Smad3 Differentially Regulates the Induction of Regulatory and Inflammatory T Cell Differentiation*

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†† The abbreviations used are: Th, T helper; TGF-β, transforming growth factor β; TGF-βRII, TGF-β receptor I; IL, interleukin; IFN, interferon; iTreg, inducible regulatory T cells; nTreg, natural Treg; WT, wild type; KO, knockout; GFP, green fluorescent protein; ELISA, enzyme-linked immunosorbent assay; CFA, complete Freund’s adjuvant; RT-PCR, reverse transcription-PCR; FACS, fluorescence-activated cell sorter; ROR, retinoid acid receptor-related orphan receptor; MOG, myelin oligodendrocyte glycoprotein.

Transforming growth factor β (TGF-β) is a crucial cytokine with pleiotropic functions on immune cells. In CD4+ T cells, TGF-β is required for induction of both regulatory T and Th17 cells. However, the molecular mechanism underlying this differential T cell fate decision remains unclear. In this study, we have evaluated the role of Smad3 in the development of Th17 and regulatory T cells. Smad3 was found to be dispensable for natural regulatory T cell function. However, induction of Foxp3 expression by TGF-β in naive T cells was significantly reduced in the absence of this molecule. On the contrary, Smad3 deficiency led to enhanced Th17 differentiation in vitro and in vivo. Moreover, Smad3 was found to interact with retinoid acid receptor-related orphan receptor γt (RORγt) and decrease its transcriptional activity. These results demonstrate that Smad3 is differentially involved in the reciprocal regulatory and inflammatory T cell generation.

Naive CD4+ T helper (Th)5 cells, upon activation, differentiate into effector cells that are characterized by their distinct cytokine production and immune regulatory functions. Under the influence of TGF-β, Foxp3 can be induced in activated T cells, leading to the generation of inducible regulatory T cells (iTregs) (1). In addition, TGF-β is also involved in the differentiation of Th17 cells, which require TGF-β and IL-6 or IL-21 (2, 3). Thus, there is not only functional antagonism but also reciprocal regulation in the generation of Th17 and iTreg cells (4, 5).

Upon binding to its receptor, TGF-β induces phosphorylation of Smad2/3 molecules, which then bind to the common Smad-Smad4, and the Smad complex accumulates in the nucleus to induce/repress the transcription of TGF-β target genes (6, 7). Interestingly, Smad2/3-dependent Smad4-independent signaling pathways have been described (8, 9).

The signaling mechanism by which TGF-β regulates iTreg and Th17 differentiation has not been clear. We found that inhibition of TGF-β receptor I (TGF-βRI) activity blocked both iTreg and Th17 differentiation (5). Moreover, deletion of Smad4 in T cells resulted in a partial defect in iTreg cell development without affecting Th17 differentiation (5). Whether distinct signaling components of TGF-β receptor differentially regulate iTreg and Th17 cell development has not been understood. In the present study, we have determined the role of Smad3 in the induction of regulatory T cells and Th17 cells. Although Smad3 is required for optimal induction of iTreg cells, it appears to negatively regulate Th17 cell differentiation, possibly by direct binding to RORγt. These results thus contribute to our understanding of the molecular antagonism of Th17 and regulatory T cells genetic programs.

EXPERIMENTAL PROCEDURES

Mice—C57BL/6 and Rag1-deficient mice were purchased from The Jackson Laboratory. Smad3 knock-out (KO) mice were kindly provided by Dr. Xiao-Fan Wang (10). Mice were housed in the specific pathogen-free animal facility at M. D. Anderson Cancer Center, and the animal experiments were performed at the age of 6–10 weeks using protocols approved by the Institutional Animal Care and Use Committee.

Cell Differentiation—CD4+CD25−CD62L−CD44hi naive T cells were FACSorted and stimulated as described (5) with plate-bound anti-CD3 (2.5 μg/ml, BD Biosciences) and anti-CD28 (2.5 μg/ml, BD Biosciences) and in the presence or absence of IL-2 (50 units/ml), 5 ng/ml TGF-β (Peprotech), 30 ng/ml IL-6 (Peprotech), 50 ng/ml IL-23 (R&D Systems), 10 μg/ml anti-IL-4 (11B11), 10 μg/ml anti-IFN-γ (XMG 1.2), 10 ng/ml IL-1β or IL-1α (Peprotech), or combination of these stimuli. 4 days after activation, cells were washed and restimulated with phorbol 12-myristate 13-acetate and ionomycin in the presence of Golgi-stop for 5 h, after which Foxp3-, IL-17-, and IFN-γ-producing cells were analyzed using intracellular staining. Intracellular staining for Foxp3 was performed by using a Foxp3 staining kit (eBioscience). Also, after differentiation, cells were restimulated with anti-CD3 overnight and cytokines were measured in the cell-free culture supernatants by ELISA.

Quantitative Real-time PCR—Total RNA was prepared from T cells using TRIzol reagent (Invitrogen). cDNA was synthesized using Superscript reverse transcriptase and oligo(dT) primers (Invitrogen), and gene expression was examined with a
Bio-Rad iCycler optical system using iQ™ SYBR green realtime PCR kit (Bio-Rad Laboratories, Inc.). The data were normalized to Actb reference. The primers for IL-17, IL-17F, IL-21, IL-22, CCR6, CCL20, IL-23R, RORγt, RORγt, IFI4, AHR, and β-actin were previously described (5, 11, 13).

**MOG Immunization** — Female Rag1 KO mice were reconstituted with T cell-depleted bone marrow cells from Smad3 KO or wild-type (WT) littermates. 8 weeks later, mice were immunized subcutaneously at the dorsal flanks with 150 μg of MOG35–55 peptide emulsified in CFA. 7 days later, cells from spleens and draining lymph nodes of the immunized mice were isolated and restimulated with MOG for 3 days, and cytokine production was determined in the culture supernatant by ELISA.

**RORE Reporter Assay** — RORγt (5), Smad3 2SD, and TGF-βRI T202D (12) were cloned into bicistronic retroviral vector pGFP-RV provided by Dr. Ken Murphy at Washington University (14) that contains IRES-regulated GFP. 293 T cells were co-transfected with 3 μg of the (RORE3-Luc reporter in the presence or absence of the indicated pGFP vectors. Cells were incubated for 16 h with complete medium and then for 24 h with 0.5% fetal bovine serum-containing medium. Then luciferase activity was analyzed with a Dual-Luciferase kit (Promega). Transfection efficiency was normalized by assayed with a Dual-Luciferase kit (Promega). Transfection efficiency was normalized by the absence of the indicated pGFP vectors. Cells were incubated for 8 h. A representative of two independent experiments is shown. Error bars indicate S.D. in panels C and E.

**RESULTS AND DISCUSSION**

**Smad3 Regulates Foxp3 Induction in Naive T Cells** — To better understand the role of the TGF-β signaling pathway in the induction of Th17 and Treg cell differentiation, we utilized mice deficient in Smad3 (10). These mice exhibit normal CD4+ and CD8+ T cells levels in vivo (data not shown). Similar to mice with a deletion of Smad4 in T cells (5), Smad3 KO mice exhibited normal numbers of CD4+ CD25+ Foxp3+ natural Treg (nTreg) cells in spleen, peripheral lymph nodes, and mesenteric lymph nodes (Fig. 1, A and B). However, Smad3-deficient mice also displayed a significant decrease of Treg cells in thymus. When their functions were assessed, peripheral nTregs from Smad3-deficient mice were as suppressive as WT nTreg cells (Fig. 1C). Thus, these results indicate that Smad3 is not required for nTreg cell suppressive activity.

We then examined whether Smad3 is necessary for the induction of iTreg cells. Naïve T cells were isolated from Smad3 KO or WT mice by FACS sorting and stimulated with plate-bound anti-CD3 and anti-CD28 in the presence of TGFβ. Smad3-deficient naive T cells had a profound defect on Foxp3 expression upon iTreg induction (Fig. 1, D and E). Moreover, induction of Treg-associated genes such as GPR83 and Ecm1 were also affected in Smad3-deficient T cells when compared with WT counterparts (Fig. 1E). Thus, these results indicate that Smad3 regulates the induction of Foxp3 and iTreg-associated genes.

**Increased Th17 Cell Differentiation in the Absence of Smad3** — Because TGF-β also regulates the differentiation of Th17 cells, we addressed the role of Smad3 in the generation of Th17 cells. When naïve cells were activated in the presence of TGF-β and IL-6, enhanced IL-17-producing cells were detected in Smad3-deficient cells (Fig. 2A). Gene expression profile analysis by real-time RT-PCR indicated an increase not only in IL-17A but also in IL-17F, CCR6, and CCL20 mRNA levels in Smad3-deficient cells when compared with WT counterparts (Fig. 2B and data not shown). Interestingly, no difference was observed in IL-21 or IL-22 expression, supporting that they are not regulated by TGF-β. Furthermore, when cells were restimulated with anti-CD3, enhanced IL-17 and IL-17F but not IL-21 cyto...
kine production was observed (data not shown). Moreover, a slight increase in RORγt/H9251 and a decrease in AHR and IRF4, but no significant change in RORγt/H9253, were detected in Smad3-deficient Th17 cells when compared with WT cells (Fig. 2B).

We have recently demonstrated that IL-1 in combination with IL-6 and IL-23 is able to initiate Th17 genetic programming. However, this Th17-polarizing condition still requires endogenous, low levels of TGF-β/H9252 (11). To evaluate whether Smad3 deficiency would affect Th17 differentiation under low TGF-β concentrations, naive T cells were stimulated with anti-CD3 and anti-CD28 in the presence of two Th17-polarizing conditions. Interestingly, although TGF-β and IL-6 stimulation led to increased Th17 differentiation in Smad3-deficient cells, the combination of IL-1, IL-6, and IL-23 stimulation induced similar numbers of IL-17-producing cells in both WT and KO T cells (Fig. 2, A and B), suggesting that Smad3 is required in the presence of high doses of TGF-β during Th17 differentiation. To assess that hypothesis, naive T cells from either WT or KO mice were stimulated with increasing concentrations of TGF-β/H9252 in the presence of IL-6 and neutralizing antibodies against IFN-γ and IL-4. At low TGF-β concentrations, similar low levels of IL-17-producing cells were observed between WT and KO cells. However, with increasing concentrations of TGF-β/H9252, Smad3-deficient cells had higher numbers of IL-17-producing cells when compared with WT counterpart (Fig. 2C). Interestingly, enhanced IL-17-producing cells were observed in Smad3-deficient T cells at TGF-β concentrations that were not sufficient to induce Foxp3 expression (Fig. 2C and data not shown), suggesting that the inhibitory role of Smad3 on Th17 cells was not dependent on Foxp3 induction.

Smad3 Directly Binds to and Decreases RORγt Transcriptional Activity—Because Smad3-deficient T cells exhibited enhanced capability to differentiate into IL-17-producing T cells independent of Foxp3 gene induction and given that RORγt levels were not affected in Smad3-deficient Th17 cells when compared with WT cells (Fig. 2B), TGF-β, Smad3-deficient cells had higher numbers of IL-17-producing cells when compared with WT counterpart (Fig. 2C). Interestingly, enhanced IL-17-producing cells were observed in Smad3-deficient T cells at TGF-β concentrations that were not sufficient to induce Foxp3 expression (Fig. 2C and data not shown), suggesting that the inhibitory role of Smad3 on Th17 cells was not dependent on Foxp3 induction.

Smad3 Directly Binds to and Decreases RORγt Transcriptional Activity—Because Smad3-deficient T cells exhibited enhanced capability to differentiate into IL-17-producing T cells independent of Foxp3 gene induction and given that RORγt levels were not affected in Smad3-deficient T cells when compared with WT T cells, we next analyzed the regulation of RORγt function by Smad3. For that purpose, HEK293T cells were transfected with an RORE luciferase reporter vector (5) in the presence or absence of Smad3-expressing vector. Although RORγt alone induced luciferase activity, co-expression of increasing concentrations of Smad3 significantly reduced its activity (Fig. 3A).

Given that Smad3 is able to inhibit RORγt transcriptional activity, we next examined the interaction of Smad3 with RORγt using co-immunoprecipitation. We found that Smad3 was able to bind RORγt when co-expressed in HEK293T cells, and this binding was
enhanced upon co-expression of a constitutively active form of rat TGF-βRI (TGF-βRI T202D) (12) (Fig. 3B), suggesting that phosphorylation of Smad3 might enhance its affinity to RORγt.

Smad3 Deficiency Enhances the Generation of Inflammatory T Cells in Vivo—To analyze the role of Smad3 in Th17 cell generation in vivo, Rag1 KO mice were reconstituted with bone marrow cells from WT or Smad3 KO mice. After 8 weeks, mice were immunized with MOG peptide emulsified in CFA. 7 days later, splenocytes and draining lymph node cells were harvested from the immunized mice and restimulated with MOG peptide for 3 days. Cytokine production was measured from cell-free supernatants by ELISA. Error bars indicate S.E.

In summary, we have investigated the role of Smad3 in the generation of iTreg and Th17 cells. Smad3 deficiency resulted in defective Foxp3 induction but enhanced Th17 cell generation in vitro and in vivo. Smad3 was found to be part of a protein complex with RORγt, leading to the inhibition of RORγt transcriptional activity. Smad3 thus differentially regulates iTreg and Th17 cell differentiation. These results may be beneficial in our further understanding of the reciprocal regulation of these two cell lineages, allowing for the development of better approaches to design immunotherapies to target each cell type individually.

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FIGURE 3. Smad3 directly binds to RORγt and inhibits its transcriptional activity. A, HEK293T cells were transfected with RORE-Luciferase and the indicated vectors. Firefly Luciferase activity was determined using a Dual-Luciferase kit (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity. The data represent at least two independent experiments with triplicate measurements with similar results. Error bars indicate S.D. B, HEK293T cells were transiently transfected with Myc-tagged Smad3, FLAG-tagged RORγt, and/or His-tagged TGF-βRI T202D. After 48 h, lysates were prepared and immunoprecipitated with an anti-FLAG monoclonal antibody (IP) followed by immunoblotting (IB) with anti-FLAG or anti-Myc (bottom two panels). The top three panels indicate Western blot of whole cell lysates (WCL).

FIGURE 4. Smad3 deficiency enhances Th17 cell generation in vivo. Bone marrow cells from Smad3 WT or KO mice were intravenously transferred to sublethally irradiated Rag1 KO mice (four mice per group). After 8 weeks, the recipient mice were immunized subcutaneously with 150 μg of MOG35–55 peptide emulsified in CFA. 7 days later, lymphoid cells from spleens were isolated and restimulated with MOG peptide for 3 days. Cytokine production was measured from cell-free supernatants by ELISA. Error bars indicate S.E.