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The Influence of Cd4+ T Cell Affinity for Self-Antigen on the Development of Inflammatory Arthritis

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The Influence of Cd4+ T Cell Affinity for Self-Antigen on the Development of Inflammatory Arthritis

Abstract
CD4+ T cells make a crucial contribution to the development of inflammatory arthritis both in humans and in mouse models. However, how the affinity with which T cells recognize target antigens might shape disease development and influence treatment modalities is poorly understood. We have examined these phenomena in mouse models of autoimmune arthritis: TS1xHACII and TS1(SW)xHACII mice express influenza hemagglutinin (HA) as a neo-self peptide and co-express transgenic TCRs that have either high affinity (TS1xHACII) or low affinity (TS1(SW)xHACII) for the HA-derived MHCII determinant, S1. Despite extensive deletion of T cells bearing autoreactive TCRs, arthritis spontaneously develops in both strains. In TS1xHACII mice, males and females develop arthritis equally. CD4+ T cells in this setting display high reactivity to the S1 self-antigen and disease is accompanied by high levels of pro-inflammatory cytokines. Arthritis was found to develop by a B cell-independent mechanism in TS1xHACII mice, although it can be suppressed by anti-IL-17 and anti-TNF treatments, the latter which prevented the accumulation of effector CD4+IL-17+ cells in the joints of treated mice. By contrast, arthritis develops with a significant female bias in the context of a more weakly autoreactive CD4+ T cell response in TS1(SW)xHACII mice and disease was accompanied by lower levels of inflammatory cytokines. IL-17 is also required for disease development in this setting as well; B cells, in this case however, were found to play a prominent role in disease pathogenesis. Rather than acting as a source of arthritogenic autoantibodies, B cells appear to serve as APCs to promote the formation of autoreactive CD4+ effector T cells (including Th17 cells). Unlike in TS1xHACII mice, anti-TNF treatment appears to modulate disease severity but was insufficient to ameliorate the onset of disease in TS1(SW)xHACII mice. These studies demonstrate that variations in the CD4+ T cell response to a single target autoantigen can play a prominent role in guiding the pathways to inflammatory arthritis development. These studies may also explain why treatment modalities targeting particular pathways (cytokines vs B cells) can exhibit different efficacies in arthritis patients.

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THE INFLUENCE OF CD4⁺ T CELL AFFINITY FOR SELF-ANTIGEN ON
THE DEVELOPMENT OF INFLAMMATORY ARTHRITIS

Olivia A. Perng

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The anti-mouse CD20 mAb was provided by Biogen Idec.

K/BxN serum was kindly provided by Dr. Laura Mandik-Nayak.
ABSTRACT

THE INFLUENCE OF CD4+ T CELL AFFINITY FOR SELF-ANTIGEN ON THE DEVELOPMENT OF INFLAMMATORY ARTHRITIS

Olivia A. Perng
Andrew J. Caton, Ph.D.

CD4+ T cells make a crucial contribution to the development of inflammatory arthritis both in humans and in mouse models. However, how the affinity with which T cells recognize target antigens might shape disease development and influence treatment modalities is poorly understood. We have examined these phenomena in mouse models of autoimmune arthritis: TS1xHACII and TS1(SW)xHACII mice express influenza hemagglutinin (HA) as a neo-self peptide and co-express transgenic TCRs that have either high affinity (TS1xHACII) or low affinity (TS1(SW)xHACII) for the HA-derived MHCII determinant, S1. Despite extensive deletion of T cells bearing autoreactive TCRs, arthritis spontaneously develops in both strains. In TS1xHACII mice, males and females develop arthritis equally. CD4+ T cells in this setting display high reactivity to the S1 self-antigen and disease is accompanied by high levels of pro-inflammatory cytokines. Arthritis was found to develop by a B cell-independent mechanism in TS1xHACII mice, although it can be suppressed by anti-IL-17 and anti-TNF treatments, the latter which prevented the accumulation of effector CD4+IL-17+ cells in the joints of treated mice. By contrast, arthritis develops with a significant female bias in the context of a more weakly autoreactive CD4+ T cell response in TS1(SW)xHACII mice and disease was accompanied by lower levels of inflammatory cytokines. IL-17 is also
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CCP</td>
<td>cyclic citrullinated peptide</td>
</tr>
<tr>
<td>CFSE</td>
<td>5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester</td>
</tr>
<tr>
<td>CIA</td>
<td>collagen induced arthritis</td>
</tr>
<tr>
<td>CTV</td>
<td>CellTrace Violet (cell proliferation dye)</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>GPI</td>
<td>glucose-6-phosphate isomerase</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
</tr>
<tr>
<td>HA</td>
<td>hemagglutinin</td>
</tr>
<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
</tr>
<tr>
<td>IBD</td>
<td>inflammatory bowel disease</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IL-17</td>
<td>interleukin 17A; IL-17A</td>
</tr>
<tr>
<td>IMDM</td>
<td>Iscove’s modified Dulbecco’s medium</td>
</tr>
<tr>
<td>jdLN</td>
<td>joint-draining lymph node</td>
</tr>
<tr>
<td>JIA</td>
<td>juvenile idiopathic arthritis</td>
</tr>
<tr>
<td>K/BxN</td>
<td>transgenic mouse strain generated by crossing KRN and C57Bl/6xNOD mice; spontaneously develops arthritis driven by CD4⁺ T cell recognition of GPI and production of anti-GPI antibodies</td>
</tr>
<tr>
<td>KRN</td>
<td>transgenic mouse expressing TCR specific for GPI (in context of I-A&lt;sup&gt;q7&lt;/sup&gt;)</td>
</tr>
<tr>
<td>LN</td>
<td>lymph node</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
</tbody>
</table>
MHCII major histocompatibility complex, class II
NOD non-obese diabetic mouse strain
PBS phosphate buffered saline
PCR polymerase chain reaction
PGIA proteoglycan induced arthritis
PMA Phorbol 12-myristate 13-acetate
PR8 influenza virus strain A/PR/8/34
RA rheumatoid arthritis
S1 site 1 determinant of PR8 influenza virus HA
S1(SW) site 1 determinant of SW influenza virus HA
SKG mouse strain described by Sakaguchi and colleagues; spontaneously develops arthritis due to altered negative selection of autoreactive thymocytes
SLE systemic lupus erythematosus
SW influenza virus strain A/SW/33
TCR T cell receptor
TNF tumor necrosis factor, alpha; TNF-α
Treg regulatory T cell (CD4⁺CD25⁺Foxp3⁺)
TS1 transgenic mouse expressing a TCR specific for the S1 determinant of PR8 influenza virus HA
TS1(SW) transgenic mouse expressing a TCR specific for the S1 determinant of SW influenza virus HA, S1(SW) (an analog of S1 peptide)
Chapter 1: Introduction and Overview

1.1 Introduction

The stochastic generation of antigen receptors expressed by cells of the adaptive immune system allows for their ability to mount a targeted response towards diverse pathogens. In generating this vast antigen receptor repertoire, specificities that are self-reactive undoubtedly appear. Dysregulation of the immune system results in the development of human autoimmune diseases. In this chapter, we give a brief overview of the adaptive immune system and the development of autoimmune diseases such as inflammatory arthritis.

1.2 The adaptive immune system

The adaptive immune system evolved to combat an enormous array of pathogens such as viruses, bacteria and parasites. It is the specificity and functional diversity of this arm of the immune system that allows for a tailored anti-pathogen response and is the hallmark of adaptive immunity. This section briefly describes the development and differentiation of cells that comprise the adaptive immune system.
1.2.1 Development of adaptive immune cells

Development of cells of the adaptive immune system begins with a common hematopoietic precursor which then either travels to the thymus and develops into T cells or remains in the bone marrow and develops into B cells (Wu et al. 1991, Spooner et al. 2009). Maturation and differentiation of these precursor cells into T or B cells occur through several steps which involve signaling through their antigen receptors and acquisition of a unique transcriptional profile (Starr et al. 2003, Pelanda and Torres 2012). The antigen receptors on T cells (T cell receptor, TCR) and B cells (B cell receptor, BCR; immunoglobulins, Igs) are produced by gene rearrangement mechanisms. Of note, the BCR of B cells can be secreted from the cell as soluble Igs (also known as antibodies) of the same specificity. Briefly, germ-line sequences of genes that comprise each subunit of the TCR (TCR α- and β-chains; and less common TCR γ- and δ-chains) and BCR (Ig-heavy and Ig-light chains) undergo gene rearrangements in a complex with the RAG 1/2 recombinase enzyme. Although each step of the gene rearrangement process is strictly and sequentially regulated, the combinations resulting from the rearrangement process are random (stochastic). Furthermore, this receptor rearrangement process occurs in each cell. As each cell bears a unique antigen receptor, this generates a pool of lymphocytes with a highly diverse receptor repertoire. Mathematically, for each lymphocyte subset approximately $10^{15}$ different receptor combinations are possible (Davis and Bjorkman 1988).

However, not all combinations are productive; cells that do not make productive rearrangements are not signaled to survive. Moreover, if a cell does generate a
functional receptor, they are subjected to repertoire selection processes. For T cells, they undergo positive and negative selection in the thymus. Thymocytes which cannot receive a signal through their TCR undergo apoptosis (positive selection) and those that are too strongly reactive to self-antigen are deleted (negative selection) (Ashton-Rickardt et al. 1994, Sebzda et al. 1994). Immature B cells that react with self-antigen with high avidity are shuttled down an apoptotic pathway (clonal deletion) whereas those with low avidity reactions are left to survive (Pelanda and Torres 2012). Additionally, B cells can undergo editing of their BCRs following a previous high avidity reaction with self-antigen (receptor editing). The end product is that each cell expresses a unique antigen receptor. Since the process of gene rearrangements used to generate TCRs and BCRs is stochastic, this results in an enormous diversity of receptors with distinct antigenic specificities.

1.2.2 Activation and effector functions of the adaptive immune system

A. T cells

T cells and B cells must receive a signal through their antigen receptors to become activated and capable of mounting an effector response. We will first address activation and differentiation of T cells. With respect to T cells, antigen must be presented in the form of a peptide bound to major histocompatibility complexes (MHC) – either MHC class I for CD8\(^+\) T cells or MHC class II (MHCII) for CD4\(^+\) T cells – to be able to be recognized by a TCR (Zijlstra et al. 1990, Cosgrove et al. 1991). MHC class I is expressed on all nucleated cells and presents peptides which are cytosolically derived.
On the other hand, MHCII expression is typically restricted to a subset of cells termed professional antigen presenting cells (APCs) and presents peptides derived from internally synthesized proteins and external proteins (both self and foreign) that were endocytosed. Cells types that are classified as APCs include dendritic cells (DC), macrophages as well as B cells.

In naïve T cells, intracellular signals converge downstream of the TCR upon interaction with antigens presented by the appropriate MHC, which leads to upregulated transcription and translation of multiple genes and entry of the cell into cell cycle (Smith-Garvin et al. 2009, Zhu and Paul 2010, Wang et al. 2011). Aside from the TCR, secondary signals, through either co-receptors and/or cytokine receptors, can instruct newly activated cells to differentiate into distinct, effector phenotypes. For CD4+ T cells, these signals would promote polarization into various T helper (Th) subsets, each capable of secreting characteristic patterns of cytokines that are tailored for specific subsets of pathogens (Mosmann et al. 1986, Cherwinski et al. 1987, Park et al. 2005). Polarization of CD4+ T cells toward the Th1 lineage has been shown to require IL-12; following TCR and IL-12 signaling, the cell then acquires and expresses the transcription factor T-bet which promotes the production of IFN-γ, the characteristic Th1 cytokine. Th1 responses are generated to effectively combat intracellular bacteria. Th2 cells develop in the presence of IL-4, express the transcription factor GATA-3, and secrete IL-4, IL-5, and IL-13; Th2 responses are generated to combat extracellular pathogens such as parasitic worms. One of the more recent T helper lineages, Th17 cells develop in the presence of TGF-β and IL-6, express the transcription factor RORγt, and secrete IL-17A.
(herein referred to as IL-17), IL-17F, IL-21 and IL-22; Th17 responses are necessary for defense against extracellular bacteria.

CD8⁺ T cells, upon activation, acquire the ability to produce various inflammatory cytokines such as IFN-γ and TNF-α (herein referred to as TNF) as well as perforin and granzyme (both cytolytic proteins, which when released, perforate and induce apoptotic damage in target cells (Sarin et al. 1997). CD8⁺ T cell responses are necessary to combat virus infections. Similar to CD4⁺ T cells, CD8⁺ T cells acquire differentiation programs which direct them to either short-lived effector cell or long-lived memory cell fates (Wherry and Ahmed 2004). When CD8⁺ T cells commit to becoming either short-lived effector or long-lived memory cells has not been agreed upon (Wiesel et al. 2009).

Notably, in addition to the stimulation through their TCR, T cells must receive a secondary signal through co-stimulatory receptors that is delivered by the same cell on which the antigen is presented. If this second co-stimulatory signal is not received, T cells fail to activate and enter into a state called functional anergy in which the cell is rendered inactive and cannot be re-stimulated through the TCR but the addition of IL-2 can reverse this block (Jenkins and Schwartz 1987, Quill and Schwartz 1987). Of note, intravenous injections of high doses of antigen can also induce functional anergy (Oki and Sercarz 1985). It can be reasoned that this mechanism is in place so that T cells can be activated by foreign antigens derived from invading pathogens, which causes upregulation of co-stimulatory ligands that are typically absent or reduced at steady state, rather than self-antigens, and constitute a mechanism of self-tolerance (Liu and Janeway 1992).
B. B cells

We next address the activation of B cells. BCRs, unlike TCRs, recognize three-dimensional structures rather than peptides (LeBien and Tedder 2008, Harwood and Batista 2010). More specifically, the structures/epitopes BCRs recognize include areas of naturally folded proteins as well as polysaccharides. Antigen recognition followed by cross-linking of BCRs on the surface of a B cell, initiates downstream signaling events resulting in their activation (LeBien and Tedder 2008, Harwood and Batista 2010). One of the main functions of B cells is the production of antibodies, which are secreted forms of the membrane bound BCR. Antibodies are useful for immunity against pathogens because they can bind and neutralize certain epitopes, bind to antigen and elicit a complement cascade, or bind to antigen to mark them for phagocytosis (LeBien and Tedder 2008, Schroeder and Cavacini 2010). In order to generate and secrete antibodies, activated B cells follow a step-wise program which directs not only the events to modify the BCR/Ig into one that is of high affinity for an antigen (somatic hypermutation), but this program also directs the Ig to “class switch”, e.g. from IgM to IgG, and directs the differentiation of B cells into a highly specialized subset called plasma cells, whose main purpose is to produce large quantities of antibodies (LeBien and Tedder 2008).

In response to many antigens, B cells require CD4+ T cell “help” in order to differentiate into plasma cells (Parker 1993). This appears to be a regulatory mechanism that needs to be fulfilled in order to license antibody production. Effector CD4+ T cells provide help to B cells through engagement of co-stimulatory receptors and through provision of cytokines. A key factor in this process is that B cells, acting as
APCs, must present the appropriate antigen (which the B cell previously acquired through its BCR and internalized) bound to MHCII on its surface to the TCR on the effector CD4⁺ T helper cell. This event, termed linked recognition, allows for the directed development of an immune response toward a certain pathogen. Of note, the epitope which the BCR recognizes does not need to be the same epitope that the TCR recognizes. However, it is crucial that the TCR epitope is part of the antigen complex in which the BCR recognizes. A further extrapolation of this concept is that a known antibody specificity can link the involvement of CD4⁺ T cells that recognize the same antigen. Of note, B cells can also be activated to produce antibodies in a T cell-independent manner (Mond et al. 1995).

Altogether, CD4⁺ T cells, CD8⁺ T cells and B cells each have unique functions but many times they elaborate their effector functions in concert to eliminate hazardous and infectious pathogens.

C. Foxp3⁺ regulatory T cells

Foxp3⁺ regulatory T cells (Tregs) constitute a unique subset of CD4⁺ T cells. Unlike conventional CD4⁺ T cells (non-Foxp3⁺), this subset mounts an immunosuppressive rather than an immunostimulatory response upon activation. The discovery of this subset and its critical impact on regulating autoimmune responses was following seminal work demonstrating that the autoimmune pathology induced by conventional CD4⁺ T cells (CD4⁺CD25⁻) can be prevented by the co-transfer of a CD4⁺CD25⁺ population (Sakaguchi et al. 1995). Further assessment has indicated that 5-10% of the peripheral CD4⁺ T cell repertoire co-express CD25 (the high affinity IL-2Ra
subunit) and that their suppressive function can be conferred by the expression of the transcription factor Foxp3 (Sakaguchi et al. 1995, Itoh et al. 1999, Fontenot et al. 2003, Hori et al. 2003). Tregs can be generated and selected for in the thymus by high affinity cognate ligands, which does not fit the paradigm for how T cells are typically selected (Jordan et al. 2001). Alternatively, Tregs can be induced from conventional CD4⁺CD25-Foxp3- cells in the periphery by various means (Chen et al. 2003, Coombes et al. 2007); although peripherally induced Tregs may resemble thymically derived Tregs these two populations differ in several respects (transcription profile, TCR repertoire) (Curotto de Lafaille and Lafaille 2009).

Tregs are reported capable of having numerous modes of action some of which include suppression of APC function, production of inhibitory cytokines such as IL-10 and TGF-β, and competition for IL-2 (Sakaguchi et al. 2008, Shevach 2009). This subset plays an important role in modulating and preventing over-exuberant immune responses mounted against pathogen. However, much work has been directed toward elucidating Treg functions (or lack of) in autoimmune settings. Moreover, Tregs are also considered an essential mechanism of peripheral tolerance since people and mice lacking Tregs develop severe autoimmune syndromes (Sakaguchi et al. 2008).

1.3 Inflammatory arthritis in humans

The adaptive immune system, evolved to efficiently control harmful pathogens, can also elicit self-destruction if not properly regulated. Due to how antigen receptors are generated, to various degrees, T and B cells present in the body are self-reactive
and can be capable of inducing autoimmunity. However, as briefly described in the previous section, multiple mechanisms of tolerance induction exist (both central and peripheral) that eliminate or functionally suppress highly self-reactive cells to prevent this from occurring (Palmer 2003, Hogquist et al. 2005, Mueller 2010). Indeed, these mechanisms of tolerance induction have proven capable of controlling autoimmunity in the majority of individuals since only 3 to 5 percent of the population develops autoimmune diseases (Marrack et al. 2001). Actually, it is through the study of autoimmune diseases that has allowed us gain insight into mechanisms of immune regulation as it would be hard to do so in healthy individuals. Many autoimmune diseases are restricted to a certain organs or tissues (organ-specific), however there are some that are classified as “systemic” (Marrack et al. 2001). One of the most prominent manifestations of systemic autoimmune diseases is inflammatory arthritis. The following section addresses the development of inflammatory arthritis in humans.

1.3.1 Characterization of inflammatory arthritis

Inflammatory arthritis is a chronic, debilitating syndrome that arises in several autoimmune disorders, including rheumatoid arthritis (RA), in which it is the most prominent manifestation (McInnes and Schett 2011). Other settings in which inflammatory arthritis appears are: psoriatic arthritis, juvenile idiopathic arthritis, ankylosing spondylitis (AS), systemic lupus erythematosus (SLE), and inflammatory bowel disease (IBD) (Gelber et al. 2010). Inflammatory arthritis by definition is the inflammation of the joints, which is more specifically characterized by hyperplasia of the synovial membranes in the joints and extensive infiltration of a variety of immune cells
including neutrophils, macrophages, T cells and B cells (McInnes and Schett 2011).
Later, more advanced stages of the syndrome, are characterized by the formation of pannus and the invasion of the synovial fibroblasts and macrophages into the cartilage which leads to the degradation of the cartilage and bone. Settings in which inflammatory arthritis presents are also associated with several systemic features including elevated levels of pro-inflammatory cytokines, such as IL-1, TNF and IL-6, and the presence of autoantibodies, such as rheumatoid factor (RF), antibodies against citrullinated peptides (anti-CCP) and antibodies against double stranded DNA (anti-dsDNA), which can be detected in the serum (McInnes and Schett 2011). The contribution of these factors to disease development will be discussed in section 1.3.3.

1.3.2 Etiology and susceptibility

The etiology of inflammatory arthritis is largely unknown. However, strong associations can be made with certain genetic and environmental factors.

A role for a genetic disposition to inflammatory arthritis was initially proposed following the observation that disease tends to occur more often in genetically related family members compared to the general populace (Lipsky 1998). Furthermore, disease association studies revealed that disease concordance in monozygotic twins was ~15% compared to ~2-5% in dizygotic twins, which was not more so than the rates between family members (Firestein 2009). Moreover, it was discovered that susceptibility to inflammatory arthritis is strongly linked to particular MHCII alleles since patients with rheumatic diseases bear certain MHCII alleles more often than healthy individuals.
These alleles are: HLA-DR4 (DRB1*401, *404, *101) in RA, HLA-DR2 (DRB1*1501) and HLA-DR3 (DRB1*0301) in SLE, and HLA-B27 in AS (Clarke and Vyse 2009).

Because there is incomplete disease concordance between monozygotic twins, it has been suggested that non-heritable factors (such as environmental agents and/or stochastic events) can also contribute to the etiology of inflammatory arthritis. Some of these candidate environmental agents include pathogens (Epstein-Barr virus, mycoplasma, retroviruses) and tobacco (Firestein 2009). Several mechanisms have been proposed to explain how these factors can initiate an autoimmune disease. Probably the most accepted explanation is that these factors trigger an overt, non-specific innate immune response which then allows for the bystander activation of autoreactive T and B cells (Fujinami et al. 2006). Another explanation is that these factors contain antigenic sites that have high homology to self-antigens, thereby activating self-reactive T cells through “molecular mimicry” (Oldstone 1998), possibly because the antigens in this case are presented by certain MHCs, such as those linked to disease susceptibility. An alternative reason for the incomplete concordance observed between twins is that stochastic events, such as those that generate TCR repertoires, can influence susceptibility. This explanation would not preclude the possibility of molecular mimicry. Variations in TCR repertoire formation is by far the hardest correlation to prove in humans due to the vast repertoire each individual possesses. However, this issue can begin to be addressed in mice (see Sections 1.4 and 1.5).
Although the etiology of inflammatory arthritis remains unclear, several lines of evidence, especially the genetic associations with disease susceptibility, implicate a central role for CD4+ T cells in disease development.

### 1.3.3 Disease pathogenesis

The involvement of multiple cellular subsets and soluble factors has been demonstrated in the development of inflammatory arthritis. The complex networks that form, contrived of these cells and factors, has made elucidating the pathways of pathogenesis in arthritis difficult yet intriguing (McInnes and Schett 2011). We examine some of the cells and factors implicated in disease pathogenesis here and explore how they can direct joint destruction.

#### A. Inflammatory cytokines

A key feature of autoimmune arthritis is the highly inflammatory nature of the disease, as compared to osteoarthritis (also a joint disease but believed to be caused by mechanical stress rather than an autoimmune reaction) (Farahat et al. 1993, Hampel et al. 2013). Not only are high levels of multiple inflammatory cytokines found directly in the inflamed joint, but elevated levels of cytokines can also be found in the serum, hence the “systemic” nature of the disease (Steiner et al. 1999, Ziolkowska et al. 2000, Feldmann 2002, McInnes and Schett 2011, Metawi et al. 2011). The cytokines that have gained the most attention include: TNF, IL-1, IL-6, and more recently, IL-17. IL-1 can induce proliferation of synovial cells and production of matrix metalloproteinases (MMPs)
that can directly lead to cartilage destruction (Pettipher et al. 1986, van de Loo and van den Berg 1990). Constitutively high levels of IL-6 can be found during active arthritis and correlates with disease severity (Md Yusof and Emery 2013). Although a pleiotropic cytokine, with regard to joint destruction, IL-6 has been demonstrated to promote MMPs and osteoclast formation which leads to bone resorption. A breakthrough in elucidating the pathways of pathogenesis in inflammatory arthritis was due to findings demonstrating that TNF is at the top of a pro-inflammatory cascade that can perpetuate as well as synergize with IL-1 and IL-6 to drive joint destruction (Feldmann 2002). In addition to TNF, IL-1 and IL-6, highly elevated levels of IL-17 have also been discovered in the synovial fluid of arthritis patients (Ziolkowska et al. 2000, Metawi et al. 2011). It has been proposed that IL-17 can synergize with TNF and IL-1 to perpetuate the production of inflammatory cytokines, induce RANKL in osteoblasts (which leads to bone erosion), as well as induce the generation and recruitment of joint-destructive neutrophils (Lubberts et al. 2005, Lubberts 2008). Interestingly, IL-6 and IL-1 appear to be important for the in vitro differentiation of Th17 cells (a distinct lineage of T helper cells that primarily produce IL-17, IL-17F, IL-21 and IL-22) (Park et al. 2005, Bettelli et al. 2006, Mangan et al. 2006).

Thus, these findings demonstrate not only how inflammatory cytokines can lead to a joint-targeted disease but that they can work in interconnecting networks to promote arthritis development.
B. CD4\(^+\) T cells

Due to the strong association between certain MHC Class II alleles and the development of RA, CD4\(^+\) T cells have been implicated to play a central role in the pathogenesis of disease (Clarke and Vyse 2009, McInnes and Schett 2011). Indeed, CD4\(^+\) T cells can be found in inflamed joints of RA patients, bearing an activated phenotype as illustrated by the expression of markers of antigen experience (CD69, CD44, CD62L\(^{lo}\)) and the expression of chemokine receptors (CCR6, CCR5, CXCR3) used to direct the migration of these cells toward their ligands expressed in the joints (Katschke et al. 2001, Firestein 2009). Importantly, joint-infiltrating CD4\(^+\) T cells were found capable of producing inflammatory cytokines such as TNF, IFN-\(\gamma\) and IL-17 (Steiner et al. 1999, Firestein 2009).

C. B cells and autoantibodies

A classic test used for the diagnosis of RA assesses the presence and level of rheumatoid factor (RF) (Firestein 2003, McInnes and Schett 2011). More recently, tests determining the presence and level of anti-cyclic citrullinated peptide (anti-CCP) have been used in conjunction with those for RF (Zendman et al. 2006). RF and anti-CCP are autoantibodies that recognize the Fc portion of self-IgG and self-proteins that have gained a citrullinated amino acid reside, respectively. The presence of these autoantibodies (patients that test positive are categorized as “seropositive”) not only indicates a break in self-tolerance but also implicates the involvement of autoantibodies in disease pathogenesis. A proposed mode of action in which autoantibodies contribute
to disease is that they form immune complexes that deposit in the joints, triggering a joint-destructive complement cascade (Firestein 2003). Moreover, “seropositive” patients tend to develop a more aggressive form of arthritis (Firestein 2009). It is notable that ~20% of patients that present with symptoms of RA do not contain detectable/elevated levels of RF or anti-CCP in their serum (categorized as “seronegative”), suggesting that autoantibodies are not required for arthritis development. Furthermore, the presence of elevated RF titers does not absolutely dictate disease onset, further opposing an essential pathogenic role for autoantibodies in arthritis development.

The extent to which B cells and autoantibodies contribute to disease pathogenesis remains unclear in humans. Nonetheless, in order to generate high affinity autoantibodies, it was likely that CD4+ T cell help was solicited. This would usually happen in the spleen or LNs, but histological analysis of joint biopsies from some arthritis patients show follicle-like structures where aggregates of B cells, T cells and follicular DC can be found and appear to function as ectopic germinal centers (Schroder et al. 1996). The appearance of these structures, however, can vary between patients ranging from diffuse infiltrates to distinct follicles (Wagner et al. 1998, Weyand and Goronzy 2003).

D. Regulatory T cells

Interestingly, several studies have indicated that autoimmune arthritis can develop despite the presence of CD4+CD25+ Tregs (de Kleer et al. 2004, Ruprecht et al. 2005, Cao et al. 2006). Moreover, it appears that Tregs were also found to be enriched
within the joints of arthritis patients. A potential explanation would be that Tregs in arthritis patients, although representatively increased, are dysfunctional. In support of this, Tregs isolated from RA patients exhibited reduced suppressor function (Ehrenstein et al. 2004, Valencia et al. 2006, Nie et al. 2013). Notably, Ehrenstein et al. showed that proliferation but not cytokine production could be suppressed with Tregs from arthritis patients. And Nie et al. showed that Tregs isolated from the joints, but not the blood, of RA patients displayed reduced suppressor function. However, a study performed with Tregs isolated from juvenile idiopathic arthritis (JIA) patients provided a contrasting result (de Kleer et al. 2004). In an in vitro suppression assay, Tregs isolated from the synovial fluid of JIA patients suppressed more effectively than those isolated from the blood. Thus, conflicting results exist on whether the dysfunction of Tregs contributes to the development of inflammatory arthritis.

1.3.4 Treatments for RA

Generally, treatment for RA begins with a course of nonsteroidal anti-inflammatory drugs (NSAIDs) and/or disease-modifying antirheumatic drugs (DMARDs) that non-specifically suppress inflammation (Isaacs 2010). However, many patients either fail to develop a response or do not maintain an adequate response to these drugs. There are currently several treatment courses available when this happens, each developed to target specific pathways believed to promote the pathogenesis of arthritis. We discuss some of these treatments and their efficacy here.
A. Anti-TNF

Since their conception and development in the 1990’s, anti-TNF regents remain the next course of treatment for RA patients following NSAID/DMARD failure. This is because anti-TNF treatments have a history of high and rapid response rates in RA patients (Lipsky et al. 2000, Feldmann 2002). Successful responses to anti-TNF treatment can be seen in upwards of 80% of patients and show efficacy as early as 2 weeks in some patients. Some mechanisms by which anti-TNF treatment is thought to work include: inhibiting the production of pro-inflammatory cytokines in the joints and reducing the expression of chemokines in the joints which prevents cell trafficking (Taylor et al. 2000, Feldmann 2002). Interestingly, several studies have demonstrated that anti-TNF treatment can also improve Treg suppressor function (Ehrenstein et al. 2004, Valencia et al. 2006, Nie et al. 2013). Valencia X et al. and Nie H et al. further demonstrated that the addition of TNF to in vitro cultures can significantly impair the suppressive ability of Tregs. Thus, anti-TNF treatment appears capable of suppressing arthritis development through multiple modes of action. However, it is also possible that these findings are an indirect consequence of the treatment. For example, although TNF can impair Treg function and Tregs were found to regain suppressor function following anti-TNF treatment, it cannot be definitively concluded that Tregs directly ameliorate disease.

Anti-TNF is by no means a universal treatment as approximate 20-40% of anti-TNF recipients do not show initial responses and some patients do not maintain long-term responses to treatment (Lipsky et al. 2000, Feldmann 2002). In the next sections, we describe additional therapies that have been developed for the treatment of arthritis.
B. Anti-B cell

Anti-B cell reagents, namely anti-CD20 or rituximab, have gained momentum as many RA patients (upwards of ~80%) have also demonstrated clinical response to this treatment (Edwards et al. 2004, Higashida et al. 2005, Cohen et al. 2006). Notably, many of the trials that have been conducted with anti-B cell therapies were with patients that showed inadequate response to anti-TNF, suggesting that different pathways of pathogenesis exist among individuals. Treatment with rituximab results in the depletion of B cells that express CD20; since long-lived plasma cells have lost this marker, they are unaffected by this treatment (Edwards et al. 2004, Higashida et al. 2005, Cohen et al. 2006). However, anti-CD20 treatment has been shown to significantly reduce RF and anti-CCP levels in some patients, suggesting that these autoantibodies are presumably produced by CD20 expressing short-lived plasma cells (Edwards et al. 2004, Higashida et al. 2005, Cohen et al. 2006, Townsend et al. 2010). Interestingly, one study has reported that the reappearance of these autoantibodies in the serum correlates with disease relapse (Cambridge et al. 2003). This may suggest that autoantibodies participate as the dominant pathogenic mechanism in RA. However, some argue that because the reduction of autoantibodies is not requisite for a clinical response and because RF and anti-CCP are not completely eliminated in treatment-responsive patients, B cells may contribute to arthritis development independently of autoantibody production, possibly as serving as APCs for pathogenic CD4\(^+\) T cells.
C. Others:

Other treatments available for RA are ones that block T cell co-stimulation (CTLA4-Ig), an IL-1 receptor antagonist (anakinra), an IL-6R blocking antibody (MRA) and an anti-IL-17 antibody (LY2439821) (Cohen et al. 2002, Moreland et al. 2002, Nishimoto et al. 2004, Genovese et al. 2010). Less is known about the efficacy of these treatments but they appear promising.

1.3.5. Summary of inflammatory arthritis in humans

Autoimmune diseases result from a breakdown in self-tolerance but the etiology of these diseases is still unclear and may differ between individuals. Autoimmune settings in which inflammatory arthritis develops are associated with activated CD4+ T cells, autoantibody production and increased levels of several pro-inflammatory cytokines, indicating that multiple cellular and soluble factors are involved in disease pathogenesis. However, treatments targeting possible disease mediators (anti-TNF and anti-B cell) has demonstrated that each treatment is effective, but only in subsets of patients. This suggest that pathways central to disease may differ between individuals. It is currently not known why some patients respond to a treatment while others fail to respond, and there are currently no reliable predictors of response. This leaves us with the following questions: What are the mechanisms that determine which pathways are involved? What underlies the heterogeneity in response? Is it because different self-antigens are being recognized in different patients? Several possible target autoantigens in inflammatory arthritis have been proposed (such as RF and citrullinated
proteins) (Bennett et al. 2003, Trouw and Mahler 2012). This is because antibodies directed toward these self-antigens can be found at increased concentrations in diseased patients relative to healthy individuals. Nonetheless, these findings in human patients only correlate with disease and cannot directly implicate them in eliciting disease. Because only correlations can be made in humans, the field has turned to mouse models of inflammatory arthritis to better assess mechanisms of disease pathogenesis.

1.4 Mouse models of inflammatory arthritis

Several mouse models of inflammatory arthritis exist, each unique with regard to how disease initiates as well as the effector pathways involved. This demonstrates that many distinct, non-overlapping pathways can lead to inflammatory arthritis.

1.4.1 Overexpression/enhanced signaling of cytokines

A key feature of RA is the highly inflammatory nature of the disease, as compared to osteoarthritis (also a joint disease but believed to be caused by mechanical stress rather than an autoimmune reaction) (Farahat et al. 1993, Hampel et al. 2013). Not only are high levels of multiple inflammatory cytokines found directly in the inflamed joint, but elevated levels of cytokines are also detected in the serum of RA patients (Steiner et al. 1999, Ziolkowska et al. 2000, McInnes and Schett 2011, Metawi et al. 2011). Indeed, transgenic mice generated to overexpress some prominent inflammatory cytokines found in human RA develop chronic and erosive arthritis.
A. hTNF transgenic

Mice generated with a gene construct encoding human TNF lacking a 3’ untranslated region (demonstrated to repress translation of the cytokine) overexpress TNF systemically and develop chronic polyarthritis (Keffer et al. 1991). Joints of hTNF transgenic mice exhibit synovial hyperplasia, inflammatory infiltrates, pannus formation, and cartilage and bone erosion, which closely resembles the human disease. Monoclonal antibodies directed against human TNF completely prevented arthritis onset in these mice, establishing the direct role of TNF in driving joint inflammation. As previously described, TNF has been shown capable of inducing and synergizing with IL-1 and IL-6 within arthritic joints (Brennan et al. 1989, Alvaro-Gracia et al. 1991). Therefore, the relationship and contribution of IL-1 and IL-6 have also been explored in this model. Arthritis development was significantly attenuated in hTNF transgenic mice either treated with a neutralizing antibody to IL-1 receptor or crossed with IL-1−/− mice, demonstrating that IL-1 is an important downstream mediator of TNF for disease pathogenesis (Probert et al. 1995, Zwerina et al. 2007). IL-6, on the other hand, did not appear required in this system since disease develops similarly in IL-6 sufficient and IL-6 deficient hTNF transgenic mice (Alonzi et al. 1998). Moreover, the adaptive immune system was found to not be required in these mice as TNF mediated arthritis is still able to develop on the RAG−/− background, demonstrating instead the requirement for TNF production and uptake by innate immune cells (Kollias et al. 1999).
B. hIL-1α transgenic

Due to the perceived involvement of IL-1 during the development of inflammatory arthritis, hIL-1α transgenic mice were generated (Niki et al. 2001). Serum from hIL-1α transgenic mice contained high levels of hIL-1α and hIL-1α mRNA was expressed in multiple tissues, including the joints. Similar to hTNF transgenic mice, hIL-1α transgenic mice also develop chronic polyarthritis with evidence of synovial hyperplasia, synovitis and articular degeneration. Thus, overexpression of IL-1α was also sufficient to induce the development of inflammatory arthritis.

C. gp130<sup>F759/F759</sup> mice

Mice generated with a homozygous point mutation in the gp130 subunit of the IL-6 receptor exhibit enhanced STAT3 signaling (downstream of the IL-6R) and develop chronic and erosive arthritis (Atsumi et al. 2002). Arthritic gp130 mice contained elevated levels of autoantibodies as well as highly activated CD4<sup>+</sup> and CD8<sup>+</sup> T cells, implicating the involvement of the adaptive immune system in the disease process. Indeed, arthritis development is dependent on lymphocytes in this model as gp130 mice crossed onto a RAG<sup>−/−</sup> background do not develop disease. Further studies in this model, however, revealed that CD4<sup>+</sup> T cells, but not CD8<sup>+</sup> T cells or B cells, are the subset of lymphocytes required for disease progression in this system (Sawa et al. 2006). Excess IL-6 signaling in gp130 mice induces IL-7 production and subsequent homeostatic proliferation of CD4<sup>+</sup> T cells which then acquire an activated phenotype and differentiate into arthritogenic Th17 cells (Sawa et al. 2006, Nishihara et al. 2007, Ogura
et al. 2008). Moreover, not only does IL-6 function to generate pathogenic Th17 cells in this system, it appears that IL-6 is also a critical downstream target of IL-17; these two processes together form a positive-feedback loop necessary to sustain inflammation and drive arthritis development (Ogura et al. 2008).

Notably, work in this model system performed by Murakami et. al. demonstrate that CD4⁺ T cell recognition of a joint-specific antigen is not required for arthritis development, however, accumulation of effector Th17 cells (irrespective of antigen specificity) directly in the joints is required (Murakami et al. 2011). Even direct injection of IL-17 into joints of gp130 mice (but not wild type mice) can induce the rapid onset of arthritis. Arthritis was originally described to develop in gp130 mice beginning around 8 months of age, whereas exogenous IL-17 can induce swollen joints as early as 3 days after injection (Atsumi et al. 2002, Murakami et al. 2011). These results implicate that non-antigen-specific activation of the IL-6 amplification loop is sufficient to elicit a joint-targeted disease.

1.4.2 Adjuvant induced models

The previous section demonstrated that overexpression/enhanced signaling of prominent pro-inflammatory cytokines found in RA was sufficient to elicit spontaneous inflammatory arthritis in mice. However, it is unclear from these models how the production of these cytokines can be elicited in a physiological (non-transgenic) setting. Mouse models of adjuvant induced arthritis allow for the investigation of this aspect, among others, of disease development.
A. Collagen induced arthritis (CIA)

CIA is one of the most widely used models for the study of inflammatory arthritis. This inducible model involves immunization of rats or mice with heterologous type II collagen (usually chicken or bovine) mixed with complete Freud’s adjuvant (Trentham et al. 1977). First and foremost, CIA recapitulates the clinical aspects of inflammatory arthritis in humans – synovial hyperplasia, synovitis, pannus formation and articular degeneration. Secondly, CIA recapitulates several inflammatory processes and pathways observed during arthritis development in humans. Notably, CIA susceptibility is limited to mice bearing certain MHCII alleles (Trentham et al. 1977, Griffiths et al. 1994, Myers et al. 1997), highlighting the existence of arthritogenic CD4$^+$ T cell clones in a given mouse’s T cell repertoire and also the central role for CD4$^+$ T cells in the disease process. Findings using this model system illustrate that under steady state conditions, autoreactive CD4$^+$ T cells (that are indeed present) are maintained tolerant. However, TCR stimulation of type II collagen-specific CD4$^+$ T cells in the presence of adjuvant allows for the break tolerance and the subsequent development of arthritis. Disease pathogenesis in CIA has both a T cell and B cell component: CD4$^+$ T cells isolated from CIA mice can transfer disease to naïve recipients (Kadowaki et al. 1994) and disease can be passively transferred by serum antibodies isolated from arthritic CIA mice (Stuart and Dixon 1983, Wooley et al. 1984). Disease induced by these methods, however, is not chronic and resides after a few days. The inflammatory cytokines TNF, IL-1, IL-6 and IL-17 have all been shown to play important roles in the development of CIA (Myers et al. 1997, Alonzi et al. 1998, Lubberts et al. 2001, Nakae et al. 2003).
Overall, CIA represents a model of inflammatory arthritis that resembles human arthritis in its clinical appearance and inflammatory pathogenic processes. Disease development in this model, however, is specifically triggered by heterologous type II collagen in the context of a strong, TLR stimulus (CFA). How well this translates into how arthritis initiates in humans is unclear, but non-concordance of arthritis development in monozygotic twins has led to speculations on the involvement of environmental factors, such as pathogens, as triggers for autoimmunity.

B. Proteoglycan induced arthritis (PGIA)

PGIA, a second inducible model of inflammatory arthritis, was established through the injection of human cartilage proteoglycan (PG) with adjuvant into genetically susceptible mice (Banerjee et al. 1992). This model was developed to assess PG as a potential autoantigen in human inflammatory arthritis, aside from type II collagen. An apparent difference between CIA and PGIA is that a single immunization can induce CIA but PGIA requires several immunizations over a course of a month to a month and a half to induce disease (Banerjee et al. 1992, O'Neill et al. 2005). Early studies in this system revealed that CD4\(^+\) but not CD8\(^+\) T cells are essential for disease induction (Banerjee et al. 1992). Later studies demonstrated that disease development in this system is also significantly dependent on B cells, which appear to serve as both APCs and as a source of pathogenic autoantibodies (O'Neill et al. 2005, Hamel et al. 2008). Furthermore, disease can be transferred with spleen and lymph node (LN) cells isolated from arthritic mice, however, successful transfer of disease requires both T and B cells (Mikecz et al. 1990). Interestingly, the inflammatory cytokine IFN-\(\gamma\), but not IL-17, was shown to play a
significant role in PGIA (Doodes et al. 2008). This result is the opposite of what was found in the CIA mouse model (Vermeire et al. 1997, Lubberts et al. 2001, Nakae et al. 2003). Thus, PGIA and CIA share both similarities and differences in the pathways by which disease develops.

1.4.3. Alterations in cells of the adaptive immune system

Due to the strong association between certain MHCII alleles and the development of inflammatory arthritis, CD4\(^+\) T cells have been implicated to play a central role in the pathogenesis of disease (Clarke and Vyse 2009, McInnes and Schett 2011). In these next models, CD4\(^+\) T cells are uniquely altered, resulting in a loss of self-tolerance; these models also demonstrate the multiple roles CD4\(^+\) T cells can play in disease development.

A. SKG mice

A spontaneously derived mutation in the signaling domain of ZAP-70 (ZAP-70\(^{W163C}\)) is the root cause of inflammatory arthritis development in SKG mice (Sakaguchi et al. 2003). ZAP-70, a protein-tyrosine kinase which associates with the phosphorylated form of the TCR zeta chain, constitutes a key molecule in TCR signal transduction (Chan et al. 1992), and T cells containing the ZAP-70\(^{W163C}\) mutation display attenuated TCR signaling, which seems paradoxical to our understanding of how T cells participate in autoimmunity (Sakaguchi et al. 2003). Extensive studies performed in SKG mice have yielded some explanations for this. One explanation that has been
proposed is that attenuated TCR signaling shifts the TCR repertoire of SKG mice to one that is highly self-reactive. This is because only highly self-specific and self-reactive T cell clones that can compensate for the TCR signaling defect can survive positive and negative selection in the thymus to populate the periphery (Sakaguchi et al. 2003, Tanaka et al. 2010). Once in the periphery, the self-reactivity of the T cells that have evaded deletion is high enough to drive T cell proliferation in response to a lymphopenic environment, despite the ZAP-70 mutation (Hirota et al. 2007). In doing so, CD4+ T cells in SKG mice acquire an activated phenotype and can also differentiate into Th17 cells. In support of the pathogenic role of CD4+ T cells in this system, adoptive transfer of CD4+ T cells from SKG mice into RAG-/- mice (B and T cell deficient) is sufficient to elicit arthritis; this experiment also demonstrates that B cells as an effector population are not required (Hirota et al. 2007). Importantly, CD4+ T cells from IL-17-/-SKG mice were incapable of transferring disease (Hirota et al. 2007). This solidified that disease develops in SKG mice by an IL-17-dependent mechanism, driven by pathogenic Th17 cells that spontaneously form in this setting. An additional and non-mutually exclusive explanation for why autoimmune arthritis develops in SKG mice is that Treg function in this system appears impaired (Tanaka et al. 2010). Therefore, elegant studies performed in this model system have attempted to reconcile the paradox that T cells with attenuated TCR signaling can lead to the development of autoimmunity and several convincing explanations have been proposed. Although CD4+ T cells clearly play a critical role in the development of arthritis in the SKG model, the exact specificities of the arthritogenic T cells in this system have yet to be elucidated.

Because Th17 cell formation is promoted and IL-17 is required for disease development in SKG mice (Hirota et al. 2007), studies were performed to elucidate the
mechanism by which this occurs. IL-6 appears required in this process as IL-6\(^{-}\) SKG CD4\(^{+}\) T cells transferred into IL-6\(^{-}\) RAG\(^{-}\) mice failed to develop into Th17 cells and also failed to initiate disease (Hirota et al. 2007). Furthermore, a following report demonstrated that upregulated CCL20 expression by synoviocytes actively recruits Th17 cells (through their expression of CCR6 – the receptor for CCL20) to the joints (Hirota et al. 2007). TNF, IL-1B and IL-17 were all shown capable of upregulating synoviocyte expression of CCL20. An anti-CCR6 blocking mAb, however, only moderately attenuated disease development.

An interesting feature of the SKG model is that arthritis only develops in mice housed in non-specific pathogen free (non-SPF) conditions, demonstrating that microbes (especially fungi) are necessary to provoke the development of arthritis in this system (Yoshitomi et al. 2005). Moreover, injections of zymosan or laminarin selectively promote the expansion/differentiation of Th17 cells (and not Th1 cells) in SKG mice (Hirota et al. 2007). Collectively, these finding argue that fungal products prime and activate innate immune cells/APCs which are capable of promoting arthritogenic Th17 cells in SKG mice.

Strikingly, effects on T cell selection and the spectrum of autoimmune manifestations that occur in SKG mice can be modulated by varying the number of mutated ZAP-70 alleles (Tanaka et al. 2010). Amazingly, these results demonstrate that the degree with which TCR signaling is altered can dramatically affect not only T cell development but also autoimmune disease manifestations in a gene dosage dependent manner. In support of this, Siggs et. al. produced similar findings in a separate ZAP-70 mutant model (Siggs et al. 2007). Mice with varying degrees of TCR attenuation were
analyzed and compared. Again, the extent of T cell deletion and of T cell activation was affected in a gene dosage dependent manner; the effect of TCR signal strength on autoimmune manifestations was not evaluated in this study. These studies begin to demonstrate that variations in the CD4\(^+\) T cell response to self-antigen(s) can indeed influence the degree of systemic immune activation and its outcome on autoimmune disease development.

**B. K/BxN mice**

This next mouse model of inflammatory arthritis was generated through mating the KRN TCR transgenic mouse with a non-obese diabetic (NOD) mouse and was named K/BxN mice (Kouskoff et al. 1996). The KRN TCR was originally known to recognize a peptide of bovine ribonuclease, when presented by MHCII I-A\(^\kappa\). However, in a NOD background, it was discovered that KRN T cells recognize the systemically distributed protein - glucose-6-phosphate isomerase (GPI), complexed with the I-A\(^\beta\) MHCII molecule as a target autoantigen, the end result being the spontaneous development of inflammatory arthritis in K/BxN mice. The importance of CD4\(^+\) T cells in K/BxN mice is evident since a TCR transgenic mouse was used to generate this model system. Unlike in the SKG model, the main role CD4\(^+\) T cells serve in this system appears to be as help for B cells to develop into plasma cells capable of secreting pathogenic autoantibodies since serum isolated from arthritic K/BxN mice rapidly induces disease (within 2-3 days) following transfer into naïve BALB/c recipients (Korganow et al. 1999). Joint swelling in serum recipients, however, is transient, often resolving after approximately 3 weeks; this suggests that constant production of the arthritogenic autoantibody is required for disease persistence. The mechanism by which
this occurs has been studied extensively, solidifying that autoantibodies represent a principle effector mechanism in K/BxN mice. Briefly, it has been proposed that arthritogenic anti-GPI autoantibodies that develop in this system preferentially deposit and accumulate along the surface of cartilage in the joints, despite the ubiquitous expression of GPI, triggering an inflammatory complement cascade and ultimately joint destruction (Matsumoto et al. 2002).

Cytokines also significantly contribute to the effector phase of disease development in this model system. Therefore, serum transfers were performed in cytokine deficient mice and disease was assessed. IL-6 did not appear required but IL-1 was essential for K/BxN serum induced arthritis (Ji et al. 2002). Anti-IL-17 treatment was also found to completely prevent disease development in K/BxN mice (Wu et al. 2010). The contribution of TNF less clear cut – the majority, but not all, of TNF−/− recipient mice were protected from developing passively transferred arthritis, demonstrating that TNF plays a significant, but not an indispensible, role in this model system (Ji et al. 2002). Why this variability was observed in a seemingly uniform experiment (same serum transfer protocol into inbred TNF−/− mice) is unclear, however, the authors suggest this variability could be due to unknown stochastic/environmental factors. Interestingly, these results are somewhat evocative of the unequal response of RA patient to anti-TNF therapy.

Strikingly, disease was significantly attenuated in K/BxN mice housed in germ free (GF) conditions, demonstrating that commensal microbes (and specifically segmented filamentous bacteria (SFB)) play a significant role in the development of arthritis in this system (Wu et al. 2010). Notably, anti-GPI titers and Th17 cell
frequencies were significantly reduced in germ free K/BxN mice relative to SPF K/BxN mice. Moreover, it was discovered that anti-IL-17 treatment and elimination of IL-17R expression specifically on B cells significantly reduced germinal center formation. Therefore from these data, the authors suggest that the contribution of commensals in the development of arthritis in K/BxN mice was to promote Th17 cell formation which in turn support germinal centers needed for the production of arthritogenic autoantibodies.

The K/BxN model of inflammatory arthritis has provided insight on how CD4⁺ T cell recognition of a ubiquitous autoantigen, the specificities of which are known, can promote a joint-targeted disease elicited by autoantibodies.

**1.4.4 Summary on mouse models of inflammatory arthritis**

The diversity of mouse models capable of recapitulating some aspects of human arthritis demonstrate that multiple, possibly disparate, pathways can lead to the development of arthritis. Although these mouse models are not completely representative of the processes that occur during inflammatory arthritis in humans, they have allowed certain aspects of the disease to be studied more carefully. The findings from mouse models demonstrate not only how inflammatory cytokines can lead to a joint-targeted disease but that they can work in interconnecting networks to promote arthritis development; they exemplify the central yet diverse roles CD4⁺ T cells can play during the development of autoimmune arthritis; and they demonstrate that the extent to which B cells and autoantibodies contribute to disease pathogenesis appears to vary
depending on the model system under evaluation. Several of the models further implicate a role for microbes for disease initiation (CIA, PGIA, SKG and K/BxN).

1.5 TS1xHACII: a spontaneous mouse model of inflammatory arthritis

TS1 mice express a transgenic TCR that is specific for the I-E\textsuperscript{d}-restricted S1 determinant of influenza virus A/PR/8/34 (PR8) hemagglutinin (HA); clonotypic T cells can be identified by the monoclonal antibody, 6.5 (Kirberg et al. 1994). HACII mice express the PR8 HA as a surrogate self-antigen under the control of an MHCII promoter, which directs the expression of HA to APCs (Reed et al. 2003). When TS1 mice are mated with HACII mice, both the TS1 TCR transgene and the PR8 HA are co-expressed in their progeny: TS1xHACII mice. As previously described, the majority of TS1xHACII mice spontaneously develop inflammatory arthritis which first becomes evident between 6-8 weeks of age (Rankin et al. 2008).

In the thymii of TS1xHACII mice, CD4\textsuperscript{+} T cells bearing the clonotypic TCR (6.5\textsuperscript{+}CD4\textsuperscript{+}) recognize the S1 self-antigen with high affinity and are subjected to extensive thymic deletion ((Rankin et al. 2008) and Fig. 1-1). However, central tolerance is incomplete as we can find a small population of 6.5\textsuperscript{+}CD4\textsuperscript{+} T cells in both the LNs and spleens of young, pre-arthritic TS1xHACII mice. These high affinity, autoreactive 6.5\textsuperscript{+}CD4\textsuperscript{+} T cells then interact with HA-expressing APCs, leading to the reciprocal activation of both T cells and APCs. Activated B cells proceed to differentiate into plasma cells and secrete high levels of IgG. However, B cells appear unnecessary for disease as B cell deficient TS1xHACII.JH\textsuperscript{−/−} mice were found to develop arthritis (Rankin
et al. 2008). The T cell-APC interaction in TS1xHACII mice was also demonstrated to promote the development of Th17-trophic inflammatory monocytes which may be one way by which Th17 cells form in this system (Simons et al. 2013). Indeed, arthritis in this system develops by an IL-17-dependent mechanism as blockade of this cytokine significantly blocks disease onset (Oh et al. 2012). Importantly, TS1xHACII mice on a RAG deficient background can develop arthritis, demonstrating that other TCR specificities besides the TS1 TCR are not necessary to initiate disease (Rankin et al. 2008).

Notably, TS1xHACII mice develop disease in the presence of high frequencies of Tregs (Oh et al. 2012). Provision of exogenously derived HA-specific Tregs was unable to ameliorate disease, but Tregs from BALB/c mice (with a polyclonal TCR repertoire) could. Furthermore, suppression of disease with polyclonal Tregs was associated with significantly reduced Th17 cell frequencies.

Thus, in TS1xHACII mice, both the target antigen and disease-eliciting T cell specificity are known and several factors involved in disease pathogenesis have been elucidated. Therefore, manipulation of the TS1xHACII mouse model of arthritis will allow us to explore how altering CD4+ T cell recognition of the target antigen can affect disease development.
FIGURE 1-1. Development of inflammatory arthritis in TS1xHACII mice: working model

1. 6.5+ thymocytes are subjected to extensive negative selection in response to S1 peptide. 6.5+CD4+ T cells that evade deletion populate the periphery and interact with S1 expressing APCs, leading to the reciprocal activation of these two populations.

2. B cells differentiate into plasma cells and secrete high levels of IgG. However, B cells and antibodies were found unnecessary for disease development.

3. Increased representations of Th17 cells are found in arthritic mice. The T cell-APC interaction promotes the generation of Th17-trophic inflammatory monocytes which may be one way by which Th17 cells form in this system.

4. Arthritis develops by an IL-17-dependent mechanism (anti-IL-17 treatment blocks disease).

5. Arthritis develops in the presence of Tregs. Provision with an exogenous source of Tregs with a polyclonal repertoire (but not with HA-specific Tregs) can suppress disease. This was associated with reduced Th17 cell frequencies.

Notably, TS1xHACII mice on a RAG deficient background can develop arthritis, demonstrating that other TCR specificities besides the TS1 TCR are not necessary to initiate disease.
Chapter 2: Materials and Methods

2.1 Transgenic mice

TS1 mice express a transgenic TCR (Vα4, Vβ8.1/8.2) that is specific for the S1 determinant of influenza virus A/PR/8/34 (PR8) HA presented by MHC class II I-E<sup>d</sup>. Clonotypic cells can be identified by the monoclonal antibody 6.5 (Kirberg et al. 1994). TS1(SW) mice express a transgenic TCR (Vα8.3, Vβ10) that is specific for an analog of the PR8 S1 peptide that is derived from the influenza virus A/SW/33 (SW) HA (Cerasoli et al. 1995, Cerasoli et al. 1995). HACII mice express the full length PR8 HA transgene driven by an MHC class II I-Eα promoter (Reed et al. 2003).

To generate TS1xHACII.JH<sup>−/−</sup> and TS1(SW)xHACII.JH<sup>−/−</sup> mice: TS1, TS1(SW) and HACII mice were first bred to JH<sup>−/−</sup> mice (Chen et al. 1993) on the BALB/c background and then TS1.JH<sup>−/−</sup> mice or TS1(SW).JH<sup>−/−</sup> mice were mated with HACII.JH<sup>−/−</sup> mice. All JH<sup>−/−</sup> mice were screened for the absence of B cells by flow cytometry.

Arthritic mice and aged-matched control mice were analyzed between 15-24 weeks of age. All mice have been backcrossed with BALB/c mice for at least 10 generations and are maintained on this background. BALB/c mice were purchased from Charles River Laboratories. All mice were housed in The Wistar Institute Animal Facility under specific pathogen-free conditions. All experiments were performed according to protocols approved by The Wistar Institutional Animal Care and Use Committee.
### 2.2 Genotyping of transgenic mice

TS1, TS1(SW) and HACII transgenic mice were genotyped upon weaning at 3 to 4 weeks of age. Tail samples (~2 mm) were digested overnight in 200 μL of tail lysis buffer at 55°C. Digestion was stopped by heat inactivation at 95°C (10 minutes). Samples were then used as template DNA for PCR. PCR was performed in 50 μL reactions: 5 μL 10X PCR amplification buffer, 50 ng of appropriate primers, 2 μL of 5 mM dNTPs, 1 unit of Taq polymerase, and 3 μL of tail DNA. PCR reactions were run in a thermocycler for 35 cycles, with each cycle consisting of a 1 minute annealing period at 50°C (for HA transgene tests) and 65°C (for TCR transgene tests), a 2 minute elongation period at 72°C, and a 1 minute denaturation period at 94°C. An additional annealing and elongation step was performed before ending the amplification cycles. PCR products were visualized on a 1% agarose gel containing ethidium bromide.

The primers used for genotyping were purchased from Integrated DNA Technologies (Coralville, IA) and their sequences are as follows:

**HA**

<table>
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<th>Primer</th>
<th>Sense</th>
<th>Antisense</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA uni-5’</td>
<td>5'-</td>
<td>CCA GCA GAT TTC ATC GAC TAT G  -3’</td>
</tr>
<tr>
<td>HA uni-3’</td>
<td>5'-</td>
<td>CCA GTA ATA GTT CAT CCT CCC  -3’</td>
</tr>
</tbody>
</table>

**TS1**

<table>
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<th>Sense</th>
<th>Antisense</th>
</tr>
</thead>
<tbody>
<tr>
<td>V2.1 CDR3</td>
<td>5'-</td>
<td>GAA CTG CTC AGC ATA ACT CCC  -3’</td>
</tr>
<tr>
<td>VβCROSS5’</td>
<td>5'-</td>
<td>GAG GCT GCA GTC ACC CAA AG  -3’</td>
</tr>
</tbody>
</table>

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2.3 Assessment of arthritis

Mice were assessed weekly for signs of arthritis. All four paws were analyzed for swelling by a blinded examiner, and each paw was assigned a score: 0, no visible swelling or discoloration; 1, visible swelling with/without discoloration; 2, severe swelling accompanied by skin discoloration. The minimum score per mouse is 0 and the maximum score per mouse is 8 (a combined score if all four limbs scored a 2).

2.4 Histology

Limbs, heart, kidneys, intestines (flushed with PBS) and lungs (perfused with formalin) were fixed in 10% formalin (Globe Scientific, Inc.). Limbs were decalcified and all tissues were then embedded in paraffin and cut at ~5 microns to generate sections, which were stained with H&E. Blinded pathological scoring of sections was performed. Grading scale: 0=not present, 1=minimal, 2=mild, 3=moderate, 4=marked.

2.5 Flow cytometry and cell sorting

Single-cell suspensions of joint-draining lymph nodes (jdLN) (pooled axillary, brachial, and popliteal LN), spleens, thymii or joints were stained with the Live/Dead
Fixable Aqua Dead Cell Stain Kit from Invitrogen (except when sorting) and then for surface markers at 4°C for 30 minutes. 1 x 10^6 to 3 x 10^6 cells were plated in 96-well V bottom plates and stained with antibodies diluted in 100 μL of FACS buffer. The following Abs were purchased from eBioscience or BD Pharmingen: anti-B220 (RA3-6B2), anti-CD3 (145-2C11), anti-CD4 (RM4-5), anti-CD8 (53-6.7), anti-CD11b (M1/70), anti-CD11c (N418), anti-CD19 (1D3), anti-CD25 (PC61.5), anti-CD44 (IM7), anti-CD45 (30-F11), anti-CD62L (MEL-14), anti-CD69 (H1.2F3), anti-CD138 (281-2), anti-Fas (Jo2), anti-Foxp3 (FJK-16s), anti-Gr-1 (RB6-8C5), anti-IFN-γ (XMG1.2), anti-IgD (11-26c), anti-IL-17 (eBio17B7), anti-IL-17RA (PAJ-17R), anti-MHCII (M5/114.15.2), and anti-Vβ10 (B21.5). Anti-6.5-biotin (Kirberg et al. 1994) and anti-Vα8.3-biotin (KT50, BD Pharmingen) were detected with streptavidin-Qdot655 (Invitrogen). Intracellular Foxp3 staining was performed according to the eBioscience protocol. Samples were collected on the LSR II flow cytometer (BD Biosciences) and data were analyzed using FlowJo software (Tree Star). Cells were sorted for use in in vitro assays on a MoFlow (DakoCytomation) or FACS Aria (BD Biosciences) cell sorter and populations obtained were of ~95% purity.

2.6 Intracellular cytokine staining

Cells were stimulated in supplemented Iscove’s Modified Dulbecco’s Medium (IMDM) plus 10% FBS with 50 ng/mL PMA (Sigma-Aldrich), 1 μM ionomycin (Sigma-Aldrich), and a 1:1000 dilution of brefeldin A (eBioscience) for 4 hours at 37°C. Following staining for surface markers, cells were fixed and permeabilized using the
Foxp3 Buffer Set (eBioscience) according to the manufacturer’s protocol and then intracellular cytokine staining was performed.

2.7 Isolation of cells from the joints

Paws from all four limbs of a mouse were isolated by dissection and fingers/toes were excised. Skin was removed from around these distal joints before incubation in 5 mL of a digestion solution consisting of 400 U/mL collagenase D (Roche) and 0.2 mg/mL DNase I (Roche) in PBS with calcium and magnesium at 37°C for 1 hour in a petri dish. Joints were then manually disrupted through a 70 µm cell strainer (BD Falcon) and these single-cell suspensions were analyzed by flow cytometry.

2.8 Labeling of cells with cell proliferation tracing dyes

For CFSE (Invitrogen) labeling: Cells to be labeled were resuspended at $1 \times 10^7$ cells/mL in sterile, serum-free 1X Dulbecco's Phosphate-Buffered Saline (DPBS) without calcium & magnesium (Mediatech, Inc.). A 5 mM stock solution of CFSE was prepared and added at a 1:1000 dilution to the cell suspension. Cells were incubated with CFSE at room temperature for 3.5 to 4 minutes. CFSE-labeling was then quenched with an equal volume of FBS at the end of the incubation period. For CellTrace Violet (CTV; Invitrogen) labeling: Manufacturer’s protocol was followed to label cells with CTV. Following either CFSE or CTV labeling, cells were washed at least twice with supplemented IMDM with 10% FBS before use in in vitro cultures.
2.9 *In vitro* proliferation assays

To assess TCR reactivity toward S1 (SFERFEIFPKE) and S1(SW) (SFEKFEIFPKT) peptides, CFSE-labeled LN cells from TS1 or TS1(SW) mice (5 x 10^4 cells/well) were co-cultured with BALB/c splenocytes as APCs (5 x 10^5 cells /well) in supplemented IMDM plus 10% FBS with or without peptide at various dilutions. After 3 days, cells were analyzed by flow cytometry for CFSE dilution. To assess CD4^+ T cell reactivity in an autologous mixed lymphocyte reaction, T cells (sorted as CD4^+CD8^- cells) from TS1xHACII or TS1(SW)xHACII mice were CFSE-labeled and cocultured with APCs (sorted as CD3^- cells) isolated from the same mouse, from HACII mice, or from BALB/c mice; each population was plated at the same numbers of cells/well as above. Anti-CD3 (145-2C11, NA/LE, BD Pharmingen) (0.1 µg/mL) was added to some wells as a positive control. After 3 days, cells were analyzed by flow cytometry for CFSE dilution.

2.10 *In vitro* suppression assay

CD4^+CD25'^ regulatory T cells (Tregs) were purified by cell sorting from spleens of non-arthritic anti-TNF treated TS1xHACII, arthritic isotype control-treated TS1xHACII, or BALB/c mice, and cocultured in varying numbers with 5 x 10^4 CTV-labeled (Invitrogen) CD4^+CD25^- responder T cells isolated from spleens of TS1xHACII mice and 2 x 10^5 CD3^- splenocytes from BALB/c mice (as APCs) in supplemented IMDM plus 10% FBS in 96-well U-bottom plates. Similarly, Tregs were sorted from spleens of non-arthritic anti-TNF treated TS1(SW)xHACII, arthritic isotype control-treated TS1(SW)xHACII, or BALB/c mice, and cocultured with CTV-labeled responder T cells isolated from spleens of TS1(SW)xHACII mice and BALB/c APCs. To stimulate both
responder cells and Tregs, anti-CD3 was added at 0.15 µg/mL. After 3 days of culture, cells were analyzed by flow cytometry for CTV dilution. Percent inhibition was determined as: \((1 - \frac{\% \text{ divided cells in each responder cells and Tregs coculture}}{\% \text{ divided cells in responder cells only culture}}) \times 100\).

2.11 In vivo antibody treatments

For anti-IL-17R treatment, mice were injected i.p. with 0.5 mg of either rat anti-mouse IL-17R blocking Ab (M751, provided by Amgen) or an isotype control Ab (MOPC-21, BioXCell) weekly from 5-14 weeks of age. For anti-CD20 treatment, mice were injected i.v. with 0.25 mg of either anti-mouse CD20 depleting Ab (18B12, provided by Biogen Idec) or an isotype control Ab (2B8, anti-human CD20 with no cross-reactivity to mouse CD20, provided by Biogen Idec) once every 3 weeks from 5-14 weeks of age (Hamel et al. 2008). For anti-TNF treatment, mice were injected i.p. with 0.5 mg of either rat anti-mouse TNF-α neutralizing Ab (XT3.11, BioXCell) or an isotype control Ab (HRPN, BioXCell) weekly from 5-14 weeks of age.

2.12 ELISAs for antibodies

Concentrations of total IgG in the serum were determined using U-bottom vinyl plates (Costar) that were coated with goat anti-mouse Ig (H+L) (SouthernBiotech), and bound Abs were detected with goat anti-mouse IgG-alkaline phosphatase (SouthernBiotech). Purified mouse IgG (SouthernBiotech) was used as a standard. To detect rheumatoid factor, plates were coated with purified mouse IgG1, lambda (BD Pharmingen), and bound Abs were detected with rat anti-mouse kappa light chain-biotin.
(SouthernBiotech) followed by streptavidin-alkaline phosphatase. Anti-type II collagen, anti-CCP and anti-dsDNA titers were determined using the anti-mouse Type II Collagen IgG (Chondrex, Inc.), QUANTA Lite CCP3 IgG (INOVA Diagnostics, Inc.), or mouse anti-dsDNA total Ig (Alpha Diagnostic International) ELISA kits, respectively, according to the manufacturer’s instructions.

2.13 Luminex assays for serum cytokines

Serum samples were analyzed on MILLIPLEX MAP Mouse Cytokine/Chemokine luminex assay kits (Millipore) by the University of Pennsylvania Human Immunology Core.

2.14 Serum transfers

Blood from donor mice was collected by heart puncture post-mortem and was allowed to coagulate for at least one hour at room temperature before centrifugation in order to isolate the serum fraction of the blood. Each recipient mouse was injected i.p. with 150 µL of donor serum on day 0 and was boosted with 100 µL of donor serum on day 3. Recipient mice were monitored for arthritis development every 3-4 days for 3 weeks.
2.15 Statistics

Statistical analyses were performed using Fisher’s Exact test, Mann-Whitney test or one-way ANOVA with Tukey post-test, as appropriate, with GraphPad Prism software (GraphPad). P values less than 0.05 were considered significant.
Chapter 3: CD4⁺ T cell affinity influences inflammatory arthritis development and extent of systemic immune activation

3.1 Introduction

The contribution of CD4⁺ T cells to the development of inflammatory arthritis has long been implicated as susceptibility to arthritis is strongly linked to certain MHC class II alleles (Clarke and Vyse 2009, McInnes and Schett 2011). Moreover, activated effector CD4⁺ T cells can be found in inflamed, arthritic joints and treatments designed to block CD4⁺ T cell co-stimulation has shown efficacy in some RA patients (Moreland et al. 2002). In mice, it has been demonstrated that an increased precursor frequency of CD4⁺ T cells specific for a known autoantigen (through the use of TCR transgenic mice), such as in K/BxN and TS1xHACII mice, and alterations to TCR signaling, such as in SKG mice, could promote autoimmune arthritis (Kouskoff et al. 1996, Sakaguchi et al. 2003). Also, in models where arthritis is induced (CIA and PGIA), susceptibility is associated with certain MHCII alleles (Trentham et al. 1977, Griffiths et al. 1994, Myers et al. 1997). Therefore, it has been appreciated that CD4⁺ T cells can play a major role in driving autoimmune arthritis.

But how CD4⁺ T cells recognize self-antigen and how this in turn affects the attributes of systemic immune activation and the outcomes of disease development is poorly understood. This is particularly difficult to assess in human patients as the self-antigens that are recognized by autoreactive CD4⁺ T cells are not well characterized (Bennett et al. 2003, Trouw and Mahler 2012). Several candidate autoantigens,
however, have been proposed, including type II collagen, IgG Fc and citrullinated proteins. Of note, several of the possible target autoantigens identified in humans so far are not joint-specific but rather ubiquitously expressed. Support of systemically expressed autoantigens as targets for autoreactive CD4⁺ T cells has been demonstrated in the aforementioned mouse models of arthritis. Developed in and previously described by our lab, the TS1xHACII spontaneous mouse model of inflammatory arthritis is a system whereby the systemic expression of an ectopic antigen in addition to the co-expression of a CD4⁺ TCR transgene for that antigen results in arthritis development (Rankin et al. 2008). Notable features of TS1xHACII mice are that the majority of mice develop arthritis and that the TCR transgene expressed recognizes the target self-antigen as a high affinity, cognate antigen. Therefore, we investigated how self-antigens are being recognized by autoreactive CD4⁺ T cells during autoimmune arthritis, and more specifically, how can the affinity of autoreactive CD4⁺ T cells for a target self-antigen affect disease development.

For this purpose, we modified the TS1xHACII model system by crossing the same antigen expressing mouse used in the TS1xHACII system with a mouse expressing a lower affinity TCR for the target self-antigen, generating TS1(SW)xHACII mice. We show here that lowering the affinity of an autoreactive TCR for a single target antigen was able to significantly modulate disease development. Significantly fewer male TS1(SW)xHACII mice developed arthritis in comparison to female TS1(SW)xHACII mice and to male TS1xHACII mice, but females from both strains developed arthritis with similar penetrance. Also, we found evidence of extra-articular manifestations of disease in arthritic TS1xHACII mice but this was rarely observed in arthritic TS1(SW)xHACII mice. The affinity with which CD4⁺ T cells recognize the target autoantigen in
TS1xHACII and TS1(SW)xHACII mice not only influenced disease susceptibility but also appeared to influence the extent of systemic immune activation. TS1xHACII mice contained CD4\(^+\) T cells that displayed a high degree of autoreactivity and high concentrations of inflammatory cytokines and IgG in the serum. Conversely, TS1(SW)xHACII mice contained CD4\(^+\) T cells that displayed a relatively low degree of autoreactivity and lower concentrations of cytokines and IgG in the serum. Collectively, these results demonstrate that an altered CD4\(^+\) T cell response to a single target autoantigen can prominently shape the development of inflammatory arthritis.
3.2 Results

3.2.1 TS1xHACII mice spontaneously develop autoimmune arthritis with high penetrance

TS1 mice express a transgenic TCR that is specific for the I-E\textsuperscript{d}-restricted S1 determinant of PR8 HA (Kirberg et al. 1994). HACII mice express the PR8 HA as a surrogate self-antigen under the control of an MHCII promoter, which directs the expression of HA to APCs (Reed et al. 2003). When TS1 mice are mated with HACII mice, both the TS1 TCR transgene and the PR8 HA are co-expressed in their progeny: TS1xHACII mice (Fig. 3-1a). As previously described (Rankin et al. 2008, Oh et al. 2012, Simons et al. 2013) and also reported here, TS1xHACII mice spontaneously develop overtly inflamed fore- and hind-paws, as represented in the photographs in Fig. 3-1b. We did not observe any evidence of inflammation in the paws of TS1 or HACII mice.

Multiple cohorts of TS1xHACII mice were assessed weekly for the development of swollen paws and from this, arthritis penetrance (percent of mice that developed at least 1 inflamed paw) and arthritis severity were determined. We found that arthritis develops in TS1xHACII mice beginning around 6-8 weeks of age, and it does so with similar penetrance and kinetics in male and female mice (Fig. 3-1c). Also, at 14 weeks of age, the combined scores of arthritis severity in all four limbs did not differ significantly between male and female TS1xHACII mice.
FIGURE 3-1. TS1xHACII mice spontaneously develop inflammatory arthritis with high penetrance. (a) Schematic depicting the generation of TS1xHACII mice through mating of TS1 mice and HACII mice. (b) Photographs show front and rear paws from representative control TS1 and arthritic TS1xHACII mice. (c) Left graph shows the mean percentages of male (n=15) and female (n=14) TS1xHACII mice that developed at least one arthritic paw over time. Right graph shows arthritis scores for individual female (F) and male (M) TS1xHACII mice at 14 weeks of age.
These results, together with extensive findings previously described in this mouse model system (Rankin et al. 2008), indicate that the co-expression of the TS1 transgene along with its cognate antigen (PR8 S1) is essential to drive disease development in TS1xHACII mice as TCR and HA transgene only mice do not develop disease. This further suggests that the development of CD4\(^+\) T cells expressing the TS1 TCR in the presence of their agonist peptide is an important component in the disease process. Indeed, TS1xHACII mice that have been mated onto a RAG deficient background also develop spontaneous arthritis (Rankin et al. 2008). Moreover, these results extend the previous findings by showing that male TS1xHACII mice develop inflammatory arthritis to the same extent as female TS1xHACII mice.

3.2.2 Affinity of TS1 and TS1(SW) T cells for S1 peptide

Numerous studies in both humans and mice expound on the contribution(s) of CD4\(^+\) T cells in the development of inflammatory arthritis. However, how autoreactive CD4\(^+\) T cells recognize and respond to self-antigens and how this can in turn lead to the development of autoimmunity is poorly understood (Riley et al. 2000, Siggs et al. 2007). This question is especially hard to study in human arthritis patients as the self-antigens recognized by autoreactive CD4\(^+\) T cells are not well characterized (Bennett et al. 2003, Trouw and Mahler 2012). Since a notable feature of TS1xHACII mice is that the TS1 TCR recognizes the S1 self-peptide as a high affinity, cognate antigen (Rankin et al. 2008), we had a unique opportunity to study how changing the affinity with which a TCR recognizes a known, target antigen could affect disease development. For this purpose, we utilized TS1(SW) mice, which express a transgenic TCR that was raised against a
variant influenza virus, called SW virus (Cerasoli et al. 1995, Cerasoli et al. 1995, Boesteanu et al. 2006). The S1 determinant within the HA protein of SW virus (termed S1(SW)) is an analog of the PR8 S1 peptide and differs from S1 by two amino acid residues (Fig. 3-2a). Clonotypic, HA-specific T cells in TS1(SW) mice recognizes S1(SW) peptide as an agonist and S1 peptide as a partial-agonist. This is demonstrated here in an in vitro proliferation assay. While CFSE-labeled CD4⁺ T cells from TS1 mice proliferated robustly in response to micromolar amounts of S1 peptide (as indicated by CFSE-dilution in red histograms), T cells from TS1(SW) mice proliferated weakly even to high amounts (3 μM) of S1 peptide and appeared unresponsive to lower concentrations of S1 peptide (Fig. 3-2b). This is a reflection of the low intrinsic affinity of the TS1(SW) TCR for the S1 peptide, because the same CD4⁺ T cells proliferated robustly upon exposure to their cognate antigen, S1(SW) peptide (blue histograms). Similarly, when splenocytes from HACII mice were used as a source of S1 peptide-expressing APCs, CD4⁺ T cells from TS1 mice proliferated strongly and TS1(SW) T cells proliferated weakly in response (Fig. 3-2c).

We next wanted to investigate how TCR affinity for the S1 self-peptide would affect CD4⁺ T cell development in TS1xHACII and TS1(SW)xHACII mice. Thymii from both TS1xHACII and TS1(SW)xHACII mice contained significantly lower frequencies of clonotypic CD4⁺ T cells (identified as 6.5⁺ in mice expressing the TS1 transgene and Vα8.3'Vβ10⁺ in mice expressing the TS1(SW) transgene) compared to TS1 mice (p=0.0007) and TS1(SW) mice (p=0.0002), respectively (Fig. 3-2d). Notably, the fold reduction of the frequency of 6.5⁻CD4⁺ T cells in TS1xHACII mice (~15 fold) is much greater than that of Vα8.3'CD4⁺ T cells observed in TS1(SW)xHACII mice (~2 fold).
FIGURE 3.2. TCR affinity for S1 self-peptide affects the degree of autoreactive thymocyte deletion. 

(a) Schematic shows relative affinity of the CD4+ T cells from TS1 and TS1(SW) mice for the S1 and S1(SW) peptide analogs. (b) Histograms show CFSE levels of CD4+ T cells from TS1 and TS1(SW) mice following incubation with indicated concentrations of S1 (red histograms) and S1(SW) (blue histograms) peptide. (c) Histograms show CFSE levels of CD4+ T cells from TS1 and TS1(SW) mice following incubation with splenocytes isolated from HACII or BALB/c mice. (d) Top panel: Dot plots show CD8 versus CD4 staining of cells from thymi of 4-6 week old TS1 (n=6) and TS1xHACII (n=8) mice, and histograms show staining of CD4+CD8+ cells with the anti-clonotypic mAb 6.5. Percentages in indicated gates are shown. Bar graphs show average thymic cellularity ± SEM and average numbers of clonotypic CD4+ T cells with the anti-clonotypic mAb 6.5. Bottom panel: As for top panel, except 4-6 week old TS1(SW) (n=6) and TS1(SW)xHACII (n=10) mice are shown, and Vα8.3 staining is used to identify clonotypic CD4+ cells. **P<0.01, ***P<0.001, Mann-Whitney test.
In line with the our findings from the *in vitro* proliferation assays performed above, these data further demonstrate the affinity of the TS1 and TS1(SW) TCR for S1 peptide. The reduced representation of clonotypic CD4⁺ T cells in both TS1xHACII and TS1(SW)xHACII mice is most likely due to thymic deletion because not only were the percentages of these cells significantly reduced compared to TCR single transgenic mice but their absolute numbers were significantly reduced as well. Of note, there was also a significant reduction in the frequency of CD4⁺CD8⁻ thymocytes in TS1(SW)xHACII mice when compared to TS1(SW) mice (p=0.0002); the CD4⁺CD8⁻ population did not differ between TS1 and TS1xHACII mice. As the TS1(SW) TCR is a better allelic excluder compared to the TS1 TCR (~75% to ~28%), the deletion of clonotypic T cells in TS1(SW)xHACII mice, which are largely CD4⁺, could account for the overall decrease in CD4⁺CD8⁻ cells. We also found that CD4⁺CD8⁻ thymocytes from TS1(SW)xHACII mice expressed lower levels of Vα8.3 compared to TS1(SW) mice. Reduced surface expression of the 6.5 TCR was observed on TS1xHACII CD4⁺CD8⁻ thymocytes relative to TS1 thymocytes as well (Rankin et al. 2008). Thus, it is possible that only clonotypic CD4⁺CD8⁻ thymocytes with low surface TCR expression are able to escape negative selection.

Collectively, these data show that the TS1(SW) TCR has a lower affinity for S1 peptide than the TS1 TCR, as demonstrated in *in vitro* proliferation assays and in the extent of clonotypic CD4⁺ T cell deletion in thymii of double transgenic mice.
3.2.3 TS1(SW)xHACII mice spontaneously develop inflammatory arthritis that is female biased

To investigate whether an autoreactive TCR with low affinity for the S1 self-peptide would be able to drive arthritis development, we generated TS1(SW)xHACII mice by mating TS1(SW) mice with HACII mice (Fig. 3-3a). Despite the lower affinity TCR, TS1(SW)xHACII mice also developed overtly visible, swollen fore- and hind-paws, similar to what was observed in TS1xHACII mice (Fig. 3-3b). Again, expression of the HA-specific TCR and HA together appear essential for disease initiation as TS1(SW) mice rarely show any indication of joint swelling even at 14 weeks of age (Fig. 3-3c). When arthritis development was assessed in TS1(SW)xHACII mice over time, we found that the large majority of female mice developed arthritis by 14 weeks of age, and the penetrance and severity of arthritis in female TS1(SW)xHACII and female TS1xHACII mice were comparable (Fig. 3-3d and Fig. 3-1c). Conversely, there was a significant delay in the development of arthritis in male TS1(SW)xHACII mice, and significantly fewer male TS1(SW)xHACII mice developed arthritis by 14 weeks of age when compared to female mice of the same lineage and to male TS1xHACII mice (p=0.007). Even after one month, arthritis penetrance in male TS1(SW)xHACII mice did not increase. Moreover, the overall severity scores for male TS1(SW)xHACII mice were significantly lower than for female TS1(SW)xHACII mice at both 14 and 18 weeks of age.

Collectively, these data demonstrate that recognition of self-antigen by CD4+ T cells bearing a low affinity TCR is able to drive the development of autoimmune arthritis. However, in the context of the low affinity TS1(SW) TCR, disease develops with a pronounced sex bias that was not observed in the high affinity TS1xHACII model.
**FIGURE 3-3.** CD4⁺ T cell affinity for a self-peptide influences the development of inflammatory arthritis. (a) Schematic depicting the generation of TS1(SW)xHACII mice through mating of TS1(SW) mice and HACII mice. (b) Photographs show front and rear paws from representative control TS1(SW) and arthritic TS1(SW)xHACII mice. (c) Graph shows arthritis scores for individual female (F) and male (M) TS1(SW) mice at 14 weeks of age. (d) Left graph shows the mean percentages of male (n=12) and female (n=14) TS1(SW)xHACII mice that developed at least one arthritic paw over time. *P<0.05, Fisher’s Exact test. Right graph shows arthritis scores for individual female (F) and male (M) TS1(SW)xHACII mice at 14 weeks of age. *P<0.05, Mann-Whitney test.
3.2.4 Histopathological examination of TS1xHACII and TS1(SW)xHACII mice

We next performed histopathological examinations of joints and tissues from arthritic TS1xHACII and TS1(SW)xHACII mice, as well as from aged-matched mice devoid of overt paw swelling (designated as “non-arthritic”). For the analysis of joints, paws from the various mice were sectioned and stained with H&E. Photographs of representative hind-paw sections were taken at 4X and 10X magnification (Fig. 3-4a). At low magnification, the extensive infiltration of immune cells present within the joints of TS1xHACII and TS1(SW)xHACII mice designated as arthritic (and the absence of such in control and “non-arthritic” mice) can be visualized. In the high magnification photographs, distinct areas of cartilage and bone erosion can be observed in sections from arthritic mice but not in sections from control or “non-arthritic” mice.

Sections from individual mice designated as either arthritic or non-arthritic were scored (on a scale of 0 – 4, with 4 being the most severe) for synovial hyperplasia, synovitis and articular degeneration, all of which are characteristic of inflammatory arthritis (McInnes and Schett 2011). Sections taken from the swollen joints of female TS1xHACII and TS1(SW)xHACII mice exhibited high degrees of hyperplasia, synovitis and articular degeneration, and generated significantly higher severity scores than did sections obtained from control TCR-only mice, supporting their designation as arthritic (Fig. 3-4b). By contrast, when we examined sections that had been obtained from TS1(SW)xHACII mice (both male and female) that did not exhibit overt joint swelling, we found that these sections did not differ from control mice with respect to all three criteria assessed, supporting and validating their designation as non-arthritic. Moreover, the synovitis and articular degeneration scores generated from arthritic male
FIGURE 3-4. Histopathological analysis of joints from TS1xHACII and TS1(SW)xHACII mice. 
(a) Photographs of representative H&E-stained joint sections of control TCR single transgenic (Tg), arthritic TS1xHACII, arthritic TS1(SW)xHACII and non-arthritic TS1(SW)xHACII mice at 4X (top row) or 10X (bottom row) magnification. (b) Graphs show scores for synovial hyperplasia, synovitis and articular degeneration (n=5-8 mice per group) with each symbol representing an individual mouse. F = female, M = male, A = arthritic, NA = non-arthritic. Grading scale: 0 = not present, 1 = minimal, 2 = mild, 3 = moderate, 4 = marked. All statistical significance determined by one-way ANOVA with Tukey post test. #P<0.05, ###P<0.01, ####P<0.001 (comparing single TCR transgenic to any set of double transgenic mice). *P<0.05, **P<0.01, ***P<0.001 (comparing between sets of double transgenic mice).
TS1(SW)xHACII mice were significantly lower than those from arthritic female TS1xHACII and TS1(SW)xHACII mice, indicating that, in addition to lower disease penetrance, the severity of arthritis was lower in arthritic male versus arthritic female mice.

We also examined extra-articular tissues for evidence of inflammation, and as previously reported, we found extensive perivascular infiltrates in the lungs of arthritic TS1xHACII mice (Rankin et al. 2008 and Fig. 3-5a and b)). By contrast, no differences were observed in the extent of perivascular infiltration in the lungs of either male or female TS1(SW)xHACII mice and control mice, irrespective of arthritis development. In addition to the lungs, mild inflammatory processes were observed in the hearts and kidneys of some TS1xHACII mice, but were either not observed, or were only rarely found in arthritic TS1(SW)xHACII mice (Fig. 3-5c). Evidence of intestinal inflammation was not observed in either arthritic lineage.

Our visual assessment of TS1xHACII and TS1(SW)xHACII mice and their subsequent designation as “arthritic” or “non-arthritic” was validated by the results presented here. Of the male TS1(SW)xHACII mice that developed arthritis, their scores for synovitis and articular degeneration were significantly lower than those from arthritic female mice; this is noteworthy since we also observed a delayed onset of disease in male TS1(SW)xHACII mice. Together, these data suggest that gender-associated factors (such as sex hormones) may play a role in promoting the extent of arthritis development and the degree of arthritis severity in the low affinity, TS1(SW)xHACII mouse model of arthritis. Lastly, the lower affinity TCR in TS1(SW)xHACII mice is
FIGURE 3.5. Histopathological analysis of non-articular tissues from TS1xHACII and TS1(SW)xHACII mice. (a) Photographs of H&E-stained sections depict perivascular infiltration in the lungs of control female TCR single transgenic (Tg), arthritic female TS1xHACII, arthritic female TS1(SW)xHACII, and non-arthritic female TS1(SW)xHACII mice at 20X magnification. Indicated scores are reflective of the degree of infiltration in each photograph. (b) Graph shows the scores for perivascular infiltration in the lungs of the indicated mice (n=3-8 mice per group) with each symbol representing an individual mouse. F = female, M = male, A = arthritic, NA = non-arthritic. Grading scale: 0 = not present, 1 = minimal, 2 = mild, 3 = moderate, 4 = marked. All statistical significance determined by one-way ANOVA with Tukey post-test. ***P<0.001 (comparing single TCR transgenic to any set of double transgenic mice). ###P<0.001 (comparing between sets of double transgenic mice). (c) Table enumerates the number of mice in which histology sections from the heart, kidneys and intestines displayed evidence of inflammation and/or immune cell infiltration.
associated with a reduction of the extra-articular inflammation that occurs in TS1xHACII mice.

3.2.5 Autoreactivity of CD4$^+$ T cells from TS1xHACII and TS1(SW)xHACII mice

Since inflammatory arthritis develops in mice co-expressing HA as a surrogate self-antigen with a transgenic TCR that recognizes the self-HA, we first wanted to assess the CD4$^+$ T cells, and importantly the clonotypic CD4$^+$ T cells, in TS1xHACII and TS1(SW)xHACII mice. Despite having been subjected to extensive negative selection in the thymus, a small population of clonotypic CD4$^+$ T cells could be found in the spleens, a systemically draining peripheral lymphoid organ, of pre-arthritic TS1xHACII and pre-arthritic TS1(SW)xHACII mice. Moreover, both 6.5$^+$$CD4^+$ and V$\alpha$8.3$^+$$CD4^+$ T cells expressed an activated phenotype, more so than the remaining fraction of non-clonotypic CD4$^+$ T cells in the same mouse and CD4$^+$ T cells from control TCR single transgenic mice, implicating the autoreactive nature of 6.5$^+$$CD4^+$ and V$\alpha$8.3$^+$$CD4^+$ T cells (Fig. 3-6a-d). Notably though, the extent of 6.5$^+$$CD4^+$ T cell activation is considerably higher than that of V$\alpha$8.3$^+$$CD4^+$ T cells. This appears to be another reflection of the affinities of the TS1 and TS1(SW) TCR for the HA self-antigen.

To measure the degree of autoreactivity of CD4$^+$ T cells in TS1xHACII and TS1(SW)xHACII mice, we performed an in vitro autologous mixed lymphocyte reaction (autologous MLR) assay. Total CD4$^+$ T cells from arthritic TS1xHACII and arthritic TS1(SW)xHACII mice were FACS sorted and CFSE-labeled. These T cells were then plated with unfractionated splenocytes from various mice as sources of APCs.
FIGURE 3.6. Clonotypic CD4+ T cells in the periphery of TS1xHACII and TS1(SW)xHACII mice display an activated phenotype. (a) Dot plots show CD8 versus CD4 staining of cells from the spleens of 4-6 week old TS1 (n=6) and TS1xHACII (n=8) mice, and histograms show staining of CD4+ cells with the anti-clonotypic mAb 6.5. Percentages in indicated gates are shown. (b) Representative histograms show expression of CD69, CD62L and CD44 on indicated splenic CD4+ T cell populations from 4-6 week old TS1 and TS1xHACII mice. (c) As for (a), except 4-6 week old TS1(SW) (n=6) and TS1(SW)xHACII (n=10) mice are shown, and Vα8.3 staining is used to identify clonotypic CD4+ cells. (d) As for (b), except 4-6 week old TS1(SW) and TS1(SW)xHACII mice are shown.
“Autologous splenocytes” were obtained from the spleens of TS1xHACII or TS1(SW)xHACII mice and were plated with CD4\(^+\) T cells sorted from the same mouse; HACII splenocytes were obtained from HACII mice where we know HA is expressed and can stimulate T cell proliferation (Fig. 3-2c); BALB/c splenocytes were from BALB/c mice (which, besides HA, should express the same self-antigens as HACII APCs); and BALB/c splenocytes plus anti-CD3 acted as a positive control. In these experiments, T cells and splenocytes were isolate from individual mice (i.e. cells from multiple mice were not pooled) as to be able to obtain independent data from separate mice. Three days after the co-culture was set up, we evaluated each stimulation condition for their ability to induce T cell proliferation (as measured by CFSE dilution). Total CD4\(^+\) T cells from both TS1xHACII and TS1(SW)xHACII mice did not proliferate in the presence of BALB/c splenocytes but proliferated robustly to anti-CD3 stimulation (Fig. 3-7). In the presence of autologous splenocytes, CD4\(^+\) T cells from TS1xHACII mice underwent division in response to self-antigens being presented, but CD4\(^+\) T cells from TS1(SW)xHACII mice underwent little to no proliferation. The lack of proliferation observed with TS1(SW)xHACII T cells could be due to the reactivity of the T cells or because HA presentation by autologous TS1(SW)xHACII splenocytes was reduced or absent. However, co-culture of CD4\(^+\) T cells from TS1(SW)xHACII mice with HACII splenocytes yielded the same results and we observed little to no dilution of CFSE in this stimulation condition as well. On the other hand, TS1xHACII T cells underwent division in the presence of HACII splenocytes, similar to what we observed with autologous splenocytes.

These data show that pre-arthritic TS1xHACII and TS1(SW)xHACII mice contain clonotypic CD4\(^+\) T cells that display an activated phenotype (Fig. 3-6). Together with
FIGURE 3-7. Autoreactivity of CD4+ T cells from TS1xHACII and TS1(SW)xHACII mice as measured by an autologous MLR. Histograms show levels of CFSE in CD4+ T cells from TS1xHACII and TS1(SW)xHACII mice following incubation for 3 days with autologous splenocytes as APCs, with HACII splenocytes, with BALB/c splenocytes alone, or with BALB/c splenocytes and anti-CD3. Percentages of divided cells are indicated.
results from an autologous MLR, we demonstrate that the extent of autoreactivity of clonotypic CD4+ T cells from TS1xHACII and TS1(SW)xHACII mice relative to each other was maintained. In other words, 6.5°CD4+ T cells from TS1xHACII mice maintained a higher level of reactivity to S1 peptide relative to Vα8.3°CD4+ T cells from TS1(SW)xHACII mice. Therefore, despite subjection to multiple mechanisms of tolerance induction, CD4+ T cells in TS1xHACII and TS1(SW)xHACII mice do not appear completely tolerized.

### 3.2.6 Th1 and Th17 cells accumulate in arthritic TS1xHACII and TS1(SW)xHACII mice

To assess whether changes in CD4+ T cell populations coincided with the presence of disease, we compared the representation of total and clonotypic CD4+ T cells in the LNs capable of draining the joints (pooled axillary, brachial and popliteal LNs; joint-draining LNs; jdLN) and spleens of TS1xHACII and TS1(SW)xHACII mice before and after arthritis development. With the exception of CD4+ T cells in the spleens and Vα8.3°CD4+ T cells in the jdLN of TS1(SW)xHACII mice, total CD4+ and clonotypic CD4+ T cell populations were either not significantly different or lower in mice after disease onset (Fig. 3-8a and b). Also, it did not appear that clonotypic CD4+ T cells specifically accumulate in the jdLN compared to the spleens of arthritic mice. Rather than in T cell populations, the most dramatic change linked to disease status is the significant increase in jdLN cellularities of both arthritic TS1xHACII and TS1(SW)xHACII mice. Spleen cellularities were also significantly increased in arthritic TS1(SW)xHACII mice and modestly increased in arthritic TS1xHACII mice.
FIGURE 3-8. Representation of total and clonotypic CD4+ T cell populations in pre-arthritic and arthritic mice. (a) Bar graphs show total cellularities and percentages of CD4+ and of clonotypic CD4+ T cells ± SEM in the jDLNs and spleens of pre-arthritic (n=8) and arthritic (n=6) TS1xHACII mice. (b) As for (a), except pre-arthritic (n=11) and arthritic (n=11) TS1(SW)xHACII mice are shown. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, Mann-Whitney test.
Although the representation of total and clonotypic CD4$^+$ T cells did not, for the most part, increase after disease onset, we wanted to examine if the CD4$^+$ T cells in arthritic TS1xHACII and TS1(SW)xHACII mice gained an effector phenotype which associates with the presence of inflammatory arthritis. Increased frequencies of Th1 and Th17 cells, but not Th2 cells, are associated with inflammatory arthritis in mice and humans (Miossec and van den Berg 1997, Mauri et al. 2003). Because of this, we compared the representation of IFN-$\gamma$- and IL-17-producing CD4$^+$ T cells in arthritic and pre-arthritic double transgenic mice as well as in non-arthritic TCR single transgenic mice. Within the total CD4$^+$ and 6.5$^+$CD4$^+$ T cell populations (isolated from the jdLN and re-stimulated ex-vivo), increased frequencies of both IFN-$\gamma^+$ and IL-17$^+$ cells were observed in TS1xHACII mice relative to control TS1 mice (Fig. 3-9a and b). In line with previous findings in this model system (Simons et al. 2013), we observed a dramatic polarization of CD4$^+$ and 6.5$^+$CD4$^+$ T cells to a Th1 phenotype in pre-arthritic TS1xHACII mice. However, in mice with active disease, we found reduced frequencies of Th1 cells compared to pre-arthritic mice and a concurrent increase in the frequency of IL-17$^+$6.5$^+$CD4$^+$ T cells.

Pre-arthritic TS1(SW)xHACII mice also contained significantly higher frequencies of IFN-$\gamma^-$ and IL-17-producing CD4$^+$ and V$\alpha8.3^+$CD4$^+$ T cells relative to control TS1(SW) mice (Fig. 3-9c and d). Examination of CD4$^+$ T cell populations isolated from arthritic TS1(SW)xHACII mice revealed even higher frequencies IFN-$\gamma^+$ and IL-17$^+$ T cells, both of which were significantly increased over that found in pre-arthritic mice. This is in contrast to what occurred in TS1xHACII mice as the reduction of Th1 cells that occurred after arthritis onset in the high affinity system was not observed in TS1(SW)xHACII mice.
FIGURE 3-9. Accumulation of Th1 and Th17 cells in arthritic TS1xHACII and TS1(SW)xHACII mice. (a) Representative dot plots show IFN-γ versus IL-17 staining of CD4+ cells isolated from the jdlN of young TS1, pre-arthritic TS1xHACII, and arthritic TS1xHACII mice. Percentages of cells in indicated gates are shown. (b) Graphs indicate the mean percentages ± SEM of CD4+ and of CD4+6.5+ cells that secrete IFN-γ or IL-17 (n=6-13). All statistical significance determined by the Mann-Whitney test. ⋆P<0.05, ⋆⋆P<0.01, ⋆⋆⋆P<0.001 (comparing TS1 mice to any set of TS1xHACII mice). (c) As for (a), except young TS1(SW), pre-arthritic TS1(SW)xHACII and arthritic TS1(SW)xHACII mice are shown. (d) As for (b), except that cells were obtained from young TS1(SW), pre-arthritic TS1(SW)xHACII and arthritic TS1(SW)xHACII mice, and CD4+Vα8.3+ cells are shown (n=6-15). All statistical significance determined by the Mann-Whitney test. #P<0.05 and ###P<0.01 (comparing TS1(SW) mice to any set of TS1(SW)xHACII mice). ***P<0.001 (comparing between sets of TS1(SW)xHACII mice).
Notably, CD4$^+$ and V$\alpha$8.3$^+$CD4$^+$ T cells in TS1(SW)xHACII mice polarized toward a Th17 phenotype more so than to a Th1 phenotype (note the difference in scales in Fig. 3-9d).

Collectively, these data show that rather than increases in the general representation of total or clonotypic CD4$^+$ T cells, the appearance of disease in arthritic TS1xHACII and TS1(SW)xHACII mice coincided with these T cell populations acquiring the ability to produce IL-17. Of note, even before the onset of disease, TS1xHACII and TS1(SW)xHACII mice contained significantly higher frequencies of Th1 and Th17 cells compared to their age-matched TCR single transgenic counterparts. Thus, these data implicate a pathogenic role for Th17 cells in the development of arthritis in both TS1xHACII and TS1(SW)xHACII mice. Indeed, treatment with an anti-IL-17 mAb abrogated arthritis development in TS1xHACII mice (Oh et al. 2012, Simons et al. 2013).

3.2.7 TCR affinity affects the extent of systemic immune activation in arthritic mice

Because we found significantly higher frequencies of IFN-$\gamma$$^+$ and IL-17$^+$ CD4$^+$ T cells in arthritic TS1xHACII and TS1(SW)xHACII mice by intracellular cytokine staining, we wanted to assess the actual levels of IFN-$\gamma$ and IL-17 present in these mice. For this purpose, we performed a Luminex assay with serum obtained from arthritic mice. In addition to IFN-$\gamma$ and IL-17, we chose to examine the levels of the pro-inflammatory cytokines TNF-a, IL-6 and IL-1B (and the anti-inflammatory cytokine IL-10) as they all were found to be upregulated in the serum and synovial fluid of RA patients (Steiner et al. 1999, Feldmann 2002, McInnes and Schett 2011, Metawi et al. 2011); as described in
FIGURE 3-10. Systemic immune activation in TS1xHACII and TS1(SW)xHACII mice. (a) Graphs show mean concentrations ± SEM of indicated cytokines in the serum of control female TCR single transgenic (TS1 and TS1(SW)) mice, arthritic female TS1xHACII mice, and arthritic female TS1(SW)xHACII mice (n=17-30). *P<0.05, **P<0.01, ***P<0.001 in a one-way ANOVA with Tukey post-test. (b) Graph shows the mean concentration and individual levels of serum IgG in control female TCR single transgenic, arthritic female TS1xHACII, and arthritic female TS1(SW)xHACII mice (n=8-14). ***P<0.001, one-way ANOVA with Tukey post-test.
Chapter 1, their contributions to disease pathogenesis have also been explored in detail. The Luminex results revealed that the serum of TS1xHACII mice, in comparison to TCR single transgenic controls, contained significantly elevated levels of all of the cytokines examined (Fig. 3-10a). On the other hand, only IL-6 was moderately elevated and IL-17 was significantly elevated in the serum of TS1(SW)xHACII mice relative to controls; levels of IL-17 were not significantly different in TS1(SW)xHACII mice compared to TS1xHACII mice. Arthritic TS1xHACII mice also contained significantly higher levels of serum IgG than control mice expressing only the TCR transgene, and while serum IgG was also elevated in arthritic TS1(SW)xHACII mice relative to controls, it was significantly lower than in TS1xHACII mice (Fig. 3-10b).

Collectively, these data demonstrate that the extent of systemic immune activation can be affected by the affinity with which autoreactive CD4^+ T cells recognize a target self-antigen. Interestingly, these results suggest that elevated levels of multiple pro-inflammatory cytokines are not necessary for the development of arthritis, as TS1(SW)xHACII mice which contain overall low levels of cytokines can develop disease; rather, the contribution of IL-6 and/or IL-17 may be important.

### 3.2.8 Inflammatory arthritis develops by an IL-17-dependent mechanism in TS1(SW)xHACII mice

Due to the previous findings showing that arthritic TS1(SW)xHACII mice contained high frequencies of IL-17^+ CD4^+ T cells and elevated levels of IL-17 in the serum (Fig. 3-9 and Fig. 3-10), we wanted to ascertain whether IL-17 is necessary for
disease development in TS1(SW)xHACII mice. IL-17 has been shown to play an important and pathogenic role in multiple mouse models of arthritis (including the TS1xHACII mouse model) (Hirota et al. 2007, Wu et al. 2010, Oh et al. 2012), and importantly, a role for IL-17 has also been implicated in RA (Ziolkowska et al. 2000, Metawi et al. 2011). For this purpose, we treated pre-arthritic TS1(SW)xHACII mice with an anti-IL-17R mAb, which has been reported to block the interaction of IL-17 with its receptor (Maitra et al. 2007), or with an isotype control mAb and monitored for disease development (Fig. 3-11a). Strikingly, we found that treatment with anti-IL-17R completely abrogates arthritis development in TS1(SW)xHACII mice. Of note, serum from anti-IL-17R treated mice contained significantly higher concentrations of IL-17 compared to both TS1(SW) and isotype control-treated TS1(SW)xHACII mice, indicating that the consumption of IL-17 was blocked with anti-IL-17R treatment (Fig. 3-11b).

Together, these results show that anti-IL-17R treatment significantly attenuates disease development in TS1(SW)xHACII mice. Therefore, inflammatory arthritis can develop by an IL-17-dependent mechanism in the low affinity mouse model (TS1(SW)xHACII mice) as well.
**FIGURE 3-11.** Anti-IL-17R treatment ameliorates disease in TS1(SW)xHACII mice. (a) Schematic shows anti-IL-17R treatment regimen in TS1(SW)xHACII mice. Graph shows the mean percentages of anti-IL-17R treated (n=8) or isotype control-treated (n=5) female TS1(SW)xHACII mice that developed at least one arthritic paw over time. *P<0.05, **P<0.01, Fisher’s exact test. (b) Graph shows mean concentrations ± SEM of IL-17 in the serum of control TS1(SW), isotype control-treated TS1(SW)xHACII and anti-IL-17R treated TS1(SW)xHACII mice (n=5-8). **P<0.01, one-way ANOVA with Tukey post test.
3.3 Discussion

Genetic associations to MHCII alleles in human patients and findings extracted from mouse models strongly implicate the central role of autoreactive CD4\(^+\) T cells in the pathogenesis of inflammatory arthritis. The data we present in this chapter demonstrate that altering the affinity of CD4\(^+\) T cells to a single target autoantigen can have profound effects on disease course and the extent of systemic immune activation.

We began this project to examine how CD4\(^+\) T cell recognition of self-antigen(s) can influence the development of autoimmunity. Using a model system of inflammatory arthritis where the specificities of the disease eliciting CD4\(^+\) T cells and target autoantigen are both known (TS1xHACII mice) ([Rankin et al. 2008] and Fig. 3-1)), we explored the outcome of altering the CD4\(^+\) T cell response to that single target antigen. Therefore, TS1(SW)xHACII mice were generated, in which the mouse expressing the target antigen from TS1xHACII mice was crossed with a mouse expressing a transgenic TCR with low (rather than high) affinity for that antigen (Fig. 3-3a). Notably, to generate TS1(SW)xHACII mice, we used an entirely different TCR transgene than was used to generate TS1xHACII mice and there are appreciable differences in these two TCRs. Besides using different \(\alpha/\beta\) chain combinations (see Materials and Methods), the TS1(SW) TCR is a much better allelic excluder than the TS1 TCR (Fig. 3-2d).

Therefore, how can we claim to have influenced disease outcome by altering the CD4\(^+\) T cell response to just PR8 HA? What if we think about it this way: there is no evidence of inflammatory arthritis development in TCR single transgenic mice, so it is the introduction of PR8 HA as a neo-self antigen that prompts disease onset in both
TS1xHACII and TS1(SW)xHACII mice. In other words, co-expression of the TCR transgene with the HA transgene is necessary to drive inflammatory arthritis in both model systems. We cannot exclude, however, the possibility that autoreactive CD4⁺ T cell specificities exist in double transgenic mice that were not present in single transgenic mice. Autoreactive α/β TCR combinations may emerge from stochastic rearrangements of endogenous TCR chains in order to compensate for the extensive thymic deletion of clonotypic T cells in each double transgenic system ((Rankin et al. 2008) and Fig. 3-2d)). It is also possible that the initial interactions of the autoreactive TCR with self-antigen leads to the bystander activation of other T cell specificities (determinant / epitope spreading) (Lehmann et al. 1992). Indeed, we can find non-clonotypic CD4⁺ T cells capable of producing IFN-γ and IL-17 in TS1xHACII and TS1(SW)xHACII mice (Fig. 3-9). Nonetheless, the autoimmune reaction that unfolds in double transgenic (but not TCR single transgenic) mice argues that, in each case, the CD4⁺ T cell response specifically to the neo-HA antigen drives the development of autoimmune arthritis.

As previously reported and repeated here, TS1xHACII mice develop a highly penetrant and severe form of inflammatory arthritis that is accompanied by extra-articular inflammatory manifestations ((Rankin et al. 2008) and Fig. 3-1, 4, 5)). The introduction of a low affinity TCR for the autoantigen still resulted in the development of arthritis in TS1(SW)xHACII mice, but compared to the high affinity model system (TS1xHACII mice), the overall penetrance of disease in TS1(SW)xHACII mice was significantly lower (due to fewer males developing arthritis) and we could find little to no evidence of inflammation in extra-articular tissues (Fig. 3-3, 4, 5). These findings were
interesting because there could have been several possible outcomes following the introduction of the low affinity TCR. Indeed, SKG mice with varying numbers of wild-type, mutant and null ZAP-70 alleles (and therefore graded extents of TCR signaling) displayed altered disease spectrums (Tanaka et al. 2010). Therefore, we might have discovered that TS1(SW)xHACII mice do not develop autoimmunity; they could have developed a different type of autoimmune disease in lieu of arthritis; or they could have developed arthritis that replicates the disease observed in TS1xHACII mice. Strikingly, these findings demonstrate that the autoreactive CD4+ T cell responses that occurs in both TS1xHACII and TS1(SW)xHACII mice promote a joint-targeted disease.

Nonetheless, tolerance is broken in both TS1xHACII and TS1(SW)xHACII mice, resulting in autoimmune arthritis. This occurs despite the subjection of CD4+ T cells in both systems to multiple mechanisms of tolerance induction. Clonotypic CD4+ T cells were negatively selected in the thymii of TS1xHACII and TS1(SW)xHACII mice in which the fold-reduction observed in each case correlated to the affinity of the T cells for the S1 autoantigen (Fig. 3-2). We also found that clonotypic CD4+ T cells that develop in the presence of S1 as a self-antigen expressed appreciably lower levels of TCRs on their surface compared to their TCR only counterparts, as demonstrated by flow cytometry (Rankin et al. 2008) and Fig. 3-2d). Furthermore, possibly due to the reduced density of TCRs on their surface, S1-specific CD4+ T cells from TS1xHACII and TS1(SW)xHACII mice displayed a heavily modulated reactivity to the S1 self-peptide compared to T cells isolated from TS1 and TS1(SW) mice, respectively (compare number of peaks in Fig. 3-2c to Fig. 3-7). These attempts at tolerance induction, however, failed and CD4+ T cells with an activated, effector phenotype can be found in the periphery of TS1xHACII and TS1(SW)xHACII mice.
It was interesting to find that, despite having a low affinity for the S1 self-antigen, the expression of the TS1(SW) TCR was sufficient to break tolerance and drive disease development in TS1(SW)xHACII mice. It is still unclear to what degree autoreactive CD4⁺ T cells need to be stimulated in order for this to happen (Siggs et al. 2007, Tanaka et al. 2010). This concept is interesting to consider because not all mice containing a transgenic TCR specific for self-antigen develop autoimmunity. An example of this from our lab is the TS1xHA104 mouse. HA104 mice were also generated with a PR8 HA transgene but its expression is driven by a SV40 early region promoter/enhancer (Caton et al. 1996, Riley et al. 2000); relative to HACII mice, HA104 mice express low levels of HA that is systemically distributed and the synthesis of HA is not limited to APCs. Of note, APCs in HA104 mice can present S1 peptide to TS1 T cells but are considerably less stimulatory than HACII APCs (Rankin et al. 2008). Similar to TS1xHACII mice, 6.5⁺CD4⁺ T cells in TS1xHA104 mice were subjected to negative selection in the thymus (the extent of their deletion was extensive but less so than in TS1xHACII mice), but can again be found in the periphery (Rankin et al. 2008). However, in striking contrast to TS1xHACII mice, TS1xHA104 mice do not develop inflammatory arthritis. One reason for why this disparate outcome in disease development may occur could be due to the amount of antigen presented in each system. This suggests that CD4⁺ T cells need to surpass a certain TCR stimulation threshold in order to break self-tolerance; simply the development of self-specific T cells in the presence of their cognate antigen does not guarantee that they will break self-tolerance. Therefore, taking the results of the TS1xHA104 mice into consideration, it would imply that CD4⁺ T cells in the TS1(SW)xHACII model system, despite expressing a low affinity TCR for the target self-antigen, were sufficiently stimulated to acquire effector functions and promote autoimmunity due to the high amounts of self-antigen that is constitutively presented by
APCs. This suggests that further reducing clonotypic TCR stimulation in TS1(SW)xHACII mice (for example, by crossing them onto a TCR signaling mutant) or removing a source of antigen (for example by crossing them onto a B cell- or DC-deficient background – see next chapter) may abrogate disease development in these mice so that they resemble TS1xHA104 mice.

Along these lines, the ability of CD4+ T cell affinity to influence the development of autoimmune arthritis does not appear to be binary – that arthritis can develop with an “intermediate” phenotype as can be observed in the incomplete penetrance of disease in TS1(SW)xHACII mice, especially in males (Fig. 3-3d). Interestingly, the extent of disease development (penetrance and severity) coincided with the extent of systemic immune activation. Not unlike how high affinity ligands can elicit a strong T helper response in other settings (Ahlers et al. 2001, Diepolder et al. 2001), CD4+ T cells bearing a high affinity TCR for the S1 self-antigen in TS1xHACII mice were associated with complementary APC activation (Rankin et al. 2008) and elevated systemic levels of IgG and multiple inflammatory cytokines ((Rankin et al. 2008) and Fig. 3-10)). In line with this, the degree of systemic immune activation in the low affinity model, TS1(SW)xHACII mice, was clearly less pronounced.

In conclusion, we have demonstrated that the affinity with which a CD4+ T cell recognizes a target self-antigen can prominently affect the development of inflammatory arthritis. Although extensive studies in humans and in mouse models support the conclusion that CD4+ T cells can promote arthritis development, how CD4+ T cells recognize self-antigen is unknown. This is especially hard to study in humans since the disease eliciting antigen(s) are not well defined. Furthermore, this issue has largely not
been addressed in other mouse models of arthritis. Therefore, we believe our work provides unique insights on how the affinity of a CD4$^+$ T cell response can direct the development of inflammatory arthritis.
Chapter 4: CD4\(^+\) T cell reactivity can influence the pathways by which inflammatory arthritis develops

4.1 Introduction

As described in detail in Chapter 1, the factors and cellular populations shown capable of participating in the development of inflammatory arthritis are numerous. Furthermore, not only can these factors work in concert to drive disease, but it can be argued that each factor could play multiple, possibly mutually exclusive, roles. For example, B cells can secrete inflammatory cytokines, serve as APCs and/or produce arthritogenic antibodies. TNF is capable of reinforcing an inflammatory cytokine cascade, inhibit Tregs and/or induce the expression of chemokines directly in the joints. In light of this, maybe it is not surprising that lineages of mice engineered to over-express inflammatory cytokines (like TNF or IL-1), found to contain mutated TCR signaling molecules (like SKG mice), or developed to co-express a transgenic TCR and a known target autoantigen (like K/BxN and TS1xHACII mice) all develop manifestations of inflammatory arthritis. These findings highlight the complexity of an autoimmune disorder which begins with a breach in self-tolerance and ultimately results in a joint-targeted disease. Furthermore, these findings suggest that the potential therapeutic targets for the treatment of arthritis are extensive.

Indeed, several reagents directed at distinct targets exist for the treatment of inflammatory arthritis. Anti-TNF reagents, which were the first biologic therapies developed for RA, have high response rates, but nonetheless, are not effective in all RA
patients (Lipsky et al. 2000, Feldmann 2002). This has led to the evaluation of antagonists targeting other pro-inflammatory cytokines (including IL-1, IL-6 and IL-17) for their therapeutic efficacy (Cohen et al. 2002, Nishimoto et al. 2004, Genovese et al. 2010). Additionally, anti-B cell reagents, such as rituximab, have proven effective in many RA patients and are usually the next line of treatment after anti-TNF fails (Edwards et al. 2004, Higashida et al. 2005, Cohen et al. 2006). These findings again highlight the complexity as well as the heterogeneity of the diseases classified as inflammatory arthritis, as there is not currently one course of treatment that is effective in all patients.

Therefore, a major hurdle for physicians in determining the appropriate treatment for arthritis patients is that they cannot predict what therapeutic reagent each patient would be responsive to; predictors of response to particular therapies have yet to be elucidated (Isaacs 2010). Progress on this issue has been limited because it is unclear what could dictate the predominant pathway(s) of pathogenesis (cytokines vs B cells) in each individual patient. A simple explanation could be that distinct autoantigens are targeted by the immune system in patients that respond to different therapeutic strategies. However, an alternative explanation is that qualitative and/or quantitative differences in the autoreactive CD4+ T cell response that drives the disease process can determine which cellular pathways take prominence. These hypotheses are difficult to assess in human patients because the self-antigens that are recognized remain poorly characterized (Bennett et al. 2003, Trouw and Mahler 2012).

As demonstrated in the previous chapter, inflammatory arthritis was found to arise in both TS1xHACII and TS1(SW)xHACII mice, despite having been generated with transgenic TCRs that have different affinities for the same S1 autoantigen. However, the development of disease, with regard to disease penetrance and the extent of systemic
immune activation, significantly differed between these two mouse models. In this chapter, we wanted to also assess the pathways by which disease develops in TS1xHACII and TS1(SW)xHACII mice. In the following experiments, we show that in the context of a highly reactive CD4\(^+\) T cell response to a single self-peptide in TS1xHACII mice, B cells are not required for arthritis development, although pro-inflammatory cytokines such as TNF are required. Conversely, eliminating B cells significantly suppresses disease development in the context of a weakly autoreactive CD4\(^+\) T cell response in TS1(SW)xHACII mice, and TNF in this system appears to play a less prominent role than B cells (as well as a less prominent role than in the TS1xHACII model system). Additional pathways appear to also be required to support arthritis development in TS1(SW)xHACII mice because disease develops with a pronounced female bias in this setting (Fig. 3-3d). Collectively, these studies reveal that fundamentally different pathways of pathogenesis exist in TS1xHACII and TS1(SW)xHACII mice. Thus, we believe we have demonstrated that the degree of CD4\(^+\) T cell reactivity for self-peptide(s) can play a prominent role in determining the cellular pathways that participate in the development of inflammatory arthritis.
4.2 Results

4.2.1 B cell elimination significantly attenuates disease development in TS1(SW)xHACII mice but not in TS1xHACII mice

To begin to assess whether differences in the degree of the autoreactive CD4+ T cell response could influence the pathways by which inflammatory arthritis develops, we first examined if B cells play a prominent role in TS1xHACII and TS1(SW)xHACII mice. As previously shown, serum IgG titers, used as a measure of B cell activity, were significantly higher in arthritic TS1xHACII mice compared to control mice expressing only the TCR transgene (Fig. 3-10b). Furthermore, B cells in the spleens of TS1xHACII mice were previously reported to express elevated levels of CD86 and MHCII (Rankin et al. 2008). All of these data indicate that highly activated B cells populate TS1xHACII mice. Serum IgG was also elevated in arthritic TS1(SW)xHACII mice relative to controls, but it was significantly lower than in TS1xHACII mice (Fig. 3-10b).

To definitively determine if B cells and/or their products are required for arthritis development, we mated TS1xHACII and TS1(SW)xHACII mice onto a JH−/− background (Chen et al. 2003) in order to generate mice that congenically lacked B cells. Cohorts of TS1xHACII.JH−/− mice, TS1(SW)xHACII.JH−/− mice, as well as B cell sufficient TS1xHACII and TS1(SW)xHACII mice, were then assessed weekly for arthritis development. As previously reported (Rankin et al. 2008) and expanded on here, the penetrance and severity of disease in TS1xHACII.JH−/− mice did not differ compared to TS1xHACII mice, indicating that B cells are not required for arthritis development in this system (Fig. 4-1a).
FIGURE 4-1. B cells promote arthritis development in TS1(SW)xHACII but not TS1xHACII mice. (a) Left graph shows mean percentages of B cell-sufficient female TS1xHACII (n=9) and B cell-deficient female TS1xHACII.JH−/− (n=6) mice that developed at least one arthritic paw over time. Right graph shows arthritis scores for mice at 14 weeks of age. (b) As for (a), except that B cell-sufficient female TS1(SW)xHACII (n=16) and B cell-deficient female TS1(SW)xHACII.JH−/− (n=17) mice are shown. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, left graph: Fisher’s Exact test; right graph: Mann-Whitney test.
Notably, we observed a marked acceleration of disease onset in TS1xHACII.JH<sup>-</sup> mice, suggesting that there may be a protective role for B cells in TS1xHACII mice. In striking contrast, when disease development was assessed in the low affinity system, we found significantly fewer TS1(SW)xHACII.JH<sup>-</sup> mice to have developed disease compared to their B cell sufficient counterparts (3/17 vs 14/16, respectively, at 14 weeks of age); and of the TS1(SW)xHACII.JH<sup>-</sup> mice that did develop disease, their severity scores were generally low (Fig. 4-1b).

Despite the presence of highly elevated serum IgG titers and systemically activated B cells in TS1xHACII mice, B cells are not a required immune cell population for the development of arthritis in this model system (Fig. 3-10b, Fig. 4-1 and ref). In striking contrast, B cells make a significant contribution to arthritis development in TS1(SW)xHACII mice - a setting where CD4<sup>+</sup> T cells recognize self-antigen with low reactivity. Therefore, interestingly, B cells significantly contribute to disease development in a setting where serum cytokines levels are low and the opposite is true in a setting with high levels of serum cytokines.

4.2.2 TS1(SW)xHACII mice do not contain elevated levels of arthritogenic autoantibody specificities

Since B cells make an important contribution to arthritis development in TS1(SW)xHACII but not TS1xHACII mice, we next examined if autoantibody specificities associated with inflammatory arthritis (Hueber et al. 2005, Isenberg et al. 2007) were present/elevated in arthritic TS1(SW)xHACII mice. To this end, we performed ELISAs to
measure the concentrations of autoantibodies in the serum of arthritic TS1(SW)xHACII mice in comparison to arthritic TS1xHACII and control TCR single transgenic mice. However, we found no differences in the levels of rheumatoid factor (RF), antibodies to type II collagen, or antibodies to double-stranded DNA (anti-dsDNA) in arthritic TS1(SW)xHACII mice compared to control TCR only mice or to arthritic TS1xHACII mice (Fig. 4-2a). Sera from TS1xHACII mice actually had higher levels of anti-cyclic citrullinated peptide (anti-CCP) antibodies relative to TS1(SW)xHACII mice. Although the presence of anti-CCP antibodies is currently the best diagnostic marker for RA, the citrullination of proteins can occur in other inflammatory settings (Vossenaar et al. 2004). Therefore, increased anti-CCP levels in TS1xHACII mice may simply reflect the high level of systemic inflammation present in the mouse (Fig. 3-10a).

To further assess if autoreactive antibodies contribute to disease development in TS1(SW)xHACII mice, we attempted to elicit disease by performing serum transfers. Serum (specifically the antibodies in the serum) from K/BxN mice has been shown to induce rapid and robust arthritis development following transfer into naïve BALB/c mice (Korganow et al. 1999). Utilizing the same protocol, we transferred serum from arthritic TS1(SW)xHACII mice into various hosts and monitored for the appearance of arthritis. We also transferred K/BxN serum into separate mice as positive controls. As expected, arthritis was induced in all recipients of K/BxN serum (Fig. 4-2b). We did not, however, observe any indication of inflammation or swelling in the paws of TS1(SW)xHACII serum recipients.
FIGURE 4-2. Serum from arthritic TS1(SW)xHACII mice does not contain elevated levels of autoantibodies associated with arthritis development. (a) Graphs show levels of rheumatoid factor, anti-type II collagen, anti-cyclic citrullinated peptide ("anti-CCP") and anti-double stranded DNA ("anti-dsDNA") in serum obtained from TS1 (n=4-10), arthritic TS1xHACII (n=10-15), TS1(SW) (n=6-10) and arthritic TS1(SW)xHACII (n=9-15) mice. Each symbol represents an individual mouse and the mean is also shown. ***P<0.0001, ****P<0.0001, one-way ANOVA with Tukey post-test. (b) Table shows the number of recipient mice of the indicated genetic background that developed arthritis following passive transfer of serum from arthritic mice of the indicated strain.
Collectively, these results suggest that the ability of B cells to promote arthritis development in TS1(SW)xHACII mice does not appear to be through a mechanism driven by arthritogenic autoantibodies.

4.2.3 B cells support clonotypic Th17 cells for the development of arthritis in TS1(SW)xHACII mice

As B cells do not seem to act as a source of pathogenic autoantibodies in TS1(SW)xHACII mice, we evaluated whether B cells provide support for effector CD4⁺ T cells as APCs in this system. To perform this analysis, we compared CD4⁺ T cell subsets from arthritic TS1(SW)xHACII, arthritic TS1(SW)xHACII.JH⁻ /⁺ and non-arthritic TS1(SW)xHACII.JH⁻ /⁺ mice that were described in Fig. 4-1b. Consistent with the absence of B cells, TS1(SW)xHACII.JH⁻ /⁺ mice had significantly smaller joint-draining LNs (jdLNs) and spleens than TS1(SW)xHACII mice; jdLN and splenic cellularities did not differ between TS1(SW)xHACII.JH⁻ /⁺ mice that did and did not develop arthritis (Fig. 4-3a). By the same token, we found significantly higher frequencies of CD4⁺ T cells in both sets of TS1(SW)xHACII.JH⁻ /⁺ mice due to the absence of B cells. Lower frequencies of Vα8.3⁺Vβ10⁺ CD4⁺ T cells were found in non-arthritic TS1(SW)xHACII.JH⁻ /⁺ mice relative to both sets of arthritic mice, however, this difference was not statistically significant. Of note, arthritic TS1(SW)xHACII.JH⁻ /⁺ mice contained similar frequencies of Vα8.3⁺Vβ10⁺ CD4⁺ T cells as arthritic TS1(SW)xHACII mice, indicating that B cells are not necessary to maintain the clonotypic CD4⁺ T cell population in this model system.
FIGURE 4-3. B cells support the differentiation of clonotypic CD4+ T cells to promote arthritis development in TS1(SW)xHACII mice. (a) Graphs show total cellularity and percentages of CD4+ and of clonotypic CD4+ T cells in the jdlNs and spleens of female arthritic TS1(SW)xHACII.JH+/- mice (n=8), female arthritic TS1(SW)xHACII.JH+/- mice (n=3), and female non-arthritic TS1(SW)xHACII.JH-/- mice (n=11). *P<0.05, ***P<0.001, one-way ANOVA with Tukey post-test. (b) Dot plots show IFN-γ versus IL-17 staining for CD4+ and for CD4+Vα8.3+Vβ10+ jdlN cells from the strains described in (a) with percentages of cytokine-secreting cells shown. (c) Mean percentages ± SEM of cytokine-secreting CD4+ and CD4+Vα8.3+Vβ10+ jdlN and spleen cells from the mice described in (a) are shown in graphs. *P<0.05, **P<0.01, ***P<0.001, one-way ANOVA with Tukey post-test.
Importantly, we wanted to evaluate whether the representation of cytokine-producing effector CD4\(^+\) T cells were altered in TS1(SW)xHACII.JH\(^{-}\) mice. Within the total CD4\(^+\) T cell population, high frequencies of IFN-\(\gamma\) and IL-17\(^+\) cells were found in both the jdLN and spleens of all sets of mice examined, irrespective of the presence or absence of B cells or disease (Fig. 4-3b and c). Within the clonotypic CD4\(^+\) T cell population, however, we observed significantly lower frequencies of IFN-\(\gamma\)\(^+\) cells in both arthritic and non-arthritic TS1(SW)xHACII.JH\(^{-}\) mice compared to B cell sufficient TS1(SW)xHACII mice. In other words, B cells appeared to support clonotypic IFN-\(\gamma\)-producing CD4\(^+\) T cells; reduced frequencies of these cells, however, did not correlate with disease status. On the other hand, reduced frequencies of clonotypic Th17 cells was observed in non-arthritic TS1(SW)xHACII.JH\(^{-}\) mice but no such reduction of these cells was found in arthritic mice.

Foxp3\(^+\) regulatory T cells (CD4\(^+\)CD25\(^+\)Foxp3\(^+\), Tregs) are regarded as an essential immune subset required for maintaining peripheral self-tolerance (Sakaguchi et al. 2008, Shevach 2009). Therefore, a large focus in the field of Treg biology has been to examine not only how Tregs are generated but also how they are maintained. Of concern to us, several studies have reported that B cells can act to expand and induce Tregs (Chen and Jensen 2007, Morlacchi et al. 2011, Ray et al. 2012). These results, however, seem contradictory to our finding that disease is ameliorated in B cell deficient TS1(SW)xHACII.JH\(^{-}\) mice. Because of this, we examined if the representation of Tregs in TS1(SW)xHACII mice was affected by the elimination of B cells. Firstly, we found that B cell sufficient TS1(SW)xHACII mice contained significantly higher frequencies of Tregs compared to control TS1(SW) mice in both the jdLN and spleens (Fig. 4-4a and b).
FIGURE 4-4. Arthritic TS1(SW)xHACII mice do not lack Tregs. (a) Dot plots show Foxp3 versus CD25 expression on CD4+ cells from the jdLN s and spleens of female TS1(SW), arthritic TS1(SW)xHACII.JH+/−, arthritic TS1(SW)xHACII.JH+−, and non-arthritic TS1(SW)xHACII.JH+− mice. Numbers indicate percentage of CD25+Foxp3+ cells. (b) Graphs show mean percentages ± SEM of CD4+ CD25+Foxp3+ cells from the strains indicated in (a). n= 3–11. *P<0.05, **P<0.01, ***P<0.001, one-way ANOVA with Tukey post-test. (c) Histograms show CFSE levels of CD4+ T cells from TS1(SW) mice following incubation with and without anti-CD3 and with or without Tregs from various sources. Percentage of undivided cells are indicated.
Secondly, we found that the frequency of Tregs in TS1(SW)xHACII.JH−/− mice did not significantly differ from TS1(SW)xHACII mice. Thirdly, arthritic and non-arthritic TS1(SW)xHACII.JH−/− mice were found to contain similar frequencies of Tregs.

Interestingly, arthritis persists in TS1(SW)xHACII mice in the presence of elevated frequencies of Tregs. It is possible, though, that Tregs in arthritic TS1(SW)xHACII mice are dysfunctional. To assess the suppressive capability of Tregs from arthritic TS1(SW)xHACII mice, we performed a classic in vitro Treg suppression assay. CD4+CD25− responder T cells from TS1(SW) mice were CFSE-labeled and cocultured with CD4+CD25− Tregs isolated from either BALB/c or arthritic TS1(SW)xHACII mice in the presence of anti-CD3 stimulation. Responder T cells proliferated robustly in response to anti-CD3 in the absence of Tregs (Fig. 4-4c). The addition of BALB/c Tregs considerably suppressed the proliferation of responder cells. Notably, in this assay, TS1(SW)xHACII Tregs were just as suppressive (and may even be more suppressive) than BALB/c Tregs.

Collectively, these data show that B cells are not required to maintain Tregs in TS1(SW)xHACII mice and an increase in Tregs does not account for the absence of arthritis in the majority of TS1(SW)xHACII.JH−/− mice. On the other hand, B cells were shown to support the differentiation and/or accumulation of IFN-γ- and IL-17-secreting effector CD4+ cells expressing the clonotypic Vα8.3\*Vβ10+ TCR. However, only the reduction of IL-17\*Vα8.3\*Vβ10+ CD4+ T cells significantly correlated with the absence of disease in TS1(SW)xHACII.JH−/− mice. Thus, since the preceding results showed that anti-IL17R blockade can also prevent arthritis, these studies suggest that B cells
promote disease in TS1(SW)xHACII mice at least in part by supporting the formation of pathogenic Th17 cells expressing the autoreactive Vα8.3′/β10′ TCR.

4.2.4 Anti-B cell treatment prevents arthritis development in TS1(SW)xHACII mice

It was possible that the reduced Th1 and Th17 cell frequencies observed in TS1(SW)xHACII.JH+ mice were a consequence of an improperly developed immune system that arose due to the congenital lack of B cells. We therefore examined the effects of eliminating B cells from mice in which B cell development had been allowed to occur. To this end, we treated 5 week old TS1(SW)xHACII mice with anti-CD20 (Hamel et al. 2008) (Fig. 4-5a). Anti-CD20 mAbs target and deplete CD20-expressing B cells, namely immature pre-B cells and all mature B cells except plasma cells (Edwards and Cambridge 2006); of note, anti-CD20 treatment was also found capable of depleting short-lived plasmablasts with autoreactive specificities (Huang et al. 2010). Therefore, we first assessed the efficacy of the reagent in depleting B cells and its effect on serum antibody titers. Indeed, 10 out of 13 TS1(SW)xHACII mice that received anti-CD20 treatment contained little to no CD19+ B cells in the spleens (Fig. 4-5b) and significantly lower concentrations of IgG in the serum (Fig. 4-5c) compared to isotype control-treated mice. Three of the anti-CD20 treated mice were, however, refractive to complete B cell depletion; although the frequencies of splenic CD19+ B cells were significantly reduced in these mice compared to isotype control-treated mice, they were still significantly higher than in the remaining anti-CD20 treated mice (Fig. 4-5b) and so these individuals were designated as "semi-depleted".
FIGURE 4-5. Anti-B cell treatment prevents arthritis development in TS1(SW)xHACII mice. (a) Schematic shows anti-CD20 treatment regimen in TS1(SW)xHACII mice. (b and c) Graphs show percentages of splenocytes that are CD19⁺ (b) and serum IgG concentrations (c) of 14 week-old female TS1(SW)xHACII mice treated either with anti-CD20 mAb (n=10) or isotype control antibody (n=12). A subset that received anti-CD20 mAb but did not achieve complete depletion (“semi-depleted”) is also shown (n=3). **P<0.01, ***P<0.001, one-way ANOVA with Tukey post-test. (d) Graphs show mean percentages of mice from (a) that developed at least one arthritic paw over time (left; *P<0.05, comparison of isotype control-treated to anti-CD20 treated mice, Fisher’s Exact test) and arthritis score of mice at 14 week of age (right; *P<0.05, Mann-Whitney test).
Weekly assessment of disease development in these treated cohorts revealed results similar to what we observed in TS1(SW)xHACII.JH⁻⁺ mice in that the majority of anti-CD20 treated TS1(SW)xHACII mice (10/13) did not develop arthritis (Fig. 4-5d). Interestingly, the number of mice that did develop arthritis after anti-CD20 treatment is the same as the number of mice in which B cell depletion was incomplete (3/13 in both cases). Surprisingly, the anti-CD20 treated mice that developed arthritis were not the "semi-depleted" mice. Indeed, none of the "semi-depleted" mice developed arthritis. The three anti-CD20 treated TS1(SW)xHACII mice that developed disease came from the group of mice in which CD19⁺ B cells and serum IgG had been significantly reduced.

Thus, these results again demonstrate that B cells are an important immune cell population that promotes the development of arthritis in TS1(SW)xHACII mice. Notably, these results support the conclusion that B cells are not required to act as a source of arthritogenic antibodies in this system as: 1) arthritis can develop in TS1(SW)xHACII mice lacking serum IgG and 2) TS1(SW)xHACII mice containing serum IgG do not always develop disease.

4.2.5 Reduced frequencies of clonotypic cytokine-producing CD4⁺ T cells correlated with the absence of disease in anti-CD20 treated TS1(SW)xHACII mice

To assess changes in immune cell populations after anti-CD20 treatment, we performed the same analysis as was previously done in TS1(SW)xHACII.JH⁻⁺ mice. Since the number of "semi-depleted" non-arthritic mice and of anti-CD20 treated arthritic mice were small (n=3 each) and the SEM of data sets from these mice were large, we
only compared data from isotype control-treated TS1(SW)xHACII mice that developed arthritis (n=12) and from anti-CD20 treated TS1(SW)xHACII mice that did not develop arthritis (n=7) (Fig. 4-6a). Similar to the findings in TS1(SW)xHACII.JH⁻/⁻ mice, the depletion of B cells in TS1(SW)xHACII mice through anti-CD20 treatment led to significantly reduced jdLN and spleen cellularities and increased percentages of CD4⁺ T cells; the frequency of CD4⁺ T cells expressing the clonotypic TCR did not differ.

Additionally, we again observed significantly lower frequencies of IFN-γ⁺ and IL-17⁺ cells within the clonotypic CD4⁺ T cell population in both the jdLN and spleens of anti-CD20 treated mice (Fig. 4-6b and c), closely resembling the results from the analysis of congenically B cell deficient TS1(SW)xHACII mice. However, we found significantly lower frequencies of IL-17⁺ and IFN-γ⁺ cells within the total CD4⁺ T cell population the jdLN and spleens, respectively, of anti-CD20 treated mice, which differed from what was observed in TS1(SW)xHACII mice where B cells were congenically ablated. However, the fold reduction of cytokine-producing cells within the total CD4⁺ T cell population was smaller than what occurred in the clonotypic CD4⁺ T cell population. Moreover, the representation of Tregs in the jdLN and spleens of anti-CD20 treated mice were not largely different from that in isotype control-treated mice (Fig. 4-6d).

Collectively, these findings provide additional evidence that B cells support the development or accumulation of cytokine-secreting effector CD4⁺ T cells, especially those expressing the autoreactive clonotypic TCR, in TS1(SW)xHACII mice.
FIGURE 4-6. B cell depletion is accompanied by decreased CD4+ effector T cell formation in non-arthritis TS1(SW)xHACII mice. (a) Graphs show total cellularity and percentages of CD4+ and of CD4+ Vα8.3+ Vβ10+ cells from jdlNs and spleens of arthritic female TS1(SW)xHACII mice that had been treated with isotype control antibody (n=12) and of non-arthritic female TS1(SW)xHACII mice that had been treated with anti-CD20 mAb (n=7). Graphs indicate mean percentages ± SEM. (b) Dot plots show IFN-γ versus IL-17 staining for CD4+ (upper row) and for CD4+ Vα8.3+ Vβ10+ (lower row) cells from the jdlNs of mice described in (a). Numbers indicate percentages of cytokine-secreting cells. (c) Graphs show mean percentages ± SEM of cytokine-secreting cells from the jdlNs and spleens of mice described in (a). *P<0.05, **P<0.01, ***P<0.001, Mann-Whitney test. (d) Dot plots show Foxp3 versus CD25 expression on CD4+ cells from the jdlNs and spleens of mice described in (a). Numbers indicate percentage of CD25+Foxp3+ cells.
4.2.6 TNF neutralization ameliorates arthritis in TS1xHACII mice

Before B cell depletion was utilized as a therapy to treat RA, treatment with anti-TNF was considered the gold standard due to the high patient response rates (Feldmann 2002). Because TNF is elevated in the serum of arthritic TS1xHACII mice and because B cells were found to not be essential in this system, we explored whether targeting the TNF cytokine pathway could affect disease development in this model. Treatment of pre-arthritic TS1xHACII mice with an anti-TNF antibody resulted in a significant reduction in arthritis penetrance; of those individuals that did develop arthritis despite receiving anti-TNF treatment, most exhibited lower arthritis scores than the majority of isotype control-treated mice (Fig. 4-7a and b).

TNF has been shown capable of participating in an inflammatory cytokine cascade which begets further elaboration of inflammatory cytokines and ultimately, joint destruction (Feldmann 2002). It is thought that because of this, the reduced levels of inflammatory cytokines, such as IL-1, IL-6 and IL-8, detected in anti-TNF treated patients (Feldmann 2002) were a direct result of removing TNF signaling. Therefore, we wanted to assess whether the neutralization of TNF in TS1xHACII mice affected serum cytokine levels, particularly in mice that did not develop disease. However, we did not find a global reduction of inflammatory cytokines in the blood of anti-TNF treated mice relative to isotype control-treated mice (Fig. 4-7c). Anti-TNF treated mice that did not develop arthritis contained lower levels of IL-6 relative to arthritic isotype control-treated mice, but not less than in anti-TNF treated mice that developed arthritis.
FIGURE 4-7. Anti-TNF treatment prevents arthritis development in TS1xHACII mice. (a) Schematic shows anti-TNF treatment regimen in TS1xHACII mice. (b) Left graph shows mean percentage of anti-TNF-treated (n=13) and isotype control-treated (n=11) TS1xHACII mice that developed at least one arthritic paw over time. *P<0.05, **P<0.01, ***P<0.001, Fisher’s Exact test. Right graph shows arthritis scores at 14 weeks of age. ****P<0.001, Mann-Whitney test. (c) Graphs show mean concentrations ± SEM of indicated cytokines in the serum of arthritic isotype control-treated, arthritic anti-TNF treated and non-arthritic anti-TNF treated TS1xHACII mice. (n=5-10). *P<0.05, one-way ANOVA with Tukey post-test.
These data show that disease can be significantly attenuated in TS1xHACII mice following treatment with a neutralizing mAb directed against TNF. Thus, although B cells are not necessary for arthritis development in this system, the pathogenic involvement of the inflammatory cytokines TNF and IL-17 (Oh et al. 2012) is evident. The prevention of disease in TS1xHACII mice treated with anti-TNF, however, did not correlate with the reduction of inflammatory cytokines that have been associated with arthritis.

4.2.7 Anti-TNF treatment did not augment Tregs in TS1xHACII mice

Studies in human RA patients have shown that a successful response to anti-TNF treatment can be associated with increased frequencies and/or activity of Foxp3⁺ Tregs (Ehrenstein et al. 2004, Valencia et al. 2006, Nie et al. 2013). We therefore examined the frequency of Tregs in anti-TNF treated TS1xHACII mice that did not develop arthritis versus arthritic TS1xHACII mice that previously received either isotype control or anti-TNF antibodies. As previously reported (Oh et al. 2012) and repeated here, the frequencies of CD25⁺Foxp3⁺ CD4⁺ T cells were higher in both the jdLN and spleens of arthritic TS1xHACII mice than in control TS1 mice (Fig. 4-8a and b). However, no significant differences were found in Treg frequencies between TS1xHACII mice that had received the anti-TNF antibody and TS1xHACII mice that had received the isotype control antibody. Thus, anti-TNF treatment did not augment Treg frequencies in TS1xHACII mice, even in mice that were protected from arthritis development.

We also performed an in vitro Treg suppression assay to evaluate the suppressive activity of Tregs isolated from anti-TNF treated TS1xHACII mice. Tregs
FIGURE 4-8. Anti-TNF treatment does not augment Treg representation or function in TS1xHACII mice. (a) Dot plots show Foxp3 versus CD25 staining on CD4+ cells isolated from the jdlNs or spleens of TS1 mice, arthritic isotype control-treated TS1xHACII mice, arthritic anti-TNF treated TS1xHACII mice, and non-arthritic anti-TNF treated TS1xHACII mice. Percentages of CD25+Foxp3+ cells are shown in plots. (b) Graphs show mean percentages ± SEM of CD4+CD25+Foxp3+ cells from the strains indicated in (a). n=4-8. *P<0.05, **P<0.01, one-way ANOVA with Tukey post-test. (c) Graph shows inhibition of in vitro proliferation of anti-CD3-stimulated responder CD4+CD25- T cells caused by addition of differing ratios of CD4+CD25+ cells isolated from BALB/c mice, from arthritic TS1xHACII mice that received isotype control antibody, or from non-arthritic TS1xHACII mice that received anti-TNF mAb. Data obtained from three independent experiments are shown with means indicated.
sorted from the spleens of control BALB/c, arthritic isotype control-treated TS1xHACII and non-arthritic anti-TNF treated TS1xHACII mice were co-cultured with CTV-labeled CD4⁺CD25⁻ responder cells obtained from arthritic isotype-control treated TS1xHACII mice at various ratios. Compared to BALB/c Tregs (which were used as a positive control), Tregs from both sets of TS1xHACII mice were neither worse nor better at suppressing the proliferation of responder cells in vitro (Fig. 4-8c). Notably, Tregs purified from non-arthritic anti-TNF treated TS1xHACII mice were no better at suppressing the proliferation of responder cells than those from arthritic isotype control-treated TS1xHACII mice.

Collectively, these data argue against the possibility that anti-TNF treatment modulates arthritis development in TS1xHACII mice through an ability to increase the representation or activity of CD25⁺Foxp3⁺ Tregs.

4.2.8 Anti-TNF treatment prevented the accumulation of Th17 cells in the joints, but not in the jdLNs or spleens, of TS1xHACII mice

We next examined if anti-TNF treatment modulates arthritis by affecting the differentiation and/or accumulation of effector CD4⁺ T cells in the jdLN and spleens of TS1xHACII mice. We again segregated data obtained from arthritic and non-arthritic TS1xHACII mice that had all previously received the same anti-TNF treatment regimen. The overall cellularity of the jdLNs (but not of the spleens) was reduced in anti-TNF treated TS1xHACII mice that did not develop arthritis (Fig. 4-9a). Although there were no significant differences in the frequencies of total CD4⁺ T cells between the sets of
FIGURE 4-9. Anti-TNF treatment does not alter the representation of Th1 or Th17 cells in the jdlNs and spleens of TS1xHACII mice. (a) Graphs show total cellularity and percentages of CD4+ and of clonotypic CD4+ T cells in the jdlN and spleens of arthritic female TS1xHACII mice that had been treated with isotype control antibody (n=8), arthritic female TS1xHACII mice that had been treated with anti-TNF (n=4) and non-arthritic female TS1xHACII mice that had been treated with anti-TNF (n=6). Graphs indicate mean percentages ± SEM. *P<0.05, one-way ANOVA with Tukey post-test. (b) Dot plots show IFN-γ versus IL-17 staining for CD4+ (upper row) and for CD4+6.5+ (lower row) joint-draining LN cells from treated mice, with percentages of cytokine-secreting cells shown. (c) Graphs show mean percentages ± SEM of cytokine-secreting cells from the jdlNs and spleens of mice described in (a).
mice examined, a small but statistically significant reduction in the representation of 6.5$^\text{+}$CD4$^+$ T cells was observed in non-arthritic anti-TNF treated mice compared to arthritic isotype control-treated mice. Furthermore, we actually found no significant differences in the representations of Th1 and Th17 cells in both the total and clonotypic CD4$^+$ T cell populations of either sets of anti-TNF treated mice relative to each other or to isotype control-treated mice (Fig. 4-9b and c).

As we could not find robust differences in the CD4$^+$ T cell subsets isolated from the jdLN and spleens of anti-TNF treated TS1xHACII mice that did not develop arthritis, we wanted to examine the immune cell populations within the actual site of inflammation, namely, the joints. The same sets of treated mice as described in Fig. 4-8 and Fig. 4-9 were examined. As might be expected, we found that the overall number of cells isolated from non-arthritic joints were fewer than that from inflamed joints (Fig. 4-10b). One of the largest populations of immune cells found within arthritic joints, CD11b$^+$ neutrophils, was found to be significantly reduced in anti-TNF treated mice that did not develop arthritis (Fig. 4-10a and b). The frequencies of CD19$^+$ B cells and CD4$^+$ T cells were, however, unaffected in anti-TNF treated TS1xHACII mice that did not develop disease relative to both sets of arthritic mice. Notably, the frequency of IL-17-secreting (but not of IFN-\gamma-secreting) CD4$^+$ T cells was significantly reduced in the joints of non-arthritic anti-TNF treated TS1xHACII mice. No such decrease in the accumulation of IL-17-secreting CD4$^+$ T cells or of CD11b$^+$ cells was found in joints of TS1xHACII mice that had developed arthritis, despite having received anti-TNF treatment.
FIGURE 4-10. Anti-TNF treatment leads to impaired recruitment of Th17 cells to the joints of TS1xHACII mice. (a) Histograms (top row) show CD11b staining of CD45+ cells from the joints of arthritic female TS1xHACII mice that had been treated with isotype control antibody, arthritic female TS1xHACII mice that had been treated with anti-TNF, and non-arthritic female TS1xHACII mice that had been treated with anti-TNF. Dot plots (middle row) show CD19 versus CD4 staining of CD45+CD11b+ cells from joints of treated mice, with percentages of CD19+ and CD4+ cells shown. Dot plots (bottom row) show IFN-γ versus IL-17 staining of CD4+ cells from the joints of treated mice, with percentages of cytokine-secreting cells shown. (b) Graphs show average joint cellularities ± SEM of treated mice or mean percentages ± SEM of various immune cell populations isolated from the joints of treated mice. n=3-6. *P<0.05, **P<0.01, one-way ANOVA with Tukey post-test.
Thus, the ability of anti-TNF treatment to prevent arthritis development in TS1xHACII mice was strongly associated with a reduced accumulation of Th17 cells and CD11b+ cells specifically in the joints, since the representation of Th17 cells in the jLN and spleens of these mice was relatively unaffected.

4.2.9 Anti-TNF treatment can modulate arthritis severity in TS1(SW)xHACII mice

Although the elimination of B cells was able to significantly reduce disease penetrance in TS1(SW)xHACII mice, we nonetheless wanted to explore whether the neutralization of TNF could also ameliorate disease development in this system. Utilizing the same treatment protocol that was performed in TS1xHACII mice, we treated pre-arthritic TS1(SW)xHACII mice with either an anti-TNF mAb or an isotype control antibody (Fig. 4-11a). At 14 weeks of age, disease penetrance and severity of anti-TNF treated TS1(SW)xHACII mice was not significantly different from isotype control-treated mice (Fig. 4-11b). Surprisingly, the arthritis that develops in isotype control-treated TS1(SW)xHACII mice scored noticeably lower than in untreated TS1(SW)xHACII mice, although disease penetrance in these two sets of mice were very similar. This seems to only occur in the TS1(SW)xHACII system as the severity of disease observed in untreated and isotype control-treated TS1xHACII mice was not noticeably different (data not shown). By 22 weeks of age, the apparent suppressive effect of the isotype control antibody on disease severity in TS1(SW)xHACII mice seemed to have been overcome. On the other hand, the arthritis scores for anti-TNF treated TS1(SW)xHACII mice at 22 weeks of age remained largely unchanged from when the mice were 14 weeks of age but were significantly lower than the arthritis scores for both age-matched isotype
FIGURE 4-11. Anti-TNF treatment modulates arthritis severity but not Treg representation or function in TS1(SW)xHACII mice.  (a) Schematic shows anti-TNF treatment regimen in TS1(SW)xHACII mice.  (b) Graph shows arthritis scores for untreated TS1(SW)xHACII mice (n=12), isotype control-treated TS1(SW)xHACII mice (n=10) and anti-TNF treated TS1(SW)xHACII mice (n=12) at approximately 14 and 22 weeks of age.  Graphs display scores from individual mice and means ± SEM.  Numbers under graph indicate the percentages of mice that developed at least one arthritic paw by each time point. *P<0.05, **P<0.01, Mann-Whitney test.  (c) Dot plots show Foxp3 versus CD25 staining on CD4+ cells isolated from the jDLNs or spleens of mice described in (a).  Percentages of CD25+Foxp3+ cells are shown in plots.  (d) Graph shows inhibition of in vitro proliferation of anti-CD3-stimulated responder CD4+CD25+ T cells caused by addition of differing ratios of CD4+CD25hi cells isolated from arthritic TS1(SW)xHACII mice that received isotype control antibody or from non-arthritic TS1(SW)xHACII mice that received anti-TNF mAb.  Data obtained from three independent experiments are shown with means indicated.
control-treated and untreated TS1(SW)xHACII mice. Although the penetrance of disease in anti-TNF treated mice did not significantly differ relative to both isotype control-treated or untreated TS1(SW)xHACII mice at 14 and 22 weeks of age, we must point out that of the anti-TNF treated mice that did develop disease, the majority displayed mild swelling in only one paw (arthritis score of 1).

As previously described, a successful response to anti-TNF treatment can be associated with increased frequencies and/or activity of Foxp3+ Tregs (Ehrenstein et al. 2004, Valencia et al. 2006, Nie et al. 2013). Therefore, similar to how Tregs in TS1xHACII mice were analyzed, we examined whether the ability of anti-TNF treatment to modulate disease in the TS1(SW)xHACII model system was through Tregs. Comparable to the findings in TS1xHACII mice, anti-TNF treatment did not seem to enhance either the representation or functionality of Tregs in TS1(SW)xHACII mice (Fig. 4-11c and d).

Collectively, these data demonstrate that anti-TNF treatment was able to attenuate but not block arthritis development in TS1(SW)xHACII mice. Notably, the ability of anti-TNF treatment to suppress disease development in TS1(SW)xHACII mice was not as robust as in TS1xHACII mice since anti-TNF treated TS1(SW)xHACII mice developed disease with reduced severity, but nonetheless, developed disease. Thus, unlike in the TS1xHACII model, TNF in the low affinity system does not appear required for diseased initiation. The modulation of arthritis development in anti-TNF treated (and isotype control-treated) TS1(SW)xHACII mice did not seem to be due to an enhanced Treg population.
4.2.10 Anti-TNF treatment exerted systemic and local effects in TS1(SW)xHACII mice

In an attempt to elucidate what may lead to the attenuation of disease in anti-TNF treated TS1(SW)xHACII mice, we examined the representation of B cells and Th17 cells as both were previously shown to be associated with disease development in this model system. The cellularity of LNs draining the joints in anti-TNF treated TS1(SW)xHACII mice were significantly smaller than in isotype control-treated mice, but this effect did not repeat with splenic cellularities (Fig. 4-12a). Notably, we did not find lower frequencies of either total or clonotypic CD4+ T cells but did find significantly lower frequencies of CD19+ B cells in anti-TNF treated mice relative to isotype control-treated mice (Fig. 4-12b and c). The reduction of B cells was restricted to the jdLNs. We also examined the representation of total and clonotypic CD4+ T cells capable of producing cytokines, despite that the overall frequencies of these T cell populations were unchanged in TS1(SW)xHACII mice after anti-TNF treatment. Although there were lower frequencies of IL-17+ cells within the total CD4+ T cell population in TS1(SW)xHACII mice treated with anti-TNF, the change that has consistently correlated with disease attenuation in the TS1(SW)xHACII model system (reduced clonotypic Th17 cells) was not observed here (Fig. 4-12c).

To complete our analysis, we examined the immune cell populations in the joints of anti-TNF treated and isotype control-treated TS1(SW)xHACII mice. Similar to what was observed in mice from the high affinity model system following treatment with anti-TNF, mice from the low affinity model system contained lower overall numbers of cells, lower frequencies of CD11b+Gr-1+ neutrophils, and lower frequencies of IL-17+CD4+ T
FIGURE 4-12. Anti-TNF treatment reduces the frequencies of CD19+ B cells and Th17 cells in TS1(SW)xHACII mice. (a) Graphs show total cellularity and percentages of CD4+ and of clonotypic CD4+ T cells in the jdlN and spleens of isotype control-treated TS1(SW)xHACII mice (n=9) and anti-TNF treated TS1(SW)xHACII mice (n=12). Graphs indicate mean percentages ± SEM. ***P<0.001, one-way ANOVA with Tukey post-test. (b) Graph shows mean percentages ± SEM of CD19+ B cells from the jdlNs and spleens of mice described in (a). ***P<0.001, Mann-Whitney test. (c) Graphs show mean percentages ± SEM of IFN-γ+ and IL-17+ cells from the jdlNs and spleens of mice described in (a). *P<0.05, Mann-Whitney test.
FIGURE 4-13. Anti-TNF treatment inhibits neutrophil and Th17 cell accumulation in the joints of TS1(SW)xHACII mice. (a) Histograms (top row) show Gr-1 versus CD11b staining of CD45+ cells from the joints of isotype control-treated and anti-TNF treated TS1(SW)xHACII mice. Dot plots (middle row) show CD19 versus CD4 staining of CD45+CD11b- cells from joints of treated mice, with percentages of CD19+ and CD4+ cells shown. Dot plots (bottom row) show IFN-γ versus IL-17 staining of CD4+ cells from the joints of treated mice, with percentages of cytokine-secreting cells shown. (b) Graphs show average joint cellularity ± SEM of treated mice or mean percentages ± SEM of various immune cell populations isolated from the joints of treated mice. n=5-6. *P<0.05, **P<0.01, one-way ANOVA with Tukey post-test.
cells in the joints following anti-TNF treatment (Fig. 4-13a and b). The frequencies of CD19⁺ B cells and of IFN-γ⁺ CD4⁺ T cells were unaffected by anti-TNF treatment; although modest, the reduced frequency of CD4⁺ T cells in anti-TNF treated mice was statistically significant.

Collectively, these data show that although anti-TNF treatment reduced B cells and Th17 cells in the jdLN and significantly prevented the accumulation of neutrophils and Th17 cells in the joints, disease still developed in treated TS1(SW)xHACII mice.

4.2.11 IL-17 can support B cells in TS1(SW)xHACII mice

In light of the findings demonstrating that IL-17 can support autoreactive germinal center B cells in the K/BxN and BXD2 mouse model systems of inflammatory arthritis (Hsu et al. 2008, Wu et al. 2010), we examined if B cells were altered in anti-IL-17R treated TS1(SW)xHACII mice. Indeed, serum IgG concentrations were significantly reduced in anti-IL-17R treated mice relative to isotype control-treated mice (Fig. 4-14a). Serum IgG titers in anti-IL-17R treated TS1(SW)xHACII mice were reduced to the point where they were comparable to the levels found in control TS1(SW) mice. Moreover, we found that the frequency and absolute numbers of total CD19⁺ B cells were significantly reduced in the jdLN of anti-IL-17R treated mice, whereas the frequency and absolute numbers of CD11c⁺ DC, another APC population, appeared unaffected (Fig. 4-14b and c). Notably, CD19⁺ B cells were only reduced in the jdLN but not the spleens of TS1(SW)xHACII mice following anti-IL-17R treatment. It is generally accepted that IL-17 participates in the maturation and recruitment of neutrophils.
FIGURE 4-14. Anti-IL-17R treatment reduced B cells and serum antibodies in TS1(SW)xHACII mice. (a) Graph shows serum IgG concentrations of control TS1(SW) (n=4), isotype control-treated TS1(SW)xHACII (n=3), and anti-IL-17R treated TS1(SW)xHACII (n=6) mice. Graph display concentrations from individual mice and means ± SEM. **P<0.01, one-way ANOVA with Tukey post-test. (b) Contour plots show CD11b versus CD19 staining (top row) and MHCII versus CD11c staining (bottom row) on total jLN and spleen cells isolated from isotype control-treated and anti-IL-17R treated TS1(SW)xHACII mice. Percentages of CD11b^CD19^, CD19^CD11b^ and CD11c^MHCII^ cells are shown in plots. (c) Total cellularities ± SEM and absolute numbers of CD19^, CD11b^ and CD11c^ cells ± SEM found in the jLN and spleens of treated mice are shown in graphs.
(Lubberts et al. 2005, Kolaczkowska and Kubes 2013). This could explain why we also found significantly lower frequencies and absolute numbers of CD11b^+ cells (which were largely neutrophils, data not shown) in anti-IL-17R treated TS1(SW)xHACII mice.

These results led us to question whether B cells and CD11b^+ myeloid cells in TS1(SW)xHACII mice express the receptor for IL-17: IL-17RA (Gaffen 2009). Consistent with published reports, we found IL-17RA expressed on multiple cell types including, but not limited to, CD11b^+/Gr-1^+ cells, B220^+ B cells, germinal center B cells, and CD138^+ plasma B cells (Fig. 4-15). Although we found fewer B cells in the jdLN (but not in the spleens) of TS1(SW)xHACII mice after anti-IL-17R treatment, we did not find a higher fraction of B cells expressing the IL-17 receptor in the jdLN compared to the spleens.

Collectively, these data show that blockade of IL-17 signaling significantly prevented the accumulation of systemic IgG, CD19^+ B cells, and CD11b^+ cells in TS1(SW)xHACII mice. Moreover, we showed that CD11b^+ and B cells can express the IL-17 receptor. Thus, these data suggest that IL-17 can directly support B cells and CD11b^+ myeloid cells in TS1(SW)xHACII mice. Importantly, these data also suggest that Th17 cells and B cells may have reciprocal roles in supporting each other leading up to the development of inflammatory arthritis in the low affinity model system. However, we recognize that anti-IL-17R treatment could have modulated other factors known to stimulate or maintain B cells (such as BAFF or IL-6) (Mackay and Ambrose 2003) and neutrophils (such as G-CSF or IL-8) (Kolaczkowska and Kubes 2013).
FIGURE 4-15. IL-17RA is expressed on multiple immune cell subsets including B cells and CD11b+ cells in TS1(SW)xHACII mice. Top and middle rows: IL-17RA versus CD11b, Gr-1, B220 or CD138 staining on total jdLN or spleen cells from arthritic TS1(SW)xHACII mice. IL-17RA versus PNA staining on B220+IgD-Fas+ cells from the jdLN or spleen of arthritic TS1(SW)xHACII mice. Bottom row: same as in middle row except that cells were stained with an isotype control antibody instead of with anti-IL-17RA.
4.2.12 Assessment of soluble factors and immune cell subsets in non-arthritic TS1(SW)xHACII mice

Based on our current findings, we believe we have identified some factors and cellular populations (mainly IL-17/Th17 cells, B cells and TNF) that are involved in the pathogenesis of disease in TS1(SW)xHACII mice. From this stance, we examined male and female TS1(SW)xHACII mice that did or did not develop arthritis (refer to Fig. 3-3d). Notably, non-arthritic female TS1(SW)xHACII mice contained significantly lower levels of IL-17 and moderately lower levels of IL-6, IL-1B and IFN-\(\gamma\) relative to females that were arthritic, while IgG titers were not significantly different (Fig. 4-16a and b). Although the jdLNs of non-arthritic female mice were moderately smaller than those from arthritic female mice, no significant differences were found in the percentages of CD4\(^+\) T cells (either total or those expressing the clonotypic TCR), of CD4\(^+\)CD25\(^+\)Foxp3\(^+\) cells, of CD4\(^+\)IL-17\(^+\) cells, or of B cells in the jdLNs between arthritic and non-arthritic females (Fig. 4-16c).

Inflammatory cytokine levels, IgG titers, and jdLN cellularities were found to be higher in female TS1(SW)xHACII mice that had developed arthritis compared to arthritic male TS1(SW)xHACII mice, consistent with the more severe inflammatory processes affecting the joints of female versus male TS1(SW)xHACII mice (Fig. 3-4b). We did not find significant difference between the representation of immune cell populations between arthritic male and female mice. It was striking in this analysis, however, that arthritic male mice were in many respects not different from non-arthritic male mice.
Along the same lines, male arthritic mice were not significantly different from female non-arthritic mice, except for possibly the percentage of CD4^{+}IL-17^{+} cells.

We performed these analyses in arthritic and non-arthritic, male and female TS1(SW)xHACII mice in an attempt to find correlates to the absence of disease development. However, the results from these analyses did not yield any clear-cut answers. The overall extent of systemic immune activation was noticeably lower in non-arthritic females compared to arthritic females. However, it is harder to explain the lack of differences between arthritic and non-arthritic males and also between non-arthritic females and males with or without disease. Perhaps comparisons must be run with multiple factors in consideration. Another possibility is that we did not account for other factors at play in this system. Some studies have tried to account for the increased prevalence of autoimmune diseases in females compared to males and have attributed this to the effect of sex hormones. Estrogen appears to be immunostimulatory whereas testosterone appears immunosuppressive and men who develop RA were found to have higher levels of estrogen and low levels of testosterone (Roden et al. 2004, Zandman-Goddard et al. 2007, Quintero et al. 2012). Because disease develops with a female bias in this model system, the level of female sex hormones (or even the balance between female and male sex hormones) may also factor into whether one littermate develops disease compared to the other and the differences observed between female and male TS1(SW)xHACII mice.
FIGURE 4.16. Assessment of serum cytokines, serum IgG and immune cell populations in arthritic and non-arthritic TS1(SW)xHACII mice. (a) Graphs show mean concentrations ± SEM of indicated cytokines in the serum of male and female TS1(SW)xHACII mice with and without arthritis (arthritic female, n=17; non-arthritic female, n=7; arthritic male, n=5; non-arthritic male, n=8). *P<0.05, **P<0.01, one-way ANOVA with Tukey post-test. (b) Graph shows the mean concentration and individual levels of serum IgG in male (M) and female (F) TS1(SW)xHACII mice with and without arthritis (n=5-8). *P<0.05, **P<0.01, one-way ANOVA with Tukey post-test. (c) Graphs show average numbers or percentages ± SEM of joint-draining LN (jdLN) cells or of indicated subpopulations isolated from female arthritic (n=9), female non-arthritic (n=7), male arthritic (n=5) and male non-arthritic (n=9) TS1(SW)xHACII mice. **P<0.01, ***P<0.001, one-way ANOVA with Tukey post-test.
4.3 Discussion

In the previous chapter, we clearly demonstrate that the affinity with which an autoreactive CD4$^+$ T cell recognizes a single target autoantigen not only dictates the extent of arthritis development but also the degree of systemic immune activation. In this chapter, we wanted to explore whether the effect of varying an autoreactive CD4$^+$ T cell response extends past affecting disease susceptibility to also influence the pathways by which disease develops in TS1xHACII and TS1(SW)xHACII mice.

As previously reported (Rankin et al. 2008) and repeated here, B cells are not required for arthritis development in TS1xHACII mice. This was surprising since TS1xHACII mice contained B cells exhibiting an activated phenotype (Rankin et al. 2008) and contained elevated levels of serum IgG, more so than in TS1(SW)xHACII mice (Fig. 3-10b). The extent of B cell activation in TS1xHACII mice seems to reflect the affinity with which autoreactive CD4$^+$ T cells recognize the S1 self-antigen presented by B cells, leading to reciprocal activation and help for B cells to class switch (Rankin et al. 2008). Nonetheless, it appears that the presentation of the S1 self-peptide by other APC subsets in this system was sufficient to drive disease. Not only are B cells and their products dispensable for disease initiation in TS1xHACII mice, they do not seem to be required to perpetuate disease as disease develops with similar penetrance and severity in TS1xHACII.JH$^{+/-}$ and TS1xHACII mice. Interestingly, disease onset was markedly accelerated in TS1xHACII.JH$^{+/-}$ mice, which suggests that B cells in this system may play a protective role. Indeed, B cells have a newly appreciated regulatory role: a subset of B cells capable of producing IL-10 (termed Bregs) have been identified and shown capable of down-modulating the extent of systemic activation in several settings.
(Fillatreau et al. 2002, Mizoguchi et al. 2002, Mauri et al. 2003). Thus, it may be of interest to examine if pre-arthritic TS1xHACII mice contain Bregs or if disease can be modulated with transfer of Bregs or IL-10.

Unlike some other models of inflammatory arthritis (Stuart and Dixon 1983, Korganow et al. 1999, Hsu et al. 2008), the requirement for B cells in TS1(SW)xHACII mice did not appear to reflect a prominent contribution of arthritogenic antibodies. Autoantibody specificities associated with arthritis in other settings (Hueber et al. 2005, McInnes and Schett 2011) were not elevated in the serum of TS1(SW)xHACII mice relative to TS1xHACII and control TCR single transgenic mice. Moreover, unlike serum isolated from K/BxN mice, serum from arthritic TS1(SW)xHACII mice did not transfer disease. Also in line with this supposition, “semi-depleted” anti-CD20 treated TS1(SW)xHACII mice (where B cell depletion was incomplete and so elevated levels of serum IgG were still found in these mice) did not develop arthritis, whereas a few anti-CD20 treated mice (containing little to no serum IgG) did develop arthritis. We understand, however, that these findings are circumstantial and cannot completely rule out a role for antibodies in this model system. Although we performed ELISAs to detect autoantibody specificities associated with arthritis, we may have not examined the right specificity, for example, anti-GPI - the pathogenic antibody specificity in K/BxN mice (Korganow et al. 1999). It is also possible that serum from arthritic TS1(SW)xHACII mice is able to transfer disease, but since the concentration of arthritogenic immunoglobulin(s) is low, not enough was transferred in the experiments previously performed. Notably, provision of TS1(SW)xHACII mice with an exogenous antibody (an isotype control IgG2a mAb) was able to delay (but not prevent) disease progression. In line with this observation, a therapy developed for some autoimmune disorders, called
IVIG, involve the transfer of exogenous polyclonal IgG into the patient (Pyne et al. 2002, Siberil et al. 2007). Several proposed mechanisms exist for how IVIG works; one of these is that the exogenous IgG saturates activating Fc receptors, limiting the binding of endogenously created immune complexes. Therefore, it would be interesting to examine the amount of immune complexes before and after isotype control-treatment. Also, since each IgG subclass binds and triggers Fc receptors differently, it might of interest to investigate the concentrations of each subclass of IgG in TS1(SW)xHACII mice. Because the contribution of antibodies to disease development in TS1(SW)xHACII mice remains unclear, we propose that an experiment better designed to probe the contribution of autoantibodies would be to cross TS1(SW)xHACII mice with the mlgM mouse developed by Mark Shlomchik in which B cells express but do not secrete their B-cell receptor as soluble immunoglobulin (Chan et al. 1999). Of note, B cells in mlgM mice express a limited BCR repertoire as these mice were generated with a fixed V\textsubscript{H} transgene. Thus, B cells in mlgM mice should be capable of presenting internally synthesized peptides complexed with MHCII on their surface normally but will have a limited capability to uptake and present external peptides. Results from these experiments should, therefore, be interpreted carefully.

Rather than act as a source of pathogenic autoantibodies, B cells appear to contribute to disease development in TS1(SW)xHACII mice by participating as APCs for Th17 cells expressing the autoreactive V\textalpha{}8.3/V\textbeta{}10 TCR. In B cell deficient TS1(SW)xHACII mice, significantly reduced frequencies of clonotypic Th17 cells was associated with the absence of disease development. Since B cells represent a large APC population, removal of this population would remove a large source of S1 antigen.
Therefore, although the overall representation of clonotypic CD4^{+} T cells was not significantly different in B cell deficient and B cell sufficient TS1(SW)xHACII mice, it is possible that limited access to antigen can result in a reduced ability of CD4^{+} T cells to differentiate into the Th17 lineage. However, it is also possible that B cells in this system act as unique APCs for the generation of IL-17^{+}V_{a}8.3^{+}V_{b}10^{+} CD4^{+} T cells. This is interesting because S1 expression in HACII mice is directed to all MHCII^{+} APCs (Reed et al. 2003). Nonetheless, these results suggest that the expression of the S1 self-antigen specifically on B cells is important in supporting the development of pathogenic Th17 cells. Although several studies have highlighted the unique contribution of B cells as APCs in several settings (including autoimmunity) (Chan et al. 1999, O'Neill et al. 2005, Crawford et al. 2006, Hamel et al. 2008, Wilson et al. 2012), not much is known on how B cells can specifically support Th17 cells (Hamel et al. 2008, van de Veerdenk et al. 2011, Wang and Rothstein 2012). Therefore, these studies demonstrate a unique finding that B cells, expressing a known target autoantigen, can play an essential role in the differentiation of autoreactive CD4^{+} T cells into the Th17 lineage.

Interestingly, B cells and Th17 cells may play reciprocal roles in supporting each other in TS1(SW)xHACII mice. In B cell deficient mice, lower frequencies of clonotypic Th17 cells were found; in anti-IL-17R treated mice, lower frequencies of B cells (and serum IgG concentrations) were found. Notably, CD19^{+} B cells were only reduced in the jDLNs, but not the spleens, of TS1(SW)xHACII mice following anti-IL-17R treatment. The expression of IL-17RA, however, was not limited to B cells in the jDLNs or specifically upregulated by B cells in the jDLNs. These pieces of data together suggest that IL-17 can directly support B cells in the jDLNs of TS1(SW)xHACII mice. Moreover,
these data imply that IL-17 may be a necessary factor for jdLN-associated B cells but other survival/growth factors (such as BAFF and IL-6) (Mackay and Ambrose 2003) can compensate for the absence of IL-17 signaling in splenic B cells. Interestingly, these results recapitulate findings in other mouse models of inflammatory arthritis whereby B cells significantly contribute to disease pathogenesis (Stuart and Dixon 1983, Korganow et al. 1999, Hsu et al. 2008).

Neutralization of TNF was able to significantly ameliorate arthritis development in TS1xHACII mice but only able to modulate disease severity in TS1(SW)xHACII mice. It was apparent that the ability of anti-TNF treatment to suppress disease in TS1xHACII mice was much more pronounced. The available literature examining the contribution of TNF in inflammatory arthritis is expansive and exemplifies the pleiotropic role the cytokine can play during disease development (Feldmann 2002). However, in TS1xHACII mice, anti-TNF treatment was not found to modulate serum cytokines levels, increase the representation/functionality of Tregs, or even prevent the differentiation of Th1/Th17 cells. Instead, in anti-TNF treated mice that did not develop disease (protected mice), the accumulation of Th17 and CD11b+ neutrophils in the joints was prevented. Taking into account that TNF has been shown capable of inducing the expression of CCL20 (the ligand for CCR6) in synoviocytes (Chabaud et al. 2001, Hirota et al. 2007), we propose the following mechanism by which TNF contributes to arthritis development in TS1xHACII mice: TNF, possibly working in concert with IL-1B and IL-17, induces joint-associated synoviocytes to upregulate the production of the chemokine CCL20. This in turn attracts Th17 cells bearing the receptor for CCL20, CCR6. Lastly, IL-17 secreted by Th17 cells that have accumulated in the joints may then attract
neutrophils capable of joint destruction. Thus, TNF appears to play a specific and possibly limited role in promoting arthritis development in TS1xHACII mice.

In anti-TNF treated TS1(SW)xHACII mice, we observed lower frequencies of B cells and IL-17^+ CD4^+ T cells in the secondary lymphoid organs. Moreover, anti-TNF treatment significantly prevented the accumulation of neutrophils and Th17 cells in the joints, similar to what occurred in anti-TNF treated TS1xHACII mice. It is unclear whether the reductions in B cells and Th17 cells seen in the jdLN and spleens of anti-TNF treated mice are required for disease attenuation in this system or whether they were a bystander consequence of a reduced inflammatory response in the mouse. Despite both systemic and local effects of the anti-TNF mAb, disease still developed in the majority of treated mice. Interestingly, although the recruitment of neutrophils and Th17 cells were significantly prevented in anti-TNF treated TS1(SW)xHACII mice, the majority of them still developed at least one swollen limb. This suggests that TNF may play an additional role besides that of recruiting cells into the joints in this model system, which is in contrast with what is found in TS1xHACII mice. It is possible, in this setting, that other pro-inflammatory cytokines such as IL-6 or IL-1 can produce a low grade inflammation in the absence of TNF, but TNF is necessary to synergize and perpetuate inflammation to progress inflammatory arthritis. If this is the case, it would suggest that TNF exerts multiple modes of action to drive inflammatory arthritis in TS1(SW)xHACII mice.

Thus, we believe we have demonstrated that the degree of CD4^+ T cell reactivity for self-peptide can play a prominent role in determining the cellular pathways that participate in the development of inflammatory arthritis. Through analysis of B cell
deficient mice and mice treated with various anti-cytokine antibodies, we have shown that TNF, but not B cells, is required for arthritis development in the TS1xHACII model system. This suggests that B cells are not necessary to either promote or act as a target of TNF in this model system. On the other hand, B cells significantly contribute to disease development in TS1(SW)xHACII mice; TNF appears to contribute to enhancing disease severity in this model but is not required for disease initiation. Thus, pathways by which disease develops in TS1xHACII and TS1(SW)xHACII mice are fundamentally different.

As shown very early on in Chapter 3, mice generated with the low affinity TCR for the S1 self-peptide (TS1(SW)xHACII mice) develop disease with a female bias. Interestingly, how disease develops in this low affinity model system is better at recapitulating RA development in humans (women develop RA 3 times more frequently than men) (Quintero et al. 2012). The sex bias observed in TS1(SW)xHACII mice suggests that gender-associated factors such as sex hormones may play a role in this model system. Indeed, in DR4.AE° mice (a humanized HLA-DR4 mouse model of induced inflammatory arthritis), arthritis develops with a female bias, but this bias was largely overcome when males were castrated, implicating the protective role of male hormones (Behrens et al. 2010). Additionally in the NZBxNZW F1 model of lupus, which develops lupus with a strong female bias, treatment with tamoxifen (an estrogen receptor modulator) attenuates disease in female mice (Sthoeger et al. 2003). Therefore, these findings suggest that the extent of the CD4+ T cell autoreactive response to self-antigens can also dictate the contribution of sex hormones in disease development.
Tregs constitute an important mechanism of peripheral tolerance induction and therefore are studied extensively in autoimmune disease settings (Sakaguchi et al. 2008, Shevach 2009). Because of this, we found it surprising that arthritic TS1xHACII and arthritic TS1(SW)xHACII mice contained high frequencies of CD4⁺ T cells expressing CD25 and Foxp3; moreover, the expression levels of CD25 and Foxp3 were not abnormal in arthritic mice compared to control mice. Furthermore, CD4⁺CD25⁺ Tregs isolated from arthritic mice of both lineages suppressed just as well as BALB/c Tregs when their functionality was compared side-by-side in *in vitro* suppression assays. From the studies performed in this chapter, the modulation of disease following treatment in each model system did not associate with an apparent enhancement in the representation or function of Tregs. It is possible that the suppression assays we performed were unable to reveal a difference (either supporting or negating Treg functionality) since we used anti-CD3 to stimulate both responders and Tregs or that the ratios of Tregs to responders used were not physiologically representative. It is also possible that effector T cells in these model systems are resistant to Treg suppression. We do not believe this to be the case since the transfer of polyclonal Tregs from wild-type mice can ameliorate disease development in both TS1xHACII and TS1(SW)xHACII mice ((Oh et al. 2012) and data not shown). Lastly, it is possible that Tregs in these two systems are not appropriately “polarized”. Recent work has demonstrated that for efficient suppression of CD4⁺ T cells of a certain T helper lineage, Tregs should adopt the same transcription factor profile (Campbell and Koch 2011). Because of this, it would be interesting to examine if we can find the presence (or lack of) Tregs in TS1xHACII and TS1(SW)xHACII mice that display a Th-17-like phenotype or enhanced STAT3 signaling (Chaudhry et al. 2009, Duhen et al. 2012). The data we show here,
however, suggest that the modulation of Tregs is not a requirement for treatment regimens to work in TS1xHACII or TS1(SW)xHACII mice.

In summary, the studies performed in this chapter demonstrate that the overall reactivity of the CD4$^+$ T cell response to a ubiquitously expressed self-peptide can play a prominent role in determining the cellular pathways that participate in the development of inflammatory arthritis. In TS1xHACII mice, an autoreactive CD4$^+$ T cell response to the S1 self-peptide was measurable in an autologous MLR, and serum in these mice contained elevated levels of both pro-inflammatory cytokines and immunoglobulin. While arthritis development could be prevented by the anti-cytokine treatments (namely anti-IL-17 and anti-TNF), it was not affected by B cell elimination. By contrast, serum cytokine levels were lower in TS1(SW)xHACII mice in a setting whereby the peripheral CD4$^+$ T cell repertoire was less responsive to the S1 self-peptide, and in this case, elimination of B cells significantly suppressed arthritis development but TNF neutralization only affected the severity with which disease develops. Notably, both anti-TNF treatment and B cell elimination appeared to prevent arthritis development in these two models at least in part by disrupting Th17 cell activity, albeit by distinct mechanisms. Collectively, these studies demonstrated that fundamentally different pathways of pathogenesis exist in TS1xHACII and TS1(SW)xHACII mice which can be attributed to the inherent reactivity of CD4$^+$ T cells in response to self-antigen in each model system. This may explain why therapies targeting distinct pathways have different efficacies in different patients.
Chapter 5: Discussion and Speculation

5.1 Impact of CD4+ T cell affinity on the development of inflammatory arthritis

It is well established that CD4+ T cells can significantly contribute to the development of autoimmune arthritis. Numerous studies examining the roles of CD4+ T cells in autoimmune settings emphasize their extensive capabilities to initiate and maintain disease. To elaborate their effector function(s), autoreactive CD4+ T cells must first be stimulated through their TCR. The spontaneous development of inflammatory arthritis in mice engineered to co-express a transgenic TCR with a known target autoantigen (like K/BxN and TS1xHACII mice) and the induced development of arthritis in mice with certain MHCII alleles (like CIA and PGIA), highlight the importance of antigen recognition by CD4+ T cells in the appropriate context in order to incite autoimmunity. Therefore, our studies were aimed at examining how CD4+ T cell recognition of a known, target autoantigen can shape the development of inflammatory arthritis. We performed these studies using the TS1xHACII mouse model of arthritis (Rankin et al. 2008) as our basis; we then modified the affinity of the transgenic TCR for the fixed target autoantigen to generate TS1(SW)xHACII mice. Briefly, TS1xHACII mice were generated to co-express PR8 HA as a surrogate self-antigen and a transgenic TCR that recognizes the S1 determinant of PR8 HA as a cognate antigen (Fig. 3-1a). We then fixed the autoantigen (PR8 S1) but changed the transgenic TCR that was co-expressed into one that recognizes S1 with low affinity to generate TS1(SW)xHACII mice (Fig. 3-3a). We demonstrated in Fig. 3-2 that the TS1(SW) TCR does indeed
recognize the S1 antigen expressed in HACII mice with low affinity. In using these two variant model systems – TS1xHACII and TS1(SW)xHACII mice – we could begin to assess how a CD4⁺ T cell response to a single target antigen can influence inflammatory arthritis development.

Overall assessment of TS1xHACII and TS1(SW)xHACII mice revealed that both of these lineages were capable of developing overtly identifiable manifestations of inflammatory arthritis (Fig. 3-1b and 3-3b). The elaboration of a destructive joint-targeted disease in these mice was further confirmed by histology (Fig. 3-4). Interestingly, although mice from both TS1xHACII and TS1(SW)xHACII lineages were found to display swollen paws, a female bias emerged in cohorts of TS1(SW)xHACII but not TS1xHACII mice (Fig. 3-1c and Fig. 3-3d). Not only did fewer male TS1(SW)xHACII mice develop disease, they also exhibited a significant delay in disease onset. Additionally, joint sections from male TS1(SW)xHACII mice that did develop disease scored significantly lower for synovitis and articular degeneration compared to arthritic female mice. These findings demonstrate that the affinity with which CD4⁺ T cells recognize a single target antigen can clearly influence the development of inflammatory arthritis with regard to disease penetrance and severity.

In both TS1xHACII and TS1(SW)xHACII mice, CD4⁺ T cells bearing the autoreactive TCR were subjected to thymic deletion; however, central tolerance was incomplete as a subset of these cells can be found in the LNs and spleens of both sets of mice (Fig. 3-2d and Fig. 3-6). The clonotypic CD4⁺ T cells that managed to escape thymic deletion appeared activated and capable of producing inflammatory cytokines (Fig. 3-6 and Fig. 3-9). If we just used these readouts as a measurement of their
autoreactivity, one might roughly estimate that the overall effector contribution of 6.5\(^{+}\)CD4\(^{+}\) and V\(\alpha\)8.3\(^{+}\)CD4\(^{+}\) T cells in each respective system might be comparable. This is taking into account that both 6.5\(^{+}\)CD4\(^{+}\) and V\(\alpha\)8.3\(^{+}\)CD4\(^{+}\) T cells expressed markers of antigen experience at levels higher than control mice (although these were generally higher on 6.5\(^{+}\)CD4\(^{+}\) T cells than on V\(\alpha\)8.3\(^{+}\)CD4\(^{+}\) T cells) and that subsets of both 6.5\(^{+}\)CD4\(^{+}\) and V\(\alpha\)8.3\(^{+}\)CD4\(^{+}\) T cells appeared capable of producing IFN-\(\gamma\) or IL-17 (the percentages of which were not dramatically dissimilar). However, from performing an autologous MLR, we discovered that CD4\(^{+}\) T cells isolated from TS1xHACII mice were appreciably more autoreactive than CD4\(^{+}\) T cells isolated from TS1(SW)xHACII mice (Fig. 3-7). It appears that the autoreactive CD4\(^{+}\) T cells that managed to escape thymic deletion in TS1xHACII mice maintained a relatively high level of reactivity as compared to those from TS1(SW)xHACII mice. In line with this finding, the extent of systemic activation (as determined by systemic cytokine levels and IgG titers) in TS1xHACII mice was not only considerably higher than in TS1(SW)xHACII mice, it was extensive (Fig. 3-10). A wide range of cytokines were elevated in the serum of TS1xHACII mice, derived from both T cells and non-T cells. Additionally, TS1xHACII mice contained significantly higher concentrations of IgG than both control and TS1(SW)xHACII mice. These results suggest that the affinity of the T cell-APC interaction, and the subsequent downstream effects of this (i.e. production of cytokines and chemokines that activate surrounding immune and non-immune cells) directly influences the extent of systemic immune activation (Ahlers et al. 2001, Diepolder et al. 2001). Thus, not only does the affinity with which autoreactive CD4\(^{+}\) T cells recognize a single target antigen influence arthritis susceptibility, there exists a positive correlation with how it affects the extent of systemic immune activation.
Our last major finding in assessing TS1xHACII and TS1(SW)xHACII mice was that differential, and shared, pathways of disease pathogenesis exist between these two models. Briefly, the pro-inflammatory cytokines IL-17 (Oh et al. 2012) and TNF\(\alpha\) were important for disease development in TS1xHACII mice, whereas B cells and their products were not required (Fig. 4-7b and Fig. 4-1a). IL-17 was also required for disease development in TS1(SW)xHACII mice (Fig. 3-11a); TNF appeared to influence the severity with which disease develops in this system but seemed unnecessary for disease initiation (Fig. 4-11b). Strikingly, in the context of a low affinity, autoreactive TCR, B cells were found necessary for arthritis to develop in TS1(SW)xHACII mice (Fig. 4-1b). A more detailed analysis of how we think each factor promotes disease in TS1xHACII and TS1(SW)xHACII mice will be discussed in the following sections. Collectively, our findings reveal that the pathways by which disease develops in TS1xHACII and TS1(SW)xHACII mice are profoundly different, demonstrating the prominent influence that the degree of CD4\(^+\) T cell autoreactivity has on directing the pathways by which inflammatory arthritis develops.

The comparison of serum cytokine levels and serum IgG titers in arthritic TS1xHACII and TS1(SW)xHACII mice were immediately surprising to us (Fig. 3-10). Not in part because these results positively correlated with the autoreactivity of CD4\(^+\) T cells found in each system, but that these two sets of mice, which developed a very similar form of autoimmune disease, displayed such a profoundly different systemic profile. Arthritic TS1xHACII mice contained elevated levels of a variety of cytokines, which was largely absent in arthritic TS1(SW)xHACII mice. Additionally, although elevated levels of IgG were found in the serum of TS1(SW)xHACII mice compared to
controls, even higher levels were observed in TS1xHACII mice. These results suggest that overt systemic inflammation is not a requisite for inflammatory arthritis development. Indeed, blockade of a single cytokine (IL-17 in particular) can ameliorate arthritis onset in both TS1xHACII and TS1(SW)xHACII mice ((Oh et al. 2012) and Fig. 3-11a)). However, we cannot rule out the possibility that in these two model systems, IL-17 sits at the top of a cytokine cascade or that IL-17 can drive arthritis development by different means. It may be worthwhile to assess the serum cytokine profiles of anti-IL-17/anti-IL-17R treated mice. Strikingly, B cells and their products were not required for arthritis to develop in TS1xHACII mice (Fig. 4-1a), despite containing B cells with an activated phenotype and exhibiting hypergammaglobulinemia ((Rankin et al. 2008) and Fig. 3-10b)). Collectively, these results argue that, although high affinity CD4⁺ T cells upon recognition of self-antigen can elicit extensive systemic immune activation, this may not be required for the development of inflammatory arthritis.

Historical work examining TCR stimulation strength and effects on T cell fate demonstrated that high affinity CD4⁺ T cells were eventually deleted or anergized following activation and proliferation (Oki and Sercarz 1985, Forster et al. 1995, Rocha et al. 1995, Yamamoto et al. 2007). Similarly with CD8⁺ T cells, high amounts of antigen promote T cell exhaustion (Wherry et al. 2003). Therefore, it might be surprising that TS1xHACII mice, which contain a high affinity TCR for self-antigen, develops a more severe form of autoimmunity than TS1(SW)xHACII mice. However, we must take into consideration that the highest self-reactive clones were most likely deleted in the thymus before entering the periphery since CD4⁺ T cells were found to express low 6.5 TCR surface densities (Rankin et al. 2008). These observations were reasoned to not be a virtue of allelic inclusion as the CD4⁺ T cells that escape negative selection to be found
in the periphery of TS1xHACII.RAG\(^+\) mice (in which only the transgenic TCR can be expressed) also express low 6.5 TCR surface densities. Moreover, the overall reactivity of HA-specific CD4\(^+\) T cells found in the periphery of TS1xHACII mice was considerably reduced compared to HA-specific CD4\(^+\) T cells isolated from TS1 mice (where TS1 T cells have not experience their cognate antigen) (compare Fig. 3-2d to Fig. 3-7). Thus, reduced TCR levels allowed for self-reactive 6.5\(^+\)CD4\(^+\) T cells to escape negative selection in TS1xHACII mice, and possibly have allowed them to escape peripheral mechanisms of tolerance such as deletion or functional anergy.

5.2 Cellular pathways underlying arthritis development dictated by the extent of CD4\(^+\) T cell autoreactivity in TS1xHACII and TS1(SW)xHACII mice: Updated working models

From our findings in B cell deficient and anti-cytokine treated mice, we have constructed working models of how various factors and cellular populations contribute to the pathways of pathogenesis in TS1xHACII and TS1(SW)xHACII mice (Fig. 5-1).

The current working model for how disease develops in TS1xHACII mice has been cultivated through work performed by previous lab members (Rankin et al. 2008, Oh et al. 2012, Simons et al. 2013) and from work performed in this thesis. In the thymii of TS1xHACII mice, CD4\(^+\) T cells bearing the clonotypic TCR recognize the S1 self-antigen with high affinity and are thus subjected to extensive thymic deletion. However, central tolerance is incomplete as we can find a small population of 6.5\(^+\)CD4\(^+\) T cells in both the LNs and spleens of young, pre-arthritic TS1xHACII mice. These high affinity,
FIGURE 5-1. Development of inflammatory arthritis in TS1xHACII and TS1(SW)xHACII mice: Updated working models
autoreactive 6.5\(^+\)CD4\(^+\) T cells then interact with HA-expressing APCs, leading to the reciprocal activation of both T cells and APCs. Activated B cells proceed to differentiate into plasma cells and secrete high levels of IgG. We believe the T cell-APC interaction in TS1xHACII mice also promote the development of Th17-trophic inflammatory monocytes which may induce the formation of pathogenic Th17 cells. Indeed, arthritis in this system develops by an IL-17-dependent mechanism as blockade of this cytokine significantly abrogates disease onset. Furthermore, disease onset in these mice could also be significantly blocked by anti-TNF treatment, but not by B cell elimination, despite containing activated B cells and high serum titers of IgG. Examination of arthritic and non-arthritic anti-TNF treated TS1xHACII mice revealed that protection from disease development did not appear to be due to the treatment’s ability to inhibit the production of inflammatory cytokines or the formation of Th1 and Th17 cells. Instead, in positive responders, anti-TNF treatment appears to prevent the specific accumulation of Th17 cells in the joints. We believe it is the accumulation of Th17 cells (whether through recruitment or local proliferation) that anti-TNF treatment prevents and not their formation since non-arthritic anti-TNF treated mice contained the same frequency of Th17 cells in the jdLN's and spleens as arthritic anti-TNF treated mice. We postulate that one mechanism by which TNF promotes disease in this model is by inducing synoviocytes to express the chemokine CCL20 which then attracts pathogenic Th17 cells (Chabaud et al. 2001, Hirota et al. 2007). Joint-localized Th17 cells would in turn secrete IL-17 to attract CD11b\(^+\) neutrophils capable of producing MMPs and reactive oxygen species (ROS) that directly lead to cartilage and bone destruction (Kolaczkowska and Kubes 2013).
We next describe our working model for how disease develops in TS1(SW)xHACII mice from results described in this thesis: Clonotypic CD4\(^+\) T cells were also subjected to thymic deletion but were again found to populate the LNs and spleens of young, pre-arthritic TS1(SW)xHACII mice. Most likely through interactions with HA-expressing APCs, self-reactive CD4\(^+\) T cells in this system acquire an activated phenotype and the ability to produce IFN-\(\gamma\) and IL-17. Notably, we found that disease develops by an IL-17-depedent mechanism in this model system as well, similar to TS1xHACII mice. However, in contrast to TS1xHACII mice, we found that B cells significantly contributed to disease development in this low affinity model system. Although elevated levels of serum IgG could be found in arthritic TS1(SW)xHACII mice relative to control mice, we do not believe disease is driven in TS1(SW)xHACII mice by arthritogenic autoantibodies. Instead, it appears that B cells function as APCs to promote the formation of clonotypic effector CD4\(^+\) T cells. Notably, the absence of disease in B cell deficient TS1(SW)xHACII mice was associated with a significant reduction of clonotypic Th17 cells. Thus, our findings suggest that B cells may promote arthritis development in TS1(SW)xHACII mice at least in part through an ability to support the differentiation and/or accumulation of clonotypic Th17 cells. Moreover, we found that TS1(SW)xHACII mice contained significantly fewer CD19\(^+\) B cells specifically in the jdLNs following anti-IL-17R treatment and that B cells (among other immune cell populations) in TS1(SW)xHACII mice can express the receptor for IL-17. Collectively, these findings lead us to postulate that B cells and Th17 cells can directly support each other to promote disease pathogenesis in this system. Lastly, we found that anti-TNF treatment has disparate effects in TS1(SW)xHACII mice compared to TS1xHACII mice. In striking contrast to TS1xHACII mice, arthritis onset was not prevented in anti-TNF
treated TS1(SW)xHACII mice, although the severity of the disease that developed was appreciably less severe relative to control mice (both isotype control-treated and untreated TS1(SW)xHACII mice). Thus TNF neutralization was also capable of modulating disease in TS1(SW)xHACII mice, but the therapeutic benefits of the treatment in this system were less prominent than in the TS1xHACII system. Also in contrast to TS1xHACII mice, effects were observed within the jdLNs in addition to the joints of anti-TNF treated TS1(SW)xHACII mice. The accumulation of B cells and Th17 cells in the jdLNs and the accumulation of neutrophils and Th17 cells in the joints were significantly reduced in TS1(SW)xHACII mice following anti-TNF treatment; whether these events are causally related or not has not yet been determined. However, we propose that in this setting anti-TNF treatment may prevent the accumulation of B cells and Th17 cells in the jdLNs by possibly disturbing their reciprocal support network, reducing the pool of Th17 cells available to be recruited to the joints. The multiple effects of anti-TNF treatment observed in TS1(SW)xHACII mice, however, appeared insufficient to completely prevent disease onset. It is possible, in this setting, that other pro-inflammatory cytokines such as IL-6 or IL-1 can produce a low grade inflammation in the absence of TNF which may be sufficient to initiate and maintain a mild form of arthritis, but in the presence of TNF, synergize to enhance inflammation and the severity of disease. If this is the case, it would suggest that TNF can play shared, but also disparate, roles dictated by the context of the CD4⁺ T cell response to self-antigen.

Notably, TS1xHACII and TS1(SW)xHACII mice develop disease in the presence of high frequencies of Tregs (Fig. 4-8a and Fig. 4-4a). We have previously demonstrated that Tregs purified from arthritic mice are as capable as BALB/c Tregs in suppressing the proliferation of responder cells in vitro (Fig. 4-8c and Fig. 4-4c).
Moreover, we demonstrated that effective disease-suppressive manipulations performed in either TS1xHACII or TS1(SW)xHACII mice (anti-TNF treatment and B cell elimination, respectively) did not enhance Treg frequencies or Treg suppressor function (Fig. 4-4, 6, 8, 11). These results would suggest that disease in TS1xHACII and TS1(SW)xHACII mice cannot be modulated through Tregs. In spite of this, we do not believe that to be the case as provision of Tregs from wild-type BALB/c mice into both TS1xHACII and TS1(SW)xHACII mice can inhibit arthritis onset (Oh et al. 2012 and data not shown). This demonstrates that effector cells in TS1xHACII and TS1(SW)xHACII mice are indeed able to be suppressed by Tregs, just not by the Tregs currently present in the mouse. Thus, we believe that the development of arthritis in both TS1xHACII and TS1(SW)xHACII mice is in part mediated by Treg insufficiency. We currently favor the idea that endogenous Tregs found in each model system are insufficient at the population level – that although seemingly “functional” on a per cell basis, they are incapable of suppressing the particular autoimmune reaction that drives disease development in TS1xHACII and TS1(SW)xHACII mice. Oh S et al. demonstrated that transfer of polyclonal Tregs, but not HA-specific Tregs, can significantly ameliorate disease onset in the TS1xHACII model system (Oh et al. 2012). These results suggest that a diverse Treg repertoire is required for the successful suppression of arthritis development in TS1xHACII mice. Therefore, it is possible that the reason why endogenous Tregs cannot control disease in TS1xHACII and TS1(SW)xHACII mice is because they do not express the appropriate TCR specificities, due to being limited by the TS1 or TS1(SW) TCR transgenes. Although we favor this hypothesis, it is also possible that endogenous Tregs in TS1xHACII and TS1(SW)xHACII mice are actually dysfunctional on a per cell basis. We are currently determining Treg functionality through use of an in vitro suppression assay which is in many respects an artificial
simulation of what may occur in vivo. Indeed, the amount and duration of TCR signaling as well as the exact environment (which is inflammatory in the case of TS1xHACII and TS1(SW)xHACII mice) found in vivo cannot be replicated in vitro. Therefore, we cannot completely rule out the possibility that Tregs in TS1xHACII and TS1(SW)xHACII mice develop inflammatory arthritis due to dysfunctional Tregs or the possibility that effective disease modulators work through bolstering Treg functionality.

In summary, we believe we have demonstrated that the degree of CD4$^+$ T cell reactivity for self-peptide can play a prominent role in determining the cellular pathways that participate in the development of inflammatory arthritis. Through analysis of B cell deficient mice and mice treated with various anti-cytokine antibodies, we have shown that TNF, but not B cells, is required for arthritis development in the TS1xHACII model system. This suggests that B cells are not necessary to either promote or act as a target of TNF in this model system. On the other hand, B cells significantly contribute to disease development in TS1(SW)xHACII mice; TNF appears to contribute to enhancing disease severity in this model but is not required for disease initiation. Thus, pathways by which disease develops in TS1xHACII and TS1(SW)xHACII mice are fundamentally different.
5.3 Speculation on how TCR affinity dictates the requirement for B cells in TS1xHACII and TS1(SW)xHACII mice.

In this next section, we would like to speculate on how TCR affinity for a self-antigen might dictate the requirement for B cells for disease development in TS1xHACII and TS1(SW)xHACII mice.

We have previously shown that B cells are not required for arthritis development in the high affinity model system – TS1xHACII mice ((Rankin et al. 2008) and Fig. 4-1a). It is notable that B cell deficient TS1xHACII.JH−/− and B cell sufficient TS1xHACII mice develop disease with equal penetrance and severity. Thus, to promote arthritis, autoreactive CD4+ T cells in this system do not require B cells to function as APCs. These results argue that the amount of self-antigen or the context in which self-antigen is presented (by other APCs such as conventional DCs, plasmacytoid DCs, macrophages, etc.) was sufficient to stimulate the high affinity autoreactive CD4+ T cells in this system to elicit a sustained autoimmune response, ultimately culminating in the development of inflammatory arthritis.

Conversely, B cells were found to significantly contribute to the development of arthritis in the low affinity model system – TS1(SW)xHACII mice; in the absence of B cells (through congenital or antibody mediate depletion) disease was significantly attenuated (Fig. 4-1b and Fig. 4-5d). Our data suggest that B cells participate as APCs rather than as a source of arthritogenic autoantibodies (Fig. 4-2, 3, 5, 6) to drive disease in this system. Although we found that B cells are capable of promoting autoreactive effector CD4+T cells (both IFN-γ- and IL-17-producing) in TS1(SW)xHACII mice, reduced
frequencies of IL-17^+ (but not of IFN-γ^+) Vα8.3^+Vβ10^+CD4^+ T cells correlated with the absence of disease in TS1(SW)xHACII.JH^+ mice. Thus, these results suggest that B cells promote disease in this system by participating as APCs for autoreactive Th17 cells. Notably, these results show that B cells in particular (and not other APCs) are necessary to generate effector CD4^+ T cells with autoreactive specificity, supporting the findings of other studies (Chan et al. 1999, O'Neill et al. 2005, Hamel et al. 2008, Wilson et al. 2012). So why are B cells required in the low affinity TS1(SW)xHACII model but not in the high affinity TS1xHACII model? It is possible that B cells promote autoreactive effector CD4^+ T cell formation in TS1xHACII mice as well. If this is the case, and as TS1xHACII.JH^+ were found to still develop severe arthritis (Fig. 4-1), it would suggest that low numbers of cytokine-producing 6.5^+CD4^+ T cells would be sufficient to drive disease. Speculating further, low numbers of autoreactive effector CD4^+ T cells may be sufficient in the high affinity but not the low affinity model system because T cells in the former are measurably more self-reactive than in the latter (Fig. 3-7). Thus, B cells may be necessary to generate sufficient numbers of autoreactive effector CD4^+ T cells in TS1(SW)xHACII mice in order to surpass a threshold, which when breached, results in the loss of self-tolerance.

We also speculate on how can B cells support Th17 cells with autoreactive specificity in TS1(SW)xHACII mice. Several reports in the literature have demonstrated that B cells can support the formation of antigen-specific effector CD4^+ T cells in response to both foreign antigens and autoantigens, including studies in the PGIA model of arthritis (Chan et al. 1999, O'Neill et al. 2005, Crawford et al. 2006, Hamel et al. 2008, Wilson et al. 2012). However, less is known about if and how B cells can specifically
promote Th17 cell differentiation (Hamel et al. 2008, van de Veerdonk et al. 2011, Wang and Rothstein 2012). It is generally accepted that a TCR stimulus in the presence of a Th17 polarizing cytokine milieu (TGF-β, IL-6, IL-23) are required for Th17 cell differentiation in vitro (Langrish et al. 2005, Park et al. 2005, Bettelli et al. 2006, Mangan et al. 2006). We do not believe a difference in cytokine milieu is the causative factor for why lower frequencies of clonotypic Th17 cells were found in TS1(SW)xHACII.JH−/− mice compared to TS1(SW)xHACII mice. This is because the frequency of IL-17+ cells within the total CD4+ T cell population was only marginally affected, if at all, in the absence of B cells (Fig. 4-3 and Fig. 4-6). So it reasons that the causative factor may be how antigen is presented by B cells. In support of this, a recent study demonstrates that naïve B-1 B cells were more capable of promoting Th17 cells than naïve B-2 B cells; however, B-2 B cells, upon activation, acquired the ability to promote Th17 cells, which they suggest was due to the upregulation of certain co-stimulatory molecules (Wang and Rothstein 2012). These findings imply that the context with which CD4+ T cells receive their antigenic stimulation may matter. Additionally, a study with human CD4+ T cells demonstrated that a low-strength TCR signal was more proficient at inducing Th17 cells compared to a high-strength TCR signal (Purvis et al. 2010). In line with this, clonotypic CD4+ T cells in TS1(SW)xHACII mice were more likely to differentiate into Th17 cells compared to clonotypic CD4+ T cells from TS1xHACII mice (Fig. 3-9). This concept is not unique as elaborate work examining TCR-pMHC interactions demonstrate that the strength and context of TCR engagement can indeed influence T cell differentiation and fate (Corse et al. 2011).
Thus, our data demonstrate that the requirement for B cells in autoimmune settings can be determined by the affinity of CD4⁺ T cells for self-antigen. Additionally, these data also attribute B cells as unique APCs necessary for the formation of self-reactive effector CD4⁺ T cells.

5.4 Summary

In conclusion, our work has demonstrated that the affinity with which CD4⁺ T cells recognize a single target self-antigen can have profound effects on the development of autoimmunity. Although the factors which promote disease development in TS1xHACII and TS1(SW)xHACII mice were distinct, both anti-TNF treatment of TS1xHACII mice and B cell elimination in TS1(SW)xHACII mice appeared to prevent arthritis development at least in part by disrupting Th17 cell activity, albeit by different mechanisms. Additionally, Th17 cells also appear to promote disease in several other mouse models of inflammatory arthritis (Lubberts et al. 2001, Nakae et al. 2003, Hirota et al. 2007, Wu et al. 2010). Therefore, outcomes of clinical trials utilizing reagents that block IL-17 activity would be of interest (Genovese et al. 2010). It is also possible, though, that the commonality of Th17 cell participation in inflammatory arthritis development is a phenomenon only observed in mice.

While there has been recent success in the use of biological therapeutics to modulate the immune system in patients with inflammatory arthritis, the factors that determine which cellular pathways are required for disease development in individual patients remain poorly understood. One reason that the therapeutic targeting of distinct cellular pathways (e.g. anti-TNF versus anti-CD20 treatment) may be effective in
different individuals could be that the cellular antigens being recognized in those individuals are different. This possibility is difficult to assess in human patients, not least because the antigens that are recognized by autoreactive lymphocytes in inflammatory arthritis (such as can occur in RA and SLE) remain poorly understood (Bennett et al. 2003, Trouw and Mahler 2012). Our studies here have used a system in which the identity of an eliciting surrogate self-peptide is known, and have shown that the overall reactivity of the autoreactive CD4$^+$ T cell response can determine whether or not B cells and TNF are required for arthritis development. Furthermore, our studies have demonstrated that stochastic events, such as TCR repertoire formation, play a larger role in determining the course of autoimmune disease development than may have been previously appreciated. Moreover, the observation that there is a female gender bias in arthritis development in TS1(SW)xHACII mice, but not in TS1xHACII mice, suggests that additional pathways (e.g. estrogen-induced immune activation) (Cutolo et al. 2002, Behrens et al. 2010) are also necessary for arthritis development in the context of a relatively weak autoreactive CD4$^+$ T cell response. As antigens that are recognized in human patients become better characterized, it will be of interest to determine whether the level of autoreactive CD4$^+$ T cell reactivity can predict the cellular pathways that are required for arthritis development and determine the efficacy of distinct classes of biological modifiers.


injection of anti-type II collagen antibody or free native type II collagen. Arthritis Rheum 27(9): 1010-1017.


