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p38 Mitogen-Activated Protein Kinase Is Activated and Linked to TNF-α Signaling in Inflammatory Bowel Disease

Georg H. Waetzig,* Dirk Seegert,* Philip Rosenstiel,*† Susanna Nikolaus,* and Stefan Schreiber*†*

Inflammatory bowel diseases (IBD)—Crohn’s disease (CD) and ulcerative colitis (UC)—are disorders of unknown etiology characterized by chronic relapsing inflammation of the gastrointestinal tract. CD and UC are multifactorial diseases caused by the interplay of genetic, environmental, and immunological factors. They are assumed to have a pathological, Ag-driven inflammatory response within a genetically susceptible individual. Both CD and UC are characterized by an imbalance between pro- and anti-inflammatory cytokines. Activated macrophages participate in the mucosal immune response, e.g., by producing proinflammatory cytokines such as TNF-α, IL-1β, and the chemokine IL-8 (3–5). TNF-α plays a central role in mucosal inflammation and is likely to be at the apex of the inflammatory cascade in CD (3, 6–8). The systemic inhibition of soluble TNF-α by a single infusion of a chimeric anti-TNF-α mAb of IgG1 isotype (infliximab) induced remission in up to 50% of CD patients and significantly improved clinical symptoms in most patients after only a short time (9, 10). Clinical responses after a single infusion of infliximab vary in duration (9, 10). In some patients, a clinical benefit of a single infusion was seen for as long as 1 year, suggesting that the underlying immunological patterns may be altered beyond the immediate effect of TNF-α removal (11). This view is supported by the relationship between mucosal production of inflammatory signaling molecules in remission and clinical relapses (7, 12). However, it appears that the increased availability of TNF-α and other proinflammatory cytokines is not the primary cause of mucosal inflammation in IBD (13). Two recent studies showed that infliximab induces apoptosis in circulating monocytes (14) as well as in lamina propria T cells of CD patients (15).

Mitogen-activated protein kinases (MAPKs) are conserved among all eukaryotes and participate in multiple cellular processes (16). Four groups of MAPKs have been identified in mammalian cells: the extracellular signal-regulated kinases (ERKs), the c-Jun N-terminal kinases (JNKs) or stress-activated protein kinases (SAPKs), the p38 kinases, and ERK5/big MAPK (16, 17). All MAPK cascades cooperate in the orchestration of inflammatory responses and extensive cross-talk to other inflammatory pathways, such as NF-κB and Janus kinase/STAT signaling, has been described (18, 19). TNF-α is one of the best-characterized agonists of the p38 and JNK pathways and is itself regulated by p38 and JNKs (20, 21). Other proinflammatory cytokines, like IL-16, which is up-regulated in IBD (22), also activate JNKs and p38 (23). The genes of p38α and ERK1 are localized in major IBD susceptibility regions on chromosomes 6 (13) and 16 (24), respectively. In a recent pilot study, the guanylylhydrazone JNKp38 inhibitor CNI-1493 strongly reduced clinical disease activity in CD patients (25). However, no systematic evaluation of the expression, activity, or signal transduction of MAPKs in IBD has been published so far.


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3 Abbreviations used in this paper: IBD, inflammatory bowel disease; ATF-2, activating transcription factor-2; MAPK, mitogen-activated protein kinase; CD, Crohn’s disease; ERK, extracellular signal-regulated kinase; HNP, human neutrophilic peptidase; JNK, c-Jun N-terminal kinase; PARP, poly(ADP-ribose) polymerase; Hsp, heat shock protein; SAPK, stress-activated protein kinase; UC, ulcerative colitis.

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The present study focused on the activity and expression of the four p38 subtypes (p38α–γ) in the inflamed mucosa of CD and UC patients in comparison with healthy normal controls. In addition, JNKs and ERK1/2 (p44/42 MAPK) were investigated. p38α showed the most substantial activation in the inflamed mucosa of both UC and CD patients; its activity and localization were further analyzed by in vitro kinase assays and immunohistochemistry, respectively. The role of p38α in the TNF-α signaling regulation loop was investigated in CD patients by assessing TNF-α secretion from inflamed mucosal tissue after in vitro treatment with the p38α inhibitor SB 203580 and by monitoring p38α activity after administration of infliximab in patients, human monocytes, and different cell lines.

Materials and Methods

Patients

Twenty-seven patients with colonic or ileocolonic CD, 16 patients with UC, and 17 age- and sex-matched normal control patients (without signs of pathology; endoscopy mainly for the exclusion of carcinoma) were included in the study (total n = 60; Table I). All IBD patients attended the outpatient clinic of the First Department of Medicine of the Christian-Albrechts-University (Kiel, Germany) because of increased clinical activity. IBD patients included in this study met several requirements: definite diagnosis of either CD or UC along established criteria (26, 27), clinical activity (CD activity index > 150 (28) or clinical activity index for UC > 4 (29)), moderate to high inflammatory activity confirmed by endoscopy and histology, and exclusion of other diseases (especially irritable bowel syndrome and infectious colitis). None of the patients was treated with cytotoxic drugs or antibiotics. Patients received either no medication, aminosalicylates, or glucocorticoids (Table I). Patients were recruited consecutively along these inclusion and exclusion criteria.

To investigate the influence of in vivo TNF-α inhibition on p38α, five additional CD patients were chosen from an infliximab study population described previously (10). These patients showed a steroid refractory, chronic active rectosigmoidal inflammatory manifestation (CD activity index > 200) and received a single infusion of infliximab, a humanized anti-TNF-α mAb. Responders were defined as patients who did not relapse during 4 wk after a single infusion with infliximab, short-responders relapsed between wk 1 and 4, and nonresponders showed no remission of disease at all. The patients included in the present study were two representative responders (patients 43 and 44), two nonresponders (patients 40 and 41), and one short-respondor (patient 42). Written informed consent was obtained from all patients at least 24 h before the procedure, and the project was granted prior approval by the institutional review board.

Samples

From each patient, at least eight colonic biopsies were taken from the same inflamed or noninflamed region. In addition, two biopsies were paraffin-embedded and used for histological examination. In eight CD patients (patients 18–20, 24, 30, 32, 34, and 38) and four UC patients (patients 45, 46, 48, and 58), several sets of biopsy specimens from the same patient including inflamed and noninflamed areas of the colonic mucosa were examined to establish the amount of variation within the samples and the influence of inflammatory activity. A biopsy was attributed to establish the amount of variation within the samples and the influence of inflammatory activity. A biopsy was attributed to infliximab from in vivo TNF-α inhibition on p38α in inflamed mucosal tissue after in vitro treatment with the p38α inhibitor SB 203580 and by monitoring p38α activity after administration of infliximab.

Processing of mucosal biopsies for Western blotting and RT-PCR

Biopsy samples were snap-frozen in liquid nitrogen at the time of removal. After mechanical homogenization in liquid nitrogen, specimens were processed for either protein or RNA extraction. Protein extracts were prepared by lysing the tissue homogenates for 5 min in boiling denaturing extraction buffer containing 1% SDS, 10 mM Tris (pH 7.4), and 1% phosphatase inhibitor mixture II (Sigma-Aldrich, St. Louis, MO). After sonication (twice for 5 s), insoluble material was removed by centrifugation for 15 min at 16,000 × g at 4°C. Protein extracts were snap-frozen in liquid nitrogen and stored at −80°C.

RNA extractions were performed using the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s recommendations. The sample obtained was quantitated by absorbance at 260 nm. RNA integrity was assessed by electrophoresis on a 1% formamide gel, and the absence of genomic DNA contamination was confirmed by PCR for β-actin.

Isolation and stimulation of human monocytes

Human monocytes were isolated from 100 ml of blood drawn from three healthy volunteers (two male and one female; age range, 24–28 years). We used a two-step density centrifugation protocol according to the respective manufacturer’s recommendations. After separation with Ficoll-Paque Plus (Amersham Pharmacia Biotech, Piscataway, NJ), mononuclear cells were collected from the interphase and washed in PBS. Monocytes were separated from lymphocytes by resuspension and subsequent centrifugation with isotonic Percoll (density, 1.065 g/cm3; Biochrom, Berlin, Germany). After two washing steps in PBS, the monocytes were suspended in monocyte medium (DMEM [Invitrogen, Carlsbad, CA] supplemented with 10% FCS and 1% penicillin/streptomycin [Biochrom]). The cell suspension was adjusted to 1 × 10⁶ cells/ml and plated on six-well plates (Falcon; Applied Scientific, San Francisco, CA). Monocytes were further enriched by 90-min adherence to the culture plates and washed twice in PBS. Enriched monocytes were allowed to rest for several hours and were subsequently incubated with infliximab (Remicade; 5 μg/ml; Centocor, Malvern, PA), a nonspecific human IgG1 mixture from myeloma patients (5 μg/ml; Calbiochem, La Jolla, CA), and/or SB 203580 (SB 203580 hydrochloride; Calbiochem) in a concentration of 1 or 10 μM. All culture reagents had endotoxin levels of < 0.01 ng/ml LPS. Viability of the monocytes was >95% as determined by trypan blue exclusion and purity was at least 85% as assessed by May-Grünewald/Giemsa staining of cytopsins (Merck, Darmstadt, Germany). After stimulation, RNA was extracted using the RNeasy Mini kit as described above.

Stimulation of cell lines and preparation of cell lysates

Human THP-1 myelomonocytes (30) and Jurkat T cells (31) were purchased from the American Type Culture Collection (Manassas, VA) and grown according to the supplier’s instructions. RPMI 8262 cells (32) were obtained from the German Collection of Microorganisms and Cell Cultures.

Table I. Clinical data for IBD patients and normal controls

<table>
<thead>
<tr>
<th>Patients</th>
<th>Diagnosis</th>
<th>Medication</th>
<th>Dose Median (min-max)</th>
<th>Age (years)</th>
<th>Sex (F/M)*</th>
<th>Location of Disease (R/S/D/T/A/C)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>1–17</td>
<td>Control</td>
<td>None</td>
<td>n/a</td>
<td>28–70</td>
<td>9/8</td>
<td>n/a</td>
</tr>
<tr>
<td>18–33</td>
<td>CD</td>
<td>None</td>
<td>n/a</td>
<td>20–43</td>
<td>7/9</td>
<td>1/3/1/0/0/4</td>
</tr>
<tr>
<td>34–39</td>
<td>CD</td>
<td>ASA*</td>
<td>3 × 1 g/day (n/a)</td>
<td>20–36</td>
<td>4/2</td>
<td>1/1/1/1/2/2</td>
</tr>
<tr>
<td>40–44</td>
<td>CD</td>
<td>GC†</td>
<td>15 mg/day (10–25)</td>
<td>23–36</td>
<td>3/2</td>
<td>4/1/1/1/1/1</td>
</tr>
<tr>
<td>45–53</td>
<td>UC</td>
<td>Infliximab*</td>
<td>5 mg/kg (n/a)</td>
<td>23–69</td>
<td>3/6</td>
<td>1/4/2/1/1/1</td>
</tr>
<tr>
<td>54–60</td>
<td>UC</td>
<td>GC</td>
<td>3 × 1 g/day (n/a)</td>
<td>21–77</td>
<td>3/4</td>
<td>1/2/1/1/1</td>
</tr>
</tbody>
</table>

* F, female; M, male.
† R, Rectum; S, sigma; D, descending colon; T, transverse colon; A, ascending colon; C, caecum.
* n/a, Not applicable.
† ASA, Aminosalicylates.
† GC, Glucocorticoids.

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Western blot analysis

Protein extracts from 16 CD patients (10 untreated or aminosalicylate-treated patients 18–22, 24, 25, and 30–32) and six glucocorticoid-treated patients (34–39), 13 UC patients (seven untreated or aminosalicylate-treated patients 45–50 and 47) and six glucocorticoid-treated patients (54–59), and 12 normal controls (patients 1–12) were used for evaluation of kinase activity (dual phosphorylation) and expression. For all Western blotting experiments, total protein concentrations were determined using a Bradford colorimetric assay according to the manufacturer’s instructions (Bio-Rad, Hercules, CA). Biopsy homogenates or cell extracts (standardized to 10 µg of total protein/lane) were separated by 12 or 15% denaturing SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Hybond-P; 0.8 mA/cm² for 60 min; Amersham Pharmacia Biotech). Unstimulated homogenates and cell lysates (20 µg each) were subjected to Western blot analysis. Membranes were blocked and incubated overnight with primary Abs: p38 MAPK, phospho-p38 MAPK, p44/42 MAPK, phospho-p44/42 MAPK, SAPK/JNK, phospho-SAPK/JNK (both monoclonal and polyclonal), activated transcription factor-2 (ATF-2), phospho-ATF2 that shock protein (Hsp27), phospho-Hsp27, and poly-(ADP-ribose) polymerase (PARP), all from Cell Signaling Technology, clones E-20 and C-16 for p38, clones N-17 and C-19 for p38α, from Santa Cruz Biotechnology (Santa Cruz, CA); ERK2 and p38α by Upstate Biotechnology (Lake Placid, NY); anti-ACTIVE JNK from Promega (Madison, WI); p38α from Zy mend Laboratories (San Francisco, CA); and β-actin from Sigma-Aldrich. After being washed in TBST (three times for 5 min), membranes were subsequently incubated for 30 min with a HRP-conjugated secondary Ab (anti-rabbit (Cell Signaling Technology), anti-goat (Sigma-Aldrich), anti-sheep (Sigma-Aldrich), or anti-mouse (Amersham Pharmacia Biotech), respectively) diluted in blocking buffer. Membranes were subsequently washed, incubated with ECL-Plus Detection Reagent, and exposed to film for varying lengths of time, and only films generating subsaturating levels of intensity were selected for densitometrical and statistical evaluation. Linearity was assured in independent experiments by using different amounts of material and multiple wash medium, single specimens were placed in 500 µl of reverse transcriptase (RT) solution. RT-PCR

For the investigation of MAPK mRNA expression in IBD patients, cDNA was synthesized from 500 ng of total RNA from five CD patients (patients 23, 25, 26, 32, and 33), five UC patients (patients 49–52 and 60), and five normal controls (patients 13–17) using the Advantage RT-for-PCR kit with oligo(dT) primers (Clontech Laboratories, Palo Alto, CA) according to the manufacturer’s protocol. The active (i.e., dual-phosphorylated) form of p38 MAPK was selectively immunoprecipitated, and kinase reactions were conducted with an ATP-2 fusion protein whose Thr71-phosphorylated form was detected by Western blotting.

In vitro p38 MAPK assay

Kinase activity of p38α in biopsies and cell lines was determined using a p38 MAPK assay kit (Cell Signaling Technology). The frozen biopsies were homogenized as described previously for Western blotting, but lysed in the presence of lysis buffer and processed according to the manufacturer’s protocol. The active (i.e., dual-phosphorylated) form of p38α was selectively immunoprecipitated, and kinase reactions were conducted with an ATP-2 fusion protein whose Thr71-phosphorylated form was detected by Western blotting.

Immunohistochemical studies

Biopsies were embedded in cryomatrix and snap-frozen in liquid nitrogen. Cryostat sections (7 µm) were thaw-mounted onto Superfrost slides (Erie, Portsmouth, NH), post-fixed for 5 min in acetone, air-dried, and stored at -20°C. Two slices of each biopsy were stained with H&E for routine histological evaluation. The other slides were permeabilized by incubation with 0.1% Triton X-100 in 0.1 M PBS, washed three times in PBS, and blocked with 0.75% BSA for 20 min. Sections were subsequently incubated with a HRP-conjugated secondary Ab (anti-rabbit (Cell Signaling Technology), anti-goat (Sigma-Aldrich), anti-sheep (Sigma-Aldrich), or anti-mouse (Amersham Pharmacia Biotech), respectively) diluted in blocking buffer. Membranes were subsequently washed, incubated with ECL-Plus Detection Reagent, and exposed to film (Hyperfilm ECL, both from Amersham Pharmacia Biotech). Between the stainings with phosphospecific Abs, kinase or target Abs, and β-actin Ab, biopsies were stored in 2% SDS, 62.5 mM Tris, and 100 mM Na-2H2O for 30 min at 50°C, washed, and blocked again. All measurements of dual-phosphorylated kinase levels and kinase protein expression were normalized by hybridization with Abs against total kinase protein and the housekeeping protein β-actin, respectively. Background values from each lane were subtracted to normalize every measurement. The bands were quantified using the densitometry program SigmaGel (Jandel Scientific, San Rafael, CA). All Western blots were exposed to film for varying lengths of time, and only films generating subsaturating levels of intensity were selected for densitometrical and statistical evaluation. Linearity was assured in independent experiments by using different amounts of material and multiple film exposures (data not shown). Each Western blotting experiment was conducted with two separate membranes in parallel to detect potential stripping artifacts.

In vitro MAPK assay

The following primers were used: ERK1, 5'-TGGGATCCTTGATGAGAAAAATGAGGCTCAC-3' (upstream) and 5'-TTCTAAGGTGCAAGTTTGTTG-3' (downstream) (amplicon: 327 bp); ERK2, 5'-AAGACCGTGTCCATGCAATG-3' (upstream) and 5'-GAAGAACCAGGGATGCTAGGAAC-3' (downstream) (amplicon: 302 bp); JNK1, 5'-TTCCCATCCCTACTTCGAGTCC-3' (upstream) and 5'-TGGCCCGGGTATAATCTCCATT-3' (downstream) (amplicon: 302 bp); JNK2, 5'-ACCTCTGTCTCACCATACAC-3' (upstream) and 5'-TCCGAGGATCAAAGCTGTGTT-3' (downstream) (amplicon: 308 bp); JNK3, 5'-CCCGGATGATATAGCTACTCTCCT-3' (upstream) and 5'-ACCTGTTGACATGCTGCCAG-3' (downstream) (amplicon: 335 bp); p38α, 5'-TTCCTCCATCCACTTTCAGTCCC-3' (upstream) and 5'-TTCTGCTCTGATAGGATCCTTG-3' (downstream) (amplicon: 306 bp); p38β, 5'-ACAGTGGTAAATGGAAGCAGG-3' (upstream) and 5'-GGACTTTAATCGGCGCCCTTGA-3' (downstream) (amplicon: 310 bp); TNF-α, 5'-ACCATGACGCTAGAAGAGATGCA-3' (upstream) and 5'-ATGAGTACAGCGCCTTCAGT-3' (downstream) (amplicon: 513 bp); JNK1, 5'-CAGACATCAGGCTGAGCCCTG-3' (downstream) and 5'-CTTAAGTGCACGAGATTCTCC-3' (downstream) (amplicon: 518 bp).
incubated in culture medium with 10 μM SB 203580 (SB 203580 hydrochloride; Calbiochem). After 4 h at 37°C and 5% CO₂, supernatants and biopsies were snap-frozen in liquid nitrogen and stored at −80°C until analysis. TNF-α levels in the supernatants were determined by standard TNF-α-ELISA (capture Ab MAB610 and detection Ab BAF210; R&D Systems). The biotinylated detection Ab was coupled to extravidin-peroxidase (Sigma-Aldrich), and immune complexes were detected by incubation with o-phenylendiamine-dihydrochloride and H₂O₂ (both from Sigma-Aldrich) according to the manufacturer’s instructions. After stopping the enzyme reaction by addition of 1 M HCl, the OD₄₉₀ was measured in an ELISA reader (Milenia/DPC, Los Angeles, CA).

Statistical analysis and replication rate

The normality of the data was checked by calculating Lilliefors probabilities based on the Kolmogorov-Smirnov test. Statistical significance of the non-normally distributed patient data was tested using the Mann-Whitney U test, the obtained p values were corrected for ties, and results were expressed as medians (quartiles). Multiple testing corrections were performed using the Bonferroni method. Measurements for each kinase were conducted three to seven times per patient, resulting in an overall average replication rate of four independent experiments per patient. Both the ELISA and cell culture data followed a normal distribution; their significance was determined by the t test for dependent (ELISA) or independent (Western blots from cell extracts) samples, and the respective results were displayed as means ± SD.

Results

Detection of active MAPKs

For activation, both a threonine and a tyrosine residue in a characteristic Thr-X-Tyr motif must be phosphorylated in MAPKs (16). Therefore, Abs specific for the dual-phosphorylated (i.e., active) forms of the MAPKs were used in Western blot analyses to determine kinase activities in the colonic mucosal biopsies.

The levels of dual-phosphorylated p38α, JNK1/2, and ERK1/2 were significantly increased in the inflamed colonic mucosa of all untreated patients with IBD (up to 3.4-, 2.2-, and 3-fold, respectively; p < 0.01; Fig. 1). As JNK3 expression is restricted to brain, heart, and testis (16, 33), the detected JNK protein represented JNK1/2. The p54 splice forms of JNK1/2 were predominantly expressed. No difference was observed between ERK1 and ERK2 (data not shown). In all patient groups, p38α showed the most pronounced activation (Fig. 1A).

While the activities of p38α and JNK1/2 were similar in IBD patients regardless of disease and treatment (data not shown), significant differences in ERK1/2 activity were observed between untreated and glucocorticoid-treated patients in CD (Fig. 2). This effect was not due to a difference in glucocorticoid dosage, as the median glucocorticoid dose in the CD patients was 15 mg/day compared with 20 mg/day in the UC patients (Table I).

FIGURE 1. Levels of dual-phosphorylated (active) MAPKs in the colonic mucosa as determined by Western blots. The results shown represent inflamed biopsies from 10 untreated or salicylate-treated CD patients (patients 18–22, 24, 25, and 30–32) and seven UC patients (patients 45–50 and 53), and biopsies from 12 normal controls (patients 1–12) with an average of four independent measurements per patient for each kinase. The data displayed were obtained by densitometrical analysis of scanned films and are expressed as medians (quartiles). Total kinase protein expression was used to normalize the signals of the dual-phosphorylated kinases.

FIGURE 2. Differential ERK1/2 activity in CD patients. In contrast to all other patient groups and MAPKs investigated, ERK1/2 activity significantly differed between untreated and glucocorticoid-treated CD patients: untreated CD patients showed a strong activation of ERK1/2 as compared with normal controls, while the amount of dual-phosphorylated ERK1/2 in glucocorticoid-treated CD patients was similar to control levels. No such differences were observed between untreated and glucocorticoid-treated UC patients. The data shown represent an average of three independent measurements in 12 normal controls (patients 1–12), 10 untreated or salicylate-treated CD patients (patients 18–22, 24, 25, and 30–32), six glucocorticoid-treated CD patients (patients 34–39), seven untreated or salicylate-treated UC patients (patients 45–50 and 53), and six glucocorticoid-treated UC patients (patients 54–59).
p38 kinase activity

p38α activation was not dependent on disease category or treatment (see above), but only on the presence and severity of inflammation, as indicated by the results of both in vitro kinase assays (Fig. 3) and Western blots for dual-phosphorylated p38 (Fig. 4A). Virtually all p38 activity could be attributed to the p38α isoform, because p38β and p38γ proteins were not found in significant amounts (see below) and phosphorylated p38β was generally not detectable (Fig. 4A). The variance of p38 activity within both the normal controls and the patients was considerable, ranging from moderate to very high activities in IBD patients and from almost none to moderate activities in normal controls, which may reflect the heterogeneity of individuals (Fig. 4A).

Expression of MAPKs

The protein expression of p38α displayed no significant differences between patients with IBD and normal controls (Fig. 4B), whereas the amount of p38β protein showed a tendency toward a lower level in the inflamed mucosa of both CD and UC patients (NS; Fig. 4C). In general, differences in protein expression were (similar to MAPK activation) more pronounced in inflamed than in noninflamed mucosal specimens of the same patient (Fig. 4C). p38β and p38γ proteins were below detection level, even when 30 μg of total protein (instead of 10 μg) were separated on each lane of the polyacrylamide gel.

Similar to p38β, JNK protein was tendentially diminished in IBD without significant differences to normal controls (data not shown). In contrast to the nonsignificant differences in p38 and JNK protein contents, ERK1/2 showed a significantly lower level of protein expression in all IBD patient groups in comparison to normal controls (CD: 39% reduction, p < 0.001; UC: 48% reduction, p < 0.0001; Fig. 5A). Similar to the phosphorylation pattern, no differences between ERK1 and ERK2 were observed in the regulation of protein expression.

Linear phase RT-PCR assessment of MAPK transcripts in IBD patients and controls showed no differences in mRNA expression (Fig. 5B). The amount of p38α and p38β mRNA was similar; in both cases, 30 cycles of the described PCR program were sufficient to produce evaluable amounts of PCR products. The low protein expression of p38β and p38γ was mirrored on the mRNA level: while 35 cycles were barely sufficient to produce detectable quantities of p38β amplicons, p38γ could not be detected even with 40 cycles. In control experiments, transcripts of all p38 isoforms were detectable in the human monocytic cell line THP-1 (data not shown). JNK1, JNK2, ERK1, and ERK2 were transcribed similarly (30–32 cycles).

Localization of p38α expression

As described above, the protein expression of p38α showed no significant differences between IBD patients and normal controls. p38α protein in the inflamed lamina propria mainly colocalized with CD68 (Ki-M6) specific for monocytes/macrophages and with HNP specific for neutrophils. Double-stained lamina propria macrophages were frequently observed near the epithelial lining of eroded crypts. Fig. 6 shows representative results obtained from
Inhibition of p38α in cultured biopsies from CD patients

From three representative CD patients (patients 27–29), whole colonic mucosal biopsies were cultured for 4 h and incubated with the p38α inhibitor SB 203580 at a concentration of 10 μM to ensure both sufficient inhibitor concentrations within the tissue and specificity of inhibition (34, 35). Concentrations of 5–20 μM showed a linear relationship between the dose of SB 203580 and TNF-α secretion (data not shown). Samples from one patient showed a low degree of inflammation (patient 27, aminosalicylate-treated), while specimens from the other two patients (patients 28 and 29, both untreated) displayed moderate to severe inflammation. From patient 29, two separate biopsy culture sets were obtained from two different anatomical locations, moderately and highly inflamed, respectively. After 4 h of incubation, TNF-α release into the supernatant was assessed by standard ELISA. All TNF-α concentrations measured were within the sensitivity range specified by the manufacturer. Biopsies from the mildly inflamed mucosa of patient 27 secreted ~5 pg TNF-α per 1-mg specimen, while the moderately and highly inflamed tissue explants of patients 28 and 29 released 47–51 pg TNF-α per 1-mg specimen. The inhibitory activity of SB 203580 was inversely correlated to the severity of inflammation (Fig. 7A). In highly inflamed tissue of patient 29, TNF-α secretion was reduced by only 8% (NS). However, a highly significant decrease of TNF-α release (p < 0.01) was observed in mildly inflamed (−88%) and moderately inflamed (−37 and −38%, respectively) mucosa of all patients.

The specificity of p38α inhibition was controlled by examining the phosphorylation levels of Hsp27, which is a specific target for p38αβ signaling (19). As p38β was barely expressed in the inflamed mucosa (see above), Hsp27 phosphorylation was a precise indicator for p38α activity. Fig. 7B shows that incubation with SB 203580 significantly reduced Hsp27 phosphorylation in both weakly and highly inflamed CD biopsies. As Hsp27 production is up-regulated by stress (36), the differences between the patients in baseline Hsp27 expression reflected the inflammatory activity.

Activation of p38α by infliximab in vivo and in vitro

Patients with CD were treated with infliximab, a mAb directed against TNF-α. Two responders to infliximab treatment, two non-responders, and one short-responder were chosen. The phosphorylation and protein expression of p38α and JNK1/2 in the affected sigmoid mucosa were determined by Western blotting experiments using denatured extracts of mucosal biopsy specimens taken immediately before, 24 h after, and 48 h (n = 3) after a single infusion of infliximab. All patients showed a highly significant increase of p38α, but not JNK1/2, dual phosphorylation (between 2- and 4-fold) 24 h after infusion (p < 0.000001; Fig. 8A). After 48 h, p38α activity dropped to a level still significantly higher than before infusion (p < 0.01), but also significantly lower than 24 h after infusion (p < 0.01).

To find out more about the mechanisms and consequences of infliximab-induced p38α activation, we stimulated freshly isolated peripheral monocytes from three healthy volunteers as well as human THP-1 (myelomonocyte), Jurkat (T lymphocyte), and RPMI
Fresh colonic biopsy specimens were obtained from three CD patients. TNF-α biopsies obtained in two separate ELISA series. The reduction of TNF-α in each patient 27, moderate (mod.) in patient 28, and moderate to high in patient 29. The membrane was successively probed with phosphospecific Hsp27 Ab (P-Hsp27) and Hsp27 Ab. SB treatment significantly reduced the protein expression of the MAPKs and ATF-2 (Fig. 8A) and SB 203580-treated (SB) biopsies from patient 27 and patient 29. The membrane was successively probed with phosphospecific Hsp27 Ab (P-Hsp27) and Hsp27 Ab. SB treatment significantly reduced Hsp27 phosphorylation in all samples.

In this study, we investigated the activity and expression of MAPKs in IBD patients and the influence of the therapeutic anti-TNF-α mAb infliximab on MAPK pathways. Our results show that p38α, JNK1/2, and ERK1/2 were significantly activated in the inflamed colonic mucosa of IBD patients, with p38α exhibiting the strongest activation in both CD and UC. In the meantime, this result (37) has been confirmed by others (25). Moreover, infliximab-induced TNF-α gene expression in human monocytes via a transient p38α activation.

The activation of p38α, JNK1/2, and ERK1/2 in IBD is consistent with previous reports implicating these enzymes in several cascades of inflammatory signal transduction (18, 21, 38). Except for ERK1/2 in glucocorticoid-treated CD patients, the activation of MAPKs in IBD was dependent only on the severity of inflammation, not on aminosalylate or glucocorticoid therapy. All p38 activity observed could be attributed to p38α. As the relative activities of JNK1/2 and ERK1/2 were lower than the activity of p38α, and as p38α was the only activated enzyme not showing a tendential (JNK1/2) or significant (ERK1/2) down-regulation on the protein level, p38α exhibited by far the highest increase of active enzyme in the inflamed mucosa, suggesting an exceptional role for this kinase in IBD.

While p38α and p38δ protein was expressed in similar amounts in all samples, p38β and p38γ protein contents were below detection level, which was mirrored by a low mRNA expression. This is consistent with several studies demonstrating that p38β expression is very low in the intestine and in peripheral leukocytes, and that p38δ is almost exclusively expressed in skeletal muscle (39–42). p38α and p38δ have been demonstrated to be the major isoforms in peripheral leukocytes, with p38α clearly emerging as the most important isoform in inflammatory cells and especially in macrophages (41–43). Therefore, p38α is a first-rate candidate enzyme for targeted inhibition. Our immunohistochemical analysis revealed that the main p38α expression observed in IBD mucosal biopsies colocalized with lamina propria macrophages and neutrophils, thus affirming the key role of these cells in IBD.

TNF-α secretion is regulated by p38α and JNK activation. In CD, TNF-α blockade by infliximab is used for therapy. We chose the model systems of SB 203580-treated CD biopsy cultures and colonic tissue from CD patients before and after infliximab treatment to investigate the interconnection of p38α and TNF-α signaling in vivo. The anti-inflammatory effects of p38αβ-inhibiting pyridinyl imidazole derivatives, such as SB 203580, have been demonstrated in several in vivo models (44–46). These effects can be attributed in part to the ability of the inhibitors to suppress monocyte/macrophage production of TNF-α, IL-1β, and other cytokines (47, 48). Several studies have demonstrated that SB 203580 inhibits TNF-α production and/or release in human monocytes (49), THP-1 cells (50), and T cells (21). To specifically inhibit p38αβ, SB 203580 had to be applied at a concentration of 10 μM or below, as influences on other kinases have been observed with higher concentrations (34, 35). As p38β is barely expressed in contrast, inhibition of p38α by 1 μM SB 203580 did not influence the infliximab-induced apoptosis of monocytes mediated by caspase-3 (14), as determined by cleavage of nuclear PARP, a main cleavage substrate of caspase-3 (Fig. 8G). This result was confirmed by FACS analysis of annexin V-stained monocytes (data not shown), which indicated a 15% increase in apoptosis by treatment with 5 μg/ml infliximab, regardless of coincubation with SB 203580.

**Discussion**

In this study, we investigated the activity and expression of MAPKs in IBD patients and the influence of the therapeutic anti-TNF-α mAb infliximab on MAPK pathways. Our results show that p38α, JNK1/2, and ERK1/2 were significantly activated in the inflamed colonic mucosa of IBD patients, with p38α exhibiting the strongest activation in both CD and UC. In the meantime, this result (37) has been confirmed by others (25). Moreover, infliximab-induced TNF-α gene expression in human monocytes via a transient p38α activation.

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8226 (multiple myeloma) cells with infliximab (5 μg/ml, corresponding to the therapeutic dose of 5 mg/kg body weight). The three cell lines were selected because THP-1 cells express TNF-α constitutively on their cell membrane, while resting Jurkat and RPMI-8226 cells do not.

Infliximab induced a highly significant (p < 0.01) increase in p38α, but not JNK1/2, dual phosphorylation in THP-1 cells (Fig. 8, B–E). This activation was visible already after 0.5 h, gradually increased toward 24 h (Fig. 8B), and was sustained until 48 h after stimulation (data not shown). In vitro kinase assays confirmed the Western blotting results (Fig. 8C). The significant activation of p38α after 24 h could not be induced by nonspecific IgG1 and did not occur in Jurkat or RPMI 8226 cells (Fig. 8D). This finding was mirrored on the transcription factor level by a strong phosphorylation of ATF-2 on Thr71 in THP-1 cells, but not in Jurkat cells (Fig. 8E). The protein expression of the MAPKs and ATF-2 (Fig. 8, A, B, and E) was not significantly altered by infliximab when compared with β-actin expression. The MAPK activity and expression patterns observed in the infliximab-treated patients (Fig. 8A) corresponded to the findings in THP-1 cells.

Treatment with infliximab (5 μg/ml) induced a significant increase in TNF-α mRNA in human monocytes (Fig. 8F) and THP-1 cells (data not shown), as determined by linear phase RT-PCR. TNF-α gene induction was observed after 6 h until 24 h after stimulation (maximum, 6-fold) and could be abrogated completely by treatment with a low dose (1 μM) of SB 203580 (Fig. 8F).
leukocytes or the intestine (see above), practically all effects observed could be attributed to p38α inhibition.

The disease status-related reduction of TNF-α secretion by specific inhibition of p38α in mucosal biopsies from CD patients demonstrated that p38α regulates TNF-α production in CD and that p38α repression can significantly diminish inflammatory activity in this system. The significant reduction of Hsp27 phosphorylation confirmed the specificity of p38α inhibition in all patients. The fact that the highly inflamed tissue of patient 29 showed a strong reduction in Hsp27 phosphorylation, but only a tendential decrease in TNF-α secretion, suggests that p38α inhibition may prove especially rewarding to avoid TNF-α production in inactive patients (i.e., remission maintenance). For induction of remission in highly active patients, the additional inhibition of JNKs could be necessary to...
reduce TNF-α secretion to normal levels (25). However, ongoing clinical studies using specific p38 inhibitors, such as BIRB 796 BS (Boehringer-Ingelheim, Ridgefield, CT), in active CD will clarify this issue.

Interestingly, induction of TNF-α by p38α was also seen after treatment with infliximab. Infliximab enhanced TNF-α gene expression in human peripheral monocytes from healthy individuals and in THP-1 myelomonocytic cells. This effect could be completely abrogated by coincubation with the p38αβ inhibitor SB 203580 (1 μM). In parallel to these findings, we demonstrated a highly significant, transient increase of p38α activity in sigmoidal biopsies of five representative CD patients during the first 48 h after a single infusion of infliximab (5 mg/kg body weight), while JNK1/2 activity was not altered. A strong increase in circulating TNF-α—most likely bound to infliximab—during the first days after treatment with a single infusion of infliximab (5 mg/kg) has been reported in rheumatoid arthritis patients (51).

To investigate the mechanisms underlying this novel signaling effect, we performed extensive in vitro studies with THP-1, Jurkat, and RPMI 8226 cells. While a constitutive secretion of TNF-α has been shown in naïve, resting THP-1 myelomonocytes (52, 53), rest 48 h after infliximab treatment (10) could be explained by an infliximab-bound inhibitor SB 203580, which was able to completely abrogate in the maintenance of an inflammatory environment, is not interfering with immune cell apoptosis.

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