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Platelet Factor 4 (CXC Chemokine Ligand 4) Differentially Regulates Respiratory Burst, Survival, and Cytokine Expression of Human Monocytes by Using Distinct Signaling Pathways

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Platelet factor 4 (PF4; CXCL4) is an abundant platelet α-granule CXC chemokine with unique functions. Although lacking a chemotactic activity, PF4 initiates a signal transduction cascade in human monocytes leading to the induction of a broad spectrum of acute and delayed functions including phagocytosis, respiratory burst, survival, and the secretion of cytokines. Surprisingly, although these monocyte functions are well defined, only very limited information exists on the specific signaling pathways that are involved in the regulation of these biological responses. By using specific inhibitors and direct phosphorylation/activation studies, we show in the present study that PF4-mediated respiratory burst is dependent on a very rapid activation of PI3K, Syk, and p38 MAPK. Moreover, monocyte survival and differentiation instead is controlled by a delayed activation of Erk, with an activity peak after 6 h of stimulation. The inhibition of Erk completely reverted PF4-mediated protection against apoptosis. Finally, even though JNK is rapidly activated in PF4-treated monocytes, it is dispensable for the regulation of survival and respiratory burst. However, PF4-induced up-regulation of chemokine and cytokine mRNA and protein requires a sustained activation of JNK and Erk. Taken together, PF4-mediated immediate monocyte functions (oxygen radical formation) are regulated by p38 MAPK, Syk, and PI3K, whereas delayed functions (survival and cytokine expression) are controlled by Erk and JNK. The Journal of Immunology, 2007, 179: 2584–2591.

Monocytes and macrophages are critically important in the generation of inflammatory mediators, cytokines, chemokines, and regulation of innate and adaptive immune responses. Although not terminally differentiated, blood monocytes are able to fulfill a variety of functions involved in the host defense against bacterial and fungal pathogens. Although monocytes are highly mobile cells transported by the bloodstream and can accumulate rapidly at inflammatory sites, macrophages, in contrast, are constitutively present in most tissues. However, representing cells of the first line of host defense against various pathogens, not only their presence at the site of inflammation is required but also fast and effective mechanisms for their activation.

Within this context, the role of platelet-derived chemokines is beginning to emerge. During acute vascular injury or chronic disease, activated platelets release a variety of mediators, and blood leukocytes are the first cellular elements that become exposed to platelet release products. A main proportion of these consist of two α-granule proteins that belong to the family of CXC chemokines, platelet factor 4 (PF4; CXCL4) and connective tissue-activating peptide III (CXCL7). Both chemokines are virtually absent in plasma, whereas they are found in serum in micromolar concentrations (1, 2). Although connective tissue-activating peptide III after conversion into its N-terminal cleavage product neutrophil-activating peptide-2 attracts mainly neutrophils (3–6), PF4 was reported to be active on a variety of different cell types including basophils, T cells, NK cells, monocytes, and endothelial cells (7–16).

Although in most cell types PF4 mediates exclusively either long-lasting or short-term biological responses, a complex spectrum of different consecutive functions is observed in monocytes. During an initial phase of up to 60 min, PF4 induces the generation of oxygen radicals and phagocytosis in these cells (11). Thereafter, PF4 initiates a cellular program, which prevents monocytes from undergoing spontaneous apoptosis and induces differentiation of these cells into a specific subtype of macrophages. Different from GM-CSF- or M-CSF-generated macrophages, PF4-treated cells lack a surface expression of HLA-DR, but show an up-regulation of the costimulatory molecule B7-2 (10). Moreover, the latter cell type displays enhanced innate immune functions such as phagocytosis and the generation of reactive oxygen species (ROS) compared with GM-CSF-generated macrophages (11).

Although chemokines typically bind to seven-transmembrane-domain G-protein-coupled receptors, binding sites for PF4 are less well defined. In a recent report, Lasagni et al. (14) described an alternatively spliced variant of CXCR3, also referred to as

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2 Address correspondence and reprint requests to Dr. Brigitte Kasper, Department of Immunology and Cell Biology, Research Center Borstel, Parkallee 22a, 23845 Borstel, Germany. E-mail address: bkasper@fz-borstel.de

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CXCR3-B, as a functional receptor for PF4 on endothelial cells. However, our own investigations revealed that CXCR3-B is not expressed on monocytes or neutrophils (11), and that PF4 binding to the latter cells is mediated by a distinct receptor, recently identified as a chondroitin sulfate proteoglycan (17, 18). Thus, taking PF4’s unusual receptors and its biological functions on monocytes into account, it becomes evident why the underlying signaling processes are poorly understood. In recent reports, we described signaling pathways involved in PF4-mediated neutrophil activation (secondary granule exocytosis and adhesion). In these cells, PF4 stimulates the activation of several members of the Src-kinase family, tyrosine kinase Syk, JNK, and monocarboxylic GTPases (19, 20).

Thus, in our study, we analyze whether PF4-mediated immediate and delayed activities on monocytes are controlled by distinct signaling pathways. By several lines of evidence, we could demonstrate that PF4-induced oxygen radical formation requires activation of PI3K, Syk, and (predominantly) p38 MAPK, whereas PF4-induced differentiation as well as protection from apoptosis is strictly dependent on Erk. Furthermore, besides Erk, we could identify JNK as a central signaling element involved in the regulation of chemokine and cytokine production.

Materials and Methods

Materials

Human natural PF4 was purified in our laboratory from release supernatants of thrombin-stimulated platelets, as described previously (17, 21). The final PF4 preparation exceeded 99% purity, containing no detectable protein contaminants as determined by mass spectrometry (performed by Dr. B. Lindner, Department of Immunochemistry and Biochemical Microbiology, Research Center Borstel, Borstel, Germany). Mononuclear cells were routinely isolated from citrated blood of healthy donors by leukapheresis from thrombin-stimulated platelets, as described previously (17, 21). Human natural PF4 was purified in our laboratory from release supernatants of thrombin-stimulated platelets, as described previously (17, 21). For signal transduction studies, monocytes (5 × 10^6/ml) were incubated for 20 min at 37°C in Ca^2+- and Mg^2+-free RPMI 1640 supplemented with 10% FCS and 2 mM L-glutamine (all from Biochrom). Monocytes were preincubated for 20 min at 37°C in Dulbecco’s PBS in the presence or absence of various inhibitors, as indicated in the text, and subsequently cultured for up to 72 h with 4 μM PF4, or left untreated. Maturation of the cells was examined routinely by determining the expression of the macrophage marker carboxypeptidase M/MAX1 (GeneID, 1368) (24) as published previously (10). For signal transduction studies, monocytes (5 × 10^6/ml) were preincubated for 20 min at 37°C in Dulbecco’s PBS in the presence or absence of various inhibitors, as indicated in the text, and supplemented with CaCl_2 and MgCl_2 to a final concentration of 0.9 and 0.5 mM, respectively. Subsequently, the cells were exposed to 4 μM PF4 at 37°C for the times indicated. Stimulation was performed under gentle agitation in a thermo-shaker (Eppendorf), and then terminated by rapid centrifugation at 4°C. Approval for these studies was obtained from the Institutional Review Board at the University of Lübeck (Lübeck, Germany), and informed consent was provided according to the Declaration of Helsinki.

Formation of ROS

Generation of ROS was determined in a microplate luminometer (LB 96V; Berthold) by measurement of chemiluminescence in the presence of 60 μg/ml luminol (5-amino-2,3-dihydro-1,4-phthalazindione; Roche Diagnostics) essentially as described elsewhere (11). In brief, monocytes were pre-treated for 20 min at 37°C in Ca^2+- and Mg^2+-free RPMI 1640 buffered with 25 mM HEPES without phenol red; Biochrom) in the presence or absence of various inhibitors, as indicated in the text, and distributed in 200-μl aliquots in an opaque 96-well microtiter plate (Nunc). Luminol was added, and the cells were preincubated for 1 h at 37°C. After stimulation with 4 μM PF4, chemiluminescence was recorded for 90 min. Individual assay backgrounds were determined in samples of unstimulated cells in the presence or absence of inhibitors run in parallel and were subtracted. Data were expressed as relative light units and quantified by integration over the time periods indicated.

Evaluation of cell viability and detection of apoptosis

Determination of apoptotic and necrotic cells was done by double-labeling with Annexin V-FITC and propidium iodide (PI). Labeling of apoptotic cells was performed by using an annexin V kit (Bender MedSystems). Briefly, monocytes were incubated with Annexin V-FITC in binding buffer (provided by the manufacturer) for 10 min on ice, washed, and resuspended in the same buffer as described by the manufacturer. PI (final concentration, 1 μg/ml) was added immediately before flow cytometry analysis (FACScan; Becton Dickinson). The population of apoptotic cells was defined by their characteristic binding pattern, annexin V^highPI^low. Syk immunoprecipitation and in vitro protein kinase assay

Activation of Syk was determined by an in vitro phosphorylation assay using MBP as exogenous substrate. Cells were stimulated for the indicated time periods and subsequently incubated in Mg^2+-containing lysis buffer (MLB; 25 mM HEPES (pH 7.5), 150 mM NaCl, 1% Nonident P-40, 10 mM MgCl_2, 1 mM EDTA, and 10% glycerol), supplemented with inhibitors (2 mM Na_3VO_4, 2 mM NaF, 500 nM okadaic acid, 4 mM Pefabloc, and 1× Complete). After extraction for 10 min at 4°C, lysates were cleared by centrifugation (10 min, 10,000 × g, 4°C). Samples of precleared cell lysate containing 300–350 μg of total protein were incubated with 1 μg of anti-Syk Ab, followed by precipitation with protein A-agarose beads. After repeated washings, beads were resuspended in kinase buffer (20 mM Tris (pH 7.4), 10 mM MgCl_2, 10 mM MnCl_2, and 1 mM DTT) containing 3 μg of MBP, and the kinase reaction was started by the addition of 10 μM ATP. The reaction was stopped after 15 min at 30°C by the addition of 4-fold concentrated sample buffer (Roth). Phosphorylated MBP was detected by Western blot analysis as described below.

Activation of AKT and MAPK

Monocytes were stimulated for the time periods indicated and lysed immediately in MLB, supplemented with inhibitors. After 10 min on ice, the lysates were cleared by centrifugation (10 min, 10,000 × g, 4°C). Phosphorylated AKT and MAPK were detected in cell lysates by Western blot analysis with Abs specific for the phosphorylated (activated) kinases. In parallel, JNK enzyme activity was examined using c-Jun fusion protein as exogenous substrate. In brief, monocyte lysates were incubated with c-Jun fusion protein beads to pull down active/phosphorylated JNK. After repeated washings, c-Jun fusion protein beads were resuspended in kinase buffer (25 mM Tris (pH 7.4), 5 mM β-glycerophosphate, 2 mM DTT, 2 mM Na_3VO_4, and 10 mM MgCl_2) supplemented with 100 μM ATP using the coupled c-Jun fusion protein as substrate. Reactions were terminated after 30 min at 30°C by the addition of 4-fold concentrated sample buffer. Phosphorylated c-Jun was detected by Western blot analysis as described below.

Western blot analysis

Cell pellets were lysed and protein concentrations were determined by the method of Bradford (25). Proteins derived from cell lysates (30 μg/lane) or immunoprecipitations were separated by SDS-PAGE (26) using 10% or 12% polyacrylamide gels and blotted onto polyvinylidene fluoride membranes (Millipore). Immunoblot analysis was performed as described (27). Briefly, membranes were incubated with the respective primary Abs and Alexa 680-conjugated goat anti-mouse IgG, or IRDye800-conjugated goat anti-rabbit IgG secondary Abs. Bands were visualized by an
Odyssey infrared imaging system (LI-COR Biosciences). Quantification of relative density of protein bands was performed using Odyssey software 1.2 (background method: median, top/bottom). Band density in unstimulated cells was set as 100% and the densities of protein bands in PF4-stimulated cells were calculated as percentage of unstimulated. For reprobing, the membranes were stripped in 62.5 mM Tris (pH 6.7), 100 mM 2-ME, and 2% SDS, for 30 min at 50°C, followed by immunodetection with the appropriate Abs.

**RNA isolation, cDNA synthesis, and real-time quantitative PCR (RQ-PCR)**

Total RNA was purified using NucleoSpin RNA II kit (Macherey-Nagel) according to manufacturer’s recommendations followed by reverse transcription into cDNA using First-Strand cDNA Synthesis kit (Fermentas). RQ-PCR was conducted on a LightCycler (Roche Diagnostics) after mixing the cDNA with SYBR Green PCR master mix (Fast Start DNA Master PLUS SYBR Green I kit; Roche Diagnostics) and appropriate primers (500 nM). Primers for human β2-microglobulin (b2M), IL-1α, and TNF genes are as follows (5’ to 3’): b2M, sense, GCT GTC CTC GCG CTA CTC TC; antisense, GCG GCA TCT TCA AAC CTC CAT; IL-1α, sense, GAT CAA TCT GTG TCT CTG AGT AT; antisense, TTG AGG GCG TCA TTC AGG ATG; TNF, sense, GCC TCC AGG CCG TGC TTG TTC; antisense, AGA CGG CGA TGC GCA TCA AAC CTC CAT. Primers for human CCL2, CCL3, CCL4 (R&D Systems), and CXCL8 (BD Biosciences) were determined in cell culture supernatants by sandwich ELISA according to manufacturer’s recommendations.

**Statistical analysis**

Data are presented as mean ± SD for the number of experiments indicated in the figures. Statistically significant (p < 0.05) differences among the treatment groups were calculated using Student’s paired t test.

**Results**

**PF4-mediated oxygen radical formation is dependent on Syk, PI3K, and p38**

To identify the signaling pathways involved in PF4-induced oxygen radical formation, in a first approach monocytes were preincubated in the presence or absence of increasing concentrations of inhibitors (SB203580, 2–50 μM; piceatannol, 1.6–40 μM; wortmannin, 2–50 nM; PD98059, 2–50 μM; SP6001250, 0.12–3 μM) directed against known molecules involved in PF4 signaling in neutrophils (20). Subsequently, the cells were stimulated with 4 μM PF4, and luminol-derived chemiluminescence was recorded for 90 min (data not shown). As shown in Fig. 1A, preincubation of the cells with inhibitors of p38 MAPK (SB203580), Syk (piceatannol), or PI3K (wortmannin) at optimal concentrations each resulted in a significant reduction of PF4-mediated respiratory burst by 73.4, 96.9, or 98.5%, respectively. These data provided first evidence that activation of PI3K, Syk, and p38 MAPK are involved in the generation of oxygen radicals in monocytes.

To understand whether the same pathways are involved in the control of PF4-mediated protection from spontaneous apoptosis in monocytes, the cells were pretreated with inhibitors as indicated in Fig. 1B and subsequently cultured for 72 h in the presence or absence of 4 μM PF4. To assess the proportion of apoptotic cells, the cultured monocytes were labeled with annexin V. Necrotic cells were identified by counterstaining with PI and excluded from further analysis. As shown in Fig. 1B, in the absence of PF4, ~67% of the monocytes developed an apoptotic staining pattern. By contrast, PF4-treated monocytes were efficiently prevented from undergoing apoptosis and only 15.1% apoptotic cells could be detected in these samples. Interestingly, the antiapoptotic effect of PF4 was not affected by Syk inhibitor piceatannol (17.5% apoptotic cells), whereas in the presence of p38 MAPK inhibitor (SB203580) or PI3K inhibitor (wortmannin) weak, and compared with PF4-treated controls statistically not significant, enhancement of apoptosis rates was observed (27.8 and 26.7% apoptotic cells). The three above-mentioned inhibitors did not modulate apoptosis in unstimulated cells (data not shown). From these data, we conclude that PF4-mediated immediate monocyte response (respiratory burst) is controlled by p38 MAPK, Syk, and PI3K, but these play no or only a minor role in the regulation of monocyte survival induced by PF4.

**PI3K, Syk, and p38 MAPK are rapidly activated by PF4 in monocytes**

Because activation of PI3K, Syk, and p38 MAPK are involved in the regulation of oxygen radical formation in monocytes, we next...
analyzed the time kinetics of their activation. Following PF4 stimulation for the time periods indicated in Fig. 2, Syk kinase activity was evaluated by analysis of its capacity to phosphorylate MBP as a model substrate. Detection of phosphorylated MBP (pMBP) was performed by Western blot analysis with anti-phospho-tyrosine Abs. Activation of p38 MAPK was determined by Western blot analysis using Abs specific for the active (dual-phosphorylated) kinase (p38), and PI3K activation was tested by probing for phosphorylated Akt (Ser473), a downstream element in PI3K pathway. As shown in Fig. 2A, phosphorylation of MBP (pMBP) was detected by Western blot analysis using anti-phospho-tyrosine Abs. In parallel experiments, activation of p38 MAPK was determined by Western blot analysis using Abs specific for the active (dual-phosphorylated) kinase (p38), and PI3K activation was tested by probing for phosphorylated Akt (Ser473), a downstream element in PI3K pathway (C). Aliquots of the same lysates were reprobed with anti-Syk (A), anti-p38 (B), or anti-AKT Abs (C) to confirm equal protein loading (A–C, lower panels). The data from one representative experiment of four are given.

These data clearly show that fast induction of reactive oxygen metabolites in PF4-treated monocytes correlates with a rapid activation of Syk, PI3K, and p38 MAPK.

**FIGURE 2.** PF4 stimulates rapid activation of Syk, p38 MAPK, and PI3K in monocytes. Monocytes were stimulated for up to 30 min with 4 μM PF4 and Syk enzyme activity was determined after immunoprecipitation of the protein by an in vitro kinase assay using MBP as model substrate. A, Phosphorylation of MBP (pMBP) was detected by Western blot analysis using anti-phospho-tyrosine Abs. B and C, In parallel samples, activation of p38 MAPK was determined by Western blot analysis using Abs specific for the active (dual-phosphorylated) kinase (p38), and PI3K activation was tested by probing for phosphorylated Akt (Ser473), a downstream element in PI3K pathway (C). Aliquots of the same lysates were reprobed with anti-Syk (A), anti-p38 (B), or anti-AKT Abs (C) to confirm equal protein loading (A–C, lower panels). The data from one representative experiment of four are given.

**FIGURE 3.** Effect of MEK/Erk and JNK inhibitors on PF4-induced oxygen radical formation and monocyte survival. A, Isolated monocytes were preincubated for 20 min at 37°C in the presence or absence of MEK/Erk inhibitor (PD098059; 10 μM), JNK inhibitor (SP600125; 3 μM), or DMSO as solvent control followed by stimulation with 4 μM PF4. Formation of oxygen radicals was quantified as described in Fig. 1. The data were integrated and shown as mean ± SD of three independent experiments. B, Alternatively, following preincubation with the inhibitors, monocytes were cultured for 72 h in the presence or absence of 4 μM PF4, and amounts of apoptotic cells were determined as described in Fig. 1. Data are shown as mean ± SD of three independent experiments. Significant differences (*, p < 0.04; **, p < 0.02) between inhibitor-treated samples and untreated controls are indicated.

**Inhibition of Erk abrogates PF4-mediated effects on apoptosis and differentiation**

In previous reports, we have demonstrated that in human neutrophils PF4-mediated activation of JNK is absolutely required for the induction of adhesion (20), whereas Erk becomes not activated in these cells (19). Therefore, we tested inhibitors directed against MEK/Erk or JNK each resulted only in a partial reduction of ROS formation (40.9 or 45.1% of inhibition, respectively) (Fig. 3A). By contrast, blocking of MEK/Erk totally reverted PF4-mediated rescue from apoptosis (67.8% apoptotic cells), whereas no difference was seen between untreated controls and samples that received the JNK inhibitor SP600125 (15.1 and 12.7% apoptotic cells, respectively) (Fig. 3B). Although the latter inhibitor was without effect on apoptosis rates of unstimulated cells, PD098059 caused a slight increase of apoptotic cells of 3–5% in these cultures (data not shown).

Furthermore, we have analyzed which signaling elements are involved in PF4-mediated monocyte differentiation into macrophages. Therefore, monocytes were preincubated with the inhibitors mentioned above and the surface expression of the differentiation marker carboxypeptidase M/MAX1 (24) was monitored.
after 72 h of PF4 treatment (10). As shown in Fig. 4, MAX1 expression is up-regulated 15.3-fold in PF4-cultured cells, whereas unstimulated cells express MAX1 comparable to isotype control (ratio, 1.4 ± 0.5). As expected, monocytes pretreated with the Erk inhibitor PD098059 did not up-regulate MAX1 indicating that these cells were unable to differentiate. Although only minimal differences to solvent treated controls (DMSO) were observed in samples that received inhibitors against p38 MAPK and JNK (SB203580 and SP600125, respectively), a partial but statistically not significant reduction of MAX1 expression was seen after pretreatment with piceatannol or wortmannin (41 and 36% inhibition, respectively). Taken together, these results clearly demonstrate that PF4-mediated protection from apoptosis as well as monocyte differentiation requires activation of Erk, whereas the role of Erk and JNK in ROS formation appears to be marginal (compare Fig. 3A). Thus, PF4-mediated monocyte differentiation is independent of Syk and PI3K activities, despite their above-described role in monocyte survival.

**PF4 induces delayed activation of Erk in monocytes**

Because PF4-mediated long term responses (rescue from apoptosis as well as differentiation) were completely reverted by PD098059, we analyzed directly time kinetics of Erk activation in PF4-treated monocytes. In comparison, we tested for JNK activity in these cells, because inhibition of JNK had at least a minor effect on PF4-stimulated ROS formation. Erk was tested in cell lysates with Abs specific for the activated (dual-phosphorylated) kinases, whereas JNK enzyme activity was determined by its capacity to phosphorylate exogenous substrate c-Jun. Aliquots of the same lysates were tested to confirm equal protein loading (Fig. 5, A and B, lower panels). In a first set of experiments, activation of both enzymes was analyzed over a period of up to 30 min of stimulation. To our surprise, we found a profound activation of JNK after first contact with the chemokine the signal became substantially amplified, and decreasing thereafter (Fig. 5A). In summary, PF4 utilizes for regulation of immediate (ROS formation) and delayed monocyte functions (survival and differentiation) not only different MAPKs, but these kinases also display different time kinetics of activation in response to the chemokine.

**Differential involvement of MAPK in PF4-induced cytokine and chemokine expression and release**

Besides the regulation of acute cellular functions, JNK and Erk are shown to play a central role in the transcriptional control of various genes, including cytokines and chemokines. Following our strategy, we analyzed whether inhibition of defined pathways differentially affects cytokine and chemokine expression. Genes of interest were selected from a whole genome microarray analysis, where gene expression of unstimulated and PF4-treated monocytes was compared (data not shown). To assess the effects of blocking Erk or JNK pathway, monocytes were pretreated with corresponding inhibitors (PD098059 or SP600125, respectively) for 20 min at 37°C and subsequently stimulated with 4 μM PF4. After 2, 4, 6, and 18 h, total RNA was isolated and transcribed into cDNA, and gene expression was tested by RQ-PCR. Based on these data, ratios of specific gene to housekeeping gene b2M were calculated.

According to our findings, the expression profiles of the different cytokines after treatment with the respective inhibitors can be divided in two subgroups. Although PF4-mediated up-regulation of CCL3 and CCL4 was affected by PD098059 as well as by SP600125, CXCL8 expression was found to be sensitive to treatment with the former inhibitor only (Fig. 6, left panels). Moreover, analysis of the release of the corresponding
played a sensitivity against both inhibitors, but also that of CCL2, CCL22, TNF, IL-1α, and IL-19. By contrast, besides expression of CXCL8 also that of CCL3, IL-1β, and IL-6 was selectively suppressed by PD098059. Interestingly, expression of CCL24 could be blocked neither by the Erk inhibitor nor by the inhibitor against JNK. From these data, we conclude that the PF4-stimulated up-regulation of various proinflammatory cytokines and chemokines requires either activation of Erk alone, or the combination of Erk and JNK activities.

Discussion

Monocytes and macrophages can respond to proinflammatory stimuli by a variety of acute and delayed defense mechanisms such as chemotaxis, formation of ROS, phagocytosis, or the presentation of Ags. Surprisingly, although most of these functions have been well characterized for >20 years, only limited information exists on the specific signaling pathways that are involved in the control of these biological responses. PF4 initiates a signal transduction cascade in human monocytes leading to the induction of a broad spectrum of acute and delayed functions including phagocytosis, respiratory burst, monocyte survival, and the secretion of cytokines (10, 11). These properties do not only underline the exceptional role of PF4 within the family of chemokines but moreover allow us to use PF4-activated monocytes as an interesting model in which the regulation of various cellular functions can be investigated in parallel.

In the present study, we show for the first time that PF4 is able to activate besides Syk and PI3K all three types of MAPK. Moreover, we demonstrate that these signaling elements initiate discrete processes leading to oxygen radical formation, cytokine secretion, as well as cell survival and differentiation.

In the last few years, the importance of MAPK in mammalian cell biology has been established in innumerable studies using a wide variety of model systems (for review, see the study by Rao (31)). However, depending on the cell type and species investigated, contradictory reports were published concerning the involvement of specific MAPK in individual functions. In the

Table 1. PF4-stimulated gene expression in the presence of MAPK inhibitors

<table>
<thead>
<tr>
<th>Gene</th>
<th>Unst. (% of Control)</th>
<th>PD098059 (% of Control)</th>
<th>SP600125 (% of Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL3 (2 h)</td>
<td>19.3 ± 7.6**</td>
<td>60.6 ± 11.9*</td>
<td>24.1 ± 14.6*</td>
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<tr>
<td>CCL4 (2 h)</td>
<td>26.1 ± 8.0**</td>
<td>79.3 ± 19.0</td>
<td>24.3 ± 15.8*</td>
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<tr>
<td>CCL2 (6 h)</td>
<td>21.3 ± 12.1</td>
<td>5.0 ± 4.5**</td>
<td>52.2 ± 28.5</td>
</tr>
<tr>
<td>CCL22 (18 h)</td>
<td>5.8 ± 4.0**</td>
<td>4.4 ± 1.0**</td>
<td>52.0 ± 22.4</td>
</tr>
<tr>
<td>TNF (2 h)</td>
<td>35.1 ± 15.3*</td>
<td>42.2 ± 19.3*</td>
<td>15.8 ± 5.6**</td>
</tr>
<tr>
<td>IL-1α (2 h)</td>
<td>36.6 ± 14.0*</td>
<td>39.5 ± 10.5*</td>
<td>43.6 ± 29.0</td>
</tr>
<tr>
<td>IL-19 (6 h)</td>
<td>8.7 ± 5.9**</td>
<td>7.7 ± 7.7**</td>
<td>11.7 ± 5.9**</td>
</tr>
<tr>
<td>CXCL8 (6 h)</td>
<td>44.1 ± 1.4**</td>
<td>28.8 ± 6.3**</td>
<td>111.3 ± 19.5</td>
</tr>
<tr>
<td>CXCL12 (4 h)</td>
<td>37.6 ± 14.0</td>
<td>38.5 ± 21.8</td>
<td>167.8 ± 75.8</td>
</tr>
<tr>
<td>IL-1β (6 h)</td>
<td>42.8 ± 12.8</td>
<td>157.6 ± 6.2**</td>
<td>179.2 ± 94.5</td>
</tr>
<tr>
<td>IL-6 (4 h)</td>
<td>26.0 ± 11.6*</td>
<td>26.3 ± 21.5*</td>
<td>112.7 ± 46.5</td>
</tr>
<tr>
<td>CCL24 (18 h)</td>
<td>15.9 ± 4.5**</td>
<td>158.6 ± 83.1</td>
<td>119.4 ± 38.9</td>
</tr>
</tbody>
</table>

*a* Freshly isolated monocytes were pretreated with MEK/Erk or JNK inhibitors for 20 min at 37°C and subsequently stimulated with 4 μM PF4 or left unstimulated. After 2, 4, 6, and 18 h, total RNA was isolated and transcribed into cDNA, and gene expression was tested by RQ-PCR. Ratio of specific gene vs 62M in PF4-stimulated samples was set at 100% (control), and gene expression in unstimulated and inhibitor-treated cells was calculated as percentage of this control. Data are shown as mean ± SD of three independent experiments.

*b* Unst., Unstimulated cells.

*c* MEK/Erk inhibitor.

*d* JNK inhibitor.

*e* Time of stimulation.

** and *** Significant differences (*, p < 0.04; **, p < 0.004) between either inhibitor-treated or unstimulated and PF4-stimulated samples based on the data from three independent experiments.

proteins into culture supernatants confirmed these findings in that the liberation of CCL3 and CCL4 was also inhibited by both inhibitors, and that of CXCL8 was reduced by PD098059 only (Fig. 6, right panels). To investigate whether these findings would be reproducible for other proinflammatory mediators, we extended our studies to a comprehensive set of different cytokines and chemokines. Because expression ratios of specific cytokine genes and b2M showed a strong variation with the single experiments, we calculated gene expression in inhibitor-treated cells in relation to those that received PF4 alone, and data were expressed as the percentage of this control. As depicted in Table I, not only expression of CCL3 and CCL4 dis-
PF4 SIGNALING IN MONOCYTE ACTIVATION

present study, we could clearly demonstrate that a delayed and sustained activation of Erk is essential for PF4-mediated rescue from apoptosis (Fig. 3B), macrophage differentiation (Fig. 4), as well as for the expression of several proinflammatory cytokines and chemokines (Fig. 6 and Table I). Although for development and survival of murine macrophages the activation of JNK was reported to be more relevant than Erk (32), our findings confirm previous reports from Monick et al. (33) and Liu et al. (34) on human cells. These authors could show that, in human alveolar macrophages in vivo and monocyte-derived macrophages in vitro, constitutive activation of Erk is required for prolonged survival of these cells. Furthermore, Erk activity was described to play a critical role for differentiation and survival of human immature dendritic cells (35). It should be mentioned that the latter three reports describe that besides Erk also the activation PI3K/AKT is involved in survival and differentiation of macrophages and dendritic cells. Although we indeed observed a rapid induction of AKT phosphorylation after PF4 treatment (Fig. 2C), no relevant effect of the PI3K inhibitor wortmannin could be detected on monocyte survival or differentiation (Figs. 1B and 4). A reason for this may be that survival of PF4-treated monocytes could be mediated by an additional redundant pathway replacing PI3K activation.

In addition to monocyte survival and differentiation, we show that PF4-mediated activation of Erk, in some cases together with JNK, is linked to the expression and secretion of several proinflammatory cytokines. Although expression of CXCL8, CXCL3, IL-1β, and IL-6 appeared to be regulated by Erk alone, CCL2, CCL3, CCL4, CCL22, as well as TNF, IL-1α, and IL-19 are controlled by both, Erk and JNK. The central role of Erk in the induction of IL-1, TNF, IL-6, and CXCL8 has been documented by several authors in the past (36–40). However, which MAPK is involved in the regulation of a specific cytokine appears to be strongly dependent on the cell type and stimulus used in the corresponding experiments. Zhu et al. (40) showed that, in LPS-stimulated murine RAW cells, the expression of TNF requires the activation of Erk, JNK, and p38 MAPK, whereas Means et al. (38) could demonstrate that, in murine alveolar macrophages but not in nonpulmonary macrophages, Erk activity is necessary for the expression of this cytokine. Furthermore, CXCL8 expression in human monocytes in response to LPS, and, as shown here, also to PF4, strictly relies on Erk, whereas in the same cell type upon stimulation with mycobacteria, production of CXCL8 was found to be independent of this kinase (36, 39). Thus, it might not be too surprising that Tuyt et al. (37) reported that IL-6 expression in okadaic acid-treated human monocytes requires Erk as well as JNK, whereas we could confirm the participation of Erk but not the latter kinase in process of IL-6 regulation. An explanation for these to some extent conflicting results may be given by the fact that MAPK share several downstream transcription factors and, thus, induce partially overlapping biological responses (for review, see Shi and Gaestel (41)). This situation may even get more complicated if one takes into consideration that different stimuli display individual time kinetics of kinase activation that could result in diverging patterns of cooperation between these signaling components.

Although intracellular signals involved in monocyte gene regulation are a matter of intensive investigation, signal transduction processes controlling acute cellular functions such as respiratory burst or phagocytosis are as yet poorly understood. In the present study, we show for the first time that PF4 induces within 30–60 s an activation of p38 MAPK, PI3K/AKT, and Syk (Fig. 2), which correlates with the release kinetics of oxygen radicals published earlier (11). Furthermore, blocking of each kinase by specific inhibitors resulted in a drastic reduction of PF4-induced respiratory burst, indicating that all three kinases participate in the transduction of this cellular function (Fig. 1A). An at least partial reduction of ROS formation was seen in cells treated with inhibitors against Erk and JNK (Fig. 3A). Because only very few reports exist on the role of MAPK and PI3K regulation in monocyte ROS production, we have to compare our results mainly with findings on neutrophils, where these mechanisms are under broad investigation. In the latter cells, stimulation with the chemotactic peptide fMLP caused a rapid activation of p38 MAPK, Erk, and PI3K. Interestingly, inhibition of p38 MAPK and PI3K, but not Erk, resulted in a drastic reduction of ROS formation and neutrophil chemotaxis (42–47). Noteworthy, PF4 activates in human neutrophils neither PI3K nor p38 MAPK and is indeed unable to induce ROS release or chemotaxis in these cells (19, 21, 48). Stimulation of monocytes and macrophages with LPS or opsonized zymosan resulted in the formation of oxygen radicals and an activation of Erk and p38 MAPK (49–51). However, pretreatment of cells with the corresponding inhibitors against these MAPK did not affect ROS production, and by several lines of evidence these authors show that Erk and p38 MAPK are located downstream of NADPH oxidase, and furthermore, MAPK activation is a consequence of oxygen radical formation. Although this pathway may be involved in the PF4-mediated delayed activation of Erk in monocytes, the strong effect of p38 MAPK, Syk, and PI3K inhibitors on ROS formation indicate that these elements are located upstream of NADPH oxidase.

Interestingly, the tyrosine kinase Syk is not involved in neutrophil respiratory burst induced by fMLP (52). However, as we have shown earlier, Syk becomes activated in these cells upon stimulation with PF4 and regulates neutrophil adhesion by controlling JNK activity (20). With regard to Syk, our data suggest that respiratory burst in monocytes may be regulated differentially from that in neutrophils.

Taken together, our results show that three MAPK family members, namely p38, Erk, and JNK become activated in PF4-stimulated monocytes. Activation of each kinase displays individual time kinetics and is required for the induction of specific monocyte function. Syk, PI3K, and p38 MAPK are rapidly activated in PF4-treated monocytes and are involved in oxygen radical formation, whereas Erk activity is required for macrophage differentiation. However, delayed and more sustained activation of JNK and Erk is required for PF4-stimulated up-regulation of chemokine and cytokine mRNA and protein. Further studies are directed to identifying signaling elements involved in the control and regulation of MAPK and PI3K.

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Disclosures

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References


