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Proinflammatory cytokines induce crosstalk between colonic epithelial cells and subepithelial myofibroblasts: Implication in intestinal fibrosis ☆

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Abstract

Background and aims: Colonic epithelial cells and adjacent subepithelial myofibroblasts are important counterparts in the pathogenesis of intestinal inflammation and fibrosis. We investigated the possible crosstalk between them, whilst focusing on the mucosal inflammation pathways that potentially trigger intestinal fibrosis.
Methods: We studied the effects of proinflammatory cytokines (IL-1α, TNF-α, IFN-γ) on human colonic epithelial cell lines and the effects of epithelial cell-conditioned media on primary human colonic subepithelial myofibroblasts isolated from normal controls or patients with inflammatory Crohn's disease along with the corresponding 18CO cell line. Readouts included production of TGF-β and TIMP-1, total collagen synthesis, matrix metalloproteinases MMP-2 and MMP-9 and myofibroblast migration/mobility.

Abbreviations α-SMA, alpha-smooth muscle actin; CD, Crohn's disease; CTGF, connective tissue growth factor; ECC, epithelial cell-conditioned; ELISA, enzyme-linked immunosorbent assay; ECM, extracellular matrix; ET, endothelin; ETR, endothelin receptor; HGF, hepatocyte growth factor; IBD, inflammatory bowel disease; IFN-γ, interferon-gamma; IL-1α, interleukin-1alpha; MMP, matrix metalloproteinase; SEMF, subepithelial myofibroblast; TF, tissue factor; TGF-β, transforming growth factor-beta; TIMP-1, tissue inhibitor of metalloproteinases-1; TNF-α, tumour necrosis factor alpha.

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Results: Proinflammatory cytokines upregulated TGF-β and TIMP-1 in colonic epithelial cells. Conditioned medium from these epithelial cell cultures induced production of MMP-9 and collagen and inhibited the migration/mobility of subepithelial myofibroblasts. MMP-9 production depended on endothelin receptor A signalling on responding myofibroblasts. Collagen up-regulation was independent of TGF-β, CTGF, TF and endothelin. Subepithelial myofibroblasts isolated from Crohn's disease patients had similar responses to those isolated from normal controls, with the exception of higher basal collagen production.
Conclusions: Our study indicates that colonic epithelial cells may respond to an inflammatory *milieu* by inducing myofibroblast functions similar to those observed during intestinal fibrosis.

1. Introduction

Intestinal subepithelial myofibroblasts (SEMFs) are alpha-smooth muscle actin (α-SMA)-positive mesenchymal cells located at the interface between the intestinal epithelium and lamina propria. Connective tissue fibrils form a connective tissue barrier called the basal lamina, through which SEMFs and the epithelium can extend interacting pseudopods.[1,2] The epithelial–mesenchymal cell interaction may play an important role in the process of intestinal extracellular matrix (ECM) remodelling and inflammation associated fibrosis. Mucosa overlying Crohn's disease (CD) strictures overexpress profibrotic transforming growth factor-beta (TGF-β) transcripts and tissue inhibitor of metalloproteinases-1 (TIMP-1),[3,4] whilst resident mesenchymal cells overproduce collagens I and III.[5–8] Matrix metalloproteinases (MMPs), including the gelatinases MMP-2 and MMP-9, have also been implicated in ECM remodelling and ulceration in inflammatory bowel disease (IBD).[9–12]

Epithelial cells have been proposed to participate in the control of ECM remodelling in the skin and lungs. In the skin, keratinocytes are partially responsible for the induction of α-SMA in fibroblasts via TGF-β and endothelin-1 (ET-1).[13] Interactions between keratinocytes and the underlying fibroblasts also seem to modulate the levels of MMP-2 and MMP-9.[14] In the lungs, mechanically stressed epithelial cells induce the incorporation of proline into matrix proteins (mostly collagen) by unstressed normal human lung fibroblasts via pathways that involve ET and TGF-β2.[15]

Our study explored possible interactions between epithelial cells and adjacent SEMFs in the gut. We studied the effects of proinflammatory cytokines on the production of profibrotic mediators by epithelial cells, and the effects of proinflammatory cytokines and epithelial cell conditioned medium on MMP activity, collagen production and mobility/migration of colonic subepithelial myofibroblasts isolated from normal controls and CD patients.

2. Materials and methods

2.1. Materials

For cell culture treatments, human interleukin-1alpha (IL-1α), tumour necrosis factor alpha (TNF-α), interferon gamma (IFN-γ) and active recombinant transforming growth factor-β1 (TGF-β1) were purchased from R&D Systems (Abingdon, UK). Mouse monoclonal anti-human antibody was also purchased from R&D Systems (Abingdon, UK) to neutralise all TGF-β isoforms (anti-pan-TGF-β). Neutralising rabbit anti-human connective tissue growth factor (CTGF) polyclonal antibody was purchased from Acris Antibodies GmbH (Hiddenhausen, Germany) and neutralising anti-human monoclonal tissue factor (TF) antibody was purchased from American Diagnostica (Stamford, CT). Human anti-thrombin-III protein (Kybernin® P) was purchased from ZLB Behring (Hattersheim am Main, Germany). We used azepane-1-carbonyl-Leu-D-Trp(For)-D-Trp-OH (BQ-610) and N-cis-2,6-dimethylpiperidinocarbonyl-β-tBu-Ala-D-Trp(1-methoxycarbonyl)-D-Nle-OH (BQ-788) as selective synthetic antagonists for endothelin receptors (ETR) A and B, respectively; these chemicals were purchased from Bachem (Weil am Rhein, Germany).[16] Goat anti-human polyclonal anti-CTGF IgG and goat anti-human monoclonal IgG1 isotype control antibodies used for immunocytochemistry studies were products of Santa Cruz Biotechnologies (Santa Cruz, CA) and DAKO (Carpinteria, CA), respectively.

2.2. Patients

Colonic tissue was obtained by endoscopic biopsy from patients with CD and normal controls undergoing colonoscopy at the Gastroenterology Department, University Hospital of Heraklion, Greece. Colonic biopsies were obtained from 10 control patients, who underwent diagnostic colonoscopy for reasons other than IBD, (e.g. abdominal pain, screening colonoscopy) and where the examination and histology were found to be normal. Biopsies of inflamed colonic mucosa were obtained from three patients with CD ileocolitis. All patients were first diagnosed, with CD proven by colonoscopy and histological examination, they had a Crohn's disease Activity Index >150 and they did not receive any treatment prior to examination. The local Research Ethics Committee has granted approval for this study, and patients gave their informed written consent prior to participation in the study.

2.3. Colonic subepithelial myofibroblast isolation and culture

Studies were performed on subepithelial myofibroblasts (SEMFs; passages 3–8) isolated from colonic tissue. Tissue was obtained by endoscopic biopsy of apparently normal mucosa from patients undergoing screening colonoscopy or inflamed colonic mucosa from the three patients with CD

ileocolitis in compliance with the Helsinki declaration and according to regulations set by our Institutional Review Board. Colonic SEMFs were isolated as previously described.[17] Mucosal specimens were collected in ice-cold Hank's balanced salt solution (HBSS) with Ca^{++}/Mg^{++} plus penicillin (100 U/ml), streptomycin (100 μg/ml), amphotericin B (2.5 μg/ml), gentamicin (50 μg/ml), vancomycin (32 μg/ml) (Gibco, Paisley, UK) and were de-epithelialised with three individual 10-min treatments with 1 mM dithiothreitol (DTT; Sigma-Aldrich, St. Louis, MO) along with three separate 30-min incubations in 3 mM ethylene-diaminetetraacetic acid (EDTA; Sigma-Aldrich, St. Louis, MO) at 37 °C. HBSS without Ca^{++}/Mg^{++} plus the same antibiotics was used for wash and DTT- or EDTA-containing media. Colonic tissue, denuded from epithelium, was ultimately incubated in Dulbecco's Modified Eagle Medium (DMEM, 4.5 g/l glucose; GIBCO, Paisley, UK) plus 10% foetal bovine serum (FBS; Gibco, Paisley, UK) and penicillin/streptomycin/amphotericin B (P/S/A) in 5% CO_2 at 37 °C. During culturing, numerous non-adherent and adherent cells appeared in the culture flasks. The cells in suspension were removed every 72 h, and the denuded mucosal tissue was maintained in culture for up to 4 weeks, until numerous foci of myofibroblasts appeared, which were attached to the bottom of the culture flask. Tissue specimens were then removed, and intestinal myofibroblasts were cultured in DMEM supplemented with 10% FBS and P/S/A. The myofibroblast phenotype was verified using immunofluorescence microscopy by confirming the expression of α-smooth muscle actin and vimentin and the absence of desmin expression.[17]

2.4. Cell culture treatments

Two human colon cancer epithelial cell lines (HT-29 and CaCO-2) and the human embryonic colonic subepithelial myofibroblast cell line 18CO were purchased from the European Collection of Animal Cell Cultures (ECACC, Porton Down, UK). HT-29, CaCO-2 and 18CO cell lines were cultured in 75 cm^2 flasks (NUNC, Roskilde, Denmark) with McCoy's, Modified Eagle Medium (MEM), and MEM-Alpha media (Gibco, Paisley, UK), respectively. Media were enriched with 10% FBS and P/S/A. Colonic epithelial cell lines were chosen after preliminary experiments showed low yields and decreased viability in primary human intestinal cells. The 18CO cell line is derived from human neonatal colonic mucosa and exhibits many of the properties of intestinal SEMFs, such as reversible stellate morphology, α-SMA expression, intracellular organelles characteristic of fibroblasts and smooth muscle cells, membrane receptors for natriuretic peptides and endothelins.[38] SEMFs and 18CO cells were passaged at a ratio of 1:2, whilst HT-29 and CaCO-2 at a ratio of 1:3. All experiments were performed with FBS-free media at 95% culture confluence with a stable ratio of supernatant:cell surface (1.5 ml:9.6 cm^2). Cultured cells were treated with the following proinflammatory cytokines: IL-1α (10 ng/ml), TNF-α (100 ng/ml), and IFN-γ (300 U/ml) used alone or in combination. To prepare epithelial cell-conditioned (ECC) media, confluent epithelial cells (HT-29 and CaCO-2) were treated in 6-well plates in the absence or presence of IL-1α, TNF-α, IFN-γ or combinations of two or three of the cytokines (3C) for 6 h. The cells were then washed, and the supernatant was replaced with cytokine-free media for another 18 h. The resulting ECC media were placed over confluent cultures of SEMFs or 18CO cells in 12-well plates for 24 h. The effects of neutralising antibodies (10 μg/ml anti-pan-TGF-β, 6 μg/ml anti-CTGF, 10 μg/ml anti-TF) or organic blocking substances (3 IU/ml anti-thrombin-III, 1 μM BQ-610, 1 μM BQ-788) were studied in ECC media. Supernatant from SEMF and 18CO cultures was collected, and cells were lysed with a commercially available lysis buffer (Promega, San Luis Obispo, CA). Supernatants and cell lysates were kept frozen at −80 °C until assayed.

2.5. Enzyme linked immunosorbent assay (ELISA)

Human DuoSet® ELISAs (R&D Systems, Abingdon, UK) were used to estimate the concentrations of the three TGF-β isoforms and TIMP-1 in supernatants. Flat 96-well plates were coated overnight with the primary antibody. The plates were then blocked with the recommended reagent for 1 h. Latent TGF-β in samples was activated with acidification (1 N HCl for 10 min). Samples were neutralised with 2.7 N NaOH/1 M HEPES for TGF-β1 or 1.2 N NaOH/0.5 M HEPES for TGF-β2 and TGF-β3. Duplicates of each sample were added in wells for 2 h, which was followed by incubation with the secondary antibody for another 2 h. Conjugation with horseradish peroxidase for 20 min and addition of tetramethylbenzidine with H_2O_2 produced differing optical densities (OD) of colour at 450 nm, which were measured after an additional 10–20 min. The results were corrected by subtraction of the OD at 540 nm. ODs of provided TGF-β1, TGF-β2, TGF-β3 and TIMP-1 standards were used to plot standard curves for the calculation of sample concentrations.

2.6. Gelatin zymography

The enzymatic activities of MMP-2 and MMP-9 were assessed by gelatin zymography.[18] Sodium dodecyl sulphate (10%; Merck, Darmstadt, Germany) gels with 0.25% bovine gelatin (Sigma-Aldrich, St. Louis, MO) were prepared. Supernatants were added to 5× sample buffer (0.2 M Tris–HCl, pH 6.8, 5% SDS, 20% glycerol, 0.1% bromophenol blue; Sigma-Aldrich, St. Louis, MO) and incubated at room temperature for 10 min. Samples were loaded onto gels and run at 20 mA with a maximum voltage of 140 V for 6 h. Afterwards, gels were washed four times (15 min each) in renaturing buffer (2.5% Triton X-100; Sigma-Aldrich, St. Louis, MO) to restore enzyme three-dimensional structure and functionality; this was followed by a 30-min incubation in development buffer (50 mM Tris–HCl, pH 7.5, 0.2 M NaCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 0.02% Brij-35; Sigma-Aldrich, St. Louis, MO). Finally, gels were incubated in new development buffer at 37 °C for 60–80 h.

After that incubation period, gels were incubated for 3 h in staining solution (0.5% Coomassie blue R-250, 30% methanol, 10% acetic acid; Sigma-Aldrich, St. Louis, MO) and were then incubated with destaining solution (50% methanol, 10% acetic acid; Sigma-Aldrich, St. Louis, MO) for 30–60 min to achieve maximum contrast between the pale areas of enzymatic activity and the blue stained areas of no enzymatic activity. MMP-9 was identified by an area of

gelatinolytic activity with an approximate molecular weight of 100 kDa, whilst pro- and active MMP-2 activity was viewed at approximately 70 kDa. Band size was indicated by the appropriate MW marker (Invitrogen, Paisley, UK or Takara, Otsu, Japan). Gelatinolytic band densities were quantified with ImageJ version 1.41 software (US National Institute of Health, Bethesda, MD).

2.7. Total intracellular collagen measurement

Collagen was measured by the Sircol assay.[19] Specifically, cell lysates were added to Sirius Red dye (Sircol; Biocolor, Carrickfergus, UK), which bound to the side chain groups of the basic amino acids that were present in collagen. After a 30-min incubation, the mixtures were centrifuged at 10,000 *g* for 10 min. The supernatant was discarded, and the collagen pellet was dissolved in 0.5 M NaOH alkali reagent for 10 min. Next, the ODs of the samples and controls of known concentration were measured at 540 nm, and the collagen concentration was calculated using a linear standard curve according to the manufacturer's instructions.

2.8. Wound healing scratch assay

Myofibroblast migration was assessed in vitro with the wound-healing scratch assay, as previously described.[20] A narrow gap devoid of cells was created on the SEMF cultures. Next, we measured the rate of gap closure. This process resembles wound healing and is dependent on myofibroblast migration.[20] The FBS concentration in the media was gradually reduced from 10% to 5% to minimise the contribution of proliferation. Subsequently, monolayers of confluent SEMF cultures in 6-well plates were given one mechanical wound per well by scoring with a 200 μl pipette tip. Wound was vertical to pre-drawn lines on the bottom of the well so that we could define more than 12 stable points per well at the junctions of those lines with the wound. Images were recorded at those fixed wound points with an inverted DMIRE2 Leica microscope equipped with a DFC300 FX Leica camera (Wetzlar, Germany). SEMFs were treated with proinflammatory cytokines (IL-1α, TNF-α, IFN-γ), TGF-β1 or ECC media from epithelial cells that were pretreated in the absence or presence of proinflammatory cytokines. Wells were reassessed at 48 h. The average percentage of gap closure after 48 h in treated wells was divided by the average percentage of gap closure in untreated wells to assess the treatment effect.

2.9. Immunocytochemistry

Immunocytochemical staining was performed according to the manufacturer's instructions with the LSAB+ System-AP kit (DAKO, Carpinteria, CA). Confluent SEMF cultures on 2-well chamber slides (NUNC, Roskilde, Denmark) were treated with or without ECC media from epithelial cells pretreated in the absence or presence of proinflammatory cytokines. After 24 h of treatment, cells were washed and fixed with ice-cold ethanol for 20 min. After rehydration, non-specific staining was blocked with a 30-min incubation in 3.3% NGS, and slides were subsequently incubated with goat anti-human CTGF antibody (1:100 dilution) at room temperature for 4 h. Next, slides were incubated in buffer that contained biotinylated anti-goat immunoglobulin. Staining was performed with streptavidin-alkaline phosphatase and the appropriate chromogens. Haematoxylin was used to counterstain nuclei.

2.10. Statistics

Data were presented as the mean ± standard error of the mean (SEM) of at least 3 independent experiments. Comparisons between groups were performed using Student's *t* test for independent samples. Statistical significance was established at $p = 0.05$.

3. Results

3.1. Colonic epithelial cells produce profibrotic mediators

We studied TGF-β1 production in colonic epithelial cell lines (HT-29 and CaCO-2). Both cell lines secreted substantial amounts of TGF-β1 into their supernatants without any stimuli with CaCO-2 having a lower steady state production (Fig. 1A and B). The addition of the proinflammatory cytokines IL-1α, TNF-α and IFN-γ into the cell culture media significantly increased the production of TGF-β1. IL-1α was the most potent single stimulator of TGF-β1 production in both cell lines ($p<0.01$), whilst TNF-α and IFN-γ had less pronounced effects (Fig. 1A and B).

When combinations of two cytokines were used as stimuli, we observed a significant additive increase of TGF-β production with some combinations. However, the highest increase in TGF-β production was observed when colonic epithelial cells were treated with a cocktail of all three cytokines ($p<10^{-11}$ for HT-29, $p<0.01$ for CaCO-2) (Fig. 1A and B). The other two TGF-β isoforms (TGF-β2 and TGF-β3) were also secreted by untreated colonic epithelial cells in considerable amounts. Their production was increased following treatment with proinflammatory cytokines in a manner similar to TGF-β1 (data not shown).

We also examined secretion of TGF-β1, TGF-β2 and TGF-β3 in cultured colonic subepithelial myofibroblasts. Primary SEMFs and the 18CO cell line secreted very small quantities of TGF-β1 after 24 h in culture. Moreover, SEMF and 18CO cultures were not responsive to IL-1α, TNF-α or IFN-γ, added in isolation or in every possible combination. TGF-β3 was also secreted at low concentrations and was not upregulated by those cytokines. We were unable to detect any TGF-β2 production in primary SEMF or 18CO cultures, even after stimulation with a cocktail of all three proinflammatory cytokines (data not shown).

Furthermore, colonic epithelial cell lines secreted substantial amounts of TIMP-1 into the cell culture supernatant. Treatment with TNF-α or IFN-γ, but not IL-1α, significantly increased TIMP-1 secretion above baseline in HT-29 ($p<0.05$) and CaCO-2 cells (TNF-α: $p<0.05$; IFN-γ: $p<0.05$) (Fig. 1C and D). Treating with two or all three cytokines together also resulted in a further increase in TIMP-1 production for HT-29 ($p<0.01$) and CaCO-2 cells ($p<0.05$) (Fig. 1C and D). Primary SEMFs and 18CO cells also exhibited substantial TIMP-1 secretion without any stimulation. However, TIMP-1

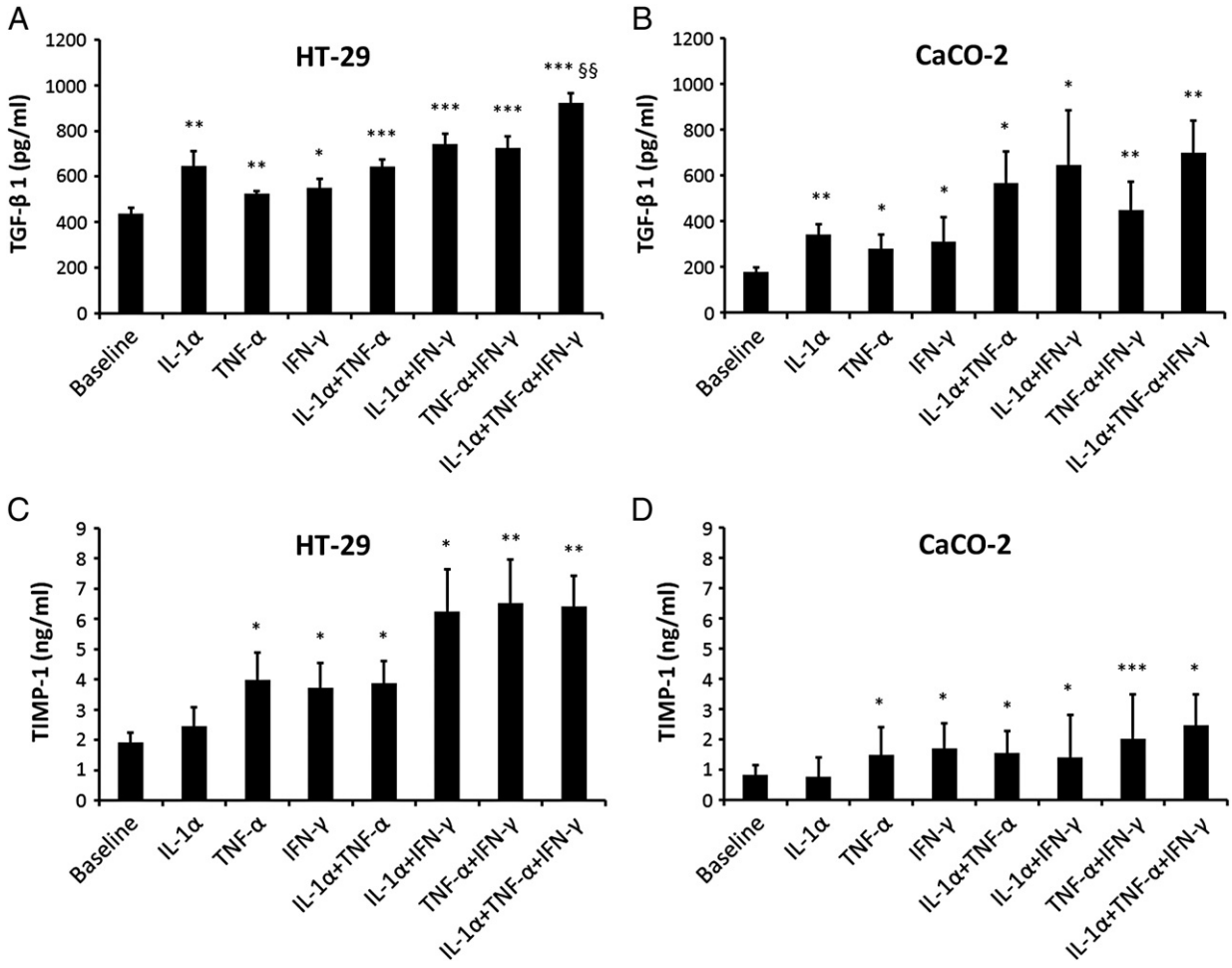


Figure 1 Proinflammatory cytokines increase TGF-β 1 and TIMP-1 production by colonic epithelial cells. TGF-β1 (A, B) and TIMP-1 (C, D) production in HT-29 (A, C) and CaCO-2 (B, D) cell lines were measured in cell culture supernatants by ELISA. The effects of stimulation with IL-1α, TNF-α and IFN-γ for 24 h on colonic epithelial cells are demonstrated. The results from = 3 experiments are shown. * $p = 0.05$, ** $p < 0.01$, *** $p < 0.001$ relative to baseline production. §§ $p < 0.01$ relative to the production induced by combinations of two cytokines.

production in SEMFs and 18CO cells was unresponsive to stimulation with IL-1α, TNF-α and IFN-γ, in isolation or in combination.

3.2. Epithelial cell-conditioned medium regulates gelatinolytic activity of colonic subepithelial myofibroblasts via the endothelin receptor A

We examined the presence of two gelatinases (MMP-2 and MMP-9) in the cell culture supernatant of colonic subepithelial myofibroblasts. Gelatinases were detected with gelatin zymography as gelatinolytic bands and discriminated by their molecular weights. MMP-2 activity was detected as one or two gelatinolytic bands (the second corresponding to active MMP-2) at approximately 70 kDa. Primary SEMFs from normal controls (Fig. 2A), CD patients (Fig. 3D) and 18CO cells (Fig. 2C) secreted MMP-2 into the cell culture supernatant without any additional stimulus after 24 h in culture. No MMP-9 activity was observed in untreated SEMFs from normal controls (Fig. 2A), CD patients (Fig. 3D) and 18CO cells (Fig. 2C), as shown by the lack of a gelatinolytic band at approximately 100 kDa.

Stimulation of primary control SEMFs and 18CO cells with TNF-α or IL-1α for 24 h induced MMP-9 activity in their supernatants (Fig. 2). Combination of both TNF-α and IL-1α had an additive effect ($p < 0.05$) (Fig. 2A, B). IFN-γ had no effect if added alone, but suppressed MMP-9 activity when added to TNF-α ($p < 0.05$), IL-1α or both ($p < 0.05$) (Fig. 2). Those cytokines in isolation or in every possible combination had no effect on MMP-2 activity (Fig. 2A, C). Colonic epithelial cell lines were also tested but showed no gelatinolytic activity despite stimulation with IL-1α, TNF-α, IFN-γ, in isolation or in combination (data not shown).

We subsequently explored the effect of epithelial cell-conditioned media (ECC) on SEMF cultures. ECC media from CaCO-2 cultures pretreated for 6 h with IL-1α, TNF-α, IFN-γ, alone or in combination, induced substantial amounts of MMP-9 activity in SEMFs from normal controls (Fig. 3A), CD patients (Fig. 3D) and 18CO cells (data not shown). Similar results were obtained when we used the HT-29 cell line as a source of ECC for both SEMFs from normal controls (Fig. 3C, E and F) and CD patients (Fig. 3C and F). Since the cytokines used for epithelial cell stimulation were washed out of the cultures and fresh medium was used to produce the ECC, we hypothesized that MMP-9 production by myofibroblasts was

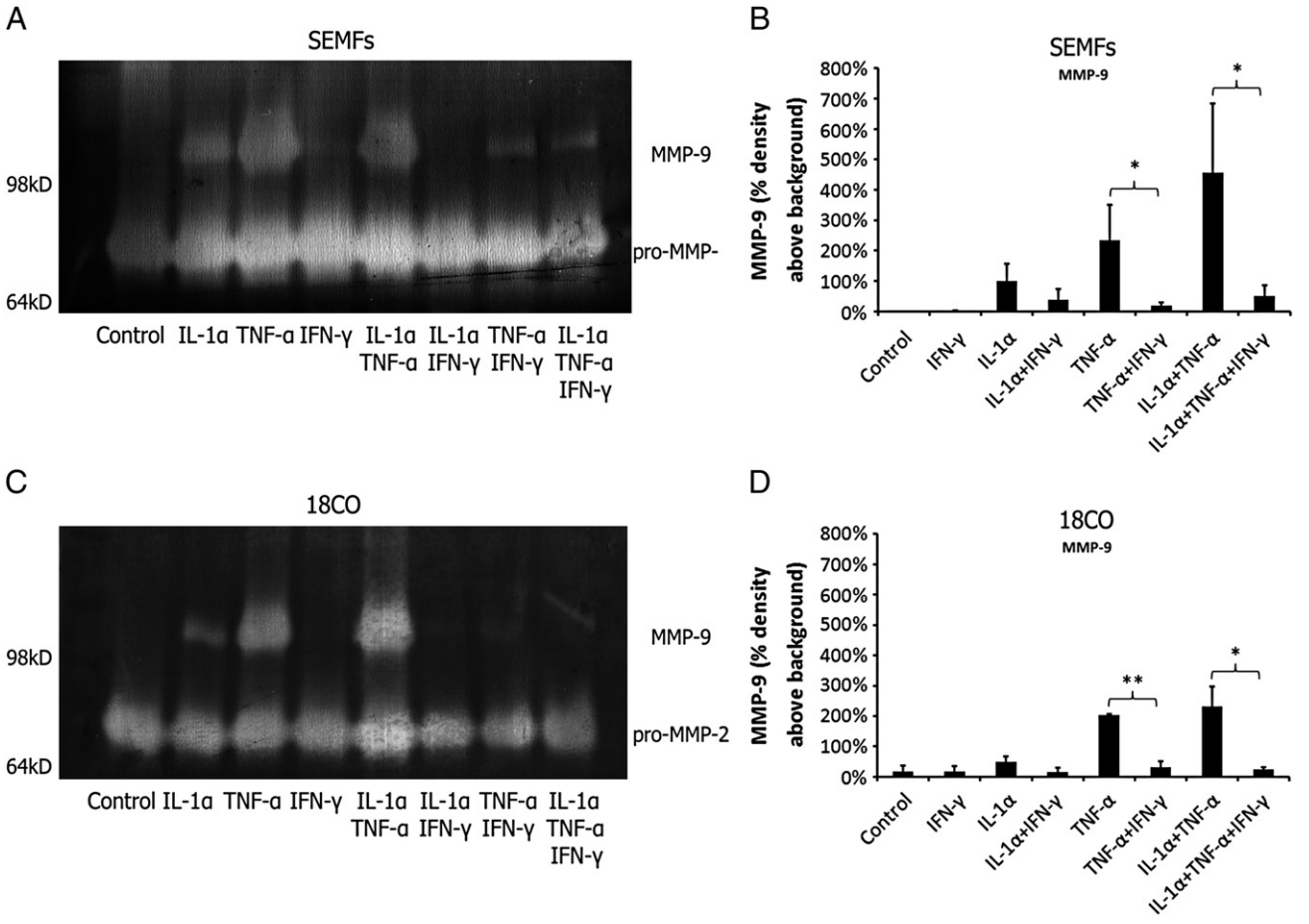


Figure 2 IFN-γ inhibits MMP-9 induction by TNF-α or IL-1α in SEMFs. Cultures of SEMFs isolated from normal controls or 18CO cells were treated with proinflammatory cytokines for 24 h. Gelatin zymography gels are representative of 3 experiments and show pro-MMP-2 (≈70 kDa) and MMP-9 (≈100 kDa) activity in SEMF (A) and 18CO (C) supernatants. MMP-9 was quantified by densitometry analysis and values are mean ± SEM of three independent experiments on SEMFs (B) and 18CO cultures (D). * $p<0.05$, ** $p<0.01$.

triggered by a soluble mediator derived from the cytokine-treated epithelial cells.

We next examined if TGF-β was responsible for the induction of MMP-9 activity in SEMFs treated with ECC medium. This hypothesis was based on the observation that exogenous TGF-β1 can induce MMP-9 activity in primary control SEMF cultures in a dose dose-dependent manner (Fig. 3B). However, neutralisation of all TGF-β isoforms did not significantly affect MMP-9 production by control or CD SEMF cultures treated with ECC media (Fig. 3C). Results were similar with ECC from CaCO-2 cells or if 18CO cells were used as the responding myofibroblasts (data not shown). Similarly, neutralisation of CTGF, which is a well-known growth factor affecting extracellular matrix turnover, did not have any effect on MMP-9 production by control (data not shown) or CD (Fig. 3D) SEMFs.[21,22]

Next, we assayed whether MMP-9 upregulation was dependent on endothelin. Inhibition of endothelin receptor A (ETR-A) signalling in SEMFs isolated from control or CD patients blocked the induction of MMP-9 by ECC media (Fig. 3E and F). In contrast, specific inhibition of endothelin receptor B (ETR-B) had no effect on MMP-9 production (Fig. 3E and F). Furthermore, there was no additional effect on MMP-9 inhibition when both ETR-A and ETR-B were blocked (Fig. 3E and data not shown).

3.3. Epithelial cell derived soluble factors increase collagen production by SEMFs

We further explored collagen production by myofibroblasts. The Sircol assay was used to measure the majority of collagen types cumulatively. SEMFs from normal controls and 18CO cells secreted minimal amounts of total collagen in cell culture supernatants in preliminary experiments, so we studied total collagen production by measuring total intracellular collagen. SEMFs derived from patients with CD produced more collagen compared to SEMFs from normal controls ($p<0.05$, Fig. 4A and B). IL-1α, TNF-α, and IFN-γ treatment alone, in pairs or all three together (3C) did not have a significant effect on collagen synthesis (Fig. 5A and B, respectively). Stimulation with TGF-β1 produced a minimal non-significant increase in collagen production at 24 h (Fig. 5C and D, respectively). We next studied the effect of ECC media on collagen production in SEMFs. ECC media equally increased collagen production in SEMFs derived from

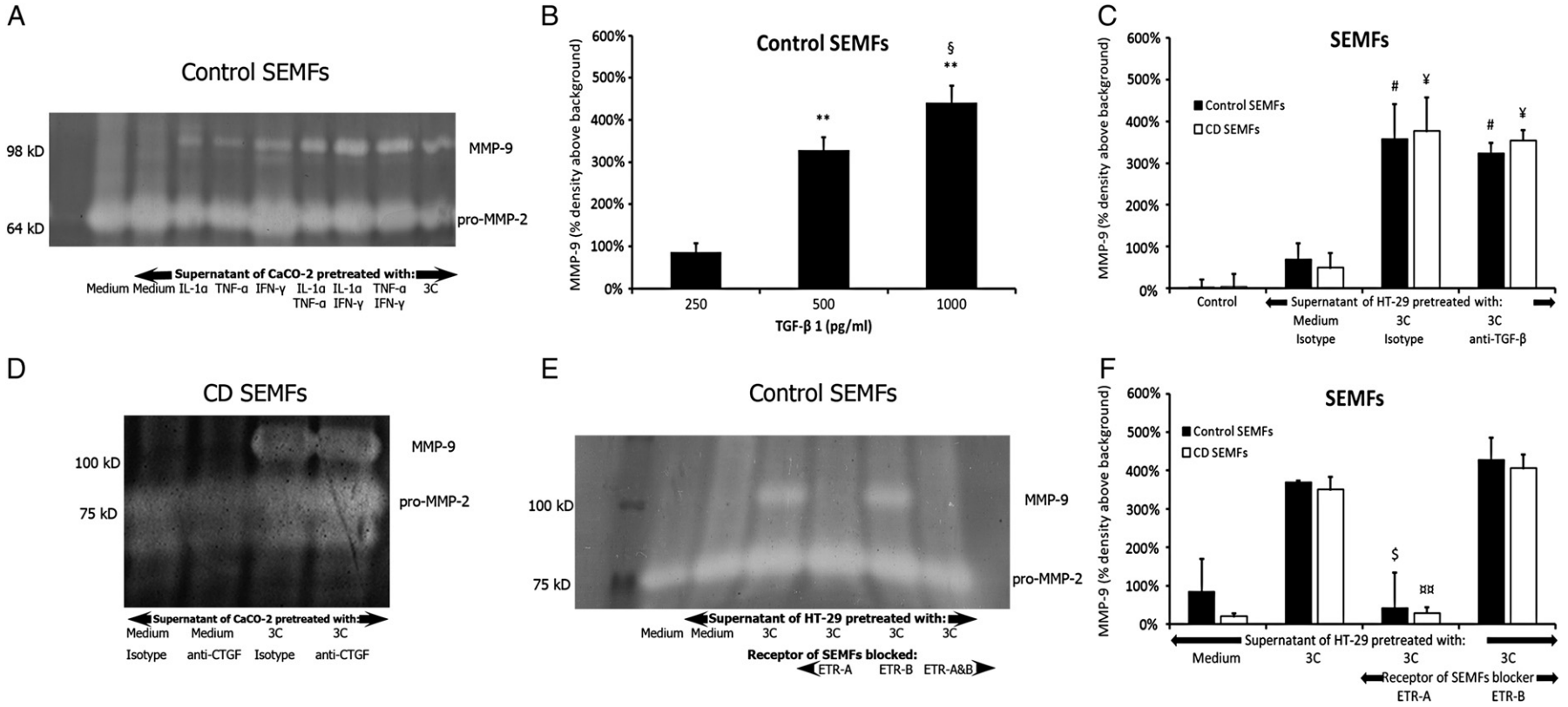


Figure 3 Conditioned medium from epithelial cells pretreated with proinflammatory cytokines induce MMP-9 in control or CD SEMFs via an endothelin receptor A dependent pathway. SEMFs isolated from normal controls or CD patients were treated for 24 h with ECC from HT-29 or CaCO-2 cultures. ECC medium was prepared by setting fresh medium devoid of cytokines over HT-29 or CaCO-2 for 18 h, after pretreating them with or not with IL-1α, TNF-α and IFN-γ in various combinations or all together (3C) for 6 h, as described in the Materials and methods section. (A, D, E) Representative gelatine zymograms showing MMP-2 and MMP-9 activity in SEMF culture supernatants. (B, C, F) Quantification of MMP-9 by densitometry analysis of gelatin zymograms. Values are mean ± SEM of three independent experiments. (A) Effects of proinflammatory cytokines on MMP production (B) Effect of TGF-β1 on MMP production; ** $p<0.01$ relative to baseline values, § $p<0.05$ from 500 pg/ml TGF-β1 (C) Effects of ECC medium and TGF-β blockage on MMP-9 production; # and ¥ $p<0.05$ from ECC of untreated HT-29. (D) Effects of ECC medium and CTGF blockage on MMP production. (E) Effects of ECC medium and ETR blockage on MMP-9 production; $ $p<0.05$ or ¤¤ $p<0.01$ relative to values of ECC media pretreated with 3 C.

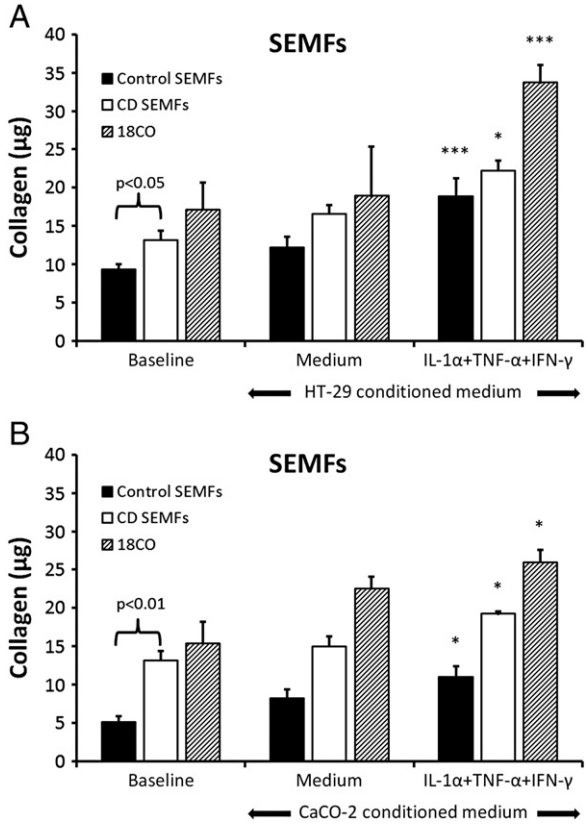


Figure 4 Baseline collagen production is higher in SEMFs isolated from CD patients compared to normal controls and is upregulated by conditioned medium from colonic epithelial cells exposed to proinflammatory cytokines. Total intracellular collagen content in primary control (black bars) or CD (white bars) SEMFs and the 18CO cell line (bars with diagonal pattern) that were untreated or treated for 24 h with ECC media. ECC media were prepared as described in the Materials and methods section. Results are the mean ± SEM of at least 3 independent experiments; * $p<0.05$, *** $p<0.001$ relative to baseline values of the same cells.

either normal controls or CD patients. The observed effect was moderate with ECC medium from untreated HT-29 or CaCO-2 (Fig. 4A and B). In contrast, ECC media from epithelial cells pretreated with IL-1α, TNF-α, and IFN-γ, induced a pronounced increase of collagen production by the SEMF cultures from normal controls, CD patients and the 18CO cell line (Fig. 4A and B).

We attempted to identify the epithelial cell-derived factors responsible for the increase in collagen synthesis by SEMFs and epithelial cell-derived TGF-β1 was a probable candidate. However, neutralisation of all three forms of TGF-β in the ECC media did not affect the induction of collagen production in control or CD SEMFs (Fig. 6A). Previous studies have shown that SEMFs can upregulate collagen production via pathways that involve ET-1, thrombin and autocrine production of CTGF and TF.[23,24] ECC medium induced CTGF production by primary SEMF isolated from normal controls, especially if colonic epithelial cells were treated with proinflammatory cytokines (Fig. 7). However, neutralisation of CTGF (Fig. 6B), anti-thrombin-III-mediated inhibition of thrombin (Fig. 6D) or neutralisation of TF (Fig. 6E) did not block collagen induction by ECC medium. Endothelin receptor antagonism had also no effect on the upregulation of collagen production in control or CD SEMFs (Fig. 6F and G).

3.4. Epithelial cell-conditioned medium inhibits SEMF's mobility/migration

SEMF mobility/migration was assessed in vitro with the wound-healing scratch assay and quantified as the percentage of wound repair. Addition of exogenous TGF-β1 in primary SEMF cultures was found to promote SEMF mobility/migration in a dose-dependent manner (Fig. 8A and B). In contrast, TNF-α ($p<10^{-7}$) and IFN-γ ($p<10^{-6}$) inhibited SEMF mobility/migration (Fig. 8C and D). In combination, these cytokines exerted an additive inhibitory effect ($p<10^{-15}$). IL-1α did not alter mobility/migration either when added alone (Fig. 8C and D) or when used in combination with TNF-α or IFN-γ (data not shown).

To study the potential impact of epithelial cells on this process, we used ECC media to treat primary SEMFs. ECC medium from untreated CaCO-2 cells had no effect on wound closure (Fig. 9). Subsequently, we treated epithelial cell cultures with IL-1α, TNF-α or IFN-γ for 6 h and then changed to fresh medium for 18 h. This ECC medium devoid of the initial inflammatory stimuli significantly inhibited wound closure (Fig. 9). Stimulating the epithelial cell cultures with all three cytokines (3C) resulted in stronger inhibition of wound closure ($p<10^{-5}$) (Fig. 9). Similar results were obtained with HT-29 ECC medium (Fig. 10). Taken together these results indicate that proinflammatory stimuli inhibit the migratory potential of SEMFs both directly and indirectly through epithelial cell-derived soluble factors.

4. Discussion

Intestinal fibrosis has been associated with local increase in TGF-β1 and TIMP-1.[4,25] TGFβ1 has been suggested to originate from inflammatory cells infiltrating the lamina propria, although previous immunohistochemical studies have detected additional TGF-β immunoreactivity at the inflamed intestinal epithelium.[3] TIMP-1 production has been attributed mostly to cells of mesenchymal origin, which is in accordance to our findings of a moderate constitutive TIMP-1 production by isolated SEMFs.[26] However, we detected substantial production of TGF-β1-3 and TIMP-1 from the two colonic epithelial cell lines. Furthermore, treatment with INF-γ, TNF-α and IL-1α had no effect on TIMP-1 production by SEMFs but significantly increased production of both TGF-β and TIMP-1 by the epithelial cells. Our findings suggest that colonic epithelial cells might be an important additional source of TGF-β and TIMP-1 during chronic intestinal inflammation. Although we haven't confirmed a major role for epithelial cell derived TGF-β in the regulation of collagen and MMP-9 production by the SEMF cultures our results might have been affected by the limited treatment times used and the lack of direct cell to cell contact.[27]

Increased MMP-9 production has been detected in the intestinal mucosa of IBD patients and has been found responsible for the bulk of the gelatinolytic activity

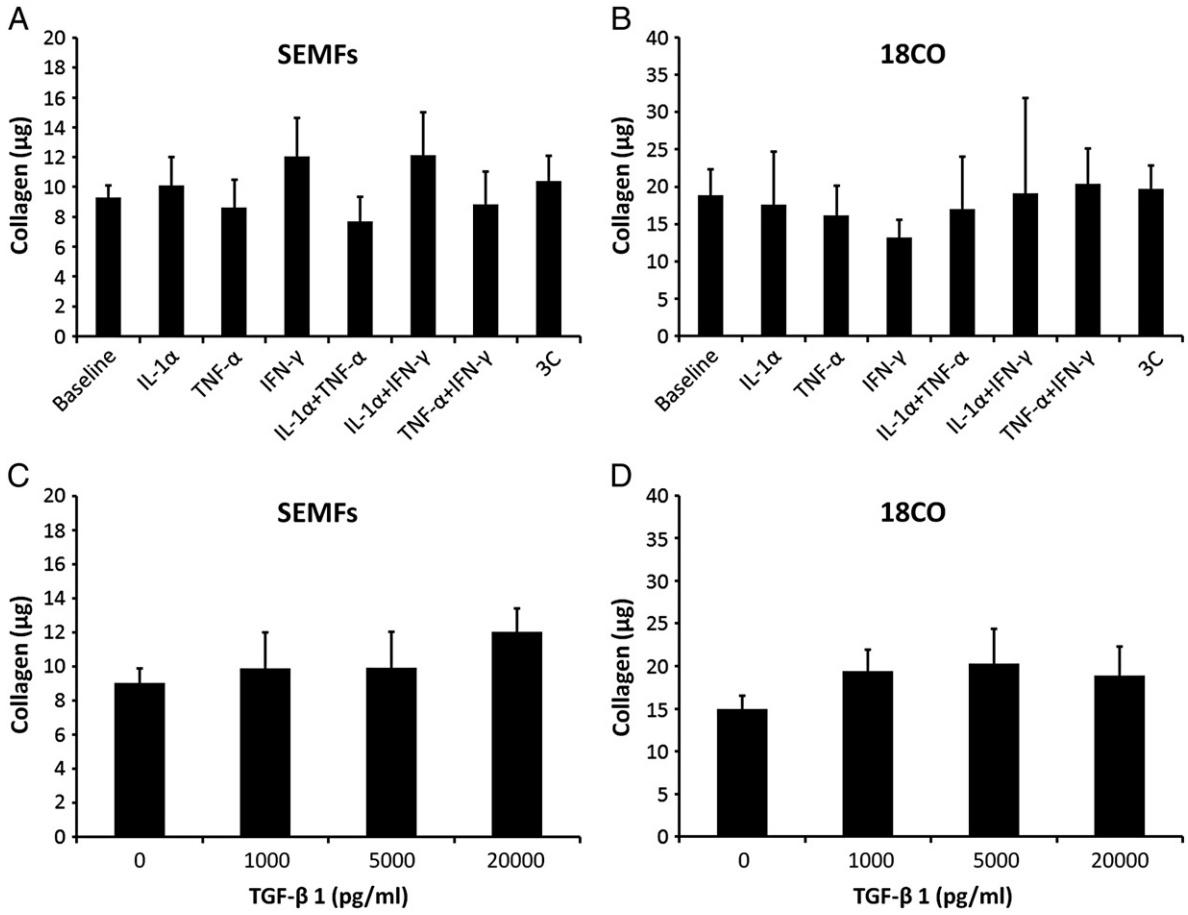
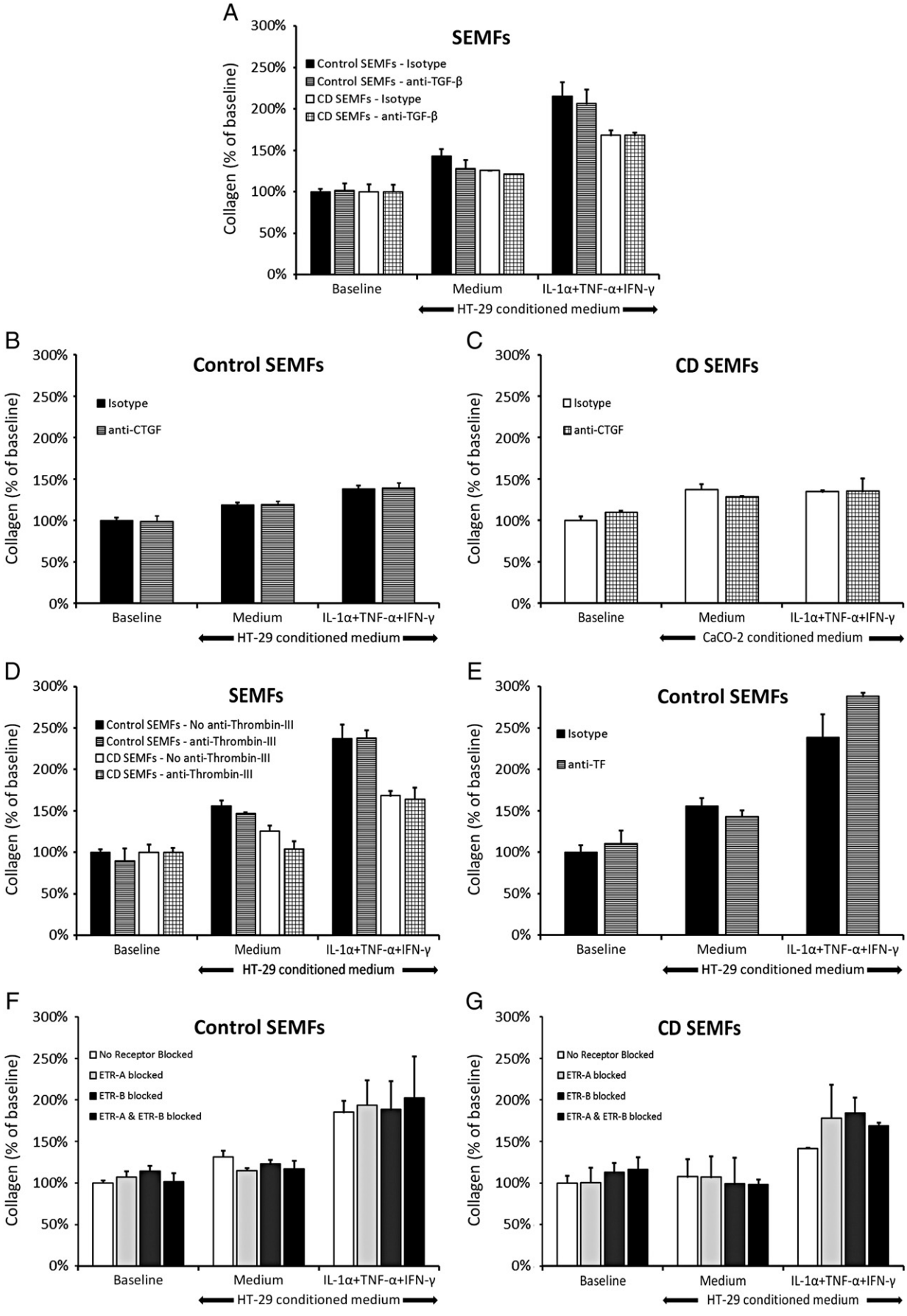


Figure 5 Collagen production by colonic subepithelial myofibroblasts (SEMFs) is not directly affected by proinflammatory cytokines or TGF-β1. Total intracellular collagen content from primary SEMFs (A) and the 18CO cell line (B) in cultures that were untreated or treated for 24 h with IL-1α, TNF-α, IFN-γ in various combinations or all three (3C). Total intracellular collagen content from primary SEMFs (C) and the 18CO cell line (D) following stimulation with TGF-β1. Values are the mean ± SEM of 3 independent experiments.

observed in IBD tissue samples.[10] Consistently with previous studies, we found that SEMFs from either normal controls or CD patients, as well as the 18CO myofibroblasts constitutively produced MMP-2 but not MMP-9.[26,28] However, treatment with IL-1β, TNF-α and TGF-β1 induced MMP-9 production, which was inhibited by IFN-γ. This effect could potentially drive the balance between pro-fibrotic and ECM catabolic factors towards collagen deposition and might represent a link between Th1 inflammation, such as CD, and intestinal fibrosis. With the exception of a single study with a small number of patients, showing relatively less MMP-9 levels in CD compared to UC tissue samples but still higher than controls, there is no published data suggesting that less MMP-9 contributes to the pathogenesis of CD-associated fibrosis.[10] The possible operation and in vivo relevance of this pathway needs to be addressed by further studies.

On the other hand, MMP-9 overproduction has been clearly associated with epithelial injury and mucosal ulceration.[12] In our study, ECC media from colonic epithelial cells conditioned with proinflammatory cytokines induced production of MMP-9 by SEMFs from normal controls, CD patients and the 18CO myofibroblasts. This effect was independent of TGF-β and CTGF and was mediated through endothelin receptor A signalling in SEMFs. ETR-A is selective for ET-1, which has been previously shown to regulate production of matrix degrading enzymes from colonic fibroblasts.[29,30] Endothelins are potentially upregulated in either epithelial cells or myofibroblast cells and therefore can exert paracrine or autocrine effects, respectively, on myofibroblasts.[16,31] Irrespective of the cellular source of endothelin-1 the existence of such a circuit operating in the gut, could represent a mechanism whereby epithelial cells facilitate the access of inflammatory cells to the intestinal epithelium via myofibroblast-mediated proteolysis of the subepithelial reticular sheet resulting in disruption of normal mucosal architecture.

Figure 6 SEMF collagen production is upregulated by unknown soluble factors derived from epithelial cells. Total intracellular collagen content from primary control (A, B, D–F) or CD (A, C, D, G) SEMF cultures that were untreated or treated for 24 h with ECC media. ECC media were prepared as described in the Materials and methods section. Effects of blocking antibodies for all TGF-β isoforms (A), CTGF (B, C) thrombin-III (D) TF (E) and chemical inhibition of endothelin receptor (ETR-) A/B with BQ-610/BQ-788 (F, G) are shown. Results are the mean ± SEM of at least 3 independent experiments.



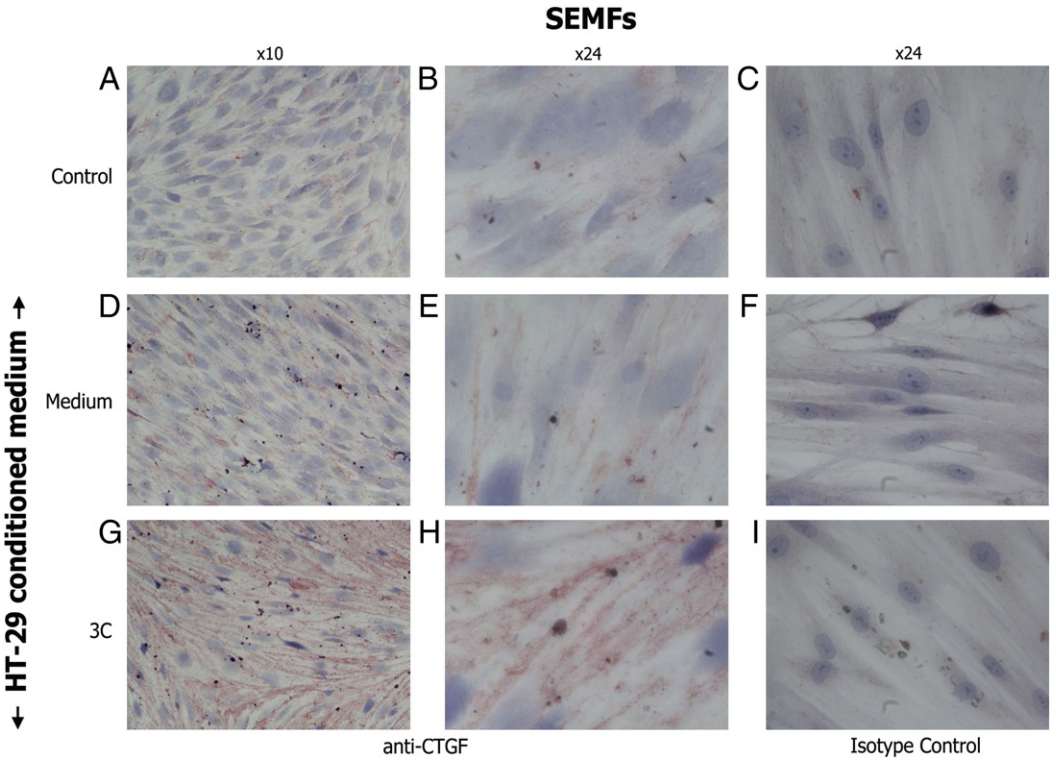


Figure 7 The effect of colonic epithelial cell conditioned media on CTGF production by primary SEMF cultures. Immunocytochemistry with goat anti-human polyclonal anti-CTGF IgG (A, B, D, E, G, and H) on confluent primary SEMF cultures. Goat IgG1 isotype control antibody is used as a control (C, F, I). SEMF cultures were treated for 24 h with ECC media obtained from HT-29 cells and prepared as described in the Materials and methods section. Left column with 10× magnification (A, D, G); middle and right columns with 24× magnification (B–C, H–I). Representative results from 3 independent experiments are shown.

The critical event that causes intestinal fibrosis is collagen accumulation. Increased collagen production has been previously reported for fibroblasts isolated from intestinal strictures of CD patients.[4,5,32] Similarly, we report significantly higher collagen production from SEMFs isolated from inflamed areas of the colon of CD patients compared to SEMFs from normal controls. This implies that subepithelial myofibroblasts may contribute to collagen accumulation during CD-associated inflammation. In contrast to studies on intestinal fibroblasts, we found that collagen production by SEMFs and 18CO myofibroblasts was not increased by exogenous TGF-β.[4,5,32] Previous studies on SEMFs have produced conflicting results concerning the regulation of collagen production by TGF-β.[33–35] This indicates that additional stimuli may be needed to induce a substantial increase of collagen production by SEMFs. Medium obtained from ECC cultures was able to increase collagen production by SEMFs from CD patients or normal controls and the 18CO myofibroblasts. Collagen production was further upregulated when the epithelial cells were exposed to proinflammatory cytokines. This finding was in accordance with results from previous experiments on the skin, where co-culturing keratinocytes with fibroblasts induced α-SMA expression and collagen production in fibroblasts. However, cell-to-cell contact and TGF-β1 signalling were required, whereas IL-1α blocked the interaction.[27]

We report that, collagen up-regulation by ECC medium was independent of TGF-β and endothelin. Despite upregulation of CTGF in cultured SEMFs by ECC-media, no effects on collagen production were observed with CTGF neutralisation. Neutralisation of TF and thrombin, recently shown to be involved in pulmonary fibrosis via up-regulation of collagen production by SEMFs, failed to inhibit ECC-induced collagen production.[23,24] This indicates that different pathways might be involved in the crosstalk between intestinal epithelial cells and SEMFs that could be unique to the intestinal microenvironment.

Finally, we studied the effects of various mediators on SEMF migration. Exogenous TGF-β1 promoted SEMF migration and enhanced wound healing, whereas TNF-α and IFN-γ had the opposite effect. These findings are in accordance with previous publications that demonstrated a migratory defect in inflammation-exposed SEMFs isolated from Crohn's disease tissue and an inhibitory effect of TNF-α and IFN-γ on SEMF migration.[4,36,37] Conditioned medium from epithelial cells exposed to proinflammatory cytokines inhibited SEMF migration, despite the fact that elevated TGF-β1 in ECC media should increase SEMF migration rate. Therefore, we propose that additional soluble factors may be responsible for the observed outcome. We agree with the suggestion by Di Sabatino et al. that the inhibition of migration potentially contributes to the retention of myofibroblasts at inflammatory foci in vivo. This reduced dispersing potential accompanied by increased collagen production, might provide a pathophysiological mechanism for enhanced intestinal fibrosis.

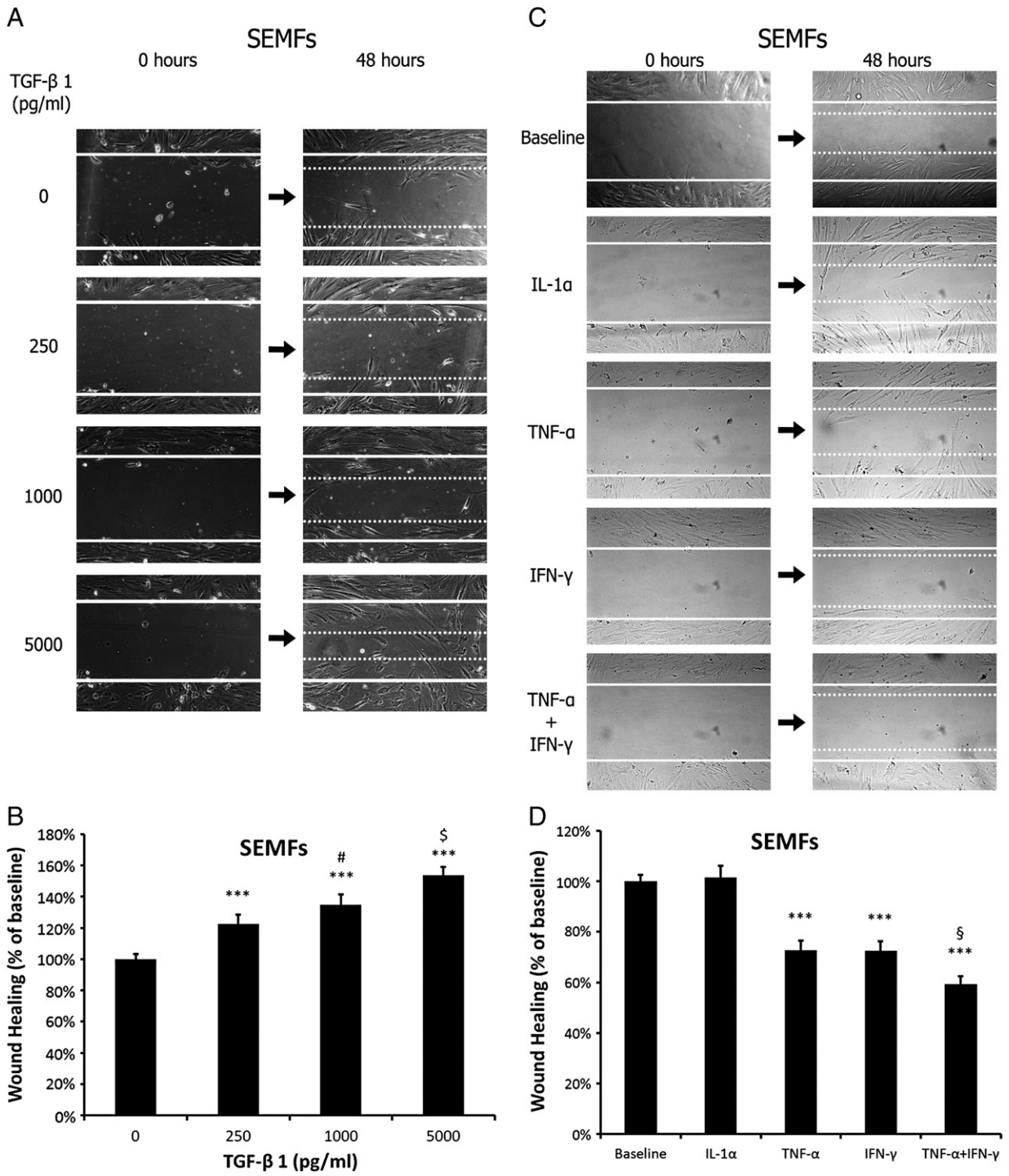


Figure 8 Wound healing by colonic subepithelial myofibroblasts (SEMFs): the effect of TGF-β1 and proinflammatory cytokines. (A, C) Representative figures of confluent primary SEMF cultures at 0 h (left column) and 48 h (left column) following a scratch with a 200-μl pipette tip. Stimuli applied are shown on the left. Front of cells at 0 h (white lines) was compared to front at 48 h (dotted lines) to determine percentage of closure. (B) The mean ± SEM of values from three independent experiments shown in (A); *** $p<0.001$ relative to baseline values. # $p<0.05$ relative to values obtained by stimulation with 250 pg/ml TGF-β 1. $ $p<0.05$ relative to values obtained by stimulation with 500 pg/ml TGF-β 1. (D) The mean ± SEM of values from 3 independent experiments shown in (C) *** $p<0.001$ relative to baseline values. § $p<0.05$ relative to values obtained by stimulation with individual cytokines.

In conclusion, this study provides evidence of a potential crosstalk between intestinal epithelial cells and SEMFs. In vitro stimulation of epithelial cells with proinflammatory cytokines, which partially mimics epithelial cell injury during intestinal inflammation, provoked changes in SEMFs from CD patients and normal controls similar to those observed during intestinal fibrosis. Our results support the necessity for further investigation and in vivo validation of the possible

cooperation between epithelial cells and adjacent myofibroblasts in the regulation of extracellular matrix remodelling and inflammation-induced intestinal fibrosis.

Authors contributions

GK, ID, VV, EK, and LB conceived of and designed the study. ID, OS, KK, and PM performed the experiments. VV, ID, and GK with help from of OS, KK, PM, analysed and interpreted

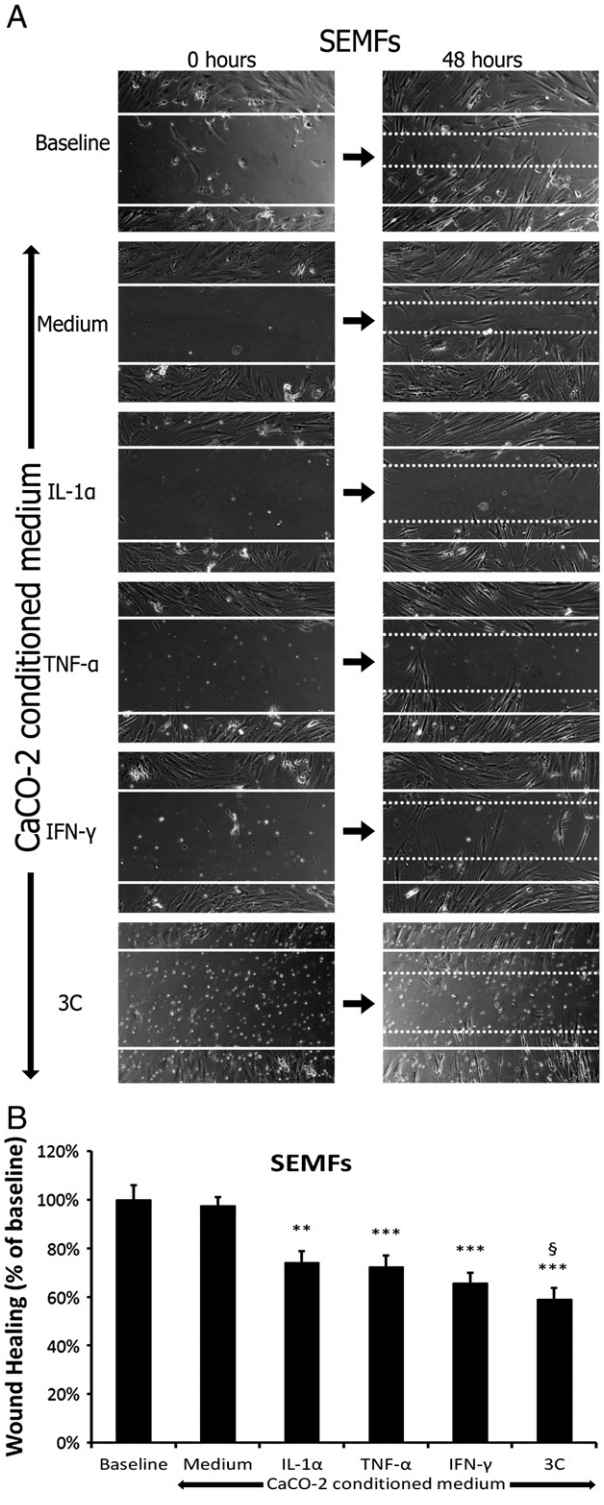


Figure 9 The effect of colonic epithelial cell conditioned media on SEMF wound-healing. (A) Representative photographs of confluent primary SEMF cultures at 0 h (left column) and 48 h (right column) following a scratch with a 200-μl pipette tip. ECC obtained from CaCO-2 cultures was used as a stimulus. ECC media were prepared as described in the Materials and methods section. Front of cells at 0 h (white lines) was compared to front at 48 h (dotted lines) to determine percentage of closure. (B) The mean ± SEM values from three independent experiments shown in (A); ** $p<0.01$, *** $p<0.001$ relative to baseline values. § $p<0.05$ relative to values obtained by pretreatment of CaCO-2 with individual cytokines.

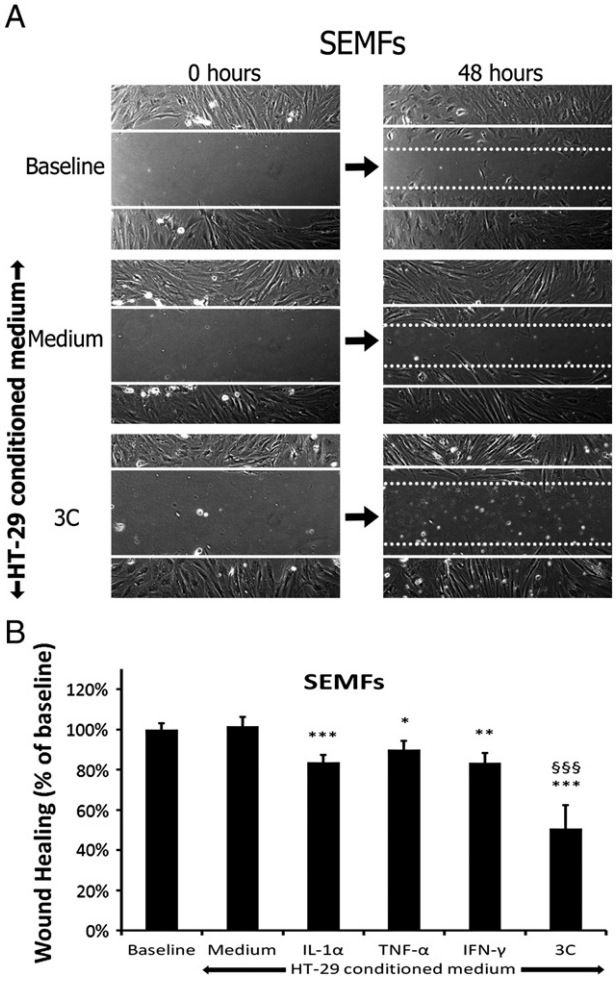


Figure 10 The effect of HT-29 epithelial cell conditioned media on SEMF wound-healing assay. (A) Representative photographs of confluent primary SEMF cultures at 0 h (left column) and 48 h (right column) following a scratch with a 200-μl pipette tip. ECC obtained from HT-29 cultures was used as a stimulus. ECC media were prepared as described in the Materials and methods section. Front of cells at 0 h (white lines) was compared to front at 48 h (dotted lines) to determine percentage of wound closure. (B) The mean ± SEM values from three independent experiments shown in (A); * $p<0.05$, ** $p<0.01$, *** $p<0.001$ relative to baseline values. §§§ $p<0.001$ relative to values obtained by pretreatment of HT-29 cells with individual cytokines.

data, wrote and revised the paper. EK supervised and co-ordinated research. KR contributed important reagents and provided important feedback on results. All authors read and approved the final manuscript.

Conflict of interest statement

The authors declare no conflict of interest.

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