238.

16. Rodriguez A, Calonge M, Pedroza-Seres M, et al. Referral patterns of uveitis in a tertiary eye care center. *Arch Ophthalmol*. 1996;114(5):593–599.
17. Zaidi AA, Ying G, Daniel E, et al. Hypopyon in patients with uveitis. *Ophthalmology*. 2010;117(2):366–372.
18. Barry RJ, Sutcliffe N, Isenberg Da, et al. The Sjögren's Syndrome Damage Index – a damage index for use in clinical trials and observational studies in primary Sjögren's syndrome. *Rheumatology*. 2008;47(8):1193–1198.
19. Skopouli FN, Dafni U, Ioannidis JP, Moutsopoulos HM. Clinical evolution, and morbidity and mortality of primary Sjögren's syndrome. *Semin Arthritis Rheum*. 2000;29(5):296–304.
20. Tempest-Roe S, Joshi L, Dick AD, Taylor SR. Local therapies for inflammatory eye disease in translation: past, present and future. *BMC Ophthalmol*. 2013;13(1):39.
21. Durrani K, Zakka FR, Ahmed M, Memon M, Siddique SS, Foster CS. Systemic therapy with conventional and novel immunomodulatory agents for ocular inflammatory disease. *Surv Ophthalmol*. 2011;56(6):474–510.
22. Denniston AK, Dick AD. Systemic therapies for inflammatory eye disease: past, present and future. *BMC Ophthalmol*. 2013;13:18.
23. Gallego-Pinazo R, Dolz-Marco R, Martínez-Castillo S, Arévalo JF, Díaz-Llopis M. Update on the principles and novel local and systemic therapies for the treatment of non-infectious uveitis. *Inflamm Allergy Drug Targets*. 2013;12(1):38–45.
24. Larson T, Nusseblatt RB, Sen HN. Emerging Drugs for Uveitis. *Expert Opin Emerg Drugs*. 2012;16(2):309–322.
25. Barisani-Asenbauer T, Maca SM, Mejdoubi L, Emminger W, Machold K, Auer H. Uveitis-a rare disease often associated with systemic diseases and infections-a systematic review of 2,619 patients. *Orphanet J Rare Dis*. 2012;7:57.
26. Gritz DC, Wong IG. Incidence and prevalence of uveitis in Northern California; the Northern California Epidemiology of Uveitis Study. *Ophthalmology*. 2004;111(3):491–500.
27. Suhler EB, Lloyd MJ, Choi D, Rosenbaum JT, Austin DF. Incidence and prevalence of uveitis in Veterans Affairs Medical Centers of the Pacific Northwest. *Am J Ophthalmol*. 2008;146(6):890–896.
28. Hwang DK, Chou YJ, Pu CY, Chou P. Epidemiology of uveitis among the Chinese population in Taiwan: a population-based study. *Ophthalmology*. 2012;119(11):2371–2376.
29. Read RW, Holland GN, Rao NA, et al. Revised diagnostic criteria for Vogt-Koyanagi-Harada disease: report of an international committee on nomenclature. *Am J Ophthalmol*. 2001;131(5):647–652.
30. Bodaghi B, Cassoux N, Wechsler B, et al. Chronic severe uveitis: etiology and visual outcome in 927 patients from a single center. *Medicine*. 2001;80(4):263–270.
31. Bloch-Michel E, Nussenblatt RB. International Uveitis Study Group recommendations for the evaluation of intraocular inflammatory disease. *Am J Ophthalmol*. 1987;103(2):234–235.
32. Holland GN. Standard diagnostic criteria for the acute retinal necrosis syndrome. Executive Committee of the American Uveitis Society. *Am J Ophthalmol*. 1994;117(5):663–667.
33. Engstrom RE, Holland GN, Margolis TP, et al. The progressive outer retinal necrosis syndrome. A variant of necrotizing herpetic retinopathy in patients with AIDS. *Ophthalmology*. 1994;101(9):1488–1502.
34. Mandeville JT, Levinson RD, Holland GN. The tubulointerstitial nephritis and uveitis syndrome. *Surv Ophthalmol*. 2001;46(3):195–208.
35. McCannel CA, Holland GN, Helm CJ, Cornell PJ, Winston JV, Rimmer TG. Causes of uveitis in the general practice of ophthalmology. UCLA Community-Based Uveitis Study Group. *Am J Ophthalmol*. 1996;121(1):35–46.
36. Heiligenhaus A, Heinz C, Edelsten C, Kotaniemi K, Minden K. Review for disease of the year: epidemiology of juvenile idiopathic arthritis and its associated uveitis: the probable risk factors. *Ocul Immunol Inflamm*. 2013;21(3):180–191.
37. Smith JA, Mackensen F, Sen HN, et al. Epidemiology and course of disease in childhood uveitis. *Ophthalmology*. 2009;116(8):1544–1551.
38. Rothova A, van Veenedaal WG, Linssen A, Glasius E, Kijlstra A, de Jong PT. Clinical features of acute anterior uveitis. *Am J Ophthalmol*. 1987;103(2):137–145.
39. London NJ, Rathinam SR, Cunningham ET Jr. The epidemiology of uveitis in developing countries. *Int Ophthalmol Clin*. 2010;50(2):1–17.
40. Wakefiled D, Chang J. Epidemiology of uveitis. *Int Ophthalmol Clin*. 2005;45(2):1–13.
41. Brewerton DA, Caffrey M, Nicholls A, Walters D, James DC. Acute anterior uveitis and HL-A 27. *Lancet*. 1973;302(7836):994–996.
42. Chang JH, Wakefield D. Uveitis: a global perspective. *Ocul Immunol Inflamm*. 2002;10(4):263–279.
43. Ohguro N, Sonoda KH, Takeuchi M, Matsumura M, Mochizuki M. The 2009 prospective multi-center epidemiologic survey of uveitis in Japan. *Jpn J Ophthalmol*. 2012;56(5):432–435.
44. Zein G, Berta A, Foster CS. Multiple sclerosis-associated uveitis. *Ocul Immunol Inflamm*. 2004;12(2):137–142.
45. Zierhut M, Foster CS. Multiple sclerosis, sarcoidosis and other diseases in patients with pars planitis. *Dev Ophthalmol*. 1992;23:41–47.
46. Yang P, Zhang Z, Zhou H, et al. Clinical patterns and characteristics of uveitis in a tertiary center for uveitis in China. *Curr Eye Res*. 2005;30(11):943–948.
47. Rathinam SR, Namperumalsamy P. Global variation and pattern changes in epidemiology of uveitis. *Indian J Ophthalmol*. 2007;55(3):173–183.
48. Chung YM, Yeh TS, Liu JH. Endogenous uveitis in Chinese – an analysis of 240 cases in a uveitis clinic. *Jpn J Ophthalmol*. 1988;32(1):64–69.
49. Kotake S, Furudate N, Sasamoto Y, Yoshikawa K, Goda C, Matsuda H. Characteristics of endogenous uveitis in Hokkaido, Japan. *Graefes Arch Clin Exp Ophthalmol*. 1996;234(10):599–603.
50. Wakabayashi T, Morimura Y, Miyamoto Y, Okada AA. Changing patterns of intraocular inflammatory disease in Japan. *Ocul Immunol Inflamm*. 2003;11(4):277–286.
51. Tugal-Tutkun I. Behçet disease in the developing world. *Int Ophthalmol Clin*. 2010;50(2):87–98.
52. Jabs DA, Busingye J. Approach to the diagnosis of the uveitides. *Am J Ophthalmol*. 2013;156(2):228–236.
53. Mandelcorn ED. Infectious causes of posterior uveitis. *Can J Ophthalmol*. 2013;48(1):31–39.
54. Guly CM, Forrester JV. Investigation and management of uveitis. *BMJ*. 2010;341:c4976.
55. De Groot-Mijnes JDF, Rothova A, Van Loon AM, et al. Polymerase chain reaction and Goldmann-Witmer coefficient analysis are complimentary for the diagnosis of infectious uveitis. *Am J Ophthalmol*. 2006;141(2):313–318.
56. Wong RW, Jumper JM, McDonald HR, et al. Emerging concepts in the management of acute retinal necrosis. *Br J Ophthalmol*. 2013;97(5):545–552.
57. Jabs DA, Rosenbaum JT, Foster S, et al. Guidelines for the Use of Immunosuppressive Drugs in Patients with Ocular Inflammatory Disorders: recommendations of an expert panel. *Am J Ophthalmol*. 2000;130:492–513.
58. Pato E, Muñoz-Fernández S, Francisco F, et al. Systematic review on the effectiveness of immunosuppressants and biological therapies in the treatment of autoimmune posterior uveitis. *Semin Arthritis Rheum*. 2011;40(4):314–323.
59. Cordero-Coma M, Yilmaz T, Onal S. Systematic review of anti-tumor necrosis factor-alpha therapy for treatment of immune-mediated uveitis. *Ocul Immunol Inflamm*. 2013;21(1):19–27.
60. Saadoun D, Bodaghi B, Bienvenu B, et al. Biotherapies in inflammatory ocular disorders: interferons, immunoglobulins, monoclonal antibodies. *Autoimmun Rev*. 2013;12(7):774–783.
61. Chakravarty K, McDonald H, Pullar T, et al. BSR/BHPR guideline for disease-modifying anti-rheumatic drug (DMARD) therapy in consultation with the British Association of Dermatologists. *Rheumatology (Oxford)*. 2008;47(6):924–925.
62. Management of DMARDS [webpage on the Internet]. National Institute of Health and Clinical Excellence Clinical Knowledge Summaries (UK); [updated January 2013; cited June 19, 2014]. Available from: http://cks.nice.org.uk/dmards. Accessed June 19, 2014.

63. Carryer HM, Koelsche GA, Prickman LE, Maytum CK, Lake CF, Williams HL. Effects of cortisone on bronchial asthma and hay fever occurring in subjects sensitive to ragweed pollen. *Proc Staff Meet Mayo Clin*. 1950;25(17):482–486.
64. Gordon DM. Effects of pituitary adrenocorticotrophic hormone (ACTH) therapy in ophthalmologic conditions. *J Am Med Assoc*. 1950;142(16):1271.
65. van der Velden VH. Glucocorticoids: mechanisms of action and anti-inflammatory potential in asthma. *Mediators Inflamm*. 1998;7:229–237.
66. Pavesio CE, Decory HH. Treatment of ocular inflammatory conditions with loteprednol etabonate. *Br J Ophthalmol*. 2008;92(4):455–459.
67. Becker B, Mills DW. Elevated intraocular pressure following corticosteroid eye drops. *J Am Med Assoc*. 1963;185:884–886.
68. Nguyen QD, Hatef E, Kayen B, et al. A cross-sectional study of the current treatment patterns in noninfectious uveitis among specialists in the United States. *Ophthalmology*. 2011;118(1):184–190.
69. Rühlmann A, Nordheim A. Effects of the immunosuppressive drugs CsA and FK506 on intracellular signalling and gene regulation. *Immunobiology*. 1997;198(1–3):192–206.
70. Ho S, Clipstone N, Timmermann L, et al. The mechanism of action of cyclosporin A and FK506. *Clin Immunol Immunopathol*. 1996;80(3 Pt 2):S40–S45.
71. Kaçmaz RO, Kempen JH, Newcomb C, et al. Cyclosporine for Ocular Inflammatory Diseases. *Ophthalmology*. 2011;117(3):576–584.
72. Masuda K, Nakajima A, Urayama A, Nakae K, Kogure M, Inaba G. Double-masked trial of cyclosporin versus colchicine and long-term open study of cyclosporin in Behçet's disease. *Lancet*. 1989;1(8647):1093–1096.
73. Nussenblatt RB, Palestine AG, Rook AH, Scher I, Wacker WB, Gery I. Treatment of intraocular inflammatory disease with cyclosporin A. *Lancet*. 1983;2(8344):235–238.
74. Graham EM, Sanders MD, James DG, Hamblin A, Kasp Grochowska E, Dumonde D. Cyclosporin A in the treatment of posterior uveitis. *Trans Ophthalmol Soc U K*. 1985;104(2):146–151.
75. Murphy CC, Greiner K, Plskova J, et al. Cyclosporine vs tacrolimus therapy for posterior and intermediate uveitis. *Arch Ophthalmol*. 2005;123(5):634–641.
76. Chan GL, Canafax DM, Johnson CA. The therapeutic use of azathioprine in renal transplantation. *Pharmacotherapy*. 1987;7(5):165–177.
77. Okada AA. Immunomodulatory therapy for ocular inflammatory disease: a basic manual and review of the literature. *Ocul Immunol Inflamm*. 2005;13(5):335–351.
78. Hoang-Xaun T, Foster CS, Rice BA. Scleritis in relapsing polychondritis. Response to therapy. *Ophthalmology*. 1990;97(7):892–898.
79. Saw VP, Dart JK, Rauz S, et al. Immunosuppressive therapy for ocular mucous membrane pemphigoid strategies and outcomes. *Ophthalmology*. 2008;115(2):253–261.
80. Hemady RK, Baer JC, Foster CS. Immunosuppressive drugs in the management of progressive, corticosteroid-resistant uveitis associated with juvenile rheumatoid arthritis. *Int Ophthalmol Clin*. 1992;32(1):241–252.
81. Newell FW, Krill AE. Treatment of uveitis with azathioprine (Imuran). *Trans Ophthalmol Soc U K*. 1967;87:499–511.
82. Moore CE. Sympathetic ophthalmitis treated with azathioprine. *Br J Ophthalmol*. 1968;52(9):688–690.
83. Yazici H, Pazarli H, Barnes C, et al. A controlled trial of azathioprine in Behcet's syndrome. *N Engl J Med*. 1990;322(5):281–285.
84. Pasadhika S, Kempen JH, Newcomb C, et al. Azathioprine for Ocular Inflammatory Disease. *Am J Ophthalmol*. 2009;148(4):500–509.
85. Cade R, Mars D, Privette M, et al. Effect of long-term azathioprine administration in adults with minimal-change glomerulonephritis and nephrotic syndrome resistant to corticosteroids. *Arch Intern Med*. 1986;146(4):737–741.
86. Singh G, Fries JF, Spitz P, Williams CA. Toxic effects of azathioprine in rheumatoid arthritis. A national post-marketing perspective. *Arthritis Rheum*. 1989;32(7):837–843.
87. Asten P, Barrett J, Symmons D. Risk of developing certain malignancies is related to duration of immunosuppressive drug exposure in patients with rheumatic diseases. *J Rheumatol*. 1999;26(8):1705–1714.
88. Penn I. Malignancies associated with immunosuppressive or cytotoxic therapy. *Surgery*. 1978;83(5):492–502.
89. Kempen JH, Gangaputra S, Daniel E, et al. Long term risk of malignancy among patients treated with immunosuppressive agents for ocular inflammation: a critical assessment of the evidence. *Am J Ophthalmol*. 2009;146(6):802–812.
90. Samson CM, Waheed N, Baltatzis S, Foster CS. Methotrexate therapy for chronic noninfectious uveitis: analysis of a case series of 160 patients. *Ophthalmology*. 2001;108(6):1134–1139.
91. Bom S, Zamiri P, Lightman S. Use of methotrexate in the management of sight-threatening uveitis. *Ocul Immunol Inflamm*. 2001;9(1):35–40.
92. Holz FG, Krastel H, Breitbart A, Schwarz-Eywill M, Pezzutto A, Völcker HE. Low-dose methotrexate treatment in noninfectious uveitis resistant to corticosteroids. *Ger J Ophthalmol*. 1992;1(3–4):142–144.
93. Kaplan-Messas A, Barkana Y, Avni I, Neumann R. Methotrexate as a first-line corticosteroid-sparing therapy in a cohort of uveitis and scleritis. *Ocul Immunol Inflamm*. 2003;11(2):131–139.
94. Shah SS, Lowder CY, Schmitt MA, Wilke WS, Kosmorsky GS, Meisler DM. Low-dose methotrexate therapy for ocular inflammatory disease. *Ophthalmology*. 1992;99(9):1419–1423.
95. Gangaputra S, Newcomb CW, Liesegang TL, et al. Methotrexate for Ocular Inflammatory Diseases. *Ophthalmology*. 2009;116(11):2188–2198.
96. Kremer JM, Alarcón GS, Lightfoot RW Jr, et al. Methotrexate for rheumatoid arthritis. Suggested guidelines for monitoring liver toxicity. American College of Rheumatology. *Arthritis Rheum*. 1994;37(3):316–328.
97. Allison AC, Eugui EM. Immunosuppressive and other effects of mycophenolic acid and an ester prodrug, mycophenolate mofetil. *Immunol Rev*. 1993;136:5–28.
98. Baltatzis S, Tufail F, Yu EN, Vredeveld CM, Foster CS. Mycophenolate mofetil as an immunomodulatory agent in the treatment of chronic ocular inflammatory disorders. *Ophthalmology*. 2003;110(5):1061–1065.
99. Sobrin L, Christen W, Foster CS. Mycophenolate mofetil after methotrexate failure or intolerance in the treatment of scleritis and uveitis. *Ophthalmology*. 2008;115(8):1416–1421.
100. Daniel E, Thorne JE, Newcomb CW, et al. Mycophenolate mofetil for ocular inflammation. *Am J Ophthalmol*. 2010;149(3):423–432.
101. Ahmed AR, Hombal SM. Cyclophosphamide (Cytoxan). A review on relevant pharmacology and clinical uses. *J Am Acad Dermatol*. 1984;11(6):1115–1126.
102. De Groot K, Harper L, Jayne DRW, et al. Pulse versus daily oral cyclophosphamide for induction of remission in antineutrophil cytoplasmic antibody-associated vasculitis: a randomized trial. *Ann Intern Med*. 2009;150(10):670–680.
103. Adu D, Pall A, Luqmani RA, et al. Controlled trial of pulse versus continuous prednisolone and cyclophosphamide in the treatment of systemic vasculitis. *QJM*. 1997;90(6):401–409.
104. Durrani K, Papaliodis GN, Foster CS. Pulse IV cyclophosphamide in ocular inflammatory disease: efficacy and short-term safety. *Ophthalmology*. 2004;111(5):960–965.
105. Carruthers DM. Rational therapy in systemic necrotising vasculitis. *Clin Med*. 2001;1:7–9.
106. Khan IJ, Barry RJ, Amissah-Arthur KN, et al. Ten-year experience of pulsed intravenous cyclophosphamide and methylprednisolone protocol (PICM protocol) in severe ocular inflammatory disease. *Br J Ophthalmol*. 2012;97(9):1118–1122.
107. Pujari SS, Kempen JH, Newcomb CW, et al. Cyclophosphamide for ocular inflammatory diseases. *Ophthalmology*. 2011;117(2):356–365.
108. Martin-Suarez I, D'Cruz D, Mansoor M, Fernandes AP, Khamashta MA, Hughes GR. Immunosuppressive treatment in severe connective tissue diseases: effects of low dose intravenous cyclophosphamide. *Ann Rheum Dis*. 1997;56:481–487.

109. Kempen JH, Daniel E, Dunn JP, et al. Overall and cancer related mortality among patients with. *BMJ*. 2009;339:b2480.
110. Blumenfeld Z, Haim N. Prevention of gonadal damage during cytotoxic therapy. *Ann Med*. 1997;29(3):199–206.
111. Newell DR, Calvert AH, Harrap KR, McElwain TJ. Studies on the pharmacokinetics of chlorambucil and prednimustine in man. *Br J Clin Pharmacol*. 1983;15(2):253–258.
112. Mudun BA, Ergen A, Ipcioglu SU, Burumcek EY, Durlu Y, Arslan MO. Short-term chlorambucil for refractory uveitis in Behcet's disease. *Ocul Immunol Inflamm*. 2001;9(4):219–229.
113. Goldstein DA, Fontanilla FA, Kaul S, Sahin O, Tessler HH. Long-term follow-up of patients treated with short-term high-dose chlorambucil for sight-threatening ocular inflammation. *Ophthalmology*. 2002;109(2):370–377.
114. Miserocchi E, Baltatzis S, Ekong A, Roque M, Foster CS. Efficacy and safety of chlorambucil in intractable noninfectious uveitis: the Massachusetts Eye and Ear Infirmary experience. *Ophthalmology*. 2002;109(1):137–142.
115. Wooley PH, Dutcher J, Widmer MB, Gillis S. Influence of a recombinant human soluble tumor necrosis factor receptor FC fusion protein on type II collagen-induced arthritis in mice. *J Immunol*. 1993;151(11):6602–6607.
116. Scallon B, Cai A, Solowski N, et al. Binding and functional comparisons of two types of tumor necrosis factor antagonists. *J Pharmacol Exp Ther*. 2002;301(2):418–426.
117. Scallon BJ, Moore MA, Trinh H, Knight DM, Ghrayeb J. Chimeric anti-TNF-alpha monoclonal antibody cA2 binds recombinant transmembrane TNF-alpha and activates immune effector functions. *Cytokine*. 1995;7(3):251–259.
118. Lin J, Ziring D, Desai S, et al. TNFa blockade in human diseases: an overview of efficacy and safety. *Clin Immunol*. 2009;126(1):13–30.
119. Braun J, Brandt J, Listing J, et al. Long-term efficacy and safety of infliximab in the treatment of ankylosing spondylitis: an open, observational, extension study of a three-month, randomized, placebo-controlled trial. *Arthritis Rheum*. 2003;48(8):2224–2233.
120. Sobrin L, Kim EC, Christen W, Papadaki T, Letko E, Foster CS. Infliximab therapy for the treatment of refractory ocular inflammatory disease. *Arch Ophthalmol*. 2007;125(7):895–900.
121. Lee FF, Foster CS. Pharmacotherapy of uveitis. *Expert Opin Pharmacother*. 2010;11(7):1135–1146.
122. Niccoli L, Nannini C, Benucci M, et al. Long-term efficacy of infliximab in refractory posterior uveitis of Behcet's disease: a 24-month follow-up study. *Rheumatology (Oxford)*. 2007;46(7):1161–1164.
123. Markomichelakis N, Delicha E, Masselos S, Fragiadaki K, Kaklamanis P, Sfikakis PP. A single infliximab infusion vs corticosteroids for acute panuveitis attacks in Behçet's disease: a comparative 4-week study. *Rheumatology (Oxford)*. 2011;50(3):593–597.
124. Sfikakis PP, Kaklamanis PH, Elezoglou A, et al. Infliximab for recurrent, sight-threatening ocular inflammation in Adamantiades-Behçet disease. *Ann Intern Med*. 2004;140(5):404–406.
125. Artornsombudh P, Gevorgyan O, Payal A, Siddique SS, Foster CS. Infliximab treatment of patients with birdshot retinochoroidopathy. *Ophthalmology*. 2013;120(3):588–592.
126. Kahn P, Weiss M, Imundo LF, Levy DM. Favorable response to high-dose infliximab for refractory childhood uveitis. *Ophthalmology*. 2006;113(5):860–864.
127. Rajaraman RT, Kimura Y, Li S, Haines K, Chu DS. Retrospective case review of pediatric patients with uveitis treated with infliximab. *Ophthalmology*. 2006;113(2):308–314.
128. Baughman RP, Bradley DA, Lower EE. Infliximab in chronic ocular inflammation. *Int J Clin Pharmacol Ther*. 2005;43(1):7–11.
129. Bodaghi B, Bui Quoc E, Wechsler B, et al. Therapeutic use of infliximab in sight threatening uveitis: retrospective analysis of efficacy, safety, and limiting factors. *Ann Rheum Dis*. 2005;64(6):962–964.
130. Suhler EB, Smith JR, Wertheim MS, et al. A prospective trial of infliximab therapy for refractory uveitis: preliminary safety and efficacy outcomes. *Arch Ophthalmol*. 2005;123(7):903–912.
131. Suhler EB, Smith JR, Giles TR, et al. Infliximab therapy for refractory uveitis: 2-year results of a prospective trial. *Arch Ophthalmol*. 2009;127(6):819–822.
132. Kaymakcalan Z, Sakorafas P, Bose S, et al. Comparisons of affinities, avidities, and complement activation of adalimumab, infliximab, and etanercept in binding to soluble and membrane tumor necrosis factor. *Clin Immunol*. 2009;131(2):308–316.
133. Rudwaleit M, Rødevand E, Holck P, et al. Adalimumab effectively reduces the rate of anterior uveitis flares in patients with active ankylosing spondylitis: results of a prospective open-label study. *Ann Rheum Dis*. 2009;68(5):696–701.
134. Dobner BC, Max R, Becker MD, et al. A three-centre experience with adalimumab for the treatment of non-infectious uveitis. *Br J Ophthalmol*. 2013;97(2):134–138.
135. Sen ES, Sharma S, Hinchcliffe A, Dick AD, Ramanan AV. Use of adalimumab in refractory non-infectious childhood chronic uveitis: efficacy in ocular disease – a case cohort interventional study. *Rheumatology (Oxford)*. 2012;51(12):2199–2203.
136. Diaz-Llopis M, García-Delpech S, Salom D, et al. Adalimumab therapy for refractory uveitis: a pilot study. *J Ocul Pharmacol Ther*. 2008;24(3):351–361.
137. Tynjälä P, Kotaniemi K, Lindahl P, et al. Adalimumab in juvenile idiopathic arthritis-associated chronic anterior uveitis. *Rheumatology (Oxford)*. 2008;47(3):339–344.
138. Calabrese LH. Molecular differences in anticytokine therapies. *Clin Exp Rheumatol*. 2003;21(2):241–248.
139. Reiff A, Takei S, Sadeghi S, et al. Etanercept therapy in children with treatment-resistant uveitis. *Arthritis Rheum*. 2001;44(6):1411–1415.
140. Reiff A. Long-term outcome of etanercept therapy in children with treatment-refractory uveitis. *Arthritis Rheum*. 2003;48(7):2079–2080.
141. Smith JR, Levinson RD, Holland GN, et al. Differential efficacy of tumor necrosis factor inhibition in the management of inflammatory eye disease and associated rheumatic disease. *Arthritis Rheum*. 2001;45(3):252–257.
142. Quartier P, Taupin P, Bourdeaut F, et al. Efficacy of etanercept for the treatment of juvenile idiopathic arthritis according to the onset type. *Arthritis Rheum*. 2003;48(4):1093–1101.
143. Smith JA, Thompson DJ, Whitcup SM, et al. A randomized, placebo-controlled, double-masked clinical trial of etanercept for the treatment of uveitis associated with juvenile idiopathic arthritis. *Arthritis Rheum*. 2005;53(1):18–23.
144. Foster CS, Tufail F, Waheed NK, et al. Efficacy of etanercept in preventing relapse of uveitis controlled by methotrexate. *Arch Ophthalmol*. 2003;121(4):437–440.
145. Galor A, Perez VL, Hammel JP, Lowder CY. Differential effectiveness of etanercept and infliximab in the treatment of ocular inflammation. *Ophthalmology*. 2006;113(12):2317–2323.
146. Treon SP, Anderson KC. The use of rituximab in the treatment of malignant and nonmalignant plasma cell disorders. *Semin Oncol*. 2000;27(6 Suppl 12):79–85.
147. Lim L, Suhler EB, Smith JR. Biologic therapies for inflammatory eye disease. *Clin Experiment Ophthalmol*. 2006;34(4):365–374.
148. Heiligenhaus A, Miserocchi E, Heinz C, Gerloni V, Kotaniemi K. Treatment of severe uveitis associated with juvenile idiopathic arthritis with anti-CD20 monoclonal antibody (rituximab). *Rheumatology (Oxford)*. 2011;50(8):1390–1394.
149. Sadreddini S, Noshad H, Molaeefard M, Noshad R. Treatment of retinal vasculitis in Behçet's disease with rituximab. *Mod Rheumatol*. 2008;18(3):306–308.
150. Davatchi F, Shams H, Rezaipoor M, et al. Rituximab in intractable ocular lesions of Behcet's disease; randomized single-blind control study (pilot study). *Int J Rheum Dis*. 2010;13(3):246–252.
151. Miserocchi E, Pontikaki I, Modorati G, Bandello F, Meroni PL, Gerloni V. Rituximab for uveitis. *Ophthalmology*. 2011;118(1):223–224.
152. Tomkins-Netzer O, Taylor SR, Lightman S. Can rituximab induce long-term disease remission in patients with intra-ocular non-infectious inflammation? *Ophthalmologica*. 2013;230(3):109–115.

153. Rubbert-Roth A, Tak PP, Zerbini C, et al. Efficacy and safety of various repeat treatment dosing regimens of rituximab in patients with active rheumatoid arthritis: results of a Phase III randomized study (MIRROR). *Rheumatology (Oxford)*. 2010;49(9):1683–1693.
154. Keating GM. Abatacept: a review of its use in the management of rheumatoid arthritis. *Drugs*. 2013;73(10):1095–1119.
155. Pasadhika S, Rosenbaum JT. Update on the use of systemic biologic agents in the treatment of noninfectious uveitis. *Biologics*. 2014;8:67–81.
156. Angeles-Han S, Flynn T, Lehman T. Abatacept for refractory juvenile idiopathic arthritis-associated uveitis-a case report. *J Rheumatol*. 2008;35(9):1897–1898.
157. Zulian F, Balzarin M, Falcini F, et al. Abatacept for severe anti-tumor necrosis factor alpha refractory juvenile idiopathic arthritis-related uveitis. *Arthritis Care Res (Hoboken)*. 2010;62(6):821–825.
158. Kenawy N, Cleary G, Mewar D, Beare N, Chandna A, Pearce I. Abatacept: a potential therapy in refractory cases of juvenile idiopathic arthritis-associated uveitis. *Graefes Arch Clin Exp Ophthalmol*. 2011;249(2):297–300.
159. Yang H, Wang J, Du J, et al. Structural basis of immunosuppression by the therapeutic antibody daclizumab. *Cell Res*. 2010;20(12):1361–1371.
160. Bhat P, Castañeda-Cervantes RA, Doctor PP, Foster CS. Intravenous daclizumab for recalcitrant ocular inflammatory disease. *Graefes Arch Clin Exp Ophthalmol*. 2009;247(5):687–692.
161. Papaliodis GN, Chu D, Foster CS. Treatment of ocular inflammatory disorders with daclizumab. *Ophthalmology*. 2003;110(4):786–789.
162. Sen HN, Levy-Clarke G, Faia LJ, et al. High-dose daclizumab for the treatment of juvenile idiopathic arthritis-associated active anterior uveitis. *Am J Ophthalmol*. 2009;148(5):696–703.
163. Webster AC, Ruster LP, McGee R, et al. Interleukin 2 receptor antagonists for kidney transplant recipients. *Cochrane database Syst Rev*. 2010;(1):CD003897.
164. Nussenblatt RB, Fortin E, Schiffman R, et al. Treatment of noninfectious intermediate and posterior uveitis with the humanized anti-Tac mAb: a phase I/II clinical trial. *Proc Natl Acad Sci U S A*. 1999;96(13):7462–7466.
165. Nussenblatt RB, Thompson DJ, Li Z, et al. Humanized anti-interleukin-2 (IL-2) receptor alpha therapy: long-term results in uveitis patients and preliminary safety and activity data for establishing parameters for subcutaneous administration. *J Autoimmun*. 2003;21(3):283–293.
166. Sobrin L, Huang JJ, Christen W, Kafkala C, Choopong P, Foster CS. Daclizumab for treatment of birdshot chorioretinopathy. *Arch Ophthalmol*. 2008;126(2):186–191.
167. Yeh S, Wroblewski K, Buggage R, et al. High-dose humanized anti-IL-2 receptor alpha antibody (daclizumab) for the treatment of active, noninfectious uveitis. *J Autoimmmun*. 2009;31(2):91–97.
168. Buggage RR, Levy-Clarke G, Sen HN, et al. A double-masked, randomized study to investigate the safety and efficacy of daclizumab to treat the ocular complications related to Behçet's disease. *Ocul Immunol Inflamm*. 2007;15(2):63–70.
169. Deuter C, Stübiger N, Zierhut M. Interferon-α therapy in noninfectious uveitis. *Dev Ophthalmol*. 2012;51:90–97.
170. Gueudry J, Wechsler B, Terrada C, et al. Long-term efficacy and safety of low-dose interferon alpha2a therapy in severe uveitis associated with Behçet disease. *Am J Ophthalmol*. 2008;146(6):837–844.
171. Kötter I, Günaydin I, Zierhut M, Stübiger N. The use of interferon alpha in Behçet disease: review of the literature. *Semin Arthritis Rheum*. 2004;33(5):320–335.
172. Becker MD, Heiligenhaus A, Hudde T, et al. Interferon as a treatment for uveitis associated with multiple sclerosis. *Br J Ophthalmol*. 2005;89(10):1254–1257.
173. Mackensen F, Max R, Becker MD. Interferons and their potential in the treatment of ocular inflammation. *Clin Ophthalmol*. 2009;3:559–566.
174. Stein MR. The new generation of liquid intravenous immunoglobulin formulations in patient care: a comparison of intravenous immunoglobulins. *Postgrad Med*. 2010;122(5):176–184.
175. Kazatchkine MD, Kaveri SV. Immunomodulation of autoimmune and inflammatory diseases with intravenous immune globulin. *N Engl J Med*. 2001;345(10):747–755.
176. Onal S, Foster CS, Ahmed AR. Efficacy of intravenous immunoglobulin treatment in refractory uveitis. *Ocul Immunol Inflamm*. 2006;14(6):367–374.
177. LeHoang P, Cassoux N, George F, Kullmann N, Kazatchkine MD. Intravenous immunoglobulin (IVIg) for the treatment of birdshot retinochoroidopathy. *Ocul Immunol Inflamm*. 2000;8(1):49–57.
178. Seider N, Beiran I, Scharf J, Miller B. Intravenous immunoglobulin therapy for resistant ocular Behçet's disease. *Br J Ophthalmol*. 2001;85(11):1287–1288.
179. González-Delgado M, González C, Blázquez JI, Salas-Puig J, Castro J, Hernández-Lahoz C. [Intravenous immunoglobulin therapy in Vogt-Koyanagi-Harada syndrome]. *Neurologia*. 2004;19(7):401–403. Spanish.
180. Weiss K, Steinbrugger I, Weger M, et al. Intravitreal VEGF levels in uveitis patients and treatment of uveitic macular oedema with intravitreal bevacizumab. *Eye (Lond)*. 2009;23(9):1812–1818.
181. Fine HF, Baffi J, Reed GF, Csaky KG, Nussenblatt RB. Aqueous humor and plasma vascular endothelial growth factor in uveitis-associated cystoid macular edema. *Am J Ophthalmol*. 2001;132(5):794–796.
182. Cordero Coma M, Sobrin L, Onal S, Christen W, Foster CS. Intravitreal bevacizumab for treatment of uveitic macular edema. *Ophthalmology*. 2007;114(8):1574–1579.
183. Mackensen F, Heinz C, Becker MD, Heiligenhaus A. Intravitreal bevacizumab (avastin) as a treatment for refractory macular edema in patients with uveitis: a pilot study. *Retina*. 2008;28(1):41–45.
184. Acharya NR, Hong KC, Lee SM. Ranibizumab for refractory uveitis-related macular edema. *Am J Ophthalmol*. 2009;148(2):303–309.
185. Lasave AF, Zeballos DG, El-Haig WM, Díaz-Llopis M, Salom D, Arevalo JF. Short-term results of a single intravitreal bevacizumab (avastin) injection versus a single intravitreal triamcinolone acetonide (kenacort) injection for the management of refractory noninfectious uveitic cystoid macular edema. *Ocul Immunol Inflamm*. 2009;17(6):423–430.
186. Bae JH, Lee CS, Lee SC. Efficacy and safety of intravitreal bevacizumab compared with intravitreal and posterior sub-tenon triamcinolone acetonide for treatment of uveitic cystoid macular edema. *Retina*. 2011;31(1):111–118.
187. Soheilian M, Rabbanikhah Z, Ramezani A, Kiavash V, Yaseri M, Peyman GA. Intravitreal bevacizumab versus triamcinolone acetonide for refractory uveitic cystoid macular edema: a randomized pilot study. *J Ocul Pharmacol Ther*. 2010;26(2):199–206.
188. Cervantes-Castañeda RA, Giuliari GP, Gallagher MJ, et al. Intravitreal bevacizumab in refractory uveitic macular edema: one-year follow-up. *Eur J Ophthalmol*. 2009;19(4):622–629.
189. Rosenfeld PJ, Brown DM, Heier JS, et al. Ranibizumab for neovascular age-related macular degeneration. *N Engl J Med*. 2006;355(14):1419–1431.
190. Brown DM, Kaiser PK, Michels M, et al. Ranibizumab versus verteporfin for neovascular age-related macular degeneration. *N Engl J Med*. 2006;355(14):1432–1444.
191. Inoue M, Takeda K, Morita K, Yamada M, Tanigawara Y, Oguchi Y. Vitreous concentrations of triamcinolone acetonide in human eyes after intravitreal or subtenon injection. *Am J Ophthalmol*. 2004;138(6):1046–1048.
192. Van Kooij B, Rothova A, de Vries P. The pros and cons of intravitreal triamcinolone injections for uveitis and inflammatory cystoid macular edema. *Ocul Immunol Inflamm*. 2006;14(2):73–85.
193. Chang-Lin JE, Attar M, Acheampong AA, et al. Pharmacokinetics and pharmacodynamics of a sustained-release dexamethasone intravitreal implant. *Invest Ophthalmol Vis Sci*. 2011;52(1):80–86.
194. Da Silva GR, da Silva Cunha A Jr, Ayres E, Oréfice RL. Effect of the macromolecular architecture of biodegradable polyurethanes on the controlled delivery of ocular drugs. *J Mater Sci Mater Med*. 2009;20(2):481–487.

195. Lowder C, Belfort R, Lightman S, et al. Dexamethasone intravitreal implant for noninfectious intermediate or posterior uveitis. *Arch Ophthalmol*. 2011;129(5):545–553.
196. Adán A, Pelegrín L, Rey A, et al. Dexamethasone intravitreal implant for treatment of uveitic persistent cystoid macular edema in vitrectomized patients. *Retina*. 2013;33(7):1435–1440.
197. Taylor SRJ, Tomkins-Netzer O, Joshi L, Morarji J, McLoone E, Lightman S. Dexamethasone implant in pediatric uveitis. *Ophthalmology*. 2012;119(11):2412–2412.
198. Jaffe GJ, Ben-Nun J, Guo H, Dunn JP, Ashton P. Fluocinolone acetonide sustained drug delivery device to treat severe uveitis. *Ophthalmology*. 2000;107(11):2024–2033.
199. Hunter RS, Lobo AM. Dexamethasone intravitreal implant for the treatment of noninfectious uveitis. *Clin Ophthalmol*. 2011;5:1613–1621.
200. Jaffe GJ, Martin D, Callanan D, Pearson PA, Levy B, Comstock T. Fluocinolone acetonide implant (Retisert) for noninfectious posterior uveitis: thirty-four-week results of a multicenter randomized clinical study. *Ophthalmology*. 2006;113(6):1020–1027.
201. Multicenter Uveitis Steroid Treatment (MUST) Trial Research Group, Kempen JH, Altaweel MM, et al. Randomized comparison of systemic anti-inflammatory therapy versus fluocinolone acetonide implant for intermediate, posterior, and panuveitis: the multicenter uveitis steroid treatment trial. *Ophthalmology*. 2011;118(10):1916–1926.
202. Callanan DG, Jaffe GJ, Martin DF, Pearson PA, Comstock TL. Treatment of posterior uveitis with a fluocinolone acetonide implant: three-year clinical trial results. *Arch Ophthalmol*. 2008;126(9):1191–1201.
203. Kane FE, Burdan J, Cutino A, Green KE. Iluvien: a new sustained delivery technology for posterior eye disease. *Expert Opin Drug Deliv*. 2008;5(9):1039–1046.
204. Campochiaro PA, Hafiz G, Shah SM, et al. Sustained ocular delivery of fluocinolone acetonide by an intravitreal insert. *Ophthalmology*. 2010;117(7):1393–1399.
205. Sanford M. Fluocinolone acetonide intravitreal implant (Iluvien®): in diabetic macular oedema. *Drugs*. 2013;73(2):187–193.
206. Focused Therapeutics/Visionary Science [webpage on the Internet]. Watertown: pSivida Corp [cited April 15, 2014]. Available from: http://www.psivida.com/. Accessed June 19, 2014.
207. Chen J. Novel regulatory mechanisms of mTOR signaling. *Curr Top Microbiol Immunol*. 2004;279:245–257.
208. Kang J, Huddleston SJ, Fraser JM, Khoruts A. De novo induction of antigen-specific CD4+CD25+Foxp3+ regulatory T-cells in vivo following systemic antigen administration accompanied by blockade of mTOR. *J Leukoc Biol*. 2008;83(5):1230–1239.
209. Zeiser R, Leveson-Gower DB, Zambricki EA, et al. Differential impact of mammalian target of rapamycin inhibition on CD4+CD25+Foxp3+ regulatory T-cells compared with conventional CD4+ T-cells. *Blood*. 2008;111(1):453–462.
210. Bruyn GA, Tate G, Caeiro F, et al. Everolimus in patients with rheumatoid arthritis receiving concomitant methotrexate: a 3-month, double-blind, randomised, placebo-controlled, parallel-group, proof-of-concept study. *Ann Rheum Dis*. 2008;67(8):1090–1095.
211. Lieberthal W, Fuhro R, Andry CC, et al. Rapamycin impairs recovery from acute renal failure: role of cell-cycle arrest and apoptosis of tubular cells. *Am J Physiol Renal Physiol*. 2001;281(4):F693–F706.
212. Tenderich G, Fuchs U, Zittermann A, Muckelbauer R, Berthold HK, Koerfer R. Comparison of sirolimus and everolimus in their effects on blood lipid profiles and haematological parameters in heart transplant recipients. *Clin Transplant*. 2007;21(4):536–543.
213. Crowe A, Bruelisauer A, Duerr L, Guntz P, Lemaire M. Absorption and intestinal metabolism of SDZ-RAD and rapamycin in rats. *Drug Metab Dispos*. 1999;27(5):627–632.
214. Heiligenhaus A, Zurek-Imhoff B, Roesel M, Hennig M, Rammrath D, Heinz C. Everolimus for the treatment of uveitis refractory to cyclosporine A: a pilot study. *Graefes Arch Clin Exp Ophthalmol*. 2013;251(1):143–152.
215. Sehgal S. Sirolimus: its discovery, biological properties, and mechanism of action. *Transplant Proc*. 2003;35(3):S7–S14.
216. Veroux M, Tallarita T, Corona D, D'Assoro A, Gurrieri C, Veroux P. Sirolimus in solid organ transplantation: current therapies and new frontiers. *Immunotherapy*. 2011;3(12):1487–1497.
217. Dasari TW, Patel B, Saucedo JF. Systematic review of effectiveness of oral sirolimus after bare-metal stenting of coronary arteries for prevention of in-stent restenosis. *Am J Cardiol*. 2013;112(9):1322–1327.
218. Javaid MM, Chowdhury S, Henderson A, Olsburgh J. Advanced native kidney renal cell carcinoma in renal transplant recipients: role of sirolimus as dual anti-cancer and anti-rejection agent. *Clin Nephrol*. 2013;79(2):154–160.
219. Shanmuganathan VA, Casely EM, Raj D, et al. The efficacy of sirolimus in the treatment of patients with refractory uveitis. *Br J Ophthalmol*. 2005;89(6):666–669.
220. Phillips BN, Wroblewski KJ. A retrospective review of oral low-dose sirolimus (rapamycin) for the treatment of active uveitis. *J Ophthalmic Inflamm Infect*. 2011;1(1):29–34.
221. Sen HN, Larson TA, Meleth AD, Smith WM, Nussenblatt RB. Suconjunctival sirolimus for the treatment of chronic active anterior uveitis: results of a pilot trial. *Am J Ophthalmol*. 2012;153(6):1038–1042.
222. Nguyen QD, Ibrahim MA, Watters A, et al. Ocular tolerability and efficacy of intravitreal and subconjunctival injections of sirolimus in patients with non-infectious uveitis: primary 6-month results of the SAVE Study. *J Ophthalmic Inflamm Infect*. 2013;3(1):32.
223. Santen Inc. Study Assessing Double-masked Uveitis Treatment (SAKURA). Available from: http://clinicaltrials.gov/show/NCT01358266. NLM identifier: NCT01358266. Accessed June 19, 2014.
224. Dumont FJ. ISAtx-247 (Isotechnika/Roche). *Curr Opin Investig Drugs*. 2004;5(5):542–550.
225. Aspeslet L, Freitag D, Trepanier D, et al. ISA(TX)247: a novel calcineurin inhibitor. *Transplant Proc*. 2001;33:1048–1051.
226. Bissonnette R, Papp K, Poulin Y, et al. A randomized, multicenter, double-blind, placebo-controlled phase 2 trial of ISA247 in patients with chronic plaque psoriasis. *J Acad Dermatology*. 2006;54(3):472–478.
227. Papp K, Bissonnette R, Rosoph L, et al. Efficacy of ISA247 in plaque psoriasis: a randomised, multicentre, double-blind, placebo-controlled phase III study. *Lancet*. 2008;371:1337–1342.
228. Anglade E, Aspeslet LJ, Weiss SL. A new agent for the treatment of noninfectious uveitis: rationale and design of three LUMINATE (Lux Uveitis Multicenter Investigation of a New Approach to Treatment) trials of steroid-sparing voclosporin. *Clin Ophthalmol*. 2008;2(4):693–702.
229. Roesel M, Tappeiner C, Heiligenhaus A, Heinz C. Oral voclosporin: novel calcineurin inhibitor for treatment of noninfectious uveitis. *Clin Ophthalmol*. 2011;5:1309–1313.
230. Schultz C. Voclosporin as a treatment for noninfectious uveitis. *Ophthalmol Eye Dis*. 2013;5:5–10.
231. Voclosporin uveitis study fails to meet primary endpoint [webpage on the Internet]. Santa Monica: Advanstar Communications, Inc.; 2013 [updated Jan 2, 2013; cited March 20, 2014]. Available from: http://ophthalmologytimes.modernmedicine.com/ophthalmologytimes/news/modernmedicine/modern-medicine-feature-articles/voclosporin-uveitis-study-fa. Accessed June 19, 2014.
232. Mesquida M, Victoria Hernández M, Llorenç V, et al. Behçet disease-associated uveitis successfully treated with golimumab. *Ocul Immunol Inflamm*. 2013;21(2):160–162.
233. William M, Faez S, Papaliodis GN, Lobo A. Golimumab for the treatment of refractory juvenile idiopathic arthritis-associated uveitis. *J Ophthalmic Inflamm Infect*. 2012;2:231–233.
234. Miserocchi E, Modorati G, Pontikaki I, Meroni PL, Gerloni V. Long-term treatment with golimumab for severe uveitis. *Ocul Immunol Inflamm*. 2014;22(2):90–95.
235. Cordero-Coma M, Salom D, Díaz-Llopis M, López-Prats MJ, Calleja S. Golimumab for uveitis. *Ophthalmology*. 2011;118(9):1892.e3–e4.

236. Faez S, Lobo AM, Sobrin L, Papaliodis GN. Treatment of seronegative spondyloarthropathy-associated uveitis with golimumab: retrospective case series. *Clin Experiment Ophthalmol*. 2014;42(4):392–395.
237. Sánchez-Cano D, Callejas-Rubio JL, Ruiz-Villaverde R, Ríos-Fernández R, Ortego-Centeno N. Off-label uses of anti-TNF therapy in three frequent disorders: Behçet's disease, sarcoidosis, and noninfectious uveitis. *Mediators Inflamm*. 2013;2013:286857.
238. Tlucek PS, Stone DU. Certolizumab pegol therapy for rheumatoid arthritis-associated scleritis. *Cornea*. 2012;31(1):90–91.
239. Dinarello CA, Simon A, van der Meer JW. Treating Inflammation by blocking interleukin-1 in a broad spectrum of diseases. *Nat Rev Drug Discov*. 2012;11(8):633–652.
240. Teoh SC, Sharma S, Hogan A, Lee R, Ramanan AV, Dick AD. Tailoring biological treatment: anakinra treatment of posterior uveitis associated with the CINCA syndrome. *Br J Ophthalmol*. 2007;91(2):263–264.
241. Mesquida M, Molins B, Llorenç V, et al. Current and future treatments for Behçet's uveitis: road to remission. *Int Ophthalmol*. 2014;34(2):365–381.
242. Issafras H, Corbin JA, Goldfine ID, Roell MK. Detailed mechanistic analysis of gevokizumab, an allosteric anti-IL-1β antibody with differential receptor-modulating properties. *J Pharmacol Exp Ther*. 2014;348(1):202–215.
243. Gül A, Tugal-Tutkun I, Dinarello CA, et al. Interleukin-1β-regulating antibody XOMA 052 (gevokizumab) in the treatment of acute exacerbations of resistant uveitis of Behcet's disease: an open-label pilot study. *Ann Rheum Dis*. 2012;71(4):563–566.
244. EyeGuard Study [homepage on the Internet]. Eyeguard; 2013 [cited March 20, 2014]. Available from: http://www.eyeguard.com. Accessed June 19, 2014.
245. Lachmann HJ, Kone-Paut I, Kuemmerle-Deschner JB, et al. Use of canakinumab in the cryopyrin-associated periodic syndrome. *N Engl J Med*. 2009;360:2416–2425.
246. Ugurlu S, Ucar D, Seyahi E, Haterni G, Yurdakul S. Canakinumab in a patient with juvenile Behcet's syndrome with refractory eye disease. *Ann Rheum Dis*. 2012;71(9):1589–1591.
247. Simonini G, Xu Z, Caputo R, et al. Clinical and transcriptional response to the long-acting interleukin-1 blocker canakinumab in Blau syndrome-related uveitis. *Arthritis Rheum*. 2013;65(2):513–518.
248. Isaacs JD, Watts RA, Hazleman BL, et al. Humanised monoclonal antibody therapy for rheumatoid arthritis. *Lancet*. 1992;340(8822):748–752.
249. Coles AJ, Twyman CL, Arnold DL, et al. Alemtuzumab for patients with relapsing multiple sclerosis after disease-modifying therapy: a randomised controlled phase 3 trial. *Lancet*. 2012;380(9856):1829–1839.
250. Isaacs J, Hale G, Waldmann H, et al. Monoclonal antibody therapy of chronic intraocular inflammation using Campath-1H. *Br J Ophthalmol*. 1995;79(11):1054–1055.
251. Dick AD, Meyer P, James T, et al. Campath-1H therapy in refractory ocular inflammatory disease. *Br J Ophthalmol*. 2000;84(1):107–109.
252. Wang J, Ibrahim M, Turkcuoglu P, et al. Intercellular adhesion molecule inhibitors as potential therapy for refractory uveitic macular edema. *Ocul Immunol Inflamm*. 2010;18(5):395–398.
253. Faia LJ, Sen HN, Li Z, Yeh S, Wroblewski KJ, Nussenblatt RB. Treatment of inflammatory macular edema with humanized anti-CD11a antibody therapy. *Invest Ophthalmol Vis Sci*. 2011;52(9):6919–6924.
254. Talamonti M, Teoli M, Botti E, Spallone G, Chimenti S, Costanzo A. Patients with moderate to severe plaque psoriasis: one year after the European Medicines Agency recommendation of efalizumab suspension. *Dermatology*. 2011;222(3):250–255.
255. Venkiteshwaran A. Tocilizumab. *MAbs*. 2009;1(5):432–438.
256. Romano M, Sironi M, Toniatti C, et al. Role of IL-6 and its soluble receptor in induction of chemokines and leukocyte recruitment. *Immunity*. 1997;6(3):315–325.
257. Simon D, Denniston AK, Tomlins PJ, et al. Soluble gp130, an antagonist of IL-6 transsignaling, is elevated in uveitis aqueous humor. *Invest Ophthalmol Vis Sci*. 2008;49(9):3988–3991.
258. Mesquida M, Leszczynska A, Llorenç V, Adán A. Interleukin-6 blockade in ocular inflammatory diseases. *Clin Exp Immunol*. 2014;176(3):301–309.
259. Tappeiner C, Heinz C, Ganser G, Heiligenhaus A. Is tocilizumab an effective option for treatment of refractory uveitis associated with juvenile idiopathic arthritis? *J Rheumatol*. 2012;39(6):1294–1295.
260. Hirano T, Ohguro N, Hohki S, et al. A case of Behçet's disease treated with a humanized anti-interleukin-6 receptor antibody, tocilizumab. *Mod Rheumatol*. 2012;22(2):298–302.
261. Oshitari T, Kajita F, Tobe A, et al. Refractory uveitis in patient with castleman disease successfully treated with tocilizumab. *Case Rep Ophthalmol Med*. 2012;2012:968180.
262. Adán A, Mesquida M, Llorenç V, et al. Tocilizumab treatment for refractory uveitis-related cystoid macular edema. *Graefes Arch Clin Exp Ophthalmol*. 2013;251(11):2627–2632.
263. Tsang AC, Roth J, Gottlieb C. Tocilizumab for severe chronic anterior uveitis associated with juvenile idiopathic arthritis in a pediatric patient. *Ocul Immunol Inflamm*. 2014;22(2):155–157.
264. Adán A, Llorenç V, Mesquida M, Pelegrín L. Tocilizumab treatment for recalcitrant uveitic macular edema. *Graefes Arch Clin Exp Ophthalmol*. 2013;251(9):2249–2250.
265. Muselier A, Bielefeld P, Bidot S, Vinit J, Besancenot JF, Bron A. Efficacy of tocilizumab in two patients with anti-TNF-alpha refractory uveitis. *Ocul Immunol Inflamm*. 2011;19(5):382–383.
266. Wendling D, Dernis E, Prati C, Frisch E, Delbosc B. Onset of inflammatory eye disease under tocilizumab treatment for rheumatologic conditions: a paradoxical effect? *J Rheumatol*. 2011;38(10):2284.
267. Sato T, Minakuchi S, Mochizuki M, Takeuchi M. Acute anterior uveitis after discontinuation of tocilizumab in a patient with rheumatoid arthritis. *Clin Ophthalmol*. 2014;8:187–190.
268. Sanofi. Phase II Study to Analyze Sarilumab in Non-Infectious Uveitis (SARILNIUSATURN). Available from: http://clinicaltrials.gov/ct2/show/NCT01900431. NLM identifier: NCT01900431. Accessed June 19, 2014.
269. Kolls JK, Lindén A. Interleukin-17 family members and inflammation. *Immunity*. 2004;21(4):467–476.
270. Xu S, Cao X. Interleukin-17 and its expanding biological functions. *Cell Mol Immunol*. 2010;7(3):164–174.
271. Fujino S, Andoh A, Bamba S, et al. Increased expression of interleukin 17 in inflammatory bowel disease. *Gut*. 2003;52(1):65–70.
272. Tzartos JS, Friese MA, Craner MJ, et al. Interleukin-17 production in central nervous system-infiltrating T-cells and glial cells is associated with active disease in multiple sclerosis. *Am J Pathol*. 2008;172(1):146–155.
273. Lubberts E, Koenders MI, van den Berg WB. The role of T-cell interleukin-17 in conducting destructive arthritis: lessons from animal models. *Arthritis Res Ther*. 2005;7(1):29–37.
274. Patel DD, Lee DM, Kolbinger F, Antoni C. Effect of IL-17A blockade with secukinumab in autoimmune diseases. *Ann Rheum Dis*. 2013;72(Suppl 2):ii116–ii123.
275. Zhou R, Horai R, Mattapallil MJ, Caspi RR. A new look at immune privilege of the eye: dual role for the vision-related molecule retinoic acid. *J Immunol*. 2011;187(8):4170–4177.
276. Zhou R, Horai R, Silver PB, et al. The living eye "disarms" uncommitted autoreactive T-cells by converting them to Foxp3(+) regulatory cells following local antigen recognition. *J Immunol*. 2012;188(4):1742–1750.
277. Hamzaoui K, Hamzaoui A, Guemira F, Bessioud M, Hamza M, Ayed K. Cytokine profile in Behçet's disease patients. Relationship with disease activity. *Scand J Rheumatol*. 2002;31(4):205–210.
278. Chi W, Zhu X, Yang P, et al. Upregulated IL-23 and IL-17 in Behçet patients with active uveitis. *Invest Ophthalmol Vis Sci*. 2008;49(7):3058–3064.
279. Amadi-Obi A, Yu CR, Liu X, et al. TH17 cells contribute to uveitis and scleritis and are expanded by IL-2 and inhibited by IL-27/STAT1. *Nat Med*. 2007;13(6):711–718.

280. Dick AD, Tugal-Tutkun I, Foster S, et al. Secukinumab in the treatment of noninfectious uveitis: results of three randomized, controlled clinical trials. *Ophthalmology*. 2013;120(4):777–787.
281. David OJ, Kovarik JM, Schmouder RL. Clinical pharmacokinetics of fingolimod. *Clin Pharmacokinet*. 2012;51(1):15–28.
282. Kurose S, Ikeda E, Tokiwa M, Hikita N, Mochizuki M. Effects of FTY720, a novel immunosuppressant, on experimental autoimmune uveoretinitis in rats. *Exp Eye Res*. 2000;70(1):7–15.
283. Commodaro AG, Peron JPS, Lopes CT, et al. Evaluation of experimental autoimmune uveitis in mice treated with FTY720. *Invest Ophthalmol Vis Sci*. 2010;51(5):2568–2574.
284. Raveney BJ, Copland DA, Nicholson LB, Dick AD. Fingolimod (FTY720) as an acute rescue therapy for intraocular inflammatory disease. *Arch Ophthalmol*. 2008;126(10):1390–1395.
285. Zarbin MA, Jampol LM, Jager RD, et al. Ophthalmic evaluations in clinical studies of fingolimod (FTY720) in multiple sclerosis. *Ophthalmology*. 2013;120(7):1432–1439.
286. Jain N, Bhatti MT. Fingolimod-associated macular edema: incidence, detection, and management. *Neurology*. 2012;78(9):672–680.
287. Lin P, Suhler EB, Rosenbaum JT. The future of uveitis treatment. *Ophthalmology*. 2014;121(1):365–376.
288. Trittibach P, Barker SE, Broderick CA, et al. Lentiviral-vector-mediated expression of murine IL-1 receptor antagonist or IL-10 reduces the severity of endotoxin-induced uveitis. *Gene Ther*. 2008; 15(22):1478–1488.
289. Tsai ML, Horng CT, Chen SL, Xiao X, Wang CH, Tsao YP. Suppression of experimental uveitis by a recombinant adeno-associated virus vector encoding interleukin-1 receptor antagonist. *Mol Vis*. 2009;15: 1542–1552.
290. Tian L, Yang P, Lei B, et al. AAV2-mediated subretinal gene transfer of hIFN-α attenuates experimental autoimmune uveoretinitis in mice. *PLoS One*. 2011;6(5):e19542.
291. Choi B, Hwang Y, Kwon HJ, et al. Tumor necrosis factor alpha small interfering RNA decreases herpes simplex virus-induced inflammation in a mouse model. *J Dermatol Sci*. 2008;52(2):87–97.
292. Hou Y, Xing L, Fu S, et al. Down-regulation of inducible co-stimulator (ICOS) by intravitreal injection of small interfering RNA (siRNA) plasmid suppresses ongoing experimental autoimmune uveoretinitis in rats. *Graefes Arch Clin Exp Ophthalmol*. 2009;247(6):755–765.
293. Iwata D, Kitamura M, Kitaichi N, et al. Prevention of experimental autoimmune uveoretinitis by blockade of osteopontin with small interfering RNA. *Exp Eye Res*. 2010;90(1):41–48.
294. He Y, Jia SB, Zhang W, Shi JM. New options for uveitis treatment. *Int J Ophthalmol*. 2013;6(5):702–707.

8.2 Association analysis of TGFBR3 gene with Behcet's disease and idiopathic intermediate uveitis in a Caucasian population (published data)

The investigation detailed in Chapter 6 was written as a scientific paper and accepted for publication in the British Journal of Ophthalmology.

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Association analysis of TGFBR3 gene with Behçet's disease and idiopathic intermediate uveitis in a Caucasian population

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ABSTRACT

Background Transforming growth factor β (TGFβ) is an important immunoregulatory cytokine in regulatory T cell (Treg) and Th17-mediated pathology, including uveitis due to Behçet's disease (BD). Of the three isoforms, TGFβ2 is found at highest levels in the aqueous humour of uninflamed eyes. TGFβ signals through a cell-surface receptor comprising three subunits (TGFBR1, 2 and 3). TGFBR3 is considered necessary for TGFβ2 signal transduction, but not for other isoforms. A polymorphism in TGFBR3 (rs1805110) has previously been identified in Han Chinese patients with BD. We investigated the frequency of this polymorphism in a Caucasian population with BD and idiopathic intermediate uveitis (IIU).

Methods The single-nucleotide polymorphism (SNP) rs1805110 in TGFBR3 was genotyped in 75 BD patients, 92 IIU disease controls and 85 disease-free controls. The association with both diseases was analysed using Fisher's exact test.

Results No significant difference in rs1805110 allele or genotype frequency was observed. A low frequency of the T allele was observed (5.88% control, 9.33% BD, 10.33% IIU) with the TT genotype absent in patients with BD and IIU (1.18% control, 0% BD and 0% IIU). Stratification analysis according to clinical features of BD did not associate with the tested SNP.

Conclusions RS1805110 is not associated with BD or IIU in Caucasian patients. The T allele frequency is consistent with that presented for Caucasian populations in the HapMap database ($p>0.05$). Our results differ from the previous analysis in Han Chinese patients ($p<0.0001$), however, the possibility of having a much smaller effect due to the low minority frequency cannot be excluded.

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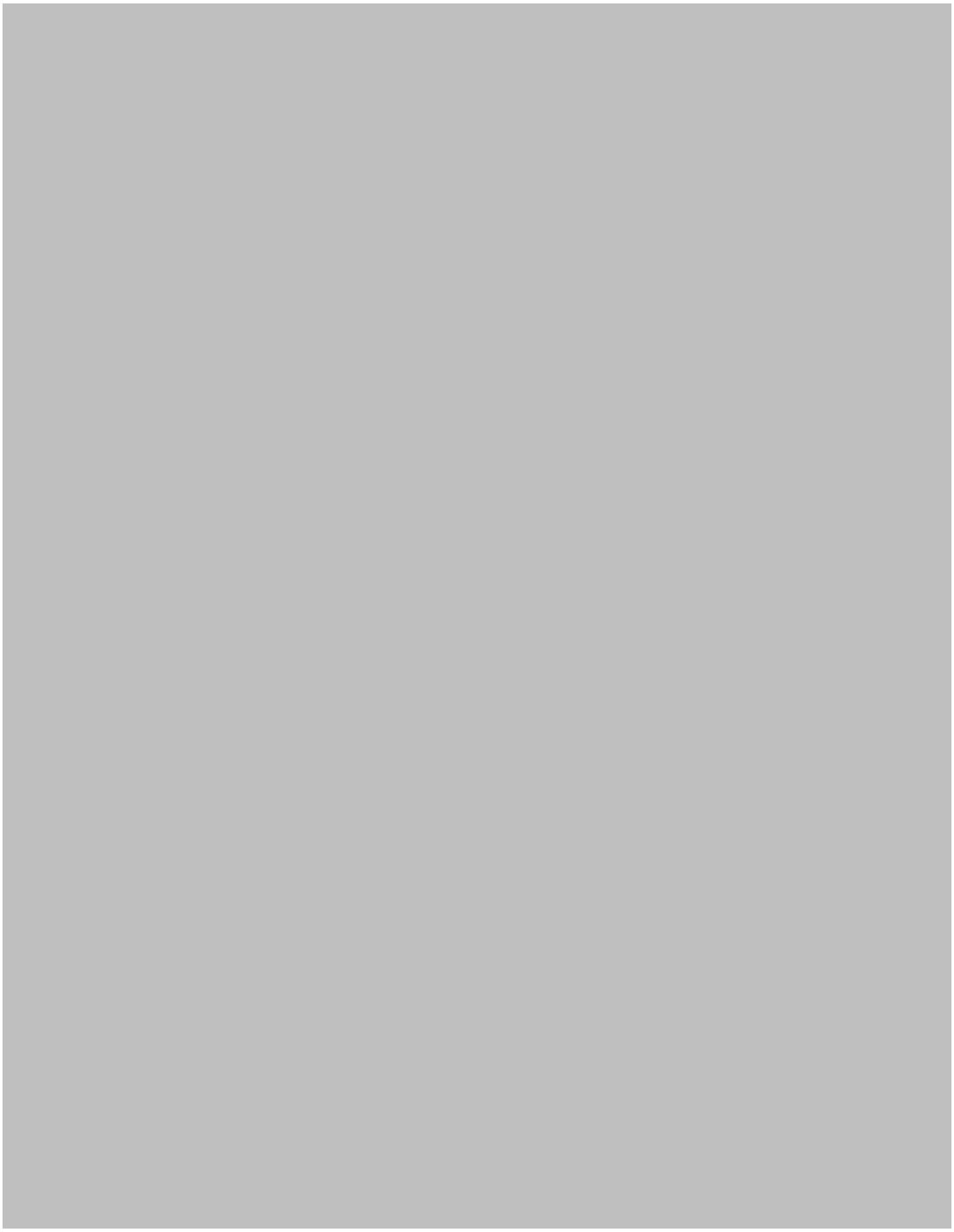

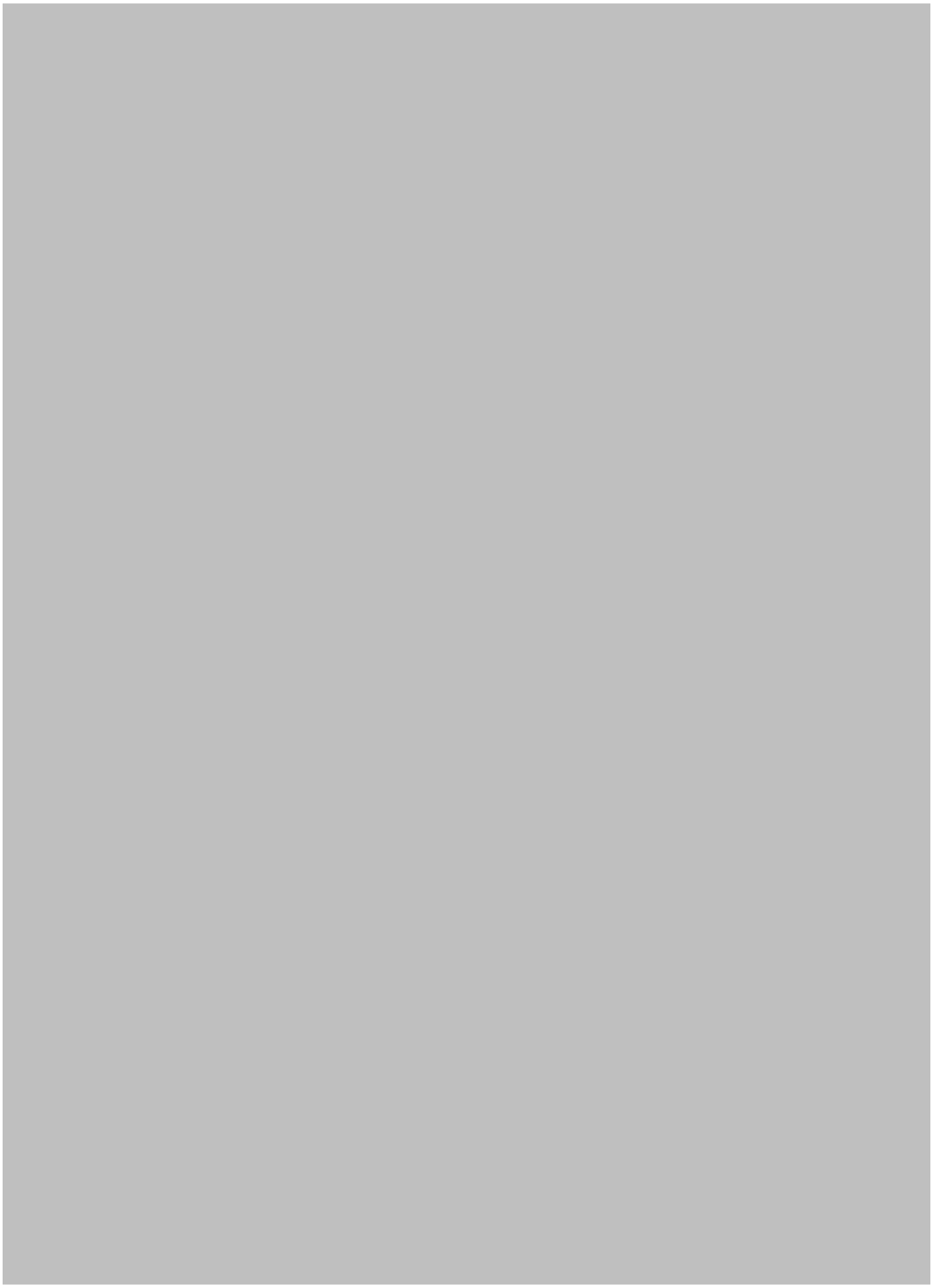

8.3 International poster presentations

Summaries of experimental data detailed within this thesis were presented as posters at the following international conferences:

1. Investigating the response of naïve T cells to the three isoforms of TGFβ. Robert J Barry, David R Withers, Philip I Murray, Peter J Lane, S John Curnow. Poster presentation at Association for Research in Vision and Ophthalmology (ARVO) Annual Scientific Meeting, May 2013, Seattle, Washington, USA.

   Summary of pilot data of *in vitro* assays as presented in chapter 5.

2. Association analysis of TGFBR3 gene with Behcet's Disease and Idiopathic Intermediate Uveitis in a Caucasian population. Robert J Barry, Jawaher A. AlSalem, Julia Faassen, S John Curnow, Philip I Murray, Graham R Wallace. Association for Research in Vision and Ophthalmology (ARVO) Annual Scientific Meeting, May 2014, Orlando, Florida, USA.

   Summary of investigation and data as presented in chapter 6.

3. Association analysis of TGFBR3 gene with Behcet's Disease and Idiopathic Intermediate Uveitis in a Caucasian population. Robert J Barry, Jawaher A. AlSalem, Julia Faassen, S John Curnow, Philip I Murray, Graham R Wallace. Poster presentation at International Society for Behcet's Disease (ISBD) Annual Meeting, September 2014, Paris, France.

   Summary of investigation and data as presented in chapter 6.

4. The TGFβ co-receptor betaglycan (TGFβRIII) is not implicated in TGFβ-dependent T cell responses. Robert J Barry, David R Withers, Philip I Murray, Peter J Lane, S John Curnow. Poster presentation at Association for Research in Vision and Ophthalmology (ARVO) Annual Scientific Meeting, May 2015, Denver, Colorado, USA.

   Summary of investigation and data as presented in chapters 3, 4 and 5.

9 REFERENCES

Acosta-Rodriguez, E. V et al., 2007. Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. *Nature Immunology*, 8(6), pp.639–46.

Akhurst, R.J. et al., 1990. TGF beta in murine morphogenetic processes: the early embryo and cardiogenesis. *Development (Cambridge, England)*, 108(4), pp.645–56.

Aleman-Muench, G.R. et al., 2012. Betaglycan (TβRIII) Is Expressed in the Thymus and Regulates T Cell Development by Protecting Thymocytes from Apoptosis. *PloS one*, 7(8), p.e44217.

Altman, D.G., 1991. *Practical Statistics for Medical Research* 1st ed., London: Chapman & Hall.

Amadi-Obi, A. et al., 2007. TH17 cells contribute to uveitis and scleritis and are expanded by IL-2 and inhibited by IL-27/STAT1. *Nature Medicine*, 13(6), pp.711–8.

Anderson, G., Lane, P.J.L. & Jenkinson, E.J., 2007. Generating intrathymic microenvironments to establish T-cell tolerance. *Nature Reviews. Immunology*, 7(12), pp.954–63.

Anderson, M.S. et al., 2002. Projection of an immunological self shadow within the thymus by the aire protein. *Science (New York, N.Y.)*, 298(5597), pp.1395–401.

Andres, J.L. et al., 1989. Membrane-anchored and soluble forms of betaglycan, a polymorphic proteoglycan that binds transforming growth factor-beta. *The Journal of Cell Biology*, 109(6 Pt 1), pp.3137–45.

Annes, J.P., Munger, J.S. & Rifkin, D.B., 2003. Making sense of latent TGFbeta activation. *Journal of Cell Science*, 116(2), pp.217–224.

Annunziato, F. et al., 2007. Phenotypic and functional features of human Th17 cells. *The Journal of Experimental Medicine*, 204(8), pp.1849–61.

Axenovich, T. et al., 2011. Linkage and association analyses of glaucoma related traits in a large pedigree from a Dutch genetically isolated population. *Journal of Medical Genetics*, 48(12), pp.802–9.

Axtell, R.C. et al., 2010. T helper type 1 and 17 cells determine efficacy of IFN-beta in multiple scelrosis and experimental encephalomyelitis. *Nature Medicine*, 16(4), pp.406–412.

Baaten, B.J.G. et al., 2010. CD44 regulates survival and memory development in Th1 cells. *Immunity*, 32(1), pp.104–15.

Babyatsky, M.W., Rossiter, G. & Podolsky, D.K., 1996. Expression of Transforming Growth Factors a and b in Colonic Mucosa in Inflammatory Bowel Disease. *Gastroenterology*, 110, pp.975–984.

Bandyopadhyay, A. et al., 1999. A Soluble Transforming Growth Factor β Type III Receptor Suppresses Tumorigenicity and Metastasis of Human Breast Cancer MDA-MB-231 Cells A Soluble Transforming Growth Factor ␢ Type III Receptor Suppresses Tumorigenicity and Metastasis of Human Breast Can. *Cancer Research*, 59, pp.5041–5046.

Baranathan, V. et al., 2007. The association of the PTPN22 620W polymorphism with Behcet's disease. *Annals of the Rheumatic Diseases*, 66(11), pp.1531–3.

Barisani-Asenbauer, T. et al., 2012. Uveitis- a rare disease often associated with systemic diseases and infections- a systematic review of 2619 patients. *Orphanet Journal of Rare Diseases*, 7, p.57.

Barry, R.J. et al., 2015. Association analysis of TGFBR3 gene with Behçet's disease and idiopathic intermediate uveitis in a Caucasian population. *British Journal of Ophthalmology*, 99(5), pp.696–9.

Barry, R.J. et al., 2014. Pharmacotherapy for uveitis : current management and emerging therapy. *Clinical Ophthalmology*, 8, pp.1891–1911.

Barry, R.J. et al., 2008. The Sjögren's Syndrome Damage Index--a damage index for use in clinical trials and observational studies in primary Sjögren's syndrome. *Rheumatology (Oxford, England)*, 47(8), pp.1193–8.

Berg, E.L. et al., 1991. The human peripheral lymph node vascular addressin is a ligand for LECAM-1, the peripheral lymph node homing receptor. *The Journal of Cell Biology*, 114(2), pp.343–9.

Bettelli, E. et al., 2006. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature*, 441(7090), pp.235–8.

Bilandzic, M. et al., 2013. Betaglycan Alters NFkB-TGFb2 Cross Talk to Reduce Survival of Human Granulosa Tumor Cells. *Molecular Endocrinology (Baltimore, Md.)*, 27(March), pp.466–479.

Bilandzic, M. et al., 2009. Loss of betaglycan contributes to the malignant properties of human granulosa tumor cells. *Molecular Endocrinology (Baltimore, Md.)*, 23(4), pp.539–48.

Bilandzic, M. & Stenvers, K.L., 2011. Betaglycan: a multifunctional accessory. *Molecular and Cellular Endocrinology*, 339(1-2), pp.180–9.

Billiau, A & Matthys, P., 2001. Modes of action of Freund's adjuvants in experimental models of autoimmune diseases. *Journal of Leukocyte Biology*, 70(6), pp.849–860.

Bleby, J., 1986. Animals (Scientific Procedures) Act 1986. *The Veterinary Record*, 119(1), p.22.

Bloch-Michel, E. & Nussenblatt, R.B., 1987. International Uveitis Study Group recommendations for the evaluation of intraocular inflammatory disease. *American Journal of Ophthalmology*, 103(2), pp.234–5.

Blumberg, R.S., Saubermann, L.J. & Strober, W., 1999. Animal models of mucosal inflammation and their relation to human inflammatory bowel disease. *Current Opinion in Immunology*, 11(6), pp.648–56.

Bodaghi, B. et al., 2001. Chronic severe uveitis: etiology and visual outcome in 927 patients from a single center. *Medicine*, 80(4), pp.263–70.

Bode, U., Tiedemann, K. & Westermann, J., 2004. CD4 + effector T cell distribution in vivo : TGF-beta 1 / TGF-beta receptor II interaction during activation mediates accumulation in the target tissue by preferential proliferation. *European Journal of Immunology*, 34, pp.1050–1058.

von Boehmer, H. & Nolting, J., 2008. What turns on Foxp3? *Nature Immunology*, 9(2), pp.121–122.

Bonewald, L.F., 1999. Regulation and regulatory activities of transforming growth factor beta. *Critical Reviews in Eukaryotic Gene Expression*, 9(1), pp.33–44.

Bradley, L.M., Watson, S.R. & Swain, S.L., 1994. Entry of naive CD4 T cells into peripheral lymph nodes requires L-selectin. *The Journal of Experimental Medicine*, 180(6), pp.2401–6.

Brewerton, D.A. et al., 1973. Acute anterior uveitis and HL-A 27. *Lancet*, 302(7836), pp.994–6.

Brunkow, M.E. et al., 2001. Disruption of a new forkhead / winged-helix protein , scurfin , results in the fatal lymphoproliferative disorder. *Nature Genetics*, 27(january), pp.68–73.

Cao, D. et al., 2012. CDKN2B polymorphism is associated with primary open-angle glaucoma (POAG) in the Afro-Caribbean population of Barbados, West Indies. *PloS One*, 7(6), p.e39278.

Caretto, D. et al., 2010. Cutting edge: the Th1 response inhibits the generation of peripheral regulatory T cells. *Journal of Immunology (Baltimore, Md. : 1950)*, 184(1), pp.30–4.

Caspi, R.R., 2003. Experimental autoimmune uveoretinitis in the rat and mouse. *Current Protocols in Immunology / edited by John E. Coligan ... [et al.]*, Chapter 15, p.Unit 15.6.

Caspi, R.R. et al., 2008. Mouse models of experimental autoimmune uveitis. *Ophthalmic Research*, 40(3-4), pp.169–174.

Cejalvo, T. et al., 2007. Bone morphogenetic protein-2/4 signalling pathway components are expressed in the human thymus and inhibit early T-cell development. *Immunology*, 121(1), pp.94–104.

Cervantes-Barragán, L. et al., 2009. TLR2 and TLR4 signaling shapes specific antibody responses to Salmonella typhi antigens. *European Journal of Immunology*, 39(1), pp.126–35.

Chang, H., 2002. Genetic Analysis of the Mammalian Transforming Growth Factor-beta Superfamily. *Endocrine Reviews*, 23(6), pp.787–823.

Chang, H.-C. et al., 2010. The transcription factor PU.1 is required for the development of IL-9-producing T cells and allergic inflammation. *Nature Immunology*, 11(6), pp.527–34.

Chang, J.C.C. et al., 1998. Adjuvant activity of incomplete Freund's adjuvant. *Advanced Drug Delivery Reviews*, 32(3), pp.173–186.

Chang, J.H.-M. & Wakefield, D., 2002. Uveitis: a global perspective. *Ocular Immunology and Inflammation*, 10(4), pp.263–79.

Chavakis, T., 2012. Leucocyte recruitment in inflammation and novel endogenous negative regulators thereof. *European Journal of Clinical Investigation*, 42(6), pp.686–691.

Cheifetz, S. et al., 1990. Distinct Transforming Growth Factor beta Receptor Subsets as Determinants of Cellular Responsiveness to Three TGF beta Isoforms. *The Journal of Biological Chemistry*, 265(33), pp.20533–20538.

Chen, C. et al., 1995. The site and stage of anti-DNA B-cell deletion. *Nature*, 373(6511), pp.252–5.

Chen, J. et al., 1993. Generation of normal lymphocyte populations by Rb-deficient embryonic stem cells. *Current Biology*, 3(7), pp.405–413.

Chen, L. & Flies, D.B., 2013. Molecular mechanisms of T cell co-stimulation and co-inhibition. *Nature reviews. Immunology*, 13(4), pp.227–42.

Chen, W. et al., 2003. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *The Journal of Experimental Medicine*, 198(12), pp.1875–86.

Chen, W. et al., 2001. Requirement for Transforming Growth Factor beta 1 in Controlling T Cell Apoptosis. *Journal of Experimental Medicine*, 194(4), pp.439–453.

Chen, X.-L. et al., 2014. IL-15 trans-presentation regulates homeostasis of CD4(+) T lymphocytes. *Cellular & Molecular Immunology*, (February), pp.387–395.

Chen, Y. et al., 2012. Association analysis of TGFBR3 gene with Vogt-Koyanagi-Harada disease and Behcet's disease in the Chinese Han population. *Current Eye Research*, 37(4), pp.312–7.

Chu, W. et al., 2011. TGFBR3, a potential negative regulator of TGF-β signaling, protects cardiac fibroblasts from hypoxia-induced apoptosis. *Journal of Cellular Physiology*, 226(10), pp.2586–94.

Chung, Y.M., Yeh, T.S. & Liu, J.H., 1988. Endogenous uveitis in Chinese--an analysis of 240 cases in a uveitis clinic. *Japanese Journal of Ophthalmology*, 32(1), pp.64–9.

Cimino, L. et al., 2010. The causes of uveitis in a referral centre of Northern Italy. *International Ophthalmology*, 30(5), pp.521–9.

Clark, E.A. & Ledbetter, J.A., 1986. Activation of human B cells mediated through two distinct cell surface differentiation antigens, Bp35 and Bp50. *Proceedings of the National Academy of Sciences of the United States of America*, 83(12), pp.4494–8.

Clement, L.T., 1992. Isoforms of the CD45 common leukocyte antigen family: markers for human T-cell differentiation. *Journal of Clinical Immunology*, 12(1), pp.1–10.

Compton, L. a et al., 2007. Coronary vessel development is dependent on the type III transforming growth factor beta receptor. *Circulation Research*, 101(8), pp.784–91.

Cooper, S.J. et al., 2010. Loss of type III transforming growth factor-beta receptor expression is due to methylation silencing of the transcription factor GATA3 in renal cell carcinoma. *Oncogene*, 29(20), pp.2905–15.

Cordero-Coma, M., Yilmaz, T. & Onal, S., 2013. Systematic review of anti-tumor necrosis factor-alpha therapy for treatment of immune-mediated uveitis. *Ocular Immunology and Inflammation*, 21(1), pp.19–27.

Corthay, A., 2009. How do Regulatory T Cells Work ? *Scandinavian Journal of Immunology*, 70, pp.326–336.

Cuppari, C. et al., 2014. Allergen immunotherapy, routes of administration and cytokine networks: an update. *Immunotherapy*, 6(6), pp.775–86.

Curnow, S.J. et al., 2004. Inhibition of T cell apoptosis in the aqueous humor of patients with uveitis by IL-6/soluble IL-6 receptor trans-signaling. *Journal of Immunology (Baltimore, Md. : 1950)*, 173(8), pp.5290–7.

Curnow, S.J. et al., 2005. Multiplex bead immunoassay analysis of aqueous humor reveals distinct cytokine profiles in uveitis. *Investigative Ophthalmology & Visual Science*, 46(11), pp.4251–9.

Dallas, S.L. et al., 2005. Preferential production of latent transforming growth factor beta-2 by primary prostatic epithelial cells and its activation by prostate-specific antigen. *Journal of Cellular Physiology*, 202(2), pp.361–70.

Dallas, S.L., Alliston, T. & Bonewald, L.F., 2008. Transforming Growth Factor- β. In J. P. Bilezikian, L. P. Raisz, & T. J. Martin, eds. *Principles of Bone Biology, 3rd Edition*. Elsevier Inc., pp. 1145–1166.

Daopin, S. et al., 1992. Crystal structure of transforming growth factor-beta 2: an unusual fold for the superfamily. *Science (New York, N.Y.)*, 257(5068), pp.369–73.

Dardalhon, V. et al., 2008. Interleukin 4 inhibits TGFb-induced-FoxP3+ T cells and generates, in combination with TGFb, FoxP3-effector T cells that produce interleukins 9 and 10. *Nature Immunology*, 9(12), pp.1347–1355.

Dardalhon, V. et al., 2008. Role of Th1 and Th17 cells in organ-specific autoimmunity. *Journal of Autoimmmunity*, 31, pp.252–256.

Darrell, R.W., Wagener, H.P. & Kurland, L.T., 1962. Epidemiology of uveitis. Incidence and prevalence in a small urban community. *Archives of Ophthalmology*, 68, pp.502–14.

Delisle, J. et al., 2013. The TGF-b-Smad3 pathway inhibits CD28-dependent cell growth and proliferation of CD4 T cells. *Genes and Immunity*, 14(2), pp.115–126.

Denniston, A.K. et al., 2011. Endogenous cortisol and TGF-beta in human aqueous humor contribute to ocular immune privilege by regulating dendritic cell function. *Journal of Immunology (Baltimore, Md. : 1950)*, 186(1), pp.305–11.

Denniston, A.K. & Dick, A.D., 2013. Systemic therapies for inflammatory eye disease: past, present and future. *BMC Ophthalmology*, 13, p.18.

Dhein, J. et al., 1995. Autocrine T-cell suicide mediated by APO-1/(Fas/CD95). *Nature*, 373(6513), pp.438–41.

Dickson, M.C. et al., 1995. Defective haematopoiesis and vasculogenesis in transforming growth factor-beta 1 knock out mice. *Development (Cambridge, England)*, 121(6), pp.1845–54.

Dignass, A.U. & Podolsky, D.K., 1993. Cytokine modulation of intestinal epithelial cell restitution: central role of transforming growth factor beta. *Gastroenterology*, 105(5), pp.1323–32.

ten Dijke, P. et al., 1988. Identification of another member of the transforming growth factor type beta gene family. *Proceedings of the National Academy of Sciences of the United States of America*, 85(13), pp.4715–9.

Dong, C., 2008. TH17 cells in development: an updated view of their molecular identity and genetic programming. *Nature Reviews. Immunology*, 8(5), pp.337–348.

Durrani, K. et al., 2011. Systemic therapy with conventional and novel immunomodulatory agents for ocular inflammatory disease. *Survey of Ophthalmology*, 56(6), pp.474–510.

Elgueta, R. et al., 2009. Molecular mechanism and function of CD40/CD40L engagement in the immune system. *Immunological Reviews*, 229(1), pp.152–72.

Ellis, E.F. & Henney, C.S., 1969. Adverse reactions following administration of human gamma globulin. *The Journal of Allergy*, 43(1), pp.45–54.

Engstrom, R.E. et al., 1994. The progressive outer retinal necrosis syndrome. A variant of necrotizing herpetic retinopathy in patients with AIDS. *Ophthalmology*, 101(9), pp.1488–502.

Escalona, R.M. et al., 2009. Reducing betaglycan expression by RNA interference (RNAi) attenuates inhibin bioactivity in LbetaT2 gonadotropes. *Molecular and Cellular Endocrinology*, 307(1-2), pp.149–56.

De Fanis, U. et al., 2007. GATA3 up-regulation associated with surface expression of CD294/CRTH2: a unique feature of human Th cells. *Blood*, 109(10), pp.4343–50.

Fantini, M.C. et al., 2006. Transforming growth factor beta induced FoxP3+ regulatory T cells suppress Th1 mediated experimental colitis. *Gut*, 55(5), pp.671–80.

Feng, X.-H. & Derynck, R., 2005. Specificity and versatility in tgf-beta signaling through Smads. *Annual Review of Cell and Developmental Biology*, 21, pp.659–93.

Fields, P., Fitch, F.W. & Gajewski, T.F., 1996. Control of T lymphocyte signal transduction through clonal anergy. *Journal of Molecular Medicine (Berlin, Germany)*, 74(11), pp.673–83.

Fietta, P. & Delsante, G., 2009. The effector T helper cell triade. *Rivista di Biologia*, 102(1), pp.61–74.

Finger, E.C. et al., 2008. TbetaRIII suppresses non-small cell lung cancer invasiveness and tumorigenicity. *Carcinogenesis*, 29(3), pp.528–35.

Forrester, J. V et al., 2008. Immune privilege or privileged immunity? *Mucosal Immunology*, 1(5), pp.372–81.

Forrester, J. V, 1991. Uveitis: pathogenesis. *Lancet*, 338(8781), pp.1498–501.

Förster, R. et al., 1999. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell*, 99(1), pp.23–33.

Gallego-Pinazo, R. et al., 2013. Update on the principles and novel local and systemic therapies for the treatment of non-infectious uveitis. *Inflammation & Allergy Drug Targets*, 12(1), pp.38–45.

Gasparin, F. et al., 2012. Experimental models of autoimmune inflammatory ocular diseases. *Arquivos Brasileiros de Oftalmologia*, 75(2), pp.143–7.

Gatza, C.E., Holtzhausen, A., et al., 2011. Type III TGF-β Receptor Enhances Colon Cancer Cell Migration and. *Neoplasia*, 13(8), pp.758–770.

Gatza, C.E., Oh, S.Y. & Blobe, G.C., 2011. Roles for the Type III TGF-β Receptor in Human Cancer. *Cellular Signalling*, 22(8), pp.1163–1174.

Gay, D. et al., 1993. Receptor editing: an approach by autoreactive B cells to escape tolerance. *The Journal of Experimental Medicine*, 177(4), pp.999–1008.

Geginat, J. et al., 2013. The CD4-centered universe of human T cell subsets. *Seminars in Immunology*, 25(4), pp.252–62.

Geiser, A.G. et al., 1993. Transforming growth factor beta 1 (TGF-beta 1) controls expression of major histocompatibility genes in the postnatal mouse: aberrant histocompatibility antigen expression in the pathogenesis of the TGF-beta 1 null mouse phenotype. *Proceedings of the National Academy of Sciences of the United States of America*, 90(21), pp.9944–9948.

Glick, B., Chang, T.S. & Jaap, R.G., 1956. The Bursa of Fabricius and Antibody Production. *Poultry Science*, 35(1), pp.224–225.

Glister, C., Satchell, L. & Knight, P.G., 2010. Changes in expression of bone morphogenetic proteins (BMPs), their receptors and inhibin co-receptor betaglycan during bovine antral follicle development: inhibin can antagonize the suppressive effect of BMPs on thecal androgen production. *Reproduction (Cambridge, England)*, 140(5), pp.699–712.

Gocho, K., Kondo, I. & Yamaki, K., 2001. Identification of Autoreactive T Cells in Vogt-Koyanagi-Harada Disease. *Investigative Ophthalmology & Visual Science*, 42(9), pp.2004–2009.

Goldstein, H., 1980. The reported demography and causes of blindness throughout the world. *Advances in Ophthalmology*, 40, pp.1–99.

Gordon, K.J. et al., 2008. Loss of type III transforming growth factor beta receptor expression increases motility and invasiveness associated with epithelial to mesenchymal transition during pancreatic cancer progression. *Carcinogenesis*, 29(2), pp.252–62.

Gorelik, L. & Flavell, R. a, 2000. Abrogation of TGFbeta signaling in T cells leads to spontaneous T cell differentiation and autoimmune disease. *Immunity*, 12(2), pp.171–81.

Goverman, J. et al., 1993. Transgenic Mice That Express a Myelin Basic Protein-Specific T Cell Receptor Develop Spontaneous Autoimmunity. *Cell*, 72, pp.551–560.

Green, D.R., Droin, N. & Pinkoski, M., 2003. Activation-induced cell death in T cells. *Immunological Reviews*, 193, pp.70–81.

Griffith, T.S. & Ferguson, T.A., 2011. Cell death in the maintenance and abrogation of tolerance: the five Ws of dying cells. *Immunity*, 35(4), pp.456–66.

Gritz, D.C. & Wong, I.G., 2004. Incidence and prevalence of uveitis in Northern California; the Northern California Epidemiology of Uveitis Study. *Ophthalmology*, 111(3), pp.491–500; discussion 500.

De Groot-Mijnes, J.D.F. et al., 2006. Polymerase chain reaction and Goldmann-Witmer coefficient analysis are complimentary for the diagnosis of infectious uveitis. *American Journal of Ophthalmology*, 141(2), pp.313–8.

Gu, A.-D. et al., 2012. Requirements of transcription factor Smad-dependent and -independent TGF-β signaling to control discrete T-cell functions. *Proceedings of the National Academy of Sciences of the United States of America*, 109(3), pp.905–10.

Guerau-de-Arellano, M. et al., 2009. Neonatal tolerance revisited: a perinatal window for Aire control of autoimmunity. *The Journal of Experimental Medicine*, 206(6), pp.1245–52.

Guly, C.M. & Forrester, J. V, 2010. Investigation and management of uveitis. *British Medical Journal*, 341, p.c4976.

Gunn, M.D. et al., 1998. A chemokine expressed in lymphoid high endothelial venules promotes the adhesion and chemotaxis of naive T lymphocytes. *Proceedings of the National Academy of Sciences of the United States of America*, 95(1), pp.258–63.

Gutcher, I. et al., 2011. Autocrine transforming growth factor-β1 promotes in vivo Th17 cell differentiation. *Immunity*, 34(3), pp.396–408.

Haddad, J.J., Saadé, N.E. & Safieh-Garabedian, B., 2002. Cytokines and neuro-immune-endocrine interactions: a role for the hypothalamic-pituitary-adrenal revolving axis. *Journal of Neuroimmunology*, 133(1-2), pp.1–19.

Handunnetthi, L. et al., 2010. Regulation of major histocompatibility complex class II gene expression, genetic variation and disease. *Genes and Immunity*, 11(2), pp.99–112.

Hanks, B. & Holtzhausen, A., 2013. Type III TGF-β receptor downregulation generates an immunotolerant tumor microenvironment. *The Journal of Clinical Investigation*, 123(9), pp.3925–40.

Harris, D.P. et al., 2000. Reciprocal regulation of polarized cytokine production by effector B and T cells. *Nature Immunology*, 1(6), pp.475–82.

Hauser, S.L. & Oksenberg, J.R., 2006. The neurobiology of multiple sclerosis: genes, inflammation, and neurodegeneration. *Neuron*, 52(1), pp.61–76.

Healy, J.I. et al., 1997. Different nuclear signals are activated by the B cell receptor during positive versus negative signaling. *Immunity*, 6(4), pp.419–28.

Heiligenhaus, A. et al., 2013. Review for disease of the year: epidemiology of juvenile idiopathic arthritis and its associated uveitis: the probable risk factors. *Ocular Immunology and Inflammation*, 21(3), pp.180–91.

Hermiston, M.L., Xu, Z. & Weiss, A., 2003. CD45: a critical regulator of signaling thresholds in immune cells. *Annual Review of Immunology*, 21, pp.107–37.

Hersh, C.P. et al., 2009. Transforming Growth Factor- b Receptor-3 Is Associated with Pulmonary Emphysema. *American Journal of Respiratory Cell and Molecular Biology*, 41(7), pp.324–331.

Hinck, A.P., 2012. Structural studies of the TGF-βs and their receptors - insights into evolution of the TGF-β superfamily. *FEBS Letters*, 586(14), pp.1860–70.

Hjelm, F. et al., 2006. Antibody-mediated regulation of the immune response. *Scandinavian journal of Immunology*, 64(3), pp.177–84.

Holland, G.N., 1994. Standard diagnostic criteria for the acute retinal necrosis syndrome. Executive Committee of the American Uveitis Society. *American Journal of Ophthalmology*, 117(5), pp.663–7.

Huseby, E.S. et al., 2005. How the T Cell Repertoire Becomes Peptide and MHC Specifc. *Cell*, 122, pp.247–260.

Hwang, D.-K. et al., 2012. Epidemiology of uveitis among the Chinese population in Taiwan: a population-based study. *Ophthalmology*, 119(11), pp.2371–6.

Ishigame, H. et al., 2013. Excessive Th1 responses due to the absence of TGF-β signaling cause autoimmune diabetes and dysregulated Treg cell homeostasis. *Proceedings of the National Academy of Sciences of the United States of America*, 110(17), pp.6961–6.

Isibasi, A. et al., 1992. Active protection of mice against Salmonella typhi by immunization with strain-specific porins. *Vaccine*, 10(12), pp.811–3.

Issazadeh, S. et al., 1996. Cytokines in relapsing experimental autoimmune encephalomyelitis in DA rats: persistent mRNA expression of proinflammatory cytokines and absent expression of interleukin-10 and transforming growth factor-beta. *Journal of Neuroimmunology*, 69(1-2), pp.103–15.

Jabbs, D.A, Nussenblatt, R.B. & Rosenbaum, J.T., 2005. Standardization of Uveitis Nomenclature for Reporting Clinical Data. Results of the First International Workshop. *American Journal of Ophthalmology*, 140, pp.509–516.

Jabs, D.A & Busingye, J., 2013. Approach to the diagnosis of the uveitides. *American Journal of Ophthalmology*, 156(2), pp.228–36.

Jabs, D.A. et al., 2000. Guidelines for the Use of Immunosuppressive Drugs in Patients with Ocular Inflammatory Disorders: Recommendations of an expert panel. *American Journal of Ophthalmology*, 130, pp.492–513.

Janeway, C.A. et al., 2001. *Immunobiology: The immune system in health and disease* 5th ed., New York: Garland Publishing.

Jellison, E.R., Kim, S. & Welsh, R.M., 2005. Cutting Edge: MHC Class II-Restricted Killing In Vivo during Viral Infection. *The Journal of Immunology*, 174, pp.614–618.

Jenkins, M.K. et al., 2001. In vivo activation of antigen-specific CD4 T cells. *Annual Review of Immunology*, 19, pp.23–45.

Kaartinen, V. et al., 1995. Abnormal lung development and cleft palate in mice lacking TGF-beta 3 indicates defects of epithelial-mesenchymal interaction. *Nature Genetics*, 11, pp.415–421.

Kang, J.S., Liu, C. & Derynck, R., 2009. New regulatory mechanisms of TGF-beta receptor function. *Trends in Cell Biology*, 19(8), pp.385–94.

Kato, T. & Nariuchi, H., 2000. Polarization of Naive CD4 + T Cells Toward the Th1 Subset by CTLA-4 Costimulation. *Journal of Immunology*, 164, pp.3554–3562.

Kehrl, B.Y.J.H. et al., 1986. Production of Transforming Growth Factor Beta by Human T Lymphocytes and its Potential Role in the Regulation of T Cell Growth. *Journal of Experimental Medicine*, 163(May), pp.1037–1050.

Khalil, N., 1999. TGF-beta: from latent to active. *Microbes and Infection / Institut Pasteur*, 1(15), pp.1255–63.

Khor, C.C. et al., 2011. Genome-wide association studies in Asians confirm the involvement of ATOH7 and TGFBR3, and further identify CARD10 as a novel locus influencing optic disc area. *Human Molecular Genetics*, 20(9), pp.1864–72.

Kim, J.-H. et al., 2011. TGFBR3 polymorphisms and its haplotypes associated with chronic hepatitis B virus infection and age of hepatocellular carcinoma occurrence. *Digestive Diseases (Basel, Switzerland)*, 29(3), pp.278–83.

Kobayashi, T. et al., 2008. IL23 differentially regulates the Th1/Th17 balance in ulcerative colitis and Crohn's disease. *Gut*, 57(12), pp.1682–9.

Kondaiah, P. et al., 1990. Identification of a Novel Transforming Growth Factor-betal (TGF-beta 5) mRNA in Xenopus laevis. *The Journal of Biological Chemistry*, 265, pp.1089–1093.

Korn, T., Bettelli, E., et al., 2007. IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. *Nature*, 448(7152), pp.484–7.

Korn, T., Oukka, M., et al., 2007. Th17 cells: effector T cells with inflammatory properties. *Seminars in Immunology*, 19(6), pp.362–71.

Kotake, S. et al., 1996. Characteristics of endogenous uveitis in Hokkaido, Japan. *Graefe's Archive for Clinical and Experimental Ophthalmology*, 234(10), pp.599–603.

Kulkarni, A.B. et al., 1993. Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death. *Proceedings of the National Academy of Sciences of the United States of America*, 90(2), pp.770–4.

Kyewski, B. & Klein, L., 2006. A central role for central tolerance. *Annual Review of Immunology*, 24, pp.571–606.

Lambert, K.E. et al., 2011. The type III transforming growth factor-β receptor inhibits proliferation, migration, and adhesion in human myeloma cells. *Molecular Biology of the Cell*, 22(9), pp.1463–72.

Lantelme, E. et al., 2001. Kinetics of GATA-3 gene expression in early polarizing and committed human T cells. *Immunology*, 102(2), pp.123–30.

Larson, T., Nusseblatt, R.B. & Sen, H.N., 2012. Emerging Drugs for Uveitis. *Expert Opinion on Emerging Drugs*, 16(2), pp.309–322.

Larsson, J. et al., 2001. Abnormal angiogenesis but intact hematopoietic potential in TGF-b type I receptor-deficient mice. *The EMBO Journal*, 20(7), pp.1663–1673.

Lebien, T.W. & Tedder, T.F., 2008. ASH 50th anniversary review B lymphocytes : how they develop and function. *Blood*, 112(5), pp.1570–1580.

Lee, J.D. et al., 2010. The type III TGF-beta receptor suppresses breast cancer progression through GIPC-mediated inhibition of TGF-beta signaling. *Carcinogenesis*, 31(2), pp.175–83.

Lee, Y. et al., 2012. Induction and molecular signature of pathogenic T H 17 cells. *Nature Immunology*, 13(10), pp.991–999.

Lee, Y.J. et al., 1997. TGF-beta suppresses IFN-gamma induction of class II MHC gene expression by inhibiting class II transactivator messenger RNA expression. *Journal of Immunology (Baltimore, Md. : 1950)*, 158(5), pp.2065–75.

Lewis, K. a et al., 2000. Betaglycan binds inhibin and can mediate functional antagonism of activin signalling. *Nature*, 404(6776), pp.411–4.

Ley, K. et al., 2007. Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nature Reviews. Immunology*, 7(9), pp.678–689.

Li, L. et al., 2006. A pathway regulated by cell cycle inhibitor p27Kip1 and checkpoint inhibitor Smad3 is involved in the induction of T cell tolerance. *Nature Immunology*, 7(11), pp.1157–65.

Li, M.O. et al., 2006. Transforming growth factor-beta regulation of immune responses. *Annual Review of Immunology*, 24, pp.99–146.

Li, M.O. & Flavell, R. a, 2008. Contextual regulation of inflammation: a duet by transforming growth factor-beta and interleukin-10. *Immunity*, 28(4), pp.468–76.

Li, M.O., Sanjabi, S. & Flavell, R.A., 2006. Transforming Growth Factor-β Controls Development, Homeostasis, and Tolerance of T Cells by Regulatory T Cell-Dependent and -Independent Mechanisms. *Immunity*, 25(3), pp.455–471.

Li, M.O., Wan, Y.Y. & Flavell, R. a., 2007. T Cell-Produced Transforming Growth Factor-β1 Controls T Cell Tolerance and Regulates Th1- and Th17-Cell Differentiation. *Immunity*, 26(5), pp.579–591.

Li, X. & Zheng, Y., 2015. Regulatory T cell identity: formation and maintenance. *Trends in Immunology*, 36(6), pp.344–353.

Lin, S.J. et al., 2006. The structural basis of TGF-beta, bone morphogenetic protein, and activin ligand binding. *Reproduction (Cambridge, England)*, 132(2), pp.179–90.

Liu, X.-L. et al., 2013. Dual role of TGFBR3 in bladder cancer. *Oncology Reports*, 30(3), pp.1301–8.

London, N.J.S., Rathinam, S.R. & Cunningham, E.T., 2010. The epidemiology of uveitis in developing countries. *International Ophthalmology Clinics*, 50(2), pp.1–17.

Looyenga, B.D. et al., 2010. Inhibin-A antagonizes TGFbeta2 signaling by down-regulating cell surface expression of the TGFbeta coreceptor betaglycan. *Molecular Endocrinology (Baltimore, Md.)*, 24(3), pp.608–20.

López-Casillas, F. et al., 1994. Betaglycan can act as a dual modulator of TGF-beta access to signaling receptors: mapping of ligand binding and GAG attachment sites. *The Journal of Cell Biology*, 124(4), pp.557–68.

López-Casillas, F. et al., 1991. Structure and expression of the membrane proteoglycan betaglycan, a component of the TGF-beta receptor system. *Cell*, 67(4), pp.785–95.

López-Casillas, F., Wrana, J.L. & Massagué, J., 1993. Betaglycan presents ligand to the TGF beta signaling receptor. *Cell*, 73(7), pp.1435–44.

Lord, G.M. et al., 2005. T-bet is required for optimal proinflammatory CD4+ T-cell trafficking. *Blood*, 106(10), pp.3432–9.

Lukacs, N.W., 2000. Migration of helper T-lymphocyte subsets into inflamed tissues. *The Journal of Allergy and Clinical Immunology*, 106(5 Suppl), pp.S264–S269.

Lukić, M.L., Cowing, C. & Leskowitz, S., 1975. Strain differences in ease of tolerance induction to bovine gamma-globulin: dependence on macrophage function. *Journal of Immunology (Baltimore, Md. : 1950)*, 114(1 Pt 2), pp.503–6.

Ma, H.-L. et al., 2008. IL-22 is required for Th17 cell-mediated pathology in a mouse model of psoriasis-like skin inflammation. *The Journal of Clinical Investigation*, 118(2), pp.597–607.

Mandelcorn, E.D., 2013. Infectious causes of posterior uveitis. *Canadian Journal of Ophthalmology*, 48(1), pp.31–9.

Mandeville, J.T., Levinson, R.D. & Holland, G.N., The tubulointerstitial nephritis and uveitis syndrome. *Survey of Ophthalmology*, 46(3), pp.195–208.

Mangan, P.R. et al., 2006. Transforming growth factor-beta induces development of the T(H)17 lineage. *Nature*, 441(7090), pp.231–4.

Marra, F. et al., 1996. Characterization and regulation of the latent transforming growth factor-beta complex secreted by vascular pericytes. *Journal of Cellular Physiology*, 166(3), pp.537–46.

Marrack, P. et al., 2011. T cell receptor specificity for major histocompatibility complex proteins. *Current Opinion in Immunology*, 20(2), pp.203–207.

Massague, J., 1992. Receptors for the TGFbeta Family. *Cell*, 69, pp.1067–1070.

Massagué, J., 1998. TGF-beta signal transduction. *Annual Review of Biochemistry*, 67, pp.753–91.

Massagué, J., 2008. TGFbeta in Cancer. *Cell*, 134(2), pp.215–30.

Massagué, J., 1990. The transforming growth factor-beta family. *Annual Review of Cell Biology*, 6, pp.597–641.

Massagué, J. & Chen, Y., 2000. Controlling TGF-β signaling. *Genes & Development*, 14, pp.627–644.

Masters, J.R., 2000. Human cancer cell lines: fact and fantasy. *Nature Reviews. Molecular Cell Biology*, 1(3), pp.233–6.

Mauri, C. & Bosma, A., 2012. Immune Regulatory Function of B Cells. *Annual Review of Immunology*, 10(30), pp.221–41.

McCannel, C.A. et al., 1996. Causes of uveitis in the general practice of ophthalmology. UCLA Community-Based Uveitis Study Group. *American Journal of Ophthalmology*, 121(1), pp.35–46.

McEver, R.P. & Zhu, C., 2010. Rolling cell adhesion. *Annual Review of Cell Biology*, 26, pp.363–396.

McGeachy, M.J. & Cua, D.J., 2008. Th17 cell differentiation: the long and winding road. *Immunity*, 28(4), pp.445–53.

Meister, M. et al., 2015. Dickkopf-3, a Tissue-Derived Modulator of Local T-Cell Responses. *Frontiers in Immunology*, 6(February), pp.1–13.

Mendes, D. et al., 2009. Behçet's disease--a contemporary review. *Journal of Autoimmunity*, 32(3-4), pp.178–88.

Millan, F. a et al., 1991. Embryonic gene expression patterns of TGF beta 1, beta 2 and beta 3 suggest different developmental functions in vivo. *Development (Cambridge, England)*, 111(1), pp.131–43.

Miller, J.F., 1961. Immunological function of the thymus. *Lancet (London, England)*, 2(7205), pp.748–9.

Miller, J.F.A.P., 1962. Effect of Neonatal Thymectomy on the Immunological Responsiveness of the Mouse. *Proceedings of the Royal Society B: Biological Sciences*, 156(964), pp.415–428.

Miserocchi, E. et al., 2013. Review on the worldwide epidemiology of uveitis. *European Journal of Ophthalmology*, 23(5), pp.705–17.

Mitchell, D.M. & Williams, M. a, 2010. An activation marker finds a function. *Immunity*, 32(1), pp.9–11.

Mizuki, N. et al., 2010. Genome-wide association studies identify IL23R-IL12RB2 and IL10 as Behçet's disease susceptibility loci. *Nature Genetics*, 42(8), pp.703–6.

Moon, J.J. et al., 2007. Naive CD4+ T cell frequency varies for different epitopes and predicts repertoire diversity and response magnitude. *Immunity*, 27(2), pp.612–625.

Mottet, C., Uhlig, H.H. & Powrie, F., 2003. Cutting edge: cure of colitis by CD4+CD25+ regulatory T cells. *Journal of Immunology (Baltimore, Md. : 1950)*, 170(8), pp.3939–43.

Na, S.Y. et al., 2013. Up-regulation of Th17 and related cytokines in Behçet's disease corresponding to disease activity. *Clinical and Experimental Rheumatology*, 31(3 Suppl 77), pp.32–40.

Nakamura, R.E.I. et al., 2007. Identification of two novel activities of the Wnt signaling regulator Dickkopf 3 and characterization of its expression in the mouse retina. *BMC Cell Biology*, 8, p.52.

Nakayamada, S. et al., 2012. Helper T cell diversity and plasticity. *Current Opinion in Immunology*, 24(3), pp.297–302.

Nemazee, D. & Buerki, K., 1989. Clonal deletion of autoreactive B lymphocytes in bone marrow chimeras. *Proceedings of the National Academy of Sciences of the United States of America*, 86(20), pp.8039–43.

Nguyen, T. & Russell, J., 2001. The regulation of FasL expression during activation-induced cell death (AICD). *Immunology*, 103(4), pp.426–34.

Nian, H. et al., 2012. Characterization of autoreactive and bystander IL-17 + T cells induced in immunized C57BL/6 mice. *Investigative Ophthalmology and Visual Science*, 53(2), pp.897–905.

Niehrs, C., 2006. Function and biological roles of the Dickkopf family of Wnt modulators. *Oncogene*, 25(57), pp.7469–81.

Nossal, G.J. & Pike, B.L., 1980. Clonal anergy: persistence in tolerant mice of antigen-binding B lymphocytes incapable of responding to antigen or mitogen. *Proceedings of the National Academy of Sciences of the United States of America*, 77(3), pp.1602–6.

Nurieva, R. et al., 2007. Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. *Nature*, 448(7152), pp.480–3.

O'Connor, R.A., Malpass, K.H. & Anderton, S.M., 2007. The Inflamed Central Nervous System Drives the Activation and Rapid Proliferation of Foxp3+ Regulatory T Cells. *Journal of Immunology*, 179, pp.958–966.

Ohguro, N. et al., 2012. The 2009 prospective multi-center epidemiologic survey of uveitis in Japan. *Japanese Journal of Ophthalmology*, 56(5), pp.432–5.

Ohkura, N., Kitagawa, Y. & Sakaguchi, S., 2013. Development and maintenance of regulatory T cells. *Immunity*, 38(3), pp.414–23.

Olsen, T. et al., 2011. TH1 and TH17 interactions in untreated inflamed mucosa of inflammatory bowel disease, and their potential to mediate the inflammation. *Cytokine*, 56(3), pp.633–40.

Oshima, M., Oshima, H. & Taketo, M.M., 1996. TGF-beta receptor type II deficiency results in defects of yolk sac hematopoiesis and vasculogenesis. *Developmental Biology*, 179(1), pp.297–302.

Ouyang, W. et al., 2010. Transforming growth factor-beta signaling curbs thymic negative selection promoting regulatory T cell development. *Immunity*, 32(5), pp.642–53.

Pakula, R. et al., 2007. Syndecan-1/CD147 association is essential for cyclophilin B-induced activation of p44/42 mitogen-activated protein kinases and promotion of cell adhesion and chemotaxis. *Glycobiology*, 17(5), pp.492–503.

Pal, A., Huang, W. & Toy, K.A., 2012. CCN6 Knockdown Disrupts Acinar Organization of Breast Cells in Three-dimensional Cultures through Up-regulation of Type III TGFb Receptor. *Neoplasia*, 14(11), pp.1067–1074.

Pamer, E.G., 2004. Immune responses to Listeria monocytogenes. *Nature Reviews. Immunology*, 4(10), pp.812–823.

Panek, R.B., Lee, Y.J. & Benveniste, E.N., 1995. TGF-beta suppression of IFN-gamma-induced class II MHC gene expression does not involve inhibition of phosphorylation of JAK1, JAK2, or signal transducers and activators of transcription, or modification of IFN-gamma enhanced factor X expression. *Journal of Immunology (Baltimore, Md. : 1950)*, 154(2), pp.610–9.

Parham, P., 2013. Putting a Face to MHC Restriction. *Journal of Immunology*, 174, pp.3–5.

Van Parijs, L. & Abbas, A.K., 1998. Homeostasis and self-tolerance in the immune system: turning lymphocytes off. *Science (New York, N.Y.)*, 280(5361), pp.243–8.

Pato, E. et al., 2011. Systematic review on the effectiveness of immunosuppressants and biological therapies in the treatment of autoimmune posterior uveitis. *Seminars in Arthritis and Rheumatism*, 40(4), pp.314–23.

Pelton, R.W. et al., 1989. Expression of transforming growth factor beta 2 RNA during murine embryogenesis. *Development (Cambridge, England)*, 106(4), pp.759–67.

Pepper, M. et al., 2010. Different routes of bacterial infection induce long-lived Th1 memory cells and short-lived Th17 cells. *Nature Immunology*, 11(1), pp.83–89.

Pepper, M. & Jenkins, M.K., 2011. Origins of CD4+ effector and central memory T cells. *Nature Immunology*, 131(6), pp.467–471.

Perez-Shibayama, C. et al., 2014. IFN-γ-producing CD4+ T cells promote generation of protective germinal center-derived IgM+ B cell memory against Salmonella Typhi. *Journal of Immunology (Baltimore, Md. : 1950)*, 192(11), pp.5192–200.

Pieper, K., Grimbacher, B. & Eibel, H., 2013. B-cell biology and development. *The Journal of Allergy and Clinical Immunology*, 131(4), pp.959–71.

Powrie, F. et al., 1994. Inhibition of Th1 responses prevents inflammatory bowel disease in scid mice reconstituted with CD45RBhi CD4+ T cells. *Immunity*, 1(7), pp.553–62.

Proetzel, G. et al., 1995. Transforming growth factor-b3 is required for secondary palate fusion. *Nature Genetics*, 11(4), pp.409–414.

Putheti, P. et al., 2010. Human CD4 memory T cells can become CD4+IL-9+ T cells. *PloS One*, 5(1), p.e8706.

Qu, Y. et al., 2010. Gamma-ray resistance of regulatory CD4+CD25+Foxp3+ T cells in mice. *Radiation Research*, 173(2), pp.148–57.

Radic, M.Z. & Zouali, M., 1996. Receptor editing, immune diversification, and self-tolerance. *Immunity*, 5(6), pp.505–11.

Ramdas, W.D. et al., 2011. Common genetic variants associated with open-angle glaucoma. *Human Molecular Genetics*, 20(12), pp.2464–71.

Ramming, A. et al., 2012. Maturation-related histone modifications in the PU.1 promoter regulate Th9-cell development. *Blood*, 119(20), pp.4665–4674.

Rangachari, M. & Kuchroo, V.K., 2013. Using EAE to better understand principles of immune function and autoimmune pathology. *Journal of Autoimmunity*, 45, pp.31–9.

Ratanji, K.D. et al., 2014. Immunogenicity of therapeutic proteins: influence of aggregation. *Journal of Immunotoxicology*, 11(2), pp.99–109.

Rathinam, S.R. & Namperumalsamy, P., Global variation and pattern changes in epidemiology of uveitis. *Indian Journal of Ophthalmology*, 55(3), pp.173–83.

Read, R.W. et al., 2001. Revised diagnostic criteria for Vogt-Koyanagi-Harada disease: report of an international committee on nomenclature. *American Journal of Ophthalmology*, 131(5), pp.647–52.

Rees, W. et al., 1999. An inverse relationship between T cell receptor affinity and antigen dose during CD4(+) T cell responses in vivo and in vitro. *Proceedings of the National Academy of Sciences of the United States of America*, 96(17), pp.9781–6.

Remmers, E.F. et al., 2010. Genome-wide association study identifies variants in the MHC class I, IL10, and IL23R-IL12RB2 regions associated with Behçet's disease. *Nature Genetics*, 42(8), pp.698–702.

Rodriguez, A. et al., 1996. Referral patterns of uveitis in a tertiary eye care center. *Archives of Ophthalmology*, 114(5), pp.593–9.

Romagnani, S., 1994. Regulation of the development of type 2 T-helper cells in allergy. *Current Opinion in Immunology*, 6(6), pp.838–46.

Rothova, A. et al., 1987. Clinical features of acute anterior uveitis. *American Journal of Ophthalmology*, 103(2), pp.137–45.

Rubtsov, Y.P. & Rudensky, A.Y., 2007. TGFbeta signalling in control of T-cell-mediated self-reactivity. *Nature Reviews. Immunology*, 7(6), pp.443–53.

Saadoun, D. et al., 2013. Biotherapies in inflammatory ocular disorders: Interferons, immunoglobulins, monoclonal antibodies. *Autoimmunity Reviews*, 12(7), pp.774–83.

Salazar-González, R.M. et al., 2004. Induction of cellular immune response and anti-Salmonella enterica serovar typhi bactericidal antibodies in healthy volunteers by immunization with a vaccine candidate against typhoid fever. *Immunology Letters*, 93(2-3), pp.115–22.

Sallusto, F. et al., 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature*, 401(6754), pp.708–12.

Sanford, L.P. et al., 1997. TGFβ2 knockout mice have multiple developmental defects that are non- TGFβ β knockout phenotypes overlapping with other TGFβ. *Development*, 2670, pp.2659–2670.

Sanjabi, S., Mosaheb, M.M. & Flavell, R. a, 2009. Opposing effects of TGF-beta and IL-15 control the number of short-lived effector CD8+ T cells. *Immunity*, 31(1), pp.131–144.

Sankar, S. et al., 1995. Expression of Transforming Growth Factor Type III Receptor in Vascular Endothelial Cells Increases Their Responsiveness to Transforming Growth Factor Beta2. *The Journal of Biological Chemistry*, 270(22), pp.13567–13572.

Santibañez, J.F., Quintanilla, M. & Bernabeu, C., 2011. TGF-β/TGF-β receptor system and its role in physiological and pathological conditions. *Clinical Science*, 121(6), pp.233–51.

Sarraj, M. a et al., 2013. Effects of TGFbeta2 on wild-type and Tgfbr3 knockout mouse fetal testis. *Biology of Reproduction*, 88(3), p.66.

Schatz, D.G., Oettinger, M. a & Baltimore, D., 1989. The V(D)J recombination activating gene, RAG-1. *Cell*, 59(6), pp.1035–48.

Schenkel, J.M. & Masopust, D., 2014. Tissue-resident memory T cells. *Immunity*, 41(6), pp.886–897.

Schlenner, S.M. et al., 2012. Smad3 binding to the foxp3 enhancer is dispensable for the development of regulatory T cells with the exception of the gut. *The Journal of Experimental Medicine*, 209(9), pp.1529–35.

Schmid, P. et al., 1991. Differential expression of TGF b1, b2 and b3 genes during mouse embryogenesis. *Development*, 111, pp.117–130.

Schmid, P. et al., 1993. TGF-beta s and TGF-beta type II receptor in human epidermis: differential expression in acute and chronic skin wounds. *The Journal of Pathology*, 171(3), pp.191–7.

Schutyser, E., Struyf, S. & Van Damme, J., 2003. The CC chemokine CCL20 and its receptor CCR6. *Cytokine & Growth Factor Reviews*, 14(5), pp.409–426.

Secundino, I. et al., 2006. Salmonella porins induce a sustained, lifelong specific bactericidal antibody memory response. *Immunology*, 117(1), pp.59–70.

Shainheit, M.G. et al., 2008. Dendritic Cell IL-23 and IL-1 Production in Response to Schistosome Eggs Induces Th17 Cells in a Mouse Strain Prone to Severe Immunopathology. *The Journal of Immunology*, 181(12), pp.8559–8567.

Shamri, R. et al., 2005. Lymphocyte arrest requires instantaneous induction of an extended LFA-1 conformation mediated by endothelium-bound chemokines. *Nature immunology*, 6(5), pp.497–506.

Sharifi, N. et al., 2007. TGFBR3 Loss and Consequences in Prostate Cancer. *The Prostate*, 67(December 2006), pp.301–311.

Shevach, E.M., 2009. Mechanisms of foxp3+ T regulatory cell-mediated suppression. *Immunity*, 30(5), pp.636–45.

Shi, Y. & Massagué, J., 2003. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell*, 113(6), pp.685–700.

Shull, M.M. et al., 1992. Targeted disruption of the mouse transforming growth factor beta 1 gene results in multifocal inflammatory disease. *Nature*, 359, pp.693–699.

Siakavellas, S.I. & Bamias, G., 2012. Role of the IL-23/IL-17 axis in Crohn's disease. *Discovery Medicine*, 14(77), pp.253–62.

Singh, M. et al., 1999. Protective efficacy and immunogenicity of Vi-porin conjugate against Salmonella typhi. *Microbiology and Immunology*, 43(6), pp.535–42.

Skopouli, F.N. et al., 2000. Clinical evolution, and morbidity and mortality of primary Sjögren's syndrome. *Seminars in Arthritis and Rheumatism*, 29(5), pp.296–304.

de Smet, M.D. et al., 2001. Human S-antigen determinant recognition in uveitis. *Investigative Ophthalmology & Visual Science*, 42(13), pp.3233–8.

Smith, J. a et al., 2009. Epidemiology and course of disease in childhood uveitis. *Ophthalmology*, 116(8), pp.1544–51, 1551.e1.

Souchelnytskyi, S. et al., 1996. Phosphorylation of Ser165 in TGF-beta type I receptor modulates TGF-beta1-induced cellular responses. *The EMBO Journal*, 15(22), pp.6231–40.

Stanford, M.R. et al., 2005. Are cytokine gene polymorphisms associated with outcome in patients with idiopathic intermediate uveitis in the United Kingdom? *British Journal of Ophthalmology*, 89(8), pp.1013–6.

Starr, T.K., Jameson, S.C. & Hogquist, K. a, 2003. Positive and negative selection of T cells. *Annual Review of Immunology*, 21, pp.139–176.

Staudt, V. et al., 2010. Interferon-regulatory factor 4 is essential for the developmental program of T helper 9 cells. *Immunity*, 33(2), pp.192–202.

Stein-Streilein, J., 2008. Immune regulation and the eye. *Trends in Immunology*, 29(11), pp.548–54.

Steinman, L. & Zamvil, S.S., 2006. How to successfully apply animal studies in experimental allergic encephalomyelitis to research on multiple sclerosis. *Annals of Neurology*, 60(1), pp.12–21.

Stenvers, K.L. et al., 2003. Heart and Liver Defects and Reduced Transforming Growth Factor beta 2 Sensitivity in Transforming Growth Factor beta Type III Receptor-Deficient Embryos. *Molecular and Cellular Biology*, 23(12), pp.4371–4385.

Stenvers, K.L. & Findlay, J.K., 2010. Inhibins: from reproductive hormones to tumor suppressors. *Trends in Endocrinology and Metabolism: TEM*, 21(3), pp.174–80.

Su, D. et al., 2013. Roles of γδ T cells in the pathogenesis of autoimmune diseases. *Clinical & Developmental Immunology*, 2013, p.985753.

Suhler, E.B. et al., 2008. Incidence and prevalence of uveitis in Veterans Affairs Medical Centers of the Pacific Northwest. *American journal of ophthalmology*, 146(6), pp.890–6.e8.

Sun, L. & Chen, C., 1997. Expression of Transforming Growth Factor Type III Receptor Suppresses Tumorigenicity of Human Breast Cancer MDA-MB-231 Cells. *Journal of Biological Chemistry*, 272(40), pp.25367–25372.

Suttorp-Schulten, M.S. & Rothova, a, 1996. The possible impact of uveitis in blindness: a literature survey. *British Journal of Ophthalmology*, 80(9), pp.844–8.

Szabo, S.J. et al., 2000. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell*, 100(6), pp.655–69.

Takeuchi, M. et al., 1997. On the mechanisms by which transforming growth factor-beta 2 alters antigen-presenting abilities of macrophages on T cell activation. *European Journal of Immunology*, 27(7), pp.1648–56.

Takeuchi, M., Alard, P. & Streilein, J.W., 1998. TGF-beta promotes immune deviation by altering accessory signals of antigen-presenting cells. *Journal of Immunology (Baltimore, Md. : 1950)*, 160(4), pp.1589–97.

Takimoto, T. et al., 2010. Smad2 and Smad3 are redundantly essential for the TGF-beta-mediated regulation of regulatory T plasticity and Th1 development. *Journal of Immunology (Baltimore, Md. : 1950)*, 185(2), pp.842–55.

Taylor, A.W. et al., 2000. Neuropeptide regulation of immunity. The immunosuppressive activity of alpha-melanocyte-stimulating hormone (alpha-MSH). *Annals of the New York Academy of Sciences*, 917, pp.239–47.

Taylor, A.W., Streilein, J.W. & Cousins, S.W., 1992. Identification of alpha-melanocyte stimulating hormone as a potential immunosuppressive factor in aqueous humor. *Current Eye Research*, 11(12), pp.1199–206.

Tempest-Roe, S. et al., 2013. Local therapies for inflammatory eye disease in translation: past, present and future. *BMC Ophthalmology*, 13(1), p.39.

The International HapMap Consortium, 2015. International HapMap Project. Available at: http://hapmap.ncbi.nlm.nih.gov/index.html.en.

The Wellcome Trust Case Control Consortium, 2007. Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. *Nature*, 447(7145), pp.661–78.

Tiegs, S.L., Russell, D.M. & Nemazee, D., 1993. Receptor editing in self-reactive bone marrow B cells. *Journal of Experimental Medicine*, 177(4), pp.1009–20.

Tone, Y. et al., 2008. Smad3 and NFAT cooperate to induce Foxp3 expression through its enhancer. *Nature Immunology*, 9(2), pp.194–202.

Townsend, A. & Bodmer, H., 1989. Antigen recognition by class I-restricted T lymphocytes. *Annual Review of Immunology*, 7, pp.601–624.

Townsend, T.A. et al., 2012. Endocardial cell epithelial-mesenchymal transformation requires Type III TGFβ receptor interaction with GIPC. *Cellular Signalling*, 24(1), pp.247–56.

Tugal-Tutkun, I., 2010. Behçet disease in the developing world. *International Ophthalmology Clinics*, 50(2), pp.87–98.

Unanue, E.R., 1984. Antigen presenting function of the macrophage. *Annual Review of Immunology*, 2, pp.395–428.

Vänttinen, T. et al., 2003. Expression of activin/inhibin signaling components in the human adrenal gland and the effects of activins and inhibins on adrenocortical steroidogenesis and apoptosis. *Journal of Endocrinology*, 178(3), pp.479–89.

Velasco-Loyden, G., Arribas, J. & López-Casillas, F., 2004. The shedding of betaglycan is regulated by pervanadate and mediated by membrane type matrix metalloprotease-1. *Journal of Biological Chemistry*, 279(9), pp.7721–33.

Veldhoen, M. et al., 2006. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity*, 24(2), pp.179–89.

Veldhoen, M. et al., 2008. Transforming growth factor-beta 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nature Immunology*, 9(12), pp.1341–6.

Verity, D.H. et al., 1999. Behçet's disease, the Silk Road and HLA-B51: historical and geographical perspectives. *Tissue Antigens*, 54(3), pp.213–20.

Vestweber, D., 2007. Adhesion and signaling molecules controlling the transmigration of leukocytes through endothelium. *Immunological Reviews*, 218(1), pp.178–196.

Vignali, D.A.A., Collison, L.W. & Workman, C.J., 2009. How regulatory T cells work. *Nature Reviews. Immunology*, 8(7), pp.523–532.

Wahl, S.M., Orenstein, J.M. & Chen, W., 2000. TGF-b influences the life and death decisions of T lymphocytes. *Cytokine Growth Factor Reviews*, 11, pp.71–79.

Wakabayashi, T. et al., 2003. Changing patterns of intraocular inflammatory disease in Japan. *Ocular Immunology and Inflammation*, 11(4), pp.277–86.

Wakefiled, D. & Chang, J., 2005. Epidemiology of uveitis. *International Ophthalmology Clinics*, 45(2), pp.1–13.

Walker, K. a et al., 2011. Betaglycan is required for the establishment of nephron endowment in the mouse. *PloS One*, 6(4), p.e18723.

Walker, L.S.K. & Sansom, D.M., 2011. The emerging role of CTLA4 as a cell-extrinsic regulator of T cell responses. *Nature Reviews. Immunology*, 11(12), pp.852–63.

Walton, R.C. & Ashmore, E.D., 2003. Retinal vasculitis. *Current Opinion in Ophthalmology*, 14(6), pp.413–9.

Wan, Y.Y. & Flavell, R.A., 2007. ' Yin-Yang ' functions of TGF-β and Tregs in immune regulation. *Immunological Reviews*, 2216(1), pp.199–213.

Wang, X.F. et al., 1991. Expression cloning and characterization of the TGF-beta type III receptor. *Cell*, 67(4), pp.797–805.

Waterhouse, P. et al., 1995. Lymphoproliferative Disorders with Early Lethality in Mice Deficient in Ctla-4. *Science*, 270(October), pp.985–988.

Wechsler, B. et al., 1990. Criteria for diagnosis of Behcet's Disease. *The Lancet*, 335, pp.1078–1080.

Wedebye Schmidt, E.G. et al., 2013. TH17 cell induction and effects of IL-17A and IL-17F blockade in experimental colitis. *Inflammatory Bowel Diseases*, 19(8), pp.1567–76.

Wiater, E. et al., 2006. Identification of distinct inhibin and transforming growth factor beta-binding sites on betaglycan: functional separation of betaglycan co-receptor actions. *The Journal of Biological Chemistry*, 281(25), pp.17011–22.

Wieser, R. et al., 1993. Signaling activity of transforming growth factor beta type II receptors lacking specific domains in the cytoplasmic region. *Molecular and Cellular Biology*, 13(12), pp.7239–47.

Wilson, N.J. et al., 2007. Development, cytokine profile and function of human interleukin 17-producing helper T cells. *Nature Immunology*, 8(9), pp.950–7.

Wong, R.W. et al., 2013. Emerging concepts in the management of acute retinal necrosis. *British Journal of Ophthalmology*, 97(5), pp.545–52.

Woodfin, A. et al., 2011. Junctional adhesion molecule-C (JAM-C) regulates polarized neutrophil transendothelial cell migration in vivo. *Nature Immunology*, 12(8), pp.761–769.

Wrana, J.L. et al., 1994. Mechanism of activation of the TGFb receptor. *Nature*, 370(4), pp.341–347.

Wrana, J.L. et al., 1992. TGF beta signals through a heteromeric protein kinase receptor complex. *Cell*, 71(6), pp.1003–14.

Xiong, D.-H. et al., 2009. Genome-wide association and follow-up replication studies identified ADAMTS18 and TGFBR3 as bone mass candidate genes in different ethnic groups. *American Journal of Human Genetics*, 84(3), pp.388–98.

Xu, H. et al., 2003. TGF- Inhibits Activation and Uveitogenicity of Primary but Not of Fully Polarized Retinal Antigen-Specific Memory-Effector T Cells. *Investigative Ophthalmology & Visual Science*, 44(11), pp.4805–4812.

Xu, H. et al., 1997. Uveitogenicity is associated with a Th1-like lymphokine profile: cytokine-dependent modulation of early and committed effector T cells in experimental autoimmune uveitis. *Cellular Immunology*, 178(1), pp.69–78.

Yamagiwa, S. et al., 2001. A role for TGF-beta in the generation and expansion of CD4+CD25+ regulatory T cells from human peripheral blood. *Journal of Immunology (Baltimore, Md. : 1950)*, 166(12), pp.7282–9.

Yang, P. et al., 2005. Clinical patterns and characteristics of uveitis in a tertiary center for uveitis in China. *Current Eye Research*, 30(11), pp.943–8.

Yoshida, Y. et al., 2014. The transcription factor IRF8 activates integrin-mediated TGF-β signaling and promotes neuroinflammation. *Immunity*, 40(2), pp.187–98.

Yoshimura, A., Wakabayashi, Y. & Mori, T., 2010. Cellular and molecular basis for the regulation of inflammation by TGF-beta. *Journal of Biochemistry*, 147(6), pp.781–92.

Zaidi, A.A. et al., 2010. Hypopion in patients with uveitis. *Ophthalmology*, 117(2), pp.366–372.

Zein, G., Berta, A. & Foster, C.S., 2004. Multiple sclerosis-associated uveitis. *Ocular Immunology and Inflammation*, 12(2), pp.137–42.

Zhang, Q. et al., 2012. No association of PTPN22 polymorphisms with susceptibility to ocular Behcet's disease in two Chinese Han populations. *PloS One*, 7(3), p.e31230.

Zhang, Z. et al., 2010. Effects of gamma radiation on bone-marrow stromal cells. *Journal of Toxicology and Environmental Health. Part A*, 73(7), pp.514–9.

Zheng, W. & Flavell, R. a, 1997. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell*, 89(4), pp.587–96.

Zheng, Y. et al., 2007. Interleukin-22, a T(H)17 cytokine, mediates IL-23-induced dermal inflammation and acanthosis. *Nature*, 445(7128), pp.648–51.

Zhou, L. et al., 2007. IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. *Nature Immunology*, 8(9), pp.967–74.

Zhou, R. et al., 2011. A new look at immune privilege of the eye: dual role for the vision-related molecule retinoic acid. *Journal of Immunology (Baltimore, Md. : 1950)*, 187(8), pp.4170–7.

Zhou, R. et al., 2012. The living eye 'disarms' uncommitted autoreactive T cells by converting them to Foxp3(+) regulatory cells following local antigen recognition. *Journal of Immunology (Baltimore, Md. : 1950)*, 188(4), pp.1742–50.

Zhu, R. et al., 2010. Aberrantly increased mRNA expression of betaglycan, an inhibin co-receptor in the ovarian tissues in women with polycystic ovary syndrome. *Journal of Obstetrics and Gynaecology Research*, 36(1), pp.138–46.

Zierhut, M. & Foster, C.S., 1992. Multiple sclerosis, sarcoidosis and other diseases in patients with pars planitis. *Developments in Ophthalmology*, 23, pp.41–7.

Zinkernagel, R.M. et al., 1997. Antigen localisation regulates immune responses in a dose- and time-dependent fashion: a geographical view of immune reactivity. *Immunological Reviews*, 156, pp.199–209.

Zinkernagel, R.M. & Doherty, P.C., 1974. Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. *Nature*, 248(5450), pp.701–2.

Zorzella-Pezavento, S.F.G. et al., 2013. Persistent inflammation in the CNS during chronic EAE despite local absence of IL-17 production. *Mediators of Inflammation*, 102(8), pp.931–6.

Zouali, M., 2014. Immunological Tolerance: Mechanisms. In *eLS*. Chichester, UK: John Wiley & Sons, Ltd, pp. 1–13.

Zouali, M., 2008. Receptor editing and receptor revision in rheumatic autoimmune diseases. *Trends in Immunology*, 29(3), pp.103–9.