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CUTTING EDGE

Cutting Edge: IL-12 Induces $CD4^+CD25^-$ T Cell Activation in the Presence of T Regulatory Cells[1]

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IL-12p40 cytokines have been implicated in the development of organ-specific autoimmune diseases as well as pathogen-specific adaptive immunity. In addition to inducing IFN-γ, IL-12 stimulates effector $CD4^+$ T cells to express adhesion molecules and homing receptors that facilitate their migration to sites of inflammation. In this study, we expand upon those observations by demonstrating an alternative pathway by which IL-12 could promote Th1 inflammatory responses in mice, namely, by restoring proliferation and cytokine expression by effector T cells in the presence of $CD4^+CD25^+$ regulatory T cells (Treg). This effect of IL-12 was not replicated by IL-23 or IFN-γ and was dependent on signaling through the IL-12R expressed on $CD25^-$ responder cells, but not on Treg. Our studies suggest that IL-12 could act in concert with other proinflammatory factors to stimulate $CD4^+CD25^-$ T cell activation in the presence of Treg. The Journal of Immunology,* 2005, 175: 641–645.

The IL-12p70 heterodimer is secreted by myeloid cells in response to proinflammatory "danger" signals such as bacterial DNA, LPS, and CD40 CD40L ligand (1). This Th1-polarizing cytokine exerts multiple effects on $CD4^+$ T cells which promote their effector functions in vivo. In addition to inducing IFN-γ, IL-12 directly stimulates effector T cells to express adhesion molecules (such as P-selectin ligand) and chemokine receptors (such as CCR5) that facilitate their infiltration into inflammatory foci within extralymphoid tissues (2, 3). Although IL-12 is a critical factor for the development of $CD4^+$ T cell-driven, macrophage-dependent immunity against infectious pathogens such as *Leishmania* and *Toxoplasma* (4, 5), it has also been implicated in a number of organ-specific autoimmune diseases (6).

Whereas IL-12 instigates Th1 immune responses, $CD4^+CD25^+$ regulatory T cells (Treg)[3] actively restrain them (7). Treg represent a subset of thymus-derived $CD4^+$ T cells that constitutively express the cell surface phenotype $CD25^+$CTLA-4^+ glucocorticoid-induced TNF receptor $(GITR)^+$ and transcribe the transcription factor Foxp3 (7). Following engagement of their TCR, Treg suppress the proliferation of conventional $CD4^+CD25^-$ T responder cells in vitro. Furthermore, they inhibit the development of $CD4^+$ T cell responses against alloantigens, tumor, microbial, and self-Ags in vivo. Although Treg are useful for prevention of spontaneous autoimmune events, their presence could potentially sabotage protective antimicrobial responses (8).

Although many studies have focused on the factors necessary for Treg function and survival, few have investigated the pathways by which conventional $CD4^+CD25^-$ T cells escape Treg-mediated suppression to mount physiological immune responses. In this study, we demonstrate that IL-12 restores the ability of $CD4^+CD25^-$ T cells to proliferate, express activation markers, and transcribe cytokine mRNA during coculture with Treg. These effects are not replicated by IL-23 or IFN-γ and are dependent on signaling through the IL-12R expressed on $CD25^-$ responder cells, but not on Treg. Therefore, in addition to its many effects on the differentiation and effector functions of Th1 cells, IL-12 promotes $CD4^+$ T cell activation in the presence of Treg.

Materials and Methods

Mice

BALB/c, C57BL/6, IL-12Rβ2 chain- deficient (IL-12Rβ$2^{-/-}$) C57BL/6 and $Thy1.1^+$ congenic BALB/c mice were obtained from The Jackson Laboratory or the National Cancer Institute (Frederick, MD) and housed under specific pathogen-free conditions. All experiments were performed under University Committee on Animal Resources-approved protocols.

Subset purification

$CD4^+$ T cells were enriched from pooled lymph nodes and spleens of naive mice by negative selection using guinea pig complement (Rockland). The resulting population was stained with anti-CD25 mAb conjugated with FITC (BD Pharmingen) and $CD25^-$ and $CD25^+$ subsets were separated using anti-FITC microbeads with the MACS system (Miltenyi Biotec). Syngeneic T-depleted splenocytes were used as APC.

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[3] Abbreviations used in this paper: Treg, regulatory T cell; GITR, glucocorticoid-induced TNFR.

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In vitro suppression assay

$CD4^+CD25^-$ T cells (5×10^4/well) were cultured with APC (5×10^4/well) in the absence or presence of $CD4^+CD25^+$ T cells at varying concentrations in RPMI 1640 (Mediatech) containing 10% FBS and standard supplements (200 μl) in 96-well plates. Anti-CD3 (0.5 μg/ml; BD Pharmingen), anti-CD28 (0.5 μg/ml; BD Pharmingen), and recombinant murine IL-2, IL-12p70, IL-23 (R&D Systems), or IFN-γ (BD Pharmingen) were added at doses indicated in the text. Plates were pulsed with [^{3}H]thymidine at 66 h and harvested at 72 h. All assays were performed in triplicate. For CFSE dilution assays, purified $CD4^+CD25^-Thy1.1^+$ or $CD4^+CD25^+Thy1.2^+$T cells were incubated in 5 nM CFSE at room temperature for 5 min and washed before culture.

Quantitative RT-PCR

Cells were harvested after 48 h of culture for RNA extraction (TRIzol; Invitrogen), DNase treatment (Invitrogen Life Technologies), and reverse transcription with oligo(dT) and Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies). cDNA was amplified using the iCycler iQ Real-Time PCR system (Bio-Rad) with the following primer and TaqMan probe sequences (Integrated DNA Technologies): IFN-γ, ATGAGTATTGC CAAGTTTGAGGTC, CTTTTCCGCTTCCTGAGGCT, and 6-FAM CCACAGGTCCAGCGCCAAGCATTC-BHQ-1; IL-2, CTACAGCGG AAGCACAGCAG, ATTTGAAGGTGAGCATCCTGGG, and 6-FAM AGCAGCAGCAGCAGCAGCAGCA-BHQ-1; and β-actin, TCTACGA GGGCTATGCTCTCC, CTTTGATGTCACGCACGATTTCC, and 6-FAM-CCTGCGTCTGGACCTGGCTGGC-BHQ-1. Samples were analyzed in triplicate and normalized to β-actin.

Statistical analysis

Lymphoproliferative responses and cytokine mRNA levels were compared using the unpaired Student's *t* test.

Results

IL-12p70 induces proliferative responses and cytokine mRNA accumulation in $CD4^+CD25^+/CD4^+CD25^-$ cocultures

IL-12p40 monokines are important factors in the generation of pathogen-specific as well as autoreactive Th1 cells (1). Other proinflammatory factors have been shown to reverse Treg-mediated immunosuppression (9). We questioned whether IL-12 has a similar effect. To test our hypothesis, we performed standard in vitro suppression assays and added recombinant murine IL-12 to some of the wells. Consistent with published data (9), coculture of Treg with $CD4^+CD25^-$ T responder cells inhibited proliferation of the latter population. The addition of rIL-12 restored proliferative responses even at high Treg:responder cell ratios (Fig. 1, *A* and *B*). Although the effects of IL-12 were dose dependent, significant reversal of suppression was seen at 0.1 ng/ml, a concentration lower than typically used to measure effects of IL-12 in vitro (3) (Fig. 1*C*). CFSE dilution

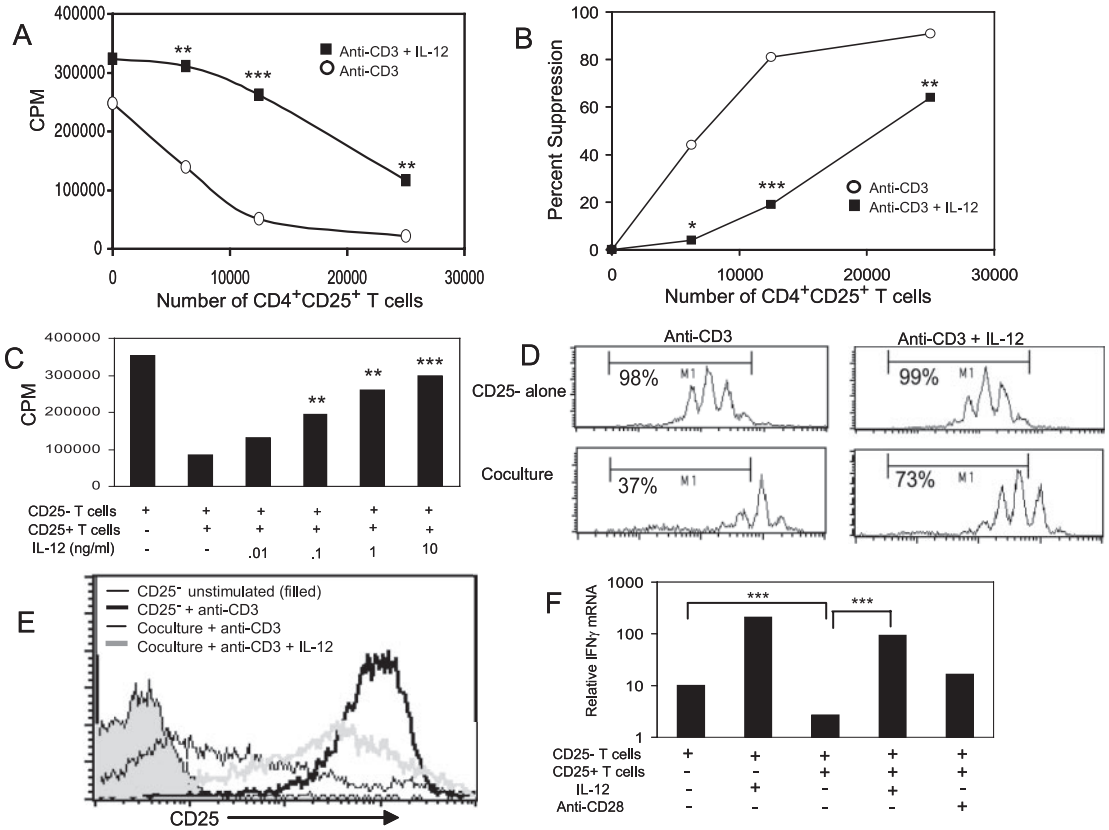

FIGURE 1. IL-12 restores proliferative and cytokine responses to cocultures of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells. *A,* $CD4^+CD25^-$ T cells were cultured for 72 h with syngeneic APC and anti-CD3 (0.5 μg/ml) with or without purified $CD4^+CD25^+$ T cells titered in across a range of ratios. rIL-12 (5 ng/ml) was added to some of the wells and [^{3}H]thymidine incorporation was measured for the final 6 h of culture. All conditions were performed in triplicate. *B,* Percent suppression was calculated by dividing the cpm of $CD25^+/CD25^-$ cocultures by the cpm of $CD25^-$ cells alone and multiplying by 100. *C,* Cocultures of $CD4^+CD25^-$ (5×10^4/well) and $CD4^+CD25^+$ T cells (1.25×10^4/well) were stimulated for 72 h with anti-CD3 in the presence of graded doses of rIL-12. *D,* CFSE-labeled $Thy1.1^+CD4^+CD25^-$ T cells (5×10^4/well) were cultured with $Thy1.2^+CD4^+CD25^+$ T cells (2.5×10^4/well) and APC in the presence of anti-CD3 with or without IL-12 (5 ng/ml) and analyzed by flow cytometry at 96 h. The histograms shown are gated on live $Thy1.1^+CD4^+$ cells. *E,* Cocultures were set up as described in *D*. At 48 h, cells were stained with anti-CD25-PE (BD Pharmingen) and analyzed by flow cytometry gating on live $CD4^+Thy1.1^+$ cells. *F,* Cultures were set up as described in *D*. Anti-CD28 (0.5 μg/ml) was added to some of the wells. Wells from each group were pooled at 48 h for RNA extraction and measurement of IFN-γ mRNA by quantitative real-time RT-PCR. All results shown are representative of at least three separate experiments (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; *A–C*, by comparison to cocultures without exogenous IL-12).

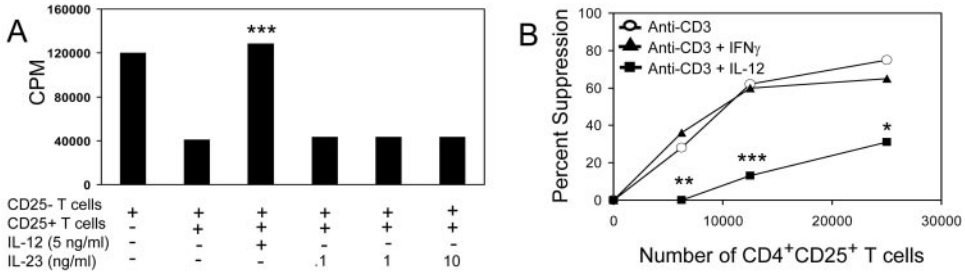

FIGURE 2. The effects of IL-12 on $CD4^+CD25^-$/Treg cocultures are not replicated by IL-23 or IFN-γ. *A,* Cocultures of $CD4^+CD25^-$ (5×10^4/well) and $CD4^+CD25^+$ (2.5×10^4/well) T cells were stimulated for 72 h with APC and anti-CD3 in the presence of rIL-12 or IL-23 at the indicated concentrations. Plates were pulsed with [^{3}H]thymidine for the final 6 h of culture. *B,* In vitro suppression assays were prepared as described in Fig. 1A to compare the effects of rIFN-γ (100 ng/ml) to IL-12 (5 ng/ml). All conditions were performed in triplicate. All results shown are representative of three independent experiments (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ by comparison to cocultures without exogenous cytokines).

studies, in which Treg and responder cells were distinguished by Thy1 congenic alleles, corroborated the results obtained with thymidine incorporation assays and directly demonstrated that $CD4^+CD25^-$ T cells expand in response to IL-12 in the cocultures. In a typical experiment, the percentage of responder cells undergoing multiple divisions in coculture rose from 37 to 73% with the addition of IL-12, approaching the percentage of dividing cells in cultures of $CD25^-$ cells alone (98%; Fig. 1*D*). IL-12 had no effect on proliferation of Treg cultured alone or with responder cells, either in the presence or absence of anti-CD3 (our unpublished data). Furthermore, IL-12 promoted $CD4^+CD25^-$ responses in cocultures as measured by up-regulation of the activation marker CD25 and IFN-γ mRNA accumulation (Fig. 1, *E* and *F*).

Unlike IL-12, neither IL-23 nor IFN-γ induces $CD4^+CD25^-$ T cell activation in cocultures

We next tested whether IL-23, another member of the IL-12p40 family that has overlapping properties with IL-12 (10), could restore proliferative responses to cocultures. Unlike IL-12, IL-23 had no effect on the in vitro suppression assays (Fig. 2*A*).

Many of the biological effects of IL-12 are realized indirectly through induction of IFN-γ (1). This raised the possibility that IFN-γ rather than IL-12 was responsible for restoring proliferative responses in $CD4^+CD25^+/CD4^+CD25^-$ cocultures. However, IFN-γ was unable to replicate the effects of IL-12 in the in vitro suppression assays (Fig. 2*B*).

IL-12 induces proliferation in cocultures by direct action on $CD25^-$ responder cells

The above studies suggest two mechanisms by which IL-12 could achieve its effects in the cocultures. First, the cytokine could signal into Treg and directly abort their suppressive functions. Second, it could directly stimulate $CD4^+CD25^-$ responder cells to resist or circumvent Treg-mediated immunosuppression. To distinguish between those two possibilities, we performed mix and match experiments in which Treg and responder cells were isolated either from wild-type or IL-12R$\beta2^{-/-}$ mice and cocultured in various combinations in the absence or presence of IL-12.

Control experiments in Fig. 3 indicate that there is no intrinsic defect in the biological functions of IL-12R$\beta2^{-/-}$ responder cells or IL-12R$\beta2^{-/-}$ Treg. Therefore, $CD4^+CD25^-$ T cells from IL-12R$\beta2^{-/-}$ mice mounted robust proliferation responses in response to anti-CD3 stimulation but were inhibited by coculture with Treg. Furthermore, IL-12R$\beta2^{-/-}$ $CD4^+CD25^+$ T cells effectively inhibited the proliferation of wild-type as well as IL-12R$\beta2^{-/-}$ responder cells.

Consistent with our earlier results, IL-12 prevented immunosuppression when both Treg and responder cells were derived from wild-type donors, but was ineffective in cocultures consisting exclusively of T cells from IL-12R$\beta2^{-/-}$ donors. When responder cells, but not Treg, express IL-12R$\beta2$, the addition of IL-12 restored proliferation responses. By contrast, IL-12 had no effect on cocultures composed of IL-12R$\beta2^{-/-}$ responder cells with wild-type Treg. The T cell growth factor IL-2 was able to reverse immunosuppression in the same cultures, suggesting that IL-12R$\beta2^{-/-}$ responder cells are capable of proliferation in the presence of Treg under appropriate conditions. Based on these findings, we concluded that, in wild-type cocultures, IL-12 significantly restores proliferation by direct signaling into $CD4^+CD25^-$ T cells, but has no discernible effect on Treg themselves.

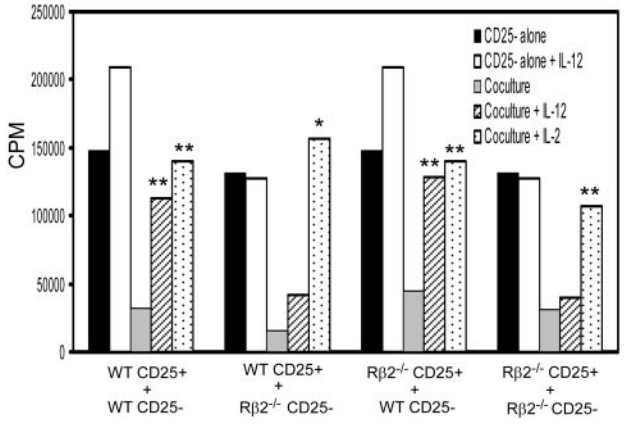

FIGURE 3. IL-12 acts directly on $CD25^-$ responder cells to restore proliferation in the presence of Treg. $CD4^+CD25^-$ (5×10^4/well) and $CD4^+CD25^+$ (2.5×10^4/well) T cells were isolated from either wild-type (WT) or IL-12R$\beta2^{-/-}$ C57BL/6 mice and cocultured in various combinations for 72 h with anti-CD3 and wild-type APC. Recombinant mouse IL-12 (5 ng/ml) or IL-2 (3 ng/ml) was added to some of the wells. Proliferative responses were measured for the final 6 h of culture by [^{3}H]thymidine incorporation. The results shown represent the average of three independent experiments with similar results (*, $p < 0.05$ and **, $p < 0.01$ by comparison to cocultures without exogenous cytokines).

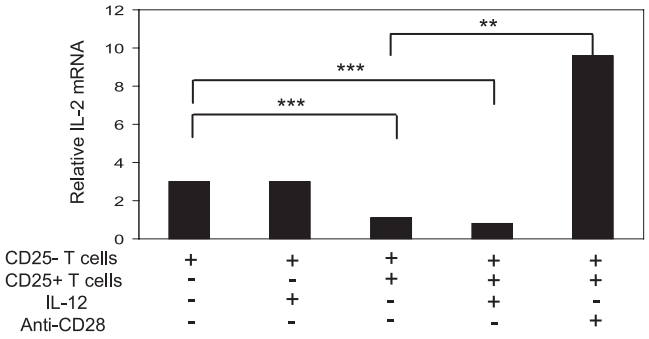

FIGURE 4. IL-12 does not enhance IL-2 mRNA expression in $CD4^+CD25^+/CD4^+CD25^-$ cocultures. Cultures were prepared as described in Fig. 1*F* and analyzed by quantitative PCR for IL-2 transcription following 48 h of culture. Each sample was run in triplicate. These results are representative of three independent experiments with similar results (**, $p < 0.01$; ***, $p < 0.001$).

IL-12 does not enhance IL-2 mRNA expression in cocultures

Treg inhibit IL-2 transcription in $CD4^+CD25^-$ responder cells (11). Although IL-2 levels are enhanced by the addition of anti-CD28 to $CD4^+CD25^+/CD4^+CD25^-$ cocultures, Treg continue to suppress IL-2 transcription in the presence of exogenous IL-2 or IL-4, which, nonetheless, restore proliferation responses. Our mix and match experiments demonstrated that IL-12 exerts its effects in cocultures via direct action on the $CD25^-$, but not the $CD25^+$, subpopulation (Fig. 3). Therefore, it was possible that Treg retain the ability to inhibit IL-2 mRNA in the presence of IL-12, but that responder cells assumed alternative (i.e., IL-2-independent) pathways to undergo clonal expansion. To investigate this possibility, we measured IL-2 mRNA expression in cocultures with or without exogenous IL-12.

Consistent with previous studies (11), IL-2 mRNA expression was significantly inhibited in $CD4^+CD25^+/CD4^+CD25^-$ cocultures in comparison to anti-CD3 stimulated $CD4^+CD25^-$ cells alone. Although anti-CD28 restored IL-2 mRNA production in the cocultures, IL-12 failed to do so at a concentration that we previously showed to be effective in stimulating responder cell proliferation (Fig. 4). Similar results were obtained when cells were harvested for RNA extraction at 8, 16, 24, 48, and 72 h from the initiation of cultures (data not shown).

Discussion

The ultimate outcome of T cell responses following in vivo challenge with foreign as well as self-Ags depends on an intricate interplay between proinflammatory and immunoregulatory pathways. Treg act to prevent spontaneous autoimmunity and to limit collateral damage to healthy tissues during adaptive immunity. However, these cells also have the potential to sabotage protective antimicrobial responses. Therefore, as stated by Powrie and Maloy (8), the regulators must be regulated themselves to ensure that the host will be able to mount appropriate immune responses while minimizing the risk of destructive autoimmunity.

There is growing evidence that Treg activity is regulated by proinflammatory mediators. For example, CpG oligonucleotides and LPS stimulate dendritic cells to inhibit immunosuppression by Treg through an IL-6-dependent pathway (12). In this study, we expand upon those observations by demonstrating that IL-12 also restores $CD4^+CD25^-$ T cell activation in the presence of Treg. Unlike other soluble and costimulatory factors such as IL-2, IL-4, and anti-GITR (9, 11, 13), IL-12 does not appear to break the anergic state of Treg. Therefore, the disruptive effects of IL-12 on Treg-mediated immunosuppression are not undermined by paradoxical effects on Treg expansion or function.

Collectively the data indicates that, in the context of an inflamed microenvironment, "danger" signals conspire to block or circumvent immunoregulatory pathways, further amplifying their role in boosting effector cell activation/differentiation. Our studies indicate that the Th1-polarizing factor IL-12 can directly contribute to this process. In fact, IL-12 and CpG oligonucleotides, administered at suboptimal doses, act synergistically to restore proliferative responses in cocultures (our unpublished observations), suggesting that they could have physiological effects even at relatively low concentrations when present together in vivo. Experiments in mouse models of *Leishmania* and malaria indicate that, during infection, activation of antimicrobial T cells within mixed Treg/effector cell populations by IL-12 and TLR ligands could translate into more efficient and rapid clearance of pathogens (14, 15). In contrast, during autoimmunity, such a phenomenon is likely to result in more severe and persistent tissue destruction.

The mechanism of action of IL-12 in restoring $CD4^+CD25^-$ T cell activation in the presence of Treg remains to be fully elucidated. Our mix and match experiments with IL-12R-deficient and wild-type donors indicate that the effects of IL-12 during in vitro suppression assays are realized solely through direct action on $CD25^-$ cells (Fig. 3). IL-12 resembles other cytokines that reverse immunosuppression (i.e., IL-2 and IL-4) in that it does not restore IL-2 transcription in $CD25^-$ cells. In contrast, IL-12 induces expression of the IL-2R α-chain on responder cells (Fig. 1*E*) and therefore could lower the threshold for IL-2-driven T cell activation. However, this in and of itself is unlikely to account for the activity of IL-12 in our experiments, since we have found that the effects of IL-2 and IL-12 in cocultures are additive rather than synergistic (our unpublished observations).

It was recently shown that the function of Treg cells requires the direct action of TGF-β on $CD25^-$ cells and that in the absence of this interaction, $CD25^-$ cells escape Treg-mediated control (16). Therefore, in future experiments we plan to investigate whether IL-12 blocks TGF-β signaling in responder cells by modulating the expression of TGF-β receptors or molecules that act downstream in the Smad pathway. Another recent study showed that suppression mediated by Treg appears to be realized, in part, by the induction of apoptosis of $CD4^+CD25^-$ effector cells via a Granzyme B-dependent mechanism (17). Therefore, we will also investigate whether IL-12 up-regulates antiapoptotic molecules, such as Bcl-2, in responder cells.

In summary, our studies suggest that, in addition to directly promoting Th1 differentiation, IL-12 can act alone or in conjunction with TLR ligands and other growth factors to restore T cell activation and effector functions in the presence of Treg. In addition, we show that IL-12 has biological properties of potential clinical importance that are not shared by IL-23. Therapeutic modalities that target the common IL-12p40 chain for the treatment of autoimmune diseases might be more effective

than those that target the unique IL-23p19 subunit by rendering autoreactive T cells more vulnerable to regulation by $CD4^+CD25^+$ T cells.

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Disclosures

The authors have no financial conflict of interest.

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