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CUTTING EDGE

Cutting Edge: IL-27 Is a Potent Inducer of IL-10 but Not FoxP3 in Murine T Cells

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The cytokine IL-27 is important for restricting inflammation in response to a wide variety of immune challenges. In this study, we demonstrate that IL-27 induces expression of the anti-inflammatory cytokine IL-10 by $CD4^+$ and $CD8^+$ T cells. IL-27 relied upon the Th1 transcription factor STAT1 to induce IL-10^+ IFN-γ^+ FoxP3$^-$ Th1 cells, which were recently shown to be key negative regulators during certain infections. $Il27ra^{-/-}$ mice generated fewer IL-10^+ T cells during both* Listeria monocytogenes *infection and experimental autoimmune encephalomyelitis. The data presented here indicate a novel mechanism for the induction of IL-10 expression by T cells and provide a mechanistic basis for the suppressive effects of IL-27. The Journal of Immunology,* 2008, 180: 2752–2756.

Interleukin (IL-27) is a heterodimeric cytokine formed by association of the subunit proteins IL-27p28 and EBV-induced protein 3 (Ebi3)[2] (1). It signals through a heterodimeric receptor that consists of IL-27Rα (WSX-1, TCCR) and gp130 (2). In vivo evidence indicates that the dominant role of IL-27 is immune suppression. Although IL-27 activates Th1 transcription factors T-bet and STAT1 and up-regulates expression of the IL-12Rβ2 chain, mice deficient in EBI3 ($Ebi3^{-/-}$) or IL-27Rα ($Il27ra^{-/-}$) do not display major defects in the ability to mount Th1 responses, even though Th1 responses are somewhat delayed in a limited number of infectious scenarios (3). Instead, these mice exhibit exacerbated inflammation in response to a wide variety of immune challenges, including pathogens that elicit Th1 and Th2 responses and inflammatory models of disease that rely on Th2 and Th17 activity (3, 4). Thus $Il27ra^{-/-}$ mice display accelerated resolution of certain infections but are more prone to develop immune-mediated pathologies (3, 4). $Il27ra^{-/-}$ mice also develop more severe symptoms in experimental autoimmune encephalomyelitis (EAE) (5) owing to the ability of IL-27 to directly suppresses Th17 cell differentiation (5, 6). Collectively, the body of evidence indicates that IL-27 has a wide-reaching role in immune suppression that cannot be explained entirely by deviations in helper T cell polarization. $Il27ra^{-/-}$ mice have no obvious defect in the development and function of natural regulatory T cells (T_{reg}) cells (5) and, hence, the mechanism by which IL-27 exerts its extensive suppressive effects remains unclear.

The phenotype of $Il27ra^{-/-}$ mice partially recapitulates the phenotype of IL-10 deficient mice, although $Il27ra^{-/-}$ mice on the C57BL/6 background do not spontaneously develop inflammatory bowel disease (7). IL-10 acts to suppress both innate leukocyte and T cell-mediated activity and, like $Il27ra^{-/-}$ mice, animals with a genetic or Ab-mediated deficiency in IL-10 signaling develop exaggerated immune responses to infection (8). Although IL-10 has long been associated with Th2 cells, it can be produced by many other cell types including Th1 cells, T_{reg} cells, B cells, and macrophages (8, 9). Recently, several reports have indicated that Th1 cells producing both IL-10 and IFN-γ play an important regulatory role during certain infections (9–11). Because IL-27 has been assigned both Th1-promoting as well as immune-suppressive functions, we investigated whether IL-27 plays a role in the development of this novel regulatory Th subtype.

Materials and Methods

Mice and reagents

$Il27ra^{-/-}$ and $Il27ra^{+/+}$ (12) mice (C57BL/6 background), $Il10^{-/-}$ (129Sv/Ev background) and DO11.10 TCR transgenic/rag2 deficient mice (DO11.10^+/$rag2^{-/-}$ on the BALB/c background) were bred in a pathogen-free facility at Genentech. $Stat1^{-/-}$ mice (129Sv/Ev background) and 129Sv/Ev control mice were purchased from Taconic Transgenics. All live animal experiments were approved by the Institutional Animal Care and Use Committee of Genentech. Unless otherwise indicated, all cytokines were purchased from R&D Systems, and all Abs were from BD Biosciences.

Isolation and in vitro stimulation of naive primary T cells

Primary $CD4^+$ and $CD8^+$ T cells were enriched from splenic mononuclear cells by magnetic separation (Miltenyi Biotec) according to the manufacturer's instructions. The purity of sorted cells ranged from 90 to 95%. Primary cells were cultured as previously described (5). Unfractionated DO11.10^+.$rag2^{-/-}$

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[2] Abbreviations used in this paper: Ebi3, EBV-induced protein 3; EAE, experimental immune encephalomyelitis; m, murine; MOG, myelin oligodendrocyte glycoprotein; rh, recombinant human; rm, recombinant murine; T_{reg}, regulatory T cell.

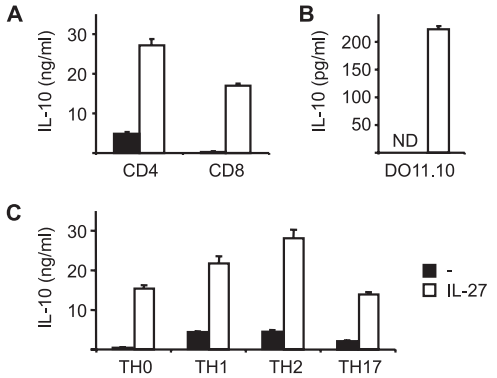

FIGURE 1. IL-27 induces IL-10 expression under all polarization conditions. $CD4^+$ or $CD8^+$ T cells from C57BL/6 spleens were stimulated with plate-bound anti-CD3 and soluble anti-CD28 Abs (*A*) and total $DO11.10^+/rag2^{-/-}$ splenocytes were stimulated with $OVA_{323–339}$ peptide (*B*) for 72 h in the presence (white bars) or absence (black bars) of rmIL-27. *C*, $CD4^+$ T cells from C57BL/6 mice were activated as in *A* under various T cell polarizing conditions (Th0, Th1, Th2, and Th17). IL-10 production in the culture supernatants was measured by ELISA. Error bars indicate SD of duplicates. These data are representative of at least three separate experiments.

splenocytes were stimulated with 0.3 μM $OVA_{323–339}$ peptide. As T cell polarizing conditions we used the following combinations of blocking Abs (all used at 5 μg/ml) and recombinant cytokines (concentration indicated; prefixes: m, murine; rh, recombinant human; rm, recombinant murine): Th0/Tc0 (anti-mIFNγ, anti-mIL-4, and anti-mIL-12), Th1/Tc1 (anti-mIL-4; 3.5 ng/ml rmIL-12), Th2 (anti-mIFNγ and anti-mIL-12; 3.5 ng/ml rmIL-4), Th17 (anti-mIFNγ, anti-mIL-4, 5 ng/ml rmIL-6, 1 ng/ml rhTGFβ1) and in the presence or absence of rmIL-27 (20 ng/ml). Before intracellular cytokine staining, PMA (50 ng/ml), ionomycin (500 ng/ml), and brefeldin A (5 μg/ml; Sigma-Aldrich) were added for the final 4 h of stimulation.

ELISA

IL-10 was detected in culture supernatants using the mouse IL-10 OptEIA ELISA set (BD Biosciences) as per the manufacturer's instructions.

Flow cytometry

Cells were treated with Fc blocking Abs and then surface stained with allophycocyanin-Cy7 conjugated anti-CD4 (GK1.5) and Pacific Blue-conjugated anti-CD8a (clone 53-6.7). The cells were stained intracellularly as previously described (5) with a combination of the following: PE-conjugated anti-mouse/rat FoxP3 (clone FJK-16s; eBioscience), PE-conjugated anti-mIL-17 (clone TC11-18H10), PE-conjugated anti-mIL-13 (clone eBio13A; eBioscience), allophycocyanin-conjugated anti-mIL-10 (clone JES5-16E3), FITC conjugated anti-mT-bet (clone 4B10), and PE-Cy7-conjugated anti-mIFNγ (clone XMG1.2).

In vivo models

EAE was induced and cells were isolated from draining lymph nodes as previously described (5). *Listeria monocytogenes* (2.5×10^4 CFU per mouse) was administered i.v. to age-matched (8–11 wk) and sex-matched groups of $Il27ra^{-/-}$ and $Il27ra^{+/+}$ mice.

Results and Discussion

IL-27 induces IL-10 expression by $CD4^+$ and $CD8^+$ T cells

We first assessed the effect of IL-27 on IL-10 expression by T cells and found that stimulation of both $CD4^+$ and $CD8^+$ T cell cultures with IL-27 resulted in strong induction of IL-10 (Fig. 1*A*). This effect was confirmed in the context of cognate Ag stimulation using splenocytes from $DO11.10^+/rag2^{-/-}$ mice (Fig. 1*B*). The activity of IL-27 in this context was specific, because no changes were observed when $Il27ra^{-/-}$ or $DO11.10^+/rag2^{-/-}/Il27ra^{-/-}$ cells were treated with rmIL-27 (data not shown). To explore the scope of this effect, we examined the regulation of IL-10 by IL-27 during the activation of $CD4^+$ T cells under a range of polarizing conditions (Th0, Th1, Th2, or Th17). Upon IL-27 stimulation, we detected high levels of IL-10 in the culture supernatants irrespective of the polarization condition (Fig. 1*C*). Similar results were observed using $CD8^+$ T cells and were reproduced in the $DO11.10^+/rag2^{-/-}$ system (data not shown). We also noted that despite eliciting strong IL-10 induction in the primary stimulation of T cells, IL-27 by itself was not sufficient to promote the formation of a stable, IL-10 producing Th cell lineage (not shown). Indeed, others have demonstrated that IL-27 suppresses IL-10 production upon repeated stimulation, while it caused induction of IL-10 production in FACS-purified naive T cells (13).

IL-27 promotes IL-10 expression by IFNγ-producing cells

To determine the status of the T cells that express IL-10 after IL-27 stimulation at the cellular level, we conducted intracellular cytokine staining (Fig. 2). Under Th0 conditions, IL-27 induced both IL-10 and IFN-γ and a considerable proportion of the cells expressed both cytokines (Fig. 2*A*). Although $IL\text{-}10^+$ cells were detected under Th1 conditions even in the absence of IL-27, stimulation with IL-27 doubled the proportion of $IFN\text{-}\gamma^+IL\text{-}10^+$ cells. Because IL-12 itself has the capacity to induce IL-10 production (14, 15), IL-27 might act by enhancing IL-12 responsiveness through up-regulation of the IL-12Rβ2 chain in

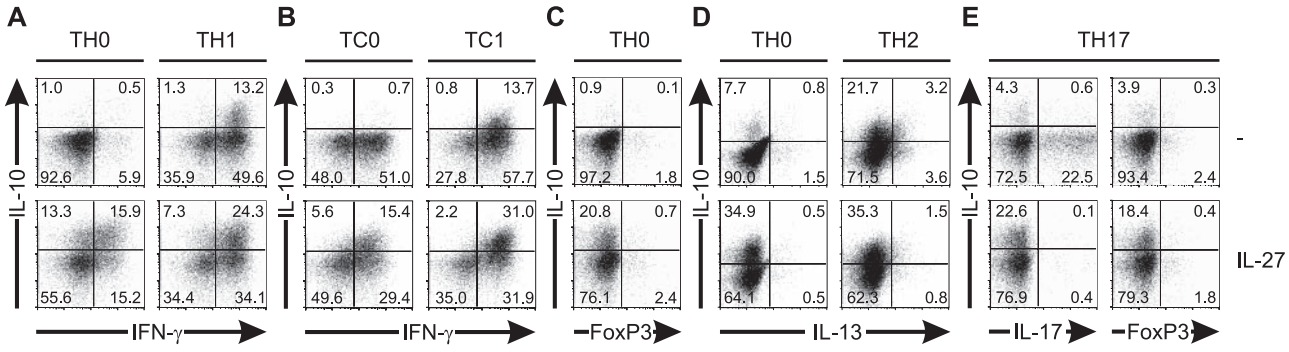

FIGURE 2. IL-27 induced $IL\text{-}10^+$ cells are $IFN\gamma^+$, $FoxP3^-$, and $IL\text{-}17^-$. $CD4^+$ or $CD8^+$ T cells from C57BL/6 spleens were stimulated with plate-bound anti-CD3 and soluble anti-CD28 Abs under various polarizing conditions for 72 h in the absence (*upper panels*) or presence (*lower panels*) of rmIL-27. Polarization conditions are shown at the *top*, whereas cytokine stains are indicated by arrows along the *x*- and *y*-axes of the graphs. Panels are gated on $CD4^+$ (*A*, *C*, *D*, and *E*) or $CD8^+$ cells (*B*), and the percentages of cytokine-producing cells are indicated in each quadrant.

this context (16). However, we found that IL-27 was very effective at inducing IL-10 in cultures of IL-12Rβ1$^{-/-}$ T cells (data not shown) and under conditions where neutralizing Abs directed against IL-12p40 are added (Th0 and Th2; Figs. 1 and 2). Therefore, IL-27 can act independently of IL-12 to induce IL-10 production. Similar to results observed in CD4$^+$ cells, IL-27 promoted IL-10 expression by IFN-γ^+CD8$^+$ cells under all polarization conditions (Tc0 and Tc1, shown in Fig. 2*B*).

Two recent publications described the critical contribution of IFN-γ-producing Th1 cells to IL-10 production during *Toxoplasma gondii* and *Leishmania major* infection (10, 11). As with the cells described in those publications, the IL-10$^+$ cells induced by IL-27 treatment were FoxP3 negative (Fig. 2*C*) and T-bet positive (data not shown). In this sense, IL-27-treated CD4$^+$ cells resemble a subset of IL-10-producing regulatory T cells termed "Tr1" cells (17). However, the cells induced to express IL-10 by IL-27 treatment continued to proliferate and could not directly suppress proliferation when cocultured with CFSE-labeled effector cells (data not shown) suggesting that they are not bona fide Tr1 cells. Nevertheless, IL-10 production by T cells, including Th1 cells, was shown to be critical for suppressing immune responses *to L. major and T. gondii* (10, 11). In those studies it was also suggested that IL-10 producing cells were not Tr1 cells because they were not anergic.

We have previously demonstrated that IL-27 can suppress expression of the Th2 transcription factor GATA3 (16). Consistent with this finding, flow cytometry revealed that in Th0 conditions IL-27-induced IL-10$^+$ cells that were negative for the Th2 cytokine IL-13. Even under Th2 conditions, IL-10 induction occurred in the IL-13$^-$ population, and the overall IL-13 level was reduced in response to IL-27 (Fig. 2*D*). Thus, IL-27 does not enhance IL-10 expression by promoting Th2 differentiation.

Interestingly, cells stimulated under Th17 polarizing conditions also produce IL-10 (Fig. 1*C* and Refs. 18 and 19), and the proportion of IL-10$^+$ cells was strongly enhanced by IL-27 treatment. However, whether cultured in the presence or absence of rmIL-27, IL-10 was predominantly expressed by cells that were negative for IL-17 (Fig. 2*E*). Murine Th17 cells develop under the influence of TGF-β along with IL-21 or IL-6, whereas TGF-β stimulation alone promotes the differentiation of FoxP3$^+$ T_{reg} cells (20–23). Because IL-27 acts antagonistically to IL-6 in the context of Th17 differentiation (5, 6), we tested whether the shift from IL-17 to IL-10 expression in response to IL-27 reflected a reconstitution of de novo T_{reg} differentiation. When staining for FoxP3 expression, we found that the IL-10-producing cells were FoxP3 negative (Fig. 2*E*). Furthermore, similar to the Th0 condition, IL-27 did not promote IL-13 and GATA3 expression in the Th17 condition (data not shown). Together, these data establish IL-27 as a cytokine that promotes expression of IL-10 under all commonly tested skewing conditions while inducing neither Th2 nor Treg differentiation.

Induction of IL-10 is STAT1 dependent

Many effects of IL-27, such as suppression of Th17 differentiation, are dependent upon the activation of STAT1 (5, 6). Therefore, to determine whether IL-27 also relies on STAT1 to induce IL-10, we used STAT1-deficient CD4$^+$ and CD8$^+$ T cells and found that IL-27-mediated induction of IL-10 was strongly reduced compared with wild-type cells (Fig. 3). However, the addition of IL-27 to STAT1$^{-/-}$ cells reproducibly induced a small amount of IL-10 expression in CD4$^+$ (Fig. 3, *A* and *B*) and CD8$^+$ (Fig. 3*C*) cells. Thus, STAT1 signaling is required for fully efficient IL-10 induction but is not absolutely necessary for IL-10 augmentation by IL-27. Interestingly, IFN-γ was a poor inducer of IL-10 despite being a well-documented activator of STAT1 phosphorylation (data not shown). Thus, STAT1 activation by itself is likely not sufficient for IL-10 induction. Indeed, a very recent publication revealed that both STAT1 and STAT3 are necessary for IL-10 induction by IL-27, whereas T-bet is dispensable (19).

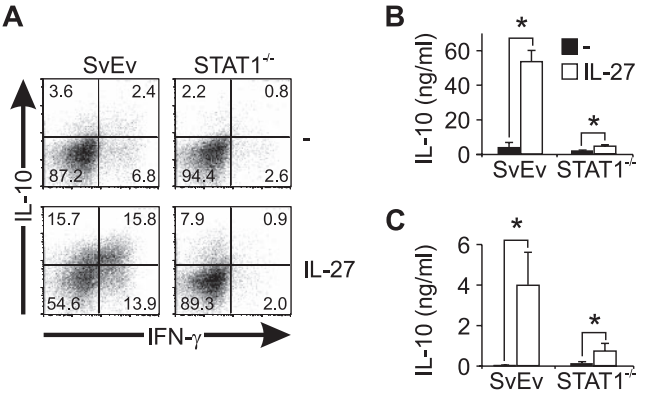

FIGURE 3. IL-27 mediated induction of IL-10 is STAT1 dependent. CD4$^+$ (*A* and *B*) or CD8$^+$ (*C*) T cells from STAT1$^{-/-}$ or control SvEv (STAT1$^{+/+}$) spleens were stimulated with plate-bound anti-CD3 and soluble anti-CD28 Abs for 72 h under Th0/Tc0 conditions in the presence or absence of rmIL-27. *A*, Intracellular IL-10 and IFN-γ staining gated on CD4$^+$ cells. *B* and *C*, IL-10 accumulation in the culture supernatants was measured by ELISA. The average cytokine concentration obtained by combining data from four independent experiments ± SEM is shown. *, $p < 0.05$.

We have previously shown that IL-27 suppresses IL-17 production, IL-6-induced T cell proliferation, and IL-6 induced IL-23R expression (5). By using IL-10$^{-/-}$ T cells in an APC-free system, we determined that these effects did not depend on the presence of IL-10 (data not shown).

Il27ra *deficiency leads to reduced IL-10 production by CD4$^+$ cells in vivo*

We previously showed that *Il27ra*$^{-/-}$ mice develop exacerbated MOG-induced EAE (5). Likewise, IL-10 is known to suppress EAE (24). To assess whether the EAE limiting effect of IL-27 could be mediated by IL-10, we investigated IL-10 production in *Il27ra*$^{-/-}$ mice upon myelin oligodendrocyte glycoprotein (MOG) immunization. At day 13 after induction of EAE, lymphocytes were isolated from the draining lymph nodes and restimulated with MOG peptide. Significantly, fewer IL-10 producing CD4$^+$ T cells were obtained from *Il27ra*$^{-/-}$ mice (Fig. 4, *A* and *B*). Furthermore, we observed a trend toward a reduction of IL-10 production in infiltrating lymphocytes in the spinal cord of *Il27ra*$^{-/-}$ mice with EAE (not shown). These data are in line with a recent publication showing that ex vivo rIL-27 treatment could reduce the pathogenicity of encephalitogenic T cells through an IL-10-dependent mechanism (25). Our data now demonstrate that IL-27 has a physiologically relevant and nonredundant role in supporting IL-10 production during EAE.

To further corroborate the importance of IL-27 for IL-10 induction in vivo, we also infected *Il27ra*$^{-/-}$ mice with a sublethal dose of *L. monocytogenes* that resulted in a similar clearance

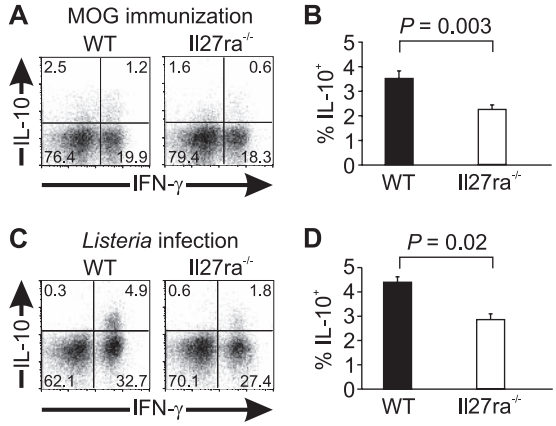

FIGURE 4. IL-27 signaling is required for the efficient generation of IL-10$^+$CD4$^+$ T cells in vivo. *A* and *B*, Draining lymph node cells from wild-type (WT) and *Il27ra*$^{-/-}$ mice were collected 13 days after MOG immunization and restimulated with MOG (35–55) ex vivo for 72 h. *C* and *D*, Splenocytes from wild-type and *Il27ra*$^{-/-}$ mice were collected 7 days after infection with *L. monocytogenes* and restimulated with heat killed *L. monocytogenes* ex vivo for 72 h. Representative FACS plots show IFN-γ and IL-10 expression in the CD4$^+$ gate (*A* and *C*) and statistical evaluation across all animals (*B* and *D*) are shown for each experiment ($n = 6$ per genotype for MOG immunization and $n = 5$ per genotype for *L. monocytogenes* infection).

of the parasite in wild-type and *Il27ra*$^{-/-}$ mice as assessed by spleen and liver CFU counts on day 7 (not shown). Upon the restimulation of splenocytes with heat-killed *L. monocytogenes*, intracellular staining for IFN-γ and IL-10 revealed a statistically significant paucity of IFN-γ$^+$IL-10$^+$ cells among the CD4$^+$ population in *Il27ra*$^{-/-}$ mice (Fig. 4, *C* and *D*). IL-10$^+$ cells observed during listeriosis were predominantly IFN-γ$^+$ (Fig. 4*C*). To account for possible variability in the proportion of IFN-γ$^+$ cells between individual mice, we also calculated the fraction of IL-10$^+$ cells as a proportion of CD4$^+$IFN-γ$^+$ cells. Even using this more stringent criterion, IL-10 production was still significantly reduced in *Il27ra*$^{-/-}$ mice ($p = 0.0017$, data not shown). We also observed significantly fewer IL-10$^+$IFN-γ$^+$CD8$^+$ cells in *Il27ra*$^{-/-}$ mice ($p = 0.0316$, data not shown). Finally, we examined the phenotype of IL-10$^+$IFN-γ$^+$ CD4$^+$ cells from *Listeria*-infected mice by intracellular staining and found that they were T-bet$^+$FoxP3$^-$IL-4$^-$ (Fig. 5). By these criteria, they were indistinguishable from the IL-10-producing cells generated in vitro in the presence of IL-27 (Fig. 2 and data not shown). Taken together, our data therefore demonstrate that IL-27 signaling is important for the physiological induction of IL-10 in T cells, even under highly inflammatory conditions.

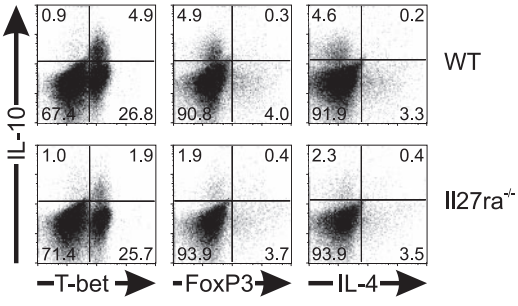

FIGURE 5. Phenotypic analysis of CD4$^+$IL-10$^+$IFN-γ$^+$ cells generated in vivo. Splenocytes from wild-type (WT) and *Il27ra*$^{-/-}$ mice were collected 7 days after infection with *L. monocytogenes* and restimulated with heat-killed *L. monocytogenes* ex vivo for 72 h. Representative FACS plots show IL-10 expression along with T-bet, FoxP3, or IL-4 (as indicated) in the CD4$^+$ gate.

It should be noted that IL-10 expression in *Il27ra*$^{-/-}$ mice was normal in several previous studies (26–30). These studies interrogated global IL-10 expression in mouse serum, by RT-PCR of unfractionated lymphoid organs, or by ELISA of culture supernatants from cells stimulated ex vivo. We have not investigated whether IL-27Ra deficiency affects global IL-10 production. However, T cell specific IL-10 abrogation phenocopied the IL-10 knockout mice in the context of *L. major* infection, suggesting that T cells are a physiologically relevant source of IL-10 (10). Furthermore, Th1-derived IL-10 is critically important in the prevention of *Toxoplasma*-induced immune pathology (11). Therefore, spatial and cellular localization of IL-10 expression is important and merits consideration when analyzing IL-10 levels in vivo.

In summary, we demonstrate that IL-27 is a potent inducer of IL-10 in T cells. The induction of IL-10 is not the result of skewing toward induced T_{reg} or Th2 differentiation but rather reflects increased IL-10 production by both CD4$^+$ and CD8$^+$ T cells in various polarization states. Importantly, IL-27 treatment promotes the emergence of IL-10$^+$IFN-γ$^+$FoxP3$^-$ T cells, and this phenotype characterizes CD4$^+$ cells that were recently described as key negative regulators of the immune response to *T. gondii* and *L. major* infection (10, 11). In the absence of IL-27 signaling in vivo, animals generate fewer IL-10$^+$ T cells during autoimmune disease and infection. Mechanistically, IL-27 depends on STAT1 but not on IL-12 receptor signaling for IL-10 induction, even though IL-12 is independently capable of having that effect. Together, these observations provide a compelling explanation for the exacerbated immune responses observed in *ebi3*$^{-/-}$ and *Il27ra*$^{-/-}$ mice.

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Disclosures

Marcel Batten, Ji Li, Noelyn Kljavin, Frederic de Sauvage, and Nico Ghilardi are employees of Genentech, Inc., a commercial biotech company.

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