

Smad7 in TGF- β -mediated negative regulation of gut inflammation

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Mice with targeted disruptions of the transforming growth factor- β 1 (TGF- β 1) gene or TGF- β 1 intracellular signalling pathways develop intestinal inflammation. Conversely, TGF- β 1-producing regulatory T cells protect against experimental colitis. Paradoxically, however, TGF- β 1 production is high in the gut of patients with chronic inflammatory intestinal disease, and yet inflammation proceeds unchecked. Here we discuss the functional role of Smad7, an intracellular inhibitor of TGF- β 1 signalling, in the control of gut inflammation by TGF- β 1. In particular, we delineate a scenario in which the high expression of Smad7 in inflammatory cells renders them unresponsive to TGF- β 1 and propose that control of Smad7, not TGF- β 1 production, is a key determinant in understanding how TGF- β 1 negatively regulates gut inflammation.

Transforming growth factor- β 1 (TGF- β 1) is a member of the large TGF- β superfamily, now comprising >40 structurally related proteins, most of which can be classified into three broad groupings: TGF- β s, bone morphogenetic proteins and the activins or inhibins [1]. Three isoforms of TGF- β , termed TGF- β 1, - β 2 and - β 3, are present in mammals. TGF- β s are synthesized as high-molecular-weight complexes made up of TGF- β , latency-associated protein (LAP) and latent TGF- β -binding proteins. Proteolytic cleavage of LAP by plasmin, or a conformational change in LAP induced by thrombospondin, are two of the ways in which active TGF- β can be produced [2,3]. Active TGF- β then binds to a serine/threonine kinase receptor, termed the TGF- β type II receptor, which in turn phosphorylates and activates the type I TGF- β receptor. Both TGF- β receptor subunits are required for signalling. The access of TGF- β s to their receptors is also a tightly controlled phenomenon and there is evidence that several molecules regulate the activation of TGF- β receptors, acting either as ligand-binding traps, sequestering the TGF- β and barring its access to the membrane receptor, or as accessory receptors or co-receptors, which enhance ligand binding to the signalling receptors [4].

Since its first biochemical characterisation in 1983, it has become evident that TGF- β 1 is able to regulate a

complex array of cellular processes, including proliferation, differentiation, motility and death, in a cell-specific manner. In epithelial cells, for example, TGF- β 1 is anti-proliferative, whereas in fibroblasts, it can induce proliferation. TGF- β 1 consistently has been linked to several human pathogenic processes, such as fibrosis, inflammation and carcinogenesis [1,5–7]. TGF- β 1 also has a major role in the regulation of immune-cell functions. Indeed, TGF- β 1-knockout mice develop severe multi-organ inflammation, which begins in the neonatal period and results in early death [8].

TGF- β 1 is produced by many different cell types, however, particular attention has been paid to its production by regulatory T (Tr) cells. There is evidence that secreted TGF- β 1 or membrane bound TGF- β 1 on regulatory cells can prevent intestinal inflammation [9]. Conversely, abrogation of TGF- β 1 signalling using dominant negative receptors in T cells alone is sufficient to break T-cell homeostasis and induce T cell-mediated inflammatory lesions in various organs, including the intestine [10]. In cases where it has been investigated, such as in mouse models of gut inflammation, the stimulus for the immune response that damages the gut is the normal bacterial flora [9,11,12]. This has led to the general notion that pathogenic T-cell responses to the flora are kept in check by Tr cells, including those producing TGF- β 1 [9]. However, TGF- β 1 is made by many mucosal cell types other than T cells and is present in abundance in inflamed tissue [13]. In this situation, it is problematic to imagine how T cell-derived TGF- β 1 could specifically downregulate ongoing inflammation in the mucosa, when T cells probably only contribute to a small fraction of the bioavailable TGF- β 1 in the tissues.

Thus, although a role for TGF- β 1 in control of gut inflammation is established and is not controversial, translation of elegant mouse experiments into therapeutic interventions in humans requires a clearer understanding of TGF- β 1 activity in the human gut. Here, we discuss the functional role of the inhibitor Smad7 in the control of gut inflammation by TGF- β 1. We delineate a scenario in which high Smad7 in inflammatory cells renders them unresponsive to TGF- β 1 and propose that control of Smad7, not TGF- β 1 production, is a key determinant in understanding how TGF- β 1 regulates gut inflammation.

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Smad signaling with particular relevance to inflammatory bowel disease (IBD)

Presence or absence of TGF- β 1 is not a particularly accurate reflection of the role of this cytokine in tissues. Between 1996 and 1999, great advances were made in elucidating the TGF- β 1-associated signalling pathway, which has informed understanding of how TGF- β 1 regulates immune responses at the mucosal level. The first clues came from genetic studies of TGF- β 1-related family members in *Drosophila* and *Caenorhabditis elegans*, which led to the functional characterisation of 'Smad' proteins [14,15]. In the canonical signalling pathway, activated TGF- β receptors phosphorylate and activate the Smad2 and Smad3 transcription factors, which form heterodimeric complexes with Smad4, enter the nucleus and regulate the activity of target genes, either directly by binding to cognate DNA consensus sites or indirectly by interaction with other transcription factors [16]. Mitogen-activated protein kinases (MAPKs) and protein kinase C can also interfere with either the nuclear translocation or the binding of Smad3/4 complexes to DNA and so regulate TGF- β 1 signalling [17,18]. Moreover, the serine-threonine protein kinase B can interact directly with unphosphorylated Smad3, thereby preventing its phosphorylation and nuclear translocation [19]. Other pathways can be directly activated by TGF- β 1. These include components of the MAPK pathway, such as Ras, Raf, ERK, p38 and JNK, the phosphatidylinositol-3 kinase cascade, as well as regulators of cadherin junctions, RhoA and Rac [17,20,21].

The important role of Smad3 as an essential mediator of the TGF- β 1-induced anti-inflammatory and suppressive activities at the mucosal level emerges from studies in mice with targeted deletion of the *Smad3* gene. The animals are viable but die from defects in mucosal immunity at 1–6 months of age. Mutant mice have diminished cell responsiveness to TGF- β 1 and massive infiltration of T cells and multiple pyogenic abscesses in the stomach and intestine [22].

When Smad signalling was studied in normal human gut mucosa, it was observed that in whole biopsies and isolated lamina propria mononuclear cells (LPMCs) (a mixture of lymphocytes, plasma cells, macrophages, dendritic cells, myofibroblasts and endothelial cells) there was a basal level of phosphorylated Smad3, which was rapidly upregulated by the addition of exogenous TGF- β 1 [23]. By contrast, samples from patients with active IBD exhibited reduced levels of endogenous phosphorylated Smad3, which was not enhanced by stimulation with exogenous TGF- β 1. Immunoprecipitation experiments showed that in IBD LPMCs there was a marked decrease in Smad3–Smad4 complexes [23]. Together, these results indicate that in patients with IBD, there is a disruption of TGF- β 1 signalling despite the abundance of TGF- β 1 in the inflamed gut.

Signal-transduction pathways have their own intracellular regulators, and in recent years evidence has accumulated that shows that an inhibitory Smad, Smad7, blocks TGF- β 1 signalling. This is due to the ability of Smad7 to physically interact with the activated TGF- β type I receptor and prevent the docking and

phosphorylation of Smad2 and 3 to the TGF- β type I receptor [24,25]. Smad7 protein is increased in IBD LPMCs and TGF- β 1 cannot prevent proinflammatory cytokine production by IBD LPMCs. However, specific inhibition of Smad7 in LPMCs and in explant tissues from patients with IBD (using an antisense strategy) restores both Smad3 phosphorylation and the ability of TGF- β 1 to inhibit cytokine production [23]. TGF- β 1 also completely prevents tumor necrosis factor- α (TNF- α)-induced NF- κ B activation in LPMCs from normal individuals by a mechanism involving the TGF- β 1-induced upregulation of IKB α [26]. In samples from IBD patients, however, NF- κ B activation proceeds unchecked and exogenous TGF- β 1 has no effect. If Smad7 is reduced with anti-sense, Smad3 activation is restored and NF- κ B activation is immediately inhibited by endogenous TGF- β 1 [26]. Upregulation of Smad7 is not, however, a specific IBD-immune defect because it is also overexpressed in *Helicobacter pylori* (Hp)-associated gastritis [27].

These studies raise the exciting possibility that resolution of chronic inflammation in the gut might be accomplished by enabling endogenous immunosuppressive mechanisms to function, rather than blocking proinflammatory pathways directly. Reducing Smad7 enables the abundant TGF- β 1 in inflamed tissues to become functional. In this context, there has never been a satisfactory explanation for the high placebo rate in clinical trials of IBD therapy, however, the role of TGF- β 1 signalling in this phenomenon seems worthy of investigation.

Control and localisation of Smad7 in cells

If Smad7 is the crucial regulator of whether or not a cell can respond to TGF- β 1, then an obvious question is what controls the expression and localisation of Smad7 in cells? The first studies suggested a simple linear pathway with Smad7 being transcriptionally regulated by external stimuli. Smad7 is induced in cell lines by the STAT1 (signal transducer and activator of T cells 1) pathway following activation with interferon- γ (IFN- γ) or interleukin-7 (IL-7), or by activated NF- κ B following stimulation of cells with TNF- α [28–30]. TGF- β 1 itself can also increase Smad7 [25]. In inflamed gut, especially Crohn's disease, IFN- γ and TNF- α are produced in excess and STAT1 and NF- κ B activity are particularly prominent [31,32]. This would therefore suggest that increased Smad7 is simply a downstream consequence of inflammation. However, a quantitative analysis of Smad7 RNA has revealed no difference between IBD and normal intestinal samples (G. Monteleone *et al.*, unpublished). In addition, Smad7 protein remains unchanged after blocking IFN- γ and Stat1 or TNF and NF- κ B activities in IBD LPMCs [26]. These findings suggest a greater complexity in the *in vivo* regulation of Smad7 than might be expected from *in vitro* studies, and that in IBD, Smad7 is regulated at the post-transcriptional level.

One important mechanism of control involves the export of Smad7 from the nucleus to the cytoplasm and the plasma membrane by the ubiquitin ligases Smurf1 and 2 [33,34]. Although Smad7 does not have nuclear export signals (NESSs), Smurf1 and 2 have functional

NESs, interact with high affinity with Smad7 and enable it to associate with the activated TGF- β type I receptor. Smurfs also induce polyubiquitination and degradation, not only of Smad7, but also of TGF- β type I receptor, in the 26S proteasome, resulting in further repression of TGF- β signalling. Whether the high expression of Smad7 seen in IBD mucosa associates with changes in the expression and/or activity of Smurfs remains, however, to be clarified.

Smad7 also undergoes dynamic post-translational modifications, which make the protein resistant to proteasome-mediated degradation in the cytoplasm. The stability of Smad7 is controlled by competition between acetylation and ubiquitination. The transcriptional coactivator p300 interacts with Smad7 and leads to its acetylation on lysine residues. Because the same residues are targeted by Smurf-mediated ubiquitination, their acetylation prevents the ubiquitination and protects Smad7 protein against proteasomal degradation [35]. Smad7 protein expression is also regulated by Arkadia, an intracellular protein widely expressed in mammalian tissues, including the adult gut [36]. Arkadia interacts with Smad7 and induces its degradation through polyubiquitination. A final level of complexity is introduced by the fact that, whereas Smad7 is itself induced by TGF- β 1, Arkadia is decreased by TGF- β 1. Thus, in a cell expressing high levels of Smad7 and unresponsive to TGF- β 1, Arkadia-mediated degradation of Smad7 is a mechanism to enable the cell to respond to TGF- β 1. Recently, it has also been shown that Smad7 expression is also regulated by Jun activation domain-binding protein 1 (JAB1), a coactivator of the transcription factor c-Jun. JAB1 is also a component of the constitutively photomorphogenic 9 (COP9) signalosome, which consists of eight subunits

exhibiting significant homologies to the eight subunits of the 26S proteasome lid complex. Indeed, the COP9 signalosome is involved in protein degradation by the ubiquitin-proteasome pathway. JAB1 interacts with Smad7 in the nucleus, enhances nuclear export and promotes ubiquitination and degradation of Smad7 [37].

We can conclude, therefore, that changes in the expression and/or activity of molecules that enhance or decrease proteasome-dependent degradation dictate the level of Smad7 inside a cell will determine whether or not the cell is able to respond to TGF- β 1 (Figure 1).

Functional relevance of Smad7 in the gut mucosa

If TGF β 1 is suppressing pathogenic T-cell responses to the bacterial flora in the gut, it would be expected that mice whose T cells overexpress Smad7 would develop an IBD. Surprisingly, however, these mice are essentially normal, despite the fact that their T cells do not respond to TGF β -1 with Smad2 phosphorylation [38]. However, given what is now known about the control of Smad7 inside cells, there is the possibility that, *in vivo*, most of the overexpressed Smad7 in the transgenic mice remains in the nucleus or is degraded by the proteasome.

Smad7 in mononuclear cells probably prevents suppression of cell division and proinflammatory cytokine production by TGF- β 1. Specific inhibition of Smad7 in mucosal tissues also results in reduced expression of T-bet, a Th1-associated transcription factor, and diminished production of Th1-type cytokines [23,27]. In normal intestinal LPMCs, TGF- β 1 stimulates the induction of I κ B α and hence inhibits the activity of NF- κ B in response to inflammatory cytokines. Conversely, the negative regulation of NF- κ B by TGF- β 1 does not occur in IBD

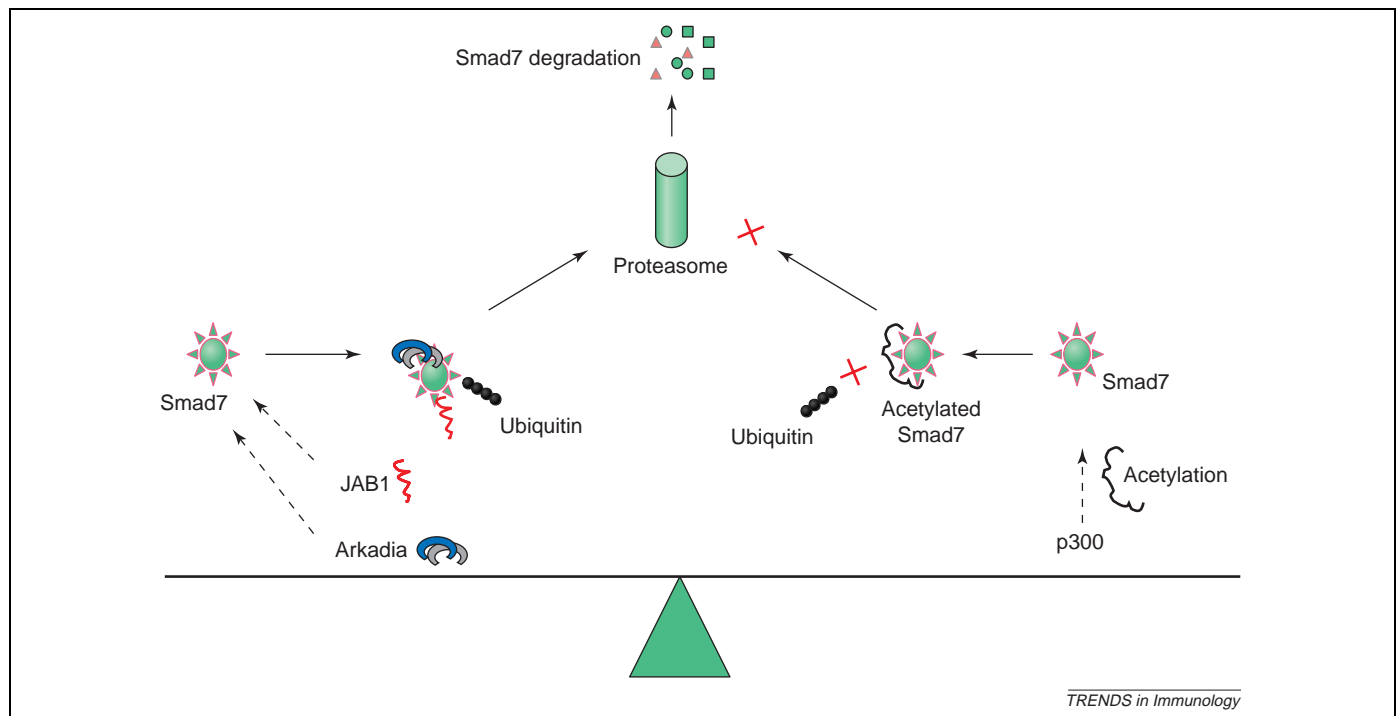


Figure 1. Smad7 protein expression is a tightly regulated phenomenon that relies on the activity of molecules, which either enhance or inhibit Smad7 degradation in the proteasome. While Arkadia and JAB1 promote Smad7 ubiquitination and subsequently proteasome-dependent degradation, p300 enhances acetylation of Smad7, thus preventing ubiquitination and degradation. Abbreviation: JAB1, Jun activation domain-binding protein 1.

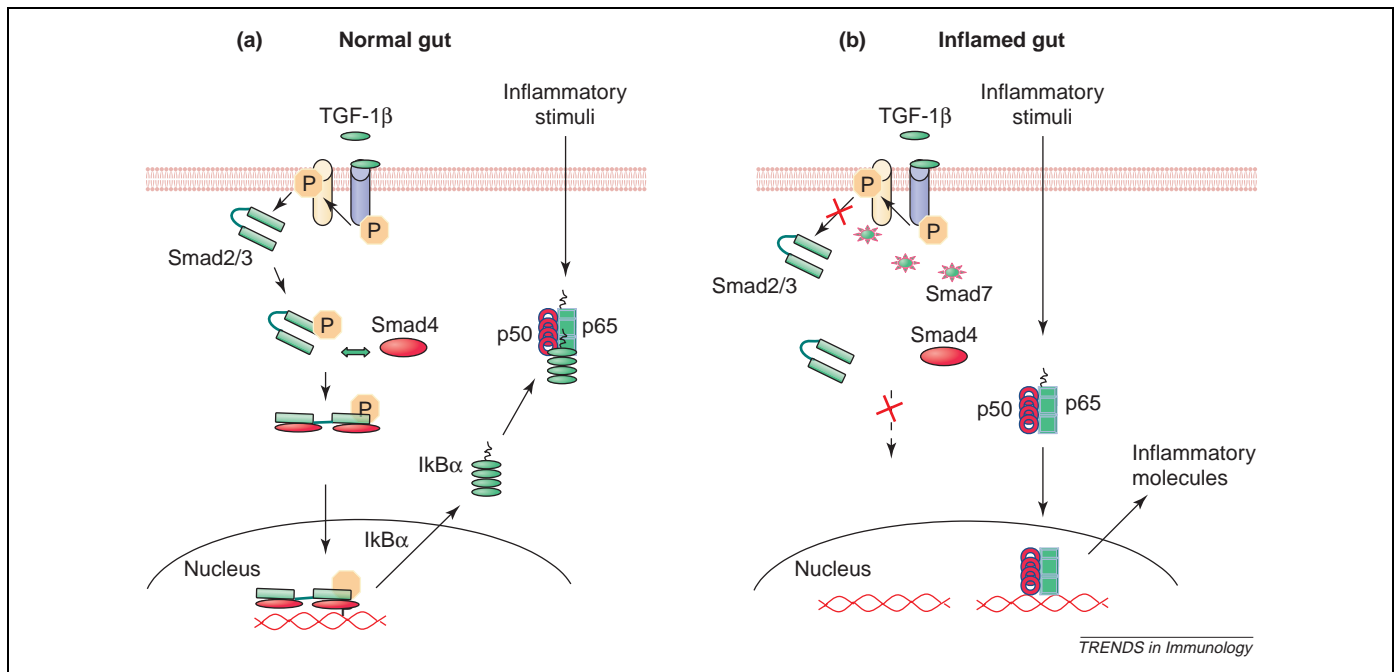


Figure 2. In normal intestinal mucosal cells (a), transforming growth factor- β 1 (TGF- β 1) signalling negatively regulates NF- κ B activation by stimulating the induction of the negative regulator I κ B α . By contrast, in inflamed gut (b), mucosal cells express high levels of Smad7. TGF- β 1 therefore cannot dampen NF- κ B activation, and thereby contributes to sustaining inflammatory chemokine and cytokine synthesis.

LPMCs unless Smad7 is inhibited [26]. These observations depict a scenario in which the TGF- β 1-associated Smad pathway helps maintain normal intestinal homeostasis by regulating NF- κ B [26,39], whereas defective TGF- β 1 signalling, due to exaggerated Smad7, sustains high NF- κ B activity, thereby expanding the local inflammatory response in IBD (Figure 2).

Smad7 and Tr cells

At the moment, there is very little evidence for Tr-cell activity as an important determinant of clinical gut inflammation. Murine studies clearly suggest a role for TGF- β 1, either secreted by T cells or membrane bound, in the control of IBD in the SCID (severe combined immunodeficiency) and RAG transfer model of colitis by CD4, CD25⁺ Tr cells [2,40]. However, the limitations of this model need to be pointed out and, even in mice, there is little evidence for Tr cells suppressing colitis in an animal with an intact immune system. Further complexity involves TGF- β control of Tr cells. TGF- β 1 induces Tr-cell activity and also Foxp3 transcripts in CD4⁺CD25⁻ cells in mouse and humans [41,42]. TGF- β 1 also induces Smad7 in these cells, however, this is abolished by Foxp3, suggesting a circuit whereby TGF- β 1 can drive the generation of Tr cells without the cells becoming unresponsive. This agrees with the demonstration that Tr cells that mediate type V collagen-induced tolerance to lung allografts in rats do not express Smad7 and, therefore, are permissive to TGF- β 1-mediated signalling [43]. However, although TGF- β 1 induces Tr cells from the naive T-cell pool with potential therapeutic implications, it remains unclear if this pathway is operating in inflamed human intestinal mucosa, where the high levels of Smad7 could render induction of Foxp3 by TGF- β 1 unlikely.

Concluding remarks

There is absolutely no doubt that TGF- β 1 is a cytokine of crucial importance in dampening tissue-damaging immune responses, especially in the gut. However, we propose that the effects of TGF- β 1 in the microenvironment are not linearly related to its relative abundance. Inhibitory Smads, such as Smad7, control the strength of the signal from the cell surface to the nucleus and thus control cell function. This, then, logically puts regulation of Smad7 inside the cell as a topic of considerable importance, reflected by the number of recent papers in this area. Initially it was considered that Smad7 was transcriptionally regulated, whereas the situation is now more complex, with important components being the control of nuclear export and Smad7 ubiquitination and degradation.

The implication from these results is, therefore, that specific pharmacological inhibition of Smad7 should enable endogenous TGF- β 1 in the inflamed gut and perhaps other inflammatory sites to negatively regulate proinflammatory pathways, which directly or indirectly promote tissue injury. Blocking Smad7 could thus represent a new and promising therapeutic approach for controlling chronic intestinal inflammation. However, cell-specific expression of Smad7 should be taken into account because TGF- β 1 has different effects on different cell types, and in some cell types Smad7 might be physiologically relevant to limiting the detrimental effects of TGF- β 1, such as its ability to induce myofibroblasts to secrete collagen and induce fibrosis.

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