Platelet Factor 4/CXCL4 Induces Phagocytosis and the Generation of Reactive Oxygen Metabolites in Mononuclear Phagocytes Independently of Gi Protein Activation or Intracellular Calcium Transients

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*J Immunol* 2004; 173:2060-2067; doi: 10.4049/jimmunol.173.3.2060

http://www.jimmunol.org/content/173/3/2060
Platelet Factor 4/CXCL4 Induces Phagocytosis and the Generation of Reactive Oxygen Metabolites in Mononuclear Phagocytes Independently of Gi Protein Activation or Intracellular Calcium Transients

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Platelet factor 4 (PF-4), a platelet-derived CXC chemokine, is known to prevent human monocytes from apoptosis and to promote differentiation of these cells into HLA-DR™ macrophages. In this study, we investigated the role of PF-4 in the control of acute monocyte proinflammatory responses involved in the direct combat of microbial invaders. We show that PF-4 increases monocyte phagocytosis and provokes a strong formation of oxygen radicals but lacks a chemotactic activity in these cells. Compared with FMLP, PF-4-induced oxidative burst was later in its onset but was remarkably longer in its duration (lasting for up to 60 min). Furthermore, in PF-4-prestimulated cells, FMLP— as well as RANTES-induced burst responses became synergistically enhanced. As we could show, PF-4-mediated oxidative burst in monocytes does not involve Gi proteins, elevation of intracellular free calcium concentrations, or binding to CXCR3B, a novel PF-4 receptor recently discovered on endothelial cells. Moreover, we found that PF-4 acts on macrophages in a dual manner. On the one hand, very similar to GM-CSF or M-CSF, PF-4 treatment of monocytes generates macrophages with a high capacity for unspecific phagocytosis. On the other hand, short term priming of GM-CSF-induced human macrophages with PF-4 substantially increases their capability for particle ingestion and oxidative burst. A comparable effect was also observed in murine bone marrow-derived macrophages, indicating cross-reactivity of human PF-4 between both species. Taken together, PF-4 may play a crucial role in the induction and maintenance of an unspecific immune response. The Journal of Immunology, 2004, 173: 2060–2067.
mechanism (15). Besides its unique receptor, PF-4-induced biological functions are intriguing. Although being structurally a chemokine, PF-4 lacks chemotactic activity on neutrophils and T cells (15, 16). Apart from inducing short term responses on PMN, PF-4 is involved in long term differentiation and regulatory processes such as the control of EC and fibroblast proliferation (17–19) and the support of the survival of hemopoietic stem cells and progenitor cells (20). Moreover, PF-4 suppresses IL-2 mRNA transcription and the release of IL-2 in activated human T cells, corresponding to its inhibitory effect on T cell proliferation and release of IFN-γ (15). Further, we could show that PF-4 prevents human monocytes from spontaneous apoptosis and induces differentiation of monocytes into a specific subtype of macrophages (21). In contrast to GM-CSF-exposed or serum-derived macrophages, these cells were characterized by a total lack of surface-expressed HLA-DR Ag, whereas the co-stimulatory molecule B7-2 was found to be significantly up-regulated on the cell membrane. The aim of this study was to investigate the role of PF-4 in monocyte-mediated innate immune responses. Here we demonstrate that PF-4 is a potent activator of phagocytosis and of reactive oxygen radical formation in human monocytes as well as in human and murine macrophages. In addition, we provide first evidence that the molecular mechanisms controlling these functions are principally different from those utilized by typical chemotactic factors.

Materials and Methods

Cytokines

Human natural PF-4 was purified to homogeneity from release supernatants of thrombin-stimulated platelets in a three-step procedure as previously described (16). The preparations contained <0.125 ng of LPS per mg of PF-4 (i.e., below 4 pg/ml at 4 μM PF-4) as determined by the Limulus amoebocyte lysate assay, ruling out possible side effects caused by contaminating LPS. PF-4 was lyophilized, stored at −80°C, and reconstituted to stock solutions of 1 mg/ml in 0.1% trifluoroacetic acid before use. Recombinant human M-CSF was obtained from R&D Systems (Wiesbaden, Germany) for monocytes or 90 μg/ml lucigenin (9,9′-bis(N-methylacridinium nitrate; Roche Diagnostics, Mannheim, Germany) for monocytes or 90 μg/ml lucigenin (9,9′-bis(N-methylacridinium nitrate; Roche Diagnostics) for macrophages essentially as described elsewhere (22). Briefly, cells (10^6/ml) in CL medium (RPMI 1640 buffered with 25 mM HEPES without phenol red; Biochrom) supplemented with either lumilin or lucigenin were distributed in 200-μl aliquots in an opaque 96-well microtiter plate (Nunc) and preincubated for 1 h at 37°C. After stimulation with PF-4, FMLP (Sigma-Aldrich), RANTES, or latex beads (7.5 × 10^6 particles in 10 μl of CL medium; Serva, Heidelberg, Germany), chemiluminescence was recorded for 30–90 min. In some cases, combinations of stimuli were used for the activation of cells. Furthermore, in some experiments, monocytes were preincubated for 90 min with pertussis toxin (PTX, from Bordetella pertussis; Calbiochem, Bad Soden, Germany) at concentrations indicated before stimulation with PF-4. Data were expressed as relative light units (RLU) and quantified by integration over the time periods indicated in the text.

For measurement of unspacific phagocytosis, 2 × 10^5 cells were resuspended in 300 μl of CL medium and preincubated for 30 min at 37°C followed by addition of 2 × 10^6 FITC-conjugated monodisperse latex beads (1.83-μm diameter; Polyscience, Eppelheim, Germany) in 10 μl of CL medium. Beads were sedimented onto the cell layer by centrifugation (1 min, 300 × g) and incubated for 30 min at 37°C. After removal of free beads by centrifugation, the relative amount of ingested particles was quantified by flow cytometry, and data were expressed as median fluorescence intensity (MFI). Dead cells were excluded by double staining of cells with propidium iodide (1 μg/ml). In some experiments, cells were incubated for 5 min with different concentrations of PF-4 before the addition of latex beads.

Statistics

All experiments were performed three to five times with cells from different individuals and data are expressed, unless indicated otherwise, as mean of the replicates ± SD. Statistical significance was analyzed by the two-tailed t test.

Results

PF-4 lacks chemotactic activity on human monocytes but induces strong oxygen radical formation and increases phagocytic activity

Early reports claimed the ability of PF-4 to induce neutrophil and monocyte chemotaxis (27, 28). However, in a more recent report
we have shown that, at least for neutrophils, highly purified PF-4 lacks such activity and that the previously observed effects were most likely due to contamination by other chemokines (16). In a first approach, we investigated the effect of a broad range of PF-4 concentrations (0.625–6.25 μM) on monocyte migration. Whereas monocytes responded vigorously to FMLP used here as a control (chemotactic index, 3.98 ± 0.67 at 10 nM FMLP), PF-4 at any concentration used failed to induce a specific or even a random migratory response above background levels (data not shown). From these results, it is evident that pure PF-4, even at high concentrations, lacks measurable chemotactic activity for human monocytes.

Because the capacity of cytokines to induce acute antimicrobial functions in monocytes is not necessarily coupled to their ability to promote chemotaxis, we performed a second set of experiments where we analyzed the capability of PF-4 to induce the formation of oxygen radicals over a wide range of stimulus concentrations. PF-4 dosages of 0.3 μM and higher provoked a dose-dependent formation of reactive oxygen metabolites (Fig. 1A). Comparison of time kinetics between the PF-4-induced reaction and the one mediated by the typical chemotaxin FMLP revealed significant differences; whereas the FMLP-induced maximal response was observed after 1 min of stimulation and decreased to background levels during the following 10 min, PF-4-mediated oxidative burst was later in its onset (maximum levels after 15–30 min) but remarkably longer in its duration (lasting up to 60 min). Although peak levels of FMLP-induced responses are much higher than those mediated by PF-4, the overall response of the chemokine based on a time period of 60 min surmounted that of FMLP by far.

An effective combat of microbial invaders does not only involve the production of reactive oxygen species but also an increased capacity of taking up particles. Consequently, we analyzed phagocytosis of FITC-labeled latex beads by monocytes after priming with increasing concentrations of PF-4. As depicted in Fig. 1B, PF-4 treatment not only increased the total number of phagocytosing cells (from 39% of unstimulated cells to 91% of cells primed with 4 μM PF-4) but also augmented the average amount of particles found within each phagocytosing monocyte (from MFI 60 with unstimulated cells to MFI 138 with cells stimulated with 4 μM PF-4).

**PF-4-induced oxidative burst does not involve CXCR3B.**

**Gi protein activation or mobilization of intracellular free calcium**

The observed differences between FMLP and PF-4 in the induction of chemotaxis and oxidative burst suggested the involvement of different molecular mechanisms activated by the two stimuli. Stimulation of phagocyte functions by FMLP, chemokines, and other chemotactic stimuli is mediated through heterotrimeric G proteins and is accompanied by a rapid and transient increase in intracellular calcium concentration. Consequently, we analyzed the effect of the G protein inhibitor PTX on FMLP- and PF-4-induced respiratory burst. Whereas treatment of monocytes with PTX at a concentration of 40 ng/ml resulted in a complete inhibition of FMLP-mediated oxygen radical formation, concentrations up to 1000 ng/ml PTX had only a minor and statistically insignificant effect on PF-4-stimulated cells (Fig. 2A). This weak reduction may be explained by side effects of the toxin on other G proteins indirectly involved in cell activation. Furthermore, different to FMLP, challenge of monocytes with PF-4 (4 μM) did not induce a mobilization of cytosolic calcium (Fig. 2B). From these data, we conclude that signaling pathways involved in PF-4-mediated monocyte activation differ principally from those of chemotaxins such as FMLP or chemokines.

Most recently, an alternative splice variant of the CXCR3, termed CXCR3B, has been described as a functional receptor for PF-4 on EC. Different to other chemokine receptors, CXCR3B is not coupled to Gi but to Gs proteins and does not provoke a chemotactic response in EC (25). However, as shown by two independent approaches (Touch Down PCR-analysis as well as examination in the LightCycler system), EC but not monocytes, macrophages, or neutrophils express detectable amounts of CXCR3B mRNA (Fig. 3). On the basis of these observations, we exclude a potential role for this receptor in PF-4-mediated processes regarding to the latter three cell types.

**PF-4 cooperates with the chemotaxins FMLP and RANTES in the generation of oxygen radicals in human monocytes**

Differences between FMLP and PF-4 at the signaling level prompted us to examine whether both stimuli may cooperate in the...
induction of reactive oxygen metabolites. Because platelets beside PF-4 contain relevant amounts of RANTES (CCL5) (29), this chemokine was included in the following set of experiments. Cells were primed for 5 min with increasing concentrations of PF-4 and subsequently challenged with FMLP or RANTES at equipotent concentrations (100 and 500 nM, respectively). Burst rates were recorded for 60 min, and data were quantified by integration over this period of time. FMLP (Fig. 4A) as well as RANTES (Fig. 4B) cooperated synergistically with the chemokine in the induction of oxygen radicals with a first superadditive effect seen at 1 μM PF-4. Interestingly, enhancement of the PF-4 response by RANTES was ~3.3-fold, whereas augmentation induced by FMLP was somewhat lower (2.1-fold). Moreover, cooperation between the stimuli also occurred when they were used in reverse order, i.e., when FMLP or RANTES were used as primers and PF-4 functioned as a second stimulus (data not shown).

In summary, our data demonstrate that PF-4 is a powerful inducer of acute proinflammatory responses in monocytes independently of Gi protein activation or calcium mobilization. Furthermore, PF-4 cooperates synergistically with chemotactic mediators like FMLP and RANTES in the release of oxygen radicals, which may play an important role in the combat against microbial invaders during an acute phase of infection.

**PF-4-differentiated macrophages are professional phagocytes**

We reported earlier that PF-4 prevents human monocytes from spontaneous apoptosis and induces the differentiation of monocytes into a specific subtype of macrophage lacking surface expression HLA-DR (21). However, the functional properties of these cells remain to be elucidated. To this end, monocytes were differentiated to different types of macrophages by 3-day culture in the presence of either GM-CSF, M-CSF, or PF-4. The phagocytic activity of the resulting cell populations was monitored measuring their capacity for ingestion of FITC-labeled latex particles as described above. All three types of macrophages displayed an enhanced capacity for phagocytosis as compared with the unstimulated control (18% positive cells with an MFI of 64) (Fig. 5). Thus, 33% of GM-CSF and 57% of PF-4-treated cells were capable of particle uptake with both populations exhibiting a comparable particle load (MFI ~100 each). The highest capacity for phagocytosis (MFI 280) was observed in M-CSF-generated macrophages, whereas the amount of active phagocytes (54%) was comparable with that found upon PF-4 stimulation.

**PF-4 induces phagocytosis and the generation of oxygen radicals in human and murine macrophages**

Host defense against microorganisms during an acute inflammatory process is not mediated by blood stream-derived monocytes alone but may also involve macrophages present in the surrounding tissue. To investigate whether PF-4 activates macrophages in a way similar to that observed for monocytes, priming experiments with GM-CSF-induced human macrophages were performed. Therefore, macrophages were primed with increasing concentrations of PF-4 for 5 min, followed by addition of latex-beads. The generation of reactive oxygen metabolites as well as latex particle uptake were recorded. Since the role of PF-4 in mice remains unclear, we evaluated a potential activity of human PF-4 on murine bone marrow-derived macrophages, which were analyzed in parallel.

Whereas in human macrophages individual oxidative burst rates induced by latex or PF-4 alone appeared to be rather low (0.9 to 1.1 × 10^6 RLU in maximum), PF-4 synergistically and significantly enhanced latex-induced oxidative burst rates from 1.3 × 10^6 RLU at 250 nM PF-4 to 9.2 × 10^6 at 8 μM PF-4 (Fig. 6A). Furthermore, PF-4 priming elevated the amount of particles ingested by the phagocytes in a dose-dependent manner. This became first visible at 250 nM and was significant from 500 nM PF-4 on (Fig. 6B). Different to human cells, PF-4 alone did not induce relevant oxidative burst in murine macrophages (Fig. 6C). However, at dosages above 500 nM, PF-4 significantly increased the formation of oxygen radicals in response to latex. Enhancement of latex particle uptake after PF-4 priming was also seen in murine cells. Nevertheless, this cell type required substantially higher concentrations (~4 μM) of PF-4 than human macrophages to induce a significant response (Fig. 6D). Interestingly, PF-4 priming of human as well as murine macrophages did not increase the total number of active

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**FIGURE 2.** Effect of PTX on respiratory burst and mobilization of intracellular free calcium in human monocytes stimulated with PF-4 or FMLP. A, Respiratory burst. Monocytes were left untreated or pretreated for 90 min with increasing concentrations of PTX followed by stimulation with 100 nM FMLP (○) or 4 μM PF-4 (●). Formation of oxygen radicals was quantified by chemiluminescence and recorded for 60 min (PF-4) or 5 min (FMLP). The data were integrated and shown as mean ± SD of three independent experiments. B, Intracellular calcium transients. Cells were stimulated first with 4 μM PF-4 followed by a second challenge with 100 nM FMLP at time points indicated by arrows. Data from one representative experiment of four are given.

**FIGURE 3.** Expression of CXCR3B mRNA in different cell types. Total mRNA was extracted from human cord vein endothelial cells (EC), neutrophils (PMN), monocytes (Mono), and M-CSF-derived macrophages (Mac) and quantitatively analyzed by real-time fluorescence RT-PCR with the LightCycler System. Data of one representative experiment of three are given and expressed as ratio of amounts of CXCR3B mRNA vs amounts of the housekeeping gene βM (n.d., no CXCR3B mRNA detectable). Inset, analysis of products obtained after Touch Down PCR by gel electrophoresis (neg., negative control; i.e., PCR in the absence of cDNA).
phagocytes but only the phagocytic activity of cells that were already capable of particle uptake. These data provide first evidence that human PF-4 activates murine macrophages in vitro at concentrations and in terms of functions comparable with those seen with PF-4 in human macrophages.

Discussion
In this study, we report on the discovery of novel biological activities of the platelet-derived CXC-chemokine PF-4 (CXCL4) for human and murine mononuclear phagocytes. On human monocytes, the chemokine mediates a strong increase in phagocytosis and a long-lasting formation of oxygen radicals, which can be synergistically enhanced in the presence of FMLP or RANTES. Furthermore, extended culture of monocytes with PF-4 results in the generation of macrophages with a high capacity for phagocytosis. Short term priming of human or murine macrophages with PF-4 resulted in a substantial increase in their ability for particle ingestion and latex-induced oxidative burst.

During acute vascular injury, successful defense of the host against microbial invaders depends on the rapid activation of neutrophils, monocytes, and macrophages. Therefore, the immediate presence of mediators initiating and coordinating the onset of an inflammatory process is required at the site of damage. A prominent source of such mediators are activated platelets, which are able to release chemokines and other factors prestored in their granules within minutes of activation (5). Although all three platelet-derived chemokines (RANTES, CTAP-III/NAP-2, and PF-4) are closely related in structure and sequence, they are able to fulfill quite different and complementary functions. Whereas NAP-2 predominantly attracts neutrophils (30, 31), RANTES is chemotactic for monocytes and T cell subpopulations (32). PF-4 activities differ principally from those of the other two chemokines. As we have shown recently, PF-4 is the only chemokine known thus far to rescue monocytes from apoptosis and induce their differentiation into a specific type of macrophage (21). In contrast to GM-CSF- or M-CSF-induced macrophages, PF-4-differentiated cells lack the expression of HLA-DR on their surface. However, the functions of this specific cell-type have remained unclear. Here we could show
that PF-4-derived macrophages display a high capacity for unspecific phagocytosis (Fig. 5). Particle uptake of these cells was comparable with that found in GM-CSF-induced cells, whereas the amount of phagocytosing cells was as high as in M-CSF-generated macrophages. Thus, PF-4-differentiated macrophages may play an important role in the initiation and maintenance of an innate immune response without activating the specific immune system.

Similar features have been reported for alveolar macrophages, which express low amounts of HLA-DR and, moreover, have the ability not to induce but instead to inhibit T cell activation (33, 34).

In contrast to growth factors like GM-CSF and M-CSF which are predominantly regulators of long term immune function, PF-4 is able to additionally stimulate immediate proinflammatory responses. As we were able to show, PF-4 directly induces a strong formation of oxygen radicals in human monocytes. However, the concentrations required for such a response (0.5–8 μM PF-4) are 5- to 50-fold higher as compared with those of other chemotactic factors like RANTES or FMLP. On the other hand, PF-4 is not secreted from cells after a prolonged stimulation but is released in extraordinary high amounts from activated platelets. Although no data are available for PF-4 concentrations directly at sites of acute platelet activation, normal serum contents of the chemokine (1–2.5 μM; Refs. 5 and 35 and our own unpublished observations) would be sufficient to induce monocyte responses. Levels of PF-4 in plasma are below 1 nM, excluding an activation of cells under normal conditions. Furthermore, induction of monocyte oxidative burst and phagocytosis was observed in the same range of PF-4 concentrations as those reported to be required for monocyte differentiation or for the modulation of T cell and neutrophil responses (11, 15, 16, 21). The time kinetics of oxygen radical release differed remarkably between FMLP and PF-4. Whereas FMLP induced a sharp increase of radical formation which reached a maximum after 1 min and declined to background levels after 5–10 min, PF-4-induced responses were later in their onset but remarkably longer in duration (up to 60 min) (Fig. 1). The latter type of kinetics is rather unusual for soluble stimuli and is normally observed in conjunction with the uptake of particles like latex or zymosan (36). Interestingly, PF-4 is able to cooperate with chemotaxins FMLP and RANTES, leading to synergistic enhancement in the generation of reactive oxygen species (Fig. 4). Because formylated peptides are present at the sites of bacterial infections, augmentation by PF-4 of the host cell reaction toward these proinflammatory agents could contribute to reinforce monocyte responses against invading microorganisms. By contrast, RANTES

**FIGURE 6.** Effect of PF-4 on phagocytosis of latex beads and latex-induced oxidative burst in human and murine macrophages. Human (A and B) or murine (C and D) macrophages were preincubated for 5 min with increasing concentrations of PF-4 or left untreated. Oxidative burst (A and C) was determined after addition of latex beads (C) in the presence of lucigenin and recorded for 30 min. Control cells received either PF-4 (●) or latex beads (– – – – – – – –) alone. The data were integrated and shown as mean ± SD of three independent experiments. Statistically significant differences between responses of PF-4-treated samples in combination with latex beads and the sum of the effects induced by either stimulus alone are indicated (*, p < 0.04; **, p < 0.018). Alternatively, PF-4-primed macrophages were incubated for 30 min with FITC-labeled latex beads (B and D), and phagocytosis was determined as described in the legend to Fig. 1. The amount of particles found in unstimulated samples was set at 100%, and data were calculated as the percentage of these controls. Data represent mean ± SD of five (B) or three (D) independent experiments. Significant differences (*, p < 0.04; **, p < 0.018) between PF-4-treated and untreated samples are indicated.
can be produced by different cell types like T cells or macrophages (37, 38). Moreover, RANTES is stored in platelet α-granules and released together with PF-4 from activated platelets (29), which strongly suggests that cooperation between these chemokines may occur under physiological conditions. Cooperation of PF-4 in the induction of oxidative stress is not limited to soluble stimuli. In human and murine macrophages, PF-4 priming led to a synergistic dose-dependent augmentation of latex-induced oxidative burst (Fig. 6). Thus, our data provide evidence that PF-4 not only increases uptake of microorganisms but also elevates and improves their intracellular killing. Such mechanisms would fit precisely into a role for PF-4 as a first-line mediator of host defense.

Apart from its function in the generation of cytotoxic oxygen radicals, PF-4 plays a prominent role in the enhancement of monocyte and macrophage phagocytosis. PF-4 not only increased the number of monocytes taking up particles but also enhanced the amount of particles ingested by the cells. This was different from effects observed after PF-4 priming with GM-CSF-differentiated human or M-CSF-treated murine macrophages, where the number of active phagocytes remained constant and only their individual phagocytic activity was augmented (Fig. 6). PF-4 treatment of human phagocytes had no effect on receptor-mediated phagocytosis of IgG-coated erythrocytes (data not shown). This may be explained by the fact that during the initial steps of immune defense, no specific Abs are present and, thus, uptake of invaders must be processed predominantly by unspecific phagocytosis.

PF-4 displays several features that render it unique within the family of chemokines. As with neutrophils and T cells (15, 16), highly purified PF-4 lacks any chemotactic activity for monocytes. Controversial results reported in the past (27) may be explained by RANTES contamination of PF-4 preparations at that time, when purification methods were much less sophisticated than nowadays. Furthermore, in contrast to chemotaxins (12), PF-4-mediated activation of monocytes is transduced independently of PTX-sensitive Gi proteins and does not involve changes of intracellular calcium concentrations (Fig. 2). These data are in line with previous findings for PF-4 with NK cells, T cells, and neutrophils (15, 16, 39). Moreover, in the latter cell type, we could demonstrate, that the coordinated activation of Src kinases is an essential step in PF-4-mediated signaling (40). The participation of Gs proteins in PF-4-induced monocyte activation was not further considered in this study. The fact that pharmacological activation of this type of G protein by cholera toxin does not induce but rather inhibits oxygen radical formation in monocytes strongly argues against a potential role for Gs proteins in this process (41).

The structure and identity of PF-4-receptors on monocytes remain unclear. A promising candidate was the most recently discovered CXCR3B, an alternative splice variant of the CXCR3, mainly unclear. A promising candidate was the most recently discovered CXCR3B, an alternative splice variant of the CXCR3, mainly unclear. A promising candidate was the most recently discovered CXCR3B, an alternative splice variant of the CXCR3, mainly unclear. A promising candidate was the most recently discovered CXCR3B, an alternative splice variant of the CXCR3, mainly unclear. A promising candidate was the most recently discovered CXCR3B, an alternative splice variant of the CXCR3, mainly unclear. A promising candidate was the most recently discovered CXCR3B, an alternative splice variant of the CXCR3, mainly unclear. A promising candidate was the most recently discovered CXCR3B, an alternative splice variant of the CXCR3, mainly unclear.

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