Smad2 Positively Regulates the Generation of Th17 Cells*

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Development of Foxp3+ regulatory T cells and pro-inflammatory Th17 cells from naive CD4+ T cells requires transforming growth factor-β (TGF-β) signaling. Although Smad4 and Smad3 have been previously shown to regulate T cell induction by TGF-β, they are not required in the development of Th17 cells. Thus, how TGF-β regulates Th17 cell differentiation remains unclear. In this study, we found that TGF-β-induced Foxp3 expression was significantly reduced in the absence of Smad2. More importantly, Smad2 deficiency led to reduced Th17 cell differentiation in vitro and in vivo. In the experimental autoimmune encephalomyelitis model, Smad2 deficiency in T cells significantly ameliorated disease severity and reduced generation of Th17 cells. Furthermore, we found that Smad2 associated with retinoid acid receptor-related orphan receptor-yt (RORyt) and enhanced RORyt-induced Th17 cell generation. These results demonstrate that Smad2 positively regulates the generation of inflammatory Th17 cells.

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EXPERIMENTAL PROCEDURES

Mice—C57BL/6 and OT-II T cell receptor transgenic mice were purchased from The Jackson Laboratory. Smad2 floxed mice (7) were bred with CD4-Cre mice (8). Mice were housed in the specific pathogen-free animal facility at the MD Anderson Cancer Center, and the animal experiments were performed at the age of 6–10 weeks using protocols approved by Institutional Animal Care and Use Committee.

*T Cell Differentiation—CD4+CD25−CD62LhiCD44lo T cells from naive CD4+CD25−CD62LhiCD44lo T cells were FACS-sorted and stimulated as described (6).

In Vitro Regulatory T Cell Suppression Assay—FACS-sorted naive CD4+CD25−CD62LhiCD44lo T cells were stimulated in the presence or absence of FACS-sorted CD4+CD25+ natural regulatory T cells (nTregs) at different ratios with 3000 Rads irradiated T cell-depleted splenocytes and soluble anti-CD3 (1 μg/ml) for 3 days. Proliferation was determined by the addition of [3H]thymidine to the culture for the last 8 h.

KLH Immunization and EAE—Mice were immunized with keyhole limpet hemocyanin (KLH) as described previously (9). EAEd induction was performed and analyzed as indicated previously (10). Disease scores from three independent experiments were combined, and p values were calculated using Student’s t test by comparing the disease scores.

Transduction of T Cells by Retrovirus—RORyt and constitutively active Smad2 (Smad2 2SD) (11) were cloned into bicistronic retroviral vector pGFP-RV (12) or pMIG-hCD2 (13) containing IRES-regulated GFP and human CD2, respectively. Naive CD4+CD25−CD62LhiCD44lo T cells from OT-II mice were infected as indicated previously (6). Four days after infection, cells were FACS-sorted based on GFP and hCD2 expression and analyzed.

TGF-β, together with IL-2, also induces Foxp3+ regulatory T (Treg) cells (3).

TGF-β, signaling through a heteromeric TGFβRII and TGFβRI complex, activates the phosphorylation of Smad2 and Smad3, which associate with the common partner Smad4, and then translocate to the nucleus (4). We have previously shown that TGF-β signaling through TGFβRI is required for generation of both Th17 and iTreg cells (5). Both Smad4 and Smad3 play an important role in the induction of Foxp3 expression upon TGF-β stimulation of naive T cells. Although Smad4 is dispensable for Th17 cell generation (5), Smad3 deficiency leads to enhanced Th17 cell development in vitro and in vivo (6). Thus, TGF-β-signaling mechanisms for Th17 cell differentiation still remain unclear.

In the present study, we have determined the role of Smad2 and found that Smad2 is required for induction of both iTreg cells as well as Th17 cells in vitro. In the experimental autoimmune encephalomyelitis (EAE) model, mice with a deficiency of Smad2 in T cells exhibited reduced disease severity and defective Th17 cell generation. Thus, Smad2 is crucial for the generation of Th17 cells.
Quantitative Real-time RT-PCR—cDNA was synthesized as reported previously (6), and gene expression was examined as described previously (5, 9, 10, 14, 15).

Co-immunoprecipitation—Expression vectors encoding 6/H11003 Myc-Smad3, 6/H11003 Myc-Smad2, 2/H9253 Myc-Smad4, FLAG-ROR and His-TGFβRI T202D were utilized to transfect HEK 293T cells. Co-immunoprecipitation was performed as indicated previously (6).

RESULTS AND DISCUSSION

Smad2 Deficiency Leads to a Partial Reduction in TGF-β-induced Foxp3 Expression—To address the role of Smad2 in T cell differentiation, Smad2 floxed mice (7) were crossed with CD4-Cre transgenic mice (8) to generate mice lacking Smad2 in T cells (Smad2 tKO), which results in the absence of Smad2 but not Smad3 protein in CD4+ and CD8+ T cells but not in B cells (supplemental Fig. 1). Interestingly, T and B cells primarily express Smad2 that is of higher molecular weight than that of Smad3, indicating that it contains exon 3 and thus has defective DNA binding capacity, unlike Smad3 (16, 17).

Smad2 tKO mice showed normal numbers of CD4+ and CD8+ T cells in thymus, as well as similar maturation markers as compared with wild-type littermates (supplemental Figs. 2 and 3). Furthermore, similar frequencies and total cell numbers T cells. Co-immunoprecipitation was performed as indicated previously (6).

FIGURE 1. Foxp3 expression is regulated by Smad2. A, CD25+ Foxp3+ cells in a CD4+ T cell gate were analyzed in the indicated tissues from Smad2fl/flCD4Cre− (Smad2 WT) or Smad2fl/flCD4Cre+ (Smad2 tKO) mice. A representative dot plot is shown for each group in each tissue (left panels), and the combined results for 10–15 mice in each group are indicated (right panel). p values were calculated using Student’s t test. *, p < 0.05; **, p < 0.01; ***, p < 0.001. LN, peripheral lymph nodes; MLN, mesenteric lymph nodes. B, CD4+ CD25+ CD62L+CD44− naive T cells from Smad2 WT mice were cultured in the presence or absence of different ratios of Smad2 KO or WT CD4+CD25+ natural regulatory T cells in triplicate wells with irradiated T cell-depleted splenocytes and stimulated with 1 g/ml anti-CD3. Proliferation was assayed 72 h after treatment by adding [3H]thymidine to the culture for the last 8 h. A representative of three independent experiments is shown. Error bars indicate mean ± S.D.

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Smad2 Is Required for Th17 Cell Generation in Vivo—To analyze the requirement for Smad2 in vivo, Smad2 tKO or WT littermate mice were first immunized with KLH emulsified in complete Freund’s adjuvant. Seven days after the immunization, spleen and draining lymph nodes were harvested, and cells were restimulated with KLH protein ex vivo to evaluate cytokine production. We found reduced frequencies and total cell numbers of IL-17-producing T cells from Smad2 tKO mice as compared with WT counterparts (supplemental Fig. 6A). Moreover, Smad2-deficient T cells produced significantly lower IL-17 and IL-17F cytokines upon ex vivo restimulation with increasing concentrations of KLH in splenocytes and draining lymph node cells, as measured by ELISA in the culture supernatants (supplemental Fig. 6B and data not shown). On the other hand, no significant differences in IL-22 or IFN-γ expression were observed between the two groups of mice, suggesting that Smad2 deficiency affects primarily IL-17 and IL-17F cytokines.

Th17 cells have been shown to be important for mediating inflammatory responses and autoimmune diseases (1). Thus, to further understand the function of Smad2 in vivo, we utilized
the EAE model. Both WT and Smad2 tKO mice showed similar disease onset and disease severity at an early stage (Fig. 3A). However, although Smad2 tKO mice started to recover by day 10, WT littermates showed sustained disease severity (Fig. 3A). At day 14 after the second immunization, we analyzed central nervous system infiltration and found significantly lower frequency of CD4$^+$ T cells in Smad2 tKO mice as compared with WT counterparts (Fig. 3B). Moreover, a reduction in the total CD4$^+$ T cell number was also observed (Fig. 3B). We further investigated the cytokine production of those CD4$^+$ T cells infiltrating the central nervous system and found a significant reduction in frequencies and total cell numbers of both IL-17$^+$ and IL-17$^+$/IFN-$\gamma^+$ cells (Fig. 3C). However, the frequencies of IFN-$\gamma$-producing CD4$^+$ T cells were not affected between the two groups (Fig. 3C), further demonstrating a specific role of Smad2 in Th17 cell generation in vivo.

The diminished disease severity in Smad2 tKO mice was not due to enhanced regulatory T cells infiltrating the CNS (data not shown). Moreover, when restimulated ex vivo with MOG peptide, splenocytes showed decreased Th17 cytokine production, albeit similar proliferation (Fig. 3D). Thus, these results showed that Smad2 is required for appropriate Th17 immune responses in vitro and in vivo.

**Smad2 Binds to and Synergizes with ROR$\gamma$**

**Th17 Cell Induction—Because Smad2-deficient T cells showed impaired Th17 cytokine expression but maintained normal levels of both RORo and ROR$\gamma$ transcription factors, we considered the possibility that Smad2 may not be required for ROR expression but is important for their function.**

Given that Smad3 can bind to ROR$\gamma$ (6), we next examined whether Smad2 can also associate with ROR$\gamma$. Similar to Smad3, Smad2 but not Smad4 was able to bind ROR$\gamma$ when co-expressed in HEK 293T cells, and this binding was increased upon co-expression of a constitutively active form of rat TGF$\beta$RI (TGF$\beta$RI T202D) (Fig. 4A) (6, 19). Because both Smad2 and Smad3 showed similar binding to ROR$\gamma$, we next evaluated their affinity to ROR$\gamma$ by doing a competitive co-immunoprecipitation experiment. We found that Smad3 competed with Smad2 for binding to ROR$\gamma$ and actually inhibited the binding of Smad2 to ROR$\gamma$, whereas Smad2 did not affect Smad3 binding. Thus, these results suggest that Smad3 might indirectly inhibit ROR$\gamma$ function by blocking Smad2 binding (supplemental Fig. 7). It has been recently suggested that PP2A differentially regulates Smad2 and Smad3 phosphorylation by directly dephosphorylating Smad3 under hypoxia conditions (20) which suggests that although TGF$\beta$R signaling might induce Smad2 and Smad3 phosphorylation similarly, other proteins might also regulate their phosphorylation status independently. Thus, this differential dephosphorylation of Smad2/3 might be the mechanism utilized in Th17 cells to favor Smad2 over Smad3 phosphorylation.

Next, we investigated whether Smad2 regulates ROR$\gamma$-dependent generation of Th17 cells. For that purpose, ROR$\gamma$ and/or constitutively activated Smad2 were overexpressed in T cells by retroviral transduction. Overexpression of ROR$\gamma$ but not Smad2 led to the generation of IL-17$^+$-producing cells and up-regulation of genes associated with Th17 phenotype (Fig. 4, B and C). Interestingly, overexpression of constitutively active Smad2 together with ROR$\gamma$ greatly enhanced the induction of IL-17$^+$-producing cells as compared with ROR$\gamma$ expression alone (Fig. 4B). In addition, Smad2 enhanced ROR$\gamma$-dependent IL-17, IL-17F, and CCL20 mRNA expression (Fig. 4C). This effect appears to be unique to Smad2 as a constitutive form...
of Smad3 did not regulate IL-17 expression in the presence or absence of RORγt in the same system (data not shown). Furthermore, we observed an increase in endogenous RORγ expression itself when RORγt was overexpressed, and such induction was further enhanced by co-expression of Smad2 (Fig. 4C). However, no differences were observed in other Th17-specific transcription factors such as RORα, BATF, and IκBζ (Fig. 4C and data not shown). Thus, these results suggest that Smad2 might act as a coactivator for RORγt, leading to enhanced Th17 cell generation.

In summary, in the present study, we studied mice with deficiency of Smad2 in T cells. We found that Smad2, like Smad4 and Smad3, was partially required for induction of iTreg cells. However, unlike Smad3 or Smad4, Smad2 not only binds but also synergizes with RORγt in the generation of Th17 cells. More importantly, Smad2-deficient T cells had reduced capability to differentiate into Th17 cells, and mice with deficiency of Smad2 in T cells showed reduced Th17 cell responses in vivo and amelioration of EAE disease symptoms. Our results provide a basis for understanding the reciprocal regulation of Th17 and regulatory T cells.

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