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RESEARCH ARTICLE

Adaptive Immunity

Roles of T Follicular Helper Cells and T Follicular Regulatory Cells in Autoantibody Production in IL-2–Deficient Mice

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ABSTRACT

Autoantibodies can result from excessive T follicular helper (Tfh) cell activity, whereas T follicular regulatory (Tfr) cells negatively regulate autoantibody production. IL-2 knockout (KO) mice on the BALB/c background have elevated Tfh responses, produce autoantibodies, and develop lethal autoimmunity. We analyzed Tfh and Tfr cells in IL-2 KO mice on the C57BL/6 (B6) genetic background. In B6 IL-2 KO mice, the spontaneous formation of Tfh cells and germinal center B cells was greatly enhanced, along with production of anti-DNA autoantibodies. IL-2 has been reported to repress Tfr cell differentiation; however, Tfr cells were not increased over wild-type levels in the B6 IL-2 KO mice. To assess Tfh and Tfr cell regulation of autoantibody production in IL-2 KO mice, we generated IL-2 KO mice with a T cell–specific deletion of the master Tfh cell transcription factor Bcl6. In IL-2 KO Bcl6 conditional KO (2KO-Bcl6TC) mice, Tfh cells, Tfr cells, and germinal center B cells were ablated. In contrast to expectations, autoantibody IgG titers in 2KO-Bcl6TC mice were significantly elevated over autoantibody IgG titers in IL-2 KO mice. Specific deletion of Tfr cells with Foxp3-cre Bcl6-flox alleles in IL-2 KO mice led to early lethality, before high levels of autoantibodies could develop. We found IL-$2^{+/+}$ Tfr cell–deficient mice produce significant levels of autoantibodies. Our overall findings provide evidence that Tfh cells are dispensable for high-level production of autoantibodies and also reveal a complex interplay between Tfh and Tfr cells in autoantibody production and autoimmune disease. *ImmunoHorizons*, 2019, 3: 306–316.

INTRODUCTION

During an immune response, CD4 Th cells can differentiate into several unique effector lineages that promote different immune responses via the secretion of distinct types of cytokines. T follicular helper (Tfh) cells are a CD4 T cell lineage whose major function is to help B cells form germinal centers (GCs) and produce high-affinity Abs (reviewed in Ref. 1, 2). Tfh cells are characterized by a high level of expression of the chemokine receptor Cxcr5, which binds the chemokine Cxcl13 expressed in B cell follicles. Cxcl13, acting on Cxcr5, promotes migration of Tfh cells to the B cell follicle. Tfh cells have an activated effector T cell phenotype and express elevated ICOS and PD-1. Tfh cells regulate memory B cell and plasma cell development, and thus Tfh cells control both the initiation as well as the outcome of the GC B cell response (1, 2). A key cytokine produced by Tfh cells is IL-21, a factor that potently promotes B cell activation and Ab secretion (1, 2). Although Tfh cells are critical for the proper production of Abs, the overproduction

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Abbreviations used in this article: AIHA, autoimmune hemolytic anemia; DKO, double KO; GC, germinal center; KO, knockout; SNR, signal-to-noise ratio; Tfh, T follicular helper; Tfr, T follicular regulatory; Treg, regulatory T cell; WT, wild-type.

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of Tfh cells can lead to autoimmunity as Tfh cells can help B cells to produce self-reactive Abs (1–10). Thus, the proper regulation of Tfh cell differentiation is essential for normal immune function as well as preventing autoimmune disease.

The Bcl6 transcriptional repressor protein is upregulated in Tfh cells and is considered the master regulator for Tfh cells (11–13). Forced Bcl6 expression promotes differentiation of CD4 T cells into Tfh cells, whereas Bcl6-deficient T cells cannot differentiate into Tfh cells. A conditional knockout (KO) mouse for Bcl6, that we have developed, allows direct analysis of Bcl6 function in specific T cell populations (14).

A subpopulation of follicular T cells can act as suppressors of the GC response (15–21). These cells express both Foxp3 and Bcl6 and have been termed T follicular regulatory (Tfr) cells. Tfr cells have a hybrid phenotype of Tfh cells and regulatory T cells (Tregs). Like Tfh cells, Tfr cells are dependent on Bcl6 and Cxcr5 for localization to the B cell follicle. Tfr cell functions are not well understood. In many model systems, Tfr cells suppress the number of Tfh cells and GC B cells during the immune response and also regulate affinity maturation of Abs (15–21). However, Tfr cells can also promote the GC and Ab response in certain model systems (22, 23). A popular model for the function of Tfr cells is that Tfr cells limit the number of Tfh cells in the GC reaction or the amount of Tfh cell help, thus preventing excessive Tfh cell help to B cells (15, 24). In the absence of Tfr cells, excessive Tfh cell activity may lead to less-stringent selection of high-affinity B cell clones, leading to the expansion of low-affinity B cell clones. Excessive Tfh cell activity may also promote the expansion of self-reactive B cells. Indeed, Tfr cells have an important role in maintaining tolerance during the B cell response and inhibiting the development of autoantibodies (10, 23, 25–30).

Many autoimmune diseases are characterized by pathogenic self-reactive or autoreactive Abs (autoantibodies) (31–33). Autoantibody-mediated autoimmune diseases, such as systemic lupus erythematosus, rheumatoid arthritis, myasthenia gravis, and autoimmune hemolytic anemia (AIHA), are a tremendous source of morbidity and mortality for the human population. Because Tfh cells can promote autoantibody production (1–9), Tfh cells are considered targets for developing therapies for autoantibody-driven autoimmune diseases, particularly systemic lupus erythematosus. However, the extent to which Tfh cells are required for autoantibody generation is not clear. Tfh cells are not strictly required to help B cells make Abs (2, 14, 34), and in some model systems, autoantibodies can even be produced in a T cell–independent manner (35, 36). Understanding the interplay of Tfh and Tfr cells in the control of the autoantibody response will expand our knowledge of the pathogenesis of diseases such as lupus and should lead to the development of new treatments.

IL-2–deficient (KO) mice develop a lethal autoimmune disease that varies with the genetic background of the mice. On a mixed 129/01a × C57BL/6 genetic background, IL-2 KO mice die early (at 9 wk on average) and almost invariably develop severe colitis (37, 38). IL-2 KO mice on the BALB/c background die between 3 and 5 wk from AIHA caused by the production of autoantibodies to RBCs (38, 39). In these mice, Tfh cells increased in number because IL-2 suppresses Tfh cell differentiation by inhibiting Bcl6 transcription (40–42). Tfr cell differentiation can be inhibited by IL-2 (25), although Tfr cells have not been examined previously in the IL-2 KO mice.

In the current study, we wondered if Tfr cells were elevated in IL-2 KO mice and if Tfh and Tfr cells played specific roles in autoantibody production. We observed that, unexpectedly, Tfr cells were not elevated, even though Tfh cells were dramatically increased in IL-2 KO mice. Furthermore, we found that in IL-2 KO mice, high levels of autoantibodies can be produced independent of Tfh cells and low levels of Tfr cells can dramatically limit autoantibody production.

MATERIALS AND METHODS

Mice

IL-2 KO mice on the C57BL/6 background and MyD88 KO mice on the C57BL/6 background were obtained from The Jackson Laboratory. CD4-cre Bcl6-flox and Foxp3-cre Bcl6-flox mice were described previously (14, 23). Crossing of strains to produce IL-2 KO CD4-cre Bcl6-flox (2KO-Bcl6TC), IL-2 KO Foxp3-cre Bcl6-flox (2KO-Bcl6FC), and IL-2 KO MyD88 double KO (IL2-MyD88) mice used published genotyping protocols (14, 23) as well as genotyping protocols from The Jackson Laboratory. All IL-2 KO mice were bred from IL-$2^{+/-}$ parents. IL-$2^{+/+}$ wild-type (WT) littermates were used as controls for IL-2 KO, 2KO-Bcl6TC, 2KO-Bcl6FC, and IL2-MyD88 double KO (DKO) mice. Both male and female mice of 5–10 wk old were analyzed. Mice were bred under specific pathogen-free conditions at the laboratory animal facility at the Indiana University School of Medicine and were handled according to protocols approved by the Indiana University School of Medicine Animal Use and Care Committee.

Flow cytometry reagents and cell staining for flow cytometry

Cell suspensions from spleens were prepared and filtered through a 40-μm cell strainer (Fisherbrand). Cells were washed and diluted in PBS with 1% FBS and were stained with Fc block (BioXCell) for 5 min, followed by surface staining for the indicated markers. The following labeled Abs were used: anti-CXCR5 (L138D7), anti–PD-1 (29F.1A12), anti-CD4 (RM4-5), anti-Foxp3 (MF-14), anti-CD38 (90), anti-B220 (RA3-6B2) (all obtained from BioLegend), and GL7 (GL7) (purchased from BD Pharmingen). For intracellular staining, after surface markers were stained, cells were fixed and stained with anti-Foxp3, and an eBioscience intracellular kit was used. All samples were acquired on an LSR2 flow cytometer (Becton Dickinson) and analyzed with FlowJo software.

Anti-dsDNA titer analysis

Anti-dsDNA IgA and IgG titers were determined by ELISA, as previously reported (14). Briefly, 96-well Nunc-Immuno plates (Sigma-Aldrich) were coated overnight at 4°C with salmon sperm DNA passed through a 45-μm filter (Invitrogen). Wells were blocked with 1% BSA for 1.5 h. Diluted serum samples were applied. ELISA plates were incubated for 1 h at 37°C for anti-dsDNA Ab detection. Peroxidase-conjugated anti-mouse IgA or

anti-mouse IgG (Sigma-Aldrich) was used as secondary Ab. Anti-dsDNA ELISA plates were washed extensively after each incubation to minimize high background signals.

Autoantibody array

Autoantibody reactivities against a penal of autoantigens were measured using an autoantigen microarray platform developed by the University of Texas Southwestern Medical Center (https://microarray.swmed.edu/products/category/protein-array/). Serum samples were incubated with the autoantigen array, and the autoantibodies binding to the Ags on the array were detected with laser wavelengths of 532 nm (cy3 labeled anti-IgG), generating Tiff images. GenePix Pro 6.0 software was used to analyze the image and generate a .GPR file (GenePix Results format). Signal-to-noise ratio (SNR = [foreground median − background median]/[SD background]) is used as a quantitative measure of the ability to resolve true signal from background noise. A higher SNR indicates higher signal over background noise. An SNR equal to or bigger than three is considered true signal from background noise.

Pristane administration

Mice were given a single i.p. injection of 0.5 ml of Pristane (Sigma-Aldrich) at 12 wk of age. After 4 mo, mice were sacrificed. The presence of anti-dsDNA Abs in the serum was tested by ELISA.

Statistical analysis

All data analysis was done using Prism GraphPad software. Unless otherwise stated, Student *t* test for pairwise comparisons or ANOVA with Tukey post hoc analysis for multivariable analysis were used. Only significant differences ($p < 0.05$) are indicated in figures.

RESULTS

Tfh cells but not Tfr cells are increased in the absence of IL-2 in vivo

To analyze the role of Tfh and Tfr cells in the IL-2 KO, we obtained IL-2 KO mice on the C57BL/6 (B6) background because this strain was genetically compatible with most conditional KO strains that are also on the B6 background. We found that IL-2 KO mice on the B6 background in our facility were healthier than reported for IL-2 KO mice on the BALB/c background (38, 39, 43). In our colony, most B6 IL-2 KO mice live longer than 5 wk and have a lifespan more similar to what was seen with IL-2 KO mice on the mixed 129-O1a × C57BL/6 background (37, 38). However, the B6 background IL-2 KO mice were nonetheless smaller than WT and IL-$2^{+/-}$ littermates, had a sickly appearance, had enlarged spleens (Supplemental Fig. 1), and were infertile. To determine if loss of IL-2 led to higher Tfh and Tfr cells in this B6 background IL-2 KO strain, we conducted flow cytometry analysis (Fig. 1). As shown in Fig. 1A and 1B, we observed a roughly 7-fold increase in Tfh cells in unimmunized IL-2 KO mice compared with unimmunized WT mice. Even more striking was the large increase (~15-fold) in PD-1^+ populations, indicating a high level of activated CD4 T cells in the unmanipulated IL-2 KO mice. In contrast to Tfh cells, Tfr cells were not significantly increased in the IL-2 KO mice (Fig. 1C, 1D), although IL-2 has been shown to inhibit both Tfh and Tfr cell development (25, 40–42). The lack of increase in Tfr cells was not due to a general loss of Tregs in these mice, as total numbers of Foxp3^+ CD4^+ T cells were not significantly different from WT (Supplemental Fig. 1). This result is different from what was seen with IL-2 KO mice on the BALB/c background, which show a marked depletion of Tregs (44). The more-severe phenotype of the BALB/c IL-2 KO mice may relate to the significant loss of Tregs seen in that strain. Even though overall numbers of Tregs were not significantly decreased in the B6 IL-2 KO mice, there was a 3-fold increase in PD-1^+ Tregs (Fig. 1C, 1D), suggesting more Treg activation in the IL-2 KO background. Our results also show that although IL-2 can be inhibitory for Tfr cell differentiation (25), in the IL-2 KO model of autoimmunity, IL-2 is also required for Tregs to fully mature into Tfr cells.

Deletion of Tfh and Tfr cells in IL-2 KO mice

To test the functional role of Tfh cells in autoimmune disease and autoantibody production in the IL-2 KO mice, we mated the mice to the Bcl6-flox CD4-cre mouse strain (termed in this article as Bcl6TC), which is unable to produce follicular T cells because of deletion of the Bcl6 gene in CD4 and CD8 T cells (14, 45). We therefore produced double mutant mice that we termed 2KO-Bcl6TC mice. The 2KO-Bcl6TC mice show a similar disease profile to IL-2 KO mice: both types of mice are smaller in size, and both have enlarged spleens, multiorgan inflammation, and similarly shortened lifespans (Supplemental Figs. 1, 2 and data not shown). However, the Bcl6TC mice did have significantly less inflammation in liver and kidney than IL-2 KO mice (Supplemental Fig. 2B). We then examined the Tfh and GC B cell compartments in 2KO-Bcl6TC mice and found both Tfh and GC B cells were completely ablated (Fig. 2). This indicates, first, that even in an IL-2–deficient environment, Bcl6 is still required for Tfh cell development. Second, these data show that Tfh cells do not correlate with overall disease in the IL-2 KO model.

Tfh cell–independent production of autoantibodies

We next asked whether Tfh cells were required for autoantibodies in IL-2 KO mice and first measured anti-dsDNA Abs by ELISA (Fig. 3). As expected, IL-2 KO mice had elevated levels of both IgG and IgA anti-DNA Abs compared with WT. Unexpectedly, loss of Tfh cells in 2KO-Bcl6TC mice led to significantly higher titers of both IgG (~3-fold higher) and IgA (~10-fold higher) anti-DNA Abs than seen in the IL-2 KO mice. To further analyze autoantibodies in these mice, we used an autoantigen microarray assay system to screen for IgG autoantibodies to a panel of mouse protein Ags. Out of 94 autoantigens analyzed on the microarray, 17 autoantigens showed a significant difference in IgG reactivity between WT, IL-2 KO and 2KO-Bcl6TC groups (Fig. 4A). All 17 of these autoantigens showed a significantly higher Ab binding score for the 2KO-Bcl6TC sera over WT and IL-2 KO. Surprisingly, IL-2 KO sera showed little increase in autoantigen binding over WT (Fig. 4). These data showed that high levels of autoantibodies could be produced in the absence of Tfh cells. To test that this result was not unique to the IL-2 KO model, we used the pristane model of

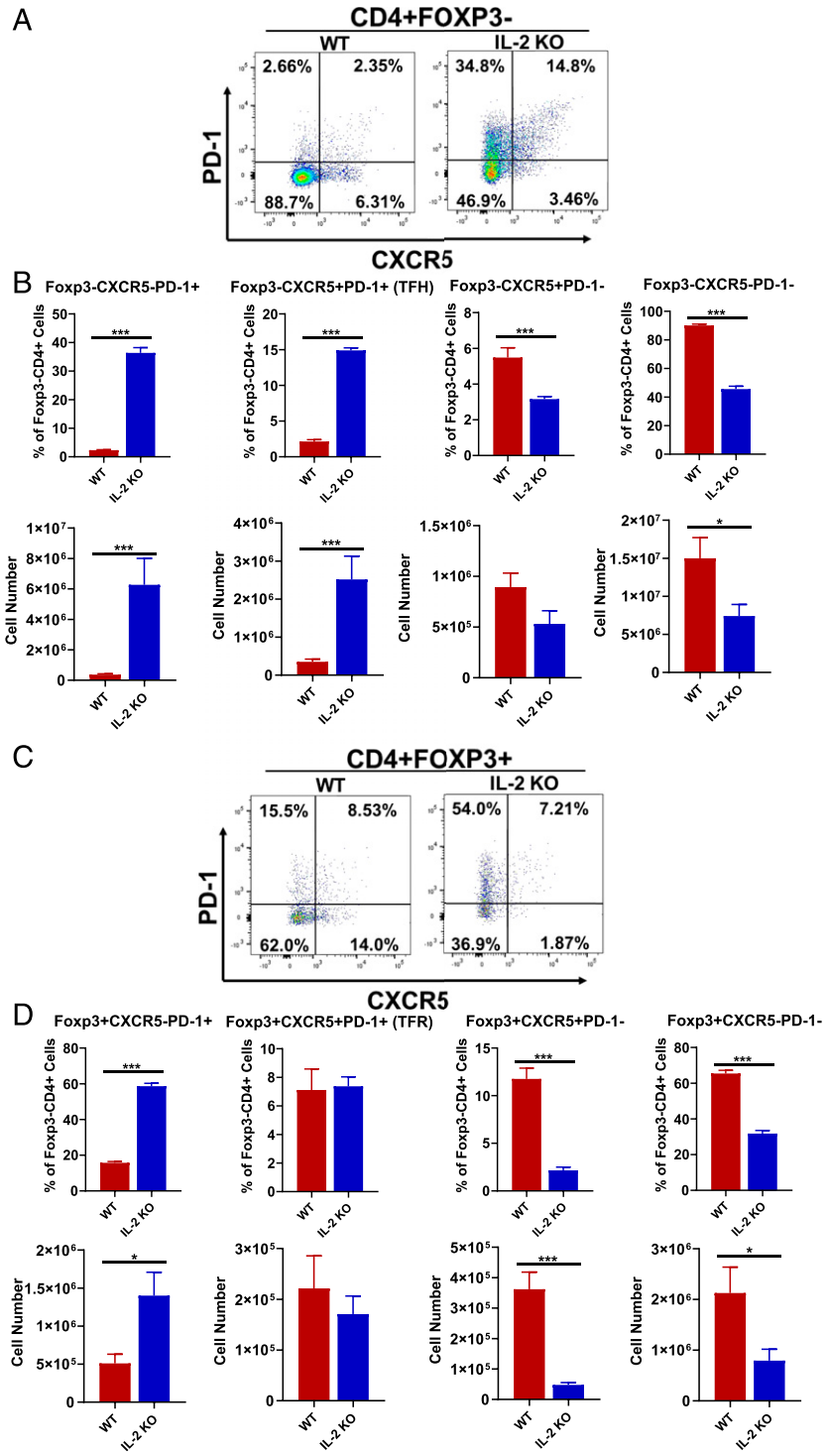

FIGURE 1. Spontaneous and strong Tfh cell but not Tfr cell development in IL-2 KO mice.

Naive 10-wk-old WT and IL-2 KO mice were used to analyze Tfh and Tfr responses. Spleens were analyzed for the indicated cell populations by flow cytometry. Representative flow cytometric dot plots for each cell stain are shown along with graphs showing average percentage of cells as a fraction of parental cell population and total yield of cells. (**A** and **B**) Analysis of $CD4^+$ $Foxp3^-$T cells as $PD\text{-}1^+Cxcr5^-$, $PD\text{-}1^+Cxcr5^+$ (Tfh), $PD\text{-}1^-Cxcr5^+$, and $PD\text{-}1^-Cxcr5^-$ populations. (B) Tfh cells and non-Tfh cell populations are quantitated as a percentage of $CD4^+Foxp3^-$ T cells and absolute number per spleen. (**C** and **D**) Analysis of $CD4^+$ $Foxp3^+$T cells as $PD\text{-}1^+Cxcr5^-$, $PD\text{-}1^+Cxcr5^+$ (Tfr), $PD\text{-}1^-Cxcr5^+$, and $PD\text{-}1^-Cxcr5^-$ populations. (D) Tfr cells are quantitated as a percentage of $CD4^+Foxp3^+$ T cells and absolute number per spleen. n = 4–6 mice, and each experiment was repeated two times. The p values were calculated by t test, in which $*p < 0.05$, $***p < 0.0001$.

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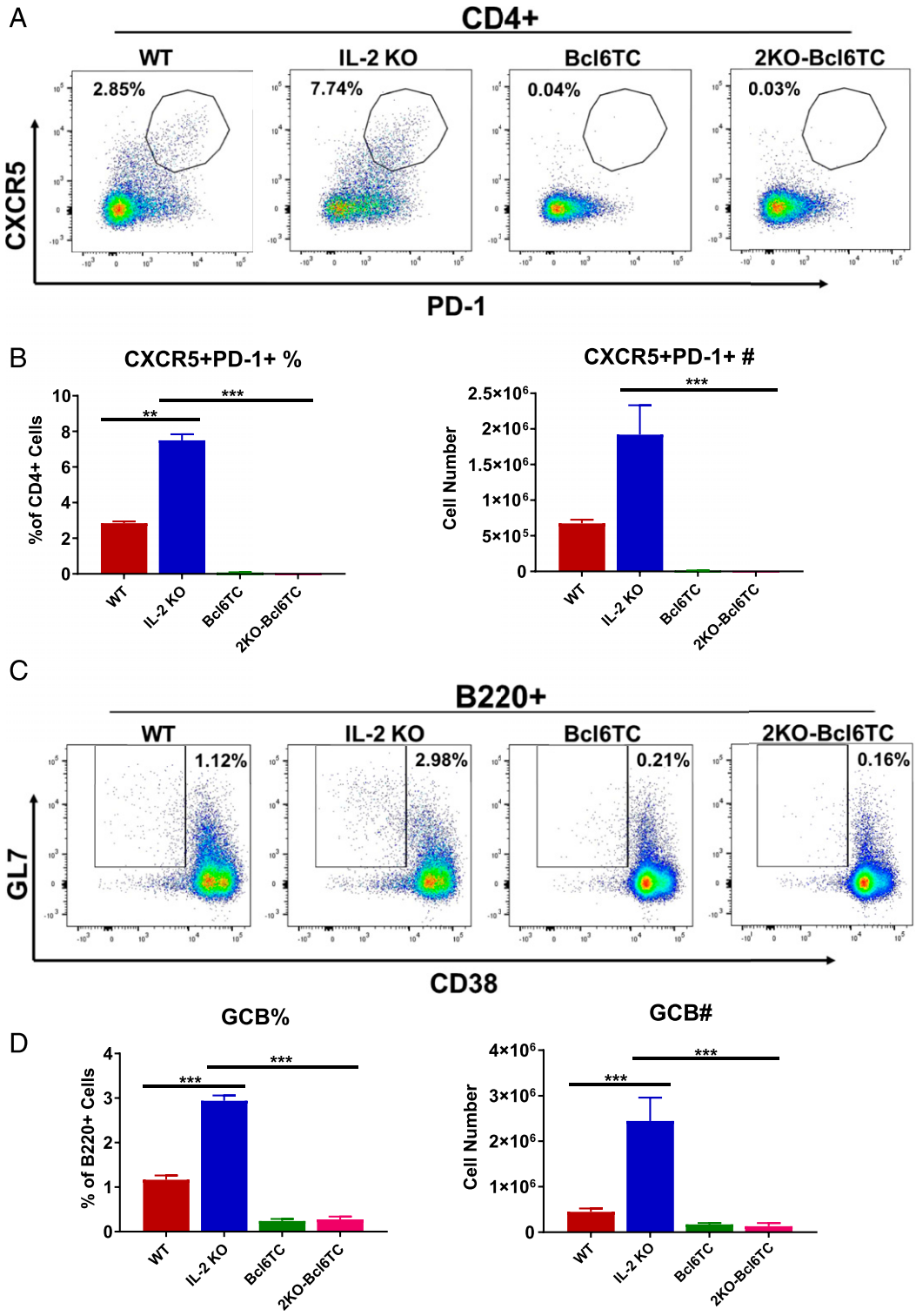

FIGURE 2. Loss of Tfh and GC B cells in 2KO-Bcl6TC mice.

Naive 10-wk-old WT, IL-2 KO, Bcl6TC (CD4-cre Bcl6-flox cKO), and 2KO-Bcl6TC (IL-2 KO and CD4-cre Bcl6-flox cKO) mice were used to analyze GC responses. Spleens were analyzed for the indicated cell populations by flow cytometry. Representative flow cytometric dot plots for each cell stain are shown along with graphs showing average percentage of cells as a fraction of parental cell population and total yield of cells. (**A** and **B**) Analysis of $CD4^+$ $PD\text{-}1^+Cxcr5^+$ follicular T cells. Follicular T cells are quantitated as a percentage of $CD4^+$ T cells and absolute number per spleen. (**C** and **D**) Analysis of $B220^+CD38^-$ $GL7^+$ GC B cells. GC B cells are quantitated as a percentage of $B220^+$ cells and absolute number per spleen. n = 4–6 mice, and each experiment was repeated two times. The p values were calculated by t test, in which $^{**}p < 0.01$, $^{***}p < 0.0001$.

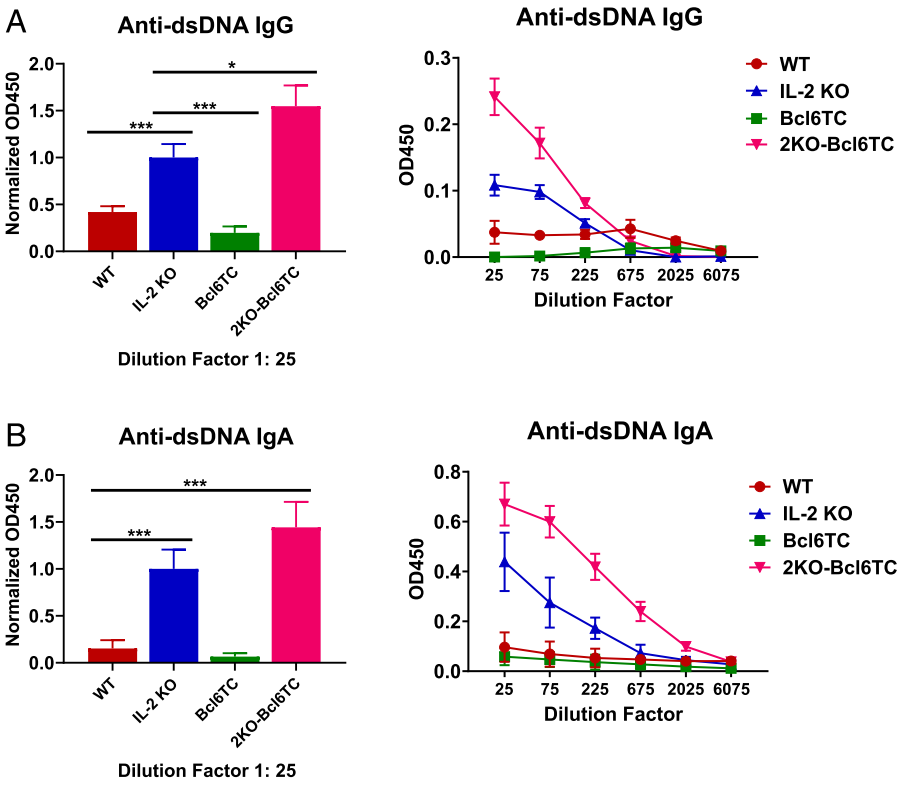

FIGURE 3. Increased anti-DNA Abs in 2KO-Bcl6TC mice.

Naive 10-wk-old WT, IL-2 KO, Bcl6TC, and 2KO-Bcl6TC mice were used to analyze serum Ab responses for reactivity to dsDNA. Bar graphs on the left show normalized results over all experiments in which OD values at 1:25 dilutions of serum are normalized relative to the average OD value for IL-2 KO serum, which is set to 1.0 for each experiment. Line graphs on the right show sample ELISA results with titrations for individual sets of sera. Serum was tested for (**A**) anti-dsDNA IgG and (**B**) anti-dsDNA IgA. n = 4–6 mice, and each experiment was repeated at least three times. The p values were calculated by t test, in which $^{*}p < 0.05$, $^{***}p < 0.0001$.

lupus (46, 47) to test the generation of anti-DNA Abs in WT and Bcl6TC mice. As shown in Fig. 5A, WT and Bcl6TC mice both produced substantial levels of anti-DNA IgG after pristane immunization, although the production of anti-DNA IgA was lower in Bcl6TC mice. Thus, significant levels of autoreactive IgG can be produced in the absence of Tfh cells, although production of autoreactive IgA appears to require Tfh cell help.

Regulation of autoantibodies by Tfr cells

We then wondered if the higher titers of autoantibodies in the 2KO-Bcl6TC mice were due to loss of Tfr cells that would normally inhibit development of strong autoantibody responses. To test this hypothesis, we mated IL-2 KO mice to Bcl6-flox Foxp3-cre mice that are specifically deficient in Tfr cells to generate double mutant (2KO-Bcl6FC) mice also on the B6 background. In 2KO-Bcl6FC mice, we found that deletion of Bcl6 in Tregs and the loss of Tfr cells dramatically worsened the early lethality in the B6 background IL-2 KO mice. The average lifespan of 2KO-Bcl6FC mice was 3.5 wk (Fig. 6A) and thus much shorter than the B6 IL-2 KO mice, which typically lived past 10 wk of age. We analyzed sera from three 2KO-Bcl6FC mice that lived to 5 wk for anti-DNA IgM and IgG levels by dsDNA ELISA (Fig. 6B, 6C). The 2KO-Bcl6FC mice had significantly less anti-DNA IgM (Fig. 6B) but had a trend toward increased anti-DNA IgG (Fig. 6C) Abs over IL-2 KO mice. We then used the autoantigen microarray assay system to screen for IgG autoantibodies but did not observe significant differences in autoantibodies between in IL-2 KO and 2KO-Bcl6FC mice (data not shown). 2KO-Bcl6FC mice also did not show significantly more organ inflammation than age-matched IL-2 KO mice (data not shown). Thus, the early death of 2KO-Bcl6FC mice is not easily explained by more autoantibodies or stronger inflammatory disease; however, we cannot rule out more-severe disease in these mice, particularly in the mice that died at a very early age. At the same time, the shortened lifespan of 2KO-Bcl6FC mice likely forestalled more-severe autoimmune disease as autoantibodies and inflammation typically develop with increased age. To better understand the role of Tfr cells in repressing autoantibody responses, we analyzed the development of autoantibodies in IL-$2^{+/+}$ Tfr cell–deficient Bcl6FC mice (Fig. 7). Bcl6FC mice are healthy, fertile, and have a normal lifespan (23). We found that significantly increased titers of anti-DNA IgG develop in older Bcl6FC mice compared with WT controls (Fig. 7A). Additionally, aged Bcl6FC mice produced significant levels of anti-DNA anti-IgA, whereas

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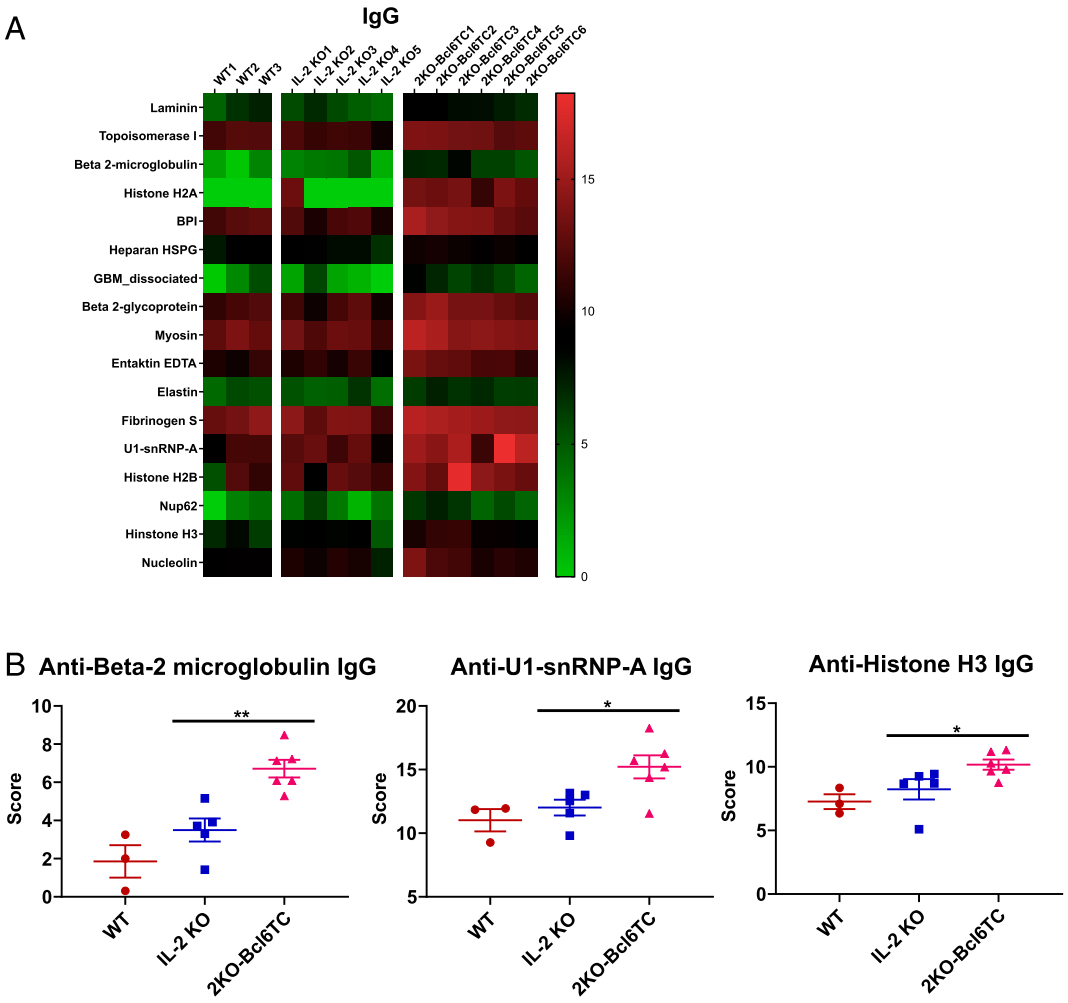

FIGURE 4. Increased autoantibodies in 2KO-Bcl6TC mice.

Serum from naive 10-wk-old WT, IL-2 KO, and 2KO-Bcl6TC mice was analyzed for IgG reactivity to an autoantigen microarray. (**A**) Heat map of 17 autoantigens with significantly higher reactive IgG between WT, IL-2 KO, and 2KO-Bcl6TC mice based on the microarray score and multicomparison statistical analysis. Seventeen autoantigens out of 94 autoantigens tested showed a significant difference in IgG reactivity between the three groups, and all 17 showed a higher score for the 2KO-Bcl6TC sera. (**B**) Graphs of selected anti-autoantigen IgG scores for anti–β-2 microglobulin, anti–U1-snRNP, and anti-Histone H3 taken from the autoantigen array data. n = 4–6 mice. The p values were calculated by t test, in which *$p < 0.05$, **$p < 0.01$.

WT mice produced undetectable levels (Fig. 7B). This finding further supports a role for Tfr cells controlling IgA production (23). We further tested autoantibodies in Bcl6FC mice by autoantigen microarray and found higher levels of IgM Abs to several autoantigens Fig. 7C. These data show that in our Bcl6FC mouse model in our mouse colony, Tfr cells have a significant role in maintaining Ab self-tolerance, consistent with recent findings using different models of Tfr cell deficiency (26, 27).

DISCUSSION

In this study, we have used the IL-2 KO mouse model of autoimmunity to probe the roles of Tfh cells and Tfr cells in the development of autoantibodies. Tfh cells are required for the GC reaction and production of high-affinity Abs to Ags, and excessive development of Tfh cells can promote autoantibody production. Tfr cell functions are less well defined because of conflicting data on whether Tfr cells have a suppressive or helper function in the GC (20). However, there is general consensus that Tfr cells repress the development of autoantibodies (23, 25–30). In our work in this study analyzing the IL-2 KO model of autoimmunity, we found unexpectedly that Tfh cells were not required for the production of high levels of autoantibodies and, further, that Tfr cells appear to potently inhibit the magnitude of autoantibody responses.

Previous work on the IL-2 KO model showed strong differences depending on the genetic background. On a mixed 129/01a × C57BL/6 background, IL-2 KO mice die at an early age, most likely from severe colitis (37, 38). Autoantibodies specific for colon cells were detected in these mixed background IL-2 KO mice (37). In

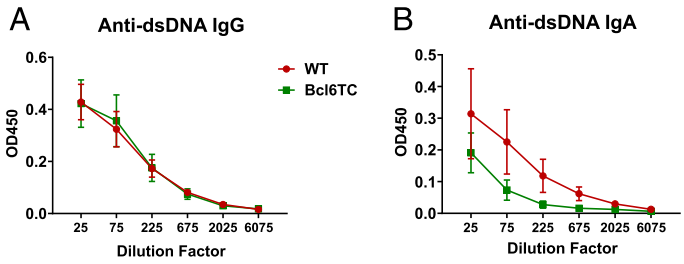

FIGURE 5. Anti-DNA Abs were not affected by the deletion of GCs in pristane-induced lupus model.

Four months after a single 0.5-ml Pristane i.p. injection, serum samples were collected from control WT and Bcl6TC mice for anti-dsDNA Ab detection by ELISA. (**A**) Anti-dsDNA IgG and (**B**) anti-dsDNA IgA titers are shown. The *x*-axis labels are dilution factors. Data shown as mean ± SEM, *n* = 5. Data are pooled from two independent experiments.

BALB/c IL-2 KO mice, high levels of autoantibodies to RBCs lead to severe AIHA and much earlier death than on a mixed background (38, 39). In BALB/c IL-2 KO mice, conventional $CD4^+$ Tfh cells are elevated as well as an unusual $CD8^+$ Tfh-like population with B cell helper activity (39). Both $CD4^+$ and $CD8^+$ cells contribute to autoantibody production and autoimmune disease in the BALB/c IL-2 KO model (39). Our B6 IL-2 KO mice live longer than what has been reported for BALB/c IL-2 KO mice and to a similar age as reported for IL-2 KO mice on a mixed background. We did not observe significant development of colitis in our B6 background IL-2 KO mice but did find significant inflammatory infiltration in the lung, liver, and kidney (Supplemental Fig. 2). Tfh cells were not analyzed in the mixed IL-2 KO mice, but we observed that Tfh cells were elevated similarly to what was reported for BALB/c IL-2 KO mice. In terms of autoantibody production, B6 IL-2 KO mice produced very little, if any, autoantibodies to self-proteins but developed significant levels of anti-dsDNA Abs. We did not assess anti-RBC autoantibodies directly; however, we did observe apparent anemia (decreased hematocrit) in the B6 IL-2 KO mice (data not shown). Overall, the B6 background IL-2 KO mice had a somewhat different phenotype from both the mixed background and BALB/c background mice, although there are many similarities (Supplemental Table I). It is not clear how many of these differences are due to the pure B6 background or the conditions of different mouse colonies and related effects, such as elimination of specific pathogens and microbiota.

Our original hypothesis for this study was that the increased Tfh cells were critical for promoting the autoimmune disease in IL-2 KO mice by driving formation of autoantibodies. However, when we deleted Tfh cells from the B6 IL-2 KO background, the 2KO-Bcl6TC mice had a similar disease phenotype as the B6 IL-2 KO strain (Supplemental Figs. 1, 2, Supplemental Table I). Note that both $CD4^+$ Tfh cells and $CD8^+$ Tfh-like cells are dependent on Bcl6 for development and are deleted in Bcl6TC cKO mice (14, 45). Even more unexpected than the similar disease phenotype of IL-2 KO mice and 2KO-Bcl6TC mice was that despite the loss of Tfh cells, 2KO-Bcl6TC mice had higher levels of not only anti-DNA IgG autoantibodies but also IgG autoantibodies to self-proteins. Among other things, these data show that Tfh cells are not required for the robust production of IgG autoantibodies in B6 IL-2 KO mice. Our data contrast with data from the BALB/c background, in which depletion of either CD4 or CD8 T cells led to lower total Ig, decreased anti-RBC Abs, and improved overall survival of the mice (39). However, it seems likely that non-Tfh cells drive autoantibody production in both the B6 and BALB/c IL-2 KO models.

The strongly increased levels of autoantibodies in 2KO-Bcl6TC mice showed that a population of T cells that would normally suppress autoantibody production was removed by the loss of T cell–specific Bcl6. The major candidates for such cells are Tfh cells and Tfr cells. Although in principle Tfh cells could play a suppressive role in driving autoantibody generation, perhaps by diverting autoreactive B cells into the GC reaction and mutating them away from autoreactivity such a mechanism is speculative. A more likely explanation is that loss of Tfr cells in 2KO-Bcl6TC mice led to a loss of suppression of autoreactive B cells. We tested this idea by generating 2KO-Bcl6FC mice, creating an IL-2 KO background in which Tfr cells are deleted without affecting Tfh cells. 2KO-Bcl6FC mice had significantly worse disease than 2KO-Bcl6TC mice, with markedly stunted growth and 80% lethality by 4 wk of age. However, 2KO-Bcl6FC mice did not have increased levels of autoantibodies nor did they have increased organ inflammation compared with IL-2 KO mice, indicating a

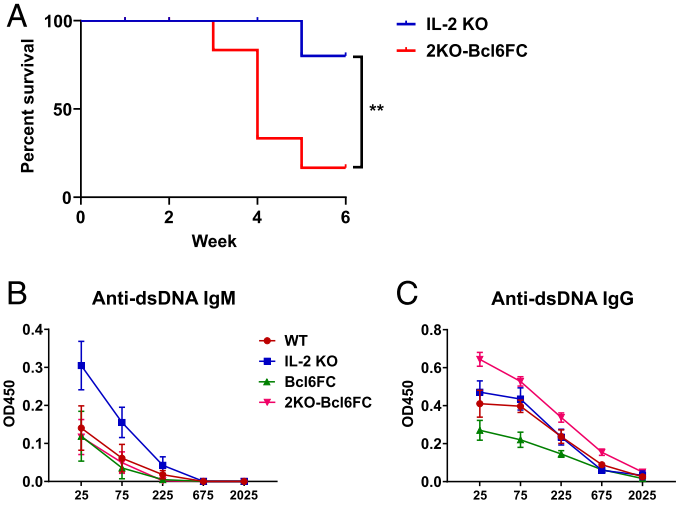

FIGURE 6. Early death of 2KO-Bcl6FC mice and autoantibody production.

(**A**) Survival curve for IL-2 KO and 2KO-Bcl6FC (IL-2 KO Foxp3-cre Bcl6-flox cKO) mice over time. (A) A Gehan–Breslow–Wilcoxon test was used to measure the statistical difference between the samples. (**B** and **C**) Serum samples were collected from 5-wk-old WT, IL-2 KO, Bcl6FC, and 2KO-Bcl6FC mice for anti-dsDNA Ab detection by ELISA. (B) Anti-dsDNA IgM and (C) anti-dsDNA IgG titers are shown. The *x*-axis labels are dilution factors. Data shown as mean ± SEM; *n* = 4 for WT, IL-2 KO, and Bcl6FC mice and *n* = 3 for 2KO-Bcl6FC mice. The *p* value was $^{**}p < 0.01$.

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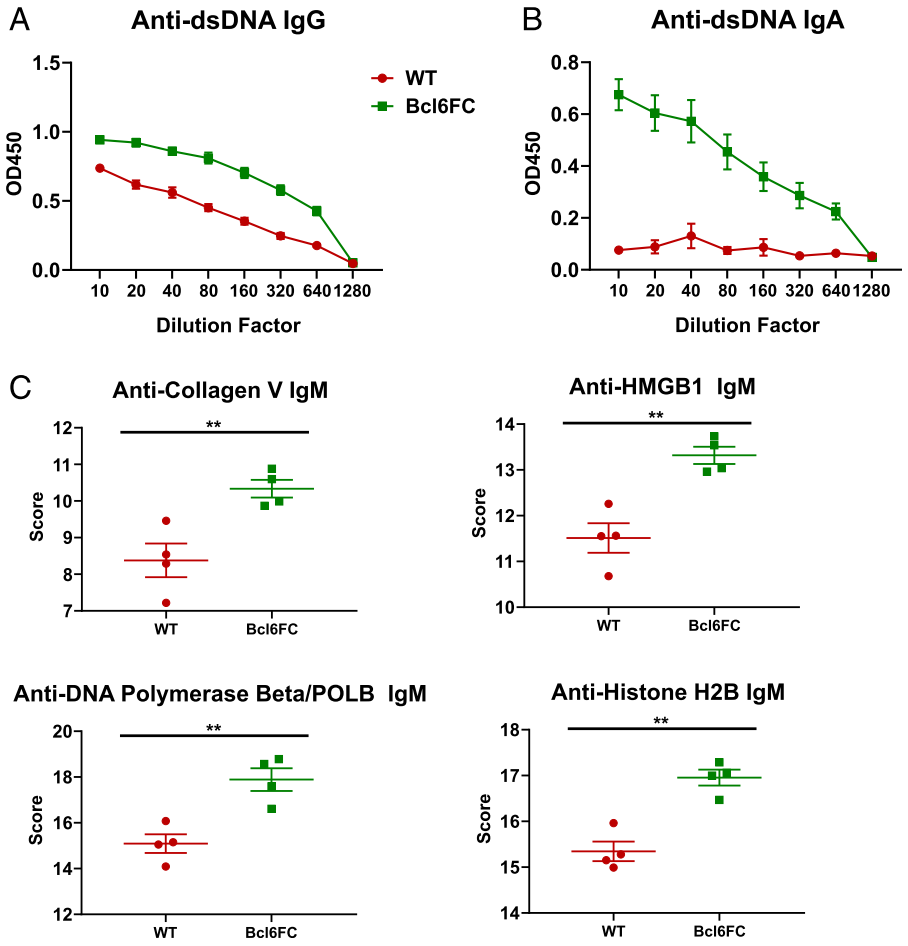

FIGURE 7. Increased autoantibodies in Bcl6FC mice.

Serum from naive WT and Bcl6FC mice was used to analyze for anti-dsDNA Abs by ELISA and autoantigen microarray. (**A** and **B**) Serum from 20-wk-old mice was tested for (A) anti-dsDNA IgG and (B) anti-dsDNA IgA. $n = 6$ mice; experiment was repeated at least two times. (**C**) Serum from 5-wk-old mice was tested for anti-autoantigen IgG by microarray. Thirty-eight autoantigens out of 118 autoantigens tested showed a significant difference in IgG reactivity between WT and Bcl6FC, and all 38 showed a higher score for the Bcl6FC sera. All graphs show selected anti-autoantigen IgG scores for anticollagen V, anti-Hmgb1, anti-DNA polymerase β, and anti-Histone H2B taken from the autoantigen array data. $n = 4$ mice. The p values were calculated by t test, in which $^{**}p < 0.01$.

more-complex pathogenesis in these mice. BALB/c IL-2 KO mice have a loss of Treg function as well as a loss of Treg numbers that correlates with autoimmune disease and that can be restored by IL-2 treatment (44). However, we did not observe decreased Tregs in the B6 IL-2 KO or IL2-Bcl6TC mice, and the normal numbers of Tregs in these mice correlated with less-severe disease than in BALB/c IL-2 KO mice. In 2KO-Bcl6FC mice, we hypothesize that loss of Bcl6 expression coupled with loss of IL-2 signals led to a disabled, depleted, or destabilized overall Treg population. A loss of Tregs or Treg function coupled with normal T effector cells can promote severe inflammatory disease and/or autoimmune disease. We did not detect stronger inflammatory disease or autoantibody production in the 2KO-Bcl6FC mice, although the early death and stunted growth of these mice made pathology difficult to elucidate.

Although deletion of Tfh cells did not inhibit the production of anti-DNA autoantibodies in the IL-2 KO mice, we did observe that deletion of MyD88 in the IL-2 KO mice did block anti-DNA autoantibodies (Supplemental Fig. 3). IL-2–MyD88 DKO mice also appeared to be healthier than IL-2 KO mice and developed to a normal size (data not shown). MyD88 is a downstream signaling protein for TLR molecules, and MyD88-dependent TLR signals are known to promote inflammation and amplify autoimmunity (48). Our data indicate that innate signals, likely nucleic acids released from dead cells (49), as well as commensal-derived TLR-ligands (50) help to promote the formation of autoimmune disease and autoantibodies in the IL-2 KO mice. Strikingly, TFR cells were increased in IL-2–MyD88 DKO mice over WT levels (Supplemental Fig. 3), suggesting that in a less-proinflammatory environment, IL-2 is indeed inhibitory for Tfr cell generation. In

other words, the loss of Tfr cells observed in IL-2 KO mice may be due to increased destabilization of the Treg population from inflammatory cytokines.

Finally, it is worth noting that aged Bcl6FC mice produced markedly increased anti-DNA anti-IgA, a finding that further supports a role for Tfr cells controlling IgA production (and particularly for suppressing autoreactive IgA responses), consistent with our earlier work (23).

In summary, our studies on the IL-2 KO model of autoimmunity have generated new (to our knowledge) insights into the roles of Tfh and Tfr cells in controlling autoantibody formation and IL-2 in regulating Tfr cell differentiation. Further work is needed to understand more completely how Tfr cells control autoimmunity and the function of Tfr cells in IL-2 KO mice.

DISCLOSURES

The authors have no financial conflicts of interest.

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