Processing and presentation of the rheumatoid arthritis candidate autoantigen aggrecan, by antigen-specific B cells

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A thesis submitted for the degree of Doctor of Philosophy

Institute of Cellular Medicine

Newcastle University

September 2010

Abstract

The proteoglycan aggrecan, which is a major structural component of cartilage, has been identified as a candidate autoantigen in rheumatoid arthritis (RA). This is principally due to its degradation early in the disease pathology, its ability to induce an RA-like disease in mouse models and the presence of elevated numbers of reactive T and B cells in RA patients. Studies have also defined an essential requirement for autoantigen-specific B cells as antigen presenting cells (APC) in RA although the cellular mechanisms involved in antigen processing and presentation of joint-derived autoantigens by B cells remains unknown.

To investigate the role of autoreactive B cells as APC in RA, I have used two complimentary approaches to generate B cells expressing an aggrecan-specific B cell receptor (BCR). The first was based on a modified monoclonal antibody production protocol and the second involved the transfection of B lymphoma cells with newly generated plasmids encoding an aggrecan-specific BCR.

Using the second approach, I have successfully generated aggrecan-specific B cell lines (C71-4C5 and C71-5F10). I have shown that these B cells specifically bind aggrecan leading to efficient processing and the generation of the immunogenic T cell epitope 84-103 that is recognised by both aggrecan-specific T cell hybridomas and CD4+ T cells isolated from an aggrecan-specific TCR transgenic mouse. The aggrecan-specific B cells are able to present aggrecan at least 10^4 fold more efficiently than non-specific B cells, 10^2 fold more efficiently than macrophages and comparable to that seen by dendritic cells.

By using a panel of inhibitors, I have also shown that the generation of the 84-103/MHC complex by aggrecan-specific B cells requires an acidic environment, proteolysis by aspartic, serine and metalloproteinases and the “classical” pathway of MHC class II biosynthesis.

During this PhD, I have highlighted a novel role for aggrecan-specific B cells as important APC involved in aggrecan-presentation, as well as elucidating a role for metalloproteinases in aggrecan processing and presentation by APC.

Acknowledgements

I would like to thank my supervisor and mentor John for all of his support, encouragement, boundless knowledge and friendship. To Andy for his endless B cell enthusiasm and helpful criticism which has helped me to develop this thesis. To James, Jane and the entire musculoskeletal research group for extending their help, support and advice. I would also like to say a special thanks to Derek and Jelena for their support during the writing of this thesis and for showing me that research really is the best job ever!

I must give heartfelt thanks to my fantastic family for supporting and encouraging me throughout all of this PhD thankyou mam, dad, John and Anne.

To my boys Andrew and James whom I love with all my heart, who make every day special and who always make me smile no matter what is thrown at me.

And finally I would like to dedicate my thesis to Claire and thank her for her unbelievably endless support, patience, love and encouragement.

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Abbreviations

APC	-antigen presenting cell
ADAMTS	-A disintegrin and metalloproteinase with thrombospondin motifs
ADCC	-antibody-dependent cell-mediated cytotoxicity
AEP	-asparagine endopeptidase
APRIL	-a proliferation-inducing ligand
BAFF	-B cell activating factor
BCA	-bicinchonic acid
BCR	-B cell receptor
BLyS	-B lymphocyte stimulator
BSA	bovine serum albumin
BMDC	-bone marrow derived dendritic cells
BMM	-bone marrow derived macrophages
BMM	-bone marrow macrophage medium
Cat	-cathepsin
CBC	-comparative biology centre
CCP	-cyclic citrullinated peptide
cDNA	-copy DNA
CH	-constant region of heavy chain immunoglobulin
CIA	-collagen-induced arthritis
CL	-constant region of light chain immunoglobulin
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
DCI	-3, 4-dichloroisocoumarin
DNA	-deoxyribonucleic acid
DMB	-dimethylmethylene blue
DMSO	-dimethyl sulfoxide
DTT	-dithiothreitol
ECM	-extracellular matrix
EDTA	-ethylenediaminetetraacetic acid
ER	-endoplasmic reticulum
ELISA	-enzyme linked immunosorbance assay
FACS	-fluorescence activated cell sorting
FBS	-foetal bovine serum
Fab	-fragment antigen binding
Fc	-fragment crystallizable
FITC	-fluorescein isothiocyanate
FoxP3	-forkhead box P3
FLS	-fibroblast-like synovial cells
FSC	-forward scatter
GAG	-glycosaminoglycan
GC	-germinal centre

GM-CSF	-granulocyte macrophage colony stimulating factor
GPI	-glucose phosphate isomerise
H	-heavy chain of immunoglobulin
HA	-hyaluronan
HAT	-hypoxanthine aminopterin thymidine
HBSS	-Hanks balanced salt solution
HEL	-hen egg lysosyme
HRP	-horse radish peroxidase
IFA	-incomplete freunds adjuvant
IFNγ	-interferon gamma
Ig	-immunoglobulin
IL	-interleukin
ITAM	-immunoreceptor tyrosine-based activation motif
ITIM	- immunoreceptor tyrosine-based inhibitory motif
Ip	-immunoprecipitation
kDa	-kilo Daltons
KS	-keratan sulphate
L	-light chain of immunoglobulin
li	-invariant chain
LPS	-lipopolysaccharide
MACS	-magnetic activated cell sorting
M-CSF	-macrophage colony stimulating factor
MHC	-major histocompatability complex
MFI	-mean fluorescence intensity
MMP	-matrix metalloproteinase
mRNA	-messenger RNA
PBS	-phosphate buffered saline
PBMC	-peripheral blood mononuclear cell
PE	-phycoerythrin
PEG	-polyethylene glycol
PerCP	-peridinin-chlorophyll proteins
PFA	-paraformaldehyde
PGIA	-proteoglycan induced arthritis
Ph	-percentage hydrogen
RA	-rheumatoid arthritis
RF	-rheumatoid factor
RNA	-ribonucleic acid
RPMI	-Roswell park memorial institute medium
SDS	-sodium dodecyl sulphate
SSC	-side scatter
TIMP	-tissue inhibitor of metalloproteinase
TLR	-toll-like receptor
TNF	-tumour necrosis factor
TTCF	-tetanus toxin c fragment
VH	-variable region of heavy chain immunoglobulin
VL	-variable region of light chain immunoglobulin

1. Introduction

1.1 Rheumatoid arthritis.

Rheumatoid arthritis (RA) is a systemic autoimmune disease affecting approximately 1% of the population and causing significant morbidity and mortality (Lee and Weinblatt 2001). Incidence increases with age and affects 3 times more women than men. (Rooney and Silman 1999). The clinical presentation of the disease varies between patients but common symptoms include joint pain and swelling predominantly in the metacarpophalangeal, proximal interphalangeal and wrist joints and accompanying feelings of malaise and fatigue (Joseph, Brasington et al.). The swelling and pain experienced in RA joints are the results of the synovitis caused by an infiltration of immune cells such as neutrophils, macrophages, B cells and T cells into the joint lining (synovium) (Firestein 2003). Local production of inflammatory mediators such as TNFα, IL-1, IL-6, IL-23 and IL-2, tissue degrading enzymes such as metalloproteinases, immune complex deposition and angiogenesis leads to hyperplasia of the synovium (pannus formation), cartilage destruction and eventually erosion of the underlying bone (**see Figure 1.1**) (Brennan and McInnes 2008). Although it is predominantly the joints which are affected by the disease, patients often develop extra-articular manifestations including the formation of rheumatoid nodules, vasculitis, anaemia and problems in other organs such as the lungs, eyes and heart (Turesson, Jacobsson et al. 1999). Once severe damage to the joints has occurred it is, in many cases irreversible. Therefore, most treatment is aimed at dealing with inflammation and preventing further damage to the joint.

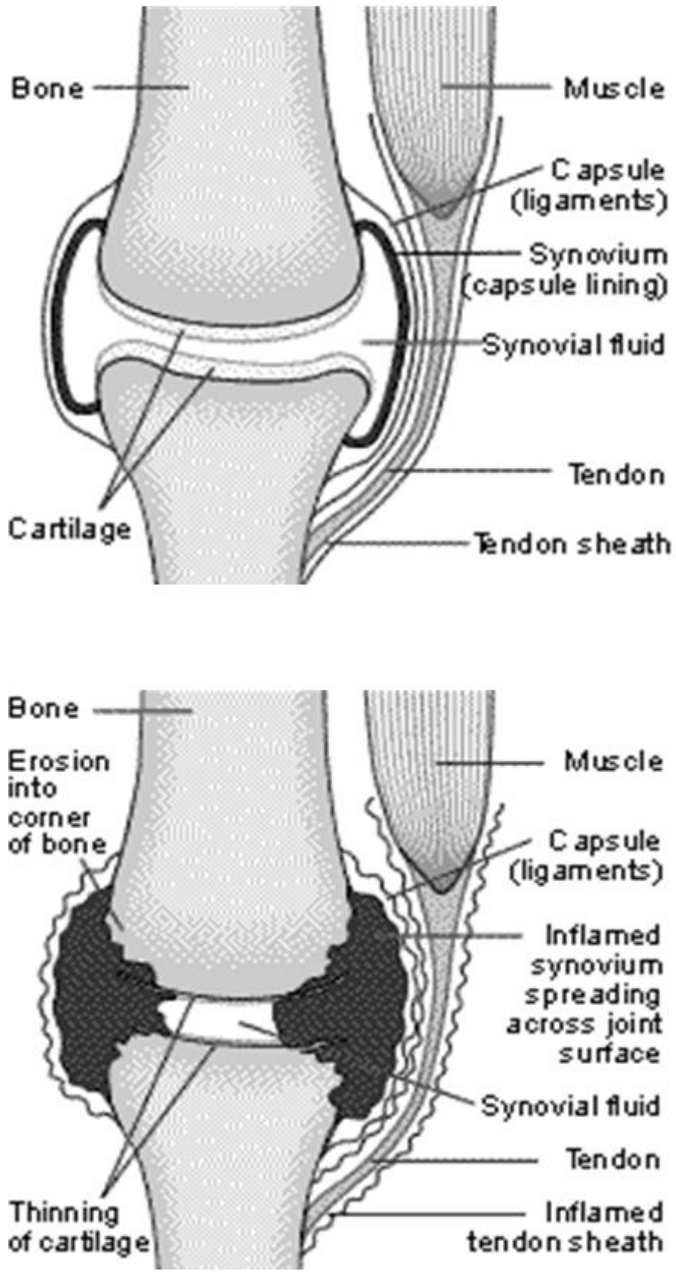

Figure 1.1 A schematic of a healthy joint (upper) and an arthritic joint (lower).
The synovium forms a thin layer encapsulating the healthy joint with no inflammation depicted in the upper picture. In the arthritic joint the synovium becomes infiltrated with immune cells, inflamed and hyperplastic encroaching on the articular cartilage and underlying bone depicted in the lower panel.
http://www.arc.org.uk/arthinfo/patpubs/6033/6033.asp

The cause of rheumatoid arthritis is not fully understood and may vary from patient to patient due to various genetic, environmental, hormonal and infectious factors.

Early findings identified that many patients with RA were seropositive for the anti-IgG autoantibodies known as rheumatoid factor (RF), leading to the view that RA is an autoimmune disease caused by autoantibodies (Franklin, Holman et al. 1957; Zvaifler 1973). It was later discovered that although RF had the potential to bind to immune complexes within the joint and potentiate inflammation it was not necessarily a marker of disease as it is also present in the serum of healthy individuals and patients with other chronic inflammatory diseases (Dorner, Egerer et al. 2004). More recently antibodies reactive against citrulline-containing proteins have been identified in the inflamed joints of RA patients. These anti-cyclic citrullinated peptide antibodies (anti-CCP) appear early on in the disease process; furthermore, they are rarely found in healthy individuals and their presence is associated with a more severe disease prognosis (van Gaalen, Ioan-Facsinay et al. 2005). The discovery of autoantibodies in RA initiated unsuccessful attempts at preventing antibody production or removing antibodies from the circulation of patients (Goronzy and Weyand 2003). However, initial studies involving the use of the anti-CD20 therapy Rituximab to deplete B cells resulted in a 70% improvement in RA disease activity (Edwards and Cambridge 2001). The role of B cells in RA is multifunctional and not limited to antibody production which may provide an explanation for the success of a B cell depletion therapy as opposed to antibody removal. Antigen-specific B cells are very efficient antigen presenting cells (APC) able to concentrate low doses of antigen generally sequestered from the immune system for presentation to autoreactive T cells (Mamula and Janeway 1993). In addition, B cells are found in abundance in the rheumatoid synovium often forming germinal centre like aggregates with T cells, implying a role for B cells in antigen-mediated T cell activation during RA pathogenesis (Weyand, Goronzy et al. 2000).

Current therapy has come a long way from the early treatment of patients with gold salts, non steroidal anti-inflammatory drugs (NSAID) and anti-malarial drugs such as chloroquine (Isaacs 2010). Disease modifying anti-rheumatic drugs (DMARDS) such as Methotrexate are also used which inhibit immune cell proliferation and induce apoptosis, inhibit pro-inflammatory cytokines and indirectly incurs effects on joint

degradatitve enzymes (Cutolo, Sulli et al. 2001). Therapies aimed at targeting pro-inflammatory cytokines such as TNFα (Etanercept, Infliximab and Adalimumab), IL-6 (Tocilizumab) and IL-1 (Anakinra) work best in combination with methotrexate (Buch; Senolt, Vencovsky et al. 2009). T and B cell directed therapies such as Rituximab and Abatacept (Orencia, Bristol Myers-Squibb) have both been relatively successful where other biological therapies such as anti-TNFα have failed, details of which are discussed in **Sections 1.3.3** and **1.4.1**. However, as yet there is no gold standard treatment for some patients and no known cure for RA. Therefore, identifying early disease markers and novel targets for RA therapy is an ongoing process.

1.2 The synovial joint

The synovial joints (diathroidal joints) are the most moveable joints in the body. They differ from the cartilaginous and fibrous joints which do not have a cavity (O'Rahilly 1957). The structure of the synovial joint is formed at the extremities of 2 spongy cancellous bones. The surfaces of the bones are coated in a layer of denser subchondral bone which provides support for the overlying articular cartilage (Li, Marshall et al. 1999).

1.2.1 Articular cartilage

Articular cartilage is of the hyaline variety, it forms a coating over the subchondral bone and its elasticity and smooth surface helps to support skeletal shape and aid freedom of movement and the loadbearing forces of the joint. Articular cartilage is avascular and is made up of extracellular matrix synthesised and maintained by a sparsely distributed homogeneous population of cartilage cells called chondrocytes. 95% of the cartilage extracellular matrix consists of the components collagen II (60%) and the major proteoglycan aggrecan (35%) (Dudhia 2005). Type II collagen provides tensile strength and a mesh network to immobilise structural proteins while the highly negative charge of aggrecan allows cartilage to have a high water content which contributes to its compressive resilience during joint loading (Dudhia 2005).

The cartilage receives nutrients from the neighbouring bone and the synovial membrane which lines the joint capsule (Gray 1918). During the progression of RA, cartilage destruction is mediated by inflammatory cytokines such as IL-1 and TNFα and cartilage degrading components such as matrix metalloproteinases (MMPs) and cathepsins which can mediate both the initiation and maintenance of inflammation and extracellular matrix (ECM) breakdown (Choy and Panayi 2001). These factors are released within the cartilage by activated chondrocytes and invading synoviocytes and within the inflamed synovium and surrounding synovial fluid by activated synoviocytes and infiltrating immune cells (Yoshihara, Nakamura et al. 2000; Pope 2002; McInnes and Schett 2007; Otero and Goldring 2007) (**Figure 1.2**)

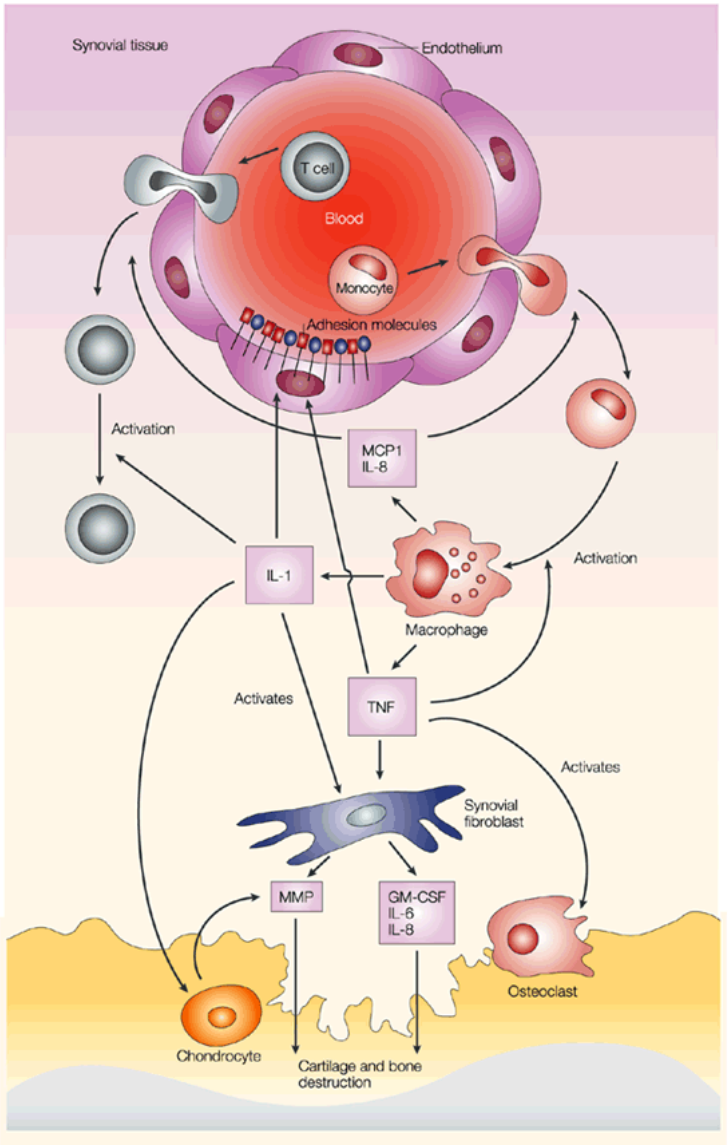

Figure 1.2 Overview of RA pathogenesis mediated by monocytes, synovial fibroblasts and activated chondrocytes. Extracted from (Pope 2002)

The extremities of the 2 bones comprising the joint are enclosed in a capsule which provides the joint with stability by limiting movement of the bones. The joint capsule consists of a fibrous outer layer which attaches to the bone and is itself lined by a softer internal layer termed the synovium (Ralphs and Benjamin 1994).

1.2.2 Synovium

The synovium is a bilayer composed of an outer layer of fat cells, blood vessels and fibroblasts and a lining layer consisting of loose connective tissue interspersed with blood vessels and a layer of 2 different types of synoviocytes 1-2 cells thick. Type a synoviocytes are macrophage like cells and type b synoviocytes are fibroblast like

cells (Lories, Derese et al. 2003; FitzGerald 2007). The lining layer of the synovium is the initiating site for RA characterized by increased vasculature, hyperplasia and an infiltration of immune cells and mesenchymal cells (Kraan, Versendaal et al. 1998; Marinova-Mutafchieva, Taylor et al. 2000; Tak and Bresnihan 2000). As the volume of the synovium increases (pannus formation) it begins to encroach on the cartilage and the pannus residing cells invade and destroy it both directly and by activating chondrocyte mediated cartilage destruction (Otero and Goldring 2007).

The synovium secretes fluid into the joint space which provides nutrients and lubrication to the avascular articular cartilage. Normal synovial fluid consists of plasma derived from synovial vessels, a few cells infiltrating from the neighbouring cartilage and synovium, and hyaluronate which gives the synovial fluid its viscosity (Freemont 1996). During RA progression both the volume and contents of the synovial fluid increases. Synovial fluid from RA patients is highly inflammatory and has been shown to contain high levels of the inflammatory mediators IL-1, IL-6, TNFα, IL-8, IL-17 and IL-21 (Brennan and McInnes 2008). In addition, the fluid contains a large influx of inflammatory cells which are predominantly neutrophils but also contains lymphocytes, granulocytes and monocytes (Harris 1990). However, variability between the cellular composition of the arthritic synovial fluid can occur in individual patients probably due to the nature of the initiating inflammatory stimulus and the individuals immune response to the causative agent (Davis, Denton et al. 1988; Freemont 1991).

1. 3 The role of T cells in RA

The role of T cells in rheumatoid arthritis has been reviewed (Lundy, Sarkar et al. 2007; Isaacs 2008; Mauri and Carter 2009). The discovery of genetic links between T cell activation and disease susceptibility have highlighted T cells as playing a central role in the disease pathogenesis.

Earlier studies involving the analysis of synovial tissues and fluid revealed the prominence of T cell infiltrates in the RA synovium and their accumulation in synovial fluid (Van Boxel and Paget 1975; Iannone, Corrigall et al. 1994). However, low levels of the Th1 cytokines IFNγ, IL-2 and IL-3 in synovial fluid and tissue suggested that T cells were not activating macrophages via IFNγ and IL-2 (Firestein and Zvaifler 1987; Firestein, Xu et al. 1988). This led to the dogma that the T cell infiltrate found in the joints of RA patients was not responsible for disease pathogenesis. However, more recently several animal models of RA have identified an essential role for T cells in the development of arthritis in RA disease models. Additionally, genetic linkage with T cell dependent factors discussed below in **Section 1.3.1** and the efficacy of RA drugs targeting T cell activation have provoked a new interest in their role as central mediators in disease pathogenesis. One possible explanation for the finding that there are low levels of IFNγ yet a prominent infiltrate of T cells in the synovium is that synovial T cells are activating macrophages independently of IFNγ. Indeed, it has been previously shown that the cytokine IL-15 which is found at increased levels in the RA synovium can activate synovial T cells to produce the RA associated cytokine TNFα. More importantly, these IL-15 activated T cells are able to induce significant TNFα by macrophages in a cell contact dependent manner which is mediated by the cell surface molecules LFA-1, ICAM1 and CD69 (McInnes, Leung et al. 1997).

1.3.1 Genetic evidence for T cell involvement

The human leukocyte antigen (HLA) DP, DQ and DR *loci* and their murine equivalent I-A and I-E encode the highly polymorphic αβ major histocompatibility complex (MHC) heterodimers found on the surfaces of antigen presenting cells (Al-Daccak, Mooney et al. 2004). A genetic association with a conserved amino acid sequence known as the 'shared epitope' from within the third hypervariable region of MHC DRβ chains is found in 90% of RA patients (Firestein 2003).

The shared epitope (^{70}QKRAA^{74} or ^{70}QRRAA^{74}) forms the fourth anchoring pocket of the peptide binding groove of complete MHC class II HLA-DR molecules which DRβ chains contribute to. Several DRB1 alleles are associated with RA including *0401, *0404, *0405 and *0101, the products of which contribute to HLA-DR molecules of the DR4 and DR1 subtypes (Tsark, Wang et al. 2002; Hill, Southwood et al. 2003; Svendsen, Andersen et al. 2004). In addition it has been shown that certain alleles such as the RA associated HLA-DR4 form less stable CLIP interactions (Patil, Pashine et al. 2001). Therefore, peptide loading of HLA-DR4 molecules may not be as tightly regulated as non-RA associated alleles providing a possible explanation for DR4 association with RA pathology.

As T cells recognise protein-derived peptide fragments in association with MHC class II molecules, this genetic association with MHC strongly implies that the disease is antigen driven and T cell mediated. In support of this, more recently identified genetic factors such as PTPN22, a gene involved in regulating T cell receptor signalling threshold and CTLA-4, which negatively regulates activated T cells, have both been implicated in RA susceptibility (Begovich, Carlton et al. 2004; Plenge, Padyukov et al. 2005). As previously described anti-CCP autoantibodies are common immunogenic determinants in patients with RA. In addition, anti-CCP antibodies are associated with a genetic linkage to the shared epitope and a more aggressive disease prognosis (Meyer, Labarre et al. 2003). The amino acid at position 71 of the shared epitope has a positive charge. However, the same amino acid in the 70-74 sequence of non-disease associated DRβ chains do not have a positive charge. Therefore, as the 70-74 sequence contributes to the peptide anchoring pocket, the charges of the amino acids at these positions will influence what peptides can bind HLA II. In contrast to non disease associated HLA II, disease associated HLA II which have shared epitope containing DRβ chains have positive charge and will favour binding of more negatively charged peptides. Citrullination removes a positive charge from peptides and makes them more negative. Therefore, individuals expressing the HLA II RA susceptibility alleles are more likely to produce responses to more negatively charged or citrullinated peptides. In support of this hypothesis, other genetic factors such as peptidylarginine deiminase 4 (PAD14), an enzyme involved in protein citrullination is another recently identified genetic determinant of RA. Furthermore, environmental factors such as smoking have been shown to increase the rate at which proteins are

citrullinated in individuals expressing the HLA II RA susceptibility alleles (Klareskog, Stolt et al. 2006). Therefore, it is possible that the association between citrullination and the HLA II RA susceptibility alleles identifies citrullinated proteins as potential RA autoantigens for T cell activation.

1.3.2 T cell depletion therapy in RA

Early attempts aimed at T cell depletion were unsuccessful due to unwanted side effects such as lymphopenia, skin rashes and in the case of the T cell superagonist monoclonal antibody (mAb) TGN142 even life threatening adverse affects (Isaacs and Waldmann 1998; Stebbings, Findlay et al. 2007; Isaacs 2010). However, the success of T cell directed therapies such as Abatacept have taught us a lot about their role in RA. Abatacept is a soluble fusion protein containing the extracellular domain of CTLA-4 linked to the modified Fc domain of human IgG targeted at switching off activated T cells and blocking the interaction between T cell CD28 and APC via its high affinity for CD80/86. In addition, Abatacept can also mediate its effects via modulation of tryptophan metabolism in APC (Grohmann, Orabona et al. 2002). Abatacept is currently an efficacious licensed therapy shown to slow disease progression and slowly ameliorate symptoms in ~50% of RA patients in which anti-TNF therapy has failed (Genovese, Becker et al. 2005, Maxwell #506). The efficiency of therapies that prevent antigen presentation to T cells uncovers a role for both APC and activated T cells in the disease process. However, it does not tell us which APC or antigen is important in T cell activation. Future possibilities for the targeting of T cells as RA therapies are the isolation, *in vitro* expansion and autologous transplantation of Tregs or cellular therapies involving autologous transplant of other regulatory cells to manipulate the T cell response (Isaacs 2008; Mauri and Carter 2009).

The processes leading to the activation of >30% of the T cell population within the synovium and also whether they are activated locally or distally is not fully understood. Genetic data in favour of T cell activation via antigen presentation is well known yet the antigen presenting cell population responsible and indeed the antigen itself is not known. Although, it is likely that T cells play a key role, the pathogenesis of RA is complex and T cells alone are unlikely to be solely responsible for the initiation and /or perpetuation of the disease.

1.3.3 CD4+ T cell activation and tolerance

CD4+, CD8+ NKT cells and CD4+ CD25+ naturally occurring T regs (nTreg) develop within the thymus. There are several CD4+ subtypes of T cells based on cytokine production including Th1, Th2, Th17 and iTreg generated in the periphery each playing a distinct role in immune responses to a variety of intracellular and extracellular pathogens, or in the case of autoimmunity, self antigens (Zhu and Paul 2008). Prolonged or serial TCR stimulation via MHC class II peptide complexes is central to the activation and differentiation of CD4+ T cells (Lanzavecchia and Sallusto 2001). In addition to this, the fate of the cognate T cell relies on the delivery of subsequent signals by the APC. A second activation signal delivered by the APC via co-stimulatory molecules such as CD80/86 via T cell CD28, CD70 via T cell CD27, or OX40L via T cell OX40 is required to prevent T cell anergy (Akiba, Oshima et al. 1999; Linton, Bautista et al. 2003). Additionally, a third 'danger signal' is required which is usually delivered to the APC by inflammatory cytokines or foreign/endogenous ligands recognised by a variety of cytokine receptors or pattern recognition receptors such as IL1r and TLRs (Curtsinger, Schmidt et al. 1999). This third signal ensures physiological changes required by the APC for efficient T cell activation are induced only when necessary. Thus, the cytokine environment and activation status of the APC/T cell dictates the type and level of CD4+ T cell response. Th1 differentiation is mediated by the cytokines IFNγ and IL-12, Th2 by IL-4 and IL-2, Th17 by TGFβ, IL-6, IL-21, IL-23 and iTreg by TGFβ, IL-2 and IL-10 (Zhu, Yamane et al. 2010).

Therefore, the location and type of APC presenting the antigen may affect the delicate balance between T cell responses and could be essential in providing the right response at the right time and with the desired kinetics (Stockinger, Zal et al. 1996).

1.3.4 T cell tolerance

The αβ TCR consists of an α and β chain generated in the thymus by somatic recombination of the variable and joining gene segments of the α chain and recombination of variable, diversity and joining gene segments of the β chain. Humans have approximately 3×10^{11} naïve T cells and TCR rearrangement leads to a possible $>10^{13}$ different TCR specificities (Jenkins, Chu et al. 2010). This diverse repertoire of T cells must be controlled to prevent autoreactive T cells entering the

peripheral circulation and causing an autoimmune response in the periphery. Central T cell tolerance involves both the positive and negative selection of T cells. TCR that recognise self MHC/peptide complexes with a low affinity within the thymus receive a survival signal and become committed to either a CD4+ or CD8+ lineage. Alternately, self-recognition of MHC class II peptide complexes with a stronger affinity can lead to the positive selection and development of forkhead box P3 (FoxP3) expressing CD4+ nTreg (10%) (Starr, Jameson et al. 2003; Josefowicz and Rudensky 2009). Negative selection of CD4+ T cells involves the deletion of immature T cells that recognize self MHC class II peptide complexes with high affinity in the thymus and this ensures that potentially self-reactive T cells are deleted during development. However, a certain percentage of self-reactive T cells may still reach the periphery. Self-reactive peripheral T cells have been identified in patients suffering from autoimmune diseases including multiple sclerosis, insulin dependent diabetes mellitus and rheumatoid arthritis as well as healthy individuals, indicating a possible lack of peripheral tolerance in autoimmune patients (de Jong, Berlo et al.; Scholz, Patton et al. 1998; Viglietta, Kent et al. 2002). Possible explanations for their escape from the thymus include, the self antigen or the specific epitope recognised by the TCR may not have been presented within the thymus for negative selection and secondly negative section may not be stringent enough to delete T cells with a low affinity for self antigen (Walker and Abbas 2002; Abbas, Lohr et al. 2004). However, positive selection of self reactive T cells may be important in maintaining a broad-specificity T cell repertoire in the periphery which is controlled by various immune mechanisms to prevent autoimmunity. It has been suggested that subtle mechanisms such as destructive antigen processing and presentation by resting APC, are in place to 'fine tune' the sensitivity of these self-reactive T cells to antigenic stimulation causing their ignorance, desensitisation or death within the periphery (Anderton and Wraith 2002).

Peripheral tolerance can be achieved by various mechanisms including T cell ignorance, anergy, suppression or apoptosis. T cell ignorance occurs when autoreactive T cells do not encounter their antigen either because it is either sequestered from the immune system or it is only available at very low levels, suppression via regulatory immune cells and to a lesser extent apoptosis via activation induced death (Walker and Abbas 2002).

T cell anergy occurs via recognition of MHC class II peptide complexes and either a lack of co-stimulation or signals via inhibitory T cell receptors such as CTLA-4 and PD-1 (Greenwald, Boussiotis et al. 2001; Latchman, Wood et al. 2001). Anergic T cells fall into two categories T cell clonal anergy which results in growth arrest and T cell adaptive tolerance which results in the inhibition proliferation and effecter functions (Schwartz 2003).

Recent evidence also highlights a role for T cell revision as a peripheral tolerance mechanism, this results from T cells whose autoreactive TCR has undergone rearrangement within the periphery thought to be due to a loss of signal via TCR, resulting in the expression of a new TCR with different specificity (Hale and Fink 2010). This tolerance mechanism is most likely in place to re-arrange self-reactive TCR that has not encountered its ligand due to mechanisms mentioned. However, it is possible that recombination may also result in the expression of a higher avidity self reactive TCR resulting in a potentially dangerous population of self-reactive cells able to cause autoimmunity. The mechanisms involved in regulating this process to prevent autoimmunity are not known and whether they are involved in promoting or preventing autoimmunity remains to be determined (Huang, Golub et al. 2002; Serra, Amrani et al. 2002; Waid, Vaitaitis et al. 2004; Wagner 2007).

Central to peripheral T cell tolerance is a group of regulatory immune cells, B regs, nTregs, iTregs, tolerogenic DC and suppressive macrophages (Mauri and Carter 2009). Tregs activated via TCR recognition of MHC class II peptide complexes on APC can subsequently suppress other autoreactive effecter T cells (Thornton and Shevach 2000). Mechanisms by which Tregs mediate suppressor function include ligation of CTLA-4 with CD86 and the production of the immunomodulatory cytokines IL-10 and IL-35 (Collison, Workman et al. 2007). IL-10 has been shown to inhibit antigen presentation, cytokine production and expression of co-stimulatory molecules on dendritic cells (Moore, de Waal Malefyt et al. 2001). The mechanisms by which IL-10 exert its effects are unclear. However, recent studies have identified that IL-10 can effect APC function via degradation of TLR adapter MyD88 dependent signalling molecules, resulting in a lack of MyD88 signalling and cytokine production (Chang, Kunkel et al. 2009). The suppressive activity of Bregs is mediated via IL-10 production and most likely by their ability to induce the differentiation of Tregs via

antigen presentation (Blair, Chavez-Rueda et al. 2009). Regulatory dendritic cells are thought to exert their effects via the differentiation of iTregs (Weiner 2001). Suppressor macrophages are thought to develop in the presence of Tregs and can suppress T cell autoreactivity via production of IL-10 and tryptophan catabolism and can also induce iTreg differentiation via antigen presentation (Mauri and Carter 2009).

Severe autoimmune conditions occur when major components of the tolerance system are missing. Examples include the autoimmune disease immunodysregulation polyendocrinopathy X-linked (IPEX) in which there is a lack of FoxP3 causing severe autoimmunity in both animals and humans (Ochs, Gambineri et al. 2007) and polymorphisms in the genes IL-10, IL-2 and CTLA-4 have been linked to the autoimmune disease primary biliary cirrhosis (Invernizzi, Selmi et al. 2005). In humans breakdown of tolerance mechanisms by combinations of genetic susceptibility such as the shared epitope and environmental triggers such as smoking can result in T cell activation in response to the presentation of self-antigens and ultimately autoimmunity.

Infection or mechanical insult can result in cartilage degradation in RA uncovering antigens from a previously immunopriviledged site (Doran, Goodstone et al. 1995). Exogenously degraded forms of antigen may be processed differently by APC producing ‘cryptic epitopes’ against which there is no T cell tolerance (Lehmann, Forsthuber et al. 1992). In these circumstances it is possible to envisage how disease could be perpetuated by auto-reactive T and B cells against joint antigens and how recognition of antigen would cause T and B cell proliferation, cytokine and immunoglobulin (Ig) production causing further damage that leads to the release of more sequestered joint antigen.

1.3.5 The role of Th17/Treg cells in RA

The low levels of Th1 cytokines found in RA synovium may be due to the involvement of Th17 cells, which have been shown to negatively regulate Th1 cell development providing a possible explanation for the low levels of IFNγ (Smeets, Dolhain et al. 1998; Harrington, Hatton et al. 2005). Th17 cells produce IL-17A, IL-21 and IL-22. Elevated levels of both IL-17A and IL-17A producing CCR6+ Th17

cells have been found in the synovial fluid of RA patients and high levels of IL-22 in synovial tissues (Chabaud, Durand et al. 1999; Ziolkowska, Koc et al. 2000; Ikeuchi, Kuroiwa et al. 2005; Hirota, Yoshitomi et al. 2007). However, results are controversial with other groups demonstrating low levels of Th17 cells within the rheumatoid synovium and the finding that other cell types such as mast cells may be the major source of IL-17A (Yamada, Nakashima et al. 2008; Hueber, Asquith et al. 2010). Studies using mouse models have shown Th17 cytokines to have effects on osteoclastogenesis, angiogenesis and inflammation and they have pro-inflammatory effects on various cells of the synovium including osteoclasts, chondrocytes and fibroblasts (Muller-Ladner, Ospelt et al. 2007; Otero and Goldring 2007; Schett 2007). However, results using animal models are contradictory with proteoglycan induced arthritis (PGIA) generating a Th17 independent disease (Doodes, Cao et al. 2008), and collagen induced arthritis (CIA) generating a Th17 dependent disease (Lubberts, Koenders et al. 2004). The contribution of Th17 cells to human arthritis is still unclear.

Another possible role for T cells in RA is the activation or deregulated activation of Treg cells whose role in central tolerance is most clearly demonstrated by the previously mentioned IPEX linked to mutations in FoxP3 causing severe autoimmunity in both animals and humans. There are 2 main types of Treg, naturally occurring FoxP3+ generated in the thymus and peripherally activated CD4+ CD25+ iTreg which can acquire FoxP3 expression upon antigen recognition in the periphery (Chen, Jin et al. 2003). CD4+ CD25+ T cells have been implicated as playing a role in RA as there are lower numbers found in patients and /or they are defective in their ability to suppress TNFα and IFNγ production by T lymphocytes and monocytes (Ehrenstein, Evans et al. 2004; Lawson, Brown et al. 2006). However, their exact mechanism of tolerance induction is not known. Experiments by van Bilsen *et al* into the mechanisms of Treg mediated suppression in RA have indicated that suppression is an antigen-specific balance between pro-inflammatory and regulatory responses. Using the candidate autoantigen human cartilage glycoprotein 39 (Hcgp39) van Bilsen *et al* showed that PBMC responses from normal individuals to Hcgp39 were biased towards anti-inflammatory IL-10 mediated Treg responses, whereas PBMC from RA patients responded in a pro-inflammatory way producing a more Th1 phenotype (van Bilsen, van Dongen et al. 2004). This data suggests that during the

initiation/perpetuation of RA there may be a dysregulation in the balance between pro-inflammatory cells and regulatory cells such as Tregs, B regs, regulatory DC and regulatory macrophages which results in a switch from self-tolerance to autoimmunity.

Th1, Th17 and Tregs have all been identified as playing a role in RA but it is not known the relative contribution of each to disease pathology. T regs and Th17 can rapidly become IFNγ producing Th1 T cells *in vitro* following the addition of IL-12 (Lee, Turner et al. 2009). Therefore, it is also possible that T cell plasticity occurs in RA and conversion from one cell type to another may upset the balance between self tolerance and autoimmunity.

1.4 The role of B cells in RA

Several autoimmune diseases are characterised by the presence of autoantibodies and hence the implications for B cell involvement in disease pathogenesis. Coupled with the successful treatment of RA patients with the B cell depleting therapy Rituximab (Rituxan, Genentech) this implicates B cells as important mediators of RA pathogenesis and excellent candidates with which to explore the potential of improved and novel drug targets.

1.4.1 B cell depletion therapy

Rituximab is an anti-CD20 therapy originally designed to treat non-Hodgkins lymphoma (Grillo-Lopez, White et al. 1999). It has shown clinical benefit in ~80% RA patients with major improvements observed in ~50% with complete resolution of inflammation observed in some cases (Edwards and Cambridge 2006). Rituximab is a chimeric IgG1 κ mAb containing mouse heavy (H) and light (L) chain variable regions and human constant regions directed against CD20 found on the surface of pre and mature B cells but not on plasma cells or pro-B cells (Edwards and Cambridge 2001; Emery, Fleischmann et al. 2006; Genentech 2007). Germinal centre marginal zone B cells also appear to be resistant to Rituximab mediated cell death possible due to protection conferred from the microenvironment (Gong, Ou et al. 2005). Although the exact mechanisms of B cell death are not known it is thought to be largely due to antibody-dependent cell-mediated cytotoxicity (ADCC) (Uchida, Hamaguchi et al. 2004). B cell depletion and clinical benefits of Rituximab last approximately 7-8 months before B cell numbers begin to increase replenished by the undepleted pre-B cell pool. Other agents aimed at targeting B cells in RA are the B-cell survival factor (BAFF) specific antibody Belimumab (human genome sciences and Glaxosmithkline) and the neutralizing recombinant fusion protein Atacicept (EMD Serono and Zymogenetics Amgen) that targets the B lymphocyte stimulator BLyS and APRIL a proliferation-inducing ligand, both involved in B cell survival, proliferation, antigen presentation and class switching (Tak, Thurlings et al. 2008). Although Belimumab was well tolerated it showed limited efficacy in clinical trials (Looney 2006). In contrast, Atacicept which contains the human IgG1 Fc fused with the extracellular portion of TACI binds to and neutralizes BLyS and APRIL has recently shown promising effects in initial small trials and larger trials are now underway (Bracewell, Isaacs et al. 2009).

1.4.2 Autoantibodies and their role in disease

As previously mentioned in rheumatoid arthritis there is evidence of autoantibodies reactive against several self-antigens including glucose-6-phosphate isomerise (GPI), a ubiquitous enzyme thought to be deposited in the synovium, post translationally modified citrullinated peptides (CCP) and the Fc region of human IgG (RF) (Silverman and Carson 2003) (Huang, Kearney et al. 2006). Mechanisms of action of autoantibodies have been shown in experiments with K/BxN mice. The K/BxN mouse models which express the TCR transgene (KRN) developed by Mathis and Benoist were crossed with non obese diabetic mice to generate the K/BxN strain (Kouskoff, Korganow et al. 1996). K/BxN mice develop a spontaneous arthritis at the age of 3-5 weeks which is localised to the joints and results from the presence of GPI specific autoantibodies and autoreactive T cells (Korganow, Ji et al. 1999). The disease was antibody dependent and could be transferred to healthy recipients by serum alone. Knockout experiments confirm that the disease is dependent on FcRyIII (Corr and Crain 2002) and anti-C5 administration confirms an essential role for complement (Ji, Ohmura et al. 2002). The K/BxN model has taught us a lot about the pathogenic potential of autoantibodies in complement activation and Fc mediated antigen uptake by APC. Whether autoantibodies play a central role in disease pathology or whether they are simply good diagnostic markers for RA is not known. However, the fact remains that populations of autoreactive B cells are present in most RA patients and are under constant activation to become antibody secreting plasma cells. The role of B cells is not limited to antibody production alone. Indeed, following their activation via antigen recognition, T cell help and TLR signalling, B cells differentiate into either antibody-secreting plasma cells or mature B cells which express surface antibody and are capable of efficiently presenting antigen to synovial T cells. Furthermore, B cells have also been shown to play a pivotal role in the production of chemokines and cytokines that influence further leukocytic infiltration into and retention within the RA synovium (Loetscher and Moser 2002). This evidence suggests that B cells have a multi-functional role in the pathogenesis of RA and indeed there are several studies which highlight the importance of B cells as APC, a role which I shall now discuss.

1.4.3 The role of B cells in lymphoid neogenesis and T cell activation

Interaction between B and T cells within the RA synovium has been extensively documented (Young, Adamson et al. 1984; Schroder, Greiner et al. 1996; Weyand, Goronzy et al. 2000). Interactions vary from patient to patient, with B and T cells forming loosely organized microstructures in some patients or aggregating with interdigitating dendritic cells to form germinal centre (GC) like structures (lymphoid neogenesis) in other patients (Silverman and Carson 2003). GC are structures essential for affinity maturation of B cells and selection by follicular dendritic cells (FDC) as well as the development of B cell memory, which relies on specific antigen and cognate T cell help (Liu, Joshua et al. 1989; Kosco and Gray 1992). Therefore, the presence of lymphoid neogenesis in the synovium indicates a strong immune response almost certainly against specific antigens. Indeed, in patients showing lymphoid neogenesis as opposed to loosely organized T and B cell interactions there is an increase in the transcription of IgG indicative of antigen-specific B cell activation and higher levels of the B cell survival factor APRIL (Seyler, Park et al. 2005). Antigen-specific B cells are efficient APC able to clonally expand and focus an immune response against a particular antigen for presentation to CD4+ T cells (Casten and Pierce 1988; Batista and Neuberger 1998; Byersdorfer, Dipaolo et al. 2004). Furthermore, self-reactive RF specific B cells are able to process different antigens complexed with IgG and thus allow effective presentation of various immune complexes (Roosnek and Lanzavecchia 1991). Indeed, it is possible that immune complexes containing TLR ligands such as chromatin or RNA associated autoantigens may be able to break peripheral B cell tolerance by providing their own danger signal and overcoming the requirement for T cell help (Christensen, Shupe et al. 2006; Herlands, Christensen et al. 2008). In support of this, immune complexes with chromatin have been proven to synergistically activate BCR uptake and TLR signalling in a MyD88 dependent manner (Leadbetter, Rifkin et al. 2002).

There are many studies using both spontaneous and antigen-induced animal models of RA that have demonstrated an essential role for B cells in lymphoid neogenesis and T cell activation (Takemura, Klimiuk et al. 2001; O'Neill, Shlomchik et al. 2005; Taneja, Krco et al. 2007). An elegant study by Takemura and colleagues demonstrated both of these roles impeccably using SCID mice transplanted with human RA synovium. T cells identified as having Th1 phenotypes were isolated from

the synovial GCs of RA patients and adoptively transferred into the transplanted synovium of these SCID mice. Adoptively transferred T cells required both cognate HLA DRβ1 alleles with the implanted synovial tissue and B cells in order to become activated. The essential role of B cells was demonstrated by the transfer of T cells into anti-CD40 mAb B cell depleted synovium or the transfer of synovium containing few or no B cells or loosely formed T and B cell aggregates which resulted in abrogated T cell activation, IFNγ and IL-1β production and GC formation (Takemura, Klimiuk et al. 2001).

Germinal centre formation within the synovium is dependent on expression of the TNF family member lymphotoxinβ (LTβ) by B cells along with other factors produced by synovial macrophages and fibroblasts such as CXCL13, CXCL12 and CXCL21 involved in lymphoid recruitment and organization (Buckley, Amft et al. 2000; Takemura, Klimiuk et al. 2001; Weyand and Goronzy 2003). B cells receive survival and activation signals from neighbouring DC, fibroblast-like synovial cells (FLS) and macrophages in the form of BAFF, APRIL as well as from T cell interactions within the germinal centre like structures found in RA synovium (Seyler, Park et al. 2005) (Ohata, Zvaifler et al. 2005).

Several antigen-specific mouse models of arthritis including PGIA, CIA and GPI induced arthritis have been used to demonstrate the role of B cells as APC in RA which suggests that their ability to efficiently present antigen is not restricted to the antigen PG or an H-2^d BALB/c restricted background.

Early studies by Brennan *et al* using the PGIA mouse model demonstrated that PG specific antibodies were present immediately prior to intense proliferation of autoreactive T cells and that B cells isolated from PG immunized mice were able to present low concentrations of aggrecan to aggrecan-specific T cells 5/4E8 (Brennan, Mikecz et al. 1995). However, this study only established that B cells were able to present PG to T cell hybridomas and didn't show a specific role for B cells in disease onset or progression. In later experiments, the same laboratory performed an elegant study demonstrating that B cells were required as both antibody producing cells and APC to induce severe PGIA. Importantly, they also demonstrated that the antigen

presenting function of the B cells could not be fulfilled by other APC. Using genetic manipulation to generate mice with membrane bound heavy chain Ig paired with an endogenous hapten specific light chain (mIg) the authors showed that T cells from these mice could transfer disease in SCID mice, only if the immunizing PG was targeted to antigen-specific B cells. In addition, it was shown that T cells transferred from B cell depleted mice failed to transfer disease. To verify the role of antibodies they showed that transfer of arthritic serum alone from mice with a normal B cell compartment induced only a mild arthritis. The data implies that the onset of severe PGIA required both antigen-specific B cells and autoantibodies highlighting the dual role of B cells in disease pathogenesis (O'Neill, Shlomchik et al. 2005). Later studies by O'neill *et al* verified that the antigen presenting function of the B cells was dependent on the co-stimulatory molecules CD80/86. Using mixed bone marrow chimeras in which CD80/86 expression was absent only on B cells, they showed that these mice were resistant to PGIA induction even though PG Ig levels were normal. T cells isolated from the PGIA mice were able to transfer disease into SCID recipients. However, when T cells isolated from chimeric mice were transferred to SCID recipients they developed a much less severe disease that took much longer to develop. *In vitro* stimulation of the T cells with PG and irradiated wt spleen cells, B cell depleted spleen cells, B cell CD80/86- or CD80/86- spleen cells verified that B cells expressing CD80/86 were required for optimal T cell activation (O'Neill S, Cao et al. 2007).

Tanjea *et al* demonstrated in 2002 that transgenic mice expressing the RA associated-allele HLA-DQ8 elicit a vigorous CD4+ response leading to CIA following collagen immunisation (Taneja, Taneja et al. 2002). To investigate the role of B cells as APC the mice were crossed with μMT mice that lack B cells due to a genetic deletion in the IgM gene. Results showed that the DQ8 μMT mice are resistant to CIA implying an essential role of B cells in disease pathogenesis. Furthermore, *in vitro* experiments demonstrated that T cells isolated from these mice have abrogated responses to collagen unless B cells were used as APC. These data suggests that the role of T cells in disease induction is at least partially dependant on the antigen presenting properties of B cells (Taneja, Krco et al. 2007). Experiments using the CIA model in rats demonstrated that B cells and not DC or macrophages are required as APC for T cell recall responses to collagen II *in vitro*. An interesting finding from this study was that

although B cells from immunized rats produced a greater proliferative T cell response, B cells from non-immunized rats were still able to function as APC, a suggested explanation for this was that alternate cell surface molecules such as B-1 integrins or CD26 may enhance collagen II uptake by tight association with the B cell surface (Catchpole, Staines et al. 2001).

Cell surface glycoprotein CD44 expressed by B cells binds to hyaluronan (HA) fragments and the chondroitin sulphate side chains of aggrecan and ligation with HA has been demonstrated to induce B cell proliferation (Rafi, Nagarkatti et al. 1997; Fujimoto, Kawashima et al. 2001). Aggrecan is made more freely available by the action of degrading proteinases such as aggrecanases and MMPs during the course of arthritis (Billington, Clark et al. 1998; Struglics, Larsson et al. 2009; Cawston and Young 2010). Therefore, it is possible that binding to CD44 may facilitate aggrecan uptake and its presentation to autoreactive T cells. In addition, aggrecan fragments containing bound HA may lead to enhanced T cell presentation by co-ligating aggrecan-specific BCR with the TLR4 receptor for which HA is a ligand (Tesar, Jiang et al. 2006).

The ability of B cells to contribute to ECM degradation via the secretion of MMPs has been demonstrated (Di Girolamo, Tedla et al. 1998; Trocme, Gaudin et al. 1998; Melamed, Messika et al. 2006). Melamed *et al* revealed that mature B cells can be stimulated to secrete the matrix degrading enzyme MMP-9 by stimulation with LPS, concanevalin A and the pro-inflammatory cytokines IL-1, IL-8, TNF-α. Secretion of MMP-9 can contribute to cartilage degradation and the breakdown of the basement membrane aiding transition into the joint tissues (Melamed, Messika et al. 2006; Chang, Lin et al. 2008). Di Girolamo *et al* demonstrated that B cells secrete MMP-3 as well as MMP-9 in response to pro-inflammatory cytokines or other proteinases and when large numbers of B cells aggregate, production of MMP-3 and MMP-9 may play a critical role in tissue destruction (Di Girolamo, Tedla et al. 1998). In addition, as B cells are competent at secreting these degrading proteinases, they may also be capable of degrading cartilage components such as aggrecan, generating self epitopes for immune recognition.

1.4.4 B cells and cytokines

B cells within the joint express the cytokines and chemokines IL-1α and or IL-1β, TNFα, IL-15, IL-10, IL-6 and LTβ all shown to play a role in the pathogenesis of RA (McInnes and Schett 2007). The various cytokines play a role in synovial fibroblast activation, FDC organisation and lymphoid neogenesis. However, the amount of these cytokines that B cells produce in relation to other synovial cells is not clear.

Besides the pathogenic role proposed for B cells in RA it is possible as with Treg cells that there is a dysregulation or dysfunction of IL-10 producing Breg cells in the disease state. Indeed treatment of certain autoimmune patients with Rituximab to deplete B cells exacerbated disease implying that B cells were conferring a protective role (Edwards, Szczepanski et al. 2004; Ardelean, Gonska et al. 2010). There is growing evidence that B cells can play a regulatory role in autoimmune disease as reviewed in (Fillatreau, Gray et al. 2008; Lampropoulou, Calderon-Gomez et al. 2010). Splenic B cells isolated during the acute phase of CIA and treated with CD40 mAb are able to prevent CIA induction when transferred into collagen immunized DBA/1 mice (Mauri, Gray et al. 2003). Later experiments identified an *in vivo* transitional 2-marginal zone precursor (T2 MZP) subset of IL-10 producing B cells able to confer protection from CIA when transferred from naïve or convalescing mice. This regulatory phenotype is dependent on IL-10 as B cells taken from IL-10 deficient mice are unable to confer protection (Evans, Chavez-Rueda et al. 2007). It is proposed that the mechanisms by which Bregs exert their activity is by regulating a Th1/Th2 balance (Mauri, Gray et al. 2003). Furthermore, it has been reported that Breg IL-10-mediated suppression of both pathogenic Th17 and Th1 subsets is critically dependent on TLR and MyD88 signalling (Lampropoulou, Hoehlig et al. 2008). Although the exact details of these mechanisms are not yet clear, a balance between where B cells encounter TLR ligands and the cytokine environment may play a role. For example, a recent study has shown a pathogenic role for TLR9 in the activation of bone marrow B cells from RA patients. Stimulation of bone marrow B cells with the TLR9 ligand bacterial CpG stimulated B cell proliferation, up-regulation of co-stimulatory molecules CD86 and CD54 and the production of pro-inflammatory cytokines IL-6 and TNFα. In addition, a significantly higher level of bacterial DNA was detected in bone marrow samples from RA patients compared to osteoarthritis controls (Rudnicka, Burakowski et al. 2009). Oedema and the formation of lymphoid

neogenesis in subchondral bone can often be seen in patients with RA particularly in those patients with early stage RA suggesting that bone marrow may provide a rich environment for pathogenic B cell activation (Bugatti, Caporali et al. 2005; Tamai, Kawakami et al. 2007).

1.5 B cells as antigen presenting cells

B cells are well documented as APC. However, their exact role in the initiation and maintenance of a CD4+ T cell response has remained controversial, due to their dependence on help from activated CD4+ T cells, the low frequency of antigen-specific B cells in the periphery and conflicting evidence from B cell depletion that conclude either normal or abrogated T cell responses in their absence (Ron, De Baetselier et al. 1981; Janeway, Ron et al. 1987; Epstein, Di Rosa et al. 1995; Liu, Wu et al. 1995; Linton, Bautista et al. 2003). Studies using B cell-deficient mice have attempted to elucidate the role of B cells in T cell activation *in vivo* using mice depleted of B cells either via the administration of anti-μ antibodies (Janeway, Ron et al. 1987), by gene disruption (μMT) (Epstein, Di Rosa et al. 1995; Liu, Wu et al. 1995; Phillips, Romball et al. 1996) or in which the B cell compartment is deficient in MHC class II (Williams, Oxenius et al. 1998; Crawford, Macleod et al. 2006). However, the conflicting evidence showing either normal or abrogated T cell responses in the absence of B cells has been demonstrated in all the above systems and is most likely due to several contributing factors including, variability between mouse strains (Rivera, Chen et al. 2001), the effect of B cell depletion on normal lymphoid architecture (Linton, Harbertson et al. 2000), the type and form of antigen (Constant, Schweitzer et al. 1995) and finally the effect that B cell depletion can have on the function of other APC (Macaulay, DeKruyff et al. 1998). Indeed, it has been shown that dendritic cells from B cell deficient mice (μMT) mice produce lower levels of IL-12 which in turn has an effect on the T cell Th1/Th2 balance, skewing production of Th1 cytokines IFNγ and IL-2 and not IL-4 (Moulin, Andris et al. 2000).

B cells play a role in both the humoral and cellular responses by organizing lymphoid architecture, producing antibodies and activating T cells. Like the other APC, B cells are capable of providing all of the required signals for T cell activation. However, unlike dendritic cells and macrophages, B cells are capable of clonal expansion in response to antigen. This additional function allows B cells to focus the T cell response around small amounts of the antigen-specific to their BCR. Indeed, B cells have been implicated as initiators of an adaptive immune response when antigen levels are low (Rivera, Chen et al. 2001; Byersdorfer, Dipaolo et al. 2004).

1.5.1 Antigen uptake via BCR

The method of antigen uptake can determine how efficiently peptide MHC class II complexes are presented to T cells (Lanzavecchia 1990). Several studies have demonstrated that although B cells are capable of taking up antigen via fluid phase uptake, this method is inefficient when compared to that of DC (Sallusto and Lanzavecchia 1994). However, antigen targeted to the BCR (and other cell surface receptors) allows for both efficient processing and presentation of antigens to CD4+ T cells (Chesnut, Colon et al. 1982; Lanzavecchia, Abrignani et al. 1988; Carel, Myones et al. 1990). Receptor-mediated antigen uptake is carried out most efficiently by the BCR. A high affinity BCR allows B cells to effectively compete with other APC for antigen capture and presentation and with other B cells for T cell survival signals (Rock, Benacerraf et al. 1984; Lanzavecchia 1985). Bias towards receptor-mediated antigen uptake, particularly via the highly antigen-specific BCR, highlights B cells as a novel group of efficient antigen-specific T cell activators.

1.5.2 The BCR complex

The B cell receptor complex is a hetero-oligomeric structure consisting of the antigen recognition component (BCR) which is a precursor of Ig and additional signalling molecules CD79α and CD79β (Hombach, Leclercq et al. 1988). The BCR are disulphide linked tetramers of two heavy (H) and two light (L) chains anchored in the cell surface (Borst, Brouns et al. 1993). The H chain consists of a single variable region and three constant regions and the L chain consists of a single variable and a single constant region. The BCR can be separated into two distinct regions the antigen recognition region or fragment antigen binding (Fab) and the fragment crystalline region (Fc) which provide different effecter functions dependent on the isotype when the BCR is secreted as Ig (**Figure 1.3**).

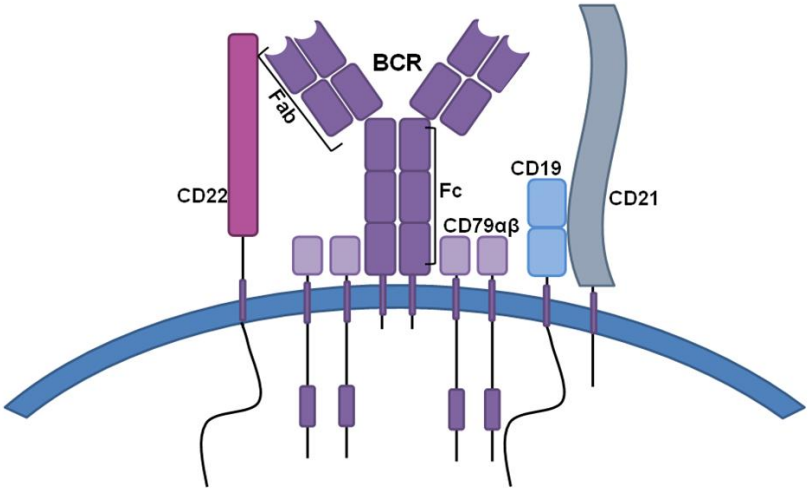

Figure 1.3 The BCR complex
Model of the BCR complex including the co-signalling molecules CD79α, CD79β, CD19, CD21 and the inhibitory molecule CD22. The BCR structure comprises of an effecter or fragment crystalline (Fc) region and an antigen recognition or Fragment antigen binding (Fab) region.

The BCR undergoes constitutive internalization mediated by the phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAM) in the Igα cytoplasmic domain (Bonnerot, Lankar et al. 1995). However, upon BCR recognition of membrane bound antigen, an increased signalling cascade leads to the formation of an immunological synapse. The immunological synapse is surrounded by co-stimulatory and adhesion molecules that enable accelerated and efficient uptake, processing and presentation of antigen to T cells (Anderson and Siahaan 2003; Carrasco and Batista 2006). Mutational studies of the BCR have revealed that BCR mediated uptake not only ensures efficient uptake of antigen but also drives the accelerated intracellular targeting of antigen for loading onto MHC class II (Aluvihare, Khamlichi et al. 1997). Peptide loading onto MHC class II molecules is mediated by the non-classical MHC class II molecule H2-DM (Ruckrich, Brandenburg et al. 2006). This is itself regulated by H2-DO binding to H2-DM at a high pH early in the endosomal pathway, favouring high affinity BCR presentation and limiting non-specific uptake via pinocytosis (Denzin, Fallas et al. 2005). In addition, as the antigen internalized via the BCR is limited to a specific protein on each individual B cell clone, there is little competition from other external antigens for processing and peptide generation that will ultimately compete for binding to the MHC class II.

The efficiency of BCR mediated uptake has been demonstrated by many laboratories using several different model antigens concluding that BCR mediated uptake allows

the B cell to present antigen around 10^3-10^4 fold more efficiently than non-specific uptake via pinocytosis (Chesnut and Grey 1981; Batista and Neuberger 1998). Indeed, Lanzavecchia demonstrated that antigen-specific B cells were able to present tetanus toxin at concentrations of 10^{-11} M when only a small fraction of BCR are occupied by antigen (~0.05%) (Lanzavecchia 1985). Batista and Neuberger demonstrated that the affinity of the BCR for its antigen has a direct effect on its ability to present antigen to T cells. In studies using lysozyme-specific B cell transfectants and mutated forms of lysozyme with differing binding affinities they showed that an antigen/BCR affinity of K_a of $>10^6$ M^{-1} was required for detectable BCR mediated antigen presentation, between a K_a of 10^6-10^8 M^-1 the presentation sensitivity was increased and at K_a $>10^{10}$ M^-1 a threshold was reached and antigen presentation no longer improved (Batista and Neuberger 1998). Indeed, differences in affinity have recently been found to relate to differences in synapse formation with high affinity BCR interactions promoting rapid synapse formation and recruitment of signalling molecules (Liu, Meckel et al. 2010).

In addition, B cells may be responsible for promoting the initial T cell responses to antigen demonstrated by Crawford *et al* who showed that T cell activation is impaired at early timepoints following immunization with OVA in bone marrow chimeric mice where B cells lack MHC class II (Crawford, Macleod et al. 2006). In support of this, other groups have identified the cell surface expression of peptide-MHC class II complexes at early timepoints following immunisation, suggesting that B cells contribute to the early activation of T cells following antigen encounter (Byersdorfer, Dipaolo et al. 2004; Catron, Itano et al. 2004; Yan, Harvey et al. 2006; Pape, Catron et al. 2007).

1.5.3 Additional B cell antigen receptors

B cells are equipped to target specific antigen via the BCR, whilst maintaining the ability to adapt the response to opsonised antigen. Indeed, the secreted form of the BCR (Ig) can both compete with the BCR for binding to its antigen and also facilitate antigen uptake by FcR (Lanzavecchia 1987).

The efficiency of BCR signalling is influenced by CR1 (CD35) and CR2 (CD21) complement receptors, CD19 of the Ig superfamily and FcγRII antibody receptors.

Antigen targeted to CD19 can upregulate co-stimulatory molecules and efficiently activates T cells independently of the BCR (Yan, Wolff et al. 2005). Additionally, CD19 and/or CD19-CR2 complexes provide signals to lower the threshold of antigenic stimulation through the BCR and augment antigen uptake (Fearon and Carroll 2000). Importantly, Rossbacher *et al* demonstrated that the BCR itself was capable of activating complement and providing the CR ligands required for the activation of this optimal antigen uptake and presentation (Rossbacher and Shlomchik 2003). However, in the absence of co-ligation with the BCR, CR2 mediated antigen uptake does not upregulate the co-stimulatory molecules required for T cell activation and may have a role in initiating T cell anergy (Brown, Barrault et al. 2006).

1.5.4 B cell Tolerance

The specificity of the BCR is determined firstly during arrangement of the V D and J segments of the H and L chains during B cell development in the bone marrow. Subsequent B cell receptor editing involving secondary V(D)J recombination occurs following BCR recognition of self-antigen to reduce affinity for self (Nemazee 2000).

Naïve B cells that either recognize multivalent membrane bound antigen in the bone marrow, are unable to edit their light chains, or fail to encounter their specific-antigen, are either eliminated or undergo follicular B cell death due to competition from other B cells for antigen-specific survival signals (Nemazee and Burki 1989; Hartley, Cooke et al. 1993). Early studies demonstrated that auto-reactive B cells expressing a BCR capable of recognising a self antigen were either deleted or made anergic (Goodnow, Crosbie et al. 1988).

The mechanisms governing whether autoreactive B cells are deleted, undergo receptor editing or become anergic are not clear. However, as with TCR tolerance factors affecting the fate of B cells involve affinity of antigen recognition with a weak recognition of self antigen resulting in anergy or ignorance and a high affinity resulting in deletion. B cells weakly crosslinked by self-antigens in the bone marrow may emerge from the follicles as ignorant in the absence of their ligand and/or autoreactive T cell help (Nemazee, Russell et al. 1991; Shlomchik, Zharhary et al. 1993; Hannum, Ni et al. 1996). B cell anergy can occur where as autoreactive antigen is not sufficient to trigger apoptosis but is sufficient to prevent follicular entry and the

recruitment of T cell help (Shlomchik 2008). In addition, to deletion, editing and anergy another proposed B cell tolerizing mechanisms is the reduction in B cell-mediated signalling via the sialic binding lectin-SHP-1 tyrosine phosphatase pathway. CD22 for example expressed at high levels by mature B cells is a prominent sialic binding, Ig domain containing lectin which associates with the BCR and delivers inhibitory signals via immunoreceptor tyrosine-based inhibitory motifs (ITIMs) on its cytoplasmic tail leading to inhibition of BCR signalling (Pillai, Cariappa et al. 2009).

1.5.5 B cell tolerance breakdown

Clonally anergic or ignorant B cells are thought to be the source of autoreactivity as up to 50% of newly emerging B cells may be anergic (Merrell, Benschop et al. 2006). In addition to the deletion of self-reactive B cells, receptor editing can also result in rearrangement at the other kappa or lambda loci resulting in a new non self-reactive BCR whilst retaining the initial autoreactive BCR (Li, Jiang et al. 2001; Liu, Velez et al. 2005). Cells expressing these dual receptors can escape into the periphery as potentially autoreactive B cells along with anergic autoreactive B cells or B cells that have escaped tolerance mechanisms by some other means such as natural leakiness or genetically mediated tolerance failure (Shlomchik 2008). So how do these potentially autoreactive B cells become activated? Studies using AM14 BCR Tg mice have elucidated AM14 autoreactive RF "IgH^{a}" allotype specific B cells are present in the periphery but are clonally anergic. However, when the AM14 BCR Tg mice were crossed onto the autoimmune prone MLR.Fas^{lpr} background the anergic B cells became activated to produce autoantibodies. This response only occurs in IgH^{a} and not IgH^{b} strains in confirming that clonally anergic B cells can be activated in the periphery and that this B cell activation is antigen dependent (Wang and Shlomchik 1999). Furthermore, subsequent studies have verified that this autoreactive B cell activation can occur at extra-follicular sites (William, Euler et al. 2002). Co-ligation of the BCR and TLR receptors have been implicated in directing autoreactive extra-follicular B cell responses possibly in a T independent manner, thus absolving the requirement for autoreactive T cell help (Leadbetter, Rifkin et al. 2002; MacLennan, Toellner et al. 2003; Herlands, Christensen et al. 2008; Shlomchik 2008). In addition, strong autoreactive T cell help has also been shown to break B cell anergy (Goodnow, Brink et al. 1991). Therefore, it is probable that a break in B cell tolerance occurs

through a combination of autoreactive B cell precursors, antigen availability and TLR stimulation and/or the activation of autoreactive T cell help.

1.6 Autoantigen processing and presentation

1.6.1 Candidate autoantigens

Genetic linkage to HLA alleles and T cell activation markers suggests that rheumatoid arthritis is an antigen-driven disease. However, attempts to consistently identify autoreactive T cells or disease specific autoantibodies have proven difficult. Studies over the years have identified several candidate joint autoantigens as potential targets leading to the development of RA, some of which are listed in **Table 1.1.** The ability of certain matrix proteins such as aggrecan and collagen to elicit disease in animals and the detection of auto antibodies and autoreactive T cells isolated from RA patients make them ideal candidates for further study. However, new proteomics and mass spectrometry analysis of RA sera is rapidly detecting novel autoantigens such as signalling molecules, citrullinated glycolytic enzymes and molecular chaperones (Auger, Balandraud et al. 2009; Goeb, Thomas-L'Otellier et al. 2009). Therefore, it is possible that the antigen responsible could vary greatly from patient to patient or indeed that several autoreactive antigens may be involved in the same patient. Another possibility is that the antigens are post-translationally modified via mechanisms such as glycosylation, or citrullination (Axford, Sumar et al. 1992; Klareskog, Ronnelid et al. 2008). In addition, as joint cartilage is progressively degraded by increased proteinase activity (including MMP) promoted by pro-inflammatory cytokines such as IL-1 and TNFα, previously immunopriviledged antigens may be uncovered or modified, suggesting that chronic disease progression could be a stepwise process involving different antigens at different stages of disease.

Antigen	Reference	Citrullinated
Aggrecan	(von Delwig, Locke et al. 2010)	yes
Collagen II	(Burkhardt, Sehnert et al. 2005)	yes
Vimentin	(Vossenaar, Despres et al. 2004)	yes
α-enolase	(Kinloch, Tatzer et al. 2005)	yes
Fibrin	(Masson-Bessiere, Sebbag et al. 2001)	yes
HCgp-39	(Verheijden, Rijnders et al. 1997)	
Chondrocyte Ag	(Bang, Mollenhauer et al. 1994)	
Human Hsp60	(van Halm, Slot et al. 2006)	
Fibrinogen	(Masson-Bessiere, Sebbag et al. 2000)	yes
MMPs	(Sedlacek, Mauch et al. 1998)	
Filagrin	(Girbal-Neuhauser, Durieux et al. 1999)	yes
Keratin	(Chang, Jian et al. 2009)	yes

Table 1.1 Table illustrating candidate autoantigens in RA.
Some of the candidate joint autoantigens identified to date are listed in the above table. If there is evidence that the autoantigens can be citrullinated then this is indicated in the right panel.

1.6.2 Antigen uptake by APC

Methods of antigen uptake (endocytosis) by different APC have been reviewed (Lanzavecchia 1996; Mellman 1996). Endocytosis is a process used by all eukaryotic cells for cellular homeostasis and recycling of cell surface components. In addition, variations of endocytosis are adopted by different APC to internalize exogenous antigens for processing and presentation to CD4+ T cells. Phagocytosis by macrophages and dendritic cells involves receptor engagement to engulf large particles >0.5 μm diameter. Varieties of pattern recognition receptors such as the mannose receptor are designed to recognize distinct patterns on bacterial components. Uptake is triggered by receptor recognition of pathogen associated molecular patterns or antigens opsonised by molecules such as Ig or complement fragments. Receptor signalling initiates F actin polymerization and pseudopod extensions that engulf the

receptor/ligand complex and form a tight phagosome (Greenberg, Burridge et al. 1990). Phagosomes rapidly fuse with endosomes and exchange their membrane and contents for processing within the endosomal pathway (Kielian and Cohn 1980; Unanue 1984). Pinocytosis is a process carried out by macrophages, dendritic cells and B cells that typically involves constitutive non-specific fluid phase antigen uptake, initiated at clathrin coated pits and resulting in the formation of smaller endocytic vesicles <0.2 μm diameter. Macropinocytosis is a clathrin independent mechanism of pinocytosis involving the formation of macropinosomes 1-5μm diameter (Mellman 1996). Macropinocytosis is performed constitutively by dendritic cells and by macrophages following stimulation with MCSF (Racoosin and Swanson 1989). However, prolonged stimulation of DC with inflammatory stimuli such as TNFα, LPS or IL-1 has been shown to dramatically reduce uptake by macropinocytosis suggesting that this mechanism of antigen uptake may be shut down in mature DC (Sallusto, Cella et al. 1995; West, Wallin et al. 2004). Macropinosomes are formed by actin polymerisation and membrane ruffling that form invaginations into the cell, that close at the outermost regions to form vesicles. Vesicles are subsequently depolymerised and enter the endocytic pathway or are recycled to the cell surface (Racoosin and Swanson 1993; Swanson and Watts 1995). Receptor mediated endocytosis of soluble antigens is clathrin dependent. Antigen receptors such as Fc receptors expressed by macrophages, dendritic cells and B cells or the B cell specific BCR complex are clustered within clathrin coated pits and rapidly internalize bound antigens delivering them to various endocytic compartments in a process dependent on signals relayed via the receptor complex (Aluvihare, Khamlichi et al. 1997). The different mechanisms of antigen uptake by B cells, macrophages and dendritic cells equips the immune system with a comprehensive set of antigen sifting cells to ensure minimal immune evasion by pathogens.

However, depending on which intracellular compartment antigen is endocytosed to, different peptide repertoires could be generated from the same antigen influenced by factors such as proteinase availability, proteinase activation and pH which differ in different intracellular compartments and possibly between different APC (Schneider and Sercarz 1997; von Delwig, Bailey et al. 2002).

1.6.3 MHC class II assembly and trafficking.

The majority of peptide epitopes recognised by CD4+ T cells are generated from antigens taken up by APC and processed within the endosomal pathway where they are loaded onto MHC class II molecules (Robinson and Delvig 2002; Watts 2004).

Partially folded MHC class II α/β heterodimers are assembled and stabilized with the type II transmembrane chaperone protein, known as the invariant chain (li) which is found within the endoplasmic reticulum (Cresswell 1996). Signalling via motifs found in the li chain cytoplasmic tail facilitates ER egress to the trans Golgi network and subsequent transport and retention into the endocytic compartment known as MHC class II containing compartment (MIIC) either directly or via the cell surface (Neefjes, Stollorz et al. 1990; Pieters, Bakke et al. 1993). A segment of the li chain occupies the MHC class II peptide binding groove and prevents premature loading of endogenous peptides and partially folded proteins (Roche and Cresswell 1990). Within the MIIC most of the li chain is degraded by cysteine and aspartyl proteinases including the cysteine proteinases cathepsins S and L which leave behind a small peptide remnant known as the class II-associated invariant chain peptide (CLIP) (Nakagawa, Roth et al. 1998; Riese and Chapman 2000). Removal of CLIP is catalyzed by the MHC class II like molecule, human leukocyte antigen-DM (HLA-DM) in exchange for antigenic peptides of ~15 amino acids either within in the endosomal compartment or at the cell surface (Denzin and Cresswell 1995). The molecular mechanisms governing MHC class II transport within the endosomal pathway and to the plasma membrane involve the ubiquitination of peptide loaded MHC class II which acts as a transport signal for MHC class II internalization from the plasma membrane and its sorting to lysosomes. Indeed, ubiquitination of MHC class II is downregulated in activated compared with immature dendritic cells by the differential expression of the ubiquitin ligase MARCH 1 resulting in an increase MHC class II cell surface expression (van Niel, Wubbolts et al. 2008). However, MARCH-1 is highly expressed in B cells and is involved in the regulation of MHC class II degradation via ubiquitination, indicating that this mechanism is central to the turnover of functional MHC class II at the cell surface. (Matsuki, Ohmura-Hoshino et al. 2007).

1.6.4 Antigenic peptide generation and loading of MHC class II molecules

The majority of antigen processing is thought to take place in the same endosomal compartments as li processing (Watts 1997). MHC class II molecules can be found in several different endocytic compartments with distinct alterations in morphology from early to late endosomes (Rocha and Neefjes 2008). Evidence suggests that MHC class II can capture antigen at various points along the pathway (Hiltbold and Roche 2002). Factors affecting peptide processing include:

1.6.4.1 Proteolysis

The requirement for antigen to be degraded into peptides prior to loading of MHC class II molecules was described by Shimonkevitz in 1983 using fixed APC and proteinase degraded ovalbumin. Additional observations from this study were that different proteinases could either preserve or destroy different antigenic epitopes (Shimonkevitz, Kappler et al. 1983). Cysteine, serine, aspartyl and metalloproteinases have all been linked to the processing of different protein antigens into peptide fragments and are capable of generating or destroying immunogenic self-peptides involved in autoimmunity (Bryant, Lennon-Dumenil et al. 2002; Honey and Rudensky 2003). See **Table 1.2** for list of enzymes implicated in antigen processing. Examples include the processing of Goodpastures autoantigen (collagen alpha3(IV)NC1). Cathepsin D diminishes responses in T cells taken from Goodpastures patients and this degradatitve processing could be blocked by the cathepsin D/E inhibitor Pepstatin A (Zou, Hannier et al. 2008).

Cysteine proteinases	**Cathepsins C,B,F,H,K,L,S,N,T,U,V,W,Z and AEP**	*(Honey and Rudensky 2003)*
Aspartyl proteinases	**Cathepsins D and E**	*(Villadangos, Bryant et al. 1999)*
Serine proteinases	**Cathepsin G, elastase, plasmin**	*(Burster, Macmillan et al.) (Honey and Rudensky 2003)*
Other enzymes involved in antigen processing	**GILT**	*(Phan, Arunachalam et al. 2000)*
Endogenous proteinase inhibitors	**Cystatins, high pH, proteinase pro-domains and p41.**	*(Kopitar-Jerala 2006) (Fineschi, Sakaguchi et al. 1996)*

Table 1.2 Proteinases involved in the antigen processing pathway.
Listed above are the major families of proteinases involved in antigen processing including some of the endogenous inhibitors known to control their activation.

Extracellular proteolysis of joint components is one of the earliest observations in rheumatoid arthritis. Endopeptidases and exopeptidases have been implicated in the cleavage of carboxyl termini of naturally occurring peptides that are loaded onto MHC class II molecules (Chicz, Urban et al. 1992). Studies have shown that cathepsin B has both exopeptidase and endopeptidase activity and is involved in the extracellular processing of the joint matrix component aggrecan. Cleavage with cathepsin B or matrix metalloproteinases in the IGD domain of aggrecan produces the small G1 fragment of aggrecan containing the neoepitope site 'VDIPEN' (Mort, Magny et al. 1998). However, the effects of this extracellular aggrecan processing on T cell recognition are not known.

Experiments using mice lacking in one of the enzymes involved in degrading the li chain, cathepsin S (CatS) revealed that the $CatS^-$ mice were resistant to CIA (Nakagawa, Brissette et al. 1999). Observations by Nakagawa *et al* revealed CD4+ T cells from $CatS^-$ mice showed abrogated responses to collagen II but normal responses to several other protein antigens. This implies a dual role for CatS in li degradation and collagen II processing. These data highlight that certain proteinases such as CatS

may be involved in generating arthritogenic peptide repertoire from joint autoantigens such as collagen II but not other antigens and therefore different proteinases must be involved in the processing of different autoantigens.

Other enzymes involved in the processing of protein antigens include the γ-interferon induced lysosomal thiol reductase GILT which reduces disulfide bonds to facilitate unfolding of proteins (Phan, Arunachalam et al. 2000) and the cysteine proteinase asparagine endopeptidase (AEP) implicated as the enzyme involved in the initial steps of li degradation and the dominant enzyme involved in processing the c terminal fragment of tetanus toxin by B cells (Manoury, Hewitt et al. 1998; Manoury, Mazzeo et al. 2003).

Further complexity exists as proteinases themselves can be regulated by endogenous inhibitors such as cystatins. Cystatin C for example, which is expressed by dendritic cells and macrophages, is an endogenous inhibitor of cathepsin S regulated by inflammatory stimuli such as TNFα and IFNγ (Barrett 1986; Pierre and Mellman 1998; Frendeus, Wallin et al. 2009). Furthermore, studies have demonstrated that the pro-inflammatory cytokines TNFα and IL-1β present at high levels in the synovium increase activity of the antigen degrading proteinases cathepsins S and B and increase antigen presentation efficiency, possibly due to indirect effects on cystatins (Fiebiger, Meraner et al. 2001). Proteinase activity within APC is also regulated by levels of proteinase chaperones such as the AEP chaperone p41 li, comparative levels of other proteinases required for the activation of proteinase precursors or chaperone degradation and intra-cellular pH (Watts 2004).

Whilst enhanced lysosomal proteolysis can increase the efficiency of antigen presentation to T cells, degradatitve proteolysis has also been demonstrated to result in less efficient antigen presentation both *in vitro* and *in vivo* (Vidard, Rock et al. 1991; Delamarre, Couture et al. 2006). Therefore, the conditions required for efficient processing and presentation of T cell epitopes will almost certainly vary depending on the stability of the antigen and access of proteinases-specific cleavage sites. In addition the repertoire of proteinases and their endogenous inhibitors (e.g. cystatins, p41) will vary dependent on APC type and activation of proteinases such as cathepsins B, D, E and L which require acidic pH will favour antigens processed in

late acidic compartments rather than early in the endosomal pathway (Goettlich-Riemann, Young et al. 1971).

1.6.4.2 Intracellular pH

Early experiments performed by Ziegler and Unanue, using ammonia and chloroquine to neutralize the pH of endocytic compartments, demonstrated the requirement of an acidic environment for efficient processing of ^{125}I-labelled listeria by macrophages (Ziegler and Unanue 1982). Endosomal compartments become progressively more acidic as they move into the cell due to vacuolar ATPase activity (Mellman, Fuchs et al. 1986). Since those early experiments by Ziegler and Unanue many studies have dissected the importance of endosomal pH and have demonstrated how the intracellular environment modulates proteinase activity, peptide generation and loading onto MHC class II molecules. Low pH has been shown to initiate the initial unfolding of antigens aiding processing for MHC class II presentation (Jensen 1993). Inflammatory triggers such as LPS can also increase the efficiency of antigen processing by increasing ATPase activity in endosomal compartments which in turn lowers the endosomal pH enhancing antigen processing and presentation(Trombetta, Ebersold et al. 2003). An acidic environment is required for the activation of proteinase precursors such as cathepsins L and B (Mason, Gal et al. 1987; Rowan, Mason et al. 1992) and for the activity of the protein reducing enzyme GILT (Phan, Arunachalam et al. 2000).

APC have an extra mechanism for regulating peptide editing in the form of the MHC class II α/β heterodimer HLA-DO. HLA-DO molecules function as HLA-DM regulators thought to be controlled by the pH of the endosomal compartments skewing peptide loading of MHC class II molecules in favour of the more acidic late endosomes (Denzin, Fallas et al. 2005). Interestingly, BCR-mediated uptake triggers rapid transport of antigen/BCR complexes to the late endosomal compartment implying that HLA-DO-HLA-DM regulation is in place so that B cells preferentially present specific antigen taken up via the BCR.

Fine tuning of intracellular pH to prevent destruction of T cell epitopes in early endosomal compartments has also been demonstrated by Savina *et al* in DC. The group demonstrated that NADPH oxidase mediates production of reactive oxygen species which causes sustained alkalization of phagosomes promoting enhanced

antigen presentation (Savina, Jancic et al. 2006). However, its relevance to antigen presentation by other APC is not yet known.

1.6.4.3 The antigen structure

The extent of antigen processing required to generate peptide fragments able to bind MHC class II molecules can differ greatly depending on the antigen structure as reviewed in (Robinson and Delvig 2002). GILT and a low pH may be required to initially reduce disulphide bonds and unfold certain antigens allowing access to the peptide by endosomal proteinases.

Partially degraded or denatured proteins may also bind directly to MHC class II without the need for further intracellular processing such as the candidate joint autoantigen fibrinogen epitope $A\alpha_{551\text{-}578}$ and the model antigen HEL (Lee, Matsueda et al. 1988; Castellino, Zappacosta et al. 1998). However, whether this minimal processing event is sufficient to enable binding of all protein antigens is not known.

Differences in the extent of processing required by the antigen can have a major effect on the survival of certain epitopes. Proteins are increasingly degraded as they travel through the endosomal pathway and some epitopes will be destroyed (termed 'degradatitve processing'). Other epitopes that require minimal processing may be generated extracellulary or within the less proteolytic early endosomes and hence will be available for MHC class II binding (Watts 1997; Delamarre, Couture et al. 2006). MHC class II molecules that recycle from the APC cell surface are the most likely source of MHC class II for binding these minimally processed epitopes as Peptide loading of recycling MHC class II molecules occurs within the mildly acidic environment of the early endosomes (Salamero, Humbert et al. 1990; Griffin, Chu et al. 1997; Accapezzato, Nisini et al. 1998; Pathak and Blum 2000). In addition, there is also evidence of exogenously processed peptides such as those derived from the MS autoantigen myelin basic protein (MBP), binding directly to MHC class II molecules present on the cell surface of APC mediated by cell surface levels of HLA-DM on dendritic cells and B cells (Arndt, Vogt et al. 2000).

Avoiding the degradatitve environment of the late endosomes will alter the peptide repertoire available for class II loading on MHC class II possibly uncovering cryptic

epitopes usually destroyed and therefore not encountered by CD4+ T cells during their development. Another possibility for the generation of an alternate peptide repertoire exists following uptake of sequestered or opsonised antigens by receptors such as the BCR, FcR or complement receptors (CR). Jacquier-Sarlin *et al* have shown that fluid phase uptake of tetanus toxin is improved when C3b has bound to it. Using C3b bound tetanus toxin the group showed that the improved processing and presentation was due to the bound C3b preventing proteolysis by cathepsin D (Jacquier-Sarlin, Gabert et al. 1995). Additionally immune complexes containing C3d can augment the signal through BCR ligation via CR2 thus improving presentation (Edwards, Cambridge et al. 1999; Barrault and Knight 2004). Antigen-capture via the BCR, Ig or CR results in the entire internalized BCR/Ig/complement-antigen complex becoming the substrate for endosomal processing (West, Lucocq et al. 1994; Jacquier-Sarlin, Gabert et al. 1995)}. The consequences of this are that antigenic epitopes masked by the BCR/Ig/complement will either be suppressed due to the physical masking of proteinase cleavage sites or enhanced due to their increased survival during transit to the MHC class II compartment (Watts and Lanzavecchia 1993; Jacquier-Sarlin, Gabert et al. 1995; Simitsek, Campbell et al. 1995). In addition, minimally processed antigen bound to the BCR can be captured by neighbouring membrane-bound MHC class II molecules in a sort of hand-over manner. The relevance of this finding is that early capture of minimally processed antigen could improve the survival of certain T cell epitopes that would normally be destroyed within the degradatitve environment of late endosomes (Moss, Tree et al. 2007).

An additional outcome of this minimal processing is that suppression of dominant MHC class II epitopes that require the proteolytic environment of late endosomes may result in the enhanced MHC class II loading and subsequent presentation of sub-dominant epitopes that would be otherwise invisible to the immune system (Dai, Carayanniotis et al. 2005). The efficiency of BCR mediated uptake and presentation is clearly influenced by the stability of the BCR-antigen complex. Rapid transit of the BCR/antigen complex may be necessary to retain antigen on the BCR long enough for delivery to the MHC class II (Aluvihare, Khamlichi et al. 1997) whereas in the absence of a high affinity interaction certain epitopes have been shown to be preferentially processed earlier in the endosomal pathway where they can be presented by recycling MHC class II molecules (Brooks and Knight 2004). The

resulting consequences of this tight affinity are the destruction of certain T cell epitopes by delivery into a more degradatitve acidic compartment or conversely protection of certain T cell epitopes from degradation by the sequestering nature of the BCR (Davidson and Watts 1989).

Post-translational modification of proteins by phosphorylation, glycosylation, methylation and citrullination leads to subtle differences in the way that antigens are processed by APC generating neoepitopes with enhanced affinity for TCR, BCR or MHC (Anderton 2004). Evidence indicates that certain T cell epitopes require specific methods of proteolysis to avoid/promote their degradation. Therefore it is possible that a particular combination of antigen modification, uptake, enzyme degradation and APC type could result in the generation and subsequent presentation of cryptic epitopes that were not previously generated by APC involved in T cell tolerance induction mechanisms (Goodman and Sercarz 1983).

1.6.5 Inhibition of the antigen processing and presentation pathway.

As detailed above differences in antigen structure will affect the extent of proteolysis required, the pH environment required for initial antigen unfolding and access to proteinase cleavage sites and which APC type is most equip to efficiently process the antigen. Over the years many studies with a variety of chemical inhibitors of various components of the antigen processing pathway have been utilised to dissect the requirement for a particular proteinase, pH, and protein synthesis. This inhibitor system can be used to uncover the processing requirements of different antigens involved in infection, cancer and autoimmunity possibly leading to the discovery of antigen-specific targeting for autoimmune or cancer therapy or improved delivery systems for targeting vaccines.

1.6.5.1 Cycloheximide and Brefeldin A

Cycloheximide is an inhibitor of protein synthesis that acts by binding to ribosomes and preventing eEF2-mediated translocation which depletes newly synthesised MHC class II molecules (Schneider-Poetsch, Ju et al.; Kerridge 1958).

Brefeldin A *is* an inhibitor of ER/Golgi transport that disrupts the delivery of newly synthesised proteins (including MHC class I and II molecules) to the cell surface or endosomal pathway. The effects of Brefeldin A are tubulation of the Golgi/ER/endosomal pathway that result in attenuated Golgi/ER transport but normal cell surface recycling of previously generated MHC class II. Newly synthesised proteins are retained in the ER of Brefeldin A treated cells and become processed by Golgi enzymes (Orci, Tagaya et al. 1991; Klausner, Donaldson et al. 1992).

Therefore, both inhibitors can be used to determine whether epitopes are presented via the classical pathway of antigen presentation on newly synthesised MHC class II molecules or via the recycling pathway loaded in the mildly acidic early endosomes on recycling class II molecules, independently of newly synthesised MHC class II and the li chain (St-Pierre and Watts 1990). Indeed, Brefeldin A and cycloheximide has been used to verify the processing pathway of several antigens including the M5 protein of *Streptococcus pyogenes*, capsular Caf1 antigen of *Yersinia pestis* and influenza hemaglutinin protein. Results from these experiments have revealed that whilst some antigenic epitopes require minimal processing and are presented by recycling MHC class II other epitopes from the same antigens require presentation via the classical pathway on newly synthesised MHC class II (Delvig and Robinson 1998; Sinnathamby and Eisenlohr 2003; Musson, Morton et al. 2006). Antigens presented via the recycling pathway require minimal processing and may be partially degraded by extracellular enzymes such as MMPs upregulated during inflammatory conditions (Accapezzato, Nisini et al. 1998; Santambrogio, Sato et al. 1999). The recycling pathway is independent of the li chain and subsequently the requirement for H-2 DM editing. Therefore, it is possible that this less stringent peptide loading and extracellular degradation may result in the presentation of immunogenic epitopes that have not been encountered during T cell development and are subsequently not tolerized against. Therefore, uncovering the presentation pathway of

immunodominant epitopes from candidate joint autoantigens such as aggrecan may help to elucidate their role in arthritic diseases such as RA

1.6.5.2 Ammonium chloride

Ammonium chloride is a weak base agent that can be used to raise the endosomal pH to pH 6.5 or above (Poole and Ohkuma 1981). Endosomal pH plays an important role in the activation of proteolytic enzymes, the initial unfolding of antigens and peptide loading of MHC class II (Jensen 1993; Riese and Chapman 2000; Denzin, Fallas et al. 2005). Most endosomal aspartyl and cysteine proteinases function optimally at acidic pH (aspartic proteinases pH 2.8-4, cysteine proteinases pH 5-6) with the exception of cathepsin S that remains functional within the pH range of most endosomal compartments (Fineschi and Miller 1997). The serine and metalloproteinases are functional closer to neutral pH and are thought to be involved in the extracellular processing of antigens (Bond and Butler 1987).

Due to the differences in the requirements of endosomal proteinases for a pH optimum, the effects of pH change on the processing of specific antigens can be determined by the use of ammonium chloride or other pH affecting agents such as chloroquine (Yoshikawa, Watanabe et al. 1987; Musson, Walker et al. 2003). In addition, the use of pH altering agents not only clarifies the requirement for a particular pH environment for proteinase activity, but it can also indicate whether an antigen is processed within early endosomal compartments implying the involvement of the recycling pathway or in late endosomes indicating presentation via the classical pathway.

1.6.5.3 Proteinase inhibitors

As described in **Section 1.6.4.1** proteinases are involved in both the removal of the li chain from MHC class II and in generating peptide fragments to fit into the MHC class II binding groove. However, the generation of some epitopes appear to be reliant on one or more of these proteinases, whereas others may not be reliant on any. Examples of this are described above in **Section 1.6.4.3**. Therefore the requirement of a specific proteinase for the generation of a particular antigenic epitope can be determined by the use of proteinase or proteinase family specific inhibitors. However, results must be interpreted with caution due to the possibility of redundancy

between proteinase families. Indeed, AEP has been implicated as playing an important role in the initial degradation of the li chain (Manoury, Mazzeo et al. 2003; Watts, Mazzeo et al. 2003). However, results from experiments using AEP deficient mice have shown that AEP is not essential for li degradation implying that other proteinases are capable of mediating this step in the absence of AEP (Maehr, Hang et al. 2005).

Pepstatin A is an aspartyl proteinase inhibitor shown to inhibit cathepsin D and E and previously used to show the involvement of aspartyl proteinases in early li degradation whilst having no effect on the peptide generation from OVA antigen (Zhang, Maekawa et al. 2000). In contrast, pepstatin A has been shown to augment responses to epitope 274-286 found in the diabetes autoantigen GAD65 (Reijonen, Elliott et al. 1999). However, using the same autoantigen Reijonen et al showed that the cysteine and serine inhibitor leupeptin inhibited T cell responses (Reijonen, Elliott et al. 1999). These data clarify that different antigens/antigenic epitopes are processed by different proteolytic enzymes and the processing requirements of autoantigens such as those implicated as playing a role in autoimmunity can be determined using proteinase inhibitors.

However, problems with inhibitors such as pepstatin A are that they are poorly soluble and inefficiently transported across the cell membrane. Therefore new studies into improving their delivery via linking to BSA or mannose and the generation of new cell permeable alternatives have been described (Mohamadzadeh, Mohamadzadeh et al. 2004; Free, Hurley et al. 2006; Zaidi, Burster et al. 2007). Indeed, cell permeable alternatives of pepstatin A demonstrate the role of aspartic proteinases in the generation of peptide fragments from tetanus toxoid and protein purified derivative by different APC.

Leupeptin is a cysteine and serine inhibitor shown to inhibit the proteinase cathepsin B. Earlier studies elucidated a role for cysteine and serine proteinases in the generation of some but not all antigenic fragments derived from hen egg lysozyme (Puri, Lonai et al. 1986) and keyhole limpet hemocyanin (Yoshikawa, Watanabe et al. 1987). However, as described above cysteine proteinases may also have a destructive role in the processing of certain antigenic epitopes as mentioned above where

leupeptin abrogated T cell responses to certain epitopes derived from the autoantigen GAD65 (Reijonen, Elliott et al. 1999). This has also been shown for the certain epitopes from the model antigen OVA, $OVA^{322-336}$ presentation is augmented in the presence of leupeptin and destroyed if digested *in vitro* with the cathepsins B and L (Rodriguez and Diment 1995). (2S,3S).trans.epoxysuccinyl-L-leucylamide-3-methylbutaneethylester (E64-d) is a broad spectrum membrane permeable cysteine proteinase inhibitor that irreversibly inhibits cathepsins S, L and B (Bond and Butler 1987). E64-d has been shown to affect the generation and presentation of some but not all epitopes derived from the recombinant protective antigen (PA) of *Bacillus Anthracis* and showed a profound negative effect on presentation of 3 epitopes derived from the V antigen from *Yersinia pestis* (Musson, Walker et al. 2003; Shim, Musson et al. 2006). Interestingly, the finding that leupeptin and E64-d do not affect the presentation of all epitopes investigated implies that cysteine proteinases are playing a role in the generation of antigenic epitopes and not in li degradation that would abrogate all peptide loading of MHC class II.

3,4-dichloroisocoumarin (DCI) is a broad spectrum serine proteinase inhibitor and Phenanthroline is a broad spectrum inhibitor of metalloproteinases. Serine and metalloproteinases are considered as extracellular non endosomal enzymes involved in antigen processing at the cell surface or in the extracellular space at neutral pH, examples include trypsin (Accapezzato, Nisini et al. 1998) the metalloproteinase (aminopeptidase N) involved in trimming of peptides bound to MHC class II (Amoscato, Prenovitz et al. 1998) and various members of the matrix metalloproteinase family involved in degrading extracellular matrix components detailed in **section 1.7.2** (Cawston and Young 2010).

Studies using DCI and phenanthroline have implicated a crucial role for serine proteinases but not metalloproteinases in the generation of PA epitopes (Musson, Walker et al. 2003) and the crucial role for metalloproteinase MMP-9 in the generation of epitopes derived from the candidate autoantigen collagen II (Van den Steen, Proost et al. 2002). B cell, macrophage and dendritic cell expression of MMP-9 has been reported therefore it is possible that these APC are involved in the extracellular processing of joint autoantigens (Di Girolamo, Tedla et al. 1998; Trocme, Gaudin et al. 1998; Bartholome, Van Aelst et al. 2001; Gough, Gomez et al. 2006).

Therapeutic benefits of targeting various cathepsins are now coming to light. Uncovering the role of cathepsin S has lead to the development of potential therapeutics for targeting immune diseases such as Rheumatoid Arthritis the efficacy of which is currently under investigation (Gupta, Singh et al. 2008). In addition, the role of MMPs in cartilage degradation during rheumatoid arthritis progression is well established. However, their role in the generation of immunogenic epitopes from candidate joint autoantigens remains to be clarified. Further understanding of how immunogenic epitopes are generated from candidate autoantigens and their effects on breaking T cell tolerance will give us a greater insight into the initiation/chronicity of potentially antigen-driven autoimmune diseases such as RA.

1.7 Proteoglycan aggrecan as a candidate joint autoantigen

MHC class II association and the presence of autoreactive T cells and antibodies in patients with RA suggest the involvement of joint autoantigens in disease pathogenesis. However, the autoantigen responsible for the onset or perpetuation of the disease remains to be elucidated. One possible candidate autoantigen is aggrecan, the large aggregating proteoglycan found in the articular cartilage, brain, meniscus, tendons and muscle.

1.7.1 Aggrecan structure and function

Aggrecan is the major proteoglycan found in articular cartilage. Articular cartilage is predominantly made up of ECM produced and maintained by resident cartilage cells (chondrocytes). ECM consists of type II collagen, proteoglycans including aggrecan, decorin, fibromodulin and biglycan, hyaluronan and various other matrix proteins and elastic fibres (Kiani, Chen et al. 2002; Poole, Kobayashi et al. 2002). Aggrecan structure and function has been reviewed and is summarized below (Hardingham, Fosang et al. 1994; Kiani, Chen et al. 2002; Dudhia 2005).

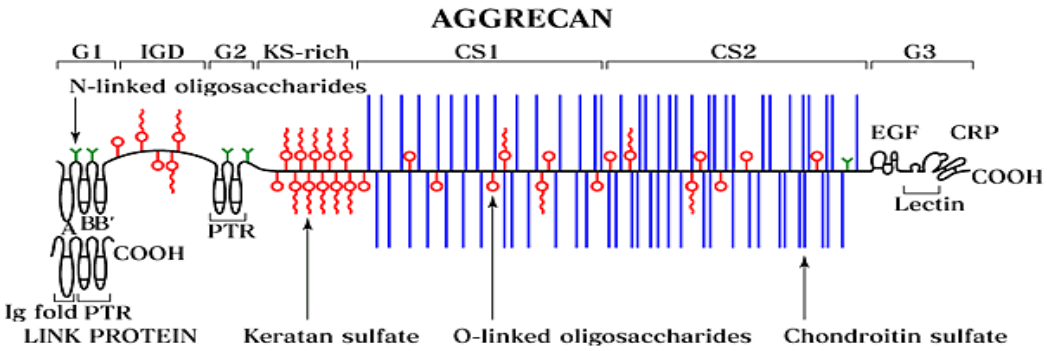

Figure 1.4 Schematic representation of the structure of aggrecan.
Aggrecan contains 3 globular domains G1, G2, G3 separated by two inter-globular domains (IGD). The IGD that separates domains G1 and G2. There are 2 chondroitin sulphate regions (CS1 and CS2) and 1 keratan sulphate region (KS-rich). The G2 region consists of a proteoglycan tandem repeat (PTR). The G3 region contains 4 different motifs, the complement regulatory protein motif (CRP), 2 epidermal growth factor regions (EGF) and a C-type lectin motif (Lectin). The structure of the link protein is very similar to that of the G1 region of aggrecan, as both regions contain an immunoglobulin fold (Ig fold) and 2 PTR. (Hardingham,T.E et al Eur J Clin Chem Clin Biochem, 1994)

The structure of aggrecan consists of a protein core of around 250 kDa containing 3 highly conserved globular (G) domains (G1, G2, and G3), an inter-globular domain (IGD) and a GAG attachment region consisting of keratan sulphate (KS) and chondrotin sulphate (CS) sidechains. The N terminal G1 domain contains an immunoglobulin fold (Ig fold) region of around 100 amino acids that forms 2 β sheets stabilised by a disulphide bond. The Ig fold promotes the interaction with the G1-like link protein responsible for stabilising aggrecan interactions with hyaluronan within the matrix (Grover and Roughley 1994). The G1 domain also contains 2 copies of a motif known as proteoglycan tandem repeat (PTR) which form 2 disulphide bonds per tandem responsible for aggrecan binding to HA (Watanabe, Yamada et al. 1998). The interaction with HA is also stabilised by an o-linked oligosaccharide side chain (Hascall and Heinegard 1974).

The G2 domain contains 2 PTR motifs and is functionally linked to aggrecan secretion (Kiani, Lee et al. 2001). The KS domain is the major site of KS attachment. Approximately 30 KS chains are attached to one aggrecan molecule predominantly at the KS domain, but also at the IGD, G1 and G2 regions (Fosang and Hardingham 1991; Barry, Neame et al. 1994). The KS attachment sequence is a repeating region of proline-serine and proline threonines found in both human and bovine aggrecan (Antonsson, Heinegard et al. 1989; Doege, Sasaki et al. 1991). The CS domain is the largest aggrecan domain and contains approximately 100 CS chains. The CS attachment sequence is proposed to be a series of ser-gly dipeptide repeats separated by an acidic residue (Bourdon, Krusius et al. 1987).

The G3 domain contains 2 epidermal growth factor like domains (EGF), a carbohydrate recognition domain (CRD) and a complement regulatory protein B domain (CRB). The function of the G3 domain is thought to be involved in mediating aggrecan secretion and in post translational modifications such as glycosylation (Kiani, Lee et al. 2001).

Aggrecan is immobilised within the extracellular matrix via its association with hyaluronan. This association aided by a 41-48 kDa link protein can produce aggregates containing over 100 aggrecan/link proteins aggregated with 1 hyaluronan chain (Dudhia 2005). Aggregates with molecular masses of up to 10^9 Da are highly

anionic due to the fixed negative charge of the glycosaminoglycan side chains; providing hydration of the cartilage, nutrient and solute transport and weight bearing resistance during joint loading (Maroudas, Muir et al. 1969; Dudhia 2005). Loss of aggrecan aggregates leads to a loss in its functionality and eventual cartilage destruction.

1.7.2 Aggrecan breakdown and turnover in disease: the role of Metalloproteinases.

Both of the major components of the extracellular matrix collagen II and aggrecan are relatively long lived and undergo considerable modification during the normal ageing process (Dudhia 2005). However, in rheumatoid arthritis one of the earliest observed changes is the depletion of cartilage aggrecan (Rousseau, Sumer et al. 2008). Aggrecan is degraded at distinct cleavage sites during the course of arthritis by the action of degrading proteinases such as a disintegrin and metalloproteinase domain (ADAMTS) 1,4,5,8,9 and MMPs 1,2,3,7,8,9,13,14,19,20, cathepsins G and B, calpain and elastase (**Figure 1.5**) (Billington, Clark et al. 1998; Struglics, Larsson et al. 2009; Cawston and Young 2010; Struglics and Hansson 2010).. In addition, aggrecan degradation is thought to occur because of the differential expression and hence loss of balance between aggrecan degrading enzymes such as MMPs and their endogenous inhibitors tissue inhibitors of metalloproteinases (TIMPS) (Tetlow and Woolley 1999; Ishiguro, Ito et al. 2001).

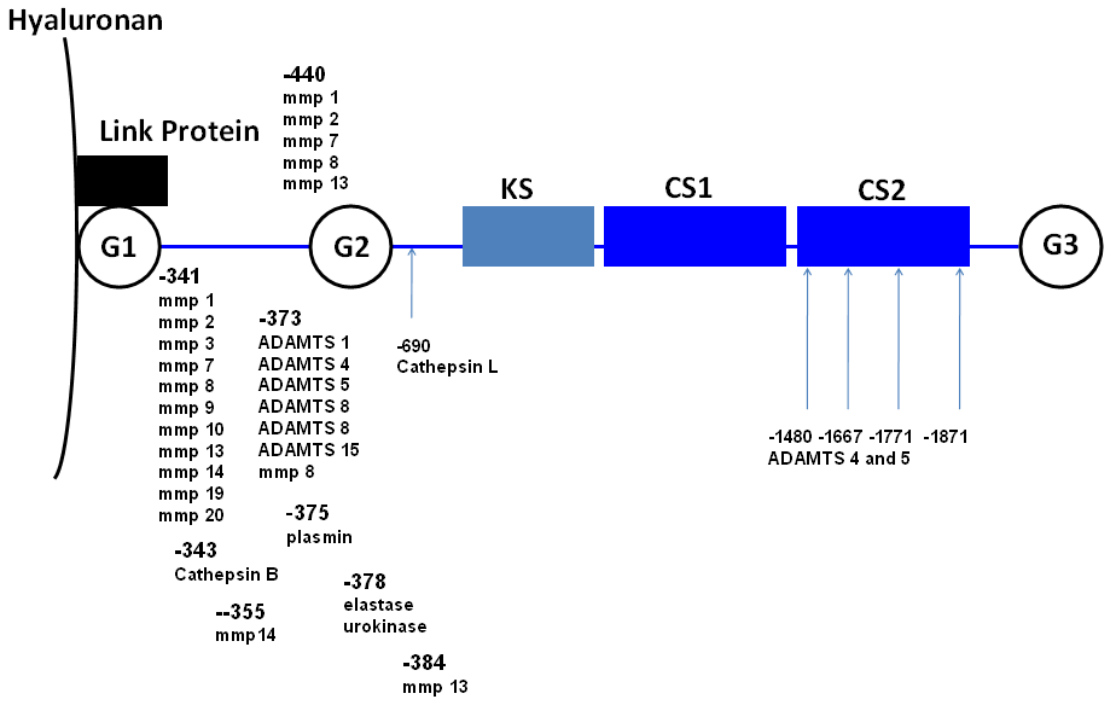

Figure 1.5 Aggrecan cleavage by extracellular proteinases.

Aggrecan cleaved by extracellular proteinases. Aggrecan core protein contains 3 globular domains G1, G2 and G3. Cleavage of aggrecan occurs most prominently within the G1 and G2 regions by matrixmetalloproteinases (MMP) or a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS). Proteinases and their corresponding cleavage sites are depicted above.

MMPs play a major role in the removal of ECM during tissue resorption and are involved in many physiological functions including normal joint turnover, wound healing, bone remodelling and angiogenesis as well as pathogenesis of diseases such as RA (Nagase and Woessner 1999). During RA pathogenesis MMPs are produced by activated fibroblast-like synoviocytes, infiltrating immune cells such as neutrophils and macrophages, osteoclasts and chondrocytes (Yoshihara, Nakamura et al. 2000; Zeisel, Druet et al. 2005; Takaishi, Kimura et al. 2008). However, their expression has also been reported by B cells and dendritic cells (Kouwenhoven, Ozenci et al. 2002; Melamed, Messika et al. 2006). MMPs are synthesised as inactive zymogens and the

difference between normal joint turnover and joint disease requires a careful balance between their activation mediated by autocatalysis, other MMPs and serine proteinases and their inhibition by TIMPs, a disruption of this balance can lead to the unwanted destruction of cartilage components including aggrecan and collagen II (Ra and Parks 2007).

Neoepitope-specific monoclonal antibodies have been developed that recognise the N and C terminus of aggrecan cleavage products and not intact aggrecan and have been useful tools in determining cartilage degradation during arthritic diseases such as RA (Fosang, Last et al. 2010). Two main cleavage sites result in the generation of aggrecan neoepitopes, C terminus VDIPEN-341 ↓ N terminus 342-FFGVGGE and NITEGE-373 ↓ 374-ARGSV (Singer, Kawka et al. 1995; Fosang, Last et al. 1996; Lark, Bayne et al. 1997).

The increased expression of MMPs and ADAMTS is thought to be predominantly mediated by the pro inflammatory environment of the inflamed synovium. Studies using bovine cartilage explants stimulated with pro-inflammatory cytokines such as IL-1 and TNFα have revealed an increased production of ARGSV neoepitopes detectable by the neoeptiope-specific monoclonal antibody BC-3 (Hughes, Caterson et al. 1995). More importantly, IL-1 and TNFα have been demonstrated to upregulate levels of MMPs and the G1 neoepitope fragments VDIPEN-341 and NITEGE-373 in cartilage and synovial fluid from rheumatoid patients (Lark, Bayne et al. 1997; Sondergaard, Henriksen et al. 2006; Larsson, Lohmander et al. 2009). The significance of this is that aggrecan cleavage by MMPs in the IGD domain results in the ARGSV and NITEGE fragments, a total loss of glycosaminoglycan sidechains from aggrecan and hence a loss of aggrecan functions. Furthermore, additional cleavage sites within the link protein which can result in the release of the entire aggrecan molecule from the cartilage, although cleavage in this domain is not as significant as that within the IGD (Wells, Davidson et al. 2003).

However, as illustrated in **Figure 1.5**, the IGD is not the only aggrecan cleavage site targeted by MMPs the G3 domain is very susceptible to proteolysis and loss of G3 can occur shortly after its secretion (Lee, Chen et al. 2002). Indeed, the heterogeneity of aggrecan fragments isolated from cartilage is partly due to the loss of the G3

domain and some of the CS region (Hardingham, Fosang et al. 1990) probably due to proteolytic attack. The IgM antibody 846 recognises chondroitin sulphate sidechains attached to amino acid 846 present within the G3 domain of aggrecan and has been utilised to detect the presence of newly synthesised aggrecan that has not yet been incorporated into the ECM (Mansson, Carey et al. 1995). The presence of 846 epitopes is barely detectable in healthy joints yet increased in synovial fluid of diseased joints of RA patients indicative of the degradation of newly synthesised aggrecan (Poole and Dieppe 1994; Mansson, Carey et al. 1995). In addition, as cleavage within the IGD domain of aggrecan results in a G1 fragment remaining bound to hyaluronan within the cartilage, this may act as a competitor for the incorporation of newly synthesised aggrecan molecules.

The increase in the concentration of aggrecan fragments found in serum and synovial fluid may trigger or potentiate the immune response within the joint. In addition, loss of the CS region as well as a loss of CS side chains from aggrecan has been reported in RA synovial fluid (Ortutay, Polgar et al. 2003). Therefore, differentially modified aggrecan fragments could result in altered processing requirements by APC, the generation and presentation of cryptic epitopes and a break in T cell tolerance. In addition the number of aggrecan molecules with intact G3 declines with age as does the incidence of RA indicating that as extracellular processing of aggrecan increases more antigen is available to break any tolerance mechanisms in place to prevent T or B cell autoreactivity (Dudhia, Davidson et al. 1996).

1.7.3 Proteoglycan induced arthritis

Animal models that mimic clinical, pathological and histological features of RA such as collagen induced arthritis (CIA) and proteoglycan induced arthritis (PGIA) allow preliminary studies of a genetically identical population of diseased animals without the complication of disease heterogeneity that hinders human RA studies. Additional benefits of mouse models of RA are that the "Day 0" of disease onset is experimentally induced and the subsequent steps in disease pathology can be followed which is not possible with human studies (Kannan, Ortmann et al. 2005).

PGIA is a murine model of RA in which disease can be induced in either, BALB/c ($H\text{-}2^d$) or HLA-DR4 or HLA-DQ8 humanized transgenic mice and also in some C3H

(H-2^k) strains, by the immunization with deglycosylated aggrecan in adjuvant (Glant, Bardos et al. 2001; Szanto, Bardos et al. 2004).

PGIA is a Th1 mediated disease and Th17 cells are not required either for its onset or severity (Hollo, Glant et al. 2000; Doodes, Cao et al. 2008). However, IL-17 is able to mediate disease in the absence of IFNγ (Doodes, Cao et al. 2010). In addition, IL-27 expressed within the joints and spleen of arthritic PGIA mice is important in mediating Th1 differentiation required for the induction of disease (Cao, Doodes et al. 2008).

The requirement for MHC class II restriction implies that predisposition to disease is governed by the polymorphic MHC class II molecules able to present autoantigenic peptides to T cells (Abraham and David 2000). Human, canine, porcine, sheep or bovine aggrecan are all capable of inducing PGIA providing both the CS and KS side chains are removed. Disease requires 2-4 intraperitoneal injections of deglycosylated aggrecan and peak of onset is around 50-70 days (Glant and Mikecz 2004).

Studies have identified that the predominant immunogenic epitopes of aggrecan are found in the G1 domain (Leroux, Guerassimov et al. 1996; Zhang, Guerassimov et al. 1998). However, the immunogenic properties of aggrecan or G1 are reliant on the removal of the CS and KS chains or KS only in the case of G1 by chondroitinase and keratanase. This observation implied that glycosylation may influence the immune responses of aggrecan-specific T cells. Indeed, *in vitro* experiments clarified that removal of the KS chains from G1 domain improves presentation of aggrecan to cloned aggrecan-specific T cell hybridomas and identified the predominant T cell epitope 70-84 located in the A loop of G1 (Zhang, Guerassimov et al. 1998). **[Note: - Epitope 70-84 is defined as 84-103 in this thesis when including the aggrecan signal sequence of 19 amino acids]**. Peptide mapping studies have uncovered 3 other epitopes identified as arthritogenic 49-63 located in the A loop of G1, 155-169 located in the B loop of G1 and 2373-2387 located in the G3 domain designated peptide p135H (Hanyecz, Bardos et al. 2003; Buzas, Vegvari et al. 2005). Indeed, this G3 epitope ^{2373}TTYKRRLQKRSSRHP^{2387} contains a highly homologous sequence motif (underlined) to that of the arthritis susceptibility shared epitope found in HLA-DR4 molecules. The arthritogenic properties of the G3 p135H were demonstrated when

SCID mice hyper-immunized with p135H developed arthritis following one subsequent immunisation with human deglycosylated aggrecan in incomplete Freunds adjuvant (Hanyecz, Bardos et al. 2003).

Disease onset is preceded by a humoral Ig response and requires both T and B cells as both are required to transfer disease to synergic irradiated BALB/c mice (Mikecz, Glant et al. 1990). T cells are required to act as 'help' in supporting autoantibody production (Angyal, Egelston et al. 2010). However, transfer of antibodies alone is not sufficient to transfer disease (Mikecz, Glant et al. 1990).

Later studies by Brennan *et al* implicated B cells as being capable of presenting immunodominant epitopes from the G1 domain to the aggrecan specific T cell hybridoma 5/4E8 (Brennan, Negroiu et al. 1995). The 5/4E8 clone was later shown to induce PGIA upon adoptive transfer into naïve BALB/c mice (Buzas, Brennan et al. 1995). The immunogenic epitope recognised by 5/4E8 was later confirmed to be G1 ^{84}ATEGQVRVNSIYQDK^{103} (Buzas, Hanyecz et al. 2003). This epitope was shown to be the dominant epitope in BALB/c, HLA DR4 and DQ8 mice (Buzas, Vegvari et al. 2005). To further analyze the role of 84-103-specific T cells 5/4E8 in PGIA a transgenic mouse was generated expressing the 5/4E8 Va1.1 and Vβ4 TCR. Mice were generated in a CBA X C57BL/6 background and backcrossed into a BALB/c background. Studies using the TCR-5/4E8 transgenic mice have shown that transfer of TCR-5/4E8 spleen cells along with human aggrecan into synergic BALB/c RAG2-/- mice induced PGIA. TCR-5/4E8 transgenic mice display increased arthritis susceptibility and developed an aggressive form of the disease upon immunisation with deglycosylated human cartilage proteoglycan (Berlo, van Kooten et al. 2005; Berlo, Guichelaar et al. 2006).

PGIA resembles the clinical features of human RA and data generated from this model uncovers the generation of aggrecan-specific antibodies and T cell mediated disease progression similar to that seen in RA patients. Using this model O'Neill *et al* demonstrated the essential role for B cells in disease pathogenesis. Importantly, they also showed that B cells were required to act both as APC as well as secreting antibodies against aggrecan (O'Neill, Shlomchik et al. 2005).

Indeed as discussed in **Section 1.4.3**, O'Neill and colleagues later demonstrated that expression of the co-stimulatory molecules CD80/86 on B cells is required for optimal activation of T cells required for T cell mediated transfer of arthritis from WT into SCID mice. (O'Neill S, Cao et al. 2007). In support of this finding another study investigating PGIA development demonstrated that there was a reduction in disease, an inability of T cells to transfer disease into SCID mice and a significant reduction in T cell recall to aggrecan following B cell depletion (Hamel, Doodes et al. 2008).

Studies using humanized mice expressing HLA DR4 and HLA DQ8 enabled peptide mapping of aggrecan epitopes without the genetic variation associated with human studies. Data from these experiments identified that 24 out of 31 HLA positive immunogenic human aggrecan peptides presented on relevant RA susceptibility HLA alleles were located in the G1 domain. Importantly, GRVRVNSAY located within the 70-84 (84-103) region was identified as 1 of 2 G1 peptides identified as dominant/arthritogenic in BALB/c mice and DR4. Ab^0 (Szanto, Bardos et al. 2004).

1.7.4 Humoral and cellular responses to aggrecan in RA patients.

A case for the role of T and B cells in the pathogenesis of RA has been demonstrated by the observations discussed in previous sections of this thesis. Aggrecan destruction during disease and its role in generating an RA like disease in animal models has also been discussed. However, both cellular and humoral immune responses to aggrecan have been detected in patients with several rheumatic diseases such as rheumatoid arthritis but also osteoarthritis, ankylosing spondylitis and juvenile idiopathic arthritis (Golds, Stephen et al. 1983; Sigal, Johnston et al. 1988; Kamphuis, Hrafnkelsdottir et al. 2006; de Jong, Berlo et al. 2010). Cellular responses to deglycosylated aggrecan, specifically epitopes within the G1 domain, have been described above.

To demonstrate T cell reactivity to G1 domain in patients suffering from RA Li *et al* generated and characterised 15 G1 specific T cell lines from rheumatoid arthritis patients and data from this study showed that 14 out of 15 patients expressed a Th1 profile. G1-specific T cells were detectable at 4.97 per 10^{-6} in peripheral blood lymphocytes and 9.85 per 10^{-6} in synovial fluid (Li, Zhang et al. 2000). Furthermore, later studies by Zou *et al* identified G1 responses in RA and AS patients and went on to define the immunodominant peptides within the G1 region as 116-133, 148-165,

252-269 and 292-309 in AS and 116-133, 148-165, 252-269 and 292-309 in RA (Zou, Zhang et al. 2003). This group tested peptide responses to G1 previously demonstrated in HLA transgenic mice and identified peptides 16-39 and 263-282 as the peptides responded to most frequently in RA patients (SI >1.8 in over 50% patients). Interestingly, the aggrecan epitope 263-282 has a high sequence homology with *Yersinia* Yop protein and RA patients responding to *Yersinia* Yop also responded to 263-282, responses to Yop were not detected in healthy controls (de Jong, Berlo et al. 2010). These data suggest a possible role for "molecular mimicry" of infectious components in the breaking of T cell tolerance in susceptible patients. This phenomenon of microbial peptides cross reacting with self-antigens has been previously described and implicated in driving RA following immunization with hepatitis B surface antigen (Pope, Stevens et al. 1998; Hill, Wang et al. 2003).

Finally, responses to modified aggrecan p84-103 have been reported recently, >60% of RA patients (total number 28 and none of the 18 healthy controls) responded to *in vitro* citrullinated but not un-citrullinated aggrecan p84-103. Interestingly, the PBMCs responding to citrullinated aggrecan p84-103 produced high levels of IL-17 in response to stimulation, this is in contrast to animal models of PGIA that are found to be independent of Th17 (Doodes, Cao et al. 2008; von Delwig, Locke et al. 2010). Indeed, increased levels of IL-17 have been reported in patients suffering from RA (Ziolkowska, Koc et al. 2000; Miossec 2004). However, contrasting data from different animal models indicate that the requirement for IL-17 is probably regulated by a balance of other mediators such as IFNγ, IL-15 and IL-27 and possibly differs dependent on the autoantigen or indeed the post translational modifications of autoantigen involved (Ziolkowska, Koc et al. 2000; Nakae, Nambu et al. 2003; Cao, Doodes et al. 2008; Doodes, Cao et al. 2008).

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In addition to the cellular responses to aggrecan observed in RA patients, increased levels of autoantibodies have also been reported in synovial fluids and sera from rheumatoid arthritis patients compared to healthy controls, particularly to the G1 domain (Karopoulos, Rowley et al. 1996; Vynios, Tsagaraki et al. 2006). This data implies that there is a localised immune response to aggrecan possibly as a result of the germinal centre like structures observed in the synovium of many RA patients.

1.8 Aims of the project

The aims of this project were to develop a system for studying aggrecan processing and presentation by aggrecan-specific B cells and to compare aggrecan processing and presentation by B cells to that seen with other APC. Specific targets were:

- To isolate the model antigen (proteoglycan aggrecan).
- To generate aggrecan-specific B cells
- To investigate the kinetics of aggrecan processing and presentation by aggrecan-specific B cells in comparison to that of dendritic cells and macrophages.
- To investigate the intracellular and extracellular enzymes involved in aggrecan processing by the different APC.
- To determine the pathway involved in the presentation of the immunodominant G1 epitope 84-103 by the different APC.

2. Materials and Methods

2.1. Antigens

2.1.1 Bovine Aggrecan Preparation

Aggrecan was prepared as previously described (Billington, Clark et al. 1998).

2.1.1.2. Dissection

Bovine nasal sections were supplied by Coast and County Meat Supply Co.Ltd, (Gateshead, Tyne & Wear) and dissected as follows: Noses were cut half way down the septum behind the nostrils, then along the top of the nostrils to give a T shaped segment, then cut along the bottom to release the septum. All soft tissue was removed and the septa were scraped until white. Septa were weighed, washed in Dulbeccos phosphate buffered saline (PBS) (Biowhittaker) and cut into approx 2x2x2 mm pieces. The pieces of nasal septum were placed in extraction buffer at 10ml/g (50 mM Na Acetate pH 6.0, 0.4 M GuHCL and 10 mM sodium salt of EDTA). 2 mM Phenylmethanesulfonyl fluoride (PMSF) in 100% ethanol, 5 mM benzamidine in 50 mM Na acetate buffer pH 6.0 and 0.1 mM 6-aminohexanoic (caproic) acid in 50 mM Na acetate buffer pH 6.0 were added to extraction buffer immediately prior to use. The septum fragments were homogenised thoroughly using an Ultra-Turrax T25 (IKA-Labortechnik, Loughborough, UK) and left stirring in the extraction mixture at 4^{o}C for 60 h.

2.1.1.3. Proteoglycan separation by caesium chloride gradient

Extraction supernatant was decanted into Oak ridge tubes (35ml/tube) (Sorvall) and centrifuged at 16,500G for 23 min (Sorvall RC5 Plus). Supernatants were carefully removed and pooled. Caesium chloride was added to give a final concentration of 609 mg/ml. The resultant solution was transferred to 35 ml Sorvall Ultracentrifuge tubes, sealed and centrifuged at 36,300G (rotor TFT 70.38 and TFT 65.38 Sorvall Ultracentrifuge) at 10^{o}C for 24 h. Following centrifugation, tubes were snap-frozen in a CO_2/methanol bath and stored at -80^{o}C. The bottom 25% of each tube was removed using a sterile hacksaw, thawed and dialysed at 4^{o}C in size 3 dialysis tubing (Sigma)

against 2 L of 50 mM Tris acetate pH 7.4, 10 mM EDTA. Dialysis buffer was changed 4 times over 48 h.

2.1.1.4. Deglycosylation

The chondroitin sulphate content of both glycosylated and deglycosylated aggrecan was measured using the Dimethyl-methylene blue (DMB) assay (Farndale, Sayers et al. 1982). 250 µl DMB solution (containing 0.32% DMB in ethanol, 0.2% formic acid, 0.2% sodium formate per 1 L and stored in dark at 4^{o}C) was added to 40 µl of aggrecan preparation (before and after enzyme treatment) along with a standard curve prepared from a 1 mg/ml (phosphate buffer) chondroitin sulphate stock in a 96 well flat-bottomed plate incubated for 30 min. Absorbance was measured at 530 nm.

Keratanase and Chondroitinase ABC (Sigma) were added to the glycosylated aggrecan preparation at 25 mU/mg and 1.25mU/mg of chondroitin sulphate content respectively. Both enzymes were dissolved in 50 mM Tris HCL pH 7.4, added to the glycosylated aggrecan simultaneously and incubated for 5 h at 37^{o}C. The deglycosylated aggrecan was then dialysed into Tris HCL pH 7.4 for 24 h with 4 buffer changes.

2.1.51. Quantification

Protein concentration of aggrecan was determined using a bicinchoninic acid (BCA) assay kit (Pierce), both before and after deglycosylation. Typical yield = 37 mg/ per septum.

2.1.1.6. SDS –polyacrylamide gel electrophoresis

Various dilutions of aggrecan were incubated for 5 min at 95^{o}C in laemelli loading buffer (4% SDS, 20% glycerol, 10% 2-Mercaptoethanol, 0.004% bromphenol blue and 0.125 M Tris HCL pH 6.8) containing 100 mM dithiothreitol (DTT) (Sigma), (along with molecular weight markers (Amersham)). Proteins were separated on a 7% polyacrylamide gel at 100 volts and visualised with 0.16% PhastGel Blue R-350 (GE Healthcare) (10% acetic acid, 10% methanol) following destaining (10% acetic acid, 10% methanol).

2.1.1.7. Viscometry

The Viscosity of the aggrecan preparation was measured using a Contraves Low Shear 30 viscometer at 37^{o}C. Viscosity was measured for both deglycosylated and glycosylated aggrecan under the following conditions: a) aggrecan alone b) aggrecan pre-treated with 10 mM DTT and 5 mM iodoacetamide to reduce disulphide bonds then incubated with Hyaluronan for 24 h at 4^{o}C c) aggrecan after incubation with Hyaluronan for 24 h and again following 15 h incubation 4^{o}C with 50 mM DTT and 5 mM iodoacetamide. Glycosylated aggrecan was measured at concentrations 0.405 mg/ml; 0.27 mg/ml, 0.135 mg/ml and deglycosylated aggrecan was measured at 1.62 mg/ml, 0.81mg/ml and 0.405 mg/ml**.**

2.1.1.8. ELISA

Anti-aggrecan antibody secreting cells (**see section 3.4**) were cultured for several days and supernatant was removed. Cells and debris were removed from the supernatant by centrifugation at 400G for 10 min. 96 well Immunosorb plates (NUNC) were coated with 50 µl coating buffer (150 mM NA_2CO_3, 350 mM $NaHCO_3$) or coating buffer containing 2 µg/ml of deglycosylated aggrecan. Plates were wrapped in parafilm and incubated at 4^{o}C overnight and then washed 3 times with PBS containing 0.1% Tween to remove any unbound material. To prevent non-specific binding of antibodies the plates were blocked by the addition of 200 µl 2% milk powder in PBS to each well and incubated for 1 h at 37^{o}C. Plates were washed 3 times and doubling dilutions of cell supernatants were added to the plate and incubated for 1 h at 37^{o}C. Plates were washed 3 times and 100 µl horseradish peroxidase (HRP)-conjugated goat anti-mouse (BD Pharmingen) (concentration 1; 2000) was added to all wells**.** Plates were incubated for 1 h at 37^{o}C and washed 6 times. Substrate 3,3',5,5'-Tetramethylbenzidine (TMB**)** (Sigma**)** 1% was subsequently added along with 0.3 µl/ml hydrogen peroxide in phosphate citrate buffer **(**0.2 M NA_2HPO_4, 0.1 M citrate) for 4 min, the reaction was stopped using 2 M H_2SO_4 and absorbance was read using a spectraMax 190 microplatereader (Molecular devices) at 450 nm**.** Controls included a positive control where mouse (IgG1) anti-aggrecan antibody (Serotec) was added to wells coated with aggrecan and negative controls where media only was added to wells coated with aggrecan and anti-aggrecan antibody or neat supernatant was added to wells coated in coating buffer only.

2.1.1.9. Biotinylation

Aggrecan (in PBS) was biotinylated according to manufacturer's instructions (Pierce EZ-Link Sulfo-NHS-LC-Biotin) at 3 different biotin/protein ratios (40-fold, 20-fold and 10-fold molar excess). Reactions were incubated for 30 min at room temperature and then dialysed overnight in PBS to remove any non-reacted and hydrolyzed biotin from the solution.

Biotinylation was determined using ELISA. 2 μg/ml of biotinylated aggrecan was adsorbed onto plates and detected either by anti- aggrecan antibody (Serotec) or supernatant from anti-aggrecan secreting hybridomas (F1.11) generated by Dr A Knight and John Robinson, followed by appropriately labelled secondary antibodies or directly using streptavidin HRP.

2.1.1.10 Reduction and alkylation of aggrecan.

An aliquot of 5 ml (1.6 mg/ml) deglycosylated aggrecan was incubated for 5 h at 37^{o}C with 10 mM DTT. The reduced aggrecan was subsequently alkylated by incubation with 30 mM iodoacetamide overnight at room temperature in the dark. Reduction and alkylation was determined using ELISA. 2 μg/ml of reduced and alkylated aggrecan was adsorbed onto plates and detected by anti-aggrecan antibody (1-C-6) that recognises a reduced and alkylated form of aggrecan (Stevens, Oike et al. 1984) followed by HRP labelled anti-mouse secondary antibodies.

2.1.2. Recombinant tetanus toxin C fragment (TTCF).

TTCF was provided by Dr A Knight and was used as a positive control antigen in several experiments. TTCF was expressed as a histidine tagged protein in *E.coli* as described in (Hewitt, Treumann et al. 1997).

2.1.3. Peptides

All peptides were purchased from Genscript and described in (Buzas, Vegvari et al. 2005). Synthetic peptide 84-103 is a 20mer peptide containing an epitope from the G1 domain of aggrecan with the sequence ^{84}VVLLVATEGRVRVNSAYQDK$_{103}$. Synthetic peptides p2373-2387 ^{2373}TTYKRRLQKRSSRHP$_{2387}$ classified as dominant/arthritogenic/cryptic and p2363–2378 ^{2363}EEPRITCTDPTTYKRR$_{2378}$

classified as conditionally immunogenic ("super-cryptic") are epitopes from the G3 domain of aggrecan.

2.2. Cell lines and culture

A20-1 is a murine B cell lymphoma, (BALB/c haplotype H-$2^{d,}$ IgG$2a^{+}$ κ; (Kim, Kanellopoulos-Langevin et al. 1979)). A20 8AGR clone 4G11 (A20-1 HS) are modified A20-1 cells, Hypoxanthine 5 mM, aminopterin, 20 mM, Thymidine 0.8 mM (HAT) sensitive (HS). A20-1 cells were grown in the presence of 8-azaguanine (8-AG); 8AGR resistant colonies were tested for aminopterin sensitivity. Anti-aggrecan antibody secreting hybridomas (F1.11) were generated by A Knight and John Robinson. (H-$2A^{d}$ restricted) murine T cell hybridoma 192, which recognise p83-104 from aggrecan as generated by Katie Lowes and provided by John Robinson. BW5147 lymphoma cells (TCRα-/β-) were provided by Dr P. Marrack, Denver. All cells unless stated otherwise were grown at 37^{o}C in a 5% CO_2 atmosphere in RPMI 1640 (Invitrogen), supplemented with 100 μg/ml kanamycin, 2 mM glutamine, 1 mM sodium pyruvate, 1 mM non-essential amino acids (Invitrogen), 50 μM β-mercaptoethanol (Sigma) and containing 10% foetal bovine serum (FBS) (First link Ltd) (cRPMI).

A20-3A5 TTCF-specific B cells were provided by A Knight (Knight, Lucocq et al. 1997) and were grown in cRPMI containing 0.5 mg/ml G418 and 0.75 mg/ml hygromycin B. The IL-2 dependent mouse T cell lymphoma HT-2 and CTLL-2 were cultured in cRPMI supplemented with 1.5 ng/ml recombinant IL-2.

Mouse fibroblasts (L cells) transfected with CD40L DNA (L47) and control untransfected fibroblasts (L5) were a kind gift from Dr Tom Barr, University of Edinburgh (Wohlleben, Gray et al. 1996). L cells were cultured in IMDM (Invitrogen) supplemented with 1 mg/ml G418, 5% FBS, 100 μg/ml kanamycin, 2 mM glutamine, 1 mM sodium pyruvate, 1 mM non-essential amino acids, 50 μM β-mercaptoethanol. HeLa cells were cultured in DMEM (Invitrogen) supplemented with 100 μg/ml kanamycin, 2 mM glutamine, 1 mM sodium pyruvate, 1 mM non-essential amino acids, 50 μM β-mercaptoethanol and containing 10% FBS.

2.3 Cryopreservation of cells.

In order to maintain consistency in both the genotype and phenotype of the generated T and B cell hybridomas, aliquots of cultured cells were frozen at the earliest opportunity.

2.3.1 Freezing cells

Cells were grown to mid-log phase in either 24 well plates (T cell hybridomas) or T_{25} tissue culture flasks (B cell hybridomas) and counted. Cells were pelleted in 15 ml sterile falcon tubes by centrifugation at 400G for 5 min. The supernatant was removed and cells slowly resuspended in ice cold freezing media (FBS containing 10% DMSO) to a final cell concentration of 1-2 x 10^6 cells/ml. The cell suspension was transferred to cold cryovials (1 ml/vial) and stored at -80^{o}C overnight. After 24 h, cell aliquots were transferred to liquid nitrogen for long-term storage.

2.3.2 Thawing cells

Cell aliquots were removed from liquid nitrogen and directly thawed in a 37^{o}C waterbath and transferred to a 15 ml tube. RF-2 was added slowly to the tube and cells were pelleted by centrifugation at 400G for 5 min. Cells were resuspended in cRPMI and transferred to either 24 well plates at various cell dilutions (T cell hybridomas) or T_{25} flasks (B cell hybridomas) at a 1:5 dilution. 24 h later cells are observed using an inverted microscope and re-seeded if necessary.

2.4 Single cell cloning

Cells were washed in RF-2 and resuspended at 3, 5 and 10 cells/ml in cRPMI. 100 μl of cell suspension was plated out onto 5 x 96 well flat bottom plates to give a final concentration of 0.3, 0.5 and 1 cell/well. Plates were incubated at 37^{o}C in a 5% CO_2 atmosphere until colonies were visible by eye. Colonies were transferred to 24 well plates and expanded accordingly.

2.5 Preparation of bone marrow derived dendritic cells for use as APC

Male BALB/c approx 6-8 weeks old were sacrificed by cervical dislocation. Femurs were removed into cRPMI and cleaned of all excess tissue. Each end of the femur was cut off and the marrow cavity was flushed with cRPMI using a 5 ml syringe and a 25 gauge needle. Washing continued until all of the bone marrow contents were removed. Bone marrow cells were washed in cRPMI by centrifugation at 400G for 5 min. Cells were resuspended in 10 ml cRPMI containing 20 ng/ml of recombinant murine granulocyte monocytes colony stimulating factor (GM-CSF) (Peprotech). Approximately 2 x 10^6 cells were seeded per sterile 100 mm petri dish and incubated at 37^{o}C in a 5% CO_2 atmosphere. Bone marrow cells were fed at day 3 by adding 10 ml of fresh cRPMI containing 10 ng/ml GM-CSF to each dish and again at days 6 and 8 by removing 10 ml of culture medium from each dish and replacing it with 10 ml of fresh cRPMI containing 20 ng/ml GM-CSF. 10 days after culture, dendritic cells were screened by flow cytometry for the surface marker CD11c and MHC class II and were used as APC in antigen presentation assays.

On the day of the experiment bone marrow dendritic cells were carefully harvested using a cell lifter. The cells were pelleted by centrifugation at 400G for 5 min, resuspended in RPMI 1640 containing 2% FBS (RF-2) and centrifuged again to wash. Cells were resuspended in cRPMI, counted and adjusted to 1 x 10^4/well for unfixed antigen presentation assays and 3 x 10^5/well for fixed antigen presentation assays to allow for cell loss during the fixation and washing process.

2.6 Preparation of bone marrow derived macrophages for use as APC

Bone marrow cells were isolated as described in **section 2.5**. To differentiate cells into bone marrow derived macrophages, cells were cultured in bone marrow macrophage medium (BMM) (cRPMI supplemented with 5% horse serum (Sigma) and 10% culture supernatant from L929 cells as a source of macrophage colony stimulating factor (M-CSF). Cells were seeded in sterile 100 mm petri dishes at approximately 4 x 10^6 per dish and incubated at 37^{o}C in a 5% CO_2 atmosphere. Macrophages were fed at day 3 by adding 5 ml of fresh BMM to each dish. 7 days after culture, bone marrow cells were used as APC in antigen presentation assays or can be maintained for an extended period by re-seeding the cells into new petri dishes with fresh BMM.

24 h prior to their use in antigen presentation experiments, bone marrow macrophages were activated by adding 100 ng/ml of recombinant murine interferon γ (IFN-γ) (R&D Systems) to each petri dish. On the day of the experiment the supernatant was removed, RF-2 was added to each petri dish and the bone marrow macrophages were harvested using a cell scraper. The cells were pelleted by centrifugation at 400G for 5 min, resuspended in RF-2 and centrifuged again to remove all residual IFN-γ. Cells were resuspended in cRPMI and screened by flow cytometry for cell surface markers F4/80 and MHC class II. 1 x 10^4/well bone marrow macrophages were seeded for unfixed antigen presentation assays and 3 x 10^5/well for fixed antigen presentation assays to allow for cell loss during the fixation and washing process.

2.7 Animals

BALB/c (H-2^d) mice were purchased from Harlan Olac 1975 Ltd, Cirencester, UK and maintained under pathogen free conditions in the Comparative Biology Centre (CBC) at Newcastle University. Tissues from female mice were used for *in vitro* studies. TCR transgenic mice (TCR-5/4E8-tg) expressing the T cell receptor Vα1.1 and Vβ4 chains specific for the dominant arthritogenic 84-103 epitope of human proteoglycan aggrecan designated (5/4E8), backcrossed into the BALB/c (H-2^d) strain were a kind gift from Willem van Eden, Institute of infectious Diseases and Immunology, Division of Immunology, Utrecht University, Utrecht, The Netherlands (Berlo, van Kooten et al. 2005; Berlo, Guichelaar et al. 2006). All experiments were carried out under the project licence of John Robinson in accordance with Home Office and Newcastle University regulations.

2.8 Immunisations

All subcutaneous or intra-peritoneal immunisations were performed by either Dr A.M. Knight or R. Stewart within the Comparative Biology Centre. Mice were immunised with an emulsion of either aggrecan or peptide in Titermax (subcutaneous) or potassium aluminium hydroxide (alum) intra-peritoneally.

2.9 Generation of immortalized aggrecan-specific B cells by fusion with A20-1 HS lymphoma cells.

2.9.1 Fusion 1 immunisation

166 μg deglycosylated aggrecan was emulsified vol/vol with 9% potassium aluminium hydroxide (alum) and adjusted to pH 7 by the addition of NaOH. The aggrecan/alum emulsion was washed and resuspended in PBS, and a final volume of 250 μl was administered per immunization. A group of 4 female BALB/c mice aged 6-8 weeks received intraperitoneal immunizations at day 1 and day 21. Two of the immunized mice were given a final boost at day 42 and spleens removed at day 47 (day 5 mice) and 2 mice were boosted at day 45 and spleens removed at day 47 (day 2 mice). Mice were bled both pre- and 10 days post-immunization and a final terminal bleed was taken under anesthetic. Antibody responses to deglycosylated aggrecan are routinely tested by ELISA and cells were used for fusion with A20-1 HS lymphoma cells.

2.9.2 Fusion 2 immunization

A group of 4 female BALB/c mice aged 6-8 weeks received a subcutaneous immunization at day 1 with a full dose of aggrecan (166 μg) emulsified vol/vol with Titermax, followed by two subsequent subcutaneous immunizations at days 21 and 42 with a 10-fold reduced dose of the aggrecan–Titermax emulsion. The mice received a final intra-peritoneal boost containing 166 μg aggrecan emulsified with alum at day 63 and spleens were removed at day 68. Mice were bled prior to immunisation and at days 10 and 31 post-immunisation and a final bleed was taken under terminal anaesthetic. Antibody responses to deglycosylated aggrecan were routinely tested by ELISA. Splenocytes were isolated and cultured with 50 μg LPS and 50 μg deglycosylated aggrecan for 48 h before fusing to A20-1HS cells.

2.9.3 Splenocyte/A20-1 HS fusion

Mice were sacrificed and splenocytes were isolated and formed into a single cell suspension via passage through sterile nylon gauze. Cells were centrifuged and red blood cells were lysed by adding 2 ml cell lysis buffer (Sigma) /spleen for 4 min at room temperature. Splenocytes and the HAT sensitive fusion partner A20-1HS were

washed and resuspended in serum free Hanks balanced salt solution **(**HBSS) (Sigma). 2.5 x 10^7 A20-1HS cells were mixed with 5 x 10^7 splenocytes and the cells were centrifuged at 400G for 5 min. Supernatant was removed by aspiration leaving the pellet dry. 25 μl serum free HBSS was added and the cells were resuspended by gentle pipetting; 0.5 ml of 37^{o}C polyethylene glycol/ Dimethyl sulfoxide (PEG/DMSO) (Sigma) was added dropwise to the cell suspension and mixed for 2 min in a 37^{o}C waterbath. The suspension was subsequently diluted with serum-free RPMI 1640 at 37^{o}C and cells were centrifuged at 400G for 5 min. The cells were resuspended in 50 ml 37^{o}C cRPMI, plated out into 24 well plates 500 μl/well and incubated at 37^{o}C in a 5% CO_2 atmosphere for 24 h before adding (500 μl/well) 2x HAT (Sigma) in cRPMI. HAT resistant colonies were usually identified at day 10 and expanded accordingly. Hybridomas were subsequently cultured in cRPMI containing 1x hypoxanthine-Thymidine (HT) and finally into cRPMI.

2.9.4 The use of CD40L transfectants to expand splenic B cells prior to fusion.

Splenocytes were isolated from aggrecan-immunized mice (using the immunisation protocol described in **section 2.9.1**) and co-cultured with monolayers of murine CD40L transfected L cells. (CD40L expression was regularly tested and compared to control L cells by flow cytometry using FITC-conjugated anti-CD40L, a kind gift from Dr Tom Barr, University of Edinburgh). CD40L transfectants were irradiated (3000 rad) and seeded in 6 well plates (4 x 10^4/well) in cRPMI and incubated overnight at 37^{o}C in 5% CO_2 to establish monolayers. Supernatant was removed from the monolayers and 2.5 x 10^5 splenocytes were added to each well in cRPMI containing 10 ng/ml murine rIL-4 (Peprotech). 5.5 x 10^{-7} M cyclosporine A (Sigma) was added to each well to prevent outgrowth of T cells. Cells were passaged and transferred to fresh irradiated monolayers every 4 days. To determine the percentage and viability of the isolated B cells, cells were screened by flow cytometry at days 0, 4, 8 and 12 both unstained and stained with FITC conjugated anti-TCR and PerCP conjugated B220. Controls for this experiment included cells co-cultured with, CD40L transfectants/ untransfected L cells only, CD40L/L cells + IL-4 only and CD40L/L cells + cyclosporine A only.

2.10. Fusion screening

2.10.1. Flow cytometry

All staining was carried out in 96 well round bottom plates at 4^{o}C in the dark in the presence of 2.4G2 Fc block (previously determined to block the binding of FITC conjugated 2.4G2 antibodies) to ensure that staining was not due to antibodies binding Fc receptors expressed on the cells. For each sample 2.5 x 10^5 cells were washed in PBS containing 2% FBS (FACS buffer), pelleted at 1400rpm at 4^{o}C and resuspended in FACS buffer. 2.4G2 Fc block was added to appropriate wells and incubated for 60 min. Primary antibodies were added and incubated for a further 60 min. Cells were then washed 4 times and resuspended in PBS 2% FBS. Secondary antibodies were added to appropriate wells and incubated for a further 60 min; cells were subsequently washed 4 times and resuspended in 400 μl PBS 2% FBS. 10,000 events were collected for each sample using a FACScan flow cytometer (BD Biosciences) and data analysed with Flowjo software (Treestar^{TM}) (**see Table 2.1** for antibodies used and concentrations).

Antibody	Concentration	Isotype	Supplier
Rat anti-mouse IgG1 FITC	1:100	IgG1, κ	BD Pharmingen
Rat anti-mouse IgG2a FITC	1:100	IgG1, κ	BD Pharmingen
Rat anti mouse IgG2b FITC	1:100	IgG2a, κ	BD Pharmingen
Rat anti mouse IgG3 FITC	1:100	IgG2a, κ	BD Pharmingen
Rat anti-mouse IgM FITC	1:100	IgG2a, κ	BD Pharmingen
Hamster anti-mouse B7.1 PE	1:100	IgG2, κ	BD Pharmingen
Rat anti mouse B7.2 PE	1:100	IgG2a, κ	BD Pharmingen
Rat anti-mouse class II I-A, I-E PE	1:1000	IgG2b, κ	BD Pharmingen
Rat anti-mouse class II I-A, I-E FITC	1:1000		BD Pharmingen
Rat anti-mouse kappa light chain PE	1:100	IgG1, κ	BD Pharmingen
Rat anti-mouse lambda light chain FITC	1:100	IgG2a, κ	BD Pharmingen
Rat anti-mouse CD16/CD32 (Fcγ III/II receptor) FITC	1:100	IgG2b, κ	BD Pharmingen
Streptavidin FITC/ Streptavidin APC	1:1000		BD Pharmingen
Rat anti-mouse F4/80 FITC	1:100	IgG2b, κ	BD Pharmingen
Rat anti-mouse CD11c PE	1:100	IgG2, κ	BD Pharmingen
Aggrecan-bio	50μg/ml		See Materials and Methods
Rat anti-mouse CD16/CD32 (Fcγ III/II receptor) 2.4G2 block and 2.4G2 bio.	75μg/ml	IgG2b, κ	(in house)
Rat anti- mouse Vβ4 PE	1:100	IgG2b, κ	BD Pharmingen
Rat anti-B220 PerCP	1:100	IgG2a, κ	BD Pharmingen
Rat anti-CD40/ Rat anti-CD40L			Dr Tom Barr (Wohlleben, Gray et al. 1996)
Rat anti-CD4 perCP	1:100	IgG2a, κ	BD Pharmingen
Rat anti-TCR FITC/PE	1:100	IgG2b, κ	BD Pharmingen
Rabbit anti-human IgG H + L biotin Fab^2	1:100		Southern Biotech. Assoc.

Table 2.1 Antibodies used for flow cytometry analysis. *Listed are all antibodies used for flow cytometry experiments***.**

2.10.2 Antigen presentation assay

Antigen was added at graded doses to duplicate wells in a 96 well flat bottomed plate together with antigen presenting cells and T cells in a total volume of 200 µl. Antigen presenting cells and T cells were added into each well at the following concentrations: $5x10^4$ T cells, $5x10^4$ non-specific B cells, $1x10^4$ aggrecan-specific B cells, $1x10^4$ bone marrow derived dendritic cells or $1x10^4$ bone marrow derived macrophages. Negative controls were medium or antigen only, medium or antigen together with T cells and medium and antigen together with antigen presenting cells. Assays were incubated at 37^oC in 5% CO_2 for 24 h and then frozen to lyse all of the cells. T cell stimulation was measured by the amount of IL-2 released into the culture supernatant. IL-2 was detected by the proliferation of IL-2 dependent T cell lines (HT-2 or CTLL-2).

2.10.3 IL-2 bioassay

HT-2 cells were used to quantify IL-2 in fusion assay supernatants. However, CTLL-2 cells were later found to be a more efficient cell line and were used to detect IL-2 levels in all subsequent experiments including all of the transfectant assays.

24 h prior to use CTLL-2 were starved of IL-2 by washing in RF-2 to remove IL-2 and resuspended in cRPMI. HT-2 cells were washed 4 times in RF-2 immediately prior to use. 50 µl of the culture supernatant was transferred to wells of a new 96 well flat bottomed plate and incubated for 24 h with 50 µl of either pre-starved $3x10^4$ CTLL-2 or HT-2. Positive and negative controls were performed for HT-2/CTLL-2 proliferation where either 50 µl cRPMI containing no IL-2 or 50 µl of rIL-2 at graded doses were added to duplicate wells along with 50 µl of $3x10^4$ HT-2 or CTLL-2. To quantify cell proliferation, 18.5 kBq ^3H thymidine (Amersham) was added per well for the last 18 h of culture. The assay was harvested onto printed glass filter mats (Wallac) using a cell harvester (Packard Micromate 196, Canberra Harwell, Didcot, UK) and counted using a liquid scintillation β-counter (1450 LSC and Luminescence Counter, Perkin Elmer MicroBeta Trilux; CT, USA).

2.11 Antigen presentation assay (fixed APC system)

The fixed APC system can be divided into two sub-sections

- The first describes how cells are pre-fixed and then used to detect pre-processed antigen in the form of peptides.
- The second system describes how cells are initially incubated with antigen +/- inhibitors of antigen processing, washed and then post-fixed to study the pathways and enzymes involved in antigen processing

2.11.1 Pre-fixation of APC

Bone marrow macrophages, dendritic cells and aggrecan-specific B cells (see **section 2.16**) were harvested, washed and $3x10^5$ cells/well were seeded in 96 well v bottomed plates. Cells were pelleted by centrifugation at 400G for 5 min, resuspended in 100 µl of ice cold 1% paraformaldehyde solution and incubated at room temperature for 5 min. 100 µl of ice cold 0.05% Gly-Gly solution was added to each well and cells were pelleted by centrifugation at 400G for 5 min. The cells were then washed 3 times and resuspended in cRPMI. $1x10^4$ cells were seeded in triplicate wells of a new 96 well flat bottomed plate in 200 µl of cRPMI containing $5x10^4$ T cell hybridoma (192) and either 10 nM p84-103 or 200nM of aggrecan. Assays were incubated at 37^{o}C in 5% CO_2 for 24 h and following freezing, 50 µl of supernatant was harvested and IL-2 levels detected by CTLL-2 proliferation as described in **section 2.10.3**.

2.11.2 Post-fixation of APC

Bone marrow macrophages, dendritic cells and aggrecan-specific B cells (**see section 2.11**) ($5x10^4$/ well) were seeded in a 96 well v bottomed plate in 200 µl cRPMI containing either 10 nM p84-103, 200nM aggrecan or medium only and incubated at 37^{o}C in 5% CO_2 for 6 h.

For inhibitor studies cells were pre-treated with inhibitors (or inhibitor solvent only,) before the addition of antigen. All of the inhibitors used and pre-treatment incubation times are shown in **Table 2.2**.

Following 6 h incubation with antigen, APC were pelleted by centrifugation at 400G for 5 min and washed 3 times in HBSS. The cells were then fixed as described above (**Section 2.11**) and $1x10^4$ fixed cells were seeded in triplicate wells of a new 96 well flat bottomed plate in 200 µl of cRPMI containing $5x10^4$ T cell hybridomas (192)

and either 10 nM of p84-103 or 200 nM of aggrecan. Assays were incubated at 37^{o}C in 5% CO_2 for 24 h and then frozen to lyse all of the cells. The supernatant was harvested and IL-2 levels detected by CTLL-2 proliferation as described previously (**section 2.10.3**). Control wells included live cells incubated with the above antigen concentrations and pre-fixed cells (**section 2.20**) subsequently incubated with the antigen.

2.12 Inhibitors of antigen processing

Details of all of the inhibitors used in the post fixation experiments are shown in **Table 2.2** All inhibitors were purchased from Sigma.

Inhibitor	Solvent	Dose range	Pre-treatment time	References
Ammonium chloride	H_2O	50-400 mM	3 h	(Conte, Petrone et al. 1996)
Brefeldin A	ethanol	0.125-2 μg/ml	3 h	(Orci, Tagaya et al. 1991)
Cycloheximide	DMSO	10-80 μM	3 h	(St-Pierre and Watts 1990)
DCI	DMSO	10-80 μM	1 h	(Harper, Hemmi et al. 1985)
E-64d	DMSO	10-100 μM	1 h	(Tamai, Matsumoto et al. 1986)
Pepstatin A	DMSO	0.5- 4 mM	1 h	(Puri and Factorovich 1988)
Leupeptin	DMSO	2.5-20 μM	1 h	(Aoyagi, Takeuchi et al. 1969)
1, 10 Phenanthroline	DMSO	200-1600 μM	1 h	(Takahashi, Cease et al. 1989)

Table 2.2 Inhibitors of antigen processing and presentation used in this study.
3, 4-dichloroisocoumarin (DCI), (2S, 3S)-*trans*-epoxylsuccinyl-L-leucylamino-3-methylbutane ethyl ester (E-64d), dimethyl sulfoxide (DMSO

2.13 Construction of plasmids encoding aggrecan-specific BCR.

2.13.1 Cell lines and plasmids

The B cell hybridoma F1.11 that produces an Ig against bovine aggrecan was generated by Dr A Knight and Prof J Robinson by fusing splenocytes from aggrecan-immunized mice with the Ig $^{-ve}$ B cell hybridoma NSO. I subsequently cloned the F1.11 hybridoma by limiting dilution as described in **section 2.4**. Resultant clones were screened by ELISA for aggrecan-specificity and to determine the antibody isotype and light chain usage (see **section 2.13.2**). The representative aggrecan-specific B cell hybridoma clone C71 ($IgG1^{+}$,κ) was chosen for further experiments. The B cell hybridoma 1-C-6 ($IgG1^{+}$,κ) was kindly donated by Prof B Caterson, Connective Tissue Biology, Cardiff School of Biosciences, Cardiff University (Caterson 1987). 1-C-6 mAb recognises repeated epitopes present in both the G1 and G2 domains of aggrecan and requires reduction and alkylation of aggrecan for the best exposure of the amino acid sequence QAAY that form the basis of recognition (Stevens, Oike et al. 1984; Caterson 1987).

2.13.2 Characterisation of the C71 and 1-C-6 antibodies by ELISA

ELISA was used to verify the antigen-specificity, heavy chain isotype and light chain usage of C71 antibodies (previously described for 1-C-6 in (Caterson 1987). C71 hybridomas were cultured for several days to allow cells to exceed optimal density and supernatant was removed. Cells and debris were removed from the supernatant by centrifugation at 400G for 10 min. ELISA was carried out as described previously in **section 2.1.1.8** using 96 well ELISA plates coated with 2 μg/ml of deglycosylated aggrecan, reduced/alkylated aggrecan, anti-IgG1, anti-IgG2a, anti-IgG3 or anti-IgM, anti-kappa light chain and anti-lambda light chain. C71/1-C-6 cell supernatant was titrated using doubling dilutions, added to the plate and incubated for 1 h at 37^{o}C. Bound C71/1-C-6 antibodies were detected following the addition of HRP-conjugated goat anti-mouse (1:2000) and substrate TMB with hydrogen peroxide in phosphate citrate buffer, optical density was read at 450 nm**.** Controls for these experiments included a positive control where anti-aggrecan antibody (Serotec) was added to wells coated with aggrecan and negative controls where media only was added to wells

coated with aggrecan and anti-aggrecan antibody or neat supernatant was added to wells coated in coating buffer only.

2.13.3 RNA extraction

Total RNA was isolated from $1x10^7$ A20-1 HS, C71 and I-C-6 cells using RNeasy RNA isolation kit according to the manufacturer's instructions (Qiagen). RNA was eluted into 50 µl of RNase free water. All work with RNA was carried out using RNase free eppendorfs, reagents and tips and gloves were worn at all times to prevent RNase contamination. RNA purity and concentration was determined using a NanoDrop ND1000 (Labtech International). In addition, 1 µg of RNA was separated on a 1.5% agarose gel along with molecular weight markers (Amersham) at 100 volts for 20 min and visualised using an AlphaImager (Alpha Innotech Corporation, GRI Ltd) at (365-nm) UV to verify integrity and purity. Total RNA was stored at -80^{o}C.

2.13.4 Rapid amplification of cDNA ends

Doenecke *et al* successfully amplified unknown sequences from the 5' end of cDNA using rapid amplification of cDNA ends (RACE) (**Figure 2.1**). To verify that this procedure was reproducible, I repeated their experiment to isolate VH cDNA from A20-1 cells and confirmed that the amplified VH sequence was identical to their already published data (Doenecke, Winnacker et al. 1997; Wang, Chen et al. 2006). Subsequently cDNAs encoding the VH and VL regions from the two aggrecan-specific monoclonal antibodies (C71 and 1-C-6) were successfully isolated using this method. 5' RACE was carried out according to the manufacturer's instructions. In brief, gene-specific primers (H chain GSP1, H chain GSP2, L chain GSP1 and L chain GSP2) for both the IgG1 H chain and kappa L chain of murine immunoglobulin were designed based on published sequences (Doenecke, Winnacker et al. 1997). First strand cDNA was synthesized from A20-1HS, C71 and 1-C-6 total RNA using H or L chain GSP1 and Superscript II reverse transcriptase (Superscript II RT). 2.5 pmoles of H or L chain GSP1, 1 µg of sample RNA and deionized, diethylpyrocarbonate (DEPC) treated water (Invitrogen) were added to a thin walled PCR tube to a final volume of 15.5 µl and RNA was denatured by heating the tube to 70^{o}C for 10 min. The tube was chilled on ice for 1 min before the addition of 2.5 µl 10x PCR buffer, 25 mM $MgCl_2$, 10 mM dNTP mix and 0.1 M DTT. The contents were mixed gently and incubated for 1 min at 42^{o}C before the addition of 1 µl Superscript II RT. The

contents were mixed and incubated for 50 min at 42^{o}C to synthesize the cDNA. The temperature was adjusted to 70^{o}C for 15 min to terminate the reaction and the tube was centrifuged for 10 seconds to collect the contents and placed at 37^{o}C. 1 µl of RNase mix was added and incubated at 37^{o}C for 30 min to degrade the RNA. cDNA was purified using a simple nucleic acid prep (S.N.A.P) column procedure to remove excess nucleotides and H or L chain GSP1 before terminal deoxynucleotidyl transferase (TdT) tailing of the cDNA. 10 µl of the S.N.A.P. purified cDNA was transferred to a new tube with 6.5µl DEPC water, 5µl 5x tailing buffer and 2 mM dCTP and incubated for 3 min at 94^{o}C before being placed on ice. 1 µl of TdT was added to the tube mixed gently and incubated for 10 min at 37^{o}C. TdT was heat inactivated by incubation at 65^{o}C for 10 min before being placed on ice prior to PCR of dc-tailed cDNA. Tailed cDNA was amplified by PCR using the H or L chain GSP2 nested primer listed in **Table 2.3**. 5 µl of dC-tailed cDNA was transferred to a thin walled PCR tube along with 31.5 µl sterilized distilled water, 5 µl 10x PCR buffer, 25 mM $MgCl_2$, 10 mM dNTP mix, 2 µl of 10 µM H or L chain GSP2 solution and 2 µl of 10 µM abridged anchor primer solution. 0.5 µl of Taq DNA polymerase was added to the tube, contents was mixed and transferred to the pre-heated PCR machine (94^{o}C). PCR programme for 35 cycles was denaturation at 94^{o}C for 1 min, annealing of primers 55^{o}C for 1 min and primer extension 72^{o}C for 1 min, followed by a final extension at 72^{o}C for 5 min. PCR products were analyzed by 1% agarose gel electrophoresis, using molecular weight standards, followed by visualization using at (365-nm) UV.

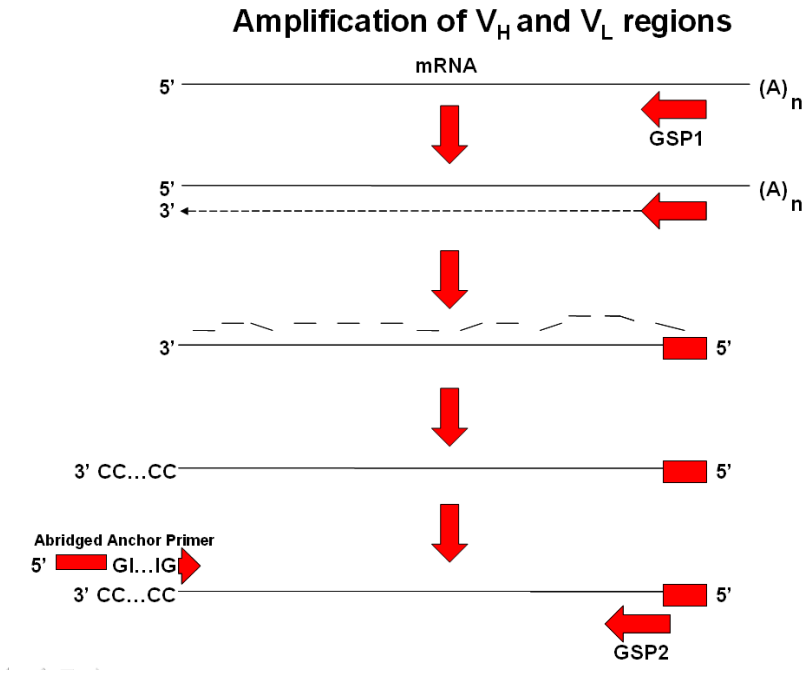


Figure 2.1 Rapid amplification of cDNA ends to amplify unknown V_H and V_L regions. *First strand cDNA is synthesized using a gene specific primer (GSP1). The RNA template is removed by the addition of RNAse. A homopolymeric tail is added to the 3' of the amplified cDNA using TdT and dCTP. PCR amplification of the unknown cDNA sequence is achieved using a nested GSP2 primer that anneals to the known sequence encoding the Ig C domain and an abridged anchor primer that recognizes the homopolymeric 5'tail. Extracted from (Invitrogen 2002)*

2.13.5 Cloning

Subsequent to RACE performed on RNA isolated from C71 and 1-C-6 the final PCR products were cloned into pCR® 2.1 plasmid vectors using TOPO TA Cloning® kit (Invitrogen) according to manufacturers instructions. In brief, 4 µl of PCR product was transferred to an eppendorf tube along with 1 µl of salt solution and 1 µl of the TOPO vector, the contents was mixed and incubated for 5 min at room temperature before being placed on ice. Top 10 competent *E.coli* (Invitrogen) were transformed with the ligation mixture and plated overnight onto pre-warmed selective LB agar plates containing 50 µg/ml ampicillin. (Negative controls for this step were Top 10 competent *E.coli* only plated onto ampicillin selection plates). The following day single colonies were picked for overnight culture and plasmid preparation.

Name	Sequence
Heavy chain GSP1	5'-ATTTTGTCGACCKYGGTSYTGCTGGCYGGGTG-3'
Heavy chain GSP2	5'-GCACACYRCTGGACAGGGATCCAGAGTTCC-3'
Light chain GSP1	5'-TTTGGGGTAGAAGTTGTT-3'
Light chain GSP2	5'-TAACTGCTCACTGGATGGTGGGAAGATGGA-3'
Abridged anchor primer (AAP)	5'-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3'
Heavy chain 3' primer including restriction site for *Apa1*	5'-GGGCCCTTGGTGGAGGCAGCAGAGACAGTGACCAGAGT-3'
Heavy chain 5' primer including restriction site for *Xho1*	5'- CTCGAGCCATGGCTGTCCTGGTGCTGTTC-3'
1-C-6 Light chain 3'primer including restriction site for *Bbs1*	5'-ATGAAGACAGATGGTGCAGCCACAGTTTTGATTTCCAGCTTGGTGCC-3'
C71 Light chain 3'primer including restriction site for *Bbs1*	5'-ATGAAGACAGATGGTGCAGCCACAGTTTTTATTTCCAGCTTGGTCCC-3'
1-C-6 Light chain 5'primer including restriction site for *Xba1*	5'-TCTAGAATGAAGTTGCCTGTTAGGCTG-3'
C71 Light chain 5'primer including restriction site for *Xba1*	5'-TCTAGAATGGATTTTCATGTGCAGATT-3'
M13 F	5´-GTAAAACGACGGCCAG-3´
M13 R	5´-CAGGAAACAGCTATGAC-3´

Table 2.3 Primers purchased for the generation of aggrecan-specific BCR heavy and light chains. *Primers all purchased from Eurofins, MWG, Operon.*

2.13.6 Plasmid DNA isolation

Plasmid DNA was prepared from overnight bacterial cultures using a QIAprep spin miniprep kit (Qiagen) according to manufacturer's instructions. In brief, bacterial colonies were grown in selective 2 ml LB broth overnight at 37^{o}C in a shaking incubator. The bacterial cells were harvested by centrifugation at 6800G for 3 min and plasmid DNA was isolated and bound to the Qiagen miniprep column. To elute the DNA, the column was placed in a clean eppendorf tube, 50 µl of water was added to the centre of the column membrane, left at room temperature for 1 min and then centrifuged at full speed for 1 min. Plasmid DNA was quantified using the NanoDrop ND1000 (A260/A280).

2.13.7 DNA sequencing

Several different preparations of mini prep and maxi prep pCR2.1 C71 and I-C-6 DNA were bidirectionally sequenced (Cogenics) using the M13F and M13R forward and reverse primers described in **Table 2.3**.

Sequencing ensured that multiple clones contained identical sequences. Subsequently, sequences were aligned with those previously described for other immunoglobulin H and L chain variable regions (Gilliland, Norris et al. 1996; Kwak, Lee et al. 1996; Karlstrom, Zhong et al. 2000; Park, Jin et al. 2000; Cauerhff, Goldbaum et al. 2004) using Entrez Nucleotide, NCBI sequence viewer v2.0.

2.13.8 Maxi preps

Following DNA sequencing to verify the quality and correct sequence of the plasmid DNA, maxi preps were carried out to amplify the amount of plasmid DNA available.
Maxi preps were prepared from 250 ml overnight bacterial cultures using Qiagen maxi plasmid kit (Qiagen) according to manufacturer's instructions. Following this, maxi prep DNA was quantified by nanodrop and used for further cloning steps, cell transfection and long term storage stocks at -80^{o}C.

2.13.9 Restriction digests.

Restriction digests were performed to remove plasmid inserts:-

- To verify that the correct sized insert has ligated with the plasmid. To do this the restriction digest is subsequently separated on a 1% agarose gel and visualized at (365-nm) UV.

or

- To remove the insert and subsequently ligate it with a different plasmid. To do this the restriction digest was separated on a 1% agarose gel, visualized at (365-nm) UV and the insert excised from the gel using a scalpel. The DNA was extracted from the gel and ligated to either an empty plasmid, or a plasmid vector already containing an insert. In the case of the latter, the unwanted insert was excised by restriction digest, separated by agarose gel electrophoresis and the linearized plasmid vector DNA isolated. In brief these procedures are detailed below.

Restriction enzymes used for cloning were; *Xho1*, *Not1*, *Kpn1*, Sac1, *Apa-1*, *Bbs-1*, *Xba1*, *BsshII (New England Biolabs).* A typical restriction digest consisted of; 10 U of restriction enzyme per 1 μg of DNA incubated for 1 h at the appropriate reaction temperature. In a total reaction volume of 50 μl, 5 μl 10x appropriate buffer was added +/- BSA and the incubation temperature of the reaction is dependent on the enzyme. Calf intestinal phosphate (CIP) (NEB) was added to remove 5' phosphatase groups when digesting vectors thus preventing linearized vectors from self re-ligation. 5 μl of CIP was added to 40 μl of digestion reaction along with 5μl NEB buffer 3 and incubated at 37^{o}C for 1 h.

De-phosphorylated DNA fragments were separated from CIP and other DNA fragments on a 1% agarose gel and visualized at (365-nm) UV, followed by the gel purification/extraction step to extract and purify the vector/insert DNA.

2.13.10 Gel extraction/purification

DNA was extracted from agarose gels using a QIAEX II gel extraction kit (Qiagen) according to manufacturer's instructions. DNA was eluted in 20 µl of H_2O and 1 µl was separated on a 1% agarose gel for 30 min at 100 volts and visualized at (365-nm) UV.

2.13.11 DNA ligation

DNA ligation was used to clone restriction fragments and generate new plasmid constructs. Ligations were performed using the enzyme T4 ligase (NEB). Typical ligation reactions included 100 ng vector and 500 ng insert, 1 µl 10x T4 DNA ligase buffer, 1 µl T4 DNA ligase made up to a total volume of 10 µl. The reaction was mixed and incubated overnight at 4^{o}C overnight.

2.13.12 PCR to introduce restriction sites required for subsequent cloning steps.

Initial clones were used to identify the sequences of the 1-C-6 and C71 V regions. Primers were then designed to amplify the H and L chain aggrecan-specific C71 and 1-C-6 V regions and to incorporate the restriction site sequences (*Xba1* /*BbS1* L chain) and (*Xho1*/*Not1* H chain) and sequences encoding the first few amino acids of the H and L chain constant regions from the anti-human tetanus toxin C fragment BCR (TTCF) (Knight, Lucocq et al. 1997). 50 µl PCR reactions consisted of 1 µl of Qiagen mini prep DNA template, 2 µl of the 5' and 3' primers (10 pmol/µl stock), 5 µl 10x PCR buffer, 3 µl 25 mM Mg2+, 1 µl deoxyribonucleotide triphosphates (dNTPs), 35.5 µl H2O and 0.5 µl Taq polymerase. PCR programme for 35 cycles was denaturation at 94^{o}C for 1 min, annealing of primers 55^{o}C for 1 min and primer extension 72^{o}C for 1 min, followed by a final extension at 72^{o}C for 5 min. Following amplification PCR products were gel purified and re-cloned into pCR2.1.

Subsequently, the variable regions from TTCF H and L were removed using the restriction enzymes (*Xba1*/*BbS1* L chain and *Xho1*/*Not1* H chain). C71/1-C-6 aggrecan-specific V regions now including compatible restriction sites and sequences encoding 12 amino acids present in the TTCF H chain C region or 20 amino acids from the TTCF L chain C region were ligated with digested C regions from the TTCF H and L to generate human/mouse chimeric aggrecan-specific H and L chains. The chimeric H and L chains were then digested from pCR2.1 and sub-cloned via several

plasmids into the final lymphocyte expression vectors pMCFR as illustrated in Chapter 4 (**Figures 4.9 and 4.11).**

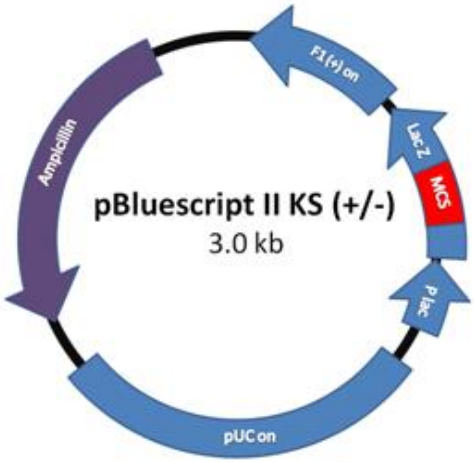
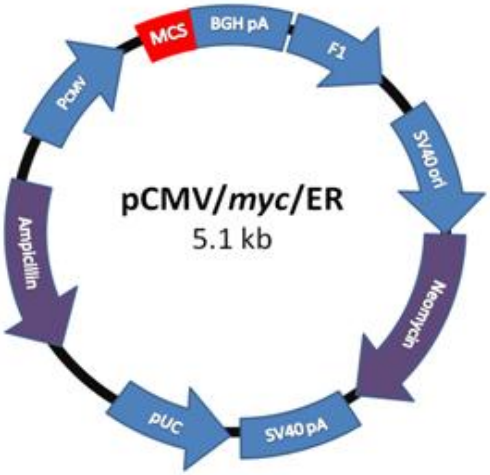
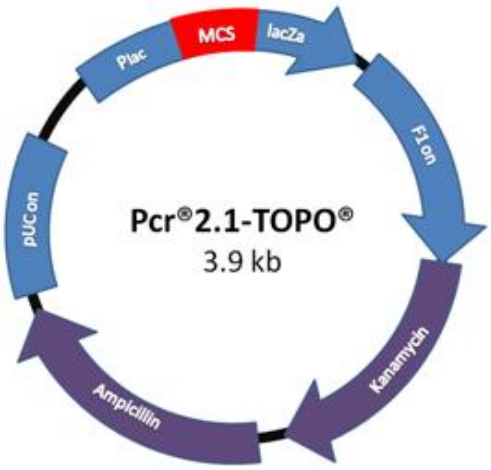
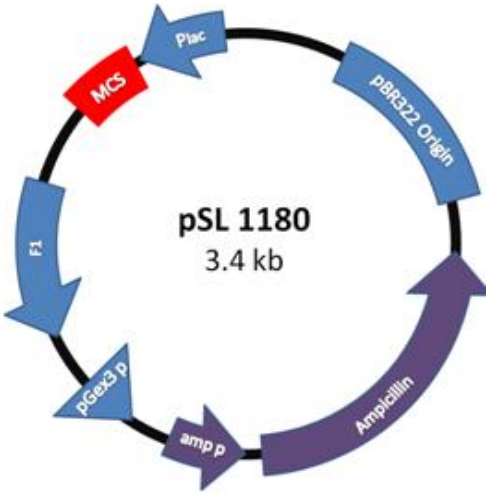
	
Stratagene www.Stratagene.com	Invitrogen www.invitrogen.com
	
Invitrogen www.invitrogen.com	Pharmacia Biotech www.lablife.org
pMCFR/hyg/NI Peter Cresswell No map available	

Table 2.4 Plasmids used for BCR H and L chain cloning. *Listed are all of the plasmids used during the generation of the H and L chains of the aggrecan-specific BCRs.*

2.14 Transfection with the aggrecan-specific BCR plasmids

Following several rounds of cloning the final 4 pMCFR constructs containing 1-C-6 H chain (pMCFR 1C6H), 1-C-6 kappa L chain (pMCFR 1C6L), C71 H chain (pMCFR C71H) and C71 kappa light chain (pMCFR C71L) were ready for transfection into cells. To verify that the constructs were assembled correctly and expressed they were firstly transfected into HeLa cells using Lipofectamine (Invitrogen). Following successful transfection into HeLa cells the construct were transfected into the A20-1 HS B lymphoma cell line by electroporation using a Nucleofector AAD-1001 (Amaxa).

2.14.1 Transient transfection of plasmids containing C71/ 1-C-6 BCR into HeLa cells.

Lipofectamine^{TM} 2000 was used to transiently transfect the final pMCFR 1C6H, pMCFR 1C6L, pMCFR C71H and pMCFR C71L plasmid constructs into HeLa cells. In brief, 24 h prior to transfection $8x10^5$ HeLa cells were seeded onto glass coverslips in 24 well plates so that cells were 90-95% confluent the following day. Approximately 1 μg of each plasmid was diluted in 50 μl Opti-MEM I reduced serum medium (Invitrogen) and mixed. 2 μl of Lipofectamine reagent was diluted in 50 μl of Opti-MEM I and incubated at room temperature for 5 min. The diluted DNA and the diluted Lipofectamine reagent were mixed together and incubated at room temperature for a further 20 min. The DNA complexes were added to the wells containing the adhered HeLa cells (100 μl/ well) and incubated at 37^{o}C in 5% CO_2 for 24 h before testing for transgene expression by Immunofluorescence using a Leica DM LB2 fluorescence microscope (Leica).

2.14.2 Amaxa electroporation

A20-1 HS cells were transfected using a Nucleofector AAD-1001 and the cell line nucleofector kit L (Amaxa Biosystems). In brief, A20-1 HS cells were washed once in PBS and then approximately $2x10^6$ cells were resuspended in 100 μl buffer L. 2 μg of plasmid DNA (detailed in **Table 5**) was added, mixed well and the cell/DNA suspension was transferred to nucleofector cuvettes. Following nucleofection using programme L-13 (optimized by Amaxa Biosystems for A20-1 transfection cells were immediately removed from the nucleofector cuvettes and transferred to 6 well plates containing 3 ml cRPMI and incubated at 37^{o}C in 5% CO_2 for 24 h. Typically 1 ml of

the cell suspension was then removed for screening by flow cytometric analysis. In addition, 1 ml was also removed for screening by antigen presentation assay and the remaining 1 ml was expanded for the generation of stable cell lines (see **Section 2.15**)

2.15 A20-1 HS generation of stable cell transfectants.

To generate A20-1 HS stable cell lines expressing the C71 or 1-C-6 aggrecan-specific BCR, cells were transfected with the C71/1-C-6 H and L chain plasmids, generated in **section 2.13**. 48 h later cells were washed in RF-2 by centrifugation at 400G for 5 min. Cells were resuspended in 50 ml cRPMI containing selection antibiotics at 50% the dose previously established to kill untransfected A20-1 HS cells (0.25 mg/ml G418 and 0.375 mg/ml hygromycin B) and plated out 500 µl/well into 4 x 24 well plates/transfection. 10 days later 500µl of cRPMI containing 2x G418 (1mg/ml) and 2x hygromycin B (1.5 mg/ml) was added to the wells. Colonies were visible by day 21, expanded accordingly in 0.5 mg/ml G418/0.75 mg/ml hygromycin B and screened using flow cytometry and antigen presentation assays. Positive colonies were later cloned using the single cell cloning method described in **Section 2.4.**

TRANSFECTION CONDITIONS
C71 heavy chain only
C71 light chain only
C71 heavy and light chain
1-C-6 heavy chain only
1-C-6 light chain only
1-C-6 heavy and light chain
No DNA negative control
TTCF heavy chain only positive control
TTCF light chain only positive control
TTCF heavy and light chain only positive control
pmaxGFP™ positive control (AMAXA)

Table 2.5 Plasmids used to transfect cells by electroporation or lipofection.

2.16 Transfectant analysis

2.16.1 HeLa transfectant Immunofluorescence

Medium was removed from transfected HeLa cells and cells were washed 3 times in PBSA (PBS containing 0.01% $MgCl_2$, 0.01% $CaCl_2$). PBSA wash was removed and 500 µl of 3% paraformaldehyde (PFA)/PBS solution was added to each well and incubated at room temperature for 20 min. Cells were washed 3 times in PBSA. 50 mM NH_4Cl was added to each well and incubated for 10 min at room temperature to quench the PFA and cells were washed 3 times in PBSA. To permeabilize the cells, 500 µl of Triton x-100 0.2% (Sigma) was added to each well and incubated at room temperature for 4 min. The cells were washed 3 times in PBSA. Primary antibodies, rabbit anti-human IgG H + L biotin Fab^2 (Southern Biotech. Assoc. Inc.) and rabbit anti-human kappa light chain bio (Serotec), were diluted (1:100) in PBSA 2% FBS and added as droplets onto a sheet of parafilm. The glass coverslips containing the permeabilized HeLa cells were placed onto the primary antibody droplet and incubated for 1 h in the dark in a dampened container. The slides were returned to a 24 well plate and washed 3 times in PBSA FBS 2%. Streptavidin FITC was diluted 1:1000 in PBSA and added in droplet form to a sheet of parafilm. The glass coverslips were placed onto the diluted streptavidin FITC and incubated for 30 min in the dark in a dampened container. Slides were returned to a 24 well plate, washed 4 times in PBSA and then washed thoroughly in a glass beaker containing H_2O to remove all traces of PBS. Coverslips were dried and mounted (Citifluor, Agar Scientific) onto a microscope slide for visualization by immunofluorescence.

2.16.2 A20-1 HS transfectant screening (Flow cytometry).

A20-1 HS cells were screened for the expression of a new aggrecan-specific BCR containing a human H chain C region, both 24 h post transfection and following the generation of stable transfectants. Cells were prepared for flow cytometry as described in **section 2.12.1**. In brief, cells were washed in FACS buffer and incubated for 1 h on ice in FACS buffer containing either primary antibody rabbit anti-human IgG H + L biotin Fab^2 (1:100) or biotinylated aggrecan 50 µg/ml. Cells were washed 3 times in FACS buffer. Cells were mixed with streptavidin FITC, diluted 1:100 and incubated for 1 h on ice, in the dark. Cells were washed 6 times and resuspended in

200 µl FACS buffer. 10,000 events were collected for each sample using a FACScan flow cytometer and data analysed using Flowjo software.

2.16.3 A20-1 HS transfectant screening (Antigen presentation).

A20-1 HS transiently transfected cells (C71 and 1-C-6) were screened (using the antigen presentation assay described in **section 2.10.2**) 24 h post transfection using graded doses of deglycosylated aggrecan, p84-103 and the p84-103-specific T cell hybridoma 192. Antigen presentation by C71 and 1-C-6 stable transfectants were compared with dendritic cells and macrophages using deglycosylated aggrecan, glycosylated aggrecan, TTCF, p84-103, p2373-2387, p2363–2378 and the p84-103-specific T cell hybridoma (192), the p2373-2387 specific T cell hybridoma (D9), the p2363–2378-specific T cell hybridoma (D11) and the TTCF $^{1120\text{-}1137}$-specific T cell hybridoma MC52 (Marzena Ciechomska unpublished).

2.17 Generation of aggrecan-specific T cell hybridomas

2.17.1 Immunisation, cell isolation and in vitro re-stimulation.

6-8 week female BALB/c mice were immunized subcutaneously in a single footpad with either 25 µl of aggrecan (27 µg) or synthetic peptides p2363-2378 or p2373-2387 (100 µg) emulsified in Titermax and popliteal lymph nodes were removed 7 days later.

Day 0: Popliteal lymph node cells were passed through a cell strainer and washed in RF-2 by centrifugation at 400G for 5 min. The cells were then resuspended in cRPMI, seeded at $3x10^6$/well in a total volume of 200 µl with either 50 µg/ml of aggrecan or 100 µg/ml of either peptide p2363-2378 or p2373-2387 (**Table 2.6**) and incubated at 37^oC in 5% CO_2 for 3 days.

Day 3: T cell lines were pooled from several wells and viable cells were separated by density gradient centrifugation. The cells were washed in RF-2 by centrifugation at 400G for 5 min and resuspended in 5 ml of cRPMI. 5 ml of Lymphoprep density separation medium of 1.077 g/ml (Axis Shield PoC AS) was loaded into a 15 ml falcon tube, the cell suspension was carefully layered on top and centrifuged at 700G for 10 min. Viable cells were recovered from the interface and washed in RF-2 by centrifugation at 400G for 5 min and resuspended in cRPMI and seeded at $6x10^5$/well in 2 ml cRPMI containing 5 ng/ml rIL-2.

Day 7: 1 ml of medium was removed and replenished with 1 ml cRPMI containing 0.1 ng/ml rIL-2.

Day 14: Resting T cells were re-stimulated with antigen (**see Table 2.6**). 1 ml of medium was removed from wells and replaced with l ml cRPMI containing irradiated splenocytes (20 grays/5 min) as a source of antigen presenting cells and either 100 µg/ml of peptide (p2363-2378 or p2373-2387) or 50 µg/ml of aggrecan.

This 14 day cycle was repeated 3 times before the T cell lines were tested for antigen-specificity using a proliferation assay and then fused to the T cell lymphoma cell line BW5147 3 days post re-stimulation

2.16.2 T cell fusion

BW5147 cells and T cell blasts were washed twice in serum free HBSS by centrifugation at 400G. Cells were then mixed at a 1:1 ratio ($2x10^6$ of each) and washed in HBBS. All of the supernatant was carefully removed and the pellet was resuspended in 0.7 ml of PEG (Sigma) added a dropwise. Cells were washed by centrifugation at 200G for 5 min in 14 ml of pre-warmed (37^oC) HBSS (added slowly to the cell suspension) and resuspended in pre-warmed (37^oC) cRPMI. The fused cells were seeded at various dilutions; 1:1, 1:3 and 1:9 in flat bottomed 96 well plates 100 μl/well and incubated at 37^oC in 5% CO_2 for 24 h before adding (100 μl/well) of 2x HAT. HAT resistant colonies were observed by day 10 and expanded accordingly.

Immunizing antigen	**Re-stimulating antigen**
Aggrecan	Aggrecan
Aggrecan	p2373-2387
Aggrecan	p2363-2378
p2373-2387	p2373-2387
p2363-2378	p2363-2378

Table 2.6 Antigens used for animal immunisation and re-stimulation of T cells.

2.17 Cytokine analysis of CD4+ T cells from TCR-5/4E8-tg mice.

2.17.1 $CD4^+$ T cell isolation

Four TCR-5/4E8-tg mice were sacrificed and cells were isolated from the spleens. Cells were pooled and passed through a 100 μm cell strainer (BD Biosciences) to form a single cell suspension. Cells were transferred to a 15 ml falcon tube, centrifuged and red blood cells lysed by adding 2 ml of red blood cell lysis buffer per spleen (Sigma) for 4 min at room temperature. Cells were washed in RF-2 by centrifugation at 400G for 5 min and resuspended in cRPMI. Cells were pelleted at 400G for 10 min and all of the supernatant removed. Cells were resuspended in ice cold MACS buffer (PBS, 0.5%BSA, 2 mM EDTA, 90 μl/10^7 cells). 10 μl of CD4 (L3T4) Microbeads (Miltenyi Biotech) were added per 10^7 cells, mixed and incubated for 15 min on ice. During the incubation period an LS separation column (Miltenyi Biotech) was prepared by placing it in the magnetic field of a VarioMacs separator (Miltenyi Biotech) and rinsing the column with 3 ml of ice cold MACS buffer. Following the 15 min incubation cells were washed by adding 2 ml of ice cold MACS buffer per 10^7 cells and centrifuged at 400G for 10 min. The supernatant was removed and cells were resuspended in 500 μl of ice cold MACS buffer/10^8 cells ready for magnetic separation. The cell suspension was applied onto the column and the unlabelled cells that passed through were collected in a 15 ml falcon tube. The column was washed 3 times in 3 ml MACS buffer and the total eluent was collected (unlabelled fraction). To isolate the magnetically labelled $CD4^+$ fraction the column was removed from the separator and placed on a 15 ml falcon tube. 5 ml of MACS buffer was added to the column and the labelled fraction was removed by applying a plunger to the column once to flush out the bound cells. The cells were washed in RF-2 by centrifugation at 400G for 5 min, resuspended in cRPMI and counted.

2.17.2 Flow cytometry to determine purity and TCR Vβ4 usage of the isolated CD4+ T cells.

p84-103-specific T cells express $V\alpha1.1^+/V\beta4^+$ TCR (Berlo, van Kooten et al. 2005). Isolated cells were incubated with anti-CD4 PerCP, anti-TCRαβ PE and anti-vβ4 PE to detect the total CD4+ and TCRαβ + population and the proportion of $V\beta4^+$ T cells. Cells were transferred to FACS tubes at approximately $2.5x10^5$/tube, pelleted by centrifugation at 400G for 5 min and washed in FACS buffer. The supernatant was

removed and the cells were resuspended in the appropriately diluted primary antibody (**Table 2.1**) and incubated at 4^{o}C for 1 h in the dark. Cells were washed 3 times in ice-cold FACS buffer at 400G for 5 min, resuspended in 200 µl of ice cold FACS buffer and placed on ice until analysis using a FACScan flow cytometer (BD Biosciences) and data analysed with Flowjo software (Treestar^{TM}). All staining was carried out in 96 well round bottom plates at 4^{o}C in the dark in the presence of 2.4G2 Fc block.

2.17.3 Cytokine analysis

Antigen presentation assays (described previously in **section 2.10.2**) were used to determine the cytokines produced by CD4+ TCR-5/4E8 T cells in response to p84-103 or aggrecan. CD4+ TCR-5/4E8 T cells (5x10^{4} /well) were incubated for 72 h with 1x10^{4} dendritic cells, macrophages, aggrecan-specific B cells (C71-4C5 and C71 -5F10) or 5x10^{4} A20-1 HS B cell hybridomas and graded doses of either p84-103 or aggrecan. Controls included each of the APC or T cells only incubated with medium, aggrecan or p84-103 and medium or antigen without any cells. The assay was frozen after 72 h to lyse the cells. 50 µl of supernatant was removed from each well to detect IL-2 by CTLL-2 proliferation, 50 µl was used to detect IL-10 and 50 µl was used to detect IFNγ levels by ELISA.

96 well ELISA plates were coated with 50 µl coating buffer containing 2 µg/ml of either anti-IL-10 or anti-IFNγ capture antibody (BD Biosciences). Plates were wrapped in parafilm and incubated at 4^{o}C overnight. Following the overnight incubation plates were washed with PBS containing 0.1% Tween to remove the unbound antibody and blocked by adding 200 µl 2% milk powder/PBS to each well and incubating for 1 h at 37^{o}C. A standard curve was determined for each plate by adding graded doses of rIL-10 or rIFNγ beginning with a top dose of 100 ng/ml to each plate containing the relevant capture antibody. 50 µl of supernatant from the antigen presentation assay was transferred to each plate containing either anti-IL-10 or anti-IFNγ and the relevant recombinant cytokine standards and incubated at room temperature for 2 h. Plates were washed 4 times with PBS containing 0.1% Tween. 100 µl of biotinylated detection antibody (0.5 µg/ml, anti-IL-10 or anti-IFNγ) (BD Biosciences) was added to each well and incubated for 1 h at room temperature. Plates were washed 6 times with PBS containing 0.1% Tween. 100 µl of extra-avidin

peroxidase diluted 1/1000 in 2% milk powder/PBS was added to each well and incubated at room temperature for 30 min. Plates were washed 6 times with PBS containing 0.1% Tween. Substrate (TMB**)** was subsequently added along with hydrogen peroxide in phosphate citrate buffer for 4 min, the reaction was stopped using 2 M H_2SO_4 and absorbance was read at 450 nm**.** Controls for this experiment included blank wells coated with capture antibody and subsequently incubated with media only.

2.18 Statistical analysis

Significant differences between groups of 3 or more was determined by one-way ANOVA using a 95% confidence interval followed by Tukeys test for multiple differences between experimental means. Experiments in which 2 groups were compared, significant differences were determined using students T test (Graphpad Prism 5.0 statistical and graphing software).

3. Generation of immortalized aggrecan-specific B cells by fusion with A20-1 HS lymphoma cells.

3.1 Introduction

B cells play a multifaceted role in the pathology of RA by the secretion of autoantibodies (Duskin and Eisenberg) and pro-inflammatory cytokines (Lund, Garvy et al. 2005) (Dorner and Burmester 2003). In addition, antigen-specific B cells are very efficient antigen presenting cells (APC) and contribute to T cell activation (Weyand, Seyler et al. 2005). Proteoglycan induced arthritis (PGIA) is a mouse model of RA, in which mice immunized with aggrecan in adjuvant develop a disease very similar to human RA. Using this model Shlomchik *et al* revealed that B cells were essential not only in providing autoantibodies but also as aggrecan-specific APC (O'Neill, Shlomchik et al. 2005).

Studies in the past few years have identified elevated levels of aggrecan cleavage products, aggrecan-specific antibodies and aggrecan-specific T cells in RA patients (Poole and Dieppe 1994) (Vynios, Tsagaraki et al. 2006) (Zou, Zhang et al. 2003). In addition, germinal centre like structures, containing proliferating B and T cells have been found in many RA patients (Weyand, Goronzy et al. 2000). Rituximab is an anti-CD20 monoclonal antibody therapy used to deplete both pre and mature B cells which has been used to successfully treat RA patients for whom other therapies have failed (Edwards and Cambridge 2006).

Taken together these data imply a role for both aggrecan and aggrecan-specific T and B cells in the induction and perpetuation of disease pathogenesis. However, these studies fail to identify the mechanisms by which B cells act as essential APC.

In the previous chapter, I described how I have isolated and modified aggrecan from bovine nasal cartilage. In this chapter I have established an antigen presentation system that could be used to compare aggrecan presentation by A20-1 HS B cells, dendritic cells and macrophages. My findings concur with previous studies that demonstrate B cells are poor at non-specific antigen uptake and presentation (Kakiuchi, Chesnut et al. 1983; Rock, Benacerraf et al. 1984). However, it has been

well documented that B cells are highly efficient at presenting antigen to T cells when they express an antigen-specific BCR (Lanzavecchia 1985; Casten, Kaumaya et al. 1988; Batista and Neuberger 1998). Therefore, in order to study the effective uptake and presentation of aggrecan by B cells I needed to generate B cells with an aggrecan-specific BCR. Methods used to isolate antigen-specific B cells involve immunization with antigen to increase the usually low numbers of specific B cells followed by isolation using a capture antigen adsorbed on a solid surface, rosetting on antigen coated particles or isolation by staining with fluorescent antigen followed by flow cytometric cell sorting (Kodituwakku, Jessup et al. 2003; Newman, Rice et al. 2003). However, these methods have had limited success and produced low yields. Therefore, my first approach was novel and based on the widely used monoclonal antibody production protocol first described by Kohler and Milstein used to boost a population of antigen-specific B cells that are usually present at a very low frequency <1% (Kohler and Milstein 1975; Oshiba, Renz et al. 1994). According to this method, it is important to give animals several immunizations with antigen in adjuvant. The resulting antigen-specific cells are then isolated from the animal and immortalised by fusion with an immunoglobulin negative tumour B cell such as the NSO mouse myeloma cell line. NSO cells lack the enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT) and are resistant to azaguanine and the "salvage pathway" of DNA synthesis. The addition of aminopterin to culture media blocks DNA *de novo* synthesis and therefore, unless NSO cells are fused with a HGPRT+ cell such as an ex-vivo B cell they will die in medium containing Hypoxanthine (5 mM), Aminopterin (20 mM), Thymidine (0.8 mM) (HAT). To identify successfully fused cells the NSO/splenic B cell fusion population are grown in HAT selection medium and resultant fusion colonies are screened for the production of antigen-specific Ig by ELISA.

Immunizing mice with antigen in adjuvant stimulates their adaptive immune system to produce both plasma cells that secrete antigen-specific Ig and memory B cells that express a surface Ig specific for aggrecan (BCR). Therefore, in principle this method could be used to expand and immortalize memory B cells expressing an aggrecan-specific BCR (illustrated in **Figure 3.1**) and the aggrecan-specific B cells could be used to understand the mechanisms by which B cells process and present the candidate rheumatoid arthritis antigen, aggrecan to aggrecan-specific $CD4^+$ T cells.

For the purpose of this study the B cell lymphoma cell line A20-1 HS was used as a fusion partner to ensure that the aggrecan-specific Ig would be expressed on the cell surface (BCR) rather than secreted as antibody (discussed in **section 3.2.1**).

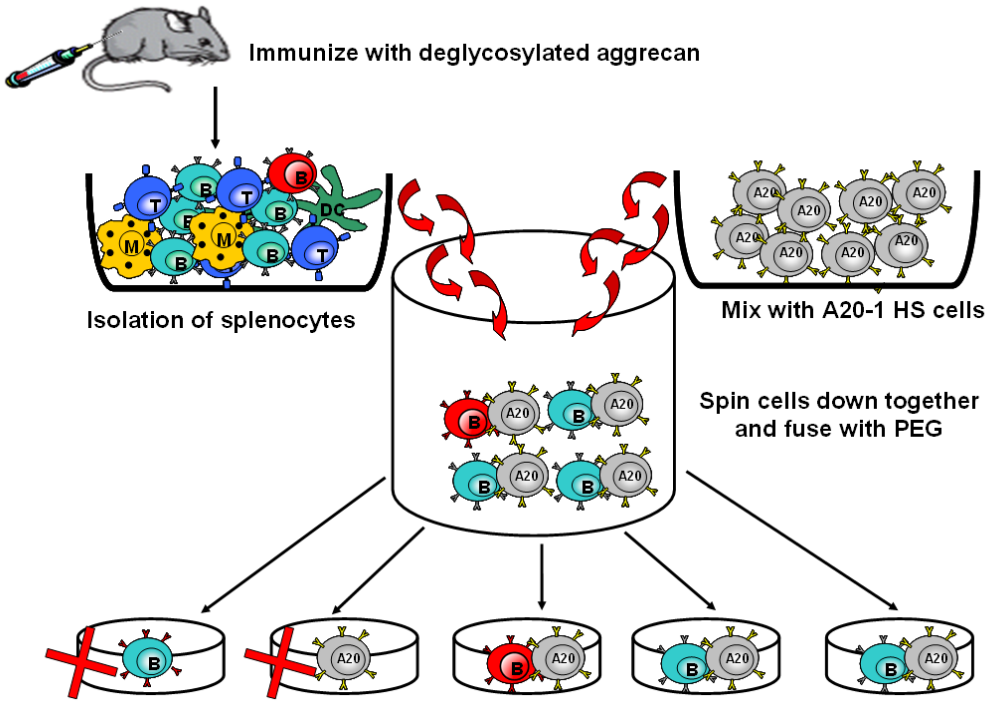


Figure 3.1 Splenocyte fusion protocol to generate aggrecan-specific B cell hybridomas. *Mice were immunised on 4 separate occasions at 3 week intervals with deglycosylated aggrecan to boost a population of aggrecan-specific B cells (shown in red). The mice were sacrificed and splenocytes removed. Splenocytes were mixed at a 2:1 ratio with A20-1 HS B lymphoma cells pelleted by centrifugation and fused with polyethylene glycol (PEG). The cells were plated out in selection media containing HAT. Cells that were not successfully fused die in the selection media and fused cell populations are expanded and screened by flow cytometry and antigen presentation assays to identify those cells that are fused with aggrecan-specific B cells.*

3.1.2 The aim of this chapter:

- To immortalize B cells isolated from aggrecan-immunized mice.
- To screen resultant colonies for B cells expressing an aggrecan-specific BCR.
- To assess their potential as antigen presenting cells.

3.2 Results

3.2.1 Immunisation and cell fusion.

Primary immunisation with antigen produces early response, low affinity Ig (Kohler and Milstein 1975). However, this first encounter with antigen allows the subsequent clonal expansion of antigen-specific T cell and B cells which are now 'primed' upon additional encounter with the same antigen. B cells with antigen-specific BCR appear at around 5-6 days post immunisation and circulating Ig appears at around day 7 peaking at day 10 (Kohler and Milstein 1975). Subsequent immunisations promote a stronger and more rapid immune response designed to allow primed lymphocytes to eliminate pathogens before they cause disease. Therefore, following a second boost, antigen-specific B cells are detectable at days 3-4 and circulating Ig at around the same time, peaking at around days 10-14, which persists for around 2-4 weeks (Kohler and Milstein 1975). Further immunisations with antigen in adjuvant will boost the affinity of Ig due to somatic mutations that occur within the variable regions of the BCR heavy and light chains during antigen induced expansion and activation (Kim, Davis et al. 1981). Somatic mutations can produce a higher affinity BCR that will bind to the antigen and induce the expansion of high affinity B cells.

Based on this strategy, groups of mice were either given 3 immunisations (fusion 1) or 4 immunisations (fusion 2). A gap of approximately 3 weeks was left between immunisations to ensure that circulating antigen-specific Ig was cleared, and the immunising antigen was not rapidly complexed with the Ig and removed from the circulation (Harlow 1988). To test the anti-aggrecan responses in the immunized mice, serum was obtained and tested for Ig levels prior to immunisation and 5 days post initial immunisation and following each subsequent boost. Results in **Figure 3.2** show that the collected serum contains no aggrecan-specific Ig prior to immunisation. However, following an initial immunisation with aggrecan, aggrecan-specific Ig was detected, with Ig levels increasing with each subsequent boost. For both fusion immunisation protocols the final aggrecan immunisations in alum were introduced intra-peritonealy to allow antigen-specific B cells to accumulate in the spleen. Cells were recovered from the spleen either 5 or 10 days post final immunisation and fused with a B lymphoma cell. Initial attempts by Dr Knight and Prof Robinson to fuse memory B cells with the B cell lymphoma NSO proved unsuccessful. We

hypothesised that NSO cells may not be a good candidate for fusion as this cell line is a non-Ig secreting, (-ve for both H and L chain) cell that may not support the cell surface expression of a BCR (Galfre and Milstein 1981). The HAT sensitive lymphoma cell line A20-1 HS expresses surface Ig, is able to present high doses of antigen to CD4+ T cells and may be a more suitable candidate for this protocol. Therefore, following a final immunisation, cells were isolated from the spleen and fused to the A20-1 HS cells to generate immortalized hybridoma cells.

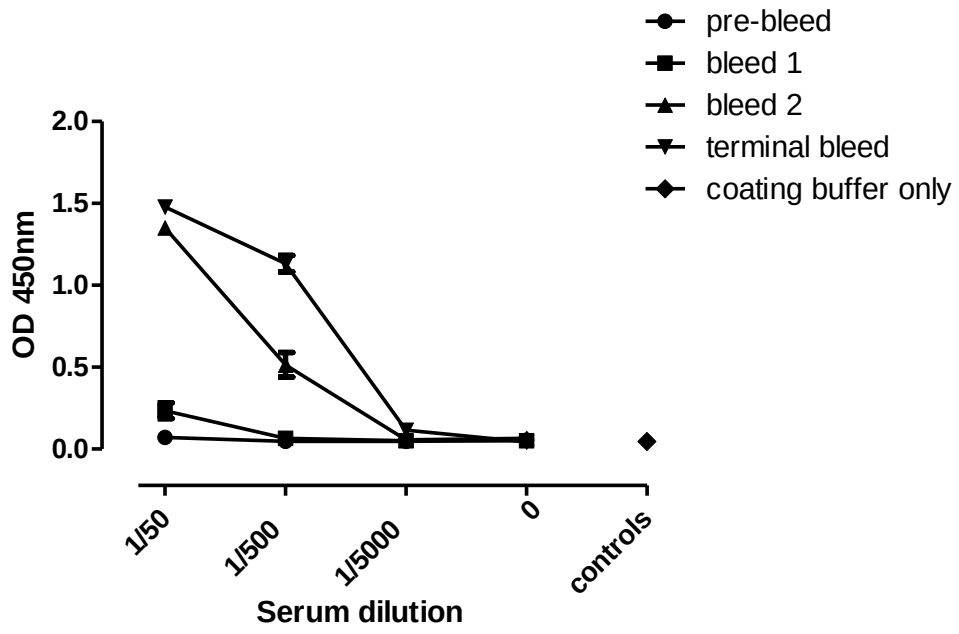


Figure 3.2 The reactivity of pre-bleed, post-bleed and terminal bleed with aggrecan detected by ELISA. *Each point represents serum pooled from 4 mice and shows anti-aggrecan antibody titres either prior to immunisation (pre-bleed), 10 days post 1st immunisation with aggrecan (bleed 1), 10 days post 2nd immunisation with aggrecan (bleed 2) and 5 days post the 3rd and final immunisation with aggrecan (terminal bleed).*

3.2.2 Characterisation of fusion cells by flow cytometry and antigen presentation.

3.2.2.1 Flow cytometry

Initial fusion of splenocytes isolated from mice immunized under the 2 described protocols (**section 2.9.1**) with A20-1 HS generated a total of 53 HAT resistant colonies. These fusions were initially screened by flow cytometry for BCR heavy chain isotype and light chain usage (Representative fusions shown in **Figure 3.3**). As expected all of the fusions expressed an IgG2a $^{+}$/κ light chain^{+} BCR at comparable levels to that seen on the fusion partner A20-1 HS. However, 7 fusions including representative fusions 13 and 24 also expressed an additional BCR H chain isotype. Subsequently, A20-1HS and all of the fusions were examined for the expression of cell surface molecules involved in antigen presentation, MHC class II and the co-stimulatory molecules B7.1 and B7.2 (**Figure 3.4**). Results show that fusion 53 expressed surface MHC class II at levels comparable to that seen on the A20-1 HS fusion partner. However, both fusions 13 and 24 express higher levels of MHC class II. Fusion 13 expresses comparable levels of the co-stimulatory molecules B7.1 and slightly higher levels of B7.2 than the A20-1 HS fusion partner. Fusions 24 and 53 express higher levels of both B7.1 and B7.2 when compared to A20-1 HS cells.

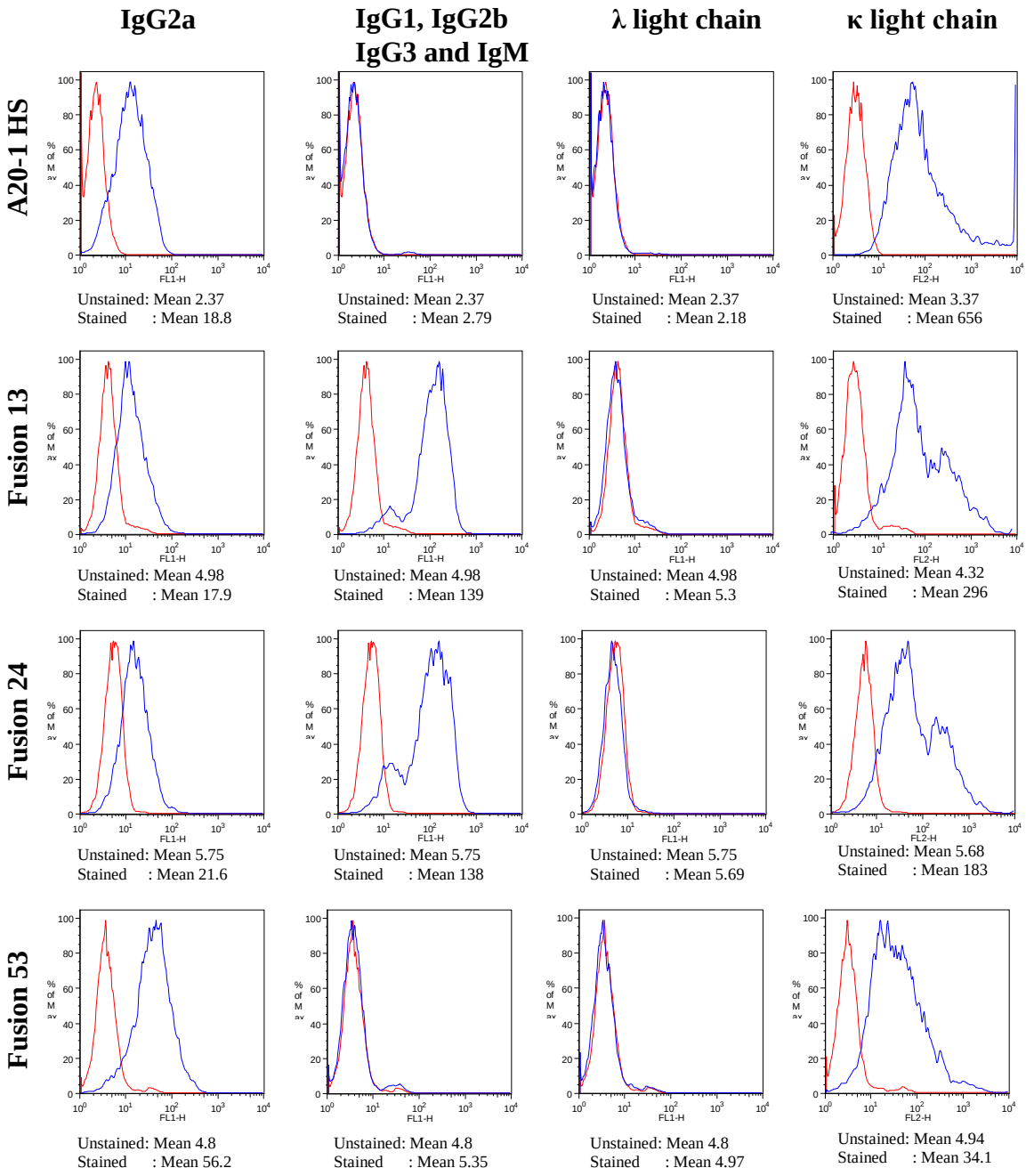


Figure 3.3 Characterisation of BCR phenotype by flow cytometry

Staining of gated A20-1HS cells and 3 representative fusion populations for various BCR isotype markers. Red histograms represent unstained cells and the blue histograms represent cells stained for the various markers. Antibodies were either FITC, PE or biotin conjugated, in the latter case streptavidin FITC was used as a secondary reagent. All experiments were carried out at 4^{o}C in the presence of Fc block. Mean fluorescence intensity values are shown below each histogram. Data is representative of 3 independent experiments.

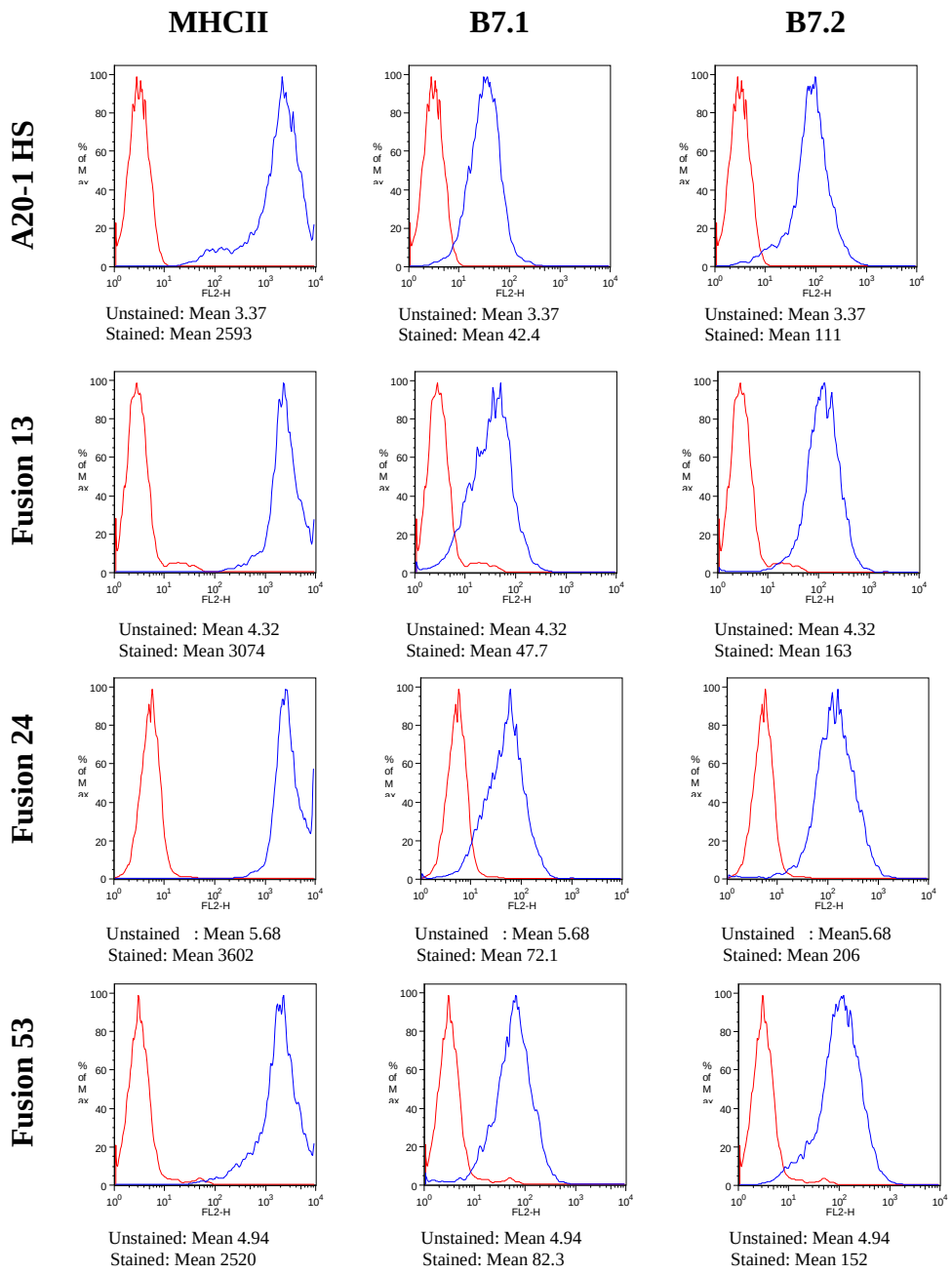


Figure 3.4 Expression of molecules involved in antigen presentation verified by flow cytometry *Staining of gated A20-1HS cells and 3 representative fusion populations for various cell surface markers involved in antigen presentation to CD4$^+$ T cells. Red histograms represent unstained cells and the blue histograms represent cells stained for the various markers. Antibodies were PE conjugated. All experiments were carried out at 4^{o}C in the presence of Fc block. Mean fluorescence intensity values are shown below each histogram. Data is representative of 3 independent experiments.*

3.2.2.2 Antigen presentation assay.

As described in **Chapter 1**, B cells would be expected to be more efficient at aggrecan presentation when they express an aggrecan-specific BCR. Therefore, if the fusions shown in **Figure 3.3** have resulted from a fusion with an aggrecan-specific memory B cell, then aggrecan presentation to T cells should be more efficient than that seen with the fusion partner A20-1 HS.

The extent of aggrecan presentation to the BALB/c ($H\text{-}2^d$) $aggrecan_{84\text{-}103}$ -specific T cell hybridoma 192 was determined using antigen presentation assays as illustrated in (**Figure 3.5**). A20-1 HS or fusion cells were cultured with the T cell hybridomas 192 for 24 h in the presence of graded doses of aggrecan. Initially, T cell hybridoma activation was measured by IL-2 production using the IL-2 dependent cell line HT-2. However, subsequently I found the IL-2 dependent cell line CTLL-2 to be more sensitive for detecting IL-2 production (data showing comparison in **Figure 3.6**). Therefore, following the screening of cells from fusion number 1, supernatants from all subsequent experiments were screened using CTLL-2. HT-2 or CTLL-2 cells were cultured with assay supernatant and proliferation in response to IL-2 was measured by the incorporation of tritiated thymidine. The efficiency of antigen presentation can then be determined by noting the minimum dose of aggrecan required to obtain an IL-2 response.

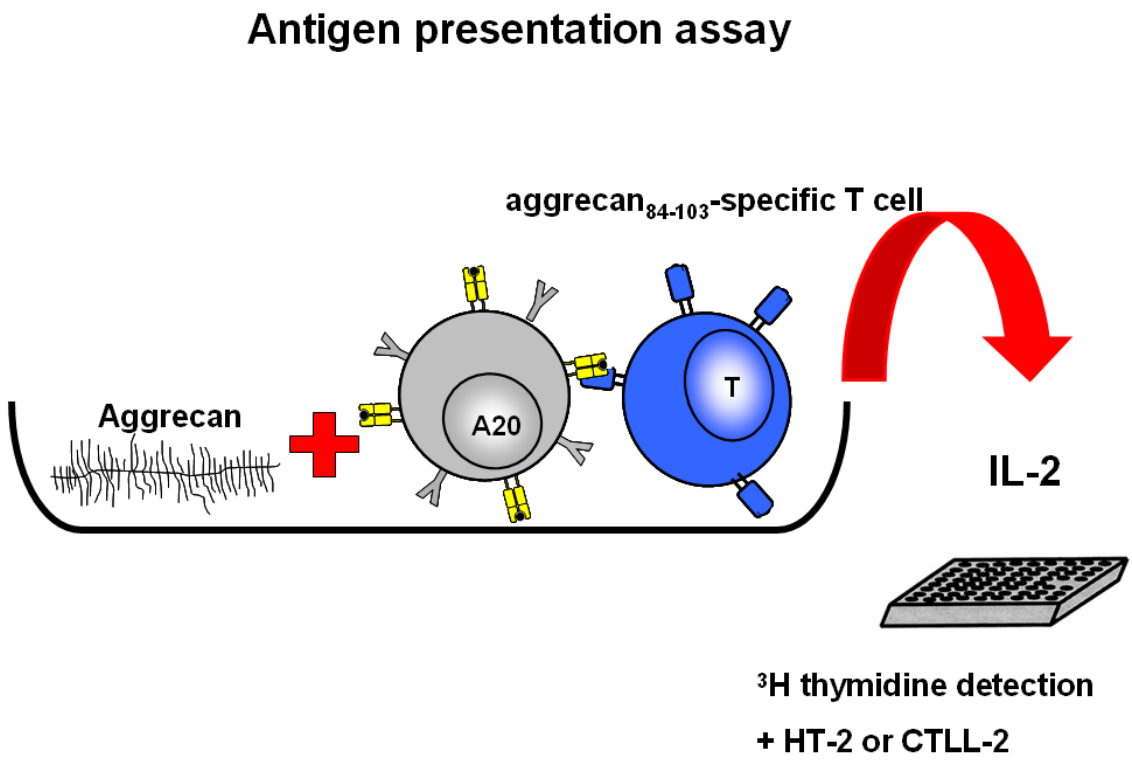


Figure 3.5 Antigen presentation assay.
A20 B lymphoma cells or other APC and T cells (T) are incubated for 24 h in a 96 well plate in the presence of graded doses of antigen (aggrecan or p84-103).T cell stimulation is measured by resultant IL-2 production. In order to quantitate IL-2 production following a single cycle of freeze/thawing supernatants were transferred to new plates and cultured with IL-2 dependent cell lines (HT-2 or CTLL-2) and ^{3}H thymidine. HT-2 or CTLL-2 proliferation in response to IL-2 can then be measured by ^{3}H thymidine incorporation.

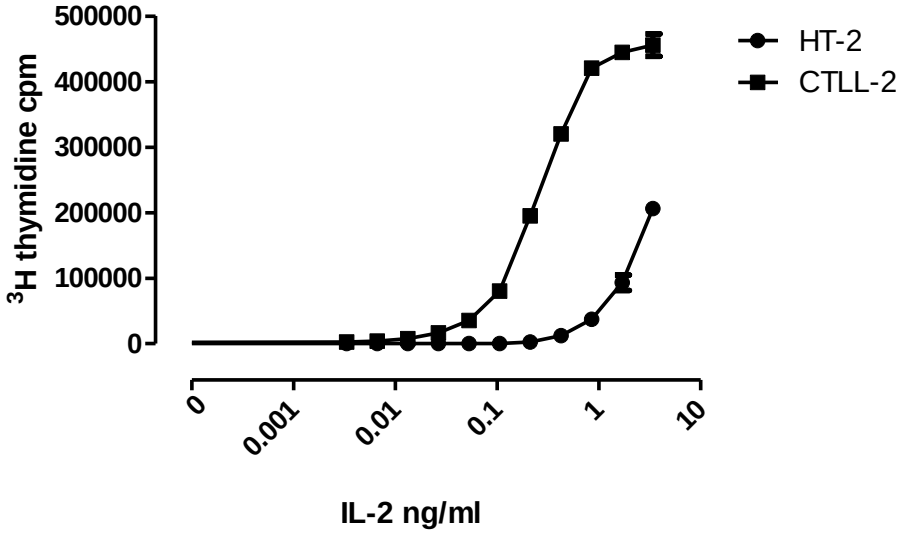


Figure 3.6 HT-2/CTLL-2 dose response curves to IL-2

.*Initial antigen presentation assays with fusions were carried out using IL-2 dependent cell line HT-2 to quantitate the amount of IL-2 present in T cell assay supernatants. All data generated from fusion 2 shows IL-2 detection by the IL-2 dependent cell line CTLL-2. HT-2 and CTLL-2 were incubated with graded doses of recombinant IL-2 for 24 hours. ^{3}H thymidine was added for 18 hours, cells were then harvested onto glass fibre filters and ^{3}H thymidine incorporation was quantified and displayed as ^{3}H thymidine cpm. Results represent the mean cpm $\pm$ S.D. of duplicate wells.*

Data from flow cytometry analysis and antigen presentation assays reveals distinct patterns by the fusion cells. Either, fusions did not present aggrecan more efficiently than the A20-1 HS fusion partner and were negative for the expression of a new isotype BCR (not shown) and were excluded from further studies, or they fell into the following categories and representative colonies were used for further examination of their potential as representative aggrecan-specific antigen presenting B cells (data shown in **Figure 3.7**).

Pattern a, 7 fusions were positive for the expression of a new BCR yet did not present aggrecan more efficiently than A20-1 HS.
Pattern b, 1 fusion (fusion 24) was positive for the expression of a new BCR and presented aggrecan more efficiently than A20-1 HS.
Pattern c, 10 fusions (including fusion 53) were negative for the expression of a new isotype BCR yet presented aggrecan more efficiently than A20-1 HS.

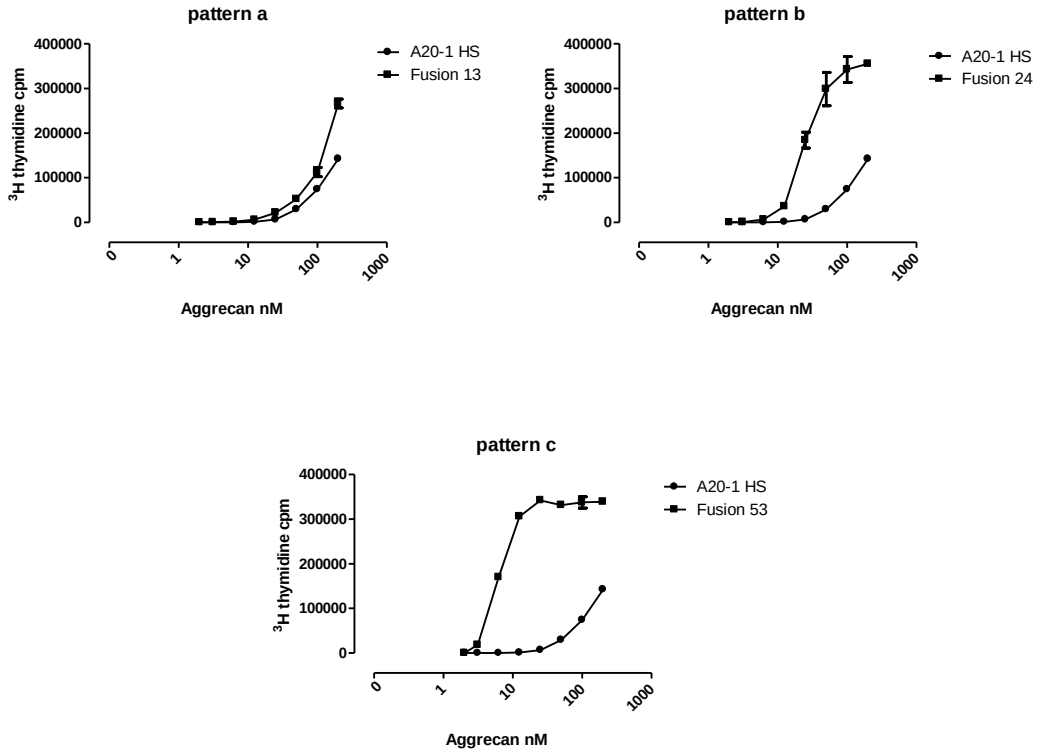


Figure 3.7 Antigen presentation by fusions generated from immunisation protocol 1. *Pattern a shows a representative fusion that expressed a new isotype BCR yet did not present aggrecan more efficiently than A20-1 HS. Pattern b shows a representative fusion that expressed a new BCR and presented aggrecan more efficiently than A20-1 HS. Pattern c shows a representative fusion that did not express a new isotype BCR and yet presented aggrecan more efficiently than A20-1 HS. A20-1 HS, or fusion cells ($5x10^4$) expressing either IgG2a and a new isotype BCR or IgG2a+ only were cultured with $aggrecan_{84-103}$-specific T cell hybridomas 192 ($5x10^4$) in the presence of graded doses of aggrecan for 24 h. Cells were killed by freezing at -80^{o}C and supernatant was removed for bioassay with CTLL-2 cells. ^{3}H thymidine was added for 18 h and T cell responses were determined and displayed as ^{3}H thymidine cpm +/- SEM. Data are representative of three experiments.*

I generated several populations of cells, which from my initial analysis, appeared to be good candidates for the expression of an aggrecan-specific BCR (patterns b and c). However, following prolonged periods of time in culture the fusions began to lose their efficiency at presenting aggrecan to the T cell hybridoma 192. I hypothesised that as the fusion cell populations were not yet clonal that this decline in antigen presentation efficiency could be due to an outgrowth of non-specific cells from a heterogeneous population. Therefore, to further investigate the candidate aggrecan-specific B cells, 44 single cell clones were generated from representative fusions 24 (pattern b) and 53 (pattern c) (**Figure 3.7**). Clones were re-screened for BCR heavy chain isotype to verify that they had retained that of the original fusion population and then examined for their ability to present aggrecan using the antigen presentation assay described. Flow cytometry confirmed that a representative clone of fusion 24 (24.14) retained the parental phenotype (BCR $IgG2a^+$ / IgM^+) and a representative clone of fusion 53 (53.28) retained the parental phenotype (BCR $IgG2a^+$) (data not shown).

Figure 3.8a, demonstrates that both clones 24.14 and 53.28 have retained their ability to show enhanced aggrecan presentation to the T cell hybridomas 192 in comparison to A20-1 HS efficiently presenting aggrecan from doses of 10nM ($p<0.01$). In all antigen presentation experiments thus far, cells had been incubated with graded doses of deglycosylated aggrecan. **Figure 3.8b** shows that A20-1 HS were unable to present aggrecan in its glycosylated form to the T cell hybridoma 192 even when incubated at high doses (200 nM). However, both fusions 24.14 (BCR $IgG2a^+$ / IgM^+) and 53.28 (BCR $IgG2a^+$) were able to efficiently present glycosylated aggrecan at doses of 50 nM ($p<0.001$).

It is possible that the enhanced presentation seen by the fusions when compared to that of A20-1 HS is not due to aggrecan uptake and processing but may reflect an overall improvement in T cell stimulation capacity due to surface presentation of the epitope 84-103 on $H\text{-}2^d$ for example. Therefore, I repeated the antigen presentation assay using graded doses of the synthetic peptide p84-103 as a pre-processed form of aggrecan. Data from **Figure 3.8c** shows that fusion 24.14 could present p84-103 approximately 10-fold more efficiently than A20-1 HS at doses of 0.003Nm ($p<0.001$) which correlates with the 10-fold improvement of deglycosylated aggrecan

seen in **Figure 3.8a**. This result may be explained by the data in **Figure 3.4** that shows relatively increased expression of MHC class II and the co-stimulatory molecules B7.1 and B7.2 by fusion 24.14 compared to 53.28 and A20-1 HS. However, fusion 53.28 did not present p84-103 more efficiently than A20-1 HS indicating that the improved presentation of both deglycosylated and glycosylated aggrecan may be due to more efficient uptake and/or processing by the 53.28 cells.

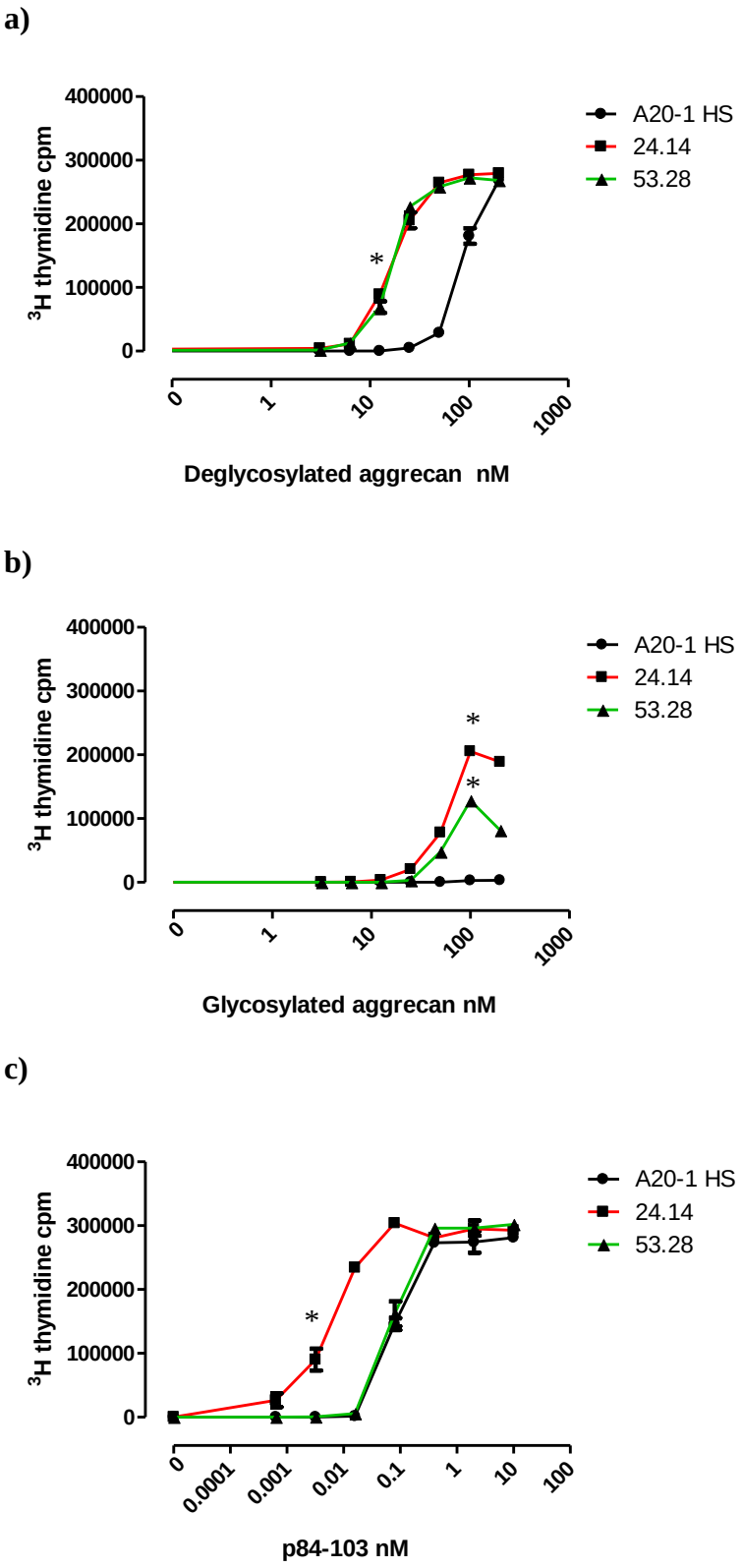


Figure 3.8 Antigen presentation by clones generated from fusions 24 and 53 generated from immunisation protocol 1. *A20-1 HS, or the fusion cells 24.14 and 53.28 ($5x10^4$) were cultured with aggrecan$_{84-103}$-specific T cell hybridomas 192 ($5x10^4$) in the presence of graded doses of **a)** deglycosylated aggrecan, **b)** glycosylated aggrecan and **c)** p84-103 for 24 h. Cells were killed by freezing at -80^{o}C and supernatant was removed for bioassay with CTLL-2 cells. ^{3}H thymidine was added for 18 h and T cell responses were determined and displayed as ^{3}H thymidine cpm +/- SEM. Data are representative of three experiments. The asterix represents statistical significance determined by one-way ANOVA where $p<0.05$.*

Data thus far was consistent with the expression of an aggrecan-specific BCR by fusions 24.14 and 53.28. Therefore, several attempts were made to verify this, before attempting more complex molecular processing studies.

3.2.2.3 Detection of aggrecan binding to fusions by flow cytometry.

The cloned fusions were screened for their ability to bind aggrecan. In order to achieve this, aggrecan was biotinylated using several different molar ratios of aggrecan/biotin. Following removal of excess biotin, the modified aggrecan was then tested by ELISA to verify a) that the biotinylated aggrecan was still recognisable by aggrecan-specific Ig and b) that the modified aggrecan contained sufficient biotin molecules to allow detection with streptavidin-HRP (**Figure 3.9**).

If the fusion clones express an aggrecan-specific BCR then I hypothesised that bound biotinylated aggrecan should be detected by flow cytometry following the addition of streptavidin-FITC. Fusion clones were incubated with 200 nM aggrecan (biotinylated at different molar concentrations) for 1 h followed by several washes and a 1 h incubation with streptavidin-FITC. Cells were kept on ice for the entire experiment to reduce internalization of the BCR and aggrecan. Data in **Figure 3.10** shows that binding of the biotinylated aggrecan by the B cell fusion clones was not detectable by flow cytometry**.** In addition, following incubation with up to 400 nM of the biotinylated aggrecan, fusion cells were still not able to retain the aggrecan at the cell surface for detection by flow cytometry (not shown).

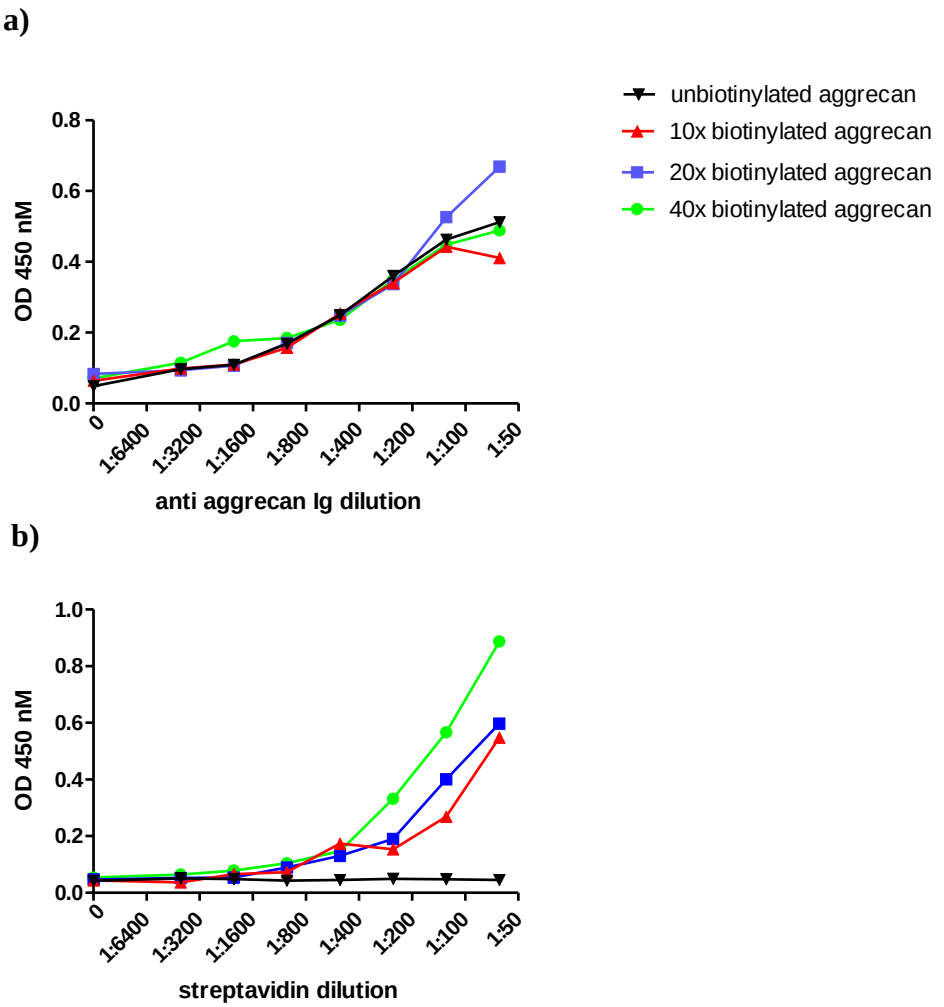


Figure 3.9 ELISA to determine the biotinylation of aggrecan and to confirm that biotinylation does not prevent recognition by aggrecan-specific antibodies.

Aggrecan was biotinylated at various molar ratios of biotin to aggrecan: 10 times biotin excess (10x), 20 times excess (20x) or 40 times excess (40x). Following dialysis, biotinylated aggrecan was bound to ELISA plates and detected by serial dilutions of either a) anti-aggrecan Ig (Serotec) followed by detection reagent HRP-conjugated anti-mouse or b) HRP-conjugated streptavidin. Data is representative of triplicate wells and 2 independent experiments.

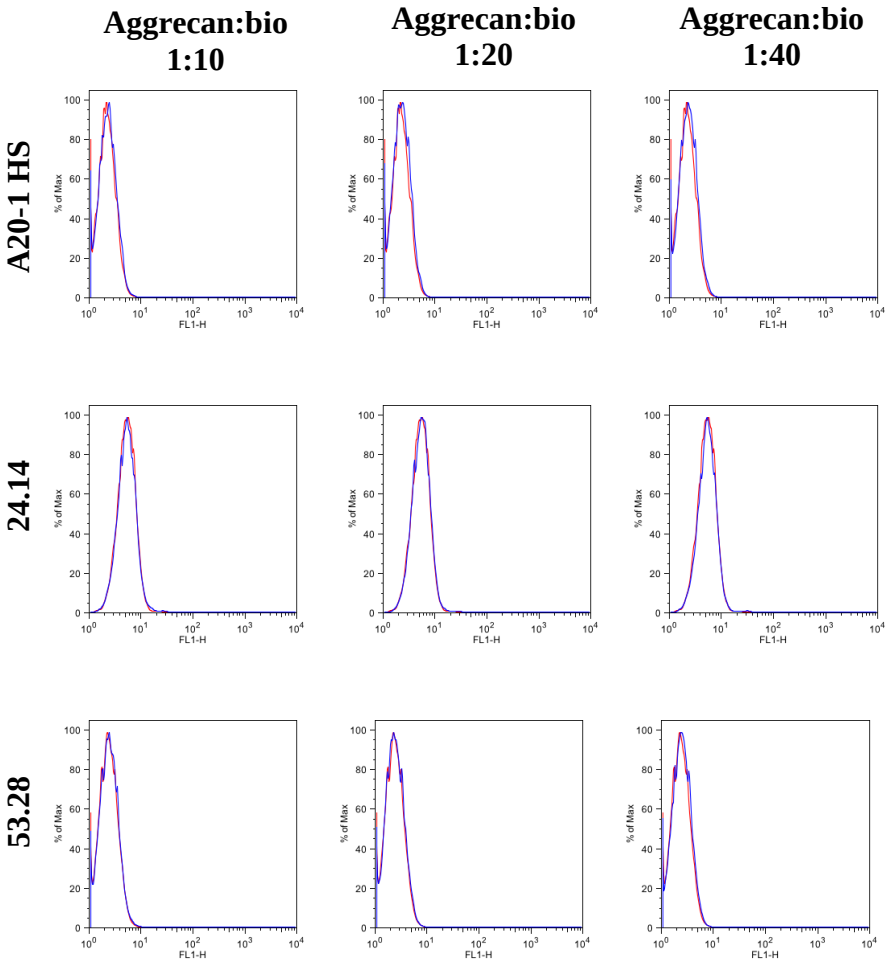


Figure 3.10 Aggrecan was not detected bound to the surface of candidate aggrecan-specific fusion clones by flow cytometry. *Staining of gated A20-1 HS cells and fusion clones 24.14 and 53.28 for surface bound aggrecan. Red histograms represent unstained cells and the blue histograms represent cells stained for the various markers. Aggrecan was biotin conjugated at either 10x, 20x or 40x molar excess and incubated with the cells for 1 h at a concentration of 200 nM. Cells were washed and then incubated with the secondary reagent streptavidin FITC for 1 h. All experiments were carried out at 4°C. Data is representative of 3 independent experiments.*

3.2.2.4 Presentation of p84-103 and deglycosylated aggrecan to T cell hybridomas 192 by fusion clones 53.28 and 24.14 in the presence of anti-Ig, anti-class II or anti-Fc to block presentation.

Can presentation via BCR mediated uptake be blocked by antibodies to the BCR?

Fusion clones were incubated with aggrecan and T cell hybridoma 192 in the presence of polyclonal anti-mouse IgG Fab^2, polyclonal anti-mouse IgM Fab^2, anti-mouse MHC class II or anti-mouse anti-FcRγII/III receptor antibodies (2.4G2) for 24 h (data shown in **Figure 3.11**.) If the observed improvement in presentation is BCR mediated, the addition of anti-Ig should have an inhibitory effect. However, I did not observe any inhibition by the addition of anti-Ig antibodies to the antigen presentation assay containing fusion clones. Furthermore, anti-Fc Ig was added to the antigen presentation assay to confirm whether Ig secreted by the fusion cells was aiding aggrecan uptake and presentation. The addition of 2.4G2 anti-Fc receptor antibodies had no effect on aggrecan presentation by the fusion cells. Anti-MHC II antibodies were used as a positive control and as expected the addition of the latter antibody had a profound inhibitory effect by preventing presentation of aggrecan peptide/MHC II complexes to the T cell hybridomas 192. Interestingly, the addition of increasing doses of both anti-IgM and anti-Fc antibodies blocked presentation of aggrecan by the A20-HS. A possible explanation for this is that the anti-IgM and anti-Fc antibodies are having a slightly toxic effect on these cells which is enough to effect antigen uptake such as that required for aggrecan presentation, but not sufficient to effect surface presentation of p84-103 that does not require uptake.

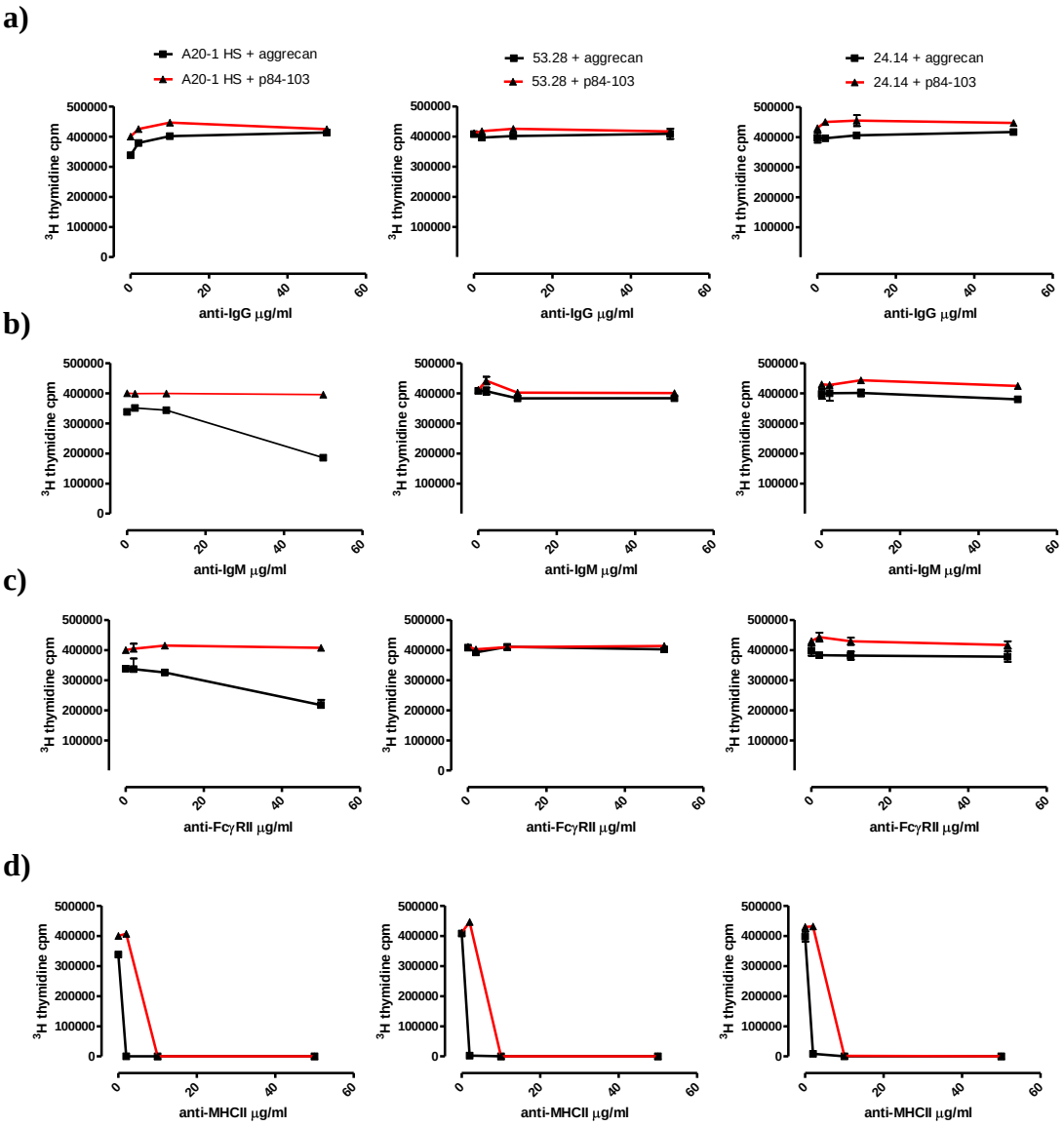


Figure 3.11 Antigen presentation by A20-1HS and fusion cells 53.28 and 24.14 in the presence of anti-Ig, Fc and MHCII antibodies. *A20-1 HS, or the fusion cells 53.28 and 24.14 were cultured with aggrecan$_{84-103}$-specific T cell hybridomas 192 and 200 nM deglycosylated aggrecan or 10 nM p84-103 in the presence of graded doses of a) anti-IgG, b) anti-IgM, c) anti-FcγRII/III or d) anti-MHCII antibodies for 24 h. Cells were killed by freezing at -80^{o}C and supernatant was removed for bioassay with CTLL-2 cells. ^{3}H thymidine was added for 18 h and T cell responses were determined and displayed as ^{3}H thymidine cpm +/- SEM. Data are representative of triplicate wells and 2 independent experiments.*

3.2.2.5 Ig characterisation by ELISA.

Do the fusions secrete aggrecan-specific Ig?

To further test the antigen-specificity of the BCR expressed by the B cell fusions 53.28 and 24.14, Ig secreted by the fusions into culture supernatant was examined by ELISA. Cells were cultured for several days to ensure a high cell density, pelleted by centrifugation and the supernatant collected. Initial experiments verified that Ig were present in the culture supernatant and detectable by ELISA. However, the Ig detected was not able to bind to aggrecan that had been bound to ELISA plates (data not shown). I hypothesised that the Ig detected in the culture supernatant may have a low affinity for aggrecan. To overcome this potential problem the Ig present in the supernatant was concentrated using vivaspin sample concentrator columns (GE Healthcare) and then tested for its ability to bind to aggrecan via ELISA (data shown in **Figure 3.12**.) Results shown in **Figure 3.12a** confirmed that IgG2a concentrated from 24.14 and 53.28 supernatant were detectable on the ELISA plates at higher levels than the control (Ig isolated from the anti–aggrecan secreting F1.11 hybridoma clone C10.3). However, 24.14 also expresses surface IgM and this was not detected in the concentrated supernatant (**Figure 3.12 b**). Data in **Figure 3.12c** shows the Ig collected from fusion clones did not bind to aggrecan using ELISA (despite their concentration in the supernatant being higher than that of the aggrecan-specific hybrid C10.3 (**Figure 3.12a**). Thus, this data demonstrates that the Ig secreted by both fusion clones 24.14 and 53.28 is not aggrecan-specific.

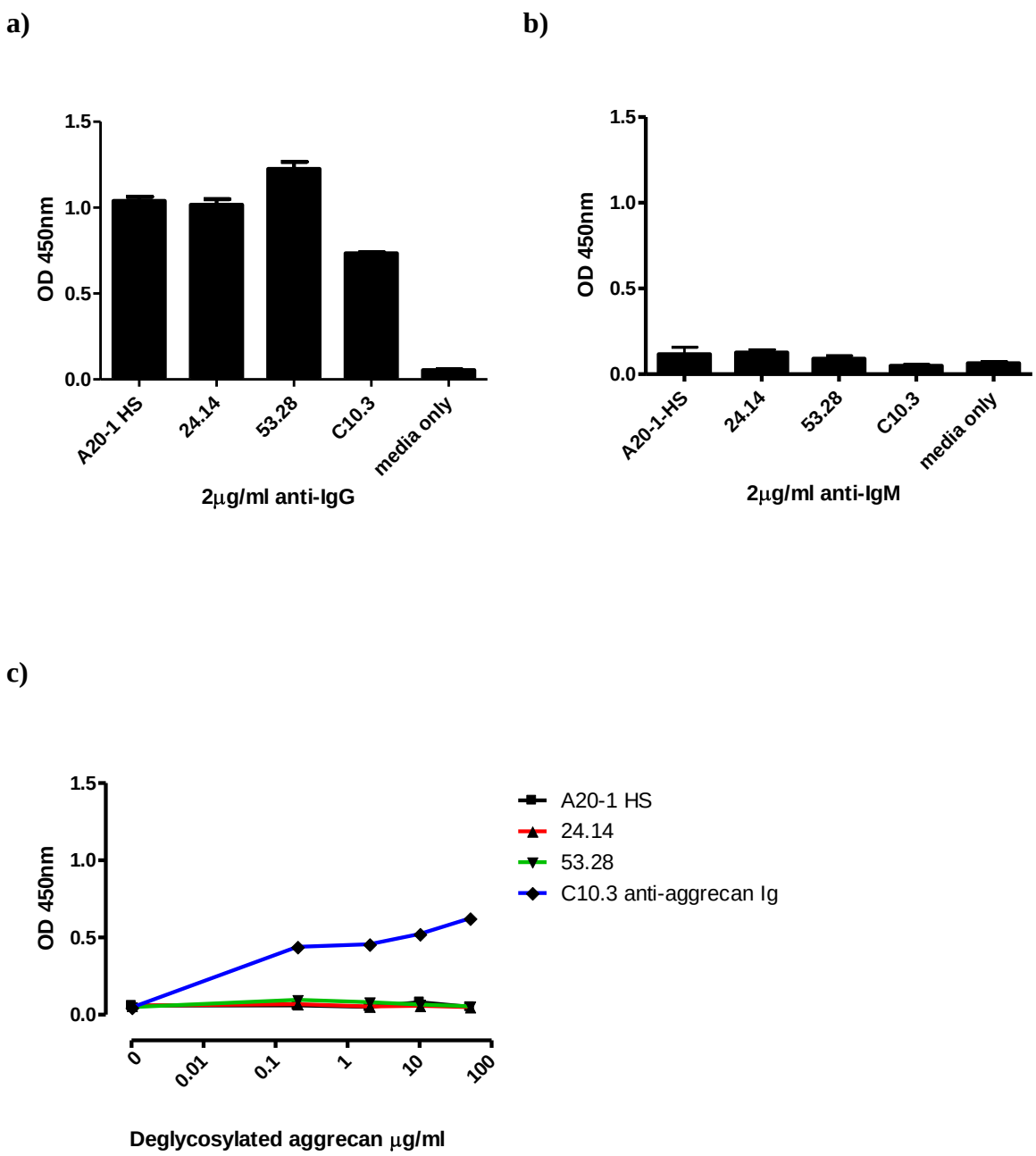


Figure 3.12 ELISA to determine the antigen-specificity of the 53.28 and 24.14 secreted Ig. *Fusions 24.14 and 53.28 were grown for several days in culture until the cells were above optimal density. A20-1 HS supernatant was used as a negative control that does not secrete aggrecan-specific Ig. C10.3 anti-aggrecan Ig was used as a positive control. Supernatant was then concentrated by vivaspin sample concentrators and used un-diluted on ELISA plates coated with either a) 2 μg/ml anti-IgG2a, b) 2 μg/ml anti-IgM or c) graded doses of aggrecan. Data is representative of triplicate wells and 2 independent experiments.*

3.2.2.6. Is aggrecan presentation improved due to the extracellular proteinases?

Do the fusions secrete proteinases that aid the presentation of aggrecan?

I wanted to investigate whether the observed improvement in aggrecan presentation by the fusions was due to the production and secretion of proteinases such as MMPs or ADAMTs demonstrated to aid extracellular processing of aggrecan (Cawston and Young 2010) (**Figure 3.13**). To examine this, the fusions were incubated for 24 h with a dose of aggrecan (12.5 nM) that is only presented by fusions and not by the A20-1 HS cells; supernatant was removed and transferred to wells containing either live or paraformaldehyde fixed A20-1 HS cells and T cell hybrids 192. If the fusions were processing aggrecan extracellularly then one would expect that after 24 h they would have processed the aggrecan sufficiently, so that it could now be presented by the A20-1 HS cells without the requirement for further processing. However, to ensure that sufficient aggrecan was available for presentation by the A20-1 HS cells, following any extracellular proteolysis and uptake by the fusion cells, an additional 12.5 nM aggrecan was added to wells along with the transferred supernatant. Controls included live and fixed A20-1 HS cells incubated with graded doses of aggrecan or p84-103 and live A20-1 HS cells incubated with graded doses of supernatant and 25 nM aggrecan. Data in **Figure 3.14** shows that there was nothing in the supernatant that could pre-process aggrecan sufficiently to allow presentation by fixed cells. Also, there was nothing present in the supernatant such as proteinases or cytokines that were able to improve the presentation of a low dose of aggrecan (12.5 nM) by live A20-1 HS cells. Positive controls A20-1 HS, live + p84-103, live + aggrecan and fixed + aggrecan showed normal dose responses similar to those observed in previous experiments.

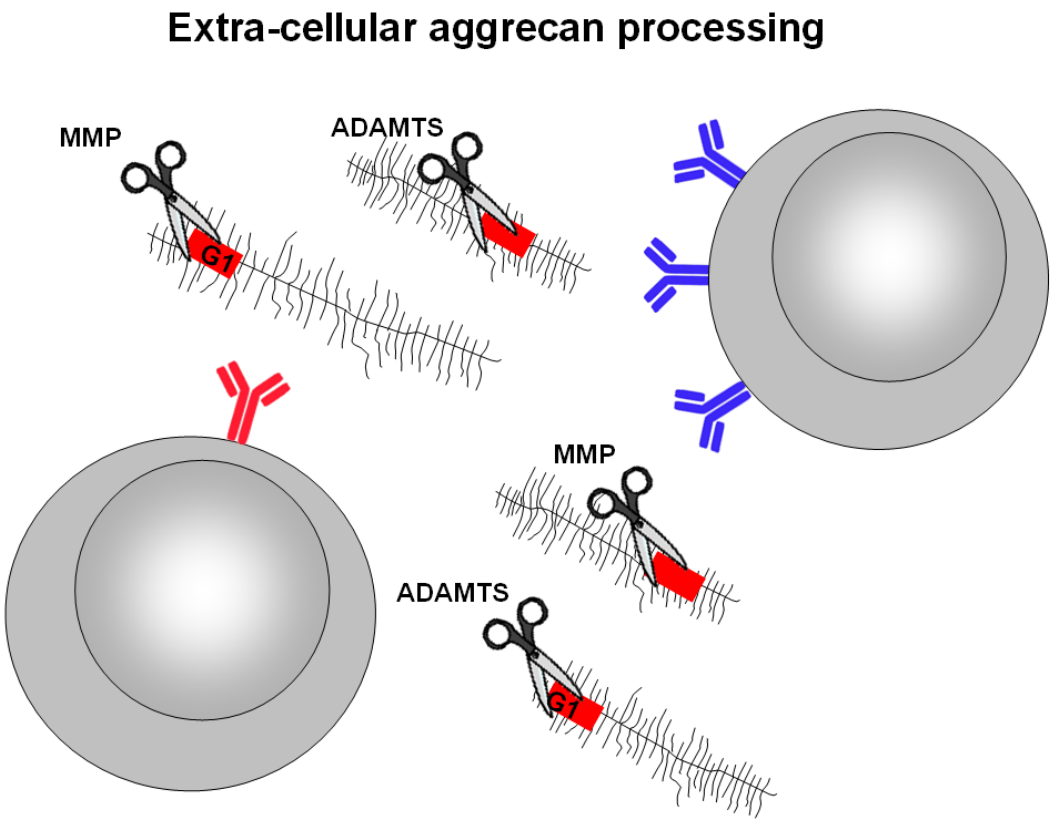


Figure 3.13 Illustration depicting the extracellular processing of aggrecan. *Aggrecan is processed in vivo by various proteinases such as matrix metalloproteinases (MMP) and A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) (Cawston and Young 2010). The cells in grey represent B cells that may secrete proteinases capable of the extracellular processing of aggrecan.*

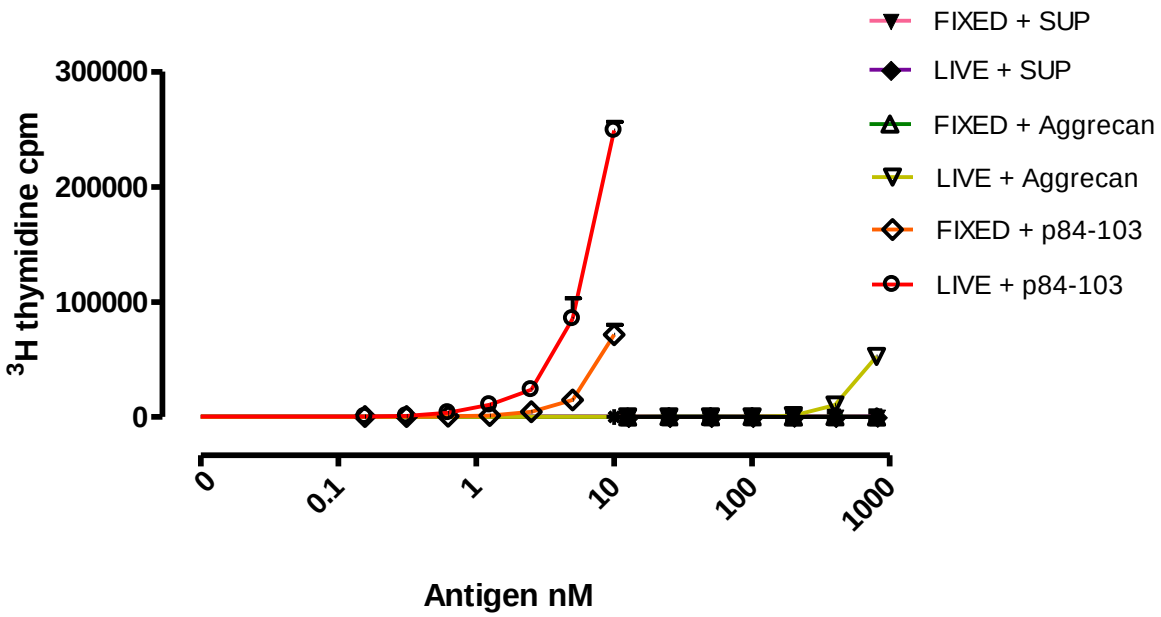


Figure 3.14 The improvement in presentation by the fusion cells is not due to the extracellular processing of aggrecan. *Fusion cells 53.28 were incubated with a low dose of aggrecan (12.5 nM) for 24 h. 12.5 nM of aggrecan is efficiently presented by the fusion cells but not A20-1 HS. Following the 24 h incubation period fusion cells were pelleted and the supernatant was removed. Fixed and live A20-1HS cells were incubated with either fusion supernatant (SUP) and an additional 12.5 nM aggrecan, graded doses of aggrecan or graded doses of peptide p84-103 for 24 h. Cells were killed by freezing at -80^{o}C and supernatant was removed for bioassay with CTLL-2 cells. Tritiated thymidine was added for 18 h and T cell responses were determined and thymidine cpm displayed as ^{3}H +/- SEM. Data is representative of triplicate wells and a single experiment.*

3.2.2.7 Aggrecan presentation to T cell hybridomas specific for epitopes within the G3 domain.

Do the fusions present an alternative aggrecan epitope more efficiently?

All of the antigen presentation assays thus far were carried out using a previously generated (John Robinson) T cell hybridoma 192 that recognises the immunodominant T cell epitope 84-103 from the G1 domain of aggrecan. Previous studies have demonstrated that BCR mediated uptake can either improve, or impede, the presentation of certain T cell epitopes by masking the epitope and preventing either its degradation or generation by endosomal proteinases (Simitsek, Campbell et al. 1995). I hypothesised that if the BCR of the recently generated B cell fusions were also specific for the immunodominant G1 domain, this may impede optimal presentation to the 192 T cells due to steric hindrance by the BCR illustrated in **Figure 3.15.**

Therefore, I aimed to generate aggrecan-specific T cell hybridomas that recognised epitopes derived from a region distinct from G1. BALB/c mice were immunized with aggrecan and Titremax in the footpad; 10 days later popliteal lymph node cells were isolated and re-stimulated in culture with the synthetic peptides spanning either amino acids 2373-2387 or 2363-2378 (previously shown to induce T cell responses in mice (Buzas, Vegvari et al. 2005). These cells were fused with BW5147 TCR $^{\alpha/\beta-}$ lymphoma cells and HAT resistant T cell fusions were tested for their ability to recognize both aggrecan and the two G3 peptides. 35 positive HAT resistant fusions were incubated with APC (J774 macrophages or 53.28/24.14 fusion cells) and graded doses of either aggrecan, G3 peptides or p84-103 and T cell activation was measured by IL-2 production (not shown). Results showed that when mice were immunized with aggrecan all of the resultant T cell population were specific for the dominant peptide 84-103 epitope from the G1 region, despite *in vitro* stimulation with G3 peptides p2373-2387 or p2363-2378.

Alternately, I took another approach in an attempt to maximize the chances of generating T cell hybridomas that recognize epitopes from the G3 region. Mice were immunized with the G3 peptides p2373-2387 or p2363-2378 and then re-stimulated *in vitro* with the corresponding peptide. Using this method I generated T cell

hybridomas D9$^{2373-2387}$ and D11$^{2363-2378}$ that respond to both of these epitopes (data shown in **Figure 3.16a.)** However, these G3 specific T cell hybridomas do not recognize whole aggrecan (**Figure 3.16b**), implying that either the G3 epitopes are cryptic and are not generated by the APC, or that the T cells will only respond when the aggrecan is given in its pre-processed form as peptide. Indeed, previous studies have shown that a subset of T cells known as type B T cells will only recognise antigen in a peptide form that is subsequently loaded onto MHC class II molecules in a different conformation to that of antigens processed within the endosomal compartments of the APC (Pu, Lovitch et al. 2004).

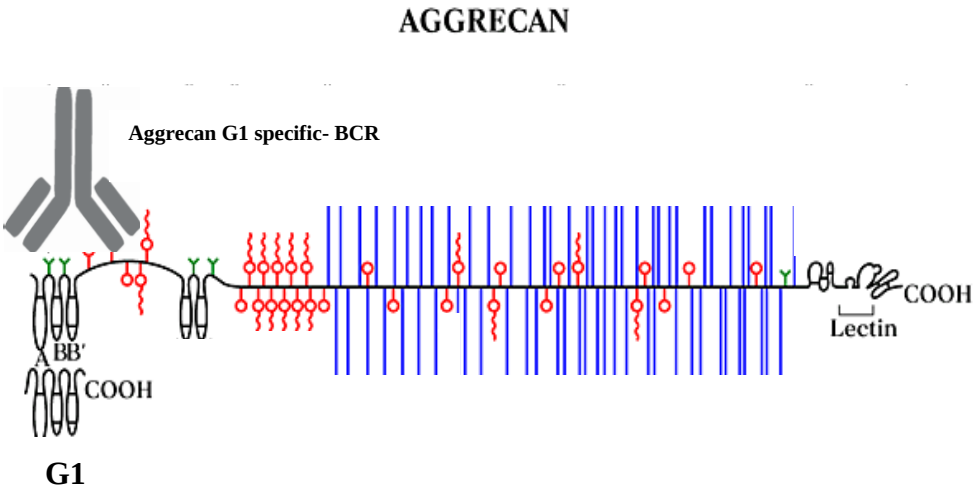


Figure 3.15 Aggrecan structure masked by an aggrecan G1 specific BCR. *Aggrecan G1 specific BCR represented in grey, bound to the G1 domain of aggrecan may impede processing and presentation of G1 derived peptides by physically preventing the access of endosomal proteinases to the G1 domain figure modified from (Hardingham, Fosang et al. 1994).*

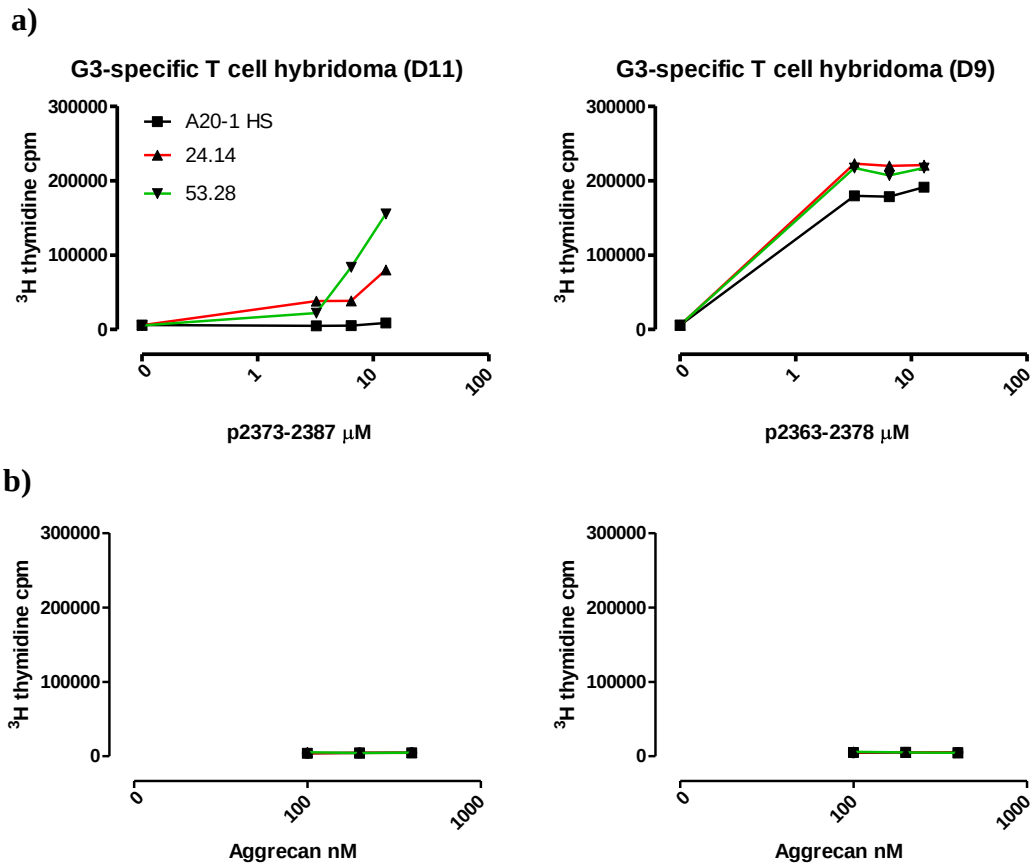


Figure 3.16 G3 specific T cell hybridomas fail to recognize APC processed aggrecan. *A20-1 HS, or the fusion cells 24.14 and 53.28 ($5x10^4$) were cultured with aggrecan$_{2373-2387}$ (D11) or $_{2363-2378}$ (D9) specific T cell hybridomas ($5x10^4$) in the presence of graded doses of* ***a)*** *peptides p2373-2387 or p2363-2378 or* ***(b)*** *aggrecan for 24 h. Cells were killed by freezing at -80^{o}C and supernatant was removed for bioassay with CTLL-2 cells. Tritiated thymidine was added for 18 h and T cell responses were determined and displayed as ^3H thymidine cpm +/- SEM. Data are representative of three experiments.*

3.2.3 Alternate immunisation and fusion protocols.

3.2.3.1 CD40L transfectants to improve B cell isolation prior to fusion.

Human CD40L- activated B cells can be successfully expanded for long periods of time *in vitro* (Schultze, Michalak et al. 1997; Coughlin, Vance et al. 2004). In addition, it was documented in 2008 that *ex vivo* murine antigen-specific B cells could be generated by hyper-immunising mice with NP-BSA then expanded *in vitro* by co-culture with CD40L transfectants and recombinant IL-4 (rIL-4) in the presence of cyclosporine A (CsA) to eliminate the T cell population (Ahmadi, Flies et al. 2008).

I attempted to repeat this finding in order to expand and improve the viability of B cells from aggrecan-immunized mice prior to fusion. To do this murine CD40L transfected L cells were grown in monolayers and cultured with splenocytes in the presence of rIL4 and CsA. Cells were harvested at various timepoints, tested for viability using trypan blue exclusion and the percentage of $B220^{+}$ B cells was determined by flow cytometry. Controls for this experiment included splenocytes co-cultured with CD40L expressing monolayers only, monolayers with IL-4 only and monolayers with CsA only. In addition, the same conditions were repeated using untransfected L cells (data is shown in **Figure 3.17**). Results from two independent experiments revealed an initial increase in the % of B cells after 4 days when co-cultured with CD40L monolayers, CsA and IL-4 (**Figure 3.17a**). However, following this period the B cell population began to steadily decline and after 12 days in culture the isolated B cell population had completely lost viability. B cells co-cultured with L cells only, either in the presence or absence of CsA and IL-4 began to steadily decline after day 1 in culture (**Figure 3.17b**). Observations from the trypan blue exclusion confirmed that the entire isolated splenocyte population became decreasingly viable in the days following isolation in line with the decreasing B cell population.

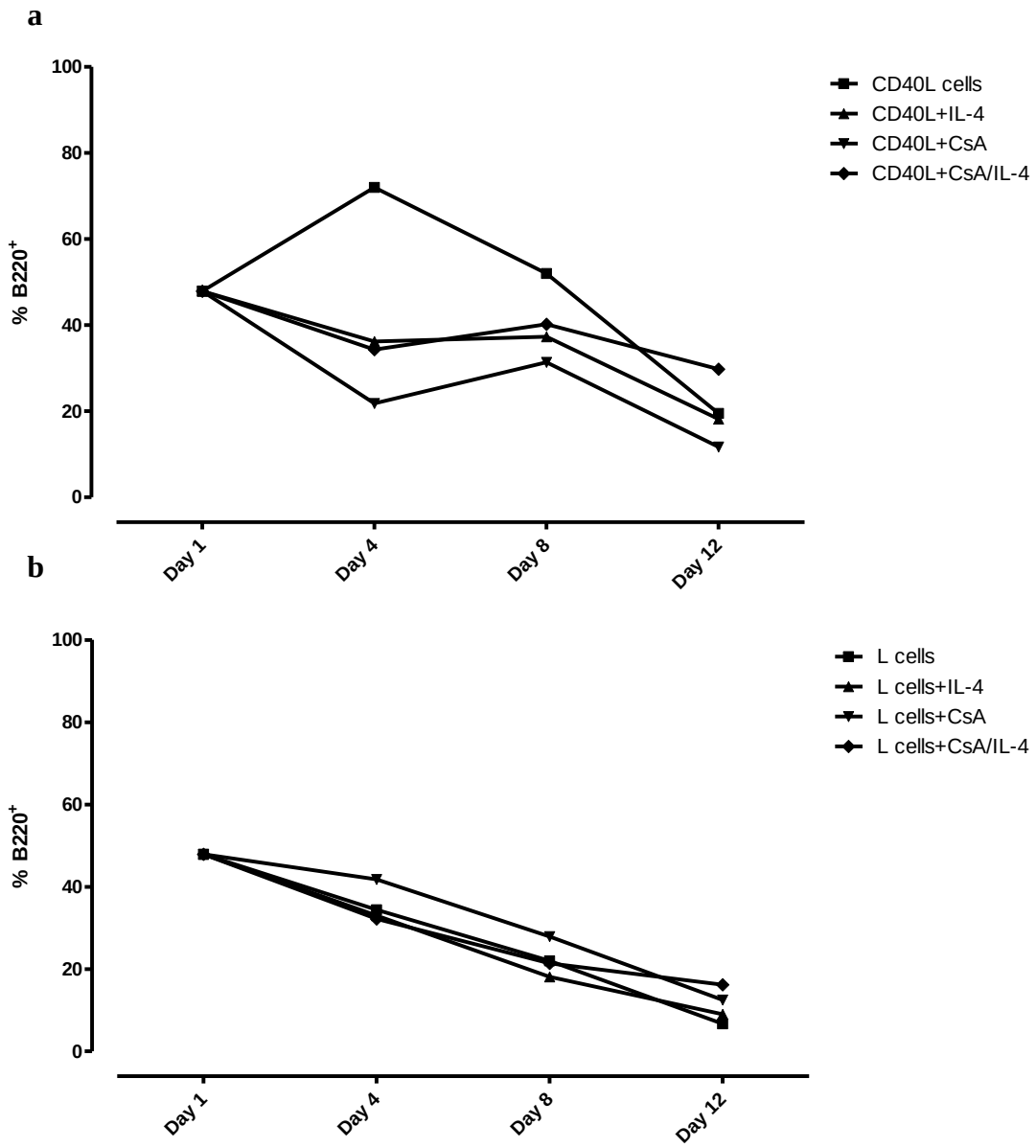


Figure 3.17 Splenic B cells could not be expanded *in vitro* by activation with CD40L and rIL-4. *Splenocytes were co-cultured in duplicate wells for 12 days with either irradiated a) CD40L-transfected L cells or b) untransfected L cells as a negative control. Cyclosporine A was added to eliminate the T cell population. Duplicate wells were harvested at days 4, 8 and 12 and percentage of B220$^+$ cells was determined by flow cytometry. Data represents the total percentage of B220$^+$ B cells in culture at various timepoints. Data is representative of 2 independent experiments.*

3.2.3.2 An alternate set of parameters for B cell fusion were carried out with slight adjustments made to the immunisation protocol

In a separate attempt to improve the aggrecan-specific population of B cells prior to fusion, three changes were made to the immunisation protocol.

1. An alternative immunogenic adjuvant was used. In the previous immunisation protocol the adjuvant used was alum (aluminium hydroxide). Alum is used to deposit the antigen when injected and prevent its rapid catabolism and is a safe choice for the intraperitoneal injections required for the final antigen boost to localise the reaction to the spleen. However, it may not be sufficiently immunogenic for this antigen. Therefore, for this protocol the adjuvant used for initial immunisations into the flank was Titermax which is a water-in-oil adjuvant that consists of squalene, an emulsifier, a patented block copolymer and microparticulate silica. Titermax is reported to be less toxic than the immunogenic adjuvant freunds complete adjuvant and yet stimulates an equal or greater antibody response (Stills 2005). As adjuvants can control Ig isotype and efficiency of antigen-specific response (Stills 2005), I thought it important to adapt the immunisation protocol and test a different routinely used immunogenic adjuvant. However, the final intra-peritoneal immunisation had to be given in alum as the mice became ill after intra-peritoneal immunisations with Titremax.
2. I hypothesised that following an initial full dose immunisation of aggrecan in Titremax; subsequent immunisations with a 10-fold reduced dose of aggrecan may boost a higher affinity population of B memory cells. Reducing the amount of aggrecan with each boost may selectively expand B cells expressing a high affinity BCR that is able to recognise small doses of immunising aggrecan. To test this theory, mice were immunized initially into the flank with a full dose of aggrecan (166 µg) in Titremax followed by two subsequent immunizations at 16.6 µg and 1.66 µg respectively. The mice were given a fourth final intra-peritoneal boost of aggrecan (166 µg) in alum and splenocytes were isolated after 5 days.
3. In order to be efficient at presenting antigen B cells must take up antigen via its antigen-specific BCR and receive co-stimulatory signals from a cognate T cell. In addition to this it has been shown by several groups that B cells must

receive a third stimulatory signal or 'danger signal' via pattern recognition receptors such as LPS via TLR4. This ensures that the B cells isolated from the aggrecan-immunized mice were fully activated and primed to present aggrecan to T cells prior to fusion. In an attempt to stimulate the aggrecan-specific B cells prior to fusion, cells were cultured with 50 µg/ml deglycosylated aggrecan and 50 µg/ml LPS for 48 h before fusing to A20-1HS cells.

46 resultant colonies were screened for their ability to present both aggrecan and p84-103 to the T cell hybridoma 192 using the antigen presentation assays described in section 2.12.2 and by flow cytometry for cell surface MHC class II expression. Results from representative fusions CW31, CW1, and CW36 are shown in **Figure 3.18**. Results revealed that fusions which presented aggrecan more efficiently than the A20-1HS fusion partner, also presented peptide more efficiently and showed increased MHC class II cell surface expression. These data indicate that although several of the fusions generated using this protocol were more efficient at presenting aggrecan to the T cell hybridoma 192 this improvement was probably due to increased availability of peptide/MHC class II complexes and not due to aggrecan-specific BCR expression.

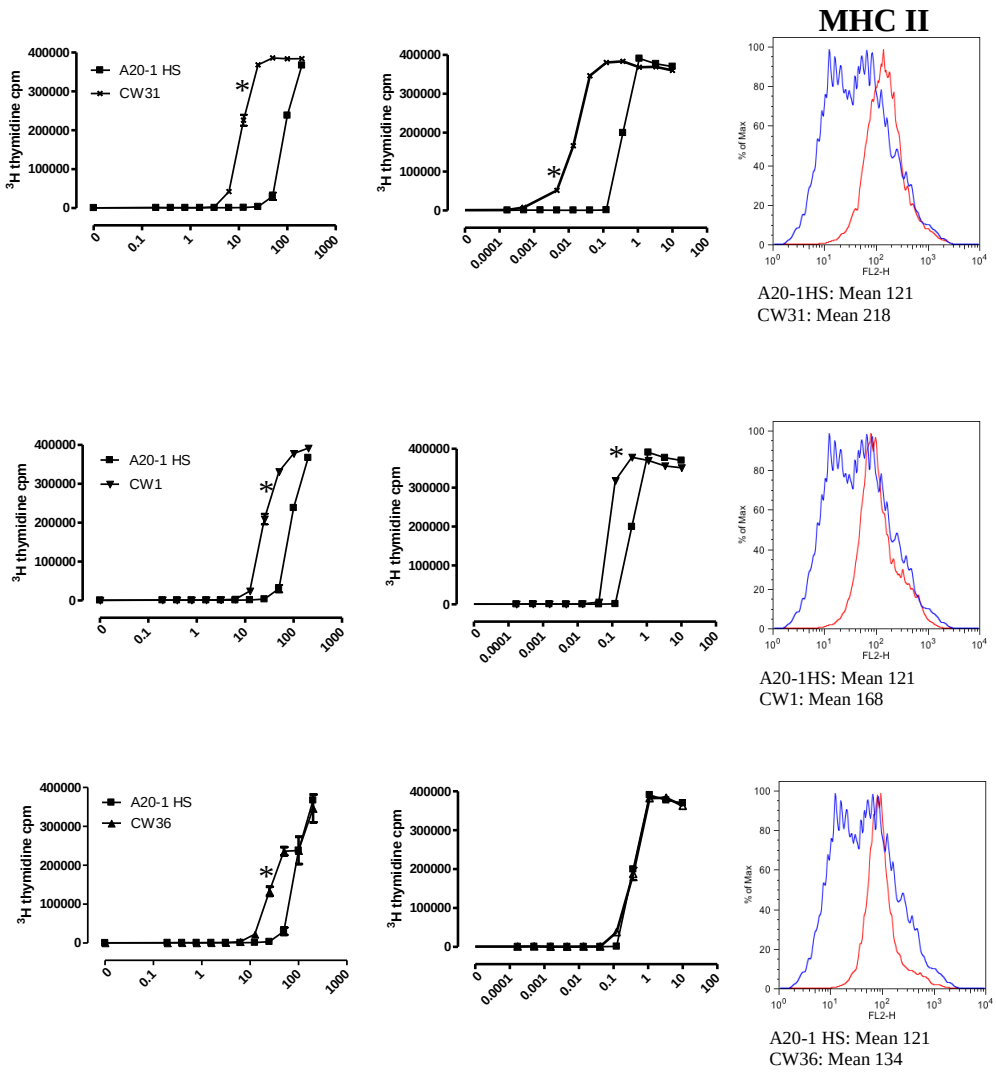


Figure 3.18 Antigen presentation by fusions generated from immunization protocol 2. *A20-1 HS or the fusion cells CW1, CW31 and CW36 were cultured with aggrecan$_{84\text{-}103}$-specific T cell hybridomas 192 in the presence of graded doses of deglycosylated aggrecan or p84-103 for 24 h. Cells were killed by freezing at -80^{o}C and supernatant was removed for bioassay with CTLL-2 cells. Tritiated thymidine was added for 18 h and T cell responses were determined and displayed as ^{3}H thymidine cpm +/- SEM. The histograms represent cells stained with anti-MHCII antibodies conjugated with PE. The blue histogram represents the gated A20-1 HS cell population and the red histogram represents different fusion populations. All experiments were carried out at 4^{o}C in the presence of Fc block. Mean fluorescence intensity values are shown below each histogram. Data are representative of three experiments. The asterix represents statistical significance determined by students T test where $p<0.05$.*

3.3 Discussion

The aims of this chapter were to generate aggrecan-specific B cells and to assess their potential as antigen presenting cells. The generation of aggrecan-specific B cells would enable me to assess their role in presenting the candidate rheumatoid arthritis autoantigen aggrecan to T cells. Detailed molecular studies could then be performed such as how aggrecan is processed by the B cells and how efficiently it is presented in comparison to presentation by dendritic cells or macrophages. In this chapter I have generated several B cell fusions from aggrecan-immunized mice. These B cell fusions have been characterised by flow cytometry to phenotype the BCR that were expressed, to assess levels of cell surface MHC class II expression and levels of the co-stimulatory molecules B7.1 and B7.2 (Pentcheva-Hoang, Egen et al. 2004). Subsequently, the fusion cells were also screened using antigen presentation assays to measure their efficiency of aggrecan presentation to several aggrecan-specific T cell hybridomas.

Representative fusions 24 and 53 were selected as potential aggrecan-specific B cells and the results obtained using these are discussed below.

3.3.1 Fusion 24

Flow cytometry analysis showed the expression of a new BCR H chain isotype on 7 of the fusion cells. All 7 fusions expressed a BCR of the IgM phenotype in addition to the IgG2a isotype BCR inherited from the A20-1 HS fusion partner. This data suggests that the new IgM BCR inherited from the B cell isolated from the aggrecan-immunised mice may confer aggrecan-specificity. However, only fusion 24 presented aggrecan more efficiently when compared to aggrecan presentation seen by the fusion partner A20-1HS. In the early stages of B cell differentiation class switching of the H chain constant region occurs by DNA recombination where a functional VDJ unit is switched from 1 constant region to another (Harriman, Volk et al. 1993). The predominant class of the early response is IgM, hence IgM antibodies / IgM+ BCR are generally not as high affinity as the subsequently predominant isotype IgG (Berek and Milstein 1988). This may provide an explanation as to why there was only a 10-fold increase in the efficiency of deglycosylated aggrecan presentation by fusion 24. Several groups have previously shown B cell antigen presentation to be improved by 10,000-fold when the B cell expresses an antigen-specific BCR (Lanzavecchia 1985; Knight, Lucocq et al. 1997; Batista and Neuberger 1998). However, these previous

studies involved antigen presentation experiments using the small model antigens TTCF (50 kDa) and HEL (14 kDa). In this study I used the candidate autoantigen aggrecan (250 kDa); this antigen is much larger, is highly glycosylated and is able to form large aggregates in the presence of small amounts of hyaluronan (Dudhia 2005). Therefore, processing and presentation of this complex molecule may never be as efficient as that seen with HEL or tetanus toxin. In addition, my findings concur with Leroux *et al* and Glant *et al* that show the multiple glycosaminoglycans hinder presentation of the 84-103 epitope and efficiency of aggrecan presentation by fusions was much improved following deglycosylation (Leroux, Guerassimov et al. 1996; Glant, Buzas et al. 1998).

It is possible, that due to the complex nature of this antigen, fusion 24 may well be aggrecan-specific but its low affinity IgM BCR is not sufficient for optimal uptake and presentation of aggrecan. An alternate explanation for the observed improvement in presentation would be that fusion 24 expresses increased levels of MHC class II. Therefore, these cells are equipped to present more aggrecan peptide/ MHC class II complexes to the T cell hybridomas improving the efficiency of presentation. Although, the levels of B7.1 and B7.2 were found to be higher on fusion 24 than the A20-1 HS cells, this may not be a contributing factor to the observed improvement in presentation as T cell hybridomas 192 are already primed and should not require further stimuli via CD28. Further attempts to verify the specificity of the BCR expressed by fusion 24 included ELISA to detect the Ig secreted by the fusions and to test its ability to bind to aggrecan. In addition, flow cytometry was used to determine if biotinylated aggrecan bound to the surface of these cells. ELISA data revealed that IgM is not secreted by fusion 24 so I could not verify whether this was aggrecan-specific or not. Data from the flow cytometry experiment revealed that aggrecan could not be detected binding to the surface of fusion 24. However, there is no positive control for this experiment and if the BCR expressed by fusion 24 is aggrecan-specific but of low affinity, then this method may not be sensitive enough to determine the specificity of the BCR. Therefore, experiments to verify that fusion 24 is an aggrecan-specific B cells were inconclusive.

3.3.2 Fusion 53

Findings from flow cytometry experiments revealed that fusion 53 expresses a BCR of the IgG2a isotype only. As IgG2a is also expressed by the A20-1 HS fusion partner, it is not possible to tell from this data whether fusion 53 expresses a new aggrecan-specific IgG2a BCR or whether this BCR is simply inherited from the A20-1 HS cell. IgG2a isotype BCR are generally of a higher affinity than IgM and represent events later in the immune response (Berek and Milstein 1988). Therefore, I would expect an aggrecan-specific IgG2a BCR to have a higher affinity for aggrecan than an IgM BCR and if fusion 53 is aggrecan-specific then it should present aggrecan more efficiently than fusion 24. However, this was not the case and fusion 53 presents both deglycosylated and glycosylated forms of aggrecan at comparable doses to that of fusion 24. An alternate interpretation of the data is that fusion 53 can present aggrecan as efficiently as fusion 24 despite expressing much lower levels of MHC class II. Therefore, uptake and processing may be more efficient when aggrecan is taken up via an IgG2a isotype BCR but presentation to T cells is less efficient due to a lower number of peptide/MHC class II complexes expressed on the cell surface of fusion 53. Attempts to isolate secreted Ig from fusion 53 and verify its antigen-specificity by ELISA were unsuccessful. Although levels of IgG2a isotype Ig were detected in the supernatant from these cells, I could not detect aggrecan-specificity using ELISA. However, this data is still inconclusive as it was not possible to determine whether the secreted Ig was inherited from the A20-1 HS cells or inherited from the B cell isolated from the aggrecan immunized mice. I have confirmed that cultured A20-1 HS cells do secrete their IgG2a Ig into the supernatant and that this is detectable by ELISA. Therefore, it is possible that only the A20-1 HS Ig and not fusion 53 Ig can be phenotyped by ELISA. Despite the possibility that fusion 53 may express a BCR of a higher affinity than fusion 24, I also failed to detect biotinylated aggrecan binding to the surface of the fusion 53 cells using flow cytometry. Therefore, attempts to verify that fusion 53 is an aggrecan-specific B cell were again inconclusive.

3.3.3 Problems with verifying the specificity of the fusion BCR.

One of the weaknesses of this system is the lack of T cell hybridomas recognising peptides derived from different regions of aggrecan. As I have described earlier aggrecan is a large complex molecule and yet the immune response is almost entirely focussed around the G1 domain (Karopoulos, Rowley et al. 1996; Leroux, Guerassimov et al. 1996; Zhang, Guerassimov et al. 1998; Li, Zhang et al. 2000). Previous studies have shown that when a BCR binds its antigen it can mask this region and may directly negatively or positively influence proteolysis once taken up inside the B cell. Therefore, if the BCR expressed by the fusions recognises an epitope in the G1 domain then it is possible that the region encompassing amino acids 84-103 is masked, thus preventing the generation of p84-103 for optimal T cell hybridoma 192 recognition. To overcome this possibility I attempted to generate T cell hybridomas that recognise peptides from an area distinct from G1 (G3). Attempts to generate T cell hybridomas that recognised epitopes within the G3 domain resulted in the generation of hybridomas that recognise G3 epitopes only when administered in their peptide form but not when derived from whole aggrecan. In addition, the majority of hybridomas generated from aggrecan-immunized mice responded to the peptide p84-103. Therefore, in accordance with previous studies, T cell responses were very much focussed around the G1 domain and the generation of alternative T cell hybridomas was unsuccessful at establishing the specificity of the B cell fusions.

Another attempt to verify that the fusions express an aggrecan-specific BCR was to block aggrecan binding to the BCR using polyclonal anti-IgG2a or anti-IgM antibodies. Fusion cells or A20-1 HS were incubated with the antibodies prior to incubation with aggrecan or p84-103. If the fusions express an aggrecan-specific BCR then prior incubation with anti-Ig antibodies would be expected to inhibit BCR-improved uptake, processing and presentation of aggrecan (Casten, Lakey et al. 1985). However, the antibodies should not have any effect on peptide presentation, as p84-103 is pre-processed and does not require uptake via the BCR. As expected, when anti-MHCII antibodies were added to the antigen presentation assays, presentation of both aggrecan and p84-103 was inhibited. However, the inclusion of either anti-IgG2a or anti-IgM antibodies did not have any effect on aggrecan presentation by the fusions. One possible explanation for this is that these isotype-specific antibodies are binding to the extracellular constant domain of the BCR and

not to the Fab, antigen binding domain and hence do not interfere with aggrecan binding or uptake. In conclusion, in the absence of a positive control to show that the antibodies are capable of blocking BCR mediated antigen uptake it was not possible to draw a positive conclusion from this experiment.

3.3.4 Subsequent fusions.

Several attempts were made to generate fusion cells following various modifications to the immunisation protocol. These included the use of a different adjuvant to improve the immune response to aggrecan, immunisation with different aggrecan doses to improve the affinity of the B cells prior to isolation and fusion, isolating cells at different timepoints after the final aggrecan boost and incubating the *ex vivo* cells with aggrecan and LPS prior to fusion. In addition, Ahmadi *et al* reported that a .population of antigen-specific B cells could be boosted *in vitro* by culturing the cells with CD40L expressing cells in the presence of CsA and IL-4 (Ahmadi, Flies et al. 2008). I attempted to repeat this result but found that the *ex vivo* B cells began to lose viability after a few days in culture. I also, tried to modify the protocol slightly by altering the number of CD40L cells used to produce the co-culture monolayer. However, I concluded from these experiments that the isolated B cells were in optimal condition when freshly isolated. The fusion with A20-1 HS cells produced considerable cell death and isolated cells must be in optimal condition for successful hybridoma generation. Therefore, isolated B cells cultured in this manner were not used for fusion or antigen presentation experiments.

3.3.5 B cells isolated from aggrecan-immunized mice used directly in antigen presentation experiments.

The generation of aggrecan-specific B cells was fundamental to this study and I was unable to study the molecular mechanisms of aggrecan processing presentation by B cells in comparison to other APC. The generation of aggrecan-specific fusions was hampered by various technical difficulties and time restraints limited screening of multiple batches of fusions. Therefore, this approach for the generation of aggrecan-specific B cells was not pursued any further.

In a separate attempt to generate aggrecan-specific B cells from mice hyper-immunized with aggrecan, splenic B cells were isolated using magnetic Microbeads

attached to anti-CD43 antibodies. This anti-CD43 isolation is a negative selection technique designed to isolate splenic B cells from a population of CD43+ cells (CD43 is expressed on all leukocytes with the exception of mature B cells) (Miltenyi, Muller et al. 1990). The isolated CD43- cells were verified as B220+ cells by flow cytometry and used directly in an antigen presentation assays along with LPS to activate the B cells, aggrecan or p84-103 and T cell hybridomas 192. A20-1 HS cells or B cells isolated from mice immunized with TTCF were used as comparison APC to B cells from aggrecan immunized mice. Although, an initial experiment demonstrated that B cells isolated from aggrecan–immunized mice showed a 10-fold improvement in aggrecan presentation when compared to the other APC, subsequent results were variable. Initial work to generate an aggrecan-specific BCR *in vitro* and transfect it into A20-1HS cells to generate stable transfectants looked promising. Therefore, focus was moved to Chapter 4 generation of aggrecan-specific B cells by transfection of a B cell line with constructed plasmids encoding an aggrecan-specific BCR.

3.4 Summary of chapter 3.

- Several cell lines and clones were generated by fusing B cells from aggrecan-immunized mice with the HAT sensitive myeloma cell line A20-1 HS in an attempt to generate immortalized aggrecan-specific B cells.

- All cell lines were analysed by flow cytometry and tested for their ability to present aggrecan to the aggrecan-specific T cell hybridomas 192 in comparison to the B lymphoma fusion partner. Several of the fusions tested, presented aggrecan (both the glycosylated and the de-glycosylated forms) at least 10-fold better than the fusion partner alone. I have established that the newly generated cells consistently present the different forms of aggrecan more efficiently than the fusion partner. Fusions 24.14 (IgM) and fusion 53.28 (IgG2a) are amongst those fusions that consistently present more efficiently than the A20-1 HS cells and were chosen for further studies.

- I have carried out an extensive series of experiments to determine if the observed improvement in aggrecan presentation was due to the expression of an aggrecan-specific BCR. Unfortunately, none of these data have confirmed that the newly generated fusions express an aggrecan-specific BCR.

4. Generation of aggrecan-specific B cells by stable transfection of a B cell line with constructed plasmids encoding an aggrecan-specific BCR.

4.1 Introduction

Previous studies have manipulated immunoglobulin (Ig) structure to examine the effects on antigen processing and presentation (Knight, Lucocq et al. 1997; O'Neill, Shlomchik et al. 2005) and to generate chimeric and/or humanized Ig for use as therapeutics (Senolt, Vencovsky et al. 2009; Beck, Wurch et al. 2010).

Therefore, as part of a complimentary approach for the generation of aggrecan-specific B cells, I wanted to generate an aggrecan-specific BCR by replacing the variable region of the heavy chain (VH) and variable region of the light chain (VL) from an existing membrane-bound tetanus toxin c fragment (TTCF)-specific BCR (Knight, Lucocq et al. 1997) with VH and VL from a newly generated mouse anti-bovine aggrecan mAb. This would result in an aggrecan-specific BCR that could be transfected into the A20-1 HS cell line to generate an aggrecan-specific stable B cell line. As the existing TTCF BCR constructs contained the entire Ig sequence isolated from an Epstein Barr Virus (EBV) clone 11.3 (Lanzavecchia 1985), this would create a chimeric BCR comprising mouse V region/human C region sequences; an approach that has previously been reported (Sahagan 1990; Fan and Leong 2007; Beck, Wurch et al. 2010).

In order to generate the aggrecan-specific BCR constructs, I had to first isolate cDNAs encoding the VH and VL regions from anti-aggrecan monoclonal antibodies for which the sequences are not known. Doenecke *et al* successfully amplified VH and VL sequences from the 5' end of cDNA using rapid amplification of cDNA ends (RACE) illustrated in **Figure 2.1**. To verify that this procedure was reproducible, I repeated their experiment to isolate the VH cDNA from A20-1 B lymphoma cells and confirmed that the isolated sequence was identical to their published data (Doenecke, Winnacker et al. 1997).

Subsequently, using the RACE protocol, I amplified the cDNA encoding the V regions of the H and L chains from two independent anti-aggrecan monoclonal antibodies. The first monoclonal antibody C71 originated from a heterogeneous

population of aggrecan-specific B cell hybridoma (F1.11), generated by A Knight and John Robinson prior to the start of this project. The second monoclonal antibody was derived from a B cell hybridoma 1-C-6 that recognizes epitopes in the G1 and G2 domains of reduced and alkylated aggrecan, kindly donated by Dr Bruce Caterson (Stevens, Oike et al. 1984; Fosang and Hardingham 1991). In order to generate an aggrecan-specific BCR, I removed the V regions from the plasmids pMCFR TTCF-H NI encoding the membrane-anchored TTCF-specific H chain and pMCFR TTCF-L hyg encoding the TTCF-specific L chain and replaced them with the C71 and 1-C-6 aggrecan-specific VH and VL regions respectively.

The plasmids encoding the aggrecan-specific BCR were then transfected into A20-1 HS and their expression verified by flow cytometry. Subsequently, these aggrecan-specific B cells could be used as a model to compare the mechanisms of aggrecan-processing and presentation by B cells to that of dendritic cells and macrophages.

4.1.2 The aims of this chapter:

- To generate chimeric plasmids encoding aggrecan-specific Ig H and L chains.
- To transfect the constructs into the A20-1 HS B cell line.
- To verify that the new aggrecan-specific BCR were expressed on the cell surface of the A20-1 HS transfectants.
- To examine whether the expression of aggrecan-specific BCR improves the presentation of aggrecan to the aggrecan-specific T cell hybridomas in comparison to untransfected A20-1 HS cells.
- To generate aggrecan-specific stable B cell lines that could be utilized for further mechanistic studies of aggrecan-processing and presentation by B cells.

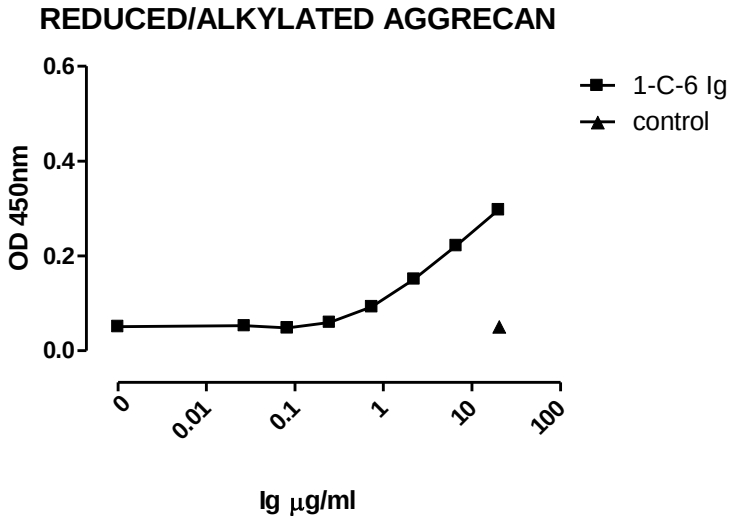
4.2 Results

4.2.1 Characterization of the monoclonal antibodies C71 and I-C-6.

4.2.1.1 1-C-6

I-C-6 Ig (IgG1) (kindly donated by Dr B Caterson) that recognises epitopes within the aggrecan G1 and G2 domains and requires antigen reduction and alkylation for the best exposure of the amino acid sequence QAAY (Fosang and Hardingham 1991). To verify the specificity of the 1-C-6 Ig, 1-C-6 cells were grown to confluence and the cell supernatant was removed and screened by ELISA for binding to both reduced/alkylated, and deglycosylated forms of aggrecan (data shown in **Figure 4.1**). Data concurs with previous studies that show 1-C-6 Ig binds the reduced/alkylated modified form of aggrecan with a higher efficiency than the deglycosylated form.

a)



b)

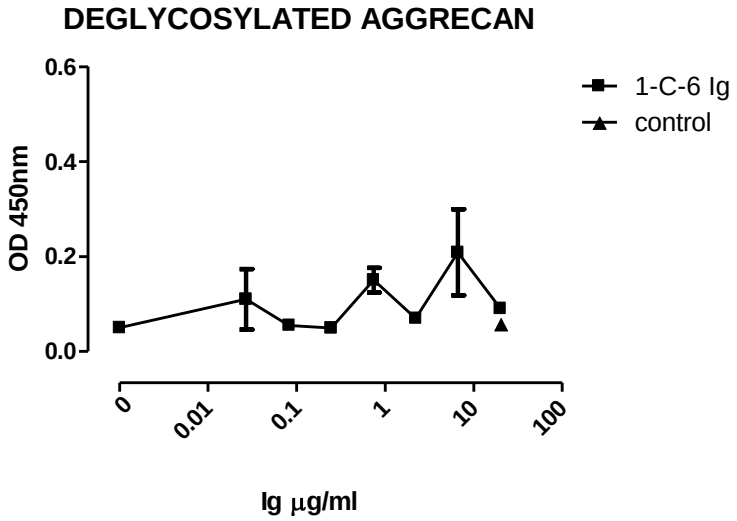


Figure 4.1 ELISA to verify the aggrecan-specificity of the 1-C-6 Ig. *1-C-6 cells were grown in 2 litres of culture media until confluent. The cells were pelleted and the supernatant was removed. Ig was purified from the culture supernatant on protein G columns and titrated on ELISA plates coated with either a) 2 μg/ml reduced and alkylated aggrecan or b) deglycosylated 2 μg/ml deglycosylated aggrecan. Wells coated with ELISA coating buffer only were used as negative controls on both plates). Data is representative of triplicate wells and 2 independent experiments.*

4.2.1.2 C71

C71 is an aggrecan-specific B cell hybridoma clone derived from the F1.11 B cell hybridoma population. F1.11 aggrecan-specific hybridomas were generated by fusing spleen cells isolated from aggrecan-immunized BALB/c mice with the mIg –ve B lymphoma NSO immediately prior to this project. F1.11 supernatant was screened by ELISA to verify aggrecan-specificity of the Ig. I subsequently transferred the F1.11 cells from HAT selection medium into HT and finally into hypoxanthine, aminopterin and thymidine free RPMI medium. In order to verify that the F1.11 cell population had retained their ability to produce aggrecan-specific antibodies I tested the ability of supernatants from these cells to bind deglycosylated, glycosylated and denatured forms of aggrecan using an ELISA (**Figure 4.2**). Results revealed that the antibodies produced by F1.11 cells bound the deglycosylated form of aggrecan most efficiently. Subsequently, the F1.11 supernatant was titrated alongside a dilution of commercially available anti-aggrecan antibody (IgG1) as a positive control and screened by ELISA (data shown in **Figure 4.3**). Results showed that the titrated F1.11 supernatant was able to bind aggrecan from dilutions ranging from 1:10 to 1:1000.

The F1.11 hybridomas are a heterogeneous population of cells. Therefore, the hybridomas were single cell cloned in order to:

- Select clones secreting anti-aggrecan antibodies,

and

- to determine whether F1.11 cells were polyclonal or monoclonal.

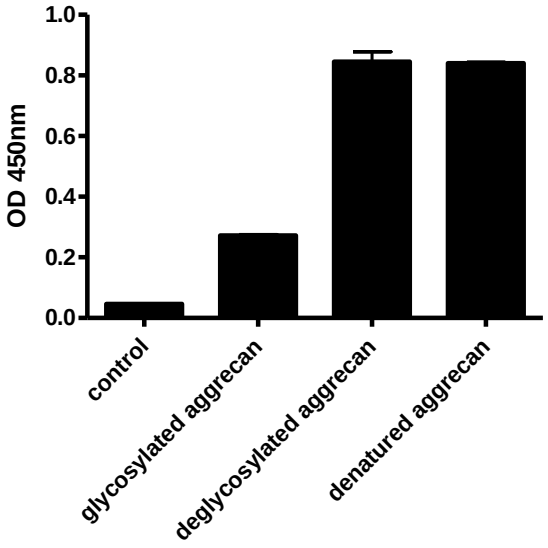


Figure 4.2 ELISA to determine the aggrecan-specificity of the F1.11 B cell hybridomas. *F1.11 cells were grown for several days in culture until confluent. Cells were pelleted and the supernatant was removed and used un-diluted on ELISA plates coated with either ELISA coating buffer only (control), 2 μg/ml glycosylated aggrecan, 2 μg/ml deglycosylated aggrecan or 2 μg/ml deglycosylated aggrecan that has been denatured by boiling at 95°C for 10 min. Data is representative of triplicate wells and 2 independent experiments.*

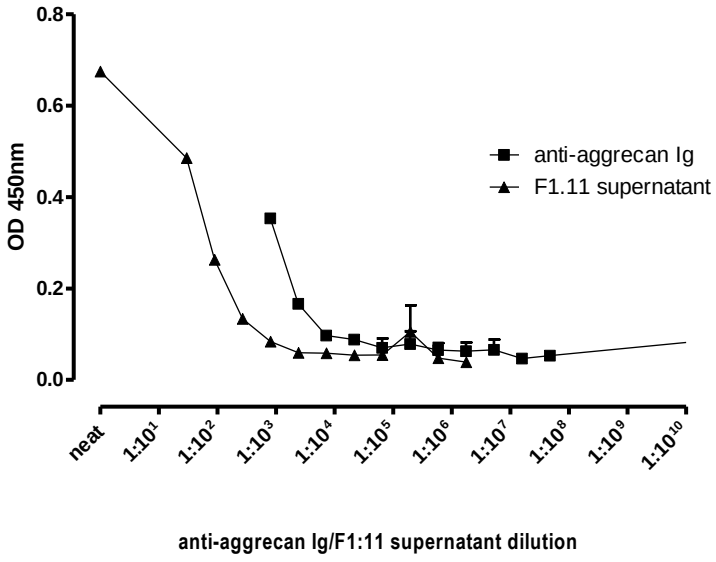


Figure 4.3 ELISA to determine the aggrecan-avidity of the F1.11 Ig. *F1.11 cells were grown for several days in culture until confluent. Cells were pelleted and the supernatant was removed and titrated on an ELISA plate coated with 2 μg/ml deglycosylated aggrecan. Anti-aggrecan IgG1 (Serotec) was titrated alongside the F1.11 supernatant as a positive control. Data is representative of duplicate wells.*

In order to clone sequences encoding VH and VL regions that bind aggrecan, I had to be confident of a monoclonal single population of cells containing a single VH and VL sequence. Therefore, 104 F1.11 single cell clones were generated by limiting dilution and the specificity of their secreted Ig was determined by ELISA to identify differences in aggrecan binding. 24 out of the 104 F1.11 clones were positive for aggrecan binding, yet no significant differences were observed in apparent affinity or judged by strength of signal (OD 450 nm). To determine if there were any differences between the 24 positive Ig in their ability to bind different forms of aggrecan, I tested their binding to deglycosylated, glycosylated and denatured aggrecan. Data shown in **Figure 4.4** shows that supernatants uniformly bound most efficiently to deglycosylated aggrecan. Surprisingly, the binding efficiency was not significantly affected by boiling the aggrecan. However, all 24 F1.11 Ig bound to the glycosylated form of aggrecan less efficiently implying that the Ig epitope could be masked by glycosylation. In a further attempt to identify differences between the Ig secreted by the 24 F1.11 clones, the H chain isotype and L chain usage was determined by ELISA (not shown). Data revealed no differences between the 24 Ig, all were identified as $IgG1^+$, κ^+.

Identifying differences in the specificity of the F1.11 clones Ig would be indicative of the presence of individual different clones and may have enabled the generation of B cells expressing aggrecan-specific BCRs that recognize different epitopes within the aggrecan structure, possibly with different affinities and avidities. By generating a panel of different aggrecan-specific B cells I could examine the effects BCR binding has on how efficiently the p84-103 epitope is generated and presented to the T cell hybridoma 192. However, taken together the results revealed that there were no obvious differences observed between the 24 F1.11 clones.

Therefore, the C71 hybridoma clone was selected as representative of the 24 F1.11 positive clones detailed above. Data verifying the aggrecan-specificity of C71 Ig by ELISA is shown in **Figure 4.5**. However, the region of aggrecan bound by C71 Ig is not known.

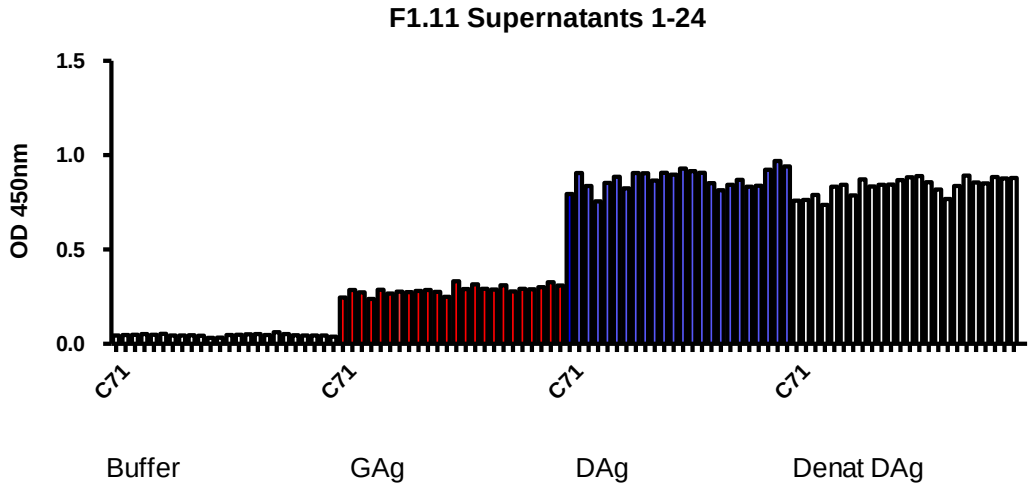
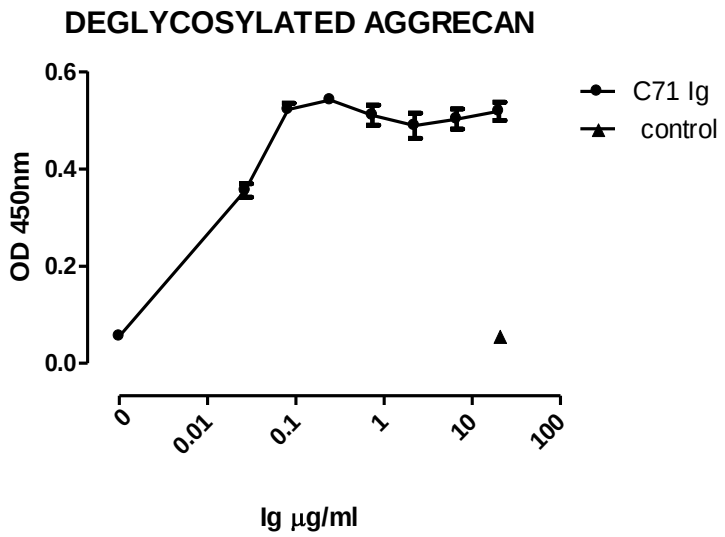


Figure 4.4 ELISA to determine the aggrecan-specificity of the 24 F1.11 clones Ig. *Clones 1-24 (C71 clone 2) were grown for several days in culture until confluent. The cells were pelleted and supernatants were removed and used neat on ELISA plates coated with either ELISA coating buffer only, 2 μg/ml glycosylated aggrecan (GAg), 2 μg/ml deglycosylated aggrecan (DAg) or 2 μg/ml deglycosylated aggrecan that has been denatured by boiling at 95°C for 10 min (Denat DAg). Data is representative of 2 independent experiments.*

a)



b)

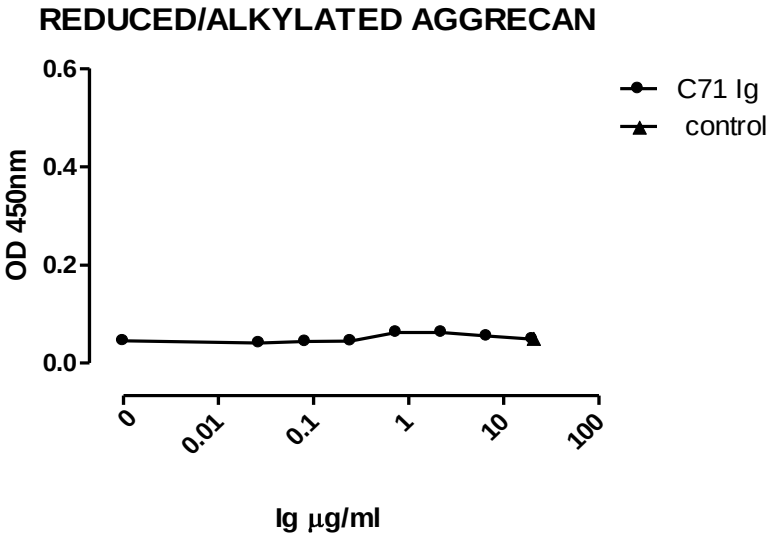


Figure 4.5 ELISA to verify the aggrecan-specificity of purified Ig from the F1.11 clone C71 Ig. *C71 cells were grown in 2 litres of culture media until confluent. The cells were pelleted and the supernatant was removed. Ig was purified from the culture supernatant on protein G columns and titrated on ELISA plates coated with either a) 2 μg/ml deglycosylated aggrecan or b) 2 μg/ml reduced and alkylated deglycosylated aggrecan. Wells coated with ELISA coating buffer only were used as negative controls on both plates. Data is representative of triplicate wells and 2 independent experiments.*

4.2.2 Amplification and sequencing of the C71/1-C-6 VH and VL regions.

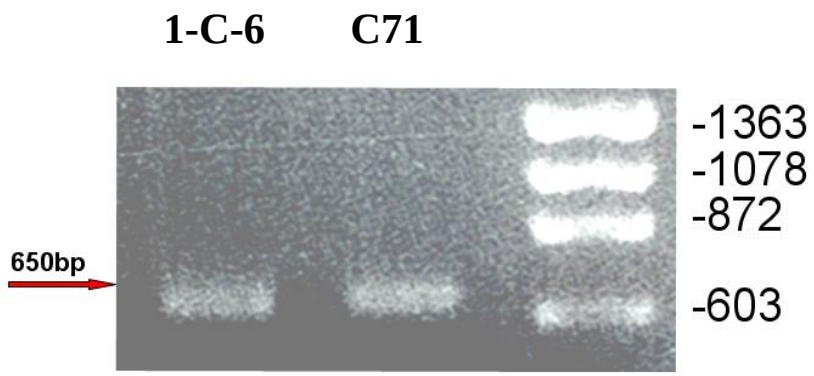
cDNAs encoding the VH and VL regions from the two aggrecan-specific monoclonal antibodies (C71 and 1-C-6) were successfully isolated using the RACE procedure detailed in **section 2.13.4**. Gene-specific primers (GSP1 and GSP2) for both the IgG1 H chain and kappa L chain of murine Ig were designed based on published sequences (Doenecke, Winnacker et al. 1997). First strand cDNA was synthesized from C71 and 1-C-6 total RNA using reverse transcriptase and the primers, GSP1 (H chain 5'-ATTTTGTCGACCKYGGTSYTGCTGGCYGGGTG-3' and (L chain 5'-TTTGGGGTAGAAGTTGTT-3'). cDNA was purified to remove excess nucleotides and GSP1 and tailed using TdT. Tailed cDNA was amplified by PCR using the GSP2 nested primers (H chain 5'-GCACACYRCTGGACAGGGATCCAGAGTTCC-3' and (L chain 5'-TAACTGCTCACTGGATGGTGGGAAGATGGA-3') and an abridged anchor primer (5'-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3') which contains a 3' sequence complementary to the homopolymeric tail of the TdT tailed cDNA. PCR products were analyzed by agarose gel separation (data shown in **Figure 4.6**.) Results show that the C71 and 1-C-6 VH region was successfully amplified at around 650 bp and the VL chain region at around 530 bp.

Subsequently, cDNA encoding the C71 and 1-C-6 VH and the VL chain regions were purified from agarose gels and ligated into the pCR®-XL-TOPO® vector using T4 ligase. The ligation mixtures were then transformed into one shot® Top 10 *E.coli* and colonies were selected on ampicillin plates. pCR®-XL-TOPO® vectors containing the VH cDNA were isolated by miniprep according to manufacturer's instructions. The correct VH insert was confirmed by restriction digest with *Sac1* and *Xho1* followed by agarose gel separation to confirm a 3.5 kb plasmid vector and 650 bp insert. pCR®-XL-TOPO® vectors containing the VL cDNA were isolated and the correct insert was verified by restriction digest with *EcoR1* followed by agarose gel separation to confirm a 3.9 kb plasmid vector and at 526 bp insert.

pCR-XL-TOPO DNA purified from clones containing both VH and VL cDNAs were sequenced using the M13 Forward and Reverse primers (M13F 5´-GTAAAACGACGGCCAG-3´, and M13R 5´-CAGGAAACAGCTATGAC-3´). Sequencing revealed 5 independent clones from C71 VH and VL vectors contained identical V sequence homology with each other and the conserved framework regions

of other published H and L chain sequences. 5 clones containing 1-C-6 VH identical regions were also selected (data shown in **Figure 4.7**). Aligned sequences of C71, 1-C-6 and other published H and L chain V regions confirm the integrity of the cDNA and that the C71 and 1-C-6 sequences contained similar framework sequences to those previously published. Therefore, the next step was to use these amplified C71 and 1-C-6 V regions to replace those present in pMCFR lymphocyte expression vectors described in (Denzin, Robbins et al. 1994) encoding the H and L chains of a membrane bound human IgG1 BCR (pMCFR TTCF-H NI and pMCFR TTCF-L hyg). pMCFR TTCF-H NI and pMCFR TTCF-L hyg have been previously shown to encode a TTCF-specific BCR and their introduction into B cell lines was subsequently shown to improve presentation of TTCF following transfection into B cell lines (Knight, Lucocq et al. 1997). Therefore, I hypothesised that exchanging the TTCF-specific V regions for aggrecan-specific V regions should enable generation of plasmid constructs encoding aggrecan-specific BCR H and L chains for stable transfection in A20-1 cells.

a)



b)

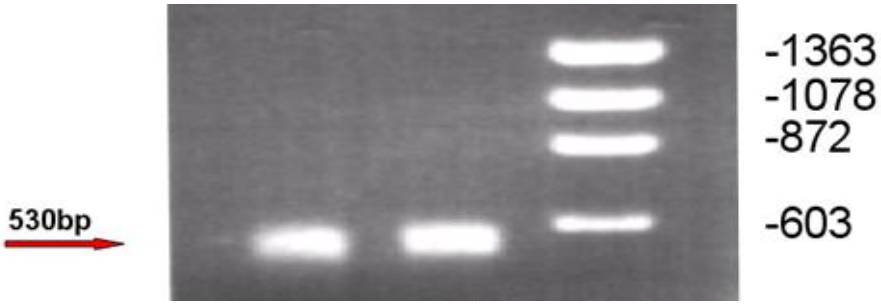


Figure 4.6 Agarose gels showing PCR amplified cDNA encoding the V regions from the a) H and b) L chains of both the 1-C-6 and C71 anti-aggrecan antibodies. *Both 1-C-6 and C71 H and L chain V regions were PCR amplified and separated by agarose gel (1%) electrophoresis to verify a correct size PCR product of approximately 650 bp (H chain) and 530 bp (L chain). on far right shows the migration of molecular weight markers (phiX 174 HaeIII).*

H chain amino acid alignment

```
            Leader                    FR1                    CDR1          FR2
1-C-6 MAVLVLFLCLVAFPSCVLS  QVQLKESGPGLVAPSQSLSITCTVSGFSLT SYGVH         WVRQPPGKGLEWLG
C71   MAVLVLFLCLVAFPSCVLS  QVQLKESGPGQVAPSQSLSITCTVSGFSLT TFGVH         WVRQPPGKGLEWLG
11.3  MDWTWSILFLVAAATGAHS  QVQLVQSGVEVKKPGASVKVSCKASGYTFS TYGMS         WVRQAPGQGLEWMG
H5    MGWSWIFLFLLSVTAGVHS  QVHLQQSGAELVRPGTSVKVSCKASGYAFT NYLIE         WVTQRPGQGLEWIG
H7    MAWVWTLLFLMAAAQSIQA  QIQLVQSGPELKKPGETVKISCKASGYTFT HYPMH         WVKQAPGKSLKWMG
17-1A MGWSRVFIFLLSVTAGVHS  QVQLQQSGAGLVRPGTSVKVSCKASGYAFT NYLIE         WVKQRPGQGLEWIG
            CDR2                    FR3                    CDR3          FR4
1-C-6 VIWAGGSTNYNSALMS  RLSISKDNSKSQVFLKMNSLQTDDTAMYYCAR  DQGYGNGGFAY   WGQGTLVTVSA
C71  VIWPGGSTNYNSALMS  RLSISKDNSKRQVFLKMSSLQTDDTAMYYCAR  EGYWFAY       WGQGTLVTVSA
11.3  WISAYNGNTNYARNFR  GRITMTTDTSTSTAYMELRSLRSDDTAVYYCAR DGRTVRGFQDLDY WGQGTLVTVSS
H5    VINPGSDFTYYNEKFKG RATLTADKSSSTAYMQLTSLTSDDSAVYFCAR  TIVTTDYFDY    WGQGTPLTVSS
H7    WINTKSGVPTYADDFKG RFAFSLETSASTACLQITNLKNEDMATYFCVR  GGLYYDYFYGVDY WGQGTSVTVSS
17-1A VINPGSGGTNYNEKFKG KATLTADKSSSTAYMQLSSLTSDDSAVYFCAR  DGPWFAY       WGQGTLVTVSA
```

L chain amino acid alignment

```
               Leader                  FR1             CDR1            FR2
1-C-6 MKLPVRLLVLMFWIPASSS  DVVMTQTPLSPVSLGDQASISR  SSQSLVHSNGNTYLH   WYLQKPGQSPKLLIY
C71   MDFHVQIFSFMLISVTVIL  SSGEIVLTQSPALMAASPGEKV  ITCSVSSSISSSNLH   WYQQKSETSPKPWIY
H5    MESDTLLLWVLLLWVPGSTG DIVLTQSPASLAVSLGQRATISC RASESVSIRGAGLMH   WYQQKPGYPPKLLIY
17-1A MESQTLVFISILLWLYGADG NIVMTQSPKSMSMSVGERVTLTC KASQNVVTYVS       WYQQKPEQSPKLLIY
H7                        DVVMTQTPLTLSVTIGQPASISC KSSQSLLDSDGETYLN  WLLQRPGQSPKRLIY
A34L                      DVLMTQTPLSLPVSLGDQASISC RSSQSIVHTNGNTYLE  WYLQKPGQSPKLLIY
            CDR2                    FR3                    CDR3      FR4
1-C-6      KVSNRFS      GVPDRFSGSGSGTDFTLKISRVEAEDLGVYFC   SQSTHVPP  FGGGTKLEIK
C71        GTSNLAS      GVPVRFSGSGSGTSYSLTISSMEAEDAATYYC   QQWSTYPLT FGGGTKLEIK
H5         AASNLES      GVPARFSGRGSGTDFTLNIHPVEEADAATYFC   QQSRRYPYT FGSGTKLEIK
17-1A      GASNRYT      GVPDRFTGSGSATDFTLTISSVQAEDLADYHC   GQGYSYPYT FGGGTKLEIK
H7         MVSKLDS      GVPDRFTGSGSGTDFFFTLKISRVEAEDLGVYYC WQGTHFPT  FGSGTKLEIK
A34L       KVSNRFS      GVPDRFSGSGSGTDFTLKISRVEAEDLGVYYC   FQGSHVPRT FGGGTKLEIK
```

Figure 4.7 C71 and 1-C-6 VH and VL chain sequence alignments. *Plasmids encoding C71 and 1-C-6 VH and VL regions were sequenced using the universal M13 F and R primers (Cogenics). Sequences were aligned using framework (FR) and complimentary determining regions (CDR) from previously published monoclonal antibody sequences 11.3 (human) (Knight, Lucocq et al. 1997), H5 (mouse) (Hackett, Hoff-Velk et al. 1998), H7 (mouse) (Park, Jin et al. 2000), 17-1A (mouse (Sun, Curtis et al. 1987), A34L (mouse) (Kwak, Lee et al. 1996).*

4.2.3 Generation of H chain plasmid constructs pMCFR C7.1/ 1-C-6 NI.

In order to generate constructs encoding a membrane-bound Ig H chain containing aggrecan-specific V regions, the next step was to remove the TTCF- specific V region from the pMCFR TTCF-H NI plasmid by restriction digestion.

To do this restriction sites *Apa1* and *Kpn1* were identified 5' of sequence encoding TTCF VH region and 3' of the VH open reading frame (ORF) respectively. However, the digest was complicated as the sequence of the pMCFR NI vector is not known. The plasmid pMCFR TTCF-H NI was digested with the enzymes *Apa1* and *Kpn1* followed by agarose gel separation. Restriction digest confirmed that the plasmid had digested into several bands indicating that it contained more than the one *Apa1* site found in the TTCF cDNA. Upon further inspection of the TTCF VH and its flanking constant region sequences I attempted to remove the V region from the pMCFR TTCF-H NI vector using several different restriction enzyme combinations. However, I was unable to find a combination that would allow me to remove the V region without digesting the vector. Therefore, as *Apa1* had been identified as an ideal restriction site with which to remove the V region and a small stretch of sequence encoding a few nucleotides from the 5' C region, I decided to remove the entire H chain from the pMCFR NI vector and ligate it into a vector that was known not to contain an *Apa1* site. pCMV 2.1 (Invitrogen) was identified as an ideal vector, compatible with the use of an *Apa1* digest to remove the tetanus toxin V region and replace it with cDNA encoding the C71 and 1-C-6 aggrecan-specific V regions. However, again there were no compatible restriction site pairs with which to remove the entire TTCF H chain cDNA from pMCFR NI and ligate it directly into the pCMV 2.1 vector. Therefore, the pKS plasmid vector was utilized as a vehicle; the H chain cDNA from pMCFR NI was removed using the restriction enzymes *Kpn1* and *Sac1* and ligated with the pKS vector.

Subsequently, cDNA encoding the entire TTCF-specific H chain region was removed from pKS (Stratagene) using the enzymes *Xho1* and *Not1*, gel purified and ligated with the vector pCMV 2.1 which does not contain an *Apa1* site allowing the TTCF V region to be subsequently removed without digesting the plasmid vector. The tetanus toxin H chain V region only was then removed from the pCMV 2.1 vector by restriction digest with the enzymes *Xho1* and *Apa1*. The vector was dephosphorylated using calf intestinal phosphate (CIP) to minimize re-ligation and then separated from the TTCF V region by agarose gel electrophoresis and gel purification. In parallel, the V regions of C71 and 1-C-6 were

amplified from the plasmid DNA pCR 2.1 C71/1-C-6 generated in section 5.3.2 to include the restriction sites *Apa1* and *Xho1*. The amplified cDNA was purified from agarose gels and ligated into the pCR®-XL-TOPO® vector, transformed into one shot® Top 10 *E.coli* and colonies were selected on ampicillin plates. Plasmids were isolated by miniprep and the correct insert was confirmed by restriction digest with *Xho1* to linearize the plasmid followed by agarose gel separation. Miniprep clones were sequenced using the M13 Forward and reverse primers (M13F 5´-GTAAAACGACGGCCAG-3´, and M13R 5´-CAGGAAACAGCTATGAC-3´). The sequencing results confirmed that the cDNA now encodes the 5' *Xho1* restriction site followed by the entire C71/1-C-6 aggrecan-specific V regions, the first few nucleotides of the 5' TTCF IgH C region and a 3' *Apa1* site (data shown in **Figure 4.8**.) Complementary sites in the *Xho1* and *Apa1* digested pCMV 2.1 vector containing the TTCF C region and at the 5' and 3' ends of the C71/ 1-C-6 V regions now enabled the V and C regions to be ligated generating a chimeric mouse V / human C H chain sequence.

The pCMV 2.1 vector containing the chimeric H chain was transformed into one shot® Top 10 *E.coli* as described previously and colonies were selected on ampicillin plates. Plasmids were isolated and amplified by overnight culture followed by maxiprep and the correct sequence was verified using the C region primer SEQCH1G3 and the C71/1-C-6 V region 5' primer including restriction site for *Xho1* 5'-CTCGAGCCATGGCTGTCCTGGTGCTGTTC-3'.

In order to move the newly generated chimeric aggrecan-specific H chain back into the lymphocyte expression vector pMCFR NI, the aggrecan-specific H chain was removed from the pCMV 2.1 vector by restriction digest with *Xho1* and *Not 1* and ligated with the pKS vector to introduce the restriction sites *Kpn1* and *Sac1* required for the final ligation with the transfection plasmid pMCFR NI. The vector was transformed into one shot® Top 10 *Ecoli*, colonies were selected on ampicillin plates then amplified and isolated as previously described. The H chain was then removed from the pKS vector by restriction digest with *Kpn1* and *Sac1* and ligated with the *Kpn1* and *Sac1* digested pCMFR NI, CIP treated vector. This final completed vector was transformed into one shot® Top 10 *E.coli* and colonies were selected on ampicillin plates, amplified and isolated by maxiprep ready for transfection. Complete H chain construction is illustrated in **Figure 4.9**.

Heavy chain nucleotide sequencing

C71 VH

CTCGAGCCATGGCTGTCCTGGTGCTGTTCCTCTGCCTGGTTGCATTTCCAAGCT
GTGTCCTGTCCCAGGTGCAGCTGAAGGAGTCAGGACCTGGCCAGGTGGCGCC
CTCACAGAGCCTGTCCATCACTTGCACTGTCTCTGGGTTTTCATTAACCACCTTT
GGTGTTCACTGGGTTCGCCAGCCTCCAGGAAAGGGTCTGGAGTGGCTGGGAG
TAATATGGCCTGGTGGAAGCACAAATTATAATTCGGCTCTCATGTCCAGACTGA
GCATCAGCAAAGACAACTCCAAGCGCCAAGTTTTCTTAAAAATGAGCAGTCTAC
AAACTGATGACACAGCCATGTACTACTGTGCCAGAGAGGGTTACTGGTTTGCTT
ACTGGGGCCAAGGGACTCTGGTCACTGTCTCTGCTGCCTCCACCAAGGGCCC

1-C-6 VH

CTCGAGCCATGGCTGTCCTGGTGCTGTTCCTCTGCCTGGTTGCATTTCCAAGCT
GTGTCCTGTCCCAGGTGCAGCTGAAGGAGTCAGGACCTGGCCTGGTGGCGCC
CTCACAGAGCCTGTCCATCACTTGCACTGTCTCTGGGTTTTCATTAACCAGTTAT
GGTGTACACTGGGTTCGCCAGCCTCCAGGAAAGGGTCTGGAGTGGCTGGGAG
TAATATGGGCTGGTGGAAGCACAAATTATAATTCGGCTCTCATGTCCAGACTGA
GCATCAGCAAAGACAACTCCAAGAGCCAAGTTTTCTTAAAAATGAACAGTCTGC
AAACTGATGACACAGCCATGTACTACTGTGCCAGAGATCAGGGGTATGGTAAC
GGCGGGTTTGCTTACTGGGGCCAAGGGACTCTGGTCACTGTCTCTGCTGCCTC
CACCAAGGGCCC

Figure 4.8 C71 and 1-C-6 VH regions amplified to include restriction sites. *C71 and 1-C-6 V regions were amplified by PCR to include the restriction sites Xho1, the Kozak sequence and 13 nucleotides of the human IgG1 constant region including an Apa1 restriction site. Xho1* site, *Kozak sequence (Kozak 1986), Human IgG1 constant region, Apa1 site.*

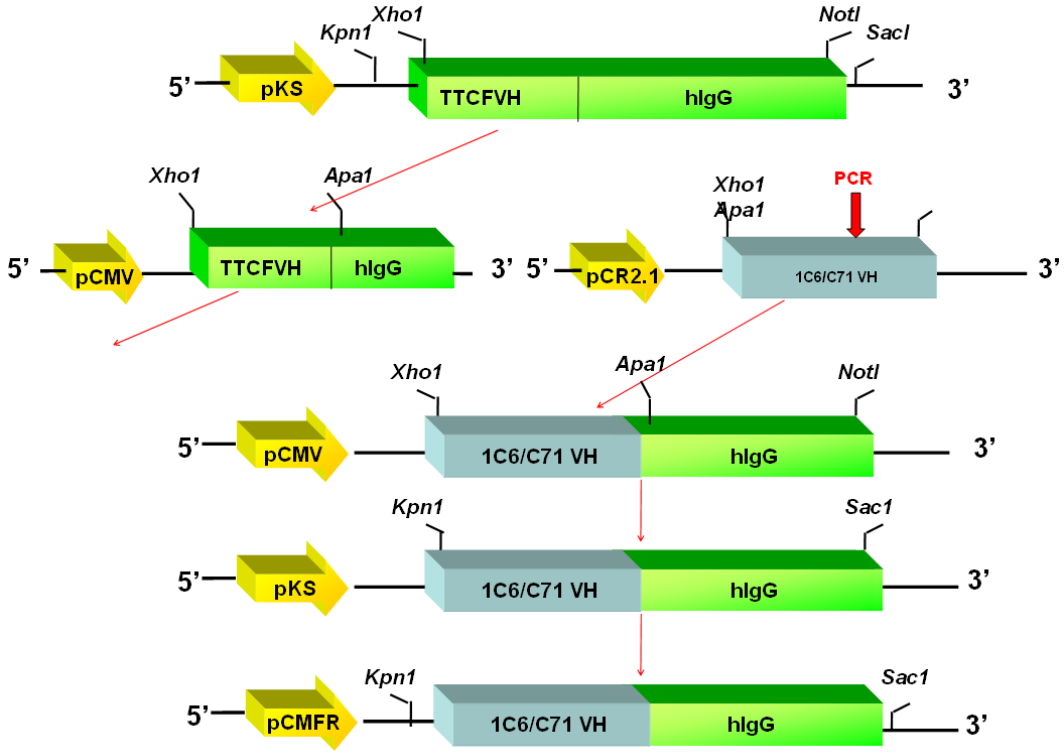


Figure 4.9 Representation of the pMCFR vector /H chain assembly. *The cDNA encoding the anti-human TTCF-specific H chain was digested from the plasmid pKSM13 which contains an Apa1 site and into the vector pCMV/myc /ER which is Apa1 negative. The cDNA encoding the V region from the TTCF H chain (TTCFVH) was removed using Xho1 and Apa1. 1-C-6 and C71 cDNA encoding aggrecan-specific VH regions (IC6/C71 VH) were PCR amplified to include Xho1 and Apa1 sites at the 5' and 3' ends respectively, cloned into the pCR2.1 plasmid and digested using Xho1 and Apa1. IC6/C71 VH cDNA were ligated with the digested pCMV/myc/ER vector containing the cDNA encoding the C region from the TTCF-specific H chain (hIgG). The assembled H chain cDNA was digested from pCMV/myc/ER with Xho1 and Not1 and ligated with pKSM13 to introduce Kpn1 and Sac1 sites at the 5' and 3' ends of the insert respectively. The H chain cDNA was digested from pKSM13 with Kpn1 and Sac1 and ligated with the final expression vector pMCFR/NI. Plasmids were transformed into E.coli competent cells and cDNA was amplified and isolated at each step by maxiprep (Qiagen).*

4.2.4 Generation of L chain plasmid pMCFR C71/1-C-6 hyg.

The pMCFR TTCF-L hyg vector contains the TTCF L chain. In order to ligate the TTCF-L human kappa C region with the C71 and 1-C-6 aggrecan-specific VL regions, the TTCF VL region and the first 13 nucleotides leading up to a restriction site in the CL region would have to be removed. Unfortunately, as with the H chain construction, this was complicated by the unknown sequence of the pMCFRhyg vector. Several possible restriction site pairs were identified to remove the TTCF VL region. However, following restriction digest and separation by agarose gel electrophoresis I found that the restriction enzymes were also present in the pMCFRhyg vector. The enzymes *Xba1* and *Bbs1* were identified as ideal candidates to remove the VL region from pMCFR hyg. However, as with the H chain, the *Bbs1* sequences present in the TTCF-L was not unique and was also found to be present in the pMCFR hyg vector. Therefore, as described in the H chain construction, the TTCF-L cDNA was removed from pMCFR hyg and ligated into the *Bbs1* negative plasmid pKS using *Xba1* and *Kpn1*.

The pKS /TTCF-L ligation mixture was transformed into one shot® Top 10 *E.coli*, colonies were selected on ampicillin plates, amplified by overnight culture and DNA was isolated. To confirm that the TTCF VL region could now be removed, the plasmids were digested with *Xba1* and *Bbs1*. The successful removal of the V region was confirmed by agarose gel separation.

In parallel, the VL regions of C71 and 1-C-6 were amplified from the plasmid DNA pCR 2.1 C71/1-C-6 generated in section 5.3.2 to include the restriction sites 5' *Xba1* and the first 20 nucleotides encoding the human kappa C region up to and including the 3' *Bbs1* site. The new pCR products were verified by agarose gel separation, ligated with the vector pCR2.1 and sequenced as described in section 5.3.2. Sequencing confirmed that the amplified C71/1-C6- VL regions now contain the 5' *Xba1* restriction site followed by the C71/1-C-6 VL region and the C kappa region up to and including the 3' *Bbs1* site (data shown in **Figure 4.10**.)

The TTCF VL region was then excised from the vector pKS by restriction digest with *Xba1* and *Bbs1,* the *Xba1* and *Bbs1* digested pKS vector was treated with CIP and separated from the TTCF VL on an agarose gel. The dephosphorylated vector was then removed from the gel, purified and ligated with the new C71/1-C-6 aggrecan-specific VL regions. As there are

no compatible restriction enzyme pairs with which to remove the newly constructed L chain from the pKS vector and into the final pMCFR hyg lymphocyte expression vector, the entire aggrecan-specific L chain was removed by restriction digest with *BsshII* and ligated into *BsshII* digested plasmid pSL1180 (Invitrogen). The L chain was then transferred from this plasmid by restriction digest with *Xba1* and *Sac1* and ligated into the dephosphorylated *Xba1* and *Sac1* digested pCMFR hyg vector. This ligation mixture was then transformed into one shot® Top 10 *E.coli* and colonies were selected on ampicillin plates. DNA from amplified colonies was then isolated for transfection. Complete L chain construction is illustrated in **Figure 4.11**.

Light chain nucleotide sequencing

C71 VL
TCTAGAATGGATTTTCATGTGCAGATTTTCAGCTTCATGCTAATCAGTGTCACAGT
CATATTGTCCAGTGGAGAAATTGTGCTCACCCAGTCTCCAGCACTCATGGCTGCA
TCTCCAGGGGAGAAGGTCACCATCACCTGCAGTGTCAGCTCAAGTATAAGTTCCA
GCAACTTGCACTGGTACCAGCAGAAGTCAGAAACCTCCCCCAAACCCTGGATTT
ATGGCACATCCAACCTGGCTTCTGGAGTCCCTGTTCGCTTCAGTGGCAGTGGATC
TGGGACCTCTTATTCTCTCACAATCAGCAGCATGGAGGCTGAAGATGCTGCCACT
TATTACTGTCAACAGTGGAGTACTTACCCACTCACTTTCGGAGGGGGGACCAAGC
TGGAAATAAAAACTGTGGCTGCACCATCTGTCTTCAT

1-C-6 VL
TCTAGAATGAAGTTGCCTGTTAGGCTGTTGGTGCTGATGTTCTGGATTCCTGCTTC
CAGCAGTGATGTTGTGATGACCCAAACTCCACTCTCCCTGCCTGTCAGTCTTGGA
GATCAAGCCTCCATCTCTTGCAGATCTAGTCAGAGCCTTGTACACAGTAATGGAA
ACACCTATTTACATTGGTACCTGCAGAAGCCAGGCCAGTCTCCAAAGCTCCTGAT
CTACAAAGTTTCCAACCGATTTTCTGGGGTCCCAGACAGGTTCAGTGGCAGTGGA
TCAGGGACAGATTTCACACTCAAGATCAGCAGAGTGGAGGCTGAGGATCTGGGA
GTTTATTTCTGCTCTCAAAGTACACATGTTCCTCCGACGTTCGGTGGAGGCACCA
AGCTGGAAATCAAAACTGTGGCTGCACCATCTGTCTTCAT

Figure 4.10 C71 and 1-C-6 VL regions amplified to include restriction sites. *C71 and 1-C-6 V regions were amplified by PCR to include the restriction sites Xba1 and 20 nucleotides of the human kappa constant region including a Bbs1 restriction site. Xba1 site, Human kappa constant region, Bbs1 site.*

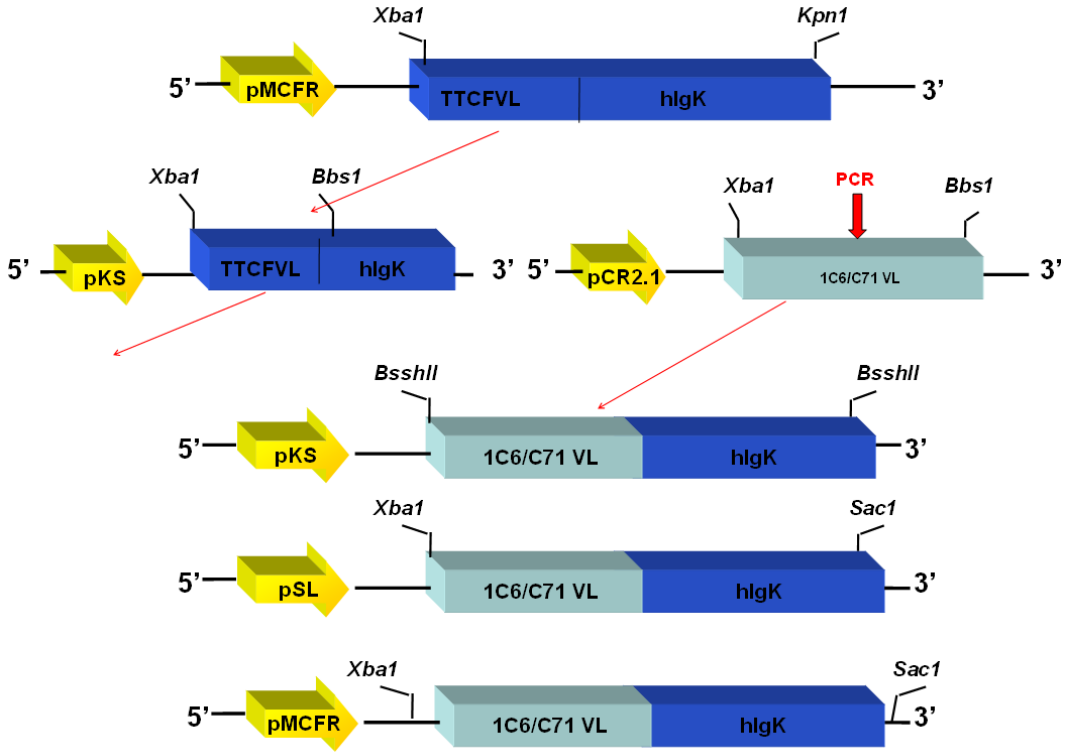


Figure 4.11 Representation of the pMCFR vector/L chain assembly. *The cDNA encoding the TTCF-specific L chain was digested from the plasmid pMCFR which contains a Bbs1 site and into the vector pKSM13 which is Bbs1 site negative. The cDNA encoding the V region from the TTCF L chain (TTCFVL) was digested using Xba1 and Bbs1. cDNA encoding the 1-C-6 and C71 aggrecan-specific VL regions (1-C-6/C71 VL) were PCR amplified to include an Xba1 and Bbs1 site at the 5' and 3' ends respectively, cloned into the pCR2.1 plasmid and digested using Xba1 and Bbs1. 1-C-6/C71 VL cDNAs were ligated with the digested pKSM13 vector containing the cDNA encoding the C region of the TTCF-specific L chain (hIgK). The assembled L chain was digested from the pKSM13 vector with BsshII and ligated with pSL1180 to introduce a Sac1 site at the 3' end of the insert. The L chain cDNA was digested from pSL1180 with Xba1 and Sac1 and ligated with the final expression vector pMCFR/hyg. Plasmids were transformed into E.coli competent cells and cDNA was amplified and isolated at each step by maxiprep (Qiagen).*

4.2.5 Transfection of plasmids encoding aggrecan-specific IgG H and L chains.

Initially I wanted to verify that the cDNAs contained within the pMCFR plasmids were capable of encoding functional H and L chains of a BCR. Our laboratory has previously demonstrated that functional IgG BCR can be expressed in non-lymphoid cells (Knight, Lucocq et al. 1997) which are comparatively simple to transfect. Therefore, constructs containing cDNA encoding both the H and L chains of the new anti-aggrecan-specific BCR were transiently transfected in HeLa cells to verify expression. HeLa cells were transfected using the Lipofectamine 2000 (Invitrogen) transfection reagent with either the C71/1-C-6 H chain only, the L chain only or both H and L. To control for transfection efficiency HeLa cells were also transfected with plasmids encoding the original TTCF-specific BCR. Data shown in **Figure 4.12** revealed that the H chain only transfected cells showed a different staining pattern to that of cells transfected with both the H and L chain as previously described. This was to be expected as the HeLa cells do not have any endogenous L chain and it is known that in the absence of the L chain the H chain is retained in the ER by BiP (Knight, Lucocq et al. 1997; Vanhove, Usherwood et al. 2001). However, when the H and L chains are co-transfected, expression was observed in cytoplasmic vesicles, indicating a correctly assembled BCR trafficking from and to the cell surface (Knight, Lucocq et al. 1997).

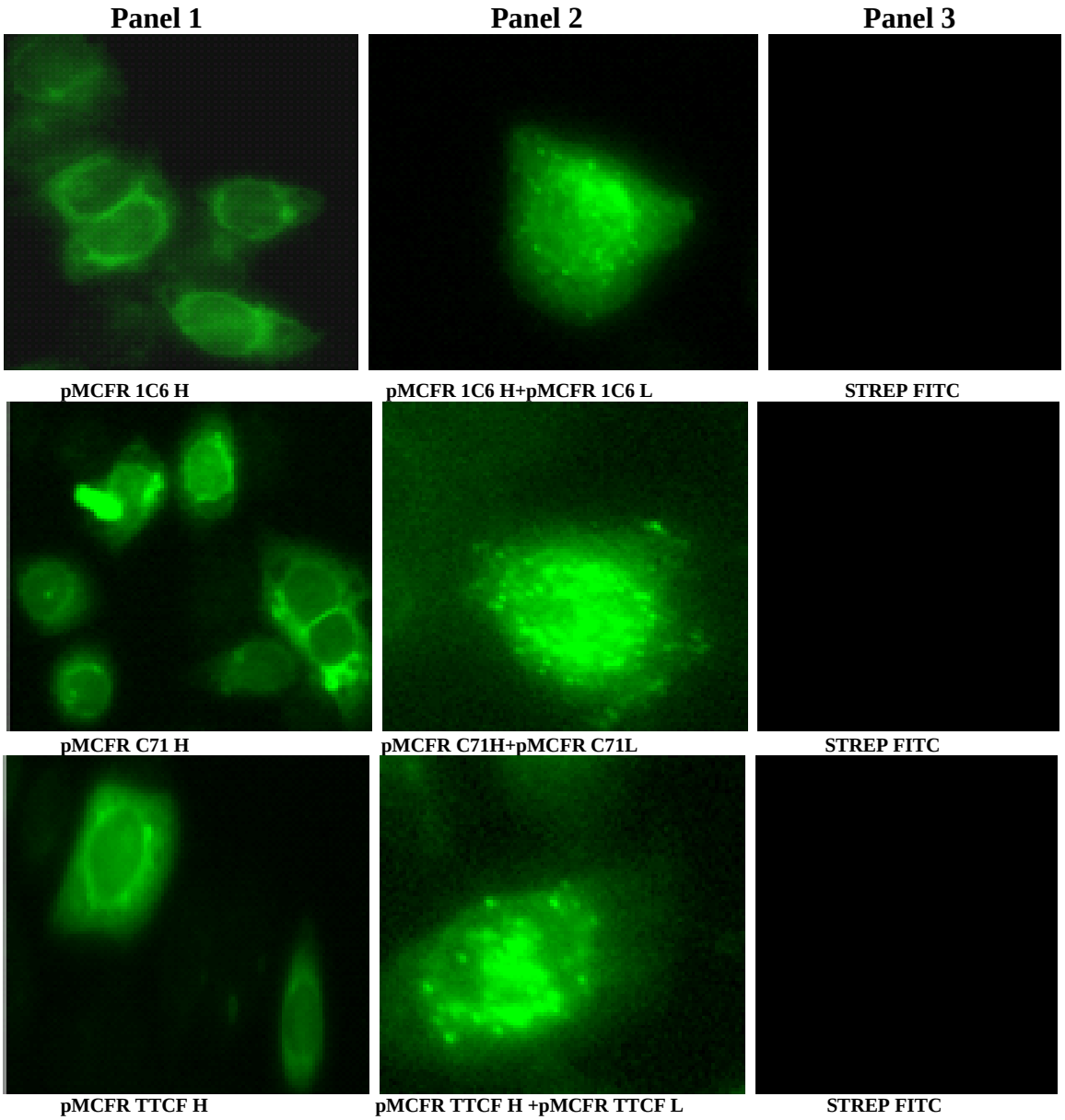


Fig 4.12 Indirect Immunofluorescence images of HeLa cells transiently transfected with C71 and 1-C-6 constructs. *HeLa cells were transiently transfected with the following H chain only (panel 1) or both H and L chain plasmid constructs (panels 1&2): positive control pMCFR TTCF H/L (anti-TTCF) originally used to create the new constructs, pMCFR 1-C-6H/L (anti-aggrecan) or pMCFR C71H/L (anti-aggrecan). Fixed, permeabilized transfectants were stained with anti-human IgG (H and L) Fab^2 biotinylated antibodies and streptavidin FITC (STREP FITC). The green fluorescence observed in the 1st panel from each transfectant shows typical endoplasmic reticulum staining. The 2nd panel shows vesicular staining. The 3rd panel control shows transfectants stained with streptavidin FITC only. Images are representative of 5 fields and 3 transfections. Magnification is 40x.*

Subsequently, several attempts were made at transfecting the plasmid constructs into the A20-1 HS cell line. Initial attempts using a squareporater and electroporater were unsuccessful as the majority of cells were killed during the electroporation process despite attempts using both linearized and circular DNA and several different μF and voltage combinations. The Amaxa nucleofector system (Lonza) combines the use of electroporation with a nucleofection buffer and a pre-programmed voltage, capacitance and resistance specific to the cell type. In addition, the Amaxa system transfects DNA directly into the nucleus where it is incorporated independent of cell division. Therefore, this system was used to transfect the A20-1 HS cells initially with a pMAXgfp plasmid control to test transfection efficiency (data shown in **Figure 4.13**), followed by transfection with the C71/1-C-6 H and L chain plasmids discussed below. Results from transfection with the pMAXgfp plasmid verified that A20-1 HS could be transfected with plasmid DNA using this method at approximately 50% transfection efficiency.

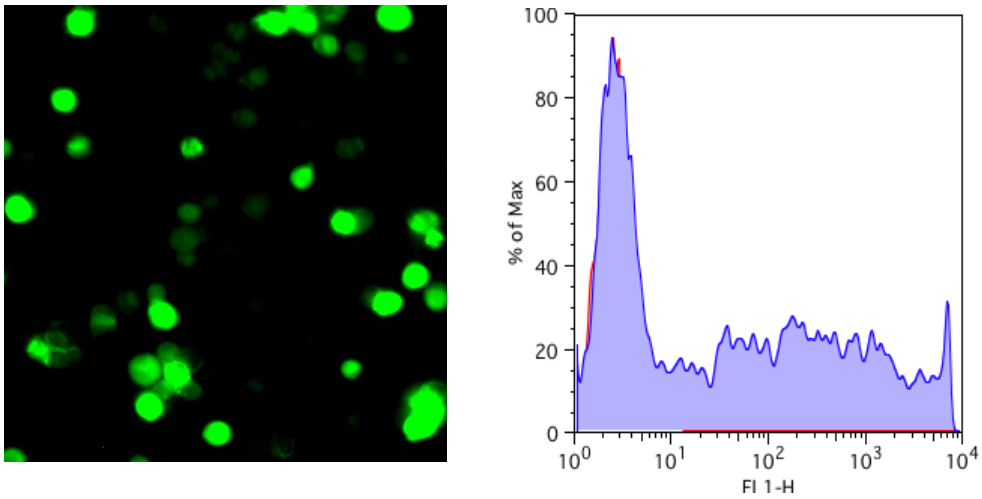


Figure 4.13. A20-1 HS pmaxGFP transfection to monitor transfection efficiency.
A20-1 HS were transfected with 3µg pmaxGFP DNA using the Amaxa nucleofector. Cells were cultured for 48 h and screened by a) Immunofluorescence microscopy and b) Flow cytometry to determine the transfection efficiency. The Immunofluorescence microscopy experiment was performed twice. The image is representative of 5 fields at 20x magnification. The flow cytometry experiment was performed 4 times.

4.2.6 Characterisation of A20-1HS C71/1-C-6 transfectants by flow cytometry and antigen presentation.

Two methods were employed to determine whether the Amaxa transfected A20-1 HS cells expressed the aggrecan-specific BCR:-

a) Flow cytometry
b) Antigen presentation assay.

4.2.6.1 Flow cytometry

A20-1 HS is a murine B cell line and the aggrecan-specific plasmids C71 and 1-C-6 contain chimeric cDNA that encode H and L chains containing human C regions. Thus the human constant regions were detected by fluorescent labelled antibody against human Ig to determine transfection efficiency by flow cytometry by differentiating the transfected BCR from the endogenous mouse IgG2a BCR. The A20-1 HS cells transfected with either the C71/1-C-6 H and L chain or C71 H or L chain only were screened by flow cytometry for the surface expression of a human IgG H+L chain using rabbit anti-human IgG H + L biotin Fab^2 followed by incubation with streptavidin FITC 24 h post Amaxa nucleofection. Results shown in **Figure 4.14** revealed that cells transfected with the C71 or 1-C-6 H and L chain constructs contained a population of cells that were now positive for the surface expression of the new chimeric aggrecan-specific BCR. However, cells transfected with either the C71 H chain or the L chain only, did not show any significant evidence of surface human BCR expression. This implies that the chimeric human H chain does not significantly pair with the endogenous mouse light chain as it is not expressed on the cell surface in the absence of the chimeric light chain. Negative controls, A20-1 HS exposed to the same transfection conditions but in the absence of DNA were also negative for surface human BCR expression as expected.

Subsequently, I attempted to visualise aggrecan binding to the surface of the transfected A20-1HS. 48 h post-transfection with the H and L chain constructs (or with the negative control DNAs described above) A20-1 HS cells were incubated with biotinylated aggrecan for 1 h at 4^oC to prevent BCR/aggrecan internalization. Cells were washed, incubated with streptavidin FITC and examined by flow cytometry. Results shown in **Figure 4.14** revealed that a population of A20-1HS transfected with either the C71 or 1-C-6 H and L chains showed an increase in fluorescence intensity indicating aggrecan binding. Importantly, this fluorescence

was not seen in A20-1 HS transfected with the C71 H chain only. Surprisingly, A20-1 HS transfected with the C71 L chain only showed a slight increase in fluorescence intensity following incubation with the biotinylated aggrecan. This indicates that C71 L chain may pair with an endogenous mouse H chain and is expressed on the cell surface at sufficient levels and with sufficient affinity as to mediate aggrecan binding.

4.2.6.2 Antigen presentation

As discussed in Chapter 5, several previous studies have shown that antigen-specific BCR expression enhances the presentation of antigen to T cells. Therefore, the extent of aggrecan presentation by A20-1 HS transfectants to the BALB/c (H-2^d) $aggrecan_{84\text{-}103}$ -specific T cell hybridoma 192, was determined using the antigen presentation assays illustrated in **Figure 3.5**. A20-1 HS transfected with both C71/1-C-6 H and L chains, C71 H or L chain only or untransfected A20-1 HS were recovered 24 h post transfection and cultured with the T cell hybridoma 192 for 24 h in the presence of either graded doses of deglycosylated aggrecan or p84-103. T cell hybridoma activation was measured by IL-2 production using CTLL-2. Data in **Figure 4.15a** revealed that A20-1 HS transfected with the C71 H and L chain presented aggrecan at least 10^3 fold more efficiently than untransfected A20-1 HS. In addition, this improvement in presentation required the expression of both the C71 H and L chain as A20-1 HS transfected with either C71 H or L chain only did not show any improvement in presentation in comparison to the untransfected A20-1 HS population. However, there was no improvement in presentation observed by A20-1 HS transfected with the 1-C-6 H and L chains in comparison with untransfected A20-1 HS.

To confirm that the improvement in presentation by the C71 H and L chain transfected A20-1 HS is due to aggrecan uptake and processing and not an improvement in surface presentation of the epitope 84-103 on H-2^d, transfected and untransfected A20-1 HS were also incubated with graded doses of the synthetic peptide p84-103. Results in **Figure 4.15b** reveal that none of the transfectants showed an improvement in presentation of p84-103 in comparison with the untransfected A20-1HS cells. Thus, confirming that the observed improvement in aggrecan presentation by the A20-1 HS transfectants is due to enhanced aggrecan uptake and/or processing and not merely an improvement in surface presentation of 84-103.

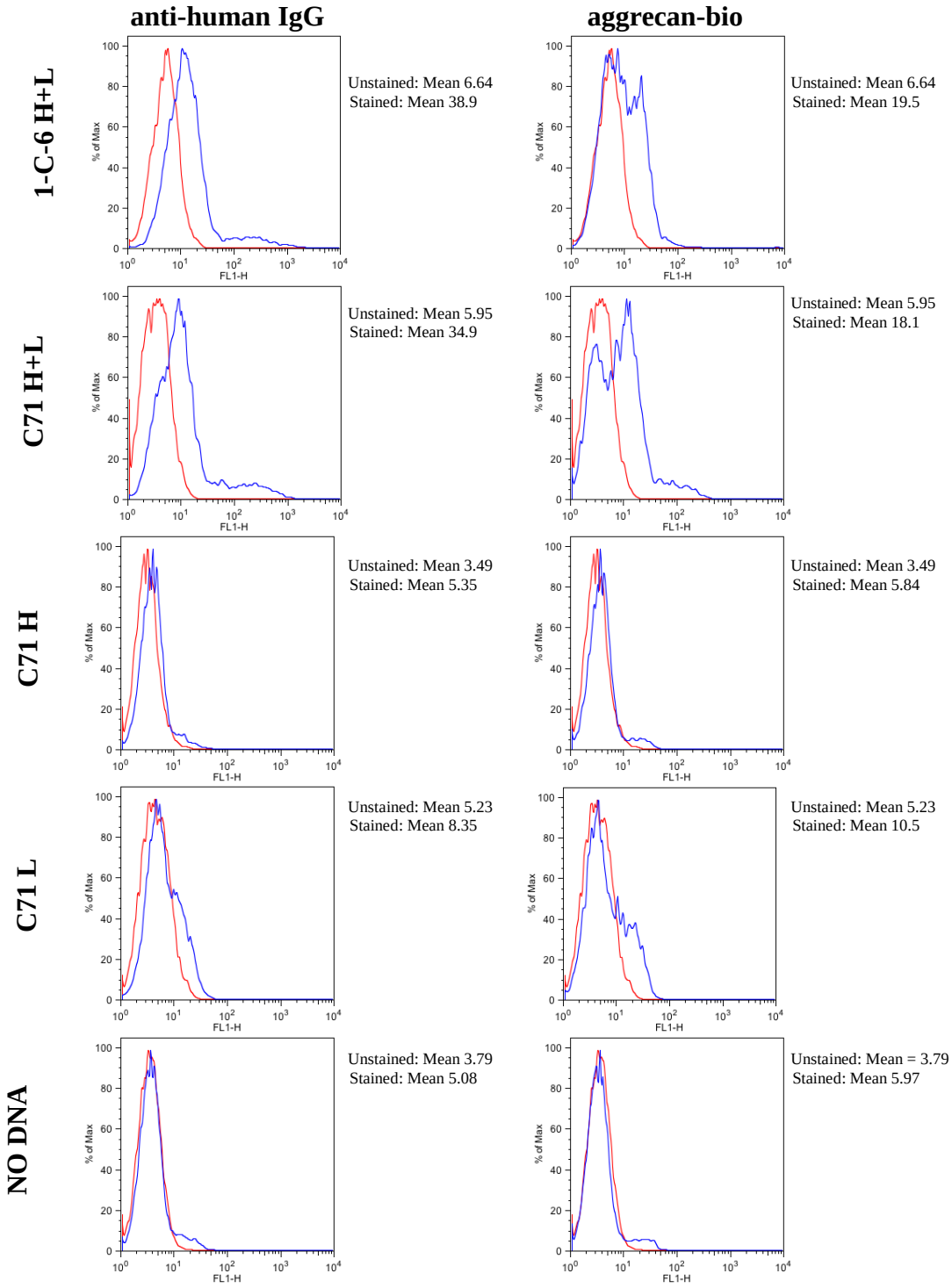
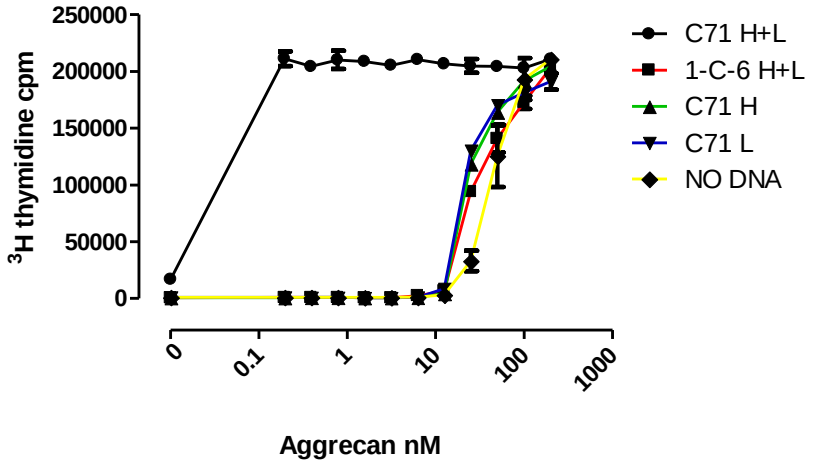


Figure 4.14 Flow cytometry following transient transfection of A20-1 HS with the C71 and 1-C-6 constructs. *Staining of gated A20-1HS cells 48 h post Amaxa nucleofection with 1-C-6 H+L chain constructs, C71 H+L chain constructs, C71 H chain construct, C71 L chain construct or no DNA controls. Cells were stained with either biotinylated anti-human IgG (H+L) Ig or biotinylated aggrecan and the secondary reagent streptavidin FITC. Red histograms represent unstained cells and the blue histograms represent cells stained for the various markers. All experiments were carried out at 4°C. Mean fluorescence intensity values are shown adjacent to each histogram. Data is representative of 3 independent experiments.*

a)



b)

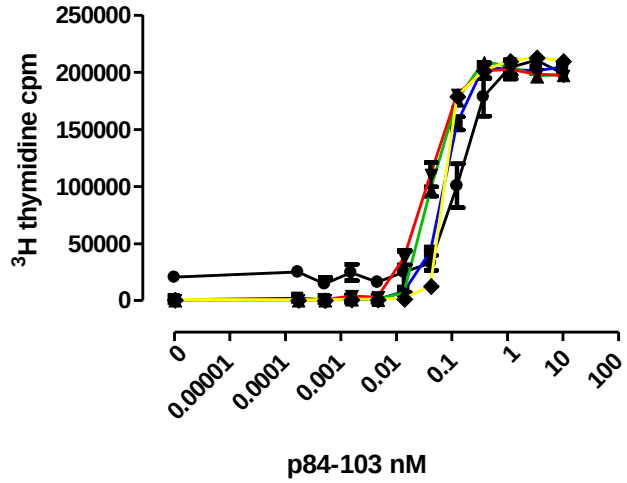


Figure 4.15 Antigen presentation assay following transient transfection of A20-1 HS with plasmids encoding- C71 and 1-C-6 BCR. *A20-1 HS were transfected with either C71 H+L chain constructs, 1-C-6 H+L chain constructs, C71 H chain only construct, C71 L chain only construct or cells underwent the Amaxa nucleofection in the absence of DNA. 24 h post transfection ($5x10^4$) cells were cultured with aggrecan$_{84-103}$-specific T cell hybridomas 192 ($5x10^4$) in the presence of graded doses of a) aggrecan or b) peptide for 24 h. Cells were killed by freezing at -80^{o}C and supernatant was removed for bioassay with CTLL-2 cells. ^{3}H thymidine was added for 18 h and T cell responses were determined and displayed as ^{3}H thymidine cpm +/- SEM. Data are representative of triplicate wells and three independent experiments.*

4.2.7 Generation of stable aggrecan-specific cell lines.

A20-1 HS were successfully transfected with the C71/1-C-6 plasmids, expression of the BCR on the cell surface was verified by flow cytometry and it was shown that the C71 transfected A20-1 HS could now present aggrecan at least 10^3 fold more efficiently. However, variation between transfection efficiencies in repeat experiments and limitations on the yield of viable cells 48 h post transfection was not optimal for studying the molecular mechanisms by which B cells act as aggrecan-specific APC. Therefore, I wanted to generate cells that were stably expressing the C71 aggrecan-specific BCR or the 1-C-6 aggrecan-specific BCR. Stable aggrecan-specific cell lines would enable me to reproducibly study downstream processing events and to examine how efficiently they present aggrecan to T cells in comparison to dendritic cells and macrophages. A20-1 HS cells were transfected with the C71 or 1-C-6 H and L chain plasmids, the bulk transfected population was screened by flow cytometry and antigen presentation 48 h post transfection and the remainder were incubated at 37^{o}C, 5% CO_2 in G418/hygromycin B selection media in 24 well plates until drug resistant colonies were visible by eye (approximately 21 days). Resultant colonies were subsequently screened for the expression of a human Ig containing BCR and their ability to retain aggrecan at the cell surface by flow cytometry and by antigen presentation assay (as previously described).

4.2.7.1 1-C-6 Transfectants

1-C-6 Ig is specific for a reduced and alkylated form of aggrecan and does not bind to the deglycosylated form in an ELISA shown in **Figure 4.1**. Therefore, as expected colonies resulting from transfection with the 1-C-6 H and L containing plasmids were unable to retain deglycosylated aggrecan at the cell surface (data not shown). Therefore, I reduced, alkylated and biotinylated aggrecan as described in the materials and methods section 2.1.10.

Subsequently, the 1-C-6 transfected cells were screened by flow cytometry for expression of a new BCR and their ability to retain reduced/alkylated aggrecan at the cell surface (**Figure 4.16**). Results show that although the 1-C-6 transfectants express a new BCR, I could not detect reduced/alkylated aggrecan binding to any of the 1-C-6 transfectants above background levels. As a final screen for the 1-C-6 cells it was important to test their ability to present the reduced/alkylated aggrecan to the T cell hybridoma 192. I have previously determined that A20-1 HS cells do not present this form of aggrecan to T cell hybridoma 192 data not shown. Therefore, for this experiment the macrophage cell line J774 was used as a

positive control. Cells were incubated with graded doses of reduced/alkylated, deglycosylated or glycosylated aggrecan or peptide p84-103 (data shown in **Figure 4.17**). Results show that the 1-C-6 transfectants did not present reduced alkylated, deglycosylated, glycosylated or the peptide form of aggrecan more efficiently than J774. J774 were the only cell line able to efficiently present the reduced /alkylated form of aggrecan to the T cell hybridoma 192 verifying that the 84-103 epitope could be generated from the reduced/alkylated aggrecan and was recognized by the T cell hybridoma 192.

As the specificity of the 1-C-6 BCR is known to be in the G1 and G2 regions it is possible that the 1-C-6 BCR is masking the 84-103 epitope and preventing its generation for presentation to the T cell hybridomas 192 (Caterson 1987). To overcome this problem 1-C-6 transfectants were incubated with graded doses of aggrecan and the G3-specific T cell hybridomas D9 and D11 generated as described in chapter 3 (data shown in **Figure 4.18**). Results show that 1-C-6 cloned transfectants were unable to generate and/or present the epitopes p2373-2387 and p2363-2378 to the G3 specific T cell hybridomas. However, data in **Figure 4.17b** shows that graded doses of the peptide form of the antigen were efficiently presented to both the T cell hybridomas D9 and D11.

To determine whether 1-C-6 was able to mask the 84-103 epitope and prevent its generation for presentation on MHC class II, deglycosylated aggrecan was incubated for 24 h with C71 transfectants, T cell hybridoma 192 and graded doses of soluble 1-C-6 Ig (data shown in **Figure 4.20**). Results show that increasing doses of 1-C-6 Ig had no effect on the efficiency of C71 aggrecan presentation to the T cell hybridoma 192 (data shown in **Figure 4.20**). Therefore, as there was no evidence that the 1-C-6 transfectants were aggrecan-specific; no further experiments were carried out using the 1-C-6 transfected B cells.

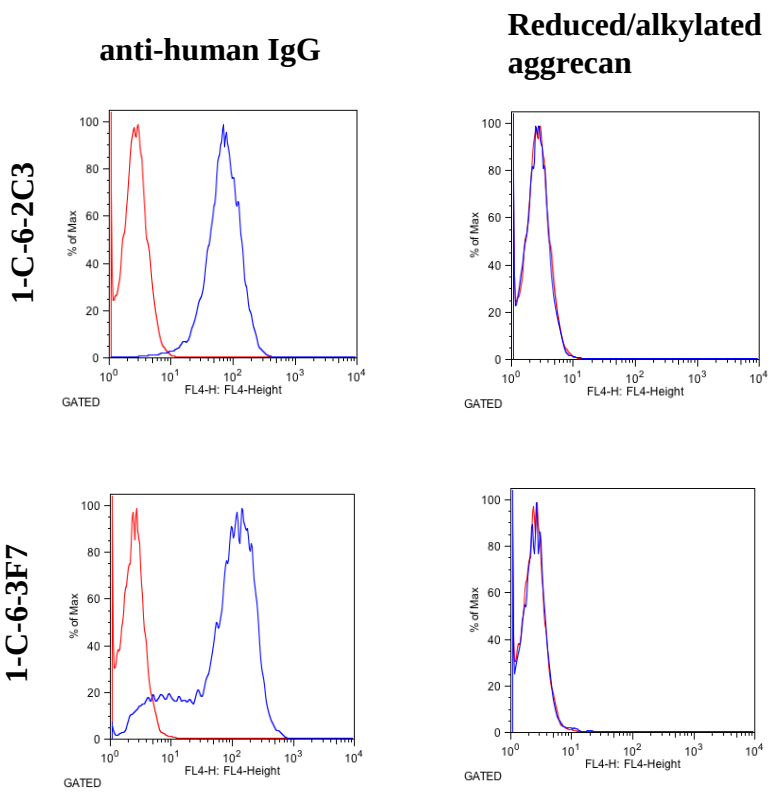


Figure 4.16 Flow cytometric analysis of representative stable A20-1 HS transfectants expressing the 1-C-6 BCR *Staining of gated representative 1-C-6 transfectants cells 1-C-6-2C3 and 1-C-6-3F7. Cells were stained with either biotinylated anti-human IgG (H+L) Ig or reduced/alkylated and biotinylated aggrecan and the secondary reagent streptavidin APC. Red histograms represent unstained cells and the blue histograms represent cells stained for the various markers. All experiments were carried out at 4°C. Data is representative of 3 independent experiments.*

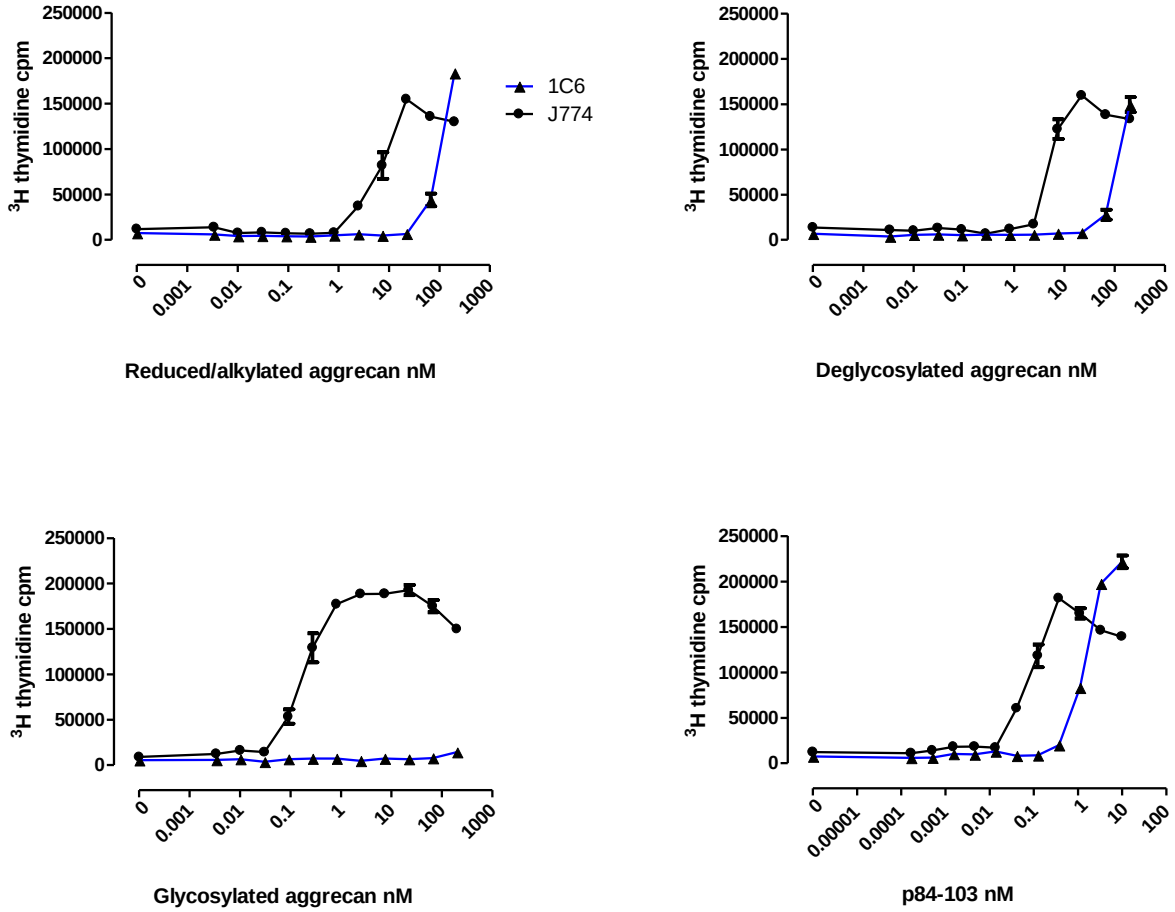


Figure 4.17 Antigen presentation by stable A20-1 HS transfectants expressing the 1-C-6 BCR. *($5x10^4$) 1-C-6 transfectant, or J774 macrophages were cultured with aggrecan$_{84-103}$-specific T cell hybridomas 192 ($5x10^4$) in the presence of graded doses of reduced/alkylated, deglycosylated or glycosylated forms of aggrecan or peptide p84-103 for 24 h. Cells were killed by freezing at -80^{o}C and supernatant was removed for bioassay with CTLL-2 cells. ^{3}H thymidine cpm was added for 18 h and T cell responses were determined and displayed as ^{3}H thymidine cpm +/- SEM. Data are representative of triplicate wells and three independent experiments.*

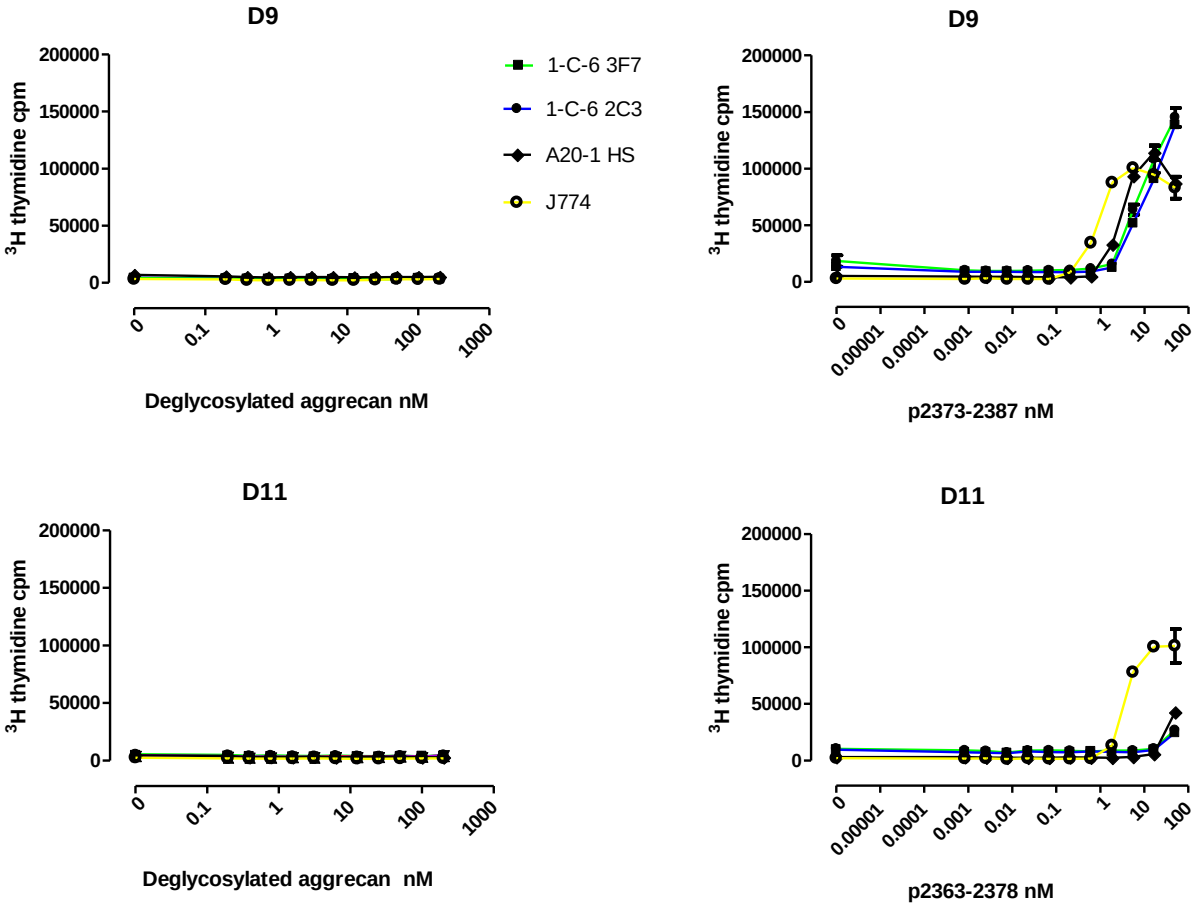


Figure 4.18 Antigen presentation assay with stable A20-1 HS transfectants expressing the 1-C-6 BCR and G3 T cell hybridomas D9 and D11. *1-C-6 cloned stable transfectants 1-C-6 3F7 and 1-C-6 2C3, A20-1 HS or J774 macrophages ($5x10^4$) were cultured with aggrecan$_{2363-2378}$-specific T cell hybridomas D9 or the aggrecan$_{2373-2387}$ -specific T cell hybridomas D11 ($5x10^4$) in the presence of graded doses of aggrecan or peptides p2363-2378 or p2373-2387 for 24 h. Cells were killed by freezing at -80^{o}C and supernatant was removed for bioassay with CTLL-2 cells. ^{3}H thymidine was added for 18 h and T cell responses were determined and displayed as ^{3}H thymidine cpm +/- SEM. Data are representative of triplicate wells and three independent experiments.*

4.2.7.2 C71 Transfectants

I confirmed by flow cytometry that several A20-1 HS transfectants generated with the C71 constructs expressed a new BCR and were able to retain aggrecan at the cell surface (data not shown). Therefore, it was important to verify that the transfectants also presented aggrecan more efficiently than the untransfected A20-1 HS. Several independent C71 transfectants were screened using an antigen presentation assay in the presence of graded doses of aggrecan and peptide p84-103 (representative transfectant data is shown in **Figure 4.19**). Data revealed that the C71 stable transfectants were able to present aggrecan and at least 10^4 fold more efficiently than the untransfected A20-1 HS cells from aggrecan doses of 0.005nM ($p<0.001$). In addition, C71 D1 transfectants did not present the pre-processed peptide form of aggrecan more efficiently than A20-1 HS, indicating that the observed improvement in aggrecan presentation is due to more efficient uptake and processing.

It was hypothesised that if the improvement in presentation of aggrecan to the T cell hybridoma 192 by the C71 transfectants was due to expression of an aggrecan-specific BCR then the observed improvement should be blocked by the addition of the soluble form of the receptor i.e C71 Ig. To test this hypothesis, C71-D1 transfectants were incubated with T cell hybridoma 192 and aggrecan in the presence of graded doses of C71 Ig. Controls wells included C71-D1 incubated with aggrecan and T cell hybridoma 192 in the absence of C71 Ig and J774 macrophages incubated with either aggrecan alone or in the presence of graded doses of C71 Ig and T cell hybridoma 192 (data shown in **Figure 4.20**) Results show that presentation of aggrecan by C71-D1 is blocked by the presence of soluble C71. However this is specific to the C71-D1 transfectants as the C71 Ig had no effect on the ability of J774 macrophages to efficiently present aggrecan to the T cell hybridoma 192. C71-D1 transfectants incubated with aggrecan and T cell hybridoma 192 in the absence of C71 Ig confirmed that the observed reduction in presentation was due to the addition of C71 Ig. Furthermore, the addition of isotype control 1-C-6 Ig (IgG1) did not block presentation of aggrecan by the C71-D1 transfectant confirming that the observed attenuation of aggrecan presentation by the C71-D1 transfectants is specific to C71 Ig.

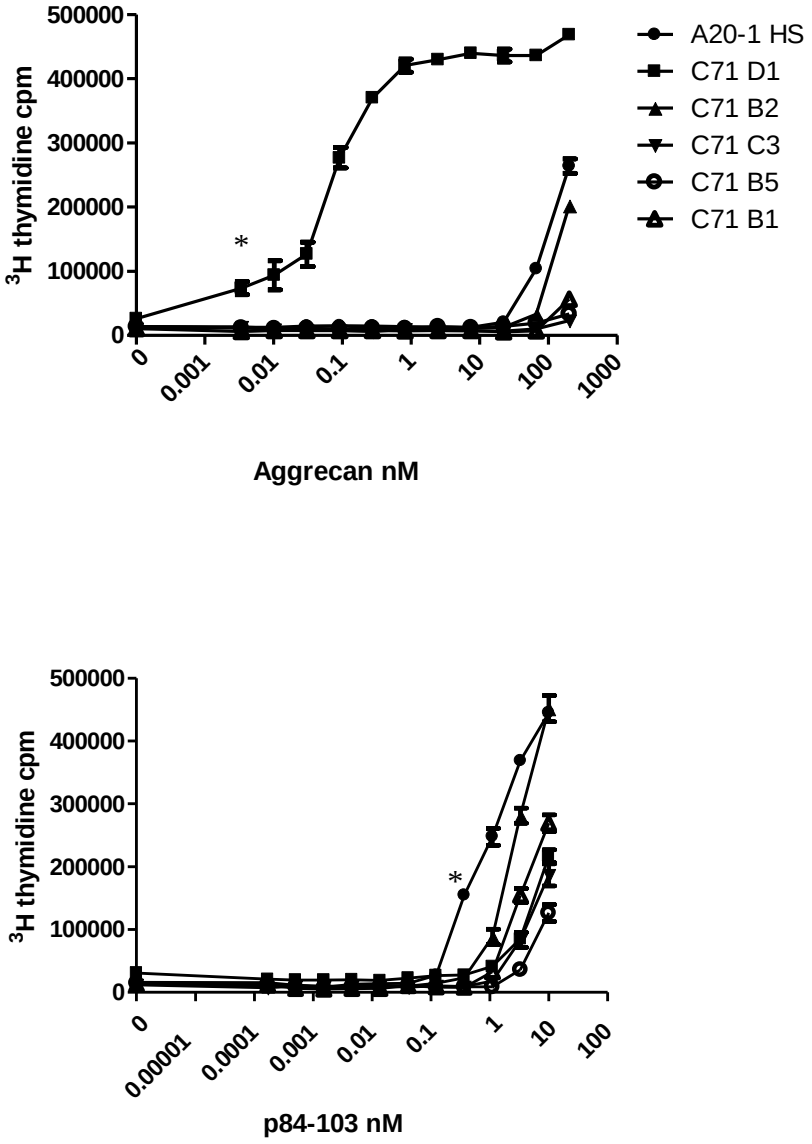


Figure 4.19 Antigen presentation assay with C71 stable transfectants and T cell hybridomas 192. *C71 stable transfectants and A20-1 HS controls were expanded and cultured ($5x10^4$) with aggrecan$_{84-103}$-specific T cell hybridoma 192 ($5x10^4$) in the presence of graded doses of aggrecan or peptide for 24 h. Cells were killed by freezing at -80^{o}C and supernatant was removed for bioassay with CTLL-2 cells. ^{3}H thymidine was added for 18 h and T cell responses were determined and displayed as ^{3}H thymidine cpm +/- SEM. Data are representative of triplicate wells and three independent experiments.*
The asterix represents statistical significance determined by one-way ANOVA where $p<0.05$.

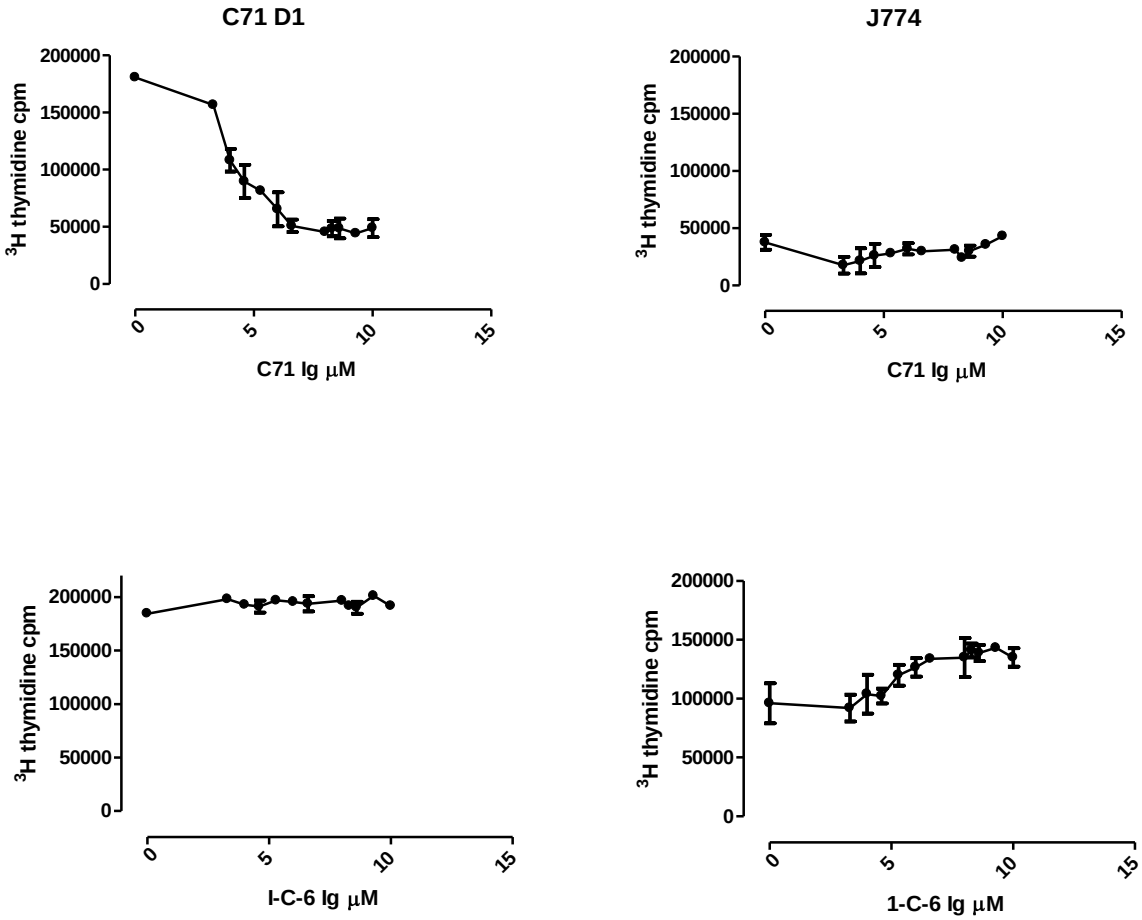


Figure 4.20 Antigen presentation assay with C71 D1 stable transfectants and J774 macrophages in the presence of graded doses of C71 or 1-C-6 Ig. *C71 D1 transfectants or J774 macrophages were incubated with 2 nM deglycosylated aggrecan and the aggrecan$_{84-103}$-specific T cell hybridomas 192 (5x10^4) in the presence of graded doses of 1-C-6 Ig for 24 h. Cells were killed by freezing at -80^{o}C and supernatant was removed for bioassay with CTLL-2 cells. ^{3}H thymidine cpm was added for 18 h and T cell responses were determined and displayed as ^{3}H thymidine cpm +/- SEM. Data are representative of triplicate wells and two independent experiments.*

The transfectant C71-D1 was chosen as a representative aggrecan-specific population and single cell cloned to ensure homogeneity.

Subsequently, resultant C71 D1 clones and A20-1 HS controls were re-screened by flow cytometry for expression of the C71 chimeric BCR, cell surface aggrecan retention, MHC class II expression and Fc receptor expression in the presence or absence of Fc block (data shown in **Figure 4.21**). C71 clones C71-4C5 and C71-5F10 were consistently positive for the expression of a new chimeric BCR and their ability to bind aggrecan. MHC class II receptor expression was at similar levels on both C71 5F10 and A20-1 HS cells. However, expression was 4 fold greater by the C71 4C5 cells. The Fc receptors were efficiently blocked by 2.4G2 block confirming that positive staining was not due to nonspecific Fc receptor binding. Therefore, C71-4C5 and C71-5F10 were chosen as representative aggrecan-specific B cell lines for further investigation.

To verify that the improvement in presentation by C71 transfectants C71-4C5 and C71-5F10 was specific to presentation of aggrecan and not an irrelevant antigen, C71-4C5 and C71-5F10 cells were screened in an antigen presentation assay with deglycosylated aggrecan, p84-103 and TTCF. The TTCF-specific B cells A20 3A5 (Knight, Lucocq et al. 1997)) were used as a positive control and presentation to the T cell hybridomas 192 and the TTCF-specific T cell hybridoma MC-52 generated by M Ciechomska in our laboratory were examined. APC and T cell hybridomas MC-52 or 192 were incubated for 24 h in the presence of graded doses of TTCF, deglycosylated aggrecan or p84-103. Data shown in **Figure 4.22a** revealed that C71-4C5 and C71-5F10 B cells are able to present aggrecan to the T cell hybridoma 192 at least 10^5 times more efficiently than the TTCF specific B cell A20 3A5 or A20-1 HS at aggrecan doses from 0.005nM ($p<0.01$). Results in **Figure 4.22b** shows that the C71-4C5 and C71-5F10 cells do not present p84-103 any more efficiently than the A20 3A5 or A20-1 HS cells. Importantly, **Figure 4.22c** shows that C71-4C5, C71-5F10 and A20-1 HS were unable to present TTCF to TTCF specific-T cells MC 52, even at doses of up to 100 nM. However, TTCF-specific B cells A20 3A5 were able to present TTCF at doses above 0.5 nM ($p<0.01$). These data verify that the C71 4C5 and 5F10 B cells are indeed antigen-specific and are highly efficient at the uptake processing and presentation of aggrecan for presentation of the 84-103 epitope to T cell hybridoma 192.

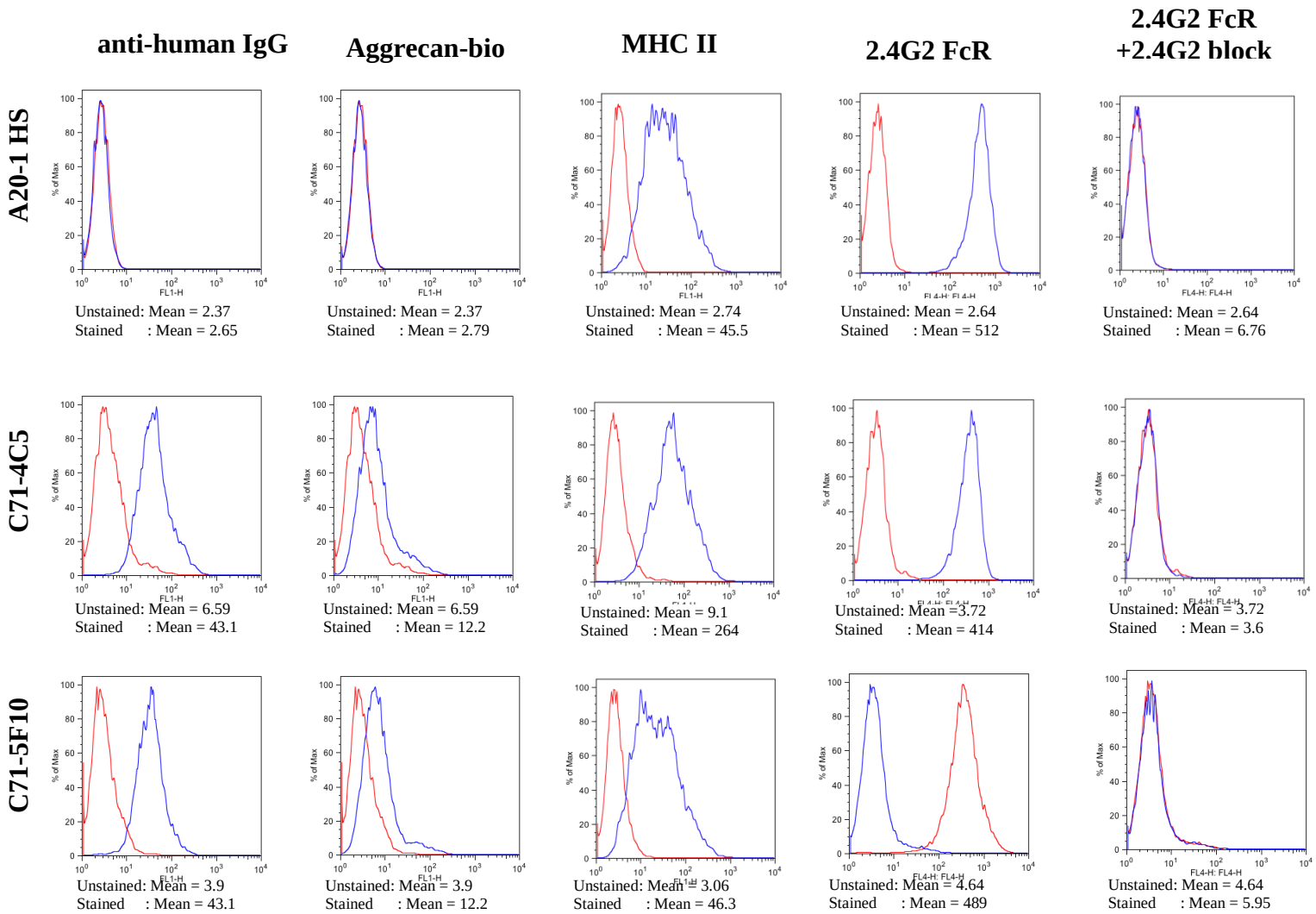


Figure 4.21 Flow cytometry with C71 clones to show BCR expression and aggrecan retention. *Staining of A20-1 HS or C71 clones C71-4C5 and C71-5F10 with either biotinylated anti-human IgG (H+L) Ig or biotinylated aggrecan and the secondary reagent streptavidin FITC. Anti-MHC II FITC 2.4G2 biotin followed by secondary reagent streptavidin APC. Red histograms represent unstained cells and the blue histograms represent cells stained for the various markers. All experiments were carried out at 4°C in the presence of Fc block unless otherwise stated. Mean fluorescence intensity values are shown below each histogram. Data is representative of 3 independent experiments.*

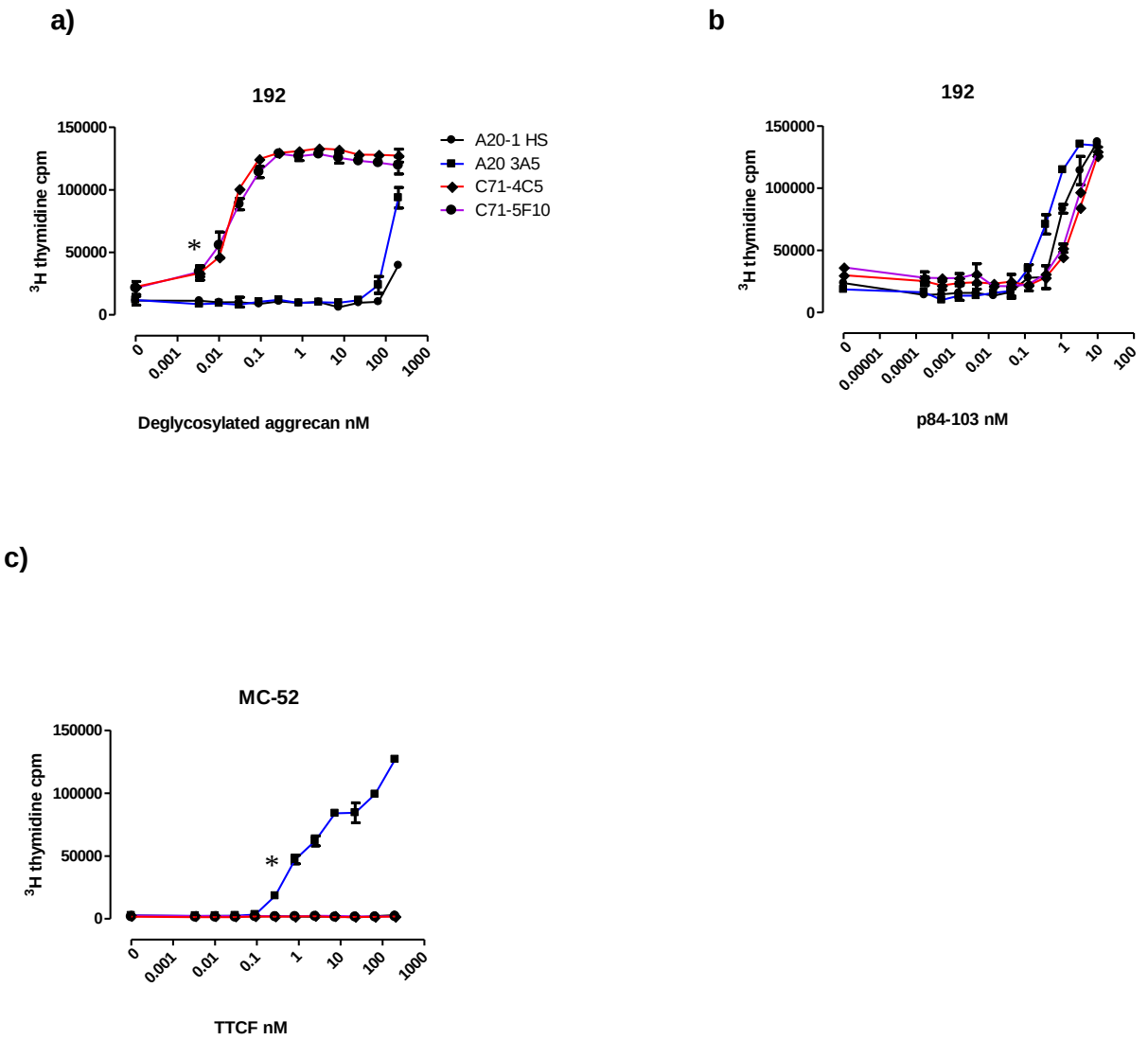


Figure 4.22 Antigen presentation assay with C71 clones and the T cell hybridomas 192 and MC-52. *C71 clones C71-4C5 and C71-5F10, A20-1 HS or the TTCF-specific B cell hybridomas A20 3A5 were cultured with aggrecan$_{84-103}$-specific T cell hybridoma 192 (5x10^4) in the presence of graded doses of a) aggrecan or b) peptide p84-103 for 24 h. C71 clones C71-4C5 and C71-5F10, A20-1 HS or the TTCF-specific B cell hybridomas A20 3A5 cultured with c) TTCF-specific T cell hybridomas MC-52 (5x10^4) in the presence of graded doses of TTCF for 24 h, Cells were killed by freezing at -80^{o}C and supernatant was removed for bioassay with CTLL-2 cells. ^{3}H thymidine was added for 18 h and T cell responses were determined and displayed as ^{3}H thymidine cpm +/- SEM. Data are representative of triplicate wells and three independent experiments. The asterix represents statistical significance determined by one-way ANOVA where p<0.05.*

4.3 Discussion

The aim of this chapter was to generate plasmid constructs containing cDNA encoding aggrecan-specific BCR H and L chains from two independent anti-aggrecan monoclonal antibodies (C71 and 1-C-6). Those plasmids were then to be transfected into the B cell line A20-1 HS in an attempt to generate aggrecan-specific B cells. Results confirm that I have successfully generated both C71 and 1-C-6 H and L chain plasmids and confirmed their cell surface expression following transfection into A20-1 HS cells. The new B cell lines have been characterised by flow cytometry, to verify the cell surface expression of a chimeric mouse/human BCR and their ability to retain biotinylated aggrecan at the cell surface. In addition, the transfectants were screened using antigen presentation assays to observe how efficiently they could present aggrecan to aggrecan-specific T cell hybridomas. The results are discussed below.

4.3.1 H and L chain plasmid construction

The H and L chain plasmids were constructed by ligating cDNAs predicted to encode aggrecan-specific VH and VL regions from the murine aggrecan-specific B cell hybridomas C71 and 1-C-6 with cDNAs encoding human H and L chain C regions (Knight, Lucocq et al. 1997) derived from the human TTCF-specific EBV transformed 11.3 (Lanzavecchia 1985).
The sequences encoding the H and L chain V regions from C71 and 1-C-6 regions were amplified by 5' RACE. This technique allows the amplification of DNA where the 5' region is unsequenced and has successfully been used previously for the amplification of cDNAs encoding VH and VL from various monoclonal antibodies. Following successful amplification of PCR products of the predicted size, cDNAs encoding both the 1-C-6 and C71 VH and VL regions were transformed into competent *E.coli* and multiple independent clones containing the amplified cDNA were sequenced and aligned with other published V region sequences.

These data showed that 5 independently isolated C71 and 1-C-6 clones contained identical sequences. In addition, similarities were observed between the structural framework regions of the C71 and 1-C-6 V H and L chains and previously published sequences. However, I did not observe any sequence similarity between the leader sequence of the C71 VL region and the leader sequence of 1-C-6 or any of the other published VL sequences shown in **Figure 4.7,** despite confirmation that 5 C71 miniprep clones contained this identical sequence. As the

leader sequence is required for endoplasmic reticulum (ER) insertion of the C71 VL translation product, I was concerned that any errors in the amplification of this region of the cDNA would prevent its correct translocation into the ER and the subsequent assembly of the C71 BCR (Bergman and Kuehl 1979; Wilkinson, Regnacq et al. 1997). However, later results confirmed that the observed improvement in aggrecan presentation by the C71 transfected clones required transfection of the A20-1 HS cells with both the C71 H and L chains; transfection with plasmids containing the H or L chain only did not improve presentation. These data indicate that the C71 L chain had successfully translocated into the ER, assembled with its corresponding H chain and was expressed on the cell surface of the A20-1 HS cells.

4.3.2 Transfection

Following several unsuccessful attempts using other methods, the Amaxa nucleofector system was adopted to transfect plasmids encoding the C71 and 1-C-6 BCR into A20-1 HS cells. Subsequently, flow cytometry was used to detect surface expression of the novel C71 or 1-C-6 chimeric BCR. Anti-human IgG (H and L) Fab^2 biotin conjugated antibodies and streptavidin FITC were used to detect human H and L C region expression on A20-1 HS transfected with the C71 or 1-C-6 H and L chain constructs. As A20-1 HS expresses an IgG2a BCR (of unknown specificity), expression of a BCR containing a human C region was used to distinguish transfected from endogenous BCR. Anti-human κ L chain antibodies were initially used to detect the expression of the introduced L chain. However, these antibodies cross-reacted with the endogenous mouse κ L chain. Therefore expression of the C71 or 1-C-6 light chain by A20-1 HS could not be verified in this way. As I could not verify the expression of both the C71 or 1-C-6 H and L chains individually it was not possible to determine from this data if the C71 or 1-C-6 H chain had paired with the endogenous A20-1 HS L chain or if the C71 or 1-C-6 L chain had paired with the endogenous H chain. I determined this was not the case by transfecting the C71 H chain plasmid alone and showing that these single-chain transfectants did not bind aggrecan at the cell surface or show improved presentation of aggrecan. However, this does not exclude the possibility that the C71 H and L transfected A20-1 HS express a variety of receptors paired with a combination of transfected and endogenous H and L chains.

Observations that A20-1 HS cells transfected with the C71 H chain alone showed no H chain cell surface expression by flow cytometry in the absence of co-transfection with the C71 L chain suggested that the H chain did not successfully pair with the endogenous A20-1 HS L chain and is retained in the ER. However, I did observe a small increase in the mean

fluorescence intensity for A20-1 HS transfected with the C71 L chain only and an increase in the mean fluorescence intensity following incubation with biotinylated aggrecan and streptavidin FITC. This data suggests that a small percentage of C71 L chains may pair with the endogenous A20-1 HS H chain but in the absence of any observed improvement in aggrecan presentation this should distract from using these transfectants as a model for aggrecan-specific uptake and processing by B cells. It is possible that the transfected A20-1 HS cells express a variety of mouse and mouse/human BCR on their cell surface. Further experiments to analyze this would have been to specifically immunoprecipitate murine Ig and Western blot for associated human-Ig.

Inevitably, the A20-1 HS transfectants are not a true reflection of an aggrecan-specific B cells *in vivo*. However, they are an excellent model with which to study the effects of BCR mediated uptake and processing and presentation of the candidate joint autoantigen aggrecan. In addition, A20-1 cells are representative of activated B cells and have been used by several investigators as a model of B cell antigen presentation (Shimonkevitz, Kappler et al. 1983; Brennan, Negroiu et al. 1995; Aoi, Nakano et al. 1997). Therefore, aggrecan-specific A20-1 HS transfectants can be used to examine the unknown processing and presentation events of the candidate joint autoantigen aggrecan by antigen-specific B cells and as a comparison to the processing and presentation by other APC.

In order to study the activation requirements, proliferation and cytokine production by autoreactive aggrecan-specific B cells, a more relevant model would be to transfect *ex vivo* B cells. However, several unsuccessful attempts were made at transfecting freshly isolated B cells, including the transfection of LPS activated ex-vivo B cells (Liu, Meckel et al. 2010). Ultimately, the generation of a BCR transgenic mouse using the C71 H and L chain constructs would enable me to identify the relevance of B cell mediated aggrecan presentation on T cell activation and PGIA development. In addition, aggrecan-specific BCR transgenic mice would also enable the study of the tolerance mechanisms involved in regulating the development of autoreactive aggrecan-specific B cells and the effects of aggrecan-specific B cell transfer on PGIA induction (Goodnow 1992) (Gay, Saunders et al. 1993). Thus, generation of a BCR transgenic mouse maybe an important next step in the future development of this study. Therefore for the purpose of this study the A20-1 HS stably transfected with the C71 and 1-C-6 H and L chain constructs were used for further

experiments to generate preliminary data on aggrecan processing and presentation by autoreactive aggrecan-specific B cells.

4.3.3 I-C-6 Cell lines.

Several stable cell lines were generated from A20-1HS transfected with plasmids encoding the 1-C-6 BCR. These cell lines were screened for the expression of a new BCR containing a human C region, for their ability to bind deglycosylated and reduced/alkylated forms of aggrecan at their surface and to present both forms of aggrecan to the T cell hybridomas 192. 1-C-6 transfectants were consistently positive for the expression of a BCR containing a human C region. However, I did not detect any binding of either forms of aggrecan at the cell surface nor did I observe any improvement in presentation of aggrecan in comparison to untransfected A20-1 HS.

Possible reasons for these findings may include

- 1-C-6 BCR may contain errors in the cDNA sequence following either V region amplification or H or L chain plasmid construction. As the 1-C-6 variable sequence has not been published I can only presume from the 12 miniprep clones sequenced that their identical sequence similarity is correct. In addition, and in contrast to the C71 sequence, both the 1-C-6 VH and VL regions contained a high percentage of sequence similarity with other published V regions. It is also possible, that there are errors in the construction of the H and L chain plasmids. However, sequencing has confirmed that the 1-C-6 V regions appear to be joined to the human Ig C regions making this unlikely.
- As there is no evidence that the 1-C-6 BCR binds with sufficient efficacy to improve presentation, it is possible that it also does not bind efficiently enough to be detected by flow cytometry. The binding of 1-C-6 Ig to reduced and alkylated aggrecan is shown in the ELISA data in **Figure 4.1**. However, an ELISA is a very sensitive assay where aggrecan is bound in a concentrated form to an ELISA plate facilitating binding at a low affinity Ig. Indeed sensitivity of ELISA to detect IgG levels was demonstrated by Lofgren and colleagues and ranges of 16ng-32μg/ml of antibodies were detected which directly correlated with affinity of the Ig (Lofgren, Dhandapani et al. 2007). 1-C-6 was detected to bind reduced/alkylated aggrecan in an ELISA at concentrations ranging from >1 μg/ml. Therefore, it is possible that 1-C-6 is a low

affinity Ig and the 1-C-6 BCR is incapable of binding sufficient reduce/alkylated aggrecan to improve uptake and processing for presentation to the T cell hybridoma 192, yet is still positively detected in a more sensitive ELISA. Batista and colleagues determined that 10^{-6}M is the lowest affinity HEL specific BCR needs to be to show an improvement in the presentation to HEL-specific T cell hybridomas (Batista and Neuberger 1998). Therefore, as described previously, competition ELISA to determine the affinity of the 1-C-6 Ig may clarify whether its affinity dictates its lack of improved aggrecan presentation and surface binding.

- It is possible that reduction and alkylation of the aggrecan may affect the processing of 84-103. Therefore, an improvement in presentation may not be detected as the 84-103 epitope is not optimally generated. C71 transfectants did not present reduced/alkylated aggrecan more efficiently than untransfected A20-1 HS. This could be due to the C71 BCR no longer recognising its epitope due to destruction of conformation or that this represents the optimal generation and presentation of the 84-103 epitope by B cells when aggrecan is in its reduced and alkylated form. Differences in the ability of other APC to present aggrecan more or less efficiently when used in a different form was shown later by macrophages that present glycosylated aggrecan 10^2-fold more efficiently than the deglycosylated form (**Figure 5.3**). Therefore altering the aggrecan structure may have different effects on processing the 84-103 epitope.
- It is possible that binding of the 1-C-6 BCR prevents the processing of the 84-103 epitope. However, experiments involving pre-incubation of deglycosylated aggrecan with 1-C-6 Ig did not affect the ability of the C71 transfectants to efficiently present it to the T cell hybridoma 192. In addition, reduced/alkylated aggrecan was efficiently presented by the J774 macrophage cell line to the T cell hybridomas 192. However, this data only confirms that the 1-C-6 Ig has no detrimental effects on reduced/alkylated aggrecan processing by macrophages and not by B cells. As there is no control for improved presentation of reduced/alkylated aggrecan by B cells it is not possible to determine whether 1-C-6 has a detrimental effect and to draw any firm conclusions on its effects on 84-103 epitope generation by B cells.
- There is no evidence that the 1-C-6 Ig efficiently recognises the deglycosylated form of aggrecan. Therefore it is reasonable to expect that I would not see an improvement in its presentation to the T cell hybridomas 192.

In conclusion, these data show no evidence for the 1-C-6 transfectant binding or presenting aggrecan to the T cell hybridomas 192 used in this study. Therefore for the next chapter all mechanistic processing and comparison with other APC experiments were carried out using the transfectants C71-4C5 and C71-5F10.

4.3.4 C71 cell lines

C71 aggrecan-specific B cells were generated and single-cell cloned to ensure homogeneity and to identify any potential differences amongst efficient presentation of aggrecan to T cell hybridoma 192. Subsequently, clones C71-4C5 and C71-5F10 were chosen as representative aggrecan-specific B cells for further studies. Antigen presentation assays confirmed that these clones presented aggrecan 10^4-10^5 fold more efficiently than the untransfected A20-1 HS whilst showing similar efficiency of p84-103 presentation (**Figure 4.22**). This data indicates that aggrecan is taken up and processed more efficiently by the C71-4C5 and C71-5F10 cells in comparison to the A20-1 HS and this improvement is not due to the ability of the C71 cells to present peptide/MHC class II complexes more efficiently.

Flow cytometry confirmed the expression of the C71 BCR on the cell surface of the clones. However, only low level biotinylated, aggrecan binding was observed by flow cytometry and the reasons for this are not fully understood. Flow cytometry experiments were performed on cells incubated on ice to prevent uptake of aggrecan once it had been bound by the C71 aggrecan-specific BCR. Therefore, once the biotinylated aggrecan had bound to the BCR it should have been retained at the cell surface and should be detectable by flow cytometry following incubation with streptavidin FITC. This data suggests that either the BCR binding site of aggrecan is masked by the biotin molecules or that the BCR is of low affinity. However, experiments to verify that biotinylated aggrecan was efficiently processed and presented by C71 transfectants were carried out (data not shown) and results showed that biotinylation of aggrecan had no effect on the ability of the C71 transfectants to process and present aggrecan to the T cell hybridoma 192. Further experiments to determine the affinity of the C71 BCR could have been used such as competition ELISA where affinity of C71 antibody is determined by the addition of various concentrations of aggrecan required to compete with the binding of biotinylated aggrecan to the C71 antibody bound to the ELISA plate (Friguet, Chaffotte et al. 1985; Batista and Neuberger 1998).

The aggrecan epitope detected by the C71 transfectants is still unknown. To clarify this epitope mapping experiments such as Western blotting with MMP digested aggrecan to produce aggrecan fragments of known molecular weight followed by incubation with the C71 Ig may have confirmed C71 specificity. Subsequently, I could have determined whether the C71 BCR a) masks the (G1 domain) 84-103 epitope and prevents its destruction and therefore improves its presentation to the T cell hybridoma 192 or b) whether it binds to a region distinct from 84-103 and improves uptake, leaving 84-103 for processing and optimal peptide generation.

Attempts to generate aggrecan-specific T cell hybridomas that recognised alternative epitopes within the G3 domain were unsuccessful. I successfully, generated two G3 specific T cell hybridomas D9 and D11. However, in antigen presentation assays using A20-1 HS, C71-4C5 or C71-5F10 as APC they were only able to recognise synthetic peptide containing their G3 specific epitopes and not those derived from deglycosylated aggrecan. This implies that the G3 epitopes are either cryptic and not generated by the B cells, possibly due to destructive processing or that the T cells only recognise their epitope when presented in a particular MHC class II /peptide conformer such as those described for type B T cells that only recognise a peptide form of antigen (Pu, Lovitch et al. 2004). Indeed, other work in our laboratory using the T cell hybridomas D9 and D11 confirmed that the macrophage cell line J774 was also only able to present peptide and not deglycosylated aggrecan to the G3-specific T cell hybridomas (Andrew Quirk unpublished data).

In summary, C71-5F10 and C71-4C5 consistently presented the candidate joint autoantigen aggrecan at least 10^4 fold more efficiently than the untransfected A20-1 HS cells and are therefore good models with which to study aggrecan-specific processing and presentation by B cells in comparison to other APC.

4.4 Summary

- Plasmid constructs were generated containing cDNAs predicted to encode aggrecan-specific H and L chains from two different aggrecan-specific B cell hybridomas C71 and 1-C-6.
- Cell lines were generated by transfecting A20-1 HS with plasmid constructs containing aggrecan-specific H and L chain cDNA.
- Expression of new chimeric human/mouse C71/1-C-6 BCRs was confirmed by flow cytometry.
- Flow cytometry confirmed that binding of biotinylated aggrecan was observed by C71 but not by 1-C-6 transfectants.
- C71 but not 1-C-6 transfectants showed a 10^4-10^5 fold improvement in aggrecan presentation in comparison to that of untransfected A20-1 HS.
- Single cell clones C71-4C5 and C71-5F10 were generated from the parental C71 D1 stable cell line population. These clones were characterised to verify aggrecan-specific BCR expression, aggrecan binding and an improvement in aggrecan presentation and were chosen for studies into the molecular mechanisms by which aggrecan-specific B cells process and present aggrecan to T cells.

5. Comparison of aggrecan processing and presentation by aggrecan-specific B cell transfectants, bone marrow derived dendritic cells and macrophages.

5.1 Introduction

The aim of this chapter was to study the mechanisms by which aggrecan is processed and presented by the aggrecan-specific B cells (C71-4C5 and C71-5F10) generated in Chapter 4 and to make comparisons between this and aggrecan processing and presentation by two other major APC, macrophages and dendritic cells.

Initial studies presented in this chapter verified that naïve splenic B cells were poor at presenting aggrecan to T cell hybridoma 192 in comparison to presentation by dendritic cells and macrophages. I wanted to elucidate whether there was a novel role for aggrecan-specific B cells in the processing and presentation of aggrecan. Therefore, I compared the kinetics of aggrecan presentation, the ability to present different forms of aggrecan, and differences in downstream processing events by the three APC.

Initial studies involved a comparison of presentation of different forms of aggrecan by the APC. As previously mentioned in the Introduction **Section 1.7.2**, aggrecan undergoes various proteolytic cleavage events and carbohydrate modifications during the course of RA progression (Ortutay, Polgar et al. 2003; Larsson, Lohmander et al. 2009). Furthermore, several studies have demonstrated that chondroitin and keratin sulphate side chains must be removed from aggrecan in order for it to be used for the induction of PGIA in mice and to generate appropriate T cell responses in humans during *in vitro* recall responses (Leroux, Guerassimov et al. 1996; Li, Zhang et al. 2000; Glant and Mikecz 2004). As both the immunogenicity and post-translational form of aggrecan is likely to be heterogeneous within the joint, I examined T cell hybridoma 192 responses to glycosylated, deglycosylated and p84-103 following presentation by the different APC.

Various studies have demonstrated that antigen-specific B cells are efficient APC both when and where B cell numbers are low (Lanzavecchia 1985; Byersdorfer, Dipaolo et al. 2004; Catron, Itano et al. 2004; Pape, Catron et al. 2007). Therefore, I wanted to investigate the nature of this requirement and the mechanisms involved in the presentation of aggrecan by

antigen-specific B cells compared to that seen with dendritic cells and macrophages. To do this I initially compared both the relative number of APC required to efficiently present aggrecan to the T cell hybridomas 192 and the concentration of aggrecan or p84-103 required to generate a T cell response. In addition, as previous studies have also shown that B cells are involved in the activation of T cells early on in the immune response (Byersdorfer, Dipaolo et al. 2004; Catron, Itano et al. 2004; Pape, Catron et al. 2007), I carried out experiments to determine the time kinetics of aggrecan processing by aggrecan-specific B cells C71-4C5 and C71-5F10 in comparison to other APC.

The classical pathway of MHC class II restricted antigen presentation involves a series of intracellular events that result in antigen degradation and the generation of antigenic determinants for loading into the peptide binding groove of newly synthesised MHC class II within the MIIC (Neefjes, Stollorz et al. 1990; Robinson and Delvig 2002; Watts 2004). MHC class II α and β chains assemble in the ER along with the invariant chain (li). Newly generated MHC class II molecules are transported from the ER to MIIC assisted by the li which is sequentially degraded by endosomal cysteine and aspartyl proteinases from the entire li chain to fragments lip22, lip10 and finally the CLIP fragment which remains bound to the peptide binding groove (Roche and Cresswell 1990; Cresswell 1992; Honey and Rudensky 2003). Subsequent release of CLIP and loading of antigenic peptides into the binding groove of MHC class II is catalyzed by the MHC class like molecule HLA-DM prior to MHC class II/ peptide cell surface presentation by APC (Denzin and Cresswell 1995). An alternate pathway involves antigen targeted to early endosomes for loading onto MHC class II molecules that are recycled from the cell surface (Pinet, Vergelli et al. 1995; Lindner and Unanue 1996; Delvig and Robinson 1998). This pathway is independent of newly synthesised MHC class II and the li chain. Cycloheximide blocks protein synthesis (St-Pierre and Watts 1990) and Brefeldin A prevents egress from the ER (Orci, Tagaya et al. 1991). Therefore, both inhibitors should block the classical pathway of antigen presentation by preventing the synthesis and transport of MHC class II molecules and should have no effect on the presentation of peptides loaded via the recycling pathway. To examine which pathways are involved in aggrecan presentation, the different inhibitors of protein synthesis and Golgi transport were used and their effects on presentation to T cell hybridoma 192 was examined.
Early studies have shown that raising the pH of endosomal compartments with ammonium chloride can inhibit the processing and presentation of several antigenic epitopes by macrophages (Ziegler and Unanue 1981), dendritic cells (Svensson, Stockinger et al. 1997)

and B cells (Casten, Lakey et al. 1985). In most cases, efficient antigen processing requires both the correct pH and the activation of the appropriate intracellular enzymes. Although intracellular enzymes are present in all endocytic compartments, most require the low pH found in late endosomes and lysosomes for removal of the N terminal pro-domain and their subsequent activation (Riese and Chapman 2000). Furthermore, HLA-DM peptide exchange is itself regulated by HLA-DO and although mechanisms of regulation are not entirely known it is thought to be pH dependent, where HLA-DO represses peptide exchange by HLA-DM in early endosomes, allowing exchange later in the pathway when pH levels are lower (van Ham, van Lith et al. 2000). HLA-DO levels differ between B cells, dendritic cells and macrophages and therefore may modulate aggrecan processing differently in the different APC (Hornell, Burster et al. 2006). To examine the requirement of endosomal pH for efficient aggrecan processing and presentation by the different APC, C71-4C5, C71-5F10, macrophages and dendritic cells were incubated with ammonium chloride to lower endosomal pH and the effects on aggrecan presentation to T cell hybridoma 192 was examined.

The functions of endosomal proteinases include the degradation of protein antigens and antigen receptors, the generation and trimming of peptide fragments for MHC class II loading and the degradation of the li chain (Honey and Rudensky 2003). The latter function is thought to primarily involve cysteine proteinases such as cathepsin S and asparagine endopeptidase (AEP) (Shi, Villadangos et al. 1999; Manoury, Mazzeo et al. 2003). MHC class II molecules are present in most endocytic compartments and can be loaded with peptide at various stages in the pathway (Robinson and Delvig 2002). Previous studies have shown that the route of antigen uptake and the type of APC can have differential effects on how antigen is processed (Nakagawa, Roth et al. 1998; Shi, Bryant et al. 2000). Therefore, it is possible that different APC may have different requirements for the generation of the aggrecan epitope 84-103. Possible differences in aggrecan processing include the route of uptake (this could determine which intracellular compartment aggrecan is targeted to), different proteinase expression and pH dependence for proteinase activation and HLA-DM control by HLA-DO (Denzin, Fallas et al. 2005). In addition, FcR or BCR uptake of antigen may have an effect on how the protein is degraded by sequestering various parts of the antigen from proteolysis (Simitsek, Campbell et al. 1995).

I have used inhibitors of the major proteinase families (cysteine, serine, aspartyl and metallo) to identify the proteinase requirements of different APC for the efficient generation and presentation of the aggrecan 84-103 epitope to the T cell hybridoma 192. Inhibitor studies have helped to elucidate which enzymes were involved in the generation and MHC class II loading of 84-103 and to determine any differences in aggrecan processing by aggrecan-specific B cells, dendritic cells and macrophages.
Elucidating the mechanisms involved in aggrecan processing by different APC may identify novel targets with which to prevent the initiation of an adaptive response against this candidate joint autoantigen.

Most of the antigen presentation assays performed in this and earlier chapters have involved T cell responses by the T cell hybridoma 192. In order to evaluate the role of the different APC in activating aggrecan-specific T cells I isolated T cells from TCR-5/4E8-tg mice that express a TCR specific for the aggrecan epitope 84-103. TCR-5/4E8-tg mice develop PGIA following immunisation with deglycosylated aggrecan and T cells isolated from TCR-5/4E8-tg mice can induce disease when adoptively transferred along with deglycosylated aggrecan into MHC class II matched SCID mice (Berlo, Guichelaar et al. 2006). PGIA is thought to be a Th1- mediated disease dependent on the cytokine IFNγ (Finnegan, Grusby et al. 2002). In addition, B cells have been implicated as important mediators of this IFNγ response in the PGIA model. Therefore, I was interested in determining whether *in vitro* presentation of aggrecan by aggrecan-specific B cells C71-4C5 and C71-5F10 is able to generate an IFNγ response when incubated with aggrecan and the aggrecan-specific T cells 5/4E8 and how this compares to IFNγ response following presentation by other APC.

5.1.2 The aims of this chapter:

- To compare aggrecan presentation to T cell hybridomas 192 and T cells isolated from TCR-5/4E8 transgenic mice by aggrecan-specific B cell clones C71-4C5 and C71-5F10 to that seen with other APC.
- To determine mechanisms of aggrecan-processing by aggrecan-specific B cell clones C71-4C5 and C71-5F10 compared to other APC.
- To determine patterns of cytokine production by T cells in response to aggrecan-presentation aggrecan-specific B cell clones C71-4C5 and C71-5F10 compared to other APC.

5.2 Results

5.2.1 Phenotype of bone marrow macrophages and bone marrow dendritic cells

Bone marrow precursor cells were isolated from BALB/c mice and differentiated into either bone marrow derived macrophages or bone marrow derived dendritic cells by culturing the cells in media containing MCSF or GMCSF respectively. Macrophages were activated overnight by the addition of 100 ng/ml IFNγ to the culture media. Macrophages and dendritic cells were screened by flow cytometry for macrophage cell surface marker F4/80, dendritic cell surface marker CD11c and MHC class II. To verify that positive staining was not due to antibodies binding to cell surface Fc receptors both macrophages and dendritic cells were stained with biotinylated 2.4G2 Fc receptor antibodies and streptavidin APC both in the presence and absence of (75 μg/ml) 2.4G2 Fc block (**Figure 5.1**). As expected macrophages expressed F4/80, MHC class II and not the dendritic cell marker CD11c and dendritic cells expressed CD11c, MHC class II and not the macrophage cell surface marker F4/80. Positive staining with biotinylated 2.4G2 Fc receptor antibodies and streptavidin APC was blocked in the presence of 75 μg/ml 2.4G2 Fc block.

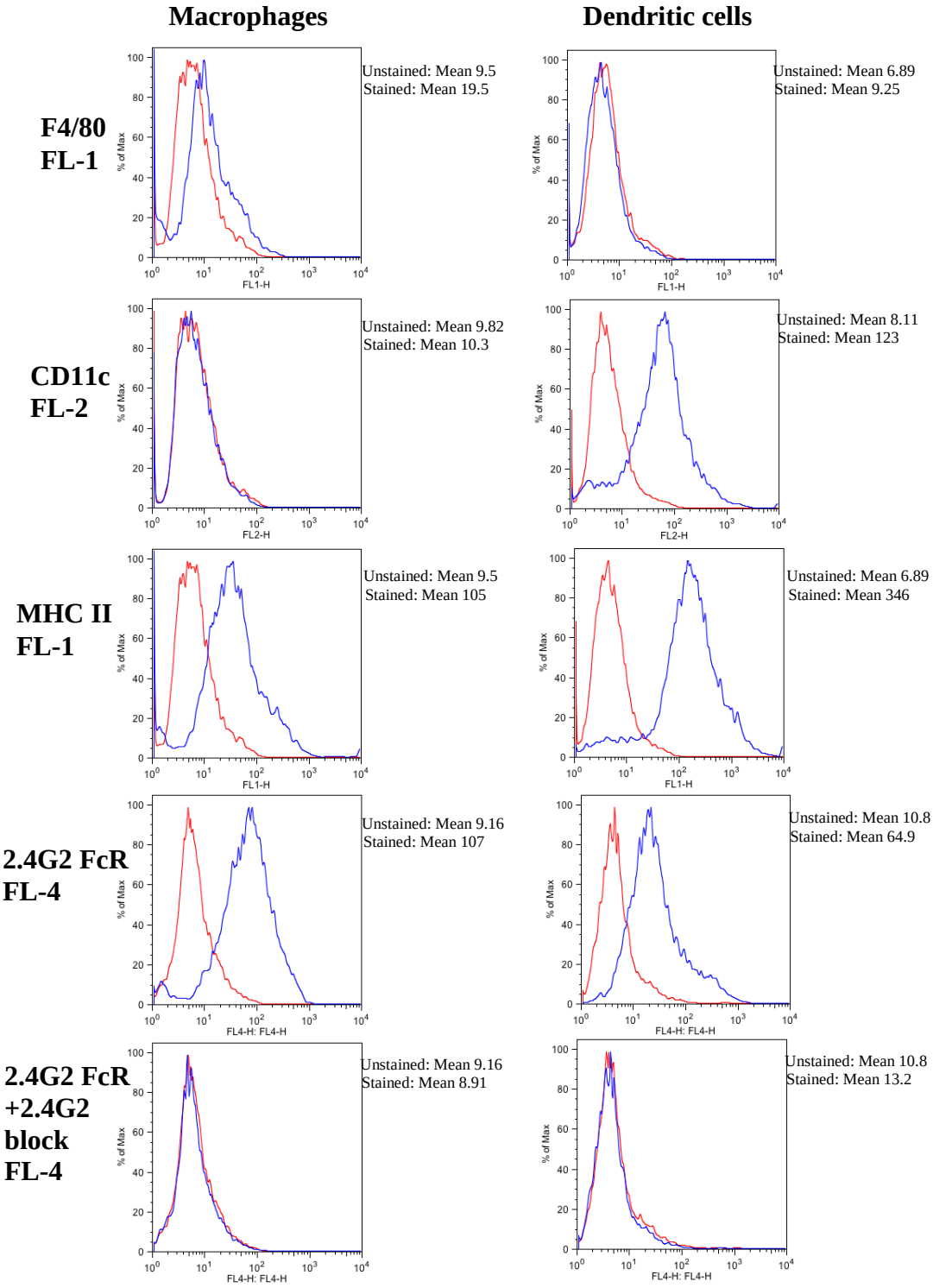


Figure 5.1 Phenotyping of bone marrow derived macrophages and dendritic cells by flow cytometry. *Bone marrow derived macrophages and bone marrow derived dendritic cells were stained with F4/80 FITC, CD11c PE or MHC II FITC. Red histograms represent gated unstained cells and the blue histograms represent gated cells stained for the various markers. All experiments were carried out at 4^{o}C in the presence of (75 µg/ml) 2.4G2 Fc block. To control for the efficiency of 2.4G2 Fc block, cells were stained with 2.4G2bio and streptavidin APC either in the presence or absence of block. Data is representative of 3 independent experiments.*

5.2.2 Kinetics of aggrecan presentation

5.2.2.1 Comparison of aggrecan presentation by dendritic cells, macrophages and splenic B cells.

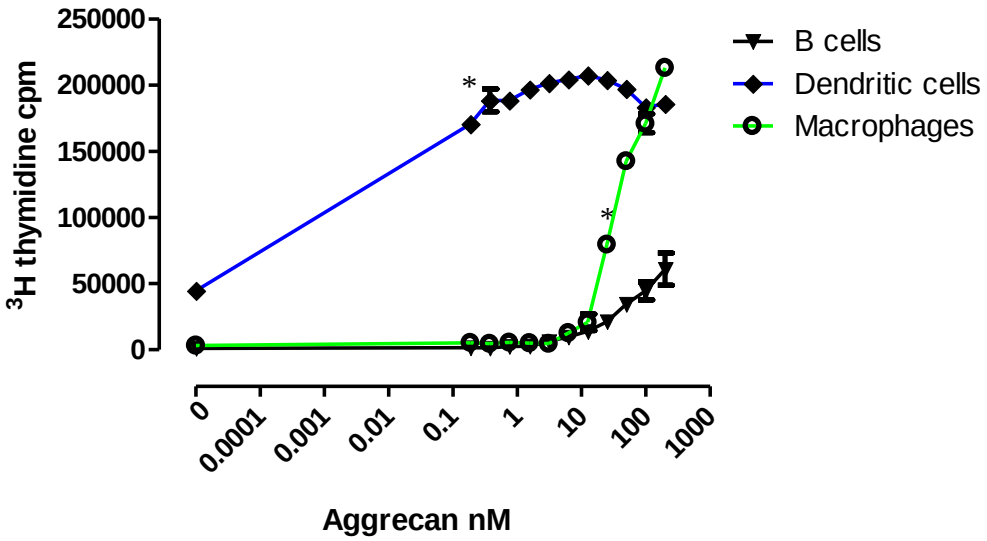
Initially I set out to compare the efficiency of aggrecan presentation by H-2^d expressing splenic B cells, bone marrow derived dendritic cells and bone marrow derived macrophages all isolated from BALB/c mice. The various APC were cultured with the T cell hybridoma 192 for 24 h in the presence of either graded doses of aggrecan or peptide p84-103. T cell hybridoma activation was measured by IL-2 production using CTLL-2 (results shown in **Figure 5.2)**. Data in **Figure 5.2a** revealed that dendritic cells are the most effective APC, presenting aggrecan at a 10^2 times lower dose than macrophages at doses from 0.3nM ($p<0.001$) and capable of inducing 192 activation at doses of aggrecan at least 10^3 times lower than that seen by splenic B cells. In addition **Figure 5.2b** shows that dendritic cells are also the most efficient APC at presenting the synthetic peptide p84-103 that requires no further processing for presentation to the T cell hybridoma 192. Dendritic cells presented p84-103 at least 10^2 times more efficiently than macrophages at doses from 0.0001nM ($p<0.001$) and at least 10^4 times more efficiently than splenic B cells.

5.3.2.2 Comparison of aggrecan presentation by dendritic cells, macrophages, aggrecan-specific B cell clones (C71-4C5 and C71-5F10) and A20-1 HS.

I wanted to determine whether the difference in aggrecan presentation between the various APC shown in **Figure 5.2a** presentation could also be observed between dendritic cells, macrophages and aggrecan-specific B cells and whether this difference in aggrecan presentation would also become apparent when using other forms of aggrecan. Dendritic cells, macrophages, two aggrecan-specific B cell clones C71-4C5 and C71-5F10 and A20-1 HS (non-specific B cell control) were cultured with the T cell hybridomas 192 for 24 h in the presence of either graded doses of deglycosylated aggrecan, glycosylated aggrecan or p84-103. Data in **Figure 5.3a** revealed that aggrecan-specific B cell clone C71-4C5 is as efficient as dendritic cells at presenting doses of deglycosylated aggrecan from 0.005nM ($p<0.01$) to the T cell hybridomas 192, 10^2 more efficient than macrophages and 10^5 times more efficient than A20-1 HS hybridomas $p<0.001$. C71-5F10 was approximately 10 times less efficient at presentation than the C71-4C5 clones. Interestingly, data in **Figure 5.3b** shows that macrophages are the most efficient at presenting the glycosylated form of aggrecan to the T cell hybridomas 192 at doses from 0.005nM ($p<0.01$), followed by dendritic cells which were

approximately 10 times more efficient than the C71-4C5 clones and 10^2 fold more efficient than C71-5F10 $p<0.001$. Data shows that A20-1 HS were not able to present this form of aggrecan to the T cell hybridomas 192 even at the highest dose of 200 nM. Data in **Figure 5.3c** shows that p84-103 which requires no further processing by the APC for presentation to the T cell hybridoma 192 was most efficiently presented by both dendritic cells and macrophages from a dose of 0.001 nM ($p<0.001$). C71-4C5, C71-5F10 and A20-1 HS all presented p84-103 approximately 10^2 fold less efficiently than dendritic cells and macrophages. These data suggest that the improved presentation of deglycosylated and glycosylated aggrecan by the C71 clones is due to the improved uptake and processing of aggrecan and not surface presentation of the 84-103 epitope.

a)



b)

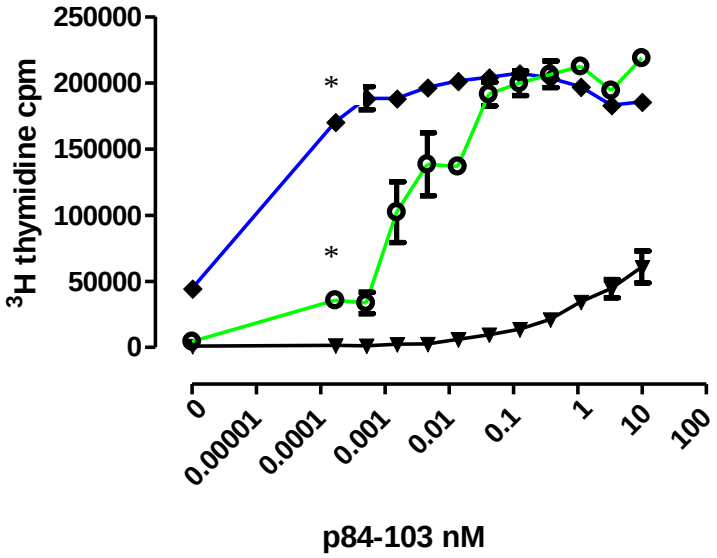


Figure 5.2 Presentation of aggrecan and p84-103 by splenic B cells, bone marrow derived dendritic cells or bone marrow derived macrophages. *APC ($5x10^4$) were cultured with aggrecan$_{84-103}$-specific T cell hybridoma 192 ($5x10^4$) in the presence of graded doses of aggrecan or peptide for 24 h. Cells were killed by freezing at -80^{o}C and supernatant was removed for bioassay with CTLL-2 cells. ^{3}H thymidine was added for 18 h and T cell responses were determined and displayed as ^{3}H thymidine cpm +/- SEM. Data are representative of three experiments. The asterix represents statistical significance determined by one-way ANOVA where $p<0.05$.*

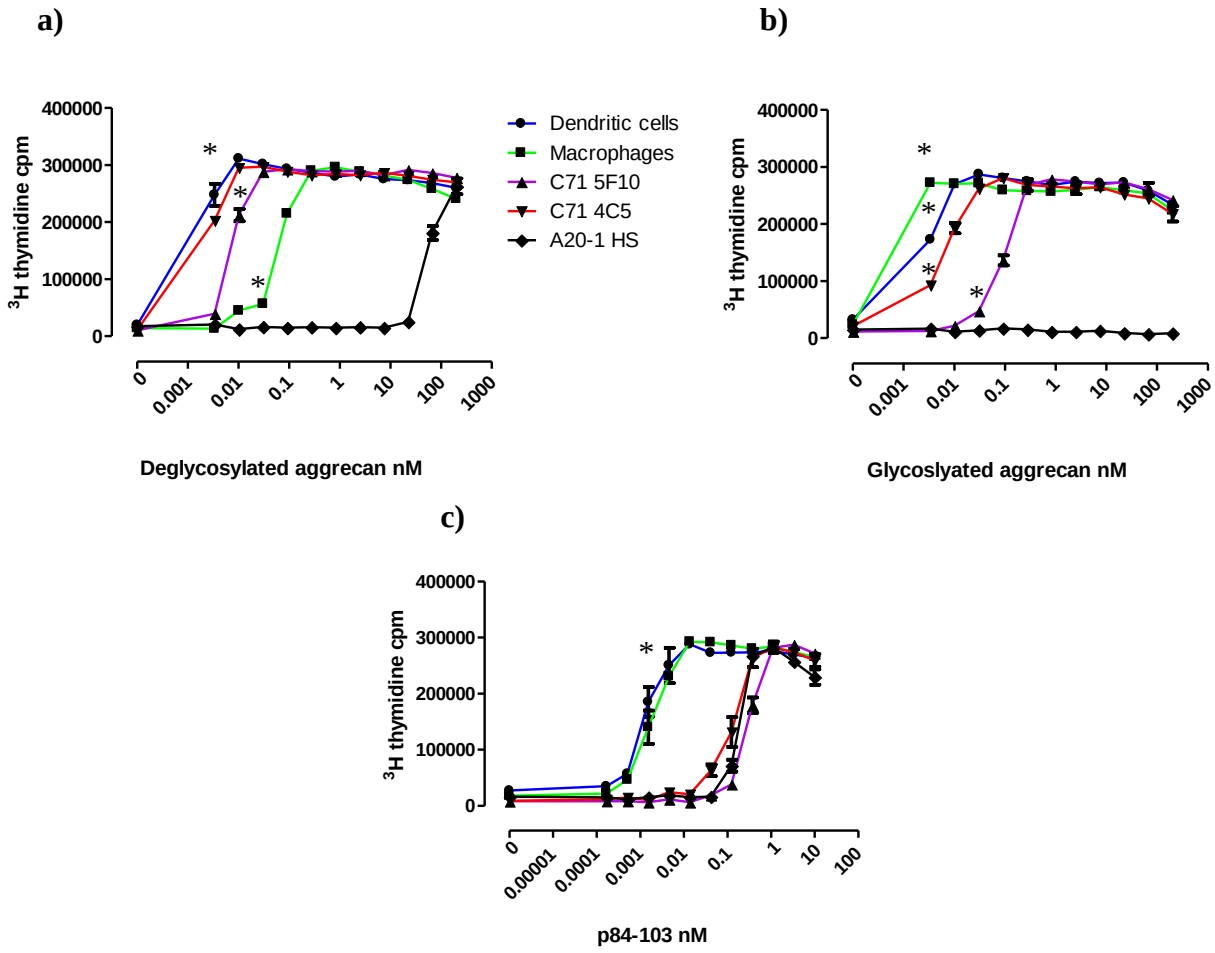


Figure 5.3 Presentation of deglycosylated and glycosylated aggrecan and p84-103 by bone marrow derived dendritic cells, bone marrow derived macrophages, aggrecan-specific B cell transfectants C71-5F10 or C71-4C5 and A20-1 HS. *APC ($5x10^4$) were cultured with $aggrecan_{84-103}$-specific T cell hybridoma 192 ($5x10^4$) in the presence of graded doses of various forms of aggrecan (deglycosylated, glycosylated) or peptide for 24 h. Cells were killed by freezing at -80^{o}C and supernatant was removed for bioassay with CTLL-2 cells. ^{3}H thymidine was added for 18 h and T cell responses were determined and displayed as ^{3}H thymidine cpm +/- SEM. Data are representative of three experiments. The asterix represents statistical significance determined by one-way ANOVA where $p<0.05$.*

5.2.2.2 Comparison of aggrecan presentation by a titrated number of dendritic cells, macrophage and aggrecan-specific B cell clones (C71-4C5 and C71-5F10).

Pape *et al* previously reported that only a small number of B cells are required to accumulate antigen following immunization and others have identified the cell surface expression of peptide-MHC class II complexes at early timepoints following immunisation suggesting that B cells contribute to the early activation of T cells following antigen encounter (Byersdorfer, Dipaolo et al. 2004; Catron, Itano et al. 2004; Pape, Catron et al. 2007). However, this finding has not been verified for aggrecan presentation. Therefore, I titrated the number of APC used in the antigen presentation assays to determine the ratio of aggrecan-specific B cells to aggrecan-specific T cells required for T cell activation in comparison with the numbers of dendritic cells and macrophages required. A20-1 HS were not included in this assay as I have previously verified that $5x10^4$ cells are required to activate the T cell hybridoma 192 (data not shown). Graded numbers of the various APC were co-cultured with the T cell hybridoma 192 ($5x10^4$) for 24 h in the presence of graded doses of deglycosylated aggrecan. Data in **Figure 5.4** revealed that when APC were cultured at a 1:1 ratio with the T cell hybridoma (50,000 APC per well) clones C71-4C5 were the most efficient at antigen presentation, presenting aggrecan from doses of 0.0001 nM $p<0.001$. Macrophages also presented aggrecan from doses of 0.0001 nM $p<0.001$. However, background ^{3}H thymidine cpm in the absence of antigen was very high in these experiments.

APC cultured at 1:3 ratio with T cell hybridomas (16,700 APC per well) was enough to reduce background ^{3}H thymidine cpm. However, this ratio increased the amount of aggrecan required to generate a T cell hybridoma response, dendritic cells and clone C71-4C5 presented aggrecan from doses of 0.001 nM whereas the clone C71-5F10 and macrophages required doses of 0.01 nM $p<0.001$.

APC cell numbers were titrated down further to a 1:9 ratio with T cell hybridomas (5600 cells per well), 1:28 ratio (1800 cells per well) and finally a 1:83 ratio (600 cells per well). Results show that as the APC numbers are titrated down the amount of aggrecan required to generate T cell activation is elevated to levels of 0.1 nM for numbers of 600 dendritic cells or clones C71-4C5 or C71-5F10 per well. Data from these experiments reveal that the aggrecan-specific B cell clones C71-4C5 and 5F10 are as efficient at presenting aggrecan at a very low cell number (600 cells per well) as dendritic cells and approximately 10 times more efficient than macrophages $p<0.01$.

It was shown above that clones C71-4C5 and C71-5F10 do not present p84-103 as efficiently as dendritic cells and macrophages (see **Figure 5.3c**). To repeat this at a reduced cell number, APC were titrated as above and cultured with T cell hybridomas 192 ($5x10^4$) in the presence of graded doses of p84-103 for 24 h (data shown in **Figure 5.5**). As shown previously for aggrecan presentation background ^3H thymidine cpm were very high when APC were cultured at a1:1 ratio (50,000 cells per well) with the T cell hybridomas 192. However, all of the APC titrations show that dendritic cells and macrophages are the most efficient at presenting the lowest doses of p84-103 to the T cell hybridomas $p<0.001$. Clones C71-4C5 and C71-5F10 required 10^3-10^4 fold more p84-103 to generate a T cell response at the entire APC/T cell ratios tested. As the APC number was reduced increasing doses of p84-103 were required to generate a T cell response at the lowest cell number of 600 APC per well dendritic cells could present 0.01 nM doses of p84-103 whereas clone C71-4C5 and C71-5F10 required doses of 10 nM $p<0.001$.

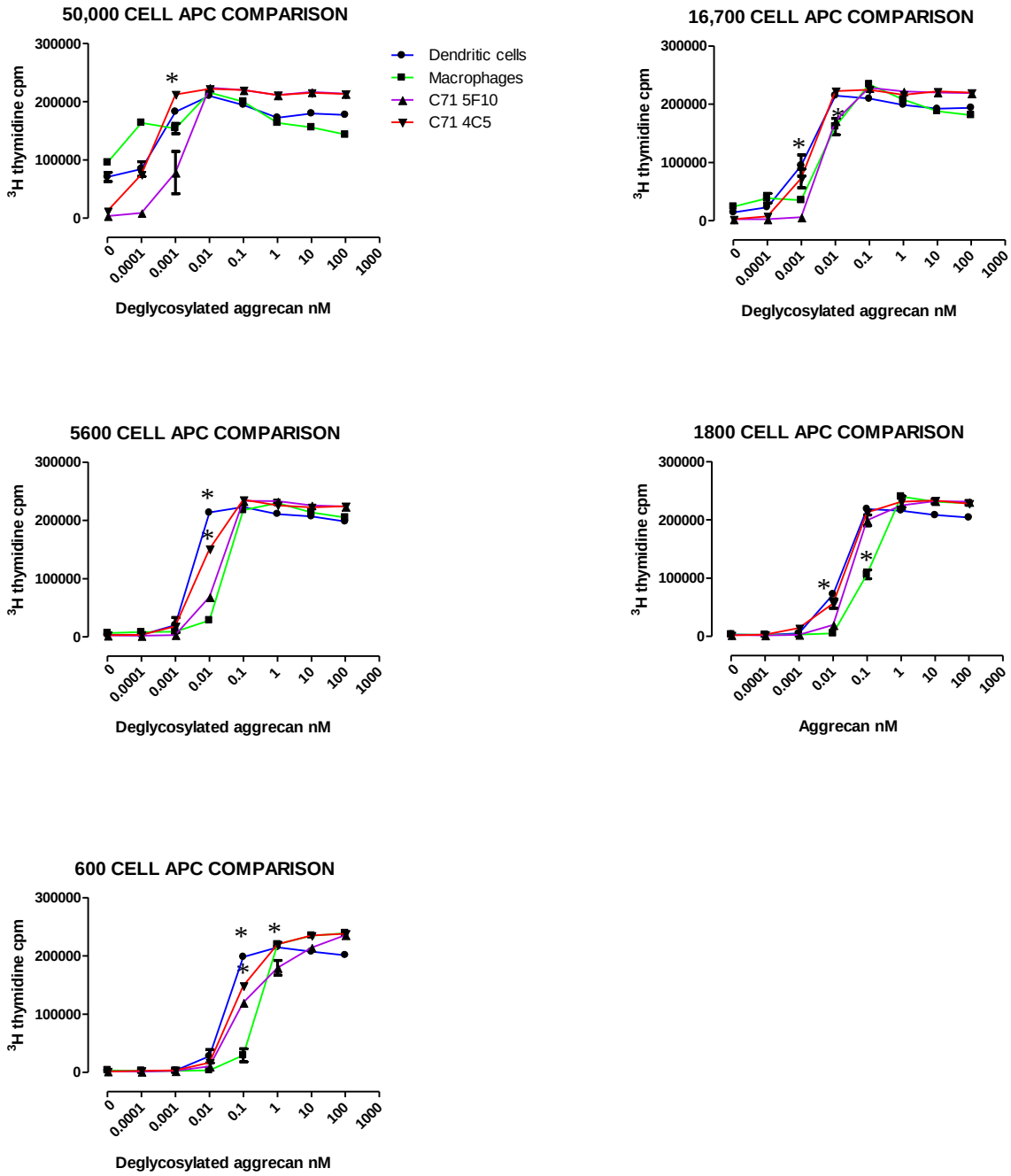


Figure 5.4 Aggrecan presentation by titrated numbers of bone marrow derived dendritic cells, bone marrow derived macrophages and aggrecan-specific B cell transfectants C71-5F10 or C71-4C5. *APC were cultured at various different cell numbers with aggrecan$_{84\text{-}103}$-specific T cell hybridoma 192 (5x10^4) in the presence of graded doses of deglycosylated aggrecan for 24 h. Cells were killed by freezing at -80^{o}C and supernatant was removed for bioassay with CTLL-2 cells. ^{3}H thymidine was added for 18 h and T cell responses were determined and displayed as ^{3}H thymidine cpm +/- SEM. Data are representative of two experiments. The asterix represents statistical significance determined by one-way ANOVA where p<0.05.*

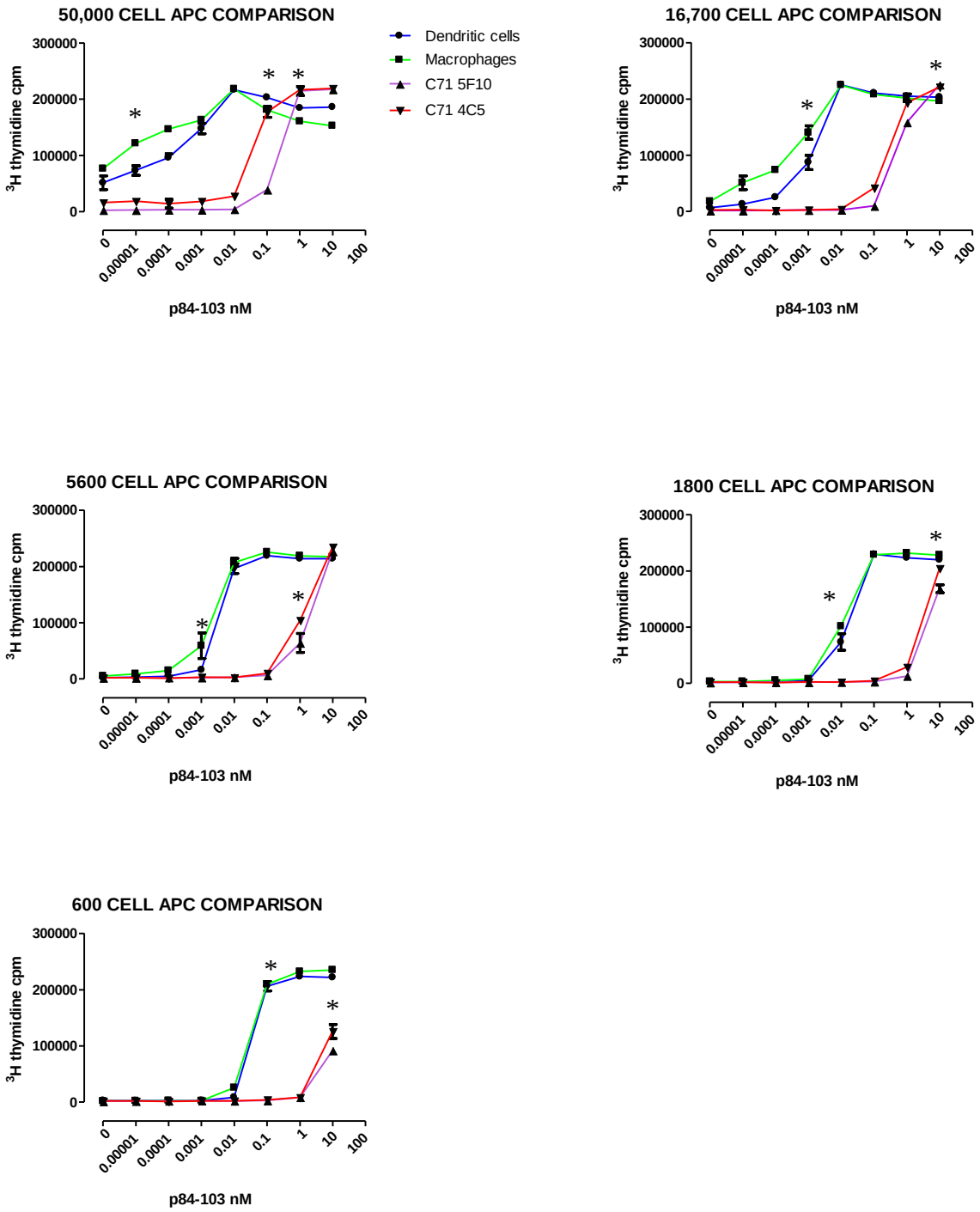


Figure 5.5 p84-103 presentation by titrated numbers of bone marrow derived dendritic cells, bone marrow derived macrophages and aggrecan-specific B cell transfectants C71-5F10 or C71-4C5. *APC were cultured at various different cell numbers with aggrecan$_{84\text{-}103}$-specific T cell hybridomas 192 (5x10^4) in the presence of graded doses of peptide for 24 h. Cells were killed by freezing at -80^{o}C and supernatant was removed for bioassay with CTLL-2 cells. ^{3}H thymidine was added for 18 h and T cell responses were determined and displayed as ^{3}H thymidine cpm +/- SEM. Data are representative of two experiments. The asterix represents statistical significance determined by one-way ANOVA where p<0.05.*

5.2.2.3 Optimisation of the pre fixed APC system.

Paraformaldehyde fixation of APC has been previously shown to block the internalization and processing of antigen (Shimonkevitz, Kappler et al. 1983). Indeed, previous studies in our laboratory have shown paraformaldehyde fixation of macrophages and dendritic cells leads to recognition of the H-2^d /p84-103 epitope by 192 (Lowes 2005).

Prior to experiments involving the pre-fixation of APC I had to optimise the percentage of paraformaldehyde required to efficiently fix antigen-specific B cells, whilst still enabling surface presentation of previously generated MHC class II/peptide complexes to the T cell hybridomas. In addition, in order for inhibitors of antigen processing (utilised in later sections) to exert their effects on their individual intracellular targets, they must be added prior to the addition of aggrecan. Inhibitors of antigen processing may also have off target or toxic effects following a prolonged incubation time with the APC or their inhibitory effects may be reversible after a certain length of time. Therefore, I had to optimise the minimum amount of time required by the APC for aggrecan uptake, processing and presentation.

Dendritic cells, macrophages, C71-4C5 and C71-5F10 B cells were incubated with different doses of antigen at different timepoints to determine the shortest amount of time required for aggrecan presentation prior to fixation with different percentage doses of paraformaldehyde. The system was found to be optimal by pre-incubating with either aggrecan or p84-103 for 5 h and APC fixation in 1% paraformaldehyde for 5 min (data not shown). Controls for each fixation experiment include APC that were fixed prior to incubation with 200nM aggrecan and T cell hybridoma 192 to verify that aggrecan can no longer be taken up and processed by the APC for presentation. APC were also fixed and then cultured with p84-103 and T cells to verify that this pre-processed form of aggrecan can still be displayed on surface MHC class II by fixed APC for presentation to T cell hybridomas and to verify that the fixation process does not produce any artefacts that lead to IL-2 production by the T cell hybridoma. Representative control data is shown in **Figure 5.6**. Data shows that all APC are able to efficiently process and present both aggrecan and p84-103 to the T cell hybridomas 192 following a 5 h incubation period prior to fixation and APC are also able to present p84-103 to the T cell hybridomas 192 post-fixation. T cell activation was minimal following culture of the T cell hybridomas with fixed APC that had been pre-incubated with media only for 5 h (fixed no antigen control) or with APC that had been fixed prior to the addition of 200 nM

aggrecan. These data verify that following fixation APC are no longer able to take up and process aggrecan for presentation to T cell hybridomas.

The positive control, un-fixed APC cultured with the T cell hybridomas 192 for 24 h in the presence of either graded doses of aggrecan or p84-103 was included alongside every fixation experiment to verify the integrity of the APC and T cell hybridomas (data not shown).

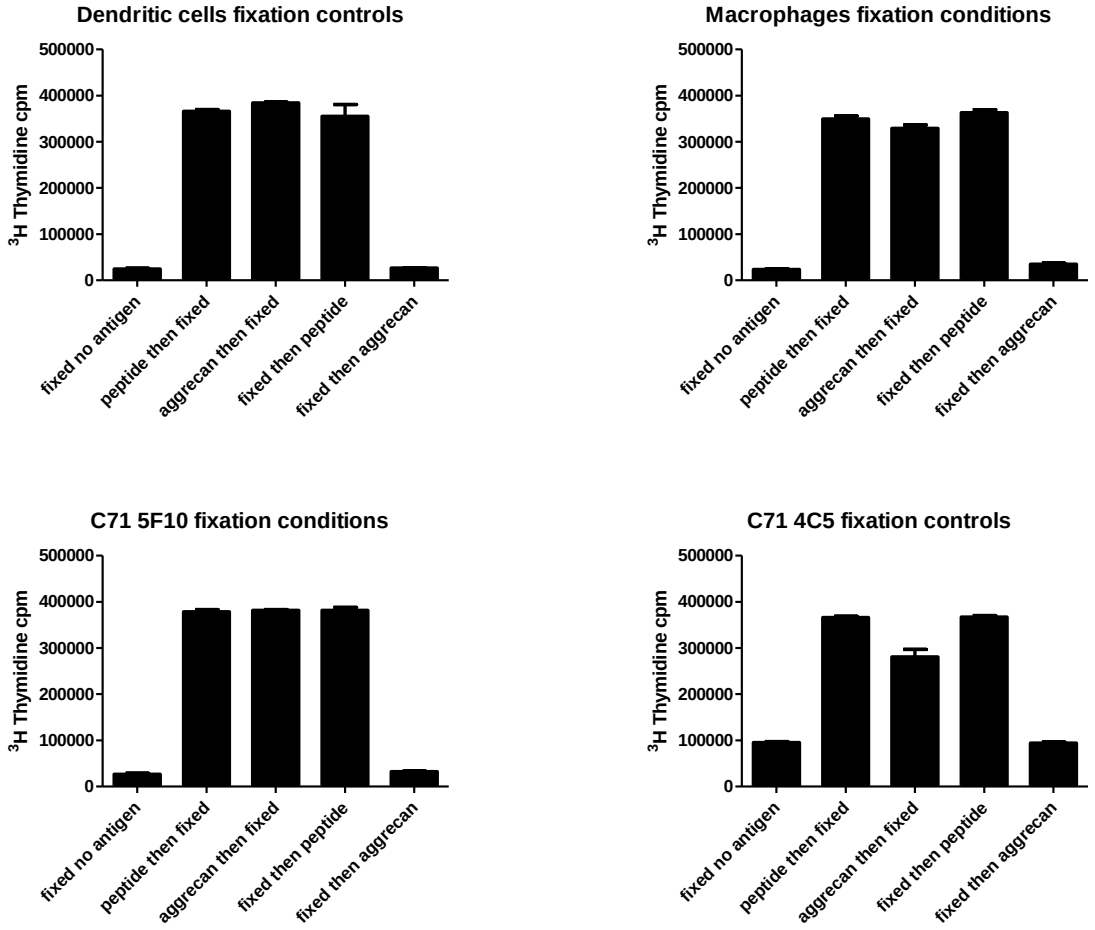


Figure 5.6 Representative controls for post fixation experiments.

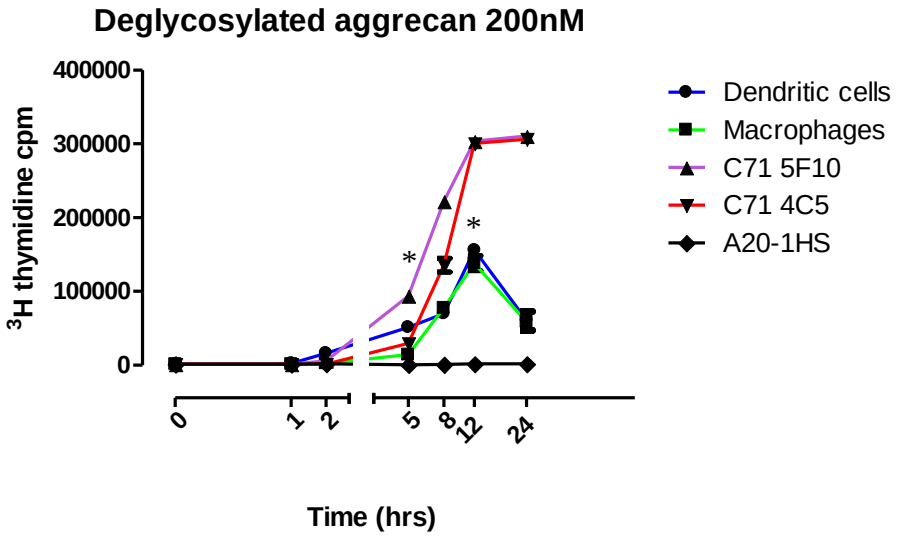
APC were cultured in media only (fixed no antigen), 10 nM p84-103 (peptide then fixed) or 200 nM aggrecan. APC were fixed in 1% paraformaldehyde followed by the addition of 0.05% Gly/Gly to halt fixation. APC were washed 3 times and cultured ($1x10^4$) with $aggrecan_{84-103}$-specific T cell hybridomas 192 ($5x10^4$) for 24 h. Alternately cells were fixed as above and cultured ($1x10^4$) with $aggrecan_{84-103}$-specific T cell hybridomas 192 ($5x10^4$) for 24 h in the presence of 10 nM p84-103 (fixed then peptide) or 200 nM Aggrecan (fixed then aggrecan). Cells were killed by freezing at -80^{o}C and supernatant was removed for bioassay with CTLL-2 cells. ^{3}H thymidine was added for 18 h and T cell responses were determined and displayed as ^{3}H thymidine cpm +/- SEM. Data are representative of >three experiments.

5.2.2.4 Kinetics of aggrecan presentation by dendritic cells, macrophages, aggrecan-specific B cell clones (C71-4C5 and C71-5F10) and A20-1 HS.

I next wanted to observe whether there were any differences in the time required by the different APC to efficiently take up and process aggrecan for presentation of the 84-103 epitope to the T cell hybridomas 192. Therefore, APC were incubated with aggrecan or p84-103 for various timepoints, fixed as described previously and cultured with the T cell hybridomas 192 for 24 h. Data in **Figure 5.7a** revealed that dendritic cells, macrophages and the C71 clones required at least 5 h incubation with aggrecan to generate a T cell response. $P<0.05$ Interestingly, A20-1 HS were not able to present aggrecan following any of the incubation timepoints post-fixation. Optimal presentation of aggrecan required 12 h incubation prior to fixation by dendritic cells, macrophages and C71 cells $p<0.001$. Surprisingly after this timepoint the efficiency of presentation by macrophages began to decline, whereas the presentation by C71 clones remained stable at 18 h and 24 h incubation prior to fixation. Data in **Figure 5.7b** shows that all APC can present p84-103 following a 1 h incubation period $p<0.05$. However, optimal presentation by dendritic cells, macrophages and C71-4C5 required a 5 h incubation period $p<0.001$.

Interestingly, A20-1HS are the first APC optimally present p84-103 following 1 h incubation, C71-5F10 required 2 h whereas the others require 5 h. This data indicates that the fixation process may work most efficiently with A20-1 HS. To ensure a minimal yet sufficient pre-incubation time is given to allow aggrecan processing by the different APC, subsequent inhibitor experiments were carried with a 6 h antigen incubation period.

a)



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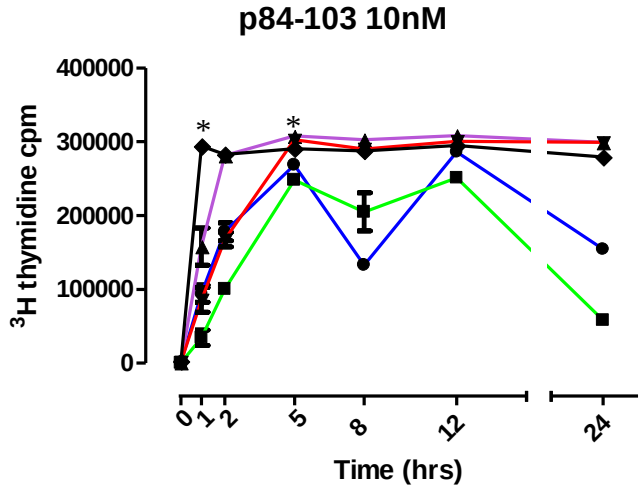


Figure 5.7 Kinetics of aggrecan and p84-103 presentation by bone marrow derived dendritic cells, bone marrow derived macrophages, aggrecan-specific B cell transfectants C71-5F10 or C71-4C5 and A20-1 HS. *APC were pulsed for various different timepoints with 200 nM deglycosylated aggrecan or 10 nM p84-103. APC were washed 3 times in HBSS to remove all unbound antigen. APC were fixed in 1% paraformaldehyde followed by the addition of 0.05% Gly/Gly to halt fixation. APC were washed 3 times and cultured ($1x10^4$) with aggrecan$_{84-103}$-specific T cell hybridomas 192 ($5x10^4$) for 24 h. Cells were killed by freezing at -80^{o}C and supernatant was removed for bioassay with CTLL-2 cells. ^{3}H thymidine was added for 18 h and T cell responses were determined and displayed as ^{3}H thymidine cpm +/- SEM. Data are representative of three experiments. The asterix represents statistical significance determined by one-way ANOVA where $p<0.05$.*

5.2.3 Effect of inhibitors on aggrecan presentation by dendritic cells, macrophages, aggrecan-specific B cell clones (C71-4C5 and C71-5F10) and A20-1 HS.

5.2.3.1 Treatment of APC with the inhibitors brefeldin A or cycloheximide to determine whether aggrecan epitope 84-103 is presented via the classical or the recycling pathway.

To determine whether 84-103 is presented via the classical or recycling MHC class II pathway dendritic cells, macrophages and aggrecan-specific B cell clones C71-4C5 or C71-5F10 were treated for 3 h with various doses of cycloheximide or brefeldin A (previously determined in our laboratory) to prevent the synthesis or transport of newly synthesised MHC class II prior to a 6 h incubation with aggrecan or p84-103. APC were fixed and $1x10^4$ were cultured with T cell hybridoma 192 (5×10^4) for 24 h. Data in **Figure 5.8** shows that treatment of all APC with 10 μM cycloheximide followed by incubation with aggrecan totally inhibited the processing and presentation of aggrecan $p<0.001$. However, doses above 10 μM cycloheximide also partially inhibited the presentation of p84-103 implying that cycloheximide at doses above this range may cause partial toxicity to the APC.

Data in **Figure 5.9** correlates with results from the cycloheximide experiments for both dendritic cells and the C71 aggrecan-specific B cells. Results show that presentation of aggrecan is totally inhibited by pre-incubation with 2.5 μg/ml brefeldin A for dendritic cells and C71-5F10 and 5 μg/ml brefeldin A for C71-4C5 $p<0.001$. Surprisingly, doses of up to 2 μg/ml brefeldin had minimal/no effect on aggrecan presentation by macrophages.

Thus, as expected brefeldin A had no effect on the presentation of the peptide p84-103 by dendritic cells, aggrecan-specific B cells C71-4C5, C71-5F10 or macrophages. Data suggest that presentation of the 84-103 epitope by dendritic cells, C71-4C5 and C71-5F10 requires newly synthesised MHC class II and presentation via the classical pathway. However, Brefeldin A data suggests that macrophages may be able to utilise both pathways for presentation of the 84-103 epitope.

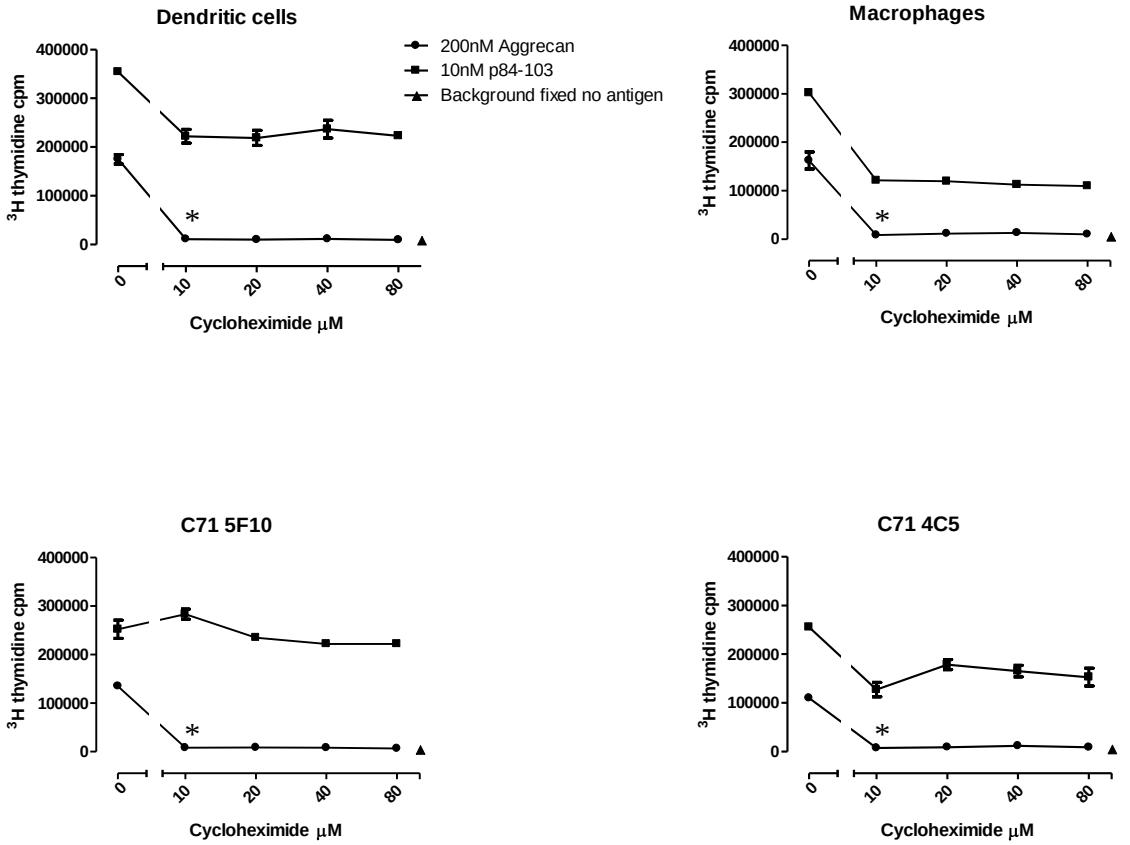


Figure 5.8 Effect of cycloheximide an inhibitor of protein synthesis, on presentation of 200 nM aggrecan or 10 nM p84-103 to T cell hybridomas 192.

APC were treated in triplicates with graded doses of cycloheximide for 3 h prior to 6 h incubation with 200 nM deglycosylated aggrecan or 10 nM p84-103. Controls included cells treated with the inhibitor only (Background fixed no antigen). APC were washed 3 times in HBSS to remove all inhibitor and unbound antigen. APC were fixed in 1% paraformaldehyde followed by the addition of 0.05% Gly/Gly to halt fixation. APC were washed 3 times and cultured ($1x10^4$) with $aggrecan_{84-103}$-specific T cell hybridomas 192 ($5x10^4$) for 24 h. Cells were killed by freezing at -80^{o}C and supernatant was removed for bioassay with CTLL-2 cells. ^{3}H thymidine was added for 18 h and T cell responses were determined and displayed as ^{3}H thymidine cpm +/- SEM. Data are representative of three experiments. The asterix represents statistical significance comparing the aggrecan response in the presence and absence of the lowest dose of inhibitor, determined by one-way ANOVA where $p<0.05$.

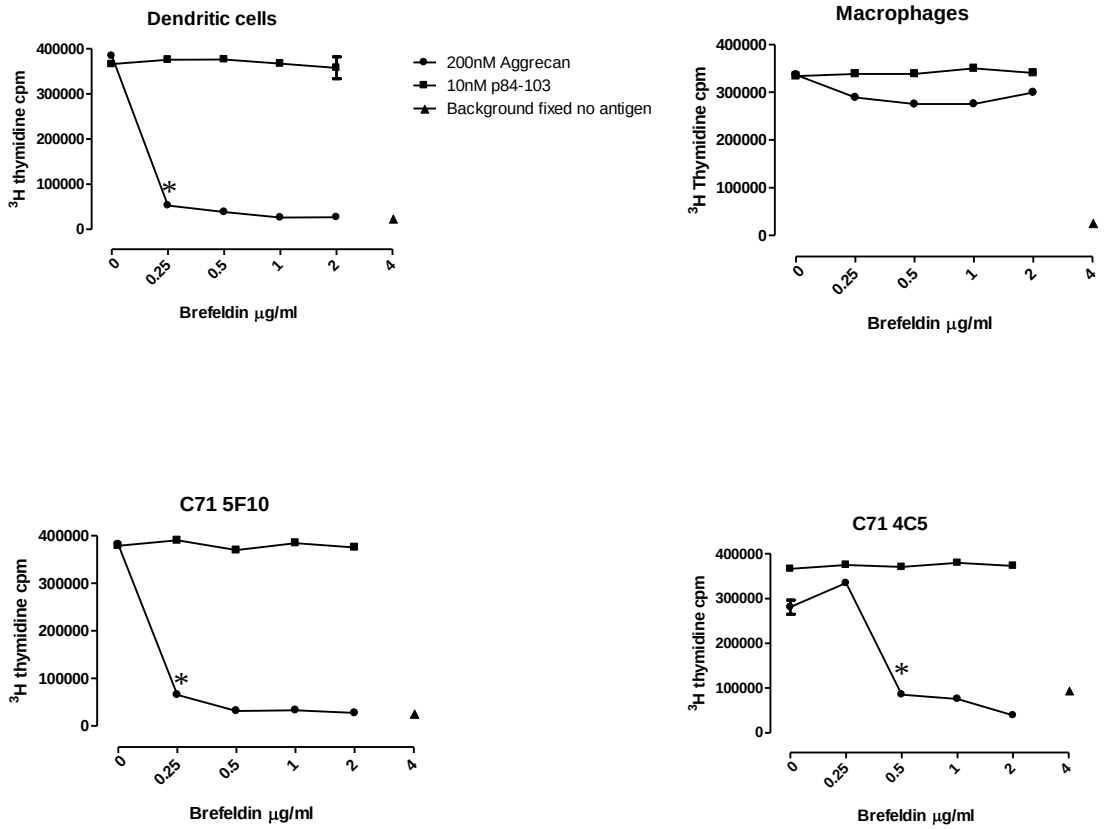


Figure 5.9 Effect of Brefeldin A, an inhibitor of Golgi transport, on presentation of 200 nM aggrecan or 10 nM p84-103 to T cell hybridomas 192.

APC were treated in triplicates with graded doses of brefeldin A for 3 h prior to 6 h incubation with 200 nM deglycosylated aggrecan or 10 nM p84-103. Controls included cells treated with the inhibitor only (Background fixed no antigen). APC were washed 3 times in HBSS to remove all inhibitor and unbound antigen. APC were fixed in 1% paraformaldehyde followed by the addition of 0.05% Gly/Gly to halt fixation. APC were washed 3 times and cultured ($1x10^4$) with $aggrecan_{84-103}$-specific T cell hybridomas 192 ($5x10^4$) for 24 h. Cells were killed by freezing at -80^{o}C and supernatant was removed for bioassay with CTLL-2 cells. ^{3}H thymidine was added for 18 h and T cell responses were determined and displayed as ^{3}H thymidine cpm +/- SEM. Data are representative of three experiments. The asterix represents statistical significance determined by one-way ANOVA where $p<0.05$.

5.2.3.2 Treatment of APC with ammonium chloride to determine the role of endosomal acidification in the processing and presentation of the 84-103 epitope.

To determine the extent of endosomal acidification required by different APC for the generation and presentation of the 84-103 epitope, APC were incubated with a dose range of ammonium chloride for 3 h (previously determined in our laboratory) to raise the intracellular pH prior to a 6 h incubation with aggrecan or p84-103. APC were fixed and $1x10^4$ were cultured with T cell hybridoma 192 (5×10^4) for 24 h. Data in **Figure 5.10** shows that doses of 50 mM ammonium chloride inhibited presentation of aggrecan by dendritic cells and both C71 clones $p<0.001$. 50 mM ammonium chloride improved the presentation of aggrecan by macrophages $p<0.05$. However, treatment of macrophages with doses of 100 mM and above totally inhibited aggrecan presentation $p<0.001$. This indicates that an acidic environment is required for efficient aggrecan processing by dendritic cells and C71 clones yet optimal presentation by macrophages may require a slightly less acidic environment implying that macrophages may begin to process aggrecan slightly earlier in the endosomal pathway.

Doses of 200 mM ammonium chloride completely inhibited peptide p84-103 presentation by the C71 clones whereas 400 mM was required to inhibit presentation by dendritic cells. Presentation of p84-103 was not inhibited by any of the ammonium chloride doses (up to 400 mM). This implies that either doses of 200 mM or 400 mM ammonium chloride are toxic to C71 clones and dendritic cells respectively or that p84-103 loading of MHC class II molecules is inhibited by increasing intracellular pH.

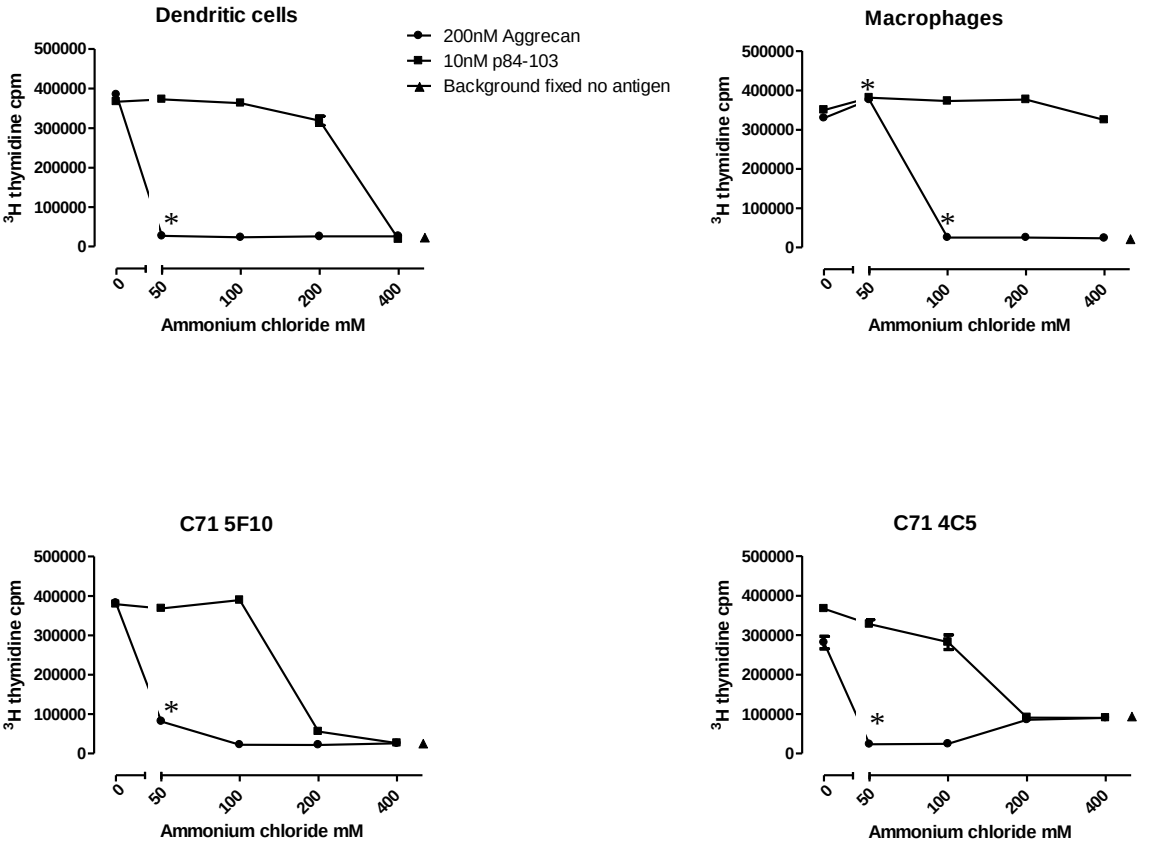


Figure 5.10 Effect of ammonium chloride, used to raise endosomal pH, on presentation of 200 nM aggrecan or 10 nM p84-103 to T cell hybridomas 192.

APC were treated in triplicates with graded doses of ammonium chloride for 3 h prior to 6 h incubation with 200 nM deglycosylated aggrecan or 10 nM p84-103. Controls included cells treated with the inhibitor only (Background fixed no antigen). APC were washed 3 times in HBSS to remove all ammonium chloride and unbound antigen. APC were fixed in 1% paraformaldehyde followed by the addition of 0.05% Gly/Gly to halt fixation. APC were washed 3 times and cultured ($1x10^4$) with aggrecan$_{84-103}$-specific T cell hybridomas 192 ($5x10^4$) for 24 h. Cells were killed by freezing at -80^{o}C and supernatant was removed for bioassay with CTLL-2 cells. ^{3}H thymidine was added for 18 h and T cell responses were determined and displayed as ^{3}H thymidine cpm +/- SEM. Data are representative of three experiments. The asterix represents statistical significance determined by one-way ANOVA where $p<0.05$.

5.2.3.3 Treatment of APC with E-64d and pepstatin A to determine the role of cysteine and aspartic proteinases in the processing and presentation of the 84-103 epitope.

I next wanted to determine the role of cysteine and aspartic proteinases in the processing and presentation of the 84-103 epitope by the different APC. E-64d is a cell permeable irreversible inhibitor of cysteine proteinases including calpain, papain and the cathepsins B, H and L (Bond and Butler 1987). Pepstatin A is a highly selective inhibitor of aspartic proteinases including cathepsin D, pepsin and rennin (Marciniszyn, Hartsuck et al. 1976).

APC were incubated with graded doses of E-64d, or pepstatin A for 1 h (previously determined by our laboratory) prior to a 6 h incubation with aggrecan or p84-103. APC were fixed and $1x10^4$ were cultured with T cell hybridoma 192 (5×10^4) for 24 h. Data in **Figure 5.11** shows that pre-treatment of the APC with doses of up to 100 μM of E-64d prior to incubation with 200 nM aggrecan had no affect on the presentation of 84-103. In addition to this, concentrations as high as 100 μM also failed to have any effect on the presentation of peptide p84-103 by all APC types. This data is consistent with experiments previously performed by our laboratory that showed pre-treatment of macrophages with up to 50 μM of E-64d had no effect on the presentation of aggrecan to 192. In addition, it was also shown in these previous studies that 1.25 μM E-64d did inhibit the presentation of the type II collagen epitope 259-273 to collagen specific hybridoma indicating that the inhibitor was functional despite having no effect on 84-103 presentation to the T cell hybridoma 192 (Lowes 2005; von Delwig, Altmann et al. 2006).

To confirm that the batch of E-64d was functional, I attempted to repeat findings by Shim *et al* from our laboratory who showed previously that the presentation of V antigen by macrophages to the V antigen-specific hybridomas 1E10, 1B4 and 2H1 is inhibited by 2.5 μM E-64d (Shim, Musson et al. 2006). Unfortunately, I was unable to demonstrate that any of the fixed APC were able to present V antigen epitopes to any of the 3 V antigen-specific T cells following prior incubation with V antigen. Therefore, although not strictly controlled, I had to speculatively conclude that cysteine proteinases are not involved in the endosomal processing of aggrecan and the generation of the 84-103 epitope.

To examine the effects of aspartic proteinases on aggrecan presentation, dendritic cells, macrophages and the aggrecan-specific B cell clones C71-4C5 and C71-5F10 were pre-treated with doses of up to 4 mM of the inhibitor pepstatin A. Data in **Figure 5.12** shows that the lowest dose of 0.5 mM totally inhibited presentation of aggrecan by both of the C71

clones $p<0.001$. Doses from 1 mM up to 4 mM pepstatin A also inhibited presentation of aggrecan by macrophages close to background levels $p<0.01$. However, doses of up to 4 mM pepstatin A only partially reduced aggrecan presentation by dendritic cells.

Pepstatin A did not have substantial affects on the presentation of peptide p84-103 by any of the APC. Therefore these data suggest that aspartic proteinases play a major role in aggrecan processing and generation of the 84-103 epitope by aggrecan-specific B cells and macrophages. However, 84-103 generation and presentation by dendritic cells was not completely abolished in the presence of pepstatin A. This implies that dendritic cells require aspartic proteinases for optimal generation and presentation of the 84-103 epitope, but are still able to process and present aggrecan at a reduced efficiency independently of aspartyl proteinases.

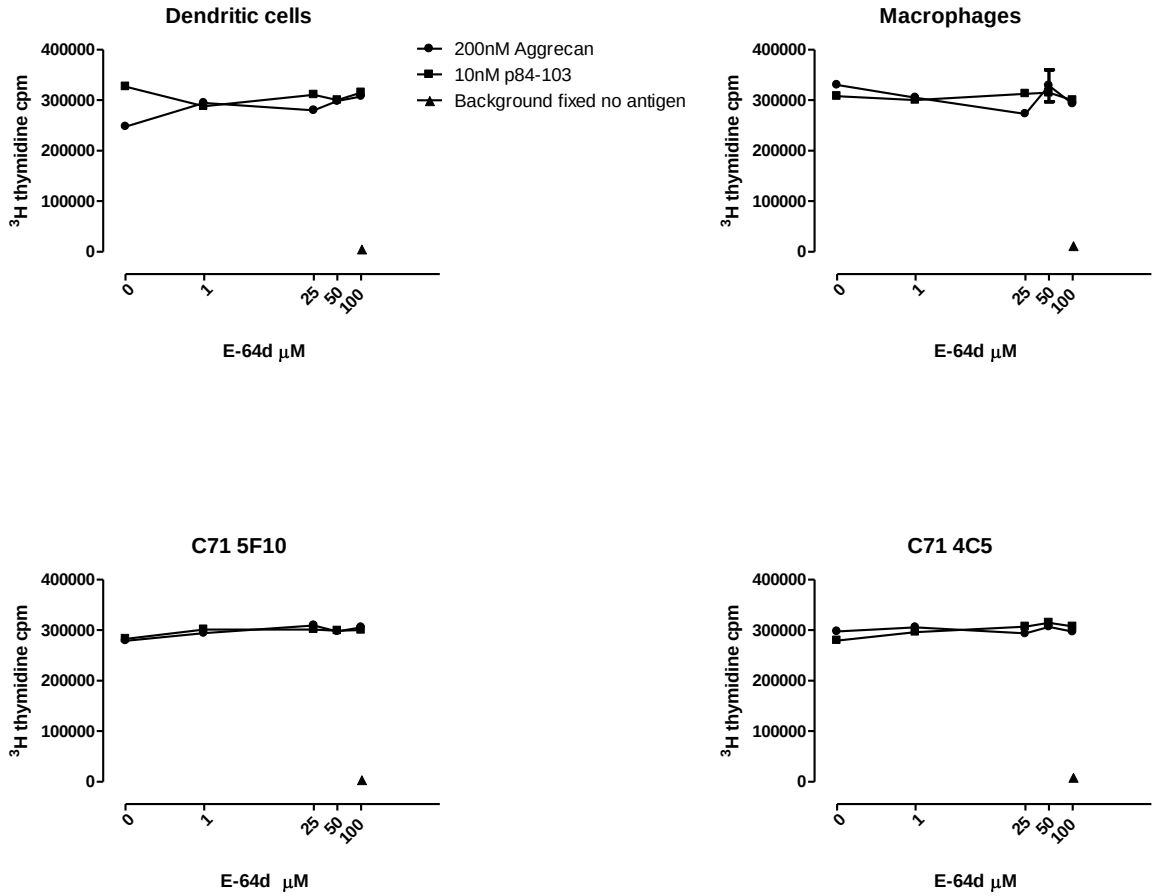


Figure 5.11 Effect of E-64d, an inhibitor of cysteine proteinases, on presentation of 200 nM aggrecan or 10 nM p84-103 to T cell hybridomas 192.

APC were treated in triplicates with graded doses of E-64d for 1 h prior to 6 h incubation with 200nM deglycosylated aggrecan or 10nM p84-103. Controls included cells treated with the inhibitor only (Background fixed no antigen). APC were washed 3 times in HBSS to remove all inhibitor and unbound antigen. APC were fixed in 1% paraformaldehyde followed by the addition of 0.05% Gly/Gly to halt fixation. APC were washed 3 times and cultured ($1x10^4$) with $aggrecan_{84-103}$-specific T cell hybridomas 192 ($5x10^4$) for 24 h. Cells were killed by freezing at -80^{o}C and supernatant was removed for bioassay with CTLL-2 cells. ^{3}H thymidine was added for 18 h and T cell responses were determined and displayed as ^{3}H thymidine cpm +/- SEM. Data are representative of three experiments.

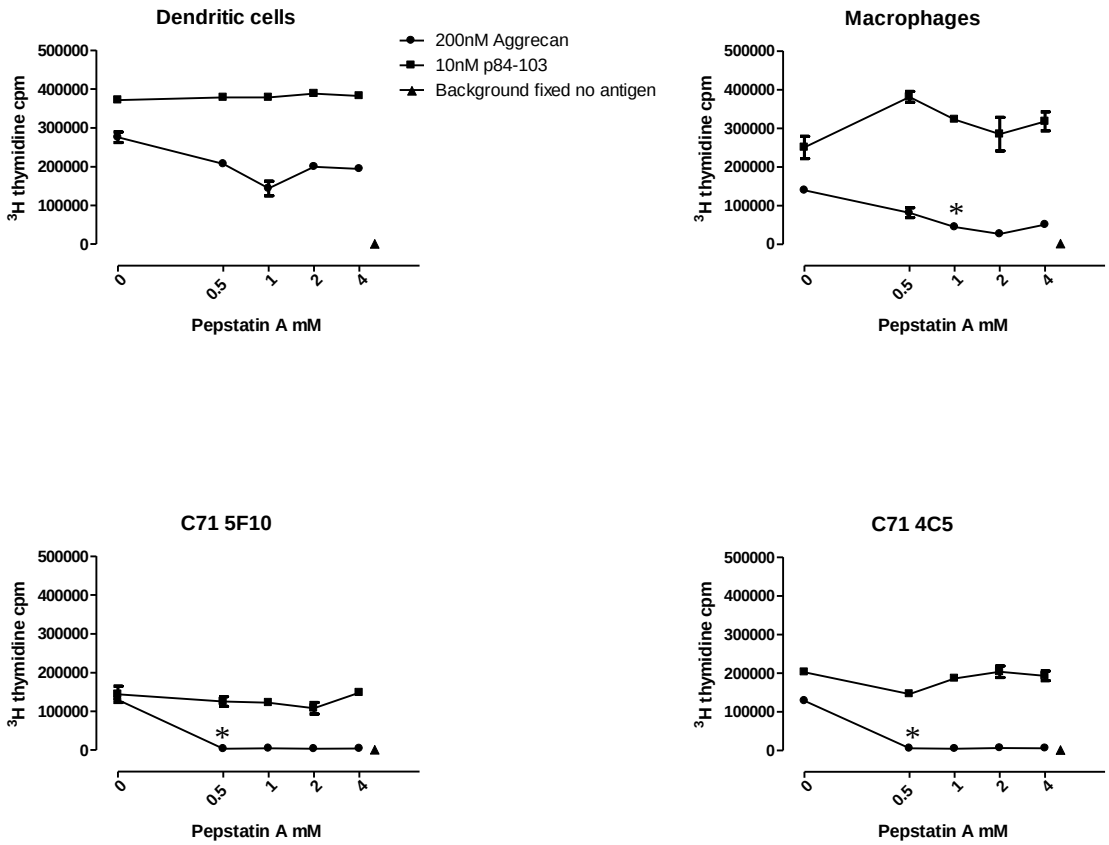


Figure 5.12 Effect of Pepstatin A an inhibitor of aspartyl proteinases, on presentation of 200 nM aggrecan or 10 nM p84-103 to T cell hybridomas 192.

APC were treated in triplicates with graded doses of pepstatin A for 1 h prior to 6 h incubation with 200 nM deglycosylated aggrecan or 10 nM p84-103. Controls included cells treated with the inhibitor only (Background fixed no antigen). APC were washed 3 times in HBSS to remove all inhibitor and unbound antigen. APC were fixed in 1% paraformaldehyde followed by the addition of 0.05% Gly/Gly to halt fixation. APC were washed 3 times and cultured ($1x10^4$) with aggrecan$_{84-103}$-specific T cell hybridomas 192 ($5x10^4$) for 24 h. Cells were killed by freezing at -80^{o}C and supernatant was removed for bioassay with CTLL-2 cells. ^{3}H thymidine was added for 18 h and T cell responses were determined and displayed as ^{3}H thymidine cpm +/- SEM. Data are representative of three experiments. The asterix represents statistical significance determined by one-way ANOVA where $p<0.05$.

5.2.3.4 Treatment of APC with 3,4-DCI and leupeptin to determine the role of cysteine and serine proteinases in the processing and presentation of the 84-103 epitope.

3,4- dichloroisocoumarin (3,4-DCI) is an irreversible broad range serine proteinase inhibitor (Harper, Hemmi et al. 1985). Leupeptin is a reversible competitive inhibitor of serine and cysteine proteinases including plasmin, trypsin, papain and cathepsin B (Aoyagi, Takeuchi et al. 1969). To determine the role of cysteine and serine proteinases in the processing and presentation of 84-103 by the different APC, APC were incubated with various doses of 3,4-DCI, or leupeptin for 1 h prior to a 6 h incubation with aggrecan or p84-103. APC were fixed and $1x10^4$ were cultured with T cell hybridoma 192 (5×10^4) for 24 h. Data in **Figure 5.13** shows that pre-treatment with doses greater than 10 μM 3,4-DCI prior to incubation with 200 nM aggrecan completely abolished presentation to T cell hybridomas 192 by both of the C71 clones $p<0.001$. Pre-treatment with >20 μM 3,4-DCI completely abolished presentation of aggrecan by dendritic cells $p<0.001$. However, macrophages required a much larger dose of 80μM 3,4-DCI to completely inhibit aggrecan presentation $p<0.001$.

Pre-treatment of dendritic cells with doses of up to 80 μM 3,4-DCI had no effect on the presentation of the peptide p84-103 by dendritic cells. Pre-treatment of C71-5F10 with 20 μM 3,4-DCI improved presentation of p84-103 yet an increased dose of 80 μM 3,4-DCI reduced p84-103 presentation. Pre-treatment of C71-4C5 with up to 40 μM had no effect on p84-103 presentation but an increased dose of 80μM reduced presentation to near background levels. Interestingly, presentation of p84-103 by macrophages was enhanced by pre-incubation with doses of 20 μM 3,4-DCI and above.

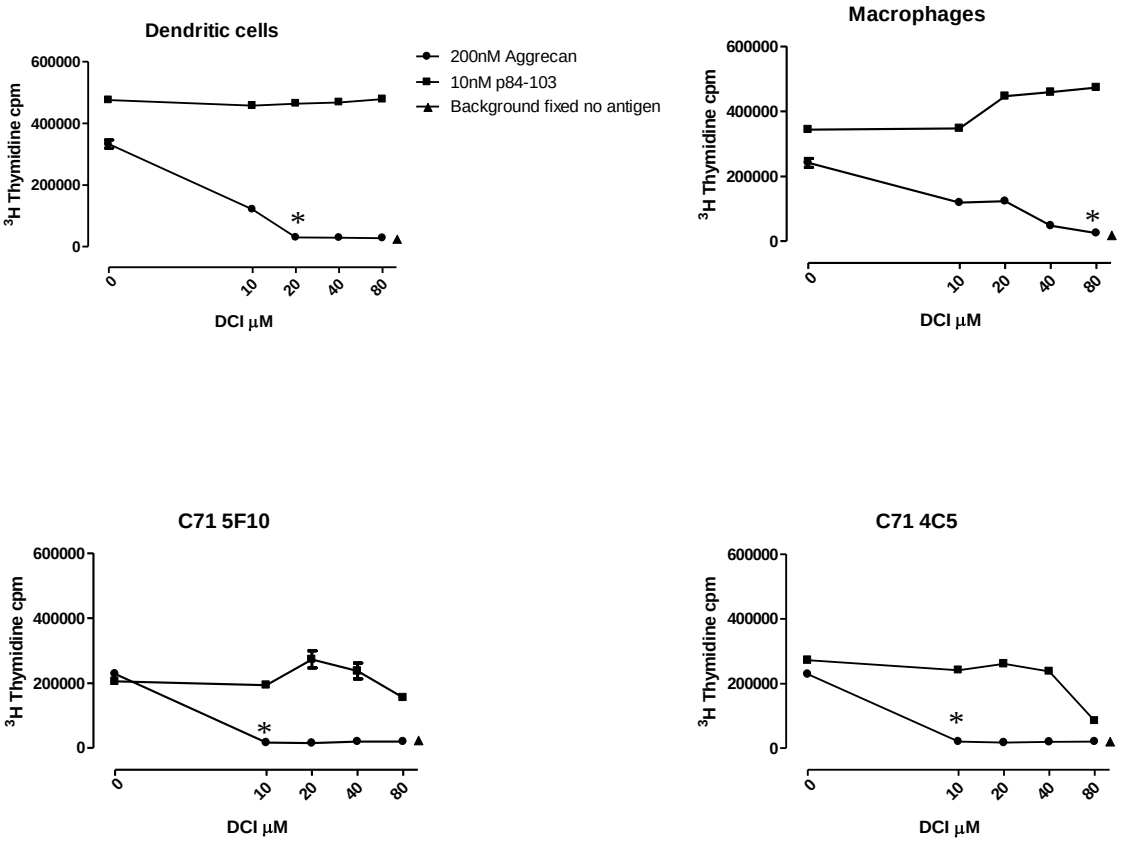


Figure 5.13 Effect of DCI, an inhibitor of serine proteinases, on presentation of 200 nM aggrecan or 10 nM p84-103 to T cell hybridomas 192.

APC were treated in triplicates with graded doses of DCI for 1 h prior to 6 h incubation with 200 nM deglycosylated aggrecan or 10 nM p84-103. Controls included cells treated with the inhibitor only (Background fixed no antigen). APC were washed 3 times in HBSS to remove all inhibitor and unbound antigen. APC were fixed in 1% paraformaldehyde followed by the addition of 0.05% Gly/Gly to halt fixation. APC were washed 3 times and cultured ($1x10^4$) with aggrecan$_{84-103}$-specific T cell hybridomas 192 ($5x10^4$) for 24 h. Cells were killed by freezing at -80^{o}C and supernatant was removed for bioassay with CTLL-2 cells. ^{3}H thymidine was added for 18 h and T cell responses were determined and displayed as ^{3}H thymidine cpm +/- SEM. Data are representative of three experiments. The asterix represents statistical significance determined by one-way ANOVA where $p<0.05$.

Data in **Figure 5.14** shows that pre-treatment of the C71 clones with 2.5 μM of the cysteine/serine inhibitor leupeptin prior to incubation with 200 nM aggrecan totally inhibited presentation to the T cell hybridomas 192 $p<0.001$. However, doses of up to 20 μM leupeptin did not reduce presentation of aggrecan by dendritic cells and macrophages to background levels. Interestingly, lower doses of 2.5 μM and 5 μM began by reducing aggrecan presentation by macrophages, and larger doses leupeptin (10 μM-20 μM) recovered and began to improve presentation.

Presentation of the peptide p84-103 was also reduced close to background levels by both of the C71 clones suggesting that leupeptin may be toxic to these cells. Presentation of p84-103 by dendritic cells was not affected by any of the leupeptin doses tested (2.5-20 μM). In correlation with the pattern of presentation of observed with the 84-103 epitope, presentation of peptide p84-103 by macrophages was reduced at doses of 5μM leupeptin followed by a recovery to untreated levels of p84-103 presentation at 20μM.

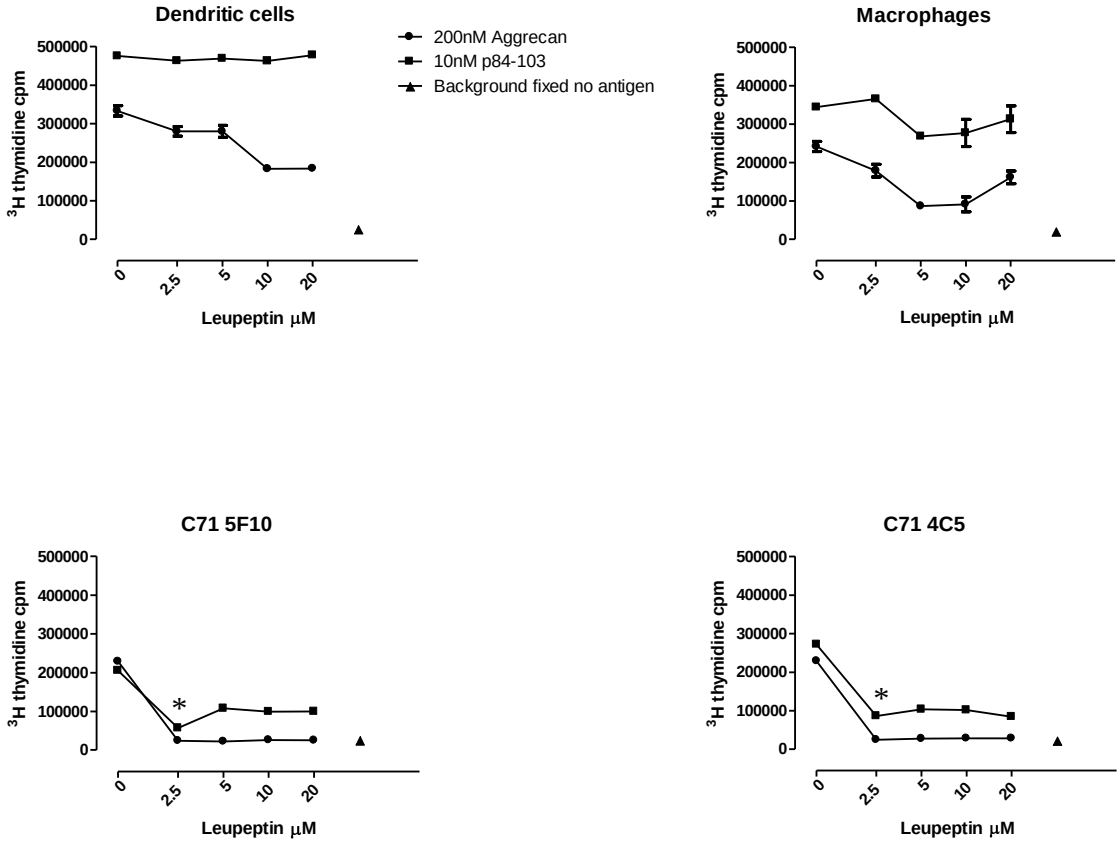


Figure 5.14 Effect of Leupeptin an inhibitor of serine and cysteine proteinases on presentation of 200 nM aggrecan or 10 nM p84-103 to T cell hybridomas 192.

APC were treated in triplicates with graded doses of leupeptin for 1 h prior to 6 h incubation with 200 nM deglycosylated aggrecan or 10 nM p84-103. Controls included cells treated with the inhibitor only (Background fixed no antigen). APC were washed 3 times in HBSS to remove all inhibitor and unbound antigen. APC were fixed in 1% paraformaldehyde followed by the addition of 0.05% Gly/Gly to halt fixation. APC were washed 3 times and cultured ($1x10^4$) with $aggrecan_{84-103}$-specific T cell hybridomas 192 ($5x10^4$) for 24 h. Cells were killed by freezing at -80^{o}C and supernatant was removed for bioassay with CTLL-2 cells. ^{3}H thymidine was added for 18 h and T cell responses were determined and displayed as ^{3}H thymidine cpm +/- SEM. Data are representative of three experiments. The asterix represents statistical significance determined by one-way ANOVA where $p<0.05$.

5.2.3.5 Treatment of APC with phenanthroline to determine the role of metalloproteinases in the presentation of the 84-103 epitope.

Phenanthroline is a broad spectrum metalloproteinase inhibitor (Takahashi, Cease et al. 1989). To determine the role of metalloproteinases in the processing and presentation of 84-103, APC were incubated with various doses of phenanthroline for 1 h prior to a 6 h incubation with aggrecan or p84-103. APC were fixed and $1x10^4$ were cultured with T cell hybridoma 192 (5×10^4) for 24 h. Data in **Figure 5.15** shows that pre-treatment of the four APC with doses of >800 μM phenanthroline prior to incubation with 200 nM aggrecan completely abolished presentation to the T cell hybridomas 192 by dendritic cells and the C71 clones C71-5F10 $p<0.001$. Increasing doses of >200 μM phenanthroline had a gradual effect on lowering the efficiency of aggrecan presentation by the C71-5F10 clone. Interestingly, phenanthroline had a slightly different effect on aggrecan presentation by the C71 clone C71-4C5. C71-4C5 clones, required doses of 1600 μM phenanthroline to completely abolish aggrecan presentation and lower doses had no effect on aggrecan presentation to the T cell hybridomas 192 $p<0.001$. Macrophages began to show a reduction in their efficiency to present aggrecan following pre-treatment with >400 μM phenanthroline. However, even when used at the highest dose of 1600 μM phenanthroline did not reduce aggrecan presentation to the T cell hybridomas 192 by macrophages back down to background levels.

Presentation of p84-103 by dendritic cells, macrophages or the C71-4C5 cells was not affected by even the highest 1600 μM dose of phenanthroline. However, presentation of p84-103 by the C71-5F10 clones was reduced from >200 μM doses of phenanthroline. Surprisingly this reduction in p84-103 presentation by C71-5F10 was recovered by pre-treatment with the highest dose of phenanthroline 1600 μM. Data suggests that metalloproteinases play a major role in the processing and presentation of 84-103 by aggrecan-specific B cells and dendritic cells and to a lesser extent in macrophages.

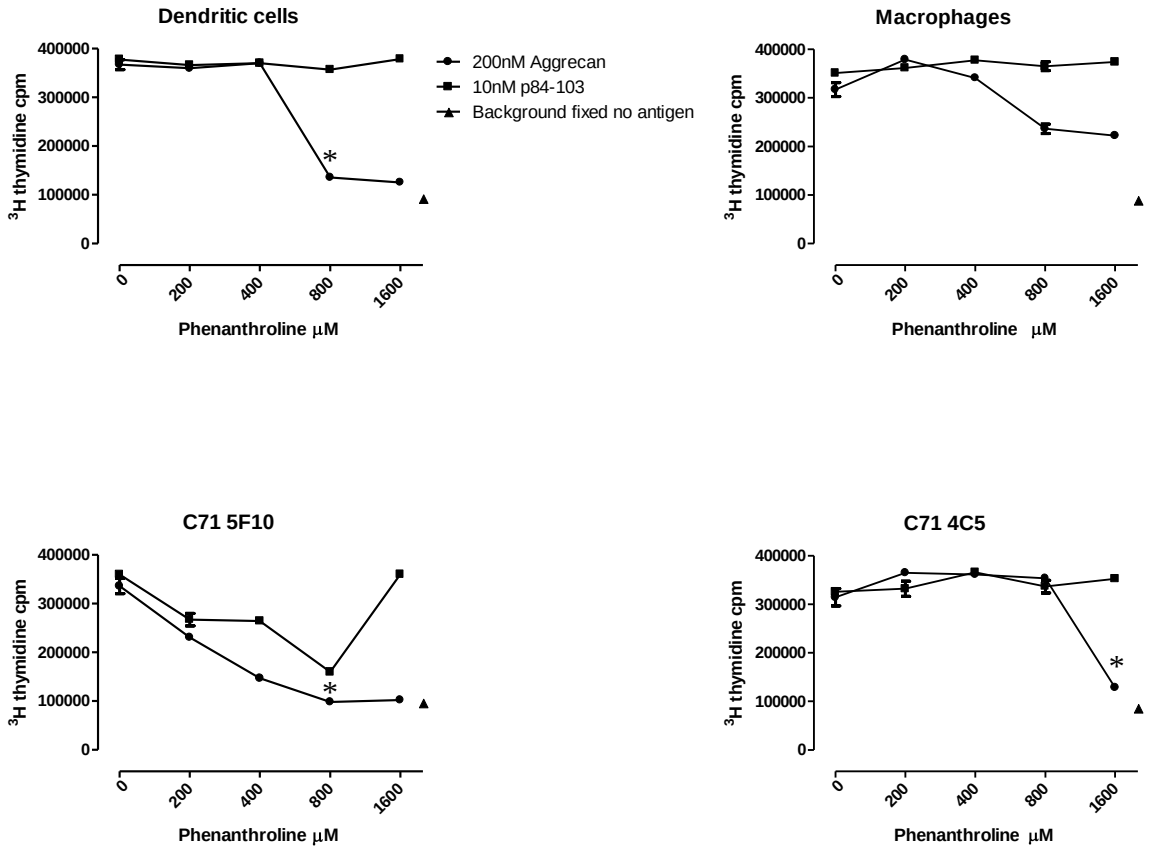


Figure 5.15 Effect of phenanthroline, an inhibitor of metalloproteinases, on presentation of 200 nM aggrecan or 10 nM p84-103 to T cell hybridomas 192.

APC were treated in triplicates with graded doses of phenanthroline for 1 h prior to 6 h incubation with 200 nM deglycosylated aggrecan or 10 nM p84-103. Controls included cells treated with the inhibitor only (Background fixed no antigen). APC were washed 3 times in HBSS to remove all inhibitor and unbound antigen. APC were fixed in 1% paraformaldehyde followed by the addition of 0.05% Gly/Gly to halt fixation. APC were washed 3 times and cultured ($1x10^4$) with $aggrecan_{84-103}$-specific T cell hybridomas 192 ($5x10^4$) for 24 h. Cells were killed by freezing at -80^{o}C and supernatant was removed for bioassay with CTLL-2 cells. ^{3}H thymidine was added for 18 h and T cell responses were determined and displayed as ^{3}H thymidine cpm +/- SEM. Data are representative of three experiments. The asterix represents statistical significance determined by one-way ANOVA where $p<0.05$.

5.2.4 A comparison of aggrecan presentation by the different APC to aggrecan-specific T cells isolated from TCR-5/4E8-tg mice.

So far I have shown that aggrecan-specific B cells are efficient APC capable of aggrecan presentation comparable to that of dendritic cells and at a far greater efficiency to that of non-specific B cells. I next wanted to examine aggrecan presentation by the different APC to another population of aggrecan-specific T cells (5/4E8) isolated from TCR-5/4E8-transgenic mice.

5.2.4.1 Phenotype of T cells from TCR-5/4E8 transgenic mice.

Therefore, I isolated 5/4E8 T cells from the spleens of TCR-5/4E8 transgenic mice. 5/4E8 CD4+ T cells express a functional TCR specific for the aggrecan epitope ATEGRVRVNSAYQDK found within the epitope 84-103. The TCR is composed of Vα1.1 and Vβ4 chains. To verify the purity of the CD4+ T cells isolated from spleens of these mice, CD4 expression was detected along with TCRαβ by flow cytometry. The percentage of Vβ4+ cells was also evaluated by flow cytometry as a marker of aggrecan-specific TCR expression **(Figure 5.16)**.

Results confirm that 97.7% of CD4+ isolated cells are indeed CD4+ and 94.2% of the isolated CD4+ cells express a Vβ+ TCR.

5.2.4.2 Aggrecan presentation to T cells from TCR-5/4E8 transgenic mice.

Dendritic cells, macrophages, C71-4C5, C71-5F10 B cells and A20-1HS B cells were cultured with the 5/4E8 T cells for 72 h in the presence of either deglycosylated aggrecan or p84-103. Isolated T cells alone (no APC) were used as a negative control to determine whether a small percentage of contaminating APC from MACS separation is enough to generate an IL-2 response to aggrecan. Activation of transgenic T cells was measured by IL-2 production using CTLL-2. Results in **Figure 5.17a** showed that C71-4C5 were the most efficient at presenting low dose (0.01nM) aggrecan to the T cells 5/4E8 $p<0.01$. Macrophages required around 10-fold more aggrecan (0.1 nM) for efficient presentation to the T cells 5/4E8 and A20-1 HS were unable to generate a T cell response above the level of the no APC control. Surprisingly, the addition of A20-1-HS generated slightly reduced IL-2 production compared to the no APC control. The no APC control required >10nM doses of aggrecan to generate an IL-2 response indicating that either the low levels of contaminating APC present in the isolated T cell population are sufficient to present high doses of aggrecan to the T cells 5/4E8 or that the T cells 5/4E8 produce IL-2 in the presence of high increasing doses of

aggrecan without the requirement for antigen presentation. Data in **Figure 5.17b** shows that dendritic cells are most efficient at presenting >0.05 nM p84-103 to the 5/4E8 T cells $p<0.001$ followed by macrophages and C71-4C5 and C71-5F10 B cells which require approximately 10 times more p84-103 for efficient presentation. The no APC and A20-1 HS controls showed weak responses to >1 nM p84-103. Surprisingly, increasing the dose of p84-103 to 10nM reduced presentation by A20-1 HS to just above background.

5.2.5 Cytokine analysis of CD4+ T cells from TCR-5/4E8-tg mice.

PGIA is thought to be reliant on a Th1 response, mediated by the cytokine IFNγ (Hollo, Glant et al. 2000). Therefore, I wanted to investigate if there were any differences in the cytokine profile of the 5/4E8 T cells when they were activated *in vitro* by the different types of APC. It is possible that APC are able to promote an anti-inflammatory response following antigen presentation to the 5/4E8 aggrecan-specific T cells. To address this possibility I wanted to determine whether any of the APC promoted a regulatory type IL-10 response following aggrecan presentation to the 5/4E8 T cells.

Therefore, supernatant from the above antigen presentation assays was examined by ELISA to determine the cytokines produced by the CD4+ TCR-5/4E8 T cells in response to p84-103 or aggrecan presentation by the different APC populations. Controls for this experiment included blank wells coated with capture antibody and subsequently incubated with media only. Supernatant from wells cultured with T cells and antigen only (no APC) and supernatant from wells cultured with APC and antigen only. The concentration of IL-10 or IFNγ in the supernatant was calculated from standard curves using recombinant IL-10 and IFNγ. Results in **Figure 5.18a** showed that increasing amounts of IFNγ (up to 9.4 ng/ml) was detected in the supernatant taken from 5/4E8 T cells cultured with C71-4C5 and doses of aggrecan >0.5 nM. IFNγ was detected from wells containing 5/4E8 T cells and C71-5F10, dendritic cells or macrophages and higher doses of 1 nM aggrecan. However, there were much higher doses of 4.7 ng/ml IFNγ detected in wells containing the C71-5F10 cells around 5 times higher than that detected in response to aggrecan presentation by dendritic cells 1.5 ng/ml or macrophages 1.3 ng/ml. No IFNγ was detected in supernatant taken from wells containing A20-1 HS and 5/4E8 T cells or the no APC control. Data indicates that aggrecan presentation by aggrecan-specific B cells C71-4C5 and C71-5F10 produces a much greater IFNγ response than presentation by dendritic cells or macrophages.

Surprisingly the same result was not obtained when 5/4E8 T cells were cultured with APC in the presence of graded doses of p84-103. **Figure 5.18b** shows that IFNγ responses are highest when 5/4E8 T cells are cultured with macrophages and p84-103. IFNγ was detected in the supernatant taken from wells containing 5/4E8 T cells cultured with macrophages and >0.1 nM p84-103. Dendritic cells produced IFNγ responses from around 1 nM, whereas C71-4C5 required 5 nM 50 times more p84-103 than macrophages to produce an IFNγ response and C71-5F10 required 10 nM 100 times more p84-103. IFNγ was not detected in supernatant taken from wells containing A20-1 HS or the no APC control. Results in **Figures 5.18 c and d** show that IL-10 was not detected in the supernatant taken from any of the antigen presentation conditions indicating that aggrecan and p84-103 presentation to 5/4E8 T cells by APC *in vitro* does not result in a regulatory IL-10 response. However, presentation of aggrecan to 5/4E8 T cells by aggrecan-specific B cells C71-4C5 and C71-5F10 results in a high level of IFNγ production indicative of a Th1 type response.

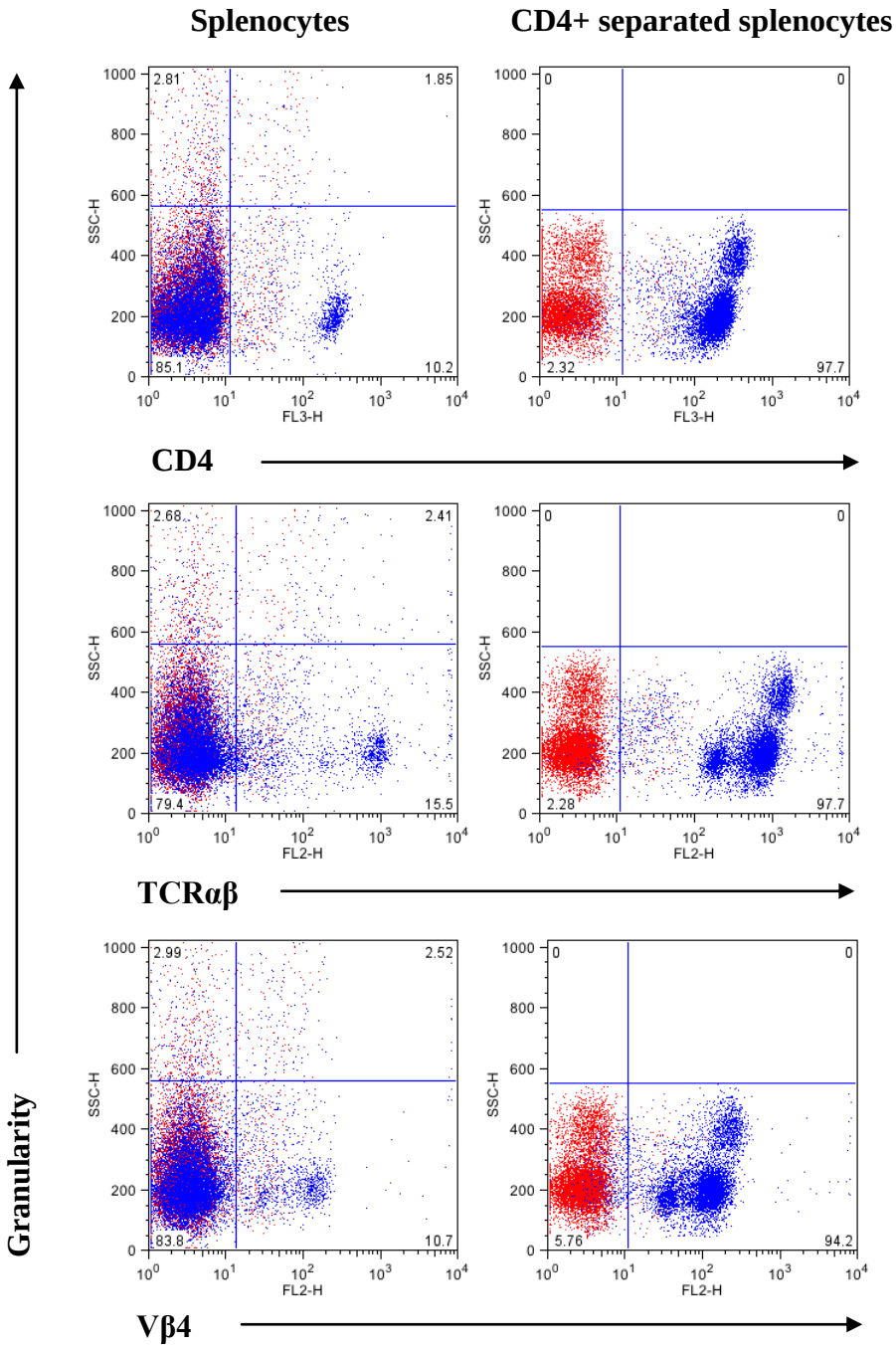
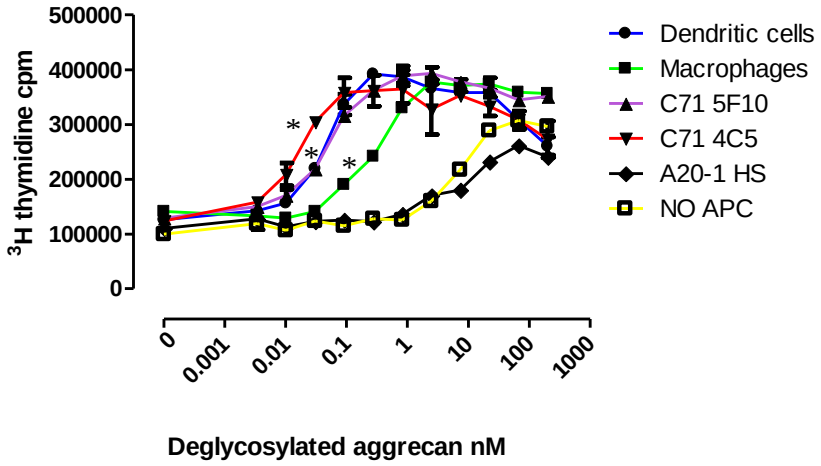


Figure 5.16 Flow cytometry of isolated spleen cells and CD4+ separated cell populations with CD4, TCRαβ and Vβ4-specific antibodies. *Staining of isolated spleen cells or CD4+ MACS separated cells with CD4, TCRαβ or Vβ4 antibodies. All experiments were carried out at 4°C in the presence of Fc block. Data is representative of 2 independent experiments. The percentage of cells per quadrant is indicated.*

a)



b)

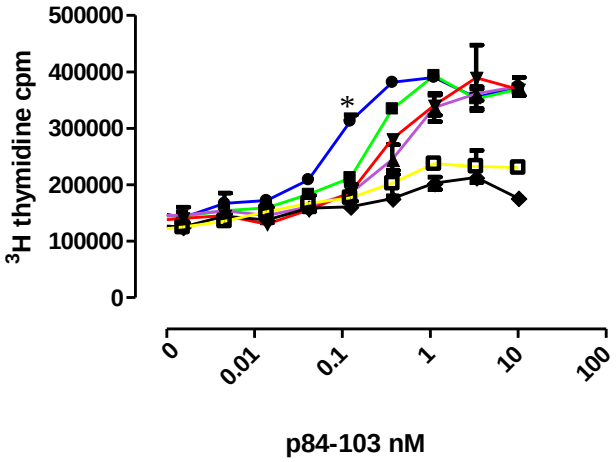


Figure 5.17 Aggrecan and p84-103 presentation to 5/4E8 CD4+ T cells by bone marrow derived dendritic cells, bone marrow derived macrophages, aggrecan-specific B cell transfectants C71-5F10 or C71-4C5 and A20-1 HS. *Aggrecan-specific 5/4E8 CD4+ T cells were isolated from the spleens of transgenic mice and separated on an LS separation column in the magnetic field of the VarioMacs separator using CD4 (L3T4) microbeads. 5x10^4 isolated T cells were cultured with APC (1x10^4) in the presence of graded doses of aggrecan or p84-103 for 72 h. Cells were killed by freezing at -80^{o}C and supernatant was removed for bioassay with CTLL-2 cells. Tritiated thymidine was added for 18 h and T cell responses were determined and displayed as ^{3}H thymidine cpm +/- SEM. Data are representative of two experiments. The asterix represents statistical significance determined by one-way ANOVA where p<0.05.*

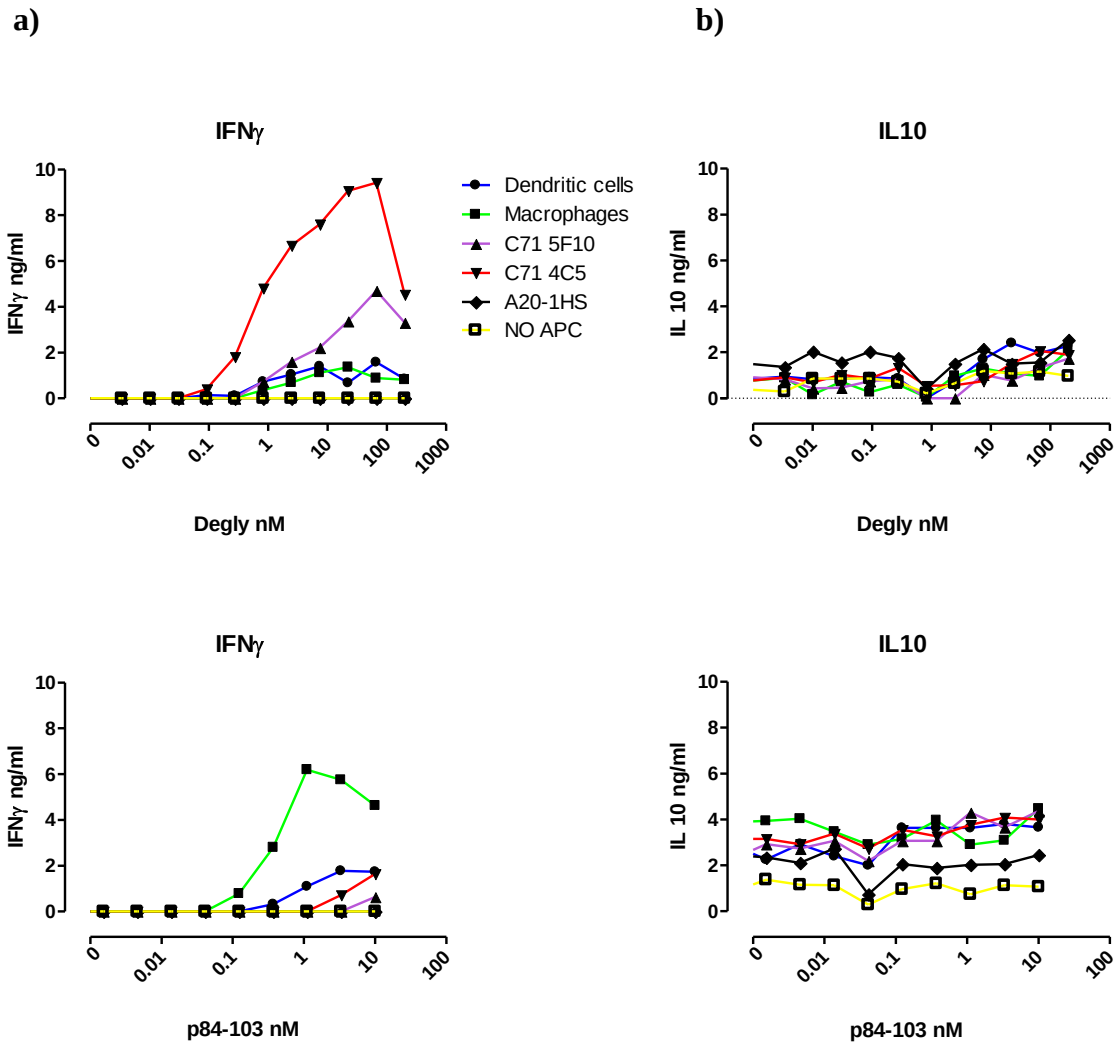


Figure 5.18 IFNγ and IL-10 levels detected in the supernatant 5/4E8 T cells cultured with aggrecan or p84-103 and the different APC. *Aggrecan-specific CD4+ T cells 5/4E8 ($5x10^4$) were cultured in the presence of graded doses of aggrecan or p84-103 and APC ($1x10^4$) for 72 h. Cells were killed by freezing at -80°C and supernatant was removed for IFNγ and IL-10 detection by ELISA. Data are representative of two experiments. The asterix represents statistical significance determined by one-way ANOVA where $p<0.05$.*

5.3 Discussion

The aim of this chapter was to examine the kinetics and mechanisms of aggrecan processing and presentation by the aggrecan-specific B cell clones C71-4C5 and C71-5F10 in comparison to that seen in dendritic cells and macrophages. Results from the kinetics study confirm the optimal time required by the various APC, the number of APC required and dose of aggrecan required for efficient presentation to the aggrecan-specific T cell hybridoma 192. In addition, the different APC were incubated with aggrecan in the presence of a number of reagents that have previously been demonstrated to have profound effects on antigen processing. This enabled dissection of the different components of the aggrecan processing and presentation pathway and established which steps were important or indeed vital in generating the 84-103 immunodominant arthritogenic epitope from this important candidate joint autoantigen. In addition to detecting 84-103 recognition by a well characterized T cell hybridoma (192), I have also examined the effects of 84-103 presentation by the various APC on naïve T cells isolated from transgenic mice expressing the 5/4E8 aggrecan 84-103-specific TCR. APC were cultured with the aggrecan-specific T cells 5/4E8 and graded doses of aggrecan or p84-103 to establish which APC were the most efficient at presenting aggrecan to *ex vivo* aggrecan-specific CD4+ T cells. These experiments also demonstrated the levels of both the pro-inflammatory cytokine IFNγ and the anti-inflammatory cytokine IL-10 produced in response to aggrecan/p84-103 presentation by the 4 different APC. The results are discussed below.

5.3.1 Kinetics of aggrecan presentation

The kinetics of antigen processing by B cells has been extensively studied. To summarize, presentation of HEL (14 kDa) is presented with different efficiencies dependent on the antigen/BCR interaction. B cells expressing anti-HEL BCR with high affinity (Ka 5 x10^{10} M^{-1}) for example, are capable of presenting HEL at concentrations as low as 0.5nM, whereas those B cells expressing a BCR with a low affinity (Ka 3 x10^{8} M^{-1}) require approximately 10 times more HEL for equivalent T cell stimulation (Batista and Neuberger 1998). Using a different model antigen (TTCF; 47 kDa) Lanzavecchia demonstrated that TTCF-specific B cells require 10^{4} fold lower concentrations of TTCF (10^{-11}-10^{-12}M) than non-specific B cells (Lanzavecchia 1985). Casten and colleagues have studied the presentation kinetics of the globular antigen pigeon cytochrome c (PCC; ~12 kDa) by non-specific B cells and when PCC is targeted to the BCR via PCC linkage to anti-Ig fragments. Data from the PCC kinetics study revealed that specific uptake via the BCR requires 10^{3} times less PCC than nonspecific

B cell for T cell presentation (Casten, Kaumaya et al. 1988). The consensus from these and other studies is that B cells that do not have an antigen- specific BCR require 10^3-10^4 more antigen for efficient presentation to T cells (Rock, Benacerraf et al. 1984; Abbas, Haber et al. 1985; Lanzavecchia 1990). Hapten-linked proteins have also been used to define the antigen uptake and kinetics of a variety of antigens using a single hapten-specific B cell line. Using this method antigens can be linked via haptens such as 2,4,6 trinitrophenol (TNP), 4-hydroxy-3-nitro-phenol-acetyl (NP) or fragments of anti-Ig antibodies (Abbas, Haber et al. 1985; Casten and Pierce 1988). Hapten-linked proteins are a good model for defining B cell uptake, activation and signalling in response to BCR-ligand binding. However, the effects on modifying antigen in an unphysiological way such as linking of haptens may have an effect on antigen processing by the APC by preventing access to various proteolytic sites.

Thus, most previously described reports have involved the study of the presentation of simple model antigens and not a large (250 kDa) complex candidate autoantigen such as aggrecan. However, there are several lines of evidence to suggest that B cells play a role in the presentation of this candidate RA autoantigen (Brennan, Mikecz et al. 1995; O'Neill, Shlomchik et al. 2005; O'Neill S, Cao et al. 2007). Indeed, elegant studies by O'Neill *et al* confirmed that B cells are essential for the induction and transfer of PGIA (O'Neill, Shlomchik et al. 2005). Furthermore, they demonstrated that the role of B cells was not only antibody production but also as an essential population of antigen-specific APC. Therefore, in an attempt to identify novel aspects of antigen presentation by aggrecan-specific B cells, I optimised a series of experiments allowing me to dissect aggrecan processing and presentation by different APC types.

As the induction of PGIA requires immunisation with deglycosylated aggrecan in adjuvant, I wanted to confirm which APC were proficient at presenting both this form of aggrecan and it's fully glycosylated form. Interestingly, results confirmed that aggrecan-specific B cells (C71-4C5) were as efficient as dendritic cells and more efficient than macrophages at presenting deglycosylated aggrecan to T cell hybridomas 192. However, it was macrophages that were the most efficient APC when presenting the glycosylated form of aggrecan. This is possibly due to the high expression of carbohydrate receptors by macrophages such as CD44 which triggers phagoctosis of large antigens (Sterling, Saginario et al. 1998; Taylor, Martinez-Pomares et al. 2005). Presentation of the synthetic peptide p84-103 by dendritic cells and macrophages was at least 10^2 times more efficient than that of aggrecan-specific B

cells (**Figure 5.3**). These findings imply that the efficient presentation of deglycosylated aggrecan by B cells is due to recognition of the aggrecan structure and efficient uptake and processing as modifications such as glycosylation reduce the presentation capacity of the aggrecan-specific B cells. In addition, as the aggrecan-specific B cells are relatively poor at presenting peptide p84-103 which does not require any further processing to be presented by APC this implies that the efficiency of aggrecan-specific B cells as APC lies in their ability to very efficiently take up and process aggrecan. Therefore it is possible that the results from O'Neill and colleagues could be explained by the fact that aggrecan-specific B cells are extremely efficient APC at presenting the immunogenic deglycosylated form of aggrecan and are required in this capacity to generate an immune response against aggrecan and a severe form of PGIA (O'Neill, Shlomchik et al. 2005; O'Neill S, Cao et al. 2007).

Furthermore, it has been shown that B cells can divert the immune response from one epitope to another by the paradigm known as 'molecular mimicry'. Molecular mimicry describes a process where foreign peptides resembling a self protein can initiate an immune response against self antigens. An example of this is rheumatic fever where B cells can recognise and promote an antibody response toward streptococcal M protein that also recognises and cross reacts with cardiac myosin (Liang and Mamula 2000). Indeed, this phenomenon is also typical for autoimmune disease models for example primary billiary cirrhosis (PBC) in mice is only induced with the antigen pyruvate dehydrogenase complex (PDC) from other species, or if mouse PDC is modified e.g. by biotinylation it will induce antibodies that cross-react to non- biotinylated mouse PDC (Palmer, Robe et al. 2004). Therefore, as PGIA is only initiated by the immunisation of deglycosylated human or bovine aggrecan and not mouse aggrecan, it is possible that B cells are involved in efficiently taking up the aggrecan and presenting different epitopes that lead to a break in self- tolerance.

Recent data has confirmed a citrullinated form of aggrecan p84-103 was able to generate responses in T cells isolated from RA patients (von Delwig, Locke et al. 2010). Therefore, time permitting it would have been interesting to investigate the processing and presentation of citrullinated whole aggrecan as an alternate modified autoantigen. This coupled with the finding that ~70% RA patients have antibodies to cyclic citrullinated peptides makes this an exciting prospect for future studies (Nishimura, Sugiyama et al. 2007).

Studies have shown that small numbers of antigen-specific B cells residing in lymphoid follicles are sufficient to present low amounts of antigen, early in the immune response (Curtsinger, Schmidt et al. 1999). To investigate the amount of aggrecan-specific B cells required to generate a T cell response in comparison to dendritic cells and macrophages, APC numbers were titrated and cultured with T cell hybridoma 192 in the presence of graded doses of either aggrecan or p84-103 (**Figures 5.4 and 5.5**). Results confirmed that 600 dendritic cells, macrophages or aggrecan-specific B cells were sufficient to present aggrecan or p84-103 to $5x10^4$ T cell hybridoma 192. Reducing the number of APC had no effect on the differences between the efficiency of aggrecan or p84-103 presentation by the different APC observed in earlier experiments (see **Figure 5.3**). What is interesting to note is that 600 aggrecan-specific B cells can efficiently process and present >0.01 nM of deglycosylated aggrecan to the T cell hybridomas 192, whereas they require >1nM p84-103 that requires no further processing for presentation. However, dendritic cells and macrophages can present >0.01 nM doses of either antigen efficiently. These data imply that optimal MHC class II loading of the 84-103 epitope requires its generation within the endosomal pathway by aggrecan-specific B cells. One possibility is that following BCR ligation there is an upregulation or more rapid turnover of MHC class II on the surface of the aggrecan-specific B cells. It has been shown that MHC class II surface expression is regulated via the ubiquitin system (Matsuki, Ohmura-Hoshino et al. 2007). Therefore, it is possible that BCR signalling is involved in this regulation. Indeed it has also been previously shown that BCR signaling triggers the re-organization of MHC class II molecules and influence the site of antigen processing (Siemasko, Eisfelder et al. 1998). An increase in MHC class II surface expression or intracellular re-organization could have been established by examining expression by flow cytometry both prior to and following aggrecan-specific B cell culture with aggrecan for 24 h.

Fixation of APC to study antigen processing by APC has been previously described (Shimonkevitz, Kappler et al. 1983). Once the APC is fixed it is metabolically inactive and can no longer process or transport antigen. Therefore, APC can be incubated with antigen and processing can be halted at various timepoints to determine the length of time required for efficient antigen processing and presentation. Alternately, APC can be pre-incubated with modulators of intracellular pH, inhibitors of protein synthesis, ER egress, and various proteinase inhibitors to examine the antigen processing pathway required by the different APC. An essential control for all the fixation experiments in this project, was to fix the APC

prior to incubation with aggrecan and p84-103, if the cell is sufficiently fixed aggrecan will not be presented yet p84-103 will bind to MHC class II already on the APC cell surface and should still be efficiently presented following 24 h culture with the T cell hybridoma 192. Using this fixation system a timecourse of aggrecan and peptide presentation by the different APC was established. I confirmed that macrophages required a minimum of 6 h to efficiently process aggrecan and generate 84-103-MHC class II complexes, yet aggrecan-specific B cells C71-4C5 and dendritic cells began to process aggrecan and generate 84-103-MHC class II complexes after 2 h incubation with aggrecan implying faster kinetics and possible peptide loading of recycling molecules. The 5 h required to present the 84-103 epitope suggests that transport of newly synthesised MHC class II that takes around this time (Delvig and Robinson 1998; Musson, Hayward et al. 2002).

Expectedly p84-103 required less time for presentation and 84-103-MHC class II complexes were generated following <1 h incubation with p84-103. Interestingly A20-1 HS were the most efficient at generating 84-103-MHC class II complexes (maximal following 1 h incubation with p84-103) despite being unable to present native aggrecan following post-fixation, whereas dendritic cells and macrophages require 5 h before 84-103-MHC class II generation is optimal. These data imply that A20-1 HS cells are less sensitive to the fixation process than dendritic cells and macrophages possibly due to the cell morphology. In support of this the aggrecan-specific B cells C71-5F10 only required 2 h. However, C71-4C5 also required 5 h for efficient p84-103 presentation and reason for the observed differences between the 2 aggrecan-specific B cell clones is not known.

Despite the observation that optimal 84-103-MHC class II generation occurs in dendritic cells, macrophages and the two aggrecan-specific B cells tested following a 12 h incubation period with aggrecan, this timepoint is too long for optimal use in these assays as the inhibitors used may loose function during this extended period prior to fixation. In support of this, Katie Lowes in our laboratory previously optimised the timepoint required for macrophage processing of aggrecan (Lowes 2005). Therefore the minimum 6 h timepoint was chosen for inhibitor studies.

5.3.2 Effect of inhibiting steps in the antigen processing pathway

Inhibitor	Dendritic cells	macrophages	C71-4C5/ C71-5F10
Cycloheximide (protein synthesis)	**inhibited**	**inhibited**	**inhibited**
Brefeldin A (Golgi transport)	**inhibited**	**Partially inhibited**	**inhibited**
Ammonium chloride (raise Ph)	**inhibited**	**inhibited**	**inhibited**
E-64d (cysteine proteinases)	**no effect**	**no effect**	**no effect**
3'4-DCI (serine proteinases)	**inhibited**	**inhibited**	**inhibited**
Leupeptin (cysteine and serine proteinases)	**Partially inhibited**	**Partially inhibited**	**inhibited**
Pepstatin A (aspartyl proteinases)	**Partially inhibited**	**inhibited**	**inhibited**
Phenanthroline (metalloproteinases)	**inhibited**	**Partially inhibited**	**inhibited**

Table 5.1 Summary of inhibitor data

The table summarizes whether the different inhibitors of antigen processing inhibited the processing and presentation of the 84-103 epitope to T cell hybridomas 192.

5.3.2.1 Inhibitors of protein synthesis and transport

In order to determine whether efficient aggrecan presentation by dendritic cells, macrophages or aggrecan-specific B cells required the synthesis and transport of newly synthesised MHC class II, APC were pre-incubated with inhibitors of protein synthesis (cycloheximide) and Golgi transport (brefeldin A). Data collected using cycloheximide, suggests that newly synthesised MHC class II molecules are required for efficient generation of 84-103/MHC class II complexes following aggrecan processing by dendritic cells, macrophages and aggrecan-specific B cells (**Figure 5.8**). However, contrasting data was obtained for macrophages using the Golgi transport inhibitor brefeldin A (**Figure 5.9**). Cycloheximide completely abolished the generation of 84-103-MHC class II complexes following aggrecan processing by dendritic cells, macrophages and aggrecan-specific B cells. However, brefeldin

A only abolished 84-103-MHC class II complex generation by dendritic cells and aggrecan-specific B cells but had minimal effects on macrophages.

One possible explanation for this observation is that the lowest dose of cycloheximide (10 μM) also had quite a profound effect on the generation of 84-103 MHC class II complexes following incubation of macrophages with p84-103 and a less profound effect following incubation of dendritic cells and aggrecan-specific B cells with p84-103. Therefore, cycloheximide may be toxic to macrophages and the observed effects on aggrecan presentation may be anomalies. Taken this into account it is possible that macrophages and not dendritic cells or aggrecan-specific B cells may present the 84-103 epitope on recycling MHC class II molecules independently of newly synthesised MHC class II. Indeed, greater molar doses of ammonium chloride used to lower intracellular pH were also required to prevent aggrecan presentation by macrophages in comparison to dendritic cells and B cells (**Figure 5.10**). This implies that macrophages may be able to process aggrecan in a higher pH environment such as those found in the early endosomes of the recycling pathway. Further titrations of cycloheximide may have determined a lower dose able to inhibit aggrecan presentation and not p84-103 presentation by macrophages. Alternately different inhibitors of the classical pathway of presentation such as emetine (protein synthesis inhibitor) or monensin (Golgi transport inhibitor) may have clarified the conflicting macrophage results.

5.3.2.2 Ammonium chloride

Raising intracellular pH is known to reduce antigen processing and MHC class II loading of epitopes derived from antigens such as HEL and the matrix protein of influenza virus (Griffin, Chu et al. 1997; Pinet and Long 1998). As the antigen travels through the various intracellular compartments the environment becomes increasingly acidic early endosomes 6.0-6.5, late endosomes 5.0-6.0 and lysosomes 4.5-5.0 (Berzofsky, Brett et al. 1988). Low pH found in late endosomes/lysosomes is required for the activation of various intracellular proteinases cathepsin D and dissociation of HLA-DO to allow peptide exchange by HLA-DM (Yamashiro and Maxfield 1987; Liljedahl, Winqvist et al. 1998). To assess the requirement for low intracellular pH for aggrecan processing dendritic cells, macrophages and aggrecan-specific B cells C71-4C5 and C71-5F10 were pre-treated with ammonium chloride to raise intracellular pH prior to culture with aggrecan. Ammonium chloride inhibited the generation of 84-103 MHC class II complexes following aggrecan and not p84-103 processing by dendritic cells (**Figure 5.10**) and aggrecan-specific B cells. This data suggests that a low

endosomal pH is required for efficient peptide generation and/or MHC class II loading in these APC. The observed effects are not due to toxicity as ammonium chloride had no effect on the presentation of p84-103 by these APC.

Interestingly, macrophages required twice as much ammonium chloride to abolish the generation of 84-103 MHC class II complexes following aggrecan processing. This concurs with my brefeldin A data that suggests that following aggrecan processing 84-103 may be loaded onto recycling epitopes earlier in the endosomal pathway where the pH is higher, thus requiring more ammonium chloride to have a substantial effect on the pH environment within this compartment. Ammonium chloride had no effect on the generation of 84-103 MHC class II complexes following incubation with p84-103 with macrophages. Therefore the observed effects were not due to ammonium chloride toxicity.

5.3.2.3 Cysteine proteinase inhibitors

Cysteine proteinases such as members of the cathepsin family have been shown to play a role in both antigen processing and removal of the li chain from the peptide binding groove of MHC class II molecules (Honey and Rudensky 2003). To determine the role of cysteine proteinases in the generation of 84-103 MHC class II complexes following aggrecan processing by dendritic cells, macrophages and aggrecan-specific B cells, APC were pre-treated with the broad spectrum cysteine proteinase inhibitor E-64d.

Results showed that pre-incubation of APC with E-64d had no effect on the (**Figure 5.11)** generation of 84-103 MHC class II complexes following incubation with either aggrecan or p84-103 by any of the APC. It has been previously shown that 2.5 μM of E-64d is sufficient to prevent the presentation of V antigen to V antigen-specific T cells by macrophages (Shim, Musson et al. 2006). However, doses up to 100 μM had no effect on the presentation of aggrecan by any of the APC. It is possible that the inhibitor was no longer active, and to test this I attempted to repeat the findings of Shim *et al* using the V antigen-specific T cells and APC pre-treated with various doses of E-64d and V antigen. However, I was unable to optimise this antigen presentation system. Although it is possible that the E64-d inhibitor was not active, this is unlikely as several different batches were tested and the observation that E64-d has no effect on aggrecan processing by macrophages has been previously shown (Lowes 2005). Therefore, these data concur that cysteine proteinases do not play a role in the generation of 84-103 MHC class II complexes by macrophages, or by dendritic cells or B cells.

5.3.2.4 Aspartic proteinases

Another of the proteinase family implicated as playing a role in both antigen processing and li chain removal are the aspartic proteinases including cathepsins D and E (Villadangos, Bryant et al. 1999). To investigate the role of aspartic proteinases in the generation of 84-103 MHC class II complexes following aggrecan processing by dendritic cells, macrophages and aggrecan-specific B cells, APC were pre-treated with the aspartic proteinase inhibitor Pepstatin A. Pepstatin A abolished aggrecan presentation by aggrecan-specific B cells and macrophages suggesting that aspartic proteinases are essential in the generation of 84-103 complexes by these APC (**Figure 5.12**). Interestingly, presentation of p84-103 was slightly increased in these APC following pre-treatment with pepstatin A. This suggests that aspartic proteinases may have a destructive effect on p84-103 processing. Presentation by dendritic cells was only partially blocked suggesting that aspartic proteinases are not absolutely required by dendritic cells for the generation of the 84-103 epitope and subsequent MHC class II loading. In addition pepstatin A did not have any effect on p84-103 presentation by dendritic cells suggesting that aggrecan processing by dendritic cells differs in its requirement for aspartic proteinases. E64-d data suggests that dendritic cells do not require cysteine proteinases for aggrecan processing. Therefore, in the absence of cysteine and aspartic proteinases there is still efficient li chain degradation to allow aggrecan presentation by dendritic cells. Future work may therefore uncover novel mechanisms of aggrecan processing by dendritic cells.

5.3.2.5 Serine proteinases

Serine proteinases have been implicated in the extracellular or cell surface processing of antigens at a neutral pH (von Delwig, Musson et al. 2003). To investigate the role of serine proteinases in the generation of 84-103 MHC class II complexes following aggrecan processing by dendritic cells, macrophages and aggrecan-specific B cells, APC were pre-treated with the broad spectrum serine inhibitor DCI or the cysteine and serine inhibitor leupeptin. Results showed that DCI abrogated aggrecan presentation by aggrecan-specific B cells at a low dose of 10 μM (**Figure 5.13**). However, dendritic cells required twice this amount to abrogate aggrecan presentation and macrophages required 4 times this amount. Despite these differences aggrecan presentation and not p84-103 presentation was eventually abolished in dendritic cells and macrophages suggesting a role for serine proteinases in the optimal presentation of aggrecan by all APC types. p84-103 presentation was slightly enhanced following the pre-treatment of aggrecan-specific B cells and macrophages but not

dendritic cells with 20 μM of DCI. These data imply that serine proteinases can mediate destructive processing of p84-103 and yet are absolutely required for the optimal processing of aggrecan in aggrecan-specific B cells and macrophages.

Results from leupeptin inhibition of serine and cysteine proteinases revealed an abrogation in both peptide and aggrecan presentation by aggrecan-specific B cells, partial abrogation of aggrecan and not p84-103 presentation by dendritic cells and partial abrogation of aggrecan and p84-103 presentation by macrophages at a lower leupeptin dose that was recovered at a higher dose of 20 μM (**Figure 5.14**). Leupeptin is a broad range inhibitor and its use is difficult to interpret. However, from my results using E64-d (cysteine) and DCI (serine) inhibitors, it is most likely that leupeptin is having its effect on serine proteinases for the abrogation of aggrecan presentation by the different APC. The finding that it also abrogates p84-103 presentation by aggrecan-specific B cells may be due to toxic effects on B cells and further optimisation of leupeptin dose may have overcome this problem. The recovery of aggrecan and p84-103 presentation by high doses of leupeptin in macrophages may be due to the inhibition of proteinases that require a high dose of leupeptin and mediate destructive processing of 84-103 in macrophages which is abolished by doses of >20 μM.

5.3.2.6 Metalloproteinases

Metalloproteinases are a class of proteolytic enzymes shown to regulate the extra-cellular environment including normal turnover of the ECM of joint cartilage at a neutral pH (Cawston and Young 2010). However, matrix metalloproteinases play a significant pathological role in the degradation of cartilage in the inflamed synovium of RA patients most likely due to a lack of balance between their own expression and that of their endogenous inhibitors TIMPs (Yoshihara, Nakamura et al. 2000). It has been previously shown that dendritic cells, macrophages and B cells can secrete these proteinases into the extracellular environment possibly contributing to the breakdown of ECM components in inflammatory conditions such as RA as well as being involved in the breakdown of the basement membrane and cell migration (Kouwenhoven, Ozenci et al. 2002; Melamed, Messika et al. 2006; West, Prescott et al. 2008; Elkington, Green et al. 2009). As these important APC are equipped to degrade joint components it is possible that they can also begin to 'process' joint autoantigens such as aggrecan extracellularly and prior to uptake for presentation to T cells.

To investigate the role of metalloproteinases in the generation of 84-103 MHC class II complexes following aggrecan processing by dendritic cells, macrophages and aggrecan-specific B cells, APC were pre-treated with the broad metalloproteinase inhibitor phenanthroline. Results showed that 1000 μM phenanthroline abrogated aggrecan and not peptide p84-103 presentation by dendritic cells and aggrecan-specific B cells, yet only partially inhibited aggrecan presentation by macrophages. These data suggest a major role for metalloproteinases in aggrecan processing by dendritic cells and aggrecan-specific B cells and an involvement in aggrecan processing by macrophages.

A full screen of MMP expression by the different APC types in response to inflammatory stimuli such as the exposure to synovial fluid from PGIA diseased mice may confirm which MMP are important in a disease scenario. Identification of MMPs involved in antigen processing is a novel concept with great scope for further investigation such as how their expression is regulated, whether the APC also secrete TIMPs and how is this balanced during inflammation. These could be studied by examining APC processing and presentation in MMP knockout mice. Furthermore, MMP cleavage sites in candidate autoantigens such as aggrecan and collagen II have been identified (see **Figure 1.5**). Therefore, by identifying specific MMP involved in antigen processing their effects on site-specific joint autoantigen cleavage can be analysed and possibly even targeted to prevent presentation of arthritogenic epitopes and induction of PGIA.

5.3.3 Presentation to the 5/4E8 transgenic T cells

In order to further investigate the efficiency and mechanisms of aggrecan presentation by dendritic cells, macrophages and aggrecan-specific B cells I wanted to investigate the consequence of antigen presentation and cytokine production in response to presentation to the aggrecan-specific TCR transgenic T cells 5/4E8. All of the previous studies had been performed using T cell hybridomas. The disadvantage of T cell hybridomas is that it is not entirely clear how typically representative T cells that form hybridomas are. In addition, the contribution of oncogenes and cell surface proteins provided by the BW5147 fusion partner may not sufficiently represent a physiological situation *in vivo*. 5/4E8 T cells are efficient at mediating PGIA in mice with a susceptible background and 5/4E8 T cells were shown to differentiate into Th1 phenotype T cells by the secretion of IL-2 and IFNγ and not IL-4 in response to stimulation with aggrecan (Buzas, Brennan et al. 1995; Berlo, van Kooten et al. 2005; Berlo, Guichelaar et al. 2006). I have confirmed that aggrecan-specific B cells C71-4C5 were the most efficient at presenting aggrecan to the 5/4E8 T cells, around 5 times more efficient than dendritic cells, around 10 fold more efficient than macrophages and 10^3 fold more efficient than the non-specific B cells A20-1 HS (**Figure 5.17**). The isolated T cell population contained a small population of class II positive cells (5%) that mediate background proliferation in response to p84-103 or aggrecan. Interestingly, the addition of A20-1 HS B cells slightly reduces this background proliferation in response to both p84-103 and aggrecan. A possible explanation for this is that the A20-1 HS do not contribute to aggrecan/p84-103 presentation to 5/4E8 T cells but are able to internalize IL-2. Alternately, proliferating A20-1 HS cells are reducing IL-2 responses by physically crowding the wells and impeding optimal antigen presentation by contaminating APC.

Cytokine production in response to aggrecan or peptide presentation by the different APC to 5/4E8 T cells was investigated (**Figure 5.18**). Low levels of the anti-inflammatory cytokine IL-10 were detected in wells containing 5/4E8 T cells and macrophages, dendritic cells or aggrecan-specific B cells. However higher levels were detected in the wells containing A20-1 HS which do not express a BCR specific for aggrecan.

Interestingly the opposite effect was found in wells containing p84-103. IL-10 production in wells containing p84-103 was higher for dendritic cells, macrophages and aggrecan-specific B cells and lowest in wells containing A20-1 HS. As the wells lacking in antigen are just as high, the levels of IL-10 were not dependent on aggrecan or p84-103 dose and therefore not incremental with antigen presentation. Therefore, it was not possible to conclude whether IL-

10 detected in the supernatant represents background levels of the assay or non-specific levels of IL-10 production from the cells.

High levels of the Th1 cytokine IFNγ were detected in wells containing aggrecan-specific clones C71-4C5 and C71-5F10 and increasing levels were detected at higher aggrecan doses (up to 10 ng/ml IFNγ), whereas IFNγ levels in wells containing dendritic cells or macrophages were low (<2 ng/ml IFNγ) with no IFNγ detected in wells containing A20-1 HS. These data imply that the major APC responsible for Th1 responses to aggrecan are aggrecan-specific B cells. This is further justified by lower production of IFNγ in response to C71-5F10 than C71-4C5 as C71-4C5 is more efficient at aggrecan presentation to the 5/4E8 T cells. A20-1 HS which are poor at aggrecan presentation to 5/4E8 T cells did not produce any IFNγ response. Interestingly, the opposite effect was observed in response to p84-103 presentation to 5/4E8 T cells. In these experiments macrophages produce the largest IFNγ response up to 6ng/ml. Dendritic cells and C71-4C5 produced low levels in response to >1nm p84-103 and A20-1 HS and C71-5F10 did not produce any IFNγ. Again IFNγ responses by macrophages, dendritic cells and C71-4C5 were dose dependent implying that production is in response to antigen presentation. Control wells containing APC and antigen only were carried out for each cytokine and no IL-10 or IFNγ was detected, implying that either the cytokines are produced by T cells or by APC following T cell interaction. Indeed, Harris and colleagues have demonstrated that B cells are able to produce IFNγ in response to Th1 cell interaction or in response to the cytokines IL-12 and IL-18 (Harris, Goodrich et al. 2005). Therefore, the IFNγ response detected in wells containing aggrecan, C71-4C5 and 5/4E8 T cells may be a result of IFNγ production by 5/4E8 T cells which is then amplified by more IFNγ produced by C71-4C5 cells. It would be interesting to extend the study to a full screen of Th1, Th2 and Th17 cytokines, and controls such as non-aggrecan-specific T cells would have confirmed that the cytokine data was dependent on aggrecan or p84-103 presentation to 5/4E8 aggrecan-specific T cells. However, aggrecan-presentation by the aggrecan-specific B cells was as efficient as that of dendritic cells and produced more of the pro-inflammatory cytokine IFNγ, highlighting an important role for aggrecan-specific B cells not only as highly efficient aggrecan presenting cells but also in mediating the IFNγ Th1 phenotype observed in the PGIA model.

5.4 Summary

- Aggrecan-specific B cells are as efficient as dendritic cells at presenting low doses <0.001 nM deglycosylated aggrecan to the T cell hybridoma 192. They are 10^2 fold more efficient than macrophages and at least 10^4-10^5 fold more efficient than A20-1 HS non-specific B cells.
- Aggrecan presentation by dendritic cells and aggrecan-specific B cells is dependent on the 'classical' pathway of MHC class II presentation. My data also provides evidence that macrophages utilise both the 'classical' and the 'recycling' pathways of antigen presentation.
- Aggrecan presentation by dendritic cells, macrophages and aggrecan-specific B cells does not require cysteine proteinases.
- Aggrecan presentation by dendritic cells and aggrecan-specific B cells is dependent on serine and metalloproteinases. Macrophages are reliant on serine proteinases yet only require metalloproteinases for optimal aggrecan presentation.
- Aggrecan presentation by aggrecan-specific B cells and macrophages is dependent on aspartyl proteinases. Dendritic cells only require aspartyl proteinases for optimal aggrecan presentation.
- Aggrecan-specific B cell clones C71-4C5 are the most efficient APC type at presenting aggrecan to T cells isolated from TCR-5/4E8 transgenic mice.
- High IFNγ responses are detected in assay supernatant when aggrecan is presented to T cells isolated from TCR-5/4E8 transgenic mice by aggrecan-specific B cells in comparison to dendritic cells macrophages and nonspecific B cells.
- IL-10 (up to 4ng/ml) is detected in assay supernatant when p84-103 is presented to T cells isolated from TCR-5/4E8 transgenic mice by macrophages, dendritic cells, aggrecan-specific B cells and A20-1HS. IL-10 produced does not appear to be dependent on p84-103 dose.

6. General discussion

Studies over the past few years have identified elevated levels of aggrecan cleavage products, aggrecan-specific antibodies and aggrecan-specific T cells in RA patients (Poole and Dieppe 1994; Zou, Zhang et al. 2003; Vynios, Tsagaraki et al. 2006). In addition, germinal centre like structures, containing proliferating B and T cells have been found in many RA patients (Weyand, Goronzy et al. 2000). Rituximab is an anti-CD20 therapy that depletes both pre and mature B cells and has been used successfully to treat RA patients where other therapies have failed (Edwards and Cambridge 2006). This evidence, taken together with an abundance of data collected from mouse models of rheumatoid arthritis implicate a role for aggrecan and autoreactive aggrecan-specific T and B cells in the perpetuation of RA. Future studies to verify the mechanisms of aggrecan processing and presentation by B cells may uncover future therapeutic targets with which to ameliorate antigen-specific autoimmune diseases.

Therefore, the aims of this project were very much focussed on investigating a role for antigen-specific B cells as APC in a model of rheumatoid arthritis and examining the mechanisms by which B cells process and present the candidate rheumatoid arthritis autoantigen aggrecan.

I have successfully isolated aggrecan from bovine cartilage and removed chondroitin and keratanase sidechains to generate both glycosylated and deglycosylated forms. In addition I have modified deglycosylated aggrecan by the addition of biotin molecules for use as a molecular tool with which to determine the binding efficiency of both aggrecan-specific antibodies and aggrecan-specific BCRs. Finally, I have generated a reduced and alkylated form of aggrecan with which to study the binding efficiency of a previously generated anti-aggrecan antibody 1-C-6 (kindly donated by Dr Bruce Caterson) and the 1-C-6 BCR construct that I have generated during this project. In addition, this reduced and alkylated form of aggrecan has enabled me to determine the importance of structural integrity of aggrecan and the effects of this loss of structure on the recognition by antibody and BCR against native intact aggrecan.

To develop and *in vitro* aggrecan presentation system I have also generated bone marrow derived dendritic cells and macrophages from BALB/c ($H\text{-}2^d$) mice to use as APC for

presentation to both previously generated as well as several newly generated T cell hybridomas that recognise the various epitopes within the G1 and G3 domains of aggrecan.

Importantly, I have also generated several cloned aggrecan-specific B cell line able to present the immunogenic deglycosylated form of aggrecan at least as efficiently as the 'professional' APC dendritic cells, 10^2 fold more efficiently than macrophages and 10^4-10^5 fold more efficiently than non-specific B cells. Upon examination of the kinetics of aggrecan presentation by these aggrecan-specific cell lines, I have determined that aggrecan-specific B cells are again as efficient as dendritic cells at presenting aggrecan at a low APC: T cell ratio of 1:83 and both require at least 5 h for optimal aggrecan presentation.

To determine the mechanisms by which APC process and present aggrecan to 84-103 specific T cell hybridomas I have optimised the fixation conditions for dendritic cells, macrophages and aggrecan-specific B cells. Using a panel of known inhibitors of antigen processing I have established that both dendritic cells and aggrecan-specific B cells present aggrecan via the 'classical pathway' on newly synthesised MHC class II molecules with a requirement for a low pH environment indicative of the classical pathway and processing within late endosomes. However, although aggrecan presentation by macrophages was inhibited by cycloheximide, implying a requirement for newly synthesised MHC class II, inhibition of presentation was less efficient using brefeldin A to prevent Golgi transport of MHC II and higher doses of ammonium chloride required to raise pH. Therefore, it is possible that in contrast to dendritic cells and aggrecan-specific B cells, macrophages are able to present aggrecan on recycling MHC class II earlier in the endosomal pathway.

Inhibitors of cysteine, aspartyl, serine and metalloproteinases were used to determine the families of proteolytic enzymes involved in aggrecan processing by the different APC types. Data from these experiments revealed the requirement of aspartyl proteinases by macrophages and aggrecan-specific B cells. However, dendritic cells showed only a partial requirement for aspartyl proteinases. In addition, none of the APC showed any requirement for cysteine proteinases for aggrecan processing and presentation. Interestingly, I have found that serine proteinases are essential for the generation of the 84-103 epitope by all APC types. Serine proteinases are extracellular enzymes that function at neutral pH, which strongly implicates a role for extracellular processing of aggrecan by all APC types. Furthermore, generation of the 84-103 epitope by dendritic cells and aggrecan-specific B cells was

completely reliant on metalloproteinases and partially required by macrophages which again are extracellular enzymes active at a neutral pH. This finding in conjunction with previous studies in our laboratory is novel and suggests that aggrecan processing begins in the extracellular environment and not following antigen uptake. Indeed, as the RA synovium is rich in inflammatory mediators it is easy to envisage a scenario where initial aggrecan cleavage by MMPs releases fragments into the extracellular milieu containing infiltrating immune cells including T cells, B cells, dendritic cells and macrophages. In this environment APC activated by the pro-inflammatory stimuli are required to secrete additional MMP to further breakdown these large matrix degradatitve fragments prior to their uptake and processing within the cell. Furthermore, as this cascade is dependent on an initial localised inflammatory episode within the joint, under non-diseased circumstances there would be limited inflammatory cell infiltrate, hence no APC activation, secretion of MMP or presentation of this important joint autoantigen. Also, it is possible that other joint components such as the candidate autoantigen collagen II are processed by similar mechanisms but further investigation is required to clarify the role of MMP in the presentation of other joint components.

Time prevailing, expression profiling of aggrecan-specific B cells, dendritic cells and macrophages to define MMP or ADAMTS production under either resting, aggrecan stimulated or proinflammatory stimuli may have given some idea of their relative contribution to aggrecan processing. Following from this particular MMP or ADAMTS shown to be significantly upregulated could be investigated more closely to determine a) The level of secretion into the extracellular environment and b) the effects on aggrecan processing in *vitro* via MMP-aggrecan co-culture followed by Western blotting. In addition, I would have liked to determine the half-life of any MMP secreted by aggrecan-specific B cells, as another possibility is that local production of MMP in B cell rich environments such as ectopic germinal centres observed in some RA patients may have a contributing effect on the breakdown of cartilage directly. This could be examined in patients with ectopic germinal centres in comparison with those that have either none or diffuse T and B cell aggregates.

Although thus far I have elucidated that aggrecan-specific B cells are as efficient as dendritic cells and appear to process aggrecan in a similar way, I have not elucidated a novel role for B cells in the processing and presentation of aggrecan. However, studies using the PGIA mouse model of arthritis have implicated B cells as essential mediators of disease required for

optimal T cell activation and severe disease progression aside from there ability to produce antibodies. Therefore, as I have examined aggrecan presentation kinetics and processing requirements, another possibility for the importance of B cells in PGIA mice was cytokine production either directly by the B cells themselves or as a result of antigen presentation to aggrecan-specific B cells.

To examine this possibility I examined aggrecan presentation by the different APC to isolated T cells from 5/4E8 TCR transgenic mice expressing an aggrecan-specific TCR for the epitope 84-103. (These T cells represent a fair approximation of a physiological scenario of aggrecan presentation to naïve aggrecan-specific T cells). Data from these experiments concurred with my findings using the T cell hybridomas 192 that confirmed aggrecan-specific B cells were as efficient at presenting aggrecan to T cells as dendritic cells. However, upon examination of the amount of pro-inflammatory cytokine IFNγ present in supernatants following aggrecan presentation by the different APC, I found that aggrecan presentation by aggrecan-specific B cells promoted a 4-fold greater IFNγ response than that of dendritic cells, macrophages or nonspecific B cells.

PGIA is reportedly dependent on IFNγ and indeed levels of this pro-inflammatory cytokine can mediate the balance between T cell responses in mouse models.
Therefore, I have found a novel role for aggrecan-specific B cells as important APC involved in aggrecan-presentation and in mediating a Th1 response *in vitro*, as well as elucidating a novel role for MMPs in aggrecan processing and presentation by APC.

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