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Anti-Inflammatory Role of IL-17 in Experimental Autoimmune Uveitis

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Previous studies have shown that IL-17 is a strong proinflammatory cytokine and that IL-17-producing autoreactive T cells play a major role in the pathogenesis of autoimmune diseases. In a previous study, we showed that injection of experimental autoimmune uveitis-susceptible mice with anti-IL-17 Abs blocked subsequent disease development. To determine whether administration of IL-17 to experimental autoimmune uveitis-susceptible Lewis rats and B10RIII mice injected with disease-inducing peptides enhanced disease susceptibility, we injected the recipient animals with various doses of human rIL-17 (hIL-17). Unexpectedly, the treated animals showed significant amelioration of disease; in addition, both the intensity of the autoreactive response and cytokine production by the autoreactive T cells induced by immunization with uveitogenic peptides were significantly decreased. Our results show that IL-17 has anti-inflammatory activity and that this cytokine can suppress the development of autoimmune disease. The Journal of Immunology, 2009, 182: 3183–3190.

Recent studies have identified an autoreactive T cell subset that produces IL-17, but not IFN-γ and IL-4 (1, 2). Other studies have demonstrated that IL-17-producing T cells are crucially involved in the pathogenesis of autoimmune diseases, such as rheumatoid arthritis, experimental autoimmune encephalomyelitis (3–5), and other allergic diseases (6–8). This is supported by the observations that IL-17-deficient mice are resistant to an arthritis-like disease (9, 10) and have impaired host defense against microbial infection and an increased incidence of acquired delayed-type hypersensitivity (9). In addition, autoimmune disease-susceptible mice become disease resistant after treatment with an IL-17R antagonist (11).

Previous studies have shown that IL-17 is functionally proinflammatory. It increases the local production of chemokines and promotes recruitment of monocytes and neutrophils (12–16). It also directly activated fibroblasts and synoviocytes, leading to the production of IL-6, IL-8, PGE2, and G-CSF, thus enhancing the inflammatory effects on the development of EAU. We therefore concluded that IL-17 has both pro- and anti-inflammatory roles in the development of EAU.

Materials and Methods

Animals and reagents

Female Lewis rats (6–8 wk old) and B10RIII mice (12–14 wk old) were purchased from The Jackson Laboratory and were housed and maintained in the animal facilities of the University of Louisville or the Doheny Eye Institute at the University of Southern California. Institutional approval was obtained and institutional guidelines regarding animal experimentation were followed. All T cells were cultured in RPMI 1640 medium (Life Technologies) supplemented with 10% FCS (Life Technologies), 5 × 10−5 M 2-ME, and 100 μg/ml penicillin/streptomycin. IFA was obtained from Sigma-Aldrich. CFA in the text refers to IFA with added Mycobacterium tuberculosis H37Ra (Difco). IRBP peptides 1177–1191 (ADGSS-AKIIRLLILHPGNTILHVD) and IRBP161–180 (SGIPYIISYLHPGNTILHVD) were synthesized by Sigma-Aldrich. hIL-17 (hIL-17) was a gift from the Research Group of Southern Biotechnology Associates. FITC-conjugated anti-mouse IL-17 and PE-conjugated anti-rat IFN-γ Abs were purchased from eBioscience.

In a previous report, we showed that IL-17+ interphotoreceptor retinoid-binding protein (IRBP)+ peptide-specific T cells play a major role in the pathogenesis of experimental autoimmune uveitis (EAU) and that injection of anti-IL-17 Abs inhibits the development of disease (24). We also showed that, while adoptive transfer of a small number of IL-17+ IRBP peptide–specific T cells induces EAU, transfer of a larger number of Ag-nonspecific IL-17+ T cells does not (24). We therefore wished to determine the separate pathogenic role of IL-17 on IL-17+ autoreactive T cells. This report shows that, unexpectedly, injection of EAU-susceptible rats or mice with small doses of IL-17 ameliorated, rather than exacerbated, disease development. In addition, IL-17-treated rats had a significantly lower T cell response to the immunizing Ag and T cells isolated from IL-17-treated rats had a suppressor effect on EAU development on adoptive transfer into EAU-susceptible rats. We therefore conclude that IL-17 has both pro- and anti-inflammatory effects on the development of EAU.

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Active induction and adoptively transferred uveitis

The active induction of uveitis in Lewis rats using peptide IRBP<sub>1177-1191</sub> (R16) has been previously described (25). Briefly, the rats were immunized s.c. with 200 μl of an emulsion containing 50 μg of R16 and 500 μg of Mycobacterium tuberculosis H37Ra (Difco) in IFA, distributed over six spots on the tail base and flank. The animals were observed three times a week for clinical signs of uveitis by fundoscopy, starting at week 2 after transfer. Fundoscopic evaluation for longitudinal follow-up of disease was performed using a binocular microscope after pupil dilation using 0.5% tropicamide and 1.25% phenylephrine hydrochloride ophthalmic solutions. The incidence and severity of EAU were graded on a scale of 0–4 in half-point increments using previously described criteria (28) based on the type, number, and size of lesions present.

Histology and immunohistochemistry

For histology, whole eyes were collected at the end of the experiment (days 11–20 after immunization) 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 processed. Fixed and dehydrated tissues were embedded in methacrylate and 5-μm sections were cut at different levels for each eye. Severity of EAU was scored on a scale of 0 (no disease) to 4 (maximum disease) in half-point increments based on the presence of inflammatory cell infiltration of the iris, ciliary body, anterior chamber, and retina, where 0 =...
normal anterior segment and retinal architecture, with no inflammatory cells in these structures; 1 = mild inflammatory cell infiltration of the anterior segment and retina; 2 = moderate inflammatory cell infiltration of the anterior segment and retina; 3 = massive inflammatory cell infiltration of the anterior segment and retina, disorganized anterior segment and retina; and 4 = as in 3, but with photoreceptor cell damage.

**Immunofluorescence flow cytometry**

Aliquots of $1 \times 10^6$ cells were incubated for 30 min at 4°C with anti-CD4 or isotype control Abs, fixed overnight with 1 ml of fixation buffer, washed, and incubated for 30 min at 4°C with anti-rat Foxp3 Abs.

For intracellular cytokine staining, cells were pretreated for 4 h with 50 ng/ml PMA, 1 μg/ml ionomycin, and 1 μg/ml brefeldin A (Sigma-Aldrich), then washed, fixed, permeabilized overnight with Cytofix/Cytoperm buffer (eBioscience), and intracellularly stained with Abs against IFN-γ and IL-17 Abs, followed by FACS analysis. The results shown are representative of those in three experiments.

**T cell proliferation assays**

APCs (irradiated syngeneic spleen cells, $2 \times 10^5$/well) were preincubated for 1 h in 96-well flat-bottom microtiter plates with an optimal dose (5 μg/ml) of R16 in the absence or presence of IL-17, IFN-γ, or LPS, then enriched T cells ($4 \times 10^5$/well), prepared from lymph nodes or spleen by nylon wool adhesion, were added and incubation was continued for 48–72 h. $[^3H]$Thymidine incorporation during the last 16 h was assessed using a
microplate scintillation counter (Packard). The proliferative response was expressed as the mean cpm ± SD of triplicate determinations.

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

Statistical analysis
The data are expressed as the mean ± SD of the results for at least three separate experiments. Statistical analyses were performed using Student’s t test. A p value of 0.05 or less was considered to be statistically significant.

Results
hIL-17 treatment protects rats against uveitis induced by R16 injection
Lewis rats immunized with 50 μg of R16 emulsified in CFA develop uveitis after 8–9 days. To determine whether hIL-17 had an effect on R16-induced uveitis, R16-injected rats received two i.p. injections of hIL-17 (3 μg/injection) on days 4 and 7 after R16 injection (day 0), while control rats received PBS. Although all control animals developed full disease, hIL-17-treated rats developed mild disease, with a delayed onset (Fig. 1A). We then determined whether delayed injection of hIL-17 had a similar protective effect. As shown in Fig. 1B, two injections of the same dose starting at disease onset (days 10 and 12) did not significantly affect EAU development, indicating that early treatment is required for a therapeutic effect.

To determine whether hIL-17 also affected the development of mouse EAU, B10RIII mice were immunized with 200 μg of IRBP161–180 peptide emulsified in CFA with or without IL-17 treatment. Mice subjected to hIL-17 treatment received three i.p. injections of hIL-17 (1 μg/mouse) on days 0, 4, and 8. The animals were examined every 2 days for clinical signs of uveitis by fundoscopy, starting on day 8 after immunization. The results showed that, although all control animals developed full disease, hIL-17-treated mice were protected (Fig. 1, C and D).

Effect of IL-17 treatment on chronic relapsing uveitis
We previously reported that adoptive transfer of R16-specific T cells into naïve Lewis rats induces chronic relapsing disease in contrast to the monophasic disease induced by immunization with R16 emulsified in CFA (29). To determine whether relapsing disease was similarly affected by IL-17 treatment, recipient rats underwent adoptive transfer of in vitro-activated R16-specific T cells (3 × 10⁶/rat) from in vivo-primed syngenic rats and IL-17 (3 μg/injection) was administered on days 0 and 3 after T cell transfer. Compared with rats treated with PBS, the IL-17-treated rats (n = 6) developed much milder relapses, both in terms of the number of relapses and the intensity of ocular inflammation (Fig. 1E).

Decreased activation of IRBP-specific T cells in immunized rats as a result of hIL-17 treatment
To determine the mechanism by which hIL-17 injection prevents the development of uveitis, we measured R16-specific T cell responses in untreated and hIL-17-treated R16-immunized animals. Groups (n = 3) of rats immunized with a uveitogenic dose of R16 (50 μg) with or without hIL-17 treatment on days 4 and 7 were sacrificed on days 10–15 and a splenic T cell-enriched preparation was prepared by nylon wool adhesion and seeded (4 × 10⁶/well) in 96-well plates to test proliferation in the presence of graded doses of R16 and irradiated APCs from naive rats. As shown in Fig. 2A, hIL-17-treated rats showed greatly decreased T cell proliferation. To exclude the possibility that the low responsiveness of T cells seen in hIL-17-treated animals resulted from dys-regulation of APCs, we performed cross-tests in which responder T cells derived from hIL-17-treated or control-treated rats were mixed with irradiated APCs from either naive or hIL-17-treated rats. As shown in Fig. 2, B and C, the hIL-17-treated rats had a low R16-specific T cell response regardless of the origin of the APCs, and the APC function of IL-17-treated animals was not appreciably altered.

IL-17 treatment has a stronger effect inhibiting the IFN-γ⁺ IRBP-specific T cells
To determine whether IL-17 treatment affected the activation of IFN-γ and/or IL-17⁺ autoreactive T cells, R16-specific T cells were prepared from the spleens and draining lymph nodes of R16-immunized rats at day 13 postimmunization (p.i.) and stimulated in vitro by immunizing Ag (10 μg/ml) and APCs for 48 h in the presence of graded doses of recombinant hIL-17 (A), LPS (B), or mouse IFN-γ (C) and T cell proliferation was assessed at 48 h by [³H]thymidine uptake. Values of p < 0.05 were labeled with * and those <0.01 were labeled with **.

FIGURE 4. IL-17 has an in vitro inhibitory effect on the activation of IRBP-specific T cells. T cells (4 × 10⁶/well) prepared from the spleens of R16-immunized rats (9 days p.i.) were stimulated in 96-well plates with R16 (10 μg/ml) and APCs for 48 h in the presence of graded doses of recombinant hIL-17 (A), LPS (B), or mouse IFN-γ (C) and T cell proliferation was assessed at 48 h by [³H]thymidine uptake. Values of p < 0.05 were labeled with * and those <0.01 were labeled with **.
and 25.1% IL-17+ cells on day 4 in culture (Fig. 3A, upper left panel). The number of IFN-γ− T cells in the IL-17-treated rats decreased more significantly with time than the number of IL-17+ T cells. The results of a cytokine assay agreed with the intracellular staining results, as T cells from IL-17-treated animals produced significantly lower amounts of IFN-γ than T cells from control animals, whereas IL-17 production was only marginally decreased (Fig. 3B).

Similar results were also seen in an in vitro study. When IL-17 was added to cultures of R16-specific T cells from non-IL-17-treated rats incubated with R16 and APCs for 48 h, it inhibited the activation of IFN-γ− R16-specific T cells in a dose-dependent manner, but did not affect the activation of Th17 cells (Fig. 3C). The proliferative response of R16-specific T cells was dose-dependently inhibited by in vitro IL-17 treatment (Fig. 4A), but not by LPS (Fig. 4B) or IFN-γ (Fig. 4C).

**IL-17 administration induces enhanced regulatory T cell activity**

One reason why IL-17 treatment decreases EAU susceptibility in recipient animals could be that it promotes the expansion of T cells with regulatory activity or enhanced regulatory T cell activity. We therefore assessed the number of Foxp3+ T cells in R16-injected rats with and without IL-17 treatment. As shown in Fig. 5A, the treated rats had significantly increased numbers of Foxp3+ T cells in T cells isolated from the spleen or the inflamed eye.

We also tested whether regulatory T cell activity was increased in the IL-17-treated animals. Splenic T cells were prepared from IL-17-treated or untreated rats immunized with peptide R16 on day 10 p.i. and 5 × 10^7 T cells were adoptively transferred to recipient rats immunized with a pathogenic dose of R16 emulsified in CFA. As shown in Fig. 5B, the recipient rats that received T cells from IL-17-treated rats developed much weaker disease, with a delayed onset, compared with those that received T cells from untreated rats.

**Discussion**

Recent studies have identified a unique CD4 T cell subset that expresses IL-17, but not IFN-γ or IL-4 (1, 2, 30) and have demonstrated that such T cells play a major role in the pathogenesis of autoimmune diseases (31–33). Previous studies have shown that the biological actions of IL-17 are proinflammatory. IL-17 increases the local production of chemokines (12–16), thereby promoting the recruitment of monocytes and neutrophils. Furthermore, IL-17 stimulates the production of the hematopoietic cytokines G-CSF and GM-CSF, which promote the expansion of these myeloid lineages (34, 35). In addition, it stimulates the production of IL-6 and PGE2, thus enhancing the local inflammatory environment (18–20). IL-17 also drives T cell responses, notably through induction of the costimulatory molecule ICAM (17, 21, 22).

In a previous report, we demonstrated the existence and pathogenic role of IL-17-producing autoreactive T cells in EAU (24, 36). Our studies have also shown that, although IRBP-specific IL-17+ T cells are uveitogenic, IL-17+ T cells expanded by anti-CD3 stimulation and thus Ag-nonspecific are not, suggesting that at least a part of the pathogenic activity of IL-17+ autoreactive T cells is not mediated by their ability to produce IL-17.

To distinguish between the role of a direct cellular effect and the effect of the cytokines produced by IL-17+ autoreactive T cells in their pathogenic and immunoregulatory activity, we examined whether administration of exogenous IL-17 to diseas susceptible mice promoted disease development and unexpectedly found that treatment of rats injected with IRBP-inducing...
peptide with recombinant hIL-17 significantly inhibited the development of EAU, rather than promoted disease development. This observation was supported by results from EAU-susceptible B10RIII mice showing that IL-17-treated mice showed significantly reduced disease susceptibility. Our results show that IL-17 has an anti-inflammatory effect in the development of EAU. In our initial studies, we used a IL-17 recombinant protein of human origin, due to that most of these studies used a rat EAU model and the recombinant rat IL-17 was not yet commercially available. With the recent availability of rat IL-17, we repeated the studies also using rat IL-17. The results reproduced well with those that used hIL-17 (Fig. 6). In addition, hIL-17 has an effect on murine cells (stimulation of fibroblasts to express ICAM-1 and produce cytokines/chemokines) and has been reported in previous studies (37, 38). Our own studies have also shown that hIL-17 induces chemokine production by rat and mouse macrophages in vitro (Fig. 7).

It is interesting to note that hIL-17 treatment ameliorated both forms of uveitis induced by Ag immunization or by adoptive transfer of R16-specific T cells. We have previously shown that, although immunization with an uveitogenic peptide induces acute and monophasic EAU in Lewis rats, adoptive transfer of a pathogenic dose of isolated uveitogenic T cells induces chronic and relapsing disease (29, 39). To determine whether IL-17 treatment affected the progression of EAU, rats induced to show relapsing EAU were injected with IL-17. Interestingly, the IL-17-treated animals showed little change in disease onset, but the number of relapses was significantly decreased, suggesting that IL-17 treatment has a long-term effect that persists after the end of treatment.

The cellular and molecular basis for the protective effect of IL-17 in this autoimmune model remains to be determined. Several possible mechanisms might be considered. Since membrane IL-17 regulates the migration of polymorphonuclear neutrophils (PMNs) in the spleen (40), it was possible that i.p. injection of IL-17 sequesters the PMNs in the injection site in the peritoneum, which may affect the development of inflammation. To examine this possibility, we examined peritoneal exudate cells in IL-17-injected mice and found that i.p. injection of IL-17 did not cause significant inflammation and PMN recruitment in the peritoneum (data not shown).

That a specific cytokine possesses both pro- and anti-inflammatory activities is not a surprising observation. For example, an anti-inflammatory effect of the classic proinflammatory cytokine TNF-α has been reported (41–43). A dual role of TNF-α in type 1 diabetes has also been observed (44). Likewise, IFN-γ has been found to be both proinflammatory (45, 46) or anti-inflammatory (47–51) and IL-6 has both anti-inflammatory (52–54) and proinflammatory (55–57) effects (56, 58–60).

It is interesting to note that a given cytokine tends to be proinflammatory during the acute phase and anti-inflammatory during the chronic phase of the same autoimmune disease (49). However, it would be of great interest to test the possibility that, while IL-17+ autoreactive T cells are pathogenic, the IL-17 produced by such T cells may exert a suppressive effect. The continuation of the study should allow us to reveal the complexity of immunoregulation.

Unfortunately, due to the limited amount of recombinant protein, we were unable to test whether larger doses of IL-17 tend to be proinflammatory and smaller doses immunosuppressive. In addition, it would also be of interest to test whether administration of IL-17 at different phases of an autoimmune disease has different clinical effects. In summary, our results document the immunosuppressive effect of IL-17 and call for a more thorough investigation of the biological function of IL-17 before approaches targeting this cytokine are applied in therapeutic trials.

Disclosures
The authors have no financial conflict of interest.

References

FIGURE 6. Protective effect of rat IL-17 on rat EAU. R16-immunized Lewis rats received two i.p. injections of hIL-17 (3 μg/injection) on days 4 and 7. Disease severity was observed using a slit lamp microscope and graded using previously described criteria. The results shown are the mean ± SD for three animals in a single experiment, which was repeated two times with similar results.

FIGURE 7. hIL-17 induces chemokine production by mouse macrophages. Mouse macrophages were cultured from bone marrow cells by medium containing M-CSF (61, 62). Monolayers of macrophages cultured in 24-well plate were exposed to graded doses of mouse IL-17 (mIL-17) or hIL-17 as indicated. Forty-eight hours later, the culture supernatants were assessed for production of CCL2 using ELISA. Values of p < 0.05 were labeled with * and those <0.01 were labeled with **.


