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DISSECTING THE CONTRIBUTION OF B CELLS IN AN EXPERIMENTAL MODEL OF RHEUMATOID ARTHRITIS

Paola Conigliaro

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Division of Immunology, Infection & Inflammation,
University of Glasgow,
Glasgow, G12 8TA

And in the

Centre for Biophotonics,
Strathclyde Institute of Pharmacy and Biomedical Sciences,
University of Strathclyde
Glasgow, G4 0RE

Summary

Rheumatoid Arthritis (RA) is a chronic systemic autoimmune disease characterised by extensive synovitis resulting in cartilage and bone erosions. Both the innate and adaptive immune pathways contribute to the initiation and the maintenance of the disease. Understanding the role of these pathways is central to develop new therapeutics. We have developed a murine model of RA where ovalbumin (OVA) specific Th1 cells induced a breach of self-tolerance and a transient monoarthritis. This thesis aimed firstly to create a model of chronic autoimmune polyarthritis and then to investigate the contribution of B cells and innate inflammation to the induction of arthritis. Relapse of arthritis was associated with the nature of the antigen (OVA) employed and the route of administration. The analysis of collagen specific B cell response revealed that anti-type II collagen antibodies titres rise during the induction of the relapse of arthritis and that they were directed against the epitope U1. Although typical RA autoantibodies were detected in OVA-mediated arthritis, a mild arthritis could be elicited in absence of antigen presenting B cells and in complete absence of mature B cells. B cells were not necessary in the induction of pathology even though their presence was associated with a higher joint histology score. Finally, this thesis describes that an innate inflammatory stimulus, such as LPS, elicited joint pathology but was insufficient to breach B and T self-tolerance. On the contrary, antigen-specific T cell activation led to arthritis and the production of several autoantibodies typical of RA. The relapse and spread of arthritis developed in this thesis provides a useful tool to investigate the contribution of the innate and adaptive immune pathways in the development of autoreactive responses. A better understanding of these mechanisms will hopefully help to design new therapeutic intervention aiming to re-establish immunological tolerance.

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Authors Declaration

"I declare that, except where explicit reference is made to the contribution of others, this dissertation is the result of my own work and that it includes work forming part of a thesis presented successfully for a degree of Specialist in Rheumatology granted by the University of Rome Sapienza in the year of 2006."

Signature

Printed Name: Paola Conigliaro

Table of Contents

LIST OF TABLES

List of Figures

Chapter 4

Chapter 5

List of Publications

1: Conigliaro P, Benson RA, Patakas A, Kelly SM, Valesini G, Holmdahl R, Brewer JM, McInnes IB, Paul Garside. Characterization of the anti-collagen antibody response in a new model of chronic polyarthritis. Arthritis Rheum. 2011 Aug;63(8):2299-308.

2: Nickdel MB, Conigliaro P, Valesini G, Hutchison S, Benson R, Bundick RV, Leishman AJ, McInnes IB, Brewer JM, Garside P. Dissecting the contribution of innate and antigen-specific pathways to the breach of self-tolerance observed in a murine model of arthritis. Ann Rheum Dis. 2009 Jun;68(6):1059-66.

List of Abbreviations

^{3}H	tritiated
ACPA	anti-citrullinated protein antibodies
AIA	antigen-induced arthritis
AID	activation-induced cytidine deaminase
AKA	anti-keratin antibodies
ANA	anti-nuclear antibodies
APC	antigen-presenting cells
APF	anti-perinuclear factor
APRIL	a proliferation-inducing ligand
BCA-1	B cell-attracting chemokine-1
BCMA	B cell maturation antigen
BCR	B cell receptor
BLC	B Lymphocyte Chemoattractant
BlyS	B lymphocyte stimulator
Breg	B regulatory
CII	type II collagen
CAIA	collagen- antibody induced arthritis
CD	clusters of differentiation
CFA	complete Freund's adjuvant
CIA	collagen-induced arthritis
Cit-C1	citrullinated C1
CTLA	cytotoxic T-lymphocyte associated antigen
DAB	diaminobenzidine
DAMP	damage-associated molecular pattern
DC	dendritic cell
DMARD	disease-modifying anti-rheumatic drug
EDTA	ethylendiaminetetraacitic acid
Edu	5-ethynyl-2´-deoxyuridine
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorter
FCS	foetal calf serum

FDC	follicular dendritic cell
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
FLS	fibroblast-like synoviocyte
G6PI	glucose-6-phosphate isomerase
GC	germinal center
GM-CSF	granulocyte-macrophage colony-stimulating factor
GWAS	genome-wide association studies
H&E	heamatoxylin and eosin
HABSA	heat aggregated bovine serum albumin
HAO	heat-aggregated ovalbumin
HBS-EP	hepes-buffered saline
HEL	hen egg lysozyme
HLA	human leukocyte antigen
HMGB1	high-mobility group box chromosomal protein-a
HRP	horseradish peroxidase
Ig	immunoglobulin
IFA	incomplete Freund's adjuvant
IFN	interferon
IHC	immunohistochemistry
IIF	indirect immunofluorescence
IL	interleukin
i.p.	intraperitoneal
IRF5	interferon regulatory factor 5
i.v.	intravenous
kDa	kilo Daltons
LN	lymph node
LPS	lipopolysaccharide
LT	lymphotoxin
MACS	magnetic-activated cell sorting
mBSA	methylated bovine serum albumin
mDC	myeloid dendritic cell
MAP	mitogen-activated protein
MHCII	class II major histocompatibility complex

MMP	metalloprotease
MyD88	myeloid differentiation primary response gene 88
NFKB	nuclear factor κ B
NHS	n-hydroxysuccinimide
NK	natural killer
NOD	non-obese diabetic
OA	osteoarthritis
OD	optical density
OVA	ovalbumin
P.	Porphyromonas
PADI	peptidylarginine deiminases citrullinatin isoenzyme
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
pDC	plasmacytoid dendritic cell
PE	phycoerythrin
PFA	paraformaldehyde
PGIA	proteoglycan-induced arthritis
PRR	pattern recognition receptor
PTPN22	protein tyrosine phosphatase non-receptor type 22
R	receptor
RA	rheumatoid arthritis
RANKL	nuclear factor κB ligand
RASF	RA synovial fibroblast
RF	rheumatoid factor
RNA	ribonucleic acid
SA	streptavidin
SAL	saline
s.c.	subcutaneous
SCID	severe combined immunodeficiency
SCW	streptococcal cell wall
SD	standard deviation
SE	shared epitope
SLE	systemic lupus erythematosus

SNP	single-nucleotide polymorphism
STAT4	signal transducer and activator of transcription 4
T1	transitional type 1
T2	transitional type 2
TACI	transmembrane activator and CAML interactor
TCR	T cell receptor
TdR	thymidine
Tg	transgenic
TGF-β	transforming growth factor-β
Th	T helper cell
TIR	Toll/Interleukin-1 receptor
TMB	3, 3', 5, 5'-tetramethylbenzidine peroxidase
TNF	tumour necrosis factor
TNFAIP3	TNF-α -induced protein
TNFR	tumour necrosis factor receptor
TolDC	tolerogenic dendritic cell
TRIF	TIR-domain-containing adapter-inducing interferon-β
TRAF1/C5	TNF receptor-associated factor 1 and C5 gene
VEGF	vascular endothelial growth factor

Chapter 1

Introduction

1.1 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is an inflammatory chronic and systemic disease characterised by extensive synovitis resulting in erosions of articular cartilage and marginal bone that lead to joint destruction (1).

RA has a prevalence estimated at 1% of the world population and an incidence of 20-50 cases every 100.000 persons/year (2). It presents a significant socioeconomic impact leading to direct and indirect costs to the health system and society (estimated €45.1 billions per year in Europe) (3).

RA pathogenesis (Figure 1.1) is a multistep process that starts with the development of autoimmunity, continues with local inflammation and finally induces bone destruction (4). Susceptible individuals, under the influence of various genetic and environmental factors, develop an underlying autoimmunity that manifests as the presence of autoantibodies, such as rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA) (4). This stage, identified as pre-articular or lymphoid phase, can precede the clinical manifestation of the disease by as much as ten years (5). It is still unclear how and why the systemic loss of tolerance is linked to a localised onset of inflammation in the joints (transitional phase of RA). Several factors may contribute including microtrauma, microvascular, neurologic and biomechanical-related mechanisms (6). The articular phase of disease is characterised by synovitis that leads to cartilage and bone damage (7). The adaptive and innate immune pathways are activated and contribute to the inflammatory process. An intricate cytokine network participates in inflammation and in perpetuation of disease by positive feedback loops promoting systemic disorders (8).

1.2 Aetiopathogenesis

RA is a multi-factorial disease of unknown aetiology. Genetic, environmental factors and deregulated immune responses contribute to the induction and maintenance of the disease (4). The following sections will overview the genetic and environmental factors involved in RA aetiopathogenesis.

1.2.1 Genes

The genetic basis of RA is extremely complex. The prevalence among siblings increases from <1%, in the general population, to 2-4% (9). Twin studies showed a concordance rate for RA of 12-15% for monozygotic twins compared to 3.5% for dizygotic twins (10). Evidence of familial clustering demonstrated prevalence from 2% to 12% in first-degree relatives of RA patients (11). The most important genetic risk factor for RA is found in the human leukocyte antigen (HLA) locus. In particular, the amino acid sequence QKRAA, QRRAA or RRRAA at positions 70-74 of the DRβ1 chain, called "shared epitope" (SE), is associated with the production of ACPA and with the disease (12-14). Multiple alleles in the DRB1 gene share the SE such as HLA-DRB1*0401, *0404, *0405, *0408, *0101, *0102, *1001 and *1402 (15). Of interest, the association between these alleles and RA has been observed only for ACPA positive patients (13). The introduction of new techniques, such as the genome-wide association studies (GWAS), has led to the identification of more than 30 alleles outside of HLA genes that contribute to RA susceptibility in the last years (16). The second most important genetic association in Caucasian population is in the gene protein tyrosine phosphatase non-receptor type 22 (PTPN22). A single-nucleotide polymorphism (SNP) encoding an arginine to tryptophan substitution increases the risk of RA by 40-80% (OR 1.4-1.8) (17, 18). The gene PTPN22 encodes a tyrosine phosphatase, Lyp, a powerful inhibitor of T cell activation. It has been hypothesized that the disease-associated allele would produce a protein affecting the threshold for B and T cell receptor signalling (19). In contrast, in the Asian population the gene peptidylarginine deiminases citrullinatin isoenzyme 4 (PADI4) appears to be the second most important susceptibility locus after HLA-DRB1 (20, 21). PADI4 is

one of several isoenzymes carrying the post-translational conversion of arginine residues to citrulline, and this may be related to the production of ACPA (22). Signal transducer and activator of transcription 4 (STAT4), is a member of the STAT family of transcription factors. The molecule plays a key role for IL-12 signalling in T cells and Natural Killer (NK) cells, leading to the production of interferon (IFN)-γ and the differentiation of Th1 and Th17 cells (23). A SNP haplotype in the third intron of STAT4 is associated with susceptibility to both RA and Systemic Lupus Erythematosus (SLE) in European populations (23). Other candidate genes associated with RA that GWAS revealed are cytotoxic T-lymphocyte associated antigen-4 (CTLA-4), the α and β chain of the IL-2 receptor (IL-2RA and IL-2RB) (24), interferon regulatory factor 5 (IRF5) (25), the locus located between TNF receptor-associated factor 1 and C5 genes (TRAF1/C5) (26), the gene near TNF-α -induced protein (TNFAIP3) (27, 28) and the co-stimulatory molecules CD40 (29) and CD28 (30).

1.2.2 Environment

Several environmental factors have been studied in RA and the interaction between genetic and environmental factors has been demonstrated in RA. Smoking, infections, sex hormones, birth weight, alcohol intake and socioeconomic status can modify the risk for RA (31). Smoking is the strongest known environmental risk factor in RA. Of interest, the association is true for ACPA-positive RA rather than ACPA-negative RA patients (32). The risk increases with amount, duration of cigarette use and it is greater in males than in females (33). The number of the SE copies further modifies the risk. Smokers who do not carry the SE have a 1.5-fold elevated risk of developing ACPA and RA compared with non-smokers who do not carry the SE. This risk increases to 21-fold in smokers carrying two copies of the SE. Moreover, smoking increases the proportion of citrulline- positive cells in the lungs (34). These findings suggested that smoking triggers citrullination in lungs through activation of PAD providing a substrate for the immune activation (34).

Other environmental factors are infectious. In particular, RA is prevalent in individuals with periodontitis (35). The periodontal pathogen *Porphyromonas* (P.)

gingivalis expresses PAD that citrullinates arginine in fibrin in periodontal tissue. The levels of antibodies against *P. gingivalis* have been correlated with the levels of ACPA in RA patients (36). Several mechanisms that characterise RA are also involved in periodontitis suggesting an association between these two conditions (37).

RA is more common in females than in males. This gender association seems related to sex hormones, supported by the fact that RA risk and exacerbation of disease increase in post-partum period and amelioration of disease is common during pregnancy (38). Moreover, oral contraceptives may have a protective effect in the development of RA, however this issue remains under debate (39). Breast-feeding was shown to protect mothers against RA in two large case-control studies (40, 41). High birth weight (> 4.5Kg) was associated with a 2-fold increased risk of RA in a large prospective study (42).

Alcohol intake decreased the risk for RA in two studies in a dose-dependent manner (32, 43). Finally, an inverse association between the socioeconomic status, measured by occupational class and education, and RA has also been demonstrated (44).

1.3 Clinical manifestations of RA

Clinically RA manifests with a symmetric polyarthritis characterised by pain, swelling, loss of function and a morning stiffness lasting more than one hour. A common onset synovitis involves the metacarpophalangeal, the proximal interphalangeal, the wrist and the metatarsophalangeal joints, although all the joints may be affected (45). Several constitutional symptoms can precede the onset of RA, such as fatigue, malaise, weight loss, fever and depression (46). Despite articular and periarticular manifestations being predominant, RA can affect many other organs and tissues (Table 1.1). Some of these extra-articular manifestations may be related to the disease itself or be a consequence of the treatment (47). Moreover, autoimmune diseases may overlap. The incidence of extra-articular manifestations varies among studies from 18% to 41% of RA patients because of the lack of consensus on how to define them (47). There are no known reliable predisposing factors for extra-articular manifestations, although there is an association with male, smokers, severe arthritis, high levels of inflammatory markers, presence of RF, ACPA, anti-nuclear antibodies (ANA), and HLA-related SE (48, 49). The most common extra-articular features and complications in RA are summarised in table 1. Rheumatoid nodule is the most common extra-articular manifestation, present to up to 30% of cases, whereas many of the other manifestations occur in 1% of RA patients (46). Sjögren syndrome, anaemia and lung manifestations are relatively common (6-10% of RA patients). Rheumatoid vasculitis affects mainly RF-positive RA patients. It can occur in any organ, although it mainly manifests with cutaneous and peripheral nerve lesions (50). Some manifestations including systemic vasculitis, Felty's syndrome, interstitial pulmonary fibrosis, neuromyopathies and amyloid may be difficult to treat. RA represents an independent risk factor for cardiovascular diseases, including myocardial infarction, cerebrovascular events and heart failure (46, 51, 52). Persistent inflammation, immune-complexes and altered lipid particles create the substrate for an accelerated atherosclerosis in RA (53, 54). RA is associated with an increased risk of non-Hodgkin's lymphoma and this risk may be further increased by the immunosuppressive treatment (55). A relationship between smoking and development of lung cancer has been reported in RA (56). Finally, high disease activity and extra-

articular manifestations have a major impact in disease outcome, morbidity and mortality (57).

1.4 Synovitis

The synovial membrane is a connective tissue formed by two main layers, the synovial lining and the synovial sublining. The synovial lining is composed by two types of synoviocytes, called macrophage-like and fibroblast-like synoviocytes (FLS), because of their surface marker expression and morphology (58). FLS are responsible for the production of constituents of the synovial matrix such as collagen types I, III, IV and V, fibronectin, laminin, chondroitin and heparan sulphate (58). Furthermore FLS produce hyaluronic acid into the joint cavity providing lubrication to its components (59, 60). The synovial sublining is a soft, loose connective tissue that facilitates smooth movement of the joints. It is formed by a network of elastic fibres and different collagens, including collagen types I, III, IV, V and VI, fibronectin, laminin and proteoglycans (61). The synovial sublining contains blood and lymph vessels, nerve fibres and few cells including macrophages, fibroblasts and adipocytes (58).

In RA the synovial membrane is characterised by cellular hyperplasia, increased vascularity and an infiltrate of inflammatory cells that invasively grow and destroy the adjacent cartilage and bone (62). The synovial hyperplasia is an increased thickening of the lining layer caused by the combination of cellular proliferation in situ, influx of cells from the circulation and disturbed apoptosis (58). The increase in synovial tissue mass, called “pannus”, results in increased oxygen demand and consequent local hypoxia. Inadequate oxygenation drives the increase in synovial angiogenesis (63). Pro-angiogenic factors are produced by macrophages and synovial fibroblasts such as CXCL8 (or IL-8), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and transforming growth factor-β (TGF-β) (64, 65). This process promotes further infiltration of inflammatory cells, production of inflammatory mediators and matrix degradation (66).

The infiltrate in RA synovitis is composed of $CD4^+$ T cells, B cells, plasma cells, NK cells, dendritic cells (DCs) and mast cells (62, 67-70). Lymphoid aggregates of variable size and organization level are present in 50-60% of RA patients even if they are not specific to the disease (71). Clear T cell/B cell segregation may be observed in larger aggregates. FLS appear to play a role in all major features of RA: hyperplasia,

inflammation and joint destruction (72). The role of most of these cells in the pathogenesis of RA will be discussed later in this chapter.

Neutrophils accumulate mainly in the synovial fluid but they can be observed at the pannus-cartilage interface, the site of active destruction of bone and cartilage (73). These cells are activated and release the contents of granules, which are implicated in the damage of the collagen matrix (74, 75). Neutrophils-derived proteases are also important mediators of inflammation activating the pro-cytokine forms of TNF-α, IL-1β and CXCL8 (76).

Mast cells in RA are activated and release mediators including histamine, heparin, cytokines (IL-6, CXCL8, TNF-α), prostaglandins and leukotrienes (70, 77). Mast cells induce oedema and contribute to the cartilage and bone destruction inducing the production of metalloproteases (MMPs) (78). Their recruitment in RA tissue and fluid is due to several chemotactic factors, such as TGF-β, C3a, C5a, serum amyloid A and platelet activating factor, all detected in RA synovial fluid (79-82).

1.5 Role of the innate immunity

The development of the inflammatory process in RA, involves many different cell types and a complex cytokine network. An overview of the cells involved in RA development will be presented in the following sections, focusing mainly on the cells of the innate immune response.

1.5.1 Innate immune cells in RA

From a functional and therapeutic point of view, the preclinical stage of RA is of great interest. It appears that before clinical sign of arthritis, the innate immune system is activated leading to the initiation of the inflammatory process (83). Cells of the myelomonocytic lineage differentiate into several cell types that are critically involved in the disease, such as monocytes/macrophages, DCs and osteoclasts.

Monocytes/macrophages: Macrophages differentiate from circulating monocytes and have primary roles in tissues as phagocytes of invading pathogens and as scavengers of apoptotic debris (84). Macrophage activation results in the expression of chemokines and cytokines that attract other cells to the site of inflammation (84). Macrophages are prominent in the inflamed synovial membrane where they are activated (85). The central role of macrophages in RA is supported by the fact that conventional therapies act to decrease the levels of cytokines mainly produced by macrophages (86). Indeed, a correlation has been demonstrated between the tissue damage and the infiltrate of macrophages in the synovial membrane (87).
Monocytes/Macrophages in RA are responsible for the:

- Production of large amount of pro-inflammatory cytokines, such as TNF-α (88), IL-1 (89), IL-6 (90), IL-15 (91), IL-18 (92), IL-23 (93) and IL-27 (94).
- Production of chemokines that promotes monocyte influx into inflamed tissue, such as CXCL8, CCL3 (or macrophage inflammatory protein 1α), CCL5 (or RANTES) and CX3CL1 (or fractalkine) (95).
- Overexpression of tissue degrading enzymes such as MMP9 (78) and MMP12 (96).

A significant part of the macrophages effector responses is mediated by cell contact-dependent signalling with inflammatory and mesenchymal cells. Fibroblast-macrophage interaction elicits the production of IL-6, GM-CSF, CXCL8 and stimulates the cartilage degradation (97). Macrophages can also be activated by cell interaction with T cells. In response to this interaction macrophages produce MMPs, IL-1α and IL-1β ((98). Moreover, stimulated T cells produce TNF-α once in contact with macrophages (99). The same process has been proven for NK cells, which can induce monocytes/macrophages to produce TNF-α upon cell-contact interaction (100).

Dendritic cells: DCs have the ability to present antigen to T cells, playing a central role in the development of both the innate and adaptive immune responses (101). Their ability to prime naïve T cells for help and cytotoxic function distinguishes them from other antigen-presenting cells (APC) (102). DCs are involved in the maintenance of central and peripheral tolerance (103). They are also essential in the generation of primary antibody response, and are powerful enhancers of NK cell cytotoxicity (103). Two major subsets of DC, known as myeloid DC (mDC) and plasmacytoid DC (pDC), are described. Both subtypes have the ability to present antigen and produce cytokines (104).
In RA mDCs and pDCs can enrich synovial tissue and fluid (105, 106). They may contribute to RA pathogenesis in several ways:

- They infiltrate synovial membrane where they may take up, process, and present antigen locally contributing to disease perpetuation (105).
- They contribute to the inflammatory process secreting pro-inflammatory mediators (102).
- They may drive the generation of ectopic lymphoid tissue in synovial membrane (102, 107, 108).
- They may prime autoimmune responses by presenting self-antigens to autoreactive T cells (109-111).

Because of their characteristics, DCs are attractive vehicles for the delivery of therapeutic vaccines. In the murine model of RA collagen-induced arthritis (CIA), immunomodulatory DCs were able to inhibit arthritis (112, 113). Moreover, in human RA clinical trials have been initiated testing DCs with "tolerogenic" (TolDCs) functions (114).

Natural Killer cells: NK cells are large, granular lymphocytes devoted to the defense against microbial agents and cancer cells, traditionally recognised as an important arm of the innate immunity, even if more recent data underpin a role also in the responses of adaptive immunity (115, 116). Several studies have led to ascertain that NK cells are involved in the pathogenesis of many immune-mediated diseases, where they may exert both protective and pathogenic roles. In RA NK cells demonstrated an impaired activity, although data on their number are controversial among the different studies (117-121). The subset of NK cells $CD56^{bright}$, showing immunoregulatory properties, has been found to accumulate in the synovial membrane and fluid from RA patients (100, 122, 123). $CD56^{bright}$ NK cell subset has the great capacity to secrete a large amount of cytokines including TNF-α, a critical mediator in RA. IL-12, IL-15 and IL-18, Th1 cytokines detected in RA, can in turn induce $CD56^{bright}$ NK cells to produce pro-inflammatory cytokines (124). In addition to the intricate cytokine milieu, cell-cell interactions between different cell types in RA synovial membrane can also contribute to the persistence of inflammation. NK cells can interact locally and/or activate different cell types, for example providing co-stimulatory signals to T and B cells (125). *In vitro* RA synovial membrane experiments demonstrated that cell contact between NK cells and FLS provided mutual stimulation supporting NK cell activation, proliferation and cytokine production. This interaction also stimulated FLS to secrete pro-inflammatory cytokines, such as IL-15 (126). NK cells can both enhance and suppress DC response (127-129). However, considering the data all together and divergent results on experimental arthritis (125, 130, 131), whether NK cells play a role in the development of RA remains unclear.

1.5.2 Other cells

Osteoclasts: Osteoclasts are multinucleated cells formed by the fusion of mononuclear progenitors of the monocyte/macrophage family (132). They are highly specialised cells in bone resorbing and remodelling throughout life (132). They have two machineries that allow them to resorb bone. The first is a proton/protein pump that creates an acidic milieu allowing the cell to solubilise calcium from bone matrix. The second is represented by matrix degrading enzymes such as MMPs and cathepsins that cleave matrix molecules (133). Synovial membrane in RA contains many monocytes/macrophages that can undergo osteoclast differentiation upon contact with the appropriate signals (134, 135). FLS and activated T cells are the cells that provide signals for monocytes to differentiate in osteoclasts. FLS express receptor activator of nuclear factor (NF)κB ligand (RANKL) that drives osteoclast formation (135, 136). Another source of pro-osteoclastogenic factors are activated T cells that produce IL-17, an important cytokine in osteoclastogenesis (137). Pro-inflammatory cytokines such as TNF-α, IL-6, IL-17 and IL-1, which are abundant in RA synovial membrane, regulate RANKL expression driving osteoclast formation (138-140). Indeed, RANKL is upregulated in human RA (135, 136). Of interest, osteoclasts in RA are strictly linked to bone damage but not to the inflammatory process as demonstrated by the therapeutic administration of bisphosphonates and osteoprotegerin, a negative regulator of RANKL (141, 142). Bone erosion in RA starts early and progresses rapidly during the first year and osteoclasts are the main actors of this process (143).

Fibroblast-like synoviocytes: FLS provide nutritive plasma proteins and lubricating molecules to the joint cavity and cartilage (65). These cells are involved in matrix remodelling by producing matrix components, such as collagen, hyaluronan and matrix-degrading enzymes (65). RA synovial fibroblasts (RASFs) show alterations in morphology and an aggressive behaviour compared with those from healthy joints (144, 145). These changes are often referred as tumour-like transformation and reflect a stable activation, long-term growth and resistance to apoptosis (61). These characteristics may be related to pro-inflammatory cytokines (TNF-α, IL-1, IL-17, IL-18) (146-148), growth factors (FGF, TGF-β) (65), hypoxia (149), up-regulation of proto-oncogenes (myc, c-fos, ras) and deficiency of tumour suppressors genes, such as p53 (150, 151).

In MRL/lpr mice, that spontaneously develop RA-like arthritis, FLS proliferate and invade joint structures before inflammatory cells migrate in the synovium (152). In another murine model, human RASFs implanted in SCID mice degrade cartilage in the absence of inflammatory cells (153). RASFs activation has been linked to microbial agents and endogenous ligands, such as RNA from necrotic cells, which may stimulate them via highly conserved receptors of the innate immune system, such as toll-like receptors (TLRs). TLRs are a family of receptors that are expressed on different cell types. TLR2, 3 and 4 are expressed on RASFs (154). Pro-inflammatory cytokines, such as TNF-α and IL-1, abundant in RA synovial fluid, enhance the expression of TLR2 in RASFs (155). TLR2 activation results in VEGF and CXCL8 production upon stimulation with the ligand peptidoglycan (156, 157). Moreover, TLR2 and 4 activation lead to the synthesis of IL-15 in RASFs (158). In addition, TLR3 ligands, such as RNA, can be released from necrotic cells acting as an endogenous stimulus for the expression of pro-inflammatory genes in RASFs (154). RASFs contribute to RA pathogenesis as effector cells in inflammation also by chemokine secretion upon cell contact with T cells and by production of pro-inflammatory mediators (61). RASF are also key mediators of cartilage and bone destruction. Indeed, they are the major source of MMPs and cathepsins that drive degradation of cartilage and bone (159). They can also contribute to bone erosion by producing RANKL and stimulating osteoclasts differentiation (160).

1.6 Role of the adaptive immunity

The genetic associations of RA and the presence of autoantibodies place the adaptive immune pathways at the center of early pathogenesis. Moreover, current therapeutic approaches targeting B and T cells, such as conventional and biologic drugs, strongly support the key role played by the adaptive immune system. An overview of T and B cell function in RA and animal models of arthritis will be presented in the following sections.

1.7 Evidence of T cells

Several issues demonstrate that $CD4^+$ T cells are involved in the pathogenesis of RA. A critical role for the adaptive immunity is supported by the genetic predisposition to develop RA. Indeed, it has been demonstrated an association with the haplotype HLADRB1 and more recently with PTPN22, CTLA4, CD40 and CD28, as previously discussed (section 1.2.1) (17, 18, 24, 29, 30, 161). HLADRB1 gene contains a sequence of amino acids in the peptide-binding pocket, the "shared epitope", that can present the antigen to the T cell receptor (TCR) on $CD4^+$ T cells (162). This issue is becoming of particular interest since it has been demonstrated that there is an association among genetic (HLA-DRB1 carriers), environmental factors (smoking) and autoimmunity (ACPA), as previously discussed (section 1.2.2) (163).

In RA T cells are activated and secrete IFN-γ, IL-2, IL-12, IL-18, TNF-α and GM-CSF, typically considered Th1 cytokines that are produced in the synovial fluid and expressed in the synovial membrane (164-168). Rapidly these cytokines activate macrophages to secrete other pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α and IL-12, which in turn can promote the differentiation of B cells and stimulate the release of matrix MMPs provoking the degradation of the cartilage and the activation of osteoclasts leading to the bone resorption (169). Macrophages are the most important source of these cytokines, however, many studies demonstrate that cell contact interactions, between synovial T lymphocytes and adjacent macrophages or fibroblasts, represent an alternative route to generate cytokines (98, 170, 171). Studies *in vitro* demonstrate that T cells may acquire an active phenotype in a bystander manner by cytokine-driven activation and not as a consequence of antigen exposure (172, 173).

The presence of activated memory $CD4^+$ T cells in the synovial membrane, synovial fluid and peripheral blood of RA patients reinforces the concept that these cells are important in the pathogenesis of RA (174, 175). Current immunosuppressive therapies that indirectly regulate T cells show a significant improvement of RA. Corticosteroids, Methotrexate, Sulfasalazine, Leflunomide and Cyclosporine A, used in the management of RA, decrease the pro-inflammatory Th1-driven cytokines and promote the shift from Th1 to Th2 immune mediated-response (176). Biological therapies can also modulate T cell activation. Abatacept is a soluble recombinant

fusion protein comprising a fragment of the Fc domain of human Immunoglobulin (Ig) G1 and the external domain of the human co-stimulatory protein CTLA-4 (177). CTLA-4 is a molecule that competes with CD28, expressed on T cells, to bind B7 molecules on APC. Different clinical trials demonstrated the efficacy of Abatacept in Methotrexate naïve RA patients (178) and in patients with moderate to severe arthritis with inadequate response to Methotrexate (179) or TNF blockers (180). One mechanism of action proposed involved the release of indoleamine-2,3-dioxygenase after the interaction between Abatacept and B7 molecules on DCs. This enzyme could modulate the function of APC, such as it should occur after the binding with regulatory $CD25^{+}CD4^{+}$ T cells (181, 182). Another recent study suggested that CTLA-4 engagement decreased the contact period between T cell and APC leading to a reduced T cell activation (183).

Several murine models of arthritis explored the role of T cells. In CIA pathology is elicited by a joint specific antigen, homologous or heterologous type II collagen (CII), presented by class II major histocompatibility complex (MHCII) to specific T cells. In this model arthritis resembles the human disease with synovial proliferation, intense infiltration of mononuclear cells resulting in cartilage damage and bone erosions (184). Other animal models of arthritis also suggest that inflammatory immune stimuli, such as adjuvants (185), or components of infectious agents, such as streptococcal cell wall (SCW) (186), can elicit arthritis. However, in both examples antigen-specific T cells seem to play a role in the chronic stage of the disease (187). Another model where the central role of T cell in the development of arthritis is demonstrated is KxB/N generated from the KRN/C57BL/6 TCR transgenic (Tg) mice crossed with the Non-Obese Diabetic (NOD) mice. Arthritis in these mice was due to the development of T-cell dependent B cell responses against glucose-6-phosphate isomerase (G6PI), a glycolytic enzyme that is ubiquitously expressed (188, 189).

Th17 cells: $CD4^{+}$ T cells, upon activation and expansion, develop into different T helper cell subsets with different cytokine profile and distinct effector functions. Until recently T cells were divided into Th1 and Th2 cells (190). A third subset of IL-17 producing T helper cells includes the Th17 cells (191). Th17 cells produce IL-17, IL-17F, IL-22 and IL-21 (192). Initially Th1 cells were speculated to play the major pathologic function in the model of CIA. This view changed after the discovery that IFN-γ receptor deficient mice were more susceptible to CIA (193, 194). Mice

deficient in IL-23, cytokine required for IL-17-producing T cells, were resistant to CIA development; in contrast mice deficient in IL-12, required for IFNγ-producing Th1 cells, showed enhanced joint pathology (195). Thus, in CIA IL-23 was an essential mediator of arthritis, in contrast IL-12 was not critical in disease generation. Moreover, CIA was suppressed in IL-17 deficient mice and this cytokine was responsible for the priming of collagen-specific T cells and collagen-specific IgG2a production (196). The role of IL-17 in arthritis was demonstrated also in other murine models of arthritis such as in SKG and IL1RA deficient mice. In both models Th17 cells were induced spontaneously and IL-17 contributed to the inflammation and the bone erosion (197, 198). In human RA IL-17 was detected in the synovial fluid (199, 200). Immunostaining of RA synovium demonstrated IL-17-producing cells not only in the T cell rich area but also within mast cells (201, 202). IL-17 may induce joint degradation by producing matrix MMPs, inducing RANKL expression on T cells (137) and up-regulation on synoviocytes (199) and leading to increased osteoclastogenesis (203). A phase I trial with a humanised anti-IL-17 monoclonal antibody in RA patients supported neutralisation of IL-17 as a potential goal for the treatment of RA (204).

1.8 Evidence of B cells

B-cell development (Figure 1.2) initiates in the bone marrow, where stem cells progress through various stages of differentiation to become immature B cells (205). In mice approximately 10% of immature B cells overcome negative selection induced by reactivity with self antigens and emerge from the bone marrow expressing surface IgM and IgD (206). Final maturation occurs in the spleen where immature "transitional" B cells can still undergo negative selection (207) Cell surface markers have been identified that enable different stage of development. In mice, CD24 is used to distinguish immature ($CD24^{hi}$) from mature ($CD24^{lo}$) splenic B cells (208). Murine splenic transitional B cells have been divided into 2 distinct populations: transitional type 1 (T1; $CD24^{hi}$, $CD21^{lo}$, $CD23^{lo}$, IgM^{hi}, IgD^{lo}) and transitional type 2 (T2; $CD24^{hi}$, $CD21^{hi}$, $CD23^{hi}$, IgM^{hi}, IgD^{hi}) (209). T1 B cells are found in the bone marrow, blood and spleen but not lymph nodes, whereas T2 B cells are restricted to

the spleen (210). Moreover, *in vivo* experiments demonstrated that T1 cells give rise to T2 cells and mature B cells (209).

Human immature transitional B cells have been identified and show similarities and differences with their murine counterparts (210). For example, the presence of circulating human T2 B cells suggested that they are not restricted to the spleen (210).

Human memory B cells are generated in germinal centers (GCs) in response to T-cell dependent antigen (211). Within a GC antigen-specific B cells undergo somatic hypermutation of Ig V genes, yielding cells with increased affinity for antigen (212). As a result, memory B cells rapidly differentiate into high-affinity plasma cells following a re-encounter with the antigen (213). In peripheral blood memory B cells represent 40-60% of all B cells (206). CD27 represents a universal marker of human memory B cells to distinguish between memory B cells ($CD27^+$) and naïve B cells ($CD27^-$) (214). In turn $CD27^+$ memory B cells can be divided into IgD^+ (unswitched memory, usually together with IgM or alone in a minor fraction of memory cells) and IgD^- (switched memory, predominately IgG^+ or IgA^+, and a small fraction of IgM^+) (214). In the last few years a population of $CD27^-$ IgG^+ memory B cells has also been described (215). The classification of human peripheral blood B cell subsets is shown in table 1.2.The relevance of B cells in RA pathogenesis is attributed to different mechanisms that can be summarised as follows:

1. B cells are a source of relevant autoantibodies in RA
2. B cells enrich RA synovial membrane
3. B cells are highly efficient APCs to stimulate T cells
4. B cells are a major source of cytokines
5. B cells as a therapeutic target in RA

1.8.1 Autoantibody Production

Classically RA is considered an autoimmune disease since the production of RF was first observed. RF is an autoantibody directed against determinants on the Fc fragment of IgG molecules. It can belong to different isotypes (IgE, IgM, IgA and IgG). RF IgM can activate the complement system due to its pentameric structure and stimulate an immune response (216). About 50-80% of patients affected by RA are positive for

RF and high serum levels are associated with an aggressive articular disease, extra-articular manifestations and a worse outcome (217-219). RF has a moderate specificity, around 66%, as it is detected in other autoimmune diseases, systemic infections, and in up to 10% of healthy subjects (220). Nevertheless, many differences exist between RF in health and disease. The former is an IgM produced by B1 cells as "natural" antibody that shows low affinity and polyreactivity, the last undergoes isotype switching and somatic hypermutation as consequence of B cells receiving help from T cells (221).

The lack of high specificity of RF has stimulated the research of other autoantibodies more specific for the diagnosis of RA (222).

Antibodies in RA can be classified as those associated with RA and those specific for RA. In the first group anti-Ra33 antibodies are the more relevant. Hassfeld described anti-Ra33 antibodies, directed against an antigen of 33 kDa, in approximately 36% of patients affected by RA (223). The molecule has been identified as the heterogeneous nuclear ribonucleoprotein A2. Anti-Ra33 antibodies are associated with early arthritis (224) and they show a high specificity (90-96%) and a low sensitivity (32%) (225). Among the RA-associated antibodies, ANA are detected in approximately 50% of RA patients and anti-dsDNA, rare in the disease, can be induced during anti-TNF treatment (226). Antibodies to collagen type II (anti-CII) are detected in about 30% of RA patients. They have a low specificity; in fact they can be found in other autoimmune diseases such as SLE, systemic sclerosis and recurrent polychondritis. It is still a matter of debate if these antibodies represent an epiphenomenon or if they play a role in human disease. To support the last hypothesis several issues have been widely discussed as the detection of high titres of anti-CII antibodies in synovial fluid compared with serum titres (227) or the demonstration of B cells producing anti-CII antibodies in RA synovia, detected also in patients lacking serum antibodies (228). The synovial production of anti-CII seems to be correlated with expression of HLA-DR4 alleles and the T cell repertoire in RA patients (229). Moreover, it has been demonstrated that serum and synovial titres of anti-CII IgG correlate with levels of acute phase proteins and pro-inflammatory cytokines, such as TNF-α and IL-6 (230). Finally, recent work also demonstrates that immune-complexes containing anti-CII in human RA sera, can induce cytokine production such as TNF-α, IL-1β, CXCL8 via Fcγ receptor IIa expressed on macrophages (231).

Among the autoantibodies specific for RA, anti-BiP antibodies and ACPA appear to be important. Antibodies against a 78 kDa protein, identified as the stress protein immunoglobulin heavy-chain binding protein (BiP), are found in 64% of RA patients. They are highly specific for RA (specificity 96%, sensitivity 40%) (232). Anti-BiP antibodies are also detected in the mouse model of CIA and pre-immunisation with BiP suppresses the onset of the experimental arthritis (233).

Finally, the most relevant autoantibodies appear to be ACPA. They were first described in 1964 as anti-perinuclear factor (APF), because they reacted with keratohyaline granules scattered around the perinuclear region of human buccal epithelial cells in indirect immunofluorescence (IIF) (234). Later on in 1979, anti-keratin antibodies (AKA) were identified by using rat or human esophagus sections for detection (235). In 1993, it became clear that the antigen recognised by AKA was filaggrin (236) and then that APF and AKA were directed against the same citrulline-containing proteins generated from the enzymatic reaction catalyzed by PAD (237). Citrullination is the critical step for the recognition of different proteins (fibrin, vimentin, fibronectin, collagen type II), highly expressed in the synovial membrane during inflammation, by ACPA (238). Anti-citrullinated vimentin antibodies are as sensitive as ACPA but slightly less specific in detecting RA (239). Interestingly, PAD enzymes were found in monocytes (PADI4) and macrophages (PADI2 and PADI4) in synovial fluid suggesting that citrullination may take place locally in the joint (240) and B cells secreting ACPA have been detected in synovial fluid from RA patients (241, 242). Another observation that strongly supports the role of ACPA in RA pathogenesis comes from genetic studies. Firstly, as previously discussed (section 1.2.1), a haplotype of the gene encoding PADI4 was shown to be associated with an increased susceptibility to develop RA (22). A second line of evidence is found in the strong association between the production of ACPA and the presence of RA susceptibility HLA-DRB1 genes (243). Indeed, SE alleles predispose for ACPA positivity rather than for RA (243). These autoantibodies show high specificity (98%) and sensitivity comparable with RF (68%) (244). ACPA positive patients with undifferentiated arthritis have a chance of 90% to progress to full-blown RA within 3 years (245). Of interest, they correlate with disease severity and with radiological progression of the disease (246). Moreover, recent studies have demonstrated the presence of both RF and ACPA up to ten years before the onset of RA (5, 247-249). The autoantibody titres increased as the onset of disease approached (5). These data

suggested that the adaptive immune response against autoantigens is initiated years before the clinical signs of the disease.

1.8.2 B cells enrich RA synovial membrane

New interest in B cells arose when it was demonstrated that B cells were represented in rheumatoid synovium (217). The histological pattern of rheumatoid synovial membrane is heterogeneous. Several studies confirmed the variable presence of T/B cell compartmentalisation, the development of high endothelial venules and follicular dendritic cells (FDCs) network (250-253). This process may recapitulate the development of lymphoid organs and therefore it has been defined ectopic lymphoid neogenesis (250). Evidence of tertiary lymphoid structure formation is not RA-specific since it has been obtained in tumours and in several chronic infectious diseases (254). A complex interaction between hematopoietic and stromal cells is responsible for the persistence and the organisation of the inflammatory process. The first study in human RA, published by Takemura (252) in a series of 64 synovial biopsies, demonstrated the presence of a diffuse infiltrate of B, T, macrophages and DCs in 23% of cases, clusters of B and T cells in 56% and GCs with a network of FDCs inside in 20.3% of biopsies. Moreover, in a given patient the histological pattern was stable over time and it was represented in different joints with synovitis (255). The histological pattern might be influenced by the disease stage and the sampling procedure (254). Recent studies in large cohort of RA patients demonstrated synovial ectopic lymphoid neogenesis in 31% (n= 103) (256) and 49% (n= 86) (250) of the specimens after arthroscopic synovial biopsies. In the first study patients with synovial lymphoid neogenesis showed increased markers of systemic inflammation but there was no association with clinical characteristics of disease severity and the presence of RF or ACPA (256). Synovial ectopic lymphoid structures expressed activation-induced cytidine deaminase (AID), the enzyme required for somatic hypermutation and class-switch recombination of Ig genes suggesting that antigen-driven antibody response may take place within GCs of secondary lymphoid tissues (257). Few studies focused on the contribution of chemokines that drive cell movement in RA synovium. The stroma has acquired in recent years the role of director of the immune response regulating the leukocyte recruitment and the

organisation within the synovium. FLS, lymphatic and blood vessels seem to exert a key role in the regulation of the inflammatory response and leukocyte recruitment (258). Of interest, FDCs, FLS and endothelial cells are the main source of CXCL13, also called BLC (B Lymphocyte Chemoattractant) or BCA-1 (B cell-attracting chemokine-1), in RA synovia (252). CXCL13 and the receptor CXCR5 promote the attraction of B cells in the GC. CCL21, which binds CCR7, is instead known for its role in the recruitment of T cells in the paracortex of the lymph node. Takemura and co-workers demonstrated the relationship between the progressive lymphoid organization in RA synovia and the expression in terms of protein and mRNA of these chemokines (252). FLS also produce TNF-α, IL-6 and IL-1 that promote immune cell recruitment and leukocyte aggregation in RA synovium. Other molecules, such as lymphotoxin (LT)-α, LT-β, and the heterotrimers LT-α1β2, promote inflammatory lymphoneogenesis and the B cell recruitment in the RA synovia. LT-β is expressed in the rheumatoid synovia by B cells in the mantle zone and in the GC, while FLS express its receptor LT-βR (255). Of interest FLS in culture, extracted from patients with active RA, produced pro-inflammatory cytokines, matrix MMPs, chemokines attracting T cells, and cell adhesion molecules after incubation with LT-α1β2 (259).
Using an adoptive transfer model, Weyand and co-workers studied whether the T cell activation could be B-dependent in the synovial membrane. These authors transplanted human RA synovia under the skin of SCID mice that, lacking T and B cells, were unable to reject the allograft. The transplanted synovia produced pro-inflammatory cytokines. The treatment with Rituximab, a monoclonal antibody that depleted $CD20^+$ B cells, provoked the disorganization of the follicular structure and the loss of $CD4^+$ T cells in the synovia together with a decrease in pro-inflammatory cytokines production (260). In the context of therapeutic intervention few studies in a small number of RA patients analysed the effect of anti-TNF treatment or Rituximab on the synovial biopsies. In particular, disease activity was associated with persistency of synovial lymphoid neogenesis in 24 RA patients treated with anti-TNF therapy, while response to treatment was accompanied by its reversal (250). The treatment with Rituximab in 13 RA patients showed a high clinical response that was associated with decreased synovial B cells and Igs (261). Another study, involving only 24 RA patients undergoing sequential synovial biopsies following Rituximab

therapy, demonstrated a reduction in plasma cells infiltrating the synovial membrane as a predictor of response to B cell depletion therapy (262).

1.8.3 Professional antigen presenting B cells

Several studies in animal models of autoimmune diseases have investigated the role of B cells as highly efficient APCs and/or autoantibody producing cells; here we report some of these studies. Naïve B cells circulate through the blood and lymph and home to secondary lymphoid organs. There, they encounter their specific antigen and T cells specific for the same antigen. In the lymph node naïve B cells are localised in the follicles and T cells in the paracortex (255). B-T cell interactions (Figure 1.3) have been observed in the edges of lymphoid follicles in the lymph node, which resulted in proliferation of both cell types and GC formation (263).

B cells can recognise the antigen, classically, through their B cell receptor (BCR). BCR is formed by membrane-bound Igs that show high affinity for a given antigen (264). The antigen, after the binding with Ig, is internalised and processed in a small peptide that, bound to an MHC class II molecule, is then presented to T cells sensitised to the same antigen. T cells can recognise the peptide through their TCR. Activation of naïve T cells requires co-stimulatory molecules such as ligation of CD40 on B cells by CD40L, a molecule expressed by activated $CD4^+$ T cells (265). On the B cell side the co-stimulatory molecules CD80/CD86, ligands for CD28, are induced after crosslinking of the BCR. The importance of CD40L-CD40 pathway is shown by the lack of antigen-presenting capacity of B cells from CD40L- or CD40-deficient mice and after blockade of the interaction with anti-CD40L antibodies (263, 266, 267). Activation of naïve T cells requires also another signal delivered by the APC such as cytokines that are involved in T cell differentiation, as described in section 1.8.4. T-B cell cognate interaction is critical in regulating T cells activation or tolerance (268), B cell clonal expansion and differentiation in antibody-secreting cells (269).

Studies from Lanzavecchia showed that B cells with RF specificity could capture a foreign antigen complexed with an antibody and present the antigen efficiently to T cells of the same specificity (270). In support of this hypothesis came the demonstration that chromatin-containing immune complexes stimulated RF^+ B cells

engaging both the BCR and TLRs (271), establishing a critical link between innate and adaptive immune systems.

Although B cells are able to prime naïve $CD4^+$ T cells, their contribution in this process is debated. Early experiments using mice rendered B cell deficient by administration of anti-IgM antibodies suggested that T cell priming was deficient in absence of B cells (272-275). Moreover, conflicting results arose when B cell deficient mice (μMT) were generated by targeted deletion of the μ region of the IgM locus. In some studies B cells were not necessary for T cell priming (276-278), supporting the notion that T cells priming was reserved for DCs, whereas in other studies an impairment in T cell priming was observed (279-281). Costant *et al*. investigated *in vivo* the ability of DCs and B cells to take up peptide or protein antigens. Mice lacking B cell (μMT mice) were impaired in their priming to protein but not peptide antigens. Indeed, peptide antigens were taken up preferentially by DCs, whereas soluble proteins were taken up by antigen-specific B cells (282).

Interesting studies on MRL/lpr mice investigated the relevance of B cells for T-cell activation by depleting B cell themselves or circulating autoantibodies (283, 284). MRL/lpr mice, that showed a lupus-like disease, developed severe nephritis, vasculitis, sialoadenitis and skin disease. These mice failed to develop activated memory T cells and pathology once rendered B cell deficient (284). On the contrary, in the total absence of circulating antibodies and normal B cells, MRL/lpr mice experienced normal T cell activation, cellular infiltration in kidney and vessels, suggesting that antibodies were not required for T-cell activation. The authors suggested that B cells, with specificity for self IgG or DNA, could present antigen and stimulate autoreactive T clones (283).

The requirement for antigen-specific B cells was investigated also in a murine model of RA, the proteoglycan-induced arthritis (PGIA), using both B cell deficient and Ig-deficient (mIgM) mice. Indeed, in PGIA model antigen-specific B cells were necessary as APCs for the activation of autoreactive T cells (285).

Interestingly, antigen presentation by resting B cells can induce T cell tolerance. In experimental models T cell tolerance was induced avoiding B cell activation, for example using an antigen without adjuvant (286), blocking CD40 signalling (266), or with an antigen expressed endogenously by B cells as a transgene (287).

In summary, these studies show that the degree to which B cells participate in T cell priming or tolerance is determined by their activation state, type of antigen used, method of administration and the function that is evaluated in each model (280, 286, 288). This probably reflects the fact that the involvement of each type of APC varies in different circumstances (268).

1.8.4 B cell-related cytokine production

The outcome of T-B interaction is influenced by the cytokine milieu (289). This is traditionally considered to be dominated by T cells polarised during the T-DC interaction (289-291). However, activated B cells can produce a variety of cytokines that may contribute to the environment such as IL-1, IL-4, IL-6, CXCL8, IL-7, IL-10, IL-12 and TGF-β (292-294). For example, Duddy *et al*. (295) demonstrated that naïve B cells, through both the BCR and CD40, proliferate and produce high levels of IL-6, TNF-α and LT, a combination that promotes GC formation and amplifies T cell responses. In contrast, CD40-mediated T cell stimulation, in absence of BCR engagement, produced high levels of the regulatory cytokine IL-10. Differently, memory B cells have a greater tendency to produce pro-inflammatory cytokines (293). Studies in animal models of autoimmune diseases demonstrated that cytokine production by B cells might either stimulate or inhibit pathogenic responses (296, 297). For example, Evans *et al*. (298) demonstrated that transitional B cells were protective against murine inflammatory arthritis via the production of IL-10. IL-10 is a regulatory cytokine that suppresses APC and T cell activation (299). IL-10 acts as B cell growth and differentiation factor promoting isotype switching and plasma cell formation (299, 300).

B cells with regulatory function (Breg) have been described in murine models of autoimmune diseases (296). In murine models of RA and multiple sclerosis, such as in K/BxN (301), CIA mice (302) and in experimental autoimmune encephalomyelitis (303, 304), Breg can both prevent the development of these diseases and reduce their severity.

A novel function of B cells in promoting lymphangiogenesis and lymph node expansion in response to immunisation has been recently described via the expression of VEGF-A (305). This is of particular interest since angiogenesis is present in RA

synovial membrane, as previously discussed (section 1.4). Moreover, B cells are able to produce either RANKL (306) or a precursor of osteoprotegerin (307), a soluble decoy receptor of RANKL and inhibitor of osteoclastogenesis, suggesting a role for B cells in regulating bone homeostasis.

A brief introduction of B-cell mediated cytokines involved in arthritis is presented.

IL-6 is a pleiotropic cytokine produced by B cells, macrophages, fibroblasts, endothelial and T cells. It exerts various effects in the inflammatory cascade and the immune responses (308, 309). Several lines of evidence showed that IL-6 is involved in RA pathogenesis (310). It is expressed in the synovial membrane of RA patients and a correlation has been found between elevated serum or synovial fluid levels and disease activity (311). IL-6 drives leukocytes activation and antibody production (309). It also stimulates osteoclast differentiation (312) and mediates systemic effects promoting acute phase response, anaemia, cognitive dysfunction and lipid-metabolism dysregulation (310, 313). Studies in CIA mice showed that IL-6 is required for the development of arthritis (314) and that an antibody (Tocilizumab) against IL-6 receptor (IL-6R) inhibit CIA development (315). Tocilizumab is a humanised monoclonal antibody that binds to soluble and membrane-expressed IL-6R (316). It is approved in Europe and in the United States for the treatment of moderate to severe RA in adult patients who have either responded inadequately or have been intolerant to previous therapy with one or more DMARDs or TNF antagonists (European Medicines Agency (EMEA). European Public Assessment Report – RoActemra. EMEA website [online], http://www.emea.europa.eu/humandocs/PDFs/EPAR/RoActemra/H-955-PI-en.pdf 2008).

TNF-α is a pluripotent cytokine produced by many cell types, including B cells, T cells and DCs Two TNF receptors have been described, TNFR1 (p55) and TNFR2 (p75) (317). TNF-α and its receptors are highly expressed in synovial tissue and fluid from RA patients (318, 319). TNF plays a central role in the inflammatory process in RA. Indeed, it induces the production of pro-inflammatory cytokines and chemokines, the expression of adhesion molecules, the synthesis and release of MMPs, the

suppression of regulatory T cells and it regulates the bone metabolism both inhibiting osteoblast differentiation and up-regulating osteoclast function RANK/RANKL-mediated (6). The development of biologic agents that block TNF-α has provided a major advance in RA treatment. Many anti-TNF drugs are currently available for the treatment of RA: Infliximab, Adalimumab, Etanercept, Golimumab and Certolizumab (320). Infliximab is a chimeric human-murine monoclonal antibody that consists of the variable region of a mouse and the Fc domain of human IgG1 (321). Adalimumab is a recombinant fully human IgG1 monoclonal antibody (322). Etanercept is a dimeric fusion protein that joints to p75 TNF receptor to the Fc domain of human IgG1 (323). Golimumab is a fully human anti-TNF IgG1 monoclonal antibody that targets and neutralises both the soluble and membrane-bound forms of TNF (324, 325). Finally, Certolizumab is a pegylated Fab fragment of a humanised anti-TNF monoclonal antibody approved for the treatment of RA in combination with Methotrexate in 2009 (326, 327). Randomised clinical trials strongly suggested that TNF inhibitors effectively reduce the clinical signs of RA and inhibit the progression of the structural damage (320). However, the biggest disadvantages are the partial or no-response to the therapy and the susceptibility to infections (328).

LT-α and -β are members of the TNF family that are required for early B cell differentiation and function (329). They promote lymphocytes compartmentalisation and GC formation (330, 331) complementary with TNF (332). In mice deficient for LT-α or LT-β the production of chemokines necessary for the recruitment of B and T cells is depressed (333). As previously described (section 1.8.2), LT–α and -β are differentially expressed in RA synovial membrane. These proteins form α1β2 heterotrimers and have been implicated in ectopic lymphoneogenesis. LT-β is produced by B cells and its receptor has been detected in FDC and FLS (252). Braun *et al.* (259) demonstrated that LT-α1β2 induced changes in the function of FLS that contributed to inflammation and T cell recruitment. In particular, it induced the production of IL-1β, MMP1 and MMP3, T cell attracting chemokines CCL2, CCL5 and CCL8, and cell adhesion molecules rendering FLS to efficient adhesion substrates for T cells.

1.8.5 B cells as a therapeutic target

The presence of autoantibodies in RA (222), the formation of lymphoid aggregates in RA synovia (255) and the studies on animal models of autoimmune diseases, demonstrating the relevance of B cells as efficient APCs (270), suggested the possibility that targeting B cells in RA could ameliorate the disease. B-cell targeting strategies have been developed such as anti-CD20 antibodies (334). Rituximab is a chimeric monoclonal antibody that binds to CD20, a surface molecule expressed on B cells at the pre-B stage of differentiation to the mature B cells excluding stem cells, pro-B cells and plasma cells (335). It is the first B cell depleting agent approved in combination with Methotrexate for the treatment of moderate to severe active RA after the failure of at least one anti-TNF (336). Rituximab, has been shown to inhibit progression of structural damage over 2 years, and continue to inhibit joint damage in the long-term treatment (337, 338). Several mechanisms of action have been hypothesized to explain the effect of B cell depletion and they may not be mutually exclusive. The antibody may activate the complement cascade promoting lysis of the target cell, activate antibody-dependent cell mediated cytotoxicity, induce apoptosis or target selected B cell subsets, such as autoreactive B cells or B cells forming extrafollicular GCs in the synovia (339). The therapy in a low percentage of cases reduce serum IgM levels although no increase in overall infection was observed in a long term follow-up (340). Other interesting potential therapeutic targets involving B cells are BlyS, APRIL, Bruton's tyrosine kinase and spleen tyrosine kinase that are implicated in B-cell activation and survival (335).

1.9 Animal models of arthritis

Animal models of arthritis have been employed extensively and generate new knowledge that can partially be applied to RA aetiology and pathogenesis. They represent an invaluable tool to identify and validate innovative drugs. Several animal models of arthritis exist, each providing unique insight into a subtype of disease. There is no universal model because of the complexity of the disease. They can be divided into induced arthritis models, whose development is based on immunising animals with an autoantigen or protein in the presence of an adjuvant, and genetically manipulated spontaneous arthritis models. These models are summarised in table 1.3.

The CIA model is the most commonly used for RA. It was first induced in rats (184) and subsequently in susceptible strains of mice (341). It is induced by intradermal injections of heterologous type II collagen in complete Freund's adjuvant (CFA) (341). CIA is MHC class II dependent and many strains have variable degree of susceptibility. Clinically it is characterised by an acute erosive polyarthritis. Autoreactive collagen-specific T and B cells play a critical role in disease progression (342). In a development of the model, injection of homologous CII caused chronic relapsing arthritis, more akin to human RA (343, 344). The importance of antibodies in arthritis development is demonstrated by the resistance of CIA induction in B cell deficient mice (345). Moreover, the transfer of collagen specific sera and monoclonal antibodies against CII induced collagen-antibody induced arthritis (CAIA) (343, 346). Relevant collagen epitopes in murine arthritis and human RA have been identified (347, 348).

Adjuvant induced arthritis was originally induced with an intradermal injection of mycobacteria cell walls suspended in mineral oil (349). However, this was not an adequate model for RA because it caused systemic acute inflammation. Thus, pristane induced-arthritis (pristane is the arthritogenic component discovered in mineral oil) was developed, and this is characterised by an acute phase with synovitis and bone erosion and a chronic relapsing phase of arthritis (350). This model is largely T cell dependent.

Streptococcal cell wall-induced arthritis is caused by a single systemic injection of an aqueous suspension of cell wall peptidoglycan-polysaccharide fragments from group

A Streptococci into susceptible rat strains. This model is characterised by an acute phase, followed by a chronic relapsing secondary phase similar to human RA (186, 351).

Proteoglycan induced model of arthritis involves immunisation with human proteoglycans in susceptible mice. These animals develop severe polyarthritis and spondylitis (352). Activation of autoreactive T cells is dependent on antigen recognition by the BCR, as previously discussed (section 1.8.3). In the absence of this recognition, the development of arthritis is inhibited (285). Treatment with anti-CD20 monoclonal antibody induced a reduction in antigen-specific T cell response and in arthritis development (353).

A limitation of these induced-arthritis models is that they rely on breaching tolerance to a single joint or systemic self-antigen based on aggressive immunisation protocols employing the same antigen.

Genetically modified spontaneous arthritis-models could be more beneficial in understanding how breach of tolerance is likely to occur in human RA. Among the genetically modified spontaneous models of arthritis, human TNF Tg model enabled investigators to study TNF-driven mechanisms of disease (354). In this model mice that over-express human TNF develop a chronic, erosive, symmetric polyarthritis. It provided a useful tool in defining the contribution of cytokines, such as RANKL, in the inflammatory process and bone destruction (355). A similar model is the IL-1 receptor antagonist-deficient model that spontaneously develops an erosive arthritis due to excess IL-1 signalling (198).

Another example of spontaneously developed arthritis comes from K/BxN mice. These mice are generated by crossing the NOD strain with the TCR Tg KNR mice that recognise the ubiquitous enzyme G6PI (189). In this model, although arthritis initiates with T cell recognition of specific antigen, anti-G6PI antibodies have arthritogenic activity when transferred in healthy or lymphocyte deficient RAG $^{-/-}$ mice (301).

The SKG model is characterised by a spontaneous mutation in the TCR signalling adapter molecule ZAP70, reflecting altered thymic T-cell selection (356). The ZAP70 mutation alone is not sufficient for triggering arthritis if mice are in a pathogen-free environment. A severe autoimmune arthritis is induced when mice are maintained in a conventional environment or by the injection of zymosan or β-glucans that activate

APCs through TLRs. Moreover, systemic manifestations such as interstitial pneumonitis and vasculitis occurred. Multiple autoreactive T cell clones are involved in the development of the SKG model and high titres of RF, ACPA and anti-CII antibodies have been demonstrated (357).

Although these models are useful for identifying the effect of specific genes in the arthritis development, they do not allow investigators to study the initial events involved in the induction of arthritis.

1.10 OVA-TCR-induced model of arthritis

In the Garside Laboratory an elegant model of experimental arthritis has been developed allowing the *in vivo* tracking of antigen-specific T cells (358). In this system $CD4^+$ T cells, that express a TCR specific for the chicken ovalbumin (OVA) peptide (323-339) (359) are purified from Tg mice DO11.10 and polarised towards Th1 phenotype (360). The adoptive transfer of Th1 cells into BALB/c recipients is then followed by the immunisation with OVA in adjuvant and challenge in the limb close to the ankle joint with heat-aggregated OVA (HAO). T cells of an irrelevant specificity were able to induce an arthritis, characterised by synovial inflammation and bone erosions (358). Although the arthritis elicited was mild, limited to the ankle joint and acute, the most interesting finding was the breach of B and T cell tolerance. This occurred even if mice were not immunised with a joint-related antigen, such as in CIA and PGIA models. Remarkably, RF, ACPA and anti-CII antibodies were detected in arthritic animals (358, 361). This finding encouraged the laboratory group to identify the timing, location and mechanisms of the breach of self-tolerance. Using this model we have demonstrated that conventional DCs mediate the breach of self-tolerance (361), while pDCs have a regulatory role, limiting self reactivity and the developing pathology (362). More recently, the importance of co-stimulation on the development of auto-reactivity was demonstrated, as the treatment with Abatacept (CTLA-4-Ig) was able to inhibit the development of autoantibodies, through the suppression of T cell follicular migration (363).

This model will be employed to answer questions relating to the role of B cells in the induction of arthritis. This thesis will start by describing the induction of a relapse of arthritis and characterising the collagen-specific B cell responses. It will continue by investigating the requirement of antigen-presenting B cells and autoantibodies in the induction of experimental acute arthritis. Finally in the last chapter the relative contribution of innate inflammation *versus* antigen-specific activation to the breach of T and B cell self-tolerance and pathology will be investigated.

The results of the chapters 3 and 5 have been published (364, 365) in peer-reviewed journals.

1.11 Thesis objectives

As highlighted above, RA pathogenesis can be subdivided in three stages: autoimmunity, inflammation and bone destruction (4). In susceptible individuals environment-gene interactions promote loss of tolerance to self-proteins (16, 31). Autoantibodies, such as RF and ACPA, can be detected in patients before the development of arthritis (pre-articular phase of RA) and their levels increase as the onset of disease approaches (5). Different mechanisms that probably involve infectious triggers, local microvascular, neurologic, biomechanical, microtrauma or other tissue-specific pathways, induce the localisation of the inflammatory process in the joints (transitional phase of RA) (6). Thus, synovitis is initiated and perpetuated by positive feedback loops and in turn promotes systemic disorders. Both the innate and adaptive immune pathways integrate to promote tissue inflammation, remodelling and damage (218). Understanding the relative contribution of innate *versus* antigen-specific pathways will help to design therapeutic strategies directed against both components of the innate and adaptive immune systems and aiming to re-establish immunological tolerance.

Experimental models of arthritis provide an invaluable tool to understand the immunological pathways underpinning the disease and evaluate potential therapeutic agents (366). Although arthritis models do cover several aspects of human disease, most models resemble the articular phase of RA ignoring early events that lead to autoimmunity. CIA is the most commonly used arthritis model because it shares many similarities with human disease (341). Collagen specific B and T cell responses have been studied in patients with RA and antibodies, immune complexes and CII-specific T cells have been detected in human joints (367).

The interest in B cells in RA has been renewed with the introduction of powerful therapeutic tools targeting B cells and related mediators (334). B cells exert several functions in RA including cytokine production, antigen presentation and the antibody production (297). Characterisation of the specific B cell responses might allow the identification of patients in the preclinical phase of disease as well as the understanding of the mechanism that leads to the establishment of chronic disease.

Recently, our group has developed a murine model where Th1 cells of irrelevant antigen specificity induced a transient arthritis and a breach of self-tolerance (358,

365). It therefore provides a useful tool to investigate the contribution of single cell populations and immune pathways to the breach of self-tolerance and pathology. However, an important limitation of this model is that arthritis was acute, self-limiting and localised to one joint unlike human disease.

The objectives of this thesis are therefore to:

1. Create a model of chronic autoimmune polyarthritis

To establish a novel model of chronic autoimmune polyarthritis that will be more applicable to human RA. This murine model will allow us to dissect the effects of single cell populations in the induction and maintenance of disease.

2. Characterise the collagen-specific antibody responses

To characterise the anti-collagen responses in terms of the major epitopes recognised and compare anti-CII responses from OVA-mediated arthritis with collagen-induced arthritis as a "gold standard" model of human disease.

3. Investigate the requirement of B cells in priming T cells and in the induction of acute OVA-mediated arthritis

To investigate the requirement of antigen presenting B cells and autoantibodies in OVA-mediated experimental arthritis employing Tg mice that exclude B cell antigen presentation and B cell deficient mice.

4. Prove the relative contribution of innate and adaptive immune responses in OVA-mediated arthritis

To confirm the relative contribution of innate *versus* antigen-specific pathways to the breach of T and B cell self-tolerance and pathology.

Table 1.1: Extra-articular manifestations and complication of RA

(Table adapted from reference (47))

Tissue or Organ	Manifestation	Complication
Skin	Nodules Raynaud's phenomenon Vasculitis	• Skin cancer • Chronic leg ulcers • Lung cancer • Hypertension • Heart failure • Ischemic heart disease • Cervical myelopathy • Atlanto-axial subluxation • Osteoporosis • Carpal tunnel syndrome • Non-Hodgkin lymphoma • Anaemia • Infections
Lung	Interstitial lung disease Pleuritis Nodules	
Heart	Valvular heart disease Myocarditis Pericarditis Nodules	
Nervous System	Mono/polyneuropathy Mononeuritis multiplex Vasculitis	
Eye	Sjögren syndrome Vasculitis Episcleritis/scleritis	
Haematological System	Felty's syndrome Lymphadenopathy Splenomegaly	
Kidney	Glomerulonephritis Interstitial nephritis Amyloid deposition	
Muscle	Myopathy Polymyositis	
Constitutional Symptoms	Fever, fatigue, weight loss, cachexia	

Table 1.2: Classification of human peripheral blood B cell subsets

(Table adapted from reference (205))

Markers			**CD27**	**IgD**	**IgM**	**IgG**	**CD38**
Transitional		T1	-	+	+++	-	+++
		T2	-	++	++	-	++
Naïve			-	++	+/-	-	+/-
Memory	Conventional CD27+ memory	unswitched	+	+	+	-	+/-
		unswitched	+	-	+	-	+/-
		switched	+	-	-	+	+/-
	Unconventional CD27- memory	unswitched	-	-	+	-	+/-
		switched	-	-	-	+	+/-

Table 1.3: Animal models of inflammatory arthritis

(Table adapted from reference (368))

Model	Species	Characteristics	Limits
CIA (184, 341)	Mouse, rat, rabbit, non-human primate	Acute/Chronic polyarthritis	Inducible in susceptible strains
CAIA (343, 346, 369)	Mouse	Polyarthritis	Self-limiting, no T and B cell involvement
Adjuvant-induced arthritis (349)	Rat	Acute symmetric polyarthritis	Acute, spondylitis not typical of RA
Pristane-induced arthritis (350)	Mouse, rat	Chronic symmetric polyarthritis	Spondylitis not typical of RA
Streptococcal cells wall-induced arthritis (186, 351)	Mouse, rat	Chronic symmetric polyarthritis	No autoantibodies
PGIA (352)	Mouse	Acute/Chronic Polyarthritis	Spondylitis
K/BxN (189)	Mouse	Chronic erosive polyarthritis	Distribution of joint involvement
SKG (356)	Mouse	Chronic erosive polyarthritis autoantibodies	Systemic manifestations not typical of RA
Human TNF transgenic (354)	Mouse	Chronic erosive polyarthritis	No autoantibodies
IL-1Ra -/- (198)	Mouse	Chronic erosive polyarthritis	No autoantibodies, tissue inflammation

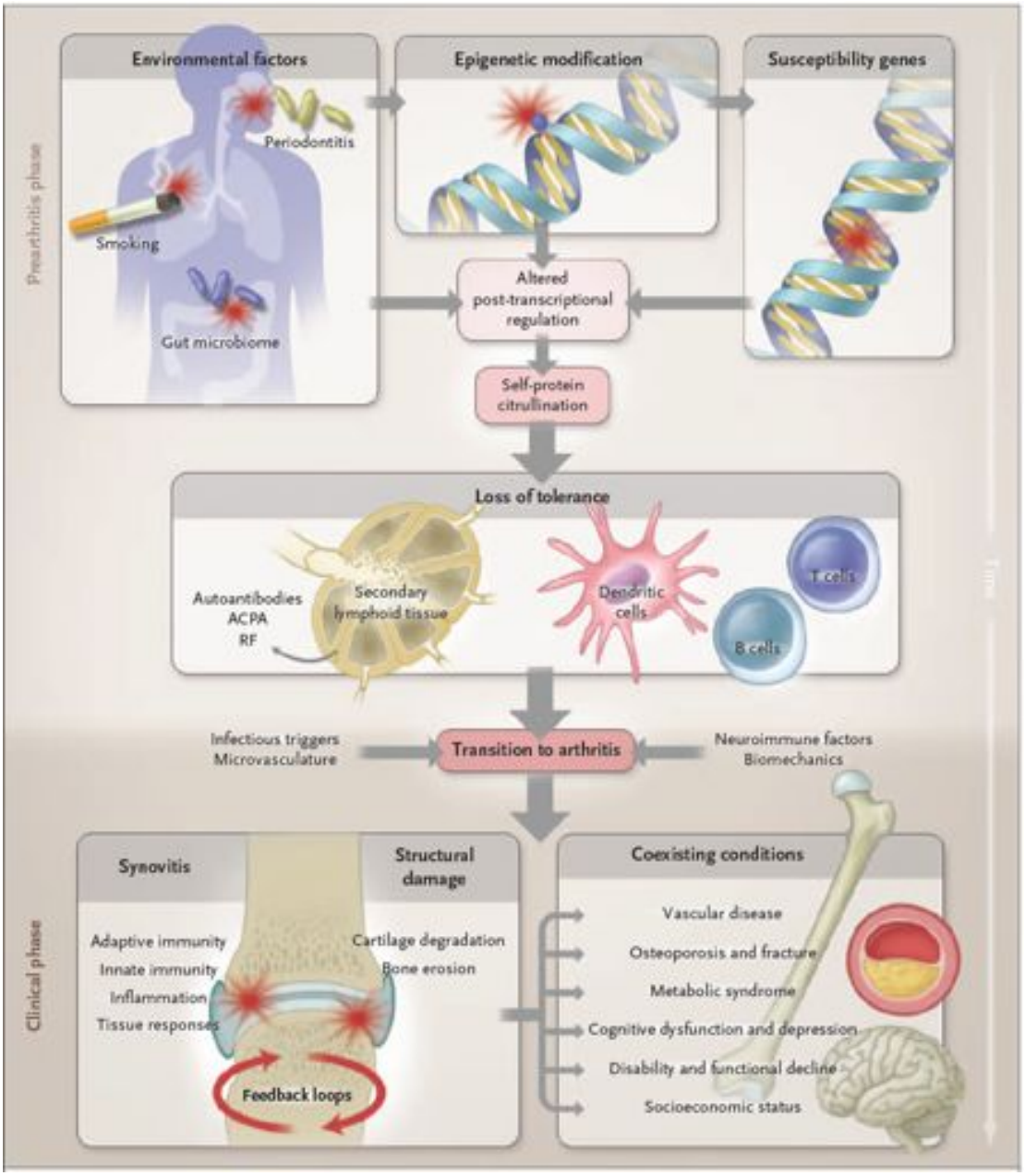


Figure 1.1 Pathogenesis of Rheumatoid Arthritis

Environment–gene interactions induce the breach of self-tolerance to different proteins and the production of RF and ACPA. Different mechanisms that probably involve infectious triggers, local microvascular, neurologic, biomechanical, microtrauma or other tissue-specific pathways, induce the localisation of the inflammatory process in the joints. The clinical phase of the disease is characterised by synovitis leading to structural damage and systemic disorders. Image reproduced from reference (6).

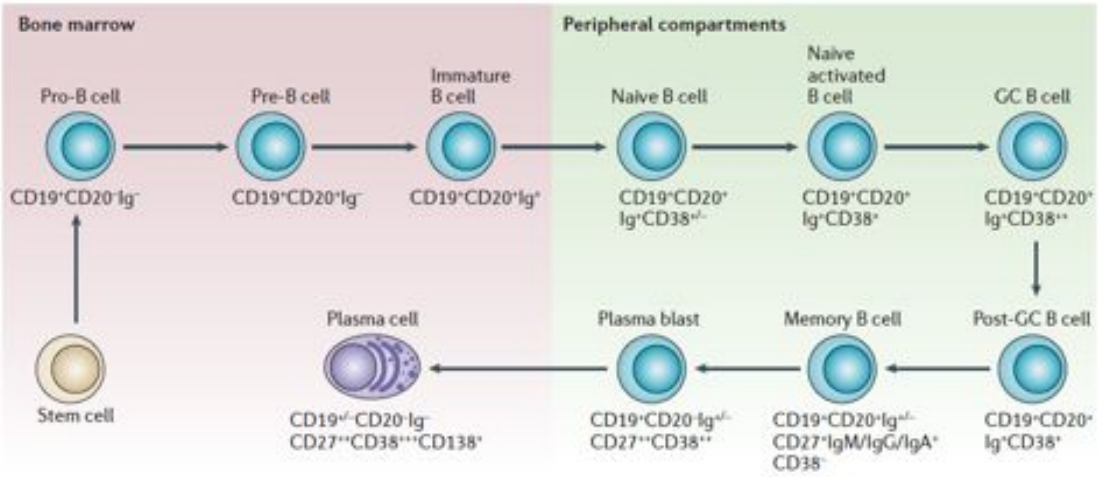


Figure 1.2 Illustration of the B cell development

Stages of the B cell maturation are indicated by their anatomical site and the expression of cell-surface markers. Image reproduced from reference (335).

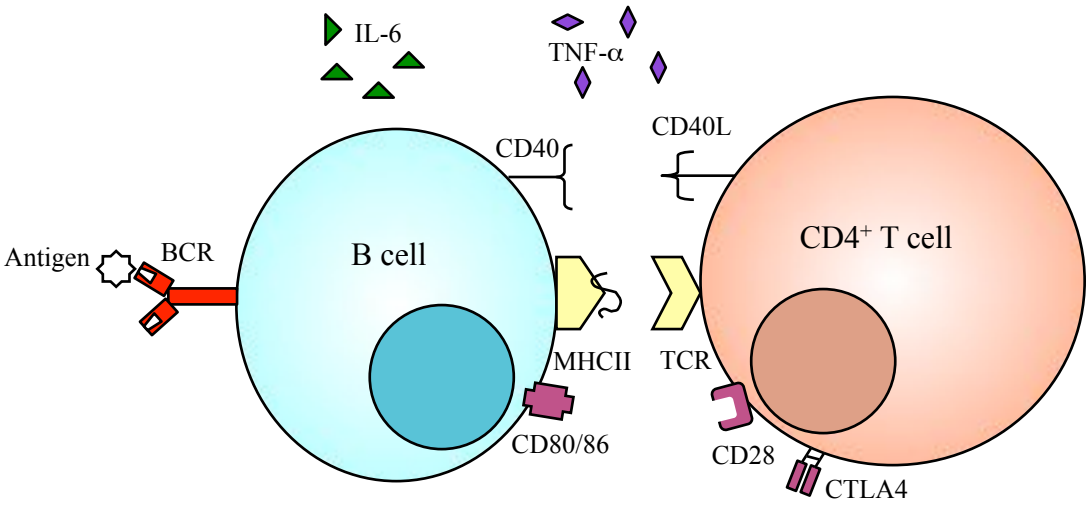


Figure 1.3 Illustration of B-T cell interaction

An illustration of B-T cell interaction. In secondary lymphoid organs B cells recognise and internalise antigen through the B cell receptor (BCR), a membrane-bound immunoglobulin that has high affinity for a given antigen. The BCR generated signals facilitate the traffic of antigen and MHCII molecules and the generation of peptide-MHC complexes. Recognition of peptide-MHC complexes on B cell triggers $CD4^+$ T cells to express CD40L that binds to CD40 on B cells. Crosslinking of the BCR by antigen induces the expression of CD86 on B cells, one of the co-stimulatory signals needed for naïve CD4 T cell activation. In this context cytokines influence the outcome of B-T cognate interaction.

Chapter 2

Materials and Methods

2.1 Mice

BALB/c (H-$2^{d/d}$) mice, between 6-12 weeks old, were either bred by the University of Strathclyde Biological Procedures Unit or purchased from Harlan, UK. Homozygous DO11.10 mice on a BALB/c (H-$2^{d/d}$) background, expressing the DO11.10 TCR specific for chicken OVA peptide 323-339/I-A^d, were used as $CD4^+$ T cell donors (359). DO11.10 Tg T cells were detectable using the KJ1-26 clonotypic antibody (359). Mice heterozygous for the antigen hen egg lysozyme (HEL) IgM^a and IgD^a transgenes on the BALB/c background (MD4 mice) were bred and screened by flow cytometry (as described in section 2.4) for their ability to bind HEL. Homozygous MD4 animals were used as recipients of DO11.10 $CD4^+$ T cells (370). JHD mice (371) on BALB/c background were kindly donated by Prof. David Gray from the University of Edinburgh. JHD mice are homozygous for the deletion of JH gene. As a result of this modification B cells cannot assemble the heavy chain genes of the Ig, and B cell differentiation is blocked at a precursor stage. A complete absence of mature B cells in the periphery and bone marrow is observed. JHD mice were used as adoptive transfer recipients. All animals were maintained at either the University of Strathclyde Biological Procedure Unit or the University of Glasgow Central Research Facility in accordance with Home Office regulations, in specified pathogen free cages, or filter-top cages, as appropriate.

2.2 Preparation of single cell suspensions from lymph nodes and spleens

Mice were killed by cervical dislocation and various lymph nodes (LNs) (cervical, inguinal, popliteal, auxiliary, brachial, cervical, mesenteric and para-aortic LNs) and spleen were extracted in RPMI complete media (for composition, refer to the table 2.1). Single cell suspensions were prepared by passing them through 100 μm nitex mesh (Cadisch and Sons) in RPMI complete media using the plunger of a sterile 5 ml syringe (BD Biosciences). Cell suspensions were washed with complete RPMI media and centrifuged at 400 x g for 5 minutes at 4°C. The pellet from spleen cell

suspension was re-suspended into 2 ml of red blood cell lysis buffer (Ebioscience) and cells were incubated for 5 minutes on ice. Spleen cells were washed with complete RPMI media, centrifuged (400 x g, 5 min, 4°C) and re-suspended in complete RPMI media. Cells were counted using a haemocytometer (Hawksley) and non-viable cells were excluded on the basis of trypan blue (Sigma) staining.

2.3 Magnetic-activated cell sorting (MACS)

$CD4^+$ T cells were isolated by negative selection using the $CD4^+$ T cell isolation kit from Miltenyi Biotec (#130-095-248) and the manufacturer's instructions were followed. In detail, spleen and LNs were made to single cell suspensions, as described in section 2.2. The cells were then centrifuged (300 x g, 10 min, 4°C) and re-suspended in 40 μl of MACS buffer (for composition refer to the table 2.1) per 10^7 cells. Biotin-antibody cocktail,10 μl of per 10^7 cells, was added and incubated for 10 minutes at 4-8°C. This antibody cocktail was directed against CD8a, CD11b, CD11c, CD19, CD45R (B220), CD49b (DX5), CD105, MHC-class II and Ter-119 (an erythroid cell marker). The incubation was followed by the addition of 30 μl of MACS buffer per 10^7 cells and 20 μl of anti-biotin labelled magnetic beads per 10^7 cells to the cell suspension for 15 minutes at 4-8°C. Cells were then washed by adding 2 ml of MACS buffer per 10^7 cells, centrifuged (300 x g, 10 min, 4°C) and re-suspended up to 10^8 cells in 500 μl of MACS buffer for the magnetic separation. LS columns (Myltenyi Biotec) were placed in the MACS separator (Miltenyi Biotec) and prepared with by rinsing with 3 ml of MACS buffer. Cell suspension was applied onto the column (up to 2 x 10^9 per column) and the effluent (negative fraction), which contained unlabelled cells representing $CD4^+$ T cells, was collected by washing the columns 4 times with 3 ml of MACS buffer. $CD4^+$ enriched fraction was used for Th1 polarisation. The positive fraction was flushed out with 5 ml of MACS buffer and used with spleen cells as a source of APCs for the Th1 polarisation. The cells in the positive and negative fraction were counted using a haemocytometer (Hawksley) and trypan blue (Sigma) for non-viable cell exclusion. Cells were washed, centrifuged (400 x g, 5 min, 4°C) and re-suspended in complete RPMI media. The percentage of $CD4^+$ $KJ1.26^+$ cells was determined by flow cytometric analysis, as described in section 2.4. The positive fraction and spleen cells were treated with 50 μg/ml of

mitomycin C (Sigma) for 1 hour at 37°C, 5% CO_2 and were then washed twice with complete RPMI media, centrifuged (400 x g, 5 min, 4°C) and re-suspended in 10 ml of complete RPMI media.

2.4 Flow cytometric analysis

Cells were isolated from the LNs of DO11.10 mice as described in section 2.2. For cell surface staining, aliquots of 10^6 cells in 12 x 75 mm polystyrene tubes (BD biosciences) were washed with 1 ml of FACS buffer (for composition refer to the table 2.1) and centrifuged (400 x g, 5 min, 4°C). Cells were re-suspended in 50 μl of Fc blocking buffer (for composition refer to the table 2.1) to reduce non-specific binding by Fc receptors and were incubated for 10 minutes at 4-8°C. Antibodies for extracellular staining or appropriately-labelled isotype controls were added to each sample at a dilution of 1:100 in Fc block and incubated for 30 minutes at 4-8°C in the dark. Cells were washed twice with 1 ml of FACS buffer and centrifuged (400 x g, 5 min, 4°C). When biotin-conjugated antibodies were used a fluorochrome-labelled streptavidin secondary reagent was necessary. The labelled streptavidin was diluted in FACS buffer and used at a concentration of 1 μg/ml for 15 minutes at 4-8°C. Cells were washed with FACS buffer, centrifuged (400 x g, 5 min, 4°C), followed by a final wash in FACS flow (BD biosciences) and centrifuged (400 x g, 5 min, 4°C). For intracellular cytokine staining, 2 x 10^5 cells per well were added in a 96-well round bottom microtitre plate (Costar) and incubated with 50 ng/ml of Phorbol-12-Myristat-13-Acetate (Sigma), 500 ng/ml Ionomycin (Sigma) for 5 hours at 37°C, 5% CO_2. Golgi-Plug (BD Biosciences) (diluted 1/1000) was added for the last 4 hours of the stimulation. After the incubation the cells were centrifuged (400 x g, 5 min, 4°C) and then stained for extracellular markers as described before. Cells were then fixed with 100 μl of 4% paraformaldehyde (PFA) for 20 minutes at room temperature in the dark, washed with 250 μl of permeabilisation buffer (for composition refer to the table 2.1), centrifuged (400 x g, 5 min, 4°C) and re-suspended in the same buffer. Cells were permeabilised for 20 minutes at 4°C in the dark, centrifuged (400 x g, 5 min, 4°C) and incubated with the antibody against the cytokine of interest. The antibodies were diluted in permeabilisation buffer at a concentration of 5 μg/ml and

cells were incubated with them for 30 minutes in room temperature in the dark. The cells were then washed with permeabilisation buffer, centrifuged (400 x g, 5 min, 4°C) and re-suspended in FACS flow (BD biosciences). Cells were passed through nitex to remove cell clumps. Reagents are listed in the table 2.2. Data were acquired on a FACSCanto (BD biosciences), using the Diva software, or FACSCalibur (BD biosciences) using Cell Quest Pro software and analysed with FlowJo software (Treestar Incorporated).

2.5 In vitro Th1 polarisation

Th1 polarisation was based on the protocol used by Maffia *et al.* (358). $CD4^+$ T cells and mitomycin C treated splenocytes, which represented the source of APCs, were isolated from DO11.10 mice as described in sections 2.2 and 2.3. In detail, $CD4^+$ T cells at a concentration of 5 x 10^5 cells/ml were co-cultured with APCs at a concentration of 5 x 10^6 cells/ml in complete RPMI media in the presence of 0.5 μg/ml $OVA_{323-339}$ peptide (Genosys), 10 ng/ml of IL-12 (PeproTech) and 2 μg/ml of anti-IL-4 (RnD Systems) (372, 373). Cells were cultured in 75T tissue culture flasks (Nunc) for 3 days at 37°C and 5% CO_2. Intracellular cytokine flow cytometric staining assessed the phenotype of the polarised population, as described in section 2.4.

2.6 Preparation of heat aggregated ovalbumin and bovine serum albumin

Chicken OVA (Sigma) or BSA (Sigma) were diluted in PBS (for composition refer to table 2.1) at a concentration of 20 mg/ml and were incubated at 100°C for 2 hours until aggregated. The denatured solidified OVA or BSA were washed with PBS and centrifuged (450 x g, 5 min, 4°C). Supernatant was discarded and aggregated OVA or BSA was re-suspended at 20 mg/ml in PBS. HAO or heat aggregated BSA (HABSA) were stored at -20°C until required. Before use, they were sonicated for 5 minutes on

ice until clumps were broken down and the mixture could pass through a 27 G needle in order to be injected.

2.7 Induction of acute OVA-mediated arthritis in BALB/c mice

The experimental model of OVA-mediated acute arthritis was developed by Maffia *et al.* (358). LNs from DO11.10 mice were pooled and $CD4^+$ T cells were purified by negative selection, as described in sections 2.2 and 2.3. Th1 cell polarisation was induced by culturing $CD4^+$ T cells with APCs in presence of $OVA_{323–339}$ (Genosys), IL-12 (PeproTech) and anti-IL-4 (RnD Systems), as described in section 2.5. The proportion of Tg Th1 IFN-γ producing cells was determined by flow cytometric analysis as described in section 2.4. Recipient mice were injected intravenously (i.v.) with 2 x 10^6 Th1 DO11.10 cells. One day following the adoptive transfer recipient mice were immunised subcutaneously (s.c.) in the scruff with 100 μg of chicken OVA (Sigma) emulsified with CFA (Sigma). Ten days after immunisation mice were challenged (primary challenge) with a s.c. injection close to the ankle joint, in one hindlimb with 100 μg of HAO in 50 μl of PBS. Control mice received PBS instead of s.c. HAO. Other control mice received an i.p injection of 100 μg HAO**.** The mice were monitored daily for clinical signs of arthritis and were scored according to table 2.3. Paw thickness was measured using a dial calliper (Kroeplin). Mice were sacrificed 7 days after challenge by cervical dislocation. Hind limbs were removed, fixed in 10% neutral-buffered formalin (Sigma) for 14 days and sent to the Histopathological Department of the Veterinary School of Glasgow University for histological analysis. They were stained with Heamatoxylin and Eosin (H&E) and toluidine blue. The joint histopathology was scored by two blinded observers based on inflammation, synovial hyperplasia and cartilage/bone erosion on a scale from 0 to 3, giving a maximum of 9 per joint (361, 374) as described in table 2.4 and showed in figure 2.1. Peripheral blood was collected into heparinised capillary tubes (Hawksley & Sons Ltd) either by cardiac puncture or venesection, the plasma separated by centrifugation at 450 *g* for 10 minutes and stored at -20°C until analysis. Serum samples were analysed for the presence of anti-OVA, anti-CII antibodies, and RF by

ELISA as described in section 2.14. When collagen-specific B cell response was compared in CIA and OVA-mediated arthritis (chapter 3, section 3.3.6), serum samples were analysed also for the presence of anti-U1, anti-J1, anti-C1 and anti-Citrullinated C1 (Cit-C1) antibodies by ELISA as described in section 2.14. Serum from mice with CIA was kindly donated by Dr Carl Goodyear from the Division of Clinical Neuroscience, University of Glasgow. Triple helical peptides U1, J1, C1 and Cit-C1 were kindly donated by Prof. Rikard Holmdahl from the Karolinska Institutet of Stockholm. Table 2.5 shows the peptide sequences.

2.8 Induction of acute OVA-mediated arthritis in mice with different B cell repertoire

When the role of B cells was analysed (chapter 4), acute OVA-mediated arthritis was induced, as described in section 2.7, in different recipient animals such as mice with normal B cells (BALB/c), in mice with B cells that could not present antigen and or induce autoantibodies (MD4), and in mice without mature B cells (JHD). Mice were monitored daily for clinical signs of arthritis and joint histology was performed at day 7 after challenge, as described in section 2.7. Serum samples were analysed for the presence of anti-OVA, anti-CII antibodies and ACPA by ELISA as described in section 2.14. $CD4^+$ T cell proliferation was assessed by flow cytometry employing the Click-iT Edu proliferation assay, as described in section 2.16.

2.9 Induction of acute OVA-mediated arthritis with innate and/or antigen specific stimulation

When the contribution of the innate and antigen-specific pathways was analysed (chapter 5) BALB/c mice, that previously received the transfer of OVA-specific Th1 cells and the immunisation with OVA (as described in section 2.7), were challenged s.c. in their limb close to the ankle joint with 100 μg of HAO, 25 μg of LPS (lipopolysaccharide, from *Salmonella enterica* serotype abortus equi (Sigma)) or a combination of HAO and LPS diluted in 50 μl saline (SAL). In some experiments

animals were challenged s.c. in their limb with 100 μg of HABSA. Control mice received a similar injection of 50 μl SAL. Seven days post challenge recipient mice were killed by cervical dislocation. Serum samples were analysed for anti-OVA, anti-CII, RF, anti-DNA, anti-KLH antibodies by ELISA and ANA by IIF, as described in sections 2.14 and 2.15. $CD4^+$ T cell proliferation was assessed by tritiated thymidine ($[^3H]$TdR) incorporation, as described in section 2.16. Hind limbs were removed and decalcified in 5.5% EDTA solution in a phosphate buffer pH 7.4 for 14 days. Tissue sections were cut and stained with monoclonal antibody KJ1.26, as described in section 2.12.

2.10 Induction of a relapse of OVA-mediated arthritis

After the development of acute arthritis, as described in section 2.7, recipient mice received a secondary challenge at day 34 after the first HAO challenge. Mice were monitored daily for the clinical signs of arthritis by the clinical score according to the table 2.3 and the paw thickness measured using a dial calliper (Kroeplin). Animals were bled via the tail vein and serum samples were stored at –70°C until they were analysed for the presence of antibodies. Mice were sacrificed by cervical dislocation, hind limbs were removed, fixed in 10% neutral-buffered formalin (Sigma) for 14 days and stained with H&E and toluidine blue.

When the secondary challenge was systemic (chapter 3, sections 3.3.2 and 3.3.3) recipient mice received an intraperitoneal (i.p.) injection with 100 μg of HAO or 200 μg of CII (Sigma) and 5 μg of LPS (from *Salmonella enterica* serotype abortus equi (Sigma)). Control mice received an i.p. injection of HAO or CII and LPS but they were not immunised and HAO-challenged. Animals were bled at day 7, 34 and 41 after the first HAO challenge and serum samples were analysed for the presence of anti-CII antibodies and RF by ELISA as described in section 2.14. Mice were sacrificed by cervical dislocation 7 days after the systemic secondary challenge.

When the secondary challenge was local (chapter 3, section 3.3.4) mice received a periarticular s.c. injection (50 μl) of 100 μg HAO or 200 μg CII (Sigma) in

incomplete Freund's adjuvant (IFA) (Sigma) in the controlateral ankle. Control animals received a local injection of 50 μl IFA in PBS or PBS. Animals were bled at day 7, 34 and 57 after the first HAO-challenge and serum samples were analysed for the presence of anti-OVA, anti-CII and anti-U1 antibodies by ELISA as described in section 2.14. Mice were sacrificed by cervical dislocation three weeks after the secondary local challenge.

2.11 Passive transfer

Sera for the passive transfer were obtained from BALB/c mice with OVA-mediated chronic arthritis. The sera were pooled and 150 μl in a single day or 200 μl in two consecutive days were injected i.v. into naïve BALB/c animals. Control mice received an i.v. injection of normal mice serum (Biosera). Recipient animals were sacrificed by cervical dislocation one day after the passive transfer and peripheral blood was collected into heparinised capillary tubes (Hawksley & Sons Ltd) by venesection, the plasma separated by centrifugation at 450 *g* for 10 minutes and stored at -20°C until analysis. Serum samples from donor arthritic mice, recipient mice and naïve BALB/c mice were analysed for the presence of anti-CII and anti-OVA antibodies by ELISA as described in section 2.14.

When 200 μl of sera were injected in two consecutive days, mice were bled at day 2 and 14 after the passive transfer, monitored daily for the clinical signs of arthritis by the paw thickness measured using a dial calliper (Kroeplin GmbH, Germany) and limbs were removed from recipient mice 14 days after passive transfer, fixed in 10% neutral-buffered formalin (Sigma) for 14 days and stained with H&E and toluidine blue.

2.12 Immunohistochemistry (IHC)

Tissue sections (6-10 μm) were cut from decalcified limbs in 5.5% EDTA solution in a phosphate buffer pH 7.4 and frozen in OCT embedding medium (Miles, Elkhart, Indiana, USA) on a cryostat microtome (ThermoShandon). Sections were mounted onto SuperFrost slides (BHD) before being allowed to air-dry and stored at –20°C until further processing. Slides were brought to room temperature, fixed in acetone for 10 minutes and the sections outlined with a wax pen to allow addition of solutions without cross contamination. The remainder of the staining process was carried out in a humidified, darkened chamber. To quench endogenous peroxidase activity, sections were incubated with 0.1% azide/3% H_2O_2 for 45 minutes, changing the solution three times. After washing in PBS, sections were stained with monoclonal antibody KJ1.26 diluted 1:1600 in TNB blocking buffer (for composition refer to the table 2.1) for 30 minutes, before being washed in TNT buffer (for composition refer to the table 2.1) (2x). Subsequently, sections were incubated with streptavidin-horseradish peroxidase (SA-HRP, from TSATM kit, Invitrogen Ltd) diluted 1:100 in TNB blocking buffer for 30 minutes before washing in TNT buffer (2x). Sections were then incubated in biotinylated tyramide diluted 1:50 in amplification diluent (both from TSATM kit, Invitrogen Ltd) for 10 minutes. Sections were washed three times in TNT buffer. SA-HRP was added again for 30 minutes before washing in TNT buffer (3x). Enzymatic activity was detected with 3,3'-diaminobenzidine (DAB) substrate (Vector) before washing in H_2O, followed by incubation with DAB enhancing solution (Vector) for approximately 10 seconds and a wash in H_2O. Harris haematoxylin (Vector) was used to counterstain before rinsing in H_2O and dipping in acid alcohol, tap water, bicarbonate then tap water. Sections were subsequently exposed to 70% ethanol, 95% ethanol (2x), then 100% ethanol for dehydration before clearing in Histoclear (BS & S Ltd) and immediate mounting in Histomount (BS & S Ltd). Multiple fields of the different joints were analysed by a blinded observer.

2.13 Biacore assay

Analysis was performed using a Biacore 2000 Surface Plasmon Resonance system, sensor chip CM5 and BIA evaluation software. Hepes-buffered saline (HBS-EP) (for composition refer to the table 2.1) was used as running buffer and maintained over the

sensor surface. Immobilisation of bovine CII (Sigma) and U1 peptide was performed according to previously described principles (375). The carboxylated dextran matrix on the sensor surface was activated with 0.2 M N-ethyl-W- (3-diethylaminopropyl) carbodiimide and 0.05 M N-hydroxysuccinimide injected for 7 minutes at a flow-rate of 5 μl/min. Specific surfaces were obtained by injecting bovine CII (20-500 μg/ml) or U1 peptide (100-500 μg/ml) in 10 mM sodium acetate buffer pH 5 for 5 minutes at a flow-rate of 5 μl/min. The immobilisation procedure was completed by injecting three times 35 μl of 1M ethanolamine hydrochloride at a flow-rate of 5μl/min to block remaining ester groups. Serum samples from OVA-mediated arthritis, CIA and control animals were diluted 1:10 in HBS buffer and injected using a flow rate of 5 μl/min. The surface was regenerated by injecting 40 μl of 10 mM glycine-HCl pH 1.5 at a flow rate of 20 μl/min. The antigen/antibody interaction generated a binding profile with an on-rate, which corresponded to the association, and an off-rate that identified the dissociation of the complex. The on-rate represents the molecular recognition and the off-rate is indicative of the stability of the complex. The antibody/antigen complex stability (% remaining) was calculated early and late in the dissociation phase following injection of the analyte by expressing the "stability late" as a percentage of "stability early". The stability late was calculated with the average response at 280 sec after buffer injection and the stability early was the average response from the first 5 sec after buffer injection (376).

2.14 Enzyme-linked immunosorbent assay (ELISA)

Immunol 2 plates (Costar) were coated with antigen, reported in table 2.6, in 0.05 M carbonate buffer pH 9.3 (50 μl per well) and incubated at 4°C overnight. Plates were then washed three times with ELISA wash buffer (for composition refer to the table 2.1). Non-specific protein binding was blocked by ELISA blocking buffer (200 μl per well) (for composition refer to the table 2.1) for 1 hour at 37 °C. Then plates were washed with ELISA wash buffer (3x) and incubated with mice serum (50 μl per well) for 1 hour at 37 °C. Serum samples were added either in serial dilution or single dilution using the ELISA sample buffer (for composition refer to the table 2.1). After the incubation, the plates were washed (4x) with ELISA wash buffer before adding

the detection antibody (100 μl per well) peroxidase-conjugated goat anti-mouse IgG2a (Southern Biotech) diluted 1:10000 or peroxidase-conjugated goat anti-mouse total IgG (Jackson InnonoResearch Laboratories) diluted 1:5000 at 37 °C for 1 hour. After washes (5x) in ELISA wash buffer plates were incubated with 3, 3',5,5'-tetramethylbenzidine peroxidase (TMB) substrate (Kirkegaard & Perry Laboratories) (50 μl per well) for the appropriate time. The reaction was stopped by the addition of 10% H_2SO_4 (50 μl per well). Absorbances were read at OD_{450} using a microplate reader (Molecular Devices).

When ACPA levels were tested, the DIASTAT Anti-CCP2 kit (Axis-Shield) was used (362). The protocol was the same of the standard ELISA described above except for the plate that was already coated with highly-purified synthetic cyclic citrullinated peptides containing modified arginine residues. The secondary antibody was substituted with alkaline phosphatase-conjugated goat anti-mouse IgG (CALTAG Laboratories) diluted 1 in 1000 in PBS.

When anti-ssDNA antibodies were tested, plates were coated with poly-L-lysine (Sigma P8920) at 50 μg/ml in carbonate buffer (100 μl/well), incubated 1 hour at 37°C, then overnight at 4°C. DNA was boiled for 10 minutes and quenched on ice to obtain single stranded DNA. Plates were washed three times in ELISA wash buffer and incubated with thymic calf DNA Type I (Sigma) at 10 μg/ml in carbonate buffer (50 μg/well) for 2 hours at 37°C. Plates were washed three times in ELISA wash buffer. Non-specific protein binding was blocked by 3% BSA in PBS (200 μl per well) for 1 hour at 37 °C. The rest of the protocol was the same of the standard ELISA described above.

The composition of the antigen, antibodies and the buffers used are listed in the tables 2.1 and 2.6.

2.15 Indirect Cellular Immunofluorescence

Cellular IIF was carried out with murine fibroblasts (clone L929) in order to detect the production of ANA. Cells were cultured in complete Dulbecco's modified Eagle Media (Sigma) (for composition refer to the table 2.1), at 37°C in a humidified atmosphere of 5% CO_2. Fibroblasts were grown to confluence (4 x 10^6 cells) and transferred at a concentration of 3 x 10^4 /well on 13 mm coverslips in 24 well tissue culture plates (Costar). Plates were incubated overnight at 37°C to allow cells to grow on the coverslips. The coverslips were removed from the wells and after washing in PBS, cells were fixed in PBS containing 1% PFA for 10 minutes at +4°C. After washing with PBS, cells were incubated with the permeabilsation buffer (for composition refer to the table 2.1) for 5 minutes at room temperature. All the following steps were performed at room temperature. Coverslips were washed with PBS and incubated with blocking reagent (for composition refer to the table 2.1) for 15 minutes. Sera from BALB/c mice challenged with HAO + LPS, HAO, LPS or SAL were diluted from 1/20 to 1/100 in blocking reagent plus 0.1 % saponin and incubated in darkness for 30 minutes. After washing the coverslips in blocking buffer, they were incubated with FITC-labelled anti-mouse IgG (SAPU) diluted 1:20 in blocking reagent with 0.1% saponin in darkness for 30 minutes. After washing the coverslips in 1% blocking buffer, they were mounted and counterstained with DAPI (Vector). Fluorescence was analysed with a Nikon, Eclipse E60 microscope. Pictures were analysed with MetaMorph software (Offline, version 4.6r3).

2.16 Proliferation assay

To measure the relative ability of $CD4^+$ T cells to proliferate in response to various antigens the incorporation of the nucleoside analogue 5-ethynyl-2´-deoxyuridine (Edu) during active DNA synthesis was employed using the Click-iT®EdU Alexa Fluor® 488 Cytometry assay kit (Invitrogen). Detection was based on a copper catalyzed reaction between an azide and an alkyne (377, 378). The Edu contained the alkyne and the Alexa Fluor® 488 dye contained the azide. Mice were killed by cervical dislocation and popliteal LNs were harvested for *in vitro* restimulation. Single cell suspensions were prepared from the popliteal LNs, as described in section 2.2. 2.5 x 10^5 cells in 200 µl were added in each well of a 96-well microtitre plate that

contained either complete RPMI media, or complete RPMI media with 1 mg/ml of chicken OVA or 50 μg/ml of CII (Sigma) and were incubated for 72 hours at 37°C, 5% CO_2, as previously described (362). After 48 hours, Edu (Invitrogen) was added to each well at a concentration of 5 μg/ml. After 72 hours the cells were centrifuged (400 x g, 5 min, 4°C), washed twice with FACS buffer, and stained for the surface marker CD4, as described in section 2.4. They were then washed twice with blocking reagent (for composition refer to the table 2.1), fixed with 4% PFA (20 minutes at room temperature in the dark), washed with blocking reagent and centrifuged (400 x g, 5 min, 4°C). Following this, they were re-suspended in the Click-iT™ reaction cocktail prepared according to manufacturer's instructions and incubated for 30 minutes at room temperature in the dark. Cells were then washed with blocking reagent, centrifuged (400x g, 5min, 4°C) and re-suspended in FACS flow (BD). Data were acquired using a FACS Canto (BD), using the Diva software, or FACSCalibur (BD) using Cell Quest Pro software, and analysed with FlowJo software (Treestar).

T cell proliferation was also assessed in a primary immune response in absence of antigen presenting B cells (chapter 4, section 4.3.3). In this experiment naïve BALB/c, MD4 and JHD mice were immunised s.c. in the scruff with 100 μg of chicken OVA (Sigma) emulsified with CFA (Sigma). Seven days later mice were killed by cervical dislocation. Peripheral LNs (pLNs) were extracted in RPMI complete media. Cells from the pLNs were made into a single cell suspension, as describes in section 2.2, and cultured for 72 hours with OVA (1 mg/ml), CII (50 μg/ml) or complete RPMI. Their ability to proliferate was assessed by flow cytometry employing the Click-iT Edu proliferation assay, as described above.

When antigen-specific proliferation was investigated in mice treated with LPS and/or HAO (chapter 5, section 5.3.4) it was assessed by [^{3}H] TdR incorporation. In detail, cell suspensions from the popliteal LNs were cultured in complete medium either alone, or with 1 mg/ml OVA or with 50 μg/ml of CII for 72-120 hours in flat-bottomed 96-well tissue culture plates. Proliferation was assessed by addition of 1 μCi/well [^{3}H] TdR for the last 18 hours of culture. DNA-bound radioactivity was harvested onto glass fibre filter mats and the thymidine incorporation was measured on a 1205 Betaplate scintillation counter. The amount of radioactivity measured in a

scintillation counter is proportional to the number of proliferating cells, and the readout is counts per minute (cpm) per well. Stimulation index was calculated with the ratio cpm experimental/ cpm background unstimulated.

2.17 Statistics

Data were analysed using the GraphPad Prism® software. To test normality of data sets the D'Agostino and Pearson omnibus test was used. To test if the means of two samples were different the Mann Whitney test was used for non-normally distributed data sets. To compare the means of more than two samples Kruskal-Wallis test was used. When the interaction of two independent variables was tested two-way ANOVA was employed. A value of $P < 0.05$ was considered as significant. The number of symbols used indicated the level of statistical significance; for example: * $p < 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

Table 2.1 Buffers

Buffer	Constituents
PBS	8 g NaCl, 1.16 g Na_2HPO_4, 0.2 g KCl, 0.2 g KH_2PO_4 in 1 L of distilled water, pH 7.4
Incomplete RPMI	RPMI-1640 medium
Complete RPMI	RPMI-1640 medium, 10% FCS, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin
MACS buffer	2% FCS, 2 mM EDTA in PBS
FACS buffer	2% FCS, 0.05% NaN_3 in PBS
Fc blocking buffer	Supernatant from 2.4G2 hybridoma cultures, 10% Mouse serum, 0.01% NaN_3
Permeabilisation buffer (for Flow Cytometry)	0.5% saponin, 1% FCS, 0.05% NaN_3, 2 mM EDTA in PBS, pH 8.0
TNB blocking buffer	0.1 M Tris-HCl pH 7.5, 0.15 M NaCl, 0.5% blocking reagent in distilled water
TNT wash buffer	0.1 M Tris-HCl pH 7.5, 0.15 M NaCl, 0.05% Tween-20 in distilled water
Hepes-buffered saline	0.01 M Hepes, 0.15 M NaCl, 3 mM EDTA and 0.005% surfactant P20, pH 7.4
ELISA wash buffer	0.05% Tween 20 in PBS
ELISA blocking buffer	10% FCS in PBS
ELISA sample buffer	0.2% FCS, 0.05% Tween 20 in PBS
Complete Dulbecco's modified Eagle	Dulbecco's modified Eagle medium, 10% FCS, 2 mM L-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin
Permeabilisation buffer (for IIF)	2% FCS, 2 mM EDTA, 0.1% saponin in PBS, pH 8.0
Blocking reagent	1% BSA in PBS

Table 2.2: Flow Cytometry reagents

Antigen	Isotype	Supplier	Label
CD4	Rat IgG2a,κ	BD	FITC, PE
B220	Rat IgG2a,κ	BD	FITC, PE
IFN-γ	Rat IgG1,κ	BD	APC
OVA-TCR (KJ1.26)	Mouse IgG2a	BD	Biotin, FITC
IgMa	Rat IgG2a,κ	BD	Biotin
HEL	N/A	N/A	Biotin
Streptavidin-FITC		BD	
Streptavidin-PE		BD	

Table 2.3: Clinical scoring system of arthritis

Score	**Clinical finding** (each limb could receive a score of ≤5 points)
0	Normal
1	Mild redness and swelling of the ankle joint
2	Moderate redness and swelling of the ankle joint
3	Severe redness and swelling of ankle joint
4	Loss of function of the ankle joint
5	Maximally inflamed limb with involvement of multiple joints

Table 2.4: Joint Histopathology Score

Score	Inflammation	Synovial Hyperplasia	Cartilage/Bone Damage
1	Mild infiltration of inflammatory cells (< 50) in synovium and periarticular tissue of affected joints	Mild infiltration of pannus in marginal zone of affected joints	Mild loss of toluidine blue staining with no obvious chondrocyte loss and/or mild small areas of marginal zone/periosteal resorption
2	Moderate infiltration of inflammatory cells (50-200) in the affected joints	Moderate infiltration of pannus (2-3 layers) in the affected joints	Moderate loss of toluidine blue staining with focal chondrocyte loss and/or moderate areas of marginal zone/periosteal resorption
3	Severe infiltration of inflammatory cells (> 200) in the affected joint	Severe infiltration of pannus (> 3 layers) in the affected joints	Severe loss of toluidine blue staining with multifocal chondrocyte loss and/or severe resorption of medullary trabecular and cortical bone, and/or destruction of joint architecture

Table 2.5 Peptide Sequences

Peptide	Peptide sequence
U1	GPBGPBGPBGPBGPBG-*LVGPRGERGFB*-GPBGPBG-εACA
J1	GPBGPBGPBGPBGPBG-*MBGERGAAGIAGPK*-GPBGPBG-εACA
C1	GPPGPPGPPGPPGPPG-*ARGLTGRBGDA*-GPPGPPG-εACA
Cit-C1	GPPGPPGPPGPPGPPG-*ACitGLTGCitPGDA*-GPPGPPG-εACA

Amino acids are abbreviated as follows: G, Glycine; P, proline; B, hydroxyproline; L, leucine; V, valine; R, arginine; F, phenylalanine; εACA, ε-amonohexanoic acid-lysine-lysine-tyrosine-glycine-OH; M, methionine; E, glutamic acid; A, alanine; I, isoleucine; K, lysine; T, threonine; D, aspartic acid; Cit, citrulline.

Table 2.6 ELISA antibodies

Antibody to be detected	Antigen coated in the plate	Detection antibody used
Anti-CII IgG2a	Bovine collagen type II (4 μg/ml)	Goat anti-mouse IgG2a-HRP (1:10000)
Anti-CII IgG	Bovine collagen type II (4 μg/ml)	Goat anti-mouse IgG-HRP (1:5000)
Rheumatoid Factor IgG2a	Purified mouse IgG1 (1 μg/ml)	Goat anti-mouse IgG2a-HRP (1:10000)
Anti-OVA IgG	Chicken Ovalbumin (20 μg/ml)	Goat anti-mouse IgG-HRP (1:5000)
Anti-OVA IgG2a	Chicken Ovalbumin (20 μg/ml)	Goat anti-mouse IgG2a-HRP (1:10000)
Anti-U1 IgG	U1 (4 μg/ml)	Goat anti-mouse IgG-HRP (1:5000)
Anti-J1 IgG	J1 (4 μg/ml)	Goat anti-mouse IgG-HRP (1:5000)
Anti-C1 IgG	C1 (4 μg/ml)	Goat anti-mouse IgG-HRP (1:5000)
Anti-Citrullinated C1 IgG	Citrullinated C1 (4 μg/ml)	Goat anti-mouse IgG-HRP (1:5000)
ACPA IgG	Citrullinated peptide	Alkaline phosphatase-labelled anti-mouse IgG (1:1000)
Anti-ssDNA IgG2a	thymic calf DNA Type I (10 μg/ml)	Goat anti-mouse IgG2a-HRP (1:10000)
Anti-KLH IgG2a	KLH	Goat anti-mouse IgG2a-HRP (1:10000)

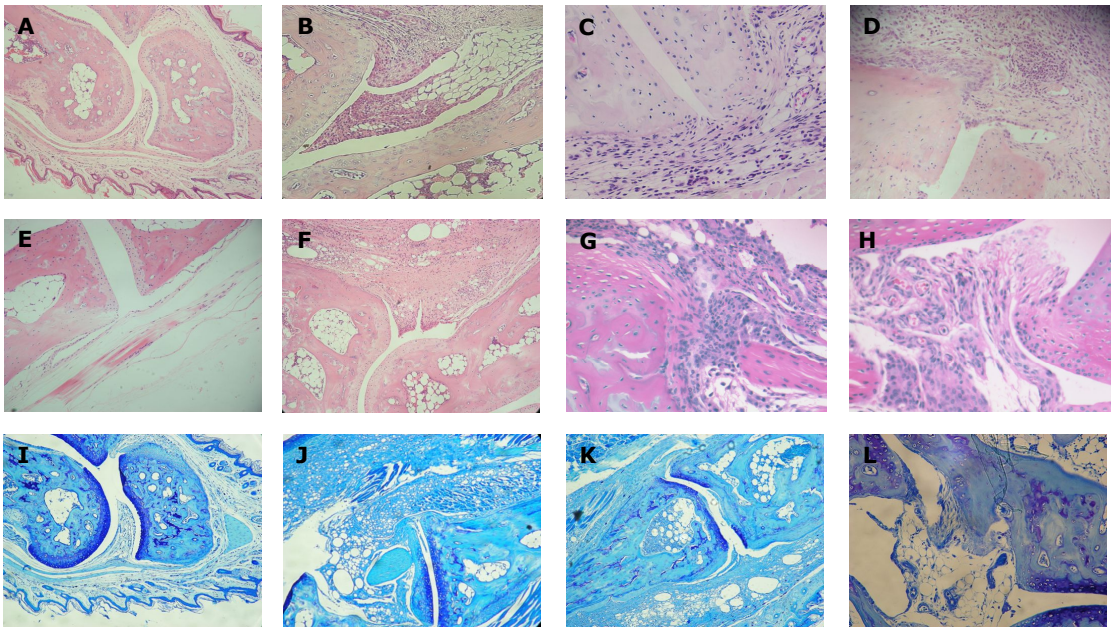


Figure 2.1 Joint histopathology scoring system

Representative haematoxylin/eosin and toluidine blue stained sections prepared from the joints of normal mice (A, E, I) and arthritic mice (B, C, D, F, G, H, J, K, L).

Panels A-D show joint inflammation with score 0 (no cell infiltration, A), 1 (mild infiltration, B), 2 (moderate infiltration, C) and score 3 (severe cell infiltration, D). Panels E-H show synovial hyperplasia with score 0 (no synovial hyperplasia, E), 1 (mild synovial hyperplasia, F), 2 (moderate synovial hyperplasia, G), and 3 (severe synovial hyperplasia, H). Panels I-L show cartilage/bone damage with score 0 (no cartilage/bone damage, I), 1 (Mild loss of toluidine blue staining with areas of marginal cartilage depletion, J), 2 (moderate loss of toluidine blue staining with focal areas of chondrocyte loss, K), and score 3 (severe bone erosion, L). Panels A, E, F, I, J, K original magnification X10; panels B and L original magnification X20; panels C, D, G, H original magnification X40.

Chapter 3

Characterisation of the anti-collagen antibody response in a model of relapsing arthritis

3.1 Aim and rationale

In this chapter a relapse of arthritis was induced in the model of OVA-mediated arthritis and the associated collagen specific B response was characterised. A model of experimental acute arthritis has been previously established where autoimmunity is elicited by antigen specific T cells (358). In this model, Tg T cells, specific for the antigen OVA, mediated the development of arthritis and the production of RF, ACPA and anti-CII antibodies, resembling the humoral features of human disease (358, 362, 365). However, a limitation of this model was that the arthritis was acute, self-limiting and localised in the ankle joint. To be more applicable to human disease, the model would ideally reach a chronic stage affecting several joints. Thus, the aims of this study were firstly, to develop a model of chronic OVA-mediated polyarthritis, rather then acute-limited arthritis; secondly, characterise the anti-CII response elicited in this model in terms of the B cell epitopes recognised; and finally to compare anti-CII responses from OVA-mediated arthritis with CIA as a 'gold standard' model of human RA.

3.2 Introduction

RA is an inflammatory chronic and systemic disease characterised by extensive synovitis resulting in erosions of articular cartilage and marginal bone leading to joint destruction (379). Animal models of RA differ in several aspects from human disease but they may reproduce some features of the disease allowing the study of particular characteristics of the pathogenesis that can be translated to human disease. They are also used to evaluate potential new therapeutic targets. Numerous animal models of RA exist and there is no "universal model" since they all differ in comparison to RA with respect to several aspects such as disease onset, chronicity, pattern of joint involvement, severity of synovitis, autoantibody profile or extraarticular manifestations (342, 366). CIA is the most widely used murine model of RA that allows the dissection of the pro-inflammatory mechanisms contributing to pathology. However, an important limitation of the CIA model is that the collagen response and the pathology are stimulated by exogenously injected collagen administered in a potent adjuvant, whereas human RA is characterised by a spontaneous breach of self-tolerance. In this regard the experimental model of OVA-mediated arthritis has been established where anti-collagen antibodies are induced by OVA-specific T cells in absence of collagen immunisation (358). In order to create chronicity different animal models of arthritis used distinct strategies. Chronicity can be induced with either protocols of immunisation with an antigen in the presence of an adjuvant or genetic manipulations. Antigen-induced arthritis (AIA), for example, is an acute arthritis induced with the intraarticular injection of an irrelevant antigen, such as mBSA, in animals pre-immunised firstly with mBSA in CFA and then with mBSA in IFA. Chronic arthritis in AIA animals is induced by a second intraarticular injection of mBSA (380). In the CIA model the development of chronic arthritis is dependent on the dose of CII, the presence of the adjuvant IFA and the generation of the collagen specific B cell response (381). Indeed, low doses of CII or IFA alone induce only acute disease. CIA can also be reactivated by the systemic injection of LPS in a dose-related fashion (382). The relevance of the collagen specific B cell response in this model has been demonstrated since B cell depleted animals are resistant to CIA and passive transfer of antibodies to healthy mice caused arthritis, an effect that was replicated by anti-CII-specific monoclonal antibodies (343, 345, 346). The anti-CII

response has been widely studied both in CIA and human RA. Synthesis of recombinant triple helical peptides and use of monoclonal antibodies have allowed the identification of predominant collagen epitopes and their relevance to disease (347, 348). The epitope J1 (aa 551-564), for example, is prevalent in murine CIA rather than human RA (383). C1 epitope (aa 359-370) is shared between human and murine RA. Antibodies against the epitopes J1 and C1 are arthritogenic since the injection of single or combination of monoclonal antibodies that bind the epitopes resulted in arthritis development in BALB/c animals (383) and in relapse of chronic arthritis in mice with mixed BALB/c and B10 backgrounds (347). The C1 epitope is also recognised in its citrullinated form (Cit-C1) in RA with a prevalence of 40% in a cohort of 286 patients (384). Moreover, citrullinated collagen has been recently demonstrated in RA synovial fluid (385). U1 epitope is formed by 11 amino acids (aa 494-504) of the triple helical CII and colocalise with $\alpha1\beta1$/ $\alpha2\beta1$ integrin binding site (386). Anti-U1 antibodies have been detected in patients with early RA, especially those with erosive arthritis (387). They correlate also with disease progression in murine CIA and the injection of a monoclonal antibody against U1 can induce both cartilage damage (387) and the relapse of arthritis in chronic arthritic mice (347). Of interest, monoclonal antibody anti-U1 reacts with the intact rat cartilage matrix *in vivo* demonstrating its accessibility by immunoglobulins (386).

3.3 Results

First, I induced the model of acute OVA-mediated arthritis and I showed that the local primary HAO challenge was necessary to stimulate the anti-collagen response. In order to establish a model of chronic arthritis, animals previously immunised with OVA and challenged with HAO, received a secondary challenge (re-challenge) once the acute arthritis had resolved. Different approaches were employed in the secondary challenge. In the first approach I elicited both the innate and adaptive immune response with a systemic (i.v.) injection of HAO or CII and LPS. In the second approach mice were injected with a local periarticular (s.c.) injection of HAO or CII in IFA in the other ankle. All mice were monitored daily for signs of arthritis, such as erythema, swelling and loss of function. Joint histology and autoantibodies were

analysed. After I induced a relapse of arthritis the anti-CII antibody response was investigated in terms of the epitopes recognised, the ability to induce arthritis by passive transfer and the affinity of these antibodies compared to that produced in murine CIA.

3.3.1 Induction of OVA-mediated acute arthritis

The experimental protocol used to induce OVA-mediated acute arthritis is shown in figure 3.1. $CD4^+$ T cells were isolated from DO11.10 mice and polarised toward a Th1 phenotype as previously described (sections 2.2, 2.3 and 2.5). Figure 3.2 shows representative FACS plots of Tg CD4+ T cells polarised toward a Th1 phenotype, identified by the antibodies KJ1.26 and IFNγ. One day following the adoptive transfer of DO11.10 Th1 cells BALB/c mice were immunised (s.c.) with OVA emulsified with CFA. Ten days later mice were challenged in one hindlimb with a s.c. injection close to the ankle joint of 100 μg HAO. The first HAO challenge induced an acute arthritis localised only to the ankle joint that persisted for approximately 7 days characterised by increased paw swelling (Fig. 3.3A) and clinical score (Fig. 3.3B) compared with un-challenged mice. Joint histology of acute arthritis displayed synovitis (Fig. 3.4A-B), cartilage depletion and bone erosion assessed by the loss of toluidine blue staining (Fig. 3.4D-E) compared with histology of un-challenged mice (Fig. 3.4C and F). The joint histopathology score of HAO challenged mice was higher compared with that of control mice (Fig. 3.4G).

3.3.2 Systemic primary challenge did not induce anti-collagen antibodies

The model of acute OVA-mediated arthritis, developed by Maffia, is characterised by the presence of anti-collagen II antibodies (358). I aimed to assess if the anti-collagen response was related to the route of antigen administration. Thus, some mice, which received the DO11.10 Th1 cells and the OVA immunisation, were challenged with a systemic i.p. injection of 100 μg HAO rather than the local s.c. injection. I compared the antibody levels in mice with acute OVA-mediated arthritis (challenged by the local HAO injection) with those in mice challenged by the systemic HAO injection.

Anti-collagen II IgG antibodies could be detected only in mice that received the challenge in the limb compared with mice that received the systemic HAO challenge and negative control mice (un-challenged mice) (Fig. 3.5A). On the contrary, anti-OVA IgG antibodies could be detected in both experimental groups compared with negative control mice. More anti-OVA antibodies could be detected in mice that received HAO in the limb compared with those in mice that received the systemic HAO injection (Fig. 3.5B).

3.3.3 Systemic secondary challenge did not induce a relapse of arthritis

In order to induce a relapse of arthritis the experimental protocol of OVA-mediated acute arthritis was modified (Fig. 3.6). Acute arthritis was induced as described in the section 2.7. At day 34 after the primary HAO challenge, some animals received a systemic i.p. secondary challenge with either 100 μg of HAO or 200 μg of CII and 5 μg of LPS. Control mice (naïve mice) received the systemic injection of OVA or CII and LPS but they were not immunised and HAO challenged. Three of five mice with a history of acute arthritis that received the systemic secondary challenge with HAO and LPS were found died in the cage the day after the secondary challenge. The other two mice of the same group appeared unhealthy because of shreking shivers and they were euthanised. These animals were observed post-mortem and an enlarged spleen was detected. No other apparent abnormalities were evident. The experiment was continued with only one experimental group of mice re-challenged with CII and LPS and the respective control group of naïve mice. The other control group of naïve mice injected systemically with HAO and LPS was not used. The secondary challenge with CII and LPS did not affect the paw thickness (Fig. 3.7A) and the clinical score (Fig. 3.7B). Histology of ankle joints was performed 7 days after the secondary challenge and it did not show any sign of synovitis (Fig. 3.8A-B). As expected control mice did not show any clinical or histological sign of arthritis (Fig. 3.7A-B and Fig. 3.8C-D). The joint histopathology score (Fig. 3.8E) did not show any difference between the two experimental groups. Serum samples were collected at day 7, 34 and 41 after the first HAO challenge and analysed for the presence of anti-CII antibodies and RF (Fig. 3.9A-B). Anti-CII antibodies were first detected at day 7 after the primary HAO

challenge compared with control mice (Fig. 3.9A). They increased with time even in the absence of arthritis (arthritic mice day 7 *vs* arthritic mice day 34). Mice that received the systemic re-challenge with CII + LPS (day 41) exhibited the highest levels of anti-CII antibodies compared with those of other animals HAO challenged (day 7 and day 34). Control mice injected with CII + LPS did not produce significant levels of anti-CII antibodies 7 days after the administration.

Mice with acute arthritis HAO-mediated (day 7) showed RF compared with control mice. RF persisted at the same levels at day 34 after the primary HAO challenge although in absence of disease. Mice that received the systemic re-challenge with CII + LPS (day 41) exhibited the highest levels of RF compared with those of other animals HAO challenged (day 7 and day 34). As expected control mice did not exhibit RF (Fig. 3.9B).

These data demonstrated that systemic secondary challenge with CII and LPS did not induce chronic arthritis. Autoantibodies produced after the primary HAO challenge, such as anti-CII and RF, were not transient but persisted in the animals even in absence of disease.

3.3.4 Induction of a relapse of arthritis after local secondary HAO challenge

Since I was not able to induce the chronic disease with the systemic challenge I developed a different experimental protocol in mice that had previously displayed acute arthritis and associated breach of self-tolerance (Fig. 3.10). At day 34 after the initial HAO challenge, animals were randomized to receive a local periarticular (s.c.) injection, close to the ankle joint in the limb that was not previously injected with HAO, CII or PBS in IFA, or PBS alone. Re-challenge with HAO or CII in IFA increased significantly paw thickness (Fig. 3.11A) and clinical score (Fig. 3.11B) compared with those of mice re-challenged with PBS + IFA or PBS. Three weeks after the secondary challenge the paw swelling and clinical score in the joints from mice treated with HAO or CII in IFA decreased; thus animals were sacrified to assess joint histology. Animals were not observed for a longer period, thus chronicity of arthritis was not verified. Re-challenge with IFA alone did not induce a significant

paw swelling compared with PBS injected mice (Fig. 3.11A). IFA secondary challenge seemed to have a similar effect of primary HAO challenge in terms of clinical score, while the paw swelling in mice that received IFA secondary challenge was higher than that observed after primary HAO challenge at days 2, 3 and 4 after the injection. Importantly, the joint swelling observed in HAO or CII in IFA re-challenged animals was localised to more than 4 joints, restricted to the injected paw and persisted for more than three weeks. Pictures of paws injected with local secondary challenge are shown in figure 3.12.

On histological examination at day 57 post primary challenge mice re-challenged with HAO + IFA exhibited an arthritis characterised by synovial hyperplasia and inflammatory cell infiltration. Cartilage damage and bone erosions were also observed in mice re-challenged with HAO + IFA, assessed by loss of toluidine blue staining (Fig. 3.13A). Injection with CII + IFA induced a mild joint infiltrate and moderate cartilage depletion (Fig. 3.13B). Injection of either IFA alone or PBS did not induce significant histopathological articular changes (Fig. 3.13C-D). The histology score of the joints from animals re-challenged with HAO + IFA was higher than that from the joints of mice re-challenged with CII +IFA, PBS + IFA, and PBS alone. There was not significant difference between the score of the joints that received CII + IFA and that of the joints injected with IFA alone or PBS (Fig. 3.14).
These data demonstrated that mice with a history of acute OVA-mediated arthritis challenged locally with HAO + IFA developed a relapse of arthritis localised to different joints in the injected paw. Joint inflammation lasted approximately three weeks. Since animals were not observed for a longer period I was not able to verify the chronicity of the model. Animals challenged with CII + IFA developed a significant paw swelling localised to the joints of the injected paw but the histopathology score was not different from that in PBS injected mice.

3.3.5 Assessment of antibody production in the new model of relapsing arthritis

After I induced a relapse of arthritis I aimed to assess the antibody response and the production of anti-collagen antibodies during the induction of the flare. For this purpose serum samples were collected at day 7, 34 and 57 after the first HAO challenge and analysed for the presence of anti-OVA and anti-CII IgG antibodies. Serum from naïve animals was used as control.

Mice re-challenged with HAO + IFA, CII + IFA, PBS + IFA or PBS had previously received OVA Tg T cell transfer, OVA/CFA immunisation, then HAO and therefore exhibited similar levels of anti-OVA IgG antibodies at day 57 post primary HAO challenge that were higher than those observed in naïve animals (Fig. 3.15A-B).

Anti-CII antibodies were first detected at day 7 after HAO challenge compared with naïve mice, which corresponded to the acute arthritis. Anti-CII antibodies at day 34 after primary HAO challenge were higher than that in mice at day 7 after challenge, even though mice were in clinical remission. At day 57 after the first HAO challenge anti-CII antibodies could be detected in all of the animals (with a history of acute arthritis) compared with naïve animals. Only mice that received the secondary challenge with HAO + IFA showed more anti-CII antibodies compared with that from mice at days 7 and 34. Moreover, these mice exhibited the highest levels of anti-CII antibodies compared with those of animals re-challenged with CII + IFA, IFA + PBS or PBS (Fig. 3.15C-D). These data demonstrated that mice with a relapse of arthritis elicited by local injection of HAO + IFA produced more anti-collagen antibodies than all other animals. In the time course experiment the titre of these antibodies increased progressively from day 7 to day 34.

3.3.6 Characterisation the anti-collagen antibody response in OVA-mediated arthritis

Type II collagen is one of the major constituents of the articular cartilage matrix proteins. Anti-CII antibodies can be directed toward different epitopes and this could be crucial for their pathogenicity because certain epitopes are more associated with arthritis than others (347, 348, 383). Some epitopes recognise privileged sites of CII that are dominant targets of CII-specific B cells. Different triple helical peptides, C1, J1, U1 and Cit-C1, identified as relevant B cell epitopes in murine CIA and human

RA (383, 386), were tested in the sera of our model of acute arthritis (day 7) and mice with CIA (Fig. 3.16). Only anti-U1 antibodies were tested also in mice with a previous history of acute OVA-mediated arthritis (day 34) and mice with a relapse of OVA-mediated arthritis (day 57) (Fig. 3.16E). Sera from mice with CIA contained high levels of anti-CII, anti-C1, anti-J1 and anti-U1 antibodies compared with those from OVA-mediated acute arthritis and naïve animals (Fig. 3.16A-D). Animals with acute arthritis induced by Th1 OVA-specific cells contained significant levels of anti-CII antibodies compared with those in naïve animals (Fig. 3.16A). Anti-C1 and anti-J1 antibodies could be detected in sera from mice with acute OVA-mediated arthritis only at one dilution point compared with naïve mice (Fig. 3.16B-C). Mice with OVA-mediated acute arthritis (day 7), those with OVA-mediated arthritis at day 34 and mice with a relapse of OVA-mediated arthritis (day 57) showed anti-U1 antibodies compared with naïve mice (Fig. 3.16D-E). In particular, anti-U1 levels increased significantly from day 7 to day 57. Mice with relapse of OVA-mediated arthritis (day 57) and mice with CIA displayed comparable titres of anti-U1 antibodies (Fig. 3.16E). Neither CIA mice nor HAO-challenged mice produced anti-Cit-C1 antibodies (Fig. 3.16F).

These data demonstrated that anti-CII antibodies in CIA and OVA mediated arthritis mice recognised different epitopes and the major epitope in OVA mediated arthritis has been identified in U1.

3.3.7 Serum passive transfer from OVA-mediated arthritis to naïve mice did not induce arthritis

Anti-CII antibodies are believed to be arthritogenic in murine CIA, in fact transfer of serum containing these antibodies can induce arthritis in healthy animals (346, 383, 387). Bovine or rat CII, used to immunise animals in model of CIA, elicits an antibody response specific for CII. These antibodies against foreign collagen are believed to cross-react with self collagen and become pathogenic (387, 388). In our model of arthritis I demonstrated the breach of self-tolerance toward collagen and the production of anti-CII and anti-U1 antibodies. Thus, I investigated first the possibility to transfer antibodies from mice with a relapse of OVA-mediated arthritis (day 57)

(donor mice) to naïve mice (recipient mice), then the ability of these antibodies to induce arthritis in naïve animals by passive transfer. Different approaches were used. In the first experiment 150 μl of serum from mice with OVA day 57 was injected i.v. in naïve BALB/c animals. Recipient animals were sacrificed one day after the passive transfer to assess serum antibodies. Anti-CII antibodies were not detected in recipient animals after the passive transfer (Fig. 3.17A). Thus, the experiment was modified and in the second experiment 200 μl of serum from mice with OVA day 57 or normal mice serum was injected i.v. in two consecutive days in naïve BALB/c animals. Anti-CII and anti-OVA antibodies were detected in recipient animals the day after the passive transfer (Fig. 3.17B-C). Once I was able to transfer antibodies to recipient mice, the experiment was repeated and antibodies were measured in the system at day 2 and 14 after passive transfer. Recipient mice were monitored for 2 weeks by paw thickness and joint histology was performed on day 14 after passive transfer. Anti-OVA could be detected in recipient mice 2 and 14 days after passive transfer, while anti-CII antibodies were detected only at day 2 because their levels decreased at day 14 (Fig. 3.18A-B). Paw thickness and joint histology in all recipient animals did not reveal significant signs of synovitis after the passive transfer (Fig. 3.19A-C).
These data demonstrated that the injection of sera from arthritic mice in healthy animals transferred transiently anti-CII antibodies and it did not induce the arthritis.

3.3.8 Affinity of anti-collagen type II antibodies in the model of OVA-mediated arthritis

Since anti-CII antibodies were not able to transfer disease I investigated the affinity of anti-collagen antibodies in our model of arthritis and compared this with those produced in murine CIA. Antibody affinity is one of the parameters affecting pathogenicity. Assays such as ELISA are routinely used to detect antibodies that bind to a specific antigen. However, this technique does not give information about the strength of the binding (the affinity of the antibodies) and the dynamic of complex formation. For this purpose the Biacore system (BIAcore 2000 instrument, GE Healthcare) (375) was used, allowing us to measure the interaction characteristics between antigen and antibody. This interaction is measured in terms of association

(on-rate) and dissociation (off-rate) of the complex. The early and late off-rate of the complex give an estimate of the antibody/antigen complex stability and the antibody affinity, as described in section 2.13.

Immobilisation of bovine CII and U1 peptide in different flow cells sensor surface chips produced a gradient surface with 400-10000 Response Unit (RU) and 170-2000 RU respectively. Binding curves generated when CII protein was used demonstrated that only serum from mice with CIA bound CII protein compared with sera from mice with a relapse of OVA-mediated arthritis (day 57) and control animals (Fig. 3.20A). Since I did not observe any binding of serum from OVA mediated arthritis with the whole collagen protein, I immobilised the U1 peptide on the flow cells sensor surface. Figure 3.20B shows the binding profile with the on-rate, the early and the late off-rate generated with the immobilisation of the U1 peptide. The stability of the complex anti-U1/U1 was similar in the serum from mice with a relapse of OVA-mediated arthritis and from CIA mice, and that was in turn greater than that observed in negative control mice (Fig. 3.20C).

These data suggested that the affinity of anti-U1 antibodies in our model of relapsing OVA-mediated arthritis might be comparable to that produced in murine CIA.

3.4 Discussion

In this chapter a relapse and widespread of arthritis was induced in the model of OVA-mediate arthritis. The anti-collagen antibody response was investigated during the induction of the flare.

Previous studies (358, 362, 365) established a model of experimental arthritis where an irrelevant antigen, OVA, drives a breach of self-tolerance toward different antigenic specificities, such as collagen, IgG and citrullinated peptides that are characteristic of RA. Interestingly, I observed that the HAO challenge must be administered s.c. proximal to the joint. Other routes of injection failed to induce anti-CII responses despite an equivalent anti-OVA reaction (389), indicating that a large Th1 response was itself insufficient and the articular environment was necessary as a source of relevant antigen. Indeed, the autoimmune response elicited in these mice is a peculiar characteristic that distinguishes this animal model from CIA where CII is administered to cause the immune response specific for the same antigen and the pathology. The model of OVA-mediated arthritis had several limits, firstly the pathology was acute and limited to the ankle joint, then arthritis did not always reach the same degree of severity in terms of paw thickness, histopathology score and antibody levels. The first aim of this chapter was to try to develop a model of chronic polyarthritis. Different animal models of RA developed chronic disease with distinct strategies. Since our model of arthritis is antigen-driven I employed two approaches with OVA or CII in the presence of an adjuvant such as LPS or IFA to induce the chronic disease. I showed that the induction of a relapse of autoimmune arthritis was dependent on the nature of the antigen used and the route of administration in the secondary challenge. In particular, systemic secondary challenge with CII and LPS was not sufficient to induce a relapse of disease. There could be a variety of reasons for this failure, such as the antigen itself or the adjuvant employed, the dose of CII or LPS, and the route of injection of the secondary challenge. Of interest, the first HAO challenge stimulated an anti-collagen response that was not transient but persisted even in absence of disease and increased after the secondary challenge. This experiment omitted two control groups of mice adoptively transferred and HAO treated with no re-challenge (PBS and LPS). Thus, it did not allow to observe mice in

absence of re-challenge and to dissect the contribution of LPS in the secondary challenge. The reason for this omission was that, as a first approach, I decided to design my controls to determine if the same treatment had some effect in naïve mice. Since the experiment failed no other mice were used for further control experiments. Of a note, arthritic mice that received the systemic challenge with HAO and LPS died two days after the injection. I hypothesized that Tg T cells OVA-specific were recalled in the peritoneum where they might be responsible, together with LPS, for a cytokine storm that caused the death of the animals. Indeed, the local secondary challenge induced a relapse of arthritis that was more severe then that observed after the first challenge. Mice were killed after 3 weeks from the local secondary challenge when the paw thickness decreased and it seemed that the paws were recovering from the arthritis. This experiment had not a control group that could be observed for a longer period of time in order to characterise the evolution of the disease in terms of chronicity. This control group will definitely be important in future experiments. Both HAO and CII in IFA induced the clinical signs of an arthritis localised in the ankle, tarsum, metarsophalangeal and proximal interphalangeal joints of the hind paws. Of interest, the other paws did not show clinical signs of arthritis and this was limited to the paws that received the secondary challenge. Ideally the involvement of joints at distant sites from injection would suggest a systemic response to the local challenge. Tertiary lymphoid structures were not observed in the joint histology although the cell infiltrate has not been characterized in this view. Local secondary challenge with HAO differed from CII re-challenge in the joint histological damage and in the autoreactive response. In fact, HAO re-challenge induced a more aggressive disease in the affected joints showed by a higher histopathological score than those in joints from mice treated with CII or IFA alone. Moreover, mice injected with CII + IFA displayed a significant paw swelling compared to control mice but the joint histopathology score was not different from control mice. It would be important in future experiments to prove the presence of DO11.10 T cells in these affected joints by immunohistochemistry. The highest autoreactive response was elicited in arthritic mice that received HAO in the local secondary challenge compared with the other control groups. I hypothesised that collagen is exposed and/or released from the inflamed joint during the primary challenge when there is the first evidence of breach of self-tolerance (358). Moreover, an increase of anti-CII antibody titre was demonstrated even in the absence of clinical signs of arthritis. When the animals

received the secondary HAO challenge autoreactive collagen B cells were elicited again and a higher antibody response toward collagen was produced together with the aggressive detectable relapse of arthritis. However, it is not possible to invoke a definitive cause and effect relationship between the secondary challenge and the autoreactive response because this experiment omits a control group of animals that did not receive the adoptive trasfer of Tg T cells. Although, human disease and the experimental model differ substantially, the onset of autoimmunity in RA (5, 247) may precede the full-blown disease in the so-called "pre-articular or lymphoid phase" (4), likewise in our model the autoimmune response was evident in absence of arthritis and preceded the relapse.

The second aim of this chapter was to characterise the collagen specific B cell response. B cells producing anti-CII are present in RA synovium and synovial fluid (390). Anti-CII antibodies have been detected both in serum and joint of patients with RA (227, 228, 390) and immune complexes containing anti-CII have been demonstrated in the cartilage (391). Anti-CII antibodies in human RA and murine models can be directed toward different epitopes. Some of these epitopes are shared between CIA and human RA, such as C1 and U1. Others are prevalent in murine CIA such as the epitope J1 (383). I demonstrated that mice with OVA-mediated acute and mice with a relapse of arthritis exhibited anti-U1 antibodies and differed from CIA in the production of anti-J1 and anti-C1 antibodies. This can be explained by the different nature of autoimmune response elicited. In fact, murine CIA is elicited by exogenous collagen in the context of specific MHC class II molecules (367, 392); on the contrary in OVA-mediated arthritis the collagen response is induced in absence of collagen injection resembling human RA. Future experiments should be performed to assess if the collagen response in this model is MHC class II dependent.

Although mice with OVA-mediated arthritis produced anti-CII and anti-U1 antibodies, passive transfer of serum to healthy animals did not transfer the arthritis. In CAIA pathology was elicited in naïve mice by CII-specific polyclonal sera (346) or purified monoclonal antibodies (393, 394). Monoclonal antibodies against the epitopes C1, J1, D3 and U1 induced a mild arthritis in naïve mice only in the presence of LPS, whereas if injected as a cocktail induced a severe arthritis (393). Thus, several variables could explain the failure of these preliminary experiments to induce arthritis in naïve mice by serum passive transfer, such as the absence of adjuvant or LPS in the experimental protocol employed, as well as the magnitude, specificity and the affinity

of the antibody response generated in the OVA-mediated arthritis model. In OVA-mediated acute arthritis, serum concentration of anti-CII and anti-U1 antibodies were lower than that detected in CIA mice. The antibody affinity is an important parameter because it determines some effector functions such as immune complex formation. I was not able to detect any stable antibody-antigen interaction in serum of OVA-mediated arthritis when the CII protein was used in the Biacore experiment. There are a number of possible explanations; firstly CII may be partially denatured on the chip making it poorly recognisable by sera from OVA-mediated arthritis. Secondly, the CII epitopes recognised by antibodies could be obscured by immobilisation on the sensor surface chip. Importantly, serum from mice with a relapse of OVA-mediated arthritis showed the same binding affinity to the antigen U1 compared with that from CIA serum. Thus, a possible explanation for the failure to induce arthritis by serum passive transfer could be related to the greater magnitude of anti-CII response than OVA-mediated arthritis and the different antibody specificity rather than the affinity. However, I cannot exclude that other autoantibodies are required to induce disease or that other cell types, such as T cells or DCs (361) may be necessary to induce arthritis. Future experiments should use different and more sophisticated approaches such as the administration of an adjuvant or LPS to further amplify the immune response in recipient mice, and/or the employment of mAb specific for the U1 peptide. A positive control with a CIA serum, from mice with a BALB/c background, would show that the model works.

In this chapter I demonstrated firstly that the administration of HAO in the primary challenge has to be local to induce anti-CII antibodies because the systemic injection does not induce autoantibodies. Thus, I can speculate that in this experimental model the local (joint) environment is essential to cause the breach of self-tolerance. Then, although I was not able to create a chronic model, I induced a relapse of OVA-mediated arthritis. In particular, only the local secondary challenge with HAO+IFA was able to induce the relapse of arthritis that was localised to more than one joint in one limb and lasted longer then the first episode of acute arthritis. The nature of the antigen used and the local environment were the main factors responsible for the relapse. This is of particular relevance since in human disease the antigen (one or more) is unknown and the contribution of the local environment is supposed, but not definitely proved, to be important. The relapse of arthritis was associated with the production of anti-CII antibodies and I characterised the collagen-specific B cell

response identifing U1 as the major epitope recognised. Antibody response may be directed towards different epitopes of the same protein (collagen) and some of these epitopes were recognised in both RA and CIA and correlated with severity. Thus, the epitope U1 seems to be shared also between the murine model of OVA-mediated arthritis and human disease. Moreover, anti-U1 antibodies from mice with a relapse of OVA-mediated arthritis showed high affinity, an ability that might confer them an advantage in the effector functions such as the immune complexes formation, the complement fixation and the tissue damage.

In the next chapter, the role of B cells in the induction of experimental arthritis will be investigated.

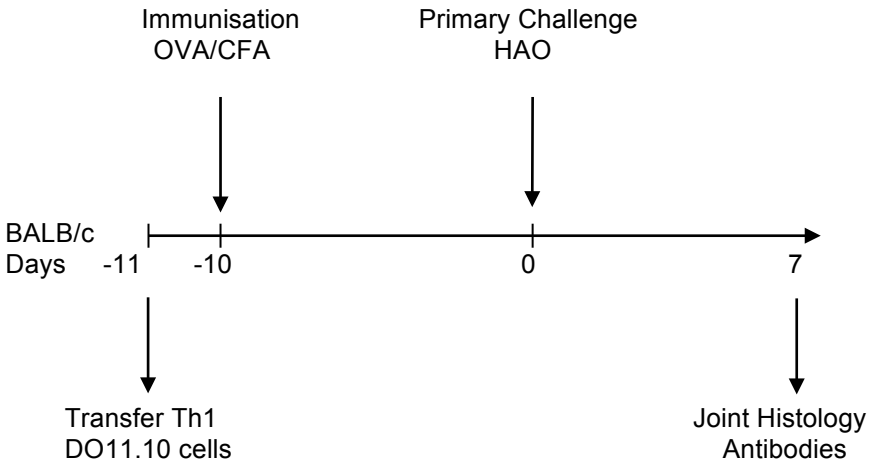


Figure 3.1 Experimental schedule for assessing the induction of acute arthritis

One day following the adoptive transfer of Th1 DO11.10 cells BALB/c mice were immunised s.c. in the scruff with OVA emulsified with CFA. Ten days later mice were challenged with a s.c. injection close to the ankle joint, in one hindlimb, with 100 μg of HAO. Control mice were immunised with OVA and received PBS in the primary challenge instead of HAO. Mice were sacrificed one week after the primary challenge (day 7) to assess joint histology and antibody production.

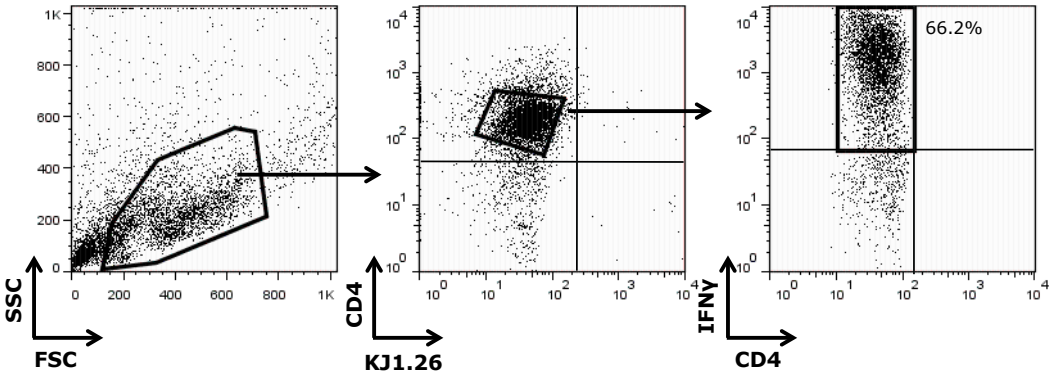


Figure 3.2 Polarisation of DO11.10 CD4$^+$ T cells

Representative FACS plots of CD4$^+$ Th1 cells. In the left panel lymphocytes from LNs were identified on the basis of size and granularity. In the middle of the figure the double positive population of KJ1.26$^+$ CD4$^+$ cells was gated. On the right side of the figure the gate identify the Th1 population represented by IFNγ$^+$ CD4$^+$ T cells.

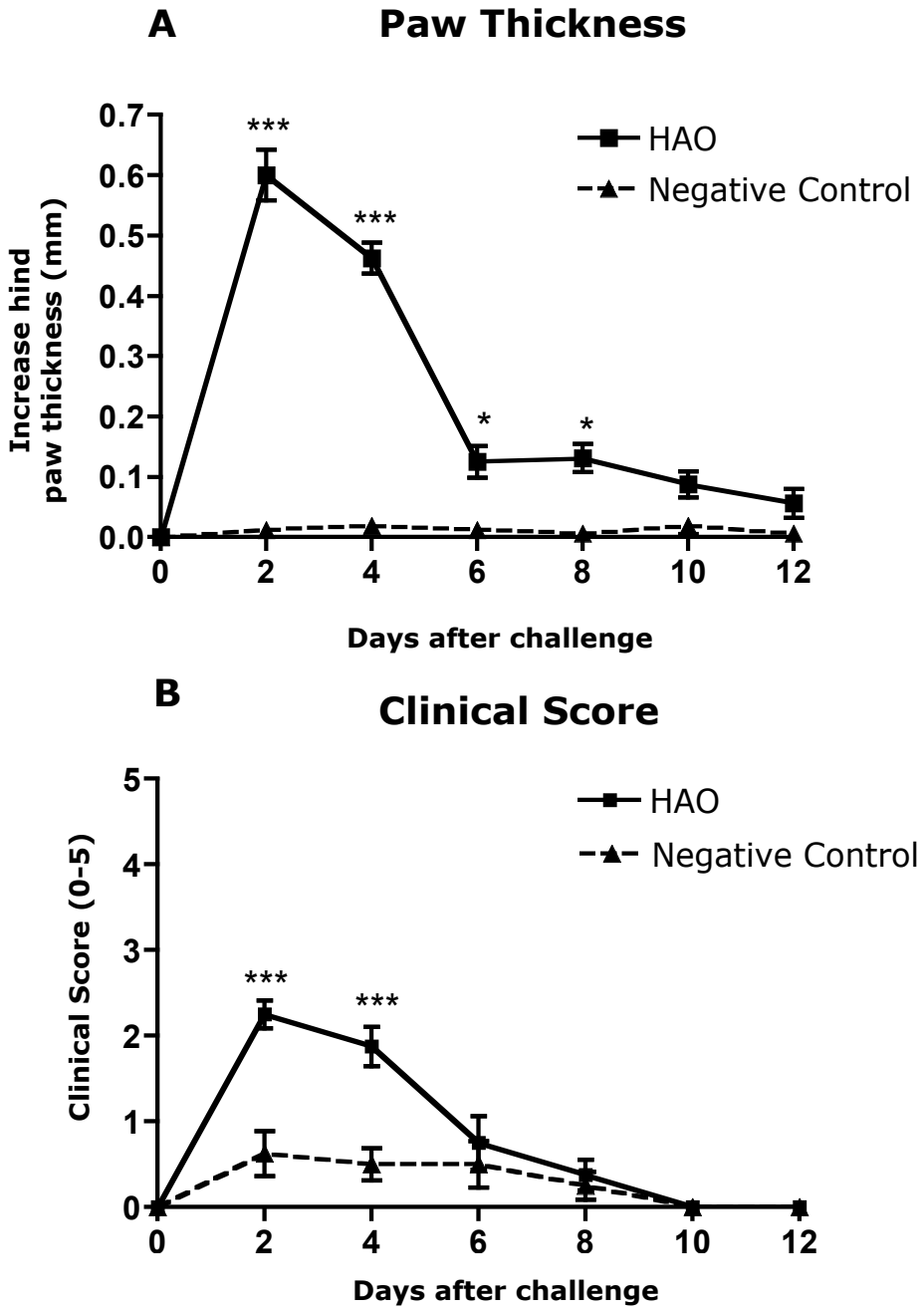


Figure 3.3 Clinical signs of acute arthritis

Hind paw thickness (A) and mean clinical score (B) in mice immunised with OVA and challenged ten days later with HAO. Control mice were immunised but they were not challenged. A significant increase in paw swelling and clinical score was observed in mice challenged with HAO compared with control mice. *, HAO *vs* Negative control. Statistical analysis was performed by Mann-Whitney U test. Data represent median and IQR (n = 3 mice per group). Similar results were obtained in 2 identical experiments (n = 12 mice in all).

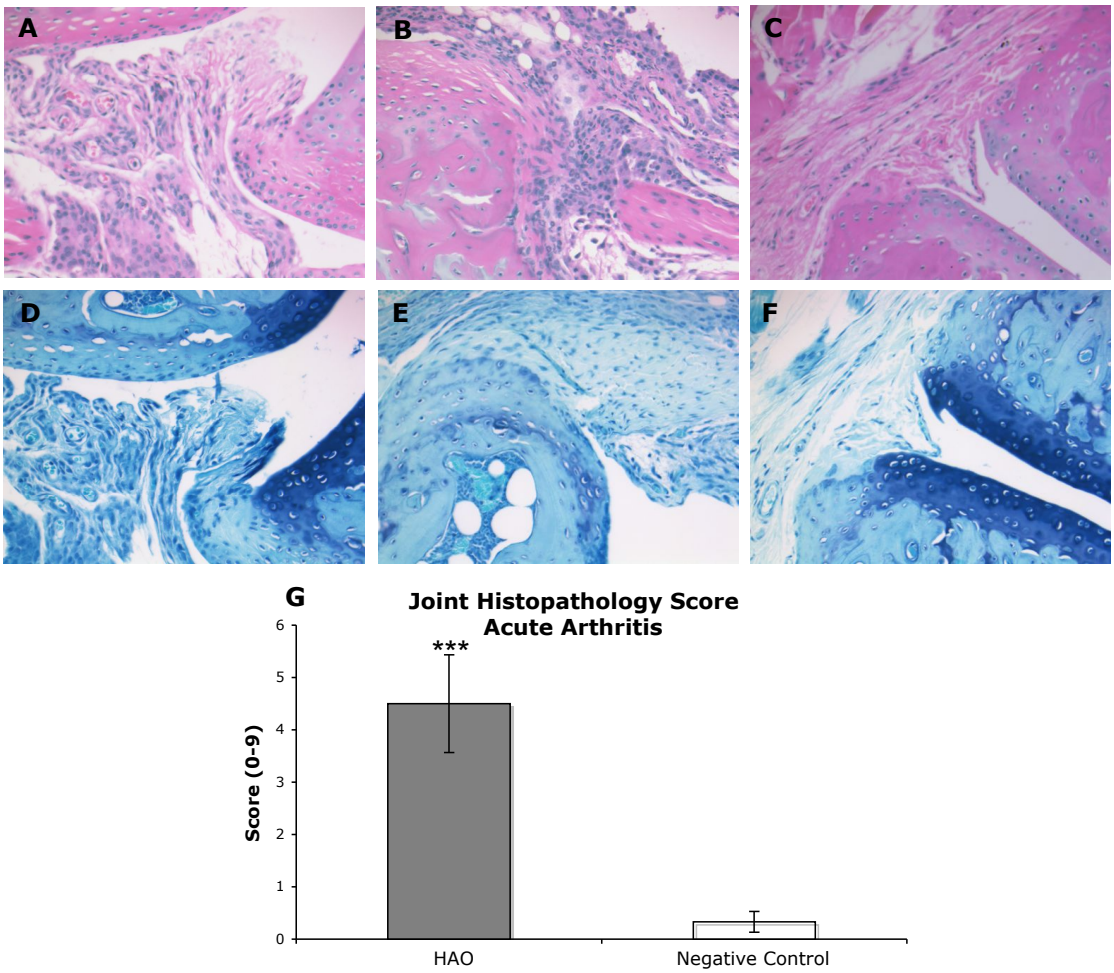


Figure 3.4 Joint histology and histopathology score of acute arthritis

Representative haematoxylin/eosin and toluidine blue stained sections prepared from the joints of BALB/c mice immunised with OVA and HAO challenged (A-B, D-E) and un-challenged mice (C and F). Images from panel A and D belong to the same section shown in figure 2.1H; image from panel B is the same shown in figure 2.1G. The histopathology score (G) was higher in HAO-challenged mice compared with that in control mice. Original magnification X40. In panel G *, HAO *vs* Negative Control. Statistical analysis was performed by Mann-Whitney U test. In panel G data represent median and IQR (n = 3 mice per group). Images and data shown are representative of 3 identical experiments (n = 12 mice in all).

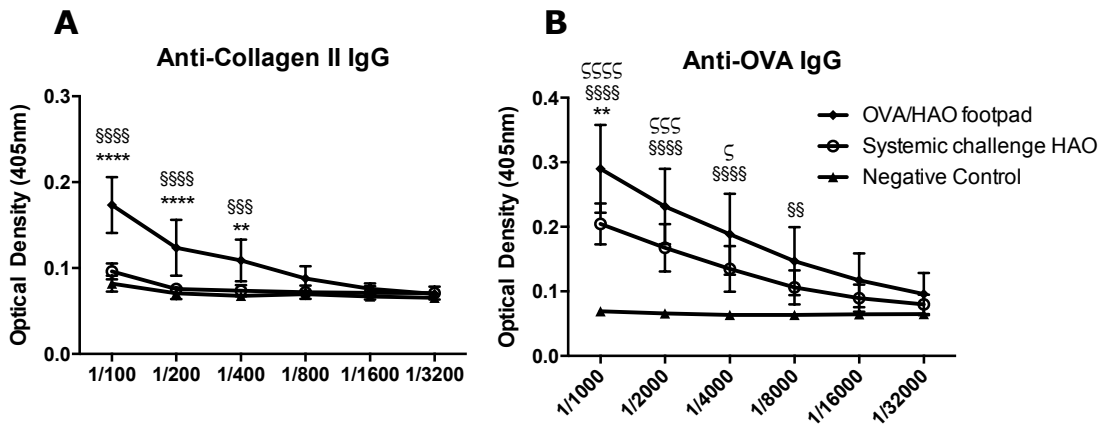


Figure 3.5 Anti-CII and anti-OVA antibodies in mice after local and systemic primary challenge

Mice were immunised with OVA and challenged ten days later with either a systemic or a local s.c. injection in one limb of HAO. Serum samples were collected at day 7 after the primary HAO challenge and analysed for the presence of anti-CII and anti-OVA antibodies. Anti-CII antibodies (A) were detected only in mice challenged locally with HAO compared with those that received the systemic challenge and negative control mice. (B) Anti-OVA antibodies were produced from both mice that received the local and systemic HAO challenge compared with negative control mice. At one dilution point mice that received the local HAO challenge displayed higher levels of anti-OVA antibodies compared with those from mice that received the systemic HAO challenge. *, OVA/HAO limb *vs* systemic challenge HAO; § OVA/HAO limb *vs* negative control; ζ, systemic challenge HAO *vs* negative control. Statistical analysis was performed by 2-way Anova with Bonferroni's multiple comparisons test. Data represent mean ± SD (n = 4 mice per group). Similar results were obtained in 2 identical experiments (n = 24 mice in all).

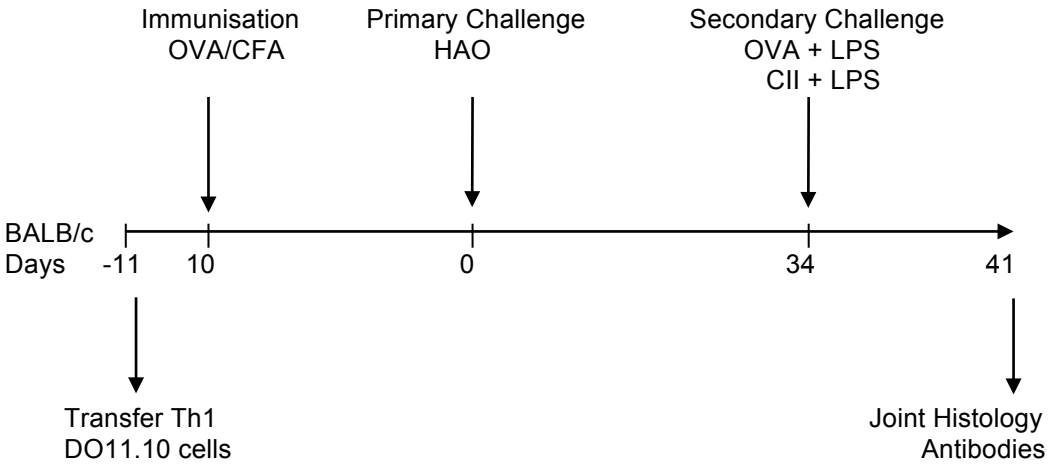


Figure 3.6 Experimental schedule for assessing the induction of chronic arthritis through the systemic secondary challenge

One day following the adoptive transfer of Th1 DO11.10 cells BALB/c mice were immunised s.c. with OVA/CFA and ten days later they were challenged s.c. with HAO in one hindlimb. At day 34 after the HAO challenge, mice received a secondary challenge with either 100 μg of HAO or 200 μg of CII and 5 μg of LPS. Control mice received the systemic injection of OVA or CII and LPS but they were not immunised and HAO challenged. Mice were sacrificed one week after the secondary challenge (day 41) to assess joint histology and antibody production.

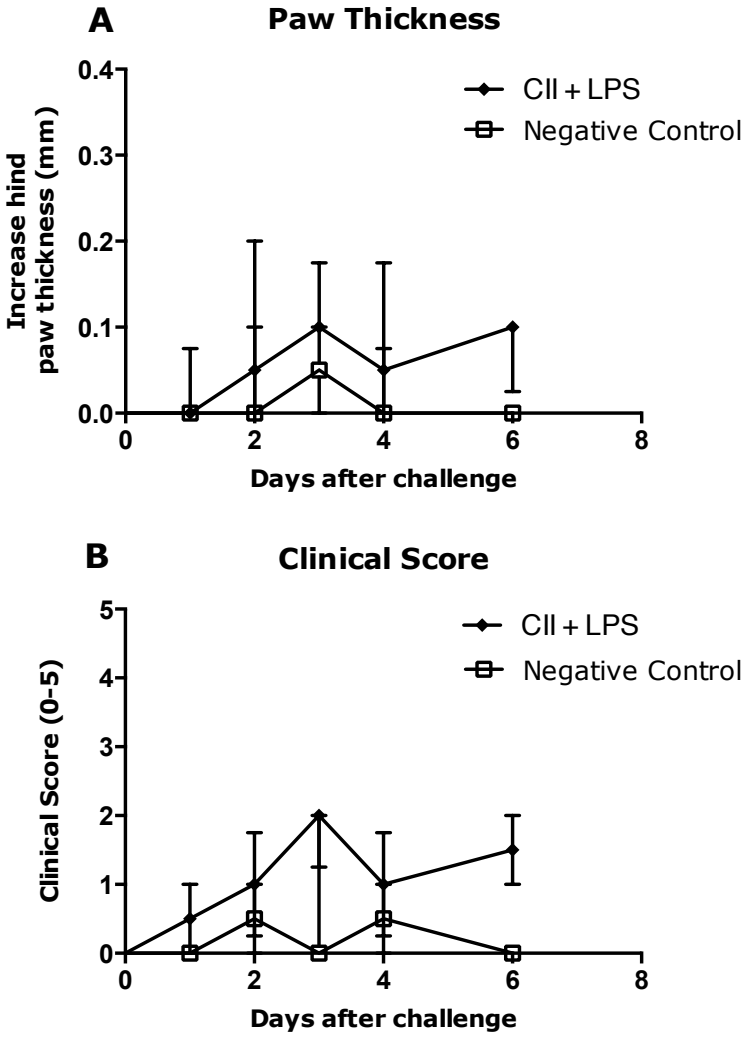


Figure 3.7 Clinical signs of arthritis in mice after the systemic secondary challenge

Hind paw thickness (A) and mean clinical score (B) in mice re-challenged with CII and LPS. No significant increase in paw swelling and clinical score was observed in mice re-challenged with CII and LPS and control mice. Mann-Whitney U test was used for the comparison between the two groups. Data represent median and IQR (n = 5 mice per group). Similar results were obtained in 2 similar experiments (n = 20 mice in all).

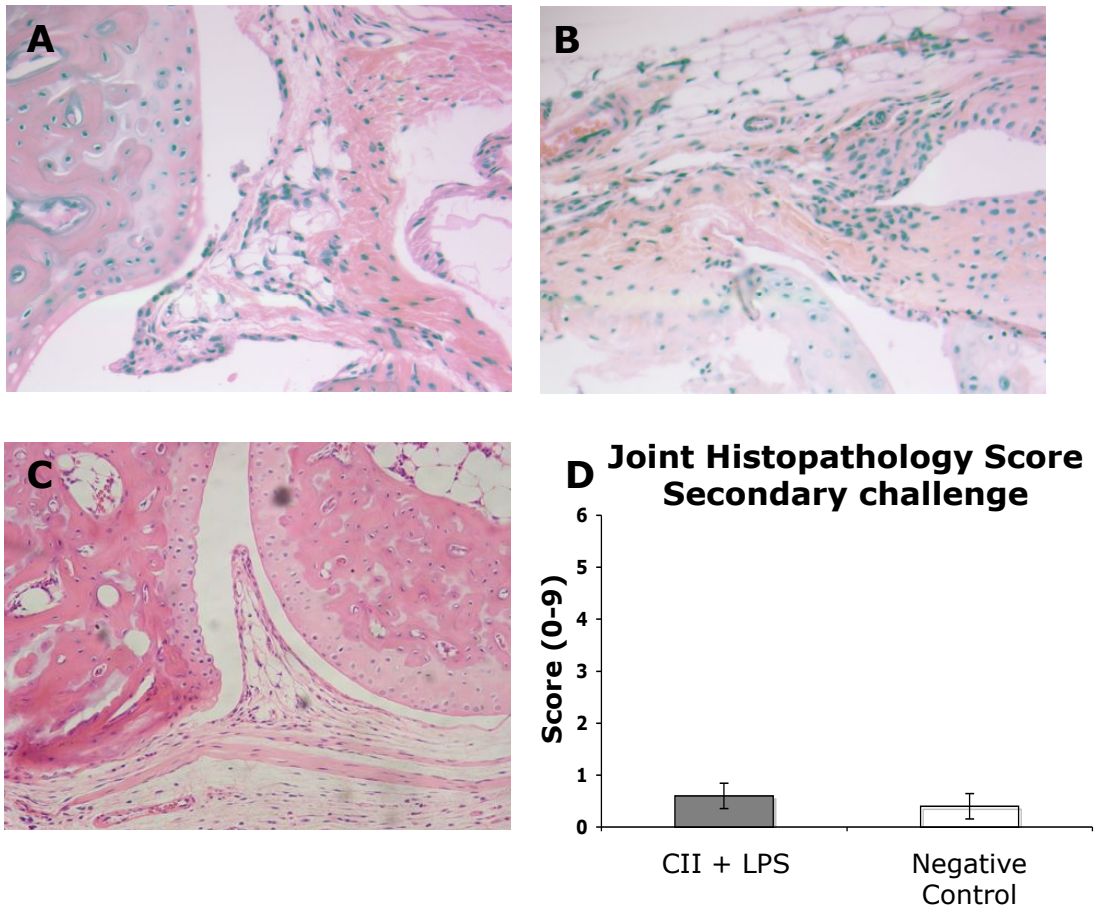


Figure 3.8 Effect of systemic secondary challenge on joint histopathology

Representative haematoxylin/eosin stained sections prepared from the joints of mice re-challenged with CII + LPS (A-B) and control mice (C). The systemic injection of CII + LPS did not induce any sign of synovitis such as cell infiltration, synovial hyperplasia and cartilage/bone erosions. The joints of control mice did not exhibit any inflammatory reaction. The joint histopathology score (D) was similar in the two experimental groups. Original magnification was X40 except panel C X20. In panel D statistical analysis was performed by Mann-Whitney U test and data represent median and IQR (n = 5 mice per group). Images and data shown are representative of two similar experiments (n = 20 mice in all).

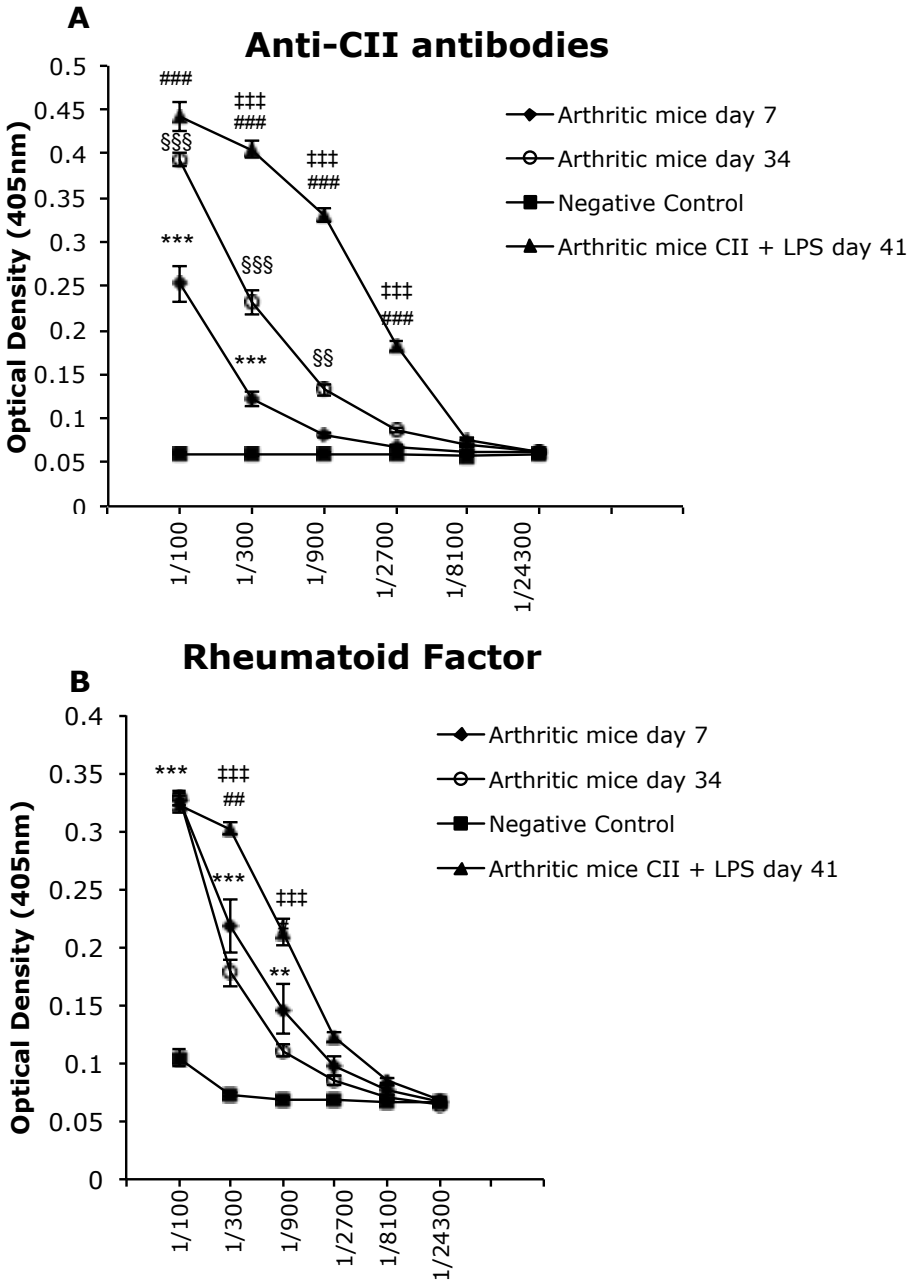


Figure 3.9 Anti-CII antibodies and Rheumatoid Factor in mice after systemic secondary challenge

Serum samples were collected at day 7, 34 and 41 after the first HAO challenge and analysed for the presence of anti-CII antibodies and Rheumatoid Factor. (A) Anti-CII antibodies were revealed at day 7 after the primary HAO challenge compared with control mice. Mice HAO challenged displayed significant levels of anti-CII antibodies even in the absence of arthritis at day 34 after primary challenge when compared with mice HAO challenged at day 7. Levels of anti-CII antibodies from mice re-challenged with CII + LPS were higher than those from mice HAO challenged (day 7 and day

34). (B) Rheumatoid factor was detected in arthritic mice HAO challenged (day 7) compared with control mice. Likewise, RF was observed in mice at day 34 after HAO challenge compared with control mice. Arthritic mice that received a systemic secondary challenge with CII + LPS showed higher levels of RF compared with those from mice 7 and 34 days after primary HAO challenge. *, Arthritic mice day 7 *vs* Negative Control; § Arthritic mice day 34 *vs* Arthritic mice day 7; # Arthritic mice CII + LPS day 41 *vs* Arthritic mice day 7; ‡ Arthritic mice CII + LPS day 41 *vs* Arthritic mice day 34. Statistical analysis was performed by 2-way Anova with Bonferroni's multiple comparisons test. Data represent mean ± SD (n = 5 mice per group). Similar results were obtained in 2 similar experiments (n = 20 mice in all).

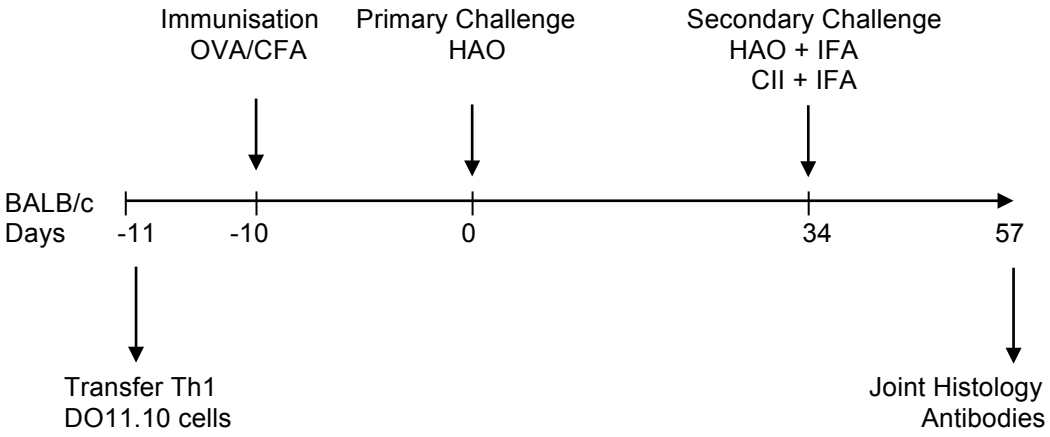


Figure 3.10 Experimental schedule for assessing the induction of chronic arthritis through the local secondary challenge

One day following the adoptive transfer of Th1 DO11.10 cells BALB/c mice were immunised s.c. with OVA + CFA (day -10) and challenged with a s.c. injection close to the ankle joint with 100 μg of HAO (day 0). At day 34 after the primary HAO challenge, mice were injected with a local (periarticular) s.c. injection of 100 μg HAO or 200 μg CII in IFA. Control mice received an injection of IFA in PBS or PBS. Mice were sacrificed 3 weeks after secondary challenge (day 57) to assess joint histology and antibody production.

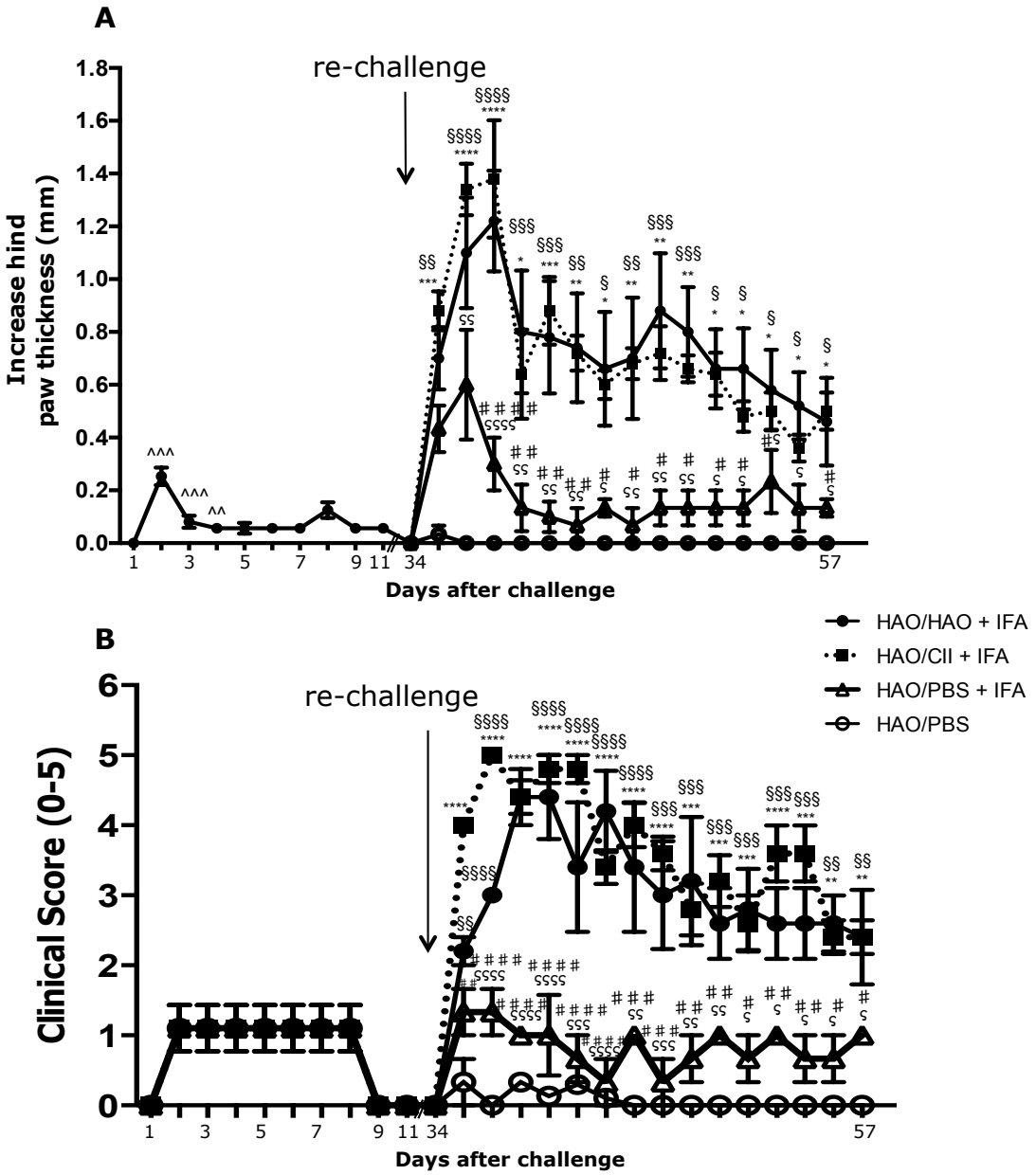


Figure 3.11 Clinical signs of arthritis in mice after the local secondary challenge

Hind paw thickness (A) and mean clinical score (B) in mice re-challenged locally with HAO, CII or PBS in IFA, or PBS alone. There was a significant difference in both paw swelling and clinical scores between mice re-challenged either with HAO or CII in IFA and those injected with IFA or PBS. The increased paw swelling and clinical score in mice re-challenged with HAO or CII in IFA persisted more than 3 weeks. * CII+IFA *vs* PBS; § HAO+IFA *vs* PBS; # CII+IFA *vs* PBS+IFA; ς HAO+IFA *vs* PBS+IFA; ^ PBS+IFA *vs* HAO primary challenge. Statistical analysis was performed by 2-way Anova with Bonferroni's multiple comparisons test. Data

represent mean ± standard error (n = 5 mice per group). Similar results were obtained in 2 identical experiments (n = 40 mice in all).

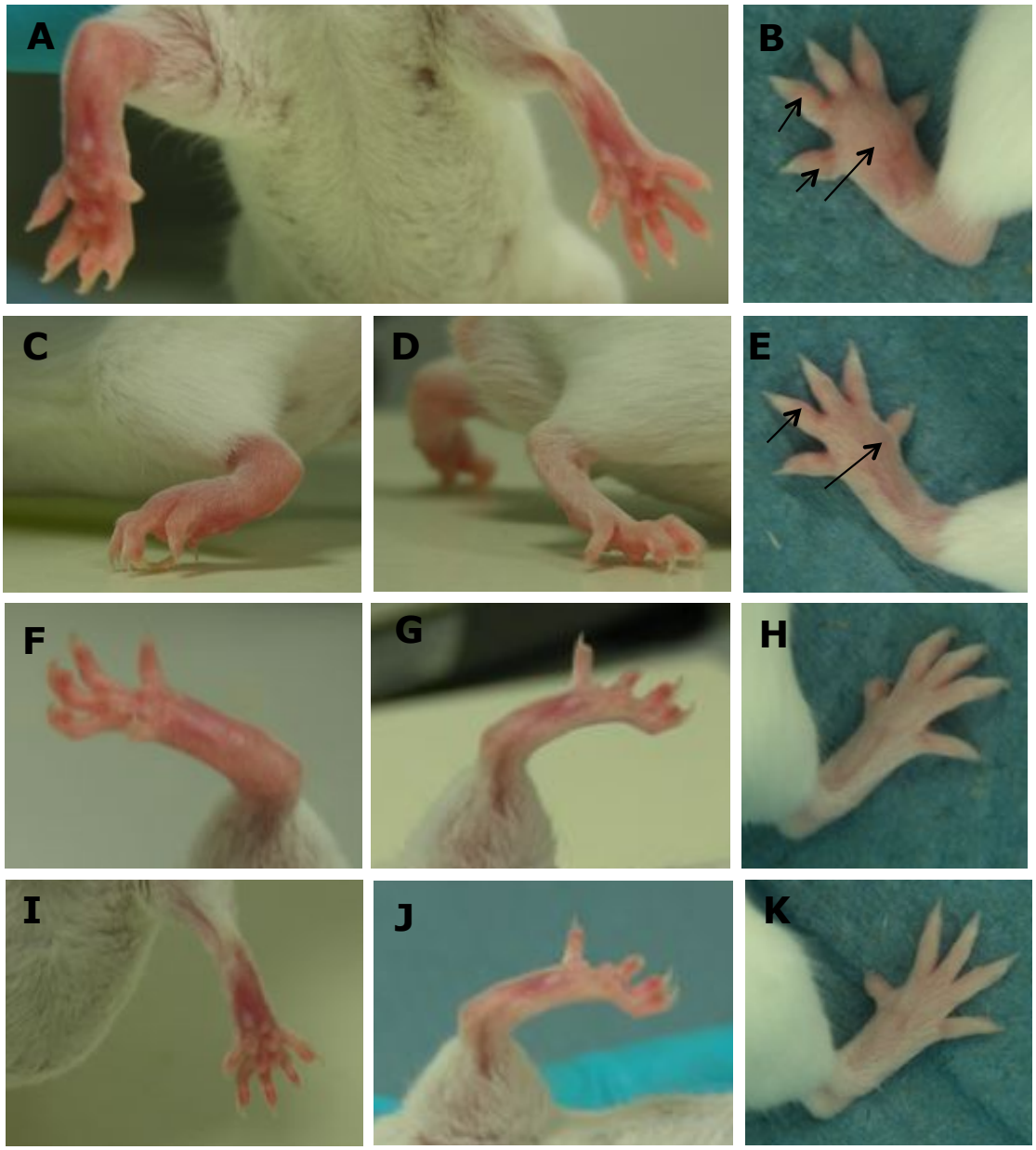


Figure 3.12 Paws of mice after local secondary challenge

Photographs of hind paws of mice re-challenged with HAO + IFA (A-B), CII + IFA (C-E), PBS + IFA (F-H) or PBS (I-K). Black arrows indicate representative sites of joint swelling. An arthritis localised to more than 4 joints is evident in one hind paw of mice re-challenged locally with HAO (A-B) or CII in IFA (C, E). Panels A, D, G and J show the controlateral paws that did not receive the secondary challenge.

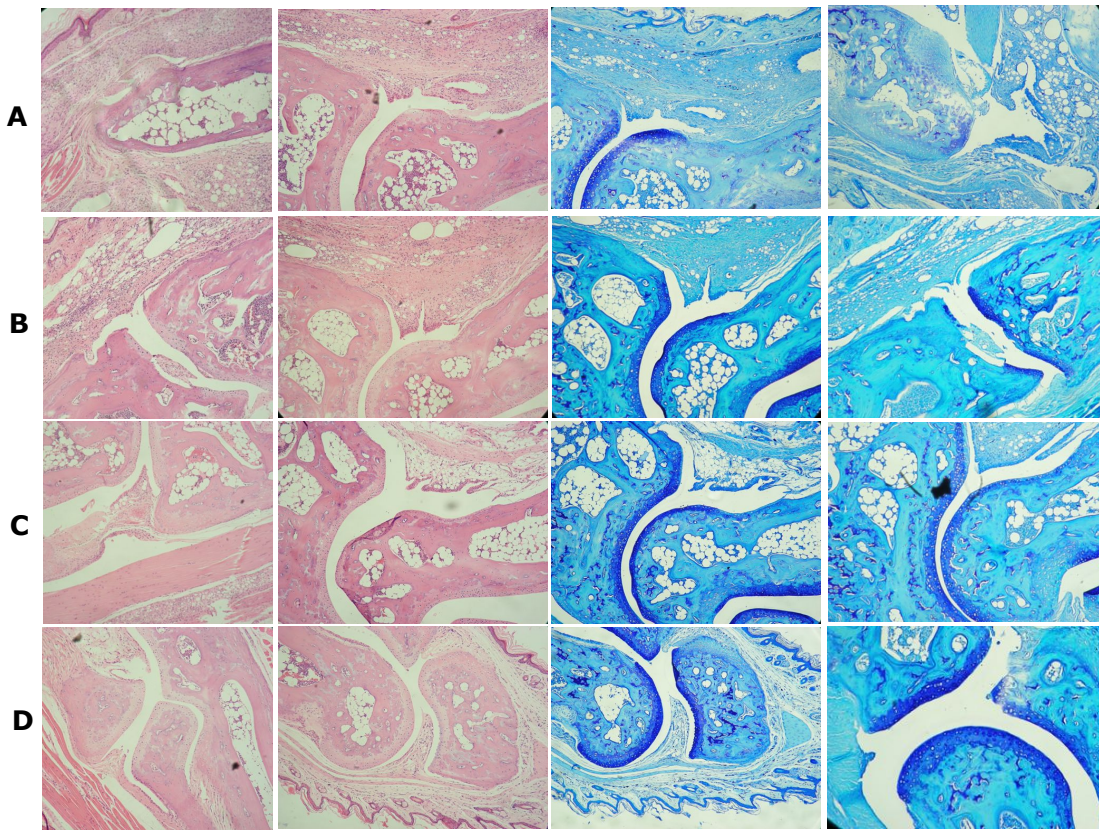


Figure 3.13 Effect of local secondary challenge on joint histopathology

Representative haematoxylin/eosin and toluidine blue stained sections prepared from the joints of mice re-challenged locally with HAO + IFA (A), CII + IFA (B), IFA + PBS (C) or PBS (D).

(A) The injection of HAO + IFA induced an arthritis characterised by cell infiltration, synovial hyperplasia and cartilage/bone erosions, assessed by loss of toluidine blue staining. Mice re-challenged with CII + IFA displayed a mild cell infiltration and moderate cartilage depletion (B). The joints of mice injected with IFA + PBS (C) or PBS (D) did not exhibit significant inflammatory reaction or cartilage erosion. Image from panel B2 is the same shown in figure 2.1F; images from panel D2 and D3 belong to the same section shown in figure 2.1A and I. Original magnification X10 except panel D4 X20. Images shown are representative of 2 identical experiments (n = 5 mice per group and 40 mice in all).

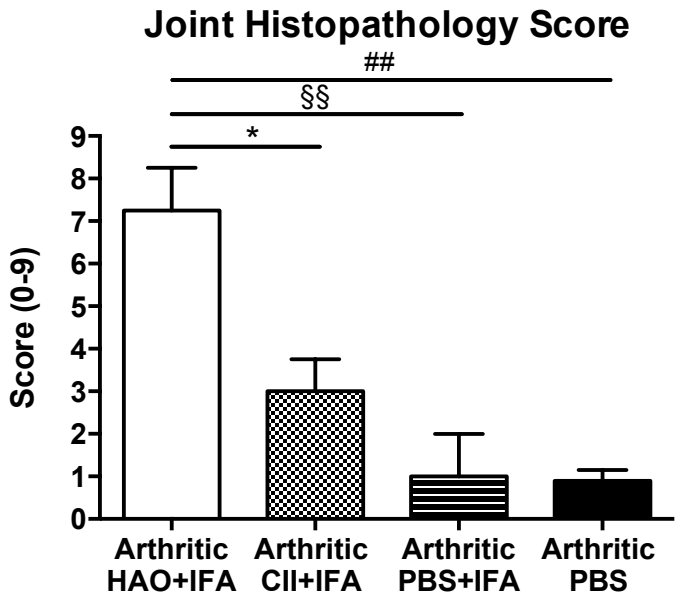


Figure 3.14 Effect of local secondary challenge on histopathology score

The histopathology score of the joints in animals re-challenged with HAO + IFA was higher than the score of the joints that received CII +IFA, PBS + IFA, and PBS alone. * HAO + IFA *vs* CII + IFA; § HAO + IFA *vs* PBS + IFA; # HAO + IFA *vs* PBS. Statistical analysis was performed by Kruskal-Wallis test. Data represent median and IQR (n = 5 mice per group). Similar results were obtained in 2 identical experiments (n = 40 mice in all).

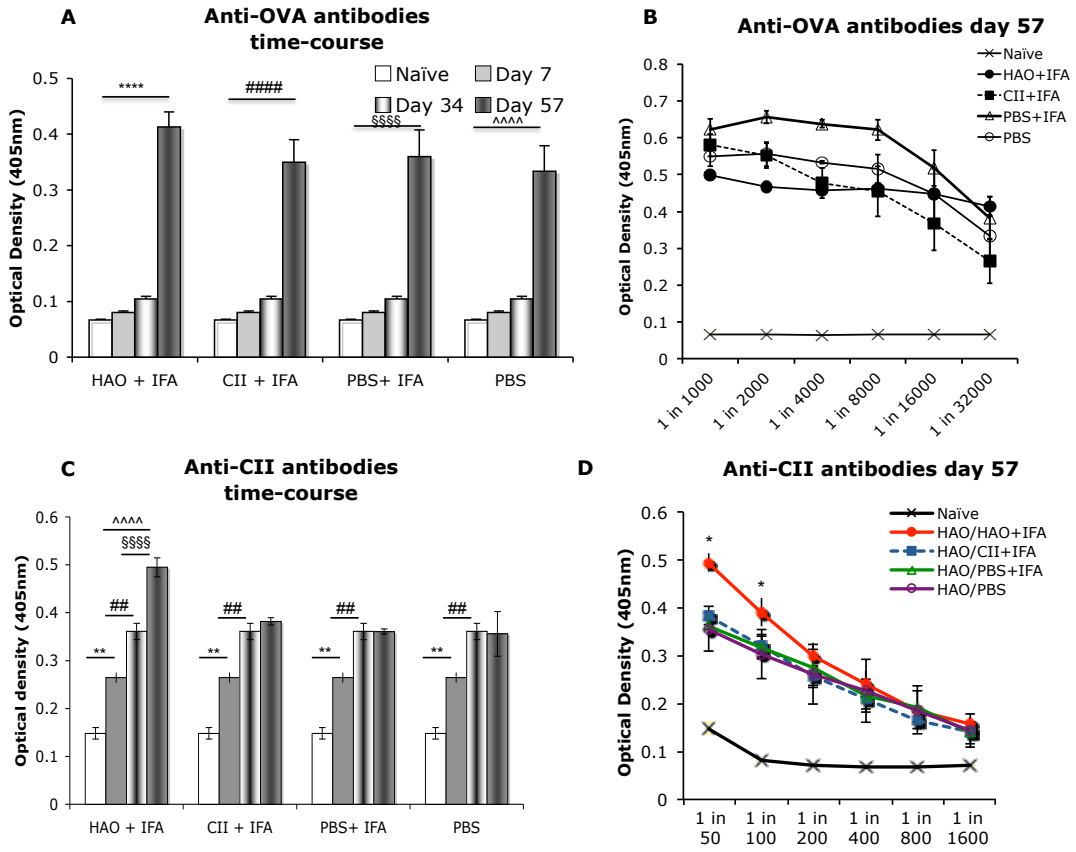


Figure 3.15 Anti-OVA and Anti-CII IgG antibodies in mice after local secondary challenge

Serum samples were collected at day 7, 34 and 57 after the first HAO challenge and analysed for the presence of anti-OVA and anti-CII IgG antibodies. Sera from mice at day 7 and day 34 are pooled because they belong to one group of 20 mice. At day 34 after primary HAO-challenge mice were randomised to receive the secondary challenge. (A) All animals immunised with OVA exhibited similar levels of anti-OVA antibodies that were higher than that in naïve mice. Sera were diluted 1 in 32000. (*, naïve *vs* day 57 HAO + IFA; #, naïve *vs* day 57 CII + IFA; § naïve *vs* day 57 PBS + IFA; ^, naïve *vs* day 57 PBS). (B) Anti-OVA antibodies from mice re-challenged with HAO + IFA, CII + IFA, PBS + IFA or PBS were all at the same levels at day 57 after the primary HAO challenge. (C) At day 7, 34 and 57 after primary HAO-challenge all mice exhibited anti-CII antibodies compared with naïve mice. Their levels increased with time even in the absence of arthritis at day 34 after primary HAO challenge. Mice that received the secondary challenge with HAO + IFA showed more anti-CII antibodies compared with that from mice day 7 and mice day 34. Sera were diluted 1 in 50. (*, naïve *vs* day 7; #, day 7 *vs* day 34; § day 34 *vs* day 57 HAO + IFA; ^ day 7 *vs* day 57 HAO + IFA). (D) At day 57 mice that received the

local secondary challenge with HAO + IFA exhibited the highest levels of anti-CII antibodies compared with those from animals re-challenged with CII + IFA, PBS + IFA or PBS (*, HAO + IFA *vs* CII + IFA, PBS + IFA or PBS). Statistical analysis was performed by 2-way Anova with Bonferroni's multiple comparisons test. Data represent mean ± SD (n = 5 mice per group). Similar results were obtained in 2 identical experiments (n = 40 mice in all).

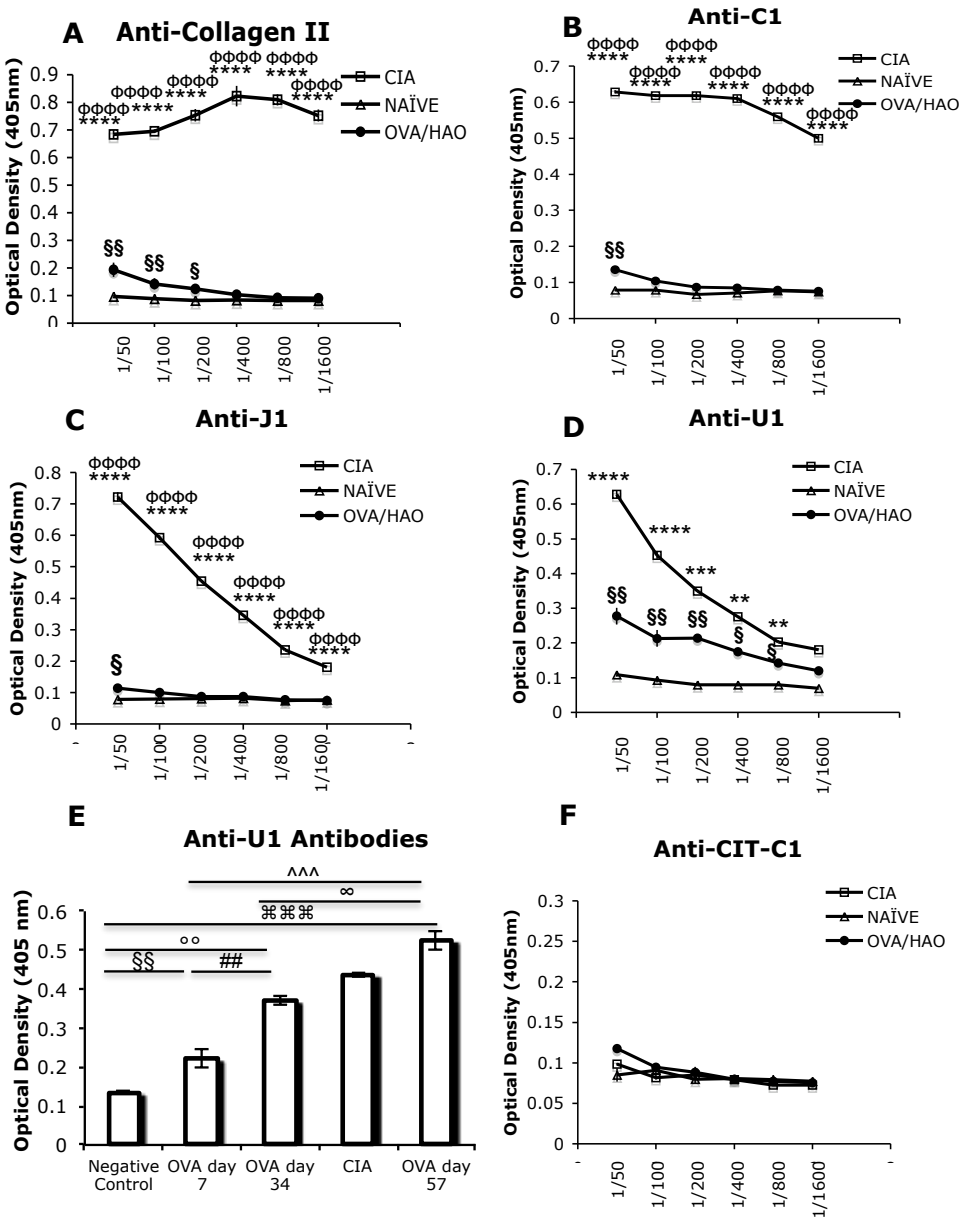


Figure 3.16 Antibody response to CII and different triple helical peptides

(A-F) Antibodies against CII and peptides C1, J1, U1, Cit-C1 were tested in sera from mice with OVA-mediated acute arthritis (day 7) (●), CIA (□) and naïve mice (△). In panel E anti-U1 antibodies were tested also in mice with previous history of acute arthritis (OVA day 34) and in mice with a relapse of OVA-mediated arthritis (OVA day 57).

(A) Sera from mice with CIA contained high levels of anti-CII compared with sera from OVA-mediated acute arthritis and naïve mice. Sera from OVA-mediated acute arthritis showed anti-CII antibodies compared with naïve mice. (B) Sera from mice

with CIA contained high levels of anti-C1 compared with naïve mice and OVA-mediated acute arthritis. Mice with OVA-mediated acute arthritis displayed a significant production of anti-C1 antibodies compared with naïve mice at the serum dilution of 1 in 50. (C) Sera from mice with CIA contained high levels of anti-J1 compared with naïve mice and OVA-mediated acute arthritis. Mice with OVA-mediated acute arthritis displayed a significant production of anti-J1 antibodies compared with naïve mice at the serum dilution of 1 in 50. (D) Mice with CIA and OVA-mediated acute arthritis produced significant levels of anti-U1 antibodies compared with naïve mice. (E) Mice with OVA-mediated acute arthritis (day 7), mice with OVA-mediated arthritis at day 34 and mice with a relapse of OVA-mediated arthritis (day 57) showed anti-U1 antibodies compared with naïve mice. Anti-U1 antibodies increased from day 7 to day 57. Mice with a relapse of OVA-mediated arthritis and mice with CIA displayed comparable titres of anti-U1 antibodies. In panel E sera were diluted 1 in 100. (F) Neither CIA mice nor OVA-mediated acute arthritis showed anti-Cit-C1 antibodies. *, CIA *vs* naïve mice; Φ, CIA *vs* OVA/HAO day 7; §, OVA/HAO day 7 *vs* naïve mice; ⌗ OVA day 7 *vs* OVA day 34; ° OVA day 34 *vs* naïve mice; ⌘ OVA day 57 *vs* naïve; ∞ OVA day 34 *vs* OVA day 57; ^ OVA day 7 *vs* OVA day 57. Statistical analysis was performed by 2-way Anova with Bonferroni's multiple comparisons test. Data represent mean ± SD (n = 5 mice per group). Similar results were obtained in 2 identical experiments (n = 30 mice).

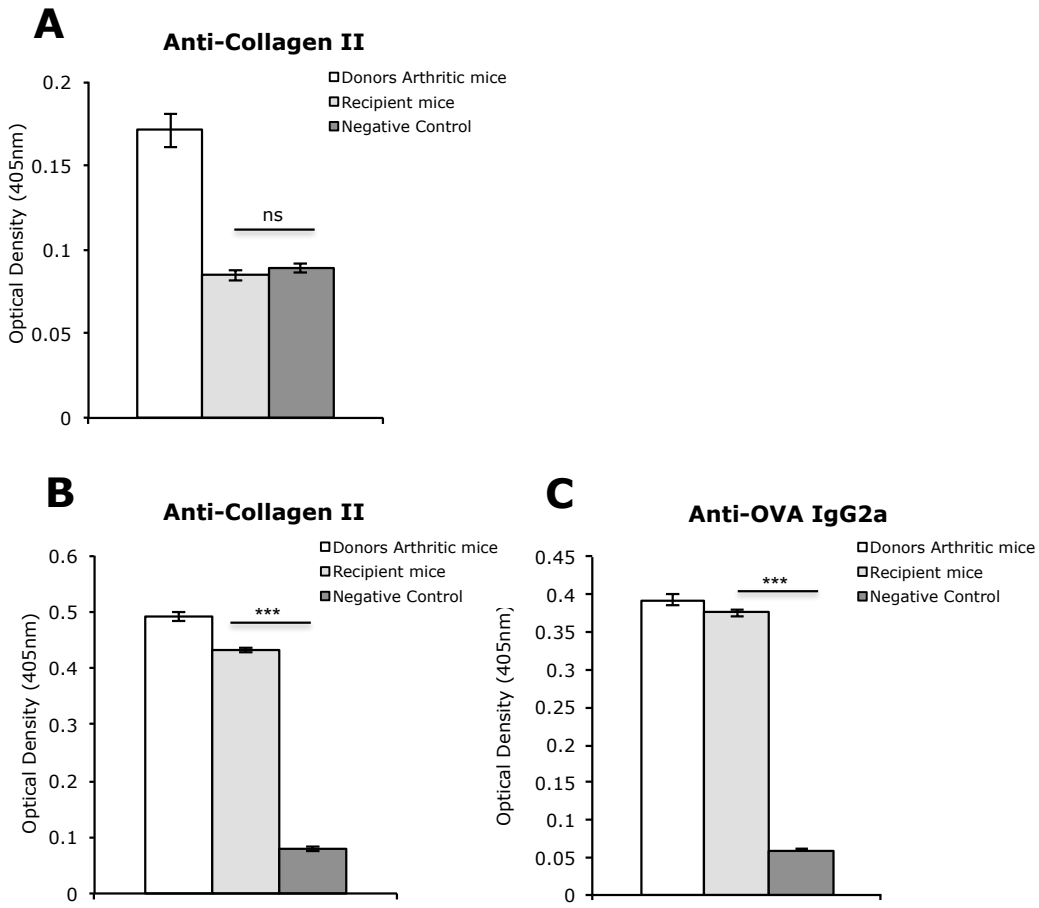


Figure 3.17 Effect of serum passive transfer on anti-CII and anti-OVA antibodies in naïve mice

(A) Anti-CII antibodies were not detected in recipient animals after passive transfer of 150 μl serum from mice with a relapse of OVA-mediated arthritis. Ns indicates not significant. (B) Anti-CII and (C) anti-OVA antibodies were detected in recipient animals after the passive transfer of 200 μl serum in two consecutive days from mice with a relapse of OVA-mediated arthritis. Sera were diluted 1 in 100 for the detection of anti-CII antibodies and 1 in 1000 for anti-OVA antibodies. *, Recipient mice *vs* negative control. Statistical analysis was performed by 2-way Anova with Bonferroni's multiple comparisons test. Data represent mean ± SD (n = 3 mice per group for each experiment).

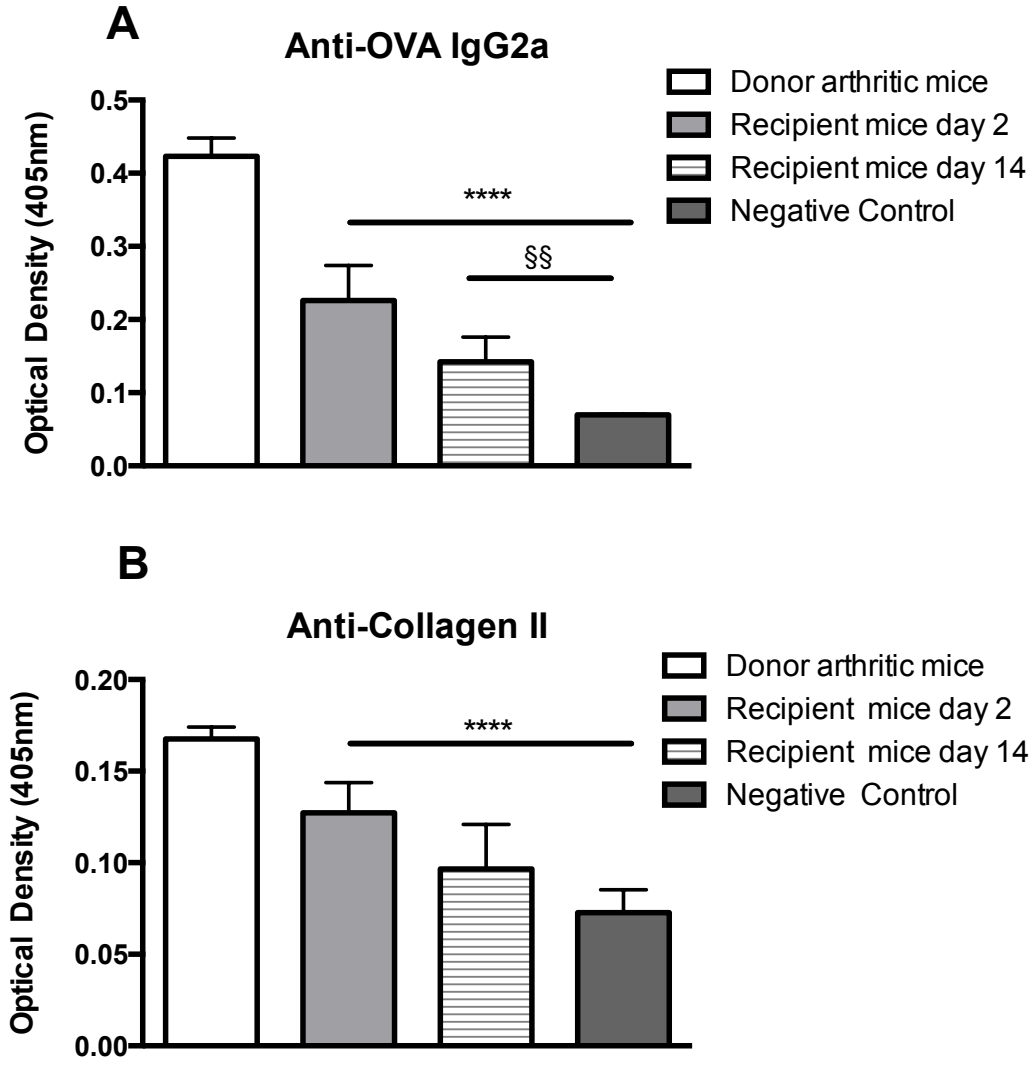


Figure 3.18 Anti-OVA and anti-CII antibodies in recipient mice after 2 and 14 days from serum passive transfer

(A) Anti-OVA antibodies were detected in recipient animals 2 and 14 days after passive transfer of 200 μl serum from mice with a relapse of OVA-mediated arthritis Sera were diluted 1 in 1000. (B) Anti-collagen II antibodies could be detected in recipient mice after 2 from passive transfer compared with control mice. Sera were diluted 1 in 100. *, Recipient mice day 2 *vs* negative control; § Recipient mice day 14 *vs* Negative Control. Statistical analysis was performed by 2-way Anova with Bonferroni's multiple comparisons test. Data represent mean ± SD (n = 3 mice per group). Similar results were obtained in 2 identical experiments (n = 18 mice in all).

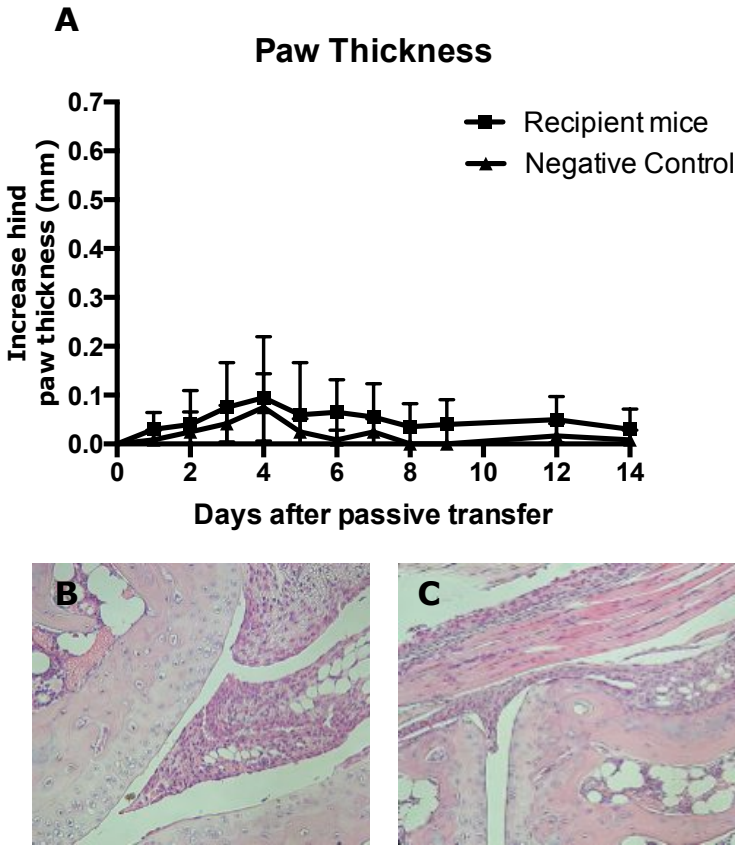
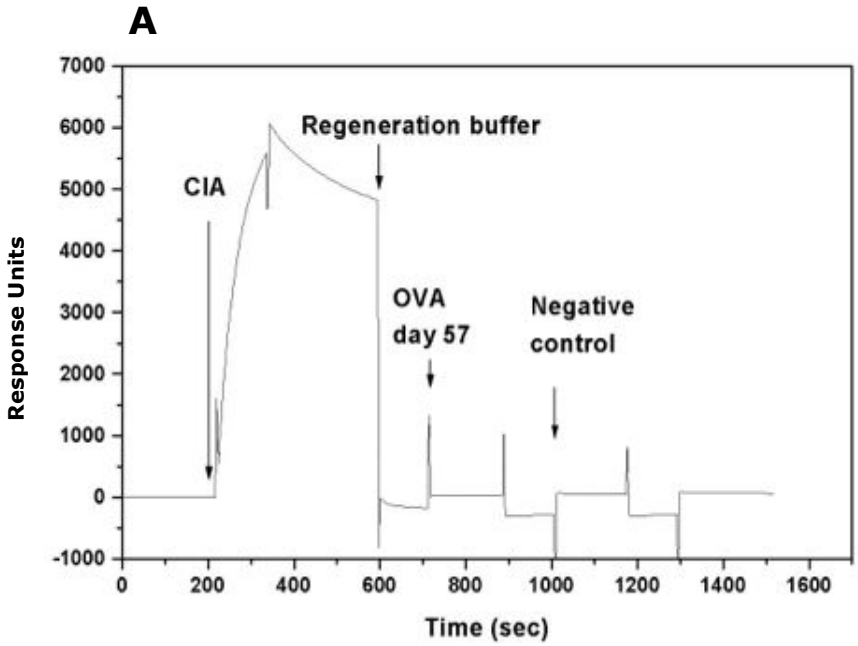


Figure 3.19 Effect of serum passive transfer on paw thickness and joint histopathology in naïve mice

(A) Hind paw thickness did not differ in mice that received serum passive transfer from mice with a relapse of OVA-mediated arthritis and in control mice that received normal mice serum. Statistical analysis was performed by Mann-Whitney U test. Data represent median and IQR (n = 3 mice per group). (B-C) Representative haematoxylin/eosin stained sections prepared from the joints of naïve BALB/c mice after the passive transfer of serum from OVA-mediated arthritis mice (B) or normal mice serum (C). The injection of serum from mice with a relapse of OVA-mediated arthritis or normal mice serum did not induce significant inflammatory reaction or cartilage erosion in naïve animals. Original magnification X20. Similar results and images were obtained in 2 identical experiments (n = 18 mice in all).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Response Units
Time (sec)
Regeneration buffer
CIA
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Negative
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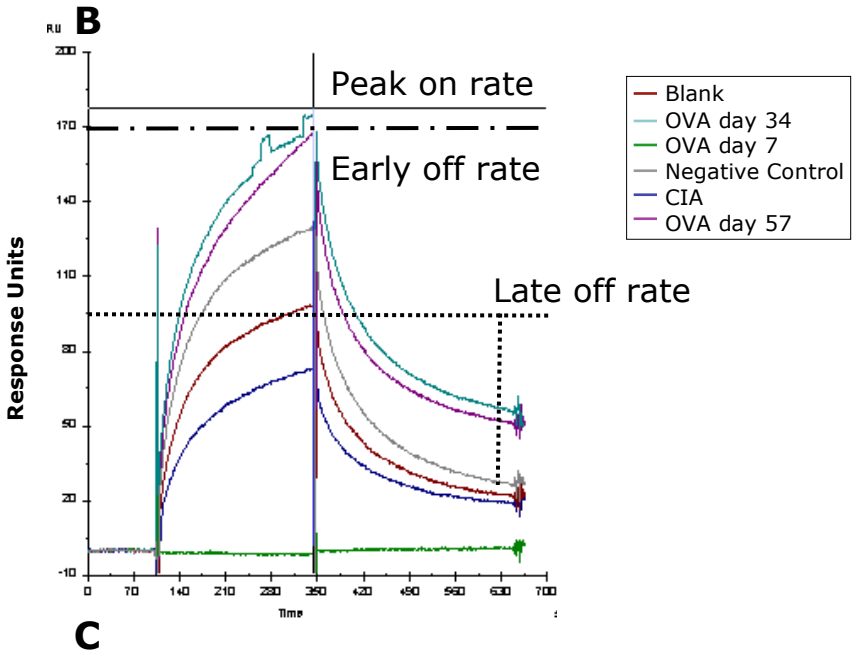
B
Response Units
Peak on rate
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Late off rate
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OVA day 7
Negative Control
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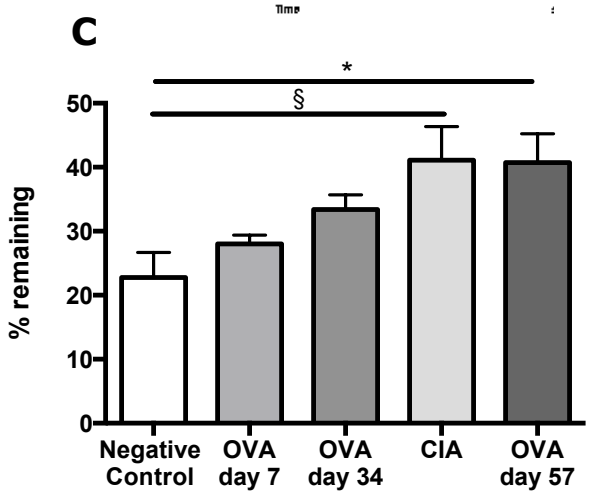
OVA day 57


C
% remaining
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Negative Control
OVA day 7
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CIA
OVA day 57

Figure 3.20 Biacore assay: Affinity of anti-CII antibodies

(A) Binding curves generated with immobilisation of CII protein and injection (arrows) on the flow cells sensor surface of serum samples from CIA, mice with a relapse of OVA-mediated arthritis (OVA day 57) and control animals. Serum from mice with CIA bound CII protein compared with sera from mice with a relapse of OVA-mediated arthritis (day 57) and control animals. (B) Binding curves generated with immobilisation of U1 peptide and the injection on the flow cells sensor surface of serum samples from CIA, OVA-mediated acute arthritis (OVA day 7), mice with a previous history of OVA-mediated arthritis (OVA day 34), mice with a relapse of OVA-mediated arthritis (OVA day 57), and control animals. (C) Sera from OVA day 57 and CIA mice exhibited the highest stability of the complex anti-U1 antibodies/U1 antigen. *, OVA day 57 *vs* negative control; §, CIA *vs* negative control. Sera were diluted 1 in 10. Statistical analysis was performed by 2-way Anova with Bonferroni's multiple comparisons test. Data represent mean ± SD (n = 3 mice per group). Similar results were obtained in 3 identical experiments (n = 27 animals in all).

Chapter 4

B cells in the induction of OVA-mediated arthritis

4.1 Aim and rationale

In this chapter Th1-OVA specific cells induced experimental arthritis in absence of antigen-specific B cells or in complete absence of mature B cells. $CD4^+$ T cell proliferation was analysed in absence of antigen presenting B cells.

The model of OVA-mediated arthritis is characterised by the breach of self-tolerance and the production of autoantibodies such as RF, ACPA and anti-CII antibodies (358, 361, 364). Despite the fact that the presence of these antibodies makes this animal model of interest for its similarity with human RA, it does not clarify the contribution of B cells in arthritis. This is of particular relevance because current therapeutic approaches aim to target B cells and B cell mediators (334, 395, 396). Thus, understanding the fine role of B cells both in human and in animal models of RA will help to design better therapeutic strategies.

Therefore, the aim of this chapter was to investigate the role of B cells in the induction of arthritis. For this purpose I modified the original model of OVA-mediated arthritis (358). In particular, the adoptive transfer of Tg Th1-OVA specific cells was performed in recipient animals with different B cell repertoires: BALB/c mice with normal B cells, MD4 mice with B cells that could not present antigen to $CD4^+$ T cells and produce antibodies (370), and JHD mice characterised by the absence of mature B cells (371).

4.2 Introduction

Several lines of evidence support an important role for B cells in the pathogenesis of RA, as previously discussed (section 1.8) (217). These can be summarised as follows:

1. B cells are a source of relevant autoantibodies in RA
2. B cells enrich RA synovial membrane
3. B cells are highly efficient APCs to stimulate T cells
4. B cells are a major source of cytokines
5. B cells are a therapeutic target in RA

RA has been considered an autoimmune disease since the production of autoantibodies was first observed (216). RF and ACPA are the most relevant antibodies in RA for their diagnostic and prognostic value. RF is detected in RA patients in about 50-80% of cases and high serum levels are associated with an aggressive articular disease, extra-articular manifestations and a worse outcome (217). ACPA show high specificity, 98%, and sensitivity comparable with RF. Of interest, they correlate with disease severity and with radiological progression of the disease (246). Moreover, recent studies have demonstrated the presence of RF and ACPA many years before the onset of RA (5, 247) suggesting that the presence of these autoantibodies can predict or interfere with the development of disease. Also anti-CII antibodies have been detected in human RA although in a low percentage of cases (227, 228).

The contribution of B cells in human RA has also been proven with the demonstration of their presence in the synovial membrane. The histological pattern of rheumatoid synovia is heterogeneous with a variable presence of synovial T-B aggregates demonstrated in 31-73% of the specimens after arthroscopic biopsy in active joints (250, 256).

Activated B cells produce pro-inflammatory cytokines, such as IL-6, TNF-α and RANKL, which may contribute to joint inflammatory environment, bone erosions and amplification of T cell responses (295).

B cells are highly efficient APCs to stimulate T cells and allow optimal $CD4^+$ T cell memory (268). Studies on mice depleted of B cells allowed analysis of T cell priming in the absence of B cells. Early experiments using mice depleted by treatment with anti-IgM suggested that naïve T cells could not be primed in absence of B cells (272, 273, 275). Later studies using mice genetically depleted of B cells, created by targeted deletion of the μ region (μMT) or J_H region (JHD) of the IgM locus, have given conflicting results concerning the importance of B cells in T cell priming (276, 397). For example, Liu *et al*. demonstrated that immunisation of JHD mice failed to prime $CD4^+$ T cells for either clonal expansion or delivery of immunological help for antibody production (397). In another interesting study, the authors showed that primed JHD LN cells proliferated poorly in response to antigen compared with primed normal BALB/c LN cells (398). The proliferative response was restored when purified JHD $CD4^+$ T cells were stimulated with antigen presented by splenic adherent cells from BALB/c, which consisted of DCs and macrophages. Moreover, JHD T cells primed by normal B cells provided little or no help for isotype switching, IgG production and failed to produce IL-4 in response to B cells compared with normal T cells (398).

Several studies dissected the contribution of B cells in murine models of autoimmune diseases. For example, in MRL/lpr mice, murine model of SLE, B cell deficient mice did not show activated or memory T cells (284). On the contrary, in the presence of normal B cells, but in the absence of circulating antibodies, MRL/lpr mice demonstrated T cell activation (283).

Similarly, the requirement for antigen-specific B cells was investigated in the experimental model of PGIA using B cell deficient mice, such as JHD and Ig-deficient (mIgM) mice (285). Of interest, both JHD and mIgM mice were completely resistant to PGIA, confirming the necessity of B cells in the initiation of PGIA. Moreover, the authors demonstrated that antigen presentation by B cells was critical for the efficient activation of autoreactive T cells. Both primed autoreactive T cells and autoantibodies were required to induce PGIA into SCID mice.

All these evidences supported the idea that targeting B cells could be effective in human RA treatment. Indeed, different therapeutic strategies that target B cells (Rituximab) or B cell mediators (Tocilizumab and Atacicept) demonstrated to be effective in RA (334, 395, 399).

Rituximab is a chimeric monoclonal antibody that binds to CD20, a surface molecule expressed on B cells at the pre-B stage of differentiation until the mature B cells excluding stem cells, pro-B cells and plasma cells (336). Rituximab is the first B cell agent approved for the treatment of adult RA patients with moderate to severe disease after the failure of an anti-TNF. Its use in combination with Methotrexate ameliorates the disease. Rituximab can also inhibit the progression of structural joint damage in RA patients over 2 years (337, 338).

Despite all of these findings, the exact contribution of B cells in the induction and maintenance of RA remains unclear (217).

The aim of this chapter was to investigate the role of B cells in the induction of OVA-mediated arthritis. For this purpose, I employed recipient animals with different B cell repertoires: MD4 and JHD mice. Homozygous MD4 Tg mice have a BCR specific for the antigen HEL (370), thus B cells could not recognise through the BCR any other antigen, such as OVA or self antigens. Inducing OVA-mediated arthritis in MD4 mice allowed me to study the development of arthritis in a situation where B cells were present, lymphoid architecture was normal but in the complete absence of antibodies and antigen-specific B cells. JHD mice contained a genetic modification, the deletion in the J gene of the heavy chain in the Ig variable region (371). As a result of this modification B cells could not assemble the heavy chain genes of the Ig and B cell differentiation was blocked at a precursor stage. A complete absence of mature B cells was observed in the periphery and bone marrow. The precursor B cell number was also affected in the bone marrow and there was no IgM or IgG in the sera of these mice. T cell development was not affected in JHD mice.

4.3 Results

As reported previously the adoptive transfer of Th1 OVA-specific cells, followed by immunisation with OVA and challenge with HAO in BALB/c mice, caused a transient arthritis characterised not only by joint inflammation and tissue damage but also by the production of RA-related autoantibodies, such as RF, ACPA and anti-CII antibodies (358, 362, 365). Moreover, in the previous chapter I demonstrated that the relapse of arthritis in this animal model was associated with increased autoreactive B

cell response (364). However, the production of autoantibodies does not clarify if these autoantibodies contribute to the induction of joint pathology or if they are only epiphenomena. Thus, I aimed to investigate if B cells were essential in the induction of arthritis. For this purpose pathology was induced in BALB/c mice, with normal B cell repertoire, in MD4 mice (370), in absence of antigen specific B cells, and in JHD mice (371), in complete absence of mature B cells.

4.3.1 Induction of arthritis in BALB/c, MD4 and JHD mice

Mice with different B cell repertoires such as BALB/c, MD4 and JHD mice were employed to study the involvement of B cells in the induction of experimental arthritis. Arthritis was elicited in different recipient animals with transfer of Th1-polarised OVA-specific Tg T cells followed by OVA immunisation and HAO challenge, as previously demonstrated (358). BALB/c mice HAO-challenged developed an increase in the paw thickness (Fig. 4.1A) and the clinical score (Fig. 4.2A) that was evident from day 1 to day 6 after challenge compared with those in un-challenged animals.

MD4 mice HAO-challenged exhibited a paw swelling evident only at day 6 after challenge (Fig. 4.1B) and a higher clinical score at days 5 and 6 (Fig. 4.2B) compared with those in MD4 un-challenged animals.

No significant increase in paw thickness and clinical score was observed in JHD mice HAO-challenged compared with those in respective un-challenged mice (Fig. 4.1C and Fig. 4.2C).

BALB/c and MD4 mice HAO-challenged showed similar levels of paw swelling and clinical score (Fig. 4.1D and Fig. 4.2D). On the contrary, a greater paw swelling and clinical score was demonstrated in BALB/c and MD4 mice HAO-challenged compared with respective JHD mice at days 4, 5 and 6 post-challenge (Fig. 4.1D and 4.2D). Of interest, BALB/c mice injected with PBS showed reduced paw thickness and clinical score compared with respective JHD and MD4 mice at days 2 and 3 after injection (Fig. 4.1E and Fig. 4.2E).

On histological examination, BALB/c mice immunised and challenged with HAO exhibited in the ankle joint cell infiltration, synovial hyperplasia (Fig. 4.3A) and

cartilage depletion (Fig. 4.3B) compared with un-challenged BALB/c mice (Fig. 4.3C-D). Of relevance, joints from both MD4 (Fig. 4.3E) and JHD mice (Fig. 4.3I) immunised with OVA and challenged with HAO showed an infiltrate and synovial proliferation. Small cartilage and bone erosions were also demonstrated in both groups by loss of toluidine blue staining after HAO-challenge (Fig. 4.3F,J). Joints from MD4 and JHD mice injected with PBS did not exhibit cell infiltrate (Fig. 4.3G,K) or cartilage/bone depletion (Fig. 4.3H,L). The joint histopathology score of BALB/c mice HAO-treated was greater than those detected in MD4 and JHD mice HAO-treated (Fig. 4.4). BALB/c, MD4 and JHD mice challenged with HAO exhibited a higher score compared with that observed in respective un-challenged mice.

These data demonstrated that HAO-challenge could elicit in the joints cell infiltration, synovial hyperplasia and cartilage/bone damage in the absence of antigen-specific B cells or in complete absence of mature B cells. However, the pathology detected was mild, in terms of histopathology score, compared with that observed in BALB/c animals with a normal B cell repertoire.

4.3.2 Antibody production in arthritic mice

After I induced the experimental arthritis I aimed to assess the antibody response in different recipient animals. For this purpose serum samples were collected at day 7 after HAO challenge and analysed for the presence of anti-OVA, anti-CII antibodies and ACPA IgG. BALB/c mice immunised with OVA and challenged with either HAO or PBS exhibited similar levels of anti-OVA antibodies (Fig. 4.5A). On the contrary MD4 and JHD mice, HAO-challenged or un-challenged, did not show significant production of anti-OVA antibodies compared with respective BALB/c mice (Fig. 4.5A). BALB/c mice HAO-challenged, as previously demonstrated (358, 361, 364, 365), produced anti-CII antibodies compared with MD4 and JHD mice HAO-challenged (Fig. 4.5B). Of a note, different experiments with the same protocol revealed that the levels of anti-CII antibodies, in terms of optical density, did not reach always the same magnitude, as showed in the two panels of figure 4.5B. Of interest, sera from BALB/c, MD4 and JHD mice HAO-treated were compared also for

the presence of ACPA, an important hallmark in human RA. I demonstrated that BALB/c mice HAO-challenged exhibited high levels of ACPA compared with MD4 and JHD mice HAO-challenged (Fig. 4.5C).

These data demonstrated that MD4 and JHD mice could not produce antibodies during the development of arthritis while arthritic BALB/c mice showed characteristic antibodies of RA.

4.3.3 Proliferative response of OVA and Collagen-specific T cells

$CD4^+$ T cell proliferation was analysed in animals with different B cell repertoire in response to antigen stimulation. For this purpose draining LN cells from BALB/c, MD4 and JHD mice were cultured *in vitro* with either medium, OVA or CII. Antigen-specific proliferation was analysed at 72 hours by flow cytometric staining for EdU incorporation as previously described (chapter 2, section 2.15). Figure 4.6A outlines a representative flow cytometric plot used to identify the lymphocyte population, which was gated on blastic $CD4^+$ cells. The gate was drawn with the backgating analysis that provides a tool to view if the gate was positioned correctly showing the final gated population within the population of its ancestors. Proliferating cells corresponded to the cells double positive for the expression of CD4 and Edu-Alexa-fluor 488 (Fig. 4.6B). Stimulation with OVA resulted in $CD4^+$ T cell proliferation from BALB/c, MD4 and JHD animals HAO challenged compared with un-stimulated cells (Medium). A higher $CD4^+$ T cells proliferation was detected from BALB/c animals HAO challenged compared to those derived from BALB/c un-challenged mice. Similar findings were observed in MD4 and JHD mice HAO challenged compared with un-challenged mice. However, no significant difference was demonstrated in the proliferation of $CD4^+$ T cells between arthritic BALB/c and MD4 or JHD mice (Fig. 4.6C).

Stimulation of draining LN cells with CII did not result in significant $CD4^+$ T cell proliferation in BALB/c, MD4 or JHD mice (Fig. 4.6).

Since the contribution of B cells in T cell proliferation could be different in a primary and secondary immune response, I investigated the ability of $CD4^+$ T cells from BALB/c, MD4 and JHD mice to proliferate in response to OVA antigen in a primary immune response. For this purpose other mice were immunised s.c. with OVA in

CFA. T cell proliferation was analysed after *in vitro* re-stimulation with OVA or medium. Of interest, $CD4^+$ T cells proliferated after OVA re-stimulation from BALB/c (Fig. 4.7A), MD4 (Fig. 4.7B) and JHD mice (Fig. 4.7C). Indeed, I demonstrated a higher proliferation of $CD4^+$ T cells from BALB/c animals compared to that derived from MD4 and JHD mice (Fig. 4.7D).

These data indicated that $CD4^+$ T cells primed in the absence of antigen presenting B cells could proliferate in response to OVA antigen. However, in a primary immune response a higher proliferation of $CD4^+$ T cells was observed in the presence of normal B cell repertoire.

4.4 Discussion

In this chapter a mild arthritis was induced in an animal model of arthritis in the absence of antigen-presenting B cells and in complete absence of mature B cells. $CD4^+$ T cell proliferation was also analysed in absence of antigen presenting B cells.

B cells are critically important in RA because of their pleiotropic effects. Indeed B cells produce pro-inflammatory cytokines, such as IL-6 and TNF-α (295), secrete antibodies that are key mediators in RA (217) and participate in synovial ectopic lymphoneogenesis (252). B cells can also regulate T cells, being essential in their ability to present antigen and regulate their expansion (273, 285). B cells can influence T cells providing other co-stimulatory (OX40L) signals that give a stimulus to survive (400). Of a note, B cells might also have regulatory function controlling T cell differentiation and autoimmune disorders (302).

Thus, the aim of this chapter was to assess the B cell requirement in the induction of experimental arthritis. For this purpose, I modified the original model of OVA-mediated arthritis (358) using the adoptive transfer into recipients animals with different B cell repertoire: BALB/c, MD4 and JHD mice. Homozygous MD4 mice contained only B specific for HEL and could not present any other antigen to $CD4^+$ T cells or produce antibodies directed against other specificities (370). On the other side, JHD mice were B cell deficient (371). I demonstrated that BALB/c mice HAO challenged showed an acute arthritis, detected by the increased paw thickness, clinical score and joint histology, compared with BALB/c mice PBS injected. JHD mice HAO treated displayed a similar paw thickness and clinical score compared with PBS treated mice. A similar finding was evident for MD4 mice in the first days after challenge. On the contrary the joint histology, performed at day 7 post-challenge, showed a mild arthritis in both MD4 and JHD mice HAO treated compared with PBS injected mice. Clinical and histological data are partially contradictory and I have hypothesized that the swelling/clinical score in all HAO and PBS injected mice might indicate two responses elicited after the injection. The first response appeared after 2-3 days post-injection and this was regardless of HAO/PBS injection. Of note, no skin oedema was evident the day after the injection. I have speculated that this initial response was not specific and caused by the trauma of the injection. The second

response appeared at days 4-6 post-injection and it might be related to the arthritis as shown by the histological analysis. However, no joint histology was performed at days 2-3 post injection to verify this hypothesis. Moreover, other issues, such as the low specificity of the clinical assessment, might be responsible for these data. Of interest, BALB/c mice PBS injected showed reduced clinical scores compared with respective MD4 and JHD mice in the first two days post-PBS injection. These reduced scores in BALB/c mice might be related to the presence of B cells with regulatory function in this context. Otherwise, different cells, such as T cells, in MD4 and JHD mice might favour the initial response.

One of the limits of OVA-mediated acute arthritis model is that the anti-CII response is variable, as highghlited previously. For this reason I described two identical experiments where the anti-CII response was different even if the levels of anti-CII antibodies were always higher than that in un-challenged BALB/c mice. These differences might be biologically relevant. As expected, MD4 and JHD mice did not produce significant amounts of autoantibodies, such as anti-CII and ACPA. Thus, autoantibodies did not contribute to the mild pathology observed in MD4 and JHD mice. These results were in contrast with other studies where antigen-presenting B cells and autoantibodies were required for the initiation of the disease (284, 285). However, several differences between the model of OVA-mediated arthritis and the other models of RA may explain this difference. For example, in the model of PGIA the nature of antigen used, human proteoglycan, and the protocol of immunisation were different from those employed in OVA-mediated arthritis. PGIA is dependent on antigen-specific B cells and autoantibodies are required for the initiation of disease but are insufficient to transfer disease (285).

I was also interested in investigating the requirement of antigen presenting B cells in this animal model. B cells could act as APC by internalising antigen through the BCR or by formation of immune complexes and their internalisation through FCγR expressed on professional APCs. Moreover, B cells with RF specificity could capture a foreign antigen complexed with an antibody via their membrane Ig receptors and present the antigen efficiently to $CD4^+$ T cells of the same specificity (270). A decrease of T cell effector and memory responses was documented in the absence of B cell antigen presentation (272, 275, 279). Weyand and co-workers demonstrated that T cell activation was B-dependent in human synovial membrane transplanted into

SCID mice (260). Thus, $CD4^+$ T cell proliferation was analysed in arthritic BALB/c, MD4 and JHD mice. Of interest, $CD4^+$ T cells from BALB/c, MD4 and JHD mice, immunised and challenged with OVA, proliferated in response to OVA antigen without significant differences among the different strains. This result could be attributed to the presence in the draining LN of other APCs rather than B cells. T cells generated a memory population after OVA stimulation and HAO challenge *in vivo*, which persisted in absence of antigen. This population was ready to proliferate after the encounter with the antigen *in vitro*. It would be relevant to analyse if the T cell population that proliferated was that one transferred (DO11.10) or if it belonged to the host. Indeed, DCs are the predominant APC population for the initial expansion of $CD4^+$ T cells (401). Our group demonstrated that DCs were the major presenter of antigen in the model of OVA-mediated arthritis (361). This cell population was also sufficient to induce B and T autoreactive responses (361). It would be interesting in future experiments to isolate DCs from the model and use them as a positive control to increase the APC function. On the contrary, previous studies in the model of OVA-mediated arthritis showed that pDCs could limit arthritis and autoimmunity (362).

I did not observe any $CD4^+$ T cell proliferation in response to CII stimulation. This result is in contrast with previous experiments that demonstrated successful induction of CII-specific T cells in arthritic BALB/c mice (361, 362, 365). Technical problems in the assay might explain these data, such as a low concentration of CII or Edu, a short duration of incubation and/or a high background in the well with medium rather than the true absence of T cell proliferation. The wells with medium alone seemed to contain proliferating $CD4^+$ T cell; this paradoxical effect might be related to the presence in these wells of APCs loaded with OVA that might stimulate $CD4^+$ T cells.

Several studies demonstrated that in a primary immune response T cells expansion and differentiation were affected in B cell deficient mice (272-274). The same finding was proven with mixed bone-marrow chimeric mice in which the B cell compartment was deficient in MHC class II (402). Moreover, an impaired $CD4^+$ T cell memory generation and cytokine production was observed in the absence of B-cell derived MHC class II (402). Thus, I aimed to confirm these data analysing $CD4^+$ T cell proliferation in BALB/c, MD4 and JHD mice immunised with OVA in CFA. Indeed a higher proliferation of $CD4^+$ T cells was detected in the presence of normal B cells compared with that observed in the absence of antigen presenting B cells, such as in MD4 and JHD mice. This result was in agreement with data from literature where

primed JHD LN cells proliferated poorly in response to the antigen compared with primed normal BALB/c LN cells (397, 398).

In this chapter I demonstrated that B cells were not necessary in the induction of OVA-mediated arthritis, however their presence was associated with a more severe arthritis confirmed by the joint histopathology score. This result could be due to the presence of autoantibodies, pro-inflammatory cytokines B cell-related that might contribute to inflammation, and/or the activation of autoreactive T cells. It would be interesting in future experiments to explore the role of B in the model previously described of relapsing arthritis.

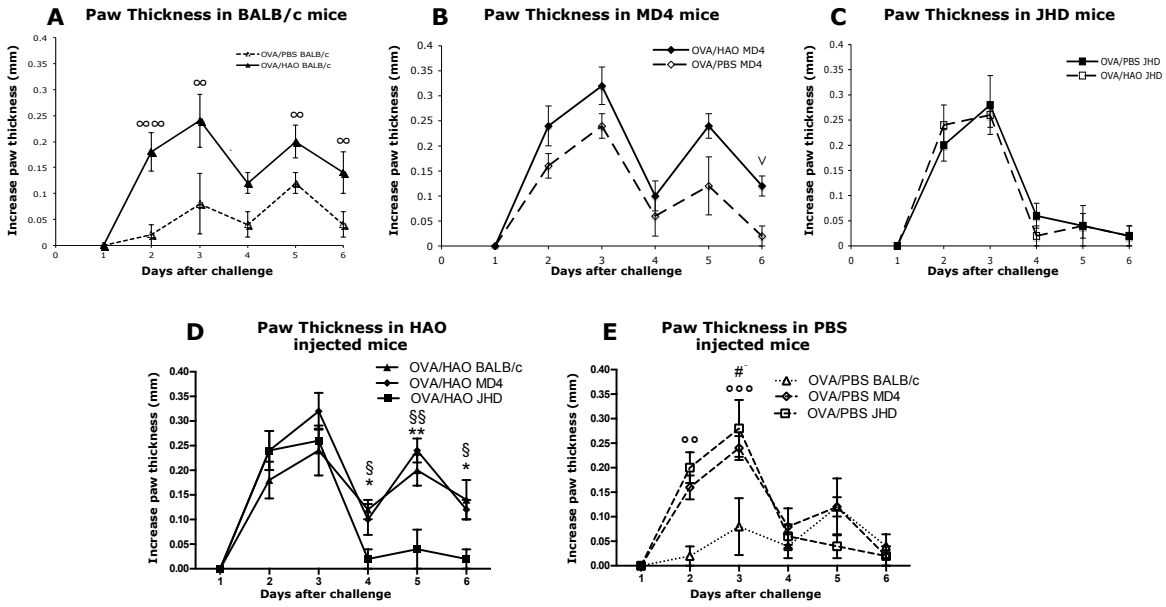

Figure 4.1 Hind paw thickness in BALB/c, MD4 and JHD mice after HAO challenge

(A) BALB/c mice HAO challenged showed increased paw swelling compared with un-challenged animals. (B) MD4 mice presented an increased paw swelling at day 6 after HAO challenge compared with MD4 un-challenged animals. (C) JHD mice HAO challenged did not exhibit a significant paw swelling compared with JHD un-challenged mice. (D) BALB/c and MD4 mice HAO challenged showed comparable levels of paw swelling that were greater than that in JHD mice. (E) BALB/c mice injected with PBS showed reduced paw swelling compared with respective JHD and MD4 mice at days 2 and 3 after the injection. ∞, BALB/c OVA/HAO *vs* BALB/c OVA/PBS; , ν MD4 OVA/HAO *vs* MD4 OVA/PBS; *, BALB/c OVA/HAO *vs* JHD OVA/HAO; § MD4 OVA/HAO *vs* JHD OVA/HAO; °, BALB/c OVA/PBS *vs* JHD OVA/PBS; ♯, BALB/c OVA/PBS *vs* MD4 OVA/PBS. Statistical analysis was performed by 2-way Anova with Bonferroni's multiple comparisons test. Data represent mean ± SD (n = 5 mice per group). Similar results were obtained in 3 identical experiments (n = 90 mice in all).

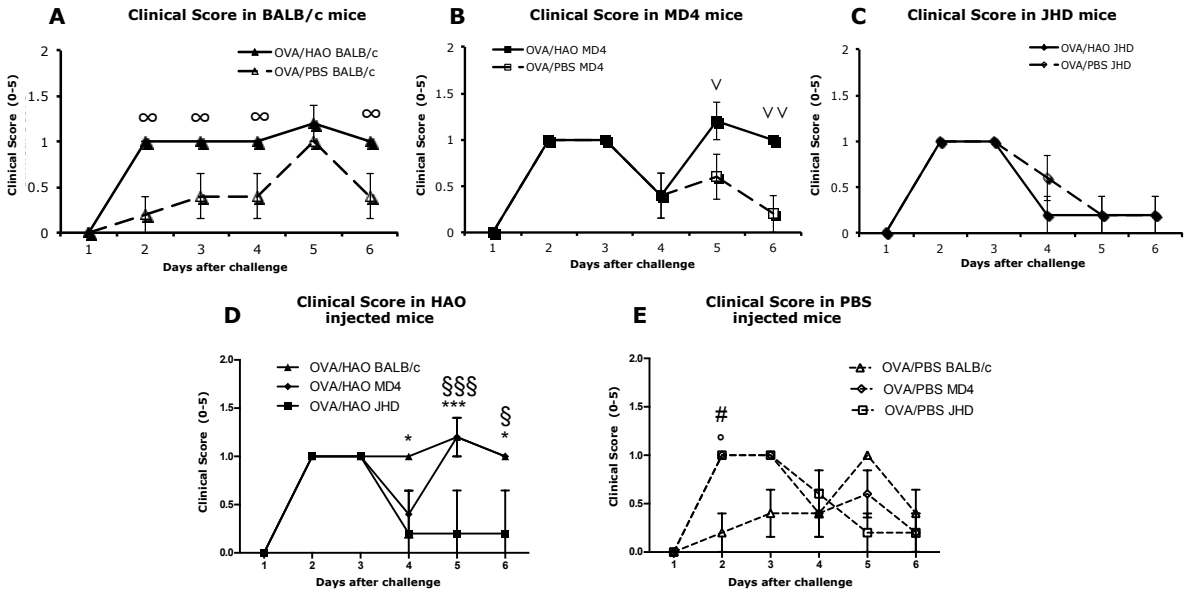

Figure 4.2 Clinical score in BALB/c, MD4 and JHD mice after HAO challenge

(A) BALB/c mice HAO challenged exhibited a higher clinical score than that in un-challenged animals. (B) MD4 mice HAO challenged showed a higher clinical score compared with that in MD4 un-challenged mice. (C) Joints from JHD mice HAO challenged and PBS treated displayed a similar clinical score. (D) BALB/c and MD4 mice HAO challenged showed similar level of clinical score and this was higher than that in JHD mice. (E) BALB/c mice injected with PBS exhibited a lower clinical score than that in JHD and MD4 mice at day 2 post-injection. ∞, BALB/c OVA/HAO *vs* BALB/c OVA/PBS; v, MD4 OVA/HAO *vs* MD4 OVA/PBS; *, BALB/c OVA/HAO *vs* JHD OVA/HAO; § MD4 OVA/HAO *vs* JHD OVA/HAO; °, BALB/c OVA/PBS *vs* JHD OVA/PBS; ♯, BALB/c OVA/PBS *vs* MD4 OVA/PBS. Statistical analysis was performed by 2-way Anova with Bonferroni's multiple comparisons test. Data represent mean ± SD n = 5 mice per group). Similar results were obtained in 3 identical experiments (n = 90 mice in all).

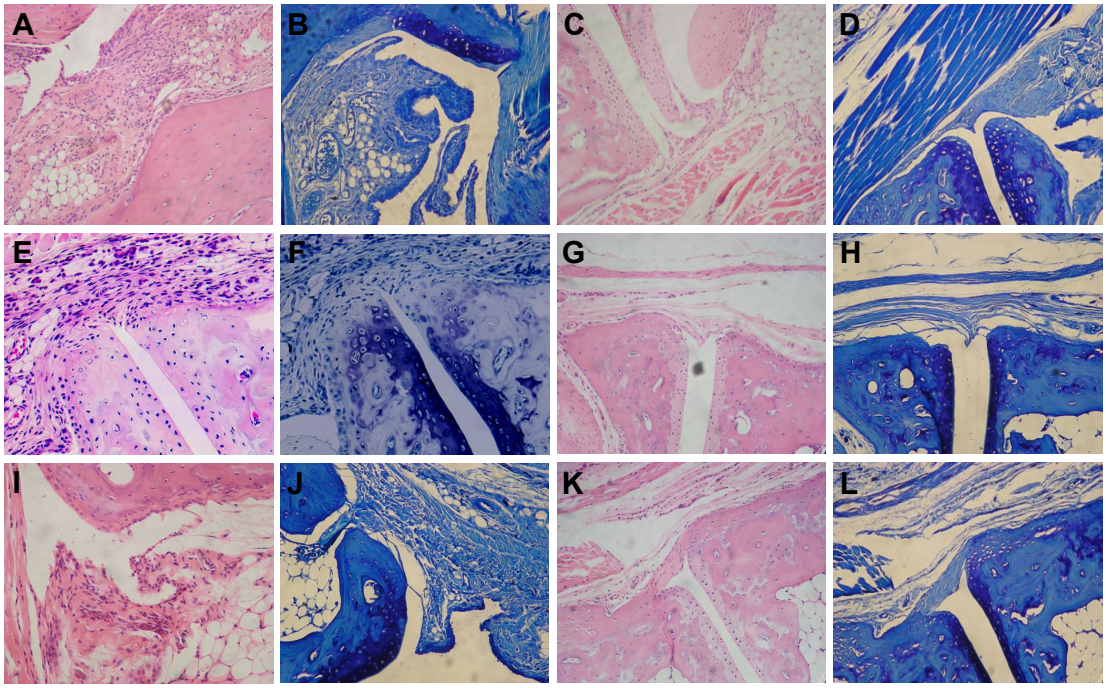

Figure 4.3 Joint histology of BALB/c, MD4 and JHD mice

Representative haematoxylin/eosin and toluidine blue stained sections prepared from the joints of BALB/c (A-D), MD4 (E-H) and JHD mice (I-L) challenged with HAO (A,B,E,F,I,J) or PBS (C,D,G,H,K,L).

Joints from BALB/c mice HAO challenged showed an inflammatory reaction with cell infiltration, synovial hyperplasia (A) and cartilage depletion (B) compared with un-challenged BALB/c mice (C-D). Both MD4 (E) and JHD mice (I) immunised with OVA and challenged with HAO displayed in their joints an inflammatory reaction and synovial hyperplasia. Bone erosions were also evident in both groups by loss of toluidine blue staining (F,J). MD4 and JHD mice injected with PBS did not exhibit joint inflammation (G,K) or cartilage/bone damage (H,L). Original magnification X20 except in panels E and F original magnification X40. Images shown are representative of 3 identical experiments (n = 5 mice per group and 60 mice in all).

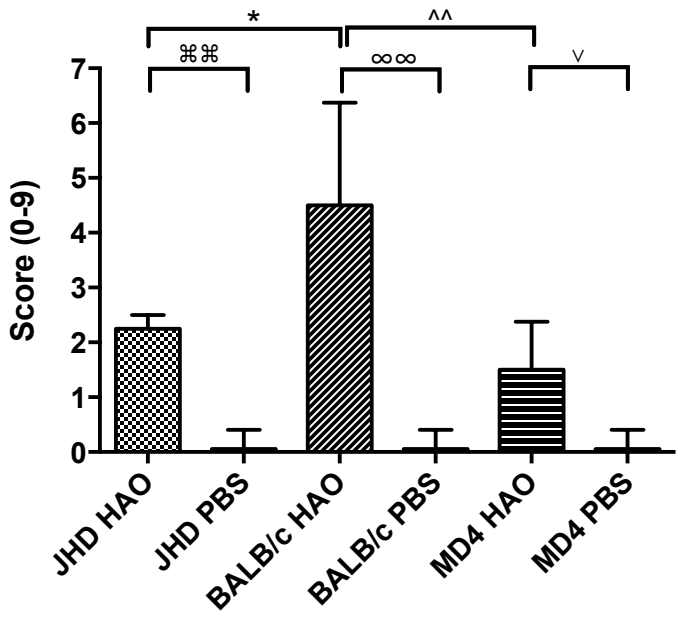

Figure 4.4 Joint histopathology score in BALB/c, MD4 and JHD mice

The joint histopathology score was higher in BALB/c mice HAO challenged compared with that of joints from MD4 and JHD mice HAO treated. BALB/c, MD4 and JHD mice HAO challenged exhibited a higher score compared with respective un-challenged mice. ^, BALB/c OVA/HAO *vs* MD4 OVA/HAO; *, BALB/c OVA/HAO *vs* JHD OVA/HAO; ∞, BALB/c OVA/HAO *vs* BALB/c OVA/PBS; ∨, MD4 OVA/HAO *vs* MD4 OVA/PBS; ⌘, JHD OVA/HAO *vs* JHD OVA/PBS. Statistical analysis was performed by Kruskal-Wallis test. Data represent median and IQR (n = 5 mice per group). Similar results were obtained in 3 identical experiments (n = 60 mice in all).

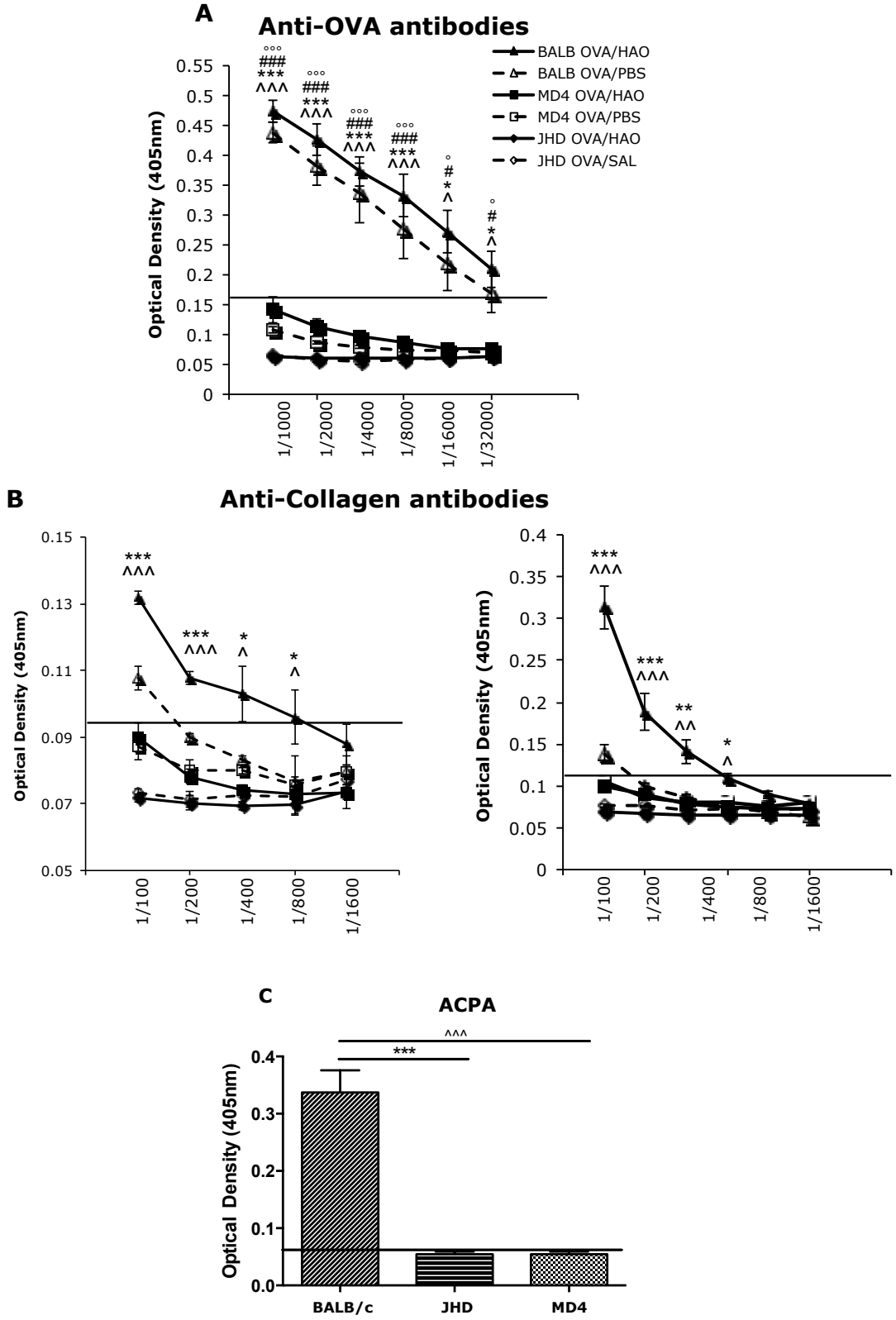

Figure 4.5 Anti-OVA, Anti-Collagen antibodies and ACPA in BALB/c, MD4 and JHD mice

(A) BALB/c mice immunised with OVA and challenged with HAO or PBS produced anti-OVA antibodies compared with respective MD4 and JHD mice. (B) Anti-CII antibodies are shown from two identical experiments. In both B panels BALB/c mice HAO-challenged produced anti-CII antibodies compared with MD4 and JHD mice HAO-challenged. (C) BALB/c mice HAO challenged showed ACPA compared with both MD4 and JHD mice HAO treated. In panel C sera were diluted 1 in 100. ^, BALB/c OVA/HAO *vs* MD4 OVA/HAO; *, BALB/c OVA/HAO *vs* JHD OVA/HAO; #, BALB/c OVA/PBS *vs* MD4 OVA/PBS; °, BALB/c OVA/PBS *vs* JHD OVA/PBS. Statistical analysis was performed by 2-way Anova with Bonferroni's multiple comparisons test. Data represent mean ± SD (n = 5 mice per group). The horizontal line in each panel represents the minimum optical density for each ELISA that is considered valid. Similar results were obtained in 3 identical experiments (n = 90 mice in all).

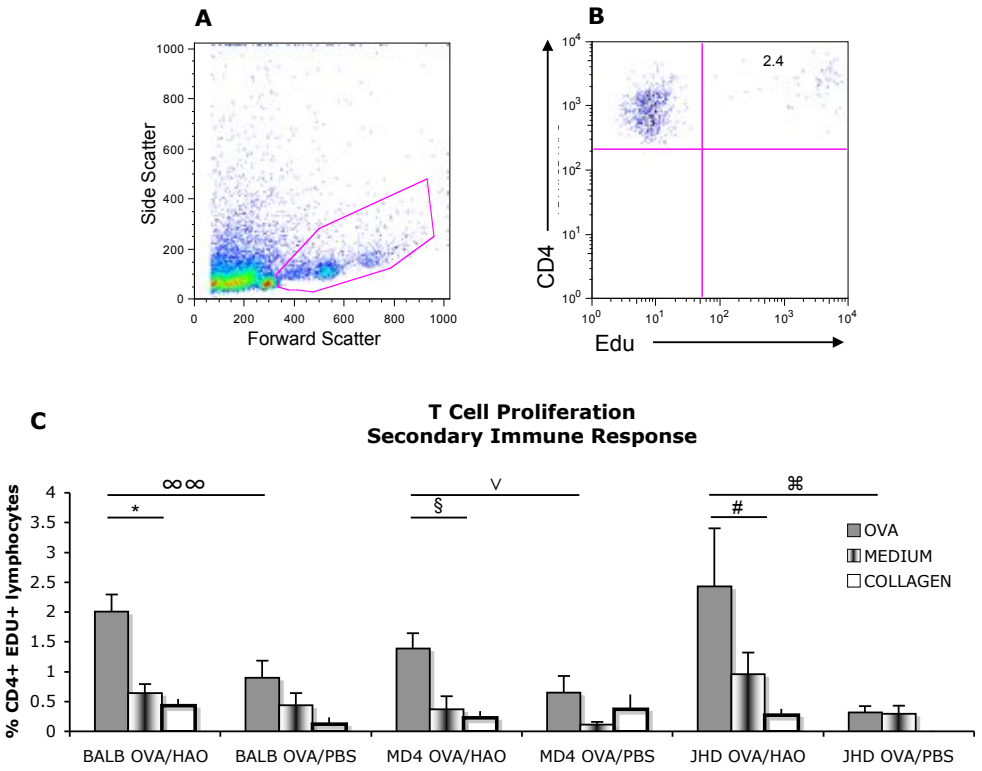

Figure 4.6 Proliferative response of OVA and Collagen-specific T cells in a secondary immune response

Representative flow cytometric plots identify a blastic population of lymphocytes (A) and a population of $CD4^+$ cells stained for Edu (B) following stimulation with OVA. (C) $CD4^+$ T cells from BALB/c, MD4 and JHD animals HAO challenged proliferated after the *in vitro* stimulation with OVA compared with un-stimulated cells (medium). A higher proliferation was observed in all animals HAO challenged compared to that in un-challenged mice. No significant difference was detected in $CD4^+$ T cell proliferation between arthritic BALB/c and MD4 or JHD mice. $CD4^+$ T cell, from all BALB/c, MD4 or JHD mice, stimulated with collagen II did not proliferate. *, BALB/c OVA/HAO: OVA vs MEDIUM; §, MD4 OVA/HAO: OVA vs MEDIUM; #, JHD OVA/HAO: OVA vs MEDIUM; ∞, BALB/c OVA/HAO *vs* BALB/c OVA/PBS; ∨, MD4 OVA/HAO *vs* MD4 OVA/PBS; ⌘, JHD OVA/HAO *vs* JHD OVA/PBS. Statistical analysis was performed by 2-way Anova with Bonferroni's multiple comparisons test. Data represent the mean of triplicate samples ± SD (n = 5 mice per group). Similar results were obtained in 2 identical experiments (n = 60 mice in all).

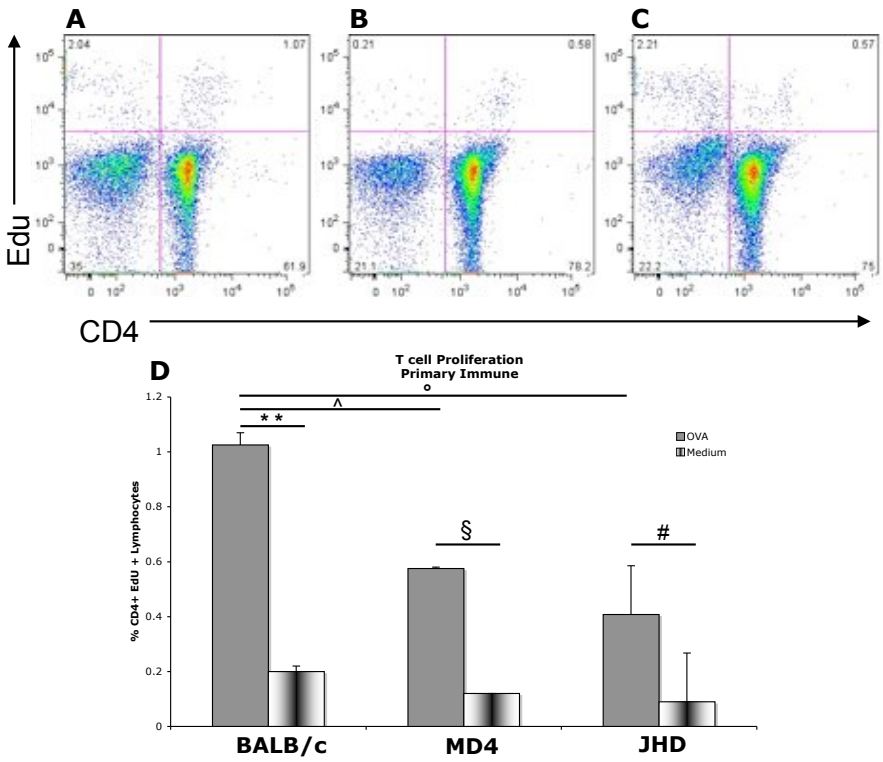

Figure 4.7 Proliferative response of OVA-specific T cells in a primary immune response

Representative flow cytometric plots identify a blastic population of $CD4^+$ cells stained for Edu following stimulation with OVA in BALB/c (A), MD4 (B) and JHD (C) mice. (D) Stimulation with OVA resulted in proliferation of $CD4^+$ T cells from BALB/c, MD4 and JHD mice compared with un-stimulated cells (Medium). A higher proliferation of $CD4^+$ T cells was detected from BALB/c animals compared to that from MD4 and JHD mice. *, BALB/c OVA *vs* MEDIUM; §, MD4 OVA *vs* MEDIUM; #, JHD OVA *vs* MEDIUM; ^ BALB/c OVA *vs* MD4 OVA; ° BALB/c OVA *vs* JHD OVA. Statistical analysis was performed by 2-way Anova with Bonferroni's multiple comparisons test. Data represent the mean of triplicate samples ± SD (n = 3 mice per group). Similar results were obtained in 2 identical experiments (n = 18 mice in all).

Chapter 5

Innate and Antigen-specific pathways to the breach of self-tolerance in OVA-mediated acute arthritis

5.1 Aim and rationale

Some of the experiments described in this chapter, such as the induction of arthritis, the joint immunohistochemistry and the proliferation assay have been performed by Dr. Mohammad Nickdel and by myself. Other experiments, such as the assessment of the antibody production, have been performed only by myself.

In this chapter the contribution of the innate and antigen-specific pathways were analysed in the murine model of OVA-mediated acute arthritis. Indeed, inflammation alone was insufficient to breach T and B cell self-tolerance. In contrast, antigen-specific T cells could induce arthritis together with autoreactive T and B cell responses (365).

The circumstances that precipitate a breach of self-tolerance and lead to autoimmune-mediated synovial inflammation are central to the understanding of the disease and the development of novel therapeutics.

In particular, there is interest in the role of antigen-specific T cell–mediated inflammation in the pathogenesis of rheumatoid synovitis (403-406) leading in turn to autoreactive B cell responses.

Previous work demonstrated that Th1 cells of an irrelevant antigen-specificity were able to break self-tolerance to joint-specific antigens and induce a transient arthritis in BALB/c mice, recapitulating several features of human disease (358). In the previous chapters I demonstrated that arthritis in this model was associated with the generation of RA-related autoantibodies, although pathology could be elicited also in the absence of B cells. Indeed, these responses were dependent on local administration of HAO that could act as both an antigen-specific and/or a general innate inflammatory stimulus. Both mechanisms could in theory promote autoimmunity in RA.

Thus, the aim of this chapter was to dissect the relative contribution of innate inflammation *versus* antigen-specific activation to the breach of T and B lymphocyte self-tolerance and pathology observed in this model. For this purpose OVA-mediated arthritis was induced either by an innate inflammatory stimulus alone (LPS), by an antigen that activate OVA-specific T cells (HAO) or a combination of both (365).

5.2 Introduction

The innate immune system is activated in arthritis. Several experiments in murine models of arthritis and human disease have demonstrated the importance of the innate immune cells in initiating the disease, promoting the adaptive immune responses and antibody production (83). Some of the critical actors of the innate immune system in RA, as previously discussed (sections 1.5.1 and 1.5.2), are summarised and described as follows:

1. Macrophages
2. Dendritic Cells
3. Toll-like receptors
4. Complement system
5. Fc receptors
6. Synovial fibroblasts

Macrophages enrich the RA synovial membrane (87). Their activation in RA results in the expression of chemokines and pro-inflammatory cytokines (83). Of interest, conventional therapies in RA, such as prednisone, Methotrexate, Sulfasalazine, Leflunomide and biologic drugs like Rituximab and anti-TNF decrease the number of macrophages in RA synovial sublining and their cytokine production (407). A significant correlation has also been found between the reduction of macrophages and the clinical improvement measured with the disease activity score (84, 407).

DCs are antigen-presenting cells to T cells, playing a major role in the development of both innate and adaptive immune responses (401). DCs contribute to the inflammatory process by the production of cytokines and differentiating naïve T cells into Th1, Th2 or Th17 cells (408). Both mDCs and pDCs are represented in RA synovial tissue with different status of maturation (105). Synovial fluid of RA patients contains significant number of mDCs and pDCs compared to blood (409). Treatment with Methotrexate and Infliximab in RA patients reduced the number, maturation and function of mDCs and increased the number of peripheral blood pDCs (410-412). In the murine model of OVA-mediated arthritis the depletion of pDCs enhanced the severity of arthritis and the autoreactive B and T responses against CII (362). On the

contrary, in the same murine model the depletion of mDCs decreased the severity of the disease and the autoimmune responses (361). The pathogenic role of DCs has also been confirmed in another murine model of arthritis where the disease was induced by the transfer of collagen-pulsed DCs into susceptible mice (413). New therapeutic strategies in RA are exploiting the "tolerogenic" function of DCs. TolDCs are generated *in vitro* by genetic or pharmacological modification (114). They are characterised by low expression of co-stimulatory molecules, low production of pro-inflammatory cytokines and high secretion of immunosuppressive molecules (114, 409). Of interest, TolDCs demonstrated beneficial effects in murine models of RA (112, 113). Thus, their adoptive transfer into RA patients represents a possible therapy, although several issues regarding their application in human disease (such as the antigen-specificity, the route, the dose and the safety) remain to be addressed (114).

Toll-like receptors are a family of receptors that are expressed on different cell types. Cell surface TLRs include TLR1, 2, 4, 5 and 6, while TLR 3, 7, 8 and 9 are inside the cell on the endosomal membrane (414). The TLR system recognises pathogen-associated molecular patterns (PAMPs) including LPS (TLR4), peptidoglycans (TLR2, 1 and 6), unmethylated CpG DNA motifs (TLR9) from bacteria and RNA (TLR3 and 7) from viruses (414). TLR signals may induce the expression of type I interferons and genes involved in the inflammation, proliferation and protection against apoptosis (415). Several TLRs are expressed in RA joint. For example, synovial fibroblasts and macrophages express TLR2 and 4 (416, 417). Studies *in vitro* demonstrated that microbial TLR ligands might activate synovial fibroblasts and macrophages and increase RANKL expression, pro-inflammatory cytokines, such as IL-6 and CXCL8, and matrix MMPs (65, 157). Endogenous TLR ligands or damage-associated molecular patterns (DAMPs), such as tenascin-C, high-mobility group box chromosomal protein-a (HMGB1) and fragments of hyaluronic acid may be released during the inflammatory process and activate innate immune cells (414). These data support the potential benefit of targeting TLR as a therapeutic approach in RA. In fact, murine models of arthritis, such as CIA and IL-1RA deficient mice, treated with a TLR4 antagonist demonstrated an amelioration of arthritis (418). Chaperonin 10 is a TLR4 antagonist that has been employed in a clinical trial in RA. This treatment appeared safe and efficacious in the short term (419).

The complement system is a key mediator of inflammation in RA (420). It is composed of a family of plasma proteins that opsonizes pathogens and dying cells, enhances phagocytosis and antigen presentation and recruits cells to the area of inflammation (421). ACPA may activate both the classic and alternative pathways of the complement cascade *in vitro* (422). In RA patients peripheral blood levels of C3 and C4 fragments are increased compared with those detected in healthy controls. Of interest, a significant reduction of these levels has been found after anti-TNF treatment (423). On the contrary, in synovial fluid of RA patients a reduction of C3 and C4 fragments has been demonstrated suggesting complement consumption (424). Moreover, levels of C5a and C5b-C9 complex are increased in the blood and synovial fluid of RA patients compared with those detected in osteoarthritis (OA) patients (425). Thus, therapies that target complement proteins may be effective in RA. Eculizumab is a monoclonal antibody that inhibits the cleavage of C5 into C5a and C5b preventing their release and the formation of C5b-C9 complex (426). In a phase II trial a significant clinical improvement in RA patients has been demonstrated (83).

Immune complexes are abundant in RA joints and they are important mediators of the inflammatory process (248). They bind to FcγRs that are capable of activating DCs and macrophages. Increased levels of activating FcγRs have been demonstrated in the plasma and the synovial tissue of RA patients compared with healthy controls (427). Indeed, suppression of FcγR pathway may be another attractive therapeutic strategy in RA.

Activated synovial fibroblasts produce several cytokines, MMPs and chemokines playing a crucial role in the maintenance of inflammation. Indeed, they are responsible for cartilage and bone damage (153, 428).

Despite all of these findings, the exact contribution of the innate immune pathways in arthritis remains unclear.

Thus, the aim of this chapter was to investigate the role of the innate and the adaptive immune responses in OVA-mediated arthritis. For this purpose mice were challenged with LPS, an innate inflammatory stimulus, the antigen HAO, or a combination of both.

5.3 Results

Both the innate immune system and the adaptive immune responses are activated in human RA and represent potential therapeutic targets. The pathology observed in the murine model of OVA-mediated arthritis was strictly dependent on the transfer of antigen-specific T cells (358). However, innate inflammation could contribute to joint pathology. Thus, I aimed to investigate the role of antigen-specific (HAO) and innate inflammation (LPS) in the pathogenesis of OVA-mediated arthritis. For this purpose BALB/c mice that received the transfer of OVA-specific Th1 cells and the immunisation with OVA, were challenged with either HAO, LPS or a combination of both. Control mice received an injection of SAL.

5.3.1 Induction of arthritis

Arthritis was induced in BALB/c mice by adoptive transfer of Tg T cells polarised toward a Th1 phenotype followed by OVA/CFA immunisation and HAO primary challenge. Control mice in the primary challenge received LPS, the combination of HAO and LPS or SAL. Mice were observed in terms of paw thickness and clinical score and killed 7 days after the primary challenge. Mice challenged with HAO, HAO + LPS or LPS displayed a significant increase in the paw thickness and clinical score compared with mice that received SAL. I did not observe any difference in the paw thickness and clinical score between HAO and LPS challenged mice or HAO and HAO + LPS treated mice (Fig. 5.1A-B).

Histology revealed a moderate infiltration of cells and moderate synovial hyperplasia in the joints of mice HAO, HAO + LPS or LPS challenged (Fig. 5.2B-D). Toluidine blue staining showed a mild/moderate loss of staining in joints from mice LPS challenged (Fig. 5.2F) and severe loss of staining with multifocal bone erosions in joints from mice HAO or HAO + LPS challenged (Fig. 5.2G-H). Joints from control mice that received SAL in the primary challenge had no cell infiltration, synovial hyperplasia or cartilage/bone damage (Fig. 5.2A and E).

Joint hisopathology score was similar in mice HAO, HAO + LPS or LPS challenged but higer than that in mice that received SAL (Fig. 5.3).

These data demonstrated that both HAO and LPS could elicit arthritis in this murine model.

5.3.2 Identification of TCR Tg T cells by immunohistochemistry

The adoptive transfer of Tg T cells in recipient mice allows localising the cells by the specific antibody KJ1.26. Using immunohistochemistry I was able to identify KJ1.26$^+$ T cells in the joints of mice challenged with the combination of HAO and LPS (Fig. 5.4D). Few KJ1.26$^+$ T cells were also found in the joints of LPS- or HAO-challenged animals (Fig. 5.4B-C). I was unable to find Tg T cells in the joints of un-challenged mice (Fig. 5.4A). However, the number of KJ1.26$^+$ T cells was not quantified in the different joints, and no statistical test was performed.
These data indicated that OVA-specific T cells could be recruited in the arthritic joints of mice challenged with antigen-specific and/or innate inflammatory stimulus.

5.3.3 Antibody production

Serum samples were collected at day 7 after the primary challenge and analysed for the presence of anti-OVA IgG2a and arthritis-associated antibodies such as anti-CII IgG2a antibodies, RF IgG2a, ANA and anti-single stranded (ss) DNA IgG2a.
All animals showed anti-OVA antibodies because they received the immunisation with OVA/CFA. Mice that received in the secondary challenge HAO or HAO + LPS displayed higher levels compared with those that received LPS or SAL (Fig. 5.5A).
Anti-CII antibodies were demonstrated in mice HAO + LPS or HAO –challenged compared with mice LPS-challenged or un-challenged mice (Fig. 5.5B). Likewise, RF was detected in the serum of mice treated with HAO + LPS or HAO compared with mice that received LPS or SAL (Fig. 5.5C).
I next investigated the presence of ANA that may be detected in human arthritis. They can be found in several autoimmune diseases like RA and SLE (222). Different nuclear staining patterns, performed on Hep2 cell lines, can be associated with different diseases. For example, a homogenous nuclear staining is characteristic of

anti-DNA or anti-histone antibodies. It is important to distinguish nuclear from cytoplasmic staining because the latter is less frequent and specific of disease. ANA were detected by indirect cellular immunofluorescence on fibroblast cultures using sera from all mice as previously described (429) (Fig. 5.6A-D). The fluorescence pattern was restricted to nuclei as revealed comparing FITC with DAPI staining. Fluorescence was not detected in the cytoplasm of fibroblasts (Fig. 5.7A-B). A staining was found in the sera of mice challenged with HAO + LPS (Fig. 5.6A) and HAO alone (Fig. 5.6B). No staining was observed on fibroblast nuclei incubated with sera from LPS-challenged (Fig. 5.6C) and un-challenged mice (Fig. 5.6D). Serial dilutions of representative sera from mice HAO-challenged with arthritis were performed and the staining was demonstrated at the dilution of 1/20 (Fig. 5.8A), 1/40 (Fig. 5.8B). A mild staining was detected when sera were diluted 1 in 80 (Fig. 5.8C). I didn't detect any staining at the dilution of 1/100 (Fig. 5.8D).

Since ANA are a family of antibodies with different specificities, they needed to be further characterised. Among these, anti-ssDNA antibodies were assessed by ELISA. Anti-ssDNA antibodies may be detected in autoimmune disease and they are less specific than anti-dsDNA for the diagnosis of SLE. Of interest, I detected only in the sera from mice challenged with HAO + LPS or HAO alone compared with mice treated with LPS or un-challenged (Fig. 5.5D). Since I demonstrated the presence of different autoantibodies in mice challenged with HAO ± LPS, I hypothesized that B cells could be polyclonally activated by the cytokine milieu in a non-antigen specific manner and therefore produce antibodies directed toward different specificities. Thus, I investigated the presence of antibody against an irrelevant antigen, KLH, in the sera of these mice. Interestingly, I could not detect anti-KLH antibodies in the sera from challenged or un-challenged mice (Fig. 5.5E).

Finally, some mice received in the primary challenge HABSA instead of HAO to further investigate if the production of anti-CII antibodies was stimulated only by the antigen HAO. Indeed, anti-CII antibodies were detected in the serum from mice HAO-challenged compared to that from mice HABSA-challenged (Fig. 5.5F).

These data demonstrated that antigen-specific stimulation of T cells of an irrelevant specificity resulted in autoantibody production compared with that induced by innate inflammation.

5.3.4 Proliferative response of OVA and Collagen-specific T cells

$CD4^+$ T cells proliferation in response to OVA and CII stimulation was assessed in BALB/c mice that received in the primary challenge HAO + LPS, HAO, LPS or SAL. Stimulation with OVA resulted in $CD4^+$ T cell proliferation from mice challenged with HAO and HAO + LPS compared with those from mice LPS challenged and un-challenged mice (SAL). On the contrary, no significant difference was observed in proliferation of $CD4^+$ T cells between mice challenged with HAO + LPS and those treated with HAO alone (Fig. 5.9A).

Stimulation of draining LN cells with CII resulted in significant $CD4^+$ T cell proliferation in mice that received HAO + LPS compared with those from mice challenged with HAO, LPS or SAL (Fig. 5.9B).

These results demonstrated that Ag-specific stimulation of T cells of an irrelevant specificity, in the presence of a second inflammatory stimulus, could induce the breach of self-tolerance with autoreactive T cell proliferation. In contrast, innate inflammation alone was insufficient to breach T cell self-tolerance.

5.4 Discussion

In this chapter the relative contribution of the innate and the antigen-specific pathways to the pathology observed in OVA-mediated acute arthritis was analysed. Innate inflammation could induce experimental arthritis but was insufficient to breach T and B cell self-tolerance. In contrast, the stimulation of OVA-specific T cells led to arthritis and the production of autoreactive T and B cells.

Current biologic therapies indirectly modulate T cells renewing the interest in this cell population in RA (430, 431). However, there are several unknowns regarding their specificity, the phenotype and function, their activation in an antigen-specific or innate inflammatory environment, the recruitment to the joint and the T-cell-dependent autoantibody production (431). Moreover, the innate immune system is activated in RA (83). Indeed, both the components of the innate immune system and T cells are potential therapeutic targets.

Therefore, the aim of this chapter was to compare the role of Ag-specific *versus* innate stimulation in the pathogenesis and the breach of self-tolerance detected in the murine model of OVA-mediated arthritis.

For this purpose experimental arthritis was induced by an inflammatory stimulus, such as LPS, HAO that stimulate Tg OVA-specific T cells or a combination of both.

Both the innate inflammatory mediator LPS and the specific antigen HAO were able in the primary challenge to induce a transient acute arthritis assessed by the increased paw thickness, clinical score and the joint histopathology without any significant difference. Of interest, the challenge with HAO and/or LPS was associated with the accumulation of OVA-specific T cells in the arthritic joints. One of the limits of this experiment is that the number of Tg T cells was not assessed in the different joints and it is not possible to definitely correlate their finding with the arthritis in LPS and HAO treated mice.

In contrast to the clinical and histopathology findings, the autoreactive response was different in LPS and HAO challenged mice. LPS-challenge had no effect on autoantibody responses; while HAO-challenge was associated with the production of different autoantibodies such as anti-CII, RF, ANA and anti-ssDNA antibodies, all immunological features of human autoimmune diseases (403). It would be interesting to confirm the ANA detection with Hep2 cells because they are the standard method

of detection, and to further characterise ANA looking for anti-dsDNA and antibodies to extractable nuclear antigens. Moreover, future experiments should verify the presence of immune complex kidney deposition because anti-DNA antibodies may be associated with them.

The combination of HAO + LPS did not induce a more severe arthritis in terms of joint histology but induced CII-specific T cell proliferation that was not evident in mice LPS-challenged. Moreover, mice treated HAO + LPS showed an increase in the autoantibodies levels, suggesting that an innate inflammatory stimuls may amplify the autoimmune response without apparent change in the pathology observed.

It has been previously demonstrated that in CIA treatment of mice with LPS without specific Ag (CII) over 140 days failed to induce arthritis. Both CII and LPS were required for the development of arthritis, and the enhanced levels of antibodies as well as the cytokines induced by LPS alone were insufficient to induce arthritis (382). In RA there are several lines of evidence of the permanent activation of the innate immune system as demonstrated for example by the expression of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1 (4). Innate immune cells, such as macrophages and DCs, are activated by the presence of pattern recognition receptors (PRRs) that recognise molecular structures conserved during evolution PAMPS and DAMPs (83). The TLR system recognises several PAMPS including LPS through TLR4 (414). TLR4 signals are mediated by MyD88 and TRIF that lead to the activation of NF-κB and the MAP kinases. The latter are involved in the transcription of genes related to inflammation, proliferation and suppression of apoptosis (414). In RA synovial tissue macrophages and fibroblasts express TLR2 and 4 compared with levels expressed in OA (416, 432). In addition, synovial fluid and peripheral blood monocytes from RA patients express TLR2 and 4 compared to negative controls (416). A number of studies have demonstrated TLR-dependent amplification of autoimmune arthritis (414). Thus, data from literature and our experiments seem to suggest that TLR driven responses alone are unable to create the necessary environment for initiation of autoreactivity. This has implications both for antigen-specific therapeutics aimed at tolerance induction and for PRR targeted therapy e.g. Chaperonin 10 (433) that seek inflammation suppression.

The more interesting aspect of the model of OVA-mediated arthritis is the generation of autoimmunity rather than the joint histopathology that reveals only a mild self-

limiting monoarthritis. The breach of self-tolerance is one of the most intriguing unexplained mechanisms in human autoimmune diseases. We have the possibility to study this aspect in a murine model where autoimmunity was elicited with a sophisticated protocol employing an irrevant antigen (such as OVA) but in complete absence of self antigens (such as collagen). The site of injection (systemic *versus* local) and the nature of the antigen (HAO *versus* HBSA or LPS) are important in this model for both the joint pathology and the autoreactive responses. Other animal models of RA rely on this approach of local immunisation protocols, such as AIA with mBSA (380, 434, 435) or zymosan-induced arthritis, the last one caused by an intrarticular injection of zymosan (436, 437). The issue that the site of injection is important to generate the autoimmune response is of great interest since in human disease the first events surrounding the breach of self-tolerance are not known.

Of interest, autoantibodies were isotype switched indicating that this B cell response was T-cell-dependent. However, how OVA-specific T cell may help collagen-specific (or IgG, ssDNA) B cell is unknown. Several hypotheses were developed such as the possibility that local damage caused the exposition of self antigens which could then activate autoreactive T and B cells. However, LPS caused the same joint damage that could create an environment responsible for the release of self antigens rendering this hypothesis unlikely. Otherwise, OVA-specific T cell might help collagen-specific B cell in a bystander manner generating locally a cytokine milieu or autoreactive B cells could acquire OVA non-specifically because it was associated with a self antigen in a 'hapten-carrier' manner (438). Such mechanisms might be relevant for B cells with RF reactivity that could bind and internalise antigens (self or foreign) in the context of an immune complex and present them to T cells while reciprocally receiving their help (270).

In this chapter I demonstrated that innate inflammatory stimulus alone was associated with joint pathology but not with T and B autoreactive responses. This mechanism could be similar to that one responsible for reactive arthritis that differs deeply from autoimmune arthritis. However, T and B cell responses were mildly amplified by the innate inflammatory stimulus in combination with HAO. Joint histology in human RA is neither specific nor diagnostic although lots of efforts in recent years tried to characterise histological modifications of synovitis during the treatments. Nevertheless, the study of the alterations of immune-mediated pathways in RA allowed generating new therapeutic interventions. In this regard murine models

provide invaluable tools to understand the events underlining immunological pathways leading to autoimmunity.

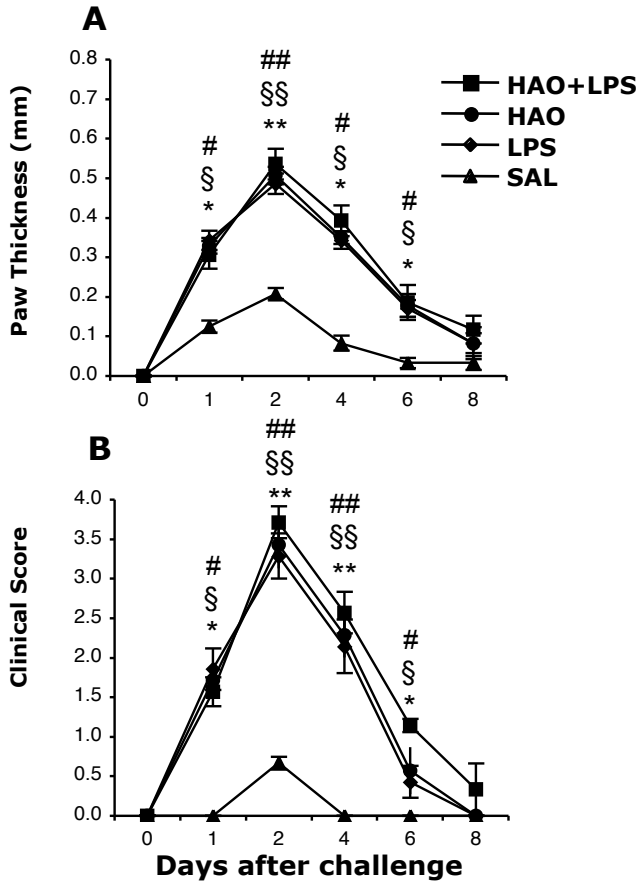

Figure 5.1 Effect of innate versus antigen-specific challenge on clinical signs of arthritis in mice

Hind paw thickness (A) and mean clinical score (B) in BALB/c mice challenged with LPS, HAO, a combination of HAO and LPS or sham injected with saline (SAL). There was a significant difference in both paw swelling (A) and clinical scores (B) between mice challenged with HAO, LPS, or both and those injected with SAL. There was not significant difference in paw swelling and clinical scores between mice challenged with HAO + LPS and HAO. *, HAO + LPS *vs* SAL; §, HAO *vs* SAL; #, LPS *vs* SAL. Statistical analysis was performed by 2-way Anova with Bonferroni's multiple comparisons test. Data represent mean ± SEM (n = 5 mice per group). Similar results were obtained in 3 identical experiments (n = 60 mice in all) that were performed in conjunction with Dr Nickdel.

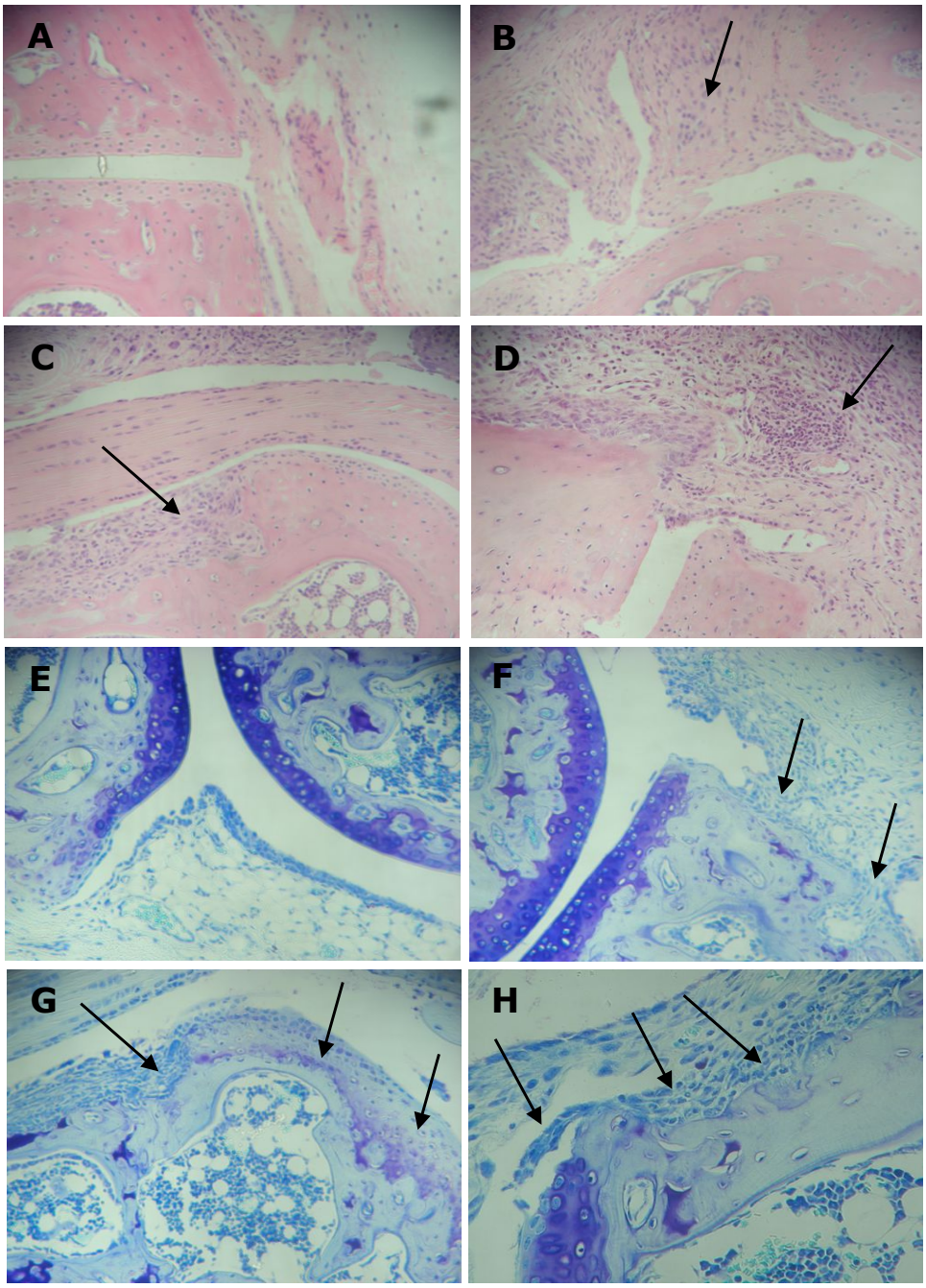

Figure 5.2 Effect of innate versus antigen-specific challenge on joint histopathology in mice

Representative haematoxylin and eosin (A-D) and toluidine blue (E-H) -stained sections prepared from the joints of recipient mice challenged with saline (A, E), LPS (B, F), HAO (C, G), or HAO + LPS (D, H). Moderate cell infiltration and synovial hyperplasia were observed in the joints from mice challenged with the combination of HAO and LPS (D). Joints from mice that received LPS (B) or HAO (C) challenge displayed mild cell infiltration and synovial hyperplasia, while those that received saline exhibited no local inflammation (A). Severe bone erosions were detected by

loss of toluidine blue staining in the joints of mice challenged with combination of HAO and LPS (H). Joints of mice challenged with either LPS (F) or HAO (G) exhibited mild/moderate loss of toluidine blue staining and joint erosions. The joints of mice that received saline displayed normal cartilage/bone integrity (E). Black arrows indicate the sites of inflammation and joint damage. Original magnification X20, except in panel H original magnification X40. Images shown are representative of 3 identical experiments (n = 5 mice per group and 60 mice in all).

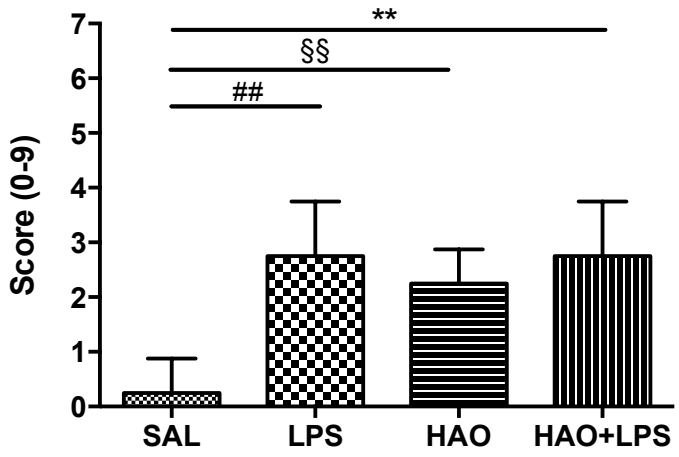

Figure 5.3 Joint histopathology score in mice following innate versus antigen-specific challenge

Histopathology score of joints from mice un-challenged (SAL), challenged with LPS or HAO alone or a combination of HAO + LPS. The histopathology score was similar in challenged mice (LPS, HAO, HAO + LPS) but it was higher when compared with un-challenged animals (SAL). *, HAO + LPS *vs* SAL; §, HAO *vs* SAL; #, LPS *vs* SAL. Statistical analysis was performed by Kruskal-Wallis test. Data represent median and IQR (n = 5 mice per group). Similar results were obtained in 3 identical experiments (n = 60 mice in all).

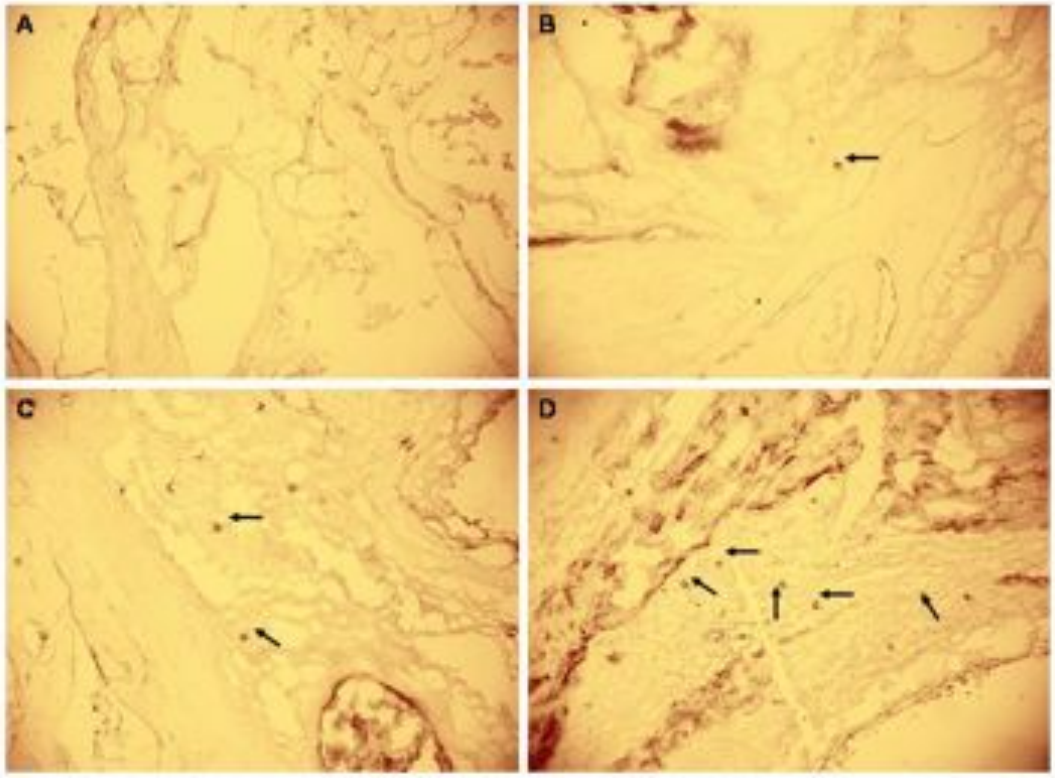

Figure 5.4 Detection of transgenic OVA-specific T cells in joints following innate or antigen-specific challenge

Representative immunohistochemical staining of the ankle joint sections prepared from the joints of recipient mice challenged with saline (A), LPS (B), HAO (C), or HAO + LPS (D). KJ1.26^{+} T cells were detected in the joints of mice challenged with HAO + LPS (D). This was also evident in mice challenged with HAO (C). Few KJ1.26^{+} T cells were detected in the joints of LPS-challenged animals (B). No KJ1.26^{+} T cells were found in un-challenged mice (A). Black arrows indicate KJ1.26^{+} T cells in the ankle joints. Original magnification X20. Images shown are representative of 3 identical experiments (n = 5 mice per group and 60 animals in all) that were performed in conjunction with Dr Nickdel.

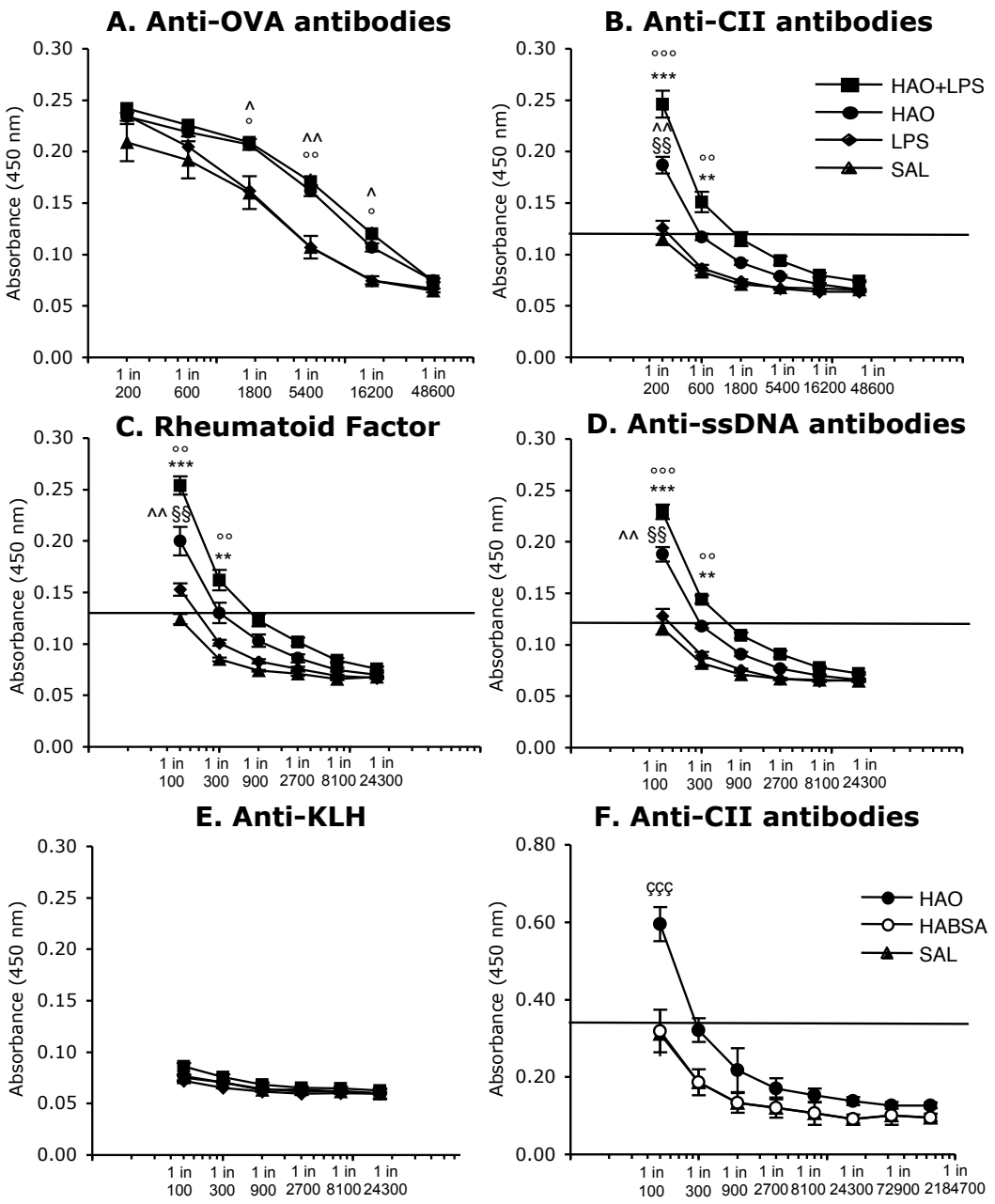

Figure 5.5 Anti-OVA, Anti-type II collagen, Rheumatoid Factor, anti-ssDNA and anti-KLH antibodies following innate or antigen-specific challenge

Anti-OVA, anti-CII, RF, anti-ssDNA, anti-KLH antibody levels were measured in the sera of mice challenged with Saline, LPS, HAO, HAO + LPS or HABSA (anti-CII only) by ELISA. (A) All mice immunised with OVA produced anti-OVA antibodies, with the highest levels in mice challenged with HAO or HAO + LPS compared with LPS-challenged mice. Mice challenged with HAO + LPS showed significant levels of anti-CII (B), RF (C) and anti-ssDNA antibodies (D) compared with LPS-challenged and un-challenged mice. Challenge with HAO alone induced anti-CII, RF and anti-ssDNA at levels that were greater than those in mice either challenged with LPS

alone, or un-challenged mice. Anti-KLH IgG2a antibodies (E) were not revealed in the sera of challenged and un-challenged mice. (F) Anti-collagen antibodies were measured in some mice challenged with HABSA. No production of significant levels of anti-collagen antibody was observed in these mice compared with un-challenged mice. ^, HAO *vs* LPS; °, HAO + LPS *vs* LPS; *, HAO + LPS *vs* SAL; §, HAO *vs* SAL; ç, HAO *vs* HABSA. Statistical analysis was performed by 2-way Anova with Bonferroni's multiple comparisons test. Data represent mean ± SEM (n = 5 mice per group). The horizontal line in the panels represents the minimum optical density for each ELISA that is considered valid. Similar results were obtained in 3 identical experiments (n = 60 mice in all).

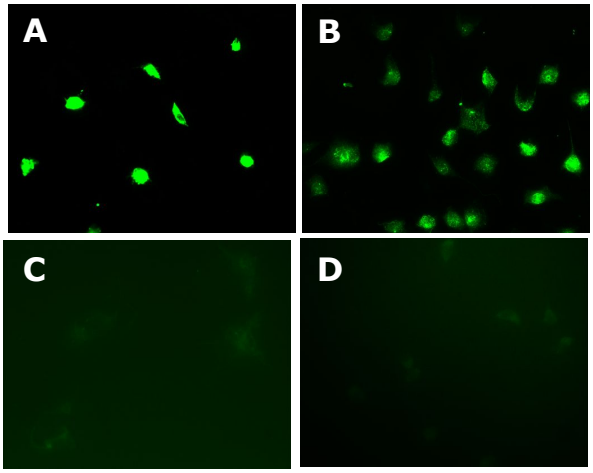

Figure 5.6 Anti-nuclear antibodies following innate or antigen-specific challenge

Intracellular staining on fibroblast cultures, using sera from BALB/c mice following challenge with HAO + LPS, HAO, LPS or saline. Sera of mice challenged with HAO + LPS (A) or HAO (B) demonstrated a considerable reaction to nuclear components of fibroblast cells. No staining was observed on fibroblast incubated with sera from LPS challenged (C) and un-challenged mice (D). Original magnification X40. Similar results were obtained in 3 identical experiments.

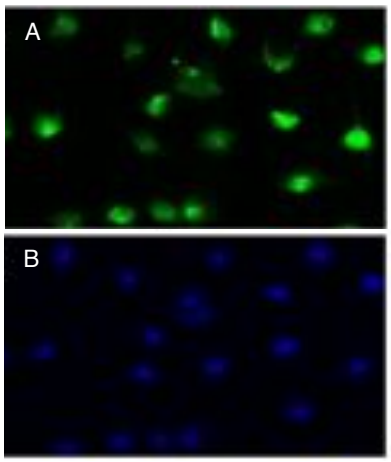

Figure 5.7 Anti-nuclear antibodies detected by intracellular staining on fibroblast cultures

Intracellular staining on fibroblast cultures, using sera from BALB/c mice HAO challenged. The fluorescence pattern was restricted to nuclei as revealed comparing FITC (A) with DAPI staining (B). Fluorescence was not detected in the cytoplasm of fibroblasts. Original magnification X40. Similar results were obtained in 3 identical experiments.

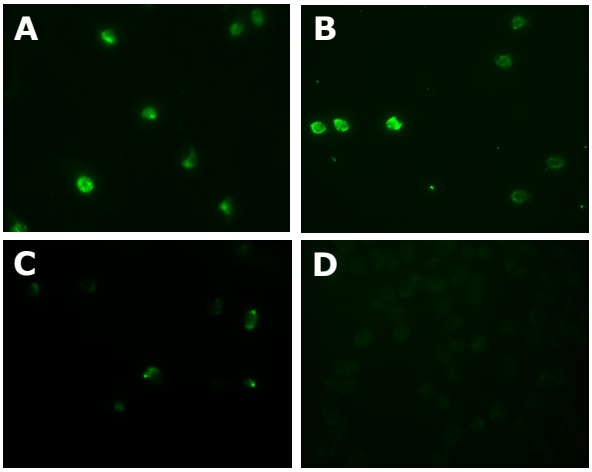

Figure 5.8 Titre of anti-nuclear antibodies detected by intracellular staining on fibroblast cultures

Intracellular staining on fibroblast cultures using serial dilutions of sera from BALB/c mice HAO-challenged. The staining was observed when serum was diluted 1 in 20 (A) 1 in 40 (B) and 1 in 80 (C). No staining was observed at the dilution of 1/100 (D). Original magnification X40. Similar results were obtained in 3 identical experiments.

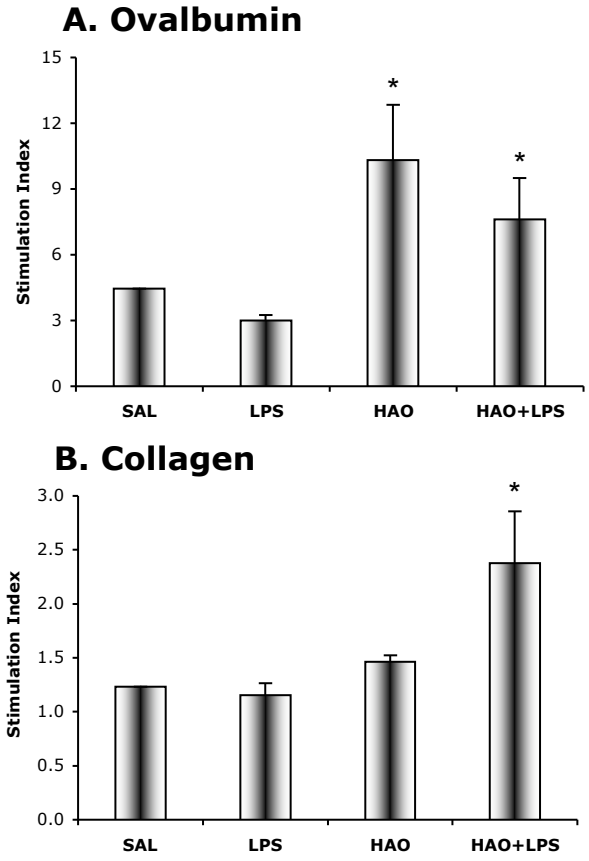

Figure 5.9 Effect of innate versus antigen-specific challenge on in vitro proliferative response of OVA- and CII-specific T cells

$CD4^+$ T cell proliferation in response to OVA and CII antigens was assessed by incorporation of tritiated thymidine from mice challenged with HAO + LPS, HAO, LPS, or saline. Stimulation with OVA (A) resulted in a higher proliferation of cells from mice challenged with HAO ± LPS compared to those from LPS-challenged or un-challenged mice. Mice challenged with HAO + LPS and mice challenged with HAO alone exhibited a similar proliferation of lymphocytes. (B) A higher proliferation of lymphocytes was observed from mice challenged with HAO + LPS compared with those from HAO-challenged, LPS-challenged or un-challenged mice after CII stimulation. Statistical analysis was performed by 2-way Anova with Bonferroni's multiple comparisons test. Data represent the mean of triplicate samples ± SEM (n = 5 mice per group). Similar results were obtained in 3 identical experiments (n = 60 mice in all) that were performed in conjunction with Dr Nickdel.

Chapter 6

Summary, Conclusions and Future Work

As highlighted in chapter 1, RA is a chronic autoimmune disease associated with progressive disability, systemic complications and socioeconomic costs (6). The innate and adaptive immune pathways contribute to the initiation and maintenance of the disease inducing the breach of self-tolerance and promoting tissue inflammation, joint remodelling and damage. B cells are critical in these processes as suggested by the presence of antibodies many years before the onset of the disease, their ability to present antigen efficiently to $CD4^+$ T cells, produce pro-inflammatory cytokines, participate to synovial ectopic lymphoneogenesis and by the efficacy of B cell depletion treatment (297). However, the fine role of these pathways is not well defined. Animal models of arthritis are invaluable tools to generate new knowledge in RA pathogenesis and validate innovative drugs (366). Previous studies established a model of acute autoimmune arthritis where antigen specific T cells drive the breach of self-tolerance and the pathology (358).
This thesis aimed to develop a novel experimental model of autoimmune chronic polyarthritis that would allow investigating the requirement of B cells in the induction of experimental arthritis and dissecting the contribution of innate inflammation *versus* antigen-specific activation to the pathology. A better understanding of the relative involvement of the innate and adaptive immune pathways will hopefully help to design novel therapeutic strategies directed against components of the innate and adaptive immune systems and aiming to re-establish immunological tolerance.

The first objective of this thesis was to create a new model of chronic autoimmune polyarthritis where the B cell responses could be assessed and characterised. The experimental model of OVA-mediated arthritis was employed because it is ideal to study autoimmunity. One of the major limitations of this model was that arthritis was mild, acute and self-limiting. Unfortunatley, I was unable to induce chronic autoimmune polyarthritis, however, I induced a severe relapse of arthritis that was localised to more than four joints in the injected paw and lasted for three weeks. The flare of arthritis was dependent on the antigen HAO in adjuvant and the route of administration. The exact nature of antigen in RA is unknown even if citrullinitated proteins seem to represent the main target of the autoimmune response. The first events that cause the breach of self-tolerance are not fully elucidated; mechanisms such as molecular mimicry with foreign antigens have been hypothesized. In our experimental model the first episode of arthritis was elicited by OVA antigen in the

context of a Th1 environment. It is peculiar that only the same antigen (HAO and not collagen) was able to induce the relapse of the disease. I can speculate that in the secondary challenge the exposition to the OVA antigen may elicit an immune response that caused a more severe arthritis than the first one. Future experiments should aim to follow the model of relapsing arthritis long enough to verify the chronicity and possibly induce arthritis at distant sites from injection.

Human RA is a chronic autoimmune inflammatory disease, thus a reliable animal model should recapitulate chronicity and autoimmunity to offer the possibility to study the pathways that lead to the breach of tolerance and the maintenance of disease. The relapse of OVA-mediated arthritis was associated with the production of autoantibodies such as anti-collagen antibodies. However, this experimental model of arthritis relies on a sophisticated protocol of Tg Th1 cells adoptively transferred in recipient mice followed by OVA immunisation, primary HAO challenge and secondary challenge; thus several factors may influence the breach of self-tolerance. Therefore, it would be important in future experiments to employ a control group of animals without the adoptive transfer of Tg Th1 cells in order to address the role of the secondary challenge in the autoreactive B cell response. The collagen specific antibody response, assessed during the induction of the relapse of arthritis, was compared with that in CIA model as a "gold standard" model of human RA. Interestingly, after the initial breach of self-tolerance, anti-CII antibody titres rise despite arthritis disappeared after the acute phase. This aspect is intrugiung since in human RA autoimmunity may appear many years before the onset of the joint disease and may be linked to the development of the chronic course of pathology. The analysis of collagen-specific B cell response revealed that anti-CII antibodies in OVA mediated arthritis and CIA mice recognised different epitopes. The major epitope in OVA-mediated arthritis has been identified in U1, while anti-CII antibodies from CIA mice recognised also J1 and C1 epitopes. Some of these epitopes are shared between human RA and murine arthritis, such as U1 and C1. Anti-U1 antibodies can bind to the intact rat cartilage matrix *in vivo* showing that the epitope is exposed to the immune system for immune complex formation in the joint (386). In future studies it would be interesting to test whether anti-U1 antibodies bind also human RA cartilage *in vitro*, this would demonstrate that the epitope U1 is not only shared between different species but also that is localised in the joint, the main tissue target of the disease. Moreover, I would verify if the injection of monoclonal antibody against U1

could induce a relapse of arthritis in OVA-mediated arthritis. This data ideally would reinforce the hypothesis that the epitote U1 is arthritogenic. The identification of the B cell epitopes on CII is relevant because it may reveal the dominant target structure of cartilage directed autoimmunity occurring in RA (386, 439). Moreover, it will hopefully help to understand if anti-CII antibodies are involved in the pathogenesis of autoimmune arthritis and whether/how they can cause the destruction of their target tissue. In conclusion, the results presented in chapter 3 provided a novel tool that will allow researchers to investigate cells and molecules involved in the induction of relapse of arthritis.

The production of autoantibodies observed in OVA-mediated arthritis does not clarify if B cells or antibodies contribute to the induction of arthritis or are only epiphenomena. To fully address the requirement of B cells in the induction of pathology, arthritis was elicited in recipient animals with different B cell repertoire: BALB/c mice with normal B cells, MD4 mice with B cells that could not present antigen to CD4+ T cells and produce antibodies (370), and JHD mice characterised by the absence of mature B cells (371). Chapter 4 shows that acute mild arthritis, investigated by joint histology, could be elicited in absence of antigen-specific B cells and in complete absence of mature B cells. Analysis of serum from MD4 and JHD arthritic mice confirmed that autoantibodies were not required for the induction of pathology. In fact, while BALB/c mice HAO-challenged produced ACPA and anti-CII antibodies, MD4 and JHD mice did not.

The next step was to analyse $CD4^+$ T cell proliferation from mice with a different B cell repertoire in the context of a primary immune response and during arthritis. As mentioned in chapter 4, previous studies in murine models showed contradictory data concerning the importance of B cells in T cell priming and pathology. The results presented in this thesis revealed that T cells primed in the absence of B cells could proliferate in response to antigen. However, in a primary immune response a higher proliferation of $CD4^+$ T cells was observed in the presence of normal B cell repertoire. Thus I can speculate that targeting B cells with current therapeutic strategies may partially affect $CD4^+$ T cell proliferation. It would be interesting in future experiments to verify the relative contribution of Tg $CD4^+$ T cells and host $CD4^+$ T cells to the proliferation observed during the induction of OVA-mediated arthritis.

It was concluded that B cells were not necessary in the induction of OVA-mediated arthritis, however their presence was associated with a higher joint histopathology score that was probably related to their pleiotropic functions. As previously mentioned the induction of the flare in OVA-mediated arthritis was associated with an increased autoreactive B cell response. Thus, future studies should investigate the B cell requirement during the relapse of arthritis.

As outlined in chapter 1, another objective of this thesis was to prove the relative contribution of innate and adaptive immune responses in OVA-mediated arthritis. Several studies demonstrated that the innate immune system is activated in RA (83). Infections have been implicated in triggering inflammation. TLRs are central players in sensing infections recognising PAMPS, including LPS, and activating synovial fibroblasts and macrophages (414). They may represent important targets for therapeutic intervention. HSP10/chaperonin 10 is a negative regulator of TLR4 signalling, suppressing cytokine production in cells derived from RA synovium (440). Indeed, the recombinant analogue of chaperonin 10, XToll, is being tested in a phase II clinical trial for RA and it seems well tolerated and efficacious in the short term (419).

Thus, experimental arthritis was induced either by an innate inflammatory stimulus, such as LPS, by an antigen that activated OVA-specific T cells (HAO) or a combination of both. Therefore, Chapter 5 outlines that LPS alone elicited an inflammatory process localised in the joint but was insufficient to breach B and T self-tolerance. On the contrary antigen-specific activation could induce both arthritis and the production of several antibodies such as RF, anti-CII, ANA and anti-DNA. The detection of anti-DNA antibodies in human disease is associated with SLE where they can form immune complexes and deposit in the kidneys. Anti-DNA antibodies can also be detected in RA patients after anti-TNF treatment in complete absence of lupus-like symptoms (441). Thus, it would be interesting to assess immune complexes deposition in kidneys of OVA-mediated arthritis mice.

Although the activation of autoreactive B and T cell responses was strictly dependent on antigen-specific stimulation, the combination of HAO and LPS caused a marked autoimmune response.

It would be interesting in future studies to test the role of TLR4 in OVA-mediated arthritis. Its expression in the synovial membrane would give new insights on the role

of the innate immune pathway in the acute and relapsing model of arthritis. The next step would be blocking *in vivo* TLR4 signalling using soluble TLR4 (442) or MD-2 mimetic peptide that interacts and inhibits TLR4 (443). The monoclonal antibody NI-0101, developed by NovImmune, has also been found to inhibit TLR4 activation (http://www.novimmune.com). Treatment of CIA with a TLR4 antagonist suppressed clinical and histological signs of arthritis without influencing the adaptive anti-CII response (418). Moreover, it could be important to investigate other TLRs, such as TLR2. Interestingly, in a murine model of arthritis, SCW model, the acute phase of arthritis was TLR2 dependent, while in the chronic phase TLR4 activation contributed to cartilage damage and bone destruction by inducing MMP release and activating osteoclasts (444). In a recent study blockade of TLR2 was found to inhibit the spontaneous release of inflammatory cytokines by intact RA synovial explant cultures (445).

The circumstances that precipitate a breach of self-tolerance are central to the understanding of the first events occurring in RA. However, not all RA patients exhibit autoantibodies before and during RA. Thus, it is possible to speculate that different mechanisms underpin the induction and development of disease in different patients. Subsets of patients, seronegative and seropositive patients affected by either acute or chronic arthritis, could benefit of different therapeutic strategies. It would be useful to identify which patients will take advantage from targeting either component of the innate and adaptive immune pathways. Moreover, it would be useful to identify patients in the pre-clinical phase of the disease, such as for example seropositive persons carrying one or more risk factors for the development of RA (smoking or a particular genotype). This could allow adopting a primary prevention consisting of measures taken to prevent disease such as stop smoking, treat/prevent comorbidities, such as infections (vaccines?), periodontitis, hypercholesterolemia and obesity.

Indeed, based on data from this thesis, it could be possible that T-B cell interactions in a secondary lymphoid organ led to the breach of self-tolerance with the induction of a systemic autoimmune response. However, this was not sufficient to induce joint pathology since a local stimulus such as HAO was required to obtain an acute arthritis. B cells in this phase were required for the autoimmune response but not for the induction of arthritis, as demonstrated in chapter 4. Nevertheless, their presence

was associated with a higher joint histopathology score compared with that in mice without antigen presenting B cells, autoantibodies or mature B cells. On the other side, the stimulation of the innate pathway could cause the induction of joint pathology but not the autoimmune response, as demonstrated in chapter 5. The activation of both innate and adaptive immune systems could elicit arthritis with an increased autoimmune response.

In conclusion, in this thesis it was elicited a relapse and spread of arthritis, in terms of more joints involved only in the injected paw, in the model of acute autoimmune OVA-mediated arthritis. Moreover, B cell requirement was investigated in the induction of the acute phase of arthritis. In addition, in this thesis the contribution of innate inflammatory stimulus to the breach of self-tolerance was dissected. This model, with some future development, will provide a useful tool to dissect the contribution of both the adaptive and innate immune pathways during the development of the chronic phase of arthritis. Understanding the roles of both the innate and adaptive immune responses hopefully will help to design new therapeutic strategies that could be validated in the autoimmune model of OVA-mediated arthritis.

6.1 Future Work

It would be interesting to try to reply to the following questions:

Is the model of relapsing OVA-mediated arthritis chronic?

- To verify the chronicity of the model: adoptive transfer of Tg Th1 cells in BALB/c mice, immunisation with OVA/CFA, primary challenge with HAO, local secondary challenge with HAO+IFA. Assess paw thickness, clinical score, joint histology and autoantibodies at different time points for the animals life-span.

Are Tg DO11.10 T cells that I adoptively transferred in recipient mice detected in arthritic joints from mice with a relapse of arthritis?

- To verify the presence of Tg T cells in joints from arthritic mice and characterise the infiltrate in terms of the presence of T cells, B cells, neutrophils, DCs by IHC.

Are Tg DO11.10 T cells that I adoptively transferred in recipient mice those cells that cause the autoreactive B cell response in the model of relapsing OVA-mediated arthritis?

- To employ a control group of animals without the adoptive transfer of Tg Th1 cells in order to address the role of the secondary challenge in the autoreactive B cell response.

May Tg DO11.10 T cells that I adoptively transferred in recipient mice cause the autoreactive T cell response in the model of relapsing OVA-mediated arthritis?

- To analyse the T cell proliferation after CII stimulation *in vitro* by incorporation of tritiated thymidine.
- To verify the source (host or donor) of proliferating CII-specific T cells *in vitro* employing the marker KJ1.26 that identifies Tg T cells from DO11.10 mice.

Is the U1 epitope arthritogenic?

- To induce a relapse of arthritis in OVA-mediated arthritis by the injection of monoclonal antibody against U1 epitope in the presence of LPS or adjuvant.
- To induce arthritis in naïve mice by passive transfer of serum from mice with a relapsing arthritis in the presence of LPS or adjuvant, or by the injection of monoclonal antibodies against U1 epitope in the presence of LPS or adjuvant.
- To test whether monoclonal antibody against U1 epitope bind *in vitro* human cartilage from patients affected by RA undergoing joint replacement.

Are B cells necessary in the induction of the relapse of arthritis?

- To induce relapsing OVA-mediated arthritis in animals with different B cell compartment: BALB/c, MD4 and JHD mice.

Considering the presence of ANA and anti-ssDNA antibodies in mice with OVA-mediated arthritis, are anti-dsDNA autoantibodies present in the sera and kidney?

- To confirm the presence of ANA employing Hep2 cells.

- To further characterise ANA evaluating the presence of anti-dsDNA and antibodies to extractable nuclear antigens by ELISA
- To assess immune complexes deposition containing anti-DNA in kidneys of OVA-mediated arthritis mice.

Is OVA-mediate arthritis TLR-driven?

- To evaluate the expression of TLR2 and 4 in the infiltrate of joints from both acute and relapsing OVA-mediated arthritis by IHC.
- To block TLR2 or TLR4 signalling using antagonists in both models of acute and relapsing OVA-mediated arthritis *in vivo*: assess paw thickness, clinical score, joint histology, autoantibodies and CII-specific T cell proliferation.

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