Gut microbiota regulates K/BxN autoimmune arthritis through Tfh but not Th17 cells

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Abstract

The bacterial community that colonizes mucosal surfaces helps shape the development and function of the immune system. The K/BxN autoimmune arthritis model is dependent on the microbiota, and particularly on segmented filamentous bacteria (SFB), for the autoimmune phenotype. The mechanisms of how the gut microbiota affects arthritis development are not well understood. Here we investigate the contribution of two T cell subsets, Th17 and Tfh, to arthritis and how microbiota modulates their differentiation. Using genetic approaches, we demonstrate that IL-17 is dispensable for arthritis. Antibiotic treatment inhibits disease in IL-17 deficient animals suggesting that the gut microbiota regulates arthritis independent of Th17 cells. In contrast, conditional deletion of Bcl6 in T cells blocks Tfh cell differentiation and arthritis development. Furthermore, Tfh cell differentiation is defective in antibiotic-treated mice. Taken together, we conclude that gut microbiota regulates arthritis through Tfh but not Th17 cells. These findings have implications in our understanding of how environmental factors contribute to the development of autoimmune diseases.

Introduction

The effects of the intestinal microbiota on health and disease have been under intense study in recent years. A diverse and balanced microbial community is required for normal development of the innate and adaptive arms of the immune system (1, 2). The microbiota modulates the immune response against pathogens as well as self-antigens (3). One example of the microbiota promoting autoimmunity is the rheumatoid arthritis mouse model K/BxN, where the microbiota is required for disease development. In specific pathogen free (SPF) colonies, K/BxN mice develop arthritis spontaneously at 4 to 5 weeks of age. Germ-free or

antibiotic-treated K/BxN mice have significantly lower serum autoantibody titers, and ameliorated disease (4). The requirement of the microbiota for arthritis development is particularly intriguing, as the disease is manifested at sites distal to the gut. While the microbiota has some effect on the effector phase of the disease mediated by innate immune cells following the production of autoantibodies (5), it also plays important roles in the initiation phase where autoreactive KRN T cells get activated and drives B cells to produce autoantibodies. Which cell types are involved at this stage and how they are affected by the microbiota are not well understood.

Autoantibodies are essential for arthritis development in K/BxN mice (6, 7). Production of autoantibodies by B cells is critically dependent on help from T cells. It has been shown that the Th2-type cytokine IL-4, but not the Th1-type cytokine IL-12, is required for K/BxN arthritis (8). However, the cytokine profile of K/BxN T cells revealed that K/BxN arthritis is not a "pure" Th2 disease. K/BxN T cells expressed much higher amounts of IFN-γ than did the conventional Th2 cells. In addition, the former expressed much lower amounts of several Th2-associated cytokines (including IL-10, IL-13, and IL-5) than did the latter (8). The exact nature of T cell subset(s) that is critical for arthritis is not clear.

Follicular helper T cells (Tfh) are a T cell subset specialized in interacting with B cells. Tfh cells require the transcription factor Bcl6 for their differentiation and function (9). B cells presenting cognate antigen to Tfh cells are driven to differentiate into germinal center B cells, somatically hypermutate and class switch, and further differentiate into plasma cells and memory B cells. This activation and differentiation requires cytokine production from T cells, namely IL-21 and IL-4. We have previously demonstrated that IL-21 produced by T cells is required by B cells for disease in K/BxN mice (10), which is consistent with the idea that Tfh cells could paly an important role in arthritis development.

Another T helper subset, Th17 cells, has been shown to be able to provide help for B cells and drive autoimmune germinal center responses (11, 12). Th17 cells and IL-17 have been implicated in a number of autoimmune diseases and animal models (13–16). The differentiation of Th17 cells is promoted by colonization with commensal bacteria. In particular, segmented filamentous bacteria (SFB) alone can potently induce Th17 cells in wild-type mice (17), and strikingly, colonization with SFB alone is sufficient to promote disease in germ-free K/BxN mice (4). It has been proposed that the link between bacterial colonization and arthritis is through induction of Th17 cells and the proinflammatory cytokine interleukin-17A (IL-17). A key experiment supporting this conclusion was that IL-17 blockade by neutralizing antibody was able to inhibit arthritis (4). However, we have shown that *Rorc* deficient KRN T cells, unable to differentiate into Th17, were able to induce arthritis as well as wild-type KRN T cells suggesting Th17 cells are not essential for arthritis development (18). Nonetheless, it is not known whether IL-17 production from non-Th17 cells such as γδ T cells, innate lymphoid cells, and neutrophils, could contribute to arthritis development.

In this paper, we used genetic approaches to test the requirement of the Th17 cytokine IL-17, as well as Tfh cells, for arthritis development and their interactions with the microbiota. We found that IL-17 deficient K/BxN mice develop arthritis in a similar manner

as IL-17 sufficient littermates. Antibiotic treatment of IL-17 deficient mice demonstrated that a replete microbiota was required for disease independent of IL-17, at the level of the initiation phase. Antibiotic treatment reduced the Tfh and germinal center B cell populations in secondary lymphoid organs throughout the body. Finally, we showed that Bcl6-deficient KRN T cells did not induce arthritis, formally demonstrating that Tfh cells are required for arthritis development. This work highlights the potent effects of microbial colonization on T helper cell differentiation beyond the induction of Th17. These findings have implications in the context of human rheumatoid arthritis, where anti-IL17 pharmaceuticals are being explored as possible treatments.

Materials and Methods

Mice

Mice were bred in-house and maintained under specific pathogen free conditions at The University of Chicago (Chicago, IL). IL-17 deficient mice ($Il17a^{tm1/1(icre)Stck}$), CD4-Cre (Tg(CD4-cre)1Cwi), C57BL/6, and NOD mice were purchased from The Jackson Laboratory (Bar Harbor, ME). KRN TCR transgenic (KRN/B6), B6 I-A^{g7} congenic (B6.H-2^{g7}), TCR C$\alpha^{-/-}$B6 and TCR C$\alpha^{-/-}$NOD mice were gifts from D. Mathis and C. Benoist (Harvard Medical School). Bcl6-floxed mice were generated as described previously (19). To produce K/g7/IL17 mice, we bred KRN/B6 mice to IL-17 deficient mice (generating K/IL-17$^{+/-}$) and bred IL-17 deficient mice to B6.H-2^{g7} mice (generating IL-17$^{+/-}$ H-2$^{g7/b}$ and introducing the I-A^{g7} allele required for disease). We intercrossed IL-17$^{+/-}$ H-2$^{g7/b}$ mice to generate IL-17$^{-/-}$ H-2$^{g7/g7}$ mice. We then crossed K/IL-17$^{+/-}$ to IL-17$^{-/-}$ H-2$^{g7/g7}$ to generate K/IL-17$^{+/-}$ H-2$^{g7/b}$ (called K/g7/IL-17$^{+/-}$ in the text and IL-17+/− in the figures) and K/IL-17$^{-/-}$ H-2$^{g7/b}$ (called K/g7/IL-17$^{-/-}$ in the text and IL-17−/− in the figures). To produce K/Bcl6 mice, we bred KRN/B6 to Bcl6-floxed mice (generating K/Bcl6$^{fl/+}$) and bred Bcl6-floxed mice to CD4-Cre mice (generating CD4-Cre/Bcl6$^{fl/+}$). We then bred Cre/Bcl6$^{fl/+}$ back to Bcl6-floxed mice (generating CD4-Cre/Bcl6$^{fl/fl}$). Finally, we crossed K/Bcl6$^{fl/+}$ mice to CD4-Cre/Bcl6$^{fl/fl}$ to generate K/CD4-Cre/Bcl6$^{fl/+}$ (called K/Bcl6+ in the text and figures) and K/CD4-Cre/Bcl6$^{fl/fl}$ (called K/Bcl6 in the text and figures). Recipient mice for T cell transfer experiments were generated by crossing B6.TCR C$\alpha^{-/-}$ to NOD.TCR C$\alpha^{-/-}$ (called C$\alpha^{-/-}$ BxN in the text). These mice were deficient in αβ T cells and expressed MHC class II H-2$^{g7/b}$, which were required to induce arthritis. All experiments were approved by IACUC of The University of Chicago.

T Cell transfer and monitoring of arthritis

Approximately 10×10^6 total splenocytes were administered via i.v. tail injection to C$\alpha^{-/-}$BxN mice. Total splenocyte numbers were normalized so that equal numbers of KRN CD4$^+$ cells were transferred between different groups in each experiment. Hind ankle joint thickness was measured every 3 to 4 days using a caliper. Left and right measurements were averaged for each mouse at each timepoint.

Antibiotic treatment

1g/L ampicillin (Sigma) and 0.5g/L vancomycin (Hospira) were added to drinking water of K/BxN or K/g7/IL17 breeding pairs and mice were allowed to drink ad libitum. Water was

changed approximately every 6 days. Offspring of these breeders were maintained on the same antibiotic water and were gavaged daily with 100 μl water containing 20 μg ampicillin and 10 μg vancomycin beginning at 21 days old and continuing until time of analysis.

Lamina propria lymphocyte isolation

Lamina propria lymphocytes were isolated from the small intestine using enzymatic digestion as described in (4). Cells were then suspended in 40% percoll, layered on an 80% percoll gradient (GE) and centrifuged for 2500 rpm for 30 minutes without brake. Lymphocytes were collected at the interface and analyzed using intracellular staining.

Abs and flow cytometry and intracellular cytokine staining

Antibodies used were against CXCR5, Bcl6, and Fas (BD Biosciences); CD45.1, TCRβ, GL-7, CD19, IL-17A, and IFN-γ (eBioscience); and CD4 and PD-1 (BioLegend). Intracellular staining for Bcl6 was performed using the Foxp3 intracellular staining kit from eBioscience, following the manufacturer's protocol. To stain cells for intracellular IL-17, cells were stimulated with PMA and ionomycin and stained using the intracellular staining kit from BD Biosciences as described (11). Multicolor flow cytometric analysis was performed using a FACSCanto and FACSCalibur (BD Biosciences). Data analysis was conducted using FlowJo (Tree Star, Inc.).

qPCR for 16S rRNA

Fresh fecal pellets were collected from mice and stored at −20°C until DNA isolation. DNA was isolated using the QIAamp Fast DNA Stool Mini Kit (Qiagen) following the manufacturer's protocol using the following modifications: pellets were lysed using a minibeater for 90 seconds with silica beads (Zymo) instead of the initial vortexing step, and the first heating step was performed at 95°C. Total bacteria (EUA) 16S and segmented filamentous bacteria specific (SFB) 16S rRNA were amplified using primers as described in (20). qPCR primers: EUA-F: 5'-ACTCCTACGGGAGGCAGCAGT-3', EUA-R: 5'-ATTACCGCGGCTGCTGGC-3', SFB-F: 5'-GACGCTGAGGCATGAGAGCAT-3', SFB-R: 5'-GACGGCACGGATTGTTATTCA-3'. Cycling conditions: 95°C for 0:40; 45 cycles of 95°C for 0:05, 60°C for 0:30; 95°C for 0:15, 60°C for 1:00. Melting curve 60°C to 95°C, reading every 5°C. Relative SFB 16S rRNA is calculated as $2^{-\Delta Ct}$ where $\Delta C_t = C_t$ SFB − C_t EUA.

ELISA for anti-GPI IgG

96-well plates were coated with 5ug/mL of recombinant GPI in PBS overnight at 4°C. Plates were blocked with 1% BSA at room temperature for two hours. Serial dilutions of serum samples were added and incubated at room temperature for one hour. Bound antibodies were detected with a biotinylated goat anti-mIgG Fc (subclasses 1+2a+2b+3) or anti-mIgG1, mIgG2b, mIgG2c, or IgG3-specific polyclonal antibodies (at room temperature for 1 hour), followed by alkaline phosphatase-conjugated streptavidin (at room temperature for 30 minutes) (all reagents from Jackson ImmunoResearch Laboratories, Inc.). Samples were developed with phosphatase substrate (Sigma) and were read at 405 nm. Data were analyzed as in (18). Serum titers were calculated as the serum dilution that gave the calculated EC50

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

Statistical analysis

Normally distributed data were analyzed by the unpaired *t* test. Welch's correction was used for testing groups with different variances. Two-way ANOVA was used to test the effect of antibiotic treatment and IL-17 genotype (Figure 4). All statistics performed using Prism 5.0b software (GraphPad).

Results

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

To test the requirement of IL-17 in arthritis development, we generated a K/BxN IL-17 deficient mouse line. The K/BxN arthritis model requires the KRN transgene and the MHC class II A^{g7} allele. The A^{g7} allele can be introduced from either the NOD strain or B6.H-2^{g7} congenic mice, the latter of which was used in our breeding. These KRN/B6 $\times$ B6.H-2^{g7} (K/g7) mice develop the same arthritis as K/BxN mice (KRN/B6$\times$NOD). We crossed an *Il17a* deficient strain (Cre recombinase is knocked into the IL-17A locus before the ATG start codon, inactivating the IL-17A expression (21)) to KRN TCR Tg and to B6.H-2^{g7} congenic mice. The IL-17 deficient offspring, KRN$^+$ $A^{g7/b}$ *Il17a$^{Cre/Cre}$* mice (referred to as K/g7/IL17$^{-/-}$ in this paper), were monitored for arthritis development. Littermates heterozygous or wild-type for IL-17 (referred to as K/g7/IL-17$^{+/-}$ or K/g7/IL-17$^{+/+}$) were used as controls. K/g7/IL-17$^{-/-}$ mice developed arthritis to the same severity as K/g7/IL-17$^{+/-}$ mice, with slightly delayed but variable kinetics (Figure 1A). A single K/g7/IL-17$^{-/-}$ mouse did not develop disease or autoantibodies, for unknown reasons. Analysis of Th17 cells by IL-17 intracellular staining from various secondary lymphoid organs confirmed that Th17 cells were absent in K/g7/IL-17$^{-/-}$ mice, and the small intestine lamina propria contained the largest population of Th17 cells in K/g7/IL-17$^{+/-}$ mice, as expected (Figure 1B).

The KRN TCR recognizes a peptide from self antigen glucose-6-phosphate isomerase (GPI) presented by A^{g7} (6). This leads to B cell production of high titers of anti-GPI IgG (7). It was reported that IL-17 regulates B cell class switching to certain IgG isotypes in some autoimmune and delayed-type hypersensitivity models (22–24). However we found no defect in serum antibody titers against glucose-6-phosphate isomerase (GPI, the autoantigen of the K/BxN model) for any IgG isotypes by ELISA (Figure 1C). This result is in agreement with a study showing no effect of IL-17 on class switching *in vitro* (25).

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

Arthritis in K/BxN mice is driven by a germinal center response and anti-GPI autoantibodies (7, 18). We analyzed the Tfh and germinal center B cells by flow cytometry in K/g7/IL-17$^{+/-}$ and K/g7/IL-17$^{-/-}$ mice. Consistent with the serum antibody titer results, there were no statistical difference in germinal center B cell (CD19$^+$Fas$^+$GL-7$^+$) and Tfh cell

(Bcl6$^+$ CXCR5$^+$ CD4$^+$) proportions and numbers in the spleen, joint draining popliteal and inguinal lymph nodes, and mesenteric lymph nodes between the two groups of mice (Figure 2A and 2B). These results demonstrate that IL-17 did not play a role in the initiation phase of arthritis development.

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

We next wanted to determine how the gut microbiota regulates arthritis in the presence or absence of IL-17. To test whether the effect of the microbiota on arthritis development was dependent on IL-17, K/g7/IL-17$^{+/-}$ and K/g7/IL-17$^{-/-}$ mice were treated with ampicillin and vancomycin in their drinking water starting from birth and daily gavage starting from weaning. This antibiotic treatment effectively prevented disease in both IL-17 sufficient and deficient mice (K/g7/IL-17$^{+/-}$ had 11.1% and K/g7/IL-17$^{-/-}$ had 12.5% disease penetrance, (Figure 3A)). This result indicates that the autoimmune arthritis promoted by the microbiota is independent of IL-17. To test if antibiotic treatment affected the initiation phase of disease, we measured anti-GPI IgG serum titers by ELISA at 5.5 weeks, the age where maximum ankle thickness is normally reached in SPF mice. Serum titers in antibiotic treated mice, regardless of IL-17 genotype, were significantly lower in antibiotic treated mice compared to untreated mice (Figure 3B). This result demonstrates that antibiotic treatment reduces disease at the level of autoantibody production, indicating a defect in T and/or B cell activation or function.

Because SFB has been associated with arthritis development and SFB mono-colonized germ free K/BxN mice develop arthritis (4), we tested whether SFB was present in our colony and whether it was eliminated by the antibiotic treatment. The DNA yield from antibiotic treated animals was significantly decreased compared to untreated mice, indicating that the antibiotics reduced the total burden of bacteria in the gut (Figure 3C). SFB was found to be present by qPCR in all untreated mice, at equal levels in K/g7/IL-17$^{+/-}$ and K/g7/IL-17$^{-/-}$ mice (Figure 3D). However, SFB was not detected in the feces of any of the antibiotic treated mice when equal amounts of total DNA were used, demonstrating the antibiotic treatment was very effective at eliminating SFB from the gut (Figure 3D).

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

To further understand how the gut microbiota regulates the production of autoantibodies, we analyzed the B and T cell populations. There was no significant change in the number of B cells in the spleen and mesenteric lymph nodes, although the percent of B cells was slightly increased (Figure 4A). The number of B cells in the joint draining lymph nodes of antibiotic treated mice was decreased by about 2 fold compared to untreated mice, which was also reflected by the sizes of lymph nodes. However, germinal center B cells were dramatically decreased in all sites in antibiotic treated mice, by both percent and number (Figure 4B). The decreased B cell response suggested to us a lack of T cell help to B cells. Overall the percent and number of CD4$^+$ T cells were reduced in all sites examined (Figure 4C). The number of Tfh cells in antibiotic treated mice was decreased in multiple sites (Figure 4D). All these observations demonstrate a defect in Tfh cell differentiation and GC response in the absence of a replete gut microbiota.

Tfh cells are required for arthritis

To formally test the requirement for Tfh cells in arthritis induction, we generated KRN T cells deficient in Bcl6, the master transcription regulator of the Tfh program (reviewed in(9)). KRN TCR Tg mice were crossed to CD4-Cre mice and *Bcl6^{fl/fl}* mice to generate a T cell specific deletion of Bcl6 (referred to as K/Bcl6). Splenocytes from these mice were transferred into TCR Cα$^{-/-}$BxN hosts. These host mice express the MHC class II allele I-A^{g7} required for disease and lack αβ T cells, which allows for the expansion of transferred KRN T cells (26, 27). Control Bcl6 sufficient KRN T cell (K/Bcl6+) recipients developed arthritis normally, whereas no arthritis was observed in K/Bcl6 recipients (Figure 5A). Consistent with the lack of arthritis there was a dramatic reduction in anti-GPI IgG serum titers (Figure 5B). K/Bcl6 T cells did not develop into Tfh cells (PD-1$^+$ CXCR5$^+$) as expected, while a substantial proportion of wild-type KRN T cells differentiated into Tfh cells in all sites examined (Figure 5C). There was a significant reduction in the total number of CD4$^+$ K/Bcl6 cells compared to that of K/Bcl6+ cells two weeks after transfer, however this decrease was less dramatic at four days post-transfer (data not shown). K/Bcl6 T cells were able to differentiate into Th1 (IFN-γ$^+$) and Th17 (IL-17$^+$) cells after transfer (Figure 5D), further supporting the conclusion that Th17 cells does not drive the antibody production and arthritis. The loss of Tfh cell differentiation resulted in the absence of germinal centers (Figure 5E). From this set of experiments, we conclude that Tfh cell differentiation is required for arthritis in the K/BxN mice. This, together with the loss of Tfh cells in antibiotic treated mice, suggests that the microbiota plays an important role in promoting autoreactive Tfh cell development.

Discussion

In this study we investigated the contribution of Tfh versus Th17 cells to K/BxN arthritis and which cell population mediates the effect of the gut microbiota. We showed that Tfh cells are essential for autoantibody production and disease by using KRN T cells deficient for Bcl6, the key transcription factor regulating Tfh cell differentiation. Consistent with earlier studies, these cells failed to differentiate into Tfh cells, but could differentiate into Th17 cells, demonstrating that these two subsets are distinct lineages and Th17 cells are not sufficient to drive arthritis. Together with our earlier finding that KRN T cells deficient in RORγt drive arthritis as well as the wild-type KRN T cells (18), we conclude that K/BxN arthritis is dependent on Tfh but not Th17 cells.

We showed that K/BxN arthritis is not dependent on the proinflammatory cytokine IL-17, and that the gut microbiota promotes disease in its absence. This finding is somewhat surprising given the numerous studies on the role of Th17 cells and IL-17 in the development of autoimmunity. However, the role of Th17 cells and IL-17 in inflammatory arthritis is multifaceted and highly dependent on disease stages, tissues involved, and the models utilized (reviewed in (28, 29)). For example, IL-17 is required for the IL1Ra$^{-/-}$ arthritis model and the collagen-induced arthritis model (14, 22, 30). In both cases, disease was greatly reduced or abolished in mice deficient in IL-17, and IL-17$^{-/-}$ T cell priming or proliferation was defective in *in vitro* assays. There were also modest decreases in antibody production for certain IgG isotypes. In contrast, in a proteoglycan-induced model of arthritis,

IL-17 deficient mice developed arthritis normally, although IL-17 was readily detectable in the joints and spleen. T cells cultured *in vitro* produced normal levels of cytokines, and serum antibodies against collagen were unchanged for the two isotypes examined (31). These differences certainly reflect the different arthritogenic mechanisms in different models. They are also mirrored in several clinical trials testing anti-IL-17 and anti-IL-17R antibodies in rheumatoid arthritis patients that demonstrated only weak to moderate efficacy (32–35).

The role of IL-17 in the T cell-independent effector phase of inflammatory arthritis has been investigated in earlier studies using the K/BxN serum transfer model. IL-17RA$^{-/-}$ mice develop less severe arthritis, and expression of several pro-inflammatory mediators are decreased in the ankle joints (36). A similar finding is shown in IL-17$^{-/-}$ mice, where neutrophils are shown to be an important source of IL-17 production (37). It is important to note that arthritis in the serum transfer model is much less aggressive than in the spontaneous K/BxN model due to the much lower titers of autoantibodies transferred. There are precedents for disease-modulating effects observed in the serum transfer model to be lost or less severe in the spontaneous model (38, 39). We focused on the initiation phase of the disease in which the germinal center response and autoantibody production are main readouts. Wu *et al* showed that an anti-IL-17 neutralizing antibody (clone MAB421) was able to inhibit arthritis in the spontaneous K/BxN model (4). This differs from our results in K/g7/IL-17$^{-/-}$ mice. We also tested two well-characterized IL-17 neutralizing monoclonal antibodies (clones TC11-18H10.1 (40, 41) and 17F3 (42, 43)) in K/BxN mice, starting treatment before disease onset. Arthritis was slightly delayed in mice treated with antibodies, however by 5 weeks of age disease severity was comparable and anti-GPI IgG serum titers were indistinguishable (data not shown). The discrepancy may be attributable to different microbiota in mouse facilities (see discussion below).

In addition to its role as a proinflammatory cytokine, IL-17 has been shown to drive germinal center responses in autoimmune BXD2 mice (12). Immunization of IL-17RA$^{-/-}$ mice on both the B6 and BXD2 background results in decreased production of high-affinity antibody specific for the hapten component of the antigen. It is proposed that IL-17 arrests the migration of B cells by suppressing B cell chemotactic response to the chemokines CXCL12 and CXCL13. Further study by the same group showed that the total numbers of Tfh cells and their ability to induce B cell responses *in vitro* were not affected in BXD2-IL17RA$^{-/-}$ mice, however, majority of the Tfh cells were not localized in the germinal center light zone (23). It was concluded that IL-17 is an extrinsic stop signal that promotes optimal localization of Tfh cells in germinal center. In K/BxN mice, *Il17ra*$^{-/-}$ B cells failed to be recruited into germinal centers when in competition with wild-type B cells (4). However, it is not clear whether *Il17ra*$^{-/-}$ B cells are defective in forming germinal centers in the absence of competition. In this context, the lack of a requirement for IL-17A in K/BxN arthritis could be due to high proportions of autoreactive T and B cells in this model, which makes their optimal localization in germinal centers unnecessary. Alternatively, IL-17F may play a redundant role as IL-17A, although IL-17F binds to IL-17RA with about 100 to 1000 times lower affinity than does IL-17A (44).

How the gut microbiota affects immune responses and autoimmunity is an actively pursued area of investigation. We have confirmed that K/BxN arthritis is critically dependent on gut microbiota using antibiotic treatment. Even though the gut microbiota plays a role in effector phase of arthritis (5), it also has a dramatic effect on the initiation phase of the disease because autoantibody titers are dramatically reduced in antibiotic-treated mice. Consistent with decreased autoantibody titers, there was a decrease in germinal center and Tfh cell populations. It was proposed that gut microbiota, specifically SFB, drives K/BxN arthritis through the promotion of Th17 cells and IL-17 production. However, our demonstration that arthritis develops in the absence of Th17 cells and IL-17, and that antibiotic treatment prevents disease independent of IL-17, points to other disease inducing effects of the microbiota.

There is evidence that microbial colonization leads to Tfh cell differentiation, with the main focus of most studies on germinal centers and IgA production against microbial antigens (45). Kubinak and colleagues have recently demonstrated that Tfh cells are dramatically reduced in the Peyer's patches of germ free mice, or mice with T cells deficient in MyD88. Furthermore, Tfh cells can be restored in germ free mice with the introduction of TLR2 ligands (46). Our observations suggest that the bacterial colonization can also play a role in promoting the differentiation of autoreactive T cells towards a Tfh phenotype.

Although it is clear that the microbiota influences autoreactive T cell activation, the site of T cell priming against self antigen is still unknown. One possibility is that T cells must traffic to the gut to be activated. There is evidence that activated cells have circulated through the gut, as KRN T cells photoactivated in the colon could later be found in the spleen and producing cytokines (47) and a subset of KRN T cells express the gut-homing integrin $\alpha 4\beta 7$ (4). Alternatively, antigen presenting cells may need to migrate from the gut to secondary lymphoid organs to prime T cells. Supporting this, using the same photoconversion technique, multiple myeloid subsets were found to migrate extensively between the gut and secondary lymphoid organs (47). Finally, it may be that neither T cells nor antigen presenting cells need to enter the microenvironment of the gut, and microbial ligands entering systemic circulation could provide necessary activating signals at sites distal to the gut (48).

Although SFB is best characterized for its ability to promote Th17 cell development (17, 49), it is highly immunopotent and is likely to regulate other cells of the immune system directly or indirectly. Our observation that SFB is undetectable in the feces of mice treated with antibiotics is consistent with a role in promoting Tfh cell differentiation and arthritis. However, antibiotic treatment also reduces the abundance of additional bacterial community members and these bacteria may contribute to or play a redundant role in Tfh cell differentiation. The variation of these additional members among different facilities may account for the differential requirement for IL-17 in arthritis development.

The K/BxN model is often used as an example of a Th17-dependent autoimmune model and as evidence that IL-17 should be a target for treatment of autoimmune disease (3, 50). However, our results demonstrate that IL-17 is not required in K/BxN arthritis and contribute to mounting evidence that IL-17 is not an effective target for some autoimmune

diseases (51). In contrast, we found that Tfh cells are critically important for the development of arthritis, and that Tfh cell differentiation is affected by the microbiota. Understanding how the commensal microbiota influences T cell activation, differentiation, and autoimmune pathogenesis will contribute to the development of new and more effective therapies for autoimmune diseases.

Acknowledgments

Abbreviations used in this article

GPI   glucose-6-phosphate isomerase

GC   germinal center

Tfh   follicular helper T cell

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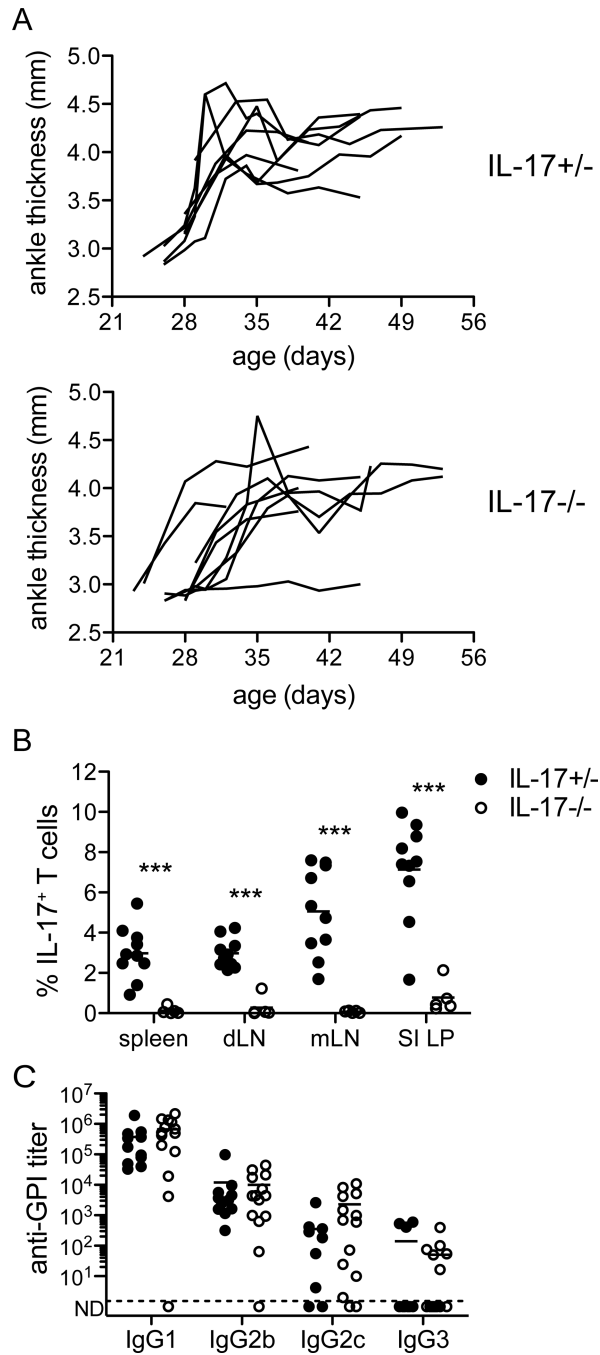
FIGURE 1.

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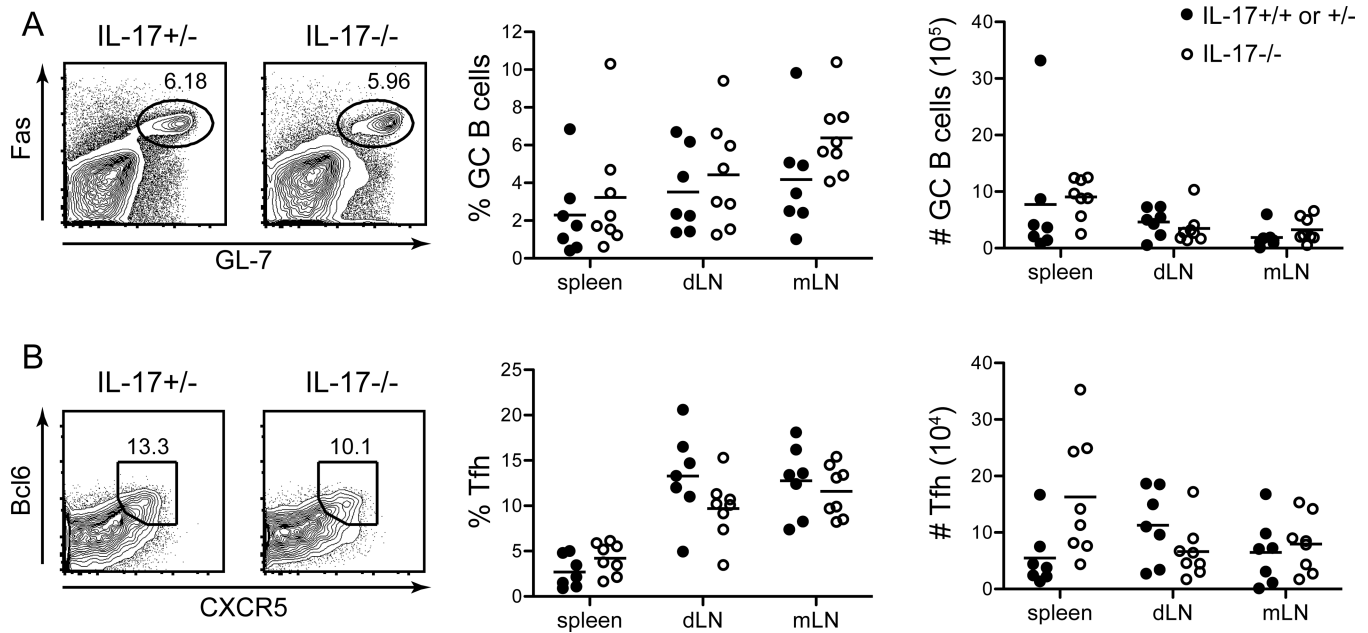
titers for the indicated isotypes in mice aged 6 to 8 weeks ($n = 11$–14 mice/group). Points below dashed line were not detectable (ND). $p > 0.05$ for all isotypes. Horizontal lines show the mean. Student's t test: $p > 0.05$ for all isotypes. ***, $p < 0.0001$.

FIGURE 2.

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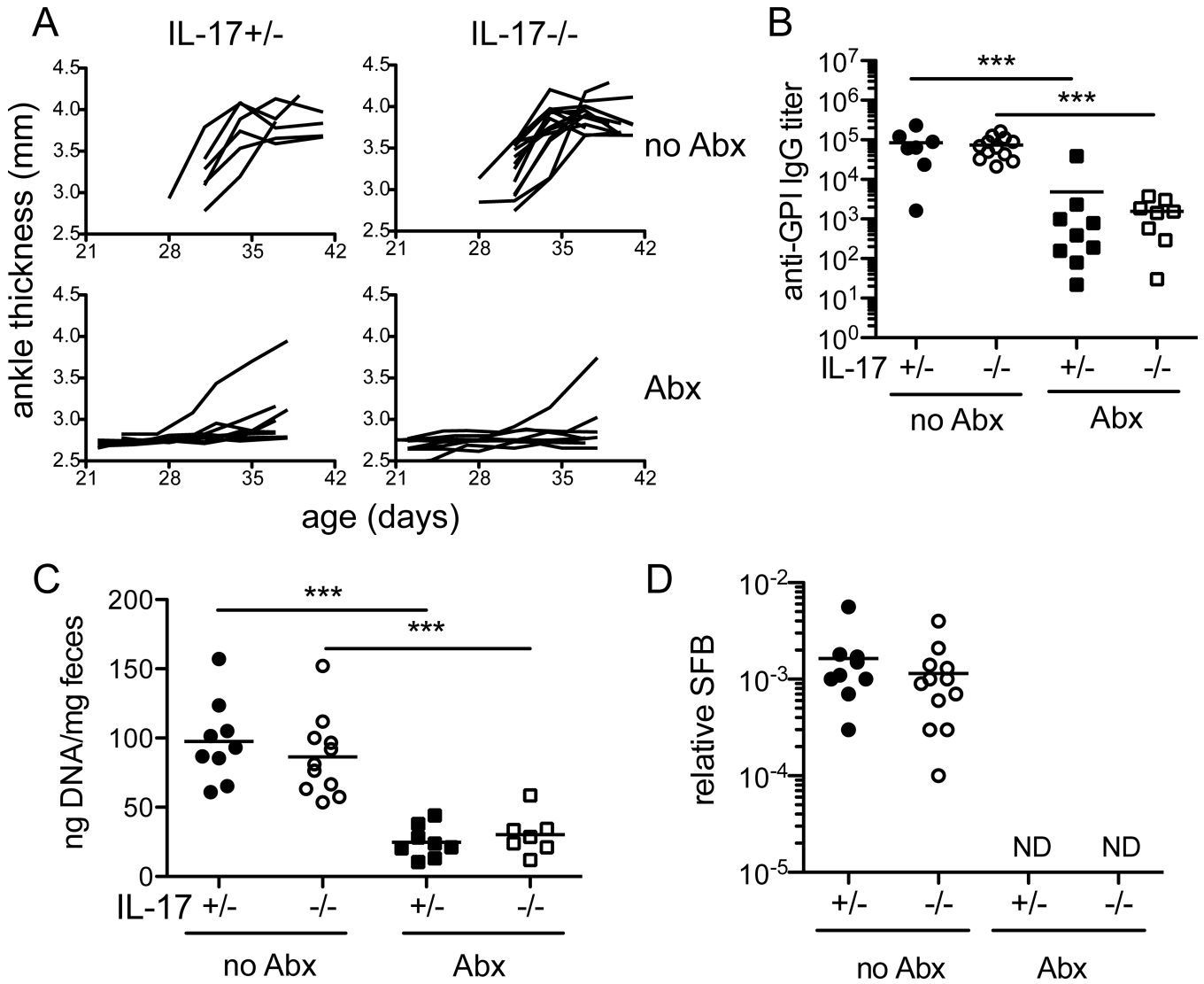significant (p > 0.05).

FIGURE 3.

Antibiotic treatment prevents arthritis independent of IL-17. (**A–D**) K/g7/IL-17$^{+/-}$ and K/g7/IL-17$^{-/-}$ littermates were untreated (no Abx) or treated with ampicillin and vancomycin in the drinking water from birth and gavaged daily with antibiotics beginning at weaning (Abx). (**A**) Ankle thickness was monitored. Each line represents a mouse. (**B**) Serum anti-GPI IgG levels were detected by ELISA at 5.5 weeks of age. (**C**) Fecal pellets were collected at 5.5 weeks of age and DNA was extracted. DNA yield was normalized to the pellet weight. (**D**) The relative amount of SFB (normalized to total bacteria) was quantified by real-time PCR. ND = not detectable for all samples of indicated group. Data shown are pooled from six no Abx litters and six Abx litters ($n = 6–13$ mice per group). Each symbol represents a mouse. Horizontal lines represent the mean. Student's t test: *** $p < 0.0001$.
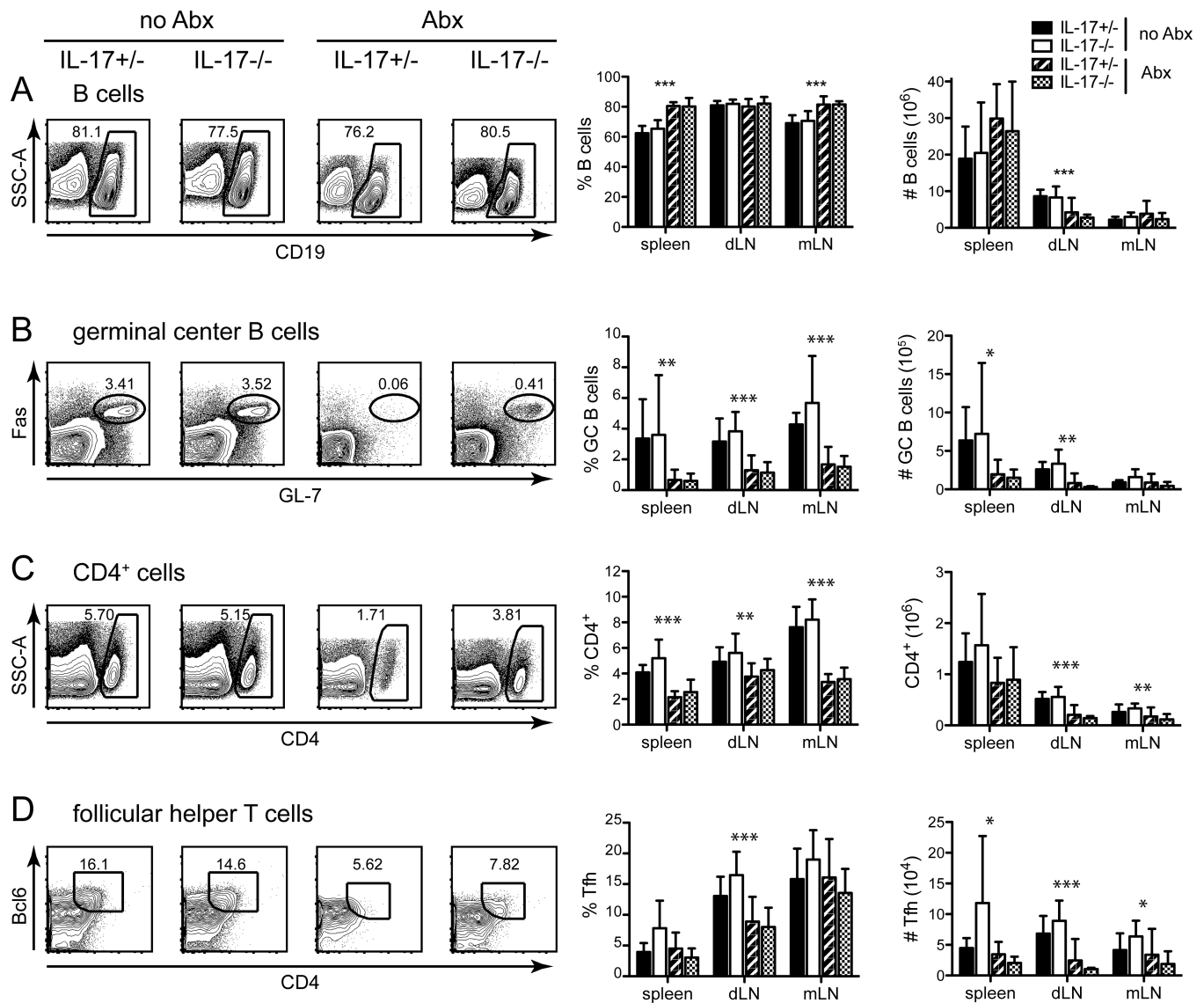
FIGURE 4.

Antibiotic treatment reduces germinal center B cells and Tfh cells independent of IL-17. (**A–D**) Percent and number of the indicated cell types in the spleen, joint draining lymph nodes (dLN, pooled popliteal and inguinal lymph nodes), and mesenteric lymph nodes (mLN) are shown for 5.5 week old K/g7/IL-17$^{+/-}$ and K/g7/IL-17$^{-/-}$ mice untreated (no Abx) or treated with antibiotics (Abx) as described in Figure 3A. Representative plots from the dLN. (**A**) B cells (CD19$^+$). (**B**) Germinal center B cells (GL-7$^+$ Fas$^+$) gated on CD19$^+$ cells. (**C**) CD4$^+$ cells. (**D**) Tfh cells (Bcl6$^+$ CXCR5$^+$) gated on CD4$^+$ cells. Data shown are pooled from six no Abx litters and six Abx litters ($n = 6$–13 mice per group). Displayed are mean + SD. Statistical difference between groups tested with 2-way ANOVA. p-values displayed are for the treatment factor, comparing no Abx vs Abx. No other factors were significant, except for percent Tfh in the spleen, where the interaction of genotype and treatment $p < 0.05$. If no p-value is displayed, $p > 0.05$. * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$.
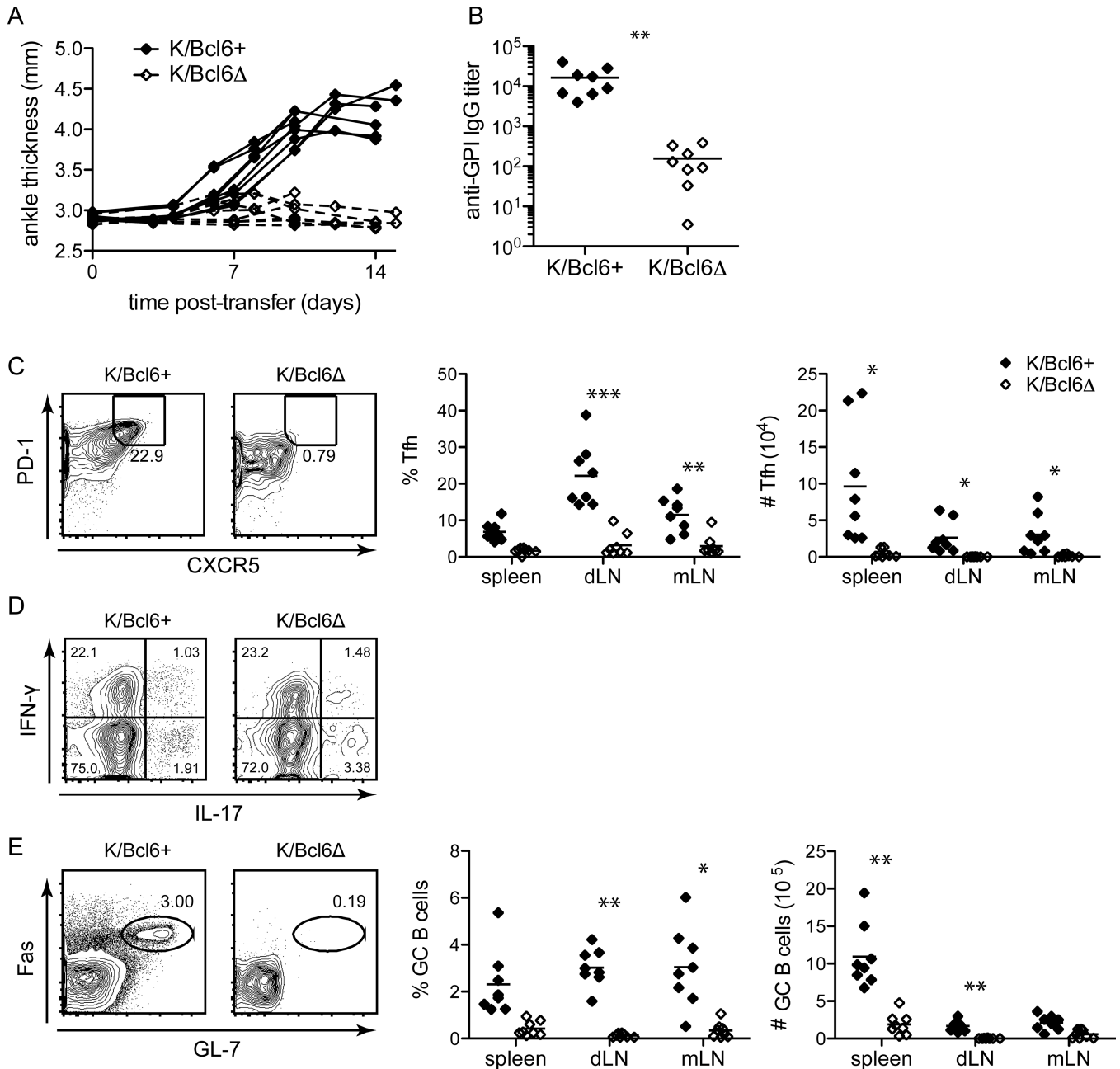
FIGURE 5.

Tfh cells are required for T cell transfer induced arthritis. (**A–E**) KRN/B6 splenocytes either sufficient for Bcl6 (K/Bcl6+) or with a T cell-specific deletion of Bcl6 (K/Bcl6∆) were transferred into Cα$^{-/-}$BxN hosts and analyzed 10–15 days later. (**A**) Ankle thickness was monitored. Each line represents a mouse. (**B**) Anti-GPI IgG serum titers. (**C**) Tfh cells (PD-1$^+$ CXCR5$^+$) of transferred T cells (CD45.1$^-$ CD4$^+$) in the spleen, joint draining lymph nodes (dLN, pooled popliteal and inguinal lymph nodes), and mesenteric lymph nodes (mLN). Shown are representative plots from the dLN. (**D**) Detection of Th1 and Th17 cells by intracellular cytokine staining gated on transferred T cells (CD45.1$^-$ CD4$^+$). Shown are representative plots from the spleen. (**E**) Germinal center B cells (GL-7$^+$ Fas$^+$) of CD19$^+$

cells in the spleen, joint draining lymph nodes (dLN, pooled popliteal and inguinal lymph nodes), and mesenteric lymph nodes (mLN). Representative plots from the dLN. Each symbol represents a mouse. Horizontal lines represent the mean. Data are pooled from two independent experiments (n = 8 mice/group). Student's t test: if no p-value is displayed, p > 0.05. * p < 0.05, ** p < 0.001, *** p < 0.0001.