Smad7 expression in T cells prevents colitis-associated cancer

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Abstract:

Patients with inflammatory bowel disease (IBD) have an increased risk of developing colorectal cancer due to chronic inflammation. In IBD, chronic inflammation relies upon a TGFß signaling blockade, but its precise mechanistic relationship to colitis-associated colorectal cancer (CAC) remains unclear. In this study, we investigated the role of the TGFß signaling inhibitor Smad7 in CAC pathogenesis. In human colonic specimens, Smad7 was downregulated in CD4+ T cells located in the lamina propria of patients with complicated IBD compared to uncomplicated IBD. Therefore, we assessed CAC susceptibility in a transgenic mouse model where Smad7 was overexpressed specifically in T cells. In this model, Smad7 overexpression increased colitis severity but the mice nevertheless developed fewer tumors than non-transgenic mice. Protection was associated with increased expression of IFNgamma and increased accumulation of cytotoxic CD8+ and NKT cells in the tumors and peritumoral areas. Moreover, genetic deficiency in IFNgamma abolished the Smad7-dependent protection against CAC. Taken together, our findings defined a novel and unexpected role for Smad7 in promoting a heightened inflammatory response that protects against CAC.

Precís:

This study illustrates that chronic inflammation in colitis does not necessary heighten risks of colorectal tumorigenesis, based on epigenetic differences in the immune microenvironment that dictate whether colitis-associated inflammation promotes or retards tumor development.
Introduction:

Chronic inflammation is believed to be a leading cause of cancer in various organs [1]. Indeed, chronic release of oxygen- and nitrogen-reactive species induces genomic damage thus contributing to tumor initiation and promotion, while proinflammatory cytokines stimulate dysplastic cell proliferation and neoangiogenesis thereby supporting tumor progression.

Transforming Growth Factor-(TGF)-β1, hereafter indicated as TGFβ, is a crucial negative regulator of inflammation and the TGFβ knockout mice develop a fatal multiorgan inflammatory disease [2]. The anti-inflammatory properties of TGFβ rely, at least in part, on its ability to suppress T lymphocyte activities. TGFβ prevents the activation and differentiation of naïve T in proinflammatory Th1 and Th2 cells, while inducing them to acquire a suppressive phenotype (i.e. Tregs) [3,4]. Moreover TGFβ suppresses proliferation and effector/memory T helper cells activity [5].

TGF β signaling is tightly regulated in T cells. The binding of TGFβ with its specific receptor complex (i.e. TGFβ receptor type I and type II), causes the phosphorylation of the cytoplasmic molecules Smad2 and Smad3 which in turn migrate into the nucleus where they regulate the expression of several proinflammatory genes. TGFβ signal transduction is negatively regulated by Smad7 which prevents Smad2/3 phosphorylation by recruiting on the intracellular domain of the TGF β receptor type I the GADD34 complex and the protein phosphatase 1 to dephosphorylate it [6]. Moreover Smad7 has been shown to promote the ubiquitination and proteosomal degradation of the TGF β receptor complex [7-10].

Smad7-mediated suppression of the TGFβ signaling has been well documented in the inflamed gut of patients with Crohn’s disease (CD) and ulcerative colitis (UC), the two major forms of inflammatory bowel diseases (IBD), which are associated with an increased risk to develop colitis-associated colorectal cancer (CAC). Downregulation of Smad7 with a specific antisense oligonucleotide restores TGFβ signaling thus suppressing proinflammatory cytokine synthesis in vitro [11]. Moreover, Smad7 overexpression makes T cells resistant to the suppressive activity of Tregs and Smad7 downregulation restores Treg-mediated...
suppression [12]. In vivo, the downregulation of Smad7 attenuates IBD-like experimental colitis in mice [13].

Although the above findings suggest that high Smad7 can facilitate colorectal cancer progression by sustaining chronic inflammation, studies in mice with a non-physiologic block of TGFβ activity in T cells operated by the dominant negative TGFβ receptor, have provided divergent results on the role of TGFβ in cancer cell growth and metastasis [14,15]. Therefore, in this study investigated the role of Smad7-induced block of TGFβ signaling on the initiation and progression of CAC. Here we demonstrate that the development of CAC in IBD patients is associated with a significant downregulation of Smad7 in CD4+ T cells. Moreover, we have identified a novel and unexpected protective role of Smad7 against CAC development in a transgenic mouse model in which Smad7 results selectively overexpressed in T cells.
Material and methods:

Cell lines: YAC-1 lymphoma cell cells were obtained from the American Type Culture Collection (ATCC-TIB-160, LGC, UK). The above-mentioned cell lines were procured more than 6 months ago and have not been tested recently for authentication in our laboratory.

Mice: C57BL/6 CD2-Smad7Tg mice, generated as previously published [12], C57BL/6 IFNγ−/− mice purchased by Jackson Laboratories (Bar Harbor, Main, USA) and IFNγ+/−Smad7Tg, were hosted in the SPF animal facility at the University of Rome Tor Vergata (Italy). See also supplemental material & methods.

Azoxymethane (AOM)/Dextran Sulphate Sodium (DSS) protocol: All mice were male and 6-8 weeks old and experimental protocols were performed according with the local institutional guidelines. To induce CAC, mice were injected intraperitoneally with a single dose (10 mg/kg) of the alkylant agent AOM (Sigma Aldrich, Milan, Italy) followed by three cycles of 2.5% DSS (MW: 36000-50000 Da; MP Biomedicals U.S.A) given in the drinking water for 3 days. Mice were kept on water for 2 weeks after each DSS cycle. In some experiments mice were treated with DSS only to induce chronic colitis but not CAC.

Endoscopic procedures: Tumor development and colitis were screened by a miniaturized endoscope (Coloview System, Karl Storz, Germany). Tumors were counted and scored as previously described [16]. Colitis was graded according to the mouse endoscopic index of colitis severity (MEICS).

Histologic Analysis and Immunohistochemistry: Colonic sections from mice were obtained for H&E staining and analyzed by light microscope (Olympus, Melville, NY, USA). Colitis was scored as previously published [17]. In some cases colonic sections were used for indirect immunofluorescence using rat anti mouse CD8 or CD4 (BD Pharmigen, Heidelberg, Germany) or CD68 (Acris, Herford, Germany) or Smad7 (Santa Crux Biotechnology, Palo Alto, CA, USA) or FoxP3 (eBioscience, Frankfurt, Germany). Antigen detection was obtained with Tyramide (Cy3 and FITC) according to the manufacturer’s guidelines (PerkinElmer). Paraffin-embedded colonic sections from patients with active IBD, IBD-related CAC, and controls were obtained.
from the Institute of Pathology (Bayreuth, Germany) as previously reported [18]. Patients’s characteristics are reported in Suppl. Table 1. Sections were used for H&E staining or stained with anti human CD4 (BD pahrmigen) and anti human Smad7 specific antibodies (R&D System, Wiesbaden, Germany) and analyzed by confocal microscope are previously published [18]. Number of positive cells was quantified by ImageJ (NIH, Bethesda, USA).

Isolation of Lamina propria mononuclear cells (LPMC) and Tumor-Infiltrating Lymphocytes (TILs): LPMC and TILs were isolated accordingly to standard protocols. See supplemental material & methods.

Flow cytometry: The following anti-mouse conjugated antibodies: CD4-FITC, CD8-PE, CD49b(DX5)-PE, CD3-FITC, IL-17A-PE, IFNγ-APC (BD Pharmigen) and AnnexinV-PE (BD Pharmigen) were used. IL-17A- and IFNγ-expressing intracellular staining on LPMC and TILs were analyzed after 5 hours stimulation with PMA (40 ng/ml) and ionomycin (1 μg/ml; Sigma-Aldrich), in the presence of monensin (2 μmol/l; eBioscience) according to standard protocols. Stained cells were analyzed by flow cytometry (FACScalibur, BD, San Jose, CA, USA).

Cytotoxicity assay: Cytotoxic activity was measured by flow-cytometry based assay as previously described [19]. (Detailed description in supplemental material & methods).

RNA extraction, cDNA preparation and real-time PCR: Total RNA was isolated using TRIzol reagent (Invitrogen, Karlsruhe, Germany) and retro-transcribed with the M-MLV reverse transcriptase (Invitrogen, Karlsruhe, Germany). Real-time PCR was performed using a SYBR Green-based PCR using iQ SYBR mix (Bio-Rad, Hercules, CA). Beta-actin was used to normalize gene expression results. Specific primers were designed on the cDNA sequence provided by ENSEMBL database (Suppl.Table 2).

Statistical analysis: Student’s t-test and the non parametric Mann-Whitney test were used to calculate statistical significance between groups. Spearman rank correlation test was used to calculate correlation between inflammation grade and tumor score.
Results:

**Smad7 is downregulated in inflamed colon of patients with IBD-related CAC.**

To assess Smad7 expression in patients with CAC, sections from IBD patients with or without CAC and controls (Fig. 1A) were stained for Smad7. A marked accumulation of Smad7 expressing cells was seen in the lamina propria of CD and UC patients without CAC in comparison to controls. In contrast, the number of Smad7-positive cells did not differ between IBD patients with CAC and controls (Fig. 1B-C). As shown in figure 1D, the majority of the Smad7-expressing cells coexpressed the CD4. These data indicate that IBD not complicated by CAC is characterized by accumulation of Smad7+CD4+ T cells in the lamina propria and that the number of these cells is significantly reduced in IBD patients with CAC.

**Smad7Tg mice develop severe DSS-mediated colitis.**

To investigate the functional role of Smad7 in CAC development, we used a T cell-specific Smad7 transgenic mouse as previously described [12]. We initially assessed whether, analogously to human IBD, high expression of Smad7 in T cells was associated with enhanced mucosal inflammation. To this end, 6-8 weeks old wild type (Wt) and Smad7 transgenic mice (Smad7Tg) received three cycles of 2.5% DSS to mimic human chronic relapsing colitis (Fig. 2A). Endoscopy performed at the end of the experimental protocol (day 75) showed colitis in both Wt and Smad7Tg mice (Fig. 2B). However Smad7Tg mice had a more severe colitis as shown by the endoscopic score of colitis (Fig. 2C). Histological analysis of colonic specimens obtained at the end of the experiment confirmed the presence of inflammation in both Wt and Smad7Tg mice (Fig. 2D) even though Smad7Tg mice had a significant higher histological score (Fig. 2E). Both DSS-treated Wt and Smad7Tg mice were characterized by high expression of the proinflammatory cytokines IFNγ, TNFα, IL-6 and IL-17A although these cytokines resulted more expressed in the Smad7Tg (Fig. 2F). Moreover Smad7Tg mice were characterized by a higher number of CD4+ and CD8+ T cells accumulating in the lamina propria as compared to Wt mice (Suppl. Fig. 1), with no differences in terms of CD68-expressing macrophages. Finally, as observed in human IBD, the stronger inflammation observed in the Smad7Tg was
associated with the accumulation of a higher number of FoxP3+ regulatory T cells [20]. These data indicate that, similarly to humans, high Smad7 expression in T cells makes mice more susceptible to colitis.

**Smad7Tg mice are largely protected against CAC.**

To investigate whether the more severe inflammation observed in Smad7Tg mice was associated with an increased susceptibility to CAC, mice underwent the AOM/DSS model of CAC (Fig.3A) which is characterized by the induction of adenomas with histological signs of high grade dysplasia by 9-10 weeks [16,21].

As expected, at the end of the experiment, Wt mice developed multiple colonic tumors (Fig.3B). As observed in humans, Smad7 was highly expressed by LPMC of peritumoral areas. In contrast, Smad7 was mostly expressed by dysplastic epithelial cells, with significantly fewer positive cells infiltrating the tumor stroma as compared to peritumoral LPMC (Suppl. Fig.2A). Surprisingly, Smad7Tg mice developed fewer tumors as compared to Wt (Fig.3C). The tumor score, which takes into account not only the number of tumors but also their size, was significantly higher in Wt than Smad7Tg mice (Fig.3D). Histological analysis of peritumoral areas showed higher inflammatory cell infiltrate in the mucosa and submucosa associated with modest mucosal hyperplasia, conserved crypt architecture and minimal goblet cell depletion in Wt mice (Fig.3Ea-c). In contrast, Smad7Tg mice were characterized by dense inflammatory cell infiltrate both in the mucosa and submucosa, mucosal hypertrophy, crypts distortion and rarefaction and strong depletion of goblet cells (Fig.3Ed-f). These differences were confirmed by the histological score of sections of Wt and Smad7Tg mice (Fig.3F) and this inversely correlated with the tumor score of both groups of mice (Fig.3G).

Accordingly, more CD4+ and CD8+ T cells in both tumor and peritumoral areas were seen in the Smad7Tg in comparison to the Wt while no differences were observed in CD68+ macrophages (Suppl.Fig.2B).

To assess whether the higher inflammation and lower tumor incidence observed in the Smad7Tg mice was related to a defect in regulatory T cells (Tregs) accumulation, the expression of the Treg-specific transcription factor FoxP3 and TGFβ was evaluated in peritumoral lamina propria cells (LPMC) and in tumor infiltrating lymphocytes (TILs). Although TGFβ expression was higher in TILs in comparison to LPMC isolated
from both the groups, there was no significant difference between Wt and Smad7Tg mice (Suppl. Fig. 3A left). FoxP3 resulted more expressed in the LPMC of highly inflamed Smad7Tg mice in comparison to the Wt while no significant difference was observed among TILs of the two groups as shown by real-time PCR and immune-fluorescent staining (Suppl. Fig.3A-C). Altogether these results indicate that the higher inflammation seen in the Smad7Tg mice might interfere with CAC despite the accumulation of FoxP3-expressing cells in the peritumoral areas.

**Smad7Tg mice exhibit a marked intratumoral Th1-mediated immune response.**

Cytokine expression analysis in the LPMC and TILs of Wt and Smad7Tg mice showed IFNγ, TNFα, IL-6 and IL-17A to be the most expressed cytokines in both Wt and Smad7Tg mice (Fig.4A-B, left). In keeping with the more severe inflammation observed in the Smad7Tg, IFNγ, IL-6 and IL-17A mRNA levels were nearly 8 fold higher in the Smad7Tg LPMC in comparison to Wt mice (Fig.4A, right panel). In contrast, in the tumors, only IFNγ was more than 4 fold higher in Smad7Tg mice while all the other cytokines were downregulated as compared to the Wt (Fig.4B, right panel). These data suggest that the peritumoral areas of both Wt and Smad7Tg mice are characterized by a similar cytokine profile while in the tumors of Smad7Tg there is a prevalent IFNγ-associated immune response. Consistently, both Tbet and RORγt, the Th1 and Th17-specific transcription factors, were overexpressed in Smad7Tg LPMC (Fig.4C), while in the Smad7Tg TILs there was a higher Tbet and lower RORγt expression as compared to Wt (Fig.4D). Consistent with the mRNA data, the number of IFNγ-expressing CD4+ T cells resulted about 10 fold higher in the Smad7Tg in comparison to the Wt at both LPMC and TILs level (Fig.4E). In contrast, the number of IL-17A-expressing cells in the Smad7Tg was higher in the LPMC but lower in the TILs as compared to Wt. These data indicate that in contrast to the Wt, a prevalent Th1 immune response develops in the tumor microenvironment of Smad7Tg mice.

**A critical role of IFNγ in mediating protection of Smad7Tg against CAC.**

Since IFNγ is upregulated in Smad7Tg mice, we next assessed the functional role of this cytokine in tumor protection seen in these mice. To this end IFNγ-deficient Smad7Tg (IFNγ−/− Smad7Tg ) mice were used in the
In the IFN$\gamma^{-/-}$/Smad7Tg mice, tumor incidence was restored to levels observed in the Wt (Fig.5A-B). However the tumors score was still significantly lower as compared to Wt (Fig.5C) thus suggesting that further factors other than IFN $\gamma$ contribute to suppress tumor growth in Smad7Tg mice. Histological analysis of colonic sections from the three groups of mice showed severe inflammation in both Smad7Tg and IFN$\gamma^{-/-}$/Smad7Tg mice although in the latter many dysplastic areas were detected (Fig.5D, outlined). TNF$\alpha$, IL-6 and IL-17A were the most expressed cytokines in both LPMC and TILs isolated from IFN$\gamma^{-/-}$/Smad7Tg mice (Fig.5E-F, left). However, TNF$\alpha$, IL-6 and IL-17A expressed by both LPMC and TILs were reduced in the IFN$\gamma^{-/-}$/Smad7Tg as compared to Wt mice (Fig.5E-F, right). These data indicate that colitis develops in Smad7Tg mice independently of IFN$\gamma$ but its expression is required for protection against CAC in these mice.

**Smad7Tg mice are characterized by IFN$\gamma$-dependent accumulation of CD8$^+$ and NKT cell into the tumors.**

To investigate how IFN$\gamma$ regulates tumor growth in Smad7Tg mice, we evaluated the percetages of CD4$^+$, CD8$^+$, CD3-DX5$^+$ Natural Killer (NK) and CD3+DX5$^+$ NK-T cells in LPMC and TILs isolated from Wt, Smad7Tg and IFN$\gamma^{-/-}$/Smad7Tg at the end of the AOM/DSS protocol by flow cytometry. Although not statistically significant, we observed an IFN$\gamma$-dependent accumulation of CD8$^+$ and NKT cells in the LPMC of Smad7Tg (Fig.6A). However the number of NKT and CD8$^+$ cells in the TILs of Smad7Tg mice was significantly higher in comparison to Wt (Fig.6B). In contrast, the percentage of both intratumoral CD8$^+$ and NKT cells resulted similar to Wt in IFN$\gamma^{-/-}$/Smad7Tg mice. No significant differences were observed in CD4$^+$ and NK cell accumulation in both LPMC and TILs of the three groups of mice. Similar results on CD4$^+$ and CD8$^+$ T cell accumulation in the tumor and peritumoral areas of Wt, Smad7Tg and IFN$\gamma^{-/-}$/Smad7Tg mice were obtained by immunofluorescent staining of colonic sections (Suppl. Fig.4).

*IFN$\gamma$ increases CD8$^+$ and NK/NK-T cell-mediated cytotoxicity in the tumoral and peritumoral areas of Smad7Tg mice.*
Th1 cells are required to activate cytotoxic lymphocytes and to generate anti-tumor immune response in different experimental systems. Accordingly with the dominant Th1-mediated immune response observed in the Smad7Tg mice, the cytotoxicity-related markers Perforin1, GranzymeB and FasL mRNA resulted upregulated in the TILs and LPMC of these mice in an IFNγ-dependent manner (Fig. 7A). To assess whether CD8+ and NK/NKT cells isolated from Smad7Tg mice were more cytotoxic, we co-cultured CD8+ and DX5+ cells sorted from mesenteric lymph nodes of Wt, Smad7Tg and IFNγ−/−Smad7 Tg mice with YAC-1 lymphoma cell line. CD8+ and DX5+ cells isolated from Smad7Tg mice induced more YAC-1 cell death (Fig. 7B). The higher cytotoxic activity of Smad7Tg effector cells was completely reverted by the loss of IFNγ. Consistently, tumors developed in the Smad7Tg mice exhibited more TUNEL positive cells than neoplastic lesions developed in Wt and IFNγ−/−Smad7 Tg mice (Fig. 7C).
Discussion:

Patients with IBD have an increased risk to develop CAC, which is related to the duration and extent of inflammation. Although circumstantial evidence suggests that severity of colitis could also affect the development of CAC, the role of inflammation in the initiation, promotion and progression of CAC remains poorly characterized [22-24].

The Smad7-dependent block of the TGFβ signaling is thought to sustain chronic inflammation in IBD [11]. Data of the present study confirm and expand our knowledge on this by showing that Smad7 expressing CD4+ T cells that highly infiltrate the intestinal lamina propria of IBD patients, are markedly reduced in CAC, thus suggesting that low Smad7 in T cells might contribute to CAC development in these patients.

In our mouse model, and similarly to human IBD, Smad7 over expression in T cells caused more severe colitis as compared to Wt. However, when we used Smad7Tg mice in the well-established AOM/DSS model of CAC surprisingly we observed a significant and highly reproducible reduction of tumor incidence even in the presence of more severe colitis. Analysis of the cytokines and transcription factors showed higher expression of the Th1-related markers IFNγ and Tbet in the peritumoral areas and even more in the tumors of Smad7Tg mice as compared to the Wt. In contrast, the Th17-related markers IL-17, IL-6 and RORγt were relatively downregulated in the tumors of Smad7Tg mice thus indicating that a Th1-mediated immune response prevails in the transgenics.

Both Th1 and Th17 T cells contribute to intestinal inflammation in different models of colitis and their differentiation is influenced by TGFβ [25-27]. TGFβ prevents the differentiation of Th1 cells directly inhibiting the expression of Tbet and IFNγ [3]. In contrast, in the presence of IL-6, TGFβ promotes Th17 cell differentiation [28,29]. Moreover, recent studies indicate that the TGFβ-mediated suppression of Th1 cells and IFNγ expression promotes the generation of Th17 [30]. These data well fit with our results showing that Smad7-mediated block of the TGFβ promotes the accumulation in the gut of Th1 cells, increases IFNγ expression while reducing the number of Th17 cells especially in the tumor microenvironment.
TGFβ has been also involved in the differentiation of Tregs, a subset of cells implicated in the suppression of the immune response against cancer. As observed in human IBD, the more severe inflammation observed in the Smad7Tg was associated with accumulation of Tregs in both the tumor and peritumoral areas thus ruling out the possibility that less FoxP3+ cells in the Smad7Tg mice may enhance the immune response against cancer [20].

Loss of IFNγ restored susceptibility to CAC in Smad7Tg. IFNγ has been shown to play a pivotal role in the immune response against cancer. In vivo, IFNγ expression protects from tumor in different tumor models [31-33]. In a murine model of CAC, Osawa et al. demonstrated that IFNγ deficient mice are more susceptible to develop CAC [34]. IFNγ expression is also essential for CD8-mediated antitumor activity. This effect can be partially explained by the capacity of IFNγ to induce Fas on dysplastic target cells surface thus inducing cell death by Fas ligand-Fas interaction [35,36]. Moreover, IFNγ increases the expression of MHC class I expression on target cells thus promoting recognition of tumor-associated antigens [37]. IFNγ might also contribute to NK and NKT cell activation and killing activity. However it is worth noting that the tumor score in the IFNγ⁻/⁻Smad7Tg was still lower than in the Wt thus indicating that even in the absence of IFNγ, Smad7 overexpression in T cells can restrain tumor growth.

In the attempt of identifying the mechanism by which IFNγ determines protection against tumors in the Smad7Tg, we observed several effects linked to high expression of this cytokine. Indeed, the higher accumulation and cytotoxic activity of CD8+ and NKT cells observed in the Smad7Tg mice was reverted in the IFNγ⁻/⁻Smad7Tg mice. The high IFNγ-dependent activation and cytotoxic activity of CD8+ and DX5+ NK/NKT cells in the Smad7Tg might be sustained by the prevalent Th1 immune environment operating in these mice. Alternatively, it might be secondary to the Smad7-mediated block of the TGFβ signaling in these subsets of cells as previously reported [38,39].
Although we were unable to provide direct demonstration of a stronger cytotoxic activity toward dysplastic epithelial cells in vivo, due to the difficulties encountered in culturing these cells ex vivo, these data strongly sustain the central role of IFNγ in the protection from colonic tumor development in our model.

We have previously demonstrated that the block of the TGFβ signaling in mice overexpressing the dominant negative form of the TGFβ receptor II (dnTGFβRII) in T cells develop more inflammation and more CAC [14]. The opposite outcomes obtained in the dnTGFβRII and Smad7 transgenic mice might be explained considering the level at which the block of the TGFβ signaling occurs. Indeed, while the expression of dnTGFβRII blunts all the downstream TGFβ-dependent intracellular signals, the result of the Smad7-mediated block at intracellular level is less clear. In agreement with published data, we have previously demonstrated that Smad7 prevents the activation of Smad2 and 3 [12]. However TGFβ is known to activate SMAD-independent pathways mediated by MAP kinases (i.e. p38, Akt, ERK) and the activation of these pathways is not affected by Smad7 expression [40]. Accordingly, while dnTGFβRII transgenic mice develop a rapidly fatal multiorgan autoimmune disease, Smad7Tg mice did not [41]. Therefore a possibility is that in primary tumors, Smad7 might modulate rather than fully inhibit the TGF-beta signaling in T cells and that the net result of Smad7 overexpression is an antitumor immune response instead of the generation of a tumor promoting environment as in the case of dnTGFβRII transgenic mice. However, it is worth noting that in a different system, the block of TGFβ signaling obtained with the overexpression of the dnTGFβRII in CD4+ T cells protected mice from liver tumor metastasis [15], thus suggesting that the TGF-beta intracellular signals required in T cells to favor tumor progression might be tumor stage dependent.

Another possibility to explain the different results obtained with the dnTGFβRII and the Smad7 Tg mice relies on the fact that Smad7 overexpression could inhibit other member of the TGF-beta superfamily (e.g. Activin and BMP) whose role in the tumor immunity is currently poorly understood.

In our system a persisting Th1 mediated immune response characterized by high expression of IFNγ resulted protective toward the development of CAC. This observation is in agreement with the emerging
concept that an indolent but chronic inflammatory process sustains cancer promotion and progression rather than a highly detrimental acute inflammation. Translating this model into humans, one could envision that while acute flares of colitis are required to generate dysplastic cells, a phase of persisting subacute inflammation, allowed by low Smad7 expression, might be more effective at sustaining CAC growth. It is tempting to speculate that the Smad7 overexpression in T cells might block the evolution of the inflammatory process thus maintaining a condition resembling an acute flare in which emerging dysplastic cells are destroyed by the immune system rather than supported in their expansion.
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References:


Figure legend:

**Figure 1:** Smad7 is downregulated in colonic samples of IBD patients complicated by CAC: (A) H&E staining of colonic samples from patients with IBD (both UC or CD) with or without CAC and control (CTRL) patients. Scale bars represent 100μm (upper) and 20μm (lower). (B-D) Immunofluorescent staining of the previously described colonic samples with anti-Smad7 and CD4 specific antibody singularly or in combination. Scale bars in (B) and (D) represent 100 μm and 7.5 μm in the left and right panels respectively. (C) Bars represent mean +/- SEM lamina propria Smad7+ cells (n=5 per group, CD and UC have been pooled n=10). Positive cells were counted in at least 5 HPF/section (*) indicates significant differences between the groups (p<0.05).

**Figure 2:** Smad7Tg mice develop more severe colitis in comparison to Wt mice in the chronic DSS model of colitis: (A) Experimental protocol of chronic DSS colitis. (B) Representative endoscopic pictures collected at day75. (C) Mouse endoscopic index of colitis severity (MEICS) applied to Wt and Smad7Tg mice at day 75. Data were obtained from two independent experiments. (D) H&E staining of representative colonic sections collected from Wt and Smad7Tg mice at the end of the experiment. Scale bars indicate 100μm. (E) Histological score of colonic sections obtained from the different groups. Horizontal bars indicate median value, (*) indicates significant differences between the groups (p<0.05). mRNA expression (F left panel) and fold change relative to the Wt (F right panel) of cytokines in Smad7Tg LPMC. Bars indicate mean value +/- SD where indicated.

**Figure 3:** Smad7Tg mice are protected from tumor development in the AOM/DSS model of colitis associated colorectal cancer (CAC): (A) Experimental protocol of AOM/DSS model of CAC. (B) Representative endoscopic pictures collected at day75. (C) Number of tumors and (D) tumor score of Wt and Smad7Tg mice at the end of the experiment. Pooled results from three independent experiments are shown. Horizontal bars indicate the median value. (*) indicates significant differences between the groups (p<0.01). (E) H&E staining of representative colonic sections of Wt (a) and Smad7Tg (d), magnification of Wt (b) and Smad7Tg (e) tumors, Wt (c) and Smad7 Tg (f) peritumoral areas. Outlining indicates dysplastic areas. Scale bars indicate 100μm. (F) Histological score of colitis was obtained from each animal of the two groups. Horizontal bars indicate median value (*) indicates significant difference between the groups (p<0.05). (G) Correlation between histological score and tumor score in the Wt and Smad7Tg mice pooled together, r²=0.72; p=0.003.
**Figure 4:** Smad7 Tg mice are characterized by Th1 immune response in the tumor microenvironment:

Relative mRNA expression of cytokines in the peritumoral LPMC (A left panel) and TILs (B left panel) isolated from Wt and Smad7Tg mice. (A) and (B) right panels show the fold change of cytokine mRNA expression in Smad7Tg LPMC and TILs relative to the Wt. (C) Tbet and RORγt mRNA relative expression (left panel) in LPMC from Wt and Smad7Tg mice and their fold change in the Smad7Tg relative to the Wt (right panel). (D) Tbet and RORγt mRNA relative expression (left panel) in TILs from Wt and Smad7Tg mice and their fold change in the Smad7Tg relative to the Wt (right panel). Bars indicate mean value +/- SD where indicated. (E) Flow cytometric analysis of LPMC and TIL from Wt and Smad7Tg mice at the end of the experiment. Frequency of IFNγ- and IL-17A-expressing cells was obtained gating on CD4+ cells. Numbers in the quadrants indicate the percentage of positive cells. Representative dot plots of three independent experiments showing similar results are shown.

**Figure 5:** IFNγ is required to protect Smad7Tg mice from tumors (A) Representative endoscopic pictures of Wt, Smad7Tg, IFNγ−/−Smad7Tg mice at the end of AOM/DSS protocol. Number of tumors (B) and tumor score (C) obtained in the different groups at the end of the experiment. Bars indicate the median value. (*) indicates significant differences between the groups as indicated (*p<0.01). (ns) not significant. Data were obtained pooling four independent experiments. (D) H&E staining of representative colonic sections collected from the different groups. Outlining indicates dysplastic areas. Scale bars indicate 100μm. mRNA expression and fold change relative to the Wt of cytokines in Smad7Tg and IFNγ−/−Smad7Tg LPMC (E) and TILs (F). Bars indicate mean value +/- SD where indicated.

**Figure 6:** IFNγ expression influences the accumulation of immune cells into colonic mucosa. FACS analysis of CD4+, CD8+, CD3+DX5+ (NKT) and CD3-DX5+ (NK) cells accumulated in peritumoral LPMC (A) and TILs (B) of Wt, Smad7Tg and IFNγ−/−Smad7Tg mice. Representative dot plots from four independent experiments are shown. Percent of the different cell populations are reported in the quadrants (left panel). Pooled results from four independent experiments are reported for each cell population analyzed (right panel). Bars indicate average +/- SEM obtained from four independent experiments. (*) indicates significant differences between the groups (*p<0.01).

**Figure 7:** Smad7Tg mice show higher IFNγ-dependent cytotoxicity. (A) Perforin1, GranzymeB and FasL mRNA relative expression in peritumoral LPMC and TILs isolated from Wt, Smad7Tg and IFNγ−/−Smad7Tg mice. Bars indicate mean value +/- SD. Representative results from one experiment of four performed obtaining similar results are shown. (B) in vitro flow cytometry-based cytotoxicity assay: (T) Target cells, (E) CD8+ or DX5+ effector cells. Killing activity is expressed as percent of Annexin-V+ target cells in the different groups. Points indicate average +/- SEM of AnnexinV+ target cells. Results from three independent experiments are
shown. (*) indicates significant differences between the groups (*p<0.05). (C) Representative TUNEL staining of colonic sections obtained from different groups of mice at the end of the experiment. Arrow heads indicate TUNEL positive cells. Scale bars indicate 100μm.
Fig. 1

A

CTRL CD UC CAC

B

Smad7

CD UC CAC CTRL

C

CD4 CD4/Smad7

IBD CAC CTRL

Smad7+ cells/HPF

\* \*
Fig. 2
Fig. 3
Fig. 4
Fig. 6

A

LPMCs

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Fig. 7
Smad7 expression in T cells prevents colitis-associated cancer

Angelamaria Rizzo, Maximilian J Waldner, Carmine Stolfi, et al.

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