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THE ROLE OF TFH CELLS, TH17 CELLS, AND THE GUT MICROBIOTA IN AUTOIMMUNE ARTHRITIS

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KATHARINE EMMA BLOCK

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Abbreviations

Abx	antibiotics
ACPA	anti-citrullinated protein antibody
BCR	B cell receptor
CFA	cellular fatty acids
DMARD	disease-modifying antirheumatic drug
EAE	experimental autoimmune encephalitis
GC	germinal center
GPI	glucose-6-phosphate isomerase
Ig	immunoglobulin
IL	interleukin
ILC	innate lymphoid cell
mAb	monoclonal antibody
MHC	major histocompatibility complex
MHC-II	MHC class II
NORA	new-onset rheumatoid arthritis
NSAID	non-steroidal anti-inflammatory drug
PRR	pattern recognition receptor
SAA	serum amyloid A
SCFA	short chain fatty acid
SFB	segmented filamentous bacteria
SPF	specific pathogen free
Tfh	follicular helper T cell
Th17	T helper 17
TCR	T cell receptor
Tg	transgenic/transgene
TLR	toll like receptor
TNF-α	tumor necrosis factor alpha
Treg	regulatory T cell
RA	rheumatoid arthritis
RAG	recombination activating genes
RF	rheumatoid factor

Abstract

Autoimmune arthritis in the K/BxN mouse model is driven by autoreactive T cells and autoantibodies. Two helper T cell subsets, Tfh and Th17 cells, have been suggested to be able to provide help to B cells. We investigated the contribution of Tfh and Th17 cells, as well as the T cell cytokines IL-21 and IL-17, to disease pathogenesis. Using genetic approaches, we found that IL-21 production from autoreactive T cells was required by B cells for the formation of germinal centers and autoantibodies. IL-21R expression on T cells was not required for Tfh cell differentiation and arthritis suggesting that IL-21 was not an autocrine factor for Tfh cells. T cells deficient in the transcription factor RORγt and incapable of Th17 cell differentiation induced disease normally. IL-17 deficient mice developed arthritis and had a normal Tfh cell population, germinal centers, and autoantibody levels.

The gut microbiota is known to promote disease in this model, and we investigated the effect of gut bacteria on T cell differentiation. Because Th17 cell differentiation is promoted by gut bacteria, Th17 cells has been suggested to mediate the effect of gut bacteria on arthritis. However, we showed that gut microbiota regulates autoimmune development independent of IL-17. In contrast, Tfh cell differentiation was defective in antibiotic treated mice. Furthermore, Bcl6 deficient T cells incapable of Tfh cell differentiation did not induce disease. We conclude that the Th17 axis is not required for K/BxN arthritis. Our studies highlight the role Tfh cells and IL-21 plays in autoantibody formation and their relationship to gut microbiota. These results have implications in treatment of human autoimmune arthritis.

Chapter I: Introduction

Introduction

The immune system is made up of a network of cells, tissues, and organs that protect an organism against pathogens. However, the role of the immune system is more complex than simply identifying foreign organisms and eliminating them. Both the innate and adaptive arms of the immune system must distinguish self from non-self but must also strike a balance of commensalism with organisms that colonize the host (Hooper et al., 2012).

The innate immune system

Cells of the innate arm of the immune system include natural killer cells, dendritic cells, eosinophils, basophils, and macrophages. Innate immune cells express germline encoded receptors evolved to sense conserved and invariant features of microorganisms (Figure 1.1). These features are generally absent in the host and therefore allow discrimination of self versus non-self. The sensed products are also usually vital to the microorganisms and are therefore relatively conserved both between microorganisms of a certain class and over time. These pattern recognition receptors (PRRs) include the membrane bound Toll-like receptors (TLRs) and dectin 1, as well as cytosolic NLRs and RIG-I (Medzhitov, 2007). TLRs are perhaps the best characterized PRRs. While members of the TLR family have an overall similar structure they reside in different parts of the cell and recognize diverse ligands, from lipopolysaccharides of gram negative bacteria to unmethylated CpG DNA from viruses and bacteria (Medzhitov, 2001). Signaling of these receptors, in the case of TLRs is through the adaptor molecules

MyD88 or TRIF, triggers the expression of a number of genes characteristic of the innate immune response (Medzhitov et al., 1998; Horng et al., 2001). PRR signaling induces, among other things, the production of cytokines, which orchestrate the immune response against a pathogen. Part of this response includes the recruitment and activation of cells of the adaptive immune system.

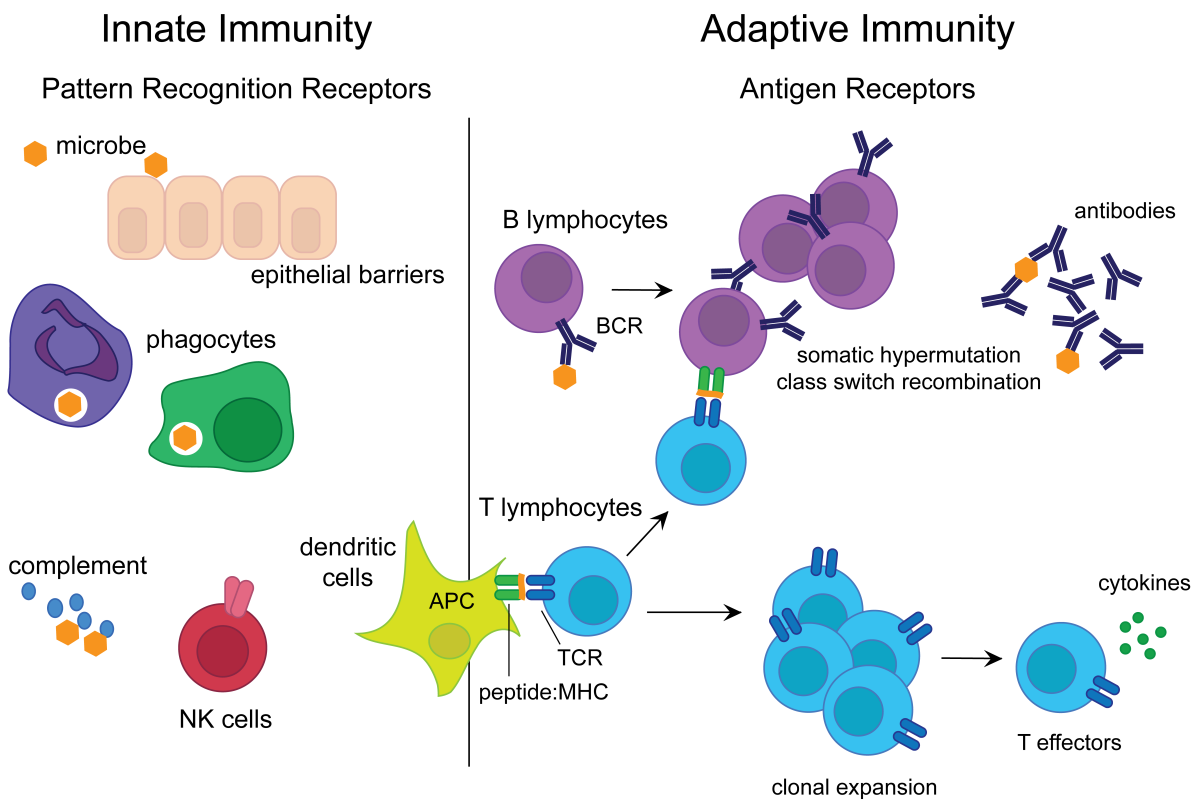

Figure 1.1. Innate and adaptive immunity. Physical barriers, such as the epithelium, keep microbes from entering the host. Innate immune cells express PRRs that recognize conserved patterns of microbes and allow for rapid responses by these cells. Cells of the adaptive immune system express somatically recombined antigen receptors that allow for specific and diverse recognition of antigens. Activated T and B lymphocytes clonally expand and have a number of effector functions. B cells recognize antigen directly with their BCR, while T cells recognize antigen as peptide:MHC. B cells produce antibodies while T cells orchestrate effector responses, partly through the secretion of cytokines.

The adaptive immune system

The adaptive immune system, comprised of T and B lymphocytes, has evolved to express somatically rearranged receptors that allow for specific recognition of an almost unlimited number of antigens (Flajnik and Kasahara, 2010). The generation of T cell receptors (TCRs) and B cell receptors (BCRs), known as V(D)J recombination, is facilitated by recombination activating genes RAG-1 and RAG-2 (Tonegawa, 1983; Oettinger et al., 1990). The nature of this random rearrangement leads to TCRs and BCRs specific to self-antigens, and therefore there are central tolerance mechanisms that cause cells expressing these autoreactive receptors to be deleted (Hogquist et al., 2005). T cell progenitors in the thymus first undergo positive selection, where only thymocytes with TCRs with affinity for peptide:MHC survive. Remaining cells can then undergo negative selection, where cells expressing TCRs with high affinity for self-peptide:MHC are clonally deleted (Palmer, 2003). Alternatively, self-reactive T cells can be diverted to the natural regulatory T cell (Treg) lineage and have effector functions that limit immune responses (Sakaguchi, 2005). B cells also undergo positive and negative selection (or deletion) processes in the bone marrow. Receptor editing is the process of recombining the second BCR light chain allele and is induced if the BCR from the first rearrangement is autoreactive (Pelanda and Torres, 2012). In the periphery, additional mechanisms, such as anergy, are in place to prevent the activation of autoreactive lymphocytes (Goodnow et al., 1988). Autoimmune disease results when these tolerance mechanisms break down (Valdor and Macian, 2013).

Naïve T and B cells in the periphery circulate through the blood, lymph, and lymphoid tissues. When lymphocytes encounter antigen, they become activated and clonally expand. T cells direct immune responses by differentiation into certain effector types. B cells produce

antibodies that can bind antigen and also direct immune responses through the Fc region. B cells can undergo class switch recombination, which changes the isotype (Fc region) of the antibody, and therefore change the function of the antibody (Murphy et al., 2011). BCRs undergo somatic hypermutation, where germline mutations alter the affinity of the BCR for antigen. T cell help selects for B cells with the highest affinity for antigen (Vinuesa et al., 2005b). Activated lymphocytes can also become memory T and B cells, allowing for fast and specific recognition of antigen and rapid elimination of pathogens upon subsequent exposure (Litman et al., 2010) (Figure 1.1).

The microbiota

Vertebrates have evolved with trillions of bacteria, as well as fungi and protozoa colonizing their mucosal surfaces. The highest density of this microbiota is in the gastrointestinal tract, and in the distal intestine in particular. The immune system does not work to eliminate all these organisms but actively works to develop a stable commensal microbial population (Hooper et al., 2012).

The presence of gut bacteria is mutually beneficial to host and microbe (Round and Mazmanian, 2009). Bacteria find a niche and an energy source from the food the host consumes. The beneficial effect of the microbiota on a host can be appreciated through the study of germ free mice. Mice derived and raised in a sterile environment must be provided supplemented food, as bacteria provide essential nutrients by metabolizing compounds indigestible by the host. Germ-free mice also have defective intestinal architecture, such as reduced number and size of Peyer's patches and mesenteric lymph nodes, and underdeveloped isolated lymphoid follicles

(Bouskra et al., 2008; Falk et al., 1998). Bacterial colonization is also important for strong antibody responses to immunization, as immunization of germ free mice with OVA in adjuvant led to dramatically decreased antigen-specific serum IgG, but colonization with normal microbiota restored this response (Lamousé-Smith et al., 2011).

Metabolites generated from components of the diet by commensal microbes have profound effects on the host. For example, short chain fatty acids (SCFA), a product of bacterial fermentation of plant derived nondigestible polysaccharides, regulate host metabolism and immune system functions (Brestoff and Artis, 2013). A number of immune and non-immune cells express GPR43, a receptor for SCFA. SCFA can influence, among other things, neutrophil migration and activation (Maslowski et al., 2009), dendritic cell function (Berndt et al., 2012), and autophagy (Donohoe et al., 2011). Germ free mice have increased inflammation in a DSS model of colitis, but administration of SCFA reduced the severity of colitis in a GPR43 dependent manner (Maslowski et al., 2009). In the same study, K/BxN serum transfer arthritis (discussed below) was more severe in mice that were deficient in GPR43 (Maslowski et al., 2009).

The Human Microbiome Project Consortium set out to sequence the microbiome of multiple areas of the body of healthy adults using 454 pyrosequencing of 16S rRNA genes (Human Microbiome Project Consortium, 2012). The healthy gut microbiota is mainly comprised of members of the phyla *Bacteroidetes* and *Firmicutes* and generally also contains members of *Proteobacteria* and *Actinobacteria*. The structure of the microbiota can be influenced by a number of factors, including host genetics, diet, and pharmaceuticals, particularly antibiotics (Buffie et al., 2012). Perturbation of the composition of resident commensal communities, known as dysbiosis, can result from loss of specific helpful bacteria,

expansion of so-called "pathobionts," or a decrease in bacterial diversity (Petersen and Round, 2014). Examples of specific bacteria that are helpful to the host are bacteria capable of inducing the development of Tregs that inhibit inflammatory responses. Polysaccharide A from the human symbiont *Bacteroides fragilis* induces Treg development in mice and protects mice from experimental colitis (Round and Mazmanian, 2010). In another study, seventeen select strains of bacteria isolated from human fecal samples could promote Treg development when given to germ free mice. A consortium of these bacteria could also protect mice from experimental colitis (Atarashi et al., 2013).

Significant changes in the gut microbial communities have been observed in a number of immune-mediated diseases. Dysbiosis has been documented in ulcerative colitis (Frank et al., 2007), diabetes (Karlsson et al., 2013), food allergy (Berni Canani et al., 2015), and asthma (Abrahamsson et al., 2014). Evidence of dysbiosis in rheumatoid arthritis is discussed below. The well-characterized role of the microbiota in shaping the immune system, along with observations made on the altered microbiota in these diseases highlight the role the microbiota may play in promoting disease in genetically susceptible individuals.

Rheumatoid arthritis

Rheumatoid arthritis (RA) is an autoimmune inflammatory disease of the synovial joints with an incompletely understood etiology. RA affects approximately 0.5 to 1% of the adult population (Silman and Pearson, 2002). The disease primarily affects the synovial joints, but may affect other organs such as the heart, kidney, and brain. Disease is thought to be triggered by an initial state of non-specific inflammation, which progresses to a stage of T cell activation, and finally a chronic inflammation stage involving autoantibodies and cytokines leading to tissue

injury. Patients with RA are classified based on serological parameters, namely whether Rheumatoid Factor (RF, antibodies against the Fc region of IgG) or anti-citrullinated protein antibodies (ACPA) are present (Choy, 2012).

Risk factors in RA

The locus encoding human leukocyte antigen (HLA) confers the single largest genetic risk for RA, with HLA-DR1 and HLA-DR4 being most associated with disease (Yarwood et al., 2014; Deighton et al., 1989). The second largest genetic association with RA is within the gene *PTPN22*, a lymphoid tyrosine phosphatase and a negative regulator of TCR signal transduction. A large number of other gene polymorphisms, many of which are immune related, are associated with RA (Lee et al., 2005; Orozco et al., 2005; Yarwood et al., 2014). While the exact nature of immune activation is unknown, there is strong evidence that the adaptive immune system is a critical player in the pathogenesis of RA.

Heritability of RA is estimated to be only 50 to 65% (MacGregor et al., 2000). In addition to genetic factors, environmental factors contribute to susceptibility to RA. Smoking is the best understood contributing risk factor, especially among individuals that harbor the HLA-DRB1 variant (Yarwood et al., 2014). Cigarette smoking is linked to a specific posttranslational modification called citrullination. The major histocompatibility complex (MHC) variant HLA-DRB1 has an enhanced ability to bind and present citrullinated proteins (Pruijn, 2015). Antibodies against citrullinated self-proteins are believed to be pathogenic, and the presence of anti-citrullinated protein antibodies (ACPA) in the serum is considered a biomarker of RA and has become part of the criteria for the classification of RA. Another risk factor for ACPA

positive RA is presence of the periodontal pathogen *Porphyromonas gingivalis*, which express enzymes capable of citrullinating proteins (Mikuls et al., 2014).

Microbiota in RA

Antibiotics have been used as a treatment for RA since the 1930s. Various types of antibiotics have been efficacious in improving signs and symptoms of RA, reinforcing the concept that the microbiota is an environmental factor contributing to the development of autoimmune disease (Ogrendik, 2013). The composition of the microbial communities that colonize RA patients has been investigated in a number of studies, discussed below. Importantly, research groups have recruited recently diagnosed RA patients, also called new-onset RA (NORA), and have examined the microbiota before patients are treated with pharmaceuticals; some of these studies are discussed below. Therefore, changes seen are not attributable to treatment, but might represent bacterial communities that could promote autoimmune development.

Over twenty years ago, the fecal microbial community in NORA patients was examined by using gas-liquid chromatography to profile the bacterial cellular fatty acids (CFA) as a proxy for measuring the diversity of bacterial species present. The CFA spectra in fecal samples from RA patients was significantly different from control patients, and the authors suggested that intestinal bacteria may contribute to the development of RA (Eerola et al., 1994). The fecal bacterial composition of NORA patients compared to fibromyalgia patients was examined with eight different oligonucleotide probes combined with flow cytometry, 16S rRNA hybridization, and DNA staining. RA patients were found to have a decrease in a number of bacterial groups, including Bifidobacteria and *Bacteroides fragilis* (Vaahtovuo et al., 2008). Another research

group also observed a decrease in *Bacteroides* in NORA patients compared to healthy controls by 16S rRNA sequencing from fecal samples (Scher et al., 2013). They also observed an overabundance of *Prevotella*, specifically an enrichment of *Prevotella copri*. One group specifically examined the Lactobacillus community from fecal samples of NORA patients and found that there was increased Lactobacillus diversity and abundance compared to healthy controls (Liu et al., 2013). Metagenomic shotgun sequencing of fecal, dental, and salivary samples from NORA patients and healthy controls revealed distinct bacterial populations between both groups in all three sites. There was a decrease in *Haemophilus* species and an overabundance of *Lactobacillus salivarious* in NORA patients in all sites as well (Zhang et al., 2015).

Several of the studies just described also found that RA patients that had been treated with disease modifying anti-rheumatic drugs (DMARDs) had communities that more closely resembled healthy patients, and response to treatment could be correlated with the composition of bacteria present (Scher et al., 2013; Zhang et al., 2015). These studies all suggest there are changes to the bacterial community structure in RA patients and a state of dysbiosis may contribute to autoimmune disease. Some results also suggest that specific individual species might be implicated in disease pathogenesis. Alternatively, the observations made could be due to increased inflammation in these patients and could represent the effect autoimmunity has on the gut microbiota, not an effect the bacterial communities play on a genetically susceptible individual. This caveat is true of almost all human studies of the microbiota. Prospective cohort studies with RA patients prior to disease onset and periodic sampling will be necessary to distinguish these possibilities.

Treatment in RA

Treatment of RA is based partly on limiting inflammation and discomfort with non-steroidal anti-inflammatories (NSAIDs) and limiting the damage mediated by the immune system with DMARDs. DMARDs are grouped into two categories, non-biologic and biologic. The former group consists of compounds that generally target metabolic pathways and are effective by blocking lymphocyte proliferation (Steiman et al., 2013). The latter group includes antibodies and decoy receptors for cytokines or immune cell surface receptors. Approved biologic DMARDs target TNF-α, IL-1, and CTLA4, and B cells (Callhoff et al., 2013). RA patients are often classified into two groups, either responsive or refractory to anti-TNF treatment. This subset of patients that do not respond to one of the most commonly prescribed treatments for RA means that identifying other targets for biologic treatments is an important avenue of research.

Animal models of RA

A number of animal models of RA have been developed in order to identify potential factors that contribute to the human disease. They can generally be categorized as induced or spontaneous disease models (Asquith et al., 2009). The induced models of arthritis rely on immunization or intra-articular injection of antigen in order to elicit an autoreactive immune response. Examples are collagen-induced arthritis of susceptible strains, intra-articular zymosan induced arthritis, or BSA immunization followed by intra-articular injection of BSA. In a subset of spontaneous models of RA, mice or rats develop autoimmunity through overexpression or deletion of immune genes that result in uncontrolled inflammatory responses. A human TNF-α transgene leads to overexpression of TNF-α and resulting inflammation leads to arthritis (Keffer

et al., 1991). A deficiency in IL-1R antagonist (*Il1ra*$^{-/-}$) leads to autoantibodies and uninhibited inflammation, which result in joint destruction (Horai et al., 2000). The SKG mouse strain carries a point mutation in the gene encoding Zap-70, a key component of TCR signal transduction. This aberrant TCR signaling leads to abnormal thymic selection and chronic autoimmune arthritis (Sakaguchi et al., 2003). The K/BxN mouse used in these studies is a spontaneous model of RA and relies on transgenic expression of an autoreactive TCR.

The K/BxN model of arthritis

The K/BxN mouse model of rheumatoid arthritis was a fortuitous discovery made in the laboratory of Benoist and Mathis in 1996 (Kouskoff et al., 1996). The KRN transgenic T cell receptor that recognizes self-antigen in this model was originally cloned from a hybridoma named R28, derived from a B10A.4R mouse that was immunized with bovine pancreas ribonuclease. The TCR recognizes the 41-61 amino acid peptide of this protein when presented on MHC class II (MHC-II) I-A^{k}. A KRN/B6 mouse line was generated and crossed onto a number of genetic backgrounds in order to understand the nature of positive selection of this T cell receptor. When the KRN/B6 line was crossed to the NOD strain of mice, the offspring displayed distal joint inflammation with 100% penetrance. The mice were characterized in detail and were found to have many similarities to rheumatoid arthritis. The arthritis model was subsequently named K/BxN, indicating a KRN/B6 mouse (K/B) that has been crossed to a NOD mouse (N). The KRN/B6 mice were crossed to other strains of mice, including a B6 congenic strain carrying the NOD-associated MHC-II allele I-A^{g7}. The presence of the MHC allele I-A^{g7}

and the KRN Tg were found to be sufficient for disease, demonstrating the disease does not require the autoimmune-prone background of the NOD strain (Kouskoff et al., 1996).

Subsequent work identified the autoantigen recognized by T and B cells in K/BxN mice (Matsumoto et al., 1999). An approximately 60-kD protein bound by IgG in diseased mice was isolated by high-performance liquid chromatography and sequenced, identifying glucose-6-phosphate isomerase (GPI). Serum from arthritic K/BxN mice bound recombinant mouse GPI by protein immunoblot and by ELISA, and only antibodies captured by a GPI-coated column induced disease by serum transfer, compared to the flowthrough antibodies. GPI was shown to be the T cell antigen as well because recombinant GPI given to APCs expressing I-A^{g7} strongly stimulated the R28 hybridoma and KRN/B6 T cells *in vitro*. The peptide recognized by KRN Tg T cells was found to be the peptide with amino acids 281-293 presented by I-A^{g7} and had some similarities to bovine RNase 41-61 peptide bound to I-A^{k} (Basu et al., 2000).

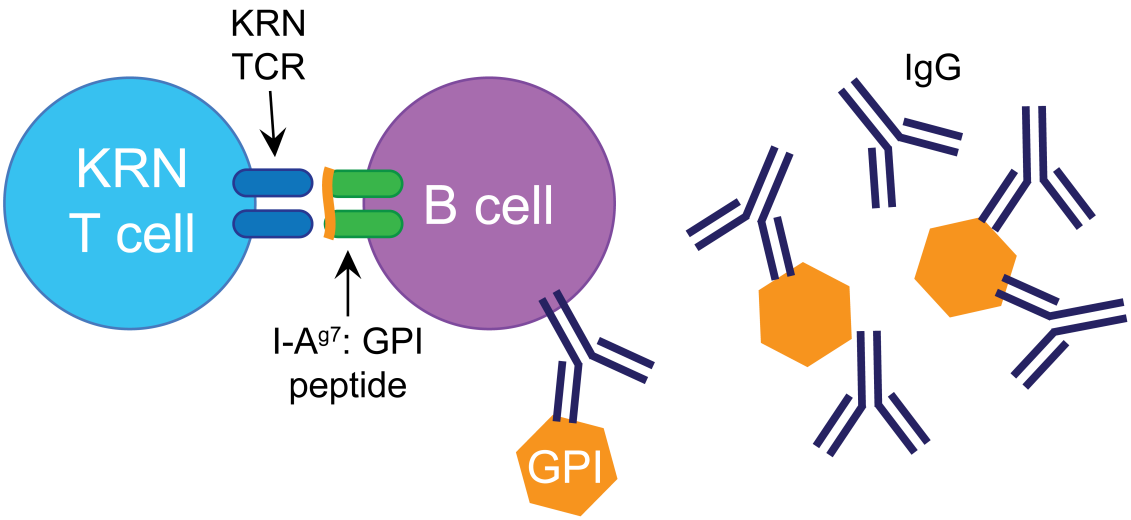

Figure 1.2. The K/BxN model of autoimmune arthritis. A transgenic TCR, KRN, recognizes glucose-6-phosphate isomerase (GPI) presented by I-A^{g7}. Once the T cell is activated, it drives B cells to undergo affinity maturation and class switch to IgG. These antibodies form immune complexes with GPI and lead to inflammatory joint destruction.

Detailed research on K/BxN mice has identified the main required components to establish arthritis in this model (Figure 1.2). Namely, the KRN TCR Tg $CD4^+$ T cells ($CD8^+$ T cells are dispensable), the MHC-II allele I-A^{g7}, the antigen GPI, and B cells capable of class switching to IgG are required for the initiation phase (Figure 1.3A) (Korganow et al., 1999). Once anti-GPI antibodies are formed, the complement alternative pathway, neutrophils, mast cells, IL-1, and TNF-α are required for arthritis (Ditzel, 2004). This phase, where autoantibodies elicit synovial joint damage, is referred to as the effector phase (Figure 1.3B). Disease can be induced through the transfer of K/BxN serum to new host mice independent of the adaptive immune system (Korganow et al., 1999). This serum transfer model has been important for establishing these requirements of the effector phase of joint destruction and has been a relatively simple approach, since serum can be transferred into any host mice deficient in a gene of interest.

Arthritis can be induced by transferring naïve KRN Tg B6 (K/B6) splenocytes or purified T cells into host mice. Host mice must express one alelle of I-A^{g7}, for presentation and recognition of GPI peptide, and lack αβ T cells, allowing space for transferred cells to expand (LaBranche et al., 2010). K/B6 mice can be crossed to mice deficient in a gene of interest. The resulting T cells can then be transferred making this model useful for testing the requirement of a gene's expression specifically in autoreactive T cells without generating conditional knockout mice. In the following studies, we use the K/BxN autoimmune arthritis model and the KRN/B6 cell transfer model to investigate helper T cell differentiation (namely Tfh and Th17 cells) and cytokine production (IL-21 and IL-17), and we look at how the microbiota influences this T cell differentiation.

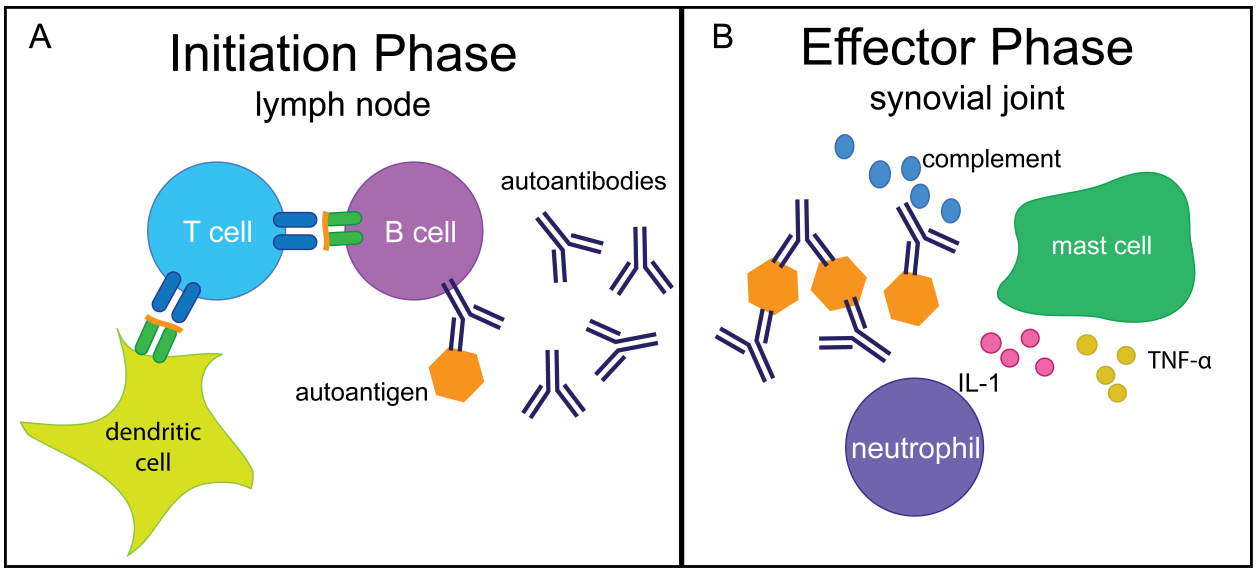

Figure 1.3. The phases of K/BxN arthritis. (**A**) The initiation phase, taking place in secondary lymphoid organs such as the lymph nodes, involves the activation of autoreactive T cells, which in turn promote B cells to undergo affinity maturation and class switching toward self-antigen. The initiation phase occurs spontaneously in K/BxN mice at approximately 4 weeks of age, or can be induced upon transfer of KRN Tg T cells into $C\alpha^{-/-}$BxN hosts. (**B**) The effector phase, occurring in the synovial joints, occurs when immune complexes of autoantibodies and antigen form. An inflammatory cascade leads to joint damage. This stage requires the alternative pathway of complement, neutrophils and mast cells, and the inflammatory cytokines IL-1 and TNF-α. This stage occurs in the spontaneous or T cell transfer models once IgG antibodies have been produced, or can be induced by transfer of K/BxN serum.

Tfh cells, IL-21, and the germinal center reaction

Follicular helper T cells (Tfh) are a relatively recently characterized subset of MHC-II restricted T helper cells. Strong TCR stimulation along with cytokines IL-6 or IL-21 induce the Tfh program, regulated by the transcription factor Bcl6 (Nurieva et al., 2009; Eto et al., 2011) (Figure 1.4). Downregulation of CCR7, which retains T cells in the T cell zone, and upregulation of the chemokine receptor CXCR5 allows T cells to migrate toward CXCL13-rich B cell follicles (Haynes et al., 2007). At the border of the T-B zone, T cells interacting with B cells presenting cognate antigen allow the further activation of the Tfh program. Entering the B

cell follicle, Tfh cells promote activation of antigen-specific B cells and establish the germinal center reaction, a concentrated factory of affinity maturation, class switching, and plasma cell differentiation (Crotty, 2014). Expression of a number of cell surface molecules by Tfh cells and germinal center B cells is indicative of the close interaction of these cell types, and the cells signal to each other to promote the differentiation and maintenance of both cell types. Loss of germinal center B cells results in Tfh cell death, and Tfh cells are required for sustained germinal centers (Crotty, 2011).

Production of the cytokine IL-21 is one of the important mechanisms by which Tfh cells promote germinal centers. IL-21 is a type I cytokine that binds to a receptor complex that includes the common γ chain. Other type I cytokines that bind to a receptor that includes the common γ chain are IL-2, IL-4, and IL15 (Leonard et al., 2008). IL-21 is produced exclusively by activated T cells, including Tfh and Th17 cells (discussed below). The receptor is expressed on a number of cell types, but the best characterized is IL-21R on B cells (Ettinger et al., 2008). IL-21R signaling on B cells can induce both Bcl6 and Blimp-1 expression, and is therefore important for germinal center B cell function (such as class switching and somatic hypermutation) and plasma cell differentiation, respectively (Ozaki et al., 2004). IL-21R signaling has also been proposed to act as a T cell autocrine factor to promote Tfh differentiation (Vogelzang et al., 2008; Nurieva et al., 2008).

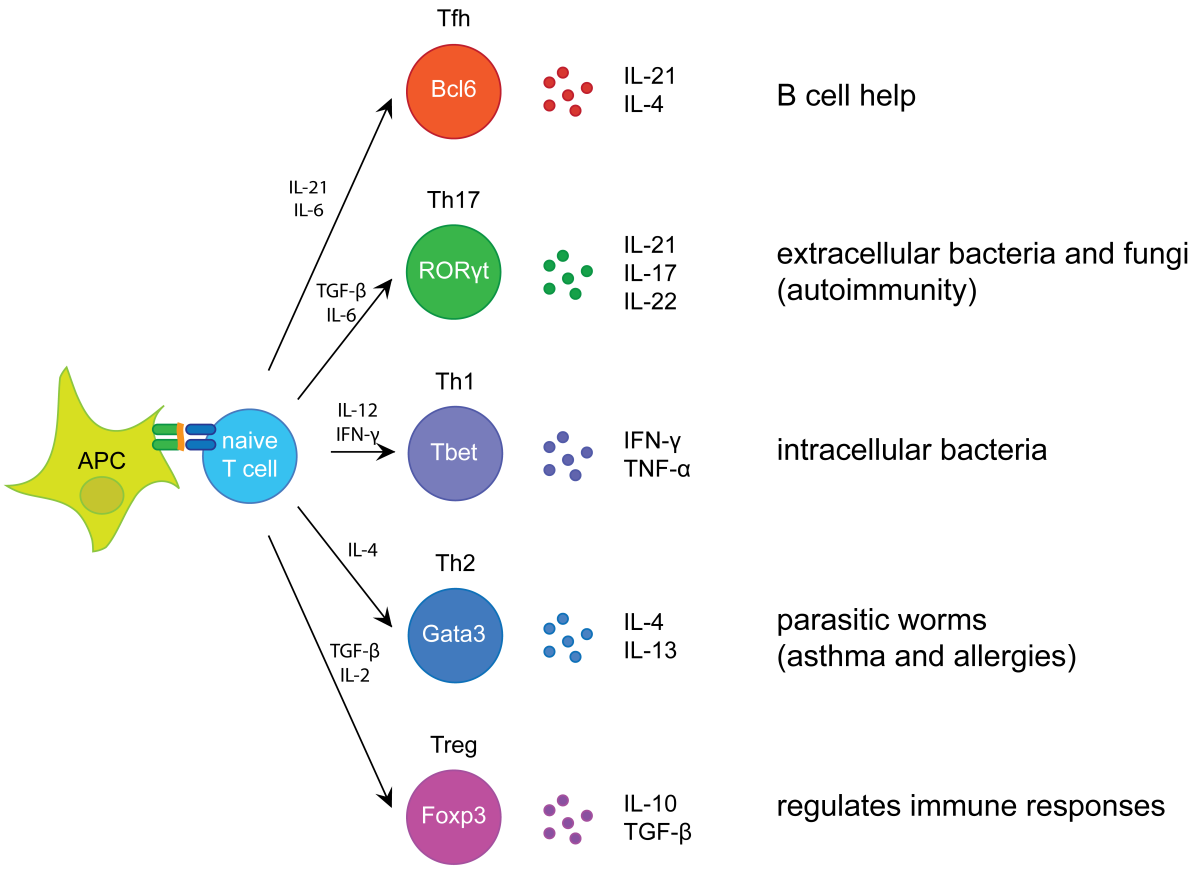

Figure 1.4. T helper cell subset differentiation. Upon TCR stimulation and coreceptor engagement, MHC-II restricted T cells differentiate into one of a number of T helper subsets. The T cell subset is determined by the cytokine environment, leading to the upregulation of a transcription factor that promotes the differentiation of the T cell. Along with the transcription factor they express, T helper subsets are also defined by the cytokines they produce, examples of which are shown.

Tfh and IL-21 in autoimmune models

Since Tfh cells and the cytokine IL-21 are potent inducers of B cell and antibody responses, it is not surprising that there has been an interest in studying their function in the context of autoantibody dependent rheumatic diseases. For example, the Roquin^{Sanroque} mouse model of systemic lupus nephritis develops spontaneous autoreactive germinal centers (Vinuesa et al., 2005a). The phenotype is due to a mutation in a RING-like ubiquitin ligase that, in a cell-

intrinsic manner, leads to spontaneous Tfh development (Linterman et al., 2009). IL-21R is also necessary for antibody production and a lupus-like phenotype in a chronic graft-versus-host model of autoimmunity (Nguyen et al., 2012). K/BxN mice deficient in IL-21R do not develop arthritis. There is a decrease in autoreactive T cell proliferation, and therefore it was proposed that the T cell autocrine function of IL-21 is important in K/BxN arthritis (Jang et al., 2009). Chapter III of this thesis investigates the cellular source and target of IL-21 in the K/BxN model.

Th17 cells and IL-17

Th17 cells are a subset of MHC-II restricted T cells thought to have evolved to fight extracellular pathogens. Their development is induced by TGF-β and IL-6 and is regulated by the transcription factor RORγt (Ivanov et al., 2006; Mangan et al., 2006; Bettelli et al., 2006) (Figure 1.4). Th17 cells are named for the cytokine IL-17, which they produce along with IL-17F, IL-21 and IL-22 (Korn et al., 2009). First thought to be exclusively a helper T cell cytokine (Rouvier et al., 1993; Yao et al., 1995), IL-17 has since been demonstrated to be secreted by γδ T cells, neutrophils, NK cells, eosinophils, and type 3 innate lymphoid cells (ILC3s) (Rouvier et al., 1993; Yao et al., 1995; Buonocore et al., 2010; Lockhart et al., 2006; Ferretti et al., 2003; Zhou et al., 2005). IL-17 acts on many hematopoietic and stromal cell types and induces the expression of a number of cytokines, chemokines, and metalloproteinases (Kolls and Lindén, 2004). IL-17 is also well characterized in its ability to recruit, activate, and retain neutrophils at sites of inflammation (Aggarwal and Gurney, 2002). There is also evidence of IL-17 acting directly on B cells and influencing class switching to certain isotypes, germinal center formation, and cell localization within the B cell follicle (Mitsdoerffer et al., 2010; Hsu et al., 2008).

Th17 cell differentiation in the intestinal mucosa is promoted by bacterial colonization (Atarashi et al., 2008). Segmented filamentous bacteria (SFB) in particular has been shown to be a potent inducer of Th17 cell differentiation (Ivanov et al., 2009; Gaboriau-Routhiau et al., 2009). SFB is a gram-positive spore forming bacterium that colonizes the distal small intestine of rodents and other vertebrates (Davis and Savage, 1974). As the name implies, SFB grow as segmented filaments and attach to the host epithelium, unlike most other commensals that are restricted to the lumen of the gastrointestinal tract.

How SFB induces Th17 cells is incompletely understood. It is known that SFB induces serum amyloid A (SAA), which promotes Th17 cell differentiation (Ivanov et al., 2009). Th17 cell differentiation promoted by SFB colonization requires antigen presentation and were found to have SFB-reactive TCRs (Goto et al., 2014; Yang et al., 2014). CX4CR1 macrophages in the intestine are required for this antigen-specific Th17 induction (Panea et al., 2015). There is now evidence that epithelial adhesion of SFB (as well as other bacteria that have this attaching capacity) is responsible for the induction of Th17 cells (Pedicord and Mucida, 2015). SFB found in rats can colonize mice, but do not attach to the epithelium and are not capable of inducing Th17 cells. Similarly, *Citrobacter rodentium* and *Escherichia coli* can attach to the epithelium of the colon and induce Th17 cells, but mutants incapable of attaching do not induce Th17 (Atarashi et al., 2015). Part of the gene signature of colonization with adherent bacteria is induction of expression of SAA in epithelial cells (Atarashi et al., 2015). Sano and colleagues provide data that suggests that type 3 innate lymphoid cells (ILC3s) must secrete IL-22 to induce SAA production from the epithelium, which acts directly on Th17 cells to promote Il-17 production (Sano et al., 2015). Adhesion of bacteria causes changes in the organization of actin

in epithelial cells, but how this is translated into immune modulating signals remains to be elucidated.

Th17 cells and IL-17 in K/BxN arthritis

Over 25 years ago adaptive immune responses were characterized as either Th1 or Th2 skewed, and for a time after autoimmune diseases were generally believed to be Th1 mediated (Mosmann and Coffman, 1989). The K/BxN mouse model was thought to be both Th1 and Th2-dependent due to the requirement of IL-4 for autoantibody formation (Ohmura et al., 2005). The discovery of Th17 cells shifted this Th1-Th2 paradigm, and in fact Th17 were first discovered and characterized in the autoimmune model experimental autoimmune encephalitis (EAE) (Park et al., 2005; Langrish et al., 2005). Th17 cells and IL-17 have been an exciting potential target for treatment of autoimmunity since this discovery (Murdaca et al., 2011).

Interest in the autoimmune-associated Th17 population led to investigation of this subset in the K/BxN model. One study compared *in vitro* polarized Th17 cells versus Th1 cells in their ability to induce arthritis (Hickman-Brecks et al., 2011). Using the T cell transfer model, it was shown that Th17-polarized cells induced arthritis better than Th1-polarized cells, and antibody blockade of IL-17 slightly delayed disease upon Th17 cell transfer. Th17 cell transfer skewed antibody class switching toward IgG2b, compared to IgG1 when naïve T cells were transferred. In another study, KRN T cells could enhance disease in serum transfer-induced arthritis. This enhancement was reduced when a monoclonal antibody against IL-17 was co-administered, suggesting that IL-17 produced by T cells was enhancing the effector phase of disease (Jacobs et al., 2009). Serum transfer into IL-17R deficient mice induced less neutrophil infiltration, reduced bone erosion, and decreased ankle thickening, again suggesting IL-17 plays a role in the

effector phase of disease (Sadik et al., 2011). However, the source of IL-17 was not addressed in this study.

Wu and colleagues published a study connecting SFB, Th17 cells, and K/BxN arthritis (Wu et al., 2010). Th17 cells were first detectable in multiple sites around the time of disease onset in K/BxN mice. Germ free K/BxN mice were largely protected from disease, and colonization with SPF microbiota restored both Th17 cell differentiation and autoantibody production. Monocolonization of mice with SFB was also sufficient to induce Th17 cells and disease (Wu et al., 2010). These results suggested that the effect of gut bacteria (particularly SFB) on disease was through the induction of Th17 cells (Figure 1.5). Blockade of IL-17 with a monoclonal antibody prevented disease and decreased autoantibody titers. IL-17 was thought to affect B cell recruitment into germinal centers because IL-17R deficient B cells were unable to enter autoreactive germinal centers in a competitive setting (Wu et al., 2010). It is therefore proposed that SFB induced Th17 cell derived IL-17 promote K/BxN arthritis by enhancing germinal center responses. We test the importance of IL-17 in K/BxN in Chapter IV.

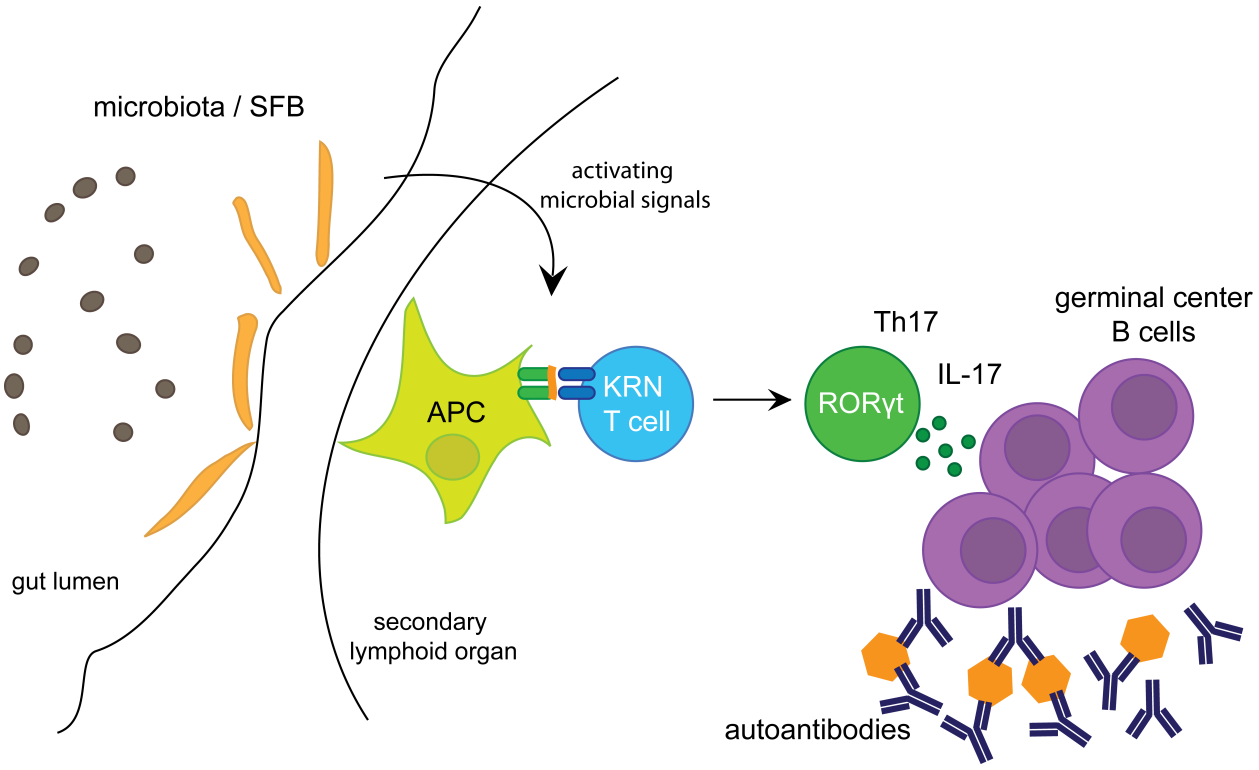

Figure 1.5. Current model of microbiota promoting K/BxN arthritis. The microbiota, and specifically SFB, promote the differentiation of Th17 cells. IL-17 from Th17 cells promotes the development of autoreactive germinal center B cells.

Summary

Autoimmune disease is a result of incompletely understood genetic and environmental triggers that lead to immune-mediated damage of self-tissues. Aberrant T cell activation and alterations of the microbiota have both been implicated in autoimmune pathogenesis. The K/BxN mouse model of autoimmune arthritis provides a system to test these factors. Activated helper T cells can have many fates, and it is unclear which subsets contribute to arthritis. In this thesis I explore the roles of two of these subsets, Tfh and Th17, and their cytokines IL-21 and IL-17, in autoimmune initiation. I also investigate how these populations are influenced by the microbiota.

Chapter II: Materials and Methods

Mice

Mice were bred in-house and maintained under specific pathogen free (SPF) conditions at The University of Chicago (Chicago, IL). The experiments described were approved by IACUC of the University of Chicago and conform to the principles set forth by the Animal Welfare Act and National Institutes of Health guidelines for the care and use of laboratory animals in biomedical research.

Mouse lines: B6 (C57BL/6J), NOD (NOD/ShiLtJ) RORγt deficient (*Rorc*^{tm2Litt}), Rag1 deficient B6 (*Rag1*^{tm1Mom}), Rag1 deficient NOD (*Rag1*^{tm1Mom}/J), IL-17 deficient (*Il17a*$^{tm1/1(icre)Stck}$), and CD4-Cre (Tg(CD4-cre)1Cwi) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). IL-21R deficient mice (TF0899) were purchased from Taconic. IL-21$^{-/-}$ mice (B6;129S5-*Il21*^{tm1Lex}) were obtained from the Mutant Mouse Regional Resource Center. KRN TCR transgenic (KRN/B6), B6 H-2^{g7} congenic (B6.H-2^{g7}), TCR Cα$^{-/-}$ B6 and TCR Cα$^{-/-}$ NOD mice were gifts from D. Mathis and C. Benoist (Harvard Medical School). Bcl6-floxed mice were generated as described previously (Hollister et al., 2013) and provided by Dr. Dent at the University of Indiana.

Spontaneous Model: K/BxN mice were generated by crossing KRN/B6 mice to NOD mice. To produce K/g7/IL17 mice (outlined in Figure 2.1A), we bred KRN/B6 mice to IL-17 deficient mice, generating K/IL-17$^{+/-}$. We bred IL-17 deficient mice to B6.H-2g7 mice, generating IL-17$^{+/-}$ H-2$^{g7/b}$. We intercrossed IL-17$^{+/-}$ H-2$^{g7/b}$ mice to generate IL-17$^{-/-}$ H-2$^{g7/g7}$ mice. We then crossed K/IL-17$^{+/-}$ to IL-17$^{-/-}$ H-2$^{g7/g7}$ to generate K/IL-17$^{+/-}$ H-2$^{g7/b}$ (called

K/g7/IL-17$^{+/-}$ in the text and IL-17+/- in the figures) and K/IL-17$^{-/-}$ H-2$^{g7/b}$ (called K/g7/IL-17$^{-/-}$ in the text and IL-17-/- in the figures).

Transfer Model, transfer mice: For T cell transfers, KRN/B6 mice were crossed to various lines to generate K/IL-21R$^{-/-}$, K/IL-21$^{-/-}$, and K/RORγt$^{-/-}$ mice. For B cell transfers IL-21R$^{-/-}$ mice were crossed to B6.H2g7 congenic mice for purified B cell transfer experiments. To produce K/Bcl6Δ mice (outlined in Figure 2.1B), we bred KRN/B6 to Bcl6-floxed mice, generating K/Bcl6$^{fl/+}$. We bred Bcl6-floxed mice to CD4-Cre mice, generating CD4-Cre/Bcl6$^{fl/+}$. We then bred Cre/Bcl6$^{fl/+}$ back to Bcl6-floxed mice, generating CD4-Cre/Bcl6$^{fl/fl}$. Finally, we crossed K/Bcl6$^{fl/+}$ mice to CD4-Cre/Bcl6$^{fl/fl}$ to generate K/CD4-Cre/Bcl6$^{fl/+}$ (called K/Bcl6+ in the text and figures) and K/CD4-Cre/Bcl6$^{fl/fl}$ (called K/Bcl6Δ in the text and figures). Whenever possible, K/WT controls in experiments were wild type or heterozygous littermates. For example, when comparing WT to IL-21R$^{-/-}$ and IL-21$^{-/-}$, the WT mouse was a littermate of IL-21R$^{-/-}$ but not IL-21$^{-/-}$ mouse. In some rare cases, a WT littermate was not available, and an age-matched control was used.

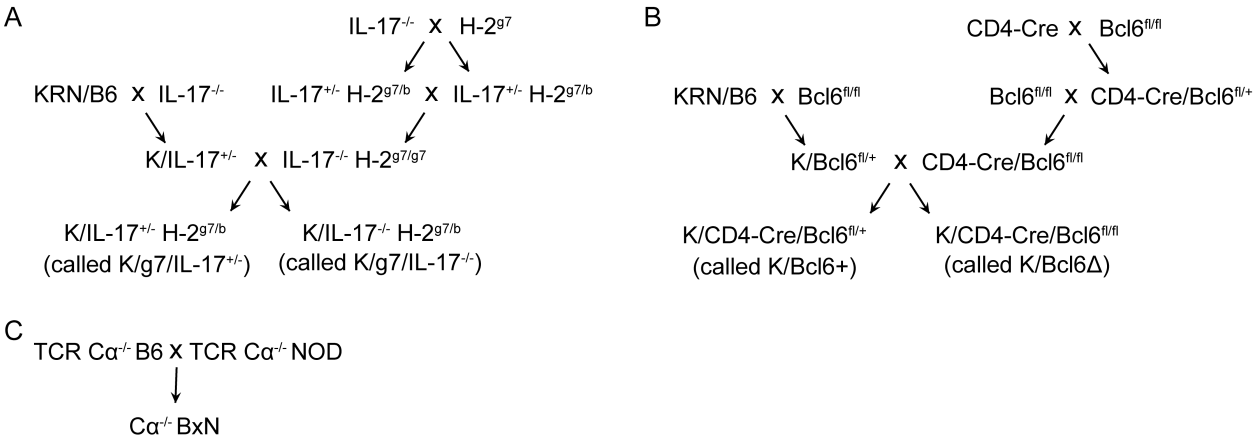

Figure 2.1. Breeding strategies. (**A**) K/g7/IL17-/- mice, (**B**) K/Bcl6Δ mice, and (**C**) Cα$^{-/-}$ BxN host mice.

Transfer Model, host mice: Host mice for T cell transfer experiments were generated by crossing TCR $C\alpha^{-/-}$ B6 to TCR $C\alpha^{-/-}$ NOD (called $C\alpha^{-/-}$ BxN in the text, Figure 2.1C). Host mice for B and T cell transfer experiments were generated by crossing $Rag1^{-/-}$ B6 to $Rag1^{-/-}$ NOD (called $Rag1^{-/-}$ BxN in the text).

Cell transfer induction of arthritis

Splenocyte transfer: Spleens were harvested from naïve mice and placed in sterile DMEM without phenol red (referred to as DMEM throughout this section). Spleens were homogenized between two frosted glass slides (Corning). Red blood cells were lysed (155 mM NH_4Cl, 0.1 mM EDTA, 12 mM $NaHCO_3$). Cells were resuspended in DMEM and filtered through a 100 μm Nytex filter, then counted using a hemocytometer. An equivalent number of cells per group were transferred intravenously into host mice with a 27 gauge needle, usually ~$10x10^6$ cells in 150 μl. Flow cytometry was also performed to confirm that the percent $CD4^+$ and $KRN\alpha^+$ cells were similar between groups.

$CD4^+$ cell transfer: Spleens were harvested from naïve mice and placed in sterile AutoMACS buffer (1x PBS, 0.5% BSA, 2mM EDTA, referred to as "buffer" throughout this section). Spleens were homogenized between two frosted glass slides and red blood cells were lysed. Cells were resuspended in buffer and filtered through a 100 μm Nytex filter, counted using a hemocytometer, and then brought to $3x10^7$ cells / 100 μl. Fc block (clone 24G.2, Fitch Monoclonal Antibody Facility) was added (final dilution 1x) and incubated on ice for 10 minutes. Biotinylated αCD4 antibody was added (final dilution 1:800) and incubated on ice for 15 minutes with occasional mixing. Cells were washed 3 times in 3-5ml of buffer. Cells were resuspended at $3x10^7$ cells/100 μl, and streptavidin microbeads were added (final dilution 1:20),

and incubated on ice for 15 minutes with occasional mixing. Cells were re-counted, resuspended at $1x10^7$/ml, and filtered again. $CD4^+$ cells were isolated from splenocytes using the AutoMACS positive selection program ("possel"). The wash program was run between each sample. Positive and negative fractions were counted to determine yield. The positive fraction was then washed in sterile DMEM and $1x10^6$ cells in 150 µl sterile DMEM were injected into the tail vein of $C\alpha^{-/-}$ BxN mice with a 27 gauge needle.

B cell transfer: Splenocytes were isolated by creating a single cell suspension and lysing red blood cells as described above. T cells were depleted by complement-mediated lysis. Briefly, splenocytes were resuspended in 2.5 mL buffer (½ RMPI, 10% FCS, ½ wash buffer (PBS, 1% FCS)) and incubated with 5 µl anti-CD90.2 antibody (clone 53-2.1, BioLegend) for 12 minutes at room temperature with slow rotation. 250 µl rabbit complement (2 mL/vial, Cedar Lane) was added, and samples incubated for 20 minutes at 37°C. Cells were washed twice in wash buffer and resuspended in sterile DMEM without phenol red. Cells were analyzed via flow cytometry to confirm the deletion of T cells. Approximately $2x10^7$ cells were combined with K/B6 T cells (isolation described above) and were injected into the tail vein of $Rag1^{-/-}$ BxN mice with a 27-gauge needle.

Antibodies and flow cytometry

Anti-KRN TCRa specific antibody (clone 3-4G-B7) was created in the laboratory (Perera et al., 2013). Commercially available antibodies used were against CXCR5, Bcl6, and Fas (BD Biosciences); CD45.1, TCRβ, GL-7, CD19, IL-17A, and B220 (eBioscience); and CD4 and PD-1 (BioLegend). Intracellular staining for Bc6 was performed using the Foxp3 intracellular staining kit from eBioscience, according to the manufacturer's protocol. To stain for

intracellular IL-17, cells were stimulated with PMA and ionomycin as described (Mitsdoerffer et al., 2010) and stained for IL-17 with the intracellular staining kit from BD Biosciences according to the manufacturer's protocol. Multicolor flow cytometric analysis was performed using a FACSCanto and FACSCalibur (BD Biosciences). Data analysis was conducted using FlowJo (Tree Star, Inc.).

ELISA for anti-GPI immunoglobulin serum titers

96-well flat bottom medium-binding plates (Corning) were coated with 100 µl of 5 µg/mL of recombinant GPI in PBS overnight at 4˚C. After a wash in wash buffer (1x PBS with 0.05% Tween-20), plates were incubated with 100 µl blocking buffer (1% BSA in wash buffer) at room temperature for 2 hours. After 3 washes, a serial dilution of each serum sample was prepared, with the highest concentration generally beginning at 1:50, and the diluted 5 to 7.5-fold for 6 to 8 wells. 100 µl of sample/well was incubated at room temperature for 1 hour, and then washed 3x. Bound antibody was detected with a biotinylated goat anti-mIgG (subclasses 1+2a+2b+3) Fcg, goat anti-mouse IgM, or IgG subclass fragment specific antibody, all diluted to 1:2500, 100 µl/well, for 1 hour at room temperature (all from Jackson ImmunoResearch Laboratories, Inc.). After 3 washes, wells were incubated for 30 minutes at room temperature with 100 µl alkaline phosphatase conjugated streptavidin (final concentration 1:2500) (Jackson ImmunoResearch Laboratories Inc). After 3 washes, samples were developed with phosphatase substrate (Sigma), 1 tablet per 5 ml of AP base buffer. Plates were read at 405 nm. A four-parameter variable slope was fitted to the data points and the EC50 (inflection point) for a standard sample was calculated from this non-linear regression. The standard sample was serum from a K/BxN arthritic mouse that was included on all plates. Serum titers were calculated as

the serum dilution (x value) that gave the calculated EC50 (y value) based on the fitted non-linear regression for each sample. Samples where the curve could not be fitted because of low signal (low antibody binding) are indicated as ND (not detectable) and a titer of 1 was assigned for statistical comparisons. All analyses were conducted using Prism 5.0b software (GraphPad).

Immunohistochemistry staining of splenic sections

Spleens were cut in half transversely, and one half was generally used for flow cytometry. The second half was cut transversely again, and pieces were placed vertically in a cryomold filled with O.C.T. Compound (Tissue-Tek) and frozen on dry ice and stored at -80°C. 5 μm sections were mounted on glass slides and stored at -80°C. Slides were thawed, rehydrated, blocked with Fc block, and then stained. Peanut agglutinin (PNA, Alexa Fluor 488), anti-mouse IgD (PE), and anti-KRN TCRα (Alexa Fluor 647) were used. Images were taken on an Axiovert200m (Zeiss) and visualized with ImageJ.

Lamina propria lymphocyte isolation

Lamina propria lymphocytes were isolated from the small intestine using enzymatic digestion as described in (Wu et al., 2010) with some modifications. To summarize, the small intestine was taken, residual mesenteric fat tissue was removed, Peyer's patches were excised, and the intestine was opened longitudinally. Intestines were cleaned with ice cold DMEM without phenol red and contents were removed. Tissue was cut into 1-2 cm pieces and placed in 10 ml epithelium removal buffer (DMEM, 5 mM EDTA, 0.145 mg/mL DTT) and shaken for 20 minutes at 37°C at 200 RPM. Tissue was then filtered and the large pieces saved, then rinsed in

wash buffer (DMEM, 3% FBS). Tissue was diced finely and placed in 5 mL digest buffer (DMEM, 5% FBS, 12.5 μg/mL liberase, 80 μg/mL DNase) and shaken for 20 minutes at 37°C at 200 RPM. Tissue was then filtered and the flow-through was washed and resuspended in 4 mL wash buffer. This sample was mixed with 4 mL 80% percoll (GE) to make 40% percoll and was then layered over 5 mL 80% percoll. Cells were separated on the percoll gradient by spinning for 30 minutes at 30°C at 2500 RPM at room temperature with no brake, and cells were collected at the interface. Cells were washed, filtered, and resuspended in a small volume. Cells were stimulated *in vitro* and stained for cytokines as described above.

Anti-IL-17 antibody blockade

250 μg of anti-IL17 neutralizing antibody clone TC11-18H10.1 (BioLegend) or clone 17F3 (BioXCell) was administered by intraperitoneal injection every 3 to 4 days beginning at 21 to 22 days of age.

Antibiotic treatment

1g/L ampicillin (Sigma) and 0.5g/L vancomycin (Hospira) were added to drinking water of K/BxN or K/g7/IL17 breeding pairs and mice were allowed to drink ad libitum. Water was changed approximately every 6 days. Offspring of these breeders were maintained on the same antibiotic water and were gavaged daily with 100 μl water containing 20 μg ampicillin and 10 μg vancomycin, beginning at 21 days old and continuing until time of analysis. This treatment was more effective at preventing disease than antibiotics in the drinking water alone, and the act of gavaging did not affect arthritis development (Figure 2.2).

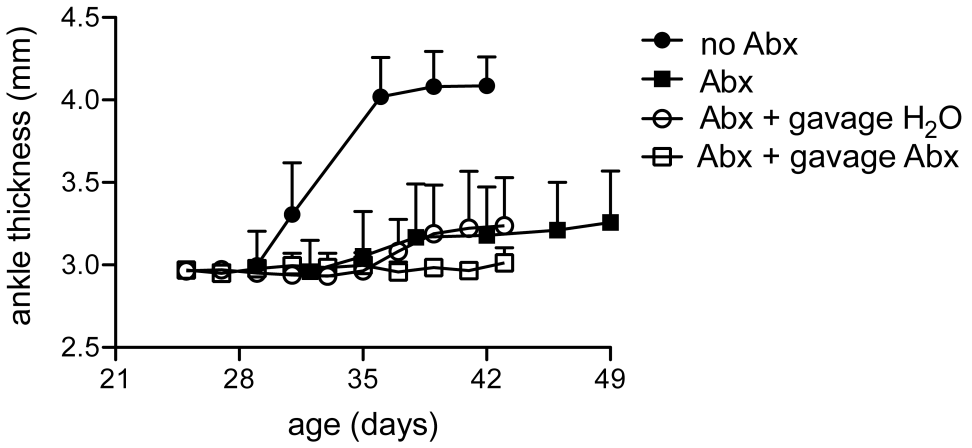

Figure 2.2. Antibiotic treatment of K/BxN mice. SPF mice (no Abx, *n* = 9) develop arthritis around 4-5 weeks of age. Treatment with ampicillin and vancomycin (Abx, *n* = 9) in the drinking water from birth reduces disease prevalence and severity. Additionally gavaging mice with Abx starting from weaning further reduces disease incidence (Abx + gavage Abx, *n* = 5). Gavage with H_2O does not alter disease (Abx + gavage H_20, *n* = 4).

qPCR for 16S rRNA

Fresh fecal pellets were collected from mice and stored at -20°C until DNA isolation. DNA was isolated using the QIAamp Fast DNA Stool Mini Kit (Qiagen) following the manufacturer's protocol using the following modifications: pellets were lysed using a minibeater for 90 seconds with silica beads (Zymo) instead of the initial vortexing step, and the first heating step was performed at 95°C. Total bacteria (EUA) 16S and segmented filamentous bacteria specific (SFB) 16S rRNA were amplified using primers as described in (Upadhyay et al., 2012). qPCR primers: EUA-F: 5'-ACTCCTACGGGAGGCAGCAGT-3', EUA-R: 5'-ATTACCGCGGCTGCTGGC-3', SFB-F: 5'-GACGCTGAGGCATGAGAGCAT-3', SFB-R: 5'-GACGGCACGGATTGTTATTCA-3'. Samples were tested in duplicate, in a volume of 25 µl (12.5 µl SYBR green, 0.5 µl each primer at 10 mM, 10 ng DNA). Cycling conditions: 95°C

for 0:40; 45 cycles of 95°C for 0:05, 60°C for 0:30; 95°C for 0:15; 60°C for 1:00. Melting curve from 60°C to 95°C, reading every 5°C. Relative SFB 16S rRNA is calculated as $2^{-\Delta Ct}$ where $\Delta C_t = C_t$ SFB – C_t EUA.

Statistical analysis

Normally distributed data were analyzed by the unpaired *t* test. Welch's correction was used for testing groups with different variances. Two-way ANOVA was used to test the effect of antibiotic treatment and IL-17 genotype (Figure 4.5). All statistics performed using Prism 5.0b software (GraphPad).

Chapter III: The cellular source and target of IL-21 in K/BxN autoimmune arthritis

This chapter is adapted from: Block, K.E., and H. Huang. 2013. The Cellular Source and Target of IL-21 in K/BxN Autoimmune Arthritis. *J. Immunol.* 191: 2948-2955.

Introduction

IL-21, a member of the common γ chain–signaling family of cytokines, plays an important role in lymphocyte activation, survival and differentiation (Spolski et al., 2008). IL-21 production is restricted to activated T cells, such as follicular helper T cells (Tfh), Th17, and NKT cells. The receptor for IL-21 is widely expressed on a variety of cell types, including B cells, activated T cells, NK cells and dendritic cells. IL-21 promotes B cell proliferation, immunoglobulin class switching and production, and plasma cell differentiation (Ettinger et al., 2008). IL-21 also enhances the proliferation of T cells stimulated through their T cell receptors (Parrish-Novak et al., 2000) and has been shown to be an autocrine growth factor for Tfh and Th17 cell differentiation (Vogelzang et al., 2008; Nurieva et al., 2008).

The Tfh cell subset, a canonical producer of IL-21, is controlled by the transcription factor Bcl6. Changes in chemokine receptor expression allow Tfh cells to migrate from the T cell zone into B cell follicles. Expression of cell surface molecules, such as PD-1, CD40L, OX40, SAP, and ICOS, promotes cell-cell contacts with B cells presenting cognate antigen. It is in these intimate interactions that IL-21 from Tfh cells is thought to act on B cells to promote germinal center and plasma cell differentiation (reviewed in (Crotty, 2011)). In addition to Tfh,

the Th17 cell subset also produces IL-21 (Wei et al., 2007). Th17 is a dominant proinflammatory T cell subset, controlled by the transcription factor RORγt, and is implicated in a number of autoimmune diseases (reviewed in (Korn et al., 2009)). Th17 cells have been shown to directly interact with and help B cells (Mitsdoerffer et al., 2010) and promote spontaneous GC formation in autoimmune BXD2 mice (Hsu et al., 2008).

IL-21 is important in a number of animal models of SLE and rheumatoid arthritis (Nguyen et al., 2012). Accordingly, an association of certain IL-21 and IL-21R alleles with risk for SLE in humans was reported (Webb et al., 2009). In the NOD mouse model IL-21 is required for the development of type I diabetes (Spolski et al., 2008; King et al., 2008). Given the complex biological functions of IL-21, it is important to understand the relevant cells producing and responding to the cytokine in the context of B cell-mediated autoimmunity.

We investigated this question of the relevant targets of IL-21 using the K/BxN model of rheumatoid arthritis. Here, we have utilized this cell transfer approach of the K/BxN model to determine the source and action of IL-21 in arthritis. We showed that T cells deficient in IL-21 did not induce GC formation or autoantibody production, but went through normal Tfh differentiation. IL-6R was more important for Tfh differentiation than IL-21R, although neither were required. However, T cells lacking IL-21R induced similar antibody titers, GC B cell frequency, and arthritis development as wild type T cells, suggesting that IL-21 is not required for Tfh differentiation and function. IL-21 must act on B cells, as IL-21R expression on B cells was required to induce disease. Surprisingly, Th17 cells are not required for arthritis development, stressing the importance of IL-21 production specifically from the Tfh subset. These results have implications for developing effective therapies for rheumatoid arthritis and other antibody-mediated autoimmune diseases.

Results

Tfh differentiation in the cell transfer model of autoimmune arthritis

To investigate the role of IL-21 in a cell specific manner, we took advantage of the cell transfer model of the K/BxN mouse. Naïve $CD4^+$ KRN T cells are isolated from healthy KRN/B6 (KRN maintained on C57/BL6 background) and are transferred into $C\alpha^{-/-}$ BxN hosts (TCR $C\alpha^{-/-}$ on B6xNOD F1 background) (Korganow et al., 1999; LaBranche et al., 2010). These hosts lack αβ T cells and express the MHC-II allele $I\text{-}A^{g7}$, which is required for the KRN TCR to recognize a peptide from the self-antigen GPI. Transferred KRN T cells are activated and induce high titers of anti-GPI IgG antibodies resulting in ankle swelling and joint remodeling. To follow autoreactive Tfh differentiation and germinal center responses, Tfh cells and germinal center B cells were characterized after T cell transfer. For comparison, naïve KRN/B6 T cells were transferred into $C\alpha^{-/-}$ B6 hosts. Because $C\alpha^{-/-}$ B6 mice do not carry the MHC-II allele $I\text{-}A^{g7}$, KRN T cells do not precipitate disease upon transfer. This allowed us to verify that Tfh cell differentiation is dependent on antigen recognition rather than lymphopenia-induced homeostatic proliferation. PD-1^+ $CXCR5^+$ or $Bcl6^+$ $CXCR5^+$ Tfh cells were identified in $C\alpha^{-/-}$ BxN hosts, but not in $C\alpha^{-/-}$ B6 hosts or KRN/B6 mice (Figure 3.1A). In the following experiments we used CXCR5 and intracellular Bcl6 to mark Tfh cells since these markers showed a more distinct pattern. Consistent with the Tfh cell staining, germinal center B cells (identified as GL-7^+ Fas^+) were induced in $C\alpha^{-/-}$ BxN hosts while $C\alpha^{-/-}$ B6 hosts had a small germinal center population, similar to what was observed in naïve KRN/B6 mice (Figure 3.1B).

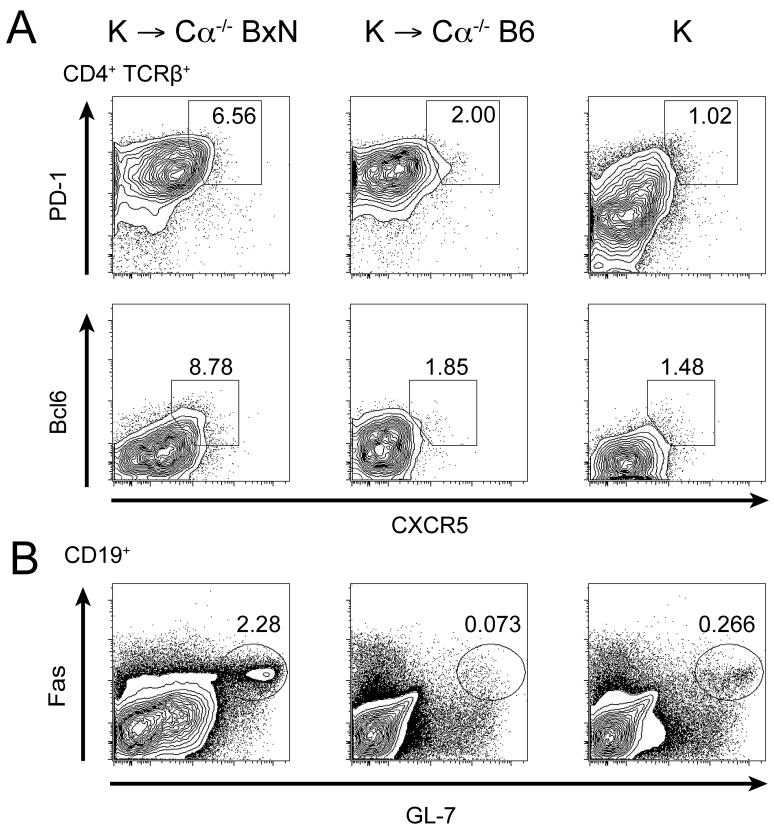

Figure 3.1. KRN T cell transfer leads to Tfh and GC B cell differentiation upon antigen recognition. (**A**) $CD4^+$ cells from KRN/B6 (K) mice were transferred into $C\alpha^{-/-}$ BxN or $C\alpha^{-/-}$ B6 hosts and splenocytes were analyzed 8 days later. Transferred cells, identified as $CD4^+$ $TCR\beta^+$, were assessed for Tfh differentiation by CXCR5 and PD-1 expression as well as CXCR5 and intracellular Bcl6 expression. Naïve $CD4^+TCR\beta^+$ cells in KRN/B6 splenocytes were used as controls. (**B**) GC B cells were assessed by Fas and GL-7 expression in the $CD19^+$ population. Results are representative of three independent experiments.

IL-21 production by T cells is required to induce arthritis

To determine whether IL-21 production by T cells acts on T cells in an autocrine manner to induce arthritis, we compared naïve $CD4^+$ KRN T cells purified from wild-type K/B6 mice (in figures denoted as K) to those purified from KRN mice deficient in IL-21 (referred to as K/IL-$21^{-/-}$) or IL-21R (referred to as K/IL-$21R^{-/-}$) after transfer into $C\alpha^{-/-}$ BxN hosts. In the

K/IL-21$^{-/-}$ transfer, KRN T cells could not produce IL-21, and in the K/IL-21R$^{-/-}$ transfer KRN T cells were able to produce IL-21 but unable to signal through the IL-21R. These two groups allowed us to test whether IL-21 production by autoreactive T cells is required and whether autoreactive T cells require IL-21 as an autocrine factor for disease. As shown in Figure 3.2A, K/IL-21$^{-/-}$ CD4^{+} T cells transferred into C$\alpha^{-/-}$ BxN hosts did not induce arthritis. In contrast, K/IL-21R$^{-/-}$ CD4^{+} T cells induced severe arthritis with the same kinetics as the wild-type KRN T cells. We determined the anti-GPI IgG titers both early (8 days) and late (29-31 days) in disease. Wild-type KRN T cells and K/IL21R$^{-/-}$ T cells induced high titers of anti-GPI IgG at both time points. In contrast, anti-GPI IgG titers were two to three orders of magnitude lower in K/IL21$^{-/-}$ T cell transfer (Figure 3.2B). These data demonstrate that IL-21 production by T cells is crucial for IgG antibody response and arthritis, but that IL-21R is not required on T cells.

To test the role of IL-21 in extrafollicular response in this model, we determined anti-GPI IgM and IgG at earlier timepoints after transferring wild-type KRN or K/IL-21$^{-/-}$ splenocytes. In wild-type KRN transfer, anti-GPI IgM titers were elevated by day four and continued to increase over time, occurring before IgG titers increased. In contrast, both anti-GPI IgM and IgG titers remained low in K/IL-21$^{-/-}$ transfers (Figure 3.2C). These results suggest that IL-21 is required in even the early extrafollicular response in this model.

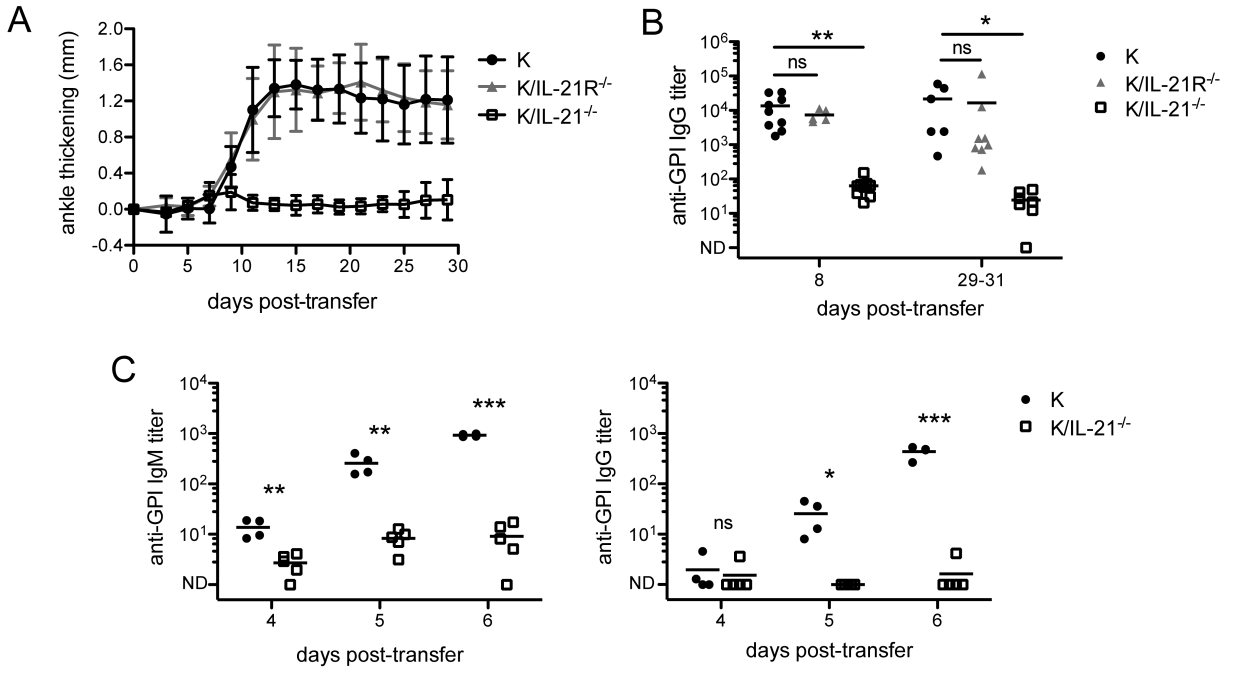

Figure 3.2. T cell production of IL-21 is required for arthritis development. (**A**) 1x10^6 $CD4^+$ cells purified from KRN/B6 (K), KRN IL-21R$^{-/-}$ (K/IL-21R$^{-/-}$), or KRN IL-21$^{-/-}$ (K/IL-21$^{-/-}$) mice were transferred into C$\alpha^{-/-}$ BxN hosts and ankle thickening was measured over time. Results shown are from two experiments (n = 6 - 8 mice per group). Mean and standard deviation are shown. (**B**) Anti-GPI IgG titers were measured by ELISA from serum collected 8 and 29 - 31 days post-transfer. Mean is shown; each symbol represents an individual mouse. Day eight results are from four experiments (n = 5 - 10 mice per group). Results for days 29-31 are from the experiment described in A. (**C**) 8x10^6 splenocytes from KRN/B6 (K) or KRN IL-21$^{-/-}$ (K/IL-21$^{-/-}$) mice were transferred into C$\alpha^{-/-}$ BxN hosts and serum was collected on the days indicated. Anti-GPI IgM and IgG titers were measured by ELISA. Mean is shown; each symbol represents an individual mouse (n = 4-5 mice per group). ND (not detectable). Student's t-test: ns, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

IL-21 is not required for Tfh cell differentiation in vivo

We next compared the fate of transferred T cells and Tfh differentiation in all three transfer settings. Congenic markers on transferred cells ($CD45.2^+$) and $C\alpha^{-/-}$ BxN host cells ($CD45.1^+/CD45.2^+$) allowed us to identify the transferred T cells as the $CD45.1^-$ $CD4^+$ population (Figure 3.3A). Eight days after cell transfer, just after disease onset, there was a small but significant increase in the percentage and number of K/IL-$21^{-/-}$ T cells compared to WT KRN T cells in the spleen. The percentage and number of K/IL-$21R^{-/-}$ T cells were comparable to WT KRN T cells. At 29 to 31 days, when disease was fully established in WT KRN and K/IL-$21R^{-/-}$ T cell transfer, there was no significant difference in percentage and numbers of transferred T cells among the three groups. These data suggest that the survival of transferred $CD4^+$ KRN T cells was not affected by their ability to produce or respond to IL-21.

To determine how Tfh cell differentiation was affected, CXCR5 and intracellular Bcl6 staining were used to identify Tfh cells. WT KRN, K/IL-$21R^{-/-}$, and K/IL-$21^{-/-}$ T cells differentiated into the Tfh subset at similar frequencies in the spleen 8 days after transfer (Figure 3.3B). The absolute number of Tfh cells from K/IL-$21^{-/-}$ animals was transiently higher than that from WT donors due to a higher total number of $CD4^+$ T cells, as shown in Figure 3.3A. However, by 29-31 days K/IL-$21^{-/-}$ Tfh cell percentage and numbers decreased to half of those in WT KRN transfer. This presumably reflects the defects in the maintenance phase of Tfh cell differentiation (Choi et al., 2011) given that there were no germinal centers formed in these mice (see below). However, there were comparable numbers of WT and K/IL-$21R^{-/-}$ Tfh cells 8 days after transfer, and a small but not statistically significant decrease in Tfh cell numbers in K/IL-$21R^{-/-}$ cells compared to in WT at days 29-31 (Figure 3.3B). These data suggest that IL-21 is not a requisite autocrine factor for KRN Tfh cell differentiation.

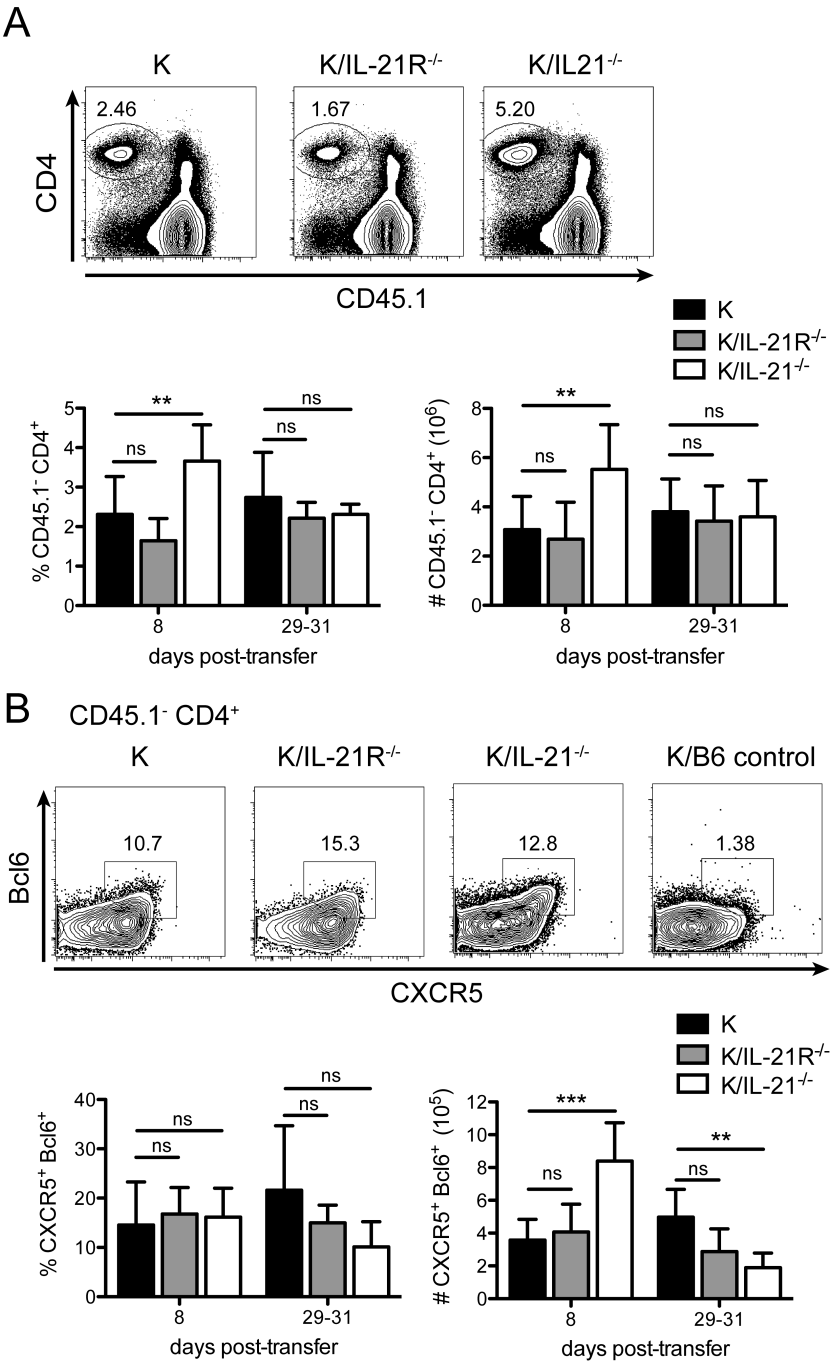

Figure 3.3. IL-21 production and IL-21 receptor signaling are not required for KRN T cell survival or Tfh cell differentiation. (**A**) Transferred KRN T cells were identified in the spleen 8 and 29-31 days post-transfer by flow cytometry as $CD45.1^{-}$ $CD4^{+}$. Percent and total number of transferred cells were shown. (**B**) Tfh differentiation of transferred cells was determined by CXCR5 and intracellular Bcl6 expression. Percent and total number of Tfh cells are shown. Day 8 results are from four experiments (n = 5 - 10 mice per group), Days 29-31 results are from two experiments (n = 6 - 8 mice per group). Mean and standard deviation are shown. Student's t-test: ns, $p \geq 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

IL-6R signaling promotes Tfh cell differentiation but is partly redundant with IL-21R signaling

Tfh cell differentiation is thought to happen through TCR signaling combined with IL-21R or IL-6R signaling (Nurieva et al., 2008; Eto et al., 2011; Nurieva et al., 2009). Both IL-21R and IL-6R signal through STAT3 (Asao et al., 2001; Zhong et al., 1994). Since IL-6R and IL-21R signaling are partially redundant for induction of Tfh cells (Eto et al., 2011), we investigated whether IL-6R deficiency or combined IL-21R and IL-6R deficiency would prevent Tfh cell differentiation and subsequently prevent autoimmune development upon T cell transfer. Transfer of T cells deficient in IL-6R alone or IL-21R and IL-6R led to arthritis similar to transfer of wild-type KRN T cells (Figure 3.4A). Anti-GPI IgG serum titers were also similar among all groups (Figure 3.4B). However, Tfh cell differentiation, as measured by percent Tfh cells among all $CD4^+$ transferred cells, at approximately 2 weeks after transfer was reduced in IL-6R deficient T cells and further reduced in the double deficient T cells. By total number of Tfh, only the double deficient cells had a statistically significant decrease in Tfh cells compared to wild type (Figure 3.4C). A similar trend was seen in germinal center B cells; the cell population was decreased with the loss of each receptor, with the percent loss being more marked than the loss in total cell number (Figure 3.4D). A loss of the cytokine receptors on T cells did not completely abolish the differentiation of Tfh or GC B cells, suggesting that additional signals promote the differentiation of Tfh cells, and that even in the absence of IL2-1R and IL-6R signaling, the Tfh cells that do develop are functional and can promote the differentiation of GC B cells capable of class switching and somatically hypermutating their B cell receptors.

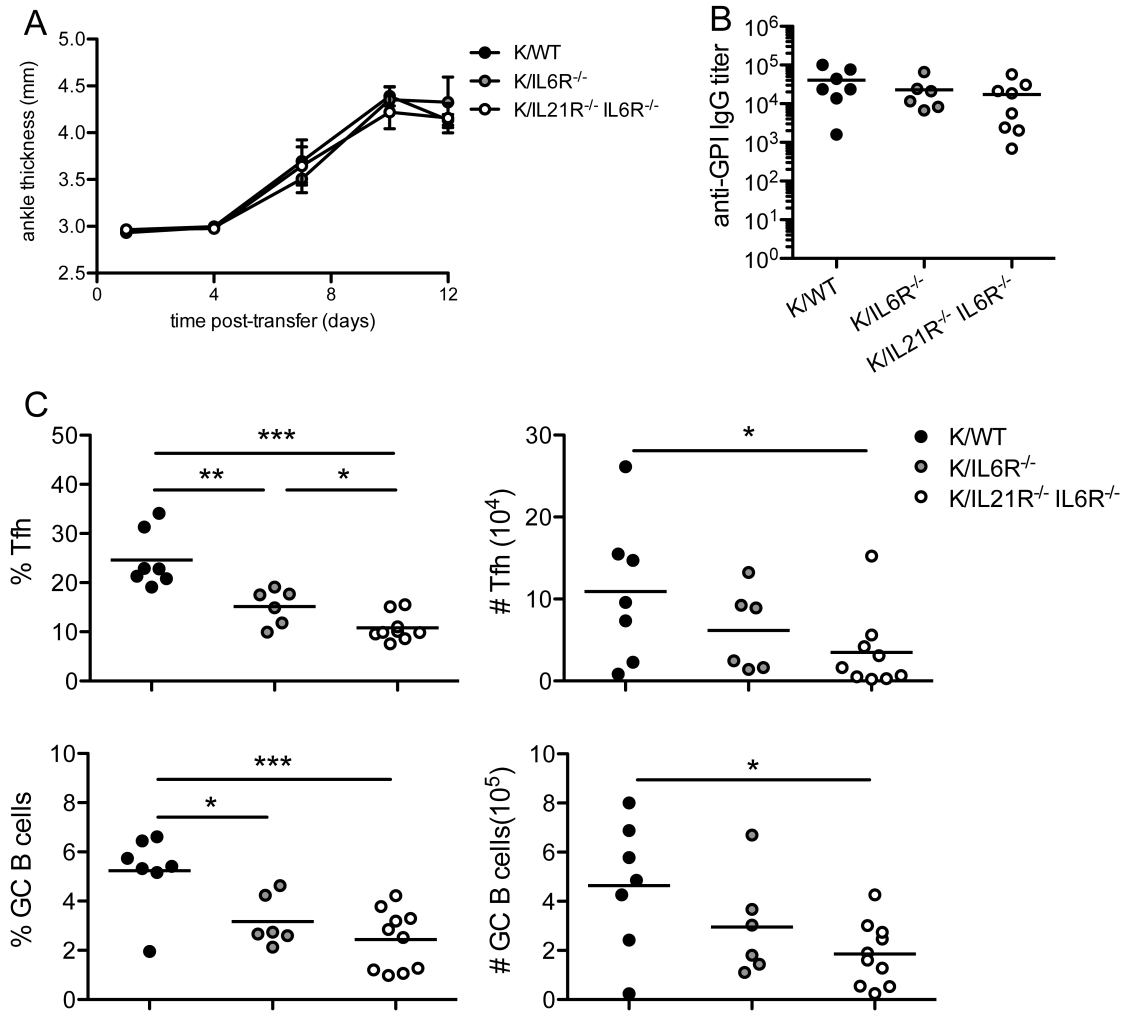

Figure 3.4. IL-6R and IL-21R signaling on T cells promote Tfh cell and germinal center differentiation. (**A-D**) Splenocytes of the indicated genotypes were transferred into $C\alpha^{-/-}$ BxN hosts. (**A**) Ankle thickening was monitored over time. Mean and standard deviation are shown. Representative of 3 experiments (n = 2 - 3/group). (**B-D**) Mice were analyzed 12-14 days after transfer. (**B**) Anti-GPI IgG titers were detected by ELISA. (**C**) Percent and number of Tfh ($CXCR5^+$ $Bcl6^+$ of $CD4^+$ CD45.1) cells in the spleen. (**D**) Percent and number of GC B cells ($GL7^+$ Fas^+ of $CD19^+$) in the spleen. Shown are from 2 experiments (n = 6 – 10 mice/group). Horizontal bars represent the mean. Student's t-test: ns, $p \geq 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

IL-21 is required for GC formation

Although Tfh cell differentiation was normal, anti-GPI IgG antibody production was severely impaired following the transfer of K/IL-21$^{-/-}$ T cells (Figure 3.2B). Therefore, we investigated the GC formation and T cell migration by immunofluorescence on spleen sections. As shown in Figure 3.5A, there were abundant GCs in both KRN WT and K/IL-21R$^{-/-}$ T cell transfers. In contrast, GCs in K/IL-21$^{-/-}$ T cell transfer were rarely observed and the few germinal centers were amorphous and dimly labeled with PNA. The number of GCs was counted from multiple spleen sections from multiple mice (Figure 3.5B). KRN WT and K/IL-21R$^{-/-}$ T cell transfer induced equivalent numbers of GCs per section. GC size was measured and KRN WT had slightly larger GCs on average compared to K/IL-21R$^{-/-}$ T cell transfer. We also assessed the GC B cells by flow cytometry. Consistent with the immunohistology, there was a significant increase in the GL-7^{+} Fas^{+} GC B cell population in KRN WT and K/IL21R$^{-/-}$ T cell transfer when compared to C$\alpha^{-/-}$ BxN hosts without transfers, whereas K/IL-21$^{-/-}$ T cells induced very few GL-7^{+} Fas^{+} GC B cells (Figure 3.5C).

To determine if the lack of GCs in the K/IL21$^{-/-}$ transfer was due to defective Tfh cell migration into the B cell follicles or defective T-B interactions, we examined spleen sections for the presence of transferred T cells in B cell follicles. As shown in Figure 3.5D, staining with an anti-KRN Vα4 specific antibody 3-4G-B7 revealed KRN T cells throughout the B cell follicle in all transfers. These data suggest that the lack of GCs in K/IL-21$^{-/-}$ is due to impaired T cell help and not T cell migration.

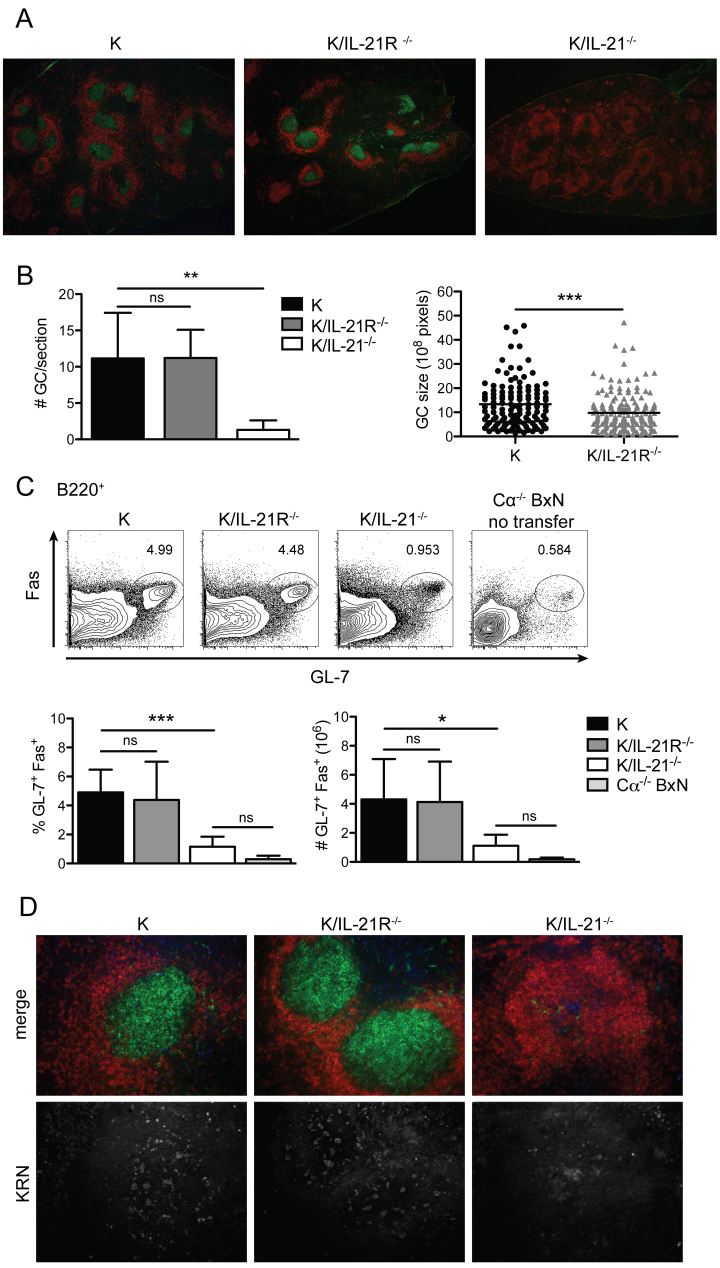

Figure 3.5. GC B cell formation is impaired in K/IL-21$^{-/-}$ T cell transfer. (**A**) Representative immunohistology of spleens 29 days after transfer of CD4^{+} KRN cells of the indicated genotype, stained with anti-IgD (red) and PNA (green), 4x magnification. (**B**) Quantification of number of GC in each spleen section (normalized by section size), and size of GCs as measured by the number of pixels (n = 6 - 8 mice per group). (**C**) Percent and total number GC B cells in the spleen (Fas^{+} GL-7^{+} of CD19^{+}). (**D**) Immunohistology of spleens as in A, labeled with anti-KRN TCRα chain specific monoclonal Ab 3-4G-B7 (blue), anti-IgD (red), and PNA (green) (top), anti-KRN staining alone (bottom), 20x magnification. For all bar graphs mean and standard deviation are shown. Results are from two experiments (n = 6 - 8 mice per group). Student's t-test: ns, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Th17 cells are not essential for GC responses and arthritis

IL-21 is not exclusively a Tfh cell cytokine. The Th17 subset produces IL-21 in addition to production of IL-17A, IL-17F, and IL-22 (Korn et al., 2009). Th17 cells were shown to drive the formation of spontaneous GCs in autoimmune responses of the BXD2 mice (Hsu et al., 2008) and Th17 cells can provide effective help to B cells (Mitsdoerffer et al., 2010; Hickman-Brecks et al., 2011). We therefore tested the contribution of Th17 cells in initiating GC responses and arthritis. K/B6 mice were crossed to RORγt$^{-/-}$ mice (Ivanov et al., 2006) to generate K/RORγt$^{-/-}$ mice. In these mice, GFP knock-in inactivates RORγt expression and Th17 cell differentiation is defective. CD4^{+} T cells were purified from either K/RORγt$^{+/+}$ or K/RORγt$^{-/-}$ mice and transferred into Ca$^{-/-}$ BxN hosts. As shown in Figure 3.6A, K/RORγt$^{+/+}$ and K/RORγt$^{-/-}$ CD4^{+} T cells induced arthritis with a similar kinetics and severity. There was also no difference in the serum anti-GPI IgG titers (Figure 3.6B). The transferred K/RORγt$^{-/-}$ cells were indeed deficient in generating Th17 cells as determined by IL-17 intracellular staining after *in vitro* stimulation (Figure 3.6C). However, there was no difference in the differentiation of Tfh cells (Figure 3.6D). These results demonstrate that Th17 cells and their production of IL-21 are not essential for GC responses and arthritis development, supporting the conclusion that Tfh cells are the major source of IL-21 production.

It has been previously shown that Th17 cell development is correlated with disease induction in K/BxN mice (Wu et al., 2010). We investigated whether there were differences in Th17 cell development and distribution in K/BxN mice versus the transfer model. Cells isolated from the spleen, draining lymph nodes (pooled popliteal and inguinal lymph nodes), and mesenteric lymph nodes stimulated *in vitro* revealed a similar profile of IL-17 production from K/BxN mice and Cα$^{-/-}$ BxN hosts after KRN T cell transfer (Figure 3.6E). IL-17 production was

detected from KRN T cells identified with the anti-KRN Vα4 specific antibody 3-4G-B7. However, non-KRN T cells were the major IL-17 producers in both K/BxN and KRN transferred mice.

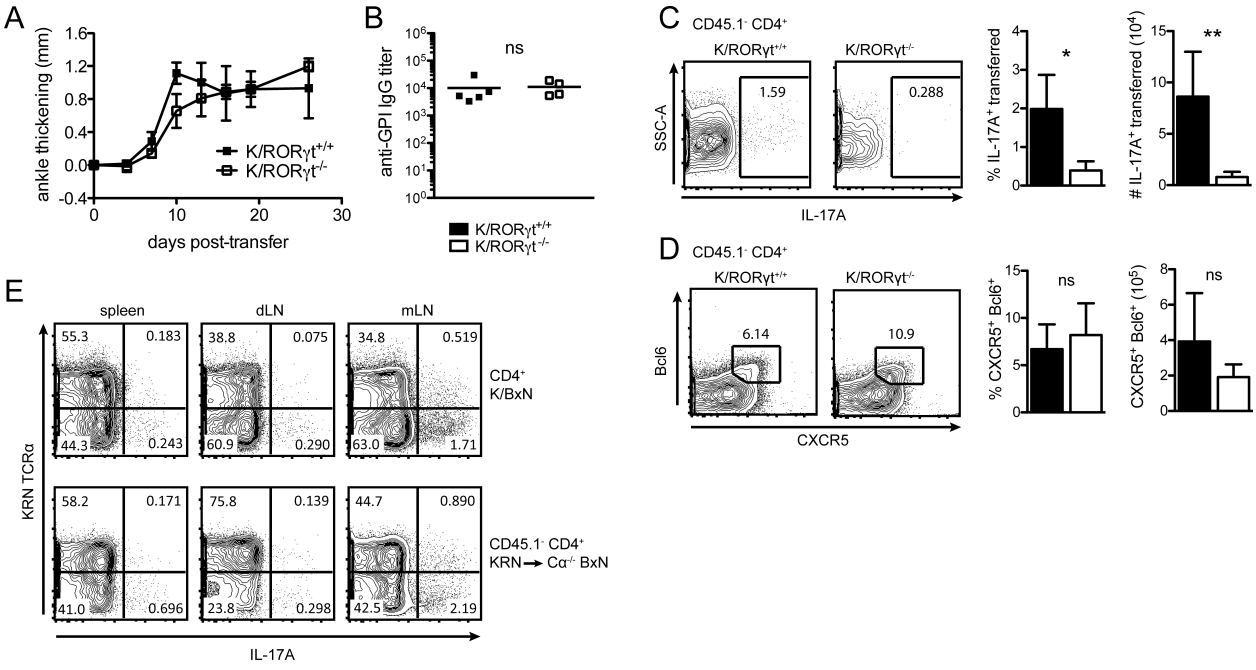

Figure 3.6. Th17 cells are dispensable for arthritis. (**A**) KRN splenocytes purified from WT KRN/B6 (K/RORγt$^{+/+}$) or KRN RORγt deficient mice (K/RORγt$^{-/-}$) were transferred into Cα$^{-/-}$ BxN hosts. Ankle thickening was monitored over time. (**B**) Anti-GPI IgG titers were detected by ELISA. (**C**) Splenocytes were cultured in PMA and ionomycin with BFA and IL-17A production was detected by intracellular staining. Percent and total number of IL-17A producing transferred cells (CD45.1$^-$ CD4$^+$) in the spleen are shown. (**D**) Percent and total number of Tfh (CXCR5$^+$Bcl6$^+$) of the CD45.1$^-$ CD4$^+$ transferred cell gate in the spleen are shown. Data are representative of two independent experiments (n = 4 - 5 mice per group). Mean and standard deviation are shown. Student's t-test: ns, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$. (**E**) Cells from the spleen, draining lymph nodes (dLN, pooled popliteal and inguinal lymph nodes), and mesenteric lymph nodes (mLN) from K/BxN mice and Cα$^{-/-}$ BxN hosts 20 days after KRN/B6 cell transfer were stimulated and assessed for IL-17A production as in (C). Data are representative of one experiment with two mice per group.

B cells require IL-21R signaling to initiate GC

Because IL-21 production by T cells is necessary for GC formation, it suggests that the target of IL-21 is the B cell. To directly test the importance of IL-21R expression on B cells, we performed a B and T cell co-transfer experiment. B cells purified from IL-21R$^{+/-}$ or IL-21R$^{-/-}$ mice expressing I-A$^{g7/b}$ MHC-II alleles were transferred along with naïve K/B6 CD4^{+} cells into Rag1$^{-/-}$ B6xNOD F1 hosts. Most mice that received IL-21R$^{+/-}$ B cells developed arthritis whereas mice that received IL-21R$^{-/-}$ B cells never showed signs of arthritis (Figure 3.7A). The disease states were reflected in the dramatic difference in anti-GPI IgG titers between these two groups of mice (Figure 3.7B). There was a moderate decrease in Tfh cells in hosts receiving IL-21R$^{-/-}$ B cells compared to those receiving IL-21R$^{+/-}$ B cells (Figure 3.7C). This result is consistent with what was observed in the K/IL-21$^{-/-}$ T cell transfer, where Tfh numbers decreased after 29-30 days, likely due to a lack of interaction with GC B cells to promote Tfh cell maintenance. There was a more dramatic decrease in GC B cells in hosts receiving IL-21R$^{-/-}$ B cells compared to those receiving IL-21R$^{+/-}$ B cells (Figure 3.7D). Immunohistological analysis of GCs on spleen section confirmed the results obtained by flow cytometry, although there were few B cell follicles and smaller GCs in general compared to T cell transfer into Cα$^{-/-}$ BxN hosts. This is not surprising considering that Rag1$^{-/-}$ mice have defective follicular structures and a much smaller population of transferred B cells. These data support the conclusion that IL-21R signaling in B cells is essential for GC formation, antibody production, and arthritis development. GC B cells are also required for Tfh cell maintenance.

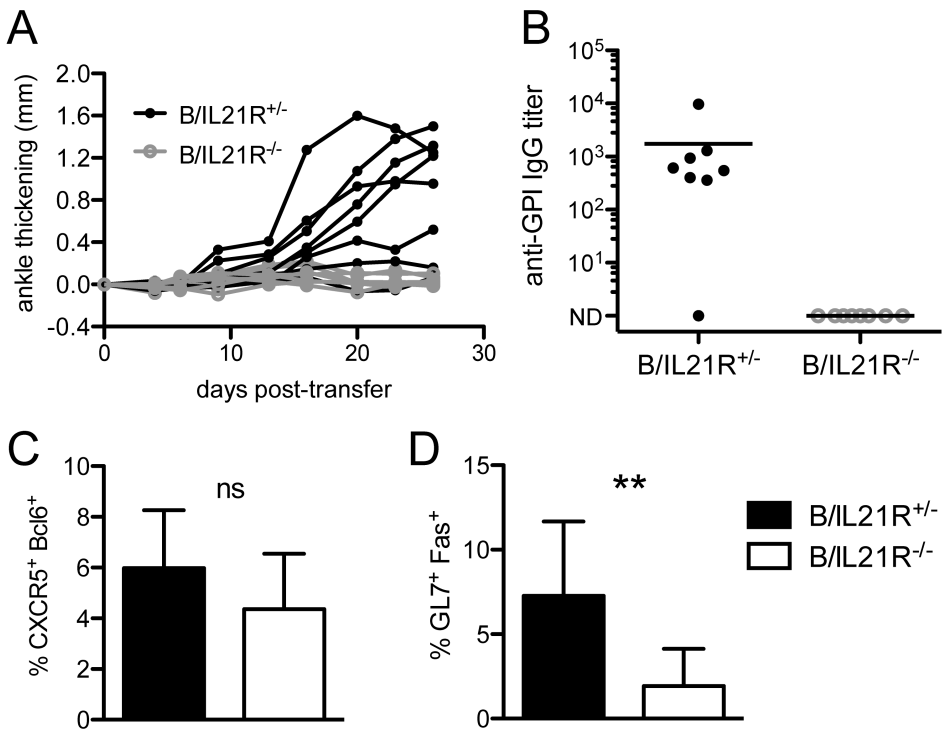

Figure 3.7. B cells require expression of IL-21R for GC formation and autoantibody production. (**A-D**) IL21R$^{+/-}$ or IL-21R$^{-/-}$ I-A$^{g7/b}$ splenic B cells and CD4^{+} KRN/B6 T cells were transferred into Rag1$^{-/-}$ BxN hosts. (**A**) Ankle thickening was monitored over time. (**B**) Anti-GPI IgG titers were detected by ELISA, analyzed 37-40 days after transfer. ND (not detectable). (**C**) Percent of GC B cells (GL-7^{+} Fas^{+}) of the B220^{+} gate and (**D**) percent of Tfh cells (CXCR5^{+} Bcl6^{+}) of the TCRβ^{+} CD4^{+} gate were determined by flow cytometry. Mean and standard deviation are shown. Results shown are from two experiments (n = 8 per group). Student's t-test: ns, $p > 0.05$; **, $p < 0.01$.

Discussion

IL-21 is a pleiotropic cytokine, affecting a diverse array of cell types (Ettinger et al., 2008). Without conditional deletion of IL-21 or IL-21R, it has been difficult to evaluate the specific roles of IL-21 in autoimmunity. We used a cell transfer system based on the K/BxN mouse model of autoimmune arthritis to address these roles in Tfh differentiation and B cell activation. Our results demonstrate that IL-21 production by T cells is required for disease

induction. However, IL-21 is not required for Tfh cell differentiation, maintenance, or function. There was no defect in T cell survival when KRN T cells were deficient in either IL-21 or IL-21R, and in fact survival was increased one week after transfer in IL-21$^{-/-}$ T cells (Figure 3.3A). A similar increase was observed in IL-21$^{-/-}$ mice after immunization with NP-KLH (Zotos et al., 2010), although the mechanism for enhanced survival is unclear. Both IL-21$^{-/-}$ and IL-21R$^{-/-}$ KRN T cells proliferated and differentiated into Tfh cells and were able to migrate into the B cell follicles in an antigen-specific manner, as this process did not take place when KRN T cells were transferred into C$\alpha^{-/-}$ B6 hosts that do not express the self-peptide:MHC complex.

In an earlier study using the IL-21R$^{-/-}$ K/BxN mice, it was shown that there were fewer CD4^{+} T cells in the spleen and joint draining lymph nodes compared to normal K/BxN mice (Jang et al., 2009). The IL-21R$^{-/-}$ K/BxN mice did not develop arthritis and it was attributed to a requirement of IL-21 by KRN T cells for homeostatic proliferation. In contrast, we found that autoreactive T cells do not require IL-21R expression, but expression is required on B cells. The different conclusions from our study and the earlier study highlight the complex biological function of IL-21 and the problem of understanding the direct versus indirect mechanisms in total knockout animals.

We found no difference in the GC B cell response between KRN WT and K/IL-21R$^{-/-}$ T cell transfer by antibody titers or GC B cell formation by flow cytometry or histology, demonstrating that there is no functional defect in Tfh in the absence of IL-21R signaling. These results contrast with a study of lupus-like disease induced in a chronic graft-versus-host model, which found that GC B cells were less frequent and GCs were smaller when IL-21R$^{-/-}$ T cells were transferred (Nguyen et al., 2012). The differential dependence on IL-21R signaling may be related to the difference in frequency or affinity of alloreactive T cells, because it was shown that

naïve antigen-specific helper T cells with TCRs of higher affinity preferentially differentiate into the CXCR5^{hi} 'resident' Tfh compartment (Fazilleau et al., 2009).

Studies on the role of IL-21 in Tfh cell differentiation and GC formation have led to different conclusions in mice immunized with protein antigens. Germinal center formation was relatively unaffected in IL-21$^{-/-}$ or IL-21R$^{-/-}$ mice in some studies (Ozaki et al., 2002; Linterman et al., 2010; Zotos et al., 2010) whereas a more profound effect on Tfh cells and GCs was found in other studies (Nurieva et al., 2008; Vogelzang et al., 2008). It was suggested that the different results obtained in these studies might be explained by the different types of antigen, adjuvant, the avidity of TCR involved for peptide:MHC, or the timing of analysis (Zotos et al., 2010; Linterman et al., 2010). Our conclusion that IL-21 is not required for Tfh cell differentiation but rather acts on GC B cells is consistent with studies in mixed bone marrow chimeras (Zotos et al., 2010; Linterman et al., 2010; Bessa et al., 2010). GC formation appears to be highly dependent on IL-21 in the K/BxN and other models of spontaneous autoimmune diseases (Bubier et al., 2007; Rankin et al., 2012), although IL-21 does not play any role in the Roquin^{Sanroque} mouse model of lupus (Linterman et al., 2009). Therapeutic intervention of the IL-21 signaling pathway has been explored in animal models (Bubier et al., 2007; Herber et al., 2007; Young et al., 2007). However, the efficacy was found to be variable and partial. For example, repeated treatment of BXSB-Yaa mice with a soluble IL-21R-Fc fusion protein had minimal effect on lupus symptoms and survival even though IL-21R$^{-/-}$ BXSB-Yaa have no sign of disease (Bubier et al., 2007). This variation could be attributed to the partial effectiveness of IL-21R-Fc in blocking IL-21 signaling or an insufficient dose of IL-21R-Fc. The evidence that Tfh cell development is not dependent on IL-21 in autoimmunity raises the possibility that an efficient inhibition of ongoing GC B cell response may be difficult to achieve in practice with partial

effectiveness of IL-21 blockade. It is tempting to speculate that IL-21 blockade is more effective in cases where Tfh is more dependent on the cytokine. IL-21 blockade together with a therapy targeting T cells might be most beneficial for treating certain antibody-mediated autoimmune diseases.

Arthritis development in K/BxN mice is dependent on gut microbiota, particularly the colonization of segmented filamentous bacteria (Wu et al., 2010). Because the KRN transfer model involves different strains of mice as source of donor cells and hosts, the potential difference in their gut microbiota might be a confounding factor in the interpretation of our experiments. However, we think it is not likely to be the case. In our experiments, we always used littermates for our cell transfer hosts, dividing the hosts housed in the same cage for different KRN genotype transfers. We used donors of different genotypes housed in the same cage when possible. Furthermore, any alteration in the gut microbiota of K/IL-21$^{-/-}$ and K/IL-21R$^{-/-}$ donor mice should be very similar because they are defective in the same pathway.

Th17 cells are now recognized to interact with antigen-specific B cells as potential B cell helpers (Hsu et al., 2008; Mitsdoerffer et al., 2010). It was therefore important to investigate whether IL-21 required for disease induction was produced by Tfh cells or from Th17 cells that enter the B cell follicle to initiate GC. By eliminating the Th17 cell subset through RORγt deficiency, K/RORγt$^{-/-}$ T cells did not dramatically alter disease kinetics or severity, suggesting that IL-21 is derived from Tfh cells but not Th17 cells.

At first glance, the result that Th17 cells are not essential for disease induction seems unexpected because Th17 cells have been suggested to play an important role in this disease model. Th17 cell induction correlated with disease onset, and neutralizing antibody against IL-17A prevented disease in K/BxN mice (Wu et al., 2010). IL-17R$^{-/-}$ B cells were defective in

differentiating into GC B cells in a competitive setting, suggesting that they are the targets of IL-17. We therefore pursued the role of Th17 cells in K/BxN arthritis in the next chapter.

Chapter IV: Gut microbiota regulates K/BxN autoimmune arthritis through Tfh but not Th17

This chapter is adapted from a manuscript under revision at *Journal of Immunology*: Block, K.E., Z. Zheng, A.L. Dent, B.L. Kee, and H. Huang. Gut microbiota regulates K/BxN autoimmune arthritis through Tfh but not Th17.

Introduction

The effects of the intestinal microbiota on health and disease have been under intense study in recent years. A diverse and balanced microbial community is required for normal development of the innate and adaptive arms of the immune system (Ganal et al., 2012; Geuking et al., 2014). The microbiota modulates the immune response against pathogens as well as self-antigens (Longman et al., 2014). One example of the microbiota promoting autoimmunity is the rheumatoid arthritis mouse model K/BxN, where the microbiota is required for disease development. In specific pathogen free (SPF) colonies, K/BxN mice develop arthritis spontaneously at 4 to 5 weeks of age. Germ-free or antibiotic treated K/BxN mice have significantly lower serum autoantibody titers, and ameliorated disease (Wu et al., 2010). The requirement of the microbiota for arthritis development is particularly intriguing, as the disease is manifested at sites distal to the gut. While the microbiota has some effect on the effector phase of the disease mediated by innate immune cells following the production of autoantibodies (Lee et al., 2014), it also plays important roles in the initiation phase where autoreactive KRN T cells

are activated and drives B cells to produce autoantibodies. Which cell types are involved at this stage and how they are affected by the microbiota are not well understood.

The T helper subset Th17 has been implicated in this initiation phase of K/BxN arthritis and the development of these cells is promoted by colonization with commensal bacteria. Segmented filamentous bacteria (SFB) alone can potently induce Th17 cells in wild-type mice (Ivanov et al., 2009), and strikingly, colonization with SFB alone is sufficient to promote disease in the K/BxN arthritis model (Wu et al., 2010). It has been proposed that the link between bacterial colonization and arthritis is through induction of the Th17 and proinflammatory cytokine interleukin-17A (IL-17). A key experiment supporting this conclusion was that IL-17 blockade by neutralizing antibody was able to inhibit arthritis (Wu et al., 2010). However, we have shown that *Rorc* deficient KRN T cells were able to induce arthritis as well as wild-type KRN T cells suggesting Th17 cells are not essential for arthritis development (Block and Huang, 2013).

Follicular helper T cells (Tfh) are a T cell subset specialized in interacting with B cells. Tfh cells require the transcription factor Bcl6 for their differentiation and function (Crotty, 2011). B cells presenting cognate antigen to Tfh cells are licensed to differentiate into germinal center B cells, somatically hypermutate and class switch, and further differentiate into plasma cells and memory B cells. This licensing requires cytokine production from T cells, namely IL-21 and IL-4. We have previously demonstrated that IL-21 from T cells is required by B cells for disease in the K/BxN arthritis model (Block and Huang, 2013). We hypothesize that Tfh cells and IL-21 are required for arthritis, and that Th17 cells and IL-17 are dispensable for disease. Since germ free and antibiotic treated mice have a reduced antibody response, we also wanted to investigate the effect of the microbiota on Tfh cell development in this model.

This chapter describes genetic approaches used to test the requirement of the Th17 cytokine IL-17, as well as Tfh cells, for arthritis development and their interactions with the microbiota. We found that IL-17 deficient K/BxN mice develop arthritis in a similar manner as IL-17 sufficient littermates. Antibiotic treatment of IL-17 deficient mice showed that a replete microbiota was required for disease independent of IL-17, at the level of the initiation phase. Antibiotic treatment reduced the Tfh and germinal center B cell populations in secondary lymphoid organs throughout the body. Finally, we showed that Bcl6-deficient KRN T cells did not induce arthritis, formally demonstrating that Tfh cells are required for arthritis development. This work highlights the potent effects of microbial colonization on T helper cell differentiation beyond the induction of Th17. These findings have implications in the context of human rheumatoid arthritis, where anti-IL17 pharmaceuticals are being explored as possible treatments.

Results

IL-17 is not required for K/BxN arthritis development

To test the requirement of IL-17 in arthritis development in K/BxN mice, we crossed an *Il17a* deficient strain to KRN TCR Tg and to B6.H-2^{g7} congenic mice (breeding strategy described in Figure 2.1A). These mice are on the B6 background, except for the MHC locus, which is from the NOD strain. KRN/B6 x B6.H-2^{g7} (K/g7) mice develop the same arthritis as K/BxN mice (B6 x NOD F1 background) because they have the essential components for disease: the transgenic KRN TCR and the MHC-II I-A^{g7} required to present self-antigen glucose-6-phosphate isomerase (GPI) peptide (Kouskoff et al., 1996). *Il17a* deficient mice from this breeding, called K/g7/IL17$^{-/-}$ in this paper, were monitored for arthritis development. Littermates heterozygous or wild-type for IL-17 (K/g7/IL-17$^{+/-}$ or K/g7/IL-17$^{+/+}$) were used as controls.

K/g7/IL-17$^{-/-}$ mice developed arthritis to the same severity as K/g7/IL-17$^{+/-}$ mice, with slightly delayed but variable kinetics (Figure 4.1A). A single K/g7/IL-17$^{-/-}$ mouse did not develop disease or autoantibodies, for unknown reasons. Analysis of Th17 cells by IL-17 intracellular staining from various secondary lymphoid organs confirmed that Th17 cells were absent in K/g7/IL-17$^{-/-}$ mice, and the small intestine lamina propria contained the largest population of Th17 cells in IL-17 sufficient mice, as expected (Figure 4.1B).

It was reported that IL-17 regulates B cell class switching to certain IgG isotypes in some autoimmune and delayed-type hypersensitivity models (Nakae et al., 2003b; Ding et al., 2013; Nakae et al., 2002). However, we found no defect in serum antibody titers against glucose-6-phosphate isomerase (GPI, the autoantigen of the K/BxN model) for any IgG isotypes by ELISA (Figure 4.1C). This result is in agreement with a study showing no effect of IL-17 on class switching *in vitro* (Shibui et al., 2012).

A previous study showed that an anti-IL-17 neutralizing antibody inhibited K/BxN arthritis (Wu et al., 2010). We tested two IL-17 neutralizing monoclonal antibodies in K/BxN mice, starting treatment before disease onset (clones TC11-18H10.1 and 17F3). Arthritis was slightly delayed in mice treated with antibodies, however by 5 weeks of age disease severity was comparable and anti-GPI IgG serum titers were indistinguishable (Figure 4.2). We cannot confirm that IL-17 was effectively blocked *in vivo*, however these results are similar to those from the IL-17 deficient mice.

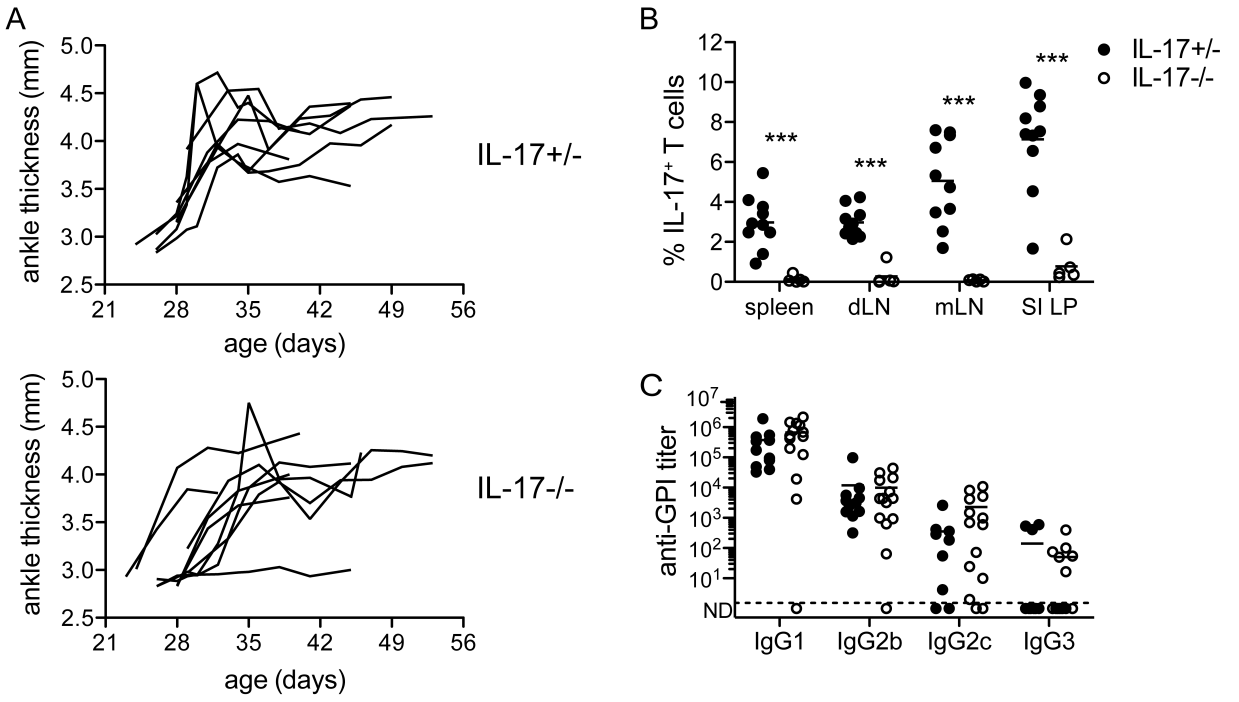

Figure 4.1. IL-17 is dispensable for K/BxN arthritis. (**A**) K/g7/IL-17$^{+/-}$ and K/g7/IL-17$^{-/-}$ mice were monitored for arthritis by measuring ankle thickness. Each line represents one mouse. Data shown are pooled from eight litters (n = 8-10 mice/group). (**B**) Th17 cells as detected by intracellular IL-17 staining in spleen, joint draining lymph nodes (dLN, pooled inguinal and popliteal lymph nodes), mesenteric lymph nodes (mLN), and small intestine lamina propria (SI LP). T cells gated as CD45$^+$ TCRβ$^+$ CD4$^+$ cells. Data shown are pooled from 3 independent experiments, mice aged 5 to 6 weeks (n = 5-10 mice/group). (**C**) Serum anti-GPI antibody titers for the indicated isotypes in mice aged 6 to 8 weeks (n = 11-14 mice/group). Points below dashed line were not detectable (ND). Horizontal lines show the mean. Student's t test: $p > 0.05$ for all isotypes. ***, $p < 0.0001$.

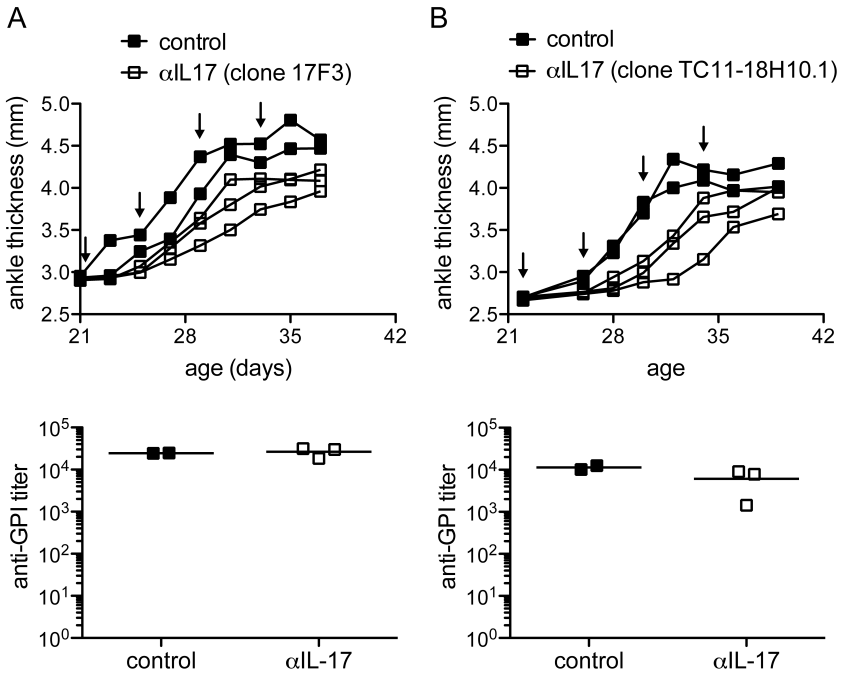

Figure 4.2. IL-17 blocking antibodies do not prevent K/BxN arthritis. K/BxN mice were injected with 250 μg of the indicated antibody clone or PBS (control) on the days indicated with arrows. (**A**) Clone 17F3. (**B**) Clone TC11-18H10.1. Ankle thickness was measured. Each line represents one mouse (top panels). Serum anti-GPI IgG antibody titers were shown (bottom panels). Each symbol represents one mouse. Horizontal lines show the mean. Data shown are two independent experiments (n = 2-3 mice/group). Student's t test: $p > 0.05$ for serum titers.

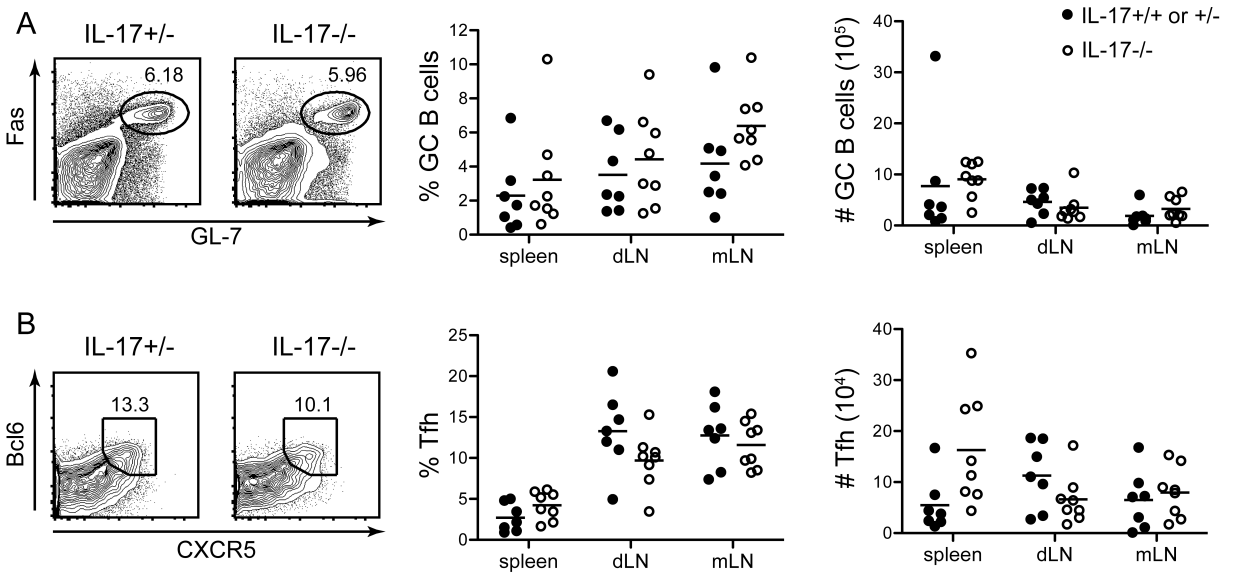

Figure 4.3. K/g7/IL-17$^{-/-}$ mice have normal germinal centers and Tfh cells. (**A**) Germinal center B cells (GL-7^{+} Fas^{+}) gated on CD19^{+} cells; (**B**) Follicular helper T cells (Tfh, Bcl6^{+} CXCR5^{+}) gated on CD4^{+} cells in the spleen, joint draining lymph nodes (dLN, pooled popliteal and inguinal lymph nodes), and mesenteric lymph nodes (mLN). Representative flow plots are from the dLN. Percent and number are displayed for 6-8 week old K/g7/IL-17$^{+/+ \text{ or } +/-}$ and K/g7/IL-17$^{-/-}$ mice. Data shown are pooled from four separate experiments (n = 7-8 mice/group). Horizontal lines show the mean. All comparisons between the two groups of mice are not significant ($p > 0.05$).

Germinal center B cells and Tfh cells are not altered in the absence of IL-17

Arthritis in K/BxN mice is driven by a germinal center response and anti-GPI autoantibodies (Matsumoto et al., 1999; Block and Huang, 2013). We analyzed the Tfh and germinal center B cells by flow cytometry in K/g7/IL-17$^{+/-}$ and K/g7/IL-17$^{-/-}$ mice. Consistent with the serum antibody titer results, there were no statistical difference in germinal center B cell (CD19^{+}Fas^{+}GL-7^{+}) and Tfh cell (Bcl6^{+} CXCR5^{+} CD4^{+}) proportions and numbers in the spleen, joint draining popliteal and inguinal lymph nodes, and mesenteric lymph nodes between the two

groups of mice (Figure 4.3A and 4.3B). These results demonstrate that IL-17 did not play a role in the initiation phase of arthritis development.

Gut microbiota regulates arthritis development in K/g7/IL-17$^{-/-}$ mice

We next wanted to determine how the gut microbiota regulates arthritis in the presence or absence of IL-17. To test whether the effect of the microbiota on arthritis development was dependent on IL-17, K/g7/IL-17$^{+/-}$ and K/g7/IL-17$^{-/-}$ mice were treated with ampicillin and vancomycin in their drinking water starting from birth and daily gavage starting from weaning. This antibiotic treatment effectively prevented disease in both IL-17 sufficient and deficient mice (K/g7/IL-17$^{+/-}$ had 11.1% and K/g7/IL-17$^{-/-}$ had 12.5% disease penetrance) (Figure 4.4A). This result indicates that the autoimmune arthritis promoted by the microbiota is independent of IL-17. To test if antibiotic treatment affected the initiation phase of disease, we measured anti-GPI IgG serum titers by ELISA at 5.5 weeks, the age where maximum ankle thickness is normally reached in conventionally housed mice. Serum titers in antibiotic treated mice, regardless of IL-17 genotype, were significantly lower in antibiotic treated mice compared to untreated mice (Figure 4.4B). This result demonstrates that antibiotic treatment reduces disease at the level of autoantibody production, indicating a defect in T and/or B cell activation or function.

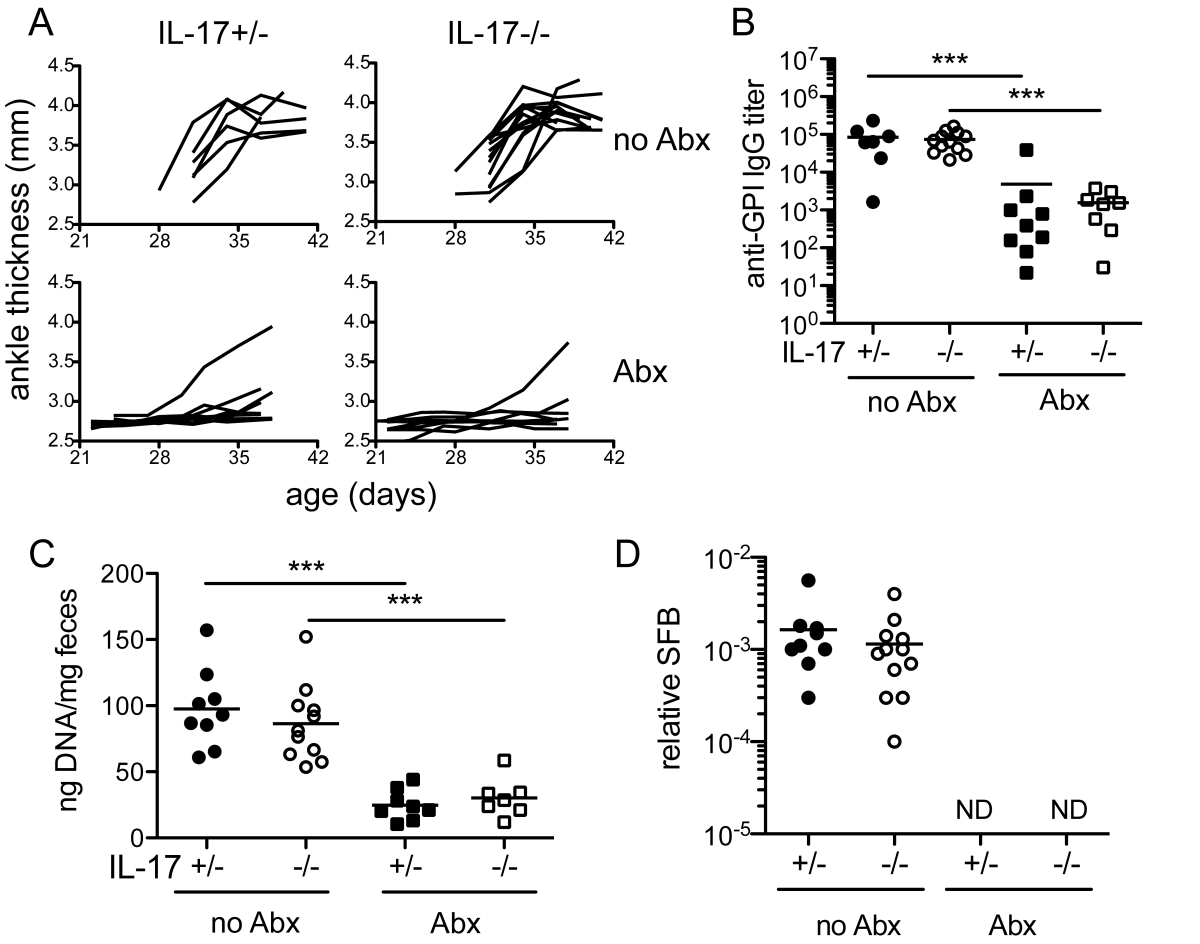

Figure 4.4. Antibiotic treatment prevents arthritis independent of IL-17. (**A-D**) K/g7/IL-17$^{+/-}$ and K/g7/IL-17$^{-/-}$ littermates were untreated (no Abx) or treated with ampicillin and vancomycin in the drinking water from birth and gavaged daily with antibiotics beginning at weaning (Abx). (**A**) Ankle thickness was monitored. Each line represents a mouse. (**B**) Serum anti-GPI IgG levels were detected by ELISA at 5.5 weeks of age. (**C**) Fecal pellets were collected at 5.5 weeks of age and DNA was extracted. DNA yield was normalized to the pellet weight. (**D**) The relative amount of SFB (normalized to total bacteria) was quantified by real-time PCR. ND = not detectable for all samples of indicated group. Data shown are pooled from six no Abx litters and six Abx litters (n = 6-13 mice per group). Each symbol represents a mouse. Horizontal lines represent the mean. Student's t test: ***, $p < 0.0001$.

SFB is sensitive to both ampicillin and vancomycin. Because SFB has been associated with arthritis development and SFB mono-colonized germ free K/BxN mice develop arthritis (Wu et al., 2010), we tested whether SFB was present in our colony and whether it was

eliminated by the antibiotic treatment. The DNA yield from antibiotic treated animals was significantly decreased compared to untreated mice, indicating that the antibiotics reduced the total burden of bacteria in the gut (Figure 4.4C). SFB was found to be present by qPCR in all untreated mice, at equal levels in K/g7/IL-17$^{+/-}$ and K/g7/IL-17$^{-/-}$ mice (Figure 4.4D). However, SFB was not detected in the feces of any of the antibiotic treated mice when equal amounts of total DNA were used, demonstrating the antibiotic treatment was very effective at eliminating SFB from the gut.

Germinal centers, T helper cells, and Tfh are reduced in antibiotic treated K/BxN mice

To further understand how the gut microbiota regulates the production of autoantibodies, we analyzed the B and T cell populations. There were no significant change in the number of B cells in the spleen and mesenteric lymph nodes, although the percent of B cells was slightly increased (Figure 4.5A). The number of B cells in the joint draining lymph nodes of antibiotic treated mice was decreased by about 2 fold compared to untreated mice, which was also reflected by the sizes of lymph nodes. Germinal center B cells were dramatically decreased in all sites in antibiotic treated mice, by both percent and number (Figure 4.5B). The decreased B cell response suggested to us a lack of T cell help to B cells. Overall the percent and number of CD4^{+} T cells were reduced in all sites examined (Figure 4.5C). The number of Tfh cells in antibiotic treated mice was decreased in multiple sites (Figure 4.5D). All these observations demonstrate a defect in Tfh cell differentiation and GC response in the absence of a replete gut microbiota.

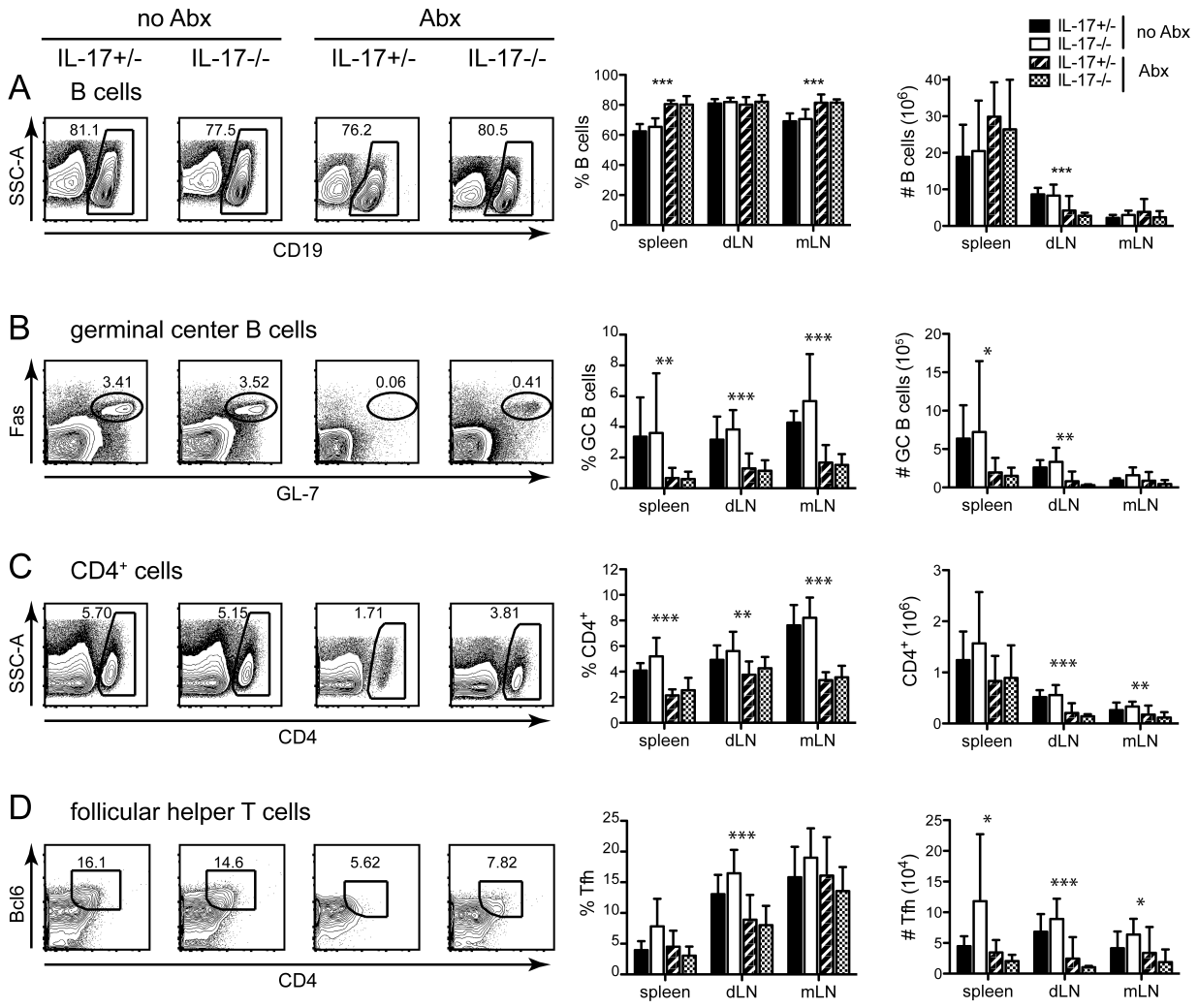

Figure 4.5. Antibiotic treatment reduces germinal center B cells and Tfh cells independent of IL-17. (**A-D**) Percent and number of the indicated cell types in the spleen, joint draining lymph nodes (dLN, pooled popliteal and inguinal lymph nodes), and mesenteric lymph nodes (mLN) are shown for 5.5 week old K/g7/IL-17$^{+/-}$ and K/g7/IL-17$^{-/-}$ mice untreated (no Abx) or treated with antibiotics (Abx) as described in Figure 4.4B. (**A**) B cells (CD19^{+}). (**B**) Germinal center B cells (GL-7^{+} Fas^{+}) gated on CD19^{+} cells. (**C**) CD4^{+} cells. (**D**) Tfh (Bcl6^{+} CXCR5^{+}) gated on CD4^{+} cells. Data shown are pooled from six no Abx litters and six Abx litters (n = 6-13 mice per group). Displayed are mean and standard deviation. Statistical difference between groups tested with 2-way ANOVA. p-values displayed are for the treatment factor, comparing no Abx vs. Abx. No other factors were significant, except for percent Tfh in the spleen, where the interaction of genotype and treatment $p < 0.05$. If no p-value is displayed, $p > 0.05$; *, $p < 0.05$; **, $p < 0.001$; ***, $p < 0.0001$.

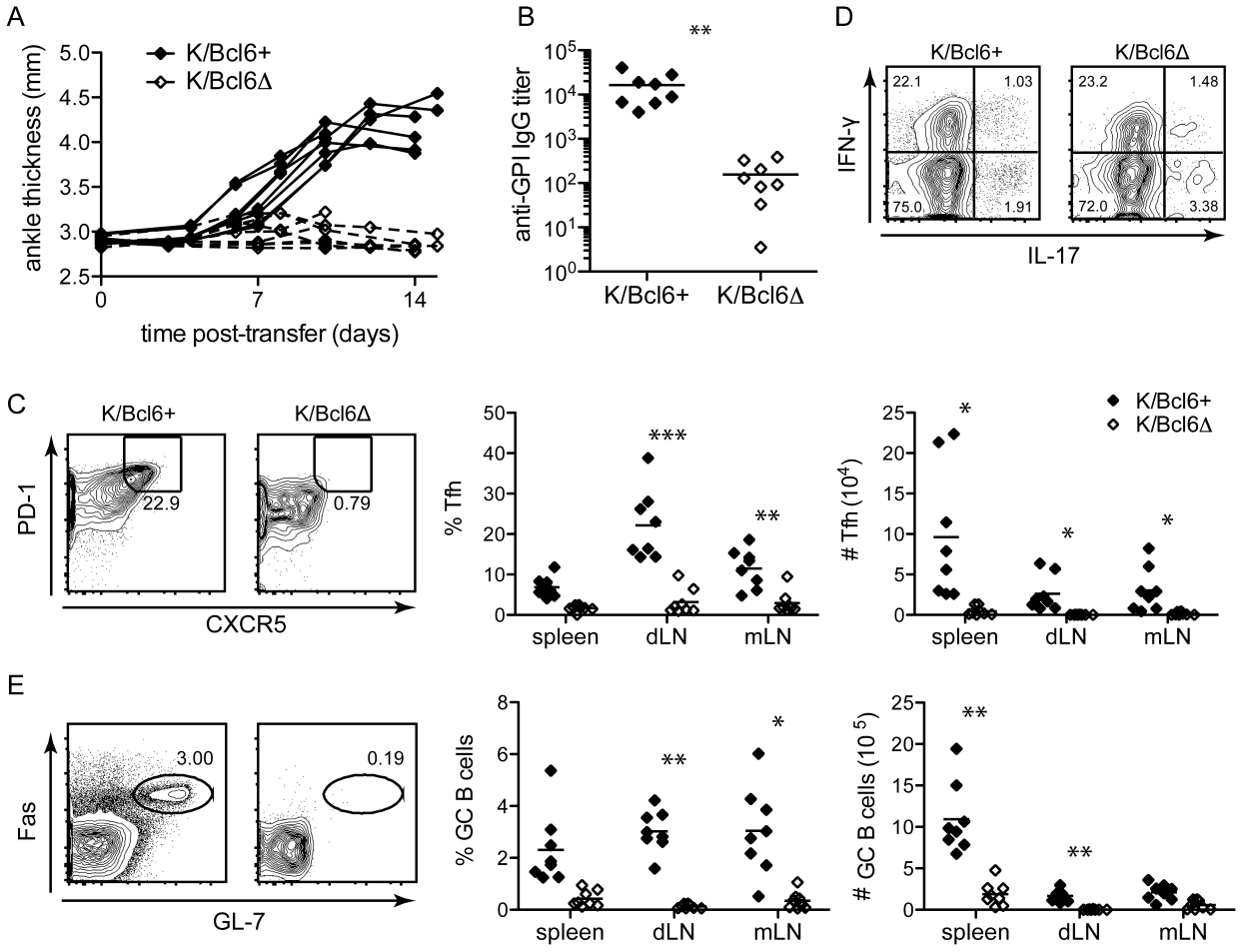

Figure 4.6. Tfh cells are required for T cell transfer induced arthritis. (**A-E**) K/B6 splenocytes either sufficient for Bcl6 (K/Bcl6+) or with a T cell-specific deletion of Bcl6 (K/Bcl6Δ) were transferred into $C\alpha^{-/-}$ BxN hosts and analyzed 10-15 days later. (**A**) Ankle thickness was monitored. Each line represents a mouse. (**B**) Anti-GPI IgG serum titers. (**C**) Tfh ($PD\text{-}1^+$ $CXCR5^+$) of transferred T cells ($CD45.1^-$ $CD4^+$). Shown are representative plots from the joint draining lymph nodes. Percent and number of Tfh are quantified in the spleen, joint draining lymph nodes (dLN, pooled popliteal and inguinal lymph nodes), and mesenteric lymph nodes (mLN). (**D**) Th1 and Th17 cells by intracellular cytokine staining gated on transferred T cells ($CD45.1^-$ $CD4^+$). Shown are representative plots from the spleen. (**E**) Germinal center B cells ($GL\text{-}7^+$ Fas^+) gated on $CD19^+$ cells. Shown are representative plots from the dLN. Percent and number of GC B cells are quantified. Each symbol represents a mouse. Horizontal lines represent the mean. Data are pooled from two independent experiments (n = 8 mice/group). Student's t test: if no p-value is displayed, $p > 0.05$; *, $p < 0.05$; **, $p < 0.001$; ***, $p < 0.0001$.

Tfh cells are required for arthritis

To formally test the requirement for Tfh cells in arthritis induction, we generated KRN T cells deficient in Bcl6, the master transcription regulator for Tfh (reviewed in (Crotty, 2011)). KRN TCR transgenic mice were crossed to CD4-Cre mice and *Bcl6*$^{fl/fl}$ mice to generate a T cell specific deletion of Bcl6 (referred to as K/Bcl6Δ, breeding strategy described in Figure 2.1B). Splenocytes from these mice were transferred into TCR Cα$^{-/-}$ BxN hosts. Control Bcl6 sufficient KRN T cell (K/Bcl6+) recipients developed arthritis normally, whereas no arthritis was observed in K/Bcl6Δ recipients (Figure 4.6A). Consistent with the lack of arthritis there was a dramatic reduction in anti-GPI IgG serum titers (Figure 4.6B). K/Bcl6Δ T cells did not develop into Tfh cells (PD-1^{+} CXCR5^{+}) as expected, while a substantial proportion of wild-type KRN T cells differentiated into Tfh in all sites examined (Figure 4.6C). There was a significant reduction in the total number of CD4^{+} K/Bcl6Δ cells compared to that of K/Bcl6+ cells two weeks after transfer, however this decrease was not significant at four days post-transfer (Figure 4.7). K/Bcl6Δ T cells were able to differentiate into Th1 (IFN-γ^{+}) and Th17 (IL-17^{+}) cells after transfer (Figure 4.6D), further supporting the conclusion that Th17 cells do not drive the antibody production and arthritis. The loss of Tfh cel differentiation resulted in the absence of germinal centers (Figure 4.6E). From this set of experiments, we conclude that Tfh cell differentiation is required for arthritis in the K/BxN mice. This, together with the loss of Tfh cells in antibiotic treated mice, suggests that the microbiota plays an important role in promoting autoreactive Tfh cell development.

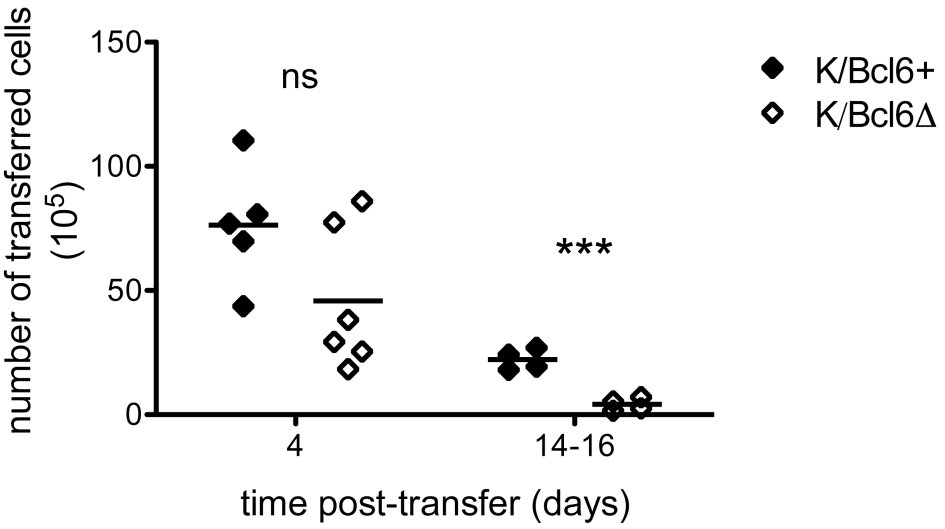

Figure 4.7. Bcl6 deficiency does not affect early proliferative or survival capacity of transferred T cells. K/B6 splenocytes either sufficient for Bcl6 (K/Bcl6+) or with a T cell-specific deletion of Bcl6 (K/Bcl6Δ) were transferred into $C\alpha^{-/-}$ BxN hosts and analyzed 4 days or 10-15 days later. Number of transferred cells ($CD45.1^-$ $CD4^+$) are shown. Data are pooled from four independent experiments (n = 4-6 mice/group). Student's t test: ns, $p > 0.05$; ***, $p < 0.0001$.

Discussion

In this study we investigated the contribution of Tfh versus Th17 cells to K/BxN arthritis and which cell population mediates the effect of the gut microbiota. We showed that Tfh cells are essential for autoantibody production and disease by using KRN T cells deficient for Bcl6, the key transcription factor regulating Tfh differentiation. Consistent with earlier studies, these cells failed to differentiate into Tfh cells, but could differentiate into Th17 cells, demonstrating that these two subsets are distinct lineages and Th17 cells are not sufficient to drive arthritis. Together with our earlier finding that KRN T cells deficient in RORγt drive arthritis as well as the wild-type KRN T cells (Block and Huang, 2013), we conclude that K/BxN arthritis is dependent on Tfh but not Th17 cells.

We showed that K/BxN arthritis is not dependent on the proinflammatory cytokine IL-17, and that the gut microbiota promotes disease in its absence. This finding is somewhat surprising given the numerous studies on the role of Th17 and IL-17 in the development of autoimmunity (reviewed in (Murdaca et al., 2011; Ouyang et al., 2008)). However, the role of Th17 and IL-17 in inflammatory arthritis is multifaceted and highly dependent on disease stages, tissues involved, and the models utilized (reviewed in (Haak et al., 2009b)). For example, IL-17 is required for the IL1Ra$^{-/-}$ arthritis model and the collagen-induced arthritis model (Nakae et al., 2003b; a; Lubberts et al., 2001). In both cases, disease was greatly reduced or abolished in mice deficient in IL-17, and IL-17$^{-/-}$ T cell priming or proliferation was defective in *in vitro* assays. There were also modest decreases in antibody production for certain IgG isotypes. In contrast, in a proteoglycan-induced model of arthritis, IL-17 deficient mice developed arthritis normally, although IL-17 was readily detectable in the joints and spleen. T cells cultured *in vitro* produced normal levels of cytokines, and serum antibodies against collagen were unchanged for the two isotypes examined (Doodes et al., 2008). These differences certainly reflect the different arthritogenic mechanisms in different models. They are also mirrored in several clinical trials testing anti-IL-17 and anti-IL-17R antibodies in rheumatoid arthritis patients that demonstrated only weak to moderate efficacy (Ruderman, 2015; Pavelka et al., 2015; Genovese et al., 2014a; b).

The role of IL-17 in the T cell-independent effector phase of inflammatory arthritis has been investigated in earlier studies using the K/BxN serum transfer model. IL-17RA$^{-/-}$ mice develop less severe arthritis, and expression of several pro-inflammatory mediators are decreased in the ankle joints (Sadik et al., 2011). A similar finding is shown in IL-17A$^{-/-}$ mice, where neutrophils are shown to be an important source of IL-17 production (Katayama et al., 2013). It

is important to note that arthritis in the serum transfer model is much less aggressive than in the spontaneous K/BxN model due to the much lower titers of autoantibodies transferred. There are precedents for disease-modulating effects observed in the serum transfer model to be lost or less severe in the spontaneous model (Choe et al., 2003; Kyburz et al., 2000). We focused on the initiation phase of the disease in which the germinal center response and autoantibody production are main readouts. Wu and colleagues showed that an anti-IL-17 neutralizing antibody (clone MAB421) was able to inhibit arthritis in the spontaneous K/BxN model (Wu et al., 2010). This differs from our results in K/g7/IL-17$^{-/-}$ mice as well as IL-17 blockade by two well-characterized antibodies (clones 17F3 (Berger et al., 2013; Valdez et al., 2012) and TC11-18H10.1 (Smith et al., 2008; Yen et al., 2006)). The discrepancy may be attributable to different microbiota in mouse facilities (see discussion below) or as yet unknown off-target effects with clone MAB421. Additional experiments should also be conducted with the clones used in our study to demonstrate that the dose used effectively blocks IL-17.

In addition to its role as a proinflammatory cytokine, IL-17 has been shown to drive germinal center responses in autoimmune BXD2 mice (Hsu et al., 2008). Immunization of IL-17RA$^{-/-}$ mice on both the B6 and BXD2 background results in decreased production of high-affinity antibody specific for the hapten component of the antigen. It is proposed that IL-17 arrests the migration of B cells by suppressing B cell chemotactic response to the chemokines CXCL12 and CXCL13. Further study by the same group showed that the total numbers of Tfh cells and their ability to induce B cell responses *in vitro* were not affected in BXD2-IL17RA$^{-/-}$ mice, however, the majority of the Tfh cells were not localized in the germinal center light zone (Ding et al., 2013). It was concluded that IL-17 is an extrinsic stop signal that promotes optimal localization of Tfh cells in germinal center. In K/BxN mice, *Il17ra*$^{-/-}$ B cells failed to be

recruited into germinal centers when in competition with wild-type B cells (Wu et al., 2010). However, it is not clear whether *Il17ra*$^{-/-}$ B cells would be defective in forming germinal centers in the absence of competition. In this context, the lack of a requirement for IL-17A in K/BxN arthritis could be due to high proportions of autoreactive T and B cells in this model, which makes their optimal localization in germinal centers unnecessary. Alternatively, IL-17F may play a role redundant with IL-17A, although IL-17F binds to IL-17RA with about 100 to 1000 times lower affinity than does IL-17A (Wright et al., 2007), and our transfer of Th17 deficient cells suggests otherwise (Block and Huang, 2013).

How the gut microbiota affects immune responses and autoimmunity is an actively pursued area of investigation. We have confirmed that K/BxN arthritis is critically dependent on gut microbiota using antibiotic treatment. Others have reported that the gut microbiota has a role in the effector phase of arthritis (Lee et al., 2014). We showed that it also has a dramatic effect on the initiation phase of the disease because autoantibody titers are greatly reduced in antibiotic treated mice. Consistent with decreased autoantibody titers, there was a decrease in germinal center and Tfh cell populations. It was proposed that gut microbiota, specifically SFB, drives K/BxN arthritis through the promotion of Th17 cells and IL-17 production (Wu et al., 2010). However, our demonstration that arthritis develops in the absence of Th17 cells and IL-17, and that antibiotic treatment prevents disease independent of IL-17, points to other disease inducing effects of the microbiota.

There is evidence that microbial colonization leads to Tfh cell differentiation, with the main focus of most studies on germinal centers and IgA production against microbial antigens (Winstead, 2014). Kubinak and colleagues have recently demonstrated that Tfh are dramatically reduced in the Peyer's patches of germ free mice, or mice with T cells deficient in MyD88.

Furthermore, Tfh cells can be restored in germ free mice with the introduction of TLR2 ligands (Kubinak et al., 2015). Our observations suggest that the bacterial colonization can also play a role in promoting the differentiation of autoreactive T cells towards a Tfh phenotype.

Although SFB is best characterized for its ability to promote Th17 cell development (Ivanov et al., 2009; Gaboriau-Routhiau et al., 2009), it is highly immunopotent and is likely to regulate other cells of the immune system directly or indirectly. Our observation that SFB is undetectable in the feces of mice treated with antibiotics is consistent with a role in promoting Tfh cell differentiation and arthritis. However, antibiotic treatment also reduces the abundance of additional bacterial community members and these bacteria may contribute to or play a redundant role in Tfh cell differentiation. Whether SFB promotes Tfh differentiation should be a topic of further study.

Our findings that Tfh cells and not Th17 cells or IL-17 drives autoimmunity in this microbiota-dependent model of autoimmunity have a number of implications. The K/BxN model is often used as an example of a Th17-dependent autoimmune model and as evidence that IL-17 should be a target for treatment of autoimmune disease (Longman et al., 2014; Wu and Wu, 2012). Our results contribute to mounting evidence that IL-17 is not an effective target for some autoimmune diseases (Haak et al., 2009b). Our findings also strengthen the link between bacterial colonization and Tfh cell differentiation, similar to differentiation of Th17 cells. Understanding how the commensal microbiota influences autoreactive T cell activation and function will contribute to our understanding of autoimmune pathogenesis.

Chapter V: Discussion

Overview

In these studies, we have investigated the contribution of two helper T cell subsets and their canonical cytokines in the initiation of autoimmunity in a mouse model of autoimmune arthritis. First, we investigated the function of IL-21 (produced by activated T cells, including Tfh and Th17 cells) in promoting autoimmunity. We found that autoreactive T cells were required to secrete IL-21 to promote autoantibody production, and that expression of IL-21R by B cells was required for the formation of germinal centers. IL-21R however was not required on T cells, demonstrating that IL-21 is not a necessary T cell autocrine factor in this model. We also demonstrated that Tfh cells are required for disease, and that Th17 cells are not necessary, pointing to Tfh as the cell type producing the required IL-21. We then tested whether IL-17 was required for arthritis and found that the cytokine was dispensable for disease.

The microbiota is known to promote arthritis in this model, and we demonstrated through antibiotic treatment that the effect of the microbiota was independent of IL-17, but a replete microbiota was required for Tfh cell development. We therefore propose that the microbiota promotes autoreactive Tfh cell differentiation, which leads to autoantibody production, and that Th17 cell differentiation induced by bacterial colonization has no essential role in this model (Figure 5.1). This work contributes to our understanding of the function of T helper cells in autoimmune disease and the influence of the microbiota on autoimmune T cell activation. It also provides further evidence that targeting Th17 or IL-17 is may not be efficacious for treating RA.

The context of these findings, as well as unanswered questions and suggested future directions are discussed below.

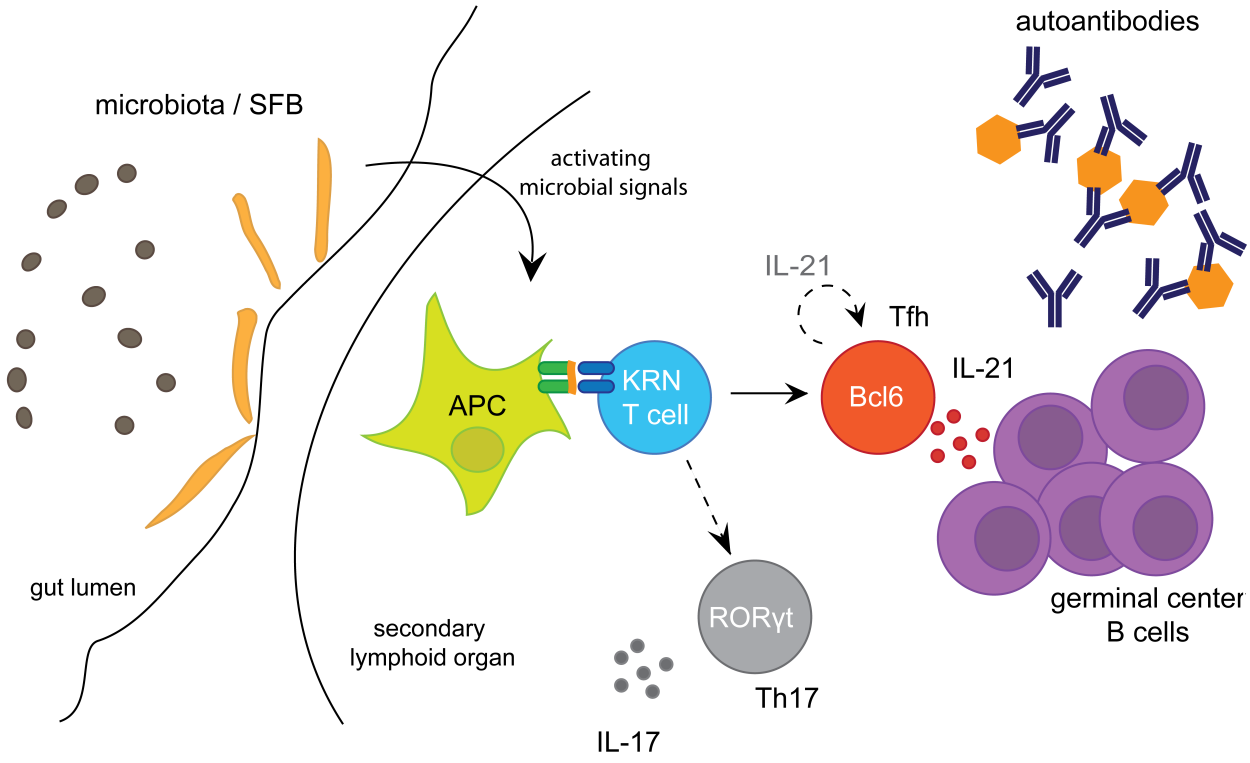

Figure 5.1. Proposed model for microbiota promoting K/BxN arthritis. Microbial signals can lead to the activation of Th17 cells and production of IL-17, however this pathway is dispensable for disease. Induction of Tfh by the microbiota promotes autoreactive germinal center formation. Germinal center induction requires T cell-derived IL-21 by B cells, but IL-21 is not required for T cell function. The precise site of T cell priming is not known but may take place in secondary lymphoid organs.

IL-21 is not an autocrine factor for T cells

The role of the cytokine IL-21 in K/BxN arthritis has been studied previously (Jang et al., 2009). Jang *et al.* found that T cells from K/BxN spleens expressed high levels of IL-21. They generated IL-21R deficient K/BxN mice and found these mice were completely protected from disease. The total number of $CD4^+$ T cells was reduced, and the percent of proliferating T cells was reduced in the spleen and joint draining lymph nodes. There was a decrease in anti-GPI antibody secreting cells and $CXCR5^+$ T cells (which the authors defined as Tfh). Because of the lack of T cell proliferation and because T cells from K/BxN mice expressed high levels of IL-21R, the conclusion was that IL-21R is required for homeostatic T cell expansion in a cell intrinsic manner (Jang et al., 2009).

IL-21R is expressed on many cell types. The roles of IL-21R signaling in promoting Tfh development as well as germinal center B cell maintenance are well characterized (Parrish-Novak et al., 2000; Ozaki et al., 2002). Therefore, we used cell transfer experiments to test the relevant sources and targets of IL-21 for disease. We found that IL-21 was indeed required for arthritis, but IL-21R expression on T cells was not required. This result demonstrates that IL-21 is not a necessary T cell autocrine factor for autoimmune development. We found that IL-21R was in fact required on B cells for autoreactive germinal center formation (Block and Huang, 2013).

The explanation for why Jang *et al.* observed a defect in T cell proliferation in IL-21R K/BxN mice could be due to the requirement of IL-21R on B cells. Without IL-21R signaling on B cells, there is a dramatic reduction of autoantibodies and the downstream inflammation that results in disease. B cells presenting cognate antigen can also promote the survival of T cells (Obst et al., 2005). Without this feed-forward inflammatory response, T cells do not proliferate

as strongly or accumulate in number. This hypothesis would best be tested by generating mice with IL-21R conditionally knocked out in B cells, however IL-21R floxed mice are not commercially available. Alternatively, mixed bone marrow chimeras could be generated, with bone marrow contributed from KRN^+ B cell deficient mice (such as μMT mice) and from K/IL-21R deficient mice (non KRN T cells inhibit KRN proliferation and so bone marrow for B cells should be KRN^+ or $Ca^{-/-}$). B cells developing in the host mice would only have B cells deficient in IL-21R. B cell sufficient bone marrow would have to be crossed to the NOD MHC $I\text{-}A^{g7}$ in order for antigen presentation. T cell proliferation and survival could be examined to determine whether the proliferative defect in IL-21R global knock out mice is due to a B cell requirement. If T cell proliferation were normal in these chimeras it would suggest that an additional cell type requires IL-21R in K/BxN mice to promote T cell proliferation. Dendritic cells express IL-21R and would be a candidate for further examination. However, most research has found that IL-21R signaling on dendritic cells inhibits functional maturation and reduces antigen presentation, and deleting IL-21R on dendritic cells might have opposite effects from deletion of the receptor on B cells (Wan et al., 2013; Brandt et al., 2003).

IL-21R and IL-6R signaling both contribute to Tfh cell differentiation

Since arthritis in this model requires T-cell dependent class switched antibody production we were interested in investigating Tfh differentiation in this model. IL-21 and IL-6 have been shown to drive Tfh differentiation. IL-21 deficient mice had decreased Tfh cell differentiation after immunization with sheep red blood cells. IL-21R signaling was shown to enhance phosphorylation of Vav1, a component of TCR downstream signaling that enhances T cell helper function (Vogelzang et al., 2008). Naïve T cells cultured *in vitro* with IL-6 or IL-21 induced

expression of Bcl6, CXCR5, and IL-21. Genetic deletion of IL-21 and blocking antibody against IL-6 reduced Tfh cell differentiation *in vivo* during infection with LCMV more than blockade of either cytokine alone (Eto et al., 2011). Immunizing IL-21 deficient mice with KLH in CFA led to reduced Tfh cells compared to wild type mice (Nurieva et al., 2008). However, another group immunized IL-21 deficient mice with NP-CGG in alum found no defect in Tfh cell differentiation (Poholek et al., 2010). IL-21R and IL-6R signaling both require STAT3. STAT3 deficient immunized mice had dramatically reduced (but not completely abolished) Tfh cells again suggesting that signaling through either cytokine receptor can promote Tfh cell differentiation (Nurieva et al., 2008). These studies have supported the conclusion that IL-21 or IL6 are required for Tfh cell differentiation and that they are partially redundant because of their shared downstream signaling pathway.

We found that IL-21R deficiency on T cells did not significantly reduce the ability of T cells to differentiate into $Bcl6^+$ $CXCR5^+$ cells and had normal Tfh cell functions insofar as they were able to migrate into B cell follicles, promote germinal center formation, and induce class-switched antibody production. We investigated whether IL-6R deficiency on T cells would have any impact on Tfh cell differentiation, and whether a double deficiency of IL-21R and IL-6R would completely abolish the differentiation of Tfh cells and prevent disease. IL-6R deletion did in fact reduce the percent of Tfh cells, and a double deficiency further reduced this population, however only the double deficiency had a significantly reduced number of Tfh cells in the spleen. The small but noticeable difference was reflected in a reduction in germinal centers, again being more dramatic in the double cytokine receptor knock out. These experiments demonstrate that IL-21 and IL-6 do redundantly promote Tfh cell differentiation, and suggest that IL-6 is in fact more important for Tfh cell differentiation, since deficiency of this receptor

alone was sufficient to reduce the proportion of the Tfh cell population while IL-21R deficiency alone showed no defect. Additionally, these results demonstrate that other factors besides IL-21 and IL-6 can promote Tfh cell differentiation, since Tfh cells were still detectable in the absence of both receptors and antibody production and class switching was normal.

Our studies took advantage of a T cell transfer model, meaning that only T cells were deficient in IL-21R and IL-6R when we tested the effect of the cytokines on Tfh cell differentiation. All of the studies described above used mice globally deficient in the cytokines and therefore the effect of IL-21 and IL6 on Tfh could be indirect. There is a large amount of literature on the effect of IL-21 on B cells, and B cells are known to feed back on Tfh cell function (Crotty, 2014; Poholek et al., 2010). Therefore, conditional deletion, or transfer of mutant cells as in our experiments, is a more rigorous approach to testing the direct cell intrinsic effect of these cytokines *in vivo*. Some of the effect of IL-21 on Tfh cell differentiation seen in the described studies could be due to the requirement of IL-21 by B cells, which then in turn support Tfh cell differentiation and survival. Experiments involving immunization and infection that were conducted with global knock out mice should be repeated with conditionally mutant mice to test the true effect of IL-21 on Tfh cell differentiation.

TCR stimulation plus IL-21R or IL-6R signaling is the best-described pathway that induces Tfh cell differentiation. However there clearly must be other mechanisms that contribute to Tfh cell commitment, since deletion of STAT3 alone does not completely abolish Tfh differentiation (Nurieva et al., 2008). Other factors have been shown to inhibit Tfh cell differentiation. STAT5 signaling, via IL-2R and IL-7R signaling in T cells, inhibits Tfh cell differentiation (Johnston et al., 2012; Nurieva et al., 2012; Oestreich, 2015). My research has also verified an increase in Tfh cell development in IL-7R deficient KRN^+ T cells. Deletion of

STAT5 in T cells enhanced Tfh cell differentiation (Nurieva et al., 2012) and a constitutively active STAT5 introduced with a retrovirus dramatically decreased Tfh cell differentiation, but again did not completely abolish Tfh cells (Johnston et al., 2012). STAT5-mediated signaling could be considered a "brake" to Tfh cell differentiation, with STAT3-mediated signaling acting as the "gas." Perhaps altering only one of these signals accounts for the incomplete phenotypes in these experiments. Experiments where the gas pedal is eliminated (STAT3 deletion) at the same time as the brake pedal is constantly engaged (constitutive STAT5) could be conducted to test this model. In this case we would predict a total block in Tfh cell differentiation.

A main function of Tfh cells is to drive germinal centers. Germinal centers function in part to promote the differentiation of plasma cells, which secrete high amounts of antibody (Shapiro-Shelef and Calame, 2005). Of note, in IL-6R IL-21R double deficient mice the changes in germinal center numbers were not reflected in the anti-GPI IgG serum titers or the overt disease. How do we explain the result that while Tfh and germinal center B cells are reduced in the IL-21R/IL-6R double deficient mice, antibody titers and disease were normal? While fewer germinal center B cells are found in the mutant mice, plasma cell differentiation could be normal and an equal amount of anti-GPI antibody may be produced. IL-21 is known to promote Blimp-1 expression, required for plasma cell development (Ozaki et al., 2004). Plasma cells should be examined by flow cytometry and by ELISPOT in double deficient mice to test for anti-GPI antibody producing cells. If there is no defect in plasma cell differentiation, this would argue that the number of germinal center B cells is not the rate-limiting step for plasma cell differentiation and that this does not require IL-21. If there is a decrease in plasma cells but no decrease in antibody titers, it implies that the plasma cells that are able to differentiate secrete higher amounts of antibody or are higher affinity.

IL-21R signaling drives germinal center formation

We established that IL-21 is critical for autoantibody production and autoimmune disease, but is not required on autoreactive T cells. Satisfyingly, deficiency of IL-21R on B cells phenocopied IL-21 deficiency from T cells and completely abolished germinal center formation and autoantibody production. This finding of IL-21R signaling being necessary for germinal center formation is somewhat surprising because other publications have demonstrated that IL-21R is important for germinal center maintenance and IgG production, but germinal center formation is normal or only slightly impaired in the absence of IL-21R (Vogelzang et al., 2008; Ozaki et al., 2002; 2004). Immunization of IL-21 deficient or IL-21R deficient mice with NP-KLH in alum resulted in normal germinal center formation early, which was lost over time, paired with a defect in plasma cell and IgG antibody formation (Zotos and Tarlinton, 2012). In a model of lupus-like chronic graft-versus-host disease, IL-21R deficient mice formed germinal centers, which decreased over time and resulted in less autoreactive antibody production (Nguyen et al., 2012).

We looked for germinal centers by flow cytometry and histology starting at four days after cell transfer and never saw any evidence of germinal centers that would have been predicted from other groups' results. There was also no evidence of anti-GPI IgM or IgG production at early timepoints, reinforcing the idea that autoreactive B cells were never being selected and activated. It is unclear why there is this discrepancy, especially since this early germinal center formation has been reported in both immunization and autoimmune models. One explanation could be that additional signals might aid in germinal center formation in certain contexts, but those signals might be absent in K/BxN arthritis compared to other models. In this case therefore IL-21R is required earlier and a block in germinal centers is more striking.

Profiling germinal center B cell gene expression in K/BxN mice compared to a model where IL-21 is required later might provide some insight.

IL-21 is a potential target in the treatment of autoimmunity

Our work demonstrates that IL-21 is critical for germinal center formation and autoantibody production. Polymorphisms within the loci encoding IL-21 and IL-21R are associated with a risk for systemic lupus erythematosus (SLE) and RA (Webb et al., 2009; Lan et al., 2014; Yarwood et al., 2014). Several studies of patients with autoimmune diseases have observed increases in Tfh cells or IL-21, described below. Because Tfh cells and IL-21 are required for autoantibody production, IL-21 is an attractive candidate for treatment of autoantibody mediated human disease.

Sjögren's syndrome is a chronic inflammatory autoimmune disease of the exocrine glands. Tfh cell populations and IL-21 levels were increased in the peripheral blood in Sjögren's syndrome patients, and Tfh cell proportions correlated with memory B cells and plasma cells (Jin et al., 2014). Levels of serum IL-21 in patients correlated with levels of autoreactive IgG (Kang et al., 2011) and IL-21 detected in tears correlated with ocular surface irritation (Lim et al., 2015). The Tfh cell population in the peripheral blood also decreased upon standard treatment with glucocorticoid and/or immunosuppressive drugs (Jin et al., 2014).

SLE is an autoimmune disorder characterized by autoantibodies and immune complex-mediated tissue and organ damage. SLE patients had a large population of Tfh cells in their peripheral blood and secreted more IL-21 compared to healthy controls. The size of the Tfh cell population also correlated with plasmablast population size and autoantibody levels. These

findings were reported in two separate studies, one with a cohort of new-onset SLE patients (Choi et al., 2015; Wang et al., 2014).

Similar findings were reported in patients with ankylosing spondylitis (AS), an autoimmune disease characterized by chronic inflammation in the spine and sacroiliac joints. Tfh, IL-21 producing Tfh cells, and IL-21 levels in the serum, were increased in AS patients compared to healthy controls. All of these parameters decreased after drug therapy (meloxicam, thalidomide, or etanercept) (Xiao et al., 2013). In another study, AS patients were stratified as having active or inactive disease; patients with active AS had increased circulating Tfh cells and plasma IL-21 compared to inactive AS, although both groups had elevated levels compared to healthy controls (Wu et al., 2015).

New-onset rheumatoid arthritis patients (NORA), who have not yet been treated with disease-modifying anti-rheumatic drugs (DMARDs), have elevated Tfh cell populations in the blood and increased IL-21 levels in the serum (Wang et al., 2013; Ma et al., 2012). Importantly, patients who responded to drug therapies at one month had decreased activated B cells and Tfh cells in their blood. IL-6 and IL-21 were elevated in the serum of RA patients in a European cohort, and the levels of these two were the only cytokines from a large panel that distinguished RA from undifferentiated arthritis (Gottenberg et al., 2012).

Clinical trials testing anti-IL-21 monoclonal antibodies (mAbs) have been undertaken, however results have not yet been presented (Burmester et al., 2014). An anti-IL6R mAb (tocilizumab) has been licensed as a therapeutic for RA. Anti-IL-6R treatment was found to be as effective as anti-TNF-α in patients not being treated with methotrexate (Gabay et al., 2013). This anti-IL-6R mAb treatment decreased IL-21 levels in rheumatoid arthritis patients, which was correlated with a slight decrease of autoantibody production (Carbone et al., 2013). Because

IL-21 acts at the stage of B cell activation, an anti-IL21 therapeutic is an attractive candidate for targeting autoimmune reactions relatively early in the disease pathway and may be attractive for treating patients with an early diagnosis, or individuals with high risk of autoimmunity. Additionally, anti-TNF-α is the most commonly prescribed treatment for RA, but as high as 40% of patients do not respond to the treatment (Siebert et al., 2015). Alternative biological pharmaceuticals, including anti-IL-21, that target other phases of disease might be efficacious for patients who do not respond to current standard treatments.

Are Th17 cells and IL-17 required in autoimmune disease models?

Th17 cells and their namesake cytokine IL-17 have been implicated in autoimmune disease almost since the discovery of IL-17. One of the most cited Th17 cell and IL-17-dependent autoimmune models is experimental autoimmune encephalitis (EAE). This model of central nervous system (CNS) autoimmune disease has similarities to multiple sclerosis. There are a number of disease-inducing protocols, but generally mice are immunized with myelin oligodendrocyte glycoprotein (MOG) with adjuvant, which leads to immune cell infiltration of the CNS and demyelination of neurons. Mice deficient in IL-23 and IL-6, both of which promote Th17 cell differentiation, are resistant to EAE (Gutcher and Becher, 2007). IL-17 secreting T cells can be found in the CNS after immunization (Park et al., 2005), and Th17 cells were found to be more encephalitogenic than Th1 cells (Langrish et al., 2005). These results, along with other supporting studies, led to the model that Th17 cells were the predominant effector cells in EAE.

There is mounting evidence that Th17 cells and IL-17 are in fact not essential mediators of EAE. While one group found that a blocking anti-IL-17 antibody dramatically reduced

disease (Langrish et al., 2005), another group could not recapitulate this result and saw only a modest decrease in disease with anti-IL-17 antibody and no efficacy with an IL-17R-Fc (Hofstetter et al., 2005). IL-17 deficient mice were found to be susceptible to EAE, but incidence was reduced in the chronic phase of disease (Komiyama et al., 2006). IL-17A deficient and IL-17F deficient mice, as well as IL-17F deficient mice treated with anti-IL-17A antibody had slightly reduced clinical scores but were fully susceptible to disease (Haak et al., 2009a). There have therefore been questions about whether EAE is truly a Th17-dependent disease model, or whether the inflammation induced by IL-17 contributes to disease but is dispensable for autoimmune damage (Haak et al., 2009b).

Similar to the story of Th17 cells and IL-17 in EAE, a number of studies have highlighted a contribution of IL-17 in autoimmune arthritis models, but not all studies agree. As mentioned in the Discussion section of Chapter IV, the collagen-induced arthritis model and the IL-1Ra$^{-/-}$ arthritis model have been characterized as Th17-mediated. In the collagen-induced arthritis model, blocking IL-17 with an IL-17R-Fc fusion protein slightly decreased disease incidence and overexpression of IL-17 with an adenovirus injected into the knee exacerbated disease (Lubberts et al., 2001). The Iwakura laboratory tested the role of IL-17 in collagen-induced arthritis by using IL-17 deficient mice. Again, disease incidence and arthritis score were reduced. A cell-intrinsic defect in T cell proliferation was also seen in IL-17 deficient mice (Nakae et al., 2003a). The same group tested the requirement of IL-17 in IL-1Ra$^{-/-}$ arthritis. IL-1Ra$^{-/-}$ mice were crossed to IL-17 deficient mice; IL-17 deficient mice had no disease, and mice heterozygous for IL-17 had an intermediate phenotype. Again, a T cell intrinsic proliferative defect was seen in IL-17 deficient mice (Nakae et al., 2003b). In contrast, IL-17 deficient mice were found to be fully susceptible to disease in a proteoglycan-induced model of arthritis, despite a robust induction of

IL-17 in the synovial fluid. In this case, proliferation of IL-17 deficient T cells was not impaired (Doodes et al., 2008).

Earlier studies suggested an important role for Th17 and IL-17 in K/BxN arthritis. T cells in the cell transfer model of K/BxN arthritis have been found to produce large amounts of IL-17. Th17-polarized T cells augmented arthritis in the cell transfer and the serum transfer models of K/BxN arthritis (Hickman-Brecks et al., 2011; Jacobs et al., 2009). Blockade of IL-17 prevented arthritis in the K/BxN model, and B cells deficient in IL-17R did not differentiate into germinal center B cells in the presence of wild type B cells (Wu et al., 2010). Therefore, it was surprising when our transfer of KRN T cells deficient in RORγt, unable to differentiate into Th17 cells and secrete IL-17, led to normal arthritis induction. In the T cell transfer model of arthritis, host mice have intact populations of other cells that can produce IL-17, namely γδ T cells and type 3 innate lymphoid cells (ILC3s), and therefore it was possible that one or more of these cell populations rather than autoreactive T cells were important contributors of IL-17. To test this, we bred the IL-17 deficient K/BxN mice and found that they too developed arthritis normally, with only a slight delay in disease kinetics. We tested two different antibody clones against IL-17 and found that the results matched the IL-17 deficient experiments.

Thus there is a discrepancy between our results in the IL-17 deficient mice and other work that found IL-17 does play a role in K/BxN arthritis. There are a number of explanations for these differences. First, the serum transfer model of disease is less severe than spontaneous arthritis and there is precedent for certain factors, such as IL-1, playing a more important role in the serum transfer model than in the spontaneous model (Choe et al., 2003). The inflammatory response caused by IL-17 may be more relevant to disease when autoantibody titers are relatively low. Second, in some cases where IL-17 or IL-17R are genetically knocked out or blocked with

antibody, conclusions based on small differences in disease outcomes tend to be overstated. While we saw a slight delay in arthritis development in IL-17 deficient mice, we would not conclude that IL-17 is required for disease. However, in some cases small differences in arthritis for a few timepoints are interpreted as IL-17 playing a crucial role in disease. Our biggest discrepancy is with the blocking antibody experiments performed by Wu *et al.* (Wu et al., 2010). Anti-IL-17 antibody injection completely prevents disease in K/BxN mice, while we found only a slight delay in disease with two different anti-IL17 antibody clones. While we did not confirm that the IL-17 clones we used did in fact block IL-17 function *in vivo*, the clones we used have been widely used and have been proven to be effective in other *in vivo* settings (Valdez et al., 2012; Berger et al., 2013; Yen et al., 2006). We suggest that the clone used by Wu and colleagues should be tested for off-target effects. The clones we used should also be tested for efficient blockade of IL-17.

IL-17 and antibody isotype class switching

IL-17 has been shown in the context of autoimmunity to impact B cell class switching to certain IgG subclasses and promote germinal center formation. *In vitro* differentiated Th17 cells specific for MOG promoted increased antibody production and class switching to IgG1 and IgG2b *in vitro* and *in vivo* compared to Th0 T cells (Mitsdoerffer et al., 2010). In the BXD2 autoimmune model, blockade of IL-17 disrupted germinal center formation and autoantibody formation, and overexpression increased class switching (Hsu et al., 2008). We therefore hypothesized that anti-GPI antibody isotypes might be skewed in the absence of IL-17, however ELISAs for individual IgG subclasses showed no alterations in the serum titers of any IgG subclasses. Germinal centers were also normal in percent and number in multiple lymphoid

organs. It is unclear why some studies found that IL-17 had an impact on germinal centers and antibody class switching, while we did not. KRN recognition of GPI peptide:MHC elicits a strong reaction and the autoantigen GPI is continually available, therefore germinal centers are not likely to have limited antigen or a limited timeframe to capture antigen. This robust and prolonged germinal center reaction is perhaps then less sensitive to the skewing effects of IL-17. In a study with K/BxN mice IL-17R deficient B cells were not recruited into germinal centers when they were in a competitive environment with wild type B cells (Wu et al., 2010). However when all B cells are in an IL-17 deficient environment, this defect may no longer be seen, as was the case in our study.

How IL-17 might influence class switching would help determine whether it might play an important role in some autoimmune settings. Hsu and colleagues propose that IL-17R signaling affects B cell responsiveness to chemokines, and therefore helps orchestrate B cell migration through the dark and light zones of the germinal center required for affinity maturation and class switching. They demonstrate that IL-17R signaling on B cells induces transcripts of proteins involved in regulating chemokine receptor expression (Hsu et al., 2008). To test this further, IL-17R deficient or wild type B cells could be labeled and tracked with intravital imaging to determine if there are migration defects within the germinal center. Although we see no evidence that IL-17 influences B cell isotypes, a direct influence of IL-17R signaling on class switching is possible. The downstream pathways of IL-17R signaling require Act1 and TRAF6, which lead to NFκB activation (Gu et al., 2013). TRAF6 and NFκB both contribute to CD40 ligation-mediated class switch recombination (Jabara et al., 2002; Dedeoglu et al., 2004). Therefore it is possible that the same mechanism is at play in IL-17R signaling.

Our focus in these studies was on helper T cells and their interactions with B cells to promote autoantibodies, and therefore we did not investigate the more downstream roles of IL-17 in the effector phase of disease. Since we observed normal induction of anti-GPI antibodies and severe arthritis in IL-17 deficient mice, we can conclude that IL-17 is not required in the initiation stage of disease. We did observe a slight delay in disease onset in IL-17 deficient mice, suggesting IL-17 does contribute to the fast kinetics in wild type animals. IL-17 deficient mice have been shown to have less severe arthritis in the serum transfer model of disease (Katayama et al., 2013), however serum transfer arthritis is much less robust than spontaneous disease. Neutrophil recruitment to the synovial joints may be delayed, but clearly any defects have minor effects on the arthritis phenotype. The joints of IL-17 deficient mice could be examined at early timepoints to ascertain whether neutrophil recruitment is indeed defective. We also did not look at histology of the joints, which might indicate a delay in bone erosion in IL-17 deficient mice.

One caveat of our studies with IL-17 deficient mice is that IL-17 is a member of a larger cytokine family. Also known as IL-17A, it shares a receptor (in mice) with IL-17F. The focus in autoimmunity has been on IL-17A. IL-17F is generally characterized as having different effects than IL-17, and has much weaker affinity for the receptor (Kolls and Lindén, 2004; Gu et al., 2013). In the IL1Ra$^{-/-}$ arthritis model, where IL-17A was found to have an effect, IL-17F played only a slight role in disease when mice were also deficient in IL-17A (Ishigame et al., 2009). We did not find any compensatory increase in IL-17F in our K/BxN mice that were deficient in IL-17A. The ideal experiment would be to generate IL-17R deficient K/BxN mice to demonstrate that no receptor signaling is required for disease. However our cell transfer studies

showed that RORγt deficient T cells could not differentiate into Th17 cells providing evidence for the conclusion that neither IL-17A or IL-17F is required for arthritis in this model.

Th17 cells and IL-17 in human autoimmunity

Th17 cells and IL-17 have been of great interest to researchers trying to understand the etiopathogenesis of RA. IL-17 can induce expression of a number of proinflammatory cytokines as well as promote joint remodeling (Kotake et al., 1999; Lundy et al., 2007; Fossiez et al., 1996). One of the first experiments that suggested IL-17 could be involved in RA was the addition of IL-17 to synoviocytes *in vitro*. This culture condition induced a number of cytokines known to be present in the joints of RA patients, including IL-6 and GM-CSF (Fossiez et al., 1996). A later study found that mRNA levels of IL-17, along with TNF-α and IL-10, in the synovial joint of RA patients were predictive of damage progression (Kirkham et al., 2006). Despite the attention IL-17 receives, no specific function of IL-17 has shown to be required for RA (Murdaca et al., 2011). In contrast to the studies implicating IL-17 in RA, another study found that IL-17$^+$ T cells in the peripheral blood of RA patients were not increased compared to healthy controls, and the synovial joints actually had a smaller percent of IL-17 producing T cells than in the blood. The percent of Th17 in the blood also did not correlate with Disease Activity Score (Yamada et al., 2007).

A number of monoclonal antibodies and inhibitors targeting IL-17, IL-17R, and other members of the Th17-axis such as IL-23 and IL-22, have been tested in clinical trials to treat RA (Krausz et al., 2012; Genovese et al., 2014a; Pavelka et al., 2015; Koenders and van den Berg, 2015). Generally, efficacy has been minimal and some rheumatologists have urged drug

developers to abandon the anti-IL17 approach to drug development and acknowledge when drug efficacy is minimal (Ruderman, 2015).

Results from human patients are reflective of the mouse model data discussed above. There are correlations between Th17 cell differentiation, IL-17 levels, and disease, but in many cases the actual effect of the cytokine on disease outcomes is not all that striking. The K/BxN mouse model of inflammatory arthritis, while admittedly just a model for human disease, has many similarities to human arthritis. The autoantigen is likely not relevant for RA, but many of the components of the immune system required for arthritis are paralleled in human RA (Mandik-Nayak and Allen, 2005). It may be that the dispensability of IL-17 in K/BxN arthritis is another way in which the model resembles human RA. At the very least, other basic researchers using the K/BxN model should not consider it a solely Th17-driven model and direct their research to other aspects of autoimmune pathogenesis.

Microbiota and Th17 versus Tfh cell differentiation

When we observed that K/BxN arthritis did not require Th17 cells or IL-17, we were naturally interested in the microbiota of these mice. K/BxN arthritis has been shown to be dependent on the microbiota, as mice derived germ-free are largely protected from disease and colonization with normal microbiota, or SFB alone, is sufficient to induce arthritis (Wu et al., 2010). It is now well established that SFB promote the differentiation of Th17 cells and production of IL-17 (Ivanov et al., 2009). This induction of K/BxN arthritis by SFB is thought to occur through the induction of Th17 cells and IL-17 production. Since we found that IL-17 was not required for disease, we were interested to know whether the microbiota was promoting

disease independent of IL-17. By altering the microbiota with antibiotics, we found that this was indeed the case.

We believed it was important to determine whether the K/BxN mice in our colony were colonized with SFB. If our mice were not colonized with these bacteria, it might indicate that different bacteria may promote disease through different mechanisms, and other Tfh-inducing bacteria exist, along with Th17-inducing SFB. However, quantitative PCR for SFB-specific 16S ribosomal RNA revealed that K/BxN mice in our colony were colonized with SFB. We also found that our antibiotic treatment completely eliminated SFB from the mice. This finding suggests that SFB could still be promoting autoimmunity in the absence of IL-17, and indeed SFB has been shown to promote other T helper subsets, although Tfh cells were not examined (Gaboriau-Routhiau et al., 2009). To test this, IL-17 deficient K/BxN germ free or antibiotic treated mice should be colonized with SFB and then monitored for disease. If arthritis is not induced, it indicates that another bacterial community member or members induce arthritis independent of the Th17 axis.

Our data suggest that Tfh cell differentiation is promoted by bacterial colonization of the gut. We have found that Tfh cells are required for arthritis by conditionally deleting the transcription factor Bcl6 in T cells and transferring KRN^{+} Bcl6 deficient T cells into hosts. The resulting loss of autoantibody production and disease development are striking and highlight the critical role Tfh cells play in promoting B cell mediated autoimmune responses. Upon antibiotic treatment, the number of Tfh cells is greatly reduced. We propose that microbial colonization can promote the differentiation of autoreactive Tfh cells, and that this is a critical way that microbial colonization promotes disease. Despite a large body of work investigating the effect of bacterial colonization on IgA production, there has been little work on the development of Tfh

cells in response to the microbiota. B cells make T cell independent "natural" IgA as well as T cell dependent "classical" IgA in response to the commensal microbiota (Slack et al., 2012). This T cell dependent IgA response is dynamic and reflects the host's current bacterial exposure (Hapfelmeier et al., 2010). This implies that the microbiota is affecting not only the B cell response, but also Tfh cell responses in the gut. Recently it was demonstrated that germ free mice have few Tfh cells in the Peyer's patches. Cell intrinsic MyD88 signaling on T cells, by administration of a TLR2 ligand, restores Tfh cell differentiation and IgA production (Kubinak et al., 2015). Our data also suggest the microbial colonization can affect sites distal to the gut, and against a non-microbial antigen. To test our hypothesis that the microbiota can promote autoreactive Tfh cell differentiation, Tfh cells and autoantibody production should be examined after colonization of germ free K/BxN mice or administration of microbial products. We have tested whether the TLR2 ligand Pam3CSK4 could induce Tfh cell activation and autoantibody production in antibiotic treated mice. There was no induction seen in our preliminary experiments, but additional ligands should be tested. We have also done pilot experiments with transfer of sorted KRN^+ Tfh cells into antibiotic treated K/BxN mice, without any effect. These experiments might not have been successful because of the small number of cells transferred and the endogenous T cells already present in the host mice. Transfer of sorted Tfh cells into T cell deficient ($C\alpha^{-/-}$ BxN) antibiotic treated hosts might yield a different result.

We suggest that the microbiota promotes Tfh cell differentiation in K/BxN mice, and that autoreactive germinal centers are promoted through T cell secretion of IL-21. It would be informative to test the levels of IL-21 in antibiotic treated mice, either by ELISA to detect IL-21 in the serum, or by intracellular flow cytometry or sorting T cells and testing mRNA levels of *Il21*. We predict that IL-21 expression will be dramatically reduced in antibiotic treated mice.

Microbiota and K/BxN mice

Monocolonization with SFB is sufficient to drive K/BxN arthritis (Wu et al., 2010). It is quite possible that many bacteria can, like SFB, induce autoimmunity in K/BxN mice, however no others have been tested. Whether monocolonization with other bacteria can induce arthritis should be tested. Our mice treated with antibiotics were still colonized with bacteria, although the total amount of bacteria in the feces was reduced. This suggests that only certain bacteria can promote disease, since arthritis was prevented despite some gut bacteria being present. SFB establish their niche close to the epithelium of the intestines, and therefore other attaching bacteria could be tested for this immunomodulatory effect.

Our antibiotic treatment regimen was very aggressive. While only two antibiotics were used (ampicillin and vancomycin), the breeding mice had to be given antibiotics in their drinking water so that experimental litters born to them would be exposed to an antibiotic-altered microbiota from birth. Additionally, gavaging the mice daily starting from weaning kept arthritis incidence low enough for us to do our experiments. If antibiotics were in the water alone without gavage, or treatment of mice started at weaning, at least some level of arthritis developed in a significant portion of the mice. This was a difficult strategy to employ for our experiments, but it may be a useful setting to conduct experiments to identify additional disease-inducing bacteria. Mice could be treated with antibiotics and then have the treatment removed. Disease incidence would go up, but not be 100%. Fecal samples, along with ankle thickness data and serum samples, could be collected. The fecal samples could be sequenced for 16S rRNA, and the bacteria present could be correlated with arthritis and autoantibody production. Bacteria present only in diseased animals could be introduced to germ-free K/BxN mice and arthritis induction could be measured.

How the microbiota specifically promotes autoimmunity in the K/BxN model is unknown. Pattern recognition receptors (PRRs) such as TLRs are likely candidates for a mechanism of a microbial product exerting this effect on the immune system. The T cell transfer system is useful for testing this, as the requirement for a PRR can be tested on T cells or on host cells (non-T cells) to help narrow down the cell type requiring microbial sensing. MyD88 is required for most TLR signaling and so testing MyD88 deficient hosts or T cells would be a good first step. MyD88 is also downstream of IL-1R signaling, which is required for optimal K/BxN arthritis development (Choe et al., 2003). Therefore, anti-GPI IgG production, determined by ELISA, should be assessed instead of ankle thickness. The T cell transfer model requires an αβ T cell deficient B6 x NOD F1 host in order for transferred KRN T cells to expand. Luckily, many knock out mice of TLR and TLR adaptor molecules are available on the NOD background, and so mutant B6 and mutant NOD mice can be intercrossed. Additionally, KRN/B6 mice have been crossed to B6 mice with a Thy1.1 congenic marker. B6 and NOD mice are Thy1.2, meaning αβ T cells in host mice can be depleted with anti Thy1.2 moncolonal antibodies, and KRN/Thy1.1 transferred cells will not be affected. This saves the step of crossing mutant host mice to $C\alpha^{-/-}$ mice.

Another major question surrounding the effect of the gut microbiota on K/BxN arthritis is that of cell migration. Arthritis is not a disease of the intestines; phenomena local to the gut have a profound effect on distal sites. There is an open question of whether autoreactive T cells must travel to the gut or gut associated lymphoid tissues before becoming responsive against self-antigens. Alternatively, antigen presenting cells might become activated in the gut and then migrate to lymph nodes where they encounter and activate naïve T cells. It is also possible that microbial products can reach lymphoid tissues outside the gut and stimulate the activation of

autoreactive T cells without any immune cell migration. There is evidence that autoreactive cells migrate to and from the gut. T cells in the spleen of K/BxN mice express the integrin α4β7 (Wu et al., 2010). α4β7 promotes the migration of cells into the gut through interactions with MaDCAM-1 on endothelial cells. Th17 cells in the gut of K/BxN mice have also been shown through *in situ* imaging to travel from the gut to the spleen (Morton et al., 2014). Both of these findings suggest a role of T cell migration to the gut, but no experiments have tested the necessity of such trafficking. We attempted to answer this question by breeding KRN/B6 mice to mice deficient in the β7 chain. Resulting T cells would lack the integrin required for gut homing, which we would transfer into host mice. These mice were never successfully generated from this cross, and the genotypes of the progeny segregated as if the two gene loci were tightly linked, despite the genes being on separate chromosomes (KRN Tg is on chromosome 2, *Itgb7* on chromosome 15). Instead of a genetic knock out of the integrin, this experiment could also be conducted with administration of an anti-α4β7 antibody. If integrin blockade prevents disease, it implies cell migration is required for disease. Not only T cells express the integrin, and therefore the results might be somewhat confounded. Incubating T cells with anti- α4β7 antibody before transfer might prevent T cell migration to the gut and allow for testing of this hypothesis. CCR9 is another gut homing receptor expressed on T cells (Kunkel et al., 2000). KRN/B6 mice could be crossed to CCR9 deficient mice to test the requirement of CCR9-mediated gut homing by T cells for disease.

Microbiota and RA

There is a growing body of evidence that rheumatoid arthritis and other inflammatory autoimmune diseases originate at mucosal sites (reviewed in (Demoruelle et al., 2014)). A

number of studies have found altered microbial composition in NORA patients, suggesting the presence or absence of certain members, or general dysbiosis, may contribute to early stages of disease (Eerola et al., 1994; Vaahtovuo et al., 2008; Liu et al., 2013). 16S sequencing of stool from NORA patients revealed that presence of *Prevotella copri* strongly correlated with disease. The *P. copri* present in the few healthy controls were found to be different strains with different metabolic pathways represented compared to NORA patients (Scher et al., 2013; Bernard, 2014). Patients that were being treated for RA (not new-onset) did not demonstrate the same overabundance of this community member, highlighting the importance of conducting studies on recently diagnosed patients or individuals that present with pre-clinical signs of RA. Since mice can be colonized with *P. copri* (and it exacerbates intestinal inflammation in the DSS colitis model (Scher et al., 2013)), it would be informative to test whether *P. copri* could drive K/BxN arthritis, by either colonizing germ free or antibiotic treated animals. Investigating the changes in these mice, whether by intestinal epithelial cell gene expression, profiling of antigen presenting cells, or phenotyping helper T cells, could help elucidate how this bacterial species might precipitate autoimmune disease. Similar gnotobiotic experiments could be conducted with *Lactobacillus salivarius*, which was over-represented in fecal, salivary, and oral microbiomes of NORA patients (Zhang et al., 2015). The whole fecal microbiota of RA patients could be introduced to germ free mice, and overall immune responses could be compared to mice colonized with feces from healthy controls. This approach could reveal how overall dysbiosis in the microbiota of RA patients could affect immune function. How dysbiosis of microbial communities, or overabundance of certain community members, could promote autoimmune development is not understood, except perhaps in the case of *Porphyromonas gingivalis* inducing

protein citrullination (Mikuls et al., 2014). The utilization of gnotobiotic methods in models of RA may give insight into potential pathways, which can then be investigated in human patients.

Conclusion

Autoimmune disease is caused by a complex interaction of genetic and environmental factors that are incompletely understood. The work in this thesis contributes to our understanding of the etiology of autoimmunity by examining helper T cell functions and the effect of the microbiota on autoimmune arthritis. Follicular helper T cells and IL-21 are both critically required for germinal center formation and autoantibody production. Our work suggests that the microbiota promote this autoreactive Tfh cell differentiation. A key area of future investigation will be to elucidate the pathway by which microbial signals may induce Tfh cells against self-antigens. K/BxN arthritis is widely cited as a Th17-mediated disease, however we have demonstrated the dispensability of Th17 cells and IL-17 for arthritis in this model. These findings contribute to the mounting evidence that Th17 cells may promote inflammation in autoimmune disorders but targeting the Th17 axis will have minimally efficacious outcomes (Ruderman, 2015). Understanding how bacterial dysbiosis in genetically susceptible individuals promotes autoreactive T cell responses will hopefully lead to better interventions for autoimmune disease.

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