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Granulocyte-Macrophage Colony Stimulating Factor Up-Regulates CCR1 in Human Neutrophils¹

Sara S. Cheng,^{*†} Joyce J. Lai,[‡] Nicholas W. Lukacs,^{*} and Steven L. Kunkel^{2*†}

Neutrophils (polymorphonuclear leukocytes; PMN) are phagocytic cells instrumental in the clearance of infectious pathogens. Human PMN are commonly thought to respond primarily to chemokines from the CXC family. However, recent findings suggest that under specific cytokine activation conditions, PMN can also respond to some CC chemokines. In this study, the effect of GM-CSF, a well-characterized PMN priming and maturation factor, on CC-chemokine receptor (CCR) expression in PMN was investigated. Constitutive expression of CCR1 and CCR3 mRNA in PMN was detected by ribonuclease protection assay. Following incubation of PMN with GM-CSF (0.01–10 ng/ml; 6 h) CCR1 mRNA expression was rapidly (~1 h) up-regulated. In contrast, no significant induction of CCR2, CCR3, CCR4, or CCR5 mRNA was observed. CCR1 protein was also up-regulated by GM-CSF stimulation. GM-CSF-induced up-regulation of CCR1 showed functional consequences because GM-CSF-treated PMN, but not control cells, responded to the CC chemokines macrophage inflammatory protein-1 α , monocyte chemoattractant protein-3, and RANTES in assays of chemotactic migration and intracellular calcium mobilization. These results suggest that PMN activated by the proinflammatory cytokine GM-CSF can change their receptor expression pattern and become responsive to CC chemokines. *The Journal of Immunology*, 2001, 166: 1178–1184.

Inflammatory processes involve the local production of cytokines that cause the selective recruitment of leukocyte subsets. Chemokines, a superfamily of chemoattractant cytokines, play a key role in leukocyte trafficking under both inflammatory and homeostatic conditions (1). These small, basic, secreted peptides are classified into four different groups (CXC, CC, C, and CX₃C) according to the number and spacing of conserved cysteines at the amino terminus. The CXC chemokines have mainly been characterized as neutrophil and T lymphocyte chemoattractants (2), while the CC chemokines act on a wider spectrum of cell types including monocytes, eosinophils, basophils, NK cells, and T lymphocytes (3). Lymphotactin, the sole member of the C chemokine group, acts on lymphoid cells (4), while the only CX₃C chemokine, fractalkine, acts on both lymphoid cells and polymorphonuclear leukocytes (PMN)³ (5).

The physiological effects of chemokines are exerted through binding of a family of seven transmembrane receptors, which couple to G-protein-mediated pathways within the cell (3). Ten human CC receptors and five human CXC receptors have been identified to date (3, 6). The expression pattern of chemokine receptors is a major factor in dictating the selectivity of chemokines for different

target cells. Traditionally, human PMN have been thought to express receptors from the CXC (2) or CX₃C (7, 8) family and to preferentially migrate to chemokine ligands from only these two families (2, 9). However, different studies have provided conflicting evidence on whether human PMN express CCRs. Although Xu et al. (10) have found that resting human PMN possess binding sites for the CC chemokines macrophage inflammatory protein (MIP)-1 α and monocyte chemoattractant protein (MCP)-3, a study by McColl et al. (11) did not detect any binding sites for CC chemokines on these cells. Furthermore, human PMN do not respond to the CC chemokine MIP-1 α in *in vitro* assays of chemotaxis (11, 12). Therefore, it is generally assumed that CC chemokines have no functional effects on resting human PMN.

In contrast to findings in human PMN, several murine models of inflammation have shown that CC chemokines do play a role in PMN chemotaxis. For example, *in vivo* studies of LPS- and endotoxemia-associated lung injury in mice have demonstrated that neutralization of MIP-1 α attenuates neutrophil infiltration into inflammatory sites (13, 14). Furthermore, PMN isolated from inflammatory exudates in mice display chemotactic migration and calcium flux responses to MIP-1 α (15).

A growing body of literature demonstrates that cytokines regulate chemokine receptor expression on a variety of different cell types. Pro-inflammatory mediators such as TNF- α , IL-1 β , and LPS down-regulate CCR2 on human monocytes (16), while the anti-inflammatory cytokine IL-10 up-regulates CCR1, CCR2, and CCR5 on these cells (17). Chemokine receptor expression on T lymphocytes is also modulated by cytokines, as studies have shown that IL-2 increases CCR1 (18), CCR2 (18), and CXCR3 (19), IL-10 decreases CCR5 (20), and IL-15 induces CCR1, CCR2, CCR4, and CCR5 on these cells (21). PMN expression of CXC chemokine receptors is also regulated by pro-inflammatory factors such as TNF (22, 23) and LPS (16, 24), which both down-regulate CXCR1 and CXCR2. In contrast, G-CSF (not GM-CSF) induces the expression of CXCR1 and CXCR2 on PMN (25).

The present study examines the effects of GM-CSF, a pro-inflammatory cytokine that is a potent activator of PMN and other granulocytes (26). This cytokine is already well known for its role

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³ Abbreviations used in this paper: PMN, polymorphonuclear leukocytes; MIP, macrophage inflammatory protein; MCP, monocyte chemoattractant protein.

in neutrophil maturation and priming (26, 27), as well as its ability to slow the apoptotic rate of PMN *in vitro* (28). In this study we demonstrate that GM-CSF induces CCR expression in PMN, suggesting that the *in vivo* maturation and priming of these cells involves expression of CC-type chemokine receptors, and that CC chemokines can exert direct effects upon activated human PMN.

Materials and Methods

Cell culture and cytokine/chemokine reagents

Recombinant human GM-CSF, MIP-1 α , MIP-1 β , MCP-1, MCP-3, MCP-4, RANTES, and IL-8 were purchased from PeproTech (Rocky Hill, NJ). Recombinant human eotaxin was purchased from R&D Systems (Minneapolis, MN). HBSS was obtained from BioWhittaker (Walkersville, MD). Complete medium consisted of RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 2 mM L-glutamine, 25 mM HEPES, 100 U/ml penicillin, 100 μ g/ml streptomycin (Hazelton Research Products, Lenexa, KS), and 10% heat-inactivated FCS (Life Technologies).

Cell isolation

PMN were isolated from human peripheral blood as previously described (29). Briefly, peripheral venous blood from healthy volunteers was drawn into heparinized syringes, mixed 1:1 with sterile saline, and layered over Ficoll-Paque (Pharmacia LKB, Piscataway, NJ) columns. These columns were centrifuged at 1500 rpm for 30 min at room temperature, the plasma and mononuclear cell layer was discarded, and the erythrocytes were eliminated by hypotonic lysis in ammonium chloride buffer. PMN were washed once in HBSS/BSA (HBSS without calcium or magnesium, with 0.1% BSA added) and resuspended in complete medium. Cell purity, assessed by Diff-Quik (Baxter, Rome, Italy) staining and light microscopy, was routinely >95% with the major contaminant being eosinophils. Viability as assessed by trypan blue exclusion was >99% for freshly isolated cells.

RNase protection assay

Total RNA was isolated from PMN using the guanidine isothiocyanate method as previously described (30) and used in the standard Pharmingen (San Diego, CA) RNase protection protocol as follows. The multiprobe template set hCR5, containing DNA templates for CCR1, CCR3, CCR4, CCR5, CCR2a+b, CCR2a, CCR2b, L32, and GAPDH, was purchased from Pharmingen. This template set was used to synthesize [α -³²P]UTP (Amersham, Buckinghamshire, U.K.)-labeled probes in the presence of a GACU pool using a T7 RNA polymerase. Probes were hybridized overnight with 5–10 μ g target RNA, followed by RNase digestion and proteinase K treatment. Samples were chloroform-extracted, ethanol precipitated in the presence of ammonium acetate, and loaded on an acrylamide-urea sequencing gel made in 0.53 M Tris-borate/EDTA (TBE) buffer. After electrophoresis at 50 watts for 1–2 h, the gel was adsorbed to filter paper, dried under vacuum, and exposed to film (X-OMAT, Kodak, Rochester, NY) with intensifying screen at -70°C . Alternatively, the dried gel blot was exposed to a phosphorimager for phosphorimager analysis using ImageQuant software. The intensity of each band was adjusted for the number of UTPs present in each of the probe sequences. These adjusted values were normalized to the adjusted values of the housekeeping gene L32, and normalized values were used to quantify expression as fold-increase over control.

Protein isolation and Western blot analysis

Protein extracts were isolated from cultured neutrophils as follows: 2×10^7 cells were pelleted and washed once with PBS and once in buffer A (10 mM HEPES, 10 mM KCl, 0.5 mM DTT). The cell pellet was lysed in 20 μ l buffer A plus 1% Nonidet P-40 and incubated for 10 min at 4°C . Samples were then spun at $16,000 \times g$ in a microcentrifuge for 2 min. The supernatant was removed, mixed with 55 μ l buffer C (20 mM HEPES, 500 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, 1.5 mM MgCl_2 , 20% glycerol), and saved as cytoplasmic extract. One-hundred micrograms of protein per sample was analyzed by Western blot. Proteins were separated by 12% SDS-PAGE and blotted onto Sequi-Blot polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA) by electrotransfer. Membranes were blocked with 5% milk and stained with rabbit polyclonal Abs raised against human CCR1 or CCR3 (Santa Cruz Biotechnology, Santa Cruz, CA), followed by a peroxidase-conjugated goat Ab against mouse IgG (Pierce, Rockford, IL). Signals were visualized by chemiluminescence using super signal reagents (Pierce).

Measurement of intracellular free calcium

Intracellular calcium levels were monitored using standard fluorometric techniques (31). Cells were loaded with the fluorescent dye fura-2AM (Sigma, St. Louis, MO) as follows: PMN were suspended in HBSS/BSA (without Ca^{+2} or Mg^{+2}) containing 2.5 μM fura-2AM and incubated at 37°C for 30 min. Cells were washed three times in HBSS/BSA and resuspended in same buffer with addition of 1 mM Mg^{+2} and 0.1 mM Ca^{+2} . Extracellular Ca^{+2} was restored to 1 mM immediately before the assay. Fluorescence was monitored at the excitation wavelengths 340 and 380 nm and emission 510 nm in a Perkin-Elmer (Norwalk, CT) LS 50B luminescence spectrometer using FL WinLab software (Perkin-Elmer). Baseline fluorescence was monitored for 30 s before the addition of agonists in small volume (<50 μ l). Calcium index is expressed as the ratio of emission intensity at excitation wavelength 340 nm divided by emission excited at 380 nm.

Chemotaxis assays

Chemotactic activity was monitored using a chemotaxis microchamber technique as previously described (32). Briefly, chemoattractant solution or control medium (HBSS containing Mg^{+2} and Ca^{+2} plus 0.1% BSA) was added to the lower wells of a 12-well chemotaxis chamber (Neuroprobe, Pleasanton, CA), a polycarbonate polyvinylpyrrolidone-free PVPF filter with a 5- μM pore size (Poretics, Livermore, CA) layered on top, and the top plate was affixed. A cell suspension of 2×10^6 PMN/ml was seeded into the upper well and the whole chamber was incubated at 37°C in air with 5% CO_2 for 60 min. Filters were removed and the upper sides were scraped clean and stained with Diff-Quik (Baxter). All treatments were performed in triplicate wells. The number of PMNs in three high-power (1000 \times) oil-immersion fields were counted for each well. Chemotactic index (CI) is defined as follows: CI = mean no. of cells counted per high-power oil-immersion field \pm SEM.

Results

GM-CSF regulates CCR mRNA and protein expression

To assess the effect of GM-CSF on CCR mRNA expression, we performed RNase protection assays on PMN treated with increasing concentrations of GM-CSF (0.01–10 ng/ml, 6 h; Fig. 1, A and B). CCR1 and CCR3 mRNA expression was detected in cells incubated in medium alone (6 h). Following incubation with GM-CSF, CCR1 expression was up-regulated, with an effect being apparent at a GM-CSF concentration of 0.01 ng/ml. In contrast, PMN treated with the same concentration range of GM-CSF did not show an increase in CCR3 expression above levels detected in control cells. CCR2, CCR4, and CCR5 expression were not detected under any of the conditions tested (Fig. 1A). Up-regulation of CCR1 expression by GM-CSF treatment (10 ng/ml) occurred rapidly, with increases being detectable by 30 min after addition of the stimulus (Fig. 2, A and B). Maximal CCR1 mRNA expression was detected between 1–2 h post-GM-CSF treatment, and declined to basal levels by 8 h. No changes in CCR1 or CCR3 expression were measured in cells treated with medium alone over this time period. Because IFN- γ has previously been shown to induce CCR1 expression in neutrophils (33), we also investigated whether GM-CSF and IFN- γ had a synergistic effect on CCR1 expression. These two cytokines did not synergize in inducing CCR1 expression in these cells, instead demonstrating additive effects (data not shown).

Because CCR1 mRNA was induced upon GM-CSF stimulation, Western blots were conducted to determine whether GM-CSF treatment also up-regulated CCR1 protein. All control cells showed detectable expression of CCR1, while treatment with 10 ng/ml GM-CSF consistently increased the intensity of this band (Fig. 3). CCR3 expression in control cells was variable in the four donors tested, with an immunoreactive band detectable in one donor (Fig. 3) and undetectable in three donors (data not shown). In all cases, treatment with GM-CSF did not yield a detectable increase in CCR3 protein expression, including the experiment in which control cells expressed CCR3 (Fig. 3). Thus, we have shown

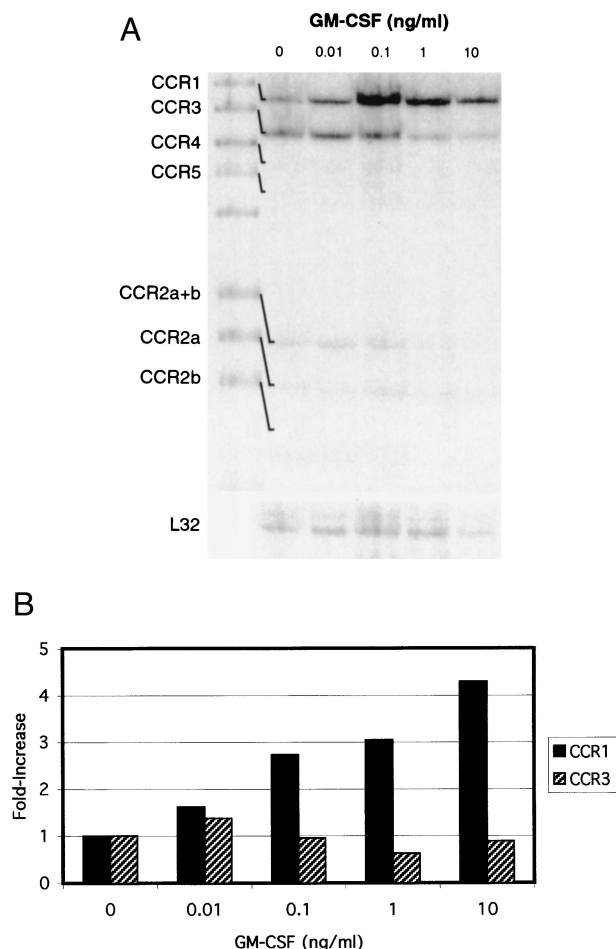


FIGURE 1. *A* and *B*, Effect of GM-CSF on the expression of chemokine receptors in PMN. Total RNA (10 μ g) was isolated from PMN after treatment for 6 h in culture medium or increasing concentrations of GM-CSF (0.01–10 ng/ml). Samples were analyzed by RNase protection assay using the template set hCR-5, which generates probes for CCR1, CCR2a, CCR2b, CCR3, CCR4, CCR5, L32, and GAPDH (see *Materials and Methods*). *A*, Blot showing expression of CCR1–5 in GM-CSF-treated PMN is representative of three individual experiments using cell preparations from different donors. The first lane shows migration of unprotected probes. Expression of the housekeeping gene L32 is shown as a measure of equal RNA loading. *B*, Quantitative analysis of CCR1 and CCR3 expression levels on blot in *A* was performed using phosphorimager analysis (see *Materials and Methods*).

that GM-CSF up-regulated CCR1 mRNA and protein, but did not affect CCR3 mRNA or protein expression.

CC chemokines induce calcium transients in GM-CSF-treated PMN

Because GM-CSF selectively up-regulated the expression of CCR1, we sought to determine whether CCR could be activated in cells treated with GM-CSF. Because chemokine-induced calcium responses are often used as a measure of chemokine receptor activation, we used standard fluorometric techniques to assess chemokine-induced calcium transients in GM-CSF-stimulated PMN. PMN cultured in GM-CSF for 16 h showed transient intracellular calcium increases in response to three known CCR1 agonists (34, 35), MIP-1 α , MCP-3, and RANTES (100 ng/ml), but not to eotaxin, MCP-1, MCP-4, or MIP-1 β (Fig. 4). Because MIP-1 α , MCP-3, and RANTES all bind to CCR1, this result correlated with GM-CSF induction of CCR1 mRNA and protein expression (Figs.

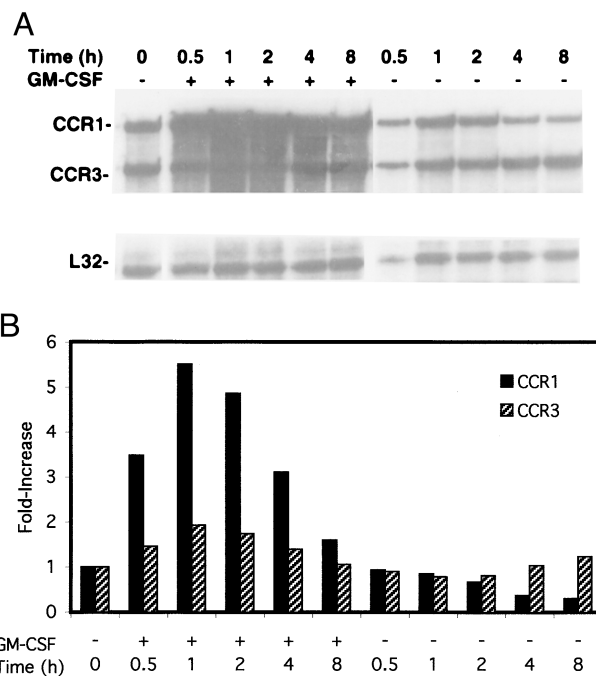


FIGURE 2. *A* and *B*, Time course of chemokine receptor expression in GM-CSF-treated PMN. Total RNA (10 μ g) was isolated from freshly isolated PMN and PMN cultured in medium alone or 10 ng/ml GM-CSF for the indicated times. *A*, Blot showing CCR1, CCR3, and L32 expression is representative of three individual experiments using cell preparations from different donors. Expression of the housekeeping gene L32 is shown as a measure of equal RNA loading. *B*, Quantitative analysis of the expression levels on blot in *A* was performed using phosphorimager analysis (see *Materials and Methods*).

1–3). Although constitutive CCR3 expression was observed in resting and GM-CSF-stimulated PMN (Fig. 1), the CCR3-specific ligand eotaxin did not stimulate intracellular calcium mobilization in this assay (Fig. 4). Control PMN cultured in medium alone for 16 h did not respond to any of the CC chemokines tested (Fig. 4, untreated). The effect of GM-CSF on calcium responses was time-dependent, as cells treated for less than 16 h did not show calcium transients in response to CC chemokines (data not shown). MIP-1 α , MCP-3, and RANTES stimulated intracellular calcium increases in a concentration-dependent fashion (100–500 ng/ml; Fig. 5), with the minimum effective concentration being ~100 ng/ml. GM-CSF treatment enhanced IL-8-induced calcium responses, an effect probably

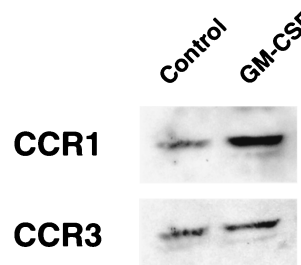


FIGURE 3. Western blot analysis of CCR1 protein expression. Cytoplasmic protein extracts (100 μ g) were isolated from PMN cultured in medium alone or 10 ng/ml GM-CSF for 16 h. Proteins were separated by SDS-PAGE, blotted onto membranes, and stained with polyclonal Abs specific for human CCR1 or CCR3. The results shown are representative of data collected from four individual experiments using cells from different donors.

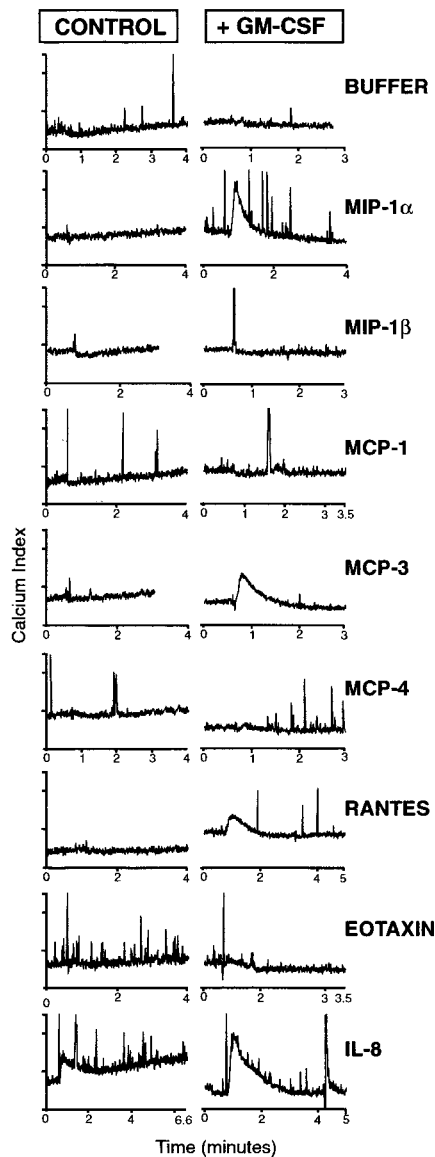


FIGURE 4. Calcium mobilization in GM-CSF-treated PMN. Cells were cultured for 16 h with or without 10 ng/ml GM-CSF, loaded with fura-2AM, and stimulated with various CC chemokines at 200 ng/ml. Responses were monitored for 5 min following the addition of chemokine. Calcium index, expressed as the ratio of emission at 340/380, is indicative of intracellular calcium concentration. Data shown are representative of three individual experiments with cells from different donors.

due to priming effects because GM-CSF has no effect on the expression of IL-8 receptors CXCR1 and CXCR2 (22).

Ligand binding to chemokine receptors is known to induce phosphorylation and internalization of the receptors (36). To ascertain whether MIP-1 α , MCP-3, or RANTES were activating intracellular calcium mobilization through the same receptor, we investigated the desensitization relationships between these three chemokines in GM-CSF-treated PMN (Fig. 6). Pretreatment of PMN with MIP-1 α (200 ng/ml) rendered the cells unresponsive to further stimulation by MCP-3 or RANTES (200 ng/ml; Fig. 6, A and B). Similarly, RANTES treatment (200 ng/ml) blocked responses to subsequent stimulation with MIP-1 α or MCP-3 (200 ng/ml; Fig. 6, C and D). In contrast, pretreatment of PMN with MCP-3 (200 ng/ml) partially blocked, but did not fully abolish, calcium responses to MIP-1 α or RANTES (200 ng/ml; Fig. 6, E and F).

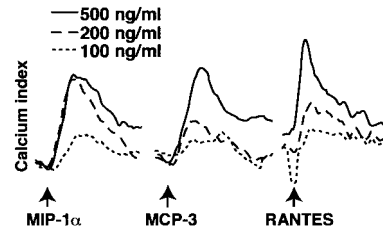


FIGURE 5. Concentration-dependent calcium responses in GM-CSF-treated PMN. Cells were cultured for 16 h with 10 ng/ml GM-CSF, loaded with fura-2AM, and treated with the indicated concentrations of MIP-1 α , MCP-3, and RANTES. Data are shown as a representative of three individual experiments with cells from different donors.

Chemotaxis of GM-CSF-treated PMN to CC chemokines

Once we had established that functional CCR were present on the surface of GM-CSF-stimulated PMN, we investigated whether any CC chemokines were chemotactic for GM-CSF-treated PMN using a chemotaxis microchamber technique. MIP-1 α , MCP-3, and RANTES were able to induce migration of cells that had been incubated for 16 h with 10 ng/ml GM-CSF, but showed no activity on cells treated with culture medium alone (Fig. 7) or freshly isolated PMN (data not shown). This result correlates with the RNase protection assay results documenting GM-CSF induction of CCR1 mRNA expression (Fig. 1 and 2), because MIP-1 α , MCP-3, and RANTES are CCR1 agonists. Migration to each of these chemokines was abolished in the absence of a concentration gradient (data not shown), indicating chemotactic (gradient-oriented) rather than chemokinetic (increased random motion) activity. Although constitutive CCR3 expression was observed in resting and GM-CSF-stimulated PMN (Fig. 1), the CCR3-specific ligand eotaxin did not induce migration in this assay (data not shown). The CC chemokines MIP-1 β , MCP-1 and MCP-4 did not show any chemoattractant activity on PMN in this assay over the concentration range used (data not shown).

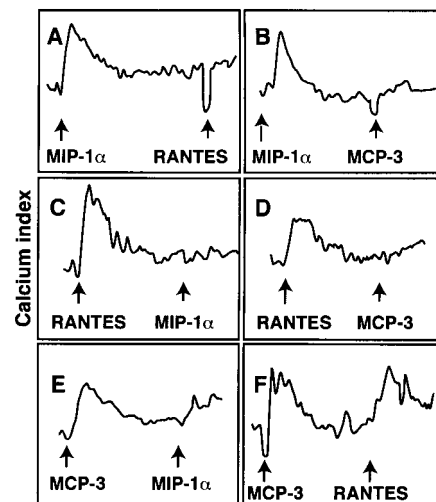


FIGURE 6. Desensitization of calcium responses by pretreatment with CC chemokines. PMN were incubated in culture medium alone or GM-CSF (10 ng/ml; 16 h), loaded with fura-2AM, and sequentially stimulated with MIP-1 α , MCP-3, RANTES (200 ng/ml) as indicated. Data are shown as representative of three individual experiments with cells from different donors.

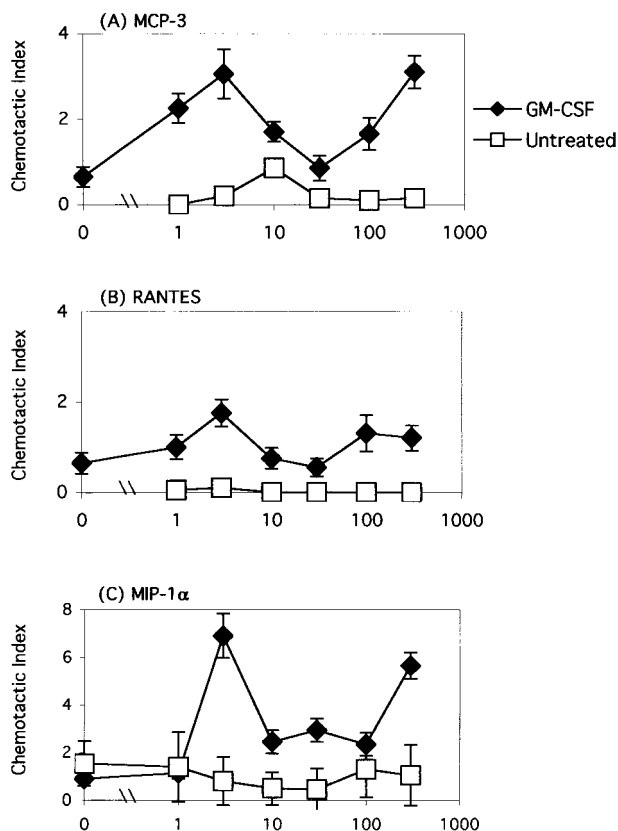


FIGURE 7. Chemotaxis of PMNs to MCP-3 (A), RANTES (B), or MIP-1 α (C) following exposure to culture medium (\square) or GM-CSF (10 ng/ml; \blacklozenge) for 16 h. Results are expressed as mean chemotactic index \pm SEM of three replicate measurements from a single experiment and is representative of three to five separate experiments.

Discussion

GM-CSF is an important pro-inflammatory cytokine expressed by epithelial cells (37–39), endothelial cells (40), fibroblasts (41), and mononuclear cells at inflammatory sites (42–44). This multifunctional cytokine plays a key role in the development of an effective neutrophilic response *in vivo*, by driving proper maturation of neutrophil precursors, priming of anti-bacterial effector functions (26, 27), and prolonging PMN longevity within inflammatory sites (26). The results obtained in the present study suggest that GM-CSF can further participate in the evolution of an inflammatory response by increasing CCR expression on PMN. We detected constitutive CCR1 mRNA expression in PMN cultured in medium alone. However, it is a formal possibility that this may not reflect CCR1 levels in resting peripheral blood PMN, as cellular activation due to the isolation procedure or adhesive interactions may act to up-regulate CCR1 mRNA. GM-CSF treatment up-regulated CCR1 mRNA expression in a concentration- and time-dependent manner. Furthermore, GM-CSF stimulation was shown to up-regulate expression of CCR1 protein as well, although this effect was smaller than the induction of CCR1 mRNA seen with GM-CSF. This moderate increase in levels of CCR1 protein was enough to confer new responsiveness to cells stimulated with GM-CSF, because stimulated cells mobilized intracellular calcium stores and chemotactically migrated in response to the CCR1 ligands MIP-1 α , MCP-3, and RANTES, while control cells did not. This is consistent with the interpretation that resting neutrophils express a level of CCR1 that is below the threshold needed to make the cells functionally responsive to the corresponding ligands, with a mod-

erate increase being enough to push this level above the threshold. A threshold model of chemoattractant receptor activation in neutrophils that supports this interpretation has been previously established (45).

Neutrophil recruitment is a critical component of the immune response to many bacterial and fungal pathogens (15, 46). Recruitment of these cells is necessary for efficient clearing of the invading pathogen, with a strong correlation between reduced neutrophil responses and increased disease severity. Several studies using neutralizing anti-MIP-1 α Abs have demonstrated that MIP-1 α is necessary for *in vivo* neutrophil recruitment in a variety of murine models of inflammation, such as experimental bacterial meningitis (46), immune complex-induced lung injury (13), mycobacterial infection (47), and endotoxemia-associated lung injury (14). In addition, targeted disruption of the murine gene encoding CCR1, a receptor known to bind MIP-1 α , has been shown to result in deficient neutrophil responses and increased morbidity in infectious aspergillosis (15), indicating that MIP-1 α -mediated neutrophil recruitment is an important component of the immune response. However, it is unclear whether MIP-1 α exerts direct effects on PMN, or works indirectly via production of classical neutrophil chemoattractants, such as CXC-type chemokines, from mononuclear or stromal cells. Murine PMN isolated from peripheral blood and inflammatory sites respond to MIP-1 α with chemotaxis and intracellular calcium transients *in vitro* (15), suggesting that direct effects are possible *in vivo*.

Although CCR1 has been shown to be a functional receptor on mouse PMN, its function on human PMN is not as clearly understood. The literature contains conflicting evidence regarding the existence of CCR on human PMN. CCR1 mRNA expression has been documented in resting human PMN (34, 48), although surface expression has been undetectable by flow cytometric staining (49). A few groups have reported that PMN do possess binding sites for MIP-1 α (10, 12), MCP-3 (10), and leukotactin (12), all of which bind to CCR1 (10, 12), although McColl et al. (11) were unable to detect MIP-1 α binding to PMN using similar methods and conditions. Although MIP-1 α does not show chemotactic activity for resting human PMN (10–12), it has been reported that other CCR1 agonists such as leukotactin (12), MCP-3 (10), CK β 8, and CK β 8-1 (32) are chemotactic for this cell type. However, other groups have not been able to demonstrate some of these results (33, 50). It is likely that resting PMN possess low levels of surface CCR1 that are insufficient for full activation of signaling pathways.

The conflicting results observed between studies on the role of CC chemokines in the chemotaxis of PMN may be explained by at least two different hypotheses. Firstly, it is possible that fundamental differences in the structure and function of the chemokine system between mouse and human may exist. For example, while the mouse has an orthologue of the human IL-8 receptor subtype CXCR2 (51), murine counterparts of IL-8 and the other human IL-8 receptor CXCR1 have not been identified. Secondly, differences in activation state of cells may affect the functional status of CCR on PMN, either by increasing the level of surface expression, or increasing availability of downstream signaling molecules. This would be consistent with findings in other cell types such as T lymphocytes, where chemokine receptor signaling in specific T cell subsets is regulated both at the level of receptor expression and receptor signaling (52). In this case, *in vivo* activation of PMN by factors such as cytokines or adhesion molecules might allow them to broaden their range of chemokine responsiveness to include those of the CC family.

The findings presented in this paper showing that PMN activated by GM-CSF up-regulate CCR1 expression suggest that cellular activation can broaden the spectrum of CC chemokine activity in vivo to include PMN. This model has been introduced in a recent study by Bonecchi et al. (33) demonstrating that CCR1 and CCR3 can be up-regulated on PMN by the pro-inflammatory cytokine γ -IFN. Our results differ from the Bonecchi study in that GM-CSF specifically up-regulated CCR1 expression without modulating CCR3 expression. Together these studies show that different cytokines have specific effects on chemokine receptor expression patterns and may be able to fine-tune the chemokine responsiveness of activated PMN. Our investigations together with the Bonecchi study also suggest that the apparent contradictions present in the literature concerning CCR expression on PMN may be due in part to different cellular activation states brought about by differences in cell handling and isolation procedures or donor variability.

In the present study, the CC chemokines MIP-1 α , RANTES, and MCP-3 induced chemotaxis in GM-CSF-treated PMN. Furthermore, our investigations have demonstrated that MIP-1 α , RANTES, and MCP-3 could induce calcium mobilization in GM-CSF-stimulated PMN. Pretreatment of PMN with MIP-1 α completely abolished responses to both RANTES and MCP-3, while pretreatment with RANTES also desensitized the cells to MIP-1 α and MCP-3. Such a reciprocal desensitization relationship suggests that these chemokines are all working through the same receptor, CCR1. However, MCP-3 treatment could not fully desensitize the calcium response to MIP-1 α and RANTES. One explanation for this is that MCP-3 might not efficiently cause receptor internalization, which is the main mechanism for chemokine receptor desensitization (36). It has been documented that MCP-3 can bind to CCR5 but is a poor stimulus for internalization of that receptor (53); it is possible that this characteristic applies to its binding to CCR1 as well.

Our data is consistent with GM-CSF up-regulation of surface CCR1, rendering the cells responsive to CCR1 ligands in assays of chemotaxis and calcium flux. This work suggests that activation of PMN can widen the spectrum of CC chemokines to include this cell type, which is traditionally thought of as responding to CXC chemokines alone. This mechanism might be functional within inflamed tissues, where GM-CSF is made locally by stromal cells and mononuclear phagocytes. Alternatively, systemic activation of endothelial cells has been shown to cause GM-CSF-dependent intravascular activation of PMN (40). It has long been known that in systemic inflammatory states, peripheral PMN lose their responsiveness to CXC chemokines (54, 55). Our work poses the question of whether these cells might gain responsiveness to CC chemokines, providing a possible mechanism for the PMN-mediated tissue damage associated with a variety of inflammatory conditions (55, 56). Such scenarios of local and systemic PMN activation and responsiveness to CC chemokines lead to new and interesting questions on PMN participation in cytokine networks and in the pathogenesis of inflammatory lesions.

Acknowledgments

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References

1. Baggiolini, M. 1998. Chemokines and leukocyte traffic. *Nature* 392:565.
2. Baggiolini, M., B. Dewald, and B. Moser. 1994. Interleukin-8 and related chemotactic cytokines: CXC and CC chemokines. *Adv. Immunol.* 55:97.
3. Premack, B., and T. Schall. 1996. Chemokine receptors: gateways to inflammation and infection. *Nat. Med.* 2:1174.
4. Kelner, G. S., J. Kennedy, K. B. Bacon, S. Kleyensteuber, D. A. Largaespada, N. A. Jenkins, N. G. Copeland, J. F. Bazan, K. W. Moore, T. J. Schall, et al. 1994. Lymphotactin: a cytokine that represents a new class of chemokine. *Science* 266:1395.
5. Bazan, J. F., K. B. Bacon, G. Hardiman, W. Wang, K. Soo, D. Rossi, D. R. Greaves, A. Zlotnik, and T. J. Schall. 1997. A new class of membrane-bound chemokine with a CX3C motif. *Nature* 385:640.
6. Legler, D. F., M. Loetscher, R. S. Roos, I. Clark-Lewis, M. Baggiolini, and B. Moser. 1998. B cell-attracting chemokine 1, a human CXC chemokine expressed in lymphoid tissues, selectively attracts B lymphocytes via BLR1/CXCR5. *J. Exp. Med.* 187:655.
7. Combadiere, C., S. K. Ahuja, and P. M. Murphy. 1995. Cloning, chromosomal localization, and RNA expression of a human β chemokine receptor-like gene. *Dev. Biol.* 174:673.
8. Combadiere, C., K. Salzwedel, E. D. Smith, H. L. Tiffany, E. A. Berger, and P. M. Murphy. 1998. Identification of CX3CR1: a chemotactic receptor for the human CX3C chemokine fractalkine and a fusion coreceptor for HIV-1. *J. Biol. Chem.* 273:23799.
9. Pan, Y., C. Lloyd, H. Zhou, S. Dolich, J. Deeds, J. A. Gonzalo, J. Vath, M. Gosselin, J. Ma, B. Dussault, E. Woolf, G. Alperin, J. Culpepper, J. C. Gutierrez-Ramos, and D. Gearing. 1997. Neurotactin, a membrane-anchored chemokine upregulated in brain inflammation. [Published erratum appears in 1997 *Nature* 389:100.] *Nature* 387:611.
10. Xu, L. L., D. W. McVicar, A. Ben-Baruch, D. B. Kuhns, J. Johnston, J. J. Oppenheim, and J. M. Wang. 1995. Monocyte chemotactic protein-3 (MCP3) interacts with multiple leukocyte receptors: binding and signaling of MCP3 through shared as well as unique receptors on monocytes and neutrophils. *Eur. J. Immunol.* 25:2612.
11. McColl, S. R., M. Hachicha, S. Levasseur, K. Neote, and T. J. Schall. 1993. Uncoupling of early signal transduction events from effector function in human peripheral blood neutrophils in response to recombinant macrophage inflammatory proteins-1 α and -1 β . *J. Immunol.* 150:4550.
12. Zhang, S., B. S. Youn, J. L. Gao, P. M. Murphy, and B. S. Kwon. 1999. Differential effects of leukotactin-1 and macrophage inflammatory protein-1 α on neutrophils mediated by CCR1. *J. Immunol.* 162:4938.
13. Shanley, T. P., H. Schmal, H. P. Friedl, M. L. Jones, and P. A. Ward. 1995. Role of macrophage inflammatory protein-1 α (MIP-1 α) in acute lung injury in rats. *J. Immunol.* 154:4793.
14. Standiford, T. J., S. L. Kunkel, N. W. Lukacs, M. J. Greenberger, J. M. Danforth, R. G. Kunkel, and R. M. Strieter. 1995. Macrophage inflammatory protein-1 α mediates lung leukocyte recruitment, lung capillary leak, and early mortality in murine endotoxemia. *J. Immunol.* 155:1515.
15. Gao, J. L., T. A. Wynn, Y. Chang, E. J. Lee, H. E. Broxmeyer, S. Cooper, H. L. Tiffany, H. Westphal, J. Kwon-Chung, and P. M. Murphy. 1997. Impaired host defense, hematopoiesis, granulomatous inflammation and type 1-type 2 cytokine balance in mice lacking CC chemokine receptor 1. *J. Exp. Med.* 185:1959.
16. Sica, A., A. Sacconi, A. Borsatti, C. A. Power, T. N. Wells, W. Luini, N. Polentarutti, S. Sozzani, and A. Mantovani. 1997. Bacterial lipopolysaccharide rapidly inhibits expression of C-C chemokine receptors in human monocytes. *J. Exp. Med.* 185:969.
17. Sozzani, S., S. Ghezzi, G. Iannolo, W. Luini, A. Borsatti, N. Polentarutti, A. Sica, M. Locati, C. Mackay, T. N. Wells, P. Biswas, E. Vicenzi, G. Poli, and A. Mantovani. 1998. Interleukin 10 increases CCR5 expression and HIV infection in human monocytes. *J. Exp. Med.* 187:439.
18. Loetscher, P., M. Seitz, M. Baggiolini, and B. Moser. 1996. Interleukin-2 regulates CC chemokine receptor expression and chemotactic responsiveness in T lymphocytes. *J. Exp. Med.* 184:569.
19. Loetscher, M., P. Loetscher, N. Brass, E. Meese, and B. Moser. 1998. Lymphocyte-specific chemokine receptor CXCR3: regulation, chemokine binding and gene localization. *Eur. J. Immunol.* 28:3696.
20. Patterson, B. K., M. Czerniewski, J. Andersson, Y. Sullivan, F. Su, D. Jiyamapa, Z. Burki, and A. Landay. 1999. Regulation of CCR5 and CXCR4 expression by type 1 and type 2 cytokines: CCR5 expression is downregulated by IL-10 in CD4-positive lymphocytes. *Clin. Immunol.* 91:254.
21. Perera, L. P., C. K. Goldman, and T. A. Waldmann. 1999. IL-15 induces the expression of chemokines and their receptors in T lymphocytes. *J. Immunol.* 162:2606.
22. Asagoe, K., K. Yamamoto, A. Takahashi, K. Suzuki, A. Maeda, M. Nohgawa, N. Harakawa, K. Takano, N. Mukaida, K. Matsushima, et al. 1998. Down-regulation of CXCR2 expression on human polymorphonuclear leukocytes by TNF- α . *J. Immunol.* 160:4518.
23. Jawa, R. S., G. A. Quaid, M. A. Williams, C. M. Cave, C. T. Robinson, G. F. Babcock, M. A. Lieberman, D. Witt, and J. S. Solomkin. 1999. Tumor necrosis factor α regulates CXC chemokine receptor expression and function. *Shock* 11:385.
24. Khandaker, M. H., L. Xu, R. Rahimpour, G. Mitchell, M. E. DeVries, J. G. Pickering, S. K. Singhal, R. D. Feldman, and D. J. Kelvin. 1998. CXCR1 and CXCR2 are rapidly down-modulated by bacterial endotoxin through a unique agonist-independent, tyrosine kinase-dependent mechanism. *J. Immunol.* 161:1930.
25. Lloyd, A. R., A. Biragyn, J. A. Johnston, D. D. Taub, L. Xu, D. Michiel, H. Sprenger, J. J. Oppenheim, and D. J. Kelvin. 1995. Granulocyte-colony stimulating factor and lipopolysaccharide regulate the expression of interleukin 8 receptors on polymorphonuclear leukocytes. *J. Biol. Chem.* 270:28188.
26. Fossati, G., I. Mazzucchelli, D. Gritti, G. Ricevuti, S. W. Edwards, D. A. Moulding, and M. L. Rossi. 1998. In vitro effects of GM-CSF on mature peripheral blood neutrophils. *Int. J. Mol. Med.* 1:943.

27. Al-Shami, A., W. Mahanna, and P. H. Naccache. 1998. Granulocyte-macrophage colony-stimulating factor-activated signaling pathways in human neutrophils. Selective activation of Jak2, Stat3, and Stat5b. *J. Biol. Chem.* 273:1058.
28. Brach, M. A., S. deVos, H. J. Gruss, and F. Herrmann. 1992. Prolongation of survival of human polymorphonuclear neutrophils by granulocyte-macrophage colony-stimulating factor is caused by inhibition of programmed cell death. *Blood* 80:2920.
29. Kasama, T., R. M. Strieter, T. J. Standiford, M. D. Burdick, and S. L. Kunkel. 1993. Expression and regulation of human neutrophil-derived macrophage inflammatory protein 1 α . *J. Exp. Med.* 178:63.
30. Kasama, T., R. M. Strieter, N. W. Lukacs, M. D. Burdick, and S. L. Kunkel. 1994. Regulation of neutrophil-derived chemokine expression by IL-10. *J. Immunol.* 152:3559.
31. McColl, S. R., and P. H. Naccache. 1997. Calcium mobilization assays. *Methods Enzymol.* 288:301.
32. Youn, B. S., S. M. Zhang, H. E. Broxmeyer, S. Cooper, K. Antol, M. Fraser, Jr., and B. S. Kwon. 1998. Characterization of CK β 8 and CK β 8-1: two alternatively spliced forms of human β -chemokine, chemoattractants for neutrophils, monocytes, and lymphocytes, and potent agonists at CC chemokine receptor 1. *Blood* 91:3118.
33. Bonocchi, R., N. Polentarutti, W. Luini, A. Borsatti, S. Bernasconi, M. Locati, C. Power, A. Proudfoot, T. N. Wells, C. Mackay, et al. 1999. Up-regulation of CCR1 and CCR3 and induction of chemotaxis to CC chemokines by IFN- γ in human neutrophils. *J. Immunol.* 162:474.
34. Neote, K., D. DiGregorio, J. Y. Mak, R. Horuk, and T. J. Schall. 1993. Molecular cloning, functional expression, and signaling characteristics of a C-C chemokine receptor. *Cell* 72:415.
35. Ben-Baruch, A., L. Xu, P. R. Young, K. Bengali, J. J. Oppenheim, and J. M. Wang. 1995. Monocyte chemoattractant protein-3 (MCP3) interacts with multiple leukocyte receptors. C-C CKR1, a receptor for macrophage inflammatory protein-1 α /Rantes, is also a functional receptor for MCP3. *J. Biol. Chem.* 270:22123.
36. Pelchen-Matthews, A., N. Signoret, P. J. Klasse, A. Fraile-Ramos, and M. Marsh. 1999. Chemokine receptor trafficking and viral replication. *Immunol. Rev.* 168:33.
37. Mullol, J., A. Xaubet, A. Gaya, J. Roca-Ferrer, E. Lopez, J. C. Fernandez, M. D. Fernandez, and C. Picado. 1995. Cytokine gene expression and release from epithelial cells: a comparison study between healthy nasal mucosa and nasal polyps. *Clin. Exp. Allergy* 25:607.
38. Mullol, J., J. N. Baraniuk, C. Logun, T. Benfield, C. Picado, and J. H. Shelhamer. 1996. Endothelin-1 induces GM-CSF, IL-6 and IL-8 but not G-CSF release from a human bronchial epithelial cell line (BEAS-2B). *Neuropeptides* 30:551.
39. Rasmussen, S. J., L. Eckmann, A. J. Quayle, L. Shen, Y. X. Zhang, D. J. Anderson, J. Fierer, R. S. Stephens, and M. F. Kagnoff. 1997. Secretion of proinflammatory cytokines by epithelial cells in response to *Chlamydia* infection suggests a central role for epithelial cells in chlamydial pathogenesis. *J. Clin. Invest.* 99:77.
40. Coxon, A., T. Tang, and T. N. Mayadas. 1999. Cytokine-activated endothelial cells delay neutrophil apoptosis in vitro and in vivo: a role for granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* 190:923.
41. Fabisiak, J. P., R. D. Weiss, G. A. Powell, and J. H. Dauber. 1993. Enhanced secretion of immune-modulating cytokines by human lung fibroblasts during in vitro infection with *Mycoplasma fermentans*. *Am. J. Respir. Cell Mol. Biol.* 8:358.
42. Andreatti, D., G. Gabbiani, and P. Neuville. 1998. Early granulocyte-macrophage colony-stimulating factor expression by alveolar inflammatory cells during bleomycin-induced rat lung fibrosis. *Lab. Invest.* 78:1493.
43. Ausiello, C. M., A. la Sala, C. Ramoni, F. Urbani, A. Funaro, and F. Malavasi. 1996. Secretion of IFN- γ , IL-6, granulocyte-macrophage colony-stimulating factor and IL-10 cytokines after activation of human purified T lymphocytes upon CD38 ligation. *Cell. Immunol.* 173:192.
44. Sallerfors, B., and T. Olofsson. 1992. Granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) secretion by adherent monocytes measured by quantitative immunoassays. *Eur. J. Haematol.* 49:199.
45. Omann, G. M., J. M. Harter, N. Hassan, P. J. Mansfield, S. J. Suchard, and R. R. Neubig. 1992. A threshold level of coupled G-proteins is required to transduce neutrophil responses. *J. Immunol.* 149:2172.
46. Diab, A., H. Abdalla, H. L. Li, F. D. Shi, J. Zhu, B. Hojberg, L. Lindquist, B. Wretling, M. Bakhiet, and H. Link. 1999. Neutralization of macrophage inflammatory protein 2 (MIP-2) and MIP-1 α attenuates neutrophil recruitment in the central nervous system during experimental bacterial meningitis. *Infect. Immun.* 67:2590.
47. Appelberg, R. 1992. Macrophage inflammatory proteins MIP-1 and MIP-2 are involved in T cell-mediated neutrophil recruitment. *J. Leukocyte Biol.* 52:303.
48. Gao, J. L., D. B. Kuhns, H. L. Tiffany, D. McDermott, X. Li, U. Francke, and P. M. Murphy. 1993. Structure and functional expression of the human macrophage inflammatory protein 1 α /RANTES receptor. *J. Exp. Med.* 177:1421.
49. Su, S. B., N. Mukaida, J. Wang, H. Nomura, and K. Matsushima. 1996. Preparation of specific polyclonal antibodies to a C-C chemokine receptor, CCR1, and determination of CCR1 expression on various types of leukocytes. *J. Leukocyte Biol.* 60:658.
50. Van Damme, J., P. Proost, J. P. Lenaerts, and G. Opendakker. 1992. Structural and functional identification of two human, tumor-derived monocyte chemotactic proteins (MCP-2 and MCP-3) belonging to the chemokine family. *J. Exp. Med.* 176:59.
51. Lee, J., G. Cacalano, T. Camerato, K. Toy, M. W. Moore, and W. I. Wood. 1995. Chemokine binding and activities mediated by the mouse IL-8 receptor. *J. Immunol.* 155:2158.
52. Rabin, R. L., M. K. Park, F. Liao, R. Swofford, D. Stephany, and J. M. Farber. 1999. Chemokine receptor responses on T cells are achieved through regulation of both receptor expression and signaling. *J. Immunol.* 162:3840.
53. Blanpain, C., I. Migeotte, B. Lee, J. Vakili, B. J. Doranz, C. Govaerts, G. Vassart, R. W. Doms, and M. Parmentier. 1999. CCR5 binds multiple CC-chemokines: MCP-3 acts as a natural antagonist. *Blood* 94:1899.
54. Solomkin, J. S., R. C. Bass, H. S. Bjornson, C. J. Tindal, and G. F. Babcock. 1994. Alterations of neutrophil responses to tumor necrosis factor α and interleukin-8 following human endotoxemia. *Infect. Immun.* 62:943.
55. Wagner, J. G., and R. A. Roth. 1999. Neutrophil migration during endotoxemia. *J. Leukocyte Biol.* 66:10.
56. Neumann, B., N. Zantl, A. Veihelmann, K. Emmanuilidis, K. Pfeffer, C. D. Heidecke, and B. Holzmann. 1999. Mechanisms of acute inflammatory lung injury induced by abdominal sepsis. *Int. Immunol.* 11:217.