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CUTTING EDGE

Cutting Edge: *Trans*-Signaling via the Soluble IL-6R Abrogates the Induction of FoxP3 in Naive $CD4^+CD25^-$ T Cells[1]

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Chronic inflammatory diseases may develop when regulatory T cells (Tregs) fail to control the balance between tolerance and immunity. Alternatively, activated immune cells might prevent the induction or activation of Tregs in such diseases. In this study, we demonstrate that* trans*-signaling into T cells via the soluble IL-6 receptor completely abrogates the de novo induction of adaptive Tregs. Mechanistically, IL-6* trans*-signaling augmented the expression of the TGF-β signaling inhibitor SMAD7. Consequently, SMAD7 overexpression in T cells using newly created transgenic mice rendered $CD4^+CD25^-$ T cells resistant to the induction of FoxP3. Finally, IL-6* trans*-signaling inhibited Treg-mediated suppression in a murine model of colitis. In summary, IL-6* trans*-signaling into T cells emerges as a key pathway for blockade of the development of adaptive Tregs and thus may play a pivotal role in shifting the balance between effector and regulatory T cell numbers in chronic inflammatory and autoimmune diseases. *The Journal of Immunology,* 2007, 179: 2041–2045.

Increasing evidence supports a role for adaptive regulatory T cells (Tregs)[5] in the control of inflammatory and autoimmune inflammation (1, 2). Recently, TGF-β signaling was identified as a potent stimulus directing the development of $CD4^+CD25^+$ Tregs from naive $CD25^-$ T cells in the periphery (3, 4). Such TGF-β-induced adaptive Tregs share most if not all properties with conventional Tregs developing in the thymus and are able to control inflammatory immune responses in vivo (3, 5). Therefore, factors that modulate the induction of Tregs in vivo are potentially interesting for the development of future therapeutic strategies aiming at restoring the balance between immunity and counter-regulatory systems.

The cytokine IL-6 is a pleiotropic cytokine with a broad range of functions on immune and nonimmune cells (for a review, see Ref. 6). Functional studies have demonstrated both proinflammatory and anti-inflammatory roles of IL-6 signaling. Classic signaling of IL-6 involves the binding of IL-6 to target cells bearing the membrane-bound receptor IL-6R. Alternatively, IL-6 can activate cells lacking the membrane-bound IL-6R when bound to a naturally occurring soluble form of the IL-6 receptor (sIL-6R) in a process called IL-6 *trans*-signaling (7). Interestingly, the soluble IL-6 receptor is produced in large amounts in patients suffering from chronic inflammatory disorders such as inflammatory bowel disease (8). Its function in these diseases is largely unknown, although experimental evidence supports a role for the sIL-6R in inducing T cell resistance against apoptosis (9). In the present article, we demonstrate for the first time that *trans*-signaling into T cells via the sIL-6R blocks tolerance induction by interfering with the development of adaptive Tregs.

Materials and Methods

Mice

Six- to 8-wk old BALB/c, SCID, and C57BL/6 mice were obtained from the animal facility of the University of Mainz (Mainz, Germany). For CD2-SMAD7 transgenic mice the SMAD7 cDNA was cloned into a vector containing a CD2 promoter/enhancer fragment. The resulting CD2-SMAD7 DNA fragment was then microinjected into the pronuclei of fertilized eggs of C57BL/6 mice. Founder mice were bred separately to generate F_1 progeny, resulting in independent lines. Transgenic integration was confirmed by a PCR of genomic DNA. Mice transgenic for the hemagglutinin (HA)-specific TCR (HA-TCRtg/$RAG1^{-/-}$) were described previously (10). All animal experiments were in accordance with the local institutional guidelines.

Isolation and culture of primary cells

$CD4^+CD25^-$ T cells were isolated from murine splenocytes by CD4-MACS negative selection followed by CD25-MACS positive selection (Miltenyi Biotech) and Treg cells were induced as previously described (4). In brief, $CD4^+CD25^-$ T cells were cultured in serum-free medium in the presence of

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[5] Abbreviations used in this paper: Treg, regulatory T cell; HA, hemagglutinin; HA-TCRtg/RAG, transgenic for HA-specific TCR (mice); HIL-6, Hyper-IL-6; HPRT, hypoxanthine phosphoribosyltransferase; iTreg, induced Treg; sIL-6R, soluble IL-6R.

plate-bound anti-CD3 (10 μg/ml), soluble anti-CD28 (2 mg/ml), and recombinant TGF-β1 (5 ng/ml). Furthermore Hyper-IL-6 (HIL-6) and IL-6 were used as indicated in the figure legends. T cells from HA-TCRtg/RAG mice were stimulated with mitomycin (50 μg/ml)-treated A20 cells at a ratio of 2:1 and HA-peptide at 2 ng/ml.

Flow cytometry analysis

Cells were stained for CD4 (BD Biosciences), CD8 (BD Biosciences), FoxP3 (eBioscience), or Caspase 3 (BD Biosciences) according to the manufacturer's instructions. Cells were analyzed by means of a FASCalibur flow cytometer (BD Biosciences).

Transfer colitis

SCID mice were i.p. administered freshly isolated $CD4^+CD25^-$ T cells from BALB/c mice (5×10^5) in the presence or absence of $CD4^+CD25^-$ T cells (5×10^5) stimulated for 5 days with anti-CD3, anti-CD28 Abs, and recombinant TGF-β1 with or without HIL-6. For the monitoring of colitis, the COLOVIEW high-resolution mouse video endoscopic system (Karl-Storz) was used as previously described (11).

Real-time quantitative PCR

Total RNA was isolated using the RNA micro kit (Qiagen). Reverse transcription into complementary DNA was performed with the Stratascript II reverse transcriptase (Invitrogen Life Technologies). PCR was performed using a TaqMan-based assay for Foxp3, SMAD-7, and hypoxanthine phosphoribosyltransferase (HPRT) (Applied Biosystems) and the ABsolute quantitative PCR master mix (ABgene) on the iCycler iQ system (Bio-Rad). FoxP3 and SMAD-7 expressions was calculated relative to the housekeeping gene *HPRT* using the delta-delta threshold cycle ($\Delta\Delta$Ct) algorithm.

Immunohistochemistry

Immunofluorescence of cryosections or cytospins was performed using the TSA Cy3 system (PerkinElmer) and a fluorescence microscope (Olympus) as described (12) using a primary Abs specific for CD4 (Santa Cruz Biotechnology).

CFSE proliferation assay

$CD4^+CD25^-$ T cells were labeled with CellTrace (Invitrogen Life Technologies) CFSE according to the manufacturer's instructions. CFSE-labeled $CD4^+CD25^-$ responder cells were cocultured at different ratios with cells preactivated for 5 days with TGF-β alone or with the addition of either IL-6 or HIL-6 and mitomycin C (Sigma-Aldrich)-treated autologous splenocytes as APCs. The proliferation of responder cells was evaluated on the basis of the CFSE dilution evaluated by flow cytometry 3 days after the setup of the coculture.

Results and Discussion

IL-6 trans-signaling via the soluble IL-6 receptor blocks the induction of FoxP3$^+$ Tregs from naive CD4$^+$CD25$^-$ T cells

Chronic inflammatory diseases such as inflammatory bowel disease are characterized by a dysregulated immune response and an excessive release of proinflammatory cytokines such as IL-6. IL-6 has recently been described as interfering with the generation and suppressive function of Tregs, suggesting that the binding of IL-6 to the membrane-bound receptor on T cells leads to a break of tolerance. Interestingly, recent data suggested that signaling via the sIL-6R can perpetuate inflammation in murine models of inflammatory bowel disease (9, 13). Given these data, we investigated whether IL-6 *trans*-signaling may block Treg cell generation. Accordingly, IL-6 or HIL-6, a recombinant molecule composed of the extracellular part of the IL6R fused to IL-6 that is able to induce *trans*-signaling in target cells (7), was added during TGF-β induced Treg generation in vitro. In agreement with previous reports (14), the addition of high amounts of IL-6 to the culture medium resulted in a partial inhibition of FoxP3 induction. In contrast, HIL-6-induced IL-6 *trans*-signaling almost completely abolished the development of FoxP3 cells from $CD4^+CD25^-$ cells (Fig. 1*A*). HIL-6 mediated suppression of FoxP3 was confirmed at the mRNA level (Fig. 1*B*) and occurred dose-dependently (Fig. 1*C*). To investigate whether IL-6 *trans*-signaling also interferes with the Ag-specific induction of Tregs, we took advantage of mice expressing a transgenic TCR specific for influenza HA on a

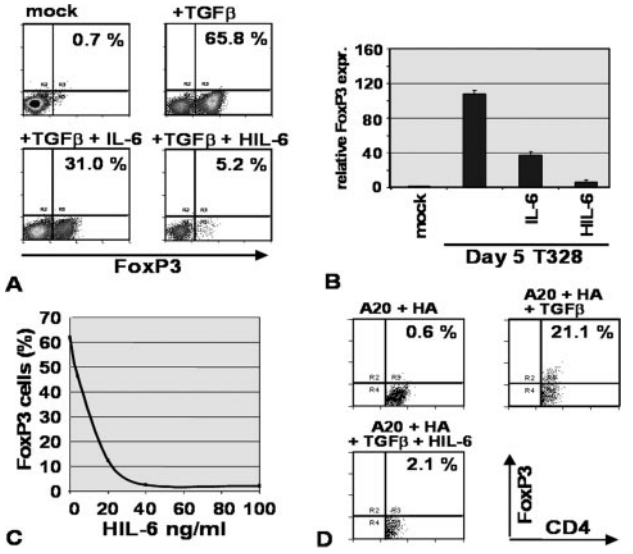

FIGURE 1. IL-6 *trans*-signaling blocks the induction of FoxP3 in naive $CD4^+CD25^-$ T cells. *A*, Freshly isolated $CD4^+CD25^-$ T cells were stimulated for 5 days with anti-CD3/anti-CD28 and TGF-β. Some samples were additionally treated with IL-6 or HIL-6. On day 5 cells were stained for FoxP3 and analyzed by flow cytometry. *B*, Cells treated as described previously (*A*) were lysed and the expression of FoxP3 mRNA relative to HPRT was measured by quantitative PCR. *C*, $CD4^+CD25^-$ T cells were stimulated for 5 days with anti-CD3/anti-CD28, TGF-β, and different concentrations of HIL-6. On day 5 cells were stained for FoxP3 and analyzed by flow cytometry. *D*, $CD4^+CD25^-$ T cells of HA-TCRtg/RAG mice were cultured for 5 days with mitomycin-treated A20 cells at a ratio of 2:1 in the presence of HA-peptide with or without TGF-β with or without HIL-6. Cells were analyzed for CD4 and FoxP3 expression using flow cytometry.

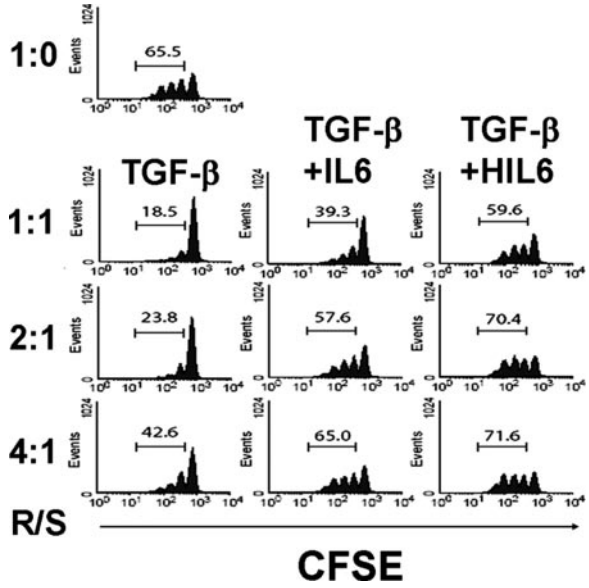

FIGURE 2. IL-6 *trans*-signaling blocks the suppressive capacity of TGF-β-induced cells. CFSE-labeled $CD4^+CD25^-$ responder cells were cocultured at different ratios with cells preactivated for 5 days with TGF-β alone or with either IL-6 or HIL-6 and mitomycin C-treated autologous splenocytes. Proliferation of responder cells was evaluated on the base of the CFSE dilution pattern evaluated by flow cytometry 3 days upon the setup of the coculture. S, Suppressor cells; R, responder cells.

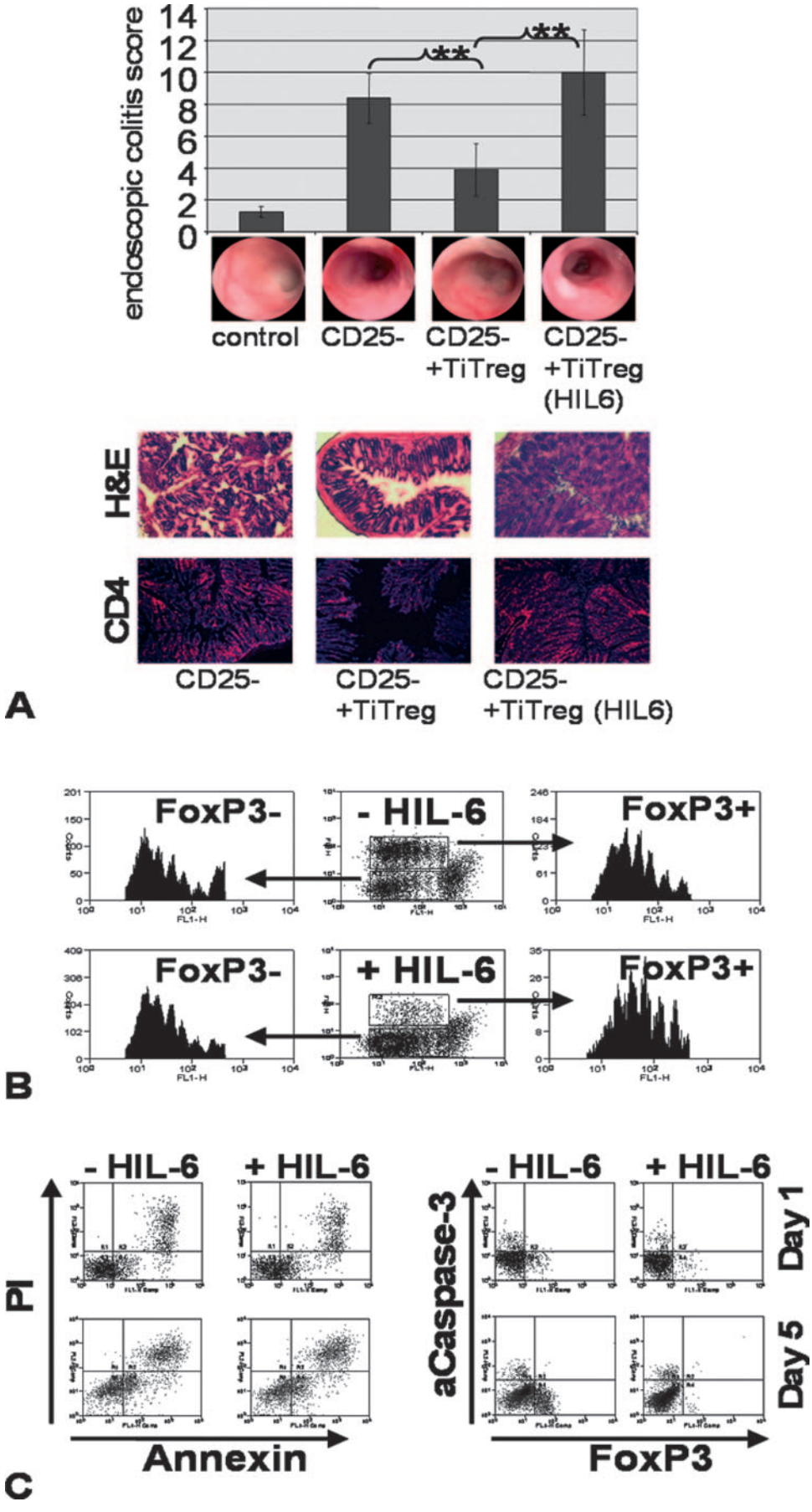

FIGURE 3. IL-6 *trans*-signaling blocks in vivo suppression of colitis. *A*, freshly isolated $CD4^+CD25^-$ T cells (5×10^5) from BALB/c mice were i.p. injected into SCID mice either alone or in combination with 5×10^5 cells stimulated for 5 days with plate-bound anti-CD3 and soluble anti-CD28 plus TGF-β with or without HIL-6. After 4 wk, the colitis score was detected. *B*, CFSE-labeled $CD4^+CD25^-$ T cells from BALB/c mice were stimulated for 5 days with anti-CD3/anti-CD28 in the presence of TGF-β alone or additionally with HIL-6. On day 5 cells were harvested and analyzed by flow cytometry. *C*, $CD4^+CD25^-$ T cells from BALB/c mice were stimulated for 5 days with anti-CD3/anti-CD28 in the presence of TGF-β or TGF-β plus HIL-6. On days 1 and 5 cells were harvested and stained for annexin-FITC and propidium iodide or FoxP3-PE and caspase-FITC.

RAG-deficient background (10). The T cell repertoire in the peripheral blood of these mice is characterized by the presence of T cells specific for a peptide from HA (HA-TCRtg/RAG mice) that exclusively show a naive phenotype. Using naive $CD4^+CD25^-$ cells isolated from HA-TCRtg/RAG mice and consistent with data obtained with wild-type cells, HIL-6 blocked FoxP3 induction when Tregs were induced with TGF-β plus HA peptide-loaded accessory cells (A20 cells), providing evidence that IL-6 *trans*-signaling can block Treg development in an Ag-specific setting (Fig. 1*D*).

To confirm that the blockade of FoxP3 induction by IL-6 *trans*-signaling correlates with a loss of suppressive activity, cell cultures obtained after 5 days of activation in the presence of TGF-β either alone or plus IL-6 or HIL-6 were challenged in a coculture suppressive assay based on CFSE dilution and measured by flow cytometry. As shown in Fig. 2 at different suppressor/responder ratios, cells preactivated in the presence of TGF-β (TGF-β-induced Tregs) strongly suppressed proliferation of $CD4^+CD25^-$ responder cells. Cells preactivated in the presence of TGF-β and IL-6 showed a partial reduction of the suppressive capacity when compared with Tregs induced by TGF-β alone. However, Treg cultures preactivated in the presence of TGF-β and HIL-6 showed almost no inhibition of responder cell proliferation. Thus, FoxP3 suppression by *trans*-signaling via the sIL-6R is accompanied by a loss of suppressive activity.

IL-6 trans-signaling prevents protection of induced Tregs (iTregs) in experimental colitis

Recent data have shown that iTregs can prevent colitis induction in an experimental model of colitis (5). To investigate whether iTregs induced in the presence of HIL-6 loose their ability to protect mice from colitis, we transferred freshly isolated colitogenic $CD4^+CD25^-$ cells with or without cells stimulated in the presence or absence of HIL-6. Mice treated with $CD4^+CD25^-$ cells only developed severe colitis after 3 wk (Fig. 3*A*). In contrast, mice coinjected additionally with iTregs generated in the absence of HIL-6 were significantly protected as indicated by reduced endoscopic colitis scores and infiltration of immune cells into the gut mucosa. Strikingly, mice cotransferred with cells that were precultured in the presence of HIL-6 were not protected and developed severe colitis similar to or even worse than that in mice treated with colitogenic cells only. In summary, our data show that *trans*-signaling via the sIL-6R efficiently abrogates the TGF-β-dependent de novo induction of Tregs.

We next investigated the molecular mechanism by which IL-6 *trans*-signaling might block FoxP3 induction in $CD4^+CD25^-$ cells. To rule out the possibility that selective proliferation or cell death within the $FoxP3^+$ or $FoxP3^-$ population could account for the diminished iTreg induction, we analyzed our cultures by flow cytometry. As shown in Fig. 3*B*, in the presence of HIL-6 the amount of $FoxP3^+$ cells was greatly diminished. The cells, no matter whether $FoxP3^+$ or $FoxP^-$, proliferated as strongly as in the cultures lacking HIL-6, suggesting that HIL-6 does not lead to a selective proliferation of T cell cultures.

To further rule out selective cell death of $FoxP3^+$-induced cells, we performed annexin and propidium iodide combined stainings. In addition, we investigated the activation of caspase-3 in the $FoxP3^+$ and $FoxP3^-$ populations by flow cytometry to analyze early induction of apoptotic pathways. As shown in Fig. 3*C*, no difference was found in the amount of apoptotic cells in the HIL-6-treated cultures on day 5 compared with control cultures without HIL-6. More importantly, active caspase-3-positive cells were present in small amounts in the $FoxP3^-$ but not the $FoxP3^+$ population. Therefore, we concluded that the diminished number of $Foxp3^+$ T cells in the culture treated with HIL-6 is not due to selective apoptosis of the $FoxP3^+$ cells.

SMAD7 hyperinduced by IL-6 trans-signaling suppresses $FoxP3^+$ Treg development

In mice engineered to express a mutated form of gp130 that resulted in hyperactivation of the STAT3 pathway, SMAD7, an endogenous inhibitor of TGF-β signaling, was strongly up-regulated (15). In addition, we have recently shown that TGF-β-induced Tregs show a diminished capacity to up-regulate SMAD7, and SMAD7 has furthermore been shown to be expressed at very low levels in naturally occurring Tregs (4, 16), suggesting a negative regulatory role of this molecule in Treg development. Given these data, we investigated whether HIL-6 could regulate SMAD7 expression in $CD4^+CD25^-$ T cells. As shown in Fig. 4*A*, low expression of FoxP3 in $CD4^+CD25^-$ T cells treated with HIL-6 plus TGF-β correlated with the up-regulation of SMAD7 mRNA, suggesting that HIL-6 may elevate the threshold of TGF-β responsiveness in naive T cells. To further demonstrate that elevated levels of SMAD7 interfere with TGF-β-induced Treg development from naive $CD4^+CD25^-$ cells, we generated mice transgenic for SMAD7

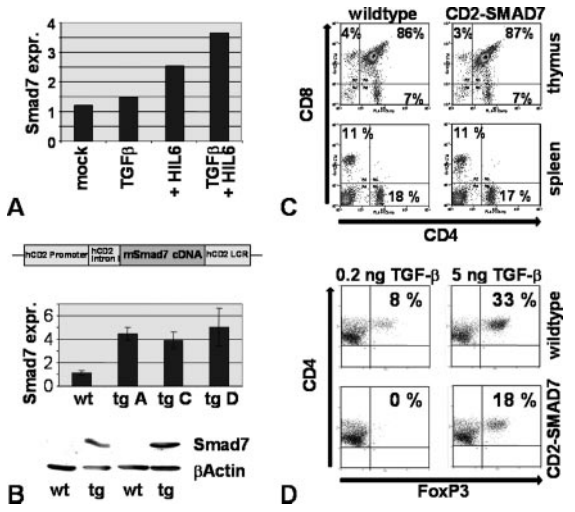

FIGURE 4. IL-6 *trans*-signaling up-regulates the TGF-β signaling inhibitor SMAD7. *A*, $CD4^+CD25^-$ T cells from BALB/c mice were stimulated for 5 days with anti-CD3/anti-CD28 in the presence or absence of TGF-β and/or HIL-6. On day 5 cells were harvested and lysed and the mRNA was isolated and reverse transcribed into cDNA. Expression (expr.) of SMAD7 mRNA relative to control HPRT was measured by quantitative PCR. *B*, Generation of CD2-SMAD7 transgenic (tg) mice. To confirm overexpression, T cells from founder lines were analyzed for SMAD7 expression by quantitative PCR or Western blotting. wt, Wild type. *C*, Freshly isolated thymocytes and splenocytes from wild-type or CD2-SMAD7-transgenic mice were double-stained for CD8-PE and CD4-FITC and analyzed by flow cytometry. *D*, Isolated $CD4^+CD25^-$ T cells from wild-type or CD2-SMAD7-transgenic mice were stimulated for 5 days with plate-bound anti-CD3 and soluble anti-CD28 plus TGF-β in two different concentrations. At day 5 cells were double-stained for CD4-PE and FoxP3-FITC and analyzed by flow cytometry.

under control of a CD2 minigene. Such mice showed strong expression of SMAD7 in T cells even in the absence of TGF-β stimulation (Fig. 4*B*). To investigate whether T cell development is influenced in these mice, we performed flow cytometric analysis of wild-type and SMAD7-transgenic animals. Accordingly, thymocytes and splenocytes were analyzed for CD4 and CD8. No significant difference was found in the T cell populations, indicating that T cell development was not impaired in SMAD7-transgenic mice (Fig. 4*C*). When $CD4^+CD25^-$ cells were isolated from CD2-SMAD7-transgenic mice and cultured in the presence of antiCD3/CD28 and TGF-β, we observed a reduced conversion of naive T cells into $CD4^+FoxP3^+$ Tregs, indicating that SMAD7, induced by IL-6 *trans*-signaling, is indeed a strong inhibitor of peripheral Treg cell development (Fig. 4*D*).

In summary, our data provide strong evidence that IL-6 *trans*-signaling via the sIL-6R provides a potent inhibitory pathway of adaptive Treg development and subsequent tolerance induction in vivo. Because IL-6 has recently been described to interfere with the suppressive function of naturally occurring, thymus-derived Tregs (17), IL-6 signaling emerges as a key regulator of Treg biology at multiple levels and IL-6 emerges as a key cytokine for the modulation of the ratio between effector T cells and Tregs in autoimmune and chronic inflammatory diseases.

Our findings are in agreement with data published recently on the development of IL-17-expressing T cells (Th-17 or Th-IL17). In these reports the authors showed that TGF-β signaling in the presence of inflammatory cytokines such as IL-6, IL-1β, and TNF-α supports the generation of Th-17 cells (18) while suppressing the induction of TGF-β-induced $FoxP3^+$ cells (15). Consistent with these observations, in cultures of cells stimulated by TGF-β and via IL-6 *trans*-signaling we also observed a transient expression of IL-17 (data not shown). Therefore, our data extend this view by demonstrating that *trans*-signaling via the sIL-6R is needed to provide a strong blockade of adaptive Treg development and tolerance induction in vivo. Because sIL-6R concentrations are elevated in patients suffering from chronic inflammatory diseases (8) and the sIL6R has a pathogenic role in murine models of inflammatory bowel disease (9, 14), our data support an important role for IL-6 *trans*-signaling in the perpetuation of chronic inflammatory diseases in humans. IL-6 *trans*-signaling may thus be a promising target in future immunotherapies targeting the immunological balance. It is noteworthy that IL-6 trans-signaling can be inhibited by a soluble form of gp130, which leaves intact IL-6 signaling via the membrane-bound IL-6R and has been shown to block the disease state in animal models of inflammatory bowel disease (9, 14).

A question arising from these findings is how Tregs can in fact reverse inflammation in mice with ongoing inflammation that are presumably producing IL-6. One may speculate that the de novo induction of Tregs may take place in tissue not inflamed or that established Tregs may be able to modulate their responsiveness toward IL-6. More investigation will be needed to clarify this issue.

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Disclosures

The authors have no financial conflict of interest.

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