The role of CCL22 (MDC) for the recruitment of eosinophils during allergic pleurisy in mice

Vanessa Pinho,* Sandra H. Oliveira,† Danielle G. Souza,* Denise Vasconcelos,* Ana Letícia Alessandri,* Nicholas W. Lukacs,‡ and Mauro M. Teixeira*

*Immunopharmacology, Departamento de Bioquímica e Imunologia, ICB, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil; †Department of Pharmacology, Faculty of Medicine of Ribeirão Preto, São Paulo, Brazil; and ‡Department of Pathology, University of Michigan Medical School, Ann Arbor

Abstract: Eosinophils are important inflammatory cells in allergic diseases. In the present study, we have investigated the effects of CCL22 on the recruitment of eosinophils in vivo and in vitro. CCL22 induced a dose- and time-dependent recruitment of eosinophils into the pleural cavity of mice, and this was dependent on the release of platelet-activating factor (PAF) and subsequent generation of CCL11. However, in an allergic pleurisy model, an anti-CCL22 polyclonal antibody given during sensitization or before challenge had no significant effect on eosinophil recruitment. CCL22 did not induce eosinophil chemotaxis in vitro but was able to induce eosinophil degranulation in vitro and in vivo. In conclusion, we show that although exogenously added CCL22 may induce eosinophil migration in vivo via release of PAF and CCL11 (eotaxin), endogenous production of CCL22 does not drive eosinophil migration during allergic inflammation. However, CCL22 may be an important activator of eosinophils once these cells have migrated into tissue. *J. Leukoc. Biol.* 73: 356–362; 2003.

Key Words: *chemokines · eotaxin (CCL11) · PAF · migration*

INTRODUCTION

Macrophage-derived chemokine (CCL22/MDC) is a CC chemokine that binds to CCR4 receptors primarily characterized on T helper cell type 2 (TH2) cells [1]. CCL22 is a potent chemoattractant to a variety of cells, including T cells, natural killer (NK) cells, monocytes, and dendritic cells (DC), and increases the bactericidal activity of macrophage [1, 2]. Earlier studies have shown that CCL22 is constitutively expressed on immune cells [3] and may be involved in the trafficking of cells, generation, and amplification of type 2 responses [1]. More recently, Lloyd et al. [4] showed an important role for CCL22 and its receptors in TH2 lymphocyte recruitment in a model of allergic airway inflammation.

There is much evidence suggesting an important role for eosinophils in the pathophysiology of allergic disease [5–7]. In allergic disease, such as asthma, eosinophils are a crucial source of cytotoxic proteins, lipid mediators, oxygen metabolites, and cytokines, which contribute to severity of disease [8]. Thus, an understanding of mechanisms involved in the recruitment of eosinophils may permit new therapies for eosinophil-associated diseases. Eosinophil recruitment in sites of allergic inflammation depends on the concerted action of a variety of molecules including the chemokines, such as CCL11 (eotaxin) and CCL5 [regulated on activation, normal T expressed and secreted (RANTES)], and lipid mediators, such as leukotriene B_4 (LTB_4) and platelet-activating factor (PAF) [9–14]. Recently, CCL22 was shown to be an activator of human eosinophil chemotaxis [15]. In this study, we have investigated the effects of CCL22 on the recruitment of murine eosinophils in vivo and in vitro and whether CCL22 works directly or indirectly to mediate eosinophil recruitment. Finally, the effects of anti-CCL22 antibodies on the recruitment of eosinophils in response to antigen challenge in sensitized mice were also investigated.

MATERIALS AND METHODS

Animals

Wild-type (WT) and PAF receptor-deficient ($PAFR^{-/-}$) male BALB/c mice (18–22 g) were used throughout these experiments. Animals were housed in a temperature-controlled room with free access to water and food. $PAFR^{-/-}$ mice were generated as described previously [16] and intercrossed for at least seven generations to establish the BALB/c strain. These animals have been kindly donated by Dr. Satoshi Ishii (University of Tokyo, Japan) and were bred in the animal facilities of this institution.

Drugs and reagents

Recombinant murine (rm)CCL22 was purchased from Peprotech (London, UK). CCL22 was diluted in phosphate-buffered saline (PBS; pH 7.4) containing 0.01% bovine serum albumin (BSA) and was stored at –70°C until use. BSA, ovalbumin (OVA), and control rabbit serum were purchased from Sigma Chemical Co. (St. Louis, MO). The LTB_4 antagonist CP105,696 and the specific and long-acting PAFR antagonist UK-74,505 were a gift from Pfizer Global Research and Development (Kent, UK). CP105,696 was dissolved in

Correspondence: Mauro Martins Teixeira, Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Av. Antonio Carlos, 6627—Pampulha, 31270-901 Belo Horizonte MG, Brazil. E-mail: mmtex@icb.ufmg.br

Received May 20, 2002; revised November 12, 2002; accepted November 19, 2002; doi: 10.1189/jlb.0502243

dimethyl sulfoxide and further diluted in PBS. UK-74,505 was dissolved in HCl 0.01N and further diluted in PBS. Control animals received drug vehicle.

Anti-CCL22 and anti-CCL11 antibodies

Rabbit anti-CCL22 antibody and anti-CCL11 antibody were prepared by multiple-site immunization of New Zealand white rabbits with rmCCL22 or CCL11 (R&D Systems, Minneapolis, MN) in complete Freund's adjuvant. Polyclonal antibodies were titered by direct enzyme-linked immunosorbent assay (ELISA) and specifically verified by failure to cross-react to m interleukin (IL)-3, mIL-1α, m tumor necrosis factor α (TNF-α), CCL3 [m macrophage-inflammatory protein-1α (MIP-1α)], IL-6, CCL2 [m homologue of monocyte chemoattractant protein-1 (MCP-1)], CCL8 (mMIP-1β), CCL2 [human (h)MCP-1], CXCL8 (hIL-8), hRANTES, hMIP-1α, hTNF. The immunoglobulin G (IgG) portion of the serum was purified over a protein A column and stored at –20°C in PBS until use.

Sensitization

Animals were immunized with OVA adsorbed to an aluminum hydroxide gel, as described previously [17]. Briefly, mice were injected subcutaneously (s.c.) on days 1 and 8 with 0.2 ml solution containing 100 μg OVA and 70 μg aluminum hydroxide (Reheiss, Dublin, Ireland).

Leukocyte migration into the pleural cavity induced by CCL22 or antigen

CCL22 (10–100 ng/cavity) was injected intrapleurally (i.pl.) in naive WT or $PAFR^{-/-}$ mice, and animals were killed at 24 h after the i.pl. injection. Sensitized mice were challenged with antigen (OVA) or PBS. The cells present in the pleural cavity were harvested by injecting 2 ml PBS and total cell counts performed in a modified Neubauer chamber using Turk's stain. Differential cell counts were performed on cytospin preparations (Shandon III) stained with May-Grumwald-Giemsa using standard morphologic criteria to identify cell types. The results are presented as the number of cells per cavity.

Anti-CCL22 or anti-CCL11 pretreatment

The role of endogenous chemokines on eosinophil recruitment induced by CCL22 or in the allergic pleurisy was investigated by using anti-CCL22, anti-CCL11, or control IgG. The IgG fraction of a rabbit polyclonal anti-CCL22 antibody or anti-CCL11 antibody or the IgG portion of serum of preimmune rabbits was prepared purified over a protein A column, as described previously [18]. Anti-CCL22 was administered intraperitoneally (i.p.) at the dose of 100 μg/mouse 60 min before the i.pl. administration of CCL22 or OVA, and anti-CCL11 was administered i.p. at the dose of 100 μg/mouse 60 min before the i.pl. administration of CCL22. Control animals received preimmune IgG.

LTB_4 receptor antagonist and PAFR antagonist pretreatment

To investigate the role of LTB_4 and PAF on the eosinophil recruitment induced by CCL22, the LTB_4 receptor antagonist CP105,696 (3 mg/Kg) or the PAFR antagonist UK-74,505 (1 mg/Kg) was administered i.p. 60 min before the stimulus. Control animals received drug vehicle.

Antigen elicited peritoneal eosinophil purification

Eosinophils were induced by the injection of thioglycolate plus soluble egg antigen (SEA) into the peritoneum of *Schistosoma mansoni*-infected mice. SEA was prepared in our laboratory by grinding isolated eggs from heavily infected *S. mansoni*-infected mice as described previously [19]. This injection induces a pool of circulating eosinophils recruited into the peritoneum in an antigen-specific manner. After 48 h, the peritoneum of mice was lavaged, and cells were collected. The initial population that is isolated from the peritoneum is ~50% eosinophils with only 2–5% neutrophils and 35–45% mononuclear cells (lymphocytes and macrophages). Adherent cell populations were removed from the population by plastic adherence in tissue-culture dishes for 1 h. The nonadherent cells were washed and resuspended in PBS/BSA (90 μl PBS/BSA per 10^7 cells), and eosinophils were purified by negative immunomagnetic bead-coupled antibodies to exclude contaminating immune cells using the magnetic cell sorter (MACS) system. The antibodies used were anti-Thy1 (for T cells), anti-B220 (for B cells) and anti-class II (for antigen-presenting cells). After the plate adherence and MACS separation, the population of cells contained >97% eosinophils with contaminating neutrophils (~1%) and mononuclear cells (1–2%).

In vitro chemotaxis

Eosinophil migration was quantitated by a modification of a Boyden chamber technique, as described previously [20]. Purified eosinophils were suspended at 3×10^6 cells/ml in Dulbecco's PBS plus 0.5% BSA and were placed in the top wells of the microchemotaxis chamber. Bottom wells were filled with CC chemokines (CCL11 and CCL22) in the final concentration of 10 and 100 ng/ml, respectively, or assay medium as negative control. A 5-μm pore-size polycarbonate filter separated the upper wells containing the cells from the control and chemokine samples in the bottom wells. The chambers were incubated for 1 h at 37°C in a 5% CO_2 moist atmosphere, and the filters were then carefully scraped of nonmigrating cells, fixed with methanol, and stained with Diff-Quik. Eosinophil migration was quantitated by counting the number of eosinophils migrating completely through the matrix-coated filter in 10 high-powered fields (HPF) in triplicate samples. The data are expressed as the average number of countable adherent cells per HPF (±SEM).

Eosinophil degranulation in vitro and in vivo

To analyze eosinophil peroxidase (EPO) release in vitro, purified murine eosinophil cells were resuspended in phenol red-free Dulbecco's modified Eagle's medium and activated with chemokines (CCL11 and CCL22 in the final concentration of 10 and 100 ng/ml, respectively, or assay medium as negative control) for 4 h. The supernatant from the activated cells (2×10^6 eosinophils) was harvested, and the EPO level in the cell-free supernatant was determined as described previously [21]. Eosinophil viability, as assessed by trypan blue exclusion, was consistently greater than 98% in control eosinophils or after stimulation by CCL11 or CCL22. To evaluate eosinophil degranulation in vivo, sensitized mice received an i.pl. injection of CCL22 or CCL11, 48 h after the challenge of the pleural cavity with antigen. After a further 2 h, cell-free supernatants were obtained from pleural cavity washes, and EPO levels were evaluated as described previously [21]. Briefly, o-phenyldiamine (OPD) (10 mg) was dissolved into 5.5 ml dH_2O, and 1.5 ml OPD solution was added to 8.5 ml of a Tris buffer (pH 8.0) followed by the addition of 7.5 μl H_2O_2. Using a 96-well plate, 100 μl substrate solution was added to a 50 μl sample. After 30 min, the reaction was quenched with 50 μl 4 M H_2SO_4, and the absorbance was read at 490 nm. The relative increase in samples was then compared. As a positive control, eosinophils (2×10^6 cells) were sonicated and measured as total EPO. A negative control was used by sonicating neutrophils (2×10^6).

Statistical analysis

All results are presented as the mean ± SEM. Normalized data were analyzed by one-way ANOVA, and differences between groups were assessed using Student-Newman-Keuls' post-test. A P value <0.05 was considered significant.

RESULTS

CCL22 induces the migration of eosinophils into the pleural cavity

The i.pl. injection of CCL22 (10–100 ng/cavity) induced a dose-dependent recruitment of eosinophils after 24 h (**Fig. 1A**). CCL22 was not specific for eosinophils, as it also induced recruitment of neutrophils and mononuclear cells (**Table 1**). Maximal eosinophil recruitment occurred between 24 and 48 h after injection of CCL22, and the number of cells decreased thereafter (Fig. 1B). In the next series of experiments, CCL22 was used at the dose of 100 ng/cavity, and recruitment of eosinophils was assessed 24 h after challenge.

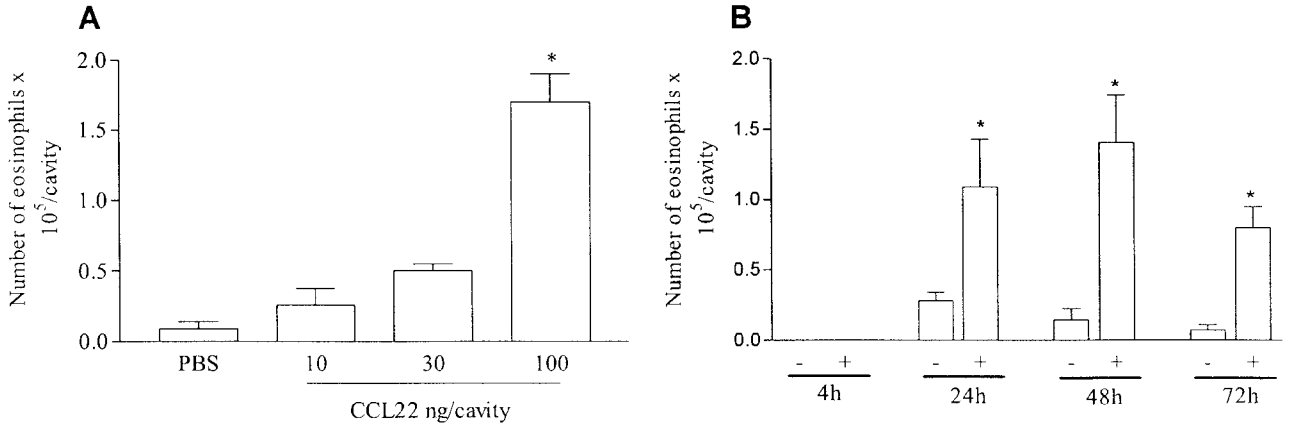

Fig. 1. Dose-response (A) and time-course (B) effects of CCL22 on the recruitment of eosinophil to the pleural cavity of mice. For the dose-response experiments (A), CCL22 was administered at the indicated doses, and the number of infiltrating eosinophil was assessed after 24 h. For time-course experiments (B), 100 ng CCL22 was administered i.pl., and eosinophil recruitment was assessed 4, 24, 48, and 72 h after injection. The results are expressed as means ± SEM of five mice in each group. *, $P < 0.05$ when compared with controls.

Role of LTB_4, PAF, and CCL11 on CCL22-induced eosinophil recruitment

We then investigated the role of endogenous LTB_4 and PAF in the eosinophil recruitment induced by CCL22. Naïve mice were pretreated with the LTB_4 receptor antagonist CP105,696 (3 mg/Kg) or the PAFR antagonist UK-74,505 (1 mg/Kg) 1 h before the i.pl. injection of CCL22, and the number of eosinophils was assessed 24 h later. The pretreatment with the PAFR antagonist UK-74,505 but not with the LTB_4 receptor antagonist CP105,696 inhibited eosinophil recruitment induced by CCL22 injection (**Fig. 2**). Similar to the effects of the PAFR antagonist, CCL22 failed to induce eosinophil recruitment in $PAFR^{-/-}$ mice (WT mice–PBS, 0.4±0.1 eosinophils×10^5/cavity; CCL22 100 ng, 3.1±1.8; $PAFR^{-/-}$ mice–PBS, 0.2±0.1; CCL22, 0.12±0.03; n=5 in each group). These results suggest that PAF is a chemotactic mediator involved in CCL22-induced eosinophil recruitment.

PAF has been shown to release CCL11 into the pleural cavity of naïve mice and to mediate the release of the latter protein following antigen challenge of sensitized mice [9]. As PAF is an intermediate in the eosinophil recruitment induced by CCL22, we examined whether the release of CCL11 was also involved in this process. Pretreatment of mice with an anti-CCL11 polyclonal antibody effectively inhibited (81% inhibition) the recruitment of eosinophils observed after injection of CCL22 (**Fig. 3**).

CCL22 is not involved in the migration of eosinophils in allergic reaction in the pleural cavity of mice

The i.pl. injection of 1 μg OVA/cavity in sensitized mice induced a PAF- and CCL11-dependent recruitment of eosinophils into the pleural cavity of mice [9, 10, 22]. As CCL22 induces and functions via the release of PAF and CCL11, we then investigated the possible involvement of endogenous CCL22 in this model of allergic inflammation. Pretreatment of animals with a purified anti-CCL22 polyclonal antibody (100 μg IgG/animal) had no significant effect on the recruitment of eosinophils to the pleural cavity of allergen-challenged animals (**Fig. 4**). Similarly, administration of higher doses of anti-CCL22 (300 μg IgG/animal) also failed to affect eosinophil recruitment after antigen challenge (OVA 1 μg, 2.62±0.92; OVA+anti-CCL22 300 μg, 2.52±0.24; $P>0.05$, n=4). However, pretreatment of animals with anti-CCL22 1 h before challenge abrogated eosinophil recruitment induced by CCL22 (Fig. 4).

As CCL22 is a chemoattractant for DC and is involved in the normal positioning of these cells in tissue and lymphoid organs

TABLE 1. Total and Differential Cell Counts (x10^5 Cells/Cavity) in Response to the i.pl. Injection of CCL22 in Naïve Mice[a]

	Time (h)							
	4		24		48		72	
	PBS	CCL22	PBS	CCL22	PBS	CCL22	PBS	CCL22
Total	5.4 ± 1.2	13.1 ± 2.9	4.0 ± 0.3	15.4 ± 3.5*	5.7 ± 0.2	17.1 ± 1.8*	6.8 ± 0.6	8.3 ± 0.6
Mononuclear	4.9 ± 1.1	6.2 ± 1.2	3.4 ± 0.2	12.1 ± 2.3*	5.5 ± 1.2	15.3 ± 1.7*	6.7 ± 0.6	7.5 ± 0.7
Neutrophils	0.5 ± 0.1	6.8 ± 1.9*	0.3 ± 0.1	2.2 ± 0.8*	0.1 ± 0.01	0.3 ± 0.1	0	0
Eosinophils	0	0	0.3 ± 0.1	1.1 ± 0.3*	0.1 ± 0.1	1.4 ± 0.3*	0.1 ± 0.03	0.8 ± 0.1*

[a] CCL22 (100 ng in 100 μl/cavity) or PBS (100 μl/cavity) was administered i.pl. in mice, and leukocyte migration was assessed at 4, 24, 48, and 72 h after injection. The results are expressed as means ± SEM of five mice in each group. * $P < 0.05$ when compared with PBS.

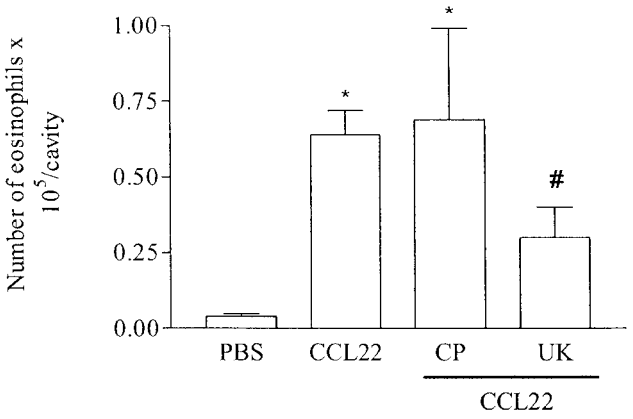

Fig. 2. Effects of the LTB_4 receptor antagonist CP105,696 and PAFR antagonist UK-74,505 on the eosinophil recruitment induced by CCL22. Mice were pretreated with CP105,696 (CP; 3 mg/Kg) and UK-74,505 (UK; 1 mg/Kg) i.p. 60 min before the i.pl. injection of CCL22 (100 ng/cavity) in naïve mice, and the number of infiltrating eosinophil was assessed after 24 h. The control group of mice was injected with vehicle. The results are expressed as means ± SEM of five mice in each group. *, $P < 0.05$ when compared with PBS; #, $P < 0.01$ when compared with vehicle-treated animals.

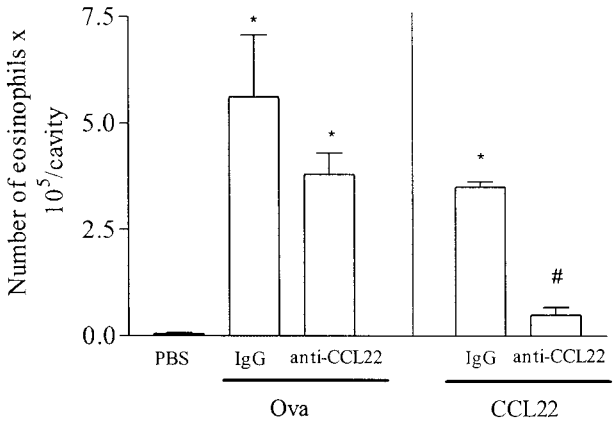

Fig. 4. Effects of anti-CCL22 pretreatment on the recruitment of eosinophil induced by antigen (Ova) challenge in sensitized mice or CCL22. Anti-CCL22 (hyperimmune Ig, 100 μg/animal) or nonimmune, purified Ig (100 μg/animal) was injected i.pl. 1 h before the i.pl. injection of CCL22 (100 ng/cavity) or OVA (1 μg/cavity), and the number of infiltrating eosinophils was assessed after 24 h injection of MDC and after 48 h injection of OVA. The results are expressed as means ± SEM of five mice in each group. *, $P < 0.05$ when compared with PBS; #, $P < 0.01$ when compared with animals injected with nonimmune IgG.

[1], we administered anti-CCL22 antibodies during sensitization. Treatment of animals 1 h before and 24 h after sensitization with antigen did not cause any changes in allergen-induced recruitment of eosinophils into the pleural cavity (**Fig. 5**). The splenic cells of these animals were stimulated in vitro, and the concentrations of interferon-γ (IFN-γ) and IL-5 in culture supernatants were measured by specific ELISA. Treatment with anti-CCL22 had little effect in the concentration of these cytokines in culture supernatant (IL-5, IgG control, 793.82±80.64 pg/ml; anti-CCL22, 832.93±39.78 pg/ml; IFN-γ, IgG control, 1018.03±96.12 pg/ml; anti-CCL22, 1837.95±178.4 pg/ml; $P>0.05$, n=4). Moreover, the antibody failed to cause any variation of IgG_1 titers measured in the serum of these animals (IgG control, 0.144±0.02; anti-CCL22, 0.155±0.01, absorbance at 492 nm; $P>0.05$, n=4).

CCL22 is involved in eosinophil activation

To determine the ability of CC chemokine CCL22 to induce chemotaxis of antigen-elicited murine eosinophils, we used a classic two-chamber assay. Antigen-elicited eosinophils migrated toward CCL11 but not to CCL22 in two different concentrations (**Fig. 6**). However, different from CCL11, CCL22 was able to induce eosinophil degranulation in a dose-dependent manner, as assessed by the release of EPO from eosinophils upon activation with CCL22 (**Fig. 7A**). The release of EPO from eosinophils upon activation with CCL22 was approximately 60% of the total eosinophil EPO content (data not

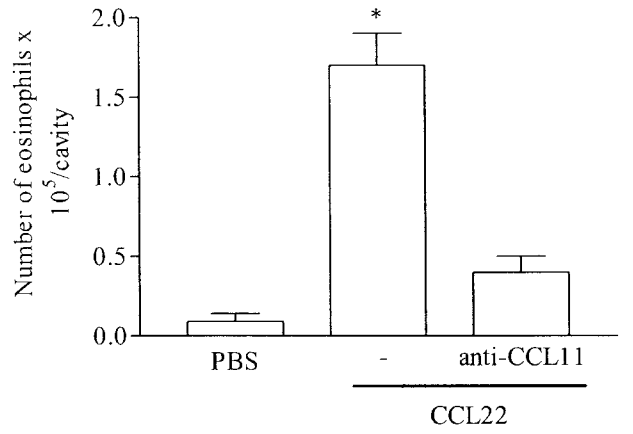

Fig. 3. Effects of an anti-CCL11 polyclonal antibody on the recruitment of eosinophils induced by CCL22. Mice were pretreated with nonimmune IgG (400 μg, i.p.) or purified anti-CCL11 polyclonal antibody (400 μg, i.p.) 60 min before the i.pl. injection of CCL22 (100 ng/cavity) in naïve mice, and the number of infiltrating eosinophils was assessed after 24 h. The control group of mice was injected with PBS. The results are expressed as means ± SEM of six mice in each group. *, $P < 0.01$ when compared with PBS.

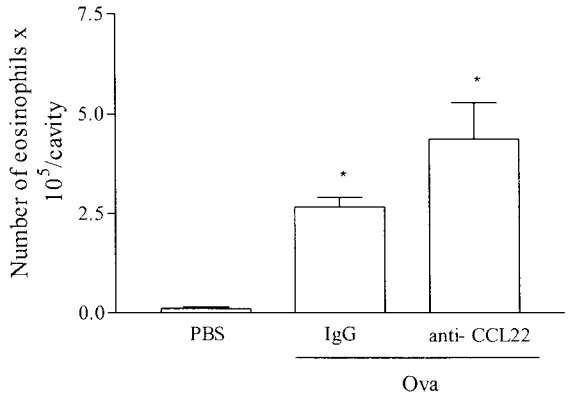

Fig. 5. Effects of treatment with anti-CCL22 antibodies during the sensitization phase on recruitment of eosinophil induced by OVA challenge in sensitized mice. Anti-CCL22 was injected 1 h before and 24 h after s.c. injection of OVA, and the number of infiltrating eosinophils was assessed after 48 h challenge with OVA. The results are expressed as means ± SEM of five mice in each group. *, $P < 0.05$ when compared with controls.

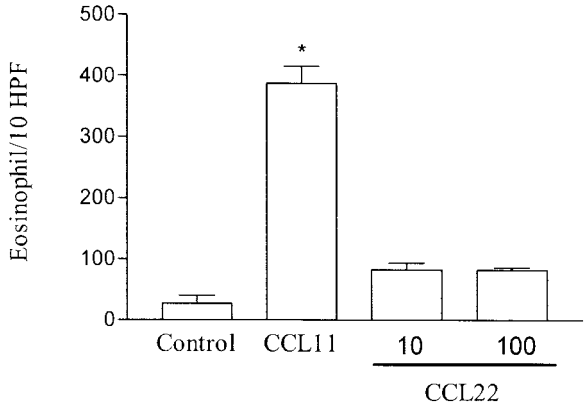

Fig. 6. Effects of CCL22 and CCL11 on the chemotaxis of antigen-elicited murine eosinophils. CCL22 and CCL11 were used at the concentrations of 10 or 100 ng/ml. Purified eosinophils were incubated for 1 h at 37°C in a 5% CO_2 moist atmosphere with chemokines. Eosinophil migration was quantitated by counting the number of eosinophils migrating completely through the matrix-coated filter in 10 HPF. The results are expressed as the means ± SEM of triplicate determinations and are representative of at least three different experiments. *, $P < 0.01$ when compared with PBS.

shown). To extend on the observation that CCL22 may be an important activator of eosinophils, experiments were performed in vivo. After 48 h, the levels of EPO in the pleural cavity of antigen-challenged mice were only slightly greater than that in pleural cavities of PBS-challenged, sensitized mice (Fig. 7B). However, if sensitized mice received an i.pl. injection of CCL22 or CCL11 at this time point (48 h after antigen challenge), and EPO levels were evaluated after a further 2 h, there was a marked increase in levels of EPO in pleural cavity washes of CCL22- but not CCL11-injected mice (Fig. 7B). These data suggest that CCL22 is a potent stimulus for eosinophil degranulation but not for eosinophil chemotaxis.

DISCUSSION

An important feature of allergic disease is the presence of a great number of eosinophils in tissue [5]. In asthma, an increased number of eosinophils have been found in bronchoalveolar lavage and bronchial biopsies [5–7, 23]. Similarly, eosinophil-derived products are commonly found in skin biopsies of patients with allergic dermatitis [24, 25]. Although the role of eosinophils in asthma is not completely understood, it is thought that the recruitment and action of eosinophils in the airways may underlie several of the pathophysiological manifestations of asthma [5, 7, 8]. An understanding of the mechanisms and role of chemokines and mediators that regulate chemokine production involved in eosinophil recruitment may be essential for the development of pharmacological therapies to control allergic disease. In this study, we evaluated the effects on and relevance of CCL22 for eosinophil migration in vivo and in vitro.

Here, we demonstrated that the injection of CCL22 caused a dose- and time-dependent recruitment of eosinophils into the pleural cavity of mice. Maximal eosinophil recruitment occurred 24 h after injection of a dose of 100 ng/cavity of the chemokine. These effects of CCL22 were qualitatively similar to the shown effects of other chemokines, including CCL3, CCL5, and CCL11 [10]. Previous studies have demonstrated the involvement of lipid mediators, such as LTB_4 and PAF, in the eosinophil recruitment induced by a range of inflammatory stimuli and following i.pl. administration of the chemokines [9, 10, 26]. Thus, we investigated whether CCL22 acted directly to induce eosinophil recruitment or did so by inducing the local release of lipid mediators. The treatment of mice with the LTB_4 receptor antagonist CP105,696 had no significant effect on the eosinophil recruitment induced by CCL22. This is in contrast with the significant inhibitory effects of the treatment with a PAFR antagonist UK-74,505 and in agreement with other studies showing the involvement of PAF in the migration of eosinophils in vivo [9, 27–29]. Thus, it appears that the ability of CCL22 to induce eosinophil recruitment is dependent on the release of PAF. More recently, we have shown that PAF-

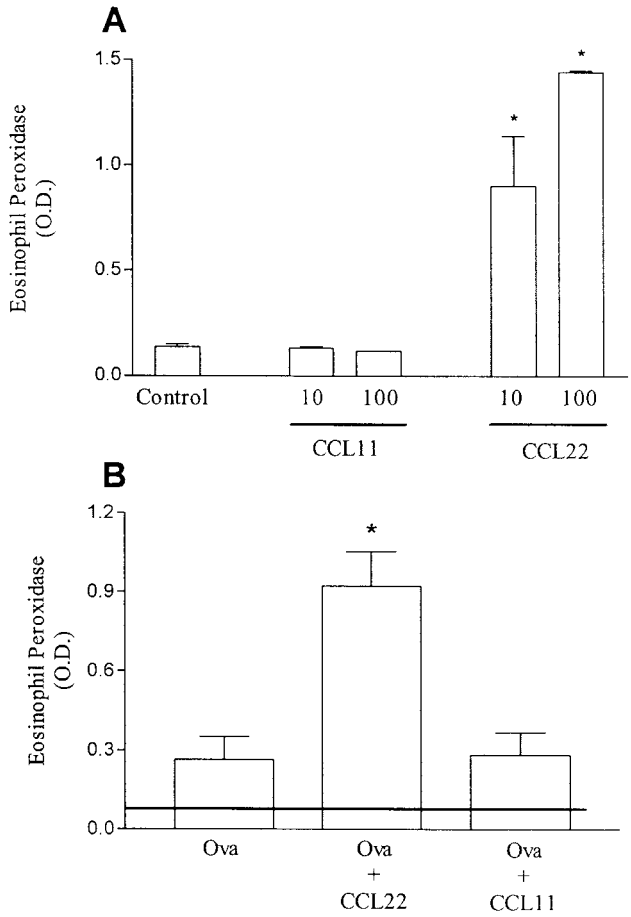

Fig. 7. Effects of CCL22 and CCL11 on the degranulation of (A) antigen-elicited murine eosinophils in vitro and (B) in the allergic pleurisy model. (A) CCL22 and CCL11 were used at the concentrations of 10 or 100 ng/ml. Purified eosinophils were activated for 4 h with the chemokines, and degranulation was assessed by measuring the EPO levels in the cell-free supernatant. (B) Sensitized mice received an i.pl. injection of PBS, CCL22 (100 ng/cavity), or CCL11 (100 ng/cavity) 48 h after antigen challenge, and EPO levels in pleural cavity washes were evaluated after a further 2 h. The line represents background levels after injection of PBS in sensitized, unchallenged mice. The results are expressed as the means ± SEM of triplicate determinations and are representative of at least three different experiments. *, $P < 0.01$ when compared with PBS or eotaxin. O.D., Optical density.

induced eosinophil recruitment is dependent on the release of CCL11 into the pleural cavity [9]. In agreement with these studies, pretreatment with anti-CCL11 antibody effectively inhibited the recruitment of eosinophils induced by CCL22. In the present study, we have been unable to detect CCL11 immunoreactivity in pleural cavity fluid of naïve animals after injection of CCL22 (data not shown). This is in contrast with the effects of the anti-CCL11 antibody in the system. However, we have previously shown that low concentrations of CCL11 may synergize with PAF to induce eosinophil recruitment [9]. It is thus possible that the amount of CCL11 present in the fluid is sufficient to cooperate with PAF to induce eosinophil recruitment but not detectable using our ELISA technique. Thus, the injection of CCL22 into the pleural cavity of naïve mice appears to trigger the release of PAF and sequential generation of CCL11, which ultimately induces eosinophil recruitment in our system. The inability of CCL22 to induce murine eosinophil chemotaxis in vitro supports the notion that intermediate mediators are needed for the in vivo effects of CCL22 on eosinophil recruitment.

As CCL22 induced eosinophil recruitment when injected exogenously and was capable of inducing PAF and CCL11, both important mediators during allergic inflammation [9, 28–32], we evaluated the relevance of CCL22 for eosinophil recruitment in our allergic pleurisy model. Pretreatment with an anti-CCL22 polyclonal antibody, at a dose that effectively blocked the effects of CCL22 itself, had no inhibitory effect on the recruitment of eosinophils induced by antigen challenge of sensitized mice. This is qualitatively similar to the lack of effects of anti-CCL22 for the migration of eosinophils into the airway lumen in an allergic model of asthma [33]. In addition, mice that are deficient in CCR4, the receptor for CCL22, showed similar lung recruitment of eosinophils to WT controls [34]. In contrast, CCL22 was relevant for the initial but not late migration of eosinophils in an animal model of asthma [33]. Thus, although CCL22 can induce the recruitment of eosinophils in a PAF- and CCL11-dependent manner when added exogenously, it appears that endogenous release of CCL22 plays only a minor role in determining eosinophil recruitment in models of allergic inflammation.

As CCL22 acts on diverse cellular targets including DC, NK cells, and T cell subsets [1], it may affect the sensitization phase of an allergic immune response. Treatment with the anti-CCL22 antibody during the sensitization phases had no significant effect on the eosinophil accumulation induced by antigen challenge. Moreover, we failed to observe any significant modification of the production of cytokines by spleen cell cultures, including IFN-γ and IL-5. In agreement with the lack of importance for CCL22 during the sensitization phase, CCR4-deficient mice were also immunized and responded normally in a model of allergic asthma [34].

Although anti-CCL22 failed to affect eosinophil migration consistently, it was found to block the development of airway hyper-responsiveness in the murine model of asthma [33]. One possibility that stems from the latter observations is that CCL22 may activate eosinophils (and possibly other cell types), which have migrated to the tissues. In support of the latter possibility, we demonstrate here that elicited eosinophils degranulated in response to activation by CCL22 but not CCL11 in vitro and in vivo. Whether CCL22 activates human eosinophils in a similar manner is not known. Of note, Bochner et al. [15] have shown that CCL22 induced the chemotaxis of eosinophils purified from blood of allergic patients. The effect of CCL22 was independent on CCR3 (blocking antibody did not reverse the effects) or CCR4 (no mRNA for CCR4 on eosinophils) and thus, was dependent on a receptor distinct from CCR1 to 7 [15]. The inability of CCL22 to induce chemotaxis of murine eosinophils suggests that this latter, distinct chemokine receptor is absent on murine eosinophils or does not respond to CCL22 in this species. Similarly, although RANTES (CCL5) stimulates human eosinophils, it fails to activate murine eosinophils [12]. Thus, it is clear that in human eosinophils, CCL22 may not only activate eosinophil degranulation (as shown here for murine eosinophils) but also their chemotaxis, suggesting a role for this chemokine in human allergic diseases.

There are other CC chemokines, including CCL17/thymus and activation-regulated chemokine, CCL8/MIP-1β, and CCL1/trichloroacetic acid-3, which are capable of preferentially inducing eosinophil activation rather than eosinophil migration (ref. [35]; and S. H. Oliveira et al., unpublished results). It is interesting that the opposite was observed with CCL11, which preferentially induced eosinophil migration over eosinophil activation, as assessed by degranulation. Thus, it appears that during the allergic process, specific chemokines may be important to induce eosinophil recruitment to sites of inflammation. Once there, eosinophils may come in contact with tissue matrix and locally produced mediators, become activated, express new chemokine receptors, and respond differentially to various chemokines [35, 36]. Moreover, an important point not to be missed is the observation that two distinct chemokines acting on the same cell type trigger distinct functions, highlighting the complexity and cooperation of chemokines in in vivo systems. Whether differential signaling transduction pathways regulate the differential response of eosinophils to the various chemokines clearly deserves further investigation.

In conclusion, we show that although exogenously added CCL22 may induce eosinophil migration in vivo via the sequential release of PAF and CCL11, endogenous production of CCL22 does not drive eosinophil migration during allergic inflammation. However, CCL22 may be an important activator of eosinophils once these cells have migrated into tissue.

ACKNOWLEDGMENTS

We are grateful to CNPq, FAPEMIG, and FAPESP for financial support.

REFERENCES

1. Mantovani, A., Gray, P. A., Van Damme, J., Sozzani, S. (2000) Macrophage-derived chemokines (MDC). *J. Leukoc. Biol.* **68,** 400–404.
2. Matsukawa, A., Hogaboam, C. M., Lucaks, N. W., Lincoln, P. M., Evanoff, H. L., Kunkel, S. L. (2000) Pivotal role of the CC chemokine, macrophage-derived chemokines, in the innate immune response. *J. Immunol.* **164,** 5362–5368.

3. Godiska, R., Chantry, D., Raport, C. J., Sozzani, S., Allavena, P., Leviten, D., Mantovani, A., Gray, P. W. (1997) Human macrophage derived chemokine (MDC) a novel chemoattractant for monocyte, monocyte derived dendritic cells, and natural killer cells. *J. Exp. Med.* **185,** 1595–1604.
4. Lloyd, C. M., Delaney, T. M., Nguyen, T., Tian, J., Martinez-A, C., Coyle, A. J., Gutierrez-Ramos, J. C. (2000) CC chemokine receptor (CCR)3/eotaxin is followed by CCR4/monocyte-derived chemokines in mediating pulmonary T helper lymphocyte type 2 recruitment after serial antigen challenge *in vivo*. *J. Exp. Med.* **191,** 265–273.
5. Cara, D. C., Negrao-Correa, D., Teixeira, M. M. (2000) Mechanisms underlying eosinophil trafficking and their relevance *in vivo*. *Histol. Histopathol.* **15,** 899–920.
6. Djukanovic, R. J., Wilson, J. W., Britten, K. M., Wilson, S. J., Walls, W. R., Roche, W. R., Howarth, P. H., Holgate, S. T. (1990) Quantitation of mast cells and eosinophils in the bronchial mucosa of symptomatic atopic asthmatics and healthy control subjects using immunohistochemistry. *Am. Rev. Respir. Dis.* **142,** 863–871.
7. Lacy, P., Moqbel, R. (2001) Immune effector functions of eosinophils in allergic airway inflammation. *Curr. Opin. Allergy Clin. Immunol.* **1,** 79–84.
8. Kita, H., Gleich, G. J. (1996) Chemokines active on eosinophils: potential roles in allergic inflammation. *J. Exp. Med.* **183,** 2421–2426.
9. Klein, A., Pinho, V., Alessandrini, A. L., Shimizu, T., Ishii, S., Teixeira, M. M. (2002) Platelet-activating factor drives eotaxin production in allergic pleurisy in mice. *Br. J. Pharmacol.* **135,** 1213–1218.
10. Klein, A., Talvani, A., Silva, P. M., Martins, M. A., Wells, T. N., Proudfoot, A., Luckacs, N. W., Teixeira, M. M. (2001) Stem cell factor-induced leukotriene B(4) production cooperates with eotaxin to mediate the recruitment of eosinophils during allergic pleurisy in mice. *J. Immunol.* **167,** 524–531.
11. Teixeira, M. M. (1998) Eosinophil-active chemokines: assessment of in vivo activity. *Braz. J. Med. Biol. Res.* **31,** 19–24
12. Teixeira, M. M., Wells, T. N. C., Lukacs, N. W., Proudfoot, A. E. E., Kunkel, S. L., Williams, T. J., Hellewell, P. G. (1997) Chemokine-induced eosinophil recruitment: evidence of a role for endogenous eotaxin in an in vivo allergy model in mouse skin. *J. Clin. Invest.* **100,** 1656–1666.
13. Schroder, J .M., Noso, N., Sticherling, M., Christophers, E. (1996) Role of eosinophil-chemotactic C-C chemokines in cutaneous inflammation. *J. Leukoc. Biol.* **59,** 1–5.
14. Teran, L. M., Noso, N., Carro, M., Davies, D. E., Holgate, S., Schroder, J. M. (1996) Eosinophil recruitment following allergen challenge is associated with the release of the chemokines RANTES into asthmatic airways. *J. Immunol.* **157,** 1806–1812.
15. Bochner, B. S., Bickel, C. A., Taylor, M. L., MacGlashan Jr., D. W., Gray, P. W., Raport, C. J., Godiska, R. (1999) Macrophage-derived chemokine induces human eosinophil chemotaxis in a CC chemokine receptor 3- and CC chemokine receptor 4-independent manner. *J. Allergy Clin. Immunol.* **103,** 527–532.
16. Ishii, S., Kuwaki, T., Nagase, T., Maki, K., Tashiro, F., Sunaga, S., Cao, W. H., Kume, K., Fukuchi, Y., Ikuta, K., Miyazaki, J., Kumada, M., Shimisu, T. (1998) Impaired anaphylactic responses with intact sensitivity to endotoxin in mice lacking a platelet-activating receptor. *J. Exp. Med.* **187,** 1779–1788.
17. Das, A. M., Flower, R. J., Hellewell, P. G., Teixeira, M. M., Perretti, M. (1997) A novel murine model of allergic inflammation to study the effect of dexamethasone on eosinophil recruitment. *Br. J. Pharmacol.* **121,** 97–104.
18. Ruth, J. H., Lukacs, N. W., Warmington, K. S., Polak, T. J., Burdick, M., Kunkel, S. L., Strieter, R. M., Chensue, S. W. (1998) Expression and participation of eotaxin during mycobacterial (type 1) and schistosomal (type 2) antigen-elicited granuloma formation. *J. Immunol.* **161,** 4276–4282.
19. Lukacs, N. W., Strieter, R. M., Shaklee, C. L., Chensue, S. W., Kunkel, S. L. (1995) Macrophage inflammatory protein-1 alpha influences eosinophil recruitment in antigen-specific airway inflammation. *Eur. J. Immunol.* **25,** 245–251.
20. Falk, W., Goodwin Jr., R. H., Leonard, E. J. (1980) A 48-well micro chemotaxis assembly for rapid and accurate measurement of leukocyte migration. *J. Immunol. Methods* **33,** 239–247.
21. Schneider, T., Issekutz, A. C. (1996) Quantitation of eosinophil and neutrophil infiltration into rat lung by specific assays for eosinophil peroxidase and myeloperoxidase application in a brown Norway rat model of allergic pulmonary inflammation. *J. Immunol. Methods* **198,** 1–14.
22. Klein, A., Talvani, A., Cara, D. C., Gomes, K. L., Lukacs, N. W., Teixeira, M. M. (2000) SCF plays a major role in the recruitment of eosinophils in allergic pleurisy in mice via the production of LTB_4. *J. Immunol.* **164,** 4271–4276.
23. Djukanovic, R. J., Roche, W. R., Wilson, J. W., Beasley, C. R. W., Twentyman, O. P., Howarth, P. H., Holgate, S. T. (1990) Mucosal inflammation in asthma. *Am. Rev. Respir. Dis.* **142,** 434–457.
24. Leiferman, K. M. (2001) A role for eosinophils in atopic dermatitis. *J. Am. Acad. Dermatol.* **45,** 521–524.
25. Martin, L. B., Kita, H., Leiferman, K. M., Gleich, G. J. (1996) Eosinophils in allergy: role in disease, degranulation, and cytokines. *Int. Arch. Allergy Immunol.* **109,** 207–215.
26. Teixeira, M. M., Reynia, S., Robinson, M., Shock, A., Williams, T. J., Williams, F. M., Rossi, A. G., Hellewell, P. G. (1994) Role of CD11/CD18 in mediating cutaneous inflammation in the guinea pig. *Br. J. Pharmacol.* **111,** 811–818.
27. Teixeira, M. M., Giembycz, M. A., Lindsay, M. A., Hellewell, P. G. (1997) Pertussis toxin reveals distinct early signalling events in platelet-activating factor, leukotriene B_4- and C5a-induced eosinophil homotypic aggregation in vitro and recruitment in vivo. *Blood* **89,** 4566–4573.
28. Henocq, E., Vargaftig, B. B. (1986) Accumulation of eosinophils in response to intracutaneous PAF-acether and allergens in man. *Lancet* **14,** 1378–1379.
29. Silva, P. M., Martins, M. A., Faria-Neto, H. C., Cordeiro, R. S., Vargaftig, B. B. (1991) Generation of an eosinophilotactic activity in the pleural cavity of platelet-activating factor-injected rats. *J. Pharmacol. Exp. Ther.* **257,** 1039–1044.
30. Fukuyama, S., Inoue, H., Aizawa, H., Oike, M., Kitaura, M., Yoshie, O., Hara, N. (2000) Effect of eotaxin and platelet-activating factor on airway inflammation and hyperresponsiveness in guinea pigs in vivo. *Am. J. Respir. Crit. Care Med.* **161,** 1844–1849.
31. Humbles, A. A., Conroy, D. M., Marleau, S., Rankin, S. M., Palframan, R. T., Proudfoot, A. E., Wells, T. N., Li, D., Jeffery, P. K., Griffiths-Johnson, D. A., Williams, T. J., Jose, P. J. (1997) Kinetics of eotaxin generation and its relationship to eosinophil accumulation in allergic airways disease: analysis in a guinea pig model in vivo. *J. Exp. Med.* **186,** 601–612.
32. Das, A. M., Flower, R. J., Perreti, M. (1997) Eotaxin-induced eosinophil migration in the peritoneal cavity of ovalbumin-sensitized mice: mechanism of action. *J. Immunol.* **159,** 1466–1473.
33. Gonzalo, J. A., Pan, Y., Lloyd, C. M., Jia, G. Q., Yu, G., Dussault, B., Powers, C. A., Proudfoot, A. E. I., Coyle. A. J., Gearing, D., Gutierrez-Ramos, J. C. (1999) Mouse monocyte-derived chemokine is involved in airway hyperreactivity and lung inflammation. *J. Immunol.* **163,** 403–411.
34. Chvatchko, Y., Hoogewerf, A. J., Meyer, A., Alouani, S., Juillard, P., Buser, R., Conquet, F., Proudfoot, A. E. I., Wells, T. N. C., Power, C. A. (2000) A key role for CC chemokine receptor 4 in lipopolysaccharide-induced endotoxic shock. *J. Exp. Med.* **15,** 1755–1763.
35. Oliveira, S. H., Lira, S., Martinez-A, C., Wiekowski, M., Sullivan, L., Lukacs, N. W. (2002) Increased responsiveness of murine eosinophils to MIP-1beta (CCL4) and TCA-3 (CCL1) is mediated by their specific receptors, CCR5 and CCR8. *J. Leukoc. Biol.* **71,** 1019–1025.
36. Lukacs, N. W., Oliveira, S. H. P., Hogaboam, C. M. (1999) Chemokines and asthma: redundancy of function or a coordinated effort? *J. Clin. Invest.* **104,** 995–999.