

# Key role for IL-21 in experimental autoimmune uveitis

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**IL-21 is a pleiotropic type 1 cytokine that shares the common cytokine receptor  $\gamma$ -chain,  $\gamma_c$ , with IL-2, IL-4, IL-7, IL-9, and IL-15. IL-21 is most homologous to IL-2. These cytokines are encoded by adjacent genes, but they are functionally distinct. Whereas IL-2 promotes development of regulatory T cells and confers protection from autoimmune disease, IL-21 promotes differentiation of Th17 cells and is implicated in several autoimmune diseases, including type 1 diabetes and systemic lupus erythematosus. However, the roles of IL-21 and IL-2 in CNS autoimmune diseases such as multiple sclerosis and uveitis have been controversial. Here, we generated *Il21*-mCherry/*Il2*-emGFP dual-reporter transgenic mice and showed that development of experimental autoimmune uveitis (EAU) correlated with the presence of T cells coexpressing IL-21 and IL-2 into the retina. Furthermore, *Il21*<sup>-/-</sup> mice were more resistant to EAU development than wild-type mice, and adoptive transfer of *Il21*<sup>-/-</sup> T cells induced much less severe EAU, underscoring the need for IL-21 in the development of this disease and suggesting that blocking IL-21/ $\gamma_c$ -signaling pathways may provide a means for controlling CNS auto-inflammatory diseases.**

IL-21 is a type 1 four- $\alpha$ -helical-bundle cytokine with major actions on a range of lymphoid populations (1). IL-21 signals via IL-21R and the common cytokine receptor  $\gamma$ -chain,  $\gamma_c$  (1), which is mutated in humans with X-linked severe combined immunodeficiency (2) and is shared by the receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 (3). IL-21 is produced mainly by CD4<sup>+</sup> T cells, including Th17 cells (1, 4), T follicular helper cells (5), and natural killer (NK) T cells (6). It promotes the function and expansion of effector CD8<sup>+</sup> T cells (7) and can activate NK cells (8). It is also critical for B-cell differentiation into plasma cells and Ig production (9, 10) and negatively regulates the function of dendritic cells (11). IL-21 plays a role in autoimmune diseases, including type I diabetes in nonobese diabetic mice (12, 13), systemic lupus erythematosus in MRL/*lpr* and BXS/B*Yaa* mice (9, 14, 15), and collagen-induced arthritis (16). In experimental allergic encephalitis (EAE), divergent effects of IL-21 have been reported related to its role in the development and progression of the autoimmune response as well as whether it is required for disease development (1, 17, 18).

The genes encoding IL-21 and IL-2 are adjacent and share similar genomic organization in humans and mice (19). Coevolution of the IL-21 and IL-2 systems is further suggested not only by their sharing of  $\gamma_c$  but also by the fact that IL-21R is most closely related to IL-2R $\beta$  (20); nevertheless, IL-21 and IL-2 have distinct functions. For example, IL-21 is implicated in the development of autoimmunity (9, 12–14, 16), but mice deficient in IL-2, IL-2R $\alpha$ , and IL-2R $\beta$  exhibit autoimmunity (21–24), suggesting that IL-2 protects against development of autoimmune disease. IL-21 can promote the development of Th17 cells, whereas IL-2 inhibits the differentiation of Th17 cells (25), although it induces the expansion of Th17 cells that mediate uveitis and scleritis (26).

Uveitis is a group of sight-threatening idiopathic intraocular inflammatory diseases including Behçet's disease, birdshot retinochoroidopathy, sympathetic ophthalmia, Vogt-Koyanagi-Harada, and ocular sarcoidosis, which may be of infectious or

autoimmune etiology (27). Experimental autoimmune uveitis (EAU) shares pathological features with human uveitis, and much of our understanding of the etiology and pathophysiology of this disease derives from studies of EAU (27, 28). EAU can be induced in susceptible rodent species by immunization with the retinal protein, interphotoreceptor retinoid-binding protein (IRBP) (*Materials and Methods*). Although the etiology of non-infectious uveitis is unknown, patients with uveitis have more IL-17-producing T cells (Th17) in peripheral blood than healthy individuals (29). Moreover, Th17 cells increase during active uveitis but decrease after treatment, suggesting that they play a role in the disease process (26). Consistent with a role for Th17 cells in CNS autoimmune diseases, mice with targeted deletion of the *Stat3* gene in T cells cannot generate Th17 cells and do not develop EAU or EAE (30), indicating the importance of Th17 cells in uveitis and multiple sclerosis. Because IL-21 is produced by Th17 cells, mediates its biological actions in part via STAT3, and promotes Th17 differentiation, IL-21 could be a potential mediator of EAU.

An impediment to the study of the role of IL-21-expressing T cells in disease has been the absence of high-quality Abs for detecting in situ expression of IL-21 in tissues by intracellular staining. In this study, we generated bacterial artificial chromosome (BAC) reporter transgenic (Tg) mice in which the promoters of the adjacent *Il2* and *Il21* genes in a BAC clone spanning both genes direct expression of emGFP and mCherry, respectively. These mice allowed us to investigate in vivo the presence of IL-21 (mCherry) and/or IL-2 (emGFP)-expressing T cells in lymph nodes (LNs) and to monitor their infiltration into the retina during EAU. We detected a substantial increase in T cells expressing *Il2*-emGFP and *Il21*-mCherry in LNs before the onset of inflammation, and these cells were subsequently detected in the retina of mice with EAU, suggesting their involvement in the pathogenic mechanism of uveitis. We also found that *Il21*<sup>-/-</sup> mice have defective development of EAU, and involvement of IL-21 in this disease was further shown by adoptive transfer experiments.

## Results

**Identification of IL-21- and IL-2-Expressing Cells in Vivo Using *Il21*-mCherry/*Il2*-emGFP Reporter Mice.** To evaluate IL-21 and IL-2 gene expression, we generated *Il21*-mCherry and *Il2*-emGFP BAC dual reporter Tg mice (Fig. 1A). For most experiments, we used a

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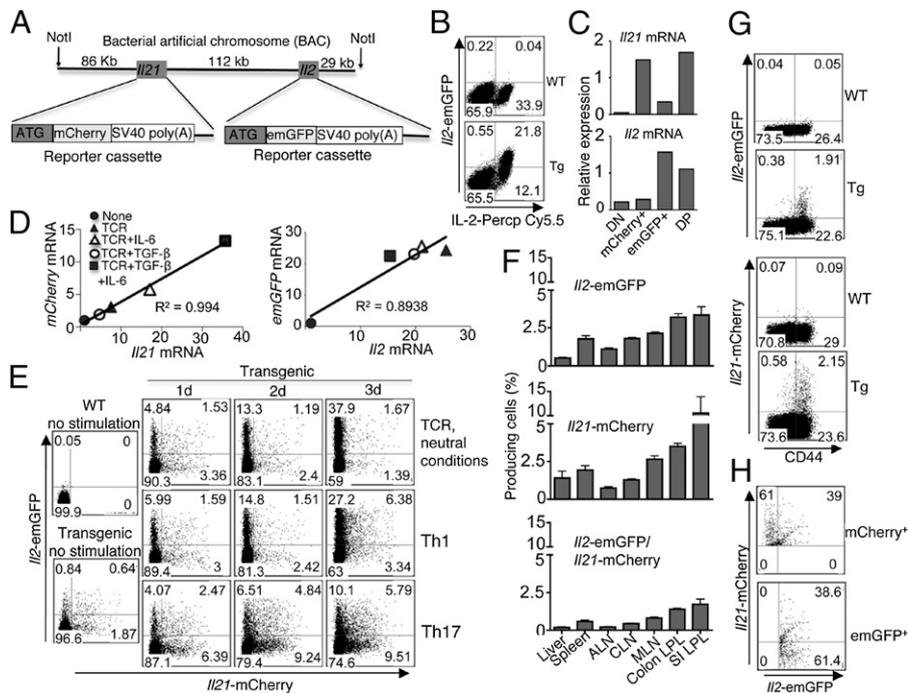
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founder line containing one integrated copy of the reporter construct, but behavior of a second independent BAC reporter mouse line was similar (Fig. S1 A and B). To validate the reporter mice, we stimulated CD4<sup>+</sup> T cells enriched from the Tg reporter and wild-type (WT) littermate mice with anti-CD3 and anti-CD28 and analyzed the expression of emGFP and IL-2 by flow cytometry (Fig. 1B). The frequency of IL-2<sup>+</sup> cells determined by intracellular staining was ~35% in both Tg reporter mice and WT controls. In the reporter mice, >95% of emGFP<sup>+</sup> cells also produced IL-2, and ~70% of IL-2-producing cells were emGFP<sup>+</sup>, even though our fixation procedure included paraformaldehyde, which can quench the emGFP fluorescence. We also stimulated CD4<sup>+</sup> T cells with anti-CD3 + anti-CD28 and sorted emGFP<sup>-</sup> and emGFP<sup>+</sup> cells. By intracellular staining, 60% of the emGFP<sup>+</sup> but only 11% of emGFP<sup>-</sup> cells were IL-2<sup>+</sup> (Fig. S2A). Moreover, we evaluated IL-2 and IL-21 mRNA expression of sorted emGFP<sup>-</sup>mCherry<sup>-</sup> double-negative (DN), emGFP<sup>+</sup> and mCherry<sup>+</sup> single-positive, and emGFP<sup>+</sup>mCherry<sup>+</sup> double-positive (DP) cells by real-time PCR. mCherry<sup>+</sup> and DP cells had ~30-fold higher IL-21 mRNA expression than DN cells. As expected, IL-21 mRNA expression in GFP<sup>+</sup> cells was weaker. emGFP<sup>+</sup> cells had higher IL-2 mRNA expression than DN and mCherry<sup>+</sup> cells, and IL-2 mRNA expression of DP cells was less than in GFP<sup>+</sup> cells but still fivefold higher than in DN cells (Fig. 1C). We also stimulated CD4<sup>+</sup> T cells with anti-CD3 + anti-CD28 with or without IL-6 and TGF- $\beta$  for 18 h and evaluated

mRNA expression of mCherry, IL-21, emGFP, and IL-2 by RT-PCR. Levels of mCherry and IL-21 mRNA were highly correlated ( $R^2 = 0.99$ ) as were emGFP and IL-2 mRNA ( $R^2 = 0.89$ ) (Fig. 1D), further validating the *I|2*-emGFP/*I|2*-mCherry reporter mice.

We next stimulated CD4<sup>+</sup> T cells with anti-CD3 + anti-CD28 under neutral conditions (presence of anti-IFN- $\gamma$  + anti-IL-4) versus Th1 or Th17 conditions and analyzed cells at days 1, 2, and 3 (Fig. 1E). emGFP as a marker for IL-2 increased under all conditions but least under Th17 conditions, whereas mCherry as a marker of IL-21 production was highest in Th17 cells. As expected, Th1 cells expressed both emGFP and mCherry, consistent with their known production of IL-21 (1) as well as of IL-2. Sorting of cells after Th1 stimulation revealed that ~28.5% of emGFP<sup>+</sup> cells produced IFN- $\gamma$  versus <2% of mGFP<sup>-</sup> cells (Fig. S2B). Similarly, sorting of cells after Th17 differentiation revealed that ~11% of the mCherry<sup>+</sup> cells produced IL-17A versus <3% of mCherry<sup>-</sup> cells (Fig. S2C).

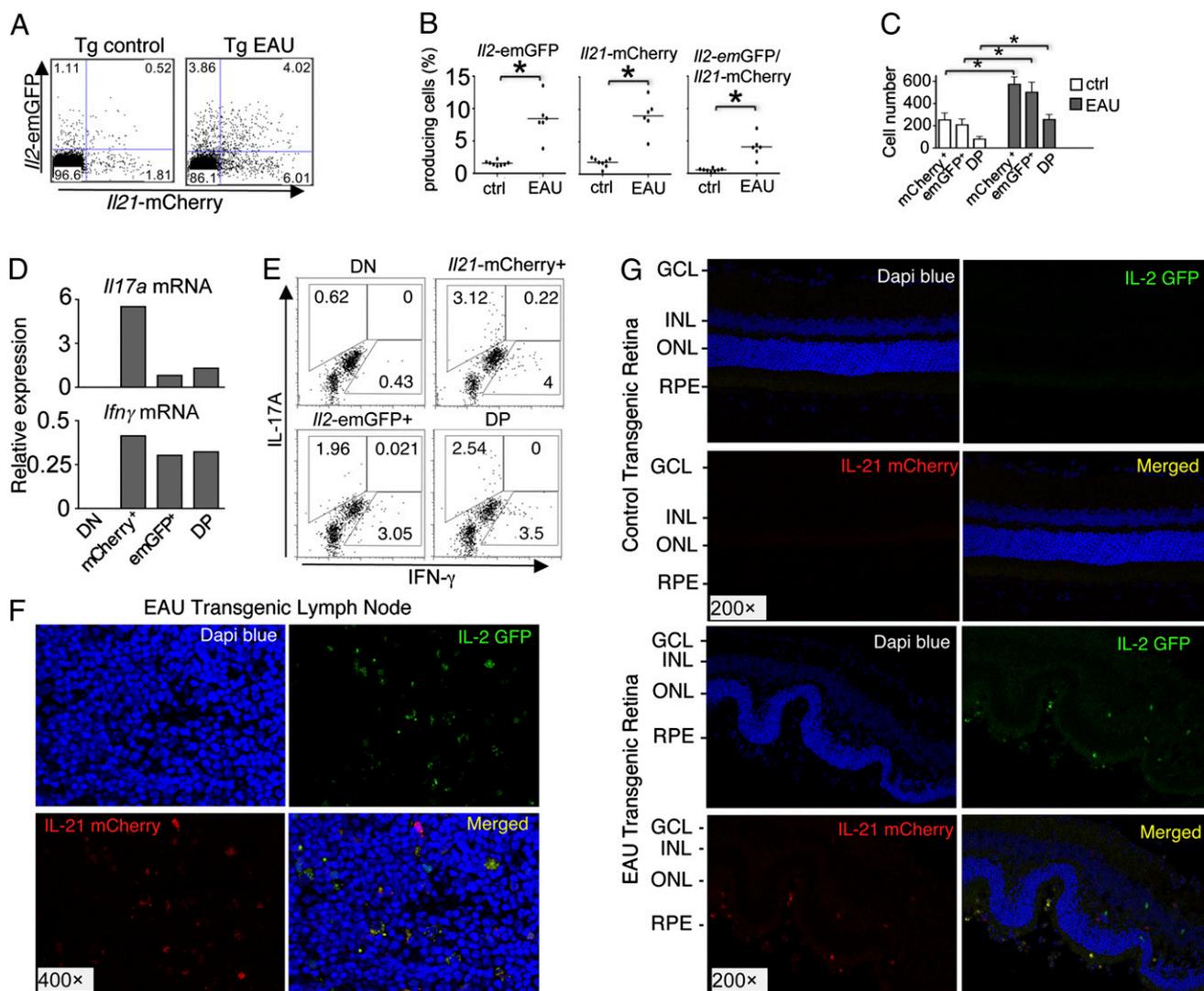
To identify IL-21- and IL-2-producing cells in vivo, we next examined *I|2*-mCherry/*I|2*-emGFP expression in liver, spleen, and other lymphoid tissues. CD4<sup>+</sup> cells that expressed mCherry, emGFP, or both were present in all tissues examined, but the frequencies of the cell subsets differed in different tissues (Fig. 1F). Approximately 1–2% of CD4<sup>+</sup> T cells in spleen, as well as axillary, cervical, and mesenteric LNs, were *I|2*-emGFP<sup>+</sup>, in contrast to ~0.5% in the liver, whereas there were more of these



**Fig. 1.** Characterization of *I|2*1-mCherry/*I|2*-emGFP reporter Tg mice. (A) Shown is a BAC containing the *I|2*1 and *I|2* genes, with mCherry and emGFP introduced. (B) Splenic CD4<sup>+</sup> T cells from control and reporter Tg mice were stimulated with anti-CD3 + anti-CD28 for 3 d, restimulated with PMA and ionomycin for 4 h, and then surface-stained for CD4 and intracellularly stained for IL-2. The plots were gated on CD4<sup>+</sup> cells. Shown is one of four experiments with similar results. (C) mCherry<sup>-</sup>emGFP<sup>-</sup> DN, single-positive (mCherry<sup>+</sup>), emGFP single-positive (emGFP<sup>+</sup>), and mCherry<sup>+</sup>emGFP<sup>+</sup> DP were sorted from CD4<sup>+</sup> T cells from the reporter Tg mice. IL-21 and IL-2 mRNAs were measured by real-time PCR. Data are from LNs pooled from 14 mice. (D) CD4<sup>+</sup> T cells from reporter Tg mice were not stimulated or were stimulated with anti-CD3 + anti-CD28, without or with IL-6, TGF- $\beta$ , or both IL-6 and TGF- $\beta$  for 18 h. IL-21, mCherry, IL-2, and emGFP mRNAs were measured by RT-PCR. Correlation of IL-21 versus mCherry mRNA and of IL-2 versus emGFP mRNA expression are shown by  $R^2$  values. Shown is one of two experiments with similar results. (E) CD4<sup>+</sup> T cells from reporter Tg mice were polarized as indicated for 3 d. Shown are *I|2*-emGFP and *I|2*1-mCherry expression at days 1, 2, and 3. Shown is one of three experiments with similar results. (F) *I|2*-emGFP and *I|2*1-mCherry expression in CD4<sup>+</sup> T cells isolated from reporter Tg mice or WT littermate controls. The bar graphs indicate data from gating on CD4<sup>+</sup> T cells. ALN, axillary lymphocytes; CLN, cervical lymphocytes; MLN, mesenteric lymphocytes; SI LPL, small intestine lamina propria lymphocytes. Shown is mean  $\pm$  SD for three independent experiments. (G) Splenocytes from reporter Tg mice were analyzed by flow cytometry for expression of CD4, CD44, emGFP, and mCherry, gated on CD4<sup>+</sup> T cells. Shown is one of three experiments with similar results. (H) Expression of mCherry and emGFP is shown. (Upper) Gated on CD4<sup>+</sup>CD44<sup>high</sup>mCherry<sup>+</sup> cells. (Lower) Gated on CD4<sup>+</sup>CD44<sup>high</sup>emGFP<sup>+</sup> cells. Shown is one of three experiments with similar results.

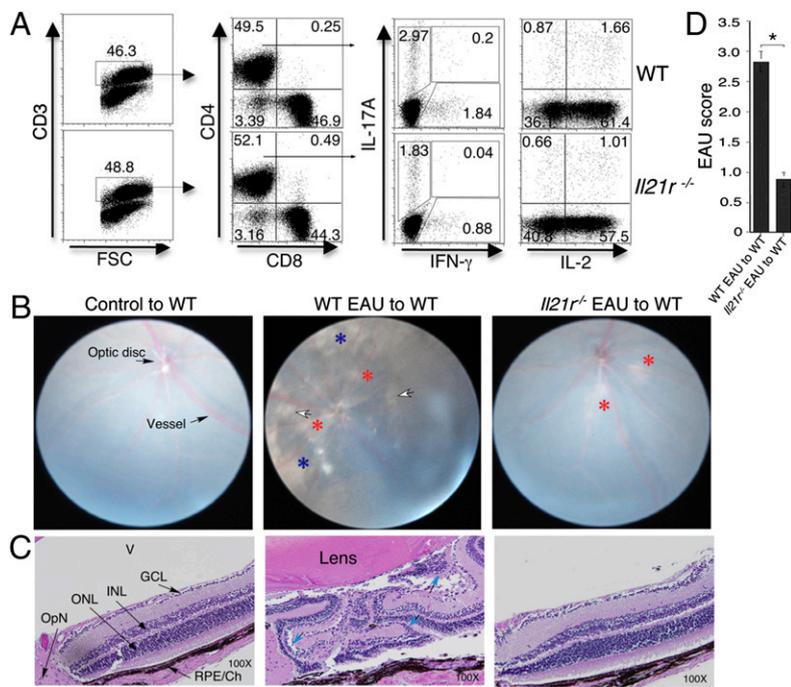
cells (3–4% of CD4<sup>+</sup> T cells) in the small intestine lamina propria lymphocytes (SI LPLs) and in the colon LPLs, consistent with higher basal stimulation in the gut (Fig. 1*F, Top*). For *Il21*-mCherry, expression tended to be lowest in liver, axillary, and cervical LNs, slightly higher in spleen, mesenteric LNs, and colon, and highest (10% of cells) in SI LPLs (*Middle*). Cells expressing both *Il2*-emGFP<sup>+</sup> and *Il21*-mCherry were detected in all tissues, but were highest in the gut (*Bottom*). Thus, *Il2*-emGFP<sup>+</sup> and *Il21*-mCherry<sup>+</sup> cells were present in all immune compartments examined, with relative enrichment in the intestine. Evaluation of splenic CD4<sup>+</sup> T cells revealed that ~80% of these *Il2*-emGFP<sup>+</sup> and *Il21*-mCherry<sup>+</sup> cells were CD44<sup>hi</sup>CD4<sup>+</sup> effector/memory type T cells (Fig. 1*G*). When we gated on CD4<sup>+</sup>CD44<sup>hi</sup>mCherry<sup>+</sup> and CD4<sup>+</sup>CD44<sup>hi</sup>emGFP<sup>+</sup> cells, 39% of *Il21*-mCherry<sup>+</sup> cells and 38.6% of *Il2*-emGFP<sup>+</sup> cells were both mCherry<sup>+</sup> and emGFP<sup>+</sup> (Fig. 1*H*).

**Augmented Expression of IL-21 and IL-2 Expression in EAU.** We next immunized the reporter Tg mice with IRBP and examined mCherry and emGFP expression at day 21, at which point mice had developed severe uveitis. After immunization, we detected *Il2*-emGFP<sup>+</sup> CD4<sup>+</sup> T cells in draining LNs (Fig. 2*A* and *Left Panel* of *B*). mCherry also increased from 2% of CD4<sup>+</sup> T cells before immunization to ~9% afterward (Fig. 2*B, Center*). After immunization, ~4% of CD4<sup>+</sup> cells were *Il21*-mCherry<sup>+</sup>/*Il2*-emGFP<sup>+</sup> DP cells (Fig. 2*B, Right*). From 50,000 LN cells for each sample, mCherry<sup>+</sup> cells increased from ~220 to ~590 cells after immunization. emGFP<sup>+</sup> cells and DP cells increased from ~200 to ~500 and from ~90 to ~210 cells, respectively (Fig. 2*C*). Similar results were obtained with a second reporter BAC Tg mouse line containing six copies of the BAC clone rather than one (Fig. *S1*). We also sorted DN, emGFP<sup>+</sup>, mCherry<sup>+</sup>, and DP cells from CD4<sup>+</sup>-draining lymphocytes 21 d after immunization



**Fig. 2.** Increased *Il2*-emGFP and *Il21*-mCherry expression after EAU induction (*A* and *B*) Expression of *Il2*-emGFP and *Il21*-mCherry in draining lymphocytes from reporter Tg mice 21 d after immunization with IRBP, gated on CD4<sup>+</sup> cells. Shown are representative mice from one of two experiments with similar results ( $n = 8$  for Tg control and  $n = 6$  for Tg EAU). (*B*) Summary of flow cytometric profiles of CD4<sup>+</sup> T cells from eight Tg control and six Tg EAU mice from the experiment in *A*. Shown are *Il2*-emGFP<sup>+</sup>, *Il21*-mCherry<sup>+</sup>, and *Il21*-mCherry<sup>+</sup>/*Il2*-emGFP<sup>+</sup> DP cells.  $*P < 0.05$ . (*C*) From the experiment in *A*, 50,000 cells for each Tg control and Tg EAU were analyzed. Shown are the number of mCherry<sup>+</sup>, emGFP<sup>+</sup>, and *Il21*-mCherry<sup>+</sup>/*Il2*-emGFP<sup>+</sup> DP cells.  $*P < 0.05$ . (*D* and *E*) mCherry<sup>-</sup> and emGFP<sup>-</sup> DN, mCherry<sup>+</sup>, emGFP<sup>+</sup>, and mCherry<sup>+</sup>emGFP<sup>+</sup> DP cells were sorted from CD4<sup>+</sup> T cells from pooled LNs from reporter Tg mice 21 d after immunization with IRBP. In *D*, IL-17A and IFN- $\gamma$  mRNAs were measured by real-time PCR. In *E*, cells were stimulated with PMA + ionomycin for 4 h, and IL-17A and IFN- $\gamma$  were measured by intracellular staining. (*F*) Frozen sections of LNs from reporter Tg mice 7 d after immunization were stained with DAPI. Red, mCherry; green, emGFP. (*G*) Frozen sections of eyes from unimmunized and immunized reporter Tg mice at day 21. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer, RPE, retinal pigment epithelial layer. Shown is one of seven experiments with similar results.





**Fig. 4.** *Il21r*<sup>-/-</sup> mice are defective in generating IRBP-specific pathogenic T cells. Draining LN cells from WT and *Il21r*<sup>-/-</sup> EAU mice were stimulated with IRBP for 4 d and then adoptively transferred into C57BL/6 WT mice. (A) WT and *Il21r*<sup>-/-</sup> EAU mice were stimulated in vitro before adoptive transfer. Percentage of CD3<sup>+</sup> and CD4<sup>+</sup> T cells are shown in the first two panels. We gated on CD4<sup>+</sup> T cells and measured the percentage of IL-17A<sup>+</sup>, IFN- $\gamma$ <sup>+</sup>, and IL-2<sup>+</sup>-producing cells (last two panels). (B) Fundoscopy was performed 14 d later. White arrows, inflammatory infiltrates; red asterisks, retinal vasculitis with cuffing; blue asterisks, severe retinal infiltrates and scars and choroiditis. (C) Histological sections of eyes of EAU-adoptively transferred C57BL/6 WT mice, as indicated. Blue arrows, retinal fold. (D) Shown is the EAU clinical score of adoptive transfer EAU based on funduscopy images by the Mann-Whitney test. Shown is mean  $\pm$  SEM. \**P* < 0.01. *n* = 4 mice in each group.

IL-17 and IFN- $\gamma$  production in *Il21r*<sup>-/-</sup> mice, but IL-2 production was similar in WT and *Il21r*<sup>-/-</sup> mice (Fig. 3 *F* and *G*). Moreover, when draining LN cells were harvested at day 21 and then stimulated with IRBP for 3 d, there was less IL-17A and IL-1 $\beta$  but more IL-10 production in *Il21r*<sup>-/-</sup> mice (Fig. 3*H*). IL-6 production tended to be lower, but the difference was not significant (Fig. 3*H*). Overall, these data indicate a possible shift from pro- to anti-inflammatory cytokines in the absence of IL-21 signaling.

**Adoptive Transfer of IRBP-Specific Uveitogenic Lymphocytes from *Il21r*<sup>-/-</sup> Mice Induces Less Severe EAU than WT Cells.** To confirm that the *Il21r*<sup>-/-</sup> mouse defect occurred in the generation of IRBP-specific pathogenic T cells, we adoptively transferred WT and *Il21r*<sup>-/-</sup> IRBP-specific lymphocytes into WT mice. We found similar CD3 and CD4/CD8 profiles (Fig. 4*A*, *Left* and *Center* panels), but the *Il21r*<sup>-/-</sup> cells produced less IL-17A and IFN- $\gamma$  but similar levels of IL-2 (Fig. 4*A*). Compared with mice receiving WT cells, animals receiving *Il21r*<sup>-/-</sup> cells exhibited less severe EAU as evaluated by funduscopy (Fig. 4*B*), histological analysis (Fig. 4*C*), and clinical EAU score (Fig. 4*D*).

## Discussion

In this study, we investigated the role of IL-21 in EAU development. The expression of IL-21 in draining LNs and retina, as revealed by the *Il21*-mCherry/*Il2*-emGFP reporter mice; the diminished development of EAU in *Il21r*<sup>-/-</sup> mice; and the less severe EAU following adoptive transfer of IRBP-specific lymphocytes from *Il21r*<sup>-/-</sup> mice together underscore the importance of IL-21 in the development of EAU. WT mice mounted a higher IL-17 response than did *Il21r*<sup>-/-</sup> mice. Given that IL-21 can promote Th17 cell differentiation (1), it is possible that the lack of IL-21 signaling protects mice from EAU through a mechanism involving decreased production of IL-17, which has been suggested to be important for the development of both EAU (26, 29) and EAE (17). Previously, IL-21 was shown to play important roles in animal models of type 1 diabetes (12, 13) and systemic lupus erythematosus (14, 15). Our data now implicate this cytokine in a model of autoimmunity causing disease at an immunologically "privileged" site as well, revealing its very broad contribution to autoimmunity.

By analyzing the location of *Il21*-mCherry<sup>+</sup> and *Il2*-emGFP<sup>+</sup> cells after inducing EAU, we infer that immunization by IRBP caused an immune response that produced both IL-21 and IL-2 in draining LNs, and both IL-21<sup>-</sup> and IL-2<sup>-</sup>-producing cells were located in the eye at day 21, when severe inflammation is evident. Previously, IL-2 was reported to enhance protection from EAU in part by stimulating production of anti-inflammatory cytokines by regulatory T cells (33). The ability of IL-2 to inhibit Th17 differentiation (25) is another mechanism by which IL-2 might inhibit EAU. The fact that there were both IL-2<sup>-</sup> and IL-21<sup>-</sup>-expressing cells in the inflammatory neuroretina in EAU is interesting, as both overlapping and distinctive actions for these cytokines have been noted (1, 34). Interestingly, IL-2 can expand Th17 cells once developed, potentially promoting uveitis and scleritis (26), and Daclizumab, a monoclonal antibody to IL-2R $\alpha$ , can control inflammation in patients with noninfectious uveitis (35, 36), suggesting a role for IL-2 in the pathogenesis of this disease. Our data showing the presence of IL-2/IL-21 double-producer T cells in EAU extend these data.

In summary, we have shown a key role of IL-21 in the development of EAU, suggesting that interfering with the action of this cytokine may have therapeutic potential for uveitis. Moreover, we have generated *Il21*-mCherry/*Il2*-emGFP double reporter Tg mice to help study the role of IL-2 and IL-21 in the pathogenesis of EAU. These mice may be valuable for studying the potential roles for IL-2 and IL-21 in a broad range of other disease models as well.

## Materials and Methods

**Mice.** *Il21r*<sup>-/-</sup> mice (10) were produced by breeding heterozygous mice. C57BL/6 mice were from the Jackson Laboratory. To generate *Il2*-emGFP/*Il21*-mCherry Tg reporter mice, we used the RP23-98115 BAC clone (Invitrogen), which contains the *Il21* and *Il2* genes (Fig. 1*A*). Recombineering (37) was used to introduce the emGFP (Invitrogen) and mCherry (38) coding sequences linked to the SV40 poly(A) site into the first exon of the *Il2* and *Il21* genes, respectively. Recombineering comprises a two-step method to create precise genetic changes: first, *amp-sacB* is placed in the DNA and then this is replaced with the emGFP or mCherry coding sequences in a second recombineering event. The targeting cassettes with *amp-sacB* were amplified using primers with  $\geq 50$  bp 5' overhangs that were homologous to the regions of the BAC just outside of the segment being replaced. The primers

are provided in *SI Materials and Methods*. BAC clones that had integrated the targeting construct were selected, and the location of the insert was confirmed by PCR and sequencing. The BAC was prepped using a Large-Construct kit (Qiagen), digested with NotI, DNA purified by phenol/chloroform extraction, and microinjected into fertilized C57BL/6J × CBA/J oocytes. Resulting pups were screened for the emGFP and mCherry reporters by PCR (see *SI Materials and Methods* for the primers). Founder lines were backcrossed at least six generations to C57BL/6 mice. Experiments were performed under protocols approved by the National Eye Institute and/or National Heart, Lung, and Blood Institute Animal Care and Use Committees.

**Analysis of CD4<sup>+</sup> T-Helper Cells.** For Th1 conditions, CD4<sup>+</sup> T cells (>98% pure) were treated with plate-bound 2 μg/mL anti-CD3 + soluble 1 μg/mL anti-CD28, 10 μg/mL anti-IL-4, and 10 ng/mL IL-12 for 3 d. For Th17 conditions, cells were treated with anti-CD3/CD28, 20 ng/mL IL-6, 2 ng/mL TGF-β1, and 10 μg/mL each of anti-IFN-γ and anti-IL-4. Cytokines and antibodies were from R & D Systems. For intracellular cytokine detection, cells were stimulated for 4 h with 10 ng/mL phorbol 2-myristate 3-acetate (PMA) + 1 μM ionomycin, Golgi-stop added in the last 2 h, and intracellular cytokine staining performed using a Cytofix/Cytoperm kit (BD Pharmingen). Flow cytometry was performed using a FACS-Calibur (BD Biosciences). All mAbs were from BD Pharmingen. Flow cytometry was performed on an LSRII (BD Biosciences). mCherry was detected using a 561-nm laser with a 605/40 filter.

**Real-Time PCR.** mRNA was isolated from 10<sup>6</sup> cells (RNeasy mini kit; Qiagen) and cDNA was prepared (Omniscript RT kit, Qiagen). Real-time PCR was performed with an ABI PRISM 7700 sequence detection system with site-specific primers and probes (Applied Biosystems).

**Induction and Evaluation of EAU.** Mice were immunized with 150 μg bovine IRBP and 300 μg human IRBP peptide (amino acids 1–20) in 0.2 mL 1:1 vol/vol emulsion with complete Freund's adjuvant (CFA) containing *Mycobacterium*

*tuberculosis* strain H37RA (2.5 mg/mL). Mice simultaneously received 0.3 μg *Bordetella pertussis* toxin. Clinical disease was scored by funduscopy (31) and histological analysis (32), as detailed in *SI Materials and Methods*. Tissue sections were also examined using a multiphoton laser-scanning confocal microscope with fluorescence emission detected with 515/30 (emerald-GFP, green) and 600/40 (mCherry, red), as described (26, 31). For adoptive transfer experiments, draining LN cells from WT and *IL21*<sup>−/−</sup> EAU mice (at day 21) were stimulated with IRBP 20 μg/mL for 4 d, and 10<sup>7</sup> cells in 200 μL of PBS were injected i.v. into sex- and age-matched recipient mice. Fundoscopic examinations were performed 14 d after adoptive transfer.

**Cytokine Analysis and Lymphocyte Proliferation Assays.** A total of 5 × 10<sup>6</sup> draining LN cells were cultured for 3 d in medium containing 10 μg/mL IRBP protein, and IL-6, IL-10, IL-1β, and IL-17 levels were measured using an ELISA kit (BD Pharmingen). For proliferation, normal control and EAU draining lymphocytes (2 × 10<sup>6</sup>/mL) were stimulated with 20 μg/mL IRBP. After 48 h, cultures were pulsed with [<sup>3</sup>H]thymidine (0.5 μCi/10 μL/well) and incorporation was measured 12 h later.

**Statistical Analyses.** Nonparametric *t* tests and Prism software (Graphpad Software) were used.

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