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The role of MAPK in governing lymphocyte adhesion to and migration across the microvasculature in inflammatory bowel disease

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Lymphocyte recruitment is a key pathogenic event in inflammatory bowel disease (IBD). Adhesion of T cells to human intestinal microvascular endothelial cells (HIMEC) is mediated by ICAM-1, VCAM-1 and fractalkine (FKN), but the signaling molecules that orchestrate this process have yet to be identified. Because MAPK play an important role in the response of many cell types to pro-inflammatory stimuli, we assessed the functional role of p38 MAPK, p42/44 MAPK and JNK in the regulation of lymphocyte adhesion to and chemotaxis across the microvasculature in IBD. We found that the MAPK were phosphorylated in the bowel microvasculature and human intestinal fibroblasts of patients with IBD but not of healthy individuals. Stimulation of HIMEC with TNF-α triggered phosphorylation of the MAPK, and up-regulation of VCAM-1, FKN and ICAM-1. Blockade of p38 decreased the expression of all MAPK by 50% ($p<0.01$), whereas inhibition of p42/44 decreased the expression of ICAM-1 and FKN by 50% ($p<0.01$). Treatment of human intestinal fibroblasts with TNF-α elicited production of IL-8 and MCP-1, which was reduced ($p<0.05$) by blockade of p38 and p42/44. Finally, blockade of p38 and p42/44 reduced lymphocyte adhesion to ($p<0.05$) and trans-migration across ($p<0.05$) HIMEC monolayers. These findings suggest a critical role for MAPK in governing lymphocyte influx into the gut in IBD patients, and their blockade may offer a molecular target for blockade of leukocyte recruitment to the intestine.

Key words: Endothelium · Inflammation · Inflammatory bowel disease · Protein kinases

Introduction

Intestinal homeostasis is the result of a rich network of reciprocal and finely orchestrated interactions among immune, epithelial, endothelial, mesenchymal and nerve cells, and the extracellular matrix [1, 2]. Dysfunction of any component of this highly integrated mucosal system may lead to a disruption in communication, and result in pathological inflammation and inflammatory bowel disease (IBD), a chronic inflammatory condition of the bowel whose etiology is still unknown [2].

The hallmark of the intestinal inflammation associated with IBD is the presence of infiltrating leukocytes in the mucosa. This process is strictly regulated and requires intercellular communication between infiltrating leukocytes, the endothelium and

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resident stromal cells [3], which is mediated by expression of adhesion molecules and production of chemokines [4, 5]. Local over-expression of chemokines results in accumulation of leukocytes at that site [6], suggesting that these molecules play a pivotal role in mucosal immunity and inflammation [3].

It is now generally accepted that the endothelium plays a fundamental pathogenic role in IBD [7, 8]. Recent reports have shown that human intestinal microvascular endothelial cells (HIMEC) derived from the mucosa of patients with IBD had a markedly greater capacity to bind leukocytes than HIMEC from individuals with a healthy mucosa [9]. This observation has been correlated with enhanced expression of the cell adhesion molecules (CAM) ICAM-1 (also known as CD54a), VCAM-1 (also known as CD106) and fractalkine (FKN) on HIMEC that had been stimulated with TNF-α. Expression was up-regulated to a much greater extent on HIMEC derived from IBD patients than those from healthy individuals [10], but the signaling molecules that orchestrate this process have not yet been defined.

MAPK are among the major signal transduction pathways and are widely used throughout all organisms in many physiological processes, including regulation of gene expression in response to extracellular stimuli, and regulation of cell proliferation, cell survival and cell motility [11]. In mammalian species, MAPK are also involved in the initiation phase of innate immunity, the activation of adaptive immunity and in cell death when immune function is complete [12].

Three major groups of MAPK have been identified in mammalian cells: the extracellular signal-regulated protein kinases (p42/44, also known as ERK), the p38 MAPK and the JNK [12]. These MAPK require activation by phosphorylation to perform their intracellular signaling task. For example, MAPK have been described to regulate the production of TNF-α by leukocytes in response to LPS activation [13–15], as well as in cellular responses to inflammatory cytokines such as TNF-α and IL-1 [16, 17]. However, the role played by the different MAPK and their activation in IBD pathophysiology and leukocytes recruitment has yet to be explored.

In this paper, we studied the functional role of the p38, p42/44 and JNK MAPK in the regulation of lymphocyte adhesion to and chemotaxis across the microvasculature in IBD. Our results show that increased phosphorylated levels of MAPK occur in the mucosa of patients with IBD by both endothelium and human intestinal fibroblasts (HIF). In addition, they are functionally important in mediating the expression of CAM, the production of chemokines and adhesion of lymphocytes to and migration across the intestinal endothelium. This study therefore identifies potential new targets for pathogenesis-driven anti-inflammatory therapeutics.

Results

Enhanced expression of the active form of the MAPK in the microvasculature of patients with IBD

Immunostaining with specific antibodies was used to identify the expression of the active, phosphorylated forms of p38 (p-p38), p42/44 (p-ERK-1/2) and JNK (p-JNK) in bowel preparations obtained from patients with Crohn's disease (CD) and ulcerative colitis (UC) and control individuals. Expression of p-p38, p-ERK-1/2 and p-JNK was either very low or absent from the microvasculature of segments from the bowels of control individuals (Fig. 1). Increased phosphorylated levels of all three MAPK were uniformly present in the microvasculature of bowel preparations of patients with CD and UC (Fig. 1). No differences were found in MAPK phosphorylation levels between controls and non-inflamed IBD mucosa (data not shown).

Recruitment of lymphocytes from the vascular compartment requires not only CAM-mediated lymphocyte–endothelial cell interactions but also the existence of several chemokine gradients to attract lymphocytes across the endothelial cells and towards the bowel interstitium [18]. However, the involvement of MAPK in this process has yet to be investigated. We therefore determined the levels of phospho-MAPK in intestinal fibroblasts. Similar to the bowel microvasculature, expression of p-p38, p-ERK-1/2 and p-JNK was very low or absent in fibroblasts located on the lamina propria and submucosa of control bowel samples (Fig. 1). In contrast, increased phosphorylated levels of all three MAPK were very high in intestinal fibroblasts in the bowel preparations from patients with CD and UC (Fig. 1). No differences were found in MAPK phosphorylated levels between controls and non-inflamed IBD mucosa (data not shown).

MAPK activation in HIMEC stimulated pro-inflammatory cytokines

HIMEC are highly specialized endothelial cells that are able to bind leukocytes, especially if derived from the mucosa of patients with IBD [9], making them a good model with which to reproduce the *in vitro* pathogenic mechanisms involved in intestinal microvasculature. We therefore investigated whether the pro-inflammatory milieu present in the IBD mucosa could be responsible for the increased endothelial phosphorylated levels of the three different MAPK. Resting HIMEC exhibited low levels of p-ERK-1/2 and p-JNK, whereas p-38 was completely absent from these cells (Fig. 2). Notably, stimulation of HIMEC with TNF-α triggered the phosphorylation of all the MAPK over time, with peak levels of phosphorylation occurring at 5–15 min for p38, p42/44 and JNK. Similar results were obtained with IL-1β (data not shown), suggesting that phosphorylation of MAPK is triggered by pro-inflammatory cytokines (Fig. 2). Phosphorylated levels of MAPK decreased after the pick but remained detectable until 2 h for all and even at 24 h for p38 and ERK.

Involvement of p38, ERK-1/2 and JNK in the expression of CAM on HIMEC

One of the most important tasks of the microvasculature during inflammation is the recruitment of leukocytes. This process starts with up-regulation of the adhesion molecules expressed on the

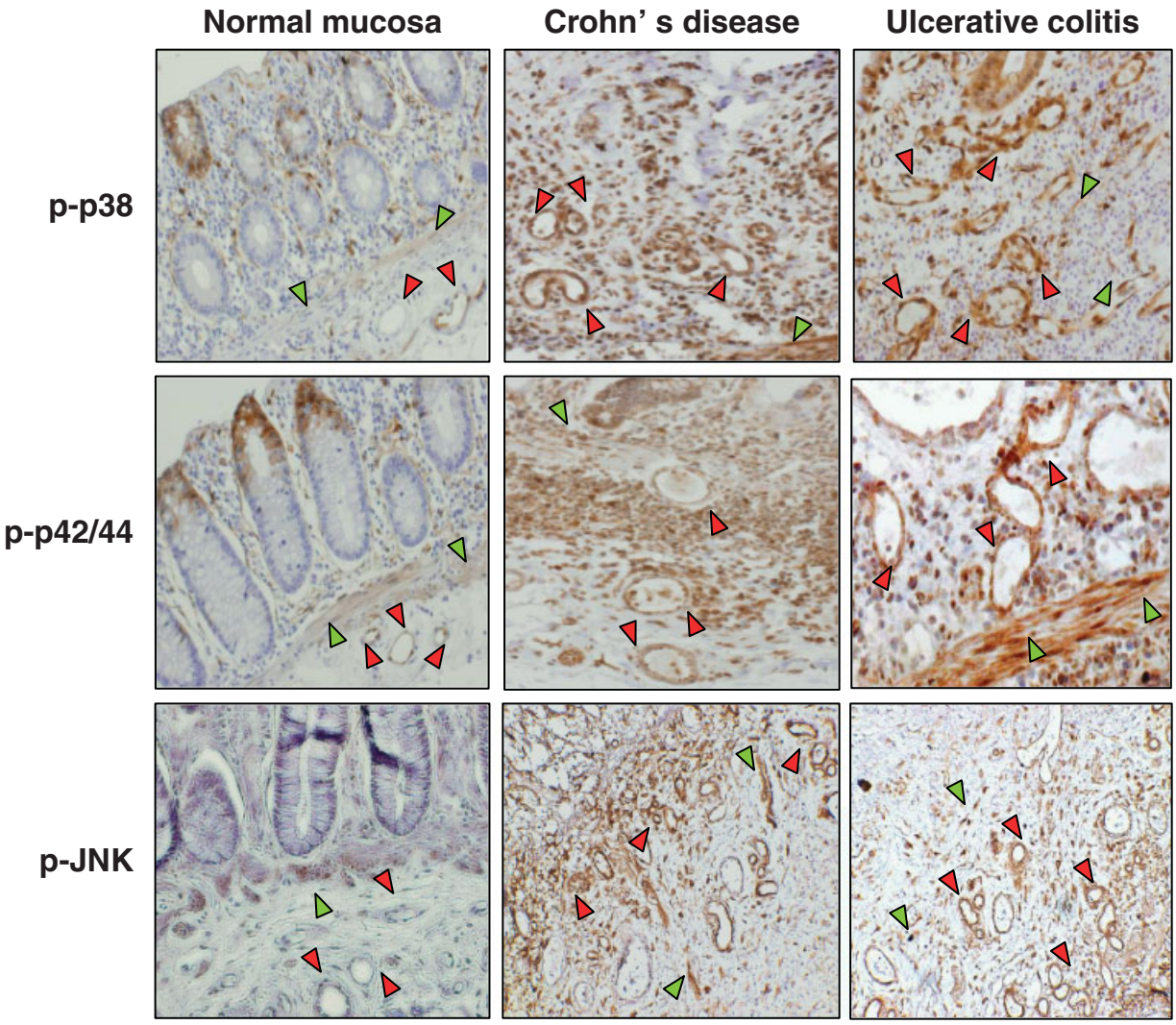

Figure 1. Enhanced expression of the active forms of MAPK in the microvasculature of patients with IBD. Immunostaining for the phosphorylated (p) forms of p38, p42/44 and JNK was performed in sections from the intestinal mucosa of patients with IBD and control individuals, using phospho-specific antibodies for each of the MAPK. Stained areas of the microvasculature are indicated by the red arrowheads and mucosal fibroblasts by green arrowheads. Panels are representative of samples from 10 patients with CD, 12 patients with UC and 10 control individuals.

endothelium. We have previously demonstrated that adhesion of lymphocytes to the HIMEC monolayer is dependent on ICAM-1, VCAM-1 and FKN [10]. Having demonstrated that MAPK are involved in the response of HIMEC to stimulation by TNF-α, we explored whether MAPK could play a role in the up-regulation of endothelial CAM. We first studied the expression of VCAM-1 and ICAM-1 on the surface of both resting and TNF-α-stimulated HIMEC. Of these, only ICAM-1 was expressed by a significant proportion of resting HIMEC, whereas VCAM-1 and FKN were present only at very low levels on these cells (Fig. 3A). On the other hand, the expression of the three adhesion molecules on the surface of HIMEC was significantly up-regulated following stimulation by TNF-α (Fig. 3A and B).

To ascertain the requirement for p38, p42/44 and JNK in the observed up-regulation of CAM, we undertook a series of experiments in which HIMEC monolayers were pre-treated for 1 h with a specific inhibitor of each of the three MAPK, prior to the stimulation with TNF-α. Flow cytometric analysis demonstrated that specific blockade of p38, using SB203580, decreased the expression of all CAM by 50% ($p<0.01$), whereas the p42/44 inhibitor PD 98059 decreased the expression of ICAM-1 and FKN by 50% ($p<0.01$) but not that of VCAM-1 (Fig. 3B). On the other hand, blockade of JNK with the specific inhibitor SP600125 had no impact on the expression of any CAM on the surface of HIMEC (Fig. 3B). In order to test whether the CAM reduction observed by the MAPK inhibitors was affecting expression and not distribution, we repeated the flow cytometry experiments by using immunofluorescence microscopy (Fig. 3C). Immunofluorescence for ICAM-1 and VCAM-1 confirmed the inhibition observed using PD 98059 and SB203580 (Fig. 3C) but not for SP600125 (data not shown). MAPK inhibitors did not affect cell number and cell viability at the end of the experiments (data not shown).

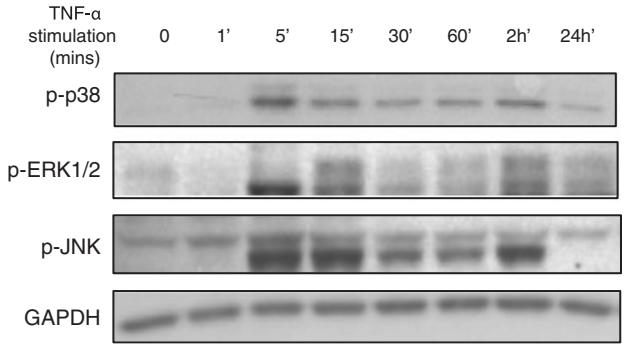

Figure 2. Activation of MAPK in TNF-α stimulated HIMEC. HIMEC monolayers were left untreated (baseline) or stimulated with TNF-α for 1, 2, 5, 15, 30 min and 1, 2 or 24 h; then total protein was extracted and analyzed by Western blot. Gels were incubated in the presence of phospho-specific antibodies for p38, p42/44 and JNK (recognizing p-p38, pERK1/2 and p-JNK, respectively). The constitutively expressed cytoplasmic protein GAPDH was used as a control for overall protein levels. This figure shows representative results from three separate experiments.

MAPK-dependent T-cell adhesion to HIMEC

Having demonstrated that MAPK, especially p38 and p42/44, are critically involved in the up-regulation of endothelial CAM on the surface of HIMEC, we next studied the functional contribution made by MAPK in the adhesion of lymphocytes to the HIMEC monolayer. As previously demonstrated [9], stimulation of HIMEC with TNF-α results in a marked and significant ($p<0.05$) increase in the number of adherent lymphocytes on the surface of HIMEC (Fig. 4). To assess whether peripheral blood T (PBT) cell adhesion is dependent on MAPK, we again used the specific inhibitors of MAPK. Blockade of p38 and p42/44 resulted in a significant ($p<0.05$) attenuation of the TNF-α-mediated increase in lymphocyte adhesion, whereas specific blockade of JNK did not modify lymphocyte adhesion to HIMEC compared with TNF-α alone (Fig. 4).

Involvement of p38, ERK-1/2 and JNK in chemokine production by HIF

Next we investigated whether pro-inflammatory cytokines could also initiate phosphorylation of MAPK in HIF. Western blot analysis did not show any expression of the phosphorylated forms of p38, p42/44 and JNK in resting HIF (Fig. 5). However, stimulation of HIF with TNF-α triggered the phosphorylation of p38, p42/44 and JNK in a time-dependent manner. Significant phosphorylation of the MAPK was apparent 1–5 min after stimulation with TNF-α; this peaked at 15 min; then phosphorylation of JNK disappeared while phosphorylation of p38 and ERK persisted with lower intensity even at 24 h (Fig. 5).

As bowel fibroblasts are a major source of several chemokines such as IL-8 and MCP-1 [19], we ascertained whether MAPK are also involved in lymphocyte chemoattraction, by modulating the production of key chemokines. As expected, stimulation of HIF with TNF-α induced a marked and significant increase in the production of both IL-8 (Fig. 6A) and MCP-1 (Fig. 6B). To dissect the individual contribution of p38, p42/44 and JNK to the production of IL-8 and MCP-1, HIF were pre-treated with the specific MAPK inhibitors prior to stimulation with TNF-α. Inhibition of both p38 and p42/44 inhibition resulted in a marked attenuation of the production of IL-8 normally triggered by stimulation of HIF with TNF-α. The effect of inhibition of JNK on the production of IL-8 was more modest and was observed only at the highest dose (Fig. 6A). Similarly, inhibition of either p38 or p42/44 significantly attenuated the increase in MCP-1 production stimulated by TNF-α. On the other hand, inhibition of JNK inhibition had no impact on the production of MCP-1 at any of the doses tested (Fig. 6B). Using all the MAPK inhibitors simultaneously did not further inhibit chemokine production by HIF (data not shown). MAPK inhibitors did not affect cell number and cell viability at the end of the experiments (data not shown).

In addition to IL-8 and MCP-1, supernatants from TNF-α-stimulated HIF were analyzed for stromal cell-derived factor (SDF)-1, RANTES, MIP-1-α and IP-10, which were released at different levels (see Table 1).

Induction of T-cell HIMEC transmigration by chemoattractants produced by activated HIF

The demonstration that MAPK-dependent activation of HIF by TNF-α can lead to the production of immune-reactive chemokines such as IL-8 and MCP-1 prompted us to investigate the functional role of this observation *in vitro*. We used a well-established model of lymphocyte chemotaxis [20] to investigate whether intestinal fibroblasts can attract T cells through the physical and functional barrier presented by endothelial cells during inflammatory conditions such as those induced by pre-treatment with TNF-α [20]. This was evaluated in a transwell system that measures transmigration of T cells from an upper chamber through a HIMEC monolayer derived from control individuals towards an HIF monolayer into a lower chamber. Compared with culture medium alone, supernatants from unstimulated HIF induced twice as many T cells to transmigrate (data not shown). As expected [20], addition of TNF-α to the HIF induced a more than threefold increase in transmigration compared with unstimulated supernatants ($p<0.01$).

Having confirmed that this phenomenon is TNF-α dependent, we then assessed whether inhibition of MAPK in HIF that had been stimulated with TNF-α could modulate the migration of T cells across HIMEC monolayers. As expected given the demonstration that p38 and p42/44, but not JNK, markedly attenuated chemokine production, HIF pre-treated with SB203580 or PD 98059, but not with SP 600125, significantly ($p<0.05$) decreased the transmigration of T cells through HIMEC monolayers (Fig. 7),

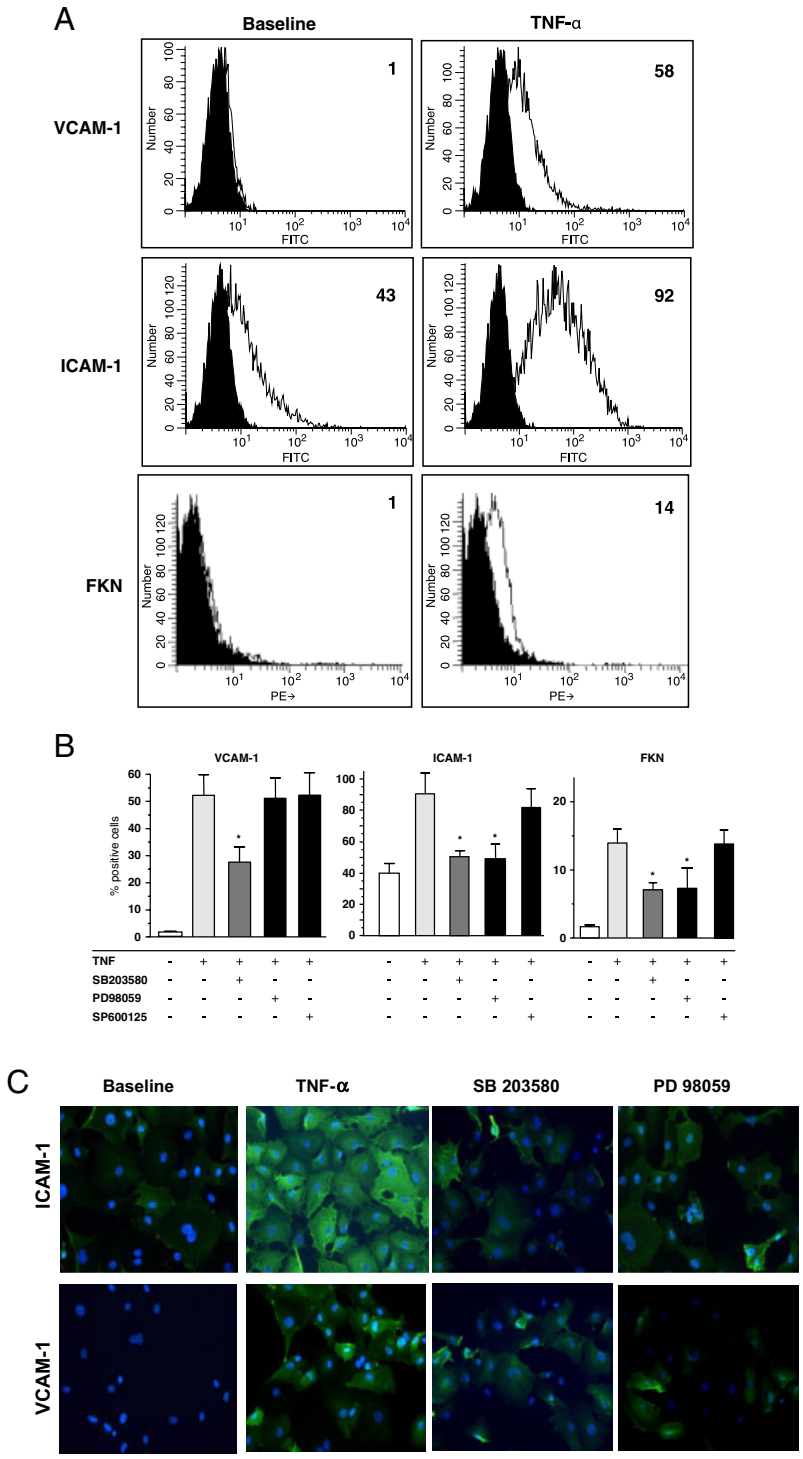

Figure 3. Involvement of p38, p42/44 and JNK in the expression of CAM by HIMEC. HIMEC were grown to subconfluence, then cultured in the presence or absence of 100 U/mL of TNF-α, with or without a 1 h pre-incubation with SB203580, PD 98059 or SP 600125. Cells were collected after 24 h and incubated in the presence of anti-VCAM-1, anti-ICAM-1 or anti-FKN Ab. (A) Cells were then incubated with FITC-conjugated secondary antibody and analyzed by flow cytometry. The black peak represents the background signal from the isotype control, and the white peak represents the cells that were stained with the FITC-conjugated antibody. The net percentage of positively stained cells is indicated. Each panel is representative of four separate experiments. (B) Quantification of the flow cytometric analysis following HIMEC stimulation with TNF-α after specific blockade of each of the MAPK for 1 h. Data are expressed as mean ± SEM of three separate experiments. *$p<0.05$ for TNF stimulated cells pre-treated with MAPK-specific inhibitors compared with TNF-treated cells. (C) HIMEC were grown to subconfluence, then cultured in the presence or absence of 100 U/mL of TNF-α alone or in combination with SB 203580 or PD 98059 for specific blockade of p38 or p42/44 respectively for 1 h, then stained for anti-VCAM-1 and ICAM-1 (green) and observed by immunofluorescence microscopy; DAPI staining is shown in blue. Each panel is representative of three separate experiments.

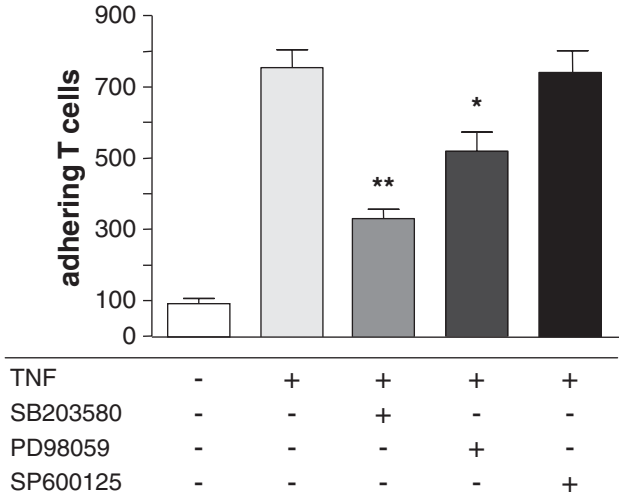

Figure 4. MAPK dependency of T-cell adhesion to HIMEC. HIMEC were plated onto fibronectin pre-coated 24-well cluster plates, then cultured in the presence or absence of TNF-α after 1 h of pre-incubation with SB203580, PD 98059 or SP 600125. After 24 h, PBT cells were labeled with calcein, added to HIMEC monolayers at 1×10^6 leukocytes/well and allowed to adhere at 37°C in a 5% CO_2 incubator for 1 h. Fluorescent-adherent leukocytes were quantified by an imaging system. The number of adherent cells in each experimental condition was expressed as mean ± SEM of three separate experiments. *$p<0.05$ and **$p<0.01$ for TNF-α- stimulated cells pre-treated with MAPK-specific inhibitors compared with cells stimulated with TNF-α alone.

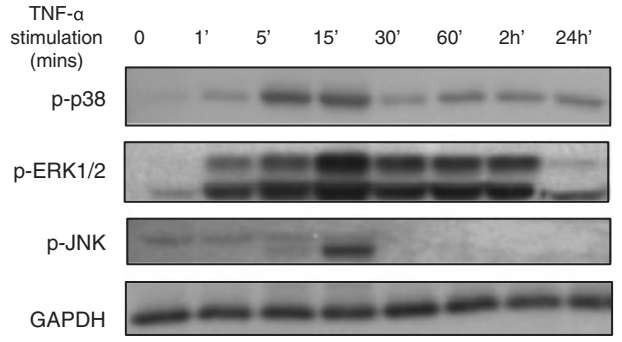

Figure 5. Activation of MAPK in HIF stimulated with TNF-α. HIF monolayers were left untreated (baseline) or stimulated with TNF 100 ug/mL for 1, 2, 5, 15, 30 min and 1, 2 or 24 h; then whole protein was extracted and analyzed by Western blot. Gels were incubated in the presence of phospho-specific antibodies for p38, p42/44 and JNK (recognizing p-p38, pERK1/2 and p-JNK, respectively), and visualized using HRP-conjugated secondary antibodies. This figure shows representative results from three separate experiments.

demonstrating that the capacity of TNF-α-stimulated HIF to attract lymphocytes is dependent on p38 and p42/44. Experiments blocking MAPK of endothelial cells alone (without fibroblasts on the bottom of the transwell) did not affect migration. On the contrary, in experiments with HIF alone (without HIMEC on the transwell) migration was inhibited at the same level as the inhibition shown in Fig. 7, suggesting that HIF are the major players in mediating leukocyte transmigration (data not shown).

Discussion

Leukocyte recruitment in intestinal inflammation plays a crucial role in IBD pathogenesis [7, 8]. Although the adhesion molecules and chemokines that regulate leukocyte recruitment are well described, the signaling molecules that orchestrate adhesion and migration of lymphocytes are still not fully characterized [21]. Our study provides important new information regarding the involvement of MAPK in mediating leukocyte recruitment to endothelium and migration into the interstitium.

Each of the three MAPK that we studied, *i.e.* p38, p42/44 and JNK, was highly phosphorylated in colonic samples from patients with IBD compared with those from control individuals. This finding is in accordance with previous studies in which Waetzig *et al.* [22] demonstrated that the activated forms of p38α, the JNK and p42/44 are up-regulated in patients with IBD. This finding is associated with a decrease in the expression of their inactive forms, particularly in lamina propria macrophages and neutrophils [22]. Mitsuyama *et al.* [23] presented similar findings, except that they identified the nucleus of epithelial and lamina propria mononuclear cells as the major source of activated MAPK in patients with IBD. These authors also described the ability of the JNK inhibitor SP600125 to prevent dextran sodium sulfate (DSS)-induced colitis in rats, suggesting a possible application of this category of drugs in the treatment of IBD.

On the other hand, Malamut *et al.* [24] found that the expression and activity of p38 and JNK were similar in patients with IBD and control individuals, indicating that neither is influenced by inflammation. These researchers also did not observe an increase in the expression or activity or p38 or JNK in mice with trinitrobenzenesulfonic acid (TNBS)-induced colitis, while SB203580 decreased the activity of p38, but did not display either a biological or a clinically therapeutic effect.

Our data provide new information regarding the role of activation of MAPK in the response of non-immune gut cells during mucosal inflammation such as that seen in patients with IBD. In particular, we demonstrate that both the endothelium and mucosal fibroblasts in the intestinal mucosa of patients with IBD express the activated forms of the MAPK *in vivo*. This observation was subsequently corroborated by *in vitro* studies. Having demonstrated that the MAPK are activated *in vitro* by stimulation of HIMEC and HIF with TNF-α, we then showed that this activation of MAPK plays a crucial role in the TNF-α-induced up-regulation of CAM such as ICAM-1, VCAM-1 and FKN on the surface of these cells, as well as the production of chemokines such as IL-8 and MCP-1 by HIF. Using MAPK-specific inhibitors, in particular, inhibitors of p38 and p42/44, we demonstrated that both the expression of CAM on intestinal endothelial cells and the production of chemokines by intestinal fibroblasts were significantly down-regulated. This suggests that the production of both CAM and chemokines in response to stimulation with TNF-α is directly dependent on the activation of MAPK.

HIMEC are highly specialized endothelial cells that have the ability to adhere to leukocytes and govern their trafficking [9]. Previously, it was demonstrated that this phenomenon is modified

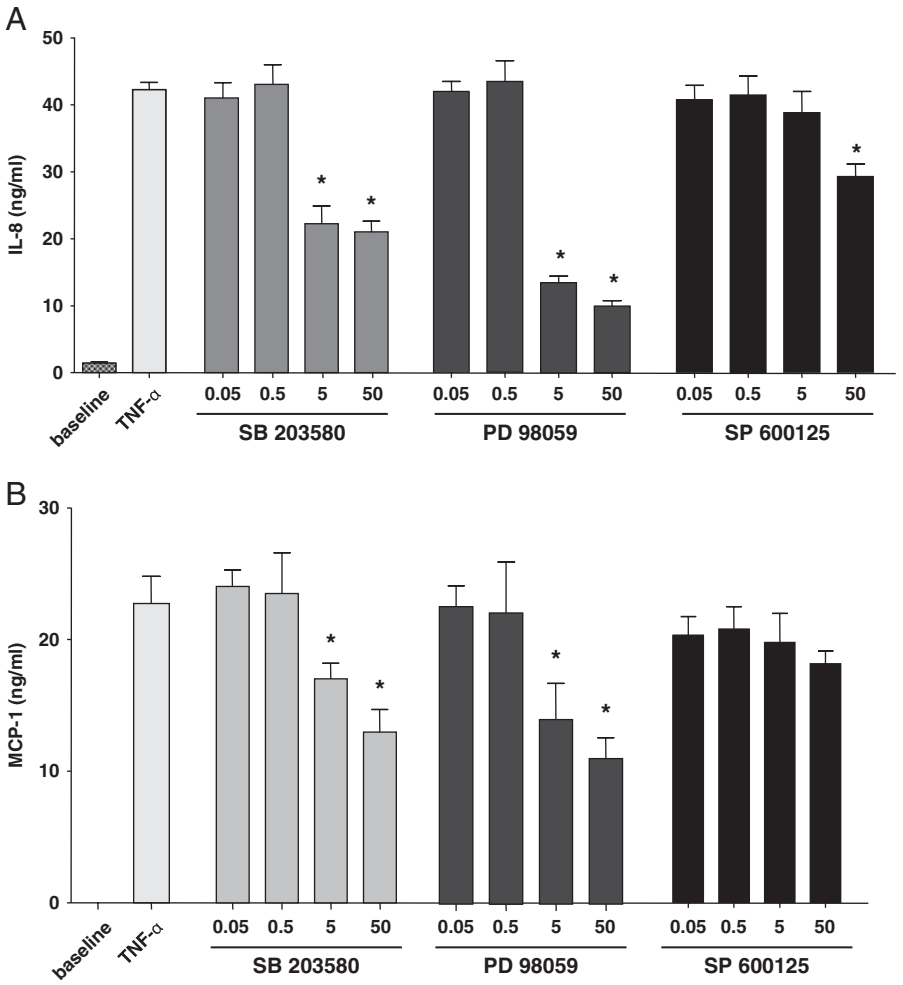

Figure 6. Involvement of p38, p42/44 and JNK in the production of chemokines by HIF. Control or IBD cells were seeded in 24-well cluster plate, then stimulated with TNF-α (100 U/mL) with or without 1 h pre-incubation with SB203580, PD 98059 or SP 600125. After 24 h of incubation, IL-8 (A) and MCP-1 (B) contents were measured by ELISA. Data are expressed as mean ± SEM of three separate experiments. $^{*}p<0.05$ for TNF stimulated cells pre-treated with MAPK-specific inhibitors compared with TNF-treated cells.

Table 1. Chemokine concentrations in TNF-α-stimulated HIMEC and HIF

Cell type	Stimulus	Chemokines (pg/mL)			
		MIP-1α	RANTES	IP-10	SDF-1
HIMEC	None	<20	47±27	<20	38±8
	TNF-α	<20	1168±362	368±74	11±2
HIF	None	<20	62±18	<20	85±34
	TNF-α	<20	328±115	1846±437	11±2

by pro-inflammatory conditions, such as after stimulation with TNF-α, or under the conditions of chronic inflammation observed in patients with IBD [9]. In this paper, we have partially elucidated the mechanism underlying this with the demonstration of a requirement for activation of MAPK. In particular, in the presence of specific inhibitors of either p38 or p42/44, the capacity of leukocytes to adhere to HIMEC was markedly decreased. In addition to adhesion to the endothelium, the chemokine gradient produced by intestinal fibroblasts is important for the recruitment of leukocytes and their migration through the endothelial monolayer. We found that chemokine production by HIF was selectively down-regulated by inhibition of p38 and p42/44, but not of JNK, in a manner similar to the effect we observed on adhesion.

Taken together, these data underline the importance of MAPK in the intestinal non-immune cell response to inflammation. Indeed, when inflammation occurs, TNF-α-induced activation of MAPK could mediate production of inflammatory chemokines such as IL-8 and MCP-1 by fibroblasts, as well as the expression of CAM such as ICAM-1, VCAM-1 and FKN, by the endothelium, thereby enhancing the recruitment of leukocytes to the inflamed gut. Our data therefore support the potential application of MAPK-specific inhibitors as a therapeutic for the treatment of IBD by describing a new specific mechanism-of-action for this category of drug.

Until the underlying causative factors have been identified for IBD, any manipulation that is found to impact the mechanisms underlying chronic inflammation, such as recruitment of leukocytes into the gut, should be considered for the development of

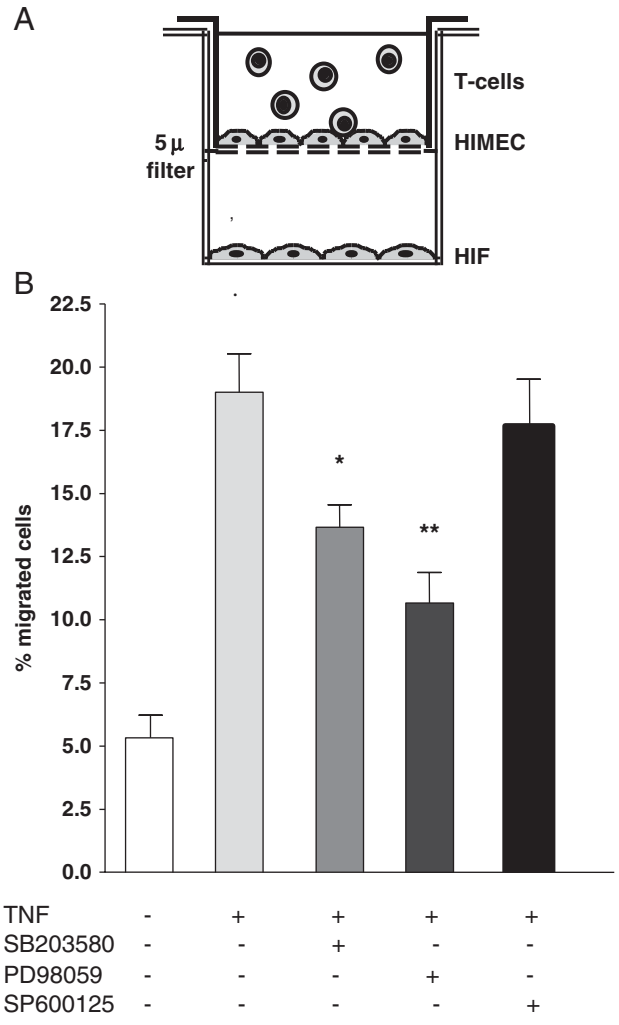

Figure 7. Induction of T-cell HIMEC transmigration by chemoattractants produced by activated HIF. HIMEC monolayers were grown to confluence on filter inserts separating the upper and lower chambers of a Transwell system. Calcein-labeled T cells were placed in the upper chamber overlaying the HIMEC monolayer, whereas medium alone, or HIF-derived supernatants were added to the lower chamber. HIF in particular were incubated in migration medium with 100 U/mL TNF-α with or without 1 h pre-incubation with SB203580, PD 98059 or SP 600125. After a 4 h incubation period, migrated cells were quantified using a computerized imaging system on an inverted fluorescence microscope. Each bar represents three separate HIMEC lines. *$p<0.05$ and **$p<0.01$ for TNF stimulated cells pre-treated with MAPK-specific inhibitors compared with TNF treated cells in the absence of inhibitors.

new therapeutic approaches. In addition, there is already proof of principle for the targeting of leukocyte–endothelial adhesion as a therapeutic intervention for IBD; the CAM inhibitor natalizumab has demonstrated efficacy in patients with CD.

There are plenty of data available regarding the application of MAPK inhibitors under conditions of experimental colitis. Of the currently available MAPK inhibitors, the greatest efficacy has thus far been demonstrated with specific inhibitors of p38. Salh *et al.* [25] demonstrated reproducible activation of p38 in intestinal lysates of dinitrobenzene sulfonic acid (DNBS)-induced colitic mice. This signal was significantly attenuated by curcumin, a component of the spice turmeric, which can inhibit the activation of NF-κB and reduce the activity of p38. Hollenbach *et al.* [26] demonstrated the efficacy of the p38 inhibitor SB203580 in the prevention of TNBS-induced colitis in mice, including improvements in the clinical condition, reductions in intestinal inflammation and suppression of the mRNA levels of pro-inflammatory cytokines that are normally elevated upon induction of colitis. The majority of the effects of SB203580 were demonstrated to be dependent on the inhibition of NF-κB and receptor-interacting caspase-like apoptosis-regulatory kinase [26]. SB203580 was also associated with an improvement of colitis in DSS-induced experimental mice, which was again associated with a reduction in the levels of the mRNA for pro-inflammatory cytokines. SB203580 was found to inhibit both the "classical" and the "alternative" NF-κB pathways during the induction of colitis in this murine model [27]. Finally, SB203580 also prevented ileitis in mice [28]. In the same study, the authors demonstrated that the ERK-specific inhibitor U0126 reduced the activity of AP-1 and decreased the expression of IL-8 and MCP-1 [28]. However, in one report [24] SB203580 did not display either a clinical or a biological therapeutic effect on colitis in mice induced with TNBS, although it did decrease the activity of p38 [24].

Only one study has thus far reported efficacy of the p-JNK inhibitor SP600125 in preventing DSS-induced colitis in mice, which was also associated with a reduction in the production of TNF-α by total colonic and mesenteric lymphocytes after stimulation with CD3/CD28 [29].

Because of the efficacy observed in pre-clinical models, MAPK inhibitors have also been tested in patients with IBD. Inhibition of JNK and p38 MAPK activation with CNI-1493 has been tested in 12 patients with CD, with encouraging results. Almost 70% of the patients responded and the authors concluded that MAPK are critically involved in the pathogenesis of CD and that their inhibition provides a novel therapeutic strategy [30]. However, thus far blockade of MAPK has only demonstrated efficacy as a therapeutic approach where the leukocyte signaling machinery and immune cells are the targets.

In contrast, our data suggest that, in addition to controlling the activity of leukocytes, MAPK play a crucial role in the control of the activity of non-immune cells. By regulating the expression of CAM on the endothelium and the secretion of chemokines by fibroblasts, blockade of specific MAPK may present a new option for prevention of aberrant leukocyte recruitment and infiltration into the mucosa of patients with CD or UC. Studies with specific endothelial or fibroblast delivery systems are now needed to test the efficacy of such a therapeutic strategy.

Materials and methods

Patient population

Ten active and ten inactive CD patients, 12 active and 8 inactive UC patients, and control individuals (ten patients admitted for bowel

resection because of colon cancer, polyps or diverticulosis) were enrolled. Patients and controls were recruited at the Division of Gastroenterology, Istituto Clinico Humanitas, Milan and at Catholic University of Rome, Italy, and the study was approved by the Institutional Review Boards of both universities. Clinical disease activity was assessed by the Harvey–Bradshaw Activity Index and the Colitis Activity Index [31, 32]. All diagnoses were confirmed by clinical, radiological, endoscopic and histological criteria. Written informed consent was obtained from all patients.

Immunostaining of paraffin-embedded colonic samples

Immunostaining for MAPK was performed as previously reported [33]. Briefly, intestinal tissues were obtained from surgical specimens of the enrolled patients and fixed in 10% formalin. Paraffin-embedded intestinal sections of 3 μm were cut, deparaffinized, hydrated, blocked for endogenous peroxidase using 3% H_2O_2/H_2O and subsequently subjected to microwave epitope enhancement using a Dako Target retrieval solution (Dako, CArpenteria). Incubation with anti-human phospho-p38, phospho-p42/44 and phospho-JNK (Cell Signaling Technology Danvers, MA) was performed at a 1:100 dilution for 30 min at room temperature. Detection was achieved using a standard streptavidin-biotin system (Vector Laboratories, Burlinghame, CA), and antigen localization was visualized with 3′-3-diamino benzidene (Vector Laboratories).

Isolation and culture of HIMEC and HIF

Isolation of HIMEC was performed as previously reported [34]. Briefly, HIMEC were obtained from surgical specimens from control individuals by enzymatic digestion of intestinal mucosal strips followed by gentle compression to extrude endothelial cell clumps, which adhere to fibronectin-coated plates. HIMEC were then cultured in MCDB131 medium (Sigma, St. Louis, MO) supplemented with 20% FBS, antibiotics, heparin and endothelial cell growth factor. Cultures of HIMEC were maintained at 37°C in 5% CO_2, fed twice a week and split at confluence. HIMEC were used between passages 3 and 12. HIF were generated as previously reported [20].

Flow cytometric analysis for evaluation of CAM expression in HIMEC

HIMEC were grown to subconfluence and then cultured in the presence or absence of 100 U/mL TNF-α (R&D Systems, Minneapolis, MN), with or without a 1 h pre-incubation with the p38 inhibitor SB203580 (10 μg/mL), the p42/44 inhibitor PD 98059 (10 μg/mL) or the p-JNK inhibitor SP 600125 (10 μg/mL) (Calbiochem, San Diego, CA). After 24 h, the confluent monolayers were thoroughly rinsed with HBSS treated with 0.5% trypsin/EDTA for 2–5 min and harvested. HIMEC were then washed twice with cold PBS containing 1.0% bovine serum albumin. Cells were then suspended in 0.1 mL of wash buffer containing mouse anti-human ICAM-1, VCAM-1 or FKN (R&D Systems). HIMEC were again washed twice and then incubated with fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulin G (Vector Laboratories). Following an additional 30-min incubation in the dark on ice, HIMEC were washed again, fixed with 0.5% paraformaldehyde and analyzed by flow cytometry (Beckman Coulter, Miami, FL).

Western blotting

Confluent HIF and HIMEC monolayers were incubated in the presence or absence of 100 U/mL of TNF-α for 0, 1, 2, 5, 15, 30 min, 1, 2 and 24 h, in regular culture medium supplemented with 5% FBS. Protein was then extracted using a lysing buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100, 50 mM protease and 50 mM phosphatase inhibitor cocktail (Sigma Chemical). The concentration of proteins in each lysate was measured using the Bio-Rad protein assay as *per* the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA). Immunoblotting was performed as previously described [35]. Equivalent amounts of proteins (20 μg) were fractionated on a 10% Tris-glycine gel and electro-transferred to a nitrocellulose membrane (Novex, San Diego, CA). Non-specific binding was blocked by incubation with 5% milk in 0.1% Tween 20/Tris-buffered saline (Fisher Scientific, Hanover Park, IL), followed by overnight incubation at 4°C with the primary antibody anti-phospho-p38, anti-phospho-p42/44 and anti-phospho-JNK. The constitutively expressed cytoplasmic protein GAPDH was used as a control for overall protein levels (Santa Cruz Biotechnology, Santa Cruz, CA).

Membranes were washed six times with 0.1% Tween 20/Tris-buffered saline, incubated with the appropriate horseradish peroxidase-conjugated secondary antibody, washed again and incubated with the chemiluminescent substrate (Super Signal; Pierce, Rockford, IL) for 5 min, after which they were exposed to film (Amersham, Arlington Heights, IL).

Induction of chemokine production by HIF

HIF were seeded in 24-well cluster plates at 3×10^4/well/mL of their respective medium and grown to subconfluence. TNF-α (100 U/mL) was then added, with or without a 1 h pre-incubation with SB203580 (0.05–50 μg/mL), PD 98059 (0.05–50 μg/mL) or SP 600125 (0.05–50 μg/mL), or fresh medium. After 24 h of incubation, supernatants were harvested and then stored at −20°C. Chemokine content in the supernatants was measured by ELISA for IL-8 and MCP-1 (R&D Systems), as previously reported [36].

Adhesion assay

The adhesion assay was performed as previously described [10]. Briefly, HIMEC were plated onto fibronectin pre-coated 24-well cluster plates (Costar, Corning, NY). After 24–48 h, the resulting monolayer was fed with fresh medium alone or medium containing 100 U/mL of TNF-α with or without a 1 h pre-incubation with SB203580 (10 μg/mL), PD 98059 (10 μg/mL) or SP 600125 (10 μg/mL). After 24 h, Jurkat or PBT cells that had been maintained under exponential growth conditions were labeled with calcein (Molecular Probes, Eugene, OR), added to HIMEC monolayers at 1×10^6 leukocytes/well and allowed to adhere to HIMEC at 37°C in a 5% CO_2 incubator. After 1 h of co-culture, wells were gently rinsed four times with PBS containing calcium and magnesium to remove all non-adherent leukocytes. Fluorescent-adherent leukocytes were quantified by an imaging system (Image Pro Plus; Media Cybernetics, Silver Spring, MD) connected to an Optronics Color digital camera (Olympus, Tokyo, Japan) on an inverted fluorescence microscope. Ten random fields were analyzed for each well and results were expressed as the number of adherent cells/mm^2.

T-cell transmigration assay

The transmigration assay was performed as previously described [10]. The method is based on a cluster of Transwell plate-containing polycarbonate porous filter inserts (3402; Costar) separating the upper and lower chambers. A HIMEC monolayer was established on the upper chamber filter by seeding 75×10^3 HIMEC in MCDB131 medium containing 20% FBS. Monolayers were grown for 7–10 days till complete confluence was reached and verified by microscopic evaluation of the histochemically stained (Diff-Quick Stain Set; Dade Diagnostics, Aguada, Puerto Rico) monolayer. The day before the assay, the HIMEC monolayer was stimulated with TNF-α with or without pre-incubation with SB203580 (10 μg/mL), PD 98059 (10 μg/mL) or SP 600125 (10 μg/mL).

On the day of the assay, cells were rinsed with MCDB131 medium to remove all sera. PBT cells were generated as previously reported (Danese, Gut 2004). T cells were suspended in 2×10^6/mL of PBS with 5% FBS and labeled at 37°C with 4 μmol/L of calcein (Molecular Probes). After 20 min, T cells were rinsed twice, resuspended in transmigration medium consisting of 50% RMPI 1640 and 50% MCDB131 medium containing 0.5% bovine serum albumin at 2×10^6/mL and added to the upper chamber at 1×10^6 cells/0.5 mL/insert. The insert was placed in a well of the cluster plate containing the HIF monolayer; then the lower chamber was stimulated for 24 h with TNF-α with or without 1 h of pre-incubation with SB203580 (10 μg/mL), PD 98059 (10 μg/mL) or SP 600125 (10 μg/mL).

After 4 h, the inserts were removed. The cell suspension in the lower chamber was allowed to settle and fluorescent cells were quantified using the same imaging system as described for the adhesion assay.

Statistical analysis

Statistical analysis was performed using the T- and chi-squared tests, and Dunn and Bonferroni's multiple comparison tests. Results are expressed as mean ± SEM, and significance was inferred at $p \leq 0.05$.

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Abbreviations: **CAM:** cell adhesion molecules · **CD:** Crohn's disease · **DSS:** dextran sodium sulfate · **FKN:** fractalkine · **HIF:** human intestinal fibroblasts · **HIMEC:** human intestinal microvascular endothelial cells · **IBD:** inflammatory bowel disease · **PBT cell:** peripheral blood T cell · **TNBS:** trinitrobenzenesulfonic acid · **UC:** ulcerative colitis

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