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IL-23 Receptor Expression on $\gamma\delta$ T Cells Correlates with Their Enhancing or Suppressive Effects on Autoreactive T Cells in Experimental Autoimmune Uveitis

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We have previously reported that, depending on their activation status, mouse $\gamma\delta$ T cells can either enhance or inhibit the activity of IL-17⁺ autoreactive T cells in experimental autoimmune uveitis. In this study, we showed that $\gamma\delta$ T cells in naive C57BL/6 (B6) mouse do not express the IL-23R, whereas in immunized mice, it is expressed on >50% of $\gamma\delta$ T cells. In vitro studies showed that IL-23R expression on $\gamma\delta$ T cells is modulated by their state of activation, as weakly activated $\gamma\delta$ T cells expressed the IL-23R, but highly activated $\gamma\delta$ T cells did not. Functional studies showed that IL-23R⁺ $\gamma\delta$ T cells had the strongest suppressive effect on IL-17⁺ autoreactive T cells, and that this effect was inhibited when the IL-23R was blocked by anti-IL-23R Ab or in the presence of excessive amounts of exogenous IL-23. We conclude that the balance between the enhancing and inhibitory effects of $\gamma\delta$ T cells is regulated by their level of IL-23R expression. The expression of variable IL-23R levels allows $\gamma\delta$ T cells to have different regulatory effects on adaptive immune responses, conceivably as a result of $\alpha\beta$ and $\gamma\delta$ T cells competing for IL-23. *The Journal of Immunology*, 2013, 191: 1118–1125.

Lymphocytes expressing $\gamma\delta$ TCR play a role in the regulation of inflammatory processes associated with infections, tumors, and autoimmunity (1–6). Studies have shown that $\gamma\delta$ T cells can either enhance (7–9) or inhibit (2, 10–12) an adaptive immune response, and that $\gamma\delta$ T cell subsets expressing distinct TCRs show functional diversity (13–16). Recent studies have shown that the regulatory effect of $\gamma\delta$ T cells is not a stable feature but fluctuates with $\gamma\delta$ T cell activation status (17, 18). The mechanisms by which $\gamma\delta$ T cells enhance or inhibit an adaptive immune response are incompletely understood, and a better understanding of the flexible regulatory effect of $\gamma\delta$ T cells should facilitate the development of $\gamma\delta$ T cell-targeted immunotherapies for related diseases.

In this study, to define the mechanism by which $\gamma\delta$ T cells regulate the autoimmune response, we examined whether the enhancing and inhibitory effects of $\gamma\delta$ T cells can be predicted based on the presence of specific biomarkers. By comparing the enhancing or suppressive activities of $\gamma\delta$ T cells activated to different extents or by different pathways, we found that the IL-23R was such a marker. Our results showed that IL-23R expression differed between $\gamma\delta$ and $\alpha\beta$ T cells, that levels of surface-expressed IL-23R were different in $\gamma\delta$ T cells activated to different extents, and that V γ 4⁺ $\gamma\delta$ T cells and V γ 1⁺ $\gamma\delta$ T cells

differed greatly in IL-23R expression. Functional studies showed that the suppressive effect of $\gamma\delta$ T cells was positively correlated with levels of IL-23R expression, both in vitro and in vivo. Manipulation of IL-23R function on $\gamma\delta$ T cells using an anti-IL-23 Ab or by the presence of high amounts of exogenous IL-23 reduced $\gamma\delta$ T cell suppressive activity. We also showed that $\gamma\delta$ T cells express the IL-23R in a biphasic fashion, as partially activated $\gamma\delta$ T cells; but those $\gamma\delta$ T cells that were either not activated or highly activated did not express the IL-23R. Weak activation of previously nonactivated $\gamma\delta$ T cells led to IL-23R expression, whereas exposure to a combination of stimulants resulted in highly activated $\gamma\delta$ T cells with no IL-23R expression. We conclude that the enhancing and inhibitory effects of $\gamma\delta$ T cells are switched on and off during $\gamma\delta$ T cell activation, and that the expression of variable IL-23R levels allows $\gamma\delta$ T cells to exert different regulatory effects on the adaptive immune response, conceivably by competition between $\alpha\beta$ and $\gamma\delta$ T cells for IL-23.

Materials and Methods

Animals and reagents

Female C57BL/6 (B6) 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 followed. Recombinant murine IL-23 and IL-12 were purchased from R&D Systems (Minneapolis, MN). FITC- or PE-conjugated Abs against mouse IFN- γ , IL-17, $\alpha\beta$ TCR, $\gamma\delta$ TCR, and anti-mouse V γ 1/V γ 4 were purchased from BioLegend (San Diego, CA). PE-anti-IL-23R Ab was purchased from R&D Systems.

Immunization procedure and in vitro stimulation of in vivo primed T cells

B6 mice were immunized s.c. over 6 spots at the tail base and on the flank with 200 μ l emulsion containing 150 μ g of the uveitogenic peptide IRBP_{1–20} (aa 1–20 of human interphotoreceptor retinoid-binding protein [IRBP; Sigma, St. Louis, MO]) emulsified in CFA (Difco, Detroit, MI). Concurrently, 200 ng pertussis toxin (Sigma) was injected i.p. At day 13 post-immunization, 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

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Abbreviations used in this article: B6, C57BL/6; IRBP, interphotoreceptor retinoid-binding protein.

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RPMI 1640 medium (Cellgro, Manassas, VA) containing 10% FCS were added to each well of a 6-well plate (Costar) and stimulated for 48 h with 10 μ g/ml IRBP₁₋₂₀ in the presence of 1×10^7 irradiated syngeneic spleen cells as APCs in the presence of either IL-12 (Th1 polarized) or IL-23 (Th17 polarized; 10 ng/ml), and then activated T cell blasts were separated by Ficoll gradient centrifugation and cultured for another 72 h in the same medium used for stimulation without the peptide.

$\gamma\delta$ T cell preparation

$\gamma\delta$ T cells were purified from IRBP₁₋₂₀ immunized B6 mice (5, 19, 20). Nylon wool-enriched splenic T cells from immunized mice were incubated for 10 min at 4°C with FITC-conjugated anti-mouse $\gamma\delta$ TCR or $\alpha\beta$ TCR Ab, then for 15 min at 4°C with anti-FITC Microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The cells were then separated into bound and nonbound on an autoMACS separator column (Miltenyi Biotec GmbH). The purity of the isolated cell as determined by flow cytometric analysis of PE-conjugated Abs against $\alpha\beta$ or $\gamma\delta$ T cells was >95%. Resting cells were harvested from this isolate after culture in cytokine-free

medium for 5–7 d, when they showed downregulation of CD69 expression. Activated $\gamma\delta$ T cells were prepared by incubating the resting $\gamma\delta$ T cells with anti- $\gamma\delta$ TCR (GL3) and anti-CD28 Abs (2 μ g/ml) for 2 d.

Assessment of in vitro effect of IL-23⁺ and IL-23R⁻ $\gamma\delta$ T cells

Responder $\alpha\beta$ T cells (1×10^6 /well) were cocultured with IRBP₁₋₂₀ (10 μ g/ml) and APCs (irradiated spleen cells) in a 24-well plate, with or without addition of 2% (2×10^4 /well) of IL-23⁺ or IL-23R⁻ $\gamma\delta$ T cells. The numbers of Ag-specific T cells expressing IL-17 or IFN- γ determined by intracellular staining followed by FACS analysis after 5 d of stimulation.

Testing the in vivo effect of injected $\gamma\delta$ T cells

TCR- $\delta^{-/-}$ mice were randomly grouped and left untreated or injected i.p. with IL-23R⁺ or IL-23R⁻ $\gamma\delta$ T cells (5×10^5), prepared as described earlier; then all the mice were immunized with a pathogenic dose of IRBP₁₋₂₀. On day 13 postimmunization, CD4⁺ $\alpha\beta$ TCR⁺ T cells were separated by magnetic sorting from lymph node and spleen cells, and stimulated with 10 μ g/ml of immunizing peptide in the presence of irradiated

FIGURE 1. Biphasic IL-23R expression by $\gamma\delta$ T cells. **(A)** More than half of $\gamma\delta$ T cells from IRBP₁₋₂₀/CFA-immunized, but not naive, mice express the IL-23R. Splenic T cells from naive (left panel) or IRBP₁₋₂₀/CFA-immunized mice at 13 d after immunization (right panel) were double-stained with Abs specific for $\gamma\delta$ T cells (GL3) and the IL-23R. **(B)** Highly activated $\gamma\delta$ T cells are IL-23Rs. Cultured $\gamma\delta$ T cells were left untreated (left panel) or exposed to a combination of cytokines (IL-1, IL-7, and IL-23) for 48 h (right panel); then IL-23R expression was examined. **(C)** IL-23R expression by $\alpha\beta$ T cells newly isolated from naive and immunized mice. **(D)** IL-23R expression by cultured $\alpha\beta$ T cells before and after in vitro activation. **(E and F)** Kinetic study of IL-23R expression on $\gamma\delta$ T cells after a single activation step using a mixture of cytokines (IL-2, IL-7, and IL-23). The cells were cultured in cytokine-free medium containing 10% FCS. IL-23R expression was measured on rested and newly activated $\gamma\delta$ T cells and at 4–18 d after activation (E), and IL-23R mRNA expression was measured by real-time PCR (F). In all panels, the results shown are from a single experiment and are representative of those obtained in more than five experiments. ** $p < 0.01$.

syngeneic spleen cells. The activated T cell blasts were then separated by Ficol 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 assessed.

Cytokine assays

Enriched T cells (3×10^4 cells/well) from the draining lymph nodes and spleens were cultured at 37°C for 48 h in 96-well microtiter plates with irradiated syngeneic spleen APCs (1×10^5) in the presence of IRBP₁₋₂₀; then a fraction of the culture supernatant was assayed for IL-17 using ELISA kits (R&D Systems).

Intracellular staining and FACS analysis

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

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 using the two-tailed *t* test.

Results

Biphasic expression of the IL-23R by $\gamma\delta$ T cells

We first measured numbers of IL-23R⁺ $\gamma\delta$ T cells in naive and immunized mice. As shown in Fig. 1A, none of the $\gamma\delta$ T cells in naive B6 mice expressed appreciable amounts of the IL-23R, whereas this receptor was expressed by a high percentage (51.4%) of $\gamma\delta$ T cells from mice immunized with IRBP in CFA. We then examined IL-23R expression on $\gamma\delta$ T cells isolated from immunized B6 mice and cultured in cytokine-free medium for 5–10 d (resting cells). As shown in Fig. 1B, resting cultured $\gamma\delta$ T cells expressed the IL-23R, but activation by 48-h exposure to a combination of cytokines resulted in a marked decrease in IL-23R expression from 69% of cells to 8%. We also compared IL-

23R expression by $\gamma\delta$ and $\alpha\beta$ T cells freshly isolated from naive and immunized mice, and found that it was expressed on 0.2% of $\alpha\beta$ T cells from naive mice and only 3–5% of $\alpha\beta$ T cells from immunized mice (Fig. 1C), contrasting with expression on 50–60% of $\gamma\delta$ T cells from immunized mice (Fig. 1A). Moreover, only a small percentage of resting cultured $\alpha\beta$ T cells expressed the IL-23R after 48-h activation by incubation with anti-CD3 Abs (increase from 2.7 to 6.1%; Fig. 1D). A kinetic study (Fig. 1E, 1F) showed that resting $\gamma\delta$ T cells expressed high levels of IL-23R protein and mRNA, whereas 2 d after in vitro activation the IL-23R was undetectable and remained so for approximately a week, then gradually reappeared and was present at high levels for at least 18 d, the longest time tested, then was again lost on reactivation. Thus, cultured $\gamma\delta$ T cells expressed high IL-23R levels until they were exposed to a new activation signal after being rested.

Weakly activated $\gamma\delta$ T cells express the highest IL-23R levels

Our studies showed that cytokines, particularly IL-1, IL-7, and IL-23, are able to activate $\gamma\delta$ T cells; in addition, these cytokines have a synergistic effect on $\gamma\delta$ activation (Fig. 2); therefore, in some cases, we used a combination of three cytokines to activate $\gamma\delta$ T cells. After exposure of resting cultured $\gamma\delta$ T cells to a single cytokine (IL-1, IL-7, or IL-23) or a combination of all three, the activated cells expressed increased levels of CD69 (Fig. 2A) and produced an increased amount of IL-17 (Fig. 2B), with the combination of three cytokines having a much greater effect. In contrast, although a single cytokine increased expression of IL-23R mRNA and surface IL-23R, the combination of all three resulted in almost complete loss of expression (Fig. 2C, 2D). These results suggest that a combination of different cytokines has a synergistic effect on $\gamma\delta$ T cell activation, but only weakly activated $\gamma\delta$ T cells show increased IL-23R levels.

The suppressive effect is restricted to IL-23R⁺ $\gamma\delta$ T cells

We then compared the regulatory effect of IL-23R⁺ and IL-23R[−] $\gamma\delta$ T cells on the IL-17⁺ IRBP-specific T cell response in vitro. As

FIGURE 2. Weakly activated $\gamma\delta$ T cells are IL-23R⁺. (A) $\gamma\delta$ T cells express high levels of CD69 after exposure to a single cytokine or a combination of cytokines. (B) ELISA test for IL-17 production. After exposure to a single cytokine or a mixture of cytokines (IL-1, IL-7, IL-23), $\gamma\delta$ T cells produce higher amounts of IL-17. (C) Real-time PCR assay for IL-23R mRNA expression. After exposure to a single cytokine (IL-1, IL-7, or IL-23), $\gamma\delta$ T cells express increased IL-23R levels, whereas after exposure to multiple cytokines, IL-23R expression is suppressed. **p* < 0.05, ***p* < 0.01. (D) Cell-surface expression of IL-23R of $\gamma\delta$ T cells after exposure to indicated cytokine or cytokines.

we reported previously (17, 18), addition of a small number of cultured $\gamma\delta$ T cells to IRBP-specific $\alpha\beta$ T cells prepared from immunized TCR- $\delta^{-/-}$ mice greatly affected the response of the $\alpha\beta$ T cells. As shown in Fig. 3A and 3B, addition of 2% of IL-23R $^{-}$ highly activated $\gamma\delta$ T cells enhanced the IL-17 $^{+}$ $\alpha\beta$ T cell response from 15.7 to 37.6%, whereas addition of 2% of IL-23R $^{+}$ resting $\gamma\delta$ T cells inhibited the response, which declined from 15.7 to 6.2%. To determine whether IL-23R $^{+}$ and IL-23R $^{-}$ $\gamma\delta$ T cells had a similar or different regulatory effect on the IL-17 $^{+}$ IRBP-specific T cell response in vivo, groups of TCR- $\delta^{-/-}$ mice ($n = 6$) were immunized with the uveitogenic peptide (IRBP₁₋₂₀/CFA) with or without i.p. transfer of 10^5 IL-23R $^{+}$ resting or IL-

23R $^{-}$ highly activated $\gamma\delta$ T cells 5 d after immunization. At 13 d postimmunization, splenic T cells were enriched and subjected to stimulation with the immunizing peptide for 5 d; then IL-17 $^{+}$ $\alpha\beta$ TCR $^{+}$ T cells were measured by cytoplasmic staining. As shown in Fig. 3C, mice that received IL-23R $^{-}$ highly activated $\gamma\delta$ T cells, but not resting IL-23R $^{+}$ $\gamma\delta$ T cells, showed a significant increase in the percentage of IL-17 $^{+}$ IRBP-specific T cells.

IL-23R $^{+}$ $\gamma\delta$ T cells treated with anti-IL-23R Ab have a reduced suppressive effect on Th17 cells

One possible mechanism for the increased suppressive activity of IL-23R $^{+}$ $\gamma\delta$ T cells was that IL-23R $^{+}$ $\gamma\delta$ T cells bind the IL-23 that is needed for the activation of IL-17 $^{+}$ autoreactive T cells. To test this possibility, we assessed the inhibitory effect of IL-23R $^{+}$ $\gamma\delta$ T cells on the activation of both Th17 and Th1 IRBP-specific T cells. The responder T cells were stimulated with the immunization Ag (IRBP) under either Th1-polarizing (Fig. 4A) or Th17-polarizing (Fig. 4B) conditions in the presence or absence of 2% $\gamma\delta$ T cells that had been previously left untreated or incubated with anti-IL-23R Ab. As shown in Fig. 4, treatment with anti-IL-23R Ab reduced the suppressive activity of IL-23R $^{+}$ $\gamma\delta$ T cells on autoreactive Th17 cells (Fig. 4A, 4C), but had only a very limited effect on autoreactive Th1 T cells (Fig. 4B), supporting the idea that competition for IL-23 is the mechanism by which $\gamma\delta$ T cells suppress Th17 cells.

Addition of excess IL-23 inhibits the suppressive effect of IL-23R $^{+}$ $\gamma\delta$ T cells

We hypothesized that the suppressive effect might be caused by competitive binding of IL-23 by the earlier responding innate $\gamma\delta$ T cells, thus preventing the later responding IL-23-dependent $\alpha\beta$ T cells from reacting. In cell activation studies under Th17-polarized conditions, we previously found that the optimal concentration of IL-23 was 10 ng/ml. If the suppressive effect was due to IL-23 binding by innate $\gamma\delta$ T cells, it should be reduced if large amounts of IL-23 were provided. We therefore set up two parallel assay groups in which Th17 $^{+}$ T cell activation was performed under Th17-polarized conditions in the presence of either the standard concentration (10 ng/ml) or a high concentration (40 ng/ml) of IL-23. As shown in Fig. 4D and 4E, addition of a high concentration of IL-23 markedly reduced the suppressive effect of IL-23R $^{+}$ $\gamma\delta$ T cells, suggesting that their ability to bind IL-23 is involved in their inhibitory effect.

IL-23R $^{+}$, but not IL-23R $^{-}$, $\gamma\delta$ T cells proliferate vigorously when added to $\alpha\beta$ responder T cells under Th17-polarized conditions

To further test the possibility that competition for IL-23 was responsible for the $\gamma\delta$ T cell suppressive effect, we assessed the number of proliferating $\alpha\beta$ and $\gamma\delta$ T cells after 5 d of in vitro stimulation of responder $\alpha\beta$ T cells derived from IRBP-immunized TCR- $\delta^{-/-}$ mice with immunizing peptide in the presence of 2% IL-23 $^{+}$ resting or IL-23 $^{-}$ highly activated $\gamma\delta$ T cells under Th17-polarized conditions. As shown in Fig. 5, although IL-23R $^{+}$ resting $\gamma\delta$ T cells did not promote the activation of $\alpha\beta$ T cells, the $\gamma\delta$ T cells themselves proliferated vigorously, in sharp contrast with the response of cultures with added IL-23R $^{-}$ activated $\gamma\delta$ T cells, in which $\alpha\beta$ T cell proliferation was dominant.

FCS contains a factor or factors that support IL-23R expression by cultured $\gamma\delta$ T cells

As mentioned earlier, $\gamma\delta$ T cells from naive mice do not express the IL-23R, whereas resting cultured $\gamma\delta$ T cells express high IL-23R levels, even after prolonged culture in cytokine-free medium when the cells no longer express activation markers, such as CD69. To examine the possibility that factors in the culture me-

FIGURE 3. IL-23R $^{+}$ $\gamma\delta$ T cells show strong suppressive activity. (**A**) Addition of 2% IL-23R $^{+}$, but not IL-23R $^{-}$, $\gamma\delta$ T cells to responder $\alpha\beta$ T cells inhibits the Th17 response. Responder $\alpha\beta$ T cells were obtained from TCR- $\delta^{-/-}$ mice immunized with IRBP₁₋₂₀/CFA and were stimulated (1×10^6 /well) for 5 d with immunizing peptide and APCs without (left panel) or with addition of 2×10^4 /well resting IL-23R $^{+}$ (center panel) or activated IL-23R $^{-}$ (right panel) $\gamma\delta$ T cells; then the activated T cells were separated, stained intracellularly for IL-17, and subjected to FACS analysis. (**B**) Summarized results for five assays showing the percentage of IL-17 $^{+}$ IRBP-specific T cells in 10^7 $\alpha\beta$ T cells from an immunized mouse after 5-d in vitro expansion in the absence or presence of 2% IL-23R $^{-}$ or IL-23R $^{+}$ $\gamma\delta$ T cells. $*p < 0.05$, $**p < 0.01$. (**C**) Groups of TCR- $\delta^{-/-}$ mice ($n = 6$) immunized with the uveitogenic peptide (IRBP₁₋₂₀/CFA) were left untreated or were injected i.p. with 10^5 IL-23R $^{+}$ or IL-23R $^{-}$ $\gamma\delta$ T cells 5 d after immunization; then at 13 d postimmunization, splenic T cells were enriched and stimulated with immunizing peptide under Th17-polarized conditions, and the activated T cells were separated and stained for intracellular expression of IL-17 (top row) or IFN- γ (bottom row).

FIGURE 4. Anti-IL-23R Ab or a high concentration of IL-23 reduces the suppressive effect of IL-23R⁺ $\gamma\delta$ T cells. (**A** and **B**) $\alpha\beta$ T cells (1×10^6 /well) from TCR- $\delta^{-/-}$ mice immunized with IRBP₁₋₂₀/CFA were stimulated with immunizing peptide and APCs under Th17-polarized (**A**) or Th1-polarized (**B**) conditions. The percentage of IL-17⁺ and IFN- γ ⁺ $\alpha\beta$ T cells generated in the absence or presence of 2% (2×10^4 /well) IL-23R⁺ $\gamma\delta$ T cells that had been previously left untreated or pretreated with anti-IL-23R Abs is shown. (**C**) Summary statistics of multiple experiments shown in (**A**) and (**B**). $**p < 0.01$. (**D**) Addition of a high concentration of IL-23 reduces the suppressive effect of IL-23R⁺ $\gamma\delta$ T cells. The protocol for in vitro activation of Th17 cells was that described in Fig. 3. The responses shown are those of IL-17⁺ $\alpha\beta$ TCR⁺ T cells with or without addition of IL-23R⁺ $\gamma\delta$ T cells in the presence of either the normally optimal concentration (10 ng/ml) or a high concentration (40 ng/ml) of IL-23. (**E**) Summary statistics of multiple experiments shown in (**D**). $**p < 0.01$.

dium other than added cytokines were able to maintain IL-23R expression on $\gamma\delta$ T cells, we tested whether FCS-containing medium helped retain IL-23R expression. As shown in Fig. 6, when we cultured $\gamma\delta$ T cells for 4 d in cytokine-free medium containing 0–10% of heat-inactivated FCS, we found that $\gamma\delta$ T cells cultured in FCS-free medium no longer expressed the IL-23R, whereas those cultured in medium containing >3% FCS

showed partial or complete retention of IL-23R expression, showing that FCS contains a factor or factors that support IL-23R expression by $\gamma\delta$ T cells.

Different IL-23R expression by V γ 1⁺ and V γ 4⁺ $\gamma\delta$ T cells

A previous study in *Listeria monocytogenes*-infected mice showed that the IL-23R is expressed by $\gamma\delta$ T cells expressing TCR-V γ 4 or

FIGURE 5. IL-23R⁺ $\gamma\delta$ T cells proliferate vigorously while they suppress the $\alpha\beta$ T cell response. In a 24-well plate, 2% (1×10^4 /well) IL-23R⁺ or IL-23R⁻ $\gamma\delta$ T cells were added to 10^6 $\alpha\beta$ responder T cells from IRBP/CFA-immunized TCR- $\delta^{-/-}$ mice. After 5-d antigenic stimulation under Th17-polarizing conditions, the activated T cells were separated by Ficoll gradient centrifugation, then surface stained with anti-mouse $\alpha\beta$ TCR or $\gamma\delta$ TCR Abs and stained intracellularly for IL-17. The results of two separate experiments are shown.

FIGURE 6. FCS contains a factor or factors that help $\gamma\delta$ T cells retain IL-23R expression. (*Upper panels*) A $\gamma\delta$ T cell line was cultured for 4 d in cytokine-free medium containing the indicated percentage of FCS and then was stained using a FITC-conjugated anti-IL-23R Ab. (*Bottom panel*) Real-time assay for IL-23 mRNA levels in the treated cells expressed relative to that in naive $\gamma\delta$ T cells. * $p < 0.05$, ** $p < 0.01$.

TCR-V γ 6, but not by $\gamma\delta$ T cells expressing TCR-V γ 1 (21). To determine whether the IL-23R-expressing $\gamma\delta$ T cells in B6 mice at 13 d after IRBP immunization belonged to different $\gamma\delta$ T cell subsets expressing different TCR segments, we compared IL-23R expression of V γ 1⁺ and V γ 4⁺ cells in naive and immunized mouse spleens. As shown in Fig. 7A, the IL-23R was expressed at high levels in V γ 4⁺ cells from immunized mice, but only at low levels in these cells from naive mice, whereas V γ 1⁺ cells from either naive or immunized mice expressed only low or very low levels. Similarly, as shown in Fig. 7B, when cultured in vitro, V γ 1⁺ cells initially expressed the IL-23R at lower levels than V γ 4⁺ cells, but on activation with either a cytokine mixture or anti-CD3 Abs, V γ 1⁺ cells showed increased IL-23R expression,

whereas V γ 4⁺ cells showed a marked reduction in IL-23R expression.

Discussion

Although a regulatory effect of $\gamma\delta$ T cells on adaptive immunity has been repeatedly observed (15, 22–24), knowledge of how these cells regulate the immune response is still limited, and the mechanisms by which they enhance an immune response in some cases, but inhibit it in others, remain largely obscure. $\gamma\delta$ T cell subsets expressing distinct $\gamma\delta$ TCR V γ segments have been found to show functional diversity (13–16). More recently, we showed that the regulatory effect of $\gamma\delta$ T cells fluctuates with their activation status (17, 18). Examining the mechanism by which $\gamma\delta$ T cells exert enhancing or inhibitory effects on autoimmune responses, we previously showed that activated $\gamma\delta$ T cells differ greatly from nonactivated $\gamma\delta$ T cells in their regulatory effect (17, 25). In this study, we found that IL-23R expression on $\gamma\delta$ T cells differed greatly from that on $\alpha\beta$ T cells, with a large percentage (~50%) of $\gamma\delta$ T cells, but only a small percentage of $\alpha\beta$ T cells (3%), in the spleens of immunized mice expressing the IL-23R. Our in vitro studies showed that IL-23R expression was seen on $\gamma\delta$ T cells exposed to a single cytokine, which provides only a partial stimulatory effect, but not on highly activated $\gamma\delta$ T cells, such as those activated by multiple cytokines or anti-CD3 Abs. Both in vitro and in vivo functional tests showed that IL-23R⁺ $\gamma\delta$ T cells had a strong suppressive effect, whereas IL-23R[−] $\gamma\delta$ T cells were far less suppressive when nonactivated and had an enhancing effect when highly activated. Because both nonactivated and highly activated $\gamma\delta$ T cells are IL-23R[−], the function of the IL-23R[−] $\gamma\delta$ T cells is complex and would depend on their activation status (17). We have determined factors that lead to increased expression of IL-23R on $\gamma\delta$ cells in immunized mice. Our results show that a synergistic effect between CFA and pertussis toxin, rather than the immunizing autoantigen, has caused this alteration, and the expression of IL-23R on $\gamma\delta$ T cells directly correlates with the activation status of the latter cells.

It appears that $\gamma\delta$ T cells can readily alter their regulatory activity in response to environmental factors. Given that IL-23 is an important growth factor for pathogenic IL-17-producing $\alpha\beta$ T (Th17) cells (21, 26–28) and that it is produced only in small amounts and only by myeloid cells activated by certain ligands (29, 30), binding of IL-23 to the earlier responding innate $\gamma\delta$ T cells might have a significant impact on subsequent $\alpha\beta$ T cell responses. This idea was supported by experiments showing the

FIGURE 7. Highly activated V γ 4⁺, but not V γ 1⁺, $\gamma\delta$ T cells lose IL-23R expression. (**A**) Enriched splenic T cells prepared by passage through nylon wool from naive B6 mice or B6 mice 13 d after immunization with IRBP/CFA were double-stained for V γ 1 or V γ 4 and the IL-23R and analyzed by FACS. (**B**) IL-23R expression by resting cultured V γ 1⁺ and V γ 4⁺ $\gamma\delta$ T cell lines measured before and after 48-h exposure to a combination of cytokines (IL-1, IL-7, and IL-23) or anti-CD3 Abs (2 μ g/ml).

suppressive effect was abolished by blockade of the IL-23R on $\gamma\delta$ T cells using anti-IL-23R Ab and attenuated in the presence of a high concentration of exogenous IL-23. In addition, IL-23R⁺ $\gamma\delta$ T cells proliferated vigorously when added to responder $\alpha\beta$ T cells under Th17-polarized conditions (culture medium containing IL-23), whereas IL-23R⁻ $\gamma\delta$ T cells proliferated only poorly.

$\gamma\delta$ T cells can be activated by multiple pathways in the absence of TCR ligation (1, 31–35). We have previously shown that $\gamma\delta$ T cells in mice with induced experimental autoimmune uveitis are not activated by the immunizing Ag, but by cytokines (20). In this article, we showed that cultured $\gamma\delta$ T cells could be readily activated by exposure to various cytokines. The degree of activation of $\gamma\delta$ T cells after exposure to different stimulatory factors varied, as judged by expression of activation surface molecules (CD69, CD62L, and CD44) and the production of cytokines (IL-17, IFN- γ , and IL-22). A synergistic effect was seen when $\gamma\delta$ T cells were exposed to a combination of stimulatory factors. An inflammatory condition might result in partially or highly activated $\gamma\delta$ T cells. In a weak inflammatory environment, $\gamma\delta$ T cells might be modestly activated and become IL-23R⁺ and gain inhibitory activity, whereas in strong inflammatory conditions or when larger amounts of proinflammatory cytokines are available, they might become highly activated, become IL-23R⁻, and lose inhibitory activity. Our findings agree with a previous observation that IL-23-activated $\gamma\delta$ T cells show increased suppressive activity (1) and further support the notion that IL-23 plays a major role in modulating the regulatory activity of $\gamma\delta$ T cells on subsequently induced adaptive responses.

We have observed that FCS contain factor(s) that helped retain IL-23R expression of cultured $\gamma\delta$ T cells, in that $\gamma\delta$ T cells cultured in FCS-free medium gradually lost surface IL-23R, whereas those cultured in medium containing >3% FCS showed partial or complete retention of IL-23R expression. To overcome potentially decreased cell viability of cultured cells in serum-free conditions, we have replaced 10% FCS in culture with a small amount (1%) of mouse serum. Remarkably, $\gamma\delta$ T cells survived much better in medium containing 1% mouse serum and the results repeated those obtained with cells cultured in serum-free medium (data not shown). These results further supported our conclusion that high FCS-containing media are able to retain $\gamma\delta$ T cell activation status.

We observed that V γ 1⁺ and V γ 4⁺ $\gamma\delta$ T cells differed significantly in IL-23R expression. For example, V γ 4⁺, but not V γ 1⁺, $\gamma\delta$ T cells in immunized mice expressed the IL-23R in vivo, even though both cell populations were able to express it after anti-CD3 activation in vitro, suggesting that these two cell populations have different requirements or thresholds for activation. Further studies should clarify how differences in activation requirements contribute to the different dominance and regulatory effects of different $\gamma\delta$ T cell subsets in infections (36, 37), tumors (6, 38), and induced autoimmune diseases (39). In this study, we observed that cultured resting $\gamma\delta$ T cells retained the IL-23R in the absence of additional stimulation by cytokines, and that FCS contained a factor or factors that are able to maintain IL-23R expression on $\gamma\delta$ T cells, suggesting that $\gamma\delta$ T cells grown in FCS-containing culture medium are constantly partially activated, even though they fail to express other activation markers, such as CD69. Expression of the IL-23R may represent an early biomarker for minimally activated $\gamma\delta$ T cells. It is likely that a large number of environmental factors are capable of causing $\gamma\delta$ T cell activation, and thus modulating the regulatory effects of $\gamma\delta$ T cells.

Disclosures

The authors have no financial conflicts of interest.

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