

Selective Suppression of Neutrophil Accumulation in Ongoing Pulmonary Inflammation by Systemic Inhibition of p38 Mitogen-Activated Protein Kinase¹

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The p38 mitogen-activated protein kinase (MAPK) signaling pathway regulates a wide range of inflammatory responses in many different cells. Inhibition of p38 MAPK before exposing a cell to stress stimuli has profound anti-inflammatory effects, but little is known about the effects of p38 MAPK inhibition on ongoing inflammatory responses. LPS-induced activation of p38 MAPK in human neutrophils was inhibited by poststimulation exposure to a p38 MAPK inhibitor (M39). Release of TNF- α , macrophage-inflammatory protein (MIP)-2 (MIP-1 β), and IL-8 by LPS-stimulated neutrophils was also reduced by poststimulation p38 MAPK inhibition. In contrast, release of monocyte chemoattractant protein-1 was found to be p38 MAPK independent. Ongoing chemotaxis toward IL-8 was eliminated by p38 MAPK inhibition, although the rate of nondirectional movement was not reduced. A murine model of acute LPS-induced lung inflammation was used to study the effect of p38 MAPK inhibition in ongoing pulmonary inflammation. Initial pulmonary cell responses occur within 4 h of stimulation in this model, so M39 was administered 4 h or 12 h after exposure of the animals to aerosolized LPS to avoid inhibition of cytokine release. Quantities of TNF- α , MIP-2, KC, or monocyte chemoattractant protein-1 recovered from bronchial alveolar lavage or serum were not changed. Recruitment of neutrophils, but not other leukocytes, to the airspaces was significantly reduced. Together, these data demonstrate the selective reduction of LPS-induced neutrophil recruitment to the airspaces, independent of suppression of other inflammatory responses. These findings support the feasibility of p38 MAPK inhibition as a selective intervention to reduce neutrophilic inflammation. *The Journal of Immunology*, 2002, 169: 5260–5269.

Rapid accumulation of neutrophils to a site of inflammation is a defining early event of innate immunity. Neutrophil recruitment to the airspaces is induced by cytokines, chemokines, and other inflammatory mediators released by resident alveolar macrophages and other cells of the lung. Neutrophils are induced to leave the pulmonary vasculature, migrate through the lung interstitium, and ultimately accumulate in the airways through a complex series of events including sequential changes in cell size and stiffness, up-regulation of adhesion molecules, cytoskeletal rearrangement, and chemotaxis (1–4). A secondary recruitment of monocytes and macrophages typically follows the initial neutrophil influx, and the neutrophil itself may serve to amplify and perpetuate the recruitment of other leukocytes to the airspaces in certain disease states (5, 6). In a number of inflammatory lung diseases such as cystic fibrosis and the acute respiratory distress syndrome, neutrophil recruitment is prolonged and likely contributes to airway injury.

Stress-induced responses of many cell types are regulated by signal transduction via the mitogen-activated protein kinase (MAPK)³ superfamily (7). Like all mammalian cells, the neutrophil contains at least three distinct families of MAPKs: the p42/44 extracellular signal-regulated kinase (ERK) MAPKs, c-Jun NH₂-terminal kinases (JNKs), and p38 MAPKs (8–10). In the setting of inflammation, cytokine release and other functional responses by pulmonary host defense cells are regulated to varying degrees by p38 MAPK. For example, inhibition of p38 α MAPK blocks TNF- α and IL-8 release by LPS-stimulated monocyte/macrophage (10–12), IL-8 release by bronchial epithelial cells, and up-regulation of the ICAM-1 in endothelial cells when exposed to inflammatory stimuli (13, 14). The response of neutrophils to these cytokines and other proinflammatory mediators is also regulated by p38 MAPK. In stimulated neutrophils, p38 α MAPK regulates distinctly different functions, including adhesion, activation of NF- κ B, synthesis of TNF- α and IL-8, superoxide anion release, chemotaxis, and apoptosis (15–18).

As a short-lived, terminally differentiated primary cell, the neutrophil appears to use fewer of the available intracellular signal transduction mechanisms, relying on the p38 MAPK cascade to regulate functional responses to nearly every type of environmental stress. For example, in monocytes or macrophage cell lines, LPS can activate p42/44 (ERK) MAPK and JNK as well as the p38

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Received for publication December 7, 2001. Accepted for publication August 21, 2002.

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¹ This work was supported by Grants HL68743, HL03657, HL-40784, HL-34303, HL-09640, and GM-30324 from the National Institutes of Health.

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³ Abbreviations used in this paper: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH₂-terminal kinase; MIP, macrophage-inflammatory protein; MCP, monocyte chemoattractant protein; ATF, activated transcription factor; BAL, bronchial alveolar lavage; M₁, McCutcheon index; NDM, nondirectional movement; MPO, myeloperoxidase; MAPKAP, MAPK-activated protein.

MAPK cascade, and LPS-induced TNF- α release can be blocked through selective inhibition of any of these kinases (10, 19–24). In contrast, LPS stimulation of neutrophils does not result in activation of the p42/44 (ERK) MAPKs or the JNKs (25–27). Reflecting the relatively greater dependence of neutrophils on signal transduction via p38 MAPK, a 1000-fold less concentration of a p38 MAPK inhibitor is required to block release of TNF- α , macrophage-inflammatory protein (MIP)-2, or KC in neutrophils compared with murine alveolar macrophages (12).

Acute aerosolized exposure to LPS serves as a model for pulmonary inflammation and is of significant clinical interest. LPS is not an effective chemoattractant for neutrophils, but it can trigger an inflammatory cascade via synthesis of cytokines and chemokines by resident alveolar macrophages, local mast cells, fibroblasts, epithelia, and endothelial cells. The subsequent release of TNF- α and neutrophil-directed chemokines such as IL-8 are essential to early LPS-mediated neutrophil recruitment, whereas other chemokines such as monocyte chemoattractant protein-1 (MCP-1) and MIP serve to orchestrate later monocyte and lymphocyte accumulation. In our single-exposure murine model of LPS-induced pulmonary inflammation, the maximal release of cytokines occurs within 4 h in the airspaces (12).

Previously we have shown that in the setting of systemic p38 MAPK inhibition, LPS-induced neutrophil accumulation was significantly reduced, whereas the secondary influx of mononuclear cells was unchanged (12). LPS-induced TNF- α release in the airspaces was also reduced through p38 MAPK inhibition. Thus, effects of systemic p38 MAPK inhibition on resident cells of the lung were clearly present as well. Using KC as a stimulus, TNF- α release was negligible and neutrophil accumulation was blocked by pretreatment of the animal with a p38 MAPK inhibitor. Combined with *in vitro* data, these results suggested that systemic p38 MAPK inhibition can inhibit neutrophil responses independent of other measured host responses. However, many potential responses to LPS were not examined, and it is likely that a more comprehensive study would have demonstrated that systemic inhibition of p38 MAPK decreased the inflammatory response, contributing to decreased neutrophil accumulation.

The present study was designed to minimize the contribution of p38 MAPK inhibition on resident cells of the airway by administering a p38 MAPK inhibitor after the inflammatory cascade has been triggered. This allows a more selective analysis of the neutrophil response, independent of the early release of cytokines and chemokines in the airways. Of equal importance, this study tests the utility of systemic p38 MAPK inhibition as a means of modifying inflammation after an initial insult, which is of much greater clinical relevance than inhibition of p38 MAPK before exposure to LPS. Inhibition of p38 α MAPK was accomplished with the compound M39, which is a highly selective and bioavailable inhibitor of p38 MAPK (28). Herein we report the effect of poststimulation inhibition of p38 MAPK on neutrophil response *in vitro* and on murine pulmonary inflammation *in vivo*.

Materials and Methods

Materials

Endotoxin-free reagents and plastics were used in all experiments. Aprotinin, leupeptin, Tris-HCl, Triton X-100, Igepal, PMSF, EDTA, EGTA, Nonidet P-40, and protein A Sepharose were purchased from Sigma-Aldrich (St. Louis, MO), and [γ - 32 P]ATP was purchased from Amersham Life Sciences (Arlington Heights, IL). M39 ((*S*)-5-[2-(1-phenylethylamino)pyrimidin-4-yl]-1-methyl-4-(3-trifluoromethylphenyl)-2-(4-piperidinyl)imidazole) was provided by Merck Research Laboratories (Rahway, NJ) and stored in DMSO at -20°C . LPS strain O111:B4 isolated from *Escherichia coli* (List Biological Laboratories, Campbell, CA) was repurified by a second phenol extraction to eliminate the possibility of contaminating

glycoproteins (29, 30). Activated transcription factor-2 $_{1-110}$ (ATF-2 $_{1-110}$) was prepared as previously described (15, 25).

Animals

Female C57BL/6 mice (Harlan Sprague Dawley, Indianapolis, IN) 6–12 wk of age and weighing 16–20 g were used in all experiments. They were given commercial pellet food and water *ad libitum*. All experiments were performed in accordance with the Animal Welfare Act and the Public Health Service Policy on Humane Care and Use of Laboratory Animals after review of the protocol by the Animal Care and Use Committee of the National Jewish Medical and Research Center. Anesthesia was provided by a single *i.p.* injection of 333 mg/kg Avertin. Avertin was prepared by mixing 10 g of tribromoethyl alcohol (Aldrich, Milwaukee, WI) with 10 ml of tertiary amyl alcohol (Aldrich) and diluting this stock to a 2.5% solution in sterile saline.

Murine bronchial alveolar lavage (BAL)

BAL was performed immediately after sacrifice of the animals as previously described (12). Cell types were determined by Wright staining of a cytocentrifuged sample. All slides were counted twice by different observers blinded to the status of the animal. Samples for cytokine analysis were immediately frozen in a dry ice/ethanol bath and stored at -70°C .

Neutrophil functional assays

Human neutrophils were isolated by the plasma percoll method (31) and suspended in RPMI 1640 culture medium (Bio-Whittaker, Walkersville, MD). All experiments were done in the presence of 1% human heat-inactivated platelet-poor plasma. Cytokine release assays were performed with human neutrophils isolated from peripheral blood resuspended in RPMI 1640 containing 10×10^6 cells/ml. One-milliliter cell suspension was contained in a 1.5-ml microcentrifuge tube (Brinkmann Instruments, Westbury, NY) and was rotated continuously for the duration of the stimulation at 37°C in the presence or absence of the p38 MAPK inhibitor, added at various time points after stimulation. At the end of the stimulation, the supernatant was removed for quantification of IL-8, MCP-1, MIP-1 β , MIP-2, or TNF- α by immunoassay (R&D Systems, Minneapolis, MN).

A Zigmond chamber chemotaxis assay was used to assess the effects of p38 inhibition on ongoing human neutrophil chemotaxis. Neutrophils (1×10^5) were loaded in a 50- μl volume onto a 22×40 -mm number one glass coverslip. The cells were allowed to adhere for 20 min at 37°C , and the coverslip was inverted onto a Zigmond chamber slide (NeuroProbe, Gaithersburg, MD). The buffer control chamber was loaded with 95 μl of Krebs-Ringer-phosphate dextrose containing 1.5% human serum albumin (right chamber), and the chemoattractant (50 ng/ml IL-8 in the same buffer) was loaded in left chamber. The relative morphology, position, orientation, and locomotion of the cells were evaluated using videomicroscopy. M39 (5 μl of 1 mM) or a buffer (5 μl of Krebs-Ringer-phosphate dextrose with 1.5% human serum albumin) was added to the left well containing 95 μl of 50 ng/ml IL-8 after ongoing chemotaxis was established (4 min).

Cells were viewed and recorded at a final magnification of $\times 750$ on a Panasonic (Secaucus, NJ) cathode ray tube monitor using a video camera (Dage-MTI, Michigan City, IN) connected to a JVC videocassette recorder (Wayne, NJ). Cell tracings were made of each field over time and the mean and peak migratory rates were calculated. In addition, from these tracings the mean path length and net displacement of the cells toward the chemoattractant were also calculated to enable assessment of relative chemotaxis and nondirectional migration in response to a gradient by calculating the McCutcheon index (M_1) (32). Cells were designated as nondirectional movement (NDM) in response to a gradient if they migrated with an M_1 of <0.6 . Cells with an $M_1 \geq 0.6$ were designated as chemotactic, because they exhibited considerable directionality. From three experiments, a total of 96 individual cells were evaluated to achieve an adequate sample size.

p38 MAPK immunoprecipitation assays

Kinase activity of p38 α MAPK was assayed from immunoprecipitated samples by the ability to phosphorylate ATF-2 $_{1-110}$ as previously described (15).

In vivo inhibition of p38 MAPK

LPS was administered to all mice simultaneously by aerosolization (300 $\mu\text{g}/\text{ml}$ for 10 min) in an aerosolization chamber (210-SR; March Manufacturing, Glenview, IL). At 4 h or 12 h, anesthetized mice were administered the p38 MAPK inhibitor by gastric intubation of a 22-gauge straight feeding needle with a 2.25-mm ball diameter (Popper and Sons, New Hyde Park, NJ). Fasting mice were placed in a semiupright position, and 100 μl

of hydroxypropylmethylcellulose (Abbott Laboratories, Abbott Park, IL) with or without M39 (3 mg/kg) was instilled.

Quantification of neutrophil accumulation of murine lung tissue

Quantification of neutrophil accumulation in the whole lung, excluding the airspaces, was performed by myeloperoxidase (MPO) assay as previously described (12). Results are expressed as units of MPO activity per gram of lung tissue.

Statistical analysis

Data were analyzed using JMP statistical software (SAS Institute, Cary, NC). Student's unpaired *t* test (two-tailed) was used to determine significance of p38 MAPK inhibition (Fig. 1) and 24-h leukocyte recovery by BAL (Fig. 7) at single time points. Dunnett's *t* test was used for pairwise comparison of means with a control group for cytokine release (Fig. 2) and chemotaxis (Fig. 3). Differences in *in vivo* cell accumulation, cytokine release, and whole-lung MPO content over time in the presence and absence of p38 MAPK inhibition (Figs. 4–6) were analyzed by two-way ANOVA. For all tests, $p < 0.05$ was considered significant.

Results

Poststimulation inhibition of p38 MAPK activation in neutrophils

Previous reports have demonstrated phosphorylation and activation of p38 MAPK in human neutrophils after stimulation with

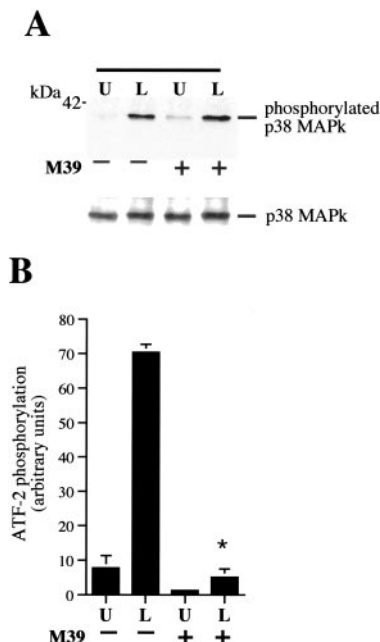
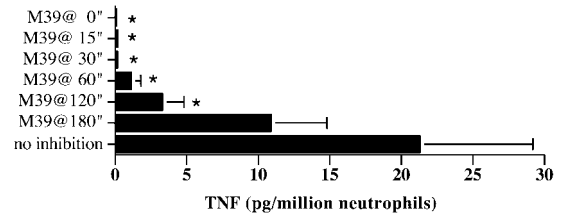
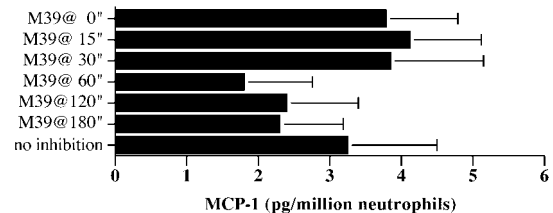


FIGURE 1. Poststimulation addition of M39 inhibits LPS-induced activation of p38 α MAPK in neutrophils. *A*, Tyrosine phosphorylation of p38 α MAPK. Human neutrophils (10×10^6 per condition) were stimulated with LPS (100 ng/ml) at 37°C (L) or were left unstimulated (U). At 15 min after addition of LPS, an unstimulated and a LPS-stimulated sample were exposed to M39 (1 μ M) for 5 min. The cells were then washed and lysed 25 min after the addition of LPS. The p38 α MAPK was immunoprecipitated and separated by SDS-PAGE. Western blots were probed with an anti-phosphotyrosine Ab capable of reacting with phosphorylated tyrosine residues of p38 α MAPK. The blots were then reprobed with an anti-p38 MAPK Ab to demonstrate an equal amount of p38 MAPK immunoprecipitated for each condition studied. Blots shown are representative of four consecutive experiments. *B*, Activation of p38 α MAPK. p38 α MAPK was immunoprecipitated from the lysates depicted in *A* and combined with ATF-2₁₋₁₁₀ in the presence of [³²P]ATP. The peptide was subjected to SDS-PAGE, and the amount of [³²P] phosphorylation of ATF-2₁₋₁₁₀ was assessed by phosphor screen autoradiography. Plots depict means \pm SEM from four consecutive experiments expressed in arbitrary units. *, $p < 0.01$ by Student's *t* test, compared with LPS-stimulated sample in the absence of M39.

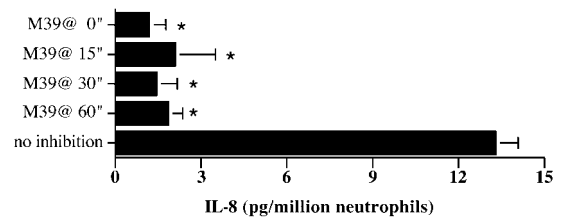
A



B



C



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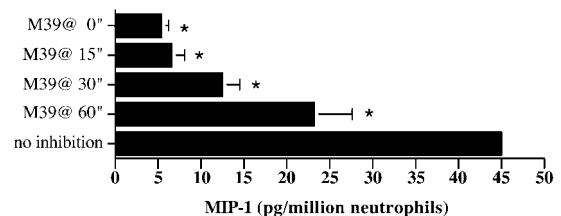
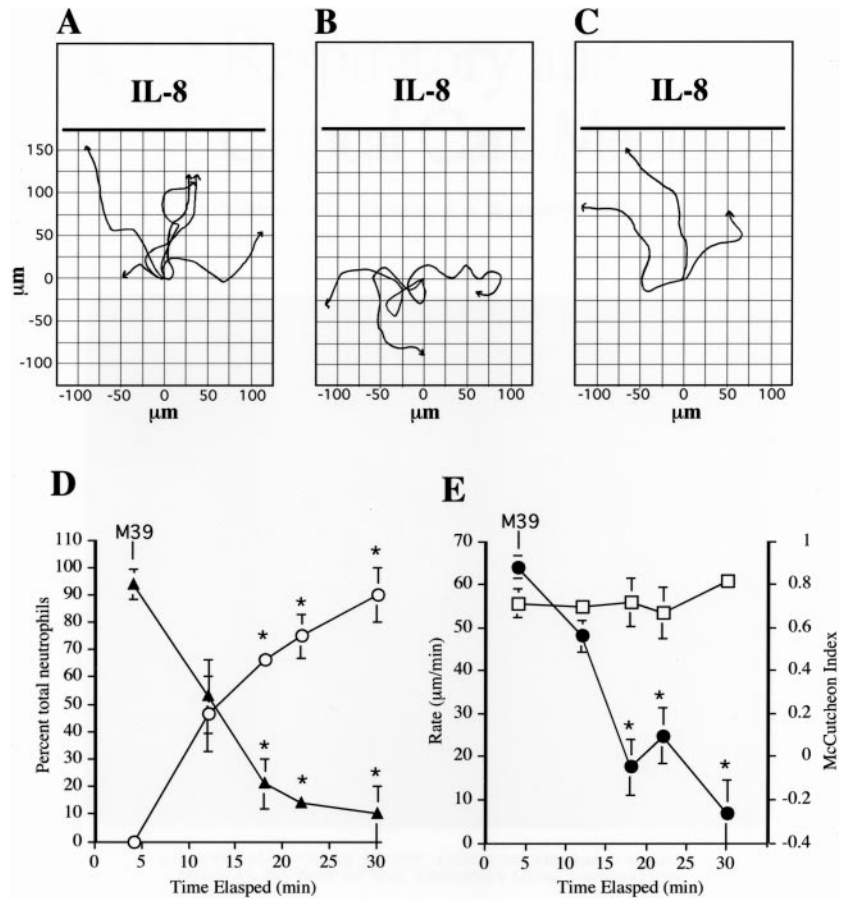


FIGURE 2. Effect of p38 MAPK inhibition on cytokine release of LPS-stimulated neutrophils. Human neutrophils stimulated with LPS (100 ng/ml) were exposed to M39 (1 μ M at 37°C) immediately before stimulation (time 0) or after stimulation over a range of times (15–180 min). Each plot depicts mean cytokine release (picograms per 10^6 cells) \pm SEM from four consecutive experiments (*, $p < 0.05$ when compared with the untreated control). *A*, Inhibition of TNF- α release by poststimulation p38 MAPK inhibition. Addition of M39 from time 0 up to 120 min poststimulation resulted in significantly reduced TNF- α release measured at 4 h. *B*, Release of MCP-1 is independent of p38 MAPK inhibition. Addition of M39 at any point did not significantly reduce MCP-1 release measured at 4 h. *C*, Inhibition of IL-8 release by poststimulation p38 MAPK inhibition. Addition of M39 from time 0 up to 60 min poststimulation resulted in significantly reduced IL-8 release at 2 h. *D*, Inhibition of MIP-1 β release by poststimulation p38 MAPK inhibition. Addition of M39 from time 0 up to 60 min poststimulation resulted in significantly reduced MIP-1 β release at 2 h.

LPS (25, 26). We have shown that LPS-induced activation of p38 MAPK occurs equally in murine and human neutrophils. M39 inhibits p38 MAPK activity in murine (as well as human) neutrophils, resulting in a loss of chemotaxis toward MIP-2 and KC and the loss of TNF- α and MIP-2 release in response to LPS (12). Because LPS-induced activation of p38 MAPK is maximal at 25

FIGURE 3. Inhibition of p38 MAPK blocks ongoing neutrophil chemotaxis. A Zigmund chamber chemotaxis assay was used to assess the effects of M39 on ongoing human neutrophil chemotaxis (see *Materials and Methods*). Neutrophils adherent to a glass coverslip were inverted onto a Zigmund chamber slide with IL-8 present in one side of the gradient chamber. The relative morphology, position, orientation, and locomotion of the cells were evaluated using videomicroscopy. Cell tracings were made of each field over time, and the mean and peak migratory rates were calculated. Center point tracings from representative fields are shown to illustrate the effects of M39 on ongoing neutrophil chemotaxis. These tracings are from one of six runs completed in two separate experiments. The position of the IL-8 gradient is at the top of the center point tracing. **A**, Untreated neutrophils demonstrated chemotaxis toward the IL-8 gradient (figure representative of three consecutive experiments). **B**, Addition of M39 to the chamber at 4 min after ongoing chemotaxis toward IL-8 was established (figure representative of three consecutive experiments). **C**, Addition of a control buffer to the chamber at 4 min after ongoing chemotaxis toward IL-8 was established (figure representative of three consecutive experiments). **D**, The percentage of neutrophils displaying chemotaxis ($M_1 > 0.6$) (▲) and NDM ($M_1 < 0.6$) (○) toward IL-8 were plotted vs time (min). Addition of M39 at 4 min resulted in a rapid shift from chemotaxis to NDM (*, $p < 0.05$, compared with 4-min time point). **E**, The rate of neutrophil migration (micrometers per minute) (□) was plotted vs time and compared with the M_1 (right axis) (●). Addition of M39 at 4 min resulted in a rapid decrease in the M_1 but did not reduce the rate of migration of the neutrophils (*, $p < 0.05$, compared with 4-min time point).



min (25), we tested whether addition of M39 after exposure to LPS could also result in p38 MAPK inhibition. Human neutrophils were stimulated with LPS or were left unstimulated, and M39 was added 15 min after stimulation. At 20 min the cells were washed to remove the M39, and at 25 min the cells were lysed. Activity and tyrosine phosphorylation of p38 MAPK were assessed simultaneously by immunoprecipitation of the kinase from the neutrophil lysates. LPS stimulation resulted in robust tyrosine phosphorylation of p38 MAPK in human neutrophils (Fig. 1A). However, p38 MAPK isolated from LPS-stimulated cells treated poststimulation with M39 had significantly reduced kinase activity (Fig. 1B). These results demonstrate that activation of p38 MAPK in the neutrophil can be significantly inhibited by M39 introduced after initiation of LPS-induced activation. Inhibition of p38 MAPK via M39 occurs independent of “upstream” signaling events in the p38 MAPK cascade; thus, tyrosine phosphorylation is not affected.

Effect of poststimulation p38 MAPK inhibition on cytokine release of LPS-stimulated neutrophils

Neutrophils have the capability to synthesize and release a limited number of cytokines (33), and under certain conditions this response may be important in perpetuating or modifying inflammation. Activation of p38 MAPK is closely associated with cytokine production by leukocytes. Although LPS-induced activation of p38 MAPK is maximal at 25 min, detectable activity persists for up to 4 h (data not shown). Thus, inhibition of p38 MAPK hours after stimulation could potentially modify p38 MAPK-regulated responses. Under conditions studied, LPS-induced release of IL-8 and MIP-1 β by neutrophils occurs rapidly, with maximal levels observed at 2 h, whereas optimal TNF- α and MCP-1 release is seen by 4 h. We compared the effects of p38 MAPK inhibition pre-

and poststimulation on LPS-induced release of TNF- α , MIP-1 β , MCP-1, and IL-8 from human neutrophils (see *Materials and Methods*). Addition of M39 before stimulation and up to 120 min after stimulation resulted in significant reduction of TNF- α release (Fig. 2A). Released MIP-1 β and IL-8 were also significantly reduced by poststimulation p38 MAPK inhibition, to nearly the same extent as cells exposed to M39 before stimulation (Fig. 2, C and D). Inhibition of p38 MAPK before or after LPS stimulation did not significantly reduce MCP-1 release at any time point studied (Fig. 2B). These results demonstrate that poststimulation inhibition of p38 MAPK effectively blocks p38 MAPK-mediated synthetic responses to LPS by the neutrophil. MCP-1 release appears largely independent of p38 MAPK in the neutrophil. These data indicate that p38 MAPK inhibition can significantly modify neutrophil responses well after maximal p38 MAPK activation has passed, suggesting that persistent low-level activation of p38 MAPK is of physiologic importance.

Poststimulation inhibition of p38 MAPK blocks chemokine-induced chemotaxis of neutrophils

Chemotaxis is a complex response involving coordination of adhesion and actin assembly. We have reported previously that inhibition of p38 MAPK results in loss of chemotaxis response by human neutrophils to fMLP (15) and of murine neutrophils toward the chemoattractants MIP-2 and KC (12). We tested whether human neutrophil chemotaxis toward IL-8 could be disrupted by p38 MAPK inhibition after chemotaxis has been initiated.

As expected, untreated human neutrophils generally demonstrate rapid chemotaxis toward an IL-8 gradient (Fig. 3A). Inhibition of p38 MAPK in neutrophils undergoing chemotaxis resulted in a complete loss of IL-8-induced chemotaxis (Fig. 3B). To

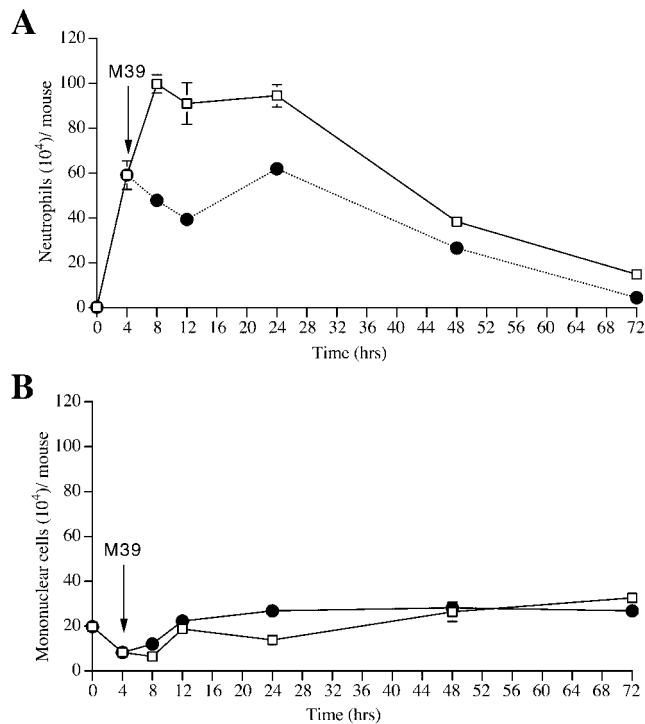


FIGURE 4. Effect of poststimulation inhibition of p38 MAPK on leukocyte accumulation in the airspaces. *A*, Neutrophil accumulation in the murine airspaces after aerosolized LPS. Mice were exposed to LPS (see *Materials and Methods*) at time 0 and then were administered M39 (●) at 4 h. Cell analysis of BAL was compared with untreated mice (□) over a series of times (see *Materials and Methods*). Each point represents mean number of neutrophils \pm SEM from three to six animals. The effect of M39 on LPS-induced neutrophil accumulation over time is significant ($p = 0.0005$) by two-way ANOVA. *B*, Mononuclear cell accumulation in the murine airspaces after LPS. Mononuclear cells from BAL samples depicted in *A* were analyzed in mice administered M39 (●) and compared with untreated mice (□) over a series of times. Each point represents mean number of cells \pm SEM from three to six animals. Poststimulation p38 MAPK inhibition resulted in a slight LPS-induced mononuclear cell accumulation over time, which was statistically significant ($p = 0.003$) by two-way ANOVA.

control for potential hydrostatic effects of injecting M39 into the chemoattractant well, the injection of buffer in the absence of M39 was found to not significantly affect ongoing chemotaxis (Fig. 3C).

Quantification of relative chemotaxis can be achieved by assigning an M_1 score to each cell (see *Materials and Methods*). Cells with an $M_1 > 0.6$ are chemotactic, whereas those with an $M_1 < 0.6$ display NDM. When p38 inhibition was initiated at 4 min, a significant shift in the behavior of the neutrophils was recorded, in that the population rapidly lost chemotaxis toward IL-8 and instead exhibited NDM (Fig. 3D). However, inhibition of p38 MAPK did not reduce the rate of movement of the cells (Fig. 3E).

Together, the data presented in Figs. 1–3 support the conclusion that *in vitro* activation of p38 MAPK in the neutrophil, as well as p38 MAPK-dependent cytokine release and chemotaxis, can be significantly blocked by administration of a p38 MAPK inhibitor well after the initiation of cell stimulation. This provides a rational basis to study the effects of poststimulation systemic p38 MAPK inhibition in a murine model, as described below.

p38 MAPK IN ONGOING PULMONARY INFLAMMATION

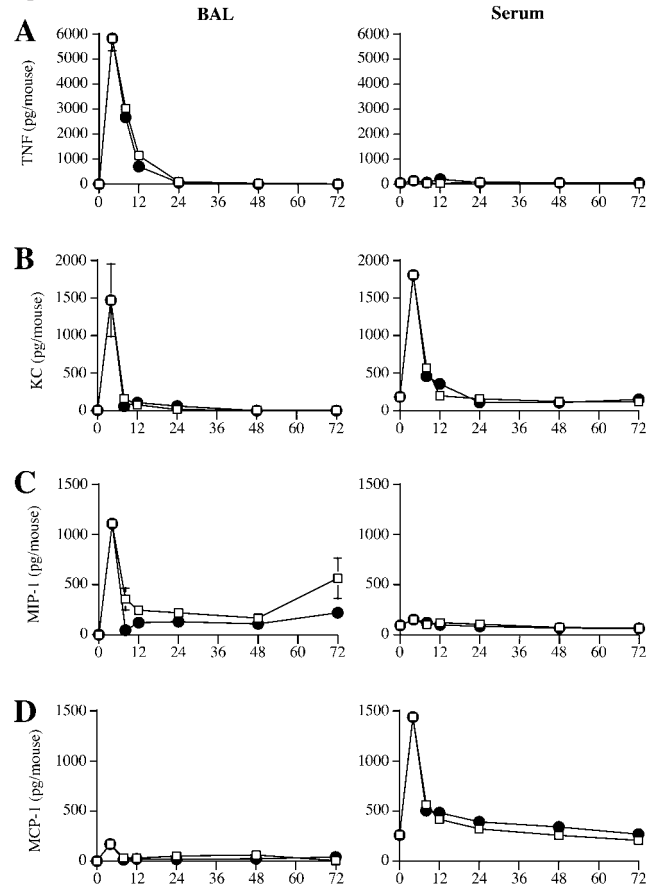


FIGURE 5. Effect of *in vivo* inhibition of p38 MAPK on cytokine release in the airspaces. *A*, TNF- α in the murine airspaces and serum in response to LPS. BAL (left panel) or serum (right panel) from Fig. 4 was analyzed for TNF- α in untreated mice (□) compared with mice administered M39 (●) from 0–72 h after LPS exposure. Each point represents the mean amount (picograms per mouse) \pm SEM of TNF- α recovered from three to six animals. The effect of M39 on LPS-induced TNF- α release in BAL ($p = 0.877$) over time is not significant by two-way ANOVA. TNF- α recovered in serum was negligible. *B*, KC in the murine airspaces and serum in response to LPS. BAL (left panel) or serum (right panel) from Fig. 4 was analyzed for KC in untreated mice (□) compared with mice administered M39 (●) from 0–72 h after LPS exposure. Each point represents the mean amount (picograms per mouse) \pm SEM of KC recovered from three to six animals. The effect of M39 on LPS-induced KC release in BAL ($p = 0.99$) or serum ($p = 0.23$) over time was not significant by two-way ANOVA. *C*, MIP-2 in the murine airspaces and serum in response to LPS. BAL (left panel) or serum (right panel) from Fig. 4 was analyzed for MIP-2 in untreated mice (□) compared with mice administered M39 (●) from 0–72 h after LPS exposure. Each point represents the mean amount (picograms per mouse) \pm SEM of MIP-2 recovered from three to six animals. The effect of M39 on LPS-induced MIP-2 release in BAL ($p = 0.175$) or serum ($p = 0.76$) over time is not significant by two-way ANOVA. *D*, MCP-1 in the murine airspaces and serum in response to LPS. BAL (left panel) or serum (right panel) from Fig. 4 was analyzed for MCP-1 in untreated mice (□) compared with mice administered M39 (●) from 0–72 h after LPS exposure. Each point represents the mean amount (picograms per mouse) \pm SEM of MCP-1 recovered from three to six animals. The effect of M39 on LPS-induced MCP-1 release in BAL ($p = 0.27$) or serum ($p = 0.83$) over time is not significant by two-way ANOVA.

Poststimulation inhibition of p38 MAPK *in vivo* results in decreased leukocyte accumulation in the airspaces

To study the effect of poststimulation p38 MAPK inhibition in the lungs, a model of nonlethal LPS-induced pulmonary inflammation was used (see *Materials and Methods*). After administration of aerosolized LPS, leukocytes were quantified from BAL samples

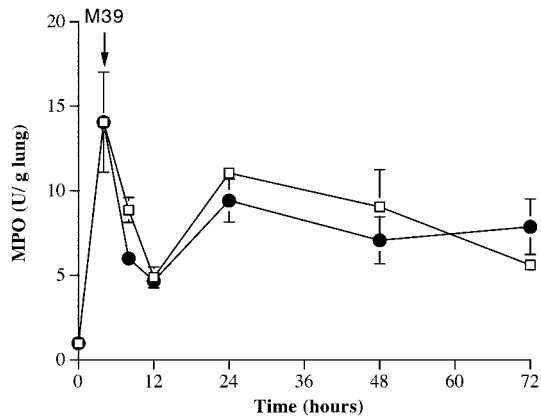


FIGURE 6. Poststimulation inhibition of p38 MAPK results in decreased accumulation of neutrophils in the airspaces, independent of the whole lung. Relative quantities of neutrophils present in the whole lungs, excluding the neutrophils accumulated in the airspaces, were determined by assaying MPO activity from the isolated lungs of animals depicted in Fig. 4 after BAL. Mean of MPO activity \pm SEM per gram of lung tissue from three to six animals is plotted over time. Mice exposed to LPS without inhibition of p38 MAPK (\square) did not have a significantly greater neutrophil content in the whole lung compared with LPS-exposed mice administered M39 poststimulation (\bullet) over a series of times ($p = 0.26$) by two-way ANOVA.

over a series of time points. LPS was dosed to elicit an exuberant neutrophil influx (8–24 h), followed by a secondary accumulation of mononuclear cells (primarily macrophages and monocytes), with near complete resolution by 72 h. Four hours after exposure to LPS, the compound M39 or the inert vehicle was administered (see *Materials and Methods*). Systemic p38 MAPK inhibition reduced neutrophil accumulation, with a maximal effect at 12 h (Fig. 4A). The cumulative effect of p38 MAPK inhibition over 72 h was a 32% reduction in neutrophils recovered from the airspaces. When total mononuclear cells were evaluated, poststimulation systemic p38 MAPK inhibition resulted in an increase of cells to the airways, which was maximal at 24 h (Fig. 4B). The cumulative effect of p38 MAPK inhibition over 72 h was a 17% increase in mononuclear cells recovered from the airspaces. Together, these data support the conclusion that poststimulation inhibition of p38 MAPK effectively reduces neutrophil accumulation, with enhancement of later recruitment of monocytes/macrophages.

Poststimulation inhibition of p38 MAPK in vivo does not block LPS-induced cytokine release in the airspaces or serum

BAL samples 0–72 h after administration of LPS were analyzed for TNF- α , MIP-2, KC, and MCP-1 in both the BAL and serum. TNF- α and MIP-2 were found predominantly in the BAL (Fig. 5, A and C), whereas MCP-1 was found predominantly in the serum (Fig. 5D). KC was found in significant quantities in both BAL and serum (Fig. 5B). As anticipated, all measured cytokines peaked within 4 h; thus, none were significantly reduced by systemic p38 MAPK inhibition 4 h after exposure to LPS. It is of interest that measured cytokines in the BAL and serum were generally equal or slightly lower in animals subjected to p38 MAPK inhibition, with the exception of MCP-1, which was consistently higher in the serum after p38 MAPK inhibition, although none of these changes were statistically significant.

Inhibition of p38 MAPK selectively blocks the accumulation of neutrophils into the airspaces

Decreased neutrophil accumulation in the airspaces in response to LPS after inhibition of p38 MAPK could possibly be due to de-

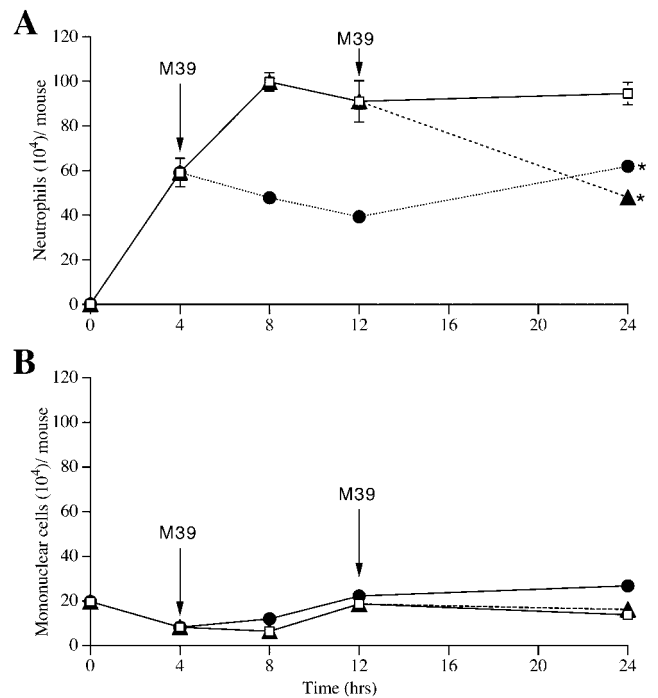


FIGURE 7. Effect of later poststimulation inhibition of p38 MAPK on leukocyte accumulation in the airspaces. *A*, Neutrophil accumulation in the murine airspaces after aerosolized LPS. Under identical conditions as depicted in Fig. 4, mice were exposed to LPS at time 0 and then administered M39 (\bullet) at 4 h or 12 h (\blacktriangle) after stimulation. Cell analysis of BAL was compared with untreated mice (\square) at 24 h (see *Materials and Methods*). Each point represents mean number of neutrophils \pm SEM from three to six animals. Inhibition of p38 MAPK at 12 h poststimulation resulted in significant reduction of neutrophil accumulation in the airways by 24 h ($p < 0.0001$), as did administration of M39 4 h poststimulation ($p = 0.002$) by Student's *t* test. *B*, Mononuclear cell accumulation in the murine airspaces after LPS. Mononuclear cells from BAL samples depicted in *A* were analyzed in mice administered M39 (\bullet) at 4 h or at 12 h after stimulation (\blacktriangle) and were compared with untreated mice (\square) at 24 h. Each point represents mean number of cells \pm SEM from three to six animals. Inhibition of p38 MAPK at 12 h poststimulation resulted in nonsignificant increase of mononuclear cell accumulation in the airways by 24 h ($p = 0.25$), as did administration of M39 4 h poststimulation ($p = 0.004$) by Student's *t* test.

creased retention of neutrophils in the pulmonary vasculature or lung interstitium or the loss of the ability of the cells to migrate into the alveoli. An MPO assay was used to quantify the total neutrophil burden in the pulmonary vasculature and interstitium. Whole lungs were excised after BAL from each animal depicted in Figs. 4 and 5, and LPS was administered (time 0) followed by systemic inhibition of p38 MAPK (time 4 h) by M39. Isolated lungs subjected to the MPO assay failed to demonstrate a significant difference in neutrophil content with or without the presence of p38 MAPK inhibition (Fig. 6), despite a significant reduction in neutrophil accumulation to the airspace (Fig. 4A). Together with the *in vitro* chemotaxis data (Fig. 3), these results support the conclusion that systemic inhibition of p38 MAPK results in a selective loss of migration of neutrophils into the airways.

Poststimulation inhibition of p38 MAPK in vivo results in decreased leukocyte accumulation in the airspaces: effects of later dosing

Data presented above support the conclusion that systemic inhibition of p38 MAPK selectively blocks ongoing neutrophil chemotaxis, independent of changes in inflammatory mediators. Thus, it

would be expected that p38 MAPK inhibition will reduce neutrophil recruitment at any point after exposure to LPS, as long as significant ongoing neutrophil migration into the airspaces is occurring. We tested whether systemic p38 MAPK inhibition at a later time point could achieve a significant reduction in subsequent neutrophil recovery from the airspaces. Under identical conditions, mice were administered M39 at 12 h after exposure to LPS, and leukocytes recovered at 24 h were compared with animals administered M39 at 4 h and with untreated animals. Neutrophils recovered at 24 h from animals with systemic p38 MAPK inhibition administered at 12 h were decreased 49% from untreated mice, compared with animals treated at 4 h, which decreased 35% (Fig. 7A). Recovery of mononuclear cells at 24 h was not changed by administration of M39 at 12 h, compared with untreated mice (Fig. 7B).

Discussion

In this report, we examined the capacity of poststimulation p38 MAPK inhibition to modify ongoing inflammatory events. In vitro studies of human neutrophils demonstrated that p38 MAPK activity can be blocked by poststimulation p38 MAPK inhibition. Release of TNF- α , MIP-1 β , and IL-8 can be significantly reduced, even after the onset of synthesis. Likewise, ongoing chemotaxis toward IL-8 (but not chemokinesis) is eliminated by introducing a p38 MAPK inhibitor. In vivo studies were conducted in a murine model using a single aerosolized dose of LPS to induce acute pulmonary inflammation characterized by a initial burst of cytokine release, followed by a transient influx of neutrophils and a secondary accumulation of mononuclear cells. The effect of systemic p38 MAPK inhibition initiated 4 h or 12 h after exposure to aerosolized LPS was to decrease neutrophil influx into the airspaces. Together, these data support the conclusion that under the conditions studied, "rescue" p38 MAPK inhibition can result in a selective modification of the inflammatory response.

By design, inhibition of cytokine release was avoided by adding M39 after the maximal release. This allowed a more selective analysis of p38 MAPK inhibition on in vivo neutrophil accumulation. Although effects of systemic p38 MAPK inhibition on pulmonary cell responses cannot be completely ruled out, our evidence supports the conclusion that decreased neutrophil accumulation occurred independently of changes in the inflammatory response of the lung. First, no significant changes in quantities of TNF- α , MIP-2, MCP-1, or KC were detected in BAL or serum (Fig. 5). In addition, no significant difference in total quantity of neutrophils in the lung was detected by MPO assay (Fig. 6). Finally, equivalent reduction in neutrophil accumulation was achieved when p38 MAPK inhibition occurred 4 h or 12 h after LPS exposure (Fig. 7). These results concur with in vitro assays of neutrophil chemotaxis to IL-8 (Fig. 3), which demonstrates that inhibition of p38 MAPK can disrupt ongoing chemotaxis independently of effects on cell motility. A recent study of KC-induced neutrophil chemotaxis through a murine muscle venule found that inhibition of p38 MAPK blocked chemotaxis independently of rolling or adhesion (34). In the presence of a p38 MAPK inhibitor, the neutrophils stayed largely within the venule or migrated a significantly shorter distance into the muscle. This observation supports our conclusion that systemic inhibition of p38 MAPK can selectively block neutrophil accumulation in the airspace by disrupting chemotaxis, but not reduce the total neutrophil burden in the lung.

Particularly intriguing is the failure of p38 MAPK inhibition to reduce synthesis of MCP-1 by neutrophils in vitro (Fig. 2B). Genomic analysis of LPS-stimulated neutrophils has demonstrated that MCP-1 is highly expressed (K. C. Malcolm, unpublished data), but that when MCP-1 is analyzed simultaneously with TNF- α , IL-8, and MIP-1 β in the neutrophil, the selective lack of

MCP-1 inhibition is striking (Fig. 2). Similar results were obtained with adherent neutrophils over a wide range of stimulation times and with macrophages (data not shown). The potential importance of these in vitro findings is observed in our model, where MCP-1 recovered in serum is consistently greater in the setting of p38 MAPK inhibition (Fig. 5D) and mononuclear cell recruitment to the airspaces is enhanced (Fig. 4B). Although the origin of MCP-1 recovered in the serum is not known, it is possible that increased quantities recovered in animals treated with M39 were released by neutrophils, in that a reduction of MCP-1 by p38 MAPK inhibition has been reported in endothelial cells (35). MCP-1 induces recruitment of monocytes and lymphocytes, which is generally viewed as a less injurious inflammatory response, associated with a "recovery phase" of inflammation. Administration of MCP-1 has been reported to be protective in a murine model of lethal bacterial infection (36). Thus, the lack of effect on MCP-1 release by neutrophils in the setting of p38 MAPK inhibition may enhance the potential usefulness of this intervention in the setting of Gram-negative bacterial infection or LPS-induced inflammation.

The selective blockade of neutrophil accumulation into the airspace is of uncertain clinical significance. It could be argued that, given the equivalent overall burden of neutrophils within the whole lung, p38 MAPK inhibition may not lead to a relevant reduction in pulmonary injury. The model of acute pulmonary inflammation used in this report was sufficiently mild that recovery occurred within 72 h, so a survival benefit could not be assessed. Studies are underway with more significant exposures to LPS to determine the physiologic benefits, if any, of reducing neutrophil accumulation in the airspaces independently of the whole lung burden.

Pretreatment of animals with p38 MAPK inhibitors has been shown to reduce inflammation in a number of models. Initial studies established the ability of p38 MAPK inhibitors to reduce neutrophil influx and cytokine release in models of peritonitis and arthritis in the absence of generalized immunosuppression (37–44). Early studies predominately used first generation p38 MAPK inhibitors such as the pyridinyl imadazoles SK&F86002 and SB203580 (39, 45–48). SB203580 has considerable inhibitory effects toward c-Raf and JNK2 α 1. Thus, high concentrations of this compound could potentially reduce signaling via the p42/44 (ERK) MAPK and JNK cascades (28). In comparison, M39 has an IC₅₀ for p38 MAPK that is 100-fold less than that of SB203580, and it has greater bioavailability and is significantly more selective (28).

Pulmonary inflammation can be reduced by administration of various p38 MAPK inhibitors before a proinflammatory stimulus or an allergic challenge. Inhibition of p38 MAPK decreases LPS-induced neutrophil influx, release of cytokines in the airspaces or serum, and expression of matrix metalloproteinase-9 in the airspaces (12, 49, 50). In two murine models of pancreatitis-induced lung injury, pretreatment with a p38 MAPK inhibitor reduced TNF- α release in the airways and reduced leukocyte accumulation, serum nitrites, and pulmonary edema (51, 52). In an ischemia and reperfusion model of pulmonary injury, a p38 MAPK inhibitor decreased serum cytokine release and lung damage, but improved oxygenation (53). Inhibition of p38 MAPK before OVA challenge in OVA-sensitized mice, rats, or guinea pigs decreased cytokine and inflammatory cell accumulation in the airways in an allergic airway model of inflammation (41, 46).

The effects of systemic p38 MAPK inhibition in the setting of ongoing inflammation have not been widely reported. In a guinea pig model of LPS-induced pulmonary inflammation, administering a p38 MAPK inhibitor 1 day after stimulation resulted in decreased neutrophil accumulation and IL-6 release in BAL at 48 h (49).

Therapeutic administration of p38 MAPK inhibitors after the initiation of focal ischemic stroke or collagen-induced arthritis is associated with an improved clinical course, but no analysis of inflammatory markers was performed (49, 54).

Several different mechanisms likely contribute to modification of ongoing inflammation by p38 MAPK inhibition. The p38 MAPK cascade may regulate a wide variety of stress responses, dependent on the cell type, various upstream regulations, and selective phosphorylation of various potential substrates (7). The regulation of synthetic responses by p38 MAPK can occur at the level of transcription and/or translation to varying degrees in a gene-specific manner. Immediate responses that are independent of protein synthesis, such as chemotaxis or superoxide anion release, are also mediated by p38 MAPK (15).

In many cells, p38 MAPK regulates transcription through phosphorylation of transcription factors such as ATF-2 (55), p53 (56), C/EBP-homologous protein (57), and muscle-specific transcription factor-2 (58). In particular, transcription of TNF- α has been linked to activation of p38 MAPK. We have reported that inhibition of p38 MAPK in neutrophils results in a 50% reduction of TNF- α mRNA 30 min after stimulation with LPS, but the effect is quite transient (59). Likewise, LPS-induced TNF- α transcription of alveolar macrophages is partially dependent on activation of p38 MAPK (60). Many other workers have found examples of p38 MAPK-regulated transcription in primary cells and cell lines, often in genes dependent to varying degrees on NF- κ B (58, 61–64).

Reduction of LPS-stimulated IL-1 and TNF- α production by p38 MAPK inhibitors can also occur at the translational level (10). Inhibition of p38 MAPK with SK&F86002 had little effect on TNF- α mRNA levels in THP-1 cells, but instead it was found to inhibit TNF- α mRNA translation by inducing a shift of TNF- α mRNA from polysomes (actively translated) to free mRNA (translationally inactive) (65). One possible mechanism by which p38 MAPK could regulate translation is through activation of MAPK-interacting kinase-1, which phosphorylates eukaryotic translation initiation factor eIF-4E (66) and is known to play a key role in the regulation of translation in mammalian cells. Several reports have demonstrated that p38 MAPK enhances mRNA stability for cytokines in various cells (67), which in some cases is mediated through activation of MAPK-activated protein-2 (MAPKAP-2) (68, 69). Based on these reports, it is likely that “rescue” p38 MAPK inhibition would be effective in reducing cytokine synthesis in a model of chronic inflammation that featured continuous release of cytokines.

Likely through the activation of other kinases, p38 MAPK can rapidly regulate many cellular responses independently of protein synthesis. Kinases such as MAPKAP kinases 2 and 3, p38-regulated/activated protein kinase, MAPK-interacting kinase-2, and mitogen- and stress-activated kinase-1 are activated by p38 MAPK under certain circumstances (66, 70–73). Neutrophils isolated from genetically modified mice lacking MAPKAP kinase 2 (MK2^{-/-}) were observed to lose directionality in movement toward fMLP, but they achieved a higher rate of migration (74). The phosphorylated form of MAPKAP kinase 2 was found to colocalize with F-actin in the leading front of the polarized neutrophils, primarily in the lamellipodia regions. Lymphocyte-specific protein-1, an F-actin cross-linking protein, has been identified as a major substrate for MAPKAP kinase 2 in the neutrophil (75). Thus, p38 MAPK could regulate neutrophil chemotaxis, in part by activating MAPKAP kinase 2, which in turn phosphorylates an F-actin cross-linking protein such as lymphocyte-specific protein-1. Inhibition of p38 MAPK was also reported to block the up-regulation of CXCR2 in monocytes (76).

Although the preponderance of in vivo and in vitro studies demonstrated anti-inflammatory effects after p38 MAPK inhibition, several reports have identified conditions in which p38 MAPK inhibitors enhance inflammatory responses. One group has found increased LPS-induced TNF- α release by the 4-4 murine macrophage cell line and isolated murine peritoneal macrophages treated with SB203580 (47). Mast cells demonstrate enhanced Ag-induced TNF- α release after p38 MAPK inhibition (77). Recovery of TNF- α by BAL in murine models of *Streptococcus pneumoniae* or *Mycobacterium tuberculosis* infection was increased after p38 MAPK inhibition (47). Although significant differences in methodology exist among these in vitro and in vivo studies, together with our results it seems clear that various cells use the p38 MAPK signaling cascade in different capacities. Thus, systemic inhibition of p38 MAPK can result in complex modification of the inflammatory response. Predicting in advance the effects of p38 MAPK inhibition in a particular model of inflammation will likely be difficult, because divergent responses to p38 MAPK inhibitors exist between cell types. Potential benefits of p38 MAPK inhibition will likely vary with the type and route of the stimulus and will require a comprehensive analysis of local and systemic markers of inflammation to fully appreciate the effect of the intervention.

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