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IL-23 Receptor Expression on γδ 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 γδ 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 γδ T cells in naive C57BL/6 (B6) mice do not express the IL-23R, whereas in immunized mice, it is expressed on >50% of γδ T cells. In vitro studies showed that IL-23R expression on γδ T cells is modulated by their state of activation, as weakly activated γδ T cells expressed the IL-23R, but highly activated γδ T cells did not. Functional studies showed that IL-23R+ γδ 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 γδ T cells is regulated by their level of IL-23R expression. The expression of variable IL-23R levels allows γδ T cells to have different regulatory effects on adaptive immune responses, conceivably as a result of αβ and γδ T cells competing for IL-23.

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Lymphocytes expressing γδ TCR play a role in the regulation of inflammatory processes associated with infections, tumors, and autoimmunity (1–6). Studies have shown that γδ T cells can either enhance (7–9) or inhibit (2, 10–12) an adaptive immune response, and that γδ T cell subsets expressing distinct TCRs show functional diversity (13–16). Recent studies have shown that 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 are incompletely understood, and a better understanding of the flexible regulatory effect of γδ T cells should facilitate the development of γδ T cell–targeted immunotherapies for related diseases.

In this study, to define the mechanism by which γδ T cells regulate the autoimmune immune response, we examined whether the enhancing and inhibitory effects of γδ T cells can be predicted based on the presence of specific biomarkers. By comparing the enhancing or suppressive activities of γδ 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 γδ and αβ T cells, that levels of surface-expressed IL-23R were different in γδ T cells activated to different extents, and that Vγ4+ γδ T cells and Vγ1+ γδ T cells differed greatly in IL-23R expression. Functional studies showed that the suppressive effect of γδ T cells was positively correlated with levels of IL-23R expression, both in vitro and in vivo. Manipulation of IL-23R function on γδ T cells using an anti–IL-23 Ab or by the presence of high amounts of exogenous IL-23 reduced γδ T cell suppressive activity. We also showed that γδ T cells express the IL-23R in a biphasic fashion, as partially activated γδ T cells; but those γδ T cells that were either not activated or highly activated did not express the IL-23R. Weak activation of previously nonactivated γδ T cells led to IL-23R expression, whereas exposure to a combination of stimulants resulted in highly activated γδ T cells with no IL-23R expression. We conclude that the enhancing and inhibitory effects of γδ T cells are switched on and off during γδ T cell activation, and that the expression of variable IL-23R levels allows γδ T cells to exert different regulatory effects on the adaptive immune response, conceivably by competition between αβ and γδ 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, αβTCR, γδTCR, and anti-mouse Vγ1/Vγ4 were purchased from BioLegend (San Diego, CA). PE-anti–IL-23 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 μl emulsion containing 150 μg of the uveitiogenic peptide IRBP1–20 (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 × 10⁶ cells in 2 ml
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 μg/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.

γδ T cell preparation

γδ 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 γδ TCR or αβ 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 αβ or γδ 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 γδ T cells were prepared by incubating the resting γδ T cells with anti-γδTCR (GL3) and anti-CD28 Abs (2 μg/ml) for 2 d.

Assessment of in vitro effect of IL-23+ and IL-23R− γδ T cells

Responder αβ T cells (1 × 10^6/well) were cocultured with IRBP1–20 (10 μg/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− γδ 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 γδ T cells

TCR-γδ− mice were randomly grouped and left untreated or injected i.p. with IL-23R+ or IL-23R− γδ 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+ αβ TCR+ T cells were separated by magnetic sorting from lymph node and spleen cells, and stimulated with 10 μg/ml of immunizing peptide in the presence of irradiated

![FIGURE 1. Biphasic IL-23R expression by γδ T cells. (A) More than half of γδ 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 γδ T cells (GL3) and the IL-23R, and the IL-23R expression was examined. (B) IL-23R expression by αβ T cells newly isolated from naive and immunized mice. (C) IL-23R expression by cultured αβ T cells before and after in vitro activation. (E and F) Kinetic study of IL-23R expression on γδ 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 γδ 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.](http://www.jimmunol.org/Downloaded from http://www.jimmunol.org/)
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 assessed.

Cytokine assays

Enriched T cells (3 × 10^6 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 IRBP (Sigma-Aldrich), 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 μl PBS) 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), intracellularly stained with Abs (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 γδ T cells

We first measured numbers of IL-23R+ γδ T cells in naive and immunized mice. As shown in Fig. 1A, none of the γδ 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 γδ T cells from mice immunized with IRBP in CFA. We then examined IL-23R expression on γδ 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 γδ 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 γδ and αβ T cells freshly isolated from naive and immunized mice, and found that it was expressed on 0.2% of αβ T cells from naive mice and only 3–5% of αβ T cells from immunized mice (Fig. 1C), contrasting with expression on 50–60% of γδ T cells from immunized mice (Fig. 1A). Moreover, only a small percentage of resting cultured αβ 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 γδ 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 γδ T cells expressed high IL-23R levels until they were exposed to a new activation signal after being rested.

Weakly activated γδ 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 γδ T cells; in addition, these cytokines have a synergistic effect on γδ activation (Fig. 2); therefore, in some cases, we used a combination of three cytokines to activate γδ T cells. After exposure of resting cultured γδ 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 γδ T cell activation, but only weakly activated γδ T cells show increased IL-23R levels.

The suppressive effect is restricted to IL-23R+ γδ T cells

We then compared the regulatory effect of IL-23R+ and IL-23R− γδ T cells on the IL-17+ IRBP-specific T cell response in vitro. As
we reported previously (17, 18), addition of a small number of cultured γδ T cells to IRBP-specific αβ T cells prepared from immunized TCR-δ−/− mice greatly affected the response of the αβ T cells. As shown in Fig. 3A and 3B, addition of 2% of IL-23R+ highly activated γδ T cells enhanced the IL-17+ αβ T cell response from 15.7 to 37.6%, whereas addition of 2% of IL-23R− resting γδ T cells inhibited the response, which declined from 15.7 to 6.2%. To determine whether IL-23R+ and IL-23R− γδ T cells had a similar or different regulatory effect on the IL-17+ IRBP-specific T cell response in vivo, groups of TCR-δ−/− mice (n = 6) were immunized with the uveitogenic peptide (IRBP1–20/CFA) with or without i.p. transfer of 105 IL-23R+ resting or IL-23R− highly activated γδ 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+ αβTCR+ T cells were measured by cytoplasmic staining. As shown in Fig. 3C, mice that received IL-23R+ highly activated γδ T cells, but not resting IL-23R− γδ T cells, showed a significant increase in the percentage of IL-17+ IRBP-specific T cells.

**IL-23R+ γδ 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+ γδ T cells was that IL-23R+ γδ 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+ γδ T cells on the activation of both Th17 and Th1 IRBP-specific T cells. The responder T cells were stimulated with the immunogen Ag (IRBP) under either Th1-polarizing (Fig. 4A) or Th17-polarizing (Fig. 4B) conditions in the presence or absence of 2% γδ 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+ γδ T cells on autoreactive Th17 cells (Fig. 4A, 4C), but had only a very limited effect on autoreactive Th1 cells (Fig. 4B), supporting the idea that competition for IL-23 is the mechanism by which γδ T cells suppress Th17 cells.

**Addition of excess IL-23 inhibits the suppressive effect of IL-23R+ γδ T cells**

We hypothesized that the suppressive effect might be caused by competitive binding of IL-23 by the earlier responding innate γδ T cells, thus preventing the later responding IL-23-dependent αβ 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 γδ 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+ γδ T cells, suggesting that their ability to bind IL-23 is involved in their inhibitory effect.

**IL-23R+, but not IL-23R−, γδ T cells proliferate vigorously when added to αβ responder T cells under Th17-polarized conditions**

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

**FCS contains a factor or factors that support IL-23R expression by cultured γδ T cells**

As mentioned earlier, γδ T cells from naive mice do not express the IL-23R, whereas resting cultured γδ 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-
medium other than added cytokines were able to maintain IL-23R expression on γδ T cells, we tested whether FCS-containing medium helped retain IL-23R expression. As shown in Fig. 6, when we cultured γδ T cells for 4 d in cytokine-free medium containing 0–10% of heat-inactivated FCS, we found that γδ 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 γδ T cells.

**Different IL-23R expression by Vγ1+ and Vγ4+ γδ T cells**

A previous study in *Listeria monocytogenes*-infected mice showed that the IL-23R is expressed by γδ T cells expressing TCR-Vγ4 or...
TCR-Vγ6, but not by γδ T cells expressing TCR-Vγ1 (21). To determine whether the IL-23R–expressing γδ T cells in B6 mice at 13 d after IRBP immunization belonged to different γδ T cell subsets expressing different TCR segments, we compared IL-23R expression of Vγ1+ and Vγ4+ cells in naive and immunized mouse spleens. As shown in Fig. 7A, the IL-23R was expressed at high levels in Vγ4+ cells from immunized mice, but only at low levels in these cells from naive mice, whereas Vγ1+ cells from either naive or immunized mice expressed only low or very low levels. Similarly, as shown in Fig. 7B, when cultured in vitro, Vγ1+ cells initially expressed the IL-23R at lower levels than Vγ4+ cells, but on activation with either a cytokine mixture or anti-CD3 Abs, Vγ1+ cells showed increased IL-23R expression, whereas Vγ4+ cells showed a marked reduction in IL-23R expression.

Discussion
Although a regulatory effect of γδ 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. γδ T cell subsets expressing distinct γδ TCR Vγ segments have been found to show functional diversity (13–16). More recently, we showed that the regulatory effect of γδ T cells fluctuates with their activation status (17, 18). Examining the mechanism by which γδ T cells exert enhancing or inhibitory effects on autoimmune responses, we previously showed that activated γδ T cells differ greatly from nonactivated γδ T cells in their regulatory effect (17, 25). In this study, we found that IL-23R expression on γδ T cells differed greatly from that on αβ T cells, with a large percentage (~50%) of γδ T cells, but only a small percentage of αβ 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 γδ T cells exposed to a single cytokine, which provides only a partial stimulatory effect, but not on highly activated γδ 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+ γδ T cells had a strong suppressive effect, whereas IL-23R− γδ T cells were far less suppressive when nonactivated and had an enhancing effect when highly activated. Because both nonactivated and highly activated γδ T cells are IL-23R−, the function of the IL-23R− γδ 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 γδ 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 γδ T cells directly correlates with the activation status of the latter cells.

It appears that γδ 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 αβ 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 γδ T cells might have a significant impact on subsequent αβ T cell responses. This idea was supported by experiments showing the
suppressive effect was abolished by blockade of the IL-23R on γδ T cells using anti-IL-23R Ab and attenuated in the presence of a high concentration of exogenous IL-23. In addition, IL-23Rγδ T cells proliferated vigorously when added to responder αβ T cells under Th17-polarized conditions (culture medium containing IL-23), whereas IL-23Rγδ T cells proliferated only poorly.

γδ T cells can be activated by multiple pathways in the absence of TCR ligation (1, 31–35). We have previously shown that γδ 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 γδ T cells could be readily activated by exposure to various cytokines. The degree of activation of γδ 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 γδ T cells were exposed to a combination of stimulatory factors. An inflammatory condition might result in partially or highly activated γδ T cells. In a weak inflammatory environment, γδ T cells might be modestly activated and become IL-23Rγδ 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-23Rγδ, and lose inhibitory activity. Our findings agree with a previous observation that IL-23–activated γδ T cells showed increased suppressive activity (1) and further support the notion that IL-23 plays a major role in modulating the regulatory activity of γδ T cells on subsequently induced adaptive responses.

We have observed that FCS contain factor(s) that helped retain IL-23R expression of cultured γδ T cells, in that γδ T cells cultured in FCS-free medium gradually lost surface IL-23R, whereas those cultured in medium containing >5% 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, γδ 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 support our conclusion that high FCS-containing media are able to retain γδ T cell activation status.

We observed that Vγ1+ and Vγ4+ γδ T cells differed significantly in IL-23R expression. For example, Vγ4+, but not Vγ1+, γδ 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 γδ T cell subsets in infections (36, 37), tumors (6, 38), and induced autoimmune diseases (39). In this study, we observed that cultured resting γδ 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 γδ T cells, suggesting that γδ 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 γδ T cells. It is likely that a large number of environmental factors are capable of causing γδ T cell activation, and thus modulating the regulatory effects of γδ T cells.

Disclosures

The authors have no financial conflicts of interest.