Characterization of Autoreactive and Bystander IL-17⁺ T Cells Induced in Immunized C57BL/6 Mice

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PURPOSE. To characterize antigen-specific and bystander IL-17⁺ T cells induced in immunized mice.

METHODS. C57BL/6 (B6) mice were immunized with the uveitogenic peptide IRBP₁₋₂₀ in either incomplete (IFA) or complete (CFA) Freund's adjuvant. In vivo-primed T cells were stimulated with syngeneic APCs, with or without the immunizing peptide, under polarizing conditions. Activated T cells were analyzed for expression and production of IL-17.

RESULTS. B6 mice immunized with the uveitogenic peptide IRBP₁₋₂₀ generated two types of IL-17⁺ T cell: one specific for the immunizing autoantigen (IRBP-Th17) and a much more abundant type (bystander-Th17) that is not reactive with the immunizing antigen. The bystander-Th17 can be demonstrated when in vivo-primed T cells are cultured in Th17-polarizing conditions in the absence of antigen stimulation. Increased expansion of both types of Th17 cells was seen in mice immunized with IRBP₁₋₂₀/CFA, but not with IRBP₁₋₂₀/IFA. Both T-cell types produced IL-17, IL-22, and IFN- γ , but only bystander Th17 cells produced IL-10. Addition to culture medium of IL-6 and TGF- β 1 caused more activation of bystander-Th17 T cells than IRBP-Th17 cells. When adoptively transferred into syngeneic naïve mice, the bystander-Th17 cells neutralized the pathogenic activity of the IRBP-Th17 cells.

CONCLUSIONS. A procedure commonly used to induce autoimmune disease promotes two functionally antagonistic types of IL-17⁺ T cells, and the pathogenic type is restricted to the population that specifically responds to the immunizing autoantigen. Molecular components of the CFA, rather than the immunizing peptide, promote the generation of both types of IL-17⁺ T cells. (*Invest Ophthalmol Vis Sci.* 2012;53:897-905) DOI:10.1167/iovs.11-8297

Although there is little doubt that autoreactive T cells expressing IL-17 are crucially involved in the pathogenesis of autoimmune diseases,¹⁻⁷ the term Th17 cells in autoimmune studies has been used inconsistently, and the heterogeneity of

IL-17⁺ T cells has not received sufficient attention. Although some studies focused on IL-17⁺ T cells that specifically react to an immunizing autoantigen, others examined IL-17⁺ T cells that are activated by anti-CD3 antibody.⁸⁻¹⁰ In this study, we isolated two types of IL-17⁺ T cells from B6 mice immunized with interphotoreceptor retinoid-binding protein peptide IRBP₁₋₂₀ and complete Freund's adjuvant (CFA), one type being IL-17⁺ T cells specific for the inducing peptide (IRBP-Th17), which expand only when stimulated with the immunizing peptide under Th17 polarized conditions, and the other IL-17⁺ T cells that can be expanded by the cytokine IL-23 in the absence of the immunizing antigen (bystander-Th17). We compared these two cell types and showed that, although both were capable of producing IL-17, IL-22, and IFN- γ , the uveitogenic T cells were exclusively contributed by the IRBP-specific IL-17⁺ T cells and the bystander IL-17⁺ T cells inhibited the function of the uveitogenic T cells.

We have reported that the microbial antigens in CFA and pertussis toxin (PTX), but not the immunizing antigen IRBP₁₋₂₀, are major contributing factors in the generation of IRBP-Th17.^{11,12} In the present study, IRBP-Th17 and bystander-Th17 T cells increased significantly in mice immunized with IRBP₁₋₂₀ in CFA, but not in those immunized with IRBP₁₋₂₀ plus incomplete Freund's adjuvant (IFA), compared to nonimmunized mice, suggesting that microbial proteins in CFA promote the activation of both types of IL-17⁺ T cell in vivo. The combination of IL-6 and TGF- β 1 was more effective in activating bystander-Th17 cells than IRBP-Th17 cells, suggesting that, although both sets of IL-17⁺ T cells share some activation requirements, there are also environmental factors that selectively activate different IL-17⁺ T cell subsets.

METHODS

Animals and Reagents

Pathogen-free female C57BL/6 (B6) (12-14 weeks old) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were housed and maintained in the animal facilities of the University of Southern California. Institutional approval was obtained, and institutional guidelines regarding animal experimentation and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research were followed. Recombinant murine IL-2, IL-6, IL-23, and TGF- β were purchased from R&D Systems. (Minneapolis, MN). FITC-conjugated anti-IL-17 antibody was purchased from Biolegend (San Diego, CA). All other antibodies were from BD Biosciences (La Jolla, CA).

Preparation of IRBP₁₋₂₀-Specific T Cells

B6 mice were immunized subcutaneously over six spots at the tail base and on the flank with 200 μ L of emulsion containing 200 μ g IRBP₁₋₂₀ (amino acids 1-20 of IRBP; Sigma-Aldrich, St. Louis, MO) in either IFA or CFA (Sigma-Aldrich). At day 13 after immunization (pi), T cells were isolated from lymph node cells and spleen cells by passage through a nylon wool column, and 1×10^7 cells in 2 mL of RPMI 1640 medium

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TABLE 1. Grading of EAU by Funduscopy

Score	Description
0.5	A few small, peripheral, focal, chorioretinal lesions and/or minimal vasculitis/vitritis
1.0	Mild vasculitis, a few small focal chorioretinal lesions, and/or linear chorioretinal lesions
2.0	Multifocal chorioretinal lesions, severe vasculitis, and/or a few linear chorioretinal lesions
3.0	Pattern of linear chorioretinal lesions; large, confluent chorioretinal lesions; and/or subretinal hemorrhage, and/or optic nerve blurring
4.0	Marked retinal detachment and/or retinal atrophy

in a six-well plate (Costar; Corning, Corning, NY) were stimulated for 48 hours with 10 $\mu\text{g/mL}$ of IRBP₁₋₂₀ in the presence of 1×10^7 irradiated syngeneic spleen cells (antigen-presenting cell [APCs]) in the presence of either IL-2 or -23 (10 ng/mL), then activated T-cell blasts were separated by density gradient centrifugation (Ficoll; GE Healthcare, Piscataway, NJ) and cultured for another 72 hours in the same medium that was used for stimulation without the peptide.

Cell Proliferation and Cytokine Assays

Enriched T cells (3×10^4 cells/well) from the draining lymph nodes and spleens prepared by nylon wool adherence were cultured at 37°C for 48 hours in 96-well microtiter plates with irradiated syngeneic spleen APCs (1×10^5) in the presence or absence of IRBP₁₋₂₀. The plates were then pulsed for 6 hours with 0.5 μCi of [³H]thymidine/well and the cells assessed for isotope incorporation (Packard Bioscience, Meriden, CT). The proliferative response was expressed as the mean counts per million $\pm$ SD of triplicate determinations.

Cytokine assays were conducted in 24-well plates, with 1×10^6 T cells/well seeded into each well, together with syngeneic APCs and IRBP₁₋₂₀ or anti-CD3 Ab. Culture supernatants were analyzed for cytokine production with ELISA kits (R&D Systems).

Test of Uveitogenic Activity of IL-17⁺ T Cells by Adoptive Transfer to Syngeneic Naïve Mice

T cells were enriched from a single-cell suspension of pooled spleen and draining lymph node cells from IRBP₁₋₂₀-immunized mice by passing through nylon wool columns, and then 1×10^7 T cells/well were seeded into six-well plates, together with syngeneic APCs (irradiated spleen cells) and either 10 $\mu\text{g/mL}$ of IRBP₁₋₂₀ (to obtain IRBP-specific IL-17⁺ T cells) or 1 $\mu\text{g/mL}$ of anti-CD3 Abs (to obtain nonspecific IL-17⁺ T cells) under Th17 polarizing conditions (culture medium supplemented with IL-23). After 2 days, activated T-cell blasts were separated on a centrifugation gradient (Ficoll; GE Healthcare) and injected (2×10^6 , IP) into naïve B6 mice.

Scoring of Experimental Autoimmune Uveitis

The mice were examined three times a week for clinical signs of experimental autoimmune uveitis (EAU) by indirect funduscopy. The pupils were dilated with 0.5% tropicamide and 1.25% phenylephrine hydrochloride ophthalmic solutions, and funduscopy grading of disease was performed using the scoring system reported by Thurau et al.¹³ (Table 1). For histopathologic evaluation, whole eyes were collected at the end of the experiment and immersed for 1 hour in 4% glutaraldehyde in phosphate buffer [pH 7.4] and transferred to 10% formaldehyde in phosphate buffer until processed. The fixed and dehydrated tissues were embedded in methacrylate, and 5- μm sections were cut through the pupillary-optic nerve plane and stained with hematoxylin and eosin. The presence or absence of disease was evaluated by blinded observers who examined six sections cut at different levels for each eye. Disease severity was graded pathologically on the basis of cellular infiltration and structural changes¹⁴ (Table 2).

Isolation of Eye-Infiltrating Cells (EICs) from Inflamed Eyes

At 10 days after infection, the animals were perfused with phosphate-buffered saline (PBS), the eyes were collected, and the lens and cornea were removed. A single-cell suspension was prepared by digestion for 10 minutes at 37°C with collagenase (1 mg/mL) and DNase (100 $\mu\text{g/mL}$) in RPMI 1640 medium, followed by gradient centrifugation on 25% low-viscosity density gradient (Percoll; GE Healthcare) and subsequent separation (Ficoll; GE Healthcare). The EICs obtained using this protocol consisted of inflammation-recruited immune cells, as well as a minor population of intraocular resident macrophages and dendritic cells.

Carboxyfluorescein Succinimidyl Ester (CFSE) Staining

As previously described,¹⁵ the cells were washed and resuspended at 50×10^6 cells/mL in serum-free RPMI 1640 medium, incubated at 37°C for 10 minutes with gentle shaking with a final concentration of 5 μM CFSE, washed twice with and resuspended in RPMI 1640 medium containing 10% FCS, stimulated with peptides and irradiated APCs, and analyzed by flow cytometry.

Intracellular Staining and FACS Analysis

For intracellular staining, T cells (2×10^5 in 100 μL) were exposed to 50 ng/mL of PMA, 1 $\mu\text{g/mL}$ of ionomycin, and 1 $\mu\text{g/mL}$ of brefeldin A (Sigma-Aldrich) for 4 hours and then were washed, fixed, and permeabilized overnight with buffer (Cytofix/Cytoperm; eBioscience, San Diego CA), intracellularly stained with antibodies against IFN- γ and IL-17, and analyzed on a flow cytometer (FACSCalibur; BD Biosciences).

Enzyme-Linked Immunosorbent Assay

IL-17 and IFN- γ were measured with commercially available ELISA kits (R&D Systems).

Statistical Analysis

Experiments were repeated at least twice, usually three or more times. Experimental groups were typically composed of four mice. The figures show data from a representative experiment. Differences between the values for different groups were examined by the two-tailed *t*-test. Statistical analyses of clinical scores were performed using one-way ANOVA with Tukey-Kramer post hoc analysis. $P < 0.05$ was considered significant.

RESULTS

Both IRBP-Specific and Bystander IL-17⁺ T Cells Are Induced in IRBP₁₋₂₀-Immunized B6 Mice

To determine the factors that are important in the generation of Th17-autoreactive T cells in EAU, B6 mice were immunized with IRBP₁₋₂₀ emulsified in CFA, and splenic T cells from the immunized mice were stimulated in vitro with syngeneic APCs (irradiated spleen cells) in the presence or absence of IRBP₁₋₂₀.

TABLE 2. Grading of EAU by Histology

Score	Cell Infiltration	Structural Changes
0.5	Mild	None
1.0	Moderate	Focal retinal folding
2.0	Medium	Retinal folding and detachment; focal photoreceptor cell damage
3.0	Heavy	Extensive retinal folding with detachment; serous exudates and subretinal bleeding; moderate photoreceptor cell damage
4.0	Very heavy	Extensive photoreceptor cell damage, retinal layer atrophy

in culture medium alone or supplemented with IL-2 or -23, and then proliferation, IL-17 production, and intracellular expression of IFN- γ and IL-17 were measured. Both proliferation (Fig. 1A) and IL-17 production (Fig. 1B) were antigen-dependent. Although neither cytokine altered the proliferative response significantly (Fig. 1A), IL-17 production was IL-23 dependent (Fig. 1B). However, even in the absence of antigenic stimulation, the frequency of IL-17⁺ T cells increased significantly among the in vivo-primed responder T cells when cultured in medium containing IL-23 (Fig. 1D), but not in the absence of IL-23 (Fig. 1F). In addition, mice immunized with either Ag/CFA or CFA alone, in the absence of specific antigen (Figs. 1I, 1J), generated an increased number of IL-17⁺ T cells, whereas the T cells from naïve mice (Fig. 1G) and Ag/IFA-immunized mice (Fig. 1H) failed to do so. It appears that activated IL-17⁺ T cells

in immunized mice are not limited to those that are specific for the immunizing antigen. They also included cells that were not reactive to the immunizing antigen and, whereas the number of both types of IL-17⁺ T cell increased (Figs. 1C, 1D) when cultured in IL-23-containing medium, the Th17⁺ cells that responded in the absence of immunizing antigen were mainly detectable by intracellular cytokines, but not by cytokine secretion or thymidine incorporation.

Next, we stimulated the in vivo-primed T cells, either with the immunizing IRBP₁₋₂₀ (10 μ g/mL) or with anti-CD3 Abs (1 μ g/mL) under Th17 polarizing conditions and separated the activated T cells by gradient centrifugation. After culture in IL-23-containing medium for 7 days, both types of T cell were tested for their responses to IRBP₁₋₂₀ and anti-CD3 antibody. As shown in Figure 2A, the IRBP-expanded Th17 cells (left) re-

FIGURE 1. IRBP-specific and IRBP-nonreactive IL-17⁺ T cells are induced in IRBP₁₋₂₀-immunized B6 mice. B6 mice were immunized with IRBP₁₋₂₀ emulsified in CFA. Splenic T cells from the immunized mice were then stimulated in vitro with syngeneic APCs (irradiated spleen cells) in the presence or absence of IRBP₁₋₂₀ in culture medium, with or without IL-2 or IL-23. (A) Thymidine incorporation test. Enriched T cells (3×10^4 /well) were cultured for 48 hours in 96-well microtiter plates with APCs (1×10^5) with or without IRBP₁₋₂₀; the plates were pulsed for 6 hours with 0.5 μ Ci [³H]thymidine/well, and the cells were assessed for isotope incorporation. The proliferative response was expressed as the mean counts per million $\pm$ SD of triplicate determinations. (B) Cytokine production assay. The 48-hour culture supernatants above were tested for IL-17 production by ELISA. (C–F) Intracellular cytokine staining (ICS) for IL-17⁺ T cells. Responder T cells (1×10^6 /well) from IRBP₁₋₂₀-immunized B6 mice were stimulated for 2 days with (C, E) or without (D, F) immunizing antigen, and APCs under Th17 polarized conditions were stimulated without (E, F) or with (C, D) 10 ng/mL IL-23. The cells were examined for cytokine staining after 3 days. (G–J) ICS showing that mice immunized with CFA alone (J) generated a comparably increased number of IL-17⁺ T cells as did those immunized with IRBP/CFA (I).

FIGURE 2. Comparison of IRBP-Th17 and bystander-Th17 cells. (A) IRBP-Th17 but not bystander-Th17 T cells responded to the immunizing uveitogenic peptide (IRBP₁₋₂₀). IRBP-Th17 cells (*left*) and bystander-Th17 cells (*right*) were stimulated in vitro with immunizing antigen (10 μ g/mL) or anti-CD3 Abs (2 μ g/mL) in the presence of syngeneic APCs (irradiated spleen cells) for 2 days. (B) Cytokine assays were conducted in 24-well plates. T cells (1×10^6) were seeded into each well, together with syngeneic APCs and IRBP₁₋₂₀ or anti-CD3 Ab, respectively. IL-17, IFN- γ , IL-22, and IL-10 levels in the culture supernatant of IRBP-expanded Th17 and anti-CD3 Ab-expanded Th17 cells measured by ELISA after 2 days of in vitro stimulation with IRBP₁₋₂₀ or anti-CD3 Ab, respectively. (C). The production of IL-17 by IRBP-expanded Th17 cells but not by anti-CD3-expanded Th17 cells relied on exogenous cytokines. The IRBP-Th17 cells (*top*) and bystander-Th17 cells (*bottom*) were stimulated with IRBP₁₋₂₀ or anti-CD3 Ab, respectively, in the presence or absence of the indicated cytokine (10 ng/mL), for 48 hours, and IL-17 in the culture supernatant was measured by ELISA.

acted both to the immunizing IRBP₁₋₂₀ and anti-CD3 Ab, whereas the anti-CD3 Ab-expanded Th17 cells responded only to anti-CD3 Ab (right). Cytokine production assays showed that both types of T cells produced IL-17, IL-22, and IFN- γ , but only the expanded Th17 cells produced IL-10 (Fig. 2B), when the cytokines were assessed after stimulation of IRBP of IRBP-Th17 T cells and anti-CD3 antibody of bystander Th17 cells, respectively. In addition, although IL-17 production by the IRBP-expanded cells relied on exogenous cytokines (Fig. 2C), that of the anti-CD3 Ab-expanded cells did not (Fig. 2C).

The Number of Both IRBP-Th17 and CD3-Th17 Cells Is Greatly Increased in Mice Injected with IRBP₁₋₂₀ in CFA, but Not in Mice Injected with IRBP₁₋₂₀ in IFA

To determine whether mice subjected to different immunizing procedures would generate IRBP-Th17 and bystander-Th17 cells in different ratios, B6 mice were immunized with IRBP₁₋₂₀

in IFA (Fig. 3B) or IRBP₁₋₂₀ in CFA, with (Fig. 3D) or without PTX (Fig. 3C; 200 ng/mouse, IP); control mice were not immunized (Fig. 3A). T cells from naïve or immunized mice were then stimulated in vitro with either IRBP₁₋₂₀ or anti-CD3 Abs (1 μ g/mL) under Th17 polarizing conditions, the frequency of the IL-17⁺ T cells in each responder T-cell group determined by intracellular staining (Figs. 3A–D), and their cytokine production measured (Figs. 3E, 3F). IRBP₁₋₂₀/CFA-immunized mice (Fig. 3C) generated a significantly larger number of IL-17⁺ T cells when stimulated in vitro with either immunizing antigen (IRBP₁₋₂₀) or anti-CD3 Ab than did mice immunized with IRBP₁₋₂₀/IFA (Fig. 3B) or naïve mice (Fig. 3A). IL-17 assays showed that cytokine production by both types of IL-17⁺ T cells depended on the use of CFA (Figs. 3E, 3F), and additional treatment with PTX further enhanced the response (Fig. 3D). These results suggest that components of the CFA and/or PTX, rather than the immunizing peptide, promote the in vivo activation of both types of IL-17⁺ T cells.

FIGURE 3. The number of both IRBP-Th17 and bystander-Th17 cells were greatly increased in mice immunized with IRBP₁₋₂₀/CFA, but not IRBP₁₋₂₀/IFA. (A–D). B6 mice were immunized with the uveitogenic peptide (IRBP₁₋₂₀) in IFA or CFA with or without PTX treatment, then, 13 days later, splenic T cells were prepared and stimulated with immunizing antigen (IRBP₁₋₂₀) (left) or anti-CD3 Ab (right) for 5 days. The activated T cells were separated by Ficoll gradient centrifugation and stained with PE-anti-IFN- γ or FITC-anti-IL-17 antibodies, followed by FACS analysis. (E). Splenic T cells (1×10^6 /24-well plate) from B6 mice immunized with the uveitogenic peptide (IRBP₁₋₂₀) in IFA or CFA were stimulated with the immunizing antigen (IRBP₁₋₂₀) (top) or anti-CD3 Ab (bottom) for 48 hours, then IL-17 in the culture supernatants was measured by ELISA.

Bystander-Th17 Cells Neutralizes the Uveitogenic Activity of IRBP-Th17 Cells

To determine whether bystander-Th17 cells play a role in the pathogenesis of EAU, we separately prepared IRBP-Th17 and bystander-Th17 cells from immunized mice, activated them, and injected them IP into naïve B6 mice, either separately or together. Splenic T cells were harvested 13 days after immunization and were adoptively transferred after 3 days of in vitro stimulation. As shown in Fig. 4, recipient mice injected with 2×10^6 IRBP-Th17-specific T cells developed EAU 8 days after cell transfer (Figs. 4A, 4D), whereas the same number or even four times more bystander-Th17 cells failed to induce EAU (Fig. 4B). In mice injected with 2×10^6 IRBP-Th17 cells and 8×10^6 bystander-Th17 cells, the symptoms of EAU were significantly delayed and ameliorated (Figs. 4C, 4D), suggesting that bystander-Th17 T cells ameliorate, rather than exacerbate, EAU.

IL-6 and TGF- β 1 Cause More Activation of Bystander-Th17 T Cells Than of IRBP-Th17 Cells

Studies have shown that cytokines, such as IL-6 and TGF- β , are important in promoting the activation of IL-17⁺ T cells.^{16–19} To determine whether the two types of IRBP-Th17 cells differ in their activation requirements, we measured their responses in the presence of the cytokines, IL-23, IL-6, and TGF- β 1. As shown in Figure 5, IL-23 pro-

moted the activation (IL-17-positivity) of both IRBP-Th17 and CD3-Th17 cells, whereas IL-6 and TGF- β 1 alone had little effect. In combination, IL-6 plus TGF- β 1 significantly promoted the activation of bystander-Th17 cells, but had only a marginal effect on IRBP-Th17 cells. However, both IL-6 and TGF- β 1 had a synergistic effect with IL-23 in promoting IRBP-Th17 and bystander-Th17 cell activation.

Mice with Transferred Bystander-Th17 T Cells Have Depressed Immune Responses

To further explore the mechanism by which transfer of bystander-Th17 cells to recipient mice ameliorates EAU, we compared immune responses in recipient mice that had received 2×10^6 IRBP-Th17 cells, alone or combined with bystander-Th17 cells. We found a significant decrease in the number of IL-17⁺ and IFN- γ ⁺ T cells in mice that had received both bystander-Th17 and IRBP-Th17 cells, compared with those that had received only IRBP-Th17 cells (Figs. 6A–C). Moreover, T cells in the mice that received both IRBP-Th17 cells and bystander-Th17 cells responded poorly to the uveitogenic peptide IRBP₁₋₂₀ (Fig. 6E) and produced less IL-17 (Fig. 6F) or IFN- γ (Fig. 6G) after in vitro stimulation with the peptide than did the mice that received only IRBP-Th17 cells. In addition, the bystander-Th17 cells contained a higher number (14.7%) of Foxp3⁺ T cells than IRBP-Th17 cells (2.3%; Fig. 6H).

FIGURE 4. Bystander-Th17 cells neutralized the uveitogenic activity of IRBP-Th17 cells. Splenic T cells enriched from B6 mice immunized with the uveitogenic peptide IRBP₁₋₂₀ were stimulated in vitro with either the immunizing antigen (IRBP-Th17) or anti-CD3 Ab (bystander-Th17) in the presence of irradiated spleen cells (APCs) under Th17 polarizing conditions (culture medium supplemented with 10 ng/mL of IL-23), and the activated T cells were adoptively transfer to groups ($n = 4$) of naïve B6 mice, either alone (A, B; 2×10^6 of IRBP-Th17 or 8×10^6 of bystander-Th17 cells) or in combination (C, D); EAU was scored by histologic examination (A-C) or funduscopy (D). The results are representative of those from three experiments.

Most IL-17⁺ T Cells among EICs in the Inflamed Eye Are Nonspecifically Recruited

We determined the eye-penetrating ability of uveitogenic T cells by adoptive transfer of a pathogenic dose (2×10^6 /mouse) of T cells to naïve mice, followed by assessment of the number of pathogenic T cells that entered the diseased eyes. The results demonstrated that, at the peak of eye inflammation (10 days after cell administration), 20% of the EICs in the diseased eyes expressed IL-17 (Fig. 7A). To distinguish the transferred pathogenic T cells from those recruited from recipient mice, we labeled the preactivated uveitogenic T cells with CFSE before injecting them into the recipient mice and found that only 1% to 2% of the $\alpha\beta$ TCR⁺ EICs were CFSE⁺ (Fig. 7B), indicating that most of the IL-17⁺ T cells found in the inflamed eyes were recruited from the recipients and were therefore nonspecific. Further analysis showed that most of the IL-17⁺ T cells among the EICs expressed the $\alpha\beta$ TCR and did not represent other cell types, such as $\gamma\delta$ T cells and NK cells (data not shown), which also express IL-17.^{20–24}

DISCUSSION

The term Th17 cell has not been used consistently in autoimmune studies. Whereas some studies have examined IL-17⁺ T cells specific for a defined autoantigen, others have investigated anti-CD3 Ab-expanded IL-17⁺ T cells.^{16,17,25} In this study, we showed that induction of EAU in the B6 mouse elicits two functionally distinct types of IL-17⁺ T cells, these being IRBP-Th17 cells, which specifically react to the uveitogenic peptide IRBP₁₋₂₀ and are pathogenic, and bystander-Th17 cells, which do not recognize the immunizing peptide and are non-uveitogenic, but ameliorate the EAU induced by IRBP-Th17. An additional approach of using a limiting dilution assay showed that the numbers of in vivo-primed bystander-Th17 cells are approximately 10 times higher than the IRBP-Th17 cells (manuscript submitted). Thus, among T cells in mice immunized with IRBP/CFA, in vivo-primed IL-17⁺ T cells were not restricted to those that are responsive to the immunizing antigen, as IL-23-containing medium significantly promoted the expansion of IL-17⁺ T cells that did not respond to the immunizing antigen, in the absence of antigen stimulation from T cells of

FIGURE 5. IL-6 and TGF-β1 have a stronger activating effect on bystander-Th17 T cells. Splenic T cells from IRBP/CFA-immunized B6 mice were stimulated with either anti-CD3 Ab (*top*) or the immunizing antigen (IRBP) (*bottom*) in the absence or presence of IL-23, IL-6, and/or TGFβ1 for 4 days, and the activated cells were separated by density centrifugation and intracellularly stained with anti-IFN-γ or anti-IL-17 antibodies. The experiments were repeated three times.

immunized, but not naïve, mice (Fig. 2). We believe that bystander-Th17 cells are a mixture of multiple cell lineages that respond to a broad range of unidentified antigens; most likely, microbial components in CFA are able to prime multiple functionally diverse IL-17⁺ T cell subsets *in vivo*. Although *in vitro* stimulation with the immunizing IRBP antigen preferentially selects the primed IRBP-specific IL-17⁺ cells, in the absence of specific antigen stimulation, microbial protein-reactive IL-17⁺ T cells become dominant, owing to their much higher abundance. Supporting such a prediction, we showed that much more abundant IL-17⁺ T cells were *in vivo*-primed in mice immunized with IRBP₁₋₂₀ emulsified in CFA, but not in IFA. We were also able to show that mice immunized with IRBP₁₋₂₀ emulsified in IFA premixed with synthetic TLR ligand(s) significantly enhanced Th17-inducing ability (manuscript in preparation). Additional treatment with PTX, a procedure routinely used in the induction of EAU^{1,26,27} and experimental allergic encephalomyelitis,²⁸⁻³¹ further promoted the generation of IL-17⁺ T cells. These observations suggest that a significant proportion of the bystander-Th17 cells are *in vivo* primed by the microbial proteins in the CFA and that microbial proteins may activate functionally distinct T cell subsets.^{32,33} It would be of interest to determine whether specific molecular components in CFA/PTX promote, or protect from, autoimmune disease by eliciting different IL-17⁺ T-cell subsets and to exam-

ine whether the balance between the pathogenic and protective IL-17⁺ T cells determines disease susceptibility.

Much remains to be determined about the functional differences between IRBP-Th17 and bystander-Th17 cells. One possibility is that excessive amounts of IL-17 contributed by bystander Th17 cells ameliorate EAU development,³⁴ which appears to be supported by both the previous finding that large quantities of IL-17 ameliorate, rather than promote, EAU development³⁴⁻³⁶ and the observations in this study that mice that had received bystander-Th17 T cells showed generally depressed immune responses (Fig. 6), similar to mice treated with excessive amounts of IL-17.³⁴ Additional studies showed that the bystander Th17 cell but not the IRBP-Th17 cells produce significant amounts of IL-10 (Fig. 2B) and the Foxp3⁺ T cells were five-times higher among the bystander-Th17 cells than the IRBP-Th17 cells (Fig. 6H). Since T-cell function is closely regulated by their activation status,^{1,37,38} and since bystander IL-17⁺ T cells may have a varying activation status depending on the availability of antigenic stimulation, the functional contribution of such bystander IL-17⁺ T cells to disease pathogenesis could be diverse, which appears to be supported by the previous reports that the use of CFA either enables induction of autoimmune diseases²⁸⁻³¹ or prevents disease progression.³⁹⁻⁴² It remains to be determined whether there are specific IL-17⁺ T-cell subsets that exert a stronger inhibi-

FIGURE 6. Administration of bystander-Th17 T cells suppressed immune responses. Mice that had received IRBP-Th17 cells only or both IRBP-Th17 and bystander-Th17 T cells were assessed for their ability to generate IRBP-specific T cells (A–D), to show a T-cell-proliferative response to the uveitogenic peptide (E), or to produce cytokines (F, G). (A–D) Splenic T cells from mice that had received 2×10^6 IRBP-Th17 cells (A, C) or IRBP-Th17 plus bystander-Th17 cells (8×10^6 of each) (B, D) were stimulated with IRBP₁₋₂₀ under Th17 polarized (A, B) or nonpolarized (C, D) conditions. The activated T cells were separated and stained with PE-anti- α TCR antibody and FITC-anti-IL-17/IFN- γ antibodies. (E) Thymidine incorporation assay. Splenic T cells from the same mice were stimulated with IRBP₁₋₂₀ in the presence of syngeneic APCs for 48 hours, and thymidine incorporation was measured. (F, G) Cytokine production assessed by ELISA. Splenic T cells from the mice were stimulated with IRBP₁₋₂₀ in the presence of syngeneic APCs for 48 hours, and then IL-17 (F) or IFN- γ (G) in the supernatants was measured by ELISA. (H) Intracellular Foxp3 staining. The bystander-Th17 cells contained a higher number of Foxp3⁺ T cells than did the IRBP-Th17 cells.

tory effect and whether there are genetic and environmental factors that can cause broader activation of a larger spectrum of IL-17⁺ T cells, leading to different outcomes of immune

FIGURE 7. Most of the IL-17⁺ T cells in the EICs of the inflamed eye are nonspecifically recruited. (A) At 10 days after transfer of a pathogenic dose (2×10^6 /mouse) of uveitogenic T cells, the recipient mice were perfused with phosphate-buffered saline (PBS), the eyes were collected and the lens and cornea removed, and a single cell suspension was prepared, followed by gradient centrifugation. The EICs were intracellularly stained with anti-IFN- γ and anti-IL-17 antibodies. (B) EICs collected from recipient mice administered CFSE-labeled uveitogenic T cells (2×10^6) and stained with PE-anti- α TCR.

responses. The identification of specific factors that can activate functionally distinct IL-17⁺ T cell subsets should help to reassess the therapeutic designs that target IL-17⁺ T cells.

On assessing the number of IL-17⁺ T cells among the inflammatory cells in the diseased eyes, we observed that 20% and 30% of the total EICs at the peak of EAU development expressed IL-17. However, in mice with adoptively transferred CFSE-labeled uveitogenic T cells, the frequency of CFSE-labeled IRBP-Th17 cells within the inflamed eye was small, indicating that host-derived cells, presumably bystander IL-17⁺ T cells, account for >95% of all IL-17⁺ cells in the diseased eye. Since EAU progresses even though bystander IL-17⁺ T cells outnumber pathogenic T cells, we thought it likely that activation determines the suppressive activity of bystander-Th17 T cells, as in our previous observation that a pathogenic T-cell population becomes pathogenic when only the cells are freshly activated by in vitro stimulation.^{37,38} As both IRBP-Th17 and bystander-Th17 cells were in vitro activated in the adoptive transfer study, whereas only the bystander-Th17 cells showed inhibitory activity, we conclude that these two types of IL-17⁺ T cells are functionally different. We originally attempted to separate IRBP-Th17 and bystander-Th17 cells directly from the EICs of the inflamed eyes of the EAU mice. However, it proved difficult to obtain sufficient EICs, and so the IRBP-Th17 and

bystander-Th17 cells had to be obtained from the spleens of the immunized mice. The question remains whether the bystander-Th17 cells in our study are indeed equivalent to those obtained from the inflamed eyes.

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