IL-27 Blocks RORc Expression to Inhibit Lineage Commitment of Th17 Cells

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IL-27 is secreted by APCs in response to inflammatory stimuli and exerts a proinflammatory Th1-enhancing activity but also has significant anti-inflammatory functions. We examined the molecular mechanism by which IL-27 regulates TGFβ plus IL-6- or IL-23-dependent Th17 development in the mouse and human systems. IL-27 inhibited the production of IL-17A and IL-17F in naive T cells by suppressing, in a STAT1-dependent manner, the expression of the Th17-specific transcription factor RORγt. The in vivo significance of the role of IL-27 was addressed in delayed-type hypersensitivity response and experimental autoimmune encephalomyelitis (EAE). By generating mice deficient for the p28 subunit of IL-27, we showed that IL-27 regulated the severity of delayed-type hypersensitivity response and EAE through its effects on Th17 cells. Furthermore, up-regulation of IL-10 in the CNS, which usually occurs late after EAE onset and plays a role in the resolution of the disease, was notably absent in IL-27p28$^{-/-}$ mice. These results show that IL-27 acts as a negative regulator of the developing IL-17A response in vivo, suggesting a potential therapeutic role for IL-27 in autoimmune diseases. *The Journal of Immunology,* **2009, 182: 5748–5756.**

Dysregulated and uncontrolled effector T cell responses can lead to autoimmunity. Th1 cells are highly proinflammatory and have been linked to the induction and progression of many autoimmune disease models, including experimental autoimmune encephalomyelitis (EAE)[2] and collagen-induced arthritis. However, mice deficient for the components of the Th1-IFN-γ pathway (*Il12a*$^{-/-}$, *Il12ra*$^{-/-}$, *IFN*$^{-/-}$, *IFNR*$^{-/-}$) are not resistant to autoimmunity; in fact, these mice are even more susceptible to inflammatory autoimmune diseases (1–4). These findings have recently been explained by the discovery of a new subset of Th cells, Th17, which has been shown to be an additional T cell mediator of autoimmune inflammation (5, 6).

IL-23, which shares the p40 subunit with IL-12, drives the expansion of a pathogenic Th17 cell population characterized by production of IL-17A, IL-17F, IL-22, TNF-α, IL-6 and diverse chemokines (5, 7). The importance of IL-23 in Th17 biology was revealed by the fact that mice deficient for IL-23p19 are resistant to EAE (5, 8). Although IL-23 is a key cytokine for Th17 effector function, recent studies have shown that early generation of Th17 cells from naive T cells requires only TGFβ plus IL-6 or IL-21 in mice (6, 9–13). In naive T cells, IL-23R expression is induced by IL-6, and restimulation with IL-23, which acts at a later stage, is needed to maintain the IL-17A-producing phenotype and to render the cells pathogenic through up-regulation of additional Th17 factors, such as IL-22 and proinflammatory chemokines (11, 14). Thus, IL-23 is critical to Th17 function. TGFβ plus IL-6 induces the transcription factor retinoid-related orphan receptor (ROR)γt, which regulates IL-23R expression and IL-23-dependent responses. Interestingly, *Rorc*$^{-/-}$ mice are resistant to EAE, and few Th17 cells are found in the CNS of these mice (15).

IL-27, which also belongs to the IL-12, IL-23, and IL-6 superfamily of cytokines, is a heterodimeric cytokine composed of p28, a p35-related polypeptide, and EBV-induced gene 3 (EBI3), a p40-related protein (16). IL-27 signals through a heterodimeric receptor consisting of IL-27R (also called WSX-1 or TCCR) and gp130, mainly by activating STAT1, but also STAT3 and to a lesser extent STAT4 (17, 18). IL-27R is expressed on monocytes/macrophages, dendritic cells, T and B lymphocytes, NK cells, mast cells, and endothelial cells (17). Protein gp130, shared with the IL-6 cytokine family, is ubiquitously expressed. Initial reports showed that IL-27 drives proliferation and differentiation of Th1 cells by induction of T-bet and up-regulation of IL-12Rβ2, thus rendering the cells sensitive to IL-12 (16). IL-27 has since also been described as an anti-inflammatory molecule able to limit Th1 or Th2 responses involved in resistance to various parasite infections. Indeed, IL-27R-deficient mice infected with *Toxoplasma gondii*, an encephalitis model, developed severe neuroinflammation associated with exaggerated T cell responses and overproduction of IFN-γ and TNF-α during the acute phase of the disease; mortality was high due to uncontrolled immune pathology (19). Additionally, an increased frequency of Th17 cells was found in the brains of *Toxoplasma*-infected IL-27R-deficient mice during the chronic phase of infection (20). The IL-27R-deficient mice are also hypersusceptible to EAE and more Th17 cells were generated in these mice as well (21). These two reports stress that IL-27 negatively regulates the development of Th17 cells in vivo. It is important to point out that these studies are based on the assumption that IL-27R is the only receptor for IL-27. Similar studies in IL-27p28 or IL-27

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Received for publication April 10, 2008. Accepted for publication February 20, 2009.

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[2] Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; LN, lymph node; DLN, draining LN; DTH, delayed-type hypersensitivity; EBI3, EBV-induced gene 3; ROR, retinoid-related orphan receptor; WT, wild type.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0801162

EBI3-deficient mice have not yet been performed to confirm this assumption.

p28 and EBI3 have been found to be highly up-regulated at the peak of the disease in $CD11b^+$ cells from the CNS, although inflammatory macrophages and resident microglia were not distinguished (22). The expression levels of p28 and EBI3 in the spinal cord seem to correlate with the EAE disease stage (23). p28 has been shown to be produced by activated astrocytes, but no assessment of EBI3 has been published. Additionally, mouse microglial cells are able to produce IL-27 in response to LPS stimulation in vitro (24). These findings suggest a role for IL-27 in regulating ongoing inflammation in the CNS. It is also possible that IL-27 regulates the differentiation of new effector T cells upon arrival in the CNS, or that IL-27 regulates de novo generation of Th17 cells in the draining lymph nodes (DLN).

In this study, we further dissect the mechanisms of action of IL-27 during regulation of Th17 cells and determine the direct contribution of IL-27 in autoimmune inflammation by using IL-27p28-deficient mice. We show that IL-27 down-regulates expression of RORγt and reduces the production of IL-17A and IL-17F in naive T cell in both human and mouse systems. IL-27 also regulates the late differentiation of pathogenic Th17 cells by inducing IL-10 expression. Hence, IL-27 regulates autoimmune inflammation by suppressing RORγt-dependent Th17 development and promotes T cell production of IL-10.

Materials and Methods

Mice

C57BL/6 mice were obtained from The Jackson Laboratory. All animal procedures were approved by the Schering-Plough Biopharma Institutional Animal Care and Use Committee Committee, in accordance with American Association for the Accreditation of Laboratory Animal Care guidelines. The IL-$27p28^{-/-}$ mice were generated through a research agreement with Xenogen Biosciences. Using the mouse p28 sequence a database search identified a BAC clone containing the p28 locus. The targeting vector was constructed with a 5′ homologous arm (3.0 kb) and 3′ homologous arm (4.7 kb) using PCR. The targeted locus has exons 2, 3, 4, and 5 replaced with an *flp* recognition target FRT-flanked neo-cassette. The targeting vector was electroporated into C57BL/6 ES cells and G418 resistant colonies were screened for homologous recombination by Southern blotting with a probe flanking the 3′ arm. The 5′ arm integration was confirmed via PCR. IL-$27p28^{-/-}$ mice were generated, and a real-time PCR genotyping strategy to distinguish +/+ vs +/− vs −/− was established. IL-$27p28^{-/-}$ and wild-type (WT) controls were maintained on a C57BL/6 background.

Cytokines and Abs

Mouse IL-12, mouse IL-23, human IL-23, mouse IL-27, human IL-27, anti-CD3, anti-IFN-γ, and anti-IL-4 were produced in Schering-Plough Biopharma. Anti-CD28 was bought from BD Pharmingen. Beads coated with anti-CD2, anti-CD3, and anti-CD28 were purchased from Miltenyi Biotec and were used at the ratio of one bead to 10 cells. Mouse IL-6, human IL-2, human IL-12, and human TGFβ1 were purchased from R&D Systems. All Abs used in flow cytometry were purchased from BD Biosciences. Mouse anti-human RORγ mAb was purchased from R&D Systems and anti-β-tubulin mAb from Sigma-Aldrich.

Naive or activated T cell purification and culture, Th17 cell generation

DLNs and spleens were isolated from naive mice; $CD4^+$ T cells were enriched using CD4 microbeads (Miltenyi Biotec), naive $CD4^+$-$CD44^{low}CD62L^{high}$ cells or activated $CD4^+CD44^{high}CD62L^{low}$ were further purified by flow cytometry by using a FACSAria sorter. These cells were cultured for 3 days with 10 μg/ml plate-bound anti-CD3 and 1 μg/ml anti-CD28 in the presence of 10 μg/ml neutralizing mAb anti-IFN-γ and anti-IL-4 with 10 ng/ml human TGFβ1, 50 ng/ml mouse IL-6, 100 ng/ml mouse IL-27 for the naive T cells and 20 ng/ml mouse IL-23 and 100 ng/ml mIL-27 for the activated T cells. Th17 cells have been established as previously described (14). These Th17 cells were then activated in the presence of anti-CD3, anti-CD28, neutralizing mAb anti-IFN-γ, anti-IL-4, 20 ng/ml mouse IL-23 and 100 ng/ml mouse IL-27 for 3 days.

Isolation and culture of human naive $CD4^+$ T cells

PBMC were prepared from buffy coats obtained from healthy donors (Stanford University Blood Center, Palo Alto, CA) by centrifugation through Ficoll (Histopaque 1077; Sigma-Aldrich). Naive $CD45RA^+CD4^+$ T cells were isolated as previously described (25), using beads and an AutoMACS instrument (Miltenyi Biotec). T cells were cultured for 5 days in Yssel's medium containing 1% human AB serum (Gemini Bio-Products) along with beads coated with anti-CD2, anti-CD3, and anti-CD28 (one bead to 10 cells, T Cell Activation/Expansion kit; Miltenyi Biotec) in the presence of no cytokine, human IL-12 at 5 ng/ml, human IL-23 at 50 ng/ml, human IL-27 at 200 ng/ml, or the combination of human IL-23 and IL-27. Cells cultured for an additional period of 6 days in the presence of various cytokines and IL-2 (100 U/ml). After a total of 11 days of culture, gene expression was analyzed by real-time PCR. Alternatively, the cells were further restimulated for 48 h with T cell activation beads in the presence of IL-2, and cytokine production was assessed in cell-free supernatants.

Gene expression analysis

RNAs were extracted from tissues or cell pellets and then treated as previously described (14). cDNA samples was normalized to the expression of the housekeeping gene ubiquitin as previously described.

Cytokine analysis

Supernatants were collected after 3-day cell culture, and cytokine protein levels analyzed by mouse IL-17A ELISA (Schering-Plough Biopharma), mouse IL-10 ELISA (R&D Systems), and LincoPlex (Linco Research). Human IL-17A, IL-10, and IL-22 ELISA were from R&D Systems. Human IL-17F ECL assay was performed as previously described (25).

Western blotting

For RORγt and β-tubulin expression, cells were lysed in an SDS sample buffer, submitted to SDS-PAGE and transferred onto an Immobilon membrane to be stained with the appropriate Ab. The reaction was visualized with chemiluminescence.

Flow cytometry

Anti-CD4, anti-CD44, anti-CD45, and anti-CD11b were used for surface staining of the cells. For intracellular staining, cells were first incubated for 4 h with 50 ng/ml PMA (Sigma-Aldrich), 500 ng/ml ionomycin (Sigma-Aldrich), and Golgi-plug (BD Pharmingen). Surface staining was then performed. After fixation and permeabilization (BD Cytofix/Cytoperm kit; BD Pharmingen), the cells were stained intracellularly with anti-IL-17A, anti-IL-10, and anti-IFN-γ. Flow cytometric analysis was performed on Canto II (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Retrovirus production and infection

RORγt cDNA or the control MIG (MSCV-IRES-GFP) cDNA was constructed following standard techniques. Cells were transfected with the indicated plasmids using Lipofectamin. Viral supernatant encoding MSCV-IRES-GFP (MIG) and RORγt-IRES-GFP (RORγt) were collected. MACS sorted or sorted naive $CD4^+$ T cells were cultured in the presence of anti-CD28 in 24-well plates coated with anti-CD3 for 24 h. On day 1, fresh retrovirus supernatant was added and the cells were spun at 2500 rpm for 1.5 h at 30°C. After spin infection, the cells were cultured with or without IL-27 (nanogram per milliliter) and harvested on day 3 for intracellular staining.

Delayed-type hypersensitivity (DTH) model

$CD4^+CD45.1^+$ OT-II T cells were transferred into WT and IL-$27p28^{-/-}$ recipients. The following day recipient mice were immunized s.c. with 100 μg of $OVA_{323–339}$ in CFA containing 100 μg of heat-killed *Mycobacterium tuberculosis* H37Ra (both from Difco). DTH response was induced on day 5 by injecting 100 μg of OVA in saline into one hind footpad, the contralateral footpad received saline as a control. Twenty-four hours after challenge, foot thickness was measured and results are expressed as mean increase in foot thickness over saline control.

EAE induction and disease scoring

EAE was induced in age-matched female mice 8- to 12-wk-old. WT and IL-$27p28^{-/-}$ C57BL/6 mice were immunized with 100 μg of myelin oligodendrocyte glycoprotein (MOG(35–55)) emulsified in 200 μl of CFA containing 100 μg of heat-killed *Mycobacterium tuberculosis* H37Ra (both from Difco) s.c. injected in four sites. Mice also received 100 ng of i.p. pertussis toxin (List Biological Laboratories). EAE was assessed according

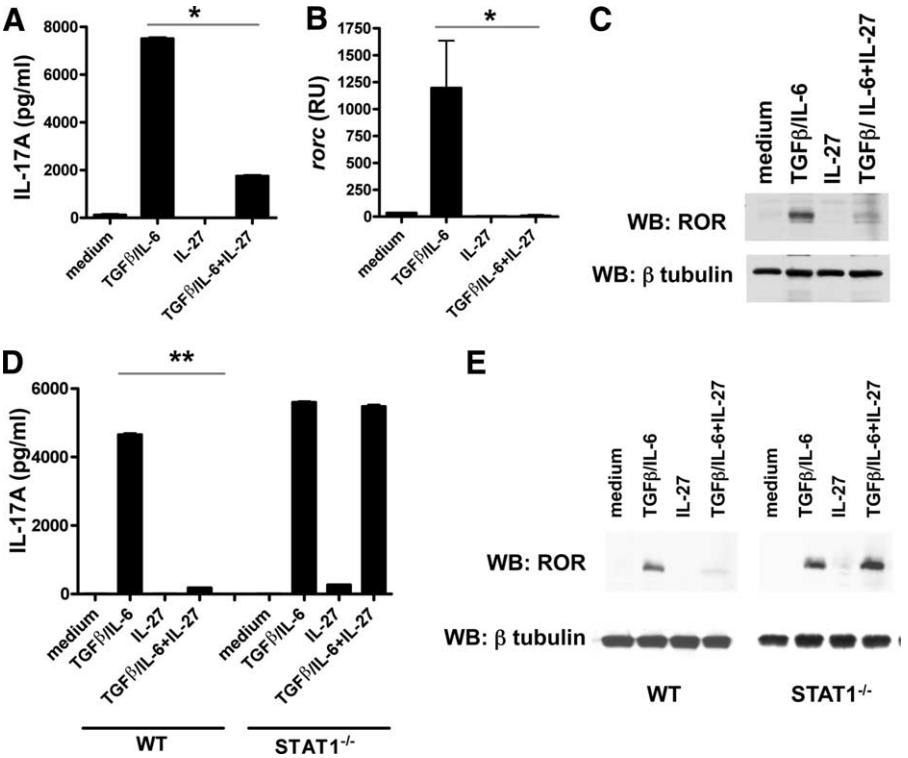

FIGURE 1. IL-27 prevents generation of Th17 cells by suppressing RORγt in a STAT1-dependent manner. Sorted mouse naive $CD4^+$ T cells ($CD44^{low}CD62L^{high}$) were cultured in the presence of anti-CD3, anti-CD28, anti-IL-4, anti-IFN-γ mAbs, TGFβ/IL-6 or IL-27. *A*, ELISA for IL-17A was performed on the supernatants after a 3-day culture period. *B*, Real-time PCR analysis of the expression of *rorc* transcript after 24-h stimulation. *C*, Naive $CD4^+$ T cells were stimulated with TGFβ/IL-6, IL-27, or TGFβ/IL-6 plus IL-27 for 2 days. The induction of RORγt was assessed by Western blot analysis. Homogeneity of protein contents was checked by Western blot for β-tubulin. MACS purified naive $CD4^+$ T cells from 129SvEv WT or 129SvEv $STAT1^{-/-}$ were cultured in the presence of anti-CD3, anti-CD28, anti-IL-4, anti-IFN-γ mAbs, TGFβ/IL-6, or IL-27. *D*, IL-17A ELISA was performed on the supernatants after a 3-day culture period. *E*, The induction of RORγt was assessed by Western blot after a 2-day period culture. Homogeneity of protein contents was checked by Western blot for β-tubulin. Data shown are representative of three (*A* and *C*) and two (*B*, *D*, and *E*) independent experiments with similar results. Data in *A*, *B*, and *D* represent mean ± SEM.

to the following clinical grades: 1, flaccid tail; 2, impaired righting reflex and hindlimb weakness; 3, partial hindlimb paralysis; 4, complete hindlimb paralysis; 5, hindlimb paralysis with partial forelimb paralysis; 6, moribund.

Generation of bone marrow chimeras

Bone marrow chimeras were prepared as previously described (26). Briefly, lethally irradiated WT C57BL/6.CD45.2 mice were reconstituted with bone marrow (1×10^7 cells) from congenic C57BL/6.CD45.1 mice. Peripheral blood was drawn from the recipients and analyzed by flow cytometry for the presence of the CD45.1 congenic marker on leukocytes. By around 8 wk after reconstitution, typically 100% of granulocytes, 100% of monocytes, 100% of B cells and 90–95% of T cells were of donor type.

Isolation of CNS mononuclear cells

T cells, macrophages, and resident microglia were isolated by digesting brain and spinal cord homogenates with collagenase and DNase followed by a Percoll gradient centrifugation as previously described (8).

CNS myeloid cell populations sorting

Myeloid cell populations were isolated from CNS of irradiation bone marrow reconstituted mice, or WT animals and highly purified into microglia or CNS-associated macrophages on the basis of their differential staining with allophycocyanin-conjugated rat anti-mouse CD11b, PE-conjugated rat anti-mouse CD45 or anti-mouse CD45.1, and FITC-conjugated anti-mouse CD45.2 using a FACSAria sorter. As a control, $CD4^+$ T cells and macrophages were FACS sorted from the LNs of immunized mice using $CD4^+$ and $CD11b^+$ stains, respectively.

Results

IL-27 inhibits Th17 development through STAT1-mediated suppression of RORγt

To investigate the effects of IL-27 on the developmental program of Th17 cells, we sorted naive $CD4^+$ T cells and activated them with anti-CD3 and anti-CD28 Abs in the presence of TGFβ and IL-6. As expected, we observed increased expression of *Il17a* (mRNA and protein) and *Il17f* (mRNA), as well as an up-regulation of *Il23ra* receptor (mRNA) (Fig. 1*A* and data not shown) (20, 21). As previously described, we also found that TGFβ plus IL-6 did not detectably induce IL-22 (data not shown), (14). The addition of IL-27 to these culture conditions strongly suppressed the expression of IL-17A protein and *Il17a, Il17f*, and *Il23ra* mRNA (Fig. 1*A* and data not shown). Importantly, IL-27 prevented the transcription of the Th17 lineage-specific transcription factor *rorc* in the presence of TGFβ plus IL-6 (Fig. 1*B*). We next confirmed the protein expression level of RORγt by Western blot. RORγt protein was strongly up-regulated in response to TGFβ and IL-6, and addition of IL-27 prevented induction of RORγt (Fig. 1*C*). Because STAT1 is activated by IL-27 in T cells (20, 21), we next investigated the role of STAT1 in the IL-27-mediated down-regulation of RORγt as well as IL-17A production using cells from $STAT1^{-/-}$ mice. As expected, IL-27 could not inhibit the production of IL-17A in the absence of STAT1 (Fig. 1*D*). Furthermore, IL-27 did not modify the level of RORγt induction in the absence

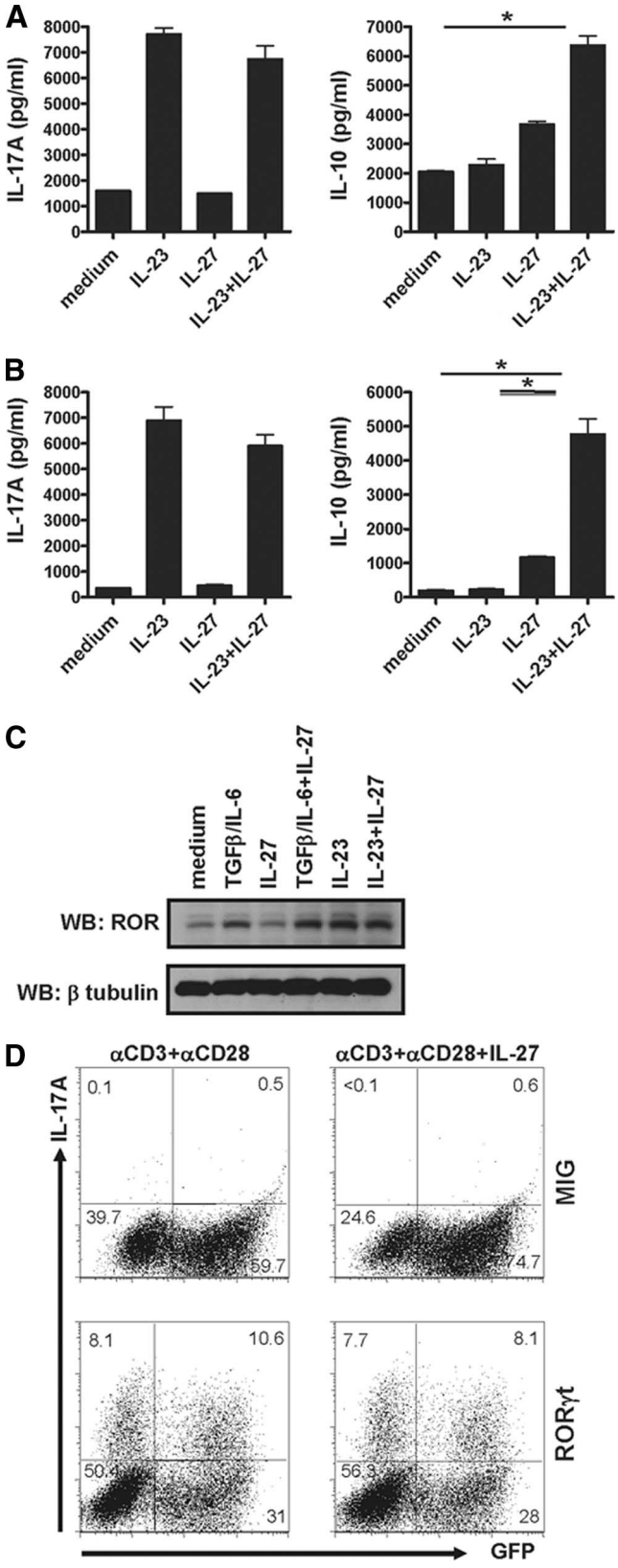

FIGURE 2. IL-27 induces IL-10 secretion. *A*, Memory/effector T cells ($CD44^{high}CD62L^{low}$) were cultured in the presence of anti-CD3, anti-CD28, anti-IL-4, anti-IFN-γ mAbs, IL-23, or IL-27 for 3 days. IL-17A and IL-10 levels in the supernatants were assessed by ELISA. *B*, Th17 cells were differentiated in the presence of TGFβ/IL-6 for 3 days and then cultured for an additional 3 days with IL-23 or IL-27. IL-17A and IL-10 levels in the supernatants were measured by ELISA. *C*, Th17 cells were stimulated with TGFβ/IL-6, IL-27, TGFβ/IL-6 plus IL-27, IL-23, or IL-23 plus IL-27 for 2 days. The induction of RORγt was assessed by Western blot analysis. Homogeneity of protein contents was checked by Western blot for β-tubulin. *D*, MACS sorted $CD4^+$ T cells from WT mice were isolated and transduced with retroviral vectors encoding MSCV-IRES-GFP (MIG) and RORγt-IRES-GFP (RORγt) in the presence of anti-CD3 and anti-CD28 with or without IL-27. IL-17A and GFP expressions were assessed by flow cytometry. Data are representative of three (*A* and *B*) and two (*C* and *D*) independent experiments with similar results and represent mean ± SEM.

of STAT1 (Fig. 1*E*). Therefore, IL-27 prevents the production of IL-17A by suppressing the Th17 transcription factor RORγt in a STAT1-dependent manner.

IL-27 induces IL-10 expression by naive and memory T cells

It has recently been shown that IL-27 can induce IL-10 production in T cells under Th1, Th2, or Th17 culture conditions (27–29). As IL-27 inhibits the secretion of IL-17A in naive T cells in the presence of TGFβ plus IL-6, we determined whether IL-27 can inhibit IL-17A expression in effector/memory T cells. To this end, we sorted naive or memory T cells and cultured them in the presence of TGFβ plus IL-6 or IL-23 respectively. We and others have shown that TGFβ induced IL-10 expression in Th17 cells even in the presence of IL-23 (14, 27), suggesting that IL-23, by itself, neither induced nor inhibited IL-10 expression in Th17 cells. Consistent with previous studies, we also found that addition of IL-27 in the presence of TGFβ plus IL-6 suppressed the production of IL-17A as well as that of IL-17F in naive cells (data not shown). We next assessed whether IL-27 could regulate effector/memory T cell production of IL-17, which was not tested in previous studies. Addition of IL-27 to IL-23-activated $CD62L^{low}$ and $CD44^{high}$ effector T cells did not modify the amounts of IL-17A (Fig. 2*A*). In contrast, IL-27 increased the amounts of IL-10 even in the presence of IL-23 (Fig. 2*A*). These results indicate that IL-27 has differential regulatory activities on naive vs memory-activated T cells.

To further support these findings, we generated Th17 cells by stimulating naive $CD4^+$ T cells with TGFβ plus IL-6 for 3 days, and then tested their response to IL-27 during second stimulation in the presence of IL-23. On these activated T cells, IL-27 was again unable to inhibit IL-17A in response to IL-23 stimulation (Fig. 2*B*), but did increase the amounts of IL-10 (Fig. 2*B*). These findings suggest that IL-27 cannot inhibit IL-17A production after initiation of the Th17 program but could still regulate pathogenicity of Th17 cells through induction of IL-10 production. Accordingly, we found that IL-27 did not change the level of the RORγt protein in the presence of IL-23 in Th17 cells (Fig. 2*C*).

Finally, we tested the ability of IL-27 to down-regulate IL-17 production in response to enforced expression of RORγt in $CD4^+$ T cells by using a retroviral vector encoding RORγt (Fig. 2*D*). As predicted, addition of IL-27 to cells already expressing RORγt did not alter the expression level of IL-17A (Fig. 2*D*), indicating that IL-27 does not interfere with the capacity of RORγt to transactivate IL-17 expression. This again supports our observation that IL-27 is only able to regulate naive T cells expression of RORγt and IL-17 production. However, the induction of IL-10 in both naive and memory cells supports the concept that IL-27 can regulate both of these cell populations, albeit by different mechanisms.

IL-27 inhibits human Th17 cell development

Although many similarities can be seen between mouse and human T cell subset development, some differences have been identified. We therefore analyzed whether human IL-27 was able to inhibit

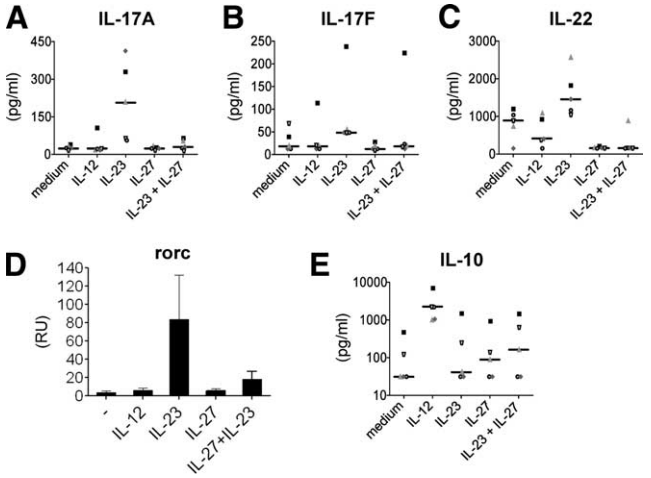

FIGURE 3. IL-27 inhibits development of human Th17 cells. Purified human naive $CD4^+CD45RA^+$ T cells were activated with beads coated with anti-CD2, anti-CD3, and anti-CD28 and cultured for 11 days in the presence of IL-12, IL-23, or IL-27. Levels of IL-17A (*A*), IL-17F (*B*), IL-22 (*C*), and IL-10 (*E*) were measured in cell-free supernatants of T cells restimulated for 48 h. *D*, *rorc* gene expression was assessed by real-time PCR after 11 days of culture. Data from five independent donors are shown. Horizontal bar represents median values. Results in *D* represent mean ± SEM of five independent donors.

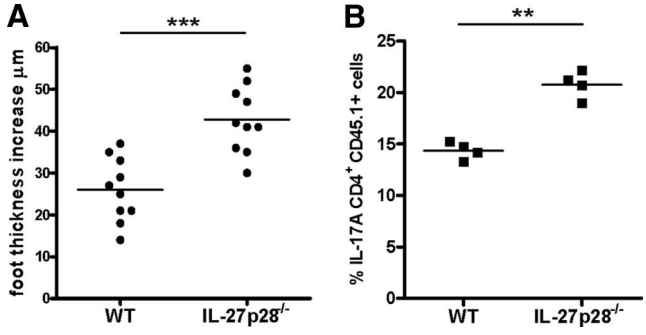

FIGURE 5. Increased DTH response in IL-27p28$^{-/-}$ mice. $CD4^+$ T cells from CD45.1 OT-II mice were transfer to WT and IL-27p28$^{-/-}$ mice by the i.v. route 1 day before Ag immunization. Mice were immunized with OVA/CFA and challenged on day 5 in the right hind footpad with OVA or in the left footpad PBS alone. *A*, Footpad swelling was measured 24 h after the challenge. *B*, Intracellular expression of IL-17A in OT-II $CD4^+CD45.1^+$ T cells from popliteal LNs was assessed by flow cytometry 24 h after OVA challenge. Each data point represents OVA-specific T cells isolated from an individual animal. Results in *A* are pooled from two independent experiments and in *B* are representative of two separate experiments.

development of human Th17 cells, which can be generated in the presence of IL-23 (25). We isolated naive $CD4^+CD45RA^+$ T cells from the peripheral blood of healthy human donors and stimulated these cells for 5 days with anti-CD3-, anti-CD28-, and anti-CD2-coated beads in the presence of IL-12, IL-23, or IL-27. These cells were thereafter maintained with IL-2 and the appropriate cytokines for an additional 6-day period before reactivation. As previously described (25), IL-23, but not IL-12, induced production of the Th17 cytokines IL-17A, IL-17F, and IL-22 (Fig. 3, *A–C*). Strik-

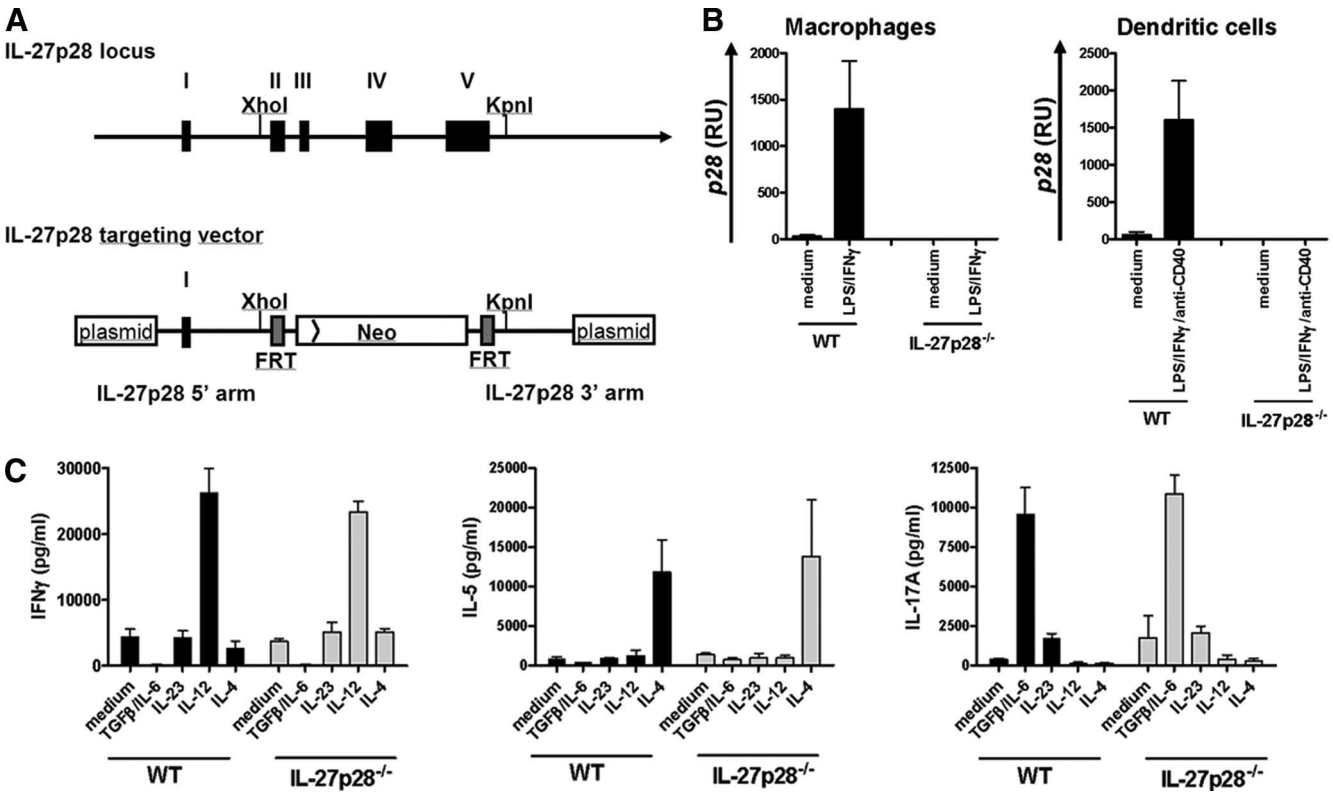

FIGURE 4. Generation of IL-27p28$^{-/-}$ mice. *A*, The IL-27p28 targeting vector contains a 5′ homologous arm (3.0 kb) and 3′ homologous arm (4.7 kb) region of homology. The targeted locus replaced exons 2, 3, 4, and 5 with an *flp* recognition target FRT-flanked neo-cassette. *B*, Macrophages and dendritic cells were derived from the bone marrow of IL-27p28$^{-/-}$ or WT mice. The mRNA expression level of *p28* and *ebi3* was analyzed by quantitative RT-PCR after 8-h activation. *C*, Naive MACS sorted $CD4^+$ T cells were differentiated with IL-12 and anti-IL-4, TGFβ/IL-6, anti-IFN-γ and anti-IL-4, or IL-4 with anti-IFN-γ for 3 days in the presence of anti-CD3 and anti-CD28. Cytokine levels in the supernatants were assessed by ELISA and Luminex. Data in *B* and *C* are representative of two independent experiments with similar results and represent ± SEM of at least three mice per group.

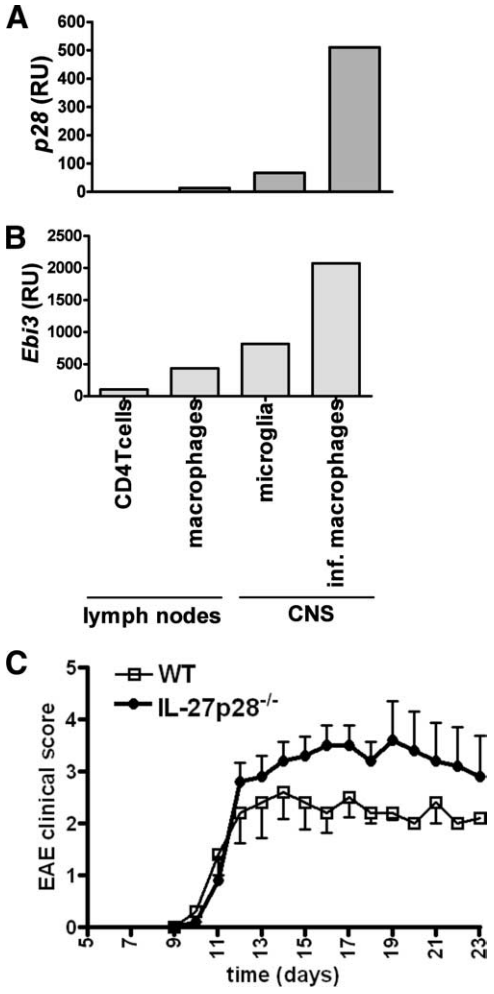

FIGURE 6. IL-27 produced by microglia and infiltrating macrophages at EAE onset protects mice from severe EAE. mRNA expression of *p28* (*A*) and *ebi3* (*B*) were assessed by real-time PCR and normalized to ubiquitin. Microglial cells and infiltrating macrophages were FACS sorted from the CNS on day 14 after EAE induction in C57BL/6 mice along with $CD4^+$ T cells and macrophages from the LN. Data are representative of two independent experiments with similar results. *C*, Average EAE disease scores in WT (□) and IL-27p28$^{-/-}$ mice (●). Data shown are five mice per group and are representative of four independent experiments shown in Table I.

ingly, the addition of IL-27 inhibited the production of these cytokines in multiple donors (Fig. 3, *A–C*). In addition, IL-27 prevented up-regulation of *rorc* expression induced by IL-23 (Fig. 3*D*). Interestingly, IL-27 induced IL-10 production in some donors (two of five donors), and the combination of IL-23 and IL-27 led to a small but consistent further up-regulation of IL-10 in the same donors (Fig. 3*E*). However, IL-27 induction of IL-10 did not reach statistical significance and further studies are required to test this concept. We found that IL-12 was a potent inducer of IL-10 secretion (Fig. 3*E*) (30). These findings show that the activities of IL-27 on Th17 cell development are similar between the human and mouse systems.

Generation of IL-27p28$^{-/-}$ mice

Th17 cells have been identified as a major pathogenic T cell subset during autoimmune diseases. To investigate the role of IL-27 during the development of Th17 cells and its function on Th17 cells in vivo, we generated IL-27p28$^{-/-}$ mice by deleting the four last exons of the gene encoding for p28 (Fig. 4*A*). These mice appear healthy and have normal cell populations in spleen and LN ($CD4^+$ T cell, $CD8^+$ T cell, B cell, NK cell, dendritic cells, monocytes, and macrophages) (data not shown). To assess the expression of p28, macrophages and dendritic cells were generated from the bone marrow of WT and IL-27p28$^{-/-}$ mice. After stimulation, both p28 mRNA and protein were not detected in IL-27p28$^{-/-}$ mice, confirming the deletion of the p28 subunit of IL-27 (Fig. 4*B* and data not shown). In addition, $CD4^+$ T cells from IL-27p28$^{-/-}$ spleen and LN showed normal Th1, Th2, and Th17 cytokine production in response to IL-12, IL-4, and TGFβ plus IL-6, respectively, confirming that there was no inherent defect in T cell subset development from these mice (Fig. 4*C*).

IL-27p28$^{-/-}$ mice develop an exaggerated DTH response

To investigate the role of IL-27 in vivo, we tested the susceptibility of IL-27p28$^{-/-}$ mice to DTH. $CD4^+$ OT-II T cells bearing the transgenic TCR specific for $OVA_{323-339}$ were transferred into WT and IL-27p28$^{-/-}$ mice and activated to become Th17 cells by immunizing with OVA in CFA. The OT-II T cells were tracked in the recipient mice by the use of CD45.1 allotypic marker. IL-27p28$^{-/-}$ mice showed significantly higher footpad swelling responses to challenge with OVA compared with WT mice (Fig. 5*A*). We then stimulated $CD45.1^+$ OT-II cells obtained from inguinal DLNs of the WT and IL-27p28$^{-/-}$ mice and assessed IL-17A production. It was clear that the OT-II T cells from IL-27p28$^{-/-}$ host mice had an increased frequency of cells producing IL-17A compared with those activated in WT mice (Fig. 5*B*). Taken together, these results indicate that IL-27 has a suppressive effect on Th17 cells, which mediate the DTH response.

IL-27 is produced by macrophages infiltrating the CNS during EAE

IL-27, like IL-12 and IL-23, is produced under proinflammatory conditions by APCs. One study reported that during EAE, a model of human multiple sclerosis, *p28* as well as *ebi3* are expressed by $CD11b^+$ cells, including both microglia and macrophages, from the CNS at onset of the disease (22). To identify more precisely the source of IL-27 during EAE, we separated microglia ($CD11b^{high}$ and $CD45^{low}$) and infiltrating macrophages ($CD11b^{high}$ and $CD45^{high}$) from the CNS of WT mice on day 14, the peak of the disease. Macrophages ($CD11b^+$) or $CD4^+$ T cells were purified from the DLN. In the CNS, the IL-27 subunits *ebi3* and *p28* were both expressed in microglia and infiltrating macrophages (Fig. 6, *A*

Table I. *Summary of EAE clinical data*[a]

Mouse Strain	Incidence	Mortality	Mean Day of Onset ± SEM	Mean Maximum Disease Score ± SEM
C57BL/6	22/25	0/25	13.59 ± 2.26	3.05 ± 1.12[b]
IL-27p28$^{-/-}$C57BL/6	24/24	1/24	12.91 ± 1.41	3.63 ± 1.04[b]

[a] Mice were immunized with MOG(35–55) and treated with pertussis toxin at day 0. Assessment of clinical EAE includes the number of mice that developed disease, the mean day of onset among mice with EAE, and the mean clinical disease grade of each group. Results of four separate experiments are shown.

[b] $p = 0.0356$, comparing the C57BL/6 group versus IL-27p28$^{-/-}$C57BL/6 group by Mann-Whitney *U* test.

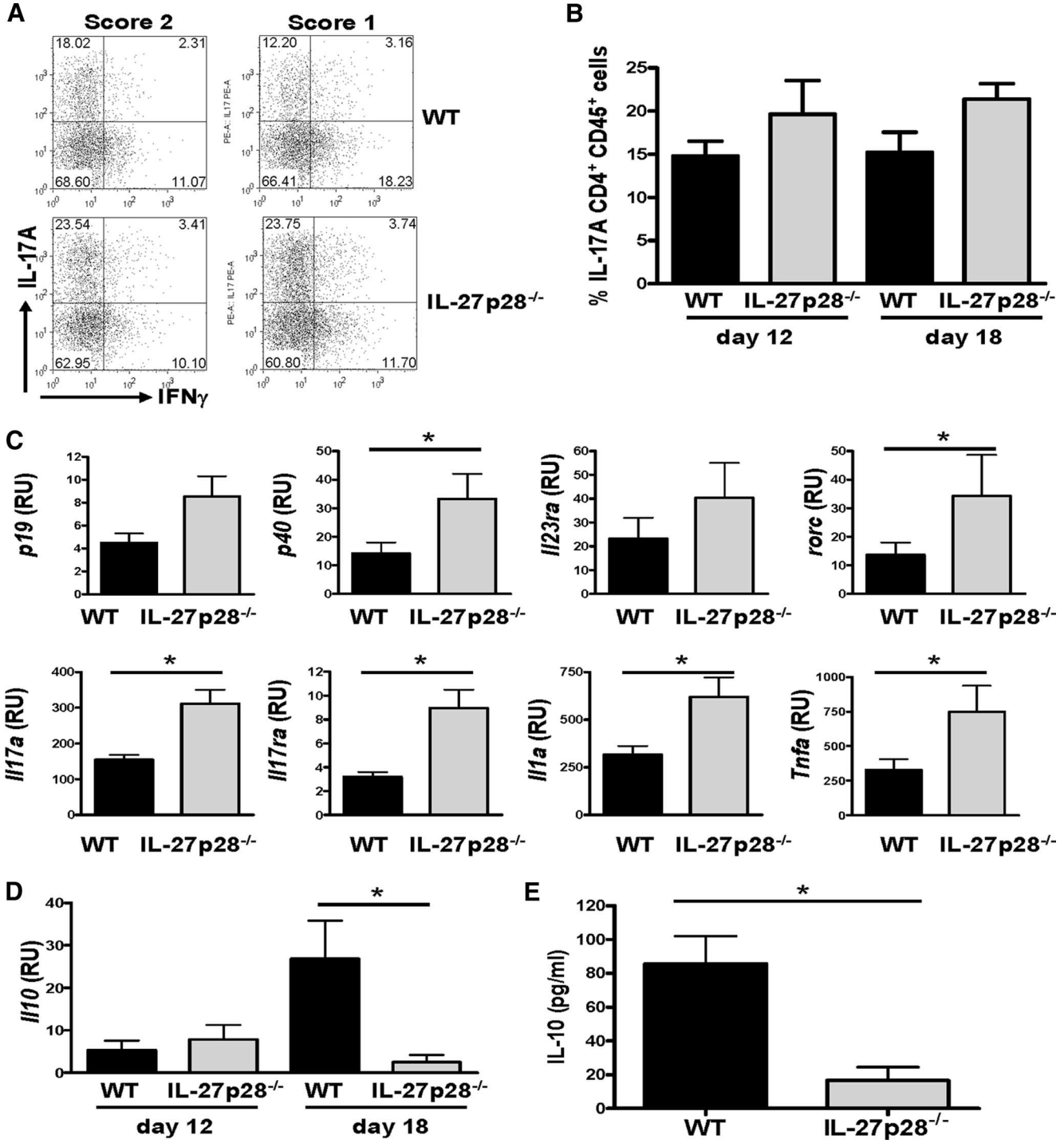

FIGURE 7. Increased Th17 and proinflammatory responses in the CNS from IL-27p28$^{-/-}$ mice. *A*, Production of IL-17A and IFN-γ in CD4$^+$ T cells from the CNS on day 12 after immunization. *B*, Percentage of IL-17A-positive CD4$^+$CD45$^+$ T cells on days 12 and 18 in the CNS from EAE-induced WT and IL-27p28$^{-/-}$ mice ($n = 3$/group). Data shown are trends and representative of two independent experiments with similar results. *C*, mRNA expression of the indicated genes was assessed by real-time PCR on the mononuclear cell fraction obtained from the CNS of WT and IL-27p28$^{-/-}$ mice on day 12 after immunization. *, $p < 0.05$ and is considered significant. *D*, *Il10* expression was performed by real-time PCR on the mononuclear cell fraction obtained from the CNS of WT and IL-27p28$^{-/-}$ mice on days 12 and 18. *E*, On day 18, mononuclear cell fractions from the CNS of WT and IL-27p28$^{-/-}$ mice were isolated and cultured for an additional 3 days. Secreted IL-10 was assessed by ELISA of culture supernatants. Data in *B* represent $n = 3$ mice/group ± SEM. Data shown in *C* and *D* represent pooled samples of at least $n = 3$ mice/group ± SEM and are representative of at least two independent experiments with similar results. Data in *E* are representative of two independent experiments of $n = 3$ mice/group ± SEM.

and *B*). Expression levels were highest in infiltrating macrophages, and because these also comprise a larger population in the inflamed CNS, it seems likely that infiltrating macrophages are an important source of IL-27.

IL-27p28$^{-/-}$ mice are more susceptible to EAE

To test and confirm the inhibitory function of IL-27 in vivo, we compared the susceptibility of IL-27p28-deficient and WT mice to

EAE, and found that IL-27p28$^{-/-}$ mice developed a more severe disease (Fig. 6*C*) as shown by a higher mean disease score in IL-27p28$^{-/-}$ mice compared with WT animals (WT score: 3.05 ± 1.12, IL-27p28$^{-/-}$ score: 3.63 ± 1.04, $p = 0.0356$) (Table I). However, the day of onset was unchanged between the two groups (WT: 13.59 ± 2.26, IL-27p28$^{-/-}$: 12.91 ± 1.41). The exacerbation of disease in IL-27p28$^{-/-}$ animals is in agreement with published results obtained with the IL-27R$^{-/-}$ mice (21).

Frequency of IL-17A$^+$ CD4$^+$ T cells is increased in the CNS of IL-27p28$^{-/-}$ mice

We have previously shown that IL-23 drives a pathogenic T cell population characterized by the production of IL-17A which in turn induces autoimmune inflammation (5). Because IL-27p28$^{-/-}$ mice are more susceptible to EAE, we first analyzed IL-17A expression level in the CNS of these mice by isolating the mononuclear fraction from spinal cords and brains of WT and IL-27p28$^{-/-}$ mice on day 12 or day 18 after disease induction and performed intracellular staining for IL-17A and IFN-γ. We found that before and after disease onset, there was a small but consistent trend in the increase of T cells producing IL-17A in the CNS from IL-27p28$^{-/-}$ mice compared with WT mice (Fig. 7, *A* and *B*).

Gene expression analyses were performed on the cells from the DLN and on the mononuclear cells from the CNS. In the DLN on days 6, 12, and 18, induction of Th17-related genes or proinflammatory cytokines in the WT or IL-27p28$^{-/-}$ mice showed little difference (data not shown). At onset of EAE, a higher gene expression of Th17-related molecules *Il23* (*p19* and *p40*), *Il-23ra*, the transcription factor *rorc*, *Il17a*, *Il17ra*, as well as the proinflammatory cytokines *Il1α* and *Tnfα*, was observed in the CNS of IL-27p28$^{-/-}$ mice compared with WT (Fig. 7*C*). By day 18, this increased gene expression in IL-27p28$^{-/-}$ mice was no longer observed (data not shown), suggesting that the direct regulatory effects of IL-27 are greatest in the earlier phases of EAE most likely due to other regulatory mechanisms taking over during the later phases. T cells entering the CNS are mostly activated cells with an effector/memory phenotype. Because we demonstrated that IL-27 induced the secretion of IL-10 in memory T cells in the presence or absence of IL-23, we also examined IL-10 expression. In contrast to many of the proinflammatory cytokines in the CNS, *Il10* was up-regulated later in the disease course in WT mice (day 18) (Fig. 7*D*). Strikingly, we observed that this late up-regulation of *Il10* failed to occur in the CNS of IL-27p28$^{-/-}$ mice (Fig. 7*D*). CNS mononuclear cells from IL-27p28$^{-/-}$ mice secreted less IL-10 in response to myelin oligodendrocyte glycoprotein MOG(35–55) than those from WT mice (Fig. 7*E*). Overall, these results suggest that the mechanisms of action of IL-27 may be mediated via the inhibition of IL-17A and proinflammatory cytokine levels in naive T cells, as well as the enhanced secretion of IL-10 in memory T cells.

Discussion

IL-27 plays an important role in the regulation of Th1, Th2, and Th17 responses (16, 20, 21). However, the mechanisms of this regulation are still being elucidated. Addition of IL-27 reduced IL-17A production by differentiating Th17 cells in the presence of TGFβ and IL-6, which correlated with an increased frequency of Th17 cells in mice deficient for IL-27R (20, 21). In this report, we show that IL-27 not only blocks IL-17A production but also inhibits Th17 differentiation by preventing up-regulation of the Th17 transcription factor RORγt in a STAT1-dependent manner. However, IL-27 had limited capacity to block the Th17 phenotype in already differentiated T cells activated in the presence of IL-23 or in T cells with forced expressed of RORγt, suggesting that IL-27 is unable to strongly inhibit the induction of IL-17 by RORγt after the initiation of the Th17 program. Hence, IL-27 appears to regulate the frequency of Th17 cells primarily at the early differentiation stage.

It has recently been demonstrated that IL-27 is able to induce production of IL-10 in T cells, which is likely one of its major regulatory functions (27–29). In addition, TGF-β regulates IL-10 expression in Th17 cells, whereas IL-23 does not suppress or enhance IL-10 expression (14, 27). IL-27 does not appear to further increase levels of IL-10 induced by TGFβ and IL-6, although the prevention of Th17 differentiation certainly changes the phenotype of the resulting cells. Interestingly, we found that although IL-27 was unable to strongly block the Th17 phenotype in already activated T cells stimulated with IL-23, it did induce IL-10, indicating that IL-27 can also act at a later stage on the pathogenic Th17 cells. However, the mechanism of IL-27 induction of IL-10 in memory activated Th17 cells is currently unknown.

TGFβ and IL-6 are the key cytokines promoting Th17 lineage commitment in the murine system. However, it is still a controversial issue whether TGFβ and IL-6 is also required to generate Th17 cells from naive human T cells (25). Given this uncertainty, it is important to test whether IL-27 is an important negative regulator of human Th17 differentiation, as in the mouse system. Our results showed that IL-27 prevents the generation of human Th17 cells, supporting a regulatory role of IL-27 in human.

The effects of IL-27 could result in regulation of the surrounding environment, explaining how this cytokine is able to regulate the outcome of IL-23-driven Th17 effector responses as it has been demonstrated by the administration of IL-27 after induction of EAE or by using the passive transfer model (23). In this study, we show that IL-27, being produced locally in the CNS by infiltrating macrophages and microglia after EAE induction, can induce the secretion of IL-10 by CD4$^+$ T cells. Very interestingly, IL-10 was not up-regulated in the IL-27p28$^{-/-}$ at a late phase of EAE in contrast with the increase observed in cells from WT mice. This finding indicates that IL-27 may drive IL-10 production during recovery phase of EAE. In the CNS, regulatory T cells are potential sources of IL-10 (31). How these different T cell populations interact and cross-talk with each other remains to be elucidated.

To determine the in vivo contribution of IL-27 on Th17, we compared the susceptibility of WT and IL-27p28$^{-/-}$ mice to EAE and demonstrated that IL-27p28$^{-/-}$ mice are more susceptible to EAE and that the infiltrating CD4$^+$ T cells expressed more IL-17A than the WT cells. We found no difference in the expression of IFN-γ that correlates with in vitro observations showing that the neutralizing effects of IL-27 on Th17 cells are T-bet- and IFN-γ-independent (20, 21). Thus IL-27 limited the severity of EAE by suppressing Th17 differentiation and inducing the expression of IL-10 in Th17 cells. These results fit perfectly with Batten et al. (21) finding that the IL-27R$^{-/-}$ mice are more susceptible to EAE due to their ability to generate a robust Th17 response. These definitive results strongly support the idea that IL-27 is the critical ligand for IL-27R/gp130 in the context of EAE.

In summary, we have demonstrated that IL-27 prevents the generation of Th17 cells through the inhibition of RORγt in a STAT1-dependent manner. It is likely that other STAT1 activators may also inhibit the function of RORγt. The IL-27p28$^{-/-}$ mice develop more severe EAE due to the presence of increased number of Th17 cells. In the context of chronic inflammation, IL-27 is secreted to suppress the pathogenic Th17 phenotype via the down-regulation of IL-17A and the induction of IL-10. Interestingly, our results show that IL-27 inhibits the generation of human Th17 cells driven by IL-23. Even if blockade of IL-12/23p40, IL-23p19 or

IL-17A ameliorates the outcome of autoimmune disease models, treatment with IL-27 may antagonize the early generation of Th17, production of Th17-related molecules and induce production of IL-10. These findings, which show that IL-27 acts as an anti-inflammatory cytokine, may have implications for a wide range of immune-mediated pathologies.

Acknowledgments

We thank Connie Rylance for careful review of the manuscript, Dr. Craig A. Murphy for isolating microglial and inflammatory macrophage cDNA for IL-27 mRNA analysis as well as Bela Desai and Steve Jungers from the FACS Facility.

Disclosures

The authors have no financial conflict of interest.

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