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Inflammation and Lymphopenia Trigger Autoimmunity by Suppression of IL-2–Controlled Regulatory T Cell and Increase of IL-21–Mediated Effector T Cell Expansion

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The dynamic interplay between regulatory T cells (T_{regs}) and effector T cells (T_{effs}) governs the balance between tolerance and effector immune responses. Perturbations of T_{reg} frequency and function or imbalances in T_{reg}/T_{eff} levels are associated with the development of autoimmunity. The factors that mediate these changes remain poorly understood and were investigated in this study in murine autoimmune arthritis. T_{regs} displayed a stable phenotype in arthritic mice and were fully functional in in vitro suppression assays. However, their expansion was delayed relative to T_{effs} (T follicular helper cells and Th17 cells) during the early stages of autoimmune reactivity. This imbalance is likely to have led to insufficient T_{reg} control of T_{effs} and induced autoimmunity. Moreover, a counterregulatory and probably IL-7–driven increase in thymic T_{reg} production and recruitment to inflamed tissues was too slow for disease prevention. Increased T_{eff} over T_{reg} expansion was further aggravated by inflammation and lymphopenia. Both these conditions contribute to autoimmune pathogenesis and were accompanied by decreases in the availability of IL-2 and increases in levels of IL-21. IL-2 neutralization or supplementation was used to show that T_{reg} expansion mainly depended on this cytokine. IL-21R$^{-/-}$ cells were used to demonstrate that IL-21 promoted the maintenance of T_{effs}. Thus, at inflammatory sites in experimental arthritis, a deficit in IL-2 hampers T_{reg} proliferation, whereas exaggerated IL-21 levels overwhelm T_{reg} control by supporting T_{eff} expansion. This identifies IL-2 and IL-21 as targets for manipulation in therapies for autoimmunity. ***The Journal of Immunology*****, 2014, 193: 4845–4858.**

Regulatory T cells (T_{regs}) are essential for immune homeostasis and the maintenance of peripheral self-tolerance. Accordingly, perturbations of T_{regs} are associated with inflammatory (1) and autoimmune diseases (2). T_{reg} development and function is controlled by the transcription factor Foxp3. Its mutation or depletion results in fatal autoimmune lymphoproliferative disease in mice (3) and immunodysregulation polyendocrinopathy enteropathy X-linked syndrome in humans (4, 5). Thus, factors that adversely affect T_{reg} numbers, stability, function, or qualitative features and result in an imbalance of increased pathogenic effector T cells (T_{effs}) over T_{regs} may contribute to the development of autoimmune disease (1, 2).

A negative correlation between T_{reg} numbers and/or function and human autoimmune rheumatoid arthritis (RA) has been shown in previous studies (6). Likewise, studies in murine models of RA clearly indicate a beneficial role for T_{regs} in reducing disease development and severity (7–9). In humans and mice, T_{regs} infiltrate the synovial tissue of inflamed joints, as well as secondary lymphoid organs, and display suppressor functions (9–13).

The protective role of T_{regs} has also been demonstrated in the well-defined murine K/BxN ([KRNtgxNOD]F1) model of RA (11–13). K/BxN mice spontaneously develop arthritis at ∼4 wk of age. A break of tolerance in this model (14) leads to the activation of KRNtg (KRN TCR transgenic C57BL/6) expressing CD4$^+$ T cells upon recognition of the autoantigen GPI that is presented in a complex with the MHC class II molecule Ag7. Interactions between KRNtg CD4$^+$ T cells and GPI-specific B cells then result in the production of anti-GPI–specific IgG1 autoantibodies, which trigger synovial inflammation (15–17). T_{regs} are also expanded in joints and lymphoid organs of arthritic K/BxN mice, proliferate well, and display full functionality in suppressing T_{effs} in vitro (11, 13, 14). Moreover, the absence of T_{regs} in K/BxN mice carrying a *scurfy* mutation led to a more rapid and aggressive arthritis (11). However, despite T_{reg} enrichment at sites of inflammation, and their full functionality, K/BxN mice still experience development of disease. This may be because numerical and/or functional imbalances within the T_{reg} and T_{eff} compartments tip the balance toward autoimmunity. This has been partially explained by increased T_{eff} resistance toward T_{reg}-mediated suppression, as well as shorter proliferative bursts and higher apoptosis rates in T_{regs} (13). However, the factors and molecular mechanisms that influence T_{reg} and T_{eff} homeostasis in an autoimmune setting are poorly under-

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Abbreviations used in this article: GC, germinal center; iT_{reg}, inducible T_{reg}; KRNtg, KRN TCR transgenic C57BL/6; LN, lymph node; Nrp-1, neuropilin-1; nT_{reg}, natural T_{reg}; PD1, programmed cell death-1; PD-L1, PD1 ligand; PSGL1, P-selectin glycoprotein ligand 1; RA, rheumatoid arthritis; rm, recombinant mouse; SP, single-positive; T_{eff}, effector T cell; T_{FH}, T follicular helper cell; T_{reg}, regulatory T cell.

stood. Inflammation and lymphopenia are prevalent in autoimmunity and may influence T_{reg}/T_{eff} stability, functionality, and homeostasis (18). Given that both these conditions are present in arthritic K/BxN mice (19, 20), we used this model to study whether and how inflammation and lymphopenia influence T_{reg} stability and function and T_{reg}/T_{eff} homeostasis, and modulate autoimmunity.

We observed full functionality of T_{regs} isolated from inflamed organs of arthritic mice in in vitro suppression assays and were able to exclude the possibility of an unstable T_{reg} phenotype that would result in a conversion of T_{regs} into T_{effs}. However, we also observed that T_{reg} expansion was delayed relative to T_{eff} expansion (T follicular helper cells [T_{FHs}] and Th17 cells) in inflamed lymphoid tissues in early stages of disease. This likely resulted in acute unhindered T_{eff} activity, which may induce disease that, once initiated, rapidly becomes uncontrolled. Inflammation and lymphopenia in arthritic mice exacerbated this delay and further increased T_{eff} proliferation. Arthritic mice also had decreased availability of IL-2 and increased levels of IL-21. Neutralization and supplementation with IL-2 showed that T_{reg} expansion was dependent on this cytokine. Transfer of wild-type and IL-21R$^{-/-}$ KRNtg T_{effs} and T_{regs} in various combinations showed that IL-21R$^{-/-}$ deficiency mainly affected T_{eff} maintenance. Increases in thymic IL-7 and CD127 expression on thymic Foxp3^{+} single-positive (SP) CD8^{-}CD4^{+} T cells indicated that enhanced T_{reg} frequencies may be because of increased IL-7–mediated thymic output and recruitment to sites of inflammation, where T_{regs}, however, fail to expand and exert their suppressive function effectively.

Materials and Methods

Mice

KRNtg mice were obtained from D. Mathis and C. Benoist (Harvard Medical School, Boston, MA), CD28$^{-/-}$.CD45.1 mice from C. Vinuesa (Australian National University, Canberra, ACT, Australia), and B6.H-2g7/g7 mice were purchased from The Jackson Laboratory, NOD/ShiLtJArc and CD45.1 congenic C57BL/6 (B6.SJL/ptprc^{a}) mice were obtained from the Animal Resources Centre (Perth, Australia), IL-21R$^{-/-}$ mice from C. King (Garvan Institute of Medical Research), and Foxp3.gfp mice from C. Vinuesa. IL-21R$^{-/-}$.KRNtg and Foxp3.gfp KRNtg mice were generated by crossing IL-21R$^{-/-}$ and Foxp3.gfp mice with KRNtg mice, respectively. CD28$^{-/-}$.Ag7$^{+/-}$ (congenic for CD45.1) mice were generated by crossing CD28$^{-/-}$.CD45.1 mice with B6.H-2g7/g7 mice. Crossing KRNtg with NOD/Lt mice generates either KRNtg-expressing arthritic K/BxN (KRNtgxNOD)F1 mice or KRNtg^{-} and healthy littermates (BxN). Crossing B6.SJL/ptprc^{a} with NOD mice generates BxN.45.1 mice (B6.SJL/ptprc^{a} × NOD)F1. Foxp3.gfp KRNtg mice were bred to NOD mice to generate Foxp3.gfp K/BxN mice; because Foxp3 is on the X chromosome, only male Foxp3.gfp K/BxN mice were analyzed. When indicated, some of the strains were further crossed with B6.SJL/ptprc^{a} mice to generate CD45.1 or CD45.1.2 congenic mice. Genotypes were assessed by genomic PCR or FACS. Experiments were approved by the Garvan-St. Vincent's and the Monash Animal Ethics Committees.

Adoptive transfer experiments and injections

Cell suspensions were prepared from pooled spleens and lymph nodes (LNs). CD4^{+} T cells and subsets were isolated to a purity of >98% by negative selection using MACS microbeads (Miltenyi Biotech) according to the manufacturer's instructions followed by FACS sorting. Purified CD4^{+} T cells were injected i.v. into recipient mice as outlined in the respective experiments. Neutralizing anti–IL-2 Abs (clone S4B6.1; WEHI, Melbourne) were injected i.p. at a concentration of 1 mg at day 0 of adoptive transfer; 250 μg neutralizing anti–programmed cell death-1 (anti-PD1; RMP1-14) and anti-PD1 ligand (anti–PD-L1; MIH5) Abs were injected i.p. at 0, 12, and 36 h after adoptive transfer; 2 μg recombinant mouse IL-2 (rmIL-2; Peprotech) was injected i.p. at −12, 0, 12, 24, and 36 h after adoptive transfer; 20 μg rmIL-21 (Peprotech) was injected i.p. at 2, 4, 6, 8, 10, and 12 d after adoptive transfer; and 100 μg LPS was injected i.p. in BxN.45.1 mice.

Arthritis scoring

Measurement of arthritis development was done as previously described (21). In brief, clinical severity of arthritis was assessed every 1–2 d for all 4 paws on a scale from 0 to 3 and indicated as cumulative score: 0, normal; 1, erythema, swelling limited to individual digits or mild ankle swelling insufficient to reverse the normal V shape of the foot; 2, swelling sufficient to make the ankle and midfoot approximate in thickness to the forefoot; 3, reversal of the normal V shape of the foot, swelling of the entire paw including multiple digits.

Flow cytometry

Cells were collected from crushed spleen, peripheral LNs (pool of inguinal, brachial, axillary, and cervical LNs), or thymus. For intracellular cytokine and nuclear Foxp3 staining, cells were fixed and permeabilized with Foxp3 Fix/Perm Buffer Set (eBioscience) according to the manufacturer's instructions. Intracellular cytokine expression was detected after restimulation of cells with 50 ng/ml PMA and 1 μg/ml ionomycin (Sigma-Aldrich) in the presence of brefeldin A and monensin (eBioscience). Cells were analyzed with BD LSRII and FACSCanto II. The following Abs were used: anti-CD4 V450 (BD), anti-CD4 PE-Cy7 (Biolegend), anti-CD103 FITC (BD), anti-CD25 PE-Cy7 (Biolegend), anti-Foxp3 allophycocyanin/FITC (eBioscience), anti-CD45.2 V450 (BD), anti-CD45.1 allophycocyanin-eFluor 780 (eBioscience), anti-CD162 PE (BD), anti-CD62L FITC (BD), anti-PD1 PE (eBioscience), anti-CXCR5 Biotin (BD), anti-streptavidin PerCP-Cy5.5 (eBioscience), anti-IgG1 FITC (BD), anti-CD45R/B220 allophycocyanin-eFluor 780 (eBioscience), anti-GL7 (T and B cell activation Ag) FITC (BD), anti-CD95 PE (BD), anti-CD196 (CCR6) allophycocyanin (BD), anti–IL-17 PE (BE), anti–IL-2 PE (eBioscience), anti–IL-21 biotin (R&D), streptavidin allophycocyanin (BD), anti–neuropilin-1 (anti–Nrp-1) allophycocyanin (R&D), anti-CD127 allophycocyanin (Biolegend), and anti-CD8 PerCP (BD).

Anti-GPI IgG1 ELISA

Anti-GPI IgG1 levels were determined in serum by ELISA as previously described (22).

Real-time quantitative PCR

Total RNA was extracted using TRIzol reagent (Invitrogen), and the QuantiTect Reverse Transcription Kit (Qiagen) was used for cDNA synthesis according to the manufacturer's instructions. Quantitative real-time PCR was performed using an ABI Prism 7900HT Real-Time PCR system (Applied Biosystems). For each sample, mRNA abundance was normalized to the amount of the housekeeping gene *Gapdh* and results expressed as arbitrary units.

Cell culture and suppression

FACS-purified T cell subsets were cultured in RPMI 1640 medium supplemented with 1% (v/v) L-glutamine, 1% (v/v) nonessential amino acids, 1% (v/v) sodium pyruvate, 1% (v/v) penicillin (10,000 U) and streptomycin (10,000 U; all from Life Technologies), 10% (v/v) FBS (Hyclone), and 55 μM 2-ME (Life Technologies). T_{effs} were stained with CellTrace Violet Component (Invitrogen) and 50,000 T_{effs} cocultured with T_{regs} at indicated ratios in the presence of 200,000 irradiated feeder splenocytes from NOD mice and anti-CD3 (clone 145-2C11, 2 μg/ml). Cell proliferation was determined 4 d later by FACS analysis.

Immunoblot

For immunoblot analysis, nuclear extracts of thymi were prepared with NE-PER Nuclear and Cytoplasmic Extraction reagents (Thermo Scientific) according to the manufacturer's instructions. Samples were analyzed by SDS-PAGE and transferred to a nitrocellulose membrane that was probed with either anti-histone 2B (Millipore, Billerica, MA) or anti–NF-κB p65 (Cell Signaling). The membranes were then probed with HRP-conjugated secondary Ab and developed with Western Lightning chemiluminescence reagent (Perkin Elmer, Boston, MA).

Statistics

Statistical significance was determined by calculating p values using an unpaired t test on Instat software (GraphPad Software, San Diego, CA). All data are means ± SD and are representative of at least two independent experiments. The p values ≤0.05 were considered significant ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$).

Results

Effector/memory T_{regs} are increased in arthritic mice and display full functionality in in vitro suppression assays

The concept that T_{regs} represent a stable T cell lineage has recently been challenged by several studies and remains controversial (23,

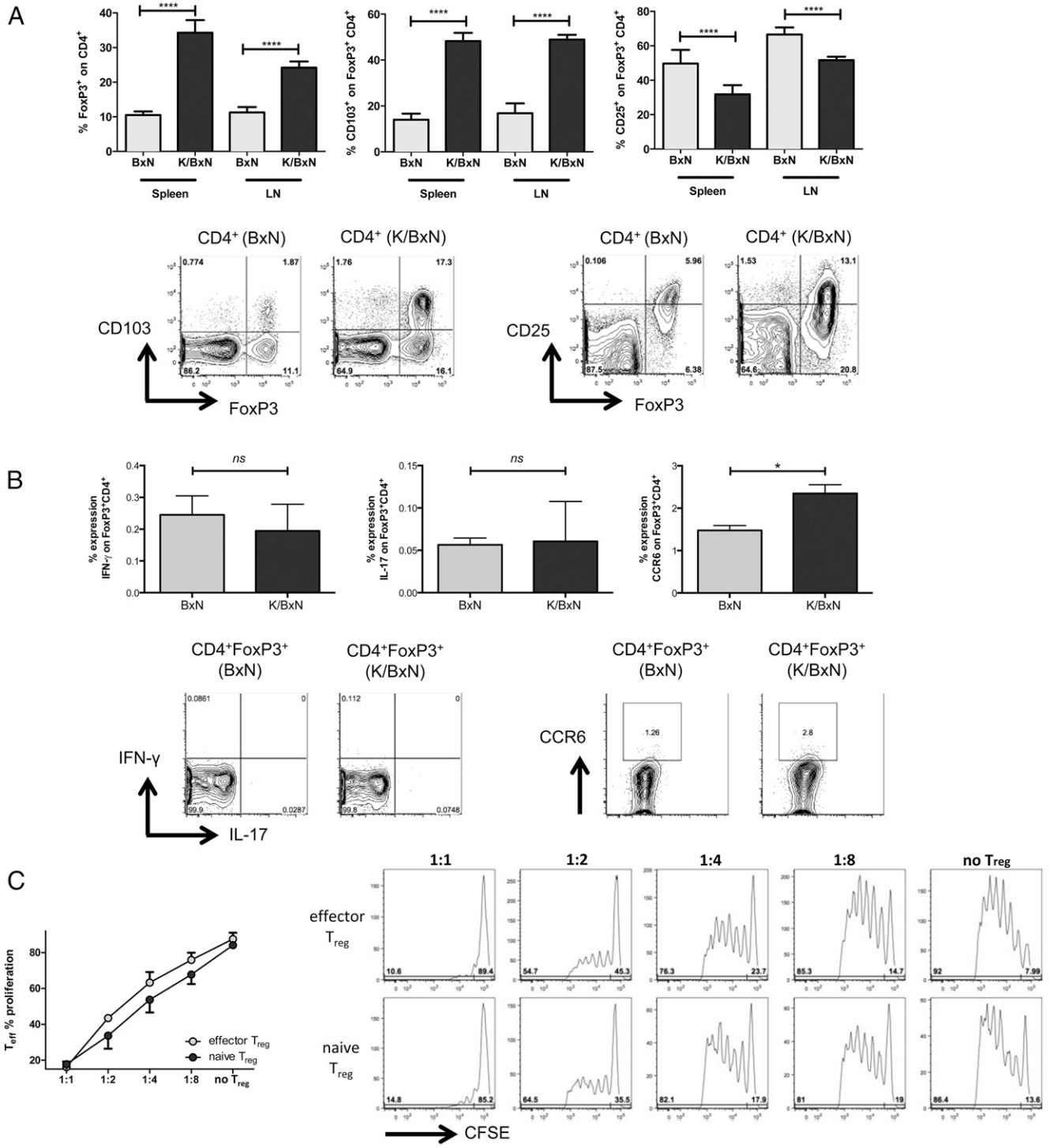

FIGURE 1. T_{regs} are increased in secondary lymphoid organs of arthritic K/BxN mice and are fully functional in in vitro suppression assays. (**A**) FACS analysis showing percentages of Foxp3 expression in $CD4^+$ T cells and of CD103 or CD25 on $Foxp3^+CD4^+$ T cells in spleens and LNs of 8-wk-old arthritic K/BxN mice ($n = 7$ mice) compared with nonarthritic $KRNtg^-$ (BxN) littermates ($n = 5$ mice). (**B**) FACS analysis showing percentages of IFN-γ, IL-17, and CCR6 expression on $Foxp3^+CD4^+$ T cells in spleens of 8-wk-old arthritic K/BxN mice ($n = 4$ mice) compared with nonarthritic $KRNtg^-$ (BxN) littermates ($n = 4$ mice). (**C**) T_{eff}/T_{reg} in vitro suppression assays of naive versus effector T_{regs}. Naive $Foxp3.gfp^-$ KRNtg T_{effs} were isolated from lymphoid organs of Foxp3.gfp KRNtg mice (purity >98%). Cells (50,000) were incubated with either naive (isolated from Foxp3.gfp KRNtg mice) or effector (isolated from Foxp3.gfp K/BxN mice) $Foxp3.gfp^+$ KRNtg T_{regs} at a ratio of 1:1, 1:2, 1:4, and 1:8, or without T_{regs} (no T_{reg}) for 4 d in the presence of 2 μg/ml anti-CD3 mAbs and 200,000 irradiated NOD splenocytes. Proliferation of $Foxp3.gfp^-$ KRNtg T_{effs} was measured by FACS using violet cell proliferation tracer. Displayed are the percentage of proliferating cells and representative FACS plots. $^*p < 0.05$, $^{****}p < 0.0001$.

24). Conditions such as lymphopenia and inflammation have been suggested to drive T_{reg} instability and their conversion into T_{effs} (i.e., Th17 and T_{FHs}) (23–25). We investigated whether an unstable T_{reg} phenotype and conversion into T_{effs} could explain why increased numbers of T_{regs} in arthritis are unable to prevent the development of disease.

We first confirmed that T_{reg} frequencies are increased in secondary lymphoid organs (spleen and peripheral LNs) of arthritic

K/BxN mice compared with KRNtg$^-$ nondiseased BxN littermates (Fig. 1A). Higher expression of CD103 was found on arthritic K/BxN compared with naive BxN Foxp3$^+$ T_{regs}, which indicates that they were effector/memory–like T_{regs} (Fig. 1A) (26). Interestingly, arthritic K/BxN T_{regs} had a higher proportion of CD25$^-$Foxp3$^+$ T_{regs} (Fig. 1A). This subset was recently reported to be less stable in a lymphopenic environment (23, 27). Moreover, it was shown previously that a proportion of CD25^{lo} Foxp3$^+$ cells lose Foxp3 expression and acquire a pathogenic Th17 phenotype within the inflammatory milieu in arthritis (2). We therefore compared nonarthritic with arthritic T_{regs} in BxN and K/BxN mice, respectively. In arthritic T_{regs} we did not find increased IL-17 or IFN-γ expression (Fig. 1B), whereas CCR6 was increased (Fig. 1B).

To examine the functionality of arthritic versus nonarthritic T_{regs}, we isolated Foxp3.gfp$^+$ CD4$^+$ cells from arthritic Foxp3.gfp K/BxN and naive Foxp3.gfp KRNtg mice, respectively, and used them in suppression assays. As shown in previous studies (13), arthritic effector T_{regs} were as efficient as naive nonarthritic T_{regs} in suppressing T_{eff} proliferation (Fig. 1C).

T_{regs} display a stable phenotype

To address whether T_{regs} are unstable and can readily convert into T_{effs}, we performed adoptive transfer experiments of Foxp3.gfp$^+$ CD4$^+$ cells isolated from Foxp3.gfp KRNtg mice. Because lymphopenia and inflammation have been shown to influence T_{reg} stability, we assessed whether this occurs in arthritis by using the following recipient animals: (*i*) lymphoreplete naive BxN.45.1 mice, (*ii*) quasi-lymphopenic CD28$^{-/-}$.Ag7$^{+/-}$ mice, and (*iii*) inflammatory arthritic K/BxN.45.1 mice. Mice were sacrificed 14 d after transfer, and adoptively transferred cells were examined for expression/downregulation of GFP in spleen and LN. Under all conditions, a small fraction (1.27–9.44%) of transferred Foxp3.gfp$^+$ KRNtg T_{regs} had become GFP$^-$. The greatest frequency of GFP$^-$ cells was found in the LN of quasi-lymphopenic CD28$^{-/-}$.Ag7$^{+/-}$ mice (Fig. 2A, 2B). Moreover, the examination of absolute numbers of recovered T_{regs} showed that T_{reg} expansion is generally hampered in the respective lymphopenic and/or inflammatory milieu of CD28$^{-/-}$ and K/BxN mice (Fig. 2C).

We next addressed whether Foxp3.gfp$^+$ T_{regs} were able to adopt a T_{eff} (P-selectin glycoprotein ligand 1 [PSGL1]^{lo}, CD62L^{lo}, PD1^{hi}, CXCR5^{hi}) or effector/memory T_{reg} (CD103) phenotype and to exert T_{eff} function (germinal center [GC] formation, anti-GPI IgG1 production, and arthritis development). Highly purified Foxp3.gfp$^-$ KRNtg T_{effs} or Foxp3.gfp$^+$ KRNtg T_{regs} from Foxp3.gfp KRNtg mice were transferred into BxN.45.1 or CD28$^{-/-}$.Ag7$^{+/-}$ recipients. CD103 was almost exclusively upregulated on transferred Foxp3.gfp$^+$ KRNtg T_{regs} (Fig. 3A, 3B). In contrast, only transferred Foxp3.gfp$^-$ KRNtg T_{effs} displayed significant downregulation of CD62L and PSGL1, and higher levels of the T_{FH} markers CXCR5 and PD1 (Fig. 3A, 3B). That CXCR5 and PD1 were also expressed on some Foxp3.gfp$^+$ KRNtg T_{regs} is in accordance with the recent description of a subset of follicular T_{regs} sharing T_{FH} phenotypic characteristics (28). Apart from CXCR5 and PD1 expression, T_{regs} or T_{effs} displayed a comparable phenotype when transferred into CD28$^{-/-}$.Ag7$^{+/-}$ or BxN.45.1 mice (Fig. 3A, 3B). Generally, in CD28$^{-/-}$.Ag7$^{+/-}$ mice, a greater proportion of transferred T_{effs} adopted a CXCR5^{hi}PD1^{hi} T_{FH} phenotype (Fig. 3A, 3B). Adoptively transferred Foxp3.gfp$^+$ KRNtg T_{regs} did not induce an autoaggressive B cell response (GL7^{hi}Fas^{hi} GC B cells and IgG1 class-switched B cells; Fig. 3C), anti-GPI IgG1, or the development of arthritis (Fig. 3D). Collectively, our data show that a small subset of cells may have converted from T_{regs} to T_{effs} in arthritis but did not induce an autoaggressive B cell response. The proportion of converted cells among transferred T_{regs}, if conversion did indeed occur, is likely to be too small to induce disease.

T_{reg} conversion is likely an artifact

We next assessed whether the observed conversion of small numbers of T_{regs} into T_{effs} may have been, as hypothesized recently (24), simply an artifact due to the outgrowth of a small number of contaminating non-T_{regs}. This seems feasible because it is impossible to sort GFP$^+$ CD4$^+$ T cells to a purity of 100%. Thus, we adoptively transferred highly purified CD45.1.2$^+$ Foxp3.gfp$^-$ KRNtg T_{effs} together with CD45.2$^+$ Foxp3.gfp$^+$ KRNtg T_{regs} into CD45.1$^+$ BxN.45.1 recipients at a ratio of 4:1 (CD45.1.2$^+$GFP$^-$: CD45.2$^+$GFP$^+$). Replicate groups of mice received ~99% pure

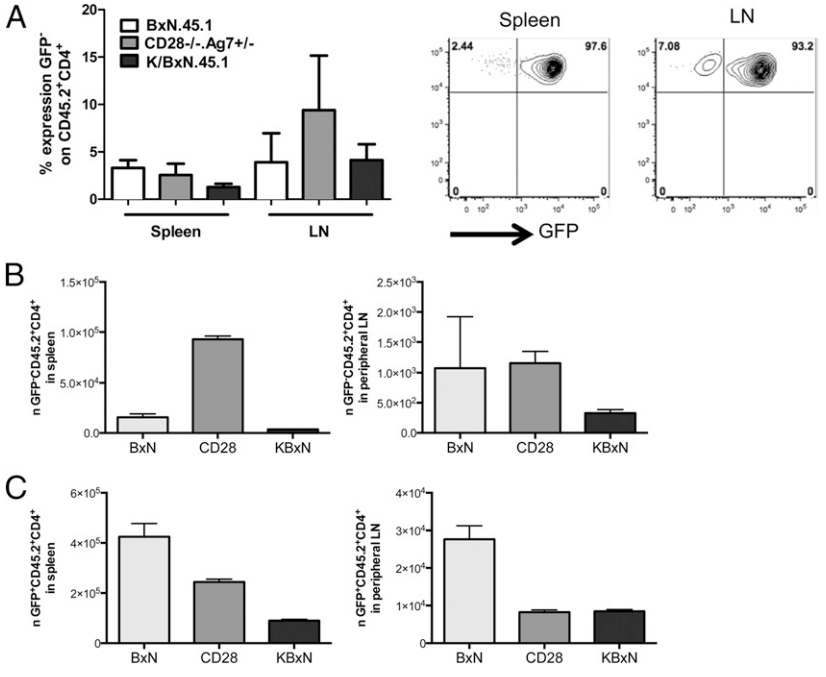

FIGURE 2. Most T_{regs} display a stable phenotype. (**A**–**C**) Foxp3.gfp$^+$ KRNtg CD4$^+$ T_{regs} were FACS purified (purity >98%) from Foxp3.gfp KRNtg mice, and 0.4 × 10^6 cells were transferred into BxN.45.1, CD28$^{-/-}$.Ag7$^{+/-}$, or K/BxN.45.1 mice. After 14 d, GFP expression was examined on transferred CD4$^+$ CD45.2$^+$ cells by FACS in spleen and LN (n = 3–4 recipient mice per group per experiment). Displayed are (A) the percentage of GFP$^-$ cells of transferred CD45.2$^+$CD4$^+$ cells, (B) the number of recovered GFP$^-$CD45.2$^+$CD4$^+$ cells per lymphoid organ(s), and (C) the number of recovered GFP$^+$CD45.2$^+$CD4$^+$ cells per lymphoid organ(s).

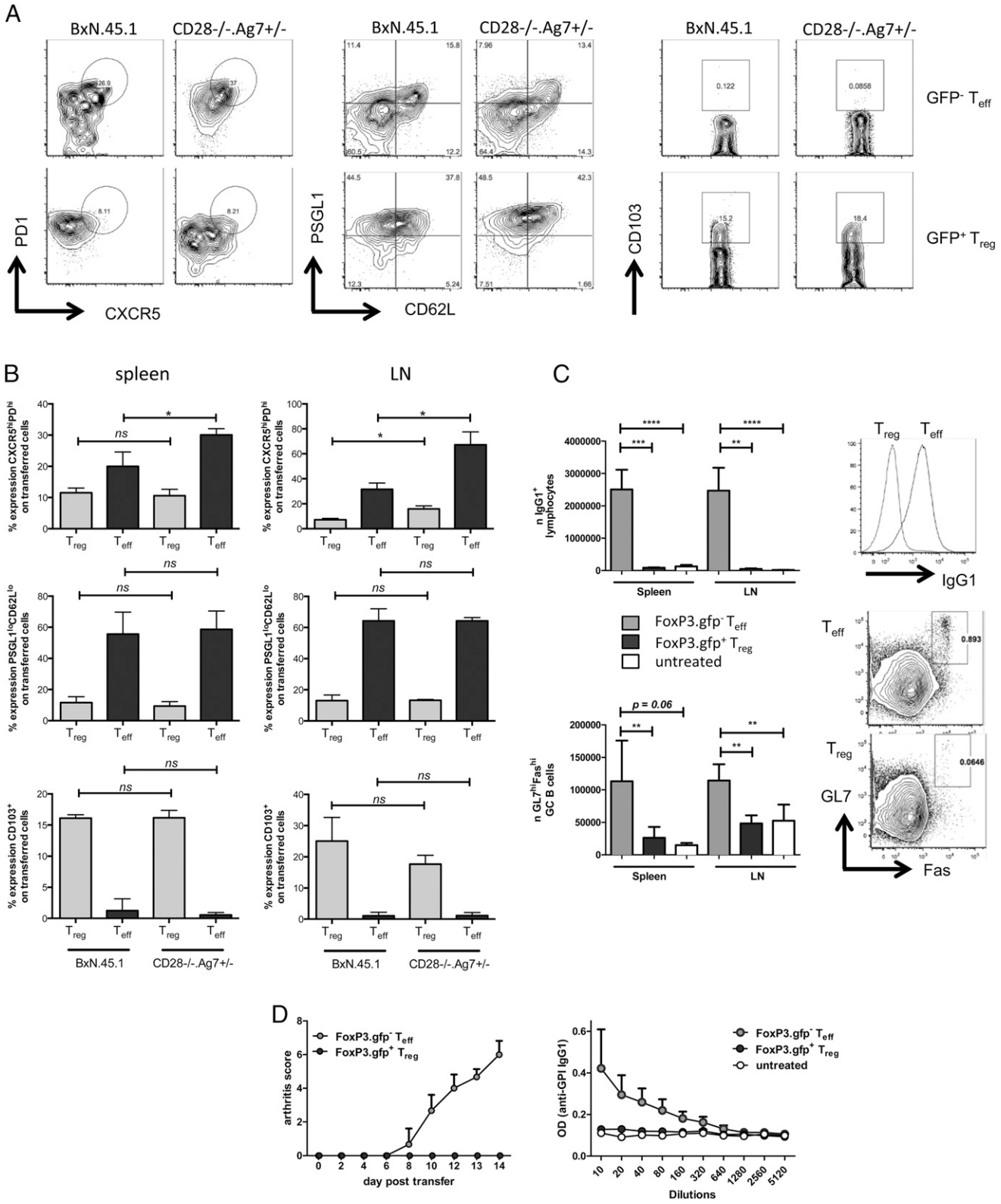

FIGURE 3. T_{regs} neither convert into T_{effs} nor exert T_{eff} function. (**A**–**D**) Foxp3.gfp$^+$ T_{regs} and Foxp3.gfp$^-$ T_{effs} were FACS purified (purity >98%) from lymphoid organs of Foxp3.gfp KRNtg mice and 0.4×10^6 cells transferred into BxN.45.1 or CD28$^{-/-}$.Ag7$^{+/-}$ recipient mice. (A and B) After 14 d, the expression of CD103, CD62L, PSGL1, CXCR5, and PD1 was determined on transferred CD45.2$^+$CD4$^+$ cells in spleen and LN. Displayed are FACS plots from transferred cells recovered from spleen (A) and graphs (B) with the percentage of CXCR5^{hi}PD1^{hi}, PSGL1^{lo}CD62L^{lo}, and CD103$^+$ expression on transferred CD45.2$^+$CD4$^+$ cells in spleen and LN. Also determined were (C) the frequencies of IgG1 switched B cells and GL7^{hi}Fas^{hi} GC B cells by FACS analysis in spleen and LN of BxN.45.1 mice that received either 0.4×10^6 purified T_{regs} or T_{effs} and (D) arthritis scores measured for 14 d after transfer, as well as serum titers of anti-GPI IgG1 determined using ELISA (n = 3–5 recipient mice/group). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

$CD45.2^+$ $Foxp3.gfp^+$ KRNtg or ~94% pure $CD45.2^+$ $Foxp3.gfp^+$ KRNtg cells. Four days after transfer, T_{effs} had proliferated to a greater extent than T_{regs} (2.49 and 4.33 times more in spleen and LN, respectively) as determined by the ratio of $CD45.1.2^+$ $Foxp3.gfp^-$ KRNtg T_{effs}:$CD45.2^+$ $Foxp3.gfp^+$ KRNtg T_{regs}, compared with day 0 (Table I and Supplemental Fig. 1A, 1B). Mathematically the increased proliferation rate of T_{effs} making up ~1 or 6% of $CD45.2^+$ $Foxp3.gfp^+$ KRNtg T_{regs} would translate into a frequency of ~2.5 or ~15% $CD45.2^+$ GFP^- cells in spleen and ~4.3 or ~26% $CD45.2^+$ GFP^- cells in LN. These values were close to the actual percentage of $CD45.2^+$ GFP^- cells determined in spleen (~4.5 ± 0.36 or ~16.1 ± 2.28%) and LN (~5.8 ± 0.91 or ~29.1 ± 3.46%) by FACS. This shows that a small population of outgrowing non-T_{regs} can proliferate and be misinterpreted as unstable and converted T_{regs}. Thus, these results suggest that the contamination of GFP^- cells could account for the observed unstable phenotype of T_{regs}. Conversion of T_{regs} into T_{effs} is not supported by our data and does not explain why numerically expanded T_{regs} are unable to prevent the development of arthritis.

T_{eff} (T_{FH} and Th17) differentiation and proliferation precedes T_{reg} expansion

Although there is an increase in T_{regs} in arthritic 8-wk-old K/BxN mice (Fig. 1A), it is possible that a T_{reg}/T_{eff} imbalance resulting in a numerical T_{eff} superiority may occur at presymptomatic stages and induce the development of disease. Thus, we examined the induction of T_{regs} ($CD4^+Foxp3^+$ cells) and T_{effs} ($CCR6^+IL\text{-}17^+$ Th17 cells and $CXCR5^{hi}$ $PD1^{hi}$ T_{FH}) during different stages of disease development in arthritic K/BxN mice.

T_{regs} and T_{effs} (T_{FH} and Th17 cells) were assessed: (*i*) when they arrive in the periphery at 17 d of age and as clonal depletion becomes incomplete; (*ii*) in prearthritic mice at 22 d and when peripheral $CD4^+$ T cells in spleen and LN appear in significant numbers; (*iii*) during acute arthritis and robust inflammation at 5 wk; (*iv*) through transition from acute into chronic arthritis at 8 wk; and (*v*) during chronic arthritis at 12 wk of age (20). T_{reg} frequencies remained the same (spleen) or were reduced (LN) at day 17 during disease onset, and increases at both sites only occurred from day 22 (Fig. 4A, 4B, and Supplemental Fig. 2A, 2B). From then T_{reg} frequencies plateaued. In line with T_{reg} frequencies, CD103 upregulation on $Foxp3^+$ $CD4^+$ T also occurred from day 22 (Fig. 4C). In contrast, T_{effs} (Th17 and T_{FH} cells) were markedly increased at both 17 and 22 d in spleen and LN, and then plateaued from 5 wk of age compared with healthy BxN mice (Fig. 4A, 4B, and Supplemental Fig. 2A, 2B). These results show that at early preclinical disease stages, altered T_{reg}/T_{eff} ratios result in T_{eff} superiority.

We next assessed whether delayed T_{reg} compared with T_{eff} expansion would also occur in adoptively transferred KRNtg $CD4^+$ T cells and how the proliferation of T_{effs} is influenced by a lymphopenic environment. To do this, we injected naive KRNtg $CD4^+$ T cells containing ~10% naive $Foxp3^+$ T_{regs} into lymphoreplete BxN.45.1 (Fig. 4D, 4E, and Supplemental Fig. 2C) or quasi-lymphopenic $CD28^{-/-}.Ag7^{+/-}$ mice (Fig. 4F and Supplemental Fig. 2D), and examined T_{eff} (T_{FH} and Th17 cells) and T_{reg} differentiation at 4, 8, and 14 d after transfer. Th17 differentiation was barely detectable in adoptively transferred cells (data not shown); therefore, we focused on T_{FH} differentiation ($CXCR5^{hi}PD1^{hi}$). As in K/BxN mice, increases in T_{regs} and $CD103^+$ T_{regs} were not observed until later (day 8) in BxN.45.1 mice, whereas T_{FHs} increased early (day 4; Fig. 4D, 4E, and Supplemental Fig. 2C). The discrepancy between T_{reg} and T_{FH} expansion was even more dramatic in quasi-lymphopenic $CD28^{-/-}.Ag7^{+/-}$ mice (Fig. 4F, Supplemental Fig. 2D), which may partly account for the observed more severe disease course (Supplemental Fig. 2E). Contrary to truly lymphopenic mice ($RAG^{-/-}$, $CD3e^{-/-}$ mice), $CD28^{-/-}$ mice have normal lymphoid cellularity and tissue organization. Nevertheless, it is likely that transferred TCR-responsive $CD4^+$ T cells behave in a similar way when transferred into quasi-lymphopenic $CD28^{-/-}$ mice or truly lymphopenic animals. Lack of competition by TCR-unresponsive endogenous $CD4^+$ T cells, reduced IL-2 levels, and a general T_{reg} defect (29, 30) may account for a generally more vigorous expansion of transferred $Foxp3^-$ T_{effs} over T_{regs} in $CD28^{-/-}$ mice.

Thus, decreased T_{reg} compared with T_{eff} (T_{FH} and Th17 cells) expansion was observed during early disease stages in K/BxN mice, as well as in adoptive transfer experiments, and was exaggerated under quasi-lymphopenic conditions. We propose that an increased autoreactive T_{eff} to T_{reg} expansion during early disease results in the uncontrolled outgrowth and activity of T_{effs} that dominate T_{regs}, and is sufficient to tip the balance of control and induce autoimmunity. The delayed increase of T_{regs} may then only suppress, but no longer contain, disease progression.

Arthritic inflammation attenuates T_{eff} and T_{reg} proliferation with T_{regs} affected to a greater extent

Although T_{reg} expansion was delayed at early disease stages compared with T_{effs}, the frequencies of both cell types were still substantially increased in secondary lymphoid organs of arthritic K/BxN compared with nondiseased BxN mice (Fig. 1A). Thus, we next examined whether and how the inflammatory milieu might influence T_{eff} and T_{reg} homeostasis. $CD4^+$ T cells were isolated from KRNtg mice, CFSE-labeled, and adoptively transferred into naive BxN.45.1 or arthritic (i.e., inflamed) 6-wk-old K/BxN.45.1 recipient mice, and proliferation was assessed 60 h later. The proliferation of both T_{effs} and T_{regs} was substantially reduced on transfer into arthritic K/BxN mice (Fig. 5A–C). However, T_{regs} were more affected than T_{effs} (Fig. 5A, 5B, 5D).

Table I. T_{reg} conversion is likely an artifact

	$CD45.1.2^+$ T_{eff}: $CD45.2^+$ T_{reg}[a]			% $GFP^-/CD45.2^+$[b]			
				≈99% Pure $CD45.2^+GFP^+$		≈94% Pure $CD45.2^+GFP^+$	
	Ratio: Day 0	Ratio: Day 4	Proliferation Increase: Day 0 versus Day 4	Calculation	Actual (Mean/SD)	Calculation	Actual (<ean/SD)
Spleen	4.1	10.24027	2.497627	2.497627	4.565 (±0.3680014)	14.985762	16.175 (±2.281858)
LN	4.1	17.77311	4.334905	4.334905	5.855 (±0.9178916)	26.00943	29.175 (±3.462207)

[a] $CD45.1.2^+$ $Foxp3.gfp^-$ KRNtg T_{eff} and $CD45.2^+$ $Foxp3.gfp^+$ KRNtg T_{regs} were mixed at a ratio of 4:1 ($CD45.1.2^+T_{eff}/CD45.2^+T_{reg}$) (day 0) and transferred into BxN.45.1 recipients. The ratio of $CD45.1.2^+T_{eff}/CD45.2^+T_{reg}$ was determined at day 4. Comparison of T_{eff}/T_{reg} ratio at day 4 to day 0 shows that T_{regs} had proliferated to a lesser extent than T_{effs} (2.49 and 4.33 times less in spleen and peripheral LN, respectively).

[b] One group of mice had received ~99% pure $CD45.2^+$ T_{regs}, another group ~94% pure $CD45.2^+$ T_{regs} in the following mixed adoptive transfer experiments. Considering the increased proliferation rate of T_{eff} (2.49 in spleen and 4.33 in LN), a contamination of $CD45.2^+$ T_{regs} with ~1 or ~6% T_{eff} translates into a calculated frequency of ~2.5 or ~15% $CD45.2^+$ GFP^- cells in spleen and ≈4.3 or ≈26% $CD45.2^+$ GFP^- cells in LN at day 4. Indicated are actual percentages of $CD45.2^+$ GFP^- cells determined in spleen (~4.5 ± 0.36 or ~16.1 ± 2.28%) and LN (~5.8 ± 0.91 or ~29.1 ± 3.46%) at day 4. Data are means ± SD. Data are representative of two independent experiments (n = 4 mice/group).

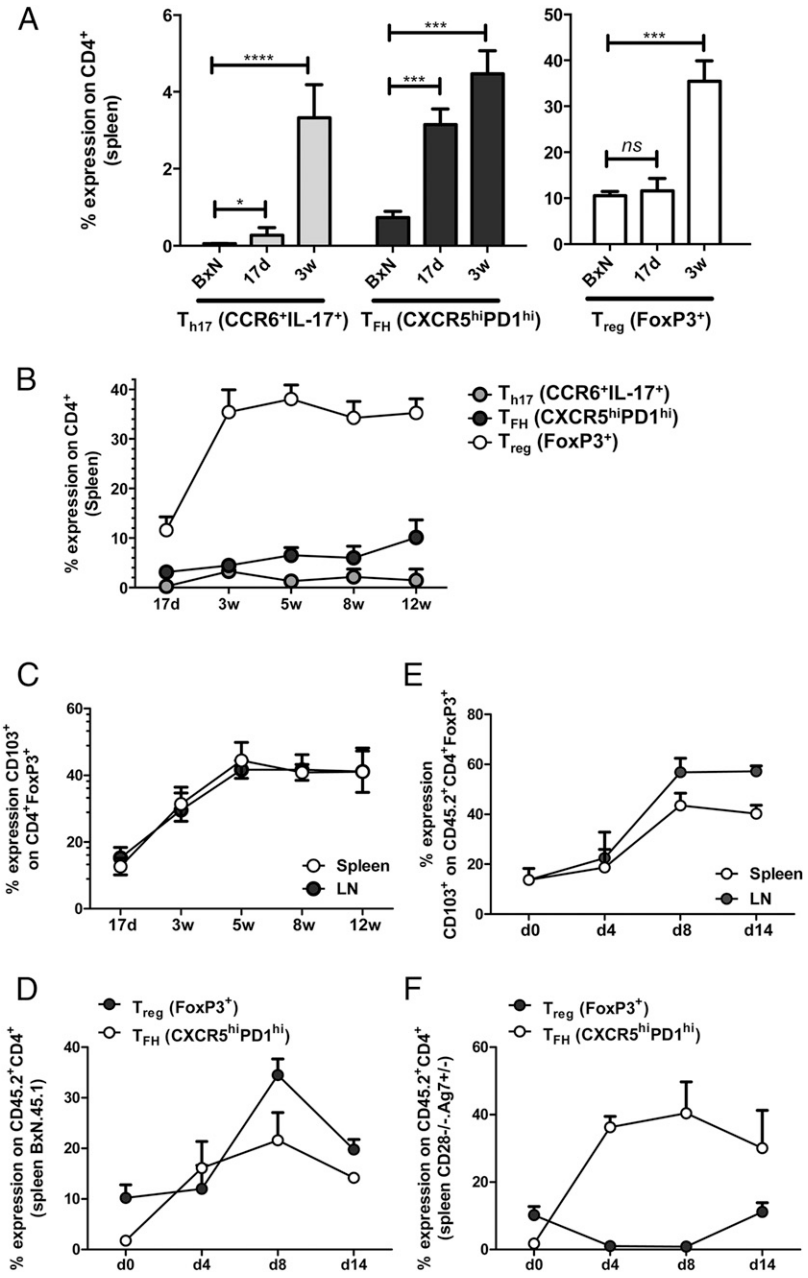

FIGURE 4. T_{regs} proliferate and expand less rapidly than T_{effs} (T_{FH} and T_{h17} cells). (**A**) FACS analysis of the percentages of $CCR6^+IL\text{-}17^+$, $CXCR5^{hi}PD1^{hi}$ and $Foxp3^+$ expression on $CD4^+$ T cells in spleens of 17d and 22d old arthritic K/BxN mice compared with naive 8w old BxN mice ($n \geq 5$ mice per group). (**B** and **C**) FACS analysis of the percentages of $CCR6^+IL\text{-}17^+$, $CXCR5^{hi}PD1^{hi}$ and $Foxp3^+$ expression on $CD4^+$ T cells (B), and of $CD103^+$ on $Foxp3^+$ $CD4^+$ T cells (C) in spleens and LN of 17d, 22d, 5w, 8w and 12w old arthritic K/BxN mice ($n \geq 5$ mice per group). (**D**–**F**) KRNtg $CD4^+$ cells were isolated from lymphoid organs of KRNtg mice (purity >98%) and 1.5×10^6 KRNtg $CD4^+$ T cells were adoptively transferred into BxN.45.1 mice (D and E) or 0.75×10^6 KRNtg $CD4^+$ T cells into $CD28^{-/-}.Ag7^{+/-}$ recipients (F) (n = 4–5 recipient mice per group). Expression of $Foxp3^+$ and $CXCR5^{hi}PD1^{hi}$ on transferred $CD45.2^+$ T cells (D and F), and of $CD103^+$ on transferred $CD45.2^+$ $Foxp3^+$ T cells (E) were determined in spleens and LN at 4, 8, and 14 d after transfer. Phenotypes of naive KRNtg $CD4^+$ T cells before transfer (day 0) are also displayed. $^*p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

IL-2 augmentation supports T_{reg} proliferation in the arthritic inflammatory milieu, and neutralization of IL-2 results in more severe disease

We then assessed the role of IL-2 within the inflamed milieu, because this cytokine is particularly required for T_{reg} homeostasis in the periphery (31). Its availability may be decreased during inflammation and lymphopenia, which would account for the reduced T_{reg}/T_{eff} proliferation and particularly the inhibition of T_{reg} expansion in inflamed tissues. We first examined IL-2 expression by splenocytes of arthritic K/BxN compared with healthy BxN mice (Fig. 6A). IL-2 levels displayed a trend toward decreased expression by arthritic K/BxN $CD4^+$ T cells as examined by FACS and quantitative PCR on sorted $CD4^+$ T cells (Fig. 6A). We were unable to detect IL-2 in serum or plasma of arthritic K/BxN or nonarthritic BxN mice by ELISA. In view of the lymphopenia (Fig. 6B) and reduced IL-2 expression by $CD4^+$ T cells in arthritic K/BxN mice (Fig. 6A), an IL-2 deficit appears, however, to be conceivable. To further assess the effects of IL-2 on early T_{eff} and T_{reg} proliferation, we transferred CFSE-labeled KRNtg $CD4^+$ T cells into arthritic K/BxN.45.1 mice treated with 2 μg rmIL-2 at −12, 0, 12, 24, and 36 h. Treatment of recipient mice markedly increased the proliferation of T_{regs} and, to a lesser extent, T_{effs} (Fig. 5A–C). We next assessed whether IL-2 availability could also influence the disease course. KRNtg $CD4^+$ T cells were adoptively transferred into nonarthritic BxN.45.1 recipients that were treated with 1 mg neutralizing anti–IL-2 Abs at day 0 (clone S4B6.1). In accordance with the favorable effects of IL-2 on T_{reg} proliferation, neutralization of IL-2 resulted in more severe disease (Fig. 6C). Unfortunately, we were unable to directly mitigate disease courses in K/BxN mice by injection of rIL-2 (data not shown). This may be the result of a whole range of explanations, including suboptimal dose and route of administration, the IL-2 not reaching the correct sites or cells, or that disease in K/BxN mice was too strong to be reversed with this treatment. Additional studies will be required to explore these issues. Regardless, IL-2 promotes T_{reg} proliferation and the suppression of arthritis.

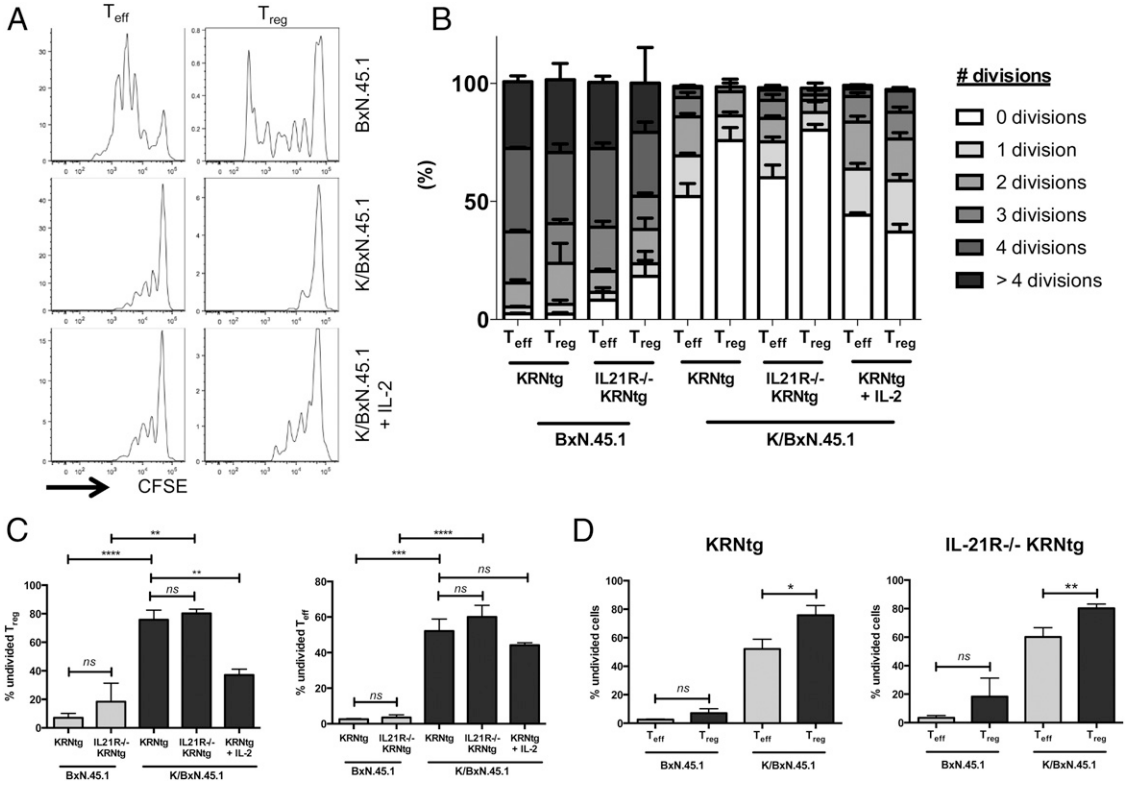

FIGURE 5. IL-2 and IL-21 modulate T_{reg}/T_{eff} homeostasis in arthritic K/BxN mice. (**A**–**C**) KRNtg or IL-21R$^{-/-}$ KRNtg CD4$^+$ cells were isolated from lymphoid organs of KRNtg or IL21-R$^{-/-}$ KRNtg mice (purity >98%). Adoptive transfer of 1.5×10^6 CFSE-labeled KRNtg CD4$^+$ T cells or IL-21R$^{-/-}$ KRNtg CD4$^+$ T cells into BxN.45.1 or K/BxN.45.1 recipient mice (n = 3–5 recipient mice per group). Where indicated, recipient mice received 2 μg rmIL-2 at −12, 0, 12, 24, and 36 h. Proliferation of transferred CD45.2$^+$CD4$^+$ cells was determined 60 h after transfer by FACS. Shown are (A) representative FACS plots of KRNtg CD4$^+$ T cells transferred into BxN.45.1 and in K/BxN.45.1 mice with and without IL-2 treatment; (B) the percentages of cells undergoing 0, 1, 2, 3, 4, or >4 cycles of proliferation; (C) percentages of undivided Foxp3$^+$CD45.2$^+$CD4$^+$ T_{regs} or Foxp3$^-$CD45.2$^+$CD4$^+$ T_{effs} after transfer into the different recipients (BxN.45.1 and K/BxN.45.1 mice ± IL-2 treatment); and (**D**) comparison of the percentage of undivided Foxp3$^+$CD45.2$^+$CD4$^+$ T_{regs} to the percentage of undivided Foxp3$^-$CD45.2$^+$CD4$^+$ T_{effs} after transfer into BxN.45.1 and K/BxN.45.1 mice. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

However, its levels are reduced in arthritis and, therefore, other pathways presumably account for the increased numbers of T_{regs} in this disease model.

Increased IL-21 dysregulates T_{reg}/T_{eff} homeostasis in arthritic K/BxN mice by supporting T_{eff} expansion

In contrast with IL-2, IL-21 levels may be increased because of the infiltration of the main IL-21 producers, T_{FH}, into inflamed organs of K/BxN mice (Fig. 4A, 4B) (32). This cytokine is important for T_{eff} homeostasis in K/BxN mice (19), and it has inhibitory effects on T_{reg} function (33–37). Thus, the role of IL-21 in T_{eff} and T_{reg} homeostasis in arthritis was investigated. We found that IL-21 expression by splenocytes of arthritic K/BxN mice was increased compared with healthy BxN mice as examined by FACS and quantitative PCR on sorted CD4$^+$ T cells (Fig. 6D). To further assess the effects of IL-21, we adoptively transferred CFSE-labeled IL-21R$^{-/-}$ Foxp3$^+$ and Foxp3$^-$ KRNtg CD4$^+$ T cells into nonarthritic BxN.45.1 and arthritic K/BxN.45.1 mice, and the proliferation of both transferred cell types was examined 60 h later (Fig. 5A–C). IL-21R deficiency had minimal effects on the proliferation of both transferred Foxp3$^-$ T_{effs} and Foxp3$^+$ T_{regs} (Fig. 5A–C). We then assessed whether IL-21 could modulate the maintenance of T_{effs} or T_{regs}, rather than early proliferation. T_{effs} and T_{regs} were sorted from CD45.2$^+$ IL-21R$^{-/-}$ KRNtg and CD45.1.2$^+$ KRNtg mice as CD25$^-$CD4$^+$ T_{effs} and CD25^{hi}CD4$^+$ T_{regs}, respectively, because these lines were not backcrossed on Foxp3.gfp reporter mice. We then transferred KRNtg T_{effs} and IL-21R$^{-/-}$ KRNtg T_{regs}, KRNtg T_{effs} and KRNtg T_{regs}, or IL-21-R$^{-/-}$ KRNtg T_{effs} and KRNtg T_{regs} (1:10 T_{reg}/T_{eff} ratio) into BxN.45.1 mice. After 14 d posttransfer there was a marked increase in the T_{reg}/T_{eff} ratio among transferred cells in mice that had received IL-21R$^{-/-}$ T_{effs} compared with other groups (Fig. 6E). In accordance with this, these animals also displayed minimal disease (Fig. 6E). That IL-21 influences disease progression by modulation of T_{reg} and T_{eff} homeostasis was further supported by the observation that injection of IL-21 into BxN.45.1 mice transferred with KRNtg CD4$^+$ T cells triggered disease. This was accompanied by increases in the percentage of recovered Foxp3$^-$ T_{effs} at the expense of Foxp3$^+$ T_{regs} (Fig. 6F).

Thus, we observed that the opposing regulation of IL-2 and IL-21 within inflamed tissue combines to impair T_{reg} proliferation and increase T_{eff} maintenance, which promoted arthritis.

PD1–PD-L1 axis does not play a major role in T_{eff} or T_{reg} expansion

Because IL-2 does not fully reconstitute hampered T_{reg}/T_{eff} proliferation and IL-21R deficiency only slightly affected the early proliferation of T_{effs} and T_{regs}, other factors within the inflammatory milieu may influence their expansion. Recently, PD1 and PD-1 ligand (PD-L1) have been described as negative regulators of T_{reg} function (38). In chronically infected hepatitis C virus patients, T_{regs} expressed higher levels of PD1 than T_{effs}; moreover, PD-L1 levels were increased at inflammatory sites. This inhibited T_{reg} expansion to a greater extent than T_{eff} ex-

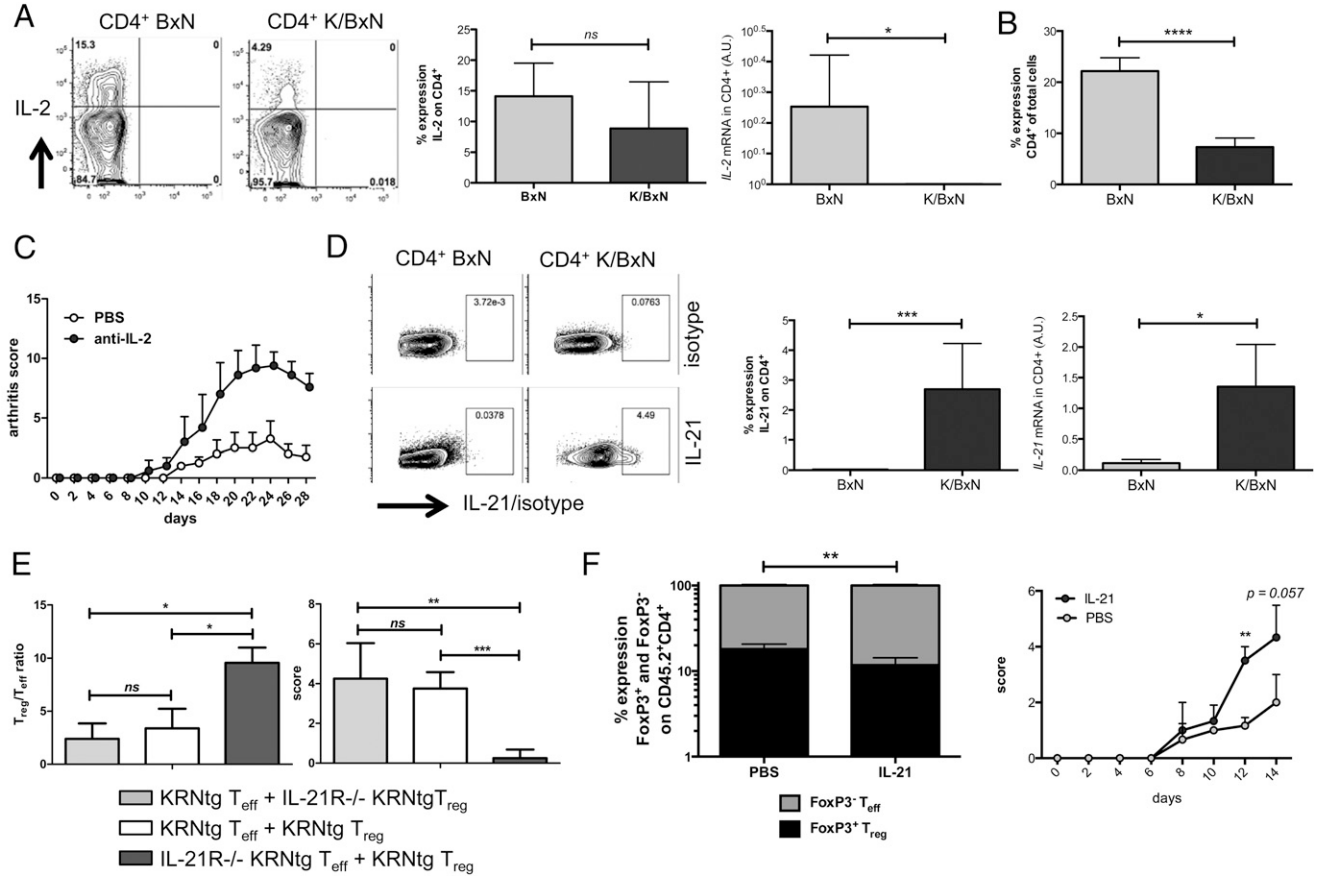

FIGURE 6. IL-2 and IL-21 modulate T_{reg}/T_{eff} homeostasis in arthritic K/BxN mice. (**A**) Expression of IL-2 on/in $CD4^+$ T cells in spleens of 8-wk-old healthy BxN and arthritic K/BxN mice ($n = 5$–6 mice/group) determined by FACS and quantitative PCR. (**B**) Percentage of $CD4^+$ T cells among total cells in spleen of 8-wk-old healthy BxN and arthritic K/BxN ($n > 5$ mice/group) determined by FACS. (**C**) KRNtg $CD4^+$ cells were isolated from lymphoid organs of KRNtg mice (purity >98%), and 1.5×10^6 KRNtg $CD4^+$ T cells were adoptively transferred into BxN.45.1 recipient mice ($n = 5$ recipient mice), treated with 1 mg neutralizing anti–IL-2 Ab (clone S4B6.1) or PBS at day 0. Arthritis scores were measured for 28 d after transfer. (**D**) Expression of IL-21 on/in $CD4^+$ T cells in spleen of 8-wk-old healthy BxN and arthritic K/BxN mice ($n = 5$–6 mice per group) determined by FACS and quantitative PCR. (**E**) T_{effs} and T_{regs} were isolated from lymphoid organs of CD45.2+ IL-21R$^{-/-}$ KRNtg and CD45.1.2+ KRNtg mice, and sorted as $CD25^{hi}CD4^+$ T_{regs} and $CD25^-CD4^+$ T_{effs}, respectively (purity >98%). KRNtg T_{eff} plus IL-21R$^{-/-}$ KRNtg T_{reg}, KRNtg T_{eff} plus KRNtg T_{reg}, and IL-21R$^{-/-}$ KRNtg T_{eff} plus KRNtg T_{reg} were mixed at a ratio of 1:10, and 1×10^6 cells were adoptively transferred into BxN.45.1 mice ($n = 4$ recipients/group). After 14 d post-transfer, the ratios of $Foxp3^+/Foxp3^-$ among transferred $CD4^+$ T cells and arthritis scores were determined. (**F**) KRNtg $CD4^+$ cells were isolated from lymphoid organs of KRNtg mice (purity >98%), and 1.5×10^6 KRNtg $CD4^+$ T cells were adoptively transferred into BxN.45.1 recipient mice ($n = 6$ recipient mice/group). Recipient mice were injected i.p. with PBS or 20 μg rmIL-21 every 2 d after adoptive cell transfer. At day 14 the percentage of $Foxp3^+$ T_{regs} and $Foxp3^-$ T_{effs} was determined on transferred $CD4^+CD45.2^+$ KRNtg cells; depicted is the difference between PBS- and IL-21–treated animals for transferred $Foxp3^-$ T_{effs} and transferred $Foxp3^+$ T_{regs}. Arthritis scores were measured for 14 d after transfer. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

pansion and made them more susceptible to PD1-dependent exhaustion (38). We therefore assessed whether, in our model of autoimmune arthritis, enhanced PD1–PD-L1 signaling could play a similar role. PD1 expression was higher on T_{regs} than T_{effs} both in naive BxN and arthritic K/BxN mice (Supplemental Fig. 3A). $CD11b^+$ macrophages/monocytes and $CD11c^+$ DCs, in arthritic K/BxN mice, as well as $CD19^+$ B cells were largely expanded (Supplemental Fig. 3B). DCs and B cells displayed a significant upregulation of PD-L1 expression (Supplemental Fig. 3C). We therefore propose that PD-L1 levels are generally increased within inflamed organs of arthritic mice and may contribute to the hampered expansion of T_{regs} because of their high PD1 expression. However, we were not able to improve T_{reg} proliferation in K/BxN recipients by treatment with neutralizing anti-PD1 or anti–PD-L1 Abs in vivo (Supplemental Fig. 3D). This does not fully exclude a role for PD1–PD-L1; however, it is likely to be minor relative to IL-2/IL-21 or other as yet unidentified factors.

Thymic natural T_{reg} production may drive increases in T_{regs} in inflamed organs in arthritic mice

The proliferation of T_{regs} was decreased in the inflammatory and lymphopenic environment of arthritic mice, and it remains unknown how the numbers of these cells are increased in this disease. We investigated whether enhanced thymic output followed by recruitment of T_{regs} to inflamed organs could account for the increase. Foxp3 expression was assessed on SP $CD8^-CD4^+$ thymocytes of 16-d-, 22-d-, and 8-wk-old arthritic K/BxN compared with naive BxN mice. In naive BxN mice, Foxp3 expression on SP $CD4^+$ thymocytes was comparable between young (16 and 22 d) and adult mice (8 w; Fig. 7A). In contrast, in arthritic mice, Foxp3 expression on SP $CD4^+$ thymocytes was significantly increased at 22 d and further increased at 8 wk (Fig. 7A). Hence T_{reg} frequencies in the thymus (Fig. 7A) correlate well with T_{reg} frequencies in lymphoid organs (Fig. 4A, 4B). Nrp-1 is a surface molecule that was recently identified as a reliable marker to distinguish natural T_{regs} (nT_{regs}) arising in thymus from inducible

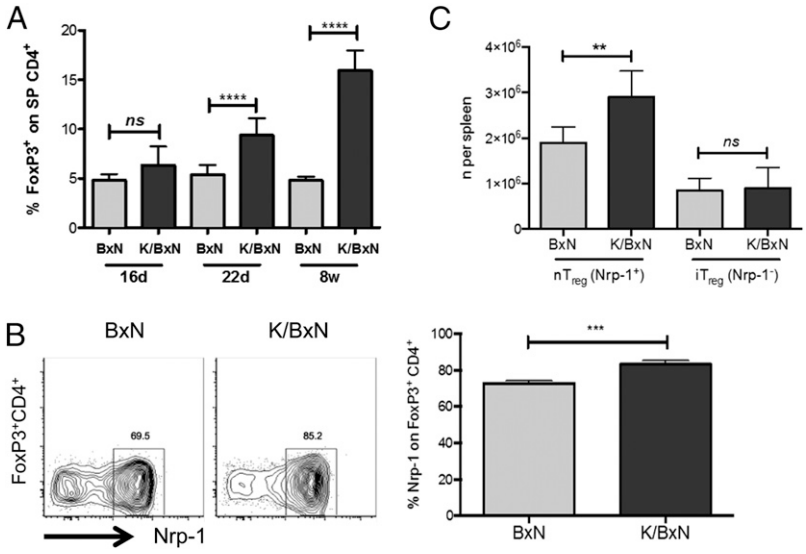

FIGURE 7. Thymic T_{reg} output may modulate T_{reg} numbers in inflamed organs of arthritic K/BxN mice. (**A**) FACS analysis of Foxp3 expression on SP $CD8^-$ $CD4^+$ thymocytes of 16-d-, 22-d-, and 8-wk-old K/BxN mice and $KRNtg^-$ littermates (BxN) ($n \geq 5$ mice per group). (**B** and **C**) FACS analysis of Nrp-1 expression on $Foxp3^+CD4^+$ T cells in spleen of 8-wk-old healthy BxN and arthritic K/BxN mice ($n \geq 5$ mice/group). Displayed are representative FACS plots plus graph showing the percentage of Nrp-1^+ on $Foxp3^+CD4^+$ T cells (B) and absolute numbers of Nrp-1^+Foxp3^+ $CD4^+$ nT_{regs} and Nrp-$1^-Foxp3^+CD4^+$ iT_{regs} per spleen of healthy BxN and arthritic K/BxN mice (C). $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

T_{regs} (iT_{regs}) generated in the periphery through the induction of Foxp3 (39). We found a higher percentage of Nrp-1–expressing T_{regs} in secondary lymphoid organs of arthritic K/BxN compared with healthy BxN animals (Fig. 7B). Moreover, the absolute cell number per lymphoid organ of Nrp-1^- iT_{regs} was comparable between arthritic K/BxN and healthy BxN mice, whereas Nrp-1^+ nT_{regs} were increased in arthritic animals (Fig. 7C). This supports our assertion that the increase in T_{regs} may be mainly because of an increased thymic T_{reg} output.

Thymic nT_{reg} production may be driven by IL-7 in arthritic K/BxN mice

We next investigated which factors may mediate increased thymic T_{reg} production. The common γ-chain cytokines IL-2, IL-7, and IL-15 are important for thymic T_{reg} homeostasis (31, 40–42). Increased NF-κB signaling in thymocytes may function as sensor of peripheral inflammation and increase the secretion of TNF-α, inducing the development of T_{regs} in the thymus (43). Thus, the mRNA expression of *IL-2*, *IL-7*, *IL-15*, and *TNFα* was determined in the thymi of 8-wk-old healthy BxN and arthritic K/BxN mice (Fig. 8A). To examine NF-κB signaling, extracts were isolated from thymi and assessed by immunoblot analysis (Fig. 8B). There were no differences in the expression of *IL-2*, *IL-15*, or *TNFα* or in the production of NF-κB between healthy BxN and arthritic K/BxN mice. However, there was an upregulation of *IL-7* in the thymi of arthritic mice (Fig. 8A, 8B). In accordance with this, $Foxp3^+$ SP $CD8^-CD4^+$ thymocytes in arthritic K/BxN mice expressed higher levels of IL-7R (CD127) compared with naive BxN mice (Fig. 8C).

These observations may point toward a potential role of IL-7 signaling in the regulation of thymic T_{reg} homeostasis (42). Because IL-7 signaling may be influenced by inflammatory cells or lymphopenia (44, 45), we compared 3-wk-old prearthritic and noninflamed animals with 8-wk-old arthritic mice. The arthritic 8-wk-old animals showed a substantially less pronounced lymphopenia in secondary lymphoid organs (Fig. 8D). They also exhibit a trend toward thymic upregulation of *IL-7* mRNA, and elevated CD127 levels (Fig. 8D) were detected on their SP $CD4^+CD8^-$ $Foxp3^+$ thymocytes (Fig. 8D). The fact that 8-wk-old animals display less severe lymphopenia but arthritic inflammation compared with 3-wk-old prearthritic mice suggests that inflammation rather than lymphopenia may drive those changes (Fig. 9).

To further examine possible effects of inflammatory signals, we injected nonarthritic BxN.45.1 animals with LPS and analyzed thymi after 12 h by FACS (Supplemental Fig. 4A) and after 4 h by quantitative PCR (Supplemental Fig. 4B). In support of our previous data, we detected increased CD127 expression on SP $Foxp3^+CD8^-CD4^+$ thymocytes (Supplemental Fig. 4A). Changes in thymic *IL-7* mRNA expression levels (Supplemental Fig. 4B) or increases in the percentage of $Foxp3^+$ among SP $CD4^+CD8^-$ thymocytes (Supplemental Fig. 4A) were not detectable. The increased CD127 expression on $CD4^+CD8^-Foxp3^+$ thymocytes further indicates a possible regulatory effect of inflammatory signals on thymic IL-7–mediated T_{reg} differentiation that is mediated by the receptor for this cytokine. That LPS treatment did not affect thymic IL-7 expression or increase the percentage of $Foxp3^+$ among SP $CD4^+CD8^-$ thymocytes may be because of a whole range of explanations such as the choice of time point, inflammatory stimulus, or the duration of inflammation, which needs to be thoroughly examined in future studies. Moreover, it will be interesting to address mechanistic details of thymic T_{reg} regulation, output, and migration to inflamed sites in future studies, as well as the influence of inflammatory stimuli and common γ-chain cytokines.

Discussion

In this article, we show that dysregulation of thymic and peripheral T_{reg} and T_{eff} homeostasis drives the induction and progression of autoimmunity in the KRN model of murine RA. We show that a short period of insufficient T_{reg} control of autoreactive T_{effs} (T_{FH} and Th17 cells) may allow the onset of autoimmunity that then becomes uncontrolled. The inflammatory milieu generated after disease initiation may then further deregulate T_{reg}/T_{eff} homeostasis and exacerbate the disease course. We identified the cytokines IL-2 and IL-21 as key players in disturbed peripheral T_{reg} and T_{eff} homeostasis. A lymphopenia-induced IL-2 deficit particularly affected T_{reg} expansion, and at the same time, increased IL-21 responses in the inflammatory milieu supported the maintenance of T_{effs}. Moreover, our data indicate that inflammation may mediate an enhanced IL-7–mediated thymic nT_{reg} output that could explain their increased frequencies at inflammatory sites. However, once they arrive in the periphery, T_{regs} fail to expand adequately, which limits their suppressive effects that are necessary to control expanding autoaggressive T cells (Fig. 9).

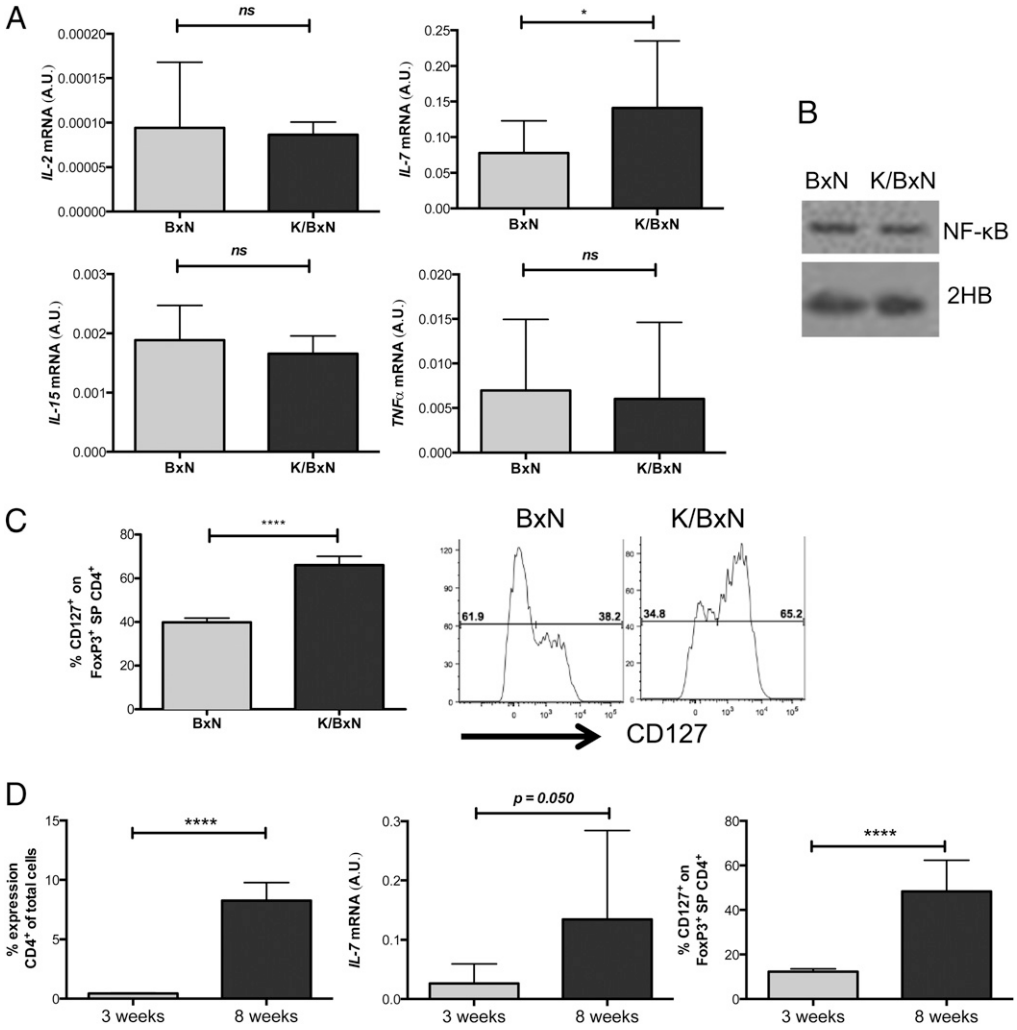

FIGURE 8. Thymic T_{reg} output may control T_{reg} numbers in inflamed organs of arthritic K/BxN mice. (**A**) The abundance of mRNA encoding for *IL-2*, *IL-7*, *IL-15*, and *TNFα* was determined by quantitative PCR in thymi of 8-wk-old BxN and K/BxN mice ($n \geq 5$ mice/group). (**B**) Immunoblot analysis of NF-κB expression in nuclear extracts of thymi from 8-wk-old BxN and K/BxN mice relative to anti-Histone 2B (2HB). Samples were run on the same gel but were noncontiguous ($n = 4$–5 mice/group). (**C**) FACS analysis of the expression of CD127 on Foxp3$^+$ SP CD8$^-$CD4$^+$ thymocytes of 8-wk-old BxN and K/BxN mice ($n \geq 5$ mice/group). (**D**) Percentage of CD4$^+$ T cells in spleen as determined by FACS, *IL-7* mRNA expression in thymi as determined by quantitative PCR, and percentage of CD127$^+$ on SP CD8$^-$CD4$^+$ thymocytes as determined by FACS in 3-wk-old prearthritic and 8-wk-old arthritic K/BxN mice. *$p < 0.05$, ****$p < 0.0001$.

Our study highlights the importance of IL-2 and IL-21 as modulators of peripheral T_{reg} and T_{eff} homeostasis, and clearly demonstrates how opposing IL-2/IL-21 regulation promotes autoimmunity. We found decreased IL-2 and increased IL-21 responses in arthritic CD4$^+$ T cells of K/BxN compared with healthy BxN mice. IL-2 deficiency particularly affected T_{reg} proliferation because IL-2 administration triggered T_{reg} expansion, whereas IL-2 neutralization promoted more severe disease. Hence our data support the concept that IL-2 is particularly required for the homeostasis and survival of T_{regs} in the periphery (46). Accordingly, another study showed that T_{regs} are the first to respond to IL-2, and IL-2 signaling particularly favored T_{reg} activity rather than promoting T_{eff} expansion (47).

Like IL-2, IL-21 is primarily produced by activated CD4$^+$ T cells, but its activity opposes the function of IL-2 (32). In terms of T_{regs}, IL-21 has been postulated to counteract T_{reg} suppression (33, 34), inhibit their de novo production (35, 36, 48), and negatively regulate T_{reg} homeostasis through a feedback loop where it reduces IL-2 production from T_{effs} (37). In addition, IL-21 has been shown to promote T_{eff} activity (37). We did not detect any inhibitory effects of IL-21 signaling on T_{reg} homeostasis. Also, IL-21R deficiency did not affect the early proliferation of T_{effs}; instead, it did reduce late T_{eff} expansion and mitigated disease. Accordingly, IL-21 administration triggered arthritis and increased the ratio of T_{effs} to T_{regs} among transferred autoaggressive cells. Mechanistically it is possible that the inductive effects on T_{eff} homeostasis are due to the fact that IL-21 may confer survival signals to T_{effs} or promote their increased resistance to T_{reg}-mediated suppression (33, 34), as reported in animal (13, 33, 34, 49) and human (50, 51) studies of autoimmunity. Data from in vitro suppression assays that the functionality of arthritic T_{regs} was equal (our studies) or increased (13) compared with naive T_{regs} may argue against direct inhibitory effects of IL-21 on the function of these T_{regs}. It seems most likely that the autocrine secretion of IL-21 enables activated CD4$^+$ T cells to expand and eventually overwhelm T_{reg} suppression. Given the frequent involvement of IL-21 in autoimmunity (32) and its predominant secretion by infiltrating Th17 and T_{FH} cells (35, 36, 52), such a scenario may be a general phenomenon that contributes to the pathogenesis of autoimmunity.

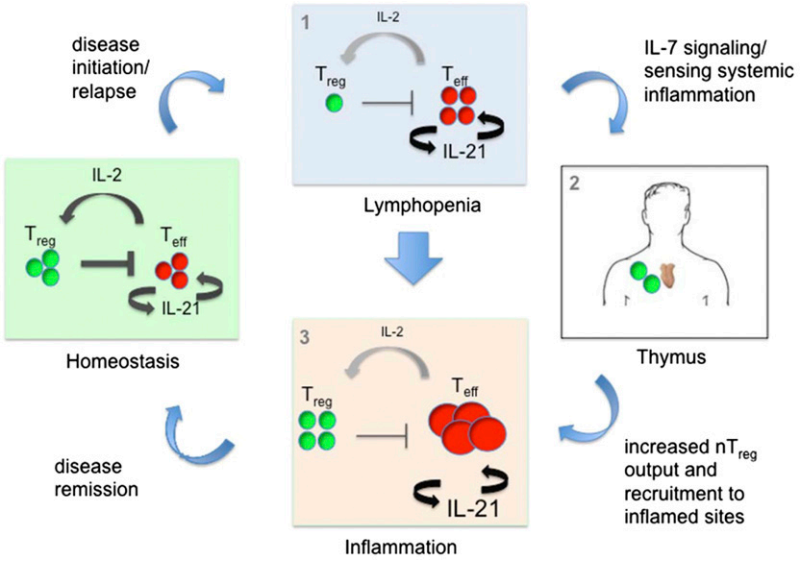

FIGURE 9. Potential model. Inflammation and lymphopenia modulate T_{reg}/T_{eff} homeostasis and drive autoimmunity by reciprocal regulation of IL-2 and IL-21 (1). Increased proliferation of T_{effs} may be caused by a lymphopenia-induced IL-2 deficit that mainly affects the T_{reg} compartment. This may result in a period of unopposed T_{eff} activity sufficient to induce disease. The resulting inflammatory milieu may sustain the IL-2 deficit (3). In addition, increased IL-21 levels may support T_{eff} expansion in an autocrine manner, by conferring survival signals or increasing T_{eff} resistance toward T_{reg}-mediated suppression (3). Subsequently, increased T_{reg} frequencies at sites of inflammation may be because of increased IL-7–triggered thymic output (2) and recruitment to inflamed tissue, where they suppress but fail to inhibit increasingly expanding and possibly more resistant T_{effs} (3).

Inflammatory cytokines, especially IL-21, are also implicated in the induction of an unstable T_{reg} phenotype. That Foxp3-expressing T cells represent a stable, terminally differentiated lineage has recently been questioned by a number of studies, where it was proposed that T_{regs}, or a subpopulation of them, retain developmental plasticity (2, 23, 27, 53, 54). In an autoimmune setting, inflammatory signals or a lymphopenic environment could destabilize the T_{reg} phenotype and allow for functional plasticity and reprogramming into Th1, Th17, or T_{FH} effector cells (23, 27, 53–59). T_{reg} instability was, however, not supported by all studies (24) and remains controversial. In our study, we did not observe a noticeable conversion of T_{regs} into T_{effs}, nor were T_{regs} able to adopt T_{eff} (T_{FH}) function that would result in GC formation, autoantibody production, and disease development. Using mixed adoptive transfer studies, we showed that a relatively stronger expansion of T_{effs} (T_{FH} and Th17 cells) together with the possibility of the outgrowth of T_{effs} containing isolated T_{regs} can be misinterpreted as T_{reg} to T_{eff} conversion. Factors, such as inflammatory cytokines or lymphopenia that support a stronger T_{eff} expansion, increase the number of "pseudo-converted cells." Accordingly, other studies indicated that T_{reg} instability was particularly observed in lymphopenic hosts (53) and within the gut microenvironment (Peyer's patches and mesenteric LN) (54, 55). This is in accordance with our own observations where the greatest numbers of pseudo-converted cells were in $CD28^{-/-}$ recipients. It is likely that reduced IL-2 availability under lymphopenic conditions hampers T_{reg} proliferation, whereas high levels of IL-21 in gut-associated lymphoid organs may support expansion of T_{effs}, and hence contaminating T_{effs} outproliferate T_{regs}.

Although it has been shown that $CD25^{hi}$ T_{regs} retain their Foxp3 expression (2, 24), $CD25^{lo}Foxp3^{+}$ T_{regs} were identified as an unstable subset (23, 27). Moreover, under the inflammatory conditions of collagen-induced arthritis, such a cell population was able to acquire a Th17 phenotype induced by IL-6 (27). However, this study also showed that these exT_{reg} Th17 cells may originate from iT_{regs} rather than nT_{regs}, with only the latter being increased in inflamed lymphoid organs in our studies. We did not find increased IL-17 or IFN-γ, but CCR6 was elevated on arthritic T_{regs}, what they may have acquired to facilitate migration to inflamed tissues.

In our studies of T_{reg} stability, it has to be taken into consideration that Foxp3.gfp reporter mice were used. For our questions, the Foxp3.gfp reporter mouse model has been a critical tool because sorting of T_{reg} cells at high purity was required. However, recent reports have indicated that the reporter may change autoimmune severity (60, 61). For our study, we would anticipate the reported phenotypic, functional, and transcriptional perturbations (60, 61) to have little or no impact, although this remains untested.

Under normal circumstances, the scenario that T_{reg} expansion follows that of T_{effs} with some delay has physiological importance. To boost their fitness and expansion, T_{regs} rely on adequate T_{eff} activation (62, 63). For this reason, it has been proposed that T_{reg} responses do not arrest a primary immune response, but rather track the T_{eff} response (64) to starve them of survival factors, such as IL-2 or TNF-α (47, 62, 63). This enables necessary immune responses, which are then controlled to prevent overexuberant immune activation. Under conditions that favor T_{eff} proliferation (inflammation and lymphopenia), there is a dysregulation in the T_{reg}/T_{eff} balance, and the T_{reg} response may be overwhelmed by T_{effs}, resulting in autoimmunity. This would rely on a more potent expansion of T_{effs} in response to inflammation or lymphopenia at different disease stages. At early or predisease stages, the resulting unopposed T_{eff} activity may tip the balance toward autoimmunity; during established disease, it may underpin phases of remittent or relapsing disease.

In summary, we demonstrate how imbalances in T_{reg} and T_{eff} homeostasis control the onset and progression of disease in RA. Given that states of inflammation or lymphopenia are a relatively common occurrence throughout life, and may also result from therapeutic regimens, it is necessary to elucidate factors and mechanisms that restore homeostasis or augment T_{reg} activity. Because IL-2 acts as a T_{reg} enhancer outweighing its simultaneous fueling of the T_{eff} pool, and IL-21 confers maintenance signals to T_{effs}, they may become new targets for novel attempts to manipulate immunological tolerance, or effector responses, to subdue or enhance immune responses. Such a concept may not only have significant implications for therapeutical use in RA, but also in other autoimmune and inflammatory diseases and cancer immunotherapy.

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Disclosures

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