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Major Role of $\gamma\delta$ T Cells in the Generation of IL-17$^+$ Uveitogenic T Cells[1]

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Abstract

We show that in vitro activation of interphotoreceptor retinoid-binding protein (IRBP)-specific T cells from C57BL/6 mice immunized with an uveitogenic IRBP peptide ($IRBP_{1–20}$) under TH17-polarizing conditions is associated with increased expansion of T cells expressing the $\gamma\delta$TCR. We also show that highly purified $\alpha\beta$ or $\gamma\delta$ T cells from C57BL/6 mice immunized with $IRBP_{1–20}$ produced only small amounts of IL-17 after exposure to the immunizing Ag in vitro, whereas a mixture of the same T cells produced greatly increased amounts of IL-17. IRBP-induced T cells from IRBP-immunized TCR-$\gamma^{-/-}$mice on the C57BL/6 genetic background produced significantly lower amounts of IL-17 than did wild-type C57BL/6 mice and had significantly decreased experimental autoimmune uveitis-inducing ability. However, reconstitution of the TCR-$\gamma^{-/-}$mice before immunization with a small number of $\gamma\delta$ T cells from IRBP-immunized C57BL/6 mice restored the disease-inducing capability of their IRBP-specific T cells and greatly enhanced the generation of IL-17$^+$ T cells in the recipient mice. Our study suggests that $\gamma\delta$ T cells are important in the generation and activation of IL-17-producing autoreactive T cells and play a major role in the pathogenesis of experimental autoimmune uveitis.

Experimental autoimmune uveitis (EAU)[3] is a T cell-mediated autoimmune disease that serves as a model for several posterior uveitides, such as Behçet's disease, Vogt-Koyanagi-Harada syndrome, birdshot retinochoroidopathy, and sympathetic ophthalmia (1, 2). EAU can be induced in animals by immunization with retinal Ags or by the adoptive transfer of

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Disclosures
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[3]Abbreviations used in this paper: EAU, experimental autoimmune uveitis; IRBP, interphotoreceptor retinoid-binding protein; MOG, myelin oligodendrocyte glycoprotein; HSP, heat shock protein.

retinal Ag-specific T lymphocytes (3–6). Among the ocular Ags known to induce EAU in rodent models are interphotoreceptor retinoid-binding protein (IRBP; Ref. 7) and the soluble retinal Ag (8, 9) The histopathology of mouse EAU is characterized by posterior retinal and choroidal inflammation, granuloma formation, vasculitis, photoreceptor damage, vitritis, and varying degrees of anterior uveitis (2).

Until recently, it was believed that the major subsets of pathogenic autoreactive T cells produce proinflammatory cytokines, including IFN-γ and IL-2, and belong to the Th1 type of CD4 T cells (10). However, recent studies have shown that a specific autore-active T cell subset that produces IL-17, but not IFN-γ and IL-4, is crucially involved in the pathogenesis of autoimmune diseases, such as rheumatoid arthritis, experimental autoimmune encephalomyelitis (11–13), and allergic diseases (14–16). IL-17$^{-/-}$ mice are resistant to an arthritis-like disease (17, 18), have impaired host defense against microbial infection (19, 20), and have an increased incidence of acquired delayed-type hypersensitivity (17). In addition, autoimmune-prone mice become disease resistant after treatment with an IL-17R antagonist (21).

In a previous report, we demonstrated that both IFN-γ^+ and IL-17$^+$ IRBP-specific T cells play a major role in the pathogenesis of EAU (22). To further examine the interrelationship between IFN-γ^+ and IL-17$^+$ uveitogenic T cells, in the present study, we determined the activation requirements for IFN-γ^+ and IL-17$^+$ IRBP-specific T cells and the factors important for the activation of IL-17$^+$ uveitogenic T cells. Our results showed that activation of IL-17$^+$ T cells in B6 mice immunized with an uveitogenic IRBP peptide (IRBP$_{1–20}$) was associated with increased expansion of T cells expressing the $\gamma\delta$TCR. Subsequent studies showed that purified $\alpha\beta$ and $\gamma\delta$ T cells produced low amounts of IL-17 after antigenic stimulation in vitro, but a mixture of these two cell types produced greatly increased amounts of IL-17. Our studies on the possible mechanism of the interaction of these $\alpha\beta$ and $\gamma\delta$ T cells further demonstrated that direct cell-cell contact is required to elicit an enhanced response. Finally, reconstruction studies showed that TCR-$\delta^{-/-}$ mice generated only a few IL-17$^+$ uveitogenic T cells and that the IRBP-specific T cells isolated from these mice had decreased uveitogenic activity, a defect that could be corrected by injecting a small number of purified $\gamma\delta$ T cells from IRBP-immunized C57BL/6 mice. Our results suggest that interactions between $\alpha\beta$ and $\gamma\delta$ T cells play a major role in the generation of IL-17$^+$ IRBP-specific T cells and in the pathogenesis of mouse EAU.

Materials and Methods

Animals and reagents

Pathogen-free female C57BL/6 (B6) and $\gamma\delta$TCR$^{-/-}$ mice on a B6 background (12–14 wk old) were purchased from The Jackson Laboratory and were housed and maintained in the animal facilities of the University of Southern California. All animal studies conformed to the Association for Research in Vision and Ophthalmology statement on the use of animals in Ophthalmic and Vision Research. Institutional approval was obtained, and institutional guidelines regarding animal experimentation followed. Recombinant murine IL-2 and IL-23 were purchased from R&D Systems. IRBP$_{1–20}$, myelin oligodendrocyte glycoprotein (MOG)$_{35–55}$, and heat shock protein (HSP)$_{180–196}$ were synthesized by, and Freund's

adjuvant obtained from, Sigma-Aldrich. FITC-conjugated anti-IL-17 Ab was purchased from BioLegend; Abs against murine TCR-δ (GL3) (23) or TCRVγ4 (UC3; Ref.24) were obtained from BD Biosciences. Anti-Vδ6.3 Ab (clone C504-17C), anti-Vδ5 Ab (clone F45-152), and anti-Vδ4 Ab (clone GL2) were provided, respectively, by Dr. Simon Carding (University of Leeds, Leeds, U.K.), Dr. Pablo Pereira (Institut Pasteur, Paris, France), and Dr. Leo Lefrançois (University of Connecticut School of Medicine, Farmington, CT). All other Abs were from BD Bioscience.

Preparation of $IRBP_{1–20}$-specific T cells

Briefly, B6 mice were immunized s.c. with 200 μl of emulsion containing 200 μg of $IRBP_{1–20}$ in CFA, distributed over six spots at the tail base and on the flank. At day 13 postimmunization, T cells were isolated from lymph node cells and spleen cells by passage through a nylon wool column; then 1×10^7 cells in 2 ml of RPMI 1640 medium in a 6-well plate (Costar) were stimulated for 48 h with 10 μg/ml IRBP1–20 in the presence of 1×10^7 irradiated syngeneic spleen cells as APCs in the presence of either IL-2 or IL-23 (10 ng/ml), then activated T cell blasts were separated by Ficoll gradient centrifugation, and cultured for another 72 h in the same medium used for stimulation minus the peptide.

Enrichment of γδ and αβ T cells from IRBP-immunized B6 mice

T cells prepared from the spleen and draining lymph nodes of $IRBP_{1–20}$– immunized B6 mice were stimulated for 2 days in vitro with immunizing Ag, followed by culturin in IL-23-containing (10 ng/ml) medium for 3 days. Then, the T cells were incubated for 10 min at 4°C with FITC-conjugated anti-mouse $\gamma\delta$ TCR or $\alpha\beta$ TCR Ab and then for 15 min at 4°C with anti-FITC Microbeads (Miltenyi Biotec) (25). The cells were then separated into bound and nonbound on an autoMACS separator column (Miltenyi Biotec) and washed with 15 ml of medium according to the manufacturer's protocol, and the bound cells ($\gamma\delta$ or $\alpha\beta$ T cells) were collected. The purity of the isolated cell fraction was determined by flow cytometric analysis using FITC-conjugated anti-TCR Abs and PE-conjugated Abs against $\gamma\delta$ T cells or $\alpha\beta$ T cells (BD Biosciences). Data collection and analysis were performed on a FACSCalibur flow cytometer using CellQuest software. The purity of the $\gamma\delta$ T cells was 95%. To further purify the $\gamma\delta$ T cells, residual $\alpha\beta^+$ T cells were removed using PE-conjugated anti-$\alpha\beta$ TCR Ab and anti-PE microbeads to give 99% pure $\gamma\delta$ T cells. $\alpha\beta$ T cells were prepared similarly after the immunized T cells were incubated with bead-conjugated Ab specific for mouse $\alpha\beta$ TCR.

Study of the αβ and γδ T cell interaction using cell culture inserts

A coculture system using purified populations $\alpha\beta$ and $\gamma\delta$ T cells separated by a cell culture insert (Falcon; BD Biosciences) was used to test whether direct cell-cell contact was required for the interaction of the two T cell types. The tests were performed in 24-well plates, in which either magnetically separated $\gamma\delta$ or $\alpha\beta$ T cells (1×10^5/well) were incubated in the lower or upper part of the cultures separated by the insert.

Scoring of EAU

The mice were examined three times a week for clinical signs of EAU by indirect fundoscopy. Pupils were dilated using 0.5% tropicamide and 1.25% phenylephrine hydrochloride ophthalmic solutions. Grading of disease was performed using the scoring systems described previously (26). For histopathological evaluation, whole eyes were collected at the end of the experiment and immersed for 1 h in 4% glutaraldehyde in phosphate buffer, pH 7.4, and then transferred to 10% formaldehyde in phosphate buffer until processed. The fixed and dehydrated tissues were embedded in methacrylate; then 5-μm sections were cut through the pupillary-optic nerve plane and stained with H&E. The presence or absence of disease was evaluated blind by examining six sections cut at different levels for each eye. Disease was graded on the basis of cellular infiltration and structural changes (5).

Immunofluorescence flow cytometry

Aliquots of 2×10^5 cells were double-stained with combinations of FITC-or PE-conjugated mAbs. Data collection and analysis were performed on a FACSCalibur flow cytometer using CellQuest software.

Intracellular cytokine flow cytometry

Unfractionated IRBP1–20-specific T cells or the corresponding purified $\gamma\delta$ T cells or $\alpha\beta$ T cells from immunized B6 mice were stimulated in vitro for 4 h with 50 ng/ml PMA, 1 μg/ml ionomycin, and 1 μg/ml brefeldin A (Sigma-Aldrich) and then washed, fixed, permeabilized overnight with Cytofix/Cytoperm buffer (eBioscience), and intracellularly stained with Abs against IFN-γ or IL-17 and analyzed on a FACSCalibur.

ELISA

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

Statistical analysis

Data are expressed as the mean ± SD for the results from at least three separate experiments.

Results

In vitro activation of Th17-polarized IRBP-specific T cells in C57BL/6 mice immunized with a uveitogenic IRBP peptide ($IRBP_{1–20}$) is associated with increased expansion of T cells expressing the γδ TCR

Fig. 1 shows the results of intracellular staining of IRBP-specific T cells isolated from B6 mice immunized with uveitogenic peptide $IRBP_{1–20}$ cultured in IL-2-containing medium (Th1 polarized) or IL-23-containing medium (Th17 polarized), after in vitro stimulation with the immunizing peptide. The overwhelming majority of the Th1-polarized IRBP-specific T cells expressed IFN-γ (Fig. 1*A*) and exclusively expressed the $\alpha\beta$ TCR (Fig. 1*B*). In contrast, when the in vivo primed T cells were stimulated in vitro, then incubated in Th17-polarized condition, the proliferating T cells predominantly expressed IL-17 (Fig. 1*A*) and, remarkably, a significant portion of the Th17-polarized cells failed to express $\alpha\beta$ TCR

(Fig. 1*B*). Further determination demonstrated that these cells were $\gamma\delta$TCR$^+$ and that the majority of the $\gamma\delta$T cells expressed IL-17 (Fig. 1*C*).

Expanded γδ T cells from immunized mice pre-dominantly express Vγ4Vδ4

$\gamma\delta$T cells accounted for only 1–1.8% of the splenic T cells in naive C57BL/6 mice, but for 5–7% of the splenic T cells from IRBP1–20-immunized B6 mice which was further increased to 10–15% when these T cells were grown in IL-23-containing medium (not shown). To determine whether the $\gamma\delta$T cells in IRBP-immunized B6 mice were homogeneous or heterogeneous, we determined the TCR usage of $\gamma\delta$T cells isolated from naive and immunized mice by staining with a panel of Abs specific for various $\gamma\delta$TCR segments (Vγ1, Vγ4, Vδ4, Vδ5, and Vδ6.3). As shown in Fig. 2, $\gamma\delta$T cells isolated from the spleen of IRBP$_{1–20}$-immunized mice predominantly expressed Vγ4 and Vδ4, whereas those from the spleen of naive mice did not. To determine whether the dominance of V$\gamma4^+$ $\gamma\delta$T cells was unique to IRBP-immu nized mice, we also injected B6 mice with an encephalitogenic peptide (MOG$_{35–55}$), or CFA alone. As shown in Fig. 2, mice immunized with MOG$_{35–55}$ or CFA alone showed a similar expansion of the V$\gamma4^+$ T cell subset, indicating that undefined Ags in the CFA, rather than the immunizing Ag, were responsible for the $\gamma\delta$T activation. Expansion of $\gamma\delta$T cells is slightly increased with addition of Ag, conceivably due to stronger immune responses in Ag-immunized mice.

Interaction of αβ and γδ T cells in the production of IL-17

To determine whether $\gamma\delta$T cells from IRBP-immunized B6 mice are able to respond to the immunizing Ag, IRBP-specific T cells of B6 mice immunized with IRBP$_{1–20}$ were stimulated in vitro with IRBP$_{1–20}$ for 2 days, then cultured in IL-23-containing medium for additional 5 days. Then, $\gamma\delta$T cells were isolated. To prepare the $\gamma\delta$T cells, the IRBP$_{1–20}$-stimulated T cells were labeled with PE (or FITC)-conjugated hamster anti-mouse δTCR Ab (GL3), followed by anti-PE (or FITC) Ab conjugated to beads; then the bound cells were separated from the nonbound using a magnetic sorter. As shown in Fig. 3*A*, this one-step positive selection procedure resulted in partially purified $\gamma\delta$T cells (95% pure). To remove more non-$\gamma\delta$T cells, a second negative selection step was performed to remove residual $\alpha\beta$ T cells using an Ab specific for the $\alpha\beta$TCR. This two-step enrichment yielded $\gamma\delta$T cells that were 99% pure (Fig. 3*B*). When the partially (95% pure) and highly purified (99% pure) $\gamma\delta$T cells (4×10^5/well) were exposed to the immunizing peptide (IRBP$_{1–20}$) or irrelevant peptides, such as HSP and MOG$_{35–55}$, or to plate-bound anti-CD3 Ab (5 μg/ml), the partially pure $\gamma\delta$T cells showed a vigorous response in terms of IL-17 production to the immunizing peptide IRBP$_{1–20}$ or to anti-CD3 Ab, but not to HSP$_{180–196}$ or MOG$_{35–55}$ (Fig. 3*D*). However, the highly purified $\gamma\delta$T cells did not react to IRBP$_{1–20}$ (Fig. 3*E*), suggesting that the small proportion of $\alpha\beta$T cells in the partially purified $\gamma\delta$T cells accounted for the Ag-specific response. To confirm this, we mixed the highly purified $\gamma\delta$T cells with highly purified $\alpha\beta$T cells from the same batch of immunized mice in various ratios and tested the response to the immunizing peptide. As shown in Fig. 3*F*, purified $\alpha\beta$ or $\gamma\delta$T cells produced only low levels of IL-17 after exposure to IRBP$_{1–20}$ and APCs, whereas the mixtures of $\alpha\beta$ and $\gamma\delta$T cells produced far greater amounts of IL-17.

Effective interaction between αβ and γδ T cells requires cell-cell contact

To determine whether the interaction between $\alpha\beta$ and $\gamma\delta$ T cells required direct cell-cell contact, we performed studies in which $\alpha\beta$ or $\gamma\delta$ T cells were cultured either together or separated by culture inserts. Once again, only low levels of IL-17 were produced by the purified $\alpha\beta$ or $\gamma\delta$ T cells, but the 90:10 mixture of $\alpha\beta$ and $\gamma\delta$ T cells generated a large amount of IL-17 (Fig. 4*A*). When the $\alpha\beta$ and $\gamma\delta$ responder T cells were added to the same culture well, but separated by the insert), IL-17 production declined substantially (Fig. 4*A*, *bottom*). In the absence of $\gamma\delta$ T cells, the purified $\alpha\beta$ T cells produced significant amounts of IFN-γ, indicating that the Th1 response is independent of $\gamma\delta$ T cells.

Transfer of γδ T cells into γδ T cell-deficient mice restores their ability to generate IRBP-specific T cells

Next, we examined whether TCR-$\delta^{-/-}$ mice have a decreased ability to generate uveitogenic T cells and whether transfer of $\gamma\delta$ T cells into these mice enhances their ability to generate uveitogenic T cells, which were tested for their ability to transfer disease to naive B6 mice, which develop only mild EAU after immunization with Ag, but more severe disease following adoptive transfer of IRBP-specific T cells (5). TCR-$\delta^{-/-}$ mice were left untreated or were injected i.p. with a single dose (2×10^5) of $\gamma\delta$ T cells 1 day before Ag immunization; then, 13 days later, cytokine production by the IRBP-specific T cells obtained from the two groups of immunized TCR-$\delta^{-/-}$ mice was assessed and the T cells were stimulated in vitro with the immunizing Ag, and the activated IRBP-specific T cells were separated and adoptively transferred into naive B6 mice. The results showed that TCR-$\delta^{-/-}$ mice that were reconstituted with $\gamma\delta$ T cells produced higher amounts of IL-17 (Fig. 5*A*) and generated increased numbers of IL17$^+$ IRBP-specific T cells (Fig. 5*B*). More importantly, IRBP-specific T cells isolated from $\gamma\delta$ T cell-injected recipient mice induced significantly more severe disease than those from donors that had not received the $\gamma\delta$ T cell injection before immunization when adoptively transferred into naive B6 mice (Fig. 5, *C* and *D*).

Discussion

T cells bearing the $\gamma\delta$ TCR represent a minor subset of human peripheral T cells and differ from $\alpha\beta$ T cells in cell surface phenotype and in limited combinatorial diversity of the TCR (27–29). The relationship between $\gamma\delta$ T cells and inflammation has been recognized for more than two decades (30 –32). Studies have shown that $\gamma\delta$ T cells are the major infiltrating cells in the virally infected lung (30, 33, 34). In autoimmune diseases, such as encephalomyelitis (35, 36) and colitis (37, 38), $\gamma\delta$ T cells are frequently found in the inflamed organ. In addition, $\gamma\delta$ T cells have been shown to play a critical role in tolerance of cytotoxic T cell responses in ocular inflammation (39, 40) and in the induction of anterior chamber-associated immune deviation (40, 41). Although there is evidence that $\gamma\delta$ T cells play a major role in immune responses against infection (30, 33, 34) and tumors (42, 43), the role of these T cells in autoimmune diseases remains elusive. In this study, we showed that $\gamma\delta$ T cells are important in the generation of uveitogenic T cells and in the pathogenesis of EAU. In the absence of $\gamma\delta$ T cells, development of IL-17$^+$ uveitogenic T cells is significantly diminished and disease susceptibility reduced.

Recent studies have identified a unique subset of pathogenic T cells in autoimmune disease that expresses IL-17, but not IFN-γ or IL-4 (44 – 46) and shown that this IL-17$^+$ autoreactive T cell subset plays a major role in the pathogenesis of autoimmune diseases (47– 49). In a previous study, we reported that both IL-17$^+$ or IFN-γ^+ IRBP-specific T cells are uveitogenic (22). In the present study, to determine the interrelationship between IL-17$^+$ and IFN-γ^+ IRBP-specific T cells in the pathogenesis of EAU, compare the pathogenic role of these subsets in different disease phases, and determine whether different pathogenic T cell subsets acted synergistically, we prepared highly purified IL-17$^+$ or IFN-γ^+ IRBP-specific T cells. Unexpect edly, we found that the expansion of Th17-polarized IRBP-specific T cells was associated with increased activation and expansion of T cells expressing the $\gamma\delta$TCR. The growth factor IL-23, which is required for expansion of Th17-polarized IRBP-specific T cells, was found to play a major role in the expansion of $\gamma\delta$T cells. Using magnetic cell sorting, we prepared highly purified $\gamma\delta$ and $\alpha\beta$T cells and compared the Ag-specific response of purified $\alpha\beta$ and $\gamma\delta$T cells. In vitro studies showed that a major proportion of the $\gamma\delta$T cells isolated from mice immunized with an uveitogenic peptide were IL-17$^+$. Neither $\alpha\beta$ nor $\gamma\delta$IRBP-specific T cells alone produced high amounts of IL-17 when exposed separately to the immunizing Ag, whereas mixed cultures of $\alpha\beta$ and $\gamma\delta$T cells at various ratios produced far greater amounts of IL-17, suggesting that the interaction between $\gamma\delta$ and $\alpha\beta$T cells plays a major role in the activation of IL-17$^+$ uveitogenic T cells.

To further study the role of $\gamma\delta$T cells in the activation of IRBP-specific uveitogenic T cells, we performed comparative studies on the proliferation and cytokine production of in vivo primed IRBP-specific T cells from wild-type C57BL/6 and $\gamma\delta$T cell-deficient mice on the same genetic background. The results showed that IRBP-specific T cells from immunized TCR-$\delta^{-/-}$ mice were poor at inducing EAU when adoptively transferred into naive mice and produced limited amounts of IL17. However, injection of the TCR-$\delta^{-/-}$ mice with a small number of $\gamma\delta$T cells (2×10^5) resulted in an increased IL17 response to IRBP and enhanced the disease-inducing ability of the IRBP-specific T cells. Thus, our studies revealed an important role of $\gamma\delta$T cells in the priming and/or activation of Ag-specific, IL-17$^+$ uveitogenic T cells.

The interaction between $\alpha\beta$ and $\gamma\delta$T cells appears to be more complex than previously thought. Earlier studies showed that $\gamma\delta$T cells may either support or suppress an immune response (27, 28). Our results showed that the $\gamma\delta$T cell subset expressing Vγ4 TCR segments had a proinflammatory effect in EAU and enhanced the generation of IL-17$^+$ IRBP-specific T cells. The mechanism remains to be determined. One possibility is that $\gamma\delta$T cells act as APCs and thus promote the activation of $\alpha\beta^+$ T cells. Given that $\gamma\delta$T cells are activated earlier than $\alpha\beta$T cells in an immune response, especially inside the inflamed organ (data not shown), and that they can be readily activated by exposure to cytokines or TLR ligands (50), we hypothesize that a prior activation of $\gamma\delta$T cells might greatly enhance the activation of $\alpha\beta$T cells. Indeed, previous studies have demonstrated that $\gamma\delta$T cells in humans (51), cattle (52), and pigs (53) had an Ag-presenting function that enhances the activation of Ag-specific T cells. Furthermore, we have recently shown that activated murine $\gamma\delta$T cells also possess an Ag-presenting function (50). Thus, it is likely that prior activation of $\gamma\delta$T cells augments the subsequent response of $\alpha\beta$T cells, whereas

proinflammatory cytokines produced by the activated $\alpha\beta$ T cells might further activate the $\gamma\delta$ T cells in a mutually augmenting and synergistic fashion. We have previously reported that $\gamma\delta$ T cells can be activated by either TLR ligands or proinflammatory cytokines (50). In fact, mixtures containing mixed cytokines strongly activate $\gamma\delta$ T cell proliferation and production of cytokine. Hence, activation of $\alpha\beta$ T cells further promotes the activation and production of IL-17 by $\gamma\delta$ T cells, leading to cascading responses.

The $\gamma\delta$ T cells isolated from the IRBP-immunized mice dominantly, if not exclusively, expressed Vγ4 in their TCR segments, and this $\gamma\delta$ T cell subset had a proinflammatory effect in EAU. Also, the $\gamma\delta$ T cells used in our reconstitution of TCR-$\delta^{-/-}$ mice with cells separated from immunized mice were mostly V$\gamma4^{+}$ $\gamma\delta$ T cells, as previously reported in an arthritis model (54). Whether the proinflammatory effect observed in this study is limited to this $\gamma\delta$ T cell subset remains to be investigated.

We also observed that, whereas the generation of IL-17^{+} uveitogenic T cells and IL-17 production were significantly compromised in the absence of $\gamma\delta$ T cells, the generation of IFN-γ^{+} uveitogenic T cells and IFN-γ production were not appreciably affected, suggesting that $\gamma\delta$ T cells in our model preferentially regulate Th17-type autoreactive T cells (our manuscript in preparation).

In summary, we have shown that $\gamma\delta$ T cells represent a critical element in the autoreactive response in IRBP-induced EAU in B6 mouse. These cells expand significantly in immunized animals. In the absence of $\gamma\delta$ T cells, development of uveitogenic T cells, particular T cell subsets expressing IL-17, is significantly diminished. In a previous report, we demonstrated that IRBP-specific T cells expressing IL-17 play a major role in the pathogenesis of EAU, and we now show that the interaction between $\alpha\beta$ and $\gamma\delta$ T cells plays a major role in the generation of the IL-17^{+} uveitogenic T cell subset.

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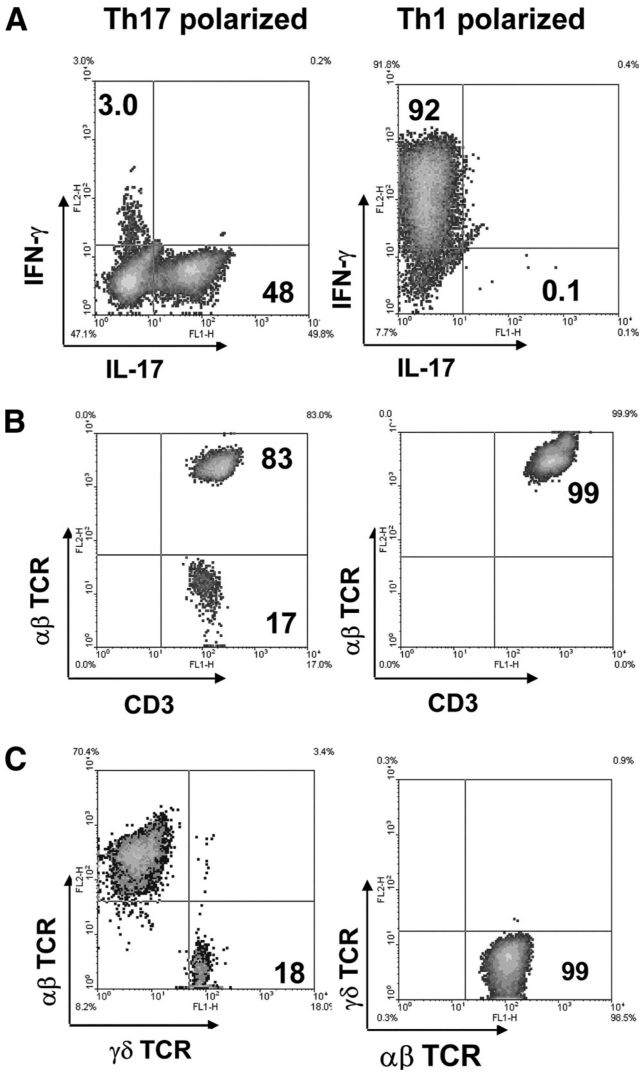

FIGURE 1.

Activation of IL-17$^+$ IRBP-specific T cells is associated with expansion of $\gamma\delta$ T cells. T cells prepared from the spleens and draining lymph nodes of $IRBP_{1–20}$-immunized B6 mice at day 13 postimmunization. were stimulated in vitro with immunizing Ag (10 μg/ml) and APCs (irradiated spleen cells) for 2 days. The activated T cell blasts were then separated by Ficoll gradient centrifugation and cultured in IL-2 (Th1-polarized)- or IL-23 (Th17-polarized)-containing medium for 5 days. Finally, the cells were stained for intracellular IL-17 and IFN-γ using FITC-anti-IL-17 and PE-anti-IFN-γ Abs (*A*), for the $\alpha\beta$ TCR and CD3 (*B*), and for the $\gamma\delta$ TCR and IL-17 (*C*). Approximately 15–20% of the Th17-polarized T cells did not express the $\alpha\beta$ TCR but expressed the $\gamma\delta$ TCR. Results are representative of multiple experiments. FL-1H and FL-2H, fluorescence.

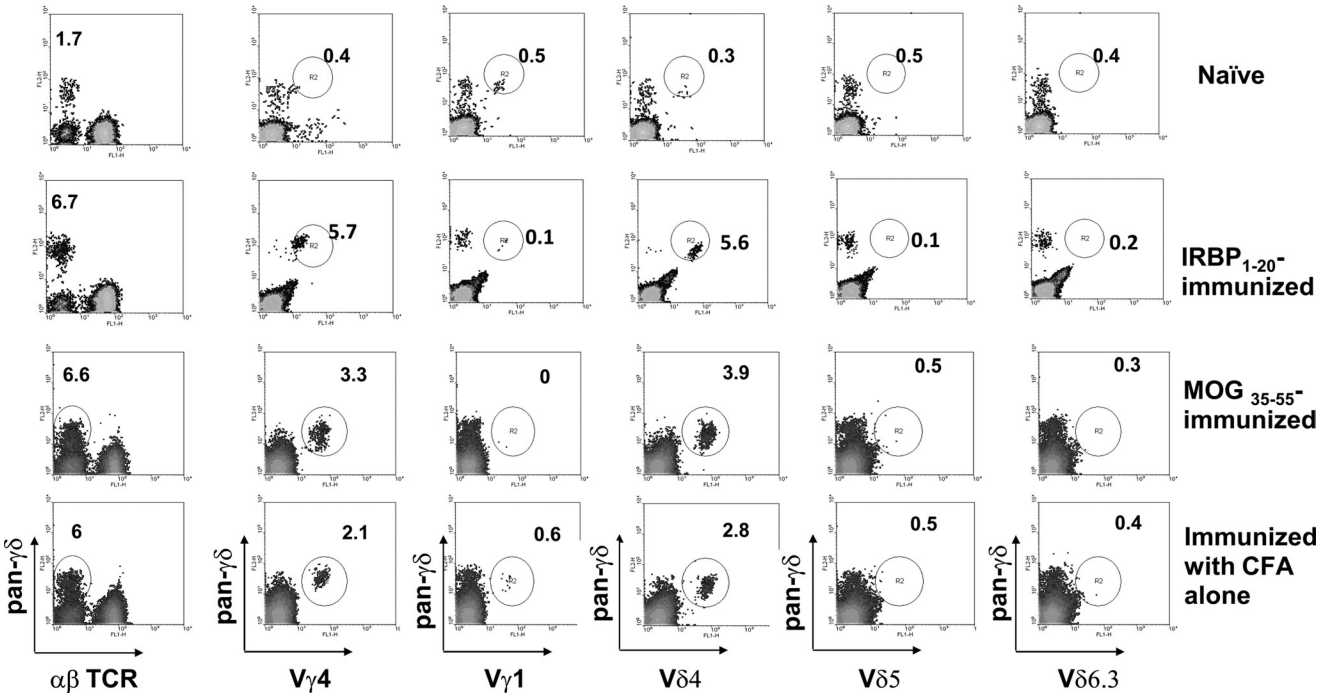

FIGURE 2.

$\gamma\delta$ T cells isolated from IRBP-immunized B6 mice predominantly express Vγ4Vδ4. Splenic T cells from naive or $IRBP_{1–20}$, $MOG_{35–55}$, or CFA-immunized B6 mice were stained using a panel of mAbs specific for $\gamma\delta$ TCR V segments (Vγ4, Vγ1, Vδ4, Vδ5, and Vδ6.3) and an Ab specific for the mouse pan-TCR δ chain (GL3), followed by FACS analysis. The results shown are representative of those in five experiments. FL-1H, fluorescence.

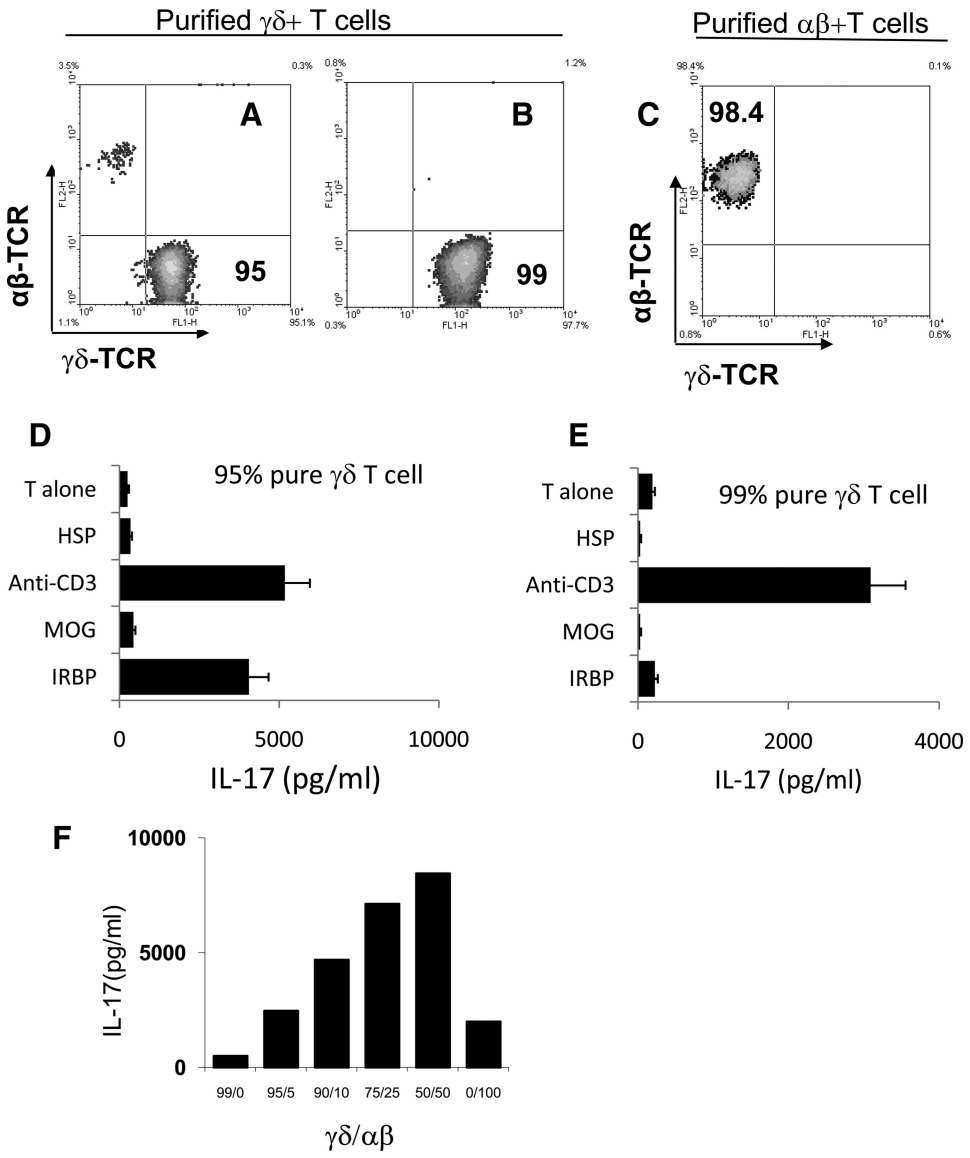

FIGURE 3.

Role of *γδ* and *αβ* T cells in the Ag-specific IL-17 response. *A–C*, Purification of *αβ* TCR^+ and *γδ* TCR^+ T cells: Purified *αβ* TCR^+ and *γδ* TCR^+ T cells were prepared from $IRBP_{1–20}$-immunized B6 mice using magnetic beads. *A*, One-step positive selection using GL3 (mAb specific for pan-mouse *γδ* TCR) resulted in 95% pure *γδ* T cells. *B*, Further depletion of *αβ* T cells resulted in 99% pure *γδ* T cells. *C*, 99% pure *αβ* T cells were prepared similarly. *D–F*, The partially purified (*D*) or highly purified (*E*) *γδ* T cells or the indicated mixtures (*F*), with the total number of cells in all 4×10^5 T cells/ well, were incubated with the indicated Ag and APCs in 96-well plates, and the culture supernatants were tested for IL-17. The results shown are representative of those in three experiments.

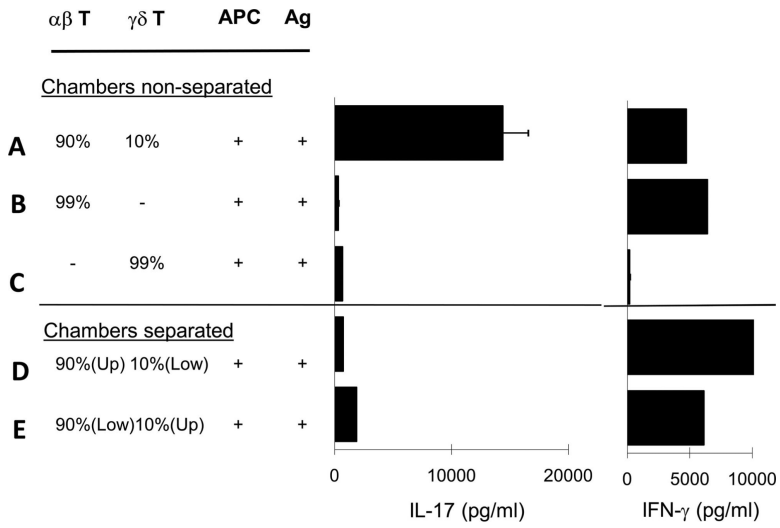

FIGURE 4.

Test of interaction between $\alpha\beta$ and $\gamma\delta$ T cells using culture inserts. *A–C*, In a 24-well plate, a total of 2×10^6/well in vivo primed $\alpha\beta$ and $\gamma\delta$ T cells from $IRBP_{1–20}$-immunized B6 mice (99% pure) were tested alone (*B* and *C*) or as a 9:1 mixture (*A*) of $\alpha\beta$ and $\gamma\delta$ TCR^+ T cells for production of IL-17 or IFN-γ after in vitro stimulation with $IRBP_{1–20}$ and APCs. Only the cells in the lower chambers are cocultured with irradiated splenocytes. *D*, 90% $\alpha\beta$ and 10% $\gamma\delta$ TCR^+ T cells were cultured in a same well but separated by inserts. $\alpha\beta$ T cells were in the upper chamber, and $\gamma\delta$ T cells were in the lower chamber; *E*, $\alpha\beta$ T cells were seeded in the lower chamber, and $\gamma\delta$ T cells were seeded in the upper chamber. Results are representative of those in three experiments.

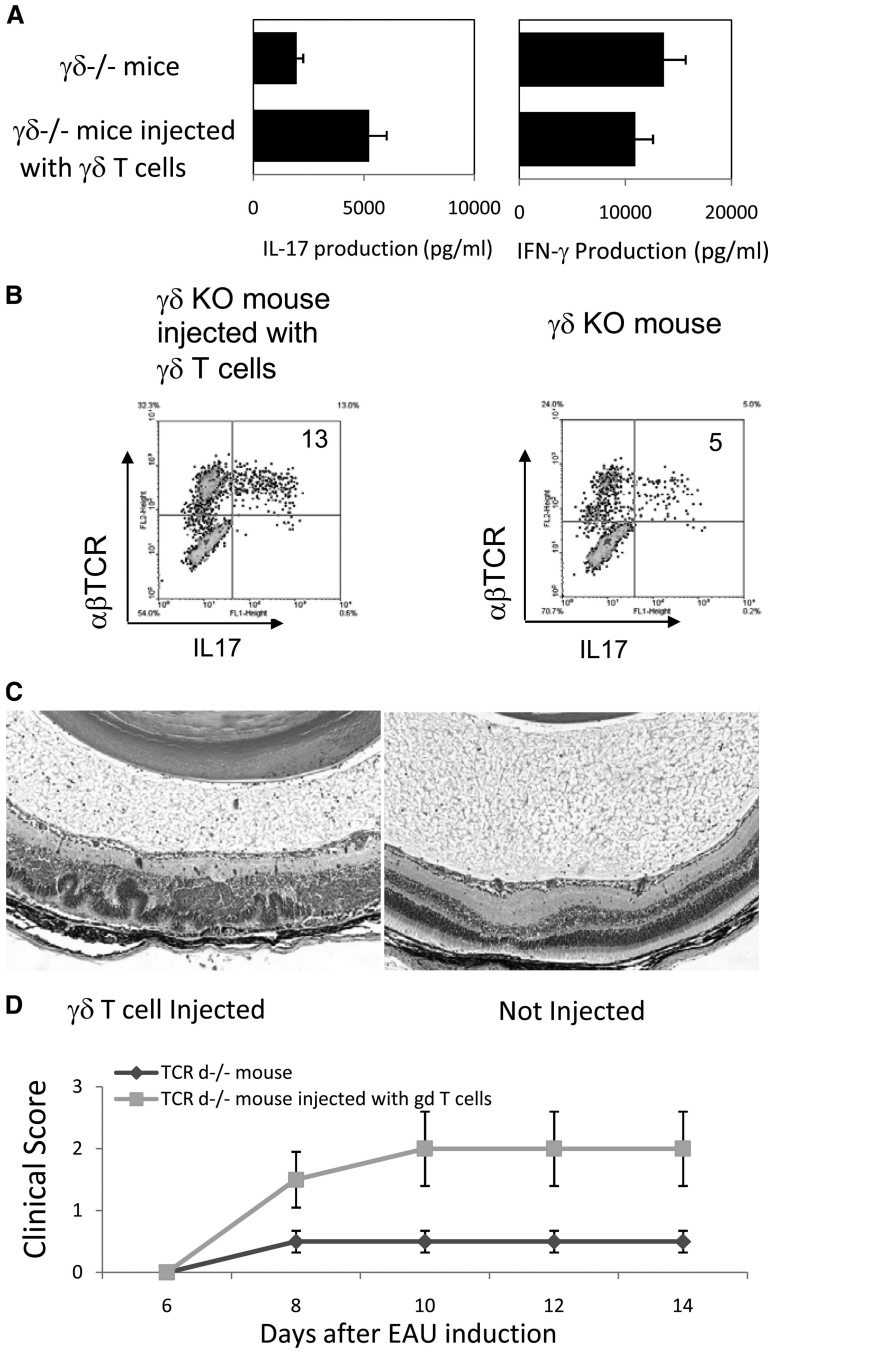

FIGURE 5.

Transfer of $\gamma\delta$T cells into TCR-$\delta^{-/-}$ mice restores their ability to generate IRBP-specific T cells. The IFN-γ- and IL-17-producing abilities of the IRBP-specific T cells of TCR-$\delta^{-/-}$ mice, either untreated or injected with 2×10^5 $\gamma\delta$T cells were determined 2 day after in vitro stimulation with immunizing Ag (*A*). Intracellular staining of IL-17$^+$ T cells were assessed after 2 days of in vitro stimulation with immunizing Ag and another 3-day culture in medium containing IL-23 (10 ng/ml; *B*). The disease-inducing ability of the IRBP-specific T cells (2×10^6) from the TCR-$\delta^{-/-}$ mice, either untreated or injected with $\gamma\delta$T cells, was also

compared after 48 h of in vitro stimulation with the immunizing Ag (*C* and *D*). Results are representative of those in two separate experiments. KO, Knockout.