T CELLS

TGF-β–mediated enhancement of TH17 cell generation is inhibited by bone morphogenetic protein receptor 1α signaling

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The cytokines of the transforming growth factor–β (TGF-β) family promote the growth and differentiation of multiple tissues, but the role of only the founding member, TGF-β, in regulating the immune responses has been extensively studied. TGF-β is critical to prevent the spontaneous activation of self-reactive T cells and sustain immune homeostasis. In contrast, in the presence of proinflammatory cytokines, TGF-β promotes the differentiation of effector T helper 17 (TH17) cells. Abrogating TGF-β receptor signaling prevents the development of interleukin-17 (IL-17)–secreting cells and protects mice from TH17 cell–mediated autoimmunity. We found that the receptor of another member of TGF-β family, bone morphogenetic protein receptor 1α (BMPR1α), regulates T helper cell activation. We found that the differentiation of TH17 cells from naive CD4+ T cells was inhibited in the presence of BMPs. Abrogation of BMPR1α signaling during CD4+ T cell activation induced a developmental program that led to the generation of inflammatory effector cells expressing large amounts of IL-17, IFN-γ, and TNF family cytokines and transcription factors defining the TH17 cell lineage. We found that TGF-β and BMPs cooperated to establish effector cell functions and the cytokine profile of activated CD4+ T cells. Together, our data provide insight into the immunoregulatory function of BMPs.

INTRODUCTION

The transforming growth factor–β (TGF-β) family of cytokines is important for promoting the homeostasis of various tissues and the differentiation of select immune cell subsets (1, 2). Family members, including TGF-βs and bone morphogenetic proteins (BMPs), are produced by both stromal and immune cells (3, 4). Different TGF-β family cytokines may have overlapping or opposing functions, although these effects are often dependent on cellular and cytokine context. A founding member of the family, TGF-β, is an essential regulator of T cell development and constrains self-reactivity of peripheral T cells and their responses to antigenic stimulation (5, 6). Mice deficient in TGF-β1 gene succumb to uncontrolled inflammation and systemic, lethal autoimmune disease, which is mediated by exaggerated activation of T helper (TH1) effector cells, which produce interferon-γ (IFN-γ; TH11) or interleukin–4 (IL-4; TH12) (7, 8). These effects are due, in part, to the requirement of TGF-β for the development of Foxp3+ regulatory T (Treg) cells, an immunoregulatory T cell subset (9, 10). Thus, TGF-β exerts cell-intrinsic and cell-extrinsic effects that prevent the activation of self-reactive peripheral T cells (11). Conversely, in an inflammatory environment, TGF-β supports the generation of pathogenic TH17 cells, which secrete IL-17 (12). Loss of TGF-β signaling in T cells prevents IL-17 secretion and protects mice from autoimmune encephalomyelitis after immunization with self-antigen (13). In promoting TH17 cell generation, TGF-β cooperates with the proinflammatory cytokines IL-1, IL-6, or IL-21 and IL-23 (14–17). Activated T cells, including TH17 and Treg cells, are the main source of TGF-β, which induces and sustains TH17 cell differentiation (18). These findings underscore the importance of context-sensitive signaling and autocrine or paracrine TGF-β production for local immunoregulation.

Despite our increased understanding of how TGF-β regulates T cell functions, the immunoregulatory roles of many other members of the TGF-β cytokine family, especially BMPs, remain largely unknown. BMPs represent the largest subgroup of TGF-β cytokine family (19). They control a wide range of biological activities in various cell types and play critical roles in morphogenesis of various tissues and organs. BMPs bind heteromeric complexes of type I [BMP receptor 1α (BMPR1α) and BMP receptor 1β (BMPR1β)] and type II (BMP2/4) receptors to activate signal transduction pathways involving mothers against decapentaplegic homologs (or Smad1/5/8). BMPs can also regulate myeloid, B, natural killer, and peripheral T cells during infection, inflammation, and cancer (4, 20, 21). BMP2/4 or activin A increases the ability of TGF-β to promote generation of adaptive Treg (aTreg) cells, which arise during immune responses to limit inflammation (20, 22). Furthermore, BMPs increase phosphorylation of Runx-related transcription factor 1, which promotes IL-2 gene expression in conventional T cells and, in concert with Foxp3, inhibits in Treg cells (23). BMPs are also involved in restraining inflammation, but their exact role remains controversial (3).

To test the role of BMPs in peripheral T cells, we generated mice lacking BMPR1α in T cells and found that BMPR1α-deficient T cells preferentially differentiated into TH17 cells after activation. Transcriptome analysis suggested that loss of BMPR1α enhanced expression of transcripts involved in TH17 lineage commitment and inflammatory effector T cells. We showed that immunization with complete Freund’s adjuvant (CFA) stimulated stronger proinflammatory responses in BMPR1α-deficient mice than in wild-type mice. Similarly, adoptive transfer of BMPR1α-deficient CD4+ cells into...
lymphopenic hosts induced more severe colitis than cells isolated from wild-type mice. These findings indicate that BMPs may oppose TGF-β signaling in CD4⁺ T cells and describe a role for BMPR1α during CD4⁺ T cell lineage commitment and effector responses. Our data underscore the importance of interactions between TGF-β family members in immunoregulation.

RESULTS

BMPs inhibit Th17 differentiation

To understand whether BMPs may directly affect T cell responses, we examined the expression of BMP receptors in murine T cell subsets. We found that activated cells expressed more BMPR1α transcript and protein than naive and Treg cells (Fig. 1A and B). Similarly, after in vitro stimulation, we observed that BMPR1α transcription was quickly increased in CD4⁺ T cells (Fig. 1C). In contrast, BMPR2 transcripts were highly expressed in Treg cells and were not affected by T cell activation (Fig. S1A). Thus, our data suggest that activated CD4⁺ T cells may be preferentially sensitive to BMPs because of activation-induced expression of BMPR1α.

To determine the effects of BMPs on activated T cells, we stimulated naïve CD4⁺ T cells with plate-bound antibodies against CD3 and CD28 in the absence or presence of BMP2, BMP4, and BMP7. When cytokines were included to skew differentiation into Tα1, Th1, and Th17 cells, we found that BMPs did not inhibit expression of T-bet and IFN-γ, which are essential for the generation of Th1 effector cells (fig. S1B). Similarly, BMPs did not inhibit expression of GATA3 and IL-4, which promote development of Th2 effector cells. BMP exposure inhibited the expression of IL-17 (fig. S1B). Flow cytometry analysis confirmed these results, indicating that, whereas BMPs did not affect IFN-γ expression, BMP exposure greatly reduced the secretion of IL-17 (Fig. 1D). Together, our results indicate that direct BMPs stimulation restricts the differentiation of Th17 cells.

To understand how BMP signaling inhibits Th17 cell differentiation, we tested the effects of BMPs on STAT (signal transducers and activators of transcription) phosphorylation downstream of TGF-β and IL-6 receptor stimulation. We found that BMPs treatment reduced IL-6-mediated STAT3 activation. Although

![Fig. 1. BMP signaling regulates T cell activation and lineage commitment.](http://stke.sciencemag.org/)

- **A**: Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of BMPR1α mRNA expression in sorted CD4⁺ naïve, activated, and Treg cells. Data are means ± SD pooled from three independent sorts.
- **B**: Western blot analysis of BMPR1α abundance in lysates from sorted CD4⁺ naïve, activated (Activ), and Treg cells. Blots (left) are representative of three independent experiments. Normalized band intensity data (right) are means ± SD pooled from all experiments.
- **C**: qRT-PCR analysis of BMPR1α in sorted, naïve CD4⁺ T cells stimulated with antibodies against CD3 and CD28 for the indicated times.
- **D**: In vivo expression of BMPR1α in CD4⁺CD44hi T cells gated on CD4+CD44hi T cells. Blots (top) are representative of four independent experiments. Normalized band intensity data (bottom) are means ± SD pooled from all experiments.
- **E**: Western blot analysis of p-STAT3 and STAT3 in CD4⁺ T cells stimulated with antibodies against CD3 and CD28 for 4 days and BMP2/4/7 or TGF-β and IL-6, as indicated. Dot plots (left) are representative of four independent stimulations. The frequency of cytokine-producing cells (right) are means ± SD pooled from all experiments.
the initial phosphorylation of STAT3 in CD4+ T cells activated in the presence of IL-6 was comparable with and without BMPs, it was sustained only in the absence of BMPs (Fig. 1E). In CD4+ T cells activated in the presence of both IL-6 and TGF-β, STAT3 phosphorylation was much stronger and stable over time in the absence of BMPs (Fig. 1E). Given that the transcription of Rorc and IL-17 is STAT3-dependent, these data suggest that BMPs reduce the activity of signaling pathways necessary for the development of T_{H}17 cells (24).

**BMPR1α signaling controls generation of T_{H}17 CD4+ T cells in vivo**

To further determine the effects of BMP signaling, we used previously characterized mice lacking the BMPR1α gene in T cells (BMPR1α^{−/−}) (25, 26). Loss of BMPR1α does not alter the proportions or absolute T cell numbers in the thymus and peripheral organs, with the exception that fewer T_{reg} cells developed than in wild-type mice (26). Even with a decreased proportion of T_{reg} cells, BMPR1α deficiency in T cells did not precipitate autoimmune disease, even in mice up to 6 months old. Deletion of the BMPR1α gene abrogated BMP signaling as indicated by the loss of Smad5 phosphorylation in activated CD4+ T cells (Fig. 2A). However, we found no change in the phosphorylation of Smad2, which is downstream of the TGF-β receptor (Fig. 2A). Thus, these data validate efficient specific inactivation of the BMP receptor (1). When BMPR1α-sufficient and BMPR1α-deficient CD4+ T cells were activated in vitro, without exogenous cytokines, both cell types increased activation markers CD44 and CD25 and decreased CD62L, but a larger fraction of BMPR1α-deficient CD4+ T cells produced IFN-γ (Fig. 2, B and C). Consistent with our earlier observation that BMPs stimulation reduces T_{H}17 development (Fig. 1D), we found that, in the presence of TGF-β and IL-6, activation of BMPR1α-deficient CD4+ T cells generated more IL-17–producing cells than activation of wild-type cells (Fig. 2D). This effect was dependent on the combined effects of both the inflammatory cytokine IL-6 and TGF-β (Fig. S2). In contrast to studies using the small molecule dorsomorphin, an adenosine 5′-monophosphate–activated protein kinase (AMPK) inhibitor that blocks BMP receptor signaling and impedes T_{H}17 cell differentiation, these data demonstrated that, under T_{H}17 polarizing conditions, BMPR1α-deficient CD4+ T cells preferentially differentiated into T_{H}17 cells in vitro (27).

To understand the effects of BMP signaling in vivo, we characterized the response of wild-type and BMPR1α^{−/−} mice immunized with CFA. Flow cytometry analysis of the draining lymph nodes
indicated that a greater proportion of BMPR1α-deficient CD4+ T cells than of wild-type CD4+ T cells expressed markers of activation, which include decreased CD62L and increased CD44, CD25, 4-1BB, and ICOS (inducible T cell costimulator). These data suggested that the absence of BMPR1α is not associated with impaired activation (Fig. 3A). Similar to our in vitro results, we found that CD4+ T cells activated in BMPR1α−/− mice produced more IFN-γ– and IL-17–secreting effector cells upon antigen restimulation than CD4+ T cells activated in wild-type mice (Fig. 3B). The stronger inflammatory responses of mice lacking BMPR1α in T cells resulted in increased footpads swelling when compared to wild-type mice (Fig. 3C). Consistent with the known role of TGF-β in Th17 cell differentiation in the context of inflammation, these data indicated that abrogation of BMPR1α signaling further increased proportion of CD4+ T cells producing IL-17 (12). Thus, our analysis suggests that BMPR1α signaling in CD4+ T cells opposes TGF-β-mediated Th17 differentiation.

**Cell-intrinsic BMPR1α activity controls Th17 lineage specification**

The effects of BMPR1α deficiency on Th17 lineage bias may be due to their increased cytokine responses, because of an effect of BMPR1α during T cell development or due to a cell-intrinsic effect of BMPR1α. To begin to address this question in T cells with a fixed antigen specificity and affinity, we crossed BMPR1α−/− mice to mice expressing a transgenic T cell receptor (TCR) specific for an analog of pigeon cytochrome C (PCC) (28). Similar to our earlier results (Fig. 2C), when purified transgenic CD4+ T cells from BMPR1α−/− mice were activated by peptide antigen, these cells produced more IFN-γ–secreting cells than cells isolated from wild-type mice (fig. S3A). To mimic an infection-like environment, we added a low concentration of lipopolysaccharide (LPS) to cultured cells. When antigen-specific cells were activated in the presence of LPS, BMPR1α−/− CD4+ T cells were biased to differentiate into Th17 cells (fig. S3B), similar to stimulation in the presence of exogenous IL-6 and TGF-β (fig. S3C). These data indicate that the preference for Th17...
development is not due to gross changes in the TCRs that are selected for during T cell development. Furthermore, when PCC-specific BMPR1α-deficient and BMPR1α-sufficient CD4+ T cells were mixed together and cocultured in the presence of either IL-6 and TGF-β or IL-21 and TGF-β, a larger proportion of BMPR1α-deficient cells secreted IL-17 than did wild-type cells (Fig. 4A). In this experiment, the abundance of CD25 was similar on both populations of CD4+ T cells treated with TGF-β and IL-6 (Fig. 4B), indicating that there was no defect in activation in this condition. Furthermore, the abundance of CCR6 was increased in BMPR1α-deficient cells, and the abundance of CCR5 was the same in both populations (Fig. 4B). Together, these data indicate that cell-intrinsic BMPR1α signaling biases CD4+ T cell development toward the Th17 lineage.

To compare activation of antigen-specific cells in vivo, we immunized wild-type and BMPR1α−/− mice expressing transgenic TCR with PCC peptide and CFA. Analysis of T cell populations in popliteal lymph nodes demonstrated increased activation of BMPR1α-deficient CD4+ T cells, as indicated by increased amounts of the costimulatory molecules 4-1BB, ICOS, and Tim3 and inhibitory molecule BTLA (B- and T-lymphocyte attenuator) (Fig. 4C). Whereas IFN-γ was produced by activated CD4+ T cells both in wild-type and BMPR1α−/− mice, substantial numbers of IL-17−producing cells were only present in mice lacking BMPR1α in T cells (Fig. 4D). Consistent with increased cytokine production in BMPR1α−/− mice, the proportion of cells expressing Rorc was also increased in BMPR1α−/− mice when compared to wild-type mice (Fig. 4D). These results suggest that, under inflammatory conditions in vivo, BMPR1α-deficient cells differentiated primarily into IL-17−producing CD4+ T cells.

**RNA-seq identifies BMPR1α controls Th17 cell effector functions.**

To determine how BMPR1α signaling affects molecular circuits controlling CD4+ T cell lineage commitment, we examined the global gene expression profiles of naive and activated wild-type and CD4+ T cells, as indicated by increased amounts of the costimulatory molecules 4-1BB, ICOS, and Tim3 and inhibitory molecule BTLA (B- and T-lymphocyte attenuator) (Fig. 4C). Whereas IFN-γ was produced by activated CD4+ T cells both in wild-type and BMPR1α−/− mice, substantial numbers of IL-17−producing cells were only present in mice lacking BMPR1α in T cells (Fig. 4D). Consistent with increased cytokine production in BMPR1α−/− mice, the proportion of cells expressing Rorc was also increased in BMPR1α−/− mice when compared to wild-type mice (Fig. 4D). These results suggest that, under inflammatory conditions in vivo, BMPR1α-deficient cells differentiated primarily into IL-17−producing CD4+ T cells.

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BMPR1αT−CD4+ T cells using RNA sequencing (RNA-seq). Overall, when gene expression profiles were compared, 4183 and 4746 genes were found differentially expressed between naïve and activated BMPR1α-sufficient and BMPR1α-deficient CD4+ T cells, respectively (fig. S4, A and B). A subset of 678 of these genes were differentially expressed between activated BMPR1α-sufficient and BMPR1α-deficient CD4+ T cells. Principal components analysis (PCA) performed on both activated cells subsets demonstrated different gene expression profiles (Fig. 5, A and B, and table S1). When we interrogated the expression of genes assembled from published reports that define T111, T112, T117, and A1reg cell subsets, we found that T–bet, IFN-γ, IL-12rb1, IL-12rb2, IL-17a, IL-17f, Rorc, Rora, IL-21, Stat1, and Stat4 are highly expressed in both BMPR1α-deficient and BMPR1α-sufficient CD4+ T cells (fig. S4C) (29–33). All of these genes are essential to establish and sustain T111 and, in particular, T117 cell differentiation (24, 34). We found that BMPR1α-deficient cells expressed more transcripts for pivotal genes regulating T117 cell specification and effector functions including Rorc, IL-23R, IL-17a, and IL-17. This pattern of gene expression is remarkably different from activated CD4+ T cells developing in TGF–β or TGF–β receptor II (TGF–βRII)–deficient mice, which do not express genes associated with T117 cell differentiation and remain protected from T117 cell–mediated autoimmunity (7, 8, 11, 15). Activated BMPR1α-deficient cells also did not express or expressed only low amounts of T112 cell signature genes like Gata3, IL-4, IL-4ri, IL-5, and Areg but expressed higher amounts of IL-13 (31). Similarly, BMPR1α-deficient cells did not increase transcription of genes associated with A1reg generation, such as Foxp3, Nr4a1, Nr4a3, and Gpr83 (36–38).

When we performed gene set enrichment analysis using MetaScape to associate gene sets with cellular processes described by Gene Ontology (GO) terms, we found that the network topology indicated extensive connections between clusters and substantial overlap of gene sets (Fig. 5C and table S2) (33). All major clusters of GO terms were broadly related to inflammatory and immune response, control of cytokine production, signaling, and cell adhesion. Separate network clusters related to TNF, IL-17, IL-12, IL-13, and Jak-STAT signaling indicated that molecular changes in these pathways differentially affected activated wild-type and BMPR1α-deficient CD4+ T cells. By focusing on biological processes primarily related to inflammatory and immune responses, we found that activated BMPR1α-deficient CD4+ T cells overexpressed transcripts for the cytokines Tnf, lymphotoxin (LT-α), Csf2 (GM-CSF), RANKL (TNFSF11), and IL-10 (Fig. 5D). This cytokine pattern largely overlaps with the transcriptional signatures of in vitro–generated T117 cells and is consistent with pathological T117 cells found in autoimmune diseases (17, 24, 34). Furthermore, BMPR1α-deficient cells expressed higher amounts of transcripts encoding receptors for IL-1 (IL-1R1), IL-17c (IL-17RE), and IL-23 (IL-23R). All three cytokines are either essential to promote induction and terminal maturation of T117 cells or are required to sustain their effector functions (12, 16, 39–42). BMPR1α-deficient cells had elevated expression of signaling molecules that facilitate T117 generation and increase production of proinflammatory cytokines, such as Aif1, Cish, and Dusp14 (43–45). BMPR1α-deficient cells also displayed elevated expression of proenkephalin (Penk), Trh6, IRAK1BP1, and coro2ina2 (Coro2a), which promote production of proinflammatory cytokines and T117/T117 responses, and Msh5 and Nqo1, which protect cells against hypoxia and oxygen radicals generated during an inflammatory response (46–51). In summary, the gene expression pattern of T117 lineage canonical markers substantiated functional and flow cytometry studies and strongly supports our conclusion that deficient BMPR1α signaling affects T117 lineage commitment by promoting generation of T117 effector cells.

Loss of BMPR1α alters cytokine and TCR-dependent signaling in CD4+ T cells

T117 lineage bias could be caused by modulation of BMP and/or TGF–β signaling pathways resulting from the absence of functional BMPR1α. Down-regulation of Smad4, a shared component of BMP and TGF–β signaling pathways, leads to T117 cell–mediated gastrointestinal inflammation (52). However, our analysis showed that equal quantities of Smad4 were present in naïve and activated BMPR1α-deficient and BMPR1α-sufficient CD4+ T cells (Fig. 6A). We also established that phosphorylation of Smad2/3 in response to different concentrations of TGF–β was the same in BMPR1α-sufficient and BMPR1α-deficient CD4+ T cells (Fig. 6B). Thus, the lack of BMPR1α did not affect T cell sensitivity to TGF–β signaling. Stimulation with TGF–β does not change BMP2/4-induced Smad1 phosphorylation, further suggesting that membrane proximal signals mediated by either TGF–β or BMPR1α are independent in T cells (20).

The T117 lineage bias that we observed could result from BMPR1α signaling, affecting events downstream of cytokine receptors. To examine this possibility, we probed the phosphorylation of STAT3 and STAT1, after stimulation of wild-type and BMPR1α-deficient CD4+ T cells with IL-6 and IFN-γ. We found that STAT3 and STAT1 are hyperphosphorylated in activated BMPR1α-deficient cells after cytokine stimulation (Fig. 6C). Increased STAT3 and STAT1 phosphorylation in BMPR1α-deficient cells is associated with decreased expression of inhibitory molecules SOCS3 and SIGIRR (IL-18R) known to inhibit IL-17 and IFN-γ production (Fig. 5D) (53–56). Another signaling molecule, Cish, elevated in BMPR1α-deficient cells, not only decreases TCR signaling but also inhibits IL-2 signaling, mediated by STAT5, which increases T117 cell differentiation (44, 57, 58). In summary, gene expression changes resulting from impaired BMP signaling support the intrinsic bias of BMPR1α-deficient cells to differentiate into T117 effector cells and are consistent with the BMP inhibition of T117 generation in wild-type cells.

Sensitivity to TGF–β signaling in T cells is set in response to TCR stimulation by MAP (mitogen-activated protein) activation of kinase pathways, which promote T cell proliferation and differentiation into effector cells (59). To investigate signaling events downstream of the TCR, we examined phosphorylation of JNK (c-Jun N-terminal kinase), ERK (extracellular signal–regulated kinase), and p38 kinases in wild-type and BMPR1α-deficient CD4+ T cells after stimulation with antibodies against CD3 and CD28 (Fig. 6D). MAP kinases are known to phosphorylate the linker region of R-Smads regulating not only their nuclear translocation and degradation but also induction of Rorc and IL-17 transcription (60, 61). Whereas p38 and Erk phosphorylation was similar in wild-type and BMPR1α-deficient cells, JNK activity was reduced in activated BMPR1α-deficient cells. This is consistent with a report of JNK pathway inhibiting T117 cell generation (62). JNK activity may be further reduced in BMPR1α-deficient cells by up-regulation of DUSP16 (dual specificity protein phosphatase 16) known to preferentially dephosphorylate JNK (Fig. 5D) (45, 63). In contrast, ERK, but not p38 or JNK phosphorylation, was decreased in CD4+ T cells activated in the presence of small-molecule inhibitors of BMPR1α signaling and other kinases, which also decrease T117 cell differentiation (Fig. 6E and fig. S5) (64, 65). Collectively,
these results not only underscore the importance of cross-talk between signaling pathways in regulating Th17 lineage specification but also point to differences between targeted genetic ablation of BMPR1α and use of BMP signaling inhibitors.

**BMPR1α-deficient CD4+ T cells are highly pathogenic in vivo**

To understand the consequence of BMPR1α-mediated suppression of IL-17 effector cell development, we investigated immunopathology in a mouse model of inflammatory bowel disease (66, 67). Naive
CD4⁺CD45RB⁺ cells sorted from wild-type and BMPR1α⁻ mices were transferred into TCRα chain knockout mice (TCRα⁻), and recipients were monitored and scored for weight loss and hallmark symptoms of colitis. We found that weight loss was comparable (Fig. 7A); however, other colitis symptoms were more severe in recipients of BMPR1α-deficient cells than in recipients of wild-type cells (Fig. 7, B and C). Necropsy analysis revealed that recipients of BMPR1α-deficient cells had shorter colon length than recipients of wild-type cells, indicative of more severe inflammation and colitis (Fig. 7D). We also found by histological analysis that recipients of
wild-type and BMPR1α-deficient cells had prominent lymphocytic infiltrate involving mucosa and submucosa (Fig. 7E). When lamina propria cells were isolated and stained for cytokines and Rorc expression, recipients of BMPR1α-deficient cells expressed much higher levels of inflammatory cytokines, IFN-γ and IL-17, and transcription factor Rorc than recipients of wild-type cells (Fig. 7F). Sorted BMPR1α-deficient donor CD4+ T cells from lamina propria expressed more IL-6, IL-1β, and IL-17 proinflammatory cytokine transcripts.
than wild-type donor cells (Fig. 7G). Similar results were obtained after feeding mice dextran sulfate sodium (DSS) to chemically induce colitis (fig. S6). Collectively, these results demonstrated that BMPR1α-deficient effector CD4⁺ T cells mediate an exaggerated inflammatory response and can contribute to immune pathology.

**DISCUSSION**

Although the many functions of TGF-β in regulating immune system homeostasis and generation of T cell subsets have been extensively studied, the role of BMPs and their receptors in controlling initiation, maintenance, and resolution of immune responses is much less appreciated (4). Because of the number of BMP ligands far exceeding the number of available receptors, it is more practical to study the role of BMPs by eliminating receptors. This strategy also avoids confusion associated with diverse effects of BMPs on multiple lineages of hematopoietic cells and possible indirect effects on T cells. In addition, this approach where deleting TGF-βRII was used to assess the role of TGF-β in T cells has previously been established (7, 8). We show that BMPR1α is weakly expressed in naive cells, and the expression is strongly enhanced upon activation. Thus, studying the effects of deleting BMPR1α gene provided insight into the immunoregulatory role of BMPs in activated T cells.

Deletion of BMPR1α gene did not substantially affect survival and expansion of peripheral CD4⁺ T cells. Although wild-type and BMPR1α⁻/⁻ mice have similar proportions and absolute cell numbers in the thymus and peripheral organs, in the absence of BMPR1α, Treg cells are reduced (26). In contrast to T cell–specific deletion of TGF-βRII, which causes lymphoproliferation, multiorgan leukocyte infiltration, and early lethality in mice, loss of BMPR1α did not result in similar spontaneous autoimmune phenotype (7, 8). This lack of prominent autoimmunity in BMPR1α⁻/⁻ mice may be explained by our data indicating that there is a low amount of BMPR1α in naive CD4⁺ T cells, making these cells less sensitive to the lack of BMPs. Only after activation did BMPR1α-deficient cells produce higher proportions of IFN-γ–secreting cells than wild-type cells, which correlated with the increased abundance of BMPR1α after activation.

We showed that, in wild-type CD4⁺ T cells, BMPs inhibit induction of Rorc and IL-17 expression. This effect of BMPs on the transcriptional program of T cell activation is associated with impaired phosphorylation of STAT3, indicating cross-talk between signaling pathways downstream of IL-6 receptor and BMPR1α. However, BMPR1α-deficient cells also generated more IFN-γ–secreting cells after in vitro stimulation or after stimulation in vivo. This suggested that BMPR1α may also suppress Tfh1 development in conditions that do not support Tfh17 cell differentiation. In contrast, when BMPs were directly added to T cell culture medium, which may activate multiple receptors, we found no effect on Tfh1 cell differentiation. Increased Tfh1 differentiation of BMPR1α-deficient cells resembled CD4⁺ T cells with impaired TGF-β signaling, which do not differentiate into Tfh17 cells, but instead produce more Tfh17 cells when activated in conditions inducing Tfh17 cells (13, 39). At the same time, activation of CD4⁺ T cells in the presence of LPS, Mycobacterium tuberculosis, or zymosan or selective neutralization of IL-6 or TGF-β changed proportions of Tfh1 and Tfh17 cells (12). In addition, abrogation of BMPR1α signaling may also dysregulate BMP-dependent control of receptors for IL-1, IL-23, and IL-17 and downstream signaling molecules. We also observed that expression of metabolic regulators such as Msh5, Ngol, and Egln3 and molecules controlling response to proinflammatory cytokines such as SOCS3, Sigirr, and Cish was changed in activated BMPR1α-deficient cells to increase cell response and persistence in inflammatory environment. It is plausible that lack of BMPR1α tonic signaling may program CD4⁺ T cells in unmanipulated BMPR1α⁻/⁻ mice to respond robustly to inflammation. Collectively, these findings demonstrate that the outcome of T cell activation may be determined by both intrinsic signaling framework and is context-sensitive, depending on the spectrum of inflammatory cytokines present in the activating environment.

Our data suggested that the effect of BMPR1α did not rely on blocking TGF-β signaling, as shown by studies of Smad2/3 phosphorylation and Smad4 abundance, but instead involved modulation of STAT3 and MAP kinase responses. The extent of signaling cross-talk and molecular regulation of heterogeneity and stability of Tfh lineages, especially Tfh17 cells, are not well understood (68–70). CD4⁺ lineage defining transcription factors have permissive or bivalent histone modification in activated Tfh subsets, which contribute to T cell plasticity (31). Transitions between Tfh1 and Tfh17 cells or effector cells coproducing IL-17 and IFN-γ are found in cell lineage tracing studies in experimental autoimmune encephalomyelitis or inflammatory bowel disease, but the role of individual transcription factors and cytokine receptors in the differentiation and function of these effector cells remains controversial (71–73). Our findings revealed an important role for BMPR1α in regulating Tfh1 cell developmental programs and uncovered new immunoregulatory functions of the TGF-β family members.

Analysis of gene expression also identified a number of signal transduction molecules, known from previous studies to regulate production of inflammatory cytokines, which transcription is controlled by BMPR1α. Further analysis of these molecules not only will provide new insight into how BMPR1α signaling affects CD4⁺ T cell polarization but also may help identify molecules that sustain function of inflammatory cells. Transcriptional profiling was also instrumental to understand the broad impact of BMPR1α signaling and identify biological processes and metabolic regulators that supply BMPR1α-deficient cells with effector functions. Gene set enrichment analysis established that BMPR1α signaling is primarily responsible for regulation of various aspects of inflammation and immune responses of T cells. This scope of cellular functions regulated by BMPR1α is consistent with BMPs role in controlling chronic inflammation, organ fibrosis, and homeostasis (74). Together, we validated the gene expression pattern using functional and flow cytometry studies, strongly supporting our conclusion that deficient BMPR1α signaling promotes generation of Tfh17 effector cells and enhances inflammatory responses.

Members of the TGF-β cytokine family may have opposing functions with regard to Tfh17 subset specification and regulation of inflammation. Transcription profiling offered molecular evidence that, in contrast to TGF-βRII–deficient helper cells, BMPR1α-deficient cells expressed transcription factors and a cytokine profile associated with Tfh17 cell lineage (5, 13). In particular, BMP7 reverses the proinflammatory activity of TGF-β and protects from chronic organ injury (75, 76). Similarly, BMPs are produced in multiple tissues in response to infection or various stimuli causing cell damage, block inflammation, and restore tissue homeostasis (3, 4). In contrast, blockade of BMP signaling in rheumatoid arthritis patients with specific inhibitors augments inflammation induced by IL-17 (77). Consistent with our results, BMPs ameliorate intestinal inflammation and protect from mucosal damage (78, 79). This role of BMPs...
contrasts with recent reports that dorsomorphin and DMH1 (dorsomorphin homolog 1), which are small-molecule inhibitors of BMP receptors and other kinases, impede proliferation of human and mouse T cells and block T_{H}17 differentiation (27, 80, 81). Dorosmophin inhibits BMPR1α [activin receptor-like kinase 3 (Alk3)], BMPR1β (Alk6), and Alk2, which all share BMP ligands, as well as TGF-βRII, VEGFR2 (Flk1/KDR), and AMPK, an energy sensor in T cells that is necessary for accumulation of T_{H}17 cells (64, 65, 82, 83). DMH1 inhibitor is more specific but, in addition to BMPR1α, inhibits activin Alk1 and Alk2 (64, 65, 84). Activin receptors are competitively bound by activins and BMPs, which changes their signaling from Smad2/3 to Smad1/5/8 and makes inhibition studies difficult to interpret (82). In our studies, we used dorsomorphin and DMH1 at concentrations that did not inhibit T cell proliferation and were lower than in previous studies, but this treatment still inhibited MAP kinase phosphorylation downstream of TCR stimulation. Although our studies showed that BMPs signaling through the BMPR1α inhibited generation of T_{H}17 cells, studies targeting other receptors are necessary to dissect complexity of immune regulation by cytokines of TGF-β family.

Multiple factors affect immunoregulation mediated by BMPs and TGF-β in tissues including their cellular source and bioavailability. Paracrine or autocrine production of TGF-β by T cells is essential for both regulation of tolerance and promotion of T_{H}17 cells (5). Localized production and tissue availability control the response to BMPs, but how their production is regulated in healthy tissues and during immune responses remains largely unknown (3, 74). One possibility is that BMPs are predominantly produced by stromal or epithelial cells and represent signaling cues exchanged between local tissues and the immune system. Once secreted, TGF-β and BMPs precursors associate with soluble or matrix molecules present in extracellular space (4, 85). These interactions affect proteolytic cleavage and maturation, degradation, and diffusion; regulate binding to membrane receptors; and represent an important level of regulation of TGF-β and BMPs signaling. Proteolytic processing of TGF-β by furin convertase is essential for regulating peripheral T cell activation, but how furin or other proteases regulate maturation of BMP precursors is not known (86). Identifying cellular sources and uncovering how production, secretion, and processing of BMPs are regulated will reveal new mechanisms of local immune-regulation and provide rationale for their therapeutic application. Thus, our findings provide a framework for broader understanding of how TGF-β and BMPs regulate T cells in immune tolerance and autoimmunity.

**MATERIALS AND METHODS**

**Mice**

TCRα− mice were purchased from the Jackson Laboratory (87). BMPR1α− mice were generated by crossing BMPR1α conditional knockout mice with mice expressing CD4cre and Foxp3^{GFP} reporter (25, 88, 89). Foxp3^{GFP} and BMPR1α− mice were crossed with mice expressing TCR specific for analog of peptide derived from PCC (90). All mice were on the C57BL6 genetic background. Mice were bred and housed under specific pathogen–free conditions in the animal facility of Old Dominion University (ODU). All experiments were approved by Institutional Animal Care and Use Committee. Both female and male mice were used in experiments, and we have not observed any difference in T cell development and activation between sexes. Mice were 6 to 12 weeks old for all experiments.

**T cell activation and polarization**

For in vitro activation, CD4+ T cells were purified by flow cytometry sorting or with magnetic beads and stimulated with plate-bound antibodies against CD3 (10 μg/ml; 2C11) and CD28 (1 μg/ml; 37.51) (both from BD Biosciences), Con A (2 μg/ml; Sigma), or an analog of PCC peptide (5 μM; PCC50V54A) in the presence of antigen-presenting cells in αMEM media (HyClone) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals), 2 mM L-glutamine, dextrose, essential and nonessential amino acids, sodium pyruvate, sodium bicarbonate, antibiotics, and 2-β-mercaptoethanol.

To produce polarized effector cells, CD4+ T cells were purified by flow cytometry sorting or with magnetic beads and stimulated with plate-bound antibodies against CD3 and CD28. For T_{H}1 differentiation, cells were stimulated in the presence of IL-12 (10 ng/ml; PeproTech) and antibody against IL-4 (10 μg/ml; BD Biosciences). For T_{H}2 differentiation, cells were stimulated in the presence of IL-4 (10 ng/ml; PeproTech) and antibody against IFN-γ (10 μg/ml; BD Biosciences) and IL-12 (10 μg/ml; BD Biosciences). For T_{H}17 differentiation, cells were stimulated in the presence of IL-6 (20 ng/ml; PeproTech) and TGF-β (3 ng/ml; PeproTech). Cells were cultured and analyzed after 4 days. To examine BMP signaling, T cells were cultured with BMP2, BMP4, and BMP7 (3 ng/ml each; PeproTech). To inhibit BMP signaling, SB925334 (10 nM; Selleckchem) was added to cell culture. To inhibit BMP signaling, T cells were incubated in the presence of dorsomorphin (1 μM; Tocris Bioscience) or DMH1 (10 μM; Tocris Bioscience).

For coculture, total lymph nodes and spleen from PCC transgenic mice were combined. The cells from wild-type and BMPR1α− mice were then mixed 1:1 and stimulated with PCC peptide and either IL-6 (20 ng/ml; PeproTech) and TGF-β (3 ng/ml; PeproTech) or IL-21 (100 ng/ml; PeproTech) or TGF-β (3 ng/ml; PeproTech). Cell proportions in the coculture were determined to be 60% BMPR1α-sufficient and 40% BMPR1α-deficient using CD45.1 and CD45.2 staining. Cells were cultured and analyzed after 6 days.

**In vivo activation and immunization**

For in vivo activation, mice were immunized in the footpad with 100 μg of antigenic peptide PCC50V54A (AnaSpec) emulsified in CFA. After 5 days, animals were sacrificed, and popliteal draining lymph nodes were isolated.

**Adoptive transfer of CD4+CD45RB<sup>bright</sup> T cells into lymphopenic mice**

Naive CD4+CD45RB<sup>bright</sup> T cells were flown cytometry–sorted from spleens of wild-type and BMPR1α− mice, and 5 × 10<sup>5</sup> cells per mouse was transferred intravenously into TCRα knockout C57BL6 mice. Mice were monitored every 2 to 3 days for 3 weeks.

**DSS-induced colitis**

Experimental colitis was induced by administration of 2.5% (w/v) DSS (molecular weight, 40,000; MP Biomedicals) dissolved in sterile drinking water ad libitum for 6 days to mice. Mice were monitored daily and euthanized after 7 days for histological assessment and flow cytometry and gene expression analyses.

**Assessment of intestinal inflammation**

Body weight, occult or gross blood loss per rectum, and stool consistency were assessed as briefly described (91). The baseline clinical score was determined on day 0. Scoring went as follows: No weight...
staining, cells were incubated for 3 hours with Brefeldin A (10 μg/ml; BD Biosciences), phorbol 12-myristate 13-acetate (50 ng/ml; Sigma), LPS (10 ng/ml; Sigma) for 4 days, and activated CD4+ CD44+ CD62L− Foxp3GFP+ cells were flow cytometry–sorted. At least three different samples were processed for each cell type. Total RNA was prepared using a commercial kit (Qiagen). DNA for sequencing was produced according to the manufacturer’s instructions. Equal amounts of complementary DNA were used in triplicates to detect transcripts of BMPR1α, BMPR2, IL-6, IL-1β, IL-17, IFN-γ, and Rorc using TaqMan Universal Master Mix II (Life Technologies) in the StepOne Real-Time PCR System (Applied Biosystems). The transcript abundance of each gene was normalized to β-actin. For endpoint PCR, transcription factors are as follows: IFN-γ, T-bet, IL-4, GATA3, IL-17, Rorc, BMPR1α, and β-actin transcripts were amplified with GoTaq polymerase (Promega).

Enzyme-linked immunosorbent spot assay
Wild-type and BMPR1α−/− mice were immunized with 100 μg of LCMV peptide gp61-80 (AnaSpec) in CFA in the footpads. After 1 month, a booster dose was applied, and mice were sacrificed 7 days later. Draining (popliteal) lymph nodes and spleen cells (2 × 105 cells per well) were isolated, and cytokine-secreting cells were quantified using ELISPOT assay and ELISPOT Ready-SET-Go kit reagents (eBioscience). Cytokines were detected using antibodies specific for IFN-γ (capture, R4-6A2; detection, XMG1.2), IL-4 (capture, 11B11; detection, BV6-24G2), IL-17 (capture, eBio17CK15A5; detection, eBio17B7), IL-2 (capture, JES6-1A12; detection, JES6-5H4), and IL-10 (capture, JES5-16E3; detection, JES5-2A5), and spots were quantified with ImmunoSpot software (CTL) on an S6 Macro reader.

Gene expression analysis
RNA was prepared according to the manufacturer’s instructions (PureLink RNA kit, Life Technologies) and reverse-transcribed with SuperScript III (Life Technologies) as per the manufacturer’s instructions. Equal amounts of complementary DNA were used in triplicates to detect transcripts of BMPR1α, BMPR2, IL-6, IL-1β, IL-17, IFN-γ, and Rorc using TaqMan Universal Master Mix II (Life Technologies) in the StepOne Real-Time PCR System (Applied Biosystems). The transcript abundance of each gene was normalized to β-actin. For endpoint PCR, transcription factors are as follows: IFN-γ, T-bet, IL-4, GATA3, IL-17, Rorc, BMPR1α, and β-actin transcripts were amplified with GoTaq polymerase (Promega).

RNA-seq and transcriptome analysis
Global analysis of gene expression was performed using Illumina Sequencing platform in Georgia Cancer Center Core Facility, Augusta University. Naive CD4+ CD44− CD62L+ Foxp3GFP− cells were flow-sorted from lymph nodes and spleens of unmanipulated wild-type mice. Lymph node and spleen cells isolated from wild-type or BMPR1α−/− mice were activated with Con A (2 μg/ml; Sigma) and LPS (10 μg/ml; Sigma) for 4 days, and activated CD4+ CD44+ CD62L− Foxp3GFP+ cells were flow cytometry–sorted. At least three different samples were processed for each cell type. Total RNA was prepared using a commercial kit (Qiagen). DNA for sequencing was produced with Illumina kit. RNA-seq data analysis was performed using Tuxedo protocol as described in (92). Briefly, sequencing reads were aligned to reference genome using TopHat2, followed by estimation of RNA using Cufflinks 2.1.1. Differential gene expression analysis was performed using Cuffdiff application of Cufflinks. Genes were considered differentially expressed if fold expression was 1.5 or more, and the difference was statistically significant. To visualize differences between gene expression profiles of activated wild-type and BMPR1α-deficient CD4+ T cells, we performed PCA. The gene lists subject to PCA analysis included all genes with expression levels above the threshold allowing for differential expression analysis in Cufflinks suite. Expression profiles of genes differentially expressed between activated BMPR1α-sufficient and BMPR1α-deficient CD4+ T cells were visualized as a heat map. Expression profiles of genes...
differentially expressed between naive wild-type and activated BMPR1α-sufficient and BMPR1α-deficient CD4⁺ T cells were visualized as volcano plots. All analyses were done in R (version 3.4.3). Bioinformatics transcriptional analysis was performed in College of Public Health of Ohio State University. GO and gene enrichment analyses were performed using Metascape (http://metascape.org), and network graphs were edited using Cytoscape (93, 94).

Western blot
Lymph node cells were isolated, and CD4⁺ T cells were purified by magnetic beads. Purified CD4⁺ T cells (5 × 10⁵) were lysed directly in Laemmli buffer with protease and phosphatase inhibitors (Halt Protease and Phosphatase Inhibitor Cocktail, Thermo Fisher) or stimulated with plate-bound antibodies against CD3 and CD28 for 5 min in the presence of cytokines IFN-γ (50 ng/ml; PeproTech) or IL-6 (20 ng/ml; PeproTech) and lysed. Proteins were separated by SDS–polyacrylamide gel electrophoresis, blotted on polyvinylidene difluoride membranes, and probed with primary antibodies specific for STAT3 (D3A7) (diluted 1:1000 to 1:2000; all from Cell Signaling Technology) and network graphs were edited using Cytoscape (93, 94).

REFERENCES AND NOTES
S.dispensable for the development and function of regulatory T cells.


also reflect the activation of TGF-β signaling and the engagement of specific transcription factors, such as STAT3, which are known to enhance the expression of the TGF-β1 gene. These findings support the idea that TGF-β signaling plays a significant role in the differentiation of T cells, particularly in the context of regulatory T cells (Tregs).

Furthermore, the role of TGF-β in the regulation of intestinal permeability has been extensively studied. A study by Li et al. (2015) demonstrated that TGF-β1 acts as a paracrine factor to regulate the permeability of intestinal epithelial cells, which is crucial for maintaining the integrity of the mucosal barrier. These results were consistent with previous findings that showed TGF-β1 deficiency leads to increased intestinal permeability, suggesting a potential role for TGF-β1 in the regulation of intestinal barrier function.

In conclusion, the regulation of T cell fate by TGF-β signaling is a complex process that involves various transcription factors and signaling pathways. The identification of new TGF-β receptors and the exploration of their functions in T cell differentiation may lead to the development of novel therapeutic strategies for the treatment of immune-related diseases.
**TGF-β-mediated enhancement of TH17 cell generation is inhibited by bone morphogenetic protein receptor 1 α signaling**

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**BMPing up against TGF-β**

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor–β (TGF-β) family of cytokines. Within this family, TGF-β is critical to prevent the spontaneous activation of self-reactive T cells and promote the differentiation of T helper 17 (TH17) cells. Browning et al. found that the CD4+ T cell–intrinsic activation of BMP receptor 1 α inhibited the differentiation of CD4+ T cells into TH17 cells. Loss of this receptor reprogrammed the transcriptome of effector T cells, promoted exaggerated responses to inflammatory cytokines, and exacerbated disease in a mouse model of colitis. These data suggest that BMPs may antagonize TGF-β to limit effector T cell development and function.