THE FUNCTIONAL CHARACTERIZATION OF INVARIANT NATURAL KILLER T CELLS AND THE CHEMOKINE RECEPTOR CXCR6 IN A MOUSE MODEL OF INFLAMMATORY ARTHRITIS

by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Rheumatoid arthritis (RA) is an autoimmune disease characterized by inflammation and an influx of immune cells, leading to irreversible joint damage. The chemokine receptor CXCR6 is highly expressed on T lymphocytes isolated from the synovium of patients with inflammatory arthritis, and the chemokine CXCL16 is present at elevated levels in both soluble and transmembrane forms. This suggests that CXCR6 and CXCL16 regulate T cell activation or infiltration in arthritic joints. This study investigates the role of CXCR6 in arthritis development by examining its effects on T cell activation and infiltration into the arthritic joints in collagen-induced arthritis (CIA), an animal model of RA. Similar to the inflamed joints in arthritis patients, the numbers of $CXCR6^{+}$ T cells were increased in the inflamed paws of arthritic mice. The incidence of arthritis and severity of disease were significantly reduced in CXCR6-deficient ($CXCR6^{-/-}$) or $CXCL16^{-/-}$ mice compared to wild-type mice. T cells from $CXCR6^{-/-}$ mice exhibited reduced RANKL expression and impaired proinflammatory cytokine polarization, resulting in decreased IL-17A– and IFN-γ-producing cells. T cell accumulation within the arthritic paws was reduced in $CXCR6^{-/-}$ mice and homing of activated $CXCR6^{-/-}$ T cells to the inflamed paws was impaired compared to recruitment of wild-type T cells. Therefore, CXCR6-CXCL16 interactions play an important role in regulating effector T cell responses and represent promising therapeutic targets in inflammatory arthritis. Invariant natural killer T (iNKT) cells are an important immunoregulatory T cell subset that express high levels of CXCR6. Defects in iNKT cell populations in RA patients suggest they may play an important role in inflammatory arthritis. Invariant NKT cells were increased in the tissues of wild-type CIA mice, whereas iNKT cell-deficient $Jα18^{-/-}$ mice were resistant to disease development. $Jα18^{-/-}$ mice were reconstituted with iNKT cells from wild-type and gene targeted mice to examine the role of iNKT cell-derived proinflammatory cytokines in CIA. Adoptive transfer of wild-type iNKT cells, but not IFN-γ– or TNF-deficient iNKT cells, restored arthritis susceptibility in $Jα18^{-/-}$ mice. Therefore, iNKT cell-targeted treatment strategies that harness the immunoregulatory potential of iNKT cells may be of therapeutic benefit in RA.

LIST OF ABBREVIATIONS USED

α-GalCer	α-galactosylceramide
ACPA	antibodies to citrullinated protein antigen
ACR	American College of Rheumatology
ADAM-10	a disintegrin and metalloproteinase 10
AIA	antigen-induced arthritis
AIRE	autoimmune regulator
ANOVA	analysis of variance
APC	antigen-presenting cell or allophycocyanin
BCA-1	B cell-attracting chemokine-1
BMDC	bone marrow-derived dendritic cell
C5	complement component 5
CAIA	collagen antibody-induced arthritis
CCP	cyclic citrullinated peptide
CCR	CC chemokine receptor
CD	cluster of differentiation
CFA	complete Freund's adjuvant
CIA	collagen-induced arthritis
CII	collagen type II
CKR	chemokine receptor
CMFDA	5-chloromethylfluorescein diacetate
CMTMR	5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine
ConA	concanavalin A
CRP	C reactive protein
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
CXCR	CXC chemokine receptor
D0/14/35	day 0/14/35
DC	dendritic cell
DMARD	disease-modifying antirheumatic drug

DN	double negative
DTH	delayed-type hypersensitivity
EAE	experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
EDTA	ethylenediamine-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FasL	Fas ligand
FBS	fetal bovine serum
FcγR	Fcγ receptor
FITC	fluorescein isothiocyanate
FoxP3	forkhead box P3
FLS	fibroblast-like synoviocytes
G6PI	glucose-6-phosphate isomerase
GATA-3	GATA binding protein 3
GFP	green fluorescent protein
GM-CSF	granulocyte macrophage colony-stimulating factor
gMFI	geometric mean fluorescence intensity
GPCR	G protein coupled receptor
HLA	human leukocyte antigen
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	human immunodeficiency virus
HRP	horseradish peroxidase
HSV-2	herpes simplex virus-2
ICAM-1	intercellular adhesion molecule 1
ICOS	inducible T cell costimulator
IDO	indoleamine 2,3-dioxygenase
IFA	incomplete Freund's adjuvant
IFN-γ	interferon-γ
Ig	immunoglobulin
IL	interleukin
IL-4R	IL-4R

IL-17RA	IL-17 receptor A
iNKT	invariant NKT
IP-10	interferon-γ-induced protein-10
IRAK	interleukin-1 receptor-associated kinase
IRF	interferon regulatory factor
I-TAC	interferon-inducible T-cell α-chemoattractant
i.v.	intravenous
JAK	Janus kinase
JIA	juvenile idiopathic arthritis
LAG-3	lymphocyte-activation gene 3
mBSA	methylated bovine serum albumin
MHC	major histocompatibility complex
MIG	monokine induced by γ-interferon
MIP	macrophage inflammatory protein
MMP	matrix metalloproteinase
MS	multiple sclerosis
MyD88	myeloid differentiation primary response gene 88
NF-κB	nuclear factor κ-light-chain-enhancer of activated B cells
NLRP3	NOD-like receptor family, pyrin domain-containing protein 3
NOD2	nucleotide-binding oligomerization domain-containing protein 2
NK	natural killer
NKT	natural killer T
PADI4	peptidyl arginine deiminase 4
PBS	phosphate buffered saline
PE	phycoerythrin
PerCP	peridinin chlorophyll protein
PGIA	proteoglycan-induced arthritis
PI3K	phosphatidylinositol-3-kinase
PMA	phorbol 12-myristate 13-acetate
PTPN22	protein tyrosine phosphatase, non-receptor type 22
PTPRC	protein tyrosine phosphatase, receptor type C

r^2	square of the sample correlation coefficient
RA	rheumatoid arthritis
RAG	recombination activating genes
RANK	receptor activator of nuclear factor κB
RANKL	receptor activator of nuclear factor κB ligand
RANTES	regulated on activation, normal T cell expressed and secreted
RORγτ	retinoic acid-related orphan receptor γτ
RPMI	Roswell Park Memorial Institute
RF	rheumatoid factor
SAP	SLAM-associated protein
SCID	severe combined immunodeficient
SCW	streptococcal cell wall
SDF-1	stromal cell-derived factor-1
SEM	standard error of the mean
SLAM	signaling lymphocytic activation molecule
SLC	secondary lymphoid-tissue chemokine
SLE	systemic lupus erythematosus
STAT	signal transducer and activator of transcription
T1D	type 1 diabetes
T-bet	T box expressed in T cells
Tc1	T cytotoxic type 1
TCR	T cell receptor
Th1/2/17	T helper type 1/2/17
TGF-β	transforming growth factor β
TLR	Toll-like receptor
TMB	tetramethylbenzidine
TNF	tumor necrosis factor
TNFAIP3	tumor necrosis factor alpha-induced protein 3
TRAF	tumor necrosis factor receptor associated factor
Treg	T regulatory cell
VEGF	vascular endothelial growth factor

XLP	X-linked lymphoproliferative disease
ZAP-70	zeta-associated protein of 70 kDa

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CHAPTER 1: INTRODUCTION

1.1.1 Autoimmune diseases

The immune system has evolved to protect the host against invading microbes, including bacteria, viruses, fungi and parasites, but also plays important roles in wound healing and tumor control. During development, cells of the immune system are 'educated' to discriminate between self and non-self, allowing these cells to recognize and respond to microbial invaders while limiting damage to healthy tissues. However, in some cases those proinflammatory responses and/or the mechanisms responsible for the resolution of inflammation become dysregulated, leading to inappropriate inflammatory reactions that contribute to tissue damage and dysfunction associated with autoimmunity. Autoimmune diseases affect at least 3% of the population in the United States (1,2), which may be viewed as a conservative estimate since it is derived from data on only 24 specific autoimmune diseases and clinical diagnoses of many autoimmune diseases have improved considerably since these data were collected.

Autoimmune disorders are classified into two types, organ-specific and non-organ-specific/systemic. Examples of organ-specific autoimmune disorders include multiple sclerosis (MS), a demyelinating disease targeting the axons of nerve cells in the brain and spinal cord, type 1 diabetes mellitus (T1D), due to destruction of insulin-producing beta cells in the pancreas, and the autoimmune thyroid diseases, Hashimoto's thyroiditis and Graves' disease. Systemic autoimmune disorders include rheumatoid arthritis (RA), a disease causing inflammation in the joints; systemic lupus erythematosus (SLE), in which the skin, joints, kidneys, heart, and other organs are affected; Sjogren's syndrome, a disease affecting mucous membranes, specifically the salivary and lacrimal glands; and systemic sclerosis, a connective tissue disease characterized by collagen accumulation and a thickening of the skin. The clinical symptoms characteristic of each of these autoimmune disorders have been thoroughly described but the underlying mechanisms that contribute to the pathophysiology associated with these conditions remain poorly understood. Indeed, major questions remain regarding the precise initiating factors in patients suffering from autoimmune disorders. It is suggested that multiple factors play roles in initiating autoimmune disease, including genetic predisposition and

environmental factors such as exposure to viruses, environmental toxins, and certain drugs. Even stress, poor diet and lack of exercise, which are appreciated to affect the proper functioning of the immune system, have been implicated in disease susceptibility. In this thesis, I explore some of the immunological mechanisms that contribute to the development and/or progression of inflammation in an animal model of RA. I focus on the contributions of the chemokine receptor CXCR6 to T cell proinflammatory cytokine production and homing to the inflamed joints as well as the role of invariant natural killer T (iNKT) cells in the development of collagen-induced arthritis (CIA) in mice.

1.1.2 Rheumatoid Arthritis

RA is a chronic autoimmune disease that affects approximately 0.2-1% of the population worldwide (3). Patients with RA experience pain and swelling of multiple joints within the body, and if not properly managed this disease may lead to irreversible damage to articular cartilage and bones (4). Despite early treatment, some patients may still develop erosive disease leading to substantial joint damage, often associated with the presence of several clinical disease activity indicators such as autoantibody production [rheumatoid factor (RF) and/or antibodies to citrullinated protein antigens (ACPAs)/anti-cyclic citrullinated peptide (anti-CCP) antibodies], and high circulating C reactive protein (CRP) levels (5). Additional extra-articular disease manifestations in RA patients may include inflammation in the lungs, pericardium, sclera, and other tissues in the body (6). These systemic complications remain a major challenge and contribute to a higher mortality rate among patients with RA than among healthy individuals (7). Other important complications of RA include fatigue, anaemia, and flu-like symptoms in addition to the characteristic joint deformity seen in patients with this disease. This leads to disability and in some cases, the need for orthopedic surgery (8). Patients with RA frequently exhibit symmetrical joint involvement, often within the small joints, which has an immense impact on hand and foot function and poses therapeutic challenges that cannot easily be overcome by joint replacement surgery (9). Presently there are no curative interventions available for RA patients. Furthermore, subsets of patients show poor clinical responses to many of the currently approved therapies and many of these established treatment strategies also have significant side effects. Therefore it is important

to further elucidate the underlying mechanisms involved in RA pathogenesis in order to provide insight for the development of new therapeutics targeting these disease pathways.

1.1.3 Genetic and environmental factors associated with RA

A number of studies have reported on the familial risks and heritability of RA, suggesting genetic factors are strongly associated with disease susceptibility. For example, siblings of RA patients have a 2-17-fold increased risk of developing RA compared with individuals with no family history (10), and a high disease concordance has been reported between monozygotic twins compared with dizygotic twins (11). However, environmental factors also play a role in RA susceptibility since despite higher observed familial risks among close relatives of RA patients, familial factors are of less importance for late-onset RA (12).

One of the strongest and best characterized associations between genetic factors and RA disease risk are of alleles within the human leukocyte antigen (HLA) region (13–15), most commonly within the *HLA-DRB1* locus (16). Recently, Raychaudhuri et al. (17) have refined the association for RA susceptibility to five amino acid positions encoded in three HLA genes: three in *HLA-DRB1* and one each in *HLA-B* and *HLA-DPB1*. Intriguingly, all five specific amino acid changes are at positions along the antigen binding groove of the folded major histocompatibility complex (MHC) proteins, suggesting these variants may have altered affinity for self-peptide antigens or may structurally change T cell recognition of those antigens. Allelic variants within MHC susceptibility loci are the strongest predictors of clinical disease phenotypes as studies have estimated that they explain ~12% of the phenotypic variance of seropositive RA compared with only ~4% for identified non-MHC loci (18).

More than 100 non-MHC RA risk loci have been identified to date (18–21). Many of these variants are near or within genes of known immune function, including those encoding the protein tyrosine phosphatases PTPN22 and PTPRC (CD45); costimulatory molecules CD40, CD28, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4); cytokine signaling interleukin-2 (IL-2), IL-2 receptor α– (CD25) and β-subunits (CD122); recombination activating genes 1 & 2 (RAG1/2), tumor necrosis factor alpha-induced

protein 3 (TNFAIP3), intercellular adhesion molecule 1 (ICAM-1); the transcription factors signal transducer and activator of transcription 4 (STAT4), autoimmune regulator (AIRE), and the interferon regulatory factors (IRFs); the signaling molecules interleukin-1 receptor-associated kinase 1 (IRAK1) and tumor necrosis factor receptor associated factors (TRAFs); and the citrullinated protein generating enzyme peptidyl arginine deiminase 4 (PADI4), among others (19) (Table 1).

Clearly RA is more than just a one gene-one disease type of autoimmune disorder. Indeed, gene interactions, such as those described between *HLA-DRB1* and *PTPN22*, further increase disease risk and highlight the complexity of the net risk across an entire genome (22). Importantly, many of the approved drugs used to treat RA patients demonstrate significant overlap with the identified biological RA risk genes. For example, abatacept is a CTLA-4-Fc fusion protein (23), tofacitinib is a small molecule inhibitor of Janus kinase 1 (JAK1) and JAK3 (24), and tocilizumab is a humanized monoclonal antibody against the IL-6 receptor (25,26). Each of these drugs target gene products or pathways for which those genes demonstrate significant RA risk within the population (19).

A population-specific genetic risk model estimated that only ~50% of disease heritability could be explained by RA risk loci (17,19). This suggests that in addition to the genetic risk, environmental risk factors also play an important role in RA susceptibility. RA is approximately three times more common in women than in men, suggesting sex hormones and gender specific reproductive factors could be involved in the etiology of RA. Consistent with this, women with RA frequently experience disease improvement during pregnancy (27), whereas the use of oral contraceptives was associated with decreased risk of RA in some studies (28,29), but not in others (30,31). Evaluation of other non-genetic risk factors for RA found enhanced risk of clinically severe RA associated with obesity, exposure to tobacco smoke, and excessive caffeine consumption (30,32). Furthermore, psychological stress (33), and dietary factors are also believed to play a role in the development of RA since a Mediterranean diet rich in fish oils, olive oil, cooked vegetables, and fruit has been associated with protection from RA (34,35).

The complex relationships between microbes and the immune system have long been of interest when studying the etiology and pathogenesis of RA. Recent studies have taken advantage of more powerful sequencing and computational capabilities to better understand the influence of the mucosal microbiome on RA onset and progression (36,37). Indeed, using a metagenome-wide association study approach, these studies have reported gut dysbiosis and perturbed oral microbiota in RA patients compared to control individuals. Interestingly, the substantial microbiome divergence between RA patients and healthy individuals changed significantly with disease-modifying antirheumatic drug (DMARD) treatment, resulting in an increase in the abundance of control-associated microbiome markers (37). The potential for shifts in the gut or oral microbiota to influence immune responses and pathogenesis in RA raises the possibility of microbiome manipulation for clinical therapeutic benefit. Exposure to infectious agents and their products has also been linked with RA. Candidate microbes suggested in an infectious etiology of RA include Epstein-Barr virus (EBV), *Escherichia coli*, *Mycobacterium tuberculosis*, *Proteus mirabilis*, and retroviruses, based on a higher frequency of detection of these microbes or specific anti-microbial antibodies within the peripheral blood and/or synovial fluid of RA patients (38–40). It is postulated that some form of molecular mimicry by microbial peptides could contribute to the activation and expansion of specific adaptive immune cells that are auto-reactive on the basis of sequence similarity (41). Alternatively microbial infection could trigger the formation of immune complexes which may induce the generation of RF (42,43), a high-affinity autoantibody against the Fc portion of immunoglobulin (Ig). The strong association of alleles within the MHC loci to RA has been explained by the presence of amino acid sequences spanning positions 70-74 in the β1 subunit, along the peptide-binding groove of the HLA-DR molecule. Intriguingly, the same amino acid motif can be found in bacterial chaperones from *E. coli* as well as on gp110 of EBV (44,45). Both T cells and B cells cross react to these amino acid sequences in gp110 and HLA-DRB1 molecules (45), suggesting that EBV or *E. coli* infection may lead to an immune response against HLA-DR molecules that share this motif, which are overrepresented among RA patients within the population.

Table 1. Select candidate genes with single nucleotide polymorphisms associated with increased risk of RA and their roles linked to lymphocyte function

Gene	Function relevant to RA pathogenesis and lymphocyte biology
HLA-DRB1	Strongest genetic risk loci associated with RA; involved in MHC molecule-based antigen presentation and contributes to selection of the T cell repertoire
PTPN22	Second genetic link described in RA; lymphocyte-specific non-receptor tyrosine phosphatase that modulates activation thresholds
PTPRC	Also known as CD45, a receptor protein tyrosine phosphatase that is an essential regulator of lymphocyte activation thresholds
CD40	Costimulatory receptor on antigen presenting cells required for their activation and is critical for T-dependent antibody production
CD28	Costimulatory receptor for T cell activation
CTLA4	Inhibitory receptor that regulates T cell activation; antagonizes CD28
IL2/*IL2RA*	Cytokine and high affinity receptor pair that regulate T cell development, differentiation and memory formation, including Tregs
RAG1/*2*	Proteins involved in antigen receptor rearrangement in lymphocytes
REL	Member of the NF-κB family of transcription factors that regulates leukocyte activation and survival, particularly in B cells
ICOSLG	Ligand for inducible T cell costimulator (ICOS) essential for T cell proliferation and function during activation
STAT4	Transcription factor required for Th1 cell development and IFN-γ production in response to IL-12
IL21	Cytokine that influences lymphocyte proliferation and differentiation
AIRE	Transcription factor expressed in thymic epithelial cells in the medulla that regulates their expression of self-antigens
IL6R	Cytokine receptor subunit that binds IL-6, an important mediator in inflammation the acute phase response; regulates Th17/Treg balance
TNFAIP3	Also known as A20, a cytoplasmic protein induced by TNF and NF-κB activation; functions as a negative regulator of NF-κB activation and TNF-mediated apoptosis
C5	Complement component 5; cleaved to form C5a anaphylatoxin and C5b fragment that forms part of the membrane attack complex
PADI4	Enzyme responsible for the conversion of arginine to citrulline; involved in the formation of citrullinated proteins

1.1.4 Synovial inflammation and leukocyte infiltration in RA

Structural changes and damage to periarticular bone and articular cartilage are hallmarks of RA and exemplify the destructive potential of chronic inflammation. Bone and cartilage damage associated with RA have a profound and largely irreversible impact on joint function (46,47). Preceding these outcomes is the development of synovitis, inflammation of the synovial membrane lining the joints. In the healthy joint, the thin synovial membrane includes a distinct intimal lining layer of 1-2 cells in thickness and consists of synovial macrophages and fibroblast-like synoviocytes (FLS) (48). In RA, the synovial lining becomes hyperplastic and leads to the formation of a pannus at the interface between synovium, cartilage, and bone. The pannus contains large numbers of macrophages, FLS, and osteoclasts that express high levels of cytokines and tissue destructive proteases (49) (Figure 1). The initiating factors and order of events to explain how synovitis develops in RA remains poorly understood. It has been suggested that activation of resident synovial macrophages and FLS occurs as a result of local hypoxia or through Toll-like receptors (TLRs) to innate stimuli such as microbial components or locally released RNA from necrotic cells (50–52). Activated synoviocytes express cytokines/growth factors, chemokines, adhesion molecules, and proteases, which participate in inflammation and the destruction of joint tissues (53). However, this does not explain the findings that autoantibodies (both RF and ACPA) are often detected in RA patients several years prior to the onset of symptoms (54,55). Therefore in many patients, inappropriate immune cell responses appear to precede synovial inflammation, suggesting that FLS may be local effectors that are activated by immune cells to express inflammatory mediators. Regardless of the initiating events, the release of cytokines, chemokines, and other factors would promote the infiltration and accumulation of immune cells in the synovium, establishing an inflammatory loop through continuous stimulation of FLS and other cells in the synovium leading to chronic inflammation and tissue destruction.

An inflamed hyperplastic synovial lining enriched with new blood vessels and infiltration of innate and adaptive immune cells within the synovial joint space are characteristic features of the active rheumatoid joint (56). There is marked accumulation

of T cells, plasma cells, monocytes/macrophages, B cells, neutrophils, mast cells, natural killer (NK) cells, and dendritic cells (DCs) in the synovial tissues (56–60) (Figure 1). Leukocyte infiltration is enabled by endothelial activation in synovial microvessels (61) and enhanced expression of adhesion molecules and chemokines (62). Each of the synovial tissue-infiltrating immune cell types have been implicated in RA pathogenesis, although RA has long been considered a T cell-dependent disease (63–69).

In the RA synovium, infiltrating T cells are mostly activated memory cells (70–72), leading to cytokine-dependent and cell-cell contact-dependent activation of other synovial cells (such as FLS, synovial macrophages, and osteoclasts), resulting in the production of high levels of inflammatory mediators including cytokines, chemokines, and tissue-destructive matrix metalloproteinases (MMPs). For example, synovial T cells in RA patients produce high levels of tumor necrosis factor (TNF) which acts on FLS and other synovial cells to increase growth and cytokine/chemokine/MMP production (73–75), whereas membrane-bound and soluble IL-15/IL-15 receptor complexes are constitutively expressed on FLS and represents a potent T cell growth factor that causes activation/proliferation and survival of effector and regulatory T cells (76). Moreover, dendritic cells, activated B cells and synovial macrophages, and FLS can also act as synovial antigen-presenting cells (APCs) to induce activation, cytokine secretion, and other effector responses of T cells infiltrating the RA synovium (77). In addition to dysregulated responses within the inflamed RA synovium, autoreactive T cells outside of the joints and T cell-mediated autoantibody generation are critical players in the pathogenesis of RA. We have learned a great deal about the pathogenesis of RA from observational studies in patients. However, animal models of arthritis have provided a means for elucidation of the underlying mechanisms responsible for disease development and progression. Despite tremendous advancements in our understanding of the immune-mediated pathways that contribute to inflammatory arthritis, additional preclinical and clinical studies are needed to further our knowledge of disease mechanisms and potential therapies.

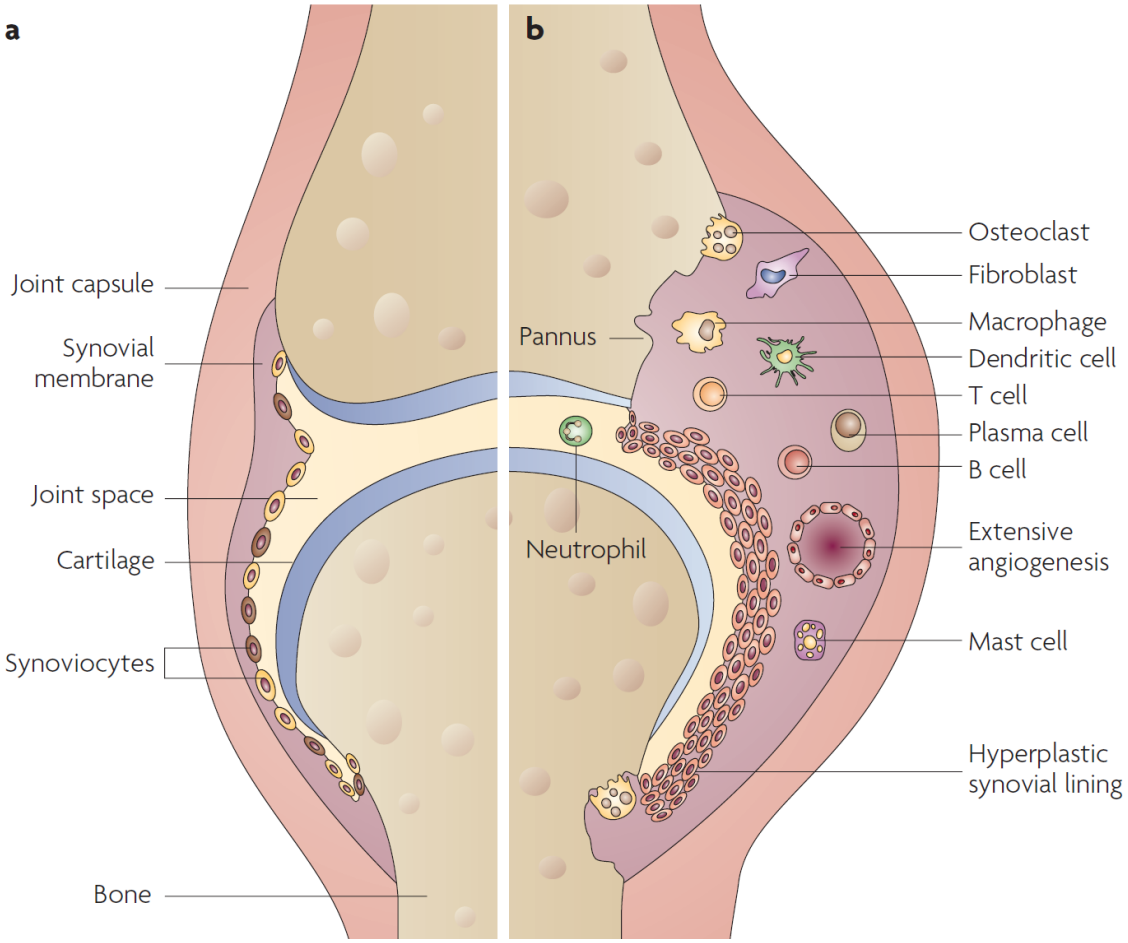


Figure 1. Diagrammatic representation of a normal joint and an inflamed joint in rheumatoid arthritis. In the healthy joint **(a)**, a thin synovial membrane consisting of synoviocytes lines the joint space. In rheumatoid arthritis **(b)**, the synovial membrane becomes hyperplastic, enriched with new blood vessels, and infiltrated by inflammatory cells. Chronic synovitis leads to the formation of a pannus that is highly destructive to the articular cartilage and bone. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Drug Discovery. Strand, V. et al. Biologic therapies in rheumatology: lessons learned, future directions. *Nat Rev Drug Discov* (2007) **6**:75-92, copyright 2007 (78).

1.2.1 Experimental models of inflammatory arthritis

Our understanding of the cellular and molecular players and pathways involved in inflammatory joint diseases has advanced considerably through the use of animal models. In addition to providing a platform for the dissection of the immune-mediated mechanisms of inflammatory arthritis, animal models have aided in the testing of potential disease-targeting pharmaceuticals, and many agents currently in clinical use or trials have been shown to be active in these models (79,80). Both induced and spontaneous models of arthritis have been employed to study immune responses in arthritis and many currently used models have one or more pathological features that are similar to those in human disease. The induced arthritis models include: CIA (81); collagen antibody-induced arthritis (CAIA), in which transfer of anti-collagen antibodies promotes the development of arthritis (82); antigen-induced arthritis (AIA) in animals following priming and challenge with an antigen [often methylated bovine serum albumin (mBSA)] in adjuvant (83); adjuvant arthritis in the rat that is induced by a single injection of adjuvant often containing mycobacterial strains (84); zymosan-induced arthritis in which intra-articular injection of the TLR2 agonist zymosan into the knee joints results in rapid onset of arthritis limited to the site of injection (85); proteoglycan-induced arthritis (PGIA) induced by the injection of proteoglycan in adjuvant (86); and arthritis induced by injection of streptococcal cell wall (SCW) fragments (87).

A number of genetically manipulated or spontaneous arthritis models have also been described. For example, the SKG mouse develops spontaneous arthritis due to a mutation in zeta-associated protein of 70 kDa (ZAP-70) that alters T cell receptor (TCR) signaling thresholds and causes abnormalities during T cell thymic selection (88). The K/BxN spontaneous arthritis model was serendipitously discovered after crossing the TCR transgenic KRN mouse line with nonobese diabetic mice, which results in transgenic TCR T cell specificity for a peptide from the ubiquitous cytoplasmic enzyme glucose-6-phosphate isomerase (G6PI) in the context of the MHC class II molecule A^{g7} (89). This recognition results in the generation of arthritogenic autoantibodies against G6PI, and passive transfer of serum or purified anti-G6PI antibodies from K/BxN mice results in arthritis in various recipient mice (90–92). Transgenic mice overexpressing

human TNF spontaneously develop chronic inflammatory arthritis that can be completely prevented using anti-TNF antibody treatment (93). Mice deficient in the endogenous IL-1 receptor antagonist represent another spontaneous arthritis model (94). A mutation in the gp130 subunit of the IL-6 receptor results in enhanced STAT3 activation and spontaneous arthritis in mice driven by altered IL-7– and Fas ligand (FasL)-dependent $CD4^+$ T cell proliferation and survival (95,96). Finally, the TS1 x HACII mouse model involves transgenic mice that express a TCR specific for hemagglutinin as well as hemagglutinin under the MHC class II promoter, leading to the activation of autoreactive $CD4^+$ T cells, systemic proinflammatory cytokine production, and spontaneous arthritis development (97).

Again, these experimental models of inflammatory arthritis share one or more pathological features with disease in RA patients. However, some important differences include the much more rapid progression through clinical stages of disease compared to human disease, and the fact that different experimental conditions can dramatically influence the type of inflammation responsible for driving disease pathogenesis in these models (98). Furthermore, relying too much on animal models can be detrimental with respect to predicting and testing the efficacy of pharmaceuticals targeting specific cellular or molecular effectors, since some therapies that are effective in treating disease in animals fail or are only moderately effective in human disease. For example, some therapies targeting T cells, such as anti-CD4 antibodies, were found to prevent or ameliorate disease in multiple animal models (99), but are no longer in development for RA due to poor clinical efficacy (100). Contrary to this, methotrexate, the 'gold standard' DMARD for treatment of RA had no effect on CIA in DBA/1 mice but did reduce clinical signs of CIA in C57BL/6 mice at a dose similar to that used in RA patients (101). This also exemplifies the importance of consistency with respect to experimental conditions, such as the mouse strain that was used, when comparing results between studies. Nonetheless, preclinical models of RA are essential for elucidating the mechanisms of disease and testing therapeutic agents. This includes assessing disease-modifying efficacy, specific side effects and toxicities associated with a particular drug, and predicting doses that show serious complications over prolonged dosing.

1.2.2 Collagen-induced arthritis

The CIA model is among the most widely studied animal model for RA due to the many pathological features and underlying disease mechanisms it shares with human RA. In addition, CIA is a useful model due to the availability of antibodies and genetically modified animals. Indeed, CIA is one of the most commonly used models for the evaluation of approved or pending RA therapies undergoing clinical trials as well as for the discovery of promising novel therapeutic targets (79). The histopathological features of CIA relevant to human RA include a proliferative synovitis that displays abundant joint infiltration by immune cells, pannus formation, cartilage degradation and bone erosions (101,102). CIA can be studied in mice, rats, and primates following immunization with collagen type II (CII) in adjuvant. In mice, immunization with heterologous CII (usually bovine CII in DBA/1 mice and chicken CII in C57BL/6 mice) in adjuvant promotes joint inflammation, likely through a breakdown of peripheral tolerance leading to immune responses against autologous collagen. This is supported by the well-defined immunodominant T cell epitopes present in CII (103), and the finding that transgenic mice overexpressing a specific TCR β-chain from a clone that recognizes murine CII develop chronic arthritis (104). Furthermore, T cells from CIA mice have been shown to respond to murine CII and anti-mouse CII autoantibodies are readily detected in mice immunized with heterologous CII, supporting a role for immune responses to endogenous CII in CIA pathogenesis (101,105).

Like RA, susceptibility to CIA is strongly associated with genes encoding MHC class II molecules since mouse strains bearing the H-2^q haplotype (e.g. DBA/1) are among the most susceptible, whereas those with an H-2^b haplotype (e.g. C57BL/6) are more resistant to disease (106). However, modification of the experimental CIA immunization protocol results in arthritic disease in C57BL/6 mice that clinically and histologically resembles CIA in DBA/1 mice (101,102,107). These modifications include the use of chicken CII, rather than bovine CII, emulsified in complete Freund's adjuvant (CFA) containing a high dose of *M. tuberculosis*, and delivery of this emulsion for both the primary and booster immunizations. The development of an immunization protocol for the reliable and reproducible induction of CIA in C57BL/6 mice has a number of

advantages over the more conventionally used DBA/1 strain. For example, CIA in C57BL/6 mice has been shown to be associated with a more robust and sustained T cell response to CII and more closely resembles RA in its response to therapeutic intervention with methotrexate (101). In addition, studies investigating various cellular and molecular pathways in CIA using DBA/1 mice were limited by the fact that most transgenic and knockout strains of mice are on a C57BL/6 background. Therefore, a protocol for inducing arthritis in C57BL/6 mice circumvents the need to backcross genetically modified strains onto the DBA/1 background, an otherwise costly and time consuming endeavor.

Other similarities exist between human RA and murine CIA with respect to the immune-mediated mechanisms involved in joint inflammation. For example, an important feature of CIA that resembles RA is the expression of proinflammatory cytokines, including TNF and IL-1β in the joints (108,109). Furthermore, antagonizing these cytokines results in a reduction in disease severity in CIA and RA (110–112). Furthermore, both T cells and B cells play critical roles in disease initiation and pathogenesis in CIA. Evidence for the pathogenic roles of T cells in RA and CIA will be introduced later. Autoantibodies are known to play an important role in the pathogenesis of both CIA and RA. Again, autoantibodies to murine CII develop in CIA mice (105). The arthritogenicity of the anti-collagen antibody response is clearly demonstrated by the CAIA model, in which passive transfer of disease is accomplished using antibodies specific for major CII epitopes (113,114). Similarly, a role for human CII autoimmunity in RA has been demonstrated since anti-CII antibodies have been associated with early disease progression in some patients (115–117).

Furthermore, ACPA/anti-CCP antibodies and RF are well characterized predictors of radiological progression in RA patients (55,118,119) and ACPA/anti-CCP antibodies have also been detected in CIA mice (120). The pathogenic role of B cells in RA is evidenced by the success of rituximab and other B cell-depleting anti-CD20 antibodies in the clinic (121,122). Importantly, in addition to reducing autoantibody titers, anti-CD20 therapy also has indirect effects on T cells which has been suggested to occur via inhibition of antigen-presentation by B cells (123). In CIA and PGIA, B cell depletion via

anti-CD20 antibody administration suppresses arthritis through reduced T cell-dependent autoantibody production, but also diminished antigen-specific T cell responses and increased T regulatory cell (Treg) activity (124–126). The induction of Tregs was reported after rituximab treatment in patients with SLE (127), but not in RA (128), highlighting the fact that although animal models such as CIA and PGIA bear many similarities to human RA they also have differences. For example, anti-CII antibodies are important for disease in CIA but this does not appear to be the case in a large proportion of RA patients since earlier studies have estimated that fewer than 15% of RA patients have antibodies to human CII (129). It is also important to note that differences in immune cell responses [e.g. T cell expansion and interferon-γ (IFN-γ) production] and response to therapeutic intervention in CIA may vary depending on the mouse strain (101). In general, CIA is a relatively acute inflammatory arthritis model that is not characterized by exacerbations and remissions like human disease. Although mouse models do not always accurately predict the mechanisms or outcomes of therapeutic interventions in RA (79,80), they complement observational and in vitro studies of human RA patients and clinical samples, and represent critical tools for understanding disease mechanisms and evaluation of novel treatment options.

1.3.1 The role of T cells in inflammatory arthritis

It is widely accepted that autoreactive T cells play a major role in the pathogenesis of RA and many of the animal models of human arthritic disease. Antigen presentation to $CD4^+$ T cells is MHC class II restricted. In RA patients carrying the HLA-DRB1 shared epitope (a five amino acid sequence present in some *HLA-DRB1* alleles), T cell responses to citrullinated self-proteins are detected more frequently than in those negative for shared-epitope containing MHC class II molecules (130). Similarly, a recent study has shown that the major T cell epitope in CII (amino acids 259-273) is preferentially presented by APCs that express *HLA-DRB1*04* (HLA-DR4) molecules, resulting in the elicitation of autoreactive T cell responses in RA patients (131). These studies provide evidence that the link between the *HLA-DRB1* locus and RA disease risk is due to the direct involvement of these HLA-DR gene products in autoreactive T cell activation. The importance of dysregulated autoreactive $CD4^+$ T cell responses in RA is further supported

by the fact that many non-HLA RA susceptibility loci are within genes with products involved in lymphocyte signaling and activation (Table 1). This is reinforced by the clinical efficacy of abatacept in RA, a CTLA-4-Ig that blocks T cell costimulation (132). Furthermore, the large numbers of T cells in the joints of RA patients suggests that they are actively involved in inflammation within the arthritic synovium. Waase et al. (133) demonstrated that multiple clonally expanded $CD4^+$ T cells are present in the peripheral blood of RA patients and contributed to the T cell infiltrate in the joint. The clonotypic composition of the multiple oligoclonally expanded $CD4^+$ T cells within the RA synovium were found to change over time in longitudinal studies (134). However, it is unclear whether this indicates that T cells bearing different TCRs are capable of participating in synovial inflammation or if this change was due to an effect of treatment on the composition of the T cell population.

Both $CD4^+$ and $CD8^+$ T cells have been characterized in the joints of RA patients. A large number of studies examining the phenotypic characteristics of $CD4^+$ T cells isolated from the synovial fluid of RA patients and animal models of arthritis support a principle role for $CD4^+$ T cells in disease pathogenesis (67,68,99,135). Indeed, CII-specific $CD4^+$ T cells from CIA mice are essential for transfer of arthritic disease into severe combined immunodeficient (SCID) mice lacking mature T and B cells (136). Moreover, numerous T cell-targeting therapies, such as anti-CD4 antibodies, genetically modified mice lacking CD4, or combination of depleting anti-CD3 and anti-TNF antibodies have shown great efficacy in preventing arthritis in animal models (137–140). Several therapies targeting T cells in RA have been developed and tested. However, clinical trials of T cell-depleting and T cell non-depleting agents in RA have been generally disappointing due to poor efficacy (100). The lack of clinical benefit with anti-T cell antibody therapy may be due to the duration of antibody treatment needed to modulate pathogenic T cell function systemically or within the arthritic synovium. Furthermore, in vivo activated CII-specific $CD4^+$ T cells have been shown to be resistant to antibody-mediated depletion and anti-CD4 antibody treatment suppressed CIA in mice when delivered early before CII immunization but not at later stages of disease (141). $CD4^+CD25^+$ forkhead box $P3^+$ ($FoxP3^+$) Tregs are enriched in the synovial fluid of patients with RA and juvenile idiopathic arthritis (JIA), through enhanced recruitment of

these cells and their expansion locally within the inflamed joints (142,143). However, high levels of proinflammatory cytokines (e.g. TNF and IL-6) and enhanced proliferative capacity of effector T cells contribute to their resistance to the suppressive functions of joint derived Tregs (142,143) (Figure 2). Nonetheless, one of the reasons for the failure of T cell-targeting therapies in RA could be because they target both Tregs as well as effector T cell subsets.

Conflicting reports characterizing $CD8^+$ T cells in the synovium of patients with inflammatory arthritis has contributed to their controversial role in joint pathology. $CD8^+$ T cells represent a large proportion of the T cells present within the synovial fluid of patients with RA and JIA (144,145). Hunter et al. (145) demonstrated that elevated synovial fluid $CD8^+$ T cells are correlated with a more severe phenotype in JIA. In line with this, synovial fluid $CD8^+$ T cells isolated from JIA patients have been shown to exhibit an effector memory phenotype and produce proinflammatory cytokines such as IFN-γ and TNF (143). In contrast, Cho et al. (144) found that effector memory $CD8^+$ T cells in the synovial fluid from RA patients were enriched for an IL-10-producing population that were inversely correlated with disease severity, suggesting that $CD8^+$ T cells may be involved in modulating inflammation. Immunoregulatory functions of $CD8^+$ T cells have also been investigated in RA synovial tissue grafts in SCID mice (146). In this study, $CD8^+$ T cell clones were isolated and expanded from synovial tissues of RA patients and adoptively transferred into SCID mice engrafted with RA synovial tissues. Adoptively transferred $CD8^+CD28^-CD56^+$ T cells exhibited strong anti-inflammatory activities, whereas $CD8^+CD28^+CD56^-$ T cell clones exhibited proinflammatory properties (146).

The importance of $CD8^+$ T cells in animal models of RA is equally controversial based on conflicting evidence on their roles in disease induction or amelioration. Depletion of $CD8^+$ T cells in the SCW model in rats resulted in enhanced severity and an earlier disease onset (147). In CIA, some studies report no effect on disease induction or severity in mice deficient in $CD8^+$ T cells (139), whereas others have shown reduced incidence in CD8-deficient mice, but no difference in disease severity (148). Interestingly, CD8-deficient mice were also more susceptible to a second induction of

CIA after remission of initial disease (148), suggesting an immunoregulatory role for $CD8^+$ T cells. Yet in the PGIA model in mice, depletion of $CD8^+$ T cells prevented disease transfer to recipient mice (149), whereas the depletion of $CD8^+$ cells in immunized mice enhanced disease severity (150), again suggesting an immunoregulatory role for $CD8^+$ T cells in established disease, but also highlighting a possible role for $CD8^+$ T cells during disease induction. To add further complexity to the roles of T cells in inflammatory arthritis, multiple T cell subsets with distinct cytokine profiles and effector functions have been described to have varying contributions to rheumatic diseases. Moreover, effector T cells possess a degree of plasticity, allowing T cells to convert between polarized subsets and alter their cytokine profiles in response to different micro-environmental stimuli. Therefore, understanding and regulating the balance between effector T cell subsets in different clinical stages of disease may be more important than targeting entire $CD4^+$ or $CD8^+$ T cell populations for providing improved clinical outcomes in RA.

1.3.2 T cell subsets in inflammatory arthritis

Classically, the $CD4^+$ T helper type 1 (Th1) subset, which is characterized by expression of the transcription factor T box expressed in T cells (T-bet) and the production of IFN-γ, were thought to drive RA pathogenesis. The proinflammatory properties of IFN-γ are attributed to many factors: its strong macrophage-activating potential, the induction of MHC class I and class II expression, enhanced antigen processing and presentation by DCs and macrophages, promotion of enhanced Th1 differentiation, activation of cytotoxic T lymphocytes (CTLs) and NK cells, stimulation of antibody subclass isotype switch, promotion of antibody-dependent cell-mediated cytotoxicity through augmented Fcγ receptor (FcγR) expression, and enhanced complement-mediated phagocytosis through increased complement/complement receptor expression (151). The initial classification of RA as a Th1-driven disease was supported by the presence of $CD4^+$ T cells and IFN-γ within the inflamed synovium (60,70,152,153). Single-cell analysis of cytokine production by $CD4^+$ T cells in synovial tissues and fluids of RA patients confirmed the presence of IFN-γ-secreting $CD4^+$ T cells in the RA synovium (154,155). More recently, epigenetic immune lineage analysis

revealed that demethylation of the *Ifnγ* locus, which is indicative of enhanced skewing towards the Th1 lineage, is significantly enhanced in $CD4^+$ T cells isolated from the synovial fluid of patients with RA (156). Consistent with this, RA synovial fluid $CD4^+$ T cells were found to predominantly produce IFN-γ in response to the human $CII_{259-273}$ peptide (131). Other important sources of IFN-γ in vivo include NK cells (157), $CD8^+$ T cells (158), and iNKT cells (159,160). However, the concept of RA as a Th1 cytokine-driven disease has been challenged by studies investigating the roles of Th1 cells and IFN-γ in mouse models. In addition, the discovery of IL-17-producing Th17 cells has also revised this concept of disease as these cells have been shown to be important in promoting inflammation and disease pathogenesis in many autoimmune diseases (161).

Multiple studies in animal models of RA have revealed contradictory roles of IFN-γ. For example, some studies on the effect of IFN-γ receptor-deficiency have demonstrated that mice were protected from CIA (162), whereas others have indicated that arthritis was more severe and disease onset was accelerated (163). Importantly, the administration of recombinant IFN-γ at the time of collagen immunization, or before disease onset, increased the incidence and severity of arthritis in mice (164–166). Similarly, IFN-γ blockade was protective when delivered early, whereas anti-IFN-γ antibody treatment after disease onset exacerbated arthritis (166). Therefore the paradoxical role of endogenous IFN-γ may be linked to opposing effects during early versus late phases of disease in the CIA model. This is supported by the presence of IFN-γ early post-immunization in the draining lymph nodes of CIA mice (167), highlighting the predominance of Th1 cells in the pre-arthritic stage of CIA, but not later in disease (168,169). However, abundant expression of IFN-γ was detected in the inflamed joints, but not the non-inflamed joints, of individual CIA mice (170), suggesting that the presence of IFN-γ within the joints may promote more severe joint destruction in CIA. Co-culture with Th1 cells, or addition of IFN-γ, activated FLS from RA patients, resulting in upregulated expression of MHC class II, CD40, and ICAM-1 (171). Increased expression of these molecules contributed to enhanced T cell-FLS interactions and bidirectional activation, since CD40 ligation promoted FLS proliferation and proinflammatory cytokine secretion (172,173), while enhanced adhesion and FLS-T cell cross-talk resulted in elevated expression of TNF, IFN-γ, IL-17, CD25, and CD69 by T

cells from RA patients (174). These conflicting reports highlight the paradoxical significance of IFN-γ and Th1 cells in the pathogenesis of inflammatory arthritis. Intriguingly, IFN-γ suppresses IL-17 production by CII-specific T cells, a function that may be relevant to the suppressive role of IFN-γ during the effector stage of CIA (175). In line with this, CIA mice treated with neutralizing antibody to IFN-γ exhibited an accelerated course of disease, which was associated with increased IL-17 levels in the serum and joints (176). The cellular mechanisms underlying the protective role of IFN-γ in CIA are inhibition of neutrophil infiltration (177), reduced expansion of $CD11b^+$ mononuclear phagocytes (178), impaired osteoclastogenesis (179), inhibition of Th17 cell differentiation (175), and increased suppressive function of Tregs (180).

Th17 cells are characterized by expression of the transcription factor retinoic acid-related orphan receptor γτ (RORγτ) (181), and the production of IL-17A (sometimes referred to as IL-17), IL-17F, IL-21, and IL-22 (182–184). Th17 cells and IL-17A are major contributors to synovial inflammation and the destruction of cartilage and bone within the RA joint (185,186). Indeed, IL-17A has been implicated as an important driver of chronic inflammation and expression of IL-17A and IL-17 receptors are elevated in patients with a variety of autoimmune diseases, including RA, MS, and Crohn's disease (187,188). Studies in inflammatory arthritis patients have identified enrichment of both IL-17A– and IFN-γ-producing T cells in the joints (189,190). Th1 cells have been reported to predominate over Th17 cells in the synovium in human disease (190), which may be a consequence of Th17 cell conversion to a Th1 phenotype within the joints of patients with inflammatory arthritis (191). However, treatment-naïve patients with early RA have an increased fraction of Th17 cells (74). Recently, anti-IL-17A monoclonal antibody therapy has shown clinical benefit in phase II clinical trials in RA patients, improving signs and symptoms in patients who were either naïve to biologics treatment or were inadequate responders to TNF inhibitors (192). Th17 cells from RA patients were shown to be potent inducers of the proinflammatory cytokines IL-6 and IL-8, and tissue-destructive MMPs from RA FLS (74,193). However, neutralization of IL-17A in co-cultures of human Th17 polarized $CD4^+$ T cells and RA FLS did not completely prevent induction of IL-6 and IL-8 production from FLS (171), suggesting that additional

cytokine-dependent or cell-cell contact dependent mechanisms contribute to the effects of Th17 cells on FLS.

The IL-17 family consists of six isoforms identified as IL-17A-F. Of these, IL-17A and IL-17F share the greatest homology and exist either as homodimers or an IL-17A/F heterodimer. Production of IL-17A and IL-17F is restricted primarily to activated Th17 polarized cells and both signal through IL-17 receptor A (IL-17RA) and IL-17RC. Other important cellular sources of IL-17 cytokine members include iNKT cells (194), γδ T cells (195), along with other innate lymphoid cells (196), mast cells (197), and possibly other myeloid cells (198). Both IL-17A and IL-17F have been implicated in RA, and have been shown to induce similar patterns of proinflammatory gene expression (IL-6, IL-8, IL-23, and various chemokines), migration and invasiveness, reduced apoptosis, and enhanced MMP secretion in RA synoviocytes, although IL-17F is less potent than IL-17A alone (199,200). Studies in the CIA model confirmed the role of IL-17 in arthritis, since disease is markedly suppressed in IL-17-deficient mice (201), and treatment with neutralizing anti-IL-17 antibody after the onset of CIA in mice reduced joint inflammation, cartilage destruction, and bone erosions (202). In CIA, IL-17 mRNA is upregulated in the synovium after the onset of arthritis, while systemic and local adenoviral gene transfer of IL-17 exacerbated disease progression and joint damage (203). In line with this, IL-$23^{-/-}$ mice, which exhibit impaired proliferation and maturation of Th17 cells (183,204), fail to develop clinical or histological disease in CIA (205). The cellular mechanisms associated with the proinflammatory effects of IL-17A include promoting the production of other inflammatory cytokines, such as IL-6, IL-8, and TNF (171,193), enhanced neutrophil chemotaxis via induction of IL-8 (206), enhanced granulopoiesis and osteoclastogenesis (206,207), and the induction of MMP production (74,87).

The other IL-17 family members seem to have distinct expression patterns, suggesting specialized biological roles. Previously, in addition to IL-17A and IL-17F, IL-17C expression in synovial fluid mononuclear cells of RA patients was reported (188), but its function in inflammatory arthritis remains unclear. Interestingly, the increased plasma levels of IL-17F in RA patients was reduced after therapy in methotrexate, anti-

TNF, and CTLA-Ig treated cohorts (208). However this was not associated with generally decreased plasma levels of all forms of IL-17, since IL-17A and IL-17A/F levels were not altered. In CIA, the expression of IL-17A and IL-17F (within $CD4^+$ T cells), IL-17B (within chondrocytes), and IL-17C (within $CD4^+$ T cells, macrophages, and DCs) were found to be upregulated in the arthritic paws of CIA mice (209). In their study, Yamaguchi et al. (209) demonstrated that IL-17B and IL-17C play important roles in arthritic disease, since adoptive transfer of IL-17B– and IL-17C-transduced $CD4^+$ T cells exacerbated arthritis, whereas antibody neutralization of IL-17B suppressed inflammation and bone destruction in CIA mice.

Th2 cells are characterized by expression of the transcription factor GATA binding protein 3 (GATA-3), and the secretion of IL-4, IL-5, and IL-13 (210). IL-4-producing Th2 cells are believed to have a protective role in inflammatory arthritis based on studies in RA patients and animal models. For example, a polymorphism in the IL-4 receptor (IL-4R) gene that resulted in weaker signaling through IL-4R was associated with rapidly erosive disease in RA patients (211). Furthermore, stimulation of RA synovial fluid mononuclear cells with human CII or citrullinated proteoglycan aggrecan resulted in increased production of IFN-γ and IL-17, but not IL-4 (130,131,212,213). In mice, IL-4-deficiency leads to more severe arthritis in the CIA model (214). In the CIA and PGIA models, administration of IL-4 prophylactically or after disease onset resulted in reduced clinical severity of disease (215,216). Additionally, genetically modified DCs, or CII-pulsed APCs, overexpressing IL-4 are able to suppress CIA (217–220). Suppression of established CIA was confirmed following administration of modified DCs or exosomes derived from those DCs expressing either soluble IL-4 or membrane-bound IL-4 (221). Furthermore, these DCs and DC-derived exosomes were shown to inhibit inflammation associated with delayed-type hypersensitivity (DTH) in mice (221). IL-4-mediated amelioration of arthritis was associated with reduced levels of CII-specific IgG2 antibodies (215,218) and regulated production of IL-17 by CII-specific T cells (220), suggesting that IL-4 may suppress CIA by affecting antigen-specific autoantibody synthesis and proinflammatory cytokine production.

Th1 and Th17 cells have both been implicated in autoimmune inflammation. However, Tregs are also present at high levels in the inflamed joints of patients with arthritis, but are ineffective in controlling effector T cells due to resistance within the synovial effector T cell population to suppression (142,143) (Figure 2). Tregs within the joints of inflammatory arthritis patients are $CD4^+CD25^+FoxP3^+$ T cells that could represent thymus derived 'natural' Tregs or peripherally induced Tregs [in the presence of transforming growth factor β (TGF-β)] (67). TGF-β plays a role in induced Treg differentiation, but also has potent anti-inflammatory effects on effector T cell expansion and cytokine production (222). The exact mechanisms through which Tregs exert their suppressive function are not fully elucidated, but multiple mechanisms are important including cell-cell contact, such as via CTLA-4 and lymphocyte-activation gene 3 (LAG-3) (223), as well as the secretion of soluble regulatory cytokines, such as TGF-β, IL-10, and IL-35 (224). Intensive research has focused on the roles of Tregs in autoimmune disease and how their numbers, phenotypes, and functions may be altered to provide therapeutic benefit to patients. In patients with autoimmune disease it remains controversial whether resistance of effector T cells to Treg control or intrinsic Treg impairment represents the major defect in peripheral tolerance. However, evidence in the literature suggests both likely contribute to uncontrolled pathogenic T cell responses in autoimmunity. For example, TNF and IL-6 present at the site of inflammation have been implicated in the resistance of synovial effector T cells to suppression in arthritis patients (143) (Figure 2). On the other hand, pathogenic Th17 responses were inhibited by Treg cells from RA patients responding to anti-TNF therapy, but not by Treg cells from patients with active disease (225), suggesting potential intrinsic defects in the suppressive capacity of Tregs from RA patients. Moreover, anti-TNF therapy in RA has been associated with increased numbers and suppressive function of peripherally induced Tregs, which was TGF-β-dependent (226).

1.3.3 T cell plasticity and cross-regulation of effector T cell subsets in arthritis

T helper cell plasticity refers to the ability of mature polarized $CD4^+$ T effector cells to trans-differentiate into alternate functional lineages. This has been demonstrated in both mouse and human T helper cell subsets that develop under physiological

conditions in vivo as well as in response to specific polarizing cytokines in vitro (227–230). For example, Th17 cells have been shown to acquire IFN-γ expression in a mouse model of colitis and in the context of experimental autoimmune encephalomyelitis (EAE), a mouse model of human MS (228,230). Furthermore, in vitro polarized human Th1 and Th17 cell subsets have been shown to exhibit a mixed phenotype with respect to cytokine secretion, with each capable of secreting IL-17 and IFN-γ (171). Bifunctional Th1-Th17 cells stably coexpressing T-bet and RORγτ and producing both IFN-γ and IL-17 have been identified in the gut of patients with Crohn's disease (231), and in synovial fluid of patients with inflammatory arthritis (191), but not in peripheral blood. Conversion of $FoxP3^+$ T cells into pathogenic Th17 cells has also been demonstrated under arthritic conditions (232) (Figure 2). Fate mapping analysis revealed IL-17-producing T cells that had undergone the conversion from $FoxP3^+$ T cells to Th17 cells in the joints and lymph nodes of CIA mice, and a similar population was detected in the synovium of RA patients (232). Therefore, dysregulated numbers and functions of T cell subsets in autoimmune disease may be a result of their trans-differentiation within inflammatory microenvironments.

Important regulatory interactions also occur between the differentiated effector T helper cell subsets. For example, induction of Th17 cell differentiation is inhibited by the Th1 cytokines IFN-γ and IL-12, as well as the Th2 cytokines IL-4 and IL-13 in vitro (182,184,233). Moreover, Th1 cytokines promote the development of additional Th1 cells while suppressing Th2 cell development, whereas Th2 cytokines suppress Th1 cell development (234,235). The cross-regulation of Th17 cells is important in arthritis susceptibility in mice. T cells from CIA or AIA immunized IFN-$\gamma^{-/-}$ mice exhibited dramatically higher levels of IL-17 production in response to CII or mBSA restimulation compared to wild-type T cells (175,236). Elevated IL-17 production directly contributed to arthritis development since anti-IL-17 antibody treatment in IFN-$\gamma^{-/-}$ mice almost completely prevented clinical signs of disease (175,236). Furthermore, neutralization of IFN-γ late during disease induction accelerated the course of CIA and was associated with increased IL-17 levels in the serum and joints (176). Anti-IL-4 antibody treatment alone had no influence on arthritis severity, but CIA immunized mice that received both anti-IFN-γ and anti-IL-4 antibodies displayed more severe arthritis, as well as an

accelerated onset and progression of disease than mice that received anti-IFN-γ alone (176). Interestingly, the therapeutic effect of IL-4-overexpressing DCs on CIA was associated suppressed IL-17 production in secondary responses to CII in T cells isolated during the early induction phase of arthritis, but had no effect on IL-17 production by T cells isolated from mice with established CIA (220). Recently IL-4 was shown to abrogate Th17 cell-mediated inflammation by STAT6-dependent attenuation of IL-23 secretion by human and mouse APCs (237). However, IL-4-mediated suppression of IL-17 production by T cells after collagen rechallenge was not due to a lack of IL-23 (220), suggesting IL-4 is suppressing IL-17 production or Th17 cell differentiation through an alternative mechanism in this system. Further investigation is required to better understand these processes in vivo, since in vivo-generated Th17 cells have been shown to be refractory to Th1– and Th2-polarizing signals, whereas in vitro-generated Th17 cells could be readily converted to a Th1 or Th2 phenotype (238). Therapeutic interventions that correct pathogenic imbalances in T helper subset ratios to restore immune homeostasis are an attractive strategy for providing clinical benefit to patients with autoimmune diseases.

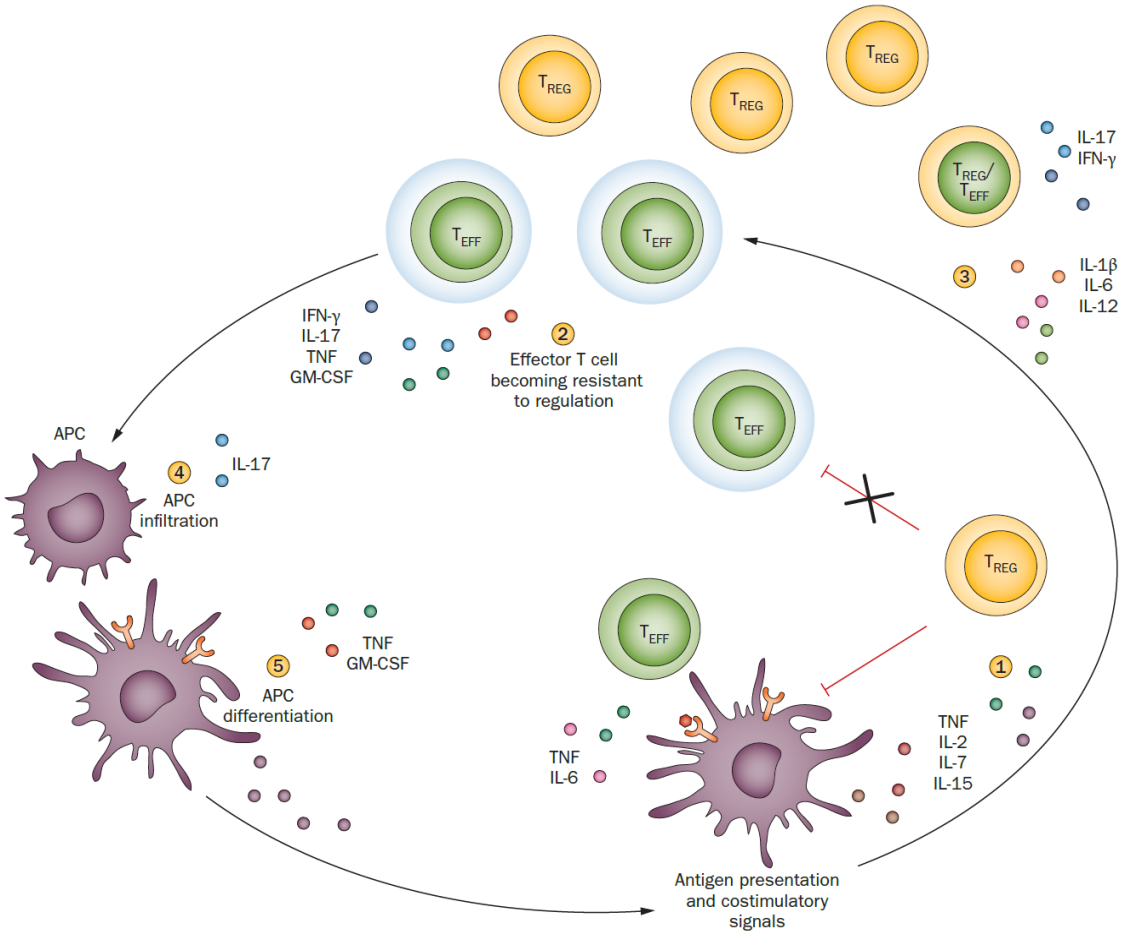


Figure 2. Uncontrolled effector T cells drive inflammation within the rheumatoid arthritis synovium. 1 and **2,** Proinflammatory cytokines and activated antigen-presenting cells (APCs) impair the function of T regulatory (T_{REG}) cells **(1)** and promote resistance of effector T cells (T_{EFF}) to Treg-mediated suppression **(2)** within the inflamed joints of autoimmune arthritis patients. Some Tregs may convert into pathogenic effector T cells in response to signals from proinflammatory cytokines such as IL-1β, IL-6, and IL-12 **(3)**. **4** and **5,** Activated effector T cells, other immune cells, and synoviocytes perpetuate inflammation within the arthritic synovium by secreting cytokines and chemokines (not depicted) leading to the recruitment of monocytes **(4)** and promoting their differentiation into activated macrophages and monocyte-derived dendritic cells **(5)**. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Rheumatology. Wehrens, E.J. et al. T cells out of control—impaired immune regulation in the inflamed joint. *Nat Rev Rheumatol* (2013) **9**:34-42, copyright 2013 (67).

1.4.1 Chemokines and chemokine receptors in cellular trafficking

Cells are continually trafficking throughout the tissues under normal physiological conditions. Leukocyte recruitment to sites of inflammation is key for the containment and sterilizing responses during infection, but is also a major contributing factor to inflammation associated with autoimmune diseases like RA. A highly regulated multistep process directs the recruitment of leukocytes into normal and inflamed tissues. This process involves leukocyte rolling on the vascular endothelium, activation and subsequent firm adhesion of leukocytes to endothelial ligands, transendothelial migration, and trafficking/positioning of cells within the surrounding tissues in response to chemotactic gradients (239,240). Members from a number of families of receptors are crucial in this process, including members of the selectin family, the integrin family, the immunoglobulin superfamily, and chemokine receptors, along with their respective ligands.

Chemokines are a family of small structurally related proteins of 8-12 kDa that can have many functions, but classically are involved in cellular activation to increase integrin adhesiveness and promote leukocyte migration and positioning within tissues (241). Chemokines are classified based on the number and spacing of conserved cysteine residues within the N-terminus into the C, CC, CXC, or CX_3C families. The CC and CXC chemokines are the two larger groups of chemokine families. CC chemokines have adjacent cysteine residues and serve as ligands for one or several of the CC chemokine receptors (CCRs). The CXC chemokines have one non-conserved amino acid between the two conserved cysteines and bind to the CXC chemokine receptors (CXCRs). Subsets of chemokines are constitutively expressed to maintain homeostasis, while others are induced during inflammation. Constitutive chemokines, such as secondary lymphoid-tissue chemokine (SLC; CCL21) and B cell-attracting chemokine-1 (BCA-1; CXCL13), are expressed in secondary lymphoid tissues and regulate the recruitment and positioning of lymphocytes expressing the receptors CCR7 and CXCR5, respectively (242). In contrast, inflammatory chemokines attract leukocytes expressing the chemokine receptors CCR5, CCR6, CXCR3, CXCR6, among others (241). Chemokines signal through G protein coupled receptors (GPCRs) containing seven transmembrane spanning helicies. Selective expression of chemokines and chemokine receptors allows for the differential

recruitment of certain immune cell subsets to sites of inflammation as well as playing a major role in tissue-specific trafficking. For example, gut homing T cells express the chemokine receptor CCR9 (243), whereas T cells homing to the skin express CCR4 (244). Chemokine receptors are attractive therapeutic targets and drugs targeting chemokine receptors have been successfully developed for clinical use in human immunodeficiency virus (HIV) infection (245) and cancer (246).

1.4.2 Chemokines and their receptors in arthritis

Elevated expression of many different chemokines have been detected in the inflamed joints of RA patients. The broad array of chemokines upregulated in the arthritic synovium is mirrored by the diverse inflammatory cellular infiltrate observed in the inflamed joints. Of particular interest among these are the ligands macrophage inflammatory protein 1α (MIP-1α; CCL3), MIP-1β/CCL4, regulated on activation normal T cell expressed and secreted (RANTES; CCL5), monokine induced by γ-interferon (MIG; CXCL9), interferon-γ-induced protein-10 (IP-10; CXCL10), interferon-inducible T-cell α-chemoattractant (I-TAC; CXCL11), CXCL16, and their receptors CCR5, CXCR3, and CXCR6, which are all upregulated within the inflamed synovium (155,247–253).

CCR5 is expressed on effector/memory T cells, iNKT cells, NK cells, monocytes/macrophages, and DCs (241,254). In the $CD4^+$ T cell lineage, CCR5 is preferentially expressed on Th1 cells, but is also present on Tregs (255,256). Consistent with the high prevalence of IFN-γ-producing Th1 cells, multiple studies have demonstrated that T cells isolated from the joints of inflammatory arthritis patients strongly express CCR5 (249,257–260). Elevated levels of the CCR5 ligands MIP-1α/CCL3, MIP-1β/CCL4, and RANTES/CCL5 have been detected in the synovial fluid and tissues of patients with inflammatory arthritis, and likely contribute to the selective recruitment of polarized T cells expressing CCR5 to the inflamed joints (155,247–250,260). In addition to its role in mediating immune cell homing into Th1-type inflammatory sites (261), CCR5 has been shown to function as a T cell costimulator, enhancing T cell proliferation and IFN-γ production following recruitment of CCR5 to the immunological synapse and ligation of MIP-1α/CCL3 or RANTES/CCL5 (262).

CCR5 also plays a role in lymphocyte survival since CCR5-dependent apoptosis has been reported in human T cells in response to high concentrations of RANTES/CCL5 (263), or ligation of CCR5 by the HIV envelop protein gp160 (264). Moreover, iNKT cells in $CCR5^{-/-}$ mice have been shown to be resistant to activation-induced apoptosis in a model of concanavalin A (ConA)-induced hepatitis (265). These studies point to a role for CCR5 in influencing lymphocyte effector responses during activation and the regulation of lymphocyte survival, in addition to its traditional role of mediating immune cell homing.

Similar to CCR5, the chemokine receptor CXCR3 is expressed on effector/memory T cells, primarily on $CD4^+$ Th1 cells, $CD8^+$ CTLs, iNKT cells, and NK cells (241,266), and contributes to the homing of these cells into Th1-type inflammatory sites (267,268). Indeed, multiple studies have demonstrated that T cells from the joints of inflammatory arthritis patients coexpress CXCR3 and CCR5 (249,257–260). Again, the prevalence of $CXCR3^+$ T cells in the inflamed joints is likely a product of the enhanced expression of the CXCR3 ligands MIG/CXCL9, IP-10/CXCL10, and I-TAC/CXCL11 in synovial fluid and tissues of arthritic patients (247,269,270). Consistent with this, blocking IP-10/CXCL10 or CXCR3 inhibited $CD4^+$ T cell infiltration into the arthritic joints in CIA mice (271) and rats with adjuvant arthritis (272). Furthermore, a phase II clinical trial recently reported beneficial responses in RA patients treated with an anti-CXCL10 antibody (273). In addition to their roles in mediating lymphocyte homing, CXCR3 ligands are highly upregulated in the lymph nodes after infection and immunization and play a role in activated effector/memory T cell positioning and interaction with APCs and other activated immune cells (274). Importantly, the CXCR3-dependent positioning of $CD4^+$ T cells within the lymph nodes was required for optimal generation of IFN-γ-producing Th1 cells in vivo (274). In addition, CXCR3 ligands have been demonstrated to have angiostatic effects by inhibiting endothelial cell proliferation (275,276), another example adding to a growing body of literature demonstrating the ability of chemokines to regulate a number of cellular functions. In addition to the chemokines and chemokine receptors described so far, expression of CXCL16 is also abundant in the inflamed joints and synovial T cells are enriched for the expression of CXCR6, suggesting that CXCL16 and CXCR6 are important in the pathogenesis of inflammatory arthritis (251–253,277,278).

1.4.3 CXCL16 and CXCR6 and their role in arthritis

CXCL16 and fractalkine/CX_3CL1 are atypical chemokines since they contain a transmembrane domain, and can be found in both membrane-bound and soluble forms (279–281) (Figure 3). CXCL16 is expressed on the surface of activated APCs such as DCs, macrophages, and B cells (279,280), and was initially discovered independently of its chemokine activity, as a scavenger receptor for oxidized low-density lipoprotein and phosphatidylserine on macrophages (282). Furthermore, CXCL16 was found to mediate phagocytosis of bacteria by macrophage and DCs (283). In its membrane-bound form, CXCL16 also mediates adhesion of $CXCR6^+$ lymphocytes (284,285), whereas cleavage of CXCL16 from the cell surface by a disintegrin and metalloproteinase 10 (ADAM-10) (286,287) releases a soluble form of CXCL16 that mediates migration of activated T cells (279,280) (Figure 3). Additionally, an alternatively spliced isoform of CXCL16 lacking the transmembrane and cytoplasmic domains has been shown to be secreted and to attract $CXCR6^+$ cells (288).

CXCR6 is expressed on T cells, iNKT cells, NK cells, and plasma cells (277,279,289–294). CXCR6 expression on T cells is restricted to effector/memory populations and defines polarized subsets of Th1 and Th17 cells (277,295,296). $CD8^+$ T cells constitutively express low levels of CXCR6 which increases dramatically on these cells following activation (277,279). The expression of CXCL16 on APCs suggests that there is a potential role for interactions between transmembrane CXCL16 and CXCR6 during T cell activation and cytokine polarization (Figure 3), similar to that previously shown for CCR5 (262). In line with this, iNKT cells in $CXCR6^{-/-}$ mice exhibit impaired cytokine production following activation (159,297), and DCs from $CXCL16^{-/-}$ mice are impaired in their ability to stimulate IFN-γ production from wild-type iNKT cells (298). Furthermore, $CXCR6^{-/-}$ mice exhibit defects in the accumulation/retention and/or survival of mature iNKT cells in the liver (159,299). These data suggest a critical role for CXCR6 in normal iNKT cell development and function, in addition to iNKT cell homing and homeostasis.

CXCR6 and CXCL16 have been implicated in multiple inflammatory conditions including intestinal inflammation (300), transplant rejection (301), microbial infection

(302,303), cancer (297,304), and inflammatory arthritis (251–253,277,278). However, the mechanisms by which CXCR6 and CXCL16 contribute to inflammation are not clear. Studies have shown that CXCR6 is highly enriched on T cells isolated from the inflamed synovium of patients with RA (251–253,277), psoriatic arthritis (277), or JIA (278). Similarly, expression of CXCL16 is elevated in the arthritic synovium and mediates the migration of synovial $CXCR6^+$ T cells in vitro (251–253). Within the arthritic synovium, CXCL16 is present in both soluble form in synovial fluid and membrane-bound form on synovial monocytes/macrophages and FLS (251–253). Nanki et al. (251) reported elevated mRNA and protein expression of CXCL16 and CXCR6 in the synovium of mice with CIA. Moreover, they demonstrated that treatment with anti-CXCL16 antibody reduced clinical disease severity scores and resulted in milder histologic changes in the joints of CIA mice (251). However, it is unclear if CXCR6 and CXCL16 function primarily to mediate cellular recruitment to inflamed synovial tissue, or whether CXCR6-CXCL16 interactions also contribute to other mechanisms that impact disease development and/or progression.

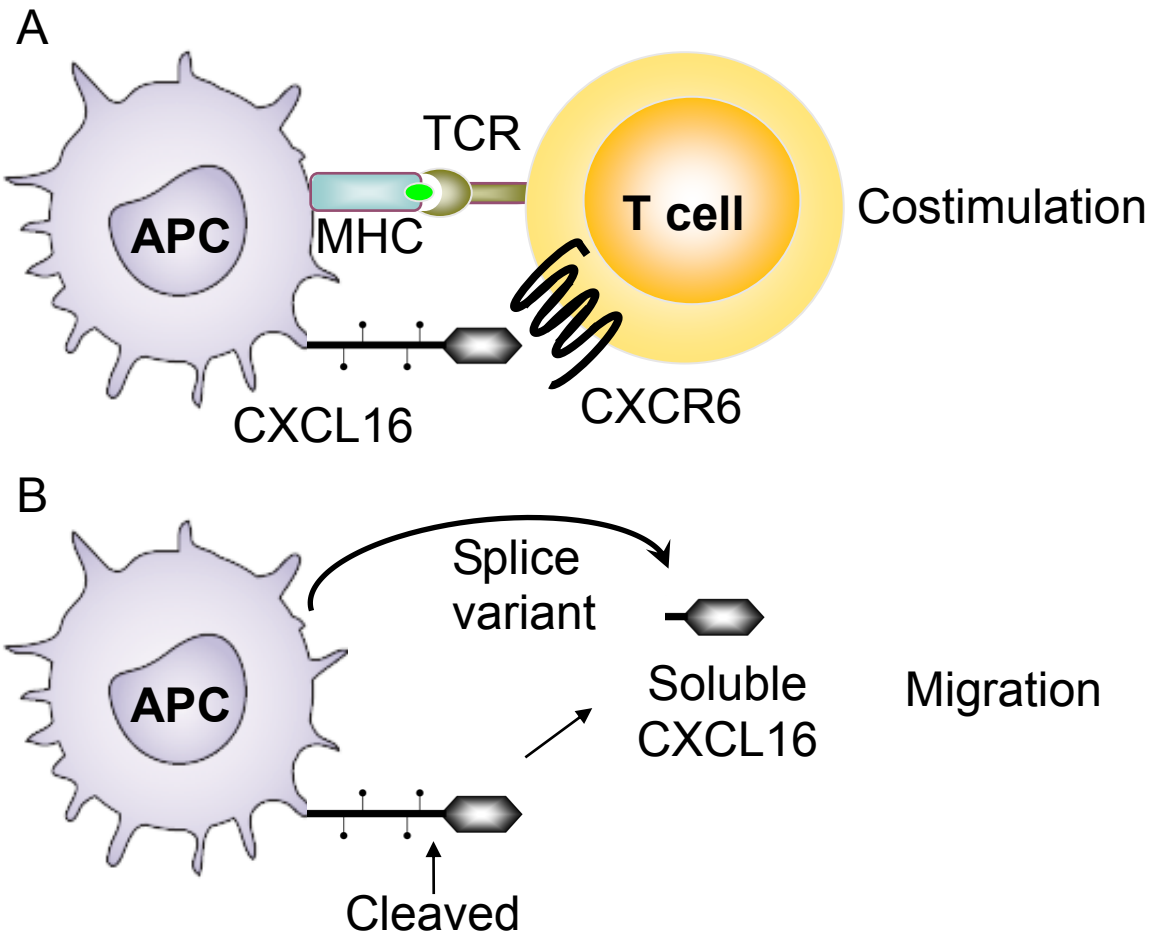


Figure 3. Cellular activities of CXCL16 could be related to distinct structural forms of the ligand. A, CXCL16 contains a transmembrane domain and can be found in a membrane-bound form expressed on the surface of antigen-presenting cells (APCs). Membrane-bound CXCL16 promotes strong adhesion of lymphocytes that express CXCR6 and has a possible role in costimulation of T cells during antigen-activation and cytokine polarization. **B,** A soluble form of CXCL16 is produced by the cleavage of native CXCL16 or as an alternatively spliced variant and acts as a chemoattractant for $CXCR6^{+}$ cells.

1.5.1 NKT cells in humans and mice

NKT cells are a subset of T cells that play a pivotal role at the interface between innate and adaptive immune responses. Unlike the diverse TCR repertoire of conventional T cells that recognize peptide antigens presented by MHC class I or class II, NKT cells express a restricted repertoire of TCR rearrangements that recognize bacterial-derived or endogenous lipid antigens in the context of the MHC-like molecule CD1d (305–307). Two major subsets of NKT cells can be distinguished based on their TCR repertoire and lipid reactivity. Type I or invariant NKT cells express an invariant TCR α-chain composed of Vα24-Jα18 rearrangements in humans and Vα14-Jα18 in mice, paired with a restricted repertoire of Vβ chains (Vβ11 in humans, and Vβ8.2, Vβ7, or Vβ2 in mice) (308,309). The glycolipid α-galactosylceramide (α-GalCer) and its analogs are potent activators of iNKT cells, and this reactivity has been exploited for the specific detection of iNKT cells though the use of CD1d tetramers loaded with α-GalCer (310,311). Type II NKT cells are also CD1d-restricted but they do not recognize α-GalCer (312). In contrast to iNKT cells, type II NKT cells are a more heterogeneous population of cells that express oligoclonal TCRs composed of a limited collection of Vα (Vα1, Vα3, Vα8) and Vβ-rearrangements (312–314). A glycolipid known as sulfatide (3-sulfated galactosylceramide) has been described as an activator of a subset of type II NKT cells (312,314,315).

Invariant NKT cells, the focus of this thesis, populate multiple tissues and overall, the tissue distribution of iNKT cells in the periphery appears to be similar between adult humans and mice. In the adult mouse, iNKT cell frequency is highest in the liver (12-30% of liver lymphocytes), whereas lower iNKT cell frequencies are detected in the lungs (5-10%), spleen (1-3%), bone marrow (0.4-8%), thymus (0.5-1%), lymph nodes (0.2-1%), intestines (0.05-0.6%), and blood (0.2%) (310,311,316–322). Despite a similar peripheral distribution, the frequency of iNKT cells in humans is much lower and subject to considerable variability among individuals, which appears to be influenced by genetic factors based on evidence from twin studies (323). Indeed, frequencies of iNKT cells range from 0.05% to 1% of liver lymphocytes in humans (324,325). Among peripheral blood mononuclear cells, iNKT cells typically account for 0.01-0.1%, but have been

observed to constitute upwards of 3% in some individuals (323,326–328). Nonetheless, iNKT cells play important roles in human health and disease, since dysfunctional iNKT cell responses and reduced circulating numbers of iNKT cells have been reported in patients with autoimmune disorders (329) and malignancies (330,331). Moreover, iNKT cells are absent in patients with X-linked lymphoproliferative disease (XLP) (332). XLP is caused by mutations in *SH2D1A*, the gene encoding signaling lymphocytic activation molecule (SLAM)-associated protein (SAP) (333), an adaptor involved in intracellular signaling elicited through multiple immune cell receptors belonging to the SLAM family. Functional SAP is essential for iNKT cell development in both humans and mice (332). Most patients with XLP are typically asymptomatic, but upon EBV exposure, many go on to develop severe infectious mononucleosis and pass away during or soon after primary infection (334). Those surviving primary EBV infection commonly develop dysgammaglobulinemia, lymphoma, and/or other lymphoproliferative disorders (335). Importantly, a subset of XLP patients have immunological abnormalities before any evidence of EBV exposure (336). In addition to lacking iNKT cells, XLP patients have deficiencies in memory B cells (despite intact B cell development), as well as impaired IL-10 production and ICOS expression by $CD4^+$ T cells (337), which likely underlie the hypogammaglobulinemia in XLP. While the degree to which iNKT cell-deficiency in XLP contributes to the clinical phenotypes in patients remains uncertain, the absence of iNKT cells may have repercussions on several immunological abnormalities in XLP, such as impaired B cell function (338), susceptibility to EBV infection, and the development of lymphoma (339,340).

1.5.2 Invariant NKT cell activation

Invariant NKT cells are activated early during a variety of infections and inflammatory diseases. Upon activation, iNKT cells rapidly generate and secrete a diverse array of cytokines and chemokines (160,297,341), allow them to influence the development of subsequent immune responses in autoimmune disease (329,342), infection (343,344), allergy (345,346), and cancer (347). Invariant NKT cells can be activated through a number of different mechanisms, including TCR-mediated activation by exogenous microbial-derived lipid antigens and cytokine-driven activation alone, or in

combination with TCR-mediated signals following recognition of CD1d-presented self-lipid antigens (305,348,349). Additional mechanisms that contribute to iNKT cell activation during inflammation include stimulation by TLR ligands (350–352), engagement of FcγR by antigen-bound IgG antibodies (353), and in response to neurotransmitters (354).

Following activation, iNKT cells are able to produce a range of cytokines including IFN-γ, TNF, IL-2, IL-4, IL-10, IL-13, IL-17, IL-21, IL-22, and granulocyte-macrophage colony-stimulating factor (GM-CSF) (160,297,341,355). The cytokine profile following activation via TCR-CD1d interactions is influenced by the nature of the stimulation. Indeed, some variants of the original α-GalCer have been shown to induce a more Th1 skewed cytokine profile, whereas others induce a more potent Th2 cytokine response by iNKT cells (356–359). Possible explanations for these differences include variable affinity or duration of TCR engagement of different glycolipid agonists, the route and kinetics of glycolipid uptake and loading onto CD1d, and the type of APC (360). In addition to these factors, the cytokine profile of activated iNKT cells may also be influenced by the subset of iNKT cells that are activated. A number of studies have identified distinct lineages of iNKT cells that emerge during development, each with a unique profile of transcription factors and cytokine production (194,361–367). Based on these profiles, iNKT cells can be subdivided into NKT-1, NKT-2, NKT-17, and NKT-10 subsets, analogous to the Th1, Th2, Th17, and IL-10 producing subsets of conventional T cells. Through their secretion of various cytokines, iNKT cells are able to activate other immune cells and contribute to NK cell transactivation (368), DC maturation (369,370), T cell polarization (371,372), and influence B cell antibody responses (373).

1.5.3 Invariant NKT cells in arthritis

Invariant NKT cells have been suggested to have either protective or harmful roles in a variety of pathological states, including autoimmunity (342,374), infection (343,344), allergy (345,346), and cancer (347). Multiple studies have suggested that reduced numbers of iNKT cells in peripheral blood correlate with active disease in RA patients (375–381). Indeed, Parietti et al. (380) noted a trend toward lower iNKT cell numbers in RA patients with higher disease activity, whereas clinical remission of RA following

rituximab therapy was associated with an increase in iNKT cell frequency (380). Similarly, iNKT cell frequency has been shown to increase as early as two weeks after initiating methotrexate therapy, but no obvious link for this increase could be made to clinical response in those patients (381). Uncertainty remains with respect to the clinical relevance of the reduced iNKT cell frequencies in patients with autoimmune disease, and whether there is value in monitoring iNKT cell deficiency as a biomarker for disease susceptibility or clinical outcomes, or even whether treating iNKT cell deficiency in these patients would provide clinical benefit.

Another important consideration in observational studies of human autoimmune disease patients is that analysis of human blood iNKT cell numbers or function may not accurately reflect the populations of iNKT cells within other tissues. Studies have determined that accumulation of iNKT cells within the inflamed synovium is unlikely to explain the reduced pool of iNKT cells within the peripheral blood in RA patients (377,379). However, assessing the reactivity of iNKT cells to α-GalCer revealed a large proportion of non-responders isolated from RA patients, whereas iNKT cells from healthy donors showed good responses in vitro (376,379,381). This suggests that the peripheral iNKT cell pool from RA patients may be functionally anergic, possibly due to chronic activation in patients with active disease. In line with this, stimulation of iNKT cells via the delivery of free α-GalCer in mice causes iNKT cell anergy for an extended period of time (382,383). It is also possible that the reduced levels of iNKT cells observed in the peripheral blood from RA patients is due to their chronic activation, since it has been shown that following stimulation with ligand, iNKT cells rapidly internalize surface receptors used to identify these cells, such as the invariant TCR (384–386). Linsen et al. (379) provided further evidence that iNKT cell responses are altered in RA patients by demonstrating that iNKT cells were biased towards a Th1-like phenotype. Taken together, these reports suggest a possible role of iNKT cells in human arthritic disease, but further investigation is required before interventions targeting iNKT cells can be considered in RA.

Much of the evidence for the role of iNKT cells in RA and other diseases comes from experimental mouse models. Two major strategies have been employed to study the

role of iNKT cells in CIA and other arthritis models. One approach involves investigating the intrinsic role of NKT cells through immunization of NKT cell-deficient $CD1d^{-/-}$ or $J\alpha18^{-/-}$ mice. The other is through evaluating disease development in mice following iNKT cell activation by administration of glycolipid agonists. From these studies it is clear that iNKT cells play a role in joint inflammation, which may vary depending on the timing of iNKT cell activation, the use of Th1– versus Th2-biased iNKT cell agonists, and the nature of the model of inflammatory arthritis used. In CIA and antibody-induced arthritis models, mice lacking iNKT cells exhibited reduced incidence and severity of disease compared to wild-type mice (353,387–391). Protection from CIA in iNKT cell-deficient mice was associated with reduced serum levels of anti-CII IgG or skewed IgG1/IgG2a ratios, reduced IL-17^+ $CD4^+$ T cells, and lower CII-specific IL-17 production, but increased IL-10 (387–390). Indeed, iNKT cells were shown to be activated early during CIA induction, resulting in their secretion of IFN-γ, IL-17, and IL-4 (392). Furthermore, CIA mice treated with a blocking anti-CD1d antibody exhibited reduced disease incidence, delayed onset, and attenuated clinical and histological severity (392).

The role of iNKT cells in inflammatory arthritis pathogenesis is complicated by studies demonstrating attenuation of arthritis in mice treated with glycolipid agonists such as α-GalCer and its analogs (393–396). Protection in CIA mice treated with OCH, an analog of α-GalCer that preferentially promotes Th2-type cytokine secretion, was abolished upon simultaneous in vivo neutralization of IL-4 or IL-10 (393), suggesting that suppression of CIA by this iNKT cell agonist was due to a shift towards protective Th2 immunity. Attenuation of CIA following treatment with α-GalCer was associated with elevated IL-10 production by $CD4^+$ T cells (394,395). Interestingly, IFN-γ produced following in vivo iNKT cell stimulation with α-GalCer was demonstrated to have a differential biphasic role during the course of disease in CIA mice, since neutralization of IFN-γ release induced by α-GalCer early during CIA resulted in reduced clinical signs of arthritis, whereas blockade of IFN-γ release later resulted in a more rapid onset of arthritis (395). In the absence of IFN-γ neutralization, early administration of α-GalCer resulted in reduced clinical severity of CIA, whereas administration of glycolipid later in the course of the CIA model (day 20 post-immunization) had no effect on disease severity but

resulted in delayed onset to clinical signs of disease (395). Therefore, reports in iNKT cell-deficient mice suggest that iNKT cells exhibit an intrinsic capacity to modulate CIA, which is clearly distinct from the suppressive ability of iNKT cells upon activation with glycolipid antigen.

1.6 Project objectives

The expression of CXCR6 and CXCL16 have been linked to autoimmune arthritis yet their role in the pathogenesis of arthritis remains unclear. The first objective of this study was to compare CIA development in wild-type mice and mice deficient in CXCR6 or CXCL16. CXCL16 can be expressed on the cell surface as a transmembrane molecule on APCs, so I hypothesized that CXCR6-CXCL16 interactions modulate the responses of effector T cells during antigen presentation and T cell cytokine polarization. Therefore, the second objective was to determine whether the absence of CXCR6 affects Th1, Th2, or Th17 cytokine polarization in immunized and/or arthritic mice. This was examined by comparing Th1, Th2, and Th17 cytokine levels in T cells isolated from the spleen, lymph nodes, and paws of wild-type and $CXCR6^{-/-}$ mice, as well as measuring cytokine production by lymph node cells following CII restimulation in vitro. Soluble CXCL16 mediates the migration of activated T cells expressing CXCR6, and expression of CXCL16 is enhanced in the arthritic synovium in both humans and mice. Therefore, the third objective was to test the hypothesis that CXCR6 is important for T cell homing to the inflamed joints in arthritis. Coadoptive transfers of wild-type and $CXCR6^{-/-}$ T cells were used to determine whether CXCR6 plays a role in activated T cell homing to arthritic joints. In addition to CXCL16 and CXCR6, other chemokines and their receptors, including CCR5 and CXCR3, have been implicated in inflammatory arthritis. Therefore, the fourth objective was to investigate whether these chemokine receptors are upregulated on T cells in the inflamed paws of CIA mice and compare arthritis development in wild-type mice and mice deficient in CCR5 or CXCR3.

Invariant NKT cell defects have been demonstrated in RA patients, whereas iNKT cells have been shown to play both protective and pathogenic roles in animal models of RA. Invariant NKT cell-deficient $J\alpha18^{-/-}$ mice are an important tool for examining the roles of iNKT cells in vivo. However, $J\alpha18^{-/-}$ mice have an estimated 60% reduction in

TCR α-chain repertoire diversity due to interference from a neomycin-resistance cassette remaining at the Jα18 locus (397). This could confound interpretation of results from studies assessing the roles of iNKT cells in disease via comparisons of Jα18$^{-/-}$ mice to wild-type mice. Therefore, the objectives of this part were two-fold. The first was to examine arthritis development in wild-type mice, Jα18$^{-/-}$ mice, and Jα18$^{-/-}$ mice reconstituted with wild-type iNKT cells. Since iNKT cells are a potent source of immunoregulatory cytokines, the second objective was to examine whether adoptive transfer of IFN-γ– or TNF-deficient iNKT cells impacted CIA development in Jα18$^{-/-}$ recipient mice. These studies provide evidence for the roles of CXCR6/CXCL16 and iNKT cells in inflammatory arthritis pathogenesis and contribute to ongoing efforts to identify promising novel therapeutics for the treatment of RA and other inflammatory diseases.

CHAPTER 2: MATERIALS AND METHODS

2.1 Mice

C57BL/6 (H-2^b) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) or Charles River Laboratories (St. Constant, QC). $CXCR6^{-/-}$ mice, in which the coding sequence for CXCR6 is replaced with that of green fluorescent protein (GFP) (398), were obtained from Dr. D. R. Littman (New York University Medical Center, New York, NY) and backcrossed 14 generations against C57BL/6 mice. $J\alpha18^{-/-}$ mice were obtained from Dr. M. Taniguchi (RIKEN Research Center for Allergy and Immunology, Kanagawa, Japan) (399). Mice deficient in both Jα18 and CXCR6 were generated by mating $J\alpha18^{-/-}$ males with $CXCR6^{-/-}$ females. All heterozygous animals appeared normal and were intercrossed to produce $J\alpha18^{-/-}CXCR6^{-/-}$ double knockout mice. IFN-$\gamma^{-/-}$, $TNF^{-/-}$, $CCR5^{-/-}$, $CXCR3^{-/-}$, and $CXCL16^{-/-}$ mice were purchased from The Jackson Laboratory. Animals were housed at the Carleton Animal Care Facility at Dalhousie University. Experiments were performed with approval from the University Committee on Laboratory Animals following guidelines of the Canadian Council on Animal Care.

2.2 Induction and clinical evaluation of CIA

Incomplete Freund's adjuvant (IFA) was prepared as a mixture of 15% Arlacel A (mannide monooleate; Fisher Scientific, Mississauga, ON) and 85% heavy paraffin (mineral) oil (Fisher Scientific). Heat-killed *M. tuberculosis* (H37Ra; Difco, Detroit, MI) was ground into IFA using a mortar and pestle to generate CFA (containing 5 mg/ml *M. tuberculosis*). Chicken CII (Sigma-Aldrich, Oakville, ON) was dissolved at 5 mg/ml overnight at 4°C in 10 mM acetic acid (BDH Chemicals, Mississauga, ON) and emulsified in an equal volume of CFA using an electric homogenizer. CII solutions, adjuvant and emulsions were generated fresh prior to injection. Mice were anaesthetized with 2% inhaled isoflurane (AErrane; Baxter, Mississauga, ON) prior to immunization. Eight to twelve week old female mice were immunized by subcutaneous injection of 100 µl emulsion (containing 250 µg CII and 250 µg *M. tuberculosis*) at two separate sites (50 µl each) on the back ~1 cm from the base of the tail. Mice were boosted with an

identical emulsion on day 21. In some experiments, mice were immunized with CFA lacking CII.

Mice were examined for onset and severity of arthritis using the following scoring system: grade 0 = normal; 1 = light swelling and redness of one to two digits, the mid foot, or the ankle joint; 2 = mild swelling and redness of the digits and/or mid foot and over the ankle; 3 = moderate swelling and redness over the ankle and midfoot extending through the digits; 4 = gross swelling and redness over the ankle and extending through the digits. Individual limb scores were summed for each mouse, with a maximum score of 16 per mouse. Paw thickness was measured using digital calipers (Mitutoyo Canada, Montreal, QC).

2.3.1 Cell isolation

Mice were anesthetized and sacrificed by cervical dislocation. Spleen and lymph node cells were obtained by pressing tissues through stainless steel wire mesh (70 μm pores). Cells were pelleted by centrifugation at 300g for 10 min at 4°C. Erythrocytes were lysed by resuspending the cells in 5 ml of lysis buffer containing 150 mM NH_4Cl (Sigma-Aldrich), 10 mM $KHCO_3$ (J.T. Baker, Montreal, QC), and 0.1 mM ethylenediamine-tetraacetic acid (EDTA; Sigma-Aldrich) for 5 min, followed by an equal volume of 2X phosphate buffered saline (PBS; Thermo-HyClone, Ottawa, ON) to inhibit further lysis. Following centrifugation, cells were resuspended in 1X PBS supplemented with 2% fetal bovine serum (FBS; Thermo-Hyclone) and counted using a hemocytometer.

Lung tissues were cut into small pieces and transferred to 15 ml conical tubes containing 1 mg/ml DNase I (Roche, Laval, QC) and 417.5 μg/ml Liberase TL (Roche) and incubated for 1 h at 37°C. Following enzyme digestion, lung tissues were pressed through stainless steel wire mesh and pelleted by centrifugation at 500g for 5 min at room temperature. Livers were dissociated by pressing through a stainless steel wire mesh and washed twice with 1X PBS supplemented with 2% FBS via centrifugation at 500g for 7 min at room temperature. Liver and lung cell pellets were resuspended in 33.7% isotonic Percoll solution (GE Healthcare, Baie d'Urfe, QC) and centrifuged at 700g for 12 min at room temperature. Lymphocyte rich pellets were collected and erythrocytes were lysed as

above. Cells were resuspended in 1X PBS with 2% FBS and counted using a hemocytometer. Bone marrow cells were isolated from mouse femurs and tibias by flushing the bones with 1X PBS + 2% FBS using a 25G needle and pressing bone marrow through a stainless steel wire mesh. Cells were pelleted by centrifugation at 300g for 10 min at 4°C and erythrocytes were lysed by resuspending bone marrow cells in 3 ml of lysis buffer for 30 sec followed by an equal volume of 2X PBS. Following centrifugation, cells were resuspended in 1X PBS with 2% FBS and counted using a hemocytometer.

2.3.2 Isolation of cells from mouse paws

Prior to removing the paw, an incision was made around the ankle and down the length of the paw using a scalpel. The skin was removed and the paws were isolated by cutting just above the ankle joint. Tissues were diced in a Petri dish containing 3 ml of Roswell Park Memorial Institute (RPMI)-1640 media supplemented with L-glutamine (Thermo-HyClone) but not FBS. Diced paw tissues and media were collected and transferred to 15 ml conical tubes containing 1 mg/ml DNase I (Roche) and 417.5 μg/ml Liberase TL (Roche) and incubated for 1 h at 37°C. Following enzyme digestion, paw tissues were pressed through stainless steel wire mesh and pelleted by centrifugation at 478g for 5 min at room temperature. Paw cell pellets were resuspended in 33.7% isotonic Percoll solution and centrifuged at 700g for 12 min at room temperature. Lymphocyte rich pellets were collected and erythrocytes were lysed as described above and cells were resuspended in 1X PBS with 2% FBS prior to counting with a hemocytometer.

2.4.1 Flow cytometry and cell sorting

The following antibodies were obtained from eBioscience (San Diego, CA), BioLegend (San Diego, CA), or BD Biosciences (Mississauga, ON) unless otherwise specified: Fluorescein isothiocyanate (FITC)-conjugated anti-CD4 (clone RM4-5), FITC-anti-CD8α (clone 53-6.7), FITC-anti-TCRβ (clone H57-597), FITC-anti-CD44 (clone IM7), FITC-anti-CD69 (clone H1.2F3), phycoerythrin (PE)-conjugated anti-CD45RB (clone C363.16A), PE-anti-CD69 (clone H1.2F3), PE-anti-CD4 (clone RM4-5), PE-anti-CD8α (clone 53-6.7), PE-anti-TCRβ (clone H57-597), PE-anti-IL-17A (clone eBiol7B7), PE-anti-IFN-γ (clone XMG1.2), PE-anti-IL-4 (clone 11B11), PE-anti-rat IgG1 (clone

eBRG1), PE-anti-RANKL (clone IK22/5), PE-anti-CCR2 (clone 475301; R & D Systems, Minneapolis, MN), PE-anti-CCR3 (clone 83101; R & D Systems), PE-anti-CCR5 (clone HM-CCR5(7A4)), PE-anti-CCR6 (clone 140706; R & D Systems), PE-anti-CCR7 (clone 4B12), PE-anti-CCR9 (clone eBioCW-1.2), PE-anti-CXCR2 (clone 242216; R & D Systems), PE-anti-CXCR3 (clone CXCR3-173), PE-anti-CXCR4 (clone 2B11), PE-anti-CXCR5 (clone 2G8), PE-rat IgG2a (clone RTK2758), PE-armenian hamster IgG (clone eBio299Arm), PE-rat IgG2b (clone A95-1), PE-goat anti-human Fcγ polyclonal antibody (Jackson ImmunoResearch, West Grove, PA), peridinin chlorophyll protein (PerCP)-Cy5.5-conjugated anti-CCR7 (clone 4B12), PerCP-Cy5.5-rat IgG2a (clone eBR2a), PerCP-Cy5.5-anti-CD3 (clone 145-2C11), PerCP-Cy5.5-anti-CD4 (clone RM4-5), PerCP-Cy5.5-anti-CD8α (clone 53-6.7), PerCP-Cy5.5-anti-TCRβ (clone H57-597), allophycocyanin (APC)-conjugated anti-CD4 (clone RM4-5), APC-anti-CD8α (clone 53-6.7), and APC-anti-TCRβ (clone H57-597). APC-conjugated mouse CD1d tetramers loaded with the α-GalCer analog PBS57, and unloaded CD1d tetramers were obtained from the National Institutes of Health Tetramer Core Facility (Emory Vaccine Center, Atlanta, GA). A rat anti-mouse CD16/32 antibody (clone 93) was used to block Fc receptors prior to surface marker staining for flow cytometry. A mouse CXCL16-human IgG1-Fc chimera fusion protein and PE-goat anti-human Fcγ polyclonal antibody were used to detect CXCR6 surface expression (159,279). Unloaded CD1d tetramers and isotype matched control antibodies were used to establish gating and quadrants. Optimal antibody concentrations for staining were determined by titration prior to use. For surface staining, cells were incubated at 4°C for 20 min, washed with 1X PBS via centrifugation at 300g for 10 min, and cells were fixed by resuspending pellets in a 4% paraformaldehyde solution (Fisher Scientific). Analysis was performed using a two laser FACSCalibur cytometer with CellQuest Pro software (BD Biosciences).

To sort iNKT cells, liver cells were isolated as described earlier, Fc receptors were blocked, and cells were incubated with FITC-anti-TCRβ antibody and APC-conjugated CD1d tetramers loaded with the α-GalCer analog PBS57. Invariant NKT cells were sorted by flow cytometry as $TCR\beta^{+}CD1d\ tetramer^{+}$ cells to greater than 95% purity. Cell sorting was performed using a FACSAria cell sorter with FACSDiva software (BD Biosciences).

2.4.2 Intracellular cytokine staining

Prior to intracellular cytokine staining, Fc receptors were blocked and surface marker staining was performed. Cells were washed and resuspended in complete RPMI-1640 medium (supplemented with 10% FBS, 2 mM L-glutamine, 100 μg/ml streptomycin, 100 units/ml penicillin, 10 mM 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES), and 50 μM 2-mercaptoethanol) in 6-well cell culture plates. Cells were stimulated for 4-6 h with 50 ng/ml phorbol 12-myristate 13-acetate (PMA; Fisher Scientific) and 1 μg/ml ionomycin (Sigma-Aldrich) at 37°C (5% CO_2). Brefeldin A (1 μg/ml; BioLegend) was added for the last 4 h to inhibit protein transport, and activated cells were transferred to 5 ml tubes for intracellular cytokine staining. Cells were fixed in 100 μl of Fixation Buffer (BioLegend) for 20 min at room temperature followed by 2X washes with 1 ml of Permeabilization Buffer (BioLegend). Cells were resuspended in 100 μl of Permeabilization Buffer and stained with fluorochrome-conjugated anti-cytokine antibodies for 30 min at room temperature. Cells were washed with 1 ml Permeabilization Buffer, centrifuged at 300g for 10 min and resuspended in 1X PBS for flow cytometry

2.5.1 In vitro analysis of T cell activity

Inguinal and popliteal lymph nodes were excised 14 or 35 days post-immunization with CII in CFA. B cells were depleted using EasySep CD19 selection kits (StemCell Technologies, Vancouver, BC) according to manufacturer's instructions. For CD19 depletion, cells were resuspended at $1x10^8$ cells/ml in 1X PBS with 2% FBS and 1 mM EDTA and incubated with PE-CD19 Labeling Reagent for 15 min at room temperature. Cell were then incubated with EasySep PE Selection Cocktail containing antibodies directed against PE and dextran bound in bispecific complexes for an equal amount of time, followed by an incubation with a suspension of EasySep magnetic dextran iron nanoparticles for 10 min at room temperature. The cell suspensions were subjected to magnetic separation for 5 min in EasySep magnets. Negatively selected cells were collected and cultured at $2x10^6$ cells/ml in flat-bottomed 96-well plates containing complete RPMI-1640 medium alone, or medium containing 50 μg/ml of denatured CII. CII was dissolved at 1 mg/ml overnight at 4°C in 0.05 M Tris-HCL/0.2 M NaCl solution

and denatured by boiling for 10 min immediately prior to addition to wells. Supernatants were collected after 48 h of culture at 37°C (5% CO_2) for analysis of IL-17A, IFN-γ, and IL-4 via enzyme-linked immunosorbent assay (ELISA). Cells were incubated for a further 18 h in the presence of 1 μCi/well of ^{3}H-thymidine (MP Biomedicals, Cleveland, OH) to quantify proliferation.

2.5.2 Cytokine ELISA

To measure cytokine levels in culture supernatants, ELISA kits for mouse IL-17A, IFN-γ, and IL-4 were used according to manufacturer's instructions (eBioscience). Corning Costar 9018 ELISA plates were coated overnight at 4°C with purified anti-mouse capture antibodies for either IL-17A (clone eBio17CK15A5) diluted 1/250 in coating buffer, IFN-γ (clone AN-18) diluted 1/1000, or IL-4 (clone 11B11) diluted 1/250. After coating, the plates were aspirated and washed using 1X PBS with 0.05% Tween-20 (Sigma-Aldrich) for a total of five washes. ELISA plates were blocked by adding 200 μl of 1X ELISA Diluent (eBioscience), followed by a 1 h incubation at room temperature and a second series of five washes. Two-fold serial dilutions of standards for IL-17A (500 pg/ml), IFN-γ (2000 pg/ml), and IL-4 (500 pg/ml) were prepared along with 1/50, 1/100, or 1/500 dilutions of supernatant samples from in vitro T cell restimulation assays. Wells received 100 μl of diluted standards or samples and were incubated overnight at 4°C.

The following day, the plates were washed for a total of five washes and 100 μl of the following diluted biotin-conjugated mouse anti-cytokine detection antibodies, anti-IL-17A - 1/250 (clone eBio17B7), anti-IFN-γ - 1/1000 (clone R4-6A2), and anti-IL-4 - 1/250 (clone BVD6-24G2) were added to each well of the appropriate plates. After a 1 h incubation at room temperature, the plates were aspirated and washed for a total of five washes. Avidin-horseradish peroxidase (HRP) was diluted 1/250 and 100 μl was added per well followed by a 30 min incubation at room temperature and a series of five washes. The enzymatic substrate tetramethylbenzidine (TMB; 100 μl/well) was added and the reaction was stopped after 15 min by addition of 50 μl/well of 2 N H_2SO_4. The plates were read at 450 nm using an Epoch Microplate Spectrophotometer (BioTek, Winooski,

VT) and sample cytokine concentrations were derived from a standard curve generated using Gen5 software (BioTek).

2.5.3 Measuring T cell proliferation by ^{3}H-thymidine incorporation

To determine the rate of T cell proliferation, cells from the activation cultures described above were incubated for a further 18 h in the presence of 1 μCi/well of ^{3}H-thymidine (MP Biomedicals). Cells were harvested using a Titertek Cell Harvester (Skatron, Sterling, VA) onto fiberglass filter mats (Skatron). Scintillation fluid was added to dried filter mats and ^{3}H-thymidine incorporation was measured in counts per minute (cpm) using a Beckman LS6000IC Liquid Scintillation Counter (Beckman Coulter, Mississauga, ON).

2.5.4 In vitro T cell activation in anti-CD3ε coated plates

Splenocytes were isolated from naïve wild-type and CXCR6$^{-/-}$ mice and 2x10^{5} cells/well were incubated for 5 days at 37°C (5% CO_2) in anti-CD3ε-coated 96-well culture plates containing complete RMPI-1640 supplemented with 2 μg/ml anti-CD28 antibodies (clone 37.51; BioLegend) and 4 ng/ml mouse IL-2 (PeproTech, Rocky Hill, NJ). Cells were collected and transferred to uncoated wells and cultured in complete RMPI-1640 supplemented with 2 ng/ml IL-2 for an additional 2 days. Cells density and viability were determined using a hemocytometer and 0.4% trypan blue dye exclusion (Lonza, Mississauga, ON). Cells were used to assess CXCR6 expression by flow cytometry, examine migration in Transwell chemotaxis assays, or to assess homing in naïve or arthritic mice.

2.5.5 Transwell chemotaxis assays

Wild-type and CXCR6$^{-/-}$ cells were collected following in vitro anti-CD3ε-activation and 1x10^{6} cells were placed in the upper chamber of Transwell inserts (5 μm pore size; Corning Costar, Corning, NY). Inserts were placed in wells containing medium alone or medium supplemented with mouse CXCL16 (1 ng/ml to 1000 ng/ml; PeproTech, Rocky Hill, NJ). After a 2 h incubation at 37°C (5% CO_2), Transwell inserts (5 μm pores) were removed and a known number of polystyrene beads (Polysciences, Warrington, PA)

were added to each well as an internal standard. Migrated lymphocyte populations were stained with fluorochrome-conjuated antibodies to identify T cell populations by flow cytometry. The frequency of migrating $CD4^+$ and $CD8^+$ T cells were determined by comparing the bead to cell ratio in the migrated and input populations (159).

$$\% \text{ Chemotaxis} = \frac{(\text{beads/migrated cells})}{(\text{beads/input cells})} \times 100\%$$

2.5.6 Labeling and adoptive transfer of in vitro activated T cells

Splenocytes from wild-type and $CXCR6^{-/-}$ mice were activated by anti-CD3ε stimulation as described above. Cell density was adjusted to $1.5x10^6$ cells/ml by adding incomplete RPMI-1640 (without FBS or any other supplementation). Wild-type T cells were labeled with 4.5 μM CellTracker Orange CMTMR (5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine; Molecular Probes, Burlington, ON), while $CXCR6^{-/-}$ T cells were labeled with 1 μM CellTracker Green CMFDA (5-chloromethylfluorescein diacetate; Molecular Probes) for 30 min at 37°C (5% CO_2). Cells were washed and resuspended in 40 ml of incomplete RPMI-1640 for 1 h at 37°C (5% CO_2). After three washes with 1X PBS, cell density and viability were determined using a hemocytometer and 0.4% trypan blue dye exclusion. Equal numbers of the two labeled populations were combined in saline (0.9% NaCl; Braun Medical, Irvine, CA) for transfer into recipient mice.

Naïve or arthritic wild-type mice at 35 days post-immunization received intravenous (i.v.) tail vein injections of ~$2.5x10^7$ cells containing a 1:1 mixture of labeled wild-type and $CXCR6^{-/-}$ cells. The spleen, popliteal and inguinal lymph nodes, and paws were removed from each recipient mouse 24 h post-transfer and single cell suspensions were generated from each tissue as described earlier. In naïve recipient mice, the liver, lungs and bone marrow were also collected. Samples were examined by flow cytometry to differentiate homing of dye-labeled wild-type and $CXCR6^{-/-}$ cells.

2.6 Measuring anti-type II collagen IgG's by ELISA

Blood samples were obtained via submandibular puncture from nonimmunized and arthritic wild-type and $CXCR6^{-/-}$ mice at day 35 post-immunization. Samples were

collected into tubes containing 10 µl of 10,000 U/ml heparin (Sigma-Aldrich). Following centrifugation at 5000g for 5 min, the serum was collected and anti-chicken CII antibodies were measured using mouse IgG1 and IgG2a anti-CII antibody ELISA kits according to manufacturer's instructions (Chondrex, Redmond, WA). Type II chicken collagen-coated 8-well strips were blocked by adding 100 µl/well of ELISA Blocking Buffer for 1 h at room temperature followed by three washes with 1X PBS containing 0.05% Tween-20 (Sigma-Aldrich). Two-fold serial dilutions of standards for IgG1 and IgG2a were prepared using ELISA Sample Dilution Buffer. Serum samples were centrifuged at 7826g for 3 min at room temperature to remove insoluble materials, and diluted 1/10,000 in ELISA Sample Dilution Buffer. Wells received 100 µl of diluted standards or samples and were incubated overnight at 4°C.

The following day, strips were washed three times and HRP-conjugated rabbit anti-mouse IgG1 or IgG2a secondary antibodies were added to each well. After a 2 h incubation at room temperature, the plates were aspirated and washed for a total of three washes. The enzymatic substrate ortho-phenylenediamine (OPD; 100 µl/well) was added and the reaction was stopped after 30 min by addition of 50 µl/well of 2 N H_2SO_4. The plates were read at 490 nm using an Epoch Microplate Spectrophotometer (BioTek) and sample IgG subtype antibody concentrations were derived from a standard curve generated using SoftMax Pro software (Molecular Devices, Sunnyvale, CA).

2.7 DTH reactions following challenge with CII

Wild-type and CXCR6$^{-/-}$ mice were immunized with CII in CFA as described earlier. At day 19 post-immunization, mice were challenged in the ear pinna with CII. Prior to injection, 0.5 mg/ml CII was dissolved in 5 mM acetic acid overnight at 4°C. Immunized mice were anaesthetized with 2% inhaled isoflurane prior to challenge. A 20 µl volume containing 10 µg dissolved CII was injected intradermally into the left ear, while 20 µl of 5 mM acetic acid alone (vehicle) was delivered into the right ear of the same mouse using a 31G needle. Ear thickness was measured at 24 h and 48 h post-challenge using digital calipers. Collagen specific inflammation was evaluated as the difference in ear thickness from the ear receiving CII compared to the ear receiving vehicle alone.

2.8.1 In vitro generation of bone marrow-derived dendritic cells

Bone marrow-derived dendritic cells (BMDCs) were prepared as previously described (400). Briefly, femurs and tibias were collected from wild-type mice by sterile dissection of the hind legs and cells were flushed from the marrow cavities with 1X PBS + 2% FBS using a syringe and 25G needle. Cells were pressed through 40 μm sterile cell strainers (Fisher Scientific), pelleted by centrifugation at 300g for 10 min at 4°C, and erythrocytes were lysed by resuspending cells in 3 ml of lysis buffer for 30 sec followed by an equal volume of 2X PBS. Bone marrow cells were cultured in 6 well plates with complete RPMI-1640 (containing 10% FBS, 2 mM L-glutamine, 100 μg/ml streptomycin, 100 units/ml penicillin, 50 μM 2-mercaptoethanol, and 1 mM sodium pyruvate) supplemented with 40 ng/ml mouse GM-CSF (PeproTech) and 10 ng/ml mouse IL-4 (PeproTech).

After 3 days at 37°C (5% CO_2), complete RPMI-1640 medium supplemented with 40 ng/ml GM-CSF and 10 ng/ml IL-4 was added to the cells for an additional 3 days. On day 6, non-adherent cells were collected, washed, and cultured in complete RPMI-1640 medium supplemented with 20 ng/ml GM-CSF for 24 h at 37°C (5% CO_2). On day 7 of culture, BMDCs were loaded overnight with 0.4 μg/ml α-GalCer ((2S,3S,4R)-1-O-(α-D-galactipyranosyl)-2-(N-hexacosanoylamino)-1,3,4-octadecanetriol); Toronto Research Chemicals, Toronto, ON). Cells were harvested from culture and cell density and viability were determined using a hemocytometer and 0.4% trypan blue dye exclusion prior to delivery of α-GalCer-loaded BMDCs into mice for in vivo iNKT cell expansion.

2.8.2 In vivo iNKT cell expansion

BMDCs loaded with α-GalCer were resuspended in saline (0.9% NaCl; Braun Medical) at $4x10^6$ cells/ml and wild-type, IFN-$\gamma^{-/-}$ or $TNF^{-/-}$ mice received i.v. injection of $6x10^5$ cells to induce in vivo expansion of iNKT cells. After 72 h, liver lymphocytes were harvested as described earlier and iNKT cells were sorted to >95% purity. Invariant NKT cells were resuspended in saline (0.9% NaCl; Braun Medical) and cell density and viability were determined using a hemocytometer and 0.4% trypan blue dye exclusion prior to reconstitution into J$\alpha18^{-/-}$ mice or J$\alpha18^{-/-}$CXCR6$^{-/-}$ mice.

2.8.3 NKT cell reconstitution of Jα18$^{-/-}$ mice for evaluation of iNKT cells in CIA

Jα18$^{-/-}$ mice or Jα18$^{-/-}$CXCR6$^{-/-}$ mice were reconstituted via i.v. injection with ~3x10^6 sorted iNKT cells (>95% purity) expanded from wild-type, IFN-γ$^{-/-}$ or TNF$^{-/-}$ mice 14 days prior to the primary CIA immunization, unless otherwise stated. In some groups, recipient Jα18$^{-/-}$ mice received a second i.v. injection of ~3x10^6 sorted iNKT cells 7 days post-immunization. Jα18$^{-/-}$ recipient mice received CIA booster immunizations 14 days after the second iNKT cell transfer and were evaluated for the development of CIA over the following weeks as described earlier.

To improve reconstitution, Jα18$^{-/-}$ mice were given sublethal whole body irradiation from a Gammacell 3000 (^{137}Cs source; Nordion) at a dose of 750 rads (7.5 Gray) in one single administration 48 h prior to adoptive transfer of sorted expanded iNKT cells (i.v. ~3x10^6 cells). Reconstituted Jα18$^{-/-}$ mice were housed for 4 weeks to ensure stable reconstitution. Non-recipient control mice were also subjected to the same irradiation protocol. After 4 weeks, irradiated non-recipient and iNKT cell-reconstituted Jα18$^{-/-}$ mice were immunized and boosted, as outlined earlier, and evaluated for the development of CIA.

2.9 Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). A non-parametric two-tailed Mann-Whitney test was used to compare between two data groups (GraphPad Instat software v3.10; San Diego, CA). Statistical analysis of arthritis incidence was performed using a log-rank significance test (GraphPad Prism software v5.04; San Diego, CA). Comparisons between three or more data groups were performed by one way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test (GraphPad Instat software v3.10). Statistical significance was set at $P < 0.05$.

CHAPTER 3: RESULTS

3.1 FUNCTIONAL CHARACTERIZATION OF CXCR6 ON T CELLS IN CIA

3.1.1 Upregulation of CXCR6 Expression on T cells in CIA

Surface expression of CXCR6 is increased on immune cells within the synovium of patients with arthritis (251–253,277,278). We examined CXCR6 expression on T cell subsets in nonimmunized control mice and arthritic mice. To induce arthritis, wild-type C57BL/6 mice were immunized with CII in CFA, followed by an identical booster 21 days later. The frequency and number of $CD4^+$ and $CD8^+$ T cells expressing CXCR6 were increased in the paws (Figures 4A and 4B), as well as the inguinal lymph nodes (draining the immunization sites), the popliteal lymph nodes (draining lymph nodes of the hind limbs), and spleen (Figures 5A and 5B) of arthritic mice compared to nonimmunized mice. A large proportion of the $TCR\beta^+$ population within the paws displayed a $CD4^-CD8^-$ [double negative (DN)] phenotype, whereas this population was detected at a much lower frequency and number within the secondary lymphoid tissues (Figure 6 and Figures 11C-E). Enzymatic tissue digestion can alter the expression of a variety of cell-surface molecules (401). However, the enzymatic digestion employed to isolate lymphocytes from the paws did not alter the relative frequencies of $CD4^+$, $CD8^+$, and DN T cells isolated from the spleen (Figure 6). The DN T cell subset could exhibit disease modifying potential through the production of proinflammatory cytokines (402), or they may represent a subset of Tregs that can suppress inflammation (403,404). The total number of $CXCR6^+$ DN T cells was increased within the paws of arthritic mice compared to nonimmunized mice (Figure 4B), whereas the frequency of $CXCR6^+$ DN T cells was not significantly different between these groups (Figure 4A).

In the paws of arthritic mice, $CXCR6^+$ $CD4^+$ T cells displayed much more dramatic increases in frequency and number compared to $CXCR6^+$ $CD8^+$ T cells (Figures 4A and 4B). Consistent with this, $CD4^+$ T cells from arthritic paws exhibited greater geometric mean fluorescence intensity (gMFI) of CXCR6 staining (Figures 7A and 7B), in addition to a greater overall accumulation within arthritic paws (Figure 11D). The geometric mean, which is defined as the ‘nth’ root of the product of ‘n’ numbers, was

used because fluorescence intensity increases logarithmically, which more readily affects or exaggerates the arithmetic mean in stained populations with significant left or right skews from a normal distribution. The gMFI is susceptible to significant shifts, but is more robust as a representation of a 'typical' cell based on flow cytometry data that follow a log-normal distribution (long right skew) (405). In the lymph nodes of both nonimmunized and arthritic mice the frequency and number of $CXCR6^+$ cells (Figures 5A and 5B), as well as the gMFI of CXCR6 staining (Figure 7C), were greater among $CD8^+$ T cells than $CD4^+$ T cells. The frequencies of $CXCR6^+$ cells (Figure 5A) and the gMFI of CXCR6 staining (Figure 7C) were similar for $CD4^+$ T cells and $CD8^+$ T cells in the spleen within nonimmunized and arthritic groups, whereas the number of $CXCR6^+$ $CD4^+$ T cells was significantly greater than the number of $CXCR6^+$ $CD8^+$ T cells in the spleen of arthritic mice (Figure 5B).

Naïve $CD4^+$ and $CD8^+$ T cells were distinguished from those with a more memory-like phenotype on the basis of $CD44^{lo}$ and $CD44^{hi}$ staining and the frequencies of $CCR7^+$, $CXCR6^+$, and $CCR7^+CXCR6^+$ populations were examined in the lymph nodes from arthritic wild-type mice (Table 2). The proportions of $CD4^+$ and $CD8^+$ T cells expressing CCR7 alone were considerably greater among $CD44^{lo}$ subsets, whereas CXCR6 expression was greater within the $CD44^{hi}$ subsets. Consistent with literature reports (279), naïve ($CD44^{lo}$) $CD8^+$ T cells constitutively express low levels of CXCR6 (~20%), whereas the level of CXCR6 expression on naïve $CD4^+$ T cells was nearly undetectable (~1%) (Table 2). The greater proportion of $CXCR6^+$ $CD44^{lo}$ $CD8^+$ T cells translated to a greater frequency of $CD44^{lo}$ $CXCR6^+$ $CD8^+$ T cells coexpressing CCR7, compared to $CD4^+$ T cells (Table 2), which may contribute to the accumulation of $CXCR6^+$ $CD8^+$ T cells in the lymph nodes (Figures 5A and 5B). However, the frequency of $CXCR6^+$ cells is considerably greater among $CD44^{hi}$ $CD8^+$ T cells compared to $CD44^{hi}$ $CD4^+$ T cells (Table 2), suggesting other mechanisms may be contributing to the accumulation of $CXCR6^+$ $CD8^+$ T cells within the lymph nodes, since the proportion of $CCR7^+$ cells is much lower within the $CD44^{hi}$ T cell population. Taken together, these results demonstrate that CXCR6 is upregulated on $CD4^+$ and $CD8^+$ T cells in the inflamed paws (and secondary lymphoid tissues) of CIA mice, similar to observations in the synovium of patients with arthritis.

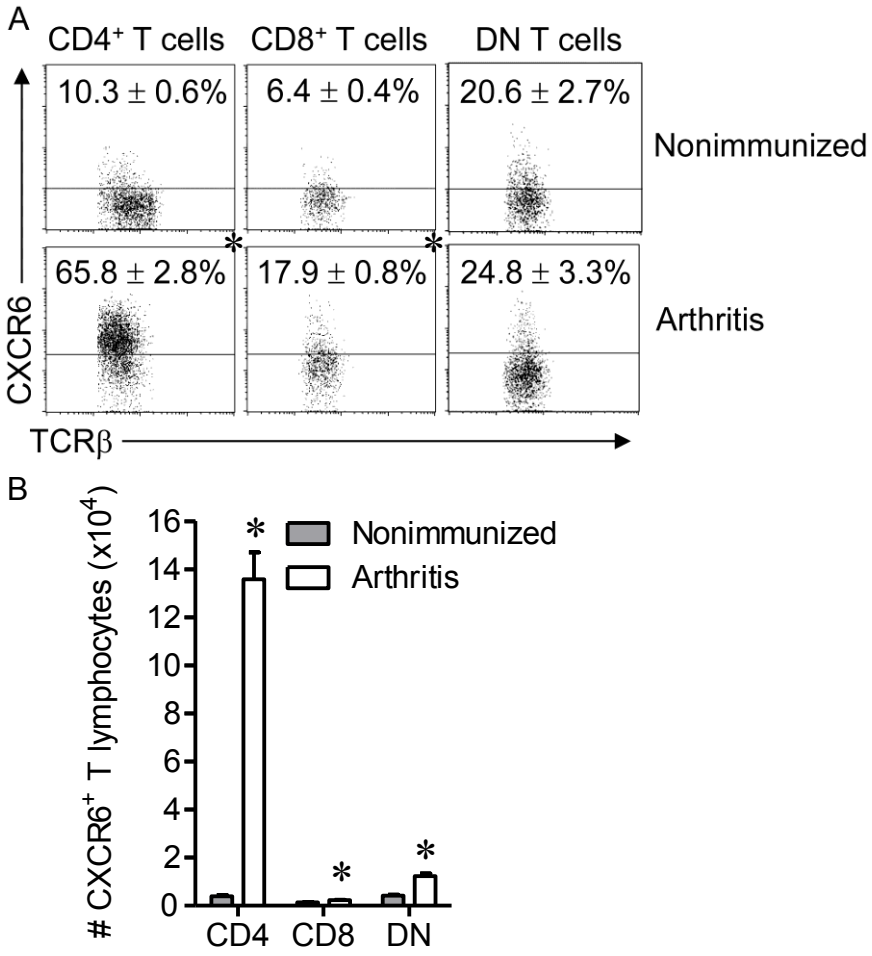


Figure 4. CXCR6$^+$ T cells are increased in the paws following arthritis development. **A,** Representative dot plots showing the expression of CXCR6 and T cell receptor β (TCRβ) on CD4$^+$, CD8$^+$, and CD4$^-$CD8$^-$ [double negative (DN)] T cells from the paws of nonimmunized and arthritic wild-type mice at day 35 post-immunization. Values are the percent of events above the isotype control threshold presented as mean ± SEM. **B,** Absolute numbers of CD4$^+$, CD8$^+$, and DN T cells expressing CXCR6 in the paws of nonimmunized and arthritic wild-type mice. Data are presented as mean ± SEM in 4-6 mice per group. * = $P < 0.05$ compared with nonimmunized mice. Adapted from Slauenwhite, D. et al. Regulation of cytokine polarization and T cell recruitment to inflamed paws in mouse collagen-induced arthritis by the chemokine receptor CXCR6. *Arthritis Rheumatol* (2014) **66**: 3001-3012, copyright 2014 (406).

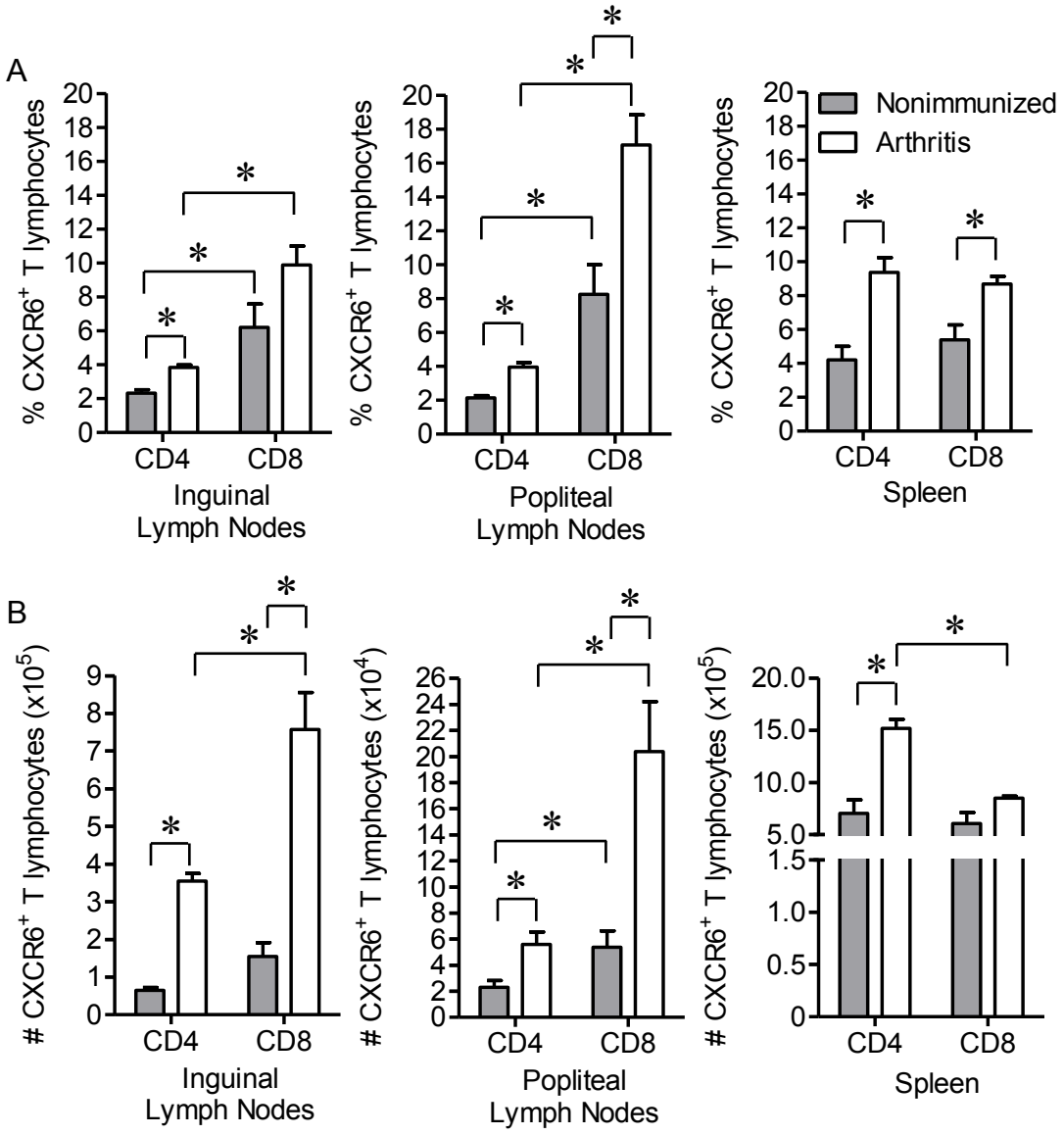


Figure 5. CXCR6$^+$ T cells are increased in the secondary lymphoid tissues following arthritis development. A and **B,** Frequency **(A)** and absolute numbers **(B)** of CD4$^+$ and CD8$^+$ T cells expressing CXCR6 in the inguinal lymph nodes, popliteal lymph nodes, and spleen of nonimmunized and arthritic wild-type mice at day 35 post-immunization. Data are presented as mean ± SEM in 4-6 mice per group. * = $P < 0.05$ for indicated comparisons. Adapted from Slauenwhite, D. et al. Regulation of cytokine polarization and T cell recruitment to inflamed paws in mouse collagen-induced arthritis by the chemokine receptor CXCR6. *Arthritis Rheumatol* (2014) **66**: 3001-3012, copyright 2014 (406).

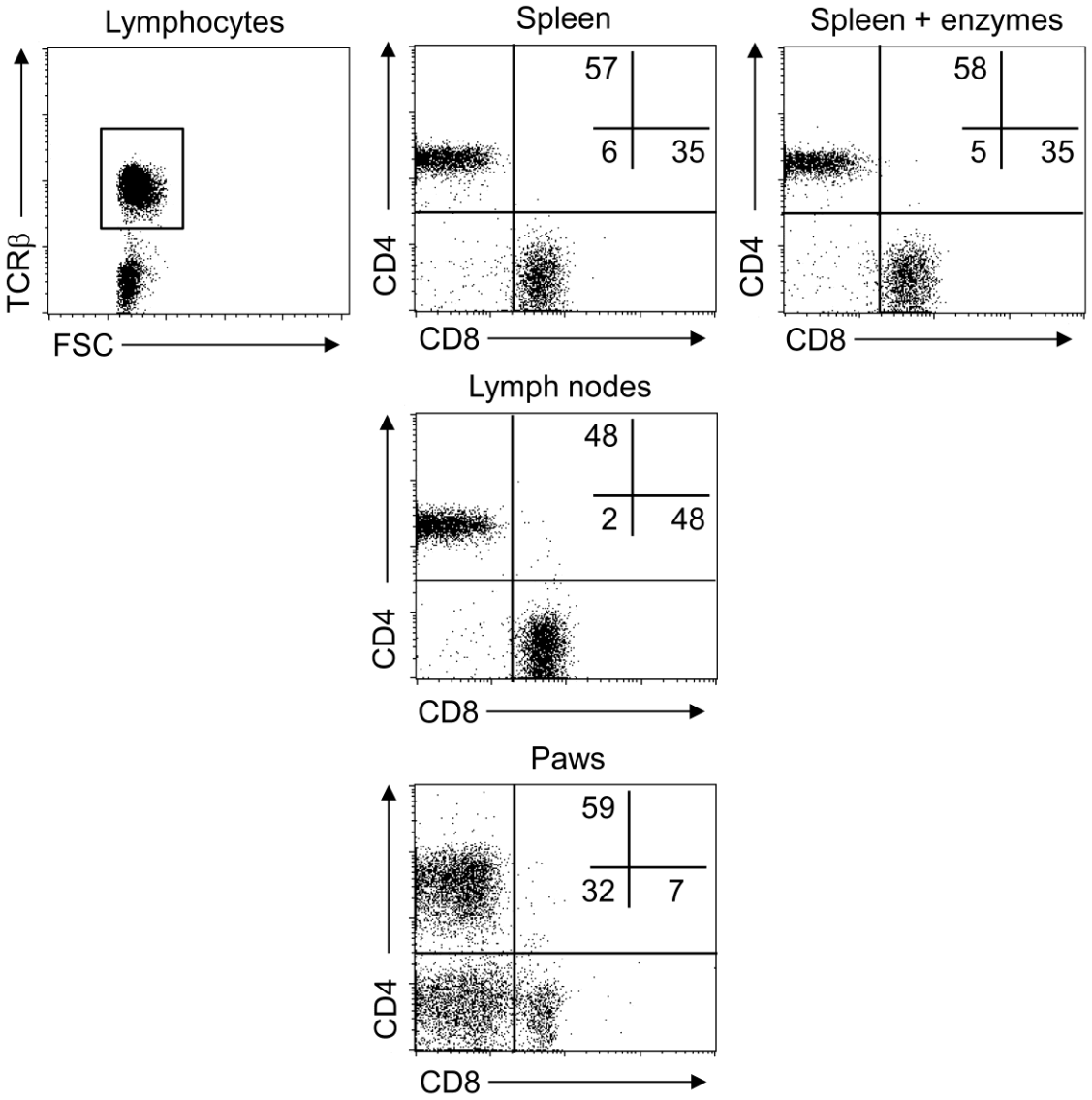


Figure 6. CD4$^+$ T cells represent the major T lymphocyte subset in arthritic paws. Representative dot plots showing the relative proportions of CD4$^+$, CD8$^+$, and CD4$^-$CD8$^-$ populations within T cell receptor β-positive (TCRβ$^+$) lymphocytes in the spleen, lymph nodes, and paws of arthritic wild-type mice at day 35 post-immunization, as well as in spleens treated with Liberase TL and DNase I digestive enzymes. Values are the percent of events represented in each quadrant.

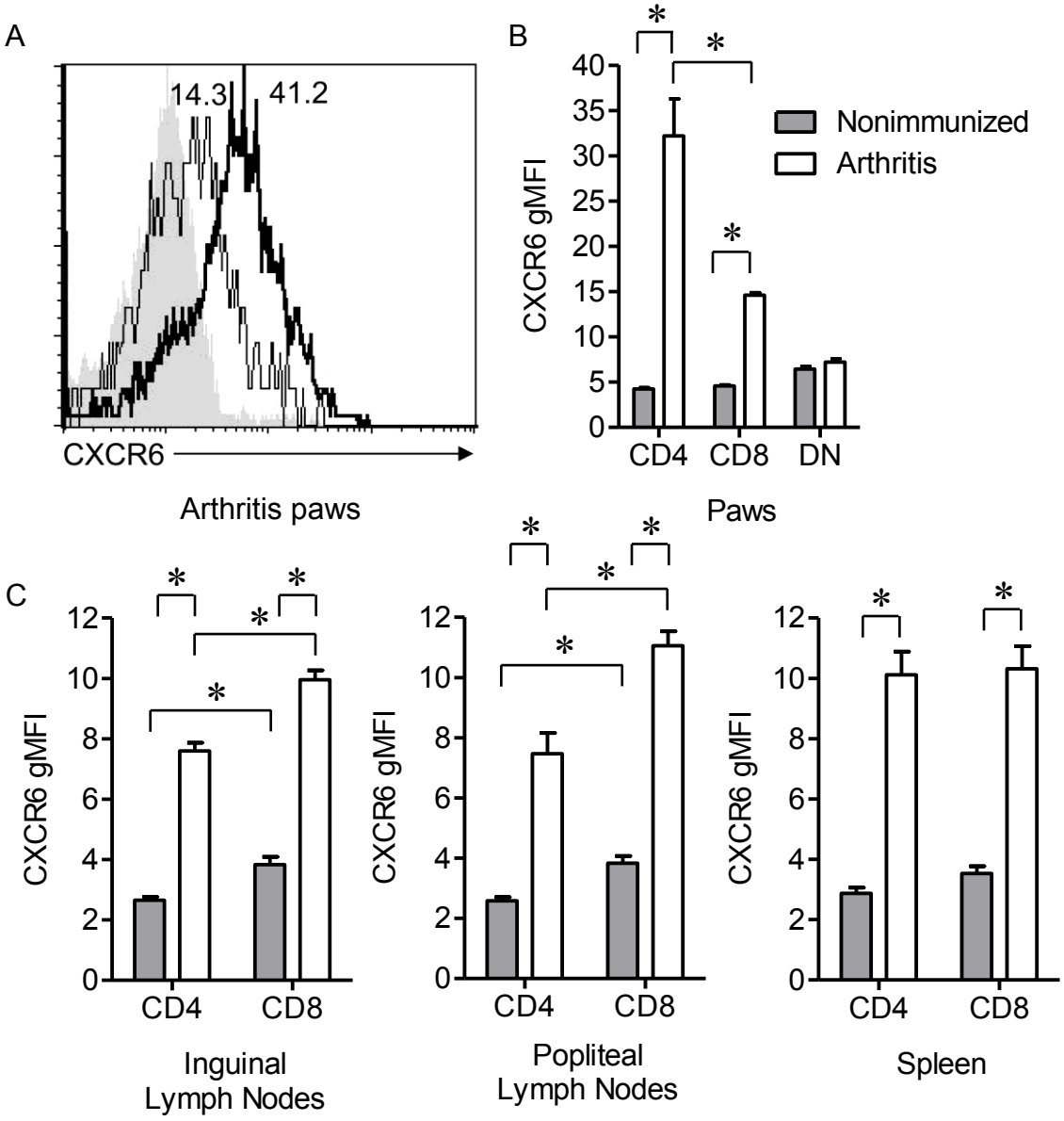


Figure 7. Increased mean fluorescence intensity of CXCR6 staining among T cells from arthritic mice. A, Representative histogram showing the expression of CXCR6 among $CD4^+$ (bold line) and $CD8^+$ (thin line) T cells within the paws of arthritic mice versus isotype control staining (grey filled line). Values are the geometric mean fluorescence intensity (gMFI) of CXCR6 staining within this representative sample. **B** and **C,** gMFI of CXCR6 staining within $CD4^+$ and $CD8^+$ (and $CD4^-CD8^-$ (DN) in paws) T cells in the paws **(B)** and inguinal lymph nodes, popliteal lymph nodes and spleen **(C)** of nonimmunized and arthritic wild-type mice at day 35 post-immunization. Data are presented as mean ± SEM in 4-6 mice per group. * = $P < 0.05$ for indicated comparisons.

Table 2. Percentages of $CCR7^+$, $CXCR6^+$, and $CCR7^+CXCR6^+$ cells among $CD4^+$ and $CD8^+$ T lymphocytes in the lymph nodes of arthritic wild-type mice (n = 3)

Lymph nodes	Subsets		$CCR7^+$	$CXCR6^+$	$CCR7^+CXCR6^+$
Inguinal	CD4	$CD44^{lo}$	69.2 ± 2.2	1.3 ± 0.1	0.81 ± 0.03
		$CD44^{hi}$	9.1 ± 0.8	12.6 ± 0.4	1.44 ± 0.08
	CD8	$CD44^{lo}$	48.9 ± 3.1	20.3 ± 1.8	12.65 ± 1.07
		$CD44^{hi}$	6.6 ± 0.4	50.4 ± 2.4	2.34 ± 0.39
Popliteal	CD4	$CD44^{lo}$	82.1 ± 2.4	1.0 ± 0.1	0.82 ± 0.08
		$CD44^{hi}$	10.0 ± 2.1	5.8 ± 0.6	1.21 ± 0.05
	CD8	$CD44^{lo}$	49.8 ± 4.0	17.6 ± 1.5	12.69 ± 0.57
		$CD44^{hi}$	11.2 ± 2.0	47.4 ± 3.8	4.11 ± 0.77

3.1.2 Bone marrow contamination does not account for changes in T cell numbers within paws of arthritic mice

To isolate cells from the paws, the whole paw was processed together, which included the small bones distal to the ankle. Therefore, to determine the contribution of cells from the bone marrow to the changes in T cell subset proportions within arthritic paws, the frequencies and absolute numbers of $TCR\beta^+$ cells within the bone marrow and paws of arthritic mice were compared to those from non-immunized mice. Absolute numbers of $TCR\beta^+$ cells were reduced within femoral bone marrow, but were increased in the paws in arthritic mice compared to nonimmunized mice (Figure 8A). Among $TCR\beta^+$ cells, the relative frequencies of $CD4^+$, $CD8^+$, and DN subsets in the bone marrow were not altered in arthritic mice compared to nonimmunized controls (Figure 8B). $CD4^+$ and $CD8^+$ subsets are each less abundant than DN cells within the low numbers of $TCR\beta^+$ cells isolated from the paws of nonimmunized mice (Figure 8A and 8B). In contrast, the $CD4^+$ subset constitutes the majority of the increased $TCR\beta^+$ population within the paws of arthritic mice, whereas the relative frequency of DN T cells is decreased in the paws compared to nonimmunized mice (Figure 8B).

These findings do not directly demonstrate the relative contribution of bone marrow contamination from the small bones during paw cell isolation. Rather, the proportions of $CD4^+$, $CD8^+$, and DN subsets among $TCR\beta^+$ cells in the paws of nonimmunized mice resembles the profile observed in the femoral bone marrow, suggesting that cells from the bone marrow may constitute some proportion of the paw-isolated cell population. However, these data suggest that $CD4^+$ T cells are specifically homing or expanding within the inflamed paws of arthritic mice, which cannot be explained by bone marrow contamination since a similar increase is not observed in the bone marrow of arthritic mice compared to nonimmunized mice.

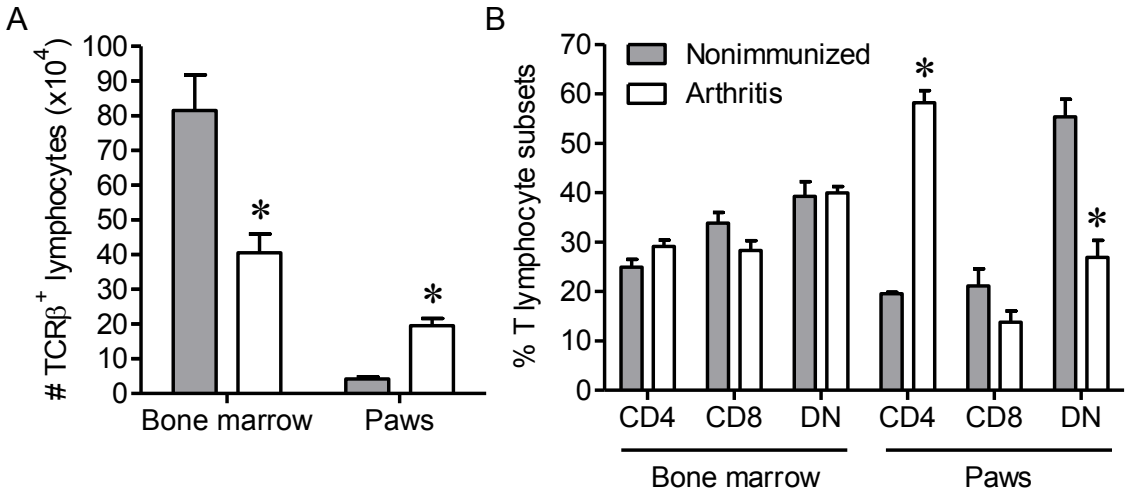


Figure 8. Absolute numbers of TCRβ$^+$ cells and the frequency of CD4$^+$ T cells are increased in the paws, but not bone marrow, from arthritic mice. A, The absolute numbers of T cell receptor β-positive (TCRβ$^+$) cells isolated from the femoral bone marrow and paws of nonimmunized and arthritic wild-type mice at day 35 post-immunization. **B,** The frequencies of CD4$^+$, CD8$^+$, and CD4$^-$CD8$^-$ [double negative (DN)] subsets among TCRβ$^+$ cells isolated from the bone marrow and paws of nonimmunized and arthritic wild-type mice. Data are presented as mean ± SEM in 5-6 mice per group. * = $P < 0.05$ compared with nonimmunized mice.

3.1.3 Reduced incidence and severity of CIA in CXCR6$^{-/-}$ and CXCL16$^{-/-}$ mice

To examine the contributions of CXCR6 and CXCL16 to CIA, wild-type, CXCR6$^{-/-}$, and CXCL16$^{-/-}$ mice were immunized and boosted with CII in CFA and monitored for the development of arthritis over an 80 day period. The cumulative incidence of arthritis in wild-type mice (49/56; 87.5%) was significantly higher than in CXCR6$^{-/-}$ mice (37/56; 66.1%) by day 80 post-immunization (Figure 9A). The mean clinical scores of arthritis severity in mice that exhibited signs of disease were significantly reduced in CXCR6$^{-/-}$ mice compared to wild-type mice (Figure 9B). The reduced mean clinical severity was apparent from day 20 and remained significant ($P < 0.05$) until observations were ended on day 80. At the peak of disease severity, mean clinical scores in wild-type and CXCR6$^{-/-}$ mice were 7.80 ± 0.52 and 6.00 ± 0.66, respectively. The mean day to onset of clinical signs of disease (from the primary immunization) was also significantly later ($P < 0.05$) for CXCR6$^{-/-}$ mice (26.97 ± 0.93) than for wild-type mice (23.80 ± 0.42). Paw measurements were performed over the course of CIA development. As shown in Figure 9C, the mean increase in paw thickness (over the paw thickness of naïve age-matched sex-matched controls) in arthritic wild-type mice was significantly greater than that of arthritic CXCR6$^{-/-}$ mice at the peak of disease severity (day 35 post-immunization). Representative images of hind paws from nonimmunized, arthritic wild-type, and arthritic CXCR6$^{-/-}$ mice are shown in Figure 9D.

Similar to CXCR6$^{-/-}$ mice, the cumulative incidence of arthritis in CXCL16$^{-/-}$ mice (11/16; 68.8%) was also significantly lower than the incident rates in wild-type mice by day 80 post-immunization (Figure 10A). The mean clinical scores in CXCL16$^{-/-}$ mice at the peak of disease severity were 5.36 ± 1.34, similar to those observed in CXCR6$^{-/-}$ mice (6.00 ± 0.66), both of which were significantly reduced compared to wild-type mice (Figure 10B). These findings indicate that CXCL16 and CXCR6 contribute to the development and progression of CIA in mice.

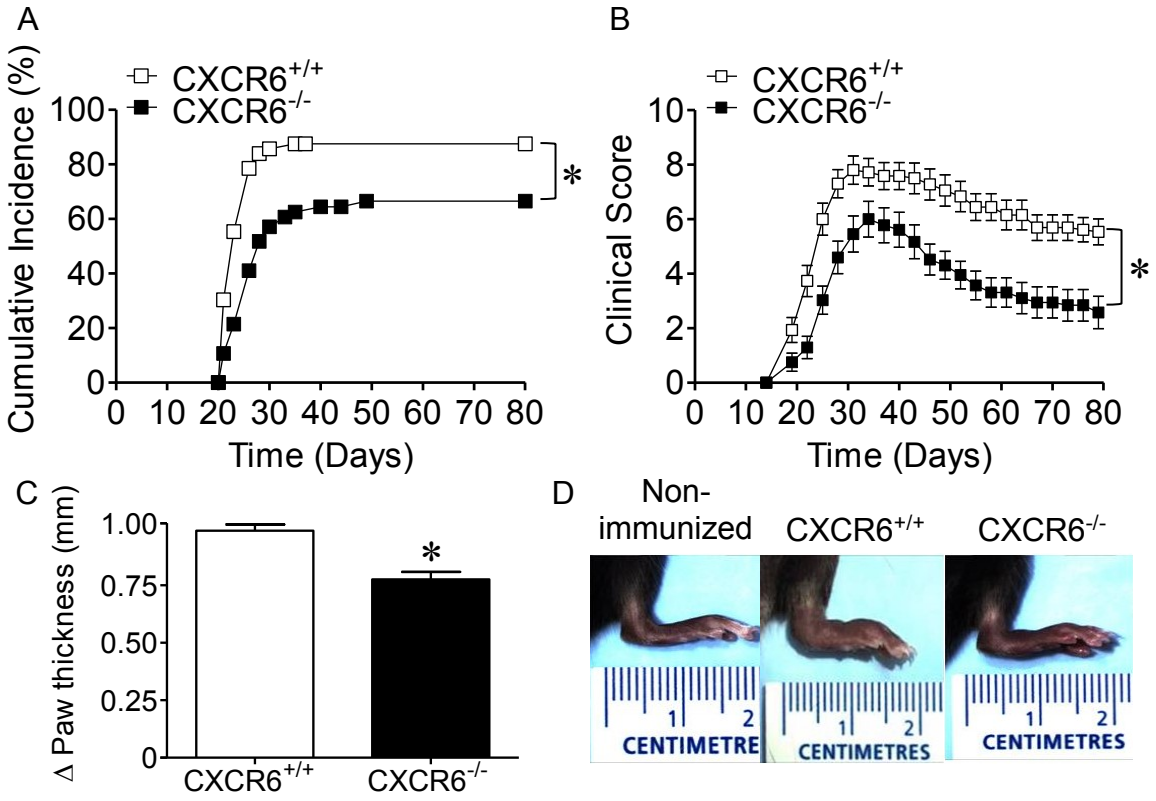


Figure 9. Reduced incidence and severity of arthritis in CXCR6$^{-/-}$ mice. A and **B,** Cumulative incidence of arthritis **(A)** and clinical disease severity scores **(B)** in wild-type (CXCR6$^{+/+}$) and CXCR6$^{-/-}$ mice over 80 days following immunization with type II collagen emulsified in complete Freund's adjuvant (n = 56 mice per group, pooled from >10 separate experiments). Only mice that had developed signs of arthritis were included in the clinical scoring. **C,** The change in paw thickness at the peak of disease severity (day 35) in arthritic wild-type (n = 49) and CXCR6$^{-/-}$ mice (n = 37), relative to paws of nonimmunized age-matched, sex-matched controls. **D,** Representative images of hind paws from nonimmunized wild-type mice, arthritic wild-type and arthritic CXCR6$^{-/-}$ mice. Data in **B** and **C** are presented as mean ± SEM. * = $P < 0.05$ compared with CXCR6$^{+/+}$ mice. Adapted from Slauenwhite, D. et al. Regulation of cytokine polarization and T cell recruitment to inflamed paws in mouse collagen-induced arthritis by the chemokine receptor CXCR6. *Arthritis Rheumatol* (2014) **66**: 3001-3012, copyright 2014 (406).

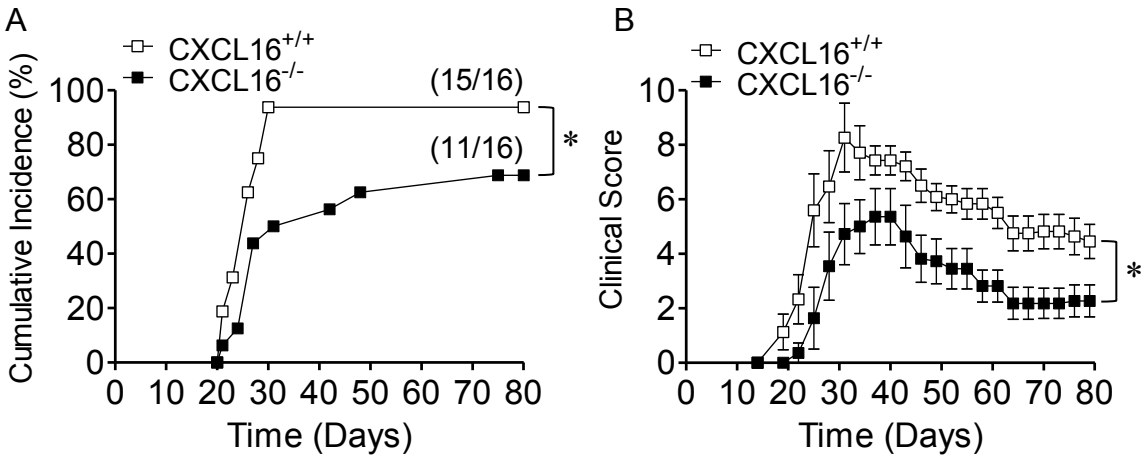


Figure 10. Reduced incidence and severity of arthritis in CXCL16$^{-/-}$ mice. A and **B,** Cumulative incidence of arthritis **(A)** and clinical disease severity scores **(B)** in wild-type (CXCL16$^{+/+}$) and CXCL16$^{-/-}$ mice over 80 days following immunization with type II collagen emulsified in complete Freund's adjuvant (n = 16 mice per group, pooled from 4 separate experiments). Only mice that had developed signs of arthritis were included in the clinical scoring. Data in **B** are presented as mean ± SEM. * = $P < 0.05$ compared with CXCL16$^{+/+}$ mice.

3.1.4 Reduced severity of arthritis in CXCR6$^{-/-}$ mice is associated with decreased cellularity and reduced CD4^{+} T cell numbers within arthritic paws.

I assessed whether the reduced severity of arthritis in CXCR6$^{-/-}$ mice was associated with altered T cell and total cell numbers within the secondary lymphoid tissues and paws. The total numbers of cells were increased in the spleen, inguinal lymph nodes, popliteal lymph nodes, and paws of arthritic wild-type and arthritic CXCR6$^{-/-}$ mice at day 35 post-immunization compared to nonimmunized groups (Figures 11A and 11B). In the spleen, inguinal lymph nodes, and popliteal lymph nodes, no differences in total cell numbers were observed between arthritic wild-type and arthritic CXCR6$^{-/-}$ mice (Figure 11A). However, consistent with the reduced clinical severity of arthritis in CXCR6$^{-/-}$ mice (Figure 9B), total cell numbers within the paws of arthritic CXCR6$^{-/-}$ mice were significantly reduced (Figure 11B).

I examined the total numbers of CD4^{+}, CD8^{+}, and DN T cells in the tissues of wild-type and CXCR6$^{-/-}$ mice and found no differences in the spleens of arthritic wild-type and arthritic CXCR6$^{-/-}$ mice or between arthritic mice and nonimmunized mice (Figure 11C). Consistent with the increased total cell numbers in the lymph nodes of arthritic mice (Figure 11A), CD4^{+}, CD8^{+}, and DN T cell subsets were increased in arthritic mice compared to nonimmunized mice (Figure 11E). However, no differences were observed between arthritic wild-type and CXCR6$^{-/-}$ mice in terms of CD4^{+}, CD8^{+}, or DN T cell numbers in the lymph nodes (Figure 11E).

The number of CD4^{+} T cells was dramatically higher in the paws of arthritic mice compared to the paws of nonimmunized mice (Figure 11D). In contrast, the numbers of CD8^{+} T cells did not increase in the paws of arthritic wild-type mice (Figure 11D), although the frequency of CD8^{+} T cells expressing CXCR6 was increased in the paw (Figures 4A and 4B). Again, this is unlikely to be attributed to an effect of the digestive enzymes on T cell CD8 expression (Figure 6). In the paws of arthritic CXCR6$^{-/-}$ mice the accumulation of CD4^{+} T cells was attenuated (Figure 11D). In addition, a reduced number of DN T cells, but not CD8^{+} T cells, was observed in the paws of arthritic CXCR6$^{-/-}$ mice compared to arthritic wild-type mice (Figure 11D). Plotting the number of TCRβ^{+} cells in the paws against the clinical disease severity scores, revealed a positive

correlation in both wild-type and CXCR6$^{-/-}$ mice ($r^2 = 0.8615$ and $r^2 = 0.8366$, respectively), as determined by linear regression analysis (Figure 11F).

Finally, nonimmunized wild-type and CXCR6$^{-/-}$ mice displayed no differences in total or absolute number of T cells in the spleen, inguinal lymph nodes, popliteal lymph nodes, or paws (Figures 11A-E). Thus the cellular composition of these tissues in naïve mice were not altered, suggesting that the differences observed in cell numbers within the paws of arthritic CXCR6$^{-/-}$ mice could be attributed either to direct impairments in the recruitment of T cells (and other inflammatory cells) to the arthritic paws or to altered immune responses and reduced inflammation in CXCR6$^{-/-}$ mice.

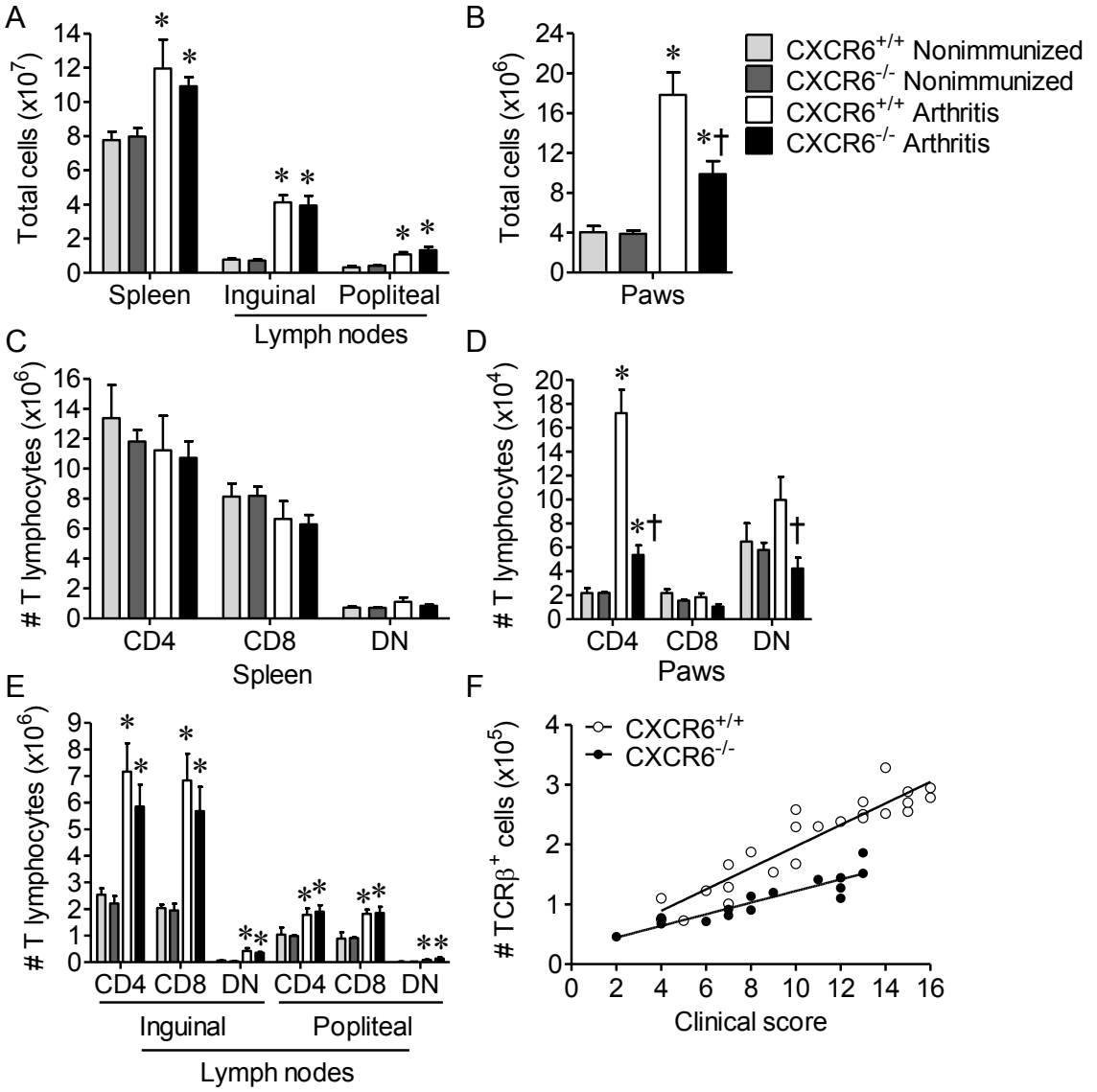


Figure 11. Absolute numbers of T cells and total cell numbers are reduced in paws of arthritic CXCR6$^{-/-}$ mice. A and **B,** Total cell numbers isolated from the spleen, inguinal lymph nodes, and popliteal lymph nodes **(A)** and paws **(B)** of nonimmunized and arthritic wild-type (CXCR6$^{+/+}$) and CXCR6$^{-/-}$ mice at day 35 post-immunization. **C-E,** The absolute numbers of CD4$^+$, CD8$^+$, and CD4$^-$CD8$^-$ [double negative (DN)] T cells in the spleen **(C)**, paws **(D)**, and lymph nodes **(E)** of nonimmunized and arthritic CXCR6$^{+/+}$ and CXCR6$^{-/-}$ mice. **F,** Correlations between the absolute numbers of T cell receptor β-positive (TCRβ$^+$) cells in the paws and the clinical disease severity scores of arthritic wild-type mice (n = 24) and arthritic CXCR6$^{-/-}$ mice (n = 15) were determined by linear regression analysis ($r^2 = 0.8615$ and $r^2 = 0.8366$ in CXCR6$^{+/+}$ and CXCR6$^{-/-}$ mice, respectively). Data in **A-E** are presented as mean ± SEM in 5-6 mice per group. * = $P <$ 0.05 compared with nonimmunized CXCR6$^{+/+}$ or CXCR6$^{-/-}$ mice. † = $P < 0.05$ compared with arthritic CXCR6$^{+/+}$ mice. Slauenwhite, D. et al. Regulation of cytokine polarization and T cell recruitment to inflamed paws in mouse collagen-induced arthritis by the chemokine receptor CXCR6. *Arthritis Rheumatol* (2014) **66**: 3001-3012, copyright 2014.

3.1.5 Altered Th1 and Th17 cytokine polarization in T cells from CXCR6$^{-/-}$ mice

CXCR6 expression defines polarized subsets of Th1, Tc1, and Th17 effector cells in vivo (277,296), and membrane-bound CXCL16 is expressed on the surface of APCs, including DCs and macrophages (279,280), suggesting the potential for CXCR6-CXCL16 interactions to modulate T cell activation and polarization during antigen presentation. Therefore, I investigated whether there were differences in cytokine polarization in T cells isolated from wild-type and CXCR6$^{-/-}$ mice on day 14 following CIA immunizations and during the peak of disease on day 35. In order to eliminate potential bias in the evaluation of cytokine production that might result from differences in severity of inflammation, wild-type and CXCR6$^{-/-}$ mice examined on day 35 were selected on the basis of having comparable levels of disease severity in terms of the number of paws involved and their relative clinical disease severity scores.

To determine whether cytokine polarization was influenced by CXCR6, intracellular cytokine staining (IL-17A, IFN-γ, and IL-4) was performed on cells isolated from the inguinal lymph nodes, popliteal lymph nodes, and spleens of nonimmunized mice [day 0 (D0)], mice that had been immunized 14 days prior but had not received a booster immunization (D14), and arthritic wild-type and CXCR6$^{-/-}$ mice at day 35 (D35). Cytokine polarization within cells isolated from the paws of arthritic wild-type and CXCR6$^{-/-}$ mice were assessed at day 35, but cell recovery was not sufficient at earlier time points. Analysis of intracellular cytokine production by CD4^{+} T cells (Figures 12 and 13), CD8^{+} T cells (Figures 14 and 15), and DN T cells (Figures 16 and 17) were performed within subsets that displayed a CD44^{hi} phenotype, as these populations were enriched for intracellular cytokine signal when examined by flow cytometry.

Examining intracellular cytokine production by CD4^{+} T cells isolated from the inguinal and popliteal lymph nodes revealed a temporal difference in the frequency and number of IFN-γ^{+} and IL-17A^{+} cells (Figures 12A & 12B and 13A & 13B). The frequency of IFN-γ^{+} cells increased in the lymph nodes of wild-type mice by day 14 and contracted to near baseline levels by day 35 (Figures 12A and 12B). In contrast, the frequency of IL-17A^{+} cells increased by day 14 and remained elevated on day 35 in wild-type mice. The numbers of IFN-γ^{+} and IL-17A^{+} CD4^{+} T cells in the inguinal lymph nodes

reinforce this temporal difference as they follow a similar distribution (Figure 13A). However, in the popliteal lymph nodes, the numbers of IFN-γ^+ and IL-17A$^+$ CD4$^+$ T cells did not increase by day 14, despite increased frequencies of cytokine producing cells, which appeared to be attributed to the absence of a robust immune reaction in the popliteal lymph nodes prior to arthritis development (Figure 13B). This is supported by the substantially increased number of IFN-γ^+ cells in the popliteal lymph nodes of arthritic mice at day 35 (Figure 13B), even though the frequency of IFN-γ^+ cells is much lower at day 35 compared to day 14 in this tissue (Figure 12B). Cytokine production within cells isolated from the spleen of wild-type mice exhibited a pattern similar to that observed in the inguinal lymph nodes, except the frequency and number of IFN-γ^+ cells did not contract to near control levels by day 35 and remained elevated after increasing by day 14 (Figures 12C and 13C).

Compared to wild-type mice, CXCR6$^{-/-}$ mice had a reduced frequency and number of IFN-γ^+ cells in the inguinal lymph nodes on day 14, but not in the popliteal lymph nodes or spleen (Figures 12A-C and 13A-C). The frequency and number of IL-17A$^+$ cells was lower in both the inguinal and popliteal lymph nodes, but not the spleen, on day 35. Analysis of intracellular cytokine staining of CD4$^+$ T cells isolated from the arthritic paws of wild-type and CXCR6$^{-/-}$ mice at day 35 post-immunization revealed reduced frequencies and absolute numbers of IL-17A$^+$ and IFN-γ^+ cells in CXCR6$^{-/-}$ mice (Figures 12D and 13D). Finally, the frequencies and numbers of IL-4$^+$ CD4$^+$ T cells were not increased in the lymph nodes, spleen, or paws of immunized mice, nor did they differ between wild-type and CXCR6$^{-/-}$ mice (Figures 12A-D and 13A-D).

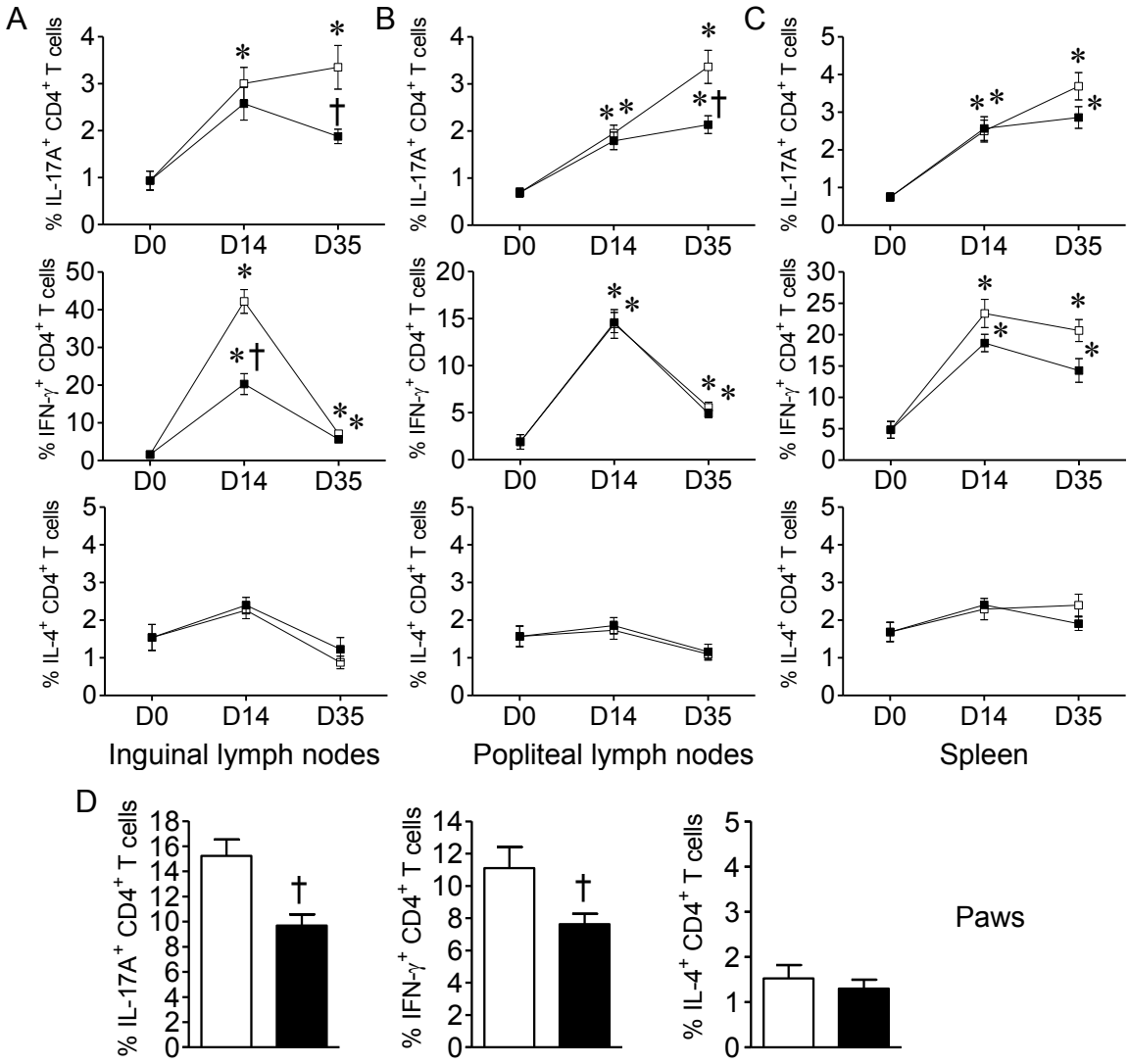


Figure 12. Reduced frequencies of IFN-γ$^+$ and IL-17A$^+$ CD4$^+$ T cells in CXCR6$^{-/-}$ mice. A-C, The frequencies of IL-17A$^+$, IFN-γ$^+$, and IL-4$^+$ CD44^{hi} CD4$^+$ T cells in the inguinal lymph nodes **(A)**, popliteal lymph nodes **(B)**, and spleen **(C)** were measured by intracellular cytokine staining following restimulation with phorbol 12-myristate 13-acetate and ionomycin in nonimmunized [day 0 (D0)], immunized non-arthritic (day 14), and arthritic (day 35) wild-type (open squares) and CXCR6$^{-/-}$ mice (filled squares). **D,** The frequencies of IL-17A$^+$, IFN-γ$^+$, and IL-4$^+$ CD44^{hi} CD4$^+$ T cells in the paws of arthritic wild-type (open bars) and CXCR6$^{-/-}$ mice (filled bars) were assessed at peak disease severity at day 35. Sufficient cell numbers could not be obtained from non-arthritic paws at earlier time points. Data are presented as mean ± SEM in 6-8 mice per group. * = $P < 0.05$ compared with nonimmunized mice. † = $P < 0.05$ compared with wild-type mice. Adapted from Slauenwhite, D. et al. Regulation of cytokine polarization and T cell recruitment to inflamed paws in mouse collagen-induced arthritis by the chemokine receptor CXCR6. *Arthritis Rheumatol* (2014) **66**: 3001-3012, copyright 2014 (406).

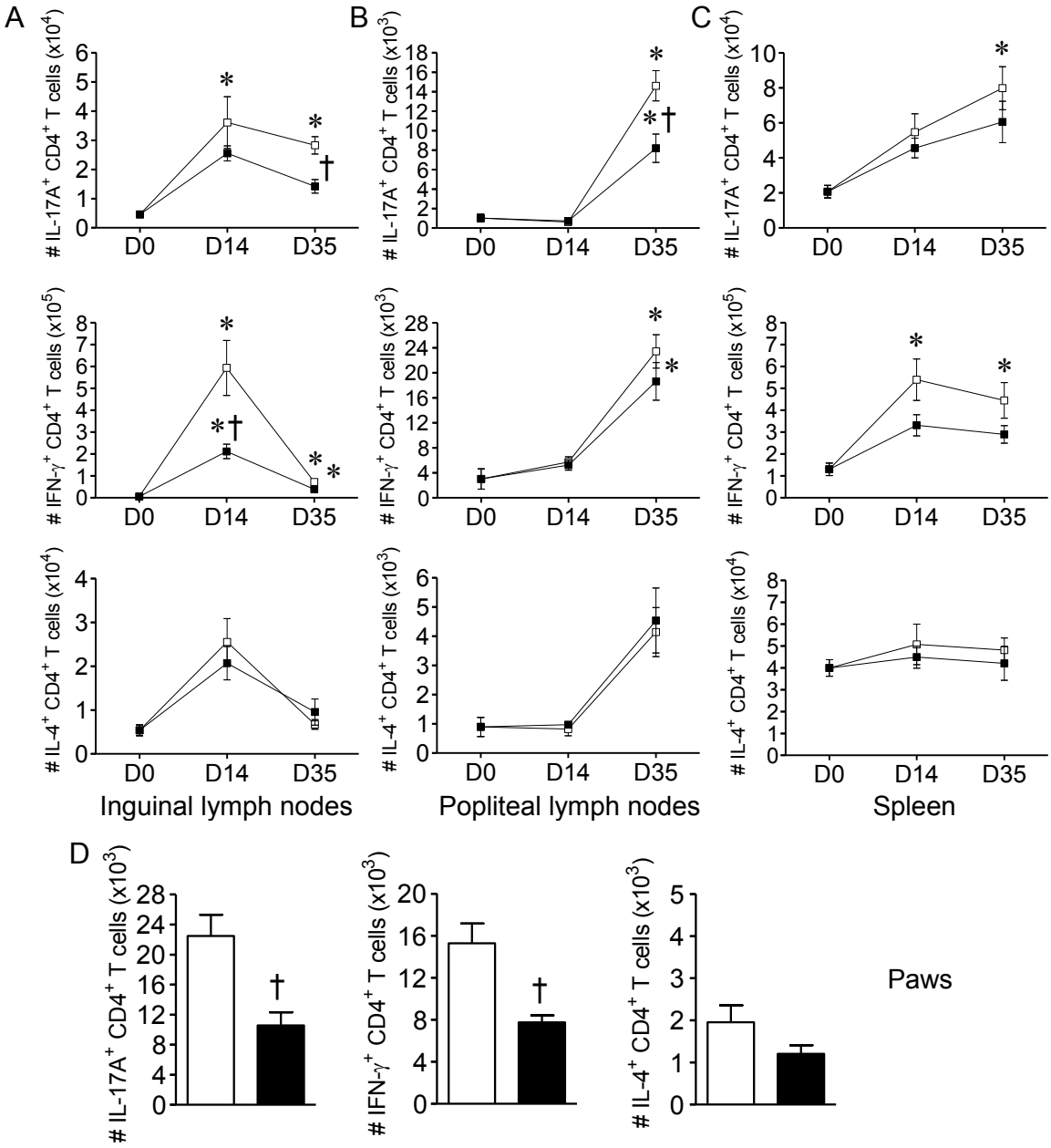


Figure 13. Reduced numbers of IFN-γ$^+$ and IL-17A$^+$ CD4$^+$ T cells in CXCR6$^{-/-}$ mice. **A-C,** The absolute numbers of IL-17A$^+$, IFN-γ$^+$, and IL-4$^+$ CD44^{hi} CD4$^+$ T cells in the inguinal lymph nodes **(A)**, popliteal lymph nodes **(B)**, and spleen **(C)** were determined by intracellular cytokine staining following restimulation with PMA and ionomycin in nonimmunized [day 0 (D0)], immunized non-arthritic (day 14), and arthritic (day 35) wild-type (open squares) and CXCR6$^{-/-}$ mice (filled squares). **D,** The absolute numbers of IL-17A$^+$, IFN-γ$^+$, and IL-4$^+$ CD44^{hi} CD4$^+$ T cells in the paws of arthritic wild-type (open bars) and CXCR6$^{-/-}$ mice (filled bars) were assessed at peak disease severity at day 35. Sufficient cell numbers could not be obtained from non-arthritic paws at earlier time points. Data are presented as mean ± SEM in 6-8 mice per group. * = $P < 0.05$ compared with nonimmunized mice. † = $P < 0.05$ compared with wild-type mice.

CXCR6 is expressed by a subset of $CD8^+$ T cells, particularly those present within the lymph nodes of arthritic mice (Figures 5A and 5B). Previously, $CXCR6^+$ $CD8^+$ T cells were found to be enriched for IFN-γ-producing Tc1 cells (277). Therefore, I examined cytokine polarization of $CD8^+$ T cells in the lymphoid tissues and paws from wild-type and $CXCR6^{-/-}$ mice. The frequencies and numbers of IFN-γ^+ $CD8^+$ T cells were increased in the inguinal lymph nodes (Figures 14A and 15A) and spleen (Figures 14C and 15C) at both 14 days post-immunization and at day 35 in arthritic mice. The frequency of IFN-γ^+ $CD8^+$ T cells was also increased in the popliteal lymph nodes at both times points (Figure 14B). However, low absolute numbers of IFN-γ^+ $CD8^+$ T cells were observed in the popliteal lymph nodes at day 14, whereas they increased in numbers by day 35 in arthritic mice (Figure 15B).

Compared to wild-type mice, $CXCR6^{-/-}$ mice exhibited reductions in the frequencies and numbers of IFN-γ^+ $CD8^+$ T cells in the inguinal lymph nodes and spleen at both 14 days post-immunization and in arthritic mice at day 35 (Figures 14A & 14C and 15A & 15C). Additionally, a reduced frequency of IFN-γ^+ $CD8^+$ T cells was observed in the popliteal lymph nodes of $CXCR6^{-/-}$ mice at 14 days post-immunization, but not in arthritic mice at day 35 (Figure 14B), and IFN-γ^+ $CD8^+$ T cell numbers were not reduced at either time point in this tissue (Figure 15B). Arthritic $CXCR6^{-/-}$ mice also exhibited reductions in the frequency and number of IFN-γ^+ $CD8^+$ T cells in the paws on day 35 post-immunization (Figures 14D and 15D). No significant IL-17A or IL-4 staining was observed within $CD8^+$ T cells in these tissues at any time point.

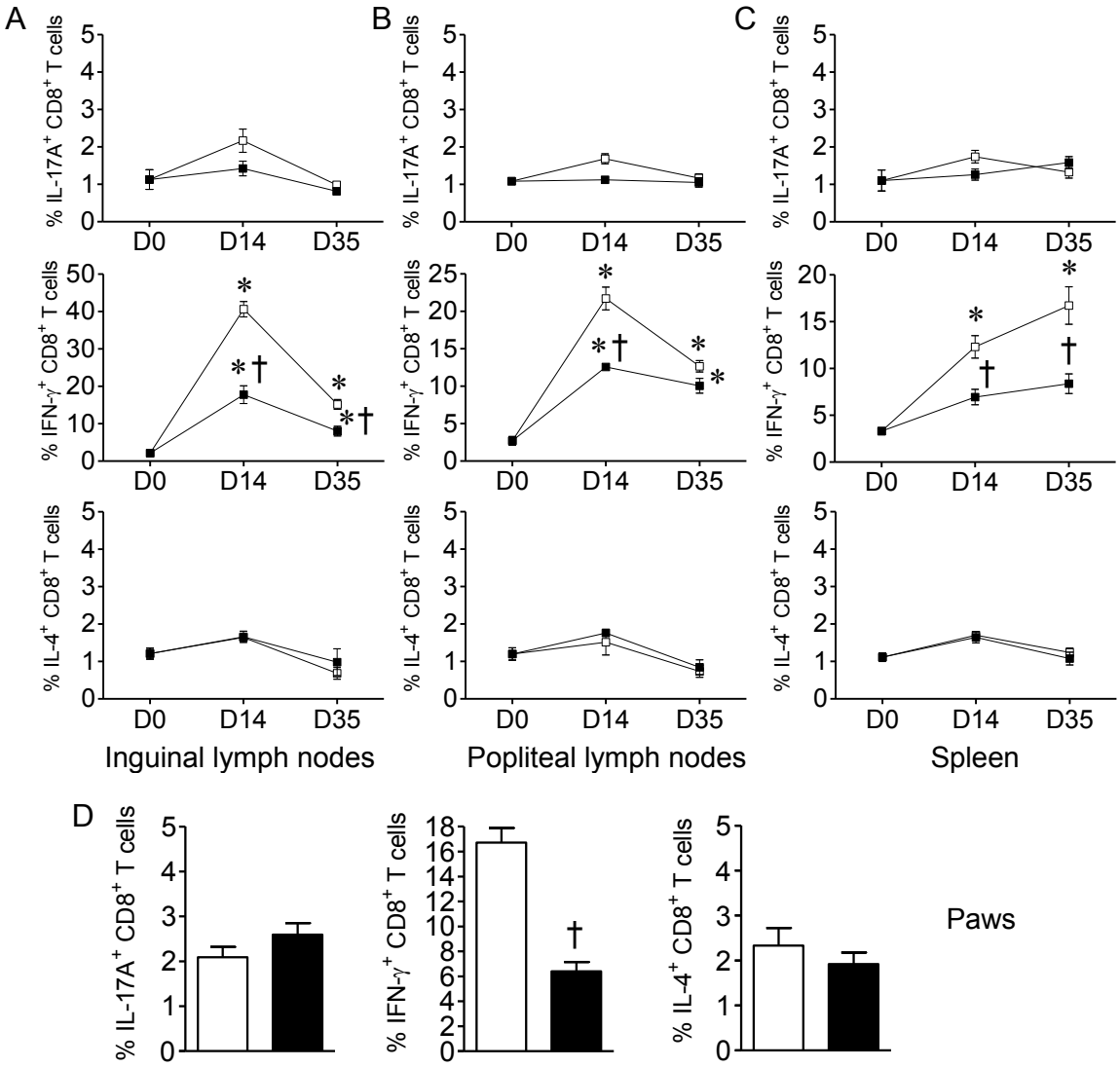


Figure 14. Reduced frequency of IFN-γ$^+$ CD8$^+$ T cells in CXCR6$^{-/-}$ mice. A-C, The frequencies of IL-17A$^+$, IFN-γ$^+$, and IL-4$^+$ CD44^{hi} CD8$^+$ T cells in the inguinal lymph nodes **(A)**, popliteal lymph nodes **(B)**, and spleen **(C)** were measured by intracellular cytokine staining following restimulation with PMA and ionomycin in nonimmunized [day 0 (D0)], immunized non-arthritic (day 14), and arthritic (day 35) wild-type (open squares) and CXCR6$^{-/-}$ mice (filled squares). **D,** The frequencies of IL-17A$^+$, IFN-γ$^+$, and IL-4$^+$ CD44^{hi} CD8$^+$ T cells in the paws of arthritic wild-type (open bars) and CXCR6$^{-/-}$ mice (filled bars) were assessed at peak disease severity at day 35. Sufficient cell numbers could not be obtained from non-arthritic paws at earlier time points. Data are presented as mean ± SEM in 6-8 mice per group. * = $P < 0.05$ compared with nonimmunized mice. † = $P < 0.05$ compared with wild-type mice.

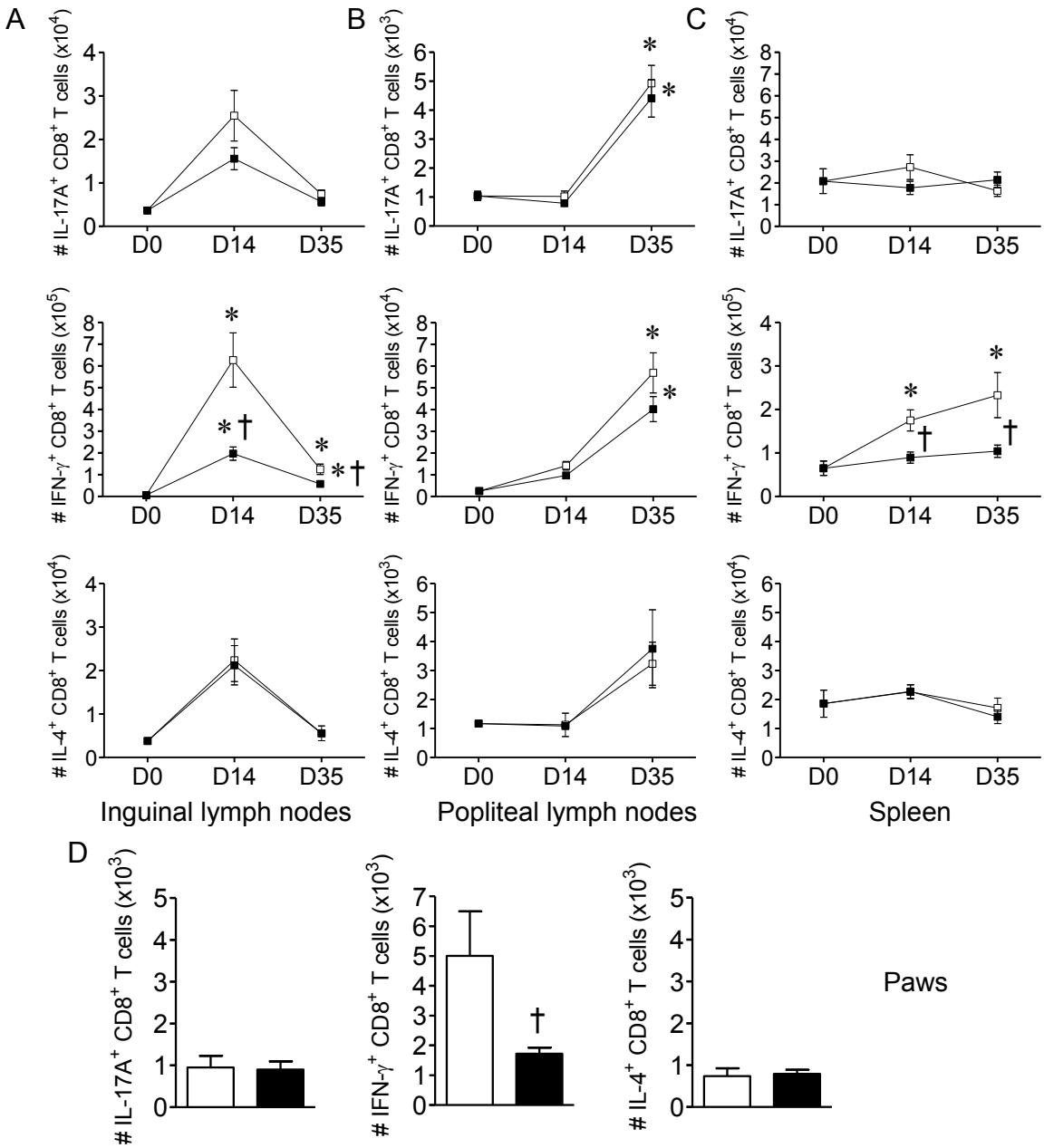


Figure 15. Reduced number of IFN-γ$^+$ CD8$^+$ T cells in CXCR6$^{-/-}$ mice. A-C, The absolute numbers of IL-17A$^+$, IFN-γ$^+$, and IL-4$^+$ CD44^{hi} CD8$^+$ T cells in the inguinal lymph nodes **(A)**, popliteal lymph nodes **(B)**, and spleen **(C)** were determined by intracellular cytokine staining following restimulation with PMA and ionomycin in nonimmunized [day 0 (D0)], immunized non-arthritic (day 14), and arthritic (day 35) wild-type (open squares) and CXCR6$^{-/-}$ mice (filled squares). **D,** The absolute numbers of IL-17A$^+$, IFN-γ$^+$, and IL-4$^+$ CD44^{hi} CD8$^+$ T cells in the paws of arthritic wild-type (open bars) and CXCR6$^{-/-}$ mice (filled bars) were assessed at peak disease severity at day 35. Sufficient cell numbers could not be obtained from non-arthritic paws at earlier time points. Data are presented as mean ± SEM in 6-8 mice per group. * = $P < 0.05$ compared with nonimmunized mice. † = $P < 0.05$ compared with wild-type mice.

Analysis of intracellular cytokine production by DN T cells from the inguinal lymph nodes, popliteal lymph nodes, spleen, and paws revealed a pattern similar to that observed from $CD8^+$ T cells, in that they produced IFN-γ, but not IL-17A or IL-4 (Figures 16A-D and 17A-D), with some notable differences. The frequencies of IFN-γ^+ DN T cells from the inguinal lymph nodes of mice at 14 days post-immunization were much lower compared to the frequencies of IFN-γ^+ $CD4^+$ or $CD8^+$ T cells at this time point (Figures 12A, 14A, and 16A). Furthermore, the frequency of IFN-γ^+ DN T cells increased in mice at 14 days post-immunization but rather than contract to near baseline levels by day 35 as seen for $CD4^+$ and $CD8^+$ T cells in the lymph nodes, a further increase was observed in arthritic mice at day 35 (Figures 16A and 16B). A significant increase in both the frequency and number of IFN-γ^+ DN T cells was detected in the spleen of arthritic mice at day 35 but not at 14 days post-immunization (Figures 16C and 17C).

Compared to wild-type mice, $CXCR6^{-/-}$ mice exhibited reductions in the frequency and number of IFN-γ^+ DN T cells in the inguinal lymph nodes and spleen of arthritic mice at day 35 (Figures 16A & 16C and 17A & 17C). However, with DN T cells accounting for such a small fraction of the total T lymphocyte population within the lymph nodes and spleen (Figure 6 and Figures 11C-E), the impact of this reduced IFN-γ^+ population within $CXCR6^{-/-}$ mice is uncertain. In contrast, DN T cells represent a sizable proportion of the T cell population isolated from the arthritic paws. Arthritic $CXCR6^{-/-}$ mice had a reduced frequency and number of IFN-γ^+ DN T cells in the paws at day 35 compared to arthritic wild-type mice (Figures 16D and 17D). Taken together, these results suggest an important role for CXCR6 in regulating cytokine polarization following T cell activation.

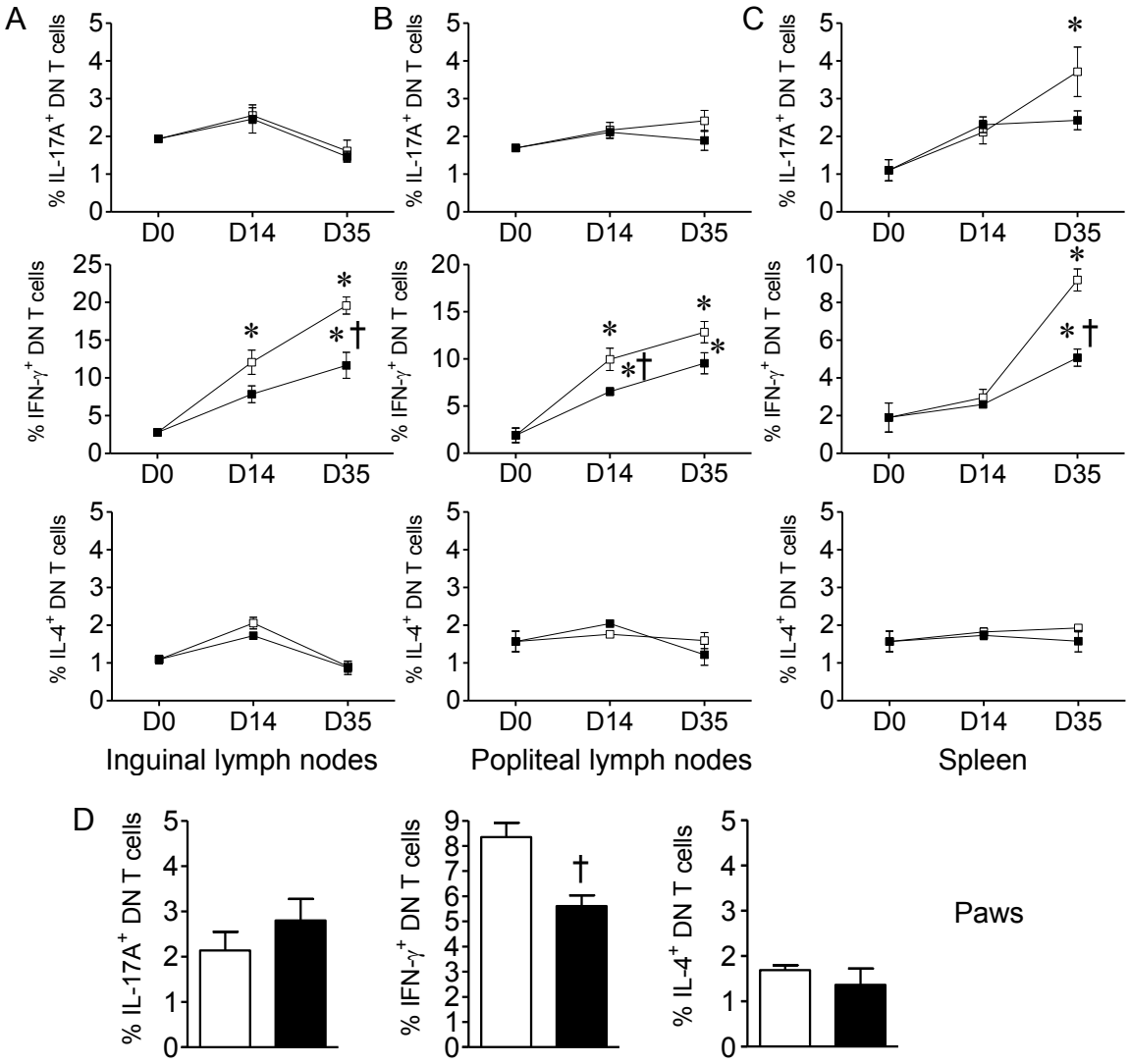


Figure 16. Reduced frequency of IFN-γ$^+$ DN T cells in CXCR6$^{-/-}$ mice. A-C, The frequencies of IL-17A$^+$, IFN-γ$^+$, and IL-4$^+$ CD44^{hi} CD4$^-$CD8$^-$ [double negative (DN)] T cells in the inguinal lymph nodes **(A)**, popliteal lymph nodes **(B)**, and spleen **(C)** were measured by intracellular cytokine staining following restimulation with PMA and ionomycin in nonimmunized [day 0 (D0)], immunized non-arthritic (day 14), and arthritic (day 35) wild-type (open squares) and CXCR6$^{-/-}$ mice (filled squares). **D,** The frequencies of IL-17A$^+$, IFN-γ$^+$, and IL-4$^+$ CD44^{hi} DN T cells in the paws of arthritic wild-type (open bars) and CXCR6$^{-/-}$ mice (filled bars) were assessed at peak disease severity at day 35. Sufficient cell numbers could not be obtained from non-arthritic paws at earlier time points. Data are presented as mean ± SEM in 6-8 mice per group. * = $P < 0.05$ compared with nonimmunized mice. † = $P < 0.05$ compared with wild-type mice.

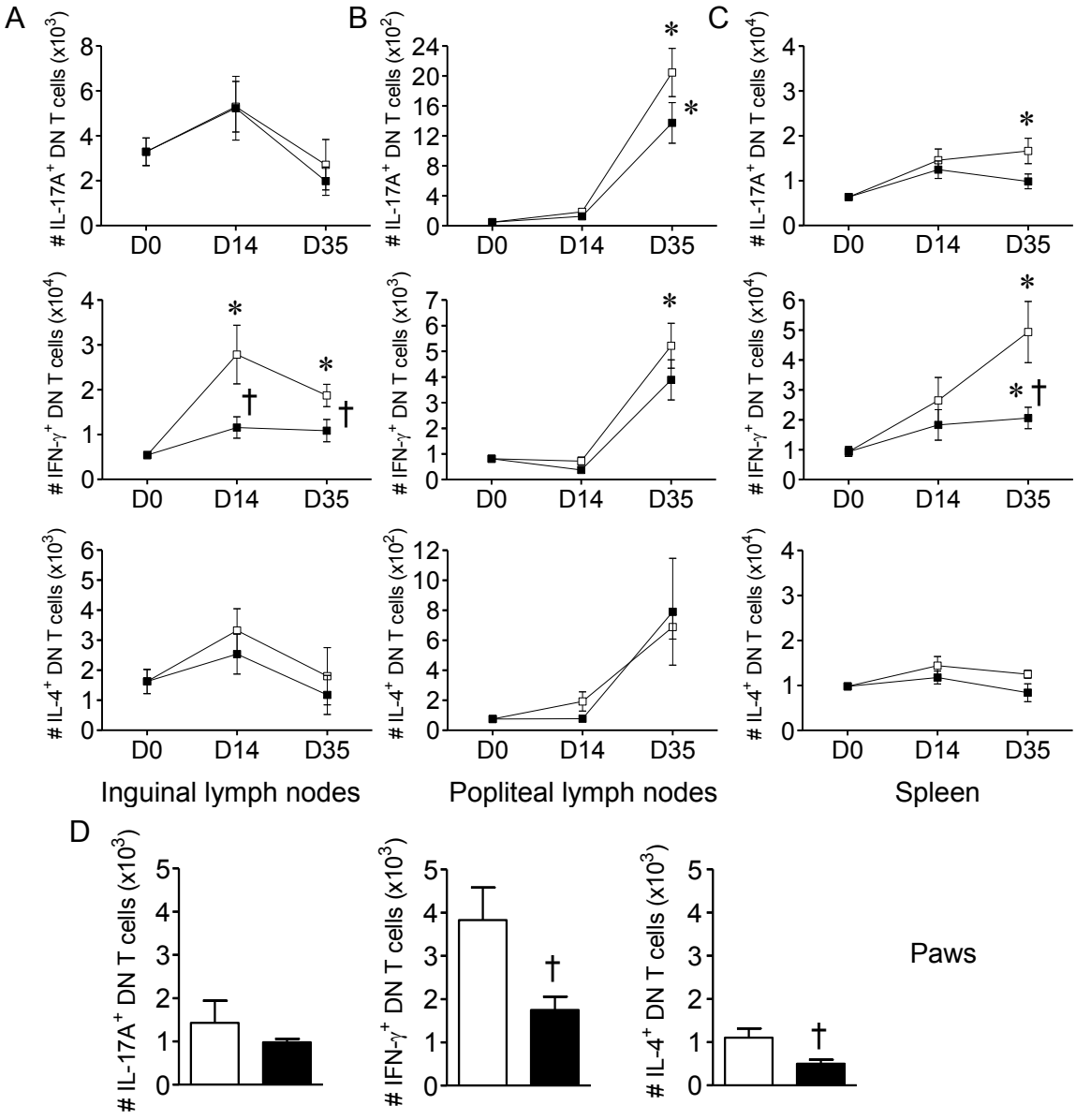


Figure 17. Reduced number of IFN-γ$^+$ DN T cells in CXCR6$^{-/-}$ mice. A-C, The absolute numbers of IL-17A$^+$, IFN-γ$^+$, and IL-4$^+$ CD44^{hi} CD4$^-$CD8$^-$ [double negative (DN)] T cells in the inguinal lymph nodes **(A)**, popliteal lymph nodes **(B)**, and spleen **(C)** were determined by intracellular cytokine staining following restimulation with PMA and ionomycin in nonimmunized [day 0 (D0)], immunized non-arthritic (day 14), and arthritic (day 35) wild-type (open squares) and CXCR6$^{-/-}$ mice (filled squares). **D,** The absolute numbers of IL-17A$^+$, IFN-γ$^+$, and IL-4$^+$ CD44^{hi} DN T cells in the paws of arthritic wild-type (open bars) and CXCR6$^{-/-}$ mice (filled bars) were assessed at peak disease severity at day 35. Sufficient cell numbers could not be obtained from non-arthritic paws at earlier time points. Data are presented as mean ± SEM in 6-8 mice per group. * = $P < 0.05$ compared with nonimmunized mice. † = $P < 0.05$ compared with wild-type mice.

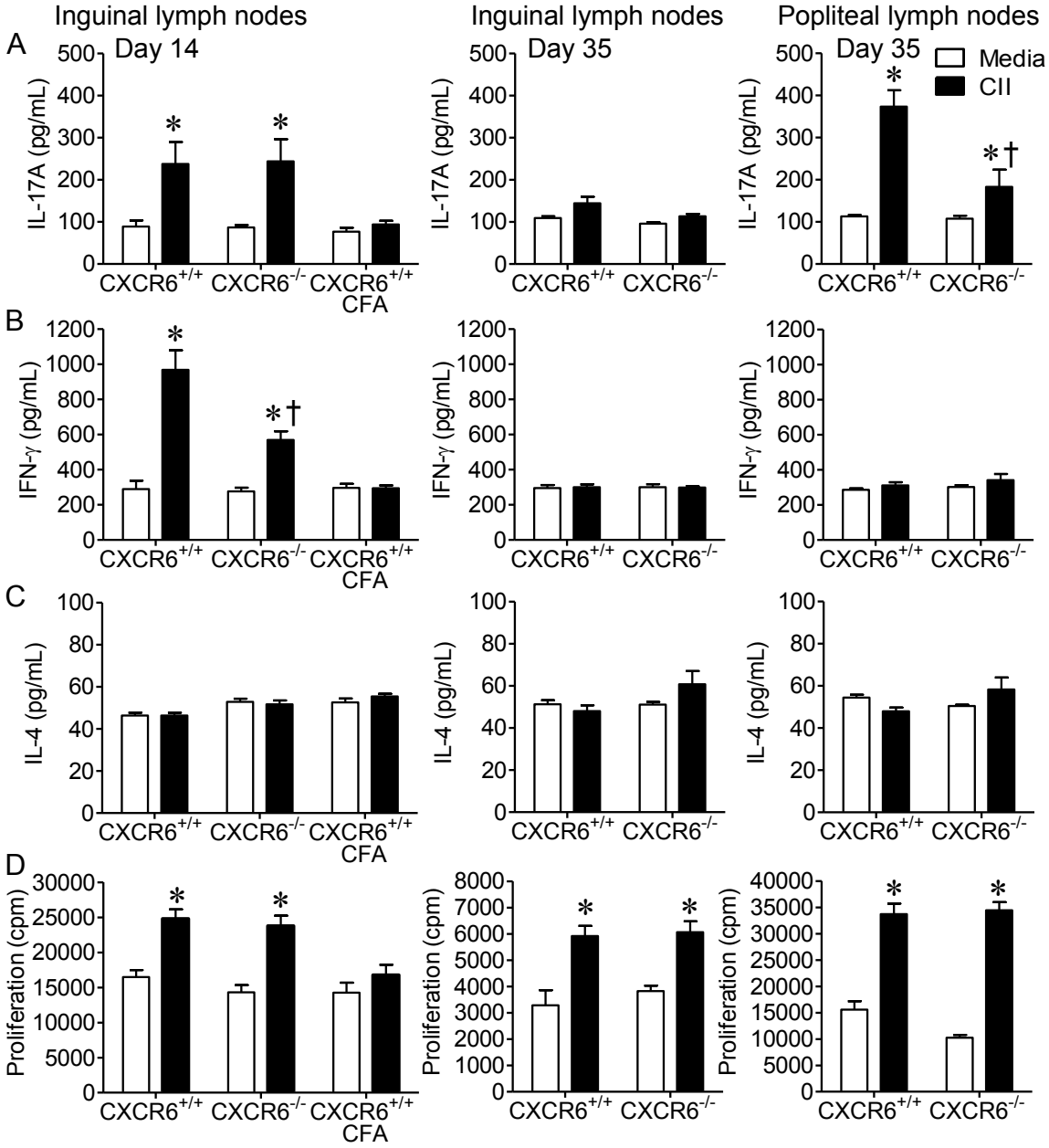
3.1.6 Collagen-specific cytokine secretion is impaired in CXCR6$^{-/-}$ mice

To assess whether reduced cytokine-producing T cells in CXCR6$^{-/-}$ mice could also be detected within antigen-specific T cell populations, I tested recall responses to CII in vitro by wild-type and CXCR6$^{-/-}$ lymph node cells on days 14 and 35 post-immunization. I measured IL-17A, IFN-γ, and IL-4 production by ELISA and cell proliferation by ^{3}H-thymidine incorporation. Consistent with the intracellular cytokine staining experiments, levels of IL-17A were elevated in the supernatants of CII restimulated wild-type and CXCR6$^{-/-}$ cells isolated from the inguinal lymph nodes 14 days after immunization (Figure 18A). However, on day 35 post-immunization, collagen-specific IL-17A production was not detected from inguinal lymph node cells, whereas IL-17A production in the supernatants from popliteal lymph node cells was increased in response to CII (Figure 18A). The collagen-specific IL-17A response was attenuated in the supernatants of popliteal lymph node cells from CXCR6$^{-/-}$ mice at day 35 (Figure 18A), consistent with a reduced frequency and number of IL-17A^{+} CD4^{+} T cells from CXCR6$^{-/-}$ popliteal lymph nodes at day 35 post-immunization (Figures 12B and 13B).

IFN-γ production in response to CII restimulation was abundant within the supernatants of wild-type cells isolated at day 14 post-immunization, but was reduced in the supernatants isolated from CXCR6$^{-/-}$ cells (Figure 18B). In contrast, production of IL-4 by lymph node cells following restimulation with CII was low and not different from background levels (Figure 18C). The intracellular staining experiments showed that IFN-γ^{+} cells increased in the lymph nodes by day 14 and contracted to near baseline levels by day 35 (Figures 12A and 12B), which is consistent with the observed decline in IFN-γ production in the supernatants of lymph node cells isolated on day 35 post-immunization (Figure 18B). Therefore, similar to the intracellular cytokine staining experiments, a shift from IFN-γ production to an increased IL-17A response was also observed in collagen-specific recall response experiments. Furthermore, the impaired production of IFN-γ and IL-17A by collagen-specific CXCR6$^{-/-}$ lymph node cells was observed despite the fact that the number of input cells was the same in each condition, and there were no differences in the proliferation of wild-type and CXCR6$^{-/-}$ cells in

response to CII (Figure 18D). Moreover, challenge with CII failed to increase cytokine production or induce proliferation of cells from wild-type mice immunized with CFA alone, which verified that the cytokine and proliferative responses were indeed collagen-specific (Figure 18A-D). Finally, production of IL-4 by lymph node cells following restimulation with CII was low and not different from background levels (Figure 18C).

Figure 18. Reduced IFN-γ and IL-17A production following collagen restimulation of cells from CXCR6$^{-/-}$ mice. Wild-type (CXCR6$^{+/+}$) and CXCR6$^{-/-}$ inguinal lymph node cells were isolated at 14 days and 35 days post-immunization, and popliteal lymph node cells were isolated at 35 days post-immunization with type II collagen (CII) in complete Freund's adjuvant (CFA). Cells from wild-type mice immunized with adjuvant alone (CXCR6$^{+/+}$ CFA) were included to verify that the responses were collagen-specific. Cells were cultured for 72 hours in medium alone or in medium with denatured chicken CII. **A-C,** Production of IL-17A **(A)**, IFN-γ **(B)**, and IL-4 **(C)** in the culture supernatants were measured by ELISA at 48 hours. **D,** Cell proliferation in the cultures was measured by assessing ^{3}H-thymidine incorporation (in counts per minute). Data are presented as mean ± SEM in 4-6 mice per group. * = $P < 0.05$ versus medium alone. † = $P < 0.05$ compared with CXCR6$^{+/+}$ mice. Adapted from Slauenwhite, D. et al. Regulation of cytokine polarization and T cell recruitment to inflamed paws in mouse collagen-induced arthritis by the chemokine receptor CXCR6. *Arthritis Rheumatol* (2014) **66**: 3001-3012, copyright 2014 (406).


Inguinal lymph nodes
Day 14
Inguinal lymph nodes
Day 35
Popliteal lymph nodes
Day 35
Media
CII
A
B
C
D
IL-17A (pg/mL)
IFN-γ (pg/mL)
IL-4 (pg/mL)
Proliferation (cpm)
CXCR6+/+
CXCR6-/-
CXCR6+/+
CFA

3.1.7 Impaired inflammatory reaction following in vivo challenge with CII in CXCR6$^{-/-}$ mice

Cells producing the proinflammatory cytokines IFN-γ and IL-17A are reduced in immunized and arthritic CXCR6$^{-/-}$ mice, respectively (Figure 12). Moreover, impaired collagen-specific IFN-γ and IL-17A responses are detected in supernatants of cells isolated from immunized and arthritic CXCR6$^{-/-}$ mice (Figure 18). Therefore, I assessed whether the reduced collagen-specific IFN-γ responses in cells isolated from CXCR6$^{-/-}$ mice influenced the development of DTH reactions in immunized mice.

Wild-type and CXCR6$^{-/-}$ mice were immunized above the base of the tail with CII in CFA 19 days prior to challenge with denatured CII injected intradermally in the ear pinna. As shown in Figure 19A, intradermal challenge with CII elicited an inflammatory response characterized by marked swelling and erythema at the site of challenge, whereas vehicle injection into the other ear induced no visible inflammatory reaction within the same mouse (Figure 19B). Collagen-specific DTH responses were determined by measuring ear thicknesses at 24 h and 48 h post-challenge of the ear that received CII and the ear that received vehicle alone (Figure 19C). The severity of swelling elicited by challenge with CII was significantly less pronounced in CXCR6$^{-/-}$ mice compared to wild-type mice at 24 h post-challenge (Figure 19C). However, at 48 h post-challenge, swelling had begun to subside in both wild-type and CXCR6$^{-/-}$ mice and no significant difference was observed. Furthermore, no differences in ear thickness were observed between wild-type and CXCR6$^{-/-}$ mice in ears that received vehicle alone. Therefore, reduced collagen-specific IFN-γ production by CXCR6$^{-/-}$ cells could be responsible for the impaired DTH reactivity observed in CXCR6$^{-/-}$ mice following in vivo challenge with CII.

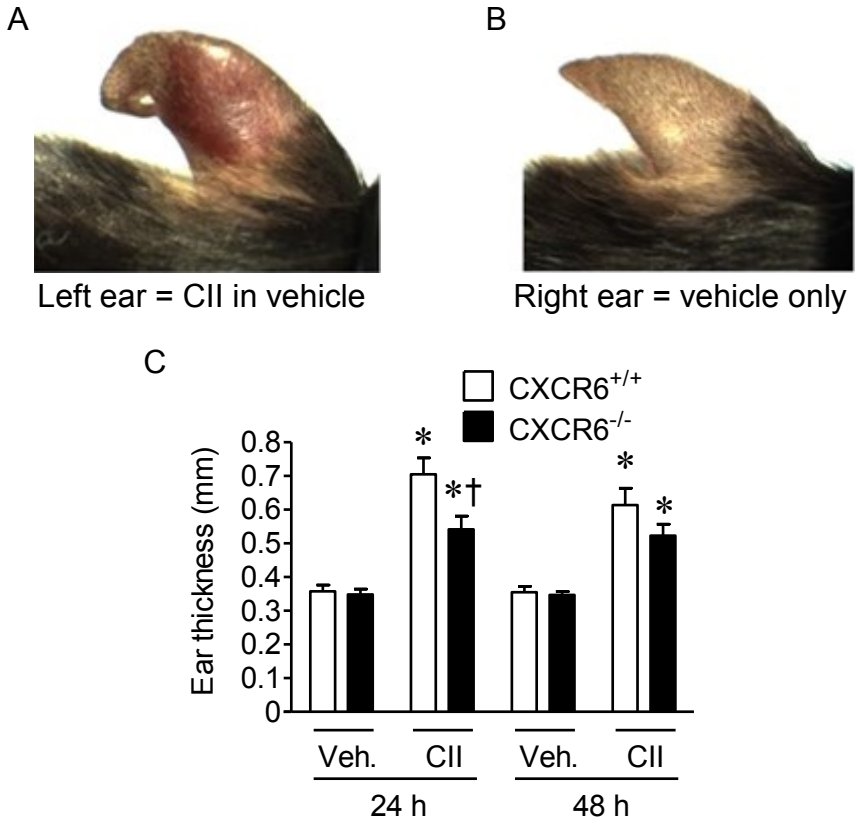


Figure 19. Collagen-specific DTH responses are less pronounced in CXCR6$^{-/-}$ mice. Wild-type (CXCR6$^{+/+}$) and CXCR6$^{-/-}$ mice were immunized with type II collagen (CII) in complete Freund's adjuvant. **A** and **B,** At 19 days post-immunization, mice were challenged with CII in vehicle in one ear **(A)** or vehicle alone (veh.) in the other ear **(B)**. **C,** Ear thicknesses were measured at 24 h and 48 h post-challenge in the ear that received CII and the ear that received vehicle alone to assess collagen-specific inflammation. Data are presented as mean ± SEM in 7-8 mice per group. * = $P < 0.05$ compared with vehicle. † = $P < 0.05$ compared with CXCR6$^{+/+}$ mice.

3.1.8 Collagen-specific IgG2a antibodies are reduced in serum from arthritic CXCR6$^{-/-}$ mice

Autoantibodies such as RF and ACPA/anti-CCP antibodies are strong predictors of radiological progression among patients with RA (118,119). Furthermore, in some patients, antibodies specific for human CII have been detected and were associated with an early inflammatory phenotype and increased radiographic damage (117). Anti-CII antibodies have been shown to be arthritogenic since disease can be induced in mice by transfer of CII-specific antibodies (114,407,408). Therefore, I analyzed sera obtained at day 35 from arthritic wild-type and CXCR6$^{-/-}$ mice for anti-CII IgG1 and IgG2a antibodies.

A high titer of anti-CII antibodies of the IgG1 subclass was detected in mice with CIA, although there was no difference in the levels observed in arthritic wild-type and CXCR6$^{-/-}$ mice (Figure 20A). However, arthritic CXCR6$^{-/-}$ mice had significantly reduced levels of anti-CII IgG2a antibodies compared to wild-type mice (Figure 20B). In contrast, anti-CII antibodies were not detected in nonimmunized wild-type or CXCR6$^{-/-}$ mice. This finding represents another mechanism likely contributing to the reduced incidence and severity of arthritis in CXCR6$^{-/-}$ mice since antibodies of the IgG2a-subclass are induced by IFN-γ and are capable of enhancing arthritis through binding to Fc receptors (409) and promotion of complement activation (407,408).

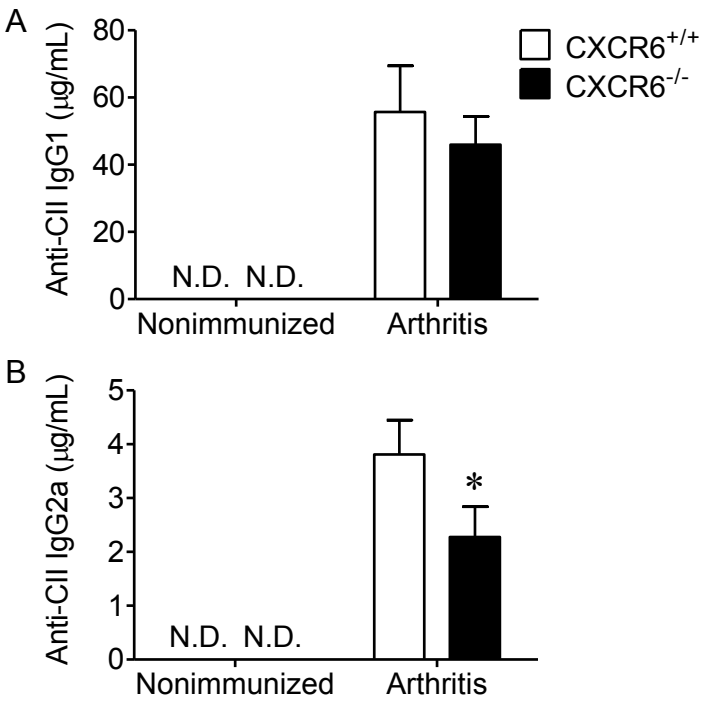


Figure 20. Reduced collagen-specific IgG2a antibody responses in CXCR6$^{-/-}$ mice. Serum was collected from nonimmunized and arthritic wild-type (CXCR6$^{+/+}$) and CXCR6$^{-/-}$ mice at day 35. **A** and **B,** The levels of anti-type II collagen (anti-CII) IgG1 **(A)** and IgG2a **(B)** were measured by ELISA. Anti-CII antibodies were not detected (N.D.) above background levels in nonimmunized mice. Data are presented as mean ± SEM in 10-14 mice per group; n = 4 nonimmunized mice. * = $P < 0.05$ compared with CXCR6$^{+/+}$ mice. Slauenwhite, D. et al. Regulation of cytokine polarization and T cell recruitment to inflamed paws in mouse collagen-induced arthritis by the chemokine receptor CXCR6. *Arthritis Rheumatol* (2014) **66**: 3001-3012, copyright 2014 (406).

3.1.9 Memory T cell populations are reduced in arthritic CXCR6$^{-/-}$ mice

In the intracellular cytokine staining experiments, analysis of cytokine production by T cell subsets was restricted to the CD44^{hi} populations, while CD44^{lo} populations exhibited no staining for IL-17A, IFN-γ, or IL-4. In separate experiments I investigated whether CXCR6$^{-/-}$ mice also had reduced frequencies and numbers of CD4^{+}, CD8^{+}, and DN T cells with a memory-like phenotype (CD44^{hi}CD45RB^{lo}) and expression of the early activation marker CD69 within the inguinal lymph nodes, popliteal lymph nodes, and spleen compared to wild-type mice.

Both the memory (CD44^{hi}CD45RB^{lo}) and naïve (CD44^{lo}CD45RB^{+}) populations were clearly discernable within CD4^{+} T cells from the spleen (Figure 21A). However, the CD44^{lo}CD45RB^{+} CD4^{+} T cell population was almost entirely absent within wild-type arthritic paws, with nearly all CD4^{+} T cells exhibiting a CD44^{hi}CD45RB^{lo} phenotype. This result was representative of what I observed within the arthritic paws of CXCR6$^{-/-}$ mice as well, and as such I did not continue to examine these populations within arthritic paws. Analysis of memory cells with a CD44^{hi} phenotype within the inguinal lymph nodes, popliteal lymph nodes, and spleen of arthritic wild-type and CXCR6$^{-/-}$ mice at day 35 revealed reduced frequencies and absolute numbers of CD44^{hi} CD4^{+} T cells (Figures 21B and 21C), CD8^{+} T cells (Figures 22A and 22B), and DN T cells (Figures 23A and 23B) in CXCR6$^{-/-}$ mice.

In the inguinal lymph nodes, popliteal lymph nodes, and spleen the absolute numbers (and frequencies in some cases) of CD4^{+} (Figures 21D and 21E), CD8^{+} (Figures 22C and 22D), and DN T cells (Figures 23C and 23D) expressing the early activation marker CD69 were significantly increased in arthritic mice compared to nonimmunized control mice. This can be appreciated from representative plots showing CD69 staining on CD4^{+} T cells isolated from the lymph nodes of nonimmunized and arthritic wild-type mice (Figure 24). The level of CD69 staining was also increased on the CD4^{+} T cell population within the arthritic paws compared to nonimmunized mice (Figure 24D). The results in Figure 24 were also representative of the levels of CD69 staining within CXCR6$^{-/-}$ mice.

In contrast to the reduced frequency and number of memory T cells observed in arthritic CXCR6$^{-/-}$ mice, in general no significant differences were observed in the frequencies or numbers of CD69^{+} cells between arthritic wild-type and CXCR6$^{-/-}$ mice at day 35. Nonetheless, I observed a reduced frequency, but not absolute number, of CD69^{+} CD4^{+} T cells (Figures 21D and 21E), and an increased frequency and number of CD69^{+} CD8^{+} T cells in the spleen in arthritic CXCR6$^{-/-}$ mice compared to arthritic wild-type mice (Figures 22C and 22D). Our laboratory has previously determined that nonimmunized wild-type and CXCR6$^{-/-}$ mice are equivalent in their expression of a range of cell surface markers (A. Sudworth and B. Johnston, unpublished data), and as shown in Figures 11A-E, total cell numbers and absolute T cell numbers are equivalent in nonimmunized wild-type and CXCR6$^{-/-}$ mice. Therefore only wild-type nonimmunized controls were included in these experiments. These findings suggest that memory T cell subsets are reduced in arthritic CXCR6$^{-/-}$ mice compared to wild-type mice.

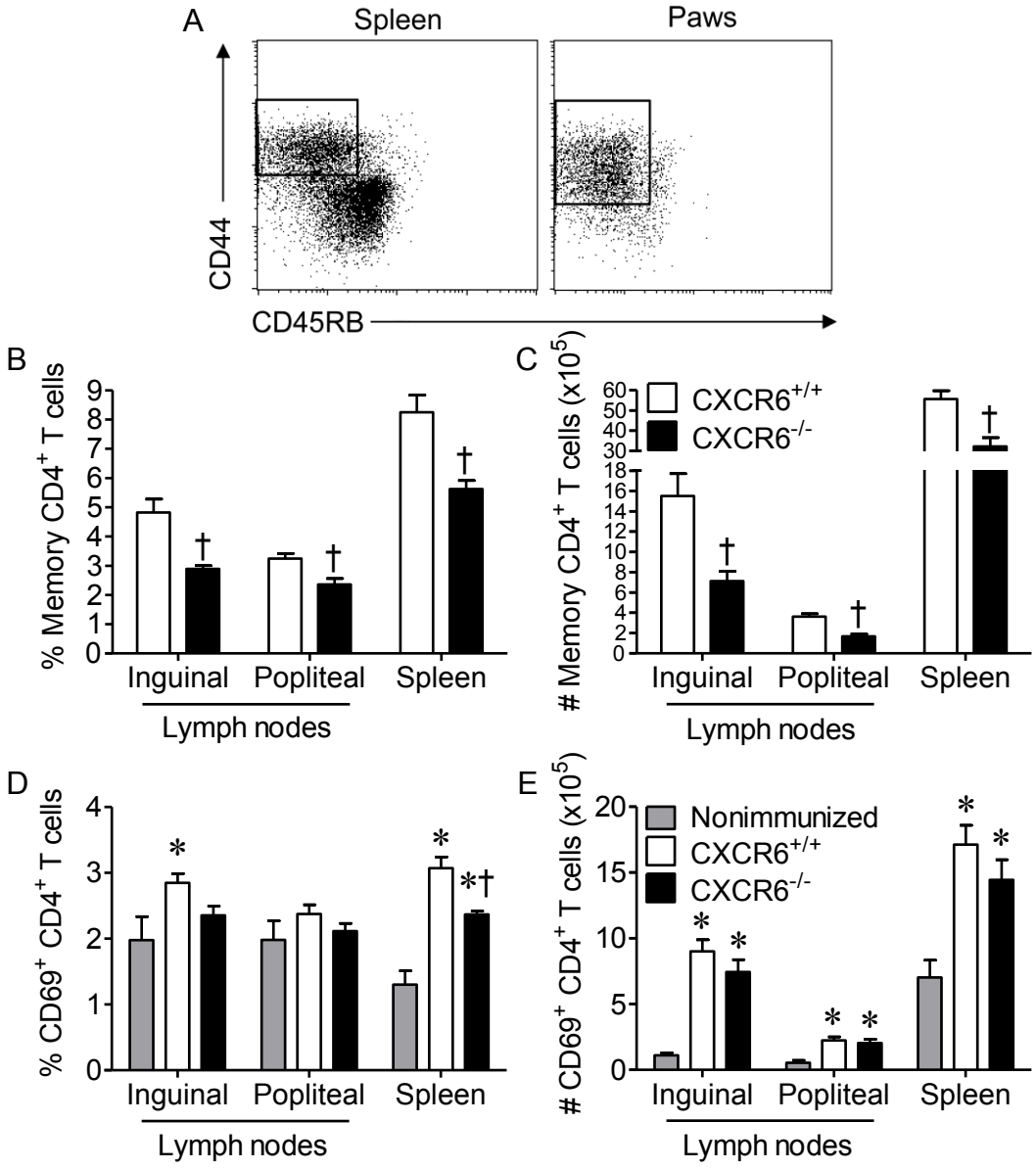


Figure 21. Memory CD4$^+$ T cells, but not CD69$^+$ CD4$^+$ T cells, are reduced in arthritic CXCR6$^{-/-}$ mice. A, Representative dot plots showing CD44 versus CD45RB staining on CD4$^+$ T cell populations isolated from the spleen and paws of arthritic wild-type mice at day 35. The rectangles represent gate placement for the CD44^{hi}CD45RB^{lo} subset. **B** and **C,** The frequencies **(B)** and absolute numbers **(C)** of CD44^{hi}CD45RB^{lo} CD4$^+$ T cells in the inguinal lymph nodes, popliteal lymph nodes, and spleen of arthritic wild-type (CXCR6$^{+/+}$) and CXCR6$^{-/-}$ mice at day 35 (n = 7 and 5, respectively). **D** and **E,** The frequencies **(D)** and absolute numbers **(E)** of CD69$^+$ CD4$^+$ T cells in the inguinal lymph nodes, popliteal lymph nodes, and spleen of nonimmunized mice (n = 5), and arthritic wild-type (n = 13) and CXCR6$^{-/-}$ mice (n = 7) at day 35. Data are presented as mean ± SEM. * = $P < 0.05$ compared with nonimmunized mice. † = $P < 0.05$ compared with CXCR6$^{+/+}$ mice.

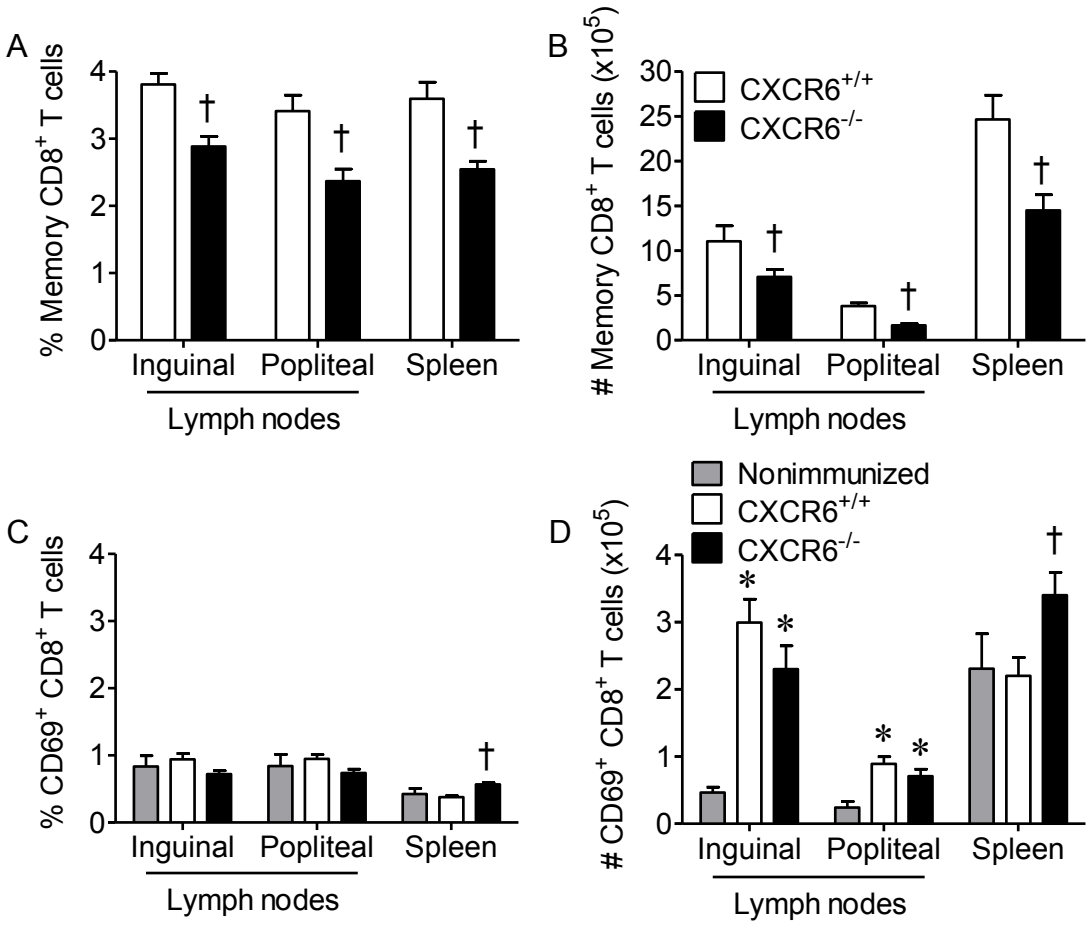


Figure 22. Memory CD8$^+$ T cells, but not CD69$^+$ CD8$^+$ T cells, are reduced in arthritic CXCR6$^{-/-}$ mice. A and **B,** The frequencies **(A)** and absolute numbers **(B)** of CD44^{hi} CD8$^+$ T cells in the inguinal lymph nodes, popliteal lymph nodes, and spleen of arthritic wild-type (CXCR6$^{+/+}$) and CXCR6$^{-/-}$ mice at day 35 (n = 7 and 5, respectively). **C** and **D,** The frequencies **(C)** and absolute numbers **(D)** of CD69$^+$ CD8$^+$ T cells in the inguinal lymph nodes, popliteal lymph nodes, and spleen of nonimmunized mice (n = 5), and arthritic wild-type (n = 13) and CXCR6$^{-/-}$ mice (n = 7) at day 35. Data are presented as mean ± SEM. * = $P < 0.05$ compared with nonimmunized mice. † = $P < 0.05$ compared with CXCR6$^{+/+}$ mice.

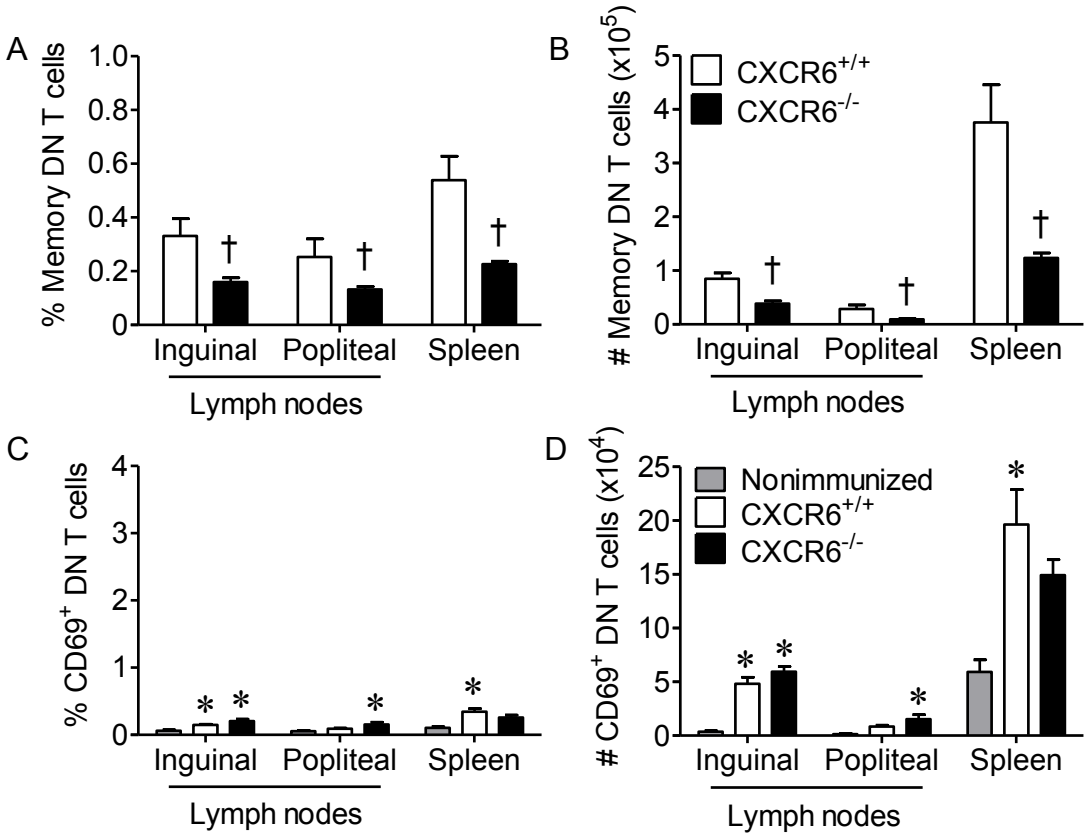


Figure 23. Memory DN T cells, but not CD69$^+$ DN T cells, are reduced in arthritic CXCR6$^{-/-}$ mice. A and **B,** The frequencies **(A)** and absolute numbers **(B)** of CD44^{hi}CD45RB^{lo} CD4$^-$CD8$^-$ [double negative (DN)] T cells in the inguinal lymph nodes, popliteal lymph nodes, and spleen of arthritic wild-type (CXCR6$^{+/+}$) and CXCR6$^{-/-}$ mice at day 35 (n = 7 and 5, respectively). **C** and **D,** The frequencies **(C)** and absolute numbers **(D)** of CD69$^+$ DN T cells in the inguinal lymph nodes, popliteal lymph nodes, and spleen of nonimmunized mice (n = 5), and arthritic wild-type (n = 13) and CXCR6$^{-/-}$ mice (n = 7) at day 35. Data are presented as mean ± SEM. * = $P < 0.05$ compared with nonimmunized mice. † = $P < 0.05$ compared with CXCR6$^{+/+}$ mice.

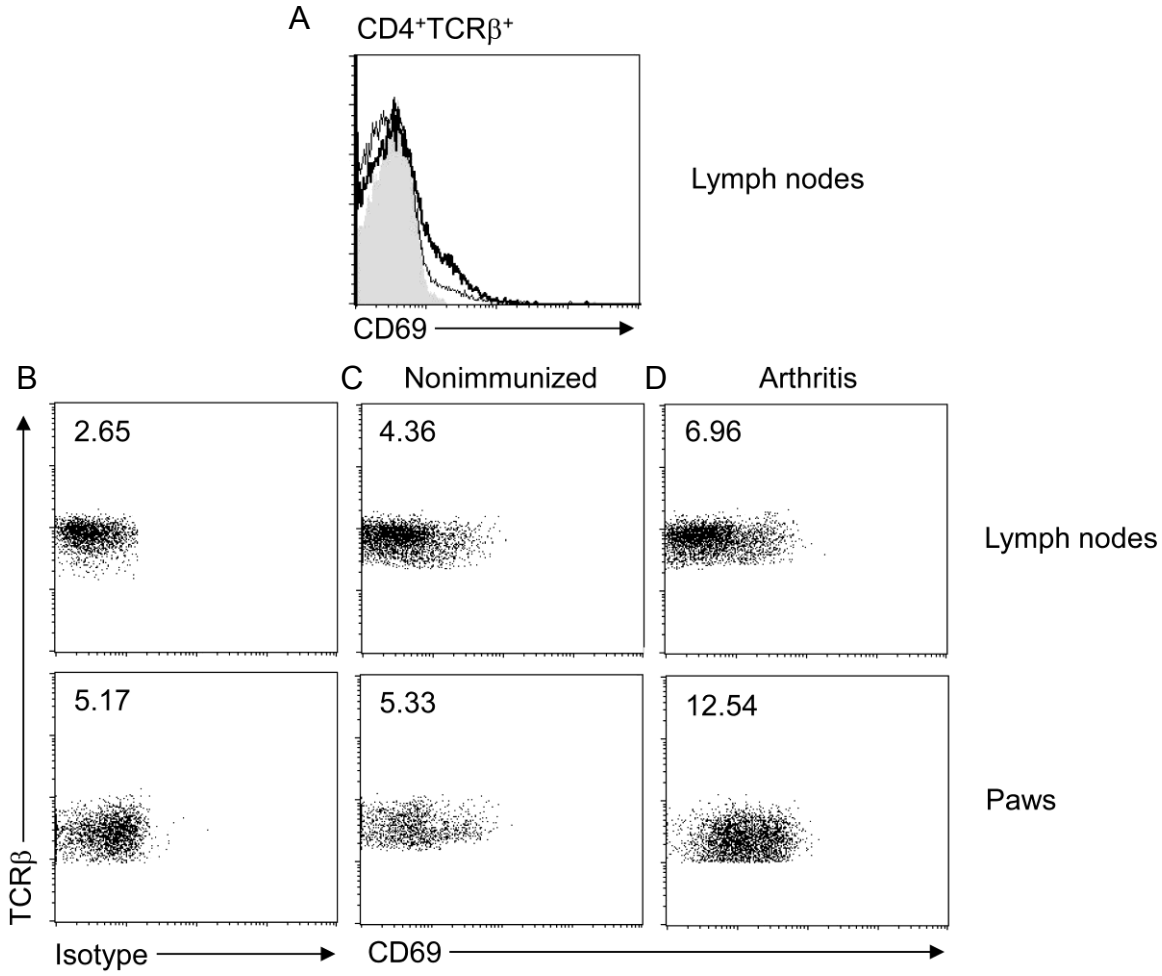


Figure 24. CD4$^+$ T cells isolated from arthritic wild-type paws display high levels of the early activation marker CD69. A, Representative histogram showing CD69 staining on CD4$^+$ T cells isolated from the lymph nodes of nonimmunized wild-type mice (thin line) and arthritic wild-type mice (bold line) at day 35 versus isotype control staining (grey filled line). **B-D,** Representative dot plots showing CD69 and T cell receptor β (TCRβ) staining among CD4$^+$ T cells isolated from the lymph nodes and paws of nonimmunized wild-type mice **(C)** and arthritic wild-type mice at day 35 **(D)** versus isotype control staining **(B)**. Values are the geometric mean fluorescence intensity of CD69 staining associated with each plot.

3.1.10 Reduced expression of RANKL on T cells in arthritic CXCR6$^{-/-}$ mice

Receptor activator of nuclear factor κB ligand (RANKL), together with its receptor RANK, act as potent drivers of osteoclast differentiation and activation (410), and plays an essential role in bone damage in arthritis (411,412). RANKL is highly expressed in the synovium of RA patients (413,414), where it is expressed by resident synovial cells and infiltrating T cells (155,413,414). Therefore, I investigated whether there were differences in RANKL expression on T cells isolated from the lymph nodes (pooled inguinal and popliteal lymph nodes) and paws of arthritic wild-type and CXCR6$^{-/-}$ mice at day 35 (Figure 25).

Compared to wild-type mice, CXCR6$^{-/-}$ mice exhibited a reduction in the frequency and number of RANKL^{+} CD4^{+} T cells in the arthritic paws, but not in the lymph nodes (Figures 25A and 25B). The reduced RANKL staining on T cells isolated from the paws of arthritic CXCR6$^{-/-}$ mice was striking, as shown by the significantly lower gMFI of RANKL staining on paw isolated T cell populations (Figures 25C and 25D). Indeed, Figure 25C shows that the RANKL staining intensity on paw isolated T cells from CXCR6$^{-/-}$ mice overlaps with the background staining obtained using an isotype control antibody. While the frequency and gMFI of RANKL^{+} CD8^{+} T cells were reduced in both the lymph nodes and paws of arthritic CXCR6$^{-/-}$ mice, the absolute numbers were not significantly different from wild-type mice (Figures 25A, 25B, and 25D). Moreover, neither the frequency nor number of RANKL^{+} DN T cells were significantly different in the lymph nodes or paws of arthritic wild-type and CXCR6$^{-/-}$ mice (Figures 25A and 25B). Since CD4^{+} T cells account for the majority of T cells accumulating in the arthritic paws (Figure 11C-E), their lower expression of RANKL in CXCR6$^{-/-}$ mice could represent another mechanism through which CXCR6$^{-/-}$ mice are protected from CIA.

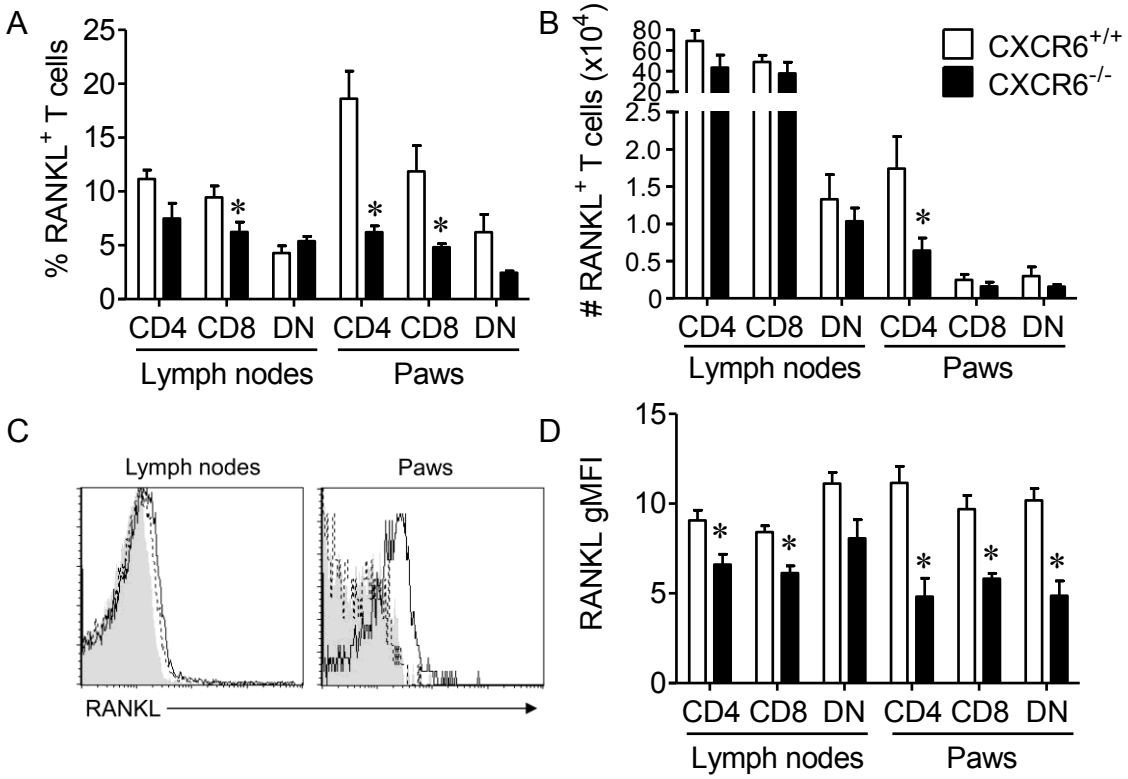


Figure 25. RANKL expression is reduced on T cells isolated from arthritic paws in CXCR6$^{-/-}$ mice. A and **B,** The frequencies **(A)** and absolute numbers **(B)** of RANKL^{+} CD4^{+}, CD8^{+}, and CD4^{-}CD8^{-} [double negative (DN)] T cells in the lymph nodes (pooled inguinal and popliteal lymph nodes) and paws of arthritic wild-type (CXCR6$^{+/+}$) and CXCR6$^{-/-}$ mice at day 35. **C,** Representative histograms showing the expression of RANKL among total T cells isolated from the lymph nodes and paws of arthritic wild-type (solid line) and CXCR6$^{-/-}$ mice (broken line) at day 35 versus isotype control staining (grey filled line). **D,** Geometric mean fluorescence intensity (gMFI) of RANKL staining on CD4^{+}, CD8^{+}, and DN T cells in the lymph nodes and paws of arthritic wild-type and CXCR6$^{-/-}$ mice at day 35. Data are presented as mean ± SEM in 6-7 mice per group. * = $P < 0.05$ compared with CXCR6$^{+/+}$ mice.

3.1.11 Diminished accumulation of CXCR6$^{-/-}$ T cells within inflamed paws

CXCR6 expression is dramatically elevated on CD4^{+} T cells isolated from the paws of arthritic mice compared to those from secondary lymphoid tissues or to those from the paws of nonimmunized mice (Figures 4, 5, and 7). Moreover, CD4^{+} T cell numbers were markedly increased in the paws of arthritic wild-type mice, but this accumulation was attenuated in the paws of arthritic CXCR6$^{-/-}$ mice (Figure 11D). Similar to the elevated CXCL16 expression detected in the synovium in RA (251–253), CXCL16 protein levels are high in the synovium of mice with CIA but are not detectable in the synovium from normal healthy mice (251). Therefore I was interested in determining whether cells lacking CXCR6 were impaired in their ability to home to the inflamed paws of mice with CIA. Consistent with previous reports (279), in vitro activation of T lymphocytes via incubation in anti-CD3ε–coated plates upregulated the surface expression of CXCR6 (Figure 26A), and in vitro activated T cells from wild-type mice functionally migrated in Transwell chemotaxis assays in response to the ligand CXCL16 (Figure 26B). In contrast, activated T cells from CXCR6$^{-/-}$ mice failed to migrate in response to the optimal CXCL16 concentration (Figure 26B).

To investigate whether CXCR6 directly contributes to the homing of activated T cells to the inflamed paws, I performed competitive adoptive transfers, in which equal numbers of in vitro activated wild-type and CXCR6$^{-/-}$ T cells were differentially labeled with CellTracker dyes and co-adoptively transferred into naïve and arthritic wild-type mice. Donor cell populations were detected by flow cytometry in various tissues isolated from recipient mice 24 h post-transfer (Figure 27A). As shown in Figure 27B, the ratio of donor wild-type T cells to donor CXCR6$^{-/-}$ T cells was approximately 1:1 in the spleen, inguinal lymph nodes, and popliteal lymph nodes. Interestingly, in the paws of recipient arthritic mice, a 2:1 ratio of wild-type to CXCR6$^{-/-}$ T cells was recovered, whereas there was no bias for the recruitment of CXCR6$^{+/+}$ T cells to uninflamed paws of naïve mice (Figure 27B). These findings demonstrate that CXCR6 is important in the homing of activated T cells to the inflamed paws of mice with CIA.

CXCR6 and CXCL16 are expressed in other organs and tissues in addition to the synovium in both human RA and mouse CIA. For example, CXCL16 is expressed in the

kidney, liver, lung, and gastrointestinal tract (279,280,300). While I did not examine all of these tissues for selective recruitment of $CXCR6^{+/+}$ donor T cells, I did observe a >2-fold difference in the accumulation of $CXCR6^{+/+}$ T cells versus $CXCR6^{-/-}$ T cells in the liver and lungs, but not the bone marrow, in recipient mice (Figure 27C). The absolute numbers of labeled wild-type and $CXCR6^{-/-}$ donor cells recovered from the tissues of nonimmunized and arthritic recipient mice at 24 h post-transfer are summarized in Table 3. Dye labeling did not affect recruitment, since homing of wild-type cells was equivalent when the cells were labeled with either dye, and the $CXCR6^{+/+}$ versus $CXCR6^{-/-}$ T cell competitive homing results were reproduced using different dyes altogether (data not shown).

A previous study has shown that most cytokine-activated $CXCR6^{+}$ T cells coexpress CCR5 and CCR6 (398), suggesting that CCR5 and CCR6 may be coordinately regulated with CXCR6. I utilized the same activating conditions described earlier and examined the expression of CCR5, CCR6, CXCR3, and CXCR6 on activated T lymphocytes by flow cytometry (Figure 28). Anti-CD3ε stimulation of wild-type T cells in vitro did not upregulate the expression of CCR5 or CCR6, but caused potent upregulation of CXCR3 and CXCR6 (Figure 28). In vitro activated T cells from $CXCR6^{-/-}$ mice exhibited similar levels of CXCR3, but like anti-CD3ε activated T cells from wild-type mice, displayed no detectable CCR5 or CCR6. Therefore, coordinated expression of CCR5 and CCR6 on $CXCR6^{+}$ T cells appears to be dependent upon the specific activating stimuli. Furthermore, impaired homing of donor $CXCR6^{-/-}$ T cells to the inflamed paws is not attributable to differences in their expression of CCR5, CCR6, or CXCR3.

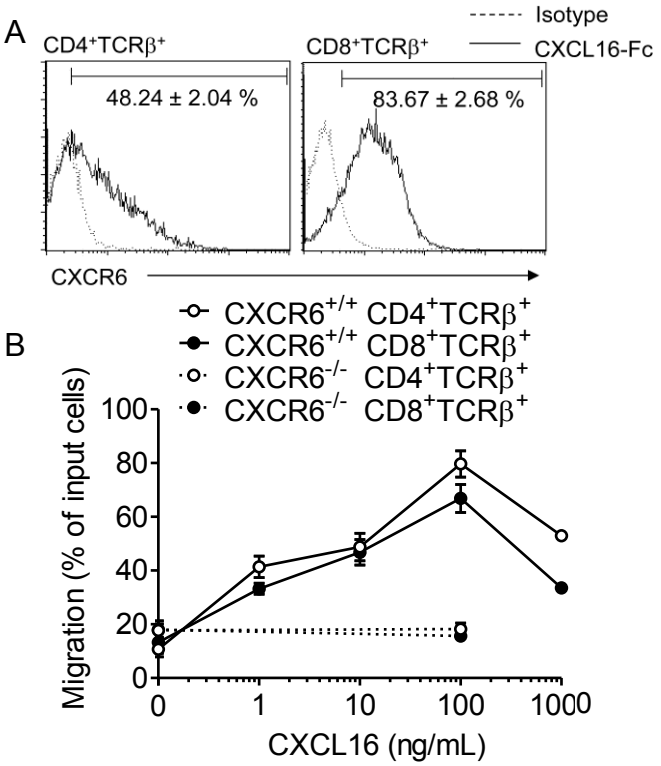


Figure 26. Functional CXCR6 is upregulated on T lymphocytes following in vitro activation via anti-CD3ε and anti-CD28 stimulation. Splenocytes isolated from naïve wild-type ($CXCR6^{+/+}$) and $CXCR6^{-/-}$ mice were incubated in anti-CD3ε–coated plates containing medium supplemented with anti-CD28 and mouse IL-2 for 5 days, and then incubated in fresh plates for an additional 2 days in the presence of IL-2 alone. **A,** Representative histograms showing CXCR6 staining on activated $CD4^+$ and $CD8^+$ T cell receptor β-positive ($TCR\beta^+$) cells from wild-type mice, determined using a CXCL16-Fc fusion construct relative to isotype control. Values are the mean ± SEM obtained from 4 independent cultures. **B,** Chemotactic migration of in vitro activated wild-type and $CXCR6^{-/-}$ $CD4^+$ and $CD8^+$ T cells toward CXCL16 was assessed in Transwell chemotaxis assays. Chemotaxis of activated T cells was determined by comparing the ratio of cells to a polystyrene bead internal standard. Data are presented as mean ± SEM of 4 separate experiments. Adapted from Slauenwhite, D. et al. Regulation of cytokine polarization and T cell recruitment to inflamed paws in mouse collagen-induced arthritis by the chemokine receptor CXCR6. *Arthritis Rheumatol* (2014) **66**: 3001-3012, copyright 2014 (406).

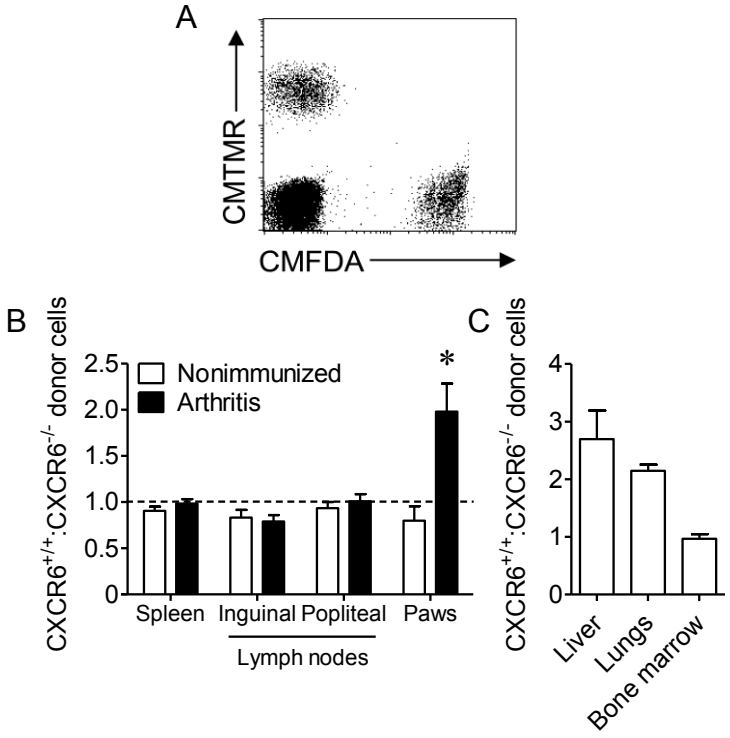


Figure 27. Impaired recruitment of CXCR6$^{-/-}$ T cells to arthritic paws. In vitro activated T cells from wild-type (CXCR6$^{+/+}$) and CXCR6$^{-/-}$ mice were differentially labeled with CellTracker dyes: activated wild-type T cells were labeled with CMTMR and activated CXCR6$^{-/-}$ T cells were labeled with CMFDA. Differentially labeled cells were combined in equal numbers and injected intravenously into wild-type recipient mice. **A,** Representative dot plot showing CMTMR labeled donor wild-type T cells and CMFDA labeled donor CXCR6$^{-/-}$ T cells within splenocytes isolated from a naïve recipient mouse at 24 h post-transfer. **B** and **C,** The ratio of labeled donor cells recovered from the spleen, inguinal lymph nodes, popliteal lymph nodes, and paws of nonimmunized or arthritic wild-type recipient mice (on days 30-35) **(B)** or from the liver, lungs, and bone marrow of nonimmunized wild-type recipient mice **(C)** were assessed by flow cytometry 24 h after adoptive transfer. Data are presented as mean ± SEM in 4-6 mice per group. * = $P < 0.05$ compared to tissue homing in nonimmunized mice. Adapted from Slauenwhite, D. et al. Regulation of cytokine polarization and T cell recruitment to inflamed paws in mouse collagen-induced arthritis by the chemokine receptor CXCR6. *Arthritis Rheumatol* (2014) **66**: 3001-3012, copyright 2014 (406).

Table 3. Absolute numbers of labelled wild-type ($CXCR6^{+/+}$) and $CXCR6^{-/-}$ donor cells recovered from tissues of recipient mice at 24 h post-transfer (n = 4-6)

Mouse	Tissue	$CXCR6^{+/+}$	$CXCR6^{-/-}$
Arthritis	Spleen	$1.51 \pm 0.24 \times 10^5$	$1.56 \pm 0.17 \times 10^5$
	Inguinal lymph node	$0.40 \pm 0.11 \times 10^5$	$0.55 \pm 0.17 \times 10^5$
	Popliteal lymph node	$0.11 \pm 0.03 \times 10^5$	$0.11 \pm 0.02 \times 10^5$
	Paws	$0.20 \pm 0.09 \times 10^5$	$0.11 \pm 0.05 \times 10^5$
Nonimmunized	Spleen	$1.06 \pm 0.04 \times 10^5$	$1.16 \pm 0.08 \times 10^5$
	Inguinal lymph node	$0.98 \pm 0.28 \times 10^3$	$1.24 \pm 0.43 \times 10^3$
	Popliteal lymph node	$0.67 \pm 0.14 \times 10^3$	$0.73 \pm 0.18 \times 10^3$
	Paws	$0.46 \pm 0.15 \times 10^3$	$0.59 \pm 0.22 \times 10^3$
	Liver	$0.50 \pm 0.09 \times 10^5$	$0.20 \pm 0.05 \times 10^5$
	Lungs	$6.41 \pm 2.08 \times 10^3$	$3.08 \pm 1.09 \times 10^3$
	Bone marrow	$0.13 \pm 0.03 \times 10^5$	$0.14 \pm 0.04 \times 10^5$

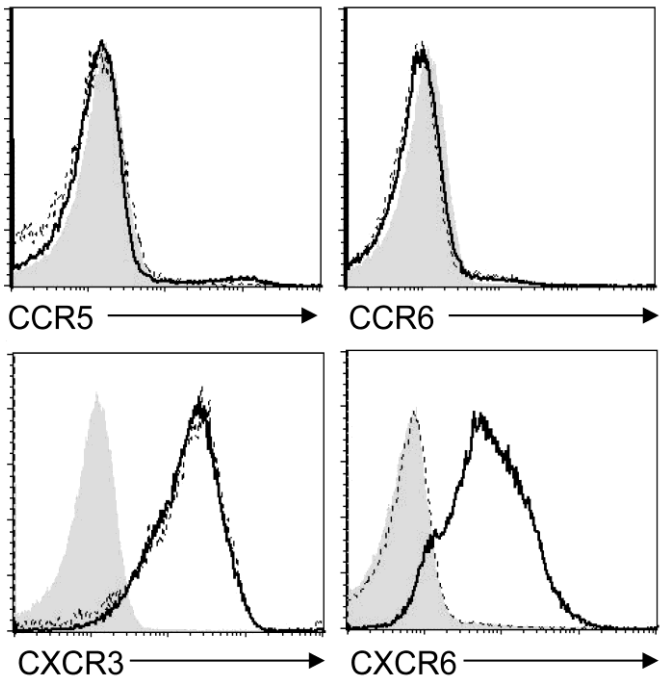


Figure 28. CXCR3 and CXCR6, but not CCR5 or CCR6, are upregulated on T lymphocytes following in vitro activation via anti-CD3ε and anti-CD28 stimulation. Representative histograms showing CCR5, CCR6, CXCR3, and CXCR6 staining on in vitro activated T cells from wild-type mice (bold lines) and $CXCR6^{-/-}$ mice (broken lines) relative to isotype controls (grey filled lines). Data are representative of 4 independent cultures.

3.1.12 CCR5, CCR6, and CXCR3 are expressed on T cells isolated from arthritic paws

The incidence and mean clinical severity scores of arthritis are reduced in CXCR6$^{-/-}$ mice but these mice are not completely protected from CIA. Despite lower numbers of CD4^{+} T cells in the inflamed paws of arthritic CXCR6$^{-/-}$ mice, there is still significant accumulation at this site, suggesting an alternative mechanism contributes to their homing in the absence of CXCR6. Therefore I examined chemokine receptor profiles on T cell populations from the lymph nodes and paws of arthritic wild-type and CXCR6$^{-/-}$ mice to assess which other chemokine receptors were highly expressed in CIA.

The frequencies (Figures 29A and 29B), absolute numbers (Figures 29C and 29D), and gMFI (Figures 29E and 29F) of CCR5^{+}, CCR6^{+}, and CXCR4^{+} T cells isolated from the lymph nodes of arthritic wild-type and CXCR6$^{-/-}$ mice were low compared to CCR7^{+} T cells. Besides CCR7, T cells expressing CXCR3 were present at the greatest frequencies and numbers in the lymph nodes of both wild-type and CXCR6$^{-/-}$ CIA mice. The lymph node DN T cell subset appeared to have the strongest expression of CXCR3 (and CCR5/CCR6) in both wild-type and CXCR6$^{-/-}$ mice as indicated by their higher frequencies (Figures 29A and 29B) and gMFI (Figures 29E and 29F) compared to CD8^{+} T cells, which exhibited intermediate levels, and the low levels of CXCR3 on CD4^{+} T cell subsets. However, DN T cells are rare in secondary lymphoid tissues and their numbers are much lower than either CD4^{+} or CD8^{+} T cells, which explains the low numbers of chemokine receptor-positive DN T cells in the lymph nodes (Figures 29C and 29D).

In contrast to lymph node T cell populations, the frequencies (Figures 30A and 30B), absolute numbers (Figures 30C and 30D), and gMFI (Figures 30E and 30F) of CCR7^{+} T cells isolated from the arthritic paws of wild-type and CXCR6$^{-/-}$ mice were low. The frequencies and gMFI of CXCR3^{+} T cells isolated from arthritic paws were much higher than those in the lymph nodes; although the frequencies, absolute numbers, and gMFI of CXCR3^{+} T cells did not match the levels observed for CXCR6^{+} T cells in the arthritic paws of wild-type mice (Figures 30A, 30C, and 30E). Interestingly, the frequencies, absolute numbers, and gMFI of CCR6^{+} CD4^{+} T cells were significantly ($P < 0.05$) greater in arthritic paws of wild-type mice than in CXCR6$^{-/-}$ mice, which is

consistent with the greater frequencies and numbers of IL-17A-producing $CD4^+$ T cells in the paws of arthritic wild-type mice (Figures 12D and 13D), since joint infiltrating Th17 cells prominently express CCR6 (415). In contrast, the frequencies of $CCR5^+$ $CD4^+$ and $CD8^+$ T cells were significantly ($P < 0.05$) higher in arthritic paws from $CXCR6^{-/-}$ mice compared to wild-type mice, whereas $CXCR3^+$ T cell frequencies were not different between wild-type and $CXCR6^{-/-}$ mice (Figures 30A and 30B). These data suggest that CCR5 and CXCR3 may contribute to T cell accumulation within the arthritic paws of $CXCR6^{-/-}$ CIA mice.

As mentioned earlier, CCR5 and CCR6 may be coordinately regulated with CXCR6 (398). While I did not observe upregulated expression of CCR5 or CCR6 following anti-CD3ε stimulation in culture (Figure 28), I was interested in determining if T cells isolated from the arthritic paws showed altered expression of other chemokine receptors depending upon their transcriptional activation of the CXCR6 gene. Therefore, I isolated T cells from the arthritic paws of $CXCR6^{-/-}$ mice, in which the coding sequence for CXCR6 is replaced with a GFP construct (398), and examined the surface expression of CCR5, CCR6, and CXCR3 on GFP^{hi} versus GFP^{lo} T cells (Figure 31). As shown in Figure 31A, the majority of $CD4^+$ T cells isolated from the arthritic paws of $CXCR6^{-/-}$ mice were GFP^{hi}, whereas fewer GFP^{hi} $CD8^+$ T cells were detected in these tissues. The frequency of GFP^{hi} cells was consistent with the frequency of $CXCR6^+$ staining in $CD4^+$ and $CD8^+$ T cells isolated from the arthritic paws of wild-type mice (Figure 4A). No differences were observed between GFP^{hi} and GFP^{lo} $CD4^+$ and $CD8^+$ T cells in terms of their expression of CCR5, CCR6, or CXCR3 (Figure 31B).

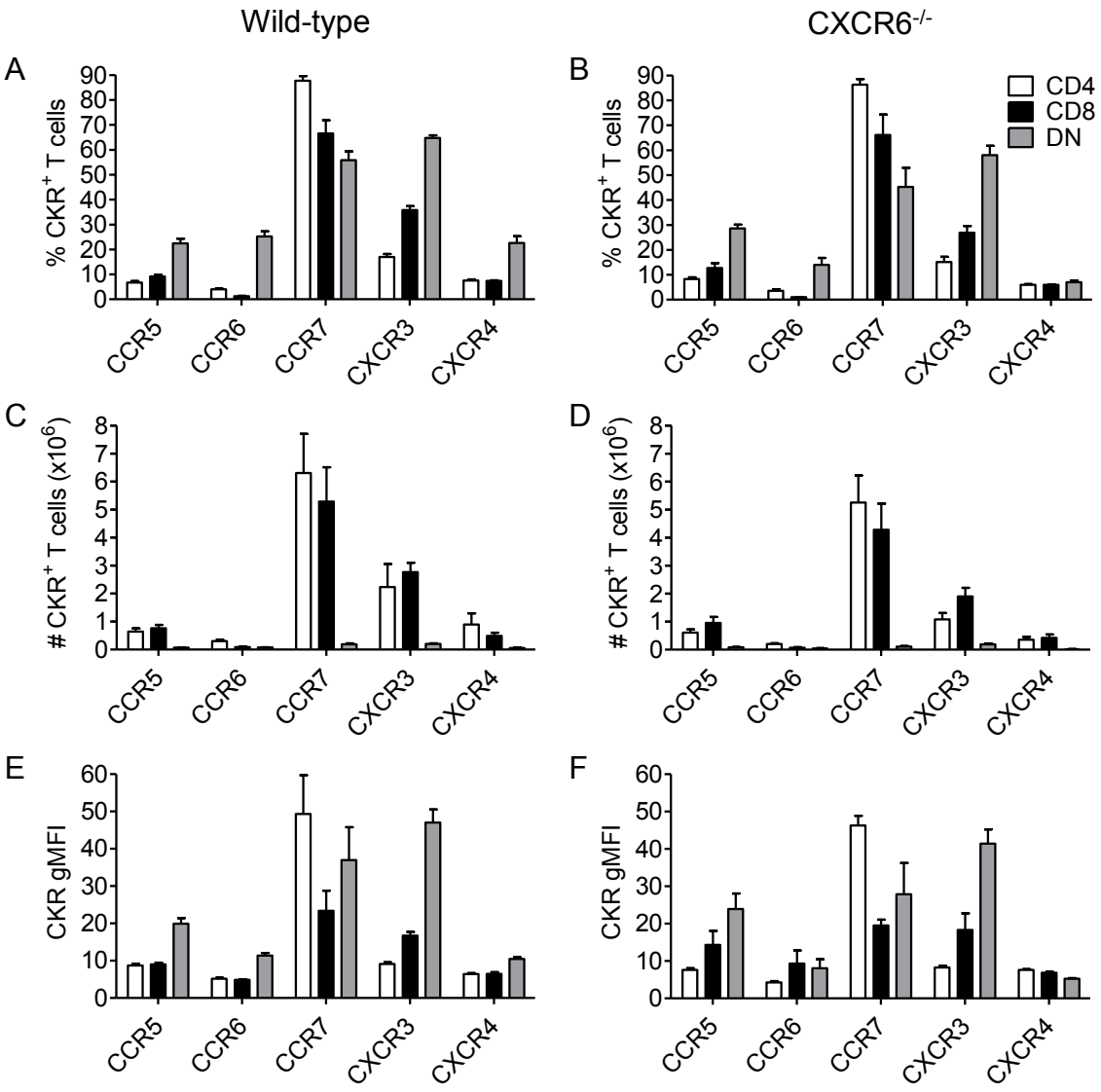


Figure 29. Chemokine receptor expression profiles on T cell subsets in the lymph nodes of arthritic mice. The frequencies (**A** and **B**), absolute numbers (**C** and **D**), and geometric mean fluorescence intensity (gMFI; **E** and **F**) of chemokine receptor-positive (CKR^+) $CD4^+$, $CD8^+$, and $CD4^-CD8^-$ [double negative (DN)] T cells in the lymph nodes (pooled inguinal and popliteal lymph nodes) of arthritic wild-type (left; **A**, **C**, and **E**) and $CXCR6^{-/-}$ mice (right; **B**, **D**, and **F**) at day 35. Data are presented as mean ± SEM in n = 7-13 (CCR5 and CXCR3), n = 6-10 (CCR6), n = 5-7 (CCR7) and n = 3 (CXCR4) mice per group.

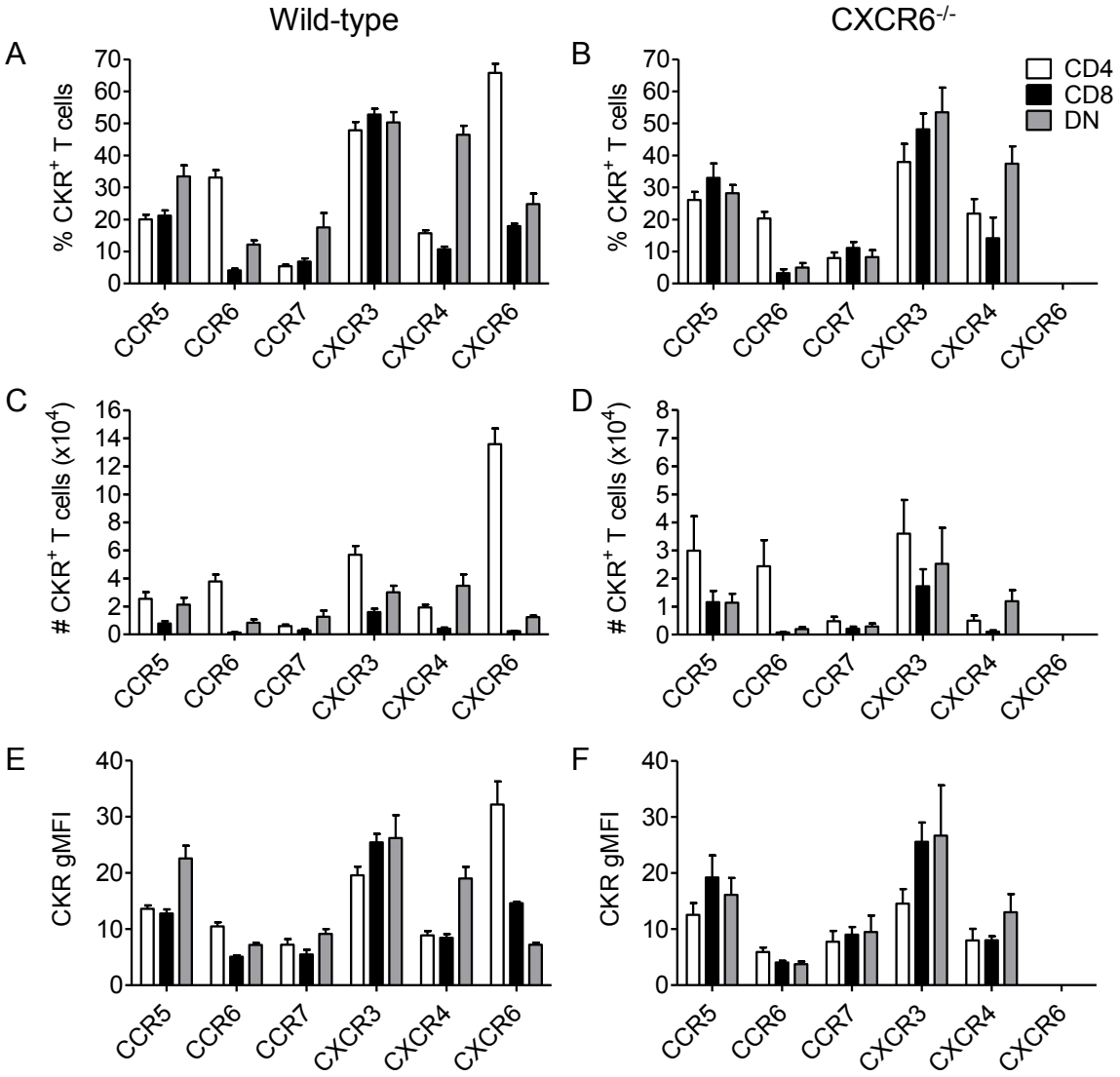


Figure 30. Chemokine receptor expression profiles on T cell subsets in the paws of arthritic mice. The frequencies (**A** and **B**), absolute numbers (**C** and **D**), and geometric mean fluorescence intensity (gMFI; **E** and **F**) of chemokine receptor-positive (CKR^+) $CD4^+$, $CD8^+$, and $CD4^-CD8^-$ [double negative (DN)] T cells in the paws of arthritic wild-type (left; **A**, **C**, and **E**) and $CXCR6^{-/-}$ mice (right; **B**, **D**, and **F**) at day 35. Data are presented as mean ± SEM in n = 7-13 (CCR5, CCR6, and CXCR3), n = 5-10 (CCR7), n = 3-8 (CXCR4), and n = 4 (CXCR6) mice per group.

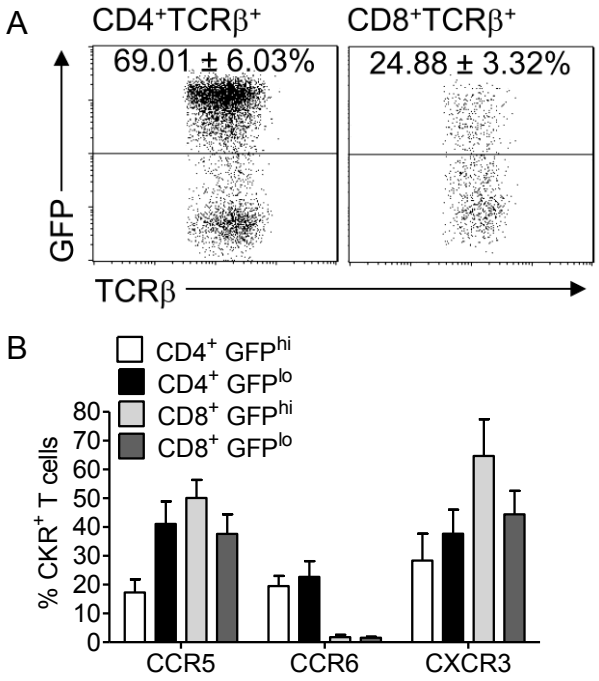


Figure 31. Chemokine receptor expression on GFP^{hi} versus GFP^{lo} T cells from CXCR6$^{-/-}$ arthritic paws. A, Representative dot plots showing the expression of green fluorescent protein (GFP) and T cell receptor β (TCRβ) on CD4^{+} and CD8^{+} T cells from the paws of arthritic CXCR6$^{-/-}$ mice on day 35. Values are percent of events above the indicated threshold presented as the mean ± SEM in 4 mice. **B,** The frequency of chemokine receptor-positive (CKR^{+}) GFP^{hi} and GFP^{lo} CD4^{+} and CD8^{+} T cells in the paws of arthritic CXCR6$^{-/-}$ mice at day 35. Data are presented as mean ± SEM in 4 mice.

3.1.13 CXCR3$^{-/-}$ and CCR5$^{-/-}$ mice are protected from CIA

In addition to CXCR6, a large proportion of T cells isolated from arthritic paws at day 35 express the receptor CXCR3, while a smaller but sizeable proportion of these cells express CCR5 (Figure 30). To examine the contributions of CXCR3 and CCR5 to CIA, CXCR3$^{-/-}$ and CCR5$^{-/-}$ mice were immunized and monitored for the development of arthritis over an 80 day period. The incidence of arthritis was significantly lower in CXCR3$^{-/-}$ mice (8/17; 47%) and CCR5$^{-/-}$ mice (6/14; 42.8%) than in matched groups of wild-type mice (14/17; 82.4% and 15/16; 93.8%, respectively) by day 80 post-immunization (Figures 32A and 33A). The mean clinical arthritis activity scores in mice that exhibited signs of disease were not significantly different in CXCR3$^{-/-}$ mice compared to wild-type mice (Figure 32B). In contrast, CCR5$^{-/-}$ mice exhibited significantly reduced ($P < 0.05$) clinical severity scores compared to wild-type mice from the time of onset to the end of disease monitoring at day 80 (Figure 33B). At the peak of disease severity, mean clinical scores in wild-type, CXCR3$^{-/-}$ mice, and CCR5$^{-/-}$ mice were 8.23 ± 0.91, 7.25 ± 1.70, and 3.33 ± 0.71, respectively. The mean day to onset of clinical signs of disease was also significantly later ($P < 0.05$) for CCR5$^{-/-}$ mice (34.00 ± 3.17), but not CXCR3$^{-/-}$ mice (30.88 ± 4.21), compared to wild-type mice (25.67 ± 0.84). These data indicate that like CXCR6, the absence of CXCR3 or CCR5 also confers protection against the development of CIA.

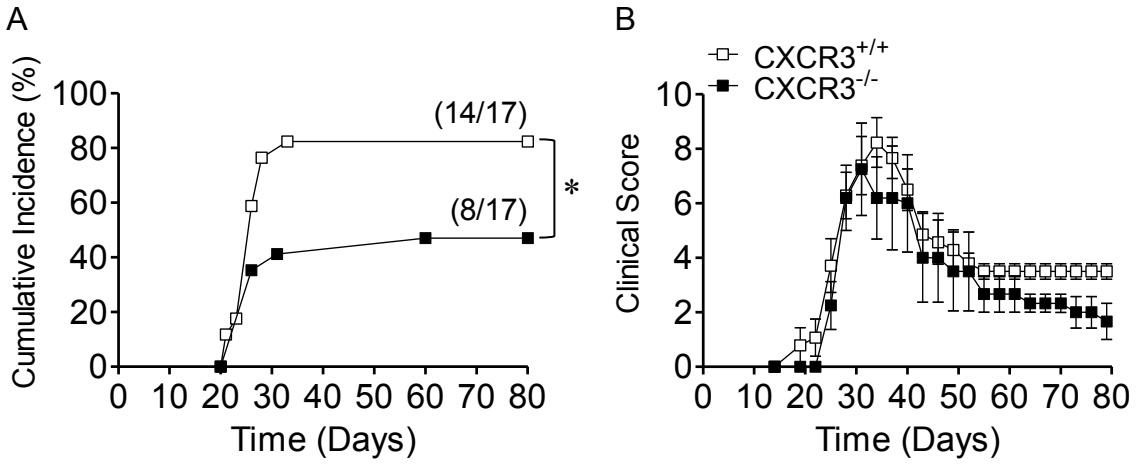


Figure 32. Reduced incidence of arthritis in CXCR3$^{-/-}$ mice. A and **B,** Cumulative incidence of arthritis **(A)** and clinical disease severity scores **(B)** in wild-type (CXCR3$^{+/+}$) and CXCR3$^{-/-}$ mice over 80 days following immunization with type II collagen emulsified in complete Freund's adjuvant (n = 17 mice per group, pooled from 5 independent experiments). Only mice that had developed signs of arthritis were included in the clinical scoring. Data in **B** are presented as mean ± SEM. * = $P < 0.05$ compared with CXCR3$^{+/+}$ mice.

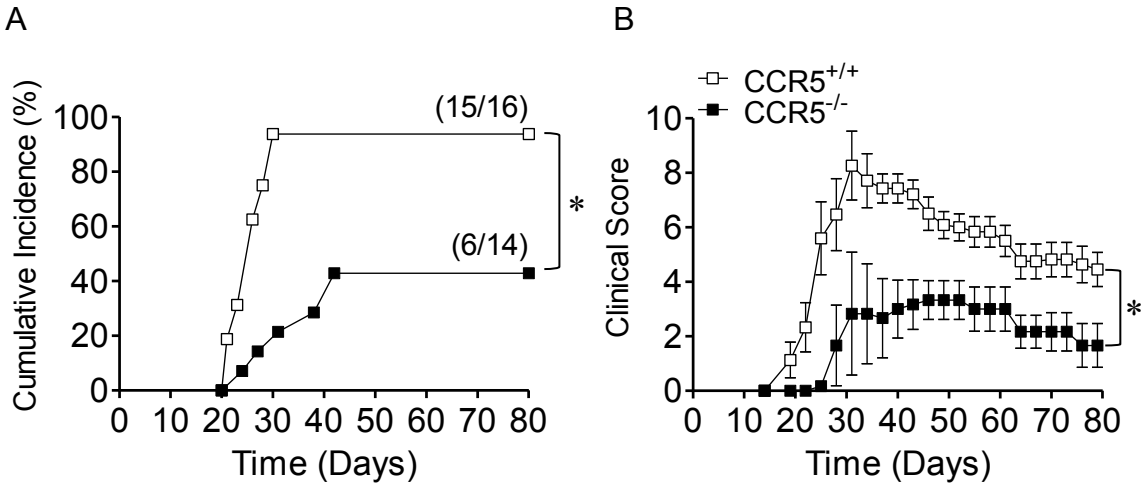


Figure 33. Reduced incidence and severity of arthritis in CCR5$^{-/-}$ mice. A and **B,** Cumulative incidence of arthritis **(A)** and clinical disease severity scores **(B)** in wild-type (CCR5$^{+/+}$) and CCR5$^{-/-}$ mice over 80 days following immunization with type II collagen emulsified in complete Freund's adjuvant (n = 14-16 mice per group, pooled from 4 individual experiments). Only mice that had developed signs of arthritis were included in the clinical scoring. Data in **B** are presented as mean ± SEM. * = $P < 0.05$ compared with CCR5$^{+/+}$ mice.

3.2 iNKT CELLS AND iNKT CELL-DERIVED CYTOKINES IN CIA

3.2.1 iNKT cells from naïve mice express CCR5, CCR9, CXCR3, and CXCR6

Human and mouse CD1d-restricted iNKT cells have been shown to express a chemokine receptor profile indicative of Th1-type inflammatory cells (160,289–291,416). For example, a majority of iNKT cells express the receptors CXCR3 and CXCR6 (160,289–291). Previously, data examining the expression of chemokine receptors on the surface of mouse NKT cells was limited to a panel of select receptors due to the lack of available reagents at the time these studies were performed. Therefore, I examined the profile of chemokine receptors on iNKT cells isolated from the spleen and liver of naïve mice.

Spleen and liver cells were stained with an anti-TCRβ antibody and PBS57-loaded CD1d tetramers to identify invariant iNKT cells, and the expression of a panel of chemokine receptors was assessed by flow cytometry (Figures 34A and 34B). Few iNKT cells were observed expressing the receptors CCR2, CCR3, CCR6, CCR7, CXCR2, CXCR4, and CXCR5. However, surface staining for these receptors was performed immediately following lymphocyte isolation with no recovery time so receptor internalization/ shedding could be responsible for the low expression of some of these receptors. Nonetheless, a majority (>50%) of iNKT cells were found to express CCR9, CXCR3, and CXCR6, while a smaller but sizable proportion of iNKT cells also expressed CCR5 in both the spleen and liver. The high frequencies of $CCR5^+$, $CXCR3^+$, and $CXCR6^+$ iNKT cells isolated from naïve mice suggests that these cells may rapidly respond to inflammatory chemokine ligands to perform effector or regulatory functions in situ or after homing to sites of inflammation within other peripheral tissues. Given that iNKT cells expressed high levels of the chemokine receptors that play important roles in the susceptibility of mice to CIA, I was interested in evaluating the contribution of iNKT cells to CIA.

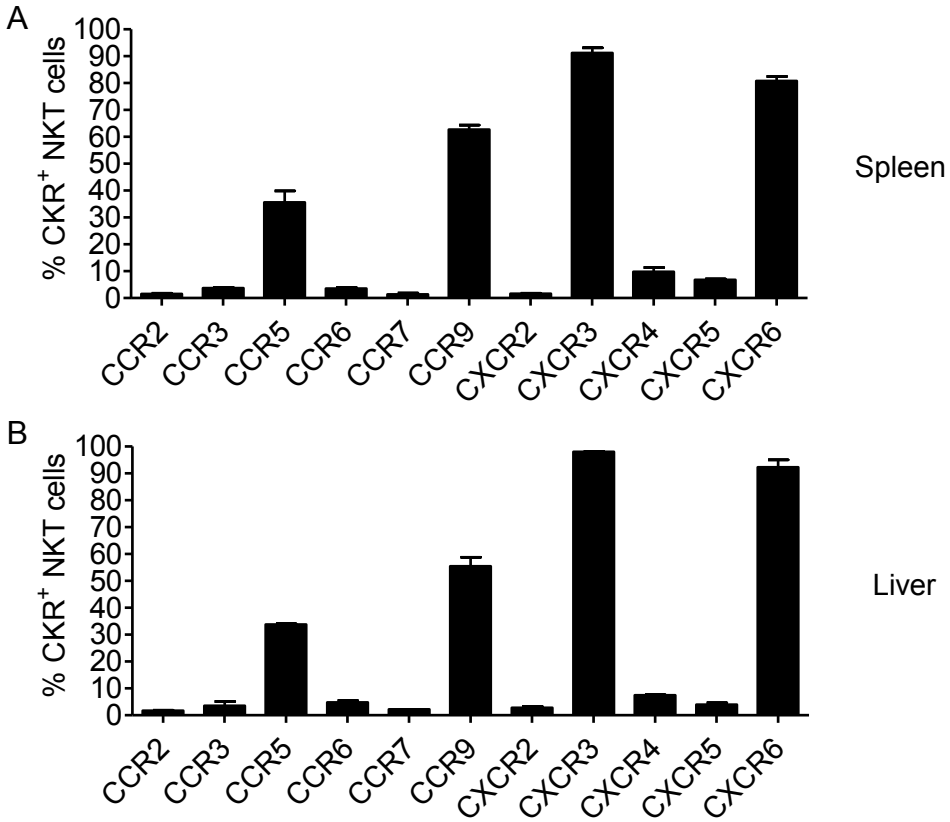


Figure 34. Chemokine receptor profile of iNKT cells isolated from naïve mice. A and **B,** Cells isolated from the spleen **(A)** and liver **(B)** of naïve mice were stained with an anti-TCRβ antibody and PBS57-loaded CD1d tetramers to identify iNKT cells and the frequencies of chemokine receptor-positive (CKR^+) iNKT cells were assessed by flow cytometry. Data are presented as mean ± SEM from 3 mice.

3.2.2 iNKT cell frequency and absolute number is increased in CIA mice

Studies in patients with RA have revealed that circulating iNKT cell numbers are significantly decreased in the peripheral blood compared to healthy donors (375–377,379). In contrast, iNKT cells were found to be significantly increased among total liver mononuclear cells and peripheral blood mononuclear cells in CIA mice on the DBA/1 background compared with control mice (387). However, iNKT cell frequencies and numbers were not examined in the paws of naïve and CIA mice. Therefore, I investigated whether iNKT cell frequencies and numbers were altered in the tissues of nonimmunized and arthritic C57BL/6 mice, including examining iNKT cell frequency and number in the paws of control and CIA mice.

Figure 35A, shows representative dot plots identifying $TCR\beta^{+}CD1d$ tetramer^{+} iNKT cells in the liver of nonimmunized and arthritic wild-type mice. The frequencies and absolute numbers of iNKT cells were increased among lymphocyte populations isolated from the liver, spleen, and paws of arthritic wild-type mice compared to nonimmunized control mice (Figures 35B and 35C). The frequency of iNKT cells was decreased in the inguinal and popliteal lymph nodes of arthritic mice (Figure 35B), but the absolute numbers of iNKT cells were increased (Figure 35C), consistent with the increased cellularity of the lymph nodes following immunization and arthritis development. The increased frequencies and absolute numbers of iNKT cells in CIA mice indicates that iNKT cell populations are expanded in CIA mice, suggesting they may have an important role in the development and/or progression of arthritis in this model.

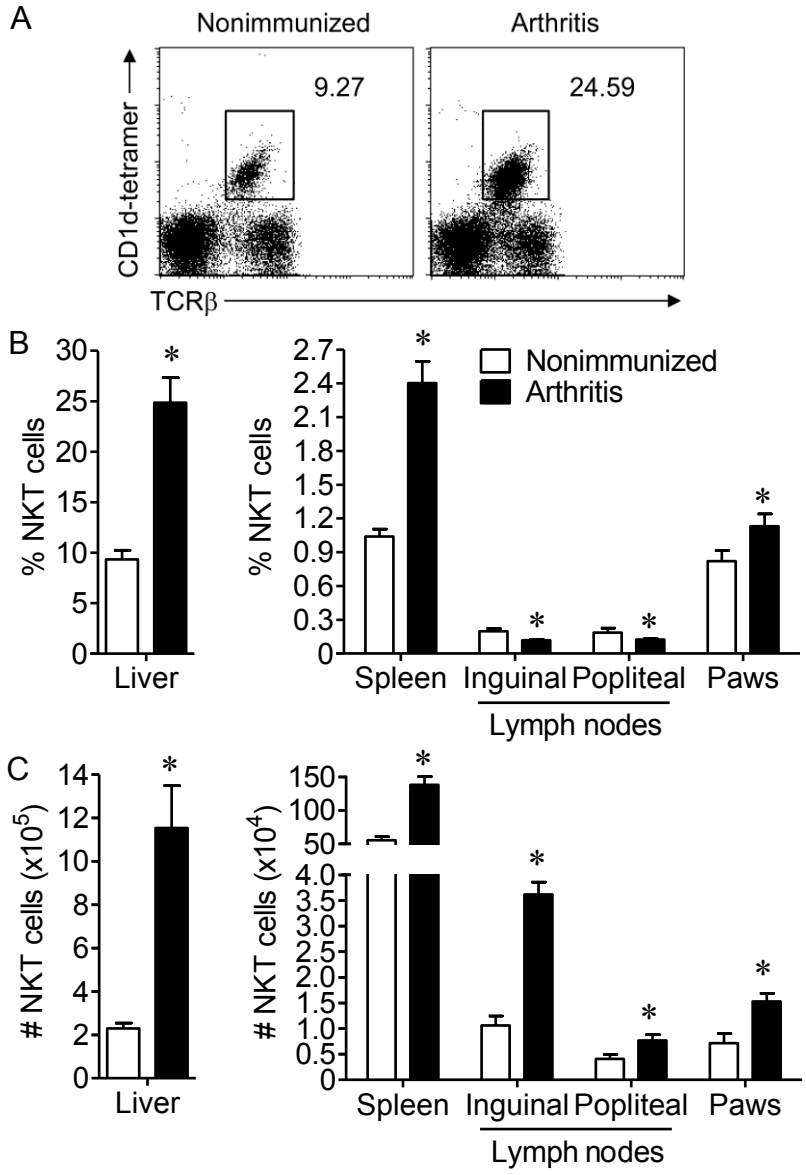


Figure 35. Invariant NKT cell frequencies and numbers are expanded in the tissues of CIA mice. A, Representative dot plots showing staining using PBS57-loaded CD1d tetramers and anti-T cell receptor β (TCRβ) antibodies in the liver of nonimmunized and arthritic wild-type mice on day 35. Values indicate the percent of events within the gated region, representing the frequency of iNKT cells within liver lymphocytes. **B** and **C,** The frequencies **(B)** and absolute numbers **(C)** of iNKT cells isolated from the liver, spleen, inguinal lymph nodes, popliteal lymph nodes, and paws of nonimmunized and arthritic wild-type mice on day 35. Data are presented as mean ± SEM in 8-11 mice per group. * = $P < 0.05$ compared to nonimmunized mice.

I also assessed iNKT cell expression of the early activation marker CD69 in nonimmunized and arthritic wild-type mice at day 35. Figure 36A shows a representative histogram of CD69 staining on iNKT cells in the spleen of nonimmunized and arthritic wild-type mice. Compared to the frequencies of $CD69^+$ T cells in nonimmunized mice (~1-2%) (Figures 21, 22, and 23), the frequencies of $CD69^+$ iNKT cells were much greater (Figure 36B), consistent with the basal activated phenotype of iNKT cells (417). Despite the large proportions of $CD69^+$ iNKT cells under basal conditions, the frequencies of $CD69^+$ iNKT cells were significantly increased in the spleen, inguinal lymph nodes, and paws, but not in the popliteal lymph nodes, of arthritic mice compared to nonimmunized control mice (Figure 36B). Greater absolute numbers of $CD69^+$ iNKT cells were observed in all tissues of arthritic mice compared to those from nonimmunized mice (Figure 36C). Thus the increase in frequency and number of iNKT cells within the paws, and other tissues (both total iNKT cells and $CD69^+$ populations) in arthritic mice compared to nonimmunized mice suggests that they may be playing an active role in arthritis development and/or progression.

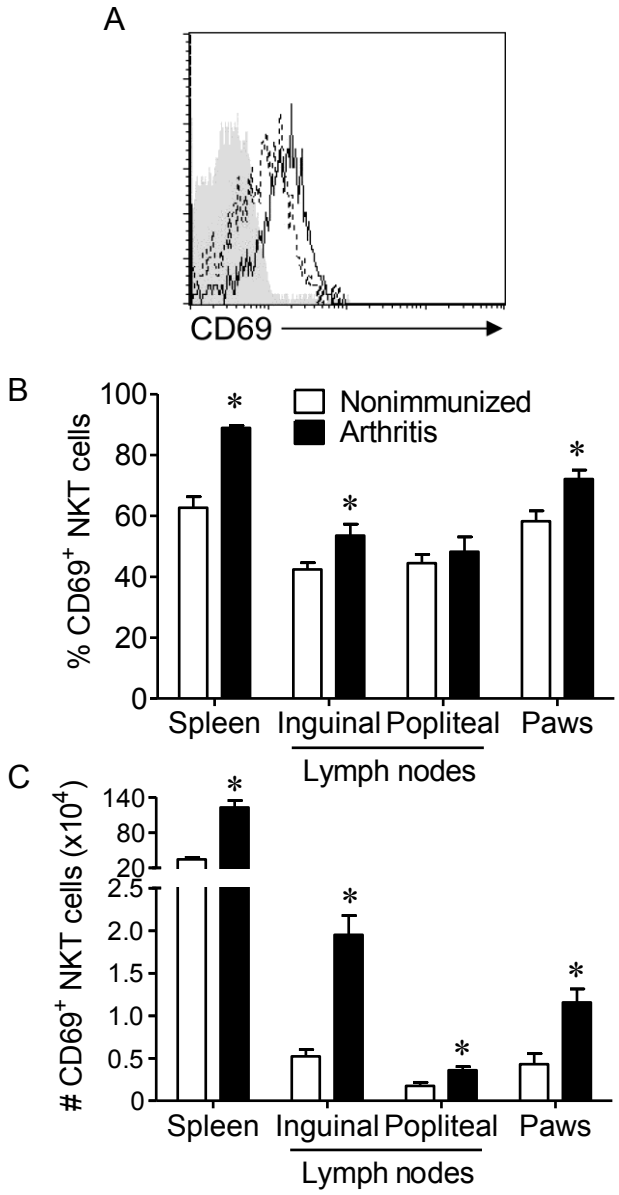


Figure 36. CD69$^+$ iNKT cell frequencies and numbers are increased in CIA mice. A, Representative histogram showing CD69 staining on iNKT cells isolated from the spleen of nonimmunized wild-type mice (broken line) and arthritic wild-type mice (solid line) at day 35 versus isotype control staining (grey filled line). **B** and **C,** The frequencies **(B)** and absolute numbers **(C)** of CD69$^+$ iNKT cells isolated from the spleen, inguinal lymph nodes, popliteal lymph nodes, and paws of nonimmunized and arthritic wild-type mice on day 35. Data are presented as mean ± SEM in 8-11 mice per group. * = $P < 0.05$ compared to nonimmunized mice.

3.2.3 Reduced incidence and severity of CIA in iNKT cell-deficient mice

To examine the contribution of iNKT cells to CIA, wild-type and iNKT cell-deficient ($J\alpha18^{-/-}$) mice were immunized and boosted with CII in CFA and monitored for the development of arthritis over an 80 day period. The incidence of arthritis in wild-type mice (35/37; 94.6%) was higher than in $J\alpha18^{-/-}$ mice (23/48; 47.9%) by day 80 post-immunization (Figure 37A). The mean clinical scores of arthritis severity in mice that exhibited signs of disease were reduced in $J\alpha18^{-/-}$ mice compared to wild-type mice (Figure 37B). The reduced mean clinical severity was apparent from day 20 and remained significant ($P < 0.05$) until observations were ended on day 80. At the peak of disease severity, mean clinical scores in wild-type and $J\alpha18^{-/-}$ mice were 8.82 ± 0.61 and 5.32 ± 0.89, respectively. The mean day to onset of clinical signs of disease was also significantly later ($P < 0.05$) for $J\alpha18^{-/-}$ mice (33.52 ± 2.03) than for wild-type mice (24.20 ± 0.50).

I also examined the development of CIA in mice which were deficient for both iNKT cells and CXCR6 ($J\alpha18^{-/-}CXCR6^{-/-}$). Consistent with earlier experiments (Figure 9), the incidence of arthritis in $CXCR6^{-/-}$ mice (25/37; 67.6%) was lower than in wild-type mice (35/37; 94.6%) (Figure 37C). However, the incidence of arthritis in $J\alpha18^{-/-}CXCR6^{-/-}$ mice (16/40; 40%) was even further reduced compared to both wild-type and $CXCR6^{-/-}$ mice by day 80 post-immunization. Again, the mean clinical scores of arthritis severity in mice that exhibited signs of disease were reduced in $CXCR6^{-/-}$ mice compared to wild-type mice, whereas the mean clinical scores of arthritis severity in $J\alpha18^{-/-}CXCR6^{-/-}$ mice were lower than wild-type mice but were also significantly lower than $CXCR6^{-/-}$ mice around the peak of disease severity (days 28-45) (Figure 37D). At the peak of disease severity, mean clinical scores in $CXCR6^{-/-}$ and $J\alpha18^{-/-}CXCR6^{-/-}$ mice were 6.43 ± 0.50 and 3.56 ± 0.85, respectively. The mean clinical scores of arthritis severity in $J\alpha18^{-/-}CXCR6^{-/-}$ mice reached a peak and appeared to plateau over the 80 day course of disease monitoring. This unusual distribution of scores can be attributed to a number of $J\alpha18^{-/-}CXCR6^{-/-}$ mice which developed clinical signs of disease much later following the delivery of their primary and booster immunizations. This is apparent within the incidence data, with a number of mice developing arthritis after day 40 post-

immunization, as well as in the mean day to onset of clinical signs of disease, which was significantly later ($P < 0.05$) for Jα18$^{-/-}$CXCR6$^{-/-}$ mice (35.56 ± 3.42) compared to wild-type mice (24.20 ± 0.50), but not compared to CXCR6$^{-/-}$ mice (28.76 ± 0.66). Taken together, these data indicate that the absence of iNKT cells confers protection against the development of CIA and reduces the clinical severity of arthritis in affected mice, both of which are further reduced when mice lack iNKT cells and CXCR6.

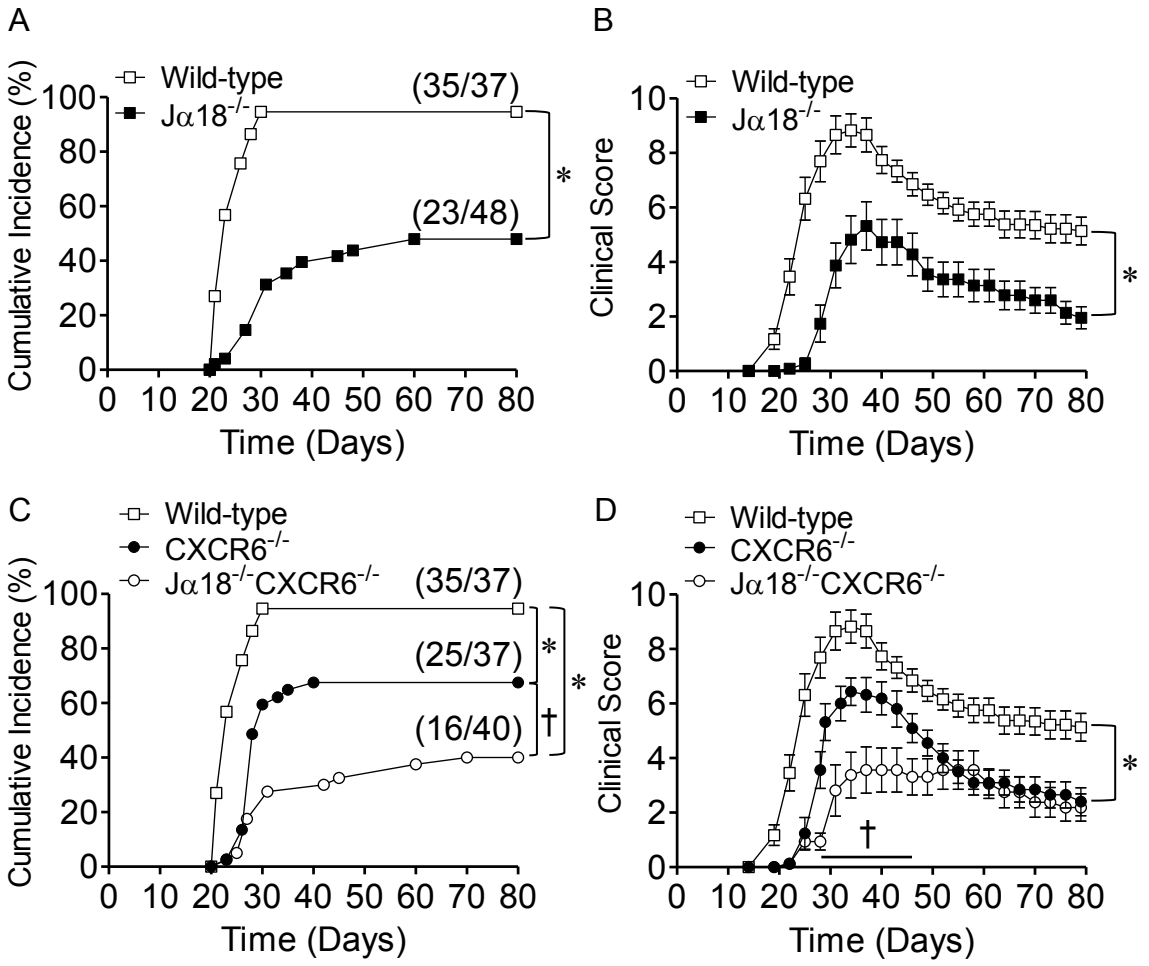


Figure 37. Reduced incidence and severity of arthritis in Jα18$^{-/-}$, CXCR6$^{-/-}$, and Jα18$^{-/-}$CXCR6$^{-/-}$ mice. A and **B,** Cumulative incidence of arthritis **(A)** and clinical disease severity scores **(B)** in wild-type and Jα18$^{-/-}$ mice over 80 days following immunization with type II collagen emulsified in complete Freund's adjuvant. Only mice that had developed signs of arthritis were included in the clinical scoring. **C** and **D,** Cumulative incidence of arthritis **(C)** and clinical disease severity scores **(D)** in wild-type, CXCR6$^{-/-}$, and Jα18$^{-/-}$CXCR6$^{-/-}$ mice over 80 days following immunization Data in **B** and **D** are presented as mean ± SEM. * = $P < 0.05$ compared with wild-type mice. † = $P <$ 0.05 compared with CXCR6$^{-/-}$ mice.

3.2.4 Irradiation of mice has suppressive effects on CIA

The lower incidence and severity of arthritis in Jα18$^{-/-}$ mice suggests that iNKT cells play an important role in the development of CIA in mice (Figure 37A and 37B). However, Jα18$^{-/-}$ mice have been shown to have an estimated 60% reduction in TCR α-chain repertoire diversity compared to wild-type mice (397). Therefore to assess the role of iNKT cells in the development of CIA directly, I explored strategies for reconstituting iNKT cell-deficient Jα18$^{-/-}$ mice through adoptive transfer of sorted iNKT cells and examined the effects on arthritis incidence and severity.

Invariant NKT cells are a rare population of immune cells, so to ensure sufficient numbers of iNKT cells could be purified from donor mice for adoptive transfer, I performed i.v. injections with α-GalCer-loaded BMDCs 72 h prior to cell sorting to expand iNKT cell populations in vivo. Invariant NKT cell expansion using α-GalCer-loaded BMDCs resulted in a dramatic increase in the frequency of iNKT cells among liver lymphocytes (Figure 38A), which translated to good numbers of sorted iNKT cells per mouse (Table 4). Furthermore, expanded liver iNKT cells in α-GalCer-loaded BMDC-treated mice exhibited a chemokine receptor profile similar to iNKT cells isolated from the liver of naïve mice, except treatment did increase the frequency of CCR5^{+} iNKT cells (Figure 38B). This iNKT cell expansion strategy was employed prior to sorting iNKT cells in adoptive transfer experiments.

Our lab has developed an approach to reconstitute iNKT cells into Jα18$^{-/-}$ mice that involves sublethal irradiation of recipient mice 48 h prior to adoptive transfer of sorted expanded iNKT cells. This results in stable long-term reconstitution of iNKT cells within Jα18$^{-/-}$ mice (418). Therefore, I established groups of iNKT cell-reconstituted Jα18$^{-/-}$ mice using this approach and immunized them for the development of CIA. To control for possible effects of prior irradiation on the development of CIA, I also established groups of irradiated wild-type and Jα18$^{-/-}$ non-recipient mice.

Arthritis incidence in irradiated Jα18$^{-/-}$ mice adoptively transferred with iNKT cells (0/6) was strikingly lower than the incidence observed in non-irradiated wild-type (7/7; 100%) and non-irradiated Jα18$^{-/-}$ mice (5/11; 45.4%) immunized within the same

groups (Figure 39A). However, the low incidence of arthritis in irradiated Jα18$^{-/-}$ recipient mice can be attributed to a suppressing effect of irradiation on CIA, as irradiated wild-type (1/6; 16.7%) and Jα18$^{-/-}$ (1/10; 10%) control groups also exhibited dramatically reduced incidence of disease compared to non-irradiated groups. The effectiveness of iNKT cell reconstitution in irradiated Jα18$^{-/-}$ mice adoptively transferred with iNKT cells was assessed at day 80 post-immunization by examining iNKT cell frequencies among liver and spleen lymphocytes by flow cytometry. The frequencies of iNKT cells in irradiated Jα18$^{-/-}$ iNKT cell recipient mice were similar to those observed in irradiated wild-type mice, but both were much lower than those observed in non-irradiated wild-type CIA mice (Figure 39B). Therefore, irradiation prior to iNKT cell transfer is useful for establishing stable long-term reconstitution of iNKT cells in Jα18$^{-/-}$ mice, but is not a viable option for evaluating the role of iNKT cells in CIA due to the suppressive effects irradiation has on CIA.

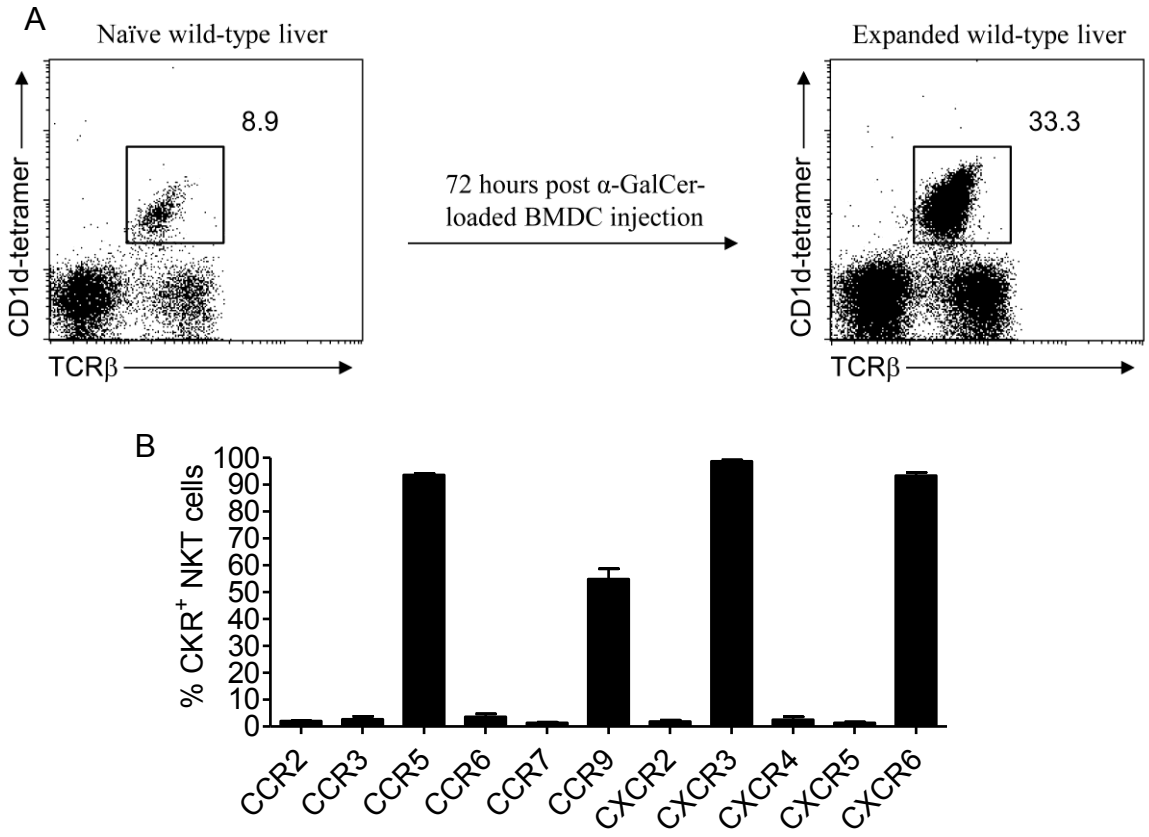


Figure 38. In vivo iNKT cell expansion following injection of α-GalCer-loaded BMDCs. A, Representative dot plots showing staining using PBS57-loaded CD1d tetramers and anti-T cell receptor β (TCRβ) antibodies in the liver of naïve wild-type mice and mice at 72 h post intravenous injection with α-GalCer-loaded bone marrow-derived dendritic cells (BMDCs). Values indicate the percent of events within the gated region, representing the frequency of iNKT cells within liver lymphocytes. **B,** Cells isolated from the liver of iNKT cell-expanded wild-type mice were stained with anti-TCRβ antibodies and PBS57-loaded CD1d tetramers to identify iNKT cells and the frequencies of chemokine receptor-positive (CKR^+) iNKT cells were assessed by flow cytometry. Data are presented as mean ± SEM from 3 mice.

Table 4. Sorted iNKT cells from expanded wild-type mice for transfer into irradiated Jα18$^{-/-}$ recipients

	Mean			
# mice	# cells post-sort	Purity (%)	# of cells delivered	Timing of transfer[a]
8 C57BL/6	10.2 x 10^6	95.8	3.6 x 10^6	4 weeks

[a]Prior to primary immunization

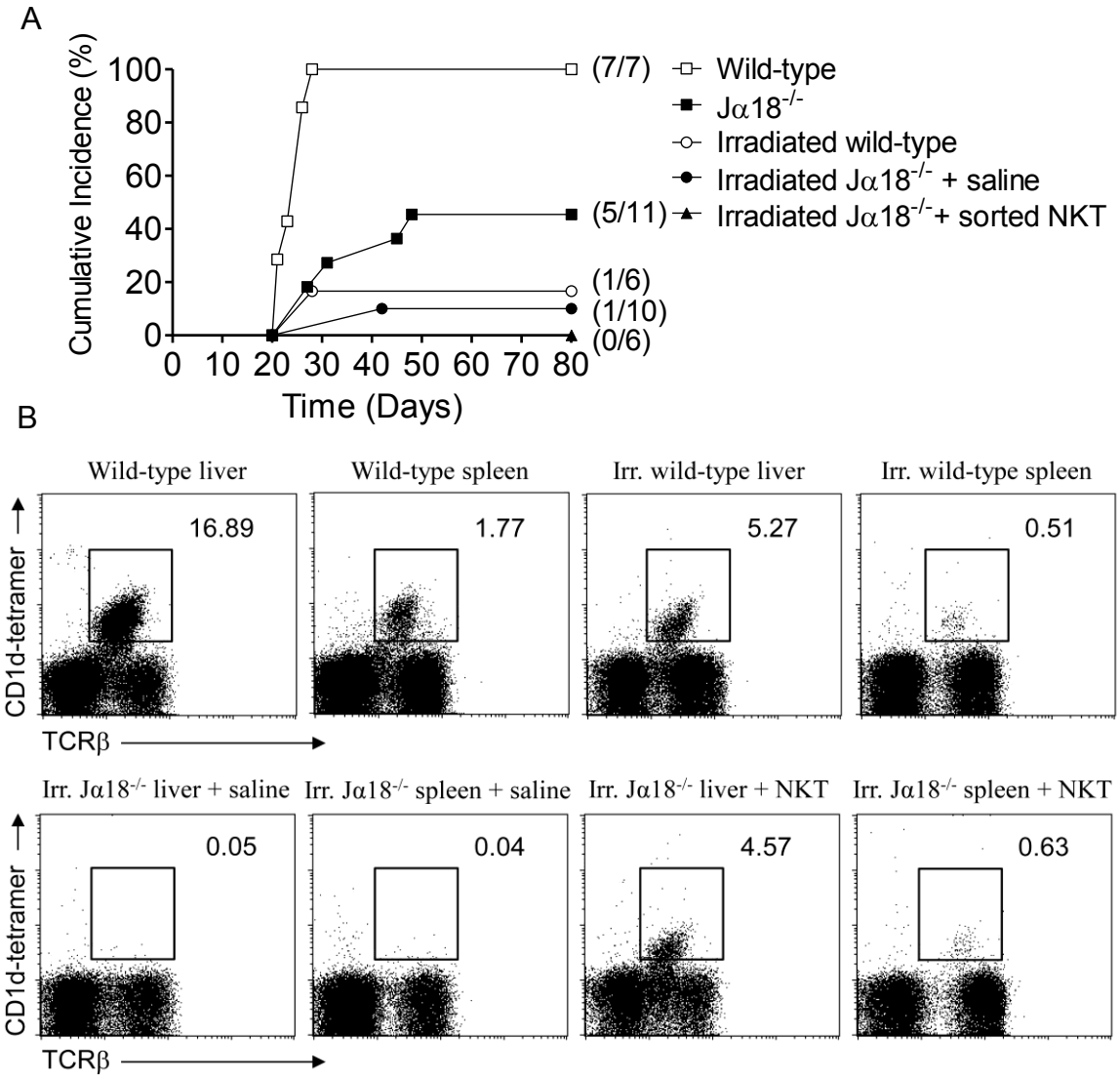


Figure 39. Suppressive effect of irradiation on CIA. A, Cumulative incidence of arthritis in wild-type, Jα18$^{-/-}$, irradiated wild-type, irradiated Jα18$^{-/-}$ control mice (Jα18$^{-/-}$ + saline), and irradiated Jα18$^{-/-}$ mice adoptively transferred with >3x10^{6} sorted wild-type iNKT cells (Jα18$^{-/-}$ + sorted NKT) over 80 days following immunization with type II collagen emulsified in complete Freund's adjuvant. **B,** Representative dot plots showing staining using PBS57-loaded CD1d tetramers and anti-T cell receptor β (TCRβ) antibodies in the liver and spleen of arthritic wild-type mice, irradiated (Irr.) wild-type mice, irradiated control Jα18$^{-/-}$ mice, and irradiated iNKT cell recipient Jα18$^{-/-}$ mice on day 80 post-immunization. Values indicate the percent of events within the gated region, representing the frequency of iNKT cells within liver and spleen lymphocytes.

3.2.5 Arthritis incidence and severity are restored in Jα18$^{-/-}$ mice receiving adoptive transfer of wild-type, but not IFN-γ$^{-/-}$ or TNF$^{-/-}$ iNKT cells

Unfortunately irradiation of recipient mice to ensure stable long-term iNKT cell reconstitution was a non-viable option for assessing the effect reintroducing iNKT cells in Jα18$^{-/-}$ mice has on arthritis incidence and severity. Studies have reported that blocking CD1d-dependent NKT cell activation in vivo using anti-CD1d antibodies starting from the booster immunization resulted in reduced clinical severity scores compared to control mice (387). In contrast, others have reported that late administration of anti-CD1d (days 27 to 39 post-immunization) did not modify clinical disease scores but early neutralization by anti-CD1d (days 0, 3, and 6) delayed onset of clinical signs and reduced clinical scores compared to control mice (392). Together with the evidence that iNKT cells undergo activation by 6 days post-immunization, which led to their release of a variety of cytokines including IFN-γ and IL-17A (392), these studies suggest that iNKT cells are important during the early stages of CIA. Therefore, I developed an adoptive transfer strategy wherein sorted iNKT cells were delivered 14 days prior to primary immunization and again on day 7 post-immunization to ensure that an iNKT cell population was established in mice during the early phases of the CIA model.

The incidence of arthritis in Jα18$^{-/-}$ mice (9/10; 90%) following two separate adoptive transfers of wild-type iNKT cells ($3.6x10^6$) (Table 5) was significantly higher than control Jα18$^{-/-}$ mice (10/20; 50%) by day 80 post-immunization (Figure 40A). Indeed, Jα18$^{-/-}$ mice receiving double adoptive transfers of wild-type iNKT cells exhibited an incidence of arthritis that was comparable to that observed in wild-type CIA mice (13/13; 100%) (Figure 40A). Invariant NKT cell-recipient Jα18$^{-/-}$ mice also had clinical disease severity scores similar to wild-type mice (Figure 40B). For example, at peak disease severity, Jα18$^{-/-}$ mice that received iNKT cells had high mean clinical scores (9.00 ± 1.55), comparable to those in arthritic wild-type mice (8.00 ± 1.28), both of which were greater than non-recipient Jα18$^{-/-}$ mice (4.90 ± 1.38) (Figure 40B). Moreover, the mean day to onset of clinical signs of disease was earlier in iNKT cell-recipient Jα18$^{-/-}$ mice (29.67 ± 2.25) compared to control Jα18$^{-/-}$ mice (34.80 ± 3.17) although this difference was not significant ($P > 0.05$).

The effectiveness of iNKT cell reconstitution in Jα18$^{-/-}$ mice adoptively transferred with iNKT cells was assessed at days 50 and 80 post-immunization by examining iNKT cell frequencies among lymphocytes in the liver (and spleen; not shown) by flow cytometry. The frequency of iNKT cells among liver lymphocytes in Jα18$^{-/-}$ iNKT cell recipient mice was greater than the background TCRβ^{+}CD1d-tetramer^{+} staining observed in Jα18$^{-/-}$ mice that received vehicle alone (Figure 40C). Indeed, an iNKT cell-recipient Jα18$^{-/-}$ mouse sacrificed at day 50 exhibited an iNKT cell frequency similar to that observed in wild-type mice, suggesting that donor iNKT cells may be lost over time in Jα18$^{-/-}$ recipient mice. These data implicate iNKT cells as direct players in the development of arthritis and the enhancement of disease severity in CIA.

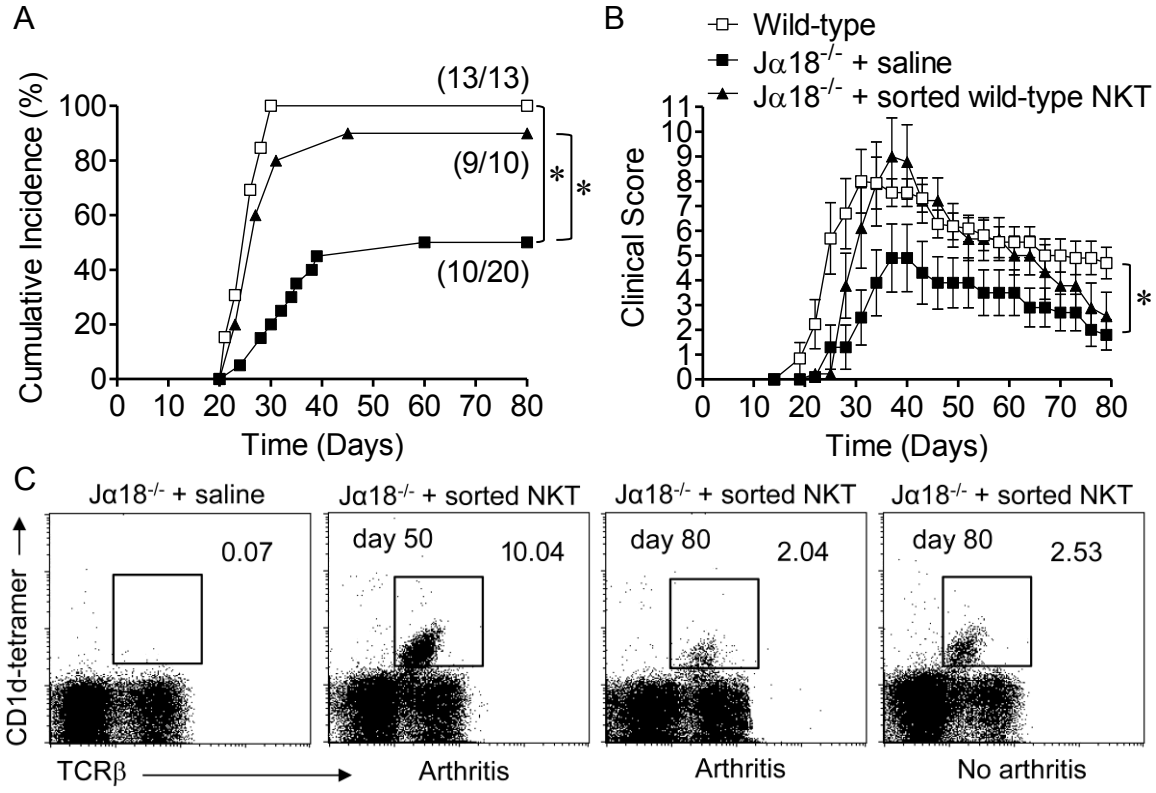


Figure 40. Arthritis incidence and severity are restored to wild-type levels in Jα18$^{-/-}$ mice receiving double adoptive transfers of wild-type iNKT cells. A and **B,** Cumulative incidence of arthritis **(A)** and clinical disease severity scores **(B)** in wild-type, Jα18$^{-/-}$ mice receiving adoptive transfers of 3.6x10^6 sorted wild-type iNKT cells two weeks prior to both the primary and booster immunizations (Jα18$^{-/-}$ + sorted wild-type NKT), and Jα18$^{-/-}$ mice receiving vehicle alone (Jα18$^{-/-}$ mice + saline) over 80 days following immunization with type II collagen emulsified in complete Freund's adjuvant. Only mice that had developed signs of arthritis were included in the clinical scoring. **C,** Representative dot plots showing staining using PBS57-loaded CD1d tetramers and anti-T cell receptor β (TCRβ) antibodies in the liver of non-recipient Jα18$^{-/-}$ mice + saline, as well as arthritic and non-arthritic iNKT cell recipient Jα18$^{-/-}$ mice on days 50 and 80 post-immunization. Values indicate the percent of event within the gated region, representing the frequency of iNKT cells within liver lymphocytes. Data in **B** are presented as mean ± SEM. * = $P < 0.05$.

An advantage of iNKT cell reconstitution in Jα18$^{-/-}$ mice is that one can assess the role of iNKT cell-derived cytokines, the mode of activation via receptor signaling pathways within iNKT cells, or other mechanisms through which iNKT cells contribute to the development of arthritis or other disease models. Therefore, I evaluated the contributions of iNKT cell-derived IFN-γ and TNF to the development of CIA by adoptively transferring sorted iNKT cells from IFN-γ$^{-/-}$ and TNF$^{-/-}$ mice into Jα18$^{-/-}$ mice. Details on the purities and numbers of sorted iNKT cell populations from these mice are summarized in Table 5. Adoptive transfer of IFN-γ$^{-/-}$ iNKT cells into Jα18$^{-/-}$ mice completely protected these mice from arthritis development (Figures 41A and 41B). However, this was not due to an inability of iNKT cells from IFN-γ$^{-/-}$ mice to reconstitute the tissues in Jα18$^{-/-}$ mice, since the frequencies of transferred iNKT cells were well above background, and in some Jα18$^{-/-}$ recipient mice, were close to that of wild-type mice (Figure 41C). These data implicate iNKT cell-derived IFN-γ as a key effector in the ability of iNKT cells to promote the development of CIA.

The incidence of arthritis in Jα18$^{-/-}$ mice that received adoptive transfers of TNF$^{-/-}$ iNKT cells (3/7; 42.8%) was significantly lower than the incidence of arthritis in wild-type mice (13/13; 100%), but closely matched that of control Jα18$^{-/-}$ mice (10/20; 50%) (Figure 42A). TNF$^{-/-}$ iNKT cell-recipient Jα18$^{-/-}$ mice exhibited mean clinical severity scores similar to those observed in control Jα18$^{-/-}$ mice, which were significantly lower than the scores in wild-type mice (Figure 42B). The effectiveness of TNF$^{-/-}$ iNKT cell reconstitutions in Jα18$^{-/-}$ mice were comparable to those achieved with wild-type and IFN-γ$^{-/-}$ iNKT cell adoptive transfers (Figure 42C). Thus, in contrast to adoptive transfer of wild-type iNKT cells (Figure 40), TNF-deficient iNKT cell transfer does not result in increased incidence and severity of arthritis in Jα18$^{-/-}$ mice, suggesting that iNKT cells are an important source of disease-promoting TNF within CIA mice.

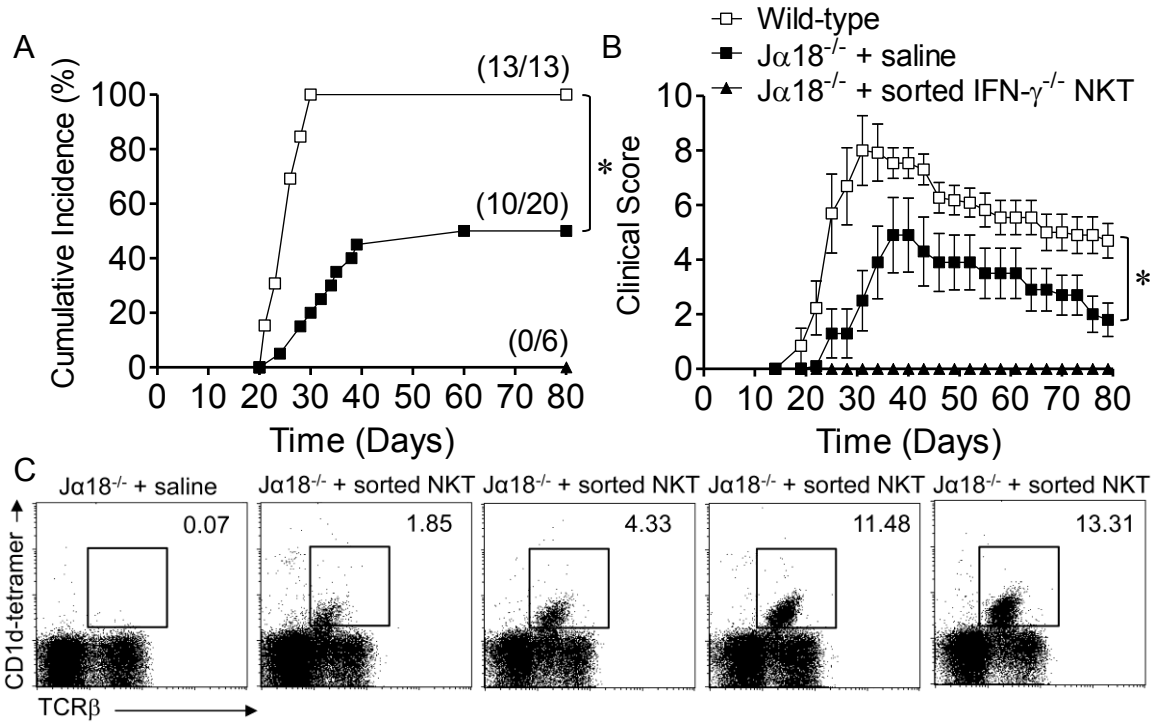


Figure 41. Jα18$^{-/-}$ mice receiving adoptive transfers of IFN-γ$^{-/-}$ iNKT cells are protected from CIA. A and **B,** Cumulative incidence of arthritis **(A)** and clinical disease severity scores **(B)** in wild-type, Jα18$^{-/-}$ mice receiving adoptive transfers of ~3x10^{6} sorted IFN-γ$^{-/-}$ iNKT cells two weeks prior to both the primary and booster immunizations (Jα18$^{-/-}$ + sorted IFN-γ$^{-/-}$ NKT), and Jα18$^{-/-}$ mice receiving vehicle alone (Jα18$^{-/-}$ mice + saline) over 80 days following immunization with type II collagen emulsified in complete Freund's adjuvant. Only mice that had developed signs of arthritis were included in the clinical scoring. **C,** Representative dot plots showing staining using PBS57-loaded CD1d tetramers and anti-T cell receptor β (TCRβ) antibodies in the liver of non-recipient Jα18$^{-/-}$ mice + saline, as well as iNKT cell recipient Jα18$^{-/-}$ mice on day 80 post-immunization. Values indicate the percent of events within the gated region, representing the frequency of iNKT cells within liver lymphocytes. Data in **B** are presented as mean ± SEM. * = $P <$ 0.05.

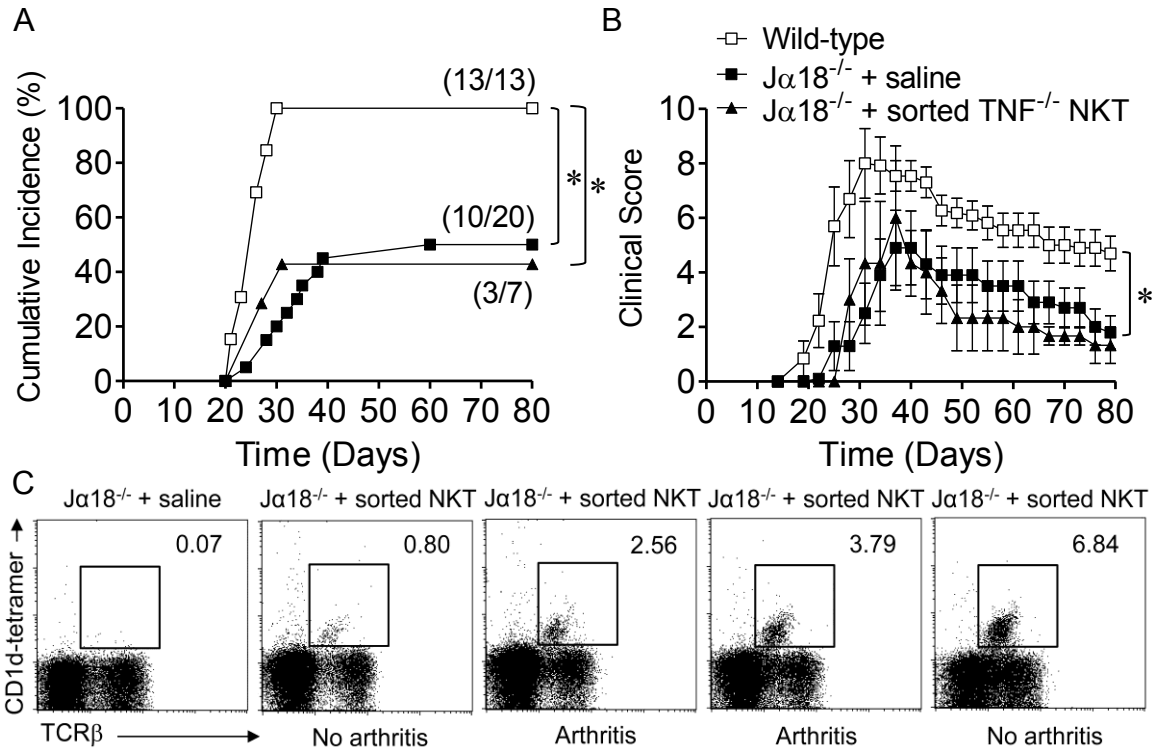


Figure 42. Arthritis incidence and severity are unaltered in Jα18$^{-/-}$ mice receiving adoptive transfers of TNF$^{-/-}$ iNKT cells. A and **B,** Cumulative incidence of arthritis **(A)** and clinical disease severity scores **(B)** in wild-type, Jα18$^{-/-}$ mice receiving adoptive transfers of ~3x10^{6} sorted TNF$^{-/-}$ iNKT cells two weeks prior to both the primary and booster immunizations (Jα18$^{-/-}$ + sorted TNF$^{-/-}$ NKT), and Jα18$^{-/-}$ mice receiving vehicle alone (Jα18$^{-/-}$ mice + saline) over 80 days following immunization with type II collagen emulsified in complete Freund's adjuvant. Only mice that had developed signs of arthritis were included in the clinical scoring. **C,** Representative dot plots showing staining using PBS57-loaded CD1d tetramers and anti-T cell receptor β (TCRβ) antibodies in the liver of non-recipient Jα18$^{-/-}$ mice + saline, as well as arthritic and non-arthritic iNKT cell recipient Jα18$^{-/-}$ mice on day 80 post-immunization. Values indicate the percent of events within the gated region, representing the frequency of iNKT cells within liver lymphocytes. Data in **B** are presented as mean ± SEM. * = $P < 0.05$.

Table 5. Sorted iNKT cells from expanded wild-type, IFN-$\gamma^{-/-}$, and $TNF^{-/-}$ mice for adoptive transfers into $J\alpha18^{-/-}$ recipients

	Mean			
# mice	# cells post-sort	Purity (%)	# of cells delivered	Timing of transfer
8 C57BL/6	11.3×10^6	96.2	3.6×10^6	2 weeks[a]
8 C57BL/6	14.5×10^6	96.0	3.6×10^6	2 weeks[b]
11 IFN-$\gamma^{-/-}$	6.5×10^6	96.2	2.9×10^6	2 weeks[a]
11 IFN-$\gamma^{-/-}$	6.2×10^6	94.1	2.5×10^6	2 weeks[b]
7 $TNF^{-/-}$	7.9×10^6	95.1	2.8×10^6	2 weeks[a]
7 $TNF^{-/-}$	7.3×10^6	94.2	2.7×10^6	2 weeks[b]

[a]Prior to primary immunization
[b]Prior to booster immunization

3.2.6 Adoptive transfer of iNKT cells impacts arthritis incidence and severity in Jα18$^{-/-}$CXCR6$^{-/-}$ mice

Invariant NKT cells express high levels of CXCR6 (279,289–291), and CXCR6$^{-/-}$ mice exhibit defects in the accumulation/retention and/or survival of mature iNKT cells in the liver, in addition to impaired cytokine production by liver and spleen iNKT cells following activation with α-GalCer (159,297). Therefore, I hypothesized that the reduced incidence and severity of arthritis in CXCR6$^{-/-}$ mice is partly due to defects in iNKT cells. To assess this, I performed adoptive transfers of sorted wild-type iNKT cells into Jα18$^{-/-}$CXCR6$^{-/-}$ mice and examined these mice for the development of CIA. Details on the purities and numbers of sorted iNKT cells used in these experiments are summarized in Table 6. Upon reconstitution, these mice would have fully functional iNKT cells but still lack CXCR6 expression on T cells and other populations.

Similar to previous experiments, mice deficient in both iNKT cells and CXCR6 (Jα18$^{-/-}$CXCR6$^{-/-}$) exhibit an incidence of arthritis (8/19; 42.1%) lower than that observed in CXCR6$^{-/-}$ mice (13/19; 68.4%) (Figure 43A). Jα18$^{-/-}$CXCR6$^{-/-}$ mice receiving adoptive transfer of wild-type iNKT cells exhibited an incidence of arthritis (4/6; 66.7%) that was higher than control Jα18$^{-/-}$CXCR6$^{-/-}$ mice and comparable to the incidence observed in CXCR6$^{-/-}$ mice. The mean clinical scores of arthritis severity in mice that exhibited signs of disease were not significantly different between these groups due to small sample size and variable disease scores between individual mice within each group (Figure 43B). However, there did appear to be a trend towards increased mean clinical scores in iNKT cell-recipient Jα18$^{-/-}$CXCR6$^{-/-}$ mice (5.50 ± 1.90) compared to control Jα18$^{-/-}$CXCR6$^{-/-}$ mice (4.25 ± 1.28) and CXCR6$^{-/-}$ mice (6.20 ± 1.14) around the peak of disease severity. These data suggest that the absence of iNKT cells and the lack of CXCR6 on other populations each confers protection against the development of CIA and reduces the clinical severity of arthritis in affected mice. However, the iNKT cell defects observed in CXCR6$^{-/-}$ mice does not fully account for their protection from CIA.

Table 6. Sorted iNKT cells from expanded wild-type mice for transfer into Jα18$^{-/-}$CXCR6$^{-/-}$ recipients

	Mean			
# mice	# cells post-sort	Purity (%)	# of cells delivered	Timing of transfer
8 C57BL/6	12.1 x 10^6	95.8	3.7 x 10^6	2 weeks[a]
8 C57BL/6	15.2 x 10^6	96.2	3.5 x 10^6	2 weeks[b]

[a]Prior to primary immunization
[b]Prior to booster immunization

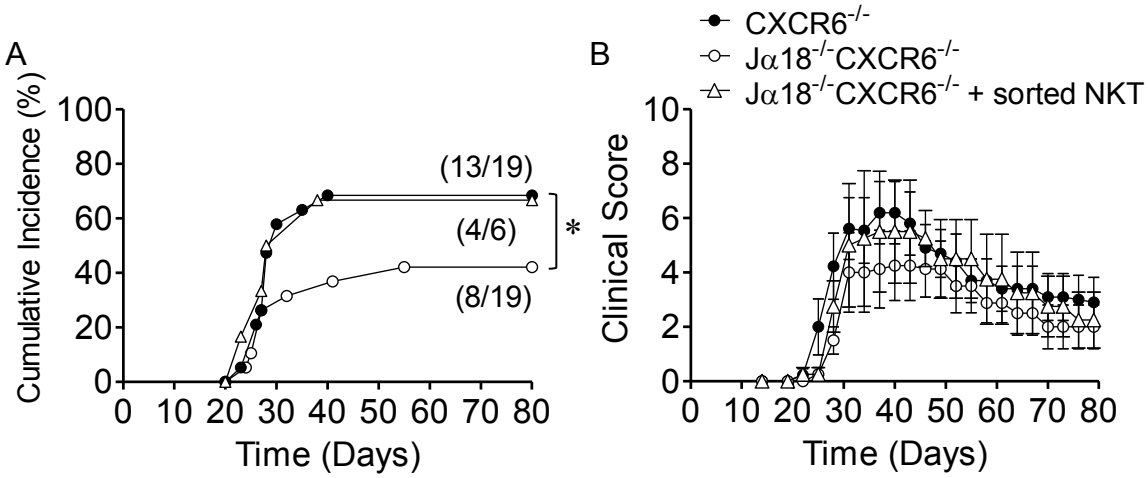


Figure 43. Influence of adoptive transfer of wild-type iNKT cells on arthritis incidence and severity in Jα18$^{-/-}$CXCR6$^{-/-}$ mice. A and **B,** Cumulative incidence of arthritis **(A)** and clinical disease severity scores **(B)** in CXCR6$^{-/-}$, Jα18$^{-/-}$CXCR6$^{-/-}$ mice receiving adoptive transfers of ~3.6x10^6 sorted wild-type iNKT cells two weeks prior to both the primary and booster immunizations (Jα18$^{-/-}$ + sorted NKT), and Jα18$^{-/-}$CXCR6$^{-/-}$ mice receiving vehicle alone (Jα18$^{-/-}$CXCR6$^{-/-}$ mice + saline) over 80 days following immunization with type II collagen emulsified in complete Freund's adjuvant. Only mice that had developed signs of arthritis were included in the clinical scoring. Data in **B** are presented as mean ± SEM.

3.2.7 Late adoptive transfer of iNKT cells protects Jα18$^{-/-}$ mice from CIA

Invariant NKT cells play an important role in the development of CIA, as evidenced by the lower incidence and severity of arthritis in Jα18$^{-/-}$ mice compared to wild-type mice (Figure 37A and 37B). The role of IFN-γ in CIA has long been debated. It is now appreciated that the effects of IFN-γ are biphasic in the CIA model, such that early during disease induction it promotes development of inflammatory responses that lead to disease onset, whereas later in disease IFN-γ has a more protective role (166). Invariant NKT cells produce large amounts of IFN-γ after stimulation. Studies have shown that in vivo neutralization of IFN-γ following iNKT cell activation protected mice from CIA when anti-IFN-γ antibodies were administered early, but accelerated disease onset upon later treatment (day 20 post-immunization) (395). Therefore, I assessed whether late adoptive transfers of wild-type iNKT cells had an influence on the development of CIA in Jα18$^{-/-}$ recipient mice.

To evaluate the effects of late adoptive transfers of iNKT cells on CIA, I modified the adoptive transfer strategy to a single delivery of sorted wild-type iNKT cells (Table 7). Immunized and boosted Jα18$^{-/-}$ mice received iNKT cells on day 28 post-immunization and were monitored for the development of clinical signs of CIA (Table 8). Jα18$^{-/-}$ mice exhibited reduced incidence (8/16; 50%) and severity of CIA and had a later time to onset of clinical signs compared to wild-type mice. Interestingly, whereas transfers of iNKT cells into Jα18$^{-/-}$ mice early during the induction phase promoted CIA (Figure 40), delivery of iNKT cells at day 28 post-immunization resulted in significantly lower ($P < 0.05$) incidence (2/9; 22.2%) of CIA, suggesting that iNKT cells are capable of playing protective roles later in the CIA model. However, onset to clinical signs of disease and clinical severity scores in the two recipient Jα18$^{-/-}$ mice that developed CIA were closer to those observed in wild-type mice than control Jα18$^{-/-}$ mice, which could indicate that iNKT cells played a disease-aggravating role rather than having a disease-limiting effect in these recipients. Further investigation is required to better understand how iNKT cells and their effector molecules influence inflammatory arthritis before the potential therapeutic benefit of iNKT cell activation can be safely and effectively translated into the clinical setting.

Table 7. Sorted iNKT cells from expanded wild-type mice for transfer into Jα18$^{-/-}$ recipients post-immunization

	Mean			
# mice	# cells post-sort	Purity (%)	# of cells delivered	Timing of transfer
8 C57BL/6	11.0 x 10^6	95.3	3.6 x 10^6	4 weeks[a]

[a]Following primary immunization

Table 8. Clinical data from Jα18$^{-/-}$ mice receiving a single transfer of sorted iNKT cells at day 28 post-immunization

			Time to Onset (days)		Maximum Score	
Group	*N*	Arthritis incidence (%)	Mean	Standard error	Mean	Standard error
C57BL/6 (B6)	13	100	25.7	0.8	8.0	1.3
Jα18$^{-/-}$ + saline	16	50	37.0	3.5	4.9	1.6
Jα18$^{-/-}$ + NKT cells	9	22.2	27.0	2.0	7.0	1.0

CHAPTER 4: DISCUSSION

4.1 CXCR6 deficiency reduces incidence and severity of arthritis

CXCR6 and CXCL16 have been linked to inflammatory arthritis in studies demonstrating high CXCL16 production in the arthritic synovium of patients with RA and JIA (251–253,278). Moreover, synovial T cells from patients with RA (251–253,277), psoriatic arthritis (277), or JIA (278) are enriched for the expression of CXCR6 and actively migrate in response to CXCL16 in vitro. Therefore, we hypothesized that CXCR6 and CXCL16 play a role in the pathogenesis of inflammatory arthritis and the first objective of this project was to investigate the mechanisms through which CXCR6 and CXCL16 contribute to arthritic disease. I have demonstrated upregulated expression of CXCR6 on T cells from the paws, lymph nodes, and spleen of mice with CIA (Figures 4, 5, and 7). Furthermore, $CXCR6^{-/-}$ mice are resistant to arthritis development and exhibit reduced joint inflammation (Figure 9). To verify the findings in $CXCR6^{-/-}$ mice, I assessed the development of clinical signs of disease in $CXCL16^{-/-}$ mice (Figure 10). Incidence and arthritis severity were similarly reduced in $CXCR6^{-/-}$ and $CXCL16^{-/-}$ mice, compared to wild-type mice. These findings implicate CXCR6 and CXCL16 in the development and progression of CIA in mice. This is consistent with a previous study showing that treatment with blocking anti-CXCL16 antibody reduced the severity of CIA in mice (251). Through the studies presented herein, I show that CXCR6 influences arthritis development through two potentially distinct mechanisms: regulation of T cell inflammatory cytokine polarization, and mediating T cell homing to inflamed joints. CXCR6 is also highly expressed on iNKT cells and is important in modulating their distribution and function in vivo, and I demonstrate an important role for iNKT cells and iNKT cell-derived cytokines in CIA pathogenesis.

Elevated CXCR6 expression was most evident on $CD4^+$ T cells isolated from the paws of arthritic mice (Figures 4 and 7), which was consistent with a greater overall accumulation of $CD4^+$ T cells within arthritis paws compared to other T cell subsets (Figure 11D). However, accumulation of $CD4^+$ T cells in the arthritic paws was attenuated in $CXCR6^{-/-}$ mice. CXCR6 expression defines subsets of effector/memory T cells in vivo (277), which, together with their predominance within the arthritic paws,

suggests that $CD4^+$ T cells are involved in the pathogenesis of inflammatory arthritis. Indeed, there is strong evidence supporting a role for $CD4^+$ T cells in both RA and CIA. For example, genetic associations of RA susceptibility are strongest for alleles within the *HLA-DRB1* locus (16,419), while susceptibility to CIA is also associated with MHC class II polymorphisms associated with certain mouse strains (i.e. $H\text{-}2^q$ bearing strains being the most susceptible, and $H\text{-}2^b$ strains amongst the least susceptible) (420), suggesting an important role for MHC-restricted $CD4^+$ T cell responses in arthritic disease. This is further supported by the detection of multiple oligoclonally expanded $CD4^+$ T cells within the RA synovium over the course of disease (134). Animal studies have shown that CII-specific $CD4^+$ T cells are essential for transferring arthritis into SCID mice (136), and mice deficient in CD4 are resistant to CIA (138,139). In addition to their recruitment to diseased joints, arthritogenic $CD4^+$ T cells have been shown to actively divide and are maintained over time in the arthritic synovium in mice (421). The high expression of CXCR6 on $CD4^+$ T cells within the arthritic paws suggests that CXCR6 and CXCL16 are involved in their homing to this site. Furthermore, the reduced severity of arthritis in $CXCR6^{-/-}$ mice may be associated with impaired accumulation of $CD4^+$ T cells in the paws of arthritic $CXCR6^{-/-}$ mice.

Unlike $CD4^+$ T cells, the elevated CXCR6 expression detected on $CD8^+$ T cells in arthritic paws was more subtle (Figures 4 and 7). Additionally, $CD8^+$ T cells failed to accumulate in the arthritic paws (Figure 11D), suggesting that their presence within the joint may not be necessary for disease pathogenesis. Higher levels of chemokine receptor expression are correlated with greater chemotactic responses (422,423), which may favor recruitment of $CD4^+$ T cells to the inflamed paws of mice with CIA. The greater frequency and number of $CXCR6^+$ $CD8^+$ T cells in the lymph nodes is likely due to the low level constitutive expression of CXCR6 on naïve $CD8^+$ T cells (279). This is consistent with a larger pool of $CXCR6^+$ $CD8^+$ T cells that coexpress CCR7 (Table 2), likely contributing to their accumulation in the lymph nodes (Figure 5). In contrast to $CD4^+$ T cells, which have been shown to be important in disease initiation and progression in multiple animal models (99), studies on the role of $CD8^+$ T cells in animal models of RA have revealed controversial results. For example, CD8-deficient DBA/1 mice were shown to have reduced incidence of CIA, but the absence of $CD8^+$ T cells had

no effect on disease severity (148). Contrary to these findings, CD8-deficiency had no impact on disease incidence or severity in B10.Q mice (139). In PGIA, depletion of $CD8^+$ T cells prevented disease transfer to recipient mice (149), whereas depletion of $CD8^+$ T cells in PGIA immunized mice exacerbated arthritis (150), suggesting $CD8^+$ T cells may play different roles during pre-clinical stages and established disease. The role of $CD8^+$ T cells in patients with RA remains relatively unknown, although it has been suggested that they may play a suppressive role based on studies of human RA synovial tissue grafts in SCID mice (146).

A relatively large population of T cells with a DN $TCR\beta^+$ phenotype was detected in the paws, but not in the lymph nodes or spleen, of nonimmunized and arthritic mice. The majority of human and mouse T cells that express an αβTCR are either $CD4^+$ or $CD8^+$ following their selection in the thymus and emigration to the periphery. However, a small proportion of T cells in the peripheral blood and lymphoid organs (~1-5%) display a DN phenotype ($CD3^+CD56^-\alpha\beta TCR^+CD4^-CD8^-$) in both healthy rodents and humans (403,424). The origin of DN T cells is not well understood. Evidence provided by Fischer et al. (403) suggests that these cells progress through a thymic developmental pathway followed by proliferation in the periphery. Alternatively, DN T cells may arise from a pathway that does not require a thymus, which is supported by findings that DN T cells can develop in thymectomized, irradiated, bone marrow-reconstituted C57BL/6 mice (424). Under conditions that remain to be determined, DN T cells may arise from peripheral T cell precursors that have downregulated their CD4 or CD8 coreceptors. It could also be envisioned that there are unique subsets of DN T cells that emerge from more than one of these pathways.

To date, no specific markers of DN T cells have been identified, and they are often defined by exclusion as $CD3^+CD4^-CD8^-$ T cells. Consequently, this distinction includes multiple specialized but rare T cell subpopulations, such as γδ T cells, DN $\alpha\beta TCR^+$ T cells, and subsets of NKT cells. I used additional markers such as TCRβ to discriminate between γδ T cells and DN $\alpha\beta TCR^+$ T cells. The phenotypic distinction between NKT cells and DN T cells was not made directly in these experiments, thus a proportion of the DN T cell population identified in the tissues of nonimmunized and

arthritic mice likely includes $CD4^-$ NKT cells. However, it is unlikely that these NKT cell subsets constitute a large fraction of the DN T cell population I detected since among $TCR\beta^+$ cells, the CD1d-$tetramer^+$ population was considerably smaller than the total DN T cell population in the paws and secondary lymphoid tissues. Furthermore, there were disparities in the expression of CXCR6 and other chemokine receptors on the DN T cell and iNKT cell populations. Finally, while it is possible that DN T cells could include other T cell subsets, such as type II NKT cells, these cells are more infrequent in mice than iNKT cells. Using CD1d-deficient mice, which are devoid of both type I and type II NKT cells, one could identify, isolate, and characterize $CD4^-CD8^-\alpha\beta TCR^+$ T cells.

Peripheral DN T cells have been shown to have roles in immune regulation and tolerance as well as in host defense and inflammation (425,426). For example, many studies have demonstrated a regulatory function of DN T cells in that they are able to suppress $CD4^+$ and $CD8^+$ T cell responses in both humans and mice (403,404). Through their suppressive activity, DN T cells have been shown to contribute to the induction of tolerance in transplantation models as well as the control of autoimmune diabetes (425). In contrast, a role for DN T cells in inflammation was demonstrated in patients with SLE (427). In this study, DN T cells (included neither γδ T cells nor NKT cells) were suggested to contribute to disease pathogenesis in patients with lupus nephritis through their production of large amounts of IL-17 and IFN-γ and their presence in diseased kidney biopsies. Interestingly, circulating levels of DN T cells have been shown to be elevated in autoimmune disease patients, most notably in those with SLE (428,429). Although the precise role of DN T cells in health and disease remains uncertain, it may vary depending on the condition. For example, in addition to their elevated circulating levels in SLE patients, DN T cells exhibit an activated phenotype, produce proinflammatory cytokines, and induce anti-DNA antibody production as efficiently as pathogenic $CD4^+$ T cells (427,428,430). However, DN T cells are unlikely to play a role in RA pathology since their circulating levels are similarly low between RA patients and healthy subjects, and are not increased in synovial fluid compared with peripheral blood (431). The presence of DN $TCR\beta^+$ cells has been previously described in the inflamed paws of CIA mice (148). Interestingly, these DN $TCR\beta^+$ cells were shown to proliferate in response to CII in a MHC-restricted fashion and produced a large amount of TNF

(138,148). However, the DN TCRβ$^+$ T cells described in these studies were detected in CD4-deficient mice (138,148), and were shown to infiltrate and accumulate within the arthritic paws (148). In contrast, I did not observe significantly increased numbers of DN TCRβ$^+$ T cells in the paws of arthritic wild-type or CXCR6$^{-/-}$ mice compared to nonimmunized controls (Figure 11D). The presence of DN TCRβ$^+$ cells within the paws of nonimmunized mice suggests they may be a resident population and it is possible that they could exhibit disease modifying potential, similar to a recently identified DN T cell population resident at articular sites that are capable of producing IL-17 and other inflammatory cytokines (402). Recently, a study demonstrated a unique epigenetic signature based on the methylation status of various genetic loci in DN T cells, with evidence suggesting a robust proinflammatory function due to strong transcriptional permissiveness in genes encoding IFN-γ, IL-17, IL-12p40, IL-18, and RANKL (432). Therefore, DN T cells were examined alongside CD4$^+$ and CD8$^+$ T cell subsets to evaluate their cytokine production and expression of activation markers.

The accumulation of activated T cells within the joints is important during inflammation and tissue destruction in inflammatory arthritis. However, autoantibody production and other immune mechanisms also play important disease promoting roles during synovial inflammation. Indeed, the detection of RF and ACPA/anti-CCP antibodies in RA patients are strong predictors of radiological progression (118,119). Moreover, the arthritogenic anti-CII antibodies raised in collagen-immunized mice (114), and anti-G6PI antibodies generated in the K/BxN mouse model (91) are sufficient for passive transfer of disease in mice. Indeed, CIA is a complex disease model and multiple immune mechanisms contribute to disease severity, in addition to T cell accumulation within the arthritic paws. Consistent with this, arthritic CXCR6$^{-/-}$ mice with similar clinical severity scores to wild-type mice exhibited lower numbers of TCRβ$^+$ cells in the paws (Figure 11F). This indicates that other disease modifying factors, such as arthritogenic antibody production and infiltration of the joint by other inflammatory cells, are contributing to clinical disease progression in wild-type and CXCR6$^{-/-}$ mice. Nonetheless, these data indicate that the absence of CXCR6 (and CXCL16) protects mice against the development of CIA and reduces the clinical severity of arthritis in affected

mice, which is associated with reduced accumulation of $CD4^+$ T cells in the arthritic paws.

4.2 Impaired Th1 and Th17 cytokine polarization in T cells from CXCR6$^{-/-}$ mice

CXCL16 and fractalkine/CX_3CL1 differ from other chemokines since they can be expressed on the cell surface as transmembrane molecules (279,280). While cleavage of CXCL16 from the cell surface releases a soluble form of CXCL16 that induces migration of activated T cells (279,280,287), the membrane bound form of CXCL16 mediates adhesion of $CXCR6^+$ cells (284). CXCR6 expression is enriched on Th1-, Tc1-, and Th17-polarized T cells in human blood (277,296) and CXCL16 is expressed on the surface of antigen-presenting cells, including DCs and macrophages (279,280). Therefore, we hypothesized that CXCL16-CXCR6 interactions modulate the responses of effector T cells during antigen presentation and T cell polarization. To assess wild-type and CXCR6$^{-/-}$ mice for differences in cytokine polarization of T cells, I isolated cells on days 14 and 35 and performed intracellular cytokine staining for IL-17A, IFN-γ, and IL-4. Wild-type and CXCR6$^{-/-}$ mice with severe disease were examined on day 35 to eliminate potential bias in the evaluation of cytokine production that might result from differences in the severity of inflammation.

Compared to wild-type mice, T cells in collagen-immunized and arthritic CXCR6$^{-/-}$ mice exhibited reduced frequencies and numbers of IFN-γ and IL-17A-generating cells (Figures 12-17). Similar to these findings, collagen-specific IFN-γ and IL-17A production was attenuated in the supernatants of CXCR6$^{-/-}$ cells after restimulation with CII in vitro (Figure 18). Importantly, the differences in cytokine production were not due to differences in the proliferation of wild-type and CXCR6$^{-/-}$ cells. These results suggest that CXCR6 plays an important role in regulating cytokine polarization, likely through interactions with $CXCL16^+$ APCs. Consistent with this, the cytokine profile of antigen-stimulated iNKT cells has been shown to be regulated by CXCR6-CXCL16 interactions by our lab and others (159,298). Moreover, other chemokines and chemokine receptors have been shown to regulate T cell activation/differentiation. Indeed, during antigen stimulation of T cells, CCR5 and

CXCR4 are recruited to the immunological synapse and function as T cell costimulators, enhancing T cell proliferation and cytokine production (262). For example, cells stimulated through the TCR and incubated in the presence of the CCR5 ligands RANTES/CCL5 and MIP-1α/CCL3, or the CXCR4 ligand stromal cell-derived factor-1 (SDF-1; CXCL12), exhibited enhanced activation marker expression (CD69, CD25, and CD40L), increased proliferation, and elevated IFN-γ and IL-2 production (262,433), whereas addition of MCP-1/CCL2 during T cell stimulation resulted in enhanced IL-4 production (434). CXCL16-dependent activation of CXCR6 in human aortic smooth muscle cells has been shown to activate NF-κB and induce proinflammatory gene transcription via $G\alpha_i$-, phosphatidylinositol-3-kinase (PI3K)-, and Akt-dependent pathways (435). Therefore, it is likely that CXCR6 delivers signals that induce proinflammatory cytokine polarization in T cells. Consequently, the absence of CXCR6-CXCL16 costimulation during T cell activation in the lymph nodes of $CXCR6^{-/-}$ mice could contribute to the reduced frequencies and numbers of IFN-γ^+ and IL-$17A^+$ T cells detected in these tissues. Interestingly, evidence suggests that T cells might express both CXCL16 and its receptor, akin to IL-2 and IL-2 receptor, providing the possibility of autocrine stimulation (436). However, further investigation is needed to confirm CXCL16 expression by T cells since CXCL16 mRNA, and not protein, expression was reported. Thus, in addition to a role in regulating T cell trafficking to the inflamed paws, CXCR6 expression on T cells could enhance their effector responses at this site in arthritic mice, where CXCL16 is abundantly expressed (251). Together this would explain the reduced frequencies and numbers of IFN-γ^+ and IL-$17A^+$ T cells observed in the arthritic paws of $CXCR6^{-/-}$ mice.

Interestingly, these experiments also revealed a temporal difference in IFN-γ and IL-17A production by $CD4^+$ T cells in immunized and arthritic CIA mice. In other words, IFN-γ production was increased significantly in lymph node cells by day 14 and contracted to near baseline levels by day 35, whereas IL-17A production increased by day 14 but remained elevated on day 35 within the lymph nodes (Figures 12 and 18). Previous studies have revealed contradictory roles of IFN-γ, which may be linked to opposing effects during early versus late phases of disease in the CIA model (166,395). For example, neutralizing anti-IFN-γ antibodies were protective when delivered early post-

immunization, but the same treatment exacerbated arthritis when delivered later during the course of CIA (166,395). Moreover, early administration of recombinant IFN-γ increased the incidence and severity of arthritis in mice (164–166). An important role for IFN-γ in the early stages of CIA is supported by our data and others demonstrating prominent IFN-γ^+ populations in the draining lymph nodes early post-immunization (Figures 12-15 and 18) (167). In line with this, others have shown that Th1 cells were predominant in the pre-arthritic stage of CIA, but not later in disease (168,169). The protective role of IFN-γ during the effector stage of CIA could be due to the suppressive effects of IFN-γ on IL-17 production (175,176,236). However, I observed greater IFN-γ production in the paws of arthritic wild-type mice, which also exhibit more severe clinical signs of disease compared to CXCR6$^{-/-}$ mice (Figures 12-17). Consistent with this, abundant expression of IFN-γ was detected in the inflamed joints, but not the non-inflamed joints, of individual CIA mice (170), suggesting that the presence of IFN-γ within the joints may promote more severe joint destruction in CIA, possibly through enhanced activation of FLS and other cells within the inflamed joints (171).

The contradictory findings regarding the role of IFN-γ in experimental arthritis led to the characterization of IL-17A in arthritis (175,176,236). In CIA, disease is markedly suppressed in IL-17-deficient mice (201), and therapeutic administration of neutralizing anti-IL-17 antibody after disease onset reduced joint inflammation, cartilage destruction, and bone erosions (202). Furthermore, IL-17 blockade almost completely prevented clinical signs of disease in IFN-$\gamma^{-/-}$ mice (175,236), and IFN-γ/IL-17 double knockout mice exhibited mild non-progressive disease (437). The success of IL-17 inhibitors in animal models has led to the development and clinical testing of anti-IL-17 antibodies in patients with RA. One IL-17A inhibitor (secukinumab) is currently under evaluation in phase III clinical trials, and two others (ixekizumab and brodalumab) are in phase II (438), and have shown some success in improving signs and symptoms in RA patients (192). However, the clinical benefit of IL-17 blockade falls short of the degree that was expected, which could be related to the multiple IL-17 members, and that the current anti-IL-17 antibodies may not efficiently neutralize all forms of IL-17 in vivo. IL-17A acts on multiple cell types to induce inflammatory cytokine and chemokine production, elevated expression of MMPs, and other inflammatory mediators (438). In addition, IL-17A has

effects on endothelial cells and contributes to angiogenesis in RA (439), and cancer (440), through inducing endothelial cell migration and vascular endothelial growth factor (VEGF) production.

Again, an early Th1 cell polarization was observed within the lymph nodes from collagen-immunized mice, followed by more pronounced IL-17A-production later after the development of clinical signs of arthritis (levels of IL-4 production were low throughout the course of CIA) (Figures 12 and 18). The increase in IL-17 production in later stages of disease may be partly due to decreased IFN-γ production as disease progresses in the CIA model, since IFN-γ has a suppressive effect on Th17 cell differentiation (182,184), and IL-17 production (175,176,236). However, contrary to these findings, IFN-γ has been shown to induce human APCs to generate IL-1 and IL-23, factors responsible for promoting the development of Th17 cells while also abating Th1 cell polarization (441). Furthermore, an IFN-γ-dependent accumulation of inflammatory monocytes in the lymph nodes of arthritic mice, which were regionally activated by autoreactive Th1 cells, has also been demonstrated to promote Th17 cell differentiation (442). These findings are consistent with the time course of IFN-γ and IL-17A polarization demonstrated in our experiments. Alternatively, it is possible that Th1 cells may convert to Th17 cells or to an intermediate Th17/Th1 phenotype, which has been previously reported in patients with inflammatory arthritis or Crohn's disease, and in EAE mice (191,230,231). Furthermore, the identification of populations of bifunctional Th1-Th17 cells producing both IFN-γ and IL-17 may provide an explanation for the high frequency and number of IFN-γ^+ and IL-$17A^+$ $CD4^+$ T cells detected in the inflamed paws of CIA mice, and the observed reductions in both of these subsets in the paws of $CXCR6^{-/-}$ mice (Figures 12D and 13D). While I did not examine which of these mechanisms may be responsible for the shift from an early IFN-γ dominated response to a higher Th17/Th1 response later, one or more may provide an explanation for how the reduced early Th1 cell polarization in $CXCR6^{-/-}$ mice translates to lower IL-17A production at later time points.

4.3 Reduced $CD8^+$ T cell– and DN T cell-derived IFN-γ in $CXCR6^{-/-}$ mice

The intracellular cytokine staining experiments revealed that, in addition to $CD4^+$ T cells, both $CD8^+$ and DN T cells were potent sources of IFN-γ in wild-type mice, whereas these IFN-γ^+ populations were reduced in the tissues of $CXCR6^{-/-}$ mice (Figures 14-17). However, the numbers of IFN-γ^+ $CD8^+$ and IFN-γ^+ DN T cells in the paws of arthritic mice at peak disease severity were much lower compared to $CD4^+$ T cells. Indeed, $CD4^+$ T cells are major producers of IFN-γ in collagen-immunized mice since treatment with anti-CD4 antibodies results in a substantial reduction in IFN-γ production in the draining lymph nodes and prevents the development or transfer of CIA (137). These findings further support an important disease promoting role for IFN-γ in the early phases in CIA. Previous reports have found $CXCR6^+$ $CD8^+$ T cells to be enriched for IFN-γ-producing cells (277). I have shown that CXCR6 is expressed by a subset of $CD8^+$ and DN T cells in the lymph nodes and paws of arthritic mice (Figures 4, 5, and 7). Similar to $CD4^+$ T cell cytokine production, IFN-γ-producing $CD8^+$ T cells and DN T cells were reduced in $CXCR6^{-/-}$ mice, suggesting that CXCR6 could deliver signals that promote IFN-γ production by $CD8^+$ and DN T cells as well.

IFN-γ-producing $CD8^+$ T cells have been detected in the synovial tissues of RA patients (154). However, as discussed earlier, the role of $CD8^+$ T cells in inflammatory arthritis is controversial. While both IL-17A and IFN-γ have been suggested to contribute to aspects of arthropathy (189,190), accumulating data have revealed the existence of diverse mechanisms by which IFN-γ counteracts inflammatory arthritis. It is possible that $CD8^+$ T cell-derived IFN-γ in the lymph nodes and paws of CIA mice is contributing to the same mechanisms described earlier regarding the role for IFN-γ in mediating a shift towards a higher Th17/Th1 response later in disease development. Alternatively, IFN-γ-producing $CD8^+$ T cells and DN T cells may exert regulatory effects through their production of IFN-γ since it has been shown to stimulate the production of the enzyme indoleamine 2,3-dioxygenase (IDO) (443). IDO can be expressed in various cells, including DCs (444), macrophages (445), and RA synovial fibroblasts (446). Expression of IDO actively reduces the availability of the essential amino acid tryptophan, generating toxic metabolites along the kynurenine pathway, which induce apoptosis of T cells (447),

but may also induce Tregs (448). Interestingly, adoptive transfer of $CD8^+$ T cells has been shown to disrupt the development of CIA, which was reversed by anti-IFN-γ antibody or co-administration of the IDO inhibitor 1-methyltryptophan (449). Consistent with this, IFN-γ has been shown to induce IDO and suppress CIA by regulating IL-17 production (437). Moreover, induction of IDO was detected in lymph node DCs following collagen-immunization, which limited IFN-γ and IL-17 production by lymph node T cells and regulated accumulation of Th1/Th17 cells in the joints of CIA mice (450).

4.4 CXCR6-deficiency is associated with reduced cellular responses to CII in vitro and in vivo

I assessed recall responses to CII on days 14 and 35 post-immunization by measuring supernatant cytokine production and proliferative responses of lymph node cells from wild-type and $CXCR6^{-/-}$ mice. As discussed earlier, the antigen-specific cytokine responses measured in these assays were similar to the cytokine polarization results observed in the intracellular cytokine staining experiments, including the shift from IFN-γ production to an increased IL-17A response (Figure 18). The potential mechanisms responsible for this shift and the possible influences it has on disease pathogenesis have already been discussed. Importantly, the differences in cytokine production were not due to differences in the proliferation of wild-type and $CXCR6^{-/-}$ cells. Notably, the proliferation of lymphocytes in these cultures was high in the absence of exogenous antigen stimulation. It has been suggested that this could result from T cell responses to xenogeneic antigens presented by self-MHC (451), such as those contained within the FBS present in the culture media. However, other possible stimulators of T cells in these autologous mixed lymphocyte reactions include apoptotic antigens released from dead or dying cells within these cultures (452). Finally, collagen-specific cytokine and proliferative responses were verified in these studies using cells from wild-type mice immunized with CFA alone. Thus, together with the intracellular cytokine staining data, these results show that IL-17A and IFN-γ production are reduced in collagen-immunized $CXCR6^{-/-}$ mice compared to wild-type mice which highlights a possible mechanism for the reduced severity of arthritis in $CXCR6^{-/-}$ mice.

For evaluation of immune responses to CII in vivo, I performed a DTH skin reactivity assay via CII challenge in the ears of immunized mice. At 24 h post-challenge, a significantly reduced inflammatory reaction was observed in CXCR6$^{-/-}$ mice compared to wild-type mice (Figure 19). Th1 cells are important drivers of DTH reactions (453), likely due to their ability to produce IFN-γ. High levels of IFN-γ are detected at the site of antigen challenge in DTH skin tests (454,455), and direct intradermal injection of IFN-γ induces a similar inflammatory infiltrate (456). Therefore, in vivo CII challenge is consistent with the results observed in the in vitro CII restimulation experiments, in which cells from collagen immunized CXCR6$^{-/-}$ mice produced significantly less IFN-γ than those from wild-type mice. This result is also consistent with what has been shown for DTH reactivity against G6PI challenge in the ear of mice previously immunized with G6PI in CFA; a reduced DTH reaction was observed in IFN-γ receptor-deficient mice when challenged early post-immunization compared to wild-type mice (457).

4.5 Reduced IgG2a anti-CII antibodies in serum from CXCR6$^{-/-}$ mice

A range of autoantibodies with various reactivities have been described in patients with RA, including anti-perinuclear (458), anti-human collagen II (115–117), anti-G6PI (459,460), ACPA/anti-CCP antibodies (461), and RF (462). Of these, seropositivity for ACPA and/or RF are among the diagnostic criteria for RA and represent strong predictors of radiological progression and clinical outcome in patients (118,119). Indeed, detection of ACPA/anti-CCP antibodies in the sera often pre-dated the onset of RA by years and accurately predicted the development of RA in those individuals (55).

Many of these autoantibodies have also been described in animal models of RA. For example, both ACPA and RF are produced in PGIA in mice (86). ACPA responses also develop in the early stages of CIA, and mice with chronic disease exhibit elevated levels of anti-CCP antibodies (120). Moreover, autoantibodies to murine CII develop in CIA mice (105), whereas anti-G6PI antibodies are largely responsible for spontaneous inflammatory arthritis in the K/BxN mouse model (90). Critically, these autoantibodies have been shown to be arthritogenic since transfer of antibodies against CII (114,407,408), whole serum and/or fractionated IgG from arthritic K/BxN mice (92), or anti-G6PI antibodies (91,463) is sufficient to induce arthritis in normal healthy animals.

Therefore, I assessed anti-CII antibody levels in serum obtained from wild-type and CXCR6$^{-/-}$ CIA mice.

Compared to wild-type mice, arthritic CXCR6$^{-/-}$ mice had reduced levels of anti-CII IgG2a antibodies, whereas the levels of anti-CII antibodies of the IgG1 subclass were not different (Figure 20). This suggests that some aspect of the T cell-B cell crosstalk involved in anti-CII antibody production in CXCR6$^{-/-}$ mice was impaired, which could be related to the reduced IFN-γ production since IFN-γ is an important inducer of IgG2a class switching (464,465). Conversely, the development of arthritis in K/BxN mice is critically dependent on IL-4, which explains the dominance of anti-G6PI antibodies of the IgG1 subclass in this model (466). Thus, the link between reduced IFN-γ and lower levels of anti-CII IgG2a in CXCR6$^{-/-}$ mice is supported by the similar levels of IL-4 and anti-CII IgG1 antibodies observed in wild-type and CXCR6$^{-/-}$ mice. This result provides an additional explanation for the reduced incidence and severity of arthritis in CXCR6$^{-/-}$ mice, since IgG2a autoantibodies have been shown to induce disease by binding activating Fc receptors (409), by contributing to complement activation (407,408), and through direct effects on cartilage matrix production by chondrocytes (467). Additionally, plasma cells have been shown to express CXCR6 and migrate in response to CXCL16 in Transwell assays (294), suggesting CXCR6 may be important for the homing and localization of plasma cells in inflamed tissues.

4.6 Reduced arthritis in CXCR6$^{-/-}$ mice is associated with decreased populations of memory T cells and reduced expression of RANKL

CXCR6 is constitutively expressed at low levels on naïve CD8^{+} T cells, whereas surface levels of CXCR6 are nearly undetectable on naïve CD4^{+} T cells (279). However, CXCR6 expression is enriched on Th1-, Tc1-, and Th17-polarized T cells with an effector/memory phenotype (277,296). Given the potential for CXCR6-CXCL16 interactions to modulate the responses of activated T cells during antigen presentation and T cell polarization, I examined whether there were differences in the proportions of activated and memory T cell populations between wild-type and CXCR6$^{-/-}$ CIA mice. Compared to wild-type mice, CXCR6$^{-/-}$ mice exhibited reduced frequencies and numbers of CD4^{+}, CD8^{+}, and DN T cells with a CD44^{hi}CD45RB^{lo} activated/memory phenotype in

the lymph nodes and spleen at day 35 post-immunization (Figures 21, 22, and 23). This is consistent with the intracellular cytokine staining data showing that $CXCR6^{-/-}$ mice had lower frequencies and numbers of IFN-γ– and IL-17A-producing T cells, since cytokine production was observed only within $CD44^{hi}$ cells. Indeed, naïve $CD4^+$ and $CD8^+$ T cells do not produce large amounts of IFN-γ but following activation, these cells can undergo differentiation into Th1 effector/memory cells (468) and IFN-γ-producing CTLs/memory $CD8^+$ T cells (469), respectively.

In contrast, no significant differences were observed in the expression of the early activation marker CD69 on T cells from arthritic wild-type and $CXCR6^{-/-}$ mice at day 35, or between arthritic mice and nonimmunized mice (Figures 21, 22, and 23). In human studies involving patients with various autoimmune and inflammatory diseases, the in vivo expression of CD69 has mainly been detected in leukocyte inflammatory infiltrates (470). Consistent with this, the level of CD69 expression detected on $CD4^+$ T cells isolated from the arthritic paws of CIA mice was appreciably higher than that observed on lymph node populations (Figure 24). However, CD69 expression on lymph node and spleen cells may not represent an accurate indicator for the proportions of T cells displaying an activated/memory phenotype in CIA mice since CD69 expression is scarce in resting lymphocytes (<2% of naïve $CD4^+$ T cells) and only 20-30% of previously activated $CD44^{hi}$ $CD4^+$ T cells in the spleen are $CD69^+$ (471). T cells rapidly express CD69 upon stimulation through the TCR (472), which is why CD69 has been used as a marker for cell activation. However, CD69 also regulates lymphocyte egress from the lymphoid organs (473). Yet, upon activation, lymphocytes will markedly alter their expression of chemokine receptors and other surface molecules, allowing for their redistribution to other peripheral tissues as specialized effectors or memory cells. Indeed, rather than residing in the secondary lymphoid organs, many types of memory cells have been shown to preferentially accumulate in the bone marrow (474). Interestingly, Shinoda et al. (471) have shown that expression of CD69 is critical in mediating the relocation of $CD4^+$ T cells from blood to the bone marrow environment, where these antigen-experienced $CD4^+$ T cells associate with IL-7-expressing stromal cells (475), possibly accompanied by their effector to memory transition. Therefore, examining $CD44^{hi}CD45RB^{lo}$ T cell populations, but not $CD69^+$ T cells, in the lymph nodes and

spleen may be more representative of reduced populations of intentionally antigen-specific and unintentionally generated effector and memory T cell pools in arthritic CXCR6$^{-/-}$ mice compared to wild-type mice in the CIA model.

RANKL is highly expressed in the synovium of RA patients (413,414), and is critical for disease-mediated bone destruction (411,412,476). RANKL-RANK signaling is essential for the regulation of bone homeostasis via acting as a potent driver of osteoclast differentiation and influencing the bone-resorbing function of mature osteoclasts (410,477). In addition to their production of proinflammatory cytokines, memory CD4^{+} T cells isolated from RA synovial tissues express the osteoclastogenic regulator RANKL (155). Consistent with this, I found that paw-isolated T cells, most notably CD4^{+} subsets, expressed elevated levels of RANKL compared to those in the lymph nodes in CIA mice (Figure 25). However, compared to wild-type mice, T cells isolated from the paws of arthritic CXCR6$^{-/-}$ mice exhibited significantly reduced expression of RANKL.

Interestingly, differences in the osteoclastogenic potential of Th17 and Th1 subsets have been suggested. For example, pathogenic Th17 cells have been shown to be potently osteoclastogenic through their own RANKL expression in addition to their ability to induce RANKL on synovial fibroblasts via IL-17, IL-1, TNF, and IL-6 cytokine signals (232,476). Critically, visualization of mature osteoclasts in intact mouse bones using intravital multiphoton microscopy revealed that Th17 cells, but not Th1 cells, could induce bone-resorptive osteoclast function via RANKL in a cell-cell contact mechanism (478). The osteoclastogenic action of Th17 cells can be counteracted by IFN-γ, since IFN-γ signaling inhibits the ability of IL-17 and TNF to induce RANKL expression on fibroblasts (206), in addition to interfering with signaling pathways downstream of RANK via proteasomal degradation of the RANK adaptor protein TRAF6 (479). In contrast, others have shown that IFN-γ functions as a pro-resorptive cytokine by stimulating antigen-dependent T cell activation and secretion of RANKL and TNF, enhancing osteoclast function and accelerating bone loss in vivo (480). Therefore, these studies highlight a possible connection between reduced IL-17A– and IFN-γ-producing T cells and RANKL expression in the arthritic paws of CXCR6$^{-/-}$ mice, which likely contributes to the attenuated clinical signs of arthritis in these mice compared to wild-type

mice. While I did not examine structural damage in the joints of CIA mice, others have shown that activated RANKL$^+$ T cells regulate bone loss and joint destruction via RANKL in animal models of RA (481,482).

Additionally, chemokines and their receptors have been shown to play critical roles in osteoclastogenesis and bone resorption through influencing osteoclast precursor cell survival and differentiation, as well as promoting increased motility and function of mature osteoclasts (483–486). Differentiating and mature osteoclasts cultured from healthy donors have been shown to express CXCR3, CXCR4, and CXCR5 (487), among other chemokine receptors, but data indicating their expression of CXCR6 is lacking. However, there is evidence suggesting that CXCR6 expression is downregulated on RAW264.7 cells cultured with RANKL during osteoclastogenesis (488). In this study, the authors show that addition of CXCL16 failed to augment RANKL-mediated osteoclast differentiation and had no effect on RANK expression on osteoclast precursors, suggesting that CXCL16-CXCR6 interactions on precursor cells are not critical during osteoclastogenesis. However, future studies should investigate whether CXCL16 signaling through CXCR6 enhances RANKL expression, since a recent study has reported that CXCL12/SDF-1 upregulated RANKL expression in RA synovial fibroblasts and CD4$^+$ T cells, and enhanced their osteoclastogenic potential (489). A similar mechanism for CXCL16-CXCR6 interactions would provide an additional explanation for the reduced expression of RANKL on T cells in the arthritic paws of CXCR6$^{-/-}$ mice, a location where CXCL16 is abundantly expressed (251).

4.7 Impaired recruitment of CXCR6$^{-/-}$ T cells to inflamed paws

Expression of CXCR6 is elevated on T cells, particularly within the CD4$^+$ subset, isolated from the paws of arthritic mice, which suggests that CXCR6 is important for the accumulation of these cells at this site (Figures 4 and 7). A role for CXCR6 in mediating recruitment of T cells to the inflamed paws of arthritic mice is supported by the dramatically reduced accumulation of CD4$^+$ T cells in the paws of arthritic CXCR6$^{-/-}$ mice (Figure 11D). Furthermore, previous studies have shown that synovial fluid T cells from RA patients can migrate toward CXCL16 gradients in vitro (252) or into RA synovial tissue grafts treated with exogenous CXCL16 (253). To investigate the role of

CXCR6 in homing of T cells to the inflamed paws of arthritic mice, I performed coadoptive transfers of wild-type and CXCR6$^{-/-}$ T cells. Prior to transfer, donor T cells were activated in vitro using anti-CD3ε antibodies to ensure surface expression of CXCR6 was upregulated and that activated wild-type cells, but not CXCR6$^{-/-}$ cells, functionally migrated toward CXCL16 in Transwell chemotaxis assays (Figure 26) (279). This in vitro activation is critical, since only a small subpopulation of splenic CD4^{+} T cells (<4%) express CXCR6 in naïve mice (Figure 5) (277,279). While constitutive low level CXCR6 expression is detected on CD8^{+} T cells, neither these nor naïve CD4^{+} T cells exhibit specific chemotaxis toward CXCL16 prior to activation (279). The absence of chemotactic responsiveness in naïve CD8^{+} T cells to CXCL16, despite their expression of low but detectable levels of CXCR6, suggests that responsiveness to CXCL16 is regulated at levels downstream from surface receptor expression, which is overcome upon cell activation. Alternatively, high levels of CXCR6 expression may be required to generate sufficient homing signals, since greater chemotactic responses are correlated with high levels of chemokine receptor expression (422,423),

To determine whether cells lacking CXCR6 were impaired in their ability to home to the inflamed paws of mice with CIA, equal numbers of in vitro-activated wild-type and CXCR6$^{-/-}$ T cells were coadoptively transferred into naïve and arthritic wild-type mice. Differential labeling allowed for clear discrimination of donor wild-type T cells from the donor CXCR6$^{-/-}$ population and dye labeling did not affect cell recruitment. At 24 h post-transfer, the ratio of labeled donor wild-type T cells to CXCR6$^{-/-}$ T cells was ~2:1 in the paws of recipient arthritic mice (Figure 27). In contrast, there was no bias for the recruitment of CXCR6^{+} T cells to uninflamed paws of naïve mice or to secondary lymphoid tissues in naïve or arthritic recipient mice (Figure 27). This greater capacity of CXCR6^{+} T cells to home to arthritic paws is supported by the elevated expression of CXCL16 observed in the synovium of mice with CIA (251). Consistent with this, accumulation of CXCR6^{+} T cells within the synovial fluid of RA patients coincided with elevated levels of CXCL16 in the arthritic synovium (251–253). CXCL16 is also expressed in other organs and tissues, including the liver, lung, and gastrointestinal tract (279,280,300). Importantly, I also observed a >2-fold difference in the accumulation of CXCR6^{+} T cells versus CXCR6$^{-/-}$ T cells in the liver and lungs of naïve recipient mice,

suggesting that the selective recruitment of donor $CXCR6^+$ T cells to these tissues, and to the inflamed paws of arthritic mice, is the result of high levels of CXCL16 present at these sites. Future studies could verify this by examining the accumulation of donor T cells in $CXCL16^{-/-}$ recipient mice.

Within the synovium of RA patients, CXCL16 is detected in both soluble and membrane bound forms. In its transmembrane form, CXCL16 has been shown to be abundantly expressed on synovial monocytes/macrophages and FLS (251–253). Cleavage of CXCL16 from the cell surface by the protease ADAM-10 (286,287), has been shown to result in elevated levels of soluble CXCL16 in RA synovial fluid (252,253). Alternatively, soluble CXCL16 can be generated and secreted through the expression of a splice variant of CXCL16 lacking the transmembrane domain (288). The immobilization of soluble chemokines on cell surface-expressed glycosaminoglycans plays an important role in their presentation to responsive cells (490). CXCL16 is expressed by endothelial cells and has been shown to be constitutively shed from the endothelial cell surface by ADAM-10 (286). For leukocyte recruitment, CXCL16 on the endothelium may initially function as an adhesion molecule and as an activation signal for leukocytes expressing CXCR6. The elevated expression and shedding of CXCL16 by synovial monocytes/macrophages and FLS within the underlying tissues in the RA joints could lead to the generation of a chemotactic gradient to direct transmigration of $CXCR6^+$ leukocytes into the underlying tissue. Intriguingly, CXCL16 in its soluble and transmembrane forms could have differing effects on $CXCR6^+$ T cell activity in the context of inflammatory arthritis. The expression of surface-anchored CXCL16 on APCs could influence proinflammatory cytokine polarization of T cells during antigen-presentation and activation within the secondary lymphoid tissues. Furthermore, the enhanced levels of soluble CXCL16 within the inflamed synovium supports my data showing that CXCR6 contributes to the accumulation of activated T cells within the inflamed paws of CIA mice; whereas in its transmembrane form on activated synovial macrophages, elevated CXCL16 levels could provide additional signals to elicit enhanced proinflammatory cytokine production by $CXCR6^+$ T cells that have infiltrated the inflamed synovium. Therefore, a possible feedback loop could contribute to the proinflammatory effects of CXCR6 and CXCL16 in arthritis pathogenesis, since IFN-γ

and TNF synergize to induce CXCL16 mRNA (286), and IFN-γ stimulation enhances the release of soluble CXCL16 (491). Thus, elevated proinflammatory cytokine production by $CXCR6^+$ T cells within arthritic tissues could feedback to promote greater levels of soluble CXCL16 within the inflamed synovium and perpetuate inflammation through recruitment of additional $CXCR6^+$ cells.

Donor $CXCR6^{-/-}$ T cells were impaired in their ability to home to the inflamed paws compared to wild-type T cells. However, despite their reduced numbers, donor $CXCR6^{-/-}$ T cells were still detected within the arthritic paws of recipient mice. This suggests that these cells utilize alternative mechanisms to accumulate within arthritic tissues in the absence of CXCR6. Activated T cells have been shown to upregulate their expression of various chemokine receptors, including CCR5, CCR6 (492), and CXCR3 (493). In many cases, depending on the activating conditions, these chemokine receptors are coordinately expressed on activated T lymphocytes (398). Indeed, multiple studies have demonstrated that T cells isolated from synovial samples of RA patients express both CCR5 and CXCR3 (249,257). Furthermore, elevated levels of the CCR6 ligand MIP-3α/CCL20 correlated with IL-17 levels in human RA synovial fluid (415), suggesting that $CCR6^+$ Th17 cells are also accumulating at this site. Therefore, I examined whether expression of these inflammatory chemokine receptors were upregulated on in vitro activated T cells, and if so, whether there were differences in their expression between activated wild-type and $CXCR6^{-/-}$ T cells. Similar to previous reports, in vitro activation of T lymphocytes via incubation in anti-CD3ε–coated plates did not upregulate the expression of CCR5 (254,494,495) or CCR6 (494,496), but caused potent upregulation of CXCR3 (494) and CXCR6 (279) (Figure 28). The lack of CXCR6 expression on in vitro activated T cells from $CXCR6^{-/-}$ mice did not influence their expression of CCR5, CCR6, or CXCR3 compared to activated wild-type T cells. Therefore, the lack of CXCR6 expression, but not other inflammatory chemokine receptors, on T cells from $CXCR6^{-/-}$ mice was responsible for the selective recruitment of donor $CXCR6^+$ T cells to the inflamed paws of arthritic mice.

4.8 Roles of CCR5, CCR6, and CXCR3 in mediating homing of T cells to joints in inflammatory arthritis

CXCR6$^{-/-}$ mice exhibited reduced incidence and clinical severity of disease compared to wild-type mice. However, CXCR6$^{-/-}$ mice were not completely protected from CIA and reduced but significant accumulation of CD4^{+} T cells was observed in the paws of arthritic CXCR6$^{-/-}$ mice. This suggests that other mechanisms were utilized by these cells to home to inflamed paws. Many studies have investigated the roles of chemokines and chemokine receptors in RA (497,498). T cells expressing the chemokine receptors CCR5, CCR6, CXCR3, CXCR6, and others are present in RA synovial tissues (155,249,250,498,499). Similarly, levels of CC and CXC chemokine ligands, such as MIP-1α/CCL3, MIP-1β/CCL4, RANTES/CCL5, MIP-3α/CCL20, MIG/CXCL9, IP-10/CXCL10, I-TAC/CXCL11, CXCL16, and others are elevated in RA synovial fluid (247,248,500). Indeed, many of these chemokine ligands have been shown to be abundantly produced by RA synovial fibroblasts and macrophages (251–253,269,270,501–503). Therefore, I examined chemokine receptor profiles on T cells isolated from the lymph nodes and paws of arthritic wild-type and CXCR6$^{-/-}$ mice to assess which chemokine receptors were highly expressed in CIA mice. I was also interested in determining whether T cells in the arthritic paws of CXCR6$^{-/-}$ mice exhibited differences in their levels of chemokine receptor expression compared to wild-type mice.

Of the chemokine receptors examined, expression of CCR7 was strongest among lymph node T cell populations (Figure 29), consistent with the requirement for CCR7 in mediating naïve T cell entry into peripheral lymph nodes (504–506). Similar to CXCR6, expression of CCR5, CCR6, CXCR3, and CXCR4 were low on T cell subsets isolated from the lymph nodes of arthritic mice. In contrast, expression of CCR7 was low on T cells isolated from the paws of arthritic mice, whereas expression of CCR5, CCR6, CXCR3, and CXCR4 were higher, as indicated by the greater frequencies and gMFI of chemokine receptor-positive cells (Figure 30). Aside from CXCR6, expression of CXCR3 and CCR5 (albeit to a lesser extent) were most notable on T cells isolated from inflamed paws of CIA mice. In agreement with these findings, the CXCR3 ligand IP-10/CXCL10 is elevated in the joints of arthritic mice (507). Blocking IP-10/CXCL10 with neutralizing

antibody or antagonizing CXCR3 reduced clinical disease severity scores and $CD4^+$ T cell infiltration into the inflamed paws in CIA (271,508), and reduced T cell recruitment to arthritic joints in an adjuvant arthritis model in rats (272). Furthermore, treatment of arthritic animals with a CCR5 peptide-antagonist led to a reduced incidence and severity of disease (509). Yang et al. (510) obtained similar results using a non-peptide antagonist of CCR5 in CIA. In this case, treatment potently inhibited $CCR5^+$ T cell infiltration in the joints of immunized mice, but did not affect induction of anti-collagen T cell responses.

A large population of $CCR6^+$ $CD4^+$ T cells was detected in the arthritic paws of CIA mice. CCR6 is found on various immune cell populations, but CCR6 and the associated chemokine ligand MIP-3α/CCL20 are prominently expressed by Th17 cells (415,511,512). Consistent with the increased frequency and number of IL-17A-producing $CD4^+$ T cells in the paws of arthritic wild-type mice (Figures 12D and 13D), the frequency, absolute number, and intensity of staining for $CCR6^+$ $CD4^+$ T cells were significantly greater in arthritic paws of wild-type mice than in $CXCR6^{-/-}$ mice (Figure 30). Th17 cells have been shown to preferentially migrate in response to MIP-3α/CCL20 produced by synoviocytes, causing the accumulation of IL-17-producing $CD4^+$ T cells in the arthritic joints of mice (415). This may also be an important mechanism for the recruitment of Th17 cells in human disease, since synovial fibroblasts from RA patients have been shown to recruit $CCR6^+$ cells through enhanced MIP-3α/CCL20 production (503). In CIA, CCR6 blockade reduced T cell infiltration into the joints and suppressed the incidence and severity of clinical disease (415).

Compared to wild-type mice, $CCR5^+$ $CD4^+$ and $CD8^+$ T cell frequencies were significantly higher in arthritic paws from $CXCR6^{-/-}$ mice, whereas $CXCR3^+$ T cell frequencies were similarly high in both groups (Figure 30). This suggests that CCR5 and CXCR3 play a role in mediating T cell accumulation within the arthritic paws of CIA mice. Multiple studies have shown that CCR5 is coexpressed with CXCR3 on synovial T cells in patients with inflammatory arthritis (257–260). Expression of CCR5 and CXCR3 are often associated with lymphocyte homing into Th1-type inflammatory sites (257,259,261) and among $CD4^+$ T lymphocytes, both CCR5 and CXCR3 have been suggested to be restricted primarily to Th1 cells (255,261). However, many have

suggested that while coexpression of some chemokine receptors together define populations highly enriched in Th1 versus Th2 cells, the individual expression of Th1– or Th2-associated chemokine receptors shows little or no preference for polarized T cell subsets in vivo (513–515). For example, CXCR3 did not correlate with IFN-γ expression and CCR5 was not expressed by all IFN-γ-producing $CD4^+$ T cells isolated from synovium of RA patients (155). While I did not examine cytokine and chemokine receptor expression together in individual T cells, I did observe reduced IFN-γ^+ T cells despite increased CCR5 expression, and no difference in CXCR3 expression, in the paws of $CXCR6^{-/-}$ mice compared to wild-type mice, suggesting that individual expression of chemokine receptors does not define physiologically relevant subsets of polarized T cells in vivo.

The fact that particular chemokine receptors are often coexpressed on polarized T cell subsets implies that expression of these chemokine receptors may be coregulated. Defects in migration of T-$bet^{-/-}$ T cells are primarily due to loss of CXCR3 expression since T-bet, the master transcription factor that activates transcription of genes important for Th1 and CTL cell function, directly induces CXCR3 (516–518). Moreover, Th1 cytokines such as TNF, IL-12, IFN-γ, and IL-2 have been suggested to cause upregulated mRNA and protein expression of CCR5 (519). This cytokine signal-dependent acquisition of chemokine receptors represents one mechanism through which T helper cell subsets can be selectively recruited to sites of inflammation. However, other mechanisms also play a role in controlling the amount of functional chemokine receptors at the cell surface. These range from complex protein-protein interactions and post-translational modifications, involved in stabilizing receptor conformation and promoting their delivery to the plasma membrane, to signaling events resulting in chemokine receptor desensitization and internalization (520,521). Furthermore, rather than functioning as monomeric units, many chemokine receptors form dimers and higher order homo– and hetero-oligomers. Oligomerization of chemokine receptors can either be constitutive, such as that observed for CXCR1 and CXCR2 homo– and hetero-oligomers, CXCR4 homodimers, and CCR5/CXCR4 hetero-oligomers, or ligand-dependent (e.g. CCR2/CCR5 heterodimers) (522,523). Currently, no evidence exists to suggest that CXCR6 forms either homo– or hetero-oligomers. Cytokine-mediated coexpression of

CXCR6 along with both CCR5 and CCR6 has been reported in human $CD4^+$ and $CD8^+$ T cells cultured in the presence of IL-2 and IL-15 (398). However, this coexpression is unlikely to indicate that CXCR6 associates with either CCR5 or CCR6 on the cell surface, since none of the ligands for CCR5 had any effect on CXCR6 surface expression, despite causing potent down-modulation of surface CCR5 (398).

Nonetheless, I examined surface expression of CCR5, CCR6, and CXCR3 on GFP^{hi} and GFP^{lo} T cells isolated from the arthritic paws of $CXCR6^{-/-}$ mice to see whether the lack of CXCR6 affected expression of these other chemokine receptors. Consistent with the frequencies of $CXCR6^+$ $CD4^+$ and $CD8^+$ T cells isolated from the arthritic paws of wild-type mice (Figure 4A), the majority of $CD4^+$ T cells isolated from the arthritic paws of $CXCR6^{-/-}$ (i.e. $CXCR6^{gfp/gfp}$) mice were GFP^{hi}, while fewer GFP^{hi} $CD8^+$ T cells were detected in these tissues (Figure 31). However, no differences were observed between GFP^{hi} and GFP^{lo} $CD4^+$ and $CD8^+$ T cells in terms of their expression of CCR5, CCR6, or CXCR3, suggesting that induced expression of the CXCR6 gene or the absence of CXCR6 from the cell surface had no impact the expression levels of these other chemokine receptors. Taken together, these data suggest that CXCR6 and CXCL16 may have chemotactic and proinflammatory potency apart from other chemokine receptors expressed in RA (499).

4.9 CXCR3 and CCR5 in inflammatory arthritis pathogenesis

CXCR3 and its ligands have been implicated in the pathogenesis of many autoimmune diseases (524,525). Blocking CXCR3-IP-10/CXCL10 interactions has been shown to control T cell accumulation and/or Th1 polarization and ameliorate disease symptoms in multiple animal models of autoimmunity (508,526–528). A large proportion of T cells isolated from the arthritic paws of CIA mice express CXCR3 (Figure 30). This, in addition to observations that IFN-γ-producing Th1 cells in vivo, and in vitro polarized Th1 cells, are almost always CXCR3 positive (513,529,530), led me to examine the contributions of CXCR3 in CIA. The incidence of arthritis in immunized $CXCR3^{-/-}$ mice was significantly lower than that observed in wild-type mice (Figure 32). However, the mean clinical severity scores of mice that developed clinical signs of arthritis were similar between wild-type and $CXCR3^{-/-}$ mice. These data suggest that mice lacking CXCR3 are

protected from disease induction, but once established, CXCR3 may not play much role in influencing the degree of inflammation within the arthritic joints. In contrast, animals treated with neutralizing antibodies to IP-10/CXCL10 or CXCR3, or a non-peptide CXCR3 antagonist (271,272,508) exhibited attenuated disease severity scores. Furthermore, blocking IP-10/CXCL10 or CXCR3 inhibited leukocyte infiltration into the synovium in CIA mice (271), and in rats with adjuvant arthritis (272). However, it is unclear if treatment had any effect on disease incidence (not reported), whereas mean severity scores were either low or highly variable, suggesting diseased and non-diseased animals may have been grouped together when comparing scores between treated and control animals.

Byrne et al. (531) found that blocking IP-10/CXCL10 with neutralizing antibodies had no effect on mean clinical scores in CIA mice or on disease scores in CAIA. Nonetheless, despite little or no effect on disease severity scores in mice that develop clinical signs of disease, CXCR3-deficiency resulted in attenuated incidence of disease compared to wild-type mice (Figure 32). This may be related to the impact CXCR3-deficiency has on T cell activation and polarization during antigen presentation within the draining lymph nodes. Recently, a requirement for CXCR3 has been demonstrated for optimal generation of IFN-γ-producing Th1 cells in vivo (274). In this study, DC-derived IP-10/CXCL10 facilitated T cell-DC interactions in lymph nodes during T cell priming, and both MIG/CXCL9 and IP-10/CXCL10 guided intranodal positioning of $CD4^+$ T cells to interfollicular and medullary zones (274). While I did not examine lymph node cells from $CXCR3^{-/-}$ mice for their production of IFN-γ following immunization, potentially impaired Th1 cell differentiation and reduced IFN-γ production during the early phase of CIA could represent one mechanism contributing to reduced incidence of CIA in $CXCR3^{-/-}$ mice.

The observation that CXCR3-deficiency did not impact the severity scores in mice that developed CIA was unexpected, given the elevated expression of CXCR3 ligands and the large proportion of $CXCR3^+$ T cells in the inflamed paws of CIA mice (507) (Figure 30). Furthermore, studies have shown that reciprocal regulation occurs between IP-10/CXCL10 and RANKL, resulting in enhanced expression of IP-10/CXCL10 and

RANKL in osteoclast precursors and $CD4^+$ T cells, respectively (271,532). The fact that Th1 cells are frequently $CXCR3^+$, and my observations that IFN-γ^+ and $CXCR3^+$ T cells are present at high frequencies and numbers in the arthritic paws of CIA mice, might imply that CXCR3 could be involved in an feedback loop leading to amplification of inflammation and tissue destruction in the diseased paws. A similar CXCR3-dependent amplification loop has been described in infection models. For example, during malarial infection and herpes simplex virus-2 (HSV-2) infection the entry of $CXCR3^+$ T cells into the infected tissues leads to increased CXCL9 and CXCL10 production in response to IFN-γ produced by the infiltrating cells (268,533). The increased secretion of CXCR3 ligands promotes additional recruitment of $CXCR3^+$ effector cells, which in turn secrete IFN-γ locally and further amplify infiltration of effector cells. As discussed earlier, blocking IP-10/CXCL10 or CXCR3 inhibited leukocyte infiltration into the synovium in animal models of RA. However, a recent study demonstrated that polarized Th1 cells from wild-type and $CXCR3^{-/-}$ mice exhibited similar capacities to accumulate within the arthritic paws of CIA mice (534). The reasons for the discrepancies in experimental results is unclear, but suggests that unlike CXCR6, CXCR3 may not be essential for T cell homing to inflamed paws in CIA.

The similar severity of arthritis in wild-type and $CXCR3^{-/-}$ mice suggests that CXCR3 may also be important in limiting inflammation. For example, CXCR3-deficiency could contribute to impaired T cell-DC cross-talk leading to reduced polarization of IFN-γ-producing T cells and lower IDO expression by DCs. Reduced IDO would relieve suppressive effects on T cell proliferation and survival, but also lead to decreased differentiation of Tregs (535,536). Alternatively, CXCR3-deficiency may have more direct effects on Treg cell suppressive capacity or tissue distribution. While most Tregs lack CXCR3 expression (534,537,538), a subset of Tregs has been identified that express CXCR3, dependent upon IFN-γ-specific signals (539). Consistent with this, IFN-γ derived from iNKT cells has been shown to promote the migration of $CXCR3^+$ Tregs into the inflamed liver (540). Therefore, a lack of CXCR3 expression may limit entry of Tregs into the inflamed paws following arthritis development and could explain the similar severity of disease between wild-type and $CXCR3^{-/-}$ mice.

Compared to $CXCR3^+$ cells, I observed much lower frequencies and numbers of $CCR5^+$ T cells in the arthritic paws of CIA mice (Figure 30). However, the incidence of arthritis in $CCR5^{-/-}$ mice was comparable to the incidence rates I observed in $CXCR3^{-/-}$ mice, both of which were significantly lower than in wild-type mice (Figures 32 and 33). Unlike $CXCR3^{-/-}$ mice, $CCR5^{-/-}$ mice exhibited reduced clinical disease severity scores compared to wild-type mice. This effect of CCR5-deficiency is consistent with previous studies demonstrating that CCR5 antagonists inhibit the development of CIA in mice and adjuvant arthritis in rats (510,541). Moreover, Met-RANTES, a dual CCR1/CCR5 antagonist, inhibited both murine CIA (509) and rat adjuvant arthritis (542). CCR5 antagonists were demonstrated to inhibit inflammatory arthritis by modulating T cell migration without affecting T cell cytokine or proliferative responses (510). Additionally, $CCR5^{-/-}$ mice have been shown to suffer less bone loss in an experimental periodontal disease model through reduced accumulation of pro-osteoclastogenic $CD4^+$ T cells (543). Contrary to these findings, CCR5 has been implicated in cartilage loss but does not affect synovium or bone in a post-traumatic osteoarthritis model (544), suggesting that CCR5 plays different roles in immune-mediated bone loss depending on the experimental conditions.

In contrast to my observations in $CCR5^{-/-}$ mice, a previous study found CIA disease incidence and severity in $CCR5^{-/-}$ mice to be similar to wild-type mice (170). However, a couple of important differences could contribute to these conflicting experimental results. For example, differences in arthritis development may be attributed to differences in mouse strain (i.e. $CCR5^{-/-}$ mice backcrossed onto the CIA susceptible DBA/1 ($H\text{-}2^q$) background in their study versus onto the C57BL/6 ($H\text{-}2^b$) background in ours) and immunization protocol. Quinones et al. (170) report <20% incidence by day 90 post-immunization and peak arthritis scores (at ~day 120 post-immunization) were <4 per mouse for both wild-type and $CCR5^{-/-}$ mice despite using a scoring system identical to the one I used. The pre-clinical data supports an important role for CCR5 in inflammatory arthritis possibly through promoting infiltration and activation of immune cells within the arthritic synovium. However, future studies should confirm this and elucidate the cellular and molecular pathways that are altered in $CCR5^{-/-}$ mice within the context of CIA, including potential effects on CCR5-dependent Treg cell accumulation or suppressive

activity since Tregs with higher CCR5 expression have been shown to be superior at suppressing activated T effector cells (545).

In human disease, CCR5 has been suggested to play an important role in disease pathogenesis due to the accumulation of $CCR5^+$ $CD4^+$ T cells in the synovial tissues of patients with inflammatory arthritis (249,258,259). However, clinical trials with CCR5 antagonists have been disappointing since all antagonists investigated so far have failed to meet the primary outcomes of phase II clinical trials in RA (546–548). In contrast, a phase II clinical trial recently reported beneficial effects of an anti-CXCL10 antibody in RA patients who had an inadequate response to methotrexate (273). Importantly, the pathophysiological relevance of CCR5 in human disease can be examined by studying individuals with the null *CCR5Δ32* allele. *CCR5Δ32* has been famously associated with a key role in HIV infection as homozygous individuals are almost totally resistant to HIV-1 infection (549–552). However, conflicting results have been reported regarding the association of *CCR5Δ32* with RA and other forms of inflammatory arthritis. Some studies have suggested that the allele provides protection against RA (553–555), whereas others have found no association (556,557). These differences are possibly due to confounding variables present between these studies such as the absence of homozygotes in some cohorts (557), differences in ethnicity (558), treatment regimens, and inclusion criteria.

4.10 Expression of chemokine receptors on iNKT cells

Human NKT cells generally express homing receptors for extralymphoid tissues with a majority of these cells expressing CCR2, CCR5, and CXCR3, and differential expression of CCR1, CCR4, CCR6, and CXCR6 on specific subsets (289–291,559). Studies examining mouse NKT cells suggest significant differences in their chemokine receptor profiles and responsiveness to chemotactic ligands compared to human blood NKT cells. For example, a majority of splenic murine NKT cells express the receptors CCR9, CXCR3, and CXCR6, but lack chemotactic responsiveness to ligands for CCR1, CCR2, CCR5, and CCR6 (290,322). Earlier studies were hindered by limited availability of reagents to examine surface expression of chemokine receptors on the surface of mouse leukocytes. Therefore, I evaluated chemokine receptor expression on murine iNKT cells isolated from the spleen and liver (Figure 34). Consistent with previous reports,

large proportions of splenic and hepatic murine iNKT cells expressed CCR9, CXCR3 and CXCR6, but not the chemokine receptors CCR2, CCR3, CCR6, CCR7, or CXCR2, for which iNKT cells were shown to lack chemotactic responsiveness to their ligands (290,322). Subsets of mouse splenic NKT cells have been described to be $CXCR4^+$ and $CXCR5^+$ and were shown to migrate in response to SDF-1/CXCL12 and BCA-1/CXCL13, respectively (290). However, I did not detect significant staining for either CXCR4 or CXCR5 on mouse iNKT cells from the spleen or liver, which could be due to receptor downregulaton/internalization/shedding induced by the manipulations involved in cell isolation, since cells were not permitted to recover prior to staining. A significant proportion of iNKT cells from naïve mice also expressed CCR5, which was dramatically upregulated on iNKT cells following in vivo activation with α-GalCer (Figure 38). Overall, most mouse iNKT cells express a chemokine receptor profile indicative of Th1-type inflammatory cells (160,289–291,416).

I have shown that mice deficient in the chemokine receptors CXCR6, CXCR3, and CCR5 exhibit reduced clinical signs of arthritis compared to wild-type mice. Large proportions of iNKT cells express these receptors, suggesting they play important roles in iNKT cell tissue distribution or function. Moreover, the impact of chemokine receptor-deficiency on CIA that I have discussed earlier could be partly due to effects on iNKT cell populations in these mice. Indeed, iNKT cells in the liver and lungs are depleted in mice lacking CXCR6 or CXCL16 (159,298,299). Invariant NKT cells from the liver of $CXCR6^{-/-}$ mice have been reported to undergo apoptosis more rapidly (299), and express lower levels of the survival factor Bcl-2, suggesting that CXCR6 plays a role in regulating survival of iNKT cells in the liver (560). Previous work in our lab demonstrated an accumulation of iNKT cells in the bone marrow, suggesting an alteration in homing in the absence of CXCR6 (159). However, iNKT cell numbers are normal in most tissues of $CXCR6^{-/-}$ mice, suggesting that CXCR6 is required for specific iNKT cell populations, or for their retention/survival within certain tissue environments (159,298,299). Our lab has also shown that iNKT cells require CXCR6 for normal cytokine production following activation with α-GalCer (159,297). Consistent with a co-stimulatory role for CXCR6-CXCL16 interactions during glycolipid recognition on CD1d-expressing APCs, DCs from $CXCL16^{-/-}$ mice are impaired in their ability to

stimulate IFN-γ production from wild-type iNKT cells (298). Taken together, these studies have highlighted a critical role for CXCR6 in iNKT cell maturation and function in addition to iNKT cell homing and homeostasis.

The expression of T-bet during thymic maturation of iNKT cells induces the expression of CXCR3 and CCR5 (561). CXCR3-deficiency had no impact on the numbers of iNKT cells in the spleen, but the peritoneal cavity of $CXCR3^{-/-}$ mice was nearly devoid of iNKT cells (562), suggesting that CXCR3 is important for the accumulation of iNKT cells within the peritoneum under normal physiological conditions. In addition to promoting iNKT cell accumulation at sites of injury, infection, or disease associated with Th1 cell-responses and high levels of IFN-γ (563), CCR5 plays a regulatory role in iNKT cell survival and function as shown by enhanced IL-4 production and resistance of $CCR5^{-/-}$ hepatic iNKT cells to activation-induced apoptosis during ConA-induced hepatitis (265). These studies describing non-traditional (i.e. independent of leukocyte recruitment) activities of chemokine receptors add to a growing body of literature demonstrating the ability of chemokine receptors to regulate a number of cellular functions. Given that iNKT cells highly expressed CXCR6, CXCR3, and CCR5, and lack of these chemokine receptors influence the susceptibility of mice to CIA, I was interested in evaluating the role of iNKT cells in CIA.

4.11 Invariant NKT cells in inflammatory arthritis

A link between iNKT cells and inflammatory arthritis has been suggested by the observations that patients with RA have reduced iNKT cell numbers and show functional iNKT cell defects (376,379–381). However, much of the evidence for the role of iNKT cells in RA and other diseases comes from studying the impact of in vivo iNKT cell activation using potent agonists such as α-GalCer or the effects of NKT cell-deficiency/blocking NKT cell activation with anti-CD1d antibodies in experimental mouse models. A number of studies have suggested that iNKT cells are able to manage the initiation and perpetuation of arthritic disease in different models of autoimmune joint inflammation (564). Prior to my investigations into the role of iNKT cells in CIA I first determined whether iNKT cell frequencies and/or numbers were altered in the tissues of arthritic mice versus control animals (Figure 35). Consistent with previous studies (387), I

observed an increased frequency and number of iNKT cells in the liver of CIA mice compared to control animals. In addition, I detected an increased frequency of iNKT cells in the spleen, but decreased frequencies of iNKT cells in the lymph nodes of arthritic mice compared to nonimmunized mice. In arthritic animals, absolute numbers of iNKT cells were increased in each of these tissues. Previous reports have shown that iNKT cell frequencies were increased in arthritic mice following K/BxN serum transfer, but were unaltered in the spleen and lymph nodes of CIA mice (387). However, others have shown that absolute numbers of iNKT cells were increased in CIA mice, reflecting increased cellularity of these tissues (389). A number of important differences between these studies and ours may account for differences in iNKT cell frequencies, or their detection. For example, iNKT cell frequency and number, or iNKT cell function, has been reported to vary between certain mouse strains (394,565). In addition, in some of the studies discussed, α-GalCer-loaded CD1d-dimers rather than CD1d-tetramers were used for the detection of iNKT cells. CD1d-tetramers have been shown to have greater staining intensity resulting in a more pronounced shift for positively stained cells and broader reactivity with CD1d-restricted iNKT cells with diverse TCR β-chains (311).

Importantly, I also observed an increased frequency and number of iNKT cells in the paws of arthritic mice compared to nonimmunized mice. However, it is unclear if this represents recruitment of iNKT cells from other sites or expansion of local iNKT cells. Studies in the K/BxN serum transfer model have suggested that iNKT cells infiltrate arthritic joint tissues since levels of Vα14Jα18 TCR mRNA increased following disease induction, and transcripts were detected arthritic joints in $CD1d^{-/-}$ mice that received iNKT cells after K/BxN serum transfer (353,391). In this study by Kim et al. (391) and others (566), no transcripts for Vα14Jα18 TCR mRNA were detected in the joint tissues of naïve mice. However, I detected iNKT cells in paw cell isolates from naïve mice by surface staining for the invariant TCR of iNKT cells (Figure 35), which is consistent with the identification of iNKT cells within the joints of control mice by immuno-histochemistry and confocal microscopy (567). Lee et al. (567) demonstrated that iNKT cells in the normal healthy joints are distributed throughout the extravascular tissue with the majority remaining in close contact with the blood vessels. Thus, the increased frequency and number of iNKT cells in arthritic paws may result from expansion of local

iNKT cells following activation by endogenous lipid ligands and/or elevated levels of proinflammatory cytokines present in the inflamed joint tissues (568–570). Interestingly, FcγRIII/CD16 engagement directly activated iNKT cells in joint tissues during antibody-induced arthritis in the absence of simultaneous TCR stimulation, which was critical for their contribution to disease progression (353). This appears to be a clinically relevant mode of cell activation since FcγRIII expression has been shown to be elevated on RA synovial macrophages and strongly correlated with TNF and MMP-1 expression in response to immune complex stimulation (571). Indeed, arthritis induced with anti-CII IgG antibodies was attenuated in FcγRIII$^{-/-}$ mice (113). Alternatively, another mode of iNKT cell activation could be through CD1d-dependent presentation of endogenous joint-derived antigens. While reports of NKT cell peptide recognition in the context of CD1d are rare, CD1d-dependent NKT cell activation has been described in response to the immunodominant epitope from endogenous mouse CII ($mCII_{707-721}$) (572). The high frequencies of expression of CCR5, CXCR3, and CXCR6 on iNKT cells suggests that these cells could respond rapidly to the production of inflammatory chemokine ligands to exert functions (i.e. cytokine production) in situ or after they home to sites of inflammation. Therefore, future studies should investigate whether iNKT cells actively infiltrate joint tissues in inflammatory arthritis and if so, which homing receptors are most important for their accumulation.

Invariant NKT cells display characteristics and functional phenotypes that classify them as immediate effectors. For example, iNKT cells constitutively express high levels of CD69 and CD44, and engagement of glycolipid in the context of CD1d stimulates the production of effector cytokines, including IL-4 and IFN-γ, within minutes (311,573). Despite their activated phenotype in naïve mice, CD1d-dependent glycolipid activation or activation by other means (i.e. FcγRIII or TLR engagement) results in enhanced expression of CD69 on iNKT cells (351,353,574). Consistent with this, iNKT cells express higher levels of CD69 following microbial infection in both mice and humans (575,576). Therefore, as a measure of iNKT cell activation in CIA mice, I examined the frequency and number of CD69$^+$ iNKT cells in nonimmunized and arthritic mice (Figure 36). Compared to iNKT cells from naïve mice, I observed a significantly greater frequency and number of CD69$^+$ iNKT cells in the tissues from arthritic animals,

including from paw-isolated iNKT cells. In agreement with these findings, iNKT cells isolated from the synovial fluid of arthritis patients have been shown to express elevated levels of CD69 (567), while Jung et al. (389) reported that iNKT cells in CIA mice expressed a slightly higher level of CD69 at day 28, but a significantly increased level of CD69 at day 35 post-immunization. Moreover, iNKT cells have been shown to undergo activation in the early phases of CIA, associated with increased CD69 expression and release of cytokines, including IFN-γ, IL-17A, and IL-4 by 6 days post-immunization (392). Taken together, iNKT cells are increased and display elevated CD69 expression in CIA mice, suggesting that they could play an active role in arthritis development and/or progression.

Again, multiple studies evaluating the role of iNKT cells in inflammatory arthritis have done so using experimental mouse models to examine the effects of NKT cell-deficiency on disease pathogenesis. Similarly, to investigate the role of iNKT cells in CIA, I examined the impact of iNKT cell-deficiency on disease incidence and severity by comparing immunized Jα18$^{-/-}$ mice to age-matched/sex-matched wild-type mice (Figure 37). Consistent with previous studies, I found that iNKT cell-deficient mice were protected from CIA as indicated by reduced incidence and severity of disease compared to wild-type mice (387–390), similar to findings in antibody-induced arthritis models (353,387,391). Furthermore, I found that mice deficient in iNKT cells, in addition to lacking CXCR6 (Jα18$^{-/-}$CXCR6$^{-/-}$), were further protected against CIA compared to CXCR6$^{-/-}$ mice (Figure 37). This suggests that iNKT cells contribute to disease in CXCR6$^{-/-}$ mice despite the altered distribution and function discussed earlier. Interestingly, this intrinsic capacity of NKT cells to modulate CIA was shown using both Jα18$^{-/-}$ mice lacking type I NKT cells and CD1d$^{-/-}$ mice lacking type I and type II NKT cells (387–389), suggesting that the suppressive capacity of type II NKT cells shown in other inflammatory disease models may not be as important in CIA (577–581).

Downstream alterations in lymph node and spleen cell cytokine polarization, CII-specific T cell responses and anti-CII antibody production have been characterized in Jα18$^{-/-}$ mice following CIA development. Some conflicting data arose from these studies, but overall, NKT cell-deficiency was associated with reduced serum levels of anti-CII

IgG or skewed IgG1/IgG2a ratios, reduced IL-17^{+} CD4^{+} T cells, and lower CII-specific IL-17 production, but increased IL-10 (387–390). Jung et al. (389) show that iNKT cells producing IFN-γ were maintained over the course of disease, whereas IL-17-producing iNKT cells steadily increased in frequency and number into later disease stages. Similarly Miellot-Gafsou et al. (392) found that hepatic iNKT cells released IFN-γ, IL-4, and IL-17 early during the course of CIA (day 6 post-immunization). The latter study further supports an early role for NKT cell activation in CIA development by showing that anti-CD1d administered early (days 0, 3, and 6) but not late (days 27-39 post-immunization) delayed onset, reduced disease incidence, and ameliorated clinical and histological severity. In contrast, Chiba et al. (387) demonstrated that anti-CD1d administered bi-weekly from day 21 post-immunization had no impact on disease incidence, but resulted in reduced clinical severity scores, suggesting NKT cell activation also plays an important role later during disease. This result could be related to impaired cross-talk between NKT cells and APCs following anti-CD1d injection, since this treatment resulted in reduced expression of CD40, CD80, and CD86 costimulatory molecules on DCs and macrophages (392), which could lessen T cell activation early and late during disease. Furthermore, the effectiveness of anti-CD1d blocking antibodies suggests that CD1d-dependent activation of NKT cells is important for their disease promoting effects in CIA. Indeed, early activation of NKT cells in this model could be attributed to the use of CFA during immunization since specific activation of NKT cells in response to *M. tuberculosis* components has been demonstrated to be CD1d-dependent, but MyD88– and NOD2-independent (582).

4.12 Reconstitution of iNKT cells in Jα18$^{-/-}$ mice following adoptive transfer

Studies have consistently shown that NKT cell-deficient mice experience reduced incidence and/or severity of arthritis in CIA and antibody-induced models. This suggests that in contrast to other models of autoimmune disease such as EAE (583), iNKT cells play a disease promoting role in inflammatory arthritis. However, results from studies using Jα18$^{-/-}$ mice may be confounded by the fact that these mice have an estimated 60% reduction in TCR α-chain repertoire diversity compared to wild-type mice (397). Therefore, I was interested in establishing groups of Jα18$^{-/-}$ that were reconstituted with

sorted iNKT cells prior to immunization for CIA. The rationale for this approach is two-fold. First, if iNKT cell reconstitution restored CIA incidence and severity in Jα18$^{-/-}$ mice to wild-type levels, this would suggest that the lack of iNKT cells is responsible for protection against CIA, not the reduced TCRα repertoire diversity in these mice. Second, this approach provides a means of assessing which iNKT cell-derived effectors contribute to their disease promoting role in CIA.

Invariant NKT cells are a rare population of immune cells in the blood and other tissues but are enriched among liver lymphocytes (310,311,316,317). However, using naïve mice as a source of donor iNKT cells proved to be an obstacle, since this approach provided low numbers of sorted iNKT cells. Thus, prior to cell sorting, I expanded iNKT cells in vivo via delivery of α-GalCer-loaded BMDCs (Figure 38). Delivery of free α-GalCer or glycolipid-loaded BMDCs each result in potent iNKT cell activation as indicated by rapid cytokine production and proliferation, which results in expanded iNKT cell populations that reach a peak around 72 h after stimulation in vivo (383). However, unlike free glycolipid, iNKT cell activation with α-GalCer-loaded BMDCs can prevent long-term iNKT cell unresponsiveness or anergy in mice (382,383), which was important considering I was interested in assessing the functional capacity of these donor iNKT cells in terms of their ability to influence the development of CIA. Furthermore, the chemokine receptor profile of glycolipid activated iNKT cells was similar to that of iNKT cells from unstimulated mice in that both strongly expressed CXCR3, CXCR6, and CCR9, except activated iNKT cells further increased their expression of CCR5 over that observed on unstimulated cells (Figures 34 and 38). The largely unaltered chemokine receptor profile suggests that tissue homing of expanded iNKT cells should resemble that of iNKT cells from unstimulated mice. This expansion approach dramatically increased the number of iNKT cells sorted per experiment and was employed for all subsequent transfer experiments (sorted populations are summarized in Tables 4-7).

One strategy for reconstitution of iNKT cells in Jα18$^{-/-}$ mice is through sublethal irradiation prior to adoptive transfer of sorted donor cells, which our lab has shown results in stable long-term maintenance of transferred iNKT cells in these mice (418). Adoptively transferred iNKT cells had not previously been shown to be maintained over

time in Jα18$^{-/-}$ mice, but irradiation of recipient mice likely provides a niche for iNKT cells by transiently depleting NK cells and other lymphocyte populations that compete for survival and expansion factors such as IL-15 (584–586). Previously, sublethal irradiation of recipients has been shown to ensure donor T cells establish as a significant population in lymphoid organs and other peripheral tissues (267). While this approach did provide a means of reconstituting iNKT cells in Jα18$^{-/-}$ mice, it was not a viable option for evaluating the role of iNKT cells in CIA due to the suppressive effects irradiation has on CIA (Figure 39). Indeed, irradiation abated CIA incidence rates not only in Jα18$^{-/-}$ iNKT cell-recipient mice but also in control Jα18$^{-/-}$ and wild-type mice. Similarly, previous studies have demonstrated a therapeutic effect for low-dose irradiation, since it was shown to attenuate disease incidence and severity in CIA (587–589) and EAE (590). The mechanisms involved in this protection were suppression of proinflammatory cytokine production, including IFN-γ, IL-17, IL-6, TNF, and reduced levels of autoantibodies, which were associated with an increased proportion and suppressive activity of CD4^{+}CD25^{+}FoxP3^{+} Tregs (587–589,591,592). Immune cells are among the most highly radiosensitive cells in the body and the effects of ionizing radiation on the immune system are widespread (593,594). However, lymphocyte subsets differ in their radiation sensitivities. For example, even lethal irradiation, for bone marrow preconditioning, does not eliminate all lymphocytes equally (595), with Tregs being among the most radioresistant (596). Indeed, increased proportions of Tregs have been reported to be maintained for up to 8 weeks following irradiation (588). Taken together, this suggests that a major mechanism of attenuation of CIA by irradiation is through enhanced Treg numbers and suppressive activity, which causes suppressed IFN-γ and IL-17 production, two mediators that are critical for the development and progression of CIA.

The advantage of irradiation prior to adoptive transfer is that it permits long-term reconstitution of donor iNKT cells, but unfortunately it also had a potent suppressive effect on CIA. Based on previous studies demonstrating that iNKT cells are activated in CIA mice during the early phase and that administration of anti-CD1d antibodies early post-immunization had a potent attenuating effect on CIA (387,392), I hypothesized that iNKT cells play an important role during CIA induction. Therefore, I developed an adoptive transfer strategy that involved two separate deliveries of sorted iNKT cells per

recipient, one 14 days prior to the primary immunization and a second on day 7 post-immunization. I speculated that two spatially separate transfers would ensure that donor iNKT cells were present around the time of primary and booster immunizations to become activated and influence the generation and polarization of CII-specific lymphocyte responses. I waited 14 days between iNKT cell transfers and recipient mouse CIA immunization to provide time for donor iNKT cells to establish within recipient tissues and rest after the manipulations involved in expansion and reconstitution.

Using this adoptive transfer strategy, I discovered that delivery of sorted wild-type iNKT cells into Jα18$^{-/-}$ mice resulted in greater CIA incidence rates and exacerbated disease severity, which were comparable to the levels observed in wild-type CIA mice (Figure 40). Furthermore, staining for CD1d-tetramer^{+} cells in the tissues of Jα18$^{-/-}$ recipients revealed effective reconstitution of donor iNKT cells for up to 50 days post-immunization, whereas after 80 days, the frequency of CD1d-tetramer^{+} cells decreased. It is unclear from these endpoint analyses whether donor iNKT cell populations are similarly maintained in all recipient mice and are gradually lost over time, or if the differences in frequency of CD1d-tetramer^{+} cells represents variable iNKT cell reconstitution efficiency between recipient mice. Nonetheless, two spatially separate iNKT cell transfers prior to and during early CIA induction phases ensured populations of iNKT cells were present to influence other immune cells following immunization.

These findings directly demonstrate the disease promoting role of iNKT cells and suggests that iNKT cell-deficiency is responsible for protection of Jα18$^{-/-}$ mice in CIA rather than the altered TCRα profile reported in these mice (397). Indeed, multiple studies have demonstrated that T cell proliferative responses to CII did not differ between Jα18$^{-/-}$ and wild-type mice (387,388,390). Furthermore, T cell cytokine responses to CII were similar for IFN-γ, but cells from Jα18$^{-/-}$ mice exhibited increased IL-10 and lower IL-17 production upon CII restimulation (387,388,390). Therefore, T cells from Jα18$^{-/-}$ mice appear to exhibit no intrinsic defects in their antigen-specific responses in CIA. However, the altered cytokine profiles upon T cell restimulation with CII suggests that the lack of iNKT cells influences early T cell polarization following immunizarion which translated to reduced IL-17-producing cells later in disease (389,390). Importantly, these

experimental findings that iNKT cell-deficiency, and not the reduced TCR α-chain repertoire, contributes to protection from severe CIA can be confirmed in a recently described Jα18$^{-/-}$ mouse strain that lacks iNKT cells while maintaining an essentially complete TCR α-chain repertoire, created by deletion of the neomycin-resistance cassette along with the Jα18 gene segment (597). In contrast to Jα18$^{-/-}$ mice, adoptive transfer of wild-type iNKT cells into Jα18$^{-/-}$CXCR6$^{-/-}$ did not restore arthritis incidence and severity to levels observed in wild-type mice (Figure 43). However, iNKT cell reconstitution in these mice did increase clinical signs of arthritis to levels similar to CXCR6$^{-/-}$ mice. This further supports an important role for CXCR6^{+} T cells in disease pathogenesis and suggests that T cells and iNKT cells act synergistically to enhance inflammation in CIA.

4.13 Influence of iNKT cell-derived cytokines on CIA

Again the power of investigating the role of iNKT cells using a reconstitution strategy stems from the ability to examine which iNKT cell-derived effectors are responsible for their effects in a particular disease model. The fact that adoptive transfer of sorted wild-type iNKT cells had such a clear and potent effect of restoring the reduced incidence and severity of CIA in Jα18$^{-/-}$ mice was advantageous since it also served as a good benchmark for comparison of the effect of transferring iNKT cells from cytokine-deficient mice. Upon activation, iNKT cells rapidly produce a wide range of cytokines including IFN-γ, TNF, IL-2, IL-4, IL-10, IL-13, IL-17, IL-21, IL-22, and GM-CSF (160,297,341,355,598). However, the cytokine profile is influenced by the nature of the stimulation and the subset of iNKT cells that are activated. In CIA, iNKT cells have been shown to produce elevated levels of IFN-γ, IL-4, and IL-17 early during the course of CIA (392) and IFN-γ-producing iNKT cells are maintained throughout disease (389). In RA patients, peripheral blood iNKT cells are biased towards a Th1-like cytokine profile, with markedly increased IFN-γ^{+} iNKT cells and decreased IL-4^{+} iNKT cells (379). IFN-γ is a key regulator of disease pathogenesis in inflammatory arthritis, so I evaluated the contribution of iNKT cell-derived IFN-γ to the development of CIA.

Adoptive transfer of IFN-γ$^{-/-}$ iNKT cells had a profound protective effect on CIA in Jα18$^{-/-}$ mice, since none of the recipient mice developed clinical signs of arthritis (Figure 41). Similar to reconstitution of Jα18$^{-/-}$ mice with wild-type iNKT cells,

reconstitution efficiency at day 80 was variable after adoptive transfer of IFN-$\gamma^{-/-}$ iNKT cells. However, the frequency of CD1d-tetramer^{+} cells in Jα18$^{-/-}$ recipient mice was close to wild-type levels in some recipients. Therefore, the difference in clinical disease after transfer of wild-type versus IFN-$\gamma^{-/-}$ iNKT cells cannot be attributed to differences in maintenance of donor iNKT cell populations. Invariant NKT cells contain preformed transcripts for a number of immunoregulatory cytokines, including IFN-γ and IL-4, allowing them to rapidly generate and secrete high levels of these cytokines following activation (311,382,385,599). The absence of IFN-γ from donor iNKT cells would be a limiting factor in their ability to restore clinical signs of arthritis in Jα18$^{-/-}$ recipients through many of the mechanisms discussed earlier. Moreover, the protective effects of adoptive transfer of iNKT cells lacking the ability to produce IFN-γ could be explained by their intact ability to produce IL-4 following activation.

Many have speculated that altering the cytokine balance in favor of a Th2 response would provide clinical benefit in 'Th1-driven' autoimmune diseases. In animal models of these diseases, the impact of iNKT cell activation has been studied by utilizing distinct glycolipid agonists to induce a Th1 or Th2 bias in iNKT cell cytokine production. For example, OCH, a synthetic analog of α-GalCer with a truncated sphingosine chain, efficiently inhibits EAE and CIA by inducing a Th2 bias in iNKT cell cytokine production (358,393). In CIA, activation of iNKT cells with OCH was associated with enhanced collagen-specific Th2 responses, whereas neutralization of IL-4 or IL-10 abolished protection by OCH (393). In contrast, α-GalCer, which stimulates iNKT cells to produce both Th1 and Th2 cytokines, has shown variable efficacy in its ability to ameliorate disease in CIA (393–395). The differential outcomes upon treatment with α-GalCer are likely attributed to differences in dosage, site, frequency, and timing of glycolipid administration in CIA mice. In line with this, the effect of iNKT cell activation in CIA was shown to be dependent upon the stage of disease, since administration of α-GalCer on day 5 resulted in reduced clinical severity of CIA, whereas a semi-therapeutic administration of glycolipid on day 20 post-immunization had no effect on disease severity but resulted in delayed onset to clinical signs of disease (395). Importantly, combined treatment of mice on day 5 with neutralizing antibodies against IFN-γ resulted in even greater reduction in clinical disease compared with α-GalCer or

anti-IFN-γ alone, whereas neutralization of IFN-γ later at day 20 led to exacerbated clinical signs of disease. This once again highlights the differential biphasic role of IFN-γ in the CIA model and demonstrates that iNKT cell-derived IFN-γ is important for the disease modulating effects of α-GalCer in early versus late disease. Consistent with a regulatory role for IFN-γ-producing iNKT cells later in CIA mice, I found that adoptive transfer of wild-type iNKT cells into $J\alpha18^{-/-}$ mice on day 28 post-immunization conferred protection against the development of disease, as indicated by the lower incidence rates in recipient mice compared to controls (Table 8).

In addition to the effects of iNKT cell-derived cytokines on downstream effector cell activation and polarization, IFN-γ secreted by iNKT cells also regulates the production of anti-inflammatory cytokines by resident cells within the joint. Administering recombinant TGF-β has been shown to suppress joint inflammation in mice (391). Invariant NKT cell-derived IFN-γ has been shown to suppress TGF-β production by synovial cells and cause exacerbated joint swelling in the K/BxN serum transfer model, which was reversed when IFN-γ was blocked (391). A similar mechanism has been reported in a model of pulmonary fibrosis, in which IFN-γ-producing iNKT cells played an anti-fibrotic role by regulating TGF-β production (600). Taken together, these findings implicate iNKT cell-derived IFN-γ as a key effector in the ability of iNKT cells to promote the development of CIA. It is plausible that adoptive transfer of IFN-γ-deficient iNKT cells provides protection against CIA in recipient mice through a combination of the mechanisms discussed here.

TNF is a major contributor to inflammation and joint destruction as evidenced by the spontaneous arthritis that develops in TNF transgenic mice (601), and the success of TNF antagonists in the treatment of patients with RA and other forms of inflammatory arthritis (602,603). Moreover, TNF blockade reduced arthritis severity in CIA mice, which was associated with reduced accumulation of Th1 and Th17 cells in the inflamed joint, despite their increased numbers in the draining lymph nodes upon anti-TNF antibody treatment (604). Invariant NKT cells rapidly produce high levels of TNF upon stimulation, as indicated by the large proportion of TNF^{+} cells and the elevated serum levels of TNF within 2 h of in vivo administration of α-GalCer (297,395,605). Therefore,

I evaluated the contribution of iNKT cell-derived TNF to the development of CIA. Unlike the opposing effects of reconstitution with wild-type and IFN-$\gamma^{-/-}$ iNKT cells, adoptive transfers of $TNF^{-/-}$ iNKT cells into $J\alpha18^{-/-}$ mice had no effect on disease incidence or clinical severity scores (Figure 42). This suggests that TNF production by iNKT cells is critical for their disease promoting effects in wild-type CIA. It is possible that iNKT cell-derived TNF is important for CIA pathogenesis through the activation of other cells and subsequent secretion of proinflammatory cytokines. Consistent with this, iNKT cell-derived TNF has been shown to be required for optimal NLRP3 inflammasome activity and IL-1β and IL-18 production by myeloid cells (605). Furthermore, in vivo activation of iNKT cells has been demonstrated to enhance osteoclast development and increase resorptive activity of mature osteoclasts, which was dependent upon iNKT cell-derived TNF (606). Therefore, these represent possible mechanisms by which iNKT cells may control early inflammation, which is lacking upon reconstitution with $TNF^{-/-}$ iNKT cells.

4.14 Clinical implications

The American College of Rheumatology (ACR) recently published an update to their recommendations for the use of DMARDs and biologic agents in the treatment of RA (607). The panel recommends the use of an anti-TNF biologic, with or without methotrexate in combination therapy, in patients with early RA and those with established RA who have high disease activity with poor prognostic features (including functional limitation, bone erosion, seropositivity for RF or ACPA, and extraarticular disease). However, not all patients exhibit positive clinical responses to these interventions and switching to another anti-TNF biologic or a non-TNF biologic is recommended in cases where disease activity remains high due to a lack of benefit or loss of benefit. Moreover, switching among biologic agents may be necessary in cases where failure is due to adverse events associated with the use of a particular biologic. Importantly, some biologics may not be safe for use in RA patients with chronic infection or malignancies. In their report, the ACR reviewed 9 biologic agents that have been frequently used or have shown clinical benefit in treating patients with RA: the TNF inhibitors adalimumab, infliximab, golimumab, etanercept, and certolizumab pegol, the IL-1 receptor antagonist anakinra, the IL-6 receptor inhibitor tocilizumab, the anti-CD20 monoclonal antibody

rituximab, and the CTLA-4 fusion protein abatacept. Despite these options available to clinicians and patients, newer therapies with improved safety and efficacy are needed to provide better outcomes for RA patients as well as to reduce health care costs incurred through managing symptoms associated with this chronic systemic autoimmune disease.

Chemokines and their receptors are attractive therapeutic targets in inflammatory disease and cancer, and chemokine receptor targeting has been successfully developed for clinical use for stem cell mobilization (the CXCR4 antagonist plerixafor) and for the treatment of HIV infection (the CCR5 antagonists maraviroc and vicriviroc) or T cell leukemia/lymphoma (the anti-CCR4 antibody mogamulizumab). Results of clinical trials in RA patients using drugs designed to target CCR2, CCR5, and CCL2 have been largely disappointing despite promising preclinical studies. However, recent and ongoing clinical trials have shown promise for novel therapies targeting CCR1 and CXCL10 (273,608–610). Previous studies have linked CXCL16 and CXCR6 to inflammatory arthritis by demonstrating high ligand levels within synovial tissues and enriched receptor expression on synovial T cells in human patients with RA, JIA, and psoriatic arthritis (251–253,277,278). Interestingly, RA patients responding to anti-TNF therapy, but not non-responders, showed dramatically decreased CXCL16 levels in serum and synovial fluid (611), and reduced CXCL16 expression on cells within the synovial lining and sublining, consistent with reports that TNF upregulates CXCL16 protein expression by monocytes/macrophages, RA FLS, and endothelial cells (251,252,286). This suggests that the clinical benefit or success of TNF targeted therapies may be partly dependent upon interfering with CXCL16-mediated inflammation in RA. I have extended the findings that CXCL16 and CXCR6 contribute to inflammatory arthritis by demonstrating that $CXCL16^{-/-}$ and $CXCR6^{-/-}$ mice are protected from CIA and have provided evidence showing that CXCR6 is important for T cell polarization and proinflammatory cytokine production and activated T cell homing to the inflamed paws in arthritic mice. Therefore, together with earlier findings that treatment with anti-CXCL16 antibody reduced clinical disease and led to milder histologic changes in the joints of CIA mice (251), the pre-clinical data suggests that blocking CXCR6-CXCL16 interactions could be of clinical benefit to patients with RA.

In addition to modulating T cell proinflammatory cytokine polarization and accumulation of activated T cells within the arthritic tissues, targeting CXCR6 and CXCL16 therapeutically may also contribute to protection against autoimmune arthritis or other inflammatory diseases through other mechanisms. For example, CXCL16 can trigger activation and degranulation of platelets through CXCR6, and this interaction has been shown to enhance platelet adhesion along the vascular wall (612). Accumulation of platelets along the $CXCL16^{+}$ endothelial lining of inflamed synovial tissues could potentiate inflammation through the release of cytokines, chemokines and other inflammatory mediators (613). Furthermore, CXCL16 has been shown to mediate angiogenesis in the K/BxN serum transfer model (614), thus blocking CXCL16-CXCR6 interactions may regulate blood vessel formation in the RA joint. Activated neutrophils play an important role in the pathogenesis of RA and are found in large numbers within RA synovial fluid and tissues (615). CXCL16 has been demonstrated to act as a significant mediator of neutrophil recruitment to acutely inflamed synovial tissues in a model of gout (616). Expression of CXCL16 is dysregulated in multiple inflammatory conditions, including RA (251–253), psoriasis (617), Crohn's disease (300), atherosclerosis (618), and differing roles for CXCR6 and CXCL16 have been demonstrated in cancer depending on the tumor type (619). However, despite the evidence linking CXCR6 and CXCL16 to a number of diseases and the strong support for the role of CXCR6 and CXCL16 in inflammatory arthritis pathogenesis, there are no published data on the development of CXCR6 antagonists or neutralizing reagents for clinical use. While anti-TNF therapies are generally well tolerated, serious adverse events that may relate to TNF antagonists include increased risk of infections and tumor development. Targeting CXCL16 or CXCR6 could potentially provide clinical benefit to patients while limiting possible complications associated with currently approved therapies.

NKT cells help orchestrate both proinflammatory and regulatory immune responses and are important players in immunity against microbial infection and cancers, as well as in allergic responses and autoimmunity. In some cases iNKT cell-based therapies have shown promise in treating patients in a clinical setting. For example, administration of α-GalCer-loaded APCs and activated iNKT cells has shown clinical

benefit in patients with advanced head and neck carcinoma and non-small cell lung cancer (620–622). In these studies, the direct clinical benefits achieved by stimulating iNKT cells was due to their potent cytokine production and ability to act as cellular adjuvants through activation of other immune cells. Alternatively, iNKT cells could be indirectly exploited in the diagnosis and/or treatment of a range of diseases through monitoring iNKT cell characteristics as biomarkers to differentiate individual patients with potentially variable prognoses or responses to therapy. Many human diseases are associated with deficiencies in circulating iNKT cells with or without associated functional defects. Together with evidence from pre-clinical studies in mice, this suggests that stimulating or adoptively transferring iNKT cells to correct defects within the iNKT cell pool or to harness their regulatory or agonistic effects could provide direct clinical benefit. Clinical trials have established that α-GalCer is safe in humans, but there are several important considerations that may preclude the use of iNKT-cell based therapies clinically.

The translation of pre-clinical findings and therapies into the clinical setting has been largely disappointing, perhaps due to the large discrepancy in iNKT cell numbers between humans and mice. Indeed, iNKT cell frequency in most healthy humans is often tenfold lower than that of wild-type mice (623). As well, the types and quantities of cytokines released by iNKT cells may differ between mice and humans (160,327,361,559). Our understanding of the roles of iNKT cells in a particular disease is complicated by conflicting reports on iNKT cell frequency or function in observational studies in patient groups versus those reported in experimental models. For example, RA patients have consistently reduced numbers of circulating iNKT cells and limited iNKT cell reactivity towards α-GalCer stimulation in vitro (375–379). Furthermore, treatment with RA-approved therapies resulted in increased iNKT cell frequency in RA patients (380,381). These findings suggest that restoring normal iNKT cell frequency through adoptive transfer may be of benefit in RA patients. In contrast, results from my studies and others have shown that iNKT cells are increased in mice with active arthritic disease and play a role in disease pathogenesis through proinflammatory cytokine production (387,391). These differences may be explained by a number of factors including circulating iNKT cell populations being a poor representation of tissue iNKT cells in

humans, discrepancies between humans and mice with respect to iNKT cell subset frequencies and/or functions, and exaggeration of the effects of iNKT cells in experimental models through the use of potent iNKT cell agonists such as α-GalCer.

Several points should be kept in mind when considering whether interventions targeting iNKT cells, such as delivery of α-GalCer or its derivatives, would be safe or effective in treating patients with RA. Most importantly, future studies should continue to examine the association of abnormal iNKT cell characteristics (i.e. altered frequency or function and subset ratios) in RA and the impact of approved therapies on iNKT cells in order to determine whether iNKT cell defects are a cause or consequence of the disease process in RA patients. Interpretation of these iNKT cell defects in RA are challenging since most studies have only examined patients on a single occasion after disease onset, which combined with the high variability of iNKT cell frequency in the blood of healthy individuals (323,326–328), makes it difficult to categorize individuals as iNKT cell deficient. If utilized in a clinical setting, a failure of iNKT cell-based therapies in RA patients could be considered as evidence to suggest that iNKT cells do not regulate the disease process. However, reports have shown that iNKT cells from RA patients exhibit variable responses following in vitro α-GalCer stimulation (376,379,381). The identification of non-responders to α-GalCer suggests that this approach may not be suitable for all RA patients. Alternatively, it could imply that iNKT cell-targeted therapies must also involve adoptive transfer of iNKT cells that have been expanded or primed in vitro. Finally, it could be worthwhile to explore the potential of combination therapy approaches that involve iNKT cells, α-GalCer, and conventional treatments. Importantly, unresponsiveness to α-GalCer may be an indication of anergy within iNKT cells from RA patients. Therefore, it is unclear whether overcoming this defect would be of benefit or harm in human inflammatory arthritis. There is convincing evidence that iNKT cells are important in disease pathogenesis in murine models of inflammatory arthritis, which combined with observational findings in RA patient groups, strongly supports further investigation of the applicability of iNKT cell-targeted therapies in human disease.

4.15 Future directions

Modulating leukocyte homing through specific drugs or molecules targeting chemokines and their receptors is a strategy with obvious therapeutic potential in RA and other inflammatory diseases. I have shown that mice deficient in CXCR6 are protected from CIA, associated with reduced T cell proinflammatory cytokine polarization and impaired homing of activated T cells to the inflamed paws in arthritic mice. Demonstrating that in vitro activated T cells or donor T cells isolated from wild-type arthritic paws exhibit no preferential recruitment (compared to donor $CXCR6^{-/-}$ T cells) to the inflamed paws of arthritic $CXCL16^{-/-}$ mice would confirm that CXCR6 expression is responsible for the enhanced recruitment that I observed upon transfer to arthritic wild-type mice. I show that wild-type and $CXCR6^{-/-}$ T cells exhibit no differences in their expression of CXCR3 or other inflammatory chemokine receptors. However, given the reports demonstrating that CXCR6 is coordinately regulated with other chemokine receptors and the complex interactions involved in chemokine receptor trafficking to the plasma membrane, formation for hetero-oligomers, regulation of signal transduction, and internalization, it will be important to show that $CXCR6^{-/-}$ T cells do not have intrinsic defects in their chemotactic responsiveness towards other ligands either in vitro or in vivo. Furthermore, the effect of CXCR6-deficiency on other immune cells in CIA should be examined. For example, CXCR6 is expressed on γδ T cells (398), and γδ T cells may represent a proportion of the IL-17-producing T cells in the arthritic joints of CIA mice (624). However, IL-17^{+} γδ T cells were unable to transfer arthritis to naïve mice and were not detected in synovial samples from RA patients so their relevance to disease pathogenesis is unclear. Future studies should also examine $CXCR6^{-/-}$ mice in other inflammatory arthritis models such as the K/BxN serum transfer model, since iNKT cells have been demonstrated to have an important role (353,391,625) and iNKT cell distribution and function are affected by CXCR6-deficiency (159,297). Another interesting model to examine the effect of CXCR6-deficiency would be in the BALB/c mutant SKG strain since they spontaneously develop T cell-mediated autoimmune arthritis dependent upon increased Th17 cell cytokine production and accumulation of these cells in the arthritic joints (415,626).

Our understanding of the role of CXCR6 on T cells and other immune cells within the highly dynamic environment of inflamed synovial tissues would be greatly advanced through the use of intravital microscopy to image cells in real-time in live arthritic animals. Heterozygous CXCR6-GFP knock-in (*Cxcr6*$^{gfp/+}$) mice could be used to visualize joint homing CXCR6^{+} cells to gain information on where within the arthritic joints these cells are accumulating, how they interact with other synovial cells and the surrounding environment, and what effects therapeutic intervention has on the number and behavior of these cells within arthritic tissues. This approach has previously been used to study the distribution and behavior of iNKT cells within liver sinusoids (299,354,627), and within the extravascular tissues of the spleen (628), and joints of mice with *Borrelia*-induced arthritis (567). An immunohistochemical approach in whole-mount and frozen joint sections could be performed alongside intravital microscopy studies to allow specific staining and identification of CXCR6^{+} cell populations and the types of cells (i.e. synovial macrophages, FLS, and osteoclasts) they are interacting with in arthritic tissues (567). Furthermore, results of imaging studies from arthritic *Cxcr6*$^{gfp/+}$ mice could be compared to those from CXCR6-deficient (i.e. *Cxcr6*$^{gfp/gfp}$) mice, or *Cxcr6*$^{gfp/+}$ mice treated with blocking antibodies to CXCL16 or CXCR6 to examine the effects on T cell (and iNKT cell) distribution and function within the arthritic joints in real-time. The combination of these imaging studies with the assessment of bone changes by micro-computed tomography and traditional histological analysis of the synovium would provide a thorough indication of the processes that are affected by the absence of CXCR6 or iNKT cells.

There is a high degree of redundancy among chemokine receptors in terms of their ability to mediate leukocyte localization within the inflamed synovium. Indeed, pre-clinical and clinical data have implicated nearly every chemokine receptor in inflammatory arthritis (498,499,610). Therefore future studies should continue to elucidate the precise effects blocking these receptors has on disease pathogenesis and the underlying mechanisms that are impacted through studies using knockout mice or specific receptor antagonists. Moreover, our lab has been generating mice deficient in both CXCR3$^{-/-}$ and CXCR6$^{-/-}$, which would allow us to assess whether these mice are further protected from the development of CIA and to perform adoptive transfer experiments to

examine whether CXCR3$^{-/-}$CXCR6$^{-/-}$ double-deficient cells exhibit reduced homing capacity to the inflamed paws compared to single knockout donor cells. Investigating the potential additive benefit of antagonizing multiple chemokine receptors in the same system is important since it has been suggested that the poor success rates of therapeutics targeting chemokines or their receptors in the treatment of RA or other autoimmune diseases may be due to chemokine receptor redundancy, among other factors (629). In other words, blocking a single chemokine receptor may fail to provide clinical benefit due to compensation by other non-targeted receptors.

Earlier reports suggested that iNKT cells were absent from normal healthy joints in mice (391). However, the presence of iNKT cells within the healthy joint and arthritic joint has been demonstrated in both mice and humans (379,567). I show that iNKT cell frequency and number are increased in the arthritic paws of CIA mice, but future studies should assess whether this is due to recruitment of iNKT cells from other sites or the activation and expansion of local resident iNKT cells. If iNKT cells are actively recruited to these inflamed tissues it is likely mediated by CXCR6, CXCR3, CCR5, or other chemokine receptors expressed on iNKT cell subsets, which warrants further investigation. Invariant NKT cells have been shown to be activated through FcγRIII (353), and cytokine signaling (625), within the arthritic joints of mice in the K/BxN serum transfer model but whether this represents a common mode of activation for iNKT cells in other inflammatory arthritis models or in RA patients is unclear. Moreover, studies investigating the effects of iNKT cell activation in experimental models of RA using α-GalCer or its derivatives have utilized a systemic activation strategy through intraperitoneal delivery of glycolipid agonists. Since glycolipid activation of iNKT cells has been demonstrated to provide protection from arthritic disease it would be interesting to compare these findings with those obtained following intra-articular glycolipid treatment. A local activation strategy may modulate severity of arthritis without causing anergy in the systemic iNKT cell pool. In addition, earlier studies have reported that derivatives of α-GalCer, such as α-C-GalCer and OCH, have differential efficacy in terms of their disease modifying potential in CIA mice on the basis of evoking differing amounts of Th1 and/or Th2 cytokines upon iNKT cell activation (393–395). This suggests that novel synthetic analogues of α-GalCer that alter this balance could

potentially provide better protection against autoimmune arthritis, as shown more recently following treatment of CIA mice with α-carba-GalCer (396).

I show that adoptive transfer of iNKT cells from wild-type, but not cytokine deficient mice, restores disease incidence and severity in Jα18$^{-/-}$ mice. Future studies should examine the efficiency of iNKT cell reconstitution using this adoptive transfer strategy by monitoring the frequency of CD1d-tetramer^{+} cells over time and comparing the success rates of iNKT cell reconstitution in nonimmunized versus CIA immunized mice. As discussed earlier, *M. tuberculosis* components present in CFA activate NKT cells and this priming following iNKT cell transfer into recipients may be responsible for efficient donor cell reconstitution. If so, this adoptive transfer strategy would be attractive for investigators interested in characterizing the roles of iNKT cell-derived effector molecules in other disease models that rely on CFA during immunization such as EAE (590).

A number of iNKT cell subsets have been described that exhibit distinct phenotypes and functions in terms of their surface marker expression and cytokine profiles. For example, an IL-17-producing iNKT cell subset that are CCR6^{+}CD4^{-}NK1.1^{-} and express RORγτ are enriched in the lymph nodes and skin (194), and a Th2 polarized iNKT cell subset has been shown to preferentially localize within the lungs (630,631). Unique transcriptional programs have been identified for NKT-1, NKT-2, NKT-17, and NKT-10 subsets of iNKT cells within the thymus (194,361–366), analogous to the Th1, Th2, Th17, and IL-10 producing subsets of conventional T cells. Future studies should determine whether these different iNKT cell subsets represent committed lineages of cells with distinct homing receptors or if these subsets exhibit plasticity and adopt various functional roles in vivo in response to micro-environmental cues. It will be important to examine which functional subsets are most important for the pro-inflammatory versus anti-inflammatory effects of NKT cells in autoimmune arthritis and other diseases. Finally, further investigations are required to determine the role of type II NKT cells in inflammatory arthritis, since type II NKT cells occur more frequently relative to type I NKT cells in humans (632).

4.16 Closing remarks

Many studies in RA, other forms of inflammatory arthritis, and experimental models, have provided strong evidence for a role of effector T cells in disease pathogenesis. T cell recruitment, localization, and retention within inflamed tissues driven by chemokine/receptor interactions is critical to their pathogenic effects in autoimmune arthritis. In this study, I evaluated the expression and function of CXCR6 in CIA, a mouse model of inflammatory arthritis. The absence of CXCR6 significantly reduced the incidence and severity of disease compared to that in wild-type mice. Furthermore, I demonstrate that CXCR6 contributes to the pathogenesis of arthritis through regulation of T cell proinflammatory cytokine polarization and accumulation of activated T cells within the inflamed synovium. Therefore, CXCR6 and CXCL16 represent promising targets for the development of novel therapeutics for the treatment of inflammatory arthritis. NKT cells are a specialized subset of T lymphocytes that can be either pathogenic or protective in inflammatory arthritis. I provide evidence for an important role of iNKT cells in promoting CIA in mice. Invariant NKT cells were increased in the tissues of wild-type CIA mice, whereas iNKT cell-deficient Jα18$^{-/-}$ mice were resistant to disease. I extend these findings through adoptive transfer experiments, demonstrating that reconstitution with wild-type iNKT cells, but not IFN-γ– or TNF-deficient iNKT cells, restored arthritis susceptibility in Jα18$^{-/-}$ mice. These results are consistent with studies showing that mice are protected from CIA following treatment with glycolipid agonists that promote a Th2 bias in iNKT cell cytokine production (393). Although I have demonstrated that iNKT cells strongly influence susceptibility to arthritis development and are important for modulating disease severity, NKT cells are a heterogeneous population that have been shown to have complex roles in RA and other autoimmune diseases. Therefore, future studies are needed to better understand how NKT cell-targeted therapies could best exploit their potential regulatory functions as a disease-modifying strategy for the treatment of patients with RA.

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