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Expression of the Chemokine Receptors CXCR1 and CXCR2 on Granulocytes in Human Endotoxemia and Tuberculosis: Involvement of the p38 Mitogen–Activated Protein Kinase Pathway

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The chemokine receptors CXCR1 and CXCR2 critically determine the functional properties of granulocytes. To obtain insight in the regulation of these receptors during infection, CXCR expression was determined on blood granulocytes by fluorescence-activated cell sorter analysis in healthy subjects intravenously injected with lipopolysaccharide (LPS) and in patients with active tuberculosis. In healthy subjects, LPS induced a transient decrease in granulocyte CXCR1 and CXCR2 expression, whereas in tuberculosis patients, only CXCR2 showed reduced levels. In whole blood in vitro, LPS, lipoarabinomannan from *Mycobacterium tuberculosis,* and lipoteichoic acid from *Staphylococcus aureus* reduced expression of CXCR2 but not of CXCR1. CXCR2 down-regulation induced by LPS or tumor necrosis factor–α in vitro was abrogated by a p38 mitogen–activated protein kinase (MAPK) inhibitor. Granulocytes may down-regulate CXCR2 and, to a lesser extent, CXCR1 at their surface upon their first interaction with mycobacterial or bacterial pathogens by a mechanism that involves activation of p38 MAPK.

Migration of granulocytes to the site of an infection is an important feature of the innate immune response to invading microorganisms [1]. Once at the infectious source, granulocytes mediate an array of antimicrobial activities, including the release of reactive oxygen species and proteolytic enzymes. These early inflammatory responses to infection are regulated by CXC chemokines, a family of small proteins with strong chemotactic activity toward granulocytes. Members of the CXC chemokine family that stimulate granulocyte functions include interleukin (IL)–8; growth-related oncogenes (GRO)–α, GRO-β, and GRO-γ; neutrophil-activating peptide (NAP)–2; and epithelial-derived neutrophil attractant (ENA)–78 [2, 3]. Granulocytes express 2 types of CXC chemokine receptors that interact with these mediators: CXCR1, which exclusively binds IL-8, and CXCR2, which, besides IL-8, can also bind GROs, NAP-2, and ENA-78 [2, 3]. Both receptor subtypes can activate degranulation and calcium flux in response to IL-8, whereas respiratory burst and phospholipase D activation are specifically mediated by CXCR1 [4].

In vitro studies have suggested that the surface expression of CXCR1 and CXCR2 is regulated differently. Both chemokine receptors are down-regulated upon stimulation with IL-8; after removal of the stimulus, CXCR1 is rapidly and almost completely reexpressed, whereas the reexpression of CXCR2 is slow and incomplete [5, 6]. Other stimuli can also down-regulate the surface expression of CXCR2, including lipopolysaccharide (LPS), tumor necrosis factor (TNF)–α, GRO-α, C5a, and FMLP [5–9]. The effect of these stimuli on CXCR1 expression was inhibitory in some studies [8, 9], whereas no effect on CXCR1 could be demonstrated in other investigations [6, 7].

Granulocyte CXC chemokine receptors also become down-regulated during in vivo infection. In patients with sepsis, only CXCR2 expression was reduced on circulating granulocytes, whereas CXCR1 levels were only modestly and nonsignificantly lower in patients than in healthy control subjects [10]. In addition, granulocytes in bronchoalveolar lavage fluid from patients with chronic lower respiratory tract infection had a lower expression of CXCR1 and CXCR2 than simultaneously obtained peripheral blood granulocytes [11]. Patients with lung tuberculosis (TB) or human immunodeficiency virus (HIV) infection (or both) also had reduced expression of CXCR1 and CXCR2 on blood granulocytes [12].

In the present study, we sought to extend these data by determining granulocyte CXC chemokine-receptor expression after in vivo exposure of healthy subjects to a low dose of LPS, after in vitro stimulation with various mycobacterial or bacterial antigens, and in patients with active pulmonary or non-

pulmonary TB. Furthermore, in additional in vitro studies, we investigated possible intracellular pathways of CXC chemokine-receptor regulation in LPS- and TNF-α–stimulated whole blood and found an important role for the p38 mitogen–activated protein kinase (MAPK) pathway.

Methods

Experimental human endotoxemia. Fifteen healthy men (mean age [±SE], 23 [±1] years) were admitted to the clinical research unit of the Academic Medical Center (Amsterdam) after documentation of good health by history, physical examination, hematological and biochemical screening, chest radiograph, and electrocardiograph. The control subjects did not smoke, used no medication, and had no febrile illness within 2 weeks before the start of the study. All study participants received a bolus intravenous injection (4 ng/kg body weight) of LPS (*Escherichia coli,* lot G; US Pharmacopeial Convention, Rockville, MD). Venous blood samples were obtained directly before the injection of LPS and 1, 2, 4, 6, and 24 h thereafter. Blood was collected in heparin-containing vials and processed immediately for flow cytometry.

Patients with TB and control subjects. Blood was obtained from 8 TB-infected patients (6 men and 2 women) attending the Academic Medical Center ($n = 5$), the Sint Lucas Hospital ($n = 2$), or the Municipal Health Center ($n = 1$) in Amsterdam. The mean age (±SE) of TB patients was 32 (±4) years and did not differ from that of 8 healthy control subjects (4 men and 4 women; mean age [±SE], 29 [±2] years). All patients had active, culture-proven TB, of whom 4 had pulmonary or extrapulmonary TB. Extrapulmonary sites for these 4 patients who had pulmonary or extrapulmonary TB included pleural tissue ($n = 2$), soft tissue ($n = 1$), and gastrointestinal tract ($n = 1$). Three TB patients were HIV seropositive and were receiving antiretroviral therapy. None of the TB patients took immunosuppressive drugs. Six patients had fever (rectal temperature >38°C). Blood for fluorescence-activated cell sorter (FACS) analysis was obtained before administration of antituberculous medication. On the same day a patient was analyzed, blood was also obtained from a healthy control subject. After collection, blood was immediately prepared for FACS analysis.

In vitro studies. For each experiment, blood was obtained from 6 healthy control subjects using a sterile collecting system consisting of a butterfly needle connected to a syringe (Becton Dickinson, Mountain View, CA) and incubated at 37°C for 8 h. Heparin (final concentration, 10 U/mL blood; Leo Pharmaceutical Products, Weesp, Netherlands) was used as an anticoagulant. Whole blood was added to sterile polypropylene tubes and diluted 1:1 with RPMI 1640 (BioWhittaker, Verviers, Belgium). LPS (from *E. coli* serotype 0111:B4; Sigma, St Louis, MO) was added for the time-course study (10 ng/mL).

In separate experiments, we added to the blood 0.01-, 0.1-, 1-, and 10-μg/mL lipoarabinomannan (LAM [kindly provided by J. T. Belisle, Colorado State University, Fort Collins, CO]; mannose-capped, isolated, and prepared from *M. tuberculosis* strain H37Rv) or 1-μg/mL lipoteichoic acid from *Staphylococcus aureus* (LTA; Sigma). Twenty minutes before being stimulated with 10-ng/mL LPS, whole blood was also incubated with inhibitors of NF-κB–dependent transcription nordihydroguaretic acid (NDGA;

Sigma) or 4-bromophenacyl bromide (4-BPB; Sigma) [13] dissolved in dimethyl sulfoxide (DMSO; Merck, Munich, Germany), until a concentration of 20 μM was reached. As a control, DMSO (in the same amount used to dissolve the inhibitors) was added to the RPMI medium.

SB203580 is a piridinyl imidazole derivative and a potent and specific inhibitor of p38 MAPK [14, 15]. SB203580 binds to the adenosine triphosphate binding site, thus preventing phosphorylation of downstream targets, including MAPK-activated protein kinase–2 and activating transcription factor–2, although not preventing phosphorylation of p38 MAPK by its upstream activators MKK3 and MKK6 [16]. PD098059 selectively inhibits the activation of p42/44 MAPK [17, 18]. SB203580 (in concentrations of 2 or 10 μM) or PD098059 (10 μM) was added to the blood 1 h before LPS stimulation. Recombinant TNF-α (provided by Knoll, Ludwigshafen, Germany) was used as a stimulus at 10 ng/mL.

Flow cytometry. Erythrocytes in blood were lysed with bicarbonate-buffered ammonium chloride solution (pH 7.4). Leukocytes were recovered after centrifugation at 20,000 *g* for 5 min and were counted. Cells (1×10^6) were resuspended in cPBS (PBS containing 100 m*M* EDTA, 0.1% sodium azide, and 5% bovine serum albumin) and were placed on ice. Triple staining was accomplished by incubation for 1 h with direct-labeled antibodies CXCR1–fluorescein isothiocyanate (CXCR1-FITC) or CXCR2-phycoerythrin (CXCR2-PE; both antibodies from R&D Systems, Abingdon, UK). Nonspecific staining was controlled for by incubation of cells with FITC- or PE-labeled mouse IgG2 (Coulter Immunotech, Marseille, France). Cells were then washed twice in ice-cold cPBS and were resuspended for flow cytofluorometric analysis (Calibrite; Becton-Dickinson Immunocytometry Systems, San Jose, CA). Data on mean cell fluorescence (MCF) intensity are represented as the difference between MCF intensities of specifically and nonspecifically stained cells.

Statistical analysis. All values are given as mean (±SE). Data for control subjects receiving endotoxin were analyzed by one-way analysis of variance. Data for TB patients and from in vitro stimulations were analyzed using Wilcoxon rank sum test. $P < .05$ was considered statistically significant.

Results

Human endotoxemia. Injection of LPS was associated with transient influenza-like symptoms, including headache, chills, vomiting, myalgia, and fever (peak temperature, 38.8°C [±0.3°C] after 3 h). Intravenous LPS induced a biphasic change in granulocyte counts in peripheral blood, characterized by an initial neutropenia after 1 h and followed by a neutrophilia (table 1). These changes were accompanied by a decrease in the expression of CXCR1 on circulating granulocytes. The MCF decreased from a baseline level of 663.0 (±67.3) to 341.3 (±44.3) ($P = .001$), reaching a nadir after 2 h and returning to the initial level of expression after 24 h (figure 1). LPS induced a more profound down-regulation of CXCR2 on circulating granulocytes. The MCF decreased from 1404.3 (±281.80) to 255.4 (±49.9) at 2 h ($P < .001$) and returned to baseline after 24 h (figure 1).

Patients with TB. Peripheral blood granulocytes of patients

Table 1. Effect of intravenous lippolysaccharide (LPS) on granulocyte counts of healthy volunteers at various hours after administration.

Time, h	Leukocytes	Granulocytes
0	5.0 ($\pm$0.4)	2.7 ($\pm$0.3)
1	2.5 ($\pm$0.1)	0.7 ($\pm$0.1)
2	3.8 ($\pm$0.3)	3.0 ($\pm$0.5)
4	7.4 ($\pm$0.7)	7.0 ($\pm$0.7)
6	14.1 ($\pm$0.7)	10.2 ($\pm$0.8)
24	6.4 ($\pm$0.5)	8.6 ($\pm$0.8)

NOTE. Data are mean ($\pm$SE) of 8 volunteers $\times 10^9$/L. LPS (4 ng/kg) was given intravenously at 0 h.

with TB demonstrated a reduced expression of CXCR2 but not of CXCR1, compared with that of healthy control subjects (figure 2).

Whole blood stimulation with mycobacterial or bacterial agents. Previous studies have documented that LPS can down-regulate the expression of granulocyte CXCR1 and CXCR2 in vitro [8, 9]. The effect of other bacterial antigens on CXC chemokine receptors is unknown. Therefore, we wished to determine the effect of LPS, LAM (a cell wall component of *Mycobacterium tuberculosis*), and LTA (a cell wall component of *S. aureus*) on the expression of CXCR1 and CXCR2 on granulocytes. In a first series of in vitro experiments, we found that LPS (10 ng/mL) induced a profound reduction in granulocyte CXCR2 expression, whereas down-regulation of CXCR1 was modest (data not shown). Since a maximal effect was observed after 1- to 2-h stimulations, further incubations were done for 1 h. All mycobacterial or bacterial stimuli induced a down-modulation of CXCR2, whereas CXCR1 levels remained unaltered (figure 3). The effect of LAM was dose-dependent, that is, LAM doses of 0.01 and 0.1 μg/mL did not produce consistent effect, but LAM at doses of 1 or 10 μg/mL reduced CXCR2 expression by 18.4% ($\pm$3.3%) and 25.8% ($\pm$4.2%), respectively, relative to incubation with RPMI.

Role of NFκB, p42/44 MAPK, and p38 MAPK. To investigate the molecular mechanism resulting in the down-regulation of CXCR2, we performed additional whole blood incubations. Recently, we have shown that NDGA and 4-BPB abrogate NFκB

activation [13]. In the current study, neither compound influenced LPS-induced down-regulation of CXCR2 on granulocytes (figure 4, *upper panel*). Also, the p42/44 MAPK inhibitor PD098059 did not prevent LPS-induced CXCR2 down-regulation. The p38 MAPK inhibitor SB203580 (2 μM), however, partially prevented the reduction in CXCR2 expression induced by LPS (figure 4, *lower panel*). This was confirmed in an additional experiment in which a higher concentration of SB203580 (10 μM) was used (figure 5). As reported elsewhere [7–9], stimulation with TNF-α also resulted in a down-regulation of CXCR2. When coincubated with SB203580, this effect was abrogated.

Discussion

The CXC chemokine receptors CXCR1 and CXCR2 play an important role in the recruitment of granulocytes to the site of an infection and in the activation of granulocyte antimicrobial effector mechanisms [19]. Here we report that in vivo administration of low-dose LPS to healthy subjects induces a down-modulation of both receptors on circulating granulocytes. Patients with active TB showed only a reduced expression of granulocyte CXCR2. In vitro, both bacterial and mycobacterial antigens could down-modulate granulocyte CXCR2 but not CXCR1, which, in the case of the LPS effect, was mediated (at least in part) by the p38 MAPK pathway. Selective down-modulation of granulocyte CXCR2 but not of CXCR1 was previously observed in patients with sepsis [10]. Granulocytes isolated from these patients demonstrated a markedly suppressed chemotactic response to the CXCR2 ligands ENA-78 and GRO-α, GRO-β, and GRO-γ, whereas the response of the CXCR1 ligand IL-8 remained intact [10]. Therefore, it seems likely that the down-regulation of granulocyte CXCR2 may have functional consequences.

Until now, the effect of LPS on granulocyte CXC chemokine receptors has been investigated in only in vitro experiments, in which LPS was found to down-modulate both CXCR1 and CXCR2 [8, 9], presumably by reducing constitutive transcrip-
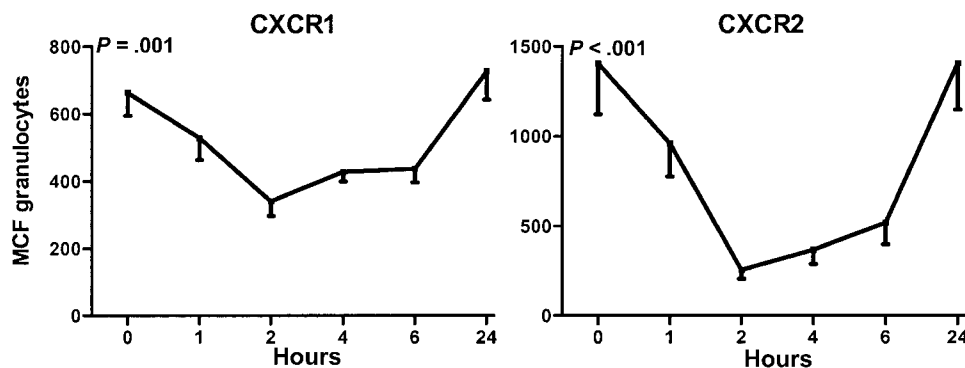

Figure 1. Expression of granulocyte CXCR1 and CXCR2 in 15 healthy subjects injected intravenously with 4 ng/kg lipopolysaccharide. Data are mean ($\pm$SE) difference between specific and nonspecific mean cell fluorescence (MCF). *P* values are for change in time.
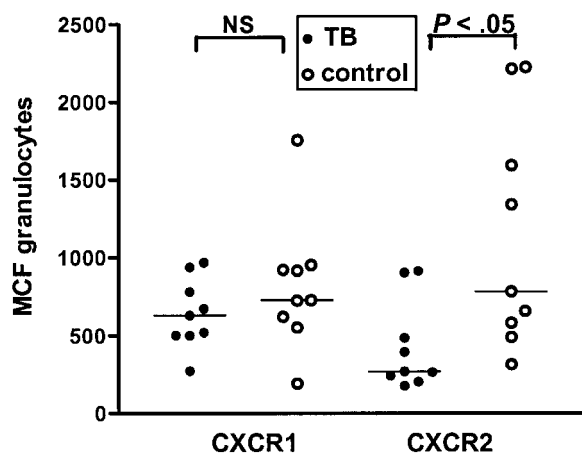
Figure 2. Expression of granulocyte CXCR1 and CXCR2 in 8 patients with tuberculosis (TB) and in 8 healthy control subjects. Data are mean ($\pm$SE) difference between specific and nonspecific mean cell fluorescence (MCF). NS, not significant.

tion of the genes that encode these receptors by a combination of transcriptional inhibition and decreasing mRNA stability [20]. We found a consistent in vitro effect of LPS only on CXCR2 expression, whereas the LPS-induced reduction of CXCR1 expression varied from experiment to experiment. The apparent discrepancy with the studies by Khandaker et al. [8, 9] may be explained by differences in the experiment design (i.e., they first isolated neutrophils and then stimulated them with 100 ng/mL LPS, whereas we stimulated whole blood with 10 ng/mL LPS and performed FACS analysis on unseparated white blood cells). It should be noted that other stimuli, such as GRO-α [5] and TNF-α [7], as well as C5a and FMLP [6], have been reported to down-regulate CXCR2 but not CXCR1 on granulocytes in vitro. Clearly, the expression of CXCR1 and CXCR2 is regulated differently, whereby CXCR1 is rapidly and virtually completely reexpressed after down-regulation by its

ligand IL-8, whereas the reappearance of CXCR2 is slow and incomplete [5, 6].

Of interest, in vivo LPS did reduce the expression of both CXC chemokine receptors, although the effect on CXCR2 was more profound. Previously, patients with sepsis were reported to have decreased granulocyte expression of CXCR2 but not of CXCR1 [10]. Conceivably, our model of human endotoxemia, during which multiple blood samples were obtained before and shortly after injection of LPS, allowed for a closer study of the kinetics of the alterations in granulocyte chemokine receptor expression. A possible change in CXCR1 expression during clinical sepsis may only be present transiently, such as that in vitro studies [5, 6], and may be missed when blood is obtained from patients on admission to the hospital (i.e., at a relatively late time point). It should be noted that FACS analysis of granulocytes obtained from healthy subjects exposed to LPS or from patients with TB only provide information about cells still present in the circulation. It is possible theoretically that decreases in CXCR expression on peripheral blood granulocytes after injection of LPS are caused by marginalization of granulocytes that do express these receptors. However, we consider this possibility less likely, since ~98%–100% of peripheral blood granulocytes stained positive for CXCR1 and CXCR2 both before and after LPS injection (data not shown) and the nadir in CXCR expression was found 2 h after LPS injection (i.e., at a time when granulocyte counts had already returned to baseline).

Granulocytes may also be of importance in the initial defense against *M. tuberculosis*. Patients with active TB have increased numbers of granulocytes in bronchoalveolar lavage fluid obtained from the site of infection [21, 22], and granulocytes are considered to contribute to granuloma formation [23]. We therefore considered it of interest to evaluate CXC chemokine receptors in patients with TB. We found that only CXCR2 displayed a reduced expression on circulating granulocytes, whereas CXCR1 levels were similar to those in healthy control
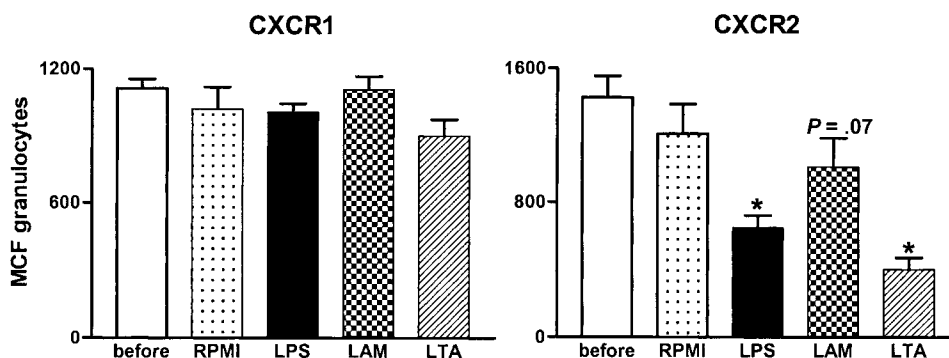
Figure 3. Expression of granulocyte CXCR1 and CXCR2 after whole blood stimulation with 10 ng/mL lipopolysaccharide (LPS), 1 μg/mL lipoarabinomannan (LAM), or 1 μg/mL lipoteichoic acid (LTA) for 1 h. Data are mean ($\pm$SE) difference between specific and nonspecific mean cell fluorescence (MCF) of 6 donors. $P < .05$ for difference with incubation of whole blood with RPMI only. "Before" refers to expression before incubation.
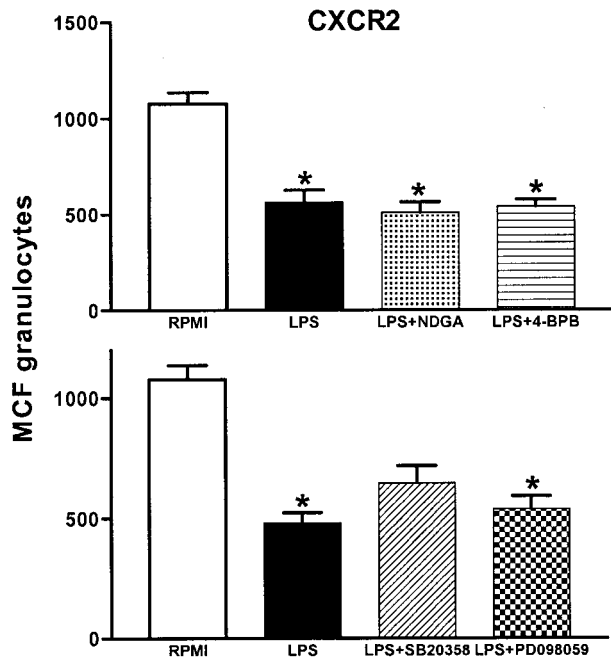
CXCR2

Figure 4. Expression of granulocyte CXCR2 after whole blood stimulation with 10 ng/mL lipopolysaccharide (LPS) coincubated with NFκB inhibitors nordihydroguaretic acid (NDGA; 20 μM) and 4-bromophenacyl bromide (4-BPB; 20 μM) or with inhibitors of the p38 (SB203580; 2 μM) or the p42/44 (PD098059; 10 μM) mitogen-activated protein kinase pathways. Data are mean ($\pm$SE) difference between specific and nonspecific mean cell fluorescence (MCF) of 6 donors. $P < .05$ for difference with incubation of whole blood with RPMI alone.

subjects. While our studies were in progress, Meddows-Taylor et al. [12] reported reduced expression of both CXCR1 and CXCR2 on granulocytes of patients with pulmonary TB with or without HIV infection [12]. The most obvious differences with our study were that in the earlier investigation, patients only had pulmonary TB and had already been treated with antituberculous drugs for various periods. Considering that both the study by Meddows-Taylor et al. [12] and our current investigation involved relatively few patients with TB, the results on granulocyte CXCR expression in TB require further confirmation in a larger population of patients with TB before and after treatment. In accordance with our in vivo findings, LAM, an immunogenic component of the cell wall of *M. tuberculosis,* only reduced CXCR2 on granulocytes in vitro.

Since little is known about the effect on CXC chemokine receptors of infectious stimuli other than LPS from *E. coli,* we also determined the influence of LTA (the cell-wall component of *S. aureus*) on granulocyte levels of CXCR1 and CXCR2 in vitro. LTA reproduced the LPS effect in vitro (i.e., down-modulation of CXCR2 but not of CXCR1). Additional studies with other bacterial products (e.g., peptidoglycan) or intact microorganisms are warranted to determine whether the down-regulation of

CXCR2 is a general response of the granulocyte to an invading microorganism.

The involvement of metalloproteinases in LPS-induced down-modulation of CXCR1 and CXCR2 on granulocytes was demonstrated recently in vitro [9], and inhibitors of tyrosine kinases also can reduce the down-regulation of CXC chemokine receptors [8, 24], presumably at least in part by abrogating metalloproteinase activation [25, 26]. Here we report the role of the p38 MAPK pathway in the regulation of CXCR2 on granulocytes. A number of inflammatory mediators, including LPS and TNF-α, has been found to activate this signaling cascade in neutrophils [27–29]. Previous studies indicated that activation of p38 MAPK may be involved in various granulocyte effector functions, such as the up-regulation of β_2 integrins [30], chemotaxis, and oxidative burst [28]. In this study, inhibition of p38 MAPK resulted in inhibition of CXCR2 down-regulation induced by either LPS or TNF-α. Inhibition of the p42/p44 MAPK pathway did not influence CXCR2 levels, which is in accordance with earlier findings that LPS does not activate this pathway in neutrophils [29].
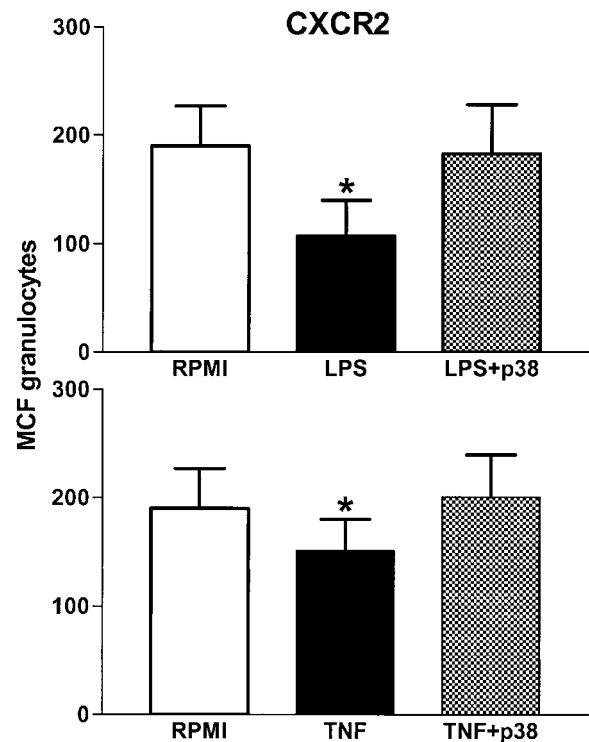
CXCR2

Figure 5. Expression of granulocyte CXCR2 after whole blood stimulation with 10 ng/mL lipopolysaccharide (LPS) or 10 ng/mL recombinant tumor necrosis (TNF)–α coincubated with 10 μM p38 mitogen-activated protein kinase pathway inhibitor SB203580. Data are mean ($\pm$SE) difference between specific and nonspecific mean cell fluorescence (MCF) of 6 donors. $P < .05$ for difference with incubation of whole blood with RPMI alone.

In general, LPS-regulated expression of cytokines and their receptors is dependent on the activation of NFκB [31]. Neither NDGA nor 4-BPB, originally identified as inhibitors of arachidonate metabolism but now also known to be potent inhibitors of NFκB-dependent transcription [13], impaired CXCR2 downregulation. Therefore, alternative pathways mediate this effect. Receptors are constantly being produced and exported to the plasma membrane and subsequently internalized and degraded in the lysosome. p38 MAPK may be implicated in each one of these processes. In this respect, it should be noted that endocytosis coincides with p38 MAPK activation [32], which may be essential for receptor degradation. More experimental work is necessary to corroborate this suggestion.

CXC chemokine receptors critically determine many proinflammatory granulocyte functions. The present study and previous observations indicate that CXCR2 and, to some extent, CXCR1 become down-regulated upon the first encounter with a bacterial or mycobacterial pathogen. We previously reported the down-regulation of TNF-α and IL-1 receptors on granulocytes upon stimulation with bacterial antigens [33, 34]. It is conceivable that these responses reflect an attempt of the host to limit excessive inflammation induced by granulocytes at the site of an infection.

Acknowledgment

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