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IL-21 Promotes Lupus-like Disease in Chronic Graft-versus-Host Disease through Both CD4 T Cell- and B Cell-Intrinsic Mechanisms

Vinh Nguyen,* Irina Luzina,† Horea Rus,‡‡ Cosmin Tegla,‡‡ Ching Chen,§ and Violeta Rus*†

T cell-driven B cell hyperactivity plays an essential role in driving autoimmune disease development in systemic lupus erythematosus. IL-21 is a member of the type I cytokine family with pleiotropic activities. It regulates B cell differentiation and function, promotes T follicular helper (TFH) cell and Th17 cell differentiation, and downregulates the induction of T regulatory cells. Although IL-21 has been implicated in systemic lupus erythematosus, the relative importance of IL-21R signaling in CD4+ T cells versus B cells is not clear. To address this, we took advantage of two induced models of lupus-like chronic graft-versus-host disease by using wild-type or IL-21R−/− mice as donors in the parent-into-F1 model and as hosts in the Bm12→B6 model. We show that IL-21R expression on donor CD4+ T cells is essential for sustaining TFH cell number and subsequent help for B cells, resulting in autoantibody production and more severe lupus-like renal disease, but it does not alter the balance of Th17 cells and regulatory T cells. In contrast, IL-21R signaling on B cells is critical for the induction and maintenance of germinal centers, plasma cell differentiation, autoantibody production, and the development of renal disease. These results demonstrate that IL-21 promotes autoimmunity in chronic graft-versus-host disease through both CD4+ T cell- and B cell-intrinsic mechanisms and suggest that IL-21 blockade may attenuate B cell hyperactivity, as well as the aberrant TFH cell pathway that contributes to lupus pathogenesis. The Journal of Immunology, 2012, 189: 1081–1093.

Interleukin-21 is a member of the type I cytokine family, with pleiotropic effects on the immune system depending on the cellular context, nature of costimulation, and cytokine environment (1). IL-21R is expressed on a variety of immune cells, including B, NK, and dendritic cells (DCs), whereas IL-21 production is restricted to activated CD4+ T cells, T follicular helper (TFH) cells, Th17 cells, and NK T cells (1, 2). IL-21 promotes the expansion of NK cells and augments their antitumor activity, enhances CD8+ T cell maturation into cytotoxic T lymphocytes, and promotes the differentiation and expansion of TFH cells (1, 3–5). In addition, within the T cell lineage, IL-21 regulates the reciprocal differentiation of Th17 cells and regulatory T cells (Tregs) by promoting Th17 cell expansion and inhibiting the generation and function of induced Tregs (iTregs) (6–9). Within the B cell lineage, IL-21 regulates B cell proliferation and survival; Ig production and class switching, particularly to IgG1; germinal center (GC) formation; plasma cell (PC) differentiation; and memory B cell responses (10–13). IL-21 can also induce B cell apoptosis when B cells are activated with LPS, CpG, anti-IgM, and IL-4 (14).

Recent evidence suggests that IL-21 may play an important role in autoimmune diseases, including systemic lupus erythematosus (SLE), rheumatoid arthritis, and Sjögren’s syndrome (15–18). In humans, an association of IL-21 and IL-21R polymorphisms with SLE, along with elevated levels of IL-21 in serum and in CD4+ T cells, was reported (16–20). Studies in murine models of lupus indicated increased production of IL-21 in MRL-Faslpr, BXSB-Yaa mice and in the knockout (KO) mouse sanroque (21–23). Furthermore, IL-21 blockade was beneficial in MRL-Faslpr mice, whereas it had a biphasic effect in BXSB-Yaa mice, negatively influencing survival early on and positively influencing survival at later stages of disease (22, 23). In addition, IL-21R−/− deficient BXSB-Yaa mice showed none of the autoimmune abnormalities characteristic of IL-21R−/− competent BXSB-Yaa mice (24).

The wide range of costimulatory and inhibitory signals delivered by IL-21 on T and B cells suggests a complex role for IL-21 in promoting autoimmunity in vivo. The relative importance of the IL-21/IL-21R interaction in promoting SLE through CD4+ T cell-dependent mechanisms that may affect TFH cells, Th17 cells, or Tregs or through B cell-intrinsic mechanisms has not yet been determined. In the absence of conditional KO mice, it has not been technically possible to investigate this issue in autoimmune-prone lupus models in vivo. Therefore, to address this question, we took advantage of the induced lupus-like model of chronic graft-versus-host disease (cGVHD) that allowed us to independently manipulate T and B cell responses and dissect the requirements of the IL-21/IL-21R interaction for the initiation and progression of the disease. To this end, IL-21R−/− sufficient and -deficient mice on the B6 background were used as donors in the parent-into-F1 (P→F1) model or as hosts in the Bm12→B6 model of cGVHD. In addition, because the exact timing of disease onset is known, these models

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Abbreviations used in this article: B6, C57BL/6; Bm12, B6.C-H2bm12/KhEg; cGVHD, chronic graft-versus-host disease; DC, dendritic cell; GC, germinal center; GN, glomerulonephritis; iTreg, induced regulatory T cell; KO, knockout; nTreg, natural regulatory T cell; P→F1, parent-into-F1; PC, plasma cell; PNA, peanut agglutinin; SLE, systemic lupus erythematosus; Tfh, T follicular helper; Treg, regulatory T cell; WT, wild-type.

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allowed us to perform a kinetic analysis of T and B cell activation, differentiation, and effector functions (25). Our results indicate that lack of the IL-21/IL-21R interaction on either B cells or Ag-specific CD4+ T cells independently impairs the development of autoimmune manifestations of cGVHD and results in an attenuated disease phenotype.

Materials and Methods

Mice

Six- to eight-week-old male B6D2DF1, B6.C-H-2b12KdEg (Bm12), and C57Bl/6J (B6) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Breeding pairs of IL-21R−/− mice on the B6 background were provided by Dr. Michael Grusby (Harvard School of Public Health, Boston, MA) (26). C57Bl/6-Tg(UBC-GFP)-30Scha mice, C57Bl/6 J transgenic mice that express GFP, were provided by Dr. David Trisler (University of Maryland School of Medicine). IL-21R−/− mice used in this study were housed and bred at the University of Maryland Animal Care Facility. All procedures were approved by the University of Maryland School of Medicine Office of Animal Welfare Assurance.

Induction of cGVHD

Single-cell suspensions of splenocytes were prepared in RPMI 1640, filtered through sterile nylon mesh, washed, and diluted to a concentration of 106 viable (trypan blue-excluding) cells/ml. P→F1 cGVHD was induced with CD4+ T cell-depleted splenocytes containing 10−15 × 106 CD4+ donor cells from either B6 wild-type (WT) or B6 IL-21R−/− donors injected i.v. into B6D2DF1, mice, as described (27). Flow cytometry was used prior to injection to confirm that equal numbers of CD4+ T cells were injected into recipient F1 mice. Donor CD8+ T cells were depleted using Dynabeads Mouse CD8 (Lyt 2) (Invitrogen, San Diego, CA). Flow cytometric analysis demonstrated <1% contaminating CD8+ T cells. Controls consisted of un.injected age- and sex-matched F1 mice. Bm12→B6 cGVHD was induced in B6 WT or B6 IL-21R−/− recipients by i.p. injection of 1×106 Bm12 donor splenocytes. Recipient and donor mice were age- and sex-matched within each independent experiment. In all experiments, we used male mice to avoid artifacts due to the sex-based difference in IL-21 gene expression (28).

Cell isolation and in vitro generation and measurement of IL-17

Spleen cells from control or P→F1 cGVHD mice were pooled (three mice/pool), and CD4+ T cells were negatively selected using MACS beads (Miltenyi Biotec). Donor and host cells from C57Bl/6 mice were further purified using biotinylated anti-H-2Kd Abs and anti-biotin MACS beads. Purified donor CD4+ T cells were cultured for 5 d with plate-bound anti-CD3 (5 μg/ml), anti-CD28 (1 μg/ml), and IL-23 (10 ng/ml) and then restimulated with PMA/ionomycin for 4 h. Supernatants were tested in duplicates for IL-17A expression by ELISA. B cells were purified from control or Bm12→B6 cGVHD mice using MACS beads.

Abs and flow cytometry

Spleen cells were first incubated with anti-murine FcγRIIIa mAb (2.4G2) for 10 min and then stained with saturating concentrations of Alexa Fluor 488-conjugated, allophycocyanin-conjugated, biotin-conjugated, PE-conjugated, FITC-conjugated, PE/Cy5-conjugated, or PE/Cy7-conjugated mAbs against CD4, CD8, B220, H-2Kd, H-2Kb, H-2Dd, I-Ab, I-Ak, CD80, CD86, CD90, CD190, Fas, FasL, PD-1, GL-7, CXCR5, PD-1, CD62L, FoxP3, Helios, RxRα, and Ki-67. Abs were purchased from BD Biosciences (San Jose, CA), BioLegend (San Diego, CA), eBioscience (San Diego, CA), and Sigma-Aldrich (Saint Louis, MO). Biotinylated primary mAbs were detected using streptavidin-allophycocyanin (BioLegend), streptavidin-FITC, streptavidin-PE, or streptavidin-PE-Cy5 (BD Biosciences). Cells were fixed in 1% paraformaldehyde before flow cytometric analysis. Intracellular staining for transcription factors was performed using the FoxP3 Buffer Staining Set from eBioscience, according to the manufacturer’s protocol. Annexin V staining was performed with the FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen), according to the manufacturer’s protocol, and analyzed by flow cytometry within 1 h of staining. Multicolor flow cytometric analyses were performed using a FACScan, Accuri C6, and LSR II flow cytometer (BD Biosciences). Lymphocytes were gated by forward and side scatter, and fluorescence data were collected for a minimum of 10,000 gated cells. Studies of donor T cells in the P→F1 model were performed using a lymphocyte gate that was positive for CD4 and negative for MHC class I of the uninfected parent (H-2Kd−).

Immunohistochemistry staining of splenic sections

Sections of formalin-fixed spleen (5 μm) were deparaffinized, rehydrated, and then stained, as previously described (29). The following Abs, with their specificities and conjugations, were used: biotinylated peanut agglutinin (PNA) from Sigma-Aldrich, unlabeled rat anti-human/mouse CD45RB (clone RA3-6B2) for eBioscience, unlabeled rabbit anti-GFP (Invitrogen), streptavidin HRP (Vector Laboratories), and alkaline phosphatase-conjugated goat anti-rabbit and alkaline phosphatase-conjugated goat anti-rat Ab (both from Jackson ImmunoResearch). Substrates used were AEC and Vector Blue Alkaline Phosphatase Kit (Vector Laboratories).

Preparation of CFSE-labeled donor cells

CFSE (Molecular Probes, Eugene, OR) labeling of donor splenocytes and analysis of donor cell proliferation by flow cytometry were performed as previously described (27). Cells were adjusted to 5 × 106/ml in PBS/0.1% BSA and then incubated in the dark for 10 min at 37°C with CFSE (10 μM stock solution diluted in DMSO) to a final concentration of 5 μM. Staining was quenched with five volumes of ice-cold RPMI 1640/10% FBS, and cells were washed three times in PBS before injection into F1 mice. Proliferating CFSE+ donor CD4+ T cells were distinguished by multiparameter flow cytometry.

ELISPOT

Ninety-six-well cellulose membrane plates (MAIP54510; Millipore) were precoated with 3% ethanol for 5 min and then coated overnight with 100 μg/ml prefiltered herminning sperm DNA (Promega) in PBS at 4°C. Two-fold serial dilutions of spleen cells or bone marrow cells were plated in duplicate overnight on DNA-coated plates, starting at 0.5 × 107/well, in 3% BSA/DMEM. After washing, the plates were incubated with anti-mouse IgG alkaline phosphatase (Sigma-Aldrich) at 1:1000 in PBS/3% BSA for 1 h at 37°C. Plates were then developed with bromochlorindolophospholipase (KPL, Gaithersburg, MD). Spots were counted using an automated reader (CTL- Europe GmbH Reader System, software version 4).

ELISA for anti-dsDNA, anti-ssDNA, total IgG, and IgG1 Abs

For quantification of serum IgG and IgG1 levels of anti-ssDNA, 96-well plates were coated with heat-denatured calf thymus DNA (Sigma-Aldrich), followed by blocking with 1% BSA/PBS and incubation with serial dilutions of experimental mouse sera, beginning at a dilution of 1/40, tested in duplicate. The plates were then incubated with alkaline phosphatase-conjugated anti-mouse IgG and anti-mouse IgG1 (Sigma-Aldrich), respectively, and OD was quantitated at 405 nm. For measurement of IgG and IgG1 anti-dsDNA Abs, plates were coated with 100 μg/ml prefiltered herminning sperm DNA (Promega). Following blocking with 3% BSA/PBS, sera were added to the plate at dilutions starting at 1/50. The respective secondary Abs were alkaline phosphatase-conjugated anti-mouse IgG and anti-mouse IgG1 (Sigma-Aldrich). For each experiment, murine MRL-Fas+/− sera were used as a standard, and the results were converted to arbitrary units.

For the IgG and IgG1 goat anti-mouse IgG or IgG1 (Southern Biotech, Birmingham AL) was coated at 5 μg/ml onto plates. Following blocking with 3% BSA/PBS, sera were added to the plate at dilutions starting at 1/50,000 and 1/25,000, respectively. Mouse IgG and IgG1 (Rockland, Gilbertsville, PA), respectively, were used as the standards. The plates were incubated with alkaline phosphatase-conjugated anti-mouse IgG (Sigma-Aldrich) and anti-mouse IgG1 (Jackson ImmunoResearch), respectively.

Real-time PCR

Total RNA isolation, quantitative and reverse transcription were performed, as described (27). 18S rRNA was used as an internal control. All primers and probes (IL-21, IL-4, IL-10, IL-6, bcl-6, RORγ, IL-17A, Pدم1, Aicda, and 18S) were purchased from SABiosciences (Frederick, MD). RT-PCR was carried out on an Applied Biosystems Step1 Plus PCR machine (Foster City, CA).

Kidney histopathology and immunofluorescence

Kidney-fixed kidney sections (4 μm) were stained with H&E. The sections were examined in a blinded fashion by C.C. and H.R.) for glomerular, tubular, and interstitial pathology. Disease was scored on a semiquantitative scale using the published criteria, with modifications (30). The severity of glomerulonephritis (GN) was graded on a 0–3 scale: 0 = normal; 1 = mild to moderate increase in cellularity with mesangial proliferation; 2 = moderate increase in cellularity with endocapillary and mesangial proliferation, increased matrix, and/or karyorrhexis; and 3 =...
marked increase in cellularity with endocapillary proliferation, crescent formation, and/or necrosis, and/or sclerosis. Scores from 20 glomeruli were averaged to obtain a mean score for each kidney section. Deposits of IgG in the glomeruli were detected by incubating acetone fixed, 5-μm-thick cryostat sections of kidney in 20% normal goat serum for 30 min, followed by a 1-h incubation with FITC-conjugated goat anti-mouse IgG (1/500; Southern Biotech). Fluorescence in glomerular capillary walls and in the mesangium was subjectively scored blindly on a scale of 0–3 (0 = none, 1 = weak, 2 = moderate, 3 = strong); 10 glomeruli/section were analyzed.

Statistical analysis
Normally distributed data were analyzed by the unpaired t test, and non-parametric data were analyzed by the Mann–Whitney U test using Prism 4.0 (GraphPad) software.

Results
Upregulation of IL-21 production and Tfh cell differentiation of donor CD4+ T cells
We first evaluated whether IL-21 is upregulated in the P→F1 and Bm12→B6 models of cGVHD. To this end, we measured IL-21 mRNA expression by RT-PCR in splenocytes of cGVHD mice at 2 wk after disease induction, when the autoimmune features are already present. Compared with normal control mice, IL-21 mRNA production increased by 30- and 28-fold in P→F1 cGVHD and Bm12→B6 cGVHD mice, respectively (Fig. 1A, 1B).

In both models of cGVHD, donor CD4+ T cells, activated by host MHC class II, expand and provide cognate help to host B cells. Because activated CD4+ T cells are known to produce IL-21, we evaluated whether donor CD4+ T cells are the major source of IL-21. We addressed this question in the P→F1 model, in which donor CD4+ T cells (H-2Kd) can be distinguished by flow cytometry and separated from H-2Kd+ host cells. As seen in Fig. 1C, IL-21 mRNA transcripts were significantly higher in purified donor CD4+ T cells than in host CD4+ T cells.

Recent studies highlighted a role for IL-21 as a growth factor for Tfh cells and as a regulator of the reciprocal differentiation of Th17 cells and Tregs (6, 9, 31). To assess the effect of IL-21R signaling on these subsets, we evaluated whether donor Tfh cells, Th17 cells, and Tregs of donor origin would be readily detectable in cGVHD mice. To this end, we first assessed whether donor CD4+ T cells differentiated into Tfh cells, with upregulation of the Tfh cell transcription factor Bcl-6 and localization in the GCs. Tfh cells detected by flow cytometry as ICOShi CXCR5hi cells among CD4+ H-2Kd+ donor cells expanded from 0.45 ± 0.02% at baseline in naive donor CD4+ T cells prior to transfer to 14 ± 2% of donor cells at day 14. Helios staining was used to distinguish FOXP3hi Helios+ nTregs and FOXP3hi Helios+ iTregs. Data are representative of two independent experiments (n = 5 mice/group). *p < 0.05, ***p < 0.001.
CD4+ T cells engrafted in cGVHD mice (Fig. 1D). Furthermore, by RT-PCR, we detected a significant upregulation of Bcl-6 mRNA in purified donor CD4+ T cells from cGVHD mice compared with naive, un.injected donor CD4+ T cell controls (Fig. 1E). Consistent with their differentiation into Tfh cells, in mice with cGVHD induced with GFP-expressing cells, donor GFP+ cells detected by immunohistochemistry were preferentially localized in GCs, as well as in the B cell area and at the T–B border (Fig. 1F). Similarly, mRNA transcripts for IL-17A and RORγt, the transcription factor required for Th17 cell differentiation, are upregulated in donor CD4+ T cells purified from cGVHD mice compared with naive, un injected CD4+ T cells (Fig. 1G). Furthermore, purified donor CD4+ T cells from cGVHD mice, cultured in vitro with anti-CD3/anti-CD28 Ab and IL-23, a cytokine known to expand differentiated Th17 cells, produced significant levels of IL-17 in supernatants (Fig. 1H). We could not detect IL-17 production from naive, un injected CD4+ T cells under the same conditions (Fig. 1H). The percentage of CD4+FOXP3+ Tregs was similar in the un injected, naive donor CD4+ T cells and in donor CD4+ T cells from cGVHD mice at 2 wk after disease induction. Furthermore, the proportions of natural Tregs (nTregs) and iTregs determined by intracellular Helios staining were comparable in naive, un injected and engrafted donor CD4+ T cells. These results indicate that, in cGVHD, donor CD4+ T cells upregulate IL-21 mRNA expression and differentiate into Tfh cells and Th17 cells while maintaining the same proportion of natural and induced Tregs.

IL-21R deficiency on donor CD4+ T cells attenuates B cell parameters and kidney disease in P→F1 cGVHD

After activation, donor CD4+ T cells provide MHC class II-restricted cognate help to host B cells, resulting in chronic B cell hyperactivity and expansion. Consequently, IgG anti-ssDNA Ab levels are elevated early (day 14) after donor cell transfer, and renal disease occurs after 2 mo (25, 32). To assess the contribution of the IL-21/IL-21R interaction on donor CD4+ T cells to the disease phenotype, we compared early and late disease parameters of P→F1 cGVHD induced with donor cells from IL-21R−/− mice, which abrogates the IL-21/IL-21R interaction on donor cells but not on host B cells, versus cGVHD induced with donor cells from WT mice.

At 7 and 14 d after cGVHD induction with WT or IL-21R−/−CD8-depleted donor cells, we determined the number of B cells, B cell MHC class II expression, the proportions of GL-7/Fas+ B220+ GC B cells, and the levels of anti-ssDNA autoantibody. After 7 d, no significant differences were observed in any of these parameters between the two groups. By comparison, after 14 d, mice injected with IL-21R−/− donor cells had a significantly lower number of B cells (Fig. 2A) and a trend toward decreased MHC class II expression, although this did not reach significance (data not shown). The proportion of GL-7/Fas+B220+ GC B cells was also significantly lower in the spleens of cGVHD mice injected with IL-21R−/− donor cells (Fig. 2B, 2C). Consistent with the flow cytometry data, GCs were smaller by immunohistochemistry (Fig. 2D–G). Furthermore, IgG anti-ssDNA Ab levels were significantly lower in mice injected with IL-21R−/− donor cells. These data suggest that the IL-21/IL-21R interaction on donor CD4+ T cells is dispensable for the initiation of the autoimmune B cell response but is required for the optimal expansion of host B cells and GC B cells and for maximal autoantibody production.

In long-term studies, mice with cGVHD induced with IL-21R−/− donor cells developed an attenuated lupus-like renal disease compared with mice with cGVHD induced with WT donor cells; they also displayed much milder GN (Fig. 3A–C, 3H), significantly lower proteinuria (Fig. 3G), and decreased deposition of IgG (Fig. 3D–F).

IL-21R–deficient donor T cells exhibit diminished Tfh cell expansion and persistence

The diminished host B cell and GC expansion and decreased anti-ssDNA Ab production at 2 wk after disease induction in the absence of the IL-21/IL-21R interaction on donor CD4+ T cells suggest a defect in sustained CD4+ Th function. We evaluated whether this effect is due to altered donor Tfh cell differentiation and/or expansion. In mice receiving WT CD8-depleted donor cells, CXC5PD−1 donor Tfh cells increased from 1.4 ± 0.5% in naive donor CD4+ T cells at the time of transfer to a peak of 18 ± 3% at day 7 and then decreased slightly to 14 ± 1.3% by day 14. In mice with cGVHD induced with IL-21R−/− donor cells, the proportion of Tfh cells was lower at both days 7 and 14 but reached statistical significance only on day 14 (Fig. 4A, Supplemental Fig. 1B). The decrease in IL-21R−/− Tfh cell frequency was mainly due to decreased proliferation, because the proportion of dividing Ki-67+ donor Tfh cells was significantly lower at 2 wk of disease (Fig. 4B, Supplemental Fig. 1D). The frequency of apoptotic donor Tfh cells was slightly higher in mice injected with IL-21R−/− donor cells at 2 wk of disease, but the difference was not statistically significant (Fig. 4C). Next, we assessed whether the decreased expansion of donor Tfh cells is specific for this T cell subset or whether it also affects non-Tfh donor CD4+ T cells. As seen in Fig. 4D, the percentage of engrafted donor CD4+ T cells is significantly lower on both days 7 and 14 in the absence of IL-21R. Similar to donor Tfh cells, in WT donor cells, the frequency of proliferating CD4+ T cells had increased 4.2-fold from baseline at day 7 and 3-fold at day 14. By comparison, in IL-21R−/− donor cells, the percentage of proliferating donor CD4+ T cells was lower, but not statistically significant, on day 7; it decreased further to significantly lower levels by day 14 (Fig. 4E, Supplemental Fig. 1C). Similar to donor Tfh cells, the apoptotic rate tended to be higher in IL-21R−/−–injected mice, although the difference was not statistically significant (Fig. 4F). These results suggest that IL-21R is important for the sustained proliferation of Tfh cells, as well as non-Tfh donor cell CD4+ T cells, from days 7 to 14. Although we were unable to demonstrate differences in apoptotic rates, altered survival of the Tfh cells, as well as non-Tfh cell donor CD4+ T cells, cannot be completely ruled out.

IL-21 and IL-4, known for their ability to promote GC development, are the main cytokines secreted by Tfh cells (33, 34). We determined whether IL-21 and IL-4 mRNA expression is altered in donor CD4+ T cells from mice injected with IL-21R−/− deficient donor cells. As seen in Fig. 5, both IL-21 and IL-4 mRNA transcripts are decreased in IL-21R−/− donor CD4+ T cells, suggesting that the expression of these cytokines parallels the decrease in donor CD4+ T cells and Tfh cells.

Donor Tregs and Th17 cells are not altered in the absence of IL-21R signaling

To assess whether the lack of IL-21R signaling on donor CD4+ T cells alters the proportion of donor Tregs and donor Th17 cells, we initially compared the percentage of FOXP3+CD4+ donor cells in cGVHD mice induced with IL-21R–sufficient and –deficient cells. At baseline, there was no difference in the frequency of CD4+FOXP3+ Tregs in the injected donor cells of IL-21R−/− and IL-21R−/− origin (data not shown). At 2 wk after disease induction, the frequency of donor CD4+FOXP3+ Tregs did not differ between IL-21R−/− and IL-21R−/− donor CD4+ T cells (Fig. 6A, 6B). Furthermore, we did not observe a significant difference between the proportions of FOXP3+Helios+ nTregs and FOXP3+Helios+ iTregs.
Helios−iTregs. Similarly, we observed no difference in either IL-17 mRNA expression or IL-17 secretion of in vitro-stimulated donor CD4+ T cells purified from mice with cGVHD induced with IL-21R+/+ or IL-21R−/− donor cells (Fig. 6C, 6D).

Decreased B cell activation, autoantibody production, and kidney disease severity in Bm12→B6 cGVHD induced in IL-21R−/− recipients

To assess the contribution of IL-21R signaling on host B cells to the disease phenotype independent of IL-21R signaling on donor CD4+ T cells, we compared early and late disease parameters in Bm12→B6 cGVHD induced in IL-21R–deficient recipients, which abrogates the IL-21/IL-21R interaction on host cells but not on donor cells, versus cGVHD induced in WT hosts.

B cell activation that characterizes cGVHD is a complex multistep process involving a nonstringent step, in which activation of donor T cells induces polyclonal activation and proliferation of all B cells, and a stringent, cognate interaction, resulting in the activation of autoreactive B cells, such as anti-dsDNA B cells, and autoantibody production (35). We first assessed whether parameters of B cell activation were altered in the absence of IL-21R signaling on host B cells. MHC class II and CD69 were upregulated to a similar extent on B cells from IL-21R+/+ or IL-21R−/− donor cells (Fig. 6C, 6D). However, at 2 wk of disease, MHC class II expression and CD69 were further upregulated on B cells from IL-21R−/− hosts but not IL-21R−/− hosts (Fig. 7). These data suggest that, in the absence of IL-21R signaling, B cells fail to reach optimal levels of activation. However, we cannot exclude the possibility that the lack of IL-21R on activated B cells impairs their survival.

Serum levels of total IgG Abs increased significantly in IL-21–sufficient hosts as early as 2 wk, peaked around week 7, and then gradually decreased by week 18 to levels reached at weeks 2 and 4. IL-21R–deficient hosts displayed significantly lower levels of total IgG at all time points before week 18, when they reached a plateau at levels similar to those detected in IL-21R–sufficient hosts (Fig. 8A). IgG anti-dsDNA autoantibody levels were only transiently detected at low titer at 2 and 4 wk in IL-21R−/− hosts compared with IL-21R+/+ hosts and were significantly lower at all time points (Fig. 8B). Because IL-21 induces isotype switching to IgG1, we assessed the levels of total and anti-dsDNA IgG1 Abs. Both total IgG1 and anti-dsDNA IgG1 Abs were detected only in IL-21R−/− hosts and displayed a kinetic similar to IgG Ab levels (Fig. 8C, 8D). IL-21R−/− hosts had total and anti-dsDNA Ab IgG1 levels similar to control groups.

The observation that cGVHD mice have greatly reduced levels of anti-dsDNA autoantibodies suggested that these mice may develop less severe renal disease. Indeed, proteinuria was significantly decreased in IL-21R−/− cGVHD mice compared with IL-21R+/+ cGVHD mice at 10 and 13 wk after disease induction (Fig. 9H).

**FIGURE 2.** IL-21R deficiency on donor CD4+ T cells attenuates B cell parameters of cGVHD. P→F1 cGVHD was induced as described in Materials and Methods. Serum and spleens were collected at the specified time points. (A) Absolute number of host B cells. (B) Frequency of GL-7+Fas+ GC B cells. (C) Flow cytometric contour plots of GL-7+Fas+ GC B cells (B cell gate). (D) Anti-ssDNA Ab levels were determined by ELISA. (E-G) Spleen sections obtained at 2 wk were stained for PNA-R+ GC B cells (brown) and B220 (blue) (original magnification ×100). Data are representative of two independent experiments (n = 5 mice/group). *p < 0.05, **p < 0.01.
formation, were observed in the kidneys of WT but not IL-21R<sup>−/−</sup> cGVHD mice (Fig. 9A–C). The latter had glomerular histological scores similar to the control groups (Fig. 9G). In addition, WT, but not IL-21R<sup>−/−</sup>, recipients of Bm12 cells displayed glomerular IgG deposition at 13 wk after disease induction (Fig. 9D–F). There was no difference between WT and KO recipient groups in the degree of perivascular lymphoid infiltration and tubulointerstitial damage (data not shown).

**Decreased GCs and PC differentiation in IL-21R<sup>−/−</sup> cGVHD mice**

In models of protein immunization, the role of the IL-21/IL-21R interaction in the initiation and/or maintenance of GCs has been controversial (5, 13, 36, 37). We assessed the importance of IL-21R signaling on B cells for GC formation and maintenance in Bm12→B6 cGVHD at 1 wk after disease induction, when GCs are initially formed, and at 3 wk, when GCs have already peaked. By flow cytometry, the percentages of GC B cells decreased by 40% at day 7 and by 60% at day 21 after disease induction in IL-21R–deficient hosts (Fig. 10G). By immunohistochemistry, splenic GCs in IL-21R<sup>−/−</sup> hosts were smaller, ill-formed, disrupted, and without apparent polarization compared with those in IL-21R<sup>+/+</sup> hosts, both at 7 and 21 d after disease induction (Fig. 10A–F). These differences were maintained at day 28 (data not shown).

These data suggest that, in cGVHD, the absence of IL-21R sig-

![FIGURE 3. IL-21R deficiency on donor CD4<sup>+</sup> T cells ameliorates lupus-like nephritis in P→F1 cGVHD. P→F1 cGVHD was induced as described in Materials and Methods. (A–C) H&E-stained kidney sections of normal F1 control mice, cGVHD mice induced with WT, or IL-21R<sup>−/−</sup> donor cells at 13 wk after disease induction. Enlarged glomerulus with crescent formation, glomerular sclerosis, and interstitial infiltrate is noted in (B) (original magnification ×400). (D–F) Immunofluorescent staining of IgG deposits (original magnification ×200). (G) Proteinuria. (H) GN pathologic scores. Data are representative of two independent experiments (n = 5 mice/group). *p < 0.05.

![FIGURE 4. IL-21R–deficient donor CD4 and T<sub>FH</sub> cells exhibit decreased expansion and proliferation in P→F1 cGVHD. P→F1 cGVHD was induced as described in Materials and Methods. Recipient mice were sacrificed at the times indicated. Mean percentage of donor T<sub>FH</sub> cells (A), Ki-67<sup>+</sup> donor T<sub>FH</sub> cells (B), annexin V<sup>+</sup> donor T<sub>FH</sub> cells (C), donor CD4<sup>+</sup> T cells (D), Ki-67<sup>+</sup> donor CD4<sup>+</sup> T cells (E), and annexin V<sup>+</sup> donor CD4<sup>+</sup> T cells (F). Data are representative of two independent experiments (n = 5 mice/group). *p < 0.05, **p < 0.01.]
naling on B cells reduces the magnitude of the initial GC response, as well as its maintenance.

Consistent with the decrease in GC B cell numbers, we observed a significant decrease in the proportion of proliferating GC B cells in the absence of IL-21R signaling. The percentage of Ki-67+Fas+GL-7+ GC B cells decreased by 1.5-fold at 7 d and by 2.8-fold at 21 d after disease induction compared with controls (Fig. 10H). Thus, although the absence of IL-21R signaling decreases GC B cell proliferation at both early and late time points, the magnitude of this reduction is greater at later time points, suggesting that IL-21R signaling has a predominant role in the maintenance of GCs. Among GC B cells, centroblasts are highly proliferative cells that express AID (encoded by Aicda). We detected significantly decreased Aicda mRNA expression in purified B cells from IL-21R−/− hosts (Fig. 10I).

The decreased level of anti-dsDNA Abs and the attenuated GC response in IL-21R−/− hosts suggest the possibility of decreased PC differentiation in the GCs. We used ELISPOT to examine the number of anti-dsDNA Ab-secreting PCs in the bone marrow at 28 d after disease induction, when PCs have already migrated out of the spleen. Anti-dsDNA-secreting PCs were significantly lower in the bone marrow of IL-21R−/− hosts for both IgG (Fig. 11A, 11B) and IgG1 (Fig. 11C, 11D) Abs. Consistent with the decrease in PC formation, mRNA expression of Prdm1, the gene encoding BLIMP-1, the master regulator of PC differentiation, was significantly decreased in purified B cells from IL-21R−/− cGVHD mice (Fig. 11E).

Normal donor CD4+ T cell priming and TFH cell formation in IL-21R−/− cGVHD mice

The diminished GC response and autoantibody levels in the IL-21R−/− recipients raise the question of the quality of the T cell help in these mice. Therefore, we assessed whether the decreased GC and autoantibody response in IL-21R−/− cGVHD mice could be due to a failure of T cell priming or to a suboptimal donor TFH cell response. T cell priming was assessed using Bm12 donor cells labeled with CFSE prior to cGVHD induction, followed by detection of activation markers and proliferation rate of CFSE+ donor cells on day 3 after cGVHD induction. CD4+CFSE+ donor cells exhibited similar expression of the activation markers CD44 and CD69 whether they were injected into WT or IL-21R−/− hosts (Fig. 12A). Similar results were obtained on day 5 (data not shown). Interestingly, as previously reported for CD8+ T cells, the proliferative response of CFSE-labeled donor CD4+ T cells was greater in IL-21R−/− hosts than in IL-21R+/+ hosts (Fig. 12B, 12C), possibly as a result of the increased concentration of IL-21 on donor cells in IL-21R−/− host mice (38). These data indicate that donor CD4+ T cells injected into IL-21R−/− mice were activated and expanded to the same, if not greater, extent as those transferred to the WT mice.

FIGURE 5. Decreased IL-21 and IL-4 mRNA expression in IL-21R−/− donor CD4+ T cells. Two weeks after P→F1 cGVHD induction, donor CD4+ T cells, purified as described in Materials and Methods, were analyzed for IL-21 (A) and IL-4 (B) mRNA expression by RT-PCR. Results were normalized to 18S RNA. Results represent mean ± SEM (n = 6 mice/group). Data are representative of two independent experiments. *p < 0.05.

FIGURE 6. Donor Tregs and Th17 cells are not altered in mice with cGVHD induced with IL-21R−/− donor cells. (A) Two weeks after P→F1 cGVHD induction, spleens from recipient F1 mice were examined by flow cytometry for the frequency of FOXP3+ donor Tregs. Helios staining was used to further identify FOXP3+Helios+ nTregs and FOXP3+Helios− iTregs. (B) Flow cytometric contour plots of FOXP3+CD4+ (donor cell gate). (C) mRNA expression for IL-17A was assessed ex vivo on day 14 from naive, un.injected CD4+ T cells and donor CD4+ T cells purified from cGVHD mice. (D) Purified donor CD4+ T cells from cGVHD mice at day 14 were stimulated for 5 d with plate-bound anti-CD3 (5 μg/ml), anti-CD28 (1 μg/ml), and IL-23 (10 ng/ml) and were restimulated with PMA/ionomycin for 4 h. Supernatants were tested for IL-17 expression by ELISA. Data are representative of two independent experiments (n = 5–6 mice/group).
Cognate interaction between primed T cells and B cells at the T–B border provide the required signals for T FH cell differentiation (5, 34, 39–41). We assessed whether the lack of IL-21R signaling on B cells impairs the differentiation of T FH cells. To this end, CFSE-labeled donor CD4 + T cells were assessed on day 7 after disease induction for the percentage of PD-1 +CXCR5+ TFH cells. The proportion of CFSE + donor T FH cells was similar in both IL-21R–sufficient and -deficient cGVHD mice (Fig. 12D). Thus, these data demonstrate that, at early time points, donor CD4 + T cells from IL-21R−/− host mice were activated, proliferated, and developed into T FH cells to the same extent as did the donor cells from WT mice. Therefore, the attenuated humoral response is not a consequence of a failure of donor T cell priming or T FH cell differentiation.

Discussion
In this study, we combined the P→F1 and Bm12→B6 models of lupus-like cGVHD to investigate the importance of IL-21R signaling on CD4+ T cells, independently of IL-21R signaling on B cells, in the initiation and progression of the disease. Previous studies addressing this issue in bone marrow chimeras and adoptive-transfer systems in recipients immunized with protein Ags reported conflicting results. Although some studies reported that the effect of IL-21 on the immune response was exclusively CD4+ T cell intrinsic, others showed a B cell-intrinsic mechanism (5, 13, 36, 37). In contrast, our data demonstrate that, in cGVHD, IL-21 promotes disease parameters, such as GC formation, PC differentiation, autoantibody production, and GN, through both CD4 cell-dependent and B cell-intrinsic mechanisms. Specifically, cGVHD induced with either IL-21R−deficient donor CD4+ T cells in the P→F1 model or with IL-21R−sufficient donor cells in IL-21R−deficient hosts (hence, IL-21R−/− B cells) in the Bm12→B6 model displayed an attenuated lupus-like phenotype with respect to GC formation, autoantibody production, PC differentiation, and renal disease.

CD4+ T cell help provided by T FH cells is the primary limiting factor for GC formation and subsequent GC B cell responses.

**FIGURE 8.** IL-21R deficiency on host B cells impairs total and anti-dsDNA IgG and IgG1 Ab production. Sera from Bm12→B6 cGVHD mice were assayed by ELISA for the presence of total IgG and IgG1 (A, C) and anti-dsDNA IgG and IgG1 (B, D). Data are representative of two independent experiments (n = 6–8 mice/group). *p < 0.05, **p < 0.01, ***p < 0.001, IL-21R–sufficient versus IL-21R–deficient cGVHD groups.
Conflicting data have been reported on the role of IL-21 in the differentiation or persistence of T<sub>FH</sub> cells. In the context of protein immunizations or viral infections, IL-21 promoted T<sub>FH</sub> cell differentiation and function in a number of studies, whereas, in others, IL-21 had a modest impact or no role (4, 5, 13, 36, 37, 42). In addition, in the autoimmune sanroque mice characterized by excessive T<sub>FH</sub> cell numbers, loss of IL-21 did not correct the increased T<sub>FH</sub> cell population (43). Our kinetic analysis of the T<sub>FH</sub> cell response in the P→F1 cGVHD model demonstrates that lack of the IL-21/IL-21R interaction on donor CD4<sup>+</sup> T cells resulted in a modest decrease in the expansion of donor-derived T<sub>FH</sub> cells at earlier time points and a more pronounced decline at later time points. These results indicate that, in our model, IL-21 contributes primarily to the persistence of T<sub>FH</sub> cells and, to a lesser extent, to their differentiation and initial expansion. Furthermore, our data suggest that IL-21 promotes the persistence of T<sub>FH</sub> cells primarily by sustaining their proliferation and, to a lesser extent, their survival. The decrease in GCs and GC B cells, as well as the levels of anti-ssDNA autoantibodies, detected after the first week of disease in cGVHD mice that received IL-21R<sup>-/-</sup> donor cells resulted in a modest decrease in the expansion of donor-derived T<sub>FH</sub> cells at earlier time points and a more pronounced decline at later time points. These results indicate that, in our model, IL-21 contributes primarily to the persistence of T<sub>FH</sub> cells and, to a lesser extent, their survival. The decrease in GCs and GC B cells, as well as the levels of anti-ssDNA autoantibodies, detected after the first week of disease in cGVHD mice that received IL-21R<sup>-/-</sup> donor cells resulted in a modest decrease in the expansion of donor-derived T<sub>FH</sub> cells at earlier time points and a more pronounced decline at later time points. These results indicate that, in our model, IL-21 contributes primarily to the persistence of T<sub>FH</sub> cells and, to a lesser extent, their survival.

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Treg-mediated suppression (6, 9). Further studies will address this question.

In the Bm12→B6 model of cGVHD, disruption of IL-21R signaling on host B cells resulted in a significant impairment of a number of B cell parameters, such as B cell activation, GC formation, GC B cell proliferation, PC formation, and autoantibody production. Lupus-like GN was also attenuated. Contrasting data were reported in different models of protein immunization with respect to the kinetics of GC formation and Ab response in the absence of IL-21R signaling. In the setting of SRBC immunization, IL-21R signaling was critical for both GC initiation and long-term maintenance, whereas following immunization with keyhole limpet hemocyanin, GC formation was comparable to control mice at day 14 but decreased significantly at day 28 due to an accelerated resolution and increased memory B cell generation (36, 37). After immunization with NP-chicken γ globulin, early GC formation and Ab production were dependent on IL-21R but not GC maintenance or long-term Ab production. Our results showing decreased GC formation and GC B cell numbers at 7 d after disease induction, which was even more striking at 21 d, indicate that in cGVHD, IL-21R signaling is important both for reaching the optimal initial GC response and autoantibody production and for their maintenance. Consistent with the attenuated GC response, we observed a decreased number of anti-dsDNA Ab-secreting cells in the bone marrow of IL-21R−/− hosts and decreased expression of BLIMP-1 (encoded by Prdm1) in spleen B cells, indicating that the differentiation of high-affinity autoantibody-secreting PCs from the follicular GC is impaired in the absence of IL-21R signaling. Although we did not examine the effect of IL-21R signaling on the extrafollicular response in...
our model, the contribution of the IL-21/IL-21R interaction on the extrafollicular pathway was reported in MRL-Fas<sup>Lpr</sup> mice, in which the extrafollicular response is the dominant pathway of autoantibody production (54).

The priming of T cells by DCs in the T cell zone, followed by T–B cell interaction at the T-B border resulting in T<sub>FH</sub> cell differentiation are the initial steps in the multistage, multifactorial process of GC formation (5, 34, 39–41). In view of reports showing an inhibitory effect of IL-21 on DC maturation and activation, we considered the possibility that the attenuation of cGVHD parameters in IL-21R<sup>−/−</sup> hosts is not due to a direct effect on B cells but to an indirect effect on the ability of DCs to prime donor T cells. Our data contradict this idea, because donor T cell priming was not impaired and was even enhanced in IL-

FIGURE 11. Lack of IL-21R on host B cells affects PC formation after Bm12→B6 cGVHD induction. Bm12→B6 cGVHD was induced as described in Materials and Methods. Bone marrow cells were collected at 28 d after immunization. The frequency of IgG (A, B) and IgG1 (C, D) anti-dsDNA Ab-secreting cells (ASC) was assessed by ELISPOT. The results are presented as anti-dsDNA ASC/million splenocytes. (E) Prdm1 mRNA expression was determined by RT-PCR in purified B cells at 14 d after disease induction. mRNA levels were normalized to 18S and reported as fold increase over normal control. Data are representative of two independent experiments (n = 5 mice/group). *p < 0.05.

FIGURE 12. Lack of IL-21R on host B cells does not alter the priming and T<sub>FH</sub> cell differentiation of donor CD4<sup>+</sup> T cells in Bm12→B6 cGVHD. Bm12→B6 cGVHD was induced using CFSE-labeled donor cells. (A) Mean percentage of CD4<sup>44<sup>hi</sup></sup> and CD69<sup>hi</sup> donor cells detected as CFSE<sup>+</sup> CD4<sup>+</sup> T cells at 3 d after disease induction. (B) Proliferation of donor CD4<sup>+</sup> T cells detected by generational analysis of CFSE<sup>+</sup> cells. (C) Percentage of proliferating donor cells. (D) Percentage of PD-1<sup>+</sup> CXCR5<sup>+</sup> T<sub>FH</sub> cells at 7 d after disease induction. Data are representative of two independent experiments (n = 5 mice/group). **p < 0.01.
more, although a number of activation markers were decreased on IL-21R–deficient and -sufficient mice (data not shown). Furthermore, MHC class II, CD80, and CD86 on CD11c + DCs was similar in IL-21R–deficient and -sufficient hosts, suggesting that, at least at the early time points examined, IL-21R deficiency on B cells did not impair Tfh cell pathway and the hyperactive B cell response. Further studies to evaluate whether in vivo blockade of the IL-21 pathway further achieves similar effects are needed.

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Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figures Legend.

Supplemental Figure 1. Decreased proliferation of donor CD4⁺ T cells and donor T_{FH} cells in cGVHD mice induced with IL-21R⁻/⁻ cells. P→F1 cGVHD was induced as described in Materials and Methods and spleen cells were examined on day 14 by flow cytometry. A-B. Flow cytometric scheme for the analysis of donor CD4⁺ T cells and donor T_{FH} cells identified as ICOS⁺CXCR5⁺ or CXCR5⁺ PD-1⁺. C-D. Representative tracings for intracellular Ki-67 are shown for donor CD4⁺ T and donor T_{FH} cells.