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. It is assumed that a pathological, Ag-driven inflammatory response within a genetically susceptible individual is triggered either by an unrecognized pathogen or by nonpathogenic bowel flora (1). The discrimination between CD and UC is based on clinical, endoscopic, radiological, and histopathological features. Both CD and UC are characterized by an imbalance between pro- and anti-inflammatory cytokines (2). 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 JNKs (18, 19). 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 JNK/p38 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.

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; 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 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-respondent (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 inflamed areas if the macroscopic appearance was confirmed by inflammatory infiltrates in the histological examination. In three additional CD patients (patients 27–29), 24–30 biopsies from the same inflamed region were taken for biopsy culture experiments (as described below).

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/cm; Biochrom, Berlin, Germany). After two washing steps in PBS, the monocytes were suspended in monocyte medium (GMEM (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-Grünwald/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 8226 cells (32) were obtained from the German Collection of Microorganisms and Cell Cultures.

Table I. Clinical data for IBD patients and normal controls

| Patients | Diagnosis | Medication | Dose Median (min-max) | Age (years) | Sex (F/M) | Location of Disease (R/S/D/T/A/C) |
|----------|-----------|------------|----------------------|-------------|----------|---------------------------------|
| 1–17     | Control   | None       | n/a                  | 28–70       | 9/8      | n/a                            |
| 18–33    | CD        | None (11 patients) | 3 × 1 g/day (n/a) | 20–43       | 7/9      | 1/3/1/1/4                      |
| 34–39    | CD        | ASA (5 patients) | 15 mg/day (10–25) | 20–36       | 4/2      | 1/1/1/2/2                      |
| 40–44    | CD        | Infliximab | 5 mg/kg (n/a)       | 23–36       | 3/2      | 4/1/1/1/1/1                    |
| 45–53    | UC        | None (7 patients) | n/a                  | 23–69       | 3/6      | 1/4/2/1/1/2                    |
| 54–60    | UC        | ASA (2 patients) | 3 × 1 g/day (n/a) | 21–77       | 3/4      | −1/1/1/1/1/1                    |

* F, female; M, male.
* ASA, Aminosalicylates.
* GC, Glucocorticoids.

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Polymerase (both from PerkinElmer, Branchburg, NJ) in a 50-μl reaction volume containing primer pairs (0.2 μM/primer) and 0.2 mM dNTPs (Amersham Pharmacia Biotech). Negative reverse transcriptase and PCR controls included reactions in the absence of RNA sample, reverse transcriptase, and cDNA, respectively. Kinase mRNA expression was normalized by amplifying and analyzing β-actin mRNA under the same reaction conditions. All MAPK primer pairs were designed to selectively amplify specific isoforms, and they were optimized to suit the following PCR program: one hold at 94°C for 5 min; 25–35 cycles at 94°C for 30 s, 60°C for 20 s, and 72°C for 30 s; and one hold of 72°C for 7 min. Resulting reactions were resolved on 2% agarose gels stained with ethidium bromide and visualized through a UV light digital imaging system. Images were evaluated using a densitometrical software (SigmaGel). For each mRNA, the number of cycles directly above detection level (linear phase) was determined and used for evaluation. The efficiency of the primers was confirmed by tests using cDNA from the human monocyte cell line THP-1. The same protocol was used for assessing TNF-α mRNA expression in human monocytes and THP-1 cells after stimulation with infliximab.

The following primers were used: ERK1, 5'-TCAGCCCTCTGAGCATCA-3' (upstream) and 5'-TCTTAAGGTTGCGAGTGGTGTT-3' (downstream) (amplonc: 327 bp); ERK2, 5'-AACAGGCTTGGTCCCAAAAT-3' (upstream) and 5'-GAAGAAACACCGTGTCCTAGGACA-3' (downstream) (amplonc: 340 bp); JNK1, 5'-TCTGCTGCTGCTGCTGCTGCTGCT-3' (upstream) and 5'-TGCCCCTGATATAACTCCATTCTC-3' (downstream) (amplonc: 302 bp); JNK2, 5'-ACCTCTGCTGCATCCACATAC-3' (upstream) and 5'-TCAGGAGCATCAAGACTGCTG-3' (downstream) (amplonc: 308 bp); p38δ, 5'-CCGAAGATGACCTTGGGAGATT-3' (upstream) and 5'-GAAGAAACACCGTGTCCTAGGACA-3' (downstream) (amplonc: 302 bp); p38β, 5'-CCCGGACATATATACCGCTTCTC-3' (upstream) and 5'-ACCTCTGCTGCATCCACATAC-3' (downstream) (amplonc: 335 bp); p38γ, 5'-TTCCTACACCTCCTCGTGTTCC-3' (upstream) and 5'-TCTGCTGCTGCTGCTGCTGCTGCT-3' (downstream) (amplonc: 306 bp); p38δ, 5'-ACAGTTGGATAGAAAGCCAGGC-3' (downstream) and 5'-GGCATTTAATCGTGCTTGTTA-3' (downstream) (amplonc: 310 bp); TNF-α, 5'-ACCATGAGACCTGAGAACATGGA-3' (upstream) and 5'-ATGAGTACAGCCTCTCGTGTTG-3' (downstream) (amplonc: 404 bp); and β-actin, 5'-GATGTGGGGACCTGGTGCA-3' (downstream) and 5'-CTTAATGCAGCAGACAGACTC-3' (downstream) (amplonc: 518 bp).

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 the primary Abs (p38 MAPK, phospho-p38 MAPK, p44/42 MAPK, phospho-p44/42 MAPK, SAPK/JNK, phospho-SAPK/JNK (both monoclonal and polyclonal), activating transcription factor-2 (ATF-2), phospho-p38 MAPK (that shock protein (Hsp)27, phospho-Hsp27, and poly-ADP-ribose) polymerase (PARP), all from Cell Signaling Technology. clones E-20 and C-16 for p38δ, clones N-17 and C-19 for p38δ, from Santa Cruz Biotechnology (Santa Cruz, CA); ERK2 and p38δ from Upstate Biotechnology (Lake Placid, NY); anti-ACTIVE JNK from Promega (Madison, WI); p38α from Zymed Laboratories (San Francisco, CA); and β-actin from Sigma-Aldrich. After being washed in TBST (three times for 5 min) and permeabilized 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 Hyperfilm ECL (both from Amersham Pharmacia Biotech). Between the stainings with phosphospecific Abs, kinase or target Abs, and β-actin Ab, the membranes were stripped in 2% SDS, 6.5 mM Tris, and 100 mM Na2CO3 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 striping artifacts.

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 provided lysis buffer and processed according to the manufacturer’s protocol. The active (i.e., dual-phosphorylated) form of p38 was selectively immunoprecipitated, and kinase reactions were conducted with an ATP-2 fusion protein whose Thr71-phosphorylated form was detected by Western blotting.

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. Resulting cDNA (5 μl of reverse transcriptase reaction) was amplified using GeneAmp PCR buffer and AmpliTaq DNA Polymerase (both from PerkinElmer, Branchburg, NJ) in a 50-μl reaction volume containing primer pairs (0.2 μM/primer) and 0.2 mM dNTPs (Amersham Pharmacia Biotech). Negative reverse transcriptase and PCR controls included reactions in the absence of RNA sample, reverse transcriptase, and cDNA, respectively. Kinase mRNA expression was normalized by amplifying and analyzing β-actin mRNA under the same reaction conditions. All MAPK primer pairs were designed to selectively amplify specific isoforms, and they were optimized to suit the following PCR program: one hold at 94°C for 5 min; 25–35 cycles at 94°C for 30 s, 60°C for 20 s, and 72°C for 30 s; and one hold of 72°C for 7 min. Resulting reactions were resolved on 2% agarose gels stained with ethidium bromide and visualized through a UV light digital imaging system. Images were evaluated using a densitometrical software (SigmaGel). For each mRNA, the number of cycles directly above detection level (linear phase) was determined and used for evaluation. The efficiency of the primers was confirmed by tests using cDNA from the human monocyte cell line THP-1. The same protocol was used for assessing TNF-α mRNA expression in human monocytes and THP-1 cells after stimulation with infliximab.

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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-phenylenediamine-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 p38α 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 p38δ 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).

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 experimental data.
one of four identical experiments conducted with biopsies from four normal controls, three CD patients, and three UC patients. In further tests, stainings with Abs against eosinophil peroxidase and CD4+ lymphocytes were performed, but none of them showed significant colocalization with the p38α signal (data not shown).

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 p38αβ 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
Half of the biopsies were incubated with the p38 inhibitor SB 203580 (SB) at a concentration of 10 μM. Results are expressed as means ± SD. Each column represents ELISA data from eight (controls) or four (SB 203580) biopsies obtained in separate ELISA series. The reduction of TNF-α secretion is very low in the intestine and in peripheral leukocytes, and p38 expression is almost exclusively expressed in skeletal muscle (39–42). As p38α is the most important isoform in inflammatory signaling in vivo, the anti-inflammatory effects of p38α 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 tendency to regulation in both CD and UC, it is postulated that the expression in IBD is consistent with previous reports implicating these enzymes in several stages 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 aminosalicylate 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 tendency (JNK1/2) or significant (ERK1/2) down-regulation on the protein level, p38α was confirmed by others (25). Moreover, p38α-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 stages 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 aminosalicylate 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 tendency (JNK1/2) or significant (ERK1/2) down-regulation on the protein level, p38α was confirmed by others (25). Moreover, p38α-induced TNF-α gene expression in human monocytes via a transient p38α activation.

While p38α and p38β 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 p38α is almost exclusively expressed in skeletal muscle (39–42). p38α and p38β 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 pyridyl 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...
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 demonstrates 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

FIGURE 8. Infliximab activates p38α both in vivo and in vitro. A, p38α is activated by infliximab in the sigmoid mucosa of CD patients (P-p38α), while JNK1/2 activity is not altered (P-JNK1/2). Biopsies were taken immediately before (b), 24 h after, and 48 h after a single infusion of infliximab (5 mg/kg). Shown are representative Western blots of the same denatured extracts from biopsy specimens of a responder (R; patient 44) and a nonresponder (NR; patient 40) to infliximab treatment. All patients featured a highly significant transient increase in dual-phosphorylated (i.e., active) p38α (P-p38α). B, Representative Western blots of denatured extracts from THP-1 cells. Infliximab (5 μg/ml) strongly enhanced p38α dual phosphorylation (P-p38α), increasing from 0.5 to 24 h. The short-term activation caused by 5 ng/ml recombinant human TNF-α (rhTNF-α) was investigated for comparison. This experimental setting was used in three independent stimulation series and produced consistent results. C, Infliximab treatment (5 μg/ml, 24 h) increased p38α kinase activity in THP-1 cells, as determined by in vitro kinase assay. After selective immunoprecipitation of the dual-phosphorylated form of p38α, kinase reactions were conducted with an ATF-2 fusion protein whose Thr71-phosphorylated form (P-ATF-2) was detected by Western blotting. The data shown are representative of four kinase assay experiments. D, Stimulation with infliximab (Ifx; 5 μg/ml, 24 h), but not with a nonspecific IgG1 mixture, induced a highly significant (p < 0.01) activation of p38α in resting THP-1 cells. In contrast, no significant activation of p38α was observed in resting Jurkat or RPMI 8226 cells. Data represent a total of seven independent stimulation series with THP-1 cells, six series with Jurkat cells, and four series with RPMI 8226 cells. E, Phosphorylation of ATF-2 (P-ATF-2) Thr71 in response to infliximab (+) was restricted to THP-1 cells. Infliximab treatment did not influence JNK1/2 activity (P-JNK1/2), which was confirmed by the use of three different primary Abs and purchased positive control cell extracts (data not shown). The Western blots displayed represent the same denatured extracts from control (-) and infliximab-treated (+; 5 μg/ml, 24 h) THP-1 and Jurkat cells, respectively. MAPK and ATF-2 protein expression were not significantly altered by infliximab (see A and B). F, Induction kinetic of TNF-α mRNA expression by infliximab (Ifx; 5 μg/ml) in monocytes obtained from a healthy individual. Coincubation with 1 μM SB 203580 completely abrogated TNF-α induction. Control IgG1 did not induce TNF-α expression after 6 or 24 h (data not shown). Data shown are representative for stimulation series with monocytes obtained from three healthy volunteers. G, PARP cleavage in response to infliximab treatment (Ifx; 5 μg/ml) was not influenced by coincubation with SB 203580 (SB; 1 μM). Nonspecific human IgG1 did not induce PARP cleavage. SB 203580 alone had no influence on apoptosis (data not shown). The Western blot shown is representative for experiments with monocytes obtained from three healthy volunteers.
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 coinoculation 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 naive, resting THP-1 myelomonocytes (52, 53), increased after addition, the ELISA used would not detect in inhibition on the apoptosis of in this cell line. The specific p38α activation also accounts for the increase in phosphorylated ATF-2, as the AP-1 controls was kindly provided by Dr. Christine Costello and Nicola Dierkes. We thank Ilka Woywod and Tanja Kaacksteen for cell culture maintenance and monitoring. High-purity total RNA from IBD patients and normal controls was kindly provided by Dr. Christine Costello and Nicola Dierkes. We thank Ilka Woywod and Tanja Kaacksteen for cell culture maintenance and monitoring.

TNF-α is an inflammatory cytokine that stimulates the expression of interleukin (IL)-8, which is a potent chemotactic factor for neutrophils and monocytes. IL-8 is produced by a variety of cell types, including endothelial cells, fibroblasts, and epithelial cells, in response to TNF-α. It plays a key role in the recruitment of leukocytes to sites of inflammation, where they mediate tissue damage and repair.

TNF-α signals through the activation of a receptor complex consisting of the TNF-α receptor 1 (TNFR1) and TNFR2. TNFR1 is a member of the TNF receptor superfamily and contains a death domain (DD) that mediates cell death. TNF-α binding to TNFR1 leads to the recruitment and activation of death domain-containing adapter proteins (e.g., Fas-associated death domain protein [FADD], Traf-2) and the recruitment of procaspase-8, which is subsequently activated and cleaved to generate active caspase-8. This leads to the activation of downstream executioner caspases, such as caspase-3 and caspase-7, resulting in cell death.

In summary, TNF-α is a pro-inflammatory cytokine that plays a central role in the pathogenesis of inflammatory bowel disease. Its role in the development and maintenance of inflammation in IBD is supported by the fact that neutralization of TNF-α in mice reduces colonic inflammation and reduces the number of CD11b+ cells, which are macrophages and neutrophils. These studies suggest that targeted inhibition of TNF-α may be a promising therapeutic strategy for the treatment of IBD.
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