MIP-1α[CCL3] acting on the CCR1 receptor mediates neutrophil migration in immune inflammation via sequential release of TNF-α and LTB_4

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Abstract: In the present study, we investigated the involvement of macrophage-inflammatory protein-1α (MIP-1α)[CC chemokine ligand 3 (CCL3)], MIP-1β[CCL4], regulated on activation, normal T expressed and secreted (RANTES)[CCL5], and CC chemokine receptors (CCRs) on neutrophil migration in murine immune inflammation. Previously, we showed that ovalbumin (OVA)-triggered neutrophil migration in immunized mice depends on the sequential release of tumor necrosis factor α (TNF-α) and leukotriene B_4 (LTB_4). Herein, we show increased mRNA expression for MIP-1α[CCL3], MIP-1β[CCL4], RANTES[CCL5], and CCR1 in peritoneal cells harvested from OVA-challenged, immunized mice, as well as MIP-1α[CCL3] and RANTES[CCL5] but not MIP-1β[CCL4] proteins in the peritoneal exudates. OVA-induced neutrophil migration response was muted in immunized MIP-1α[CCL3]$^{-/-}$ mice, but it was not inhibited by treatment with antibodies against RANTES[CCL5] or MIP-1β[CCL4]. MIP-1α[CCL3] mediated neutrophil migration in immunized mice through induction of TNF-α and LTB_4 synthesis, as these mediators were detected in the exudates harvested from OVA-challenged immunized wild-type but not MIP-1α[CCL3]$^{-/-}$ mice; administration of MIP-1α[CCL3] induced a dose-dependent neutrophil migration, which was inhibited by treatment with an anti-TNF-α antibody in TNF receptor 1 (p55$^{-/-}$)-deficient mice or by MK 886 (a 5-lipoxygenase inhibitor); and MIP-1α[CCL3] failed to induce LTB_4 production in p55$^{-/-}$ mice. MIP-1α[CCL3] used CCR1 to promote neutrophil recruitment, as OVA or MIP-1α[CCL3] failed to induce neutrophil migration in CCR1$^{-/-}$ mice, in contrast to CCR5$^{-/-}$ mice. In summary, we have demonstrated that neutrophil migration observed in this model of immune inflammation is mediated by MIP-1α[CCL3], which via CCR1, induces the sequential release of TNF-α and LTB_4. Therefore, whether a similar pathway mediates neutrophil migration in human immune-inflammatory diseases, the development of specific CCR1 antagonists might have a therapeutic potential. *J. Leukoc. Biol.* 78: 167–177; 2005.

Key Words: *chemokines · chemokine receptors · chemotaxis*

INTRODUCTION

Neutrophil migration is a complex process, which results mainly from the release of neutrophil chemotactic factors by resident cells, inducing rolling and adhesion of neutrophils on endothelial cells, followed by their transmigration to the extravascular space [1, 2]. Apart from its importance in host defense, the migration of neutrophils to the inflammatory site is, at least in part, responsible for tissue damage observed in several inflammatory diseases such as rheumatoid arthritis, glomerulonephritis, autoimmune vasculitis, and inflammatory bowel disease [3–5], highlighting the importance of understanding the mechanisms involved in leukocyte migration. Interleukin-1β (IL-1β), tumor necrosis factor α (TNF-α), complement fragment C5a, lipid mediators, such as platelet-activating factor 4 and leukotriene B_4 (LTB_4), and more recently, chemokines are the main chemotactic mediators involved in neutrophil recruitment to sites of inflammation [6].

Chemokines are important mediators involved in the migration and activation of the various subsets of leukocytes [7]. Chemokines are divided into four subfamilies, based on the position of one or two cysteine residues located near the N terminus of the protein: C (lymphotactin[XCL1]), CC {i.e., monocyte chemoattractant protein-1 (MCP-1)[CC chemokine ligand 2 (CCL2)], macrophage-inflammatory protein-1α (MIP-1α)[CCL3], MIP-1β[CCL4], regulated on activation, normal T expressed and secreted (RANTES)[CCL5], eotaxin[CCL11], pulmonary and activation-regulated chemokine/dendritic cells (DC)-chemokine 1[CCL18])}, CXC {i.e., growth-related onco-

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gene-α (GRO-α)[CXC chemokine ligand 1 (CXCL1)], IL-8-[CXCL8], monokine induced by interferon-γ (IFN-γ)[CXCL9], IFN-inducible protein 10 (IP-10)[CXCL10])}, and CX_3C (fraktalkine[CX3CL1]). The CXC subfamily is subdivided according to the presence or the absence of the Glu-Leu-Arg (ELR) motif positioned in the NH_2-terminal region before the first cysteine residue. ELR^+CXC chemokines cause, preferentially, the recruitment of neutrophils, and ELR^-CXC chemokines are more specific for lymphocytes [8]. The release of ELR^+CXC chemokines such as MIP-2[CXCL2], keratinocyte-derived chemokine[CXCL1], GRO-α[CXCL1], epithelial-derived neutrophil-activating factor-78[CXCL5], or IL-8[CXCL8] correlates with neutrophil infiltration observed in experimental models of inflammation and in human inflammatory diseases, such as rheumatoid arthritis, inflammatory bowel disease, sarcoidosis, and psoriasis [9–12]. Furthermore, inhibition of these chemokines with antibodies or the use of knockout mice for these chemokines or their receptors reduces neutrophil migration in different models of inflammation [13–16]. In human models, the chemokines induce neutrophil migration mainly via the activation of the CXC chemokine receptor 1 (CXCR1) and CXCR2 [17, 18]. In this context, substances that inhibit these chemokines receptors, such as repertaxin, have been tested to inhibit human neutrophil migration [19, 20].

The CC chemokines have been described as preferential chemoattractants and activators of mononuclear cells and eosinophils [21–23]. However, recent studies demonstrate that this chemokine subtype may also be involved in neutrophil migration in murine models [24, 25]. MIP-1α[CCL3] is produced by a variety of cells, including lymphocytes, monocytes/macrophages, mast cells, basophils, epithelial cells, and fibroblasts, and it binds to CC chemokine receptor 1 (CCR1) and CCR5 with high affinity to exert its biological effects [26–30]. MIP-1α[CCL3] also induces calcium influx in a dose-dependent manner and the chemotaxis of murine neutrophils in vitro [31]. Its administration in mice induces neutrophil influx and protected mice against *Cryptococcus neoformans* infection [32]. Furthermore, in 1999, Das et al. [33] demonstrated that neutrophil recruitment observed in a model of immune inflammation was inhibited partially by anti-MIP-1α[CCL3] treatment. However, the CCR subtype(s) that this chemokine uses as well as the mechanisms by which MIP-1α[CCL3] induces neutrophil recruitment have not been characterized. It is important to mention that although the MIP-1α[CCL3] production is also increased in patients with rheumatoid arthritis and sarcoidosis [34, 35], its participation in neutrophil migration in these and other human diseases was not described.

Recently, our laboratory showed that the neutrophil recruitment observed after ovalbumin (OVA) challenge in immunized mice was dependent on the endogenous release of TNF-α, which induces neutrophil migration through LTB_4 synthesis. OVA-induced neutrophil accumulation was completely inhibited by the treatment of OVA-immunized mice with an antibody against TNF-α in OVA-immunized TNF-α receptor ($p55^{-/-}$) knockout mice, in OVA-immunized mice treated with MK 886, a leukotrienes synthesis inhibitor [36], or with CP 105,696, an LTB_4 receptor antagonist [37]. Furthermore, TNF-α-induced neutrophil migration in wild-type (WT) animals was inhibited by MK 886 or CP 105,696, reinforcing that TNF-α induces neutrophil migration via a LTB_4-dependent mechanism [38].

In the present study, we investigated the role of MIP-1α[CCL3], MIP-1β[CCL4], and RANTES[CCL5] during OVA-induced neutrophil migration. We demonstrated that OVA-induced neutrophil migration in immunized mice was mediated by MIP-1α[CCL3] but not by RANTES[CCL5] or MIP-1β[CCL4] via the release of TNF-α and LTB_4. In addition, we showed that MIP-1α[CCL3] activated CCR1 but not CCR5 to promote neutrophil migration.

MATERIALS AND METHODS

Animals

Male BALB/c and C57Bl/6 (WT) mice and mice with a targeted disruption of the MIP-1α (C57Bl/6 MIP-1α$[CCL3]^{-/-}$), TNF-α receptor type I (C57Bl/6 $p55^{-/-}$), CCR1, or CCR5 (BALB/c $CCR1^{-/-}$ and C57Bl6 $CCR5^{-/-}$, respectively), weighing 18–22 g, were housed in temperature-controlled rooms (22–25°C) and received water and food ad libitum in the animal facility of the Department of Pharmacology or Immunology, School of Medicine of Ribeirão Preto, University of São Paulo (Brazil).

Breeding pairs of MIP-1α$[CCL3]^{-/-}$, $p55^{-/-}$, and $CCR5^{-/-}$ were purchased from the Jackson Laboratories (Bar Harbor, MA). Breeding stocks backcrossed to C57Bl/6 were obtained and housed in a sterile laminar flow cabinet until experiments were conducted. The breeding pairs of $CCR1^{-/-}$ mice were a gift of Prof. Craig Gerard (Children's Hospital, Harvard Medical School, Boston, MA).

Active sensitization

BALB/c, C57Bl/6, MIP-1α$[CCL3]^{-/-}$, $CCR1^{-/-}$, and $CCR5^{-/-}$ mice were immunized as described previously [34]. Briefly, on day 0, the animals received a single subcutaneous (s.c.) injection of OVA (100 μg) in 0.2 mL of an emulsion containing 0.1 mL phosphate-buffered saline (PBS) and 0.1 mL complete Freund's adjuvant (CFA; Sigma Chemical Co., St. Louis, MO). The animals were given booster injections of OVA dissolved in incomplete Freund's adjuvant (Sigma Chemical Co.) on days 7 and 14. Control mice were injected s.c. with 0.2 mL of an emulsion containing equal volumes of PBS and CFA, followed by boosters containing PBS and incomplete Freund's adjuvant. Twenty-one days after the initial injection, the immunized and control animals were challenged by intraperitoneal (i.p.) injection with OVA (10 μg/cavity) dissolved in 0.2 mL PBS or PBS alone, and the neutrophil migration was determined 2, 4, and 8 h after, as described below. In addition, the peritoneal exudates were collected 1.5 h after PBS or OVA injection to measure the TNF-α, MIP-1α[CCL3], MIP-1β[CCL4], and RANTES[CCL5] by enzyme-linked immunosorbent assay (ELISA; see below). The MIP-1α[CCL3] concentration was also measured 30 min, 2 h, 4 h, and 8 h after OVA injection. Alternatively, the animals were used as a source of peritoneal or spleen cells. CFA was used in the immunization protocol to drive the immune response after OVA challenge to a T helper cell type 1 pattern with a predominance of neutrophil migration in the acute phase [38, 39].

Leukocyte migration

OVA (1, 3, and 10 μg/cavity), MIP-1α[CCL3] (1, 3, 10, or 30 ng/cavity), or PBS (0.2 mL/cavity) was injected i.p. in OVA-immunized, control, or $p55^{-/-}$, $CCR1^{-/-}$, or $CCR5^{-/-}$ mice, and leukocyte migration was evaluated at indicated times. The animals were killed, and the peritoneal cavity cells were harvested with 3 mL PBS containing 1 mM EDTA. Total cell counts were performed in a cell counter (Coulter® A^C T, Coulter Corp., Miami, FL), and differential cell counts (100 cells total) were carried out on cytocentrifuge (Cytospin® 3, Shandon Lipshaw Inc., Pittsburgh, PA) slides stained with Rosenfeld. The differential count was performed under light microscope, and the results were presented as number of neutrophils per cavity.

RNase protection assay (RPA) for chemokines and CCRs

Total RNA was extracted from peritoneal exudate cells obtained from immunized mice at 1 h after challenge with 0.2 mL PBS or OVA (10 μg/cavity) using Trizol reagent (Life Technologies, Gaithersburg, MD), according to the manufacturer's instructions. Multiprobe template sets of mCK-5b, containing DNA templates for lymphotactin[XCL1], RANTES[CCL5], eotaxin[CCL11], MIP-1β[CCL4], MIP-1α[CCL3], MIP-2[CXCL2], MCP-1[CCL2], trichloroacetic acid (TCA)-3[CCL1], L32, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), or of mCR-5, containing DNA templates for CCR1, CCR2, and CCR5, were purchased from PharMingen (San Diego, CA). A DNA template was used to synthesize the α-[^{32}P]uridine 5′-triphosphate (3000 Ci/mmol, 10 mCi/mL, Amersham Life Science, Buckinghamshire, UK)-labeled probes in the presence of a GTP-ATP-CTP-UTP (GACU) pool using a T7 RNA polymerase. Hybridization with 10 μg each target RNA was performed overnight, followed by digestion with RNase A and T1 according to the PharMingen standard protocol. The samples were treated by a proteinase K-sodium dodecyl sulfate mixture and then extracted with chloroform and precipitated in the presence of ammonium acetate. The samples were loaded on an acrylamide-urea sequencing gel along with labeled DNA molecular weight markers and labeled probes and run at 50 watts with 0.5× Tris-borate/EDTA electrophoresis buffer. The gel was adsorbed to filter paper and dried under vacuum, and radioactivity of [α-^{32}P]-labeled probes was measured by phosphorimaging.

ELISA for IL-2, TNF-α, MIP-1α[CCL3], MIP-1β[CCL4], RANTES[CCL5], and LTB_4

TNF-α, MIP-1α[CCL3], MIP-1β[CCL4], and RANTES[CCL5] levels in the exudates from OVA-injected immunized mice, IL-2 levels in cell-free supernatants from OVA-stimulated spleen cells (5×10^5/mL), and MIP-1α[CCL3] concentration in the peritoneal exudates from MIP-1α[CCL3]-injected mice were detected by ELISA, based on a previously described protocol [40]. Briefly, microtiter plates were coated overnight at 4°C with an immunoaffinity-purified polyclonal sheep antibody against TNF-α (2 μg/mL), IL-2 (5 ng/mL), MIP-1α[CCL3] (0.5 μg/mL), MIP-1β[CCL4] (3 μg/mL), or RANTES[CCL5] (1 μg/mL). After blocking the plates, recombinant murine TNF-α, IL-2, MIP-1α[CCL3], MIP-1β[CCL4], or RANTES[CCL5] standards at various dilutions and the samples were added in duplicate at room temperature overnight. Rabbit biotinylated, immunoaffinity-purified polyclonal antibody anti-TNF-α (1:1000), anti-IL-2 (0.5 μg/mL), anti-MIP-1α[CCL3] (0.2 μg/mL), anti-MIP-1β[CCL4] (0.5 μg/mL), or anti-RANTES[CCL5] (0.2 μg/mL) was added, followed by incubation at room temperature for 1 h. Finally, 50 μl avidin-horseradish peroxidase (1:5000 dilution, DAKO A/S, Denmark) was added to each well; after 30 min, the plates were washed, and the color reagent o-phenylenediamine (200 μg/well, Sigma Chemical Co.) was added. After 15 min, the reaction was interrupted with 1 M H_2SO_4, and the optical density was measured at 490 nm. The results were expressed as pg/mL TNF-α, IL-2, MIP-1α[CCL3], MIP-1β[CCL4], or RANTES[CCL5], based on the standard curves.

The concentrations of LTB_4 in the peritoneal exudates from OVA- or MIP-1α[CCL3]-injected mice were detected by the enzyme immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, MI), according to the manufacturer's instructions.

Effect of antichemokine/TNF-α antibodies and LTB_4 synthesis inhibitor on neutrophil migration induced by MIP-1α[CCL3] or OVA

Preimmune serum (35 μL/cavity; control), sheep antiserum against murine RANTES[CCL5], an unrelated immunoglobuolin G (IgG)1 (15 μg/cavity; control), or a monoclonal IgG1 against MIP-1β[CCL4] (15 μg/cavity) was injected i.p. in WT OVA-immunized mice 15 min before challenge with OVA (10 μg/cavity). To confirm the efficacy of the antibodies, the respective antibodies were injected into the peritoneal cavity of the animals, and 15 min after, recombinant RANTES[CCL5] (30 ng/cavity) or MIP-1β[CCL4] (10 ng/cavity) was injected. To investigate the possible role of MIP-1α[CCL3] on OVA-induced neutrophil migration in immunized mice, MIP-1α[CCL3]$^{-/-}$ mice were immunized and challenged with OVA as described above. The effect of TNF-α and LTB_4 on MIP-1α[CCL3]-induced neutrophil migration was also investigated. First MIP-1α[CCL3] (1, 3, 10, or 30 ng/cavity/0.2 mL sterile PBS) was administrated via i.p. injection to determine the ideal dose that induces neutrophil migration. Next, sheep antiserum against murine TNF-α or the preimmune serum (35 μL/cavity) was injected i.p. 15 min prior to MIP-1α[CCL3] (10 ng/cavity) administration. MIP-1α[CCL3] (10 ng/cavity) was injected i.p. in p55$^{-/-}$ mice. To investigate the involvement of leukotrienes in MIP-1α[CCL3]-induced neutrophil migration, mice were treated 1 h before MIP-1α[CCL3] injection with MK 886 (1 mg/kg; orally). MK 886 was dissolved in 0.1% of methylcellulose in PBS. Neutrophil migration was measured 4 h after OVA or chemokine administration (see above). In addition, the peritoneal exudate of WT or p55$^{-/-}$ mice injected with MIP-1α[CCL3] was also collected 2 h after chemokine injection to measure the LTB_4 level by EIA (see above).

Role of CCR1 and CCR5 on MIP-1α[CCL3]-induced neutrophil migration

MIP-1α[CCL3] (10 ng/cavity) or PBS (control) was injected in the C57Bl/6 (control), CCR1$^{-/-}$, or CCR5$^{-/-}$ mice, and neutrophil migration was evaluated 4 h after chemokine injection (see above).

Statistical analysis

Data are reported as mean ± SEM and are representative of two or three separate experiments. The means from different treatments were compared by ANOVA, followed by the Bonferroni *t*-test for unpaired values. Statistical significance was set at $P < 0.05$.

RESULTS

OVA induces neutrophil migration, chemokines, and CCR1 RNA expression

As previously reported, the i.p. injection of OVA in immunized but not in control (nonimmunized) mice induced a dose- and time-dependent neutrophil migration (**Fig. 1, A** and **B**). OVA challenge induced a significant neutrophil migration 2 h after the challenge, which peaked at 4 h, declining thereafter, but it was still statistically significant 8 h after the challenge (Fig. 1B). The neutrophil migration returned to basal line 24 h after the challenge (data not shown). To investigate whether CC chemokines were mediating the OVA-induced neutrophil migration, we first used RPA to detect chemokines and CCR gene transcripts in cells harvested from the peritoneal cavities of OVA-immunized and -challenged mice. One hour after the injection of OVA in immunized mice, a significant increase in the expression of mRNA for the CC chemokines MIP-1α[CCL3], MIP-1β[CCL4], RANTES[CCL5], and CXC chemokine MIP-2[CXCL2] was observed compared with animals injected with PBS alone (Fig. 1C). However, the expression of mRNA for the C chemokine lymphotactin and for CC chemokines such as eotaxin[CCL11], MCP-1[CCL2], and TCA-3[CCL1] was not detected. Moreover, increases in the expression of mRNA for CCR1, but not CCR2 and CCR5, were also observed (Fig. 1D). The expression of L32 and GAPDH (controls) was similar in OVA- and PBS-injected mice (Fig. 1, C and D).

Production of CC chemokines in the OVA-challenged immunized mice

Subsequently, the levels of MIP-1α[CCL3], MIP-1β[CCL4], and RANTES[CCL5] protein were analyzed in peritoneal exudates after OVA challenge. The concentration of MIP-1α[CCL3] peaked at 1.5 h after the challenge, reducing there-

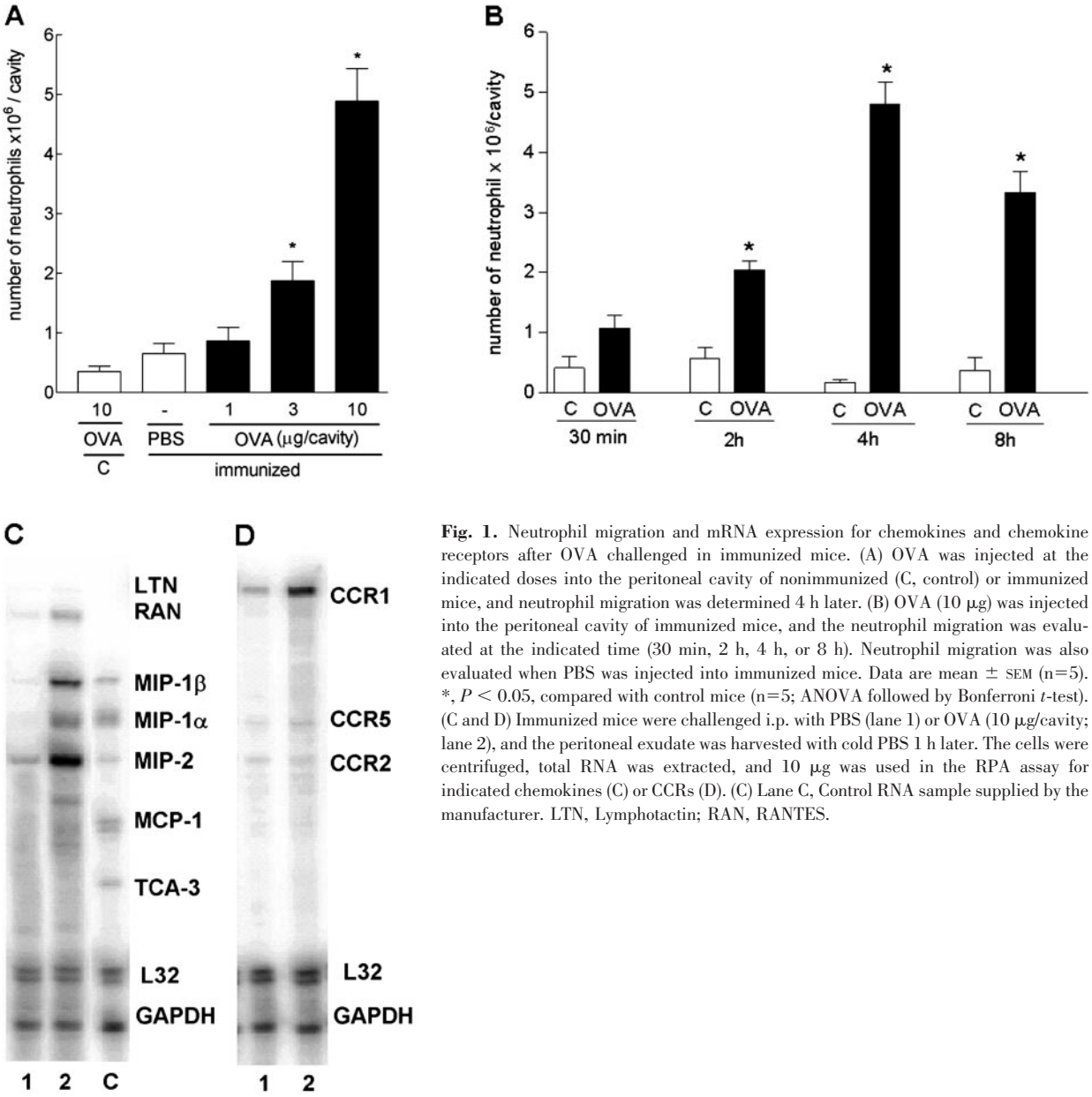

Fig. 1. Neutrophil migration and mRNA expression for chemokines and chemokine receptors after OVA challenged in immunized mice. (A) OVA was injected at the indicated doses into the peritoneal cavity of nonimmunized (C, control) or immunized mice, and neutrophil migration was determined 4 h later. (B) OVA (10 μg) was injected into the peritoneal cavity of immunized mice, and the neutrophil migration was evaluated at the indicated time (30 min, 2 h, 4 h, or 8 h). Neutrophil migration was also evaluated when PBS was injected into immunized mice. Data are mean ± SEM (n=5). *, $P < 0.05$, compared with control mice (n=5; ANOVA followed by Bonferroni *t*-test). (C and D) Immunized mice were challenged i.p. with PBS (lane 1) or OVA (10 μg/cavity; lane 2), and the peritoneal exudate was harvested with cold PBS 1 h later. The cells were centrifuged, total RNA was extracted, and 10 μg was used in the RPA assay for indicated chemokines (C) or CCRs (D). (C) Lane C, Control RNA sample supplied by the manufacturer. LTN, Lymphotactin; RAN, RANTES.

after, but still significant at 2 h after OVA administration and returned to levels similar to that observed in control 4 h later (**Fig. 2**). RANTES[CCL5], but not MIP-1β[CCL4], was also detected in the peritoneal exudates 1.5 h after OVA injection in immunized mice (PBS, 0.04±0.025; OVA, 0.521±0.1 ng RANTES/cavity, n=3).

MIP-1α[CCL3], but not MIP-1β[CCL4] or RANTES[CCL5], is involved in the neutrophil migration induced by OVA

In contrast to that observed in WT-immunized mice, the administration of OVA in immunized MIP-1α[CCL3]$^{-/-}$ mice did not induce neutrophil recruitment (**Fig. 3A**). The absence of neutrophil migration in these animals was not a result of inefficient immunization of these animals, as the concentration of IL-2 in the supernatant of OVA-stimulated spleen cells from MIP-1α[CCL3]$^{-/-}$ was similar to that observed in the WT mice (**Table 1**). Furthermore, unlike nonimmunized animals, immunized WT and MIP-1α[CCL3]$^{-/-}$ mice had a high serum titer of IgG against OVA (data not shown). The possible involvement of RANTES[CCL5] and MIP-1β[CCL4] on OVA-induced neutrophil migration into the peritoneal cavity of immunized mice was also investigated. It was observed that OVA-induced neutrophil migration in immunized mice was not inhibited by treatment of the animals with antibodies directed against murine RANTES[CCL5] or MIP-1β[CCL4] (Fig. 3B). The efficiency of these antibodies in neutralizing their respective chemokines was confirmed by the ability to inhibit the neutrophil migration induced by an i.p. injection of RANTES[CCL5] (30 ng/cavity; inhibition of 58.7%) or MIP-1β[CCL4] (10 ng/cavity; inhibition of 31%).

Involvement of TNF-α and LTB_4 in neutrophil migration induced by MIP-1α[CCL3]

As we have previously demonstrated that OVA-induced neutrophil migration in immunized mice is mediated by TNF-α

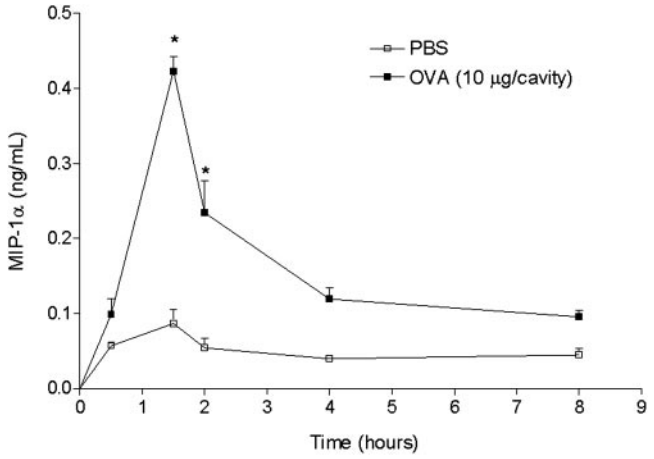

Fig. 2. MIP-1α[CCL3] production in the peritoneal exudate of OVA-injected mice. The figure indicates the concentration of MIP-1α[CCL3] in the peritoneal exudates collected 30 min, 1.5 h, 2 h, 4 h, or 8 h after injection of PBS (□) or OVA (10 μg/cavity; ■) in immunized mice. Data are mean ± SEM (n=3). *, $P < 0.05$, compared with respective control group (ANOVA followed by Bonferroni *t*-test).

and LTB_4 acting sequentially [38], here, we addressed whether MIP-1α[CCL3] was also involved in the OVA-induced release of TNF-α and LTB_4 in immunized mice. OVA challenge in immunized WT mice induced the production of TNF-α and LTB_4 in the peritoneal exudates 2 h after antigen challenge, which did not occur in immunized MIP-1α[CCL3]$^{-/-}$ mice (**Fig. 4**).

We next investigated whether MIP-1α[CCL3] induces neutrophil migration by a mechanism dependent on the sequential release of TNF-α and LTB_4. First, we observed that the i.p. injection of MIP-1α[CCL3] induced neutrophil migration dose- and time-dependently. The lower dose of MIP-1α[CCL3], which induced significant neutrophil migration, was 3 ng/cavity, and the peak of neutrophil migration was observed with the dose of 10 ng/cavity (**Fig. 5A**). The MIP-1α[CCL3]-induced neutrophil migration peaked 4 h after chemokine injection, decreasing at 8 h (Fig. 5B), and retuned to control levels 12 h later (data not shown). The concentration of MIP-1α[CCL3] in the exudates of mice injected i.p. with 10 ng recombinant MIP-1α[CCL3] was determined 5 min, 1.5 h, 4 h, and 8 h after the chemokine injection. It was observed that 5 min after MIP-1α[CCL3] injection, its concentration in the exudates was unpredictable of only 0.74 ± 0.01 ng/cavity, n = 5 (i.e., 7% of the amount injected), decreasing to 0.24 ± 0.04, 0.10 ± 0.02, and 0.10 ± 0.01 ng/cavity at time-points 1.5 h, 4 h, and 8 h, respectively. The concentration of MIP-1α[CCL3] in the exudates of PBS-injected mice (control, 5 min after injection) was 0.11 ± 0.02 ng/cavity, n = 5. These results suggest that the process of harvesting of MIP-1α with PBS from peritoneal cavity was not efficient (i.e., only 7% of injected was recovered). It could be a result of the capacity of the injected chemokine to hardly bind to extravascular tissues. Chemokines are basic proteins and bind avidly to negatively charged glycosaminoglycans via heparan sulfate-binding domains of extracellular matrix [6–8]. It might explain the fact that levels of measured MIP-1α[CCL3] in the peritoneal exudates from OVA-injected mice do not match with the dose of injected MIP-1α, which induced significant neutrophil migration.

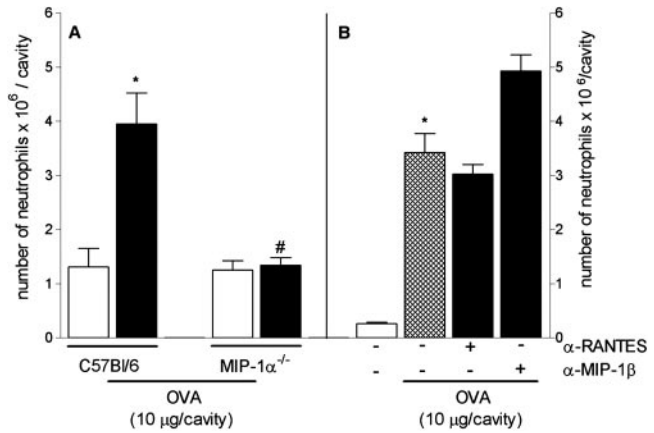

Fig. 3. Inhibition of OVA-induced neutrophil migration in MIP1-α[CCL3]$^{-/-}$. (A) Control (open bars) and immunized (solid bars) WT or MIP-1α[CCL3]$^{-/-}$ mice were challenged with OVA (10 μg/cavity), and 4 h later, the neutrophil migration was determined. (B) Immunized BALB/c mice were injected with control serum (–; cross-hatched bar) or with anti-RANTES/CcL5 serum (35 μL/cavity) or with a monoclonal anti-MIP-1β[CCL4] (15 μg/cavity) 15 min after the animals were injected with OVA (10 μg/cavity). The neutrophil migration was evaluated 4 h after the stimuli injection. Data are mean ± SEM (n=5). *, $P < 0.05$, compared with control mice; #, $P < 0.05$, compared with the group treated with WT mice (n=5; ANOVA followed by Bonferroni *t*-test).

TABLE 1. Concentration of IL-2 in the Supernatants of RPMI(–)- or OVA(+)-Stimulated Spleen Cells Obtained from Control or Immunized C57Bl/6 or MIP-1α$^{-/-}$ Mice

Spleen cells source	OVA (10 μg/mL)	IL-2 (pg/mL) 24 h
C57 Bl/6 control	–	ND
C57 Bl/6 control	+	ND
C57Bl/6 immunized	–	ND
C57Bl/6 immunized	+	52.37 ± 9.52*
MIP-1α–/– control	–	ND
MIP-1α–/– control	+	ND
MIP-1α–/– immunized	–	ND
MIP-1α–/– immunized	+	56.07 ± 11.37*

Spleen cells (5×10^5) were incubated as described in Materials and Methods. Data are the mean ± SEM of three samples; ND, Not detected. *$P <$ 0.05 compared with control group (ANOVA followed by Bonferroni *t*-test).

The MIP-1α-induced neutrophil migration was blocked by treatment of the animals with anti-mouse TNF-α antiserum, administrated 15 min before the MIP-1α[CCL3] (10 ng/cavity) challenge (**Fig. 6A**). Moreover, i.p. administration of MIP-1α[CCL3] in TNF-α receptor p55-deficient mice failed to induce neutrophil recruitment (Fig. 6B). Furthermore, the neutrophil migration induced by MIP-1α[CCL3] (10 ng/cavity) was blocked by the 5-lipoxygenase-activating protein inhibitor MK 886 treatment (1 mg/Kg, oral; Fig. 6C). Finally, it was observed that the i.p. injection of MIP-1α[CCL3] in p55$^{-/-}$ mice did not induce LTB_4 formation in the peritoneal exudates (Fig. 6D). Together, these results suggest that OVA-induced neutrophil migration in immunized mice depends on the release of MIP-1α[CCL3], which acts via the sequential release of TNF-α and LTB_4.

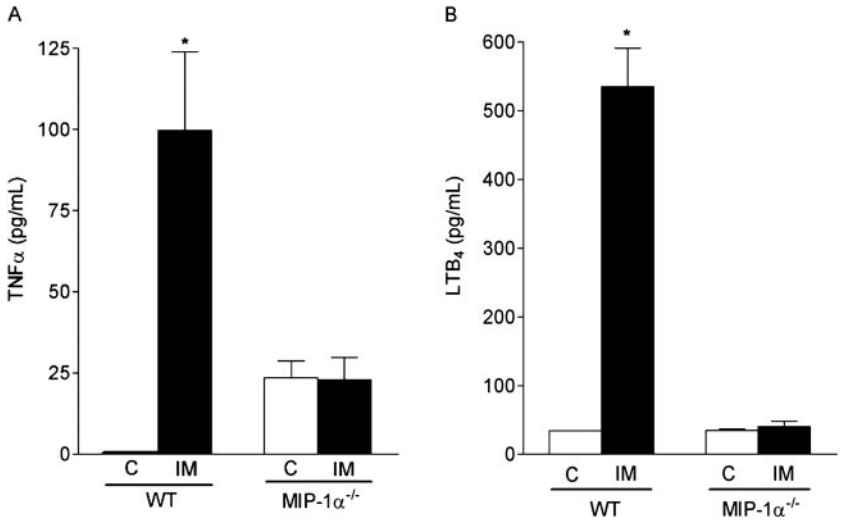

Fig. 4. Production of TNF-α and LTB_4 in the OVA-injected WT C57Bl/6 and MIP-α[CCL3]$^{-/-}$. Control (C; open bars) and immunized (IM; solid bars) C57Bl/6 (WT) or MIP-1α[CCL3]$^{-/-}$ mice were challenged with OVA (10 μg/cavity), and 1.5 h later, the TNF-α (A) and LTB_4 (B) concentration in the peritoneal exudate was determined. Data are mean ± SEM (n=3). *, $P < 0.05$, compared with respective control group (ANOVA followed by Bonferroni *t*-test).

MIP-1α[CCL3] induces neutrophil migration via CCR1

As MIP-1α[CCL3] binds to CCR1 and CCR5, we investigated the identity of the receptor that mediated the MIP-1α[CCL3] effect on neutrophil migration. To this end, we examined the effect of i.p. injection of OVA in immunized WT, CCR1$^{-/-}$, and CCR5$^{-/-}$ mice. OVA induced higher neutrophil migration into the peritoneal cavities of immunized WT mice than that induced by MIP-1α in naïve mice. Furthermore, neither i.p. OVA injection in immunized CCR1$^{-/-}$ mice nor MIP-1α[CCL3] in naïve CCR1$^{-/-}$ mice induced neutrophil migration into peritoneal cavities (**Fig. 7**). In contrast, OVA and MIP-1α[CCL3] promoted a marked neutrophil migration into peritoneal exudates from WT C57Bl/6 and CCR5$^{-/-}$ mice. It is interesting that the neutrophil migration induced by OVA or MIP-1α[CCL3] was higher in CCR5$^{-/-}$ mice than in C57Bl/6 WT mice (**Fig. 8**). The efficiency of the immunization procedure was confirmed by measuring IL-2 levels in the supernatants of OVA-challenged (10 μg/mL) spleen cells from all immunized mouse groups (data not shown). Thus, these results show that neutrophil migration induced by OVA is dependent on MIP-1α[CCL3] activation via CCR1, and this effect promotes the release of TNF-α and LTB_4.

DISCUSSION

Neutrophil activation and migration to a pathologic lesion are associated with inflammatory diseases such as glomerulonephritis, rheumatoid arthritis, autoimmune vasculitis, and inflammatory bowel disease. At the inflammatory site, this cell releases proteolytic enzymes and oxygen- and nitrogen-derived, free radicals, thereby causing tissue damage [41, 42]. Therefore, strategies that limit neutrophil trafficking and/or activation have received attention as novel treatments of these diseases. In this context, taking into account the literature describing that neutrophil migration induced by chemokines in human models is mediated by CXCR1 and CXCR2, substances

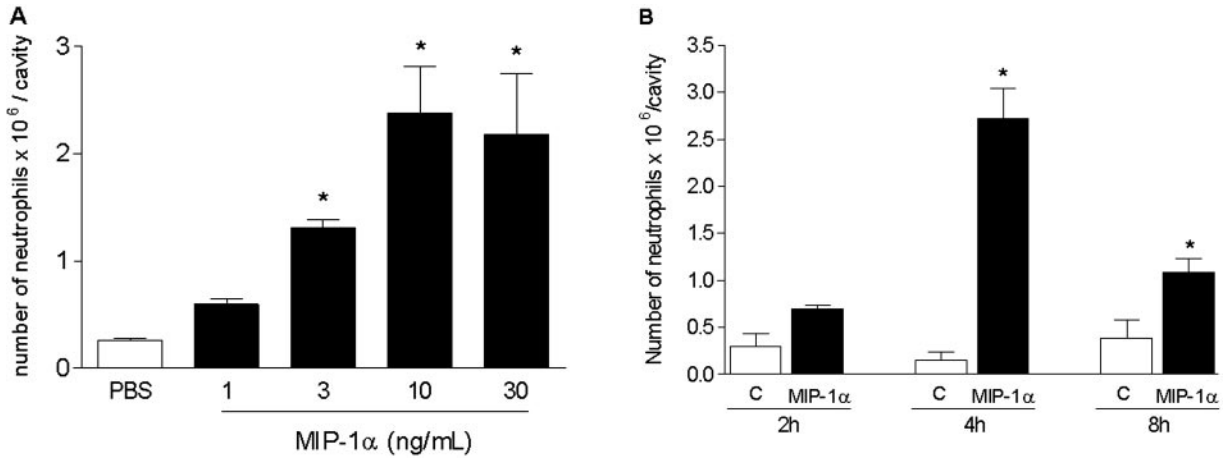

Fig. 5. MIP-1α[CCL3] induces a dose- and time-dependent neutrophil migration into peritoneal cavities. (A) The bars indicate the neutrophil migration into peritoneal cavities induced after injection of PBS (control; open bar) or MIP-1α[CCL3] (1, 3, 10, and 30 ng/cavity; solid bars) in mice 4 h previously. (B) MIP-1α (10 ng/cavity) was injected into the peritoneal cavity of BALB/c, and the neutrophil migration was evaluated at the indicated time (2 h, 4 h, or 8 h). Data are mean ± SEM (n=5). *, $P < 0.05$, compared with injection of PBS (ANOVA followed by Bonferroni *t*-test).

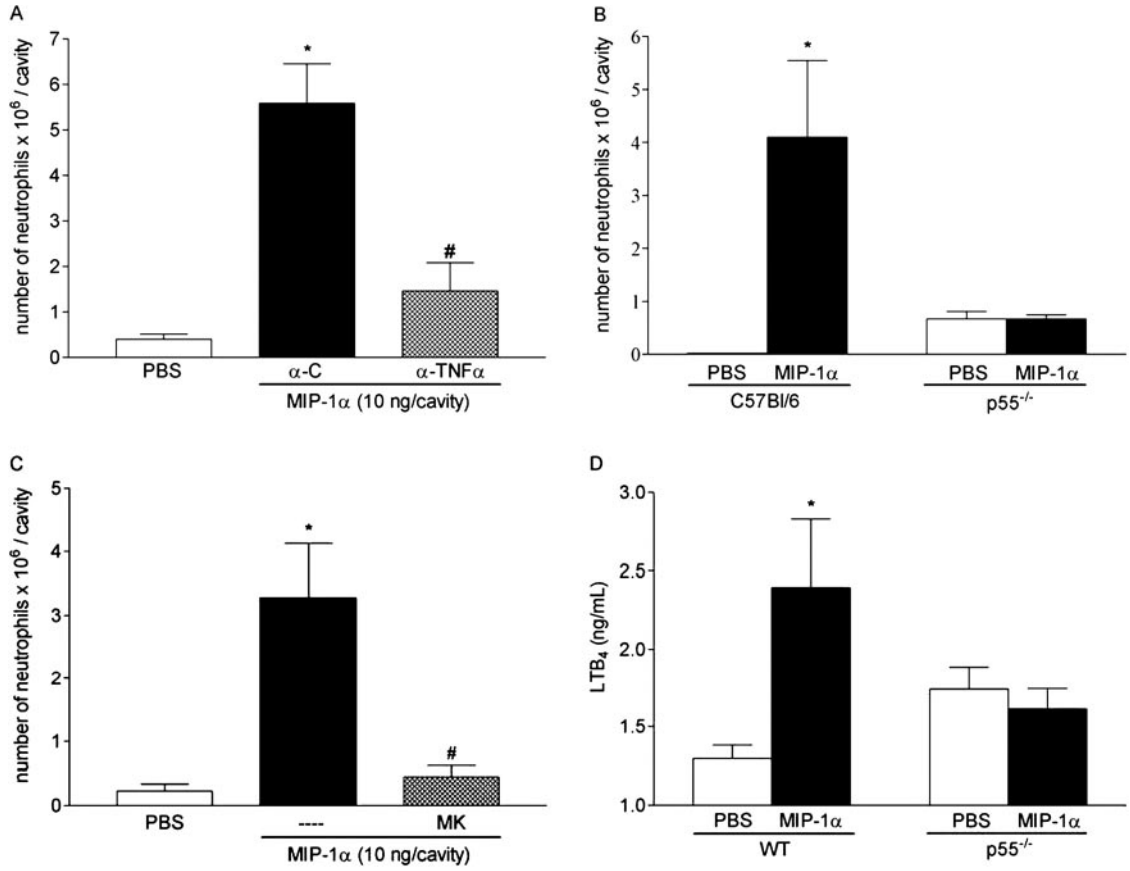

Fig. 6. Neutrophil migration induced by MIP-1α[CCL3] is dependent on TNF-α and LTB_4. (A) BALB/c mice were injected with PBS (open bar), control serum (α-C; solid bar), or with anti-TNF-α serum (α-TNFα; 35 μL/cavity; cross-hatched bar) 15 min before the injection of MIP-1α[CCL3] (10 ng/cavity), and the neutrophil migration was evaluated 4 h later. (B) WT C57Bl/6 or $p55^{-/-}$ mice were injected with PBS (open bar) or MIP-1α[CCL3] (10 ng/cavity; solid bars), and the neutrophil migration was evaluated 4 h later. (C) Neutrophil migration at 4 h after injection of PBS (open bar) or MIP-1α[CCL3] in the MK 886 (MK; 1 mg/Kg, orally, 1 h before; cross-hatched bar) or vehicle-treated (—-; solid bar) mice. (D) C57Bl/6 (WT) or $p55^{-/-}$ mice were injected with PBS (open bars) or MIP-1α[CCL3] (10 ng/cavity; solid bars), and 4 h later, the LTB_4 concentration in the peritoneal exudate was determined. The values are mean ± SEM (n=5). *, $P < 0.05$, compared with respective control group; #, $P < 0.01$, compared with C57Bl/6 mice injected with MIP-1α[CCL3] (ANOVA followed by Bonferroni *t*-test).

that antagonize these receptors might to be tested in immune-inflammation diseases.

In the present study, we further investigated a model of immune aseptic inflammation in mice, in which neutrophil migration is a relevant event. We confirm and extend previous findings [38], showing that the i.p. injection of OVA in immunized mice induces a dose- and time-dependent neutrophil migration into peritoneal cavities, which is MIP-1α[CCL3]-

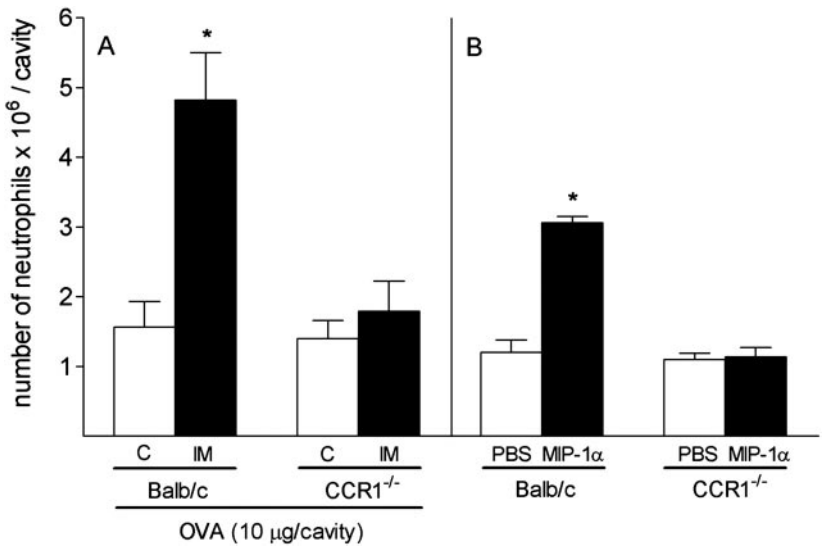

Fig. 7. OVA and MIP-1α[CCL3] do not induce neutrophil migration in the CCR1-deficient mice. (A) Neutrophil migration induced by OVA (10 μg/cavity) injected i.p. in the nonimmunized (C, control; open bars) and immunized (IM; solid bars) WT BALB/c or $CCR1^{-/-}$ mice. (B) Neutrophil migration induced by PBS (open bars) or MIP-1α[CCL3] (10 ng/cavity; solid bars) injected i.p. in the normal WT BALB/c or $CCR1^{-/-}$ mice. Data are mean ± SEM (n=5). *, $P < 0.05$, compared with respective control (ANOVA followed by Bonferroni *t*-test).

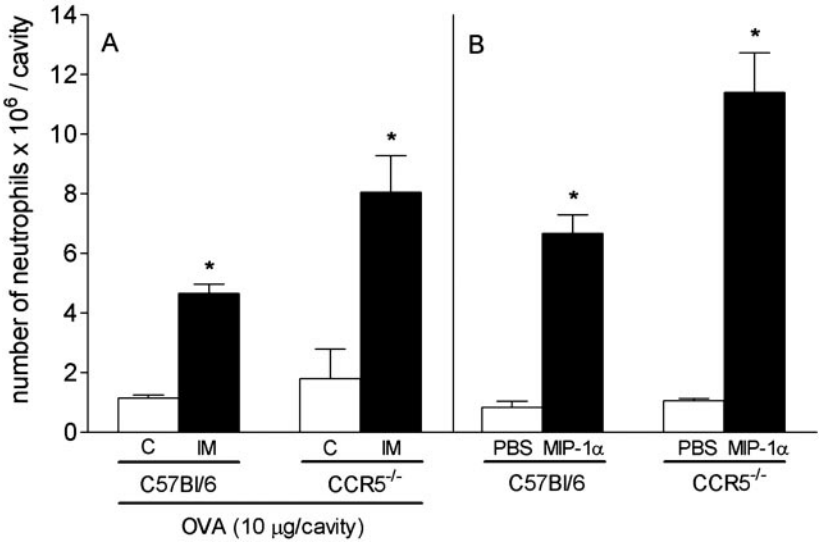

Fig. 8. Neutrophil migration induced by OVA and MIP-1α[CCL3] in the CCR5-deficient mice. (A) Neutrophil migration induced by OVA (10 μg/cavity) injected i.p. in nonimmunized (C, control; open bars) and immunized (IM; solid bars) WT BALB/c or $CCR5^{-/-}$ mice. (B) Neutrophil migration induced by PBS (open bars) or MIP-1α[CCL3] (10 ng/cavity; solid bars) injected i.p. in the normal WT BALB/c or $CCR5^{-/-}$ mice. Data are mean ± SEM (n=5). *, $P < 0.05$, compared with respective control (ANOVA followed by Bonferroni *t*-test).

and CCR1-dependent. These findings are novel, as CXC chemokines, mainly ELR^+ motif subtypes, are well-known neutrophil chemotactic mediators in different animals models of inflammation and in human inflammatory diseases, as mentioned above [43–45], but the effect of CC chemokines on neutrophil migration is less well-characterized [33, 46, 47]. Therefore, although a mouse model of immune inflammation has limited homology to the human diseases, the possible participation of MIP-1α in neutrophil migration in human models might also be investigated. In this context, it was demonstrated that human neutrophils express CCR1 [48, 49].

Peritoneal cells harvested from immunized mice at 1 h after OVA challenge showed increased mRNA expression for MIP-1α[CCL3], MIP-1β[CCL4], RANTES[CCL5], and CCR1. Confirming that the mRNAs to MIP-1α[CCL3] and RANTES[CCL5] were translated, significant amounts of MIP-1α[CCL4] (Fig. 2) and RANTES[CCL5] protein were observed in the peritoneal exudates harvested after OVA challenge. Conversely, we failed to detect MIP-1β[CCL4] in the exudates at this time-point, despite the increase in mRNA expression. A possible explanation for this failure is that the translation kinetics of mRNA for MIP-1β[CCL4] may differ from those for MIP-1α[CCL3] and RANTES[CCL5]. Furthermore, we found an increase in mRNA for the CXC chemokine MIP-2[CXCL2]; however, its role in OVA-induced neutrophil migration in immunized mice was not addressed by this study.

MIP-1α[CCL3] appears to be involved in OVA-induced neutrophil recruitment in immunized mice, as OVA failed to induce neutrophil migration into peritoneal cavities of MIP-1α$[CCL3]^{-/-}$-immunized mice, unlike WT mice. Furthermore, the peak of neutrophil migration induced by OVA was preceded by MIP-1α[CCL-3] production. These data are consistent with previous studies showing that neutralizing antibodies directed against MIP-1α[CCL3] blocked the neutrophil migration observed during acute s.c. inflammation induced by staphylococcal superantigens or endotoxin [50, 51]. Moreover, despite the fact that MIP-1α[CCL3] is a relatively weak chemoattractant for neutrophils in vitro [52], its administration into the footpads of mice induced a marked neutrophil infiltration [53].

Recently, we described that TNF-α and LTB_4, acting sequentially (TNF-α released by $CD4^+$ lymphocytes induced the release of LTB_4), are also involved in OVA-induced neutrophil migration in immunized mice [38]. It was observed that the inhibition of LTB_4 synthesis (MK 886) or its receptor (CP 105,696) or the neutralization of TNF-α by a specific antibody or genetic inhibition of TNF receptor 1 expression ($p55^{-/-}$ mice) inhibited neutrophil migration in OVA-challenged immunized animals. Moreover, OVA-induced LTB_4 synthesis was not observed in mice treated with antibody against TNF-α or in $p55^{-/-}$ mice [38].

These previous findings prompted us to test the hypothesis that MIP-1α[CCL3] is required for the release of TNF-α and LTB_4 in OVA-challenged, immunized mice. It was observed that in contrast to WT mice, OVA challenge in immunized MIP-1α$[CCL3]^{-/-}$ mice did not induce the production of TNF-α and LTB_4 in peritoneal exudates, indicating a requirement for MIP-1α[CCL3] in TNF-α and LTB_4 production and the subsequent neutrophil recruitment. To confirm this assumption, we found that MIP-1α[CCL3] induced a dose- and time-dependent neutrophil migration with a peak similar to that observed in OVA-injected mice (4 h later), and it was preceded by the release of TNF-α (data not shown) and of LTB_4. Treatment of mice with an antibody against TNF-α or the use of $p55^{-/-}$ mice or treatment with leukotriene synthesis inhibitor (MK 886) led to a marked inhibition of MIP-1α[CCL3]-induced neutrophil migration. Moreover, MIP-1α[CCL3] was not able to induce significant production of LTB_4 in the inflammatory exudates of $p55^{-/-}$ mice. Previous studies support our findings, as it has been shown that MIP-1α[CCL3] triggers TNF-α release during acute lung injury induced by the IgG immune complex or by lipopolysaccharide (LPS) [54]. Moreover, the intradermal injection of LPS into rat skin induced a marked leukocyte recruitment accompanied by significant levels of TNF-α and of CC chemokines MCP-1[CCL2] and MIP-1α[CCL3], and the treatment with a functional chemokine inhibitor (NR58-3.14.3) inhibited the release of TNF-α and leukocyte recruitment completely [51]. However, the authors did not investigate whether TNF-α was the final

mediator involved in the observed neutrophil migration. It is important to mention that our results do not discard the possibility that MIP-1α[CCL3] may also be acting by itself to induce neutrophil migration. In fact, there is evidence in vitro that MIP-1α is able to induce neutrophil migration by itself and that neutrophils express CCR1 on their cell membrane [31, 48, 49]. The fact that OVA induced higher neutrophil migration than that induced by MIP-1α[CCL3] suggests that besides MIP-1α[CCL3], OVA is also inducing the release of additional chemotactic mediators, including other chemokines, which could be synergizing with MIP-1α[CCL3] to induce the neutrophil recruitment. Reinforcing this suggestion, we observed that the OVA challenge in immunized mice induced a significant expression of MIP-2[CXCL2] mRNA, which has been described as a potent neutrophil chemoattractant [15].

MIP-1α binds to G-protein-coupled receptors CCR1 and CCR5 with high affinity. Besides MIP-1α[CCL3], CCR1 also binds other CC chemokines including RANTES[CCL5] and MCP-3[CCL7; refs. 55, 56]. CCR1 is expressed by T lymphocytes, natural killer (NK) cells, neutrophils, eosinophils, basophils, DC, and platelets [55, 57–59]. CCR5 shares ~55% amino acid sequence homology with CCR1 [60] and is expressed by T lymphocytes, NK cells, and macrophages [61]. To investigate whether MIP-1α[CCL3] bound to CCR1 or CCR5 to mediate the OVA-induced, neutrophil accumulation in immunized mice, we analyzed the neutrophil recruitment after OVA or MIP-1α[CCL3] injection in $CCR1^{-/-}$ and $CCR5^{-/-}$ mice. The injection of OVA or MIP-1α[CCL3] induced a significant neutrophil recruitment in $CCR5^{-/-}$ but not in $CCR1^{-/-}$ mice. These results demonstrate that MIP-1α[CCL3] acts through CCR1 to promote neutrophil recruitment. Reinforcing this conclusion, we noted that OVA challenge in immunized mice induced the expression of CCR1 mRNA but not CCR5 mRNA. It is interesting that the neutrophil migration induced by OVA or MIP-1α[CCL3] in $CCR5^{-/-}$ was greater than that observed in WT mice. This may be explained by enhancement of CCR1 expression or the release of other chemotactic mediators in $CCR5^{-/-}$ mice. In fact, $CCR5^{-/-}$ mice, submitted to a protocol of immune neuritis, produced more IP-10[CXCL10] and MIP-1β[CCL4] than did WT mice [62]. Alternatively, activation of CCR5 might mediate the release of mediators that down-modulate neutrophil migration.

In summary, we demonstrate a functional relationship between MIP-1α[CCL3] and neutrophil accumulation in an experimental model of allergen-induced immune inflammation in the peritoneum. The neutrophil migration induced by OVA in immunized mice depended on a cascade of mediators, in which MIP-1α[CCL3] was pivotal: MIP-1α[CCL3] → TNF-α → LTB_4. We postulate that MIP-1α[CCL3] bound to CCR1 present on $CD4^+$ lymphocytes, a finding that is consistent with our previous studies showing that this lymphocyte subtype is the source of TNF-α after OVA challenge [38]. Although a mouse model of immune inflammation has limited homology to the human diseases, our findings support the need to investigate whether similar mechanisms are participating in neutrophil migration in human immune-inflammatory diseases. If this were the case, the development of specific CCR1 antagonists might hold potential therapeutic benefits in the treatment of such diseases.

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