The Evolution of CD4+ T Cell Clonality in a Murine Model of Inflammatory Arthritis

Shaima Mazin Jawad Al Khabouri

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Institute of Infection, Immunity, and Inflammation

College of Medical, Veterinary & Life Sciences

University of Glasgow

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Abstract

Immunological tolerance is an important aspect of immunity preventing responses being mounted against self-peptides and other innocuous foreign antigens. A breach in self-tolerance can lead to the development of autoimmune diseases such as Rheumatoid arthritis (RA). RA is a chronic inflammatory autoimmune disease that is characterised by synovial inflammation and joint erosion. CD4+ T cells have been shown to play a key role in disease progression, and their ability to infiltrate joints is associated with perpetuation of local and systemic inflammatory responses. A diverse range of T cell receptor (TCR) usage has been demonstrated in RA patients, however how such diversity arises and is shaped remains unclear. Understanding the development of CD4+ T cell antigen specific responses will therefore be important for the development and application of antigen-specific therapeutic tolerance regimes.

To investigate these antigen specific responses, CD4+ T cell clonality was examined using the OVA induced breach of tolerance model of experimental arthritis, allowing the assessment of developing antigen specific responses in the early stages of arthritis. The initial articular CD4+ T cell response was found to be oligoclonal in nature, with enrichment of several TCRVβ families in the inflamed joint. Moreover, the enrichment for some families is associated with joint derived antigens. Next-generation sequencing analysis of CDR3β sequences of CD4+ T cell clones revealed the dynamics of clonal responses between the inflamed joint and its associated draining lymph node and how these responses change with the progression of the disease. Inflamed joints displayed similar CD4+ T cell repertoire diversity at early and late stages of the disease, while inflamed lymph nodes displayed increased repertoire diversity with disease progression. Moreover, the number of CD4+ T cell clones shared between the inflamed joint and lymph node decreased with time. However, correlation analyses of highly abundant clones between inflamed joints and lymph nodes suggested continued migration of CD4+ T cell clones from inflamed lymph nodes to the joints. The decreased diversity in inflamed lymph nodes at the later time point may be a reflection of epitope spreading to the initial inciting antigen as well as development of new responses to neo antigens released as a result of continued joint damage. The hypothesis is that the reduced CD4+ T cell diversity in the inflamed lymph nodes will eventually be mirrored in the joint if the disease continues untreated.

This research provides insight on the dynamics of the antigen specific response between the inflamed tissue and its draining lymph node with disease progression, highlighting important site specific and temporal differences in clonal diversity with disease development and also highlights the role autoreactive CD4+ T cell responses play in disease progression. By understating the evolution of $CD4^+$ T cell responses in RA, more informed decisions can be made on how antigen-specific therapeutics should be applied and will help develop more effective regimes to reinstate self-tolerance, with the ultimate goal of moving towards drug free remission and a cure.

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Author’s Declaration

I declare that, except where reference is made to the contribution of others, this thesis is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Signature:

Name: Shaima Mazin Jawad Al Khabouri

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Abbreviations

Abbreviation	Definition
ACPA	Anti-citrullinated protein antibodies
AIRE	Autoimmune regulator
APC	Antigen presenting cell
BCR	B cell receptor
BMDC	Bone marrow derived dendritic cell
CDR3β	Complimentarity determining region 3 of the T cell receptor β chain
CFA	Complete Freund's adjuvant
CIA	Collagen induced arthritis
CII	Collagen type II
CMV	Cytomegalovirus
cTEC	Thymic cortical epithelial cells
CTLA-4	Cytotoxic T-lymphocyte associated protein 4
DC	Dendritic cell
DMARD	Disease modifying anti-rheumatic drug
EAE	Experimental allergic encephalomyelitis
EBV	Epstein-Barr virus
GFP	Green fluorescent protein
GM-CSF	Granulocyte macrophage colony stimulating factor
GWAS	Genome-wide association studies
HAO	Heat aggregated ovalbumin
HIV	Human immunodificiency virus
HLA	Human leukocyte antigen
HSV-1	Herpes simplex virus 1
HVH-1	Herpesvirus hominus type 1
ICOS	Inducible T cell costimulator
IFA	Incomplete Freund's adjuvant
IFNγ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
LCK	Lymphocyte protein tyrosine kinase
LCMV	Lymphocytic choriomeningitis virus
MBP	Myelin basic protein
MTX	Methotrexate
MHC	Major histocompatibility complex
MS	multiple sclerosis
mTEC	Medullary thymic epithelial cell
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NFAT	Nuclear factor of activated T cells
NGS	Next generation sequencing
OVA	Ovalbumin
PAD	Peptidylarginine deaminase
PD-1	Programmed death protein 1
PKC-γ	Protein kinase C gamma
pLN	Popliteal lymph node

PTPN22	Protein tyrosine phosphatase non -receptor type 22
RA	Rheumatoid arthritis
RF	Rheumatoid factor
SIV	Simian immunodificiency virus
SNP	Single nucleotide polymorphism
TCR	T cell receptor
TdT	Terminal deoxynucleotidyl transferase
TGF-β	Tumour growth factor beta
TID	Type I diabetes
TNFα	Tumour necrosis factor alpha
Treg	Regulator T cell
Vβ	Variable region of the TCR β chain
ZAP-70	Zeta-chain associated protein kinase

Chapter 1 Introduction

1.1 Overview

The immune system is composed of innate and adaptive components that work in concert to provide protection against pathogens and infection. However, this process can go awry and can result in unwanted responses to self. Mechanisms such as immunological tolerance have evolved to prevent responses being mounted against self-peptides and other harmless foreign antigens and are a crucial aspect of a functioning immune system **(section 1.3)**. However, mechanisms of tolerance can also breakdown, resulting in breach of tolerance and the development of autoimmunity and allergy. Breach of tolerance is coordinated mainly by cells of the adaptive immune system, such as CD4+ T cells **(section 1.3.3)**, which have been strongly implicated in coordinating, maintaining, and progressing autoimmune diseases such as Rheumatoid Arthritis (RA) **(section 1.4)**.

RA is a chronic debilitating inflammatory disease affecting 1.5-2.5% of the global population that results in cartilage degradation and joint destruction. RA therapeutics have relied on broad immunosuppression strategies, but more recently have aimed to target specific components of the adaptive immune system, such as T and B cell activation, to provide a more effective form of therapy **(section 1.4.5)**. Unfortunately, a significant proportion of RA patients fail to respond to current therapeutics and eventually have a relapse in disease. More effective forms of therapy are therefore required to achieve long lasting, drug-free remission in RA patients.

One potential line of therapy currently being investigated for use in RA is antigen specific therapy, which relies on using antigens to specifically target autoreactive CD4+ T cells and induce mechanisms of peripheral tolerance to reinstate tolerance to self-antigens **(sections 1.3.2 and 1.5)**. By targeting the pathogenic autoreactive CD4+ T cells, the hope is to prevent perpetuation of immune responses that lead to joint destruction and ultimately restore tolerance to self-antigens implicated in the disease. One of the main setbacks presented by autoimmune diseases in general is that they are diagnosed long after disease onset, which makes it difficult to pinpoint the CD4+ T cell specificities that were potentially involved in disease initiation, and ultimately, makes it difficult to choose the antigens required for

tolerogenic therapy. This is particularly relevant for RA, as patients display clinical symptoms up to 15 years after breach of tolerance has occurred[1].Moreover, events resulting from chronic inflammation further complicate the process of finding the right cells and specificities to target to effectively ameliorate the disease and reinstate self-tolerance. The delay between disease onset and the presentation of clinical symptoms has made it difficult to investigate how antigen specific responses develop during RA; specifically, from when breach of tolerance occurs to when clinical symptoms are manifest.

In an effort to investigate the development of antigen specific responses in RA, numerous studies have focussed on inspecting CD4+ T cell clonality and diversity in RA patients. This requires examining the T cell receptor (TCR) sequence and identifying shifts in frequencies of T cell clones, thus identifying the presence of antigen specific responses. The advantage of this method is it circumvents the necessity in knowing the antigens involved in these responses. However, the delay between breach of tolerance and the presentation of clinical symptoms still poses an issue when investigating CD4+ T cell clonality in RA patients as it difficult to establish when breach of tolerance has occurred in humans. And so, it is very difficult to monitor the initial antigen specific responses at the point when breach of tolerance occurs in humans. Thus, a system whereby antigen specific responses can be monitored from when they first occur is necessary to understand how antigen specific responses develop with disease progression.

The research I have presented here aims to provide a detailed examination of the evolution of CD4+ T cell clonality and antigen specific responses in the OVA induced breach of tolerance model of experimental arthritis. These responses were examined from the very early stages of experimental arthritis to a later more chronic stage of the model. This model uses a joint irrelevant antigen to induce arthritis and allows for the tracking of endogenous antigen specific responses in conditions where autoreactive responses have been shown to occur. The results and conclusions outlined in this thesis aim to further our understanding of the development of antigen specific responses and may emulate the antigen specific responses observed in RA patients. This will potentially contribute to the advancement of more effective tolerogenic antigen specific therapies in RA and toward therapies providing drug-free remission.

1.2 The role of T cells in the adaptive immune system

The hallmark of the adaptive immune system is antigen specificity and immunological memory – a long lasting remnant of the primary response against a pathogen that allows for a quicker and more intensive response upon pathogen re-encounter. The adaptive immune system is composed of B and T lymphocytes which are both able to recognise specific epitopes of proteins, giving the adaptive immune system the unique property of mounting a response against specific antigens. B and T lymphocytes are able to do this via their B and T cell receptors respectively. This introduction will concentrate on T cells and discuss the generation of the T cell receptor (TCR), TCR diversity, and factors that affect this diversity which influence the degree of protection against infections and also influence the development of autoimmunity.

1.2.1 The T cell receptor (TCR) and TCR clonality

The T cell receptor (TCR) is a protein complex found on both CD4+ and CD8+ T cells which allows them to recognise peptides in the context of MHC molecules[2]. Generally, TCR engagement results in a cascade of signalling events leading to T cell activation and onset of effector functions. TCRs are highly diverse molecules allowing T cell populations to recognise an immense range of peptide/MHC (major histocompatibility) complexes. Generation of a diverse T cell repertoire is an important attribute of the adaptive immune system resulting in the protection of the host from numerous potential pathogens. The following sections discuss how this diversity is achieved by detailing the structure of the TCR and the mechanism by which TCRs are generated.

1.2.1.1 Structure and generation of a functional TCR

The TCR is a heterodimeric transmembrane protein that is made up of an α and β chain that are joined together by a disulphide bond. A smaller subset of T cells express TCRs made up of a γ and a δ chain and are adequately named γδ T cells. These cells have a limited antigen recognition repertoire and function differently to conventional αβ T cells and will not be discussed in this introduction. Each α and β chain are made up of constant (C), variable (V), and joining (J) segments with the addition of a diversity (D) segment in the β chain. Each TCR chain is divided into two regions; the invariable constant region made up of the C segment, and the

variable region which consists of the V, (D), and J segments. The constant region, as the name implies, is invariant across different TCRs and is encoded by one C segment gene for the α chain and 2 for the β chain. The V region segments are randomly selected from a large range of genes (human TCRα locus: 43 V genes, and 58 J genes, human TCRβ locus: 42 V genes, 12 J genes, and 2 D genes) and combined in a process called V(D)J recombination[3,4]. Each segment gene used to synthesise the V region of a TCR chain is flanked by a conserved recombination signal sequence (RS) that is utilised by recombination activating genes 1 and 2 (RAG1 and RAG2) and non-homologous end joining (NHEJ) machinery to initialise the recombination event. The RS sequence is an AT rich sequence that is separated by either 12 or 23 non-conserved base pairs (bps). Recombination events only occur using genes that contain an RS sequence followed by 12 bps with those containing an RS sequence followed by 23 bps and is known as the 12/23 rule. This ensures that the segments assemble in the correct order; for the TCRβ chain for instance, V segment genes are flanked by RS sequences with a 23 bp spacer and therefore can only be joined to D genes but not J genes, as these TCR segment genes have RS sequences with 12 and 23 bp spacers respectively. Moreover, recombination events only occur between genes from the same chromosome, which guards against cross recombination events between TCRα and TCRβ chain genes. The RS sequences are precisely aligned during recombination to maintain an in-frame coding region. The alignment of the RS sequences between different gene segments creates loops in the chromosome that are excised during recombination along with the RS sequences. The variable region is then imprecisely joined by having nucleotides added or removed by the terminal deoxynucleotidyl transferase (TdT) enzyme or by exonucleases respectively. The variable region is then ligated by DNA ligase to leave an exon coding for the variable region. This rearranged DNA V region is then transcribed and spliced to a C segment to generate a mature mRNA molecule that encodes a complete α or β TCR chain[5,6] **(Figure 1-1)**.

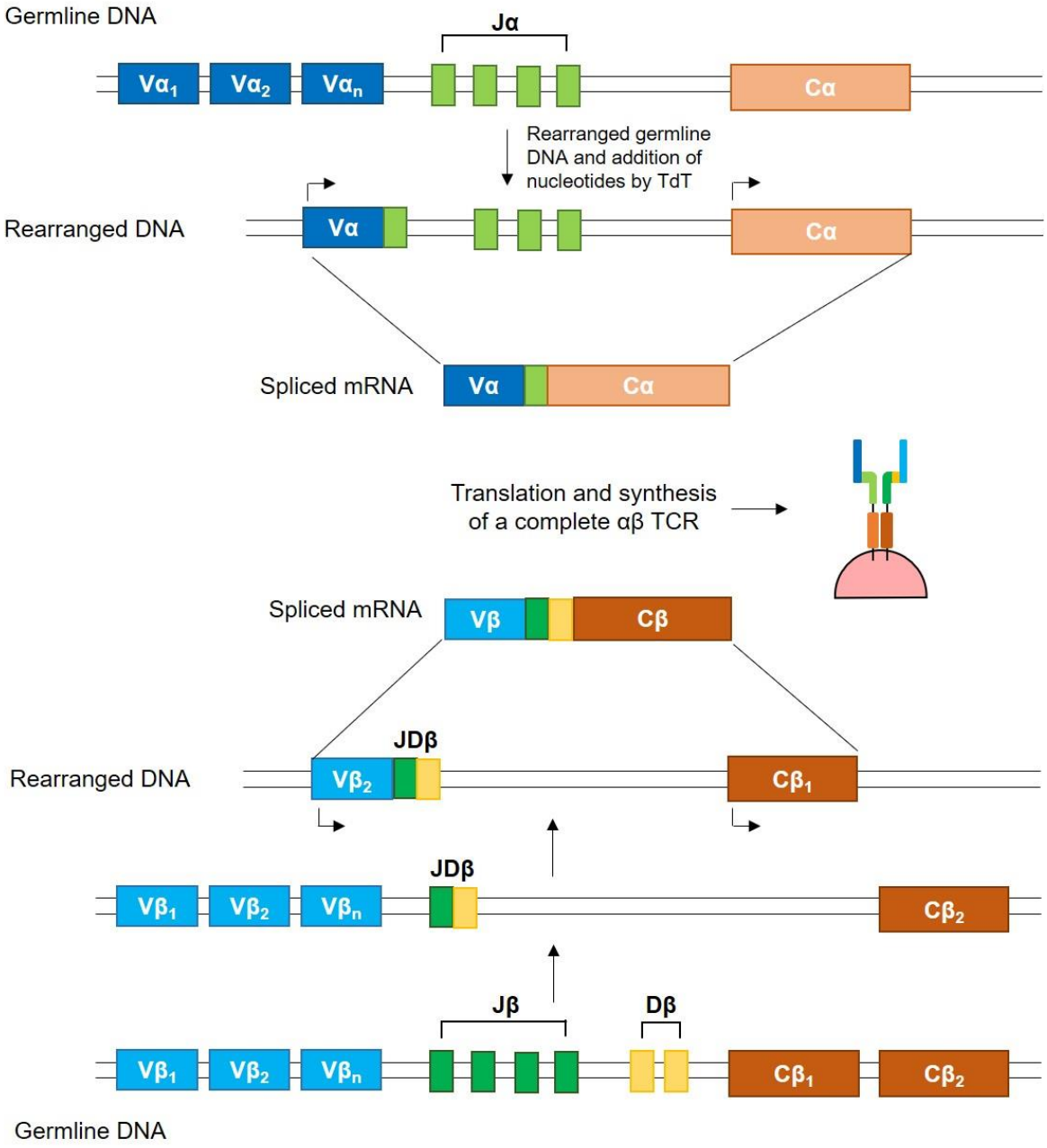


Figure 1-1 Generation of a functional αβ TCR
Schematic representing gene rearrangements and molecular mechanisms to generate an αβ TCR chain. VJ or VDJ gene segments rearrange from a large range of segment genes in a process called V(D)J recombination. Due to the imprecise mechanism of segment joining, base pairs are added or removed by TdT to ensure the coding region remains in-frame. Once the VJ or VDJ genes are rearranged, the mRNA is spliced to mRNA transcribed from the constant genes, which are then translated to form a complete αβ TCR.

Crystal structures of TCRs interacting with peptide/MHC complexes have revealed TCR interaction loops known as complementarity-determining regions (CDRs) that serve as contact points between the TCR, peptide, and MHC molecule[7,8]. CDRs 1 and 2 are encoded by V gene segments and form contacts with the MHC molecule with CDR1 also contacting the outer edges of the peptide. The CDR3 regions are formed from the VJ and VDJ junctions of α and β TCR chains respectively and were found to contact the centre of the peptide. The CDR3 region was predicted to be the

most variable region of the TCR due to the junctional diversity of the VJ and VDJ regions[2] and was confirmed by mutagenesis studies directed against the CDR3 sequence resulting in altered response to peptide or abolishing it altogether[9,10]. The variability in the CDR3 region is therefore critical in recognising specific peptide sequences within the context of MHC[11,12] **(Figure 1-2).**

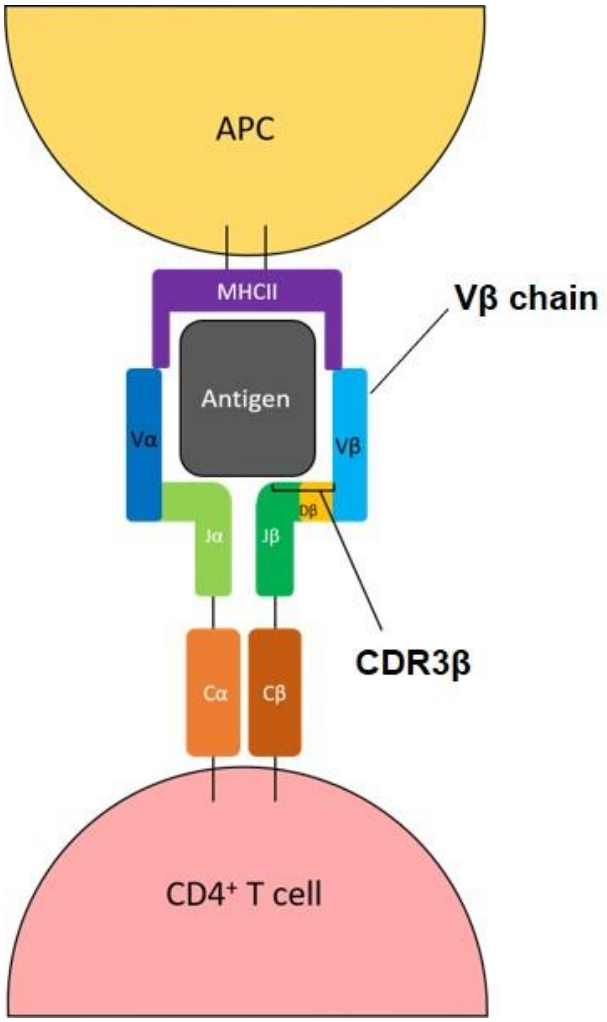


Figure 1-2 αβ TCR structure
Schematic of an αβ TCR chain highlighting the CDR3β region. A functional αβ TCR consists of an α and β chain joined together by a disulphide bond. The TCR contacts the peptide/MHC complex at several contact via complementary determining regions (CDRs) present on the TCR. CDRs 1 and 2 are encoded by V gene segments mainly contact the MHC molecule, with CDR1 also forming contacts with the outer edges of the peptide. The CDR3 regions are formed from the VJ and VDJ junctions of α and β TCR chains respectively and were found to contact the centre of the peptide. The CDR3 region was predicted to be the most variable region of the TCR due to the junctional diversity of the VJ and VDJ regions. The Vβ chain and the CDR3β region are highlighted in this schematic, as antibodies against the Vβ chain and NGS methods targeting the CDR3β sequence were used in the research presented in this thesis.

1.2.1.2 Generation of TCR and T cell repertoire diversity

Rearrangement of TCR genes generates diversity in 3 ways; firstly the recombination of different V region gene segments generates what is known as combinatorial diversity. Secondly, junctional diversity is generated in the V region during the addition and subtraction of nucleotides between V and J or between V and DJ regions during V(D)J recombination. Thirdly, different combinations of TCRα and β chains creates a remarkable range of unique TCR complexes. Due to these mechanisms, theoretical TCR diversity is estimated to achieve $1x10^{15}$ possible unique TCR complexes that is reduced to $1x10^{13}$ after thymic selection[2,13]. Theoretical TCR diversity therefore has the ability to generate an immense TCR repertoire pool that can essentially recognise any possible peptide sequence[14]. In reality, this diversity is limited by the physical number of T cells available in an organism, which in humans is approximately $2x10^{7}$ T cells[15]. Due to the immense number of possible TCR sequences, the probability of organisms having the same TCR repertoire is very low. Indeed, a study by Bousso et. al. found that the naïve T cell repertoire between individual mice of the same strain overlaps by only 20-25%[16] and genetically identical mice infected with the lymphocytic choriomeningitis virus (LCMV) each develop unique responses to the virus[17], known as private responses. Having a varied and more diverse T cell repertoire has been shown to be more effective in an organism's capability of responding to a given pathogen and also reduces the possibility of mutated pathogens evading the immune system. A study in rhesus macaques infected with simian immunodeficiency virus (SIV) by Price et. al[18]. demonstrated that the virus evades immune detection in individuals with a TCR repertoire with a restricted CDR3β sequence. In addition, a study by Messaoudi et. al[19]. demonstrated how a polyclonal but not an oligoclonal CD8+ T cell repertoire – T cell populations that have varied versus restricted ranges of CDR3 sequences respectively – protected mice against the *Herpesvirus hominus* type I (HVH-1). Together, these studies provide evidence for the value in having a diverse TCR repertoire for immune protection.

However, there are instances where the same TCR sequence occurs across individuals in response to a pathogen and this is known as a public TCR response[20]. Evidence of public responses have been found in cases of viral infection – such as Epstein-Barr virus (EBV), and influenza virus[21,22]- and autoimmunity[23,24]. Given the rarity in generating the same TCR sequence in two different individuals, a potential

explanation for having public responses is the possibility of specific TCR sequences posing a selective advantage to a given response. Indeed, deletion of the Dβ region from C57BL/6 mice practically ablated the CD8+ T cell response to Herpes simplex virus 1 (HSV-1) and reduced the cellular response to infection[25]. Recent evidence has suggested that public TCRs are positively selected in the thymus due to their increased ability to cross-react with different antigens[26] potentially overcoming the restriction in TCR diversity presented by the finite number of T cells produced in an organism[13]. Although, evidence for the benefits of TCR cross-reactivity is controversial[25,27], and the association of public TCR sequences to autoimmune diseases[23,24] perhaps undermines the benefits of public TCR sequences.

1.2.1.3 Factors affecting TCR and T cell repertoire diversity

There are several factors that can affect TCR diversity. These are thymic selection, outgrowth of specific clones during an initial immune response and during persistent or chronic infection, and whether there is a public or private response against a pathogen.

Several studies have demonstrated how thymic selection influences diversity of the T cell repertoire and have suggested that this process can reduce T cell diversity by 3 to 100 fold[13]. Zerrahn et. al.[28] demonstrated how this selection bias may in fact be encoded in germline TCR gene segments prior to any form of selection. Indeed, a study by Fazilleau et. al.[29] demonstrated a bias in TCR gene segment selection in mice deficient for the TdT enzyme and found repeated selection of certain V, D, and J segment genes across TdT deficient animals. They and others hypothesised that this bias is to enrich the T cell precursors for self-MHC restriction[29,30]. MHC polymorphic restriction heavily influences TCR diversity, as precursor T cells selected during positive selection are only selected if they bind to the MHC molecules expressed by thymic cortical epithelial cells. Indeed, a complex between the TCR, peptide, and MHC molecule forms upon peptide recognition – named the trimolecular complex – which is facilitated and perhaps stabilised by TCR contacts with the MHC molecule[8,31,32]. Studies have demonstrated that mutations in MHC molecules have a profound impact on T cell responses and peptide recognition[33,34]. An example of the influence of MHC II restriction on the TCR repertoire perhaps occurs in cases of rheumatoid arthritis (RA). Particular HLA DRB1 alleles – antigen presenting molecules in humans – strongly correlate with the prevalence of RA.

Indeed, over 80% of RA patients carry one or more HLA DRB1 alleles that have been found to be associated with RA[35,36], suggesting that these alleles may affect both positive and negative selection during T cell development and bias the T cell repertoire generated. Another example of the influence of MHC restriction was shown by Dyall et. al. They demonstrated that mutations in MHC molecules of C57BL/6 mice selected for TCR molecules against an HSV epitope while TCRs specific for an ovalbumin (OVA) epitope were lost[37]. Moreover, some mouse models of autoimmunity are restricted to specific mouse strains. For instance the CIA model of arthritis is restricted to mouse strains with the H-2^q MHC restriction such as the DBA/1 and the C57BL/6N.Q strains[38,39]. And, models of EAE are reported to have higher susceptibility in C57BL/6 mice compared to Balb/c mice[40], which may be a reflection of the MHC restriction between these two strains. This again has implications on MHC restriction and TCR repertoire formation in patients with autoimmune diseases as the presence of HLA-DQ2/DR3, DQ6/DR2, and DQ8/DR4 account for 90% susceptibility to autoimmune diseases[41].

Thymic selection affects the diversity of the naïve T cell repertoire. Antigen presentation and activation of T cells on the other hand affects the effector and memory T cell repertoires. When T cells encounter their antigen they clonally expand and proliferate. This naturally leads to the outgrowth of certain T cell clones compared to others and skews the repertoire to reflect the antigen encounter. Numerous studies have utilised this phenomenon to monitor the progression or resolution of diseases such as leukaemia[42], infections such as human immunodeficiency virus (HIV)[43] and CMV[44], as well as autoimmune diseases like coeliac disease[23], type I diabetes[45], and RA[46–48]. Several studies have shown that the outgrowth of particular clones during primary infection is due in part to high affinity interactions between the TCR peptide-MHC complex[49,50] demonstrating the effect a primary infection has on the resulting T cell repertoire. Chronic infections have been shown to narrow the T cell repertoire in several cases of infection such as in CMV[51] and EBV[21] where a selection bias during infection results in narrowing of the repertoire. Although, a phenomenon that does arise during chronic or persistent infections is epitope spreading, where an initial dominant response to a specific epitope diversifies into a response against subdominant epitopes of a either a foreign or self-protein[52]. Epitope spreading would therefore broaden the T cell repertoire in terms of the epitopes recognised evidence of which is suggested in cases of multiple sclerosis[53,54].

A phenomenon known as convergent recombination is also a factor to consider when thinking about TCR diversity. Convergent recombination describes a situation where TCR CDR3 sequences have different DNA coding sequences but result in the same amino acid sequence due to the degeneracy in the DNA code. So, although there might appear to be greater diversity in the CDR3 TCR DNA sequence, this diversity would be reduced if the CDR3 regions code for the same amino acid sequence. This would also be considered as a public TCR sequence, as the CDR3 DNA sequences code for the same amino acid sequence. The importance of converged TCR sequences in an immune response is yet to be determined.

1.2.2 T cell development and selection

T cells originate from haematopoietic stem cells located in the bone marrow that produce T cell precursors which migrate to the thymus to become mature T cells. T cell precursors undergo meticulous selection processes to ensure the production of a self-MHC restricted T cell population as well as a population that is tolerant to self-peptides, known as positive and negative selection respectively. In order to be selected, T cells need to express a functional TCR that can bind to MHC molecules with a degree of affinity without compromising their ability to ignore self-peptides. The following sections detail the processes by which T cells are selected and demonstrate how these selection processes begin to shape the T cell repertoire.

1.2.2.1 Positive selection

T cell progenitors enter the thymus and migrate to the outer thymic cortex and begin to proliferate. Generation of a functional TCR begins with rearrangement of the β chain locus to form a complete β TCR chain. This chain then binds to an α chain precursor to form the pre-TCR that then provides signals and results in the expression of both CD4 and CD8 co-receptor molecules, thus producing double positive thymocytes. These double positive thymocytes then rearrange the α chain locus to form a complete α TCR chain, completing the formation of an αβ TCR complex. Once a complete TCR is formed, the double positive thymocytes encounter thymic cortical epithelial cells (cTECs) that express both MHC-I and MHC-II molecules. Double positive T cell precursors that bind to either MHC-I or MHC-II molecules via the TCR begin to proliferate and upregulate expression of the TCR. In addition, these precursors begin to upregulate one co-receptor while downregulating the expression of the other depending on their encounter with MHC-

I or MHC-II; MHC-I cells upregulate expression of CD8 and downregulate CD4, while cells encountering MHC-II molecules do the opposite. Double positive thymocytes that fail to interact with thymic cortical epithelial cells or that interact with the MHC complexes on these cells with high affinity undergo apoptosis. Selection of cells via this process is called positive selection.

1.2.2.2 Negative selection

Single positive thymocytes, expressing either CD4 or CD8, then undergo negative selection in both the thymic cortex and medulla. Negative selection is a process that ensures the deletion of immature T cells recognising self-peptides. Medullary thymic epithelial cells (mTECs) express a protein called autoimmune regulator (AIRE) that allows them to express self-peptides from numerous tissues and present them to immature T cells. Immature T cells that bind strongly to mTECs as well as thymic dendritic cells presenting a self-peptide undergo apoptosis and are eliminated. T cells that survive negative selection exit the thymus as mature T cells.

Together, positive and negative selection strike a balance in producing a T cell repertoire that is self-tolerant and protective against foreign pathogens.

1.2.3 Antigen recognition and clonal expansion

T cells successfully selected by positive and negative selection mechanisms are now mature and exit the thymus and migrate to secondary lymphoid organs. They then circulate between the secondary lymphoid organs situated throughout the body increasing the chances of encountering their antigen and providing immunosurveillance. Once naive T cells encounter their antigen via antigen presenting cells (APCs), they become activated, proliferate, and migrate to tissues to execute their effector functions. T cells can only be activated once a sufficient signalling threshold is achieved. In order to become activated naïve T cells require 3 signals; the first signal is provided when the TCR binds to its cognate antigen through an MHC molecule, which is reinforced by either the CD4 or CD8 molecule binding to the MHC molecule. Signal 2 is provided by co-stimulatory molecules, such as CD28 on the T cell interacting with CD80/86 on MHC. And finally signal 3 is provided by cytokines released from APCs which interact with their respective receptors on T cells, influencing the type of effector T cell the activated T cell will differentiate into. Together these signals initiate a signalling cascade resulting in the

up regulation of T cell activation genes to initiate proliferation and T cell differentiation **(Figure 1-3)**.

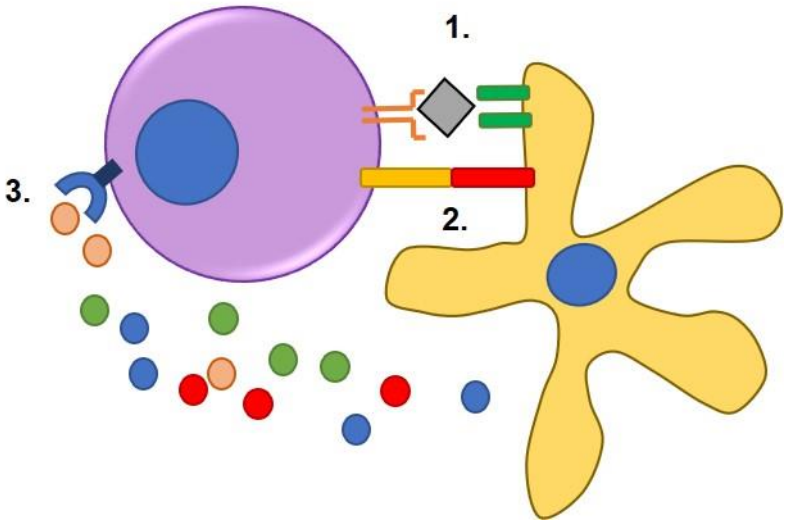


Figure 1-3 Signals required for complete T cell activation
A naïve T cell requires 3 signals in order to become fully activated, proliferate, and execute their effector functions. The first signal is provided when the TCR binds to its cognate antigen on the appropriate MHC molecule. Signal 2 is provided by co-stimulatory molecule CD28 on the T cell interacting with CD80/86 on MHC. And finally signal 3 is provided by cytokines released from APCs which interact with their respective receptors on T cells, influencing the type of effector T cell the activated T cell will differentiate into. Together these signals initiate a signalling cascade resulting in the up regulation of T cell activation genes to initiate proliferation and T cell differentiation.

Once the TCR is engaged, a signal transduction cascade is initiated via the TCR complex. The TCR complex consists of a TCR and CD3 proteins which in turn are made up of gamma, delta, epsilon, and zeta chains[55]. Engagement of the TCR causes cross-phosphorylation of the transmembrane chains of the complex, known as ITAMs (immunoreceptor tyrosine-based activation motifs). This causes recruitment of tyrosine receptor kinases such as LCK and ZAP-70, which in turn recruit other tyrosine kinases to continue the signalling cascade. An important enzyme in this cascade is phospholipase C-γ (PLC-γ). whose recruitment is reinforced by the binding of co-stimulatory molecules. PLC-γ causes generation of secondary messengers such as diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3) which result in the influx of Ca^{2+} and the eventual activation of the transcription factor NFAT. Activation of PKC-γ also activates protein kinase C-theta (PKC-θ) and Ras eventually leading to the release of transcription factors NFκB and AP-1. Together, NFAT, NFκB, and AP-1 induce transcription of specific genes that lead to T cell proliferation and specific differentiation.

Activation of T cells via their TCR ensures clonal expansion of that T cell clone, generating many more “copies” of that T cell, which enables the immune system to tackle the incoming pathogen quickly and effectively.

1.3 Mechanisms of T cell tolerance

As described in section 1.2.2, T cells undergo rigorous selection processes to ensure the production of a T cell population capable of recognising foreign antigens, but are also tolerant to self-peptides. In order to achieve this balance, the immune system has put mechanisms in place to restrict production of T cells recognising self-peptides while maximising the number of T cells recognising foreign peptides both at the development stages as well as when the cells have already exited the thymus and are mature. These processes will be described in detail in the following section.

1.3.1 Central tolerance

Central tolerance describes the thymic process of eliminating developing T cells that can potentially mount an immune response against self-peptides. Central tolerance encompasses the processes of positive and negative selection, described in **sections 1.2.2.1 and 1.2.2.2**, which aims to delete these autoreactive precursor T cells, preventing them becoming part of the T cell repertoire. Conversion of T cells recognising self-antigens into regulatory T cells (Tregs) is another mechanism of tolerance. These cells modulate immune responses once they leave the thymus. Although generated in the thymus, these cells function to suppress the immune response in the periphery **(see section 1.3.2)** by producing anti-inflammatory cytokines and tempering the activation state of APCs[56]. Generation of thymic Tregs (tTregs) is dependent on a complex interplay between TCR peptide-MHC binding affinity[57], duration of binding, and a culmination of intracellular signalling pathways generated from TCR peptide engagement, binding of co-stimulatory molecules[58] and cytokines[59,60] **(Figure 1-4)**.

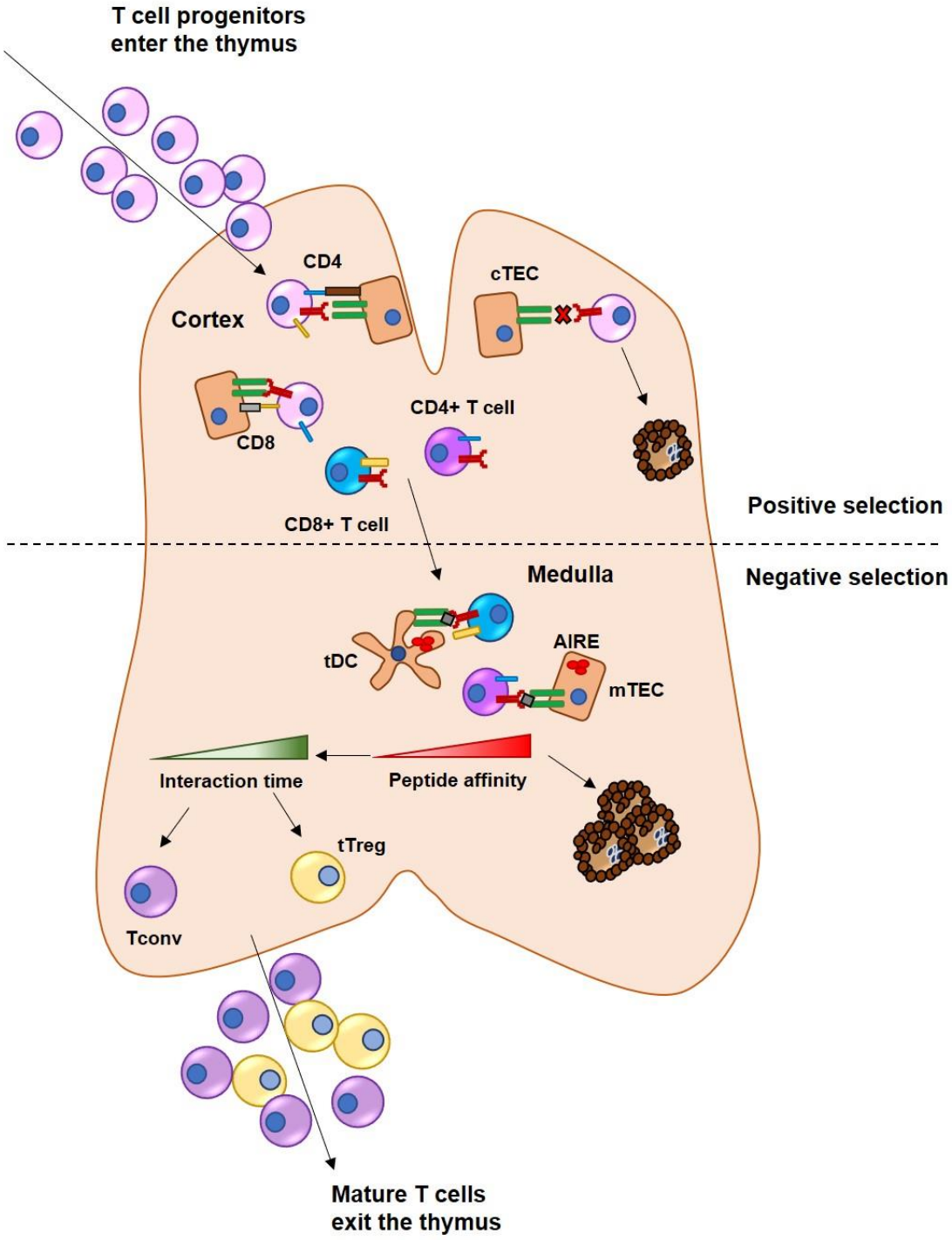


Figure 1-4 Mechanisms of central tolerance
Central tolerance eliminates developing T cells with the potential to recognise self-peptides through the mechanisms of positive and negative selection, described in **sections 1.2.2.1 and 1.2.2.2**. Briefly, progenitor T cells enter the thymic cortex, synthesise a TCR chain, then bind to MHC molecules on cTECs which generate either single positive CD4 or CD8 immature T cells depending on the MHC molecule bound. Cells failing to bind to MHC molecules or those that bind too strongly undergo apoptosis. Selection by this process is called positive selection. Immature T cells that are selected for then undergo negative selection in both the cortex and the thymic medulla. mTECs and thymic DCs (tDCs) express the autoimmune regulator (AIRE), allowing them to present self-peptides to immature T cells. T cells that bind to self-peptides with high affinity undergo apoptosis and are prevented from becoming part of the T cell repertoire. Cells that bind to self-peptides with medium to low affinity survive negative selection. Binding time also plays a role on T cell fate, as thymic Tregs (tTregs) are generated from a complex interplay of peptide affinity, binding time, and culmination of intracellular signalling pathways. Both tTregs and conventional T cells (Tconv) exit the thymus as mature T cells.

1.3.2 Peripheral tolerance

Peripheral tolerance is the second mode of tolerance that occurs once T cells mature and exit the thymus. This mechanism of tolerance serves to protect an organism from the cells that have escaped mechanisms of central tolerance. In addition, it also protects the organism from self-peptides that were not encountered in the thymus, as well as innocuous foreign antigens, such as antigens derived from foods. Numerous mechanisms of peripheral tolerance exist including induction of T cell death, induction of T cell anergy, and production of induced Tregs (iTregs) **(Figure 1-5)**.

Clonal T cell deletion in the periphery occurs when T cells are constantly stimulated by self or foreign antigens[61,62]. Constant stimulation of T cells activates Fas and Bim dependent pathways resulting in the activation of apoptosis machinery and T cell death. This mechanism therefore eliminates over-active T cells from the immune system.

Anergy is a state of T cell hyporesponsiveness resulting from engagement of the TCR without receiving co-stimulatory signals, referred to as signals two and three, as discussed in **section 1.2.3**. T cell anergy can also be achieved when the T cell receives inhibitory signals via ligands binding to inhibitory receptors on T cells such as CTLA-4 and PD-1[63]. The absence of co-stimulatory molecules and activation of inhibitory pathways represses TCR signalling and eventually prevents the upregulation of activation genes[64]. These signals are delivered when antigens are presented to T cells by tolerogenic DCs (tolDCs), which are thought to be DCs that have not completely matured due to the absence of danger signals from the environment[65]. Although, there is also evidence of mature DCs providing immune regulatory signals to T cells[66].

In addition to anergy, tolDCs are also responsible for converting T cells to Tregs, which are called induced Tregs (iTregs) to differentiate them from thymic Tregs (tTregs). iTregs are differentiated from naïve T cells and are programmed to synthesise inhibitory molecules such as IL-10 and TGF-β[67]. Unlike anergic T cells, iTregs are functionally programmed to be immunosuppressive. They also impart their functions on DCs, preventing them from maturing and promoting the expression of immunosuppressive molecules[67]. Generation of iTregs is dependent

on both peptide affinity for the TCR[68], lack of co-stimulatory signals[69], and the presence of cytokines such as IL-2 and TGF-β[70] **(Figure 1-5)**.

Together, these mechanisms of peripheral tolerance aim to complete immunoregulation of potentially self-reactive T cells that have escaped central tolerance mechanisms in the thymus, as well as preventing autoreactivity against antigens expressed in the periphery.

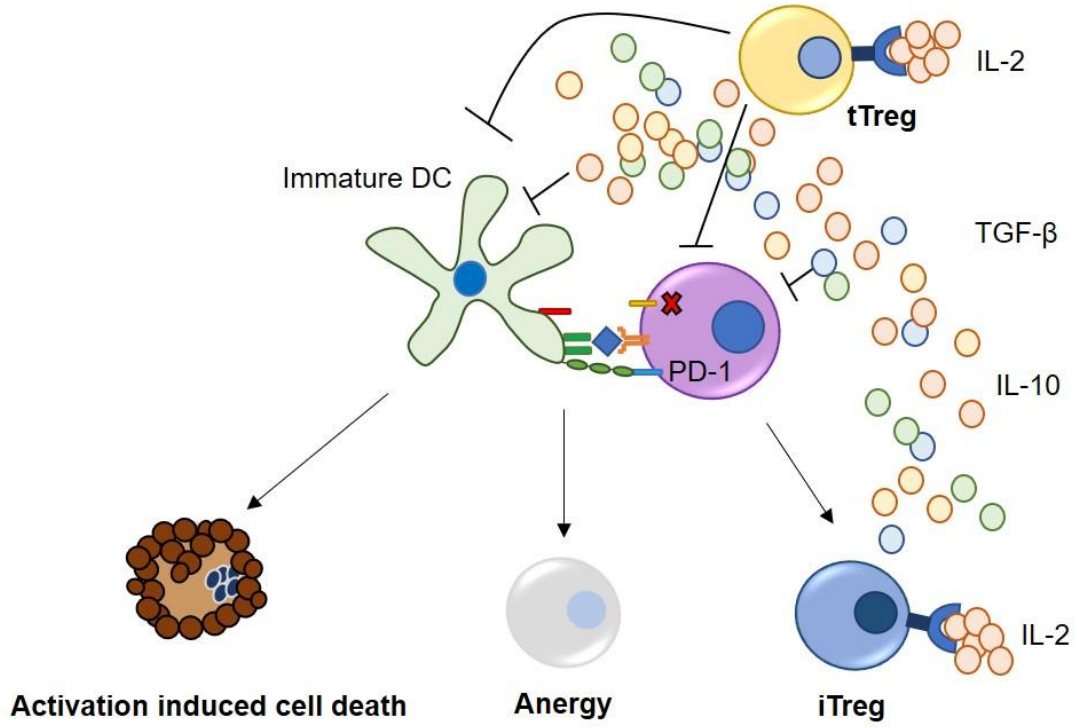


Figure 1-5 Mechanisms of peripheral tolerance
Several mechanisms of peripheral tolerance exist to control cells that have escaped mechanisms of central tolerance and from harmless peptides not encountered in the thymus. Constant stimulation of T cells leads to activation induced cell death and clonal deletion by apoptosis. An anergic or hyporesponsive state is achieved when the TCR is engaged without receiving co-stimulatory signals, like engagement of CD28 by CD80/86, or when inhibitory receptors are engaged, such as the interaction of PD-1 on the T cell with PD-L1 on an APC. T cells interacting with immature or tolerogenic DCs also results in a state of anergy, but also causes induction of iTregs, which secrete inhibitory cytokines like IL-10 and TGF-β, and also sequester IL-2, preventing effector T cell proliferation. In addition, iTregs supress DCs, preventing them from maturing and promoting DC production of immunosuppressive molecules. Thymic Tregs also function in the periphery and supress T cell and DC activation by the production of immunosuppressive molecules. Antigen specific tolerogenic therapies, discussed in section 1.5, propose to take advantage of these mechanisms to re-instate self-tolerance.

1.3.3 T cells in breach of tolerance and autoimmunity

Breach of tolerance occurs when mechanisms of central and peripheral tolerance fail. There are several reasons for breach of tolerance, but how this phenomenon

arises is not fully understood. Complex interplay between genetic susceptibility, environmental stimuli, and immune dysregulation is thought to contribute to the development and progression of autoimmune diseases. Numerous studies have shown the importance of T cells in autoimmunity as the genetic and environmental factors implicated in autoimmune diseases indicate how the selection, aberrant activation, and dysregulation of T cells all contribute to the development and progression of autoimmunity. The following section will discuss the potential mechanisms of autoimmunity and the diseases in which they are manifest.

1.3.3.1 Potential mechanisms of autoimmunity

There are several suggested mechanisms of autoimmunity which encompass genetic and environmental factors. One of the most important genetic factors contributing to autoimmunity is the occurrence of certain major histocompatibility (MHC) alleles, or human leukocyte antigens (HLA) in humans, which are complexes that bind and present peptides to T cells[71] . Many autoimmune diseases are associated with particular HLA alleles such as coeliac disease[23], type I diabetes (TID)[72], and RA[73], which highlights the importance of antigen presentation and T cell activation in these diseases. Selection of certain HLA alleles would suggest that T cells that would normally be eliminated by mechanisms of central tolerance **(see section 1.3.1)** are in fact selected for. Indeed, studies have shown how changes in MHC alleles presented in the thymus affect the T cell repertoire and change the diversity of the resulting repertoire[37]. Moreover, C57BL/6N.Q mice are more susceptible to collagen induced arthritis (CIA) than C57BL/6 mice due to differences in MHC restriction ($I\text{-}A^q$ vs. $I\text{-}A^b$ for C57BL/6N.Q and C57BL/6 mice respectively), and develop T cell responses against the collagen II epitope CII260-270 which are absent in C57BL/6 mice[39,74]. This would suggest that mice with the $I\text{-}A^q$ haplotype have collagen II reactive T cells in their repertoire. A similar observation can be made in non-obese diabetic (NOD) mice, a mouse model of type I diabetes where mutations in MHC II alleles, akin to the mutations found in diabetic patients, confer a major risk to diabetes onset[75].

Genetic association studies have also revealed mutations in which aberrant immune regulation is implied including mutations associated with defects in lymphocyte activation and those affecting cytokines and cytokine receptors. Mutations in the *PTPN22* locus, which is involved in B and T cell activation[76], are found across a

number of autoimmune diseases including RA, multiple sclerosis (MS), TID, and systemic lupus erythematosus (SLE)[71]. The PTPN22 gene codes for a tyrosine phosphatase and is involved in attenuating T cell activation by inhibiting parts of activation pathways[77]. Mutations in the gene are therefore associated with T cell hyperactivity and aberrant T cell activation in autoimmunity. Other mutations causing aberrant lymphocyte activation include those found in CTLA-4 and CD2/CD58 loci are associated with RA, TID, and MS. In terms of cytokines and cytokine receptors, the IL12/23 pathway has a clear role in inflammatory bowel disease (IBD) and psoriasis[78,79] with genetic wide association studies (GWAS) identifying strong genetic links of this pathway to both autoimmune diseases[80,81]. Other mutations in cytokine related genes affecting autoimmune diseases are mutations in the TNF region which were found to be associated with SLE, psoriasis, and RA[71]. A combination of defective HLA alleles and mutations in T cell regulatory genes together increase the chances of autoimmune disease onset. Indeed, autoimmune diseases are generally not governed by single mutations, but rather are a culmination of many SNPs[82,83].

Genetics play an important role in the disposition of autoimmune diseases and the examples above demonstrate that genetic mutations causing immune dysregulation are attributable to autoimmune diseases. However, genetics alone are not sufficient to initiate autoimmune diseases and environmental factors have been shown to contribute to the onset of autoimmunity. Indeed, studies involving identical (monozygotic) and non-identical (dizygotic) twins have demonstrated the degree to which different autoimmune diseases are affected by environmental factors. For instance, twin studies to determine the degree of genetic association with TID have shown the concordance rate of TID in monozygotic twins is in the range of 13-47% and is between 4-12% in dizygotic twins[84]. While in RA, the concordance rate of the disease is in the range of 12-21% in monozygotic twins and 4-9% in dizygotic twins[84,85]. These studies therefore indicate that genetics have a stronger influence on the onset of TID than in RA, and the environment plays a bigger role in initiating RA than in TID.

One of the environmental mechanisms that potentially contributes to the development of autoimmunity is cross reactivity due to molecular mimicry. This occurs when a TCR cross-reacts with other peptides due to structural similarities between the peptides[86]. One postulated mechanism for the initiation of

autoimmunity is B and T cell priming to bacterial and viral antigens that have structural similarities to self-peptides, thus priming the immune system to react to self-peptides. This mechanism has been postulated to explain the occurrence of flares in multiple sclerosis (MS) patients[87,88] and has been shown in animal models to be sufficient in triggering experimental allergic encephalomyelitis (EAE), a mouse model of MS[89]. Viral infections have also been implicated in triggering SLE; EBV epitopes have been found to have amino acid sequence similarities to ribonuclear proteins Sm B/B', Sm D1, and Ro, proteins to which autoantibodies are generated against in SLE[90]. In addition, strong correlations have been demonstrated between SLE patients and previous EBV infections[91,92].

Another mechanism implicated in the initiation of autoimmunity is epitope spreading. As mentioned in **section 1.2.1.3,** epitope spreading is a phenomenon that occurs during chronic inflammation where an initial dominant response to a specific epitope diversifies into a response against subdominant epitopes of a either a foreign or self-protein[52]. This can result from the release of self-peptides as a result of tissue damage, presentation of sub-dominant epitopes in an inflammatory environment, and the size of the T cell population that can recognise these epitopes[93,94]. Chronic viral infections for instance can create an environment to encourage epitope spreading to self-peptides which is also a postulated mechanism for the development of MS . Indeed, Lehmann et. al[95] demonstrated epitope spreading to other MBP peptides in chronic EAE and, when Vanderlugt et. al[96] tolerised naïve mice using relapse associated peptides in the relapsing EAE model, mice were protected against EAE relapse . Epitope spreading has also been postulated in RA and a study by van der Woude et. al[1]. demonstrated that an increase in epitope spreading correlates with disease onset.

An important mechanism thought to contribute to the development of autoimmunity is bystander activation. This entails the indirect activation of effector and memory T cells by cytokines and chemokines found in an inflammatory environment without necessarily recognising their cognate antigen. Effector and memory T cells are implicated in bystander activation as these cells subsets have a reduced threshold for activation and are less reliant on the presence of their cognate antigens to fulfil their effector functions[97,98]. Thus, in an inflammatory environment, cytokines released from other cell types can activate effector and memory T cells of irrelevant specificity to the pathogen. The relevance of this phenomenon in autoimmunity has

been demonstrated by Lee et. al[99] in the EAE model. The study found that when OT-II cells polarised to the Th17 subtype were co-transferred with naïve MOG specific CD4+ T cells into RAG knockout mice, the mice exhibited greater EAE severity. In addition, the Th17 OT-II cells were able to infiltrate the spinal cord and produced more effector cytokines such as IL-17A, IFNγ, and GM-CSF when co-transferred with MOG specific CD4+ T cells than if transferred on their own. What this experiment also showed was the requirement of the activation of antigen specific T cells for bystander activation as the Th17 polarised OT-II cells did not produce significant amounts of IFNγ, IL-17A, and GM-CSF when CFA was administered[99]. Bystander activation has also been implicated in RA as Brennan et. al[100] found that T cells isolated from arthritic synovia had striking similarities to T cells activated by cytokines alone in terms of TNFα production and not to T cells activated by TCR stimulation. Moreover, a study by Fazou et al[101] found that memory T cells with specificities irrelevant to joint antigens are enriched in the arthritic synovium and have suggested a chemokine driven mechanism of recruitment for the their accumulation in the joint. This is a plausible mechanism as Patel et. al.[102] demonstrated significantly high levels of chemokines such as macrophage inflammatory protein (Mip)1α, Mip-1β, and IFNγ inducible protein 10 (IP-10) in the synovial fluid of RA patients. However, whether these joint irrelevant memory T cells execute their effector functions and propagate an inflammatory response has not yet been determined.

Finally, recognition of post-translationally modified self-proteins as foreign is yet another mechanism for the development of autoimmunity. Although self-peptides are presented in the thymus to ensure self-tolerance (**see section 1.2.2.2**), it is possible that not all peptides and the various post-translational options were expressed in the thymus, therefore allowing potential autoreactive T cells to exit the thymus. Evidence for the recognition of post-translationally modified peptides have been found in RA, SLE, and MS. The presence of the post-translational molecule citrulline on several peptides in RA have in fact made antibodies directed against citrullinated proteins (ACPA) a biomarker for the disease[103,104]. Post-translational modifications of peptides alter their biochemistry and, can bind more strongly to TCRs than the native peptide, and in some cases, the TCR recognising the modified peptide does not cross-react with the native peptide. This was demonstrated by Zamvil et. al[105]. who showed that only the acetylated version of myelin basic protein (MBP) could induce EAE in mice.

The mechanisms of autoimmunity discussed, which include both genetic and environmental factors, highlight that the development of autoimmunity is a complex process with many possible aetiologies. Moreover, the genetic and environmental factors involved demonstrate the pivotal role the adaptive immune system, specifically T cells, has in coordinating and propagating autoimmune diseases. The next section will focus on the development of RA and discuss the factors involved that predispose individuals to the onset of RA, the mechanisms involved in its development, and strategies to control and eradicate the disease. This section will also discuss the contribution of the adaptive immune system, with a specific focus on CD4+ T cells, to the development and evolution of the disease.

1.4 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease that is characterised by synovial inflammation and joint and cartilage degradation[106]. It has a global prevalence of 0.24% and affects females twice as much as it does males[107]. RA primarily affects the joints, but is classified as a systemic disease as extra-articular manifestations, involving for example the cardiovascular and respiratory systems, are prevalent in RA patients[108]. Moreover, not only does RA inflict chronic pain and disability, it has also been shown to reduce life expectancy by 10 years. RA is diagnosed by the presence of class switched anti-citrullinated protein antibodies (ACPA) and Rheumatoid Factor (RF) – an antibody generated against the Fc portion of an antibody. These autoantibodies are the primary biomarkers of RA, as they have been strongly correlated with disease severity and increase in titre before disease onset[1,109,110]. However, RA is also classified as either seropositive or seronegative, which reflects the respective presence or absence of ACPAs and RF[111]. The risk factors associated with seropositive and seronegative RA are different and seronegative RA patients respond differently to therapeutics than those with seropositive RA[111]. Increasing evidence would suggest that seronegative RA is clinically different to seropositive RA and should be considered a distinct disease[82,111,112]. Therefore, the focus of this introduction will be on seropositive RA.

RA aetiology is complex, heterogenous, and poorly understood, although genetic and environmental factors have shown strong links to the immune system, mainly adaptive immunity – as a key factor in the initiation and development of the disease[82]. Like all autoimmune diseases, a breach of tolerance occurs in RA by an

unknown mechanism. All potential mechanisms of autoimmunity mentioned in **section 1.3.3.1** have been found in different cases of RA[113–115]. The following sections will discuss RA disease aetiology and development, the role adaptive immunity- specifically CD4+ T cells – play in disease progression, and current therapeutics available to treat RA patients.

1.4.1 Genes implicated in RA pathogenesis

RA has long been associated with mutations in HLA alleles, specifically to the HLA-DRB alleles where 80-90% of RA patients display mutations[35]. More specifically, the HLA-DRB alleles 0401, 0404, 0101, and 1402 have a shared sequence known as the shared susceptibility epitope which has been found in the majority of RA patients[73,82]. This shared epitope is a 5 amino acid motif with a QKRAA sequence[73]. It is not fully understood how this shared epitope contributes to RA development, but it is thought that it may result in more efficient presentation of self-peptides[116,117] and may alter T cell selection[37,118]. Indeed, the HLA-DRB alleles associated with RA were found to bind more efficiently to altered self-peptides[117,119].

Genetic wide association studies (GWAS) have highlighted the importance of other SNPs outside the HLA-DRB alleles in implicating RA susceptibility. These mutations cluster around genes associated with immunity and suggest strong associations with immune dysregulation and RA[120]. As mentioned in **section 1.3.3.1**, there are many SNPs that lead to aberrant lymphocyte activation and dysregulation of cytokine signalling pathways in autoimmunity including in RA. The PTPN22 gene is the second most associated genetic risk factor for RA[77] and is a gene involved in T cell receptor signal attenuation which ultimately regulates T cell activation. Another SNP associated with RA is in the CTLA-4 gene which is also involved in T cell signal modulation[121]. Other SNPs involving signal transduction pathways found were related to TNF signalling and include the *TNFAIP3* and *TRAF1* loci[122]. Mutations in the *STAT4* locus were also associated with RA[123] which is an important factor when considering cytokine signalling pathways as STAT4 is involved in integrating signals triggered by IL12 and IL23[124].

1.4.2 Environmental factors contributing to RA development

Genetic predisposition alone is not sufficient for driving the development of RA. As mentioned in **section 1.4.1**, twin studies have shown concordance rates of the

disease is in the range of 12-21% in monozygotic twins and 4-9% in dizygotic twins[84], implying that environmental factors are significant contributors to RA pathogenesis.

One of the most important environmental contributors to RA is smoking, as smoking was found to be associated with an increased production of RF[125] and ACPA[126]. A study by Klareskog et. al[126]. demonstrated that smokers have an increased amount of citrullinated proteins in lung macrophages than non-smokers and also showed a 21 fold increased risk of RA susceptibility in smokers who carry HLA-DRB RA risk alleles. A molecular basis for this association was provided by Scally et. al. who demonstrated that citrullinated peptides bound better to the product of the HLA-DRB1*0401 allele[117]. In addition, smoking was found to increase the production of peptidylarginine deaminase (PAD) 2 - an enzyme that adds citrulline to arginine residues in proteins – in the lungs of smokers, providing a functional link to smoking and RA susceptibility[127,128]. Together, these studies provide evidence for the association of smoking with RA and plausible mechanisms as to why such a strong association exists.

Due to the association of HLA-DRB alleles with citrullinated peptides in RA, it would follow that the generation of citrullinated peptides in general would increase the susceptibility to RA. Indeed, citrullinated peptides and PAD have been found in synovia of RA patients[129]. Furthermore, periodontitis (PD) is also an associated risk factor and its prevalence is approximately 2-fold higher in RA patients[130]. It was found that patients with PD have citrullinated proteins in the periodontium[131] which has been associated with the presence of *Porphyromonas gingivalis* a pathogen associated with PD known to express PAD[132].

1.4.3 Stages of RA and clinical manifestations

In an effort to better stratify RA patients at different phases of the disease, the European League Against Rheumatism (EULAR) have set up guidelines for identifying the stages of RA development[133]. The first phase is the pre-RA or at-risk phase where breach of tolerance occurs. This phase is characterised by the presence of ACPA and RF as mentioned earlier, but also by the presence of acute-phase reactants such as C-reactive protein (CRP), and proinflammatory cytokines and chemokines in peripheral blood[134,135]. Patients who eventually develop RA

remain in this stage for an average of 5 years before progressing to clinical stages[136]. Moreover, increasing levels of ACPA concomitant with joint pain, as well as the diversity of ACPAs present, place the patients at a much greater risk for progressing to clinical phases[1,137].

The transition from susceptibility or at-risk phases of RA to one where clinical symptoms develop is poorly understood. It is thought that the disease may have in fact a mucosal origin, as ACPAs develop before any joint damage is seen and ACPA generation is associated with increased PAD in the lungs and periodontium as discussed in the previous section[82,138]. How the generation of ACPA in mucosal sites link to inflammation and cellular infiltration in the joint is not understood. Moreover, a study by Rodríguez-Carrio et. al[139]. demonstrated how patients at the at-risk phase have altered inguinal lymph nodes compared to healthy controls in that the innate like cells (ILCs) in these lymph nodes shifted from a homeostatic to an inflammatory profile. It would have been interesting to see whether other lymph nodes in these at-risk patients displayed the same skewed ILC profile. It is thought that a second hit or re-exposure event, not necessarily to the original antigen, is required to push the system from the pre-articular to articular stage[82]. This was demonstrated in a mouse model of RA developed by Maffia et. al[140]. where immunisation with OVA in the footpad resulted in anti-collagen responses and cartilage and bone degradation after the transfer of Th1 polarised OT-II cells and subsequent systemic immunisation with OVA in adjuvant. In addition, several studies have suggested a link between EBV and RA as sera isolated RA patients react with EBV nuclear antigens, have higher antibody titres against EBV epitopes, and have higher amounts of EBV DNA in their synovia[141]. This would suggest that mounting responses against EBV may exacerbate the onset of RA.

Clinical phases can be divided into early clinical and established RA. At the early clinical phases cells from both the innate and adaptive immune systems begin to infiltrate the joint and cause synovial inflammation. A cascade of proinflammatory events are instigated by the immune cell infiltrate and involve the release of pro-inflammatory cytokines such as TNFα, IL-1, and IL-6, which perpetuate a proinflammatory environment and result in the recruitment and retention of cells. In addition, the inflammatory environment affects other cells associated with the joint, such as synovial fibroblasts and osteoclasts, which in turn also perpetuate the

inflammatory environment. Eventually, this leads to cartilage degradation and joint destruction, propelling the patient into the established phase of RA[82].

1.4.4 Role of the CD4+ T cells in RA

The studies discussed throughout this introduction provide ample proof for the role of the adaptive immune system in the development and progression of RA. Specifically, the evidence provided highlights the importance of antigen presentation and activation of adaptive immune responses, which ultimately facilitate the production of class switched autoantibodies – ACPA and RF– by B cells. Between antigen presentation and production of autoantibodies, lies the CD4+ T cell which coordinates adaptive immune responses. In this section evidence will be provided highlighting the importance of CD4+ T cells in initiating and propagating RA.

1.4.4.1 Evidence of CD4+ T cells in the initiation, coordination, and maintenance of RA

The evidence implicating CD4+ T cells in RA pathogenesis is compelling. As discussed in **sections 1.3.3.1 and 1.4.1**, many genetic factors associated with RA implicate antigen presentation and T cell dysregulation in the perpetuation of RA. Moreover, infiltration of memory CD4+ T cells into arthritic joints is a common observation[46–48,142,143] and therapeutics like Abatacept that modulate CD4+ T cell interactions with APC are an accepted and effective line of therapy in RA[144,145].

Experimental mouse models of arthritis have provided strong evidence for the importance of CD4+ T cells in disease onset and development. Kadowaki et. al[146]. demonstrated the transfer of CD4+ T cells and not CD8+ T cells from collagen induced arthritis (CIA) mice into severe combined immunodeficiency (SCID) mice initiated arthritis. Also, a study by Mauri et. al. show that monoclonal antibodies against CD4+ T cells reduced inflammation in the CIA model of murine arthritis[147]. A model of spontaneous arthritis known as the SKG model of arthritis develop chronic autoimmune arthritis due to a mutation in ZAP-70, which reduces T cells signalling[148,149]. Also, peripheral CD4+ T cells or bone marrow transferred from these mice to histocompatible mice were found to initiate arthritis, implying an inherent problem with T cell selection and the resulting T cell repertoire.

Different CD4+ T cell subsets have also been implicated in RA. The earliest subset identified in synovial tissues were Th1 CD4+ T cells as these cells were found to produce IFNγ which is characteristic of Th1 cells[150], while no significant increase in cytokines suggestive of a Th2 response, such as IL-4, was observed in RA patients[151,152]. In addition, James et. al[153] observed that approximately 40% of CD4+ T cells specific for citrullinated peptides in blood collected from RA patients were CXCR3+, which is another marker for Th1 cells[154]. Finally, the presence of IgG1 and IgG3 class switched antibodies to collagen II and citrullinated fibrinogen in RA patients again indicates the importance of Th1 CD4+ T cells in the pathogenesis of RA. Together, these data would suggest that RA is a Th1 driven disease. However, attempts to target Th1 responses to control the progression of RA did not yield the intended results; clinical trials investigating the efficacy of humanised anti-IFNγ antibody Fontolizumab in RA patients were terminated at phase II (ClinicalTrials.gov)[155] as endpoint goals were not met, and IFNγ knock-out mice displayed an accelerated development of CIA[156]. Interestingly, acceleration of CIA in these mice was thought to result from an increase in IL-17 producing T cells and IFNγ has been suggested play a role in regulating Th17 responses[157,158]. Although clinical trials with Fontolizumab failed, anti-TNFα therapy is an efficacious line of therapy in RA[159], which again is associated with Th1 responses. Hence, Th1 CD4+ T cells may play two opposing roles in RA which may be important during different phases of the disease; one by facilitating tissue damage through the production of TNFα and perhaps IFNγ at the early stages of the disease, and second by regulating pathogenic Th17 responses which have also been found contribute to joint destruction.

The hallmark of the Th17 CD4+ T cell subset is the production of IL-17 which in turn has been found to stimulate the production of inflammatory cytokines IL-6, and IL-8, as well as matrix metalloproteases MMP-1 and 3 from synovial fibroblasts isolated from patients with juvenile idiopathic arthritis[160]. In addition, IL-17 producing CD4+ T cells as well as Th17 inducing cytokines like IL-6, TGF-β, IL-21, and IL-23, were found in synovial tissues of arthritic patients[161,162]. IL-17 has also been associated with the initiation of neutrophil development[163] as well as recruitment of neutrophils[164], which is another hallmark of RA[165]. In addition, ablation of IL-17 was also found to reduce CIA in mice[166,167]. However, like attempts to temper Th1 activity clinically, therapeutics like Secukinumab and Brodalumab that target IL-17A and the IL-17 receptor respectively were not as effective in RA patients as they were in

patients with other diseases such as psoriasis, psoriatic arthritis and ankylosing spondylitis[168–170]. Results from clinical trials suggest that Th17 cells perhaps play a more complex role in the pathogenesis of RA, as they can also be involved in anti-inflammatory processes[171]. Indeed, Th17 cells isolated from patients that have undergone anti-TNFα treatment acquired IL-10 production, implying that these cells may in fact participate in dampening inflammatory processes in RA[172].

One of the key features of RA is the production of antibodies against self-antigens and CD4+ T cells are likely to provide help to B cells to produce these antibodies. T cells isolated from synovia of RA patients were found to induce Ig production from B cells *in vitro*[142] and germinal centres – where B cells undergo affinity maturation to generate high-affinity antibodies – were also found in synovial tissues in RA patients[173,174]. CD4+ T follicular helper cells (Tfh) are a specialised subset of CD4+ T cells that play a prominent role in forming germinal centres and in directly providing co-stimulation to B cells, resulting in their selection, proliferation, and survival[175]. Tfhs have been implicated in the progression of RA, as this subset was increased in peripheral blood of RA patients and Tfh frequencies were found to correlate with ACPA titres and disease severity[176].

In addition to the presence of proinflammatory CD4+ T cells subsets, regulatory CD4+ T cells have also been found in RA patients. However, the observed frequencies have been controversial with studies reporting higher, or lower frequencies of Tregs in RA patients[177–179]. Despite this, Tregs isolated from patients have been shown to be functionally impaired in some cases, and this may be a result of high TNFα levels in arthritic synovial tissues[177,180]. Indeed, a study by Valenica et. al[181] showed that TNFα signalling inhibits Treg suppressive activity and studies by Ehrenstein et. al[180] and Toubi et. al[182] have shown that anti-TNFα therapy reverses these impaired Treg functions. Although, some studies have also reported that Tregs are in fact capable of supressing effector T cells, but the effector T cells found in the inflamed synovium were resistant to Treg suppression[178,179].

Together, these data reinforce the importance of CD4+ T cells in the pathogenesis of RA and demonstrate the complex interplay of the various CD4+ T cell subtypes involved in RA initiation and development. Furthermore, these studies highlight the fact that the varying effector responses involved in RA may be important at different stages of the disease and effective modulation of RA may rely on understanding

when these different effectors come into play. Indeed, RA therapeutics have been developed that target various cytokine pathways associated with different effector responses, details of which can be found in **section 1.4.5**.

1.4.4.2 CD4+ T cell clonality in RA

Numerous CD4+ T cell subsets are involved in propagating RA and targeting different subsets at different stages of the disease may help abate its progression. Additionally, understanding which antigen specific responses drive the effector responses observed will also provide a target for modulating the disease. Moreover, identifying antigen specific responses at different stages of disease may provide insight on where immune responses against certain antigens originate and how different tissue sites contribute to the propagation of RA. The majority of CD4+ T cells isolated from synovia of RA patients were shown to have an antigen experienced, memory phenotype[142], indicating that the presence of antigen may explain why these cells accumulate in arthritic joints. It also suggests the joint may be an important tissue site for the development of antigen specific responses. Moreover, Wagner et al[48] demonstrated that both memory and naïve T cells from RA patients show reduced TCR diversity, implying that RA patients have a skewed TCR repertoire generated from thymic selection processes. This again demonstrates that certain antigen specificities have been selected for in RA patients and that antigen presentation is clearly a factor in disease initiation and development. Despite this, 'arthrogenic' T cell clones have not been identified, and T cell clones common to RA patients have not been found[46] . Identification of specific CD4+ T cell clones has also been impeded by the fact that many antigens have been implicated in RA such as citrullinated vimentin, α-enolase, aggrecan, and collagen II[114,153,183,184]. Furthermore, not all of the antigens implicated in the disease are present across patients as the ACPA profiles of RA patients were found to differ between individuals[183,185]. These factors combined have made it difficult to pinpoint the autoreactive CD4+ T cells involved in RA initiation and propagation and have also impeded finding where these cells are primed.

One approach of determining the occurrence of an antigen specific response is to look for clonal expansion of T cell clones. The presence of clonal populations provides evidence of antigen driven clonal expansion, albeit in the absence of information pertaining to antigen identity. Again, this especially useful in RA as there

are many antigens implicated in the disease that are not necessarily the same across patients. Clinical observations have revealed the presence of an oligoclonal CD4+ T cell repertoire in the synovia of RA patients[46,186,187] **(Table 1-1)**. These expanded CD4+ T cell clones are likely to play a role in disease pathogenicity as they were found to persist two years after first being identified[143]. Also, the CD4+ TCR repertoire differs between peripheral blood and synovial tissue, indicating that tissue sites play a role in the retention or accumulation of CD4+ T cells possibly in an antigen specific manner[46,187]. Indeed, studies on TID have shown that islet specific CD4+ T cells localise to the pancreas at very early stages of the disease due to antigen encounter[188,189] and may in fact expand due to presentation of insulin[190]. This mechanism may therefore also be relevant to RA development.

Although important, antigen driven accumulation of CD4+ T cells in arthritic joints may not be the only mechanism of retention. Indeed, Prendergast et. al[191] demonstrated that joint irrelevant CD4+ T cells can be recruited to an inflamed joint in a mouse model of arthritis. In addition, joint irrelevant antigens such as BiP (immunoglobulin binding protein) have been implicated in RA[192]. Studies by Brennan et. al[100,193] have shown that CD4+ T cells activated in a non-antigen dependent manner resembled CD4+ T cells isolated from arthritic synovial tissues and that these cells are capable of producing TNFα without the requirement of cognate antigen. Moreover, Iannone et. al[194] demonstrated that non-antigen activated CD4+ T cells could be preferentially recruited to the inflamed synovium. These cells can therefore act as bystander cells **(see section 1.3.3.1)** propagating and maintaining an inflammatory environment in arthritic tissues and can skew the antigen driven CD4+ TCR clonality observed in arthritic joints.

Given the possible antigen and non-antigen driven mechanisms of CD4+ T cell retention in arthritic joints, an important question to ask is whether the initial recruitment of CD4+ T cells to the inflamed joint occurs in an antigen driven manner, and whether the recruitment and accumulation of these antigen specific cells then creates a permissive environment for other CD4+ T cells to enter. Understanding how these antigen specific responses arise, develop, and are maintained can highlight their role in RA and provide an avenue for the development of antigen specific therapies, which will be discussed in detail in **section 1.5**.

Table 1-1 Summary of T cell clonality studies in RA

Author	Method used	Key findings	Comment
Stamenkovic, I et. al, *Proc. Natl. Acad. Sci. USA* (1988)[186]	Southern blot analysis of TCR β chains	Limited number of IL-2 responsive T lymphocytes clones dominate the synovium, but not PB of same patients. Oligoclonality found in both RA and osteoarthritis patients.	Oligoclonality not distinguished between CD4 and CD8 T cells.
Ikeda, Y et. al *Arthritis Rheum* (1996)[187]	RT-PCR and single strand conformation polymorphism (SSCP) analysis, nucleotide sequencing	Distinct expansion of clonal T cells was detected in the synovium of RA patients. At least 60% of the same T cell clones found in two different samples in the same joint of each patient and 40% of those clones were found in PBL of the same patient.	HLA type for each patient was also noted.
Wagner, U. G et. al, *Proc. Natl. Acad. Sci. USA* (1998)[48]	TCRβ chain sequence cloning and sequencing	TCRβ frequency analysis showed a significant contraction of CD4+ T cell clones isolated from PBMCs of RA patients compared to hep C and healthy controls. This loss in diversity was found in both naïve and memory CD4+ T cell subsets.	Robust study investigating multiple factors affecting the T cell repertoire in RA, hep C, and healthy controls, although no samples from the synovium were tested
VanderBorght, A. et. al *Rheumatol. Oxf. Engl* (2000)[143]	TCRα and β specific PCR amplification and subsequent analysis by ELISA	TCR V gene expression was compared between cells isolated from synovial fluid, synovial tissue, and PB of early RA, chronic RA, OA, and healthy controls. TCR V genes were overexpressed in joints but not PB of RA patients. Moreover, the over represented TCR V genes varied across RA patients, but were similar within different joints of the same patient. In addition, overrepresented V genes persist for at least 2 years.	Excellent and well controlled study comparing T cell repertoires in different tissue types with increasing disease chronicity.
Klarenbeek, P.L, et. al *Ann. Rheum. Dis.* (2012)[46]	CDR3β sequencing	Clonality of CD4+ T cells were compared between synovial fluid and PB of early and established RA patients. Highly expanded clones comprised 56% of the T cell repertoire in the joints of early RA patients, while highly expanded clones only contributed to 9.8% of the T cell repertoire. Moreover, highly expanded clones were shared between joints in the same patient.	First study conducting CDR3β sequencing analysis in RA
Zhang, F et. al. *Nat. Immunol.* (2019)[195]	Single cell RNAseq and mass spectometry	Synovial tissue obtained from RA and OA patients were compared and specific profiling of fibroblasts, autoimmune associated B cells, T peripheral and follicular helper cells were identified. Specific C8 T cell subsets were also identified in RA patients.	Bulk analysis of T and B cells, monocytes and fibroblasts. No clonality analysis.

1.4.4.3 Methods of measuring T cell receptor diversity and T cell clonality to assess T cell clonality in RA

There are several approaches to determine T cell clonality experimentally. One method is to amplify regions of the TCR via PCR using region specific primers to capture the CDR3 region, which are then distinguished by the length of the products[196,197]. This method is rapid and robust and has been used to examine T cell repertoire diversity in cancers[198,199] and autoimmune diseases[48,200], including RA[46,48,143,201].

Another method of assessing T cell clonality is to use antibodies directed against the TCR. An example of this is the KJ1.26 antibody that specifically recognises the TCR of DO11.10 cells[202] that are specific for the chicken ovalbumin peptide 323-339[203]. TCR transgenics, such as the OTI and II systems, only express one type of Vα and Vβ chain and can therefore be tracked by using antibodies specifically against these regions[204]. Indeed, behaviour of clonal cells have been tracked using this method[205,206]. Because the antigen specificities of expanded T cells in RA are unknown, using antibodies against specific T cell clones is futile. Instead, antibodies against V regions of the TCR α and β chains can be used to quantify the presence of different V regions by flow cytometry, which would highlight biases in V region usage, and consequentially, identify antigen associated expansion. Quantification of V regions has been performed in healthy controls[87,207], in patients with leukemia[208], and in RA[209]. In studies where non healthy samples were used, biases in V region usage were observed[208,209].

Both the methods of determining T cell clonality mentioned have advantages of providing rapid, global assessments of changes in the T cell repertoire[207,210,211]. These methods rely on the skewed presence of different V regions to reflect changes in the T cell repertoire. T cells undergo clonal expansion when they recognise their cognate antigen via the TCR[212,213]. Determining frequencies of V chain usage does not provide clonal information per se, but it still highlights biases in the usage of these chains, implicating antigen driven accumulation of T cells[210,214]. Historically, the V region of the β chain of the TCR has been examined more than the α chain. This is because the β chain has higher combinatorial potential due to the presence of the D region[215], and it is possible for T cells to have two α chains, but only one β chain[216,217]. This makes examining the β chain more reliable when assessing frequency of Vβ regions.

A more robust method of determining T cell clonality is to do so on a sequence resolution level with techniques utilizing next generation sequencing (NGS) methods[218]. This allows one to determine the sequence of the CDR3 region, the most variable region of the TCR that is unique in clonal T cells[12,219,220] **(see section 1.2.1)**

1.4.5 Current RA therapeutics

Treatments for RA traditionally relied on relieving inflammation using nonsteroidal anti-inflammatory drugs (NSAIDs) and glucocorticoids, while the use of disease modifying anti-rheumatic agents (DMARDs) aimed to slow down or halt disease progression. Important DMARDs that were found to reduce extra-articular manifestations and joint damage are methotrexate (MTX) and sulphasalazine, but despite these drugs, a significant proportion of RA patients either did not tolerate or respond to these DMARDs. As a result, alternative targets were sought in an effort to push for more effective therapies.

Biologics are a class of drug that were introduced to treat RA in the early 1990s[221]. Biologics are specific in that they are designed to target cell types, receptors, or cytokine signalling to modulate specific immune responses that are relevant to RA. These therapeutics have shown a lot of promise in the treatment of RA as well as other inflammatory diseases. Examples of biologics used in RA are TNFα inhibitors such as Adalimumab, Etanercept, and Infliximab, which have proven successful in RA[222,223]. Other biologics targeting cytokine pathways include Tocilizumab and Anakira, which target the IL-6 and IL-1 pathways respectively[221]. An example of a biologic targeting a specific cell type is Rituximab, which results in B cell depletion. Abatacept is a CTLA-4 fusion protein which interrupts T cell APC interactions and co-stimulation by targeting CD80/86 on APCs and is yet another effective RA therapeutic.

Despite the successes of biologics, there still remains a significant number of patients who do not achieve remission and only partially respond to this line of therapy. As such, a number of groups have focussed on developing alternative therapies. One approach being developed is to target kinases such as JAKs which control numerous cytokine pathways[224]. Another therapeutic approach gaining interest is antigen specific therapy which has the potential of achieving drug free

remission for RA and permanent cessation of the disease. This line of therapy will be discussed in the next section.

1.5 Antigen specific therapy

Antigen specific therapies aim to utilise the mechanisms of peripheral tolerance described in **section 1.3.2** to target CD4+ T cells of a certain antigen specificity via the TCR. The strong interest in these therapies arises from the highly attractive prospect of specifically targeting the pathogenic CD4+ T cells while leaving the rest of the immune system intact, thus reinstating tolerance towards self or innocuous antigens like allergens. Both animal studies and historical treatments for allergies in humans have shown successes for antigen specific therapy. As CD4+ T cells have strongly been implicated in RA (discussed in **sections 1.4.1 and 1.4.4**), antigen specific therapy is considered a viable therapeutic option for treating the disease.

Antigen specific therapy has been practiced for over 100 years for treating allergies[225,226]. Administering an escalating dose of peptides derived from the inciting allergen is thought to modulate the immune response to the peptides. A study trialling the efficacy of peptide immunotherapy to grass pollen found the allergic immune response to switch from an IgE to an IgG antibody response and also reported an increase in the regulatory cytokine IL-10[227]. Another study determining the efficacy of antigen specific therapy to bee venom also reported the same antibody isotype switch[228] In addition to modulating the allergic response, a study by Wambre et. al[229] found that peptide immunotherapy to grass pollen causes deletion of the antigen specific T cells.

Antigen specific immunotherapy to autoimmune disease has proven difficult as the antigens involved in autoimmune diseases are poorly defined. And, targeting pathogenic CD4+ T cells in an antigen specific manner is further hampered by epitope spreading which arises as a consequence of disease chronicity. Despite these setbacks, antigen specific therapies have been attempted for some autoimmune diseases but have yielded conflicting results. Ruiz et. al[230] administered a DNA vaccine encoding a peptide from myelin proteolipid protein (PLP) and observed a reduction in paralysis and production of Th1 cytokines from CD4+ T cells in mice undergoing the EAE model of MS. A phase I/II trial where a peptide derived from myelin basic protein (MBP) was administered to MS patients

delayed disease progression[231]. However, a phase III trial with the same peptide failed to be effective in patients with progressive MS[232], and another MBP peptide in an MHC complex also failed to show any efficacy in a phase I trial[233]. Similar results were observed in antigen specific therapy trials for TID[234].

Several studies have demonstrated the efficacy of oral tolerance in models of RA. For instance, administration of collagen II was shown to delay onset and severity of CIA in mice and rats[235–237]. Administration of collagen II orally to RA patients was well tolerated[238], however, there was only a small improvement in RA patients undergoing oral tolerance trials to collagen II[239]. Other attempts of peptide tolerogenic therapy for RA was the administration of a peptide from the dnaJ protein, a protein that has been implicated in RA due to sequence similarities with the RA shared epitope discussed in **section 1.4.1**. The dnaJ peptide was well tolerated in the trial and resulted in reduced production of TNFα from T cells and a simultaneous increase in IL-10 production[240]. However, administration of another antigen implicated in RA – human cartilage glycoprotein-39 – did not have an effect on RA progression[241]. Other methods of inducing tolerance to self-peptides in RA involve the use of tolerogenic DCs (tolDCs), which have been shown to stimulate regulatory responses from T cells[242]. tolDCs pulsed with collagen II significantly reduced disease severity in the CIA model of arthritis[243] and tolDCs loaded with antigens from synovial fluid of arthritic patients were tolerated when re-administered, and patients did not show any signs of worsening symptoms[244]. However, no changes in levels of regulatory cytokines nor in T cell phenotypes were observed in these patients, and so, the mechanism of action of these tolDCs in the patients is not understood.

1.5.1 Considerations for developing more effective antigen specific therapies to treat RA

One possible reason for the conflicting results of antigen specific therapies is the difference in tolerising naïve CD4+ T cells to tolerising effector or memory cells. Unlike naïve CD4+ T cells, effector and memory subsets can be activated with lower levels of antigen and do not necessarily require costimulation to generate an effector response[97,98]. Considering the chronicity of autoimmune diseases, the CD4+ T cells that are involved and targeted in antigen specific therapies are likely to be effector and memory T cells. As mentioned in **section 1.4.4,** the majority of CD4+ T cells

isolated from synovial tissues of arthritic patients have an activated phenotype. Thus antigen specific therapies targeting naïve cells are unlikely to work for effector or memory CD4+ T cells and may in fact result in memory CD4+ T cell activation.

Another factor to consider is timing of interventions using antigen specific therapies. A study by Mackenzie et. al[245] demonstrated that effector memory CD4+ T cells had reduced capacity for proliferation and cytokine production compared to central memory CD4+ T cells after administration of tolerogenic peptide in a mouse model of allergic airway inflammation. This highlights the importance of considering the phenotype of cells targeted for antigen specific therapies and when these cell types dominate the immune response to an antigen.

Anatomic location of the cells being targeted for therapy is another factor to consider when designing tolerogenic therapies. A study by David et. al[61] demonstrated that memory CD4+ T cells isolated from the spleen require two rounds of antigen specific activation to induce death of antigen specific cells. In contrast, ongoing work by Gray et. al (unpublished data – personal correspondence with Dr. Joshua Gray) demonstrated that effector cells tolerised with NP peptide derived from influenza in the periphery do not undergo apoptosis and can in fact be reactivated upon re-exposure to flu.

Developing antigen specific therapies for RA is not an easy task as many factors need to be considered from the antigen specificities that would be best to target, the phenotypes of cells to be targeted, and the location for optimal induction of tolerance. As mentioned in **section 1.4.4.2** there is strong evidence of antigen specific responses being involved in the propagation of RA which may create a permissive environment for other cells to enter the joint, thus perpetuating autoreactivity. Identifying the antigen specific CD4+ T cell clones that create these permissive environments will provide targets for tolerogenic therapies and provide more specific treatments to the ones currently available[244,246].

1.6 The OVA induced breach of tolerance model of experimental arthritis

Understanding the evolution of antigen specific responses requires the use of animal models as the role of antigen in RA onset is not understood and difficult to

pinpoint in humans. Animal models provide a setting whereby initiation and evolution of antigen specific responses can be observed with the progression of experimental arthritis. As RA is a heterogenous disease with complex aetiology, many models of RA have been developed to emulate different aspects of the disease. One of the most widely used animal models to study different aspects of RA is the collagen induced model of arthritis (CIA). This model requires an immunisation of collagen II in Freund's complete adjuvant (CFA) in genetically susceptible mice which causes collagen erosion in feet and paws[247,248]. Other models include SKG mice and K/BxN that develop RA spontaneously as well as models targeting cytokine pathways such as the TNFα transgenic mouse line[249]. However, it is difficult to monitor the development of antigen specific responses in these models for several reasons. Firstly, autoreactive responses cannot be distinguished from those resulting from immunisation with the initial peptide. For instance, in the CIA model, one cannot distinguish autoreactive responses from responses induced by collagen II in CFA, as collagen II is an antigen found in the joint. While in models that spontaneously develop arthritis, it is difficult to pinpoint where and when the disease initiates, thus making it difficult to track the development of antigen specific responses.

A good model to use to monitor the development of antigen specific responses is the OVA induced breach of tolerance model of experimental arthritis, which will also be referred to as the OVA model of experimental arthritis, developed by Maffia et. al[140] which relies on the use of a joint irrelevant antigen to initiate experimental arthritis (described in detail in **section 2.2.5** of methods). Specifically, the model uses TCR transgenic CD4+ T cells of known antigen specificity but of irrelevant specificity to the joint that have been polarised to a Th1 phenotype. Maffia et. al. demonstrated that transient arthritis was initiated by the transfer of Th1 polarised DO11.10 cells into BALB/c mice followed by immunisation with OVA in CFA and subsequent challenge using heat aggregated OVA (HAO) in the footpad, which was characterised by synovial hyperplasia and cartilage degradation. More importantly, the pathology observed is concomitant with breach of tolerance, evidenced by the production of collagen specific T and B cells responses. Experimental arthritis also developed when using C57BL/6 mice, but to a lesser extent[250]. Despite this, the advantage of using C57BL/6 mice as hosts is the availability of numerous knockouts, transgenic, and reporter strains on this background, which ultimately allows one to address a wider variety of questions. One other advantage of using this model to monitor the development of antigen specific responses is that the

transferred Th1 cells are trackable as they express a different congenic marker to the host strain (CD45.1 vs CD45.2 on OT-II and C57BL/6 mice respectively, or track the DO11.10 cells in BALB/c mice using the clonotypic KJ1.26 antibody) and therefore can be separated from endogenous CD4+ T cells. Thus, the development of antigen specific responses endogenous to the host strain can be monitored in conditions where autoreactive responses to joint antigens are most permissive, conditions that are difficult to ensure in RA patients and other animal models.

The OVA model of arthritis has also been used extensively over the past decade to examine the various roles the adaptive immune system may play in the early phases of RA and in conditions favouring breach of tolerance. These include the importance of Th1, but not Th2 cells in breach of tolerance[140], the roles conventional and plasmacytoid DCs play in initiating autoreactive T cells responses and their potential roles in early RA[250,251], the development of B cell responses with the progression of experimental arthritis[252], the impact of early therapeutic intervention on breach of tolerance[253], and the contribution of components of the innate and adaptive immune system to the breach of tolerance observed[254]. More recently, Prendergast et. al[191] showed interaction of endogenous CD4+ T cells with DCs in inflamed joints, thus demonstrating CD4+ T cells recognising their cognate antigen in the inflamed joint tissue itself.

1.7 Project aims and objectives

Despite the advancements made in RA therapeutics, there still remains an unmet clinical need for patients who fail or show partial remission to RA. And for those who do achieve remission, they are required to take the prescribed drugs indefinitely. Thus, the critical therapeutic objective now is to restore self-tolerance to self-peptides and achieve drug-free remission. To achieve this, the first steps are to understand how antigen specific responses develop in RA, the role antigen specific responses play in the early phases of disease, and how these responses develop with disease chronicity. Moreover, it is also important to establish where these antigen specific responses occur in the early phases of the disease. The hypothesis is that antigen specific CD4+ T cell responses in the early phases of disease occur in the joint and create a permissive environment for cells of other specificities to enter, perpetuating an inflammatory environment resulting in joint damage. Using the OVA model of experimental arthritis, the aims of this thesis are:

1. To assess the TCR clonality of CD4+ T cells in the OVA model of experimental arthritis with the aim of finding evidence supporting antigen specific accumulation of CD4+ T cells in inflamed joints.

2. To monitor the evolution of CD4+ T cell clonality from the early stages to a later more chronic phase of experimental arthritis in both inflamed joints and joint draining popliteal lymph nodes (pLNs).

3. To identify whether antigen specific responses occur in the inflamed joint and whether these responses are to self-antigens.

Addressing these aims should further our understanding of antigen specific responses in RA patients and identify the role of these autoreactive CD4+ T cells in the early phases of the disease. In addition, monitoring the antigen specific responses and specifically CD4+ T cell clonality can serve as a biomarker for specific disease stages facilitating patient stratification for more effective therapy. Furthermore, CD4+ T cell clonality can be used as a method to monitor response to therapeutics, as has been done to monitor leukemia[208]. Ultimately, understanding the development of antigen specific CD4+ T cell responses will contribute to the advancement of more effective tolerogenic antigen specific therapies in RA and toward therapies providing drug-free remission.

Chapter 2 Materials and Methods

2.1 Animals

Seven to 12-week-old male and female C57BL/6 mice were purchased from Harlan (now Envigo), UK and used as adoptive transfer recipients. Nur77GFP mice described by Moran et. al[57] were purchased from Jackson Laboratories (cat. no. 016617) and were also used as adoptive transfer recipients. OT-II TCR transgenic mice contain CD4+ T cells that only express a TCR recognising peptide 323-339 from chicken ovalbumin (OVA) in the context of MHC II molecule I-A^{b} [204]. Mice were bred and maintained at the University of Glasgow's central research facility and housed under standard housing conditions specified by the UK Home Office. All protocols were conducted under licences issued by the UK Home Office under the Animals (Scientific Procedures) Act of 1986 and approved by the University of Glasgow Ethical Review Committee.

2.2 OVA Models of Experimental Arthritis and Associated Components

The OVA model of experimental arthritis was conducted as described by Maffia et. al.[140]. Preparation of the required components and induction of the models will be described in the following subsections.

2.2.1 Isolation of Cells from Lymphoid Organs

Cells were isolated from spleens, popliteal lymph nodes, peripheral lymph nodes (inguinal, brachial, and axillary), superficial cervical lymph nodes, mesenteric lymph nodes, and lumbar lymph nodes of OT-II transgenic mice. Spleens and lymph nodes were mashed separately using a 70µm EASYStrainer (Greiner-Bio-One) fitted onto a 50mL centrifuge tube (Corning) using the flat end of a plunger from a 1mL syringe (Fisher Scientific). Strainer was washed with 1X MACS buffer (phosphate buffered saline (PBS) (Gibco) and 2% foetal calf serum (FCS) (Gibco)) to get maximum cell yield. Cells were then pelleted by centrifugation at 400xg for 5 minutes at 4°C. Splenocytes were further treated with Red Blood Cell (RBC) Lysis Buffer (eBioscience) using 500µL of RBC lysis buffer per spleen, incubated at room temperature for 3 minutes, washed then pelleted. Cells were then resuspended in MACS buffer to achieve the concentration required for CD4+ T cell purification.

2.2.2 CD4+ T Cell Purification and Th1 Polarisation

CD4+ T cells were purified by negative antibody selection (Miltenyi Biotech, 130-104-454, Surrey, UK) from OT-II mice spleens and LNs according to the protocol outlined in the kit. $25x10^6$ CD4+ OT-II T cells were cultured with complete media (RPMI 1640, Gibco, Thermofisher; 10% FCS; 1% Penicillin and streptomycin; 1% L-glutamine) in a 1:5-1:10 ratio of CD4+ OT-II T cells: mitomycin C (Sigma-Aldrich) treated APCs. APCs were collected from leftover cells following CD4+ T cell purification and from C57BL/6 spleens. APCs were treated with 0.05mg/mL mitomycin C (500µL 1mg/mL of mitomycin C in PBS per 10mL of cells) for 45-60 minutes at 37°C and 5% CO_2 and washed twice in PBS before being cultured with OT-II T cells. OT-II T cells and mitomycin C treated APCs were cultured with complete media containing the following Th1 polarising reagents: 1µg/mL OVA peptide 323-339 (Sigma-Aldrich), 10ng/mL IL-12 (Bio-Techne), and 2µg/mL murine monoclonal anti-IL4 antibody (2BScientific, clone 11B11). Cells were cultured in $75cm^2$ vented flasks (Corning) at a tilt at 37°C, 5% CO_2 for 72 hours. After 72 hours, percentage of Th1 polarised cells was determined by flow cytometry using antibodies against Vα2, Vβ5, and CD4, details of which can be found in **Table 2-3**.

2.2.3 Preparation of OVA in Freund's Adjuvant (CFA)

Tissue culture grade OVA (Code OAC – Worthington Biochemicals) was used to make a 1µg/µL emulsion of OVA in CFA. OVA was prepared in PBS and was added to CFA diluted 1:1 in PBS. Using a 1mL syringe (Fisher Scientific) an emulsion was created by quickly and repeatedly taking the mixture up and releasing it back into the vessel. The emulsion was ready once a solid pellet formed, with no oily waves dissipating from it, when dropped into a dish with water or PBS.

2.2.4 Preparation of Heat Aggregated OVA (HAO)

HAO was prepared as 20mg/mL stocks using a solution of OVA (Sigma-Aldrich) in PBS. 200µL of the 20mg/mL OVA solution was placed in 1.5mL Eppendorf tubes (Greiner Bio-One) and incubated at 100°C for 2 hours. Tubes were then spun at 13,300 rpm for 5 minutes and supernatant removed. Aggregated OVA was washed with PBS and spun again. Supernatant was again removed, and 200µL of PBS was added. Aliquots were frozen until required for use in the model.

2mg/mL HAO was required for the model. Stock HAO aliquots were thawed and contents placed in GentleMACS c-tubes (Miltenyi Bio). 1.8mL of PBS was added to the c-tube per HAO stock aliquot. HAO was homogenised using a gentleMACS Dissociator (Miltenyi Biotec) using a customised program, details of which are in **Table 2-1**. The program was run 4-6 times and HAO was deemed ready when the solution passed through a 25 or 26 gauge needle.

Table 2-1 HAO Dissociation Program

Time (sec)	RPM	Time (sec)	RPM	Time (sec)	RPM	Time (sec)	RPM
90	0	67	+ 1000	44	+ 1800	21	- 1400
89	+ 200	66	- 200	43	+ 1000	20	+ 1500
88	+ 500	65	- 600	42	- 200	19	- 1400
87	+ 900	64	- 1000	41	- 1700	18	+ 1500
86	+ 900	63	- 1300	40	- 2100	17	- 1400
85	+ 1300	62	- 1500	39	- 3000	16	+ 1500
84	+ 1600	61	- 2600	38	- 3700	15	0
83	+ 2000	60	- 3000	37	- 3900	14	0
82	+ 2500	59	- 3400	36	- 3700	13	0
81	+ 2800	58	- 3800	35	- 3200	12	0
80	+ 3100	57	- 3800	34	- 2800	11	+ 1100
79	+ 3300	56	- 3200	33	- 2000	10	+ 1100
78	+ 3300	55	- 2000	32	- 400	9	+ 1100
77	+ 3500	54	- 800	31	- 200	8	+ 1200
76	+ 3700	53	+ 1000	30	+ 600	7	+ 1100
75	+ 3800	52	+ 1600	29	+ 1200	6	+ 1100
74	+ 3900	51	+ 2200	28	+ 1500	5	+ 500
73	+ 3800	50	+ 2700	27	+ 1300	4	+ 500
72	+ 3500	49	+ 3600	26	0	3	+ 300
71	+ 3100	48	+ 3900	25	- 1400	2	+ 700
70	+ 3100	47	+ 3900	24	+ 1500	1	0
69	+ 2500	46	+ 3900	23	- 1400	0	0
68	+ 1900	45	+ 3900	22	+ 1500		

2.2.5 Induction of the OVA Model of Experimental Arthritis (Early Timepoint)

2-3x10^6 Th1 polarised OT-II CD4+ T cells were injected i.v in C57BL/6 or Nur77GFP recipients. This was followed by immunisation with OVA protein (Sigma-Aldrich) in Freund's complete adjuvant (CFA) (Sigma-Aldrich) subcutaneously in the scruff the next day. 10 days later the mice were injected subcutaneously in both footpads close to the ankle joint with 50µL PBS, 100µg heat aggregated OVA in 50µL (HAO),

or 25µg of lipopolysaccharide (LPS) (from *E. coli* O111 (Sigma-Aldrich)) in PBS in 50µL. Mice were sacrificed 4 days after footpad injections, as this timepoint was found to have the highest number of T cell infiltration into the joints (Drs. Robert Benson and Catriona Prendergast unpublished data). Joints and pLNs were then collected for processing and cell isolation.

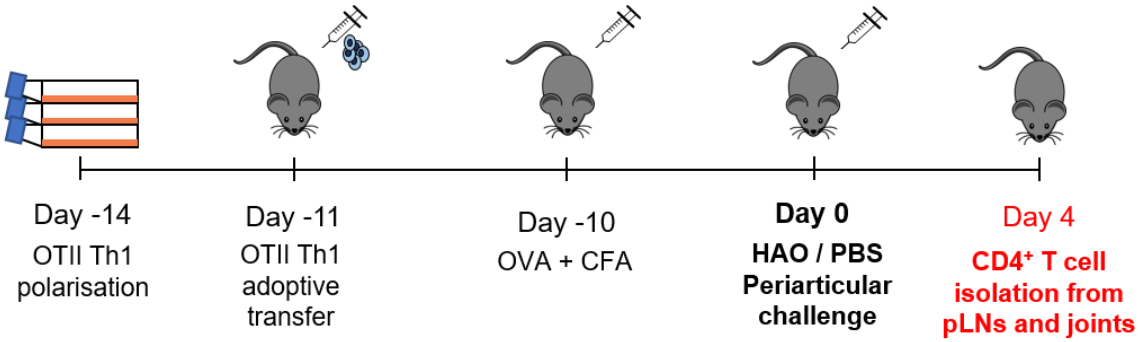


Figure 2-1 Experimental timeline of the early timepoint of the OVA model of experimental arthritis
Illustration of the experimental timeline of the OVA model of experimental arthritis terminating at the early timepoint. Timeline is centred around day of periarticular challenge. Model setup begins with the polarisation of OT-II cells to the Th1 phenotype for 72 hours. 2-3x10^6 Th1 polarised OT-II cells are transferred into C57BL/6 or Nur77GFP recipients, which is followed by an immunisation of OVA in CFA in the scruff. 10 days later mice are challenged with HAO in the periarticular tissue to induce breach of tolerance and the initiation of experimental arthritis. PBS or LPS are administered to other mice as controls. Mice were sacrificed 4 days after periarticular challenge and joint draining popliteal lymph nodes (pLNs) and joints were collected for processing and cell isolation.

2.2.6 Induction of Inflammation in Non-Arthritic Tissues (Ears)

The OVA model of experimental arthritis was adapted to induce inflammation in non-articular tissues to determine differences in TCR use in these tissues compared with articular tissues. This allows assessment of whether a bias in TCR use is due to accumulation of CD4+ T cells that have breached tolerance and now react to articular antigens. Again, 2-3x10^6 Th1 polarised OT-II CD4+ T cells were injected i.v in C57BL/6 recipients, which was followed by immunisation with OVA in CFA in the hind limbs. 10 days later mice were injected with 100µg HAO in 25µL in the ear pinnae. Control mice were injected with 25µL of PBS. Mice were sacrificed 4 days later to be consistent with the OVA model of arthritis. Super cervical lymph nodes (scLNs) and ears were then collected and processed for cell isolation.

2.2.7 Induction of the OVA Model of Experimental Arthritis (Late Timepoint)

One of the aims of the project is to determine the evolution of the antigen specific response with the progression of experimental arthritis. To do this, a model of relapsing arthritis, developed by Conigliaro et. al.[252] was modified for the purposes of project. The model of relapsing arthritis is also based on the OVA model developed by Maffia et. al. and will be referred to as the late model of OVA experimental arthritis. The late model of experimental arthritis was developed to investigate the anti-collagen antibody response in a more chronic setting. In the study, Conigliaro et. al determined that the model begins to resolve after 21 days and so, the endogenous CD4+ T cells response was examined within that time frame and is discussed in **Chapter 3 section 3.2.1.5**. The initial model set up is the same as that for the early timepoint up to the point of periarticular administration of either HAO or PBS. A second periarticular challenge of 100µg of HAO in incomplete Freund's adjuvant (IFA) (Sigma-Aldrich) is administered 30 days after the primary HAO challenge. Mice were then sacrificed, 4, 11, or 17 days after the second periarticular challenge and pLNs and joints were then collected for processing and cell isolation. In order to verify that the observed response is due to the second periarticular challenge, several controls were required at each timepoint, details of which can be found in the following table:

Table 2-2 Experimental and control groups in the late model of experimental arthritis

Group name	Group type	Primary periarticular challenge	Secondary periarticular challenge
Late inflamed	Experimental	HAO	HAO+IFA
Non-specific (NS) inflammation	Control	HAO	IFA
Primary	Control	PBS	HAO
Memory	Control	HAO	PBS
Baseline	Control	PBS	PBS

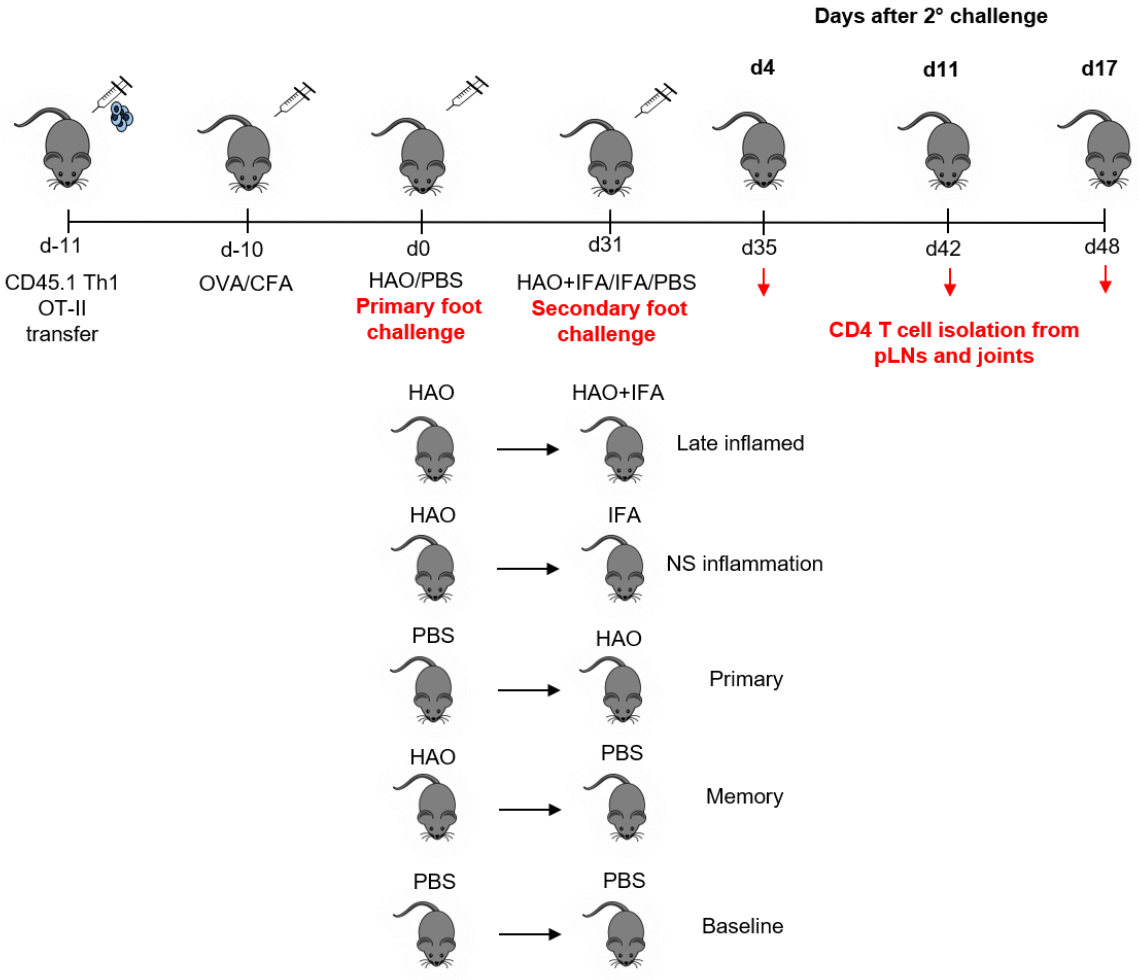


Figure 2-2 Experimental timeline of the late timepoint of the OVA model of experimental arthritis and controls
Illustration of the experimental timeline of the OVA model of experimental arthritis to determine a late timepoint. Timeline is centred around day of primary periarticular challenge. Model setup begins with the polarisation of OT-II cells to the Th1 phenotype for 72 hours. 2-3x10^6 Th1 polarised OT-II cells are transferred into C57BL/6 or Nur77GFP recipients, which is followed by an immunisation of OVA in CFA in the scruff. 10 days later mice are challenged with HAO or PBS in the periarticular tissue to induce breach of tolerance and the initiation of experimental arthritis. Mice were given a second periarticular challenge of either HAO+IFA, IFA, HAO, or PBS depending on the experimental or control group 30 days after primary periarticular challenge. Mice were sacrificed 4, 11, or 17 days after secondary challenge and pLNs and joints were collected for processing and cell isolation.

2.2.8 Power calculations

To determine the number of animals required for each experiment, the following power calculations were performed using G*Power software version 3.1.9.6. Power calculations indicate that 5 animals per group are required to detect large effects (d=2.34) with 95% power and alpha set to 0.05 using Student's t-test to determine differences between two independent means. Using one-way ANOVA,

power calculations indicate that 9 animals per group are required to detect an effect size of f=2 with 80% power and an alpha set to 0.05.

2.3 Assessment of Joint Inflammation

Inflammation was assessed by measuring footpad thickness after HAO, PBS, or LPS administration daily until experiment termination using C-Serie IP67 electronic callipers (Kroeplin GmbH).

2.4 Isolation of Cells from Tissues

2.4.1 pLNs and scLNs

Popliteal lymph nodes (pLNs) or super cervical lymph nodes (scLNs) were forced through 40µm Nitex mesh (Cadisch Precision Meshes) to obtain single cell suspensions and shaken at 110 rpm at 37°C for 25 minutes with 2.68mg/mL collagenase D (Roche). Collagenase D reaction was quenched using complete media (RPMI 1640, Gibco, Thermofisher; 10% FCS; 1% Penicillin and streptomycin; 1% L-glutamine). Single cell suspensions were washed in PBS and pelleted by centrifugation at 400xg for 5 mins at 4°C. Dead volumes of pLN samples were measured and cells counted by trypan blue (Sigma Aldrich) exclusion using a haemocytometer (Fisher Scientific), and subsequently stained for flow cytometry. pLNs and scLNs were pooled per mouse for analysis.

2.4.2 Spleens

Spleens were forced through 40µm Nitex mesh (Cadisch Precision Meshes) or through a 70µm EASYstrainer mesh (Greiner-Bio-One) to obtain single cell suspensions and shaken at 110 rpm at 37°C for 25 minutes with 2.68mg/mL collagenase D (Roche). Collagenase D reaction was quenched using complete media. Single cell suspensions were washed with FACS buffer (PBS + 2mM EDTA + 2% FBS) and pelleted using centrifugation at 400xg for 5 mins at 4°C. Splenocytes were the treated with RBC lysis buffer (eBioscience) using 500µL of RBC lysis buffer per spleen. Samples were briefly vortexed and incubated for 3 mins at room temperature, then washed in FACS buffer. Samples were resuspended in 10mL PBS, cells were counted by trypan blue (Sigma Aldrich) exclusion using a haemocytometer (Fisher Scientific), and subsequently stained for flow cytometry.

2.4.3 Joints

Skin and muscle tissue were removed from mouse feet. Ankle and joints were teased apart and shaken at 110 rpm at 37°C for 25 minutes with 2.68mg/mL collagenase D (Roche) in RPMI 1640 (Gibco, Thermofisher). Collagenase D reaction was quenched using complete media. Tibias and fibulas were removed and joint tissue was then homogenised in gentleMACS C tubes (Miltenyi Biotech) using a gentleMACS Dissociator (Miltenyi Biotech) set at 3000 rpm for 15 seconds 3 times. Homogenised tissue was then strained using 70µm EASYstrainer mesh (Greiner) and pelleted by centrifugation at 400xg for 5 mins at 4°C. Dead volumes were measured and cells counted by trypan blue exclusion using a haemocytometer. Single cell suspensions were then washed in PBS and subsequently stained for flow cytometry. Joints were pooled per mouse for analysis.

2.4.4 Ears

Ears were cut up into small pieces and shaken at 110rpm at 37°C for 25 minutes with 2.68mg/mL collagenase D (Roche) and 2mg/mL hyaluronidase (Sigma Aldrich) in RPMI 1640 (Gibco, Thermofisher). Collagenase D and hyaluronidase reactions were quenched using complete media. Tissues were then homogenised in gentleMACS C tubes (Miltenyi Biotech) using a gentleMACS Dissociator (Miltenyi Biotech) set at 3000 rpm for 15 seconds 3 times. Homogenised tissue was then strained using 70µm EASYstrainer mesh (Greiner) and pelleted by centrifugation at 400xg for 5 mins at 4°C. Dead volumes were measured and cells counted by trypan blue exclusion using a haemocytometer. Single cell suspensions were then washed in PBS and subsequently stained for flow cytometry. Ears were pooled per mouse for analysis.

2.5 Flow Cytometry

Background staining was controlled for using both isotype and fluorescence minus one (FMO) controls. For Vβ antibodies, antibodies were used, for other extracellular antibodies e.g. ICOS, an FMO was used.

2.5.1 Extracellular Staining

Cells resuspended in PBS were transferred to 96 well round-bottom plates (Corning) for staining. All cells from pLNs, joints, scLNs, and ears were transferred to plates. For spleens, approximately $5x10^6$ cells were transferred for staining. Cells were pelleted by centrifugation at 400xg for 5 mins at 4°C, resuspended in 100µL viability dye, and incubated for 30 mins at 4°C. Cells were then washed in FACS buffer (PBS + 2mM EDTA + 2% FBS) and resuspended in 50µL FcR block (generated in house using supernatant from the 2.4G2 cell line) and incubated at 4°C for 10 mins. Appropriate flow cytometry antibodies were added in FACS buffer and samples were incubated for a further 15 mins at 4°C. Cells were then washed in FACS buffer and filtered through Nitex mesh (Cadisch Precision Meshes) prior to running on BD LSR II, BD Fortessa, or BD FACS Celesta analysers (BD Biosciences). Data was then analysed using FlowJo software (Treestar version 10, Oregon, USA). Details of antibodies used are listed in **Table 2-3**

2.5.2 Intracellular Staining

Cells were stained intracellularly after samples were incubated with antibodies staining extracellular proteins. Samples were washed in PBS before staining for intracellular proteins. To account for background staining, isotype controls were included for all intracellular stains used, except for anti-GFP, where a fluorescence minus one (FMO) control was used.

2.5.2.1 Intracellular Cytokine Staining

To stain for intracellular cytokines, cells were fixed and permeabilised using 100µL of Cyotofix/Cytoperm solution (BD Bioscience) for 1 hour at 4°C then washed in 100µL Perm/Wash buffer (BD Bioscience). Cells were then stained with 100µL of the appropriate antibodies prepared in Perm/Wash buffer and stained for 1 hour at 4°C. Cells were then washed once in Perm/Wash buffer and then in FACS buffer, filtered through Nitex mesh and analysed on flow cytometers.

2.5.2.2 Staining of Transcription Factors and Other Nuclear Proteins

Staining of transcription factors and nuclear proteins were performed on Nur77GFP cells and to maintain the GFP signal, staining protocols were adjusted depending on the antibodies used. When Nur77GFP cells were stained with an anti-GFP

antibody as well as antibodies staining transcription factors, the staining protocol used was adapted from methods developed by Grupillo et. al. and Heinen et. al.[255,256]. Cells were fixed using 100µL of 2% formaldehyde (10% formalin with 4% formaldehyde (Sigma Aldrich) diluted 1:1 in PBS) for 45-60 mins at 4°C. Cells were then washed twice in 1X permeabilisation buffer (eBioscience) and stained with the appropriate antibodies prepared in permeabilisation buffer at 4°C overnight. Samples were then washed once in permeabilisation buffer then in FACS buffer and analysed on flow cytometers.

2.5.3 Vβ Antibody Titration

Optimal dilutions of the Vβ antibodies for flow cytometry were determined by testing 1:50, 1:100, and 1:200 antibody dilutions prepared in FACS buffer and tested on lymphocytes collected from naïve C57BL/6 mice. Some Vβ antibodies were conjugated to biotin, therefore optimal streptavidin dilutions were also determined for biotin conjugated Vβ antibodies. Streptavidin dilutions were optimal when matched with the dilutions used for the biotin conjugated Vβ antibodies. Dilutions used are outlined in **Table 2-3**.

Table 2-3 List of Antibodies Used for Flow Cytometry

Marker	Fluorochrome	Clone	Concentration in sample	Manufacturer	Cat. No.
CD4	BUV395	GK1.5	1:200	BD Biosciences	563790
CD4	BUV737	GK1.5	1:200	BD Biosciences	564298
CD4	eVolve 605	RM4-5	1:20	eBioscience	83-0042-41
CD4	eFluor 450	RM4-5	1:200	eBioscience	48-0042-82
CD44	APC eFluor 780	IM7	1:200	eBioscience	47-0441-82
CD44	PerCP Cy5.5	IM7	1:200	eBioscience	45-0441-82
CD45	PE Cy7	30-F11	1:200	eBioscience	25-0451-82
CD45.1	eFluor 450	a20	1:200	eBioscience	48-0453-82
CD278 (ICOS)	BV785	C398.4A	1:200	BioLegend	313539
GFP	AlexaFluor 647	Polyclonal	1:400	ThermoFisher	A31852
IFNγ	PE	XMG1.2	1:400	BD Biosciences	554412
IL-2	BV711	JES6-5H4	1:200	BioLegend	503837
MHC-II	APC	M5/114.15.2	1:800	eBioscience	17-5321-82
RORγT	PE	AFKJS-9	1:100	eBioscience	12-6988-82
Streptavidin	PerCP Cy5.5	-	1:200	eBioscience	45-4317-82
Tbet	PE Cy7	4B10	1:100	eBiosceince	25-5825-82
TNFα	BV605	MP6-XY22	1:100	BioLegend	506329
Vβ2	FITC	B20.6	1:50	BD Pharmingen	553280
Vβ3	Biotin	KJ25	1:200	BD Pharmingen	553207
Vβ4	Biotin	KT4	1:200	BD Pharmingen	553364
Vβ4	FITC	KT4	1:200	BD Pharmingen	553365
Vβ4	PE	KT4	1:200	BD Pharmingen	553366
Vβ5.1/5.2	APC	MR9-4	1:100	BioLegend	139506
Vβ5.1/5.2	FITC	MR9-4	1:200	eBioscience	11-5796-82
Vβ6	PE	RR4-7	1:200	BD Pharmingen	553194
Vβ7	FITC	TR310	1:200	BD Pharmingen	553216
Vβ8.3	FITC	8C1	1:200	BioLegend	118603
Vβ9	PE	MR10-2	1:200	BD Pharmingen	553201
Vβ10[b]	PE	B21.5	1:200	BD Pharmingen	553285
Vβ11	Biotin	RR3-15	1:200	BD Pharmingen	553196
Vβ11	FITC	RR3-15	1:200	BD Pharmingen	553197
Vβ12	Biotin	MR11-1	1:200	BD Pharmingen	553299
Vβ13	APC	MR12-3	1:200	BD Pharmingen	561542
Vβ14	Biotin	14-2	1:200	BD Pharmingen	553257
Viability	eFluor 506	-	1:1000	eBiosceince	65-0866-14

2.6 CDR3β Amplification by PCR

Vβ CDR3 clonality was assessed using SuperTCR*Express*™ (BioMed Immunotech, Florida, US) on endogenous $CD4^+$ T cells sorted from inflamed joints and popliteal LNs using the BD FACS ARIAIII. Sorted cells were lysed and the RNA extractions,

cDNA synthesis and PCR performed as per the manufacturer's instructions. PCR products were run on a 4% agarose gel and visualized on a GelDoc system (BioRad, Hertfordshire, UK).

2.7 Fluorescence Activated Cell Sorting (FACS) and CDR3β Sequencing

2.7.1 Sorting Endogenous CD4+ T Cells from Tissues

Cells were isolated from pLNs, joints, scLNs, and ears as described in **section 2.2.1**. Cells were stained for viability and with antibodies against CD4, CD45, CD45.1, and CD44 and sorted using either the BD AriaIIU or BD Aria III FACS sorters (BD Biosciences) using a 100µm nozzle. Sorters were prepared for an RNA free sort by cleaning with nuclease free water Samples were run in FACS buffer and gated on live, single cells that were CD4+, CD45+, and CD45.1- to ensure only the endogenous CD4+ T cells were collected. Endogenous CD4+ T cells were then further gated on CD44 and CD44hi cells were collected. Sorters were set up for a 4-way sort, 4-way purity precision, and on a continuous setting. Cells were collected in 1.5mL Eppendorf tubes (Greiner-Bio-One) containing Lysis Buffer (Purelink RNA micro kit, Thermofisher, cat.no. 12183-016) with 1% β-mercaptoethanol (Sigma-Aldrich) and lysed by passing 3 times using 29G insulin syringes (VWR, 613-4897). Sorting chamber was cleaned with nuclease free water (Roche) using the 'sample line backflush' program once before every sample. Cells were then frozen at -80°C before RNA purification.

2.7.2 RNA Purification

RNA was purified using the Purelink RNA micro kit (Thermofisher) as per manufacturer's instructions. RNA was eluted from columns using 10µL of Elution Buffer. For all joint and ear samples, and pLN PBS samples, carrier RNA was added as per manufacturer's instructions. RNA concentration was determined using a Nanodrop 1000 spectophotometer (Thermofisher) and quality was determined by measuring A260/280. Purified RNA was frozen at -80°C before CDR3β sequencing. RNA concentrations ranged from 1.3-48ng/µL.

2.7.3 CDR3β Sequencing

CDR3β sequencing was performed by iRepertoire Inc. (Huntsville, AL, USA) on the Vβ TCR chain. Frozen RNA samples were shipped on dry ice, and each sample was PCR amplified using barcoded primers covering the V-J TCR region, enabling sequencing data to be tracked back to a specific sample. Amplified samples were pooled together and sequenced using the Illumina MiSeq platform using 150 paired end reads (PER) primers. PBS joint and PBS pLN samples did not pass iRepertoire's quality control and these samples were generated again and sequenced using the same parameters. Unlike the other samples, PBS pLN and PBS joint sample replicates were pooled, resulting in having one biological replicate for each PBS challenged tissue.

2.8 CDR3β Sequencing Analysis

Basic data analysis was performed by iRepertoire (Huntsville, AL, USA) and data was available on iRepertoire's data web viewer iRweb. Sequencing data was also prepared for analysis using MiXCR developed by Bolotin et. al.[257] and analysed using tcR developed by Nazarov et. al.[258].

2.8.1 Calculation of D50 diversity index using iRepertoire generated data

Repertoire diversity can be calculated in several ways, but the diversity index (DI) used in this thesis is based on the calculation used by iRepertoire and is called D50 (from iRepertoire's data analysis guide). The D50 value represents the cumulative percentage of T cell clones that accounts for 50% of the total CDR3β sequence reads. The higher the diversity, the closer the value is to 50. Two formulae exist to calculate the D50 value depending on the number of total unique CDR3β sequences generated; 10,000 unique CDR3β and above, or below 10,000 unique CDR3β sequences, which are found below:

No. of unique CDR3β sequences ≥ 10,000:

1. Obtain the total number of reads of the top 10,000 unique CDR3β sequences.

2. Calculate 50% of this value

3. Find the rank of unique CDR3β sequence up to which the total value of reads approximately matches the value obtained in point 2. 50% of the total number of reads usually falls between 2 ranks, and the lower rank is chosen to calculate the D50 value. I.e. if 50% of total number of reads falls between unique CDR3β sequences at positions 14 and 15, 15 is chosen to calculate D50.

4. Calculate D50:

   (rank of unique CDR3β sequence where 50% of the top 10,000 total reads falls x 100) / 10,000 = D50

No. of unique CDR3β sequences <10,000:

1. Calculate the total number of reads of all unique CDR3β sequences

2. Calculate 50% of the total number of reads

3. Find the rank of unique CDR3β sequence up to which the total value of reads approximately matches the value obtained in point 2. 50% of the total number of reads usually falls between 2 ranks, and the lower rank is chosen to calculate the D50 value. I.e. if 50% of total number of reads falls between unique CDR3β sequences at positions 14 and 15, 15 is chosen to calculate D50.

4. Calculate D50:

   (rank of unique CDR3β sequence where 50% of the total reads falls x 100) / no. of unique CDR3β sequences = D50

2.8.2 Preparation of Sequencing Data by MiXCR

Demultiplexed sequencing data was downloaded onto University of Glasgow servers and prepared for analysis using MiXCR using the following Linux code:

```
#!/bin/bash

read1=$1
read2=$2
```

```
name=$3

/export/projects/bioinfo3/to16r/bin/mixcr-2.1.5/mixcr align -f -t 12 -s mmu -r $name.align.report.log $read1 $read2 $name.vdjca

/export/projects/bioinfo3/to16r/bin/mixcr-2.1.5/mixcr assemble $name.vdjca $name.clns

/export/projects/bioinfo3/to16r/bin/mixcr-2.1.5/mixcr exportClones $name.clns $name.txt
```

This code aligns, assembles, and creates .clns (clone) files. .clns files were then converted to .txt files for subsequent analysis using the tcR R package[258].

2.8.3 Analysis of Sequencing Files with the tcR R package

The tcR R package was specifically designed to analyse sequencing data generated by several software including MiXCR. The package was used in the R statistical software framework (R version 3.4.3) and the code detailed below was used for visualising and analysing TCR sequencing data.

2.8.3.1 Organisation of Data

Individual sample text files were compiled into lists using the following code:

```
List_name <- list(sample_name1=sample_name1, sample_name2=sample_name2,…,sample_nameN=sample_nameN)
```

2.8.3.2 Top proportion charts

To obtain figures such as **Figure 4-3 A**, use the following code:

```
vis.top.proportions(“name of list”, c(10, 25, 500, 3000, 10000), .col = "Read.count")
```

2.8.3.3 PCA Plots

To make a PCA plot of V-gene usage such as **Figure 4-7**, use the following code:

```
vis.pca((pca.segments(“name of list”, .cast.freq.seg = T, .text = F, .do.plot = F)),
```

```
        .groups = list(“name of group within list_1” = c(1, 2, 3, 4, 5), “name of group within list_2” = c(6, 7, 8, 9, 10))
```

2.8.3.4 Heatmaps Showing CDR3β Sequence Overlap

```
vis.heatmap(repOverlap(“name of list”, 'exact', 'aa', .vgene = F, .norm = T, .verbose = F), .title = "No. Shared Clonotypes pLN Samples",
.scientific = T, .labs = c(" "," "), .legend = "# clonotypes", .size.text = 2.5, .signif.digits = 3)
```

2.8.3.5 Searching for specific CDR3β Sequences Across Data Frames

This code was used to look for the top 10 clones in joint samples and to check whether the same CDR3β sequences could be found in respective pLN samples, and how those sequences ranked in the pLN samples. In order to do this, the data set being searched needed to be ranked using this code:

```
“List_name” <- set.rank(“List_name”)
```

Then, the top 10 clones in a joint sample were found and the sequences searched in the respective pLN sample:

```
new_variable_name <- find.clonotypes(“name of pLN dataset to be searched”, .targets = “name of joint data set” [1:10, c('CDR3.amino.acid.sequence', 'V.gene')],
.method = c("exact"), .col.name = c("Read.count", "Rank"), .target.col = c("CDR3.amino.acid.sequence", 'V.gene'), .verbose = T)
```

The results were then written into tab delimited text files:

```
write.table(“new variable name”, file = "’Desired name to be given to file’.txt", sep = "\t")
```

2.9 *In vitro* T Cell Stimulations

To test for Nur77GFP expression *in vitro,* 96 well round-bottom plates were coated with 50µL 0.1-10 µg/mL anti-CD3 antibody (NA/LE hamster anti-mouse CD3e, clone 145-2C11, BD Biosciences) prepared in PBS and incubated overnight at 4°C. The

anti-CD3 solution was removed by flicking and tapping the plates onto tissue paper. Lymphocytes from Nur77GFP mice were collected and forced through 70µm EASYstrainer mesh (Greiner-Bio-One) and washed with PBS. Cells were then counted by trypan blue exclusion using a haemocytometer and resuspended in PBS to obtain a concentration of $1.5x10^6$ cells/mL. 200µL of the cell suspension was added to respective wells in triplicate and incubated for either 3 or 24 hours for Nur77GFP expression and Nur77GFP time-course experiments respectively. Cells were cultured at 37°C and 5% CO_2.

2.10 T Cell DC Co-cultures

2.10.1 Generation of BMDCs

BMDCs were generated by culturing bone marrow obtained from femurs and tibias of C57BL/6 mice in complete media supplemented with 5% GM-CSF (RPMI 1640, Gibco, Thermofisher; 10% FCS; 1% Penicillin and streptomycin; 1% L-glutamine, GM-CSF made in house from X63 GM-CSF cell line). Bone marrow was removed from bones by forcing Hank's balanced salt solution (HBSS, No Ca^{2+}, No Mg^{2+}, no phenol red, ThermoFisher) into bones using a 25G needle. Bone marrow was then pipetted several times then pelleted by centrifugation at 400xg for 5 mins at 4°C. Cells were then treated with 1mL RBC lysis buffer per mouse and incubated for 2 mins at RT. Cells were then washed, pelleted, and counted by trypan blue exclusion using a haemocytometer. $1.5x10^6$ cells were added to 6-well non-TC treated plates (Corning) and cultured for 7 days at 37°C and 5% CO_2. Cells were fed with complete media supplemented with GM-CSF on days 3 and 6.

2.10.2 T cell DC Co-culture for Testing Nur77GFP Re-stimulation with OVA

BMDCs were pulsed with OVA prior to culture with T cells. On day 7 of BMDC culture, BMDCs were cultured with complete media supplemented with 100µg/mL OVA protein (Code OAC – Worthington Biochemicals) for 24 hours at 37°C at 5% CO_2. DCs were harvested by incubating cells with cold HBSS supplemented with 2mM EDTA for 10 mins at room temperature, then washed in HBSS, and resuspended in complete media. CD4+ T cells were purified from brachial and axillary lymph nodes of mice (as described in **section 2.2.1**) immunised with 1µg/mL OVA in CFA (described in **section 2.2.3**) in the scruff 7 days prior, and from naïve

Nur77GFP mice. CD4+ T cells were purified by negative antibody selection (Miltenyi Biotech, 130-104-454, Surrey, UK), and counted by trypan blue exclusion using a haemocytometer. $1x10^6$ purified CD4+ T cells were co-cultured with OVA pulsed DCs in a 5:1 ratio in 48 well plates (Corning) for cytokine analysis, or in 24 well plates for proliferation analysis. For cytokine analysis T cell DC co-cultures were cultured for 6 hours at 37°C and 5% in complete media. 2 hours into the culture, Golgistop (BD Biosceinces) diluted 1:1000 in complete media (BD Biosciences) was added to the wells. For positive controls, cells were stimulated with 10ng/mL PMA (Sigma Aldrich) and 500ng/mL ionomycin (Sigma Aldrich) and cultured with complete media supplemented with 1:1000 Golgistop. Cells were then analysed by flow cytometry. Method of measuring proliferation is detailed in the following section.

2.10.3 Cell Proliferation Assay

Purified CD4+ T cells were pelleted and resuspended in warmed PBS (37°C) supplemented with 2% FCS to a concentration of $1x10^6$ cells/mL. Approximately $1x10^6$ CD4+ T cells were incubated for 20 mins at 37°C and 5% $CO_2$with 1uL/mL of CellTrace Violet (Invitrogen), prepared according to manufacturer's instructions. 10mL complete media was added and the cells were incubated for a further 3 mins. Cells were then washed, pelleted, and resuspended in complete media and cultured with OVA pulsed DCs in a 5:1 ratio in 24 well plates. Cells were cultured for 96 hours at 37°C and 5% CO_2 and then prepared for analysis by flow cytometry.

2.10.4 CD4+ T Cell DC Co-culture with Joint and Candidate RA Antigens

Cells were isolated from pLNs and joints of Nur77GFP mice as described in **sections 2.4.1 and 2.4.3**. Cells were pooled per sample per group prior to CD4+ T cell purification. CD4+ T cells were purified by negative selection using EasySep Mouse CD4+ T cell Isolation Kit (Stemcell Technologies) as per manufacturer's instructions. CD4+ T cells isolated from joint samples needed to go through two rounds of purification before use in co-culture experiments. 20,000-30,000 purified CD4+ T cells were cultured with DCs in a 5:1 ratio which were simultaneously pulsed with either 100µg/mL OVA protein (Code OAC – Worthington Biochemicals), a combination of 100µg/mL bovine collagen II (Sigma Aldrich) and 100µg/mL bovine aggrecan (Sigma Aldrich), or 50µL joint extract, which is the supernatant of LPS challenged or IFA challenged homogenised joints. Non-pulsed DCs were also

included to determine baseline Nur77GFP expression. Each group was cultured in duplicate. Cells were cultured in 48 well plates for 6 hours at 37°C and 5% CO_2. GolgiStop (containing Brefeldin A) (BD Biosciences) was added to each well to obtain a final dilution of 1:1000. Cells were cultured for a further 6-12 hours before preparation for flow cytometry analysis.

2.11 Enzyme-Linked Immunosorbent Assays (ELISAs)

Anti-OVA and anti-collagen II antibody concentrations were determined from serum samples of mice undergoing the early and late models of OVA experimental arthritis. Blood was collected from each mouse and allowed to clot at room temperature for up to 6 hours. Samples were then spun at 13,300 rpm for 5 mins. Supernatants were then transferred to 1.5mL Eppendorfs (Greiner-Bio-One) and frozen at -80°C. 96 transparent flat bottom plates (Costar) were coated with 50µL of 20µg/mL OVA (Sigma Aldrich) or 4µg/mL bovine collagen II (Sigma Aldrich) diluted in carbonate buffer (Sigma Aldrich) and incubated at 4°C overnight. Plates were then washed in 0.05% PBS-T (PBS + Tween20), blocked with 50µL animal free serum (Vector labs SP5030) for 1 hour at 37°C, then washed again in 0.05% PBS-T. Serum samples were diluted to the appropriate concentration in PBS and added to the plates in duplicate. Samples were incubated at 37°C for 1 hour. Plates were washed in 0.05% PBS-T and incubated with an HRP conjugated rabbit anti-mouse total IgG antibody (Sigma Aldrich) diluted to 1:10,000 in PBS at 37°C for 1 hour then washed again in 0.05% PBS-T. Antibodies were detected using 100µL of SigmaFAST OPD solution (Sigma Aldrich) for 10-30 mins at room temperature. Plates were covered in aluminium folder during the detection period. Reaction was stopped using 10% sulphuric acid. Absorbance was measured at 492nm using a Tecan Sunrise plate reader (Tecan). Values were subtracted from blank controls and normalised to a standard control well.

Chapter 3 Assessment of CD4+ T cell clonality in a mouse model of experimental arthritis

3.1 Introduction

As discussed in **section 1.4.4** of the introduction, CD4+ T cells play an important role in the initiation, coordination, and maintenance of Rheumatoid Arthritis (RA) and their ability to infiltrate joints is associated with perpetuation of local and systemic inflammatory responses. Several research groups have investigated the diversity of these cells in RA patients to better understand how antigen specificity and CD4+ T cells are involved in disease progression. Oligoclonal CD4+ T cell populations have been found in joints of RA patients, demonstrating that antigen specific CD4+ T cell responses have occurred in these patients[46,201,259]. Such expanded CD4+ T cell populations are envisioned to persist with the progression of disease; indeed expanded clones were found in RA patients more than 2 years after they were first identified[143]. However, the antigen specificities of these cells remain unclear, and how such T cell responses arise and evolve with disease progression remains poorly understood. Investigating the antigen specificities of these T cells has important implications for antigen specific tolerogenic therapies, as these pathogenic cells represent the targets that will lead to prevention of further damage and disease remission. Indeed, several studies employing antigen specific tolerogenic therapies have shown promise in dampening allergic and autoimmune responses[229,240,243,260]. Moreover, understanding how these responses evolve with disease chronicity will better inform where and when these therapies will be most effective.

Antigen specific responses can be assessed by determining the presence of biases in TCR usage, which can be done on both a sequence and protein level. Methods utilising PCR to amplify regions of the TCR transcript have demonstrated changes in TCR diversity in cancers[198,199] and autoimmune diseases[48,200], including RA[46,48,143,201]. Using antibodies directed against the TCR is also a valid method for assessing T cell clonality and can be used to identify specific T cell clones[202 203]. Because the antigen specificities of expanded T cells in RA are unknown, antibodies against relevant, specific T cell clones do not exist. Instead, antibodies against V regions of the TCR β chain can be used to quantify the presence of different V regions by flow cytometry, which would highlight biases in TCR frequencies, and

consequentially, suggests antigen associated retention and accumulation. Quantification of V regions has been performed in healthy people[87,207], in patients with leukemia[208], and in RA patients[209]. Both these methods have the advantage of providing rapid, global assessments of changes in the T cell repertoire[207,210,211], which rely on the skewed presence of different V regions to reflect changes in the T cell population. Determining frequencies of V chain usage does not provide clonal information per se, but it still highlights biases in the usage of these chains, implicating antigen driven proliferation of T cells[210,214].

As initiation of the antigen specific responses are poorly understood in patients, a murine model of early arthritis was used to investigate the clonality of the initial CD4+ T cell infiltrate and provide evidence for antigen specific responses at the earliest stages of disease. The OVA model of early experimental arthritis uses a joint irrelevant antigen to cause breach of tolerance and recapitulates the pre-articular phase of RA[140], details of which can be found in **section 1.4.3** of the introduction. This model is therefore ideal for monitoring the dynamics of the initial CD4+ T cell joint infiltrate during conditions for which developing autoreactivity to articular antigens is most permissive.

3.1.1 Chapter aims and summary

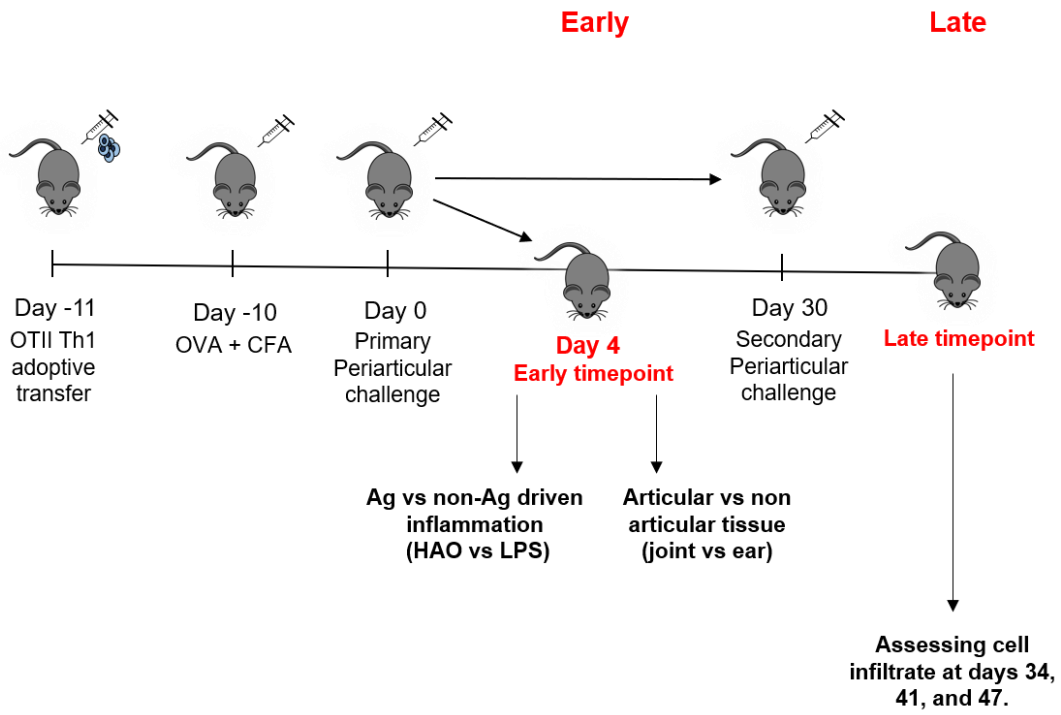
The aims of this chapter are twofold: Firstly, to assess the clonality of CD4+ T cells in a model of early experimental arthritis with the aim of finding evidence supporting antigen specific accumulation of CD4+ T cells in the joints during early phases of the disease. Secondly, to monitor how these antigen specific responses evolve with disease progression. In order to do this, I have established the models, techniques, tissues, and time points necessary for assessing CD4+ T cell clonality. These populations were subsequently analysed for biases in TCR Vβ usage using both the PCR and flow cytometry techniques mentioned earlier.

CD4+ T cells were isolated from both joints and joint draining popliteal lymph nodes (pLNs), analysed, and compared to assess differences in antigen specific responses between these two sites. The CD4+ T cell population was also analysed in the context of non-antigen driven inflammation to determine the degree an inflammatory environment contributes to the recruitment of CD4+ T cells, and how this influences biases in the T cell repertoire. Previous studies have demonstrated

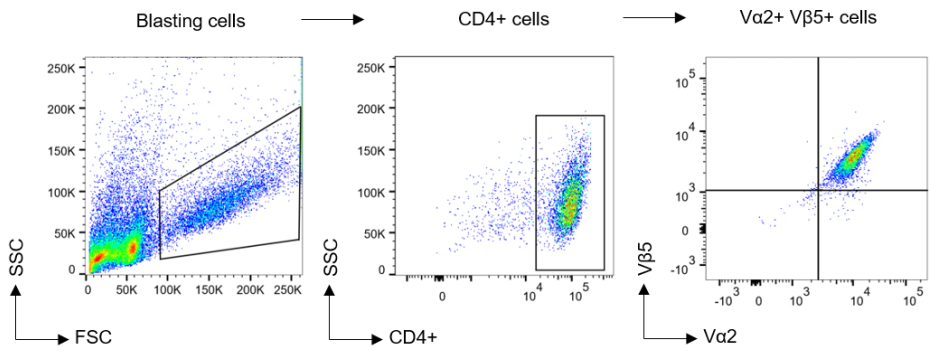
the ability of antigen experienced CD4+ T cells to infiltrate and accumulate in tissues in the absence of their cognate antigen[191,193,194] and so changes to the CD4+ T cell repertoire could arise as a result of the inflammatory environment as well as the presence of antigen driven responses. Comparisons were also made between articular and non-articular tissues to evaluate the degree joint derived antigens shape the CD4+ T cell repertoire. All of this was done in an early model of experimental arthritis. To address the evolution of the antigen specific response, a model of relapsing polyarthritis, developed by Conigliaro et al[252] was used and a time point for disease chronicity was established, and CD4+ TCR clonality assessed **(Figure 3-1 A)**.

Overall my findings indicate the presence of a bias in the CD4+ T cell repertoire in early experimental arthritis that is associated with an antigen driven response and is influenced by joint derived antigens. Moreover, the biases in the TCR repertoire found at the later timepoint mirror those found in the early phases, suggesting the presence of clones in early arthritis that play role in propagating the disease.

A)


Early
Late
Day -11
OTII Th1
adoptive
transfer
Day -10
OVA + CFA
Day 0
Primary
Periarticular
challenge
Day 4
Early timepoint
Day 30
Secondary
Periarticular
challenge
Late timepoint
Ag vs non-Ag driven
inflammation
(HAO vs LPS)
Articular vs non
articular tissue
(joint vs ear)
Assessing cell
infiltrate at days 34,
41, and 47.

B)



C)

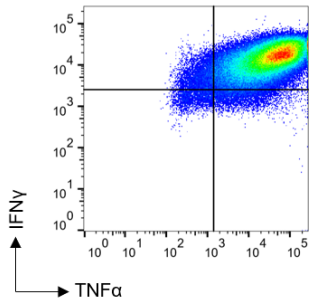


Figure 3-1 - The OVA experimental arthritis model illustrating the early and late model timepoints and modifications and assessment of Th1 polarised OT-II cells used for adoptive transfer

A) Early and late model timepoints, described in detail in methods **sections 2.2.5 and 2.2.7** are adaptations of the OVA experimental arthritis model described in introduction **section 1.6**. Briefly, Th1 polarised OT-II cells are transferred in to C57BL/6 mice which is followed by immunisation of OVA in CFA in the scruff 24 hours after the adoptive transfer. 10 days after OVA+CFA immunisation, mice are given a periarticular immunisation of either HAO or PBS as a control. To assess the effects of an inflammatory environment on CD4+ clonality, an additional group of mice were challenged with LPS in the joints. To compare the influence of articular antigen on the CD4+ T cell population, a group of mice were immunised with HAO or PBS in the ear. For the early timepoint, mice are sacrificed 4 days after this primary periarticular challenge. To establish the late timepoint, mice are given a 2nd periarticular challenge 30 days after the primary HAO challenge with either HAO in IFA or IFA alone as a control. The mice are then sacrificed 4, 11, or 17 days after the 2nd periarticular challenge. **B)** Representative flow cytometry plots illustrating identification of Th1 polarised OT-II cells, described in detail in methods **section 2.2.2**. Briefly, 72 hours after naïve OT-II cells were cultured with IL-12, anti IL-4, and OVA peptide 323-339, percentage of Th1 polarisation was determined by gating on blasting, CD4+ T cells that were Vα2, Vβ5 double positive. For the adoptive transfer, the cell concentration was adjusted – using the percentage of blasting, Vα2, Vβ5 double positive CD4+ T cells – to ensure 2-3x10^6 Th1 polarised cells were transferred into C57BL/6 or Nur77GFP hosts. The percentage of Th1 polarised OT-II cells ranged from 75%-97% across experiments. **C)** A representative flow cytometry plot of Th1 polarised cells stimulated with PMA/Ionomycin. Briefly, a sample of Th1 polarised cells was taken for to determine production of IFNγ and TNFα and reaffirm polarisation to the Th1 phenotype. Cells were cultured with 20ng/mL PMA and 10µg/mL ionomycin for 1 hour at 37°C in complete media, then for a further 3 hours after addition of GolgiStop diluted 1:1000 in complete media. Cells were then stained for flow cytometry analysis. Polarised CD4+, Vα2+, Vβ5+ cells were 92% IFNγ and TNFα double positive on average.

3.2 Results

3.2.1 Establishing the OVA arthritis model for CD4+ T cell clonality assessment

Before investigating CD4+ T cell clonality and determining the presence of antigen specific responses in the OVA model of experimental arthritis, conditions for the assessment of CD4+ T cell clonality first needed to be established. This would allow for the accurate evaluation of CD4+ TCR clonality and antigen specific responses in early experimental arthritis in addition to assessing how CD4+ T cell clonality evolves with disease severity.

3.2.1.1 Isolation and Identification of endogenous CD4+ T cell populations and Vβ families from pLNs and joints

Evaluating the presence of any biases in Vβ chain use is dependent on the accurate identification of the endogenous CD4+ T cell population in joints and the joint draining popliteal lymph nodes (pLNs). The congenic marker CD45.1 was used to distinguish the transferred OT-II cell population from the endogenous CD4+ T cell population and was sufficient in doing so in both pLNs **(Figure 3-2 A)** and joints **(Figure 3-2 B)**. Moreover, the naïve (CD44lo) and antigen experienced (CD44hi) populations were identified within the endogenous CD4+ T cell population of pLNs, and the naïve population was then used to identify the different Vβ families **(Figure 3-3)**. The different gates were then applied within the antigen experienced populations in pLNs and joints.

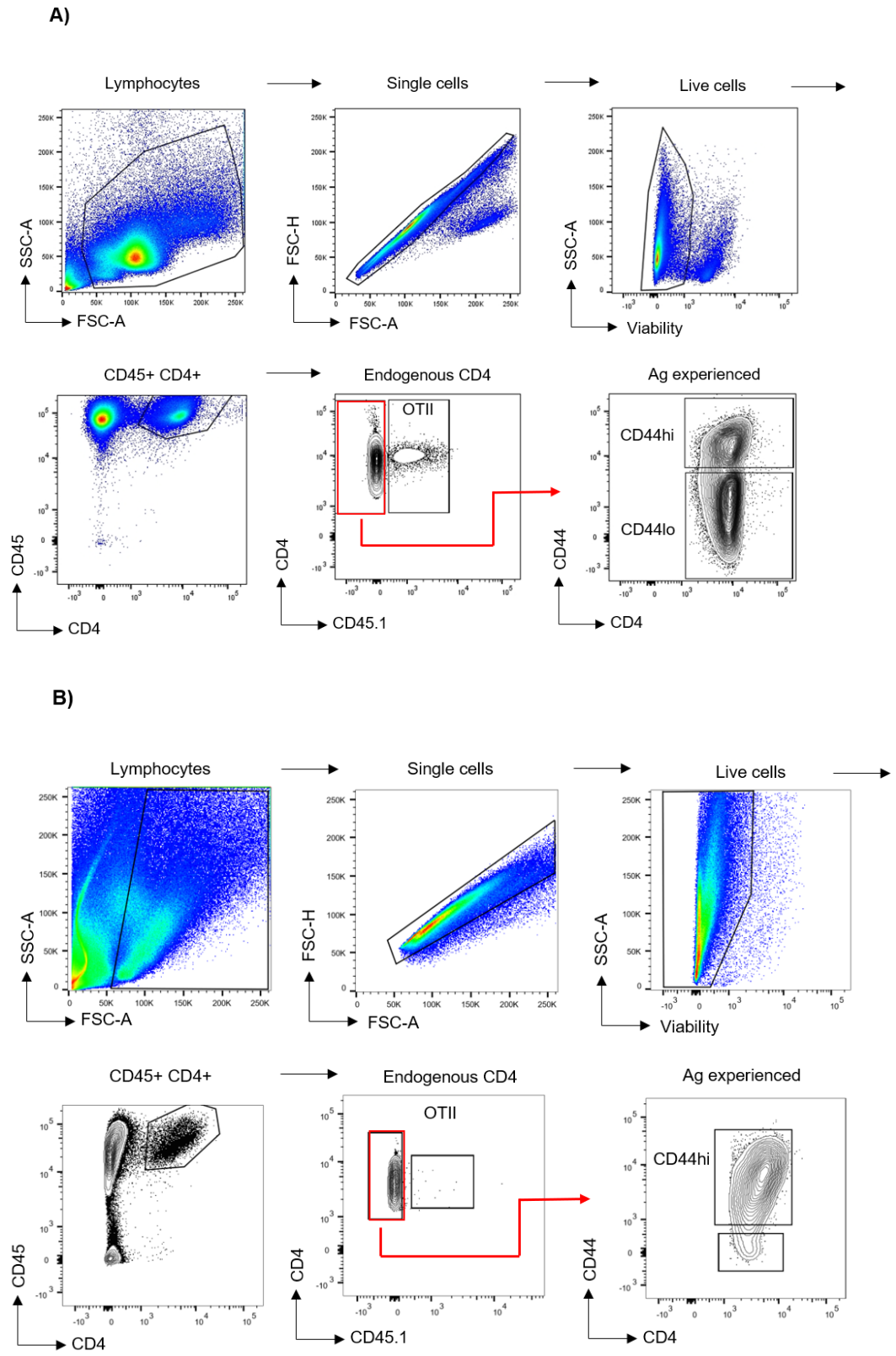


Figure 3-2 - Gating strategy to identify endogenous antigen experienced CD4+ T cells from pLNs and joints.
Cells were isolated from **A)** pLNs, and **B)** joints as described in methods and stained with antibodies for flow cytometry also described in methods. Endogenous antigen experienced cells are identified as CD44hi CD4+ CD45- cells and were isolated by gating on lymphocytes, single cells, live cells, CD45+ CD4+ cells, CD4+ CD45.1- cells, and finally CD44hi cells. flow cytometry plots are representative of pLNs and joint cells.

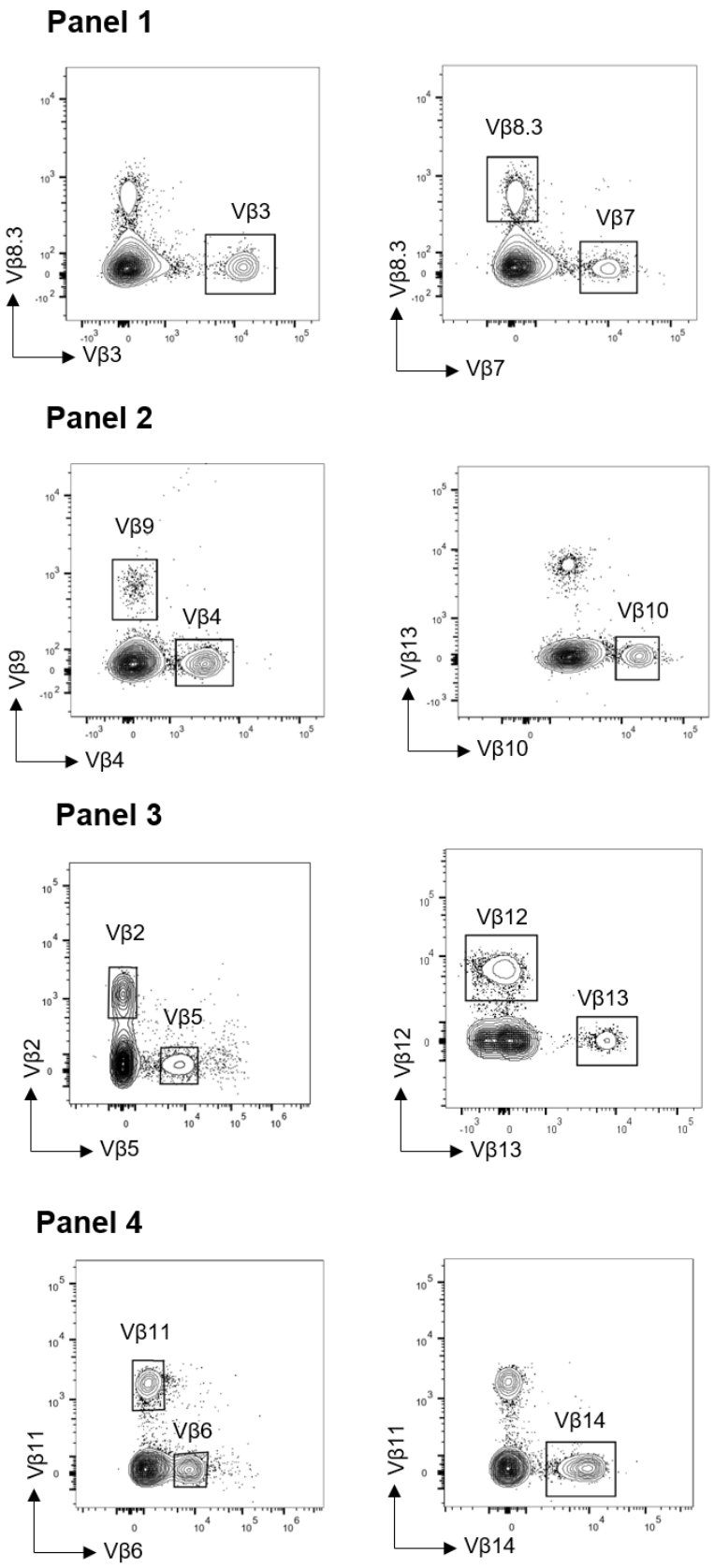


Figure 3-3 - Identification of Vβ families from the naïve (CD44lo) population from pLNs
Vβ populations were identified from the CD44lo population of pLNs as the naïve population should contain cells representing all Vβ chains and also be present in high enough numbers to be accurately detected by flow cytometry. These gates were then applied and adjusted to the CD44hi repertoire in both pLNs and joint samples. Vβ antibodies were split amongst 4 panels and 2 panels were used per sample. Panel one Vβ3, 7, and 8.3. Panel two Vβ4, 9, and 10. Panel 3 Vβ2, 5, 12, and 13. Panel 4 Vβ6, 11, and 14. flow cytometry plots are representative of pLN samples.

3.2.1.2 HAO challenge induces joint inflammation, and accumulation of antigen experienced endogenous CD4+ T cells

To determine whether the model was successful in inducing experimental arthritis, joint thickness was measured each day after administration of HAO or PBS until experiment termination **(Figure 3-4)**. In addition, the total cell number, total number of endogenous CD4+ T cells, and the number of antigen experienced CD4+ T cells (CD44hi) cells was determined in both the joints and respective pLNs **(Figure 3-5)**. HAO challenged mice footpads were significantly larger than PBS controls on all days following immunisation until experiment termination **(Figure 3-4)**. Mice challenged with HAO also showed a significant increase in cell number in both the joint and pLN samples compared to PBS challenged controls **(Figure 3-5)** The number of endogenous CD4+ T cells is also greater than the number of OT-II cells in both HAO challenged pLNs and joints **(Figure 3-5 B)**, which demonstrates that HAO challenge elicits an endogenous response. Taken together, this data suggests that HAO causes local inflammation, and accumulation of antigen experienced (CD44hi) endogenous CD4+ T cells in both the joints and pLNs.

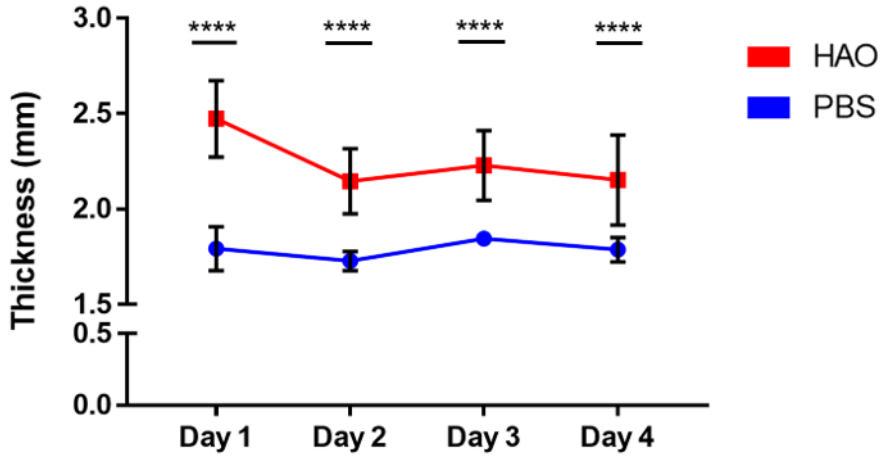


Figure 3-4 - Footpad thickness of HAO and PBS challenged mice.
Both footpads were measured daily after HAO or PBS administration for 4 days until experiment termination. Graphs represent 1 experiment with n=4 for both HAO and PBS groups. Groups were compared using repeated measures 2 way ANOVA. Bars represent mean ±SD. Stars represent the following p values: **** <0.0001

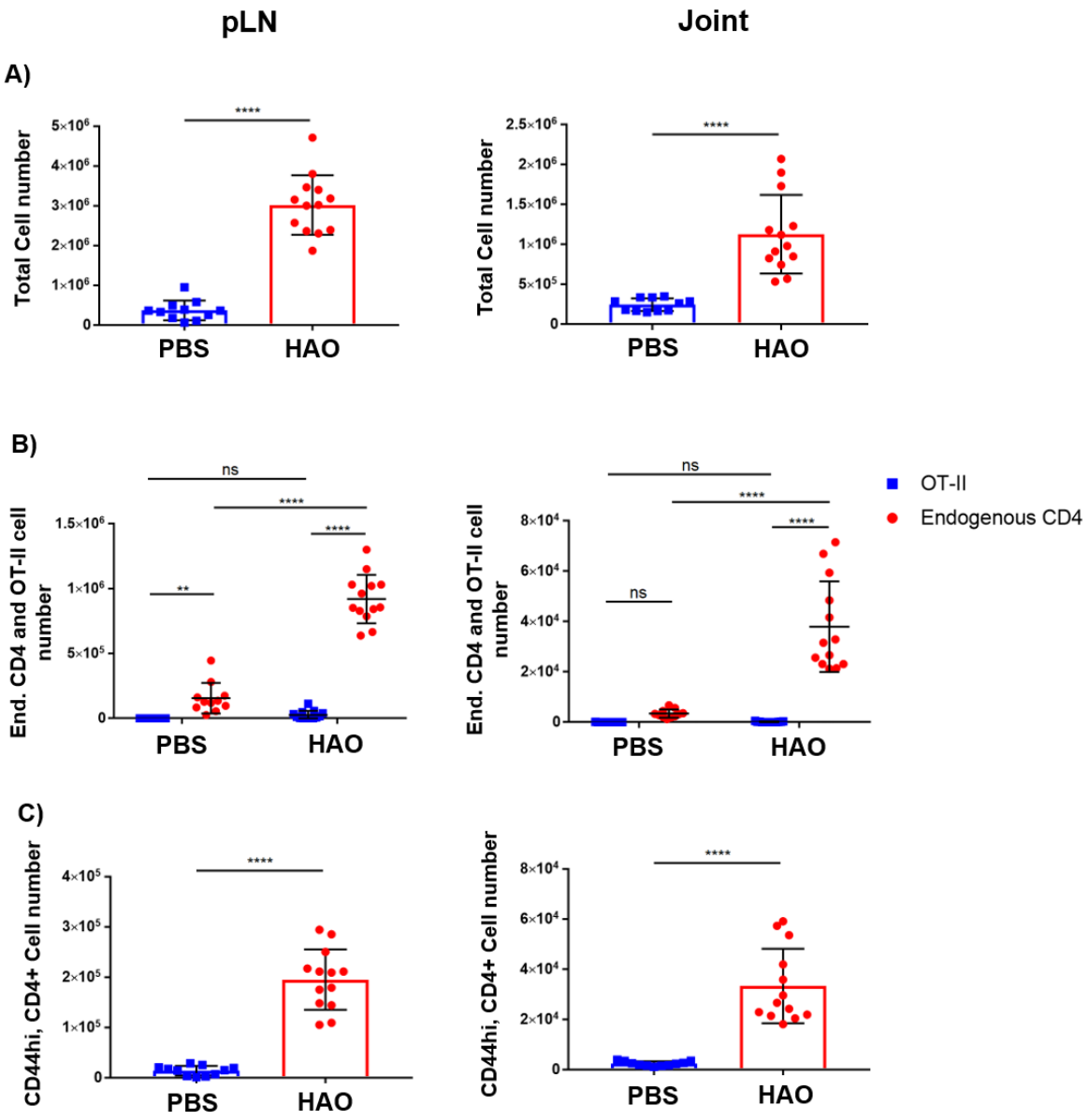


Figure 3-5 - Number of cells in the joints and respective pLNs in HAO and PBS challenged mice.

Cells were isolated from both joints and respective pLNs of HAO and PBS challenged mice. Transferred cells were identified using the CD45.1 marker and were excluded from analysis, leaving only the endogenous CD4+ T cells **A)** Total cell number, **B)** Total endogenous CD4+ T and OT-II cells, **C)** Total CD44hi, CD4+ T cells in HAO and PBS challenged joints and pLNs. Each symbol represents one mouse. Graphs represent 3 experiments with n=13 and 10 for the HAO and PBS groups respectively. Graphs with two groups were compared using unpaired Student's t test. Graphs with more than two groups were compared using 2 way ANOVA. Bars represent mean ±SD. Stars represent the following p values: ** <0.01; **** <0.0001; ns: not significant

3.2.1.3 Assessing the influence of an inflammatory environment on the accumulation of CD4+ T cells in inflamed pLNs and joints

Previous studies have shown how an inflammatory environment is capable of recruiting activated CD4+ T cells without their cognate antigen[191,193,194]. To accurately determine the presence of antigen specific responses in the early phases of experimental arthritis, conditions where inflammation is induced in the absence of antigens needed to be established, as this would determine the relative contributions of antigen specific and non-specific inflammation to the initial CD4+ joint infiltrate. To address this, mice were challenged with either HAO, PBS, or LPS. LPS is a non-specific inflammatory mediator that causes the release of proinflammatory cytokines via the TLR4 receptor, and so any recruitment or retention of CD4+ T cells would not be due to antigen since the TCR is not engaged[261]. A previous study by Nickdel et. al. demonstrated how innate responses initiated by LPS contribute to the inflammatory environment in early experimental arthritis and facilitate damage to the joint without the development of an autoreactive response[254]. By administering an LPS footpad challenge, the initial CD4+ T cell infiltrate could be investigated in the context of a non-antigen driven inflammatory environment and in the absence of conditions permissive to the development of autoreactive responses, thereby clarifying the clonality of endogenous CD4+ T cells involved in propagating breach of tolerance and the development of an autoreactive response.

Two doses of LPS isolated from *E.coli* (see methods **section 2.2.5**) were initially tested in the model to evaluate which dose was better at causing a significant infiltration of cells in pLN and joints. It was found that 5µg of LPS failed at inducing cell accumulation in the joints (data not shown), but 25µg was sufficient. Moreover, in the same model Nickdel et. al. demonstrated that 25µg of LPS lead to cell infiltration and moderate cartilage erosion[254]. Considering this, 25µg of LPS was used in all experiments requiring the use of LPS. Mice were challenged with either HAO, PBS, or LPS and their footpads were measured 1, 2, and 4 days after periarticular challenge to assess footpad swelling **(Figure 3-6)**. LPS did not increase footpad swelling on days 1 and 3 compared to PBS controls, but by day 4 footpads were more swollen than PBS controls. The total cell number, the number of CD45+ CD4- cells, endogenous CD4+ T cells, and the number of CD44hi CD4+ endogenous T cells from joints and pLNs were also compared to determine the

effect LPS has on cell accumulation in both these sites **(Figure 3-7)**. The total cell number and the number of CD45+ CD4- cells were significantly higher in the LPS group than PBS controls in both pLNs and joints **(Figure 3-7 A and D)**, but the number of endogenous CD4+ T cells, as well as the number of CD44hi CD4+ T cells were comparable to PBS controls **(Figure 3-7 B and C)**. Despite this, the increase in total cell number and CD45+ CD4- cells indicates that the LPS was functioning as expected and the increase in cell number observed may be attributed to an increase in the number of innate cells. Indeed, LPS has been shown to attract neutrophils, macrophages and DCs[261]. Administration of 25µg of LPS was therefore deemed sufficient for evaluating the clonality of the CD4+ T cell infiltrate in the context of non-antigen induced inflammation.

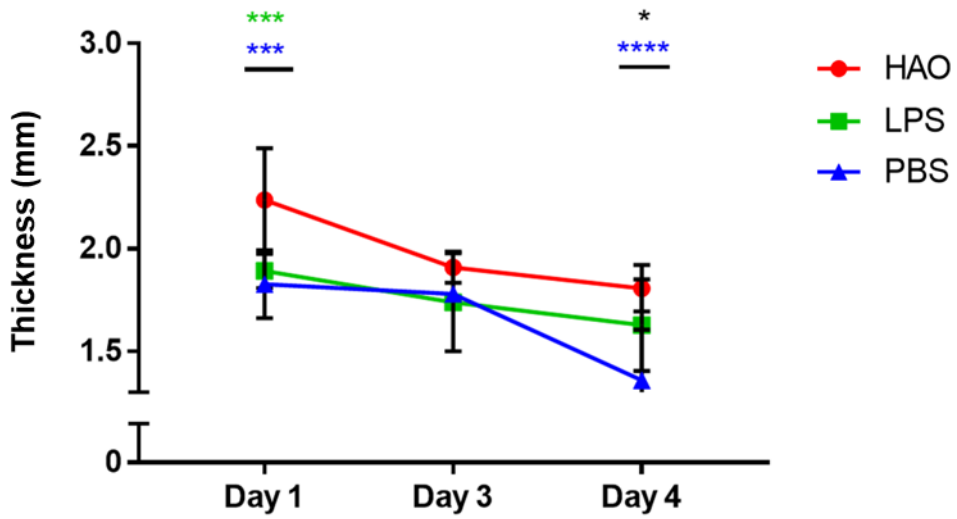


Figure 3-6 - Footpad thickness of HAO, PBS, and LPS challenged mice.
Both footpads were measured one, three, and four days after HAO, PBS, or 25µg of LPS administration until experiment termination. Graphs represent 1 experiment with n=5 for HAO, PBS, and LPS groups per experiment. Groups were compared using repeated measures 2 way ANOVA. Bars represent mean ±SD. Stars represent the following p values: * <0.05; *** <0.001; **** <0.0001. Green stars represent comparisons between HAO and LPS groups, blue stars represent comparisons between HAO and PBS groups, and black stars represent comparisons between LPS and PBS groups.

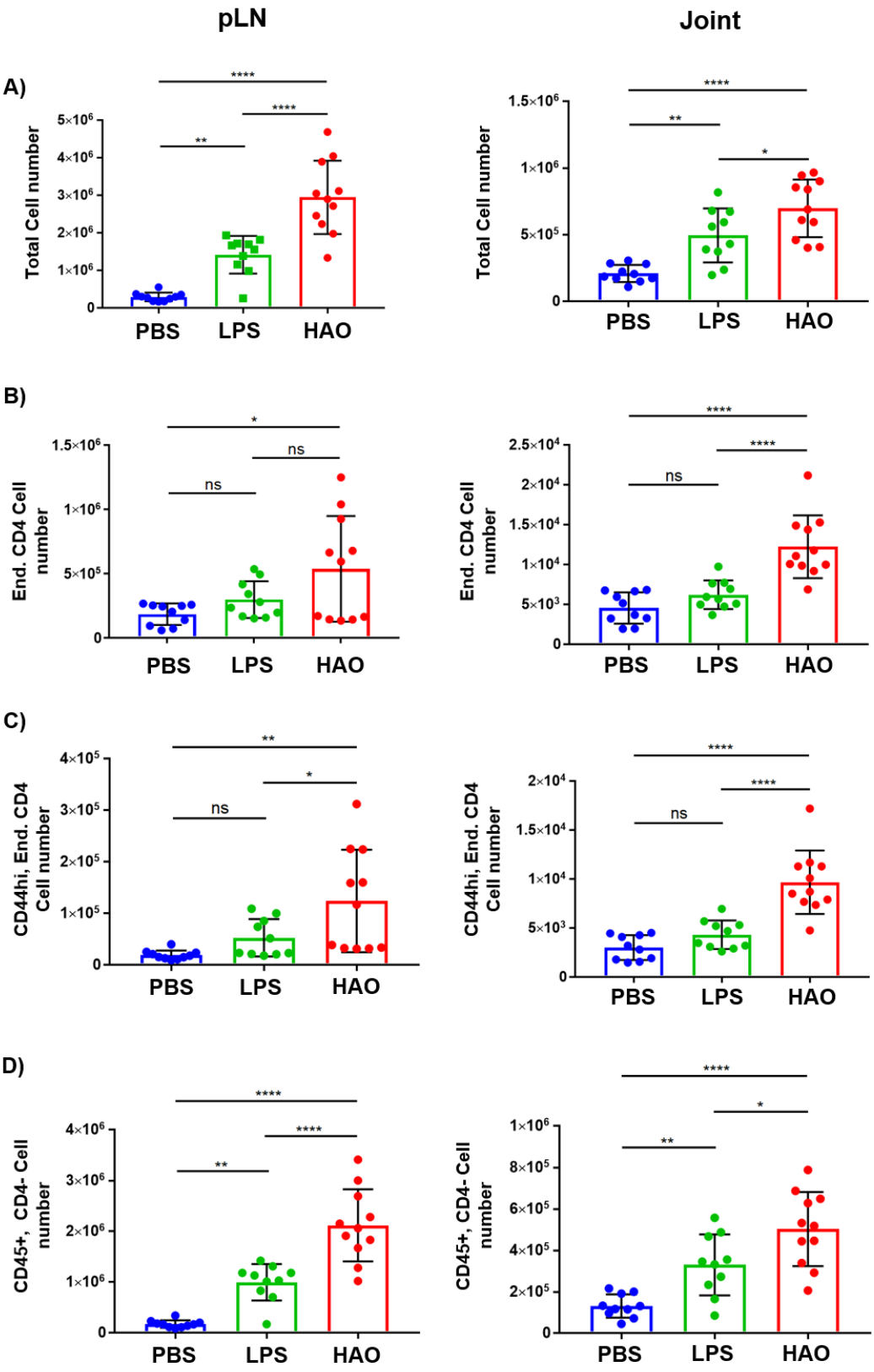


Figure 3-7 - Number of cells in the foot joints and respective pLNs of HAO, PBS, and LPS challenged mice.

Cells were isolated from both foot joints and respective pLNs of HAO, PBS, and LPS (25µg) challenged mice. Transferred cells were identified using the CD45.1 marker and were excluded from analysis, leaving only the endogenous CD4+ T cells **A)** Total cell number, **B)** endogenous CD4+ T cells, **C)** CD44hi, CD4+ T cell number, and **D)** CD45+ CD4- cell number in HAO, PBS, and LPS challenged joints and pLNs. Each symbol represents one mouse. Graphs represent 2 experiments with n=11 for the HAO groups, and n=10 for the PBS and LPS groups. Groups were compared using one way ANOVA. Bars represent mean ±SD. Stars represent the following p values: * <0.05; ** <0.01; **** <0.0001; ns: not significant

3.2.1.4 Assessing the influence of articular antigens on the accumulation of CD4+ T cells in inflamed pLNs and joints

Another aim of the chapter was to determine whether breach of tolerance to joint derived antigens plays a role in initiating and propagating antigen experimental arthritis via autoreactive responses. A number of studies provide evidence for the role of articular antigens, such as collagen and human cartilage gp-39 (HC gp-39), as the targets of antigen specific T cell responses in RA [262,263]. A method to determine whether antigen specific responses observed are restricted to the joint is to immunise a site other than the joint, such as the ear. The composition of ear cartilage is different to the joint, and the ear is not affected in RA[264,265], so the damage that is caused by HAO should – in theory – expose a different set of antigens. Other members of the lab have immunised the ear to compare endogenous CD4+ T cell retention mechanisms between the inflamed joint and ear in the model. The OVA experimental arthritis was initiated as normal, but instead of periarticular administration of HAO, mice were injected with 100µg of HAO in the ear pinnae. Ears and the superficial cervical lymph nodes (scLNs) that drain the ear[266] were collected and cell numbers determined **(Figure 3-8)**. The total, endogenous CD4+ T cell, and endogenous CD44hi cell numbers were all significantly higher than their PBS counterparts in both the scLNs and ears **(Figure 3-8 A-C)**. In addition, the endogenous CD4+ T cell number is significantly greater than the OT-II cell numbers in HAO challenged scLNs and ears **(Figure 3-8 B)**. This indicates that HAO challenge in the ear results in an increase in antigen experienced endogenous CD4+ T cells similar to the joint.

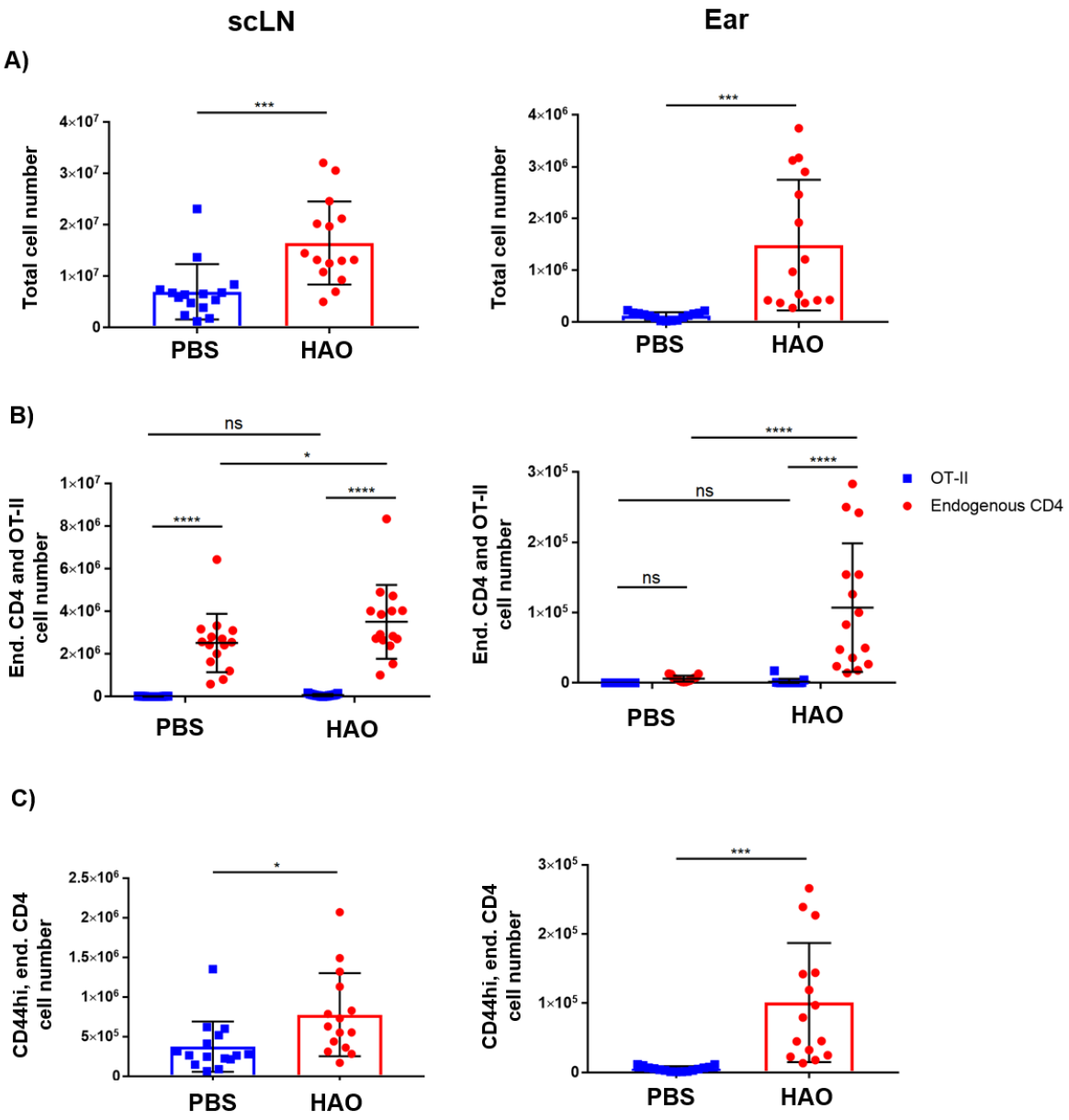


Figure 3-8 - Number of cells in ears and respective draining superficial cervical lymph nodes (scLN) in HAO and PBS challenged mice.
Cells were isolated from both ears and respective scLNs of HAO and PBS challenged mice. Transferred cells were identified using the CD45.1 marker and were excluded from analysis, leaving only the endogenous CD4+ T cells **A)** Total cell number, **B)** Total endogenous CD4+ T and OT-II cells, **C)** Total CD44hi, CD4+ T cells in HAO and PBS challenged ears and scLNs. Each symbol represents one mouse. Graphs represent 3 experiments with n=15 in total for the HAO and PBS groups respectively. Graphs with two groups were compared using unpaired Student's t test. Graphs with more than two groups were compared using 2 way ANOVA. Bars represent mean ±SD. Stars represent the following p values: * <0.05; *** <0.001; **** <0.0001; ns: not significant

3.2.1.5 Establishing a late timepoint to assess the evolution of the antigen specific CD4+ T cell response in the late model of inflammatory arthritis

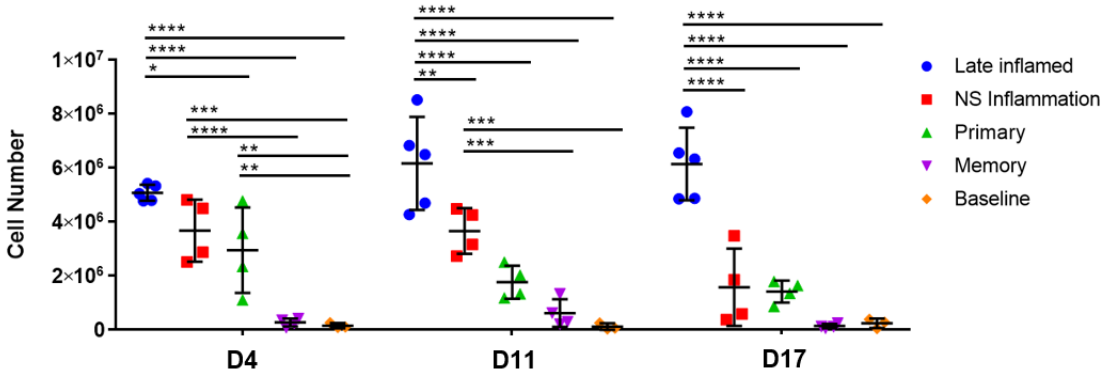
Another aim of the chapter is to investigate how CD4+ T cell clonality would change with the progression of experimental arthritis, which suggests how antigen specific responses may change with the development of the disease. The OVA model of

experimental arthritis represents an early timepoint of RA and models the initial cellular infiltrate into the joint. To monitor progression of antigen specific responses, a model of relapsing arthritis was used – hereafter referred to as the late model of inflammatory arthritis. The late model of inflammatory arthritis was developed by Dr. Paola Conigliaro to more resemble human RA and to investigate the anti-collagen antibody response in a more chronic setting[252]. The development of the CD4+ T cell response has not yet been examined in this model. As such, a time point for chronicity needed to be established before attempting to assess CD4+ T cell clonality in this late setting.

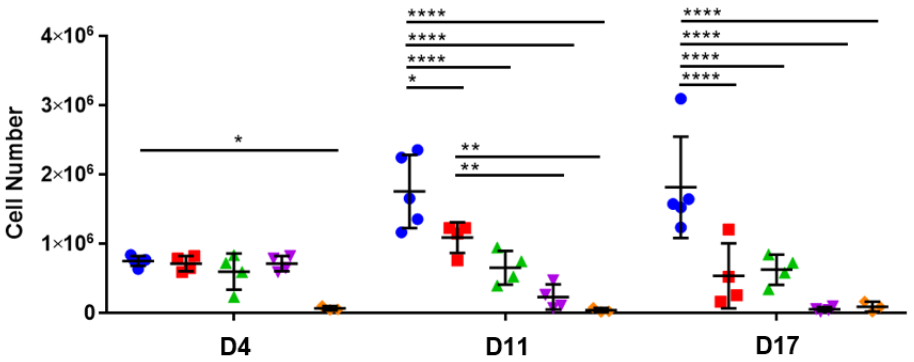
Mice display clinical signs of disease for greater than 3 weeks in this late model of inflammatory arthritis[252]. As such, the T cell response was examined within that time frame at 4, 11, and 17 days (referred to as D4, D11, and D17 respectively) after secondary periarticular challenge (**Figure 3-1** and **Chapter 2 Figure 2-2 for details**). A number of controls needed to be included to evaluate whether the inflammation and cell accumulation observed is due to the 2nd periarticular challenge with antigen (see **section 2.2.7** for clarification). In this chapter I will refer to the experimental group that received a 2nd periarticular challenge of HAO in IFA as “late inflamed”.

Cell numbers were determined at all three time points in late inflamed pLNs and joints and controls. The pLNs of late inflamed mice displayed the highest number of total, endogenous CD4+ T cell, and endogenous CD44hi CD4+ T cell numbers at all three time points compared to controls **(Figure 3-9 A-C)**. The same was observed in late inflamed joints, but only at D11 and D17 **(Figure 3-9 D-F)**. This increase in total and endogenous CD4+ T cell numbers is sustained by D17 in pLNs **(Figure 3-10 A and B)** and joints **(Figure 3-10 D and E)** of the late inflamed group, while the control groups show an overall decrease in cell numbers by D17. This data therefore suggests that D17 is the best timepoint to use to determine changes in CD4+ T cell clonality with the knowledge that the cells retained at this point are assumed to be antigen specific This timepoint will be referred to as the late timepoint from now on.

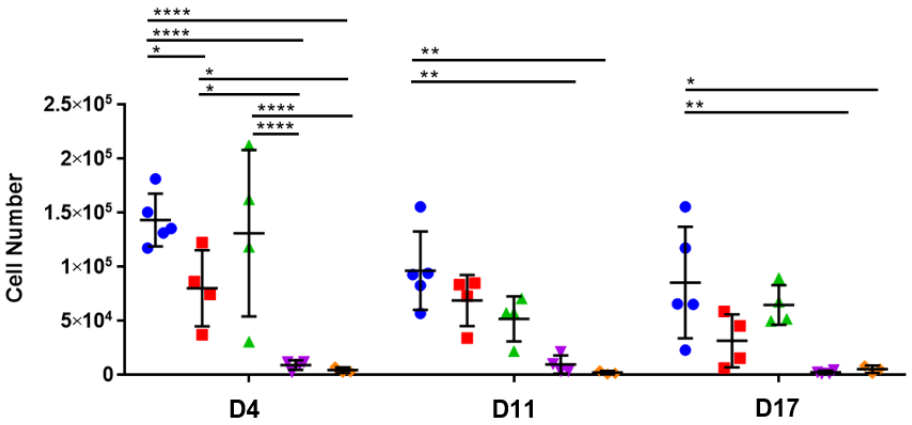
A) pLN total cell number



B) pLN end. CD4 cell number



C) pLN CD44hi, end. CD4 cell number



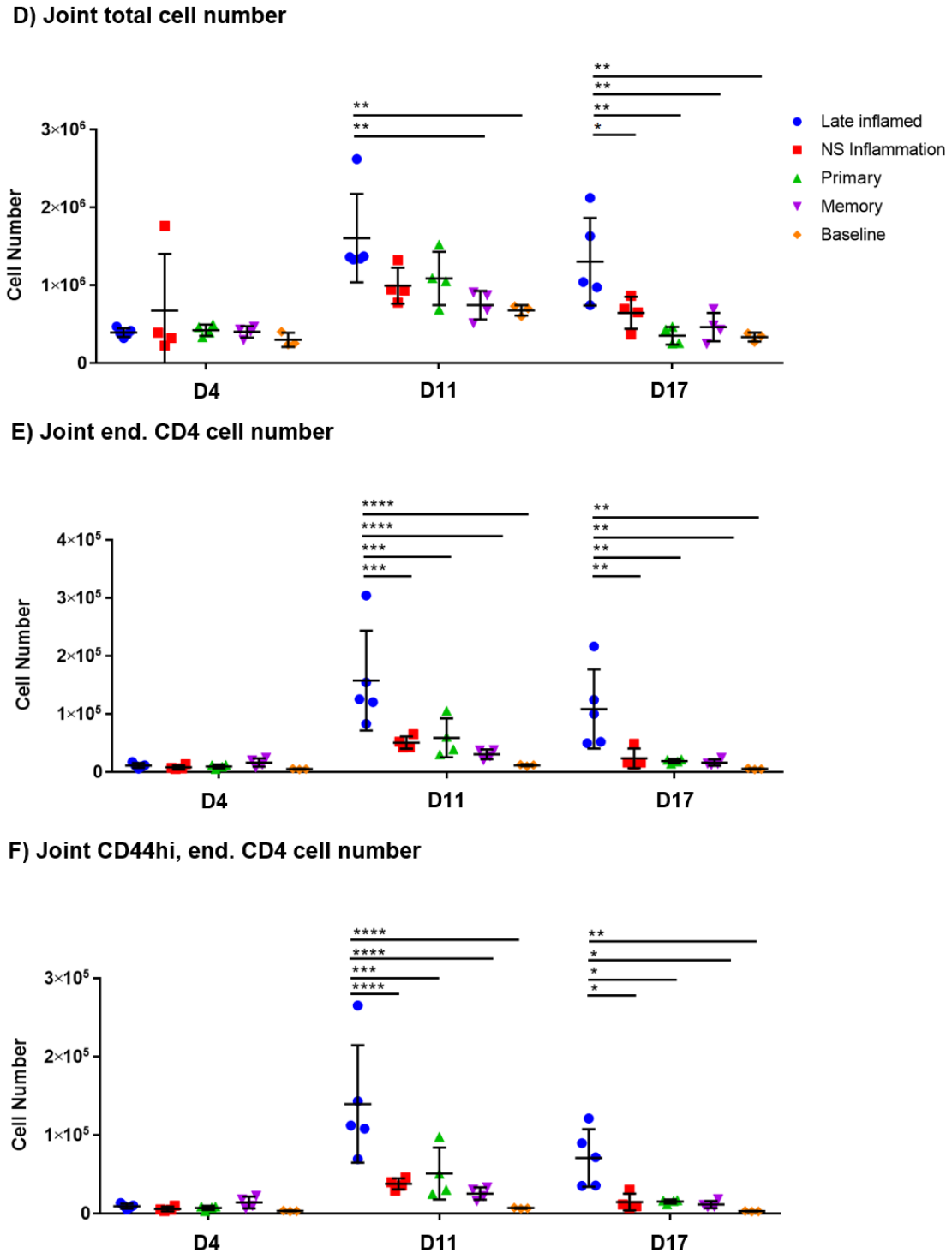
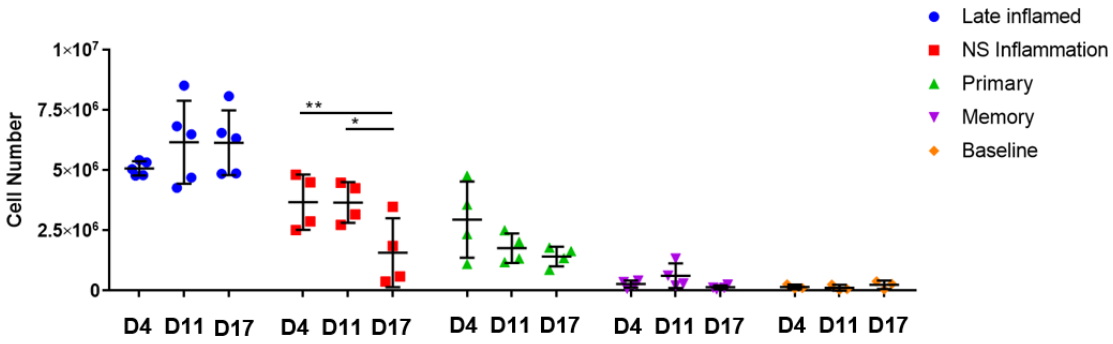


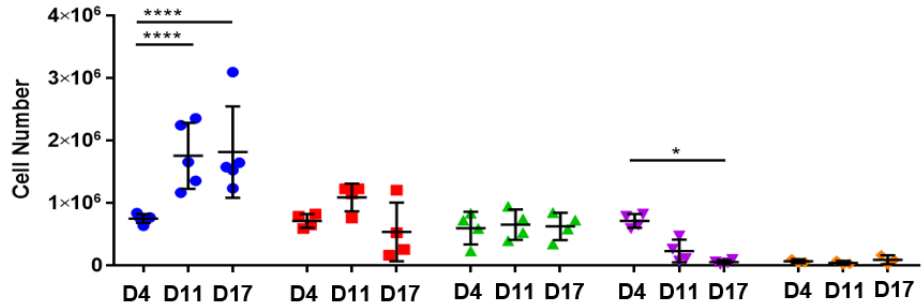
Figure 3-9 - Number of cells from pLNs and joints of late inflamed mice and controls within each timepoint

The late model of inflammatory arthritis was induced as described in methods. Cells were isolated from both pLNs and joints at 4 (D4), 11 (D11), or 17 (D17) days after secondary challenge. Groups are named depending on the 1st and 2nd periarticular challenges represented as 1st periarticular immunisation/2nd periarticular immunisation: late inflamed (HAO/HAO+IFA); non-specific (NS) inflammation (HAO/IFA+PBS); primary (PBS/HAO); memory (HAO/PBS), and baseline (PBS/PBS). Number of **A)** total cells, **B)** endogenous CD4+ T cells, and **C)** endogenous CD44hi, CD4+ T cells from mouse pLNs and number of **D)** total cells, **E)** endogenous CD4+ T cells, and **F)** endogenous CD44hi CD4+ T cells from joints . Graphs represent 1 experiment with n=5 for the late inflamed group, n=4 for the NS inflammation group, primary, and memory groups, and n=3 for the baseline group at each timepoint. Groups were compared using 2 way ANOVA Data presented as mean ±SD. Stars represent the following p values: * <0.05; ** <0.01; *** <0.001; **** <0.0001

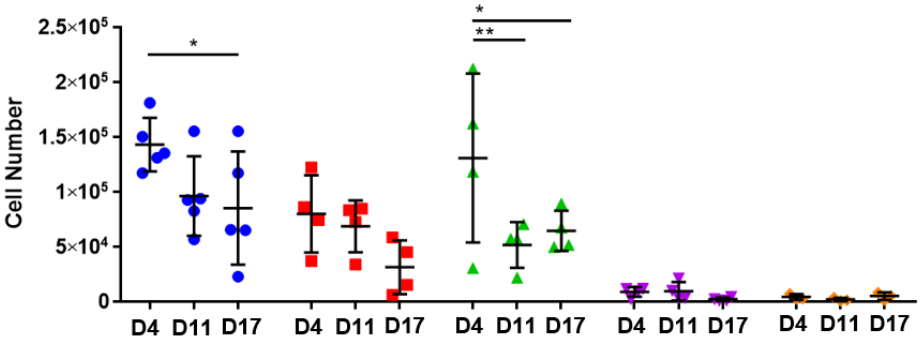
A) pLN total cell number



B) pLN end. CD4 cell number



C) pLN CD44hi CD4 cell number



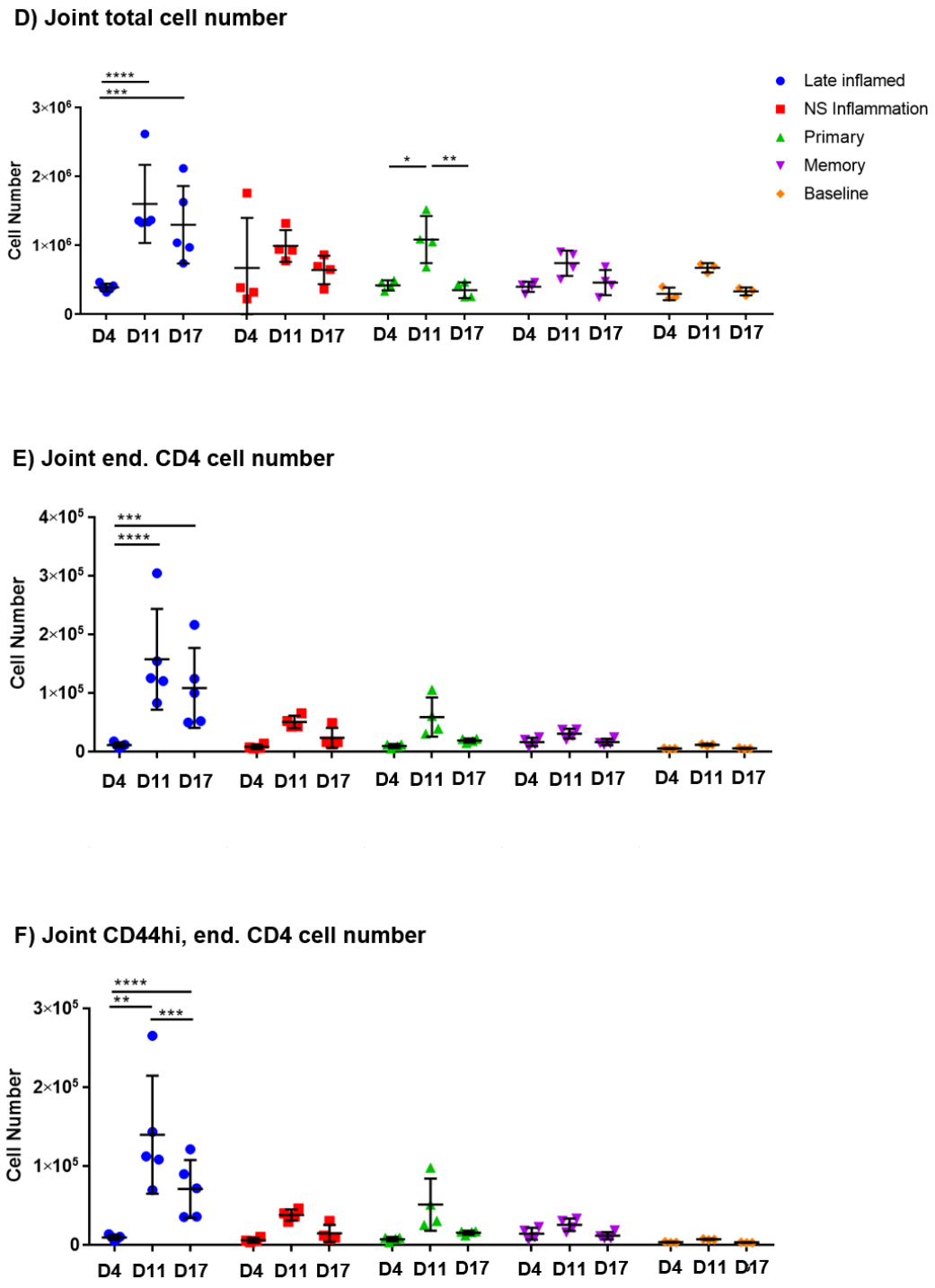


Figure 3-10 - Number of cells from pLNs and joints from late inflamed mice and controls across three timepoints.
The late model of inflammatory arthritis was induced as described in methods. Cells were isolated from both pLNs and joints 4 (D4), 11 (D11), or 17 (D17) days after secondary challenge. Groups are named depending on the 1st and 2nd periarticular challenges represented as 1st periarticular immunisation/2nd periarticular immunisation: late inflamed (HAO/HAO+IFA); non-specific (NS) inflammation (HAO/IFA+PBS); primary (PBS/HAO); memory (HAO/PBS), and baseline (PBS/PBS). Number of **A)** total cells, **B)** endogenous CD4+ T cells, and **C)** endogenous CD44hi, CD4+ T cells from mouse pLNs and number of **D)** total cells, **E)** endogenous CD4+ T cells, and **F)** endogenous CD44hi CD4+ T cells from joints. Graphs represent 1 experiment with n=5 for the late inflamed group, n=4 for the NS inflammation group, primary, and memory groups, and n=3 for the baseline group at each timepoint. Groups were compared using 2 way ANOVA Data presented as mean ±SD. Stars represent the following p values: * <0.05; ** <0.01; *** <0.001; **** <0.0001

3.2.2 Assessment of CD4+ T cell clonality in the OVA model of experimental arthritis

In the previous section I established the OVA model of experimental arthritis as well as the conditions in which changes in CD4+ T cell clonality can be attributed to joint associated antigen driven responses. In addition, I have established a timepoint to investigate how antigen specific responses change with the progression of experimental arthritis. Now, CD4+ T cell clonality can be assessed in these contexts and evidence for the presence of antigen specific responses in the early phases of experimental arthritis, as well as their development over time, can be evaluated.

3.2.2.1 Oligoclonal presence of CDR3β regions in the endogenous CD4+ T cell population in arthritic mice

As an increase in endogenous CD4+ T cells was observed in **section 3.2.1.2**, the question that followed is whether there is a restriction in clonality in the CD4+ T cells present in the affected joints and pLNs. This in turn would indicate the presence of antigen specific responses at the early stages of experimental arthritis. CD4+, CD44hi T cell were isolated from joints and pLNs and were sorted via FACS as described in methods. cDNA was collected from these cells and a PCR was performed to determine the diversity of CDR3β regions in the antigen experienced CD4+ T cell repertoire from pLNs and joints **(Figure 3-11 A and B)**. The CDR3β region, as mention in **section 1.2.1.2** of the introduction, is the most diverse region of the TCR and is unique to each T cell clone[220]. As a control, PCR was also performed on CD44hi cells isolated from the pLNs of a naïve C57BL6 mouse **(Figure 3-11 C)**. As expected, the CD44hi cohort in the naïve C57BL6 mice displayed a polyclonal phenotype, whereby all Vβs are represented and multiple CDR3β bands can be seen per Vβ chain. In the CD44hi cells isolated from the joints and pLNs of arthritic mice, a clear reduction in the number of CDR3βs per Vβ chain is observed, but each Vβ chain is represented apart from Vβ13 in both joint and pLN, and Vβ17 in the joint. This reduction in CDR3β regions, or oligoclonality, suggests a bias in the CD4+ TCR repertoire present in the joint and the pLN and could be an indication antigen specific retention, recruitment, or expansion at the tissue site.

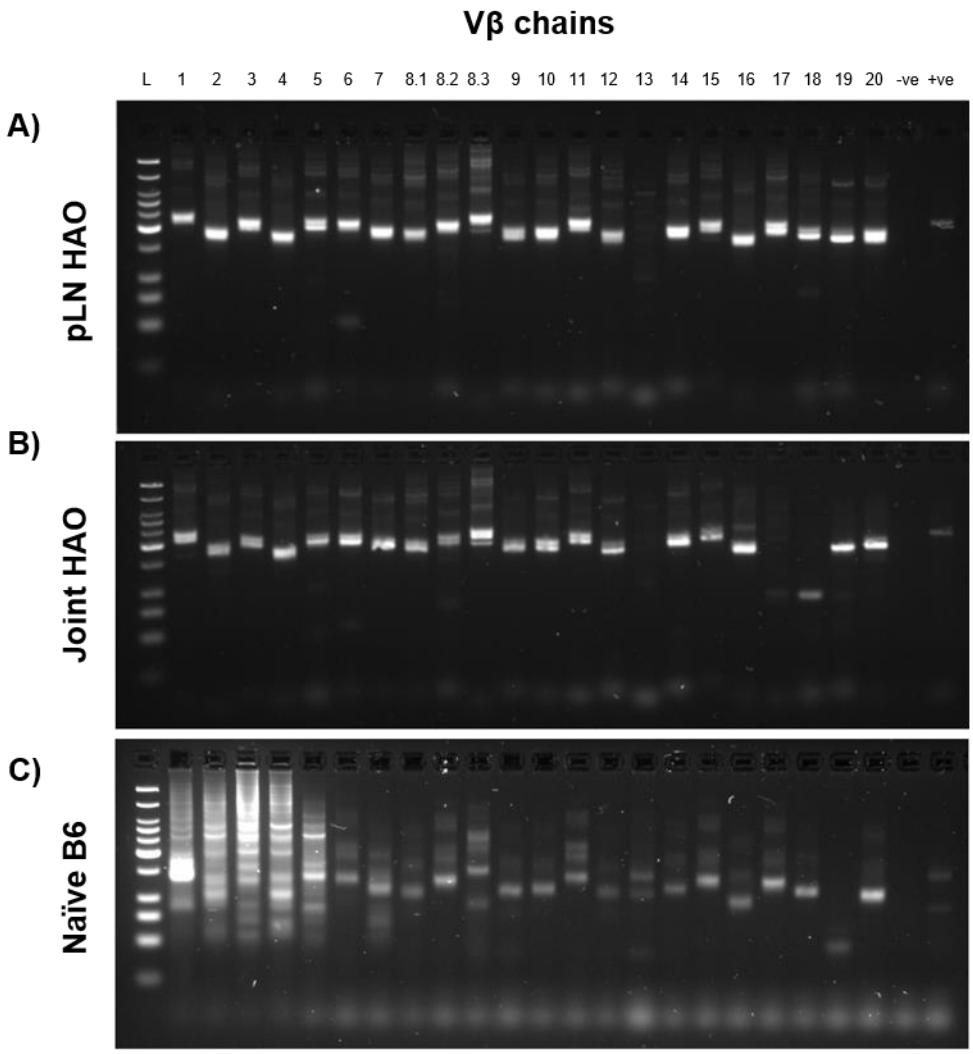


Figure 3-11 - CDR3β amplification of endogenous CD44hi, CD4+ T cells in HAO challenged and C57BL6 mice.
TCR diversity in HAO challenged mice was determined by CDR3β analysis of CD44hi, CD4+ T cells. **A)** CD44hi, CD4+ T cells from HAO challenged mouse pLNs and **B)** joints were sorted by FACS. **C)** CD44hi, CD4+ T cells from C57BL6 pLNs were also isolated as controls. CDR3β analysis was done by end point PCR using the SuperTCRExpress Mouse T Cell Receptor Vβ Repertoire Clonality Detecting Kit from BioMed Immunotech. Endogenous CD4+ T cells were pooled for each PCR (Naïve C57BL6 n=3; pLN HAO n=20; joint HAO n=20).

3.2.2.2 Increased number and frequency of the Vβ4 TCR chain in HAO challenged mice

The PCR performed was an end point PCR, which can only provide semi quantitative information on the presence of a given clone rather than the degree to which it is represented within the population. As a result, I opted to determine frequencies of cells with a given Vβ TCR chains via flow cytometry. This enabled me to determine the degree each Vβ chain contributes to the CD44hi repertoire of CD4+ T cells. Although this approach does not provide clonal information per se, it still highlights biases in Vβ usage, implicating preferential antigen driven recruitment, retention, or expansion of endogenous CD4+ T cells. The pLNs of HAO challenged mice showed an increase in the number of CD4+ T cells for

approximately half of the Vβ chains examined compared to PBS challenged mice, while in the joint, Vβ4 is the only chain that is present in significantly higher numbers than PBS challenged mice **(Figure 3-12 A and B)**. This is further demonstrated when looking at the percentage contribution of each Vβ chain to the CD44hi repertoire **(Figure 3-12 C and D)**; in both pLNs and joints of HAO challenged mice Vβ4 is present at a higher frequency compared to PBS controls. One thing to consider is how any bias in Vβ chain frequency in the naïve repertoire contributes to the CD44hi pool of cells and whether this influences the increases observed in HAO challenged mice. To address this, Vβ chain frequencies were determined in the naïve repertoire in pLNs. The naïve repertoire was in fact found to have unequal representation of TCR Vβ chains in both HAO and PBS challenged mice, with Vβ14 contributing the most, and Vβ9 the least **(Figure 3-13)**. Vβ4 is present at a high frequency in the naïve repertoire, but not higher than Vβ14, which demonstrates that the naïve repertoire does not influence the increase in Vβ4 usage in HAO challenged mice Together with the increase in cell number and frequency, this data indicates that Vβ4 specifically accumulates in HAO challenged mice.

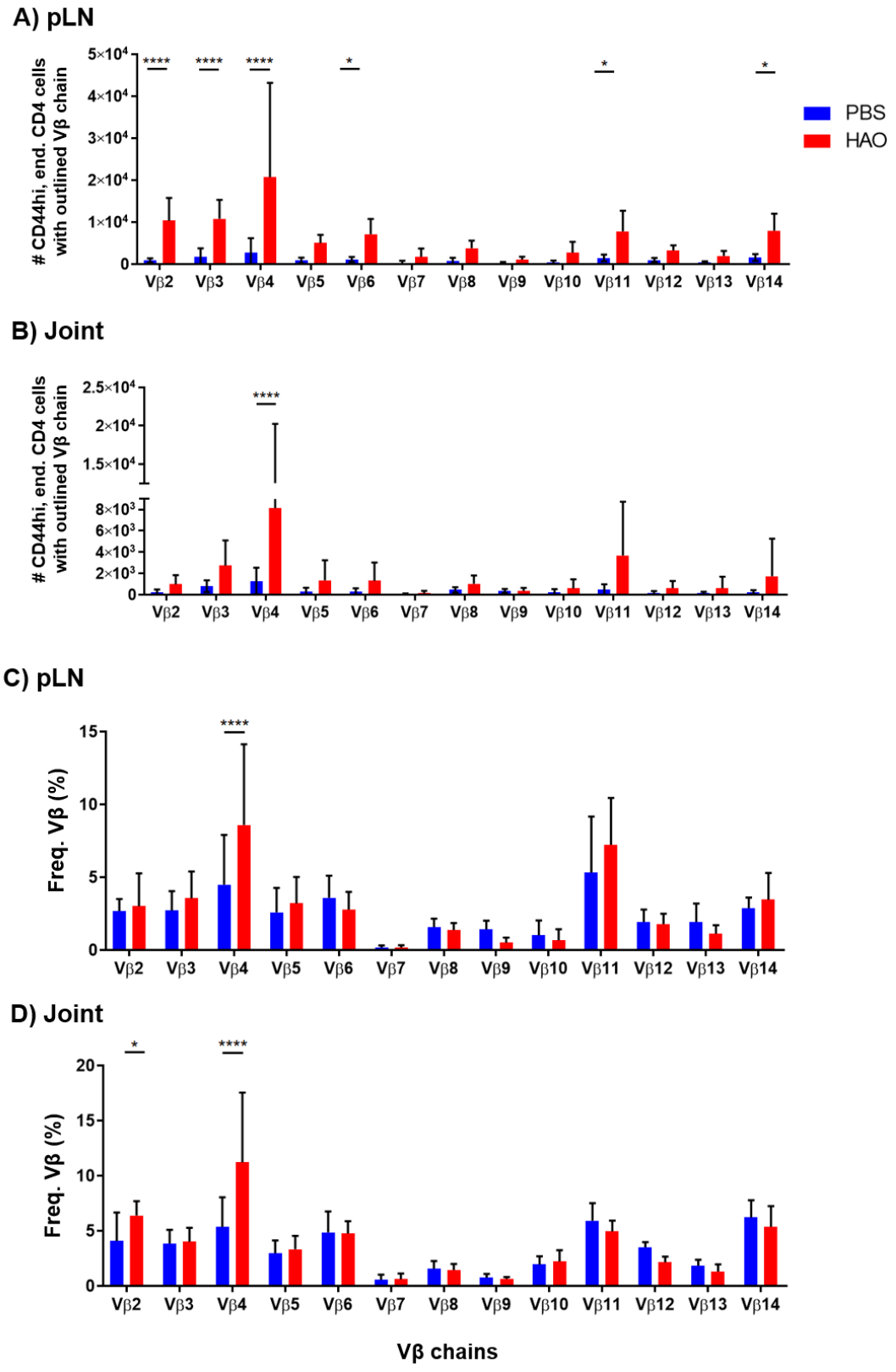


Figure 3-12 - Number and frequency of CD44hi, CD4+ T cells expressing a given Vβ TCR chain in pLNs and joints of HAO and PBS challenged mice.
Cells were isolated from both foot joints and respective pLNs of HAO and PBS challenged mice and Vβ chains were identified by flow cytometry as described in methods. Number of CD44hi, CD4+ T cells with the outlined Vβ TCR chains isolated from **A)** pLNs, and **B)** joints from the same mice, and percentage of Vβ TCR chains in **C)** pLNs and **D)** joints. Bars represent 3 experiments with n=5 for each of HAO and PBS groups (total n=15), except for Vβ2, 12, 5, 13, 11, and 6 where total n= 14 and 13 for the HAO and PBS groups respectively. Vβ groups were compared using 2 way ANOVA. Bars represent mean ±SD. Stars represent the following p values: * <0.05; ** <0.01; *** <0.001; **** <0.0001

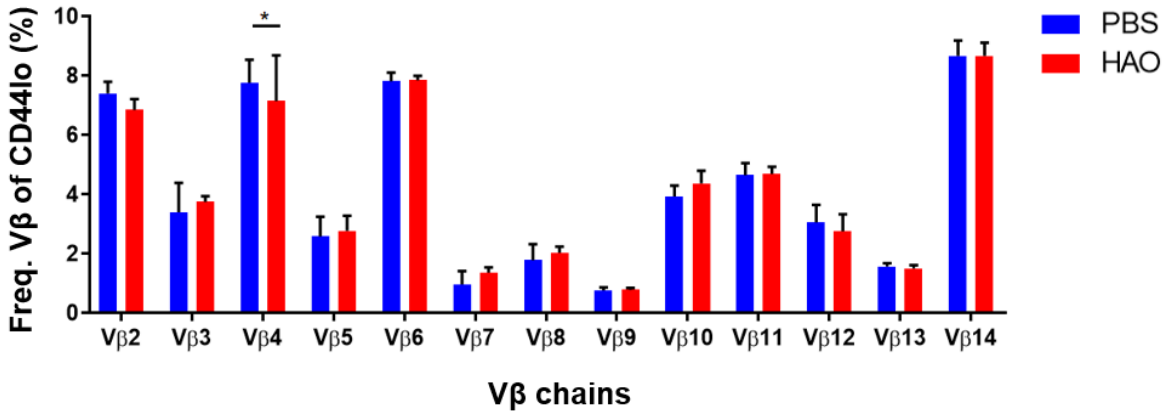


Figure 3-13 - Percentage contribution of the Vβ TCR chains to the CD44lo, CD4+ T cell subset in pLNs of HAO and PBS challenged mice.
Frequencies of Vβ TCR chains from the CD44lo, CD4+ T cell repertoire were determined in HAO and PBS challenged mice. Bars represent 3 experiments with n=5 for each of HAO and PBS groups (total n=15), except for Vβ2, 12, 5, 13, 11, and 6 where total n=14 and 13 for the HAO and PBS groups respectively. Groups were compared using 2 way ANOVA. Bars represent mean ±SD. Stars represent the following p values: * <0.05.

3.2.2.3 Vβ chain usage is due to HAO challenge and is associated with the joint

Part of the model involves using Freund's adjuvant (CFA) in combination with OVA protein as the primary OVA challenge. One thing to consider is whether the observed enrichment is in fact due to the induction of arthritis and not an artefact of the model i.e. the observed Vβ chain usage is simply the response to mycobacterial antigens in the CFA component. To verify this, pLNs were isolated from the PBS controls of the OVA arthritis model as well as naïve C57BL6 mice, and their Vβ profiles were compared **(Figure 3-14 A)**. The data of each mouse is presented as a ratio of CD44hi cells to CD44lo to normalise any biases in the number of cells with a given Vβ chain introduced by the naïve repertoire. No significant accumulation of Vβ chains in the PBS mice were observed when compared to C57BL6 mice, indicating that the accumulation of Vβ chains in the HAO challenged mice is due to HAO challenge. This accumulation in the joint does not appear to reflect a systemic change as no Vβ chains were enriched in spleens of HAO and PBS challenged mice **(Figure 3-14 B).**

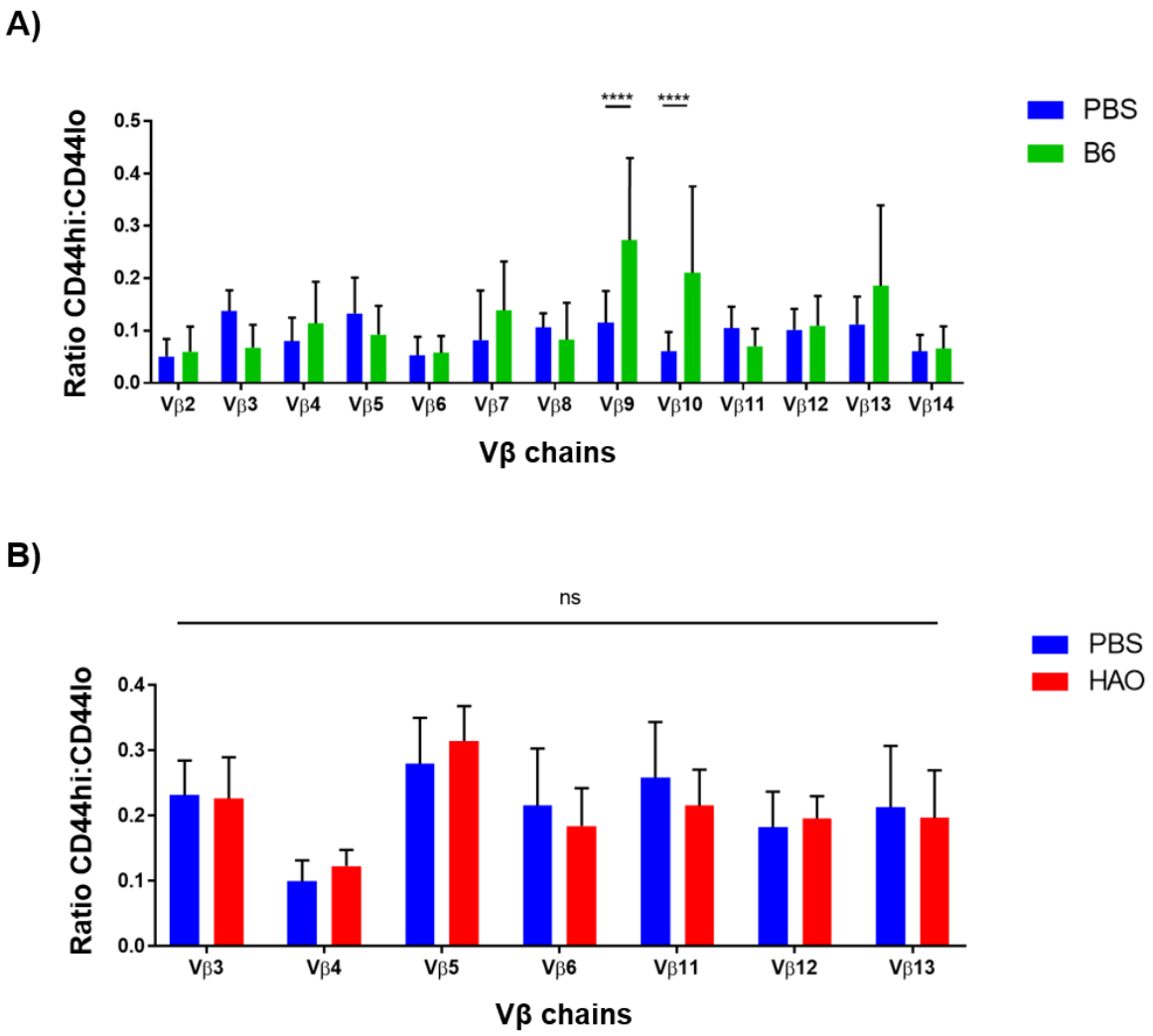


Figure 3-14 - Vβ chain enrichment in C57BL6 mouse pLNs and spleens of HAO and PBS challenged mice
Enrichment was determined by dividing the number of CD44hi, $CD4^+$ T cells of a particular Vβ chain by the respective CD44lo, $CD4^+$ T cell cohort (CD44hi:CD44lo) isolated from pLNs per mouse. This ratio of CD44hi:CD44lo cells were compared between **A)** PBS challenged mice and unchallenged (naïve) C57BL6 pLNs, **B)** spleens of HAO and PBS challenged mice. For figure A, bars represent 3 experiments with n=3 and 5 for the C57BL6 and PBS groups respectively. For figure B, bars represent 1 experiment with n=5 for both the HAO and PBS challenged groups. Groups were compared using 2 way ANOVA. Bars represent mean ±SD. Stars represent the following p values: **** <0.0001; ns: not significant.

3.2.2.4 Accumulation of endogenous Vβ4 CD4+ T cells is associated with an antigen driven response

As mentioned in **section 3.2.1.3**, antigen experienced CD4+ T cells are capable of infiltrating and accumulating in tissues in the absence of their cognate antigen[191,193,194]. An enrichment of Vβ chains, in particular Vβ4, was found in HAO challenged mice and whether this enrichment is driven by antigen specificity or is a consequence of an inflammatory environment is unknown. Using the conditions established in **section 3.2.1.3**, mice were challenged with either HAO, PBS, LPS and the Vβ profile of the joints and pLNs was compared to address the influence of

an inflammatory environment to the Vβ enrichment observed. In both the pLN and joint, the number and frequency of endogenous CD4+ T cells with the Vβ4 TCR chain is significantly higher than in LPS challenged mice **(Figure 3-15 A-D)**, suggesting that Vβ4 accumulation is associated with an antigen driven response.

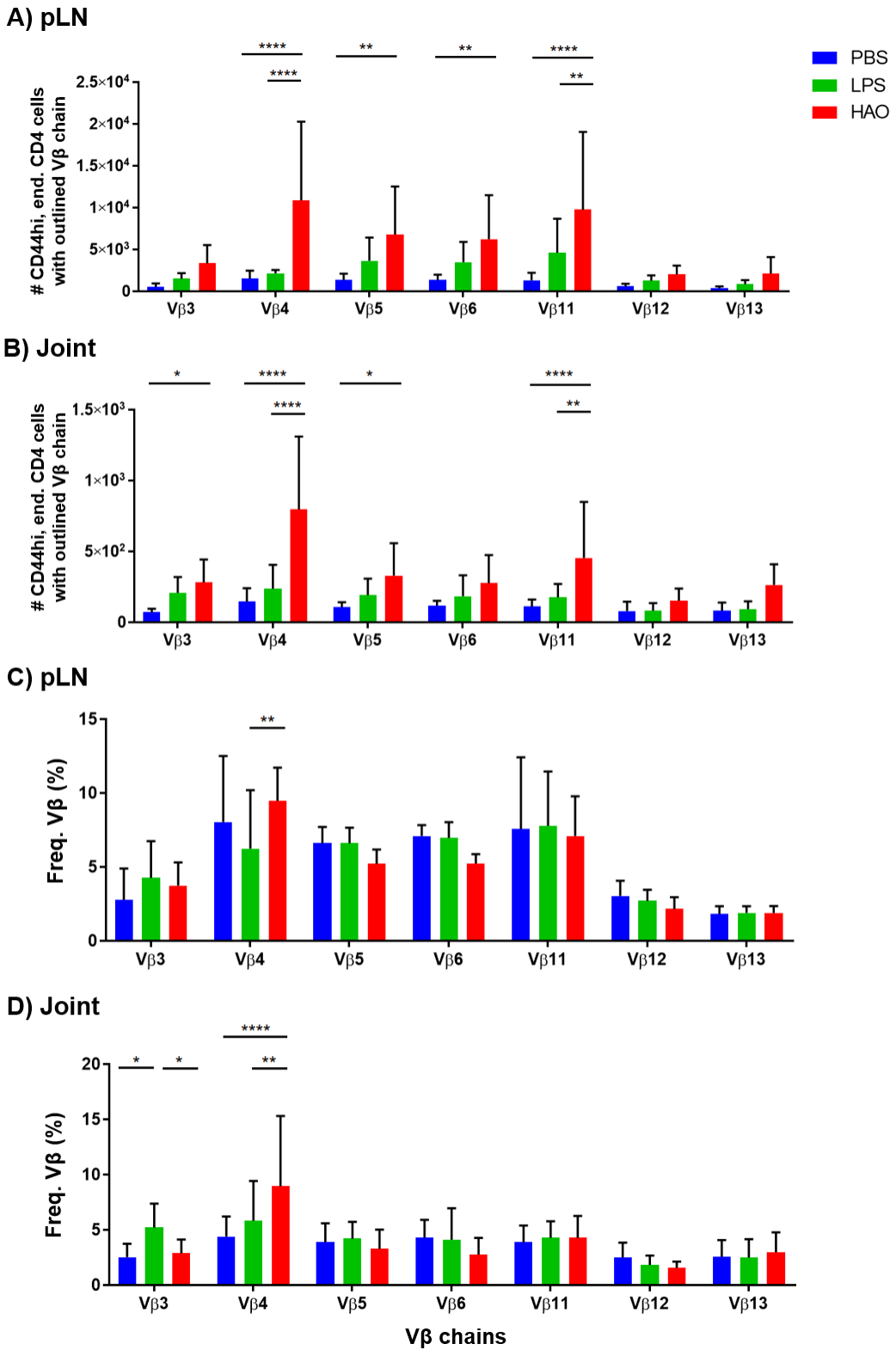


Figure 3-15 - Number an frequency of CD44hi, CD4+ T cells expressing a given Vβ TCR chain from joints and pLNs of HAO, PBS, and LPS (25µg) challenged mice.
Cells were isolated from both foot joints and respective pLNs of HAO, PBS, and LPS (25µg) challenged mice. Transferred cells were identified using the CD45.1 marker and were excluded from analysis, leaving only the endogenous CD4+ T cells. Vβ chains were identified by flow cytometry as described in methods. Number of CD44hi, CD4+ T cells with the outlined Vβ TCR chains isolated from **A)** pLNs, and **B)** joints from the same mice, and frequency of Vβ chains isolated from **C)** pLNs, and **D)** joints. Graphs represent 2 experiments with n=11 for the HAO groups, and n=10 for the PBS and LPS groups. Groups were compared using 2 way ANOVA. Bars represent mean ±SD. Stars represent the following p values: * <0.05; ** <0.01; *** <0.001; **** <0.0001.

3.2.2.5 Vβ4 usage is partly associated with joint derived antigens

So far, it has been demonstrated that Vβ4 usage is due to HAO challenge and associated with an antigen driven response. Whether the antigen recognised is joint tissue specific, OVA specific, or both remains unknown. One approach to narrow down the antigens the Vβ4 family may recognise is to immunise a site other than the joint, such as the ear as mentioned in **section 3.2.1.4**. As with the pLNs and joint, Vβ chain numbers and frequencies were determined in the scLNs and ears to assess the presence of any biases in Vβ use **(Figure 3-16)**. In both HAO challenged scLNs and ears, endogenous CD4+ T cells with the Vβ4 TCR chain were present in the highest numbers **(Figure 3-16 A and B)** and frequencies **(Figure 3-16 C and D)** compared to PBS controls, similar to that observed for HAO challenged pLNs and joints. But when comparing Vβ chain frequencies between the two HAO challenged tissues, all Vβ chains are present in similar frequencies between those sites, except for Vβ4, which is present at a higher frequency in the joint compared to the ear **(Figure 3-17 and Figure 3-18 B)**. These observations indicate that the Vβ4 family may contain clones that specifically recognise joint derived antigens. However, it is important to note that despite the significant increase in Vβ4 frequency in the inflamed joint compared to the ear, the overall Vβ usage pattern is in fact similar between the two sites. This indicates that antigen specific responses between these two sites may be similar.

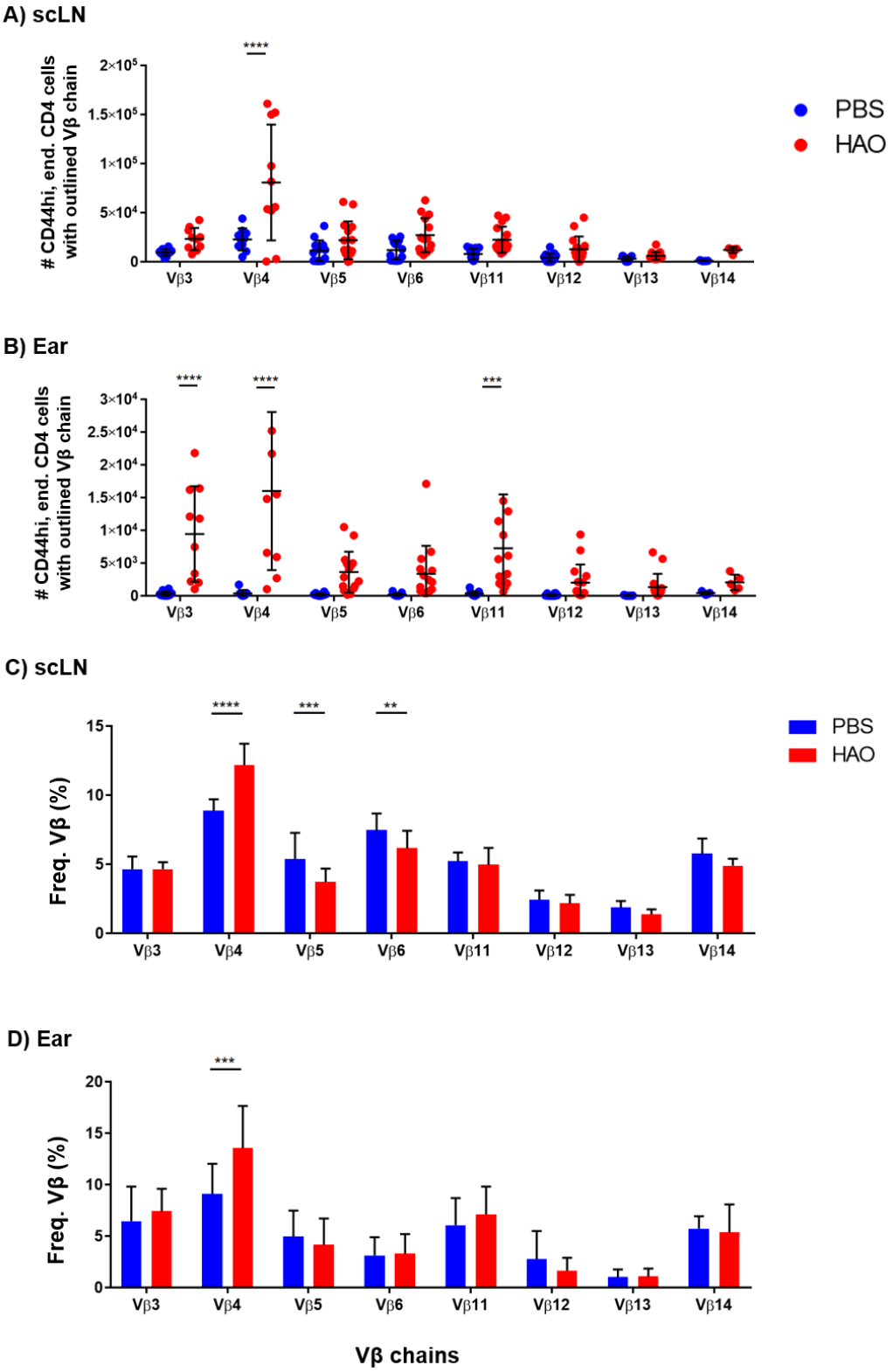


Figure 3-16 - Number and frequency of CD44hi, CD4+ T cells with the outlined Vβ TCR chains from scLNs and ears of HAO and PBS challenged mice.
Cells were isolated from both ears and respective scLNs of HAO and PBS challenged mice and Vβ chains were identified by flow cytometry as described in methods. Number of Vβ TCR chains in CD44hi, CD4+ T cells isolated from **A)** scLNs, and **B)** ears from the same mice, and percentage of Vβ TCR chains in **C)** scLNs, and **D)** ears of HAO and PBS challenged mice. For Vβs 5-13 graphs represent 3 experiments with n=15 for the HAO groups, n=15 (Vβs 6, 11, and 13), and n=14 (Vβs 5 and 12) for the PBS groups. For Vβs 3 and 4, graphs represent 2 experiments with n=10 and 9 for the HAO and PBS groups respectively. For Vβ14, graphs represent 1 experiment with n=5 for each of the HAO and PBS groups. Vβ groups were compared using 2 way ANOVA. Bars represent mean ±SD. Stars represent the following p values: ** <0.01; *** <0.001; **** <0.0001

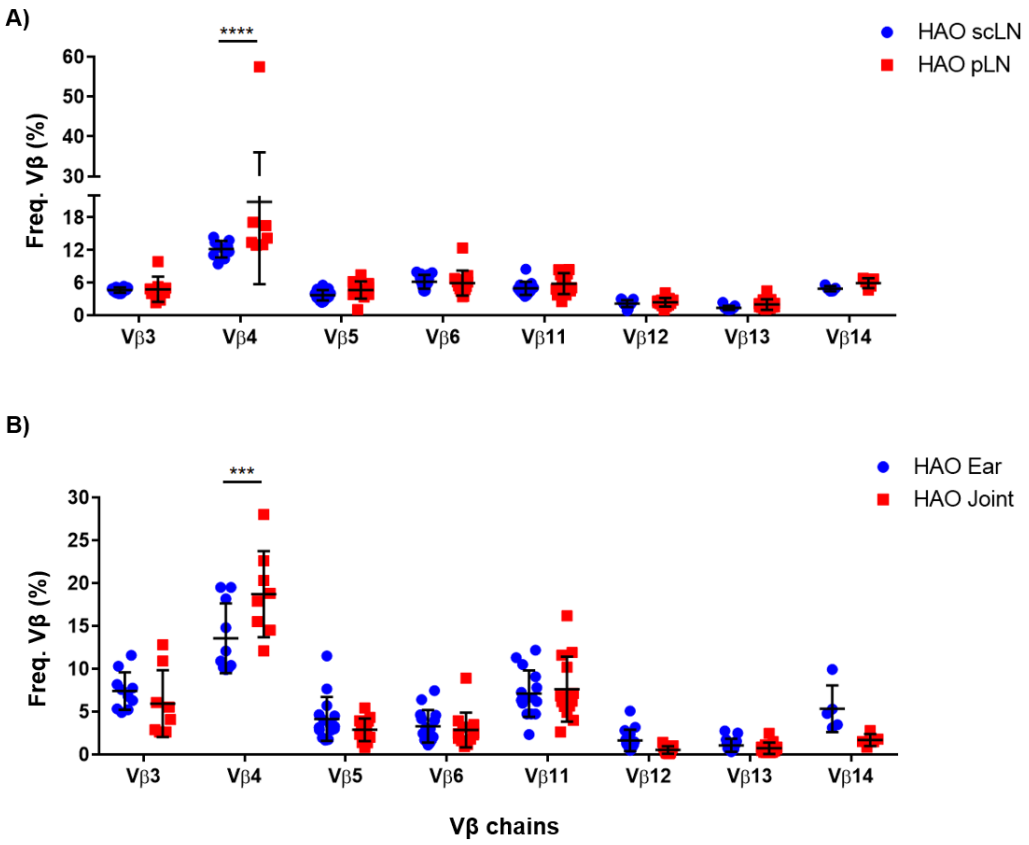


Figure 3-17 - Percentage contribution of the Vβ TCR chains to the CD44hi, CD4+ T cell subset in HAO challenged mouse ears, joints, and respective dLNs.
Cells were isolated from both HAO challenged ears and joints and their respective scLNs and pLNs. Vβ chains were identified by flow cytometry as described in methods. Percentage of Vβ TCR chains in CD44hi, CD4+ T cells isolated from **A)** scLNs and pLNs and **B)** ears and joints from HAO challenged mice. For Vβs 5-13 graphs represent 3 experiments with n=15 for ears and scLNs and n=13 for joints and pLNs. For Vβs 3 and 4, graphs represent 2 experiments with n=10 ears and scLNs and n=8 for joints and pLNs. For Vβ14, graphs represent 1 experiment with n=5 for ears, scLNs, joints, and pLNs. Vβ groups were compared using 2 way ANOVA. Bars represent mean ±SD. Stars represent the following p values: **** <0.0001

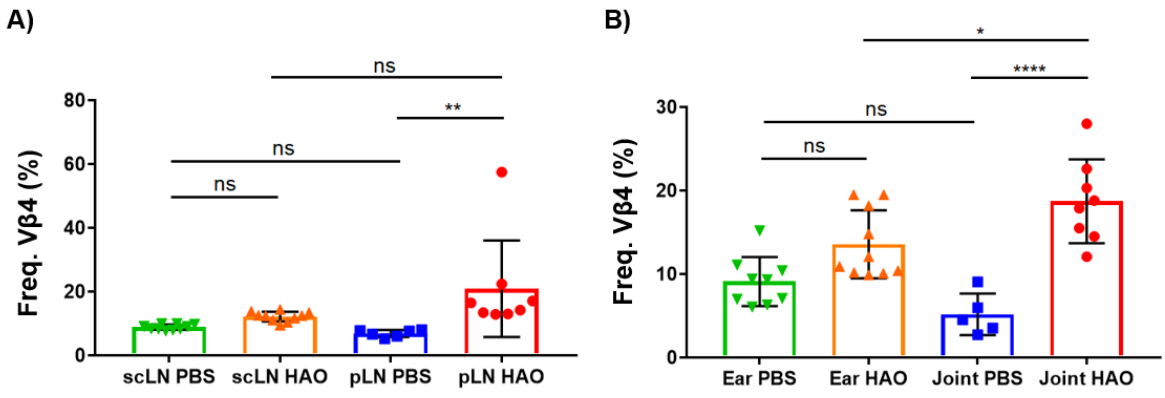


Figure 3-18 - Percentage contribution of the Vβ4 TCR chain to the CD44hi, CD4+ T cell subset in ears, joints, and dLNs of HAO and PBS challenged mice.
Cells were isolated from both HAO and PBS challenged ears and joints and their respective scLNs and pLNs. Vβ4 was identified by flow cytometry as described in methods. Percentage of the Vβ4 TCR chain in CD44hi, CD4+ T cells isolated from **A)** scLNs and pLNs and **B)** ears and joints from HAO and PBS challenged mice. Graphs represent 2 experiments with n=10 for HAO and PBS challenged ears and scLNs and n=8 and 5 for joints and pLNs challenged with HAO and PBS respectively. Groups were compared using one way ANOVA. Bars represent mean ±SD. Stars represent the following p values: * <0.05; ** <0.01; **** <0.0001; ns: not significant.

3.2.2.6 Inflamed joints at the late timepoint continue to display a biased accumulation of Vβ4 CD4+ T cells while the pLNs at the same timepoint do not

The data suggests that the Vβ4 TCR chain dominates the response in this model and may be associated with joint derived antigens. As this model represents an early arthritic time point, it would be interesting to see how the response develops with time and disease chronicity, and how much the initial response, namely Vβ4, contributes to the progression of the disease. Using the late model of experimental arthritis established in **section 3.2.1.5**, Vβ use was assessed and compared to the enrichment observed at the early timepoint to determine how the antigen specific responses evolves with disease progression.

The number of cells with a certain Vβ chain remined unchanged between pLNs at the early and late timepoints, except for Vβ6, which increased in late inflamed pLNs **(Figure 3-19 A)**. This is reflected when looking at frequencies of the Vβ chains, where Vβ6 also appears at a higher frequency in late pLNs. **(Figure 3-19 B)**. Interestingly, Vβ4 appeared at a lower frequency in pLNs at the late timepoint compared to pLNs at the early timepoint, indicating that number of cells with the Vβ6 are indeed increasing and therefore suggests skewing of the endogenous CD4+ T cell antigen specific response in the pLN. In contrast, the late inflamed joints have a significantly higher number of CD4+ T cells with the Vβ4 TCR chain compared to HAO challenged joints at the early timepoint, while the remainder of the Vβ chains were present in similar numbers **(Figure 3-19 C)**. The frequencies of the Vβ chains remained unchanged between inflamed joints from the early and late timepoints **(Figure 3-19 D)**. This “switch” in Vβ usage in the late inflamed pLNs indicates an evolution in the inflammatory response and a possible change in the antigens being recognised in late inflamed pLNs compared to those in early pLNs. In contrast, the joints seem to maintain the same Vβ usage as their early counterparts, suggesting that the cells may be recognising the same antigens that are present in the early stage. The continued increase in Vβ4 numbers in the joint though, is evidence that cells with this TCR chain are specifically retained with disease progression and may suggest that the Vβ4 family contains the CD4+ T cell clones that initiate and propagate inflammatory arthritis. The data also suggests divergent endogenous CD4+ T cell responses between the pLN and the inflamed joint with the progression of experimental arthritis. Whether this divergence is maintained, or whether the joint will eventually mirror the clonality observed in the pLN remains to be elucidated.

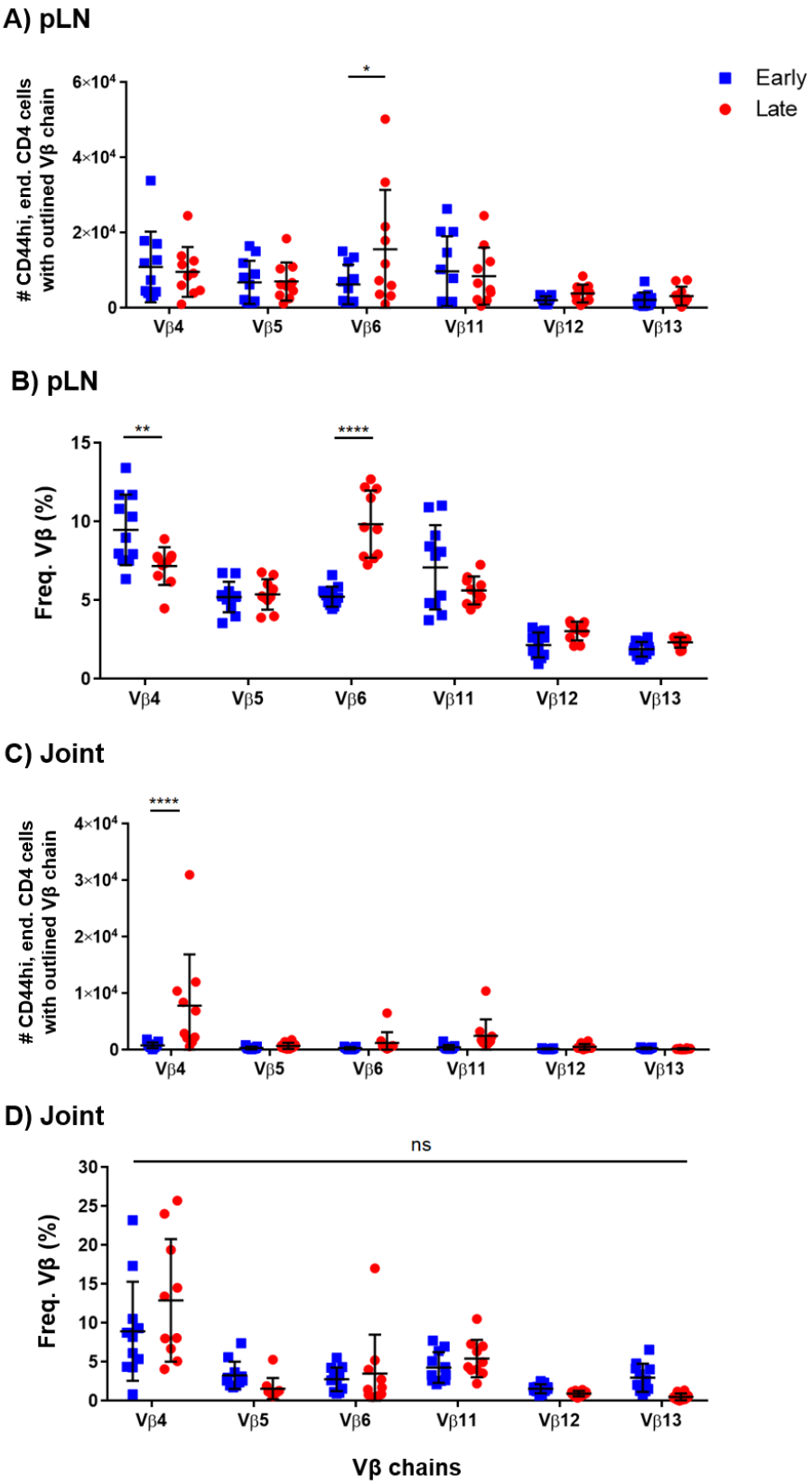


Figure 3-19 - Number and frequency of CD44hi, CD4+ T cells with the outlined Vβ TCR chains from pLNs and joints of acute and chronic arthritic mice.
Cells were isolated from both pLNs and joints of acute and chronic (D17 after secondary HAO challenge) arthritic mice and Vβ chains were identified by flow cytometry as described in methods. Number and frequency of CD44hi, CD4+ T cells with the outlined Vβ TCR chains isolated from pLNs **(A and B)** and joints **(C and D)**. Graphs represent 2 experiments with n=10 and 11 for the chronic and acute groups respectively. Each dot represents one mouse. Vβ groups were compared using 2 way ANOVA. Bars represent mean ±SD. Stars represent the following p values: ** <0.01; **** <0.0001; ns: not significant

3.3 Discussion

The early events leading to RA pathogenesis remain poorly understood. More specifically, the roles that antigen specificity and CD4+ T cells play in early stages of disease are also unknown. The main objective of this chapter was to provide evidence for the presence of antigen specific responses in the early phases of experimental arthritis, as this this has important implications for the development of antigen specific tolerogenic therapies. To investigate the development of these antigen specific responses, the OVA experimental arthritis model - developed by Maffia et. al.[140]- was established to assess antigen associated changes CD4+ T cell clonality as well as the influence of an inflammatory environment and joint derived antigens to the CD4+ T cell repertoire. Once this was achieved, the model was also adapted to investigate how CD4+ T cell clonality changes with the progression of experimental arthritis. The CD4+ T cell clonal response was monitored by assessing biases in TCR Vβ chain use, as a bias would indicate the presence of antigen specific responses. Overall, a bias in Vβ chain usage was found early after the initiation of experimental arthritis, with the Vβ4 TCR chain being present in the largest numbers and highest frequency in inflamed mouse pLNs and joints at the early timepoint, demonstrating preferential accumulation of CD4+ T cells with the Vβ4 TCR chain in these tissues. Vβ4 was also found to be associated with an antigen driven response, and increases in number with disease progression. Together, this data shows that this family may contain clones that initiate and propagate inflammatory arthritis and provides evidence for the presence of an antigen specific response in the early phases of disease.

The model was successful in inducing local inflammation and CD4+ T cell accumulation at articular sites where HAO was administered. Moreover, endogenous CD4+ T cells were present at much higher numbers than transferred OT-II cells, implying that endogenous CD4+ T cells could be playing a greater role in perpetuating the inflammatory response by recognising antigens in addition to OVA. Indeed, it has been shown that when a high number (approximately 1x10^6) of naïve OT-I cells are transferred into C57BL6 mice, the endogenous CD8+ T cell response to OVA is inhibited[205]. Similarly, a large monoclonal naïve or effector CD4+ T cell population can inhibit proliferation of naïve CD4+ T cells with the same TCR[206].

Evidence of antigen experienced CD4+ T cells in infiltrating inflamed sites without the need of their cognate antigen has been reported in the OVA model used here[191] and in RA patients[100,193]. An inflamed site can therefore obscure any evidence of antigen specific accumulation of CD4+ T cells. Considering this, an LPS challenge was administered to assess the non-antigen induced inflammatory accumulation of CD4+ T cells in the inflamed joint. LPS challenge was sufficient in causing an influx of cells into inflamed pLNs and joints, but the number of total and antigen experienced endogenous CD4+ T cells were comparable to PBS controls. This is not unusual as LPS is known to recruit innate immune cells such as DCs macrophages and neutrophils[261] and are likely to make up the majority of the infiltrate in the LPS inflamed joint. It is important to note that although the number of CD4+ T cells is comparable to controls, the composition of the repertoire may not be the same. Similarly, how much the increase in endogenous CD4+ T cells in HAO challenged pLNs and joints can be attributed to antigen specific responses is unknown. Comparing the CD4+ T cell repertoire from LPS and HAO challenged mice will therefore elucidate how much of the response is antigen specific.

The model was also adapted to investigate the contribution of joint derived antigens to the clonality of the CD4+ T cell infiltrate. Mouse ears were immunised with HAO and the CD4+ T cells were isolated from ears and ear draining scLNs. The total and antigen experienced number of endogenous CD4+ T cells is greater in HAO challenged ears and scLNs than PBS controls, mirroring the observations seen in HAO challenged pLNs and joints. This would indicate that HAO challenged in the ear induces similar inflammatory responses observed in the joint.

Examining the CD4+ T cell infiltrate in these contexts allows for the accurate assessment of antigen specific CD4+ T cell responses in inflamed joints and pLNs at an early timepoint when conditions are most permissive for developing autoreactivity to articular antigens. Observing any changes in the evolution of these antigen specific responses can therefore be attributed to joint derived antigen specific responses.

The late model of experimental arthritis was developed to establish a time point representing chronicity and to monitor the evolution of the initial articular response observed. The late timepoint – 17 days after the second periarticular challenge – was found to be the best timepoint of the late model, as the difference in

endogenous CD4+ T cell number was greatest between the late inflamed groups than the rest of the controls. This would indicate that the cells present at this timepoint are accumulating due to an antigen specific response rather than being artefacts of general inflammation. The bias seen in Vβ usage at this timepoint would therefore more reflect an antigen driven bias rather than a bystander or "background" response (see **section 1.3.3.1** of the introduction), which has been demonstrated in several autoimmune diseases, and implicated in RA[46,100,101]. This endogenous CD4+ T cell population was also maintained in the late inflamed groups with time, while the numbers decreased in the control groups over time. This further indicates that the response observed is more likely antigen associated. One thing to note is the reduction in the number of antigen experienced cells at this timepoint compared with both the early and intermediate time points. CD44hi is a receptor of extra cellular matrix proteins and is found on a variety of leukocyte populations[267]. It plays many important roles in T cell survival, migration, and proliferation, and is used as a marker of antigen stimulated memory T cells[268,269]. The reduced number of CD44hi cells at the late timepoint is unexpected, considering that the maintained endogenous CD4+ T cell accumulation is considered to be a result of antigen recognition. A reduction in CD44hi cells could be a result of cell death, although that was ruled out considering the number of endogenous CD4+ T cells is not reduced. Furthermore, there is evidence for CD44 playing a role in T cell survival[270]. A possible explanation is the downregulation of CD44 once the cells are retained in the tissue. CD44 is involved in T cell extravasation into tissues, but it is not necessary[270], and T cells can be retained in tissues via CD44 independent mechanisms, such as selectins[271,272]. Therefore, CD44 expression could be dependent on the other roles it plays. It could simply be that number of CD44 molecules are reduced due cleavage of the molecule, which is a mechanism used by cancer cells to regulate migration[273]. The late model is a resolving model, and the mice do recover. So, reduction of CD44 could reflect the model resolving and that the T cells are just getting ready to leave the site. It is clear that investigating the biological function of these cells is necessary in understanding the role the play in propagating the inflammatory response.

The PCR data demonstrated CDR3β oligoclonality in arthritic pLNs and joints, while maintaining broad Vβ chain usage. This is consistent with observed clinical data where an oligoclonal CD4+ T cell repertoire was found in the synovia of RA patients[46]. It is important to note that a substantial amount of DNA was required for

the CDR3β PCR and samples from 20 HAO challenged mice were pooled to obtain enough DNA. So, the diversity observed may have resulted from multiple private responses as opposed to the presence of a diverse public response (see introduction **section 1.2.1.3**). It has been shown that mice immunised with OVA display private responses but have some CDR3β sequence similarity[274]. In addition, the CD4+ T cell response was found to be private across RA patients[46,143,275].

In order to overcome the limitations of PCR, Vβ chain usage was determined from individual mice using flow cytometry. An overall increase in Vβ chain use was found in HAO challenged pLNs and joints and the Vβ4 TCR family was present in the largest numbers and frequency in these tissues. It is important to note that approximately 50% of the antigen experienced repertoire remains unaccounted for in terms of Vβ chain usage – as reagents for only 13 out of the 22 Vβ TCR mouse chains were available. Therefore, the presence of other dominant chains cannot be ruled out. Other Vβ TCR chains were present at significantly higher numbers than PBS controls in pLNs - namely Vβ2, 3, 6, 11, and 14, while other remained unchanged. This disproportionate increase again reflects a change in Vβ usage depending on the challenge type and also reflects narrowing of the response, confirming the oligoclonality observed using PCR.

On examining Vβ use in LPS challenged mice, the results show that LPS contributes to the accumulation of several Vβ chains, but Vβ4 is the only chain associated with an antigen driven response. This highlights the possibility of an antigen specific response playing an early role in the accumulation of CD4+ T cells in the inflamed joint, which may create an ideal inflammatory environment for other cells to infiltrate. Indeed, HAO challenge results in a larger influx of endogenous CD4+ T cells than LPS challenged mice. It also reiterates how general inflammation can bring cells into the joint in a non-antigen specific manner.

Vβ4 is also present in the largest numbers and the highest frequency in joints and ears of HAO challenged mice compared to their respective PBS controls. However, the percentage contribution of cells expressing Vβ4 is higher in HAO challenged joints than in the ear, suggesting that cells with the Vβ4 TCR chain are retained at a higher degree in the joint than the ear, despite the increased cell number observed in the ear. This preferential recruitment in the joint may suggest that the Vβ4 family contains clones that possibly recognise joint antigens.

The fact that the Vβ4 chain is also present in large numbers and in the highest frequency in the ear suggests that the Vβ4 family also contains CD4+ T cells clones that recognise OVA, proteins common to the joint and ear, or both. A more direct way of determining what these cells may recognise is to isolate them and re-stimulate them with joint derived antigens and determine their cytokine production. This experiment has been carried out and will be elaborated on in **Chapter 5**.

The data presented here demonstrates that the initial articular response is restricted and could be initiated by the recognition of joint associated antigens. Monitoring the evolution of the initial responses in this experimental model could help elucidate the events that lead to development of the responses observed in RA. On comparing Vβ usage between the early and late models, it was found that late inflamed pLNs displayed a different response to early, HAO challenged pLNs, while the joints seemed to maintain the same Vβ usage with disease progression. The switch in Vβ frequency between pLNs at the early and late timepoints may indicate the beginnings of other responses and may reflect epitope spreading, a phenomenon whereby both B and T cells react to epitopes other than the dominant epitope of a protein[52]. In addition, the reduced Vβ4 frequency and the maintained cell number could indicate the influx of cells with other Vβ chains that have not been examined in these experiments. The joints on the other hand show an increased accumulation of cells with the Vβ4 TCR chain in the late model, while the frequency of the Vβ chains remain unchanged. Again, this indicates that other cells with other Vβ chains may be accumulating in the joint. The degree of the changing response cannot be fully appreciated as only a subset of the Vβ chains were examined

The different responses observed between the lymph node and joint may indicate that changes in antigen responses occur in the lymph node before they are reflected in the joint. Indeed, studies have shown changes in the pre-articular lymph node occur before any clinical manifestations are observed in the joint[139]. Whether these changes in the model reflect epitope spreading (see **section 1.3.3.1** of introduction) and a continued propagation of proinflammatory responses or the development of regulatory responses is unknown, as the function of these cells has not been examined. Finally, the increasing number of cells with the Vβ4 TCR chain in the joint suggest that this family contains CD4+ T cell clones that initiate and propagate inflammatory arthritis.

Detecting biases in Vβ usage by flow cytometry allows for a quick assessment of the changes occurring due to antigen specific responses and has proven its usefulness in elucidating the events shaping the oligoclonal CD4+ T cell repertoire in this model of early arthritis. The drawback with this technique comes with the assessment of small or rare changes in the repertoire, as flow cytometry is more accurate for assessing large changes in population frequencies[276–278]. In addition, approximately 50% of the Vβ chains remain unaccounted for due to lack of available reagents.

It is important to remember that biases in Vβ chain use are only a proxy for clonality and cannot provide information on a clonal level. In order to find the actual clones that initiate and propagate arthritis, a technique with higher resolution is required. The CDR3β sequence of the TCR, which lies at the interface of the VDJ regions of the β chain, is the most variable region of the TCR and is the region that confers peptide specificity[220]. Each CD4+ T cell clone will therefore have a CDR3β sequence specific to their cognate peptide. Next generation sequencing (NGS) methods can be utilised to sequence CDR3β regions of T cells and the contribution of individual clones can be detected in different tissue sites[279]. In order to fully appreciate the evolution of the articular response, NGS methods were used in the model to more accurately detect clonal CD4+ T cell dynamics between the pLN and joint and will be discussed in the following chapter.

Chapter 4 A detailed examination of the evolution of CD4+ T cell clonality in the OVA model of experimental arthritis

4.1 Introduction

In the previous chapter CD4+ TCR clonality was determined using PCR and flow cytometry techniques which targeted the Vβ region of the TCR. Evidence was found to support the presence of an antigen specific response in the early phases of inflammatory arthritis which may represent autoreactive T cell clones that may be involved in initiating and/or propagating the disease. In this chapter, we employed next generation sequencing (NGS) techniques[279] to investigate the evolution of CD4+ T cell clonality in the early and late models of OVA mediated inflammatory arthritis. **(see sections 2.7 and 2.8, and Figure 4-2)**.

Enrichment of specific T cell clones within the repertoire may represent previous encounters with specific antigens, and so, provide historical evidence of antigen specific responses that have occurred throughout an individual's lifetime. As such, T cell repertoires have been examined to evaluate these antigen specific responses which in turn would mirror progression or cessation of diseases[23,42,280]. Indeed, TCR repertoire analysis has been used to monitor the development of leukaemia[42], coeliac disease[23], and systemic lupus erythematosus[281]. With the advent of NGS methods, in depth analyses can be made of the TCR repertoire and the expansion of individual clones can be detected and tracked[23,46]. Several clinical studies have utilised NGS methods to detect expanded clones in RA patients at different stages of disease[46] and have attempted to functionally characterise them as well[282]. To date, there have not been any studies – clinical or otherwise – investigating the spatiotemporal dynamics of CD4+ T cells between arthritic tissues and their draining lymph nodes. Understanding these dynamics will provide further insight into the development of the antigen specific response in RA and determine the role autoreactive CD4+ T cells play in the initial phases of the disease and whether they create a permissive environment for other CD4+ T cells to enter. As noted in **section 1.5.1** of the introduction, understanding the development of autoreactive responses in RA is paramount for the development of more effective tolerogenic therapies.

T cell repertoires can be characterised by the number of species or clones that make up the repertoire and the degree to which each clone contributes to the repertoire, referred to as species richness and diversity respectively[283,284]. Together, these attributes describe the clonality of the T cell repertoire **(Figure 4-1)**. Employing NGS methods described in **section 2.7.3** of methods, species richness and clonal diversity of the antigen experienced i.e. CD44hi CD4+ T cell repertoires were characterised and compared from inflamed joints and pLNs from the early and late model timepoints, established in **Chapter 3 sections 3.2.2.2 and 3.2.2.6 and Figure 4-2**. V-gene sequence frequencies and clonal overlap were also assessed to determine similarities in antigen specific responses and the degree specific clones were shared between early and late inflamed joints and pLNs. The distribution of high frequency clones was also compared between the two sites to assess the dynamics of these CD4+ T cell clones with the progression of experimental arthritis.

Overall, I have demonstrated that the antigen experienced CD4+ T cell repertoire in pLNs shows enhanced clonality and diversity with disease progression, while inflamed joints maintain similar CD4+ T cell clonality and diversity over time. This enhanced diversity seen in inflamed pLNs at the late timepoint is a result of changes in TCR usage and may reflect changes and broadening of antigen specific responses. Furthermore, the number of clones shared between the inflamed pLN and joint was found to decrease with time, but the correlation of clones occurring at the highest frequency between the inflamed pLN and joint is indicative of ongoing migration and communication between these sites. Together these analyses demonstrate the presence of spatiotemporal differences in the antigen specific CD4+ T cell response with the progression of experimental arthritis and may reflect the evolution of these responses in RA. By understating the evolution of CD4+ T cell responses in RA, more informed decisions can be made on where and when antigen-specific therapeutics should be applied and will help develop more effective regimes to reinstate self-tolerance.

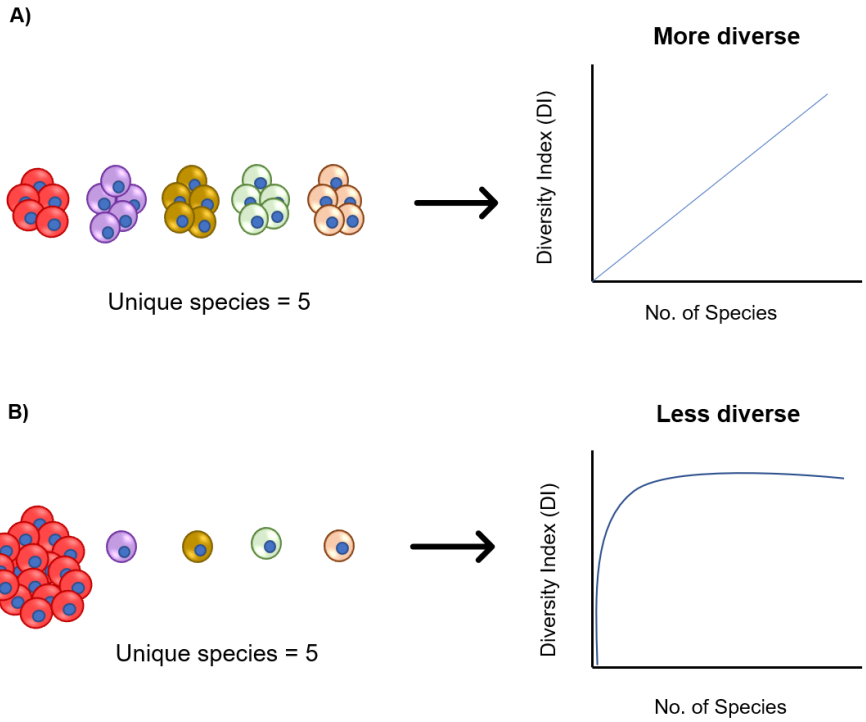


Figure 4-1 Illustration of species richness and diversity to describe T cell clonality
Descriptors like species richness and diversity are used to describe the composition of T cell repertoires. Species richness refers to the number of species, or in this case clones, that make up a T cell population. The diversity takes into account the relative contribution of each clone to the population. In this study the D50 value is used to describe repertoire diversity and is calculated as described in **section 2.8.1** of methods In **A)** the number of unique species, or species richness equates to 5, as illustrated by the 5 different coloured species. Each species is represented in equal frequencies and so this population is said to be diverse. In **B)** the number of unique species is also 5, but each species is not represented equally and one species (red) makes up the majority of the T cell repertoire. In this case the population shows less diversity. Together these descriptors describe the clonality of a T cell population.

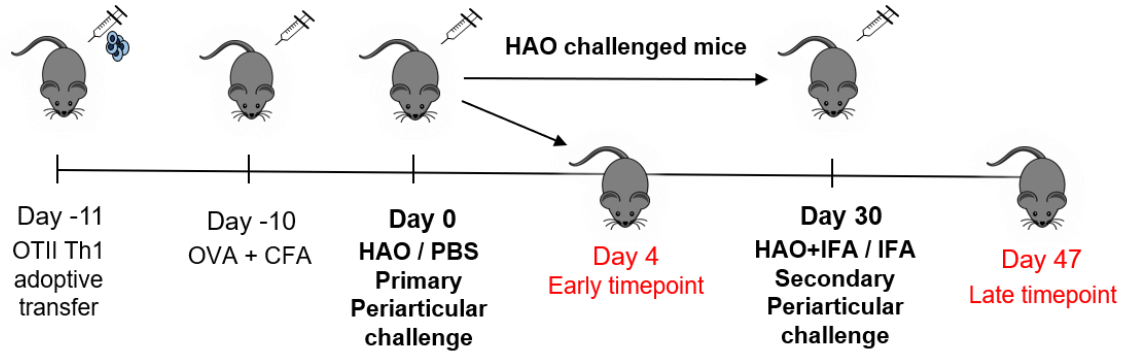


Figure 4-2 Illustration of early and late model timepoints.
Early and late model timepoints, described in detail in **sections 2.2.5 and 2.2.7** are adaptations of the OVA experimental arthritis model described in introduction **section 1.6**. Briefly, Th1 polarised OT-II cells are transferred in to C57Bl/6 mice which is followed by immunisation of OVA in CFA in the scruff 24 hours after the adoptive transfer. 10 days after OVA+CFA immunisation, mice are given a periarticular immunisation of either HAO or PBS as a control. For the early timepoint, mice are sacrificed 4 days after this primary periarticular challenge. For the late timepoint, HAO challenged mice are given a 2nd periarticular challenge 30 days after the primary HAO challenge with either HAO in IFA or IFA alone as a control. The mice are then sacrificed 17 days after the 2nd periarticular challenge.

4.2 Results

4.2.1 CD4+ T cell repertoire displays enhanced clonal diversity in late inflamed pLNs compared to early inflamed pLNs

In order to investigate the evolution of the CD4+ T cell response in the breach of tolerance model of inflammatory arthritis, endogenous antigen experienced CD4+ T cells i.e. excluding the transferred OT-II cells, were sorted from pLNs and joints of mice undergoing the early and late models of OVA mediated inflammatory arthritis. Total RNA was isolated from the endogenous CD4+ T cells and sent for CDR3β sequencing to iRepertoire Inc. Endogenous CD4+ repertoires were examined and compared in mouse pLN samples from early and late model timepoints. The number of unique CDR3β were determined to assess the number of different clones present in each sample in each group **(Figure 4-3 D)**. pLNs at the late timepoint have a higher number of unique CDR3β sequences than their early counterparts, which is expected because late pLNs also have a greater overall number of endogenous CD4+ T cells **(Figure 4-3 C)**. Repertoires were characterised by examining the degree that expanded clones contributed to the composition of each repertoire and a clear difference was observed between repertoire compositions of early and late pLNs **(Figure 4-3 A and B)**. The top 10 clones – represented by the dark orange blocks in Figure 4-3 A and B – make up on average 18% of the early inflamed pLN repertoire, while the top 10 clones only contribute 2% to the total repertoire in late inflamed pLNs **(Figure 4-3 E)**. Moreover, the biggest difference between the two groups is the contribution of low abundance clones (blue blocks) to the CD4+ T cell population from pLNs at the late timepoint, which on average make up the majority of the antigen experienced population in late inflamed pLNs. This is also reflected when looking at the diversity of each repertoire in each group; late pLNs display higher repertoire diversity compared to early inflamed pLN samples **(Figure 4-3 F)**, which indicates that the clones in late pLNs are represented more equally, while early pLNs have a repertoire skewed towards high abundance clones.

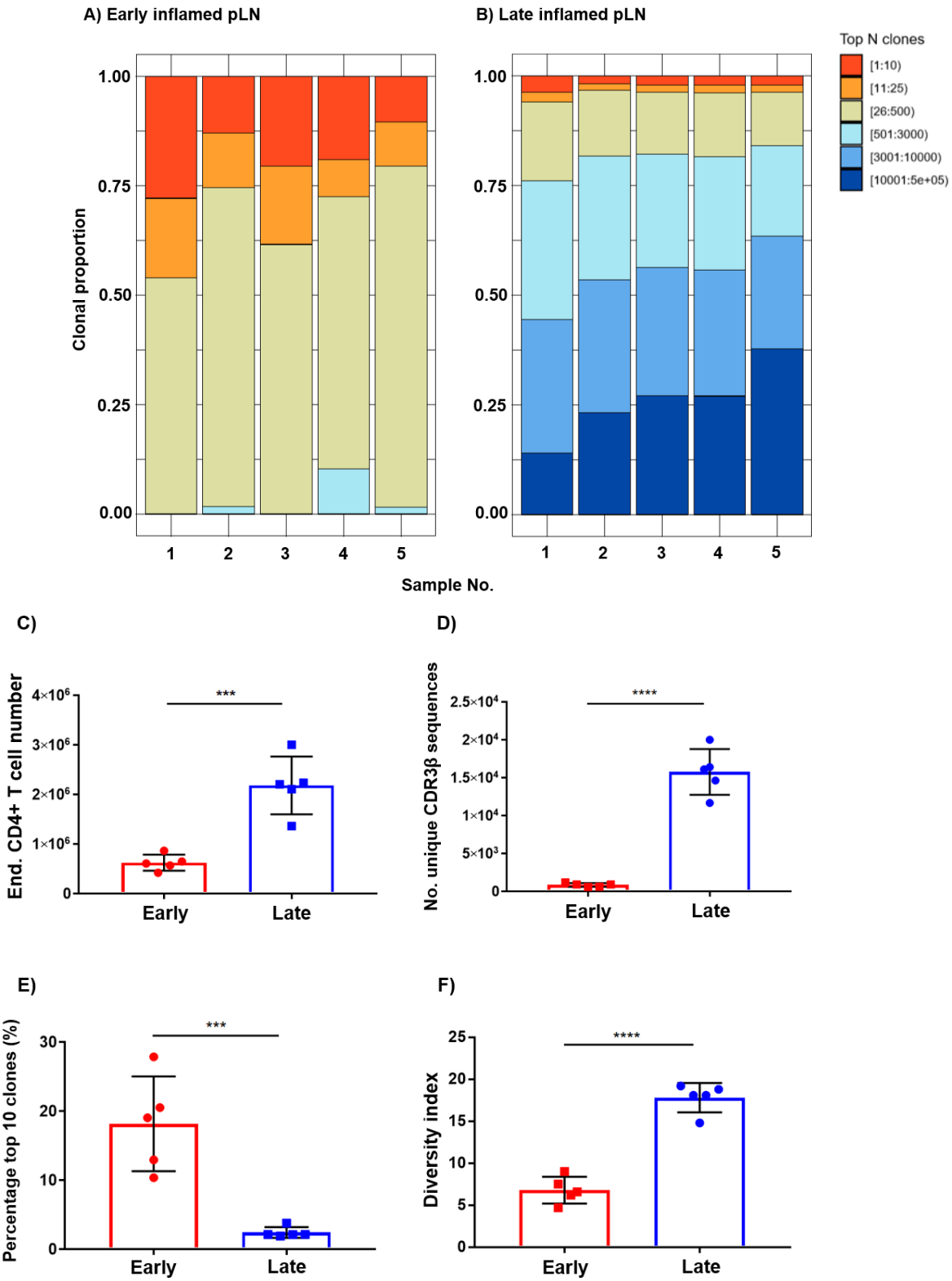


Figure 4-3 Characterisation of the antigen experienced CD4+ T cell repertoire in early and late inflamed pLNs.

Proportion of the top 10 most frequently occurring clones, followed by the next 25, 500, 3000, 10,000, and 100,000 most frequent clones contributing to the endogenous antigen experienced CD4+ T cell repertoire in **A)** early, and **B)** late inflamed pLNs per sample. **C)** Number of endogenous CD4+ T cells, **D)** number of unique CDR3β sequences in early and late inflamed pLNs. **E)** Percentage contribution of the top 10 most frequently occurring clones, and **F)** diversity indices (D50) of the antigen experienced endogenous CD4+ T cell repertoire in early and late inflamed pLNs. Graphs represent 1 experiment with n=5 for the early and late inflamed groups. Top proportion charts were generated using the tcR R package[258]. Groups were compared using unpaired Student's t-test. Data presented as mean ± SD. Stars represent the following p values: *** <0.001; **** <0.0001.

4.2.2 CD4+ T cell repertoires of early and late inflamed joints are comparable

In contrast to observations between early and late inflamed pLNs, early and late inflamed joints display very skewed repertoires, where the top 25 clones dominate the antigen experienced CD4+ T cell population and make up on average 60% and 56% of the repertoire **(Figure 4-4 A and B)**. Interestingly, the repertoire composition is similar between early and late inflamed joints; the number of unique CDR3β sequences, the percentage contribution of the top 10 clones, and the diversity of the repertoires are similar **(Figure 4-4 D-F)**, despite the late inflamed joints having a higher number of endogenous CD4+ T cells **(Figure 4-4 C)**. This suggests that early and late inflamed joints display low repertoire diversity, having similar numbers of clones that are present in similar frequencies.

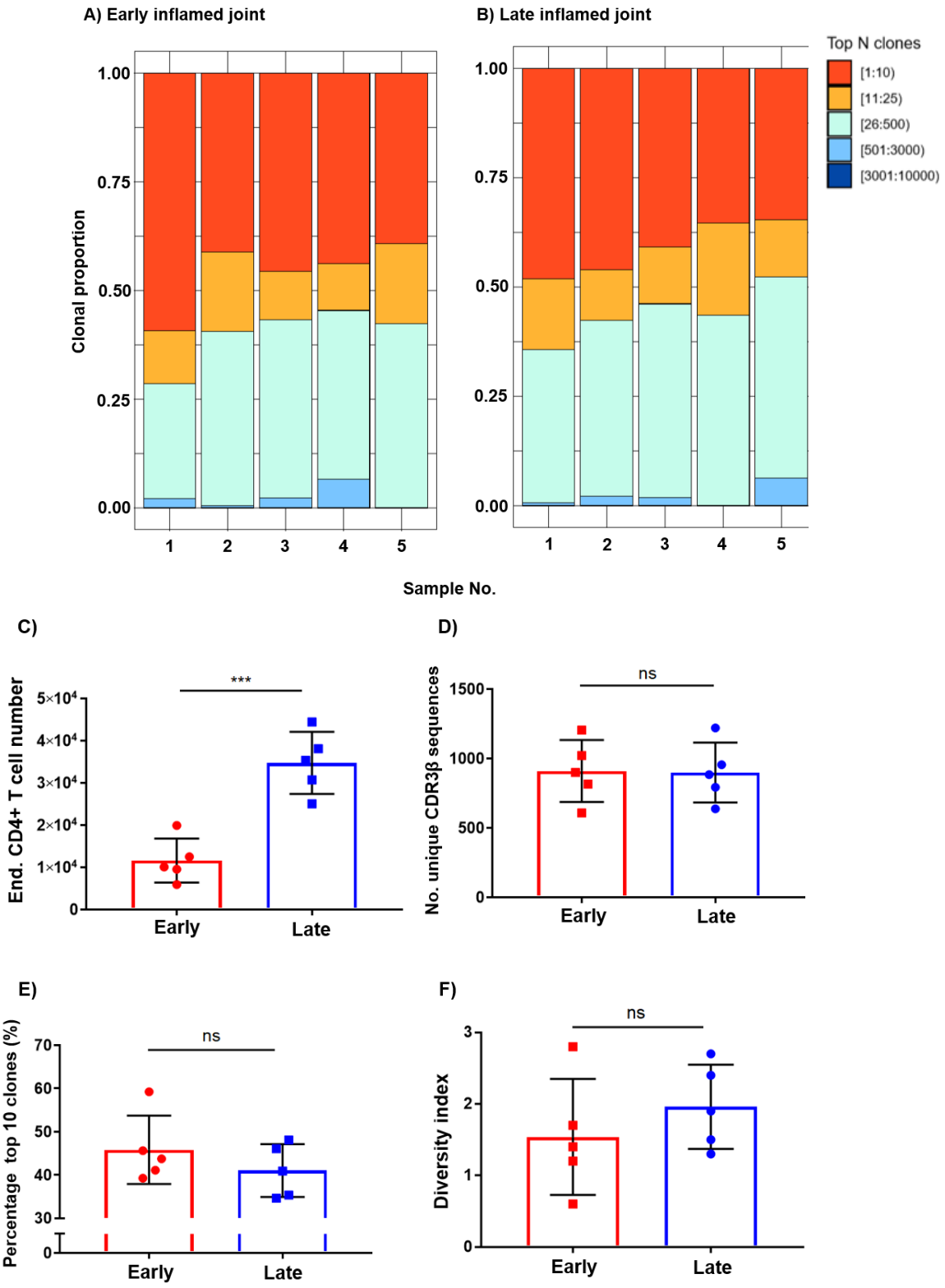


Figure 4-4 Characterisation of the antigen experienced CD4+ T cell repertoire in early and late inflamed joints.

Proportion of the top 10 most frequently occurring clones, followed by the next 25, 500, 3000, and 10,000 most frequent clones contributing to the endogenous antigen experienced CD4+ T cell repertoire in **A)** early, and **B)** late inflamed joints. **C)** Number of endogenous CD4 cells, **D)** number of unique CDR3β sequences, and **E)** percentage contribution of the top 10 most frequently occurring clones in early and late inflamed joints. **F)** Diversity indices (D50) of the antigen experienced endogenous CD4+ T cell repertoire in early and late inflamed joints. Graphs represent 1 experiment with n=5 for the early and late inflamed groups. Top proportion charts were generated using the tcR R package. Groups were compared using unpaired Student's t-test. Data presented as mean ± SD. Stars represent the following p values: *** <0.001; ns: not significant.

4.2.3 Early inflamed pLNs and joints display accumulation of select clones compared to PBS controls

Due to low cell recovery from PBS pLN and joint controls, samples needed to be combined before being sequenced. Pooling samples changes the composition of the repertoire and affects T cell repertoire diversity, so comparisons of the PBS CD4+ T cell populations were made against artificially pooled early inflamed joint and pLN samples using the tcR R package and repertoire statistics were compared **(Table 4-1)**. The data clearly shows that select clones accumulate in early inflamed pLNs and joints as the repertoire diversity in these samples is much lower compared with controls. This is further highlighted when comparing the number of unique CDR3β sequences to the number of endogenous CD4+ T cells in each of the groups. HAO and control pLNs have a similar number of unique CDR3β sequences but the total number endogenous CD4+ T cells is approximately 10 times greater in HAO pLNs. HAO joints have a higher number of endogenous CD4+ T cells and unique CDR3β sequences compared to controls, and yet low repertoire diversity. Together this is evidence for the selection and accumulation of a specific subset of clones in early inflamed pLNs and joints.

Table 4-1 Descriptive statistics of the antigen experienced CD4+ T cell repertoire in early inflamed pLNs, joints, and PBS controls.

Sample	pLN HAO	pLN PBS	Joint HAO	Joint PBS
No. of End. CD4+ T cells	3,113,218	224,978	58,041	1424
No. unique CDR3β	4181	4347	4549	885
Diversity index (D50)	6.4	16.3	0.72	9.3

HAO pLN samples and HAO joint samples were combined in R and compared with pooled pLN and pooled joint PBS samples. Number of endogenous CD4+ T cells were calculated using Flowjo. Number of unique CDR3β sequences and diversity indices (D50) of the antigen experienced CD4+ T cell repertoire of PBS samples were obtained from iRepertoire provided data and calculated for the combined HAO samples using iRepertoire's iRweb data analysis guide. Data from early inflamed tissues represent combined data of 5 samples for each of the pLN and joint samples. Data for PBS samples represent pooled pLN and pooled joint samples from 3 mice.

4.2.4 Late pLNs show no differences in clonality and diversity compared to IFA controls

It is widely accepted that disease progression in RA results in increased joint damage, and with that comes exposure of new antigens[82,285] To confirm whether the changing composition of late pLNs reflects a change in the antigen specific

response, the repertoires of late inflamed pLNs were compared to pLNs from mice challenged with IFA alone i.e. in the absence of OVA. Looking at the overall repertoire composition **(Figure 4-5 A and B)**, there is no significant difference between the two groups when comparing the contribution of the top 10 clones (average of 2% for each group) nor when comparing the contribution of the lowest frequency clones **(Figure 4-5 E)**. Antigen specific responses cause an expansion of T cell clones, so to check for antigen driven expansion, clones with a frequency of two or more were compared between late pLNs and IFA controls. This frequency was selected as it represents the median frequency of “expanded” clones in the pLN PBS sample. No difference was found between the number of expanded clones between the two groups (*p value 0.32 Welch two sample t-test*). This test was repeated with a threshold frequency of >10, as this is the median frequency in the joint PBS sample. Again, no difference was found between the number of expanded clones in late pLNs and IFA controls (*p value 0.23 Welch two sample t-test*). Moreover, both groups have the same number of endogenous CD4+ T cells, similar number of unique CDR3β sequences, and have repertoires of similar diversities **(Figure 4-5 C, D, and F)**. Together this data suggests an absence of expansion or accumulation of antigen specific CD4+ T cell specific clones in late pLNs and the changes observed are probably due to a chronic inflammatory environment. Whether the same observations can be said about late inflamed joints needs to be determined, and it is what I sought to do next.

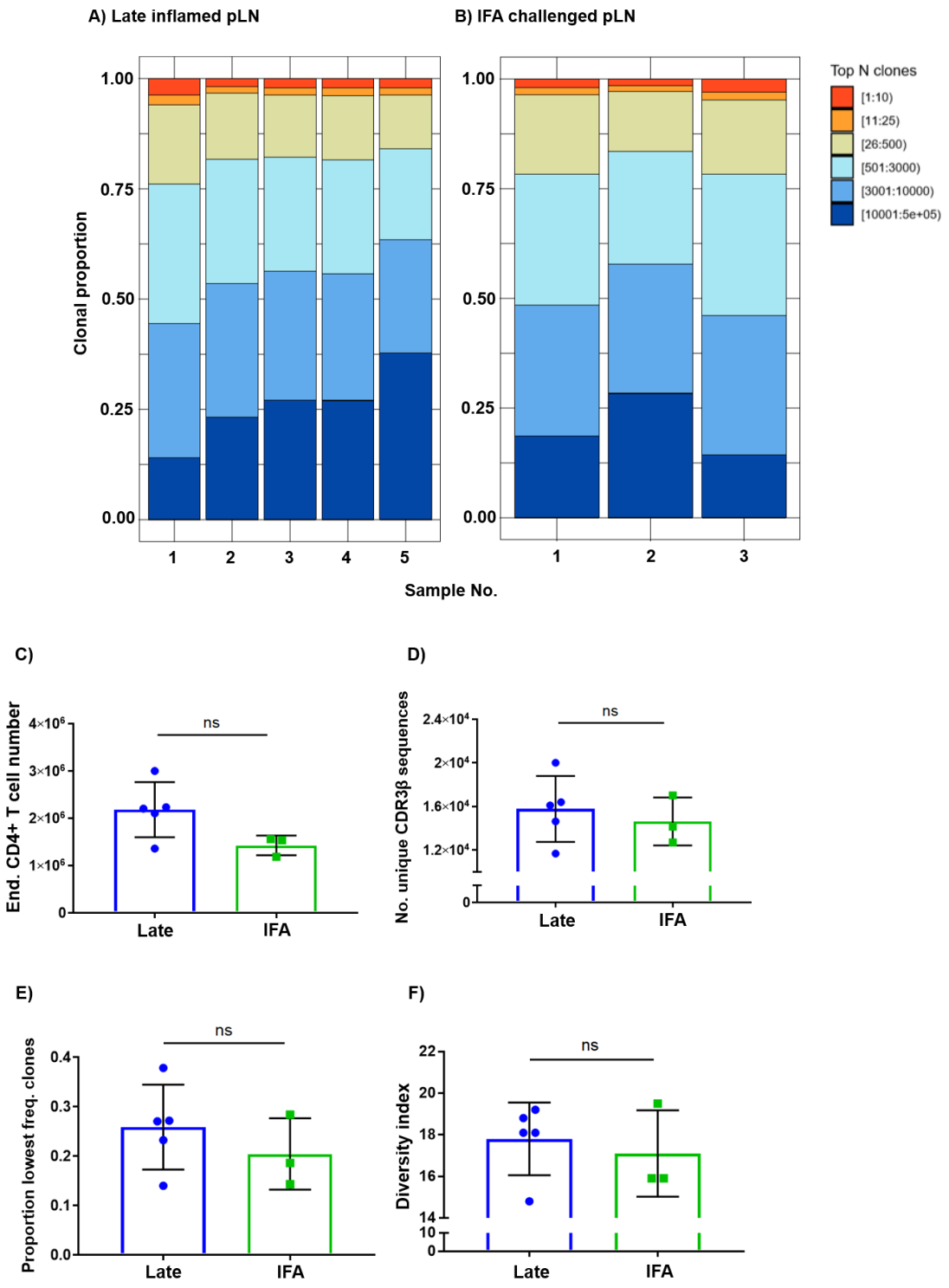


Figure 4-5 Characterisation of the antigen experienced CD4+ T cell repertoire in late inflamed pLNs and IFA control pLNs.
Proportion of the top 10 most frequently occurring clones, followed by the next 25, 500, 3000,10,000, and 100,000 most frequent clones contributing to the endogenous antigen experienced CD4+ T cell repertoire in **A)** late inflamed pLNs, and **B)** IFA challenged mouse pLNs. **C)** Number of endogenous CD4+ T cells in late inflamed and IFA challenged mouse pLNs. **D)** Number of unique CDR3β sequences in the CD44hi, endogenous CD4+ T cell repertoires of late inflamed pLNs and IFA controls. **E)** Proportion of the least frequently occurring clones (dark blue in top proportions charts A and B) in late inflamed and IFA challenged pLNs. **F)** Diversity indices (D50) of the antigen experienced endogenous CD4+ T cells from late inflamed and IFA administered mouse pLNs. Graphs represent 1 experiment with n=5 and 3 for the late inflamed pLNs and control IFA challenged pLNs respectively. Top proportion charts were generated using the tcR R package. Groups were compared using unpaired Student's t-test. Data presented as mean ± SD. ns: not significant.

4.2.5 Late inflamed joints display antigen associated accumulation of CD4+ T cells compared to IFA controls

To address whether the observed clonality of the CD4+ T cell repertoire from inflamed joints at the late timepoint is due to a prolonged inflammatory environment or is due to antigen specific responses, I compared the repertoires of CD4+ T cell populations isolated from antigen inflamed joints at the late timepoint to joints inflamed without the presence of antigen i.e. with IFA only **(Figure 4-6 A and B)**. The top 10 clones make up approximately 41% of the repertoire in late inflamed joints, while in IFA controls, the top 10 clones make up 29% of the population **(Figure 4-6 E)**. Interestingly, the number of unique CDR3β sequences is comparable between late joints and IFA controls **(Figure 4-6 D)**, despite the late inflamed joints having a much greater number of endogenous CD4+ T cells **(Figure 4-6 C)**. Repertoires from inflamed joints display lower population diversity compared with controls **(Figure 4-6 F)**, which would indicate that select clones accumulate in late inflamed joints, while CD4+ T cells accumulate in more of a random fashion in controls. These observations would suggest that antigen specific responses can in fact be detected in the inflamed joint, in contrast to what was observed in late inflamed pLNs. Moreover, these observations also suggest that specific CD4+ T cell clones may be expanding locally in the joint tissue. Whether these endogenous CD4+ T cell clones are OVA specific is yet to be determined. However as discussed in **Chapter 3 section 3.3**, the endogenous response to OVA may be significantly reduced given the large number of transferred OVA specific OT-II cells required for the model[205,206]. Thus the antigen specific accumulation of certain endogenous CD4+ T cell clones observed may be a reflection of breach of tolerance to joint specific antigens.

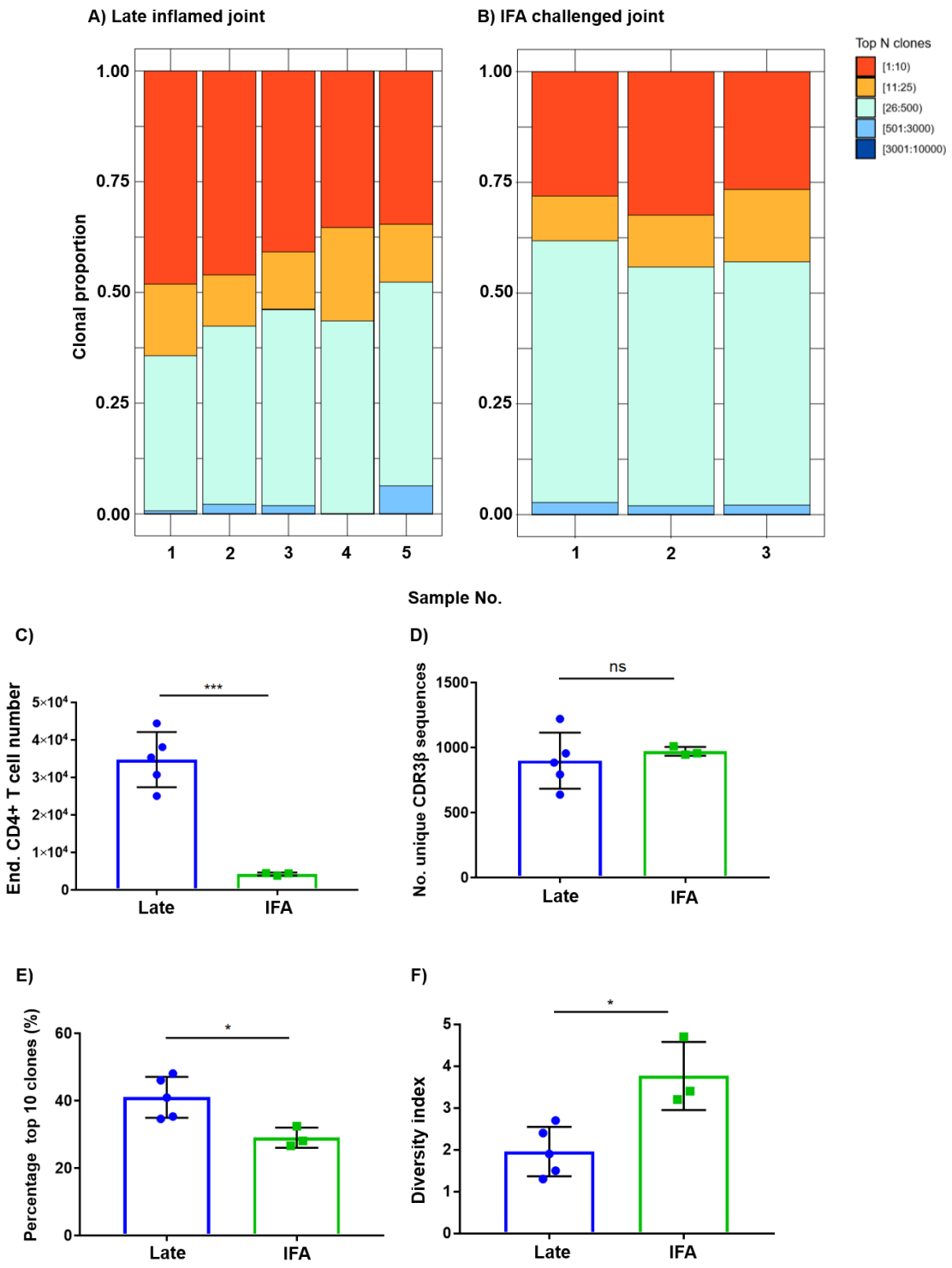


Figure 4-6 Characterisation of the antigen experienced CD4+ T cell repertoire in late inflamed joints and IFA controls.
Proportion of the top 10 most frequently occurring clones, followed by the next 25, 500, 3000, and 10,000 most frequent clones contributing to the endogenous antigen experienced CD4+ T cell repertoire in **A)** late inflamed joints, and **B)** IFA challenged mouse joints. **C)** Number of endogenous CD4+ T cells in late inflamed and IFA challenged mouse joints. **D)** Number of unique CDR3β sequences in the antigen experienced endogenous CD4+ T cell repertoires of late inflamed and IFA administered mouse joints. **E)** Percentage of the top 10 most frequently occurring clones (orange in top proportions charts A and B) in late inflamed and IFA challenged joints. **F)** Diversity indices (D50) of the antigen experienced endogenous CD4+ T cells from late inflamed and IFA administered mouse joints. Graphs represent 1 experiment with n=5 and 3 for the late inflamed joints and control IFA challenged joints respectively. Top proportion charts were generated using the tcR R package. Groups were compared using unpaired Student's t-test. Data presented as mean ± SD. Stars represent the following p values: * <0.05; *** <0.001; ns: not significant.

4.2.6 The CD4+ T Cell Repertoire in late inflamed pLNs diverges from late inflamed joints and early inflamed joints and pLNs in terms of V-gene usage

Repertoires of pLNs and joints at the early and late timepoints have been characterised in terms of the overall composition,, but this does not provide any details of the antigen specificities of these clones. Looking at the frequency of V-gene sequence usage can quickly identify how the antigen specific response changes in the early and late groups. A PCA analysis was performed on V-gene frequency in each sample in each group **(Figure 4-7)** and the late inflamed pLNs were found to have the largest range in V-gene usage, while late inflamed joints, and early inflamed pLNs and joints use a smaller range of V-genes, which were present with similar frequencies. Indeed, when quantifying these differences, late pLNs have significantly increased presence of V-genes 1, 2, 3, 5, and 19, while V-genes 12-1, 12-2, and 13-1 are present in the highest frequencies in early inflamed pLNs **(Figure 4-8)**. Where the individual samples are plotted on the PCA plot reflects differences in V-gene use and frequency between each sample i.e. the closer the samples are to each other on the plot, the more similar they are in terms of the V-gene sequences used and in the frequency at which they occur and vice versa. The distance of the samples from the late inflamed pLN group on the PCA plot suggests that V-gene use is different between the samples themselves. This indicates that not only are the late pLNs diverging from the other groups in terms of V-genes usage, but the response also diverges across individuals with progression of experimental arthritis.

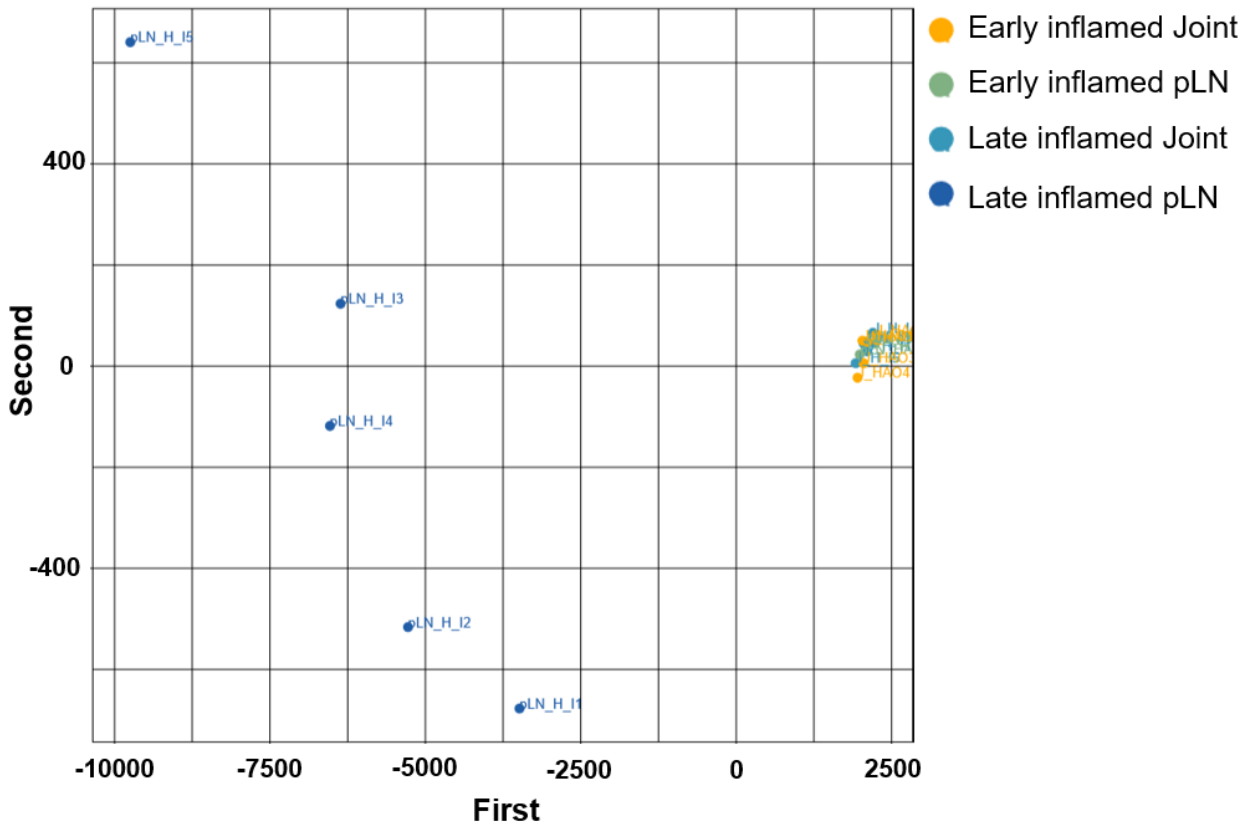
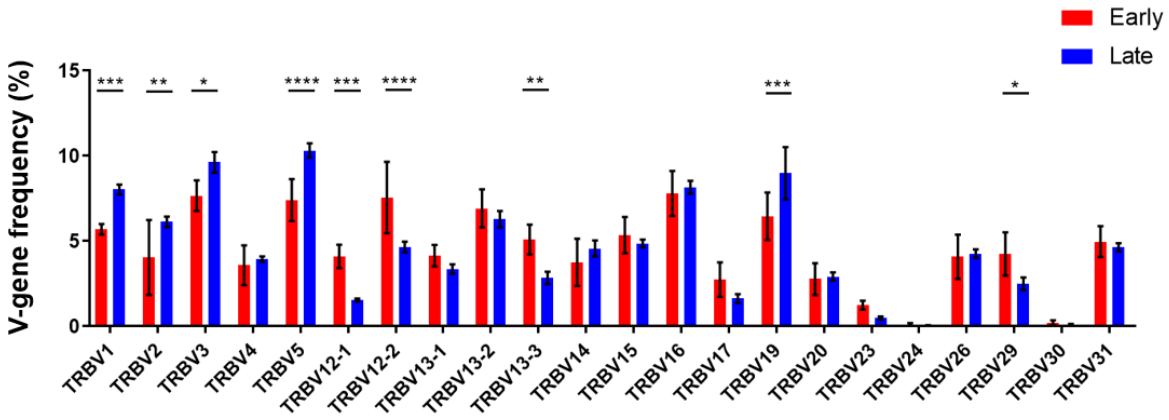


Figure 4-7 PCA plot of V-gene usage in the antigen experienced CD4+ T cell repertoire from inflamed joints and pLNs at the early and late model timepoints.
PCA plot was generated using the tcR R package and separates samples according to V-gene frequency. Samples compared were from early and late inflamed joints and pLNs. Each group represents 1 experiment with n=5 in each group.



Timepoint in model	Significantly increased Vgene freq.
Early	TRBV12-1, TRBV12-2, TRBV13-3, TRBV29
Late	TRBV1, TRBV2, TRBV3, TRBV5, TRBV19

Figure 4-8 V-gene frequencies in early and late inflamed mouse pLNs.
V-gene frequencies were determined from sequencing data using the gene usage function in the tcR R package. Graphs represent 1 experiment with n=5 for both the early and late inflamed groups. Groups were compared using ordinary 2 way ANOVA with Sidak's multiple comparisons test. Data presented as mean ±SD. Stars represent the following p values: * <0.05; ** <0.01; *** <0.001; **** <0.0001; ns: not significant. Table summerises significantly different V-gene frequencies between early and late pLNs.

4.2.7 Reduction in number of shared clones between pLNs and joints with the progression of inflammatory arthritis

The contrast in repertoire composition and V-gene usage in late inflamed pLNs compared with late inflamed joints and the early inflamed groups may have interesting implications for the development of antigen specific CD4+ T cell responses between joints and their draining lymph nodes. Changes in the antigen specific response can be addressed more accurately by determining the distribution of specific clones between the sites and between mice at the early and late model timepoints. Because each sample was barcoded before being sequenced (see methods **section 2.7** and **Figure 4-9**), clones isolated from joints can be matched with those from their respective draining lymph nodes – via their CDR3β amino acid sequence – and the distribution of CD4+ T cell clones can be monitored between the two sites within each animal **(Figure 4-9)**. When comparing the degree of CDR3β amino acid sequence overlap between inflamed joints and their draining lymph nodes in early and late mice, the number of sequences shared is reduced with the progression of arthritis **(Figure 4-10 A and B)**. This is indicated by the intersect of the pLN and joints samples in the bottom left square in Figure 4-10 A and B and is quantified in Figure 4-10 C. Sequence overlap between samples remains unchanged in early and late joint repertoires **(Figure 4-10 D)**, but reduces significantly between samples when comparing inflamed pLNs at the early and late timepoints **(Figure 4-10 E)**. The reduction in CDR3β amino acid sequence overlap with time suggests that the antigen specific responses start out by being similar between the sites, but then diverge as the disease progresses.

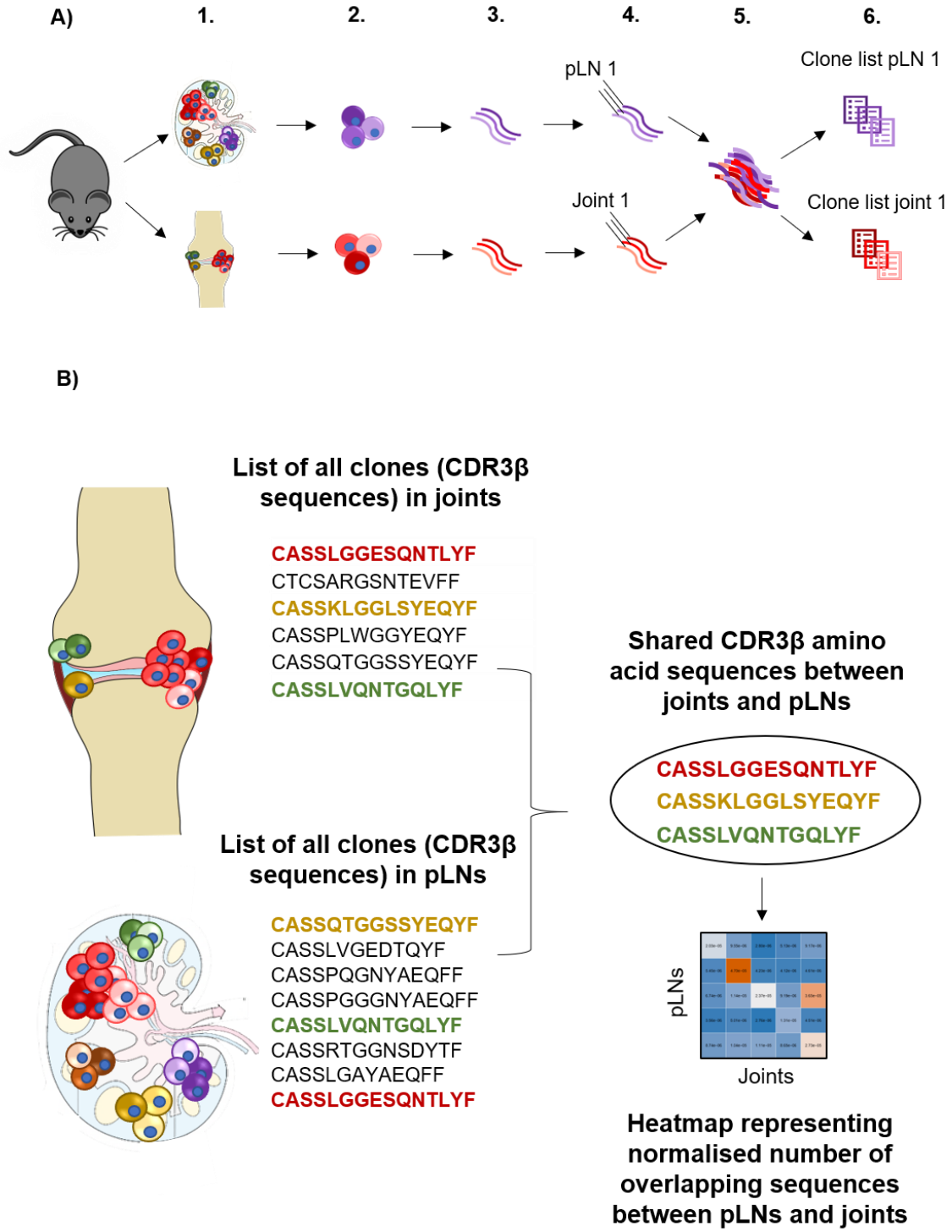
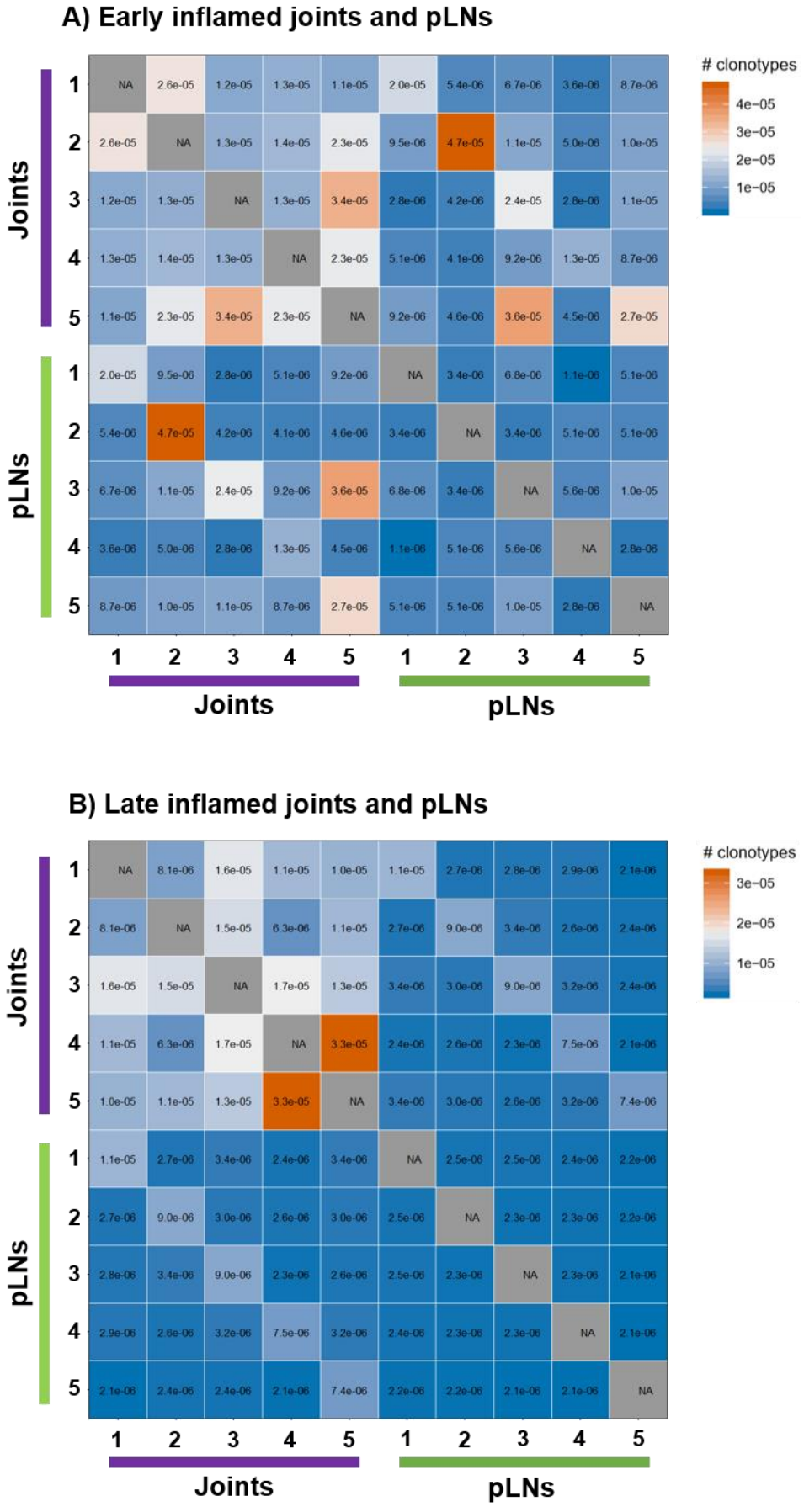


Figure 4-9 Illustration of barcoded sample sequencing and evaluation of clonal overlap between pLNs and joints

A) Illustration of barcoded sample sequencing. Antigen experienced endogenous CD4+ T cells (CD4+ CD44hi CD45.1-) were sorted separately from pLNs and joints of animals undergoing model of experimental arthritis (1 and 2). Total RNA was isolated and a Vβ specific PCR was performed with barcoded primers (3 and 4). Each sample was amplified with primers with a different set of barcodes. Samples were then pooled and sequenced using the Illumina Miseq platform (5) and sequences were backtracked to the original samples vi the barcodes (6). **B)** Evaluation of clonal overlap between pLNs and joints. CDR3β amino acid sequences from samples were compared using the rep.overlap function in the tcR R package. The number of sequences shared between 2 repertoires found is then normalised to the product of the repertoire sizes. Multiple pairwise comparisons are plotted as a heatmap representing normalised degree of overlap between 2 samples.


A) Early inflamed joints and pLNs
clonotypes
4e−05
3e−05
2e−05
1e−05
Joints
pLNs
1 2 3 4 5 1 2 3 4 5
Joints
pLNs
B) Late inflamed joints and pLNs
clonotypes
3e−05
2e−05
1e−05
Joints
pLNs
1 2 3 4 5 1 2 3 4 5
Joints
pLNs

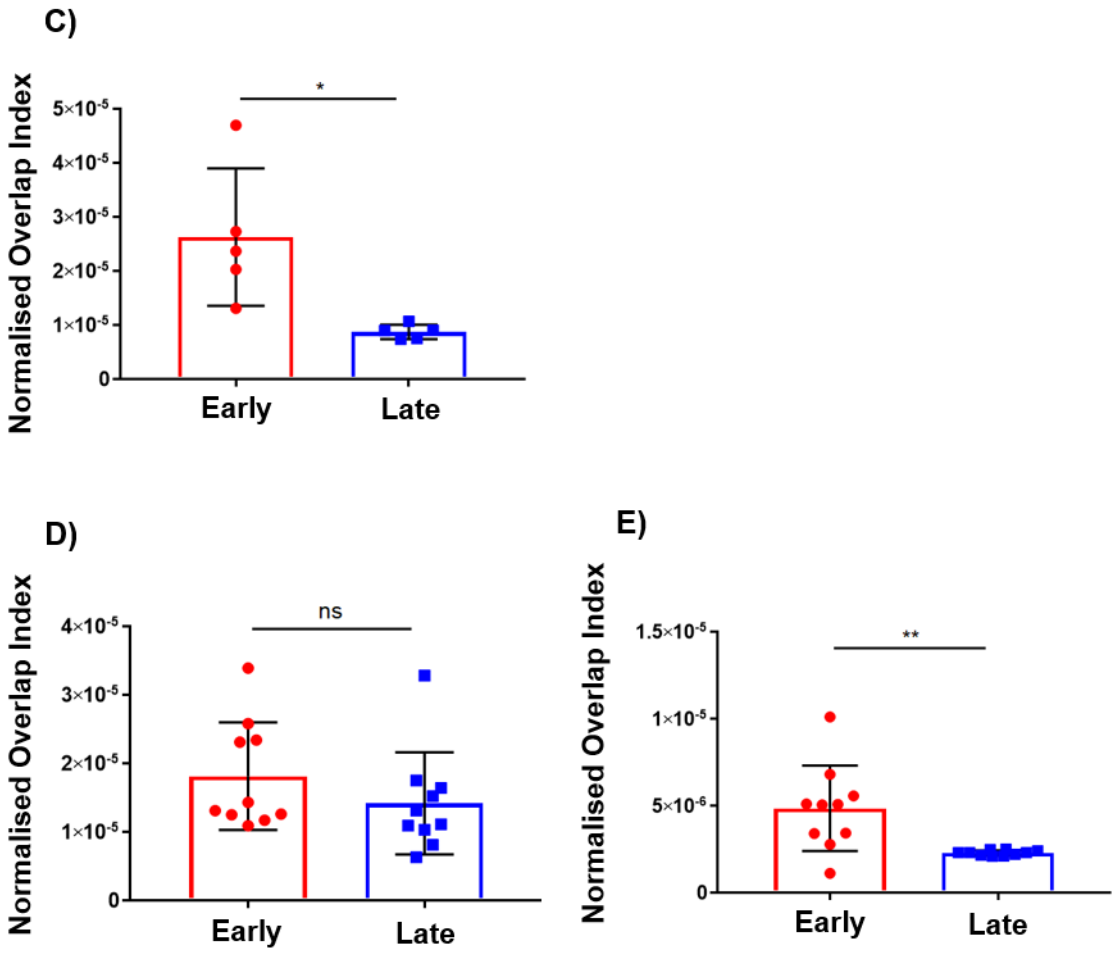


Figure 4-10 Degree of CDR3β amino acid sequence overlap between inflamed pLNs and joints at the early and late model timepoints.
Number of shared CDR3β amino acid sequences were normalised by dividing the number of shared clones found between two samples by the product of the repertoire sizes of the two samples. Heatmaps of normalised CDR3β amino acid sequence overlap counts in **A)** early inflamed joints and pLNs, and **B)** late inflamed joints and pLNs. **C-E** Normalised values obtained from the heatmap were plotted as bar graphs. **C)** Normalised overlap values between joints and the respective draining pLNs from the same mouse. **D and E** Normalised overlap values between samples within the early and late groups. **D)** Overlap values between joint samples of early and late groups, and **E)** between pLN samples of early and late groups. Graphs represent 1 experiment with n=5 for the early and late groups. Heatmaps were generated using the tcR R package. Groups were compared using unpaired students t-test. Data presented as mean ±SD. Stars represent the following p values: * <0.05; ** <0.01; ns: not significant.

4.2.8 Frequency of top 10 clones in joints correlate with high frequency clones in pLNs in late inflamed mice, but not in early inflamed mice

A reduction in the number of overlapping sequences between late inflamed pLNs and joints would suggest that the antigen specific response changes between these two sites and these antigen specific responses may diverge even further with the progression of arthritis. To verify whether the antigen specific response truly diverges between the inflamed pLN and joint and to address the dynamics of individual CD4+ T cell clones between these sites, I looked for a correlation between

high frequency clones in inflamed pLNs and joints at both the early and late model timepoints. When searching for the top 10 ranked clones from the early inflamed joint samples in the respective pLNs, not all the clones are returned. By contrast, the top 10 clones in late inflamed joint samples are all present in their lymph node counterparts **(Table 4-2 A and B)**. Moreover, the ranks of the clones correlate more between late inflamed joints and pLNs than in early inflamed joints and pLNs **(Figure 4-11 A and B)**. Interestingly the top 10 clones from joints immunised with IFA alone (i.e. without OVA) do not correlate with highly ranked clones in the respective lymph nodes **(Figure 4-11 C)**, suggesting that the observed dynamics between the joint and lymph node are due to antigen specific responses.

Together, these results reveal ongoing communication between late inflamed joints and their draining lymph nodes which appears to be antigen driven rather than due to chronic inflammation. The reduction in the number of overlapping clones between the late inflamed pLNs and joints and the changes in CD4+ T cell clonality in the late inflamed pLN may not represent a divergence in the antigen specific response, but rather could reflect changes that may eventually occur in the inflamed joint, as suggested by clinical data examining repertoire CD4+ T cell repertoire changes in early and established RA patients[46].

Table 4-2 The top 10 frequently occurring clones in early and late joint samples ranked in terms of frequency in respective pLNs.

A)

Sample	CDR3β amino acid sequence	V-gene	Read count in pLN	Rank in pLN	Rank in joint
Top 10 early joint 1 in early pLN 1	CASSPGQGTGERLFF	TRBV26	4276	2	1
	CASSQTGGSYEQYF	TRBV2	275	95	7
	CASSNRDWGQDTQYF	TRBV16	267	98.5	3
	CASSFAGHYEQYF	TRBV3	94	196	5
Top 10 early joint 2 in early pLN 2	CASSLVGEDTQYF	TRBV16	1727	2	9
	CASSPQGNYAEQFF	TRBV2	1705	3	4
	CASSPGGGNYAEQFF	TRBV2	1384	6	10
	CASSLVQNTGQLYF	TRBV16	1004	15	7
	CASSRTGGNSDYTF	TRBV2	628	39	1
	CASSLGAYAEQFF	TRBV3	616	40	8
	CASSKLGGLSYEQYF	TRBV19	340	98	3
Top 10 early joint 3 in early pLN 3	CASSPLGGRNTGQLYF	TRBV5	2307	2	1
	CASSLGGESQNTLYF	TRBV12-2	1250	11	8
	CASSNREGTQYF	TRBV16	753	36	3
	CGAGQGNTEVFF	TRBV20	101	193	9
Top 10 early joint 4 in early pLN 4	CASSLGGESQNTLYF	TRBV12-2	14841	1	2
	CASSQGTGGQDTQYF	TRBV2	280	129	3
	CASSFGLEGQNTLYF	TRBV3	228	154	1
	CTCSARGSNTEVFF	TRBV1	209	174	6
	CASSPGQGVNTEVFF	TRBV4	61	506	8
Top 10 early joint 5 in early pLN 5	CASSQAGGYAEQFF	TRBV2	631	45	1
	CASSQTGVNAEQFF	TRBV2	250	153.5	5
	CASSQNTGQLYF	TRBV16	198	198.5	3

B)

Sample	CDR3β amino acid sequence	V-gene	Read count in pLN	Rank in pLN	Rank in joint
Top 10 late joint 1 in late pLN 1	CASSLDLGGRGEQYF	TRBV16	976	2	7
	CASSQTGGAREQYF	TRBV2	849	3	3
	CASSRQGNSDYTF	TRBV12-2	807	4	1
	CASSLSGLGGGAEQFF	TRBV26	764	5	2
	CASGGSGTGETLYF	TRBV13-2	456	7	4
	CASSQAQNTEVFF	TRBV2	330	11	8
	CASSQTGANTEVFF	TRBV2	153	38	10
	CASSLSLGGLEQYF	TRBV26	142	47	5
	CASSSLRGSNQAPLF	TRBV16	95	98	6
Top 10 late joint 2 in late pLN 2	CASSFSGQTEVFF	TRBV14	530	1	1
	CASSWGQGRDTQYF	TRBV16	267	6	3
	CASGDLDKYEQYF	TRBV13-2	167	18	2
	CTCSTHSDYTF	TRBV1	90	69.5	7
	CASSQNGALYEQYF	TRBV2	89	71.5	5
	CASSLQSSAETLYF	TRBV16	85	77.5	10
	CASQFRQDNYAEQFF	TRBV14	84	81	9
	CASSPPYINSDYTF	TRBV3	60	171	4
	CASSQAGGYAEQFF	TRBV2	52	226.5	6
	CASSKGPYNSPLYF	TRBV19	26	950	8
Top 10 late joint 3 in late pLN 3	CASSLETGGASEQYF	TRBV12-2	879	1	2
	CASRTASGNTLYF	TRBV19	798	2	4
	CARSTRAYNSPLYF	TRBV19	257	7	7
	CASSSNTGQLYF	TRBV16	217	12	1
	CTCSASGFSNERLFF	TRBV1	199	16	6
	CASSQTGGSYEQYF	TRBV2	164	25	5
	CASSQDSGGRAEQFF	TRBV2	158	31	8
	CASGRNYAEQFF	TRBV13-2	134	39	10
	CASSQDTGGLNTLYF	TRBV2	107	51.5	3
	CASSQPGTNQAPLF	TRBV2	52	197	9
Top 10 late joint 4 in late pLN 4	CASSKPGYAEQFF	TRBV19	435	3.5	1
	CASSQEAGRNTGQLYF	TRBV5	435	3.5	10
	CASSQTGAETLYF	TRBV5	260	14	4
	CASSRGPYNSPLYF	TRBV19	203	20	7
	CASSQAGGYAEQFF	TRBV2	170	26.5	2
	CASSSNTGQLYF	TRBV16	109	59.5	3
	CASSLAGGGYAEQFF	TRBV3	102	68	5
	CASSFGSSAETLYF	TRBV29	55	209.5	6
	CASSGQGWSTGQLYF	TRBV2	35	528	8
	CASSDRASSYEQYF	TRBV3	32	621.5	9
	CASSSNTGQLYF	*TRBV16*	*6*	*8460*	*3*
	CASSLAGGGYAEQFF	*TRBV3*	*1*	*30109.5*	*5*
Top 10 late joint 5 in late pLN 5	CASSLAWGGMAEQFF	TRBV16	515	3	4
	CASSHRLANSDYTF	TRBV14	496	4	1
	CTCSRDRGSGNTLYF	TRBV1	255	13	3
	CASSSNQDTQYF	TRBV16	217	19	7
	CASSPLGNYAEQFF	TRBV2	196	23	5
	CASSPPGQNQAPLF	TRBV2	76	95.5	6
	CASSQMTISNERLFF	TRBV2	76	95.5	10
	CASSLDGRSQNTLYF	TRBV16	34	377	8
	CASSQGQQDTQYF	TRBV2	34	377	9
	CASSQGQQDTQYF	*TRBV2*	*5*	*10330*	*9*
	CASSQGQQDTQYF	*TRBV2*	*3*	*17765.5*	*9*
	CASSQGQQDTQYF	*TRBV2*	*1*	*39595*	*9*

The top 10 frequently occurring clones in the antigen experienced CD4+ T cell repertoire were found in each of the **A)** early and **B)** late joint samples and searched for in respective pLN samples using the find.clonotypes function in the tcR R package. The CDR3β amino acid sequence, associated V-gene, read count, and rank of the clone in the pLN sample was obtained. The rank each of the top 10 clones in the joint were also included in the table. Data from early and late tissues represent 1 experiment with n=5 for each group.

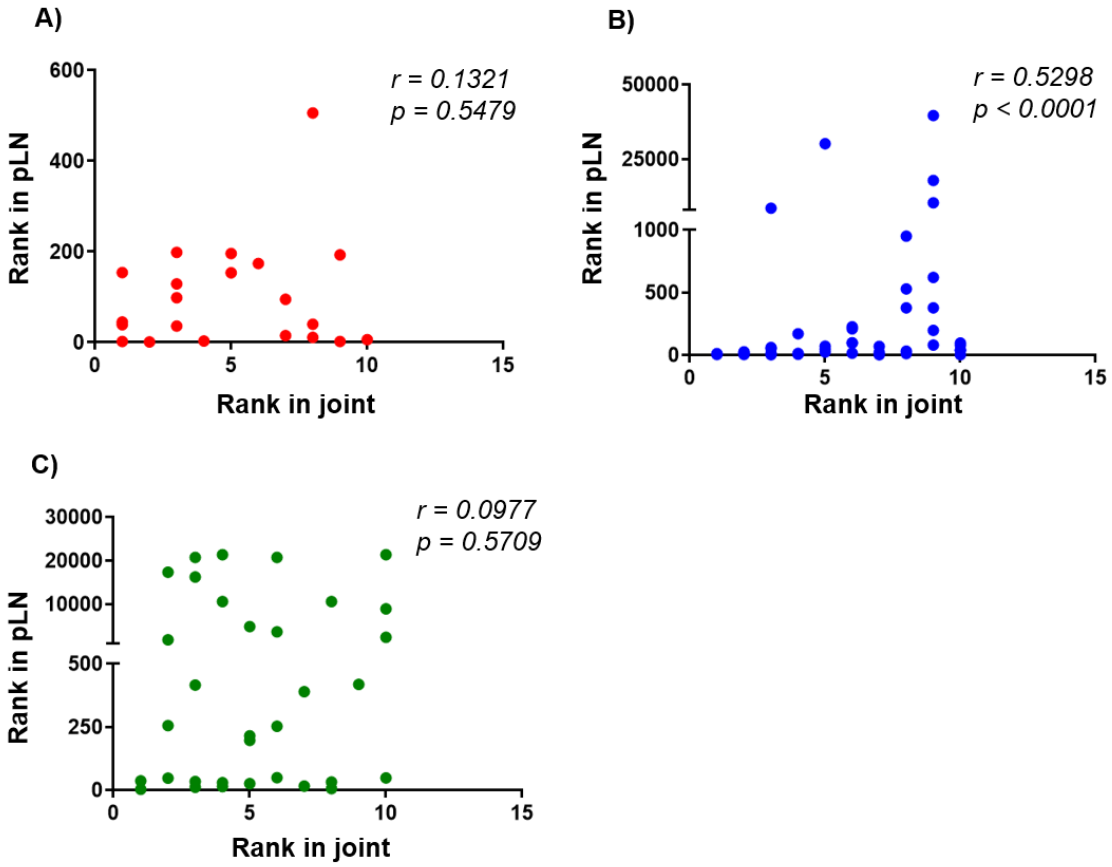


Figure 4-11 Correlation of the top 10 ranked clones in early and late joints with their rank in respective pLN samples.
The top 10 frequently occurring clones in the antigen experienced CD4+ T cell repertoire were found in each of the early and late joint samples and searched for in respective pLN samples using the find.clonotypes function in the tcR R package. The rank of each of the top 10 clones in **A)** early inflamed joint, **B)** late inflamed joint, and **C)** IFA control joint samples were plotted against the rank of that clone in respective pLN samples. Data from early and late tissues represent 1 experiment with n=5 for each group. Correlations were calculated using Spearman's correlation after testing for normality of the data using the D'Agostino-Pearson test.

4.3 Discussion

TCR repertoire characterisation has been used to distinguish naive immune profiles from diseased states in cases of infection[286], cancers[42], and autoimmunity[23,287], including RA[46,48]. Although clonality of CD4+ T cell repertoires have been determined in RA patients at different stages of disease[46,143], how the antigen specific response develops from the very early stages, before clinical symptoms are manifest, to one where inflammation is propagated and continues is unknown. In this chapter, NGS methods were utilised to investigate the evolution of the antigen specific response in early and chronic models of inflammatory arthritis. Moreover, changes in CD4+ T cell clonality were monitored between the inflamed joint and its draining lymph node to better understand the dynamics of antigen specific CD4+ T cell responses between these sites with disease progression.

The antigen experienced CD4+ T cell repertoire was found to be similar between early and late inflamed joints; both repertoires displayed low T cell diversity and were skewed by the presence of a low number of high frequency clones. By contrast, early and late inflamed pLNs have quite a distinct repertoire composition, where late inflamed pLNs have a more diverse repertoire mainly composed of low frequency clones. This indicates that late pLNs take on a more polyclonal phenotype compared to early inflamed pLNs, which may reflect changes in antigen specific responses due to the release of novel antigens resulting from continued joint damage, or is a reflection of epitope spreading - an occurrence where responses against a subdominant epitope of a protein develop[52], a phenomenon which has been demonstrated in RA[288]. If this is the case, then it is striking that late joints and pLNs have very different repertoire compositions. Specifically, late pLNs have, comparatively, a very low number of highly expanded clones.

Although late inflamed joints have similar repertoire compositions compared with their early inflamed counterparts, it is not known whether these repertoires are made up of the same clones. Specific sequences cannot be compared between animals because individual animals display different immune responses, and so, clones from one animal is unlikely to be found in another animal[13,220]. Indeed, when clonality analyses are conducted in human studies, the same individual has samples taken throughout the course of the study to track specific clones[23,46], or comparisons are made on different criteria such as CDR3 length to describe differences between

individuals[287]. So, although repertoires from early and late joints are similar in terms of relative composition, the late joint may be made up of entirely different clones from those present in early inflamed joints. Given that the number of endogenous CD4+ T cells in late inflamed joints is greater than in their early counterparts, and given the similarity in V-gene sequence usage in both the CD4+ T cell repertoires in early and late inflamed joints, one could suggest that, in fact, the CD4+ T cell clones present in both these tissues recognise similar antigens. Additionally, the clones found in early inflamed joints could continue to expand and accumulate with disease progression, a phenomenon that has also been reported in RA patients[143,289].

Individual joint and pLN samples from individual animals were barcoded before being sequenced which allowed me to determine the distribution of individual clones between these two sites. In order to investigate how specific clones contribute to each of the repertoires from the inflamed tissues, CDR3β amino acid sequences were compared between these two sites in both early and late inflamed groups and normalised. The amino acid sequence rather than the nucleotide sequence was compared due to a phenomenon known as convergent recombination, where different CDR3 nucleotide sequences encode the same amino acid sequence and is postulated to feature in the generation of public T cell responses[290]. The amino acid sequence of the CDR3 region reflects the type of peptide this region, and therefore the TCR, has an affinity for. An overlap in amino acid sequences between two different CD4+ T cell repertories would indicate the degree those repertoires recognise the same peptides and highlight similarities in the antigen specific response in those populations. The CD4+ T cell repertoires of late inflamed joints and pLNs share fewer clones compared to early inflamed samples, indicating that the antigen specific responses differ between these sites with time. Moreover, the divergence of V-gene usage in late pLNs suggests that the difference in response is due to changes in late pLNs rather than late joints. It is also interesting to note that the late pLN samples not only diverge from the other groups in terms of V-gene usage, but also deviate from each other, illustrating the development of individual responses with disease chronicity. This has also been observed in RA patients; highly expanded clones within a patient with a unique CDRβ sequence were only found in different joints within the same individual but not shared across patients[46,143]

Comparisons of repertoires from early inflamed mice with those from PBS controls demonstrate that the restriction in diversity and clonality is a result of an antigen specific response in the early stages of arthritis. Interestingly, late pLNs show no difference in repertoire compositions compared to IFA controls, which indicates that pLNs mainly reflect chronic inflammation rather than the development of an antigen specific response. This is unexpected given that the top 10 highly expanded clones in the late inflamed joint correlate with highly expanded clones in the lymph node, while this does not occur between IFA joints and pLNs. This suggests that the lymph nodes in fact reflect the presence of antigen specific responses. Interestingly, there is no correlation between highly expanded clones in the early inflamed joint with highly abundant clones in respective pLNs. Whether this observation is a reflection of the dynamics of CD4+ T cells between the joint and pLN is also something to be considered and may hint to antigen specific CD4+ T cells expanding locally in the joint. Indeed, antigen specific expansion of memory CD8+ T cells has been demonstrated in sites where infections were localised[290,291]. Additionally, the differences in correlation in top ranking clones between early and late joints and lymph nodes may also reflect a proliferation burst in pLNs and subsequent migration of effector cells to the inflamed joint, which would have occurred prior to analysis of the repertoires.

The general contrast in CD4+ T cell clonality between early and late inflamed mice - where early inflamed pLNs reflect the clonality in early inflamed joints, while late pLNs do not - demonstrate that CD4+ T cell repertoires can be different between tissues and their draining lymph nodes at different points in disease. Moreover, the changing composition of the late inflamed pLN may predict the responses that will occur in the joint. Indeed, Klarenbeek et al[46] demonstrated that repertoires from synovial fluid of patients with chronic or established RA have fewer highly expanded clones compared to the synovial fluid taken from patients 6 months after showing clinical symptoms. This suggests that the joint may eventually mirror the polyclonality observed in the lymph node. This delayed response in the joints has important implications for antigen specific therapies, as specific pathogenic clones could potentially be targeted in the lymph node before accumulating in the joint. Indeed, Rodríguez-Carrio et. al.[139] obtained inguinal lymph node samples of at-risk and early RA patients to determine whether lymph node composition changes prior to RA onset. Moreover, analysing clonal compositions early in the disease will be more effective in terms of identifying common antigen specific responses between

individuals, demonstrated by the divergence of pLN samples with disease development. Analysis of clonal compositions can also serve as a biomarker to monitor disease development and assess the therapeutic impact on changes in CD4+ T cell clonality.

Together this data highlights the importance of site specific and temporal dynamics of antigen specific CD4+ T cell responses in inflamed tissues and their respective lymph nodes and is a step forward in understanding the development of the antigen specific response in RA. Identifying key developments in these responses will aid in the development of more efficient regimes to reinstate self-tolerance.

Chapter 5 Assessment of autoantigens and location of antigen recognition using the Nur77GFP transgenic system

5.1 Introduction

Evidence of antigen presentation in RA is supported by the association of certain HLA-DRB alleles with the disease; over 80% of RA patients express the HLA-DRB1 allele[35,36], making HLA-DRB loci the most important risk association for developing RA. The presence of a CD4+ T cell TCR bias in RA patients is further evidence of antigen specific responses playing an role in maintaining and propagating the disease[46,143,201,289] and these CD4+ T cells may in fact be selected for as a result of mutations in the HLA-DRB alleles. As such, CD4+ T cells have been proposed as a therapeutic target for RA[292–294]. However, one of the biggest hurdles in specifically targeting these pathogenic CD4+ T cells is that their antigen specificities are unknown. Moreover, data from several studies have suggested that the antigens the autoreactive T cells recognise are heterogenous and vary across RA patients[82,292,295,296] (see **section 1.4** of introduction), making it more difficult to identify the antigens these CD4+ T cells recognise. An additional impediment is the lack of a clearly defined location where these autoreactive responses originate and where exactly autoreactive CD4+ T cells recognise their cognate antigen [82,297]. The unknown nature of antigens recognised by pathogenic CD4+ T cells and the paucity of information of where these CD4+ T cells encounter their antigens have impeded the development of antigen specific therapies for RA.

However, the arthritic joint has been found to provide a niche for expanded CD4+ T cells[46], and the RA synovium is enriched with citrullinated peptides[183] to which CD4+ T cell responses have been demonstrated[114,298]. This would suggest that the CD4+ T cells infiltrating the arthritic joint may in fact encounter their cognate antigens at the inflamed joint. Moreover, behaviour of endogenous CD4+ T cells in the OVA model of arthritis would suggest that these cells also interact with their antigen in the inflamed joints[191]. A study of type I diabetes, also an autoimmune disease, has shown that islet specific CD4+ T cells localise to the pancreas at very early stages of the disease because of antigen encounter[188,189], suggesting that the same mechanisms could be responsible for CD4+ T cell infiltration into inflamed synovial tissues at early stages of arthritis. Therefore, in this chapter, I wished to determine

whether CD4+ T cells encounter their cognate antigen in the joint itself, and whether these antigens originate from the joint. This will address the possible location of antigen recognition as well as the nature of the antigens recognised in the early phases of RA. To do this, the OVA model of experimental arthritis was employed in Nur77GFP transgenic mice, which report TCR stimulation events[57].

The Nur77GFP transgenic system developed by Moran et al is a reporter of TCR stimulation and was originally developed to understand how TCR stimulation strength affected Treg development and selection in the thymus[57]. Nur77 is a gene product of the *Nr4a1* locus which is upregulated upon TCR stimulation in T cells[299]. Unlike the early activation marker CD69[300], Nur77 has been found to specifically report on T and B cell activation via engagement of the TCR and BCR respectively, as opposed to cytokine driven activation[57,301]. This makes Nur77 ideal for distinguishing antigen driven activation of T cells from “bystander” activation, where lymphocytes are activated by the inflammatory environment. Indeed, evidence for cytokine activated CD4+ T cells in RA has been previously shown[100,193]. The distinction between antigen driven and bystander T cell activation is important for antigen specific therapies and ensures that the correct CD4+ T cells are targeted.

There are three main aims of this chapter. Firstly, to provide evidence for antigen recognition in the early phases of experimental arthritis and to complement the findings of the first results chapter. Secondly, to determine whether CD4+ T cells recognise their cognate antigen specifically in the joint, and finally, to establish whether the antigens the CD4+ T cells recognise are joint specific, providing evidence of their autoreactivity. To achieve these aims, the feasibility of the Nur77GFP transgenic system as a reporter of antigen recognition in the early model of experimental arthritis was first established and I found that this system reported TCR stimulation events that occurred within 48 hours of TCR engagement. Endogenous CD4+ T cells were then collected from pLNs and joints of Nur77GFP mice undergoing the model and Nur77GFP expression was assessed. Finally, endogenous CD4+ T cells were isolated from pLNs and joints of mice undergoing the early and late models of arthritis and co-cultured with BMDCs pulsed with OVA, inflamed joint extract, and candidate RA antigens collagen II and aggrecan. This was done to determine which antigens these cells recognised and to demonstrate whether the endogenous CD4+ T cells recognised antigens that have been implicated in RA, namely collagen II[302–304] and aggrecan[305]. I found that a greater

number of endogenous CD4+ T cells with high levels of Nur77GFP were isolated from inflamed pLNs and joints, but these cells did not respond to the joint derived antigens tested, nor to the candidate RA antigens. Despite this, the data reveals interesting differences in activation profiles of endogenous CD4+ T cells between pLNs and joints and at the early and late stages of experimental arthritis.

5.2 Results

5.2.1 In vitro stimulation of Nur77GFP CD4+ T cells with anti-CD3 results in GFP upregulation and correlates with CD69 expression

The primary goal of this experiment was to establish whether the Nur77GFP system reports T cell activation as required for the purposes of this project. A simple way of testing the system is to determine whether GFP expression can be detected upon TCR stimulation. Lymphocytes were isolated from Nur77GFP mice - along with C57BL/6 and Nur77GFP transgenic negative controls - and cultured overnight in anti-CD3 coated plates then analysed by flow cytometry **(Figure 5-1)**. GFP was significantly upregulated in Nur77GFP CD4+ T cells cultured with anti-CD3 compared to unstimulated controls. Furthermore, GFP expression was significantly greater compared to stimulated non transgenic controls. Moreover, GFP expression correlated with increasing amounts of anti-CD3 and reached maximum expression at 5µg/mL anti-CD3 **(Figure 5-1 E and F)**. GFP upregulation was also compared to the expression of the early activation marker CD69 and it was found that both GFP and CD69 expression increased in a dose dependent manner **(Figure 5-2)**. Upregulation of Nur77GFP can therefore be associated with CD4+ T cell activation.

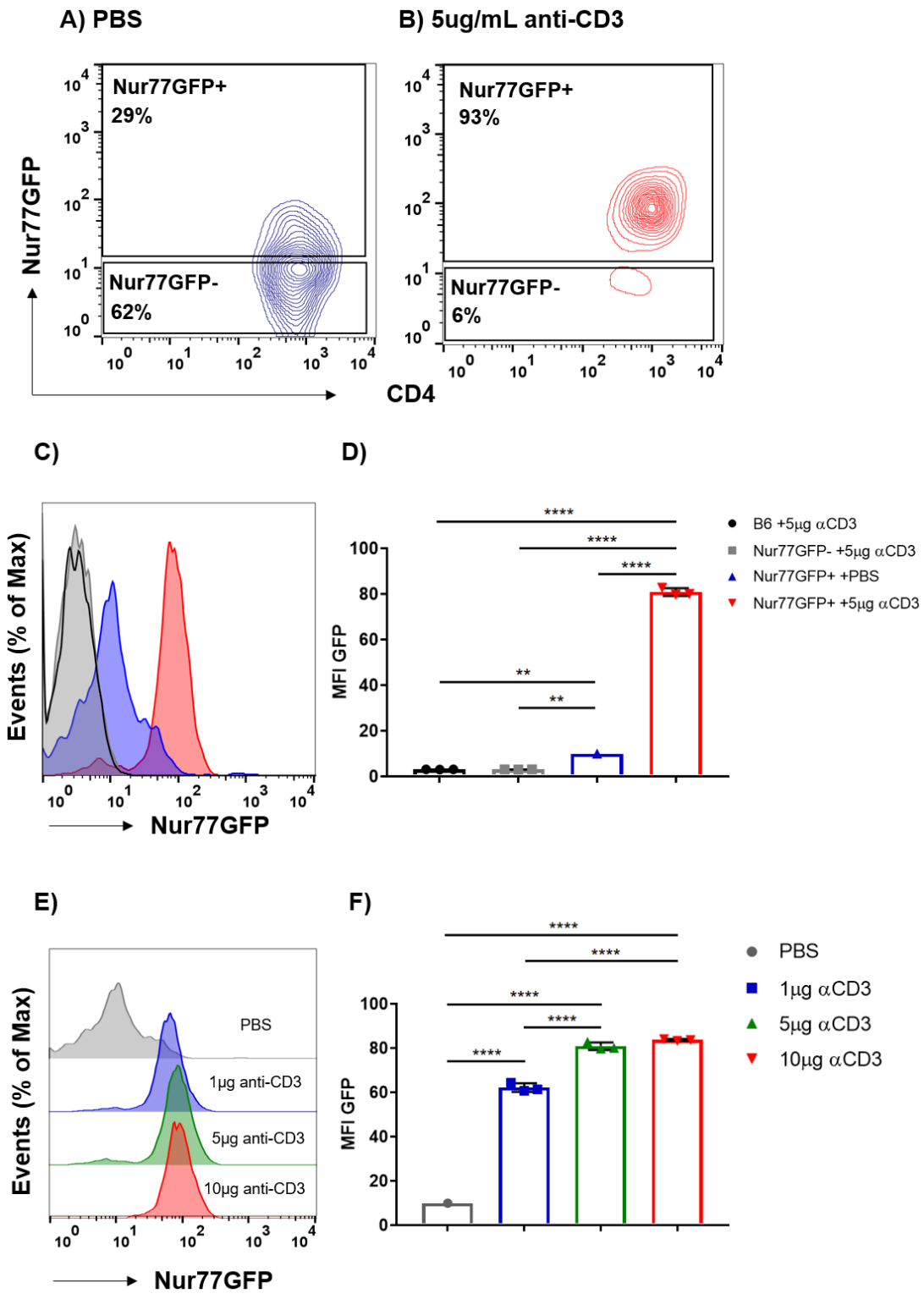


Figure 5-1 In vitro stimulation of Nur77GFP CD4+ T cells with 1, 5, and 10µg of anti-CD3
Lymph nodes were collected and lymphocytes isolated from Nur77GFP mice as well as C57BL/6 and Nur77GFP negative transgenic mice as controls. Approximately 3x10^5 cells were cultured for 24 hours in triplicate with varying amounts of anti-CD3 or PBS then analysed for GFP expression. Representative Flow Cytometry plots of Nur77GFP CD4+ T cells cultured with **A)** PBS and **B)** 5µg anti-CD3. **C)** Representative Nur77GFP MFI (median fluorescence intensity) histograms of C57BL/6, Nur77GFP transgenic negative, Nur77GFP positive CD4+ T cells cultured with PBS and 5µg anti-CD3, and **D)** quantification of the MFI values from C. **E)** Representative Nur77GFP MFI histograms of Nur77GFP CD4+ T cells cultured with PBS, 1, 5, and 10µg anti-CD3, and **F)** quantification of the MFI values from E. Graphs represent 1 experiment with cells isolated from 1 mouse and plated in triplicate, except for cells cultured with PBS which was done as a singlet. Groups were compared using one-way ANOVA. Stars represent the following values: ** ≤0.01; **** <0.0001.

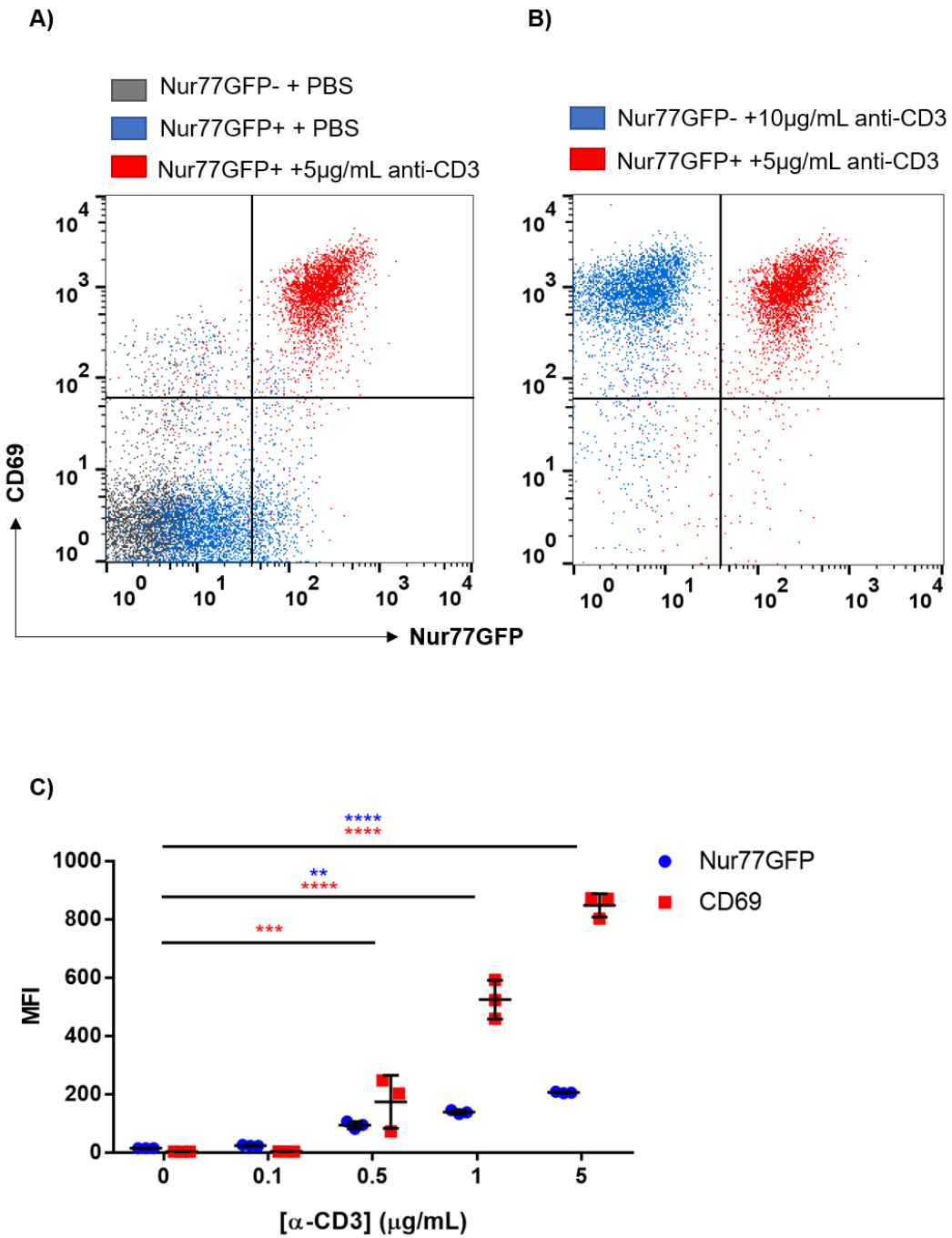


Figure 5-2 Nur77GFP and CD69 expression of Nur77GFP CD4+ T cells after In vitro stimulation with anti-CD3

Lymph nodes were collected and lymphocytes isolated from Nur77GFP. Approximately 3x10^5 cells were cultured for 24 hoursin triplicate with 0.1-5mug/mL anti-CD3 or PBS then analysed for GFP and CD69 expression. **A)** Representative Flow Cytometry plot of Nur77GFP CD4+ T cells cultured with 5ug/mL anti-CD3 and PBS and Nur77 transgenic negative controls cultured with PBS. **B)** Representative flow cytometry plot of Nur77GFP and Nur77GFP transgenic negative CD4+ T cells cultured with 5µg/mL and 10µg/mL anti-CD3 respectively. **C)** Nur77GFP and CD69 MFI (median fluorescence intensity) values from Nur77GFP CD4+ T cells cultured with 0.1-5µg/mL anti-CD3 along with a PBS control. Graphs represent 1 experiment with cells isolated from 1 mouse and plated in triplicate. Groups were compared using two-way ANOVA. Stars represent the following values: ** ≤0.01; *** ≤0.001; **** <0.0001. Red and blue stars represent statistics of the CD69 and Nur77GFP groups respectively.

5.2.2 *In vitro* stimulation time course of Nur77GFP CD4+ T with anti-CD3 reports on TCR stimulation events occurring within 48 hours of TCR engagement

As per previous experiments, CD4+ T cells were purified at day 4 after periarticular administration of HAO for the assessment of TCR clonality in the OVA model of experimental arthritis **(see sections 2.2.5 and 3.2.2.2)**. To determine whether the Nur77GFP system can report T cell stimulation within this time frame, an *in vitro* time course experiment was conducted to investigate how long GFP expression remains upregulated after TCR stimulation. According to Moran et al. a 3 hour *in vitro* stimulation of T cells was sufficient in inducing GFP protein expression[306], and so cells were also stimulated for 3 hours in the *in vitro* culture. Lymphocytes isolated from Nur77GFP mice were stimulated for 3 hours with 1 or 5µg/mL anti-CD3 then washed, re-plated, and cultured for 24-96 hours. At each timepoint, cells were analysed by flow cytometry and GFP expression in CD4+ T cells was determined **(Figure 5-3)**. A PBS control was also included for the same time frame to monitor background levels of GFP. Cells were also analysed immediately after the 3-hour stimulation to determine peak GFP expression, represented by the red, 0 hour bar in Figure 5-3. GFP levels in CD4+ T cells stimulated with 1µg/mL anti-CD3 steadily declined over 3 days and plateaued between 3 and 4 days post stimulation. In contrast, CD4+ T cells stimulated with 5µg/mL anti-CD3 sustained GFP levels 24 hours post stimulation and reduced slightly by 48 hours. By 72 hours, GFP levels drastically decreased and plateaued again by 96 hours. Levels of GFP from CD4+ T cells stimulated with 1µg/mL are comparable with those of unstimulated cells at the 96 hour time point, while GFP expression from CD4+ T cells stimulated with 5µg/mL is still significantly higher compared to PBS controls at the 96 hour time point. This indicates that strongly stimulated CD4+ T cells will still retain some remnant GFP 4 days after the initial stimulation event. This data would therefore suggest that Nur77GFPhi expressing CD4+ T cells reflect TCR stimulation events that have occurred within 48 hours. This system can therefore reflect CD4+ T cell antigen recognition events occurring after periarticular administration of HAO.

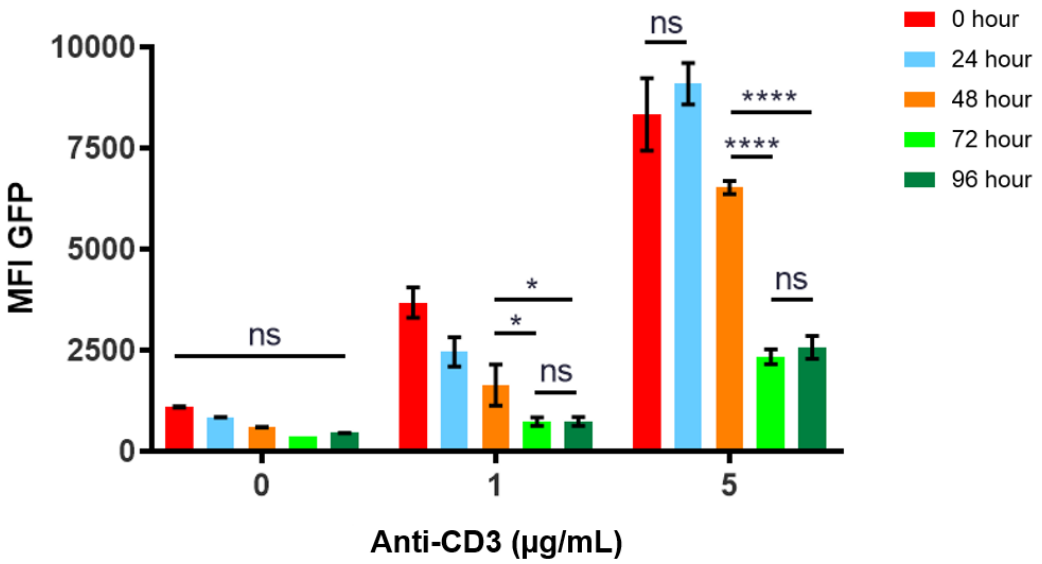


Figure 5-3 Nur77GFP expression 24-96 hours after in vitro stimulation of Nur77GFP CD4+ T cells with anti-CD3
Lymph nodes were collected and lymphocytes isolated from Nur77GFP. Approximately 3x10^5 cells were cultured in triplicate for 3 hours with 0, 1, or 5µg/mL anti-CD3 at 37C. Cells were then washed and transferred to new plates, and taken after 24-96 hours of culture. Cells were analysed at each timepoint by flow cytometry and GFP expression in CD4+ T cells was determined. Columns represent Nur77GFP MFI (median fluorescence intensity) of CD4+ T cells cultured with PBS (0µg/mL anti-CD3), 1µg/mL, and 5 µg/mL anti-CD3 taken immediately after 3 hour anti-CD3 stimulation (0 hours) to 96 hours after stimulation in 24 hour increments. Graphs represent 1 experiment with cells isolated from 1 mouse and plated in triplicate. Groups were compared using two-way ANOVA. Stars represent the following values: * ≤0.05; **** <0.0001; ns: not significant.

5.2.3 Evidence of antigen recognition in early inflamed joints

The data presented so far suggests that the Nur77GFP system reflects recent antigen recognition events – more specifically, events that have occurred no more than 48 after TCR stimulation. This timeframe is adequate for determining TCR stimulation events and T cell activation following periarticular challenge with HAO, which allowed us to assess whether CD4+ T cells can potentially recognise their cognate antigen in the joint. The OVA model of experimental arthritis was therefore carried out and the number and frequency of endogenous CD4+ T cells with high Nur77GFP expression was determined **(Figure 5-4 A and B)**. The number of CD4+ Nur77GFPhi cells were significantly higher in inflamed pLNs and joints compared to PBS controls **(Figure 5-4 B)**. The frequency of Nur77GFPhi cells was also significantly greater in HAO challenged joints compared to controls, but not in the pLNs. The greater overall number of Nur77GFPhi cells would suggest that more cells in HAO challenged mice have had their TCR engaged. To verify that this increase in Nur77GFP expression reflects T cell activation due to TCR engagement rather than a consequence of non-specific environmental inflammatory stimuli , mice were also challenged with 25µg of LPS and Nur77GFP expression was determined

in pLNs and joints **(Figure 5-5)**. The number of Nur77GFPhi endogenous CD4+ T cells was lower in pLNs from LPS challenged mice compared to pLNs from HAO challenged mice and was also comparable to PBS controls. This could suggest that of the endogenous CD4+ T cells entering the HAO challenged joint, more of them have seen their cognate antigen in HAO than in LPS challenged joints.

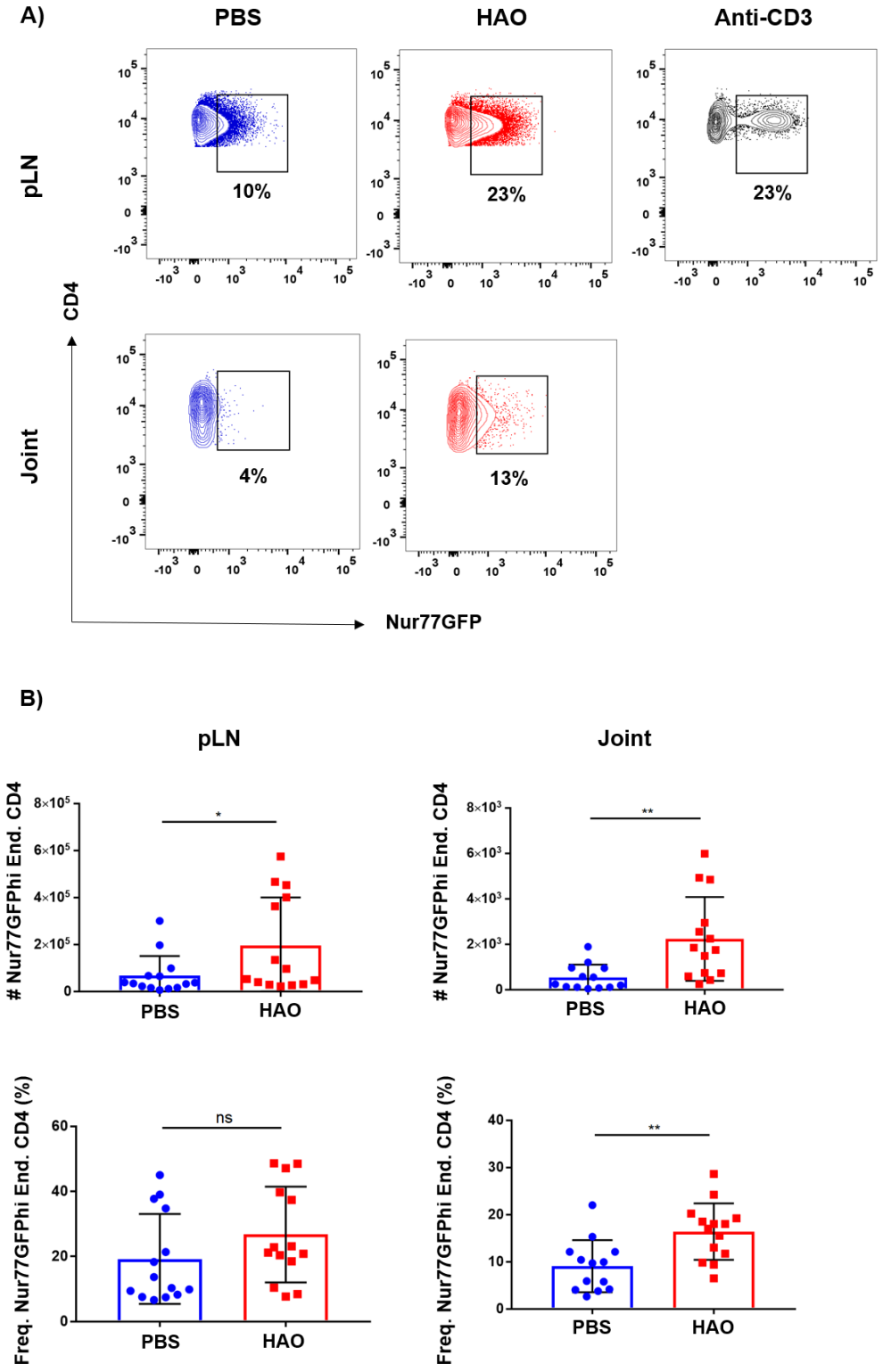


Figure 5-4 Number and frequency of Nur77GFPhi endogenous CD4+ T cells from pLNs and joints of HAO challenged mice and PBS controls

Cells were isolated from pLNs and joints 4 days after periarticular challenge and stained for flow cytometry analysis. Transferred cells were identified using the CD45.1 marker and were excluded from analysis, leaving only the endogenous CD4+ T cells. **A)** Representative flow cytometry plots of Nur77FP expression in endogenous CD4+ T cells isolated from pLNs (top) and joints (bottom) of HAO (red) and PBS (blue) challenged mice. Lymphocytes isolated from pLNs treated with anti-CD3 as a positive control for GFP expression (black). **B)** Number (top) and frequency (bottom) of endogenous CD4+ T cells expressing high levels of Nur77GFP from pLNs and joints of HAO and PBS challenged mice. Graphs represent 3 experiments with n=14 for HAO challenged pLNs and joints, and PBS challenged pLNs, and n=13 for PBS challenged joints. Groups were compared using Student's t-test. Stars represent the following values: * ≤0.05; ** <0.01; ns: not significant.

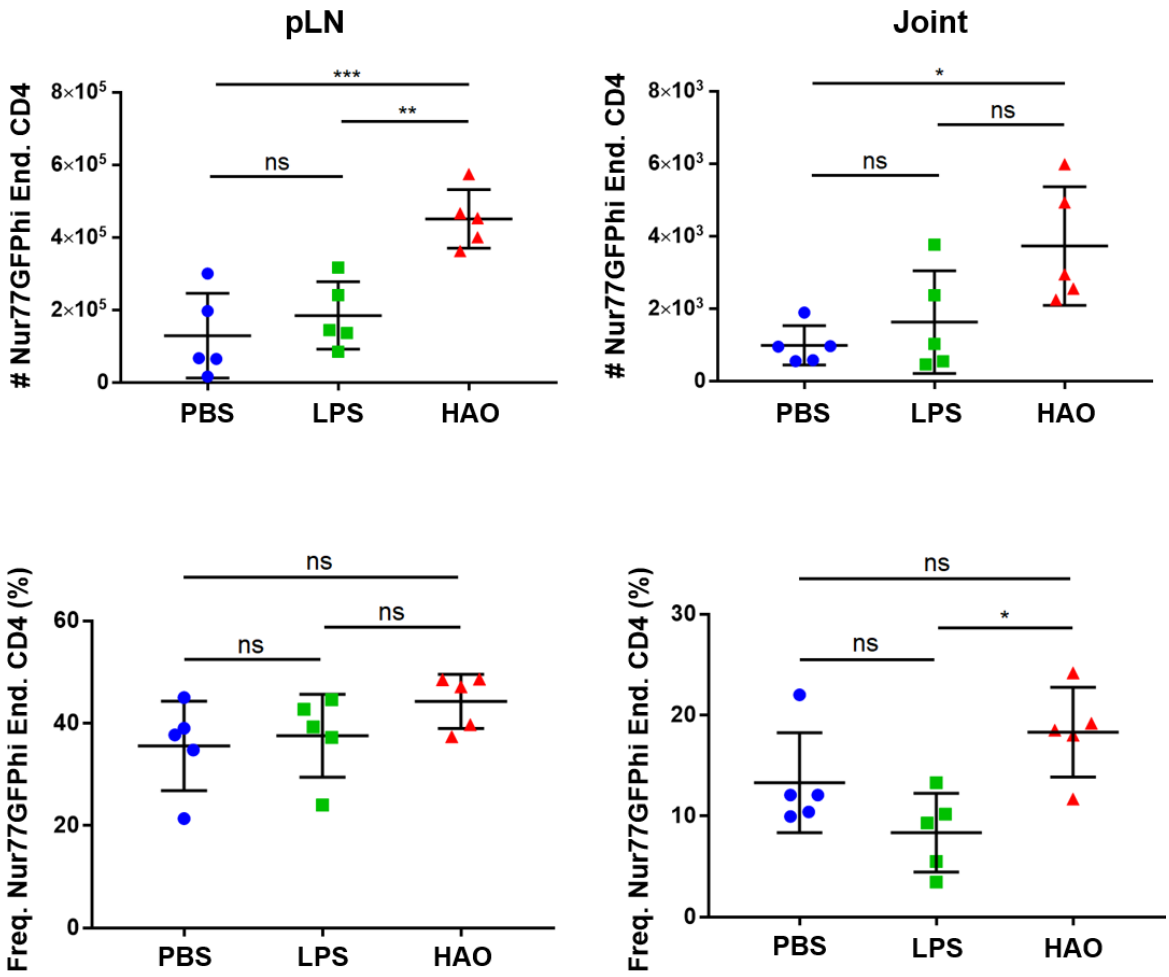


Figure 5-5 Number and frequency of Nur77GFPhi endogenous CD4+ T cells from pLNs and joints of HAO, LPS, and PBS challenged mice
Cells were isolated from pLNs and joints 4 days after periarticular challenge and stained for flow cytometry analysis. Transferred cells were identified using the CD45.1 marker and were excluded from analysis, leaving only the endogenous CD4+ T cells. Number (top) and frequency (bottom) of endogenous CD4+ T cells expressing high levels of Nur77GFP from pLNs and joints of HAO, LPS and PBS challenged mice. Graphs represent 1 experiment with n=5 for all groups. Groups were compared using one-way ANOVA. Stars represent the following values: * ≤0.05; ** <0.01; *** <0.001; ns: not significant.

5.2.4 Endogenous CD4+ Nur77GFPhi cells isolated from HAO challenged pLNs are not enriched for Vβ4 expressing cells

One of the aims of the project was to provide evidence of antigen recognition in the early phases of experimental arthritis. In results Chapter 3, I demonstrated that Vβ4 was preferentially enriched in HAO challenged pLNs and joints and that Vβ4 may be joint antigen specific **(see sections 3.2.2.2 and 3.2.2.5)**. To determine whether cells with the Vβ4 TCR chain are enriched in HAO challenged pLNs and joints as a consequence of TCR engagement with their cognate antigen, and to further refine where these cells are seeing their cognate antigen, the OVA experimental model was carried out in Nur77GFP mice. The number and frequencies of Nur77GFPhi endogenous CD4+ T cells with Vβ4, 6, and 13 TCR chains were then determined

from HAO and PBS challenged mouse pLNs **(Figure 5-6)**. The number of Nur77GFPhi cells was too low in the joints to accurately determine frequencies and number of cells with a given Vβ chain (data not shown). The number and frequencies of Nur77GFPhi endogenous CD4+ T cells with Vβ4, 6, and 13 TCR chains were similar in both HAO and PBS challenged pLNs. This data suggests that endogenous CD4+ cells with the Vβ4 TCR chain did not have their TCR engaged within 48 hours of analysis. This implies that these cells did not see their cognate antigen within that time frame and their enrichment in the inflamed pLN may not be due to antigen recognition.

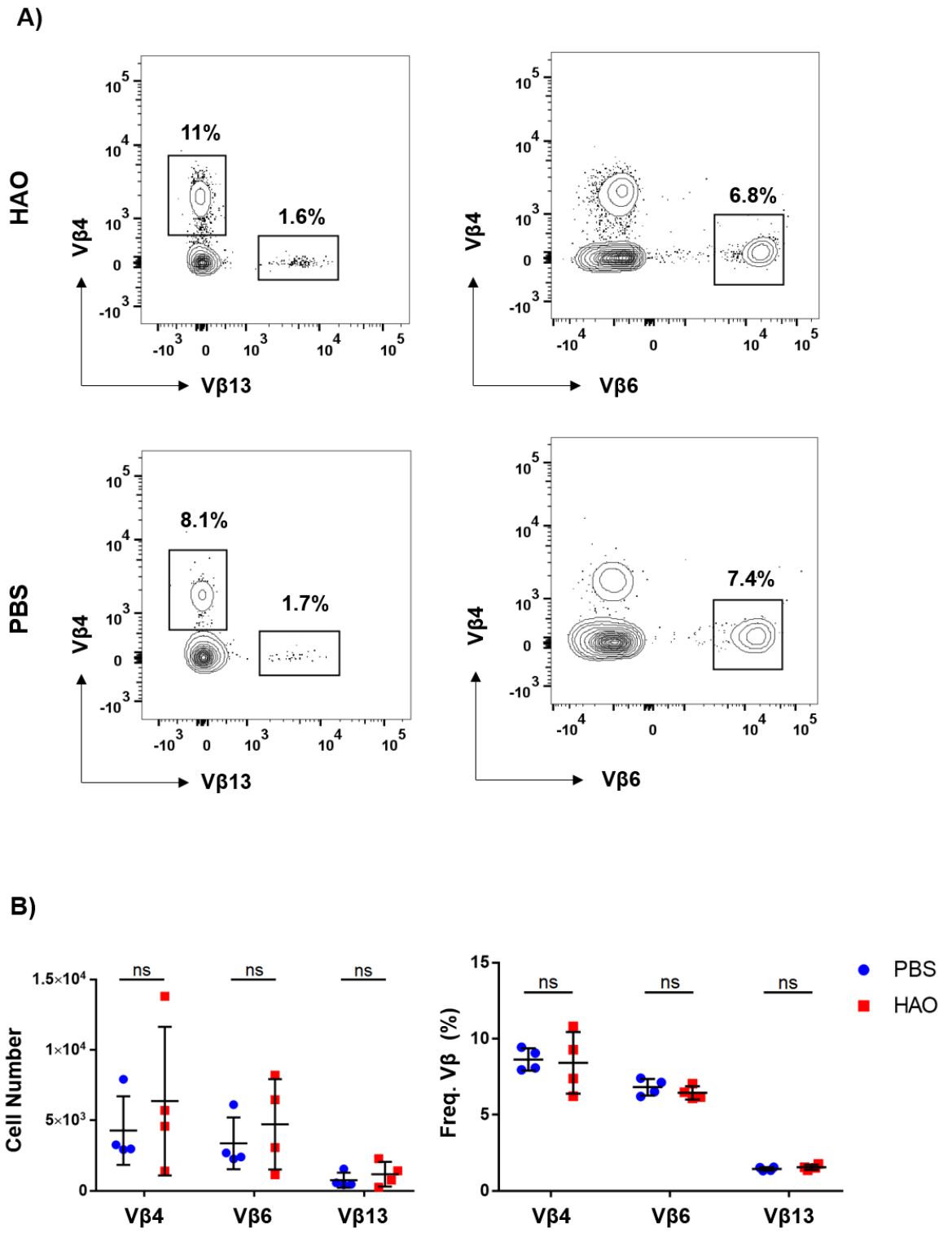


Figure 5-6 Number and frequency of cells with a given Vβ chain from the Nur77GFPhi endogenous CD4+ T cell population isolated from pLNs of HAO and PBS challenged mice
Cells were isolated from pLNs and 4 days after periarticular challenge and stained for flow cytometry analysis. Transferred cells were identified using the CD45.1 marker and were excluded from analysis, leaving only the endogenous CD4+ T cells. **A)** Representative flow cytometry plots of Vβ4, Vβ6, and Vβ13 populations gated on Nur77GFPhi endogenous CD4+ T cells isolated from HAO and PBS challenged mice. **B)** Number and frequency of cells expressing a given Vβ chain from endogenous CD4+ T cells expressing high levels of Nur77GFP from pLNs of HAO and PBS challenged mice. Graphs represent 1 experiment with n=4 for all groups. Groups were compared using 2-way ANOVA. ns: not significant.

5.2.5 Establishing optimal conditions for Nur77GFP CD4+ T cell co-coculture experiments

Endogenous CD4+ T cells with the Vβ4 TCR chain isolated from pLNs did not show an increase in Nur77GFP expression, despite being enriched in HAO challenged joints and pLNs and being associated with joint derived antigens **(see Chapter 3 section 3.2.2.5)**. Another method of determining whether endogenous CD4+ T cells recognise joint antigens was to purify the endogenous CD4+ T cells from Nur77GFP mice undergoing the OVA arthritis model from pLNs and joints and co-culture them with BMDCs pulsed with joint antigens. The aim of using this approach was to refine the cohort of antigens that these cells potentially recognised, and to also demonstrate the possibility of joint endogenous CD4+ T cells being activated in the joint itself if their cognate antigens are presented there. This approach would address the third aim of this chapter which was to provide evidence of autoreactive CD4+ T cell clones that could potentially recognise their cognate antigen in the inflamed joint.

Before attempting to investigate the possible antigens CD4+ T cells recognise in pLNs and joints of mice undergoing the experimental arthritis model, an assay to measure Nur77GFP expression as a result of re-stimulation with cognate antigen needed to be established. To develop this assay, brachial and axillary lymph nodes were collected from Nur77GFP mice immunised with OVA/CFA as well as from unimmunised mice. CD4+ T cells were then co-cultured with BMDCs pulsed with or without OVA and Nur77GFP expression, proliferation, and cytokine production was evaluated.

CD4+ T cells were purified from brachial and axillary lymph nodes 7 days after Nur77GFP mice were immunised with OVA/CFA, as well as from control naive mice and cultured for 6 hours with BMDCs pulsed with or without OVA protein. Nur77GFP expression, degree of proliferation, and cytokine production of CD4+ T cells were then determined by flow cytometry. The percentage of Nur77GFPhi cells was similar in the naïve samples compared to OVA immunised mice in the cytokine expression experiments **(Figure 5-7)** and comparable across all samples in the proliferation experiments (data not shown). This may be a reflection of the high background of GFP in LNs due to continuous TCR/MHC interactions[306] and together with the high number of Nur77GFPmid-lo CD4+ T cells, the frequency of Nur77GFPhi cells was

probably not great enough in OVA/CFA challenged mice to be detected. Although, when gating on the Nur77hi CD4+ cells, the cells isolated from OVA immunised mice that were then co-cultured with OVA pulsed BMDCs showed the greatest proliferation compared to controls **(Figure 5-8 A)** and also had increased expression of ICOS, IL-2, IFNγ, and TNFα **(Figure 5-8 B and C)**. Interestingly, CD4+ cells isolated from OVA immunised mice continue to proliferate despite the absence of their cognate antigen **(green histogram in Figure 5-8 A)**. When evaluating ICOS expression and cytokine production, cells isolated from OVA immunised mice seem to show a clearer difference in ICOS and overall cytokine expression when co-cultured with OVA pulsed BMDCs compared to controls **(Figure 5-8 C)**. This data would suggest that evaluating ICOS and cytokine expression is more accurate for reporting T cell activation and was therefore used to evaluate antigen recognition in co-culture experiments of cells isolated from the model. Moreover, cytokine expression can provide information on the type of T helper responses and therefore elaborate on the function of CD4+ T cells in response to antigen at the tissue site. Unfortunately, both proliferation and cytokine expression experiments could not be performed with endogenous CD4+ T cells from the model, as the yield of CD4+ T cells from the joints is so low that it was not feasible to do both.

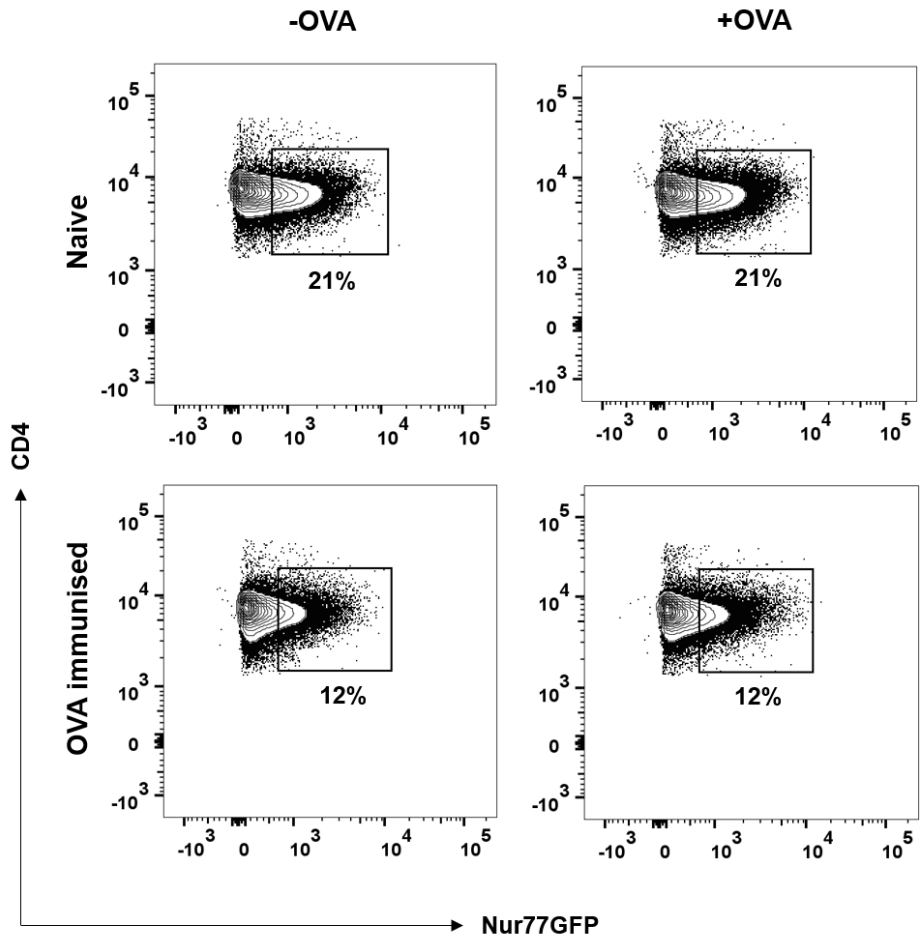
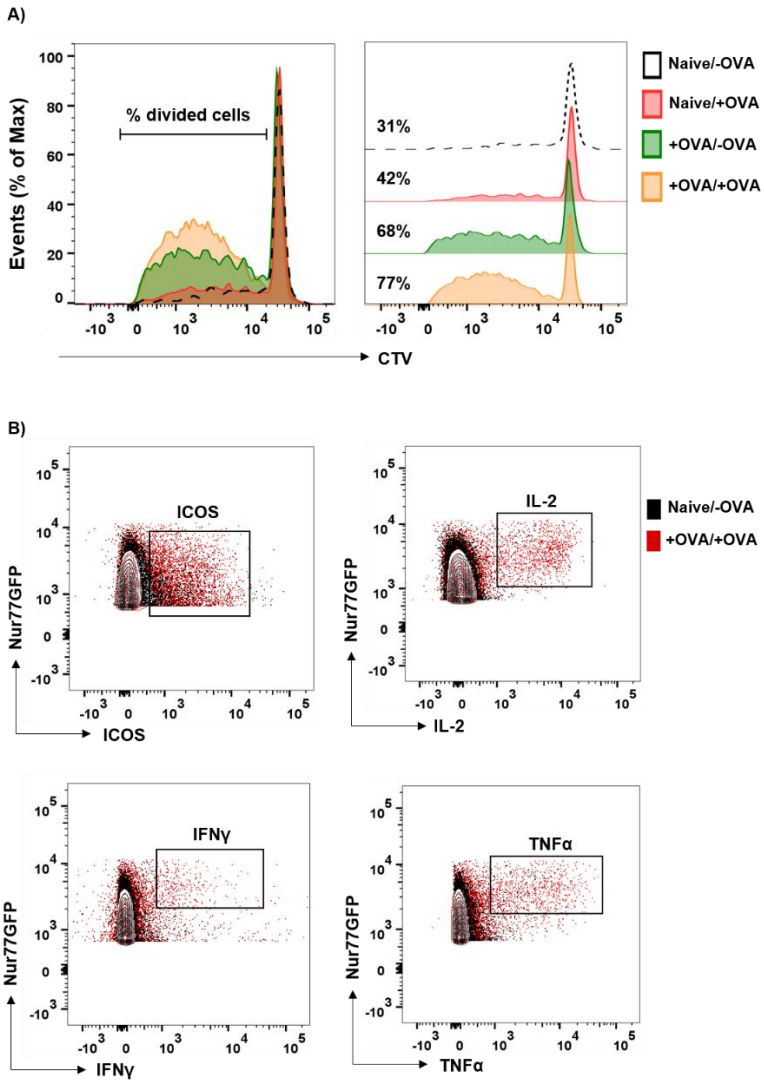


Figure 5-7 Assessment of Nur77GFP expression in CD4+ T cells isolated from OVA immunised mice co-cultured with OVA

Brachial and axillary lymph nodes were collected from a naïve Nur77GFP mouse and Nur77GFP mice immunised with OVA in CFA in the scruff 7 days after immunisation. 1x10^6 purified CD4 T cells were co-cultured in a 5:1 ratio with BMDCs pulsed with OVA protein 24 hours prior to co-culture. Purified CD4+ T cells were also cultured with non-pulsed BMDCs as controls. Cells were cultured for 6 hours then stained for flow cytometry analysis. Plots are representative flow cytometry plots of the frequency of Nur77GFPhi cells.



C)

Sample	% ICOS+	% IL-2+	% IFNγ+	% TNFα+
Naïve/-OVA (-OVA/-OVA)	2.5	0.11	0.06	0.17
Naïve/+OVA (-OVA/+OVA)	2.3	0.18	0.10	0.26
OVA Immunised/-OVA (+OVA/-OVA)	5.9	0.15	0.08	0.18
OVA immunised/+OVA (+OVA/+OVA)	7.3	2.2	0.62	1.6

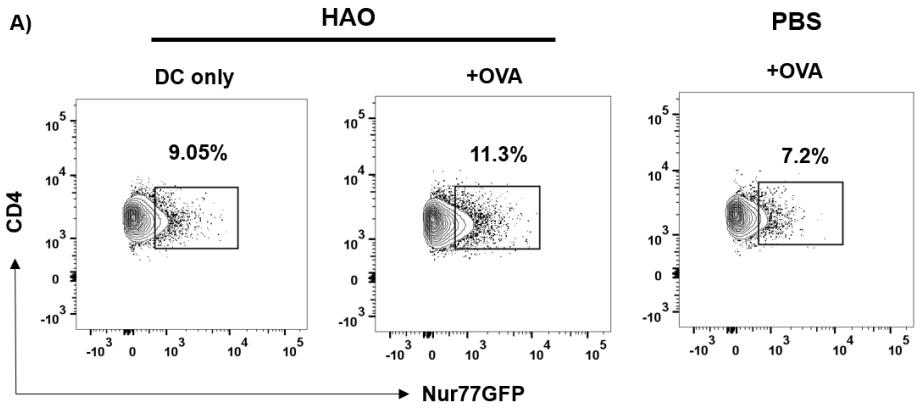
Figure 5-8 Proliferation and cytokine expression of Nur77GFPhi CD4+ T cells isolated from OVA immunised mice co-cultured with OVA

Brachial and axillary lymph nodes were collected from a naïve Nur77GFP mouse and Nur77GFP mice immunised with OVA in CFA in the scruff 7 days after immunisation. 1x10^6 purified CD4 T cells were co-cultured in a 5:1 ratio with BMDCs pulsed with OVA protein 24 hours prior to co-culture. Purified CD4+ T cells were also cultured with non-pulsed BMDCs as controls. Cells were co-cultured for 6 and 96 hours for cytokine expression and proliferation respectively, then stained for flow cytometry analysis. **A)** Proliferation of Nur77GFPhi cells co-cultured with or without OVA pulsed BMDCs. Proliferation was determined by the dilution of Cell Trace Violet dye (CTV). Histograms represent the following: -OVA/-OVA: naïve CD4+ T cells with DCs –OVA; -OVA/+OVA: naïve CD4+ T cells with DCs +OVA; +OVA/-OVA: OVA immunised CD4+ T cells with BMDCs –OVA; +OVA/+OVA: OVA immunised CD4+ T cells with DCs +OVA. Percentages are frequency of divided cells. **B)** Representative ICOS and cytokine (IL-2, IFNγ, and TNFα) staining of Nur77GFPhi cells, and **C)** percentages of ICOS+, IL-2+, IFNγ+, and TNFα+ Nur77GFPhi cells from naïve and OVA immunised mice co-culture with BMDCs pulsed with or without OVA. Plots and table represent 1 experiment with n=1.

5.2.6 Evaluating endogenous CD4+ T cell activation in response to joint antigen recognition in animals undergoing the early model of experimental arthritis

To determine whether the endogenous CD4+ T cells isolated from pLNs and joints of HAO challenged mice recognise joint derived antigens, co-cultures were set up and BMDCs were pulsed with joint extract from LPS challenged mice (see methods **section 2.2.5** for details), as this would emulate inflammatory conditions without the presence of HAO, ensuring that the antigen specific responses observed were not due to recognition of OVA. BMDCs were also pulsed with OVA or with a combination of bovine collagen II and aggrecan, which served as candidate RA antigens. Bovine collagen II and aggrecan were used as a candidate RA antigens as evidence for antibody production and T cell proliferation in response to collagen II has been reported in other studies using the model[140,252,254]. Aggrecan is a component of collagen to which CD4+ T cell responses have also been demonstrated[305]. Having these antigens may also refine what type of joint antigens the endogenous CD4+ T cells may recognise, if the CD4+ T cells do indeed show evidence of antigen recognition when co-cultured with joint extract. Nur77GFP expression was determined in endogenous CD4+ T cells isolated from both pLNs and joints of HAO and PBS challenged mice to evaluate the degree of antigen recognition after the co-cultures **(Figure 5-9)**. Under all conditions, there was a higher frequency of Nur77GFPhi endogenous CD4+ T cells isolated from HAO challenged pLNs compared to PBS pLNs. When comparing co-culture conditions across CD4+ T cells isolated from HAO challenged pLNs, cells co-cultured with OVA show a significant increase in Nur77GFP expression compared to the DC only control, while cells cultured with LPS joint extract and candidate RA antigens show a decrease or no significant increase in Nur77GFP expression respectively. Endogenous CD4+ T cells isolated from the joint show no difference in Nur77GFP expression between PBS and HAO challenged mice, nor across any of the co-culture conditions **(Figure 5-9 C and D)**.

pLN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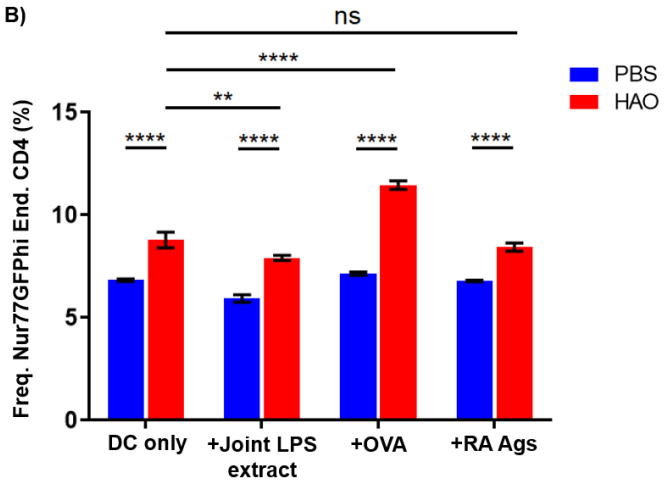
HAO
PBS
DC only
+OVA
+OVA
9.05%
11.3%
7.2%
CD4
Nur77GFP


B)
ns
****
**
PBS
HAO
Freq. Nur77GFPhi End. CD4 (%)
DC only
+Joint LPS extract
+OVA
+RA Ags

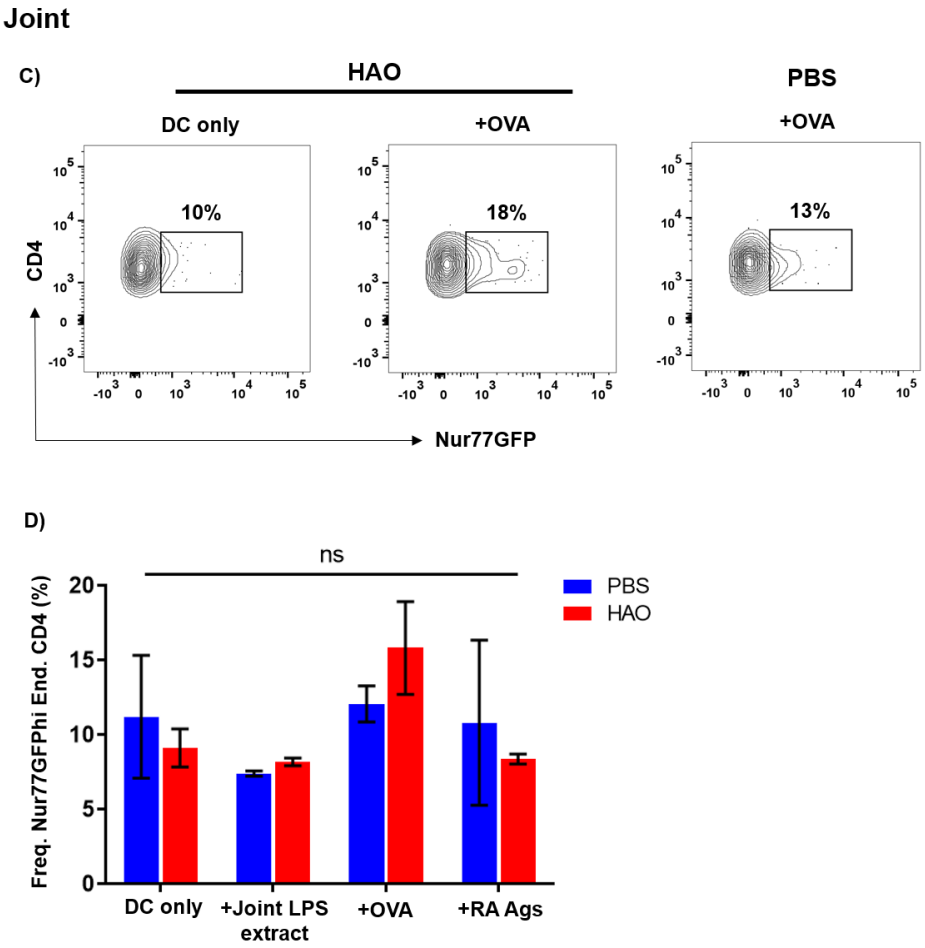


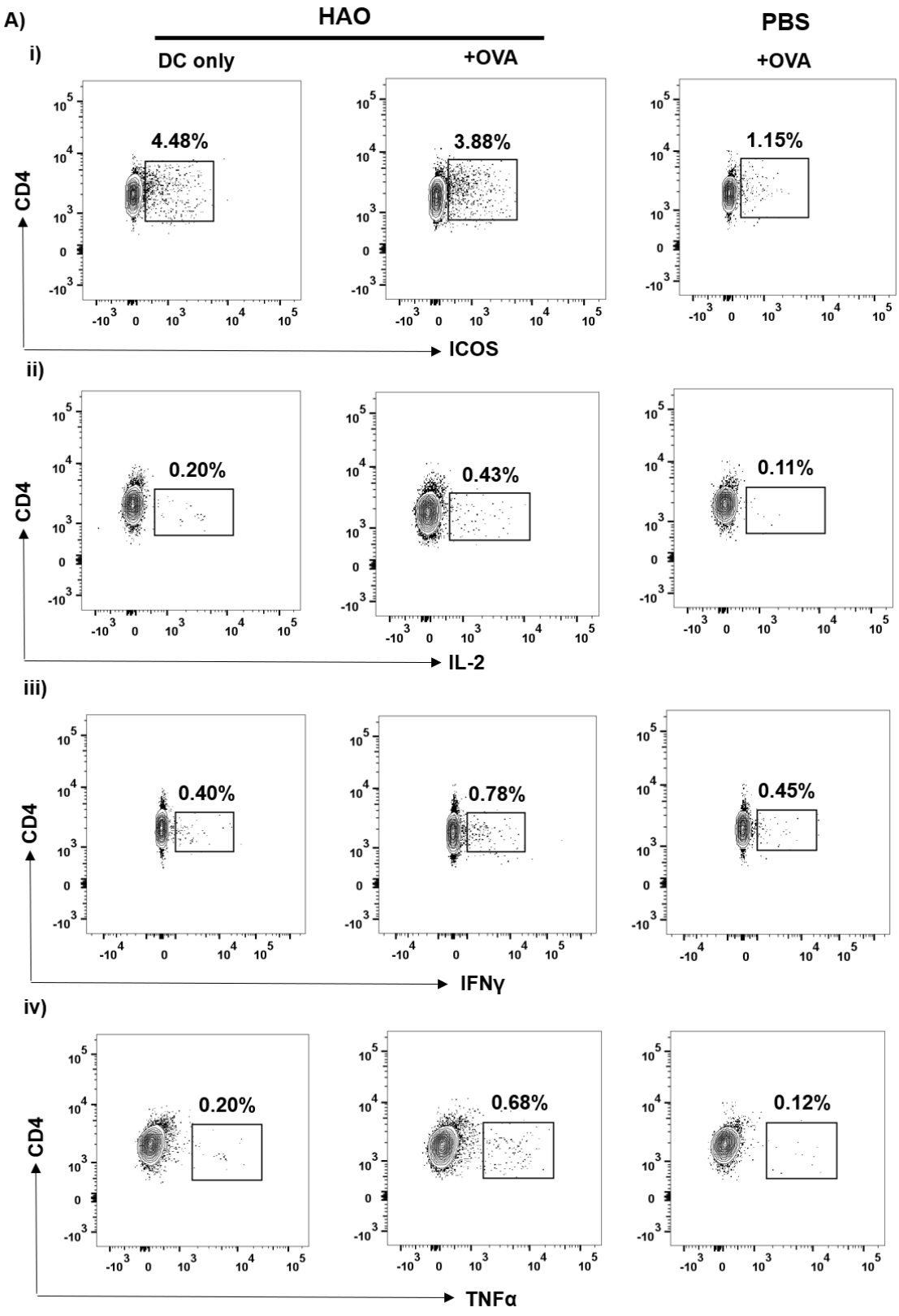
Figure 5-9 Percentage of Nur77GFPhi endogenous CD4+ T cells isolated from pLN and joints of HAO and PBS challenged mice co-cultured with joint extract, OVA, or candidate RA antigens

pLNs and joints were collected from HAO and PBS challenged mice. CD4+ T cells were purified and co-cultured with BMDCs pulsed with joint extract from an LPS challenged mouse, OVA, or candidate RA antigens bovine collagen II and bovine aggrecan. Cells were also cultured with non-pulsed BMDCs to determine baseline Nur77GFPhi frequency. BMDCs were pulsed and simultaneously cultured with CD4+ T cells. Golgi plug was added 6 hours after co-culture set up and cells were cultured for a further 6 hours at 37C. Cells were collected and stained for flow cytometry analysis. Cells were cultured in duplicate for each condition. Representative flow cytometry plots of **A)** pLN and **C)** joint Nur77GFPhi endogenous CD4+ T cells isolated from HAO challenged mice co-cultured with and without OVA, and from PBS challenged mice co-cultured with OVA. Frequency of **B)** pLN and **D)** joint Nur77GFPhi endogenous CD4+ T cells from each experimental condition. Graphs represent 1 experiment with n=1 (pLNs and joints from 7 HAO and 6 PBS challenged mice were pooled per tissue per group. Values from each technical replicate plotted). Groups were compared using 2-way ANOVA. Stars represent the following values: ** ≤0.01; **** <0.0001; ns: not significant

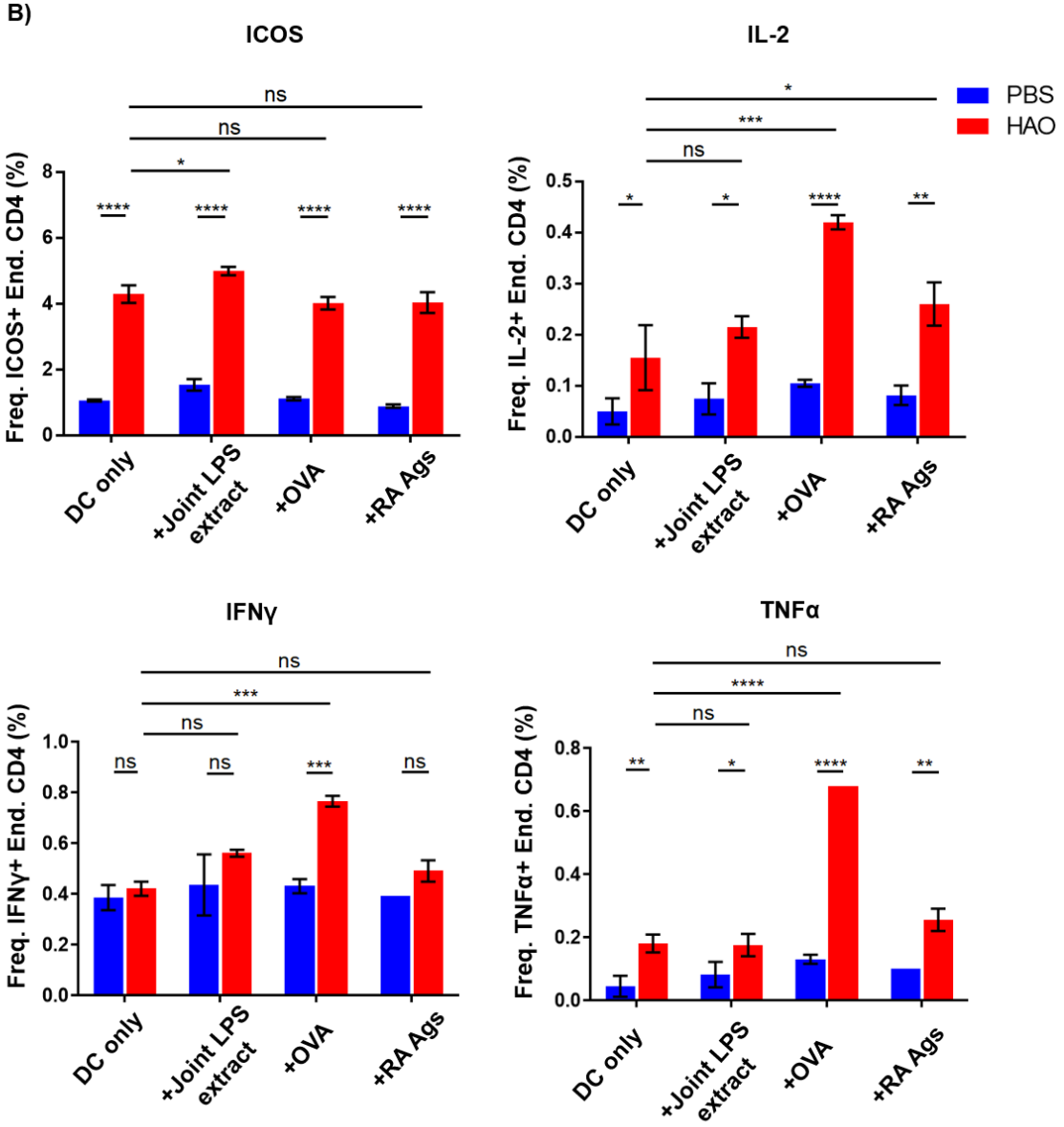
ICOS expression and cytokine production of endogenous CD4+ T cells from HAO and PBS pLNs show a similar pattern to Nur77GFP expression **(Figure 5-10 A and B)**. ICOS, IL-2, and TNFα expression were all greater in cells isolated from HAO challenged mice compared to PBS challenged mice, but only OVA cultured HAO CD4+ T cells showed an increase in IFNγ production. Again, cells isolated from HAO challenged pLNs show an increase in IL-2, IFNγ, and TNFα production compared to the DC only group when cultured with OVA. Cells cultured with joint extract show an increase in ICOS expression, and cells cultured with candidate RA antigens show

an increase in IL-2 production when compared to the DC only controls. On the other hand, endogenous CD4+ T cells isolated form the joint show an increase in ICOS expression when cultured with joint extract, OVA, and RA candidate antigens compared to DC only and PBS controls **(Figure 5-10 C and D)**. However, there is no significant difference in IL-2, IFNγ, and TNFα production.

Overall this data shows that endogenous CD4+ T cells isolated from pLNs and joints of HAO challenged mice did not respond to the joint derived antigens tested in the assay. Moreover, a significant percentage of these cells recognise OVA antigens in the assays I employed and show effector responses when re-stimulated with OVA. However, although significant, only 15% of the total endogenous CD4+ T cell population purified from pLNs recognised OVA, and an even lower percentage produced cytokines in response to OVA, indicating that only a minority of the endogenous CD4+ T cells recognise OVA at this timepoint.


A)
HAO
PBS
i)
DC only
+OVA
+OVA
4.48%
3.88%
1.15%
CD4
ICOS
ii)
0.20%
0.43%
0.11%
CD4
IL-2
iii)
0.40%
0.78%
0.45%
CD4
IFNγ
iv)
0.20%
0.68%
0.12%
CD4
TNFα

B)


ICOS
IL-2
IFNγ
TNFα
PBS
HAO
Freq. ICOS+ End. CD4 (%)
Freq. IL-2+ End. CD4 (%)
Freq. IFNγ+ End. CD4 (%)
Freq. TNFα+ End. CD4 (%)
DC only
+Joint LPS extract
+OVA
+RA Ags
ns
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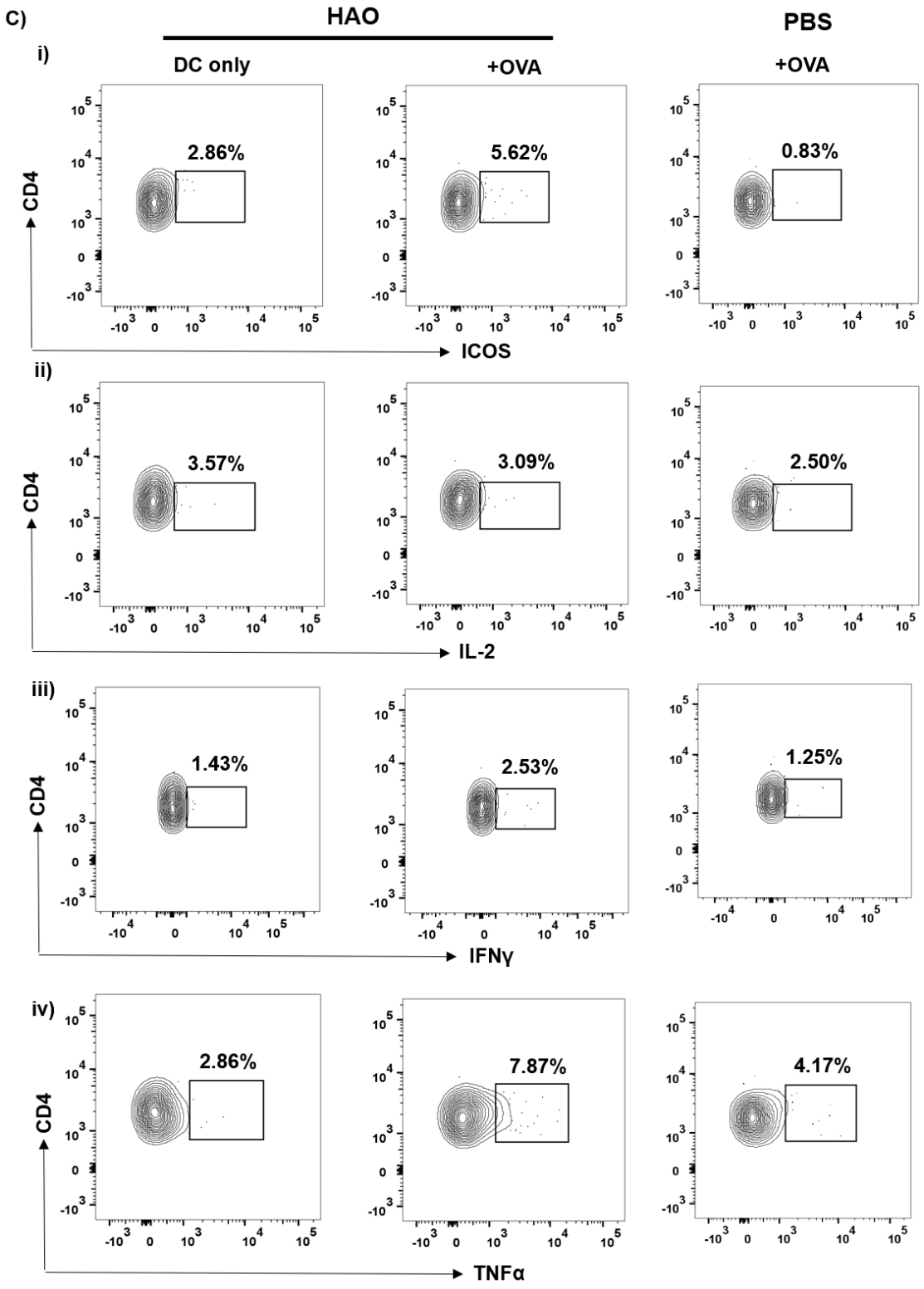
C)
HAO
PBS
i)
DC only
+OVA
+OVA
2.86%
5.62%
0.83%
CD4
ICOS
ii)
3.57%
3.09%
2.50%
CD4
IL-2
iii)
1.43%
2.53%
1.25%
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iv)
2.86%
7.87%
4.17%
CD4
TNFα

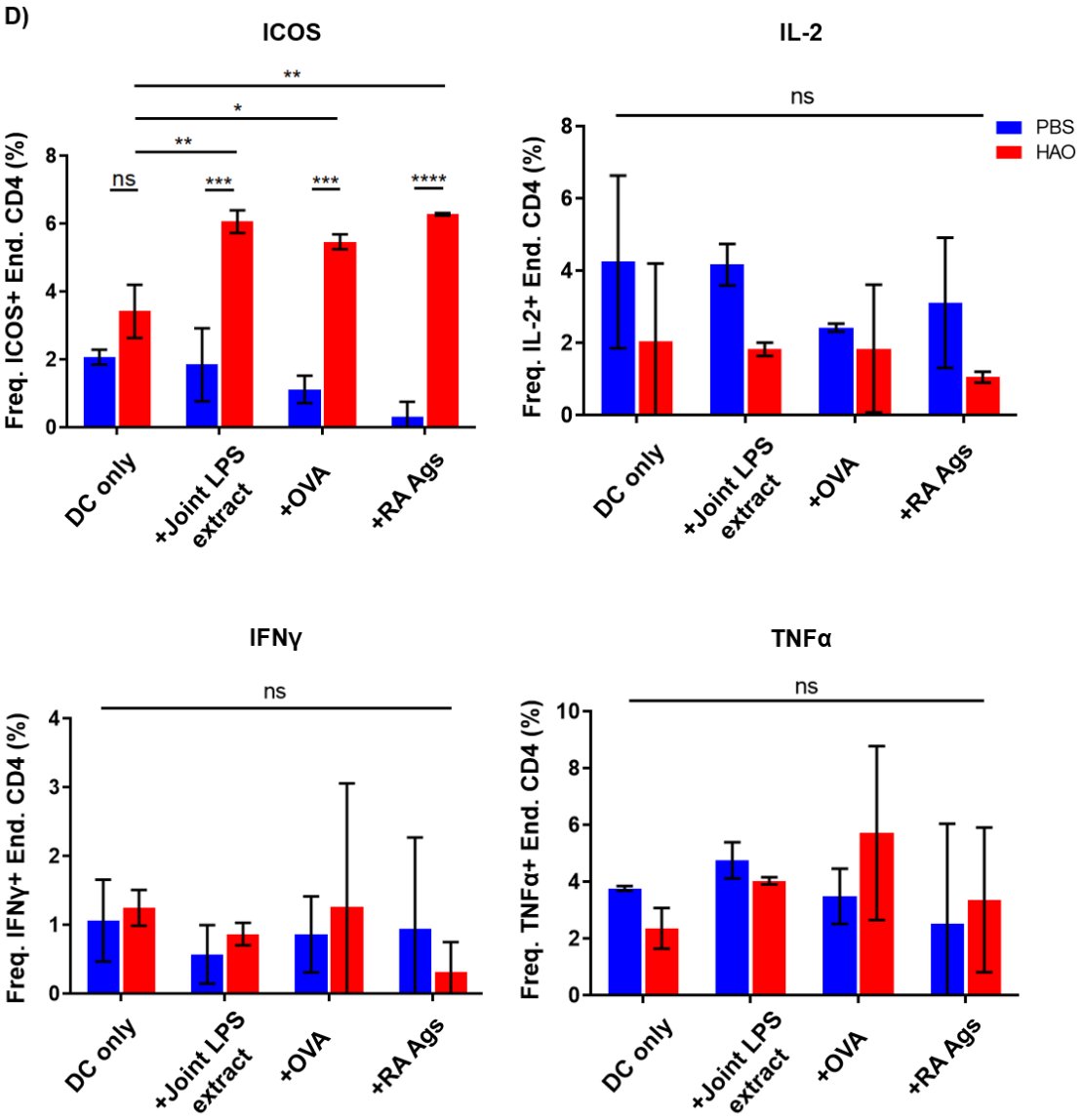


Figure 5-10 Percentage of cytokine producing endogenous CD4+ T cells isolated from pLNs and joints of HAO and PBS challenged mice co-cultured with joint extract, OVA, or candidate RA antigens

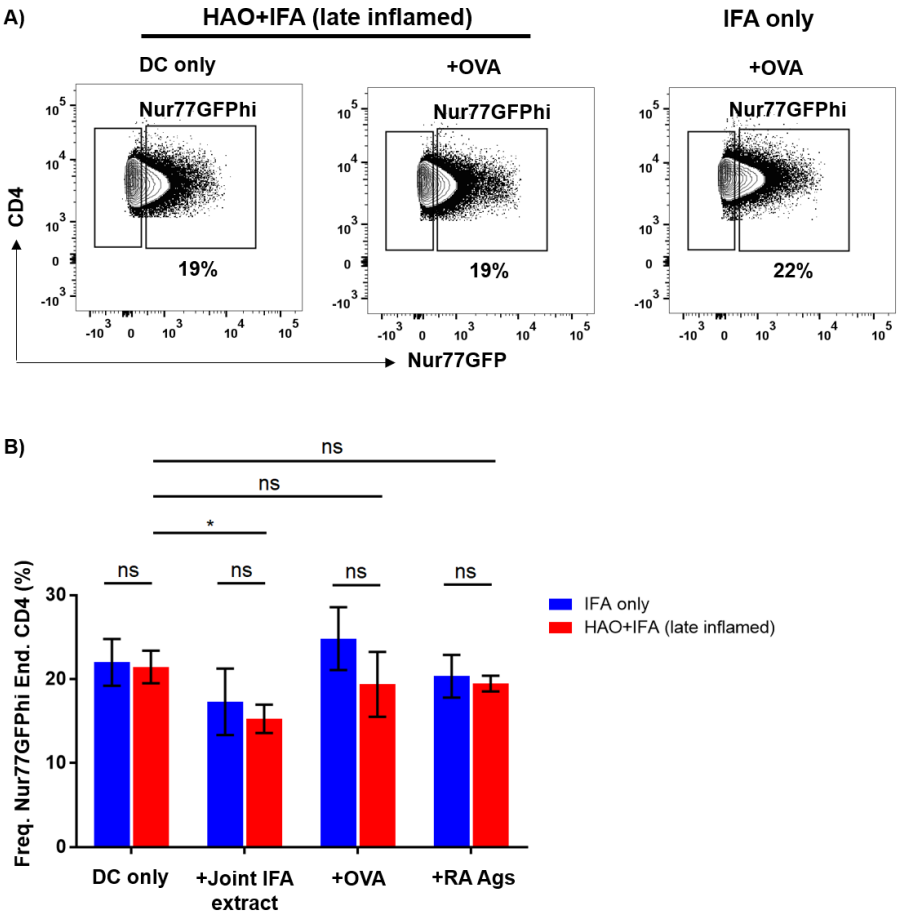
pLNs and joints were collected from HAO and PBS challenged mice. CD4+ T cells were purified and co-cultured with BMDCs pulsed with joint extract from an LPS challenged mouse, OVA, or candidate RA antigens bovine collagen II and bovine aggrecan. Cells were also cultured with non-pulsed BMDCs to determine baseline cytokine expression. BMDCs were pulsed and simultaneously cultured with CD4+ T cells. Golgi plug was added 6 hours after co-culture set up and cells were cultured for a further 6 hours at 37C. Cells were collected and stained for flow cytometry analysis. Cells were cultured in duplicate for each condition. Representative flow cytometry plots of **A)** pLN and **C)** joint endogenous CD4+ T cells from HAO and PBS challenged mice showing **i)** ICOS, **ii)** IL-2, **iii)** IFNγ, and **iv)** TNFα expression when co-cultured with and without OVA. Frequency of ICOS+, IL-2+, IFNγ+, and TNFα+ in **B)** pLN and **D)** joint endogenous CD4+ T cells from each experimental condition. Graphs represent 1 experiment with n=1 (pLNs and joints from 7 HAO and 6 PBS challenged mice were pooled per tissue per group. Values from each technical replicate plotted). Groups were compared using 2-way ANOVA. Stars represent the following values: * ≤0.05; ** ≤0.01; *** ≤0.001; **** <0.0001; ns: not significant.

5.2.7 Evaluating endogenous CD4+ T cell activation in response to joint antigen recognition in animals undergoing the late model of experimental arthritis

In **Chapter 3 section 3.2.2.6** and results **Chapter 4** it was demonstrated that an antigen specific response appears to evolve over time, with the inflamed lymph node and joint showing differences in antigen specific responses with the progression of experimental arthritis. One of the questions that I asked is whether the changes in antigen specific responses is due to an increased response to joint antigens. To address this, co-culture experiments were performed with endogenous CD4+ T cells isolated from pLNs and joints of Nur77GFP mice undergoing the late model of experimental arthritis (see **section 2.10** for more details). In these experiments, BMDCs were pulsed with joint extract made from IFA challenged joints, OVA, and the same candidate RA antigens used in the co-culture experiments discussed in the previous section. Nur77GFP expression was evaluated from endogenous CD4+ T cells isolated from pLNs and joints and ICOS and cytokine expression in response to the various antigens was also evaluated.

Endogenous CD4+ T cells isolated from late inflamed and IFA only pLNs did not show a difference in Nur77GFP expression between the two experimental groups, nor in response to OVA or RA candidate antigens **(Figure 5-11 A and B)**. Interestingly, Nur77GFP expression was reduced in endogenous CD4+ T cells cultured with joint IFA extract. By contrast, the T cells isolated from late inflamed and IFA only joints showed a significant increase in Nur77GFP expression when cultured with OVA – 35% and 28% of the endogenous CD4+ T cells isolated from late inflamed and IFA only joints respectively respond to OVA **(Figure 5-11 C and D)**. No significant increase in Nur77GFP expression was found when cultured with joint IFA extract or candidate RA antigens. It is also important to note that the overall GFP expression is greater in pLNs than it is in the joints.

pLN


A)
HAO+IFA (late inflamed)
IFA only
DC only
+OVA
+OVA
Nur77GFPhi
Nur77GFPhi
Nur77GFPhi
19%
19%
22%
CD4
Nur77GFP
B)
ns
ns
*
ns
ns
ns
ns
Freq. Nur77GFPhi End. CD4 (%)
30
20
10
0
IFA only
HAO+IFA (late inflamed)
DC only
+Joint IFA extract
+OVA
+RA Ags

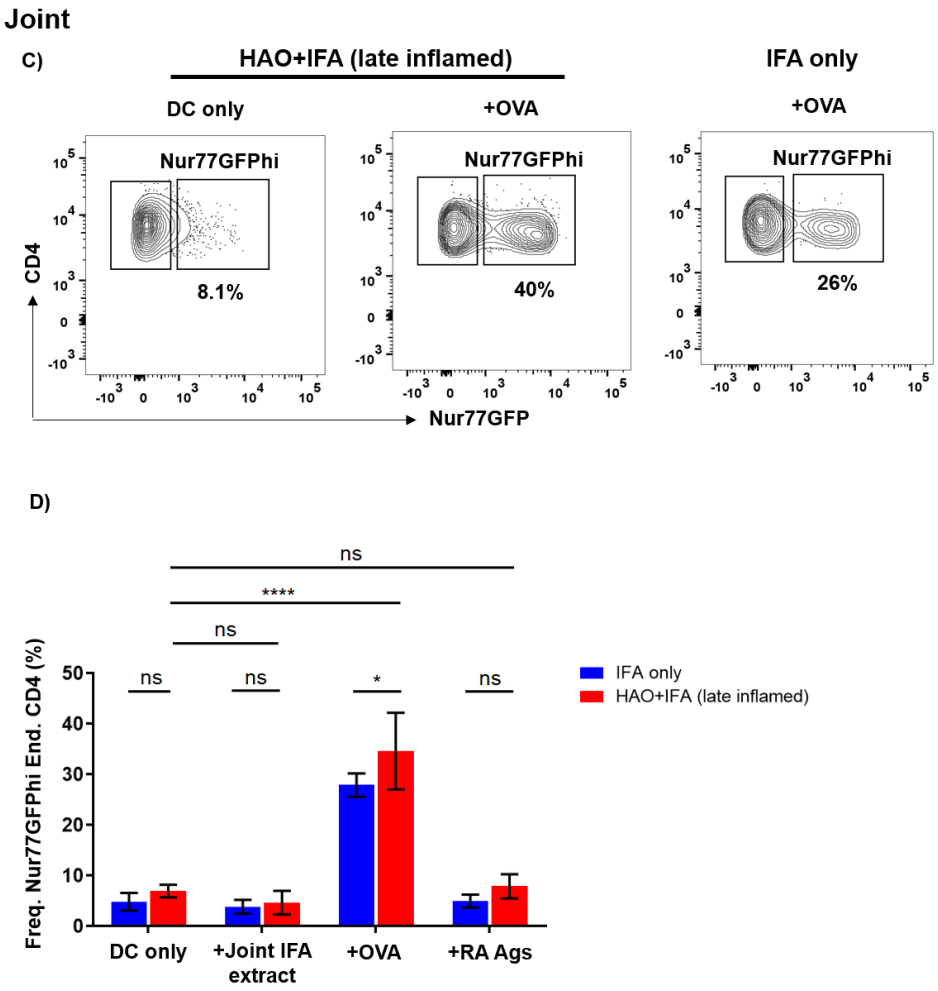
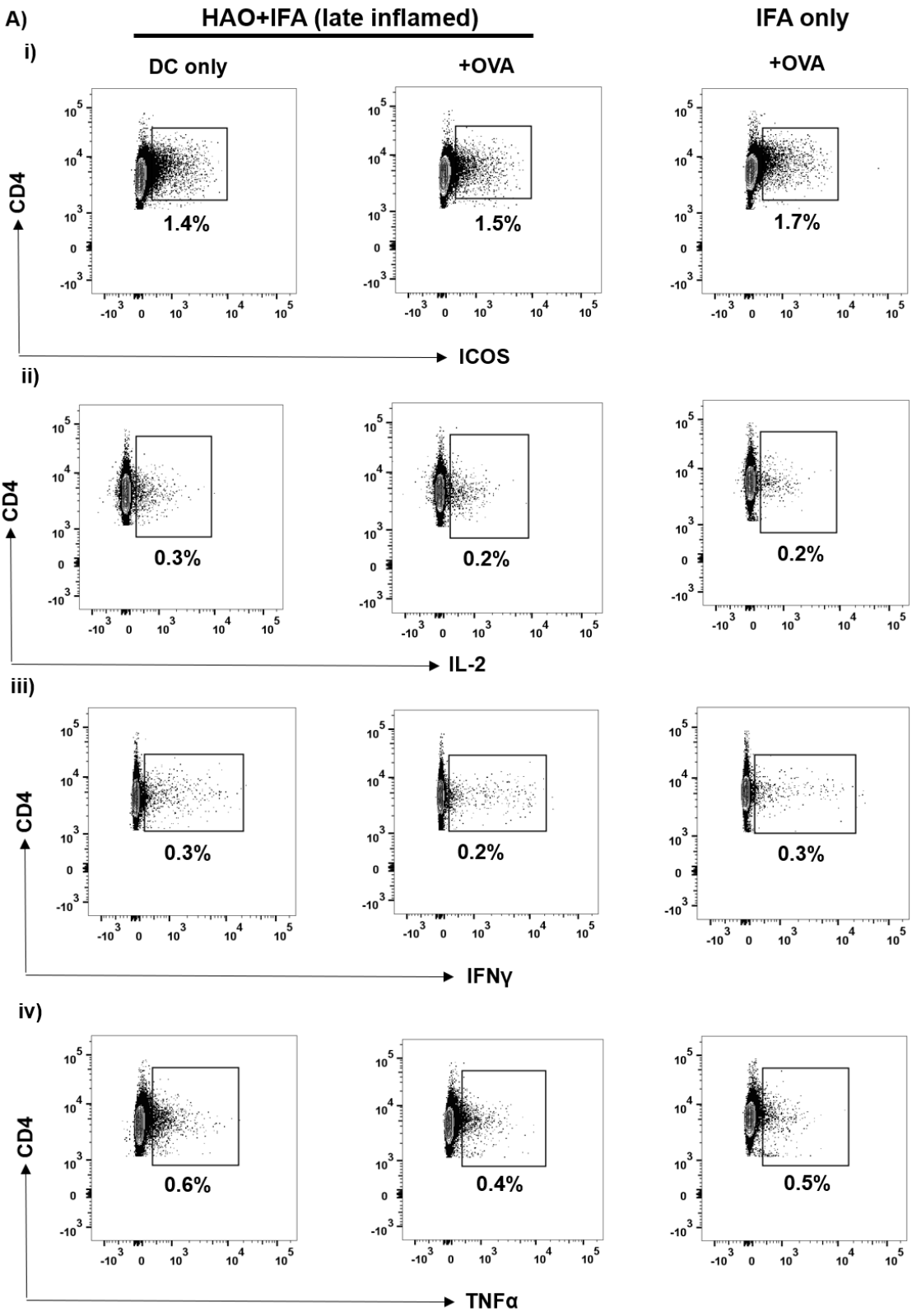


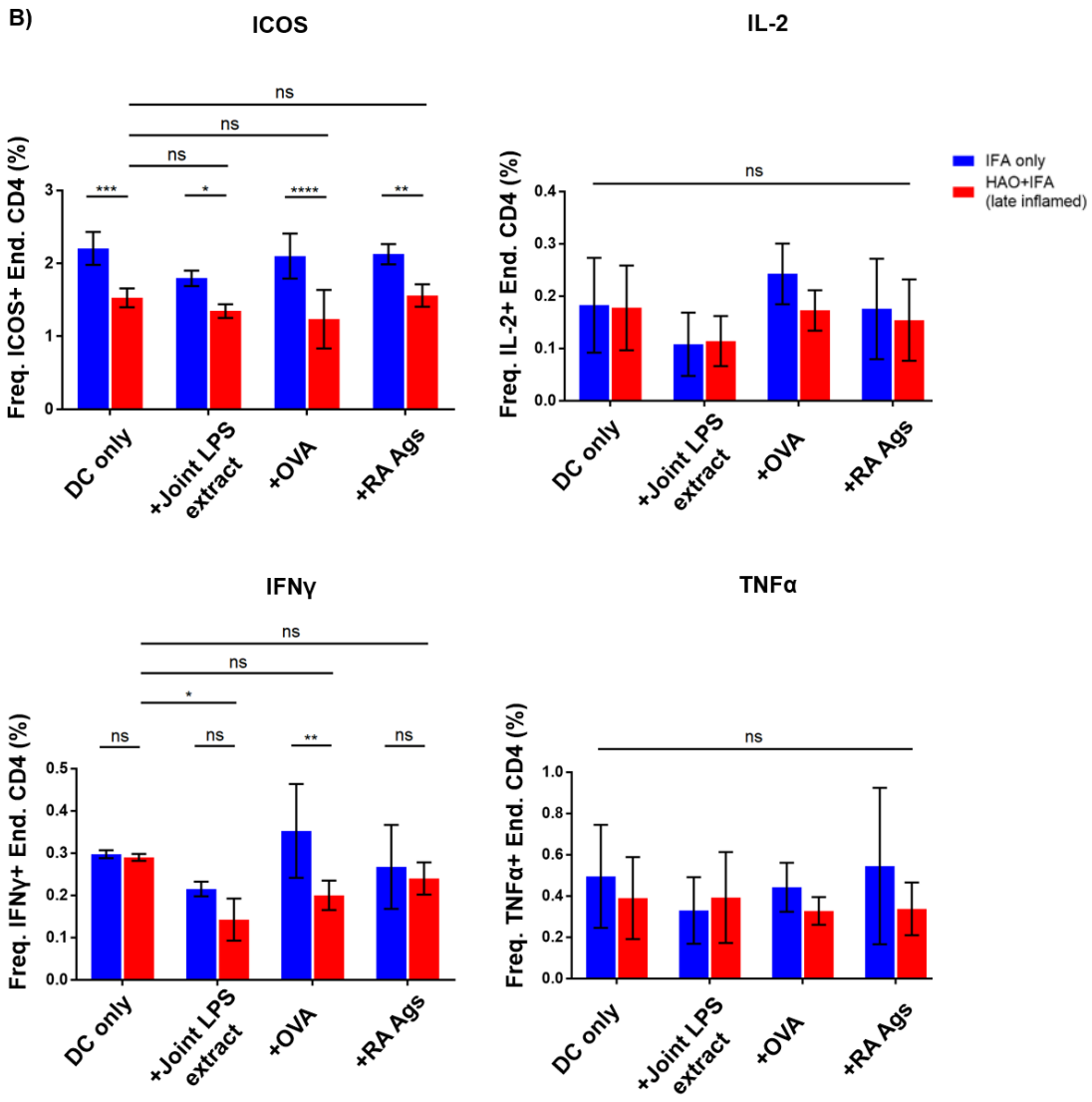
Figure 5-11 Percentage of Nur77GFPhi endogenous CD4+ T cells isolated from pLNs and joints of HAO+IFA (late inflamed) and IFA only challenged mice co-cultured with joint extract, OVA, or candidate RA antigens

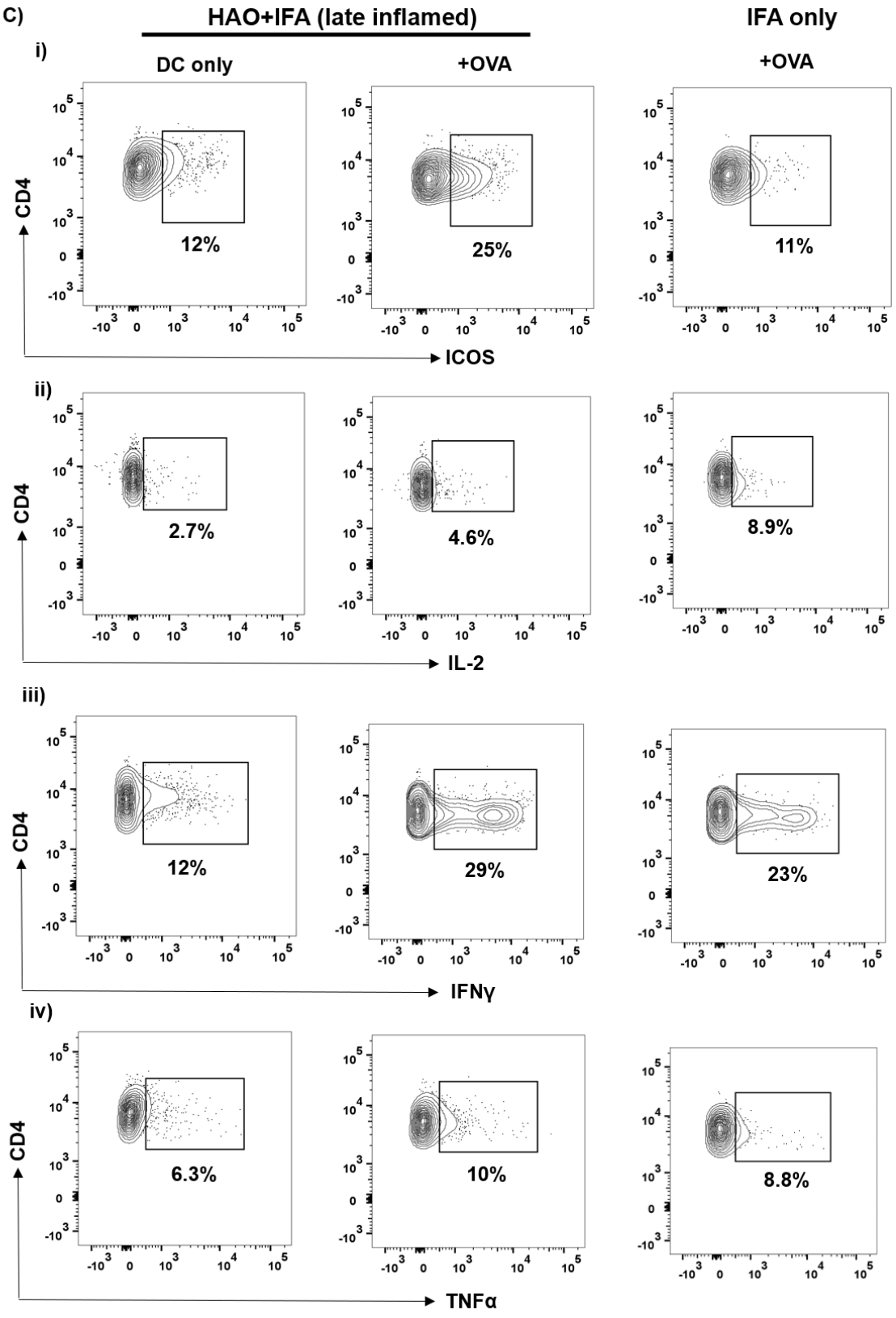
pLNs and joints were collected from HAO+IFA and IFA only challenged mice. CD4+ T cells were purified and co-cultured with BMDCs pulsed with joint extract from IFA challenged mice, OVA, or candidate RA antigens bovine collagen II and bovine aggrecan. Cells were also cultured with non-pulsed BMDCs to determine baseline Nur77GFPhi frequency. BMDCs were pulsed and simultaneously cultured with CD4+ T cells. Golgi plug was added 12 hours after co-culture set up and cells were cultured for a further 6 hours at 37C. Cells were collected and stained for flow cytometry analysis. Cells were cultured in duplicate for each condition. Representative flow cytometry plots of **A)** pLN and **C)** joint Nur77GFPhi endogenous CD4+ T cells isolated from HAO+IFA challenged mice co-cultured with and without OVA, and from IFA challenged mice co-cultured with OVA. Frequency of Nur77GFPhi endogenous CD4+ T cells from **B)** pLNs and **D)** joints for each experimental condition. Graphs represent 2 experiment with n=1 for each experiment (pLNs and joints from 6 and 5 HAO+IFA challenged mice were pooled for the 1st and 2nd experiments respectively and pLNs and joints from 5 IFA challenged mice were pooled for both experiments. Values from each technical replicate plotted). Groups were compared using 2-way ANOVA. Stars represent the following values: * ≤0.05; **** <0.0001; ns: not significant.

Interestingly, endogenous CD4+ T cells from late inflamed pLNs display reduced ICOS expression compared to IFA only controls and showed no overall difference in cytokine expression **(Figure 5-12 A and B)**. However, endogenous CD4+ T cells isolated form the late inflamed joints contrast the pLNs in terms of activation profile **(Figure 5-12 C and D)**; ICOS expression is significantly greater than in IFA only cells, and again, cells co-cultured with OVA have higher ICOS expression compared

to DC only controls. Moreover, IL-2, IFNγ, and TNFα are all increased in late inflamed isolated endogenous CD4+ T cells co-cultured with OVA compared to DC controls. It is interesting to note that IL-2 production decreases when late inflamed endogenous CD4+ T cells are cultured with OVA compared to cells isolated from IFA only joints, which may suggest that these T cells have a reduced proliferative capacity. It is also important to note that the IFA only cells co-cultured with OVA also show increased production of IL-2, IFNγ, and TNFα compared to IFA only DC controls, suggesting that these cells may be memory effector CD4+ T cells. Another method of determining the presence of responses against joint derived antigens is to check for IgG responses against joint antigens such as collagen II, which would indicate the presence of autoreactive collagen II specific endogenous CD4+ T cells providing help to collagen specific B cells to generate class-switched antibodies against collagen II. This method has been used to detect autoreactive responses in the model[140,252,254]. As expected, no differences in the OVA response was observed between the baseline, IFA, and late inflamed mice, as all these mice received immunisations of OVA/CFA. However, the absence of a response against joint antigens was verified in an ELISA against bovine collagen II **(Figure 5-13)** which showed no significant difference in collagen II antibody titres between mice that only received periarticular PBS immunisations (baseline), IFA only and late inflamed mice.


A)
HAO+IFA (late inflamed)
IFA only
i)
DC only
+OVA
+OVA
CD4
1.4%
1.5%
1.7%
ICOS
ii)
CD4
0.3%
0.2%
0.2%
IL-2
iii)
CD4
0.3%
0.2%
0.3%
IFNγ
iv)
CD4
0.6%
0.4%
0.5%
TNFα


B)
ICOS
IL-2
IFNγ
TNFα
Freq. ICOS+ End. CD4 (%)
Freq. IL-2+ End. CD4 (%)
Freq. IFNγ+ End. CD4 (%)
Freq. TNFα+ End. CD4 (%)
IFA only
HAO+IFA (late inflamed)
ns
*
**
***
****
DC only
+Joint LPS extract
+OVA
+RA Ags


C)
HAO+IFA (late inflamed)
IFA only
i)
DC only
+OVA
+OVA
CD4
12%
25%
11%
ICOS
ii)
CD4
2.7%
4.6%
8.9%
IL-2
iii)
CD4
12%
29%
23%
IFNγ
iv)
CD4
6.3%
10%
8.8%
TNFα

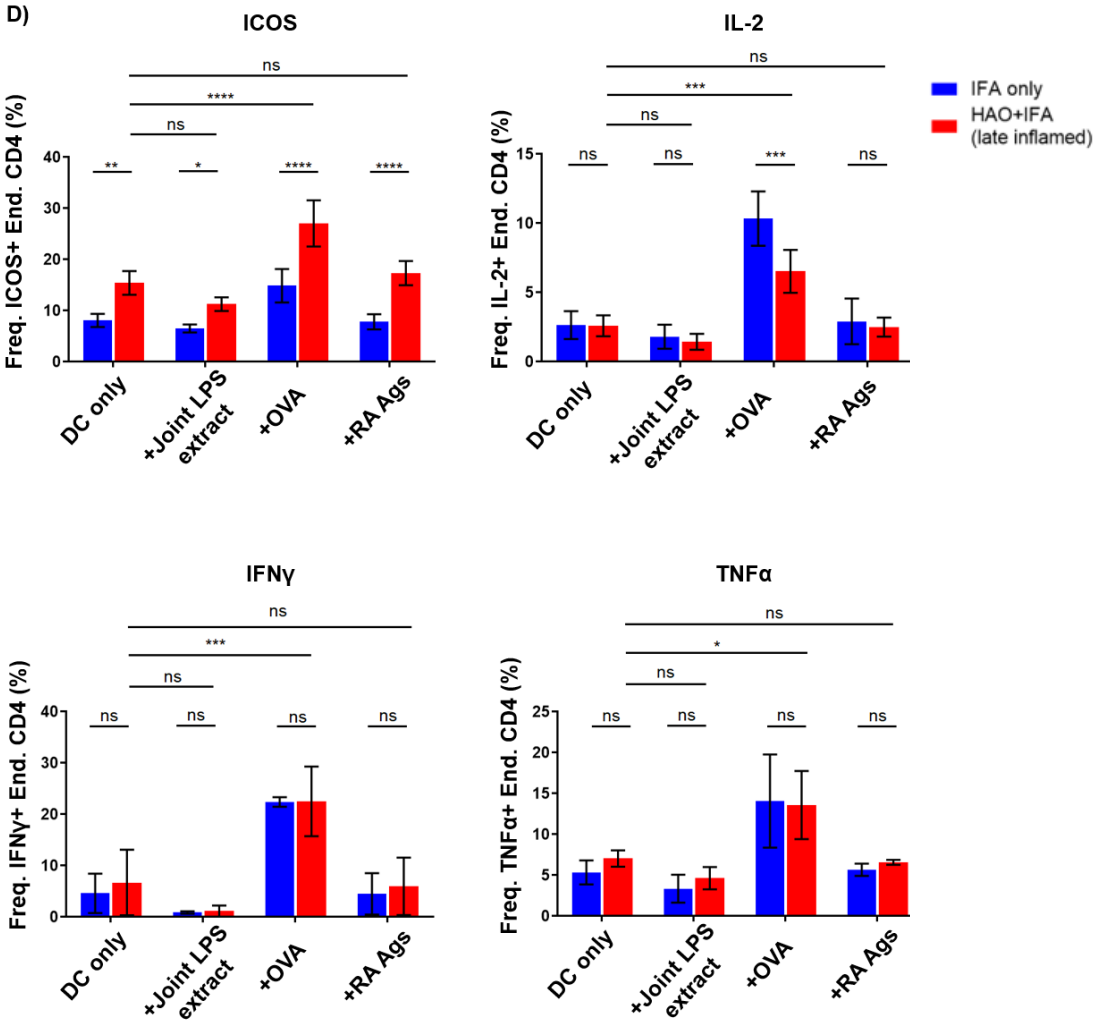


Figure 5-12 Percentage of cytokine producing endogenous CD4+ T cells isolated from pLNs joints of HAO+IFA (late inflamed) and IFA only challenged mice co-cultured with joint extract, OVA, or candidate RA antigens

pLNs and joints were collected from HAO+IFA and IFA only challenged mice. CD4+ T cells were purified and co-cultured with BMDCs pulsed with joint extract from IFA challenged mice, OVA, or candidate RA antigens bovine collagen II and bovine aggrecan. Cells were also cultured with non-pulsed BMDCs to determine baseline cytokine expression. BMDCs were pulsed and simultaneously cultured with CD4+ T cells. Golgi plug was added 12 hours after co-culture set up and cells were cultured for a further 6 hours at 37C. Cells were collected and stained for flow cytometry analysis. Cells were cultured in duplicate for each condition. Representative flow cytometry plots of **A)** pLN and **C)** joint endogenous CD4+ T cells from late inflamed and IFA only challenged mice showing **i)** ICOS, **ii)** IL-2, **iii)** IFNγ, and **iv)** TNFα expression when co-cultured with and without OVA. Frequency of ICOS+, IL-2+, IFNγ+, and TNFα+ in **B)** pLN and **D)** joint endogenous CD4+ T cells from each experimental condition. Graphs represent 2 experiment with n=1 for each experiment (pLNs and joints from 6 and 5 HAO+IFA challenged mice were pooled for the 1st and 2nd experiments respectively and pLNs and joints from 5 IFA challenged mice were pooled for both experiments. Values from each technical replicate plotted). Groups were compared using 2-way ANOVA. Stars represent the following values: * ≤0.05; ** ≤0.01; *** ≤0.001; **** <0.0001; ns: not significant.

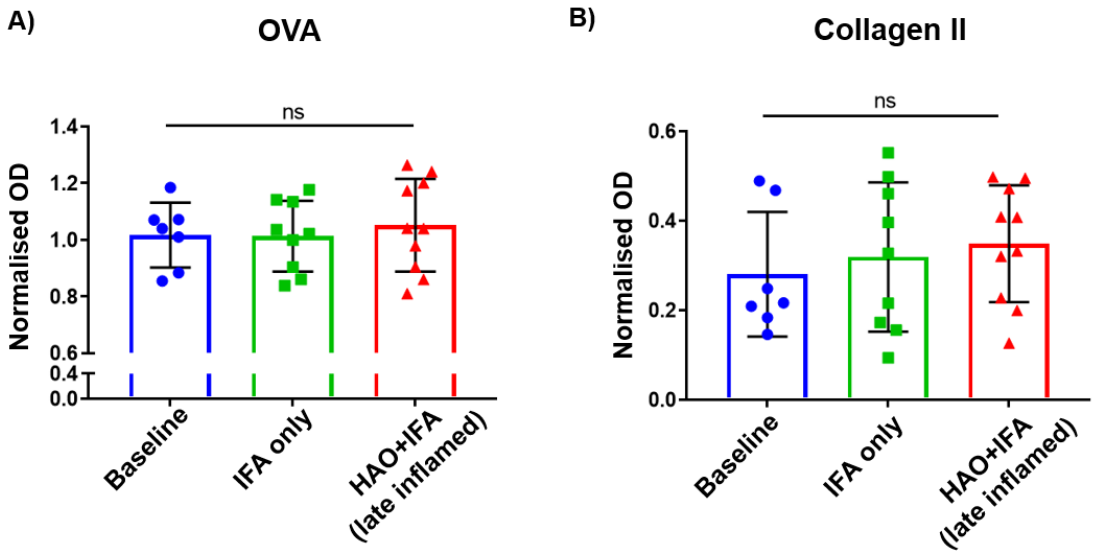


Figure 5-13 OVA and collagen II serum ELISAs of mice undergoing the late model of experimental arthritis
Bloods were collected from mice undergoing the late model of experimental arthritis and sera were isolated from each sample. Serum ELISAs were performed to detect serum antibodies against **A)** chicken ovalbumin (OVA) and **B)** bovine collagen II. Samples were screened in duplicate. Readings were normalised to a standard, averaged per sample, and plotted. Graphs represent 2 experiments with a total of n=9 for the HAO+IFA (late inflamed) and IFA only groups, and n=7 for the baseline group. Groups were compared using one-way ANOVA. ns: not significant.

Overall, the same conclusions can be drawn from the late timepoint data as with the early timepoint data; the endogenous CD4+ T cells do not appear to be responding to the joint derived antigens tested and based on the parameters measured, a significant and higher proportion of these cells responded to OVA at this timepoint than at the early timepoint. However, the contrast in Nur77GFP and cytokine expression between late inflamed pLNs and joints is interesting and indicates that endogenous CD4+ T cells isolated from pLNs could have more of an exhausted phenotype compared to those isolated from the joint and also compared to endogenous CD4+ T cells isolated from early HAO challenged pLNs. This needs to be verified, as no exhaustion markers were included in the flow cytometry panel. These observations again highlight the differences in CD4+ T cell responses between the inflamed tissue and its draining lymph node at different stages of disease as noted in **Chapter 4**.

5.3 Discussion

Evidence for antigen specific responses has been demonstrated in RA[46,201,289]. Despite this, it is contentious as to where autoreactive CD4+ T cells encounter their antigen, especially in the early phases of disease[82]. Moreover, the range of antigens postulated to be involved in RA is large and heterogenous across patients[82,183,295], making it more difficult to pinpoint where autoreactive responses may be occurring and originating from. Nevertheless, the presence of expanded cells in the inflamed arthritic joint[46], the use of synovial fluid extract for RA tolerogenic therapies[244], and behaviour of endogenous CD4+ T cells in the OVA model of experimental arthritis[191], all point to the joint being an important site in which autoreactive CD4+ T cells may encounter their cognate antigen. In this chapter, the Nur77GFP system was used to further probe for evidence of antigen recognition in early phases of disease and determine whether antigen recognition occurs in the joint, specifically in response to joint derived antigens.

The Nur77GFP system was established as an accurate reporter of TCR engagement and T cell activation suitable for evaluating those responses in the early model of experimental arthritis. Nur77GFP expression levels increased upon stimulation with anti-CD3 in a dose dependent manner and correlated with the early activation marker CD69. Moreover, expression of GFP was significantly reduced 48 hours after strong TCR stimulation, which fits well within the timeframe of detecting TCR engagement of endogenous CD4+ T cells after HAO administration, as mice are culled 4 days after periarticular challenge (see methods **section 2.2.5**). This would imply that CD4+ T cells with high levels of Nur77GFP have seen their cognate antigen in the past 48 hours.

A greater number of endogenous CD4+ T cells with high levels of Nur77GFP were purified from HAO challenged pLNs and joints, indicating that these cells had seen their cognate antigen recently in both pLNs and joints. Moreover, the higher frequency of these cells in HAO challenged joints compared to LPS challenged joints verified that these cells are activated due to TCR engagement, complementing the observations in **Chapter 3 section 3.2.2.4**. This increased endogenous Nur77GFPhi CD4+ T cell frequency may in fact reflect the breach of tolerance associated with HAO but not LPS challenge, demonstrated by Nickdel et. al.[254], and may reflect recognition of autoantigens.

Although, an increase in endogenous Nur77GFPhi CD4+ T cells in the joint is not definitive evidence for TCR engagement in the joint itself; having Nur77GFPhi cells indicates that the cells have seen cognate antigen in the past 48 hours, but whether these cells recognised their antigen in the lymph node then migrated to the joint cannot be distinguished from the cells that have seen their cognate antigen in the joint. Several studies have demonstrated that effector T cells can migrate to inflamed sites within a timeframe spanning hours[188] to days[307,308].

Migration of cells between the pLN and inflamed joint in the model has been examined in Kaede mice by members of the lab (unpublished data). Kaede mice developed by Tomura et al express a fluorescent protein that changes from green to red upon exposure to UV light[309], thus allowing one to track cells migrating from the UV exposed site. When the OVA experimental arthritis model was conducted in Kaede mice, approximately 50% of photoconverted cells migrated out of the joint 24 hours after the inflamed joint was exposed to UV light, a small proportion of which were CD4+ T cells (personal correspondence with Dr. Robert Benson). This would suggest that the CD4+ T cells that migrated to the inflamed joint were still retained at the site after 24 hours. Moreover, several studies have shown that antigen recognition impedes T cells egress from the inflamed site[310,311] and a study by Prendergast et. al. has shown that a proportion of endogenous CD4+ T cells in the joint behave in a manner implying antigen recognition[191]. This evidence, together with the increased number of endogenous CD4+ Nur77GFPhi cells in the joint, suggests a strong possibility of endogenous CD4+ T cells recognising their cognate antigen in the joint. Stronger evidence for this phenomenon can be provided by combining a system of cell tracking with a reporter of TCR engagement; a cross between Kaede and Nur77GFP mice. Theoretically this would overcome the issue of identifying where CD4+ T cells have seen their cognate antigen, however, GFP and Kaede green have the same excitation and emission wavelengths making this cross not feasible in practise. This obstacle can be overcome, however, by performing RNA sequencing experiments on endogenous CD4+ T cells retained in the joint and on those that have migrated out of the inflamed joint to the draining lymph node. By performing differential gene expression analysis on genes involved in TCR engagement - including Nur77 - between these two groups, stronger evidence of CD4+ T cells engaging with their cognate antigen in the joint itself can be obtained. Although, a limitation to this type of experiment is recovering cell numbers sufficient for accurate analysis.

In Chapter 3, cells with the Vβ4 TCR were shown to be enriched in the early model of experimental arthritis, be associated with joint antigens, and possibly contain the clones for the progression of the disease. Too few endogenous CD4+ Nur77GFPhi cells were recovered from joints to accurately quantify the number of Vβ4+ CD4+ T cells, and cells isolated from pLNs of HAO challenged mice showed no difference in the number and frequency of cells with the Vβ4, 6, or 13 TCR chains. It was especially unexpected that endogenous CD4+ T cells with the Vβ4 TCR did not show increased Nur77GFP expression as it was postulated that these cells are retained in the joint as a consequence of antigen recognition. This perhaps is a reflection on the mechanism by which endogenous CD4+ T cells are retained in the lymph node and may not need continued antigen stimulation to be retained. The increase in antigen experienced endogenous CD4+ T cells expressing the Vβ4 TCR chain observed in **Chapter 3 section 3.2.2.5** could in fact be a result of cell proliferation which does not necessarily require continued antigen simulation, as demonstrated by proliferation experiments to establish the Nur77GFP re-stimulation assays discussed in **section 5.2.5**. Moreover, Nickdel et. al. also demonstrated that once autoreactive responses are established, they can be perpetuated by non-antigen specific inflammatory stimuli[254]. It is important to note that the observations of Vβ4 endogenous CD4+ T cells found in Nur77GFP mice were isolated from pLNs, and the mechanisms that may explain absence of Nur77GFP upregulation in Vβ4 endogenous CD4+ T cells may not reflect the retention mechanisms in the inflamed joint.

To substantiate recognition of joint antigens by endogenous CD4+ T cells, DC/T cell co-culture experiments with joint extract, OVA, and candidate RA antigens collagen II and aggrecan were conducted with cells isolated from pLNs and joints of mice undergoing the early and late model of experimental arthritis. It is important to note that the co-culture experiments were only performed once with one biological replicate when looking endogenous CD4+ T cell responses at the early timepoint, and twice at the late timepoint, due to the number of mice that needed to be pooled to recover sufficient number of cells. Therefore, the conclusions drawn from these experiments need to be verified with additional experimental repeats.

Endogenous CD4+ T cells isolated from pLNs from HAO challenged mice at the early timepoint had a higher Nur77GFP expression overall and only showed significant increase in GFP expression when cultured with OVA. This increase is

verified as IL-2, IFNγ, and TNFα were also higher when endogenous CD4+ T cells were cultured with OVA. In contrast, endogenous CD4+ T cells isolated from the joint showed no significant increase in Nur77GFP expression nor in cytokine production when cultured with any antigens. However, increased ICOS expression in the T cells co-cultured with joint extract, OVA and candidate RA antigens indicates that these cells are activated and have received co-stimulatory signals from APCs[312], which implies that these cells have in fact seen their cognate antigen, but not necessarily within the 48 hour timeframe that the Nur77GFP system was found to report. The contrast in ICOS and Nur77GFP expression of joint endogenous CD4+ T cells may reflect a limitation of the Nur77GFP system, in that it is only effective for reporting strong TCR stimulation events. Indeed, CD69 seemed to be more sensitive to TCR triggering than Nur77GFP; CD69 expression increased at a lower dose compared to Nur77GFP and increased more rapidly, meaning that it may be more sensitive in reporting T cell activation than the Nur77GFP system. In addition, high background of GFP expression resulting from continued T cell/MHC interactions[306] , especially in endogenous CD4+ T cells isolated from pLN samples, has proved difficult in effectively determining low to medium strength TCR stimulation. As a result, the use of other indicators of activation, such as ICOS expression and production of inflammatory cytokines, was required to corroborate the observed GFP expression. And so, lack of cytokine production from endogenous CD4+ T cells isolated from the joint may in fact corroborate absence of Nur77GFP expression from these cells. It is important to note that in absolute terms the percentage of cytokine producing endogenous CD4+ T cells, cultured with OVA or otherwise, is low ranging from 0.20-0.78% and 1.4-7.9% in pLNs and joints respectively and whether these are biologically relevant values is something to consider. Moreover, this would indicate that the majority of the cells did not in fact respond to OVA or the other antigens tested in the assay and the antigen specificities of the endogenous CD4+ T cells still remains unknown.

Chapter 4 discussed the evolution of the antigen specific response in the model and eluded to differences in endogenous CD4+ TCR diversity between late inflamed pLNs and joints, suggesting a change in the antigens recognised. To investigate whether the development of these differences resulted from endogenous CD4+ T cells recognising and responding to a different set of joint specific antigens, co-culture experiments were also conducted with endogenous CD4+ T cells isolated from mice undergoing the late model of experimental arthritis. Nur77GFP

expression in endogenous CD4+ T cells isolated from late inflamed pLNs showed no significant increase in Nur77GFP expression nor in cytokine production compared to IFA only controls. In fact, whenever there was a significant difference, it was lower compared to the IFA only and DC only controls. The pattern of expression indicates T cell exhaustion which occurs when T cells are overstimulated[313] and has been reported in cases of chronic viral infection[314,315].This would need to be verified by the inclusion of antibodies against exhaustion markers, such as PD-1 and CTLA-4, in flow cytometry panels. In contrast, endogenous CD4+ T cells isolated from both late inflamed and IFA only joints show increased Nur77GFP and ICOS expression, as well as increased IL-2, IFNγ, and TNFα production when co-cultured with OVA pulsed BMDCs. The fact endogenous CD4+ T cells isolated from IFA only joints had a similar or greater percentage of cytokine producing cells indicates that these cells may be OVA specific effector memory T cells which are known to be more sensitive to activation signals and produce high levels of different cytokines[316]. This may explain the difference in activation profiles observed of endogenous CD4+ T cells isolated from pLNs compared to joints at this late timepoint, and also compared to the endogenous CD4+ T cells isolated from the early timepoint.

Although it could not be shown that the cells responded to joint derived antigens tested, the interesting observation is the contrast in responses between the late inflamed pLN and joint, and how that compares to the responses observed at the early timepoint. Endogenous CD4+ T cells isolated from pLNs and joints at both the early and late model timepoints responded to OVA; 12-15% of the cells at the early timepoint and 20-35% at the late timepoint in pLNs and joints respectively. The increase in proportion of cells by the late timepoint may reflect increased proliferation of OVA stimulated cells, which is supported by the increase in IL-2 production compared to CD4+ T cells cultured with non-pulsed BMDCs. Or this increase could be a reflection of epitope spreading with respect to OVA, a phenomenon where cells respond to other sub-dominant epitopes of the protein in addition to the dominant epitope[52].

Again, approximately 30% and 35% of the endogenous CD4+ T cells from IFA only and late inflamed joints respectively responded to OVA. Although this is a substantial increase from endogenous CD4+ T cells that responded at the early

timepoint, the majority of cells present in the joint did not in fact recognise OVA and the antigens these endogenous CD4+ T cells recognise still remains unknown.

In summary, the experiments presented in this chapter aimed to provide evidence for antigen recognition in the early phases of experimental arthritis and to determine whether antigen recognition occurs in the joint and to joint specific antigens. The data demonstrated the possibility of CD4+ T cell antigen recognition in the joint itself and that this antigen recognition may be associated with the breach of tolerance resulting from periarticular administration of HAO. Although a significant percentage of endogenous CD4+ T cells were found to respond to OVA *ex vivo*, the antigen specificities of the majority of the endogenous CD4+ T cells cultured remains unknown, suggesting that these cells may indeed respond to autoantigens. In addition, the data presented elaborates on the evolution of CD4+ T cell clonality observed in the previous results chapter **(Chapter 4)** by providing information on the functional state of the CD4+ T cells in pLNs and joints at different stages of the disease. This again highlights the importance of site specific and temporal differences of antigen specific CD4+ T cell responses during the development of inflammatory arthritis. Moreover, the different functional states of the CD4+ T cells in the pLNs and joints can perhaps inform us of how these cells should be targeted – in their respective tissue sites - in order to effectively evoke the desired immunological responses. This would result in more efficient tolerogenic therapies and a firm step towards drug-free remission.

Chapter 6 General Discussion

Treatments targeting RA have improved drastically over the past two decades, however, a significant proportion of patients still fail to improve or show partial remission to current therapeutics. Moreover, those who do achieve remission are required to take the prescribed medication indefinitely, which is not only impractical and unpleasant for the patient, but also places a large economic burden on society. Consequently, current therapeutic objectives have been to develop more effective therapies to achieve long lasting, drug-free remission for all RA patients. One approach to achieve this is to develop antigen specific tolerogenic therapies to reinstate tolerance to self-antigens, which has shown promise in mouse models of autoimmune diseases as well as in some clinical trials, as discussed in **section 1.5** of the introduction. However, the unknown nature of the antigens recognised, a poor understanding of which CD4+ T cells to target, and the delay between breach of self-tolerance and presentation of clinical symptoms in RA patients have impeded development of effective tolerogenic therapeutics for RA. The current requirement, therefore, is to investigate how antigen specific CD4+ T cell responses develop and evolve with the progression of RA and the role autoreactive CD4+ T cell responses play in shaping the antigen specific response in RA patients. This will further our understanding of the roles autoreactive CD4+ T cells play in RA progression, potentially contributing to the advancement of more effective tolerogenic therapies.

6.1 Summary of key findings

The aims of this study were to investigate the development of antigen specific CD4+ T cell responses after the induction of breach of tolerance, to examine how these responses evolved with disease progression and to determine whether accumulation of CD4+ T cells is a result of breach of tolerance to joint antigens in the joint itself. To achieve this, TCR clonality of CD4+ T cells was examined in the OVA induced breach of tolerance model of experimental arthritis, which allows for the investigation of antigen specific CD4+ T cell responses under conditions where autoreactive responses have been shown to develop. The initial CD4+ T cell infiltrate was found to be oligoclonal in nature, enriched with CD4+ T cells expressing the Vβ4 TCR chain. Moreover, this enrichment was found to be associated with antigen specific responses, specifically implying the contribution of joint derived antigens to the observed repertoire. CDR3β sequencing analysis

highlighted the dynamics of clonal responses between the inflamed joint and its associated draining lymph node and how these responses evolved with the progression of experimental arthritis. Inflamed joints displayed similar CD4+ T cell repertoire diversity at early and late stages of the disease, while inflamed lymph nodes displayed increased repertoire diversity with disease progression. Moreover, the number of CD4+ T cell clones shared between the inflamed joint and lymph node decreased with time. However, correlation analyses of highly abundant clones between inflamed joints and lymph nodes suggested continued migration of CD4+ T cell clones from inflamed lymph nodes to the joints.

To address whether the changes in CD4+ T cell clonality observed was due to antigen recognition in the inflamed joint itself and whether the endogenous CD4+ T cells recognise joint autoantigens, the Nur77GFP reporter mouse was employed. A greater number of Nur77GFPhi endogenous CD4+ T cells were isolated from early inflamed pLNs and joints, which may reflect antigen recognition in the joint, but more specifically, reflect recognition of joint autoantigens as a consequence of the breach of tolerance induced. To verify this, endogenous CD4+ T cells isolated from Nur77GFP mice undergoing the early and late OVA models of experimental arthritis were co-cultured with BMDCs either pulsed with non-antigen induced inflamed joint extract, with OVA, or candidate joint antigens collagen II and aggrecan. At the early timepoint Nur77GFP, ICOS, and cytokine expression was significantly higher in endogenous CD4+ T cells isolated from inflamed pLNs only when cultured with OVA, while no significant increase was observed in CD4+ T cells isolated from inflamed joints when cultured with any antigens. However, the opposite was observed in endogenous CD4+ T cells isolated from inflamed pLNs and joints at the late timepoint; expression of Nur77GFP, ICOS, and cytokines was increased in cells isolated from joints cultured with OVA, while no increase in expression was observed from cells isolated from late inflamed pLNs.

6.2 Development of autoreactive responses in RA

Overall, my results have a number of potential implications for understanding the fundamentals of the development of articular inflammation and potential implications for clinical practice. These are summarised in **Figure 6-1**, which depicts a potential mechanism for the role antigen specific autoreactive responses in initiating and propagating RA. The disease begins with priming or reactivation of autoreactive

CD4+ T cells in joint draining lymph nodes to antigens potentially originating from the joint. The inciting antigen is unlikely to be new and has probably been encountered for a second or third time at this time point, as suggested by Firestein and McInnes[82]. The Th1 polarised OT-II cells used in the model, and the subsequent immunisations with OVA/CFA and HAO, behave as cells primed and reactivated by the inciting antigen in RA. This results in an expansion of oligoclonal CD4+ T cell populations in the lymph node **(Figure 6-1 A)**. Expanded autoreactive CD4+ T cells then migrate to and accumulate in the joint, due to recognition of cognate antigen, and potentially execute their effector functions and cause damage **(Figure 6-1 B)**. This is supported by data obtained at the early timepoint where both Vβ analysis using flow cytometry and CDR3β sequencing analysis of endogenous CD4+ T cells revealed similar CD4+ TCR clonalities between the inflamed pLN and joint. Moreover, the seeming lack of correlation between expanded clones in the early inflamed pLN compared with the joint could indicate that a small number of autoreactive clones that have breached self-tolerance initiate the damage inducing proinflammatory responses. The expansion and migration of autoreactive CD4+ T cells from the lymph node to the joint would eventually lead to increased joint damage and the subsequent release of neo antigens, and/or cause epitope spreading to the primary inciting antigen(s) due to perpetuation of proinflammatory responses. This would further prime other autoreactive CD4+ T cells in the lymph node, changing the CD4+ T cell diversity and repertoire clonality in the lymph node **(Figure 6-1 C)**. This process can be postulated when analysing data obtained from the late inflamed timepoint as both Vβ and CDR3β analysis revealed changes in endogenous CD4+ T cell clonality; Vβ analysis showed that the clonality of endogenous CD4+ T cells isolated from late inflamed pLNs displayed a “switch” in clonality compared to those isolated from early inflamed pLNs, with endogenous CD4+ T cells with Vβ6 TCR chain dominating the response compared to Vβ4 in early inflamed pLNs. In addition, CDR3β sequencing analysis clearly demonstrated that late inflamed pLNs had highly diverse CD4+ T cell repertoires composed of a large number of clones present in low frequencies compared to early inflamed pLNs. Simultaneously, CD4+ T cells from the initial wave of migrating cells continue to migrate and be retained in the joint in an antigen dependent manner and/or expand locally in the joint due to antigen recognition. This is indicated by the increasing numbers of Vβ4 endogenous CD4+ T cells in the joint at the late timepoint as well as correlation data at the late timepoint, suggesting continued migration of CD4+ T

cells from late inflamed pLNs to late inflamed joints. This phenomenon is further highlighted when considering the endogenous CD4+ T cell/BMDC co-culture data showing a large increase in OVA specific endogenous CD4+ T cells at the late timepoint. Both mechanisms of continued migration of cells responding to the primary inciting antigen(s) and local expansion of these cells in the inflamed joint would cause an overall increase in the number of CD4+ T cells in the joint while maintaining the clonality observed in the earlier phases of the disease. As the disease progresses, autoreactive CD4+ T cells of different clonalities that have been primed in the lymph node eventually migrate to the joint, thus mirroring the increased diversity and clonality of the lymph node CD4+ T cell repertoire **(Figure 6-1 D)**. Although this final step was not observed in the experiments presented due to the resolving nature of the model, Klarenbeek et. al[46] demonstrated a reduced number of highly expanded CD4+ T cell clones in synovia of patients with established RA compared to those classified as early RA patients, implying an increase in the CD4+ T cell clonal and repertoire diversity. An increase in TCR diversity with disease severity was also reported by Bucht et. al[317], reiterating that CD4+ T cell repertoire diversity in the joint may eventually reflect the diversity observed in the lymph node.

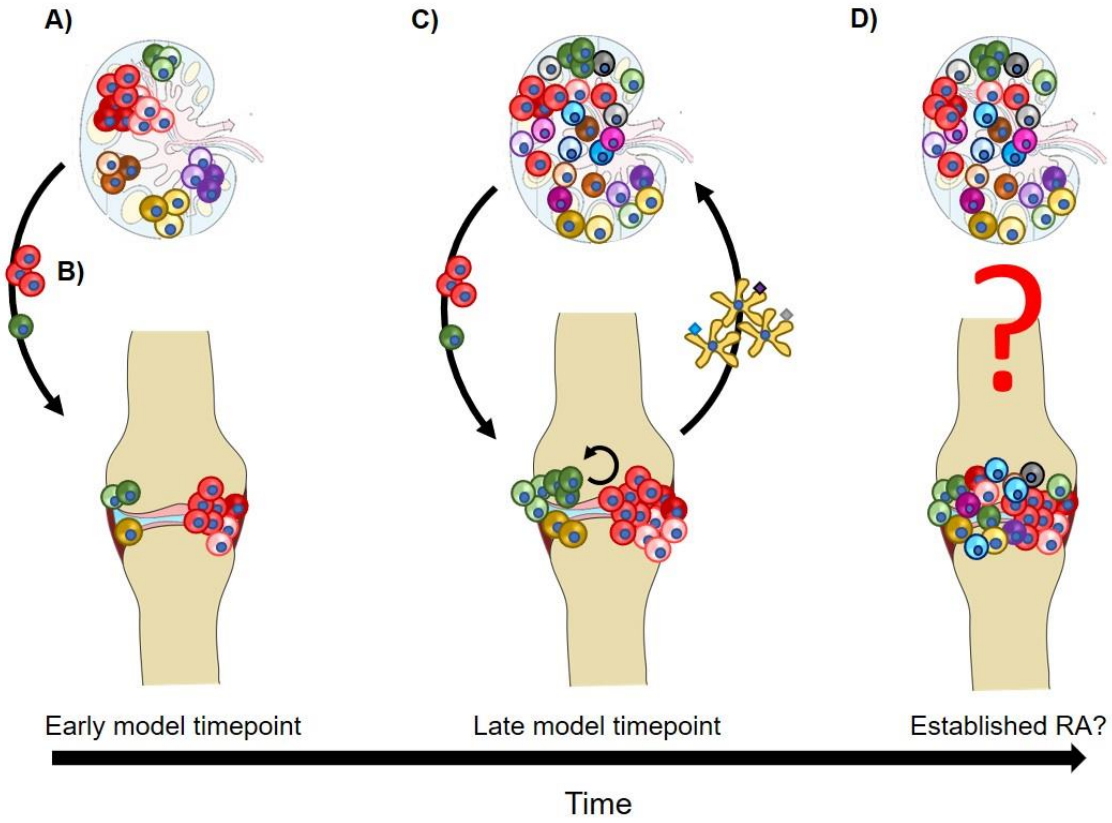


Figure 6-1 Development of the antigen specific CD4+ T cell response in RA.
Schematic representing potential mechanism of the development of antigen specific CD4+ T cell responses in RA. **A)** Autoreactive CD4+ T cells are primed or reactivated in the lymph node by an unknown antigen. This antigen is unlikely to be new, and is likely to have been encountered a 2nd or 3rd time at this point, as suggested by Firestein and Mcinnes[82]. **B)** Expanded autoreactive CD4+ T cells then migrate to the joint and are accumulate there by antigen induced retention mechanisms, increased survival, and/or proliferate in the joint itself due to antigen recognition. The CD4+ T cells possibly execute their effector function in the joint and perpetuate joint damage. **C)** Increased joint damage results in the release of neo antigens, or cause epitope spreading to the primary inciting antigen(s) due to the perpetuation of proinflammatory responses, which further primes other autoreactive CD4+ T cells in the lymph node. This causes a change in the CD4+ TCR clonality and diversity in the lymph node. Simultaneously, CD4+ T cells from the initial wave of migrating cells continue to migrate and be retained in the joint in an antigen dependent manner and/or expand locally in the joint due to antigen recognition. **D)** As the disease progresses, autoreactive CD4+ T cells of different clonalities that have been primed in the lymph node then migrate to the joint, thus mirroring the increased diversity and clonality of the lymph node CD4+ T cell repertoire, resulting in a more polyclonal CD4+ T cell repertoire in the joint.

6.3 Implication for the development of antigen specific therapies

These results suggest that antigen specific therapies would be best targeted to autoreactive CD4+ T cells in joint draining lymph nodes as quickly as possible after diagnosis to prevent the development of a polyclonal CD4+ T cell repertoire, which may be indicative of epitope spreading to other epitopes of the protein from which the primary inciting antigen was derived, and/or priming of autoreactive CD4+ T cells to neo self-antigens originating from different proteins. Targeting pathogenic CD4+

T cells early would curb development of autoreactive responses and subsequent damage to joint tissue. There are several hurdles to implementing this approach in humans however, one of which is identifying at-risk patients who would benefit from antigen specific tolerogenic therapies. Currently, at-risk patients are identified as those who have increased levels of ACPA and RF without clinical signs of joint damage[318–320]. However, using these antibodies as biomarkers for RA prognosis is unreliable and controversial as patients display differing antibody responses that cannot be correlated to disease activity[319]. Thus more reliable biomarkers to stratify at-risk patients are required as this would better identify where along the at-risk phase patients stand in order to provide more effective antigen specific therapies tailored for that individual. Perhaps a biomarker relying on CD4+ T cell clonality may be more effective in identifying where on the "at-risk spectrum" these patients stand. Given the results of this research, observing changes in CD4+ TCR clonality in human inguinal lymph nodes would be a good place to start monitoring changes in CD4+ T cell clonality and how that correlates with disease activity. Ultrasound guided lymph node core biopsies have been as diagnostic tools for lymphomas for over 20 years[321] and have also been evaluated for use in RA as a method for monitoring disease progression[322]. In a study by Rodriguez-Carrio et. al[139] RA patients and at-risk individuals who were classified as those with significant titres of IgM RF and ACPA, but who do not display any clinical symptoms of RA, had their inguinal lymph nodes screened for changes in ILC subsets. They found that ILC profiles of at-risk individuals and early RA patients displayed more of an inflammatory phenotype compared with healthy controls. The results of these studies indicate two important things: firstly, it is feasible to screen lymph nodes of at-risk, pre-RA patients for cellular changes. Secondly, the lymph node does in fact change in at-risk individuals before any clinical symptoms are manifest. It would be interesting to monitor changes in CD4+ T cell clonality in LNs of at-risk patients, as well as in LNs and joints of early and established RA patients and determine whether the changes in CD4+ T cell clonality mirror those observed in this study. Moreover, monitoring changes in clonality can also be used to determine a patient's response to therapeutics and to determine whether current therapeutics can "reset" the CD4+ TCR clonality to reflect the clonality observed at earlier stages of disease, as depicted in **Figure 6-1 A**. This is beneficial as the application of antigen specific therapies would more likely be easier at a stage where the CD4+ T cell response is oligoclonal, increasing the chances of targeting the dominant autoreactive CD4+ T

cell population and prevent subsequent damage possibly caused by the migration of these cells to the joint where they can execute their effector functions.

This brings us to the next hurdle of applying antigen specific therapies which is knowing the antigens the autoreactive CD4+ T cells respond to in order to target them with antigen specific therapies. In an effort to identify what antigens the endogenous CD4+ T cells recognise, I attempted to generate CD4+ T cell hybridomas from endogenous CD4+ T cells isolated from pLNs and joints of Nur77GFP mice undergoing the OVA model of experimental arthritis, with the aim of using the Nur77GFP reporter to identify when a TCR bound to its cognate antigen. By having a library of endogenous CD4+ T cells isolated from inflamed joints, numerous peptides can be screened without the hindrance of having a limited number of cells for screening, as hybridomas can be maintained indefinitely. Unfortunately, attempts to generate these hybridoma cell lines failed. However, progress has been made in identifying antigens of unknown TCRs. Studies by Birnbaum et. al[323] and Gee et. al[324] used yeast-display of peptide/HLA libraries to identify cross-reacting peptides of known TCRs and identify antigens of “orphan” TCRs from tumour infiltrating lymphocytes. In addition, this method was used by Salingrama et. al[325] to identify antigens recognised by a CD8+ T cell population from mice undergoing the EAE model, which when immunised with the synthetic peptide, resulted in the expansion of the regulatory CD8+ T cells, resulting in resistance to EAE[325]. An important point raised by this study is the fact that the protein from which these antigens were derived was never identified, but synthetic peptides were used to target a specific clonal population of T cells. This would be particularly beneficial for RA as antigens priming autoreactive CD4+ T cells have been difficult to identify and are also likely to be different across patients. Designing synthetic peptides for RA tolerogenic therapies would therefore overcome the necessity in knowing the peptide for targeting the autoreactive CD4+ T cells. Moreover, synthetic peptides can be modulated to bind to the TCR with different affinities and can potentially be tailored to deliver the desired response. An example of the use of an artificial peptide to treat an autoimmune disease is glatiramer acetate (GA) - a multimer with the same amino acid sequence as an MBP epitope - which is a first line therapy for relapsing remitting MS. GA was found to modulate the immune response from a Th1 to a Th2 driven response via inhibition of pro-inflammatory responses from DCs[326,327].

6.4 The function of endogenous CD4+ T cells in early arthritis

Changes in CD4+ TCR repertoire clonality were observed in this study, however, this research raises questions about the function of the endogenous CD4+ T cells accumulating in inflamed joints. Growing evidence suggests the presence of an antagonistic Th1-Th17 relationship in RA patients as well as in mouse models of RA[155–158], with some suggesting that Th1 cells may in fact regulate pro inflammatory Th17 responses in RA. In addition, unpublished work by Clay et. al. (personal communication) demonstrated reciprocal dynamics of Th17 and Th1 responses, governed by different Treg subsets, correlated with the progression of a model of colitis. Thus, changes in CD4+ TCR clonality may in fact be a reflection of changing functional responses and reflect regulation rather than the development of pro-inflammatory responses. Without understanding the function of CD4+ T cells in the inflamed joint and with no knowledge of the phenotype of expanding CD4+ T cells in the lymph node, therapies targeting these cells may exacerbate rather than ameliorate symptoms. Indeed, a study by Saligrama et. al[325] demonstrated the development of regulatory CD8+ responses as a result of induction of autoreactive CD4+ T cells responses in the EAE model. Moreover, Treg populations have been identified in RA patients[177–179], suggesting that regulatory responses do develop to control disease progression.

Another consideration is that cells with the same TCR can result in the generation of cells with heterogenous functional phenotypes. Indeed, a study by Gerlach et. al[328] demonstrated that individual naïve CD8+ T cells yield both effector and memory T cell subsets. Moreover, in a different study, Gerlach et. al[329] also demonstrated how individual cells from a monoclonal population displayed varied capacities for expansion, differentiation, and recall during an infection. In addition, studies published from Steven L Reiner's lab have shown evidence of asymmetric CD8+ T cell division upon pathogen re-encounter and demonstrated the asymmetric distribution of CD25 and Tbet during cell division[330,331], suggesting that asymmetric division of a single cell may give rise to progeny with different effector functions. Further evidence of the "one cell, multiple fates" theory has been provided by Becattini et. al[332]. who demonstrated that a monoclonal population of human naïve CD4+ T cells can produce Th1, Th2, and Th17 CD4+ T cell subsets.

And so, experiments that simultaneously look at T cell clonality and function are required to better understand the relationship between changing CD4+ TCR repertoire clonality and the resulting effector responses. With the advent of single cell RNA sequencing technologies, such experiments are feasible and have been conducted to analyse clonality and function of T cells isolated from colorectal cancer patients[333]. Single cell RNA sequencing has also been conducted on cells isolated from RA synovia[195,334], however, neither of these studies looked at T cell clonality, nor did they focus on CD4+ T cells – an important aspect to consider for a more complete representation of the CD4+ T cell repertoire. Thus, the method developed by Han et. al[333],which was optimised to specifically amplify TCR sequences and key gene transcripts relevant to T cell function and specific T cell subtypes, could be adapted to investigate the effector function of clonally expanded CD4+ T cells in inflamed joints and pLNs in the model, as well as in arthritic joints and lymph nodes in humans. The advantage of such experiments is that they can also address the function of bystander CD4+ T cells in arthritic lymph nodes and joints and dissect the contribution antigen specific and bystander CD4+ T cells to the inflammatory environment and to tissue damage. Indeed, Prendergast et. al[191] showed that although a subset of the endogenous CD4+ T cell population in the OVA model of experimental arthritis displayed behaviour indicative of antigen recognition, the remainder of the endogenous CD4+ T cells displayed scanning behaviour. In the same study, Prendergast et. al. also demonstrated that activated CD4+ T cells of known antigen specificity irrelevant to joint antigens and to OVA infiltrated the joint. Additionally, data presented in **Chapter 5** showed that the average frequency of Nur77GFPhi CD4+ T cells is approximately 15%, consistent with the data presented by Prendergast et. al. Why these bystander cells infiltrate the joint and how they contribute to the development of inflammatory responses would indeed be an interesting question to ask and would provide a more complete picture of the development of CD4+ T cell responses in arthritic joints.

6.5 Final conclusions

The research presented in this thesis provides a detailed examination of the evolution of CD4+ T cell clonality and antigen specific responses in the OVA induced breach of tolerance model of experimental arthritis and how this may emulate development of antigen specific responses in the early phases of RA. In addition, the results presented highlight the important differences in the diversity of site

specific and temporal responses during disease. Further work is warranted to understand the function of these autoreactive CD4+ T cells in joints and LNs of RA patients, which will ultimately guide the development of more effective antigen specific tolerogenic therapies for RA and bring treatments closer to providing drug-free remission.

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