Antifungal and Airway Remodeling Roles for Murine Monocyte Chemoattractant Protein-1/CCL2 During Pulmonary Exposure to Aspergillus fumigatus Conidia

Kate Blease, Borana Mehrad, Nicholas W. Lukacs, Steven L. Kunkel, Theodore J. Standiford and Cory M. Hogaboam

*J Immunol* 2001; 166:1832-1842; doi: 10.4049/jimmunol.166.3.1832
http://www.jimmunol.org/content/166/3/1832

**References**
This article cites 56 articles, 22 of which you can access for free at: http://www.jimmunol.org/content/166/3/1832.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Antifungal and Airway Remodeling Roles for Murine Monocyte Chemoattractant Protein-1/CCL2 During Pulmonary Exposure to Aspergillus fumigatus Conidia

Kate Blease,* Borna Mehrad, ‡ Nicholas W. Lukacs,* Steven L. Kunkel,* Theodore J. Standiford, ‡ and Cory M. Hogaboam*†

Aspergillus fumigatus spores or conidia are quickly eliminated from the airways of nonsensitized individuals but persist in individuals with allergic pulmonary responsiveness to fungus. A. fumigatus-induced allergic airway disease is characterized by persistent airway hyperreactivity, inflammation, and fibrosis. The present study explored the role of CCR2 ligands in the murine airway response to A. fumigatus conidia. Nonsensitized and A. fumigatus-sensitized CBA/J mice received an intratracheal challenge of A. fumigatus conidia, and pulmonary changes were analyzed at various times after conidia. Whole lung levels of monocyte chemoattractant protein-1 (MCP-1/CCL2), but neither MCP-3/CCL7 nor MCP-5/CCL12, were significantly elevated at days 3 and 7 after conidia in nonsensitized mice. MCP-1/CCL2 was significantly increased in lung samples from A. fumigatus-sensitized mice at days 14 and 30 after a conidia challenge. Administration of anti-MCP-1/CCL2 antiserum to nonsensitized mice for 14 days after the conidia challenge attenuated the clearance of conidia and significantly increased airway hyperreactivity, eosinophilia, and peribronchial fibrosis compared with nonsensitized mice that received conidia and normal serum. Adenovirus-directed overexpression of MCP-1/CCL2 in A. fumigatus-sensitized mice markedly reduced the number of conidia, airway inflammation, and airway hyperresponsiveness at day 7 after the conidia challenge in these mice. Immunoneutralization of MCP-1/CCL2 levels in A. fumigatus-sensitized mice during days 14–30 after the conidia challenge did not affect the conidia burden but significantly reduced airway hyperreactivity, lung IL-4 levels, and lymphocyte recruitment into the airways compared with the control group. These data suggest that MCP-1/CCL2 participates in the pulmonary antifungal and allergic responses to A. fumigatus conidia. The Journal of Immunology, 2001, 166: 1832–1842.

Pulmonary responses to A. fumigatus conidia span a wide clinical spectrum, including a rapid clearance of the conidia without lung injury, allergic lung disease with or without fungus colonization (1), and lung destruction due to invasive pulmonary aspergillosis (2). These diverse consequences of pulmonary exposure to A. fumigatus appear to be directly related to the persistence and/or colonization of this fungus in the airways (3). In immunocompetent individuals, the coordinated activities of alveolar macrophages and infiltrating platelets and neutrophils effectively prevent the retention of A. fumigatus in the airways (4, 5). In contrast, patients with cutaneous and serologic evidence of A. fumigatus hypersensitivity typically exhibit the persistence of A. fumigatus conidia in airways, and this prolonged exposure to this fungus can cause airway hyperreactivity and peribronchial inflammation and fibrosis (3, 6, 7). Severe allergic responses are observed in patients with allergic bronchopulmonary aspergillosis, a syndrome characterized by A. fumigatus colonization, recurrent episodes of wheezing, mucus production, pulmonary infiltrates, and elevated levels of serum IgE, bronchiectasis, and fibrotic lung disease (3, 8, 9). Finally, invasive pulmonary aspergillosis is characterized by profound destructive fungal growth in the airways (10). Although the development of invasive aspergillosis is a complication of immunosuppression and/or neutropenia (11), an explanation for the persistence of A. fumigatus in the lungs of immunocompetent individuals sensitized to this fungus is not presently known.

Our recent studies showed that A. fumigatus-sensitized mice lacking CCR2 due to gene knockout failed to clear A. fumigatus spores from their lungs like their wild-type counterparts and consequently developed severe allergic lung disease (12). Given that CCR2 binds a number of major CC chemokines such as monocyte chemoattractant protein (MCP)1-1 (MCP-1/CCL2) (13), MCP-3/CCL7 (also known as MARC in the mouse) (13) and MCP-5/CCL12 (14), it was not clear from our previous study which chemokine was necessary for conidia clearance from the lungs of A. fumigatus-sensitized mice. All three CCR2 ligands have been detected in the context of clinical and experimental allergic airway disease. MCP-1/CCL2 is detected in abundance during clinical asthma (15–18) and mediates bronchial hyperreactivity in murine models of allergic airway disease (19) in a CCR2-dependent manner (20). MCP-3/CCL7 protein sequence exhibits 74% identity with human MCP-1/CCL2 (21), but MCP-3/CCL7 chemotactically most leukocytes due to its ability to bind CCR1, CCR2, and CCR3 (22–24). Clinical studies suggest that allergen-induced rhinitis (25) and atopic asthma (26) are associated with increased expression of...
MCP-3/CCL7, and it has a major role in the allergen-induced eosinophilic inflammation of the airways of mice sensitized to OVA (27). MCP-5/CCL7 is a mouse chemokine that is homologous with human MCP-1/CCL2 (66% amino acid identity) and is markedly increased during allergic responses (14) and Th1- and Th2-type pulmonary granulomatous responses (28) in mice. Immunoneutralization of MCP-5/CCL7 abolishes airway hyperreactivity by altering the trafficking of leukocytes through the lung interstitium (19).

We have previously shown that the intratracheal instillation of A. fumigatus conidia into mice previously sensitized to soluble A. fumigatus Ags results in the persistence of A. fumigatus conidia, airway hyperresponsiveness to spasmogens, goblet cell hyperplasia, and subepithelial fibrosis (29). However, nonsensitized mice quickly cleared conidia from their airways and failed to develop allergic airway disease (29). Thus, the purpose of the present study was 3-fold: 1) to determine whether the CCR2 ligands MCP-1/CCL2, MCP-3/CCL7, and MCP-5/CCL12 were elevated in nonsensitized and A. fumigatus-sensitized mice following their pulmonary exposure to A. fumigatus conidia; 2) to determine whether CCR2 ligands were involved in the regulation of the conidia burden in the lungs of nonsensitized and A. fumigatus-sensitized mice; and 3) to determine whether these CCR2 ligands were involved in the development of lung injury in nonsensitized and A. fumigatus-sensitized mice following an A. fumigatus conidia challenge.

Materials and Methods

Mice

Specific pathogen-free female CBA/J mice (The Jackson Laboratory, Bar Harbor, ME) were housed in the University Laboratory Animal Medicine facility at the University of Michigan Medical School. Prior approval for mouse usage was obtained from the University Laboratory Animal Medicine facility. Mice were sensitized to a commercially available preparation of soluble A. fumigatus Ags as previously described in detail (29). Unless stated otherwise, nonsensitized and A. fumigatus-sensitized mice received 5.0 × 10⁵ A. fumigatus conidia suspended in 30 μl of 0.1% Tween 80 via the intratracheal route (29).

Chemokine and cytokine ELISA analysis

Murine MCP-1/CCL2, MCP-3/CCL7, MCP-5/CCL12, and IL-4 levels were determined in 50-μl aliquots of whole lung homogenates using a standardized sandwich ELISA technique (30). Nunc-immuno ELISA plates (MaxiSorp; Nunc, Naperville, IL) were coated with the appropriate polyclonal capture Ab (R&D Systems, Minneapolis, MN) at a dilution of 1–5 μg/ml coating buffer (0.6 M NaCl, 0.26 M H₃BO₄, and 0.08 M NaOH, pH 9.6) overnight at 4°C. The unbound capture Ab was washed away and each plate was blocked with 2% BSA-PBS for 1 h at 37°C. Each ELISA plate was then washed three times with PBS/Tween 20 (0.05% v/v), and 50 μl of undiluted or diluted (1:10) whole lung homogenate was added to duplicate wells and incubated for 1 h at 37°C. Following the incubation period, the ELISA plates were then thoroughly washed and the appropriate biotinylated polyclonal detection Ab (3.5 μg/ml) was added. After washing the plates 45 min later, streptavidin-peroxidase (1:5000 dilution; Bio-Rad, Richmond, CA) was added to each well for 30 min and then thoroughly washed again. A chromagen substrate solution (Bio-Rad) was added and optical readings at 492 nm were obtained using an ELISA plate scanner. Recombinant murine chemokines and cytokines (R&D Systems) were used to generate the standard curves from which the concentrations present in the samples were derived. The limit of ELISA detection for each cytokine was consistently above 50 pg/ml. Each ELISA was screened to ensure the specificity of each Ab used.

Immunoneutralization studies

Antiserum containing polyclonal Abs directed against mouse MCP-1/CCL2 were generated in multiple-site immunized New Zealand White rabbits using a well-established protocol (30). The specificity of the anti-MCP-1/CCL2 antiserum was rigorously screened before its use in an experiment,
and it was found to lack cross-reactivity with all other chemokines and cytokines. In passive immunoneutralization experiments, each nonsensitized CBA/J mouse was injected i.p. with either 0.5 ml of preimmune rabbit serum or an equivalent amount of anti-MCP-1/CCL2 immune serum 2 h before the conidia challenge. Subsequent to this first injection and the conidia challenge, nonsensitized mice were injected with 0.5 ml of normal serum or anti-MCP-1/CCL2 antiserum every 48 h for 14 days. BAL cells were dispersed onto microscope slides using a cytosin, and macrophages, eosinophils, lymphocytes, and neutrophils were differentially stained with Wright-Giesma stain. A minimum of 15 high-powered-fields or 300 cells was examined in each cytosin. A total of $1 \times 10^6$ BAL cells was cytopsin onto each slide to compensate for differences in cell retrieval from each mouse. Values are expressed as mean ± SE. **, $p \leq 0.01$; *, $p \leq 0.001$ compared with BAL counts in nonsensitized mice that received normal serum and conidia 7 days previously.

Measurement of bronchial hyperresponsiveness

Bronchial hyperresponsiveness in individual mice was assessed using a Buxco plethysmograph (Buxco, Troy, NY) (29). Briefly, sodium pentobarbital (0.04 mg/g of mouse body weight; Butler, Columbus, OH) was injected into each mouse before their intubation and ventilation with a Harvard pump ventilator (Harvard Apparatus, Reno, NV) (29). After a baseline period of 5 min, the mouse received 1 μg of methacholine by tail vein injection. Airway hyperresponsiveness was calculated via the division of the transpulmonary pressure by the change in inspiratory volume (29). Immediately following the assessment of airway hyperresponsiveness, the mouse was euthanized and a bronchoalveolar lavage (BAL) was performed using 1 ml of normal saline, and a 500-μl aliquot of blood was also removed from each mouse. The BALs were centrifuged at 2000 rpm for 5 min, the supernatants were discarded, and the pelleted cells were transferred to a Cytospin (Shandon Scientific, Runcorn, U.K.). Serum was obtained from each blood sample after centrifugation at 9000 rpm for 10 min. Finally, whole lungs were dissected from each mouse and snap frozen in liquid N$_2$ or prepared for histological analysis (see below).

Morphometric analysis of leukocyte accumulation in BAL samples

Neutrophils, macrophages, eosinophils, and lymphocytes were quantified in BAL samples cytospun onto coded microscope slides. Each slide was stained with a Wright-Giesma differential stain, and the average number of each cell type was determined after counting a total of 300 cells in 10–20

![FIGURE 4](http://www.jimmunol.org/)

Leukocyte counts in BAL samples from nonsensitized CBA/J mice before and at day 7 after an intrapulmonary challenge with live *A. fumigatus* conidia. Nonsensitized CBA/J mouse were injected i.p. with either 0.5 ml of preimmune rabbit serum or an equivalent amount of anti-MCP-1/CCL2 immune serum 2 h before the conidia challenge. Subsequent to this first injection and the conidia challenge, nonsensitized mice were injected with 0.5 ml of normal serum or anti-MCP-1/CCL2 antiserum every 48 h for 14 days. BAL cells were dispersed onto microscope slides using a cytosin, and macrophages, eosinophils, lymphocytes, and neutrophils were differentially stained with Wright-Giesma stain. A minimum of 15 high-powered-fields or 300 cells was examined in each cytosin. A total of $1 \times 10^6$ BAL cells was cytopsin onto each slide to compensate for differences in cell retrieval from each mouse. Values are expressed as mean ± SE. **, $p \leq 0.01$; *, $p \leq 0.001$ compared with BAL counts in nonsensitized mice that received normal serum and conidia 7 days previously.

and it was found to lack cross-reactivity with all other chemokines and cytokines. In passive immunoneutralization experiments, each nonsensitized CBA/J mouse was injected i.p. with either 0.5 ml of preimmune rabbit serum or an equivalent amount of anti-MCP-1/CCL2 immune serum 2 h before the conidia challenge. Subsequent to this first injection and the conidia challenge, nonsensitized mice were injected with 0.5 ml of normal serum or anti-MCP-1/CCL2 antiserum every 48 h for 14 days. BAL cells were dispersed onto microscope slides using a cytosin, and macrophages, eosinophils, lymphocytes, and neutrophils were differentially stained with Wright-Giesma stain. A minimum of 15 high-powered-fields or 300 cells was examined in each cytosin. A total of $1 \times 10^6$ BAL cells was cytopsin onto each slide to compensate for differences in cell retrieval from each mouse. Values are expressed as mean ± SE. **, $p \leq 0.01$; *, $p \leq 0.001$ compared with BAL counts in nonsensitized mice that received normal serum and conidia 7 days previously.

Adenoviral-mediated overexpression of MCP-1/CCL2 in *A. fumigatus*-sensitized mice

To increase the levels of MCP-1/CCL2 in the lungs of *A. fumigatus*-sensitized mice, a recombinant adenovirus encoding for murine MCP-1/CCL2 (AdMCP-1/CCL2) was used. The construction of the recombinant Ad-MCP-1/CCL2 is explained in detail elsewhere (32). An adenovirus containing the *LacZ* gene (AdLacZ) was employed in the control group for this experiment. Groups of *A. fumigatus*-sensitized mice received $5.0 \times 10^8$ PFU of one of the recombinant viruses mixed with $5 \times 10^6$ conidia at day 0. Lung MCP-1/CCL2 levels, airway hyperresponsiveness, inflammation, and remodeling were examined on days 3 and 7 after the adenovirus and conidia challenge. MCP-1/CCL2 transgene expression persists for 7 days in the lungs of mice challenged intratracheally with $5.0 \times 10^6$ PFU of AdMCP-1/CCL2 (T. J. Standiford, unpublished observations).
high-powered fields (×1000) per slide. A total of 1 × 10⁶ BAL cells was cytospun onto each slide to compensate for differences in cell retrieval.

Whole lung histological analysis

Whole lungs from nonsensitized and *A. fumigatus*-sensitized mice before and after *A. fumigatus* conidia challenge were fully inflated with 4% paraformaldehyde and dissected and placed in fresh paraformaldehyde for 24 h. Routine histological techniques were used to paraffin embed the entire lung, and 5-μm sections of whole lung were stained with hematoxylin and eosin, Masson trichrome, periodic acid-Schiff (PAS), and Gomori methanamine silver (GMS). Inflammatory infiltrates and structural alterations were examined around blood vessels and airways using light microscopy at a magnification of ×200.

Hydroxyproline assay

Hydroxyproline levels were determined using a previously described assay (29). Briefly, whole lungs were homogenized in 2 ml of normal saline with a Tissue Tearor, and a 500-μl sample was added to 1 ml of 6 N HCl for 8 h at 120°C. To a 5-μl sample of the digested lung, 5 μl of citrate/acetate buffer (5% citric acid, 7.2% sodium acetate, 3.4% sodium hydroxide, and 1.2% glacial acetic acid, pH 6.0) and 100 μl of chloramine-T solution (282 mg of chloramine-T, 2 ml of n-propanol, 2 ml of distilled water, and 16 ml of citrate/acetate buffer) were subsequently added. These samples were added in triplicate to 96-well plates and then incubated at room temperature for 20 min before the addition of 100 μl of Ehrlich’s solution (Aldrich Chemical, Milwaukee, WI), 9.3 ml of n-propanol, and 3.9 ml of 70%.

![FIGURE 5. Representative photomicrographs of hematoxylin and eosin (A and B)-, Masson trichrome (C and D)-, and GMS (E and F)-stained whole lung sections from nonsensitized CBA/J mice at day 14 after an intrapulmonary challenge with live *A. fumigatus* conidia. Nonsensitized CBA/J mouse were injected i.p. with either 0.5 ml of preimmune rabbit serum or an equivalent amount of anti-MCP-1/CCL2 immune serum ~2 h before the conidia challenge. Subsequent to this first injection and the conidia challenge, nonsensitized mice were injected with 0.5 ml of normal serum or anti-MCP-1/CCL2 antiserum every 48 h for 14 days. Peribronchial inflammation and fibrosis were not prominent in lung sections from control CBA/J mice that received normal serum and conidia (A and C, respectively) compared with mice that received anti-MCP-1/CCL2 and conidia (B and D, respectively). GMS-stained conidia were absent in whole lung sections from control mice (E) but were prominent in lung sections from anti-MCP-1/CCL2-treated mice at day 14 after the conidia challenge (F; see arrows). Original magnification, ×200 for A–D and ×400 for E and F.](http://www.jimmunol.org/)
perchloric acid. The samples were subsequently incubated for 15 min at 65°C and cooled to room temperature before the 96-well plate was read at 550 nm in an ELISA plate scanner. Hydroxyproline concentrations were calculated from a standard curve of known hydroxyproline concentrations of 0–100 μg/ml.

Statistical analysis
All results are expressed as mean ± SEM (SE). ANOVA and Dunnett’s test for multiple comparisons were used to determine statistical significance in both groups at various times after the conidia challenge; \( p < 0.05 \) was considered to be statistically significant.

Results
Differential MCP-1 levels in nonsensitized and A. fumigatus-sensitized mice challenged with conidia
ELISA analysis of MCP-1/CCL2 in whole lung homogenates from nonsensitized and A. fumigatus-sensitized mice challenged with A. fumigatus conidia are shown in Fig. 1. Whole lung levels of MCP-1/CCL2 in nonsensitized mice were significantly elevated at days 3 and 7 after conidia compared with levels measured immediately before the conidia challenge (i.e., baseline levels; Fig. 1). At days 14 and 30 after the conidia challenge in nonsensitized mice, MCP-1/CCL2 levels were similar to baseline levels. In the A. fumigatus-sensitized group, significantly elevated levels of MCP-1/CCL2 in the lung were observed at days 14 and 30 after the conidia challenge (Fig. 1). MCP-3/CCL7 levels were below the limits of detection of this ELISA at all times after conidia challenge in both groups of mice (data not shown). Baseline MCP-5/CCL12 levels in nonsensitized and A. fumigatus-sensitized mice were ~4-fold lower than baseline levels of MCP-1/CCL2, and levels of this chemokine did not exceed 0.75 ng/ml in lung homogenates. In addition, temporal differences in MCP-5/CCL12 levels in the lungs of nonsensitized and A. fumigatus-sensitized mice were not observed. Thus, these data show that MCP-1/CCL2 was the only CCR2 ligand that was significantly increased during conidia challenge in CBA/J mice. In addition, the timing of lung MCP-1/CCL2 synthesis following an intratracheal challenge with A. fumigatus conidia appeared to be dependent on the allergic status of the mouse.

FIGURE 6. Hydroxyproline levels in whole lung homogenates from nonsensitized CBA/J mice at days 7 and 14 after an intrapulmonary challenge with live A. fumigatus conidia. Nonsensitized CBA/J mice were injected i.p. with either 0.5 ml of preimmune rabbit serum or an equivalent amount of anti-MCP-1/CCL2 immune serum ~2 h before the conidia challenge. Subsequent to this first injection and the conidia challenge, nonsensitized mice were injected with 0.5 ml of normal serum or anti-MCP-1/CCL2 antiserum every 48 h for 14 days. Hydroxyproline levels were measured as described in Materials and Methods. Values are expressed as mean ± SE; \( n = 4–5 \) group/time point. *, \( p \leq 0.05 \) compared with the values measured in CBA/J mice that received normal serum and conidia at the same time.

FIGURE 7. Whole lung levels of MCP-1/CCL2 in A. fumigatus-sensitized CBA/J mice before and at days 3 and 7 after a live A. fumigatus conidia challenge combined with \( 5.0 \times 10^6 \) PFU of either AdLacZ or AdMCP-1/CCL2. MCP-1/CCL2 was measured in both groups using a specific ELISA as described in Materials and Methods. Data are expressed as mean ± SEM; \( n = 4–5 \) group/time point. *, \( p \leq 0.05 \) compared with levels measured in the appropriate group of mice before the conidia and adenovirus challenge.

Immunoneutralization of MCP-1/CCL2 accelerated the onset of airway hyperresponsiveness in nonsensitized mice challenged with A. fumigatus conidia
The importance of the early increase in MCP-1/CCL2 in nonsensitized mice challenged with A. fumigatus conidia was addressed using an immunoneutralization approach. Two hours before A. fumigatus conidia challenge, mice received either anti-MCP-1 antiserum or normal rabbit serum and dosing continued every 2 days for 14 days after conidia challenge. To verify that MCP-1 levels were diminished by this treatment, whole lung homogenates were examined for the presence of immunoreactive MCP-1/CCL2 at days 7 and 14 after the conidia challenge. As shown in Fig. 2, the anti-MCP-1/CCL2 antiserum treatment abolished MCP-1/CCL2 levels in whole lung homogenates from nonsensitized mice at both times.

Airway hyperresponsiveness in nonsensitized mice before and at days 7 and 14 after the conidia challenge is shown in Fig. 3. Before the conidia challenge, an i.v. methacholine challenge in nonsensitized mice did not provoke a major increase in airway hyperresponsiveness above the baseline response (Fig. 3, dashed line). At day 7 after the conidia challenge, airway hyperresponsiveness was significantly increased above the baseline response in the group of nonsensitized mice treated with anti-MCP-1/CCL2.
antiserum (Fig. 3). The control group did not exhibit similar increases in airway hyperresponsiveness at day 7. Nonsensitized mice were also analyzed at day 14 after the conidia challenge, and both groups displayed identical airway hyperresponsiveness following a methacholine challenge. Thus, the administration of anti-MCP-1/CCL2 antiserum markedly accelerated the onset of significant airway hyperresponsiveness in nonsensitized mice.

Immunoneutralization of MCP-1/CCL2 significantly increased eosinophil recruitment into the airways of nonsensitized mice challenged with A. fumigatus conidia

Given the significant increase in airway hyperresponsiveness at day 7 after the conidia challenge in mice treated with anti-MCP-1/CCL2 antiserum, we next examined leukocyte numbers in BAL samples. The data from this analysis are shown in Fig. 4. Lymphocyte and neutrophil numbers in the BAL did not differ between the control and anti-MCP-1/CCL2-treated groups. However, a significant 2-fold decrease in macrophage numbers was observed in the BAL samples removed from the anti-MCP-1/CCL2 group compared with the control group (Fig. 4). Conversely, significantly more eosinophils were detected in BAL samples from the anti-MCP-1/CCL2 group compared with the normal serum control. Taken together, these data suggested that the immunoneutralization of MCP-1/CCL2 attenuated monocyte but augmented eosinophil recruitment into the airways of nonsensitized mice following a conidia challenge.

Immunoneutralization of MCP-1/CCL2 markedly augmented peribronchial inflammation and fibrosis and the presence of conidia in nonsensitized mice challenged with A. fumigatus conidia

The immunoneutralization of MCP-1/CCL2 in nonsensitized mice markedly exacerbated the degree of peribronchial inflammation at day 14 (Fig. 5B) compared with the normal serum group at the same time after the conidia challenge (Fig. 5A). The increased airway inflammation was characterized by marked increases in the numbers of peribronchial eosinophils and lymphocytes. The anti-MCP-1/CCL2 antiserum-treated mice also had evidence of markedly increased peribronchial fibrosis as revealed by trichrome staining in Fig. 5D. A similar degree of peribronchial fibrosis was not observed in the nonsensitized group that received normal serum (Fig. 5C). Finally, there was no evidence that A. fumigatus conidia had been retained in the lungs of nonsensitized mice that received normal serum over the 14-day period after the conidia challenge. A representative GMS stain from the control group is shown in Fig. 5E. In contrast, numerous conidia were detected in the lungs of nonsensitized mice treated with anti-MCP-1/CCL2 antiserum over this same time. Together, these histological findings suggested that the immunoneutralization of MCP-1/CCL2 in nonsensitized mice during a conidia challenge prompted the development of allergic airway disease due in part to the retention of conidia in the lungs of these mice.
Quantitative analysis of peribronchial fibrosis in both groups of nonsensitized mice is shown in Fig. 6. At day 7 after conidia challenge, nonsensitized mice that received anti-MCP-1/CCL2 antiserum exhibited significantly greater amounts of hydroxyproline in whole lung samples compared with the normal serum control. Similarly, at day 14, whole lung samples from the anti-MCP-1/CCL2 group contained significantly greater quantities of hydroxyproline compared with the control group. Therefore, the biochemical analysis of peribronchial fibrosis confirmed that the immunoneutralization of MCP-1/CCL2 greatly enhanced the development of allergic airway remodeling in nonsensitized mice.

Adenoviral-mediated overexpression of MCP-1/CCL2 accelerated the clearance of A. fumigatus conidia from A. fumigatus-sensitized mice and inhibited allergic airway disease at day 7 after the conidia challenge

The role of MCP-1/CCL2 was next explored in mice sensitized to soluble A. fumigatus Ags and then challenged with A. fumigatus conidia. As shown in Fig. 1, the whole lung levels of MCP-1/CCL2 were significantly elevated above baseline levels at days 14 and 30 after conidia but not at earlier times. We have also noted that A. fumigatus conidia persist in A. fumigatus-sensitized mice in contrast to nonsensitized mice, which effectively clear conidia by day 7 after the conidia challenge (29). Therefore, we next examined whether the adenovirus-mediated overexpression of MCP-1/CCL2 (AdMCP-1/CCL2) during the first week of the conidia challenge in A. fumigatus-sensitize mice had an impact on the clearance of the conidia and the development of allergic airway disease. As shown in Fig. 7, the presence of AdMCP-1/CCL2 but not AdLacZ significantly increased the immunoreactive levels of MCP-1/CCL2 in whole lungs from A. fumigatus-sensitized mice at days 3 and 7 after the conidia challenge. AdMCP-1/CCL2- and AdLacZ-treated mice exhibited similar airway hyperresponsiveness at day 3 after the conidia challenge, but the former group of mice had significantly lower airway hyperresponsiveness at day 7 (Fig. 8). Also on day 7 after the conidia challenge, the lungs of AdMCP-1/CCL2-treated mice exhibited significantly less airway inflammation and contained fewer conidia compared with the AdLacZ-treated controls (Fig. 9). Thus, these data demonstrated that the increased expression of MCP-1/CCL2 in A. fumigatus-sensitized mice during the first week of the conidia challenge markedly reduced the conidia burden and consequently impaired the development of allergic airway disease.

**MCP-1/CCL2 promotes airway hyperresponsiveness in A. fumigatus conidia challenge in A. fumigatus-sensitized mice**

We next determined the role of MCP-1/CCL2 in A. fumigatus-sensitized mice challenged with conidia. Accordingly, A. fumigatus-sensitized mice received anti-MCP-1/CCL2 antiserum or normal serum beginning at day 14 and continuing to day 30 after the conidia challenge, corresponding to the period of significantly elevated MCP-1/CCL2 in the lungs of these mice. Airway inflammation, hyperresponsiveness and histological appearance were then examined at day 30, and notable differences were evident between the treatment groups at this time. There was a significant reduction in airway hyperresponsiveness in the anti-MCP-1/CCL2 antisera-treated group compared with the normal serum group.

**FIGURE 10.** Airway hyperresponsiveness in A. fumigatus-sensitized CBA/J mice at 30 days after an intrapulmonary challenge with live A. fumigatus conidia. A. fumigatus-sensitized CBA/J mice received an i.p. injection of 0.5 ml of anti-MCP-1/CCL2 antiserum or normal serum starting at day 14 after conidia and every 48 h after that for 16 days. Values are expressed as mean ± SE; n = 4–5/group/time point. *p ≤ 0.05 compared with the values measured in CBA/J mice that received normal serum and conidia at the same time.

**FIGURE 11.** Macrophage and lymphocyte counts in BAL samples from A. fumigatus-sensitized CBA/J mice at day 30 after an intrapulmonary challenge with live A. fumigatus conidia. A. fumigatus-sensitized CBA/J mice received an i.p. injection of 0.5 ml of anti-MCP-1/CCL2 antiserum or normal serum starting at day 14 after conidia and every 48 h after that for 16 days. BAL cells were dispersed onto microscope slides using a cytoospin, and macrophages, eosinophils, lymphocytes, and neutrophils were differentially stained with Wright-Giesma stain. A minimum of 15 high-powered-fields or 300 cells was examined in each cytoospin. A total of 1.1 × 10⁶ BAL cells was cytoospun onto each slide to compensate for differences in cell retrieval from each mouse. Values are expressed as mean ± SE. **p ≤ 0.01; ***p ≤ 0.001 compared with BAL counts in nonsensitized mice that received normal serum and conidia 7 days previously.
(Fig. 10). The decrease in airway hyperresponsiveness in the anti-MCP-1/CCL2 group was also associated with a significant decrease in lymphocytes in the BAL (Fig. 11) and IL-4 levels in whole lung samples (Fig. 12); however, IFN-γ levels in the whole lung were not altered. Histological analysis of anti-MCP-1/CCL2-treated mice revealed that subepithelial fibrosis was not diminished (Fig. 13B), but the presence of goblet cell numbers was markedly reduced (Fig. 13D) compared with the control group (Fig. 13, A and C). Surprisingly, the anti-MCP-1/CCL2 antiserum treatment did not enhance the retention of A. fumigatus conidia in the lungs of A. fumigatus-sensitized mice (Fig. 13F). Thus, these data suggest that MCP-1/CCL2 has distinct temporally dependent roles during the development of allergic airway disease in A. fumigatus-sensitized mice challenged with conidia.

Discussion

The pulmonary defense against fungus is very efficient in healthy individuals, but it is impaired in immunocompetent patients with allergic responsiveness to Aspergillus (3) and it is absent in immunocompetent patients (33). Although boosting the antifungal activity of available immune cells has been shown to improve the outcome of invasive aspergillosis in mice (34), similar strategies have not been previously examined in the context of allergic responsiveness to Aspergillus. Further impetus for the present study came from our previous observations that A. fumigatus-sensitized mice lacking CCR2 due to homologous recombination developed severe allergic disease due to their inability to clear conidia from the lung (12). Thus, in the present study, we examined changes in the production of major CCR2 ligands, including MCP-1/CCL2, MCP-3/CCL7, and MCP-5/CCL12, during the course of conidia challenge in nonsensitized and A. fumigatus-sensitized mice. Unlike the latter two ligands, MCP-1/CCL2 levels were significantly increased in both groups of mice and major temporal differences in MCP-1/CCL2 production were detected between these groups. The clearance of conidia from the lungs of nonsensitized mice was significantly impaired when anti-MCP-1 antiserum was administered to these mice. In addition, the nonsensitized mice challenged with conidia developed many features of allergic airway disease in the context of impaired MCP-1 function. This study also showed that MCP-1/CCL2, through AdMCP-1/CCL2 transgene expression, was responsible for the clearance of conidia from the airways of A. fumigatus-sensitized mice. However, when A. fumigatus-sensitized mice received anti-MCP-1/CCL2 antiserum beginning at day 14 of the conidia challenge, decreased airway hyperresponsiveness and airway remodeling were observed at day 30. Taken together, these data demonstrate that MCP-1/CCL2 exerts a number of major effects on the pulmonary defense and allergic responsiveness during pulmonary exposure to A. fumigatus conidia.

Aspergillus fumigatus is a clinically important pathogen that invokes a range of distinct pulmonary diseases (2). The primary lung defense against A. fumigatus conidia is mediated by the alveolar macrophage which has been shown to engulf and kill A. fumigatus conidia before these spores have a chance to germinate in the lung (35). Recruited platelets and neutrophils also contribute to fungal killing (5). Previous experimental studies have shown that cytokines such as TNF-α and GM-CSF are required for the recruitment of neutrophils in the airways during fungal challenge (36, 37). More recently, a significant role for the CXC chemokine macrophage-inflammatory protein (MIP)-2 (MIP-2) and KC (11, 38) and the CC chemokine MIP-1 (MIP-1α/CCL3) (34) in the recruitment of neutrophils and macrophages, respectively, and the clearance of conidia from the airways was revealed. Much of the data described above are pertinent to models of invasive aspergillosis characterized by fungal colonization in immunodeficient mice. However, less is known about the soluble mediators that regulate the clearance of A. fumigatus conidia from A. fumigatus-sensitized and immunocompetent airways. In many cases, A. fumigatus persists but fails to colonize the airways of individuals with Aspergillus hypersensitivity (3). Our previous study showed that A. fumigatus conidia persisted (but did not colonize) in the airways of A. fumigatus-sensitized CCR2ko mice, suggesting that CCR2 ligands are required for the clearance of conidia from A. fumigatus-sensitized mice (12). The present study confirmed this hypothesis since the CCR2 ligand MCP-1/CCL2 was required for the early and rapid clearance of conidia from the lungs of nonsensitized mice. MCP-1/CCL2 levels were significantly elevated within 3 days after the conidia challenge, and immunoneutralization of MCP-1/CCL2 levels in these mice was associated with the persistence of conidia. Further compelling evidence that MCP-1/CCL2 was necessary for the clearance of conidia was observed in A. fumigatus-sensitized mice. These mice exhibited a clear deficit in MCP-1/CCL2 levels during the first 2 wk after the conidia challenge, and conidia were prominent in the lungs of these mice during this time. Conversely, the transgene-directed overexpression of AdMCP-1/CCL2 in A. fumigatus-sensitized mice markedly accelerated the clearance of conidia from these mice. However, the antifungal effects of MCP-1/CCL2 appeared to be limited to the

FIGURE 12. Whole lung levels of IL-4 (A) and IFN-γ (B) in A. fumigatus-sensitized CBA/J mice at day 30 after a live A. fumigatus conidia challenge. A. fumigatus-sensitized CBA/J mice received an i.p. injection of 0.5 ml of anti-MCP-1/CCL2 antiserum or normal serum starting at day 14 after conidia and every 48 h after that for 16 days. IL-4 and IFN-γ levels were measured using a specific ELISA as described in Materials and Methods. Data are expressed as mean ± SEM; n = 4–5/group/time point. *, p ≤ 0.05 compared with levels measured in the appropriate group of mice before the conidia challenge.
2-wk period following the conidia challenge since the immuno-neutralization of MCP-1/CCL2 from days 14–30 after the conidia challenge in
*A. fumigatus* -sensitized mice did not appear to affect the retention of conidia. Taken together, these findings demonstrate that MCP-1/CCL2 has a prominent role in the regulation of the conidia burden in nonsensitized and *A. fumigatus* -sensitized mice.

Chemokines have emerged as a prominent group of factors in the initiation and maintenance of several features of asthma and allergic airway disease, most prominently the reversible airway obstruction due to bronchoconstriction (39). A number of investigators have shown that MCP-1/CCL2 has a prominent role in the development of airway hyperreactivity in normal (20) and allergic mice (19, 20, 40). In the present study, the contribution of MCP-1/CCL2 to airway hyperreactivity appeared to follow its antifungal role but impairment of the antifungal role of MCP-1/CCL2 clearly aggravated airway hyperresponsiveness. This effect was observed in both nonsensitized and *A. fumigatus* -sensitized mice during the 2-wk period following the conidia challenge. The temporally disparate roles for MCP-1 in the conidia-challenged airways are presumably a consequence of changes in CCR2 expression on immune cells and smooth muscle cells during the course of the lung disease. It is also interesting that anti-MCP-1/CCL2 treatment of *A. fumigatus*-sensitized mice significantly decreased whole lung IL-4 levels at day 30 after the conidia challenge. This finding coincides with findings from our laboratory (41, 42) and others (43, 44) that have shown that MCP-1 controls the Th2 response. Furthermore, although IL-4 does not mediate the lung pathology, it has a major effect on the airway hyperresponsiveness associated with another murine model of allergic aspergillosis (45, 46). Thus, subsequent to its antifungal effects, MCP-1/CCL2 contributes to the airway hyperresponsiveness-associated conidia-induced allergic airway disease.

There is growing evidence that the asthmatic airway can remodel to the point of irreversible airway obstruction (47). The significance of airway remodeling is highlighted in the elderly asthmatic population in which ~80% of these patients succumb to complications of irreversible obstruction (48). The remodeled asthmatic airway is characterized by increased goblet cell number, airway smooth muscle mass, and subepithelial fibrosis (39, 47). Although the mechanisms leading to irreversible airway obstruction are poorly understood, it is recognized that the airway’s aggressive reparative processes contribute to this process (49). The present study showed that the anti-MCP-1/CCL2 treatment of *A. fumigatus*-sensitized mice eliminated histological evidence of goblet cell hyperplasia; however, this treatment had no effect on the peribronchial fibrosis. The latter finding is perplexing in light of previous documentation that MCP-1/CCL2 is increased during clinical (50) and experimental (51–53) fibrotic responses, and a profibrotic role for MCP-1 has been demonstrated in models of

**FIGURE 13.** Representative photomicrographs of Masson trichrome (A and B)-, PAS (C and D)-, and GMS (E and F)-stained whole lung sections from *A. fumigatus*-sensitized CBA/J mice at day 30 after an intrapulmonary challenge with live *A. fumigatus* conidia. *A. fumigatus*-sensitized CBA/J mice received an i.p. injection of 0.5 ml of anti-MCP-1/CCL2 antiserum or normal serum starting at day 14 after conidia and every 48 h after that for 16 days. Peribronchial fibrosis was similar in lung sections from both treatment groups of *A. fumigatus*-sensitized CBA/J mice that received conidia 30 days previously (A and B). Unlike the control group (C; see arrows showing staining of goblet cells), no PAS staining of goblet cells was apparent in lung sections from anti-MCP-1/CCL2-treated mice (D). Compared with control mice that received normal serum and conidia (E), lung sections from anti-MCP-1/CCL2-treated mice lacked GMS-positive material associated with collections of mononuclear cells (F). Original magnification, ×200 for each photomicrograph.
crescentic glomerular nephritis (54) and granulomatous lung disease (28). Nevertheless, in the present study, the immunoneutralization of MCP-1/CCL2 in nonsensitized mice markedly increased peribronchial fibrosis at day 14 after the conidia challenge. The immunoneutralization of MCP-1/CCL2 in A. fumigatus-sensitized mice failed to reduce the peribronchial fibrosis at day 30 after the conidia challenge. The reason for this discrepancy is not immediately apparent, but our previous studies suggest that CCR1 agonists such as MIP-1α/CCL3 and RANTES/CCL5 are major mediators of airway remodeling but not airway hyperreactivity in A. fumigatus-sensitized mice challenged with conidia (55). Taken together, the present study highlights the fact that a number of divergent chemokines and their receptors contribute at different levels of the pathophysiological process associated with asthma and allergic airway disease (19).

Thus, the present study shows that MCP-1/CCL2 contributes to multiple facets of the antifungal and allergic pulmonary responses to A. fumigatus. Given its complex role in the experimental diseases described herein, manipulation of MCP-1/CCL2 during allergic responses to A. fumigatus may promote an exacerbation of pulmonary disease (56). At the same time, the results from the present study are motivation to examine the effect of current antifungal and antiallergic therapies on the generation of MCP-1/CCL2 in the lung.

References


