

# Interleukin-25 production is differently regulated by TNF- $\alpha$ and TGF- $\beta$ 1 in the human gut

D Fina<sup>1</sup>, E Franzè<sup>1</sup>, L Rovedatti<sup>2</sup>, GR Corazza<sup>2</sup>, L Biancone<sup>1</sup>, PP Sileri<sup>3</sup>, G Sica<sup>3</sup>, TT MacDonald<sup>4</sup>, F Pallone<sup>1</sup>, A Di Sabatino<sup>2</sup> and G Monteleone<sup>1</sup>

An altered balance between effector and regulatory factors is supposed to sustain the tissue-damaging immune response in inflammatory bowel disease (IBD). We have recently shown that in IBD, there is a defective synthesis of the counter-regulatory cytokine, interleukin (IL)-25. In this study we investigated factors that control IL-25 production in the gut. IBD patients produced less IL-25 when compared with normal controls. Stimulation of normal intestinal explants with tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), but not interferon- $\gamma$  (IFN- $\gamma$ ) or IL-21, reduced IL-25 synthesis. Consistently, IL-25 production was enhanced by anti-TNF- $\alpha$  both *in vitro* and *in vivo*. Upregulation of IL-25 was also seen in normal colonic explants stimulated with transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1). As in IBD, TGF- $\beta$ 1 activity is abrogated by Smad7, we next assessed whether inhibition of Smad7 with an antisense oligonucleotide enhanced IL-25 expression. Knockdown of Smad7 was accompanied by an increase in IL-25 production. Data show that IL-25 production is differently regulated by TNF- $\alpha$  and TGF- $\beta$ 1 in the human gut.

## INTRODUCTION

In recent years, it has become evident that inflammatory bowel disease (IBD) results from a deregulated and excessive immune reactivity within the intestinal wall that is directed against luminal antigens.<sup>1,2</sup> Nonetheless, mechanisms that contribute to amplify and maintain the mucosal inflammation are not yet fully understood. An intriguing hypothesis is that in IBD, the tissue-damaging immune-inflammatory reaction is partly sustained by a defective activity of counter-regulatory mechanisms.<sup>3,4</sup>

Interleukin (IL)-25 (also known as IL-17E) is a recently described member of the IL-17 cytokine gene family.<sup>5</sup> Distinct from other IL-17 cytokine family members, IL-25 has been shown to facilitate pathogenic T helper (Th)-2 cell responses.<sup>6,7</sup> Indeed, studies in mice have shown that both transgenic expression of IL-25 and systemic administration of recombinant IL-25 increase the production of IL-4, IL-5, and IL-13, cause epithelial cell hyperplasia, and facilitate the recruitment of inflammatory cells into inflamed tissues.<sup>6,8-10</sup> In contrast, IL-25-deficient mice infected with the parasitic helminthes *Nippostrongylus brasiliensis* and *Trichuris muris* are unable to sustain Th2 cell responses and fail to expel the parasites from the gut.<sup>11,12</sup>

More recent studies have shown that IL-25 can regulate the outcome of other immune responses. For example, studies in murine models of autoimmunity have shown that IL-25 can negatively regulate the development and/or amplification of Th17-mediated pathology and suppress the production of bacteria-driven IL-23 production in the gut.<sup>13,14</sup> In line with this, we have recently shown that CD14<sup>+</sup> cells isolated from the inflamed gut of patients with Crohn's disease (CD) or ulcerative colitis (UC), the major forms of human IBD, respond to IL-25 by downregulating the synthesis of multiple inflammatory cytokines.<sup>15</sup> Consistently, *in vivo* in mice, administration of IL-25 prevented and cured experimental colitides.<sup>15,16</sup> Interestingly, analysis of IL-25 expression in the human gut revealed that IL-25 is mostly made by subepithelial macrophages and that patients with IBD produce significantly less IL-25 compared with controls.<sup>15</sup> It is thus conceivable that in IBD, the defective IL-25 production can contribute to sustain and/or amplify the pathogenic response. However, the molecular mechanisms that account for IL-25 downregulation during intestinal inflammation are not known. Hence, in this study, we analyzed factors that regulate IL-25 in the human gut.

<sup>1</sup>Department of Internal Medicine, University "Tor Vergata" of Rome, Rome, Italy. <sup>2</sup>Department of Internal Medicine, Fondazione IRCCS Policlinico, San Matteo, Università di Pavia, Pavia, Italy. <sup>3</sup>Department of Surgery, University "Tor Vergata" of Rome, Rome, Italy. <sup>4</sup>Institute of Cell and Molecular Science, Bart's and the London School of Medicine and Dentistry, London, UK. Correspondence: G Monteleone (G.Monteleone@Med.uniroma2.it)

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## RESULTS

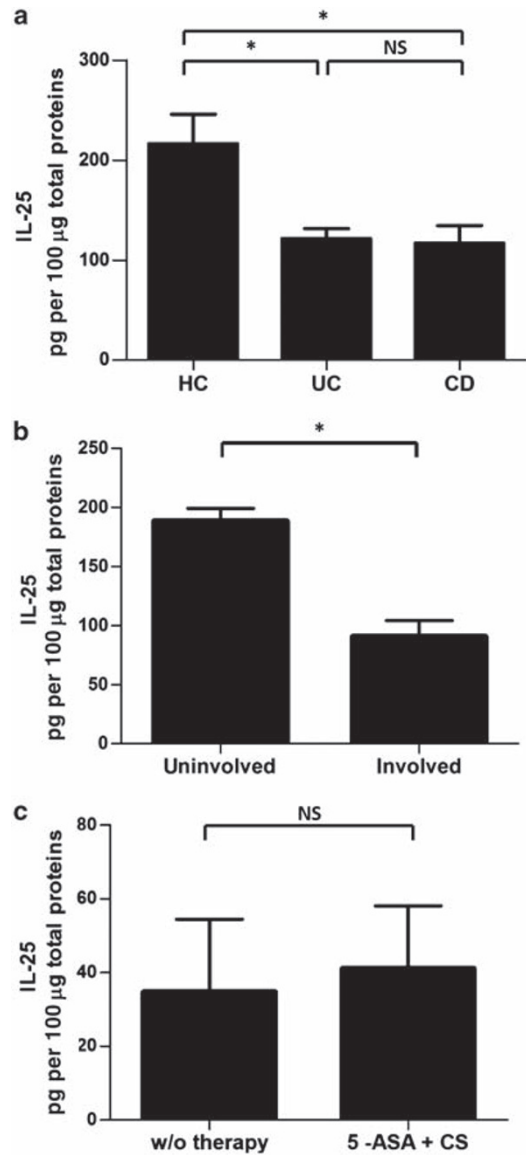
**IL-25 is downregulated in IBD but not celiac disease mucosa**

Initially, we sought to confirm that in IBD there is a defective IL-25 production. To this end, we measured IL-25 quantitatively in total proteins extracted from whole mucosal samples by enzyme-linked immunosorbent assay. A diminished expression of IL-25 was found in CD and UC samples when compared with controls (**Figure 1a**). IL-25 was also analyzed in biopsy samples taken from uninvolved and involved mucosal areas of further eight IBD patients. IL-25 protein expression was significantly diminished in the inflamed tissue when compared with uninvolved tissue (**Figure 1b**). Total extracts prepared from ileal and colonic biopsies of CD patients showed no significant difference in terms of IL-25 production (not shown). Moreover, there was no difference in IL-25 production between IBD patients taking no drugs and those who were receiving mesalazine or mesalazine + steroids (**Figure 1c**).

To assess whether IL-25 expression is downregulated in other chronic intestinal inflammatory diseases, we next measured IL-25 in duodenal biopsies of patients with active celiac disease and normal controls. A more pronounced expression of IL-25 was seen in celiac disease patients when compared with controls (**Figure 2a**). Moreover, analysis of IL-25 in biopsies taken from three patients before and after 1 year of gluten-free diet revealed that induction of remission was associated with a reduced synthesis of IL-25 (**Figure 2b**), thus suggesting that in celiac disease, gluten-induced immune response sustains IL-25 production. This hypothesis is supported by the demonstration that treatment of duodenal biopsies of inactive celiac disease patients with peptic-tryptic digest of gliadin enhanced IL-25 production (**Figure 2c**).

**TNF- $\alpha$  negatively regulates IL-25 production in the gut**

Next, we determined which factors control IL-25 production in the gut. To this end, we stimulated colonic biopsies taken from normal controls with various inflammatory cytokines that are produced in excess in IBD tissue.<sup>17–19</sup> Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), but not interferon- $\gamma$  (IFN- $\gamma$ ) or IL-21, significantly reduced IL-25 synthesis (**Figure 3**). To confirm these data, we cultured biopsies taken from IBD patients with infliximab or control IgG. Treatment of IBD explants with infliximab led to a significant increase in IL-25 production (**Figure 4a**). In contrast, incubation of IBD samples with neutralizing IFN- $\gamma$  or IL-21 antibody did not alter IL-25 production (not shown). Subsequently, IL-25 protein expression was evaluated in colonic biopsies taken from six IBD patients before and after a successful treatment with infliximab. Induction of clinical and endoscopic remission by infliximab was accompanied by enhanced synthesis of IL-25 (**Figure 4b**). Taken together, these data suggest that TNF- $\alpha$  is a negative regulator of IL-25 in the human gut. To confirm that TNF- $\alpha$  is overexpressed in IBD, biopsies taken from the same IBD patients and controls, who were investigated for IL-25 expression, were analyzed for the content of TNF- $\alpha$  RNA transcripts by real-time PCR. As expected, a more pronounced TNF- $\alpha$ - $\beta$ -actin RNA relative expression was seen in

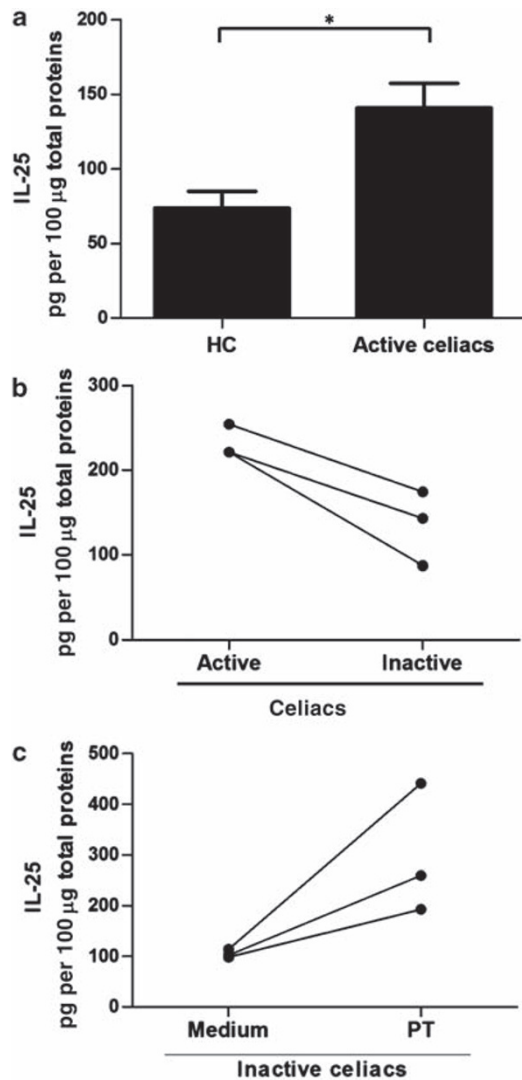


**Figure 1** Interleukin (IL)-25 protein synthesis is diminished in the inflamed gut of patients with inflammatory bowel disease (IBD). **(a)** Quantitative analysis of IL-25 protein expression in mucosal samples taken from 18 healthy controls (HC), 23 patients with ulcerative colitis (UC), and 15 patients with Crohn's disease (CD) as measured by enzyme-linked immunosorbent assay (ELISA). Data are expressed as mean $\pm$ s.d.; \* $P$ <0.001. **(b)** Total proteins extracted from IBD mucosal biopsies taken from involved and uninvolved areas of seven patients with UC and two patients with CD were measured for IL-25 protein content by ELISA. Data are expressed as mean $\pm$ s.d.; \* $P$ <0.004. **(c)** IL-25 protein was quantified in total proteins extracted from inflamed colonic biopsies of five IBD patients receiving no therapy (w/o therapy) and seven IBD patients treated with mesalazine (5-ASA) or mesalazine + steroids by ELISA. Data are expressed as mean $\pm$ s.d. NS, not significant.

CD ( $140\pm 48$  arbitrary units) and UC ( $51.13\pm 15.68$  arbitrary units) in comparison with normal controls ( $11.78\pm 7.34$  arbitrary units) ( $P$ <0.01).

**TGF- $\beta$ 1 enhances IL-25 production**

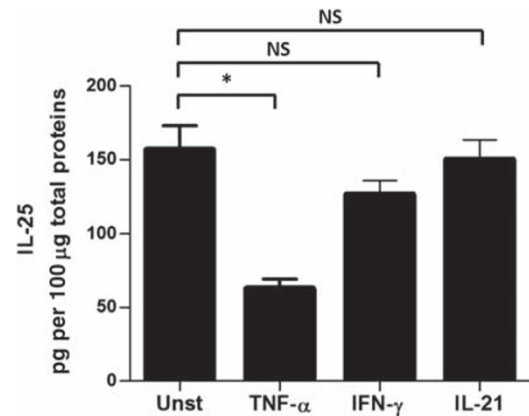
IL-25-expressing cells are enriched in the normal colonic mucosa.<sup>15</sup> However, factors/mechanisms that induce and sustain



**Figure 2** Interleukin (IL)-25 production is increased in active celiac disease. (a) Mucosal samples were collected from the duodenum of 13 healthy controls (HC) and 10 patients with active celiac disease and measured for IL-25 protein content by enzyme-linked immunosorbent assay (ELISA). Data are expressed as mean $\pm$ s.d.; \* $P=0.003$ . (b) IL-25 was measured in total proteins extracted from duodenal biopsies taken from 3 celiac disease patients before (active) and after (inactive) 1 year of gluten-free diet. (c) IL-25 protein synthesis in duodenal biopsies taken from inactive celiac disease patients and cultured in the presence or absence (medium) of peptic-tryptic digest (PT) of gliadin for 24 h.

IL-25 in the human gut remain unknown. Therefore, the next series of experiments were conducted to evaluate whether IL-25 expression is regulated by transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1), a cytokine that is highly produced in the normal human gut, where it contributes to maintain mucosal homeostasis.<sup>20,21</sup> Stimulation of normal colonic biopsies with TGF- $\beta$ 1 significantly enhanced IL-25 production (Figure 5a).

In IBD, there is a defective TGF- $\beta$ 1 activity because of high levels of Smad7, an intracellular protein that inhibits TGF- $\beta$ 1-driven intracellular signaling.<sup>3,4,22</sup> Indeed, analysis of Smad7 protein expression in our samples by western blotting confirmed

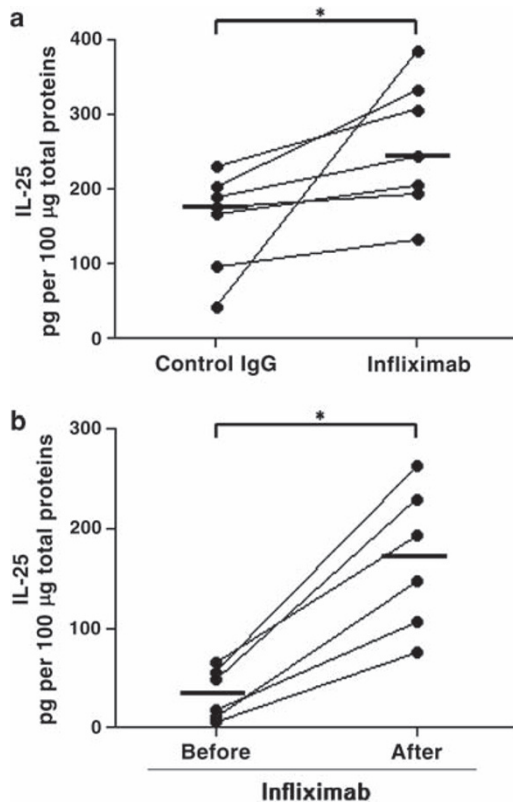


**Figure 3** Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) reduces interleukin (IL)-25 production in normal intestinal biopsies. IL-25 was measured in total proteins extracted from normal colonic explants cultured with or without (unstimulated (unst)) TNF- $\alpha$ , interferon- $\gamma$  (IFN- $\gamma$ ), or IL-21 for 24 h. Data are expressed as mean $\pm$ s.d. of six experiments; \* $P=0.035$ ; NS, not significant.

that Smad7 protein was increased in IBD patients in comparison with normal controls. In particular, patients with CD and UC had a median of Smad7- $\beta$ -actin ratio of 0.75 and 0.56 densitometry arbitrary units, respectively (range 0.4–0.98 and 0.26–0.7, respectively) that was significantly higher than that found in normal controls (median 0.12; range 0.001–0.29;  $P < 0.002$ ). Therefore, we next determined whether in IBD, the diminished production of IL-25 was partly related to Smad7. To address this issue, we incubated IBD mucosal explants with Smad7 sense or antisense oligonucleotides, and then analyzed IL-25 protein by enzyme-linked immunosorbent assay. Treatment of IBD samples with Smad7 antisense oligonucleotide led to a significant upregulation of IL-25 synthesis (Figure 5b). The effective reduction of Smad7 protein expression in whole mucosal biopsies incubated with Smad7 antisense oligonucleotides was confirmed by western blotting (not shown).

## DISCUSSION

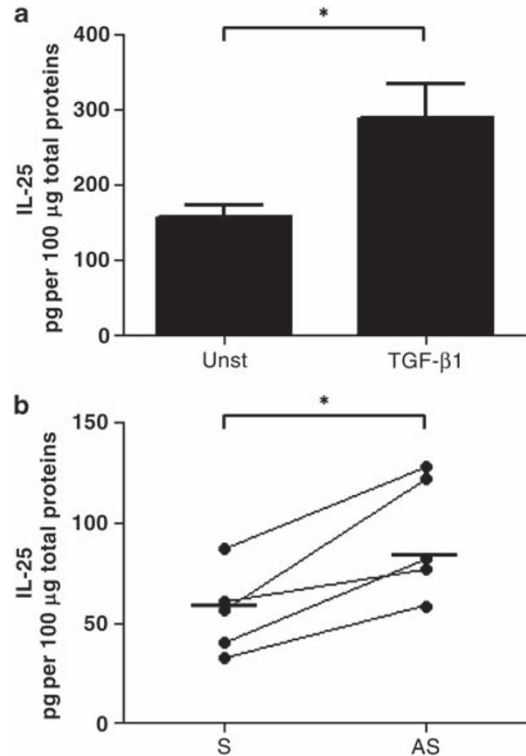
Initially considered as a factor that expands Th2 cell responses,<sup>6,7</sup> IL-25 has been recently shown to regulate additional immune pathways. For example, it is known that IL-25 inhibits the synthesis of cytokines associated with Th1 and Th17 immunity and that, *in vivo* in mice, administration of IL-25 prevents and cures experimental colitides.<sup>13–16</sup> IL-25 is constitutively produced in the human intestine, but its expression is markedly reduced in IBD.<sup>15</sup> Interestingly, a recent study showed that IL-25 is produced by endothelial cells in the lumbar spinal cord of normal mice and that IL-25-producing cells are reduced after the development of experimental autoimmune encephalomyelitis, a murine model of multiple sclerosis.<sup>13</sup> IL-25 administration ameliorated experimental autoimmune encephalomyelitis induced by passive transfer of myelin oligodendrocyte glycoprotein-reactive CD4<sup>+</sup> T cells in recipient mice.<sup>13</sup> A decrease of IL-25 expression in brain capillary endothelial cells was also seen in patients with active multiple sclerosis lesions.<sup>23</sup> Taken together, these findings support the anti-inflammatory action



**Figure 4** Anti-tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) treatment enhances interleukin (IL)-25 production in inflammatory bowel disease (IBD) tissue. **(a)** Mucosal samples were collected from the inflamed colon of three patients with Crohn's disease (CD) and four patients with ulcerative colitis (UC) and cultured with infliximab or control IgG for 24 h. Total proteins were then extracted and analyzed for IL-25 content by enzyme-linked immunosorbent assay (ELISA). Each point represents the value of IL-25 protein in mucosal samples taken from a single patient and treated with control IgG or infliximab. Horizontal bars indicate the median; \* $P=0.015$ . **(b)** Mucosal samples were collected from five CD patients and one UC patient immediately before and after a successful treatment with infliximab and used to extract total proteins. IL-25 was analyzed by ELISA. Each point represents the value of IL-25 protein in mucosal samples taken from a single patient before and after infliximab therapy. Horizontal bars indicate the median; \* $P=0.030$ .

of IL-25, and suggest that defects in IL-25 production can contribute to sustain inflammatory responses in various organs.

In this study we analyzed factors that regulate IL-25 in the human gut. Initially, we confirmed that IL-25 production is downregulated in IBD tissue, by measuring the cytokine in whole mucosal extracts of IBD patients and controls. Interestingly, in IBD, IL-25 expression was reduced in the involved areas when compared with uninvolved tissues, whereas the expression of the cytokine in samples taken from uninvolved mucosa did not differ from that seen in the colon of controls (**Figure 1a, b**). Moreover, IL-25 production did not differ between IBD patients taking no therapy and those who were receiving anti-inflammatory drugs. These observations, together with our previous follow-up studies showing that medically induced remission enhances IL-25 production,<sup>15</sup> indicate that downregulation of IL-25 in IBD is not caused by current therapy.



**Figure 5** IL-25 is positively regulated by TGF- $\beta$ 1 signalling. **(a)** Transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) enhances interleukin (IL)-25 production in normal colonic explants. Mucosal colonic samples were cultured in the presence or absence (unstimulated (unst)) of TGF- $\beta$ 1 for 24 h. Total proteins were then extracted and analyzed for IL-25 content by enzyme-linked immunosorbent assay (ELISA). Data are expressed as mean $\pm$ s.d. of four experiments; \* $P=0.03$ . **(b)** Treatment of inflammatory bowel disease (IBD) mucosal explants with Smad7 antisense oligonucleotide upregulates IL-25 synthesis. Mucosal samples of active IBD patients (4 Crohn's disease (CD) and 1 ulcerative colitis (UC)) were cultured with Smad7 sense (S) or antisense (AS) oligonucleotides for 48 h, and then used to extract total proteins. IL-25 was analyzed by ELISA. Each point represents the value of IL-25 protein in mucosal samples taken from a single patient and treated with Smad7 S or AS. Horizontal bars indicate the median; \* $P=0.03$ .

Another important finding of this work is that IL-25 is highly expressed in the inflamed gut of patients with celiac disease. Although further experimentation is necessary to ascertain how IL-25 is expressed in other gastrointestinal inflammatory conditions, the above data would seem to suggest that the defective production of IL-25 seen in IBD is not an epiphenomenon of the ongoing inflammation, but it might rather rely on factors/mechanisms that act selectively in IBD tissue. One such factor could be TNF- $\alpha$ , because it is highly produced in IBD but not in celiac disease mucosa.<sup>17,24,25</sup> Indeed, stimulation of normal colonic biopsies with TNF- $\alpha$  reduced IL-25 expression, whereas anti-TNF- $\alpha$  upregulated IL-25 synthesis in IBD mucosa both *in vivo* and *in vitro*. These data fit with the study by Sonobe *et al.*<sup>23</sup> showing that TNF- $\alpha$  inhibited IL-25 RNA expression in the brain capillary endothelial cell line, MBEC4. In the same work, Sonobe *et al.*<sup>23</sup> also documented a negative effect of IFN- $\gamma$  on IL-25 mRNA expression in MBEC4 cells. In contrast, we were not able to see any significant change in IL-25 expression

in normal mucosal explants following IFN- $\gamma$  exposure. The reason for this apparent discrepancy is not known, although it is conceivable that IL-25 production is regulated in a cell- and tissue-dependent manner. In line with this, it is the demonstration that eosinophils, but not mast cells, from allergen-challenged mice significantly upregulate IL-25 expression when exposed to stem cell factor.<sup>26</sup> The demonstration that IL-25 is enhanced in celiac disease but not CD mucosa, despite that active lesions in both diseases are characterized by excessive synthesis of IFN- $\gamma$ ,<sup>18,27</sup> supports this hypothesis further.

As IL-25 is constitutively produced by gut mucosal cells, and evidence suggests that IL-25 contributes to dampen intestinal inflammation,<sup>15</sup> we conducted further experimentation to identify molecules, which positively regulate IL-25 production. We focused our work on TGF- $\beta$ 1, because this cytokine is highly synthesized in the gut where it has a key role in promoting counter-regulatory mechanisms.<sup>20,21</sup> Our *ex vivo* organ culture studies indicate that in the normal gut, IL-25 production can be enhanced by TGF- $\beta$ 1. This scenario is however somewhat different from what we can image in IBD tissue, where the high levels of Smad7 inhibit TGF- $\beta$ 1 signaling.<sup>3,4</sup> If so, one could hypothesize that knockdown of Smad7 could eventually increase IL-25 synthesis. Indeed, treatment of IBD biopsies with Smad7 antisense oligonucleotide led to a significant upregulation of IL-25 production.

A caveat of these studies is that analysis of IL-25 expression was however performed in biopsies and not in purified cell types. We think it would be biologically relevant to examine Smad7 and IL-25 in single mucosal cell populations in order to ascertain if inhibition of Smad7 by antisense oligonucleotide directly leads to IL-25 production. This should be performed using subepithelial macrophages, because these are the cell sources of IL-25 in the human gut.<sup>15</sup> Unfortunately, we were not able to purify sufficient cells to carry out mechanistic studies. Hence, at the present time, we cannot exclude the possibility that the increased synthesis of IL-25 seen in IBD biopsies treated with Smad7 antisense is secondary to a downregulation of inflammatory pathways (e.g., TNF- $\alpha$ ) rather than the result of a direct effect of Smad7 on IL-25 expression. As TNF- $\alpha$  does not seem to be involved in the regulation of Smad7 in IBD,<sup>3-4</sup> we can exclude the possibility that TNF- $\alpha$ -mediated downregulation of IL-25 expression that we observed in IBD tissue is secondary to induction of Smad7.

In conclusion, our data confirm that IL-25 is downregulated in IBD and suggest that such a defect can be perpetuated and amplified by inflammatory pathways acting in IBD tissue.

## METHODS

**Patients and samples.** Mucosal biopsies were taken from the inflamed areas of 15 patients with CD and 23 patients with UC. In 9 IBD (2 CD and 7 UC) patients, biopsies were taken from both involved and uninvolved mucosal areas. In 9 out of 15 CD patients, the primary site of involvement was the terminal ileum, whereas the remaining patients had a colonic disease. Two CD patients were receiving mesalazine, 9 patients were receiving steroids and mesalazine, and the remaining were taking azathioprine. Among UC patients, 17 were on mesalazine, whereas the remaining were taking mesalazine and steroids.

Additional mucosal samples were taken from intestinal resection specimens of eight CD patients undergoing surgery because of a chronic active disease poorly responsive to medical treatment. At the time of surgery, four patients were receiving steroids and four patients were on antibiotics and steroids. Moreover, biopsies were collected from five CD patients and one UC patient before and 10 weeks after a successful therapeutic treatment with anti-TNF- $\alpha$  antibody (i.e., infliximab). To evaluate whether in IBD, IL-25 expression is influenced by therapy, colonic biopsies were taken from 5 active patients (4 CD and 1 UC) taking no drug and 7 patients (3 CD and 4 UC) treated with mesalazine ( $N=4$ ) or mesalazine and steroids ( $N=3$ ).

Healthy controls for the IBD study included colonic mucosal samples taken from macroscopically and microscopically unaffected areas of 18 subjects undergoing colonoscopy for colorectal cancer screening ( $N=13$ ) or colon resection for colon cancer ( $N=5$ ).

To evaluate how IL-25 is expressed in other chronic intestinal inflammatory diseases, biopsies were taken from the duodenum of ten patients with active celiac disease. The histopathological diagnosis of celiac disease was based on typical mucosal lesions with crypt cell hyperplasia and villous atrophy. All active celiac disease patients were positive for antiendomysial and antitransglutaminase antibodies at the time of diagnosis. Of these ten celiac disease patients, three underwent a second endoscopy at 1 year after the initial diagnosis for the appearance of dyspeptic symptoms. All these patients were on gluten-free diet, in histological remission, and negative for antiendomysial and antitransglutaminase antibodies at the time of the second endoscopy. Controls for celiac disease study included duodenal biopsies taken from ten individuals who underwent upper endoscopy for dyspeptic symptoms, but had normal histology, no increase in inflammatory cells, and were negative for antiendomysial and antitransglutaminase antibodies. Informed consent was obtained from all patients and controls, and the study protocol was approved by the local ethics committee.

**Ex vivo organ cultures.** All of the reagents were from Sigma-Aldrich (Milan, Italy) unless specified. Freshly obtained mucosal samples were cultured as described elsewhere.<sup>3</sup> Briefly, intestinal samples were placed on iron grids with the mucosal face upward in the central well of an organ culture dish in RPMI-1640 medium containing 10% fetal bovine serum, and supplemented with penicillin ( $100 \text{ U ml}^{-1}$ ) and streptomycin ( $100 \mu\text{g ml}^{-1}$ ) (all from Lonza, Verviers, Belgium). Dishes were placed in a tight container with 95% O<sub>2</sub>/5% CO<sub>2</sub> at 37 °C, at 1 bar. Control biopsies were incubated in the presence or absence of TNF- $\alpha$  ( $20 \text{ ng ml}^{-1}$ ), TGF- $\beta$ 1 ( $5 \text{ ng ml}^{-1}$ ), IL-21 ( $100 \text{ ng ml}^{-1}$ ), all from R&D Systems, Minneapolis, MN), and IFN- $\gamma$  ( $100 \text{ ng ml}^{-1}$ ; Peprotech, London, UK). Mucosal samples taken from the inflamed colon of four patients with CD and three patients with UC were incubated with infliximab or control human IgG (both at  $50 \mu\text{g ml}^{-1}$ ). Additionally, samples taken from three patients with CD were incubated in the presence or absence of a neutralizing IFN- $\gamma$  ( $5 \mu\text{g ml}^{-1}$ ; R&D Systems) or IL-21 (antibody  $5 \mu\text{g ml}^{-1}$ ).<sup>28</sup> After 24 h of culture, mucosal samples were snap frozen and stored at  $-80^\circ\text{C}$  until tested.

To examine the effect of Smad7 inhibition on the expression of IL-25, mucosal samples taken from four patients with CD and one patient with UC were cultured in X-vivo, a serum-free culture medium, supplemented with penicillin-streptomycin in the presence or absence of Smad7 antisense or sense oligonucleotides ( $10 \mu\text{g ml}^{-1}$ ). These concentrations were selected on the basis of our previous studies showing that at these concentrations Smad7 antisense oligonucleotide was effective in reducing Smad7 expression in whole mucosal biopsies.<sup>3</sup> Both Smad7 antisense and sense oligonucleotides were combined with Lipofectamine 2000 Reagent according to the manufacturer's instructions (Invitrogen Italia, San Giuliano Milanese, Italy). After 48 h, intestinal mucosal specimens were snap frozen and stored at  $-80^\circ\text{C}$  until tested.

Duodenal biopsies were taken from three inactive celiac disease patients and cultured with a peptic-tryptic digest of gliadin ( $1 \text{ mg ml}^{-1}$ ) for 24 h as previously described.<sup>29</sup>

**Total protein extraction, IL-25 enzyme-linked immunosorbent assay, and Smad7 western blotting.** Intestinal mucosal samples were lysed on ice in buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.2 mM EGTA, and 0.5% Nonidet P40, supplemented with 1 mM dithiothreitol, 10 mg ml<sup>-1</sup> aprotinin, 10 mg ml<sup>-1</sup> leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na<sub>3</sub>VO<sub>4</sub>, and 1 mM NaF. Lysates were clarified by centrifugation at 12,000 g for 30 min at 4 °C. Extracts were analyzed for IL-25 content using a sensitive commercial enzyme-linked immunosorbent assay kit (Peprotech) according to the manufacturer's instructions. Smad7 expression was analyzed by western blotting as previously described<sup>3</sup> using a specific rabbit polyclonal antibody (final dilution 1:500, Imgenex, San Diego, CA).

**Real-time PCR.** RNA was extracted using TRIzol reagent according to the manufacturer's instructions (Invitrogen). A constant amount of RNA (1 µg per sample) was retro-transcribed into complementary DNA, and 1 µl of complementary DNA per sample was then amplified using the following conditions: denaturation 1 min at 95 °C, annealing 30 s at 62 °C for TNF-α and at 60 °C for β-actin, followed by 30 s of extension at 72 °C.

β-actin (FWD: 5-AAGATGACCCAGATCATGTTTGAGACC-3 and REV: 5-AGCCAGTCCAGACGCAGGAT-3) and TNF-α (FWD: 5'-AGGCGGTGCTTGTTCCTCAG-3' and REV 5'-GGCTACAGGCTTGCTACTCG-3) expressions were evaluated using IQ SYBR Green Supermix (Bio-Rad Laboratories, Milan, Italy). Real-time PCR was performed using a CFX96 thermal cycler (Bio-Rad Laboratories). Gene expression was calculated using the ΔΔCt algorithm.

**Statistical analysis.** Differences between groups were compared using the paired *t*-test, the Mann-Whitney *U*-test, and one-way analysis of variance test.

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#### DISCLOSURE

G.M. has filed a patent entitled "A treatment for inflammatory diseases" (patent Nr. 08154101.3). The remaining authors declared no conflict of interest.

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