SMAD2 Is Essential for TGFβ-mediated Th17 Cell Generation

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TGFβ is the quintessential cytokine of T cell homeostasis. TGFβ signaling is required for the efficient differentiation and maintenance of CD4+FOXP3+ T cells that inhibit immune responses. Conversely, in conjunction with the inflammatory cytokine IL-6, TGFβ promotes Th17 cell differentiation. The mechanism by which TGFβ signals synergize with IL-6 to generate inflammatory and maintenance of CD4+Foxp3+T cells that inhibit immune responses. Conversely, in conjunction with the inflammatory cytokine IL-6, TGFβ promotes Th17 cell differentiation. The mechanism by which TGFβ signals synergize with IL-6 to generate inflammatory versus immunosuppressive T cell subsets is unclear. TGFβ signaling activates receptor SMADs, SMAD2 and SMAD3, which associate with a variety of nuclear factors to regulate gene transcription. Defining relative contributions of distinct SMAD molecules for CD4 T cell differentiation is critical for mapping the versatile intracellular TGFβ-signaling pathways that tailor TGFβ activities to the state of host interaction with pathogens. We show here that SMAD2 is essential for Th17 cell differentiation and that it acts in part by modulating the expression of IL-6Rα on T cells. Although mice lacking SMAD2 specifically in T cells do not develop spontaneous lymphoproliferative autoimmunity, Smad2-deficient T cells are impaired in their response to TGFβ in vitro and in vivo, and they are more pathogenic than controls when transferred into lymphopenic mice. These results demonstrate that SMAD2 is uniquely essential for TGFβ signaling in CD4+ T effector cell differentiation.

Transforming growth factor β (TGFβ) is an immune modulatory cytokine that is critical for the development and homeostasis of the immune system (1). The absence of TGFβ activity in mice leads to deregulated, hyperactive T cell activations resulting in early onset fatal multiorgan autoimmunity. Recent studies have highlighted the dominant function of TGFβ in the differentiation of T cell subsets. TGFβ can initiate transcription of Foxp3 in naive conventional CD4+ T (Tconv)2 cells to generate induced regulatory T (iTreg) cells. Moreover, in conjunction with the pro-inflammatory cytokine IL-6, TGFβ promotes differentiation of Tconv cells to Th17 cells (2–4) that contribute to the pathogenesis of autoimmune disorders. However, Th17 cells are also necessary for combating infectious diseases such as oral candidiasis and enteritis, caused by Candida albicans and Citrobacter rodentium, respectively (5). Hence, for CD4+ T cells, TGFβ controls the balance between inflammation and quiescence.

Although extensive progress has been made in understanding the effect of TGFβ on T cell differentiation and function, detailed characterization of the biochemical basis of TGFβ signaling in T cells has lagged significantly. In common with other cell types, TGFβ binds to TGFβ receptor I on T cells, which leads to the recruitment of TGFβ receptor I to the complex and activation of cytoplasmic transcription factors SMAD2 and SMAD3. In most cells, activated SMAD2 and SMAD3 translocate to the nucleus in a complex with SMAD4 to regulate target gene transcription (6). Some genes in the TGFβ pathway are redundantly regulated by SMAD2 and SMAD3, but unique target genes of specific SMADs also exist, suggesting that each possesses distinct functions. Hence, differential activation of SMADs may biochemically account for context-dependent pleiotropic effects of TGFβ.

Using mice deficient in individual SMADs, it has been shown that Smad3 and Smad4 are necessary for efficient TGFβ-mediated generation of FOXP3+ T cells but not for the differentiation of naive CD4+ T cells to the Th17 lineage (7–9). However, results from a recent study led to the contrasting conclusion that receptor SMADs are not necessary for TGFβ signaling in T cells (10). Here, analysis of T cell-specific Smad2-deficient mice indicates that in contrast to SMAD3 and SMAD4, SMAD2 plays a non-redundant role in the generation of Th17 cells in vitro and in vivo. The diminution in IL-17 production by CD4+ T cells correlates with accelerated loss of Il6ra expression and a corresponding decrease in STAT3 activation in Smad2-deficient T cells, suggesting that SMAD2 specifically modulates the cross-talk between TGFβ and IL-6 in Th17 cell differentiation.

EXPERIMENTAL PROCEDURES

Mice—Smad2fl/fl mice (11) generated in the Robertson laboratory were provided by Dr. Richard Flavell. These mice were backcrossed six times to the C57BL/6 background before analysis. Smad2 conditional KO (CKO) mice were generated using hCD2 Cre Tg+ mice (C57BL/6 background). Rag1−/− and C57BL/6 mice were purchased from The Jackson Laboratory. For C. rodentium infection, 1010 cfu of C. rodentium (DBS 100) in 10% sodium bicarbonate was administered by oral gavage. All experiments used mice of 6–12 weeks of age and were approved by Institutional Animal Care and Use Committee.

Abs, Flow Cytometry, and Cell Sorting—Cells were stained for surface markers, intracellular cytokines, and transcription factors using monoclonal antibodies (mAbs) and intracellular kits purchased from BD Biosciences and eBioscience. Anti-activin
CD4+ T cells at the CD25−Rag1−/− mice (supplemental Fig. 1D) and are able to inhibit Tconv cell proliferation in vitro (data not shown).

**Smad2 Regulates CD4+ T Cell Differentiation into iTreg and Th17 Cells**—To determine whether TGFβ-activated Smad2 mediates unique function in CD4+ T cell proliferation and differentiation, we first assayed for TGFβ-mediated suppression of CD4+ Tconv cell division and differentiation to iTreg and Th17 subsets. TGFβ suppresses Tconv cell proliferation by inducing cell cycle arrest. To assay TGFβ sensitivity of Smad2 KO CD4+ Tconv cells, naive CD4+ T cells were labeled with the cell cycle dye CFSE and activated with or without TGFβ. As expected, WT CD4+ T cells showed diminished proliferation in the presence of TGFβ, with the proportion of divided cells (CFSElo) decreased by ∼50% as compared with cultures without TGFβ. In contrast, Smad2 KO T cells were relatively insensitive to TGFβ, as indicated by the limited difference in the proportion of divided cells with TGFβ (Fig. 1A). However, when the concentration of TGFβ in the cultures was increased, Smad2 KO T cells responded to TGFβ, and proliferation was reduced. These results suggest that there is a dose-dependent impairment in TGFβ signaling in Smad2 KO T cells.

CD4+ T cells stimulated with TGFβ in vitro convert to FOXP3-expressing CD4+ T cells that resemble nTreg cells. We observed that in Smad2 KO T cells, there is a partial, but significant, decrease in the TGFβ-induced differentiation to FOXP3+CD4+ T cells (Fig. 1B). Smad3−/− T cells were reported to also have impairment in the FOXP3 induction in vitro (8, 9). The extent of impairment in those studies is similar to that observed here with Smad2 KO T cells, suggesting that each regulatory SMADs contribute to FOXP3 induction in T cells.

In the presence of IL-6 and TGFβ, CD4+ T cells differentiate into Th17 cells. Smad2 KO T cells were significantly impaired in Th17 lineage differentiation (Fig. 1, C and D), as indicated by decreases in IL-17A+ T cells in various culture conditions. Critically, the differentiation of Smad2-deficient CD4+ T cells to Th17 lineage was dictated by the concentration of both TGFβ and IL-6. Smad2 KO T cells have a more substantial impairment in converting to the Th17 lineage at lower concentrations of TGFβ and/or IL-6 than at higher concentrations of both of these cytokines (Fig. 1, C and D). This suggests that alterations in both TGFβ and IL-6 signaling pathways in Smad2 KO T cells are responsible for the reduced efficiency of Th17 cell generation. Differentiation of Smad2 KO T cells in the presence of IL-1β and IL-6 to Th17 lineage cells (14) was also less efficient than with control T cells (Fig. 1E). IL-2 signaling is inhibitory for IL-17 production by CD4 T cells, and blocking IL-2 enhances Th17 differentiation in vitro (15). However, the defect in Th17 differentiation of Smad2 KO T cells was not restored by the blockade of IL-2 signaling in Th17-inducing cultures (Fig. 1F).

We next determined whether other cytokines that activate SMAD2 are also impaired in function in T cells from Smad2 KO mice. Activins are members of the TGFβ family of cytokines, and activin A has been shown to exhibit a marked pref-
SMAD2 is necessary to efficiently induce TGFβ-dependent FOXP3+ and IL-17A+ T cell subsets. A, CFSE-labeled WT and Smad2 CKO naive CD4 T cells were activated (anti-CD3/CD28 cross-linking in all panels) with varying concentrations of TGFβ for 2 days. The extent of proliferation was measured by the loss of CFSE using flow cytometry. Data shown are representative of three independent experiments (a minimum of three mice/genotype/experiment) with similar results. Error bars are S.E. B, CD4 T cells were cultured under iTreg conditions with varying concentrations of TGFβ for 3 days. FOXP3 expression was analyzed by intranuclear staining (three independent experiments with similar results). C and D, CD4 T cells were cultured in varying doses of TGFβ and IL-6 with mitomycin C-treated splenocytes in Th17 conditions for 4 days. Intracellular staining (ics) results for IL-17A and IFNγ are shown. Flow cytometric profiles shown in C are representative of three independent experiments, and D shows the average frequency of IL-17A+ Smad2 CKO T cells relative to controls in varying culture conditions. Statistics are based on Student’s t test, with *, p < 0.05; **, p < 0.01. E, CD4 T cells were cultured with IL-1 and IL-6 in 3 day-activated CD4 T cells (right). H, WT and Smad2 CKO peripheral lymph node cells were stimulated with phorbol 12-myristate 13-acetate and ionomycin for 5 h and analyzed for cytokine expression in γδ TCR+ cells (two independent experiments).
IL-6 but is dependent on TGFβ and SMAD3 (17). Ex vivo γδT cells from lymph nodes of Smad2 CKO mice were not different from control γδT cells in IL-17A secretion (Fig. 1H), indicating that SMAD2 is dispensable for innate IL-17A production. In sum, SMAD2 is uniquely required to efficiently induce IL-17 in adaptive αβT cells downstream of the TGFβ family of cytokines and IL-6.

Smad2 Regulates IL-6Rα Expression and STAT3 Phosphorylation—The finding that Th17 differentiation by IL-6 and TGFβ is SMAD2-dependent led us to examine the alterations in the IL-6-signaling cascade in Smad2-deficient T cells. It has been reported that TGFβ up-regulates IL-6Rα expression in activated CD4+ T cells (18). Further, increased and sustained STAT3 phosphorylation in activated CD4+ T cells was observed in the presence of both TGFβ and IL-6, as compared with IL-6 alone (19). These results suggest that one function of TGFβ in promoting Th17 cell generation is to enhance and/or prolong IL-6 signaling in T cells. To investigate whether the IL-6-signaling pathway is altered in Smad2 CKO T cells, we measured the amounts of Il6ra transcripts in stimulated T cells. In our hands, available Abs to IL-6Rα are not sufficiently sensitive to be useful for flow cytometry, so other methods were used to quantify Il6ra expression. By quantitative PCR analysis, we observed a dramatic down-modulation of Il6ra mRNA expression in activated Smad2 CKO T cells (Fig. 2, A and B), as well as in Th17 culture conditions (data not shown). When Smad2 CKO CD4+ T cells were cultured with IL-6 alone, there was a significant decrease in intracellular phosphorylated STAT3 at early time points (15 and 30 min) as compared with control CD4+ T cells (Fig. 2, C and D; supporting Western blots are in supplemental Fig. 2) in an IL-6 concentration-dependent manner. In contrast to the alteration in IL-6 signaling, no changes in expression of two essential factors of Th17 differentiation, RORγt (Fig. 2B) and RUNX1 (data not shown), were observed in Smad2 CKO T cells. These results suggest that Smad2 CKO CD4+ T cells have a decreased capacity to respond to IL-6 and that the synergy between TGFβ and IL-6 in promoting Th17 differentiation is likely to involve SMAD2 regulation of IL-6R expression.

Smad2-deficient Tconv Cells Cause More Severe Colitis—It has been shown that IL-17A is protective during colitis induction, and CD4+ T cells that cannot produce IL-17A cause more aggressive colitis in Rag1−/− recipients (20). To determine the in vivo relevance of the in vitro defects in IL-17 production by Smad2 CKO CD4+ T cells, we assayed whether naive Smad2 CKO CD4+ T cells produce IL-17 when transferred to lymphopenic Rag1−/− recipients. Three weeks after T cell transfer, there was a significant decrease in IL-17A+ CD4+
The IL-17 family of cytokines is required for efficient clearance of the gut pathogen C. rodentium. To determine whether pathogen-driven IL-17 production by CD4+ T cells also requires SMAD2, we infected Smad2 CKO mice with C. rodentium. In C57BL/6 mice, the infection reaches maximal pathogen load at 7–9 days, and by 14 days, it is resolved. Ten days after infection, Smad2 CKO mice had comparable numbers of activated T cells in the mesenteric lymph nodes and spleen as WT infected mice (Fig. 3D and data not shown). However, there was a significant reduction in the frequency of Th17 cells in the lymphoid organs of infected Smad2 CKO mice (data not shown). In Smad2 CKO mice, IL-17+ CD4+ T cells accumulated on average to ~50% of the numbers seen in control infected mice (Fig. 3E). These results demonstrate that during infection, optimal Th17 cell generation requires SMAD2.

Our results show that SMAD2 is specifically utilized for optimal IL-17 production from CD4+ T cells downstream of the TGFβ family of cytokines. A similar finding using independently derived T cell-specific Smad2-deficient mice was just published (21). In comparison, Smad3−/− or Smad4 CKO mice do not exhibit significant impairments in Th17 generation (7, 9). Smad4-independent Th17 differentiation suggests the possibility that the unique TRIM33 interaction with SMAD2 is central to the process (22). The reduction in Th17 cell differentiation of Smad2 CKO T cells in vitro is most obvious at lower concentrations of TGFβ and/or IL-6. That TGFβ dose is a critical determinant in regulatory versus inflammatory T cell subset generation has been proposed (23), and it is likely that under saturating cytokine culture conditions, normal biochemical circuits can be subverted and masked. An essential function of SMAD2 for Th17 cell differentiation leaves open the possibility that selective regulatory SMAD activation may underpin the varied fate of TGFβ-signalized T cells. Further work is required to dissect how and in what in vivo conditions TGFβ-activated SMAD2 intersects with TCR- and IL-6-signaling pathways to dictate Th17 subset differentiation.

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