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Cutting Edge: IL-27 Induces the Transcription Factor c-Maf, Cytokine IL-21, and the Costimulatory Receptor ICOS that Cooperatively Act Together to Promote Differentiation of IL-10-Producing Tr1 Cells

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IL-27 has recently been identified as a differentiation factor for the generation of IL-10-producing regulatory type 1 (Tr1) T cells. However, how IL-27 induces the expansion of Tr1 cells has not been elucidated. In this study we demonstrate that IL-27 drives the expansion and differentiation of IL-10-producing murine Tr1 cells by inducing three key elements: the transcription factor c-Maf, the cytokine IL-21, and the costimulatory receptor ICOS. IL-27-driven c-Maf expression transactivates IL-21 production, which acts as an oncogene growth factor for the expansion and/or maintenance of IL-27-induced Tr1 cells. ICOS further promotes IL-27-driven Tr1 cells. Each of those elements is essential, because loss of c-Maf, IL-21-signaling, or ICOS decreases the frequency of IL-27-induced differentiation of IL-10-producing Tr1 cells. *The Journal of Immunology, 2009, 183: 797–801.

Interleukin-27, a member of the IL-12/IL-23 heterodimeric family of cytokines produced by APCs, is composed of two chains, IL-27p28 and EBV-induced gene 3 (1). Activated T cells and NK cells have the highest expression of IL-27R, which is composed of two chains, a specific IL-27Ra chain (WSX-1 or TCCR) and a signaling chain, gp130, that it shares with IL-6R (1). Initial studies have suggested that, similarly as IL-12, IL-27 induces the expansion of proinflammatory Th1 cells by activating the STAT1-mediated T-bet pathway (1). However, analysis of the IL-27Ra−/− (WSX-1−/−) mice infected with various pathogens resulted in clearance of the parasites with exaggerated T cell responses and enhanced proinflammatory cytokine production (1). Furthermore, IL-27Ra−/− mice developed severe experimental autoimmune encephalomyelitis with enhanced Th17 responses (1), and treatment with rIL-27 suppressed disease and decreased the frequency of Th17 cells (1). These paradoxical observations led to the hypothesis that IL-27 may not be necessary for the generation of proinflammatory T cells (Th1 or Th17), but rather play a crucial role in regulating T cell responses. Subsequently, three groups, including ours, reported that IL-27 not only inhibited the generation of Th17 cells but also induced differentiation of IL-10-producing regulatory T cell type 1 (Tr1)3 from naive T cells (2–4).

Tr1 cells are a subset of T cells that have strong immunosuppressive properties, predominantly produce IL-10 with variable amounts of IFN-γ, but do not express Forkhead box gene 3 (Foxp3) (5). Adoptive transfer of Tr1 cells has been shown to suppress autoimmunity, colitis, graft-vs-host disease, and tissue inflammation (6). Although initial studies suggested that Tr1 cells are induced by repetitive antigenic stimulation of T cells in the presence of IL-10 (6), T cells differentiated in the presence of IL-10 could not be propagated long term in culture. The identification of IL-27 as a differentiating factor for the generation of Tr1 cells provided a means by which they could be grown in large numbers and facilitated their functional analysis. However, the molecular mechanisms by which IL-27 mediates the generation and/or expansion of Tr1 cells are not well understood. Thus, we analyzed the expression of various key cytokines and transcription factors induced by IL-27. Our results show that IL-27 is a potent inducer of three essential elements: the transcription factor c-Maf, the cytokine IL-21, and the costimulatory factor c-Maf, Cytokine IL-21, and the Costimulatory Receptor ICOS that Cooperatively Act Together to Promote Differentiation of IL-10-Producing Tr1 Cells

Cutting Edge

TNF, T follicular helper cell; WT, wild type.

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receptor ICOS, which coordinately work together to promote differentiation of Tr1 cells.

**Materials and Methods**

**Mice and reagents**

IL-10-enhanced GFP reporter mice (Vert-X), Foxp3.gfp “knock-in” mice, ICOS−/− mice on C57BL/6 background, were generated as described (7, 8). WSX-1−/− mice on C57BL/6 background were obtained from C. Saris (Amgen, Thousand Oaks, CA), and IL-21R-deficient mice on NOD background (9) from N. Sarvetnick (Scripps Research Institute, La Jolla, CA). c-Maf−/− mice on an N5 BALB/c background have been described (10). Mice were housed in conventional, pathogen-free facilities at the Harvard Institute of Medicine (Boston, MA). All experiments were undertaken in accordance with guidelines from the Committee on Animals at Harvard Medical School (Boston, MA).

**T cell differentiation and proliferation in vitro**

Naive CD4+ T cells (CD4+ CD62L+CD25−), pooled from both spleen and lymph nodes, or memory cells (CD4+ CD44+), obtained from lymph nodes, were purified by flow cytometry and stimulated with plate-bound Ab against CD3 (145-2C11; 1 μg/ml−1) and CD28 (PV-1; 1 μg/ml−1). Cells were cultured as previously described (8). Monoclonal anti-TGF-β1 Ab (10 μg/ml−1), mouse IL-21 (80 ng/ml−1), mouse IL-21 (25 ng/ml−1), and anti-mouse IL-21 Ab (25 μg/ml−1) were all purchased from R&D Systems. Proliferation assay was performed as previously described (8).

**Measurement of cytokines**

Secreted cytokines were measured after 48 h by cytometric bead array (BD Biosciences) or ELISA. Intracellular cytokine staining was performed as previously described (8).

**Quantitative real-time PCR**

RNA was extracted with RNeasy mini kits (Qiagen) and analyzed by real-time PCR according to the manufacturer’s instructions (Applied Biosystems). The following primers/probe mixtures were purchased from Applied Biosystems: IL-10 (catalog no. Mm 00439615_g1); ICOS (catalog no. Mm004497600_m1); c-Maf (catalog no. Mm 02581355_S1); IL-21 (catalog no. Mm00600319_M1); TGF-β1 (catalog no. Mm004497600_m1); and IL-27 acted synergistically to generate Tr1 cells. However, under such culture conditions low Foxp3 expression was also induced by TGF-β. Using IL-10-enhanced GFP reporter mouse (Vert-X), we developed culture conditions under which IL-27 alone could induce Tr1 cells. These in vitro derived Tr1 cells were as suppressive as natural Foxp3+ regulatory T (Treg) cells in inhibiting T cell proliferation in vitro (supplemental Fig. 1).

To understand the molecular mechanisms by which IL-27 induces and expands Tr1 cells, we analyzed the expression of genes up-regulated by IL-27 at multiple time points following activation. Real-time PCR of IL-10 (A), c-Maf (B), IL-21 (C), IL-21R (D), and ICOS (E) induction by IL-27 is shown. F and G, IL-10 and IL-21 production was measured by cytokine bead array as induced by IL-27 in WT (open bars) and IL-27Ra−/− (WSX-1−/−) (filled bars) CD4+CD62LhiCD25− cells. H, Intracellular cytokine staining of IL-10 and IL-21 by T cells following activation in the presence of IL-27.

*FIGURE 1. IL-27 induces c-Maf, IL-21, IL-21R, and ICOS. A–E, RNA isolated from naive CD4+ CD62LhiCD25− cells cultured with IL-27 (open squares) or without IL-27 (closed squares) were subjected to real-time PCR relative to the expression (rel. expression) of mRNA encoding B-actin (2−ΔΔCT × 100,000; where Ct is cycle threshold) to examine the expression of cytokines at different time points following activation. Real-time PCR of IL-10 (A), c-Maf (B), IL-21 (C), IL-21R (D), and ICOS (E) induction by IL-27 is shown. F and G, IL-10 and IL-21 production was measured by cytokine bead array as induced by IL-27 in WT (open bars) and IL-27Ra−/− (WSX-1−/−) (filled bars) CD4+CD62LhiCD25− cells. H, Intracellular cytokine staining of IL-10 and IL-21 by T cells following activation in the presence of IL-27.*

*The online version of this article contains supplemental material.*
with our previous observation that c-Maf regulates IL-21 expression in Th17 cells (8), we observed that IL-27 also induced IL-21 mRNA and that IL-10 and IL-21 showed similar kinetics of mRNA expression (Fig. 1, A and C). Although IL-21R expression was low in unactivated T and B cells, TCR-driven activation up-regulated this expression and IL-27 further up-regulated IL-21R expression in activated T cells (Fig. 1D). We (8) and others (11) have shown that the c-Maf transcription factor is downstream of ICOS, and because IL-10-producing T cells were first shown to be preferentially costimulated by ICOS (12), we examined the expression of ICOS mRNA and observed that the addition of IL-27 indeed induced higher ICOS expression than T cell activation without IL-27 (Fig. 1E). Thus, IL-27, in addition to inducing IL-10 production, induced c-Maf, IL-21, IL-21R, and ICOS expression. At the protein level, T cells activated in the presence of IL-27 produced both IL-10 and IL-21, thus confirming mRNA expression (Fig. 1, F and G). Besides the mRNA expression, we also observed an increase in ICOS expression induced by IL-27 at the protein level (data not shown). Furthermore, IL-27Ra/−/− (WSX-1/−/−) mice produced no detectable IL-10 or IL-21 and did not show an increased ICOS expression upon activation, indicating that IL-10 and IL-21 production and increased ICOS expression were specifically induced by IL-27 (Fig. 1, F and G, and data not shown). To analyze the cells that produce both IL-10 and IL-21, we undertook intracellular cytokine staining for IL-10 and IL-21 after 3 days of culture in vitro with IL-27 and found that the IL-10-producing cells also produced IL-21 (Fig. 1H).

Because IL-21 belongs to the IL-2 cytokine family and uses the common γ-chain receptor, we hypothesized that IL-27-driven IL-21 production from T cells may be an autocrine growth factor for the generation of Tr1 cells. To test this, we first added a neutralizing IL-21 Ab in the presence of IL-27 and found that blocking IL-21 reduced the frequency of IL-10-producing T cells significantly by >75% (Fig. 2A) and IL-10 cytokine production in the culture supernatants by >50% (Fig. 2B). Further addition of IL-21 together with IL-27 increased IL-10 production, but this increase with exogenous IL-21 was modest (Fig. 2B). These data raised the issue of whether IL-21 could directly expand IL-10-producing T cells. However, activation of T cells from mice lacking IL-27Ra (WSX-1) signaling in the presence of IL-21 and IL-27 did not expand Tr1 cells (supplemental Fig. 2). We further confirmed the role of IL-21 in the expansion of Tr1 cells using CD4+ T cells from IL-21R-deficient mice. Loss of IL-21 signaling resulted in the inhibition of IL-27-driven generation of IL-10-producing T cells by >75% (Fig. 2C) and IL-10 cytokine production in the culture supernatants by over 90% (Fig. 2D). However, loss of IL-21 signaling had no effect on IL-27-driven IFN-γ production (data not shown). Furthermore, IL-21R-deficient CD4+ T cells stimulated with IL-27 expressed lower levels of c-Maf and IL-21, as determined by real-time PCR (Fig. 2E). These data suggest that IL-21 may be an important growth factor induced by IL-27 to expand Tr1 cells without affecting the expansion of IFN-γ-producing cells. Because IL-27 not only induces IL-21 production but also induces IL-21R expression, these data suggest that IL-27-mediated IL-21R up-regulation might be required for IL-21 to expand Tr1 cells.

To study the relevance of IL-21 in expanding Tr1 cells in vivo, we examined the frequency of IL-10-producing Tr1 cells generated in vivo in IL-21R−/− mice. We found that the fraction of IL-10-producing CD4+CD44− memory T cells was significantly reduced in IL-21R−/− mice, which showed only 10% as many IL-10-producing T cells compared with wild-type (WT) mice (Fig. 3A). In contrast, the frequency of IFN-γ producers was similar in WT and IL-21R−/− cells. To further determine whether IL-27 could correct the defect in Tr1 cell development, CD4+CD44− memory T cells were activated in the presence of IL-27, but IL-21R−/− mice continued to show a significant reduction in IL-10-producing T cells (data not shown). CD4+CD44+ T cells purified from IL-21R−/− mice also showed a lower expression of c-Maf and IL-21 mRNA (Fig. 3B), highlighting the importance of this amplification loop in generating Tr1 cells. In addition to the induction of c-Maf, IL-21, and IL-21R, IL-27 also enhanced the expression of ICOS. Because IL-10-producing T cells were first shown to be preferentially costimulated by ICOS (12), we analyzed the effect of a lack of ICOS signaling on the induction of Tr1 cells by IL-27. In vitro differentiation of T cells from ICOS-deficient mice demonstrated that IL-10 production by ICOS−/− T cells was similar to that by WT T cells at 48 h, but by 72 h there was a significant defect in IL-10 production induced by IL-27, as determined by cytometric bead array (Fig. 3C) and by intracellular staining (data not shown). In the plate-bound Ab system used here, the ICOS ligand (ICOS-L) is most likely provided by the CD4+ T cells, because T cells can express ICOS-L when activated (13). When Tr1 cells were differentiated from
ICOS<sup>−/−</sup> mice, it was clear that ICOS<sup>−/−</sup> mice had a defect in sustaining growth/expansion of Tr1 cells in vitro.

Our results clearly demonstrated that IL-27 induces expression of c-Maf and that expressions of c-Maf and IL-21 mRNA appeared to be coexpressed in differentiating Tr1 cells under various differentiation conditions (Figs. 1, 2E, and 3B). Therefore, we compared c-Maf expression in T cell subsets (Th0, Th1, Th2, Th17, and Tr1), and observed that Tr1 cells had ~500-fold higher expression than Th1 or Th2 cells (Fig. 4A). We have observed that c-Maf and IL-21 mRNA were coexpressed by Tr1 cells, suggesting that c-Maf may be a transcription factor for IL-21, which in turn expands Tr1 cells. Our analysis of the IL-21 promoter revealed four putative conserved binding sites located 1070 bp (half MARE), 370 bp (v-MARE), 260 bp (half MARE) and 200 bp (v-MARE) upstream of the transcriptional start site (where MARE is Maf recognition element and v-MARE is v-Maf recognition element). To test this, an IL-21 promoter-luciferase reporter construct was cotransfected with a c-Maf expression plasmid into HEK 293T cells. Interestingly, c-Maf could transactivate IL-21 promoter-luciferase in a dose-dependent manner, but the transcription factors T-bet and GATA3, which are involved in Th1 and Th2 differentiation, could not (Fig. 3B), suggesting that c-Maf may expand Tr1 cells by inducing IL-21 production. Indeed, we have previously shown that c-Maf-deficient mice have a defect in IL-21 production (8). To address whether c-Maf-deficient mice have a defect in the IL-27-mediated IL-10 and IFN-γ production, we activated c-Maf-deficient T cells in the presence of IL-27 and analyzed the expression of IL-10 and IFN-γ. IL-27 was not able to induce either IL-10 or IL-21 production in c-Maf-deficient CD4<sup>+</sup> T cells (Fig. 4C and data not shown), but the IL-27-mediated IFN-γ response was not affected (data not shown). As c-Maf has been described to directly transactivate the IL-10 promoter (14, 15), we further added IL-21 to IL-27-activated c-Maf-deficient CD4<sup>+</sup> T cells and showed that exogenous IL-21 can partially rescue IL-10 production in c-Maf<sup>−/−</sup> T cells (Fig. 4D), highlighting the importance of IL-21 transactivation by c-Maf.

These data clearly show that IL-27 induces c-Maf, a transcription factor previously identified in Th2 cells, to transactivate IL-21, which then drives the expansion of Tr1 cells. Therefore, similar to Th17 and T follicular helper (TFh) cells, Tr1 cells express c-Maf and IL-21 and use IL-21 for autocrine growth and expansion. It is interesting to note that three different T cell subsets, Tr1, Th17, and TFh cells, which express c-Maf and IL-21, also produce IL-10, albeit at different levels (8, 16, 17). IL-21 acts as an autocrine growth/differentiation factor for all three subsets of T cells. It stands to reason that IL-21, which belongs to the family of IL-2 growth factors and uses the common γ-chain for signaling, may act as an expansion/growth factor for cells that do not produce IL-2. Consistent with this idea, loss of IL-21 or IL-21 signaling results in a defect in all the three T cell subsets, Th17, TFh, and Tr1 (8, 16). Our data are consistent with a recent study showing that IL-21 mediates its inhibitory effects by inducing IL-10 production (18). Similar to IL-6, which induces IL-21 by inducing phospho-STAT-3, IL-27 also induces phospho-STAT-3 and IL-21, most likely due to the fact that IL-6 and IL-27 both share the gp130 chain for signaling.
IL-27-enhanced expression of ICOS is of interest because ICOS was initially shown to costimulate IL-10-producing T cells (12). We and others have shown that ICOS/ICOS ligand interaction induces c-Maf expression (8, 11) which may further enhance stable IL-21 production from developing Tr1 cells. Like Th17 and Tfh cells, ICOS appears to be crucial in maintaining IL-27-driven Tr1 cells. These results are supported by the observation that ICOS−/− mice indeed have a defect in IL-10-producing T cells and therefore develop more severe autoimmunity (8, 19). IL-27 also up-regulates ICOS for maintenance and survival of Tr1 cells.

In summary, we have demonstrated that IL-27 drives the expansion and differentiation of Tr1 cells by inducing expression of three key elements: the transcription factor c-Maf, the growth factor IL-21, and the costimulatory receptor ICOS, which coordinately act to mediate Tr1 differentiation (supplemental Fig. 3). Loss of any of these factors results in a defective IL-27-driven, IL-10-producing Tr1 cells.

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Disclosures
The authors have no financial conflict of interest.

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