THE ROLE OF IL-21 AND IL-17 IN REGULATING FOLLICULAR T HELPER CELLS IN GERMINAL CENTER RESPONSE OF AUTOIMMUNITY

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A DISSERTATION

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Overreactivity of the germinal center (GC) is a central feature of autoantibody-mediated autoimmune diseases. Follicular T helper (Tfh) cells are the primary T helper subset that migrates to the incipient GC and forms close contacts with GC-B cells to promote GC formation and help GC-B cell differentiation, resulting in high-affinity antibody production. Increased levels of interleukin (IL)-21 and aberrant accumulation or function of Tfh cells have been associated with autoimmune disease severity in humans and lupus-prone mice. Follicular regulatory T (Tfr) cells are the regulatory T cell subset that also localizes in the GC and inhibits GC B cell differentiation. IL-21 plays a fundamental role for Tfh cell development but negatively regulates conventional regulatory T (Treg) cells. In addition, the pro-inflammatory cytokine IL-17 also has been shown to promote GC development and autoantibody production.

In the current study, we demonstrated increased levels of serum IL-21 and accumulation of Tfh cells in autoimmune BXD2 mice, which have a high level of IL-17 in sera and spontaneously develop GC containing autoantibody-producing cells. Deficiency of IL-21 or IL-17 resulted in impaired GC formation in BXD2 mice. Tfh cells in BXD2 mice consisted of distinct IL-21- and IL-17-producing subpopulations. IL-21 primarily promoted Tfh cell development but inhibited Tfr cell commitment, thereby leading to an imbalance of Tfr/Tfh in BXD2 mice. IL-21 also converted Tfr cells into
non-Tfr cells and counteracted Tfr-mediated inhibition on Tfh cells and B cells. Therefore, Tfr cells in BXD2 mice were defective in frequency and suppressive function that may regulate GC formation in the mice.

In addition, IL-21 promoted IL-17 production and IL-17R expression by Tfh cells, which prepared Tfh cells for the IL-17 regulation. IL-17 then sequentially up-regulated regulator of G-protein signaling (RGS)-16 and co-stimulatory molecules to exert a unique function by stabilizing Tfh cells in GC light zone (LZ) to form close contact with GC B cells.

The study suggests a novel two-checkpoint model for regulating Tfh cells in murine autoimmunity, namely the number of differentiated Tfh cells and their physical stabilization in GC LZ. IL-21 and IL-17 acted at each checkpoint to enable pathogenic GC development in BXD2 mice.

Keywords: IL-21, IL-17, Follicular T helper (Tfh) cell, Follicular regulatory T (Tfr) cell, Germinal center, Autoimmunity
DEDICATIONS

Dedicated to my parents who brought me up, my sister who supports me forever, my husband, Ning Zheng, for being a joyful companion and for his unreserved support throughout the tough times and good times
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INTRODUCTION

Autoimmune diseases are characterized by self-tissue damage mediated by autoantibodies and/or effector T cells and can be classified as either systemic diseases or organ-specific diseases (1). Genetic and environmental factors can trigger or accelerate the breakdown of self-tolerance, which initiates the activation of multiple immune components and promotes the onset of the diseases (1, 2). Although autoimmune diseases are traditionally categorized as primarily autoantibody-mediated diseases or T cell-mediated diseases, increasing evidence indicates that autoantibody-mediated immune reactions are involved in the pathogenesis of most autoimmune diseases, not only in the autoantibody-mediated diseases, such as systemic lupus erythematosus (SLE), but also in the diseases previously considered to be strictly T cell-mediated, such as Type 1 Diabetes (3).

SLE is a systemic, remitting, relapsing autoimmune disorder that can involve many organs and tissues in the body but principally affects the skin, kidneys, joints, heart and serosal membranes (4, 5). The prevalence of SLE may be as high as 1 in 2000 in certain populations and there is a strong female preponderance of approximately 9:1 (female : male), especially among child-bearing females (6). Recent data on the survival rate for SLE patients is approximately 95% for 5 years, 91% for 10 years, 85% for 15 years and 78% for 20 years in Europe and North America (7). To date, unfortunately, the therapeutic options for SLE patients are still limited. SLE is highly heterogeneous disease
and the affected individuals may present variable and diverse clinical manifestations (8). Serologically, the most important characteristic of SLE is the presence of elevated levels of serum antibodies to nuclear constituents (antinuclear antibody, ANA), including anti-double-stranded DNA antibodies and anti-histone antibodies (9). Similar to other autoantibody-mediated autoimmune diseases, SLE can be considered a germinal center (GC)-derived disease, considering that the autoantibodies are produced by autoreactive B cells arising in the GC. Autoantibody-producing B cells are well-known key players in the pathogenesis of SLE, which has been further confirmed by the clinical benefit of B cell depleting therapies in SLE patients (10). In addition to B cells, aberrant T cell homeostasis, such as an imbalance of Th1 and Th2 cells, plays a pivotal role in the development of the disease (11-13). Dys-regulation of regulatory T (Treg) cells and pro-inflammatory effector T cells, such as Th17 cells, are also associated with the disease activity in SLE patients and lupus-prone mouse models (14-17). Furthermore, cytokine milieu imbalances, which could be the cause or consequence of the T cells imbalances, are critical to drive the immune response towards disease according to the context of challenges (11, 16).

Germinal Center (GC)

Germinal centers are the sites where intense B cell proliferation and differentiation occur, and where high affinity antibody producing plasma cells and memory B cells are generated. When naive B cells have bound antigen in the primary lymphoid follicles, they can migrate to the border of the resting B cell and T cell areas,
where they may encounter and interact with the activated cognate T helper cells and undergo proliferation. Some of these activated B cells may move back to the follicles together with their associated T cells, and continue to proliferate to form the GCs (18). Therefore, the GCs are similar to islands surrounded by a sea of resting B cells and T cells.

Structurally, GCs primarily consist of proliferating B cells, activated T cells, as well as follicular dendritic cells (FDC) and stromal cells. Highly proliferative B cells comprise the dense dark zone (DZ) of a GC (Figure 1). These B cells are named centroblasts and primarily express CXCR4. Expression of CXCR4 enables centroblasts to be recruited to the DZ by CXCL12 generated locally by stromal cells (19, 20). Centroblasts can down-regulate CXCR4 but up-regulate CXCR5 and become centrocytes with a lower rate of division. Centrocytes move to the light zone (LZ) of the GC where they co-localize with activated T cells and FDCs. FDCs in the LZ primarily produce CXCL13 and aggregate centrocytes and T cells that express CXCR5 (19, 21). Centrocytes may potentially move back to the DZ by re-expressing CXCR4 (22). In addition to B cells as the main component of GC, T cells constitute approximately 5 -20% of the GC cells (23, 24). The Ag-specific T cells must be recruited and remain in the GC (25), where they play a pivotal role in helping B cell development. T cell subsets that reside within the GC include follicular T helper (Tfh) cells, NK Tfh cells, follicular regulatory T (Tfr) cells, CD8+ Treg cells (26), and potentially others.

Under the regulation of T cells, GC founder B cells undergo a series of events in the germinal center (27-30), including proliferation, differentiation, somatic hypermutation (SHM), affinity maturation and selection, class switch recombination (CSR), as
well as necessary apoptosis. The B cells that are positively selected may ultimately develop into antibody producing plasma cells or memory B cells. Abnormalities in the cellular components and the checkpoints during the process of GC formation can cause breakdown of self-tolerance or loss of immune homeostasis, which may further lead to over-reactivity of the GC response and the development of autoantibody-mediated autoimmune diseases.

There are some variations of the structure of GC in different secondary lymphoid organs, such as lymph node (LN) and spleen, but the basic cell components and compartments are similar. The common structure and cellular composition of a GC in a mouse spleen follicle is illustrated in Figure 1.

![Figure 1. Schematic Diagram of a Follicle with a Germinal Center in Mouse Spleen](image-url)
Follicular T Helper (Tfh) Cells

Follicular T helper (Tfh) cells are a recently defined T helper subset that is independent of the other T helper subsets (31-34) (Figure 2). This T helper subset primarily provides help for B cells and its main function is to regulate T-dependent antibody responses. As the name implies, the most remarkable difference between Tfh cells and other T helper cells is the primary localization of Tfh cells in germinal centers of the follicle, where they play a critical role in regulating GC B cell development and antibody production (31).

![Figure 2. Major Th Subsets](image)

The cytokines and transcription factors involved in development of the subsets, as well as the cytokines produced and the major functions of the subsets are depicted.
Characteristics of Tfh Cells

Conventional Tfh cells are considered to be CXCR5+ and/or ICOS+ and/or PD-1+ and Bcl6+ CD4 T helper effectors that are located in the GC. Tfh cells express co-stimulatory molecules and predominantly secrete interleukin (IL) 21 (IL-21) to help GC B cell maturation to produce high affinity antibodies and long-lived plasma cells. However, the definition of Tfh cells, to date, is still elusive. First, the key surface marker of Tfh cells is CXCR5, however, CXCR5 is also up-regulated to intermediate levels upon T cell priming in vivo, regardless of whether the immune response is mediated by Th1, Th2, Th17 or Tfh cells (35, 36). High expression of ICOS is a distinguishing feature of human Tfh cells, however, in mice, Tfh cells and activated non-Tfh cells can express comparable levels of ICOS (37, 38), whereas expression of PD-1 on GC Tfh cells has been consistently found in both human and mice (30, 39, 40). Second, Bcl6 is considered to be the master transcription factor for Tfh cells to direct Tfh development (37, 41, 42); yet the expression of Bcl6 is not exclusively found in Tfh cells because other CD4 T cell development programs also require expression of Bcl6 (43). For example, Bcl6 is expressed during development of Treg cells and it plays a role in Treg-mediated control of Th2 driven inflammation and in Tfr-mediated suppression of GC responses (44, 45). Nevertheless, CXCR5hi PD-1hi Tfh cells express the highest amount of Bcl-6, compared with other Bcl6+ T cell subsets (46, 47). Furthermore, CD4 T cells located in the B cell follicles are highly heterogeneous (48, 49). Not all CD4 T cells that reside in the follicular area are specialized in providing help to B cells. For example, the CD25+Foxp3+ cells share some phenotypic features with Tfh cells, including high expressions of CXCR5, ICOS and PD-1 (50, 51). But instead of executing helper
function, these cells appear to be specialized in the suppression of T cells and of Ig production by B cells, at least in vitro (50, 52). Moreover, IL-21 is the well-known signature cytokine for Tfh cells (37, 42). Tfh cells have been shown to produce high levels of IL-21 RNA and protein, with variable levels of other cytokines, such as IL-4 (34, 46, 53, 54). However, IL-21 can also be produced by NKT cells. Among CD4 T cells, IL-21 can be detected together with IFN-γ, IL-4 or IL-17 in the same cells (55), however, other studies have suggested that terminally differentiated Tfh cells produce large amount of IL-21 but not IFN-γ or IL-4. Therefore, Tfh cells are best characterized as \( \text{CXCR5}^{hi} \text{ICOS}^{hi} \text{PD-1}^{hi} \text{Bcl6}^{hi} \text{IL-21}^{hi} \) (Figure 3).

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**Figure 3. The Characteristics of Tfh Cells**

- **High**: Bcl6.
- **Low**: T-bet, Gata-3, RORγt, Foxp3.
- **High**: CXCR5, ICOS, PD-1.
- **High**: IL-21 and IL-17?, IL-4?
- **Located** in GC light zone.

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*Migration of Pre-Tfh Cells to the GC*

Tfh cell development originates in the CD4 T cell area. After antigen primed T cells encounter antigen bound B cells in the T-B cell border area, the selected pre-Tfh...
cells begin to down-regulate the expression of CCR7, the chemotactic receptor for CCL19/12, which are enriched in the CD4 T cell area (56), and up-regulate the expression of CXCR5. At the very early stage, the commitment of primed-naïve T cells to pre-Tfh cell development is suggested to be determined by cytokines, particularly IL-6 and IL-12 (57). The effect of IL-12 is associated with the low level expression of Bcl6 and CXCR5, which initiate the differentiation of Tfh cells. IL-21 then induces the high level expression of Bcl6, which is important for the sustained expression of CXCR5 and the full development of Tfh cells (37, 41, 58). Sustained expression of CXCR5 facilitates migration of these Tfh cells into the GC via recruitment by CXCL13 which is abundant in GC LZ (39). After migration to the GC, Tfh cells form close contact with GC B cells and acquire terminal differentiation. During this process, IL-21 continues to promote the proliferation and survival of Tfh cells in an autocrine fashion (34, 53). Mature Tfh cells then proceed to secret functional cytokines, primarily IL-21, as well as present co-stimulatory signals to help GC B cells. (Figure 4)
Figure 4. Migration of Pre-Tfh Cells into GC (Top) and the Location of Mature Tfh Cells in LZ (Bottom)

(Top) CXCR5+ Pre-Tfh cells migrate into GC mediated by CXCL13 and differentiate into mature Tfh cells that secrete functional cytokine(s) and form interaction with GC B cells; (Bottom) mature Tfh cells (yellow color) mainly localize in GC LZ to exert their B cells help function.
Regulation of Tfh cells

Transcription factors are essential to direct the differentiation and lineage commitment of specific T helper cell subsets. For example, T-bet is specific for Th1, GATA3 is critical for Th2, retinoid orphan receptor γt (RORγt) is particular for Th17 while forkhead box protein 3 (FoxP3) is indispensable for Treg lineage commitment (59). Similarly, Bcl6 is unique for Tfh programming (37, 41, 42), whereas Blimp-1 is the counteracting factor for Bcl6 and it negatively regulates the development of Tfh cells (41).

Cytokines also play a critical role in regulating the development of Tfh cells, although their influence is still not fully understood. IL-21 together with IL-6 have been shown to be fundamental in Tfh development (34, 53, 60, 61) and have been implicated in the generation of autoimmune diseases. After being activated by dendritic cells (DC) in the CD4 T cell area, and under the influence of IL-6, primed CD4 T cells develop along the Tfh pathway. They subsequently begin to express low level of Bcl6 which up-regulates CXCR5 expression and down-regulates the expression of the chemokine receptor CCR7. This enables the cells to migrate to the follicle, mediated by the chemokine CXCL13. During this process, IL-21 significantly enhances Bcl6 expression (34, 37, 53, 57) and promotes Tfh survival (34, 53). In tandem, IL-21 promotes GC B cell survival and differentiation as well in the later stage of GC formation (62-64). Recently, IL-27 has been found to induce IL-21 production to regulate Tfh development (65). However, some studies in IL-6 and IL-21 deficient mice indicate that both IL-6 and IL-21 are redundant for the development of Tfh cells (66). Therefore, regulation of the
development of Tfh cells continues to be an important and challenging area of investigation.

Although the signals that direct the development of Tfh cells are still not completely clear, some of the signals may result from the interactions between T cells and antigen presenting cells (APC) including B cells and DCs (34, 67-70). The strength of T cell antigen receptor binding has been shown to affect the function of Tfh cells in vivo (71). In addition, ICOS-mediated co-stimulatory signal augments IL-21 production and promotes accumulation of Tfh cells in sanroque mice which exhibit features of human SLE (72, 73). Micro-RNA has also been suggested to be a fine-tuning mechanism to suppress CXCR5 expression and thereby regulate the formation of Tfh cells (37).

Recently, it has been shown that other counterparts of T cells located in the GC can also determine the fate of Tfh cells. In mice, Qa-1+ CD8+ T cells can negatively regulate Tfh cells in vivo, whereas in humans, CD69+CD25+CD4 T cells capable of migrating into the GC are found to suppress the GC B cell responses (50, 52, 74). A new subset of follicular regulatory T cells (Tfr) has been shown to localize in the GC and functionally regulate the Tfh population and GC B cell differentiation in vivo (44, 45). However, in certain microenvironments like Peyer’s patches, the Foxp3+ Treg cells can be converted into Tfh-like cells to promote the GC response (75). These results indicate the plasticity of the Treg subset and the complex relationship of this subset with Tfh cells.
**Tfh Cells in B Cell Differentiation and GC Development**

Tfh cells promote GC formation by regulating B cell survival, activation, differentiation, affinity maturation, SHM, class switch recombination (CSR) and their ultimate maturation into antibody producing plasma cells or memory B cells. In the nascent germinal center, Tfh cells primarily provide help to GC founder B cells by forming conjugates with B cells via interactions of surface co-stimulatory molecules such as ICOS, CD40L, OX40, SLAM associated protein (SAP) family and CD28 on Tfh cells with the corresponding molecules on B cells.

The physical conjugate formation between Tfh cells and GC B cells induces mutual regulation for both cell types (34, 67-70). Insufficient contact between Tfh cells and GC B cells leads to impaired GC development (69), which suggests that formation of close contact between Tfh cells and B cells is critical for GC development. The important role of Tfh cells to provide selection and survival signals to GC B cells and GC has been demonstrated by administration of anti-CD40 L neutralization antibodies (76). The survival of Tfh cells, in return, requires the CD80 signal provided by B cells (77). Furthermore, the SLAM family receptor CD84 on Tfh cells is required for SAP-dependent adhesion between Tfh cells and B cells. Such signals are used to promote optimal GC responses and long-term humoral immunity (78, 79).

Cytokines produced by Tfh cells are another important mechanism to regulate GC B cells and GC development. Most importantly, IL-21 is the signature cytokine of Tfh cells and has been shown to act on GC B cells to promote their survival (34, 53, 62), whereas IL-4 secreted from Tfh cells plays a critical role in SHM and immunoglobulin
CSR in B cells (54). In addition, IFNγ produced by Tfh cells has been shown to regulate autoantibody response (80). IL-17 produced by Tfh cells or other effector T cells is also associated with GC reactions in various autoimmune conditions (81, 82).

A Schematic Diagram for the B cell help function of Tfh cells is shown in Figure 5.

![Diagram showing the role of Tfh cells in GC B cells]

Figure 5. Tfh Cells Help GC B Cells

Tfh in Autoimmune Diseases
Aberrant accumulation of Tfh cells has been correlated with disease severity in SLE, Sjogren’s syndrome and autoimmune arthritis (83-86). Treatment of SLE patients with corticosteroids decreases circulating Tfh cells (87). Increased number and frequency of Tfh cells are also found in several lupus-prone mouse models, such as BXSB-Yaa (88), MRL-Fas$^{lpr/lpr}$ (89) and NZB/W (90). The sanroque mice exhibit excessive Tfh cells with high expression of ICOS as well as elevated levels of IL-21 (72, 73). In mouse models of lupus, the elimination or defective function of GC Tfh cells reduces or even abolishes the disease (85, 91).

Tfh cells contribute to autoantibody-driven autoimmune diseases in a manner analogous to a normal T-dependent immune response (92) in that Tfh cells provide cognate help to B cells for high-affinity antibody production in the GC. During this course of events, antigen stimulation from follicular dendritic cells (FDCs) or macrophages is important, however, not indispensable for B cell activation in a T-dependent immune response. By contrast, accumulation of Tfh cells that can produce functional cytokines and are capable of forming close interaction with B cells is essential for GC formation. The interaction between Tfh cells and B cells can ultimately lead to maturation of GC B cells and generation of autoantibodies that play an important pathogenic effector role for autoimmune diseases.

Follicular Regulatory T (Tfr) cells

_Treg and Tfr cells_
Conventional Treg cells are a subset of FoxP3+ CD4 T cells that can limit the activity of effector T cells and maintain immune homeostasis. Generally, the Treg subset is composed of natural Treg (nTreg) cells that originate from the thymus, and inducible Treg (iTreg) cells that are generated in the periphery, induced by antigen and specific cytokines (51, 93). CD25+CD4+ Treg cells have been found to regulate maturation of autoantibody responses (94). More recent studies, however, have shown that these CD25+CD4+ Treg cells can migrate into the GC to repress Tfh cells and suppress the GC response (50, 52, 95). A new subset of regulatory T cells, the follicular regulatory T (Tfr) cell, has been shown to localize in the GC and regulate Tfh population and GC B cell differentiation in vivo (44, 45, 96). These new findings suggest the potential to develop novel regulatory cell therapy strategies to suppress the reactivity of immune responses in autoimmune diseases. The contrasting effects of Tfr and Tfh cells are illustrated in Figure 6.

Figure 6. Counteraction of Tfh cells and Tfr Cells on GC B Cells
Characteristics of Tfr cells

It is still unclear if the previously observed CD25+CD4+ Treg cells in GC are the same as the newly defined FoxP3+ Tfr cells. The latter subset, Tfr cells, has been shown to share many phenotypic features of both Tfh cells and Treg cells in that they are CXCR5+PD-1+FoxP3+ (44, 45). Tfr cells also express markers that are commonly found on Treg cells including CD25, GITR and CTLA-4. However, unlike Tfh cells, Tfr cells do not express CD40L. Similar to the development of Tfh cells, the differentiation of Tfr cells also depends on Bcl6, SAP, CD28 and B cells (44). Bcl6 is critical for the expression of CXCR5 by Tfr cells (45) and is expected to be expressed in Tfr cells. Interestingly, Blimp-1, an antagonist of Bcl6, is highly expressed by Tfr cells as well (44).

Role of Tfr cells in the GC reaction

Tfr cells have been shown to suppress the number of Tfh cells and GC B cells and Tfr cells deficiency results in greater GC responses and antibody maturation (44, 45) as well as outgrowth of GC B cells that are non-antigen specific (44). These results suggest the possibility of specific suppression by Tfr cells of autoreactive B cell responses. It is, however, unclear if Tfr cells act directly on B cells and/or Tfh cells to suppress the GC response.
The unconfirmed features of Tfr cells

The origin and plasticity of Tfr cells are still unknown. Previous studies suggest that Tfr cells can be generated from natural Foxp3+ precursors, but not from naïve or Tfh cells (44, 45). However, blockade of TGF-β or iTreg cells in vivo can lead to excessive GC responses in mice (97, 98), suggesting that Tfr cells can also be derived from iTreg cells. A recent report further shows that Treg cells can be converted into Tfh cells in certain inflammatory environments (75). Therefore, it is unknown if Tfr cells are terminally differentiated or are transitional cells that can further develop into non-Tfr cells in the presence of other lineage stimuli in the microenvironment.

Regulation of Treg and Tfr cells

Treg cells have been shown to be regulated by TGF-β and IL-2. Initially, TGF-β was reported to induce differentiation of CD4+CD25− T cells into CD4+CD25+ T cells in vitro (99). Later studies demonstrate that TGF-β can induce FoxP3 expression by iTregs (100, 101). TGF-β alone can direct the programming of Treg cells, whereas in the presence of other cytokines, such as IL-6, IL-21 or IL-23, TGF-β predominantly induces the development of Th17 cells (102, 103). IL-2 and TGF-β are important in Treg development and are especially essential for the programming of iTreg cells (104), however, both cytokines are redundant for the development of nTreg cells, as demonstrated in IL-2 and TGF-β deficient mice (105). Interestingly, nTreg cells are
thought to be unstable in phenotype and are easily affected by pro-inflammatory cytokines. For example, IL-6 and other inflammatory cytokines have been shown to convert nTreg cells into Th17 cells (106, 107). In addition, IL-21 negatively regulates the number of conventional Treg cells *in vitro*, as demonstrated by studies of cells from IL-21 deficient mice (108).

However, for Tfr cells, very little is known about how this subset is regulated. It has been reported that PD-1 and PD-L1 interaction can negatively regulate the survival of Tfr cells and compromise their suppressive function in a T-B co-culture system, thereby inhibiting the number and function of Tfr subset in the lymph nodes and blood (109). There has been no further study showing the influences of the cytokine milieu on Tfr lineage commitment, differentiation and stability. However, given the GC-specific location of Tfr cells, it is highly possible that GC-associated cytokines including IL-21, IL-6 and TGF-β may play an important role to regulate the lineage development of Tfr cells.

*Tfr cells in Autoimmune Diseases*

Deficiency in the number and function of Treg cells in autoimmune conditions has been demonstrated *in vivo* and *in vitro* (16, 110-112). Imbalance between Treg and pro-inflammatory effector T cells, such as Th17, is associated with disease progression in lupus prone mice and SLE patients (16). Similarly, imbalance between Tfr and Tfh cells may be critical in driving the development of autoimmune diseases. As aforementioned, Tfr cells play an important role in regulating Tfh cell and GC B cell differentiation and
may counteract with functions of Tfh cells in GC development. Therefore, the ratio of Tfr cells to Tfh cells could be used as a meaningful biomarker for diagnosis and prognosis of GC-derived autoimmune diseases.

The mechanism that regulates the balance between Tfh cells and Tfr cells in the pathogenesis of autoimmunity has not been explored. Understanding how the Tfr subpopulation is regulated and what controls the balance between Tfh cells and Tfr cells is critical for elucidating the pathogenesis and progression in GC-derived autoimmunity. Such findings may ultimately help develop effective therapeutic strategy for the diseases.

Interleukin-21 (IL-21)

*IL-21- IL-21R and the Signaling Pathway*

IL-21 is a member of one subfamily in the set of type I four-α-helical-bundle cytokines and is closely related to IL-2, IL-4 and IL-15 (113). IL-21 is primarily produced by Tfh cells in human tonsil and mouse spleens or lymph nodes (34, 37, 53), although Th17, Th2 and NKT cells are also known to produce IL-21 (55). The IL-21 receptor shares a common gamma chain, called γc, with the other receptor complexes of this subfamily and each cytokine receptor in this subfamily has its own private components. For IL-21, the functional receptor is γc plus the private component IL-21R, which also exhibits high homology to IL-2Rβ and IL-4R (114). IL-21R is broadly expressed by various types of lymphohematopoietic cells. The cytokine receptors
containing the common γ chain and the corresponding cytokines including IL-21 are depicted in Figure 7.

![Diagram of cytokine receptors containing γc and the cytokine subfamily]

Figure 7. Cytokine Receptors Containing γc and the Cytokine Subfamily

Jak/STAT3 is the major signaling pathway of IL-21R activation upon binding with IL-21. Generally, IL-21 firstly activates the Janus kinases Jak1 and Jak3, which in turn, activate STAT1, STAT3 or STAT5, leading to the expressions of target genes and the consequent downstream effects. STAT3 normally acts as a growth factor or an anti-apoptotic factor (115, 116). By contrast, STAT1 may mediate cell apoptosis (117, 118).
Activation of Jak/STAT signaling by IL-21, especially strong activation of STAT3, results in survival, growth or differentiation of B cells (119). Furthermore, PI3K/Akt and RAS/MAPK pathways may also contribute to IL-21 mediated biologic effects. MAPK/ERK1/2 phosphorylation induced by IL-21 has been implicated in the growth and survival of a human myeloma cell line (120). IL-21-mediated phosphorylation of Shc and Akt are associated with the proliferation of murine T cells whereas all of the Jak/STAT, RAS/MAPK and PI3K/Akt pathways are involved in proliferation of other cells promoted by IL-21 (121). The potential singling pathways involved in IL-21-IL-21R-mediated effects are illustrated in Figure 8.

Figure 8. Signaling Pathways for the IL-21-IL-21R

IL-21 has diverse effects on the entire immune system, including survival, proliferation and differentiation of B cells, proliferation and development of Th cells, survival and function of CD8 T cells, NK cells, NKT cells and DC (55, 113, 122). (Figure 9)

Figure 9. Diverse Effects of IL-21 on Cells of the Immune System

IL-21 in Development of GC B, Th17, TfH and Treg Cells
IL-21 has been shown to act directly on B cells to promote maximal expression of Bcl6 and support the survival and differentiation of B cells (62, 63), as well as the formation of GCs (64). However, the effect of IL-21 on the fate of B cells depends on other context stimulating signals. IL-21 inhibits proliferation and may trigger apoptosis of B cells that receive strong signals from toll-like receptors (TLR) or receive stimulation from BCR binding only, without Th cell help (123, 124), whereas when co-existing with anti-CD40 signaling, IL-21 promotes survival and proliferation of B cells. With appropriate co-stimulation, IL-21 also regulates CSR by inducing the expression of activation-induced deaminase (AID) in B cells (125, 126). Defects in IL-21-IL-21R signaling impair CSR in B cells (55, 127). IL-21 has been shown by multiple studies to promote CSR from IgM to IgG1, IgG3 and IgA in human B cells (128, 129). Together with IL-4, IL-21 can also promote class switch to IgE and IgG4 (130).

One unique aspect of IL-21 is its positive effect on Th17 development. It has been shown that IL-21 induced by IL-6 through the STAT3-mediated pathway can further form an autocrine loop to promote the differentiation of Th17 cells, whereas a deficiency of IL-21-IL21R leads to a defect in Th17 generation in mice (102, 108, 131). IL-21 also up-regulates IL-23R expression on CD4 T cells (102, 108) to promote maintenance and expansion of Th17 cells (132). In addition, both IL-21 and IL-23 can induce the expression of RORγt, which is the essential transcription factor for Th17 cells (133). Taken together, IL-21 is important for initiating Th17 development and lineage maintenance.

The association of IL-21 with Tfh cells has been extensively explored. Although there are controversial observations on the essential role of IL-21 in Tfh development (63,
IL-21 is generally considered as the signature cytokine of Tfh cells (37, 88, 135). IL-21 acts in an autocrine fashion to stimulate the proliferation and survival of Tfh cells, and hence has a direct effect on Tfh cell accumulation and the Tfh-mediated GC formation (34, 53). By contrast, a lack of IL-21 or IL-21R in mice results in reduced Tfh cells. IL-21 directly induces high expression of Bcl6, the master transcription factor for Tfh cells, which is important for the expressions of Tfh cell surface markers including CXCR5 and other co-stimulatory molecules for supporting cognate help to B cells (37, 41, 58). Taken together, in most of experimental conditions, IL-21 is critical in the differentiation of Tfh cells, and its redundant effect on Tfh cells may be related to influences of other stimulatory factors.

In addition to its effects on Tfh cells and Th17 cells, IL-21 can negatively regulate conventional Treg cells. It has been shown that there is an approximately 3-4 fold increase in the number of Treg cells in IL-21 deficient mice (108).

**IL-21 in Autoimmune Diseases**

Increased levels of IL-21 have been found in sera of SLE patients (136) and lupus-prone mice. IL-21 is extremely high in serum of BSXB-Yaa lupus prone mice, which leads to a dramatic increase of Tfh cells and high titers of IgM and IgG (124). The sanroque mice that exhibit overexpression of ICOS and excessive production of IL-21 also develop severe SLE-like disease (72). In MRL\(^{lpr}\) mice, IL-21 contributes to the increased generation of pathogenic B cells, T cells and autoantibodies (89, 137). Blockade or deficiency of IL-21R in these mice abrogates the SLE-like manifestations of
the disease (88, 89, 137). This suggests that the IL-21-IL-21R axis is critical in the pathogenesis of autoimmunity in lupus-prone mice. In human rheumatoid arthritis (RA) patients, IL-21R but not IL-21 expression can be detected on synovial macrophages and fibroblasts (138). In mouse models of collagen-induced arthritis and adjuvant-induced arthritis (139), blockade of IL-21 using an IL-21R-Fc fusion protein reverses the disease activity. These studies indicate that, in addition to SLE, IL-21 may be directly or indirectly involved in the pathogenesis of RA.

Interleukin-17 (IL-17)/Th17

**IL-17-IL-17R and the Signaling Pathway**

The IL-17 cytokine family includes IL-17A (IL-17), IL-17B, IL-17C, IL-17D, IL-17E and IL-17F (140, 141). With exception of IL-17E (also known as IL-25), which is produced by Th2 cells (142), all other IL-17 cytokine proteins are mainly produced by Th17 cells. However, IL-17A and IL-17F can also be produced by other cell types including NKT cells, NK cells and neutrophils (143-145). It has been demonstrated that IL-17A and IL-17F are largely co-expressed by CD4 T cells (146, 147) and both are pro-inflammatory cytokines (148). The origins of IL-17 cytokine family proteins and their binding with IL-17 receptor (IL-17R) family proteins are summarized in Table 1.

IL-17 receptors form a large cytokine receptor family that includes IL-17RA, IL-17RB, IL-17RC, IL-17RD and IL-17RE (149). The ligands for IL-17RD and IL-17RE are unknown. IL-17RB is the receptor for IL-17E (IL-25) and IL-17B (150, 151). 17RA
and IL-17RC are the receptors for IL-17A and IL-17C, respectively (152-154). IL-17RA is the cognate receptor for IL-17A and IL-17F but with a higher affinity for IL-17A (149). IL-17RC is the cognate receptor for IL-17F (153) and can also bind to IL-17A in human but not in mouse. IL-17RA can form heterodimer with IL-17RC to bind to both IL-17A and IL-17F (154). IL-17RA is highly expressed on hematopoietic cells whereas IL-17RC is highly expressed on non-hematopoietic cells.

Currently, both MAPK and NF-κB appear to be involved in the IL-17-IL-17R activation signaling pathway (155-157).

Table 1. Binding of IL-17 Cytokine Family with the IL-17 Receptor Family Proteins

<table>
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<tr>
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<th>Origin</th>
<th>Binding affinity</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>IL-17RA</td>
<td>IL-17RB</td>
<td>IL-17RC</td>
<td>IL-17RC</td>
<td>IL-17RD</td>
<td>IL-17RE</td>
</tr>
<tr>
<td>IL-17A</td>
<td>Th17, γδ T, NKT, NK, Neutrophils Eosinophils</td>
<td>high</td>
<td>-</td>
<td>+(human)</td>
<td>-(mouse)</td>
<td>+(human)</td>
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<tr>
<td>(IL-17)</td>
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<tr>
<td>IL-17B</td>
<td>Th17</td>
<td>-</td>
<td>low</td>
<td>-</td>
<td>-</td>
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<td>unknown</td>
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<tr>
<td>IL-17C</td>
<td>Th17</td>
<td>-</td>
<td>-</td>
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<tr>
<td>IL-17D</td>
<td>Th17</td>
<td>-</td>
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<tr>
<td>IL-17E</td>
<td>Th2</td>
<td>+</td>
<td>high</td>
<td>-</td>
<td>-</td>
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<tr>
<td>(IL-25)</td>
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<tr>
<td>IL-17F</td>
<td>Th17, γδ T, NKT, NK, Neutrophils Eosinophils</td>
<td>+</td>
<td>-</td>
<td>high</td>
<td>+(human)</td>
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Th17

Th17 cells are named due to their ability to produce a large amount of IL-17A. The critical transcription factor for Th17 is RORγt although STAT3 and RORα may also contribute to the differentiation of Th17 cells. The development of Th17 cells has been shown to be regulated by multiple cytokines including IL-6, IL-21, IL-23, and TGF-β (102, 108, 133). To date, there is no specific surface marker for Th17, however, expressions of CCR2, CCR4 and CCR6 are all associated with Th17 cells in different inflammatory conditions (158, 159). In addition to secretion of IL-17A, Th17 cells also produce other cytokines, such as IL-17F, IL-22, IL-21, GM-CSF and others. (146, 147). Th17 cells can be highly involved in both innate and adaptive immunity, including clearance of bacteria and fungi during host defense reactions, and production of antibodies.

IL-17/Th17 in Autoimmune Diseases and GC Development

High levels of IL-17 have been found in a large range of human autoimmune diseases, such as SLE, inflammatory bowel disease, psoriasis, rheumatoid arthritis and multiple sclerosis (160-165). The IL-23/IL-17 axis plays an important pathogenic role in mouse models of EAE (166-170).

IL-17 exhibits direct effects on B cells. IL-17 alone or in combination with B-cell activating factor (BAFF) can enhance human B cell survival and differentiation into
antibody-producing plasma cells (165). In addition, IL-17 has been demonstrated to have the potential to induce generation of autoantibody following administration of polarized Th17 cells in several mouse models of autoimmune diseases (171, 172).

B cell activation and antibody production are initiated via interactions between the founder B cells and the T-helper (Th) cells, which occur in the GC LZ. Previous investigators have established that GC B cells need a specific period of prolonged interaction with Th cells to acquire effective stimulation, suggesting that both GC B cells and Th cells need to be able to remain in the GC LZ for sufficient interactions (25). Recent studies have shown that IL-17 can play a critical role in the formation of spontaneous GCs by stabilizing the initial interactions of B cells with the T-helper (Th) cells through the canonical NF-κB phospho-p65 pathway to up-regulate regulator of G protein signaling (RGS) 13 and RGS16 in B cells (81, 157). These findings suggest a novel function of IL-17 in regulating B cells migration to facilitate GC formation and B cell maturation in the GCs.

Remaining Questions

In autoimmune diseases, strong interactions mediated by co-stimulatory molecules or pro-inflammatory cytokines are critical in generating autoreactive Tfh and autoreactive B cells. Although SLAM associated protein (SAP) expression on T cells has been shown to be crucial for the late-stage T-B interaction and GC formation (69, 78), little is known about the factors that regulate the localization of Tfh in GCs and that regulate the close conjugate formation between Tfh cells and GC B cells. IL-17 plays an
important role in regulating GC formation and autoimmune diseases development; however, thus far, there has been no investigation of the specific effect of IL-17 on Tfh cells, in terms of development, migration or conjugating ability. The original report that described the cloning of \textit{Il17ra} gene, however, clearly demonstrated that IL-17R is expressed by both T cells and B cells, suggesting that both cell types are subject to IL-17 regulation (152).

RGS and Chemokine-Mediated Lymphocyte Trafficking

\textit{GPCR and RGS}

After binding with their ligands, G-protein-coupled receptors (GPCRs) function through heterotrimeric G proteins by inducing substitution of GDP on G-protein \(\alpha\) (G\(\alpha\)) with GTP, which leads to dissociation of the GTP bound G\(\alpha\) subunit from the \(\beta\gamma\) components of the previous heterotrimeric complex, thereby activating the downstream signaling cascades.

There are two mechanisms for regulating the GTP-bound status of G\(\alpha\). The intrinsic GTPase activity of G\(\alpha\) can hydrolyze the GTP, which promotes the reunion of G\(\alpha\) with \(\beta\gamma\) subunits and deactivates the downstream signaling events (173). The second and a more efficient one is mediated by regulator of G-protein signaling (RGS), which can extensively increase the GTPase activity of G\(\alpha\) and terminate the signaling cascades initiated by GPCRs (174). The GPCR mediated signaling pathway and its regulation are shown in Figure 10.
Figure 10. GPCR Associated Signaling Pathway Regulated by RGS

Chemokine Receptors and Lymphocyte Migration

Trafficking of lymphocytes is important for the normal development of lymphoid organs and tissues as well as normal immune responses. Lymphocyte trafficking is usually mediated by chemokines or chemoattractants upon binding to the corresponding receptor. Chemokine receptors are GPCRs and almost all the chemokine receptors signal through heterotrimeric G proteins and, therefore, are subject to regulation by the RGS family. Because the GPCR signaling strength is tightly regulated by the expression of cell type specific RGS proteins, hence, the availability of RGS in the cells could dramatically affect the ability of chemokine receptors on lymphocytes to respond to their ligands, and consequently affect cell migration.
The RGS proteins that have been found to be expressed by lymphocytes include RGS1, RGS3, RGS4, RGS10, RGS13, RGS14, RGS16 and RGS19. Overexpression of RGS proteins can limit chemokine-mediated signaling in lymphocytes (175-179). It has been shown that B cells are mainly regulated by RGS1, RGS3, RGS4, RGS13 and RGS16, whereas T cells are regulated by RGS16 (175-179).

B cell trafficking in the GC is primarily mediated by CXCL12 and CXCL13 through binding to the chemokine receptors CXCR4 and CXCR5, respectively. Previous study has established that up-regulation of RGS13 and RGS16 in GC B cells is important to arrest the migration of B cells mediated by CXCL12 and that this retains founder B cells in the GC for sufficient interaction with Th cells, thereby promoting GC formation (81). Interestingly, overexpression of RGS13 and RGS16 in B cells can be mediated by IL-17 through the canonical NF-κB phospho-p65 pathway (157).

As aforementioned, in the process of GC formation, Tfh cells also migrate from T cells area to the GC LZ mediated by CXCL13. Similar to GC B cells, Tfh cells need to remain in the GC LZ to exert their help on GC B cells via interaction. However, whether the trafficking of Tfh cells is also regulated by IL-17-mediated RGS signaling or whether this cascade is critical to retain Tfh cells in the GC LZ remains unexplored.

Autoimmune Mouse Model and BXD2 Mice

General Autoimmune Mouse Models
Currently, there are several autoimmune mouse models available. Most of these mouse models are either lupus prone mice or specialized for arthritis.

The one mouse strain that can develop both spontaneous arthritis and lupus is the MRL-Fas\textsuperscript{Ipr} strain, but the pathogenesis is due to a single mutation of the Fas gene. A causative single mutant gene is unusual in most of human autoimmune diseases, although the rare autoimmune lymphoproliferative syndrome (ALPS) is caused by mutations in the FAS gene (180); so far, there has been no Fas or Fas ligand gene mutation found in SLE patients. Therefore, there are limits for the application of this mouse model to investigating the pathogenesis of human autoimmune diseases (181). In MRL-Fas\textsuperscript{Ipr} mice, however, IL-21 contributes to increased production of multiple pathogenic B cells, T cells and autoantibodies. In addition, the extra-follicular Tfh cells are involved in the disease development (89, 137).

The lupus prone-BXSB/Yaa mouse model is a recombinant inbred strain created by cross-breeding C57BL/6 females with SB/Le males. Almost all BXSB mice spontaneously develop lethal SLE-like disease. With an accelerating factor in males contributed by the male parent, the disease affects males predominantly with an accelerated onset compared to females. IL-21 is extremely high in sera of male BSXB-Yaa mice, which leads to a dramatic increase of Tfh cells and high titers of IgM and IgG autoantibodies (88, 124).

The Sanroque mutant mouse is another mouse model that can develop early onset of SLE-like disease. This mouse model is generated by mutating the ROQUIN protein with a M199R substitution (72). ROQUIN is the protein that suppresses the mRNA of
*Icos* and *Ifng* post-transcriptionally (3, 73, 80). Mutation of ROQUIN therefore causes overexpression of ICOS and excessive accumulation of Tfh cells, as well as increased levels of IL-21, which leads to autoreactive germinal center formation. Notably, mutated ROQUIN-mediated overexpression of ICOS in the mice can completely act to induce a T-dependent immune response in place of CD28 (182).

The transgenic K/BXN mouse model is generated by crossing KRN TCR-trangenic mice on the B6 background to non-obese diabetic mice. Expression of the transgenic TCR is required for the development of extensive erosive arthritis resembling the human rheumatoid arthritis (183). The pathogenesis is mediated by KRN CD4 T cells that can recognize glucose-6-phosphate isomerase (GPI) expressed in the joint tissue. In this model, non-self-tolerant Tfh cells have been shown to contribute to the development of aggressive arthritis by helping B cells produce high titers of autoantibodies. However, K/BXN mice have not been shown to develop lupus-like disease.

There are also multigenetic spontaneous autoimmune mouse models, such as NZB/W and (MRL-Fas<sup>lpr</sup> x B6-Fas<sup>lpr</sup>) F2 mice. NZB/W mice have been extensively used to study SLE-like disease. Tfh cells are also increased in this mouse model (90). Both NZB/W and (MRL-Fas<sup>lpr</sup> x B6-Fas<sup>lpr</sup>) F2 mice do not spontaneously develop arthritis.

**BXD2 Mouse Model**

BXD2 is one of the recombinant inbred strains derived by breeding the intercross progeny between DBA/2J and C57BL/6J (B6) mice for more than 20 generations (184).
Both the MHC gene (H2b) and Ig genes of BXD2 mice are derived from the parental B6 strain. Therefore, B6 mice have been used as healthy control in the study of BXD2 mice.

Spontaneous autoimmune diseases, including glomerulonephritis and arthritis, in BXD2 mice were first described in 2005 (185). Unlike other lupus prone BXSB/Yaa, MLR-Fas<sup>lpr</sup> and sanroque mice, there is no identified gene mutation in the BXD2 mouse model (185), however, the Quantitative trait loci (QTL) influencing the titers of anti-DNA and RF autoantibodies in the mice locate close to the previously mapped autoimmune susceptibility loci (185), which suggests that this is a polygenic autoimmune mouse model. The multi-genetic pathogenesis background of the BXD2 mouse is similar to that of most human autoimmune diseases, which renders it as an attractive mouse model to elucidate the mechanism of the immunopathogenesis of complex human autoimmune diseases.

BXD2 mice develop spontaneous GCs that produce pathologic B cells and high-titers of autoantibodies, which arise at the age of 2 months and peak at 6-12 months (185, 186). Transfer of cloned multi-reactive antibodies induces glomerulonephritis and arthritis in BALB/c (H<sup>2d</sup>) and B6 (H<sup>2b</sup>) nude mice(186).

Several studies have been carried out to investigate the mechanism of autoreactive GC formation in BXD2 mice. Overexpression of AID in B cells has been associated with the production of pathogenic autoantibodies (187), whereas suppression of the function of AID in these mice inhibits the GC formation and autoimmunity development (188). Consistent with reports showing that IL-17 is increased in human autoimmune diseases (160-162), elevated levels of serum IL-17 and an increased number of Th17 cells have
been shown to promote the autoreactive GC formation (81). In addition, type I interferon is involved in the GC development in BXD2 mice by regulating antigen delivery through a subset of B cells named the marginal zone-precursor (MZ-P) B cells (189).

Previous studies have shown that CD4 T cells in BXD2 mice have increased expression of CD28. Furthermore, \textit{in vivo} administration of AdCTLA-4-Ig to block CD28-CD86 interactions leads to a dramatic decrease in GC formation in BXD2 mice (187). In addition, high expression of CD86 in MZ-P B cells provides stronger costimulation to CD4 T cells, and a deficiency of CD86 causes compromised GC formation (190). All these findings together suggest that the pathogenic autoantibody development in BXD2 mice is predominantly dependent on T cell help. Therefore, the interaction between GC–Tfh cells is a crucial mechanism in this mouse model. However, whether there is accumulation of Tfh cells in this mouse model and how Tfh cells are regulated in this mouse model have not been determined. This is one of the major endeavors that we have undertaken in the current work.

\textbf{Questions and Hypothesis}

BXD2 mice develop spontaneous germinal centers (GCs) that produce pathogenic B cells and high-titer autoantibodies capable of inducing glomerulonephritis and arthritis (81, 186, 187). Previous results have shown that IL-17 is essential for GC development and autoantibody production (171, 172). IL-17 regulates autoreactive GC formation by arresting the migration of GC B cells through up-regulation of RGS13/16 (81). Tfh cells primarily provide help to GC B cells to promote GC formation. Accumulation of Tfh
cells and increased levels of IL-21 have been reported to be involved in the pathogenesis of human autoimmune diseases and in other lupus-prone mice (73, 80, 89, 137). IL-21 is the critical cytokine to promote Tfh cell development (34, 53) and it also negatively regulates conventional Treg (108). Tfr cells are a new regulatory T cell subset that has been shown to inhibit Tfh cells and GC formation in immunized mouse models (44, 45). Several questions are raised based on these previous observations:

1. Does autoreactive GC formation and autoimmune disease development require IL-21 in BXD2 mice?

2. Is there dysregulation in the number or the function of Tfh cells and their regulatory counterpart Treg and Tfr cells in autoimmune BXD2 mice? Does abnormal regulation of these T cell subsets correlate with the development of spontaneous GCs in BXD2 mice?

3. Does development of spontaneous GCs in BXD2 mice requires both IL-17 and IL-21? How do these two cytokines act together to induce pathogenic autoantibodies?

We propose a hypothetical model in which IL-21 and IL-17 exert distinct effects leading to enhanced development, localization and function of Tfh cells in GCs: (1.) IL-21 may up-regulate the critical transcription factor Bcl6 and promote the development of Tfh cells. (2.) IL-21 may inhibit Treg cells or Tfr cells to promote Tfh cells and GC formation. (3.) IL-17 may anchor of Tfh cells in the GC light zone (LZ), leading to prolonged contact and interaction between Tfh cells and GC B cells by up-regulating RGS protein and co-stimulator molecules. (Figure 11)
Significance

The novel autoimmune BXD2 mouse model that was used in the study presents human autoimmune disease-like manifestations, including spontaneous GC formation, nephritis and arthritis, which are accompanied with high titer autoantibodies (81, 186, 187) and elevated IL-17 in the serum (81). These features are consistent with reports in several human autoimmune diseases (160-162). The immune defects in this mouse model have been extensively characterized, and sufficient numbers of key pathogenic immune cell populations are available for analysis. Unlike most of the other autoimmune mouse
models, the BXD2 mouse is similar to most human autoimmune diseases. Therefore, investigation using this novel mouse model would be of special value to elucidate the mechanism of the immunopathogenesis in human autoimmune diseases. The dissertation study was carried out with BXD2 mice and several genetically modified BXD2 mice with deficiencies in IL-21, IL-21R, IL-17RA or RGS16. All these different strains of mice were utilized to investigate their specific effects on Tfh cell development and GC formation.

Aberrant accumulation of Tfh cells has been correlated with disease severity in human autoimmune diseases and in mouse models of lupus (72, 73, 83-86, 88-90). IL-21 is the critical cytokine to promote the development of Tfh cells (34, 53) and may negatively regulate conventional Treg cells (108). Increased levels of IL-21 have been found in sera of SLE patients (136) and lupus-prone mice (73, 80, 89, 137). Tfr cells are a new regulatory T cell subset that has been shown to inhibit Tfh cells and GC formation (44, 45). It is not known if IL-21 inhibits Tfr cells. In the current study, we determined the frequency of Tfh cells and the levels of IL-21 in BXD2 mice and confirmed that IL-21 is essential for Tfh development. In addition, the role of IL-21 in Treg cells and Tfr cells as well as the regulation by Tfr cells of Tfh cells and B cells were determined in BXD2 mice. The study enhances our understanding in the various regulatory effects of IL-21 on Tfh cells and provides novel insights for the pathogenesis of autoimmune diseases.

Imbalance between inflammatory cells and Treg cells has been associated with disease activity in lupus prone mice and SLE patients (16). Tfr cells play an important role in regulating Tfh cells and GC development (44, 45). The balance between Tfr cells
and Tfh cells is critical in determining whether an immunogenic autoimmune response or a tolerogenic self-limited response will be mounted. The ratio between Tfr cells versus Tfh cells could be a useful biomarker for the diagnosis and prognosis of GC-derived autoimmune diseases. The mechanism that regulates the balance between Tfr cells and Tfh cells has not been explored. In the first study of the current dissertation work, we investigated the balance between Tfh and Tfr subsets in BXD2 mice and determined that IL-21 plays an important role to shift the balance between Tfh and Tfr subsets. The study improves the understanding on how the Tfr cell subset is regulated and what controls the balance between Tfh and Tfr cells in murine autoimmunity.

Similar to IL-21, IL-17 regulates GC development and autoantibody production (171, 172). However, the role of IL-17 in regulating Tfh cells is unexplored. Previous studies have shown that autoantibodies produced by the BXD2 mice are highly pathogenic, and have undergone extensive SHM and CSR (186, 187). The results suggest that there must be undergoing important pathogenic mechanisms occurring in the GC to enable the formation of these antibody producing B cells. Results provided by the previous studies clearly showed that IL-17 was elevated in BXD2 mice and it inhibited the migration of GC B cells by up-regulation of RGS13/16 (81). However, I hypothesized that stabilization of GC B cells may only be one component of the IL-17-promoted GC response as successful conjugate formation requires extended interactions between both GC B cells with Tfh cells. The second study of this thesis work determined whether successful autoreactive GC responses require stabilization of both Tfh cells and GC B cells and if IL-17 provides the necessary signals to stabilize both cell types in the spontaneous GCs of BXD2 mice. During the process of GC formation, Tfh cells migrate
from T cell area, mediated by CXCL13, and finally localize in the GC LZ to form close conjugates with GC B cells. SLAM associated protein (SAP) expression on T cells has been shown to be crucial for the late-stage T-B interaction and GC formation (69, 78). In the current work, we found that IL-17 regulates migration of Tfh cells and their conjugate formation with GC B cells via the induction of RGS16 and co-stimulatory molecules. The study improves our understanding of the contribution of IL-17 to Tfh cells and Tfh-GC B cells conjugate formation in chronic autoimmunity.

Overall, the current dissertation work is of significance in providing novel insights into Tfh-mediated autoreactive GC formation in terms of IL-21 and IL-17 regulation, and thereby provides a reasonable strategy for therapeutic intervention in pathogenic Tfh-B cell interactions.
IL-21 PROMOTES GERMINAL CENTER REACTION BY SKEWING THE TFR/TFH BALANCE IN AUTOIMMUNITY

by

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Abstract

Objective: Follicular regulatory T (Tfr) cells act as the regulatory counterpart of follicular T helper (Tfh) cells to suppress germinal center (GC) B cell differentiation. We recently identified that interleukin 21 (IL-21) promotes Tfh differentiation in autoimmune BXD2 mice that develop spontaneous GCs. The objective of this study is to determine the modulatory effects of IL-21 on Tfr and the Tfr/Tfh balance in BXD2 mice.

Methods: The percentage and phenotype of Tfr were determined in BXD2 and BXD2-II21−/− mice. The effects of IL-21 on Tfr and the ratio of Tfr/Tfh were evaluated. Sorted Tfr cells from BXD2-II21−/− mice were co-cultured with Tfh and B cells, or transferred into BXD2 mice to determine their function.

Results: GC B cells and Tfh cells were significantly reduced, but the percentage of Tfr cells was 2-fold higher in BXD2-II21−/− mice than in WT. AdIL-21 administration to BXD2-II21−/− mice decreased Tfr and the ratio of Tfr/Tfh in spleen but increased GC B cells. rmIL-21 suppressed Foxp3 and significantly reduced Tgfb, Gitr and Il2 but enhanced Il21 and Il10 in Tfr cells. IL-21 also counteracted Tfr-mediated inhibition of antibody secretion in Tfh-B cells co-culture system. Transfer of Tfr cells into BXD2 mice reduced GC size and decreased serum IgG and IgM autoantibodies.

Conclusion: IL-21 selectively enhanced Tfh differentiation but inhibited Tfr commitment and compromised their suppressive function on Tfh and B cells. The study suggests that IL-21 skews the balance from Tfr to Tfh to promote autoreactive GC reactions in BXD2 mice.
Introduction

Abnormal selection and development of high affinity auto-Ab producing B cells in germinal centers (GC) is central for initiating autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis. Many cell types contribute to the formation of GC, including antigen presenting cells (APC), pro-inflammatory T helper cells and regulatory T (Treg) cells. Most importantly, the development of Ab producing plasma cells within the GC requires help from CXCR5⁺ICOS⁺ PD-1⁺ follicular T helper cells (Tfh) and the differentiation of Tfh is Bcl6-dependent and IL-21-mediated (1-3). An increase in numbers or activity of Tfh cells has been correlated with the pathogenesis and severity of disease in GC-dependent autoimmune conditions including SLE (4-8). “Regulatory” cells within the GC control the number and the function of Tfh and GC B cells. In mice, Qa-1⁺ CD8⁺ T cells can regulate the Tfh cells in vivo, whereas in humans, CD69⁻CD25⁺CD4 T cells capable of migrating into GCs were found to suppress GC B cells response (9-11). Notably, a recently described subset of follicular regulatory T cells (Tfr) which share features of Bcl6-dependent Tfh and Treg cells have been found to locate in GC and regulate the Tfh population and the differentiation of GC B cells in vivo (12, 13). However, little is known about how Tfr are regulated, although it was reported that PD-1-PD-L1 interactions can inhibit the number and function of these cells in the lymph nodes and blood (14).

Aberrant T cells homeostasis also contributes to the development of autoimmune diseases. Imbalance between Treg and pro-inflammatory effector T cells, such as Th17, is
associated with disease activity in lupus prone mice and SLE patients (15). However, the mechanism that regulates the balance between Tfh and Tfr cells in the pathogenesis of autoimmunity has not been explored.

Cytokine milieu is critical parameter that controls the development of pathogenic and non-pathogenic immune responses. Increased level of IL-21 have been detected in sera from SLE patients (16) and lupus prone mice (17). IL-21 has multiple effects including promoting the survival of GC B cells, affinity maturation, and plasma cell differentiation (18, 19). IL-21 also acts in an autocrine manner to promote the generation of T follicular helper cells (3, 20) and is considered the signature cytokine of Tfh cells (2, 21, 22). Conversely, IL-21 has also been shown to negatively regulate the number of conventional Treg in IL-21 KO mice (23). However, whether IL-21 affects Tfr cells is unknown. In this study, we report that, in the autoimmune BXD2 mice that develop spontaneous autoreactive GC in spleen, IL-21 plays a critical role in promoting autoimmunity by selectively enhancing Tfh development and inhibiting Tfr cell programming, as well as counteracting the suppressive function of Tfr in vitro and in vivo. These imbalances in Tfh and Tfr differentiation serve to promote GC reactions resulting in the accumulation of autoantibodies.
Materials and Methods:

Mice

C57BL/6 (B6) and BXD2 recombinant inbred mice were obtained from The Jackson Laboratory, and B6-Il21−/− mice were obtained from the Mutant Mice Regional Resource Center (Davis, CA). B6-Il21−/− mice were backcrossed with BXD2 mice for seven generations by a marker-assisted speed congenic approach. All mice were housed in the University of Alabama at Birmingham (UAB) Mouse Facility under specific pathogen-free conditions in a room equipped with an air-filtering system. The cages, bedding, water, and food were sterilized. All mouse procedures were approved by The UAB Institutional Animal Care and Use Committee.

Flow cytometry analysis

Single-cell suspensions of whole-spleen cells, cultured cells or PBMC cells were labeled by surface staining with the following antibodies: Pacific blue– or Alexa488– (RM4-5, GK1.5, Biolegend); Pacific blue–anti-CD19 (6D5, Biolegend); Alexa647–anti-GL-7 (GL7, eBioscience); PE–Cy7-anti-CXCR5 (2G8, BD Biosciences); FITC- or PE- anti-ICOS (398.4A, 7E.17G9, eBioscience). Antibodies against PD-1 (RMP1-30, Biolegend), CD44 (IM7, Biolegend), CD25 (pc61.5, eBioscience), GITR (DTA-1, eBioscience), TGF-β1 (TW7-16B4, Biolegend), CTLA-4 (UC10-4F10-11, BD Pharmingen) and Fas (15A7, eBioscience) were all conjugated with PE.

For nuclear transcription factor staining, cells were labeled with surface markers, then fixed and permeabilized with the Foxp3 Staining Buffer Set (eBioscience), according to
the manufacturer's instruction. Permeabilized cells were stained with PE–anti-Bcl6
(K112-91, BD Biosciences) and PE–anti-Foxp3 (FJK-16s, eBiosciences).

For phosphoflow staining, cells were collected after treatment, fixed and permeabilized
with the BD Phosflow™ Fix Buffer and Perm Buffer, according to the manufacturer's
instruction. Cells were stained for surface markers and intracellular staining with Alexa
647-rabbit anti-phospho-Akt (Ser 473) (Cell signaling) or Pacific blue-mouse anti-Stat3
(p-Y705) (4/p-Stat3, BD Bioscience).

Samples were acquired with an LSR II FACS analyzer (BD Biosciences), and data were
analyzed with FlowJo software (Tree Star, Inc. Ashland, OR, USA). All flow cytometry
analysis was carried out using a combination of FSC and SSC height, area, and width
parameters to exclude aggregated cells.

**Immunofluorescent staining of frozen sections and confocal imaging**
Spleens from mice were collected, embedded in Frozen Tissue Media (Fisher Scientific),
and snap-frozen in 2-methylbutane. Frozen sections (8-µm thick) were fixed and
processed as previously described (24). Unless specified, all reagents and antibodies were
purchased from Invitrogen: Biotin–PNA (Vector Laboratory) followed by SA–Alexa 350;
Alexa 555–anti-IgM; Alexa 647–anti-CD4 (GK1.5, Biolegend); Application of rat anti-
mouse Foxp3-biotin (FJK-16s, eBiosciences) and SA–HRP was followed by the tyramide
signal amplification method (TSA Kit, T20931) and SA–Alexa-488. Sections were
mounted in the Slow Fade Gold Antifade reagent and images were captured with a Leica
DMIRBE inverted Nomarski/epifluorescence microscope outfitted with Leica TCS NT laser confocal optics.

**Real-time quantitative RT-PCR**

RNA isolation, cDNA synthesis, and real-time PCR reactions were carried out as we described previously (24). The following primers were used: *Gitr*, 5’-

GGAACAAGACCCACAATG -3’ (F), 5’- ATGACCAGGAAGATGACA-3’ (R); *Ii2*, 5’- TAAAAGGGCTTGACAACA-3’ (F), 5’- GGCTATCCATCTCCAG-3’ (R); *Tgfβ1*, 5’-CTACTATGCTAAAGAGGTAC-3’ (F), 5’-CATCGTGCCTCCACACTTG-3’ (R); *Gapdh*, 5’-AGGTCGGTGATAACGGATTGTG-3’ (F), 5’-

TGTAGACCATGTTGAGATGTCA-3’(R). *Icos*, 5’-

CGTGTCTTTGCTTTCTGCTCC-3’ (F), 5’-TTCGTCCTGCTGGTTTGGTGTG-3’ (R); *Cxcr5*, 5’-GAGATTCCCCCTACAGGACAGT-3’ (F), 5’-

CCAGCACCAGGATGGTTCCC-3’(R); *Il21*, 5’-ATGCCCCTTCTGTAGTCGATATCGTATG-3’ (F), 5’-CGGCCTTCCTCTCAGTCCTCCTC-3’ (R); *Il10*, 5’-AATCCCTGGGTTGAGAAG-3’ (F), 5’-CATTCATGGCCTTTGTAGAC-3’ (R).

**ELISA to detect antibodies and cytokines**

Serum levels of autoantibodies and total IgG, IgM levels in cultured supernatant were determined by ELISA, as we described previously (25). BiP was purchased from Assay Designs, Inc. and all other autoantigens from Sigma-Aldrich. IgG (1030-01) and IgM (1030-01) capture antibodies were purchased from SouthernBiotech.
Serum levels of IL-21 and TGF-β1 were measured using mouse IL-21 ELISA Ready-SET-Go!® (88-8210, eBioscience) and Human/Mouse TGF beta 1 ELISA Ready-SET-Go! (88-7344, eBioscience) kits, respectively, according to the manufacturer’s instructions.

**ELISPOT quantification of autoantibody-producing B cells**

The ELISPOT procedure was carried out as previously described (26). Briefly, polyvinylidene difluoride-backed 96-well plates (Millipore) were coated overnight at 4°C with 5 mg/ml of the indicated autoantigen. BiP was purchased from StressMarq Biosciences Inc. (Victoria BC, Canada), and other autoantigens were purchased from Sigma-Aldrich. Total spleen cells in 100 µl complete medium, containing 5x10^5 cells per well for detecting IgM and 2x10^6 per well for detecting IgG, were cultured overnight in a 37°C incubator. Plated cells were then incubated at RT with HRP-labeled goat anti-mouse IgG/M (Southern Biotechnology) in PBS with 1.5% BSA for 3–4 h. Spots were developed with 3-amino-9-ethylcarbazole. Plates were read by an automatic ELISPOT reader (CTL) and analyzed with Immunospot 3.1 software (CTL).

**Administration of AdLacZ and AdIL-21**

AdIL-21 and AdLacZ (2x10^9 p.f.u. per mouse), generous gifts from Dr. Oliver Wildner (Paul-Ehrlich-Institute, Langen, Germany), were administered intravenously (i.v.). Spleen tissue and cells were analyzed 7 days later.
**Proliferation and functional assay of responder cells co-cultured with Tfr cells**

B cells from BXD2 mice were purified by Anti-CD19 MACS columns (Miltenyi Biotech). Tfr cells (CXCR5^+^GITR^+^CD4^+^ T cells) and Tfh cells (CXCR5^+^GITR^+^CD4^+^ T cells) from BXD2-Il21^−/−^ mice, BXD2-Il21r^−/−^ mice or BXD2 mice were sorted by FACS Aria II flow cytometer. Tfh cells were labeled with CFSE and either left untreated or stimulated with anti-CD3 (1 µg/ml) and anti-CD28 (2µg/ml). Tfh cells (1x10^5/well) were then cultured alone or with a 1:1 ratio of Tfr cells or other control cells and either left untreated or treated with IL-21(50 ng/ml) for 3 days in 96-well round bottom plate. B cells (3x10^5/well) were either left untreated or stimulated with anti-IgM (10µg/ml) and anti-CD40 (10µg/ml), then cultured alone or with Tfr or other control cells at a 3:1 ratio together with IL-21(50 ng/ml) in volume of 250ul in round-bottom 96-well-plate for 5 days. In certain experiments, B cells were co-cultured with Tfh and Tfr at a ratio of 3:1:1 together with IL-21, anti-CD3 and anti-CD28 stimulation for 5 days.

For analysis of Tfh cells proliferation on day 3, CFSE dilution was determined by flow cytometric analysis. For B cell functional assay, supernatants from the co-cultures were collected on day 5 and the total IgG antibody secretion levels were measured by ELISA.

**Adoptive transfer of Tfr cells**

Tfr cells (CXCR5^+^GITR^+^CD4^+^ T cells) from BXD2-Il21^−/−^ mice were sorted using a FACS Aria II flow cytometer and injected intravenously into BXD2 mice (8x10^5^ cells in 200 µl PBS per mouse). Recipients were sacrificed 17 days after transfer and splenic responses analyzed.
Statistical analysis

All results are shown as the mean ± standard deviation (s.d.). A two-tailed t test was used when two groups were compared for statistical differences. An ANOVA test was used when more than two groups were compared for statistical differences. P values less than 0.05 were considered significant.
Results

IL-21 promotes Tfh, but inhibits Tfr development in BXD2 mice

We previously showed that autoimmune BXD2 mice exhibited an increase in autoantibody-producing B cells and an accumulation of Tfh cells in spleens (26, 27). Consistent with the previous reports that increased serum levels of IL-21 are associated with the development of autoimmune diseases (16, 17), we found that sera levels of IL-21 were significantly increased in 3-mo-old BXD2 mice compared with B6 mice, and as expected, it was not detectable in BXD2-Il21−/− mice (Fig. 1A). A decrease in Treg cells has also been demonstrated in lupus prone mice and SLE patients (15) and IL-21 has been shown to negatively regulate the number of conventional Treg in IL-21 KO mice (23). To determine the role of T regulatory cells in autoimmune BXD2 mice, we evaluated the FoxP3+ CD4+ Treg cells in B6, BXD2, and BXD2-Il21−/− mice at 3 months of age. There was no significant difference in the percentage of total Treg cells among the three stains of mice (Fig. 1B). Somewhat surprisingly, there was an increase in the total number of Treg cells in the spleens of BXD2 mice and the ratios of Treg versus non-Treg CD4 T cells were nearly equivalent among the three stains of mice (Fig. 1B), despite the high level autoantibody production in BXD2 mice and the defected autoantibody production in BXD2-Il21−/− mice (27). Together, these results suggest that the total number of Treg cells is not correlated with suppression of GC development or autoantibody production in BXD2 mice.
As expected, the frequency of ICOS⁺CXCR5⁺CD4⁺Tfh was low in the spleen of BXD2-Il21⁻/⁻ mice compared with WT-BXD2 mice (Fig. 1C left top), which was consistent with the reports that IL-21 plays a fundamental role in Tfh development (3, 20). However, the percentage of Foxp3⁺ cells within the ICOS⁺CXCR5⁺CD4⁺ subset was greatly increased in BXD2-Il21⁻/⁻ mice compared with BXD2 mice. Thus, almost half of the ICOS⁺CXCR5⁺CD4⁺ cells in BXD2-Il21⁻/⁻ mice display a Tfr phenotype (Fig. 1C left bottom). The ratio of Foxp3⁺Tfr cells to Foxp3⁻Tfh cells (Foxp3⁺ICOS⁺CXCR5⁺CD4⁺T) was significantly higher in both the spleens and the peripheral blood (PBMCs) of BXD2-Il21⁻/⁻ mice compared with BXD2 mice (Fig. 1C right). Although it is not known whether the Tfr cells act directly on B cells or indirectly through inhibition of Tfh, Tfr cells have been shown to locate in nascent GC and suppress further GC B cells differentiation in vivo (12, 13). Consistently, we found that the percentage of GC B cells were significantly lower in BXD2-Il21⁻/⁻ mice compared with WT-BXD2 mice (Fig. 1D left). Accordingly, the ratio of Tfr relative to GC B cells was dramatically higher in BXD2-Il21⁻/⁻ compared with BXD2 mice (Fig. 1D right). These results indicate that IL-21 promotes Tfh development and suppresses the formation of Tfr cells but does not affect the conventional Treg population in BXD2 mice.

**Increased Tfr cells in GC of BXD2-Il21⁻/⁻ mice**

Tfr cells are a regulatory T cell subpopulation that share features of Tfh and Treg cells and are located in the GC (12, 13). To further characterize the Tfr cells, we compared the expression of molecular markers that have been previously associated with Foxp3⁻ Tfh
and Foxp3⁺Tfr cells. Compared with Tfh cells, Tfr cells expressed higher levels of Treg markers including CTLA-4, membrane TGF-β1, GITR and CD25, whereas they shared similar high levels of expression of PD-1, CD44 and Bcl6 (Fig. 2A). The Tfr and Tfh cells from BXD2 mice show a similar pattern of expression (data not shown). In addition to the significantly increased frequency of Foxp3 expression in ICOS⁺CXCR5⁺CD4 T cells (Fig. 1C left bottom), the expression of CTLA-4, membrane TGF-β1 and GITR in ICOS⁺CXCR5⁺CD4 T cells were all higher in BXD2-Il21⁻/⁻ mice compared with WT-BXD2 mice (Fig. 2B). The results further confirm that Foxp3⁺ ICOS⁺CXCR5⁺CD4 T were Tfr cells and show that this population is increased in BXD2-Il21⁻/⁻ mice.

The location of Tfr cells in BXD2 and BXD2-Il21⁻/⁻ mice was determined by confocal microscopy analysis of CD4, Foxp3, and PNA staining in spleen tissue. The GC, indicated by PNA staining, was much smaller in BXD2-Il21⁻/⁻ mice, however, there was a significant increase in the number of CD4⁺Foxp3⁺ Tfr in the GC and entire follicle of BXD2-Il21⁻/⁻ mice compared with BXD2 mice (Fig. 2C). A higher magnification view of the area of GC showed that the nuclear expression of Foxp3 (green color) was surrounded by surface expression of CD4 (red color). Together, these results suggest that there was increase in the relative number of Foxp3⁺ Tfr subset in BXD2-Il21⁻/⁻ mice which consisted predominately of cells with shared characteristics of Treg and Tfh cells, and these cells were also located in GC.
**IL-21 suppressed Tfr development in vivo**

The aforementioned results suggest that IL-21 promotes Tfh development and reduces the percentage of Tfr cells in BXD2 mice. In contrast, a deficiency of IL-21 led to an increased frequency of Tfr cells in BXD2-Il21−/− mice. To determine if exogenous IL-21 could suppress Tfr cells and promote Tfh cells development in BXD2-Il21−/− mice, adenovirus vectors producing IL-21 (Ad-IL21) or control Ad-LacZ were administered and mice analyzed 7 days later. There was a significant induction of IL-21, but suppression of TGF-β1 in the sera of mice administered Ad-IL21 compared to the Ad-LacZ treated control groups (Fig. 3A). In the spleens of mice administered Ad-IL21 there was a significant increase in the frequency of CXCR5+ICOS+CD4 T cells (Fig. 3B top), which was associated with a significant decrease in the percentage of Foxp3+ Tfr cells (Fig. 3B middle). Consistent with decreased level of TGF-β1 in sera, the expression of membrane TGF-β1 in CD4+FoxP3+CXCR5+ICOS+ (Tfr) cells was reduced in Ad-IL21 treated mice (Fig. 3B bottom). The ratio of Tfr to FoxP3+ Tfh cells was therefore decreased in these mice (Fig. 3C) and this was associated with increases in Fas+GL7+ GC-B cells (Fig. 3D).

**IL-21 down regulates Foxp3 via inhibiting p-AKT in CXCR5+ICOS+CD4 T cells**

To further confirm the suppression of Tfr development by IL-21, we analyzed the effect of in vitro IL-21 stimulation (50ng/ml) on CXCR5+ICOS+CD4 T cells from BXD2 and BXD2-Il21−/− mice. Without IL-21 stimulation, there was a significant increase in the Foxp3+ population of CXCR5+ICOS+CD4 T cells in BXD2-Il21−/− mice compared with
BXD2 mice (Fig. 4A top). This Foxp3+ population from BXD2-Il21+/− mice was significantly down regulated following a 16 hour co-culture with IL-21 (Fig. 4A bottom). Similarly, IL-21 treatment also led to a decreased expression of membrane TGF-β1 and GITR in the CXCR5+ICOS+CD4 T cells (Fig. 4B).

The suppressive effects of IL-21 on Tfr cells were also confirmed by co-culture of sorted Tfr cells (CXCR5+GITR+CD4 T) either with IL-21(100 ng/ml) or control media for 4 hours. RT-PCR analysis showed that there was a significant decrease in expression of Treg-cell-related markers Tgf-β1, Il2, and Gitr in cells cultured with IL-21 compared to control (Fig. 4C). In contrast, the inflammatory-cell-related cytokines Il21, Il6 and Il10 were all significantly increased in cells stimulated with IL-21 compared with control. Intriguingly, there were also increased expressions of the Tfh cells signature surface markers Cxcr5, Icos and Pd1 on Tfr cells treated with IL-21 (Fig. 4C). These results indicate that IL-21 suppresses the phenotype of Tfr cells and can convert Tfr cells into non-regulatory T cells or even inflammatory T cells.

IL-21 signals through Stat3 to promote differentiation of Th17 and Tfh cells (23, 28) and Stat3 phosphorylation is required for the loss of Treg function (29). The PI3K- Akt pathway plays a key role in IL-21-mediated proliferation in primary CD8 T cells (30), and the PI3K-Akt-mTOR pathway is involved in context dependent regulation of Foxp3 expression and accessibility according (31-33). To determine how IL-21 signals to suppress of Tfr cells, CD4 T cells from spleens of BXD2 and BXD2-Il21+/− mice were
cultured with either IL-21 or control media for 15 minutes. Cells were then gated for CXCR5^+ICOS^+CD4 T cells and analyzed for intracellular expression of Stat3 (pY705), and p-Akt (Serine 473). IL-21 induced significant up-regulation of p-Stat3 in CXCR5^+ICOS^+CD4 T cells from both BXD2-Il21^-/- mice and WT-BXD2 mice (Fig. 4D left bottom). Interestingly, Akt phosphorylation was higher on the majority of CXCR5^+ICOS^+CD4 T cells from BXD2-Il21^-/- mice than on cells from BXD2 mice. Co-culture of IL-21 for 15 minutes significantly down regulated the phosphorylation level of Akt in these T cells from BXD2-Il21^-/- mice. The p-Akt in CXCR5^+ICOS^+CD4 T from BXD2 mice was also inhibited to a lesser extent by IL-21 treatment (Fig. 4D left top).

CD4 T cells were cultured with different doses of the p-Akt inhibitor, LY294002, to determine whether p-Akt regulates the expression of Foxp3 by CXCR5^+ICOS^+CD4 T cells. There was a significant dose-dependent decrease in the frequency of Foxp3^+ cells in CXCR5^+ICOS^+CD4 T subset (Fig. 4D right). These results suggest that IL-21 may prevent Foxp3 expression and Tfr cells commitment by inhibiting the phosphorylation of Akt but activating Stat3.

**IL-21 acts directly on Tfr cells to counteract their suppression on Tfh cells and IgG production from B cells**

To determine the suppressive function of Tfr cells, Tfr cells were sorted from BXD2-Il21^-/- mice (Fig. 5A) and then co-cultured with Tfh cells, B cells only or with both Tfh and B cells. Tfr from BXD2-Il21r^-/- mice suppressed the proliferation of Tfh cells. This
suppressive effect was counteracted by the addition of IL-21 (Fig. 5B). Notably, Tfr cells from WT-BXD2 mice did not suppress but instead promoted the proliferation of Tfh cells.

In the Tfr and B cells co-culture assay, in which B cells were stimulated with anti-IgM and anti-CD40, Tfr from BXD2-Il21r<sup>−/−</sup> inhibited IgG secretion from B cells (Fig. 5C), and the presence of IL-21 restored IgG secretion. Again, Tfr cells from WT-BXD2 mice did not suppress the IgG production from B cells. These results show that Tfr cells from BXD2-Il21r<sup>−/−</sup> mice can inhibit proliferation of Tfh cells and IgG production by B cells and that IL-21 can counteract these suppressive effects.

To further determine whether Tfr cells inhibit B cell and Tfr responses co-culture assays were performed with anti-CD3 and anti-CD28 stimulation. In the absence of IL-21, IL21<sup>−/−</sup> Tfr cells suppressed IgG production and the effect was positively associated with the numbers of Tfr cells; however, addition of IL-21 restored IgG level despite the presence of the IL21<sup>−/−</sup> Tfr cells (Fig. 5D). To investigate if IL-21 acts directly on Tfr cells to counteract their suppressive effect, Tfr cells from BXD2-Il21r<sup>−/−</sup> mice were added to replace the IL21<sup>−/−</sup> Tfr cells in the co-culture. Intriguingly, these IL21r<sup>−/−</sup> Tfr cells suppressed IgG production even in the presence of IL-21. This result suggests that IL-21 primarily acts directly on the Tfr cells to “convert” these cells into non-Tfr, which then enables IgG production in the co-culture system.
Suppression of GC formation by transfer of Tfr cells into BXD2 mice

To directly demonstrate that the Tfr cells from BXD2-II21−/− mice suppress GC development and autoantibody production in vivo, Tfr cells were sorted from BXD2-II21−/− mice as described in Figure 5A and transferred into 2-mo-old BXD2 mice (Fig. 6A). By 17 days after transfer a significant decrease in Fas+GL7+ GC B cells and Foxp3−CXCR5+ICOS+Tfh was apparent in recipient mice that received Tfr cells (Fig. 6B). Transfer of Tfr cells also decreased the spleen size and confocal image analysis also revealed that the PNA+ GCs in spleens were much smaller than those in control mice (Fig. 6C left). The localization of transferred Tfr cells in GCs was confirmed by confocal microscopy analysis of CD4, Foxp3 and PNA staining in spleen sections (Fig. 6C right).

ELISPOT assay showed that there was a significant decrease in autoantibody-producing B cells of the IgM but not IgG subpopulation in Tfr transferred recipients (Fig. 6D left). However, ELISA assay indicated that both the levels of IgG and IgM autoantibodies to DNA, rheumatoid factor (RF), histone and Bip in sera were significantly decreased in Tfr transferred recipients compared with control mice (Fig. 6D right). The results demonstrate that the Tfr (CXCR5+GITR+) subpopulation of CD4 T cells that is increased in BXD2-II21−/− mice exhibited the regulatory properties of being able to home to the GC, inhibit Tfh cells, and suppress GC development and autoantibody production in vivo.
Discussion

An imbalance between Treg and inflammatory cells has been observed in autoimmune diseases (15). Increased Th17 and Tfh cells have been shown in autoimmune patients and lupus prone mouse models (4-8, 24). In contrast, deficiencies in the number and function of Treg cells in autoimmune conditions have been demonstrated in vivo and in vitro (15, 34-36). Tfr cells are the relatively new regulatory T cell subset that locates in GC and suppresses GC B cells differentiation (12, 13). The balance between Tfh cells and Tfr cells in autoimmunity has not been explored. In the current study, we demonstrated that in autoimmune BXD2 mice, IL-21 regulated GC formation by preferentially promoting the development of Tfh cells but inhibiting the commitment of Tfr cells, thereby, skewed the balance between Tfr cells versus Tfh cells to favor autoreactive GC response and autoantibody production.

IL-6, TGF-β, IL-17, IL-21 alone or cooperatively have been shown to promote the commitment of specific effector T cells or Treg cells (3, 20, 37, 38), while IL-6 and IL-21 were shown to inhibit differentiation of conventional Treg (23, 39, 40). Here, we showed that, compared with WT-BXD2, a deficiency in BXD2-Il21−/− mice led to significant decrease in the frequency of Tfh cells and significant increase in the frequency of Tfr cells but not conventional Treg cells, which therefore resulted in an dramatically increased ratio of Foxp3+Tfr versus Foxp3'Tfh cells in the BXD2-Il21−/− mice. This suggests that in BXD2 mice, IL-21 primarily affects the ratio of Foxp3+/FoxP3− CD4 T cells with CXCR5+ICOS+ phenotype, but not affect conventional Treg cells, which is in
contrast to the previous report that absence of IL-21 enhanced the development of conventional Treg in an ex-vivo cell culture (23). However, the frequency of Tfr cells and the balance of Tfr versus Tfh (Tfr/Tfh) cells appeared to be closely associated with GC formation and antibody production in BXD2 mice. In BXD2-Il21−/− mice, the ratio of Tfr/Tfh was high while the GC formation was almost absent. In contrast, AdIL-21 administration in BXD2-Il21−/− mice promoted Tfh cells development and GC formation while the frequency of Tfr cells and the ratio of Tfr/Tfh were significantly decreased. In vitro stimulation of spleen cells with rmIL-21 also reduced the frequency of Tfr cells. The result indicated that IL-21 inhibited the ratio of Tfr/Tfh and promoted autoreactive GC formation in BXD2 mice. A balance between Tfr and Tfh cells could be a useful biomarker to determine the progression of autoimmunity.

IL-21 is essential for the development of Tfh cells (3, 20). In BXD2-Il21−/− mice, the frequency of Tfr cells was significantly increased, but the total cell count of Tfr cells was not more than that in the WT-BXD2 mice, which suggested that the increased ratio of Tfr/Tfh in the BXD2-Il21−/− mice may be primarily due to the decreased number of Tfh cells but not the increased number of Tfr cells resulted from deficiency of IL-21. However, the decrease of the total spleen cells number and CD4 T cells number in BXD2-Il21−/− mice may explain the less Tfr cell count. In addition, the further study indeed showed that IL-21 could directly suppress Tfr cells in BXD2 mice. rmIL-21 in the in-vitro culture system inhibited the expression of Foxp3 on CXCR5+ICOS+CD4 T cells and TGF-β1 on Tfr cells, both are the critical markers for Tfr cells. AdIL-21 administration also decreased the number of Tfr cells in BXD2-Il21−/− mice. Furthermore,
IL-21 also converted the sorted Tfr cells into non-Tfr by inhibiting the expression of Treg functional molecules Tgfb1 and Il2 but promoting expression of Il21, Il10, Il6 and Tfh markers. These results suggest that IL-21 can inhibit commitment of Tfr. Interestingly, in addition to the result from in vitro study, we found that extra IL-21 in vivo led to increased levels of IL-10 in sera of BXD2-Il21−/− mice. IL-10 is generally thought to execute the suppressive function of Treg and other regulatory cells of the immune system. Also, previous study has indicated that IL-21 mediate suppressive effects by inducing IL-10 (41). However, CSR for IgA production requires IL-10. In addition, IL-10 is dramatically increased in SLE patients (42), and has been associated with autoantibody formation (43). Therefore, we propose that IL-21 may induce IL-10 to enhance B cell response and antibody production in autoimmune BXD2 mice. The present study also showed that IL-21 counteracted the inhibition of Tfr on Tfh cells and B cells. Thus, IL-21 not only promotes the development of Tfh cells but also inhibits the commitment and function of Tfr cells, thereby exerts its inflammatory function on GC formation in BXD2 mice. This is the new finding on the regulation of Tfr cells.

IL-21 signals through Stat3 to promote T helper cells differentiation (23, 28) and reverse Treg suppression function (29) by p-Stat3. IL-21 also mediated proliferation of primary CD8 T cells through PI3K-Akt pathway (30) whereas Akt phosphorylation was shown to regulate programing and commitment of T helper cells including Th17 but was still controversial (44-46). In addition, PI3K-Akt-mTOR pathway was also involved in either negatively or positively regulating Foxp3 expression or accessibility according to different stimulation context (31-33). Our in vitro culture study showed that rmIL-21
inactivated Akt by down-regulating the phosphorylation of Akt at Ser-473 in ICOS⁺CXCR5⁺CD4 T cells from BXD2-*Il21⁻/⁻* mice while in contrast, enhancing Stat3-p in this cell subset. Inactivation of Akt by LY294002 down-regulated Foxp3 expression in ICOS⁺CXCR5⁺CD4 T cells in a dose-dependent manner. These suggest that IL-21 could up-regulate p-Stat3 to promote the differentiation of effector T helper cells or the resistance of T cells to Treg suppression and that IL-21 also may down-regulate Akt-p to inhibit the commitment of Foxp3⁺Tfr cells.

Tfr cells has been shown to suppress GC B cells differentiation (12, 13), however, there is no evidence indicating whether Tfr cells can act directly on Tfh cells or B cells. In the current study, we demonstrated that sorted Tfr cells from BXD2-*Il21⁻/⁻* mice exhibited the ability to inhibit the proliferation of Tfh cells and the IgG production from B cells, as well as suppressing the IgG production from Tfh-B cells co-culture system. IL-21 counteracted the inhibition of Tfr on Tfh cells and B cells by directly acting on Tfr cells since 21R⁺ Tfr cells, which were unable to response to IL-21, maintained their suppressive function on IgG production from Tfh-B cells co-culture system despite the presence of IL-21. Interestingly, unlike Tfr cells from BXD2-*Il21⁻/⁻* mice, Tfr cells from BXD2 mice failed to inhibit Tfh and B cells. The result suggested that, in addition to the low ratio of Tfr/Tfh in BXD2, the suppressive function of Tfr cells was also defective, which together with accumulation of Tfh cells, contributed to the excessive GC formation and autoantibody production in BXD2 mice.
The suppressive function of Tfr cells was further demonstrated by in vivo transfer of Tfr cells from BXD2-Il21⁻/⁻ mice into WT BXD2 mice. The Tfr cells homed to preexisting GCs and reduced the Tfh cells and GC B cells, the size of GCs and the autoantibodies titers in sera to a significant but not dramatic extent. This is in line with previous reports that CXCR5-mediated migration of Treg cells to the GC was necessary to limited the amount of secreted Ag-specific IgM, IgG1, IgG2b, and IgA (47) while CXCR5⁻/⁻ Treg cells were unable to gain access to the GC follicles, and that the Treg phenotype is durable in vivo (48). However, it is inconsistent with the previous observation that Treg cells may convert into Tfh-like cells in certain inflammatory environment (49) and also inconsistent with our aforementioned that IL-21 may convert Tfr cells into non-Tfr cells in vitro. We suggest that the maintenance of suppressive function of Tfr cells in vivo may be due to the lower level of IL-21 compared with the experimental level of IL-21 (50ng/ml) during cell culture in vitro. In addition, we still cannot exclude the conversion of transferred Tfr cells in the animals. It is possible that blockade of IL-21 signaling may lead to even better suppressive effect of the transferred Tfr cells.

Overall, the current study showed that IL-21 promotes autoreactive GC reactions in autoimmune BXD2 mice by selectively promoting development of Tfh and inhibiting programing and commitment of Tfr cells as well as compromising the suppressive function of Tfr cells on B cells and Tfh cells. The study suggests the Tfr cells transfer together with IL-21 blockade as a possible therapeutic strategy. However, the mechanisms by which Tfr cells exert their suppressive function need further investigation.
Acknowledgments

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**Abbreviations used in this paper**

<table>
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<tr>
<td>B6</td>
<td>C57BL/6</td>
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<tr>
<td>BXD2-WT</td>
<td>wild-type BXD2</td>
</tr>
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<td>Foxp3</td>
<td>forkhead box protein 3</td>
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<td>germinal center</td>
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<td>programmed cell death-1</td>
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<td>peanut agglutinin</td>
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<td>Treg</td>
<td>regulatory T cells</td>
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Figure Legend

**Fig. 1  IL-21 deficiency in autoimmune BXD2 mice results in defective Tfr but not conventional Treg responses.** A. ELISA assay of serum IL-21 level in BXD2 and B6 mice. B. Flow cytometry analysis show the mean percentage ± s.d. of conventional Foxp3+ Treg, plots are gated on lymphocytes; bar graphs show the total numbers of conventional Treg (Foxp3+ CD4 T) cells and ratio of Treg to non-Treg CD4 T cells. C. Flow cytometry analysis of CXCR5+ICOS+ Tfh cells and Foxp3+ expression by CXCR5+ICOS+CD4 T cells in the spleens of mice, numbers beside the boxed region report the mean frequency ± s.d; bar graphs show the ratios of Foxp3+CXCR5+ICOS+Tfr cells versus Foxp3+CXCR5+ICOS+Tfh cells in the spleen and PBMC. D. Flow cytometry analysis of GL-7+Fas+ GC B cells in the spleens of female 2.5-3-mo-old mice, gated CD19+ B cells or CD4 T cells are plotted; bar graph show the ratios of Tfr versus GC B cells. Data are representative of the analysis of n >= 5 mice per group (B, C, D), or are the mean ± s.d. of n >= 5 mice per group (A, B, C, D); *p < 0.05, ** p<0.01, ***p<0.005 compared between the different groups.

**Fig. 2 Increased Tfrs in BXD2-Il21−/− mice ** A. Flow cytometry analysis of the expression of the indicated molecules in Tfr and Tfh cells from spleens of BXD2-Il21−/− mice, dash line indicates the gating B. Flow cytometry analysis of the expression of the indicated Treg markers in CXCR5+ICOS+CD4 T cells from WT-BXD2 and BXD2-Il21−/− mice, dash lines indicate the gating. C. Immunofluorescence staining and confocal imaging of Foxp3+ CD4 T cells in spleen follicles of WT-BXD2 and BXD2-Il21−/− mice,
original 20x with equal magnification, the white rectangle region in the middle image of each panel was further magnified to show the staining pattern of Foxp3 in CD4 T cells in the right image of each panel. Data are representative of the analysis of n >= 5 mice of 3 mo-old per group (A, B, C).

**Fig. 3** IL-21 promotes GC formation but inhibits Tfr in vivo. (A-D) IL-21 producing Adenovirus (Ad-IL21) or control Ad-LacZ were administred to 2.5 month old BXD2-II21−/− mice. Mice were analyzed 7 days later. (A) ELISA assay of serum IL-21 and TGF-β1 level in the mice. (B) Flow cytometry analysis of the of CXCR5+ICOS+Tfh cells (top panels), the frequency of Foxp3+ CXCR5+ICOS+ CD4 T cells (middle panels), and the expression of membrane TGF-β1 on Tfr cells (bottom panels) in the spleens of mice. Cells were first gated on CD4 T cells. (C) Bar graph show the numbers of Tfr cells and the ratios of splenic Foxp3+CXCR5+ICOS+Tfr cells versus Foxp3+CXC5+ICOS+Tfh cells. (D) Flow cytometry analysis of GL-7+Fas+ GC B cells in the spleens of mice. Gated CD19+ B cells are shown. Representative data or mean values ± s.d. are shown from two repeated experiments analysing 5 mice per group. ** p<0.01, ***p< 0.005.

**Fig. 4** IL-21 inhibits Tfr programing in vitro by preventing p-Akt. (A-B) Spleen cells from WT-BXD2 and BXD2-II21−/− mice were cultured in media only or with IL-21 for 16 hours. Flow cytometric analysis of Foxp3 (A) or membrane TGF-β1 and GITR (B) on CXCR5+ICOS+CD4 T cells. Numbers besides the gating indicated mean frequency ± s.d. (A). (C) Quantitative RT-PCR analysis of the indicated gene expression on sorted Tfr
cells from BXD2-Il21−/− mice after IL-21 stimulation for 4 hours. (D) Flow cytometry analysis of the phosphorylation of Akt (left panels top) and Stat3 (left panels bottom) in CXCR5+ICOS+CD4 T cells after IL-21 stimulation for 15 minutes; flow cytometry analysis of Foxp3 expression in CXCR5+ICOS+CD4 T cells after treatment of LY294002 (p-Akt inhibitor) for 16 hours (right panels top); bar graphs show the frequency of Foxp3 after the treatment (right panels bottom). Representative or mean values ± s.d. from two experiments using 3 mice at 3 months of age per group is shown. *p < 0.05, ** p<0.01, ***p< 0.005 compared with the unstimulated control group (A, C) or compared with control group and different concentration group (D right panels bottom).

**Fig.5**  IL-21 counteracts the Tfr-mediated inhibition of Tfh proliferation and B cell functions in vitro. (A-D) FACS sorted Tfr cells from spleens of BXD2-Il21−/− mice (A) were co-cultured with responder B cells or Tfh cells from spleens of WT-BXD2 mice with or without IL-21 (50ng/ml) for 3 to 5 days under B cells or T cell stimulating conditions. The ratio of B cells: Tfh: Tfr is 3:1:1 unless otherwise stated. Tfh from BXD2-Il21−/− or Tfr from BXD2 or BXD2-Il21r−/− mice were used in control co-cultures with responder cells. (B) Plots and bar graphs show the percent of divided Tfh cells indicated by CFSE dilution on day 3. (C) ELISA assay for IgG level in supernatant on day 5 in B cells and Tfr co-culture system. (D) ELISA assay for IgG level in supernatant on day 5 in B cells, Tfh and Tfr co-culture system. Representative results or mean values ± s.d. are shown from three experiments with pooled cells from 5 mice of 3-4 mo-old per group. *p < 0.05 compared between the indicated groups or with all other groups.
Fig. 6  IL-21−/− Tfr cells decreased GC formation and autoantibody production in BXD2 mice. FACS sorted Tfr cells from BXD2-IL21−/− mice were transferred into young BXD2 mice of 2-month age by i.v. (8x10⁵ cells per mouse). Mice without cell transfer were used as control. Mice were sacrificed on day 17 (A). (B) Flow cytometry analysis of the percentage of GL-7⁺Fas⁺ GC B cells (top panels) and CXCR5⁺ICOS⁺Tfh cells (bottom panels) in the spleens of mice. Cells were first gated on CD19⁺ B cells or CD4 T cells. Numbers in the plots indicated mean frequency ± s.d. (C) Immunofluorescence staining and confocal imaging of GC and Foxp3⁺CD4 T cells in GC of mice spleen tissue. Indicated markers were presented by the indicated colors in the image. Dash-oval circles indicate the border of GC. Small pictures in the lower right of each image represent the spleen size of each mouse group (left panels). (D) Bar graphs show the ELISPOT analysis of the IgG and IgM-autoantibody producing B cells from the spleens of mice (left panels) or show the IgG and IgM autoantibody levels in sera by ELISA assay (right panels). Representative results or mean values ± s.d are shown from duplicate experiments analyzing 4 mice per group. *p < 0.05, ** p<0.01 compared with the control group without cell transfer (B, D).
Figure 1. IL-21 Deficiency in Autoimmune BXD2 Mice Results in Defective Tfr but not Conventional Treg Responses
Figure 2. Increased Tfr cells in BXD2-II21- Mice
Figure 3. IL-21 Promotes GC Formation but Inhibits Tfr in vivo
Figure 4. IL-21 Inhibits Tfr Programming \textit{in vitro} by Preventing p-Akt
Figure 5. IL-21 Counteracts the Tfr-Mediated Inhibition of Tfh Proliferation and B Cell Functions \textit{in vitro}
Figure 6. IL-21⁺ Tfr Cells Decreased GC Formation and Autoantibody Production in BXD2 Mice
IL-17RA IS ESSENTIAL FOR OPTIMAL LOCALIZATION OF FOLLICULAR T HELPER CELLS IN THE GERMINAL CENTER LIGHT ZONE TO PROMOTE AUTOANTIBODY-PRODUCING B CELLS

by

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Abstract

Germinal centers provide a microenvironment that promotes and regulates the interactions of B-cells with follicular T-helper cells (T\textsubscript{FH}). Here we show that there are significantly higher frequencies of CXCR5\textsuperscript{+}ICOS\textsuperscript{+} T\textsubscript{FH} cells in autoimmune BXD2 mice, and these cells express both interleukin (IL)-21R and IL-17RA. Although IL-17 and IL-21 are both important for the formation of spontaneous GCs and development of pathogenic autoantibodies, IL-21, but not IL-17, is required for the proper development of T\textsubscript{FH} cells in BXD2 mice. The total numbers of T\textsubscript{FH} cells and their ability to induce B cell responses in vitro were not affected by a deficiency of IL-17RA in BXD2-Ill17ra\textsuperscript{−/−} mice, the majority of CXCR5\textsuperscript{+} T\textsubscript{FH} cells from BXD2-Ill17ra\textsuperscript{−/−} mice were, however, not localized in the GC light zone (LZ). Interruption of IL-17 signaling, either acutely by AdIL-17R:Fc or chronically by Ill17ra\textsuperscript{−/−}, disrupted T\textsubscript{FH}–B interactions and abrogated the generation of autoantibody-forming B cells in BXD2 mice. IL-17 up-regulated the expression of regulator of G-protein signaling (RGS)16 to promote the ability of T\textsubscript{FH} to form conjugates with B cells which was abolished in T\textsubscript{FH} cells from BXD2-Rgs16\textsuperscript{−/−} mice. The results suggests that IL-17 is an extrinsic stop signal that acts on post-differentiated IL-17RA\textsuperscript{+} T\textsubscript{FH} to enable its interaction with responder B cells in the LZ niche. These data suggest a novel concept that T\textsubscript{FH} differentiation and its stabilization in the LZ are two separate checkpoints and that IL-21 and IL-17 act at each checkpoint to enable pathogenic GC development.
**Introduction**

Germinal centers (GCs) are essential in promoting T-dependent immune responses (1, 2). They provide a microenvironment that promotes and regulates the interactions of B-cells with follicular T-helper cells (T\textsubscript{FH}), which provide the cognate help required for the generation of high-affinity, antibody-producing plasma cells and memory B cells (3, 4). Spontaneous GC formation has been demonstrated in various mouse models of autoimmune disease (5), and dysregulated GC formation or function may contribute to autoimmune disease in humans (6, 7). Dysregulation of GCs is often caused by aberrant accumulation or function of T\textsubscript{FH} cells (8, 9), and an increase in the numbers of T\textsubscript{FH} cells has been correlated with the severity of disease in several autoimmune conditions, including systemic lupus erythematosus (SLE) (10-12). In mouse models of lupus, the elimination or defective function of GC T\textsubscript{FH} cells reduces or even abolishes the disease (5, 13).

High levels of IL-21 are found in the circulation of patients with SLE (14), and disruption of IL-21 signaling can inhibit GC formation and autoantibody production as well as ameliorate autoimmune disease in mice (15). Although IL-21 is important but not necessarily essential for T\textsubscript{FH} development (16-18), IL-21 is considered a signature cytokine of T\textsubscript{FH} cells (15, 19, 20). IL-21 acts in an autocrine manner to stimulate the proliferation and survival of T\textsubscript{FH} cells, and hence has a direct effect on their accumulation and the associated formation of GCs (21, 22). IL-21 directly induces high expression of Bcl6, the master transcription factor for T\textsubscript{FH} differentiation, which is important for the sustained expression of CXCR5 and also promotes the expression of other T\textsubscript{FH} co-
stimulatory molecules supporting cognate help to B cells (20, 23, 24). The CXCL13–CXCR5 interaction mediates the migration of TFH cells to the GC light zone (LZ) where the affinity maturation of B cells takes place (25, 26).

Until recently, the potential role of IL-17A in GC formation and function received less attention, as TFH cells has been shown to not to produce IL-17A (19). High levels of IL-17 and Th17 cells are commonly found in patients with autoimmune disease (27) and have now been shown to drive GC and ectopic lymphoid follicle development in mouse models of autoimmune disease (28, 29). Using the BXD2 model of systemic autoimmune disease, we established that Th17 cells can be identified in the spontaneous GCs (28, 30) and that IL-17 promotes the formation of GCs in these mice (28). Analysis of the effects of IL-17 on B cells in the GCs indicate that it modulates the expression of regulators of G protein signaling (RGS) proteins, which promote desensitization of B cell chemotaxis to chemokines CXCL12 and CXCL13 and stabilization of interactions of the GC B cells with nearby T-helper cells (31).

IL-17A signals through the IL-17RA–IL-17RC heterodimeric receptor complex (32). The original cloning of IL-17RA revealed that T cells as well as B cells can express high levels of Il17ra and can be responsive to IL-17 stimulation (33). Although it has been demonstrated that IL-17 is not required for the development of TFH cells (22) but may be involved in antibody production (29, 34), the possibility that IL-17 can act cooperatively with IL-21 to affect the function of TFH cells in regulating GC responses has not been explored. Here, we provide evidence that, compared with normal B6 mice, there are
significantly higher frequencies of \( \text{TF}_{\text{FH}} \) cells in autoimmune BXD2 mice, and these cells express high levels of both IL-21R and IL-17RA. Although \( \text{TF}_{\text{FH}} \) was dramatically deficient in BXD2-\( \text{Il21}^{-/-} \) mice, the percentage and the direct B-cell support function of \( \text{TF}_{\text{FH}} \) were not affected by IL-17RA deficiency. We have established that the IL-17RA signal is important for securing the localization of \( \text{TF}_{\text{FH}} \) in the GC LZ in BXD2-\( \text{Il17ra}^{-/-} \) mice. Such anatomic stabilization is needed for \( \text{TF}_{\text{FH}} \) to induce its function and hence is a unique \( \text{TF}_{\text{FH}} \) checkpoint in autoreactive GCs.
Materials and Methods

Mice.
C57BL/6 (B6) and BXD2 recombinant inbred mice were obtained from The Jackson Laboratory, B6-II17ra<sup>−/−</sup> mice were obtained from Amgen, Inc. (Thousand Oaks, CA), B6-Rgs16<sup>−/−</sup> mice were a gift from Dr. Kirk M. Druey, and B6-Il21<sup>−/−</sup> mice were obtained from the Mutant Mice Regional Resource Center (Davis, CA). These mice were backcrossed with BXD2 mice for seven generations by a marker-assisted speed congenic approach. All mice were housed in the University of Alabama at Birmingham (UAB) Mouse Facility under specific pathogen-free conditions in a room equipped with an air-filtering system. The cages, bedding, water, and food were sterilized. All mouse procedures were approved by The UAB Institutional Animal Care and Use Committee.

Administration of AdLacZ and AdIL-17R:Fc.
AdIL-17R:Fc and AdLacZ (2x10<sup>9</sup> p.f.u. per mouse), generous gifts from Dr. Jay Kolls (University of Pittsburgh) (35), were administered intravenously (i.v.). Spleen tissue and cells were analyzed 10 days later.

T cell adoptive transfer.
Effector T cells (CD62L<sup>−/lo</sup> CD4<sup>+</sup>) from BXD2 and BXD2-Rgs16<sup>−/−</sup> mice were sorted by FACS Aria II and i.v. injected into BXD2-II17ra<sup>−/−</sup> mice (3x10<sup>6</sup> cells in 200 µl PBS per mouse). Recipients were sacrificed on Days 6 and 21. Spleen tissue and spleen cells were collected for the indicated analysis.
NP-CGG immunization.

B6 and B6-Il17ra<sup>−/−</sup> mice were immunized i.p. with 50 μg of NP–chicken gamma globulin (NP-CGG; BioSearch Technologies, Novato, CA) adsorbed to 1.3 mg alum (Sigma-Aldrich) in a total volume of 100 μl NP-CGG alum/ PBS. Mice were sacrificed on day 6, 9 and 12. Spleen tissue and spleen cells were collected for the indicated analysis.

Flow cytometry analysis.

Single-cell suspensions of whole-spleen cells or migrated cells collected from the lower chamber in a cell migration assay were labeled by surface staining with the following antibodies: Pacific blue– or Alexa488– (RM4-5, GK1.5, Biolegend) or PE–anti-CD4 (RM4-5, Biolegend); Pacific blue–anti-CD19 (6D5, Biolegend); Alexa647–anti-GL-7 (GL7, eBioscience); APC–anti-B220 (RA3-6B2, Biolegend); PE–Cy7-anti-CXCR5 (2G8, BD Biosciences); FITC– or PE– (398.4A, 7E.17G9, eBioscience) or PE–Cy5-anti-ICOS (15F9, Biolegend). Antibodies against PD-1(RMP1-30, Biolegend), CD28 (37.51, eBioscience), CD40L (MR1, eBioscience), CD62L (MEL-14, BD Biosciences), CD44 (IM7, Biolegend), IL-21R (eBio4A9, eBioscience), IL-17RA (PAAJ-17R, eBioscience), and Fas (15A7, eBioscience) were all conjugated with PE. Peanut agglutinin (PNA) was conjugated with biotin (Vector Laboratory, Burlingame, CA) and was detected by Alexa450-conjugated streptavidin (Invitrogen).

For nuclear transcription factor staining, cells were labeled with surface markers, then fixed and permeabilized with the FoxP3 Staining Buffer Set (eBioscience), according to
the manufacturer's instruction. Permeabilized cells were stained with PE–anti-Bcl6 (K112-91, BD Biosciences) and PE–anti-RORγt (Q31-378, BD Biosciences).

For cytokine-producing T cell analysis, cells were stimulated for 5 h with phorbol myristate acetate (PMA; 50 ng/ml; Sigma-Aldrich) and ionomycin (750 ng/ml; Sigma-Aldrich) in the presence of GolgiPlug (BD Biosciences). Cells were stained for surface markers and then fixed and permeabilized with Cytofix/Cytoperm solution (BD Biosciences) before intracellular staining with APC–anti-IL-17 (eBio17B7, eBioscience) and rmIL21R/h-Fc chimera (R&D Systems), followed by R-PE-conjugated goat anti-human IgG (Jackson Immunoresearch) (36). Samples were acquired with an LSRII FACS analyzer (BD Biosciences), and data were analyzed with FlowJo software (Tree Star, Inc. Ashland, OR, USA).

Except for T/B conjugates analysis, all flow cytometry analysis was carried out using a combination use of FSC and SSC height, area, and width parameters to exclude aggregated cells.

**Immunofluorescent staining of frozen sections.**

Spleens from mice were collected, embedded in Frozen Tissue Media (Fisher Scientific), and snap-frozen in 2-methylbutane. Frozen sections (8-µm thick) were fixed and processed as previously described (28). Unless specified, all reagents and antibodies were purchased from Invitrogen: Biotin–PNA (Vector Laboratory) followed by SA–Alexa 350; Alexa 555–anti-IgM; Alexa 488–anti-CD35/21 (clone 8C12, BD Bioscience); Dylight
649–goat-anti-mouse IgG (Poly4053, Biolegend); Alexa 647–anti-CD4 (GK1.5, Biolegend); rat anti-mouse-CXCR5 (2G8, BD) followed by goat anti-rat IgG–biotin (Southern Biotech) and SA–Alexa488; rat anti-IL-17 (TC11-18H10.1, Biolegend) and Alexa 555–goat-anti-rat IgG (A21434); goat anti-mouse IL-21 (AF594, R&D Systems) and anti-IL-17R (AF448, R&D Systems) followed by Alexa 647-donkey-anti-goat IgG. Application of chicken anti-mouse RGS16 (NB300-350, Novus Biologicals) and goat anti-chicken IgY–HRP (ab6877, Abcam) was followed by the tyramide signal amplification method (TSA Kit, T20931) and SA–Alexa-488. Sections were mounted in the Slow Fade Gold Antifade reagent and images were captured with a Leica DMIRBE inverted Nomarski/epifluorescence microscope outfitted with Leica TCS NT laser confocal optics. Confocal imaging intensity analysis was carried out using ImageJ software, version 1.4, developed by the U.S. National Institutes of Health (and available on the Internet at http://rsb.info.nih.gov/nih-image/), with background intensity subtracted from each image.

**Analysis of T_{FH}–B cell conjugates.**

Cells were labeled with fluorochrome antibodies against CD3, CD4, B220, CD19, CXCR5, ICOS, and IL-17RA as needed. For T/B conjugates analysis, the doublets based on FSC and SSC height, area and width were included before gating on CD3^+CD4^+ cells. The phenotypes of CD4 T cells forming conjugates with B cells were then identified by FACS sorting of CD4^+B220^+ or CD4^+CD19^+ doublets. The collected conjugates were dissociated with 2 mM EDTA/PBS, as described (37). Cells were then washed and resuspended for flow cytometry acquisition of the cell population distribution.
Real-time quantitative RT-PCR to detect Rgs16.

RNA isolation, cDNA synthesis, and real-time PCR reactions were carried out as we described previously (28). The following primers were used: Rgs16, 5’-GGTACTTGGCTACTCGCTTTTCC-3’ (F), 5’-CAGCCCGGTCTTTGAACTCTCT-3’ (R); Gapdh, 5’-AGGTCGGTGTGAACGGATTTG-3’ (F), 5’-TGTAGACCATGTAGTTGAGGTCA-3’ (R).

Western blot analysis for RGS16 in CD4 T cells.

Spleen CD4+ T cells from BXD2 mice were purified using a MACS column (Miltenyi Biotech) and cultured with either medium alone or with rmIL-17 (30 ng/ml, R&D Systems) for 1 or 24 h. Collected cells were subjected to protein extraction and western immunoblot analysis using the method we described previously (31). Chicken anti-mouse RGS16 (NB300-350, Novus Biologicals) was used at 1:2000 followed by goat anti-IgY–HRP (ab6877, Abcam) at 1:1000. Rabbit anti-mouse GAPDH was used at 1:5000 followed by anti-rabbit HRP-conjugated Ab (Cell Signaling Technology) at a 1:3000 dilution. HRP Abs were detected using chemiluminescence reagent (Pierce).

ELISA.

Serum levels of autoantibodies and total IgG levels in cultured supernatant were determined by ELISA, as we described previously (38). BiP was purchased from Assay Designs, Inc. and all other autoantigens from Sigma-Aldrich. Urinary albumin was
analyzed using the competitive Albuwell M ELISA kit (Exocell, Inc.), as described previously (38).

**ELISPOT quantification of autoantibody-producing B cells.**

The ELISPOT procedure was carried out using the method we previously described (30). Briefly, polyvinylidene difluoride-backed 96-well plates (Millipore) were coated overnight at 4°C with 5 mg/ml of the indicated autoantigen. BiP was purchased from StressMarq Biosciences Inc. (Victoria BC, Canada), NP7-BSA was purchased from BioSearch Technologies, and all other autoantigens were purchased from Sigma-Aldrich. Total spleen cells in 100 µl complete medium, containing 5x10^5 cells per well for detecting IgM and 2x10^6 per well for detecting IgG, were cultured overnight in a 37°C incubator. Plated cells were then incubated at RT with HRP-labeled goat anti-mouse IgG/M (Southern Biotechnology) in PBS with 1.5% BSA for 3–4 h. Spots were developed with 3-amino-9-ethylcarbazole. Plates were read by an automatic ELISPOT reader (CTL) and analyzed with Immunospot 3.1 software (CTL).

**Cell migration assay.**

Single-cell suspensions of spleen CD4 T cells were purified using a MACS column (Miltenyi Biotech) and stimulated with medium alone or with mrIL-17 (30 ng/ml, R&D Systems) for 3 h. The cell migration assay was carried out as described (39). The stimulated cells (2 x 10^6) were loaded into the upper well insert (5-µm pore size) of a Transwell system (Costar), and medium alone or medium with CXCL13 (100 ng/ml, R&D Systems) was added to the bottom chamber. After incubation for 2 h at 37°C in an
incubator, the cells that had migrated down to the lower chamber were collected and counted, and the percentage of CXCR5^+ICOS^+ CD4 T cells (T_{FH}) was determined by flow cytometry in some experiments. The chemotaxis index of total CD4 T cells or T_{FH} cells was calculated by dividing the number of cells that migrated in the presence of CXCL13 by the number of cells that migrated in the absence of CXCL13 (39).

**T_{FH}-B cell coculture, proliferation assay by the Click-iT® EdU assay and IgG secretion detection.**

Anti-CD19 MACS column (Miltenyi Biotech)-purified B cells from BXD2 were co-cultured in a 3:1 ratio with FACS sorted T_{FH} cells and other CD4 T subsets based on CXCR5 and ICOS expression. T cells were stimulated with anti-CD3 (1 µg/ml) and anti-CD28 (2 µg/ml). T cells and B cells were co-cultured for 2–3 days. One hour before collection, 5-ethynyl-2′-deoxyuridine (EDU, Invitrogen) was added to the cell culture. EDU intensity together with anti-B220 and anti-Thy1.2 staining were determined by flow cytometry analysis, according to the manufacturer’s instructions. For analysis of Ig secretion, T cells and B cells were co-cultured for 3 days, supernatant was collected and total IgG were detected by ELISA.

**Histology assessment of kidney tissue sections.**

Kidney tissues collected from the mice were fixed in 10% buffered formalin and then processed to make a paraffin-embedded block. Tissue sections (5 µm) were stained with hematoxylin and eosin (H&E) and examined by light microscopy.
Statistical analysis.

All results are shown as the mean ± standard deviation (s.d.). A two-tailed t test was used when two groups were compared for statistical differences. An ANOVA test was used when more than two groups were compared for statistical differences. P values less than 0.05 were considered significant.
**Results**

**T<sub>FH</sub> cells express the IL-17RA in BXD2 mice**

BXD2 mice spontaneously develop a lupus-like syndrome with high titers of autoantibodies and lupus nephritis (30, 40). The disease manifests with age and correlates with the formation of GCs in the spleen (31, 41). At 10 to 12 weeks of age, the mice have well-defined GCs with a prominent LZ and dark zone (DZ) (28, 41). Analysis of the CD4<sup>+</sup> T cells in the spleens of BXD2 mice of this age indicated that the frequency and cell count of CXCR5<sup>+</sup>ICOS<sup>+</sup>CD4<sup>+</sup> T cells was significantly higher than in B6 mice (Fig.1A, 1B, Supplementary Fig.1A, 1B). This subpopulation of cells expressed higher levels of PD-1, CD28, CD40L, CD44, IL-21R, (Fig.1C, Supplementary Fig.1C), and Bcl6 (Fig. 1D) but lower levels of CD62L than CXCR5<sup>-</sup>ICOS<sup>-</sup> and CXCR5<sup>+</sup>ICOS<sup>-</sup>CD4<sup>+</sup> T cells. Approximately 60–70% of the CXCR5<sup>-</sup>ICOS<sup>+</sup>CD4<sup>+</sup> T cells expressed Bcl6, and the highest frequency of cells expressing IL-21 was found in this subpopulation of CD4<sup>+</sup> T cells (Fig.1E, Supplementary Fig.1D, 1E). A subpopulation of the CXCR5<sup>+</sup>ICOS<sup>+</sup>CD4<sup>+</sup> T-cell population expressed RORγt (Fig. 1D) and IL-17 (Fig. 1E). The CXCR5<sup>-</sup>ICOS<sup>+</sup> subpopulation of CD4 T cells predominately expressed IL-17 and contained a population of RORγt<sup>+</sup> cells (Fig. 1D, 1E, Supplementary Fig. 1E). However, very few cells in either subpopulation expressed both cytokines (Fig. 1E). The percentage of IL-17<sup>+</sup> or IL-21<sup>+</sup> cells from either subpopulation was significantly higher in BXD2 mouse spleen compared with B6 (Supplementary Fig. 1D, 1E). The current results show that IL-17 and IL-21 are produced from two distinct populations of CD4 T cells and that a subset of T<sub>FH</sub> cells in the GCs of BXD2 mice may express IL-17 rather than IL-21.
On analysis of the CXCR5+ CD4+ cells in the spleens of BXD2 mice, we found that a very high percentage (~80–90%) of these cells expressed IL-17RA (Fig. 1C, Supplemental Fig. 1C), which indicates that the T_{FH} cells in the spleen of BXD2 mice can potentially respond to IL-17. By confocal microscopic analysis of spleen sections, we found that the LZ contained an apparent population of CD4+ T cells in close proximity to CD35+ follicular dendritic cells (FDCs), with a distinct CD4 T cell zone located adjacent to the dark zone (DZ) of the GC (Fig. 1F). The majority of CD4 T cells localized in the LZ end of the GC were CXCR5+ (arrows indicated, yellow color, Fig. 1G). CD4 T cells localized to the LZ of the GC also expressed IL-21 (Fig. 1H), IL-17 (Fig. 1I), and IL-17RA (Fig. 1J). These results demonstrate that there was a distinct population of CXCR5+CD4+ T cells in the LZ that express IL-17RA and either IL-21 or IL-17 in the BXD2 mice. Such IL-17 producing T cells may represent a unique population of IL-17 producing T_{FH}, as identified by their surface phenotype and location.

**Deficiency of either IL-21 or IL-17RA compromises GC development**

Although it is shown that blockade of IL-21 or IL-17 signaling can affect GC formation and ameliorate autoimmune disease, it is not known whether the effects of the two cytokines on these aspects of the immune response are identical in BXD2 mice. To answer this question, we generated BXD2-Il17ra−/− and BXD2-Il21−/− mice. Compared with wild-type BXD2 (BXD2-WT) mice, there were lower levels of anti-histone and anti-DNA autoantibodies of the IgM, IgG2b, and IgG2c isotype in both BXD2-Il17ra−/− and BXD2-Il21−/− mice (Fig. 2A). Kidney disease, as determined by the levels of urinary albumin (Fig. 2B), the extent of inflammatory infiltrates (Fig. 2C), and IgG deposition in
kidney glomeruli (Fig. 2D, 2E) was alleviated in both BXD2-Il17ra−/− and BXD2-Il21−/− mice. Splenomegaly, which is a key autoimmune feature of BXD2-WT mice, was also reduced in both BXD2-Il17ra−/− and BXD2-Il21−/− mice (Supplementary Fig. 2A, 2B). The frequency of GC B cells (Fas+PNA+CD19+ cells) was significantly lower in BXD2-Il17ra−/− and BXD2-Il21−/− mice than in BXD2-WT mice (Fig. 2F). Defective formation of GCs in BXD2-Il17ra−/− and BXD2-Il21−/− mice was apparent by confocal imaging analysis (Fig. 2G) and quantitation of the fractional area of PNA+ B cells in each IgM+ spleen follicle by ImageJ analysis (Fig. 2H). These data indicated that both IL-17RA and IL-21 are required for optimal GC responses and autoimmune disease development in BXD2 mice.

High numbers of functional CXCR5+ICOS+ T<sub>FH</sub> cells in BXD2-Il17ra−/− mice

IL-21 is important for the development of T<sub>FH</sub> (21, 22), especially in chronic autoimmune response (15), whereas IL-17 is not required for the development of T<sub>FH</sub> cells in normal mice (22). The total CD4+ T-cell counts in the spleens of BXD2-Il21−/− and BXD2-Il17ra−/− mice were lower than in BXD2-WT mice (Supplementary Fig. 2C). There were significantly fewer CXCR5+ICOS+ T<sub>FH</sub> cells in the spleens of BXD2-Il21−/− mice than in BXD2-WT mice (Fig. 3A, 3B, Supplementary Fig. 2D, 2E). The deficiency in IL-21, however, also resulted in a reduction in the frequency of IL-17+ CXCR5+ICOS+ CD4+ T cells (Fig. 3C), which is consistent with the report that IL-21 can act to promote the formation of T<sub>H</sub>17 cells (42, 43).
Surprisingly, the frequency of CXCR5+ICOS+ $T_{FH}$ cells was significantly higher in the spleens of BXD2-$Il17ra^-/-$ mice than in BXD2-WT mice, which resulted in an equivalent cell count for this subset in both strains of mice (Fig. 3A, 3B and Supplementary Fig. 2D, 2E). The frequency of IL-21+ cells within the CXCR5+ICOS+ CD4 T subset from BXD2-$Il17ra^-/-$ mice was equivalent to that in the BXD2-WT mice, whereas the frequency of IL-17-expressing cells was significantly higher (Fig. 3C). The expression of CD28, CD40L, CD62L, CD44, and IL-21R was similar in CXCR5+ICOS+ $T_{FH}$ cells from BXD2-WT and BXD2-$Il17ra^-/-$ mice, although the expression intensity of PD-1 was slightly, but not significantly, lower (Fig. 3D, Supplementary Fig. 2F).

To test the B-cell helper function of CXCR5+ICOS+ CD4+ T cells from BXD2-$Il17ra^-/-$ mice, we co-cultured anti-CD3- plus anti-CD28-stimulated CXCR5+ICOS+ $T_{FH}$ cells and other CD4 T subsets based on CXCR5 and ICOS expression from either BXD2-WT or BXD2-$Il17ra^-/-$ mice with B cells from BXD2-WT mice. The ability of CXCR5+ICOS+ CD4 T cells from BXD2-$Il17ra^-/-$ to promote B cell activation and IgG secretion was equivalent to those from BXD2-WT mice (Fig. 3E, upper, 3F). The ability of CXCR5+ICOS+ CD4 T cells from both groups to promote IgG secretion was greater compared with other subsets of CD4 T cells, supporting the role of CXCR5+ICOS+ CD4 T cells as the $T_{FH}$ subset (Fig. 3F). The proliferative response of CXCR5+ICOS+ CD4+ T cells from BXD2-$Il17ra^-/-$ mice to anti-CD3 plus anti-CD28 stimulation was even higher than those from BXD2-WT mice (Fig. 3E, lower). Thus, despite the dramatically reduced development of spontaneous GCs in the BXD2-$Il17ra^-/-$ mice, the numbers of $T_{FH}$ cells
from these mice were not reduced, and these cells exhibited equivalent ability, compared to those from WT mice, to support the activation and IgG secretion of B cells \textit{in vitro}.

**Mislocation and reduced contact of T\textsubscript{FH} cells with B cells in BXD2-Il17ra\textsuperscript{−/−} mice**

Confocal imaging analysis confirmed that, in the spleen follicles of BXD2-WT mice, the majority of CXCR5\textsuperscript{+}CD4\textsuperscript{+} T cells were localized in the LZ of the PNA\textsuperscript{+} GC area (Fig. 4A, left panels). Although intact white pulp follicles were found in the spleens of BXD2-Il21\textsuperscript{−/−} mice, PNA\textsuperscript{+} GC structures could not be identified, and almost no CXCR5\textsuperscript{+} T\textsubscript{FH} cells were present (Fig. 4A, right panels). By contrast, marked mis-localization of the CXCR5\textsuperscript{+} CD4\textsuperscript{+} T cells was apparent in the spleens of BXD2-Il17ra\textsuperscript{−/−} mice. In these mice, many CXCR5\textsuperscript{+} CD4\textsuperscript{+} T cells were located outside the PNA\textsuperscript{+} GC area and were not in immediate contact with PNA\textsuperscript{+} GC B cells (Fig. 4A, middle panels).

To determine whether IL-17RA signaling is required for aggregation of T\textsubscript{FH} in GC LZ, we neutralized endogenous IL-17 in WT BXD2 mice by administration of AdIL-17R:Fc. Following such treatment, there was a dramatic reduction in the localization of CXCR5\textsuperscript{+}CD4\textsuperscript{+} T cells to the GC LZ, and the majority of these cells were not in close contact with PNA\textsuperscript{+} GC B cells (Fig. 4B, right). By contrast, the frequency and location of CXCR5\textsuperscript{+} CD4\textsuperscript{+} T cells in spleens of control AdLacZ-treated BXD2 mice were similar to those observed in untreated BXD2 mice, with the majority of these cells found in the LZ of the GCs (Fig. 4B, left). Consistent with our previous results (25), the size, weight, and cellularity of the spleen and the numbers of autoantibody-producing B cells were significantly reduced in mice treated with AdIL-17R:Fc, compared with mice treated with
AdLacZ (data not shown). These results suggest that neutralization of IL-17 also disrupted the interaction of T_{FH} and GC B cells and perturbed the generation of autoantibody-producing B cells.

We next determined whether mis-location of T_{FH} cells was associated with loss of CD4^{+} T–B cell conjugate formation in the spleens of BXD2-Il17ra^{-/−} mice. A significantly higher percentage of B cell–CD4 T cell doublets was isolated from the spleens of BXD2-WT mice than of B6 mice (Fig. 4C upper panel and Fig. 4D) and the frequency of CXCR5^{+}ICOS^{+} T_{FH} and CXCR5^{−}ICOS^{+} CD4 T cells in the disassociated conjugates was higher in BXD2-WT mice than in B6 or BXD2-Il21^{-/−} mice (Fig. 4C, lower panel and Fig. 4E). Interestingly, although there was an increased frequency of CXCR5^{+}ICOS^{+} T_{FH} and CXCR5^{−}ICOS^{+} CD4 T cells in the spleens of BXD2-Il17ra^{-/−} mice, the frequency of these CD4 T cells forming conjugates with B cells was lower in BXD2-Il17ra^{-/−} than in BXD2 mice (Fig. 4C, lower panel and Fig. 4E). These results indicate that T_{FH} cells in BXD2-Il17ra^{-/−} mice were unable to locate to the GC LZ and that their ability to interact with B cells was compromised.

**IL-17 up-regulates RGS16 to mediate T_{FH} location in GC LZ**

It has been reported that up-regulation of RGS16 is associated with a desensitization of both B cells and CD4^{+} T cells to CXCL12 and CXCL13 migratory cues (39, 44), thereby prolonging retention of both cells in the GC for efficient interaction. IL-17 can induce up-regulation of RGS16 proteins in B cells, resulting in migration arrest (31). We investigated the association between IL-17 signaling and RGS16 expression in CD4^{+} T cells.
cells. IL-17 stimulation of CD4$^+$ T cells isolated from the spleens of BXD2 mice resulted in a significant increase in RGS16 at both the mRNA (Fig. 5A) and protein levels (Fig. 5B). Analysis of IL-17 modulation of the T$\text{FH}$ chemotactic response to CXCL13 showed that pretreatment with IL-17 inhibited CXCL13-induced migration of T$\text{FH}$ cells from BXD2-WT mice in response to CXCL13 in the transwell assay (Fig. 5C). A similar result was found in CXCR5$^+$ICOS$^-$ CD4 T cells that also expressed both CXCR5 and IL-17RA (data not shown), suggesting that the migration desensitizing effect of IL-17 is not limited to CXCR5$^+$ICOS$^+$ T$\text{FH}$ cells. In contrast, pretreatment with IL-17 did not inhibit migration of T$\text{FH}$ from BXD2-Rgs16$^{-/-}$ mice (Fig. 5C). The possibility that T$\text{FH}$ cells from BXD2-Rgs16$^{-/-}$ mice expressed lower levels of IL-17RA compared with T$\text{FH}$ cells from BXD2-WT mice was ruled out by FACS analysis, which showed an equivalent intensity of IL-17RA (Supplementary Fig. 3A). These results suggest that RGS16 is involved in the migration arrest of T$\text{FH}$ cells stimulated by IL-17.

To further determine the function of IL-17-induced up-regulation of RGS16 in the localization of T$\text{FH}$ in vivo, we performed confocal imaging analysis. These studies revealed that RGS16 was highly expressed by cells in spleen follicles, including CD4 T cells, in the GC LZ of BXD2-WT mice but not in BXD2-Il17ra$^{-/-}$ mice (Fig. 5D, upper panels). Moreover, localization of RGS16$^+$CD4 T cells in GC LZ mirrored that of CXCR5$^+$ CD4 T cells in the GC LZ in the spleens of BXD2 mice (Fig. 5D, lower left). By contrast, in BXD2-Il17ra$^{-/-}$ mice, few RGS16$^+$ cells were detected in spleen follicles, and this was associated with fewer CXCR5$^+$CD4 T cells localized in the GC LZ (Fig. 5D, lower right). We also observed reduced numbers of CXCR5$^+$ CD4 T cells in the spleen
GC LZ of BXD2-Rgs16−/− (Fig. 5E, right) compared with BXD2-WT mice (Fig. 5E, left). FACS analysis revealed a lower frequency of T_{FH} cells in the spleens of BXD2-Rgs16−/− mice than in the spleens of BXD2-WT mice (Supplementary Fig. 3B), and confocal imaging demonstrated that the size of the GCs in the spleens of BXD2-Rgs16−/− mice was also smaller than the size of the GCs formed in BXD2 mice (Fig. 5E). Analysis of T cell–B cell conjugates further showed that there was reduced in vivo formation of CD4 T cell–B cell conjugates (Fig. 5F), with a lower frequency of CXCR5+ICOS+ CD4 T and CXCR5−ICOS+ CD4 T cells in the conjugates isolated from the spleens of BXD2-Rgs16−/− compared with those isolated from BXD2 mice (Fig. 5G, Supplementary Fig. 3C). Collectively, these results suggest that T_{FH} cells stimulated by IL-17 increased expression of a desensitizing regulator of chemotaxis, RGS16, resulting in migration arrest in the splenic GC LZ of BXD2 mice.

**IL-17 acts directly on effector CD4 T cells to promote the GC response**

We previously showed that IL-17 acts through the canonical NF-κB pathway to up-regulate RGS16 in B cells, thereby modulating B-cell migration responses (31). To rule out the possibility that the IL-17-induced stabilization of T_{FH} cells in the GCs is secondary to its effects on B cells, effector CD4 T cells (CD62L−/lo) from BXD2-WT and BXD2-Rgs16−/− mice were adoptively transferred into BXD2-Il17ra−/− mice. This strategy enabled us to discriminate between IL-17RA+RGS16+ and IL-17RA+RGS16− T_{FH} cells in an IL-17hi but IL-17RA-deficient microenvironment. Six days following transfer, it was apparent that donor effector CD4+ T cells from BXD2-WT mice caused an increased percentage of Fas+GL7+GC B cells and splenomegaly in BXD2-Il17ra−/−
recipients. By contrast, donor cells from BXD2-\textit{Rgs16}^{-/-} mice did not lead to significant changes in these phenotypes in the BXD2-\textit{Il17ra}^{-/-} recipients (Fig. 6A, Supplementary Fig. 4A). ELISPOT analysis of autoantibody-producing cells on day 21 also indicated that BXD2-WT, but not BXD2-\textit{Rgs16}^{-/-}, donor cells promoted autoantibody production associated with dramatically elevated numbers of autoantibody-forming B cells in BXD2-\textit{Il17ra}^{-/-} recipients (Fig. 6B, Supplementary Fig. 4B).

Analysis of conjugate formation further revealed that donor IL-17RA\,^{+} CD4\,^{+} T cells and CXCR5\,^{+}ICOS\,^{+} T_{FH} cells from BXD2-WT mice had a significantly enhanced ability to form conjugates with recipient B cells compared with the same population of CD4\,^{+} T cells from either BXD2-\textit{Rgs16}^{-/-} donors or from endogenous recipient BXD2-\textit{Il17ra}^{-/-} cells (Fig. 6C, Supplementary Fig. 4C). Real-time RT-PCR showed that, on day 6 post-transfer of BXD2-WT effector CD4\,^{+} T cells, there was higher expression of \textit{Rgs16} in conjugated donor IL-17RA\,^{+} CD4\,^{+} T cells than in singlet donor cells and in recipient IL-17RA\,^{-} CD4\,^{+} T cells (Fig. 6D). These results further indicate that intact RGS16 in the IL-17RA\,^{+} CD4\,^{+} T effector cells is needed to promote the formation of spontaneous GCs, even in an environment where all other cells are IL-17RA-deficient.

\textbf{IL-17RA regulates T\textsubscript{FH} development and function during a T-dependent (TD) response in normal B6 mice.}

It is unknown whether IL-17RA-IL-17 signaling affects T\textsubscript{FH} in a similar pattern in the TD response in non-autoimmune mice. To address this, we analyzed the effect of IL-17 on CD4 T cells and T\textsubscript{FH} cells in normal B6 mice. CXCR5\,^{+}ICOS\,^{+} T\textsubscript{FH} cells from B6
expressed the highest level of IL-17RA followed by CXCR5+ICOS− CD4 T subset, whereas, the other CD4 T subsets expressed low levels of IL-17RA (Fig. 7A). IL-17 stimulation of CD4+ T cells isolated from the spleens of B6 mice resulted in a significant increase in Rgs16 at the 4 hour time point (Fig. 7B) and also inhibited migration of CD4 T cells in response to CXCL13 in the transwell assay (Fig. 7C). To further investigate TFH response in B6-Il17ra−/− mice during a TD response, B6-Il17ra−/− mice and WT B6 mice were immunized with NP(21)-CGG and sacrificed on day 6, 9 and 12. The results indicate that the frequency of TFH peaked on day 9, and began to decrease by day 12 after NP-CGG immunization. The Tfh frequency was synchronized with that of anti-NP IgG antibody level in sera, whereas the GC B population peaked on day 12. On day 9, the frequency of CXCR5+ICOS+ Tfh was approximately two folds higher in B6-Il17ra−/− mice, compared with WT B6 (Fig. 7D, left). Although IL-21 production was comparable, production of IL-17 by CXCR5+ICOS+ Tfh cells was significantly increased in B6-Il17ra−/− mice, compared with WT B6 (Fig. 7D, middle). The frequency of GC B cells was, however, higher in WT compared with B6-Il17ra−/− mice (Fig. 7D, right), which is consistent with the lower number of high affinity anti-NP IgG antibody producing B cells detected by ELISPOT in B6-Il17ra−/− mice (Fig. 7E). Confocal image analysis showed that, on day 9, CXCR5+ CD4 T cells could be localized in the LZ of the GCs in the spleen of WT B6 mice (Fig. 7F, left, yellow color). By contrast, although many CXCR5+ CD4 T cells were identified in the spleen of B6-Il17ra−/− mice, these CXCR5+ CD4 T cells were not localized to the LZ of the smaller GCs found in these mice (Fig. 7F, right, yellow color). These results indicate that, even in normal mice, IL-17RA-IL-17
signaling can regulate $T_{FH}$ cell localization in GC LZ via up-regulation of $Rgs16$ in $T_{FH}$ cells to promote GC development and high affinity antibody production.
Discussion

Analysis of the CD4\(^+\) T subpopulations in the spleens of BXD2 mice provided several unique insights into the regulation of T\(_{FH}\) cell function. First, we found that the majority of cells that expressed the high levels of CXCR5 typical of T\(_{FH}\) cells also expressed high levels of IL-17RA, and thus were potentially capable of responding to IL-17. Second, our data suggest that in BXD2 mice, a subset of CD4\(^+\) T cells with phenotypic characteristics of T\(_{FH}\) cells was capable of producing IL-17 rather than IL-21. CD4\(^+\) T cells with the characteristics of T\(_{H17}\) cells (CXCR5 \(^{\text{ICOS}^+}\)) were also present. Third, the loss of IL-17RA affected the stabilization of T\(_{FH}\) in GC LZ. These results together suggest that IL-17 plays an essential role in stabilizing T\(_{FH}\) location and close contact with B cells in GCs. IL-17 thus complements the effects of IL-21, and both cytokines are needed for pathogenic GC development in BXD2 mice, as loss of either cytokine dramatically abrogated this response.

The parameters that should be used to define T\(_{FH}\) cells are currently a matter of debate. Moreover, the relationship of T\(_{FH}\) cells to other CD4\(^+\) T cell populations, including T\(_{H17}\) cells, is unclear and it has been suggested that there is a considerable degree of plasticity (19, 45). A recent study shows that, in human tonsils, while IL-21 was the major cytokine produced by CXCR5\(^{hi}\) T\(_{FH}\) cells, IL-17 was produced mainly by CXCR5\(^{lo}\)-CD4 T cells (24). However, in autoimmune BXD2 mouse spleen, IL-17 and IL-21 could be produced by two separate subsets of CXCR5\(^{i}\)ICOS\(^+\) T\(_{FH}\) cells and IL-17 could be further identified in CXCR5 \(^{\text{ICOS}^+}\) CD4 T cells. Interestingly, these IL-17 producing T\(_{FH}\) cells are different from the conventional T\(_{H17}\) by their surface phenotype and location.
In agreement with previous studies (22), we found that IL-21, but not IL-17, was important for the proper development of T_{FH} cells in this autoimmune mouse model. Although there were almost no GCs in BXD2-II21^{-/-} mice, the deficiency of IL-21 did not appear to perturb the structure of the follicles in the spleens in that there was a normal distribution of CD4^{+} T cells surrounded by IgM^{+} B cells. Development of T_{H17} cells was significantly reduced in BXD2-II21^{-/-} mice, which is also consistent with the previous finding that IL-21 exhibits a unique effect in promoting T_{H17} differentiation (42, 43). In contrast, the frequency and total numbers of T_{FH} cells were not reduced by lack of IL-17RA expression in the BXD2-II17ra^{-/-} mice as well as in NP-CGG immunized B6-II17ra^{-/-} mice. The proliferative response of T_{FH} cells from BXD2-II17ra^{-/-} and their ability to induce B cell proliferation and IgG secretion in vitro were also intact.

Surprisingly, the sera titers of IgG autoantibodies were similar in the BXD2-II17ra^{-/-} and BXD2-II21^{-/-} mice. Confocal imaging analysis confirmed our previous report (28) of a dissipation of B cells in the follicles of the BXD2-II17ra^{-/-} mice and further indicated that the majority of CXCR5^{+} T_{FH} cells were not localized in the GC LZ. The present study suggests that IL-21 acts at an early checkpoint to cue the development of T_{FH}, whereas IL-17 acts at a later checkpoint at the LZ to enable prolonged interaction of differentiated T_{FH} to help GC B cell maturation.

Under optimal conditions for generating antibody-forming B cells, both GC B cells and T_{FH} exhibit a tendency to migrate towards the same LZ compartment, thereby enhancing their opportunity for cognate interactions (46). Although recruitment to the GC LZ is
enabled by CXCL13, special migration “stop” signaling events are required to induce conjugate formation. Prolonged interactions between CD4 T cells and APCs have been shown to enable the generation of autoreactive T cells and such immobilization is associated with TCR signals and can affect the future activation fate of T cells (47). Similarly, stable interaction between CD4 T cells and cognate B cells mediated by SAP is another intrinsic mechanism to retain CD4 T cells in a nascent germinal center for sustain GC reaction (48). The present study, however, suggests that IL-17 is an extrinsic stop signal that it acts on post-differentiated IL-17RA⁺ T_{FH} as a “braking trigger” to enable its interaction with responder B cells in the LZ niche. This unique property is especially important for a GC response for which T-cell help is proposed to be the limiting factor in the establishment of stable conjugates (49). This braking effect of IL-17 in BXD2 mice may preferentially occur in the LZ because IL-17⁺ CD4 cells are mainly located in this region. It has been reported that GC CD4⁺ T cells utilize the RGS13 and RGS16 signaling proteins to modulate their trafficking and that GC B cells utilize RGS1, RGS13, and RGS16 during CXCL12/13-induced formation of GCs (39, 44). Here we show that, in the BXD2 mouse, RGS16 is responsible for the IL-17-mediated migration arrest of T_{FH} cells in the GC LZ, providing the necessary cell anchorage for cognate B–T cell interactions and GC development. We found that IL-17 stimulated the expression of RGS16 in CD4 T cells at both the transcriptional and protein levels and that IL-17 stimulation inhibited CXCL13-mediated chemotaxis of T_{FH} cells isolated from RGS16⁺ BXD2 mice but not BXD2-Rgs16⁻/⁻ mice. The lack of aggregating T_{FH} cells in GC LZ in the spleen of BXD2-Il17ra⁻/⁻ mice was recapitulated in BXD2-Rgs16⁻/⁻ mice. Furthermore, T_{FH} cells from BXD2-WT, but not BXD2-Rgs16⁻/⁻ mice that were
adoptively transferred into BXD2-Il17ra−/−, formed more conjugates with B cells and supported the resumption of GC formation in the recipients. These observations provide further evidence that IL-17-induced up-regulation of RGS16 plays a dominant role in the proper localization of T<sub>FH</sub> cells in GC LZ. Under these experimental conditions, the B cells as well as all other host cells were IL-17RA-deficient. Together with our previous findings concerning the effects of IL-17 on B cells (28, 31), the current results suggest that T<sub>FH</sub>–B conjugate formation in GCs can be regulated by modification of the chemotactic responses of either cell type at the post-receptor level.

Why is regulation of the location of T<sub>FH</sub> cells and their prolonged interactions with GC B cells important for development of highly pathogenic autoantibodies in BXD2 mice that can passively transfer autoimmunity (30)? T<sub>FH</sub> cells located within GCs drive positive selection of B cells that have acquired high-affinity receptors (3, 4). The cognate contact between T<sub>FH</sub> and GC B cells, mediated by a series of cell-surface co-stimulator interactions, such as CD28–CD86, ICOS–ICOSL, and CD40L–CD40, is required for class-switch recombination (CSR) and somatic hypermutation (SHM) (25). New evidence further suggests that the ability to induce SHM plays an important role in the survival of self-reactive B cells in the GC (50). The present results indicate that perturbation of IL-17 signaling, either acutely by AdIL-17R:Fc or chronically by Il17ra−/−, disrupts T<sub>FH</sub>–B interactions and abrogates the generation of autoantibody-forming B cells in BXD2 mice. As a soluble mediator, IL-17 potentially defies TCR specificity and unselectively arrests all IL-17R<sup>+</sup> T<sub>FH</sub> cells in the LZ niche, enhancing the opportunity of cognate interactions of T<sub>FH</sub>–B cells to drive the development of
pathogenic polyreactive autoantibody-forming B cells in BXD2 mice (28, 30). Because the T<sub>FH</sub> phenotype and numbers are normal in BXD2-<i>Il17ra</i>−/− mice, the present study further suggests that T<sub>FH</sub> cell differentiation can be dissociated from GCs but that their anatomical location in the GC LZ is indispensable for their function in supporting autoreactive GC B cell maturation.
Acknowledgments

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**Abbreviations used in this paper**

<table>
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<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>B6</td>
<td>C57BL/6</td>
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<tr>
<td>BXD2-WT</td>
<td>wild-type BXD2</td>
</tr>
<tr>
<td>EDU</td>
<td>5-ethyl-2'-deoxyuridine</td>
</tr>
<tr>
<td>GC</td>
<td>germinal center</td>
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<tr>
<td>NP-CGG</td>
<td>4-Hydroxy-3-nitrophenylacetyl hapten conjugated to chicken gamma globulin</td>
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<tr>
<td>PD-1</td>
<td>programmed cell death-1</td>
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<tr>
<td>rm</td>
<td>recombinant murine</td>
</tr>
<tr>
<td>RORγt</td>
<td>retinoic acid–related orphan receptor γt</td>
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<tr>
<td>SA</td>
<td>streptavidin</td>
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<tr>
<td>TD</td>
<td>T-dependent</td>
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<td>LZ</td>
<td>light zone</td>
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<td>PNA</td>
<td>peanut agglutinin</td>
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<tr>
<td>T&lt;sub&gt;FH&lt;/sub&gt;</td>
<td>follicular T helper cells</td>
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<tr>
<td>RGS</td>
<td>regulator of G-protein signaling</td>
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Figure Legends

Figure 1. Increased CXCR5+ICOS+ T\textsubscript{FH} cells producing IL-21 or IL-17 in spleen GCs in naïve BXD2 mice.

(A-E) Flow cytometry analysis of CD4 T subsets based on the expression of CXCR5 and ICOS in the spleens of female 10-12-week-old mice. Cells were first gated for CD4 T cells. (A) Analysis of CXCR5 and ICOS. (B) Frequency of the indicated CD4 T subsets. (C) Analysis of the indicated cell surface markers or (D) intranuclear Bcl6 and RORγt on CD4 T subsets in BXD2 mice. Cells were gated based on the differential expression of CXCR5 and ICOS. (E) Analysis of intracellular IL-21 and IL-17 produced by the indicated CD4 T subsets from BXD2 mice. (F-J) Confocal imaging analysis of frozen spleen sections from 3-mo-old BXD2 mice. Sections were stained with fluorochrome-conjugated reagents or antibodies. Abbreviations used: FDC, follicular dendritic cell (yellow arrows, F); FO B, follicular B cells; DZ, dark zone; LZ, light zone. The GC LZ vicinity is marked by a white dashed line (G). A higher-magnification view of the boxed area is shown (H, I, J, lower). White arrows indicate representative CXCR5+ CD4 T cells (G), IL-21+ CD4 T cells (H), IL-17+ CD4 T cells (I), or IL-17RA+ CD4 T cells (J) in the GC LZ. Data are representative of the analysis. Numbers in dot plots indicate the percentage of cells in each quadrant (A, E). Bar graph results from (B) are shown as mean ± s.d.; n ≥ 5 per group; **p<0.01 and ***p<0.005 between B6 and BXD2 (B).

Figure 2. GC development and autoimmune disease severity were alleviated in BXD2-\textit{Il17ra}\textsuperscript{−/−} and BXD2-\textit{Il21}\textsuperscript{−/−} mice.
(A) ELISA of IgM, IgG2b, and IgG2c autoantibodies specific for histone and DNA in the sera of 4–5-mo-old mice. (B) ELISA of urinary albumin in 4–5-mo-old mice. (C) H&E staining in paraffin-embedded kidney sections from 5-mo-old mice. (D) Immunofluorescence analysis for IgG (red) deposition in glomeruli in frozen kidney sections from 5-mo-old mice. Magnification, x20. (E) ImageJ quantitation of the intensity of IgG deposition per glomerulus in frozen kidney sections from 5-mo-old mice. (F) Flow cytometry analysis of the percentage of PNA\textsuperscript{+}Fas\textsuperscript{+} GC B cells in the spleens of 2–3-mo-old mice. Cells were gated on CD19\textsuperscript{+} B cells. Numbers above the boxed region represent the frequency of PNA\textsuperscript{+}Fas\textsuperscript{+} within CD19\textsuperscript{+} cells (mean ± s.d.). (G, H) Confocal imaging analysis of PNA\textsuperscript{+} GC B cells in frozen spleen sections from 2–3-mo-old mice. (G) Sections were stained with anti-IgM (red), anti-CD4 (green), and PNA (blue). Magnification, x10. (H) ImageJ quantitation of the area of PNA\textsuperscript{+} staining versus the area of each IgM\textsuperscript{+} spleen follicle. All data are representative or an average of n ≥ 5 female mice per group; *p < 0.05, **p < 0.01, ***p < 0.005 compared with BXD2-WT mice.

**Figure 3. Increased frequency of T\textsubscript{FH} cells in the spleens of BXD2-Il17ra\textsuperscript{−/−} mice.**

Single-cell suspensions were prepared from the spleens of female 10–12-week-old mice. (A) Flow cytometry analysis of CXCR5 and ICOS on CD4 T cells. (B) Bar graph showing the frequency (left) and the total cell count of CXCR5\textsuperscript{+}ICOS\textsuperscript{+} T\textsubscript{FH} cells per spleen (right). (C) Upper: Flow cytometry analysis of intracellular IL-21 and IL-17 produced by CXCR5\textsuperscript{+}ICOS\textsuperscript{+} T\textsubscript{FH} cells. Cells were gated on CD4\textsuperscript{+} T cells first. Lower: Quantitation of the percentage of CXCR5\textsuperscript{+}ICOS\textsuperscript{+} T\textsubscript{FH} that expressed either IL-17 or IL-21. (D) Flow cytometry analysis of the indicated cell surface markers on CXCR5\textsuperscript{+}ICOS\textsuperscript{+}
CD4 T cells. (E, F) Functional assay of T cells in vitro. Sorted T<sub>FH</sub> cells and other CD4 T subsets based on CXCR5 and ICOS expression from BXD2-WT or Il17ra<sup>−/−</sup> mice, either unstimulated or stimulated with anti-CD3 plus anti-CD28, were co-cultured with B cells from BXD2 mice. Flow cytometry analysis of the proliferative responses indicated by EDU<sup>+</sup> staining was carried out on day 3 for B cells (upper) and day 2 for T cells (lower). (F) ELISA analysis of IgG secretion in the coculture supernatant on day 3 after subtraction of the background levels without anti-CD3 and anti-CD28 stimulation. Numbers in quadrants or above gate indicate the percentage of cells (A, C) or the mean percentage ± s.d. (E, F). Data are representative of the analysis of n = 5 mice per group (A, C upper panel, D), two independent experiments with n = 3 mice per group (E, F), or are the mean ± s.d. of n = 5 mice per group (B, C lower panel); *p < 0.05, **p < 0.01, ***p < 0.005 compared with BXD2-WT mice (B, C), the unstimulated group (E), or the CXCR5<sup>+</sup>ICOS<sup>+</sup>CD4 T subset (F).

**Figure 4. Abnormal localization of T<sub>FH</sub> cells and defective T<sub>FH</sub>–B cell conjugates in the spleens of BXD2-I17ra<sup>−/−</sup> mice.**

(A-B) Immunofluorescence microscopy of frozen sections from spleens of naive 3-mo-old mice (A) or from spleens of 2.5-mo-old mice 10 days post-administration of either AdLacZ or AdIL-17R:Fc (B). Sections were stained with anti-CXCR5 (red), anti-CD4 (green), and PNA (blue). The GC border is marked by a white dashed line (B, upper), and the white rectangle regions from the upper panels were further magnified to show the distribution of CXCR5<sup>+</sup> CD4 T cells (yellow, arrows). The GC LZ vicinity is marked by a white dashed line (all lower panels). (C-E) Analysis of conjugates formed between
CXCR5^ICOS^CD4 T and B cells in the spleens of 3-mo-old mice. (C) FACS sorting of CD4–B220 cell conjugates. Upper: Cells gated on CD3^CD4^+ T cells prior to acquisition of CD4^B220^ doublets. Middle: Conjugated cells were dissociated with EDTA/PBS as described (37) to yield CD4 and B200 single cells. Lower: Analysis of expression of ICOS and CXCR5 within the dissociated CD4 T cells; numbers in quadrants indicate frequency. (D) Frequency of CD4^B220^ doublets in the CD3^CD4^+ T cells in each strain. (E) Frequency of CXCR5^ICOS^ or CXCR5^ICOS^ cells in dissociated CD4 T cells. Data are representative of the analysis of n = 5 mice per group (A), two independent experiments with n = 3 mice per group (B, C), or indicate mean ± s.d.; *p < 0.05, ***p< 0.005 compared with BXD2-WT mice (D, E).

Figure 5. RGS16 is involved in IL-17-induced location of CXCR5^+ CD4 T cells.

(A-C) MACS-purified CD4 T cells from spleens were cultured with/without IL-17 (30 ng/ml) in vitro. (A) qRT-PCR analysis of Rgs16 and (B) western blot and ImageJ quantitation of RGS16 at the indicated times. (C) Chemotactic response of CXCR5^ICOS^ CD4 T cells. Culture medium or IL-17-pre-incubated CD4 T cells in response to CXCL13 or medium were analyzed. Migrated cells were counted and the percentage of CXCR5^ICOS^ T_{FH} cells was determined by flow cytometry. The chemotaxis index was calculated as previously described (39). (D) Immunofluorescence microscopy of frozen spleen sections of naïve mice. For both strains, the same spleen follicle is presented for each staining. Arrows indicate representative RGS16^+ CD4 T cells (yellow, upper) or CXCR5^CD4 T cells (yellow, lower) in the GC LZ. (E) Immunofluorescence microscopy of frozen spleen sections showing the localization of
CXCR5+CD4 T cells in naïve mice. Magnification, x20. GC LZ vicinity is marked by a white dashed line (D, E). (F, G) B cell–T cell conjugate analysis of the indicated strains. (F) Frequency of CD4+CD19+ doublets in spleen cells. (G) Frequency of CXCR5 ICOS+ or CXCR5+ICOS+ CD4 T cells dissociated from the CD4+CD19+ doublets. Data are representative of the analysis of 3–4 mice (2.5–3-mo-old) per group (D, E) or indicate mean ± s.d. of 2–3 mice per group for three repeated experiments;*p < 0.05, ** p<0.01, ***p< 0.005 between the groups (A, B, C, F, G).

Figure 6. RGS16−IL-17RA+, but not RGS16−IL-17RA+, CD4 T cells resumed GC formation in BXD2-Ill7ra−/− mice.

Effector CD62L−flo CD4 T cells from 2.5-mo-old BXD2 or BXD2-Rgs16−/− mice were FACS-sorted and transferred into age/sex-matched BXD2-Ill7ra−/− mice by i.v. injection (3x10⁶ cells in 200 µl PBS per mouse). (A) Flow cytometry analysis of GL-7+Fas+ GC B cells from the spleens of recipients on day 6. (B) ELISPOT assay of the autoantibody-producing B cells from the spleens of recipients on day 21. (C) The ratio of CD4+B220+ doublets versus CD4+B220− singlets (left) and the ratio of CXCR5+ICOS+CD4+B220+ doublets versus CXCR5+ICOS+ CD4+B220− singlets (right) determined by flow cytometry sorting and analysis on day 6 in recipient spleens. Donor IL-17RA+ CD4 T cells and CXCR5+ICOS+ Tfh cells were separated from IL-17RA− recipient cells by IL-17RA staining. Ratios were calculated using the absolute cell count. (D) qRT-PCR analysis of Rgs16 in conjugated and non-conjugated FACS-sorted CD4 T cells from BXD2 donor or BXD2-Ill7ra−/− recipients on day 6. Data are representative (A) or
indicate mean ± s.d. of two independent experiments with n = 3 mice per group; *p<0.05, **p<0.01, ***p<0.005 between the groups (B-D).

Figure 7. The increased percentage of T_{FH} cells in B6-Ill17ra^{−/−} mice also exhibited a lower ability to promote a TD immunization response.

(A) FACS analysis of IL17RA in the indicated subsets. Cells were first gated for CD4 T cells. (B) qRT-PCR analysis of Rgs16 in purified CD4 T cells with/without IL-17 (30 ng/ml) stimulation. (C) CXCL13 mediated chemotactic response of CD4 T cells with/without IL-17-pretreatment. (D-F) 2.5-mo-old B6 and B6-Ill17ra^{−/−} mice were immunized with NP(21)-CGG by i.p.. Mice with vehicle injection were used as control. (D) Flow cytometry analysis of T_{FH} (left), expression of IL-21 and IL-17 in T_{FH} (gated on CXCR5^{+}ICOS^{+} CD4 T cells, middle) and GC B cells (gated on CD19^{+} cells, right) on day 9 after NP-CGG immunization in the indicated mice. Numbers in quadrants indicate mean frequency ± s.d.. (E) ELISPOT quantitation of the IgG anti-NP_{7} antibody producing B cells at the indicated days (D) post NP-CGG immunization. (F) Confocal imaging on spleen tissue for GC B cells and T_{FH} on day 9. GC LZ is indicated by a white dashed line. Representative data (A, D, F) or indicate mean ± s.d. (B, C, E) are shown; *p < 0.05, ** p<0.01, ***p< 0.005. All experiments were carried out using 3 mice per group for two experiments (2.5 mo-old).
Figure 1. Increased CXCR5\(^+\)ICOS\(^+\) Tfh Cells Producing IL-21 or IL-17 in Spleen GCs in Naïve BXD2 Mice
Figure 2. GC Development and Autoimmune Disease Severity Were Alleviated in BXD2-Il17ra<sup>−/−</sup> and BXD2-Il21<sup>−/−</sup> Mice
Figure 3. Increased Frequency of Tfh Cells in the Spleens of BXD2-Il17ra⁻/⁻ Mice
Figure 4. Abnormal Localization of Tfh Cells and Defective Tfh–B Cell Conjugates in the Spleens of BXD2-*Il17ra*" Mice
Figure 5. RGS16 was Involved in IL-17-Induced Location of CXCR5⁺ CD4 T Cells
Figure 6. RGS16^{+}IL-17RA^{+}, but not RGS16^{−}IL-17RA^{+}, CD4 T Cells Resumed GC Formation in BXD2-{*-Il17ra^-^} Mice
Figure 7. The Increased Percentage of Tfh Cells in B6-Il17ra<sup>−/−</sup> Mice Also Exhibited a Lower Ability to Promote a TD Immunization Response
S1. CD4 T Cell Subsets are Differentiated by the Expression of CXCR5 and ICOS

Single-cell suspensions prepared from spleens of the indicated strains at 10-12 weeks of age. (A, B) Cells were counted with a hemocytometer and cellular markers were analyzed by flow cytometry. (A) Total CD4 T cell count was obtained by multiplying its frequency with the total spleen cell count of each strain. (B) The CXCR5+ICOS+CD4+ T cell count was obtained by multiplying its frequency with the CD4 T cell count of each strain. (C) Flow cytometry analysis of the frequency of the indicated markers on CD4 T subsets based on the expression of CXCR5 and ICOS in the spleens of BXD2 mice. (D, E) Frequency of IL-21-producing or IL-17-producing cells in CXCR5+ICOS+ T_{FH} cells (D) or in CXCR5 ICOS+ CD4 T cells (E) from the spleens of the indicated strains. Data are presented as the mean ± standard deviation (s.d.); n ≥ 5 per group; *p<0.05, **p<0.01, and ***p<0.005 between B6 and BXD2 (A, B, D, E). Data indicated with different letters are significantly different from each other (C).
S2. Distribution of ICOS- and CXCR5-Expressing CD4 T Cells in the Spleen

(A) Gross anatomic image of a representative spleen from the indicated strains of 2–3-month-old female mice. (B) Total spleen cell count from the indicated strains. (C-F) Single-cell suspensions prepared from the spleens of female 10–12-week-old BXD2, BXD2-Il17ra<sup>−/−</sup>, and BXD2-Il21<sup>−/−</sup> mice were counted with a hemocytometer and cellular markers were analyzed by flow cytometry. (C) Counts of total CD4 T cells per spleen in the indicated strains. Results were obtained by multiplying the frequency of CD4<sup>+</sup> cells with the total spleen cell count for each strain. (D) Frequency of the indicated CD4 T subsets, as determined by flow cytometry. (E) Cell count per spleen of the indicated CD4 T subsets with the total CD4 T cell count of each strain. (F) Frequency of indicated surface markers on CXCR5<sup>+</sup>ICOS<sup>+</sup> T<sub>FH</sub> cells, determined by flow cytometry analysis. Data are representative or shown as mean ± s.d.; n = 5 mice per group; *p<0.05; **p<0.01; ***p<0.005 compared with BXD2-WT mice.
S3. Immune Properties of Tfh Cells in the Spleens of BXD2-Rgs16−/− Mice

(A) Representative flow cytometry histogram plots showing the expression of IL-17RA on CXCR5+ICOS+ Tfh cells from the spleens of the indicated strains at 2.5 months of age. (B) Flow cytometry analysis of ICOS and CXCR5. Cells were gated for CD4 T cells from the spleens of female 3-mo-old mice. (C) FACS sorting and analysis of the phenotype of CD4 T cells forming conjugates with CD19+ B cells in the spleens of 3-month-old BXD2 and BXD2-Rgs16−/− mice. CD4+CD19+ doublets were first sorted by FACS (left). Following in vitro EDTA treatment to dissociate the sorted doublets, the dissociated CD19+ or CD4+ single cells are shown (middle). The dissociated CD4+ cells were further analyzed for the expression of CXCR5 and ICOS to determine the frequency of CXCR5+ICOS+ and CXCR5 ICOS+CD4 T subsets (right). Numbers in the quadrants indicate the mean percentage or the mean ± s.d. of the gated cell population; **p<0.01 between BXD2 and BXD2-Rgs16−/− (C). Data are representative of the analysis for 3–4 mice per group (A, B) or 2–3 mice per group for three repeated experiments (C).
S4. RGS16<sup>+</sup>IL-17RA<sup>+</sup>CD4 T Cells Enhanced Autoantibody-Forming B Cells in BXD2-Il17ra<sup>−/−</sup> Mice

Effector T cells (CD62L<sup>−/lo</sup> CD4 T cells) from 2.5-mo-old BXD2 and BXD2-Rgs16<sup>−/−</sup> mice were sorted by FACSARia II and were administered intravenously into age-matched BXD2-Il17ra<sup>−/−</sup> mice (3x10<sup>6</sup> cells in 200 µl PBS per mouse). Recipients were sacrificed on days 6 or 21. (A) Gross anatomic images of representative spleens from mice that received effector CD4 T cells from either BXD2 or BXD2-Rgs16<sup>−/−</sup> mice on day 6. The control group, indicated as “no cell ctrl”, is the spleen from a representative naïve BXD2-Il17ra<sup>−/−</sup> mouse. (B) ELISPOT assay of IgG autoantibody-producing B cells in the spleens of recipients on day 21. (C) Flow cytometry gating and analysis of CD4 T cells or CXCR5<sup>+</sup>ICOS<sup>+</sup>CD4 T cells that were either IL-17RA<sup>+</sup> or IL-17RA<sup>−</sup> in CD4<sup>+</sup>B220<sup>+</sup> doublets (left, EDTA dissociated) or CD4<sup>+</sup>B220<sup>−</sup> singlet cells (right) in spleens of recipients on day 6. All cells were sorted by FACS to obtain either CD4<sup>+</sup>B220<sup>+</sup> doublets or CD4<sup>+</sup>B220<sup>−</sup> singlets before the subsequent analyses were carried out. Numbers above outlined areas indicate the percentage of gated cells within the parent population of cells. Data are representative of two independent experiments with n = 3 mice per group.
SUMMARY AND CONCLUSIONS

Summary

Background

Autoantibody-mediated autoimmune diseases, including SLE, are germinal center (GC)-derived diseases. Autoantibody-producing B cells are the key players in the pathogenesis of these diseases (10), although the CD4 T cell subsets, such as Th1, Th2, Th17 and Treg cells, are also associated with the disease activity (11-17). Germinal centers are the structures that accommodate B cell proliferation and differentiation, leading to generation of high affinity antibody producing plasma cells and memory B cells. In addition to B cells, GCs also consist of activated T cells, follicular dendritic cells (FDC) and stromal cells. Both B cells and Ag-specific T helper cells must be recruited by chemokine-mediated migration into the nascent GC and be retained there (25) to form close interactions with each other, thereby initiating the development of GCs. During the process of GC formation, defects in any of the cell components and checkpoints may cause breakdown of self-tolerance and result in over-reactivity of the GC response that leads to development of autoantibody-mediated autoimmune diseases. Therefore, understanding the mechanisms that drive the hyperreactivity of GCs is critical for developing effective therapeutic strategies for the diseases.
Tfh cells are the primary T helper cells that migrate from the T cell area into GCs to interact with B cells and provide B cell help; therefore, Tfh cells play a critical role in high affinity antibody production by GC B cells. Aberrant number and function of Tfh cells have been correlated with the severity of autoimmune diseases in humans and lupus-prone mice (72, 73, 83-86, 88-90). In contrast to Tfh cells, Tfr cells are a newly defined regulatory T cell subset that can inhibit Tfh cells and GC formation (44, 45). Loss of T cell homeostasis is crucial in driving GC-dependent autoimmunity, hence, imbalance between inflammatory T cells and Treg cells has been associated with disease activity in lupus prone mice and SLE patients (16). However, the factors that regulate the balance between Tfr cells and Tfh cells under normal conditions, as well as factors that disrupt this balance in autoimmune disease are not clear.

Increased levels of IL-21 have been found in sera of SLE patients (136) and lupus-prone mice (73, 80, 89, 137). IL-21 is thought to be essential for the development of Tfh cells (34, 53); however, there are also controversial observations about the redundant effect of IL-21 on Tfh cells (63, 66, 134). IL-21 has been shown to negatively regulate conventional Treg cells (108). Considering that both IL-21 and Tfr cells play an important role to regulate the development of GCs, we therefore determined the effect of IL-21 on Tfh cells and Tfr cells in autoimmune BXD2 mice in the first study of this dissertation.

BXD2 mice develop spontaneous GCs that produce autoreactive B cells and high-titer autoantibodies to induce glomerulonephritis and arthritis (81, 186, 187). The serum level of IL-17 is significantly higher in BXD2 than in B6 mice (81). IL-17 has been shown to play an important role in regulating GC development and autoantibody
production (171, 172). During the process of GC formation, both GC B cells and Tfh cells migrate into follicles to interact with each other and their interactions require sufficient duration (25) and strength to generate effective cognate help. Expression of SLAM associated protein (SAP) on T cells is crucial for the close conjugate formation between T-B cells (69, 78). A previous study suggests that IL-17 induces retention of GC B cells in GCs by up-regulation of RGS13/16 (81), leading to large GC formation and strong AID expression in BXD2 mice. However, factors that regulate the localization of Tfh cells in GC LZ and their ability to form conjugates with GC B cells are currently unexplored. Meanwhile, the regulatory effects of IL-17 on Tfh cell migration in a specific GC niche are also unknown. Therefore, we determined the effect of IL-17 on regulating mature Tfh cells in autoimmune BXD2 mice in the second study of this dissertation.

**Results**

We found that the serum levels of IL-21 were elevated in BXD2 mice compared with those in B6 mice and BXD2-Il21-/- mice, indicating that IL-21 is involved in the pathogenesis of BXD2 mice. There. The number of CXCR5^+ICOS^+ Tfh cells in the spleens of autoimmune BXD2 mice was significantly increased compared to healthy control B6 mice. This result is consistent with previous observations made in other lupus-prone mouse models and in human autoantibody-driven autoimmune diseases (83, 85, 191). The accumulation of Tfh cells highly correlated with disease progression as mice aged. Interestingly, the Tfh cells in BXD2 mice consist of an IL-21-producing subpopulation and an IL-17-producing subpopulation. The majority of Tfh cells express
both IL-21R and IL-17RA, indicating that these cells are potentially subject to the regulation by both cytokines.

Indeed, the frequencies of Tfh cells in BXD2-Il21−/− mice were significantly reduced compared to BXD2 mice, suggesting that IL-21 is important for the development of Tfh cells. GC B cell development and GC formation were also significantly diminished in BXD2-Il21−/− mice. The present work suggests that IL-21 can influence the formation of autoreactive GCs via regulation of the composition and function of cells in the GCs: (1) Composition: The present work demonstrates that manipulation of IL-21 can dramatically influence the ratio between Tfh cells and Tfr cells. Interestingly, the frequency and cell number of conventional Treg (Foxp3+ CD4 T) cells in BXD2-Il21−/− mice were equal to or less than those in BXD2 mice. Surprisingly, the frequency of Tfr (CXCR5+ICOS+Foxp3+ CD4 T) cells was 2-fold higher, which led to a significant increase in the ratio of Tfr cells versus Tfh cells in the spleens and PBMCs of the BXD2-Il21−/− mice. Administration of IL-21-producing adenovirus (AdIL-21) to BXD2-Il21−/− mice increased Tfh cells and GC B cells, but decreased Tfr cells and the ratio of Tfr versus Tfh in spleen. (2) Function: rmIL-21 suppressed Foxp3 and significantly reduced the expression of Tgfβ, Gitr and Il2 but enhanced the expression of Il21, Il6, Cxcr5 and Icos in Tfr cells. While Tfr cells directly inhibited proliferation of Tfh cells, as well as antibody production by B cells and antibody secretion in the Tfh-B cell co-culture system, IL-21 primarily acted directly on Tfr cells to counteract the Tfr-mediated suppression. Notably, unlike Tfr cells from BXD2-Il21−/−, Tfr cells from BXD2 failed to exert their suppressive function on Tfh cells and B cells. Transfer of IL-21−/− Tfr cells into BXD2 mice reduced GC size and decreased the levels of IgG and IgM autoantibodies in sera.
These results suggest that IL-21 played a critical role in skewing the GC CD4 T cell balance to increase the Tfh/Tfr ratio and promote GC reaction in autoimmune BXD2 mice.

Similar to IL-21, IL-17 is also important for the pathogenesis of autoimmune disease in BXD2 mice. A deficiency of IL-17RA in BXD2-Il17ra−/− mice led to compromised spontaneous GC formation and pathogenic autoantibody production. However, IL-17 was not required for the development of Tfh cells in BXD2 mice; the total number of Tfh cells and their ability to induce B cell responses in vitro were not affected by the deficiency of IL-17-IL-17RA signaling. However, the majority of CXCR5+ Tfh cells in BXD2-Il17ra−/− mice were not localized in the GC LZ. In addition, interruption of IL-17 signaling, either acutely by AdIL-17R:Fc, or chronically in Il17ra−/− mice, disrupted Tfh–B interactions and abrogated the generation of autoantibody-forming B cells in BXD2 mice. IL-17 up-regulated the expression of regulator of G-protein signaling (RGS)16 in Tfh cells and promoted Tfh:B cell conjugate formation. This conjugate forming ability was significantly impaired in BXD2-Rgs16−/− mice. These results indicate that IL-17 is an extrinsic stop signal and it acts on IL-17RA+ Tfh cells to enable their retention and interaction with responder B cells in the LZ niche.

Discussion

The Critical Role of IL-21 in Development of Tfh Cells
IL-21 is thought to play a fundamental role in the development of Tfh cells (34, 53) and is the signature cytokine of these cells. However, other investigators previously showed that IL-21 and IL-6 are redundant for the differentiation of Tfh cells in immunized mouse models (63, 66, 134). In the current work, we demonstrate that IL-21 levels are elevated in sera of autoimmune BXD2 mice, a finding consistent with what has been reported in other lupus prone mice (73, 80, 89, 137) and SLE patients (136). There is an accumulation of Tfh cells in BXD2 mice compared with normal healthy B6 mice. By contrast, the frequency of Tfh cells in BXD2-Il21−/− is 3-4 fold lower than in BXD2 mice, equal to or even lower than in B6 mice. However, CXCR5+ICOS+ Tfh cells are not completely absent in the BXD2-Il21−/− mice. These results suggest that additional factors exist to support the differentiation/expansion of Tfh cells in BXD2 mice. Interestingly, further study showed that up to 70% of the CXCR5+ICOS+ CD4 T cells in BXD2-Il21−/− mice that previously appeared to be Tfh cells are Foxp3+, indicating that the majority of CXCR5+ICOS+ CD4 T cells in BXD2-Il21−/− mice are not GC facilitating Tfh cells but are Tfr cells that can suppress GC formation and GC B cell differentiation (44, 45). Therefore, the results confirm that IL-21 is essential for development of the conventional GC-promoting Tfh cells (Foxp3+CXCR5+ICOS+ CD4 T) in BXD2 mice.

_The Role of IL-21 in Inhibiting Tfr but not Conventional Tregs in BXD2 Mice_

IL-21 also has been shown to negatively regulate conventional Treg cells (108). However, considering the unique function and anatomic location of IL-21 producing CD4 T cells, the present study determined the specific role of IL-21 in regulating the GC
associated Tfr cells. In the current study, we showed that, although GC development was almost absent in the BXD2-Il21−/− mice, the frequency and number of conventional Treg cells were equal to or even less than those in WT-BXD2 mice, suggesting that IL-21 deficiency may not affect the frequency of Treg cells in BXD2 mice. Interestingly, the frequency of Tfr cells, gating on CXCR5+ICOS+ CD4 T cells, was two-fold higher in BXD2-Il21−/− mice than in WT. Notably, the ratio of Foxp3+ Tfr cells versus Foxp3+ Tfh cells in BXD2-Il21−/− mice was increased although the total cell number of Tfr cells remained less than that in WT BXD2 mice. These results suggest that the primary regulatory role of IL-21 on CD4 T cell lineage development is in supporting Tfh cells and not suppressing Tfr cell formation. However, the results also provided the following evidence that IL-21 had a unique ability to suppress Tfr commitment: (i) IL-21 deficiency reduced the total number of CD4 T cells in BXD2-Il21−/− mouse spleens; (ii) AdIL-21 administration decreased both the number and the frequency of Tfr cells in BXD2-Il21−/− mice; (iii) In vitro studies showed that rmIL-21 decreased expression of Foxp3 and Treg surface markers including GITR and TGF-β on CXCR5+ICOS+ CD4 T cells; and (iv) IL-21 decreased the Tgfb, Gitr and Il2 mRNA levels but enhanced the expression of Il21, Il6, Cxcr5 and Icos by sorted Tfr cells in vitro. Taken together, these results suggest that IL-21 has the potential to inhibit the commitment of Tfr cells and it may convert Tfr cells into non-Tfr cells or effector T helper cells, at least in vitro.

Interestingly, we found that augmentation of IL-21 levels in vivo and in vitro promoted the production of IL-10 but inhibited the production of TGF-β both at the protein level and at the mRNA level. IL-10 is generally thought to be the major suppressive cytokine produced by Treg cells and other regulatory cells of the immune
system. It has been reported that IL-21 can also mediate suppressive effects by inducing IL-10 (192). However, IL-10 is also critical for IgA production during normal immune responses. In addition, IL-10 levels are highly increased in SLE patients (193) and is required for autoantibody production by stimulating B cell differentiation (194). Therefore, the role of IL-10 in the pathogenesis of autoimmunity is controversial. By contrast, membrane bound and secreted TGF-β have been confirmed to mediate the suppressive function of Treg cells (195, 196). Taken together, the inflammatory cytokine IL-21 may induce IL-10 as a negative feedback mechanism to maintain immune homeostasis. Meanwhile, based on the current study in autoimmune BXD2 mice, it seems quite likely that, IL-21 enhances B cell response and antibody production partially by inducing IL-10, as well as by inhibiting TGF-β to abrogate the immunosuppressive function.

In the current work, IL-21 was shown to counteract the inhibitory effect of Tfr cells on both Tfh cells and B cells. Thus, IL-21-promoted Tfh cell development and IL-21-inhibited Tfr commitment are two parallel mechanisms used by IL-21 to support GC formation in BXD2 mice. This IL-21-mediated suppression is a novel mechanism to regulate Tfr in addition to the previously reported PD-1-PD-L-1 induced suppression by Tfr cells (109).

The conversion of Tfr cells into non-Tfr cells by IL-21 in vitro is consistent with the finding that transferring Treg cells into T cell-deficient CD3ε−/− mice led to conversion of the Tregs into Tfh-like cells in gut Peyer’s patches (75). However, the present in vivo study showed that Tfr cells from BXD2-Il21−/− mice transferred into young BXD2 mice displayed suppressive effects on Tfh cells, GC formation and autoantibody
production in recipient mice, despite the higher levels of serum IL-21 in recipient mice. There was no apparent conversion of Tfr cells into non-Tfr cells by IL-21 in BXD2 mice in the present in vivo study. These findings are contradictory to a previous in vivo report by others (75) as well as our own in vitro studies reported here. We proposed that the relatively low levels of IL-21 in vivo compared with the high levels (50 ng/ml) in vitro may contribute to the observed differences. Thus, we do not completely exclude the possibility that IL-21 can promote the conversion of transferred Tfr cells into non-Tfr or Tfh cells in vivo. Blockade of IL-21-IL-21R signaling may demonstrate better suppressive effects of Tfr cells in the mice. We propose that this could be best resolved by future experiments in which IL-21R−/− Tfr cells are transferred.

Inhibition of Tfh and B Cells by Tfr

Conventional Treg cells are central to limit T cell responses and maintain immune homeostasis. The mechanisms of Treg cell-mediated immunosuppression include secretion of immunosuppressive cytokines such as IL-10, TGF-β and IL-35; delivery of granzymes and perforin; and provision of cell contact-mediated immunosuppressive molecules including CD39 and CTLA-4. Although the effect of IL-10 is controversial as aforementioned (193, 194), membrane bound or secreted TGF-β is a well confirmed molecule to mediate the suppressive function of Treg cells (195, 196). Similar to what has been found by other groups (44, 45), in the current study, we demonstrated that transfer of Tfr cells into young BXD2 mice suppressed GC formation and autoantibody production. Furthermore, our in vitro study showed that Tfr cells from BXD2-Il21−/− mice
acted directly to inhibit the proliferation of Tfh cells and antibody production by B cells. We propose that TGF-β but not IL-10 is the critical suppressive cytokine produced by Tfr cells, however, the mechanisms that mediate the inhibitory effect of Tfr cells is still unconfirmed and need further investigation.

The Balance of Tfh and Tfr in GC Development

Loss of T cell homeostasis is critical in driving autoimmunity. For example, imbalance between inflammatory cells and regulatory cells, such as Th17 and Treg cells, has been associated with disease activity in lupus prone mice and SLE patients (16). In the current study, we found that the ratio of Foxp3+ Tfr cells to Foxp3- Tfh cells was much higher in BXD2-II21-/- mice than in WT-BXD2 mice, although the total number of Tfr cells was less in the BXD2-II21-/- mice. In vitro stimulation of spleen cells with rmIL-21 decreased the ratio of Tfr/Tfh in both WT and BXD2-II21-/- mice. Consistently, in vivo administration of AdIL-21 to BXD2-II21-/- mice also induced a reduction in the Tfr/Tfh ratio. GC B cell development and GC formation were greatly enhanced in the AdIL-21 treated BXD2-II21-/- mice. Thus, the Tfr/Tfh ratio appears to be negatively correlated with GC formation. Overall, the results suggest that, compared with the number or frequency of Tfr cells, the ratio of Tfr/Tfh is a better biomarker of disease progression in BXD2 mice. The present work is the first report showing that IL-21 is a key cytokine to regulate the balance between Tfr cells and Tfh cells.
The Role of IL-17 on Mature Tfh Cells — A Novel Two-Checkpoint Model of Tfh Regulation in Murine Autoimmunity

IL-17 plays an important role in regulating GC development and autoantibody production (171, 172). IL-17 also regulates the migratory behavior of GC B cells during the GC formation process (81). The formation of GC is initiated by migration of both GC B cells and Tfh cells into follicles, a process mediated by chemokines and chemokine receptors down-stream signals. B and T cells are committed to localize in the GC LZ, where they interact with each other. Furthermore, the interaction requires sufficient duration (25) and strength to generate effective stimulation of both cell types. By up-regulation of RGS13 and RGS16, IL-17 induces migration arrest of GC B cells mediated by CXCL12 and CXCL13 to secure their retention in the GC and thereby promotes the formation of large GCs in BXD2 mice (81).

In the current work, we have demonstrated consistent evidence that GC formation and autoantibody production were impaired by IL-17RA deficiency in BXD2-Il17ra−/− mice. Surprisingly, the percentage and number of Tfh cells and their direct B-cell support function in vitro were unaffected. Further investigation indicated that the loss of IL-17RA affected the in vivo localization of Tfh cells in the GC LZ. Interruption of IL-17 signal via administration of AdIL-17R:Fc disrupted Tfh–B interactions and abrogated the generation of autoantibody-forming B cells in BXD2 mice. The effect of IL-17 on Tfh cells was mediated by up-regulation of RGS16 in Tfh cells, which extinguished the CXCL13-mediated chemotaxis of Tfh cells and facilitated their appropriate localization.
in the GC LZ. This promoted Tfh cells to form extended conjugates with B cells. These results suggest that IL-17 provides an extrinsic stop signal that acts on IL-17RA+ Tfh cells to enable their retention and interaction with responder B cells in the LZ niche.

The interactions between GC B cells and Th cells are executed by co-stimulatory molecules. Among these, SLAM associated protein (SAP) expression on T cells is crucial in the late stage of close conjugate formation between T-B cells (69, 78). In addition to the evidence provided by the second study in this dissertation, we also found that the expression of SLAM on Tfh cells in BXD2-Ill17ra−/− mice was lower than on WT BXD2 Tfh cells (Figure 1A). Interestingly, RGS16 deficiency in BXD2-Rgs16−/− mice also impaired the expression of this co-stimulatory molecule (Figure 1B). Interestingly, Slam expression correlated with the expression of IL-17RA, as transferring of Ill17ra+/+ CXCR5+ICOS+ or CXCR5+ICOS+ cells into Ill17ra−/− mice resulted in significantly elevated expression of Slam in donor IL-17R+ T cells compared with recipient T cells (Figure 1C). In vitro stimulation with rmIL-17 increased Sap mRNA levels in Tfh cells (Fig. 1D). This suggests that IL-17-IL-17R signaling is important to promote the expression of co-stimulatory molecules such as SLAM and SAP on Tfh cells, and thereby prepare Tfh cells for close conjugation formation with B cells. RGS16 may also be associated with this process. Thus, IL-17-mediated up-regulation of RGS16 may also promote Tfh-GC B cell interactions through a Tfh intrinsic mechanism.
Figure 1. IL-17 Promotes SLAM and SAP on Tfh Cells.

**A-B.** Flow cytometry shows the expression of SLAM on Tfh cells. **C.** IL-17R⁺ CD4 T cells were transferred into BXD2-17r⁻/⁻ mice. Real-time PCR shows the expression of *Slam* on both donor IL-17RA⁺ CD4 T cells and recipient IL-17RA⁻ CD4 T cells. **D.** Real-time PCR shows the expression of *Sap* on Tfh cells after rmIL-17 (50 ng/ml) treatment.

The second study of the current dissertation suggests a novel concept - that Tfh cell differentiation and their physical stabilization in the LZ are two separate checkpoints. Complete differentiation of Tfh cells may be sufficient for their B cell helper function *in vitro*, however, *in vivo*, the correct physical localization and formation of stable close contacts with GC B cells are also required for Tfh cells to effectively help B cell maturation and differentiation into antibody producing plasma cells. In BXD2 mice, IL-21 and IL-17 act at each checkpoint to enable pathogenic GC development. IL-21 is critical for full differentiation of Tfh cells, whereas IL-17 supports the effects of IL-21.
via stabilization of Tfh cells in the GC LZ to promote their close contact with B cells. Therefore, both cytokines are needed for pathogenic GC development in BXD2 mice, and loss of either cytokine dramatically abrogates the disease.

*Suggested Definition of Tfh and Tfr cells*

The present work supports the concept that the Tfh subset is a heterogeneous population of CD4 T cells (49) with elusive characteristics. To date, the most used biomarkers to define Tfh cells are Bcl6\(^+\)CXCR5\(^+\)ICOS\(^+\)PD-1\(^+\) and IL-21\(^{hi}\).

As aforementioned, both cell development and physical location are essential to secure the normal function of Tfh cells to promote the GC reaction *in vivo*. Therefore, based on the results of studies in this dissertation, we propose that the physical location of Tfh cells in the GC LZ should be included as one major defining characteristic of Tfh cells, and that this characteristic clearly distinguishes Tfh cells from other subsets of T helper cells.

The Tfr subset, the counteracting partner of Tfh cells, is also characterized by surface markers CXCR5\(^+\)ICOS\(^+\)PD-1\(^+\) and localization in GCs, thus resembling Tfh cells (44, 45). However, Tfr cells also express high levels of CTLA-4, GITR, CD25 and BTLA. The current study suggests that CXCR5\(^+\)ICOS\(^+\)Foxp3\(^+\) Tfr cells sustain expression of membrane TGF-\(\beta\)1, which together with the secreted form of TGF-\(\beta\), have been confirmed to mediate the suppressive function of Treg cells (195, 196). We suggest that, among all the surface markers, TGF-\(\beta\)1 and GITR are the two most important signatures to classify CXCR5\(^+\)ICOS\(^+\)PD-1\(^+\) CD4 T cells as Tfr cells.
The signature transcription factor for Tfr cells is Foxp3, whereas Tfh cells do not express Foxp3. Instead, Bcl6 is the critical transcription factor that directs the differentiation of Tfh cells (37, 41, 42) and this distinguishes the Tfh subset from other T helper subsets. However, the differentiation of Tfr cells also depends on Bcl6 (44, 45). The current study shows that Foxp3\(^+\) Tfr and Foxp3\(^-\) Tfh cells express equal levels of Bcl6. This suggests that Bcl6 is not a specific transcriptional factor to differentiate Tfh cells from Tfr cells. In addition to expressing Bcl6, Tfr cells also expressed high levels of Blimp-1 (44), an antagonistic transcription factor for Bcl6 which can inhibit the differentiation of Tfh cells (41), suggesting that Blimp-1 expression may be an important factor to delineate Tfr cells from Tfh cells.

Our study suggests that, in BXD2 mice, the CXCR5\(^+\)ICOS\(^+\) Tfh subset consists of an IL-21 producing subpopulation and an IL-17 producing subpopulation, and both subpopulations are located in the GC LZ. IL-17-producing CXCR5\(^+\)ICOS\(^+\)Tfh cells have a different phenotype compared with the conventional Th17 (CXCR5\(^-\)ICOS\(^+\)) cells found in BXD2 mice, which produce large amounts of IL-17. The conventional Th17 cells are thought to express ICOS, CCR6, CCR4, or CCR2 but not CXCR5; and their localization is not restricted to the GC. By contrast, the IL-17-producing CXCR5\(^+\)ICOS\(^+\)Tfh cells express CXCR5, which enables their localization in the GCs. We propose that the specific function of this subset is to induce Tfh-GC B stabilization and interaction in the GC LZ. Therefore, in addition to IL-21, IL-17 is another functional cytokine produced by Tfh cells. For Tfr cells, it is confirmed that they do not secrete detectable levels, if any, of inflammatory cytokines; however, TGF-\(\beta\), IL-2 or IL-10 could be produced by Tfr cells.
Based on these results, we suggest that the current definition of Tfh cells and Tfr cells should be revised as described in the following table to more precisely reflect the specific function of each subset (Table 2). This revision is important for understanding of Th-mediated autoimmune diseases, but may also be applicable to other induced immune responses.

Table 2. Summary of the Characteristics of Tfh Cells and Tfr Cells

<table>
<thead>
<tr>
<th>Cell subset</th>
<th>Surface markers</th>
<th>Transcriptional factor level</th>
<th>Cytokine level</th>
<th>Localization</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tfh</td>
<td>CXCR5, ICOS, PD-1, CD40L, IL-21R, IL-17R</td>
<td><strong>High:</strong> Bcl6 &lt;br&gt; <strong>Medium:</strong> RORγt? &lt;br&gt; <strong>Low:</strong> T-bet, Gata-3, Foxp3, Blimp-1</td>
<td><strong>High:</strong> IL-21, IL-17, IL-10? &lt;br&gt; <strong>Low:</strong> TGF-β, IL-4, INF-γ</td>
<td>GC light zone</td>
<td>Help: GC B cells &amp; GC formation</td>
</tr>
<tr>
<td>Tfr</td>
<td>CXCR5, ICOS, PD-1, CTLA-4, GITR, CD25, membrane TGF-β1, IL-21R, IL-17R? BTLA?</td>
<td><strong>High:</strong> Foxp3, Blimp-1, Bcl6 &lt;br&gt; <strong>Low:</strong> T-bet, Gata-3, RORγt</td>
<td><strong>High:</strong> TGF-β, IL-2, IL-10? &lt;br&gt; <strong>Low:</strong> IL-6, IL-21, IL-17</td>
<td>GC</td>
<td>Inhibit: Tfh cells &amp; GC B cells &amp; GC formation</td>
</tr>
</tbody>
</table>
The Sequential Effect of IL-21 and IL-17 in Regulating Tfh Cells

It has been shown that IL-21 alone or together with IL-6 and IL-23 can program Th17 differentiation (102, 108, 131, 197, 198). IL-21 drives RORγt activation and IL-17 production in a STAT3-dependent manner (199). All of these studies suggest that IL-21 is the upstream factor in driving Th17 differentiation and IL-17 production. However, most of these studies were carried out in vitro or using a mouse model of experimental autoimmune encephalomyelitis (EAE), which relies heavily on effector CD4 T cell inflammation. The biologic significance of this IL-21-IL-17 axis has not been demonstrated in other autoimmune conditions and thus, it is not clear as to why IL-17 is needed if IL-21 but not IL-17 is critical to drive Tfh differentiation.

In the current work, we demonstrate that BXD2 mice have increased numbers of CXCR5hiICOShi Bcl6+ Tfh cells that produce either IL-21 or IL-17. A deficiency of IL-21 in BXD2-Il21-/- mice results in a defect in development of the Tfh subpopulation as well as lower levels of serum IL-17 (Figure 2A) and lower IL-17 expression by Tfh cells. Here, we also show that AdIL-21 administration in BXD2-Il21-/- mice dramatically increases IL-17 levels in sera (Fig. 2B). rmIL-21 stimulation of CD4 T cells enhances the expression of IL-17R, RORγt (Fig. 2C) and IL-17 by Tfh cells. By contrast, a deficiency of IL-17RA-mediated signaling in BXD2-Il17r-/- mice does not suppress the development of Tfh cells. BXD2-Il17r-/- mice have an increased number of Tfh cells that produce higher levels of IL-17 and equal levels of IL-21 compared with those from WT BXD2 mice. The serum levels of IL-17 in BXD2-Il17r-/- mice are dramatically higher than those in BXD2 mice, whereas the IL-21 levels are comparable (Fig. 2A). These observations
suggest that IL-21 is an upstream cytokine for promoting IL-17, whereas IL-17 does not affect the production of IL-21.

Figure 2. IL-21 Promotes IL-17 Production

A. Serum levels of IL-21 and IL-17 detected by ELISA in 4 strains of mice. B. Serum levels of IL-17 in BXD2-II21−/− after administration of AdIL-21. C. Expression of IL-17R and RORγt by Tfh cells after rmIL-21(50 ng/ml) stimulation in vitro. D. Bar graph shows expression of the indicated genes in CD4 T cells after rmIL-21 (50 ng/ml) stimulation.

A deficiency of either IL-21 or IL-17 signaling, however, leads to impaired pathogenic GC formation and autoantibody production. As aforementioned, we suggest that IL-21 and IL-17 act on two separate Tfh cell checkpoints to promote maturation of
autoantibody forming B cells in the spontaneous BXD2 GCs. Together with the additional results shown here in Figure 2, we propose that the two checkpoints for Tfh cells are inter-related by the critical effect of IL-21 in promoting IL-17 production. In this model, during the process of Tfh differentiation, IL-21 is critical for Tfh cell development and it also promotes production of IL-17 and expression of the IL-17R on Tfh cells. These latter events prepare Tfh cells for IL-17 stimulation. IL-17 then signals through IL-17RA to stabilize well-developed Tfh cells in the GC LZ to exert their help for GC B cell maturation.

Pathogenic Factors in Autoimmune BXD2 Mice

The autoimmune BXD2 mouse model used in the present studies exhibits many manifestations of autoimmune disease similar to human autoimmunity. The mice spontaneously develop large GCs and a very high frequency of autoreactive GC B cells at a very early age. Cloned polyreactive pathogenic autoantibodies derived from these mice were shown to be capable of inducing nephritis and arthritis in non-autoimmune mice (81, 186, 187). High levels of IL-17 in the sera of BXD2 mice have been shown to be critical in promoting GC formation and autoantibody production (81), while IFN-α has been shown to regulate antigen delivery by MZ-P B cells and the MZ-P B cell-T cell interaction to promote GC formation in BXD2 mice (189, 190).

In the current studies, we found that BXD2 mice also produce high levels of IL-21 in the sera compared with B6 mice. The high level of IL-21 is associated with aberrant accumulation of Tfh cells in the mice. IL-21 also promotes IL-17 production in vivo.
Further investigation into the role of IL-17 on Tfh cells showed that IL-17 is not required for Tfh cells development but is essential for the appropriate localization and stabilization of Tfh cells in GC LZ to promote GC formation.

In contrast to the accumulation of Tfh cells, the frequency of Tfr cells and the ratio of Tfr/Tfh in BXD2 mice are significantly decreased in BXD2 mice compared with those in healthy B6 mice or BXD2-Il21−/− mice. Intriguingly, unlike Tfr cells from BXD2-Il21−/− mice, Tfr cells from BXD2 mice do not show suppressive function on Tfh cells and B cells, and this is proposed to be associated with the high levels of IL-21 in the mice that may induce Tfr cell anergy. Therefore, IL-21 in BXD2 mice also inhibits the commitment of Tfr cells, and the IL-21-mediated defects in frequency and function of Tfr cells are involved in the pathogenesis of autoimmunity in BXD2 mice.

To summarize, the pathogenic factors that have been reported by previous studies and the current dissertation work include IL-17, IFN-α, MZP, IL-21, Tfh and Tfr cells. These factors act at different anatomic sites to break immune checkpoints, leading to excessive GC formation, pathogenic autoantibody production and the development of autoimmune diseases in BXD2 mice. Given the similar multi-genetic background and the disease manifestation in BXD2 mice to those of many human autoimmune diseases, these investigations in BXD2 mice provide significant insights to elucidate the immunopathogenesis of complex human autoimmune diseases.
Conclusions

The current dissertation study demonstrates that the spontaneous formation of GCs in BXD2 mice can be considered as the net outcome of interactions among at least 3 unique subsets of CD4 T cell within the GCs (Fig 3). There is aberrant accumulation of Tfh cells in autoimmune BXD2 mice. The CXCR5+ICOS+ Tfh subset consists of an IL-21 producing subpopulation and an IL-17 producing subpopulation; both are located in the GC LZ. The IL-21 producing CXCR5+ICOS+ Tfh subset is the conventional Tfh specific for GC B cell help. The IL-17 producing CXCR5+ICOS+ Tfh subset is needed to promote Tfh-GC B stabilization and interaction, and thus, should be considered as a novel and unique subpopulation of Tfh cells that has not been previously described. The follicular regulatory Tfr cells are a group of regulatory CD4 T cells that directly suppress Tfh cells and GC B cells in the GCs. Our study indicates that IL-21 is essential to promote differentiation of Tfh cells but inhibits commitment of Tfr cells. IL-21 also enhances production of IL-17 and expression of IL-17R on Tfh cells, thereby preparing the Tfh cells for response to and regulation by IL-17. IL-17 then sequentially acts on Tfh cells to promote their localization in the GC LZ and secure their stable interaction with GC B cells by up-regulating RGS16 and co-stimulatory molecules. Overall, both IL-21 and IL-17 are critical in regulating Tfh cells and the strong combined effects of IL-21 and IL-17 overwhelm the regulatory effects of Tfr cells, thereby promoting the formation of autoreactive GC and the generation of pathogenic autoantibodies in BXD2 mice.
In addition to their effects on CD4 T cells in follicles, IL-21 and IL-17 also regulate B cells. The previous study indicates that IL-17 regulates autoreactive GC formation in BXD2 mice by up-regulating RGS13 and RGS16 in B cells. The current work continues to demonstrate a similar effect of IL-17 on Tfh cells via RGS16. We therefore conclude that IL-17 mainly plays an extrinsic role in modulating the migration of B cells and Tfh cells to prolong the autoreactive GC responses in BXD2 mice.

A similar effect of IL-17 on Tfh cells was also demonstrated in an NP-CGG-induced GC reaction in B6. However, sheep red blood cell (SRBC)-induced strong GC reactions in B6 mice are not affected by IL-17 deficiency. This suggests that some cytokine, such as IL-17, can function to affect chronic or low dose of antigen-stimulated GC responses, including autoantigen-mediated spontaneous GC responses, but may not affect the process of acute or strong immunogen-induced GC responses.

Taken together, the current study provides novel insights into the role of IL-21 and IL-17 in Tfh cell mediated pathogenesis of autoimmunity.
Figure 3. Proposed Model Based on the Current Study in BXD2 Mice
REFERENCES


11. Wong CK, Ho CY, Li EK, Lam CW. Elevation of proinflammatory cytokine (IL-18, IL-17, IL-12) and Th2 cytokine (IL-4) concentrations in patients with systemic lupus erythematosus. Lupus. 2000;9(8):589-93.


25. Fuller KA, Kanagawa O, Nahm MH. T cells within germinal centers are specific for the immunizing antigen. Journal of immunology. 1993;151(9):4505-12.


APPENDIX

APPROVAL FORM FROM INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC)

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF RENEWAL

DATE: December 13, 2012
TO: JOHN D MOUNTZ, M.D., Ph.D.
    SHEL-307 2182
    FAX: (205) 996-6788

FROM: Judith A. Kapp, Ph.D., Chair
      Institutional Animal Care and Use Committee (IACUC)

SUBJECT: Title: Suppression of Pathogenic Autoantibodies in Lupus by Inhibition of AID
         Sponsor: NIH
         Animal Project Number: 130108350

As of January 25, 2013, the animal use proposed in the above referenced application is
renewed. The University of Alabama at Birmingham Institutional Animal Care and Use
Committee (IACUC) approves the use of the following species and numbers of animals:

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<th>Species</th>
<th>Use Category</th>
<th>Number in Category</th>
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Animal use must be renewed by January 24, 2014. Approval from the IACUC must be obtained
before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate
granting agency.

Refer to Animal Protocol Number (APN) 130108350 when ordering animals or in any
correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this
study. If you have concerns or questions regarding this notice, please call the IACUC office at
(205) 934-7692.