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Gut Commensal Segmented Filamentous Bacteria Fine-Tune T Follicular Regulatory Cells to Modify the Severity of Systemic Autoimmune Arthritis

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Abstract

Autoantibodies play a major pathogenic role in rheumatoid arthritis (RA). T follicular helper (Tfh) cells promote germinal center (GC) B cell and antibody responses. Excessive Tfh cell responses lead to autoimmunity and therefore counter-regulation is crucial. T follicular regulatory (Tfr) cells, mainly differentiated from T regulatory cells (Tregs), can negatively regulate Tfh and GC B cells. Dysbiosis is involved in RA's pathogenesis. We previously demonstrated that the gut microbiota, segmented filamentous bacteria (SFB), promote autoimmune arthritis by inducing Tfh cells. However, little is known regarding whether gut microbiota influence systemic (non-gut) Tfr cells, impacting gut-distal autoimmunity. Here, using SFB in autoimmune arthritic K/BxN mice, we demonstrated that SFB-induced arthritis is linked to the reduction of Tfrs' CTLA-4, the key regulatory molecule of Tfr cells. This SFB-mediated CTLA-4 reduction is associated with increased Tfr glycolytic activity, and glycolytic inhibition increases Tfrs' CTLA-4 levels and reduces arthritis. The surface expression of CTLA-4 is tied to T cell receptor (TCR) signaling strength, and we discovered that SFB-reduced CTLA-4 is associated with a reduction of Nur77, an indicator of TCR signaling strength. Nur77 is known for repressing glycolytic activity. Using a loss-of-function study, we demonstrated that Nur77$^{+/-}$ haplodeficiency increases glycolysis and reduces CTLA-4 on Tfr cells, which is associated with increased arthritis and anti-GPI titers. Tfr-specific deletion (KRN.Foxp3CreBcl-6$^{fl/fl}$) in autoimmune condition reveals that Tfr cells repress arthritis, Tfh cells, and autoantibody responses, and that SFB can mitigate this repression. Overall, these findings demonstrated that gut microbiota distally impact systemic autoimmunity by fine-tuning Tfr cells.

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease that causes chronic inflammation of the joints. Autoantibodies (auto-Abs) play critical roles in the pathogenesis of RA as they contribute to immune complex formation and complement activation, leading to tissue

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damage in the joints (1–6). T follicular helper (Tfh) cells are $CD4^+$ T cells that specialize in B cell help and are differentiated from naive $CD4^+$ T cells in the follicles of secondary lymphoid organs (7–9). The function of Tfh cells is to help germinal center B cells produce high-titer, high-affinity, isotype-switched Abs and differentiate into long-lived plasma cells. Therefore, an excessive Tfh cell response can lead to over-productive auto-Ab responses and autoimmune conditions. Thus, a counter-regulator for the Tfh cell response is crucially required (10–12).

$CD4^+Foxp3^+$ regulatory T cells (Tregs) are potent suppressors of immune responses and are crucial for preventing autoimmune disease (13–16). Under inflammatory conditions, Tregs acquire characteristics of the effector T helper cells they constrain, thus becoming effector Tregs. One such example are T follicular regulatory (Tfr) cells, observed to be potent regulators of the germinal center (GC) response (17–19). Tfr cells express not only the Treg master regulator Foxp3 but also other Treg-related molecules such as CD25, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), and glucocorticoid-induced tumor necrosis factor receptor (GITR) (19–21). In addition, Tfr cells express Tfh cell-associated molecules including CXCR5, programmed cell death 1 (PD-1), and the master transcription factor of Tfh cells, B-cell lymphoma 6 (Bcl-6). By simultaneously obtaining the characteristics of both Treg and Tfh cells, Tfr cells have been shown to maintain a suppressive function and gain access to B-cell follicles (17–19). However, recent studies unexpectedly found positive regulation of Tfr on GC B cell and antibody responses (22–24).

Genetics play an important role in RA, and the disease concordance rate between monozygotic twins with RA is ~15% (25, 26). However, this also suggests that environmental factors are critically involved in RA pathogenesis. These include smoking, infection, and a more recently appreciated factor, the composition of gut microbiota (27–30). We have previously demonstrated that the gut microbiota constituent segmented filamentous bacteria (SFB) promotes autoimmune arthritis by inducing Tfh cell responses (31, 32). However, little is known regarding whether gut microbiota can influence the systemic Tfr cell response to impact gut-distal autoimmunity. Here, we investigate whether gut SFB can indirectly enhance Tfh cell and/or Ab responses by modifying systemic (non-gut) Tfr cells. One of the key regulatory effector molecules of Tfr is CTLA-4. Two studies have shown that in the absence of Treg cell-expressed CTLA-4, large numbers of Tfr cells were present but were unable to confine Tfh cell and GC formation (33), and loss of CTLA-4 on Tfr cells resulted in defective suppression of antigen-specific antibody responses (34). We thus hypothesized and tested whether and how SFB may regulate CTLA-4 to impact the function of systemic Tfr cells and autoimmunity.

Here, we use the K/BxN [KRN T cell receptor (TCR) transgenic mice on the C57BL/6 (B6) background x NOD] model to test our hypothesis. The K/BxN model is a murine autoimmune arthritis model in which KRN T cells recognize glucose-6-phosphate isomerase (GPI), the self-antigen (self-Ag) presented by MHC class II $I\text{-}A^{g7}$ from NOD mice (35). These activated T cells can in turn activate B cells to produce anti-GPI auto-Abs. Disease can also be induced by transferring KRN T cells into $TCR\alpha^{-/-}$.BxN (T cell deficient B6xNOD) mice (36). The K/BxN mouse model shares many clinical and histologic features with human RA patients (37). As in many human autoimmune diseases including RA,

auto-Abs play important pathological roles in K/BxN disease development (37). By using the K/BxN.*Foxp3*^{sf} mice which carry the *scurfy (sf)* mutation, a null mutation of the *Foxp3* gene that results in impaired Tregs, Nguyen et al., discovered that *scurfy* mutation impacts the immunologic initiation phase as the K/BxN.*Foxp3*^{sf} mice have increased pathogenic autoantibodies (38). We now know that this is likely contributed by the Tfr deficiency in the K/BxN.*Foxp3*^{sf} mice. Additionally, Tregs may also directly suppress the inflammatory phase of arthritis in the joints of K/BxN mice, as ~50% of the joint synovial fluid T cells in K/BxN mice expressed Foxp3. Here, we focused on the effect of gut commensal SFB on systemic Tfr cells and showed that SFB-induced arthritis is linked to a significant reduction of CTLA-4 expression on Tfr cells. SFB-mediated CTLA-4 expression on Tfr cells is associated with an increase of Tfr glycolytic activity, and inhibition of glycolysis can increase CTLA-4 expression on Tfr cells. Additionally, as CTLA-4 expression has been tied to TCR signal strength (39), we examined and showed that SFB modify CTLA-4 expression by altering TCR signaling strength. Finally, by using Foxp3Cre.Bcl-6$^{fl/fl}$ mice in the K/BxN model background, we generated Tfr deficient (Tfr KO) mice and demonstrated a gut-microbiota dependent role for Tfr cells in regulating the development of auto-Ab and autoimmune arthritis. Overall, these findings demonstrate that gut microbiota can alter systemic Tfr cell activity, impacting autoimmune status.

Materials and Methods

Mice

K/BxN mice were generated by crossing KRN TCR transgenic mice on the C57BL/6 (B6) background with NOD mice. BxN mice were generated by crossing B6 mice with NOD mice. Nur77 deficient mice (B6;129S2-Nr4a1^{tm1Jmi}/J) were obtained from JAX and crossed to KRN mice to generate Nur77$^{+/-}$.KRN mice. TCRα$^{-/-}$.BxN mice were generated by crossing TCRα$^{-/-}$.B6 with TCRα$^{-/-}$.NOD mice as previously described (31). KRN.FoxP3Cre.Bcl-6$^{fl/fl}$ (Tfr KO) mice are generated by crossing KRN mice to Foxp3Cre mice (Stock No: 016959, JAX). Then, KRN.Foxp3Cre mice are further crossed to Bcl-6$^{fl/fl}$ mice (Stock No: 023727, JAX) to generate KRN.Foxp3Cre.Bcl6$^{fl/fl}$ mice with deletion of Bcl-6 specifically in Foxp3^{+} T cells. Ankle thickness was measured with a caliper (J15 Blet micrometer) as described previously (40). Ankle thickness indicates the average value of two ankles from one mouse while ankle thickening indicates the delta; i.e. the ankle thickness of a later time point minus the initial baseline thickness in any individual mouse. All mice were housed at the animal facility at the University of Arizona. All experiments were conducted according to the guidelines of the Institutional Animal Care and Use Committee at the University of Arizona.

Antibodies and flow cytometry

For surface staining, fluorophore-conjugated monoclonal Abs (mAbs) specific for CD3ε(17A2), TCRβ(H57–597), CD4(GK1.5 or RM4–5), PD-1 (RMP1–30), GITR (DTA-1), and CTLA-4 (UC10–4B9) were obtained from BioLegend. The mAb recognizing CXCR5 (2G8) was from BD Pharmingen. The mAb recognizing TCR Vβ6(RR4–7) was from eBioscience. For intranuclear staining, buffers from a Foxp3 Staining Buffer Set (eBioscience) were used to stain with Abs recognizing Foxp3 (FJK-16s, eBioscience),

Helios (22F6, Biolegend), and/or Nur77 (12.14, eBioscience). Cells were run on an LSRII (BD Biosciences), and analyses were performed with FlowJo (TreeStar) software.

Microbiota reconstitution and antibiotic treatment

Our mouse colony and SFB colonization was described previously (31). We matched gender and age of K/BxN mice for the experiments. Briefly, to generate SFB positive (SFB+) mice, SFB negative (SFB−) mice were weaned at 21 days old, then orally gavaged for 3 consecutive days starting at 22 days old with SFB-containing feces collected in house. The SFB− mice were ungavaged littermate controls. The SFB colonization status was examined on day 10 after SFB gavage or equivalent by SFB-specific 16S rRNA quantitative PCR as previously described (31). For experiments involved both SFB− and SFB+ K/BxN mice, mice were analyzed at 5–7 week old (2–4 weeks after gavage). For the antibiotic treatment and 2-DG experiments, as all experimental mice were inherently SFB+, we directly used littermates that were maintained as an SFB+ K/BxN colony by vertical transmission from SFB gavaged mothers, rather than individually gavaged experimental mice. For the antibiotic treatment, SFB+ K/BxN mice were treated with 0.5g/L vancomycin (Hospira) in drinking water containing sweetener (Equal, 2.5 g/300 mL) or left untreated, starting at 22 days of age for 2 weeks before analysis taken place at 5 week old (41).

2-deoxy-D-glucose and 2-NBDG administration

For 2-deoxy-D-glucose (2-DG) administration, SFB colonized K/BxN mice were treated i.p. with 2-DG (Sigma) at a concentration of 15–30 mg/mouse in PBS, every other day for 4–5 injections starting at 28 days of age before analysis took place at 5–6 weeks old. Control mice received PBS alone. To determine cellular glucose uptake, 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG, Invitrogen) was administered retro-orbitally at 100μg/mouse 30 minutes prior to tissue collection.

ELISA

Anti-GPI Ab titers were measured as described (40). In brief, ELISA plates were coated with recombinant mouse GPI at 5 μg/ml, and diluted mouse sera were added. Subsequently, plates were washed and alkaline-phosphatase (AP)-conjugated anti-mouse IgG Abs were added. After the final wash, AP substrate was added and titers were quantified as optical density values via an ELISA reader. Ab titers were expressed as arbitrary units, which were calculated from serial dilutions of sample serum and defined as the reciprocal of the highest dilution that gave a background Optical Density (OD_{405}) value set as 0.1 (42).

Adoptive transfer

For the K/BxN transfer model, splenic and lymph node-derived $CD4^+$ T cells from SFB− $Nur77^{+/-}$.KRN, KRN.FoxP3Cre.Bcl-$6^{fl/fl}$ (Tfr KO), or KRN mice were enriched using CD4-conjugated MACS beads (Miltenyi) and adoptively transferred by retro-orbital (r.o.) injection into SFB− and/or SFB+ TCR$\alpha^{-/-}$.BxN recipients as indicated in the corresponding figures. Two weeks after transfer, organs were collected for flow cytometry and sera were collected and analyzed by ELISA.

In vitro stimulation

TCR stimulation for Nur77 expression was performed by adding a final concentration of 5 μg/ml GPI (282–294) peptide (LSIALHVGFDHFE, GenScript) to splenocytes at 10^6 cells/well in 96 well plate for a total duration of 0, 2, 4, or 6 hours at 37°C before FACS analysis.

Statistical analysis

Differences were considered significant when $p < 0.05$ by Student's t test (two-tailed, unpaired) or two-way analysis of variance (ANOVA) (Prism 6, Graph-Pad Software). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Results

The impact of autoimmunity and the gut commensal SFB on Tfr cell responses

By TCR analysis, Maceiras et al. demonstrated that Tfr cells expressed potentially autoreactive TCRs, as the TCR repertoire of Tfr cells is close to that of Treg cells (43). However, in this study, no direct evidence was shown to demonstrate that Tfr cells recognize self-Ag. Moreover, we have previously demonstrated that SFB enhance the Tfh cell response in both systemic (spleen) and gut-associated lymphoid tissue (Peyer's patches/PPs) of K/BxN mice (31). We are thus interested in examining whether SFB also impact self-reactive Tfr cells to indirectly affect the Tfh cell response. We used the autoimmune TCR transgenic strain K/BxN and its non-transgenic background B6xNOD (BxN) strain in both SFB− and SFB+ conditions to ask how the SFB and self-Ag may impact the Tfr cell responses. We observed that the percentage of splenic Tfr cells was highly upregulated in K/BxN mice compared to BxN mice regardless of SFB status (Fig 1A, gating scheme and representative data depicted in Suppl. Fig. 1A and 1B). While this may be due to a lack of active GCs in the non-autoimmune BxN mice, this phenotype still holds in Peyer's Patches (PPs), a site with active GCs. Interestingly in both BxN and K/BxN mice, SFB reduced the percentage of Tfr in total $CD4^+$ T cells from PPs but not spleen. Based on our previous study, this is likely a secondary effect due to the dramatic increase of Tfh cells in PPs which reduces the percentage of non-Tfh cell types (31). Using Treg depletion and adoptive transfer, it was reported that Tfr cells are mostly derived from Tregs (18, 44, 45). Therefore, we examined the possibility that SFB may alter the proportion of Tfr relative to total $Foxp3^+$ Tregs. We found that SFB did not alter the proportion of Tfr cells in the total $CD4^+Foxp3^+$ T cell population (Fig. 1B). This was true regardless of autoimmune condition or tissue site as we found SFB did not alter Tfr/$CD4^+Foxp3^+$ T cell ratio in either spleen and PPs of BxN and K/BxN mice. However, there was a much higher Tfr/$CD4^+Foxp3^+$ ratio in autoimmune K/BxN than control BxN strains in both spleen and PPs. In addition to the percentage data, we also show the cell number data for $CD4^+$ T cell, Tfr, Tfh and $CXCR5^{lo}PD\text{-}1^{lo}$ Treg (Suppl. Fig. 1C and 1D). However, many reports have shown that it is not the absolute numbers of Tfr or Tfh cells and rather it is the ratio of Tfr/Tfh cells dictates the magnitude of Ab responses with higher Tfr/Tfh ratio correlated with lower Ab responses. Therefore, we also calculate the ratio of Tfr/Tfh and found that SFB reduced the Tfr/Tfh ratio in spleen, and this reduction was even more obvious in PP (Fig. 1C) (46–48). Foxp3 levels of the cells in Tfrs and $CXCR5^{lo}PD\text{-}1^{lo}$ Tregs were also examined and no difference

was observed between SFB− and SFB+ groups in both cell types in the K/BxN mice (Suppl. Fig. 2A).

The use of Helios as a marker to define thymus-derived Tregs has been controversial (49–51). Nevertheless, Helios has been shown to regulate effector Treg fitness and to be required by Tfr cells to control Tfh cell responses in an immunization study (52). Therefore, we examined Helios expression in the Tfr cells of autoimmune K/BxN mice. As expected, Helios was highly expressed (~80%) in splenic Tfr cells similar to the level observed in $CXCR5^{lo}PD\text{-}1^{lo}$ Treg cells (Fig. 1D and Suppl. Fig. 2B). However, unlike $CXCR5^{lo}PD\text{-}1^{lo}$ Tregs whose expression of Helios was reduced to between 40–50% in PPs, the expression of Helios on Tfr cells remained relatively high (~75%) in PPs. Finally, SFB did not impact the expression of Helios except in the PP $CXCR5^{lo}PD\text{-}1^{lo}$ Treg group. Interestingly, there was an increase of $Helios^{+}$ cell numbers in Tfrs and $CXCR5^{lo}PD\text{-}1^{lo}$ Tregs in spleen and PPs of SFB+ compared to SFB− K/BxN mice (Suppl. Fig. 2C). The auto-Ab isotype in the K/BxN model is IgG1 (31). Previously, we have demonstrated that spleen, but not PPs, is the major producing site of anti-GPI IgG1 Abs and that SFB-induced PP Tfh cells did not induce anti-GPI of the IgA isotype (31). Here, we also measured serum anti-GPI in IgG1 and IgA isotype and plotted these against ankle thickness (Fig 1E). There was a positive correlation between anti-GPI IgG1 auto-Ab and ankle thickness but the IgA titers were undetectable even in SFB+ group. Hence, despite the fact that SFB generally induce stronger immunophenotypes in PPs, we focused on spleen as the target organ for the Tfr cell studies for the rest of this paper.

Surface CTLA-4 expression on systemic Tfr cells can be modulated by gut microbiota

Cytotoxic T lymphocyte-associated protein 4 (CTLA-4) is a co-inhibitory molecule expressed by T cells (53). It has previously been shown that Tfr cells potently suppress GC B cell responses through CTLA-4 (33). We therefore investigated whether gut SFB may modulate systemic Tfr cell function by altering the expression of CTLA-4 on systemic Tfr cells. It is known that most CTLA-4 is localized in intracellular compartments (54). We have chosen to focus on surface CTLA-4 expression because it is the optimal expression of surface CTLA-4 that is crucial for the balance of maintaining immunotolerance and preventing autoimmunity (55). For example, one interesting study shows that surface CTLA-4 can remove CD80 and CD86 from dendritic cells. After removal, these costimulatory ligands are degraded inside CTLA-4 expressing cells, resulting in impaired costimulation via CD28 (56). Throughout this paper, the CTLA-4 examined are surface CTLA-4 except for Suppl. Fig 4A and 4B which show intracellular CTLA-4 expression. Notably, we found that gut SFB reduced CTLA-4 expression on systemic (splenic) Tfr cells (Fig. 2A). Interestingly, this effect was more significant in Tfr cells than $CXCR5^{lo}PD\text{-}1^{lo}$ Tregs. The SFB-induced CTLA-4 reduction on Tfr cells was associated with an increase of arthritis development (Fig 2B). To confirm our finding using a different approach, we treated SFB+ mice with vancomycin, an antibiotic targeting gram-positive bacteria such as SFB. Vancomycin is poorly absorbed from the gut (57), thus its effect when delivered by the oral route is mainly limited to the gastrointestinal tract. Indeed, SFB depletion by vancomycin rescued CTLA-4 expression on Tfr cells (Fig. 2C) and reduced arthritis severity (Fig. 2D). CTLA-4 is highly expressed on Tfr cells, and this expression

is required for Tfr cells to negatively regulate humoral immune responses (33, 34). In the K/BxN mice, higher CTLA-4 expression on Tfr cells also strongly correlated with reduced arthritis in the gavaging (Fig. 2E) and antibiotic treatment (Fig. 2F) experiments.

SFB reduces CTLA-4 surface expression in Tfr cells by promoting a glycolytic metabolic program

Recently, metabolic status in T cells has been shown to play a key role in their function (58, 59). Additionally, the germinal center is known for its hypoxic environment, which promotes glycolysis (60). However, little is known regarding whether glycolysis will impact effector molecules such as CTLA-4 on Treg or Tfr cells. As SFB is known to induce a prominent GC response in both spleen and PPs (31, 61), we tested whether the Tfr cells in SFB+ mice shift their metabolism to a pro-glycolytic program. 2-DG is a glucose analog; however, unlike glucose, 2-DG cannot be fully metabolized, and as a result 2-DG acts to inhibit glycolysis (62–64). Fluorescently-labeled forms of 2-DG such as 2-NBDG can accumulate in cells and serve as a good marker for tissue glucose uptake and glycolytic activity (62–64). To assess acute glucose uptake, we injected SFB− and SFB+ K/BxN mice with 2-NBDG 30 minutes prior to tissue collection. As 2-NBDG staining requires intact cells, we used GITR, a Treg marker, as the surface marker to identify $Foxp3^+$ cells, and confirmed that $GITR^+$ cells were 92–96% $Foxp3^+$ in the Tfr and $CXCR5^{lo}PD\text{-}1^{lo}$ Treg cell populations (Suppl. Fig. 3A). Our data showed that, compared to $CXCR5^{lo}PD\text{-}1^{lo}$ Tregs, Tfr indeed displayed an increase in 2-NBDG uptake in both SFB− and SFB+ groups, suggesting that Tfr cells are more glycolytic than $CXCR5^{lo}PD\text{-}1^{lo}$ Tregs (Fig. 3A). Interestingly, SFB colonization further enhanced 2-NBDG uptake in Tfr cells, suggesting that Tfr in SFB+ mice have further shifted their metabolism to a pro-glycolytic program.

Given the prior observation that SFB reduced CTLA-4 expression on Tfr cells (Fig. 2A), we hypothesized that the SFB-mediated increase in glycolytic metabolism may contribute to reduced CTLA-4 expression in the Tfr population. To examine the effect of glycolysis on Tfr cell CTLA-4 levels, we treated SFB+ K/BxN mice with 2-DG *in vivo* to inhibit glycolysis. Our data showed that there was a significant reduction of arthritis and anti-GPI titers in 2-DG treated compared to PBS treated SFB+ K/BxN mice (Fig. 3B and 3C). However, systemic inhibition of glycolysis can impact many cell types, and we cannot therefore directly correlate the reduction in arthritis and autoantibodies to Tfr cells. In this regard, we found that there was no significant difference in lymphocyte numbers, including both Tfr and $CXCR5^{lo}PD\text{-}1^{lo}$ Tregs, and there was some reduction in non-lymphocytes in 2-DG treated mice compared to PBS treated controls (Suppl. Fig. 3B and 3C). To directly examine whether the glycolytic inhibition may impact the effctor molecules on Tfr cells, we examined the CTLA-4 expression and found that inhibition of glycolysis by 2-DG caused an increase of CTLA-4 expression on Tfr cells(Fig. 3D). These results, together with data from Fig 2 suggest that SFB colonization, reduced the CTLA-4 expression on Tfr cells by promoting their glycolytic activity.

SFB decrease T cell receptor (TCR) signal strength indicated by the increase of Nur77^{lo} Tfr cells

Next, we searched for early events during T cell activation that would explain the observed CTLA-4 reduction on Tfr cells in SFB colonized mice. It has been reported that CTLA-4 expression on the T cell surface is proportional to TCR signal strength (39). Therefore, we tested whether the SFB-dependent CTLA-4 reduction on Tfr cells is due to reduced TCR signaling in SFB+ compared to SFB− Tfr cells. Nur77 (a.k.a. Nr4a1) is a transcription factor whose expression is induced rapidly upon T cell activation, in a manner representative of TCR signaling intensity (65). Thus, Nur77 reporter mice, as well as direct detection of endogenous Nur77 using antibodies, have been used as methods to evaluate TCR signal strength (65, 66). To our surprise, TCR signal strength in Tfr cells was increased relative to CXCR5^{lo}PD-1^{lo} Tregs *ex vivo* (the levels of Nur77 were measured in freshly isolated T cells without further TCR stimulation *in vitro*) (Fig. 4A). Importantly, there was a significant increase of Nur77^{lo} cells in the SFB+ group of both splenic Tfr and CXCR5^{lo}PD-1^{lo} Treg cell populations. We found there was a similar trend in PPs, though the significance in Tfr cell remained and the difference between SFB− and SFB+ groups in CXCR5^{lo}PD-1^{lo} Treg cells became non significant, suggesting this SFB effect is more pronounced in Tfr cells (Suppl. Fig. 3D). Moreover, we have shown that, similar to the study by Egen et al. showing CTLA-4 surface expression is proportional to the TCR signal strength, there was a strong positive correlation of surface CTLA-4 with Nur77 in autoimmune K/BxN mice (Fig. 4B) (39). However, when we performed the same experiments by staining intracellular CTLA-4, there was actually a negative correlation between intracellular CTLA-4 and Nur77, and SFB further increased the retention of CTLA-4 within intracellular compartments (Suppl. Fig. 4A and 4B). Thus, SFB reduce surface CTLA-4, and increase intracellular CTLA-4 expression. These data suggest that SFB may condition Tfr cells to express a reduced level of CTLA-4 through modifying TCR signaling strength.

K/BxN mice express a transgenic TCR, and therefore all T cell populations express the same TCR toward self-antigens. Accordingly,we hypothesized that the mechanism controlling Nur77 levels in these cell types is the relative amount of TCR expressed on the cell surface. To quantify the TCR level, we stained T cells with antibodies against TCR Vβ6, the β chain for the KRN TCR transgene. We found that Tfr cells express the highest TCR Vβ6 level, followed by Tregs, and conventional T cells (Tconv) with the least TCR expression (Fig. 4C). Indeed, our data demonstrated that the TCR Vβ6 levels on Tconv, Treg and Tfr cells positively correlated with these cells' Nur77 level in both SFB− and SFB+ hosts (Fig. 4D). However, between SFB− and SFB+ groups within the same cell type, we did not observe any reduction of TCR Vβ6 levels by SFB (Fig 4B). Since Nur77 expression was reduced by SFB in these populations, these data raise the question of how SFB reduces TCR signal strength independently of TCR expression.

Nur77 haplodeficiency increases glycolysis and decreases CTLA-4 expression on Tfr cells, enhancing arthritis development

After its rapid induction post-TCR activation, Nur77 gets down-regulated after just a few hours (66, 67). We therefore wondered if SFB colonization promotes faster Nur77 degradation after TCR stimulation, leading to reduced Nur77 detection *ex vivo* seen in Fig.

4A. To test this, we examined the temporal characteristics of endogenous Nur77 protein expression in GPI peptide stimulated T cells (containing Tconv, Treg and Tfr cells). In Tfr cells, Nur77 protein levels increased similarly between the SFB− and SFB+ groups up to 4 hours after stimulation with GPI peptide (Fig. 5A). However, there was a small but significant reduction of Nur77 in SFB+ Tfr cells by the 6 hour time point. There is no difference in cell numbers of Tfr cells at 6 hrs compared to 0 hrs (Suppl. Fig. 5A). No significant difference was observed in Tconv cells and $CXCR5^{lo}PD\text{-}1^{lo}$ Treg cells (Fig. 5A). Overall, these data suggest that SFB colonization facilitates the decline of Nur77 levels (Fig 4A and 5A).

Next we addressed whether SFB's reduction of Nur77 in Tfr and $CXCR5^{lo}PD\text{-}1^{lo}$ Tregs would explain the reduction of CTLA-4 in these populations. To mimic the Nur77 reduction by SFB, we transferred control KRN T cells or $Nur77^{+/-}$.KRN T cells that that were heterozygous for deletion of Nur77 into SFB− $TCR\alpha^{-/-}$.BxN hosts (T cell deficient mice on a B6xNOD background and thus, bearing NOD MHC class II, $I\text{-}A^{g7}$, for self-Ag presentation). Notably, this K/BxN T cell transfer model allows for manipulation of the gene of interest specifically in T cells, and host mice spontaneously develop disease symptoms after T cell transfer (35, 36). The haplodeficiency of Nur77 in $Nur77^{+/-}$.KRN T cells reduce Nur77 gMFI level to around 50% when compared to WT KRN T cells in both Tfr and $CXCR5^{lo}PD\text{-}1^{lo}$ Treg groups (Suppl. Fig. 5B). When KRN T cells were transferred into SFB− $TCR\alpha^{-/-}$.BxN recipients, only minimal ankle thickening was observed (Fig. 5B). Importantly, when $Nur77^{+/-}$.KRN T cells were transferred, arthritis and auto-Ab development was enhanced. Additionally, CTLA-4 expression was reduced in both $CXCR5^{lo}PD\text{-}1^{lo}$ Treg and Tfr cells in the $Nur77^{+/-}$ deficient background compared to WT (Fig. 5C). Therefore, this suggests that Nur77 levels act upstream of CTLA-4 expression in both $CXCR5^{lo}PD\text{-}1^{lo}$ Tregs and Tfr cells, and that the sped-up degradation of Nur77 in SFB+ Tregs may underlie their reduced CTLA-4 expression, leading to an exacerbated disease state.

Similar to Fig 2E and 2F, in the $Nur77^{+/-}$.KRN T cell transfer system, we also found a negative correlation of Tfr's CTLA-4 expression and arthritis severity, and the reduction of CTLA-4 in $Nur77^{+/-}$.KRN Tfr cells is correlated with a stronger arthritis development (Fig. 6A). Additionally, there is also a negative correlation between surface CTLA-4 expression of Tfr versus anti-GPI titers (Fig. 6B). Nur77 expression regulates metabolic activity of T cells including glycolysis (68). As inhibition of glycolysis increases CTLA-4 expression levels in Tfr (Fig. 3D), and Nur77 deficiency results in the reduction of CTLA-4 expression, we investigated whether Nur77 deficiency may increase glycolytic activity that could explain the reduction of CTLA-4 in Nur77 deficient T cells. Our data demonstrated an increase of $2\text{-}NBDG^{+}$ Tfr cells in Nur77 deficient mice compared to WT controls (Fig. 6C). This data is inconsistent with a previous report showing that the general CD4+ T cell population in Nur77 KO mice display increased glycolytic activity (68). It is interesting that we found a similar result with only $Nur77^{+/-}$ haplodeficiency and in Tfr cells, a cell type derived from Treg, which are traditionally viewed as cell type relies more on oxidavtive phosporylation than the glycolysis pathway.

Tfr deficiency enhances autoimmune arthritis and autoantibody development dependent on microbiota status

As mentioned earlier, many studies support the hypothesis that Tfr cells are a negative regulator of humoral immunity that act by restraining excessive Tfh and GC B cell responses (17–19, 45). In support of this idea, the loss of CTLA-4 on Tfr cells resulted in defective suppression of antigen-specific antibody responses (34). However, recent studies have challenged the suppressor model of Tfr cell function. In two of these studies, which were conducted in murine immunization and infection models, Tfr cells were found to promote GC B cell responses and Tfr deficiency actually resulted in reduced IgG Ab titers (24, 69). We have thus far demonstrated that alteration of CTLA-4 expression on Tfr cells by SFB, antibiotics, and Nur77 deficiency generate a negative correlation between Tfr CTLA-4 levels and arthritis severity, which is suggestive of a negative regulatory role for Tfr cells in autoimmune K/BxN mice (Figs. 2E, 2F and 6A). Finally, we would like to definitively determine the positive or negative effect of Tfr cells on autoimmune arthritis and auto-Ab responses in K/BxN mice, and how SFB may further modulate this positive or negative effect. To address these questions, we first crossed KRN to Foxp3Cre and Bcl-$6^{fl/fl}$ mice to generate KRN.Foxp3Cre.Bcl-$6l^{fl/fl}$ mice that are deficient in Tfr cells (to simplify, referred to as Tfr KO). Similar to many reported cell-targeted deletions, the Tfr in our Tfr KO model is not depleted by 100% (only ~75% reduction) (Suppl. Fig. 6). Nevertheless, as we had achieved a large reduction of Tfr cells, we tested our hypothesis by transferring $CD4^+$ T cells from Tfr KO or KRN mice into SFB− and/or + $TCR\alpha^{-/-}$.BxN recipients. Our results showed an increase in arthritis and autoantibodies in SFB− mice receiving Tfr KO T cells compared to mice receiving control KRN T cells (Fig. 7A and 7B). However, we did not observe differences between the WT and Tfr KO groups in SFB+ mice. Interestingly, we also observed a significant induction of Tfh cells in the hosts receiving Tfr KO T cells compared those receiving WT KRN T cells under SFB− but not SFB+ conditions (Fig. 7C). This indicates that, under SFB− conditions, Tfr cells act to restrain the Tfh response, thereby inhibiting the autoimmune state and that Tfr cells are unable to assert their repressive function under SFB+ conditions. Overall, our data show that Tfr-mediated control over autoimmune arthritis and autoantibodies is operated in a microbiota dependent manner.

Discussion

Kawamoto *et al.* have shown that Tfr cells support IgA selection in the GCs of PPs, resulting in diversification of gut microbiota (70). These findings have crucial implications because they suggest that Tfr cell deficiency may present a less diverse microbiota, which can later contribute to inflammatory diseases such as colitis in the gut (70). Inspired by this finding, we set to examine whether the gut microbiota status can also modify Tfr cells, in turn leading to systemic autoimmune disease. Our data showed that SFB do not alter the proportion of Tfr cells among total Treg cells in both systemic and gut-associated lymphoid tissues. Helios is expressed by 70–80% of Treg in systemic lymphoid tissues and by a somewhat lower percentage (50–60%) of mucosal derived Treg (15). Interestingly, our data show that, unlike Tregs, Tfr cells in PPs, a mucosa-associated lymphoid tissue, still express high level of Helios. Our results have further revealed that the gut microbiota, SFB, reduces the effector molecule CTLA-4 on systemic Tfr cells, by increasing glycolysis and reducing

TCR signal strength on Tfr cells. There have been functional discrepancies reported in Tfr cells as studies show Tfr cells either positively or negatively regulate humoral immune responses (17–19, 24, 45, 69). Our data support a negative regulatory role for Tfr cells on the humoral immune response in the autoimmune condition. Interestingly, we showed that this negative role can be diminished in a gut microbiota-dependent manner.

CTLA-4 is highly expressed on Tfr cells and its expression is crucial for Tfr cells to contain humoral immune responses (33, 34). For an efficiently controlled immune response, CTLA-4 surface localization is tightly regulated by restricted trafficking to the cell surface and rapid internalization (55). Our data suggested SFB reduce surface CTLA-4 expression and increase CTLA-4 inside the cell. This could be achieved through a few pathways controlling CTLA-4 trafficking (71, 72). This is a very interesting observation and warrants future study. Previously, CTLA-4 ligation has been shown to reduce glucose uptake and glycolysis in T cells (73). However, little is known regarding the reverse situation, whether glycolytic activity will impact CTLA-4 expression. Our 2-DG experiment indicates that glycolytic inhibition can act as a positive regulator of CTLA-4 surface expression. This suggests that the increase in glycolysis by SFB may impair Tfr function and enhance autoimmunity by decreasing CTLA-4 expression on Tfr cells. Tregs have been shown to preferentially use oxidative phosphorylation (OXPHOS) pathways and glycolysis has been shown to inhibit the differentiation of Foxp3+ Treg cells (74). However, recent studies demonstrate that in a special environment, such as a tumor, Tregs can engage in both glycolysis and OXPHOS for their expansion (75). Our new data show that in the unique hypoxic environment of GC, Treg-derived Tfr cells also adapt and increase their glycolytic activity.

Several reports show that Nur77 deficiency is associated with autoimmune conditions (68, 76, 77). Nur77 deficiency may contribute to autoimmunity through a few potential mechanisms involving many different T cell types. During thymocyte development, Nur77 deficiency has been shown to impair clonal deletion (76). Nur77 deficiency expands immature T cells as Nur77 has an overlapping role with Bim, a dominant player in negative selection induced apoptosis (76). However, in mature T cells, Nur77 deficiency does not impact apoptosis (67, 68, 77). Instead, Nur77 restricts activation and proliferation of mature T cells to prevent autoimmunity. The data in our study suggest another mechanism whereby Nur77 deficiency contributes to autoimmunity—by downregulating CTLA-4, a dominant regulatory molecule of peripheral Treg and Tfr cells. Indeed, haplodeficiency of Nur77 in T cells is sufficient to enhance autoimmune arthritis severity and autoantibody titers. Our data also show that SFB may exert its pro-autoimmune activity partly by increasing the proportion of $Nur77^{lo}$ Tfr and $CXCR5^{lo}PD\text{-}1^{lo}$ Treg cells, leading to the increased glycolysis, and reduced surface expression of CTLA-4 and enhanced arthritis severity. Activation of Nur77 has been shown to faithfully reflect the TCR strength in T cells (65). There are many factors that can alter the levels of Nur77 in T cells. One obvious candidate is a change in TCR signaling pathways. For example, temporal differences in Nur77 expression have been documented between naïve and memory T cells (66), and reports have shown that naive T cells display greater TCR signaling and calcium mobilization than memory T cells (78–80). Our data is unique in showing that even within the same T cell type, the environmental stimulus of SFB colonization can condition T cells to lower their

Nur77 level. We have shown SFB can skew cells toward dual TCR expression by increasing SFB-specific TCRVβ14 expression on KRN TCRVβ6 expressing Th17 cells (41). It will be very interesting to see if SFB also promotes dual TCR expression in other cell types, thereby modulating TCR signaling. Our results suggest that despite similar induction of Nur77 after GPI peptide stimulation, Nur77 is degraded more rapidly in SFB+ than SFB− Tfr cells. This may result in the increase of $Nur77^{lo}$ Tfr cells observed in SFB+ than SFB− mice. Nur77 has a relatively short half-life, between 20–40 mins (81). It will be interesting to explore the underlying mechanism that further reduces the half-life of Nur77 in SFB+ Tfr and Treg cells compared to their counter parts in future studies.

Although Tfr cells have mainly been shown as a negative regulator for GC B cell and antibody responses, several recent studies indicate that the role of Tfr cells may vary based on models of studies (17–19, 22–24). In general, a suppressive role of Tfr cells on B cells in immunization or infection models has been observed. For example, in sheep red blood cells (SRBC) and NP-KLH immunization, $Foxp3Cre.Bcl6^{fl/fl}$ mice display increased anti-SRBC IgA (but reduced IgG) compared to WT controls (22). In another study, $Foxp3Cre.Bcl6^{fl/fl}$ mice display an increase of IgG2c Ab during influenza infection (but normal IgG1 and IgM) response (82). Another independent study found that, during influenza infection, the lack of Tfr cells did not change Tfh cell or the GC B-cell responses, but promoted the accumulation of $CD138^{+}$ ASCs (83). However, there are examples of Tfr as a positive regulator of GC B cell responses. For example, Tfr can promote GC responses by IL-10 in lymphocytic choriomeningitis virus infection (24). Tfr, by suppressing cytotoxic Tfh cells, help boost the GC B cell response in a mouse model of peanut allergy (23). Additionally, as mentioned earlier, reduced IgG was found in mice injected with SRBC and NP-KLH (22).

With this variation in previous studies, we therefore decided to examine the role of Tfr cells in the K/BxN autoimmune model. In the low inflammatory condition of SFB− hosts, we found that Tfr cells play a negative role in auto-antibody responses. However, when SFB were added to Tfr KO mice, there was no further enhancement in arthritis severity, auto-antibody, and Tfh cell responses compared to SFB+ Tfr WT group (Fig. 7). One possibility is that in the Tfr WT mice, SFB repress CTLA-4 on Tfr cells, leading to an impaired function of Tfr cells similar to the effect of Tfr KO that enhances auto-antibody and Tfh cell responses. However, the niche for Tfh cell expansion is unlikely to be the same between SFB− and + mice, and a substantial increase of Tfh cells caused by Tfr cell deficiency may not be observed in a system where Tfh cell expansion has already been taken to a close to a plateau capacity (i.e. SFB+ mice). Only one pioneering study has focused on the role of Tfr cells in a spontaneous autoimmune disease model, which revealed a similar suppressive effect to what we observed in SFB− K/BxN mice (22, 82). Additionally, using the pristane-induced lupus model, a study also found that Tfr play a suppressive role in producing anti-DNA of IgA isotypes (22).

Here, our study suggests that a constituent of the gut microbiota, SFB, modifies TCR signaling that reduces Nur77 and induces glycolytic metabolism, leading to a lower surface expression of CTLA-4 on Tfr cells, which is correlated with an increase in autoimmune arthritis disease. The data presented in this study show a Tfr suppressive function towards B cell responses in an autoimmune arthritis model. Our data further reveal a new twist that

gut microbiota SFB could modify Tfr function in the same disease model to eliminate this suppressive effect. In the future, it will be interesting to study whether or not Tfr can better or more consistently repress self-reactive Tfh cells and B cell responses than foreign-Ag reactive counterparts. It has been shown that Tfr cells expressed potentially autoreactive TCRs, as the TCR repertoire of Tfr cells is closer to that of Treg cells (43). If Tfr cells do repress self-reactive responses better than foreign-Ag, it will be interesting to learn whether that is a result of Tfr cells expressing a TCR repertoire that is more geared toward recognition of self-antigen (43). Finally, it will be equally interesting to determine whether gut microbiota may also alter Tfr cell function in these contexts, which may contribute to the variable Tfr function observed—from negative to positive effects on antibody responses—in many of the previous reports (17–19, 22–24).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Key points

- SFB-mediated CTLA-4 reduction is associated with increased Tfr glycolytic activity
- Reduced Nur77, an indicator of TCR signal strength, is linked to SFB-reduced CTLA-4
- Nur77 deficiency causes an increased glycolysis and decreased CTLA-4 in Tfr cells

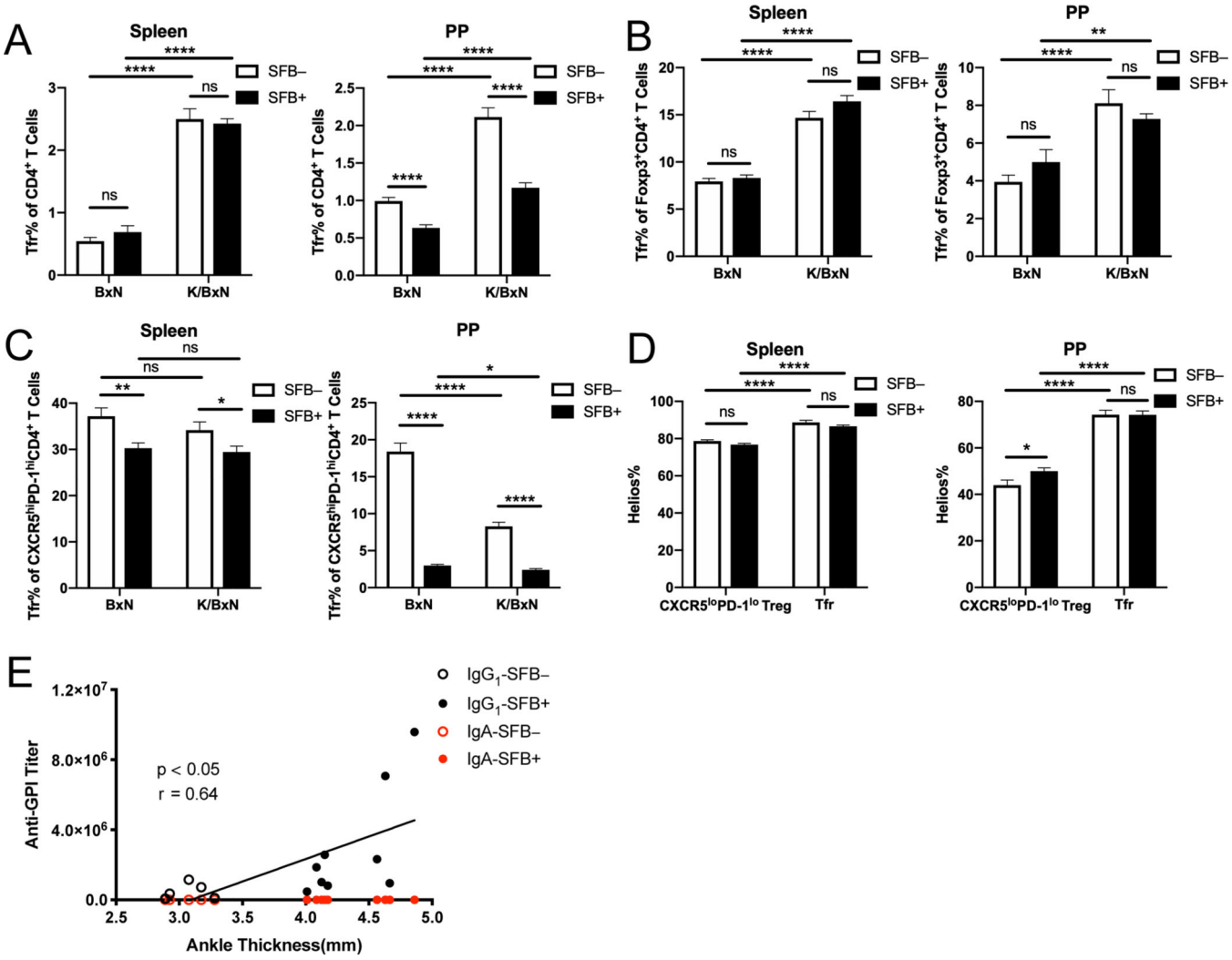

Figure 1. The impact of gut microbiota SFB and self-Ag on the Tfr cell population.

(A) Splenocytes and PP cells from both SFB− and SFB+ BxN and K/BxN mice were stained with Abs against TCRβ, CD4, PD-1, CXCR5, and Foxp3. The quantitative percentage of Tfr cells among total CD4+ T cells is shown as mean + SEM (n=12–19/group, data combined from 4–7 independent assays).

(B) Splenocytes and PP cells from both SFB− and SFB+ BXN and K/BxN mice were stained with Abs as in 1A. The quantitative percentage of Tfr cells among $Foxp3^+$ Tregs is shown as mean + SEM (n=12–19/group, data combined from 4–7 independent assays).

(C) The Tfr/Tfh ratio from experiments in 1A was calculated as the percentage of Tfr cell numbers among $CXCR5^{hi}PD\text{-}1^{hi}CD4^+$ T (Tfh) cell numbers. Data is shown as mean + SEM (n=12–19/group, data combined from 4–7 independent assays).

(D) Splenocytes and PP cells from both SFB− and SFB+ K/BxN mice were stained with Abs against TCRβ, CD4, PD-1, CXCR5, Helios, and Foxp3. The quantitative percentages of Helios+ cells in $CXCR5^{lo}PD\text{-}1^{lo}$ Tregs and Tfr cell populations are shown as mean + SEM (n=12–16/group, data combined from 4 independent assays).

(E) The anti-GPI titers in IgG1 and IgA isotype from each individual mouse were plotted against its ankle thickness in SFB− and SFB+ K/BxN mice (n=5–9/group, data combined from 2 independent assays).

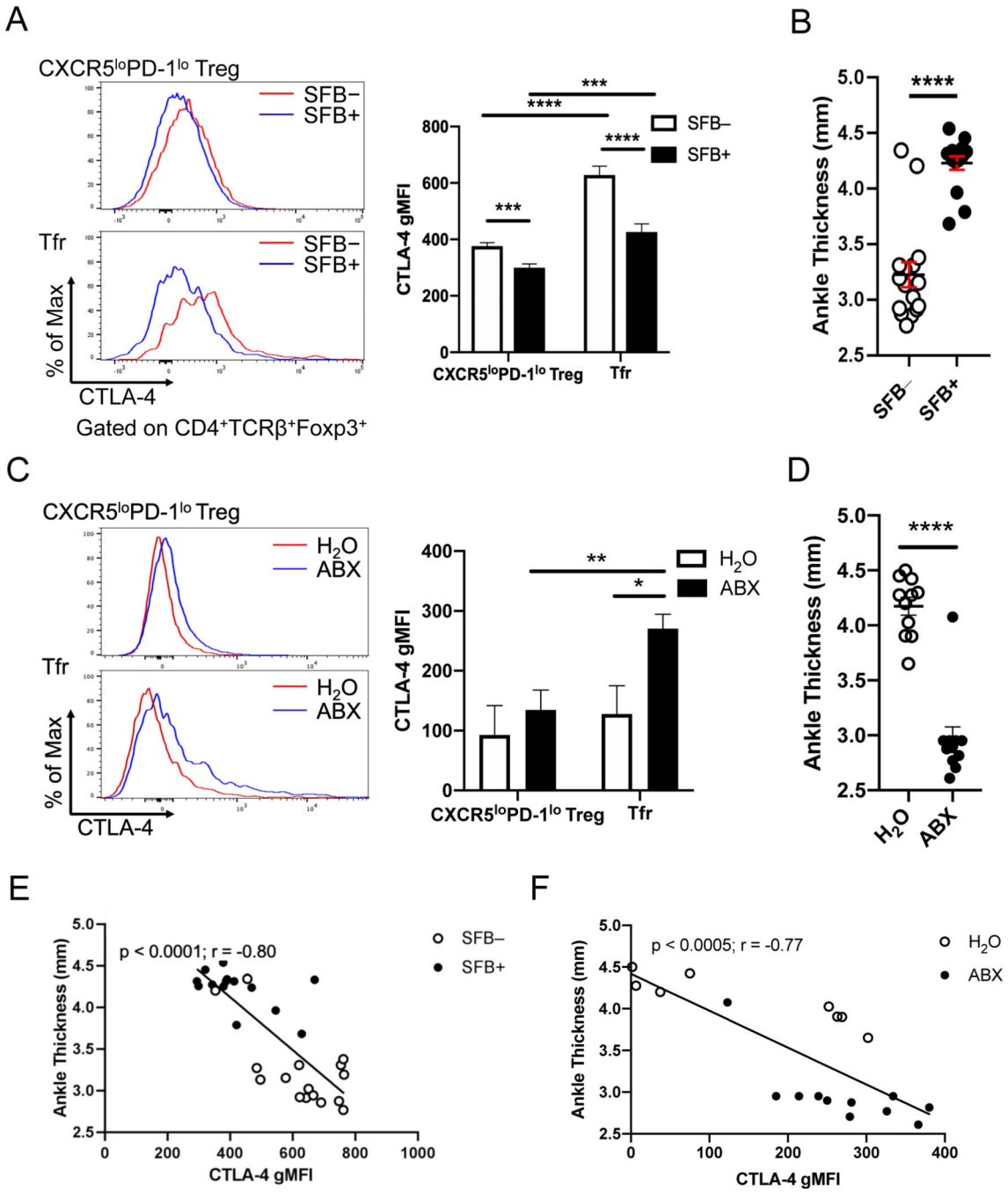

Figure 2. Manipulating the gut microbiota alters the systemic (splenic) CTLA-4 expression on Tfr cells, which is negatively associated with arthritis severity.

(**A)** Histogram overlays of CTLA-4 in CXCR5^{lo}PD-1^{lo} Tregs and Tfr cell populations from spleen of SFB− and SFB+ K/BxN mice are shown. Quantitative gMFI of CTLA-4 in SFB− and SFB+ CXCR5^{lo}PD-1^{lo} Tregs and Tfr cell populations are also shown (n=15–16/group, 4 independent assays).

(B) The ankle thickness data of SFB− and SFB+ K/BxN mice from experiments in 2A are shown.

(**C)** Histogram overlays of CTLA-4 in $CXCR5^{lo}PD\text{-}1^{lo}$ Tregs and Tfr cell populations from spleen of SFB+ (H_2O) and antibiotic treated (and thus SFB−) K/BxN mice are shown. Quantitative gMFI of CTLA-4 in SFB+ and antibiotic treated (ABX) $CXCR5^{lo}PD\text{-}1^{lo}$ Tregs and Tfr cell populations are also shown (n=15–16/group, 4 independent assays).
(D) The ankle thickness data of SFB+ and ABX K/BxN mice from experiments in 2C are shown.
(E) The CTLA-4 expression level from each individual mouse of experiments in 2A was plotted against its ankle thickness in SFB− and SFB+ K/BxN mice.
(F) The CTLA-4 expression level from each individual mouse of experiments in 2C was plotted against its ankle thickness in ABX (SFB−) and H_2O (SFB+) K/BxN mice.

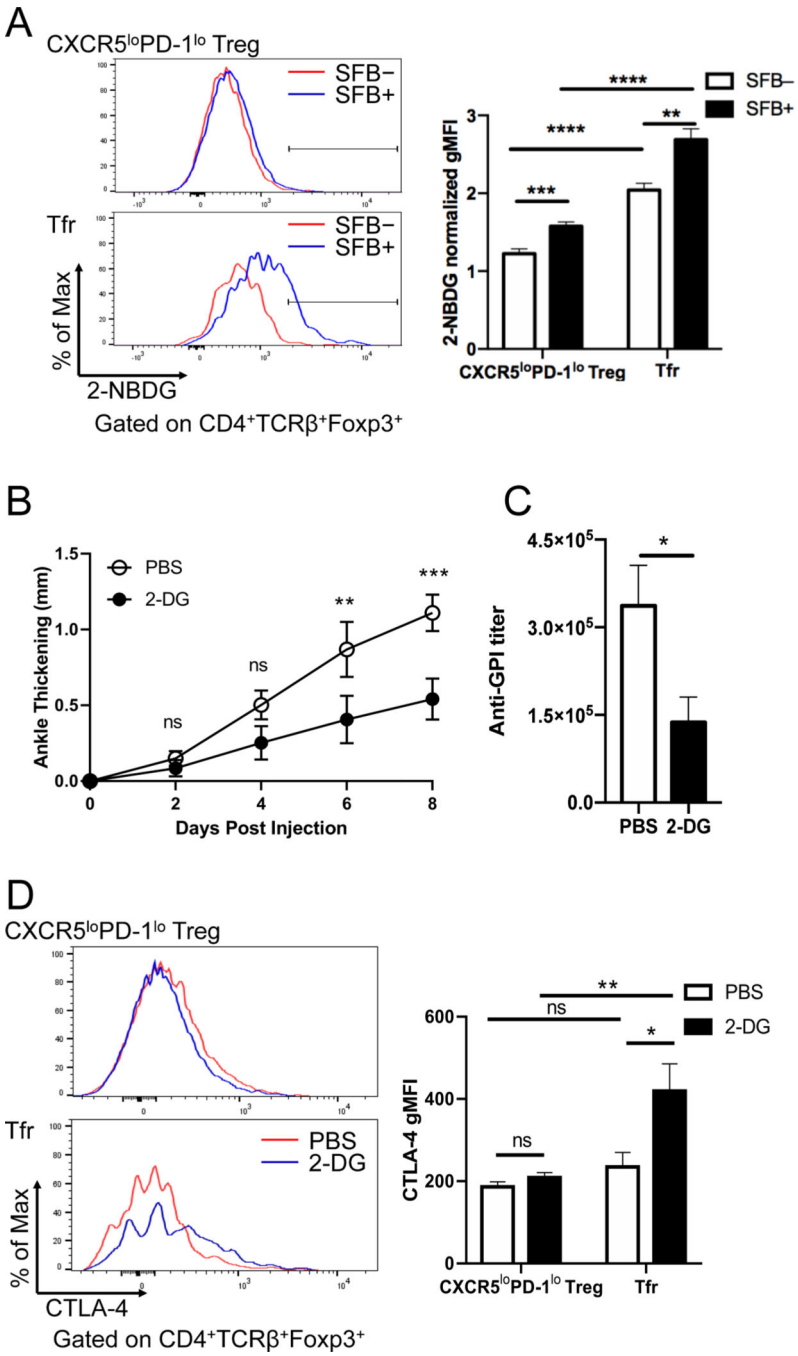

Figure 3. SFB+ Tfr cells display an increased 2-NBDG level and the glycolysis inhibitor 2-DG enhances CTLA-4 expression on Tfr cells.

(A) 2-NBDG uptake in CXCR5^{lo}PD-1^{lo} Tregs and Tfr cells in SFB− and SFB+ K/BxN mice. As the 2-NBDG intake varies moderately from experiments to experiments, we normalized the 2-NBDG in each experiment by setting the 2-NBDG value in any cell group or SFB status of any mouse that expressed the lowest level of 2-NBDG as 1. Representative plots and quantitative normalized gMFI data of 2-NBDG^{+} cells in CXCR5^{lo}PD-1^{lo} Tregs

and Tfr cell populations from spleen of SFB− and SFB+ K/BxN mice are shown (n=8–9/group, 2 independent assays).

(B) SFB+ K/BxN mice were injected every other day with 2-DG from 4 weeks of age. The ankle thickening of PBS and 2-DG treated K/BxN mice are shown (n=7–9/group, data combined from 2 independent assays).

(C) The anti-GPI titers of PBS and 2-DG treated K/BxN mice are shown (n=8–15/group, data combined from 3 independent assays).

(D) Histogram overlays of CTLA-4 in $CXCR5^{lo}PD\text{-}1^{lo}$ Tregs and Tfr cell populations from spleen of PBS and 2-DG treated K/BxN mice from experiments in 3B are shown. Quantitative gMFI of CTLA-4 are also shown (n=10–13/group, 3 independent assays).

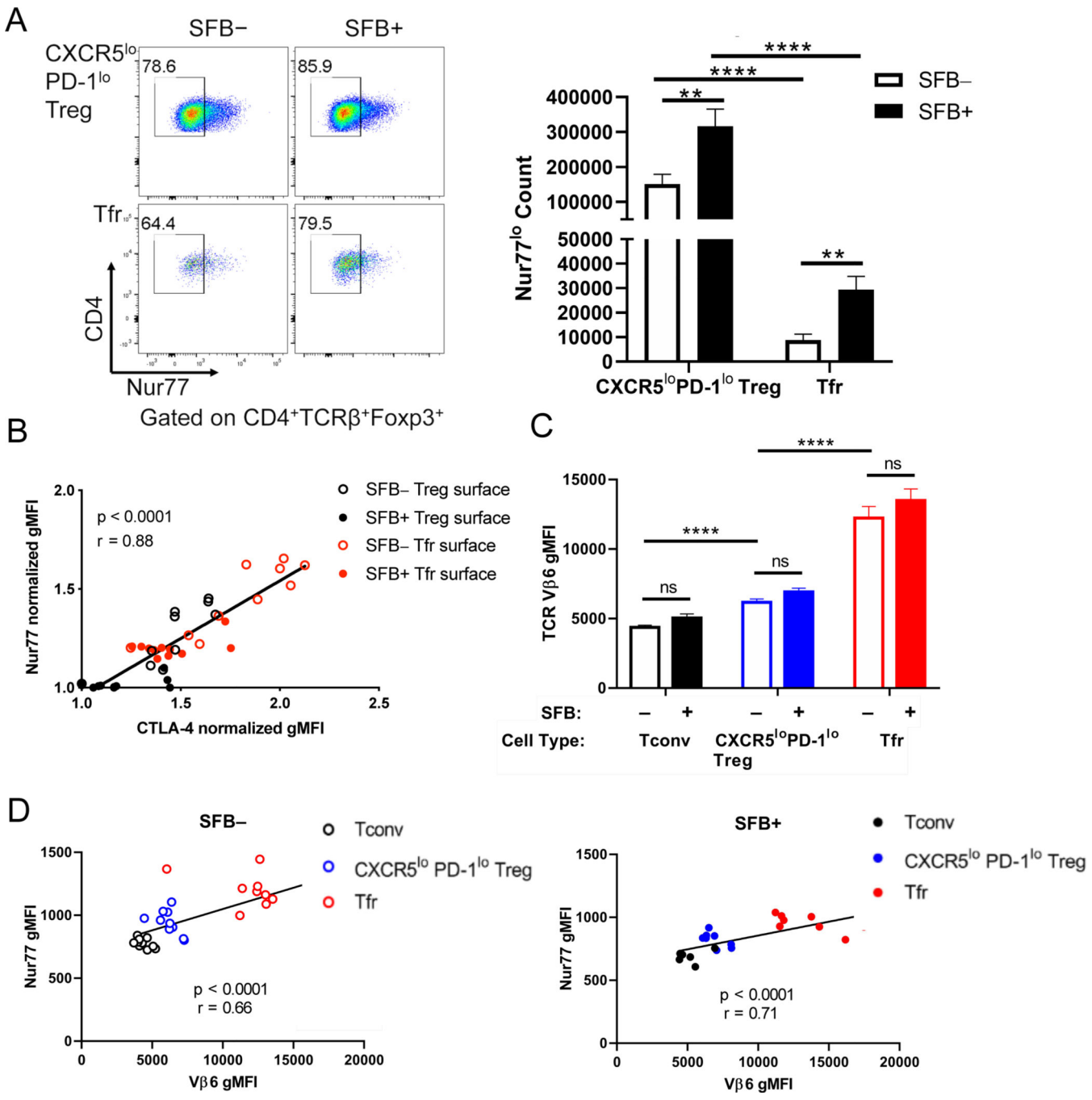

Figure 4. SFB-reduced TCR signaling strength indicated by increased Nur77^{lo} Tfr and Treg cells are independent of the TCR expressing level.

(A) Representative percentage plots and quantitative cell number data of Nur77^{lo} T cells in CXCR5^{lo}PD-1^{lo} Tregs and Tfr cell populations from spleen of SFB− and SFB+ of K/BxN mice (n=11–13/group, 3 independent assays).

(B) The Nur77 level in Tfr and CXCR5^{lo}PD-1^{lo} Tregs from individual mouse in experiment 4A is plotted against its surface CTLA-4 level. In each independent experiment, we set the

Nur77 and CTLA-4 from the mouse with lowest Nur77 and CTLA-4 gMFI as value of 1 (n=11–13/group, 3 independent assays).

(C) The gMFI of TCRVβ6, the transgenic TCR Vβ gene of KRN mice, are shown in 3 T cell types including Tconv, $CXCR5^{lo}PD\text{-}1^{lo}$ Tregs, and Tfr cells from both SFB− and SFB+ K/BxN mice (n=8–10/group, data combined from 2 independent assays).

(D) The gMFI of TCRVβ6 is plotted against gMFI of Nur77 in Tconv, $CXCR5^{lo}PD\text{-}1^{lo}$ Tregs, and Tfr cells (each symbol represents one mouse). The data from SFB− and SFB+ K/BxN mice are shown in two plots for simple visualization (n=8–10/group, data combined from 2 independent assays).

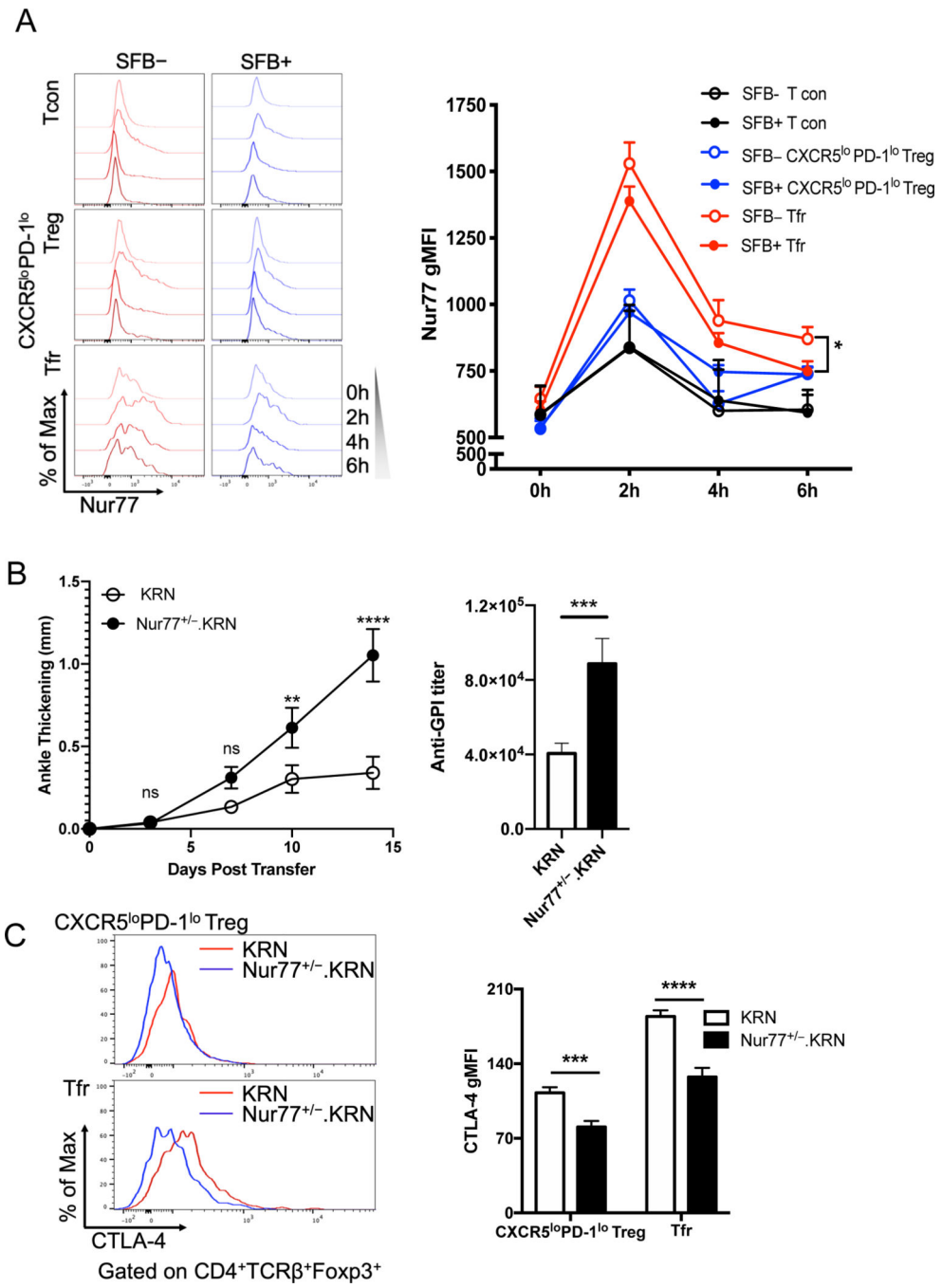

Figure 5. SFB promote a swifter degradation of Nur77 after TCR ligation and Nur77 haplodeficiency leads to enhanced arthritis and reduced CTLA-4 expression.

(A) Nur77 levels in the Tconv, CXCR5^{lo}PD-1^{lo} Tregs, and Tfr cell populations after TCR ligation over time. The splenocytes of SFB− and SFB+ K/BxN mice were incubated at 37°C with plate bound 5 μg/ml of GPI peptides for the indicated time period (ranging from 0 to 6 hours). Both representative plots and compiled data are shown (n=14–22/group, data combined from 3 independent assays).

(B) SFB− KRN or Nur77$^{+/-}$.KRN donor CD4^{+} T cells were transferred into SFB− TCRα$^{-/-}$.BxN recipients (T cell deficient mice on a B6xNOD background and thus, bearing NOD MHC class II, I-A^{g7}, for self-Ag presentation). Ankle thickening of recipient mice is shown as mean ± SEM. Day 0 indicates the day of cell transfer (n=10–13/group, data combined from 3 independent assays).

(C) CTLA-4 expression on Tfr cells from experiments in **5B** is shown as representative plots as well as compiled data (mean + SEM).

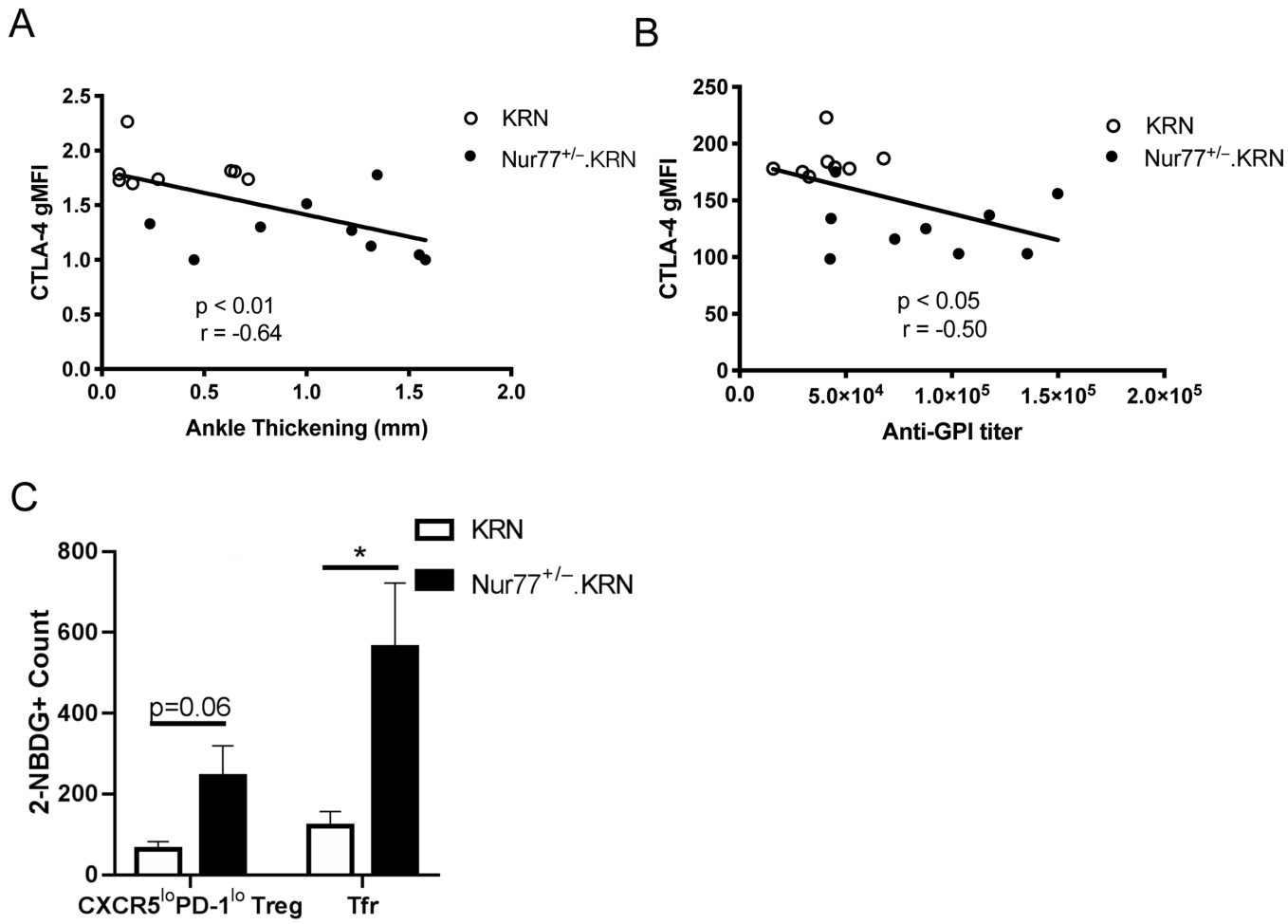

Figure 6. Nur77 deficiency increases glycolysis in Tfr cells and the Nur77 deficiency-associated Tfr's CTLA-4 reduction is associated with increased ankle thickening and anti-GPI titers.

((A, B) SFB− KRN or Nur77$^{+/-}$.KRN donor CD4^{+} T cells were transferred into SFB− TCRα$^{-/-}$.BxN recipients. The CTLA-4 expression level from each individual mouse was plotted against its ankle thickening (A) or anti-GPI titers (B) (n=10–13/group, 3 independent assays).

((C) 2-NBDG uptake in CXCR5^{lo}PD-1^{lo} Tregs and Tfr cells were examined in mice from experiment 6A and the total 2-NBDG^{+} cell numbers in CXCR5^{lo}PD-1^{lo} Tregs and Tfr cell populations from spleen are shown (n=10–13/group, 3 independent assays).

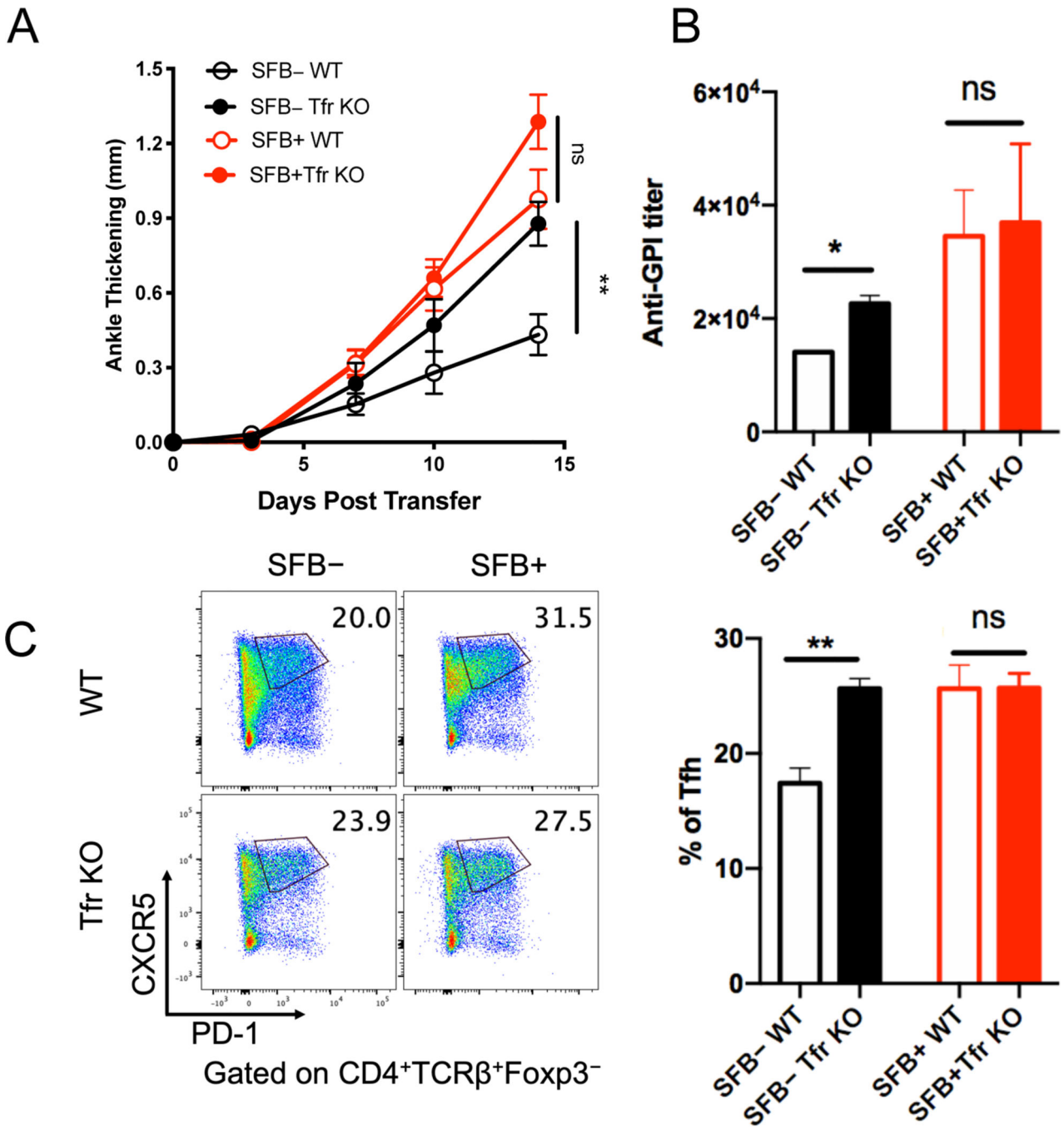

Figure 7. Deletion of Tfr cells increase the development of arthritis and autoantibodies in SFB− but not SFB+ mice.

((A) SFB− KRN or KRN.Foxp3Cre.Bcl-6$^{fl/fl}$ (Tfr KO) donor CD4^{+} T cells were transferred into SFB− and SFB+ TCRα$^{-/-}$.BxN recipients. Ankle thickening of recipient mice is shown as mean ± SEM. Day 0 indicates the day of cell transfer (n=9–15/group, data combined from 6 independent assays).

((B) Sera of mice in 6A were collected on day 14 after T cell transfer. Anti-GPI titers are shown.

((C) The representative and quantified percentage data of Tfh cells among Tconv cells ($CD4^+TCR\beta^+Foxp3^-$) are shown (n=7–10/group, data combined from 4 independent assays).