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γδ T Cells Activated in Different Inflammatory Environments Are Functionally Distinct

Deming Sun,* Nymph Chan,* Hui Shao,† Willi K. Born,‡ and Henry J. Kaplan§

γδ T cells are important immunoregulatory cells in experimental autoimmune uveitis (EAU), and the activation status of γδ T cells determines their disease-enhancing or inhibitory effects. Because γδ T cells can be activated via various pathways, we questioned whether the nature of their activation might impact their function. In this study, we show that γδ T cells activated under different inflammatory conditions differ greatly in their functions. Whereas anti-CD3 treatment activated both IFN-γ$^+$ and IL-17$^+$ γδ T cells, cytokines preferentially activated IL-17$^+$ γδ T cells. γδ T cells continued to express high levels of surface CD73 after exposure to inflammatory cytokines, but they downregulated surface CD73 after exposure to dendritic cells. Although both CD73^{high} and CD73^{low} cells have a disease-enhancing effect, the CD73^{low} γδ T cells are less inhibitory. We also show that polarized activation not only applies to αβ T cells and myeloid cells, but also to γδ T cells. After activation under Th17-polarizing conditions, γδ T cells predominantly expressed IL-17 (gdT17), but after activation under Th1 polarizing conditions (gdT1) they mainly expressed IFN-γ. The pro-Th17 activity of γδ T cells was associated with gdT17, but not gdT1. Our results demonstrate that the functional activity of γδ T cells is strikingly modulated by their activation level, as well as the pathway through which they were activated. ***The Journal of Immunology*****, 2022, 208: 1–8.**

Playing a role in the regulation of inflammation associated with infections, tumors, and autoimmunity (1–6), γδ T cells can either enhance (7–9) or inhibit (2, 10–12) an adaptive immune response. γδ T cell subsets expressing distinct TCRs show functional diversity (13–16). The regulatory effect of γδ T cells is not a stable feature but fluctuates with γδ T cell activation status (17, 18). The mechanisms by which γδ T cells enhance or inhibit an adaptive immune response remain unclear, and a better understanding of the variable regulatory effect of γδ T cells should facilitate the development of γδ T cell–targeted immunotherapies for related diseases. Given our previous observation that the regulatory effect of γδ T cells is closely related to their activation status (17, 19, 20), and that γδ T cells can be activated via multiple pathways even in the absence of TCR ligation (1, 21–23), this study investigated whether γδ T cells activated via different pathways are functionally distinct.

We show that γδ T cells activated by cytokines are phenotypically and functionally distinct from the γδ T cells activated by dendritic cells (DCs). Cytokine-activated γδ T cells (cytokine-γδs) retained high levels of CD73 expression (CD73^{high}), whereas γδ T cells activated by DCs (DC-γδs) downregulated CD73 (CD73^{low}). Moreover, although cytokine-γδs can either enhance or inhibit T cell function, they are preferentially disease enhancing. Therefore, it appears that CD73 expression is fundamentally important for the immunoregulatory function of γδ T cells (20).

Previous studies have shown that αβ T cells activated under different polarizing conditions are phenotypically and functionally distinct (21, 24–29). Likewise, macrophages activated under polarizing conditions are also functionally different (30, 31). We therefore examined whether such treatment would also polarize γδ T cells. Our results showed that γδ T cells activated under Th1- or Th17-polarizing conditions are functionally distinct. γδ T cells dominantly expressed IL-17 (gdT17) when they were activated under Th17-polarizing conditions, and they exhibited greatly increased pro-Th17 activity. In contrast, under Th1-polarizing conditions they dominantly expressed IFN-γ (gdT1) with poor pro-Th17 activity. Our results demonstrate that the regulatory potential of γδ T cells is determined not only by the level of their activation but also by the nature or pathway of their activation. A better understanding of these mechanisms should improve development of γδ T cell–targeted immunotherapies and the treatment of autoimmune diseases.

Materials and Methods

Animals and reagents

Female C57BL/6 (B6), TCR-δ$^{-/-}$, and CD73$^{-/-}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME); 12- to 16-wk-old mice were used in all studies. All mice were housed and maintained in the animal facilities of the University of California, Los Angeles. All protocols in this study were approved by the Committee on the Ethics of Animal Experiments of the University of California, Los Angeles (Institutional Animal Care and Use

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Abbreviations used in this article: B6, C57BL/6; BMDC, bone marrow–derived DC; cytokine-γδ, cytokine-activated γδ T cell; DC, dendritic cell; DC-γδ, DC-activated γδ T cell; EAU, experimental autoimmune uveitis; gdT1, γδ T cells expressing IFN-γ; gdT17, γδ T cells expressing IL-17; IRBP, interphotoreceptor retinoid-binding protein.

Committee permit ARC 2014-029-03A), in compliance with the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health.

Recombinant murine IFN-γ and IL-23 were purchased from R&D Systems (Minneapolis, MN). FITC-, PE-, or allophycocyanin-conjugated Abs against mouse CD3, CD4, αβ TCR, γδ TCR (GL3), CD69, and CD62L and their isotype control Abs were purchased from BioLegend (San Diego, CA).

Immunization, experimental autoimmune uveitis induction, and anti–IFN-γ treatment

Experimental autoimmune uveitis (EAU) was induced in B6 mice by s.c. injection of 200 μl of emulsion containing 200 μg of human interphotoreceptor retinoid-binding protein $(IRBP)_{1–20}$ (Sigma-Aldrich, St. Louis, MO) in CFA (Difco, Detroit, MI) at six spots at the tail base and on the flank, and by i.p. injection with 300 ng of pertussis toxin. Mice were then examined three times a week until the end of the experiment (day 30 postimmunization). To examine mice for clinical signs of EAU by indirect fundoscopy, the pupils were dilated using 0.5% tropicamide and 1.25% phenylephrine hydrochloride ophthalmic solutions. Fundoscopic grading of disease was performed using the scoring system described previously (32). For histology, whole eyes were collected at the end of the experiment and prepared for histopathological evaluation.

EAU evaluation

The mice were examined three times a week for 30 d postimmunization. The clinical signs of EAU were evaluated using fundoscopic examination. Fundoscopic grading of disease was performed using the scoring system described previously (32). At 30 d postimmunization, the mice were euthanized and the eyes were collected for histological examination. For histology, whole eyes were collected at the end of the experiment and prepared for histopathological evaluation. The eyes were immersed for 1 h in 4% phosphate-buffered glutaraldehyde, then transferred to 10% phosphate-buffered formaldehyde until they were processed. Fixed and dehydrated tissues were embedded in methacrylate, and 5-μm sections were cut through the pupillary-optic nerve plane and stained with H&E. The eyes were fixed overnight at 40°C in Davison's solution and then processed as paraffin-embedded blocks.

T cell preparations

Responder $CD3^+$ T cells were purified from B6 mice immunized with the human $IRBP_{1–20}$ peptide (5, 19, 33). Nylon wool–enriched splenic T cells from naive or immunized mice were incubated sequentially for 10 min at 4°C with FITC-conjugated anti-mouse γδ TCR or αβ TCR Abs and for 15 min at 4°C with anti-FITC MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The cells were then separated into bound and non-bound fractions on an autoMACS separator column (Miltenyi Biotec). To obtain a sufficient number of cells, we routinely pool the cells obtained from all six mice in the same group before the T cells are further enriched using a MACS column. The purity of the isolated cells, as determined by flow cytometric analysis using PE-conjugated Abs against αβ or γδ T cells, was >95%.

γδ T cells were purified from $IRBP_{1–20}$-immunized B6 mice. Nylon wool–enriched splenic T cells from immunized mice were incubated for 10 min at 4°C with FITC-conjugated anti-mouse γδ TCR or αβ TCR Abs, then for 15 min at 4°C with anti-FITC MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) (25). Resting cells were harvested from this isolate after culture in cytokine-free medium for 3–5 d, when they showed downregulation of CD69 expression. Activated γδ T cells were prepared by incubating the resting γδ T cells with anti–γδ TCR (GL3) and anti-CD28 Abs (2 μg/ml) for 2 d.

Testing the in vivo effect of injected γδ T cells

TCR-$\delta^{-/-}$ mice were randomly grouped and left untreated or injected i.p. with Th1- or Th17-polarized γδ T cells (5×10^5), prepared as described above, then all of the mice were immunized with a pathogenic dose of $IRBP_{1–20}$. On day 13 postimmunization, $CD3^+$ T cells were separated by magnetic sorting from lymph node and spleen cells and stimulated with 10 μg/ml immunizing peptide in the presence of irradiated syngeneic spleen cells. The activated T cell blasts were then separated by Ficoll gradient centrifugation and cultured in polarizing culture medium for 3 d, then cytoplasmic expression of IFN-γ and IL-17 by the responder T cells was determined and cytokine levels in the culture supernatants were assessed.

Intracellular staining and FACS analysis

For intracellular staining, T cells (2×10^5 in 100 μl) were incubated for 4 h with 50 ng/ml PMA, 1 μg/ml ionomycin, and 1 μg/ml brefeldin A (Sigma-Aldrich), then were washed, fixed, permeabilized overnight with Cytofix/Cytoperm buffer (eBioscience, San Diego, CA), and intracellularly stained with Abs against IFN-γ and IL-17 and analyzed on a FACSCalibur flow cytometer.

Assessment of Th1- and Th17-polarized responses

Responder $CD3^+$ T cells (3×10^6) were cocultured for 48 h with $IRBP_{1–20}$ (10 μg/ml) and with irradiated spleen cells (2×10^6/well) as APCs in a 12-well plate under either Th17-polarized conditions (culture medium supplemented with 10 ng/ml IL-23) or Th1-polarized conditions (culture medium supplemented with 10 ng/ml IL-12). The responder αβ T cells were collected from $IRBP_{1–20}$-immunized B6 mice on day 13 postimmunization. Forty-eight hours after stimulation, IL-17 and IFN-γ levels in the culture medium were measured using ELISA kits (R&D Systems) and the percentage of IFN-γ^+ and IL-17^+ T cells among the responder T cells was determined by intracellular staining after 5 d of culture, followed by FACS analysis, as described below (19, 34).

Immunofluorescence flow cytometry for surface and cytoplasmic Ags

In vivo–primed T cells were stimulated with the immunizing Ag and APCs for 5 d. The T cells were then separated using Ficoll gradient centrifugation and stimulated in vitro for 4 h with 50 ng/ml PMA, 1 μg/ml ionomycin, and 1 μg/ml brefeldin A (all from Sigma). Aliquots of cells (2×10^5 cells) were then fixed, permeabilized overnight with Cytofix/Cytoperm buffer (eBioscience), and intracellularly stained with PE-conjugated anti-mouse IFN-γ Abs or FITC-labeled anti-mouse IL-17 Abs. Data collection and analysis were performed on a FACSCalibur flow cytometer using CellQuest software.

Cytokine assays by ELISA

Cytokine (IL-1, IL-6, IL-12, and IL-23) levels in the culture medium were measured by ELISA. Purified αβ T cells (3×10^4 cells/well; 200 μl) from the draining lymph nodes and spleens of $IRBP_{1–20}$-immunized B6 mice were cultured in complete medium at 37°C for 48 h in 96-well microtiter plates with irradiated syngeneic spleen APCs (1×10^5) in the presence of 10 μg/ml $IRBP_{1–20}$. A fraction of the culture supernatant was then assayed for IL-17 and IFN-γ using ELISA kits (R&D Systems).

Statistical analysis

The results in the figures are representative of one experiment, which was repeated five times. The statistical significance of differences between groups in a single experiment was initially analyzed by two-way Student *t* tests, and when statistical significance was detected, the Student–Newman–Keuls post hoc test was subsequently used. A p value <0.05 was considered statistically significant.

Results

In vivo–activated murine γδ T cells express either IFN-γ and/or IL-17, whereas cultured γδ T cells dominantly express IL-17

γδ T cells constitute 1–2% of total splenic $CD3^+$ cells in naive B6 mice but the percentage increases significantly in immunized EAU-prone B6 mice at the peak of the inflammatory response (13–15 d postimmunization) (Fig. 1A). Assessment of cytoplasmic expression of IFN-γ and/or IL-17 showed that γδ T cells of naive mice (Fig. 1A) do not express either IFN-γ or IL-17, whereas a larger portion of γδ T cells of immunized mice (Fig. 1B) express either IL-17 or IFN-γ. Fig. 1C compares the total number as well as the number of IFN-γ^+ and IL-17^+ γδ T cells in naive and immunized mouse. Intracellular staining showed that ~60% of the γδ T cells freshly isolated from immunized mice expressed IL-17 and 30% of the cells expressed IFN-γ (Fig. 1D). When the fresh γδ T cells from immunized mice were cultured in vitro, a rapid shift of cytokine gene expression was observed. As seen in Fig. 1E, 30% of the freshly isolated $CD3^+$ splenic γδ T cells expressed either IL-17 or IFN-γ, and 16.3% of the cells dually expressed IL-17 and IFN-γ. After a 4-d culture in medium, IL-17^+ cells became overwhelmingly dominant on day 7 (Fig. 1E), indicating that in vitro culture favors expansion of IL-17^+ γδ T cells. However, stimulating the cultured γδ T cells with anti-CD3 Ab (2 μg/ml) restored levels of IFN-γ^+ cells (Fig.1F), suggesting that both IFN-γ^+ and IL-17^+ γδ T cells coexist, and the dominance of one subset is promoted by the culture conditions.

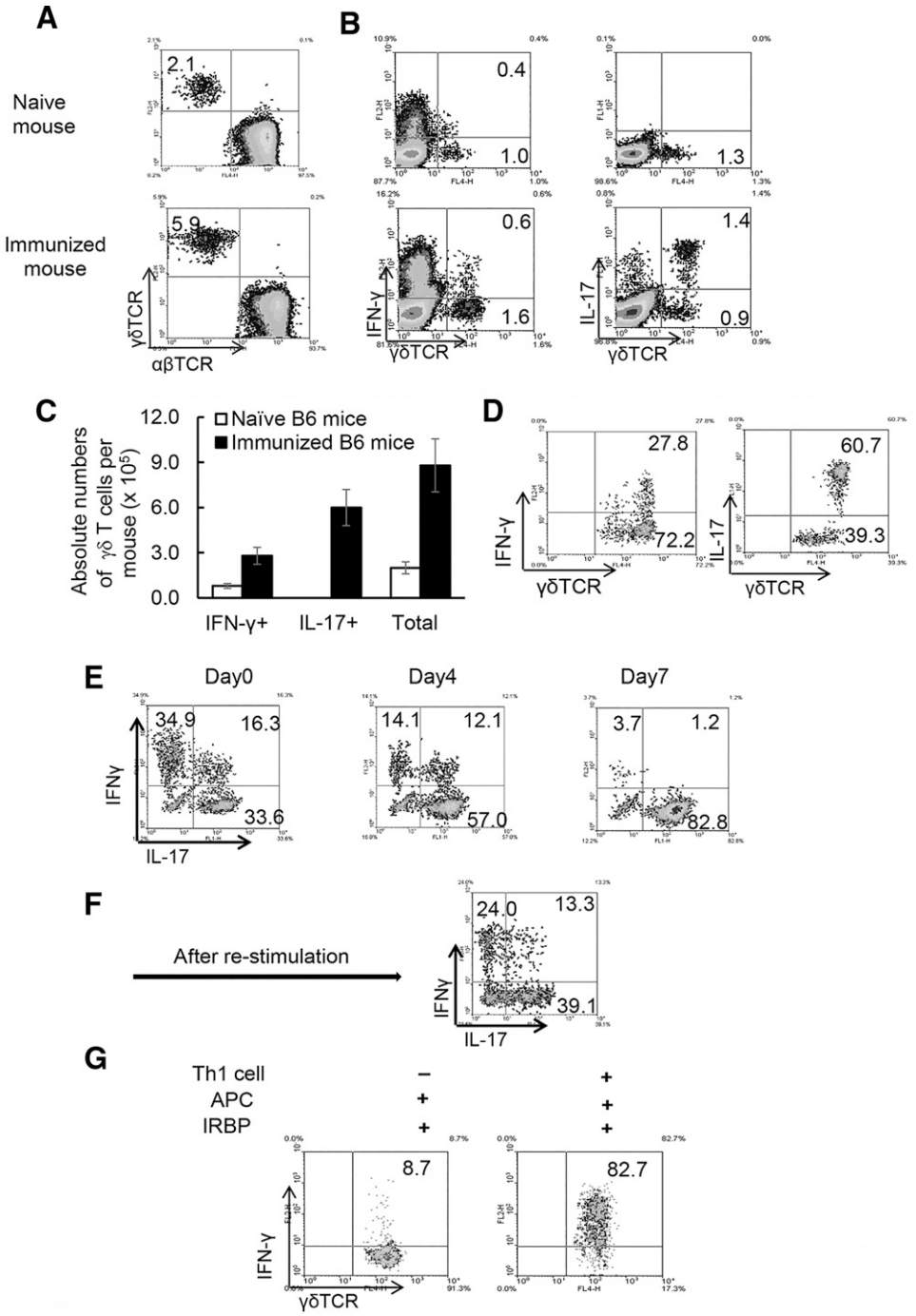

FIGURE 1. In vivo–activated murine γδ T cells expressed either IFN-γ and/or IL-17, whereas cultured γδ T cells dominantly expressed IL-17. (**A**) Analysis of gated $CD3^+$ splenic cells of naive and immunized mice stained with FITC-αβ TCR and PE-anti–γδ TCR Abs. (**B**) Splenic cells of naive and immunized mice stained with FITC-anti–γδ TCR and intracellularly stained with PE-anti–IFN-γ/IL-17 Abs. (**C**) Total number and IFN-γ^+ and IL-17^+ γδ T cell numbers of naive and immunized mice were estimated. (**D**) γδ T cells were gated and stained with FITC-anti–γδ TCR and intracellularly stained with PE-anti–IFN-γ/IL-17 Abs. (**E**) γδ T cells were separated from splenic cells of immunized mice and cultured in medium containing IL-7 and IL-23 (10 ng/ml). Sample cells at 4 and 7 d after culture were stained with FITC-anti–γδ TCR and intracellularly stained with PE-anti–IFN-γ/IL-17 Abs. (**F**) After 7 d of culture γδ T cells were stimulated with anti-CD3 for 2 d, then stained with FITC-anti–γδ TCR and intracellularly stained with PE-anti–IFN-γ/IL-17 Abs. (**G**) Cultured γδ T cells (1×10^6/well) were stimulated with the immunizing Ag $IRBP_{1–20}$ and splenic APCs, with or without the addition of IRBP-reactive Th1 (IFN-γ^+) T cells (5×10^5/well) for 2 d before staining with FITC-anti–γδ TCR and intracellularly stained with PE-anti–IFN-γ/IL-17 Abs.

IFN-γ^+ αβ (Th1) cells promote activation of IFN-γ^+ γδ T cells

Because in vivo–activated murine γδ T cells expressed either IFN-γ and/or IL-17, whereas cultured γδ T cells were predominantly IL-17^+, we questioned whether coactivation of αβ T cells in vivo accounts for the activation of IFN-γ^+ γδ T cells. To address this question, we restimulated cultured γδ T cells in vitro, with or without IRBP-reactive Th1 (IFN-γ^+) T cells (Fig. 1G), prepared as previously reported by us (34). The results showed that in the absence of αβ TCR^+ Th1 cells, γδ T cells could not be activated by the immunizing Ag (IRBP) and APCs. However, a great number of the γδ T cells turned to express IFN-γ when αβ TCR^+ Th1 cells were added during in vitro stimulation. These results suggest that γδ T cells did not directly respond to the immunizing Ag but could be activated and expressed IFN-γ as bystanders when αβ T cells were activated by the immunizing Ag.

Cytokine-activated γδ T cells produce only IL-17; anti–CD3-activated γδ T cells produce both IFN-γ and IL-17

A number of cytokines are able to trigger γδ T cell activation (1, 19, 21). Because the medium that maintains growth of in vitro–cultured γδ T cells contains mixed cytokines, including IL-1, IL-7, and IL-23 (19, 20), we questioned whether cytokines in the culture medium can bias growth of γδ T cell subsets. To test this hypothesis, we enriched γδ T cells by MACS separation and observed that a one-step separation yields 95% pure γδ T cells, whereas a two-step separation enriched γδ T cells to 99% purity (Fig. 2A). The γδ T cells were cultured either in plates prebound with anti-CD3 Ab or in medium containing a mixture of IL-1, IL-7, and IL-23 (10 ng/ml). Forty-eight hours later we measured cytokine production of γδ T cells by ELISA. Our results showed that whereas γδ T cells activated by

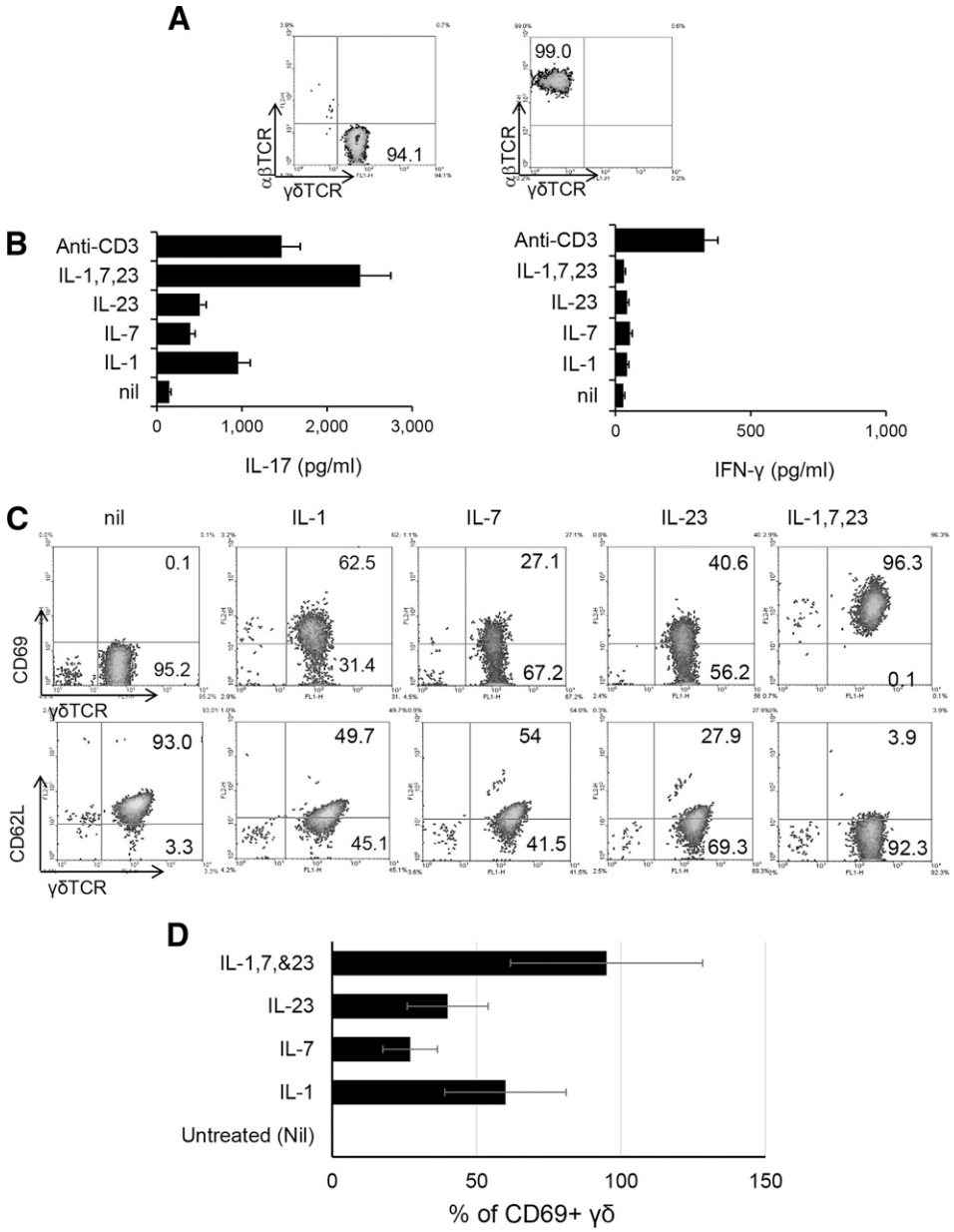

FIGURE 2. Exposure of γδ T cells to IL-1, IL-7, or IL-23 increased T cell activation molecules CD69/CD44 and enhanced γδ T cell cytokine production, including IL-17. (**A**) Purified γδ T cells in the right panel were twice enriched using a MACS column. (**B**) Separated γδ T cells were cultured in growth factor–free medium for 7 d before exposure to anti-CD3 Ab (2 μg/ml) or cytokine (10 ng/ml). Two days later, supernatants were sampled and assessed for IL-17 and IFN-γ by ELISA. (**C**). Separated γδ T cells were cultured in growth factor–free medium for 7 d before exposure to indicated cytokines (10 ng/ml). Two days later the cells were stained with FITC-anti–γδ TCR and anti-mouse CD69 (upper panels) or anti-mouse CD62L (lower panels). (**D**) Data from three independent experiments were pooled.

anti-CD3 Ab produced both IL-17 and IFN-γ, cytokine-exposed γδ T cells produced only IL-17 and not IFN-γ (Fig. 2B). Phenotypic tests measuring expression of T cell activation markers CD69 and CD62L showed that cultured γδ T cells expressed elevated levels of CD69 but decreased levels of CD62L after exposure to individual cytokines (IL-1, IL-7, or IL-23), but that a combination of these cytokines had a synergistic stimulating effect (Fig. 2C, 2D).

γδ T cells activated by cytokines or by DCs are phenotypically and functionally distinct

Our previous studies showed that DCs are also important in γδ T cell activation (35–37). Bone marrow–derived DCs (BMDCs) acquired an increased stimulating effect on γδ T cells after exposure to TLR ligand (37). In this study, we compared the phenotype and function of γδ T cells activated by cytokines or BMDCs. Our results showed that cytokine-activated γδ T cells (cytokine-γδs) expressed CD73 at decreased levels, whereas γδ T cells activated by DCs (DC-γδs) retained CD73 at high levels. However, both sets of γδ T cells exhibited a similar increase in the CD44 T cell activation (Fig. 3A, lower panels) and produced similar amounts of IL-17 after stimulation with either cytokines or DCs (Fig. 3B).

Analysis of in vitro pro-Th17 activity showed that pure αβ responder T cells generated a low percentage of IL-17^{+} cells in the absence of γδ T cells from TCR-δ$^{-/-}$ mice. Addition of either cytokine-γδs or DC-γδs increased the percentage of IL-17^{+} cells, with the DC-γδs having a stronger effect (Fig. 3C, upper panel). Next, we compared the suppressive effect of the two γδ T cell phenotypes. We previously reported a synergistic effect of AMP and γδ T cells in γδ-mediated suppression (20). AMP did not directly suppress TCR-δ$^{-/-}$ responder T cells when cultured alone (Fig. 3C, left panels), but it did with added cytokine-γδs (i.e., DC-γδs). The suppressive effect of cytokine-γδs was abolished in TCR-δ$^{-/-}$ mice, suggesting that CD73 expression by γδ T cells was essential for their suppressive function, but it was not essential for the pro-Th17 activity of γδ T cells. Results of multiple repeated assays are summarized in Fig. 3D. This conclusion was further supported by demonstrating that the CD73 blocker APCP (38, 39) could augment the enhancing effect of cytokine-γδs, but not that of DCs-γδ (Fig. 3E, 3F). These results suggest that the combined effect of CD73^{high} and cytokine-γδs can exert

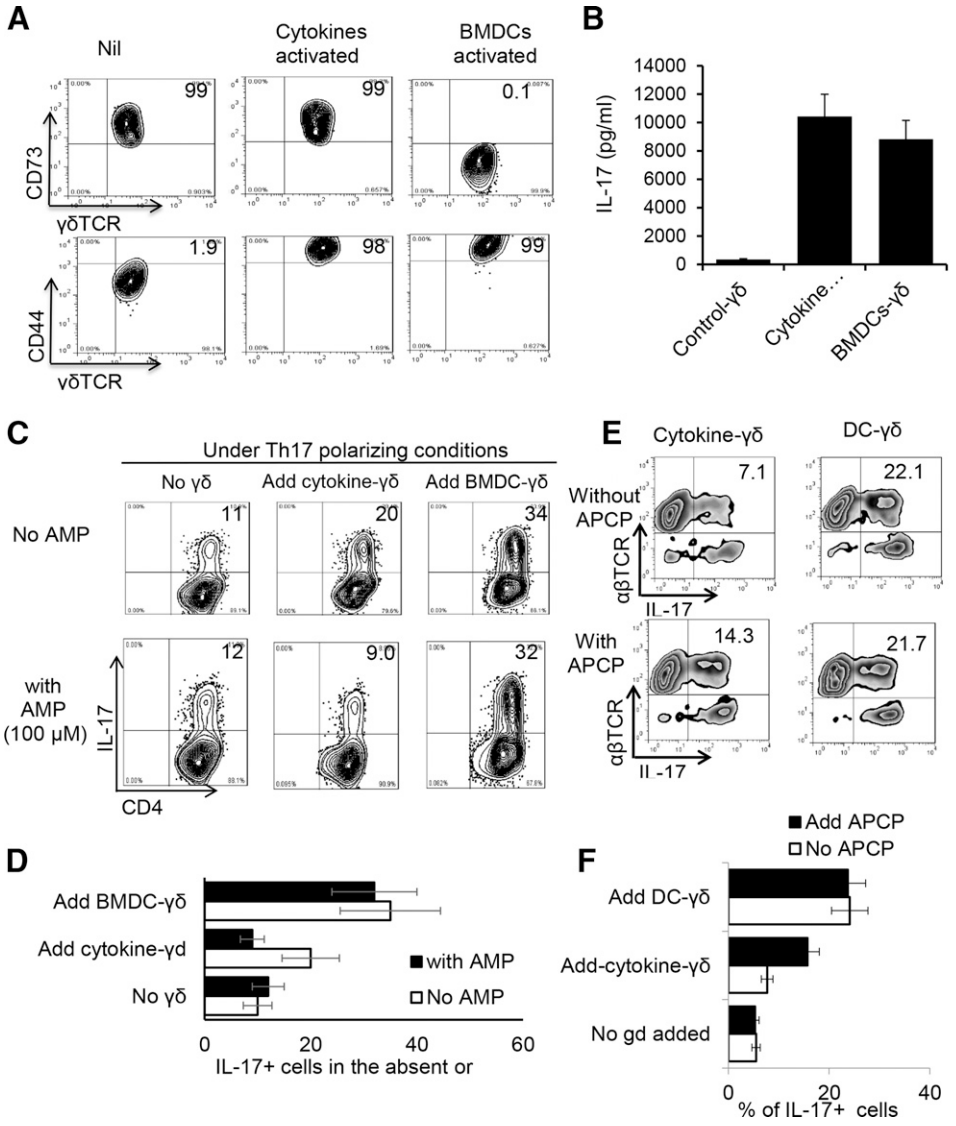

FIGURE 3. γδ T cells are phenotypically and functionally distinct after activation by cytokines or DCs. (**A**) γδ T cells purified from $IRBP_{1–20}$-immunized B6 mice on day 13 postimmunization were cultured for 48 h in cytokine-free medium (Nil), or with a cytokine mixture [10 ng/ml each of IL-1, IL-7, and IL-23 (middle column), or with BMDCs (right column)], and then the γδ T cells were separated and double-stained with anti–TCR-γ and anti-CD73 Abs (upper panels) or anti–TCR-δ and anti-CD44 Abs (lower panels). (**B**) Supernatants of cytokine-activated and DC-activated γδ T cells were assessed for IL-17 production by ELISA 48 h after stimulation. (**C**) Aliquots of responder γδ T cells prepared from $IRBP_{1–20}$-immunized TCR-$\delta^{-/-}$ mice (1×10^6/well) were stimulated for 5 d in 24-well plates with the immunizing peptide and splenic APCs, in the absence of γδ T cells (5% of total responder T cells) (left panels), with cytokine-activated γδ T cells (center panels), or with DC-activated γδ T cells (right panels). Proliferating γδ T cells were measured by FACS analysis after cytoplasmic staining with anti–IL-17 Abs and surface staining with anti-CD4 Abs in the absence (upper panel) or presence (lower panel) of AMP. (**D**) Data pooled from three independent experiments are also shown. (**E**) Responder T cells of immunized TCR-$\delta^{-/-}$ mice (1×10^6/well) were stimulated for 5 d in 24-well plates with the immunizing peptide and splenic APCs, in the presence of either cytokine-activated or DC-activated γδ T cells and with or without addition of CD73 blocker APCP (3 μM). (**F**) Data pooled from three independent experiments are shown.

both enhancing and inhibiting function, while the blockade of CD73 function alone abolished the suppressive effect of cytokine-γδs and augmented its enhancing effect. Blockade of CD73 had little effect on $CD73^{low}$ DC-γδs.

Polarizing γδ T activation

We were able to show that γδ T cells are phenotypically and functionally distinct when activated under Th1 or Th17 polarizing conditions. Abundant numbers of IL-17^+ γδ T cells (gdT17) were seen when activated under Th17 polarizing condition (culture medium containing IL-23). In contrast, IFN-γ^+ γδ T cells (gdT1) prevailed when activated under Th1 polarizing conditions (culture medium containing IL-12). Fig. 4A shows that IL-17^+ γδ T cells accounted for 65% of total γδ T cells after 5 d of in vitro stimulation under Th17 polarizing conditions. In contrast, the IFN-γ^+ γδ T cells accounted for 69.2% of total γδ T cells after activation under Th1-polarized conditions. Summarized data of three independent experiments are shown in Fig. 4B. Cytokine production tests showed that gdT17 γδ T cells produced abundant amounts of IL-17 whereas IFN-γ production prevailed with gdT1 γδ T cells (Fig. 4C).

Functional comparisons between gdT17 and gdT1 initially were made in vitro (5, 17), where γδ-deficient TCR-$\delta^{-/-}$ responder T cells generated low numbers (11.1%) of IL-$17^+\alpha\beta$ TCR^+ (Th17) T cells after antigenic stimulation (Fig. 5A); when small numbers of gdT17 γδ T cells (5% of the total responder T cells) were added to the responder T cells this number rose 3-fold (33%) (Fig. 5C), whereas addition of gdT1 hardly augmented the Th17 responses (Fig. 5B). In contrast, neither γδ T cell had an effect on Th1 responses (Fig. 5A–C, right panel). These results suggest that gdT17 and gdT1 are both pro-Th17, but neither has pro-Th1 activity. Summarized data of three independent experiments are shown in Fig. 5D.

To determine whether the pro-Th17 effect also could be demonstrated in vivo, three groups ($n = 6$) of TCR-$\delta^{-/-}$ mice were injected i.p. with gdT1 or gdT17 γδ T cells (5×10^5/mouse recipient) or left untreated, and then immunized with a pathogenic dose (150 μg/mouse) of the uveitogenic peptide $IRBP_{1–20}$. EAU development of the mice was monitored by fundoscopic examination. The results showed that TCR-$\delta^{-/-}$ mice preinjected with gdT17 developed more severe EAU, whereas those injected with gdT1 did not (Fig. 6A, 6B).

Cytokine assays of the culture medium performed after 48 h of in vitro stimulation showed that T cells from recipient mice injected with gdT17 γδ T cells, but not gdT1 γδ T cells, produced significantly higher amounts of IL-17 than did controls when stimulated under Th17-polarized condition (Fig. 6C) and that IFN-γ production was only marginally affected by γδ T cell administration.

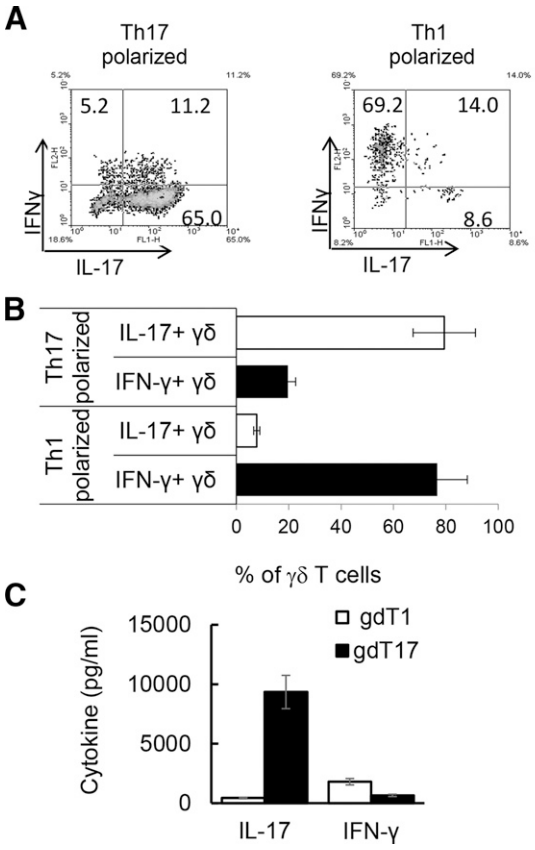

FIGURE 4. IL-17$^+$ γδ T cells activated in Th17 polarizing conditions and IFN-γ$^+$ γδ T cells activated in Th1 polarizing conditions. (**A**) Splenic T cells from IRBP$_{1-20}$/CFA-immunized B6 mice were stimulated with the immunizing Ag and APCs under Th1- or Th17-polarized conditions. After staining with PE-anti–IFN-γ Abs and FITC-anti–IL-17 Abs, the gated γδ T cells were FACS analyzed. (**B**) Data are from three separate experiments. (**C**) γδ T cells were purified from splenic T cells after 2 d of in vitro stimulation with Ag under Th1- (gdT1) or Th17-polarized (gdT17) conditions, stimulated for 48 h with anti-CD3 Ab (2 μg/ml), and stained for cytokine expression, and culture supernatants were assessed for IFN-γ and IL-17 production by ELISA.

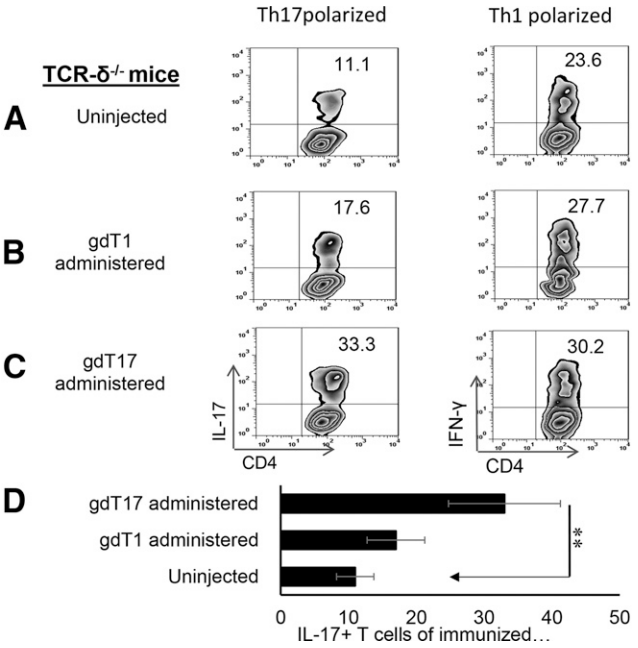

FIGURE 5. Pro-Th17 activity of gdT1 and gdT17. (**A**–**C**) CD3$^+$ responder T cells (1×10^6) prepared from TCR-δ$^{-/-}$ mice immunized with the uveitogenic peptide IRBP$_{1-20}$ were stimulated in vitro with IRBP$_{1-20}$ in the presence of syngeneic APCs (irradiated spleen cells) either alone (top panels) or after addition of 5×10^4 (5%) of gdT1 (center panels) or gdT17 (bottom panels) γδ T cells from immunized B6 mice. The activated T cells were then stained with PE-conjugated anti-IFN-γ Abs (Th1-polarized) or anti-IL-17 Abs (Th17-polarized) and analyzed by FACS. The results shown are from a single assay. (**D**) Compiled data from five separate experiments. $^{**}p < 0.01$.

Discussion

γδ T cells play an active role in the regulation of inflammation associated with infectious disease and autoimmunity (40–42). Studies have shown that γδ T cells can either enhance (7–9, 43) or inhibit (10–12) immune responses. Clinical approaches have been developed to modulate immune responses by targeting γδ T cells (44–46). Functional diversity of γδ T cells has been previously observed between γδ T cell subsets expressing distinct TCRs (13–16). Recent studies showed that the regulatory effect of γδ T cells fluctuated when γδ T cell activation status changes (1, 17, 19, 20, 47, 48). Knowledge of how γδ T cells modulate immune responses and why these cells can either enhance or inhibit inflammation are very much needed for development of therapeutic applications.

We have previously reported that γδ T cells play a major role in regulating the IL-17$^+$ autoreactive T cell response in EAU (5, 17, 33) and that the regulatory effect of γδ T cells changed depending on their level of activation (17, 19, 20). γδ T cells can be activated through multiple pathways (1, 21–23); for example, γδ T cells can be activated by bacterial products, such as tetanus toxoid (49), staphylococcal enterotoxin A (50), heat shock protein 65 (HSP65) (51), DCs (37, 52, 53), and cytokines (1, 19, 21, 47). In this study, we asked whether γδ T cells activated via different activation pathways are functionally distinct. Our results demonstrate that the function of γδ T cells is not only dependent on the level of their activation, but also on the pathway through which they were activated.

Factors that contribute to the generation of IFN-γ$^+$ or IL-17$^+$ γδ T cells have been examined previously (54–62). For example, it was shown that Ag-naive cells produce IL-17 whereas Ag-experienced cells produce IFN-γ (58). It was also shown that human γδ T cells can be polarized into Th17 (producing only IL-17), Th1/17 (producing both IFN-γ and IL-17), and Th22 (producing only IL-22) populations (56, 60). It has also been reported that γδ T cells expressing different levels of surface CD27 (54, 57) or CCR6 (57) are capable of producing different levels of IL-17 or IFN-γ (58). Our results show that in vivo–activated murine γδ T cells express either IL-17 and/or IFN-γ, whereas cultured γδ T cells predominantly express IL-17$^+$. We also found that a number of environmental factors influence the activation of IFN-γ$^+$ and/or IL-17$^+$ γδ T cells. Among those, the T cell–polarizing cytokines IL-12 and IL-23 are important. However, cytokine-activated γδ T cells produced only IL-17, whereas anti–CD3-activated γδ T cells produced both IFN-γ and IL-17.

Susceptibility to dissociated or polarizing activation appears to be a common property of immune cells. αβ T cells activated under different polarizing conditions are phenotypically and functionally distinct (21, 27–29). Likewise, macrophages activated under polarizing conditions are functionally different (30, 31). NK cells produced higher amounts of IFN-γ upon cross-linking of NK receptors, whereas their stimulation with IL-2 induced proliferation without IFN-γ production (63–65). NKT cells produced IFN-γ upon NK receptor cross-linking but secreted both IFN-γ and IL-4 upon TCR cross-linking (63, 64, 66). We therefore examined the function of γδ T cells activated under Th1 or Th17 polarizing conditions and found that more abundant IL-17$^+$ γδ T cells are activated and

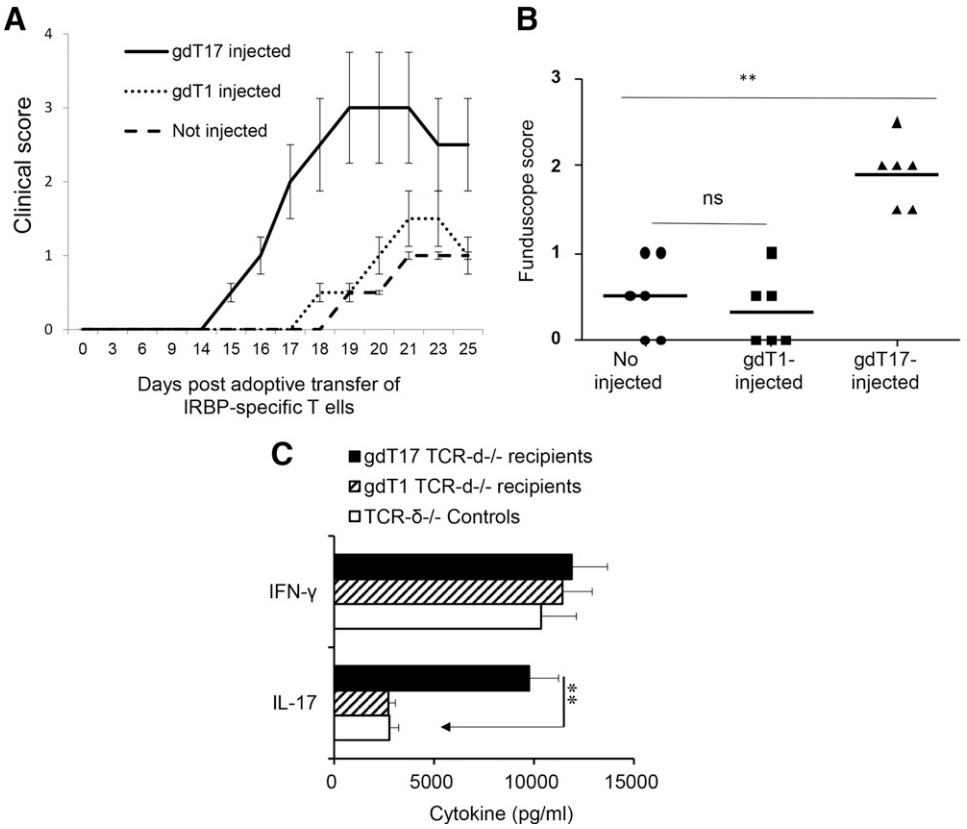

FIGURE 6. gdT17, but not gdT1, cells have enhanced pathogenic activity and IL-17 production. (**A**) Three groups ($n = 6$) of TCR-$\delta^{-/-}$ mice were left untreated or were injected with gdT1 or gdT17 γδ T cells (5×10^5/mouse recipient). All mice were immunized with uveitogenic peptide $IRBP_{1-20}$ and examined three times a week by indirect fundoscopy until day 25. (**B**) $CD3^+$ T cells were separated from each group on day 13 after immunization, after stimulated with $IRBP_{1-20}$ in vitro under Th17-polarized conditions and activated T cells adoptively transferred to naive B6 mice. Mice were examined three times a week by indirect fundoscopy until day 15 postimmunization. (**C**) IL-17 and IFN-γ levels in the culture medium were assessed by ELISA after stimulation of responder T cells with the immunizing Ag and splenic APCs for 2 d. Data from three independent experiments are pooled (C). $^{**}p < 0.01$.

expanded when activated under Th17-polarizing conditions, whereas IFN-γ–expressing cells prevailed when activated under Th1-polarizing conditions. Strikingly, IL-17^+ and the IFN-γ^+ γδ T cells differed greatly in regulating Th17 responses. Only IL-17^+ γδ T cells are able to promote Th17 responses, both in vitro and in vivo.

γδ T cells regulate autoimmune responses via diverse pathways. We previously reported that activated murine γδ T cells can express high levels of MHC class II Ags, are effective APCs, and stimulate Ag-specific T cell activation (67). Our previous work also demonstrated that expression of CD73 at different levels plays an essential role in the balance of disease-enhancing and inhibiting functions of γδ T cells (20). In addition, γδ T cells enhance the autoimmune response, in part, because they inhibit the Foxp3 T cell response more effectively (68). We were also able to show that reciprocal interactions between γδ T cells and DCs play a critical role in Th17 responses (37). A better understanding of the functional conversion of γδ T cells under the conditions that lead to their activation could enhance our ability to develop γδ T cell–targeted immunotherapies in the treatment of autoimmune diseases.

In conclusion, our results demonstrate that although γδ T cell activation is a key pathogenic event causing disease progression, and although pro-Th17 activity is restricted to IL-17^+ γδ T cells, the balance of IL-17^+ and IFN-γ^+ γδ T cells is influenced by the environmental factors that mediate their activation.

Disclosures

The authors have no financial conflicts of interest.

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