

B cells as antigen-presenting cells in a model of rheumatoid arthritis

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Abstract

Rheumatoid arthritis (RA) is a multifaceted inflammatory autoimmune disease characterised by the infiltration of leukocytes into synovial joints leading to the destruction of articular cartilage and bone. Autoreactive CD4⁺ T cells producing inflammatory cytokines are implicated in disease pathogenesis. The clinical efficacy of B cell-depletion therapy is not dependent on clearance of autoantibodies suggesting a critical role for B cells as antigen presenting cells (APC).

To elucidate the role of B cells in activating CD4⁺ T cells in RA, this project examined B cell-mediated antigen presentation to CD4⁺ T cells isolated from T cell receptor (TCR)-transgenic mice specific for the major arthritogenic epitope of the candidate cartilage autoantigen, aggrecan. This system allows for direct comparison of aggrecan-specific B cells to dendritic cells (DC) and other APC in order to identify any unique consequences of B cell antigen presentation and modulation of CD4⁺ T cell effector cytokine production.

The data presented here show the activation, proliferation and effector function of CD4⁺ T cells co-cultured with different APC in the presence of aggrecan and demonstrate key differences in B cell and DC production of disease-relevant cytokines. Crucially, aggrecan-specific B cells induced greater CD4⁺ T cell production of the pathogenic cytokine IFN- γ than DC, despite equivalent IL-2 production. The role of aggrecan-mediated activation of several pattern recognition receptors in this process was excluded using an in vitro detection system. However, the identification of a differential requirement for CD80 and CD86 during antigen presentation by B cells and DC suggested a role for co-stimulatory molecules at the APC-T cell interface in mediating B cell induction in CD4⁺ T cell IFN- γ production.

In summary, this work highlights the role of the CD80/86-CD28 pathway in mediating the observed differential induction of CD4⁺ T cell IFN- γ production by antigen-specific B cells and DC following aggrecan presentation.

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Abbreviations

Agg	Aggrecan
APC	Antigen presenting cell or allophycocyanin (fluorophore)
ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
AID	Activation-induced cytidine deaminase
AIRE	Autoimmune regulator
BAFF	B cell activating factor
BCR	B cell receptor
B _E 1	B effector 1 cell
B _E 2	B effector 2 cell
B _{reg}	B regulatory cell
BSA	Bovine serum albumin
ACPA	Anti-cyclic citrullinated peptide antibodies
CD	Cluster of differentiation
CIA	Collagen-induced arthritis
CLIP	Class II-associated invariant chain peptide
CPM	Counts per minute
cRPMI	Complete RPMI medium
CS	Chondroitin sulphate
CTLA-4	Cytotoxic T lymphocyte antigen 4
CTLL-2	Cytotoxic lymphocyte line-2
dAgg	Deglycosylated aggrecan
DAMP	Damage-associated molecular pattern
DDA	Dimethyldioctadecylammonium
DNA	Deoxyribonucleic acid
DMB	Dimethylmethylene blue

DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbance assay
ER	Endoplasmic reticulum
ERAD	ER-associated protein degradation
FACS	Fluorescence activated cell sorting
F(ab') ₂	Fragment antigen-binding
Fc	Fragment crystallisable
FcR	Fc receptor
FCS	Foetal calf serum
fDC	Follicular dendritic cell
FITC	Fluorescein isothiocyanate
FoxP3	Forkhead box P3
GAG	Glycosaminoglycan
GM-CSF	Granulocyte macrophage colony stimulating factor
H-chain	Heavy-chain of immunoglobulin
HA	Hyaluronan
HEK	Human embryonic kidney
HRP	Horse radish peroxidase
ICOS	Inducible T cell costimulator
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif

JAK	Janus kinase
KS	Keratan sulphate
L-chain	Light-chain of immunoglobulin
LBP	LPS-binding protein
LFA-1	Lymphocyte function-associated antigen 1
LT	Lymphotoxin
LPS	Lipopolysaccharide
MACS	Magnetic activated cell sorting
M-CSF	Macrophage colony stimulating factor
MHC	Major histocompatibility complex
MFI	Median fluorescence intensity
NLR	NOD-like receptor
MMP	Matrix metalloproteinase
mTEC	Medullary thymic epithelial cell
MTX	Methotrexate
NOD	Non-obese diabetic (mice) or nucleotide-binding oligomerization domain (PRR)
ODN	Oligonucleotide
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PD-1	Programmed cell death 1
pDC	Plasmacytoid dendritic cell
PE	Phycoerythrin
PerCP	Peridinin-chlorophyll protein
PGIA	Proteoglycan induced arthritis
PMA	Phorbol 12-myristate 13-acetate
pMHC	Peptide-MHC

PRR	Pattern-recognition receptor
RA	Rheumatoid arthritis
RAG	Recombination activating genes
RANKL	Receptor activator of NFκB ligand
RF	Rheumatoid factor
RNA	Ribonucleic acid
RPMI	<i>Roswell Park Memorial Institute</i> medium
SCID	Severe combined immunodeficiency
SLE	Systemic lupus erythematosus
SMAC	Supramolecular activation cluster
STAT	Signal transducer and activator of transcription
TCR	T cell receptor
TGF	Transforming growth factor
T _{CM}	T central memory cells
T _{EM}	T effector memory cells
T _{H1}	T helper 1 cell
T _{H2}	T helper 2 cell
T _{H17}	T helper 17 cell
TIMP	Tissue inhibitor of metalloproteinase
T _{FH}	T follicular helper cell
TLR	Toll-like receptor
TNF	Tumour necrosis factor
T _{reg}	T regulatory cell
TSA	Tissue-specific antigen
TTCF	Tetanus toxin C-fragment

1. Introduction

1.1. Rheumatoid arthritis

1.1.1. Symptoms and phenotype

Rheumatoid arthritis (RA) is a prevalent and multifaceted inflammatory autoimmune disease characterised by extensive damage to the articular cartilage of synovial joints. RA affects approximately 1% of the population and is more prevalent in women with incidence increasing with age (Lee and Weinblatt, 2001). Symptoms include joint pain and swelling predominantly caused by synovitis. Extra-articular manifestations of RA, particularly cardiovascular co-morbidity, can cause significant mortality amongst patients (Hochberg et al., 2008; Turesson et al., 2003). The pathogenesis of RA involves infiltration of B cells, T cells and other leukocytes into the synovium. Leukocyte migration may be facilitated by the dense network of capillaries associated with the synovium, combined with vasculature leakage induced by activation of Fc receptors on mast cells and neutrophils by immune complex deposition (Binstadt et al., 2006; Knight and Levick, 1984). Leukocyte infiltration occurs in conjunction with upregulation of articular cartilage turnover, synovial lining hyperplasia and the formation of an invasive destructive tissue called *pannus* (Firestein, 2003).

The infiltrating leukocytes secrete cytokines which form part of a complex network which sustains the inflammatory microenvironment within the joint. High levels of proinflammatory cytokines such as tumour necrosis factor- α (TNF- α), interleukin (IL)-1 β , IL-6 and IL-23 are produced by local cell types in conjunction with T cell-derived interferon (IFN)- γ and IL-17. These inflammatory mediators are not adequately balanced in RA synovium by cytokines which regulate T helper-1 (T_H1) activity, such as IL-4 and IL-10 (Firestein, 2004). As a result, accelerated production of tissue metalloproteinases occurs alongside angiogenesis and *pannus* formation, which together contribute to cartilage and bone erosion (Brennan and McInnes, 2008).

1.1.2. Current therapies

A number of therapies have been developed for the treatment of RA. Some focus on treating the symptoms such as joint pain but those that target cytokines involved in the inflammatory process have been particularly effective. However, these therapies have critical limitations and are not sufficient to cure disease (Nurmohamed and Dijkmans, 2005).

Methotrexate (MTX) inhibits *de novo* synthesis of DNA and RNA precursors required for cell proliferation and was originally used to treat lymphoproliferative diseases. MTX was therefore proposed to be useful in the treatment of RA through inhibition of lymphocyte proliferation in the joint (Cutolo et al., 2001). However, MTX was shown to induce rapid clinical remission in many RA patients even at low doses, with disease flaring following discontinuation of treatment, which is more indicative of an anti-inflammatory effect (Weinblatt et al., 1985). MTX was later shown to have profound effects on monocyte and macrophage cytokine production. In particular, production of IL-1, IL-6 and TNF- α is reduced and expression of T cell cytokines such as IL-4 and IFN- γ are also suppressed following MTX administration (Chan and Cronstein, 2002). Furthermore, MTX also prevents cartilage destruction by inhibiting osteoclast formation and cartilage-degrading enzyme production by local cells (Wessels et al., 2008). Due to the efficacy of MTX in targeting a range of immunological functions associated with RA pathology it has formed the foundation of RA treatment up to the present.

The cytokine TNF- α exerts inflammatory effects by binding and signalling through the receptor TNFR1 which is expressed in RA synovium (Alsalameh et al., 1999). TNF- α expression and signalling is thought to be defective in many autoimmune diseases (Vinay and Kwon, 2011). TNF-transgenic mice, expressing a modified form of the human gene, develop an inflammatory arthritis which can be abolished by treatment with monoclonal antibodies against TNF- α or TNFR1 (Alexopoulou et al., 1997; Keffer et al., 1991; Mori et al., 1996). Indeed, anti-TNF- α has been shown to be highly beneficial in treating RA and other autoimmune diseases and has subsequently proven to be the most successful biological therapy (Taylor and Feldmann, 2009). Anti-TNF- α probably interferes with the established feedback loops that maintain the network of inflammatory cytokines produced by local and infiltrating cells. This hypothesis is supported by evidence showing that anti-TNF- α reduces IL-1 and IL-6 production by synovial cells (Feldmann and Maini, 2001). This has the additional consequence of reducing angiogenesis and levels of cartilage-degrading enzymes. While anti-TNF- α represents the principal treatment option for a large proportion of RA

patients, the use of anti-TNF- α is limited due to the need for continuous treatment to prevent disease flares and the fact that 10-20% of patients do not respond (Feldmann, 2002).

Other therapies targeting alternative cytokines have achieved mixed results. For example, IL-1R antagonists were trialled in order to mitigate the proinflammatory effect of IL-1 in RA. However, this treatment had limited efficacy when used alone or in combination with methotrexate (Bresnihan et al., 1998; Cohen et al., 2002; Nuki et al., 2002). IL-6 is another proinflammatory cytokine produced by a range of infiltrating leukocytes in RA synovium and has been demonstrated to induce osteoclast differentiation. In contrast to IL-1R, blockade of IL-6R has been shown to be more effective when used in conjunction with standard disease-modifying anti-rheumatic drugs (Genovese et al., 2008; Maini et al., 2006). In addition, murine models of RA have validated both IL-15 and IL-17 as putative targets for therapy. Initial clinical trials have shown clinical benefits from using monoclonal antibodies to neutralise these cytokines in combination with other therapies (Baslund et al., 2005; Genovese et al., 2010).

The medical limitations and financial cost of biological agents such as anti-TNF- α have dictated the need for other therapies. The use of TNF blockers carries the risk of a range of side effects such as infections and tumours due to their immunosuppressive effects. In addition, it has been suggested that the heterogeneous nature of RA phenotypes found in patients necessitates more advanced tailoring of specific therapies based on genetic and phenotypic distinctions in order to maximise clinical effectiveness (Moreland et al., 2012). Taking this into account, a more complete understanding of the elements of the adaptive immune system involved will provide opportunities that can be exploited for therapeutic benefit to patients expressing a range of disease phenotypes. The identification of environmental or genetic precursors to disease and accurate biomarkers which predict outcomes are both particularly useful clinically. However, it is crucial to distinguish between processes associated with initiation of disease and those responsible for perpetuation of inflammation to facilitate targeting of the most relevant mechanisms at different stages of disease.

1.2. Autoimmunity in the context of rheumatoid arthritis

1.2.1. The role of autoantibodies

Antibodies play an important role in RA pathogenesis, diagnosis and treatment. Rheumatoid factor (RF), an antibody which binds the Fc region of IgG, was an early discovery identified as a marker for onset of RA and RF serum titres correlate with disease severity (Franklin et al., 1957; Rantapaa-Dahlqvist et al., 2003; van Zeben et al., 1992). RF has been proposed to act via generation of immune complexes leading to the activation of complement and recruitment of immune cells into the synovium. Antigens contained within complexes of RF are efficiently presented to T cells by RF-specific B cells following uptake via membrane-bound immunoglobulin. This occurs independently of any antigens contained within the complex and does not occur using non-specific B cells using Fc receptor (FcR) uptake (Roosnek and Lanzavecchia, 1991). Interestingly, FcR capable of binding immune complexes have also been implicated as a potential pathogenic pathway in the K/BxN mouse model of RA by localising the immune response to the joint (Mandik-Nayak and Allen, 2005). This opens up the possibility that T cells with a range of specificities could be activated by immune complexes containing joint autoantigens (Mauri and Ehrenstein, 2007).

Citrullination is the post-translational conversion of a peptidyl arginine to a citrulline residue by the enzyme peptidyl arginine deiminase (PAD) (Eggleton et al., 2008). Expression of PAD can be found in the joint synovial fluid of both arthritic mice and patients and is co-localised with citrullinated joint proteins (De Rycke et al., 2005; Vossenaar et al., 2003). Citrullination can generate 'altered self' neoantigens which, by appearing to be novel antigens, may be recognised by the immune system as foreign. Citrullination is generally enhanced by inflammation but, crucially, synovial fluid is an important site and antigen source for this process and an RA-specific antibody response is seen in a large subset of patients (Kinloch et al., 2008). Antibodies reactive with cyclic citrullinated peptides (ACPA) are excellent biomarkers for patients showing symptoms of arthritis who are likely to go on to develop RA, especially when observed in conjunction with RF (Klareskog et al., 2008; Lundberg et al., 2005; Rantapaa-Dahlqvist et al., 2003). A combination of genetic factors, particularly RA-associated human leukocyte antigen (HLA) alleles and environmental causes, such as smoking, are being explored as mechanisms for the link between autoimmunity and citrullinated peptides (Klareskog et al., 2011; Mahdi et al., 2009; Willemze et al., 2011).

Kuhn *et al.* demonstrated that fibrinogen-binding ACPA could be found prior to disease onset and in the synovium of arthritic mice. These antibodies enhanced arthritis even when a submaximal dose of anti-collagen type II antibodies were co-administered. However, ACPA were not necessary or sufficient to induce disease but could enhance mild arthritis established using a cocktail of collagen type II-specific antibodies, suggesting that their physiological role is complementary to other agents (Kuhn *et al.*, 2006). More recently, it has been shown that immune complexes containing citrullinated antigens are present in a subset of RA patients. These antigens and immunoglobulins co-localise with complement component 3 (C3) in RA *pannus* tissue sections implicating both immune complexes and citrullinated antigens in initiation of a complement cascade and synovitis (Zhao *et al.*, 2008). This process may be analogous to the immune complex-mediated activation of the complement alternative pathway in the K/BxN mouse model or engagement of FcR on macrophages from RA patients and subsequent secretion of TNF- α (Clavel *et al.*, 2008; Matsumoto *et al.*, 2002). In addition, T cells specific for citrullinated variants of collagen and aggrecan epitopes can be found in patients with RA (Von Delwig *et al.*, 2010; Uysal *et al.*, 2009). Stimulation of T cells from these patients using citrullinated peptides, of which aggrecan peptides were the most immunogenic, leads to production inflammatory cytokines such as IL-6, IL-17 and TNF- α (Law *et al.*, 2012). Dendritic cells (DC), macrophages and B cells are all capable of presenting citrullinated peptides to T cells and in some cases do so preferentially when compared to unmodified peptides. This has been shown to occur via autophagy, a catabolic process whereby cellular components can be degraded in lysosomes. However, B cells required stimulation via the B cell receptor (BCR) in order to present citrullinated peptides (Ireland and Unanue, 2011). It has been speculated previously that T cells specific for citrullinated epitopes are critical for activating B cells to produce ACPA (Hill *et al.*, 2008). In contrast to the majority of healthy controls, peripheral blood B cells from patients with RA produce high levels of ACPA when cultured *in vitro* in the presence of cells expressing CD40L (Bellatin *et al.*, 2012). It is notable that, in this study, B cells had a greater potential for ACPA reactivity when isolated from RA patients carrying RA-associated HLA alleles which implies a link between genetic susceptibility and autoantibody production.

Taken together, these studies indicate that citrullination may be a critical step in the cycle of events that perpetuate joint inflammation and facilitate leukocyte infiltration (Uysal *et al.*, 2010; van Venrooij and Pruijn, 2008). It follows that the PAD enzyme responsible for citrullination may be an appropriate target for preventing inflammation and this therapeutic avenue is currently being explored further (Auger *et al.*, 2010; Makrygiannakis *et al.*, 2012).

1.2.2. T cell involvement

Early work showed that severe combined immunodeficiency (SCID) mice that lack both B and T lymphocytes develop inflammatory arthritis following transfer of serum from arthritic mice suggesting that autoantibodies were the principal cause of arthritis (Stuart et al., 1983). Subsequently, the serendipitous discovery of the autoantibody-mediated K/BxN spontaneous mouse model of arthritis appeared to confirm this pathogenic effect of antibodies, in this case specific for ubiquitous self-antigen (Korganow et al., 1999; Kouskoff et al., 1996). Despite the many potential roles for autoantibodies in these models, experiments have since shown that disease induction in mice following autoantibody transfer is exacerbated by the presence of presensitised antigen-specific T cells (Nandakumar et al., 2004). Furthermore, the aforementioned K/BxN mouse model requires the presence of antigen-specific T cell receptor (TCR)-transgenic T cells to provoke B cell autoantibody production and is dependent on major histocompatibility complex (MHC) class II (Kyburz and Corr, 2003; Matsumoto et al., 1999). These findings have highlighted the significance of cellular factors for providing B cell help and producing inflammatory cytokines in RA. CD4⁺ T cells infiltrate the synovium in RA and diverse TCR specificities are typically present in the synovial T cell population (Bennett et al., 2003; Struyk et al., 1995). Characterisation of these T cells in patients has revealed that infiltrating synovial T cells from different joints share TCR β -chain sequences and express high levels complementarity-determining region 3 nucleotide sequence homology (Striebich et al., 1998). This suggests that such T cells may be selected and expanded by presentation of a common group of joint-derived antigens. Various animal models have provided support for this concept by showing that joint components are arthritogenic and may initiate autoimmunity via autoantigen-specific T cells (Brand et al., 2003; Glant and Mikecz, 2004; Qian et al., 2010).

1.2.3. Genetic evidence for antigen presentation to T cells

Early studies using both antigen-driven and non-specific approaches to induce arthritis in rats identified numerous MHC and non-MHC susceptibility loci which were thought to provide candidate genes for investigation of the genetic foundations of autoimmunity across species (Griffiths and Remmers, 2001). Since then, association of various genetic loci with RA have been established by genome-wide association studies (GWAS). In particular, strong associations of *HLA-DR*, *CD40*, *CTLA4* and *PTNP22* genes with various autoimmune diseases including RA have been identified (Raychaudhuri et al., 2008; Stahl et al., 2010; Zhernakova et al., 2011). Recent evidence from mouse models is also accumulating in support of the notion that attenuation of TCR signalling caused by mutations in *ZAP70* may be key to promoting positive selection of autoreactive T cell cells in the thymus (Sakaguchi et al., 2012). It is notable that many of the aforementioned genes linked to RA are involved in antigen presentation and TCR signalling. This suggests that the genetic component of RA may confer an increased risk of autoantigen presentation and aberrant T cell activation.

HLA genes encode for MHC class II and other associated molecules involved in antigen presentation. The contribution of HLA-DR molecules is one of the strongest and most intriguing findings from genetic studies of RA (Gonzalez-Gay et al., 2002; Nepom et al., 1989). More specifically, several *HLA-DRB1* alleles contain a sequence referred to as the *shared epitope* (De Vries et al., 2002). Patterns of variation found from amino acid positions 67-74 of *HLA-DRB1* have been demonstrated to influence disease protection, susceptibility and severity (Gourraud et al., 2006; du Montcel et al., 2005). A recent study has conducted exhaustive genotyping of candidate genes identified through the aforementioned GWAS and confirmed the well-established strong association of RA with *HLA-DR* genes and other genes related to T cell activation (Eyre et al., 2012). In addition, this study supports data showing different allelic associations at the *HLA* and *PTPN22* genes in ACPA-positive and ACPA-negative patients and goes on to identify novel differences in other loci (Padyukov et al., 2011). The genetic association of the *shared epitope* with RA, in conjunction with T cell infiltration of joint tissue, has formed the basis for the hypothesis that antigen-specific T cell interactions may mediate disease through pathogenic cytokine production.

1.2.4. T cell contributions to pathology

CD4⁺ T cells are activated by TCR binding of short antigen-derived peptides in the context of MHC class II molecules that are 'presented' on the surface of antigen-presenting cells (APC). APC also provide additional ligands that act as co-stimulatory signals initiating cytokine production and differentiation of T cell subsets (Watts, 2001). The production of proinflammatory cytokines such as TNF- α , IL-6 and IL-12 by activated APC drives T cell production of pathogenic cytokines (Aarvak and Natvig, 2001). Activated T cells differentiate into various effector T cell subsets which each produce a distinct cytokine profile. Data from human disease and animal models show that T_H1 cells are the principal T cell phenotype found in arthritic synovium (Yamada, 2008). IFN- γ is the major cytokine produced by this subset but is found at low levels in RA synovium (Feldmann et al., 1996). However, T_H1 cells are also capable of exerting effects on tissue-resident macrophages through contact-dependent mechanisms following activation by IL-15 (McInnes et al., 1997).

An alternative cytokine, IL-17, is present at higher levels in arthritic synovium and is also a potent inducer of proinflammatory cytokine production along with cartilage matrix breakdown and bone destruction. IL-17-producing helper T cells, designated T_H17 cells, have also been described and implicated in RA (Kirkham et al., 2006; Stamp et al., 2004). The cytokine milieu and chemokine content of arthritic joints in RA and animal models enable infiltration and activation of T_H17 cells (Hirota et al., 2007a, 2007b). Interestingly, the interactions of T_H17 cells isolated from early RA patients with RA synovial fibroblasts generate higher metalloproteinase and autocrine IL-17 production than T_H1 cells from the same patients (Van Hamburg et al., 2011). Furthermore, in an animal model of RA in which the humoral response is the dominant pathogenic force, T_H17 cells can help B cells to produce autoantibodies via IL-17 production and enhance arthritis (Hickman-Brecks et al., 2011; Jacobs et al., 2009). In addition, IL-17 has been shown to influence the formation of ectopic lymphoid follicles in chronically inflamed tissue such as the synovium in RA (Grogan and Ouyang, 2012). Despite this finding, T_H1 cells outnumber T_H17 cells in arthritic synovium and recent evidence suggests that other local cell types, such as mast cells for example, may be responsible for the majority of the IL-17 production (Hueber et al., 2010; Yamada, 2008). Furthermore, the stability of CD4⁺ T cell subsets has been shown to be dependent on the strength and duration of polarisation and adoption of a particular cytokine profile may be stochastic under some circumstances (Kelso et al., 1995; Murphy et al., 1996). Evidence is now emerging the T_H17 phenotype is unstable and T_H17 cells recruited into inflammatory conditions are not committed to solely producing cytokines associated with the T_H17 lineage but may be sufficiently 'plastic' in their

effector phenotype to express the T_H1 transcription factor T-bet and switch to producing IFN- γ (Hirota et al., 2011; Nistala et al., 2010). Nevertheless, IL-17 still remains an attractive target for immunotherapy and early trials have shown that treatment with anti-IL-17 monoclonal antibodies suppresses RA symptoms (Van den Berg and Miossec, 2009; Genovese et al., 2010).

The exact manner in which $CD4^+$ T cell tolerance is broken in RA leading to self-reactive $CD4^+$ T cells to enter the periphery is unknown. In respect to central tolerance, an immunological 'self-shadow' consisting of a diverse range of tissue-specific antigens (TSA) derived from self-proteins which are presented via MHC class II within the thymus by medullary thymic epithelial cells (mTEC) (Anderson et al., 2002; Derbinski et al., 2001). Thymocytes which bind self-antigen with a high affinity are deleted by negative selection forming the basis for T cell tolerance (Anderson et al., 2005). Expression of TSA is regulated by the autoimmune regulator (AIRE) transcription factor and absence of this protein leads to systemic autoimmunity (Anderson et al., 2002). It is possible that antigens associated with autoimmunity, such as joint antigens in RA, may not be presented by mTEC in genetically susceptible individuals or the affinity of self-reactive TCR does not exceed the threshold required for deletion (Liston et al., 2003).

KRN TCR-transgenic T cells from the K/BxN mouse model of spontaneous arthritis are probably an example of T cells escaping central tolerance due to insufficient expression of their target antigen, glucose-6-phosphate isomerase, by thymic APC (Mandik-Nayak and Allen, 2005). However, several mechanisms exist to control T cell activity in the periphery (Walker and Abbas, 2002). The immune system can remain ignorant of self-antigen sequestered in immunoprivileged sites or if the quantity of self-antigen does not exceed the threshold required to trigger an immune response (Hoglund et al., 1999). Furthermore, if antigen is presented by APC lacking expression of co-stimulatory molecules, T cells enter a state termed 'anergy' which leads to functional inactivation (Jenkins and Schwartz, 1987). Unlike antigens derived from pathogens, self-antigens are not cleared over time. Therefore, self-reactive T cells probably undergo repetitive TCR engagement which has been shown to lead to activation-induced cell death. This provides an effective, T cell-intrinsic means to delete cells specific for self-antigen from the T cell repertoire (Lenardo, 1991; Singer and Abbas, 1994).

Since their discovery, regulatory T cells (T_{reg}) have been shown to form a key component of peripheral T cell tolerance (Sakaguchi et al., 1995; Wing and Sakaguchi, 2010). T_{reg} expressing a $CD4^+CD25^+$ phenotype can control inflammatory responses and maintain peripheral tolerance (Leipe et al., 2005). This is achieved via production of tumour growth factor- β (TGF- β), IL-10 and inhibitory surface molecules (Piccirillo et al., 2008). Recent evidence also points to the cell-extrinsic function of cytotoxic T lymphocyte antigen-4 (CTLA-4) as a key mechanism of preventing APC from activating effector T cells by depriving them of the required CD80/86 co-stimulatory molecules (Qureshi et al., 2011). IL-10 is also particularly important due to its suppression of T_H17 cells and promotion of further T_{reg} differentiation (Heo et al., 2009). IL-10 also inhibits APC activation by targeting innate immunoreceptor signalling molecules for destruction which may contribute to its suppressive effects (Chang et al., 2009).

By crossing mice with a loss-of-function mutation for FoxP3, the transcription factor associated with the T_{reg} lineage and required for their function, with mice which develop spontaneous arthritis, Benoist and Mathis *et al.* have shown that absence of functional T_{reg} spreads the influence of arthritis over a wider spectrum of joints and increases overall disease severity (Nguyen et al., 2007). However, T_{reg} are both prevalent and deficient at different stages of RA in the peripheral blood and synovium of patients (Han et al., 2008; Lawson et al., 2006; Mottonen et al., 2005). This has led to confusion over their role in the disease. Fortunately, enlightening data from Ehrenstein *et al.* suggest that T_{reg} in RA may have a compromised ability to suppress cytokine production by activated T cells and monocytes. The success of anti-TNF- α therapy may partly be due to restoration of this function (Ehrenstein et al., 2004). Furthermore, the same group reported that a reason for this disruption of a specific T_{reg} function is due to reduced T_{reg} expression of CTLA-4 (Flores-Borja et al., 2008).

Receptor activator of nuclear factor κ B ligand (RANKL) is essential for osteoclast differentiation and activation of their bone-resorbing capacity. B cells and synovial fibroblasts have been shown to express RANKL and contribute to bone resorption (Tunyogi-Csapo et al., 2008; Yeo et al., 2011). In addition, T_H1 and T_H17 cells are also capable of inducing osteoclastogenesis via expression of RANKL (Takayanagi et al., 2000). Interestingly, T cells can act as mediators for the osteoclast-forming action of cytokines such as IL-18, which increases T cell production of soluble RANKL (Dai et al., 2004). RANKL-expressing T_H1 cells can induce osteoclastogenesis from monocyte precursors in an IFN- γ -dependent manner (Kotake et al., 2005). Similarly, T_H17 cells can modulate osteoclast formation through IL-17-mediated upregulation of RANKL on mesenchymal cells (Sato et al., 2006). This mechanism has been linked to the early phase of RA pathogenesis and is thought to induce osteoclast development from monocyte precursors (Miranda-Carus et al., 2006).

Finally, the highly proinflammatory environment seen in arthritic joints may also activate bystander T cells in an antigen-independent manner. This can lead to additional T cell release of IFN- γ and initiation of TNF- α production by infiltrating monocytes (Brennan et al., 2002, 2008; Sattler et al., 2009). In particular, local IL-6 production has been shown to be important in regulating T cell responses in the synovial microenvironment. It has been proposed that the IL-6 signalling pathway may be part of an amplification loop capable of attracting T cells without a requirement for antigen-specific interactions (Murakami et al., 2011). Furthermore, animal models of spontaneous arthritis support the model that T cell autoreactivity and cytokine production are the consequence of interactions between multiple cell types from both the innate and adaptive immune system (Kouskoff et al., 1996; Lundy et al., 2007). Overall, innate immune responses may play a role in recruiting effector T cells but antigen-specific activation and clonal expansion of these cells is probably necessary for the autoimmune pathology associated with RA (Nickdel et al., 2009). This balance between autoantigen-mediated clonal expansion of T cells and intricate exchanges with local cells has replaced formerly polarised views seeking to pin responsibility on a particular cell type.

1.2.5. T cell-directed therapies

The combination of synovial infiltration, genetics, inflammatory cytokine production and their involvement in animal models presents a strong case for T cell involvement in RA pathogenesis. This rationale led to the development of therapies which aim to induce T cell tolerance, prevent activation of T cells by APC or temporarily remove them entirely. Despite this solid foundation, therapies using cyclosporin, anti-CD4 and anti-CD52 monoclonal antibodies have not generated promising results (Keystone, 2003). Such failures may be due to an incomplete depletion of the entire CD4⁺ T cell compartment or, conversely, removal of the essential T_{reg} required to suppress the on-going immune response. However, these studies did reveal that, while depletion of CD4⁺ cells was not sufficient to prevent disease, persistent inhibition of residual T cell activation by coating of CD4 by non-depleting antibodies was predictive of a therapeutic response (Choy et al., 1996; Connolly et al., 1992; Ruderman et al., 1995). However, unacceptable side effects have thwarted further application of anti-CD4 therapies (Choy et al., 2002). Despite this, newer therapies which act to prevent the co-stimulation events necessary to activate T cells have proven to be more effective and biologically tolerated (Genovese et al., 2005; Weyand and Goronzy, 2006).

Alternatively, protein kinases responsible for transmission and downstream amplification of T cell activation signals provide an abundance of potential targets. Furthermore, use of protein kinase inhibitors circumvents the clinical limitations of current biological therapies as such small molecules are comparatively cheap, biologically tolerated and can be administered easily. Mitogen-activated protein kinases (MAPK) have garnered particular attention. However, despite promising results in animal models, MAPK inhibitors have been largely unsuccessful during preliminary clinical trials (Cohen et al., 2009; Damjanov et al., 2009). Several explanations have been offered for these failures (reviewed in Hammaker and Firestein, 2010). Briefly, signalling networks contain redundancy which may compensate for blockade of specific molecules. Furthermore, the effects of MAPK are complex and are likely to be involved in the control of anti-inflammatory mechanisms. In light of this, recent studies have focussed on targeting upstream MAPK kinases (MKK) in an attempt to bypass some of these limitations. Indeed, following proof-of-concept studies using MKK-deficient mice, novel inhibitors for MKK have achieved success in ameliorating arthritis in mice (Guma et al., 2012; Hammaker et al., 2012). Many cytokine receptors rely on JAK (Janus kinase) to activate STAT (signal transducer and activator of transcription) and initiate downstream T cell activation. Therefore, members of the JAK family have emerged as promising targets. For example, inhibition

of JAK3 decreases T cell infiltration of synovium and reduces joint damage in animal models of RA (Milici et al., 2008).

In conclusion, a diverse range of T cell-derived cytokines clearly play a role in RA through activation of synovial cells which contribute to cartilage and bone destruction. These cytokines are the product of pathogenic effector subsets found in RA synovium. However, therapies seeking to mitigate T cell activation and proliferation have either failed or achieved only moderate success. Furthermore, small molecule inhibitors of signalling pathways are still in their infancy and have generated mixed results during development. As a result, a renewed effort to understand T cell activation is needed to determine more appropriate sites for intervention. As APC are required for activating CD4⁺ T cells and have a major effect on their subsequent effector function, elucidation of this process may prove fundamental to understanding how T cells are involved in RA and simultaneously provide new targets for therapy.

1.3. The role of antigen presentation in rheumatoid arthritis

1.3.1. T cell antigen recognition and immune synapse formation

To initiate the process of naïve T cell activation and cytokine production, antigen-specific T cells establish contact with APC loaded with cognate antigen and form an immune synapse (Dustin and Depoil, 2011). The T cell-APC immune synapse is evolutionary adapted to detect low levels of peptide-MHC (pMHC) complexes. The stability and duration of APC-T cell interactions, and the quantity of pMHC complexes present on the APC surface, is critical in determining the level of T cell activation. During activation T cell surface molecules accrue in a microcluster termed the supramolecular activation cluster (SMAC) characterised by a central cluster of TCR molecules involved in antigen recognition surrounded by a peripheral ring of adhesion molecules such as leukocyte function-associated antigen 1 (LFA-1) involved in maintaining cell-cell contact (Smith et al., 2005). Below this a layer of tyrosine kinases propagates TCR signals to enact genetic changes involved in determining T cell function (Deindl et al., 2009; Nika et al., 2010). In addition, adaptor proteins link the TCR to a cytoskeletal network of actin, centrosomes and microtubules which mediate morphological and mechanical polarisation important in T cell activation (Hashimoto-Tane et al., 2011; Ilani et al., 2009; Stinchcombe et al., 2006). T cells have been shown to selectively reorganise their intracellular machinery to favour polarisation towards activated APC when interacting with multiple cells (Duchez et al., 2011). During this process, in addition to contributing to the local inflammatory milieu in general, T cells directionally secrete cytokines such as IL-2 and IFN- γ towards the APC face of the immune synapse during the course of antigen-specific interactions in order to communicate at the cytokine level without activating bystander cells (Huse et al., 2006). Various forms of T cell polarisation may be crucial for allowing T cells to navigate a potentially 'noisy' immunological environment in order to respond to the appropriate signals (Krummel and Macara, 2006).

1.3.2. Co-stimulatory molecule modulation of T cell function

Co-stimulatory molecules are an essential additional signal required for activation of T cells and their absence prevents the aberrant activation of T cells without provision of additional 'danger' detected by APC. The presence of APC-derived co-stimulatory molecules serves to amplify TCR signals (Chambers, 2001). Naïve T cells receiving appropriate levels of co-stimulation can respond to lower doses of antigen and require less time commitment than those receiving TCR stimulation alone. For previously activated effector or memory T cells, TCR signalling is efficiently coupled to the relevant signalling pathways required for induction of proliferation and cytokine production even in the absence of strong TCR activation or co-stimulation (Lanzavecchia and Sallusto, 2001). In addition to lowering the threshold for T cell activation, APC-derived co-stimulatory signals also serve to direct subsequent T cell differentiation thus fine-tuning the response. Co-stimulatory molecules can have either stimulatory or inhibitory effects on T cell activation and cytokine production and their ligands may induce different effects depending on the receptor they encounter making narrow definitions of their function difficult (Chen, 2004; Croft, 2003).

Most co-stimulatory molecules fall into two groups: the immunoglobulin superfamily and the TNF receptor superfamily. The immunoglobulin superfamily includes CD28, CTLA-4 and inducible T cell co-stimulator (ICOS). CD28 co-localises with the TCR within the immune synapse where it meets its primary ligands, APC-derived CD80 and CD86, as part of the initial priming event. Ligation of CD28 by CD80/86 is essential for lowering the threshold for TCR activation, inducing T cell proliferation and IL-2 production and promoting T cell survival (Boise et al., 1995). This is achieved through recruitment of lipid rafts containing kinases and adapter molecules which aid tyrosine phosphorylation within TCR-CD28 microclusters and initiate transcriptional programs. Absence of signalling through CD28 leads to functional unresponsiveness of T cells and anergy (Wells et al., 2001, 2003). CD28 signalling also has a qualitative effect on T cell function through modulation of T cell differentiation into effector cytokine-producing subsets. Substantial evidence points to combined strong TCR and CD28 signalling inducing T_H2 differentiation (Howland et al., 2000). This favouring of T_H2 responses has implications for disease regulation in the context of T_H1 and T_H17 -mediated autoimmunity by shifting the response away from these subsets to non-pathogenic T_H2 cells (Bouguermouh et al., 2009; Lenschow et al., 1996; Purvis et al., 2010). In contrast to CD28, OX40 is an example of the TNF receptor superfamily found on T cells. Its ligand, OX40L is not constitutively expressed on APC like CD80/86 but is instead induced following activation. OX40 is important for generation of effector and memory T cells as

demonstrated by OX40-deficient mice. Evidence suggests OX40 is predominantly concerned with the late stages of T cell activation and prolongs the survival of effector T cells in contrast to CD28-derived signals involved in T cell priming (Redmond et al., 2009).

Following T cell activation there is an upregulation of T cell-derived CTLA-4, which is a negative regulator of T cell activation in the late stage of T cell priming, in order to dampen T cell responses and prevent autoimmunity (Krummel and Allison, 1995; Walunas et al., 1994). CTLA-4 binds CD80/86 with a higher affinity than CD28 and therefore out-competes CD28 for these ligands. The importance of CTLA-4 is highlighted by knock-out mice which develop a lethal lymphoproliferative disease characterised by severe tissue injury following infiltration of organs with polyclonal T cells (Tivol et al., 1995; Waterhouse et al., 1995). CTLA-4 is also involved in T cell differentiation as demonstrated by CTLA-4-deficient mice exhibiting a pronounced T_H2 phenotype and antibody blockade of CTLA-4 leading to increased T_H17 differentiation (Bour-Jordan et al., 2003; Ying et al., 2010). Programmed death-1 (PD-1) is another negative regulator of immunity expressed on T and B cells. PD-1 is critical for prevention of autoimmunity through limiting IFN- γ production and proliferation while promoting T cell apoptosis (Brown et al., 2003; Freeman et al., 2000). PD-1 knock-out mice develop symptoms resembling cardiomyopathy, arthritis and glomerulonephritis (Sandner et al., 2005). Although they share similar functions, CTLA-4 and PD-1 appear to act at different stages of T cell development and activation. While CTLA-4 acts early on in the T cell response to control T cell proliferation within lymphoid structures, PD-1 seems to play a more general role in maintaining long-term tolerance and preventing tissue-specific injury thus emphasising the sequential sequence of co-stimulation (Fife and Bluestone, 2008).

The exact mechanisms of modulation of T_H1 and T_H2 differentiation and cytokine production by distinct co-stimulatory molecules in autoimmunity is controversial due to conflicting data from various animal models. However, a key paper by Kuchroo *et al.* suggested that CD80 and CD86 differentially affected T helper cell development. Blockade of CD80 was shown to increase T_H2 cytokines while blockade of CD86 led to an increase in T_H1 cytokines (Kuchroo et al., 1995). Further work supported these observations to an extent but later clarified the effect by demonstrating that the antigenic experience of T cells prior to activation by APC lacking CD80/86 is an important factor in determining whether cytokine production is affected. Specifically, naïve T_H2 differentiation and IL-4 production was highly dependent on CD80/86 but lack of CD80/86 primarily affected antigen-experienced T_H1 proliferation and IL-2 production instead of IFN- γ production (Schweitzer and Sharpe, 1998; Schweitzer et al., 1997). In models of type I diabetes, dysregulation of both CD80 and CD86 have been shown to be potentially relevant to the

development of inflammatory insulinitis. CD80 is overexpressed in comparison to CD86 on B cells in autoimmune-prone NOD mice and is linked to exacerbation of T cell-driven inflammation and prevention of T_{reg} differentiation. Furthermore, blockade of CD80/86 protects against disease development (Hussain and Delovitch, 2005). Similarly, in another study, a loss of CD86 has been shown to mediate a reduction in islet-reactive CD4⁺ T cells due to a combination of reduced T cell priming and induction of hyporesponsiveness or cell death (Yadav et al., 2004).

Overall, signals received by T cells through co-stimulatory molecules are not only important for controlling the process of T cell activation and tolerance but also influence T cell differentiation and, therefore, are implicated in modulating the prevalence of pathogenic T cell subsets linked to autoimmune disease.

1.3.3. Pattern-recognition receptor modulation of APC function

In addition to pMHC-TCR interactions during antigen presentation, the provision of a secondary co-stimulatory signal by APC is critical for T cell activation. Importantly, the upregulation of co-stimulatory molecules on APC has been shown to be dependent on ligation of pattern-recognition receptors (PRR) by ligands that contain certain motifs, designated as either damage-associated molecular patterns (DAMP) or pathogen-associated molecular patterns (PAMP) (Bianchi, 2007; Medzhitov and Janeway, 1997). This concept was outlined in a seminal prediction by Janeway suggesting that APC possess PRR that recognise pathogen-derived molecularly-conserved motifs as a signal to upregulate T cell-stimulatory functions (Janeway, 1992). This preceded the discovery of a mammalian homologue of the *Drosophila* Toll protein which had already been shown to be involved in microbial immunity (Lemaitre, 2004; Medzhitov et al., 1997). This particular form of PRR was termed a Toll-like receptor (TLR). TLR are a family of type I transmembrane receptors characterised by variable leucine-rich extracellular domains that determine specificity for different pathogen-derived ligands.

Some TLR bind intermediate molecules attached to their target. For example, lipopolysaccharide (LPS) is extracted by LPS-binding protein (LBP) and presented by the CD14 accessory molecule to a heterodimer of TLR4 and MD-2 (Miyake, 2006). In this instance, MD-2 acts a surrogate hydrophobic chamber for the binding of the lipid A region of LPS (Park et al., 2009). In addition, evolutionarily conserved Toll-IL-1R (TIR) intracellular domains are linked by adapter molecules, such as Myeloid differentiation primary response gene 88 (MyD88) and TIR domain-containing adapter protein (TIRAP), to transcription of genes mediated by the protein NF- κ B (Fitzgerald et al., 2001; Werling et al., 2009). In particular, NF- κ B upregulates transcription of IL-1, IL-6 and CD80, which is crucial for antigen presentation (Medzhitov et al., 1997). The critical nature of this pathway is illustrated by experiments using mice lacking functional MyD88 that show abrogation of responses to PAMP (Hoshino et al., 1999; Kawai et al., 1999; Poltorak et al., 1998; Qureshi et al., 1999). In addition, TLR signalling is required for APC production of IL-12 and effective T_H1 responses (Imanishi et al., 2007; Schnare et al., 2001).

A significant body of evidence has also been assembled supporting a role for TLR in autoimmunity (Groom et al., 2007; Krieg and Vollmer, 2007; Liu et al., 2006). Microbial-derived ligands for multiple TLR have been implicated due to their role in activating APC either directly or, in the case of RA, via the activation of intermediaries such as synovial cells (Waldner et al., 2004). Inhibition of IL-1 production in inflammatory arthritis can be achieved through antagonising TLR4 (Abdollahi-Roodsaz et al., 2007). Furthermore, TLR3 and TLR7 are upregulated in RA synovium and co-ligation of TLR2 and TLR4 on DC derived from patients induces higher TNF- α and IL-6 production than healthy controls (Roelofs et al., 2005). The role of TLR2 and TLR4 is further supported by data from mice lacking these receptors. While TLR2^{-/-} mice display a marked reduction in T_{reg} and increased IFN- γ production, TLR4^{-/-} mice are protected from severe arthritis due to a reduction in T_H17 development (Abdollahi-Roodsaz et al., 2008a, 2008b). This evidence suggests a complex interaction between these receptors but outlines TLR4 as an important upstream mediator of pathogenic T cell cytokine production in arthritis. Recently, elevated levels of TLR5 have also been described in RA on synovial macrophages alongside a concurrent increase in synovial TNF- α . Expression of TLR5 is mediated by IL-8 and IL-17 which have both been connected to RA. Interestingly, blockade of TLR5 was sufficient to drastically reduce TNF- α transcription (Chamberlain et al., 2012).

The triggering of DC maturation by TLR sensing of microbial ligands is one of the principal features of antigen presentation leading to effector T cell activation (Napolitani et al., 2005). Therefore, in addition to playing a role in the activation of local cells such as those found in the synovium, TLR signalling is also an essential regulator of DC-mediated aspects of autoimmunity. This is exemplified by the exacerbation of systemic lupus erythematosus (SLE)-like disease following overexpression of the TLR7 gene in transgenic mice and subsequent dysregulation of DC proliferation and proinflammatory cytokine production (Deane et al., 2007). Furthermore, TLR9 ligands are thought to activate DC through interactions with secreted intermediaries to augment IFN- α secretion and enhance SLE (Means and Luster, 2005; Tian et al., 2007).

Ligation of TLR is also required, in conjunction with BCR signalling and T cell help, to initiate B cell driven T cell-dependent responses (Christensen et al., 2005; Pasare and Medzhitov, 2005). The immune system can therefore focus innate signals towards the appropriate antigen-specific B cells during the primary response (Ruprecht and Lanzavecchia, 2006). Numerous studies have linked BCR and TLR stimulation and demonstrated synergistic roles that influence a range of B cell functions. For example, a combination of BCR and TLR4 signalling is capable of inducing activation-induced cytidine deaminase (AID) and class-switching which are processes essential to antibody responses (Pone et al., 2012). Furthermore, in contrast to TLR9 stimulation by ligands internalised by macropinocytosis, which is inefficient in B cells, ligands that co-ligate BCR and TLR9 induce enhanced B cell proliferation and plasma cell formation (Eckl-Dorna and Batista, 2009). BCR-acquired ligands are targeted to intracellular TLR9 through BCR-mediated recruitment of TLR9-containing endocytic vesicles (Chaturvedi et al., 2008).

The expression of TLR on different APC may help determine their relative importance in autoimmune pathology (other factors are discussed further in section 1.4). When compared to DC, B cell subsets have been shown to respond to unique patterns of TLR stimulation. In particular, although upregulation of activation markers such as CD86 can be induced by individual TLR ligands for TLR2 and TLR9, IFN- γ production by follicular B cells is only induced by a combination of TLR ligands. In contrast, IL-10 is produced following addition of individual ligands for TLR2, TLR4 and TLR9. Furthermore, CD40, TLR2 and TLR4 behave synergistically and co-ligation leads to enhanced IL-10 production (Barr et al., 2007). These experiments complement previous work and clearly demonstrate that TLR ligands can modulate B cell cytokine production to either regulate or enhance T cell-driven responses, a finding which has profound implications for autoimmunity (Fillatreau et al., 2002).

Shlomchik *et al.* have shown that activation of autoreactive B cells and subsequent antibody production prior to T cell involvement is dependent on MyD88 with TLR7 and TLR9 playing a key role (Herlands et al., 2008). However, although further work on TLR7 supports the notion that B cells have an intrinsic ability to respond to TLR7, which promotes germinal centre reactions and plasmablast development, they still exhibit a requirement for T cell-derived signals during the course of disease (Walsh et al., 2012). LPS can directly stimulate TLR4 on B cells to enhance the formation of germinal centres by increasing B cell aggregation and proliferation. TLR4 signalling can also override the processes that normally prevent non-specific B cells from entering germinal centre dark zones and induce expansion of memory and plasma cells (Hwang et al., 2009). The authors of this study suggest that TLR activation may play a role in the maintenance of ectopic lymphoid structures observed in some autoimmune patients. Activation of B cells through TLR9 during the early stages of maturation in the bone marrow leads to increased expression of CD86 and could enhance the APC capabilities of B cells once they reach circulation. Bone marrow samples from RA patients contain higher levels of bacterial DNA ligands for TLR9 than controls, highlighting a potential pathway for B cell activation in the early stages of RA pathogenesis (Rudnicka et al., 2009). In addition to direct signalling, the synovial environment may contribute to B cell survival via TLR activation. TLR3 stimulation of synovial cells can drive the production of the survival factor BAFF (Bombardieri et al., 2011). Similarly, host-derived TLR2 ligands can induce expression of CXCL13, a B cell chemoattractant, by acting on DC and macrophages and exacerbate B and T cell-driven autoimmunity (Moreth et al., 2010; Popovic et al., 2011).

It is also worth noting that NOD-like receptors (NLR), PRR that recognise peptidoglycans, are also expressed on B cells and can function synergistically with BCR triggering to induce CD80/86 expression. Importantly, proliferation of B cells in response to TLR ligands could be enhanced by addition of NLR ligands (Pettersson et al., 2011). NOD1 is also expressed in RA synovium on synovial fibroblasts. Addition of NOD1 ligands upregulates expression of IL-6; an effect amplified by co-ligation of TLR2 or TLR4. As IL-6 is a key inflammatory mediator, this could represent another pathway through which inflammation and long-term B cell activation is maintained (Yokota et al., 2012). The addition of the NOD2 ligand muramyl dipeptide (MDP) to a mouse model of inflammatory arthritis induces more joint inflammation and appears to mediate these effects by enhancing IFN- γ production by antigen-specific T cells. However, although MDP can induce inflammation in the absence of other stimuli, the arthritis is not NOD2-dependent suggesting a complementary role in disease (Rosenzweig et al., 2009).

Activation of TLR on APC is not exclusively involved in the generation of effector T cell responses. In addition to their role in endowing APC with co-stimulation they can also mediate the generation of regulatory cytokines. A combination of lineage, maturation status, antigen dose, TLR stimulation and the cytokine environment confers some DC with the ability to induce tolerogenic T cell responses (Rutella et al., 2006; Steinman et al., 2003). In the absence of TLR stimulation DC can adopt a partially mature tolerogenic phenotype remarkably similar to the migratory DC within the lymphatic system which continuously tolerise T cells to self-antigens. In contrast, generation of mature DC requires PRR ligation and their immunogenicity appears to be conferred primarily by the secretion of proinflammatory cytokines (Lutz and Schuler, 2002). However, in some scenarios TLR stimulation can induce tolerogenic DC properties. For example, the restimulation of DC primed with TLR2 and TLR4 ligands has been shown to induce enhanced IL-10 production in comparison to naïve DC (Yanagawa and Onoé, 2007). A similar effect has been observed using established tolerogenic DC. Despite expressing similar TLR as conventional DC, tolerogenic DC upregulate TLR2 following triggering of TLR4 by LPS and respond to TLR2 activation by expressing IL-10 while secreting only low levels of proinflammatory cytokines IL-12 and TNF- α (Chamorro et al., 2009). Thus, tolerogenic DC have a reduced capacity to induce T_H1 cells and, through TLR2 signalling and IL-10 production, may drive T_{reg} induction (Abdollahi-Roodsaz et al., 2008b; Dillon et al., 2006; Netea et al., 2004). Interestingly, a more subtle role for NF κ B in the TLR signalling pathway has been revealed when immature and mature DC are compared. NF κ B was recently shown to be essential for maintaining the resting state of DC. In NF κ B^{-/-} mice, DC exposed to self-antigen more readily activated T cells and induced autoimmunity even in the absence

of maturation stimuli (Dissanayake et al., 2011). This suggests NF κ B may have a dynamic role in maintaining the DC resting state, similar to the role of other transcriptional regulators in controlling lymphocyte quiescence (Ouyang et al., 2009).

B cells activated through TLR can also suppress T_H1 and T_H17-mediated autoimmunity. Mice with B cells lacking MyD88^{-/-} develop an exacerbated form of experimental autoimmune encephalomyelitis (EAE) and exhibit increased T cell activation. MyD88 appears to mediate the opposite effect in other APC, as it is required for disease induction (Lampropoulou et al., 2008). Expansion of IL-10-producing B cell subsets is also facilitated by TLR9 activation by immune complexes found on apoptotic cells or co-ligation of CD40 and TLR4 (Miles et al., 2012; Yanaba et al., 2009). B cell-derived IL-10 in these models counterbalances DC IL-12 production in the later stages of the immune response. Cross-talk between receptors has also been demonstrated to be important in determining the B cell cytokine response. Not only does TLR4 induce IL-10 production while TLR2 mediates TNF- α and IL-8 synthesis but a feedback system also allows TLR2 and TLR9 to regulate TLR4 expression. An elegant example of the role of TLR cross-talk has been described in models of SLE. TLR7 is crucial for disease but, in contrast, TLR9 appears to have a regulatory effect despite the fact these receptors utilise analogous signalling pathways (Christensen et al., 2006). Importantly, these responses and cross-talk mechanisms have been shown to be unique to B cells in comparison to other APC (Jagannathan et al., 2009).

In summary, the role of PRR in RA is of significant interest as their ligands can be found at the site of inflammation and have a striking ability to influence the activation of T cells by APC. For example, fibrinogen is a joint component that has recently emerged as a candidate autoantigen following experiments demonstrating that immunisation with fibrinogen can induce an antibody and T cell-dependent form of arthritis in mice (Ho et al., 2010). Interestingly, in patients with RA, citrullinated forms of fibrinogen have been found trapped within immune complexes and are capable of stimulating TLR4 on macrophages (Sokolove et al., 2011; Zhao et al., 2008).

1.4. Candidate antigen-presenting cells

The aforementioned evidence strongly supports a role for antigen-specific CD4⁺ T cells in RA. It is clear that APC provide a range of activatory and regulatory signals to CD4⁺ T cells which are derived from the specific antigen and concurrent TLR stimulation and communicated via co-stimulation and cytokine production. It is evident that understanding the role APC such as DC, macrophages and B cells perform is critical. However, the relative importance of each APC in RA must be elucidated to fully understand RA pathogenesis. In addition, novel treatments now aim to expand on the ability of biological agents to mitigate disease symptoms by re-establishing long-term immune tolerance through targeting the interactions between APC and T cells (Kremer et al., 2003). Ultimately, knowing which APC are the most important and which molecular targets are the most appropriate will drastically increase the chances of these therapies being successful.

Both DC and macrophages have evolved to efficiently acquire, process and present antigen to T cells. The processes of pinocytosis, macropinocytosis and phagocytosis allow DC and macrophages to maximise internalisation of soluble antigen from the environment where it may only be present at low concentrations (Lim and Gleeson, 2011). In addition, these cells express a range of surface receptors that facilitate endocytosis of different types of antigen complexed with either complement or antibodies (Aderem and Underhill, 1999; Bajtaj et al., 2006).

Characterisation of synovial APC populations in RA patients initially identified DC with the potential to present antigen and activate T cells (Pettit et al., 2000; Zvaifler et al., 1985). Subsequently, more detailed analysis has also revealed the presence of immature DC in the synovial lining with mature DC in ectopic lymphoid infiltrates in the joint. This work has led to the suggestion that DC may migrate into the tissue following chemokine gradients and mature locally in the presence of other lymphoid cells (Page et al., 2002). During typical immune responses directed towards pathogens encountered in non-joint tissue, DC acquire antigen at the site of inflammation and migrate to lymph nodes where they activate antigen-specific T cells. Taken together, these findings suggest that, in RA patients, DC may undergo this entire process within the synovium as a result of activation following joint autoantigen acquisition and the formation of local ectopic lymphoid tissue similar to that seen in lymph nodes after encountering pathogens.

Several subsets of DC may be involved in generating the inflammatory environment observed in RA synovium without directly presenting antigen to CD4⁺ T cells. Follicular DC (fDC) are essential for disease in the K/BxN autoantibody-mediated animal model of arthritis due to their ability to recruit T follicular helper cells (T_{FH}) and display immune complexes to B cells and authorise germinal

centre reactivity (Victoratos and Kollias, 2009). Plasmacytoid DC (pDC) are typically involved in inhibition of viral infections through natural production of type I IFN and can be found in synovial tissue in RA patients (Colonna et al., 2002; Lande et al., 2004). Interestingly, pDC isolated from the synovial fluid are more capable producers of TNF- α , IFN- γ and IL-10 than peripheral blood pDC in response to incubation with allogeneic T cells or TLR stimulation (Cavanagh et al., 2005; Jongbloed et al., 2006). The role of synovial myeloid DC in comparison to pDC has been clarified following experiments demonstrating that pDC produce IFN- α/β and IL-18 but myeloid DC are the key producers of IL-23 which is crucial for the expansion of T_H17 cells proposed to be involved in RA (Lebre et al., 2008). Despite this, studies assessing the contribution of different APC to autoimmune pathology have suggested that B cells may surpass DC as activators of T cells at the sites of inflammation. DC in NOD mice produce only low levels of IL-12, a defect which prevents T_H1 differentiation and is compensated for by high macrophage production of IL-12 and B cell antigen presentation to islet-specific T cells (Marleau et al., 2008). Synovial fluid DC isolated from RA patients express only low levels of CD80 and CD86 and show defects in upregulation of these molecules following culture with IFN- γ or GM-CSF. The authors of this study speculate that the T cell activatory function of synovial fluid DC is modulated by the presence of regulatory cytokines such as IL-10 in the joint (Summers et al., 1996).

Macrophages form a prominent component of the inflamed synovium in RA and contribute to *pannus* formation (Mulherin et al., 1996). In addition to their primary role in scavenging for particulate antigens, pathogens and immune complexes, macrophages also have the capacity to phagocytose and present joint-derived antigens or synovial fluid components to T cells via MHC class II (Michaëlsson et al., 1995; Tsark et al., 2002). However, other evidence suggests that many T cell interactions with macrophages are largely antigen-independent processes. T cells activated by the cytokine milieu, such as that found in the inflamed joint, induce macrophages to produce pathogenic TNF- α but not regulatory IL-10 (Sebbag et al., 1997). Indeed, the expansive role of macrophages in RA as effector cells involved in tissue destruction and chronic production of inflammatory cytokines has been the primary focus of research. Macrophages certainly serve a role in amplifying and perpetuating chronic inflammation in response to soluble stimuli or cell-cell contact with T cells and synovial fibroblasts which overshadows their potential for acting as an initiating agent (Kinne et al., 2007).

1.4.1. B cells

The concept that the APC and T cell activation function of B cells may be important to autoimmune pathogenesis has returned to the forefront of study largely due to the success of B cell-directed therapies in RA and other autoimmune diseases (Townsend et al., 2010). In addition to the aforementioned production of autoantibodies, B cells also serve a range of other effector functions including antigen presentation to T cells. Therefore, the development of autoreactive B cells and their effector functions are now considered critical areas of interest.

1.4.2. B cell tolerance

Exactly how B cell tolerance is broken in RA is currently unknown but there are many potential mechanisms open to further elucidation. Normal B cell development involves the genetic recombination of V(D)J segments in the BCR heavy-chain (H-chain) immunoglobulin locus at the pro-B cell stage followed by light-chain (L-chain) recombination at the pre-B cell stage. The H-chain and L-chain eventual pair in immature B cells to form a BCR. As B cells do not express a complete BCR during the early stages of development the recombination processes which generate antigen receptor diversity occur independently of any feedback from antigen recognition. Therefore, the potential for generating self-reactive B cells is high and some estimates place the frequency of autoreactive clones as high as 50% (Nemazee, 1995). This necessitates mechanisms to remove autoreactive B cells to prevent systemic autoimmunity. B cell central tolerance leads to the removal of immature B cells which recognise mostly membrane-bound self-antigen expressed in the bone marrow (Cornall et al., 1995). However, it has subsequently been shown that this preliminary defence removes only a small fraction of the autoreactive B cell population and many autoreactive B cells exit the bone marrow and migrate into peripheral lymphoid tissues (Goodnow et al., 1989; Klinman, 1996; Merrell et al., 2006). Alternatively, immature B cells can be redirected to the pre-B cell stage to undergo receptor editing. Receptor editing involves another rearrangement of the immunoglobulin genes in order to generate a novel BCR (Tiegs et al., 1993). A failure to revise the BCR at this stage and retention of autoreactivity results in apoptosis and clonal deletion (Von Boehmer and Melchers, 2010; Monroe and Dorshkind, 2007).

Similar to autoreactive T cells which escape negative selection, many autoreactive B cells emerge from the bone marrow anergic, a state of non-responsiveness maintained by uninterrupted BCR signalling leading to a form of desensitisation. This results in diminished BCR expression and compromised signalling in these cells. As a consequence, antigen trafficking to the endocytic compartments required for proteolytic processing is reduced. This generates a lower frequency of pMHC complexes and, therefore, inhibits antigen presentation (Gauld et al., 2005; Goodnow et al., 1988; O'Neill et al., 2009). Furthermore, the strong competition for the necessary survival signals, such as B cell-activating factor (BAFF), in the follicular compartments of the spleen provides a cellular basis for B cell censoring. This competition is biased against self-reactive clones due to weaker BCR signals, which lower BAFF receptor expression, and the relative absence of T cell help (Cyster et al., 1994; Freitas et al., 1995; McLean et al., 1997). Therefore, anergic B cells are excluded from follicular niches and undergo accelerated apoptosis (Smith et al., 2010; Stadanlick and Cancro, 2008; Stadanlick et al., 2008). During activation of B cells, the expression of CD22, a BCR-associated lectin, negatively regulates BCR and CD40 signalling via immunoreceptor tyrosine-based inhibitory motifs (ITIM) (Pillai et al., 2009; Poe et al., 2004a, 2004b). Interestingly, mutations in CD22 have been associated with a range of autoimmune diseases suggesting that this molecule plays a key role in suppressing potentially autoreactive B cells which may not be anergic (Surolia et al., 2010).

Anergic B cells in the periphery can be saved from apoptosis by entering germinal centre reactions and undergoing receptor editing similar to that in the bone marrow. However, despite the efficiency of this process, autoreactive B cells can occasionally avoid deletion by simultaneously expressing the novel BCR alongside the original autoreactive BCR. Such cells can progress to autoantibody-producing plasma cells following high-avidity interactions with ubiquitous self-antigen (Liu et al., 2005). Furthermore, dual-specific B cells may have a greater propensity for autoreactivity due to their prevalence in autoimmune-susceptible mice and overrepresentation in activated memory and plasmablast populations (Fournier et al., 2012). Interestingly, recent work has shown that, amongst the polyclonal repertoire of B cells, suppression of autoreactive B cells by deletion or anergy is limited to those with a high affinity for membrane-bound self-antigen. In contrast, B cells specific for soluble self-antigen are proposed to be regulated by an absence of T cell help (Taylor et al., 2012). Similar to membrane-bound antigen, several joint-derived autoantigens such as aggrecan are immobilised in the ECM and so may only be accessible to autoreactive B cells capable of acquiring them via BCR-dependent mechanisms (Ciechomska et al.; unpublished). Therefore, if such B cells are important in acquiring and presenting such antigens in RA this implies that the mechanisms for deleting B cells or rendering them anergic are defective or overwhelmed. One potential hypothesis suggests that autoreactive T cells provide help to anergic B cells by initiating a cycle of reciprocal activation signals (Seo et al., 2002). However, studies seeking to elucidate this mechanism have suggested an alternative to T cell-initiated autoimmunity. As mentioned previously, antigen-TLR complexes have been shown to bind both BCR and TLR simultaneously leading to B cell activation in a T-independent manner (Leadbetter et al., 2002). As a result, TLR-mediated activation represents a mechanism for breaking B cell tolerance which emphasises the role of B cells as initiators of autoimmunity leading to formation of a positive-feedback loop between autoreactive T and B cells and maintenance of the immune response (Herlands et al., 2008).

1.4.3. B cell antigen presentation

Autoreactive B cells that escape tolerance mechanisms have the potential to play several roles in RA including autoantibody production, lymphoid neogenesis and activation of autoreactive T cells. In order to focus B cell antibody responses to pathogen-derived antigens that are not efficiently recognised by PRR, the provision of T cell help is essential. In addition, B cells are capable APC and possess numerous characteristics that differentiate them from other APC. The study of B cells as APC has highlighted numerous scenarios where B cells appear to be crucial APC for the presentation of certain antigens and induction of CD4⁺ T cell activation and differentiation.

Marrack and Kappler demonstrated in 1980 that specific and non-specific B cells were capable of displaying antigen on their surface and interacting with T cells to induce B cell antibody production. This study outlined the link between B cell expression of MHC genes and presentation of soluble antigen to T cells while also suggesting a role for the immunoglobulin receptor in focusing the response to antigen-specific B cells when antigen is limited in the environment (Marrack and Kappler, 1980). This was later confirmed by the comparison of B cell presentation of non-specific and BCR-specific antigen. In these experiments, only antigen targeted to the BCR was capable of eliciting strong T cell proliferation responses from B cell cultures while both antigens were presented similarly by splenocytes (Chesnut and Grey, 1981). Further reports demonstrated that a homogenous population of B lymphocyte tumour cells specific for a known antigen could present soluble antigen to antigen-specific T cells via MHC (McKean et al., 1981). Since then, the anatomical location of antigen-specific B cell interactions with CD4⁺ T cells has been visualised and described in detail. Following systemic challenge with antigen, B cells have been shown to meet T cells at the peripheral regions of lymphoid follicles resulting in proliferation of both cell types (Garside et al., 1998; reviewed in Germain et al., 2008). A number of studies have attempted to gauge the importance of B cells activation of T cells through the use of B cell-depleting antibodies. Janeway *et al.* showed that lymph node B cells were an important antigen-presenting cell but this data was later contradicted by later studies implicating non-B cell APC such as DC as the initiating APC (Janeway et al., 1987; Ron and Sprent, 1987). Furthermore, immunisation of μ MT B cell-deficient mice with various protein antigens suggested that B cells were unnecessary for T cell priming in response to these antigens (Epstein et al., 1995; Phillips et al., 1996). However, studies using alternative antigens and B cells lacking MHC class II demonstrated that B cells were capable of providing unique signals that could not be replicated by other APC, particularly in the area of memory T cell generation (Constant et al., 1995a; Crawford et al., 2006; Linton et al., 2000;

Macaulay et al., 1998). This conflicting data may be explained by the complex interaction of a plethora of factors including mouse strain genetics, the type and form of antigen used and the influence of B cells on both lymphoid structure and cytokine production by other APC (Constant et al., 1995b; Linton et al., 2000; Macaulay et al., 1998; Rivera et al., 2001; Williams et al., 1998).

For example, aside from any direct involvement in antigen presentation, B cells also occupy important subsidiary roles in the immune system. B cells have been shown to be critical in the development and maintenance of the splenic marginal zone through production of lymphotoxin- β (LT- β) and induction of chemokine release (Dingjan et al., 1998; Gonzalez et al., 1998; Nolte et al., 2004). Absence of B cells affects the presence and organisation of both DC and T cell subsets within the spleen (Crowley et al., 1999; Ngo et al., 2001). B cells are responsible for guiding the migration of FcR⁺ DC bearing antigen into the splenic follicles and therefore facilitate antigen specific responses (Yu et al., 2002). B cells also maintain the T_H1/T_H2 balance as their absence leads to increased DC release of IL-12 and skewing of the T cell repertoire towards IFN- γ production (Moulin et al., 2000). Finally, B cell-deficient mice develop a lower frequency of memory CD4⁺ T cells and numbers can only be restored following addition of activated B cells. B cells activated with an irrelevant antigen are still capable of restoring the memory population demonstrating that this particular process is not dependent on antigen presentation (Linton et al., 2000).

The prerequisite for B cells for development of splenic architecture and T cell activation is particularly pertinent to arthritis as B cells interact with T cells within the synovium and formation of aggregates or microstructures that resemble germinal centres has been observed in RA patients (Takemura et al., 2001a). The presence of these structures has been associated with a more severe inflammatory phenotype (Thurlings et al., 2008). Germinal centres are a specialised and dynamic cellular structure with interdigitating fDC and T_{FH} cells which display antigen for capture by B cells or provide T cell help respectively. Animal models have revealed that LT- β production by synovial B cells is essential for lymphoid neogenesis, working in conjunction with CXCL13 and B cell survival signals (e.g. BAFF), which are released by germinal centre and synovial cells (Weyand and Goronzy, 2003). In addition, B cells can produce chemokines, such as CCL3 which facilitate leukocyte infiltration (Iwamoto et al., 2008; Loetscher and Moser, 2002). Studies using transplanted human synovial tissue have shown that cognate interactions between T cells and activated B cells are essential for the formation of ectopic lymphoid structures and pathogenic T cell IFN- γ production (Takemura et al., 2001b). From the presence of terminally-differentiated plasma cells in the synovium of RF⁺ and ACPA⁺ patients one could infer these structures are functional, as germinal centres are generally required for affinity maturation and the generation of fine-tuned antigen-

specific B cell responses, although fibroblast-like synoviocytes treated with IFN- γ and TNF- α can also support plasma cell differentiation through expression B cell survival factors (Burger et al., 2001; Ohata et al., 2005). Taken together, these findings suggest a process whereby B cells can recruit CD4⁺ T cell help to drive the generation of antigen-specific autoreactive clones which can proceed to traffic to and infiltrate distal joints and establish new lesions (Voswinkel et al., 1999).

In addition to autoantibody production and lymphoid neogenesis, work in animal models has emphasised the requirement of B cell antigen presentation in RA (reviewed in Rodriguez-Pinto, 2005). Critically, antigen-specific B cells that have been genetically engineered to be incapable of secreting antibody have been shown to be essential APC for the development of severe experimental arthritis (O'Neill et al., 2005). In addition, this study also demonstrated that the ability of autoreactive T cells to initiate disease in this model was significantly enhanced following priming by these antigen-specific B cells as opposed to that seen with other APC. However, development of severe arthritis was only possible if the antibody-producing function was restored. This strongly implicates B cells as both producers of pathogenic antibodies and as APC. These differing functions may develop at different stages during the course of the disease. Intriguingly, RF-specific B cells, and to some extent other antigen-specific B cells, have been shown to present immune complexes to T cells very effectively. These complexes may contain a multitude of antigens and thus lead to the activation of T cells with a diverse range of specificities (Roosnek and Lanzavecchia, 1991). Furthermore, in a mouse model of SLE, B cell antigen presentation has been shown to precede that of other APC, including DC (Yan et al., 2006). The authors of this study suggested that this might allow B cells to focus the immune response to particular autoantigens early in disease development.

The three core mechanisms of B cell antigen presentation contain features unique to these cells in comparison to other APC which may explain the aforementioned observations:

a) Acquisition of soluble and membrane-bound antigen

Whilst B cells can obtain antigen via fluid phase uptake mechanisms *in vitro*, this process is highly inefficient compared to that seen in APC. In contrast, B cell antigen uptake through the internalisation of antigen bound to the BCR is highly efficient (Lanzavecchia, 1985; Rock et al., 1984; Stoddart et al., 2002). Each B cell clone expresses a unique BCR and processing and presentation of antigen bound to this receptor following its uptake into the B cell endocytic pathway is also highly efficient (Chesnut et al., 1982; Lanzavecchia et al., 1988). Due to their ability to undergo clonal expansion, along with the selective advantage given to daughter clones expressing somatically mutated, higher affinity BCR, B cells can concentrate relatively small quantities of

antigen for presentation to CD4⁺ T cells in a MHC-restricted manner (Byersdorfer et al., 2004; Guernonprez et al., 1998; Lanzavecchia, 1987; Rivera et al., 2001). A pivotal study by Batista and Neuberger showed that BCR affinity for antigen imposes restrictions on signalling. In particular, BCR-antigen affinity was required to exceed a threshold in order for signalling to take place. High affinity antigens were capable of triggering BCR signalling at lower concentrations than those with low affinity. Interestingly, above a certain limit the affinity made no difference to BCR triggering (Batista and Neuberger, 1998). The authors emphasise that the selection of B cells based on antigen affinity is probably due to the limited amount of antigen available *in vivo* which makes serial stimulation of the BCR impractical, in contrast to T cells where the density of pMHC complexes is paramount.

The BCR consists of a tetramer comprised of two H and two L-chains linked by disulphide bonds and anchored in the cell plasma membrane (Borst et al., 1993). The BCR exists within a complex including dimers of the CD79 α and CD79 β signalling molecules and a range of co-receptors including CD19, CD21 and CD22 which both positively and negatively modulate the signals transmitted via the CD79 heterodimer (**Figure 1.1**) (Hombach et al., 1988). Downstream signalling leading to subsequent B cell activation and entry into the cell cycle is mediated by spleen tyrosine kinase (Syk) (Cheng et al., 1995; Cornall et al., 2000; Turner et al., 1995). Syk is capable of self-regulating BCR signalling and modulates trafficking and processing of antigen following internalisation (Heizmann et al., 2010; Le Roux et al., 2007). Membrane-bound IgM endocytosis is mediated by phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAM) in the CD79 α cytoplasmic domain. This enables BCR-antigen complex targeting to MHC class II-rich endosomal compartments. Dependence on CD79 phosphorylation is abrogated following class-switching to IgG (Amigorena et al., 1994; Knight et al., 1997). The actin cytoskeletal network restricts BCR motility within the membrane in order to prevent aberrant signalling. In particular, the cytoplasmic domain of CD79 β is important in confining BCR diffusion within actin-defined boundaries (Treanor et al., 2010). The IgG tail-segment has been shown to enhance intracellular calcium responses and prevent inhibition of genes responsible for B cell differentiation into specialised memory cell such as plasma cells (Horikawa et al., 2007). Pierce *et al.* have expanded on this knowledge by demonstrating the BCR-intrinsic ability of IgG on naïve and memory B cells to recruit Syk and enhance calcium responses when compared to IgM (Davey and Pierce, 2012; Liu et al., 2010a).

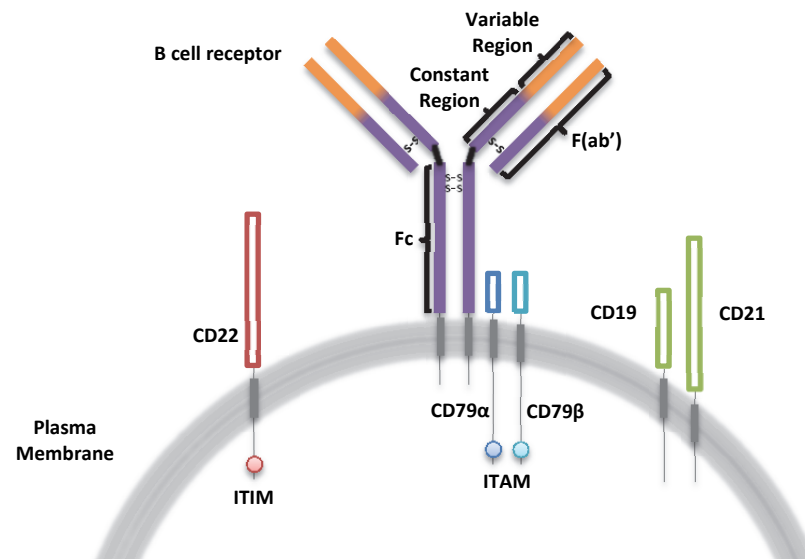


Figure 1.1 *The structure of the BCR and co-signalling molecules.*

The BCR consists of two heavy chains and two light chains linked by disulphide bonds. The variable regions contain the antigen-binding domains. The structure can be enzymatically cleaved into two fragments denoted fragment antigen binding F(ab') and fragment crystalline (Fc) based on their functional characteristics. The BCR can be membrane-bound or secreted. When membrane-bound, the BCR is associated with CD79α/β dimer. The BCR complex includes the co-receptor CD19 and complement receptor CD21 along with inhibitory molecules such as CD22.

Importantly, in conjunction with uptake of soluble antigen, membrane-bound antigen can also be internalised via the BCR. The acquisition of such antigens has been proposed to be a major feature which distinguishes B cell antigen presentation from other APC (Carrasco and Batista, 2006). Unlike other APC, some evidence suggests that B cells may acquire the majority of antigen in a membrane-bound form from the surface of macrophages and DC during the initial phase of primary responses in spleen follicles (reviewed in Batista and Harwood, 2009; Carrasco and Batista, 2007; Harwood and Batista, 2009). The binding of membrane-bound antigen to the BCR leads to the formation of an immune synapse between the antigen-displaying cell and the B cell which gathers antigen and organises surface co-stimulatory molecules and cytoplasmic effectors. Notably, similarly to processes in T cell-APC synapses, the BCR is segregated from co-receptors such as CD45 which negatively regulate BCR responses (Batista et al., 2001). The interaction of multiple antigen ligands fixed on a surface serves to increase the avidity of the BCR-antigen interaction and has been shown to mediate the formation of BCR microclusters within the immune synapse (Liu et al., 2010b). The dependence of immune synapse formation in B cells on *Dock8*, a *Rho-Rac* guanine exchange factor involved in intracellular G-protein signalling and cytokinesis, emphasises the importance of polarisation of B cells towards the immune synapse (Randall et al., 2009). High-avidity BCR-antigen interactions facilitate B cell spreading over the target membrane and initiate signalling cascades which induce internalisation of antigen. The spreading response of the B cell serves to increase formation of microclusters and thus is important in locating and aggregating antigen during the subsequent contraction phase (Fleire et al., 2006). The CD19 co-receptor is responsible for stimulating efficient microcluster formation and B cells lacking CD19 exhibit both defective spreading responses and intracellular signalling (Depoil et al., 2008).

The BCR is phosphorylated by *Lyn* following binding and microcluster formation leading to continuation of signalling and synapse formation. Recruitment of *Lyn* embedded in lipid rafts has been shown to be a BCR-intrinsic mechanism that occurs independently of further downstream signalling events (Sohn et al., 2008). Furthermore, a coordinated *Lyn* and *Syk*-dependent downstream signalling pathway initiated within BCR microclusters is also essential for propagation B cell spreading (Weber et al., 2008). A range of adapter molecules recruit dynein to enable movement of microclusters along sub-surface microtubules to facilitate antigen gathering at a focal point in the immune synapse (Schnyder et al., 2011). Overall, this process represents a novel mechanism by which B cells may be able to efficiently extract membrane-bound antigens that are inaccessible to other APC. In addition, as many intracellular antigens are relocated to the cell surface

during differing forms of cell death, recent studies from our laboratory have shown that B cells are also capable of acquiring and presenting these previously unavailable antigens (Ciechomska et al., 2011). In the context of RA, recently acquired data also demonstrates that antigen-specific B cells are capable of acquiring joint autoantigens immobilised within cartilage explants (Ciechomska et al., unpublished).

B cells also express the complement receptors CD21 and CD35 which modulate BCR signalling. When complexed with CD19, which in itself can upregulate co-stimulatory molecule expression, ligation of CD21 is capable of lowering the threshold for BCR signalling (Fearon and Carroll, 2000; Yan et al., 2005). Indeed, membrane-bound IgM has been shown to be able to fix complement sufficiently to co-ligate CD21 and CD35 (Rossbacher and Shlomchik, 2003). In contrast, CD21 activation without BCR co-ligation fails to upregulate B cell co-stimulatory molecules and, as a result, may induce T cell anergy due to the lack of a second signal (Brown et al., 2006). Similarly, other receptors such as FcγRIIB1 can negatively regulate B cell responses following Fc binding (Wagle et al., 1999).

Additionally, it has been demonstrated that uptake of antigen linked to TLR ligand is mediated by the BCR (Eckl-Dorna and Batista, 2009). As previously described in section 1.3.3 B cells are also activated by antigen complexes containing TLR ligands (Leadbetter et al., 2002). This is particularly interesting as TLR9 ligands have been shown to induce a transient form of arthritis in experimental models. Furthermore, joint components such as fibrinogen and hyaluronan are known to be TLR4 ligands and can be found in arthritic synovium (Deng et al., 1999; van der Heijden et al., 2000).

b) Antigen processing

Binding of antigen by the BCR results in the propagation of activation signals and initiates traffic of the antigen-BCR complexes to MHC class II-rich compartments (Aluvihare et al., 1997; Lankar et al., 2002). Elegant studies using chimeric BCR specific for different antigens have shown that BCR binding confers an advantage to antigen processing by accelerating targeting and delivery to MHC class II-rich compartments (Aluvihare et al., 1997). The high-affinity nature of the BCR-antigen binding ensures stability of the interaction at the high endosomal pH (Denzin et al., 2005). Processing of antigen regions masked by high-affinity interactions with the BCR may also be affected, thus enhancing presentation of an alternative range of epitopes (Watts and Lanzavecchia, 1993). Due to this masking, the epitopes presented through BCR-mediated antigen processing may be altered in comparison to other methods of uptake. As a result, antigens bound by antibodies or internalised via the BCR contribute to the presentation of a differential repertoire of epitopes to T

cells. This process can enhance the MHC class II loading of a specific T cell epitope by 10-fold in some cases and, therefore, lower the threshold of antigen required for T cell activation (Simitsek et al., 1995).

MHC class II molecules are assembled in the endoplasmic reticulum and stabilised by binding the invariant chain chaperone. The cytoplasmic tail of the invariant chain contains signalling motifs that facilitate egress of MHC class II into the endocytic compartment (MIIC) for subsequent peptide loading (Neeffes et al., 1990; Pieters et al., 1993). The invariant chain is essential for prevention of premature loading of endogenous peptides (Roche and Cresswell, 1990). The invariant chain is trimmed in the MIIC by proteinases leaving the class II-associated invariant chain peptide (CLIP) occupying the MHC class II binding groove (Nakagawa et al., 1998). CLIP is exchanged with antigenic peptides in a process catalysed by HLA-DM (Denzin and Cresswell, 1995; Roche and Cresswell, 2011).

In contrast to other APC, the MHC-like molecule HLA-DO is expressed in B cells. Importantly, HLA-DO inhibits loading of antigen peptide fragments onto MHC class II molecules by HLA-DM at high pH. BCR cross-linking leads to acidification of MIIC and, therefore, a reduction in HLA-DO activity (Alfonso et al., 2003; Chen and Jensen, 2004). This ensures that MHC class II loading in B cells occurs more efficiently in later endocytic compartments. As described previously, the efficiency of antigen access to these endocytic compartments increases with BCR affinity. Therefore, the regulated expression of HLA-DO in B cells acts as one of the molecular mechanisms involved in B cell competition for T cell help based on BCR affinity. This is borne out by the finding that B cells process antigen more efficiently via BCR internalisation in comparison to other mechanisms such as macropinocytosis. There is evidence that B cells use HLA-DO to avoid superfluous entry of low-affinity B cells into germinal centres. For example, naïve B cell expression of HLA-DO inhibits entry into germinal centres, thus setting a high threshold for antigen presentation which favours BCR-mediated uptake (Chen et al., 2002; Draghi and Denzin, 2010). B cells within the germinal centre downregulate HLA-DO, perhaps to expedite B cell antigen presentation and survival once the initial checkpoint has been passed (Glazier et al., 2002).

c) MHC class II antigen presentation to T cells

BCR cross-linking and CD40-derived signals also upregulate B cell expression of co-stimulatory molecules leading to induction of APC characteristics. These changes include expression of CD80 and CD86 with a concurrent increase in MHC class II molecules trafficked to the cell membrane (Von Andrian and Mempel, 2003; Evans et al., 2000). These molecules are stabilised and organised

to allow B cells to present antigen to naïve CD4⁺ T cells (Clatza et al., 2003). However, activation of B cell antigen presentation is dependent on CD40-CD154 interaction, as highlighted by loss of their antigen-presenting function in CD40^{-/-} and CD154^{-/-} mice (Buhlmann et al., 1995; Hollander et al., 1996). Indeed, a lack of CD40 expression on B cells presenting antigen induces anergy in naïve T cells, rather than activation. As a result, B cells require help from CD154-expressing activated CD4⁺ T cells, in contrast to other APC (Lee et al., 2003; Wu et al., 1995). In light of this, and further evidence comparing B cell antigen presentation to other APC, DC were initially advocated as the primary APC responsible for T cell priming (Byersdorfer et al., 2004; Epstein et al., 1995; Phillips et al., 1996; Williams et al., 1998).

Despite this, B cells have been shown to be capable of inducing CD154 expression on CD4⁺ T cells and, therefore, a chain of events resulting in activation of both cell types (Constant, 1999). This mechanism is mediated initially by pMHC-TCR cross-linking. Further stabilisation is achieved by CD80/86-CD28 interactions. The combination of these interactions results in CD154 upregulation on the T cell (Lindgren et al., 2001). Subsequently, co-stimulation via CD40 is sufficient to maintain MHC class II and CD80/86 expression. Thus, B cells are capable of priming naïve T cells and receiving the necessary activation signals to achieve activation without prior involvement of other APC. Furthermore, B cells have been implicated as an important APC due to their superior ability to present certain protein antigens to T cells in comparison to DC. Constant *et al.* have elegantly shown this in two studies. The first demonstrated that B cell-deficient mice exhibited less T cell priming following challenge with several protein antigens while the second showed that B cells were responsible for the processing of these antigens, not DC (Constant et al., 1995a, 1995b).

The evidence presented above demonstrates that B cell antigen presentation contains many unique mechanisms, such as the highly efficient acquisition of membrane-bound antigen, which may permit presentation of antigens denied to other APC. B cells are also capable of interactions with CD4⁺ T cells which lead to reciprocal activation events thus bypassing the requirement for other APC to initiate T cell-driven immune responses. In conjunction with their ability to expand in response to 'danger' and T cell-derived activation signals, and therefore continue to present antigen throughout the course of an immune response, a strong case can consequently be made for B cell antigen presentation potentially forming an integral part of chronic inflammatory diseases such as RA. As a result, an improved understanding of B cell induction of CD4⁺ T cell cytokine production, especially in the context of autoimmune disease, is crucial for the development of more effective therapies.

1.4.4. B cell modulation of CD4⁺ T cell effector function

The range of cytokines released by APC, either as a result of CD4⁺ T cell recognition of pMHC complexes or independently of TCR engagement, in combination with the activation status of the APC involved, are important in determining the generation of effector CD4⁺ T cell responses (Zhu and Paul, 2008). B cells appear to maintain a dual role in mediating CD4⁺ T cell responses. Different B cell populations have been shown to induce activation and proliferation of both effector and regulatory CD4⁺ T cell subsets depending on the stage of disease (Matsushita et al., 2008).

B cells found within the joint in RA produce a wide range of cytokines many of which, such as TNF- α , IL-1 β and IL-6, are associated with disease pathology. B cells have also been shown to adopt distinct cytokine production profiles based on the surrounding cytokine milieu and CD4⁺ T cell interactions. When cultured with T_H1 cytokines, B cells have been shown to produce proinflammatory T_H1 cytokines and similarly for T_H2 cytokines. Harris *et al.* designated these B cell 'effector' populations as B_E1 and B_E2 respectively (Harris et al., 2000). B cells did not require BCR ligation for cytokine production but optimal responses were achieved in the presence of both antigen and CD4⁺ T cells. Such cytokine production by activated B cells has been implicated in directing CD4⁺ T cell responses. B cells can drive differentiation of naïve CD4⁺ T cells into T_H1 and T_H2 effector subsets due to production of cytokines such as IL-12 and IL-4 respectively (Johansson-Lindbom et al., 2003; Wojciechowski et al., 2009). This discovery has implications for the study of RA, as CD4⁺ T cell subsets producing key proinflammatory cytokines are involved in orchestrating pathology and polarised B cells could contribute to the amplification or perpetuation of a T cell-directed immune response.

In contrast to the induction of proinflammatory responses, B cells may also function as regulators of CD4⁺ T cells in an antigen-specific manner. B cells can promote expansion of CD4⁺CD25⁺T_{reg} and following CD40-ligand activation can also selectively expand these cells from a population of naïve CD4⁺ T cells via IL-2 production (Chen et al., 2009; Tu et al., 2008). Moreover, studies into induction of peripheral tolerance to self-antigen have shown that specific B cells can respond to tolerogenic antigen and produce IL-10 in order to enhance the generation of T_{reg} (Sun et al., 2008). The production of IL-10 by B cells serves as an example of a cytokine-producing subset with an antagonistic role to the aforementioned effector B cells. Regulatory B cells (B_{reg}) have been shown to stall the progress of autoimmune disease in various mouse models, including arthritis (Mauri and Ehrenstein, 2008). The characterisation of these cells has revealed a number of possible phenotypes described at various stages of B cell development, including transitional 2 (T2) marginal zone

precursor B cells, but secretion of IL-10 is a common factor (Rawlings et al., 2012). B_{reg} have been shown to be compromised in both mouse models of autoimmune disease and patients (Blair et al., 2010; Yanaba et al., 2008). The exact signalling mechanisms leading to the development of B_{reg} *in vivo* have yet to be clarified although numerous activation signals have been recognised as promising candidates. Initial reports demonstrated that B cell-restricted TLR signalling is required for resolution of EAE in mice through suppression of T_H1 and T_H17 responses (Lampropoulou et al., 2008). Tedder *et al.* later confirmed this requirement for MyD88 signalling and LPS was shown to be capable of initiating clonal expansion of IL-10-producing B cells (Yanaba et al., 2009). Interestingly, this study and others have demonstrated that CD40 ligation induces B cell IL-10 production. Indeed, Mauri *et al.* have identified that targeting splenic B cells with agonistic CD40 is sufficient to initiate expansion of B_{reg} derived from the T2 population which are capable of suppressing T_H1 differentiation and ameliorating experimental disease (Blair et al., 2009; Mauri et al., 2003). Recent work seeking to explain how B_{reg} are controlled *in vivo* has thoroughly demonstrated that cognate interactions with T cells provide IL-10-producing B cells with the necessary CD40 and IL-21R signals necessary to promote B cell expansion and immune suppression (Yoshizaki et al., 2012). Additional *in vitro* evidence has also shown that marginal zone B cells stimulated with BAFF can produce IL-10 due to binding of the BAFF-inducible transcription factor AP-1 to the IL-10 promoter (Yang et al., 2010). This implies that BAFF is also required to maintain the IL-10-producing B cell population *in vivo*.

The paradoxical role of B cells in both promotion and suppression of T cell responses may be explained by a model in which the inflammatory microenvironment generated during autoimmune disease, such as the joint synovium in RA, prevents expansion of regulatory B and T cell subsets and leads to enhancement of effector T cell responses by activated B cells (Lund and Randall, 2010). Ultimately, this information can be exploited to generate new treatments for RA by seeking to restore the normal balance of effector and regulatory cell subsets.

1.4.5. B cell-targeted therapies

In contrast to T cell-directed therapies, B cell depletion is highly effective in mitigating the symptoms of RA (Edwards et al., 2004). In patients with disease refractory to anti-TNF- α therapy, targeting of naïve and memory B cells using chimeric anti-CD20 monoclonal antibodies leads to alleviation of disease (Cohen et al., 2006). CD20⁺ B cells are subject to antibody-dependent cell-mediated cytotoxicity via Fc receptor-expressing cells, such as natural killer cells and macrophages. Some B cell populations exist within structural niches that are resistant to depletion and require recruitment of other mechanisms such as complement for deletion (Hamaguchi et al., 2005). Interestingly, the aforementioned lymphoid aggregates found in some RA patients appear to offer refuge for B cells from the effects of anti-CD20 and their presence correlates with the persistence of ACPA and RF antibodies (Rosengren et al., 2008). This may be due to the limited ability of the anti-CD20 to penetrate the synovial tissue. Plasma cells, which do not express CD20 and often exist in survival niches, are not targeted (Chu and Berek, 2013; DiLillo et al., 2008). Therefore, long-lived antibody responses are not significantly affected. The clinical benefits of therapy are limited to 7-8 months before the B cell population is replenished from surviving pro-B cells (Uchida et al., 2004). Repopulation of the B cell compartment is a consistently ordered process involving the initial return of immature B cells followed by plasmablasts and naïve B cells (Roll et al., 2006). Low levels of plasmablasts prior to initiation of treatment and successful deletion of memory B cells appear to be reliable predictors of a positive outcome indicating that removal of established autoreactive B cells is critical (Nakou et al., 2009; Vital et al., 2010). B cell depletion appears to be effective in inducing RA remission independent of changes in autoantibody titres, suggesting that antigen-specific B cells mediate disease progression via an alternative pathway, such as antigen presentation (Liossis and Sfrikakis, 2008). As a result, the overall picture from the use of B cell depletion therapy in RA supports the concept that the clinical efficacy of B cell depletion is due to ablation of synovial tissue-resident B cells and prevention of repopulation of the synovium with autoreactive B cells (Silverman and Boyle, 2008; Townsend et al., 2010; Voswinkel et al., 1999).

B cell depletion has also been scrutinised in mouse models of RA which allow for a more reductionist approach in order to elucidate the mechanism by which B cell depletion alleviates disease. In an antigen-induced arthritis model, not only is a reduction in autoantibody titres observed following B cell depletion, but also a diminished number of CD4⁺ T cells producing key inflammatory cytokines such as IFN- γ and IL-17 (Hamel et al., 2008). The observed effects of this therapy may be due to disruption of B and T cell responses to antigen (Bouaziz et al., 2007).

Interestingly, further work from Hamel *et al.* has suggested that pathogenic B cell subsets also inhibit the activity of T_{reg} suggesting that B cell depletion may be critical in allowing T_{reg} to control inflammation (Hamel *et al.*, 2011). Work in the EAE model has identified that removal of pathogenic IL-6-producing B cells is critical to alleviation of disease. Importantly, following B cell depletion in multiple sclerosis patients, previously elevated B cell production of IL-6 was normalised following reconstitution of the B cell population (Barr *et al.*, 2012). The modest reduction in autoantibody titres seen following B cell depletion in both RA patients and animal models cannot be explained by removal of long-lived plasma cells as previously discussed (Edwards and Cambridge, 2006). However, B cell depletion can remove cells at the pre-plasma cell stage and short-lived autoreactive plasma cells. Such plasmablasts are found in the spleen of arthritic mice and are preferentially depleted by anti-CD20 over long-lived bone marrow-resident antimicrobial plasma cells (Huang *et al.*, 2010). This finding may explain why B cell depletion reduces autoantibody levels without significantly affecting total serum antibody titres.

Work in B cell-deficient mice has suggested that B cells are required for the induction of $CD4^+$ T cell proliferation shortly after encountering antigen, independent of their antibody-producing role. However, most of this work has been done in the context of infection and has yet to be fully characterised in models of autoimmune disease (Whitmire *et al.*, 2009; Wojciechowski *et al.*, 2009). However, Shlomchick *et al.* have shown that, in the *lpr/lpr* model of autoimmune nephritis, B cells are critical for disease development. *Jh* knockout mice, expressing a mutation which prevents B cell maturation, were crossed with *lpr/lpr* mice. These mice did not develop the disease while, in contrast, *lpr/lpr* mice with an intact B cell population showed spontaneous autoimmunity (Shlomchik *et al.*, 1994). Further work in the MRL/*lpr* model showed that T cell infiltration, considered to be essential for disease pathogenesis, was totally lacking in B cell-deficient mice (Chan and Shlomchik, 1998). The same group sought to isolate the exact B cell function involved in the pathogenesis. They found that mice with membrane-bound but not soluble immunoglobulin could still develop nephritis as characterised by a cellular infiltrate (Chan *et al.*, 1999). This evidence suggests that B cells may influence T cell effector functions via autoantigen presentation or cytokine production (Shlomchik, 2008). This study echoes previously described work by O'Neill *et al.* using a mouse model of arthritis (O'Neill *et al.*, 2005). Similarly, in chimeric mice implanted with inflamed human synovial tissue, $CD4^+$ T cell activation is dependent on B cells as mice depleted of B cells could not support activation of adoptively transferred T cells. Furthermore, use of anti-CD20 monoclonal antibodies led to the inhibition of IFN- γ production (Takemura *et al.*, 2001b). This suggests that B cells were key factors in provoking $CD4^+$ T cell activation and cytokine production.

B cell depletion is not the only B cell-directed therapy currently being investigated. New monoclonal antibodies targeting BAFF have been developed and have achieved modest success in early clinical trials (Bracewell et al., 2009; Dillon et al., 2010; Looney, 2006; Tak et al., 2008; van Vollenhoven et al., 2011). *Syk* is localised to the cytoplasmic domains of FcγR and BCR-associated CD79α and serves to activate pathways involved in cell survival and cytokine production. Genetic deficiency of *Syk* in mice completely abrogates autoantibody-induced arthritis (Jakus et al., 2010). Similarly, *Syk* inhibitors have also been shown to be effective in suppressing inflammation in RA patients and animal models of the disease (Pine et al., 2007; Weinblatt et al., 2008). One proposed mechanism suggests that blocking FcγR signalling prevents activation of macrophages by immune complexes. Furthermore, the key role of *Syk* in BCR signalling and B cell survival further implicates these cells in RA pathology (Brasemann et al., 2006).

The aforementioned mechanisms by which B cells can modulate CD4⁺ T cell responses may explain the relative success of B cell-directed therapy in autoimmune disease. Furthermore, removal or suppression of autoreactive B cells may allow for the establishment of regulatory subsets and alleviation of disease (Lund and Randall, 2010). Consequently, this information provides a compelling foundation for further work into elucidation of B cell interactions with CD4⁺ T cells in RA.

1.5. Animal models of rheumatoid arthritis

Pioneering work into elucidating the mechanisms of B and T cell involvement in RA was facilitated by the development of various mouse models of the disease. A subset of these models, including collagen-induced arthritis (CIA) and proteoglycan-induced arthritis (PGIA), rely on immunisation of mice with cartilage components to induce autoimmunity. Such models in particular have emphasised the role of T cells in disease pathogenesis. These models offer the benefit of controlling the onset of disease pathogenesis and working with a genetically homogenous population of animals. Together, these features allow for comparatively simple replication of disease and a reductionist approach not possible with human subjects expressing complex and diverse phenotypes (reviewed in Kannan et al., 2005).

PGIA has been studied less extensively than CIA but has several distinguishing features. Notably, the use of aggrecan as an immunising antigen is relevant as proteoglycans are degraded in the early stages of inflammatory arthritis (Nagase and Kashiwagi, 2003). As a result, aggrecan epitopes may represent the first autoantigens that activate the immune response. Furthermore, intriguing evidence for the role of B cells in autoimmune arthritis has been revealed using this model.

1.5.1. Origins of candidate joint autoantigens

The synovium, which lines the joint cavity and is responsible for the production of the lubricating synovial fluid, is the principal site of inflammation in RA (Tak and Bresnihan, 2000). In the healthy joint, synovial fibroblasts secrete synovial fluid containing plasma and hyaluronate which provide nutrients and lubrication essential for joint maintenance and function (Freemont, 1996). However, increased vascularisation and synovial hyperplasia during RA pathogenesis are responsible for invasive *pannus* formation which destroys articular cartilage directly or through upregulation of cartilage-degrading enzymes (Choy, 2012; Otero and Goldring, 2007). Articular cartilage encapsulates the subchondral bone surface within synovial joints and is vital for enabling movement and absorbing structural load. Cartilage is a form of extracellular matrix (ECM) which serves as a structural scaffold for the joint architecture and contains enzymes, growth factors and cells that respond dynamically to developmental and environmental changes. Cartilage ECM consists primarily of a network of collagen fibrils interspersed with aggrecan and is synthesised and maintained by a sparsely distributed population of chondrocytes (Dudhia, 2005).

During the course of RA this connective tissue is degraded and the underlying bone is eroded. Subsequently, antigens derived from cartilage components such as aggrecan are made more accessible to recognition by the immune system. Evidence from both patients and animal models indicates that autoreactive immune cells responding to such autoantigens mediate RA pathogenesis (Goronzy and Weyand, 2009).

1.5.2. Structure, function and turnover of aggrecan

The proteoglycan aggrecan is a fundamental component of the cartilage ECM (Kiani et al., 2002). Aggrecan has several structural characteristics which allow it to fulfil an instrumental role in countering the elastic force of the collagen fibrils found in the ECM. This is made possible by the charged glycosaminoglycan (GAG) chains which generate an osmotic pressure and induce hydration of the matrix. Due to its size and association via link protein with hyaluronan (HA), aggrecan is static within the matrix and this prevents the osmotic gradient from being resolved. Furthermore, in conjunction with the collagen fibrils, these properties allow aggrecan to contribute to the structural integrity of the joint by resisting compressive loads (**Figure 1.2**) (Mow et al., 1989). The protein core of the aggrecan molecule consists of three globular domains (G_{1-3}). The inter-globular domain (IGD) separates the G_1 and G_2 domains and a large number of keratan sulfate (KS) and chondroitin sulfate (CS) side chains extend from the protein (Kiani et al., 2002). The core is post-translationally modified to incorporate the covalently attached GAG side chains (Wight et al., 1992). The structure of the G_1 domain has been shown to be essential for aggrecan binding to HA via link protein to form stabilised aggregates (Hardingham et al., 1994). The IGD is the region where proteolytic enzymes cleave aggrecan leading to the release of C-terminal fragments into the matrix. This forms the basis of aggrecan turnover. Turnover is essential for maintenance of the ECM in healthy joints but is dysregulated in RA. In contrast, the G_2 and G_3 domains are involved in aggrecan secretion and glycosylation (Kiani et al., 2002).

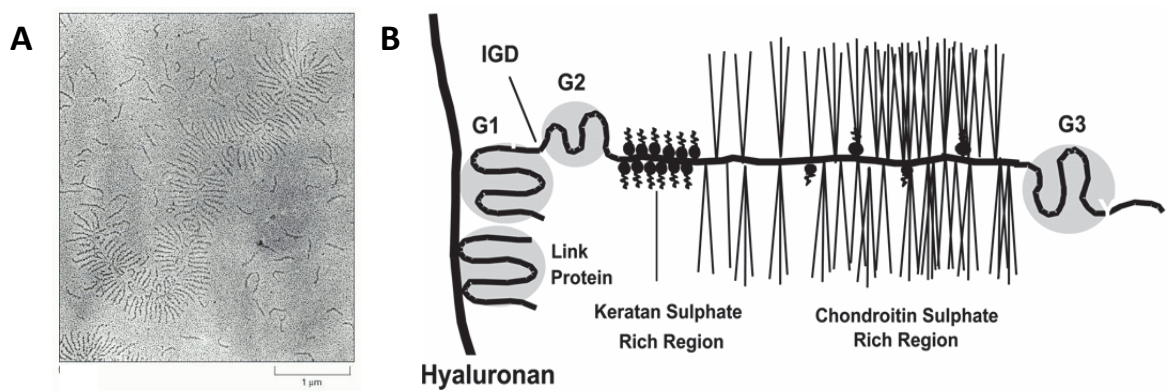


Figure 1.2 *The structure of aggrecan.*

(A) Electron micrograph of a hyaluronan chain and a large number of attached aggrecan molecules. (B) Schematic of the aggrecan molecule showing the link protein and core globular domains.

Adapted from Porter *et al.* (2005) and Alberts *et al.* (2002).

Chondrocytes are isolated cells found embedded in the cartilage ECM responsible for the maintenance of cartilage by constant turnover of the ECM components, including aggrecan (Maroudas et al., 1998). In arthritis, the net synthesis of ECM components is exceeded by degradation due to an imbalance in the levels of matrix metalloproteinases (MMP) and counterbalancing tissue inhibitors of matrix metalloproteinases (TIMP) leading to excessive cartilage breakdown (Otero and Goldring, 2007). Loss of aggrecan is considered to be an early event in arthritis which precedes collagen breakdown (Rousseau et al., 2008). Furthermore, aggrecan has been shown to protect collagen from degradation by collagenases, emphasising the importance of this step in disease pathogenesis (Pratta et al., 2003).

Aggrecan metabolism is mediated by proteolytic enzymes including MMP and aggrecanases (Nagase and Kashiwagi, 2003). Increased levels of MMP can be found in the joints of RA patients and MMP production by chondrocytes has been implicated in disease pathogenesis (Tetlow et al., 2001). Specifically, aggrecan is acted upon by ADAMTS (a disintegrin and metalloproteinase with a thrombospondin type 1 motif) hylectanases. These are zinc metalloproteinases and cleave the IGD domain (Sandy et al., 1991). Other proteolytic enzymes can cleave aggrecan but the ADAMTS family is particularly important in arthritis (Lohmander et al., 1993; Sandy and Verscharen, 2001). This notion is supported by evidence from ADAMTS-4 and ADAMTS-5 knockout mice which, following induction of inflammatory arthritis, were protected against aggrecan degradation (Stanton et al., 2005). The targeting of the enzymes responsible for joint destruction may have therapeutic benefits in arthritis patients but inflammatory diseases such as RA also include a leukocyte-mediated aspect of pathogenesis (Arner, 2002; Ishiguro et al., 2001). As a result, the turnover of aggrecan is a facet of the disease that may play an important role in the generation of autoimmune epitopes that instigate or propagate a cellular immune response.

1.5.3. Aggrecan as a key autoantigen

Although studies into joint autoantigens in RA have often focused on cartilage collagens, aggrecan has now emerged as a prominent candidate. Aggrecan has been shown to cause arthritis in mice via immunisation and, similar to collagen, adoptive transfer of leukocytes from these mice can induce disease in naïve recipients (Glant et al., 1987; Mikecz et al., 1990). This work in animal models has been supported by human research demonstrating the presence of immunogenic aggrecan epitopes in the synovial fluid of RA patients (Goodstone et al., 1996). Furthermore, T cells from RA patients can be activated by common aggrecan epitopes (De Jong et al., 2009). In particular, epitopes of the G₁ domain of the aggrecan core protein have received substantial interest as targets of cell-mediated autoimmunity with evidence for the presence of both B and T cell epitopes (Brennan et al., 1995a; Li et al., 2000; Zou et al., 2003). Indeed, the immunodominant arthritogenic CD4⁺ T cell epitope has been identified the amino acids spanning positions 84-103 within the G₁ domain (Guerassimov et al., 1998). As a result, a number of studies have focused on elucidating the mechanisms by which aggrecan can be processed and presented by APC leading to the development of autoimmunity.

Poole *et al.* determined that removal of the KS side-chains associated with the G₁ domain was required for aggrecan immunogenicity. Crucially, in a deglycosylated form, the G₁ domain alone was sufficient for inducing severe arthritis in mice leading the authors to conclude that this was the arthritogenic domain (Guerassimov et al., 1998; Leroux et al., 1996; Zhang et al., 1998). In support of this, Zou *et al.* examined CD4⁺ T cell responses to antigen-specific stimulation with the G₁ domain and found that >50% of RA patients developed a cellular immune response (Zou et al., 2003). Taken together, this work suggests that deglycosylated aggrecan is more arthritogenic than glycosylated aggrecan. This is supported by previous research showing that foetal aggrecan, which has fewer KS chains to mask the immune epitopes, is also more arthritogenic than the adult equivalent. Interestingly, removal of the CS chains, found at greater levels on foetal aggrecan, left stubs on the molecule which aided B cell recognition and presentation of aggrecan in combination with antibody production (Glant et al., 1998a; Zhang et al., 1998). This led to the hypothesis that, during arthritis, the adoption of a developmental phenotype by chondrocytes, in conjunction with increased turnover in the joint, leads to fragments of aggrecan being distributed into the synovial fluid. Consequently, cryptic epitopes, normally masked by carbohydrate side-chains may be exposed to the immune system and initiate an autoimmune response (Glant et al., 1998a).

B cells have been implicated in both aggrecan degradation and acquisition in arthritis. B cells have been shown to produce MMP-9, as modulated by stimulation with LPS or proinflammatory

cytokines such as IL-1 β and TNF α , which is capable of degrading ECM and, therefore, contributing to leukocyte infiltration of joint tissue (Melamed et al., 2006; Trocmé et al., 1998). Proinflammatory cytokines can also induce MMP-3 and MMP-9 production from plasma cells, which may be located in proximity to sites of inflammation in RA (Di Girolamo et al., 1998). Expression of these enzymes may facilitate exposure of aggrecan components to recognition by B cells. Furthermore, B cells express CD44, a cell-surface glycoprotein whose ligands include HA and aggrecan CS side chains (Fujimoto et al., 2001; Rafi et al., 1997). B cell proliferation and differentiation can be induced by HA binding to CD44 independent of BCR or TLR stimulation. HA can also act as an agonist for TLR2 and TLR4 opening up the possibility that aggrecan and HA may cross-link both the BCR and innate immune receptors leading to enhanced B cell activation and antigen presentation (Tesar et al., 2006) as described earlier.

The identification of the G₁ domain of aggrecan as a source of the immunodominant B and T cell epitopes has occurred in parallel with the development of a mouse model of RA which utilises joint proteoglycan as the arthritogenic agent. The PGIA model has subsequently proven to be a valuable resource for studying the relevance of aggrecan to RA pathogenesis and exposing the role of both B and T cells in this process.

1.5.4. Proteoglycan-induced arthritis

Glant *et al.* have induced arthritis by immunising BALB/c mice with cartilage proteoglycan and observed the development of a severe polyarthritis closely resembling the human disease (Glant and Mikecz, 2004; Glant et al., 1987). This was a BALB/c strain-specific response, implicating the H-2^d haplotype, and more prevalent in female mice (Glant et al., 1998b, 2003). A follow-up study confirmed that arthritic mice generated a combination of proteoglycan-specific antibodies and a cellular immune response (Glant et al., 1987). The PGIA model is dependent on cross-reactivity between various forms of aggrecan used for immunisation and the murine equivalent (Glant et al., 1998b). The globular domains of aggrecan are highly conserved across species, notably in humans and mice (Doege et al., 1991; Walcz et al., 1994; Watanabe et al., 1995). In addition, B and T cell epitopes have been identified in the bovine form of aggrecan which is commonly used to induce arthritis (Glant and Mikecz, 2004; Zhang et al., 1998). As a result, the PGIA model can exploit this cross-reactivity when immunising with cartilage proteoglycan derived from bovine or human tissue.

Peptides from the N-terminal G₁ domain of aggrecan have also been used to induce arthritis in disease-susceptible mice (Zhang et al., 1998). In addition, T cell hybridoma derived from the PGIA model can induce mild disease in naïve mice following adoptive transfer (Mikecz et al., 1990). This form of arthritis has been shown to be T_H1 CD4⁺ T cell-dependent, comparable to the human disease (Finnegan et al., 1999; Hollo et al., 2000; Li et al., 2000). The relevance of this model is supported by evidence suggesting that T cells specific for aggrecan are present in the synovium in RA patients and also produce T_H1-type cytokines (Zou et al., 2003). Although both IFN- γ and IL-17 contribute to PGIA, IFN- γ is the major proinflammatory cytokine involved. This is exemplified by the finding that IL-17^{-/-} mice are still susceptible to PGIA, albeit developing a somewhat milder form of disease (Doodes et al., 2008). This is in contrast to CIA, in which IL-17 is essential and loss of IFN- γ exacerbates symptoms due to its ability to suppress the action of IL-17. As a result, the levels of IFN- γ regulate the contribution of IL-17 to the inflammation seen in PGIA (Doodes et al., 2009).

Numerous studies have also implicated B cells in PGIA. Brennan *et al.* have demonstrated that fragments of cartilage proteoglycan can be presented to T cells via the MHC II complex and that the predominant APC involved were B cells (Brennan et al., 1995a, 1995b). B cell depletion using anti-CD20 monoclonal antibodies has been shown to suppress PGIA when treatment was initiated after antigen-specific T cell activation, but before the onset of severe arthritis. A subsequent reduction in proteoglycan-specific CD4⁺ T cells producing IFN- γ and IL-17 was observed (Hamel et al., 2008).

Furthermore, following targeting antigen to the BCR using a proteoglycan-hapten conjugate, O'Neill *et al.* have shown that autoreactive T cells are capable of transferring arthritis to recipient mice, even when the immunoglobulin was restricted to a membrane-bound form (O'Neill *et al.*, 2005). This suggests that B cells are acting as APC in order to effectively prime antigen-specific T cells. The APC function of B cells is further accentuated by a subsequent study using chimeric mice in which a genetic CD80/86 deficiency was restricted to B cells. These mice were resistant to the development of PGIA. As CD80/86 interactions with CD28 on T cells form an important part of B cell antigen presentation and reciprocal activation of both cell types, the authors concluded that an autoantibody-independent mechanism was required for induction of arthritis (O'Neill *et al.*, 2007). Interestingly, experiments inhibiting the capacity of T cells to migrate into joints have shown this has little effect on arthritis development. This suggests that one possible scenario may see the primary function of autoreactive T cells to provide help for B cell autoantibody production within the lymph nodes rather than the site of inflammation (Angyal *et al.*, 2010). These experiments do not address the initial APC responsible for autoreactive T cell activation but do point toward persistent autoantibody production being a key factor in the establishment of chronic disease.

1.5.5. Strengths and limitations of animal models

The wealth of data obtained from CIA and PGIA models, especially regarding the involvement of T cells and B cells, demonstrates the usefulness of mouse models of RA in isolating key characteristics of the disease. In particular, these models have aided identification of key proinflammatory T cell cytokines and elucidated the function of B cells as APC. These findings are of vital significance for understanding RA pathogenesis. Furthermore, these models help to overcome the significant limitations of using cell lines to study the interactions between APC and T cells. The use of *ex vivo* T cell populations allows for the direct measurement of T cell activation, proliferation and differentiation in response to antigen. This is in contrast to the use of cloned T cells or T cell hybridoma which may represent differentiated T cells or effector cells in a state of non-physiological activation. Furthermore, oncogenes derived from fusion partners may contribute expression of cell surface proteins not representative of the *in vivo* situation (Woodland et al., 1987).

However, animal models of RA in general do not fully replicate the complex pathology of the disease. There are multiple candidate autoantigens in RA while most models involve spontaneous immunity towards, or immunisation with, a single antigen. In addition, models of arthritis are limited by their dependence on the genetic background of the mice used (Asquith et al., 2009). For example, CIA is restricted to mice bearing the I-A^q, I-A^r and H-2^q MHC class II subtypes and is most efficiently induced in mice on the DBA/1 background (Courtenay et al., 1980). Study of long term disease is also constrained by the lack of chronicity, as DBA/1 mice enter remission after peak disease severity at approximately 35 days associated with an increase in serum IL-10 and a subsequent reduction of T_H1-associated cytokines (Mauri et al., 1996). This limitation is of particular significance as genetically modified transgenic mice are often generated on the C57BL/6 background which is resistant to CIA. Modified protocols have been developed to induce arthritis in C57BL/6 mice albeit with reduced disease incidence (Inglis et al., 2008). For example, in an effort to overcome these limitations, one group has recently developed a chronic arthritis protocol which induces disease in both C57BL/6 and BALB/c mice by first immunising with methylated bovine serum albumin (BSA), a potent immunogen, prior to co-administration of methylated BSA and bovine type II collagen one week later. Although arthritis remained less severe in C57BL/6 mice than BALB/c mice, this model did yield the interesting discovery of a 'periarticular' draining lymph node for the knee joint restricted to the BALB/c strain (Baddack et al., 2013). This observation, in conjunction with enhanced MHC class II presentation of autoantigens and higher serum anti-

collagen antibodies in the BALB/c strain, serves to highlight the effect of strain-specific features in animal models.

The situation is complicated further as some mouse models of arthritis also show conflicting results in regards to the relative importance of the T cell cytokines IFN- γ and IL-17. For example, IFN- γ is essential for the development of PGIA while IL-17 is an integral component of CIA pathogenesis (Doodes et al., 2008). Experiments modulating IFN- γ levels in CIA highlight the complexity of the cytokine microenvironment by demonstrating that IFN- γ can exacerbate disease in early arthritis but develop a protective function during later phases (Rosloniec et al., 2002). Furthermore, the different adjuvants used in conjunction with each antigen have been shown to affect the ratio of T_H1 to T_H17 cells present highlighting a the type of adjuvant as a confounding factor in the interpretation of animal studies of arthritis (Stoop et al., 2012). Interestingly, the levels of IFN- γ and IL-17 produced are not drastically altered suggesting a complex relationship involving feedback between these cytokines. It is clear that using CIA or PGIA as a model of arthritis can have a significant impact on the nature of the cytokines that drive disease. However, this distinction is informative as it suggests that the multitude of effector cytokines involved in human RA may have different roles at different stages in disease. Due to these factors, results obtained from models such as CIA and PGIA must be initially interpreted in context and extrapolations to human disease must be cautious. Despite this, it is important to note that key components of the immune response must be isolated in order to allow for the study of such a complex disease.

1.6. Knowledge gaps

In summary, the complex nature of RA has yielded many questions in regards to the interactions between T cells and other infiltrating cells. The hypothesis that presentation of joint antigens to autoreactive T cells is responsible for driving disease denotes that one or more key autoantigens must be present. However, despite several promising candidates, the significance of each potential joint component has not been fully clarified. This study aims to evaluate aggrecan as a candidate autoantigen for presentation to autoreactive CD4⁺ T cells. However, models of arthritis that rely on immunisation with joint autoantigens are often restricted by the lack of an accessible population of antigen-specific T cells. TCR-transgenic mice offer a solution to this problem by providing a homogenous population specific for a known antigen epitope. As a result, the present study uses an approach based on the use of the TCR-5/4E8-transgenic mouse line possessing a CD4⁺ T cell population skewed towards specificity for the immunodominant arthritogenic p84-103 epitope of cartilage aggrecan. This tool is used extensively to answer key questions regarding APC interactions with CD4⁺ T cells within the context of RA (Berlo et al., 2005, 2006). Furthermore, the relative importance of the APC responsible for presentation of autoantigens has not been fully elucidated. Evidence associates B cells with this role but this must be assessed in context with other APC. Therefore, this study incorporates an aggrecan-specific B cell line into an *ex vivo* antigen-presentation assay in order to compare aggrecan presentation by antigen-specific B cells to non-specific B cells and other APC (Wilson et al., 2012). Finally, the identification of a plethora of T cell subsets with unique cytokine profiles necessitates further characterisation of involvement in RA. Therefore, this study sought to identify the production of disease-associated cytokines by TCR-5/4E8-transgenic CD4⁺ T cells following presentation of aggrecan by different APC, including aggrecan-specific B cells.

1.7. Aims

In summary, this project aimed to achieve the following:

- 1) Characterisation of an *ex vivo* population of aggrecan-specific CD4⁺ T cells isolated from the TCR-5/4E8 -transgenic mouse.
- 2) Generation of a co-culture assay to assess CD4⁺ T cell activation and proliferation.
- 3) Comparison aggrecan presentation by different APC, including an antigen-specific B cell line, to CD4⁺ T cells.
- 4) Identification of disease-associated cytokine production by different effector T cell subsets.
- 5) Transfection of an *ex vivo* B cell population with an aggrecan-specific BCR to generate an entirely *ex vivo* system.
- 6) Investigation of the ability of aggrecan to act as a TLR ligand.

The literature suggests that the role of B cells as APC and modulators of CD4⁺ T cell function requires further elucidation in order to understand the pathogenesis of RA and to improve upon current B cell-directed therapies. Taken together, these experiments will illustrate the relative importance of antigen-specific B cells as APC. The use of the TCR-5/4E8-transgenic mouse to acquire a CD4⁺ T cell population specific for a RA candidate autoantigen will put this work in the context of autoimmunity. Furthermore, the identification of important pathogenic T cell subsets and their respective cytokine profiles will expand on previous work and help focus attention on the relevant effector populations involved in RA.

2. Methods

2.2. Antigens

2.2.1. Aggrecan

Bovine nasal aggrecan (Agg) was prepared as previously described (Wilson et al., 2012) and dissolved in 50 mM TRIS acetate, 10 mM EDTA (pH 7.4).. Deglycosylation (removal of GAG) was performed by the addition of 25 mU keratanase (Sigma) and 1.25 mU chondroitinase ABC (ICN/MP Biomedicals) per mg protein and incubated for 5 h at 37°C. Deglycosylated aggrecan (dAgg) was then dialysed into 50 mM Tris-HCl (pH 7.4), aliquoted and routinely stored at -20°C.

To determine the efficiency of aggrecan deglycosylation, a dimethylmethylen blue (DMB) assay was performed. DMB was prepared by dissolving 16 mg of solid DMB in 5 ml of ethanol, left for 12 h in the dark prior to addition of 2 ml of formic acid and 2 g sodium formate before dilution to 1 L with deionised water. 1 mg/ml chondroitin sulphate stock was diluted in 0.1 M phosphate buffer ($\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$; pH 6.5) over a range of standard concentrations from 0 to 40 $\mu\text{g}/\text{ml}$. Untreated Agg and dAgg samples were diluted in 0.1 M phosphate buffer (1:10, 1:50, 1:100, 1:500 and 1:1000) and 40 μl of each standard or sample was added to 250 μl of DMB solution in a 96-well plate (NUNC Maxisorp). Absorbance was read at 530 nm immediately. Based on the standard values, the glycoaminoglycan concentrations of Agg and dAgg were determined. dAgg from the preparations used in this study was routinely shown to contain approximately 90% less GAG than Agg. In addition, the BCA protein assay (Pierce) was used to determine the dAgg protein concentration. 2 mg/ml stock of BSA was diluted in PBS to standard concentrations ranging from 0 to 0.6 mg/ml. The dAgg stock was then diluted in PBS (1:5, 1:10, 1:20, 1:40, 1:80 and 1:160). 10 μl of the standard or sample to be tested was added to each well. The BCA reagent was then prepared by mixing 50 parts of Pierce reagent A with 1 part Pierce reagent B. 200 μl of this solution was added to each well and incubated at 37°C for 30 min. The plate was cooled to room temperature and the absorbance immediately read at 562 nm. Based on the standard values, the sample total protein concentrations were determined. If a higher concentration was required for further experiments, the sample was lyophilised and resuspended in an appropriate volume of PBS. To confirm similar deglycosylation and ensure estimated concentrations were accurate, all new dAgg preparations were compared to the previous stock in a proliferation assay utilising A20-Agg B cell line and the CD4⁺ T cell hybridoma 192 (details of the cells used can be found in section 2.4.1) (Figure 2.1).

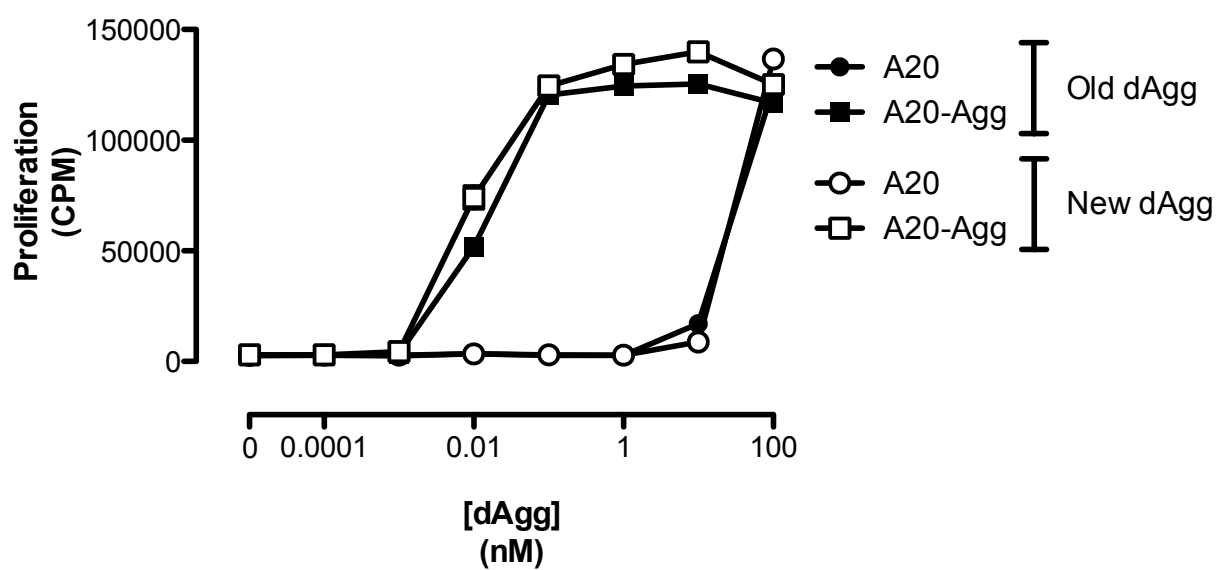


Figure 2.1 *Comparison of B cell responses to old and new stocks of dAgg.*

5×10^4 APC and 5×10^4 T cells were co-cultured for 24 h in the presence of graded doses of antigen in a flat-bottomed 96-well plate. Supernatants were extracted and added to 3×10^4 CTLL-2. Cultures were pulsed with radioactive thymidine (^3H) and DNA harvested onto a glass fibre mesh 18 h later. ^3H incorporation was measured using a scintillation counter and counts per minute (CPM) recorded. Each data point represents a triplicate with error bars showing $\pm$ SEM.

2.2.2. Streptavidin labelling of deglycosylated aggrecan

Streptavidin-conjugated dAgg (dAgg-SAv) was generated using the Lightning-Link™ streptavidin conjugation kit (Innova Biosciences) according to the manufacturer's instructions. Briefly, 100 µL of 1 mg/mL dAgg in PBS was added directly to lyophilised Lightning-Link™ mix containing 10 µL of LL-Modifier reagent and mixed gently. The vial was left to stand at RT for 3 h and 10 µL of LL-Quencher reagent added.

Analysis of labelling was performed by ELISA. 50 µL of 2 µg/mL biotinylated tetanus toxin C-fragment (TTCF) (generated using the EZ-Link Sulfo-NHS-LC-Biotin kit (Pierce) accordingly to the manufacturer's instructions) diluted in PBS was bound to a 96-well Immunosorb plate (NUNC) overnight at 4°C. The plate was washed 3 times with excess PBS + 0.1% Tween and blocked for 2 h at RT with PBS + 1% BSA. 100 µL of 2 µg/mL dAgg-SAv was added and incubated for 2 h at 4°C. The plate was washed again and 2 µg/mL of mouse anti-aggrecan monoclonal antibody (Wilson et al., 2012) added for a further incubation of 2 h at 4°C. The plate was washed again and 100 µL anti-mouse HRP (1:1000 dilution; BD Biosciences) added for 1 h at 4°C. Finally, the plate was washed thoroughly and 100 µL of 1% tetramethylbenzidine (TMB) substrate diluted in 0.3 µL/mL hydrogen peroxide phosphate-citrate buffer (0.2 M Na_2HPO_4 ; 0.1 M citrate) was added per well and allowed to develop for 5-15 min. The reaction was stopped using 50 µL of 2 M H_2SO_4 and absorbance read at 450 nm using a spectrophotometer.

2.2.3. Aggrecan peptide⁸⁴⁻¹⁰³

The p84-103 peptide (Cambridge Peptides) based on the bovine aggrecan sequence (Zou et al., 2003) was specified to be 80-95% pure using mass spectrometry and contained the sequence:

⁸⁴VLLVATEGRVRVNSAYQDK₁₀₃

The truncated citrullinated variant (JPT) contained the sequence:

⁸⁹ATEG-Cit-VRVNSAYQDK₁₀₃

Peptide stocks were stored at -20°C at a concentration of 2 mM.

2.2.4. Concanavalin A

Concanavalin A (ConA) (Sigma) was used as a positive control for lymphocyte activation, proliferation and IL-2 production and was included at a final concentration of 5 µg/ml.

2.3. T cell receptor-transgenic mice

TCR-5/4E8-transgenic mice backcrossed onto the BALB/c background (subsequently referred to as TCR-5/4E8-transgenic mice) were developed by Berlo *et al.* contain a CD4⁺ T cell population expressing transgenic TCR Vα1.1 and Vβ4 conferring specificity for the immunodominant arthritogenic p84-103 aggrecan epitope (Berlo et al., 2005; Berlo et al., 2006). All work using these mice was carried out under the project licence (PPL: 60/3281) of Prof. J. H. Robinson. Original breeding pairs were a gift from W. van Eden and used to establish a colony housed in Newcastle University's Comparative Biology Centre.

2.3.1. TCR-5/4E8-transgenic CD4⁺ T cell phenotyping

TCR-5/4E8-transgenic mice phenotypes were determined using flow cytometry. Approximately 50 µl tail-bleed blood samples from 5-8 wk old mice were incubated with 1 ml of red blood cell (RBC) lysis buffer (BD Biosciences) at 37°C for 10 min. The samples were washed in excess PBS before incubation with anti-CD4, anti-TCRβ and anti-Vβ4 antibodies (**Table 2.2**). Mice expressing a >80% Vβ4⁺CD4⁺ T cell population were deemed to be TCR-5/4E8-transgenic (TG) while littermates expressing a normal proportion of Vβ4⁺CD4⁺ T cells were identified as wild-type (WT) (**Figure 2.2**). Unless otherwise stated, all mice used for experiments were 8-16 wk old.

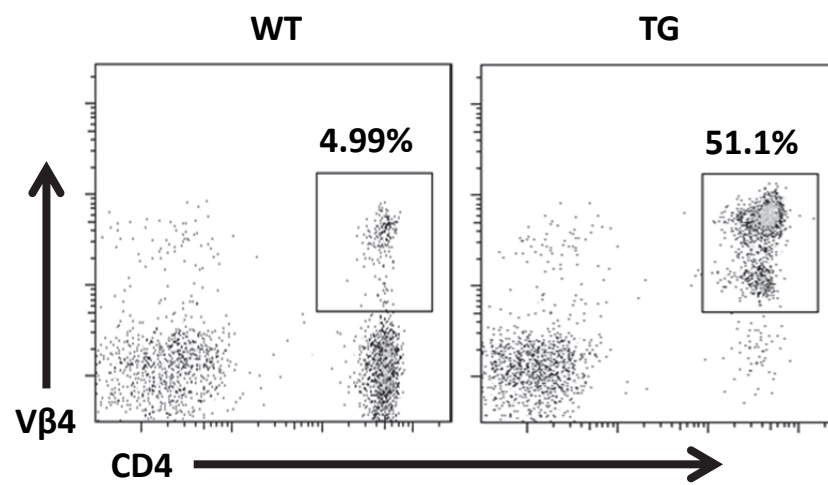


Figure 2.2 *Flow cytometric analysis of wild-type and TCR-transgenic CD4⁺ T cell TCR Vβ4 expression.*

Peripheral blood from wild-type (WT) and TCR-transgenic (TG) mice were treated with red blood cell lysis buffer and labelled with CD4 and TCR Vβ4 antibodies. Mice were determined to be TG when >80% of CD4⁺ cells (~50% of total population) expressed TCR Vβ4.

2.3.2. Characterisation of infiltrating lymphocytes in proteoglycan-induced arthritis

PGIA was induced in 12 wk old male TCR-5/4E8-transgenic mice by subcutaneous injection at the tail base with 100 µg of dAgg emulsified in complete Freund's adjuvant containing 1 mg/ml heat-killed *M. tuberculosis* (Difco, Detroit, MI) on d 0, followed by a subcutaneous boost with 100 µg of dAgg in incomplete Freund's adjuvant (Sigma-Aldrich) on d 21. Alternatively, mice were immunised by intraperitoneal injection with 100 µg of dAgg emulsified in dimethyldioctadecylammonium (DDA) (Sigma-Aldrich, Poole UK) on d 0, and again on d 21. Mice were sacrificed on d 28 following establishment of arthritis.

All four (arthritic and non-arthritic) feet were isolated from each mouse, skinned to remove subcutaneous tissue and cut into fine sections using a scalpel. Sections were then transferred to a glass homogeniser containing 1-2 mL of cRPMI to extract the cells from the joint tissue. The total cell yield recovered was pooled and counted. Cells were subsequently stained for expression of CD4 and B220.

2.4. Cell culture

2.4.1. Cell lines

The aggrecan-specific B cell line A20-C71/4C5 used throughout this study (subsequently termed A20-Agg) is a cloned A20 B cell transfectant that was previously constructed in our laboratory by Dr. Caroline Wilson (Wilson et al., 2012). The aggrecan-specific BCR is encoded by plasmids containing chimeric cDNA for the heavy (H) and light (L)-chain variable regions from a recently generated mouse anti-aggrecan monoclonal antibody (C71) spliced to a human membrane-bound IgG1/κ BCR. Previous experiments with this and other representative clones, have shown that the expression of this BCR is stable leading to specific binding and enhanced presentation of both soluble (Wilson et al., 2012) and artificially immobilised aggrecan (Ciechomska et al.; unpublished). To maintain expression of these plasmids, A20-Agg cells were continuously cultured in cRPMI supplemented with 0.5 mg/ml G418 and 0.75 mg/ml hygromycin B. Other cell lines used in this study are described in Table 2.1.

Name	Cell Type	Notes	Reference
192	T cell hybridoma	H-2A ^d -restricted p84-103-specific TCR	Prof J. H. Robinson
CTLL-2	T cell hybridoma	IL-2-dependent	(Gillis and Smith, 1977)
J774	Macrophage	-	(Ralph et al., 1976)
A20	B cell lymphoma	BALB/c H-2 ^d IgG2a/k	(Kim et al., 1979)
A20-Agg	Transfected B cell lymphoma	Aggrecan-specific BCR	(Wilson et al., 2012)
HEK-Blue	Human embryonic kidney	Transfected with PPR + reporter enzyme	InvivoGen

Table 2.1 *Description of cell lines utilised.*

2.4.2. Culture conditions

Cells were routinely cultured in complete RPMI [cRPMI: RPMI 1640 with 25 mM HEPES (Invitrogen) supplemented with 10% FCS (First Link), 2 mM L-glutamine (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 0.1 mM MEM non-essential amino acids solution, 100 µg/mL kanamycin (Invitrogen) and 0.05 mM 2-mercaptoethanol (Sigma)] at 37°C in a 5% CO₂ atmosphere in a humidified incubator and sub-cultured approximately every 48 h according to confluence. CTLL-2 were maintained by supplementing cRPMI with supernatant collected from an IL-2-expressing transfectant (IL-2T8) to a final concentration of 1.5 ng/ml IL-2. All antigen presentation assays involving cell lines were performed in serum-free medium (SFM; Invitrogen).

HEK-Blue cells were cultured in growth medium [DMEM, 4.5 g/L glucose (Invitrogen), 10% FCS (First Link), 2 mM L-glutamine (Invitrogen) and 100 µg/mL kanamycin (Invitrogen)] according to the manufacturer's specifications. Cells were passaged at 70-80% confluency and detached in the presence of PBS using a cell scraper. HEK-Blue cells undergo genotypic changes over multiple passages in normal cell culture conditions resulting in hyporesponsiveness. Therefore, HEK-Blue cells were not passaged greater than 10 times to ensure optimal efficiency and a large number of frozen stocks were prepared from early passages (1-4).

2.4.3. Irradiation

1x10⁶/mL A20 or A20-Agg in cRPMI were transferred to a 15 mL tube and irradiated with graded doses (0-50 Gy) of γ-radiation prior to addition of 100 nM p84-103 or dAgg. Cells were immediately pulsed with radioactive thymidine (³H) as described in section 2.9.

2.4.4. Cell preservation

For cell storage, batches of 1-2x10⁶ cells were resuspended in 1 ml of cold FCS + 10% DMSO (Sigma) and transferred to -80°C for 24 h prior to storage in liquid nitrogen. The cells were recovered from liquid nitrogen by rapid warming to 37°C followed by addition of pre-warmed cRPMI dropwise. The cells were then centrifuged at 300 G and resuspended in cRPMI for subsequent culture.

2.5. Splenocyte preparation

5-8 wk old male or female BALB/c or TCR-5/4E8-transgenic mice were sacrificed via a schedule 1 procedure and washed in 70% ethanol. Spleens were removed into 2 ml cRPMI. A single-cell suspension was prepared by forcing the spleen through a 100 µm sieve (BD Biosciences) immersed in 5 ml of cRPMI. Aggregates were removed by passing the cells through a 19-gauge needle prior to centrifugation at 300 G for 5 min. The splenocytes were resuspended in 2-3 ml of RBC lysis buffer (Sigma) and incubated at 37°C for 10 min. The cells were washed in 10 ml of cRPMI and centrifuged at 300 G for 5 min. Following resuspension in 5 ml of cRPMI, an aliquot of cells were counted using a haemocytometer in the presence of trypan blue (Sigma) to exclude dead cells. Following organ extraction from TCR-5/4E8-transgenic mice, mesenteric lymph node (MLN) cells or thymocytes were prepared using the same procedure.

2.6. TCR-5/4E8-transgenic CD4⁺ T cell isolation

CD4⁺ T cell yields and assay reproducibility were improved by pooling splenocyte populations from two or more TCR-5/4E8-transgenic mice prior to isolation. Splenocytes were resuspended in cRPMI and centrifuged at 300 G for 10 min. The supernatant was removed and the cells resuspended in 90 µl of cold MACS buffer (PBS; 0.5% BSA; 2 mM EDTA) per 10⁷ cells. 10 µl of anti-MHC II (M5/114.15.2) microbeads (Miltenyi-Biotec) were added per 10⁷ cells and incubated at 4°C for 15 min. The cells were washed in cold MACS buffer and centrifuged before resuspension in 500 µl of cold MACS buffer. LD columns were magnetised by placement in a Midi MACS separator (Miltenyi-Biotec) and prepared by washing with cold MACS buffer. The cells were passed through the column and the total MHC class II⁺ eluent was collected. The eluent was centrifuged, resuspended in 90 µl of MACS buffer per 10⁷ cells and incubated at 4°C for 15 min with 10 µl of anti-CD4 (L3T4) microbeads (Miltenyi-Biotec) per 10⁷ cells. The cells were washed, centrifuged and passed through an LS column. The CD4⁺ fraction was eluted by de-magnetising the column before washing with 5 ml of cold MACS buffer and applying a plunger to flush out the cells. The cells were centrifuged and resuspended in appropriate volumes of either SFM for cell culture or PBS + 2% FBS for flow cytometry.

2.7. Splenic CD43⁻ B cell isolation

Microbeads coated with anti-CD43 (Miltenyi-Biotec) were used to isolate CD43⁻ splenic B cells from BALB/c mouse splenocytes using an identical negative selection protocol to that previously described for the isolation of MHC class II⁻ cells prior to CD4⁺ T cell isolation from TCR-5/4E8-transgenic mice (described in section 2.6).

2.8. Bone marrow-derived cells

5-8 wk old male or female BALB/c mice were sacrificed via a schedule 1 procedure and placed in 70% ethanol. The femur and tibia were extracted and the muscle cut away before being placed in cRMPI until further processing. Heavy-duty scissors were used to cut the ends off the bones and the remaining bone was flushed through with cRMPI using a 3 mL syringe and 25-gauge needle. The collected bone marrow was then gently aspirated using a pipette to generate a single-cell suspension.

2.8.1. Dendritic cells

Bone marrow-derived cells were seeded onto 100 mm bacteriological Petri dishes at a density of 2×10^6 cells per plate in 10 ml of cRMPI containing 20 ng/ml GM-CSF (PeproTech). At d 3 of culture, 10 ml of GM-CSF-supplemented medium was added to the plate. At d 6, 9 ml of medium was removed carefully and replaced gently with 10 ml of fresh GM-CSF-supplemented medium and cultured until d 8 before this process was repeated. At d 10, 13.5 ml of medium was removed from each plate and the cells lifted. The DC were counted and re-suspended in SFM containing antigen for maturation for 18 h prior to the addition of T cells.

2.8.2. Macrophages

Bone marrow cells, prepared as above were seeded onto 100 mm bacteriological Petri dishes at a density of $2-4 \times 10^6$ cells per plate in 20 ml of cRMPI containing 10% M-CSF (monocyte colony-stimulating factor; L929 cell line supernatant) and 5% horse serum (Sigma). At d 6 of culture, the cells were lifted using a cell lifter and re-plated in new Petri dishes at a density of $2-4 \times 10^6$ cells per plate in 20 ml of medium. At d 10, 20 μ l of 100 ng/ml IFN- γ (R&D Systems) was added and the macrophages cultured for a further 24 h. The remaining medium was completely removed and replaced with fresh serum-free media (SFM) (Invitrogen) and the macrophages lifted using a cell scraper, washed thoroughly in cRMPI to remove the IFN- γ and counted prior to incorporation into antigen-presentation assays.

All bone marrow-derived cells were routinely analysed using flow cytometry for expression of MHC class II and co-stimulatory molecules before use in antigen-presentation assays.

2.9. Antigen presentation assays

Unless otherwise stated 2×10^5 MLN, spleen or thymocyte single-cell suspensions were cultured in 200 μ L cRMPI in a 96-well U-bottomed plate for 24 h in the presence of graded doses of antigen. 0.018 MBq methyl- 3 [H]thymidine (74 GBq/mmol) (Perkin-Elmer) was added to each well and incubated for a further 18 h at 37°C. The cells were lysed using a cell harvester (TomTec Harvester 96 Mach III M) and DNA collected on a glass-fibre capture mesh (Perkin-Elmer). The mesh was dried, soaked with scintillation fluid (Perkin-Elmer) and sealed in a plastic wallet (Perkin-Elmer). A scintillation counter (Perkin-Elmer MicroBetaTrilux) was used to record the number of counts per minute (CPM) for each well.

For comparison of different APC, 1.5×10^4 TCR-5/4E8-transgenic CD4⁺ T cells and 3×10^4 APC were co-cultured in a total volume of 200 μ L SFM containing graded doses of antigen in a flat-bottomed 96-well plate and incubated at 37°C in a 5% CO₂ atmosphere for 72 h. All cell types were routinely cultured alone with or without the maximal antigen dose as negative controls. Supernatants were collected and frozen at -20°C for cytokine analysis.

2.9.1. CTLL-2 bioassay

CTLL-2 were used to detect IL-2 production from activated T cells. After culture in the absence of IL-2 for 24 h, 3×10^4 CTLL-2 were added to 50 μ L of thawed supernatant. To confirm the CTLL-2 response was IL-2-dependent, a titration of recombinant IL-2 (0.05% IL-2T8 transfectant supernatant; estimated concentration = 3.4 ng/mL) was set up in parallel wells (**Figure 2.3**). Proliferation was measured as described in section 2.9.

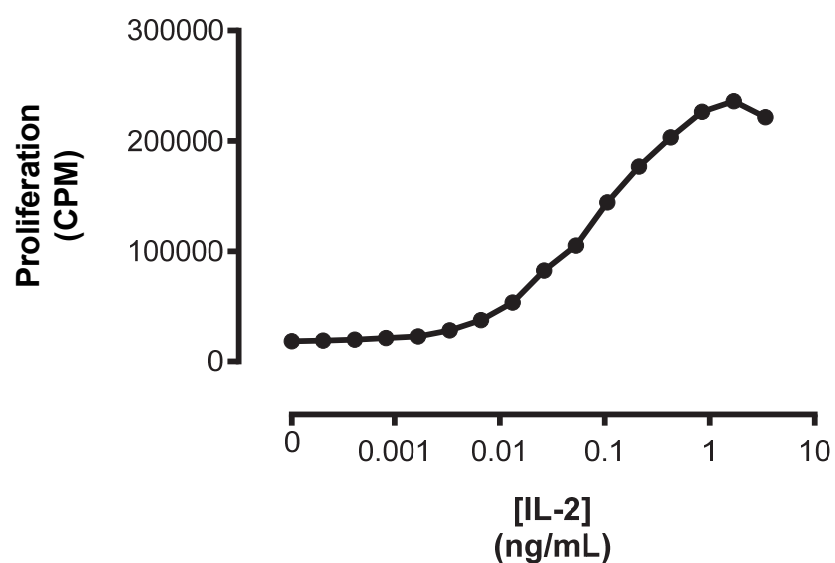


Figure 2.3 *Representation of the response of the IL-2 dependent cell line CTLL-2 to recombinant IL-2.*

3×10^4 CTLL-2 were incubated with a titration of IL-2 in a 96-well flat-bottomed plate and pulsed immediately with radioactive thymidine (^3H). DNA was harvested onto a glass fibre mesh 18 h later. ^3H incorporation was measured using a scintillation counter and counts per minute (CPM) recorded. Each data point represents a triplicate with error bars showing $\pm\text{SEM}$.

2.9.2. Antigen-loading assay

5 µg/ml biotinylated anti-mouse IgG F(ab')₂ monoclonal antibody (Jackson) was incubated with 1x10⁵/mL CD43⁻ isolated *ex vivo* splenic B cells for 20 min at 4°C in a 15 mL tube. 5x10⁴ or 1x10⁶ B cells were then added to graded doses of dAgg-SAv for 3 h at 37°C followed by the addition of 5x10⁴ T cell hybridoma 192 (200 µL culture volume; flat-bottomed 96-well plate, 24 h culture period) or 5x10⁵ TCR-5/4E8-transgenic CD4⁺ T cells (2 mL culture volume; 24-well plate, 72 h culture period). Supernatants were subsequently collected for cytokine analysis.

2.10. Flow cytometry

Approximately 1x10⁵ cells were added to FACS tubes and centrifuged at 400 G for 5 min. The supernatant was removed and the cells resuspended in 1 mL of cold wash buffer (PBS + 2% FCS). The cells were centrifuged at 400 G for 5 min and the supernatant removed. The cells were then resuspended in the remaining volume of wash buffer. 100 µL of 0.1 µg/mL 2.4G2 Fc block was added per 1x10⁶ cells and incubated at 4°C for 20 min. 50 µL of the relevant antibody was added to each tube and incubated at 4°C for 40 min. Primary biotinylated antibodies and secondary streptavidin-conjugated fluorochromes were added sequentially with separate incubations and wash steps. The cells were then washed again and resuspended in 100 µL of wash buffer. Alternatively, cells were fixed in 1% paraformaldehyde (Sigma) for 1 h and stored in the dark at 4°C for up to 24 h prior to acquisition. Flow cytometry data (approximately 15,000 events for characterisation experiments and 50,000 events for ICS experiments) was acquired on a FACS Canto II using the FACSDiva acquisition software (BD Biosciences). FlowJo (Tree Star) software was used to analyse the data and CD4⁺ T cells were routinely analysed using the gating strategy described in **Figure 2.4**.

2.10.1. Antibodies

All antibodies were diluted in PBS + 2% FCS and stored at 4°C. TCR Vβ typing was performed using a mouse TCR Vβ screening panel (BD Pharmingen) containing pre-diluted FITC-conjugated monoclonal antibodies for Vβ 2, 3, 4, 5.1/5.2, 6, 7, 8.1/8.2, 8.3, 9, 10^b, 11, 12, 13, 14, and 17^a. For details of the other antibodies used in this study see **Table 2.2**.

Target	Conjugate	Working Conc. (µg/ml)	Source
IgG	Biotin	1	Jackson
Biotin	Streptavidin-APC	2	BD Pharmingen
Biotin	Streptavidin-eFluor450	4	eBioscience
B220	PerCP	1	BD Pharmingen
CCR7	AlexaFluor647	2	BD Pharmingen
CD4	PerCP-Cy5.5	1	BD Pharmingen
CD11c	APC	1	BD Pharmingen
CD40	FITC	1	BD Pharmingen
CD44	Biotin	5	BD Pharmingen
CD62L	PE	1	BD Pharmingen
CD69	PE	1	BD Pharmingen
CD80	PE	2.5	BD Pharmingen
CD86	PE	2.5	BD Pharmingen
CD154	Biotin	5	BD Pharmingen
CXCR5	Biotin	5	BD Pharmingen
FcγIIIR (Fc Block)	Purified	0.1	BD Pharmingen
FoxP3	PE	N/A	Miltenyi Biotec
F4/80	PE-Cy7	1	BD Pharmingen
IFN-γ	FITC	20	BD Pharmingen
MHC class II	Biotin	5	M5/114.15.2
PD-1	PE	2	BD Pharmingen
Vβ4	Biotin	1	BD Pharmingen
TCRβ	FITC	5	BD Pharmingen

Table 2.2 *Antibodies used for flow cytometric analysis.*

The M5/114.15.2 anti-mouse MHC class II (I-A/I-E) monoclonal antibody (Bhattacharya et al., 1981) was purified in-house using a HiTrap Protein G HP column (GE Healthcare).

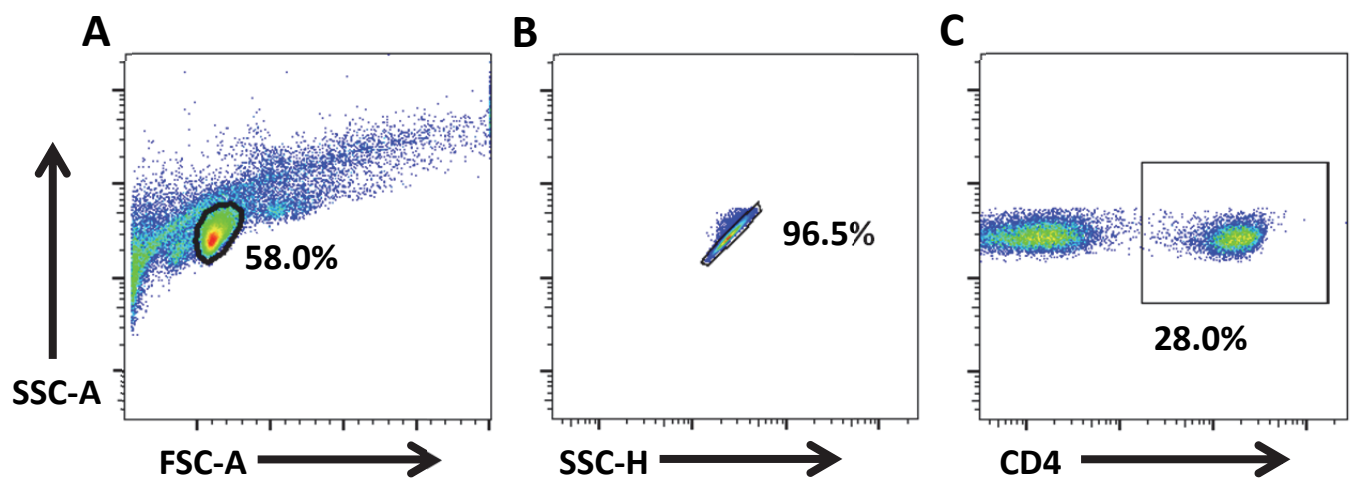


Figure 2.4 *Example gating strategy for flow cytometric analysis of CD4⁺ T lymphocytes.*

(A) The lymphocyte population was identified using a side scatter (SSC) and forward scatter (FSC) density plot. (B) Doublets were excluded by gating cells found on the 45° angle on a SSC-area (SSC-A) versus SSC-height (SSC-H) plot. (C) CD4⁺ lymphocytes were identified using a mouse monoclonal anti-CD4 antibody.

2.10.2. Intracellular cytokine staining

1×10^6 APC and 5×10^5 TCR-5/4E8-transgenic CD4⁺ T cells per well were resuspended in 2 mL of SFM containing antigen and co-cultured in 12-well plates for 72 h at 37°C. For the final 5 h, co-cultures were resuspended at 1×10^6 /ml and stimulated with phorbol myristate acetate (PMA; 50 ng/ml; Sigma) and ionomycin (500 ng/ml; Sigma) for 1 h at 37°C. 10 µg/ml Brefeldin A (Sigma) was subsequently added and the cells cultured for a further 4 h at 37°C. Cells were washed in cold PBS + 2% FBS and treated with anti-mouse FcγIIIR (Fc Block; BD Pharmingen) for 20 min at 4°C prior to staining with anti-mouse CD4 (BD Pharmingen) for a further 40 min at 4°C. The cells were washed in cold PBS, centrifuged and resuspended in 200 µl Fix/Perm buffer (eBioscience) for 30 min at 4°C before being washed with Perm buffer (eBioscience). Intracellular staining was performed using anti-mouse IFN-γ (clone XMG1.2; BD Pharmingen) and/or anti-mouse FoxP3 (Miltenyi Biotec) in 50 µl Perm buffer. The cells were washed again with Perm buffer and cold PBS before re-suspension in PBS and storage at 4°C for up to 18 h until flow cytometric analysis.

2.11. Enzyme-linked immunosorbent assay

Supernatants were harvested from co-cultures after 72 hours and cytokine levels were determined by sandwich ELISA (R&D Systems). The ELISA was performed according to the manufacturer's instructions. Briefly, 100 μ L of 4 μ g/mL capture antibody diluted in PBS was bound to a 96-well Immunosorb plate (NUNC) overnight at 4°C. The plate was washed 3 times with excess PBS + 0.1% Tween and blocked for 2 h at RT with PBS + 1% BSA. 100 μ L thawed assay supernatant or an 8-point 1:2 titration of the standard recombinant cytokine (top concentration for each cytokine as recommended by manufacturer) was added and incubated for 2 h at RT. The plate was washed again and 100 μ L of 200 ng/mL detection antibody added for a further incubation of 2 h at RT. The plate was washed again and 100 μ L streptavidin-HRP (1:200 dilution) added for 20 min at RT. Finally, the plate was washed thoroughly and 100 μ L of 1% tetramethylbenzidine (TMB) substrate diluted in 0.3 μ L/mL hydrogen peroxide phosphate-citrate buffer (0.2 M Na_2HPO_4 ; 0.1 M citrate) was added per well and allowed to develop for 5-15 min. The reaction was stopped using 50 μ L of 2 M H_2SO_4 and absorbance read at 450 nm using a spectrophotometer.

Duplicate or triplicate readings for each sample or control were averaged and a standard curve plotted using the standard recombinant cytokine results. A four-parameter logistic curve-fit was drawn and sample concentrations generated by interpolating the data. If the samples were diluted prior to the ELISA the interpolated values were subsequently multiplied by the dilution factor.

2.12. Transfections

2.12.1. Plasmids

One Shot® TOP10 (Invitrogen) chemically competent *E.coli* were transformed according to the manufacturer's instructions. Briefly, 10-50 µL of chemically competent *E.coli* were thawed on ice and 1-5 µL containing 1-10 ng of plasmid DNA was added directly to the *E.coli* and mixed gently. The cells were incubated on ice for 30 min before pulsing for 30 seconds in a 42°C water bath. 250 µL S.O.C medium pre-warmed to RT was then added and the cells transferred to a shaking incubator for 1 h at 37°C. The cultures were then spread onto LB agar plates and inverted in a 37°C incubator for 12-18 h. Individual colonies from these plates were then inoculated in 2 mL LB broth liquid cultures overnight in a shaking incubator at 37°C.

Plasmid DNA was isolated from overnight bacterial cultures using the QIAprep Miniprep kit (Qiagen) according to the manufacturer's instructions. Following harvesting by centrifugation at 13000 G for 2 min, proteins and genomic DNA was removed by precipitation and the plasmid DNA bound to the Miniprep column. DNA was eluted by addition of 50 µL endotoxin-free water to the column which was allowed to stand for 1 min in a sterile Eppendorf tube before centrifugation at 7000 G for a further 1 min. Plasmid DNA was quantified and purity estimated by measuring absorbance at 260/280 using a Nanodrop ND1000 system. Plasmid DNA isolated from Miniprep columns were stored at -20°C. Using a similar process, plasmid DNA was isolated from 250 mL bacterial cultures using the Endotoxin-free Maxiprep kit (Qiagen) and long-term plasmid stocks stored at -20°C.

The aggrecan-specific BCR H and κ L-chain plasmids containing chimeric cDNAs encoding the signal peptide followed by the variable VDJ and VJ respective regions from the C71 mouse (IgG1/κ) anti-aggrecan monoclonal antibody fused to the human IgG1 and κ constant regions were previously generated in the lymphocyte expression vectors pMCFR-HphI (Hygro) and pMCRF-NI (Wilson et al., 2012). In some experiments, expression plasmids (pMCFR-HphI (Hygro) and pMCRF-NI (Knight et al., 1997) or pCDNA3.1 (Invitrogen) containing cDNA encoding the human IgG1/κ anti-TTCF BCR derived from the TTCF-specific EBV clone 11.3 were also used (Demotz et al., 1989). The pmaxGFP plasmid was included in the B cell Nucleofector™ kit (Lonza). All plasmids were validated by appropriate restriction enzyme digestions followed by analysis by agarose gel electrophoresis using ethidium bromide (0.5 µg/mL) for visualisation under UV light.

2.12.2. Electroporation

2.12.2.1. A20 B cell line

12-well plates containing 1 ml of cRPMI supplemented with 1% ITS (Insulin-Transferrin-Sodium selenite; Sigma) were equilibrated in an incubator at 37°C with a 5% CO₂ atmosphere. 3x10⁶ A20 cells were added per sample tube and centrifuged at 300 G for 5 min and the supernatant removed ensuring that no residual medium remained. 100 µl PBS was added to each sample and 2 µg of plasmid DNA was added. The samples were mixed gently and immediately transferred to a cuvette. The cuvette was placed in a Nucleofector I device (Lonza) and program L-13 activated. 500 µL cRPMI pre-warmed to 37°C was then added to the cuvette. The cells were then immediately removed using a Pasteur pipette and transferred into a 6-well plate. The cells were analysed for transient cDNA expression 18 h later using flow cytometry.

2.12.2.2. Splenic CD43⁻ B cells

Following isolation, CD43⁻ B cells were stimulated with 50 µg/ml LPS for 24 h prior to transfection according to the Amaxa™ protocol. The A20 transfection protocol was then used except with the replacement of PBS with B cell Nucleofector™ Solution (Lonza) and the use of program Z-01.

2.12.3. Reverse transfection

2.12.3.1. HEK-Blue cells

Cells were lifted using a cell scraper, washed in PBS and resuspended at 1x10⁶ cells/mL in transfection medium (DMEM, 10% FBS, 2mM L-glutamine). 10 µL Lipofectamine™ (Invitrogen) was diluted in 40 µL DMEM per sample. 4 µg pmax GFP or 4 µg BCR H-chain + 4 µg BCR L-chain plasmid DNA were diluted in 50 µL DMEM. Each solution was left to rest for 5 min before the Lipofectamine™ and DNA solutions were combined and vortexed gently prior to incubation at RT for a further 5 minutes. 100 µL Lipofectamine™/DNA solution was scattered dropwise into a 6-well plate. 2 mL of HEK-Blue cells were immediately seeded on top of the transfection mixture. Plate was swirled clockwise for 30 seconds and incubated at 37°C for 48 hours. The cells were then lifted with a cell scraper, washed in PBS and either analysed by flow cytometry or incorporated into assays.

2.13. Measurement of ligand interactions with PRR using HEK-Blue colorimetric assay

Assays were performed according to the manufacturer's instructions. Briefly, 20 μ L of each sample was added in triplicate to each well in a flat-bottomed 96-well plate. HEK-Blue cells were prepared at the relevant concentrations. In parallel cultures, HEK-Blue cells were also incubated with graded doses of known ligands for 24 h to obtain a dose-response titration and standard curve (SC) (Table 2.3). PRR activation in the cultures stimulated with antigen was estimated by interpolating data from this dose-response titration.

180 μ L of cell suspension was added to each well and plates were incubated at 37°C for 20-24 h in the presence of antigen. 20 μ L of supernatant was then collected from each well and added to 180 μ L of QUANTI-Blue™ in a flat-bottomed 96-well plate and incubated at 37°C for a further 1 h. The plate was then read at 650 nm using a spectrophotometer.

Transfected TLR + Co-receptors	Concentration (cells/mL)	Ligand	SC Dose Range (ng/mL)
Mouse TLR2 + CD14	280,000	PGN	0 – 500,000
Mouse TLR4 + MD-2/CD14	140,000	LPS	0 – 100
Mouse TLR9	220,000	ODN	0 – 50,000
Mouse NOD2	220,000	MDP	0 – 500,000

Table 2.3 Description of HEK-Blue transfectants with cell concentration, known ligand and standard curve range for assays.

2.14. Statistics

Data were analysed using the PRISM software package (GraphPad Software). Differences between two groups were determined by applying the Student's two-tailed t -test for unpaired data assuming equal variance. When comparisons between multiple means were required the one-way analysis of variance (ANOVA) test was used. Results were considered significant if $p \geq 0.05$ (NS = not significant; * $p = \geq 0.05$; ** $p = \geq 0.005$; *** $p = \geq 0.005$ etc.). For HEK-Blue assays, background activation was considered equal to the (MEAN PBS) + (3 X SD) as indicated by the dashed red/blue line. Above-background values were considered significant if a p -value ≤ 0.05 was obtained when compared to background.

3. Characterisation and isolation of CD4⁺ T cells from the T cell receptor-5/4E8-transgenic mouse

3.1. Introduction

Antigen-specific T cells have been strongly implicated in RA pathology. T cells which respond to joint components have been found in arthritic synovium and genetic evidence suggests a role for cognate interactions with APC (Bennett et al., 2003; Eyre et al., 2012). Furthermore, mouse models of the disease have validated the role of joint-derived antigens by establishing models of arthritis induction using joint components such as collagen and aggrecan (Kannan et al., 2005).

The TCR found on T lymphocytes is responsible for recognising specific antigen bound to MHC and initiating intracellular signalling which leads to T cell activation. This mechanism has evolved to allow the immune system to specifically recognise and respond to foreign antigen. Likewise, TCR are also integral to the processes which, due to failures in either central or peripheral T cell tolerance, can lead to the immune system reacting destructively to self-antigen (Rojo et al., 2008). TCR must be highly diverse in order to recognise the range of potential antigens derived from pathogens. As a result, TCR are formed from an α -chain and β -chain which are arranged from a limited array of V, D and J gene segments and recombined in a process regulated by the lymphocyte-specific recombination-activating genes (RAG-1 and RAG-2). This finite combinatorial diversity is further expanded upon by random insertions and deletions that occur at the junctions between rearranged gene segments. Taken together, these mechanisms ensure a potentially massive repertoire of TCR specificities (10^{15} - 10^{20}) through modification of the antigen binding pocket (reviewed in Market and Papavasiliou, 2003).

The insertion of pre-arranged immunoglobulin genes into the germline of mice to generate BCR transgenic animals preceded the generation of development of TCR transgenic mice (Goodnow et al., 1988; Grosschedl et al., 1984; Rusconi and Köhler, 1985). However, since von Boehmer *et al.* first began to use TCR transgenic mice they have provided a valuable model in which to examine the mechanisms of selection, central tolerance and deletion within the context of T cell development in the thymus (Von Boehmer et al., 1989). Furthermore, these transgenic models have been a significant source of *in vivo* evidence for the role of peripheral self-reactive T cells in mediating the pathogenesis of autoimmune disease. Through the use of TCR transgenic mice disease progression can be monitored in an environment enriched in T cells possessing a TCR of a known specificity. TCR-transgenic mice allow researchers to assess the relevance of T cell subsets, potential autoantigens, molecular mimicry and the role of pathogens. As a result, these models have been successful as a reductionist method allowing for the independent study of different elements of this complex process.

Development of TCR-transgenic mice was made possible by optimisation of powerful methods of genetic modification. Pronuclear microinjection was developed in the 1980s and demonstrated to be effective for genetic transformation by gene integration and expression (reviewed in Wight and Wagner, 1994). Extracted embryo pronuclei are injected with isolated DNA fragments and transferred into the oviduct of pseudopregnant foster mothers. Transgenic mice in the subsequent litters are then identified by genetic analysis (Palmiter and Brinster, 1985; Pinkert, 2002). Expression of a transgenic TCR gene partially inhibits further germline recombination of the TCR genes via allelic exclusion (Brady et al., 2010; Lacorazza and Nikolich-Zugich, 2004; Uematsu et al., 1988). As a result, these mice have a significantly reduced TCR repertoire that includes a substantial T cell population expressing a single TCR α/β heterodimer specific for a known peptide sequence. This system allows experimenters to perform *ex vivo* studies with naïve antigen-specific T cells which are otherwise impossible to isolate from normal animals. This technology has proven to be essential in the production of the diverse experimental models that have allowed the study of T cell development and the pathology of autoimmunity.

In order to study antigen-specific T cell interactions in the context of RA, mice which express a transgenic TCR specific for a particular epitope of aggrecan have been developed and subsequently bred onto the PGIA susceptible, BALB/c (H-2^d) background (Berlo et al., 2005). The transgenic CD4⁺ T cell population found in the TCR-5/4E8-transgenic mouse line express an I-A^d-restricted TCR derived from the 5/4E8 T cell hybridoma which is specific for the immunodominant and arthritogenic p84-103 epitope from the G₁ domain of the RA candidate autoantigen, aggrecan. These T cells make up the vast majority (approximately 90%) of the CD4⁺ T cell population. The phenotype of the remaining 10% of CD4⁺ cells was not thoroughly characterised in the original research papers but will be addressed in this chapter. The TCR-5/4E8-transgenic T cells express a activation profile skewed towards a T_H1 phenotype (Berlo et al., 2006). This phenotype reflects the characteristics 5/4E8 T cell hybridoma which was previously shown to secrete IL-2 and IFN- γ following p84-103 stimulation but not IL-4 (Berlo et al., 2005). Accordingly, initial studies demonstrated that these TCR-5/4E8-transgenic CD4⁺ T cells proliferate *in vitro* in response to p84-103 and undergo a dynamic polarisation during the course of arthritis development *in vivo* to preferentially produce IFN- γ (Berlo et al., 2006).

Immunisation of TCR-5/4E8-transgenic mice with human aggrecan leads to development of a severe polyarthritis with symptoms and histology closely resembling the human disease. Importantly, the multiple immunisations required when using WT BALB/c mice were found to be unnecessary for arthritis induction when using TCR-5/4E8-transgenic mice. This highlights the efficiency of using a T cell population restricted to a known epitope (Glant et al., 1987). Furthermore, adoptive transfer of naïve TCR-5/4E8-transgenic splenocytes into syngeneic immunodeficient recipients leads to development of arthritis, in contrast to naïve WT BALB/c splenocytes (Berlo et al., 2005, 2006). As the 5/4E8 TCR is specific for human aggrecan, the induction of T cell-mediated arthritis in TCR-5/4E8-transgenic mice is based on cross-reactivity between homologous sequences from human or bovine aggrecan and equivalent found in the murine host cartilage aggrecan (Holler and Kranz, 2004). The induction of arthritis by adoptively transferred naïve TCR-5/4E8-transgenic cells without prior stimulation with human aggrecan supports the notion that arthritis in this model is due to T cell activation by host peptides generated by cartilage turnover or inflammation. This is further reinforced by evidence from the PGIA model indicating that T cell proliferation in response to the mouse homologue of p84-103 is only detectable in cultures from arthritic mice previously immunised with human aggrecan (Berlo et al., 2006). In addition, T cells transferred from PGIA mice exhibit homing behaviour to synovial tissue in naïve hosts in contrast to T cells from non-arthritic donors (Mikecz and Glant, 1994).

CD4⁺ T helper cell subsets determine the severity of arthritis in both mouse models and humans due to their production of pathogenic and regulatory cytokines such as IFN- γ and IL-10. Therefore, as this study aimed to assess the relative importance of different APC in presentation of aggrecan, it is crucial that the cytokine-production by the CD4⁺ T cells used in this study can be modulated by APC-derived signals. By using CD4⁺ T cells isolated from TCR-5/4E8-transgenic mice, this study aimed to bypass the limitations of antigen-specific T cell hybridoma which, due to their terminally differentiated state, are incapable of skewing towards different CD4⁺ T cell subsets in response to environmental and co-stimulatory signals.

The experiments presented in this chapter aimed to characterise further the phenotype of TCR-5/4E8-transgenic CD4⁺ T cells. This is a key objective as any unexpected idiosyncrasies in the phenotype of this population could have significant consequences in later experiments seeking to examine T cell skewing and cytokine production. Furthermore, in preparation for development of an *ex vivo* assay to compare the ability of different APC to activate CD4⁺ T cells, experiments were performed to:

- a) Determine the kinetics of TCR-5/4E8-transgenic CD4⁺ T cell activation.
- b) Establish the optimum conditions for proliferation assays.
- c) Isolate TCR-5/4E8-transgenic CD4⁺ T cells in the absence of APC.

This final goal is particularly vital as any contaminating APC in the CD4⁺ T cell preparation would potentially invalidate results obtained comparing different APC populations.

3.2. Phenotypic characterisation of the T cell compartment of the TCR-5/4E8-transgenic mouse

3.2.1. TCR V β -chain expression on wild-type BALB/c and T cell receptor-5/4E8-transgenic CD4⁺ T cells

To confirm that the expected proportion of CD4⁺ T cells from TCR-5/4E8-transgenic splenocyte preparations expressed the transgenic TCR, an array of TCR V β -chain antibodies were first utilised to examine the CD4⁺ T cell population by flow cytometry (**Figure 3.1**). The variable region of the transgenic TCR consists of V α 1.1 and V β 4 chains (Berlo et al., 2005). Therefore, in this study, TCR-5/4E8-transgenic mice were identified using an antibody specific for V β 4 as to date no antibody for V α 1.1 has been developed.

Figure 3.1A shows superimposed histograms for WT BALB/c and TCR-5/4E8-transgenic splenocytes stained for CD4 and an array of TCR V β -chains. Cells were gated on CD4 before being analysed for TCR expression. Interestingly, the TCR-5/4E8-transgenic CD4⁺ T cell population was divided into cells expressing high (V β 4^{hi}) and low (V β 4^{lo}) levels of TCR which was not explored previously by Berlo *et al.* during initial studies with these mice (Berlo et al., 2005, 2006). Later work in this chapter will address whether this issue is related to other aspects of the T cell phenotype (**Figure 3.3**) or responses to cognate antigen (**Figure 3.8**).

Figure 3.1B represents pooled data from duplicate experiments directly comparing expression of each TCR V β -chain on WT and TCR-5/4E8-transgenic CD4⁺ splenocytes. The data show that, as expected, the TCR-5/4E8-transgenic CD4⁺ population expresses predominantly V β 4 (94.4 $\pm$ 2.2%; n = 2) with other TCR V β -chains under-represented in comparison to the WT population which displays a range of TCR V β -chains and a substantially lower percentage of V β 4⁺ cells (7.85 $\pm$ 0.35; n = 2).

These experiments indicated the presence of the transgenic 5/4E8 TCR on the vast majority of CD4⁺ splenocytes and suggested that this highly homogenous population was suitable for experiments examining T cell activation by aggregan.

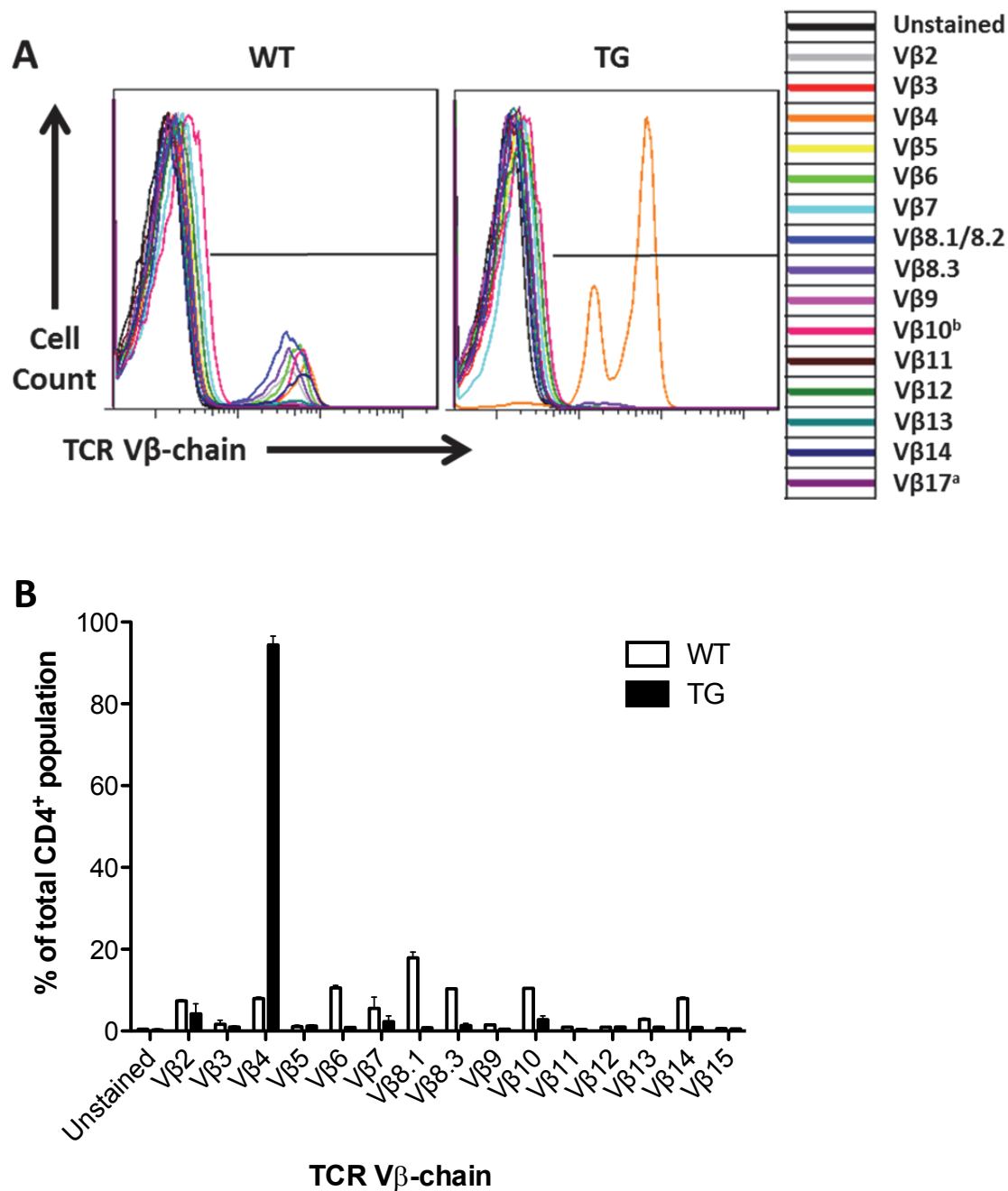


Figure 3.1 Flow cytometric analysis of wild-type BALB/c and TCR-5/4E8-transgenic CD4⁺ T cell TCR Vβ-chain expression.

(A) 5×10^5 splenocytes from wild-type (WT) and TCR-5/4E8-transgenic (TG) mice were labelled with an array of TCR Vβ-chain antibodies and gated on CD4⁺ T cells for analysis. (B) Data showing % positive CD4⁺ T cells for each TCR variable region antibody was pooled from two independent experiments.

3.2.2. Expression of activation, follicular helper and memory markers on wild-type

BALB/c and T cell receptor-5/4E8-transgenic CD4⁺ T cells

Further investigation of the TCR-5/4E8-transgenic splenocyte CD4⁺ T cell population was undertaken using multi-parameter flow cytometry to demonstrate phenotypic similarity with WT BALB/c CD4⁺ T cells. Of particular importance to this project was confirming that the TCR-5/4E8-transgenic CD4⁺ T cells expressed a predominantly naïve T cell phenotype, as it was thought that an abnormally enlarged memory compartment may affect T cell cytokine responses in later experiments. Furthermore, this analysis also presented an opportunity to determine whether the two populations of TCR-5/4E8-transgenic CD4⁺ T cells distinguished by high and low TCR expression (**Figure 3.1A**) could not be further discriminated by memory marker expression. These populations have yet to be fully characterised and a more complete understanding would benefit future work using the TCR-5/4E8-transgenic mice.

As can be seen in **Figure 3.2A**, a small percentage of CD4⁺ cells from either WT BALB/c (2.79±1.01%) or TG (2.99±0.61%) expressed either CD69 or CD154 (WT = 4.58±0.74%; TG = 5.80±0.62%) and the levels of expression of both markers were low, thus confirming that the CD4 T cells from the TG mice demonstrated a similar naïve phenotype to those seen in WT BALB/c mice. Furthermore, no evidence was found for the presence of increased numbers of T_{FH} cells, which express a PD-1⁺CXCR5⁺ phenotype, in either the WT BALB/C or TCR-5/4E8-transgenic populations (**Figure 3.2B**). Enriched numbers of T_{FH} cells are associated antigen-specific activation and subsequent formation of germinal centre reactions in the spleen which form the site of mature B cell class-switching (Fazilleau et al., 2009).

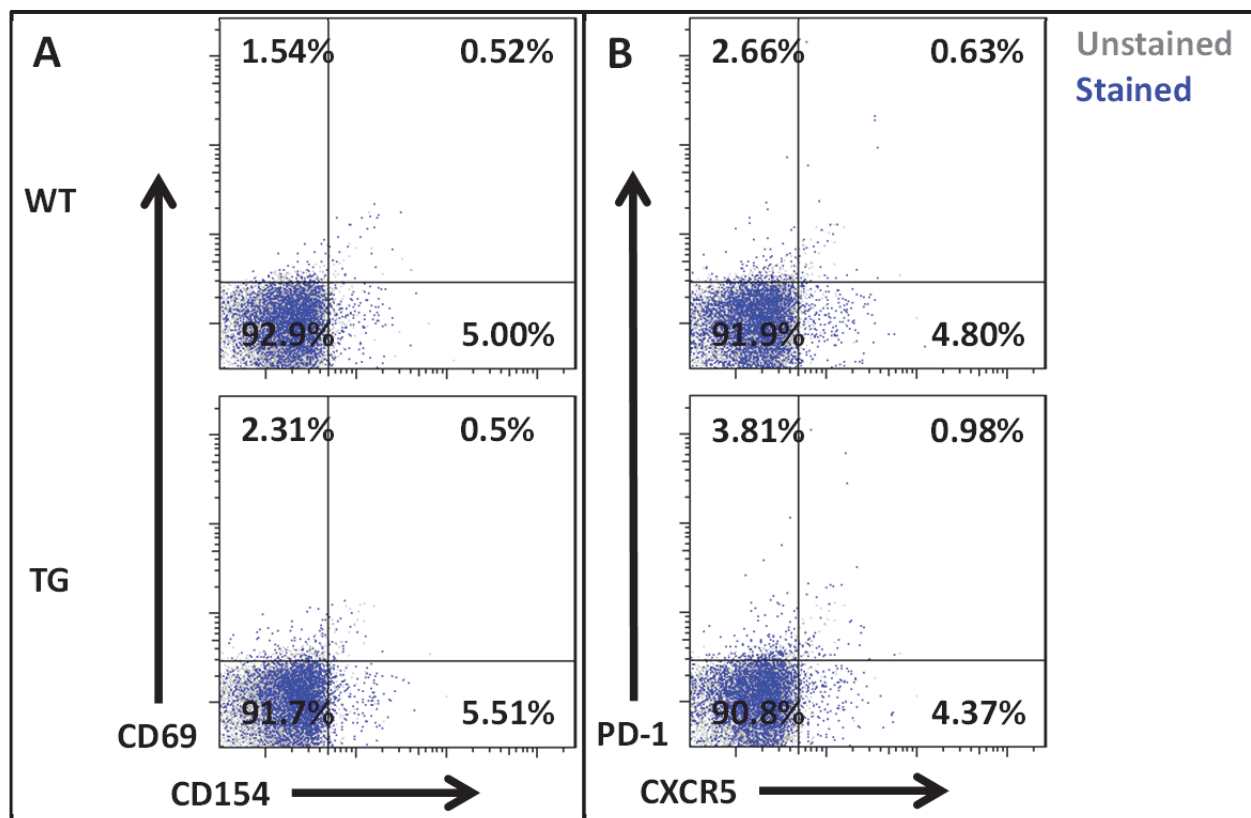


Figure 3.2 Flow cytometric analysis of wild-type BALB/c and TCR-5/4E8-transgenic CD4⁺ T cell expression of activation and follicular helper T cell markers.

Splenocytes from wild-type (WT) and TCR-5/4E8-transgenic (TG) mice were labelled with an array of antibodies designed to identify (A) activated and (B) follicular helper CD4⁺ T cells. Graphs show gated CD4⁺ T cells. Data representative of three separate experiments.

Memory T cells can be divided into at least two subsets: effector memory (T_{EM}) and central memory (T_{CM}) T cells. CD62L is expressed primarily on naïve cells and is downregulated following activation allowing T cells to enter secondary lymphoid organs via the high endothelial venules before migration to non-lymphoid sites of inflammation (Pepper and Jenkins, 2011). In addition to a reduction in CD62L expression, T_{EM} also express increased levels of CD44 which serves to prevent apoptotic cell death (Baaten et al., 2010). Unlike T_{EM} , T_{CM} express both CD62L and CCR7 which is thought to aid migration and circulation through lymphoid organs where these cells may encounter antigen again and undergo a secondary response (Pepper and Jenkins, 2011).

Therefore, to establish if the memory compartment was altered in $CD4^+$ TCR-5/4E8-transgenic $CD4^+$ T cells, the expression of CD62L, CD44 and CCR7 memory T cell markers was compared to the WT BALB/c population. **Figure 3.3** shows both the TCR-5/4E8-transgenic and WT BALB/C $CD4^+$ T cell populations expressed similar levels of all three markers. While $88.9 \pm 2.2\%$ ($n = 3$) of the TCR-5/4E8-transgenic $CD4^+$ cells were $CD62L^+$, only low levels of CCR7 were expressed on $8.1 \pm 2.2\%$ ($n = 3$) of $CD62L^+$ cells suggesting that the majority of cells are naïve and only a small proportion represent a T_{CM} phenotype (**Figure 3.3A**). Almost all of $CD4^+$ T cells were $CD44^+$ ($99.5 \pm 0.1\%$; $n = 3$). However, $CD62L^-$ cells expressed higher levels of CD44 (MFI = 9950 ± 1307 ; $n = 3$) than $CD62L^+$ cells (MFI = 4231 ± 447 ; $n = 3$) suggesting the presence of a $CD62L^-CD44^{hi}$ T_{EM} population. In addition, both the $CD4^+V\beta4^{hi}$ and $CD4^+V\beta4^{lo}$ TCR-5/4E8-transgenic T cell populations were shown to express similar levels of CD62L. However, it was apparent that the $V\beta4^{hi}$ population contained the majority of $V\beta4^+CD62L^-$ cells (**Figure 3.3B**). This suggests the $V\beta4^{lo}$ T cell phenotype may be constrained to naïve T cells due to defective memory T cell development in this population or, alternatively, the $V\beta4^{hi}$ population may contain an increased number of activated cells.

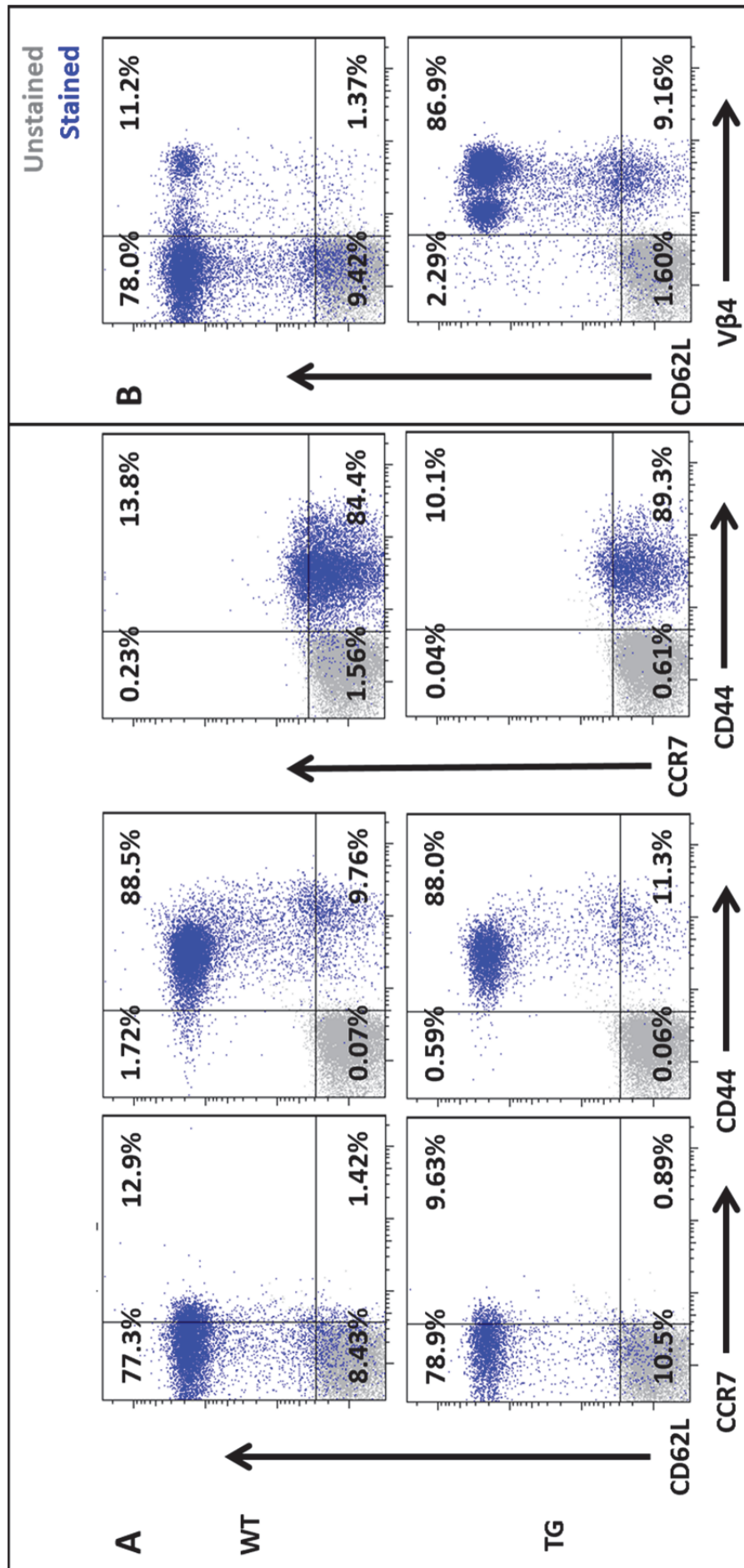


Figure 3.3 Flow cytometric analysis of wild-type B6.129/SvEv and TCR-5/4E8-transgenic CD4⁺ T cell expression of memory T cell markers.

Splenocytes from wild-type (WT) and TCR-5/4E8-transgenic (TG) mice were labelled with an array of antibodies designed to identify memory CD4⁺ T cell subsets. Graphs show gated CD4⁺ T cells. Data representative of three separate experiments.

3.3. T cell receptor-5/4E8-transgenic splenocyte responses to antigen

3.3.1. Proliferation of TCR-5/4E8-transgenic lymphocyte populations in response to aggrecan and p84-103

In order to establish the most appropriate tissue from which to isolate TCR-5/4E8 CD4⁺ T cells for use in experiments comparing antigen presentation by different APC, the responses of mesenteric lymph node (MLN), splenocyte and thymus cell preparations from TCR-5/4E8-transgenic mice to graded doses of glycosylated (gAgg), deglycosylated aggrecan (dAgg) and p84-103 were measured (Figure 3. 4).

As expected, both MLN cells (Figure 3.4A) and splenocytes (Figure 3.4B) from TCR-5/4E8-transgenic mice responded to p84-103, although MLN cells demonstrated a reduced maximum proliferation in comparison to splenocytes, which showed substantial proliferation responses even at low doses (1 nM). In contrast, thymocytes exhibited comparatively poor proliferation following p84-103 stimulation (Figure 3.4C). Low-level proliferation was induced by dAgg in the splenocyte cultures between 1 and 200 nM but both MLN cells and thymocytes did not respond to dAgg even at high doses. Interestingly, all lymphocyte populations failed to respond to gAgg. In light of this information, splenocytes were determined to be the most suitable population for future study into TCR-5/4E8-transgenic T cell activation and proliferation due to their robust response to antigen *ex vivo*.

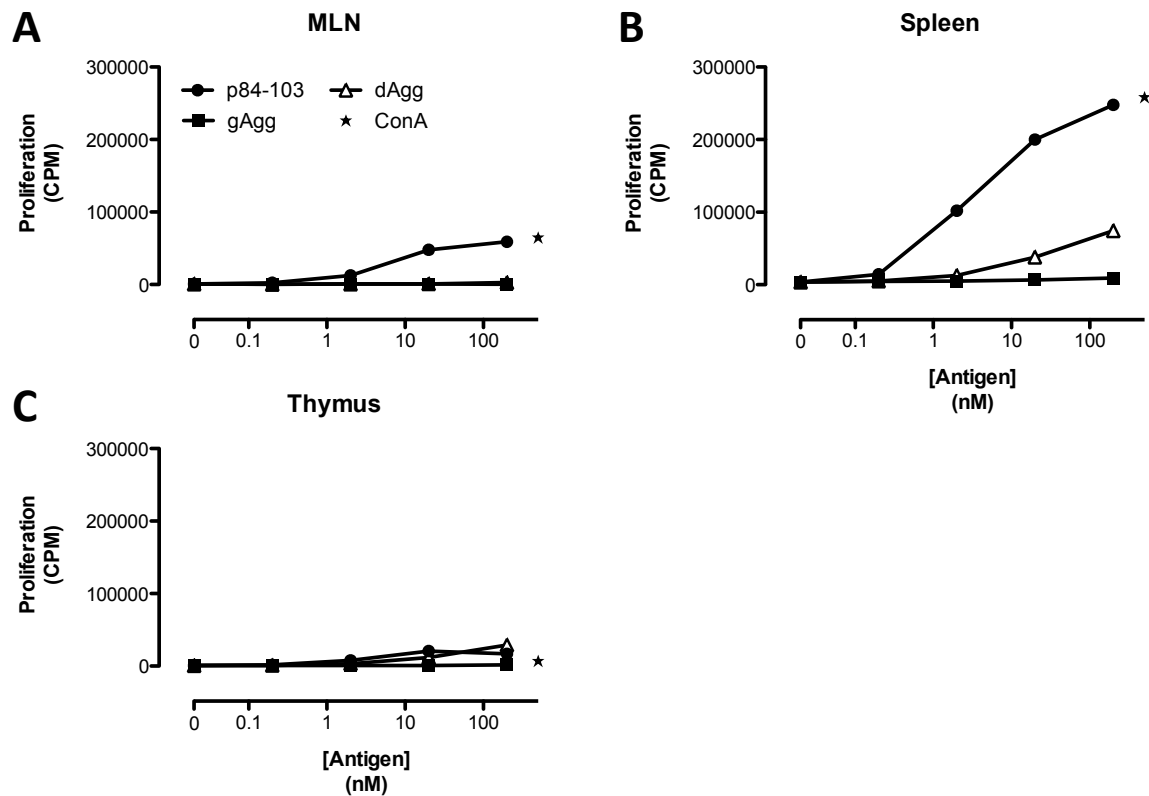


Figure 3.4 Proliferation of TCR-5/4E8-transgenic lymphocyte populations in response to graded doses of glycosylated (gAgg) and deglycosylated (dAgg) aggrecan and p84-103.

5×10^4 (A) mesenteric lymph node (MLN), (B) spleen and (C) thymus single-cell suspensions were cultured for 24 h in the presence of graded doses of antigen in a U-bottomed 96-well plate. The cultures were pulsed with radioactive thymidine (^3H) and DNA harvested 18 h later. ^3H incorporation was measured using a scintillation counter and counts per minute (CPM) recorded. ConA-stimulated cells were included as positive controls. Each data point represents a triplicate with error bars showing the SEM. Data representative of 2 independent experiments.

3.3.2. Proliferation of TCR-5/4E8-transgenic splenocyte in response to a citrullinated variant of p84-103

Having confirmed the specificity of the TCR-5/4E8-transgenic splenocyte population, it was of interest to examine the response of CD4⁺ T cells from these animals to modifications of the p84-103 peptide. Citrullination is a post-transcriptional modification capable of generating 'altered self' peptide sequences which may be recognised as foreign antigens by the host immune system. Many RA patients have antibodies to citrullinated peptides in their serum and high titres are associated with an increased probability of disease progression (Klareskog et al., 2008). In addition, CD4⁺ T cells in patients may express citrullinated peptide-specific TCR that have escaped central tolerance mechanisms due to a lack of citrullinated self-antigens presented in the thymus. Indeed, T cells from patients have been shown to respond to citrullinated peptides, including those derived from aggrecan, and producing inflammatory cytokines (Uysal et al., 2010). Interestingly, the immunogenicity of self-antigens, particularly aggrecan, can be increased by citrullination due to an increase in antigen affinity for HLA-DR molecules containing the shared epitope. Furthermore, preliminary studies suggest responses to a larger diversity of citrullinated antigens occurs during the later stages of RA, indicating a dynamic change in T cell epitopes throughout disease (Law et al., 2012). Taken together, this work suggests citrullinated antigens have the potential to change the affinity of CD4⁺ T cells for antigens such as aggrecan in RA. Therefore, a preliminary experiment was performed to determine whether TCR-5/4E8-transgenic splenocytes demonstrate modified proliferation responses to a truncated citrullinated variant of p84-103 (cit.p89-103).

Figure 3.5 shows that, in comparison to the unmodified p84-103 peptide, TCR-5/4E8-transgenic splenocytes proliferated only when stimulated with cit.p89-103 at high doses (10-1000 nM). However, this proliferation was not as substantial as the response seen to the unmodified peptide and plateaued at 100 nM. Furthermore, the unmodified peptide induced a similar response at approximately 10⁵-fold lower doses.

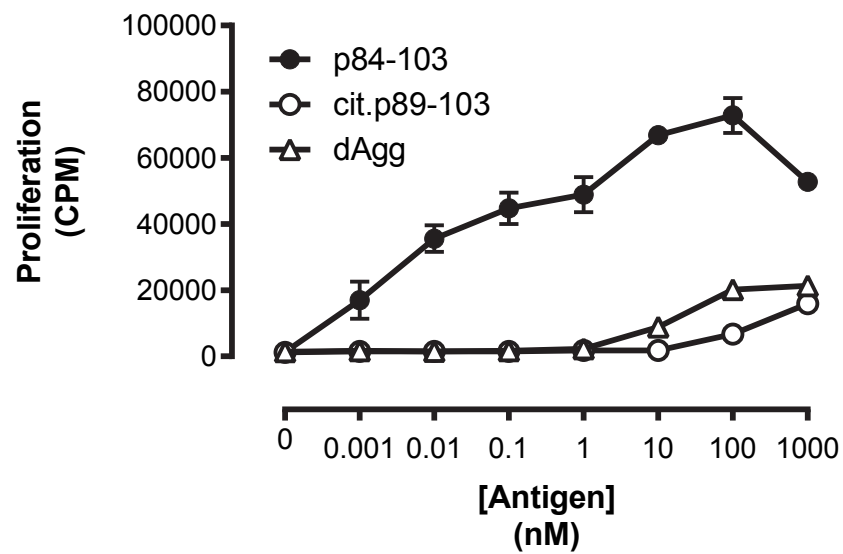


Figure 3.5 *TCR-5/4E8-transgenic splenocyte proliferation in response to the citrullinated form of p84-103 aggrecan peptide antigen (cit.p89-103) in comparison to the unmodified p84-103.*

2×10^5 splenocytes were cultured for 24 h in the presence of graded doses of antigen in a flat-bottomed 96-well plate. Co-cultures were pulsed with radioactive thymidine (^3H) and DNA harvested onto a glass fibre mesh 18 h later. ^3H incorporation was measured using a scintillation counter and counts per minute (CPM) recorded. Each data point represents a triplicate with error bars showing $\pm\text{SEM}$.

3.3.3. Kinetics of TCR-5/4E8-transgenic splenocyte responses

TCR-5/4E8-transgenic splenocyte proliferation in response to aggrecan and p84-103 was studied further in **Figure 3.6** by using an extended time course to determine the optimum antigen concentration and time to measure T cell proliferation. ^3H incorporation was measured at 24 h intervals following stimulation with graded doses of both dAgg and p84-103.

The TCR-5/4E8-transgenic splenocytes exhibited increased proliferation in response to p84-103 at lower doses over time (**Figure 6A & B**). By 72 h, with 1000 nM p84-103, the proliferation was seen to reach a peak (**Figure 3.6C**). However, at 96 h proliferation reached an early plateau of approximately 200,000 CPM at low doses (**Figure 3.6D**). Leaving the assay for further time points (up to 168 h) resulted in a deterioration of the proliferative response (data not shown).

In contrast, the response to dAgg failed to improve over the extent of the time course. This suggests that, while an increased time frame allows the resident APC population to undergo more rounds of peptide antigen presentation, the processing of aggrecan is not improved. This may be due to a lack of APC specificity for aggrecan leading to low uptake. Intriguingly, when the TCR-5/4E8-transgenic splenocytes were cultured with the TCR V β 4 superantigen concanavalin A (ConA) as a positive control T cell stimulus, their proliferation did not significantly increase with extended assay time points. Indeed, in later times points at (**Figure 3.6C & D**), their proliferation was seen to be reduced, compared to that seen with low doses of p84-103.

In conclusion, these data reveal that the optimum time point for measuring the peak proliferation of TCR-5/4E8-transgenic splenocytes is 72 h. Doses of p84-103 between 1 and 1000 nM are sufficient to induce high levels of proliferation. However, responses to dAgg are shown to remain poor even at later time points. Similarly, proliferation to the ConA control is demonstrated to be largely unaffected up to 72 h but also deteriorates beyond that point. As a result, ConA remains suitable as a positive control for assays up to but not exceeding 72 h in duration.

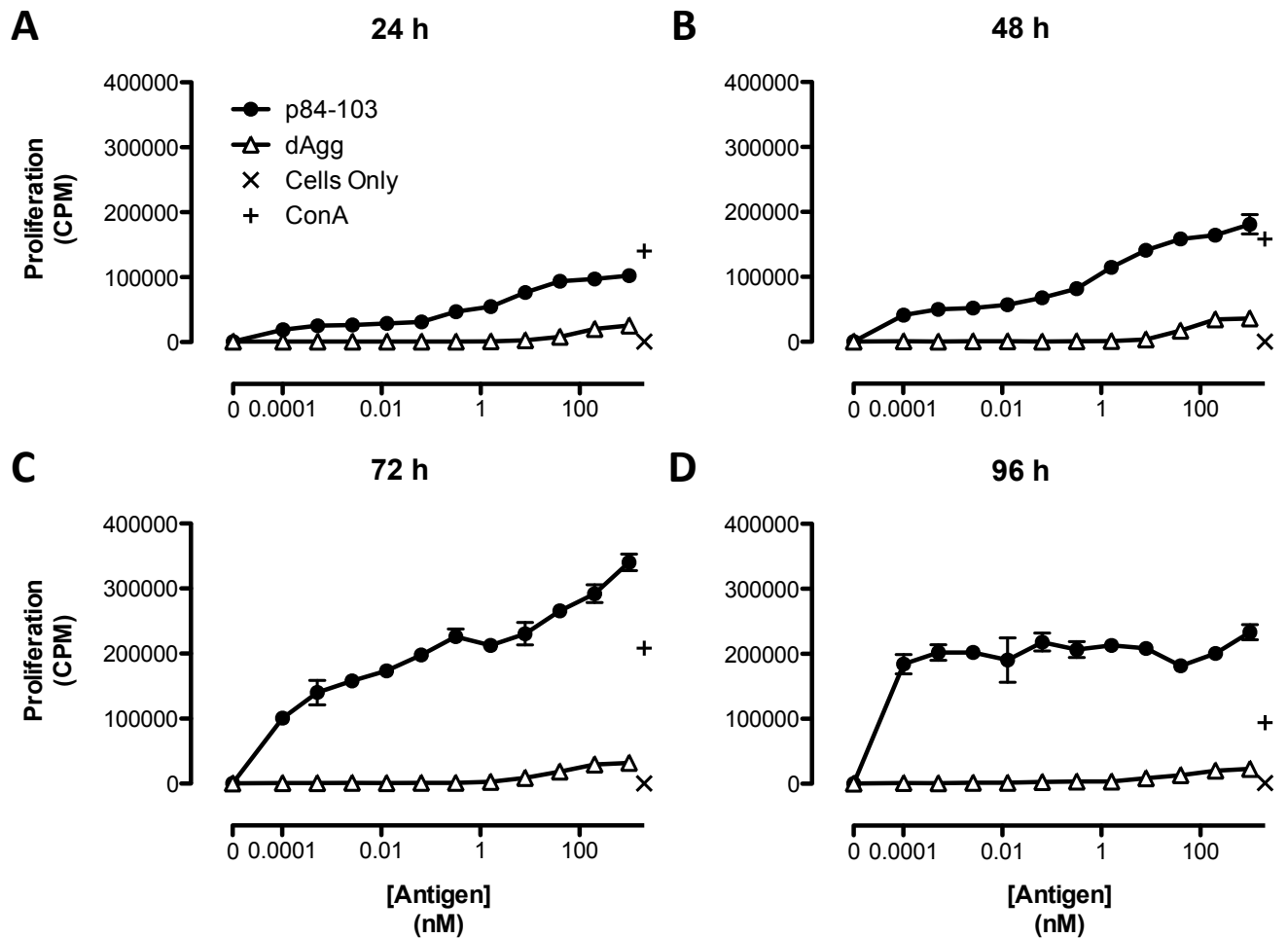


Figure 3.6 *TCR-5/4E8-transgenic splenocyte proliferation in response deglycosylated aggrecan (dAgg) and p84-103 at extended time points.*

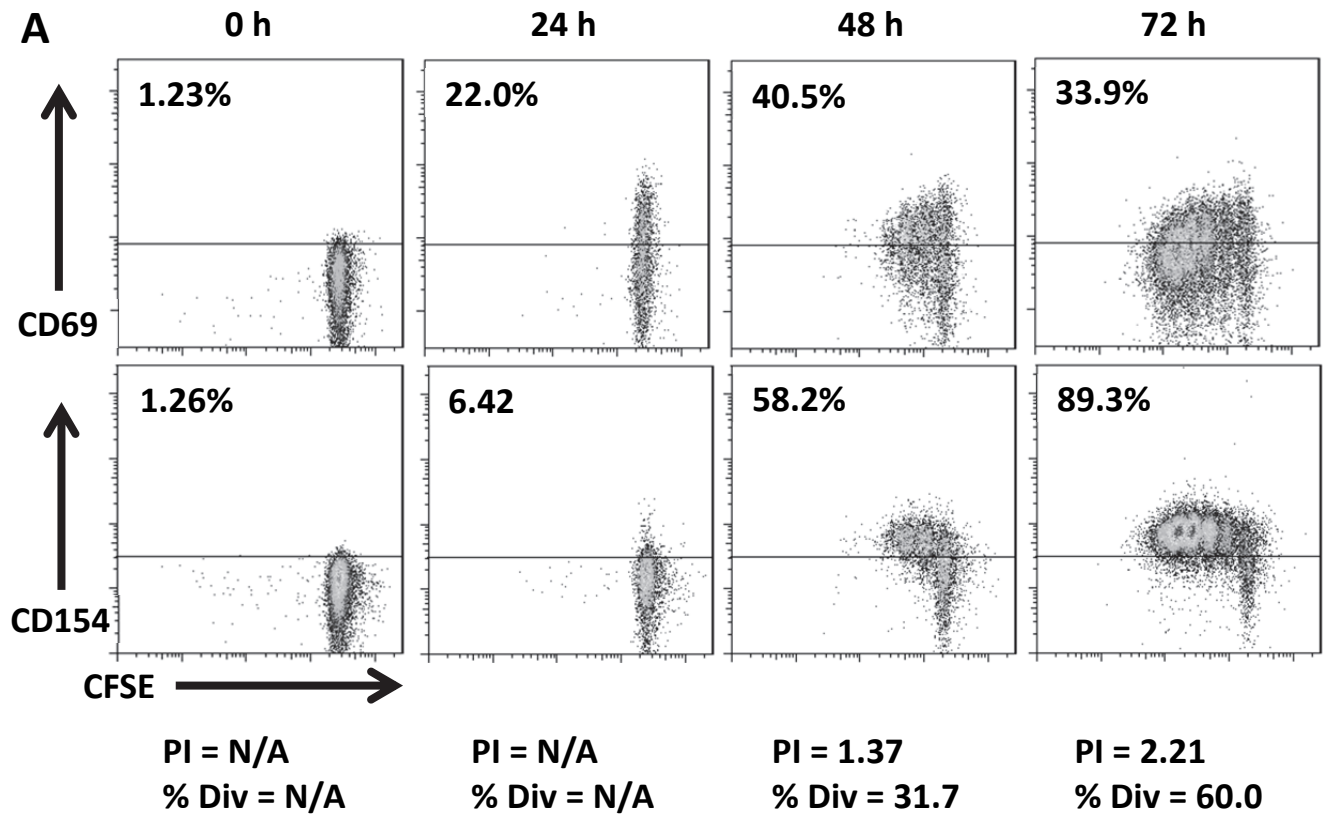
2×10^5 splenocytes were cultured for (A) 24, (B) 48, (C) 72 or (D) 96 h with graded doses of antigen in a flat-bottomed 96-well plate. Co-cultures were pulsed with radioactive thymidine (^3H) and DNA was harvested onto a glass fibre mesh 18 h later. ^3H incorporation was measured using a scintillation counter and counts per minute (CPM) recorded. Each data point represents a triplicate with error bars showing $\pm\text{SEM}$. Data representative of 2 independent experiments.

3.3.4. Stimulation of TCR-5/4E8-transgenic splenocytes with p84-103 and visualisation of proliferation and activation

Figure 3.7 shows further characterisation of TCR-5/4E8-transgenic splenocyte proliferation in response to p84-103, flow cytometry was used to specifically observe CD4⁺ T cell responses. In contrast to the radiolabelling assay described, which provides a measure of the proliferation of the entire splenocyte population, the measurement of the dilution of the fluorescent intracellular dye carboxyfluorescein succinimidyl ester (CFSE) in conjunction with the measurement of surface expression of CD69/CD154 allows for a more detailed analysis of TCR-5/4E8 CD4⁺ T cell proliferation kinetics and their subsequent activation status at various time points. These observations should allow the identification of the time point when the T cell population is adopting a phenotype typical of proliferating cells and, therefore, corroborate those findings from previously described radiolabelling assays as well as confirming that the majority of T cell activation and proliferation has occurred 72 h post-stimulation. Flow cytometric analysis of p84-103-stimulated splenocytes was performed at 24 h intervals to monitor CFSE dilution and cell-surface expression of CD69 and CD154.

24 h following stimulation with 1000 nM p84-103, CD4⁺ TCR-5/4E8-transgenic splenocytes were seen to upregulate the expression of CD69 and CD154 (Figure 3.7A). The CD4⁺ splenocytes began to divide at 48 h, as indicated by CFSE dilution, and further upregulated expression of CD154. However, while total CD69 upregulation continued, later generations can be seen to downregulate expression of CD69. By 72 h 84.7±6.5% (*n* = 2) CD4⁺ cells express CD154. A marked CD69 downregulation within later generations persisted as the assay progressed into 72 h (Figure 3.7A). Unstimulated control splenocyte cultures showed no sign of CFSE dilution or activation molecule expression over the course of the assay (data not shown).

These results indicate that the TCR-5/4E8-transgenic CD4⁺ splenocytes undergo activation as early as 24 h following p84-103 stimulation and this change encompasses the majority of cells by 72 h. Furthermore, CFSE dilution clearly shows that multiple rounds of division occur in this population leading to 56.8±4.6% (*n* = 2) of T cells having surpassed >1 division cycle by 72 h. Data pooled from two independent experiments demonstrate that the CD69 and CD154 upregulation is significant at 48 h (CD69 *p* = 0.03; CD154 *p* = 0.001) and 72 h (CD69 *p* = 0.03; CD154 *p* = 0.005) post-stimulation (Figure 3.7B).



B

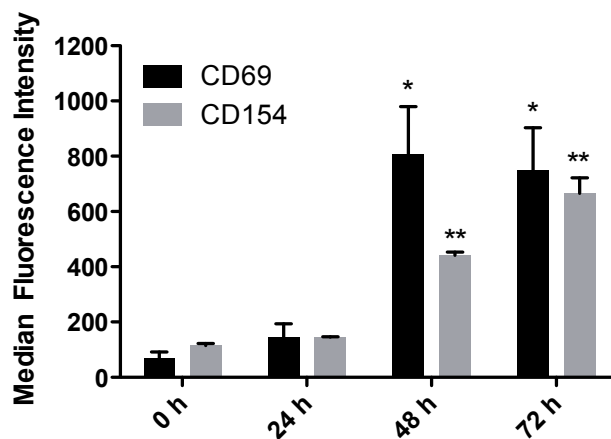


Figure 3.7 Flow cytometric analysis of TCR-5/4E8-transgenic splenocyte proliferation and activation marker expression following stimulation with p84-103.

(A) 2×10^6 splenocytes were labelled with $1 \mu\text{M}$ CFSE and allowed to rest for 18 h prior to stimulation with 1000 nM p84-103 and cultured for 24, 48 and 72 h in a 24-well plate. Gated CD4^+ T cells were analysed by flow cytometry for CFSE dilution and expression of CD69 and CD154. Data representative of two independent experiments. PI = proliferation index; % Div = % of CD4^+ T cells divided after one generation. (B) Pooled analysis of CD69 and CD154 expression on CD4^+ T cells quantitated by median fluorescence intensity values. Each bar represents data from two independent experiments with error bars showing $\pm\text{SEM}$. * indicates a *t*-test *p*-value ≤ 0.05 when data are compared to 0 h.

In addition, the CD4⁺ V β 4^{hi} and V β 4^{lo} T cell populations described in **Figure 3.1** were also identified and proliferation of each subset analysed. As shown in **Figure 3.8**, the V β 4^{lo} subset upregulated expression of the TCR in response to p84-103 stimulation over the course of the assay. By 72 h all CD4⁺ T cells had merged into the V β 4^{hi} gate and undergone multiple rounds of proliferation (**Figure 3.8**). In summary, **Figure 3.7** clearly demonstrates that the majority of TCR-5/4E8-transgenic CD4⁺ splenocytes undergo activation and proliferation 72 h after stimulation with p84-103. Furthermore, data from **Figure 3.8** confirms that both the V β 4^{hi} and V β 4^{lo} T cells found in the CD4⁺ population are functionally similar in regards to proliferation in response to antigen.

3.3.5. Titration of TCR-5/4E8-transgenic splenocytes into a wild-type BALB/c population and responses to p84-103

Figure 3.9 shows experiments performed to determine the optimum number of CD4⁺ T cells required for proliferation independent of the number of APC present, TCR-5/4E8-transgenic splenocytes were titrated into a population of WT BALB/C splenocytes maintaining a total number of 2×10^5 splenocytes (as used for previous kinetics experiments, see Figure 3.6) in the presence of 1 nM or 1000 nM doses of p84-103. Proliferation was measured using ³H-thymidine incorporation after 48 and 72 h.

At 48 h, 1×10^5 TCR-5/4E8-transgenic cells were required to induce the maximal response to the 1000 nM p84-103 dose (Figure 3.9A). However, at 72 h, 2.5×10^4 cells were sufficient to induce comparable proliferation at 1000 nM providing further evidence that the extended time course allows the APC to undergo more rounds of antigen presentation to the limited number of T cells present (Figure 3.9B). A similar trend can be seen at the 1 nM dose, albeit with <50% of the proliferation seen with the 1000 nM dose, at both 48 (Figure 3.9A) and 72 h (Figure 3.9B).

This experiment suggests that, at 72 h following antigen stimulation, between 1×10^4 and 5×10^4 TCR-5/4E8-transgenic CD4⁺ T cells per well are sufficient to yield high responses to p84-103 in an environment rich with APC. These data were used to inform the design of future experiments using *ex vivo* APC and T cell co-cultures.

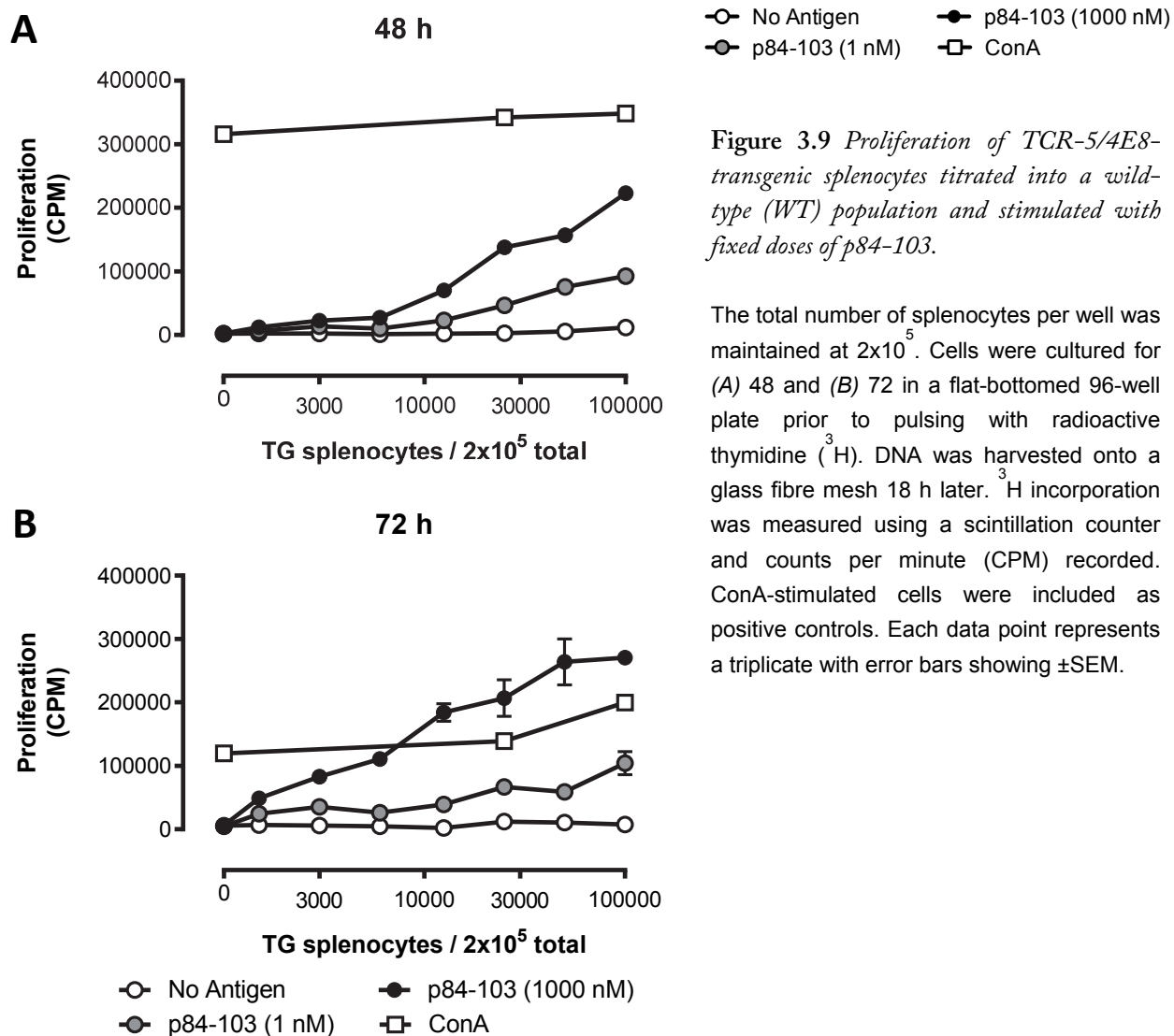


Figure 3.9 Proliferation of TCR-5/4E8-transgenic splenocytes titrated into a wild-type (WT) population and stimulated with fixed doses of p84-103.

The total number of splenocytes per well was maintained at 2×10^5 . Cells were cultured for (A) 48 and (B) 72 in a flat-bottomed 96-well plate prior to pulsing with radioactive thymidine (^3H). DNA was harvested onto a glass fibre mesh 18 h later. ^3H incorporation was measured using a scintillation counter and counts per minute (CPM) recorded. ConA-stimulated cells were included as positive controls. Each data point represents a triplicate with error bars showing $\pm\text{SEM}$.

3.4. Isolation of CD4⁺ T cells from TCR-5/4E8-transgenic mice in the absence of contaminating antigen-presenting cells

3.4.1. Isolation of CD4⁺ T cells by CD4 positive selection

Experiments involving co-culture of TCR-5/4E8-transgenic CD4⁺ T cells with different purified APC populations necessitate isolation of *ex vivo* CD4⁺ T cells in the absence of contaminating APC. This will allow us to confidently attribute induction of CD4⁺ T cell cytokine production to distinct APC populations.

Figure 3.10 shows an example of preliminary positive selection experiments undertaken using CD4⁺ magnetic microbeads and a MACS column to purify CD4⁺ T cells from a TCR-5/4E8-transgenic splenocyte population. Flow cytometry was used to analyse the initial splenocyte population and the CD4⁺ microbead eluate. A combination of TCR β and CD4 was used to identify the CD4⁺ T cells in conjunction with B220 and MHC class II to identify B cells and other APC. TCR-5/4E8-transgenic splenocytes routinely contained approximately 20% CD4⁺ T cells and 70% APC expressing B220 and/or MHC class II (**Figure 3.10A**). In the representative experiment shown, the CD4⁺ microbead eluate was shown to contain 86.7% CD4⁺ T cells but approximately 7% of these purified cells expressed MHC class II. In addition, approximately 7% of the purified cells were CD4⁺TCR β ⁻ (**Figure 3.10B**), maybe representing CD4⁺ DC or lymphoid tissue inducer cells (Lane et al., 2012). These data suggested that whilst a single-step separation achieved a reasonably high level of CD4⁺ T cell purity, there was a significant contaminating APC population still present. Therefore, to assess the ability of the isolated CD4⁺ T cells to proliferate, cells in the CD4⁺ eluate were cultured with graded doses of p84-103 for 72 h.

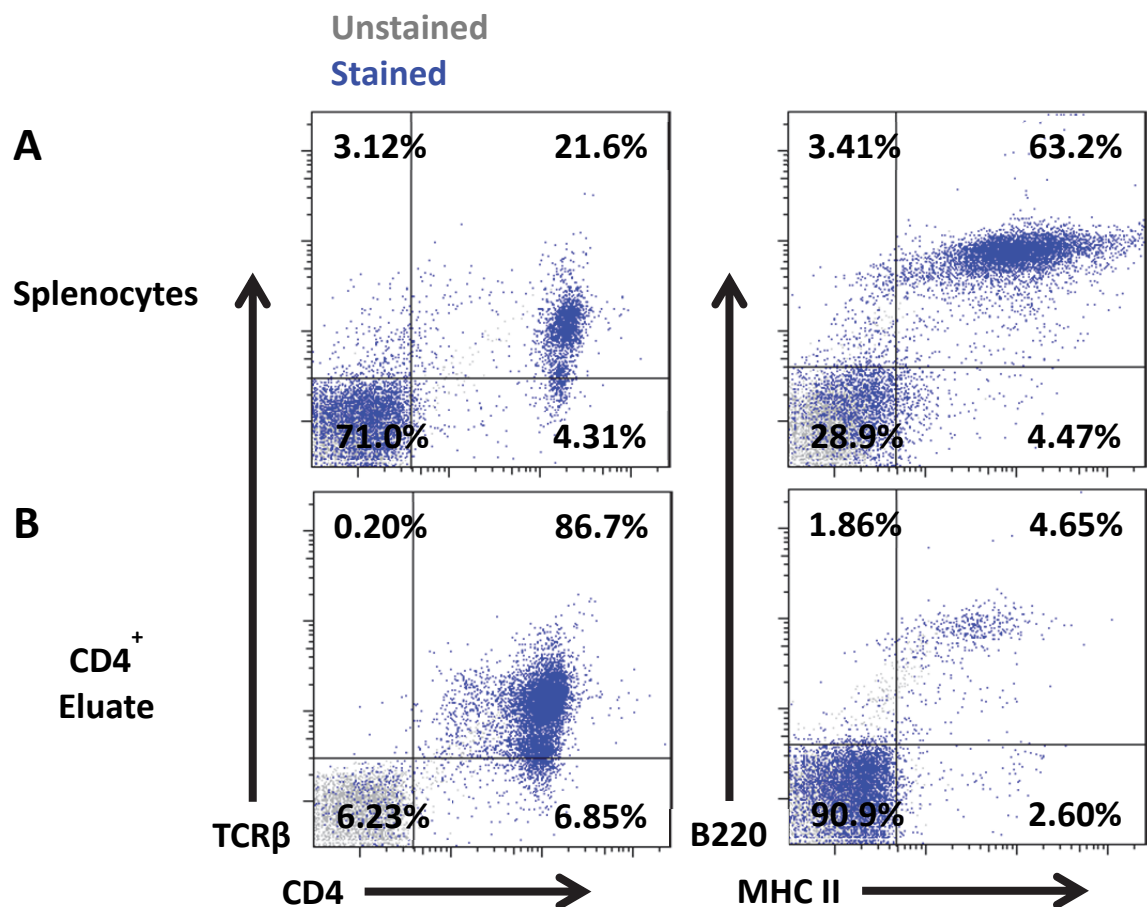


Figure 3.10 Flow cytometric analysis of TCR-5/4E8-transgenic splenocytes and the CD4⁺ microbead-labelled fraction following MACS positive selection with T cell and APC markers.

1×10^8 splenocytes labelled with CD4⁺ magnetic microbeads were passed through a MACS column and the bound CD4⁺ fraction eluted. Flow cytometry was performed on 2×10^5 cells from (A) the initial splenocyte population and (B) the eluted CD4 fraction. Cells were stained for TCRβ and CD4 or B220 and MHC class II to identify CD4⁺ T cells or APC respectively. Data representative of two separate experiments.

As predicted, **Figure 3.11** shows that the eluted CD4⁺ cells proliferated in response to p84-103 (**Figure 3.11A**) indicative of the contaminating APC still present following this single-step purification process. However, the response was impaired at higher doses in comparison to a whole splenocyte preparation. This may be due to saturation of the available APC with antigen within the 72 h time frame or an insufficient APC:T cell ratio. Interestingly, the unlabelled filtrate did not proliferate at low doses and showed a very poor response at high doses (**Figure 3.11A**). This indicates that, despite the abundant APC, T cell numbers appears to be the primary limiting factor, in accordance with previous data (**Figure 3.9**). The CD4⁺ eluate was passed through the MACS column for an additional two rounds of separation. Whilst a further diminishing response was observed (approximately 50% of the whole splenocyte preparation response at 1000 nM), sufficient contaminating APC still remained inducing detectable proliferation (**Figure 3.11B**). Thus, even multiple rounds of isolation using CD4-labelled microbeads is not stringent enough to allow the isolation of CD4⁺ cells lacking contaminating APC.

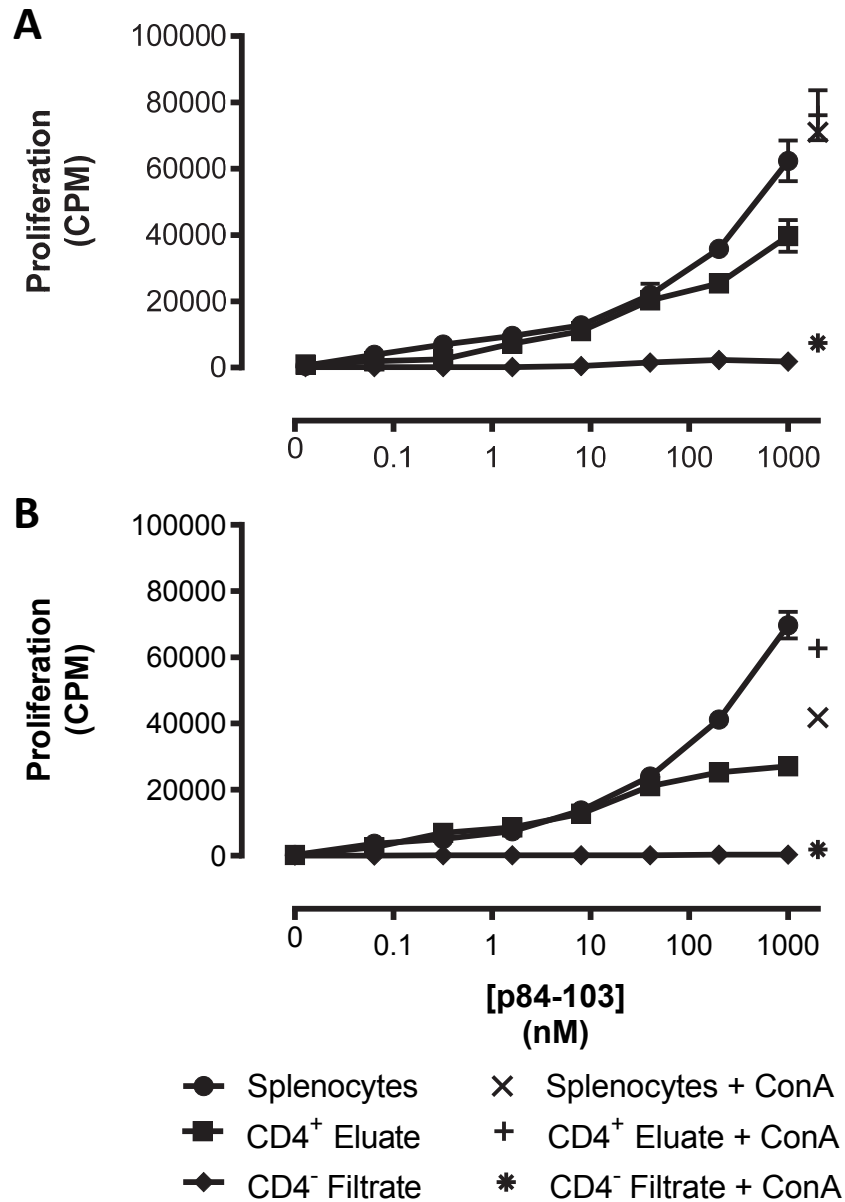


Figure 3.11 Isolation of TCR-5/4E8-transgenic CD4⁺ T cells from a splenocyte preparation using CD4⁺ magnetic microbeads followed by assessment of purity by proliferation assay.

CD4 selection: 1×10^8 splenocytes labelled with CD4⁺ magnetic microbeads were passed through a MACS column and the bound CD4⁺ cells eluted either (A) once or (B) three times. 5×10^4 splenocytes or isolated CD4⁺ T cells were cultured for 72 h in the presence of graded doses of p84-103 in a flat-bottomed 96-well plate. Cells were pulsed with radioactive thymidine (^3H) and DNA harvested onto a glass fibre mesh 18 h later. ^3H incorporation was measured using a scintillation counter and counts per minute (CPM) recorded. ConA-stimulated cells were included as positive controls. Each data point represents a triplicate with error bars showing $\pm\text{SEM}$.

3.4.2. Isolation of CD4⁺ T cells by MHC class II depletion followed by CD4 positive selection

In an attempt to remove contaminating APC from isolated CD4⁺ TCR-5/4E8-transgenic T cells, a precursor round of MHC class II depletion was used to prior to positive selection of CD4⁺ cells. Flow cytometry was used to analyse both the initial splenocyte population (**Figure 3.12A**) and the isolated CD4⁺ eluate (**Figure 3.12B**). Crucially, by using both MHC class II depletion and CD4 selection, this process yielded a CD4⁺ eluate which was routinely >99% in purity (**Figure 3.12B**). Furthermore, the CD4⁺ T cells were again stimulated with p84-103. In contrast to cells isolated by CD4 positive selection alone, the CD4⁺ T cells purified via MHC class II depletion followed by CD4 positive selection did not proliferate in response to any of the doses of p84-103 but did respond to the ConA (**Figure 3.12C**). These data suggest that viable CD4⁺ T cells were purified to an extent that contaminating APC were not present in sufficient numbers to induce a response to antigen stimulation.

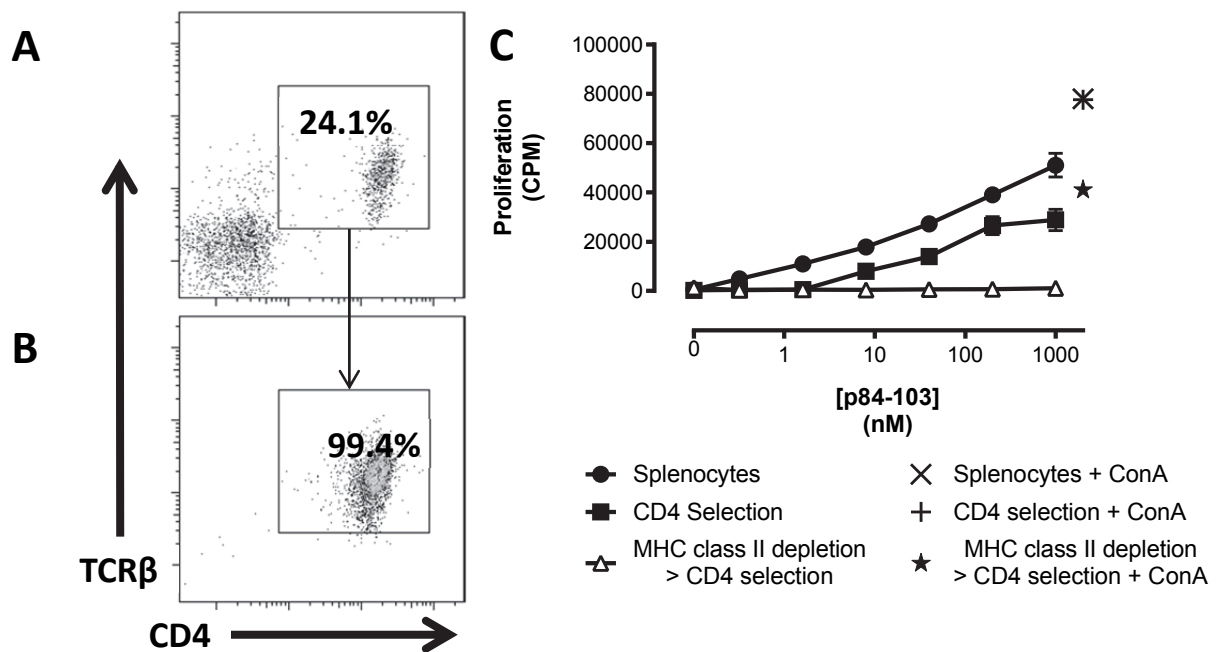


Figure 3.12 Isolation of TCR-5/4E8-transgenic $CD4^+$ T cells from a splenocyte preparation using MHC class II⁺ and $CD4^+$ magnetic microbeads followed by assessment of purity by flow cytometry and proliferation assay.

CD4 SELECTION: 1×10^8 splenocytes labelled with $CD4^+$ magnetic microbeads were passed through a MACS column and the bound $CD4^+$ cells eluted. **MHC CLASS II DEPLETION > CD4 SELECTION:** 1×10^8 splenocytes labelled with MHC class II⁺ magnetic microbeads were passed through a MACS column and the filtrate collected. The MHC class II⁻ filtrate was then passed through a $CD4^+$ selection column. Flow cytometry was performed on 2×10^5 cells from either (A) the initial splenocyte population and (B) the final $CD4^+$ population following MHC class II depletion > $CD4^+$ selection. Cells were stained for TCRβ and CD4 to identify $CD4^+$ T cells. (C) 5×10^4 splenocytes or isolated $CD4^+$ T cells were cultured for 72 h in the presence of graded doses of p84-103 in a flat-bottomed 96-well plate. Cultures were pulsed with radioactive thymidine (3H) and DNA harvested onto a glass fibre mesh 18 h later. 3H incorporation was measured using a scintillation counter and counts per minute (CPM) recorded. ConA-stimulated cells were included as positive controls. Each data point represents a triplicate with error bars showing $\pm$ SEM.

As shown in **Figure 3.13**, repetition of this isolation method confirmed that this level of purity was highly consistent (**Figure 3.13A**). In addition, examination of the activation state of the CD4⁺ T cells following isolation in this manner was not altered in comparison to naïve CD4⁺ T cells (**Figure 3.13B**) and was similar to that seen of unmanipulated CD4 T cells described earlier (**Figure 3.2A**). This indicates that magnetic microbead binding to CD4 does not subsequently modify the activation status of the CD4⁺ T cells prior to their co-culture with p84-103. In addition to their measured response to ConA, the titration of WT BALB/C splenocytes into a fixed number (5x10⁴) of CD4⁺ TCR-5/4E8-transgenic cells, isolated using this two-step process, was seen to restore a p84-103 dose-dependent proliferation response (**Figure 3.14**).

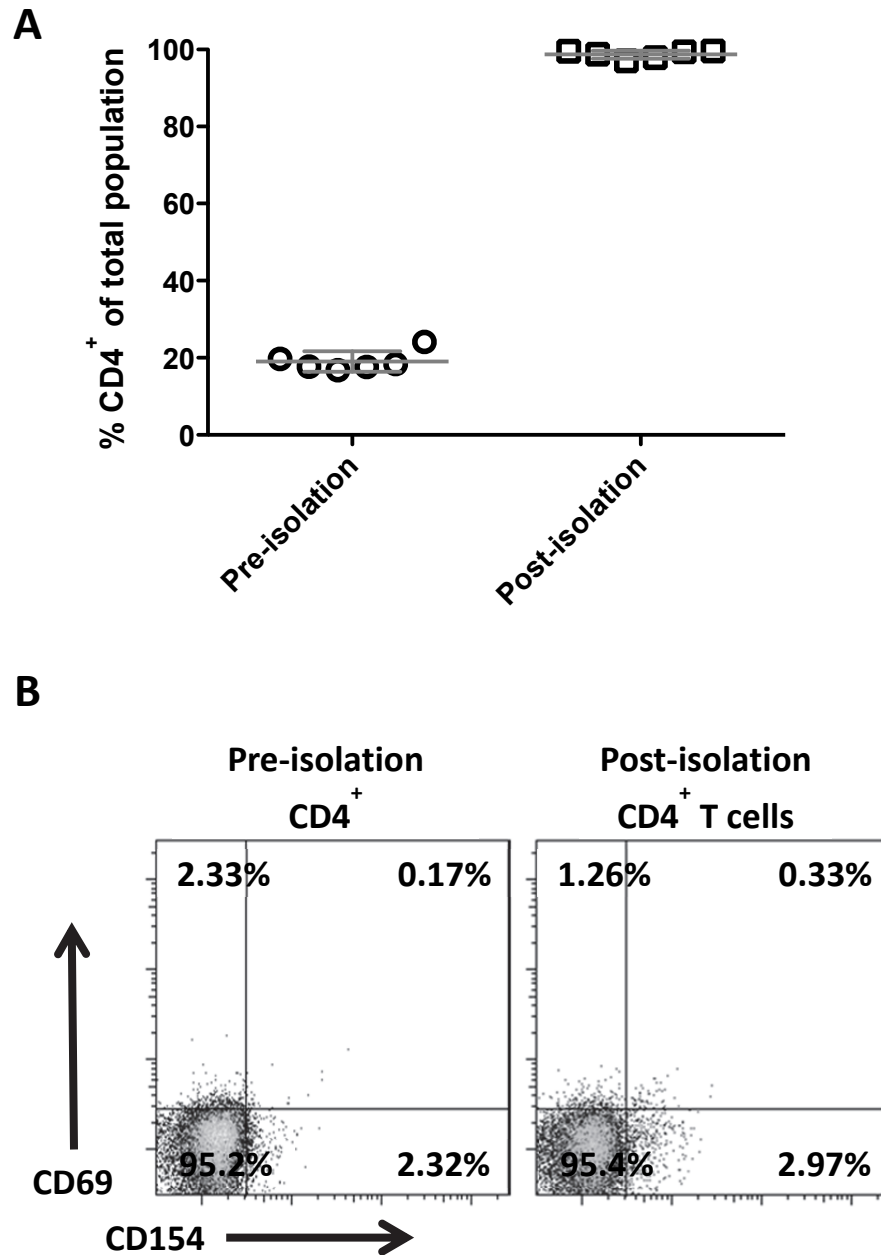


Figure 3.13 *Assessment of purity and activation state of TCR-5/4E8-transgenic CD4⁺ T cells isolated using MHC class II⁺ and CD4⁺ magnetic microbeads by flow cytometry.*

(A) 2×10^5 cells from six representative CD4⁺ T cell isolations were stained for CD4 expression immediately following isolation and % CD4⁺ T cells determined by flow cytometry. (B) Expression of CD69 and CD154 on CD4⁺ T cells pre/post-isolation as assessed by flow cytometry. Data representative of three independent experiments.

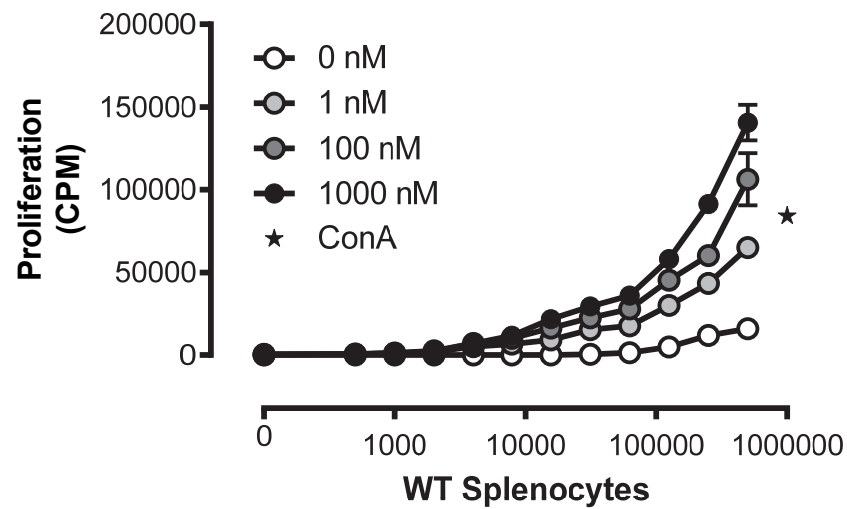


Figure 3.14 *Reconstitution of isolated TCR-5/4E8-transgenic $CD4^+$ T cells with titrated numbers of WT splenocytes prior to stimulation with fixed doses of p84-103.*

5×10^4 isolated $CD4^+$ T cells were co-cultured for 72 h in the presence of titrated numbers of WT splenocytes and 1, 100 and 1000 nM doses of p84-103 in a flat-bottomed 96-well plate. Co-cultures were pulsed with radioactive thymidine (3H) and DNA harvested onto a glass fibre mesh 18 h later. 3H incorporation was measured using a scintillation counter and counts per minute (CPM) recorded. Each data point represents a triplicate with error bars showing $\pm$ SEM.

3.5. Discussion

The TCR-5/4E8-transgenic mice described here form a key component of a robust *in vitro* system designed to compare the presentation of aggrecan by different APC. Over 90% of CD4⁺ T cells isolated from these mice were shown to express the V β 4 component of the transgenic 5/4E8 T cell hybridoma TCR specific for p84-103 (Figure 3.1) and, in accordance with previous reports (Berlo et al., 2005, 2006), exhibit a predominantly resting (Figure 3.2) and naïve (Figure 3.3) phenotype similar to that seen in control BALB/c littermates. This suggests that responses measured with isolated TCR-5/4E8-transgenic CD4⁺ T cells are representative of naïve T cell responses to antigen with only a limited number (<10%) of non-specific T cells involved. The use of an anti-V β 4 antibody to detect CD4⁺ T cells expressing the TCR-5/4E8-transgenic TCR in this study is not an ideal solution as a proportion of non-transgenic T cells will express V β 4 as part of the normal TCR repertoire. A superior method of detecting the transgenic population would involve the use of a fluorescent peptide-MHC class II tetramer (Altman et al., 1996). In contrast to the anti-V β 4, this molecular tool would provide highly accurate identification of p84-103-specific T cells and allow for *in vivo* tracking opportunities. Despite this, the presence of a minority of non-p84-103 reactive T cells in the splenocyte population and isolated CD4⁺ T cells utilised in this study may potentially be more representative of the disease situation as T cells found in the joints of arthritic patients express a range of specificities (Bennett et al., 2003; Struyk et al., 1995). Consequently, non-cognate bystander T cells may be activated in the joint environment by inflammatory cytokines and contribute to pathology in the absence of antigen-specific interactions (Brennan et al., 2008; Sattler et al., 2009).

In regards to further characterisation of the 5/4E8-TCR-transgenic mice, tissue-specific differences between WT BALB/c and TCR-5/4E8-transgenic lymphocyte populations, including APC, could be investigated. The initial characterisation of the TCR-5/4E8-transgenic mice by Berlo *et al.* did examine CD4⁺ T cell expression of CD25 and CD44 in spleen and lymph nodes and found them comparable with WT BALB/c controls. Furthermore, the T:B cell ratio was also found to be similar. In contrast, the CD4:CD8 T cell ratio was shown to be significantly higher than WT BALB/c mice in spleen and thymus populations although this was expected due to the 5/4E8 TCR being MHC class II-restricted (Berlo et al., 2006). Nevertheless, this characterisation did not include other APC, such as DC, or other T cell markers and was not extended to other tissues such as bone marrow. However, as this project is primarily concerned with use of *ex vivo* CD4⁺ T cells derived from splenocytes, these potential avenues of further research were not pursued.

Experiments revealed that TCR-5/4E8-transgenic splenocytes generated the highest proliferation responses when compared to mesenteric lymph node or thymus cell populations suggesting that the spleen-resident T cell population had the greatest potential for activation and proliferation (**Figure 3.4**). The variation in responses from different lymphocyte populations are probably the result of different numbers and ratios of stromal cells, parenchymal cells, APC and T cells present. Furthermore, the functional characteristics of each organ could augment the response. For example, T cells in the thymocyte population may undergo activation-induced cell death or be rendered anergic in response to high doses of antigen.

These initial experiments also demonstrated robust splenocyte responses to p84-103 but poor responses to aggrecan. Such results were expected with gAgg, due to the interference of glycosylated side-chains which mask the immunodominant epitopes found on the aggrecan molecule with large GAG residues leading to inhibition of antigen acquisition and processing (Leroux et al., 1996; Zhang et al., 1998). However, the poor responses to dAgg were not expected. Instead, these data may be explained by the low frequency of 'professional' APC such as DC and antigen-specific B cells in the splenocyte population which is made up predominantly of polyclonal B cells (**Figure 3.10**).

The potential effect of citrullination of p84-103 was also explored but revealed that a truncated citrullinated variant of the peptide was less immunogenic than the unmodified form (**Figure 3.5**). This effect may be due to the nature of the citrullination event. The arginine-citrulline conversion occurs at the 10th amino acid residue of the p84-103 peptide used in this experiment. This residue may be involved in interactions with the MHC class II molecule or TCR. As a result, the CD4⁺ T cells from this TCR-5/4E8-transgenic mouse may not respond as well to this version of the antigen due to reduced MHC class II loading or augmented signalling through the TCR. Due to the drastic reduction in splenocyte proliferation observed when using the cit.p89-103, presumably leading to a reduction in total T cell cytokine production, further assays utilised the unmodified p84-103 peptide sequence in order to maximise T cell responses. It is plausible that, despite a reduction in total CD4⁺ T cell activation, a peptide with a lower TCR affinity caused by citrullination may have other pathogenic effects through modulation of T cell cytokine production. For example, low strength T cell activation via CD3 has been shown to favour T_H17 responses (Purvis et al., 2010). Alternatively, low dose foreign or self antigen can also induce T_{reg} generation (Long et al., 2011). However, further elucidation would require examining the effects of a library of modified peptides and is beyond the scope of this project. Therefore, informed by these findings, unmodified p84-103 and deglycosylated aggrecan were used as antigens for the duration of this study.

In order to optimise the conditions for future assays, experiments were undertaken to identify optimal time-points, cell numbers and antigen doses at which to measure TCR-5/4E8-transgenic T cell proliferation and activation. The culture of TCR-5/4E8-transgenic splenocytes in the presence of graded doses of p84-103 and aggrecan revealed that the maximum levels of proliferation were attained at the 72 h time point at doses >100 nM (**Figure 3.6**). After this time point the response declined, perhaps due to limited antigen availability or activation-induced cell death. These results indicate that, unlike data previously generated in our laboratory using a p84-103 specific CD4⁺ T cell hybridoma (Wilson et al., 2012), *ex vivo* CD4⁺ T cells appear to require over 24 h to become activated and achieve peak proliferation responses.

In support of this, CFSE labelling data show that after 24 h post-stimulation with p84-103, TCR-5/4E8-transgenic CD4⁺ splenocytes transiently upregulate the early activation marker CD69, a key molecule in T cell activation which mediates retention of lymphocytes in lymph nodes during the early stages of immune responses, and begin proliferation at 48 h (**Figure 3.7**) (Cosulich et al., 1987; López-Cabrera et al., 1993; Shiow et al., 2006). Importantly, expression of the T cell activation marker CD154 becomes almost ubiquitous at 72 h when the majority of CD4⁺ cells are proliferating (**Figure 3.7**) in accordance with data from radioactive thymidine incorporation proliferation assays (**Figure 3.6**). CD154 is required for activation of APC by T cells through binding its ligand, CD40, and inducing APC expression of co-stimulatory molecules (Bennett et al., 1998; Schoenberger et al., 1998). As a result, the observed expression of CD154 on TCR-5/4E8-transgenic CD4⁺ splenocytes highlights 72 h as a key time point when the majority of T cells are activated and capable of reciprocal interactions with APC. The CD4⁺Vβ4⁺ splenocyte population was found to be divided into two populations expressing different levels of TCR (**Figure 3.1A**) designated Vβ4^{hi} and Vβ4^{lo} which had remained unexamined by previous studies (Berlo et al., 2005, 2006). Stimulation of these populations with p84-103 induced upregulation of TCR expression on the smaller Vβ4^{lo} population which proceeded to mimic the phenotype of the majority of cells found the Vβ4^{hi} population after 24 h. Following this, the enriched Vβ4^{hi} population proliferated 48 h post-stimulation as visualised with CFSE labelling (**Figure 3.8**). This suggests that both populations are capable of responding to antigen and do so according to similar kinetics. Due to this unified response to antigen, these populations were not separated prior to incorporation into antigen-presentation assays and treated as a single p84-103-reactive transgenic CD4⁺ T cell population during the extent of this study.

The titration of TCR-5/4E8-transgenic splenocytes into a WT BALB/c population and subsequent stimulation with p84-103 revealed that a CD4⁺ T cell number between 1x10⁴ and 5x10⁴ was capable of generating robust proliferation responses detectable via radioactive thymidine incorporation (**Figure 3.9**). However, these data were obtained from T cells responding in an environment rich with APC derived from the WT BALB/c splenocyte population. As a result, the need for a high density of APC was taken into account when designing further experiments.

Isolation of TCR-5/4E8-transgenic CD4⁺ T cells is integral to investigation of the role of different APC in presentation of aggrecan. Contaminating APC in the isolated CD4⁺ T cell population may prevent accurate assignments of potential effects attributable to specific APC subsets. Although cell-sorting was shown to be effective at isolating T cells, low viable cell yields made repeated use of this technique impractical. Initial attempts presented here, using up to three rounds of MACS positive selection to isolate CD4⁺ T cells, demonstrate that this method is also insufficient as contaminating APC (identified by flow cytometric analysis (**Figure 3.10**)) are capable of activating the CD4⁺ T cells following addition of antigen (**Figure 3.11**). Furthermore, further experiments using a T cell purification kit (Miltenyi Biotec) containing a cocktail of APC markers to negatively select for CD4⁺ T cells did not significantly improve the final purity over the positive selection method (data not shown). However, a combination of negative selection of MHC II⁺ cells followed by positive selection of CD4⁺ cells was successful at achieving high purity isolation of CD4⁺ T cells (**Figure 3.12**).

This method was shown to be highly repeatable (**Figure 3.13A**) and did not have an effect on the activation state of the CD4⁺ T cells (**Figure 3.13B**). Furthermore, the potential for the purified CD4⁺ T cells to proliferate is restored by reconstitution with WT BALB/c splenocytes containing MHC class II⁺ cells thus confirming the viability of the isolated CD4⁺ T cells for incorporation into proliferation assays (**Figure 3.14**). Conclusions drawn from this experiment are constrained, however, by the potential for the WT BALB/c splenocytes to respond to p84-103. Separate experiments clearly demonstrated that WT BALB/c splenocytes do not proliferate following stimulation with high doses of p84-103 (data not shown) but this does not exclude the possibility that WT BALB/c cells may be activated in a non-cognate, cytokine-dependent manner following activation of the TCR-5/4E8-transgenic splenocytes in the co-culture.

As previously mentioned, a combination of peptide-MHC class II tetramers and cell-sorting could provide a more accurate method of isolation exclusively transgenic CD4⁺ T cells. Furthermore, in conjunction with additional antibodies specific for memory cell markers, cell-sorting would allow for separation of naïve and memory T cells prior to incorporation into antigen presentation assays. However, as investigation of these factors is not the primary aim of this study, the success of the MACS method at isolating sufficient yields of CD4⁺ T cells to a required purity to prevent activation in the presence of peptide antigen was deemed to be satisfactory for further experiments.

In conclusion, the results shown in this chapter demonstrate via two independent methods that the splenocytes from TCR-5/4E8-transgenic mice contain significantly increased numbers of naïve CD4⁺ T cells specific for the immunodominant aggrecan epitope p84-103 that undergo activation and proliferation in response to both peptide and protein antigen. In addition, this chapter also documents the extensive characterization of the activation of these cells using a number of different forms of antigen over a range of doses, time courses and cell numbers thus allowing the optimum conditions for the TCR-5/4E8-transgenic CD4⁺ T cell activation to be established. The successful isolation of viable naïve TCR-5/4E8-transgenic CD4⁺ T cell population in the absence of APC, provides the primary tool with which to compare the antigen-presentation and T cell activation capabilities of different APC, including antigen-specific B cells.

4. Presentation of aggrecan by aggrecan-specific B cells to T cell receptor-5/4E8-transgenic CD4⁺ T cells in comparison to other antigen-presenting cells

4.1. Introduction

A key feature of RA is the migration of leukocytes into the affected synovial joints. CD4⁺ T cells form part of this infiltrate and produce proinflammatory cytokines such as IFN- γ and IL-17. These contribute to a network of factors including TNF- α and IL-6 produced by local synoviocytes which together generate a persistent inflammatory microenvironment (Lundy et al., 2007). Studies of both the human disease and animal models have demonstrated that T_H1 and T_H17 cells are found in arthritic synovium and their signature cytokines, IFN- γ and IL-17 respectively, have both been implicated in RA pathology (Kirkham et al., 2006; Stamp et al., 2004). Conversely, it has also been suggested that immune regulation by T_{reg} may be compromised in RA (Ehrenstein et al., 2004). The disruption of T_{reg} function has been proposed to be caused by reduced expression of CTLA-4, which is key for the suppression of T cell responses, or FoxP3 dephosphorylation induced by the inflammatory cytokine TNF- α (Flores-Borja et al., 2008; Nie et al., 2013). Consequently, the study of CD4⁺ T cell cytokine production and the mechanisms required for the differentiation of these key cells are of significant importance.

The T cell response in RA may be driven by T cells specific for joint-derived antigens. However, in the spontaneous K/BxN mouse model of arthritis, the autoantigen required to activate CD4⁺ T cells and drive autoantibody production is ubiquitous and not joint-specific (Korganow et al., 1999; Kouskoff et al., 1996). Furthermore, other models have suggested that CD4⁺ T cells may infiltrate the synovium and become activated in response to the inflammatory cytokine environment rather than due to antigen-specific interactions (Murakami et al., 2011). In contrast, in models such as CIA and PGIA where the pathology is thought to be predominantly mediated by CD4⁺ T cell cytokine production, the disease is induced by immunisation with joint-derived autoantigens (Corrigall and Panayi, 2002). In addition, CD4⁺ T cells adoptively transferred from mice immunised with joint autoantigens can also induce disease in naïve recipients (Glant et al., 1987; Mikecz et al., 1990). The relevance of these models is supported by the discovery of immunogenic epitopes derived from the joint autoantigen aggrecan in RA synovial fluid which can activate T cells derived from patients (Guerassimov et al., 1998; de Jong et al., 2009). Due to this compelling combination of evidence

from animal models and patients, this study focuses on aggrecan as a candidate autoantigen (Kiani et al., 2002).

Experiments described **Chapter 3** examined the activation and proliferation of aggrecan-specific CD4⁺ T cells from the TCR-5/4E8-transgenic mouse model. These experiments defined the characteristics and kinetics of TCR-5/4E8-transgenic splenocyte responses to both the synthetic form of the immunodominant aggrecan peptide, p84-103, as well as purified aggrecan. TCR-5/4E8-transgenic splenocytes were shown to proliferate and upregulate activation markers in response to peptide antigen. While the TCR-5/4E8-transgenic splenocytes were seen to proliferate following splenocyte APC processing of aggrecan, this was less efficient than that seen following stimulation with the pre-processed p84-103 peptide. This was probably due to the poor uptake and processing capacity of non-specific APC present in the splenocyte population (predominantly polyclonal B cells). Therefore, in order to study antigen-specific CD4⁺ T cell activation following presentation of aggrecan by more proficient APC populations relevant to disease, it was essential to establish a methodology that would allow for isolation of TCR-5/4E8-transgenic CD4⁺ T cells in the absence of contaminating splenocyte APC. In particular, it was deemed necessary for the purification to be of a sufficient extent that proliferation of the isolated CD4⁺ T cell population following culture with peptide antigen was minimal. This method, combined with the optimisation assays, contributed to the generation of an *in vitro* system for comparison of antigen presentation and CD4⁺ T cell activation by various APC which will be utilised in this chapter.

Recently, the success of B cell-depletion therapy in the treatment of RA has provoked interest in B cell antigen presentation as a potentially key facet of pathogenesis (Edwards et al., 2004). Anti-CD20 monoclonal antibodies subject naïve and memory B cells to antibody-dependent cell-mediated cytotoxicity via Fc-receptor-expressing cells (Uchida et al., 2004). B cell depletion has been shown to function therapeutically independent of a reduction in antibody titres, suggesting B cells mediate pathology via a different mechanism (Lioussis and Sfrikakis, 2008). Evidence from the PGIA model of RA has supported the hypothesis that B cells recognise aggrecan epitopes and are essential for disease development. B cell depletion leads to a reduction in both autoantibody titres and CD4⁺ T cells producing pathogenic cytokines (Hamel et al., 2008). Intriguingly, even when only the antibody-secreting function of B cells was abrogated, arthritis will still develop in mice immunised with aggrecan (O'Neill et al., 2005). This suggests that an autoantibody-independent mechanism is required for arthritis induction and that B cells are essential for priming of antigen-specific T cells. This evidence supports the hypothesis that removal of autoreactive B cells in patients may provide

the conditions required for the establishment of a more regulatory environment in the joint and subsequent alleviation of disease (Lund and Randall, 2010).

The study of B cell modulation of T cell function indicates that B cells can promote the activation and expansion of both effector and regulatory T cell subsets. Via production of IL-12 and IFN- γ or IL-4 antigen-presentation by B cells can direct the differentiation of naïve CD4⁺ T cells into T_H1 and T_H2 effector subsets respectively (Johansson-Lindbom et al., 2003). Skewing events such as these have implications in the context of RA as T_H1 cell cytokines contribute to disease. In contrast, B cells can also produce IL-2 in order to selectively expand CD4⁺CD25⁺ T_{reg} from a population of naïve T cells (Chen et al., 2009; Tu et al., 2008). In an antagonistic role to B cell generation of effector T cells, B_{reg} can produce IL-10 in response to tolerogenic antigen and subsequently regulate CD4⁺ T cell responses in autoimmunity (Blair et al., 2010; Sun et al., 2008; Yanaba et al., 2008). Crucially, removal of endogenous IL-10-producing B_{reg} leads to an increase in pathogenic CD4⁺ T cell subsets and a reduction in T_{reg} leading to increased disease severity (Carter et al., 2011). Harnessing the ability of B cells to attenuate autoreactive T cell responses could facilitate the development of new therapies.

In order to compare the consequences of aggrecan presentation by antigen-specific B cells with that seen by other APC types, this study utilises a cloned, aggrecan-specific, B cell transfectant (Wilson et al., 2012), in conjunction with purified APC-free TCR-5/4E8-transgenic CD4⁺ T cells as described in the previous chapter. A20-Agg clone 4C5 is a previously characterised, representative A20 B cell lymphoma transfectant that was selected for the expression of a chimeric BCR consisting of both H and L-chain constant regions derived from a human IgG1/ κ anti-tetanus toxin C fragment (TTCTF) BCR (Knight et al., 1997) fused to the corresponding variable regions from a recently generated mouse IgG1/ κ anti-aggrecan monoclonal antibody (Wilson et al., 2012). Previous studies have shown that such human/mouse chimeric BCR are biologically active, particularly with respect to their ability to recruit mouse CD79 α/β , maintaining both signalling and antigen presentation function when expressed in mouse B cells (Barrault and Knight, 2004; Hackett et al., 1998; Sun et al., 1987; Wilson et al., 2012).

Using these tools, the aims of this chapter were to:

- a) Culture and characterise *ex vivo* splenocyte and bone marrow-derived APC populations for comparison to the antigen-specific A20-Agg B cells and its non-specific parent B cell line A20.
- b) Test the ability of various APC, including A20-Agg B cells, to induce TCR-5/4E8-transgenic CD4⁺ T cell activation and cytokine production following culture with aggrecan. In particular, the induction of the key pathogenic cytokine in PGIA, IFN- γ , was to be investigated in detail.
- c) Establish any differences in TCR-5/4E8-transgenic CD4⁺ T cell production of arthritis-associated cytokines and outline the kinetics the response and phenotype of the cytokine-producing cells.

4.2. Presentation of aggrecan to TCR-5/4E8-transgenic CD4⁺ T cells by aggrecan-specific B cells in comparison to other antigen-presenting cells

4.2.1. B cell infiltration of joint tissue during proteoglycan-induced arthritis

Evidence from animal models of RA suggests that B cells may infiltrate the joint and present joint autoantigens to T cells in the synovial environment (O'Neill et al., 2005). The TCR-5/4E8-transgenic PGIA model was used to ascertain the presence of B and T cells in arthritic joints to provide a foundation for further experiments seeking to compare B cells to other APC when presenting aggrecan to TCR-5/4E8 CD4⁺ T cells. **Figure 4.1** shows the lymphocyte content of skinned TCR-5/4E8-transgenic mice feet and lower ankle joints following immunisation with dAgg emulsified in CFA or DDA, a synthetic non-irritant adjuvant that has been shown previously to activate innate immunity and favour T_H1 dominance (Hanyecz et al., 2004). The cellular infiltrate was quantified and stained for B and T cell markers 28 d later.

Flow cytometric analysis (**Figure 4.1A**) revealed that the cellular content of the foot and lower ankle joints of TCR-5/4E8-transgenic mice following immunisation with either dAgg+CFA or dAgg+DDA contained significantly higher B cell numbers compared to those from unimmunised control animals (dAgg+CFA $p = 0.02$; dAgg+DDA $p = 0.0001$). In the case of dAgg+DDA but not dAgg+CFA immunised mice, T cell numbers were also significantly increased (dAgg+CFA $p = 0.4$; dAgg+DDA $p = 0.002$). Taken together, these results suggested infiltration of lymphocytes into the synovium in mice immunised with dAgg and either adjuvant (**Figure 4.1B**). However, when the proportion of B and T cells isolated from the joints of animals immunised with dAgg was compared, dAgg+DDA mice had significantly higher proportion of B cells ($p = 0.0004$) and T cells ($p = 0.06$) than those immunised with dAgg+CFA (**Figure 4.1B**). Immunisation of TCR-5/4E8-transgenic mice with dAgg in conjunction with either adjuvant induced higher total cell counts than untreated controls (**Figure 4.1C**).

These data demonstrate that both B and T cells infiltrate the joints of arthritic TCR-5/4E8-transgenic mice suggesting that both cell types contribute to autoimmune inflammation in this system. Furthermore, the type of adjuvant used was shown to affect both the total number of infiltrating cells and the ratio of T and B cells present.

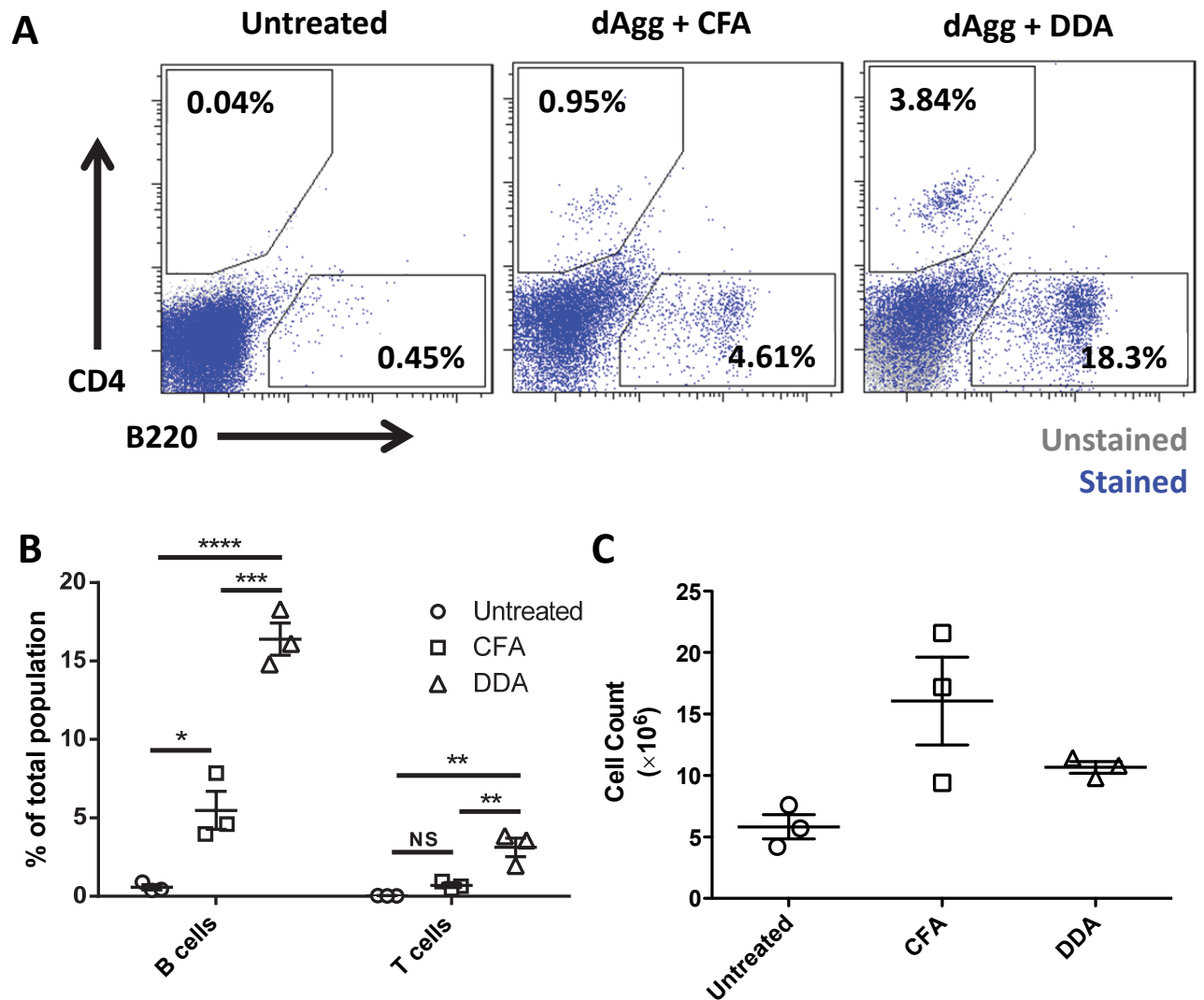


Figure 4.1 Characterisation of cells isolated from the joints of TCR-5/4E8-transgenic mice immunised with aggrecan.

TCR-transgenic mice were immunised with dAgg emulsified in CFA or DDA and sacrificed 28 d later. Three mice were included per group. (A) Cells were extracted from the joints of four feet per mouse and stained for expression of CD4 and B220. (B) Pooled data comparing % CD4⁺ or B220⁺ from the total population recovered. (C) Total cell yield was pooled and counted. Error bars show $\pm$ SEM. * indicates an ANOVA test p -value ≤ 0.05 .

4.2.2. Aggrecan-specific A20-Agg B cell presentation of aggrecan to T cell hybridoma 192 in comparison to other APC lines

Initial experiments were performed in order to confirm that the aggrecan-specific A20-Agg B cells generated previously in our laboratory were more effective at presenting dAgg than non-specific B cells and macrophages. This provides a basis for the use of this cell line in comparison assays involving *ex vivo* APC and TCR-5/4E8-transgenic CD4⁺ T cells. **Figure 4.2** shows IL-2 production by the p84-103-specific CD4⁺ T cell hybridoma 192, following co-culture with the J774 macrophage-like cell line, non-specific A20 B cells or aggrecan-specific A20-Agg B cells in the presence of graded doses of dAgg or p84-103. IL-2 production was used to assess T cell activation as the cell lines used in this and further assays undergo unrestricted division making direct measurement of T cell proliferation using incorporation of ³H-thymidine impossible. Therefore, IL-2 production was determined by recording the proliferation of the IL-2-dependent CTLL-2 T cell hybridoma when cultured with assay supernatants.

J774, A20 and A20-Agg were all shown to equivalently present p84-103 with high efficiency with levels as low as 0.0004 nM inducing IL-2 production from 192 (**Figure 4.2A**). In contrast, approximately 100-fold higher doses of dAgg were required for J774 and A20 to achieve the same level of T cell activation (**Figure 4.2B**). This observation was expected as, unlike p84-103, dAgg requires processing prior to loading onto MHC class II for TCR binding. In contrast, A20-Agg B cells presented dAgg more efficiently than both J774 and A20 (**Figure 4.2B**). Indeed, the presentation of dAgg by the A20-Agg B cells was almost as efficient as that seen with p84-103. This indicates that the aggrecan-specific BCR confers a major advantage to A20-Agg B cells when processing and presenting aggrecan in protein form. All cell types were cultured alone with or without the maximal antigen dose as negative controls. These conditions demonstrate both APC and T cells did not produce sufficient IL-2 in the absence of APC-T cell interactions to induce CTLL-2 proliferation (**Figure 4.2A & B**). Co-culture of 192 T cells and APC with the superantigen ConA showed CTLL-2 proliferation similar to that seen with the maximal antigen doses (**Figure 4.2B**).

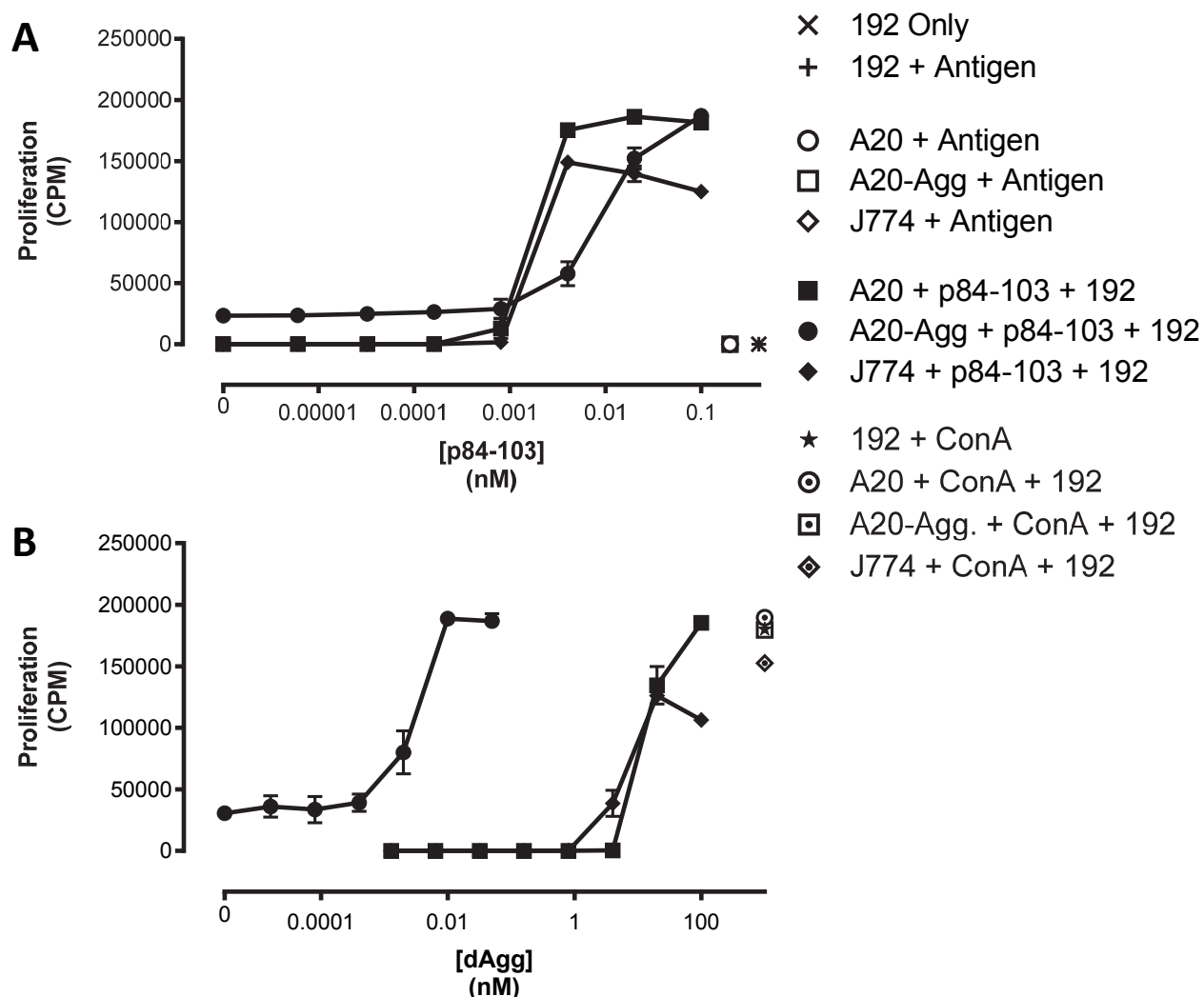


Figure 4.2 J774, A20 and A20-Agg B cell presentation of deglycosylated aggrecan and p84-103 to T cell hybridoma 192.

5×10^4 APC and 5×10^4 T cells were co-cultured for 24 h in the presence of graded doses of antigen in a flat-bottomed 96-well plate. Supernatants were extracted and added to 3×10^4 CTLL-2. Cultures were pulsed with radioactive thymidine (^3H) and DNA harvested onto a glass fibre mesh 18 h later. ^3H incorporation was measured using a scintillation counter and counts per minute (CPM) recorded. Unstimulated and ConA-stimulated co-cultures were included as negative and positive controls respectively. Each data point represents a triplicate with error bars showing $\pm$ SEM.

As described previously for aggrecan and other antigens (Constant, 1999; Constant et al., 1995a, 1995b; Wilson et al., 2012), improved presentation mediated by the expression of an aggrecan-specific BCR is almost certainly derived from a combination of improved dAgg uptake via the BCR along with the subsequent targeting of internalized dAgg to intracellular pathways involved in efficient presentation via MHC class II (Amigorena et al., 1994). Wilson *et al.* have also shown that, similar to other APC, BCR-mediated presentation of aggrecan is dependent on newly-synthesised MHC class II and the presence of H2-M in the endocytic compartment (Wilson et al., 2012). Therefore, these data confirm that, as previously reported, the dAgg-specific B cell line A20-Agg is more efficient at processing and presenting dAgg to aggrecan-specific T cells than non-specific APC and, therefore, is a valid tool for comparing aggrecan-specific B cell presentation of dAgg to other APC.

4.2.3. Isolation of antigen-presenting cells and phenotypic characterisation

Having established A20-Agg B cells as an antigen-specific B cell population with efficient aggrecan uptake and presentation capabilities, various *ex vivo* APC populations were isolated to determine the best candidate for comparison to A20-Agg B cells when presenting aggrecan to TCR-5/4E8-transgenic CD4⁺ T cells. DC and macrophages were cultured from WT BALB/c bone marrow preparations and CD43⁻ B cells were isolated from WT BALB/c splenocytes. Phenotypic characterisation was performed to confirm APC maturation status and expression of specific APC markers and molecules required for antigen presentation, such as MHC class II and co-stimulatory molecules. This was also undertaken to ensure that both the generation of bone marrow-derived cells and the separation of CD43⁻ B cells from each preparation was comparable and thus standardise the APC incorporated into each assay.

Figures 4.3 and **4.4** show typical examples of routine flow cytometric analysis of various APC prior to co-culture with TCR-5/4E8-transgenic CD4⁺ T cells. All cells were incubated with 0.1 µg/mL Fc block before staining. Both the A20 (**Figure 4.3A**) and A20-Agg B cells (**Figure 4.3B**) cultures were MHC class II⁺ (A20 99.6%; A20-Agg 99.8%). Furthermore, both populations contained a similar percentage of CD40⁺ (A20 90.9%; A20-Agg 91.4%) and CD80⁺ (A20 52.9%; A20-Agg 66.0%) cells. As expected, the A20-Agg B cells expressed the transfected chimeric C7.1 aggrecan-specific BCR as detected using an anti-human IgG antibody while the A20 B cells were negative (A20 1.98%; A20-Agg 92.6%). Surprisingly, the A20 B cell population did contain a greater number of CD86⁺ cells than the A20-Agg B cells (A20 95.5%; A20-Agg 50.8%). This difference in CD86 expression was unexpected as A20-Agg B cells are a stable cloned transfectant derived from the parental A20 B cell line. It is possible that the cloning process may have inadvertently selected a clone that expresses lower levels of CD86.

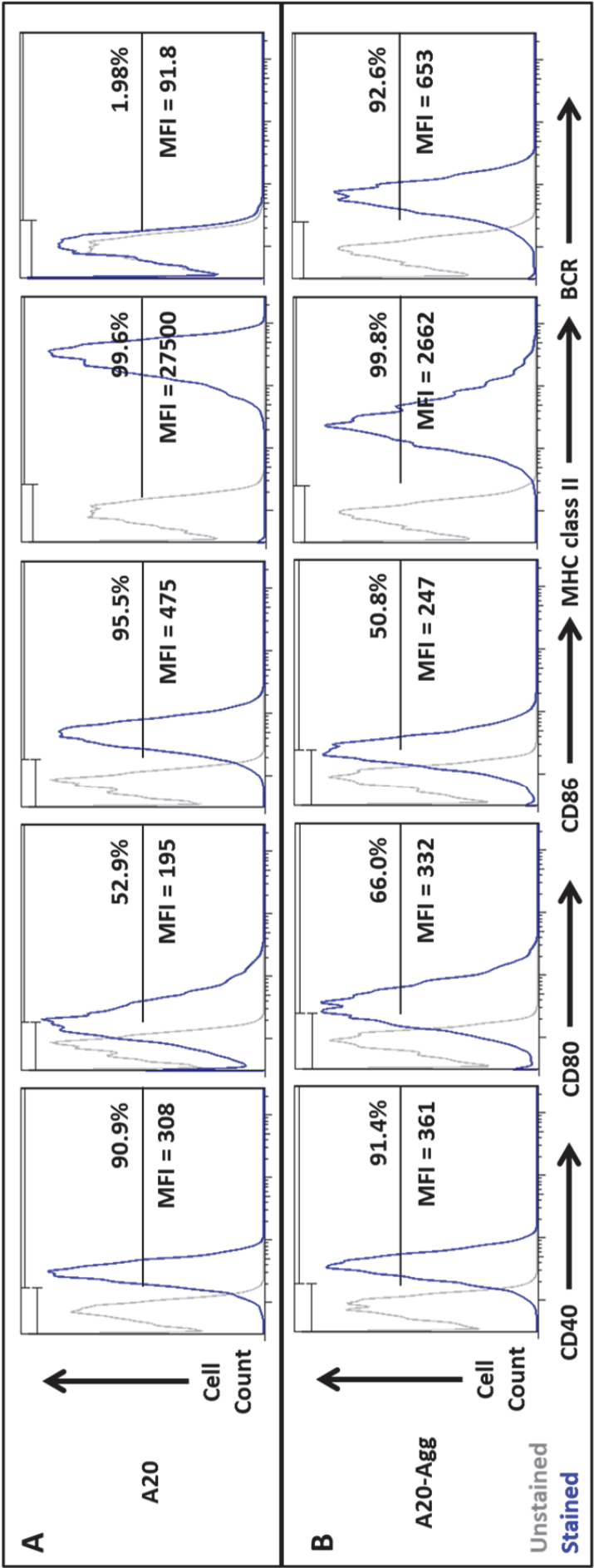


Figure 4.3 Flow cytometric analysis of non-specific A20 and aggrecan-specific A20-Agg B cell lines.

(A) A20 and (B) A20-Agg were incubated with an array of antibodies to determine expression of phenotypic markers, co-stimulation molecules and MHC class II. Data representative of at least 3 routine screening experiments conducted prior to incorporation of cells into assays. MFI = median fluorescence intensity.

Resting splenic B cells were isolated via negative selection using CD43⁺ microbeads. The resulting population was shown to be of a high purity (98.7%) and contained predominantly CD43⁺B220⁺ cells (93.6%). The CD43⁺ B cells were MHC class II⁺ (95.1%) and the majority of cells were also CD40⁺ (93.5%). However, CD80⁺ (11.4%) cells were less numerous than CD86⁺ (55.1%) cells (**Figure 4.4A; top panels**). After 10 d of culture, DC preparations formed a heterogeneous population but the majority of cells were CD11c⁺ (63.1%) and F4/80⁺ (61.7%). The DC population also contained CD40⁺ (26.0%), CD80⁺ (72.0%), CD86⁺ (71.9%) and MHC class II⁺ (74.5%) cells (**Figure 4.4B; middle panels**). The macrophage population was F4/80⁺ (93.1%) and MHC class II⁺ (98.7%) but, in contrast to the DC, most did not express CD11c (6.49%) and CD40 (1.18%) and were comprised of a comparatively lower percentage of CD80⁺ (12.0%), CD86⁺ (29.7%) cells (**Figure 4.4B; bottom panels**).

The cell lines and *ex vivo* cell preparations examined all demonstrated some expression of MHC class II but showed varying levels of co-stimulatory molecule expression. As a result, this combination of cell lines and *ex vivo* cell preparations represented a range of APC types with which to study the consequences of aggrecan presentation to the TCR-5/4E8 transgenic CD4⁺ T cells.

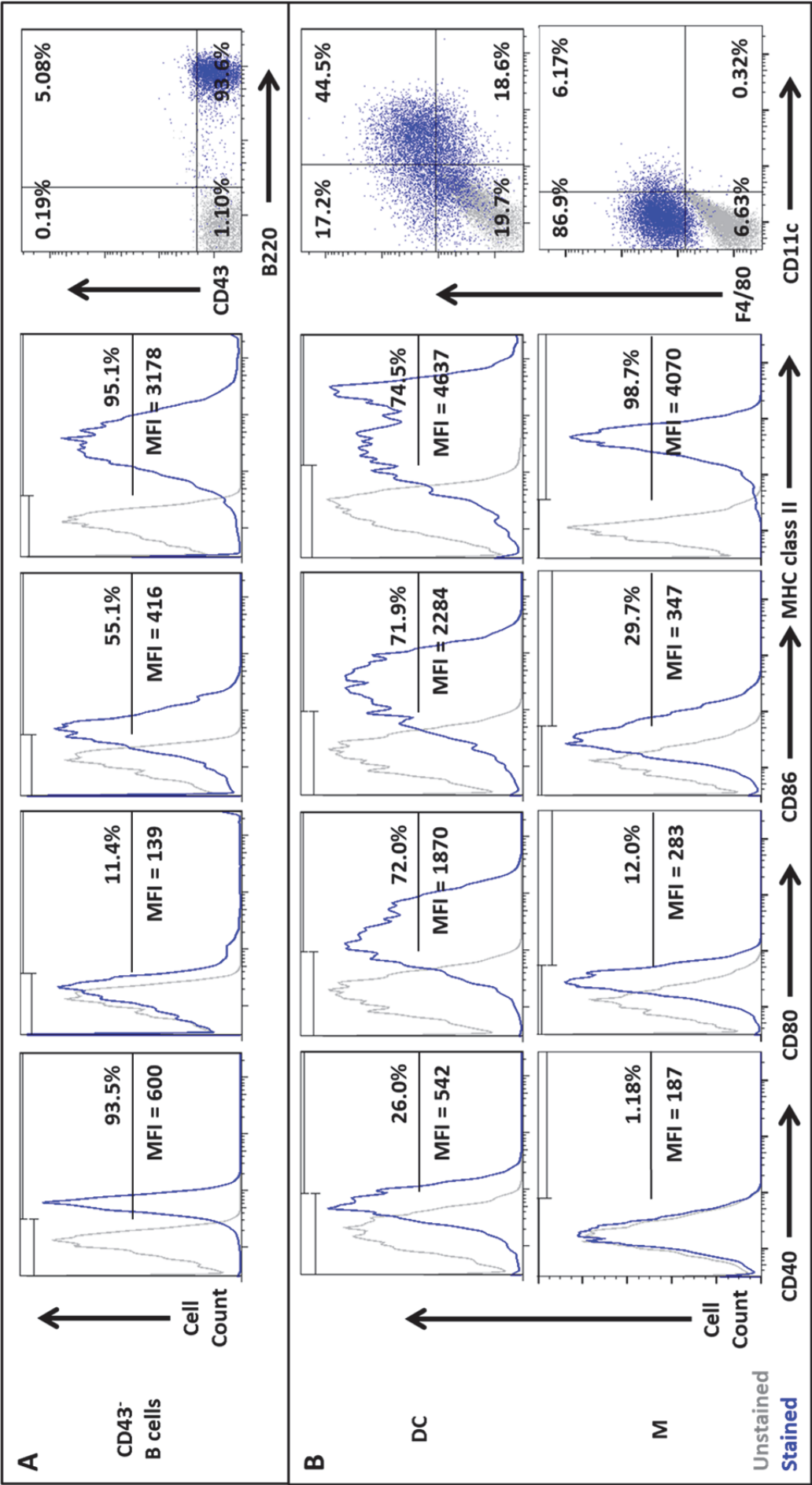


Figure 4.4 Flow cytometric analysis of ex vivo APC populations.

(A) CD43⁻ B cells were isolated by CD43⁺ depletion: 1x10⁸ splenocytes labelled with CD43⁺ magnetic microbeads were passed through a MACS column and filtrate collected. (B) DC and macrophages (M) were cultured from WT BALB/c bone marrow preparations. APC were then incubated with an array of antibodies to determine expression of phenotypic markers, co-stimulation molecules and MHC class II. Data representative of at least 3 routine screening experiments conducted prior to incorporation of cells into assays. MFI = median fluorescence intensity.

4.2.4. Effects of lipopolysaccharide stimulation at different stages of dendritic cell maturation on antigen presentation

DC are capable of capturing antigen efficiently and subsequently process and present peptide fragments to T cells (Sallusto and Lanzavecchia, 1994). Antigen capture occurs through macropinocytosis, a cytoskeleton-dependent form of fluid-phase uptake, or via receptor-mediated endocytosis. In response to inflammatory stimuli, such as TNF- α , IL-1 and pattern recognition receptor ligation (e.g. LPS binding TLR4), DC undergo a transient phase of enhanced macropinocytosis and actin-mediated endocytosis. DC then undergo a shift away from antigen capture and instead migrate into lymphatics and focus on activation of T cells. This is largely achieved by the downregulation of antigen uptake mechanisms, including macropinocytosis and Fc receptor expression, in combination with increased transport of pMHC complexes to the cell surface and upregulation of chemokine, co-stimulatory and adhesion molecule expression (Roake et al., 1995; Sallusto et al., 1995). Therefore, in addition to their phenotypic characterisation, DC uptake and presentation of aggrecan was also assessed under different maturation conditions to ensure that optimal presentation to TCR-5/4E8-transgenic CD4⁺ T cells was measured.

DC were matured in four different conditions prior to incorporation into T cell activation assays (Figure 4.5). DC were either pulsed with antigen for 3 h (- LPS), with antigen and 0.1 μ g/ml LPS for 3 h (+ LPS), with antigen for 18 h (+ Antigen - LPS) or with antigen and LPS for 18 h (+ Antigen + LPS) before the addition of T cells. When cultured with p84-103 or dAgg, DC treated in all conditions induced similar CD4⁺ T cell IL-2 production in response to both p84-103 (Figure 4.5A) and dAgg (Figure 4.5B). However, DC matured with antigen and LPS induced slightly higher background IL-2 than those matured in other conditions.

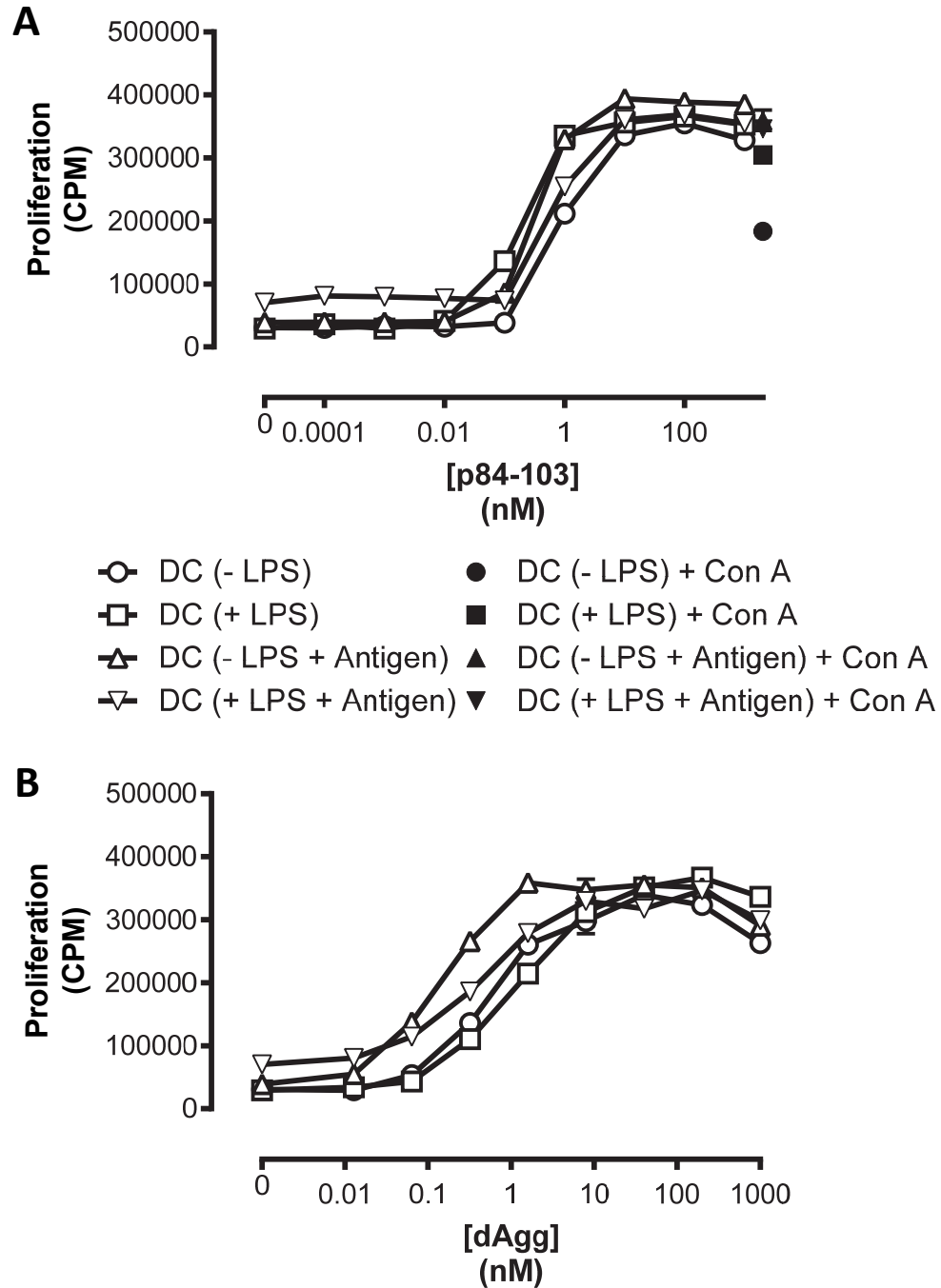


Figure 4.5 *IL-2 production in co-cultures of DC matured with different combinations of antigen and LPS and TCR-5/4E8-transgenic CD4⁺ T cells in response to graded doses of p84-103 and aggrecan.*

DC were pulsed with antigen for 3 h (- LPS), antigen + 0.1 µg/ml LPS for 3 h (+ LPS), antigen for 18 h (+ Antigen - LPS) or antigen + LPS for 18 h (+ Antigen + LPS). 3×10^5 DC and 1.5×10^5 CD4⁺ T cells were added to each well and cultured for 72 h. Supernatants were extracted and added to 3×10^4 CTLL-2. Cultures were pulsed with radioactive thymidine (³H) and DNA harvested onto a glass fibre mesh 18 h later. ³H incorporation was measured using a scintillation counter and counts per minute (CPM) recorded. Each data point represents a triplicate with error bars showing ±SEM. Data representative of three independent experiments.

Since the original observations documenting the substantial changes that take place in the process of antigen acquisition and presentation following the activation of DC via PRR ligands, more recent experiments have shown that DC do in fact increase antigen acquisition for short periods of time following TLR activation prior to the previously documented reduced antigen uptake and a shift in focus to increased antigen loading and subsequent pMHC expression (West et al., 2004). However, previous flow cytometric data (**Figure 4.4B; middle panels**) demonstrated that DC lifted from Petri dishes already expressed MHC class II and co-stimulation molecules prior to any further stimulation suggesting that the traditional requirement for the addition of LPS may not be required for bone marrow-derived DC to efficiently present antigen and activate CD4⁺ T cells. In light of both this and the experiments described in **Figure 4.5**, all further experiments with bone marrow-derived DC involved their detachment and re-plating with antigen for 18 h to allow efficient antigen uptake prior to co-culture with T cells. This was done in the absence of LPS to reduce background IL-2 production and any potential non-specific effects of LPS.

4.2.5. Optimisation of proliferation assay APC:T cell ratio and total cell number

Figure 4.6 shows further optimization of the experiments shown in Chapter 3 in order to establish a 96-well assay (200 μ l culture volume) for comparing the ability of different APC to present aggrecan to TCR-5/4E8-transgenic CD4⁺ T cells. While previous experiments (Figures 3.4–3.9) were performed with splenocyte APC, it was essential that assays that would be used to compare different APC types were also optimised. Preliminary experiments were performed where the numbers of A20-Agg B cells and isolated TCR-5/4E8-transgenic CD4⁺ T cells/well were examined. Therefore, A20-Agg B cells and TCR-5/4E8-transgenic CD4⁺ T cells were simultaneously titrated 1:2 from 50,000 to 400 cells per well and co-cultured with 100 nM dAgg for 72 h.

In accordance with previous data (Figure 3.9), IL-2 production was shown to increase with total APC and T cell number. IL-2 production was shown to be exquisitely sensitive to the number of APC. When <12,500 T cells were present only 400 APC were capable of inducing >50,000 CPM of CTLL-2 proliferation. In contrast, at <12,500 APC per well, increasing numbers of T cells did not generate >100,000 CPM of CTLL-2 proliferation until T cell numbers also reached 12,500 per well. Taken together this suggested that optimal conditions would involve at least $\geq$ 12,500 of each cell type present preferably with an abundance of APC in order to maximise antigen presentation and T cell activation. As the isolation of CD4⁺ T cells in the absence of splenic APC (previously shown Figure 3.12) resulted in significantly reduced yields, future assays were performed in the 96-well format, where 15,000 T cells were co-cultured with 30,000 APC at a 2:1 APC:T cell ratio to maintain a balance between total cell number and the potential for T cell activation (the black arrow on Figure 4.6 shows proliferation using the same ratio and similar cell numbers). For some experiments, where larger cell numbers were required, culture volumes and cell numbers were scaled-up appropriately maintaining the APC:T cell ratio at 2:1.

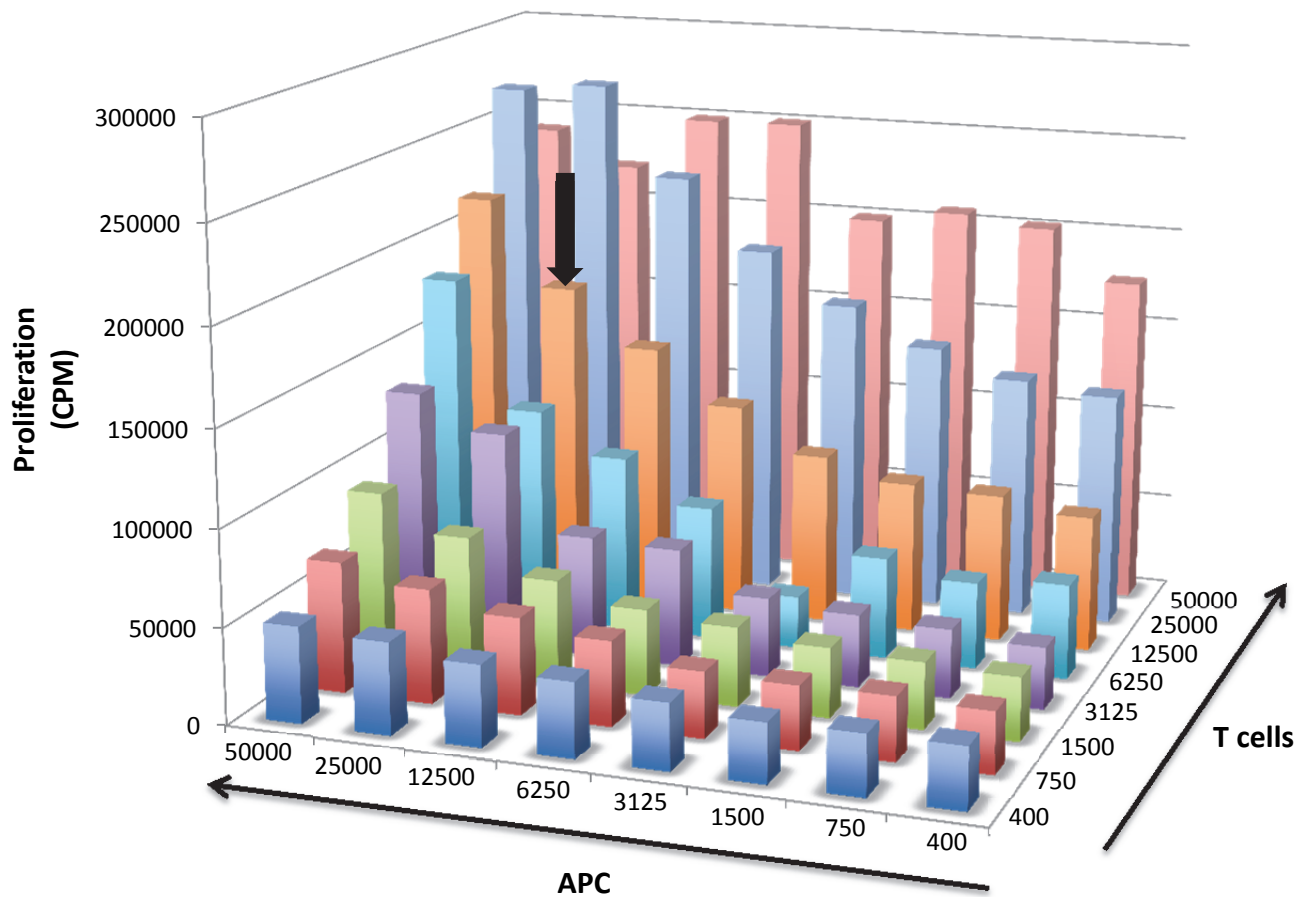


Figure 4.6 *IL-2 production by titrated numbers of TCR-5/4E8-transgenic CD4⁺ T cells following incubation with titrated numbers of A20-Agg B cells and a fixed dose of dAgg.*

Titred numbers of CD4⁺ T cells and A20-Agg B cells were co-cultured for 72 h in the presence of 100 nM dAgg. Supernatants were extracted and 3×10^4 CTLL-2 added. The cells were then pulsed with radioactive thymidine (^3H) and DNA was harvested 18 h later. ^3H incorporation was measured using a scintillation counter and the number of counts per minute (CPM) measured for each well. Each data point represents a duplicate. Arrow indicates approximate cell number and APC:T cell ratio used for subsequent assays. Black arrow indicates data point closest to cell ratio utilised in assays.

4.2.6. IL-2 production by CD4⁺ T cells following co-culture with different *ex vivo* antigen-presenting cell populations

Having established a procedure for the isolation of a functionally pure, predominantly naïve TCR-5/4E8-transgenic CD4⁺ T cell population, and characterised several candidate populations of *ex vivo* APC, the ability of the various APC populations to induce CD4⁺ T cell proliferation in response to both peptide and protein antigen was next examined. **Figure 4.7** shows T cell activation when WT splenocytes, CD43⁻ B cells, DC and macrophages were co-cultured with TCR-5/4E8-transgenic CD4⁺ T cells in the presence of either graded doses of p84-103 or dAgg.

In comparison to other APC, DC were shown to induce both the highest levels of IL-2 production as well as at the lowest dose of both p84-103 (**Figure 4.7A**) and dAgg (**Figure 4.7B**). The TCR-5/4E8-transgenic CD4⁺ T cells achieved a stable peak response at 1 nM p84-103 or dAgg demonstrating that DC are efficient APC in both situations. In contrast, the macrophages did not induce IL-2 production from TCR-5/4E8-transgenic CD4⁺ T cells in response to either p84-103 (**Figure 4.7A**) or dAgg (**Figure 4.7B**) even at doses as high as 100 nM.

The induction of TCR-5/4E8-transgenic CD4⁺ T cell IL-2 production by both CD43⁻ B cells and splenocytes showed similar characteristics. This was expected, as the most abundant APC population in the spleen is B cells. Although the B cells and splenocytes were not as sensitive as the DC, they did induce IL-2 production in response to high doses of p84-103 in the range of 1-1000 nM (**Figure 4.7A**). In contrast, both splenocytes and CD43⁻ B cells failed to induce IL-2 production in response to dAgg (**Figure 4.7B**).

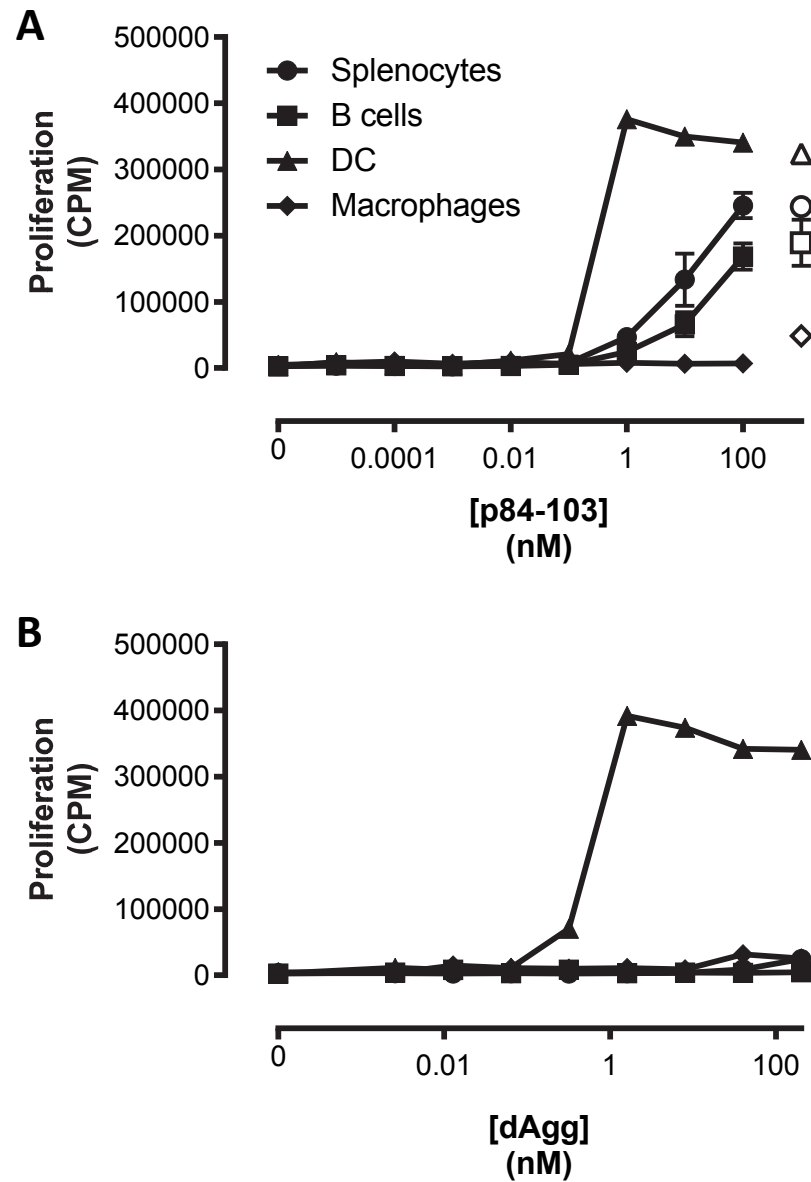


Figure 4.7 *IL-2 production in co-cultures of different ex vivo APC populations and TCR-5/4E8-transgenic CD4⁺ T cells in response to graded doses of p84-103 and aggrecan.*

3×10^5 APC and 1.5×10^5 CD4⁺ T cells were added to each well and cultured for 72 h in a flat-bottomed 96-well plate. Supernatants were extracted and added to 3×10^4 CTLL-2. Cultures were pulsed with radioactive thymidine (³H) and DNA harvested onto a glass fibre mesh 18 h later. ³H incorporation was measured using a scintillation counter and counts per minute (CPM) recorded. Each data point represents a triplicate with error bars showing $\pm$ SEM. Data representative of three independent experiments.

4.2.7. Differential cytokine production by TCR-5/4E8-transgenic CD4⁺ T cells following co-culture with A20-Agg B cells or dendritic cells in the presence of graded doses of aggrecan

Comparison of antigen-presentation by different *ex vivo* APC revealed that DC were the most efficient at presenting peptide or protein antigen to the TCR-5/4E8-transgenic CD4⁺ T cells inducing IL-2 production (Figure 4.7). Therefore, DC were selected as APC for further comparison to the A20-Agg B cells. Previous work from our laboratory has examined the enzyme usage during aggrecan processing and presentation by different APC and found no significant differences. In addition, the kinetics of antigen presentation to the aggrecan-specific T cell hybridoma 192 and the effects of the APC:T cell ratio were also studied and, similarly, showed no significant differences between A20-Agg B cells, DC or macrophages when using protein antigen. Consequently, having ruled out differences in antigen uptake or processing, DC were compared to aggrecan-specific A20-Agg B cells with respect to their ability to induce naïve TCR-5/4E8-transgenic CD4⁺ T cell activation and cytokine production in response to both high and low doses of antigen (Figure 4.8). For reasons discussed earlier, the production of key proinflammatory (IFN- γ and IL-17) and regulatory (IL-4 and IL-10) cytokines was analysed (Firestein, 2004).

DC induction of TCR-5/4E8-transgenic CD4⁺ T cell IL-2 production following culture with 100 or 1 nM p84-103 was shown to be greater than that seen following co-culture with A20-Agg B cells. In contrast, both APC induced similar IL-2 production following co-culture with dAgg at both doses (Figure 4.8A). This confirms previous observations using the T cell hybridoma 192 (Wilson et al., 2012). DC also induced greater levels of IFN- γ than A20-Agg B cells when presenting high doses of p84-103. Remarkably, however, the contrary was shown following incubation with dAgg. A20-Agg B cells consistently demonstrated an ability to induce greater IFN- γ production than DC in response to both 100 and 1 nM dAgg (Figure 4.8B). Only very low levels of IL-4 were detected following culture with all antigen doses with no significant differences observed between A20-Agg B cells and DC (Figure 4.8C).

Co-cultures containing TCR-5/4E8-transgenic CD4⁺ T cells and A20-Agg B cells were shown to contain low levels (<100 pg/mL) of IL-10 following incubation with p84-103 and, interestingly, this was similar to that seen in the absence of antigen. Despite this, control wells where both A20-Agg B cells and TCR-5/4E8-transgenic CD4⁺ T cells were incubated in isolation did not contain any IL-10 suggesting that the presence of both cell types was necessary for IL-10 production (Appendix A). The addition of dAgg did, however, induce production of higher levels of IL-10 in A20-Agg B cell co-cultures but DC co-cultures did not contain similarly high levels IL-10 in response to any stimuli (Figure 4.8D).

In contrast to IL-10, IL-17 was only detected in p84-103-stimulated DC co-cultures, albeit at low levels. Cultures stimulated with dAgg contained very low levels of IL-17. Similarly, co-cultures containing A20-Agg B cells were also shown to contain very low levels of IL-17 following incubation with either p84-103 or dAgg (Figure 4.8E).

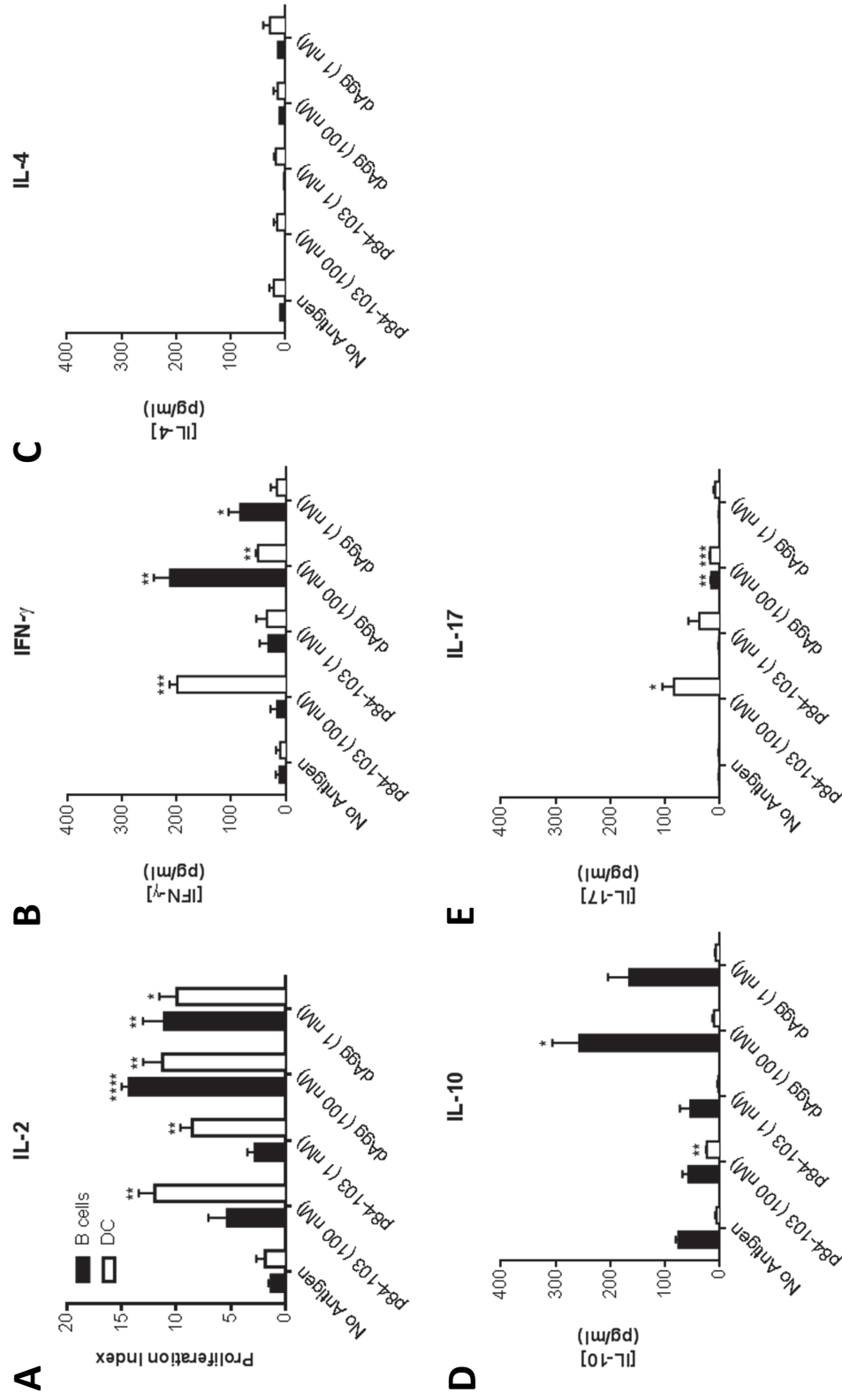


Figure 4.8 Cytokine production by TCR-5/4E8-transgenic CD4⁺ T cells in co-cultures with A20-Agg or DC following stimulation with fixed doses of p84-103 and dAgg.

3x10⁵ APC and 1.5x10⁵ CD4⁺ T cells were added to each well and stimulated with 100 nM or 1 nM of p84-103 or dAgg and cultured for 72 h in a flat-bottomed 96-well plate. (A) IL-2 levels were determined by bioassay. Supernatants were extracted and 3x10⁴ CTLL-2 added. The cells were then pulsed with radioactive thymidine (³H) and DNA was harvested 18 h later. ³H incorporation was measured using a scintillation counter and the number of counts per minute (CPM) measured for each well. T cell proliferation indices = (CPM following CTLL-2 culture with supernatants from co-cultures stimulated with 100 nM antigen)/(CPM following CTLL-2 culture with medium only). Supernatants were tested for (B) IFN- γ , (C) IL-4, (D) IL-10 and (E) IL-17 by ELISA. Data were pooled from three independent experiments. Each data point represents a triplicate with error bars showing $\pm$ SEM. * indicates an ANOVA test p -value ≤ 0.05 when compared to unstimulated controls.

4.2.8. IL-2 production by CD4⁺ T cells following co-culture with A20-Agg or dendritic cells and stimulation with graded doses of aggrecan

Previous experiments established that cytokine production by TCR-5/4E8-transgenic CD4⁺ T cells, following either presentation of pre-processed aggrecan p84-103 or following the uptake and processing of aggrecan by aggrecan-specific B cells was different to that seen with DC (Figures 4.8A, B, D & E). In order to further characterise the process responsible for these differences, the IL-2 response was firstly characterised in more detail utilising an extended range of antigen doses. In addition, TCR-5/4E8-transgenic CD4⁺ T cell IL-2 production was also measured following presentation by the non-specific A20 B cells as APC for comparison (Figure 4.9).

Incubation of TCR-5/4E8-transgenic CD4⁺ T cells with graded doses of p84-103 revealed that both the aggrecan-specific A20-Agg and non-specific A20 B cells induced T cell responses at concentrations >1 nM (Figure 4.9A). However, DC were seen to induce increased levels of IL-2 than that seen with both B cell lines at doses ranging from 0.01-100 nM (Figures 4.9A & C).

However, when parallel co-cultures were incubated with dAgg, A20-Agg B cells showed a similar ability to process and present antigen as DC, achieving T cell activation at concentrations <0.01nM. Both the A20-Agg B cells and DC were capable of inducing IL-2 production at much lower doses than the A20 B cells, which did not induce IL-2 production until the antigen concentration exceeded 10 nM (Figure 4.9B). This direct comparison to DC confirms that antigen-specific B cells are highly efficient at presenting T cell epitopes derived from their cognate antigen. However, the difference in T cell activation between A20-Agg B cells and DC was not significant (Figure 4.9C). IL-2 production by T cells is used as a marker for T cell activation. However, production of cytokines such as IFN- γ and IL-10 are associated with specialised CD4⁺ T cell subsets and, therefore, are indicative of T cell differentiation. As a result, these experiments exclude differences in the efficiency of dAgg uptake and processing by A20-Agg B cells and DC as an explanation for the differential cytokine production observed in Figure 4.8B.

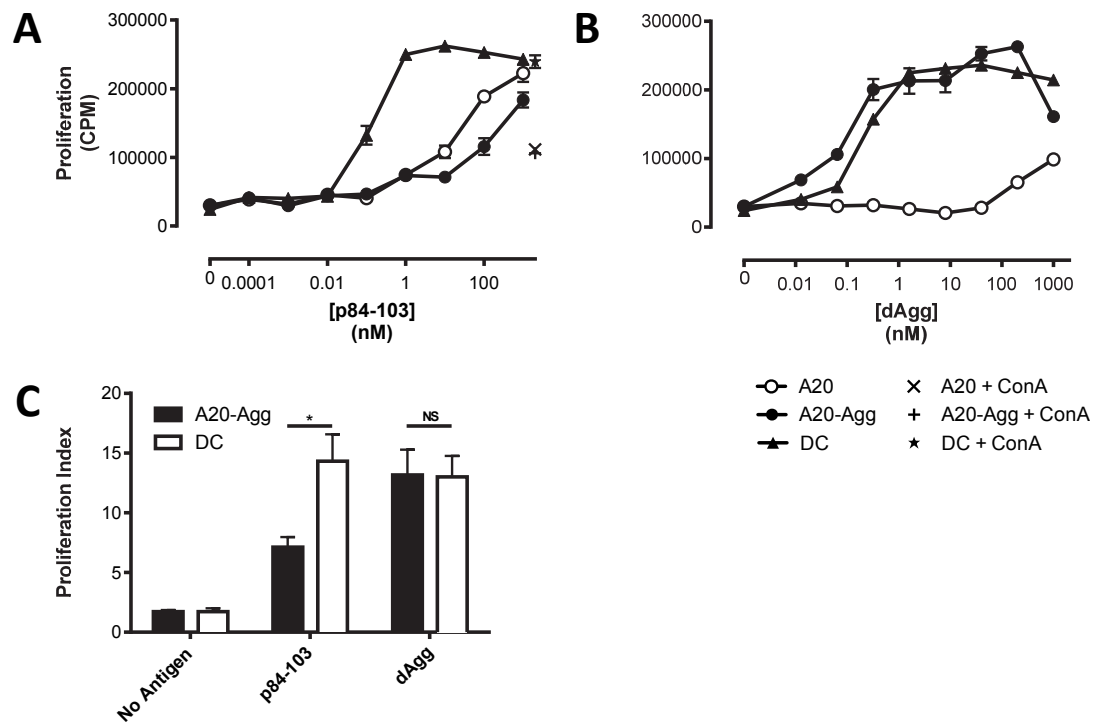


Figure 4.9 IL-2 production by TCR-5/4E8-transgenic $CD4^+$ T cells in co-cultures with A20, A20-Agg or DC following stimulation with graded doses of p84-103 and aggrecan.

3×10^5 APC and 1.5×10^5 $CD4^+$ T cells were added to each well, stimulated with graded doses of (A) p84-103 or (B) dAgg and cultured for 72 h in a flat-bottomed 96-well plate. IL-2 levels were determined by bioassay. Supernatants were extracted and 3×10^4 CTLL-2 added. The cells were then pulsed with radioactive thymidine (3H) and DNA was harvested 18 h later. 3H incorporation was measured using a scintillation counter and the number of counts per minute (CPM) measured for each well. Each data point represents a triplicate with error bars showing $\pm$ SEM. Data representative of three independent experiments. (C) Summary of the IL-2 proliferation index from A20-Agg and DC co-cultures compared at 100 nM p84-103 or dAgg. T cell proliferation indices = (CPM following CTLL-2 culture with supernatants from co-cultures stimulated with 100 nM antigen)/(CPM following CTLL-2 culture with medium only). Data were pooled from three independent experiments. Error bars show $\pm$ SEM. * indicates a t -test p -value ≤ 0.05 .

4.2.9. IFN- γ production by TCR-5/4E8-transgenic CD4⁺ T cells following co-culture with A20-Agg or dendritic cells in the presence of graded doses of aggrecan

Although **Figure 4.9** demonstrated that antigen-specific B cells were similar to DC in their ability to activate TCR-5/4E8-transgenic CD4⁺ T cells in the presence of very low doses of dAgg, this finding alone does not provide a compelling explanation for the requirement for B cell-mediated antigen presentation in a number of mouse models of RA or the success of B cell depletion therapy in RA patients. As a result, future experiments shifted the focus to explore the differences shown by the various APC on TCR-5/4E8-transgenic CD4⁺ T cell production of the proinflammatory cytokine IFN- γ . IFN- γ has been shown to be a key inflammatory molecule in RA and the mouse model PGIA and so presents a logical target of interest for study. In addition, previous experiments using a limited range of antigen doses suggested that A20-Agg B cells may induce greater IFN- γ production from the TCR-5/4E8-transgenic CD4⁺ T cells than that seen with DC (**Figure 4.8B**).

Figure 4.10 revealed that following incubation with graded doses of p84-103 only DC generated detectable levels of IFN- γ production from TCR-5/4E8-transgenic CD4⁺ T cells at low doses while both A20 and A20-Agg B cells failed to induce detectable IFN- γ production even at high doses (**Figure 4.10A**). This experiment confirms that seen with the two antigen doses used in **Figure 4.8B** and clearly demonstrates that DC are significantly more effective than B cells when presenting pre-processed peptide antigen (**Figure 4.10C**). Whilst DC also were shown to induce IFN- γ production in an dose-dependent manner when co-cultured with TCR-5/4E8-transgenic CD4⁺ T cells in the presence of dAgg, it was evident that co-cultures containing A20-Agg B cells were seen to contain enhanced levels of IFN- γ (**Figure 4.10B**). Remarkably, when the levels of IFN- γ produced following incubation with 100 nM aggrecan from three independent experiments were compared, it was seen that the A20-Agg B cells consistently induced significantly greater IFN- γ production than that seen following DC co-culture (**Figure 4.10C**).

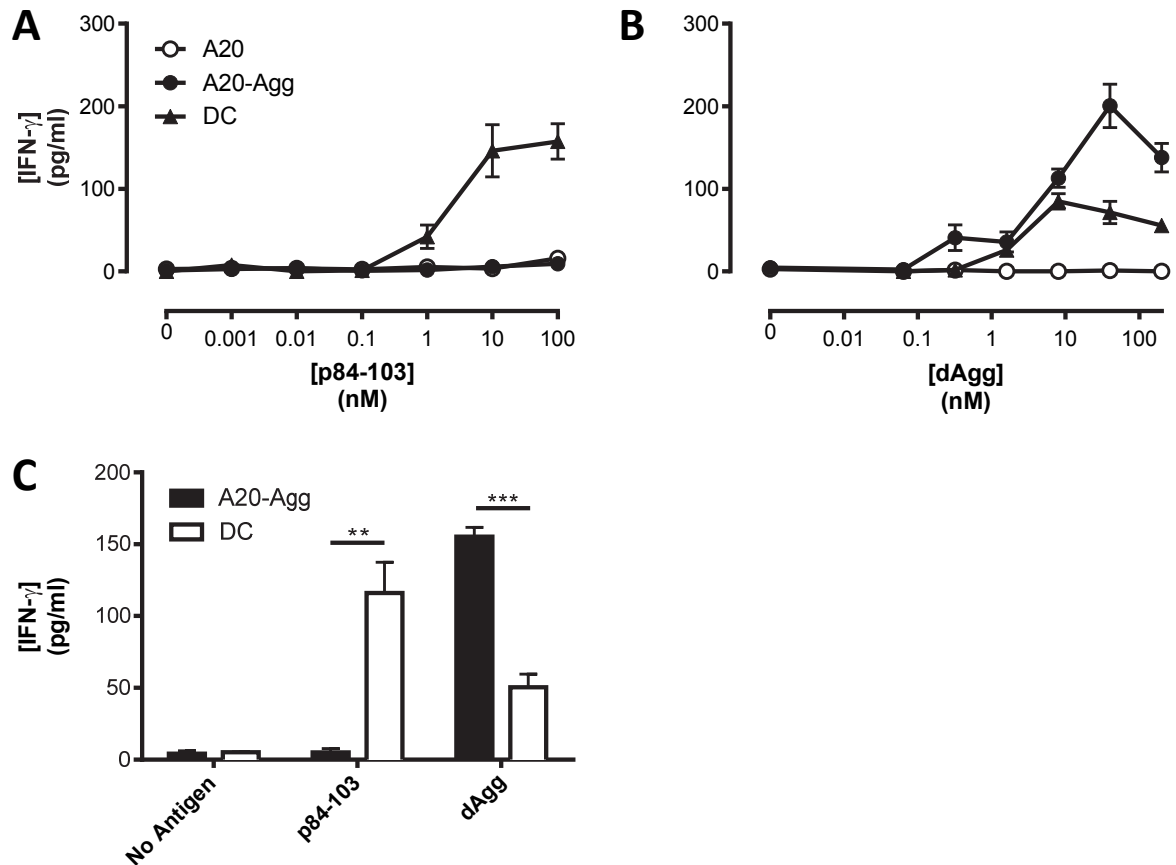


Figure 4.10 *IFN- γ production by TCR-5/4E8-transgenic CD4⁺ T cells in co-cultures with A20, A20-Agg or DC following stimulation with graded doses of p84-103 and aggrecan.*

3×10^5 APC and 1.5×10^5 CD4⁺ T cells were added to each well and stimulated with graded doses of (A) p84-103 or (B) dAgg and cultured for 72 h in a flat-bottomed 96-well plate. Supernatants were extracted and tested for IFN- γ by ELISA. Each data point represents a triplicate with error bars showing $\pm$ SEM. Data representative of 3 independent experiments. (C) Summary of IFN- γ induction in A20-Agg and DC co-cultures compared at 100 nM p84-103 or dAgg. Data were pooled from three independent experiments. Error bars show $\pm$ SEM. * indicates a *t*-test *p*-value ≤ 0.05 .

4.2.10. Intracellular staining for expression of IFN- γ by CD4⁺ T cells following co-culture with A20-Agg or dendritic cells and stimulation with aggrecan

To corroborate the ELISA data showing enhanced IFN- γ production following the co-culture of the TCR-5/4E8-transgenic CD4⁺ T with aggrecan-specific B cells as well as to simultaneously identify the cellular origin of the IFN- γ , larger-scale cultures were then established with TCR-5/4E8-transgenic CD4⁺ T cells co-cultured with DC or with A20-Agg B cells in the presence of either 100 nM p84-103 or dAgg. During the final 5 h of the 72 h incubation, PMA/ionomycin and Brefeldin A were added into these cultures prior to staining with lineage-specific antibodies. Cells in the co-cultures were then permeabilised and stained with an anti-IFN- γ antibody and analysed by flow cytometry. Importantly, to enable the IFN- γ staining to be differentially attributed to either of the two APC types or to the TCR-5/4E8-transgenic CD4⁺ T cells present in the co-cultures, a gating strategy detailed in **Figure 4.11** was used. Co-cultures were stained with anti-CD4 to identify TCR-5/4E8-transgenic CD4⁺ T cells. Anti-B220 or anti-CD11c was used to identify A20-Agg B cells or DC respectively. A20-Agg co-cultures were shown to contain a large proportion of B220⁺ cells (**Figure 4.11A**) while the DC co-cultures contained a sparser population of CD11c⁺ cells (**Figure 4.11B**). All B220⁺ and CD11c⁺ cells were shown not to express IFN- γ and were excluded prior to analysis of CD4⁺ T cell IFN- γ expression (**Figure 4.11A & B**). A small number of events were dual positive (CD4⁺B220⁺ or CD4⁺CD11c⁺) and may represent interacting APC and T cells or, in the case of the DC co-cultures, CD4⁺ DC. These events were also excluded from further analysis. Using this gating strategy, it was therefore possible to calculate the percentage of TCR-5/4E8-transgenic CD4⁺ T cells that were IFN- γ ⁺ in each co-culture.

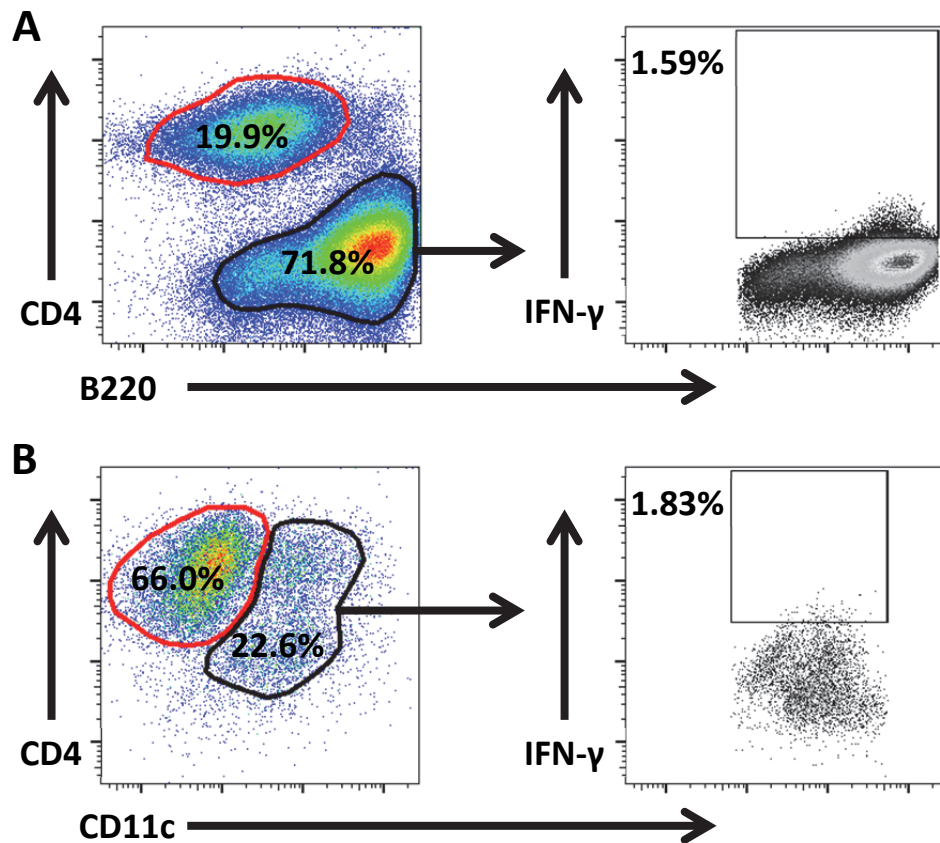


Figure 4.11 Gating strategy for flow cytometric analysis of intracellular staining.

1×10^6 (A) A20-Agg B cells or (B) DC and 5×10^5 CD4⁺ T cells were added to each well, stimulated with 100 nM p84-103 and cultured for 72 h. Co-cultures were stained with anti-CD4 and anti-B220 or anti-CD11c. The CD4⁺ (red) gate indicates cells analysed in all intracellular cytokine staining experiments. Cells in the APC (black) gate were shown not to express IFN- γ and excluded from further analysis.

The results shown in **Figure 4.12** demonstrate that addition of 100 nM p84-103 to DC co-cultures induced a higher proportion of IFN- γ ⁺ TCR-5/4E8-transgenic CD4⁺ T cells than 100 nM dAgg (**Figure 4.12A; bottom panels**). In contrast, A20-Agg co-cultures showed an increased percentage of IFN- γ ⁺ TCR-5/4E8-transgenic CD4⁺ T cells when incubated with dAgg compared to incubation with p84-103. Furthermore, dAgg-stimulated A20-Agg B cell co-cultures also had a higher proportion of IFN- γ ⁺ TCR-5/4E8-transgenic CD4⁺ T cells compared to DC co-cultures (**Figure 4.12A; top panels**). When data from several repeat experiments was pooled (**Figure 4.12B**), these findings were confirmed as DC were shown to be significantly more effective at inducing CD4⁺ T cell differentiation into IFN- γ -producing cells when cultured with p84-103, whilst A20-Agg B cells were shown to be significantly more effective than DC at generating IFN- γ -producing T cells in the presence of dAgg. Thus, these data are in accordance with the previous results quantifying greater secretion of IFN- γ in DC co-cultures following addition of p84-103 but, conversely, higher levels of IFN- γ in A20-Agg co-cultures containing dAgg (**Figure 4.10**).

Therefore in combination, these two sets of experiments show that the increased levels of IFN- γ detected in A20-Agg B cell/TCR-5/4E8-transgenic CD4⁺ T cell co-cultures following the addition of dAgg results from enhanced polarization of the transgenic CD4⁺ T cells into an IFN- γ -producing subset, classically defined as T_H1.

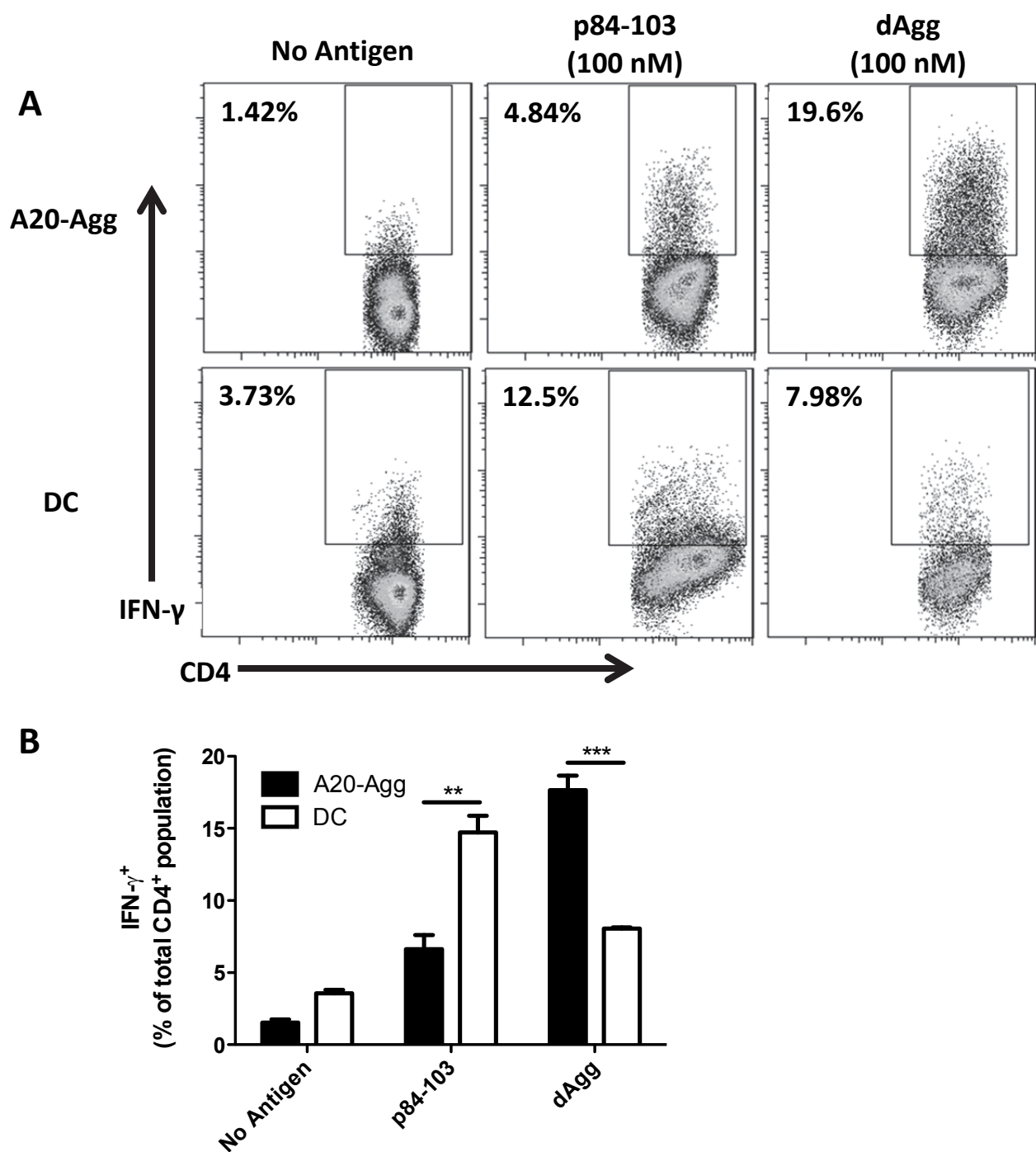


Figure 4.12 Flow cytometric analysis of intracellular IFN- γ expression by TCR-5/4E8-transgenic CD4⁺ T cells following co-culture with A20-Agg B cells or DC in the presence of 100 nM p84-103 or aggrecan.

1×10^6 APC and 5×10^5 CD4⁺ T cells were added to each well and cultured for 72 h in a 24-well plate. Co-cultures were then re-stimulated with PMA/ionomycin for the final 5 h of culture. APC were excluded from the analysis using the gating strategy described in **Figure 11**. (A) Numbers represent number of IFN- γ ⁺ cells as a percentage of the total CD4⁺ population. (B) Data was pooled from three independent experiments and the number of IFN- γ ⁺ cells as a percentage of the total CD4⁺ T cell population was calculated with error bars showing $\pm$ SEM. * indicates a *t*-test *p*-value ≤ 0.05 .

4.2.11. Intracellular staining for expression of IFN- γ by CD4⁺ T cells following co-culture with irradiated A20-Agg and stimulation with aggrecan

One potential explanation for the increased frequency of IFN- γ ⁺ TCR-5/4E8-transgenic CD4⁺ T cells following co-culture with A20-Agg B cells and aggrecan was the possibility that, as the B cells are immortalized transfectants, their numbers are likely to have increased over the course of the 72 h incubation, hence increasing the APC:T cell ratio. To determine if this was the case, additional experiments were performed, in which the proliferation of the A20-Agg B cells was reduced by irradiation prior to their culture with TCR-5/4E8-transgenic CD4⁺ T cells in the presence of either 100 nM p84-103 or dAgg.

To establish the optimal dose of irradiation required to significantly reduce the proliferation of the A20-Agg B cells over a 72 h period (in the presence or absence of antigen), a series of experiments were conducted over a range of irradiation doses. **Figure 4.13** shows an example of such an experiment comparing proliferation of control and irradiated A20 and A20-Agg B cell lines over a 72 h period. It was established that the highest dose of 50 Gy was required to maximally inhibit proliferation. Although, as expected, the presence of 100nM p84-103 was shown to have no effect on B cell proliferation, the presence of 100nM dAgg did somewhat reduce the proliferation of both A20 and A20-Agg. The reasons for this are unknown but may be caused by some component of the aggrecan preparation process that is mildly toxic and not completely removed following dialysis into PBS. Alternatively, cell growth over the 72 h may be inhibited by the dilution of the medium by the dAgg solution required when using high antigen concentrations.

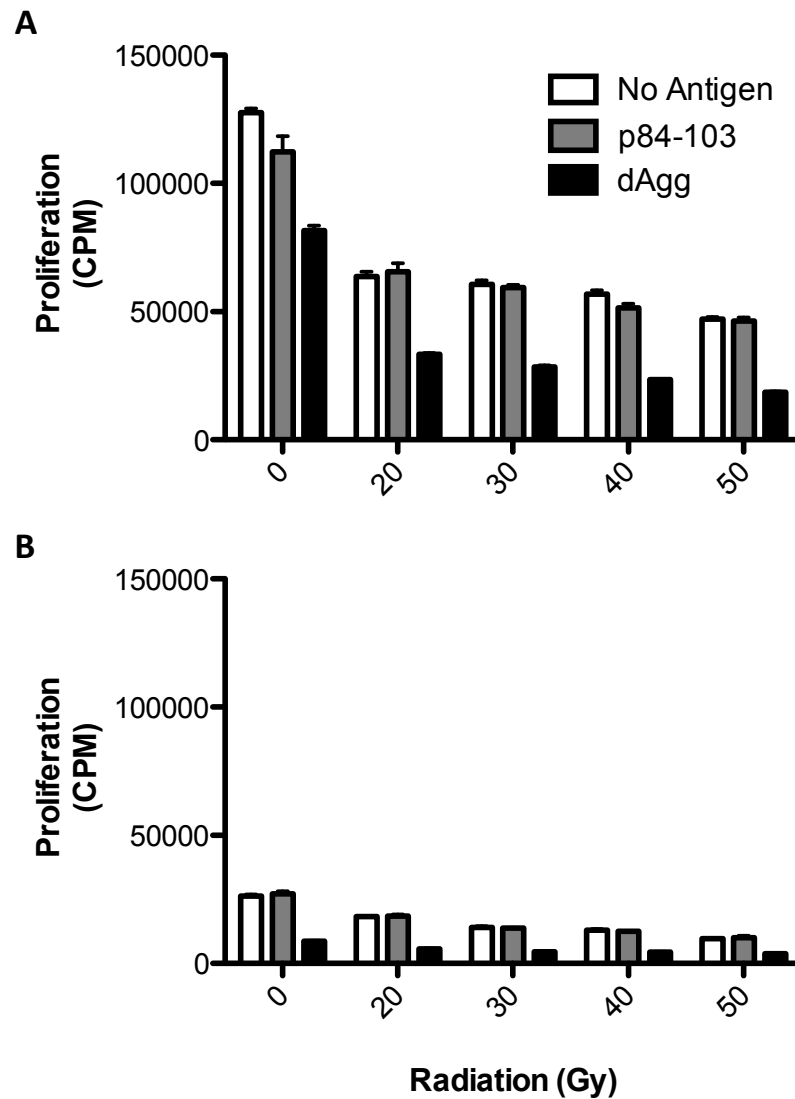


Figure 4.13 *Irradiation of A20 and A20-Agg B cells and measurement of proliferation over a 72 h period in the presence of antigen.*

5×10^4 (A) A20 or (B) A20-Agg B cells were irradiated with graded doses of γ -radiation prior to addition of 100 nM p84-103 or dAgg and culture in a flat-bottomed 96-well plate. Cells were immediately pulsed with radioactive thymidine (^3H) and DNA was harvested onto a glass fibre mesh 72 h later. ^3H incorporation was measured using a scintillation counter and counts per minute (CPM) recorded. Each data point represents a triplicate with error bars showing $\pm\text{SEM}$.

Interestingly, these experiments also revealed that the A20-Agg B cells proliferated substantially less (**Figure 4.13B**) than the untransfected control A20 B cells over the 72 h time period. However, **Figure 4.14** shows that when irradiated A20-Agg B cells were directly compared to viable A20-Agg B cells in co-cultures with TCR-5/4E8-transgenic CD4⁺ T cells similar numbers of IFN- γ ⁺ TCR-5/4E8-transgenic CD4⁺ T cells were detected. Thus the higher proportion of IFN- γ ⁺ TCR-5/4E8-transgenic CD4⁺ T cells in co-cultures containing A20-Agg B cells versus DC in the presence of dAgg does not appear to result from increased APC:T cell ratios as a result of A20-Agg proliferation during the culture period (**Figure 4.14**).

In conjunction with the IFN- γ ELISA data, these experiments are therefore consistent with the hypothesis that an essential component of B cell function in RA pathology may be derived from the unique properties of joint-reactive B cell presentation of arthritogenic antigens to autoreactive CD4⁺ T cells leading to enhanced inflammatory cytokine production.

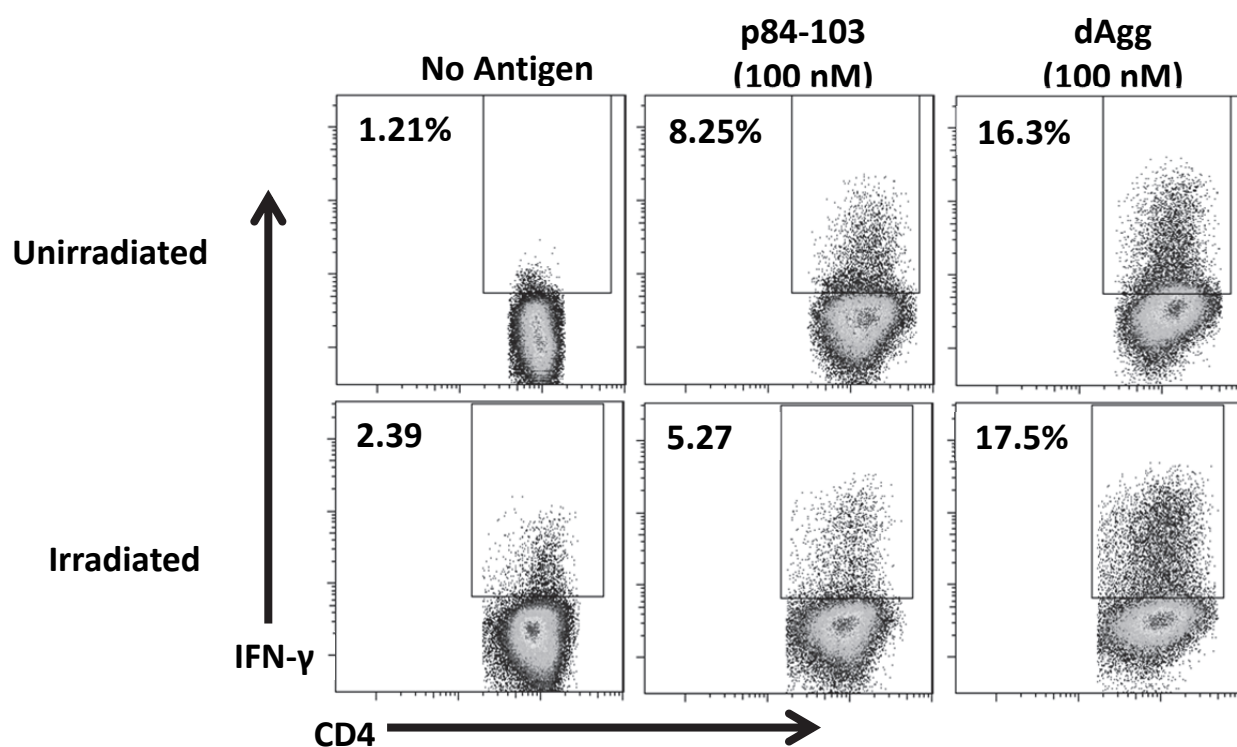


Figure 4.14 Flow cytometric analysis of intracellular IFN- γ expression by TCR-5/4E8-transgenic CD4⁺ T cells following co-culture with control or irradiated A20-Agg B cells in the presence of 100 nM p84-103 or aggrecan.

1×10^6 A20-Agg B cells were irradiated with 50 Gy of gamma radiation prior to culture with 5×10^5 CD4⁺ T cells for 72 h in a 24-well plate. Co-cultures were re-stimulated with PMA/ionomycin for the final 5 h of culture. Numbers represent IFN- γ ⁺ cells as a percentage of the total CD4⁺ population.

4.2.12. Generation of CD4⁺FoxP3⁺ TCR-5/4E8-transgenic T cells following co-culture with A20-Agg or dendritic cells and stimulation with aggrecan

In addition to activation of effector T cell functions, several lines of evidence also implicate APC in the polarisation of antigen-specific T cells into distinct regulatory populations during the course of an immune response (Walker et al., 2003; Zhu et al., 2010). In mice, T_{reg} can be reliably identified by the expression of the transcription factor FoxP3 (Ziegler, 2006). A previous experiment (**Figure 4.8D**) had showed that as well as the detection of IFN- γ in co-cultures of TCR-5/4E8-transgenic CD4⁺ T cells with A20-Agg B cells in the presence of aggrecan, IL-10 was also present at increased levels than that seen in co-cultures containing DC. T_{reg} produce IL-10 in order to exert immunosuppressive effects on APC and effector T cells (Chang et al., 2009; Heo et al., 2009). It was therefore of considerable interest to establish if the ability of A20-Agg B cells to skew TCR-5/4E8-transgenic CD4⁺ T cells into IFN- γ -producing effector cells more efficiently than DC was complemented by a concomitant increase in T_{reg} generation in equivalent co-cultures.

Following co-culture with DC the percentage of TCR-5/4E8-transgenic CD4⁺ T cells that expressed of FoxP3⁺ cells did not alter substantially even in the presence of antigen (**Figure 4.15A; bottom panels**). However, co-culture of TCR-5/4E8-transgenic CD4⁺ T cells with A20-Agg B cells in the absence of antigen resulted in lower numbers of TCR-5/4E8-transgenic CD4⁺ T cells expressing FoxP3 (compared to that seen in co-cultures with DC) which increased following incubation with 100 nM p84-103. Interestingly, the numbers of TCR-5/4E8-transgenic CD4⁺ T expressing FoxP3 further increased in co-cultures with A20-Agg B cells in the presence of 100 nM dAgg (**Figure 4.15A; top panels**).

Thus it appears that the profile of FoxP3 expression by the TCR-5/4E8-transgenic CD4⁺ T cells following incubation with the different forms of antigen and APC mirrors that of IFN- γ (**Figure 4.12**). These data imply that aggrecan presentation by A20-Agg B cells not only promotes TCR-5/4E8-transgenic CD4⁺ T cells differentiation into IFN- γ producing effector cells, thus defining the requirement for B cell APC function in both animal models of disease and in RA itself, but that this process similarly generates a proportionate regulatory population to manage this response. However, DC co-cultures showed similar induction of CD4⁺FoxP3⁺ T cells following p84-103 or dAgg stimulation (**Figure 4.15B**). Taken together, these data indicate that, when compared to DC, A20-Agg B cells induce increased expansion of both pathogenic and regulatory CD4⁺ T cell subsets following processing and presentation of extra-cellular matrix components such as aggrecan.

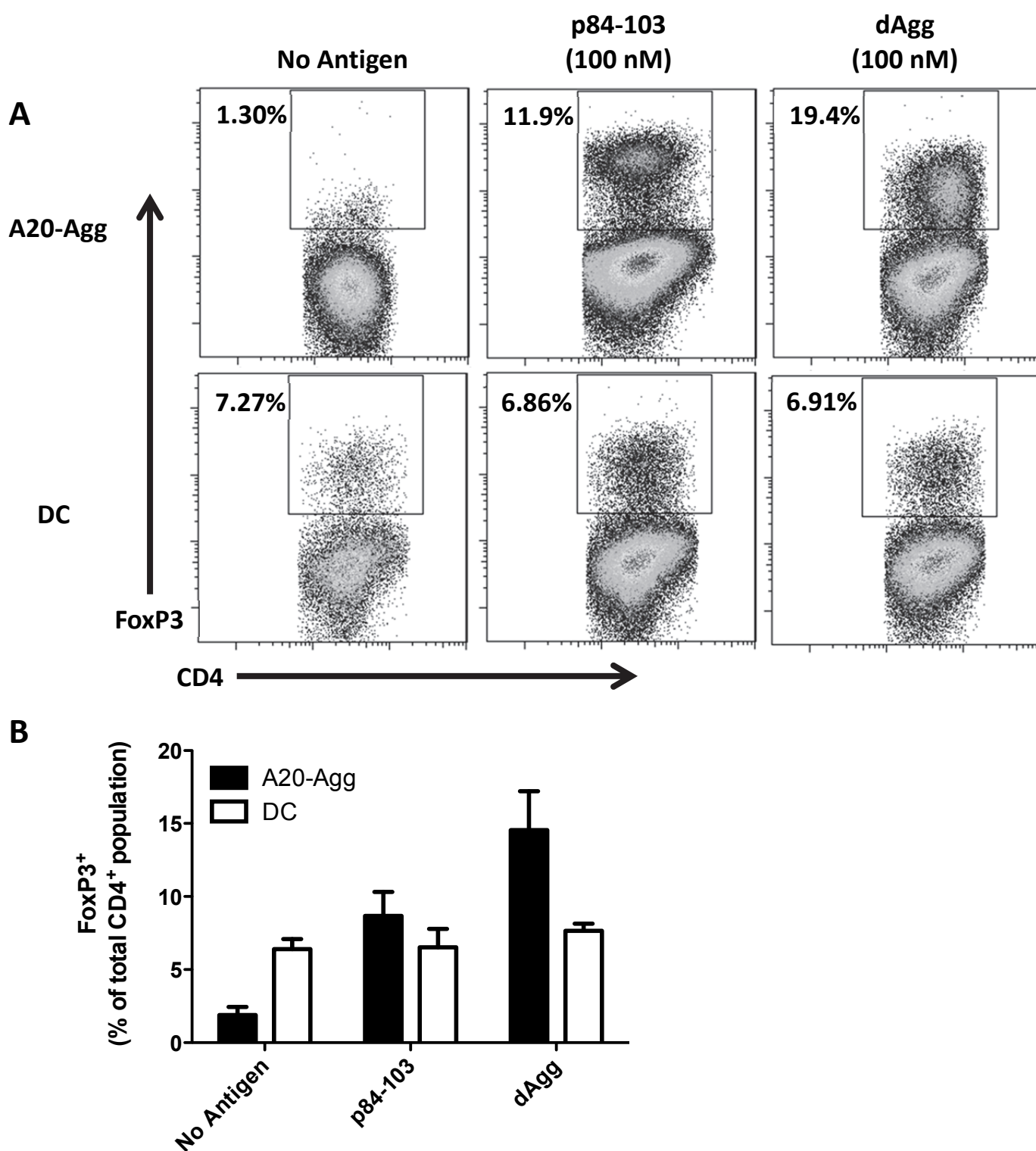


Figure 4.15 Flow cytometric analysis of intracellular FoxP3 expression by TCR-5/4E8-transgenic $CD4^+$ T cells following co-culture with A20-Agg B cells or DC in the presence of 100 nM p84-103 or aggrecan.

1×10^6 APC and 5×10^5 $CD4^+$ T cells were added to each well and cultured for 72 h in a 24-well plate. Co-cultures were then re-stimulated with PMA/ionomycin for the final 5 h of culture. (A) Numbers represent number of FoxP3⁺ cells as a percentage of the total $CD4^+$ population. (B) Data were pooled from three independent experiments and the number of FoxP3⁺ cells as a percentage of the total $CD4^+$ T cell population was calculated with error bars shown mean $\pm$ SEM.

4.3. Discussion

The work presented in this chapter provides *in vivo* evidence that both B and T cells infiltrate the joints of arthritic mice and, through the development of an *in vitro* system, demonstrates that antigen-specific B cells are more capable than other APC at inducing greater pathogenic cytokine production by autoreactive CD4⁺ T cells. Intravital microscopy of the ankle joints of arthritic mice has visualised leukocyte trafficking into the joint compartment. However, the majority of cells in the joint in healthy and arthritic joints are local synoviocytes such as macrophages and fibroblasts which are responsible for production of cytokines such as TNF- α and IL-6 in disease (Firestein, 2003). Furthermore, although infiltrating T cells were detected they are vastly outnumbered by granulocytes (Gal et al., 2005). Despite this, in the present study, higher levels of B and T cells are detected in the joint tissue of arthritic TCR-5/4E8-transgenic mice in comparison to untreated controls suggesting that autoreactive T cells may encounter B cells *in situ* rather than in draining lymphoid organs (**Figure 4.1**). Furthermore, evidence from other studies with RA patients suggests that autoreactive T cells and mature B cells can be found in this compartment as part of an on-going immune response (Bennett et al., 2003; Michelutti et al., 2011). Other work with the PGIA model utilising inhibitors of lymphocyte egress from lymph nodes has cast doubt on the role of T cells in this scenario. PGIA has been shown to progress even in the absence of joint-infiltrating T cells. As T cells are required for disease, these results strongly implicate T cells as providers of T cell help to B cells in lymph nodes rather than localised contributors to joint pathology (Angyal et al., 2010). However, the results from **Figure 4.1** show that a significantly higher proportion of B cells are present in arthritic joints than T cells indicating that they may play a more direct role outside of secretion of autoantibodies in the periphery (**Figure 4.1**).

Differences were also detected in the proportion of T and B cells in joint tissue preparations when using alternative adjuvants. Immunisation of aggrecan with DDA, a synthetic adjuvant, resulted in detection of significantly higher levels of B and T cells in joints in comparison to mice immunised with aggrecan and CFA (**Figure 4.1**). Previous studies have shown that CFA and DDA are capable of modulating the proportion of T_H1 and T_H17 cells in PGIA but overall disease severity is not affected (Stoop et al., 2012). However, interpretation of data from **Figure 4.1** must be cautious as a control group of mice were not immunised with adjuvant only and, therefore, the effect of the adjuvant alone cannot be discounted as a reason for the presence of lymphocytes in the synovium. In addition, combination of the flow cytometric analysis with joint histology and *in vivo* imaging techniques could further elucidate the differences between using CFA and DDA adjuvants and determine whether B cells interact directly with autoreactive T cells in the joints of mice with PGIA.

Initial experiments in this Chapter confirmed that the aggrecan-specific B cell line, A20-Agg efficiently presents aggrecan to the p84-103-specific CD4⁺ T cell hybridoma 192. A20-Agg B cells were capable of presenting aggrecan at approximately 10³ times lower doses of aggrecan than non-specific A20 B cells (**Figure 4.2**). Based on evidence from other studies (Aluvihare et al., 1997; Batista and Neuberger, 1998), this effect is very likely to be a combination of significantly improved uptake of dAgg via the BCR, and its subsequent focusing to the intracellular pathways involved in pMHC assembly (Wilson et al., 2012).

In order to identify candidate APC for comparison to A20-Agg B cells, several *ex vivo* APC populations were isolated from splenocytes or differentiated from bone marrow. The expression of MHC class II and various co-stimulatory molecules was measured on these different cell types as indicators of their APC capacity. Both the A20 and A20-Agg B cell lines (**Figure 4.3**) along with the *ex vivo* APC (**Figure 4.4**) were shown to consistently express MHC class II and a range of co-stimulatory molecules (with the possible exception of macrophages which did not express CD40, see **Figure 4; bottom panels**) and, therefore, were considered suitable for use in antigen-presentation assays with APC-free (**Figure 3.12**), purified TCR-5/4E8-transgenic CD4⁺ T cells which had been shown to represent a population of unstimulated naïve CD4⁺ T cells (**Figure 3.2 & 3.3**). As observed by other groups, the DC produced using the method described here, which utilises GM-CSF to promote maturation, produced a heterogeneous population. It has also been suggested that inclusion of IL-4, Flt3L or LPS during the culture period modulates DC co-stimulatory molecule expression and their potential to activate T cells (Weigel et al., 2002; Wells et al., 2005). The same work has also identified that DC cultured in all the conditions tested are capable of taking up antigen efficiently by macropinocytosis. Similarly, further experiments to characterise the DC generated in

this chapter showed no difference in DC induction of T cell IL-2 production when cultured with LPS during the final stages of maturation (**Figure 4.5**). However, the work presented here does not directly address whether the use of the aforementioned different culture conditions or use of splenocyte-derived DC can affect DC induction of cytokine production. Given the diverse culture conditions used in different studies, future work could make use of splenocyte-derived DC isolated using more precise methods such as cell-sorting (Inaba et al., 2009). This could potentially reduce any inter-experimental variability involved with differentiating bone marrow-derived DC as splenocyte-derived DC may have a more consistent phenotype.

Prior to experiments comparing the consequences of aggrecan presentation by different APC, efforts were made to establish optimal cell numbers and APC:T cell ratios required for efficient activation of isolated TCR-5/4E8-transgenic CD4⁺ T cells (**Figure 4.6**). When using A20-Agg B cells as an example APC, TCR-5/4E8-transgenic CD4⁺ T cell activation was shown to be highly sensitive to the number of APC. Efficient activation of low numbers of TCR-5/4E8-transgenic CD4⁺ T cells could be achieved by increasing the number of APC. Therefore, due to the reduced yield of CD4⁺ T cells following the stringent isolation process outlined in **Chapter 3**, a 2:1 APC:T cell ratio was used for further assays. As the ratio of aggrecan-specific CD4⁺ T cells to B cells in the arthritic joint is unknown, it is not clear whether the ratio used in this study is representative of disease. Evidence from patients suggests only a low number of CD4⁺ T cells specific for joint autoantigens will be present, particularly during the initial stages of pathology (Struyk et al., 1995). Similarly, although there is evidence in some cases of ectopic lymphoid development in arthritic joints from which selection of antigen-specific B cell clones could be inferred, isolation of large numbers of B cells of a single specificity is not possible (Takemura et al., 2001a; Thurlings et al., 2008). However, both T and B cells have the potential to rapidly proliferate in response to cognate antigen and other inflammatory stimuli. As a result, these populations may be present in large numbers at the onset of RA following an initial antigen-specific inflammation event. This is supported by some animal models of RA in which disease is driven by a rapid influx and proliferation of antigen-specific CD4⁺ T cells (Kannan et al., 2005).

Comparison of antigen presentation by isolated CD43⁻ B cells, DC and macrophages to WT BALB/c splenocytes revealed that DC were superior APC when presenting both peptide and protein aggrecan to TCR-5/4E8-transgenic CD4⁺ T cells (**Figure 4.7**). As the splenocyte population is predominantly made up of non-specific B cells it was not surprising that unfractionated splenocytes presented antigen with similar efficiency to that seen with the isolated CD43⁻ B cells. Interestingly, the macrophage population failed to induce T cell activation in response to antigen.

This is somewhat surprising as the macrophages were shown to express high levels of MHC class II. However, this may be explained by the lack of macrophage CD40 expression, which ligates T cell CD154 and is essential for reciprocal activation events required for T cell activation. Previous assays with aggrecan presentation by macrophages have utilised the p84-103-specific T cell hybridoma 192 as responders (Wilson et al., 2012) which do not generally require co-stimulation for IL-2 production (Bennett et al., 1998; Schoenberger et al., 1998). This indicates that the macrophage population may require additional innate activatory signal(s) in order to become competent APC in this context. However, in order to minimise the presence of reagents in downstream antigen presentation assays which might activate macrophages but also modulate T cell function, DC were used for future experiments due to their robust responses even in the absence of additional innate stimuli such as LPS.

Presentation of aggrecan by A20-Agg B cells and DC was initially compared using fixed doses of antigen and a broad range of proinflammatory and regulatory cytokines were analysed in order to identify any major differences (**Figure 4.8**). Differential T cell activation, as measured by IL-2, and effector cytokine production was detected under several conditions. Notably, the largest differences between the different APC types were observed in regards to IFN- γ and IL-10 production, especially when comparing peptide and protein antigen. However, background levels of IL-10 in the A20-Agg B cell cultures were high, possibly due to constitutive A20-Agg B cell production of IL-10 as this occurred even in the absence of antigen (O'Garra et al., 1990). Future work could modulate the parameters of these experiments to clarify the exact relationship between aggrecan dose and subsequent T cell cytokine responses. The strength of TCR stimulation and antigen dose can have downstream effects on CD4⁺ T cell fate (Corse et al., 2011). For example, weaker TCR signals derived by pMHC complexes with a high off-rate can favour T_H2 differentiation (Tao et al., 1997) while T cells with a high affinity for antigen differentiate into T_{FH} more efficiently (Fazilleau et al., 2009). More recently, it has been shown that low strength T cell activation can have profoundly increase IL-17 production by T cells (Purvis et al., 2010). In order to address this in the context of PGIA, the system described here could also be altered to include a greater range of antigen doses and additional APC:T cell ratios. Furthermore, the use of truncated or citrullinated peptide antigen, which has been shown earlier in this study to induce less T cell proliferation than unmodified peptide (**Figure 3.4**), may reveal substantial differences in T cell skewing and cytokine production at different phases of the culture period. However, due to the significance of IFN- γ in PGIA pathology, further experiments in this study focused on extended antigen titrations to further characterise the response.

PGIA is dependent on both B cells and T cell-derived IFN- γ (Doodes et al., 2008; O'Neill et al., 2005, 2007). Work presented in this chapter, which has also been published recently (Wilson et al., 2012), corroborates these findings by suggesting that B cell antigen-presentation plays a unique role in expanding pathogenic T_H1 cells. The aggrecan-specific B cell line A20-Agg induced equivalent aggrecan-specific CD4⁺ T cell activation as DC in response to graded doses of dAgg when measured by IL-2 production. In contrast, non-specific A20 B cells require much higher doses to achieve the same levels of IL-2 production (**Figure 4.9**). DC primarily acquire antigen non-specifically via macropinocytosis (Lim and Gleeson, 2011) and with a wide range of innate antigen receptors, including Fc receptors, complement receptors (Bajtay et al., 2006), DEC205 (Jiang et al., 1995; Mahnke et al., 2000), and MARCO (Grolleau et al., 2003), and also express high levels of MHC class II and co-stimulatory molecules (**Figure 4.4**) which contribute to efficient T cell activation (**Figure 4.9**) (Kleijmeer et al., 1995; Nijman et al., 1995). The failure of both non-specific A20 B cells (**Figure 4.2**) and *ex vivo* B cells (**Figure 4.7**) to induce high levels T cell activation at low doses of protein antigen in this system is to be expected as the vast majority of B cells will not express a BCR capable of binding and acquiring dAgg for uptake and, therefore, will not efficiently process and present antigen to CD4⁺ T cells. In contrast, antigen-specific B cells such as A20-Agg are capable of highly efficient antigen uptake via the BCR (**Figure 4.2**) and subsequently are proficient at activating T cells in response to protein antigen (**Figure 4.9**) (Batista and Neuberger, 1998). Previous studies have outlined differences in the requirement for B cells during immune responses towards different forms of antigen. Consequently, aggrecan presentation may be highly dependent on the presence of B cells to elicit strong T cell responses (Constant et al., 1995a, 1995b).

Crucially, in this study, the A20-Agg B cells are also shown to induce significantly greater IFN- γ production from the TCR-5/4E8-transgenic CD4⁺ T cells than both DC and non-specific B cells following the presentation of dAgg (**Figure 4.10**). This is supported by intracellular cytokine staining indicating that co-cultures containing T cells and A20-Agg treated with 100 nM dAgg have significantly increased numbers of CD4⁺IFN- γ ⁺ T cells in comparison to DC co-cultures (**Figure 4.12**). In addition, the potential effects of B cell proliferation were negated as A20-Agg were shown not to proliferate extensively in culture following aggrecan stimulation (**Figure 4.13**). Such low levels of proliferation in comparison to untransfected A20 may be explained by the antibiotic-based selection of the particular cloned transfectant during the generation of the A20-Agg B cells. Irrespective of these differences, irradiated A20-Agg B cells showed an identical ability to induce high levels of TCR-5/4E8-transgenic CD4⁺IFN- γ ⁺ T cells to that seen with viable A20-Agg B cells (**Figure 4.14**).

In summary, these results suggest a difference in the way B cells activate antigen-specific CD4⁺ T cells in comparison to DC when processing and presenting dAgg. This finding may represent an important initial step in understanding why B cell depletion is a successful therapy for RA and further work must focus on elucidating the exact molecular mechanism through which B cells exert this effect. Previous work from our lab has demonstrated that antigen-specific B cells process dAgg similarly to other APC (Wilson et al., 2012). As a result, as-yet-unidentified differences in co-stimulatory molecule expression, immune synapse organisation and signalling properties, APC cytokine production and cell morphology may instead be responsible for the differences observed in T cell cytokine production in this study (**Figure 4.8**) (Friedl et al., 2005; Thauland and Parker, 2010).

For example, Harris *et al.* have reported that, through reciprocal interactions with T cells, B cells can adopt effector phenotypes characterised by production of T_H1 or T_H2 cytokines (Harris et al., 2000). Based on this evidence, it could be postulated that the cytokine microenvironment surrounding the A20-Agg B cells, or directed into the immune synapse during antigen presentation, may aid skewing of the TCR-5/4E8-transgenic CD4⁺ T cells towards a T_H1 phenotype following the processing and presentation of aggrecan. In addition, B cell induction of T_H1 cytokine production in response to infection has been linked to expression of membrane-bound TNF- α which acts through contact-dependent mechanisms (Menard et al., 2007). Similarly, A20-Agg B cells may amplify T cell IFN- γ production by up-regulating expression of TNF- α in response to dAgg. B cells and DC express different levels of co-stimulatory molecules. The application of blocking monoclonal antibodies to these APC prior to incorporation into T cell assays may reveal differences in the key molecules involved in T cell activation and/or required for induction of IFN- γ production. In line with this notion, O'Neill *et al.* have demonstrated that the expression of B cell CD80 and CD86 is required for the development of full PGIA and further investigation of this interface is crucial to greater understanding of B cell antigen presentation (O'Neill et al., 2007). The activation of B cells and DC and expression of such co-stimulatory molecules may differ due to a unique aspect of the dAgg preparation in comparison to the p84-103. For example, a particular aggrecan domain or the presence of a particular component of the aggrecan preparation could lead to activation of PRR found on B cells and subsequent upregulation of the requisite co-stimulation.

Fate-mapping studies may offer an alternative explanation for the differences observed between A20-Agg B cell and DC-mediated antigen presentation. In particular, a population of T_H17 cells have been identified in the EAE model which respond to IL-23 by expressing IFN- γ during the course of the inflammatory response (Hirota et al., 2011). This may be due to stimuli-dependent epigenetic modifications at lineage-specific loci (Mukasa et al., 2010; Wei et al., 2009; Yamanaka et al., 2013). Designated 'ex-T_H17' cells, it was considered possible that the IFN- γ ⁺ TCR-5/4E8-transgenic CD4⁺ T cells seen in A20-Agg B cell or DC co-cultures shared a similar differentiation pathway. Notably, low levels of IL-17 were detected in CD4⁺ T cell and DC co-cultures stimulated with p84-103 suggesting the possibility that a burst of IL-17 precedes IFN- γ production in this scenario (**Figure 4.8D**). However, flow cytometric analysis of TCR-5/4E8-transgenic CD4⁺ T cells for the IFN- γ ⁺IL-1R⁺ phenotype synonymous with the 'ex-T_H17' phenotype did not reveal the presence of these cells suggesting that IFN- γ or IL-17 production originated from discrete T_H1 and T_H17 populations (Appendix B). Despite this, intracellular cytokine staining is limited due to its destructive endpoint preventing further tracking of the dynamics of T cell cytokine secretion beyond the selected time-point. More advanced techniques have been utilised to longitudinally monitor cytokine secretion by trapping individual cells in nanowells. These studies have indicated that, following polyclonal stimulation, T cells tend to secrete single cytokines sequentially and simultaneous secretion of multiple cytokines is only a brief transitory phase (Han et al., 2012). Although this method has yet to be tested using antigen-specific stimulation, it may prove useful in identifying if and when IL-17 or other T cell-derived cytokines are produced over an extended time-course and the level of heterogeneity within the TCR-5/4E8-transgenic CD4⁺ T cell population.

A20-Agg B cells and DC were also capable of inducing the expansion of T_{reg} populations, as identified by expression of the transcription factor FoxP3, in response to protein antigen (**Figure 4.15**). Interestingly, these data replicate the trend revealed when observing IFN- γ ⁺CD4⁺ T cell generation in this system. Co-culture with A20-Agg B cells in the presence of aggrecan induced increased numbers of TCR-5/4E8-transgenic CD4⁺ T cells that were positive for FoxP3⁺ cells when compared to co-cultures containing DC. This suggests that T_{reg} expansion occurs in parallel with effector CD4⁺ T cell induction in these co-cultures. There was a noticeable reduction in T_{reg} co-cultured with A20-Agg B cells in the absence of antigen, in contrast to DC, which may be due to A20-Agg B cells not providing basal levels of survival signals, such as IL-2, required for maintenance of T_{reg} in the absence of antigen (Josefowicz et al., 2012). The presence of a substantial T_{reg} population in the A20-Agg co-cultures may offer an explanation for the paucity of IL-17

detected in these co-cultures (**Figure 4.8E**) as T_{reg} are instrumental in suppressing the differentiation to T_H17 cells and subsequent IL-17 production by this population (Josefowicz et al., 2012).

It is tempting to speculate that the enlarged T_{reg} population in the A20-Agg co-cultures was also responsible for the increase in IL-10 observed by ELISA (**Figure 4.8**). However, T cells can switch to IL-10 production without a change in lineage as part of the regulatory processes associated with an effector response. For example, a recent report has highlighted the role of CD46, a complement receptor, in the switching of $IFN-\gamma^+$ T cells to IL-10 production in response to elevated levels of IL-2. Defects in this regulatory pathway were also found in small cohorts of RA and multiple sclerosis patients suggesting CD46 dysregulation is linked to T_H1 -mediated autoimmunity (Astier et al., 2006; Cardone et al., 2010). However, although this pathway appears promising as a candidate mechanism for the IL-10 induction observed in A20-Agg B cell co-cultures, IL-2-dependent switching of T cell phenotype via CD46 has not currently been replicated in murine models making testing this scenario difficult in the TCR-5/4E8-transgenic model (Kemper and Atkinson, 2007).

However, B cells have been shown to induce T_{reg} populations when co-cultured at different APC:T cell ratios and have even been presented as a potential means to expand T_{reg} for immunotherapy (Chen et al., 2009; Mann et al., 2007; Reichardt et al., 2007). Furthermore, when presenting self-antigen, B cells preferentially induce T_{reg} populations instead of anergy and so may contribute to expansion of autoantigen-specific T_{reg} (Morlacchi et al., 2011). However, the ability of A20-Agg B cells specifically to expand T_{reg} must be approached cautiously as B cell lymphomas, including A20, have been shown previously to induce T_{reg} as a survival mechanism aiming to down-regulate anti-tumour immune responses (Elpek et al., 2007). It has been suggested that uncommitted $FoxP3^+$ T cells not only demonstrate a level of plasticity but can also produce inflammatory cytokines in autoimmunity (Ehrenstein et al., 2004; Esposito et al., 2010; Hori, 2011; Zhao et al., 2011). Therefore, these cells may be capable of reverting into effector phenotypes upon entering highly inflammatory microenvironments such as those found in the arthritic joint and contributing to disease. In contrast, exploratory experiments in the present study did not identify $IFN-\gamma^+FoxP3^+$ cells in A20-Agg B cell or DC co-cultures using either p84-103 or dAgg (Appendix C). This is unsurprising as the hypothesis that T_{reg} are functionally plastic *in vivo* is not currently considered to be an established feature of this lineage and remains controversial due to potential contamination of isolated human T_{reg} cultures with residual populations of effector T cells due to promiscuous and transient expression of $FoxP3$ (Hori, 2011; Miyao et al., 2012). Such populations can lose their $FoxP3$ expression and go on to proliferate following a change in conditions and as such do not represent true T_{reg} plasticity.

Nevertheless, the identification of both CD4⁺IFN- γ ⁺ and CD4⁺FoxP3⁺ T cells in these co-cultures provides a compelling reason to consider both the effector and regulatory subsets involved in autoreactive CD4⁺ T cell responses to aggrecan. As a result, this may provide the foundation for further analysis of T_{reg} in this context. More advanced phenotyping of the FoxP3⁺ population, such as staining for CD25, over an extended time-course would ensure the observed phenotype is stable. This could be combined with T_{reg} isolation and functional assays to compare B cell and DC-primed T_{reg} suppression of CD4⁺ T cell activation through IL-10 in supernatants or direct contact mechanisms. TCR-5/4E8-transgenic T_{reg} are of particular interest as they are being explored as a mechanism for therapeutic delivery of IL-10 in autoimmune arthritis (Guichelaar et al., 2008a, 2008b; van Herwijnen et al., 2012; Wieten et al., 2009). The relative importance of CD4⁺IFN- γ ⁺ or CD4⁺FoxP3⁺ IL-10-producing T cells in disease could be established *in vivo* using the PGIA model. For example, naïve TCR-5/4E8-transgenic mice or those with established PGIA could be immunised with B cell versus DC-primed CD4⁺ T cells and induction or modulation of arthritis observed. Finally, as B cells and DC are shown to induce different responses to peptide and protein antigen, it would also be interesting to determine whether these APC can behave synergistically when both are included in co-culture with CD4⁺ T cells. Different APC:T cell ratios may also regulate the activation and effector function of CD4⁺ T cells by determining the availability of antigen, density of accessible co-stimulation or the concentration of T cell skewing cytokines in the surrounding medium.

Despite many options for further study using A20-Agg B cells, in order to fairly assess the full range of effects B cells exert on CD4⁺ T cells it is necessary to go beyond the limitations of cell lines and utilise *ex vivo* B cells. In contrast to cell lines, *ex vivo* resting B cells can be activated by PRR ligation and skewed into their putative effector and regulatory subsets. Furthermore, aggrecan-specific B cell subsets could be adoptively transferred into the PGIA model in order to assess their effect *in vivo* on disease pathogenesis. Overall, this would facilitate for elucidation of any unique features of B cell activation of CD4⁺ T cells and characterisation of the events involved in these interactions.

5. Generation of an *ex vivo* aggrecan-specific B cell and the role of pattern-recognition receptors and co-stimulation in antigen presentation

5.1. Introduction

Based on the findings discussed in **Chapter 4**, specifically experiments showing A20-Agg B cells induce greater TCR-5/4E8-transgenic CD4⁺ T cell IFN- γ production than DC in response to aggrecan stimulation, three distinct approaches were subsequently taken to shed light on the possible PRR or co-stimulatory molecule-mediated mechanisms by which aggrecan can preferentially induce TCR-5/4E8-transgenic CD4⁺ T cell differentiation and cytokine production following presentation by A20-Agg B cells. These three strands of experiments can be summarised as follows:

- a) Investigation of aggrecan-induced PRR activation.
- b) Establishing a protocol for the transfection of *ex vivo* B cells with an aggrecan-specific BCR and incorporation of these B cells into antigen-presentation assays with TCR-5/4E8-transgenic CD4⁺ T cells.
- c) Determining the requirement for the expression of the key co-stimulatory molecules CD80 and CD86 on B cells and DC to identify any differential requirement for each molecule for induction of CD4⁺ T cell IFN- γ production.

The rationale for each of these approaches is outlined prior to the description of each set of experiments and the results are discussed together at the end of this chapter.

5.2. Activation of pattern-recognition receptors by joint components in rheumatoid arthritis

The differential activation of PRR may represent one possible explanation for the difference in A20-Agg B cell and DC induction of TCR-5/4E8-transgenic CD4⁺ T cell production of IFN- γ . Both B cell and DC antigen presentation is modulated by the presence of ligands for PRR, including members of the TLR and NLR families. This is achieved through PRR-induced upregulation of co-stimulatory molecule expression and inflammatory cytokine production via NF- κ B (Barr et al., 2007; Medzhitov and Janeway, 1997). Interestingly, many TLR are expressed at high levels in RA synovium and on resident synovial cells and infiltrating APC (Tamaki et al., 2011). Synovial fibroblasts from RA patients have been shown to overexpress TLR 2, 3 and 4 in the lining layer and respond to TLR ligation by producing more IL-6 and MMP-13 than osteoarthritis and skin fibroblast controls. Notably, this overexpression was present in early-stage RA patients as well as those with longstanding disease suggesting this is an early event in RA pathology (Ospelt et al., 2008). In regards to APC, monocyte-derived DC from RA patients have been shown to induce more TNF- α and IL-6 following TLR2 and TLR4 stimulation than healthy controls. Furthermore, a synergistic increase in cytokine production was also observed following simultaneous stimulation with TLR4 and TLR7/8 agonists. Importantly, a cell-based detection system identified the presence of TLR4 ligands in serum and synovial fluid from RA patients with active disease providing evidence for a DC activation pathway mediated by TLR4 (Roelofs et al., 2005). A role for TLR in RA is also strongly supported by abundant evidence provided by animal models (reviewed in Huang and Pope, 2009). However, the abundance of studies suggesting a role for TLR in RA pathology, a contradictory consensus has emerged in the field of genetics suggesting that polymorphisms in TLR genes are not associated with either susceptibility to RA or responses to common therapies such as anti-TNF- α (Coenen et al., 2010; Sánchez et al., 2004; Wellcome Trust Case Control Consortium, 2007).

It has been hypothesised that either an initial microbial trigger or the release of DAMP-containing self components as a result of local tissue damage may provide PRR ligands responsible for the initiation of an inflammatory loop leading to RA pathology (Brentano et al., 2005; Rashid and Ebringer, 2007; Santegoets et al., 2011). Studies have shown that both ECM and synovial fluid contain several products capable of acting as endogenous ligands for a number of PRR. Thus, the understanding of the role these ligands play in the activation of APC is crucial in forming a complete picture of joint-related disease pathology (Ho et al., 2010). Indeed, the aggrecan-

associated molecule hyaluronan has previously been demonstrated to be a TLR4 ligand (Bazin et al., 2008). Similarly, the endogenous ECM glycoprotein Tenascin-C can also engage TLR4 on synovial fibroblasts and macrophages or synovial tissue explants from RA patients. This induces synthesis of proinflammatory cytokines which are essential pathology in mice (Midwood et al., 2009). This research, along with other studies, strongly suggests TLR4 activation via this mechanism is important in joint disease (Choe et al., 2003; Lee et al., 2005). Through this mechanism, aggrecan and other ECM components found in the antigen preparation could modulate T cell activation and differentiation following autoantigen presentation by B cells and other APC.

Different DC subsets express distinct sets of TLR and respond to activation by producing unique cytokine profiles suggesting that each subset has evolved to respond to specific groups of microbial antigens (Kadowaki et al., 2001). For example, myeloid DC preferentially express all TLR except TLR7 and TLR9 which are limited to pDC (Jarrossay et al., 2001). Mouse splenic B cells have been shown to express mRNA for all TLR ranging from TLR1-9 and B cell cytokine production can be induced following activation through these receptors (Barr et al., 2007). In particular, activation of TLR2, TLR4 or TLR9 by individual ligands induced B cell production of IL-6 and IL-10. However, only PMA/ionomycin activation or stimulation through all three of these TLR could elicit IFN- γ production, similar to the synergistic effect of multiple TLR ligands observed previously to induce a T_H1-polarising program in DC (Napolitani et al., 2005). Several TLR are expressed in RA synovial tissue and the TLR adaptor molecules MyD88 and TIRAP have both been linked to the inflammation observed in arthritic joints. Interestingly, disruption of MyD88 and TIRAP downregulates the production of TNF- α , IL-6 and multiple MMP involved in inflammation and ECM degradation respectively (Sacre et al., 2007). Overall, this research has highlighted the potential role for TLR2 and TLR4. Synovial membrane cell cultures have also indicated that ligands for these receptors may be secreted by into the RA synovial fluid during the course of inflammation. Furthermore, expression of these receptors is regulated by the T_H1-associated cytokines IL-12 and IFN- γ which are present in RA synovial fluid (Radstake et al., 2004). TLR4 stimulation of DC from RA patients can induce TNF- α , IL-6 and IL-12 and functions synergistically with TLR3, TLR7 and TLR8 to increase cytokine production (Roelofs et al., 2005). In addition to induction of cytokine production, it is probable that TLR2 activation contributes to the production of chemokines which mediate infiltration of leukocytes into the inflamed synovium (Pierer et al., 2004).

In addition to the ability of various TLR ligands to modulate arthritis, the NOD2 ligands are also capable of mediating an increase in IFN- γ production and development of a more severe form of B cell-dependent arthritis in mice following immunisation with aggrecan (Rosenzweig et al., 2009). NLR are typically involved in the sensing of microbial products and structures such as cell wall peptidoglycans (Kaparakis et al., 2007). In addition, when coupled with BCR signalling, NLR can induce CD80/86 expression on B cells (Pettersen et al., 2011). Given the importance of CD80/86 expression on B cells in PGIA, NOD2 was hypothesised to be a potentially important modulator of B cell presentation of aggrecan to autoreactive CD4⁺ T cells (O'Neill et al., 2007).

Before establishing if interactions between aggrecan and PRR expressed by B cells and DC are in some way involved in the differential induction of TCR-5/4E8-transgenic CD4⁺ T cell IFN- γ production by different APC a reductionist approach was first taken to determine whether the aggrecan preparation used in previous experiments has the capability to activate PRR. Many cells, including APC, express a multitude of different PRR (both at the plasma membrane and intracellularly). Therefore, it was decided to initially use a simple commercially available cell expression system consisting of human embryonic kidney (HEK) cells expressing individual PRR in order to identify aggrecan-mediated PRR activation. Importantly, this system has been optimised by the co-transfection of PRR and several accessory molecules required for efficient ligand binding and subsequent PRR signalling. Thus, this system reconstitutes the minimal requirements for the activation of the NF κ B signalling pathway which is linked to secretion of an enzyme that can be detected using a colorimetric assay.

5.2.1. Aggrecan as a pattern-recognition receptor ligand

The potential for ECM components to act as PRR ligands in RA suggested that aggrecan may fulfil this role in the TCR-5/4E8-transgenic system. It was hypothesised that differential expression of PRR which bind aggrecan on A20-Agg B cells or DC may contribute to APC activation and facilitate the induction of CD4⁺ T cell IFN- γ production. Initially, a panel of HEK-Blue cells expressing mouse (m)TLR and NOD receptors and their associated co-receptors were used to test the ability of the aggrecan antigen preparation to induce PRR activation. Graded doses of either p84-103 or dAgg were added to a culture of HEK-Blue cells and incubated for 24 h and PRR activation interpolated from a reference curve generated using a known ligand. Stimulation of HEK-Blue cells results in activation of NF- κ B and AP-1 leading to expression of a secreted reporter enzyme, embryonic alkaline phosphatase (SEAP) which is detected using a colorimetric assay. This simple system was utilised to determine the potential of the peptide or protein forms of aggrecan used in the previous assays to activate PRR. If this was shown to be the case, this would act as a proof-of-concept for further experiments seeking to limit PRR activation in B cell and DC co-cultures in order to manipulate levels CD4⁺ T cell cytokine production and activation.

Ligands for TLR2 consist of peptidoglycan (PGN) and other cell wall components of gram-positive bacteria. TLR2 signalling is dependent on the presence of intracellular TLR1 and TLR2 domains and co-operation with CD14 amplifies the ability of TLR2 to recognise its ligands (Girard et al., 2003; Lotz et al., 2004; Sandor et al., 2003). Accordingly, the commercially acquired HEK-Blue mouse (m)TLR2 cells are supplied transfected with both mouse TLR2 and CD14. As shown in **Figure 5.1**, following incubation with PGN for 24 h, HEK-Blue mTLR2 cells respond in a dose-dependent manner at doses above 177.0 ng/mL (**Figure 5.1A**). Incubation of HEK-Blue mTLR2 cells with p84-103 at levels up to 1000 nM did not induce any significant mTLR2 activation above background levels, media only controls or addition of ligand and antigen solvents (water or PBS). In contrast, the addition of 1000 nM dAgg induced significant levels of TLR2 activation (equivalent to 4355 $\pm$ 830 ng/mL PGN; $n = 3$) However, this effect did not titrate in accordance with dose suggesting a threshold of 1000 nM dAgg must be reached in order to generate detectable activation (**Figure 5.1B**).

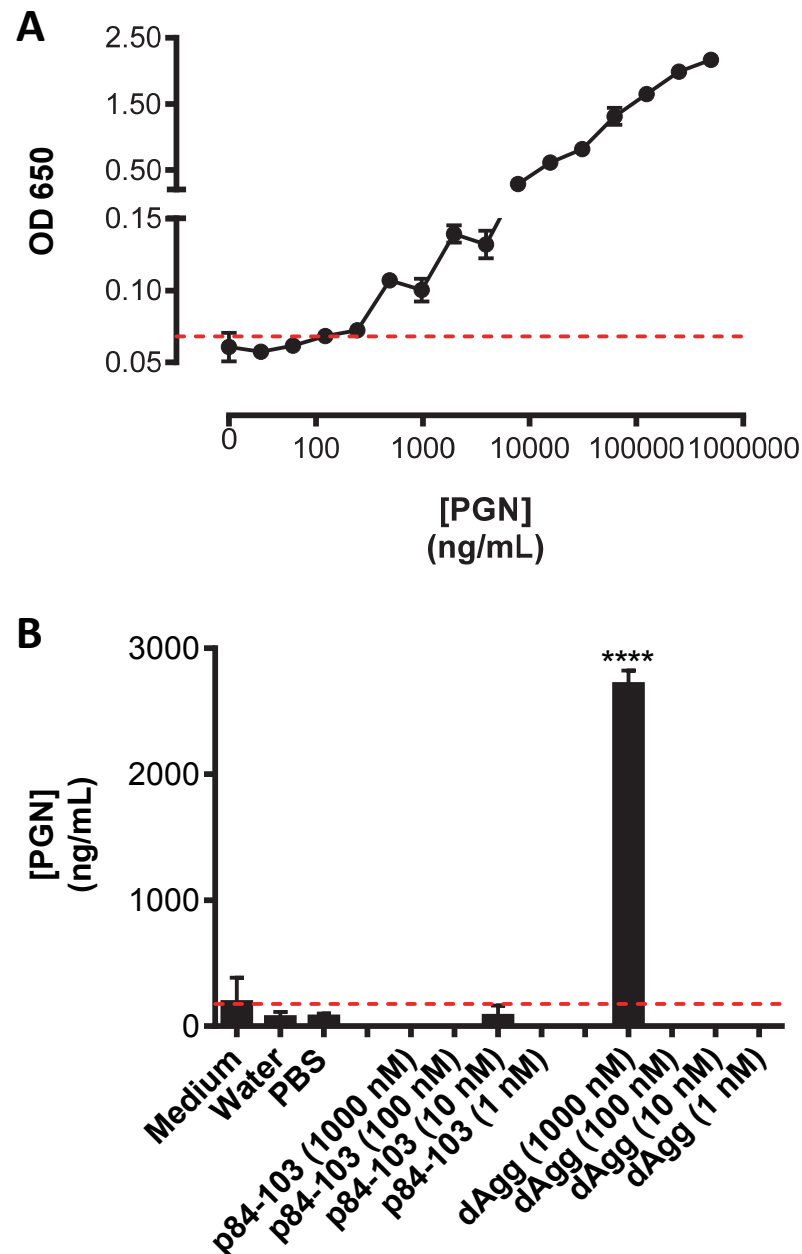


Figure 5.1 *Activation of HEK-Blue by aggrecan via TLR2.*

(A) 5×10^4 HEK-Blue mTLR2 cells were incubated with graded doses of PGN for 24 h in a flat-bottomed 96-well plate to obtain a dose-response titration. Supernatants were extracted and incubated for 1 h with QUANTI-Blue prior to measurement of absorbance at 650 nm using a spectrophotometer. (B) Parallel cultures were incubated with p84-103 or dAgg and TLR2 activation estimated by interpolating data from a PGN dose-response titration. Each data point represents a triplicate with error bars showing $\pm$ SEM. Background activation was considered equal to the (MEAN PBS) + (3 x SD) as indicated by the dashed red line shown on both graphs. Above-background values were considered significant if a *t*-test *p*-value ≤ 0.05 was obtained when compared to background. Data representative of two independent experiments. * indicates a *t*-test *p*-value ≤ 0.05 .

TLR4, in conjunction with MD-2, interacts with and responds to the endotoxin LPS found in the outer membrane of many gram negative bacteria (Chow et al., 1999). In a similar mechanism to that seen with TLR2, CD14 is a receptor for complexes of LPS and LPS-binding protein and facilitates LPS presentation to MD-2 (Wright et al., 1990). The resulting complex of MD-2, TLR4 and LPS leads to downstream recruitment of TIR domain-containing adaptors such as MyD88 and activation of NF κ B (Shimazu et al., 1999; Shuto et al., 2005). Therefore, HEK-Blue mTLR4 cells are transfected with mouse TLR4 and its associated molecules MD-2 and CD14. HEK-Blue mTLR4 cells were stimulated for 24 h with graded doses of LPS which induced above-background activation at doses >71.3 pg/mL which plateaued at 10,000 pg/mL (**Figure 5.2A**). Stimulation of mTLR4 cells with p84-103 did not induce TLR4 activation. However, similar to that seen with HEK-Blue mTLR2 assays, stimulation with dAgg was capable of inducing significant levels of TLR4 activation (equivalent to 187 ± 21 pg/mL LPS; $n = 3$) at 1000 nM (**Figure 5.2B**).

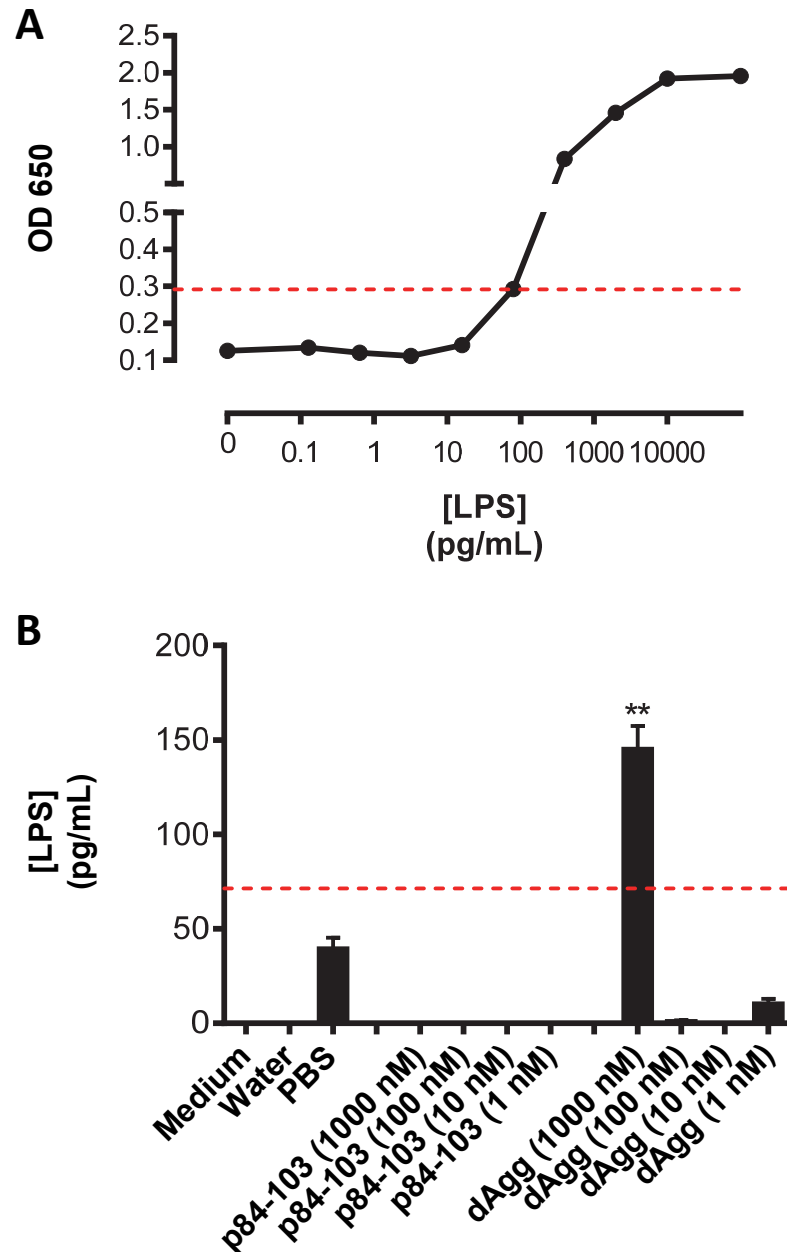


Figure 5.2 *Activation of HEK-Blue by aggrecan via TLR4.*

(A) 2.5×10^4 HEK-Blue mTLR4 cells were incubated with graded doses of LPS for 24 h in a flat-bottomed 96-well plate to obtain a dose-response titration. Supernatants were extracted and incubated for 1 h with QUANTI-Blue prior to measurement of absorbance at 650 nm using a spectrophotometer. (B) Parallel cultures were incubated with p84-103 or dAgg and TLR4 activation estimated by interpolating data from a LPS dose-response titration. Each data point represents a triplicate with error bars showing $\pm$ SEM. Background activation was considered equal to the (MEAN PBS) + (3 x SD) as indicated by the dashed red line shown on both graphs. Above-background values were considered significant if a *t*-test *p*-value ≤ 0.05 was obtained when compared to background. Data representative of two independent experiments. * indicates a *t*-test *p*-value ≤ 0.05 .

TLR9 is found in the ER and translocates to the compartments of the endocytic pathway to interact with specific unmethylated CpG-ODN (oligonucleotide) motifs unique to single-stranded bacterial DNA sequences (Chuang et al., 2002). This interaction is pH-dependent and occurs predominantly in the acidic conditions found in endosomes and lysosomes (Rutz et al., 2004). HEK-Blue mTLR9 cells are transfected with mouse TLR9 and stimulation with ODN induced above-background activation at doses >8.5 ng/mL (**Figure 5.3A**). However, stimulation with exogenously added p84-103 and dAgg failed to reach this activation threshold at all the doses tested (**Figure 5.3B**). Thus, unlike the results obtained with TLR2 and TLR4, high doses of dAgg were not capable of activating TLR9.

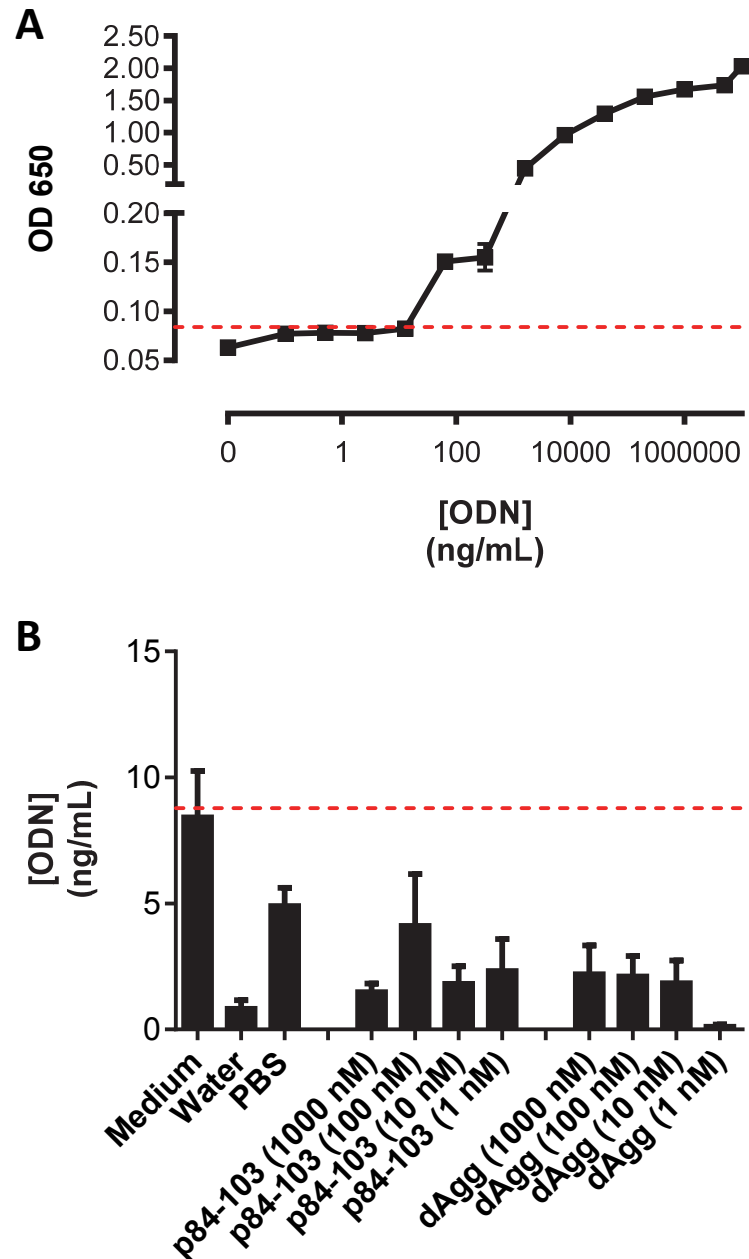


Figure 5.3 *Activation of HEK-Blue by aggrecan via TLR9.*

(A) 4×10^4 HEK-Blue mTLR9 cells were incubated with graded doses of ODN for 24 h in a flat-bottomed 96-well plate to obtain a dose-response titration. Supernatants were extracted and incubated for 1 h with QUANTI-Blue prior to measurement of absorbance at 650 nm using a spectrophotometer. (B) Parallel cultures were incubated with p84-103 or dAgg and TLR9 activation estimated by interpolating data from a ODN dose-response titration. Each data point represents a triplicate with error bars showing $\pm$ SEM. Background activation was considered equal to the **(MEAN PBS) + (3 x SD)** as indicated by the dashed red line shown on both graphs. Above-background values were considered significant if a *t*-test *p*-value ≤ 0.05 was obtained when compared to background. Data representative of two independent experiments.

Similar to TLR9, NOD2 is also an intracellular PRR which recognises bacterial structural motifs within molecules such as PGN and MDP (Hsu et al., 2007; Kobayashi et al., 2005). Unlike many TLR molecules which utilise MyD88, NOD2 signals through RICK/RIP kinases in order to activate IKK and, subsequently, NF- κ B (Hasegawa et al., 2008). NOD2 activation is linked to antigen processing through induction of autophagy which facilitates lysosomal degradation of bacterial antigen. Peptide loading onto MHC class II and pMHC expression the surface of APC is also increased following NOD2 activation (Cooney et al., 2010). HEK-Blue mNOD2 cells are transfected with mouse NOD2 and a titration of MDP induced above-background activation at doses >94.0 ng/mL (**Figure 5.4A**). However, HEK-Blue mNOD2 cells exhibited high levels of background activation. Such activation was detected even when HEK-Blue mNOD2 cells were incubated with media alone or media supplemented with water or PBS resulting in OD_{650nm} values of approximately 0.2, in contrast to <0.1 seen for the other HEK-Blue cell lines. The FCS in the culture media was excluded as a source of NOD2 ligands as antigen titrations performed using SFM showed similar background activation levels (data not shown). Furthermore, maximal activation levels were also compressed as OD_{650nm} values rarely exceeded 1.0 while other HEK-Blue cell lines typically plateaued between 2.4 and 2.5 limiting the detection range of responses to MDP to 150-1000 ng/ml (**Figures 5.1–5.4**). Similar to the results seen with HEK-Blue cells expressing TLR9, both p84-103 and dAgg failed to induce significant levels of HEK-Blue NOD2 activation above background, even at doses of 1000nM (**Figure 5.4B**).

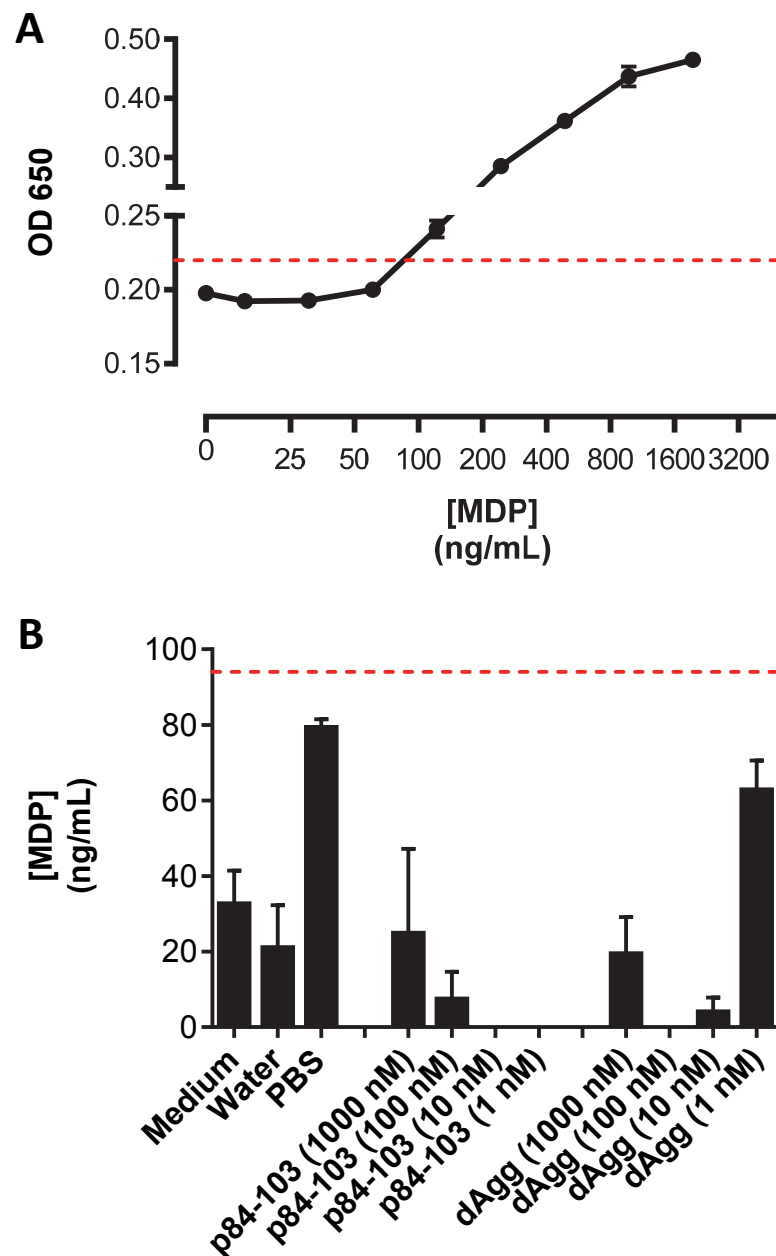


Figure 5.4 Activation of HEK-Blue by aggrecan via NOD2.

(A) 4×10^4 HEK-Blue mNOD2 cells were incubated with graded doses of MDP for 24 h in a flat-bottomed 96-well plate to obtain a dose-response titration. Supernatants were extracted and incubated for 1 h with QUANTI-Blue prior to measurement of absorbance at 650 nm using a spectrophotometer. (B) Parallel cultures were incubated with p84-103 or dAgg and NOD2 activation estimated by interpolating data from a MDP dose-response titration. Each data point represents a triplicate with error bars showing $\pm$ SEM. Background activation was considered equal to the (MEAN PBS) + (3 x SD) as indicated by the dashed red line shown on both graphs. Above-background values were considered significant if a *t*-test *p*-value ≤ 0.05 was obtained when compared to background. Data representative of two independent experiments.

5.2.2. Role of the aggrecan-specific B cell receptor in mediating pattern-recognition receptor activation

In contrast to cell surface-associated TLR2 and TLR4, TLR9 and NOD2 are expressed in intracellular compartments. However it would be expected that the higher doses of p84-103 used in the previous experiments should be able to penetrate to these locations following their exogenous addition in the cell culture media. In contrast, it is possible that the addition of soluble dAgg to the culture medium does not lead to efficient entry either into the cytosol or to the endocytic pathway where it would be able to interact with NOD2 or TLR9 respectively (Hsu et al., 2007; Latz et al., 2004). Interaction of dAgg with these intracellular PRR may therefore require interaction with receptors capable of allowing delivery to these compartments. For example, following BCR-mediated acquisition antigen is efficiently targeted to intracellular endocytic compartments where it undergoes proteolysis prior to association with MHC class II molecules and expression on the cell surface. Previous work has shown that BCR-mediated antigen acquisition also leads to the recruitment of TLR9-containing endosomes to autophagosomes where they may encounter BCR-bound antigen (Chaturvedi et al., 2008). Therefore, to test if exogenously added dAgg could activate these intracellular PRR following binding and internalisation by an antigen-specific plasma membrane receptor, HEK-Blue mTLR9 and mNOD2 cells were transiently transfected with plasmids containing the chimeric C7.1 aggrecan-specific BCR used previously (Figure 5.5). BCR-transfected HEK-Blue cells were used in the assays previously shown (Figures 5.3 & 5.4) to re-test responsiveness to dAgg. Firstly, in order to monitor transfection efficiencies, HEK-Blue cells were transfected with pmaxGFP previously used to monitor B cell transfection. In addition, to control for any potential non-specific BCR uptake of dAgg, HEK-Blue cells were also transfected with plasmids encoding the TTCF-specific BCR used previously.

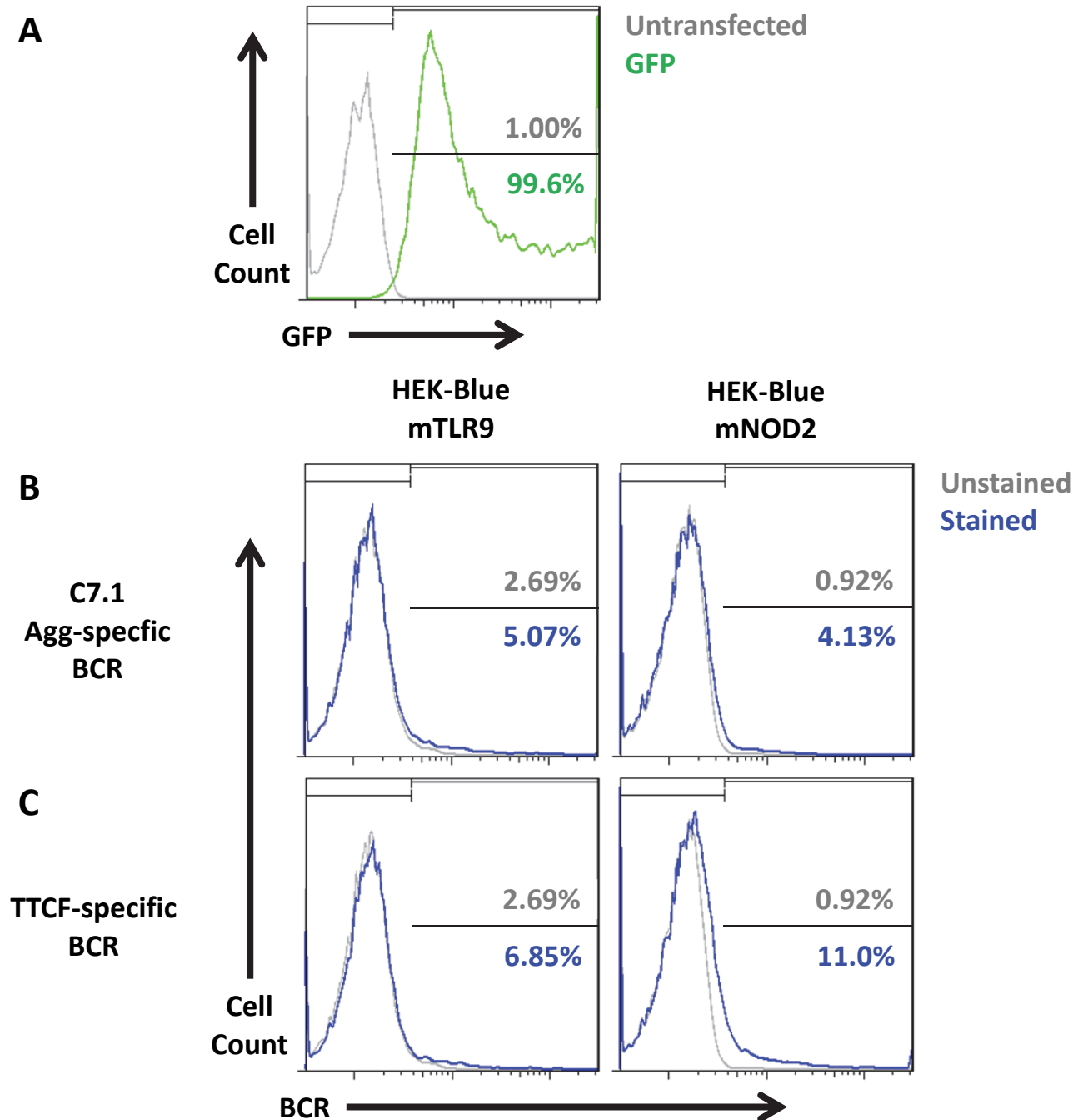


Figure 5.5 *Transfection of HEK-Blue cells with GFP or an aggrecan-specific BCR.*

2×10^6 HEK-Blue were reverse-transfected with Lipofectamine and plasmid DNA encoding either (A) pmaxGFP (4 μ g total) or co-transfected with the H and L-chain of (B) C7.1 aggrecan (Agg) or (C) tetanus toxin C fragment (TTCF)-specific BCR (8 μ g total). Transfected BCR was detected using anti-human IgG F(ab')₂. Plasmid-encoded gene expression was analysed 48 h later by flow cytometry.

Flow cytometry was used to determine transfection efficiency by analysing the percentage positive cells over untransfected controls. Transfection with pmaxGFP resulted in a high transfection efficiency of 98.6% (**Figure 5.5A**). However, co-transfection of HEK-blue cells with plasmids containing the C7.1 aggrecan-specific BCR H and L-chain cDNAs resulted in a substantially lower transfection efficiency but BCR expression was detected on both HEK-Blue mTLR9 (5.07%) and mNOD2 (3.21%) transfected cells (**Figure 5.5B**). Likewise, co-transfection with plasmids encoding the TTCF-specific BCR H and L-chain cDNAs resulted in similar transfection efficiencies for both HEK-Blue mTLR9 (4.16%) and mNOD2 (10.1%) cells (**Figure 5.5C**). It was postulated that the both the Lipofectamine[™] and (*E.coli*-derived) plasmid preparations used in the transfection process could themselves activate either HEK-Blue mTLR9 or mNOD2 cells prior to the subsequent addition of dAgg. To test this, both HEK-Blue mTLR9 and mNOD2 cells were cultured separately with Lipofectamine[™] and the individual aggrecan or TTCF-specific BCR H and L-chain plasmids at concentrations equivalent to those used for transfection and subsequent activation was measured by extrapolation from standard curves using established with ODN and MDP as previously described. **Figure 5.6** shows that Lipofectamine[™] failed to induce above-background activation in either HEK-Blue mTLR9 (**Figure 5.6A**) or mNOD2 (**Figure 5.6B**) cultures. However, both the aggrecan and TTCF-specific BCR H and L-chain plasmids did induce low but significant activation of HEK-Blue mTLR9 cells (**Figure 5.6A**). This was in contrast to HEK-Blue mNOD2 cell cultures in which no above-background activation was observed (**Figure 5.6B**). Due to this, all subsequently transfected HEK-Blue mTLR9 cells were cultured for at least 48 h following transfection to allow for a return to baseline activation and washed thoroughly in medium prior to incorporation into detection assays.

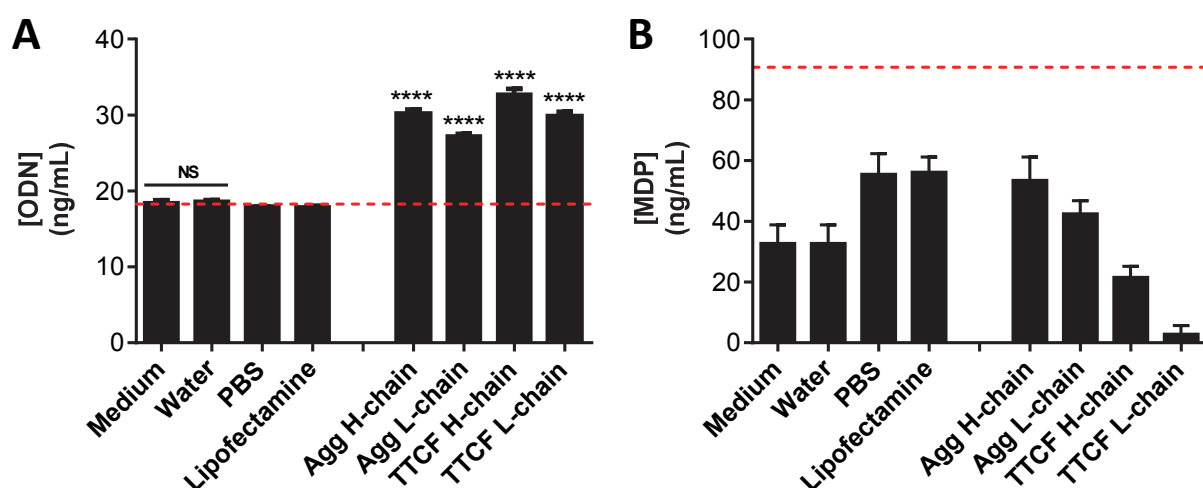


Figure 5.6 Activation of HEK-Blue by aggrecan or TTCF-specific BCR H and L-chain plasmids via TLR9 or NOD2.

4×10^4 HEK-Blue mTLR9 or mNOD2 cells were incubated with graded doses of ODN or MDP respectively for 24 h in a flat-bottomed 96-well plate to obtain a dose-response titration. Supernatants were extracted and incubated for 1 h with QUANTI-Blue prior to measurement of absorbance at 650 nm using a spectrophotometer. Parallel HEK-Blue (A) mTLR9 and (B) mNOD2 cultures were incubated with aggrecan or TTCF-specific BCR H and L-chain plasmids at the concentration used for transfection with Lipofectamine™ and activation estimated by interpolating data from the dose-response titrations. Each data point represents a triplicate with error bars showing $\pm$ SEM. Background activation was considered equal to the (MEAN PBS) + (3 x SD) as indicated by the dashed red line shown on both graphs. Above-background values were considered significant if a *t*-test *p*-value ≤ 0.05 was obtained when compared to background. Data representative of two independent experiments. * indicates a *t*-test *p*-value ≤ 0.05 . NS = not significant.

Figure 5.7A shows stimulation of aggrecan-specific BCR-transfected HEK-Blue mTLR9 cells with graded doses of ODN in comparison to untransfected controls. The transfected HEK-Blue mTLR9 population showed higher background and a slightly depressed peak activation level compared to the untransfected HEK-Blue mTLR9 cells. However, as seen previously (**Figure 5.3**) incubation with ODN for 24 h did induce above-background activation at doses >21.1 ng/mL with no apparent differences observed between untransfected and BCR-transfected HEK-Blue mTLR9 cells. Similar to the previous experiments performed with untransfected HEK-Blue mTLR9 cells (**Figure 5.3B**), graded doses of p84-103 also failed to induce above-background activation of aggrecan-specific BCR-transfected HEK-Blue mTLR9 cells (**Figure 5.7B**). However, the addition of dAgg to aggrecan-specific BCR-transfected HEK-Blue mTLR9 cells did generate some above-background activation and at doses ≥ 10 nM (**Figure 5.7B**). However, these values were low and were not significant.

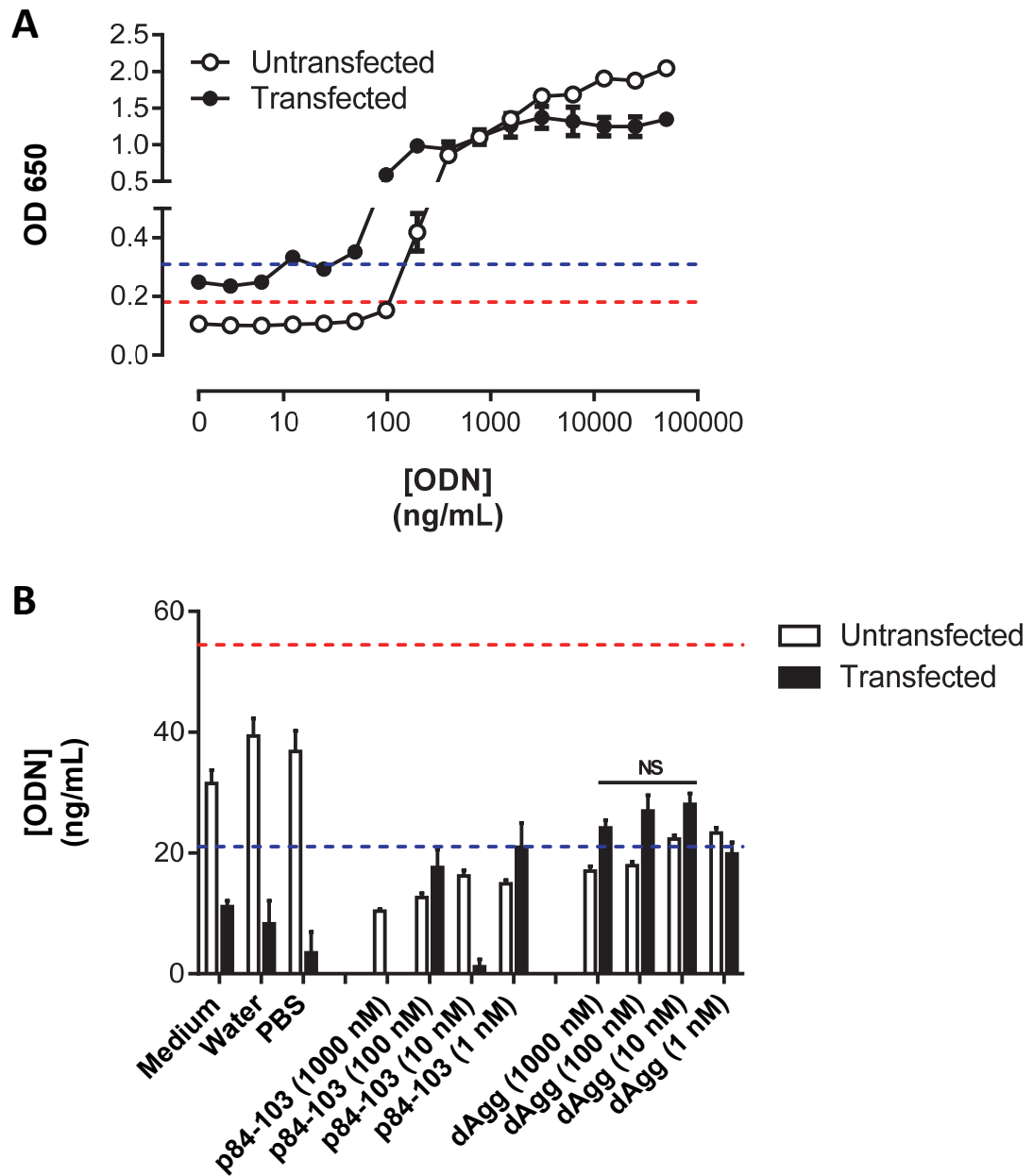


Figure 5.7 Activation of HEK-Blue by aggrecan via TLR9 following transfection with an aggrecan-specific BCR.

(A) 4×10^4 untransfected or transfected HEK-Blue mTLR9 cells were incubated with graded doses of ODN for 24 h in a flat-bottomed 96-well plate to obtain a dose-response titration. Supernatants were extracted and incubated for 1 h with QUANTI-Blue prior to measurement of absorbance at 650 nm using a spectrophotometer. (B) Parallel transfected HEK-Blue mTLR9 cultures were incubated with p84-103 or dAgg and TLR9 activation estimated by interpolating data from a ODN dose-response titration. Each data point represents a triplicate with error bars showing $\pm$ SEM. Background activation was considered equal to the (MEAN PBS) + (3 $\times$ SD) as indicated by the dashed red line (untransfected) or blue line (transfected) shown on both graphs. Above-background values were considered significant if an ANOVA test p -value ≤ 0.05 was obtained when compared to background. Data representative of two independent experiments. NS = not significant.

Figure 5.8A shows the stimulation of aggrecan-specific BCR-transfected HEK-Blue mNOD2 cells with MDP in comparison to untransfected HEK-Blue mNOD2 control cells. As seen previously with untransfected HEK-Blue mNOD2 cells (**Figure 5.4**) the levels of background activation remained high following transfection of HEK-Blue mNOD2 cells and response to the addition of graded doses of MDP closely matched that observed with the untransfected HEK-Blue mNOD2 control cells (**Figure 5.8A**). Similar to untransfected HEK-Blue mNOD2 cells, graded doses of p84-103 did not induce above-background activation of BCR-transfected cells (**Figure 5.8B**). Similarly, the addition of dAgg did not induce above-background activation despite the presence of the BCR (**Figure 5.8B**).

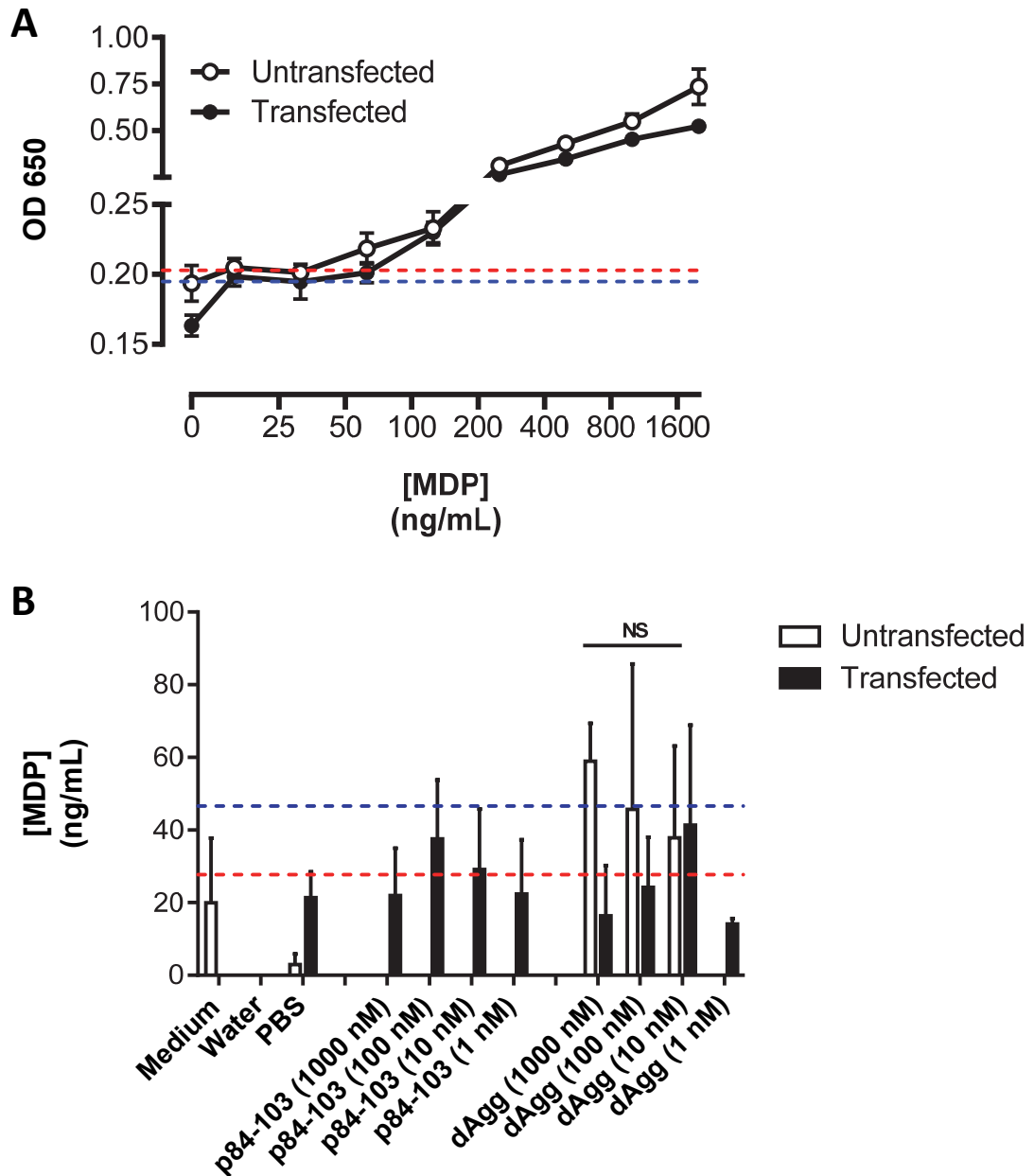


Figure 5.8 Activation of HEK-Blue by aggrecan via NOD2 following transfection with an aggrecan-specific BCR.

(A) 4×10^4 untransfected or transfected HEK-Blue mNOD2 cells were incubated with graded doses of MDP for 24 h in a flat-bottomed 96-well plate to obtain a dose-response titration. Supernatants were extracted and incubated for 1 h with QUANTI-Blue prior to measurement of absorbance at 650 nm using a spectrophotometer. (B) Parallel transfected HEK-Blue mNOD2 cultures were incubated with p84-103 or dAgg and NOD2 activation estimated by interpolating data from a MDP dose-response titration. Each data point represents a triplicate with error bars showing $\pm$ SEM. Background activation was considered equal to the (MEAN PBS) + (3 $\times$ SD) as indicated by the dashed red line (untransfected) or blue line (transfected) shown on both graphs. Above-background values were considered significant if an ANOVA test p -value ≤ 0.05 was obtained when compared to background. Data representative of two independent experiments. NS = not significant.

5.3. *In vitro* generation of an *ex vivo* antigen-specific B cells

A20-Agg B cells differ from *ex vivo* B cells in several ways which limit their usefulness for the study of antigen presentation and modulation of *ex vivo* CD4⁺ T cell activation. Their primary advantage is their homogenous expression of an aggrecan-specific BCR which is not reflected in a normal polyclonal *ex vivo* B cell population. However, *ex vivo* B cells are capable of proliferating and producing class-switched antibodies in response to various stimuli including T cell-derived signals through CD40-CD154 interactions (Buhlmann et al., 1995; Hollander et al., 1996; Wu et al., 1995) and PRR ligands (Ruprecht and Lanzavecchia, 2006). This is in contrast to A20-Agg B cells which, being a mouse B cell lymphoma, do not exit the division phase of the cell cycle. Unlike A20-Agg B cells, resting CD43⁺ B cells also express physiological baseline levels of co-stimulation (as shown in **Figure 4.4**) and will upregulate expression of these molecules when activated by environmental factors (Barr et al., 2007; Hathcock et al., 1994; Stack et al., 1994) or following antigen binding to the BCR and reciprocal interactions with CD4⁺ T cells (Lenschow et al., 1994; Rodriguez-Pinto, 2005). Similarly, the use of A20-Agg B cells for evaluating the role of different B cell subsets is also restricted as these cells are terminally differentiated.

Due to these limitations, this project also sought to employ a population of *ex vivo* splenic B cells expressing an aggrecan-specific BCR *in vitro*. Having already established that a range of PRR are not stimulated by aggrecan, this would facilitate further study of alternative explanations for the differences in B cell and DC induction of TCR-5/4E8-transgenic CD4⁺ T cell IFN- γ production. Whilst electroporation has been successfully used in our laboratory to introduce plasmids containing cDNAs encoding BCR into both mouse (Barrault et al 2004; Wilson et al 2012) and human B cell lines (Knight 1997), a single recent report has documented the use of the Amaxa® Nucleofector™ electroporation system for the successful introduction of plasmids encoding individual BCR H-chain cDNAs into LPS-activated *ex vivo* splenic B cells (Liu et al., 2010a).

Utilising an *ex vivo* B cell population enables investigation of B cell responses to the cytokine environment. Evidence suggests that *ex vivo* B cells are capable of producing cytokines in a way that reflects T_H1 and T_H2 profiles in response to activation by the corresponding $CD4^+$ T cell subset (Harris et al., 2000). This could have profound implications for autoimmune disease. For example, such effector B cells skewed towards a production of pathogenic cytokines by the inflammatory environment in the arthritic joint may proceed to similarly activate aggrecan-specific $CD4^+$ T cells and trigger a cycle which perpetuates disease (Lund and Randall, 2010). In addition, the role of B_{reg} is well-documented and the ability to generate B_{reg} from naïve B cells and observe their ability to inhibit activation of TCR-5/4E8-transgenic $CD4^+$ T cells could inform the development of new therapies (Mauri and Ehrenstein, 2008). *In vitro* generation of effector or regulatory B cells and incorporation into antigen-presentation assays containing either naïve or differentiated TCR-5/4E8-transgenic $CD4^+$ T cells would determine whether these cells are capable of significantly altering the $CD4^+$ T cell activation in a model system. Furthermore, adoptive transfer of effector or regulatory B cells or co-cultured TCR-5/4E8-transgenic $CD4^+$ T cells into PGIA mice would shed light on whether these cells can alter pathology *in vivo*.

5.3.1. Transfection of *ex vivo* B cells with an aggrecan-specific B cell receptor

Establishing an *ex vivo* population of aggrecan-specific B cells is an important next step in the development of an *ex vivo* system for comparison of antigen presentation to TCR-5/4E8-transgenic CD4⁺ T cells by different APC. B cells transfected with an aggrecan-specific BCR would replace the A20-Agg B cell line used in previous assays and offer a more physiologically representative population of B cells comparable to those found *in vivo*. Previous experiments from our laboratory have shown that following the electroporation of A20 B cells, transient expression of an aggrecan-specific BCR for periods of up to 72 hours is sufficient for enhanced aggrecan presentation similar to that seen with stably transfected A20 B cell clones (Wilson et al., 2012). Thus, it was envisaged that if successful transient transfection of an *ex vivo* population of B cells was achieved, that it may be possible to modulate this population into putative effector and/or regulatory B cell subsets. This would then permit the examination of the capabilities of different B cell subsets in modulating TCR-5/4E8-transgenic CD4⁺ T cell activation within the time frame of the transient expression of the aggrecan-specific BCR.

Based on the report by Liu *et al.*, *ex vivo* splenic B cells (negatively isolated as described previously in sections 2.7 and 4.2.3) were transfected using the Amaxa[®] Nucleofector[™] electroporation system (Liu et al., 2010a). This report indicated that LPS activation of the CD43⁺ B cells was required to induce proliferation was a prerequisite of successful transfection of plasmid DNA. Therefore following MACS isolation, splenic CD43⁺ B cells were incubated with 50 µg/mL LPS for 24 h. To check on the activation status of these cells they were then analysed by flow cytometry for expression of CD69 and MHC class II levels (**Figure 5.9**). As expected, in comparison to control CD43⁺ splenic B cells that were unstimulated (**Figure 5.9A**), LPS-stimulated B cells were seen to proliferate in culture (data not shown) and showed an increased proportion of activated cells characterised by upregulated CD69 and MHC class II expression (**Figure 5.9B**).

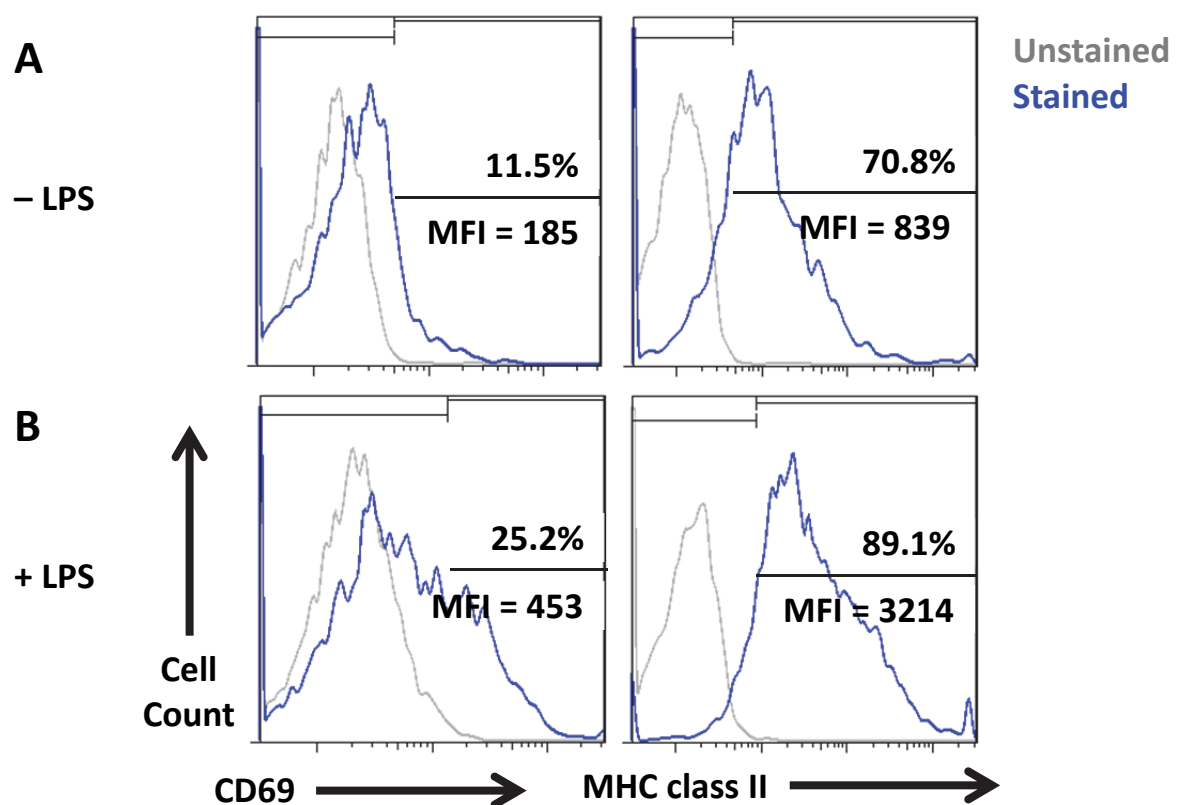


Figure 5.9 Flow cytometric analysis of $CD43^-$ B cells before and after stimulation with LPS.

$CD43^-$ B cells were incubated with (A) medium only or (B) medium supplemented with 50 $\mu\text{g/mL}$ LPS for 24 h prior to staining with anti-CD69 or anti-MHC class II and flow cytometric analysis. MFI = median fluorescence intensity.

LPS-stimulated splenic CD43⁺ B cells were then transfected with a plasmid encoding GFP (pmaxGFP) or co-transfected with plasmids containing the C7.1 aggrecan-specific BCR (described in section 2.12) previously used to generate the A20-Agg B cell transfectant. 24 h later, cells were stained with a biotinylated F(ab')₂ fragment of an anti-human-IgG monoclonal antibody (mAb) followed by streptavidin APC and analysed by flow cytometry. For comparison, A20 B cells were also transfected with the same plasmids. As expected, transfection of A20 B cells with pmaxGFP resulted in high transfection efficiency (93.9%) after 24 h (**Figure 5.10A**). In addition, co-transfection of A20 B cells with the C7.1 aggrecan-specific BCR containing plasmids resulted in expression of the BCR on a substantial minority (18.0%) of cells in accordance with previous work (Wilson et al., 2012) (**Figure 5.10B**).

Transfection of splenic CD43⁺ B cells proved to be far less efficient, however. Following transfection with pmaxGFP only low levels of GFP expression could be detected on a small percentage (4.03%) of B cells (**Figure 5.10A**). Furthermore, co-transfection of the C7.1 aggrecan-specific BCR plasmids did not result in expression of the BCR (**Figure 5.10B**). In order to examine if the murine thymotropic retrovirus MCF-13 (MCFR) promoter that is present in the BCR plasmids was not optimal for *ex vivo* B cell expression alternative plasmids (pCDNA3.1) containing the H and L-chain cDNA encoding a human TTCF-specific, IgG1/κ BCR under the control of the cytomegalovirus (CMV) promoter were also tested using this method. Similarly, co-transfection of splenic CD43⁺ B cells with the TTCF-specific H and L-chain plasmids also failed to induce expression of the introduced BCR (**Figure 5.10C**).

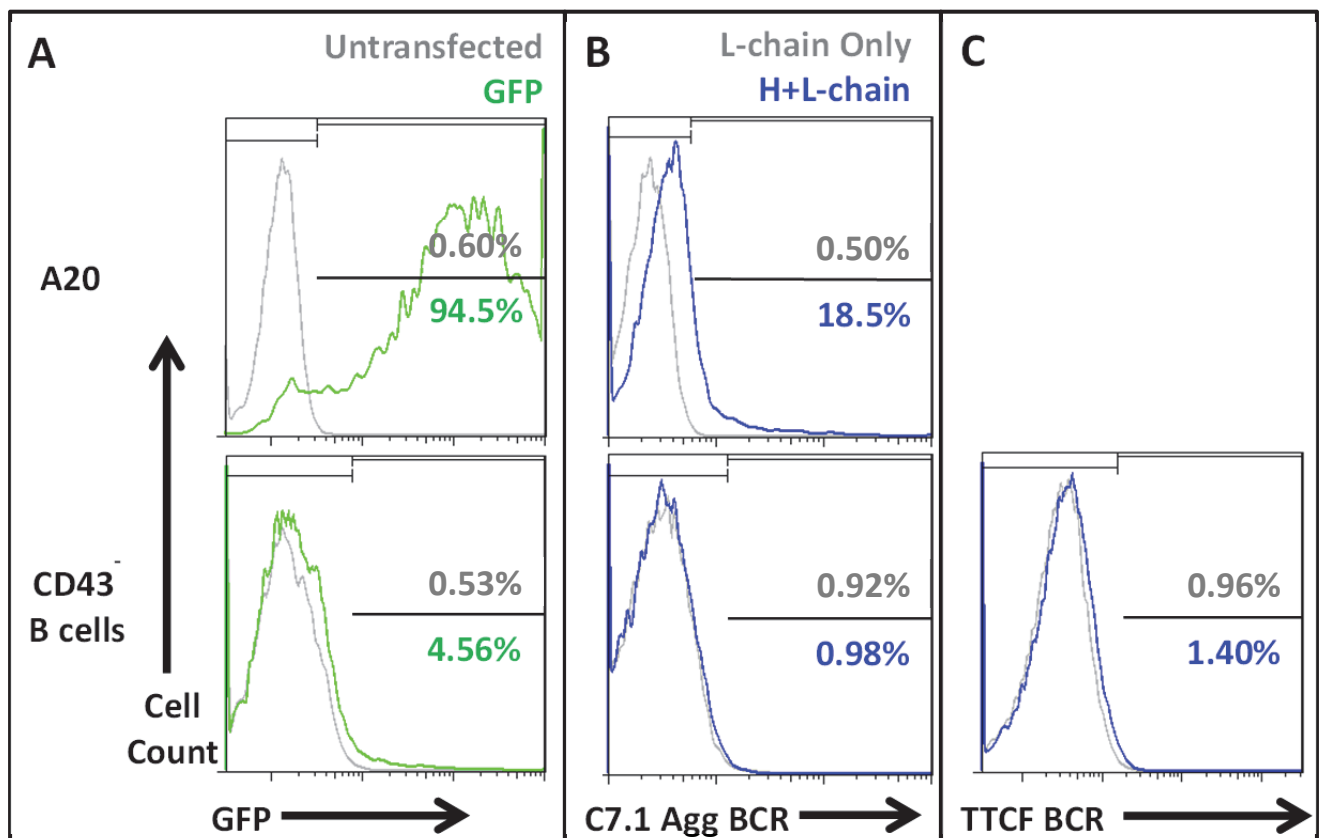


Figure 5.10 Transfection of A20 and *ex vivo* CD43⁻ B cells with GFP or an aggrecan-specific BCR.

3×10^6 A20 or CD43⁻ B cells were transfected using the Amaxa Nucleofector I electroporation system. Cells were transfected with a total of 2 μ g of plasmids (A) encoding pmaxGFP or co-transfected with plasmids encoding the H or L-chain of (B) C7.1 aggrecan (Agg) or (C) tetanus toxin C fragment (TTCF)-specific BCR. Transfected BCR was detected using anti-human IgG F(ab')₂. Plasmid-encoded gene expression was analysed 48 h later by flow cytometry.

5.3.2. Antibody-mediated antigen-loading of *ex vivo* B cells as a method for efficient B cell antigen acquisition and presentation to CD4⁺ T cells

Following the failure of electroporation an alternative approach for efficiently loading antigen into non-specific splenic CD43⁺ B cells was developed (see **Figure 5.11** for a schematic representation). Freshly isolated splenic CD43⁺ B cells were first incubated with a biotinylated F(ab')₂ fragment of an anti-mouse IgG mAb on ice (**Figure 5.11A**), followed by a shift to 37°C in the presence of graded doses of streptavidin-labelled dAgg (SAv-dAgg) (**Figure 5.11B**). It was predicted that any resulting biotinylated anti-mouse F(ab')₂/SAv-dAgg complexes would be internalised via the BCR leading to efficient dAgg processing and presentation similar to that previously seen with the aggrecan-specific A20-Agg B cells. The antigen-loaded *ex vivo* splenic B cells were therefore subsequently co-cultured with CD4⁺ T cells and T cell activation and cytokine production measured (**Figure 5.11C**).

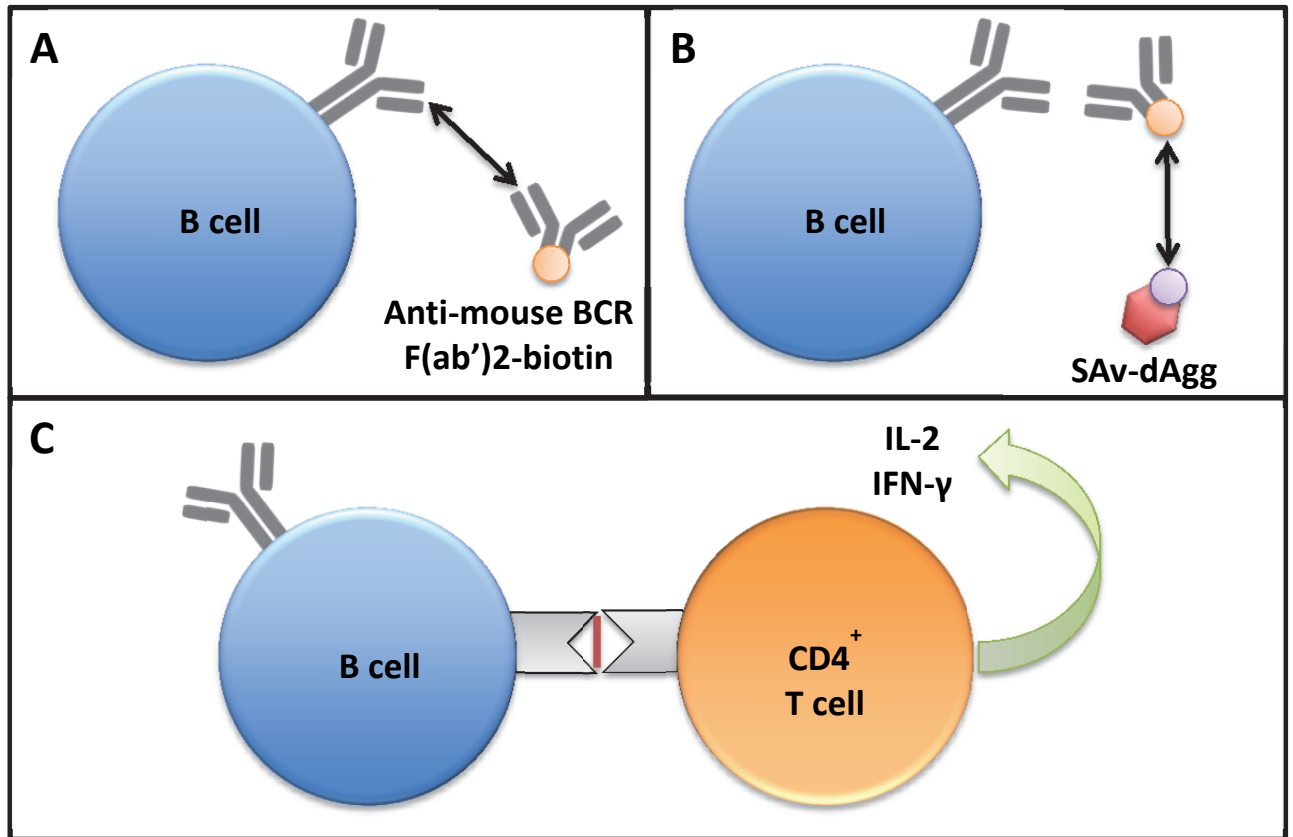


Figure 5.11 *Schematic representation of the antigen-loading methodology.*

(A) 1×10^6 CD43⁻ B cells were incubated with 5 $\mu\text{g}/\text{mL}$ anti-mouse BCR-biotin for 20 min at 4°C. (B) Cells were transferred to wells containing graded doses of SAV-dAgg and incubated for 3 h at 37°C. (C) 5×10^5 CD4⁺ T cells were added to each well and cultured for 72 h.

Initially, antigen-loaded splenic CD43⁻ B cells were co-cultured with the p84-103-specific T cell hybridoma 192 due to their enhanced sensitivity compared to TCR-5/4E8-transgenic CD4⁺ T cells (Figure 5.12). As expected (see Figure 4.2), A20-Agg B cells included as a positive control induced robust T cell responses at doses of dAgg less than 1 nM irrespective of the inclusion of the anti-mouse IgG mAb. In addition, the presentation efficiency of SA_v-dAgg by A20-Agg B cells was identical to that seen of dAgg, demonstrating that the biochemical modification of dAgg had not impaired the generation of the p84-104 epitope. In contrast, when splenic CD43⁻ B cells were cultured with graded doses of dAgg (either in the presence or absence of the anti-mouse IgG mAb) they induced poor 192 T cell activation. However, when splenic CD43⁻ B cells were pre-incubated with anti-mouse IgG before culture with graded doses of SA_v-dAgg they were seen to induce improved presentation to the T cell hybridoma 192 compared to when cultured with unmodified dAgg. However, despite this improvement, presentation was not as efficient as that seen by A20-Agg B cells (an approximately 10-fold higher dose of SA_v-dAgg was required). Despite this, 192 T cells incubated with antigen-loaded splenic CD43⁻ B cells were capable of equivalent IL-2 production as A20-Agg B cells at doses ≥ 1 nM (Figure 5.12). Taken together, these results indicate that this method represents a novel and effective way to load antigen into non-specific B cells for presentation to T cells.

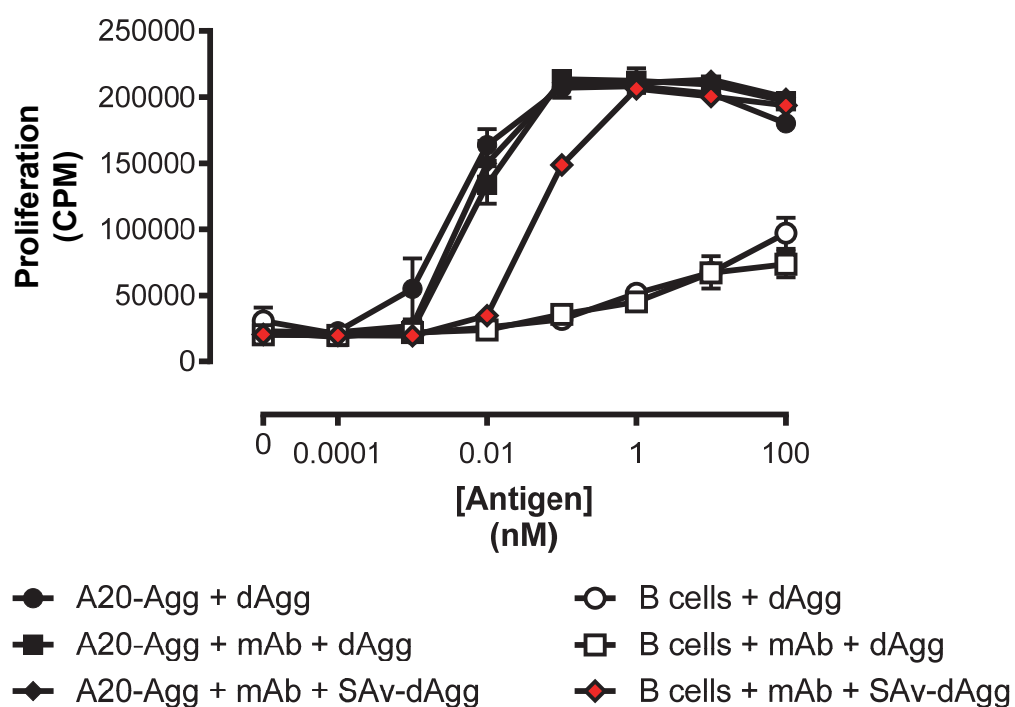


Figure 5.12 *IL-2 production following antibody-mediated loading of aggrecan into CD43⁻ B cells and co-culture with T cell hybridoma 192.*

5×10^4 APC were incubated with or without 5 $\mu\text{g/mL}$ anti-mouse BCR-biotin (mAb) and transferred to a flat-bottomed 96-well plate containing graded doses of either dAgg or streptavidin (SAv)-dAgg. 5×10^4 T cells were added to each well and cultured for 18 h. Supernatants were extracted and added to 3×10^4 CTLL-2. Cultures were pulsed with radioactive thymidine (^3H) and DNA harvested onto a glass fibre mesh 18 h later. ^3H incorporation was measured using a scintillation counter and counts per minute (CPM) recorded. Each data point represents a triplicate with error bars showing $\pm\text{SEM}$.

Following the demonstration of efficient presentation to the aggrecan-specific T cell hybridoma 192 by splenic CD43⁻ B cells following antigen-targeting to the BCR, the next step was to incorporate TCR-5/4E8-transgenic CD4⁺ T cells into this assay. This would then provide an *ex vivo* assay in which both APC and responding T cells were naïve and capable of physiological responses to antigen-presentation and reciprocal activation, including the production of effector cytokines such as IFN- γ . Therefore, A20-Agg B cells or splenic CD43⁻ B cells (with or without anti-IgG mAb pre-treatment) were incubated with purified TCR-5/4E8-transgenic CD4⁺ T cells in the presence of graded doses of SAv-dAgg for 72 h and IL-2 and IFN- γ production measured (**Figure 5.13**). Initial assays resulted in reduced T cell activation in the supernatants of CD43⁻ B cell co-cultures when stimulated with low antigen doses and a suppressed peak response at 100 nM SAv-dAgg in comparison to A20-Agg controls (Appendix D).

Due to the 72 h culture period and the lower sensitivity of the *ex vivo* TCR-5/4E8-transgenic CD4⁺ T cells in comparison to the T cell hybridoma 192, it was hypothesised that an increased number of antigen-loaded B cells would be required to activate the T cell population. It was hoped that this would serve to counterbalance the depletion of available mAb during the extended incubation period and subsequent scarcity of mAb+SAv-dAgg complexes required for efficient antigen presentation in the early stages of the response. Subsequent assays utilising increased APC (1×10^6) while retaining the same APC:T cell ratio (2:1) showed a similar trend to T cell hybridoma 192 assays with CD43⁻ splenic B cells incubated with mAb+SAv-dAgg demonstrating improved T cell activation in comparison to CD43⁻ splenic B cells without mAb pre-treatment that was almost as efficient as the presentation seen by A20-Agg B cells at doses ≥ 1 nM (**Figure 5.13A**). However, although this approach led to efficient IL-2 production, it did not result in significant IFN- γ induction from the TCR-5/4E8-transgenic CD4⁺ T cells. Splenic CD43⁻ B cells pre-incubated with or without mAb both failed to induce levels of IFN- γ production by TCR-5/4E8-transgenic CD4⁺ T cells when compared to levels measured following A20-Agg B cell presentation (**Figure 5.13B**). Background levels of IFN- γ were increased in CD43⁻ B cell co-cultures stimulated with mAb+SAv-dAgg suggesting that the anti-mouse IgG mAb potentially induces B cell cytokine production. Data pooled from duplicate experiments confirmed a lack of IFN- γ production following CD43⁻ B cell co-cultures with 100 nM SAv-dAgg, both in the absence or presence of the anti-IgG F(ab')₂ (**Figure 5.13C**).

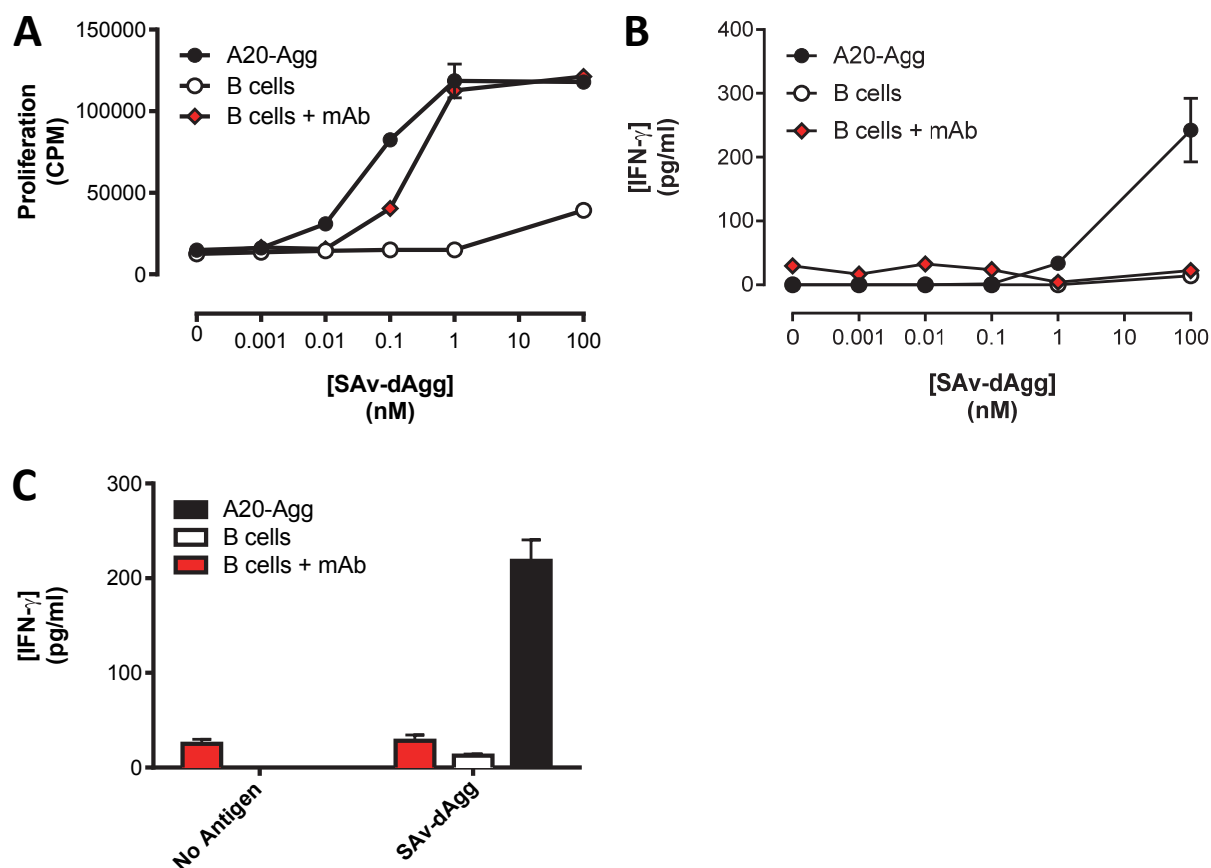


Figure 5.13 *IL-2 and IFN- γ production following antibody-mediated loading of aggrecan into CD43⁻ B cells and co-culture with TCR-5/4E8-transgenic CD4⁺ T cells.*

1×10^6 APC were incubated with or without 5 $\mu\text{g}/\text{mL}$ anti-mouse BCR-biotin and transferred to a flat-bottomed 96-well plate containing graded doses of SAv-dAgg. 5×10^5 CD4⁺ T cells were added to each well and cultured for 72 h. (A) Supernatants were extracted and added to 3×10^4 CTLL-2. Cultures were pulsed with radioactive thymidine (^3H) and DNA harvested onto a glass fibre mesh 18 h later. ^3H incorporation was measured using a scintillation counter and counts per minute (CPM) recorded. Each data point represents a triplicate with error bars showing $\pm\text{SEM}$. (B) Supernatants were extracted and tested for IFN- γ by ELISA. (C) Induction of IFN- γ in the co-cultures was compared at 100 nM antigen using data pooled from two independent experiments. Error bars show $\pm\text{SEM}$

5.4. Modulation of aggrecan presentation by blockade of co-stimulation

Following the absence of PRR activation induced by either p84-103 or dAgg an alternative explanation for the differences in IFN- γ production observed following antigen presentation by DC in comparison to A20-Agg B cells was explored. It was hypothesised that increased TCR-5/4E8-transgenic CD4⁺ T cell IFN- γ production following co-culture with A20-Agg B cells in comparison to DC was due to differential expression of co-stimulatory molecules. The use of *ex vivo* splenic CD43⁻ B cells loaded with aggrecan via an antibody complex failed to induce equivalent IFN- γ production (**Figure 5.13**). This suggested that A20-Agg B cells possessed unique characteristics which facilitate the generation of CD4⁺ T cell IFN- γ production. Therefore, as an alternative approach, the role of co-stimulation on A20-Agg B cells in comparison to DC was compared utilising antibody blockade of CD80 and CD86.

CD80 and CD86 are crucial co-stimulatory molecules which, in conjunction with signalling through the TCR, are required for efficient activation of naïve CD4⁺ T cells and differentiation into effector subsets such as T_H1 cells (Boise et al., 1995; Lanzavecchia and Sallusto, 2001). Some evidence indicates that differential CD80 and CD86 signalling may qualitatively affect the cytokine profile adopted by CD4⁺ T cells. CD80 signalling has been associated with production of T_H1 cytokines and CD86 with T_H2 cytokines (Kuchroo et al., 1995). Furthermore, aberrant expression of CD80 on B cells has been linked with T cell-dependent inflammation in autoimmune-prone NOD mice (Hussain and Delovitch, 2005). The cause of the enhanced T cell pathology observed in this *in vivo* model may represent a parallel situation to the co-culture experiments described in **Chapter 4**. For example, variations in the levels of CD80 and CD86 expression on A20-Agg B cells and DC during presentation of dAgg may contribute to differences in TCR-5/4E8-transgenic CD4⁺ T cell IFN- γ production. Accordingly, the relative importance of CD80 and CD86 expression for efficient aggrecan presentation by A20-Agg B cells was compared to that seen with DC.

5.4.1. Differential effects of blocking B cell or DC CD80/86 co-stimulation on CD4⁺ T cell activation and cytokine production

Previous experiments in Chapter 4 (Figures 4.10/12) demonstrated that dAgg presentation by aggrecan-specific B cells induced increased numbers of IFN- γ secreting TCR-5/4E8-transgenic CD4⁺ T cells than that seen with DC despite showing similar levels of TCR-5/4E8-transgenic CD4⁺ T cell activation measured by IL-2 production. It was hypothesised that this enhanced ability of the aggrecan-specific B cells was due to a differential effect of CD80 or CD86 expression on the TCR-5/4E8-transgenic CD4⁺ T cells when responding to p84-103/MHC complexes. To test this hypothesis experiments were therefore performed in which monoclonal antibodies specific for CD80 and/or CD86 were added to APC/TCR-5/4E8-transgenic CD4⁺ T cell co-cultures and the activation and differentiation of the TCR-5/4E8-transgenic CD4⁺ T cells was measured as performed previously.

Firstly, the effects of anti-CD80 (clone 16-10A1) or anti-CD86 (clone GL1) mAb on the activation of the TCR-5/4E8-transgenic CD4⁺ T cell by the two APC types were examined. Both 16-10A1 and GL1 mAb have previously been shown to functionally block CD80 and CD86 respectively when administered together, resembling the effect of CTLA-4-mediated inhibition on CD4⁺ T cell activation (Hancock et al., 1996; Lang et al., 2002; Suvas et al., 2002). **Figure 5.14** shows the effects of the addition of these two antibodies on the activation of TCR-5/4E8-transgenic CD4⁺ T cells, following co-culture with the A20-Agg B cells in the presence of 100 nM dAgg. Incubation of co-cultures with 10 μ g/mL anti-CD80 mAb decreased T cell activation, measured by IL-2 production, by 2.9 fold (**Figure 5.14A**). Furthermore, reducing the concentration of the anti-CD80 mAb to 2 μ g/mL resulted in a 2.2 fold reduction in IL-2 production. In contrast, treatment with anti-CD86 mAb did not replicate this effect even at 10 μ g/mL. Instead, 10 μ g/mL anti-CD86 mAb only achieved a 1.4 fold reduction in IL-2 production (**Figure 5.14B**). Adding the two antibodies together at 2 μ g/mL resulted in a synergistic 3.6 fold reduction in T cell IL-2 production that was increased to a 4.5 fold reduction at higher doses of 10 μ g/mL (**Figure 5.14C**). Control co-cultures containing A20-Agg B cells and TCR-5/4E8-transgenic CD4⁺ T cells in the presence of 100 nM dAgg with 10 μ g/mL hamster IgG2/ κ or rat IgG2a/ κ isotype control antibodies had no significant reduction in IL-2 production (**Figure 5.14D**).

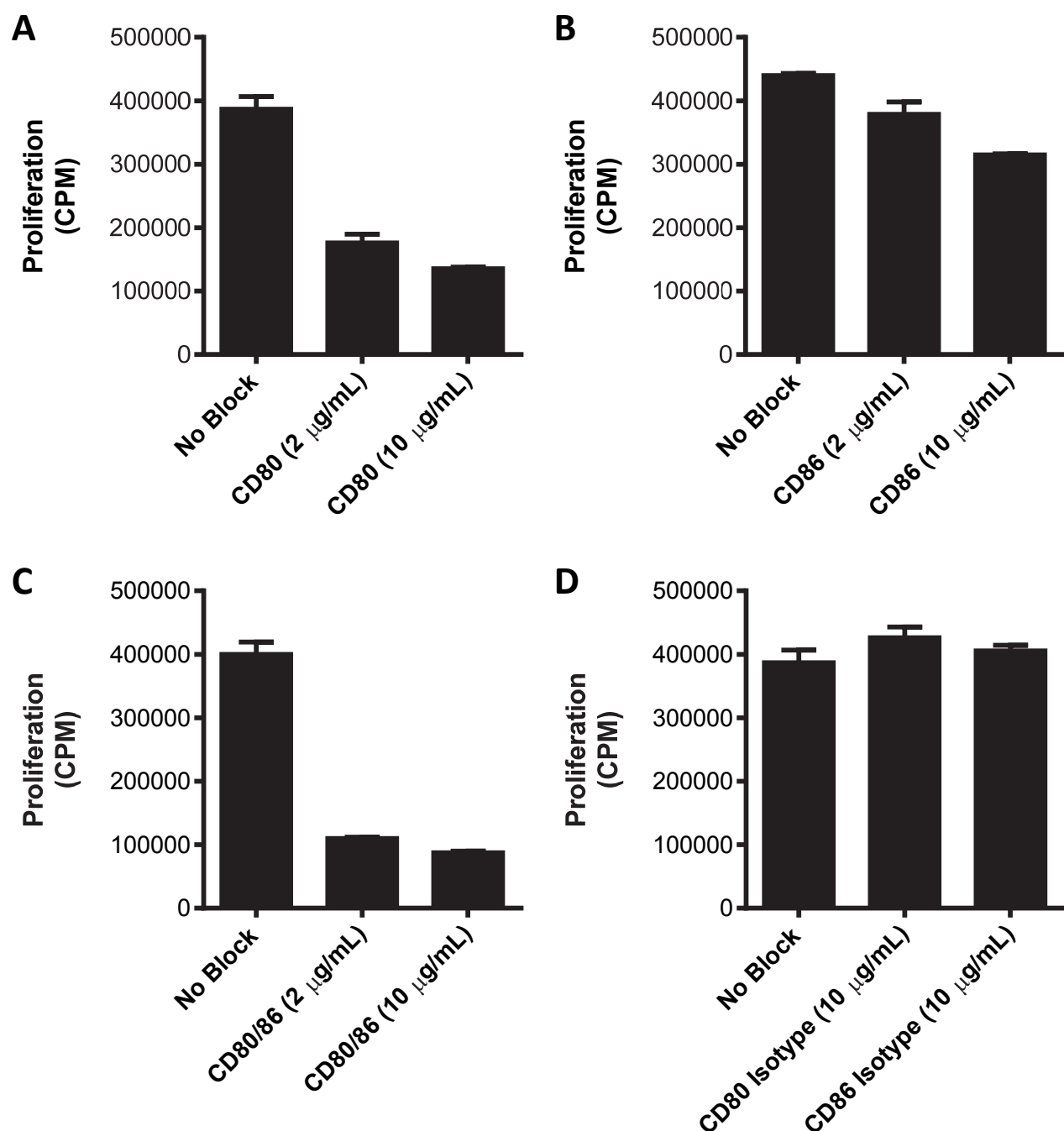


Figure 5.14 *IL-2 production in A20-Agg co-cultures treated with blocking monoclonal antibodies for CD80 and CD86 incubated with TCR-5/4E8-transgenic CD4⁺ T cells in the presence of a fixed dose of aggrecan.*

3×10^4 A20-Agg were incubated with 10 or 2 µg/mL anti-mouse (A) CD80, (B) CD86, (C) CD80/86 or (D) isotype controls and cultured with 1.5×10^4 CD4⁺ T cells in the presence of 100 nM dAgg in a flat-bottomed 96-well plate for 72 h. Supernatants were extracted and added to 3×10^4 CTLL-2. Cultures were pulsed with radioactive thymidine (^3H) and DNA harvested onto a glass fibre mesh 18 h later. ^3H incorporation was measured using a scintillation counter and counts per minute (CPM) recorded. Each data point represents a duplicate with error bars showing $\pm$ SEM. Data representative of three independent experiments.

In contrast, when similar experiments were performed with co-cultures containing DC (**Figure 5.15**) the addition of both anti-CD80 (**Figure 5.15A**) and anti-CD86 (**Figure 5.15B**) mAb showed a similar dose-dependent reduction in TCR-5/4E8-transgenic CD4⁺ T cell IL-2 production. However, similar to that seen in the A20-Agg B cell co-culture, the inclusion of both mAb was seen to act synergistically, almost abolishing T cell IL-2 production (**Figure 5.15C**). As seen for A20-Agg B cells, the presence of isotype controls for anti-mouse CD80 and CD86 respectively in DC co-cultures had no significant effect on T cell activation (**Figure 5.15D**).

Thus, whilst both CD80 and CD86 were required for optimal TCR-5/4E8-transgenic CD4⁺ T cell activation following presentation of dAgg by DC, presentation in co-cultures containing A20-Agg B cells appeared not to be less effected by the inclusion of high doses of antibodies specific for CD86. Taken together, these data suggest that CD80, rather than CD86, plays a more substantial role in the activation of TCR-5/4E8-transgenic CD4⁺ T cells following presentation of dAgg by A20-agg B cells.

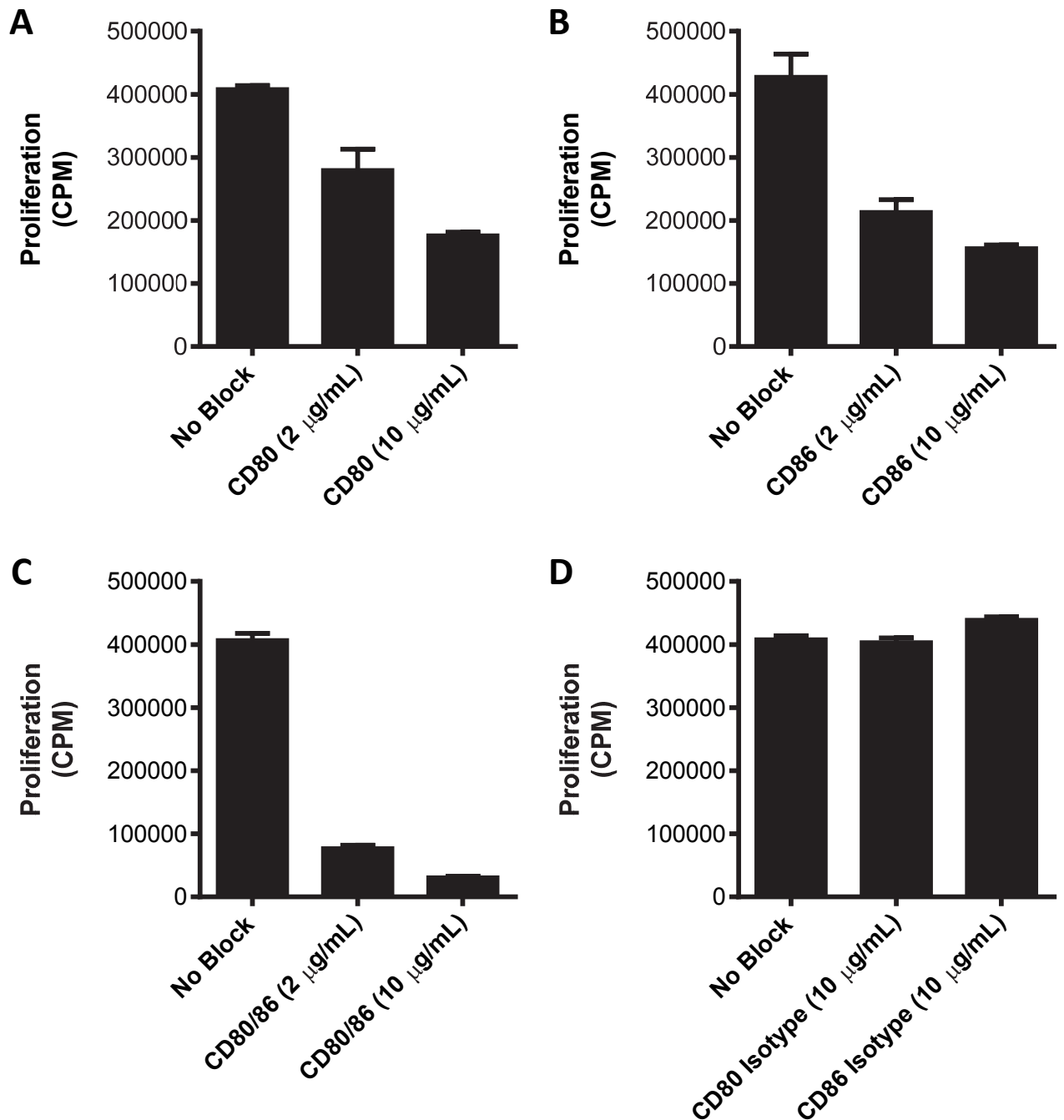


Figure 5.15 *IL-2 production in DC co-cultures treated with blocking monoclonal antibodies for CD80 and CD86 incubated with TCR-5/4E8-transgenic CD4⁺ T cells in the presence of a fixed dose of aggrecan.*

3×10^4 DC were incubated with 10 or 2 µg/mL anti-mouse (A) CD80, (B) CD86, (C) CD80/86 or (D) isotype controls and cultured with 1.5×10^4 CD4⁺ T cells in the presence of 100 nM dAgg in a flat-bottomed 96-well plate for 72 h. Supernatants were extracted and added to 3×10^4 CTLL-2. Cultures were pulsed with radioactive thymidine (^3H) and DNA harvested onto a glass fibre mesh 18 h later. ^3H incorporation was measured using a scintillation counter and counts per minute (CPM) recorded. Each data point represents a duplicate with error bars showing $\pm$ SEM. Data representative of three independent experiments.

This potential difference in APC/CD4⁺ T cell interactions was thought to offer a plausible explanation for the differences observed in A20-Agg and DC induction of IFN- γ production following the presentation of dAgg. Therefore, supernatants from similar co-cultures were screened for levels of IFN- γ . **Figure 5.16** shows that the inclusion of the anti-CD80 mAb to co-cultures containing A20-Agg B cell reduced IFN- γ production by 7.7 fold when added at 2 μ g/mL and completely abolished IFN- γ production at 10 μ g/mL (**Figure 5.16A**). In contrast, whilst the inclusion of the anti-CD86 mAb at 2 μ g/mL was also seen to reduce IFN- γ production by 1.6 fold, addition of this mAb was much less effective than the equivalent concentration of the anti-CD80 mAb (**Figure 5.16B**). This result was similar to that seen when IL-2 production was measured. As expected, the inclusion of both anti-CD80 and anti-CD86 mAb completely abrogated the IFN- γ response (**Figure 5.16C**) in accordance with that seen measuring T cell activation (**Figure 5.14C**). Addition of antibodies to CD80/86 on DC, as shown in **Figure 5.17**, demonstrates that treatment with 2 μ g/mL anti-CD80 (**Figure 5.17A**) or anti-CD86 (**Figure 5.17B**) caused equivalent reduction in IFN- γ production (2.3 and 2.2 fold respectively). Similarly, the 10 μ g/mL concentration was capable of severely diminishing IFN- γ levels in both instances. In addition, concomitant addition of anti-CD80 and CD86 acted to eliminate IFN- γ production even at the 2 μ g/mL concentration (**Figure 5.17C**).

In conclusion, A20-Agg B cell activation of TCR-5/4E8-transgenic CD4⁺ T cells and subsequent induction of IFN- γ production following dAgg presentation appears to exhibit a preferential requirement for CD80 rather than CD86 although both molecules are shown to be necessary. In contrast, IFN- γ production from the TCR-5/4E8-transgenic CD4⁺ T cells following presentation of dAgg by DC does not appear to show this preferential co-stimulatory molecule requirement.

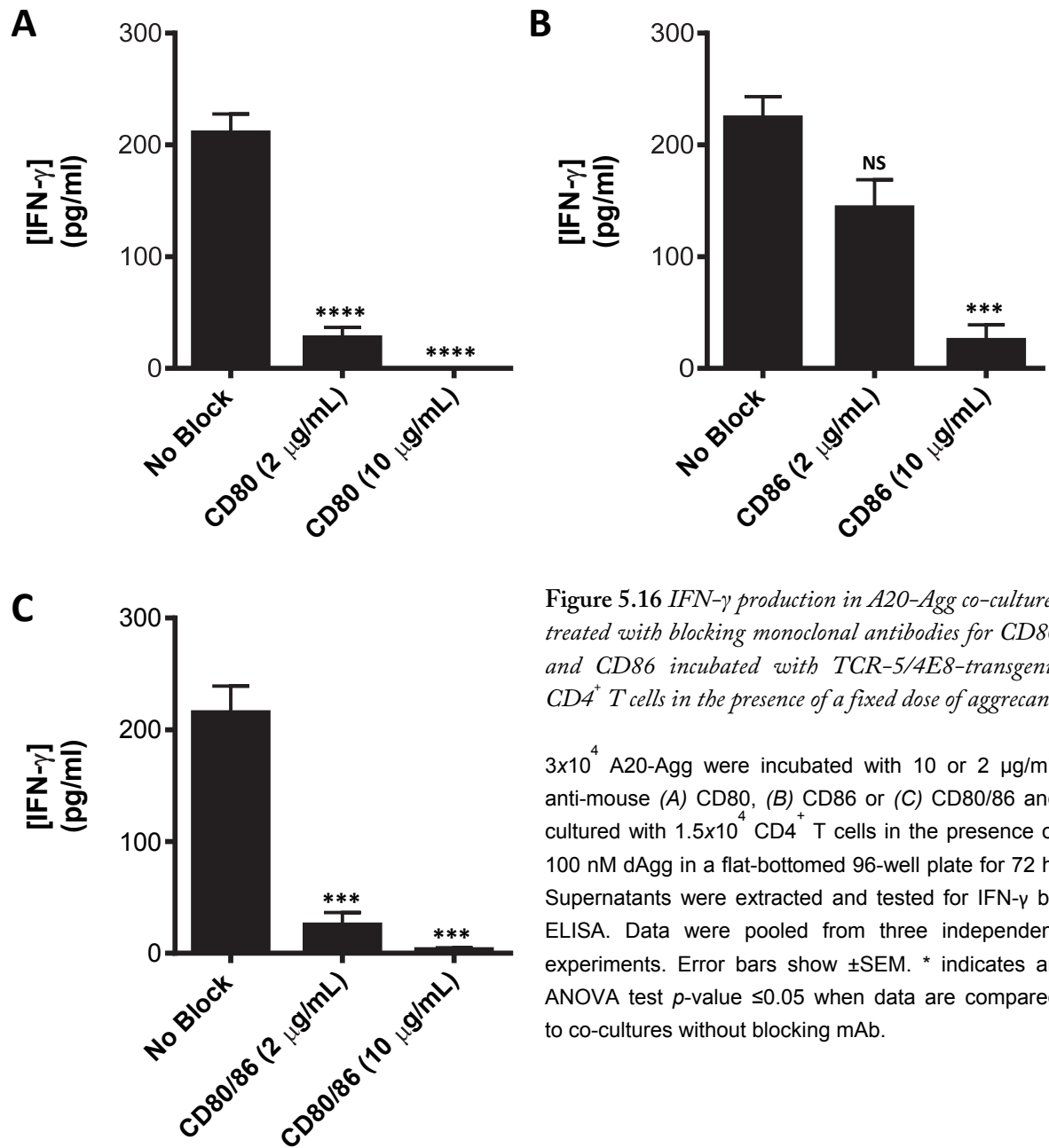


Figure 5.16 *IFN- γ production in A20-Agg co-cultures treated with blocking monoclonal antibodies for CD80 and CD86 incubated with TCR-5/4E8-transgenic CD4⁺ T cells in the presence of a fixed dose of aggrecan.*

3×10^4 A20-Agg were incubated with 10 or 2 μ g/mL anti-mouse (A) CD80, (B) CD86 or (C) CD80/86 and cultured with 1.5×10^4 CD4⁺ T cells in the presence of 100 nM dAgg in a flat-bottomed 96-well plate for 72 h. Supernatants were extracted and tested for IFN- γ by ELISA. Data were pooled from three independent experiments. Error bars show $\pm$ SEM. * indicates an ANOVA test p -value ≤ 0.05 when data are compared to co-cultures without blocking mAb.

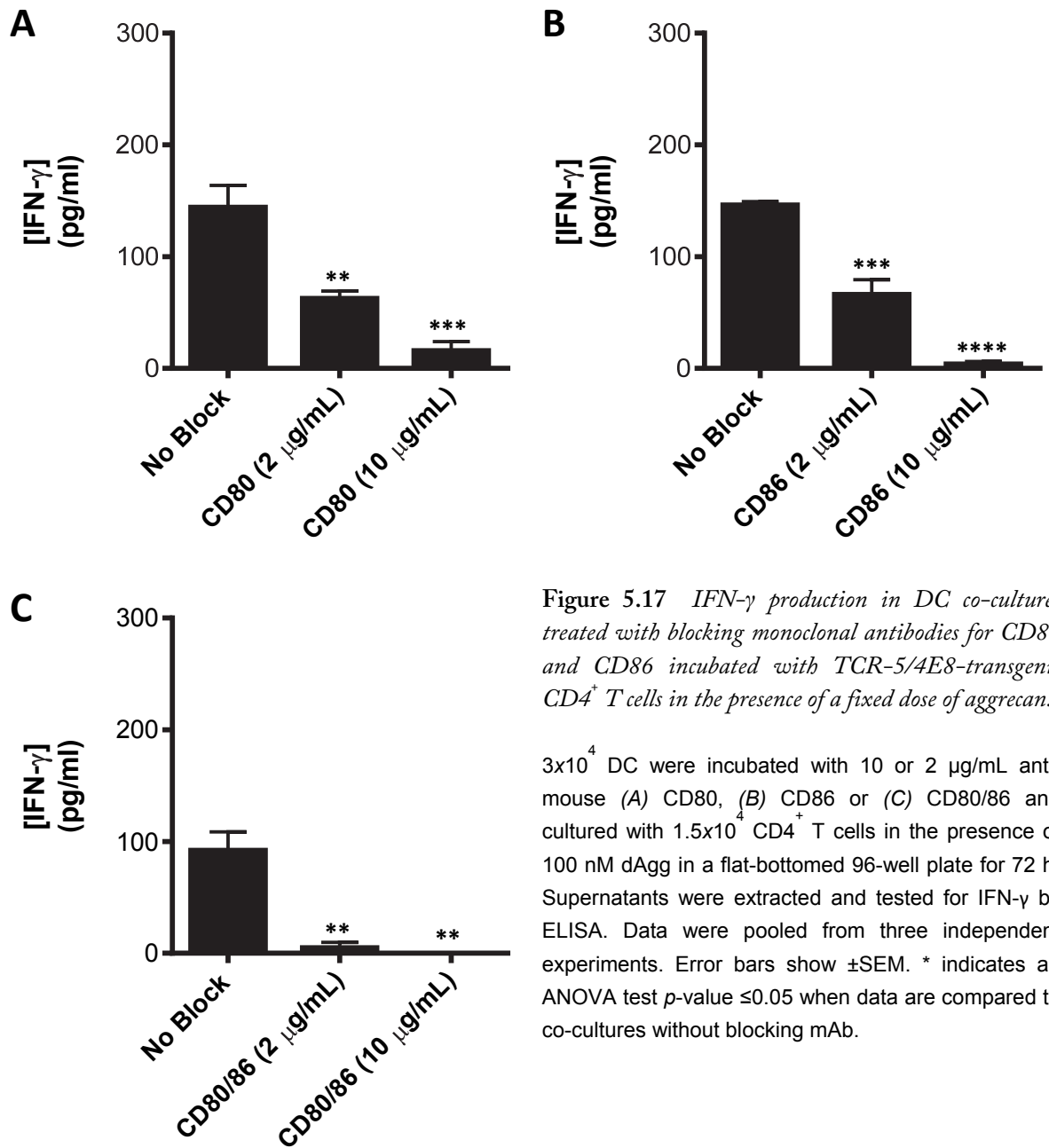


Figure 5.17 *IFN- γ production in DC co-cultures treated with blocking monoclonal antibodies for CD80 and CD86 incubated with TCR-5/4E8-transgenic CD4⁺ T cells in the presence of a fixed dose of aggrecan.*

3x10⁴ DC were incubated with 10 or 2 μ g/mL anti-mouse (A) CD80, (B) CD86 or (C) CD80/86 and cultured with 1.5x10⁴ CD4⁺ T cells in the presence of 100 nM dAgg in a flat-bottomed 96-well plate for 72 h. Supernatants were extracted and tested for IFN- γ by ELISA. Data were pooled from three independent experiments. Error bars show $\pm$ SEM. * indicates an ANOVA test p -value ≤ 0.05 when data are compared to co-cultures without blocking mAb.

5.5. Discussion

Differential aggrecan stimulation of PRR expressed on B cells was hypothesised to be a potential mechanism facilitating the increased induction of TCR-5/4E8-transgenic CD4⁺ T cell IFN- γ production than that seen following co-culture with DC. Subsequently, p84-103 peptide and dAgg were therefore added to cultured HEK-Blue reporter cells expressing individual TLR and NLR considered to be candidate receptors either for aggrecan itself or for possible ECM components likely to be found in the purified nasal aggrecan preparations used in these experiments.

These experiments did demonstrate that unlike p84-103, high doses of purified dAgg induced significant TLR2 (**Figure 5.1**) and TLR4 (**Figure 5.2**) activation. Activation of TLR2 by dAgg at these levels may result from direct binding of dAgg or other ECM components in the preparation to TLR2. As TLR2 typically recognises bacterial peptidoglycans this could be due to the presence homologous structural motifs found within aggrecan. Equally, this could be due to contamination of the dAgg preparation with bacterial components which form the natural ligands for TLR2. TLR4 activation was also observed in response to a high concentration of dAgg (**Figure 5.4**). This may be due to the binding of fragments of HA, a major ECM component associated with aggrecan, which has been shown previously to induce maturation of DC via TLR4 (Termeer et al., 2002). The potential for this mechanism to provide endogenous ligands for TLR4 on APC is particularly relevant to this model as breakdown of ECM is upregulated during the inflammation associated with RA.

However, similar to TLR2, the TLR4 activation observed in response to dAgg could be explained by low concentrations of bacterial TLR4 ligands present in the dAgg preparation. LPS is a common contaminant found in protein preparations (Petsch and Anspach, 2000). For example, murine IgG prepared from a cell culture filtrate can contain up to 100 endotoxin units (EU)/mL (equivalent to 120 pg/mL of LPS from *E.coli* O111:B). The dAgg used in this study was prepared in our laboratory from bovine nasal cartilage resulting in the possibility that the dAgg was contaminated with LPS during the procedure which was not subsequently removed or destroyed during the isolation or deglycosylation process. However, based on the findings presented in this chapter, 1000 nM dAgg (approximately 250 μ g/ml) contains only 187 $\pm$ 21 pg/mL LPS. This is ten times the dAgg concentration used to induce TCR-5/4E8-transgenic CD4⁺ T cell IFN- γ production in the experiments described in **Chapter 4** and is only marginally above the lower limit of detection for the HEK-Blue mTLR4 detection assay (**Figure 5.2**). Indeed, the quantity of endotoxin equivalent to the TLR4 activation detected in the HEK-Blue assay is below the concentration required to induce

significant upregulation of macrophage IL-6, B cell antibody production or DC maturation (Mattern et al., 1994; Morris et al., 1992; Sibley et al., 1988; Termeer et al., 2000). The *Limulus* amoebocyte lysate (LAL) assay, which can detect LPS at concentrations as low as 0.01-0.001 EU/mL, may provide a useful tool for corroborating the HEK-Blue data and ascertaining whether endotoxins are the causative factor. However, despite being a high-sensitivity assay, this method is known to be non-specific and therefore of limited usefulness for determining the exact agent responsible for PRR activation (Elin and Wolff, 1973).

As previously discussed, B cell IL-6 and IL-10 production can be induced by stimulation of TLR2 or TLR4 (Barr et al., 2007). If this mechanism is also active in A20-Agg B cells this may offer a potential explanation for the induction of T_{reg} in co-cultures stimulated with aggrecan (**Figure 4.15**) as CD4⁺ T cells have been shown in different scenarios to differentiate into T_{reg} in response to IL-6 (Gu et al., 2008; McGeachy et al., 2007) and also require IL-10R signalling in order to suppress inflammation (Chaudhry et al., 2011; Josefowicz et al., 2012). Although our hypothesis was based on APC activation via TLR, it is also possible that the TCR-5/4E8-transgenic CD4⁺ T cells were directly stimulated by aggrecan or associated ECM components found in the dAgg preparation. Interestingly, both TLR2 (**Figure 5.1**) and TLR4 (**Figure 5.2**), which aggrecan was shown to activate on HEK-Blue cells at high antigen doses, are expressed by CD4⁺ T cells. TLR2 has been shown to be a specific inducer of T_H1 activation and can be enhanced further in the presence of IL-2 and IL-12 (Imanishi et al., 2007). Furthermore, loss of TLR2 or TLR4 on CD4⁺ T cells can ameliorate EAE through suppression of T_H1 and T_H17 proliferation and survival (Reynolds et al., 2010, 2012). Therefore, future studies could extend the study of the potential role of PRR in this model to also investigate TCR-5/4E8-transgenic CD4⁺ T cell activation through TLR using the *in vitro* system described here.

In contrast to TLR2 and TLR4, HEK-Blue cells expressing the predominantly intracellular mTLR9 (**Figure 5.3**) and mNOD2 (**Figure 5.4**) failed to respond to both p84-103 or dAgg even at high doses. It was hypothesised that this lack of activation may be due to an inability of exogenously added dAgg to access the intracellular compartments where these PRR reside. TLR9 undergoes trafficking to endolysosomes via the membrane chaperone UNC93B1 (Kim et al., 2008). TLR9 ligands enter these endocytic compartments following internalisation from the surrounding medium (Latz et al., 2004). It has been demonstrated that some antigens may be exported from phagosomes into the cytosol by the action of the ER-associated protein degradation (ERAD) complex following partial degradation (Mantegazza et al., 2013; Rodriguez et al., 1999). Therefore, it is speculated that NOD2 may encounter antigens transported to the cytosol in this manner. Such antigens can then

return to phagosomes or endosomes for loading onto MHC prior to transport to the cell surface (von Delwig et al., 2003; Ramachandra et al., 1999).

As BCR binding has previously been demonstrated to facilitate the co-localisation of antigen/BCR complexes to PRR-rich endosomes (Chaturvedi et al., 2008), it was hypothesised that transfection of HEK-Blue cells with plasmids containing cDNA encoding the C7.1, aggrecan-specific BCR would confer enhanced antigen delivery to TLR9 containing intracellular compartments. Similar experiments were also performed with BCR transfected HEK-Blue NOD2 cells to test if endocytosed aggrecan would also be able to access NOD2 in intracellular compartments. HEK-Blue mTLR9 and mNOD2 cells were reverse-transfected using Lipofectamine™ with plasmids containing cDNA encoding aggrecan-specific BCR H and L-chains resulting in a typical transfection efficiency ranging from 5-15% (**Figure 5.5**). Although Lipofectamine™ alone had no effect on the activation status of the cells, HEK-Blue mTLR9 incubated (in the absence of Lipofectamine™ or any other transfection reagents) with only plasmids containing cDNA encoding either aggrecan or TTCF-specific BCR H and L-chains did show low levels of above-background activation which may affect the results of downstream assays (**Figure 5.6A**). Indeed, background levels of HEK-Blue mTLR9 activation were slightly increased in comparison to untransfected controls. As a result, interpretation of HEK-Blue mTLR9 data was approached cautiously. Transfected HEK-Blue mTLR9 and mNOD2 cells were stimulated with graded doses of p84-103 and dAgg. Despite successful transfection with the aggrecan-specific BCR, addition of aggrecan, even at concentrations of 1000 nM did not lead to significant activation of either HEK-Blue mTLR9 (**Figure 5.7**) or mNOD2 (**Figure 5.8**) cells.

Although these results may indicate that dAgg does not interact with TLR9 or NOD2 there still remains a possibility that dAgg did not actually encounter either of these PRR during the course of the experiments performed. In order to address this possibility fluorescence microscopy could be used to visualise the intracellular localisation of dAgg following its addition to HEK-Blue mTLR9 or mNOD2 cells, in particular with respect to the endogenous intracellular locations of TLR9 and NOD2. By comparison of untransfected HEK cells with those transfected with an aggrecan-specific BCR this may also ascertain whether the BCR plays a role in facilitating dAgg uptake leading to co-localisation with these PRR. TLR9 has been shown to both exacerbate and suppress autoimmunity in different models of autoimmunity (Christensen et al., 2005, 2006; Rudnicka et al., 2009; Stoeckl et al., 2011) and administration of NOD2 ligand can promote T_H1-mediated inflammation in PGIA (Rosenzweig et al., 2009). Therefore, it would be interesting to artificially stimulate B cell or

DC with TLR9 or NOD2 ligands prior to co-culture with aggrecan and TCR-5/4E8-transgenic CD4⁺ T cells to observe any changes in cytokine production.

In conclusion, these data indicate that although dAgg can induce TLR2 and TLR4 activation this only occurs at doses >100 nM which is in excess of the dose utilised in assays investigating A20-Agg B cell induction of CD4⁺ T cell IFN- γ production (**Figures 4.8-4.15**). This suggests that TLR2 or TLR4 activation is not required for this effect. However, it is possible that the detection method used in this instance (the HEK-Blue reporter cell line) is not sensitive enough to detect the levels of ligand involved in activation of TLR2 or TLR4 on A20-Agg B cells or other APC. In addition, B cells have been shown to express other TLR in addition to those described here (Barr et al., 2007) and future work could investigate aggrecan activation of these receptors utilising the HEK-Blue detection system. Treatment of A20-Agg B cells and DC with inhibitors of the MyD88 (or other PRR adapter molecules) signalling pathway prior to culture with aggrecan and TCR-5/4E8-transgenic CD4⁺ T cells may reveal the requirement of this pathway without specifically identifying the individual PRR involved (Bartfai et al., 2003; Loiarro et al., 2005, 2007). Similarly, APC could be isolated from tissue derived from MyD88-deficient mice (Hou et al., 2008). If TCR-5/4E8-transgenic CD4⁺ T cell differentiation and cytokine production is affected in either scenario this would suggest PRR involvement. These approaches are limited, however, as they do not provide information regarding any synergistic effects that may occur when multiple TLR are stimulated. In contrast, the addition of graded doses of PRR ligands, particularly those found in arthritic synovium, to APC and TCR-5/4E8-transgenic CD4⁺ T cell co-cultures could be used to determine whether activation of specific PRR combinations can differentially modulate TCR-5/4E8-transgenic CD4⁺ T cell activation and cytokine production by B cells or DC. Addition of multiple ligands simultaneously, or synovial fluid extracted from patients, could also be used as a poly-PRR stimulus (Huang and Pope, 2009). This is of particular interest as co-ligation of TLR2, TLR4 and TLR9 has been shown to induce B cell production of IFN- γ (Barr et al., 2007) and, therefore, could be critical for induction of T_H1 differentiation which has been shown to be important in this *in vitro* model (**Chapter 4**) and PGIA pathology (Finnegan et al., 1999).

A20-Agg B cells have been demonstrated to be effective antigen-presenting cells and a useful tool for studying the ability of aggrecan-specific B cells to activate TCR-5/4E8-transgenic CD4⁺ T cells (**Chapter 4**). Despite this, A20-Agg B cells are a terminally differentiated mouse lymphoma cell line and are therefore not suitable for experiments designed to investigate the role of specific B cell subsets due to their fixed activation status (**Figure 4.3**) and unregulated proliferation (**Figure 4.13**). This prevents A20-Agg B cells from responding to the cytokine environment or signals derived from

PRR or T cells in a way that is representative of the *in vivo* situation. To overcome these limitations, initial experiments presented in this chapter aimed to transiently transfect splenic CD43⁻ B cells with an aggrecan-specific BCR.

Liu *et al.* have previously transfected mouse splenic CD43⁻ B lymphocytes with mutant BCR H-chains using the Amaxa® Nucleofector™ system following LPS stimulation (Liu *et al.*, 2010a). Therefore, this protocol was replicated in this study. Freshly isolated CD43⁻ B cells were shown to upregulate CD69 and MHC class II following LPS stimulation (**Figure 5.9**). LPS-activated *ex vivo* CD43⁻ B cells were then successfully transfected with a plasmid encoding GFP indicating that this is a viable method for transiently transfecting B cells and demonstrating expression of plasmid-encoded genes (**Figure 5.10A**). However, attempts to co-transfect BCR H and L-chains have been unsuccessful using the pMCFR plasmid vector (**Figure 5.10B**). This vector has been used previously for successful transfection and gene expression in both human and mouse B cell lines (Wilson *et al.*, 2012). It was hypothesised that its failure in this scenario may be due to inefficient gene expression in *ex vivo* B lymphocytes when using the MCFR promoter. However, the use of the alternative plasmid vector pcDNA3, which contains the well-characterised and almost ‘universal’ CMV promoter, also failed to yield transfected CD43⁻ B cells (**Figure 5.10C**). It is also possible that the transfected aggrecan-specific H and L-chains paired with endogenous H and L-chains and, therefore, diluted the presence of the aggrecan-specific BCR. Thus detection of the transfected BCR on the cell surface of the mixed population of *ex vivo* transfectants using an anti-human IgG-specific reagent may have been reduced due to competition with the native BCR expressed by the transfected B cell pool. This may also explain the disparity between the A20-Agg B cell line and *ex vivo* splenic CD43⁻ B cells as the former expresses a single endogenous H and L-chain while the latter population will express a range of potential BCR.

As an alternate approach to transient BCR transfection, loading antigen into non-specific B cells was investigated. By targeting antigen to the BCR irrespective of inherent specificity this method aimed to bypass the need for transfection to achieve efficient antigen uptake and was hoped to also induce BCR-mediated CD4⁺ T cell activation (**Figure 5.11**). Importantly, as shown in **Figure 5.12**, modification of aggrecan by the biochemical addition of streptavidin was shown to have no effect on aggrecan presentation by A20-Agg B cells. Furthermore, it was demonstrated when utilising the T cell hybridoma 192 as responder cells that this approach was highly successful, leading to enhanced aggrecan presentation by splenic CD43⁻ B cells (**Figure 5.12**). Similarly, when increased cell numbers were utilised this method remained effective when antigen presentation was measured using the activation of TCR-5/4E8-transgenic CD4⁺ T cells (**Figure 5.13A**). The requirement for

higher APC and T cell numbers may be due to the limited window of opportunity for APC uptake of antigen-antibody complexes which may deteriorate during the extended 72 h co-culture period required for measurement of *ex vivo* CD4⁺ T cell activation compared to the 24 h period required for the measurement of T cell hybridoma activation. However, despite inducing efficient T cell activation (measured by IL-2 production), streptavidin targeting of aggrecan to the BCR of *ex vivo* B cells failed to induced detectable TCR-5/4E8-transgenic CD4⁺ T cell IFN- γ production equivalent to that seen following aggrecan presentation by A20-Agg B cells (**Figure 5.13B & C**).

There are several possible reasons for this. The use of an anti-mouse IgG may have limited binding to naïve B cells as many express large quantities of IgM which would represent another route for antigen-loading. In addition, direct engagement of the BCR by antigen may be necessary for the signalling events required to promote B cell induction of TCR-5/4E8-transgenic CD4⁺ T cell IFN- γ production. Similarly, the use of an antibody complex and streptavidin link protein to deliver the antigen may obstruct engagement of PRR or BCR co-receptors by the antigen and, therefore, prevent concurrent activation events (Rawlings et al., 2012). However, as modified aggrecan/antibody complexes lead to similar levels of TCR-5/4E8-transgenic CD4⁺ T cell IFN- γ production as that detected with unmodified aggrecan, following co-culture with A20-Agg B cells, if these mechanisms are responsible for CD4⁺ T cell IFN- γ induction, they are probably unique to *ex vivo* CD43⁻ B cells. The dissociation kinetics of the antibody-antigen complexes may also play an important role in defining the time frame for BCR engagement and/or the subsequent signalling events required for detectable CD4⁺ T cell IFN- γ production.

As a substitute to the approaches described here, the use of plasmids containing lentivirus (LV) sequences could be utilised to deliver the BCR H and L-chain. These types of vectors have been shown previously to be highly effective when transfecting *ex vivo* lymphocyte and APC populations (Naldini et al., 1996; Vigna and Naldini, 2000). In addition, one recent report has shown these plasmids may also be used to successfully transfect unstimulated CD43⁻ B cells and induce functional reprogramming (Calderón-Gómez et al., 2011). Although experiments performed in our laboratory using plasmids containing a control cDNA in conjunction with those containing LV sequences have also resulted in significant numbers of transfected CD43⁻ B cells, experiments including plasmids containing BCR H and L chain cDNAs have yet to lead to BCR expression (Ramesh, Floudas and Knight, unpublished). However, even if this approach delivered high transfection efficiency, the expression of the transfected BCR would remain transient. Therefore, a significantly improved scenario would involve the use of a monoclonal population of *ex vivo* B cells. Such B cells can be obtained from BCR-transgenic mice which contain B cells which express a single BCR derived from

germline immunoglobulin genes inserted during development (Goodnow et al., 1988). Therefore, the development of a BCR-transgenic mouse in which the majority of B cells express an aggrecan-specific BCR would allow the isolation of aggrecan-specific *ex vivo* B cells. Such B cells would have more physiologically relevant responses to PRR and T cell-derived signals and would be amenable to culture in the presence of cytokines predicted to induce differentiation into putative effector and regulatory B cell subsets for further investigation. Alternatively, different aggrecan-specific pathogenic or regulatory B cell subsets, such as B1 cells and B_{reg}, could be isolated based on phenotypic markers and utilised in *ex vivo* antigen-presentation assays (Blair et al., 2010; Griffin and Rothstein, 2011; Moshkani et al., 2012; Zhong et al., 2007).

CD80 and CD86 are key co-stimulatory molecules expressed by APC that, through interactions with CD28 on T cells, enhance TCR stimulation and promote T cell survival and proliferation (Lanzavecchia and Sallusto, 2001). Based on evidence suggesting that dysregulation of CD80 and CD86 expression on APC may be responsible for autoimmune pathology observed in mice (Hussain and Delovitch, 2005; Takemura et al., 2001b), we sought to determine the requirement for these molecules on A20-Agg B cells or DC in order to activate TCR-5/4E8-transgenic CD4⁺ T cells and induce IFN- γ production. CD80 and CD86 were of specific interest as evidence suggests that differential modulation of CD4⁺ T cell differentiation into T_H1 and T_H2 subsets may be mediated by these molecules (Kuchroo et al., 1995; Schweitzer and Sharpe, 1998; Schweitzer et al., 1997). Furthermore, CD80/86 expression on B cells is critical for T cell activation in the PGIA model of autoimmune arthritis suggesting that the essential role of B cells in disease progression a result of antigen presentation to autoreactive T cells (O'Neill et al., 2005, 2007). Therefore, blockade of CD80 and CD86 on A20-Agg B cells or DC using mAb was used to determine any divergence in the requirement for these molecules when presenting aggrecan to the TCR-5/4E8-transgenic CD4⁺ T cells.

Figure 5.14 demonstrated that A20-Agg B cell co-cultures show a greater reduction in TCR-5/4E8-transgenic CD4⁺ T cell activation following incubation with anti-CD80 mAb in comparison to anti-CD86. This difference was not apparent in co-cultures containing DC which showed an equivalent reduction in TCR-5/4E8-transgenic CD4⁺ T cell activation following incubation with anti-CD80 or CD86 mAb (**Figure 5.15**). However, blockade of either molecule alone was not sufficient to abrogate T cell activation which required the presence of both anti-CD80 and anti-CD86 mAb. These results were shown to be independent of any non-specific effects associated with mAb. Strikingly, the importance of CD80 on A20-Agg B cells was mirrored when supernatants were screened for IFN- γ (**Figure 5.16**). However, similar to T cell activation, inclusion of both mAb

was required for complete abolition of IFN- γ production by both A20-Agg B cells and DC (**Figure 5.16**). This is in accordance with previous work indicating the essential role of CD80/86 on B cells in order to induce PGIA (O'Neill et al., 2007). These data also suggest that CD80 plays a more critical role in A20-Agg B cell activation of TCR-5/4E8-transgenic CD4⁺ T cells than CD86, in contrast to DC. This would not only underscore the critical requirement for the CD80/86-CD28 pathway in induction of T_H1 responses but also the importance of CD80 expression on A20-Agg B cells in order to achieve maximal IFN- γ production could be linked to early work showing a specific role for CD80 in the induction of T_H1 responses (Kuchroo et al., 1995; Lang et al., 2002). However, the blocking mAb approach used in this study has several limitations. The binding affinities of the anti-CD80 and CD86 may differ significantly resulting in variable levels of blockade with each mAb. Therefore, any conclusions derived from direct comparison of the effects of these mAb must be cautious and must be supported by further evidence using other methods. Furthermore, the maximum dose used in this study (10 μ g/mL) was not confirmed to be saturating for either CD80 or CD86 leaving the potential for further effects at different higher doses. This could also be addressed as part of future work by using an extended titration of mAb and demonstrating CD80/86 blockade of surface molecules with flow cytometry.

Results from **Chapter 4** demonstrated that expression of CD80 and CD86 was only marginally lower on A20-Agg B cells than DC and the ratio of CD80:CD86 expression (approximately 1:1) was comparable on both cell types (**Figure 4.3 & 4.4**). This suggests that any differential expression of these molecules prior to incorporation into antigen presentation assays does not explain A20-Agg B cell induction of greater TCR-5/4E8-transgenic CD4⁺ T cell IFN- γ production than DC. Furthermore, this also excludes the possibility that the drastic reduction in A20-Agg B cell activation of TCR-5/4E8-transgenic CD4⁺ T cells following CD80 blockade is due to a much lower initial expression of CD80 leading to enhanced sensitivity to blockade in comparison to CD86. However, it may be the case that the levels of CD80 and CD86 expressed on A20-Agg B cells and DC change during the course of the 72 h co-culture following stimulation with p84-103 or dAgg. Flow cytometric analysis of the APC populations during different phases of the culture period may reveal distinct changes in expression of either molecule on A20-Agg B cells and DC. The partitioning of molecules at the immune synapse can also influence the trajectory of T cell development (Yamanaka et al., 2013). Therefore, combining the flow cytometric approach with microscopy or high-throughput imaging techniques may reveal differences in the molecular composition of B-T cell synapses in comparison to DC which affects downstream cytokine production.

Although CD80 and CD86 show some functional redundancy, numerous studies have highlighted differential roles for CD80 and CD86 in APC-T cell interactions (Borriello et al., 1997; McAdam et al., 1998). For example, multiple reports have suggested that although CD80 and CD86 share qualitative properties, CD80 is a more potent inducer of T cell proliferation and IL-2 production than CD86 (Fields et al., 1998; Olsson et al., 1998). This is in alignment with the data obtained in **Figure 5.14** showing a more exaggerated reduction in IL-2 production in B cell co-cultures following addition of mAb targeting CD80 in comparison to CD86. In addition, immune suppression by T_{reg} has been shown to be enhanced following blockade of CD86 on allogeneic DC cultured with $CD4^+$ T cells while blockade of CD80 induced greater $CD4^+$ T cell activation through the inhibition of T_{reg} (Zheng et al., 2004). Although this particular study focused on DC, the suppressive role of CD86 in this scenario may shed light on the increased levels of IL-10 (**Figure 4.8**) and T_{reg} (**Figure 4.15**) observed in A20-Agg B cell and TCR-5/4E8-transgenic co-cultures stimulated with dAgg.

Differences in the role of CD80 and CD86 are not limited to $CD4^+$ T cell differentiation but can also affect the initial phases of the APC-T cell interaction. For example, blockade of CD86 results in a greater reduction in the interaction force between DC and T cells than blockade of CD80 (Lim et al., 2012). However, similar to the results observed in **Figures 5.14–5.18**, interactions could not be abolished by disruption of either molecule alone. Therefore, this study and others demonstrate that the strength and duration of APC-T cell contact appears to be governed in part by CD80/86 and can have profound consequences on T cell activation (Friedl et al., 2005; Gunzer et al., 2000; Mempel et al., 2004; Okada et al., 2005). Future experiments may expand on the findings presented in this chapter by combining the use of mAb with an approach based on visualising B cell and DC interactions with $CD4^+$ T cells. For example, fluorescence microscopy or ImageStream® technology would reveal any differences in the accumulation of CD80 or CD86 (or other co-stimulatory molecules) in the immune synapses formed by B cells or DC.

Previous reports have suggested that the monoclonal antibodies used for CD80/86 blocking in this study may have other effects on APC activation. For example, when utilising the same mAb used in this project, cross-linking of CD80 inhibits the growth proliferation of B cell lymphoma while cross-linking CD86 has the opposite effect (Suvas et al., 2002). However, isotype control antibodies had no significant effect on IFN- γ production (**Figures 5.14D & 5.15D**). Furthermore, the inhibition of A20-Agg proliferation following irradiation (**Figure 4.14**) had also had no effect on the induction of IFN- γ ⁺CD4⁺ T cells. It also remains to be seen whether the observed reduction in IFN- γ is due to induction of anergy, as demonstrated in previous reports, or deletion (Lang et al., 2002; Via et al., 1996).

Other co-stimulatory molecules expressed by APC or ligands expressed by the TCR-5/4E8-transgenic CD4⁺ T cells may complement signalling through CD80 or CD86 and be valid targets for further blocking experiments. For example, OX40 is expressed on activated T cells (Murata et al., 2000) and its ligand, OX40L, can be induced on CD40-activated B cells or DC (Croft et al., 2009; Ohshima et al., 1997). T cell stimulation through OX40 promotes effector T cell expansion and survival. In the absence of an on-going T_H1 response, OX40 signalling preferentially induces T_H2 differentiation through autocrine production of IL-4 (Linton et al., 2003). However, this can be overcome in the presence of IL-12 and antigen. Interestingly, OX40L behaves synergistically with CD80 to prolong naïve CD4⁺ T cell proliferation and IL-2 production upon encountering antigen (Gramaglia et al., 1998). Indeed, simultaneous ligation of OX40 and CD28 lowers the antigen dose required to induce CD4⁺ T cell IFN- γ production. This supportive mechanism could be active in the TCR-5/4E8-transgenic model system and experimental blockade may reveal a distinct requirement for OX40L on A20-Agg B cells or DC (Rogers and Croft, 2000). However, another potential role for OX40 in this system is the suppression of T_{reg} activity. The induction of T_{reg} and IL-10 production was detected in A20-Agg B cell and TCR-5/4E8-transgenic CD4⁺ T cell co-cultures following aggrecan stimulation (**Figures 4.8 & 4.15**). This suggests a limited role for OX40 in these cultures as it has been shown to strongly antagonise the induction of FoxP3⁺CD4⁺ T cells and the subsequent production of IL-10 (Ito et al., 2006; So and Croft, 2007). Importantly, a role for the OX40 pathway in autoimmunity, including RA, is supported by data showing that both constitutive expression of OX40L on T cells leads to organ-specific autoimmunity (Murata et al., 2002). Furthermore, blockade of OX40 can inhibit CD4⁺ T cell IFN- γ production in CIA and EAE making it a potential therapeutic target (Boot et al., 2005; Weinberg et al., 1999; Yoshioka et al., 2000).

Similarly, inducible T-cell co-stimulator (ICOS) is another member of the CD28-B7 family of co-stimulatory molecules (Coyle et al., 2000; Hutloff et al., 1999) and blockade of its ligand, B7 homologous protein (B7h) (Swallow et al., 1999), has been shown to ameliorate CIA. In particular, there was a reduction in the expression of inflammatory cytokines such as IL-6 in the synovium and secondary T cell proliferation responses and production of IFN- γ and IL-10 were significantly inhibited (Iwai et al., 2002). Although initially linked with primarily T_H2 responses (Dong et al., 2001; McAdam et al., 2001; Tafuri et al., 2001), ICOS has subsequently been associated with T_H1-mediated autoimmune diseases such as EAE making it a logical target of interest in PGIA (Rottman et al., 2001; Sporici et al., 2001). ICOS appears to play a significant role in the effluent phase of CD4⁺ T cell responses suggesting that this molecule may be better studied by observing secondary T cell responses *in vitro* or by blockade in PGIA mice, in contrast to the methods used in the present study.

In a contrasting role, CTLA-4 is expressed on T_{reg} and has recently been shown to function by physically extracting CD80/86 from the surface of APC in order to suppress activation of T cells following antigen presentation (Qureshi et al., 2011). Indeed, T_{reg} were found to be expanded in A20-Agg B cell co-cultures stimulated with dAgg (**Figure 4.15**) suggesting blockade of CTLA-4 could drastically enhance TCR-5/4E8-transgenic CD4⁺ T cell IFN- γ production by preventing the action of T_{reg}. The suppressive potential of CTLA-4 is supported by data from the multiple models of autoimmunity showing amelioration of disease following blockade of CD80/86 by soluble CTLA-4 (Arima et al., 1996; Finck et al., 1994; Lenschow et al., 1995; Webb et al., 1996). Further work has shown that an absence of CD28 also leads to resistance to CIA demonstrating the importance of the CD80/86-CD28 activation pathway (Tada et al., 1999). The therapeutic potential of inhibiting T cell activation using soluble CTLA-4 has been explored in RA patients with some success (Kremer et al., 2003). As a result, further study of the role of this molecule in the TCR-5/4E8-transgenic system reveal an integral role in the regulation of B cell-mediated CD4⁺ T cell activation.

Engagement of CD40 is a crucial step in B cell activation, differentiation and antigen presentation (Buhlmann et al., 1995; Clatza et al., 2003; Harnett, 2004; Ishida et al., 1995). Genetic variants in the CD40 signalling pathway have been associated with RA and additional evidence suggests that CD40-mediated activation of NF- κ B may be involved (Raychaudhuri et al., 2008; Xie et al., 2006). This is supported by animal models of arthritis and other autoimmune diseases. Blockade of CD154, the T cell ligand for CD40, prevents CD4⁺ T cell activation, synovial inflammation and B cell antibody production and, therefore, inhibits induction of CIA (Durie et al., 1993). The requirement for CD40-CD154 interactions was replicated in NOD mice where antibody blockade prevented priming of islet-specific CD4⁺ T cells and lead to a drastic reduction in IFN- γ independently of a decrease in IL-4 or a concomitant increase in regulatory cells (Balasa et al., 1997). Therefore, it seems probable that CD40-CD154 signalling is crucial for A20-Agg B cell induction of TCR-5/4E8-transgenic CD4⁺ T cells and blocking experiments using a range of anti-CD40 or anti-CD154 concentrations may reveal different levels of involvement for this pathway on B cells and DC.

In summary, aggrecan-mediated activation of the candidate PRR investigated in this chapter does not appear to be involved in A20-Agg B cell induction of TCR-5/4E8-transgenic CD4⁺ T cell IFN- γ production as aggrecan failed to activate PRR at concentrations equivalent to those used in antigen-presentation assays. However, A20-Agg B cell and DC activation of TCR-5/4E8-transgenic CD4⁺ T cells was shown to be dependent on CD80/86. Furthermore, blockade of CD80 or CD86 alone revealed a preferential requirement for CD80 over CD86 on A20-Agg B cells suggesting a critical role for this molecule in generating enhanced TCR-5/4E8-transgenic CD4⁺ T cell IFN- γ production in comparison to DC. Additional experiments investigating the role of other co-stimulatory molecules may reveal synergistic or antagonistic effects in combination with CD80/86. In conjunction with experiments using *ex vivo* B cells isolated from an C7.1 aggrecan-specific BCR-transgenic mouse (currently under construction), these experiments may further elucidate the unique features of B cell antigen presentation to CD4⁺ T cells and the generation of pathogenic and regulatory B and T cell subsets capable of modulating autoimmune diseases.

6. General Discussion

Genetic evidence has implicated antigen-specific interactions between APC and autoreactive T cells in RA pathology (Eyre et al., 2012; de Vries et al., 2002). Activated CD4⁺ T cells subsequently release inflammatory cytokines such as IFN- γ and IL-17 which contribute to both the early stages of RA and perpetuation of the inflammatory environment throughout disease (Lubberts et al., 2005; Raza et al., 2005; Schulze-Koops and Kalden, 2001). Several lines of evidence link the candidate autoantigen aggrecan to this process including the presence of aggrecan-specific antibodies and aggrecan-specific CD4⁺ T cells in RA patients (Vynios et al., 2006; Zou et al., 2003). The generation of autoimmune responses to aggrecan is probably facilitated by the inflammatory environment in arthritic joints which upregulates cartilage degradation and leads to the release of aggrecan cleavage products into the synovial fluid (Poole et al., 1994).

The hypothesis that B cells present such autoantigens and drive pathogenic T cell activation is supported by the success of anti-CD20 B cell depletion therapy in the treatment of patients refractory to other biological therapies such as anti-TNF α monoclonal antibodies (Edwards and Cambridge, 2006). Anti-CD20 monoclonal antibodies do not specifically target long-lived antibody-producing plasma cells (Edwards et al., 2004). Therefore, studies in both patients and mouse models have suggested that the therapeutic effect of B cell depletion may be specifically derived from the negation of B cell antigen presentation (O'Neill et al., 2005, 2007; Townsend et al., 2010). When this is considered alongside circumstantial evidence, such as the presence of ectopic lymphoid tissue in the synovium and B-T cell aggregates in the bone marrow, there is a strong case for the role of B cells as APC in RA (Jimenez-Boj et al., 2005; Weyand and Goronzy, 2003). However, it remains unknown why B cells are particularly critical when other APC, such as DC and macrophages, are also present in the arthritic joint.

This project aimed to compare the ability of aggrecan-specific B cells to present aggrecan to CD4⁺ T cells in comparison to other APC. The TCR-5/4E8-transgenic system was used to obtain a CD4⁺ T cell population specific for the immunodominant arthritogenic epitope of aggrecan. Inflammatory cytokines derived from effector CD4⁺ T cells are important inflammatory agents responsible for activating infiltrating leukocytes and local synoviocytes leading to cartilage and bone destruction in RA (Firestein, 2004). Importantly, *ex vivo* TCR-5/4E8-transgenic CD4⁺ T cells are capable of differentiation into such cytokine-producing effector subsets. Therefore, this project focused on differential production of key pathogenic cytokines such as IFN- γ by TCR-5/4E8-transgenic CD4⁺ T cells following antigen presentation by aggrecan-specific B cells or other APC.

The development of a system for comparing APC required a thorough characterisation of the TCR-5/4E8-transgenic mouse from which aggrecan-specific CD4⁺ T cells were to be derived. Therefore, the presence of the aggrecan-specific VB4⁺CD4⁺ T cell population in TCR-5/4E8-transgenic splenocytes was confirmed by flow cytometry and shown to express characteristics typical of naïve CD4⁺ T cells. Following incubation with a synthetic version of the p84-103 aggrecan peptide, these splenocytes demonstrated robust proliferation responses and the VB4⁺CD4⁺ T cell population was shown to upregulate activation markers. The activation kinetics of TCR-5/4E8-transgenic splenocytes were also investigated to establish the conditions for assays comparing activation of CD4⁺ T cells by various APC. The optimum time point for measurement of proliferation was shown to be approximately 72 h for CD4⁺ T cell numbers ranging from 1x10⁴ to 5x10⁴ per well. Importantly, a novel two-step magnetic cell separation method was used to consistently isolate TCR-5/4E8-transgenic CD4⁺ T cells in the absence of contaminating APC. MHC class II⁺ cells were depleted prior to the subsequent positive selection of CD4⁺ cells. This resulted in the isolation of a high purity, viable CD4⁺ T cell population which did not respond to antigen stimulation without further reconstitution with APC. Taken together, this work expanded on previous attempts to characterise the TCR-5/4E8-transgenic mouse and established a robust method for isolating CD4⁺ T cells from a splenocyte preparation to a level of purity necessary for further experiments comparing presentation of aggrecan by different APC populations.

Several *ex vivo* APC populations were cultured and the efficiency of aggrecan presentation studied using T cell activation assays. It was determined that DC were the optimal cell type for comparison to the aggrecan-specific A20-Agg B cell line. These experiments confirmed previous work from our laboratory using the aggrecan-specific T cell hybridoma 192 by demonstrating that A20-Agg B cells were equally capable of activating CD4⁺ T cells as DC (Wilson et al., 2012). In contrast to this similarity, it was also revealed that A20-Agg B cells and DC induced differential CD4⁺ T cell cytokine production. Of these differences, the most remarkable was the discovery that A20-Agg B cells induced significantly more IFN- γ production than DC when stimulated with aggrecan. This has profound consequences as IFN- γ is a key pathogenic cytokine in the TCR-5/4E8-transgenic PGIA model of RA and is also thought to play a significant role in the human disease (Doodes et al., 2008, 2009). Further experiments demonstrated that dAgg presentation by A20-Agg B cells induced the differentiation of TCR-5/4E8-transgenic CD4⁺ T cells into IFN- γ ⁺ T_H1 cells with no evidence suggesting this was via an IL-17-secreting subset. High levels of IL-10 and T_{reg} were also found in A20-Agg B cell co-cultures highlighting the dichotomy between pathogenic and regulatory modulation of CD4⁺ T cell cytokine production by B cells. To explore this further the scope of this

project could be expanded in the future to isolate these T_{reg} and determine whether they are long-lived and capable of functional inhibition *in vitro* and *in vivo*. In addition, the role of inflammatory cytokines such as $TNF-\alpha$ in inhibiting the action of these cells could also be explored by *in vitro* culture within a cytokine milieu similar to that found in the arthritic joint (Nadkarni et al., 2007). Other pathogenic $CD4^+$ T cell subsets besides T_H1 cells have been strongly implicated in other animal models (Brennan and McInnes, 2008). Therefore, future work could take the opportunity to differentiate TCR-5/4E8-transgenic $CD4^+$ T cells into various effector subsets and observe the ability of A20-Agg B cells to alter their phenotype and cytokine production following aggrecan presentation. However, the work presented here provides a solid foundation from which to study the effects of adoptive transfer of $CD4^+$ T cells activated by B cells into the PGIA animal model and to determine whether these results translate into other systems using alternative antigens such as CIA.

The role of aggrecan as a PRR ligand was explored as a potential mechanism for mediating the differences seen between B cell and DC induction of TCR-5/4E8-transgenic $CD4^+$ T cell cytokine production. For this purpose, a detection assay was used consisting of cell lines transfected with candidate PRR, selected on evidence for their role in RA. Although aggrecan was seen to induce TLR2 and TLR4 activation at high doses, this was not the case at the lower doses previously shown to induce significant TCR-5/4E8-transgenic $CD4^+$ T cell $IFN-\gamma$ induction. Furthermore, TLR9 and NOD2 were not activated by aggrecan even when aggrecan uptake into HEK cells was facilitated by transfection with an aggrecan-specific BCR.

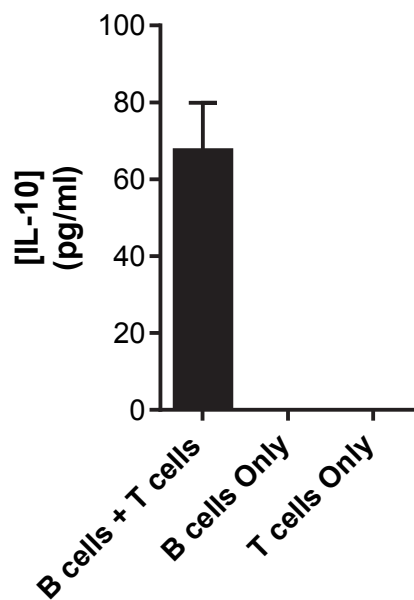
Having excluded a role for these PRR, further experiments focused on the generation of an aggrecan-specific *ex vivo* B cell population. These cells were considered a more physiologically representative population with which to test the hypothesis that B cell cytokine production or co-stimulatory molecule expression may be responsible for differential induction of $CD4^+$ T cell $IFN-\gamma$. However, electroporation transfection previously shown to be effective using the A20 B cell line (Wilson et al., 2012) failed to generate *ex vivo* B cells transiently expressing the aggrecan-specific BCR H and L-chains. An aggrecan-specific BCR-transgenic mouse is currently under development which will bypass the requirement for transfection as the majority of the B cell population will express a single aggrecan-specific BCR. However, this tool was not available at during this project. Instead, a novel method of antibody-mediated antigen-loading was developed. A biotinylated anti-mouse IgG monoclonal antibody was used as an intermediary to bind streptavidin-linked aggrecan to the BCR of *ex vivo* B cells. Remarkably, this method successfully improved B cell aggrecan uptake and was sufficient to induce activation of both T cell hybridoma 192 and TCR-5/4E8-transgenic $CD4^+$ T cells. This suggests that the antigen-loading method could be adapted for

targeting any streptavidin-linked antigen to non-specific B cells making it a potentially useful tool in other *in vitro* experiments using alternative antigens. However, this assay failed to replicate the TCR-5/4E8-transgenic IFN- γ production seen with A20-Agg B cells. It was therefore hypothesised that A20-Agg B cells possess unique properties which facilitate this induction of IFN- γ production. In particular, the CD80/86 co-stimulatory molecules were thought to play a role in this observation. Therefore, blocking monoclonal antibodies were used to individually or simultaneously interfere with A20-Agg B cell or DC co-stimulation of TCR-5/4E8-transgenic CD4⁺ T cells. Intriguingly, CD80 monoclonal antibodies were shown to be a more potent inhibitor of T cell activation in A20-Agg B cell rather than DC co-cultures. Therefore, CD80 plays a critical role in mediating A20-Agg B cell antigen presentation and induction of CD4⁺ T cell cytokine production. This suggests that CD80 may be a promising target for future RA therapies. This is particularly interesting as the most successful T cell-directed therapies have targeted co-stimulation through addition of a CTLA-4-immunoglobulin Fc fusion protein (Genovese et al., 2005). Additional experiments blocking other co-stimulatory molecules in the TCR-5/4E8-transgenic system may reveal other differences between B cell and DC antigen presentation and highlight more therapeutic opportunities.

Taken together, these results suggest that future work would be aided by the development of an aggrecan-specific BCR-transgenic mouse which would obviate the need to transfect *ex vivo* B cells. Putative effector and regulatory B cell subsets could then be isolated or generated using *in vitro* methods. Aggrecan presentation to CD4⁺ T cells by these B cell subsets in comparison to other APC could be investigated in order to assess differences in T cell differentiation. If significant differences were observed, it would then be possible to both assess their potential relevance to disease by adoptive transfer of B and T cell subsets from antigen-presentation assays into the PGIA model of arthritis and observation of disease progression. The role of PRR ligands could also be explored further by documenting the effects of spiking antigen-presentation assays with individual PRR ligands or patient synovial fluid shown to contain a milieu of ligands. As different APC subsets express variable levels and combinations of PRR, any differences in APC activation and CD4⁺ T cell differentiation in these cultures may yield new insights into the importance of PRR ligands in RA in activating APC and CD4⁺ T cells. Similarly, the role of co-stimulatory molecules, including CD80/86 and other candidates, in aggrecan presentation to CD4⁺ T cells by B cells and DC could be elucidated further by a combination of techniques, especially visualisation of differences in the localisation and interactions of co-stimulatory molecules in the APC-T cell immune synapse and the effect of functional inhibition of co-stimulatory molecule expression using blocking antibodies or genetic modification.

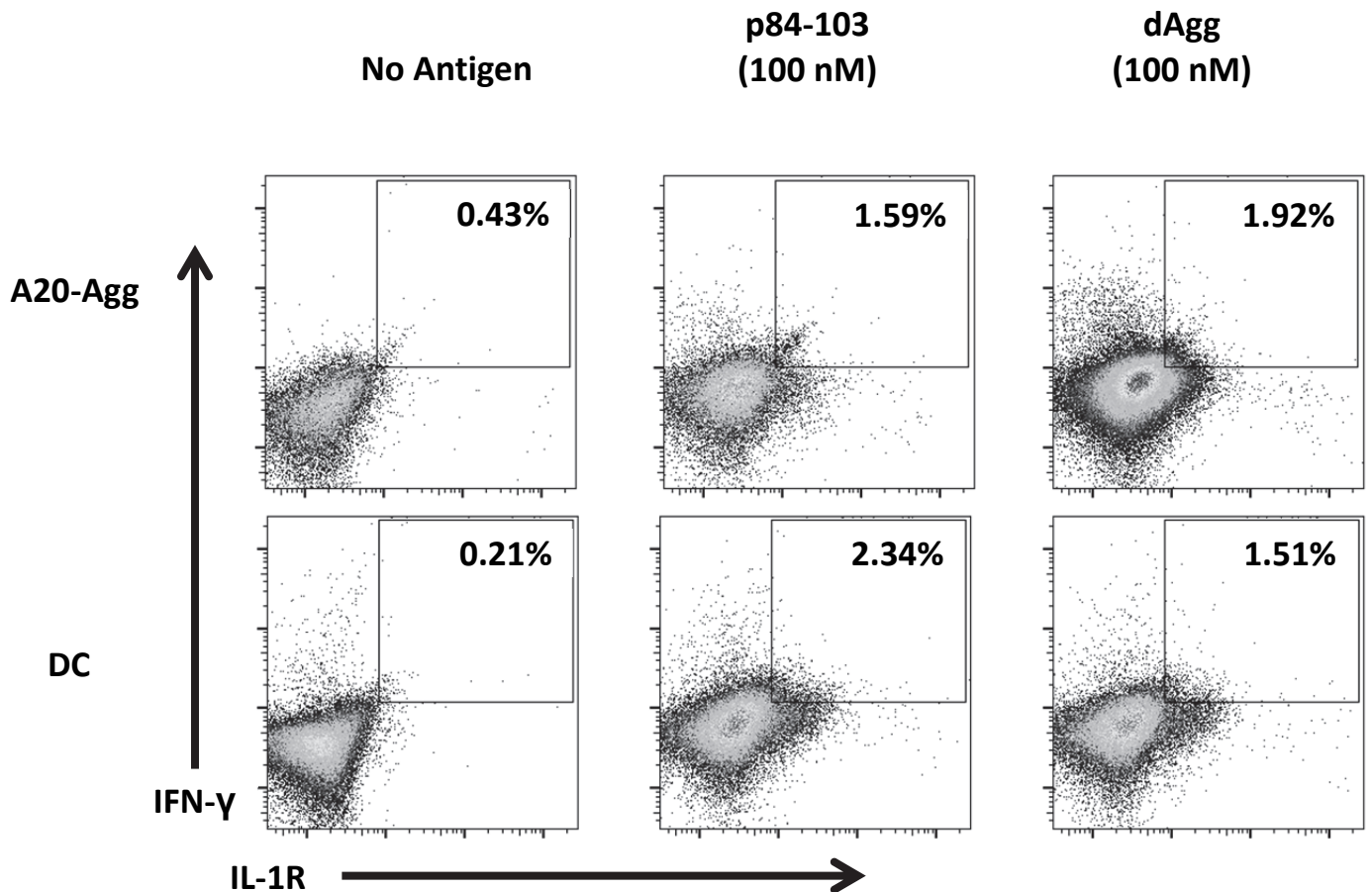
In conclusion, using a TCR-transgenic mouse model of RA, this project has demonstrated an increased efficiency in the induction of CD4⁺ T cell IFN- γ production following aggrecan presentation by aggrecan-specific B cells compared to that seen following presentation by DC. An *in vitro* detection assay has discounted the ability of aggrecan to bind several PRR at assay concentrations and, therefore, modulate APC function. However, CD80 and CD86 blockade using monoclonal antibodies demonstrated a greater requirement for CD80 on B cells in comparison to DC in order to induce maximal CD4⁺ T cell activation and cytokine production.

Appendices



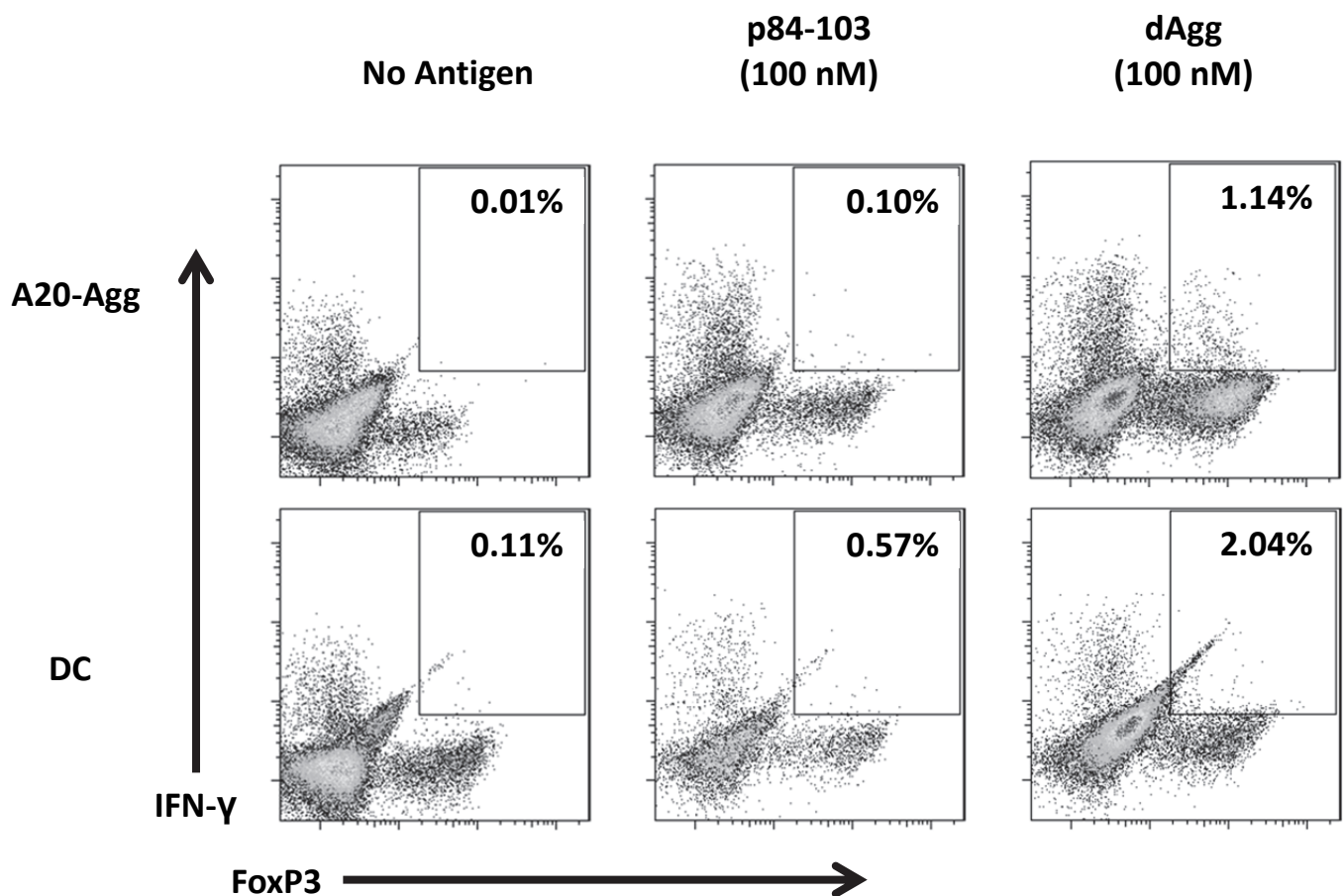
Appendix 1 Cytokine production by A20-Agg cultured alone or in the presence of TCR-5/4E8-transgenic CD4⁺ T cells.

3×10^5 A20-Agg B cells were cultured with or without 1.5×10^5 TCR-5/4E8-transgenic CD4⁺ T cells for 72 h in a flat-bottomed 96-well plate. Supernatants were tested for IL-10 by ELISA. Data were pooled from two independent experiments. Each data point represents a duplicate with error bars showing $\pm$ SEM.



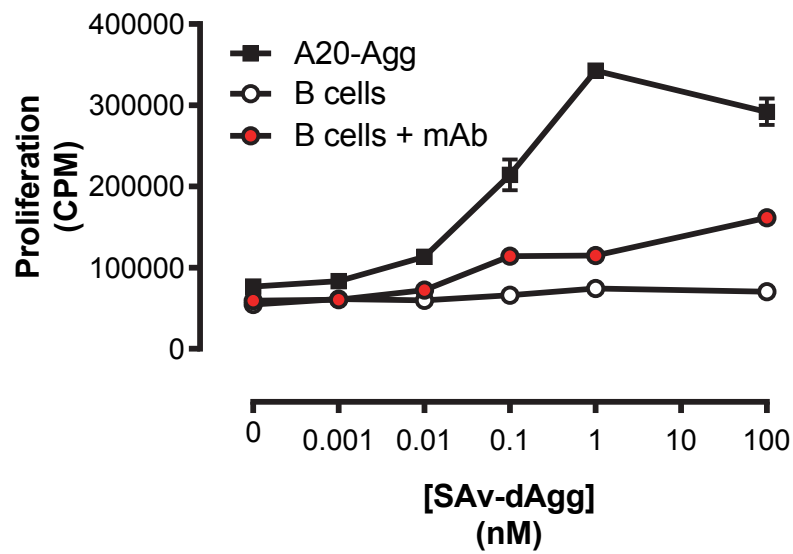
Appendix 2 Flow cytometric analysis of intracellular IFN- γ and surface IL-1R expression by TCR-5/4E8-transgenic CD4⁺ T cells following co-culture with A20-Agg B cells or DC in the presence of 100 nM p84-103 or aggrecan.

1×10^6 APC and 5×10^5 CD4⁺ T cells were added to each well and cultured for 72 h in a 24-well plate. Co-cultures were then re-stimulated with PMA/ionomycin for the final 5 h of culture. Numbers represent IFN- γ IL-1R⁺ cells as a percentage of the total CD4⁺ population.



Appendix 3 Flow cytometric analysis of intracellular IFN- γ and FoxP3 expression by TCR-5/4E8-transgenic CD4⁺ T cells following co-culture with A20-Agg B cells or DC in the presence of 100 nM p84-103 or aggrecan.

1×10^6 APC and 5×10^5 CD4⁺ T cells were added to each well and cultured for 72 h in a 24-well plate. Co-cultures were then re-stimulated with PMA/ionomycin for the final 5 h of culture. Numbers represent IFN- γ ⁺ FoxP3⁺ cells as a percentage of the total CD4⁺ population.



Appendix 4 *IL-2 production following antibody-mediated loading of aggrecan into CD43⁻ B cells and co-culture with TCR-5/4E8-transgenic CD4⁺ T cells.*

3×10^4 APC were incubated with or without 5 $\mu\text{g/mL}$ anti-mouse BCR-biotin and transferred to a flat-bottomed 96-well plate containing graded doses of SAv-dAgg. 1.5×10^4 CD4⁺ T cells were added to each well and cultured for 72 h. Supernatants were extracted and added to 3×10^4 CTLL-2. Cultures were pulsed with radioactive thymidine (^3H) and DNA harvested onto a glass fibre mesh 18 h later. ^3H incorporation was measured using a scintillation counter and counts per minute (CPM) recorded. Each data point represents a triplicate with error bars showing $\pm\text{SEM}$.

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