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The Serine Protease Plasmin Triggers Expression of MCP-1 and CD40 in Human Primary Monocytes via Activation of p38 MAPK and Janus Kinase (JAK)/STAT Signaling Pathways*

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The mechanism of proinflammatory activation of human monocytes by plasmin is unknown. Here we demonstrate that in human primary monocytes, plasmin stimulates mitogen-activated protein kinase (MAPK) signaling via phosphorylation of MAPK kinase 3/6 (MKK3/6) and p38 MAPK that triggers subsequent DNA binding of transcription factor activator protein-1 (AP-1). The AP-1 complex contained phosphorylated c-Jun and ATF2, and its DNA binding activity was blocked by the p38 MAPK inhibitor SB203580. In addition, plasmin elicits Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling, as detected by phosphorylation of JAK1 tyrosine kinase and STAT1 and STAT3 proteins. Plasmin-induced DNA binding of STAT1 and STAT3 was blocked by SB203580 and AG490, inhibitors of p38 MAPK and JAK, respectively, but not by U0126, an inhibitor of MKK1/2. DNA binding of NF-κB remained unaffected by any of these inhibitors. The plasmin-induced signaling led to expression of monocyte chemoattractant protein-1 (MCP-1) and CD40, which required activation of both p38 MAPK and JAK/STAT signaling pathways. Additionally, signaling through both p38 MAPK and JAK is involved in the plasmin-mediated monocyte migration, whereas the formylmethionylleucylphenylalanine-induced chemotaxis remained unaffected. Taken together, our data demonstrate a novel function of the serine protease plasmin in a proinflammatory signaling network.

Most blood cells, including monocytes, bind plasmin and plasminogen with similar affinity. A number of membrane-associated macromolecules including annexin II, gangliosides, α-enolase, and TIP49a are able to bind plasmin and plasminogen, reflecting the heterogeneous nature of these binding sites (1–3). Specifically on blood cells, the functional consequences of membrane-bound plasmin have usually been regarded in terms of amplification of the fibrinolytic activity (1, 2). However, the serine protease plasmin might have functions beyond fibrinolysis. Thus, plasmin induces neutrophil aggregation and platelet degranulation, implying activity as a proinflammatory agonist (4, 5). Moreover, plasmin was found to be a potent and selective stimulus for human monocytes eliciting release of lipid mediators, such as chemotactic leukotriene B_4, as well as a chemotactic response equipotent to that of fMLP,[1] and proinflammatory cytokine and tissue factor expression (6–9). Monocyte stimulation with plasmin requires binding of plasmin via its lysine binding sites and an active catalytic center (6–9). The stimulatory effect is specific for plasmin because it cannot be mimicked by the zymogen plasminogen or other serine proteases such as α-chymotrypsin, neutrophil elastase, or cathepsin G (6, 9). The importance of plasmin for inflammatory reactions was further demonstrated in plasminogen-deficient mice, in which monocyte recruitment to sites of inflammation as well as the development of atherosclerosis was severely impaired (10, 11).

One of the major signaling pathways controlling expression of different proinflammatory genes is the MAPK pathway. The MAPK family consists of three subgroups, the extracellular signal-regulated kinases 1/2 (ERK1/2), c-Jun NH_2-terminal kinases (JNKs), and p38 MAPK; all of them require dual (Tyr/Thr) phosphorylation for activation (12). ERK kinases are typically activated by growth factor receptor activation of the Ras/Raf and ERK1/2 kinases signaling pathway. MAPKs regulate several transcription factors involved in cell proliferation and differentiation (12). A variety of stimuli elicit stimulation of JNK and p38 MAPK through activation of upstream MKK4/7 and MKK3/6, respectively (12). Activated JNK and p38 kinases translocate to the nucleus, where they activate transcription factors of the Jun family, which regulate expression of genes of proinflammatory molecules such as c-Fos, MCP-1, and tumor necrosis factor (TNF)-α (12).

JAKs have been reported to mediate signaling of some G protein-coupled receptors (GPCRs), supposedly including the chemokine receptors for stromal cell-derived factor-1α and MCP-1 (13). Likewise, thrombin-mediated activation of protease-activated receptor 1, another GPCR, leads also to phosphorylation of JAKs (14). Four members of the JAK family were identified so far, JAK1, JAK2, JAK3, and TYK2 (15). Following activation of chemokine receptors, JAK kinases

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[1] The abbreviations used are: fMLP, formylmethionylleucylphenylalanine; MAPK, mitogen-activated protein kinase; MKK, MAPK kinase; ERK1/2, extracellular signal-regulated kinase 1/2; JNK, c-Jun NH_2-terminal kinase; JAK, Janus kinase; AP-1, activator protein-1; ATF2, activating transcription factor-2; STAT, signal transducer and activator of transcription; NF-κB, nuclear factor κB; MCP-1, monocyte chemoattractant protein-1; GPCR, G protein-coupled receptor; LPS, endotoxic lipopolysaccharide; TNF, tumor necrosis factor; CREB, cAMP-response element-binding protein; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole; SB202474, 4-(ethyl)-2-(4-methoxyphenyl)-5-(4-pyridyl)1H-imidazole; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene; AG490, α-cyano-(3,4-dihydroxy)-*N*-benzylcinnamide; CTA, Committee on Thrombolytic Agents.

phosphorylate tyrosine residues in the cytoplasmic domain of the receptor, subsequently leading to a conformational change that creates a docking site for STATs (15). After phosphorylation by JAKs, STAT transcription factors form homo- or heterodimers and translocate into the nucleus, where they regulate gene expression of proteins of the acute phase such as MCP-1 and CD40 (15–17). MCP-1 is expressed by different cell types including LPS-stimulated human monocytes (18–20). It is chemotactic for monocytes as well as for activated T cells and natural killer cells (21, 22); its major role is therefore recruitment of immunocompetent cells to sites of inflammation.

Activation of monocytes by immunocompetent cells is dependent both on cytokine- and cell contact-mediated signals. CD40, a member of the tumor necrosis factor receptor superfamily, is expressed on many cells associated with inflammatory reactions including monocytes, endothelial cells, and vascular smooth muscle cells (23). Interaction of CD40 with its ligand CD154 (presented for example by activated T cells) triggers cell-mediated stimulation of monocytes, leading to expression of adhesion molecules, release of proinflammatory cytokines, and increased nitric oxide production (23). Dysregulation of the CD40/CD154 signaling system may have severe consequences for the immune response, and persistent stimulation may indeed contribute to chronic inflammatory diseases such as rheumatoid arthritis and atherosclerosis (23–26).

Plasmin-induced monocyte chemotaxis proceeds via pertussis toxin-sensitive G proteins and can be blocked by inhibitors of protein kinase C (9). Because G proteins and protein kinase C may induce MAPK activation (12, 27), the role of MAPKs in plasmin-triggered signaling and monocyte chemotaxis was investigated. Here we show that in addition to p38 MAPK, plasmin activates the JAK/STAT signaling pathway and that both signals cooperate in the induction of MCP-1 and CD40 gene expression as well as in the plasmin-mediated monocyte migration.

EXPERIMENTAL PROCEDURES

Materials—Human plasmin was from Fluka (Deisenhofen, Germany). Kinase-specific inhibitors and control compounds SB203580 (4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole), SB202474 (4-(ethyl)-2-(4-methoxyphenyl)-5-(4-pyridyl)1H-imidazole), U0126 (1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio-)butadiene), and AG490 (α-cyano-(3,4-dihydroxy)-*N*-benzylcinnamide) were purchased from Calbiochem. Antibodies against MKK3 and phosphorylated forms of MKK3/6, p38 MAPK, and anti-phosphotyrosine and against phosphorylated ATF2 and c-Jun (Ser-63) were from New England Biolabs (Beverly, MA). Antibodies against total and phosphorylated forms of Tyk2, JAK1, JAK2, JAK3, STAT1α, and STAT3 were from BioSource International (Nivelles, Belgium). Anti-CD40 monoclonal antibody was obtained from BD PharMingen. RPMI 1640, *Limulus* amebocyte lysate assay, fMLP, and endotoxic lipopolysaccharide (LPS; *Escherichia coli* serotype 055:B5) were purchased from Sigma. Percoll was from Amersham Biosciences. Oligo(dT)$_{25}$ magnetic beads were from Dynal (Oslo, Norway). All other chemicals were of analytical grade.

Monocyte Preparations—Human primary monocytes were isolated by Percoll gradient centrifugation as described previously (8, 9). Preparations with 94% or more CD14$^+$ cells were used. Contaminating cells were lymphocytes (2–6%). Flow cytometry of cells stained additionally with anti-CD41 antibodies did not show any platelets associated with monocytes.

Monocytes were generally incubated in lysine-free RPMI 1640 in the presence or absence of LPS-free human plasmin (0.043–0.86 Committee on Thrombolytic Agents (CTA) units/ml). All plasmin batches were routinely tested with the *Limulus* amebocyte lysate assay for LPS contamination. Occasionally monocytes were stimulated with either LPS or UVC irradiation (254 nm, 500 J/m^2 for 2 min) as control stimulus.

Immunoprecipitation and Western Blot Analysis—Freshly isolated human primary monocytes were cultured for 16 h in lysine- and serum-free media on hydrophobic PetriPerm membranes to reduce stress signaling by the isolation procedure. Monocytes (2×10^6 cells/assay) were then stimulated as indicated. Cells were lysed at 4 °C in 500 μl of buffer containing 10 mM Tris-HCl, pH 7.6, 100 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, 5 mM $MgCl_2$, 1% Triton X-100 (v/v), 8% glycerol (v/v), and mammalian protease and phosphatase inhibitor cocktails (Sigma), precleared by normal rabbit antiserum (Sigma) with 20 μl of protein-G agarose (Invitrogen) for 1 h at 4 °C, and then immunoprecipitated with 1 μg of anti-phosphotyrosine antibody and the same amount of protein-G agarose for 2 h at 4 °C. After three washes (10 min at 4 °C) with lysis buffer, proteins bound to the beads were solubilized in sample lysis buffer and resolved by 8% Tricine-SDS-PAGE and electroblotted onto nitrocellulose membranes (28). Alternatively, whole cell lysates were directly resolved by PAGE. Blots were immunostained with specific antisera, and antibody complexes were detected using the SuperSignal West Pico enhanced chemiluminescence detection kit (Pierce) with subsequent exposure to Hyperfilm ECL (Amersham Biosciences).

Electrophoretic Mobility Shift Assays—Monocytes were preincubated for 30 min with or without inhibitors before stimulation with plasmin (0.43 CTA units/ml) for the indicated periods of time. Nuclear extracts for electrophoretic mobility shift assay were prepared as described (8). AP-1 and NF-κB DNA-protein interactions were assayed by incubating 5 μg of nuclear extract with 50,000 cpm of ^{32}P-end labeled site-specific DNA probes in the presence of 2 μg of poly(dI-dC) (Amersham Biosciences) in 15 μl of binding buffer (8). In supershift experiments, nuclear extracts were incubated with corresponding antibodies (2 μg, Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at 4 °C (8). STAT activation in monocytes stimulated with plasmin for 25 min was assayed with sis-inducible element (SIE)-oligonucleotide from the *c-fos* promoter according to Kube *et al.* (29). Antibodies against STAT1 (clone E-23) and STAT3 (clone H-190) (Santa Cruz Biotechnology) were added 10 min after addition of the radiolabeled oligonucleotide, and incubation proceeded for another 20 min at 24 °C.

Reverse Transcription-Polymerase Chain Reaction Analysis—mRNA isolated from monocytes (0.5×10^6 cells/assay) with Oligo(dT)$_{25}$ magnetic beads was used for cDNA synthesis using SuperScript II reverse transcriptase (Invitrogen). cDNA was subsequently amplified by PCR using primers specific for human MCP-1 and CD40 (30, 31). Conditions were such that the PCR reactions did not reach the saturation phase. Electrophoresis in 2% TBE-agarose gels showed amplification products of the appropriate size. Control experiments without reverse transcriptase showed no DNA contamination. HLA(B) served as internal standard for normalization of PCR reactions (8). The identity of the PCR products was confirmed by direct sequencing (ABI Prism 310; Applied Biosystems, Foster City, CA).

Expression of MCP-1 and CD40—MCP-1 was assayed in cell-free supernatants using enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN). For flow cytometric analysis of CD40 expression, monocytes plated on hydrophobic PetriPerm membranes (In Vitro Systems, Osterode, Germany) were stimulated with plasmin (0.43 CTA units/ml) or LPS (1μg/ml) for 4 or 8 h. Cells were detached by scraping, stained with monoclonal anti-CD40 or control IgG mouse antibodies and anti-CD-14 monoclonal antibodies (BD PharMingen), and analyzed by FACScan (BD PharMingen).

Migration Assays—Cell migration was analyzed in triplicate using tissue culture-treated 24-well Transwell plates (Costar, Cambridge, MA) with polycarbonate membranes of pore size of 5 μm (9). Monocytes were suspended in Hank's balanced salt solution containing 0.4% (w/v) bovine serum albumin at a concentration of 2×10^6 cells/ml, and 100 μl was added to the upper compartment of each well. Chemoattractants or solvents were added to the lower compartments, and cells were allowed to migrate at 37 °C for 90 min. Polycarbonate membranes were fixed, and then stained with hematoxylin (Accustain; Sigma). Migrated cells were counted in seven high power oil-immersion fields (×100) (9). fMLP (10 nM) was used as standard chemoattractant. Pharmacological inhibitors were added to both upper and lower compartments 30 min before the addition of chemoattractants.

Statistical Analysis—Values shown represent mean ± S.E. where applicable. Statistical significance was calculated with the Newman-Keuls test. Differences were considered significant for $p < 0.05$.

RESULTS

Plasmin Activates the p38 MAPK Signaling Pathway—Based on previous studies showing plasmin-induced and G protein-mediated proinflammatory stimulation of human primary monocytes, we now investigated whether plasmin might elicit activation of MAPKs. Stimulation of human monocytes with 0.43 CTA units/ml plasmin led to time-dependent stimu-

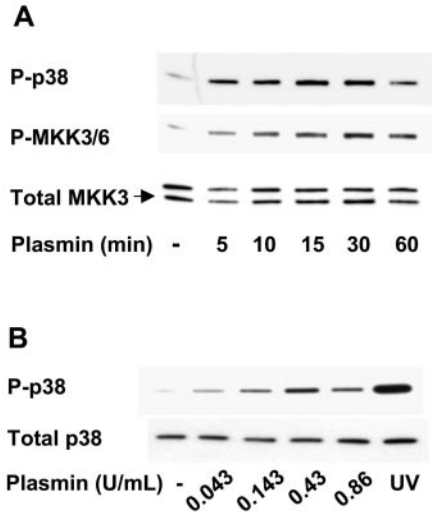

FIG. 1. **Plasmin activates the p38 MAPK signaling pathway in human primary monocytes.** *A*, time-dependent activation of MKK3/6 and p38 MAPK. Monocytes (2×10^6/assay) were incubated in serum-free media in the presence of plasmin (0.43 CTA units/ml) for the indicated time. After washing, cells were lysed, and 40-μg protein aliquots were subjected to Western blot analysis using either phospho-specific antibody against the activated form of p38 MAPK or MKK3/6. Blots were stripped and developed with anti-total-MKK3 antibodies as a control of equal protein loading. Specific signals were detected by ECL and autoradiography. *B*, concentration-dependent activation of p38 MAPK by plasmin. Monocytes were incubated with plasmin (0.043–0.86 CTA units/ml) for 30 min or exposed to UVC (254 nm, 500 J/m^2) for 2 min and incubated for an additional 30 min. Blots were stripped and developed with anti-p38 antibodies as a control of equal protein loading. Results of one of three independent experiments are shown in each case.

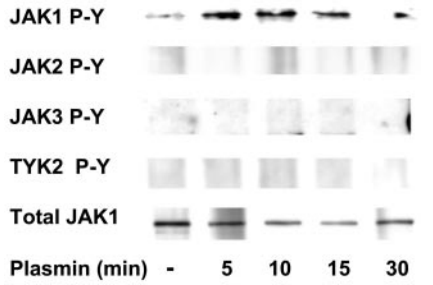

FIG. 2. **Plasmin induces phosphorylation of JAK1 protein kinase in human monocytes.** Monocytes (2×10^6/assay) were incubated in serum-free media in the presence of plasmin (0.43 CTA units/ml) for the indicated time. Whole cell lysates were prepared and immunoprecipitated with anti-phosphotyrosine (*P-Y*) monoclonal antibodies. After blotting, phosphorylated proteins were detected using specific rabbit polyclonal antibodies. The amount of total JAK1 protein in lysates before immunoprecipitation is shown on the *lower panel*. Results of one of three independent experiments are shown.

lation of p38 MAPK (Fig. 1*A*). A detectable increase in phosphorylation of p38 MAPK occurred as early as 5 min after plasmin exposure with a maximum at 30 min and a decrease at 60 min.

In addition, plasmin was found to stimulate time-dependent activation of MKK3/6, kinases upstream from p38 MAPK, as shown by immunoblot of phosphorylated MKK3/6 (Fig. 1*A*). Both MKK3/6 and p38 MAPK were phosphorylated as early as 5 min after plasmin stimulation, showing closely correlating activation kinetics, although activation of p38 MAPK occurred somewhat earlier than that of MKK3/6, suggesting some additional mechanism of p38 phosphorylation. Plasmin-induced phosphorylation of p38 MAPK followed a classical bell-shaped concentration-response curve with the highest response at 0.43 CTA units/ml plasmin, a concentration that had previously triggered maximum responses in terms of chemotaxis and cytokine and lipid mediator release (6–9). At this concentration, p38 phosphorylation level was somewhat lower than that seen after UVC irradiation (254 nm, 500 J/m^2) used as positive control (Fig. 1*B*).

Activation of p38 MAPK is often associated with stimulation of ERK1/2 and JNK MAPK signaling pathways (12, 32–34). However, stimulation of these MAPKs could not be detected by Western blotting in plasmin-stimulated human monocytes (data not shown).

Plasmin Induces Phosphorylation of JAK1 Tyrosine Kinase—GPCRs do not possess intrinsic tyrosine kinase activity for downstream signaling. In light of the pertussis toxin sensitivity of plasmin-induced signaling (6, 9) and the recent demonstration that JAK kinases can mediate signaling from different GPCRs (13, 27), tyrosine phosphorylation of different JAK kinases was studied in plasmin-stimulated monocytes. Whole cell lysates were immunoprecipitated with anti-phosphotyrosine antibodies and subjected to Western blot analysis with antibodies against JAK1, JAK2, JAK3, and TYK2. Exposure of monocytes to 0.43 CTA units/ml plasmin resulted in strong stimulation of tyrosine phosphorylation of JAK1 (Fig. 2). The increase in JAK1 phosphorylation was observed already 5 min after stimulation and rapidly decreased so that after 30 min, no phosphorylated JAK1 protein could be detected any more. Plasmin stimulation did not induce phosphorylation of any of the other members of the JAK family, JAK2, JAK3, or TYK2 (Fig. 2).

Plasmin Elicits Phosphorylation of STAT1 and STAT3—To address the question of whether plasmin-mediated activation of JAK1 kinase leads to tyrosine phosphorylation of STAT proteins, membranes used for detection of activated JAKs were stripped and reprobed with anti-STAT1 or -STAT3 antibodies. As shown in Fig. 3*A*, after stimulation with 0.43 CTA units/ml plasmin, both STAT1 and STAT3 were phosphorylated at tyrosine residues (known to be Tyr-701 or Tyr-705 for STAT1 and STAT3, respectively). Maximum levels of tyrosine phosphorylation occurred between 5 and 10 min after stimulation; although STAT1 activation had vanished already after 15 min, the phosphorylated form of STAT3 still remained detectable at this time point.

Dual phosphorylation of STAT proteins at both tyrosine and serine 727 residues is generally required for maximum transcriptional activity (35). Therefore, besides tyrosine phosphorylation, changes in serine phosphorylation were additionally investigated using phospho-specific antibodies recognizing STAT1 and STAT3 phosphorylated at serine 727. Indeed, Western blot analysis of lysed monocytes showed that 0.43 CTA units/ml plasmin induced rapid and transient Ser-727 phosphorylation of STAT1 and STAT3 within 5 min after stimulation (Fig. 3, *B* and *C*). Ser-727 phosphorylations of both STAT proteins (Fig. 3, *B* and *C*) corresponded favorably with the activation kinetics of p38 MAPK (Fig. 1*A*) that might act as an upstream kinase phosphorylating STAT proteins (35–37). The significance of both JAK and p38 MAPK activity for the phosphorylation of STAT1 and STAT3 was confirmed by experiments demonstrating that phosphorylation was inhibited by the respective inhibitors AG490 (50 μM) and SB203580 (1 μM) but not by U0126 (1 μM), an inhibitor of MKK1/2 (Fig. 4). U0126 (1 μM) was, however, effectively inhibiting the LPS (100 ng/ml)-induced TNF-α generation (data not shown), which is MKK1/2-ERK1/2-dependent (38), confirming inhibitory efficacy at the concentration used. Thus, via activation of a so far unidentified receptor, plasmin stimulates p38 MAPK and JAK/STAT signaling pathways, implying activation of further cellular responses by these signaling cascades.

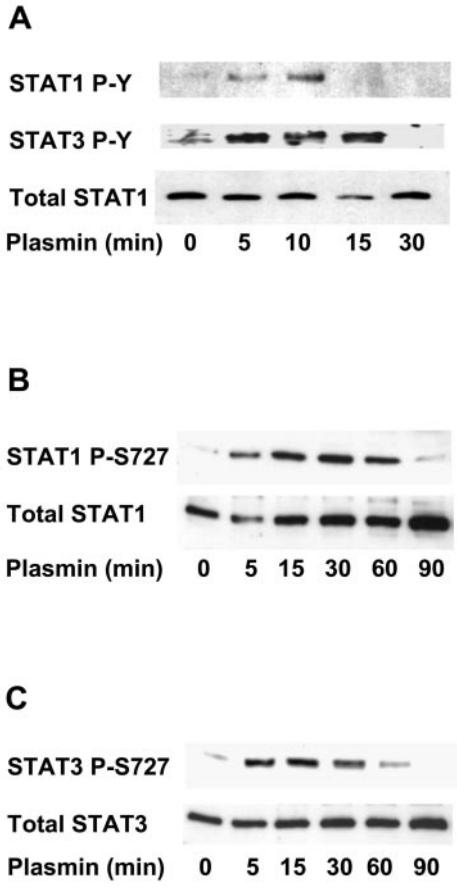

FIG. 3. **Plasmin elicits phosphorylation of STAT1 and STAT3 at both tyrosine and serine 727 residues.** Human monocytes (2×10^6/assay) were incubated in the presence of plasmin (0.43 CTA units/ml) for the indicated time. *A*, tyrosine phosphorylation. Whole cell lysates were prepared and immunoprecipitated with anti-phosphotyrosine (*P-Y*) antibodies. Blots stained with anti-JAK antibodies were stripped and reprobed with anti-STAT1 and -STAT3 antibodies. *B*, Western blot analysis of whole cell lysates with phospho-specific antibodies against STAT1 P-S727. *C*, Western blot analysis of whole cell lysates with phospho-specific antibodies against STAT3 P-S727. For the control of loading, blots were stripped and reprobed with modification-independent antibodies (total). Results of one of at least three independent experiments are shown.

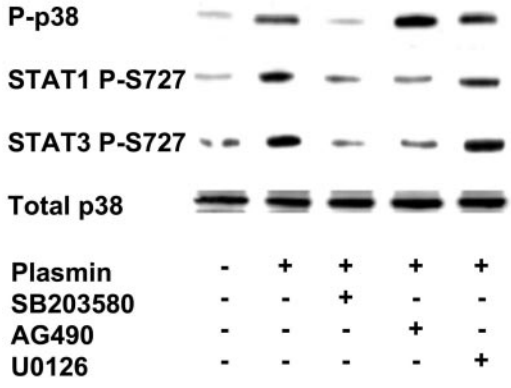

FIG. 4. **Plasmin-induced phosphorylation of STAT1 and STAT3 depends on p38 MAPK and JAK activity.** Human monocytes (2×10^6/assay) were preincubated in the presence or absence of the p38 MAPK inhibitor SB203580 (1 μM), the JAK inhibitor AG490 (50 μM), or the MKK1/2 inhibitor U0126 (1 μM) for 30 min; subsequently, cells were stimulated with 0.43 CTA units/ml plasmin for 30 min. Whole cell lysates were prepared, and Western blot analysis was performed with antibodies against phosphorylated p38 MAPK, STAT1 P-S727, and STAT3 P-S727. For the control of equal protein loading, blots were stripped and reprobed with modification-independent anti-p38 antibodies. Results of one of three independent experiments are shown.

Plasmin-induced Activation of JAK1 and p38 Triggers DNA Binding of STAT1, STAT3, and AP-1 Transcription Factors—Based on the plasmin-induced phosphorylation of STAT proteins, DNA binding activity of STATs was investigated by electrophoretic mobility shift assay. Although no DNA binding activity recognizing the sis-inducible element of the *c-fos* gene, a known response element for STAT1 and STAT3, was detected in nuclear extracts from untreated monocytes, formation of DNA-protein complexes was observed in extracts from plasmin (0.43 CTA units/ml)-stimulated monocytes (Fig. 5*A*). Preincubation of nuclear extracts with specific antibodies revealed that the protein-DNA complex with lower mobility contained STAT3, whereas the complex with higher mobility contained STAT1. The specificity of STAT binding was confirmed in competition experiments; a 25-fold molar excess of unlabeled STAT, but not of AP-2 consensus oligonucleotides, abolished binding of the nuclear extracts to the labeled STAT binding site sequence. Preincubation of monocytes for 30 min with either 1 μM SB203580 or 50 μM AG490 as inhibitors of p38 MAPK and JAK, respectively, totally abolished the DNA binding activity, indicating that the dual phosphorylation of STATs elicited by p38 and JAK1 is essential for the plasmin-induced STAT activation. U0126 (1 μM), the inhibitor of the MKK1/2-ERK1/2 pathway, had no effect on STAT activation (Fig. 5*A*).

The p38 MAPK is believed to play an important role in AP-1-dependent gene expression through phosphorylation of activating transcription factor 2 (ATF2), which may form heterodimers with phosphorylated c-Jun (12, 39). Consistent with our previous findings (8), stimulation of monocytes with 0.43 CTA units/ml plasmin for 1 h led to binding of nuclear extracts to AP-1 response elements (Fig. 5*B*). Treatment of the extracts with antibodies directed against the phosphorylated forms of ATF2 or c-Jun abolished the DNA retardation, indicating that the DNA-binding complex was indeed composed of phosphorylated ATF2 and c-Jun. This effect was specific because phospho-specific antibodies directed against ERK1/2 had no significant effect on AP-1-DNA binding (Fig. 5*B*). Moreover, binding of nuclear extracts to the labeled AP-1 binding site sequence was abolished by a 100-fold molar excess of unlabeled AP-1 but not by AP-2 consensus oligonucleotides. The p38 inhibitor SB203580 (1 μM) totally ablated the AP-1 binding activity, whereas the JAK inhibitor AG490 (50 μM) and the MKK1/2 inhibitor U0126 (1 μM) remained ineffective (Fig. 5*B*). Thus, p38-dependent phosphorylation of ATF2 is essential for the plasmin-mediated AP-1 activation.

Because previous findings had shown plasmin-induced nuclear translocation of NF-κB (8), we investigated whether NF-κB activation is affected by p38 or JAK/STAT activation. Stimulation of monocytes with plasmin (0.43 CTA units/ml) for 1 h induced an NF-κB-dependent DNA binding of nuclear extracts that could be antagonized by a 100-fold molar excess of unlabeled NF-κB consensus oligonucleotides but not by AP-2 oligonucleotides (Fig. 5*C*). Preincubation of monocytes with either the p38, JAK, or MKK1/2 inhibitor did not affect binding of the nuclear extracts to the NF-κB binding site, indicating that p38, JAK, and ERK kinases are dispensable for NF-κB nuclear translocation and DNA binding activity (Fig. 5*C*).

Plasmin Stimulates Expression of MCP-1 and CD40—As monocytes play a key role in the orchestration of inflammatory reactions, it was tested whether plasmin triggers expression of proinflammatory genes that may be regulated by MAPK and/or JAK/STAT signaling pathways. Reverse transcription-PCR revealed increased expression of both MCP-1 and CD40 genes in monocytes stimulated with 0.43 CTA units/ml plasmin (Fig.

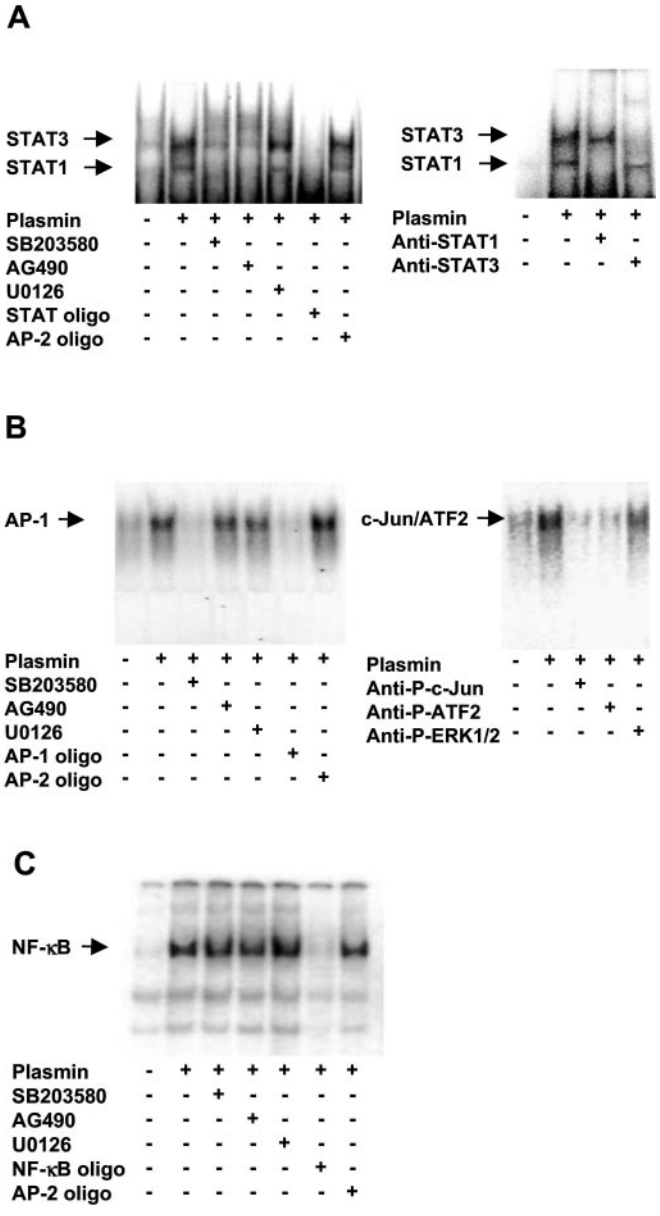

FIG. 5. **DNA binding of STAT1/STAT3, AP-1, and NF-κB in plasmin-stimulated monocytes.** Human monocytes were preincubated in the presence or absence of the p38 MAPK inhibitor SB 203580 (1 μM), the JAK inhibitor AG490 (50 μM), or the MKK1/2 inhibitor U0126 (1 μM) for 30 min. After stimulation, cells were lysed, and nuclei were isolated. Nuclear extracts (5 μg) were subjected to electrophoretic mobility shift assay with a ^{32}P-labeled DNA probe containing the appropriate binding sites. *A*, STAT1/STAT3. Monocytes were stimulated with plasmin 0.43 CTA units/ml for 25 min. For competition studies, nuclear extracts were incubated for 30 min with unlabeled STAT or AP-2 specific oligonucleotides (*oligo*; 25-fold excess); for supershift experiments, extracts were incubated with anti-STAT1 or anti-STAT3 antibodies. *B*, AP-1. Monocytes were stimulated with plasmin 0.43 CTA units/ml for 60 min. For competition studies, nuclear extracts were incubated for 30 min with unlabeled AP-1 or AP-2 specific oligonucleotides (100-fold excess); for supershift experiments, extracts were incubated with anti-phospho c-Jun or anti-phospho ATF2 antibodies. Anti-phospho ERK1/2 antibodies served as control for the phospho-specific antibodies. *C*, NF-κB. Monocytes were stimulated with plasmin 0.43 CTA units/ml for 60 min. For competition experiments, nuclear extracts were incubated for 30 min with unlabeled NF-κB or AP-2 oligonucleotides (100-fold excess). Results of one of three experiments are shown.

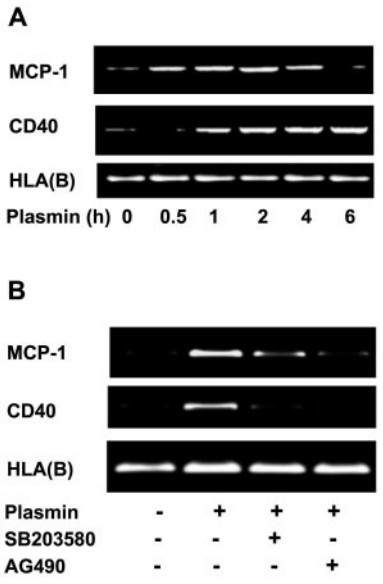

FIG. 6. **Plasmin stimulates expression of MCP-1 and CD40 mRNA in human monocytes.** Monocytes (0.5×10^6/assay) were incubated in the presence or absence of 0.43 CTA units/ml plasmin. mRNA was extracted at the indicated times and subjected to reverse transcription-PCR. HLA(B) transcripts were used for normalization. *A*, time-dependent mRNA expression. *B*, effects of the inhibitors of p38 MAPK, SB203580 (1 μM), and JAK kinase, AG490 (50 μM). Monocytes were preincubated for 30 min in the presence of the indicated inhibitors and exposed to plasmin for additional 4 h before reverse transcription-PCR was performed. Results of one of three experiments are shown.

6*A*). Levels of MCP-1 mRNA were increased as early as 30 min after plasmin stimulation with maximum levels at 2 h after stimulation. After 6 h, MCP-1 transcripts returned to baseline levels again. By contrast, CD40 gene expression responded more slowly to plasmin; transcripts started to rise after 1 h and continued to increase up to 6 h after plasmin stimulation (Fig. 6*A*). Cells incubated for 6 h in the absence of plasmin did not show any significant induction of MCP-1 or CD40 (data not shown).

To characterize the relevance of p38 MAPK and/or JAK/STAT signaling pathways for the regulation of MCP-1 and CD40 gene expression, monocytes were pretreated with 1 μM SB203580 or 50 μM AG490 for 30 min before stimulation with 0.43 CTA units/ml plasmin for 4 h. As shown in Fig. 6*B*, pharmacological inhibition of either p38 MAPK or JAKs resulted in significant reduction of MCP-1 and CD40 transcript levels. It should be noted that besides JAK2, AG490 is also effectively inhibiting JAK1 (40). These data suggest that gene expressions of MCP-1 and CD40 is similarly regulated by JAK1- and p38 MAPK-dependent signaling, although with different efficacy.

To verify whether plasmin is truly able to induce an increase in MCP-1 and CD40 at the protein level, we have assayed MCP-1 protein levels in the media and CD40 cell surface expression by FACS analysis. Plasmin triggered concentration-dependent release of MCP-1 from monocytes (Fig. 7*A*). Maximum MCP-1 release (6.2 ± 0.8 ng/10^6 cells) induced by 0.43 CTA units/ml plasmin was somewhat lower than that released by LPS (1 μg/ml)-stimulated monocytes (9.7 ± 1.3 ng/10^6 cells). Flow cytometric analysis of monocytes stimulated with 0.43 CTA units/ml plasmin showed a slight increase of surface CD40 antigen after 4 h of stimulation, which was more prominent after 8 h of stimulation (Fig. 7*B*). CD40 expression induced by 0.43 CTA units/ml plasmin was almost identical to that induced by 1 μg/ml LPS at both time points. These data indicate that in human monocytes, plasmin is able to induce MCP-1 release as well as CD40 antigen expression, thereby promoting recruitment and intercellular communication of immunocompetent cells.

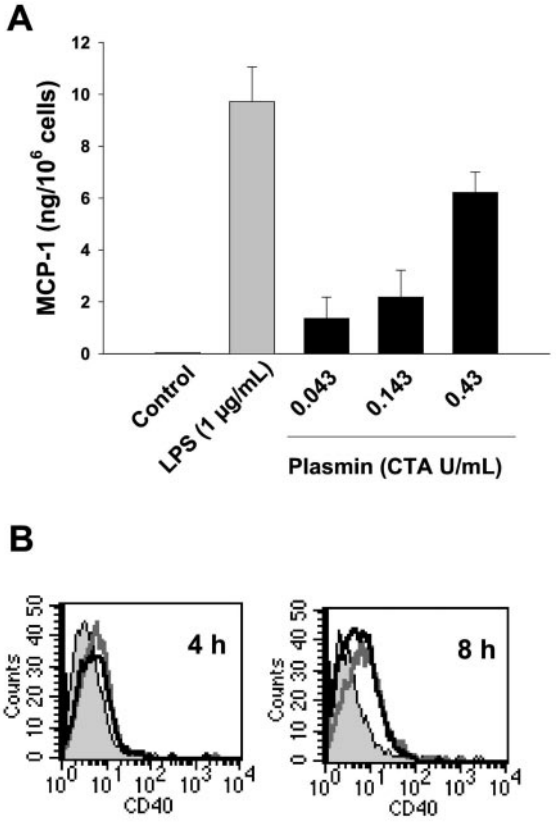

FIG. 7. **Induction of MCP-1 and CD40 expression in plasmin-stimulated monocytes.** *A*, concentration-dependent stimulation of MCP-1 release from human monocytes. Cells were incubated in the presence of the indicated plasmin concentrations or LPS for 20 h before MCP-1 release was analyzed in the media by enzyme-linked immunosorbent assay. Results are the mean ± S.E. of three independent experiments. *B*, flow cytometric analysis of CD40 protein expression on the surface of human monocytes incubated with plasmin (0.43 CTA units/ml; *black*) or LPS (1 µg/ml; *gray*) for the indicated time. The *gray-filled* area represents the amount of CD40 in unstimulated controls. Results of one of three independent experiments are shown.

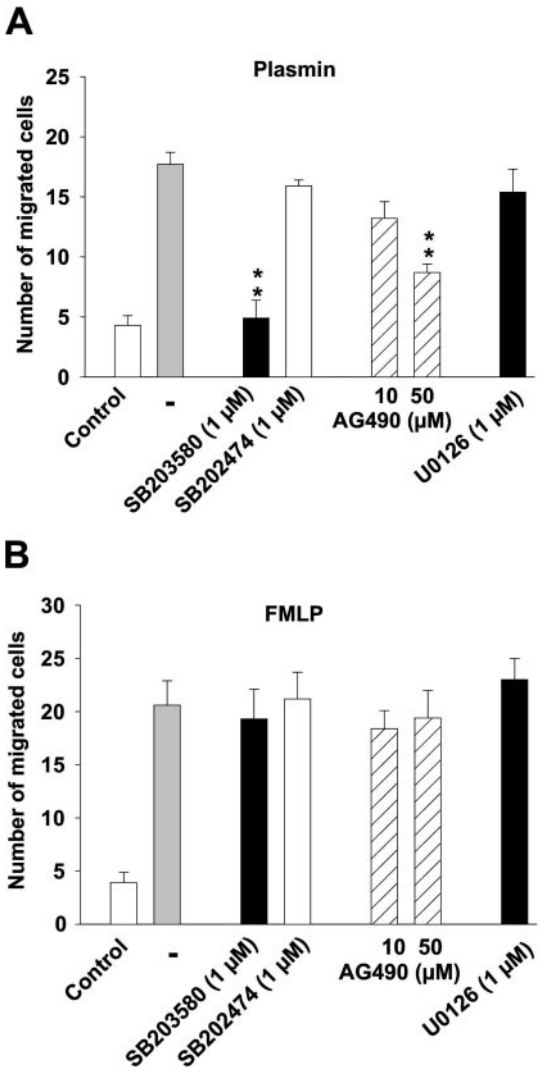

FIG. 8. **Plasmin-mediated monocyte chemotaxis requires activation of p38 MAPK and JAK.** Human monocytes were preincubated for 30 min with the indicated compounds, 1 µM SB203580, 1 µM SB202474, 1 µM U0126 , or 10 or 50 µM AG490 and allowed to migrate for another 90 min across polycarbonate membranes (pore size of 5 µm) toward the chemoattractants plasmin 0.43 CTA units/ml (*A*) or fMLP 10 nM (*B*). Seven high power fields were counted. Results are the mean ± S.E. of four independent experiments, each performed in triplicate. **, $p < 0.01$ as compared with plasmin controls.

p38 MAPK and JAK/STAT Signaling Pathways Are Involved in Plasmin-induced Chemotaxis of Human Monocytes—Plasmin acts as a potent chemoattractant for human primary monocytes (9). It was therefore investigated whether p38 MAPK and JAK kinases may play a role in the chemotactic signaling of monocytes. Thus, monocyte locomotion was studied in the presence of kinase inhibitors. Monocyte migration toward 0.43 CTA units/ml plasmin was not affected by the MKK1/2 inhibitor U0126 (1 µM) (Fig. 8*A*). By contrast, pretreatment of monocytes with the JAK inhibitor AG490 at 10 and 50 µM resulted in a concentration-dependent inhibition of the plasmin (0.43 CTA units/ml)-induced locomotion by 33.6 ± 10.4% and 67.2 ± 5.2%, respectively. The most profound inhibition was observed with the p38 MAPK inhibitor SB203580 (1 µM) that virtually blocked plasmin-mediated monocyte migration by 95.5 ± 3.7%. By contrast, pretreatment with the structurally related control compound SB202474 (1 µM) did not show any significant effect (Fig. 8*A*). Interestingly, when monocyte migration was elicited by 10 nM fMLP as standard chemoattractant, none of the kinase inhibitors had any significant effect on the chemotactic response (Fig. 8*B*). It should be noted that the protein kinase inhibitors at the concentration used had no effect on cell viability (data not shown); this is also proven by the uncompromised reactivity of monocytes to the standard chemoattractant fMLP.

DISCUSSION

In the present study, we have shown that in human primary monocytes, plasmin activates MKK3/6 and p38 MAPK as well as JAK/STAT signaling pathways, which subsequently lead to MCP-1 and CD40 expression. Thus, the serine protease plasmin is able to stimulate multiple signaling pathways, a critical prerequisite for effective induction of gene expression.

Plasmin-induced effects in monocytes such as chemotaxis and proinflammatory mediator release are highly sensitive to pertussis toxin (6, 7), implying participation of G proteins such as $G\alpha_i/G\alpha_0$ in the plasmin-mediated signal transduction. Therefore, it is conceivable that plasmin conveys cell activation through a yet unidentified receptor belonging to the family of GPCR. Only recently it has been recognized that GPCR may signal through novel mechanisms including the MAPK signaling network, which might link GPCR to the nucleus (27, 41). Consistent with these findings, it has been reported that MAPKs including p38 can be activated by stimulation of several G proteins including $G\alpha_i$ (27, 42, 43). Our data showing

plasmin-dependent phosphorylation of p38 MAPK and of upstream kinase MKK3/6 clearly demonstrate that plasmin triggers such a signaling in human monocytes.

Phosphorylation and thereby activation of MAPK including p38 is tightly regulated at the level of the upstream MKK kinases as well as at the level of phosphatase activity (12, 44). We observed a slight lag of MKK3/6 phosphorylation behind that of p38 MAPK, suggesting additional mechanisms contributing to the initial activation of p38 MAPK. c-Abl tyrosine kinase, known to activate the p38 MAPK pathway (45–47), is an unlikely candidate since it seems to act via MKK6 stimulation (48), which would have been recognized by the phospho-specific antibodies used. Alternatively, rapid inhibition of protein phosphatase activity can also contribute to p38 MAPK activation in the absence of MKK3/6 activation (44, 49). Indeed, inhibition of protein tyrosine phosphatase activity may occur in a cAMP-dependent protein kinase-dependent manner (50); this is intriguing because cAMP-dependent protein kinase plays a critical role in the cellular response to chemoattractants (51) such as plasmin (9). However, the precise nature of this early phase of p38 MAPK activation in human monocytes remains to be determined.

Activity of p38 MAPK is crucial for the induction of numerous genes including MCP-1 and CD40 (12, 52, 53). Indeed, activated p38 MAPK can phosphorylate various transcription factors including cAMP-response element-binding protein (CREB) and ATF2, which are known to bind to the positive regulatory element in the promoter region of the c-*fos* gene and activate its transcription. Besides, ATF2 and c-Fos can heterodimerize with c-Jun, forming AP-1 transcription factor complexes that bind to AP-1 binding sites, which activate transcription of numerous proinflammatory genes (12, 39, 54). In line with such a signaling cascade and complementing our previous findings of nuclear translocation of AP-1 in plasmin-stimulated monocytes (8), both phosphorylated ATF2 and c-Jun were identified as components of the AP-1 complex after plasmin stimulation.

The AP-1 composition varies in the cell as a function of time and stimulus and the functional consequences of AP-1 family proteins depend on the cell type in which they are expressed (55). Generally AP-1 dimers may be regarded as constituents of the transcriptional effector system of the ERK, JNK and p38 MAPK (55, 56). However, because AP-1 proteins may modulate gene transcription also independent of preceding MAPK activation, the significance of MAPK activity was investigated using kinase inhibitors. Our results demonstrate that DNA binding activity of phosphorylated ATF2-c-Jun complexes is clearly dependent on p38 MAPK activity as it was blocked by the p38 inhibitor SB203580 (12) but not by U0126 and AG490, inhibitors of MKK1/2 and JAK (38, 40), respectively. From the four known subtypes of p38 MAPK, monocytes harbor primarily p38α and only low levels of the p38δ isoform (57). Of those isoforms, it is only p38α that by specific interaction with its ATP binding pocket is sensitive to the pyridinyl imidazole inhibitor SB203580 (12, 57), suggesting that this isoform, which is preferentially activated by MKK3, is important for the plasmin-induced signaling in monocytes.

Transcriptional activation of c-Jun depends on its phosphorylation at serine 63 and 73 residues, thereby recruiting the coactivator protein CBP (CREB-binding protein) (55). Taking into account that c-Jun is a primary substrate of JNK (12, 39, 55), phosphorylation of c-Jun in the absence of detectable JNK activity is somewhat surprising. However, evidence has been presented that phosphorylation of the c-Jun serine residues can also be regulated by other kinases (55). Thus, in THP-1 macrophages as well as in murine macrophages, p38 MAPK activity seems to phosphorylate c-Jun directly; in addition and perhaps more important for the AP-1-driven transcription, it also modulates activation of the TATA-binding protein necessary for transcriptional activation (56, 58). Moreover, inositol 1,3,4-trisphosphate 5/6-kinase and the COP9 complex may also phosphorylate transcription factors c-Jun and ATF2, facilitating cross-talk with inositol phosphate signaling (59).

Signaling activity of many GPCR also depends on tyrosine phosphorylation, and since they do not possess internal phosphorylation activity, they associate with some of the intracellular non-receptor tyrosine kinase such as JAKs (27, 41, 60). JAK-dependent STAT1 and STAT3 activation occurs in G protein-mediated signaling of thrombin in vascular smooth muscle cells (14). Likewise, sublytic C5b-9 complement complexes trigger pertussis toxin-sensitive and therefore G protein-dependent tyrosine phosphorylation and activation of both JAK1 and STAT3 in human aortic endothelial cells (61). In monocytes, plasmin similarly induces rapid phosphorylation of JAK1, which subsequently phosphorylates tyrosine residues of STAT1 and STAT3 proteins (tyrosine 701 and 705 for STAT1 and STAT3, respectively). Surprisingly, JAK2, JAK3, and TYK2, which are often coactivated with JAK1, remained unaffected in plasmin-stimulated monocytes. We further identified plasmin-dependent dual phosphorylation of STAT1 and STAT3, *i.e.* additional phosphorylation at serine 727 residues, which, despite a few exceptions, is generally required for full transcriptional activity of STAT proteins (35). The kinetics of serine phosphorylation corresponded well to the activation of p38 MAPK, suggesting that STAT proteins might serve as a substrate for p38 MAPK in plasmin-stimulated monocytes as it has been observed in LPS or TNF-α-stimulated macrophages (35, 62). The plasmin-mediated serine phosphorylation was indeed critically dependent on p38 MAPK and to a lesser extent on JAK activity as it was blocked by SB203580 and inhibited by the tyrphostin AG490, inhibitors of p38 MAPK and JAK activity, respectively, but not by the MKK1/2 inhibitor U0126 (12, 35, 40), a constellation implying a signaling network. Indeed, p38 MAPK is able to phosphorylate Ser-727 of STAT1 and STAT3 *in vitro* directly, and p38-dependent STAT1 and STAT3 Ser-727 phosphorylation has been observed in response to cellular activation in various experimental settings (35–37), providing a molecular basis for cross-talk between the p38 MAPK and STAT signaling pathway.

Besides transcription factors AP-1 and STAT1/STAT3, plasmin also triggers activation of NF-κB through IκB kinase β-mediated degradation of the endogenous inhibitors IκB and p105 (8). Although cooperation between NF-κB and MAP kinase activity has been repeatedly suggested, these complex interactions, which have primarily been described for JNK, are far from being understood (12). Although in human endothelial cells p38 MAPK activity has been linked to NF-κB activation (52), no such link was observed in B lymphocytes, indicating cell-specific networking (63). Neither could we detect any plasmin-induced activation of JNK and ERK1/2, nor did any of the kinase inhibitors used affect activation of NF-κB, suggesting that in plasmin-stimulated human monocytes, there is no direct cross-talk between NF-κB and JAK/STAT or MAPK signaling. However, this does not exclude cooperation at the promoter level; only recently, it has been reported that p38 MAPK-dependent phosphorylation of histone H3 may mark promoters of inflammatory genes for increased NF-κB recruitment (64).

LPS was used as a positive control for plasmin because LPS is a potent activator of MAPKs and NF-κB and a strong activator of MCP-1 and CD40 gene expression; for the expression of both genes, the activity of p38 MAPK is critical in human monocytes and monocyte-derived dendritic cells (53, 65). Con-

sistent with its activation of p38 MAPK, plasmin induced MCP-1 release from primary monocytes, similar to that triggered by LPS. Local contact activation leading to plasmin generation occurs in inflammatory reactions (7, 66). Therefore, plasmin-induced expression of MCP-1 could have important implications for the recruitment of immunocompetent cells, such as additional monocytes, but also T lymphocytes and NK cells (20–22). Moreover, the fact that activation of p38 MAPK is critical for maturation of monocyte-derived dendritic cells (53) suggests another putative role for plasmin generated at sites of inflammation.

The finding that plasmin induces gene expression and increase in cell surface expression of CD40, a key effector of cell contact-mediated proinflammatory activation of monocytes, sheds new light on the complex proinflammatory network activated by plasmin stimulation. It implies that monocytes in inflammatory lesions would become more susceptible to CD40/CD154-mediated activation, which during continuous overexpression might contribute to the development of chronic inflammatory disorders such as rheumatoid arthritis or atherosclerosis (23).

Analysis of the plasmin-induced chemotactic activity in human primary monocytes revealed that both JAK/STAT and p38 MAPK signaling pathways are actively involved in this complex cellular reaction. Inhibition of p38 MAPK by the inhibitor SB203580 had a critical impact on monocyte migration, leading to nearly complete blockade of plasmin-induced locomotion. Specificity of the inhibitory effect was ensured by the structurally related control compound SB202474, which did not affect the plasmin-mediated monocyte chemotaxis. Likewise, activity of p38 MAPK is also required for chemotaxis of monocytic THP-1 cells elicited either with lysophosphatidylcholine or MCP-1 but not for IL-8-induced neutrophil migration (67–69). On the other hand, it has been reported that monocyte chemotaxis to MCP-1 is inhibited by PD98059, implying involvement of the ERK pathway in this response (70). Although the downstream target of p38 MAPK activity leading to chemotaxis remains to be determined, it is of interest that p38 phosphorylates via downstream kinases heat shock protein 27 (HSP27). HSP27 may, depending on its phosphorylation state, interact with the F-actin polymerization, thereby affecting cell motility (12, 71). Such interaction could provide a link between p38 activation and monocyte chemotaxis.

By contrast, inhibition of JAK phosphorylation only partially inhibited monocyte migration. The nature of the JAK-mediated effect on the cell migration can only be speculated. However, it should be noted that JAKs supposedly carry a band 4.1/JEF domain, which might target them together with focal adhesion kinases to the focal adhesion complex, which is crucial for the cytoskeletal organization and signal transduction (72).

Our cell migration data clearly indicate that the activation of both p38 MAPK and JAK/STAT signaling pathways plays an important role in the plasmin-mediated monocyte chemotaxis. In contrast, fMLP induces monocyte migration through different signaling pathways because it remained unaffected by p38 MAPK or JAK kinase inhibition, which further supports the notion that the pharmacological inhibitors act specifically on the plasmin-mediated stimulation of human monocytes. In conclusion, our results shed new light on an intricate plasmin-triggered signaling system that involves activation of the p38 MAPK and JAK/STAT signaling pathways with subsequent induction of gene expression of MCP-1 and CD40, both of which are important effectors of inflammation.

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