ORIGINAL ARTICLE

Interleukin-13 and transforming growth factor β synergise in the pathogenesis of human intestinal fistulae

Michael Scharl,[1,2] Sandra Frei,[1] Theresa Pesch,[1] Silvia Kellermeier,[1] Joba Arikkat,[1] Pascal Frei,[1] Michael Fried,[1,2] Achim Weber,[3] Ekkehard Jehle,[4] Anne Rühl,[5] Gerhard Rogler[1,2]

► Additional materials are published online only. To view these files please visit the journal online (http://dx.doi.org/10.1136/gutjnl-2011-300498).

[1]Division of Gastroenterology and Hepatology, University Hospital Zurich, Zurich, Switzerland
[2]Zurich Center for Integrative Human Physiology, University of Zurich, Zurich, Switzerland
[3]Department of Pathology, Institute of Surgical Pathology, University Hospital of Zurich, Zurich, Switzerland
[4]Department of Surgery, Oberschwaben-Klinik, Ravensburg, Germany
[5]Novartis Pharma AG, Basel, Switzerland

Correspondence to
Dr Michael Scharl, Division of Gastroenterology and Hepatology, University Hospital Zurich, Rämistrasse 100, Zurich 8091, Switzerland; michael.scharl@usz.ch

Revised 3 January 2012
Accepted 4 January 2012
Published Online First 27 January 2012

ABSTRACT

Objective Epithelial to mesenchymal transition (EMT) seems to play an important role in the pathogenesis of fistulae, a common clinical complication of Crohn's disease (CD). TGFβ and interleukin-13 (IL-13) have been correlated with the onset of EMT-associated organ fibrosis and high levels of TGFβ have been shown in transitional cells (TCs) lining CD fistula tracts. This study investigated whether IL-13 could be involved in the pathogenesis of CD-associated fistulae.

Design Protein or mRNA levels in HT29 intestinal epithelial cells (IECs) or colonic lamina propria fibroblasts (CLPFs) were studied by western blotting or real-time PCR. CLPFs were isolated from non-inflammatory disease controls or patients with CD with or without fistulae and IL-13 levels were analysed in surgically removed fistula specimens by immunohistochemistry.

Results TGFβ induced IL-13 secretion in CLPFs from patients with fistulising CD. In fistula specimens high levels of IL-13 were detected in TCs covering fistula tracts. In HT29 IEC monolayers, IL-13 induced SLUG and β6-integrin mRNA, which are associated with cell invasion. HT29 spheroids completely disintegrated when treated with TGFβ for 7 days, whereas IL-13-treated spheroids did not show morphological changes. Here, TGFβ induced mRNA expression of SNAIL1 and IL-13, whereas IL-13 elevated SLUG and β6-integrin mRNA. An anti-IL-13 antibody was able to prevent IL-13-induced SLUG expression in HT29 IECs.

Conclusions TGFβ induces IL-13 expression and an EMT-like phenotype of IECs, while IL-13 promotes the expression of genes associated with cell invasion. These findings suggest that TGFβ and IL-13 play a synergistic role in the pathogenesis of fistulae and inhibition of IL-13 might represent a novel therapeutic approach for fistula treatment.

Significance of this study

What is already known about this subject?

- Epithelial to mesenchymal transition (EMT) is involved in fistula pathogenesis in patients with Crohn's disease.
- Transforming growth factor β (TGFβ) and interleukin-13 (IL-13) are associated with the onset of EMT in fibrotic organ lesions.
- Molecules being associated with invasive cell growth, such as SLUG or β6-integrin, are detectable in cells lining the fistula tracts.

What are the new findings?

- IL-13 and its receptor, IL-13α_1, are highly expressed in cells lining the fistula tracts.
- IL-13 induces the expression of SLUG and β6-integrin in intestinal epithelial cells and in in vitro models of EMT.
- Inhibition of IL-13-induced effects by a specific antibody inhibits the cytokine-induced effects in intestinal epithelial cells.

How might it impact on clinical practice in the foreseeable future?

- These data show that IL-13 can be induced by TGFβ and is upregulated in cells lining the fistula tracts.
- IL-13 then induces the expression of molecules being associated with invasive cell growth and/or EMT that can be observed in fistula pathogenesis.
- Inhibition of IL-13 could provide a successful approach for the treatment of Crohn's disease-associated fistulae.

BACKGROUND AND AIMS

Fistulae represent a pathological connection between two epithelium-covered organs. Patients with Crohn's disease (CD), a subtype of inflammatory bowel disease (IBD) and a common form of chronic intestinal inflammation, frequently suffer from fistulae. Between 17% and 50% of patients with CD develop mostly perianal, entero-enteric or entero-cutaneous fistulae, which frequently represent a recurring condition.[1–3] Though new and more effective medications, such as anti-tumour necrosis factor (TNF) antibodies, have been developed for intestinal inflammation and CD respectively, the outcome of medical fistula therapy is still poor.[4 5] Therefore there is an obvious need for a better understanding of fistula pathogenesis to find more promising treatment options.

We have recently demonstrated that epithelial to mesenchymal transition (EMT) takes place in and around the majority of CD-associated fistulae.[6 7] EMT represents the transformation from a differentiated, polarised epithelial cell to a

mesenchymal-like cell featuring a myofibroblast phenotype. As a specific characteristic, EMT cells display epithelial markers, such as E-cadherin or cytokeratines 8 and 20, and mesenchymal markers, such as vimentin or α smooth muscle actin.[8 9] On a functional level, EMT is essential for embryogenesis, organ development and wound repair, but is also associated with tissue fibrosis, tumour growth and metastasis.[8–10]

About two-thirds of the CD-associated fistulae are non-epithelialised fistulae and covered by myofibroblast-like 'transitional cells' (TCs).[6] In or around the fistula tracts, we detected nuclear localisation of the transcription factors SNAIL1 and SLUG, indicative of their activation. We further found elevated levels of β6-integrin, transforming growth factor β (TGFβ) and TNF, but decreased levels of epithelial markers, such as E-cadherin.[6] TGFβ is well known as a key mediator of EMT and induces, similar to TNF, EMT in vitro.[11–15] Of note, β6-integrin and SLUG have been positively correlated with the invasive potential of tumour cells and the extent of EMT.[16–18]

The cytokine IL-13 is thought to be mainly secreted by immune cells, especially T helper 2 cells.[19] While the IL-13-receptor α1 (IL-13Rα$_1$) is regarded as the signal-transducing receptor, IL-13Rα$_2$ acts as a decoy receptor.[20] IL-13 has been implicated in the pathogenesis of tissue fibrosis in organ systems, such as the lung or liver.[21 22] One of the fibrosis-inducing effects of IL-13 involves the secretion and activation of TGFβ, indicating that the growth factor could be a downstream mediator of the cytokine.[23] However, data on the role of IL-13 in tumour growth and invasion are conflicting because IL-13 has recently been associated with increased invasiveness and metastasis of ovarian and pancreatic cancers.[24–27] However, IL-13 can also inhibit tumour growth, for example the growth of breast or renal cell cancer.[28 29]

Here, we demonstrate that TGFβ induces SNAIL1 and IL-13 mRNA expression in primary human colonic lamina propria fibroblasts (CLPFs) derived from patients with CD. High levels of IL-13 and IL-13Rα$_1$ were detected in TCs lining the tracts of CD-associated fistulae. In an intestinal epithelial cell (IEC) model of EMT, IL-13 induced SLUG and β6-integrin levels, whereas chronic TGFβ administration resulted in concomitant elevation of SNAIL1 and IL-13 mRNA expression. Because anti-IL-13 antibody treatment prevented the IL-13-induced effects, this could provide a novel therapeutic approach for fistula treatment.

MATERIALS AND METHODS

Material

Anti-human-IL-13 antibody was provided by Novartis AG (Basel, Switzerland). A list of additional antibodies and cytokines is provided in the online supplementary methods.

Cell culture

Human HT29 cells were grown as monolayers or spheroids. Please find additional information in the online supplementary methods.

Patient samples

Perianal fistulae specimens from patients with CD for immunohistochemistry were prospectively collected from men and women with and without CD. We investigated seven fistulae in formalin-fixed tissue samples from seven patients with fistulising CD, and intestinal specimens were tested from six patients with active CD without fistulae, seven patients with CD in remission without fistulae, six non-IBD controls and five patients with active ulcerative colitis (UC). Tissue samples were surgically resected and immediately transferred to 4% formalin and stored at 4°C until further analysis. Primary CLPF cultures were obtained from fistulising areas of the intestinal mucosa of eight patients with CD (mean age 55±5 years), from the intestinal mucosa of five patients with non-fistulising CD (mean age 45±13 years) and from the intestinal mucosa of seven patients without IBD (mean age 47±9 years). Samples were collected from men and women and CLPF cultures were collected from surgical specimens. Written informed consent was obtained before specimen collection and studies were approved by the local ethics committee.

Isolation and culture of human CLPFs

Procedures were performed as described previously and given in detail in the online supplementary methods.[30] CLPFs from patients with non-fistulising disease were taken from actively inflamed areas.

Immunohistochemistry

Procedures were performed as described previously and given in detail in the online supplementary methods.[30]

Real-time PCR

The experimental setup is described in detail in the online supplementary methods.

Western blotting

The experimental setup is described in detail in the online supplementary methods.

ELISA

The experimental setup is described in detail in the online supplementary methods.

siRNA transfection

The experimental setup is described in detail in the online supplementary methods.

Statistical analysis

Data are presented as means±SEM for a series of n experiments. Statistical analysis was performed by analysis of variance followed by Student–Newman–Keuls post hoc test or Student t test, in which appropriate p values <0.05 were considered significant.

RESULTS

TGFβ induces IL-13 secretion from CD fistula CLPFs

We first studied whether TGFβ could induce SNAIL1 levels in CLPF derived from patients with CD. In CLPFs from patients without IBD or with non-fistulising CD, treatment with TGFβ induced a fourfold to fivefold increase in SNAIL1 mRNA expression and this effect was potentiated in CLPFs from patients with fistulising disease (about sixfold increase; figure 1A). It has to be noted that CLPFs derived from fistulae always represent a mixture of 'true' fibroblasts and EMT (epithelial) derived mesenchymal-like cells. In contrast, SLUG mRNA levels were not affected by TGFβ treatment in CLPFs from either non-IBD controls or patients with non-fistulising CD. However, the growth factor caused a significant decrease in SLUG mRNA levels in CLPFs derived from patients with fistulising CD (figure 1B). In CLPFs from non-IBD controls or patients with non-fistulising CD, TGFβ was not sufficient to induce the secretion

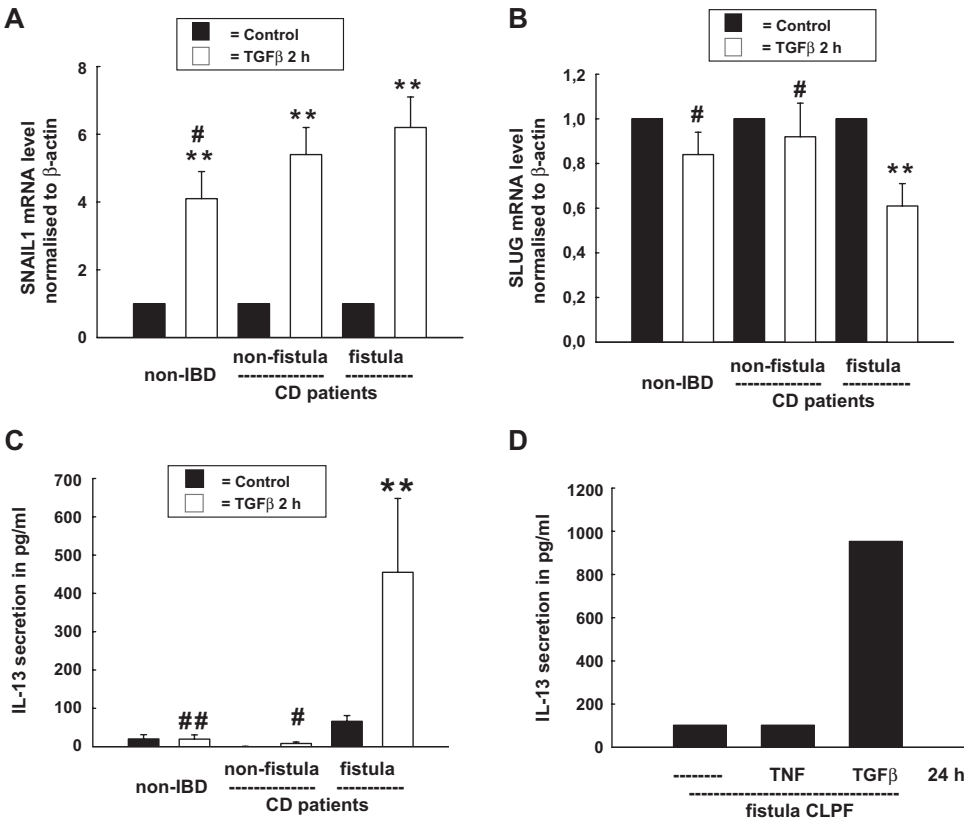

Figure 1 Transforming growth factor β (TGFβ) induced the secretion of interleukin-13 (IL-13) from fistula colonic lamina propria fibroblasts (CLPFs). (A,B) CLPFs derived from non-inflammatory bowel disease (IBD) controls (n=7), patients with non-fistulising Crohn's disease (CD) (n=3) or fistulising disease (n=8) were treated with TGFβ (50 ng/ml) for 2 h. Histograms show mRNA levels of (A) SNAIL1 or (B) SLUG relative to the respective untreated controls. (C) CLPFs from non-IBD controls (n=6), patients with non-fistulising disease (n=3) or fistulising CD (n=5) were treated with 50 ng/ml TGFβ for 24 h. Histogram demonstrates IL-13 concentration in cell supernatants in pg/ml. (D) Fistula CLPFs were treated with TGFβ (50 ng/ml) or tumour necrosis factor (TNF) (100 ng/ml) for 24 h (n=1). Histogram represents IL-13 concentration in the cell supernatant of the cells shown in pg/ml. Significant differences from the respective controls are represented by **p<0.01, #p<0.05, ##p<0.01, versus TGFβ treatment of fistula CLPFs.

of IL-13. Fistula CLPFs had a clearly elevated basal secretion of IL-13 (66±15 pg/ml vs 20±11 pg/ml) and addition of TGFβ for 24 h resulted in a strong and significant increase in IL-13 secretion in the cell supernatant (455±193 pg/ml) (figure 1C). Of note, treatment of fistula CLPFs with TNF was, in contrast to TGFβ, not sufficient to elevate IL-13 secretion (figure 1D).

IL-13 and IL-13 receptor α_1 (IL-13Rα_1) are strongly expressed in TCs

Since we have demonstrated that TGFβ induces IL-13 secretion from fistula CLPFs we analysed whether IL-13 would be expressed in and/or around fistula tracts in patients with CD. First, we studied whether IL-13 would be expressed in the intestinal mucosa of non-IBD controls. By immunohistochemistry we could almost detect no IL-13 staining in these tissue samples (figure 2A). Also, we found only a very weak staining for IL-13 in intestinal tissue specimen of patients with active UC (figure 2B). In intestinal tissue specimens from patients with non-fistulising CD that was either in remission (figure 2C) or active (figure 2D), we detected a certain amount of IL-13 staining. However, the staining was restricted to small areas and mainly limited to the IECs. In contrast, we observed a strong staining for IL-13 in TCs covering the fistula tracts of patients with fistulising CD (figure 2E). Additionally, we found IL-13 staining in epithelial cells of deformed crypts directly next to the fistula tracts and in inflammatory, mainly lymphocytic, infiltrates adjacent to the fistula tracts (figure 2E). It is noteworthy that IECs of regular colonic crypts showed hardly any staining for IL-13 protein (figure 2E, online supplementary figure 1A). The IL-13Rα_1 showed a strong staining intensity in TCs of CD fistulae and in epithelial-like cells lining crypt-like structures adjacent to the fistulae (figure 2F). IECs of regular colonic crypts also expressed a strong staining of the cytokine receptor (online supplementary figure 1B). Interestingly, basal mRNA levels of the decoy receptor, IL-13Rα_2, did not significantly differ in CLPFs from non-IBD controls or patients with fistulising CD or non-fistulising CD (online supplementary figure 2). These findings demonstrate that IL-13 and IL-13Rα_1 are expressed in TCs and epithelial cells of deformed crypts alongside and next to CD-associated fistulae and suggest an involvement of IL-13 in the pathogenesis of such fistulae.

IL-13 induces SLUG and β6-integrin expression in HT29 IECs

We assessed whether IL-13 could be involved in EMT-associated effects in IECs. HT29 IECs were treated with 100 ng/ml IL-13 for 30 min or 24 h, respectively. Administration of IL-13 induced mRNA levels of SLUG and β6-integrin by 24 h treatment (figures 3A,B), but had no effect on SNAIL1 or TGFβ mRNA expression at any tested time point (figures 3C,D). To study how IL-13 could affect β6-integrin levels, we performed SLUG knock-down studies using SLUG-specific siRNA constructs. While IL-13 induced SLUG mRNA in cells transfected with non-specific control siRNA constructs, SLUG-specific oligonucleotides caused a clear reduction of SLUG mRNA in untreated and in IL-13-treated HT29 IECs (figure 3E). In cells transfected with control siRNA constructs, IL-13 induced β6-integrin mRNA levels by 24 h of treatment. This effect was, at least partially, diminished in SLUG knock-down cells (figure 3F). On a protein level, IL-13 induced phosphorylation, indicative of activation of its signalling intermediates, signal transducer and activator of transcription 6 (STAT6) and extracellular signal-regulated kinase 1/2 (ERK1/2) (online supplementary figure 3A), and caused an increase in levels of β-catenin, indicating increased signal transduction via this pathway and claudin-2, while protein levels of E-cadherin and occludin were not affected (online supplementary figures 3B–D). These data indicate that IL-13 induces the expression of genes that are involved in cell invasion in

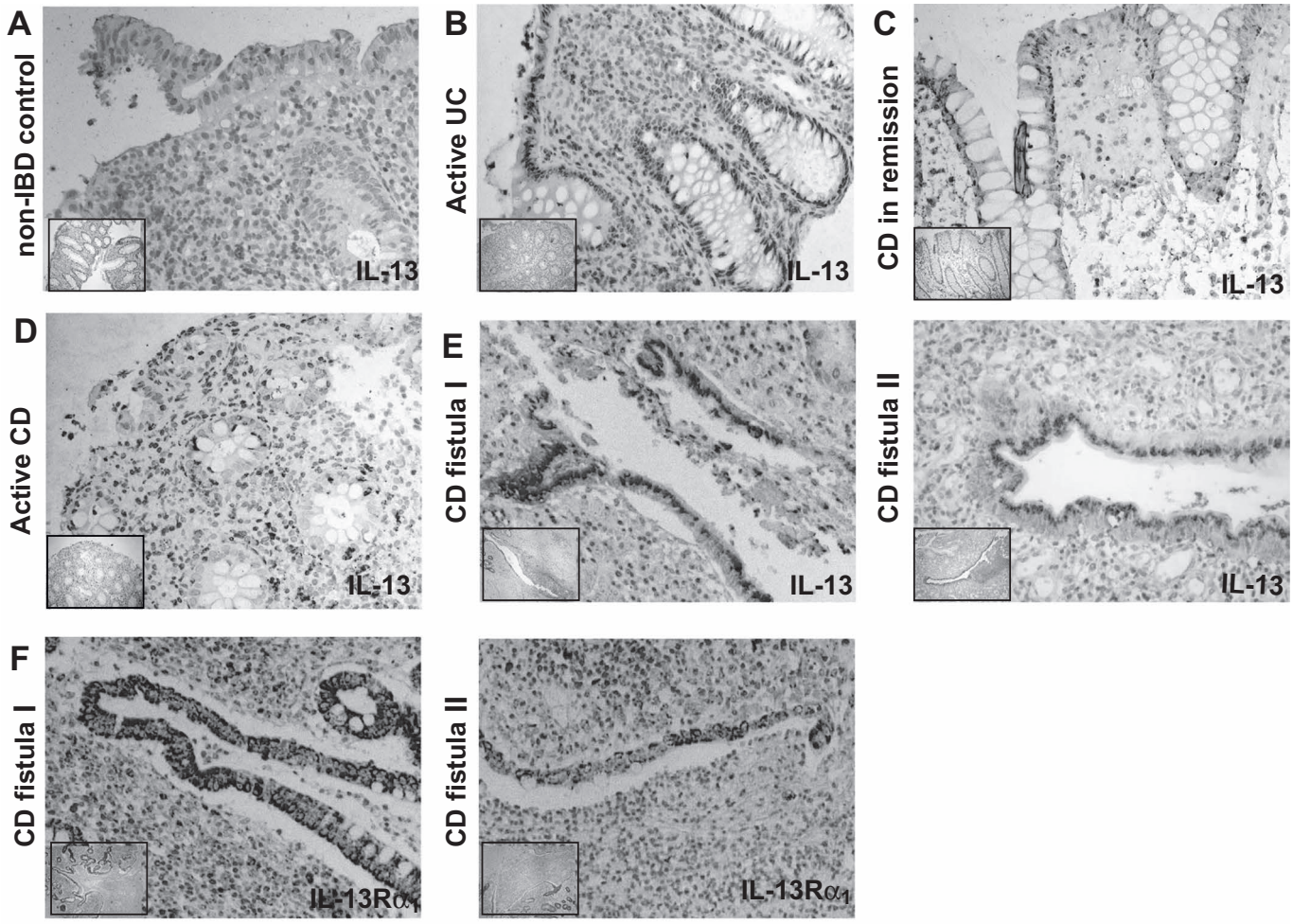

Figure 2 Interleukin-13 (IL-13) and IL-13Rα_1 protein in fistula specimens from patients with Crohn's disease (CD). Intestinal tissue specimen from non-inflammatory bowel disease (IBD) controls (n=6), patients with active ulcerative colitis (UC) (n=5), patients with CD in remission without fistulae (n=7), patients with active non-fistulising CD (n=6) or patients with fistulising CD (fistula resectates) were immunohistochemically stained for IL-13. (A) Representative image shows IL-13 expression in the intestinal mucosa of non-IBD controls. (B) IL-13 protein expression in intestinal tissue samples of patients with active UC. (C) Representative picture demonstrating IL-13 levels in intestinal mucosa of patients with CD in remission (non-fistulising disease). (D) Representative image shows IL-13 levels in intestinal tissue specimens of patients with CD with active, non-fistulising disease. (E) IL-13 is clearly visible in cells lining the fistula tracts and in intestinal epithelial cells (IECs) of deformed crypts adjacent to the fistulae. IECs of crypts with normal appearance feature almost no IL-13 staining. Representative images from two patients are shown. (F) IL-13Rα_1 reveals a similar staining pattern as IL-13. It is clearly detectable in cells lining the fistula tracts and in IECs of deformed crypts adjacent to the fistulae. Representative images from two patients are shown. Magnification: 40-fold. Inserts represent the respective overview image (magnification: fivefold).

HT29 IECs and might hereby contribute to the invasiveness of CD-associated fistulae.

TGFβ, but not IL-13, induces EMT in a HT29-spheroid cell model

We studied whether IL-13 would be able to induce EMT in vitro. We seeded HT29 IECs as spheroids for 7 days. Then, spheroids were either left untreated or treated with TGFβ (20 ng/ml) or IL-13 (100 ng/ml) for an additional 7 days. All of the studied spheroids presented as compact cell formations after 7 days of seeding. Untreated HT29 spheroids showed a similar appearance even after 14 days with a clear black line indicating the border of the cell formation. In contrast, TGFβ treatment resulted in the almost complete disassembly of the cell formation after 7 days of growth factor treatment, indicative of the onset of EMT. IL-13-treated spheroids featured no obvious morphological signs of the onset of EMT, since almost no disintegrated, single cells were detectable. However, the rim of the cell formation was somewhat diffusely confined compared with untreated control spheroids (figure 4A). These findings suggest that IL-13 alone, in contrast to TGFβ, is not sufficient to induce EMT in our IEC model.

TGFβ induces IL-13 and SNAIL1 mRNA expression in HT29 spheroids

We investigated mRNA levels of IL-13, SNAIL1, β6-integrin and SLUG in response to TGFβ treatment in the spheroid cells. We found that TGFβ treatment resulted in a time-dependent increase in IL-13 mRNA reaching statistical significance after 7 days of treatment (figure 4B). We also found that TGFβ increased mRNA expression of the EMT-associated transcription factor, SNAIL1, reaching a peak after 7 days (figure 4C). As shown above, at that time point, HT29 spheroids had completely disintegrated, indicative of the onset of EMT in these cells (figure 4A). These findings also correlated with our observations in fistula specimens from patients with CD showing that TCs lining the fistula tracts, that are thus currently undergoing EMT, feature strong staining for IL-13 (figure 2E) and SNAIL1.[7] In contrast, TGFβ treatment resulted in a significant

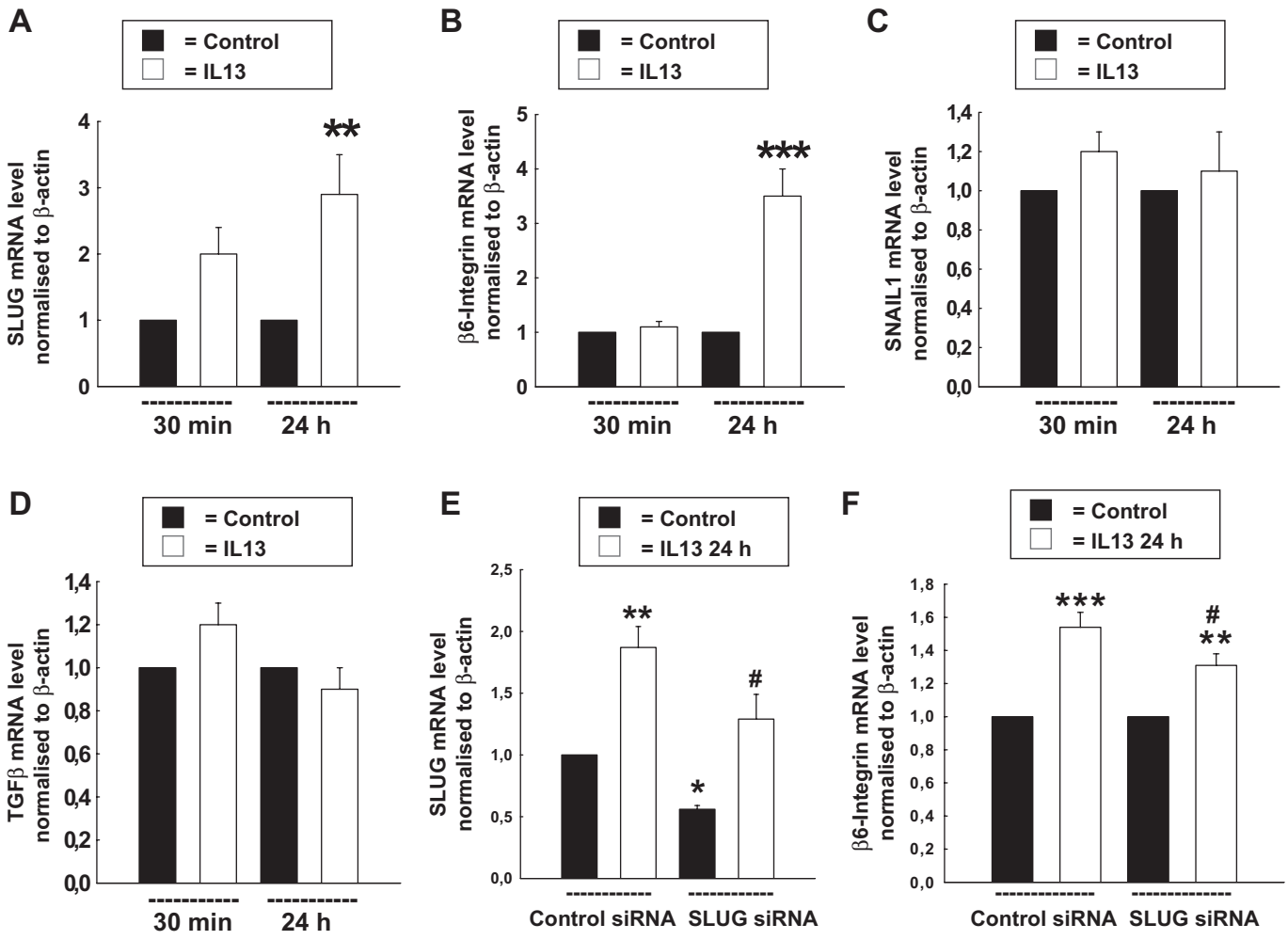

Figure 3 Interleukin-13 (IL-13) induces mRNA levels of SLUG and β6-integrin in HT29 intestinal epithelial cells (IECs). HT29 cells were treated with IL-13 (100 ng/ml) for 30 min or 24 h. Histograms represent the mRNA levels of (A) SLUG (n=7), (B) β6-integrin (n=7), (C) SNAIL1 (n=6) and (D) transforming growth factor β (TGFβ) (n=6) relative to the respective controls. (E,F) HT29 cells were transfected with either non-specific or SLUG-specific siRNA constructs and treated with IL-13 (100 ng/ml) for 24 h. Histogram shows the mRNA level of (E) SLUG and (F) β6-integrin relative to the respective controls. Significant differences compared with the respective controls are represented by *p<0.05, **p<0.01, ***p<0.001, #p<0.05, versus 24 h IL-13 treatment of control siRNA cells.

decrease in β6-integrin expression after 7 days of treatment (figure 4D) and did not (significantly) affect SLUG mRNA at any investigated time point (figure 4E). These data indicate that TGFβ induces EMT-related transcription events in HT29 spheroids and provide a further hint that the growth factor is responsible for the observed IL-13 expression in TCs alongside CD fistula tracts.

IL-13 induces SLUG and β6-integrin expression in an in vitro model of EMT

We analysed mRNA levels in IL-13-treated spheroids. Cytokine treatment induced SLUG mRNA levels up to sixfold after incubation for 1 day. After 5 days and 7days, SLUG expression levels were comparable to those in untreated control cells (figure 5A). In contrast, mRNA expression of β6-integrin was increased at any investigated time point in response to IL-13 (figure 5B). Of note, SNAIL1 mRNA levels were also induced in response to IL-13 treatment for 7 days (figure 5C). On a protein level, TGFβ, but not IL-13 treatment, resulted in a slight but significant reduction in E-cadherin levels after 7 days of treatment (figure 5D, online supplementary figure 4A). While TGFβ administration did not affect claudin-2 protein, IL-13 caused a strong increase in claudin-2 protein levels (figure 5D, online supplementary figure 4B). These data support the hypothesis that TGFβ induces EMT-specific gene expression pattern in IECs, whereas IL-13 induces the expression of genes associated with cell invasion.

Levels of SLUG, β6-integrin and MMP-13 are increased in CD fistulae CLPFs

We assessed basal mRNA expression levels of SLUG and β6-integrin in CLPFs isolated from non-IBD controls or patients with either fistulising or non-fistulising CD. We found that SLUG mRNA levels were higher in CLPFs derived from patients with fistulising CD than in CLPFs from non-IBD controls or patients with non-fistulising CD (figure 6A). A comparable finding was obtained for β6-integrin mRNA levels (figure 6B). We then treated CLPFs with IL-13 to study potential differences in the responsiveness of these cells to the cytokine. However, at the studied time points no differences in the ability of IL-13 to stimulate the phosphorylation of the signalling intermediates, STAT6 and ERK1/2, could be detected. Additionally, no obvious differences in their baseline phosphorylation could be observed (figure 6C). We then investigated baseline and IL-13-induced

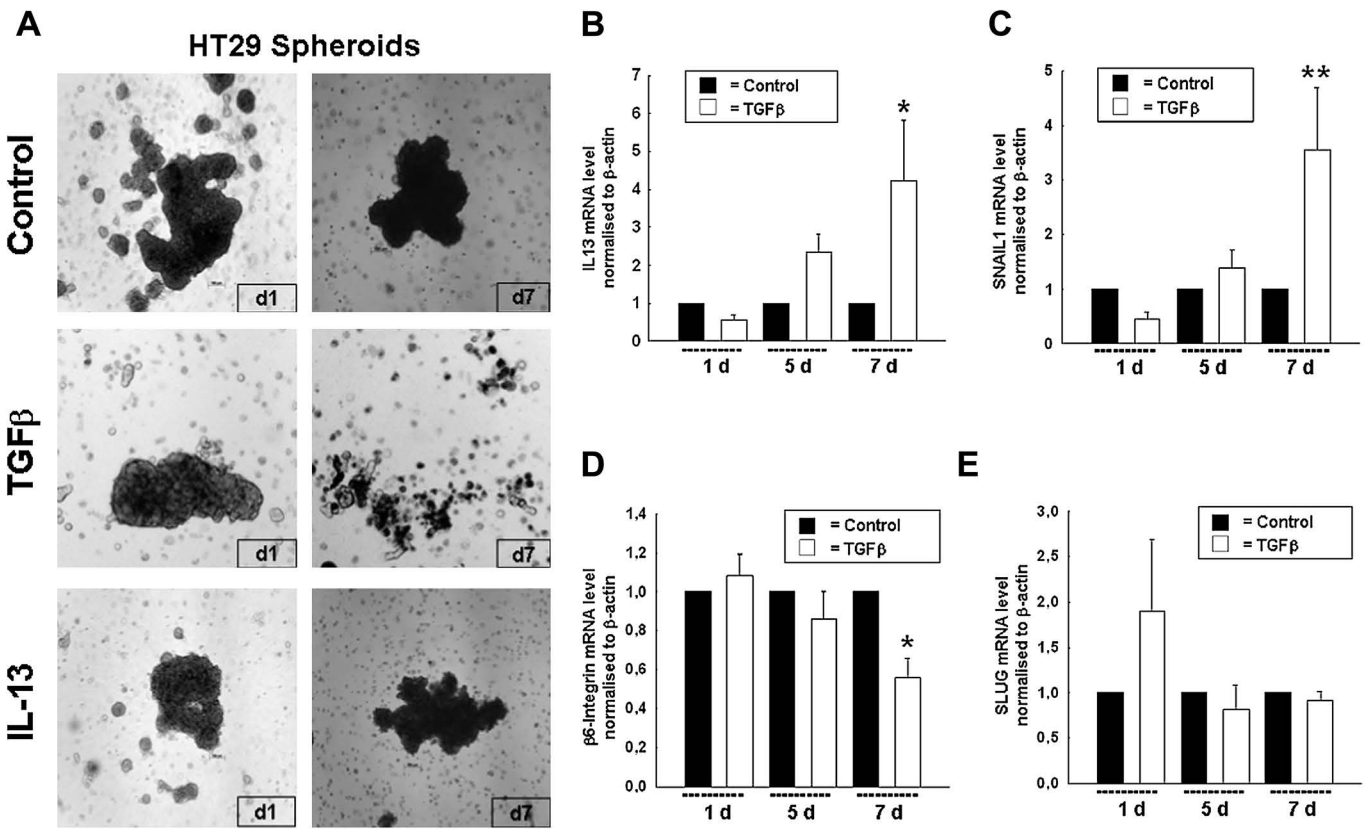

Figure 4 Chronic administration of transforming growth factor β (TGFβ), but not of interleukin-13 (IL-13), induced epithelial to mesenchymal transition (EMT) in a HT29 intestinal epithelial cell (IEC) spheroid model. HT29 cells were seeded as 'hanging drops' for 7 days. Then cells were either left untreated, treated with TGFβ (20 ng/ml) or treated with IL-13 (100 ng/ml) for a further 7 days. Untreated spheroids do not show any alteration in cell formation. TGFβ-treated spheroids almost completely disassemble after 7 days, indicative of the onset of EMT in these cells. In contrast, IL-13 treatment does not cause an obvious cell disassembling, suggesting that IL-13 does not induce EMT in this cell model. Each image is representative of three similar experiments per condition. (B–E) TGFβ induces mRNA levels of IL-13 and SNAIL1 in HT29 spheroids. HT29 spheroids were treated with TGFβ (20 ng/ml) for up to 7 days. Histograms represent the mRNA levels of (A) IL-13, (B) SNAIL1, (C) β6-integrin and (D) SLUG relative to the respective controls (n=3 each). Significant differences compared with the respective controls are represented by *$p<0.05$, **$p<0.01$.

levels of full-length and cleaved matrix metalloproteinase 13 (MMP-13, collagenase-3). The cleaved isoform of MMP-13 represents the activated protein, which is involved in ECM degradation and is associated with tumour metastasis, for example in breast cancer.[31] Basal levels of full-length and cleaved MMP-13 were higher in fistula CLPFs than in CLPFs from patients with non-fistulising disease. However, IL-13 treatment did not cause any obvious alterations in levels of the MMP-13 isoforms (figure 6D). These data demonstrate that CLPFs derived from patients with fistulising CD exhibit higher levels of molecules associated with cell invasion than CLPFs from patients with non-fistulising disease.

Anti-IL-13 antibody treatment prevents IL-13-induced STAT6 phosphorylation and SLUG expression in HT29 IECs

We have demonstrated that IL-13 treatment results in the induction of genes, being involved in fistula pathogenesis, such as SLUG. To study whether antibody-mediated targeting of IL-13 could be a reasonable approach to fistula treatment, we treated HT29 IECs with IL-13 (100 ng/ml) and administered different concentrations of an anti-IL-13 antibody (1–1000 μg/ml) for 30 min. As expected, IL-13 clearly induced STAT6 phosphorylation, and this effect was dose-dependently inhibited by anti-IL-13 antibody co-treatment (figure 7A). We found that an anti-IL-13 antibody concentration of 100 μg/ml caused an optimal inhibitory effect on IL-13-induced STAT6 phosphorylation that could not be further enhanced by higher anti-IL-13 antibody concentrations. Similarly, we found that the optimal inhibitory concentration of the anti-IL-13 antibody with respect to SLUG mRNA levels was also 100 μg/ml at the 24 h time point (figure 7B) and the inhibitory effect of the anti-IL-13 antibody on IL-13-induced SLUG mRNA expression reached statistical significance at that time point (figure 7C). In contrast, IL-13 did not alter mRNA expression of the transcription factor and anti-IL-13 antibody co-treatment also had no effect (figure 7D). We then co-treated HT29 IECs with IL-13 and TGFβ for 24 h. As before, the growth factor had no obvious effect on SLUG mRNA levels and IL-13-induced SLUG expression was not further enhanced by TGFβ co-treatment. Of note, the anti-IL-13 antibody in this approach was also able to considerably diminish IL-13-induced expression of SLUG mRNA in HT29 IECs (figure 7E). We did not detect unspecific effects of the anti-IL-13 antibody in all of our experiments. Of note, administration of an anti-IL-13 antibody did not prevent spheroid formation of HT29 cells (data not shown).

DISCUSSION

In this study we demonstrated that IL-13 is detectable in TC lining fistula tracts and in IECs of deformed crypts adjacent to CD-associated fistulae. TGFβ, the most powerful inducer of EMT, is capable of inducing IL-13 secretion from CLPFs derived from patients with fistulising CD. In an EMT cell model using

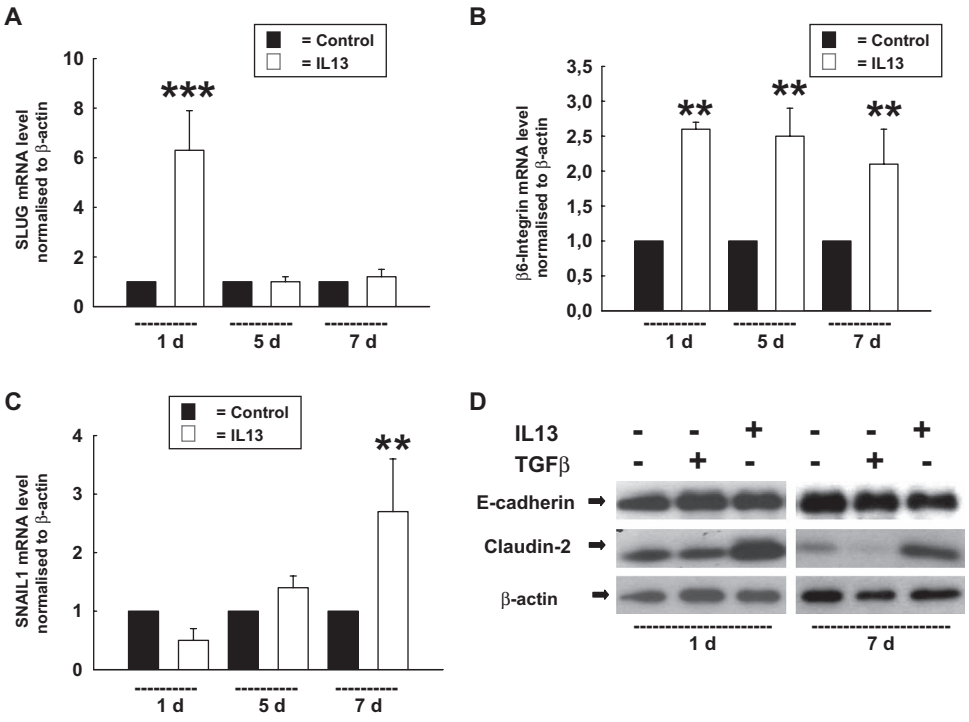

Figure 5 Interleukin-13 (IL-13) induces mRNA levels of SLUG and β6-integrin in HT29 spheroids. HT29 spheroids were treated with IL-13 (100 ng/ml) for up to 7 days. Histograms represent the mRNA levels of (A) SLUG, (B) β6-integrin and (C) SNAIL1 (n=6 each). (D) Representative western blots show protein levels of E-cadherin, claudin-2 and the loading control, β-actin, in spheroids either left untreated or treated with IL-13 (100 ng/ml) or TGFβ (20 ng/ml, n=3 each). Significant differences compared with the respective controls are represented by **p<0.01, ***p<0.001. TGFβ, transforming growth factor β.

HT29 IECs, TGFβ also induces IL-13 mRNA by chronic exposure. On a functional level, IL-13 causes increased expression of genes associated with cell invasion, indicating a role for IL-13 in the pathogenesis of CD fistulae.

As we have shown previously TCs lining the tracts of CD-associated fistulae feature several aspects that strongly support the onset of EMT. In particular they express high levels of transcriptionally active SNAIL1, a downregulation of E-cadherin and the concomitant expression of epithelial (cytokeratine 8 and 20) and mesenchymal markers (vimentin). Additionally, considerable levels of TGFβ are detectable in TCs lining the fistula tracts.[6] A further hint of the onset of EMT during fistula pathogenesis is the fact that fistula CLPFs strongly upregulate SNAIL1 mRNA levels, which could not be observed in CLPFs derived from patients with non-fistulising CD.

We demonstrated strong staining for IL-13 and its receptor, IL-13Rα$_1$, in TCs lining the fistula tracts and in epithelial cells of deformed crypts adjacent to the fistulae. This observation

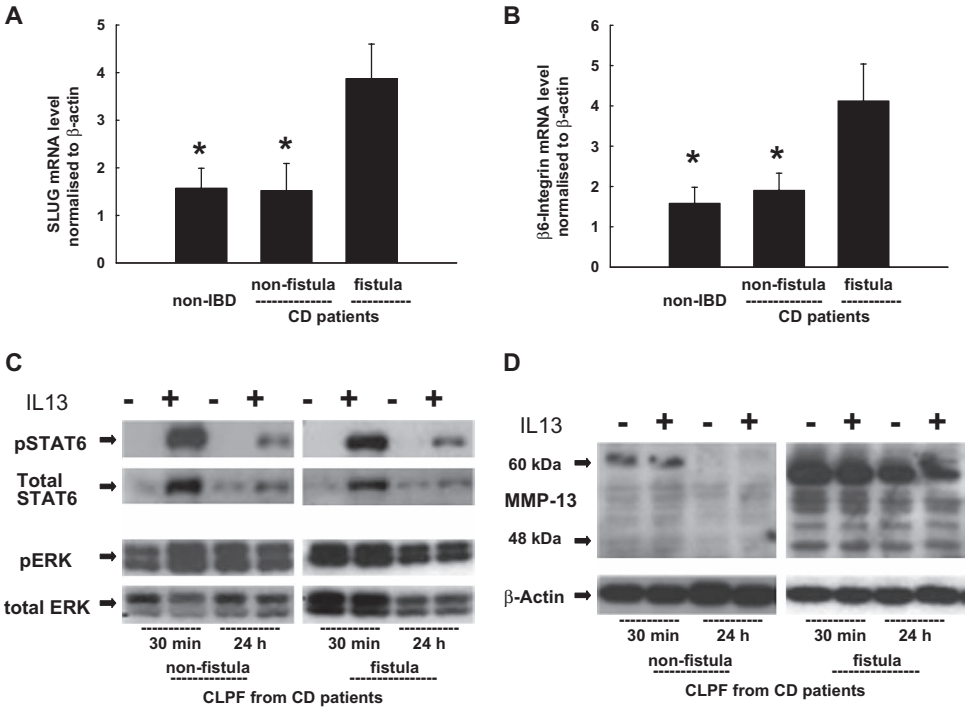

Figure 6 Basal levels of SLUG, β6-integrin and matrix metalloproteinase 13 (MMP-13) are elevated in fistula colonic lamina propria fibroblasts (CLPFs). Histograms show mRNA levels of (A) SLUG and (B) β6-integrin in CLPFs derived from non-inflammatory bowel disease (IBD) controls (n=8) and patients with non-fistulising Crohn's disease (CD) (n=5) or fistulising CD (n=8). These CLPFs were then treated with interleukin-13 (IL-13) (100 ng/ml) for 30 min or 24 h. (C) Representative western blots show levels of phosphorylated (Tyr^{641}) and total signal transducer and activator of transcription 6 (STAT6) and phosphorylated (Thr^{202}/Tyr^{204}) and total extracellular signal-regulated kinase 1/2 (ERK1/2) in CLPFs derived from patients with non-fistulising (n=3) or fistulising (n=3) CD. (D) Representative western blots show protein levels of full-length and cleaved (activated) MMP-13 and the loading control, β-actin, in CLPFs derived from patients with non-fistulising (n=3) or fistulising (n=3) CD. Significant differences compared with CLPFs from patients with fistulising disease are represented by *p<0.05.

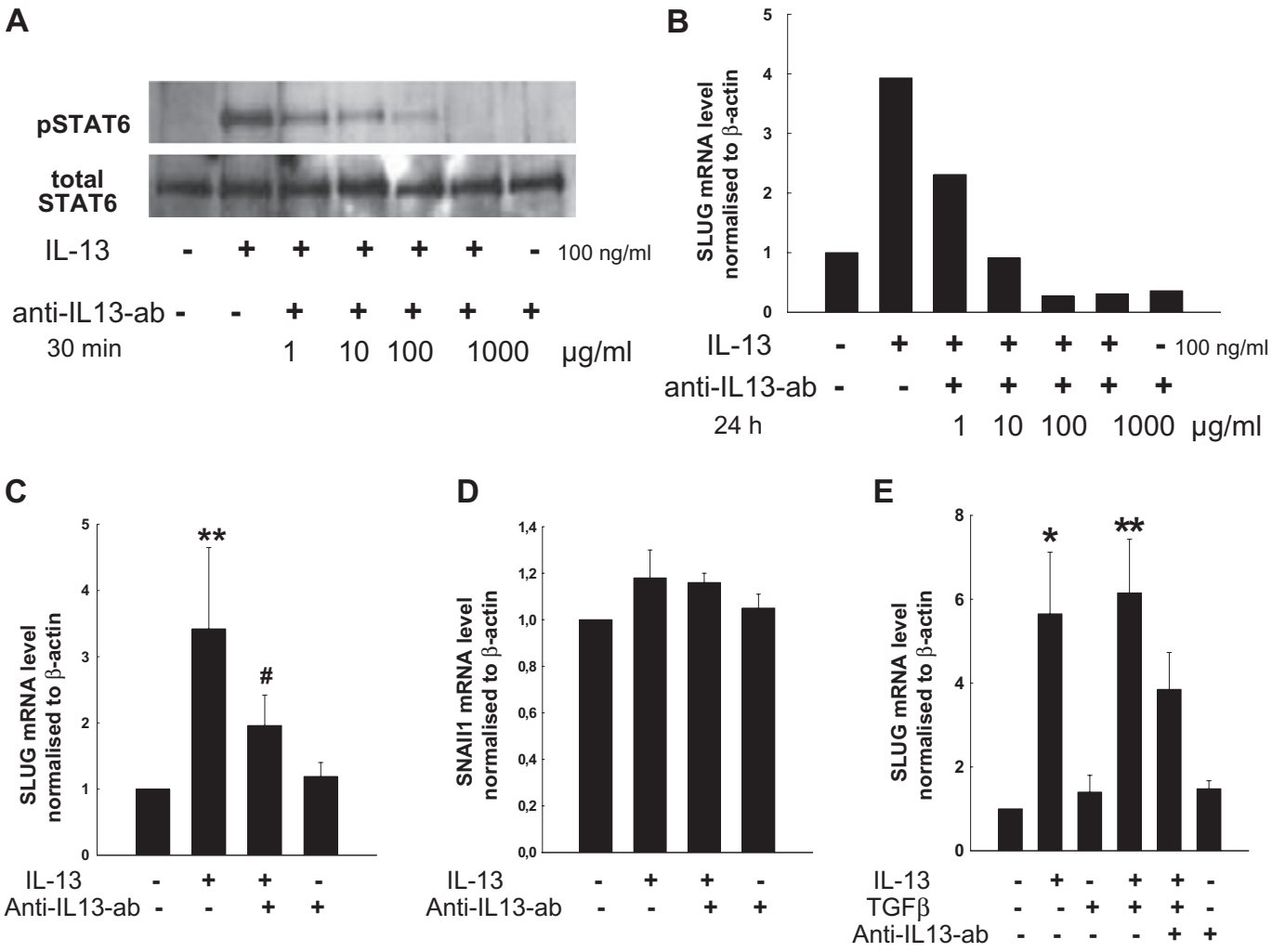

Figure 7 Anti-interleukin-13 (IL-13) antibody treatment prevents IL-13-induced signal transducer and activator of transcription 6 (STAT6) phosphorylation and SLUG mRNA expression in HT29 intestinal epithelial cells (IECs). HT29 IECs were either treated with IL-13 (100 ng/ml) or anti-IL-13 antibody (100 µg/ml) alone or in combination. (A) Representative western blots show levels of phosphorylated (Tyr^{641}) and total STAT6 in HT29 IECs after 30 min of treatment (n=1). (B) Histogram shows mRNA levels of SLUG in HT29 IECs treated with IL-13 (100 ng/ml) and/or anti-IL-13 antibody (100 µg/ml) for 24 h. (C) Histograms represent mRNA levels of SLUG and (D) SNAIL1 in HT29 IECs treated with IL-13 (100 ng/ml) and/or anti-IL-13 antibody (100 µg/ml) for 24 h (n=3 each). (E) Histogram represents mRNA level of SLUG in HT29 IECs treated with IL-13 (100 ng/ml), transforming growth factor β (TGFβ) (50 ng/ml) and/or anti-IL-13 antibody (100 µg/ml) for 24 h (n=3 each). Significant differences compared with the respective controls is represented by *p<0.05, **p<0.01, #p<0.05 versus 24 h treatment of IL-13-treated cells.

was somewhat unexpected because IL-13 was thought to be mainly expressed by immune cells, especially T helper 2 cells.[19] Though IL-13 has not been associated with EMT so far, it has been clearly correlated with the onset of tissue fibrosis, such as pulmonary fibrosis, hepatic fibrosis or systemic sclerosis.[21 22 32] We showed high levels of IL-13 in cells that feature invasive and penetrating cell growth into vicinal tissue layers, such as TCs. Interestingly, we also found high levels of IL-13Rα_1, suggesting that IL-13 itself causes effects on these cells in an autocrine manner.

We then investigated the possible driving force for the expression of IL-13 in TCs or IECs. By stimulating CLPF cultures with TGFβ, we found that the growth factor induced the secretion of IL-13 from fistula CLPFs, but not from non-fistula CLPFs. Additionally, CLPFs isolated from patients with fistulising CD also featured higher basal levels of IL-13 secretion than cells from patients without fistulising CD. In contrast, TNF, which has been widely shown to play a pivotal role for CD pathogenesis,[33] was not sufficient to further elevate IL-13 secretion from fistula CLPFs. Nevertheless, since TGFβ was not sufficient to induce IL-13 secretion from non-fistula CLPFs, additional events seem to be necessary to allow TGFβ to stimulate the secretion of IL-13 from IECs, possibly the concomitant expression of SNAIL1 transcription factor or even epigenetic modifications in these cells following long-term exposure to TGFβ (though we admit that this has not been formally demonstrated).

TCs represent original IECs that underwent EMT. However, in addition to their migratory potential, they obviously exhibit a considerable ability to penetrate into adjacent tissue layers, since they can be found at the invasive top of the fistulae. We have previously found that SLUG and β6-integrin are expressed in TCs or mesenchymal-like cells around the fistula tracts.[6 7] Both of these genes have been associated with tumour invasiveness and cell invasion.[16–18] Therefore, we speculated that IECs should upregulate the expression of both of these molecules during EMT. However, although chronic treatment of HT29 spheroids with TGFβ resulted in an EMT-like

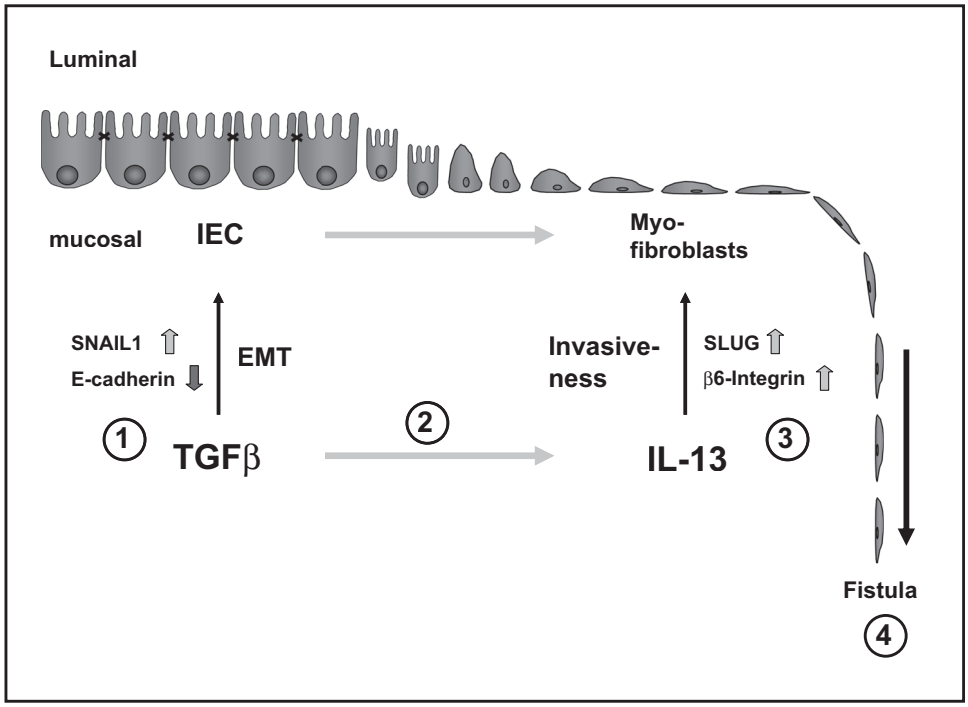

Figure 8 Transforming growth factor β (TGFβ) and interleukin-13 (IL-13) exert synergistic effects on fistula formation in the human intestine. (1) TGFβ induces epithelial to mesenchymal transition (EMT) in intestinal epithelial cells (IECs), possibly as part of the regular wound healing processes during chronic intestinal inflammation. Characteristic markers for the onset of EMT are the upregulation of SNAIL1 and the downregulation of E-cadherin in IECs. EMT finally results in the transformation of IECs into intestinal myofibroblasts featuring epithelial and mesenchymal markers. (2) Those myofibroblasts are, in contrast to IECs, able to express high levels of IL-13 upon stimulation with TGFβ. (3) IL-13 induces the expression of genes being associated with cell invasion, such as SLUG and β6-integrin, finally resulting in an invasive phenotype of the former IECs/ myofibroblasts (4) leading to the development of fistulae.

disintegration of the epithelial cell formation and the EMT-typical upregulation of SNAIL1 mRNA, it was not sufficient to induce mRNA expression of SLUG or β6-integrin but resulted in increased mRNA levels of IL-13. Since we had found strong staining for IL-13 and IL-13Rα_1 in TCs and elevated levels of SLUG and β6-integrin in CLPFs from patients with fistulising CD compared with those from non-fistulising CD, we hypothesised that IL-13 could act on TCs in an autocrine manner to induce the expression of genes associated with cell invasion. To test this assumption, we treated HT29 IECs with IL-13. We found that the cytokine was able to induce mRNA levels of SLUG and β6-integrin in HT29 monolayers and in the spheroid model. However, though in the spheroid model IL-13 was not sufficient to induce an EMT phenotype of these cells, these observations are in line with recent findings showing that IL-13 is associated with increased cell invasion in pancreatic cancer.[24–27] Altogether, these observations strongly support a previously unknown role for IL-13 in the pathogenesis of CD-associated fistulae.

TGFβ treatment of HT29 spheroids resulted in a time-dependent upregulation of SNAIL1 mRNA but downregulation of β6-integrin and SLUG, reaching a peak for all described effects after 7 days. Vice versa, IL-13 induced SLUG and β6-integrin levels after 1 day of treatment and their expression levels declined to control levels after 7 days. Interestingly, SNAIL1 mRNA expression was threefold higher after 7 days of IL-13 treatment than in control cells. These observations suggest that SNAIL1 could act as a repressor of SLUG and β6-integrin expression in IECs. These findings could make sense in a way that TGFβ/SNAIL1-induced EMT acts as a mechanism of wound healing and tissue regeneration at sites of chronic inflammation and tissue destruction, as present during active CD,[34] but does not feature an invasive potential which can be observed in CD fistulae. In contrast, IL-13, which is expressed after chronic exposure of IECs to TGFβ, drives the invasive potential of EMT cells in an autocrine manner. This observation seems to be in contrast to previous findings because, during fibrogenesis, IL-13 acts upstream of TGFβ, whereas in the setting of cell invasion, the cytokine seems to be regulated by the growth factor.

Of particular interest with respect to the therapy of fistulae is the finding that an anti-IL-13 antibody was able to block IL-13-induced events in HT29 IECs, in particular STAT6 phosphorylation and SLUG mRNA expression. Since IL-13 and SLUG are strongly expressed in and around CD-associated fistulae and SLUG expression has been clearly associated with the invasive potential of transformed epithelial cells, the blockade of IL-13-induced SLUG expression might provide a novel and reasonable approach for the treatment of such fistulae.

In summary, our data show for the first time a functional role for TGFβ and IL-13 in the pathogenesis of CD-associated fistulae. Both mediators seem to collaborate in a synergistic step-by-step process whereby TGFβ induces EMT by causing disintegration of the epithelial cell formation, possibly as part of regular wound healing during chronic intestinal inflammation, and IL-13 finally enables the EMT cells to penetrate into deeper tissue layers (figure 8). These findings suggest that dysregulation of TGFβ and/or IL-13-induced effects plays a pivotal role in the pathogenesis of CD-associated fistulae. They also emphasise the importance of further investigations into the detailed mechanisms resulting in the onset of fistulae during the course of CD. In addition, our observations might open up new avenues for the development of more effective therapeutic strategies, for example the use of an anti-IL-13 antibody, for the treatment of such fistulae.

Funding This work was supported by an educational grant from Essex Chemie, Switzerland to MS, a research credit from the University of Zurich to MS, a grant from the Broad Medical Research Program to GR (grant no. IBD-0241R1), a grant from the Swiss National Science Foundation (grant no. 310030-120312) to GR, the Zurich Center for Integrative Human Physiology and the Swiss IBD cohort (SIBDC).

Competing interests None.

Ethics approval Ethics committee of the Canton Zurich, Switzerland.

Contributors MS, GR, AR: conceived the study; MS study concept and design, data acquisition, analysis and interpretation, statistical analysis; GR: study concept and design, data interpretation; TP, JA, SK, SF: Western blotting, immunohistochemistry, cell culture, transfection/nucleofection procedures; EJ, GR, MF, PF: acquisition of

human tissue specimen; AW: pathological assessment of tissue samples; MS, GR: obtained funding. All authors wrote, discussed and revised the manuscript.

Provenance and peer review Not commissioned; internally peer reviewed.

REFERENCES

1. **Solomon MJ.** Fistulae and abscesses in symptomatic perianal Crohn's disease. *Int J Colorectal Dis* 1996;**11**:222—6.
2. **Michelassi F,** Stella M, Balestracci T, *et al*. Incidence, diagnosis, and treatment of enteric and colorectal fistulae in patients with Crohn's disease. *Ann Surg* 1993;**218**:660—6.
3. **Schwartz DA,** Loftus EV Jr, Tremaine WJ, *et al*. The natural history of fistulizing Crohn's disease in Olmsted County, Minnesota. *Gastroenterology* 2002;**122**:875—80.
4. **Bourikas LA,** Koutroubakis IE. Anti-TNF and fistulizing perianal Crohn's disease: use in clinical practice. *Curr Drug Targets* 2010;**11**:187—97.
5. **Judge TA,** Lichtenstein GR. Treatment of fistulizing Crohn's disease. *Gastroenterol Clin North Am* 2004;**33**:421—54, xi—xii.
6. **Bataille F,** Rohrmeier C, Bates R, *et al*. Evidence for a role of epithelial mesenchymal transition during pathogenesis of fistulae in Crohn's disease. *Inflamm Bowel Dis* 2008;**14**:1514—27.
7. **Scharl M,** Weber A, Furst A, *et al*. Potential role for snail family transcription factors in the etiology of Crohn's disease-associated fistulae. *Inflamm Bowel Dis* 2011;**17**:1907—16.
8. **Kalluri R,** Neilson EG. Epithelial-mesenchymal transition and its implications for fibrosis. *J Clin Invest* 2003;**112**:1776—84.
9. **Arias AM.** Epithelial mesenchymal interactions in cancer and development. *Cell* 2001;**105**:425—31.
10. **Thiery JP,** Sleeman JP. Complex networks orchestrate epithelial-mesenchymal transitions. *Nat Rev Mol Cell Biol* 2006;**7**:131—42.
11. **Zavadil J,** Bottinger EP. TGF-beta and epithelial-to-mesenchymal transitions. *Oncogene* 2005;**24**:5764—74.
12. **Kasai H,** Allen JT, Mason RM, *et al*. TGF-beta1 induces human alveolar epithelial to mesenchymal cell transition (EMT). *Respir Res* 2005;**6**:56.
13. **Margetts PJ,** Bonniaud P, Liu L, *et al*. Transient overexpression of TGF-{beta}1 induces epithelial mesenchymal transition in the rodent peritoneum. *J Am Soc Nephrol* 2005;**16**:425—36.
14. **Bates RC,** Mercurio AM. Tumor necrosis factor-alpha stimulates the epithelial-to-mesenchymal transition of human colonic organoids. *Mol Biol Cell* 2003;**14**:1790—800.
15. **Peinado H,** Quintanilla M, Cano A. Transforming growth factor beta-1 induces snail transcription factor in epithelial cell lines: mechanisms for epithelial mesenchymal transitions. *J Biol Chem* 2003;**278**:21113—23.
16. **Bates RC.** Colorectal cancer progression: integrin alphavbeta6 and the epithelial-mesenchymal transition (EMT). *Cell Cycle* 2005;**4**:1350—2.
17. **Bates RC,** Bellovin DI, Brown C, *et al*. Transcriptional activation of integrin beta6 during the epithelial-mesenchymal transition defines a novel prognostic indicator of aggressive colon carcinoma. *J Clin Invest* 2005;**115**:339—47.
18. **Alves CC,** Carneiro F, Hoefler H, *et al*. Role of the epithelial-mesenchymal transition regulator SLUG in primary human cancers. *Front Biosci* 2009;**14**:3035—50.
19. **Wynn TA.** IL-13 effector functions. *Annu Rev Immunol* 2003;**21**:425—56.
20. **Donaldson DD,** Whitters MJ, Fitz LJ, *et al*. The murine IL-13 receptor alpha 2: molecular cloning, characterization, and comparison with murine IL-13 receptor alpha 1. *J Immunol* 1998;**161**:2317—24.
21. **Zhu Z,** Homer RJ, Wang Z, *et al*. Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. *J Clin Invest* 1999;**103**:779—88.
22. **Chiaramonte MG,** Donaldson DD, Cheever AW, *et al*. An IL-13 inhibitor blocks the development of hepatic fibrosis during a T-helper type 2-dominated inflammatory response. *J Clin Invest* 1999;**104**:777—85.
23. **Lee CG,** Homer RJ, Zhu Z, *et al*. Interleukin-13 induces tissue fibrosis by selectively stimulating and activating transforming growth factor beta(1). *J Exp Med* 2001;**194**:809—21.
24. **Kornmann M,** Kleeff J, Debinski W, *et al*. Pancreatic cancer cells express interleukin-13 and -4 receptors, and their growth is inhibited by Pseudomonas exotoxin coupled to interleukin-13 and -4. *Anticancer Res* 1999;**19**:125—31.
25. **Fujisawa T,** Nakashima H, Nakajima A, *et al*. Targeting IL-13Ralpha2 in human pancreatic ductal adenocarcinoma with combination therapy of IL-13-PE and gemcitabine. *Int J Cancer* 2010;**128**:1221—31.
26. **Vallera DA,** Stish BJ, Shu Y, *et al*. Genetically designing a more potent antipancreatic cancer agent by simultaneously co-targeting human IL13 and EGF receptors in a mouse xenograft model. *Gut* 2008;**57**:634—41.
27. **Shimamura T,** Fujisawa T, Husain SR, *et al*. Interleukin 13 mediates signal transduction through interleukin 13 receptor alpha2 in pancreatic ductal adenocarcinoma: role of IL-13 Pseudomonas exotoxin in pancreatic cancer therapy. *Clin Cancer Res* 2010;**16**:577—86.
28. **Obiri NI,** Husain SR, Debinski W, *et al*. Interleukin 13 inhibits growth of human renal cell carcinoma cells independently of the p140 interleukin 4 receptor chain. *Clin Cancer Res* 1996;**2**:1743—9.
29. **Serve H,** Oelmann E, Herweg A, *et al*. Inhibition of proliferation and clonal growth of human breast cancer cells by interleukin 13. *Cancer Res* 1996;**56**: 3583—8.
30. **Brenmoehl J,** Miller SN, Hofmann C, *et al*. Transforming growth factor-beta 1 induces intestinal myofibroblast differentiation and modulates their migration. *World J Gastroenterol* 2009;**15**:1431—42.
31. **Freije JM,** Diez-Itza I, Balbin M, *et al*. Molecular cloning and expression of collagenase-3, a novel human matrix metalloproteinase produced by breast carcinomas. *J Biol Chem* 1994;**269**:16766—73.
32. **Hasegawa M,** Fujimoto M, Kikuchi K, *et al*. Elevated serum levels of interleukin 4 (IL-4), IL-10, and IL-13 in patients with systemic sclerosis. *J Rheumatol* 1997;**24**:328—32.
33. **Reinecker HC,** Steffen M, Witthoeft T, *et al*. Enhanced secretion of tumour necrosis factor-alpha, IL-6, and IL-1 beta by isolated lamina propria mononuclear cells from patients with ulcerative colitis and Crohn's disease. *Clin Exp Immunol* 1993;**94**:174—81.
34. **Rieder F,** Brenmoehl J, Leeb S, *et al*. Wound healing and fibrosis in intestinal disease. *Gut* 2007;**56**:130—9.

Interleukin-13 and transforming growth factor β synergise in the pathogenesis of human intestinal fistulae

Michael Scharl, Sandra Frei, Theresa Pesch, et al.

Gut 2013 62: 63-72 originally published online January 27, 2012
doi: 10.1136/gutjnl-2011-300498