Immunosuppressive Effects of CCL17 on Pulmonary Antifungal Responses during Pulmonary Invasive Aspergillosis

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Aspergillus fumigatus-sensitized CCR4-deficient (CCR4^{-/-}) mice exhibit an accelerated clearance of conidia during fungal asthma. In the present study, we examined the roles of CCL17 and CCL22, two CCR4 ligands, during pulmonary invasive aspergillosis in neutropenic mice. Kaplan-Meier survival curve analysis revealed that wild-type C57BL/6 (CCR4^{+/+}) mice were significantly protected from the lethal effects of *Aspergillus* compared with their wild-type controls following systemic neutralization with anti-CCL17 but not anti-CCL22 antibodies. Systemic neutralization of CCL17 significantly increased whole-lung CCL2 levels. Mouse survival and histological analysis revealed that the receptor mediating the deleterious effects of CCL17 was CCR4 since mice genetically deficient in CCR4 (CCR4^{-/-}) did not develop invasive aspergillosis. Enzyme-linked immunosorbent assay analysis of whole-lung samples at day 2 after conidial challenge in neutrophil-depleted CCR4^{-/-} and CCR4^{+/+} mice revealed that whole-lung IL-12 levels were significantly increased in the CCR4^{-/-} group compared with the wild-type group. Also at day 2 after conidial challenge, significantly greater numbers of CD11c⁺ F4/80⁺ and CD11c⁺/CD86⁺ but fewer CD3/NK1.1⁺ cells were present in the lungs of CCR4^{-/-} mice compared with their wild-type counterparts. Thus, CCL17-CCR4 interactions dramatically impair the pulmonary antifungal response against *A. fumigatus* in neutropenic mice.

Aspergillus fumigatus is a major clinical menace in developed countries given its prevalence and ability to elicit a number of diseases, including asthma, hypersensitivity pneumonitis, allergic bronchopulmonary aspergillosis, aspergilloma, saprophytic involvement of infarcted tissue, chronic necrotizing pulmonary aspergillosis, and invasive aspergillosis (25). A number of factors participate in the development of *Aspergillus*-associated diseases, and these include the virulence of the fungal strain, the magnitude or duration of the exposure, and, probably most important, the status of host defense mechanisms and the type of exposure (25). All organs appear to be susceptible to infection, but pulmonary aspergillosis is the most common disease associated with this airborne saprophytic fungi (14). The spores or conidia from *Aspergillus* pose few problems for immunocompetent individuals; however, any perturbation to the systemic or pulmonary mucosal immune systems can lead to severe and usually fatal invasive aspergillosis (40), the incidence of which has increased alarmingly in this era of modern medicine (41).

Considerable research attention has been directed toward understanding the major immune factors that are required to contain the growth of *A. fumigatus*, particularly in the immunosuppressed host (56). Some very exciting antifungal strategies which work in the immunocompromised host have been elucidated, and they include manipulation of cytokine (16, 19, 47, 52) and chemokine levels (48, 51); anti-idiotypic monoclonal antibodies (18); local delivery of adjuvants (i.e., unmethylated CpG oligodeoxynucleotides and *Aspergillus* allergens) (10); and various vaccination strategies involving T cells (15), NK cells (51), and immu-

nocompetent (11, 12, 54) or immature (6) dendritic cells. Although impressive progress has been made, further experimental work is required to fully elucidate the mechanisms responsible for protective pulmonary immunity against *A. fumigatus*.

Previous studies demonstrated that *A. fumigatus*-sensitized mice lacking CCR4, the chemokine receptor that binds thymus and activation-regulated chemokine (TARC/CCL17) (34) and macrophage-derived chemokine (MDC/CCL22) (35), due to gene deletion rapidly cleared *A. fumigatus* conidia from their lungs, whereas wild-type mice did not (57). This finding was surprising in light of other studies demonstrating the major enhancing effect of CCL22 on innate immune responses directed by monocytes, dendritic cells, and NK cells (29, 37, 44). However, CCL17, which is predominately generated by non-immune cells (27, 34), does not share CCL22's effects on the innate immune response. In fact, enhanced production of this chemokine is driven by Th2 cytokines (26) and has a major enhancing role in highly skewed Th2-type immune responses, including asthma (7, 9, 39, 59), atopic dermatitis (61, 62), fulminant liver failure (63), and *Schistosoma mansoni* egg-induced pulmonary granulomatous responses (37). The differential roles of these two chemokines in immune responses may be due to the ability of CCL17 but not CCL22 to bind CCR8 (8). In addition, we recently observed that, unlike CCL22, CCL17 had a major immunosuppressive effect in a murine model of *S. mansoni*-induced pulmonary granuloma formation (37).

Thus, the present study addressed the roles of CCL17, CCL22, and their common chemokine receptor in a well-characterized murine model of invasive aspergillosis. The neutralization of CCL17 or the lack of CCR4, but not CCR8, markedly enhanced mouse survival following the bolus administration of *A. fumigatus* conidia to neutrophil-depleted mice on a C57BL/6 background. This protective effect was associated with significant increases in the antifungal chemokine

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CCL2 (51) and interleukin-12 (IL-12). In addition, the numbers of activated CD11c⁺ F4/80⁺ and CD11c⁺ CD86⁺ cells were significantly higher in the lungs of neutrophil-depleted CCR4^{-/-} mice at day 2 after conidial challenge compared with neutrophil-depleted CCR4^{+/+} mice at the same time after conidial challenge. Thus, CCL17 via CCR4 activation markedly impaired the innate mononuclear cell response to *Aspergillus* conidia, thereby permitting the development of invasive pulmonary aspergillosis.

MATERIALS AND METHODS

Mice. Specific-pathogen-free female C57BL/6 (wild type, CCR4^{+/+}, or CCR8^{+/+}) mice (6 to 8 weeks of age) were purchased from Taconic (Germantown, NY). CCR4^{-/-} mice were generated as previously described in detail (22) and backcrossed 10 generations onto the C57BL/6 genetic background. Tassie Collins (Amgen Inc., South San Francisco, CA) kindly provided breeding pairs of the appropriately backcrossed CCR4^{-/-} mice. CCR8^{-/-} mice were generated at previously described in detail (21) and backcrossed 10 generations onto the C57BL/6 genetic background. Sergio Lira (Mount Sinai School of Medicine) kindly provided breeding pairs of the appropriately backcrossed CCR8^{-/-} mice. Breeding colonies of CCR4^{-/-} and CCR8^{-/-} mice were maintained under specific-pathogen-free conditions in the University Laboratory of Animal Medicine (ULAM) facility. As previously described, CCR4^{-/-} (22) and CCR8^{-/-} (21) mice were born at the expected Mendelian ratios and showed no evidence of abnormal growth patterns. Prior approval for mouse usage in the present study was obtained from the ULAM.

Murine model of invasive aspergillosis. All mice used in the present study were depleted of neutrophils with an intraperitoneal injection of 100 µg of RB6-8C5 (anti-Ly6G) as previously described in detail (46). At 24 and 72 h after RB6-8C5 antibody injection, peripheral blood neutrophil count was reduced to less than 50 cells/µl (46). In the present study, all mice were anesthetized with a mixture of ketamine and xylazine (53) prior to receiving 5.0×10^6 *A. fumigatus* conidia suspended in 30 µl of 0.1% Tween 80 via an intratracheal (i.t.) injection (32) at 24 h after RB6-8C5 administration. *A. fumigatus* strain 13073 (American Type Culture Collection, Manassas, VA) was used since this is a highly virulent *Aspergillus* strain (49) which elicits a reproducible form of invasive aspergillosis in RB6-8C5-treated mice (46).

In the first series of experiments, neutrophil-depleted wild-type mice received goat immunoglobulin G (IgG), goat anti-mouse polyclonal anti-CCL17, or goat anti-mouse polyclonal anti-CCL22 (all at 20 µg/dose; R&D Systems, Minneapolis, MN) beginning 2 h prior to the i.t. conidial challenge and IgG or specific antibodies were readministered every 48 h thereafter. We have previously used anti-CCL17 and anti-CCL22 antibodies for effective neutralization of each chemokine in mice (37).

In the second series of experiments, groups of wild-type, CCR4^{-/-}, and CCR8^{-/-} mice were rendered neutropenic and challenged with 5×10^6 conidia. In both series of experiments, groups of seven mice were monitored for survival after the i.t. injection of conidia for 6 days. Additional groups of five mice were killed at designated time points after conidial challenge by anesthesia overdose and bled, and bronchoalveolar lavage (BAL) and whole-lung samples were processed as described below. The designated time points in this study were days 0 (i.e., 24 h after RB6-8C5 and immediately prior to conidial challenge), 2, and 6 after conidial challenge.

ELISA analysis. Murine CCL1, CCL17, CCL22, CCL2, CCL3, tumor necrosis factor alpha (TNF-α), gamma interferon (IFN-γ), IL-10, and IL-12 levels were determined in 50-µl samples from whole-lung homogenates by a standardized sandwich enzyme-linked immunosorbent assay (ELISA) technique provided by the supplier (R&D Systems, Minneapolis, MN). Each ELISA was screened to ensure the specificity of each antibody used. Recombinant murine cytokines or chemokines (R&D Systems, Minneapolis, MN) 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. Cytokine and chemokine levels were normalized to total protein levels with the Bradford assay.

Chitin analysis. Chitin is a major component of the hyphal wall and is not present in conidia, nor is it present in mammalian tissues. This hyphal wall component is often quantified as a measure of the fungal load in the lung, rather than relying on the dilution and culture of lung homogenates, which may underestimate the number of hyphae present in these samples. Whole-lung samples were processed as described in detail elsewhere (51). The chitin content in each sample was expressed as µg of glucosamine per lung.

Whole-lung histological analysis. Whole lungs from neutrophil-depleted CCR4^{+/+} and CCR4^{-/-} mice at days 0, 2, and 6 after *A. fumigatus* conidial challenge were fully inflated with 10% formalin, dissected, and placed in fresh formalin for 24 h. Routine histological techniques were used to paraffin embed the entire lung, and 5-µm whole-lung sections were stained with either Gomori methenamine silver (GMS) or hematoxylin and eosin (H&E). Using light microscopy at a magnification of $\times 200$, GMS staining was used to localize fungal material in whole-lung tissue sections, whereas inflammatory and structural alterations were determined in similar H&E-stained whole-lung sections.

Whole-lung cytometric analysis. Whole lungs were aseptically removed from neutrophil-depleted CCR4^{+/+} and CCR4^{-/-} mice at day 2 after conidial challenge. Briefly, lung tissues were pooled, macerated, shaken in 0.2% (wt/vol) collagenase (type IV) in RPMI with 10% fetal bovine serum for 45 min at 37°C, and washed in a balanced salt solution to remove aggregates. Lung cell suspensions were treated with ammonium chloride to lyse red blood cells, and the remaining cells were counted with a hemocytometer. Approximately 5×10^4 lung cells were transferred to clean tubes for staining. Fc binding was blocked via a 10-min incubation with anti-murine CD16/CD32 (Fc γ III/II receptor; BD Pharmingen, San Diego, CA), and cells were stained with anti-murine CD11c, anti-murine F4/80, anti-murine CD86, anti-murine CD3, anti-murine NK1.1, or the appropriate isotype control (all BD Pharmingen) for 30 min at 4°C in phosphate-buffered saline supplemented with 5% fetal calf serum. All cells were washed thoroughly and finally fixed in 2% paraformaldehyde for 30 min. Each cell population was analyzed by flow cytometry (Beckman Coulter EPICS XL, Miami, FL). The isotype control was used to gate the population of interest and as the negative control. The total population of each cell type was corrected against the control, and total cell counts were calculated.

Statistical analysis. All results are expressed as the mean $\pm$ standard error of the mean (SEM). Kaplan-Meier survival curve analysis was completed using a log rank test. Analysis of variance and the Tukey-Kramer test for multiple comparisons were used to determine statistical significance in both groups at various times after the conidial challenge; $P < 0.05$ was considered statistically significant.

RESULTS

Bronchoalveolar lavage levels of the CCR4 agonists CCL22 and CCL17 were significantly elevated at day 2 after conidial challenge in neutrophil-depleted CCR4^{+/+} mice. Prior to the *A. fumigatus* conidial challenge, baseline whole-lung levels of CCL22 and CCL17 in CCR4^{+/+} mice were similarly below 250 pg/ml, whereas immunoreactive CCL1 levels were below the level of ELISA detection (Fig. 1A). Both CCL22 and CCL17 were elevated at days 2 and 6 after conidial challenge in neutrophil-depleted CCR4^{+/+} mice. Elevations in whole-lung levels of CCL22 and CCL17 at days 2 and 6 after conidial challenge reached statistical significance compared with immunoreactive levels of both chemokines at day 0. At all three time points, CCL1 levels were consistently below the limits of ELISA detection in whole-lung samples.

In BAL samples, CCL22, CCL17, and CCL1 were not detected immediately prior to *A. fumigatus* conidial challenge in CCR4^{+/+} mice (Fig. 1B). However, at day 2 after conidial challenge, both CCL22 and CCL17 were significantly increased compared with day 0 levels. At this time, BAL CCL17 levels were approximately threefold greater than BAL CCL22 levels (Fig. 1B). At day 6 after conidial challenge, BAL levels of both CCR4 agonists were again below the limits of ELISA detection. Again, ELISA did not detect CCL1 in BAL samples either before or after conidial challenge.

Neutrophil-depleted C57BL/6 mice were highly resistant to invasive pulmonary aspergillosis following neutralization of CCL17. The roles of CCL22 and CCL17 during invasive aspergillosis in CCR4^{+/+} mice were examined next. As shown in Fig. 2 (top panel), the neutralization of CCL17 significantly ($P \leq 0.05$; log rank test) improved survival of neutrophil-depleted CCR4^{+/+} mice compared with neutrophil-depleted

FIG. 1. ELISA analysis of CCL1, CCL17, and CCL22 levels in whole-lung (A) and bronchoalveolar lavage (B) samples from neutropenic wild-type mice prior to (i.e., day 0) and at days 2 and 6 after an i.t. challenge with 5×10^6 conidia. To induce neutropenia, all mice were injected with 100 μ g of RB6-8C5 antibody 24 h prior to the conidial challenge. Data shown are means $\pm$ SEM ($n = 5$ mice/group/time point). ***, $P \leq 0.001$ compared with appropriate chemokine levels at day 0 after conidial challenge; $\tau\tau$, $P \leq 0.01$ compared with appropriate chemokine levels at day 2 after conidial challenge.

CCR4^{+/+} mice that received IgG at the same time points. At day 6 after conidial challenge, approximately 85% of CCR4^{+/+} mice receiving anti-CCL17 were alive. In contrast, 57% and 43% of mice that received anti-CCL22 antibody and IgG, respectively, were alive at the same time point (Fig. 2, top panel). Thus these data suggested that CCL17 exerted an inhibitory effect on the innate antifungal response.

Significantly elevated whole-lung CCL2 levels were present in anti-CCL17 antibody-treated mice. Whole-lung ELISA analyses for TNF- α , IL-12, CCL2, and CCL3 are shown in Fig. 2, bottom panel. At day 6 after conidial challenge in neutropenic wild-type mice, whole-lung levels of TNF- α , IL-12, and CCL3 did not differ among the treatment groups examined. These groups included mice that received 20 μ g of IgG, anti-CCL17, or anti-CCL22 at 2 h prior to and at days 2, 4, and 6 after conidial challenge. Whole-lung levels of CCL2 were significantly greater in the anti-CCL17-treated group than in the other two groups of treated mice at day 6 after conidial challenge (Fig. 2, bottom panel). Thus, the neutralization of CCL17 appeared to promote the generation of CCL2 during pulmonary invasive aspergillosis.

Neutrophil-depleted CCR4^{-/-}, but not CCR8^{-/-}, mice exhibited a marked reduction in the histological and biochemical presence of fungal material at days 2 and 6 after conidial challenge. We next determined whether the immunomodulatory effect of CCL17 during invasive aspergillosis was mediated through CCR4 or CCR8. Although it is widely accepted that CCL17 binds and signals through CCR4 (64), controversy persists as to whether this chemokine also binds and signals through CCR8 in vivo (8). In the present study, neutropenic wild-type (C57BL/6), CCR4^{-/-}, and CCR8^{-/-} mice were challenged with conidia and whole-lung samples were assessed histologically at days 2 and 6 after conidial challenge. GMS staining of histological tissue sections highlighted the presence of *Aspergillus* conidia and hyphae in the wild-type and CCR8^{-/-} groups at day 2 (Fig. 3A and C, respectively). In contrast, intact conidia and a few fungal elements were present in whole-lung samples from the CCR4^{-/-} group (Fig. 3B). Fungal hyphae and invasive growth were prominent in histological sections from the wild-type and CCR8^{-/-} groups at day 6 after conidial challenge (Fig. 3D and F, respectively). No GMS-staining material was present in the CCR4^{-/-} group at this time point after conidial challenge (Fig. 3E). Analysis of chitin levels in whole-lung samples from all three groups at day 2 after conidial challenge confirmed that significantly less fungal growth was present in the CCR4^{-/-} group compared with those in the wild-type and CCR8^{-/-} groups (Fig. 3, bottom panel). Thus, neutropenic CCR4^{-/-} mice exhibited a markedly enhanced ability to clear an intrapulmonary *Aspergillus fumigatus* challenge.

Neutrophil-depleted CCR4^{-/-} mice were significantly protected from the lethal effects of an intratracheal *A. fumigatus* conidial challenge. The uncontrolled growth of *Aspergillus* in the lungs of neutrophil-depleted mice wreaks tremendous destruction to the overall architecture of the lung, and this damage contributes to the mortality induced by this fungus. The i.t. instillation of 5×10^6 *A. fumigatus* conidia into CCR4^{+/+} mice resulted in 100% mortality in this group at day 3 after the conidial challenge, whereas 75% of CCR4^{-/-} mice were alive at this time point (Fig. 4). This difference in survival between the CCR4^{+/+} and CCR4^{-/-} groups was statistically significant ($P \leq 0.05$; log rank test).

Although the administration of RB6-8C5 did not alter the histological appearance of lungs of either CCR4^{-/-} (Fig. 5A) or CCR4^{+/+} (Fig. 5B) mice, the impact of neutrophil depletion lead to much greater lung injury and destruction in the wild-type groups at days 2 (Fig. 5C) and 6 (Fig. 5E) after an i.t. bolus challenge with 5×10^6 conidia compared with the CCR4^{-/-} groups at the same times after conidial challenge (Fig. 5D and F, respectively). Thus, the genetic deletion of CCR4 provided a major protective advantage to neutrophil-depleted mice subjected to an intrapulmonary challenge with *A. fumigatus* conidia.

Significantly elevated whole-lung TNF- α , IL-12, CCL2, and CCL3 levels in CCR4^{-/-} mice. TNF- α (47, 52, 55) and IL-12 (54, 60) have been shown to have protective effects in murine models of invasive aspergillosis. In the present study, ELISA analysis of whole-lung samples at days 2 and 6 after conidial challenge revealed marked differences in the temporal changes of these two important innate immune effector cytokines. As shown in Fig. 6, TNF- α levels were increased at day 2 after conidial challenge in both the CCR4^{+/+} and CCR4^{-/-} groups,

FIG. 2. (Top) Kaplan-Meier survival analysis of neutropenic wild-type mice after an i.t. challenge with 5×10^6 conidia. To induce neutropenia, all mice were injected with 100 μ g of RB6-8C5 antibody 24 h prior to the conidial challenge. Groups of seven mice were intraperitoneally injected with 20 μ g of IgG, anti-CCL17, or anti-CCL22 polyclonal antibody immediately prior to the conidial challenge and every 48 h thereafter. Data are representative of two separate experiments. (Bottom) Whole-lung ELISA analysis of TNF- α , IL-12, CCL2, and CCL3 at day 6 after an i.t. challenge with 5×10^6 conidia in neutropenic wild-type mice. To induce neutropenia, all mice were injected with 100 μ g of RB6-8C5 antibody 24 h prior to the conidial challenge. Mice were intraperitoneally injected with 20 μ g of IgG, anti-CCL17, or anti-CCL22 polyclonal antibody immediately prior to the conidial challenge and every 48 h thereafter. Data shown are means $\pm$ SEM ($n = 5$ mice/group). *, $P \leq 0.05$ compared with the IgG- and anti-CCL22 antibody-treated groups.

but the increase in TNF- α in CCR4 $^{-/-}$ mice reached statistical significance (compared with TNF- α levels in the group immediately prior to conidial challenge; i.e., day 0) (Fig. 6). The temporal patterns of expression of whole-lung IL-12 also differed between the two groups of mice. Specifically, IL-12 levels dropped significantly at day 2 after conidial challenge in the CCR4 $^{+/+}$ group. Conversely, whole-lung IL-12 levels in the

CCR4 $^{-/-}$ group at days 2 and 6 after conidial challenge did not differ from levels detected in whole-lung samples removed immediately prior to conidial challenge. However, a comparison between the CCR4 $^{+/+}$ and CCR4 $^{-/-}$ groups at day 2 after exposure to conidia revealed that whole-lung IL-12 levels were significantly increased in the knockout group relative to the wild-type group (Fig. 6).

FIG. 3. (Top) GMS staining of whole-lung tissue sections from neutropenic wild-type (A and D), CCR4^{-/-} (B and E), and CCR8^{-/-} (C and F) mice at days 2 (A, B, and C) and 6 (D, E, and F) after an i.t. challenge with 5×10^6 conidia. To induce neutropenia, all mice were injected with 100 μ g of RB6-8C5 antibody 24 h prior to the conidial challenge. *Aspergillus* conidia and hyphae stained black with this staining procedure. The original magnification was $\times 200$. (Bottom) Whole-lung chitin analysis in neutropenic wild-type (C57BL/6), CCR4^{-/-}, and CCR8^{-/-} mice at day 2 after an i.t. challenge with 5×10^6 conidia. To induce neutropenia, all mice were injected with 100 μ g of RB6-8C5 antibody 24 h prior to the conidial challenge. Data shown are means $\pm$ SEM ($n = 5$ /group). *, $P \leq 0.05$ compared with the C57BL/6 group; τ , $P \leq 0.05$ compared with the CCR8^{-/-} group.

CCL2 (51) and CCL3 (45) exert prominent protective roles in the lung during invasive aspergillosis. Major differences in the expression of both CC chemokines were observed in CCR4^{+/+} and CCR4^{-/-} mice, particularly at day 2 after conidial challenge. Whole-lung CCL2 levels were significantly increased at day 2 after conidial challenge in CCR4^{-/-} mice but not CCR4^{+/+} mice compared with whole-lung CCL2 levels measured at the day zero time point in these groups of mice (Fig. 6). Whole-lung CCL3 was significantly increased in both groups at day 2 after conidial challenge, but markedly less variability in whole-lung CCL3 levels was observed in the CCR4^{-/-} group relative to the CCR4^{+/+} group. Accordingly, CCL3 levels were significantly higher in the CCR4^{-/-} group at the day 2 time point compared with the other two time points analyzed in this group. Thus, together the whole-lung ELISA results demonstrated that protective cytokines such as TNF- α , CCL2, and CCL3 were significantly induced in the lungs of CCR4^{-/-} mice at day 2 of invasive aspergillosis compared to whole-lung levels in neutropenic CCR4^{-/-} mice before the conidial challenge. CCR4^{+/+} mice only exhibited a significant increase in whole-lung levels of CCL3 at day 2 after the conid-

FIG. 4. Kaplan-Meier survival analysis of neutropenic wild-type (C57BL/6) and CCR4^{-/-} mice after an i.t. challenge with 5×10^6 conidia. To induce neutropenia, all mice were injected with 100 μ g of RB6-8C5 antibody 24 h prior to the conidial challenge. Groups of seven mice were monitored for 6 days after conidial challenge. CCR4^{-/-} mouse survival was significantly ($P \leq 0.05$) greater than that of the wild-type group (C57BL/6). Data are representative of two separate survival experiments.

FIG. 5. Representative H&E-stained whole-lung sections from wild type (A, C, and E) and $CCR4^{-/-}$ (B, D, and F) mice at days 0 (A and B), 2 (C and D), and 6 (E and F) after an i.t. challenge with 5×10^6 conidia. To induce neutropenia, all mice were injected with $100 \mu\text{g}$ of RB6-8C5 antibody 24 h prior to the conidial challenge. The original magnification was $\times 200$.

ial challenge compared with day 0 levels in the wild-type group. Most importantly, whole-lung IL-12 levels were significantly greater in the knockout group at day 2 of invasive aspergillosis than those in the wild-type group at the same time in this lung infection.

Enhanced mononuclear but not lymphocytic cell recruitment into the lungs of $CCR4^{-/-}$ mice during invasive pulmonary aspergillosis. Previous studies have highlighted the importance of mononuclear (45, 48), dendritic (4, 12, 54), and NK (51) cells in the protective antifungal response against *A. fumigatus* conidial challenge in neutropenic or immunocompromised mice. Prior to the conidial challenge, whole-lung samples from neutropenic $CCR4^{+/+}$ and $CCR4^{-/-}$ mice contained similar numbers of dendritic cells ($CD11c^{+} CD86^{+}$) but significantly fewer macrophages ($CD11c^{+} F4/80^{+}$) compared with respective $CCR4^{+/+}$ and $CCR4^{-/-}$ groups not treated with RB6-8C5 (data not shown). At day 2 after challenge with saline alone,

the quantities of pulmonary dendritic cells ($CD11c^{+}$ cells expressing CD86) and pulmonary macrophages ($CD11c^{+}$ cells expressing F4/80) were similar in the $CCR4^{+/+}$ and $CCR4^{-/-}$ groups (Fig. 7). Conversely, both cell types were significantly increased in whole-lung samples from the $CCR4^{-/-}$ group at day 2 after conidial challenge compared with the $CCR4^{+/+}$ group at the same time after conidial challenge (Fig. 7).

Analysis of $CD3^{+}$ and $NK1.1^{+}$ cell populations revealed that both cell types were detected in similar numbers immediately prior to conidial challenge in neutropenic $CCR4^{+/+}$ and $CCR4^{-/-}$ mice (data not shown). A day 2 after saline challenge alone, similar numbers of $CD3^{+}$ and $NK1.1^{+}$ cells were observed in the lungs of $CCR4^{+/+}$ and $CCR4^{-/-}$ mice (Fig. 7). However, both cell types were significantly decreased in whole-lung samples from the $CCR4^{-/-}$ group at day 2 after conidial challenge compared with the $CCR4^{+/+}$ group at the same time after conidial challenge (Fig. 7). Thus, the absence of CCR4

FIG. 6. Whole-lung ELISA analysis of TNF- α , IL-12, CCL2, and CCL3 at days 0, 2, and 6 after an i.t. challenge with 5×10^6 conidia in neutropenic wild-type (CCR4^{+/+}) and CCR4^{-/-} mice. To induce neutropenia, all mice were injected with 100 μ g of RB6-8C5 antibody 24 h prior to the conidial challenge. Data shown are means $\pm$ SEM ($n = 5$ mice/group). *, $P \leq 0.05$; **, $P \leq 0.01$; and ***, $P \leq 0.001$ between the groups indicated by the horizontal bars.

during invasive pulmonary aspergillosis was associated with increased dendritic cells and macrophages and decreased T-cell or NK cell recruitment into the lung.

DISCUSSION

The immunopathogenesis of invasive pulmonary aspergillosis remains enigmatic, although considerable progress has been made examining the soluble and or soluble factors that inhibit or enhance immune activation. Inflammatory cytokines such as TNF- α (47, 52, 55), IL-12 (54, 60), and IL-6 (16) protect, whereas IL-4 (19) predisposes immunocompromised mice to the pulmonary ravages of invasive growth by *A. fumigatus*. CC chemokines such as CCL2 (through its effects on NK cell recruitment (51) and CCL3 and CXCL1 (through their effects on monocyte/macrophage recruitment) (45, 48) exert prominent protective effects in the context of invasive pulmonary aspergillosis. In the present study, we demonstrate that the de novo generation of CCL17 in the lung has deleterious effects on the innate immune response during experimental aspergillosis in neutropenic mice. The effects of CCL17 appeared to be mediated through CCR4 and not CCR8. Finally, the immunosuppressive effects of CCL17 appeared to be directed, in part, toward the modulation of lung mononuclear cell recruitment into the *Aspergillus*-infected lung.

Despite the presence and upregulation of both CCR4 ligands in whole-lung and BAL samples from wild-type mice with invasive pulmonary aspergillosis, it was clear from the present study that CCL17 and CCL22 had differential roles in the context of this model. Previous studies have demonstrated

that although often concomitantly expressed in a variety of noninflamed and inflamed tissues, the regulation and action of these CCR4 ligands may differ markedly. For example, CCL17 and CCL22 are produced by a variety of immune cells, including dendritic cells, B cells, macrophages, NK cells, monocytes, and CD4 T cells (1, 2), but the expression of CCL17 alone has been documented in non-immune cells, including bronchial epithelial cells (58), keratinocytes (61), airway smooth muscle cells (26), and fibroblasts (5, 27, 37). In isolated monocytes, CCL22, but not CCL17, expression is inhibited by IL-10 (2) while corticosteroids appear to inhibit both chemokines regardless of the cell of origin (58). Differential roles for CCL17 and CCL22 have been reported in a number of experimental models of disease, including bacterium-induced fulminant hepatic failure (CCL17 alone) (63), cardiac allograft rejection and contact hypersensitivity (both CCL17 alone) (1), pulmonary fibrosis (CCL17 but not CCL22) (5), pulmonary granulomatous inflammation (CCL22 > CCL17) (37), and allograft transplant rejection (CCL22 > CCL17) (42). However, it has been uniformly reported that both chemokines contribute to Th2-dominated inflammatory responses such as atopic dermatitis (62) and allergic airway disease or asthma (30, 39, 43). In the present study, we noted a clear demarcation between the roles of CCL17 and CCL22 during invasive pulmonary aspergillosis in that the neutralization of CCL17 but not CCL22 improved mouse survival during the course of invasive pulmonary aspergillosis.

Part of the impetus to pursue the role of CCL17, CCL22, and CCR4 in invasive pulmonary aspergillosis stemmed from our previous study on the role of CCR4 during chronic fungal

FIG. 7. Flow cytometric analysis of CD11c⁺ F4/80⁺, CD11c⁺ CD86⁺, CD3⁺, and NK1.1⁺ cells in whole-lung samples from neutropenic wild-type (CCR4^{+/+}) and CCR4^{-/-} mice at day 2 after an i.t. challenge with 5×10^6 conidia. To induce neutropenia, all mice were injected with 100 μ g of RB6-8C5 antibody 24 h prior to the conidial challenge. Data shown are means $\pm$ SEM ($n = 5$ mice/group). ***, $P \leq 0.001$ compared with the CCR4^{+/+} group that received conidia; τ , $P \leq 0.05$ compared with the CCR4^{+/+} group that received conidia.

asthma induced by *A. fumigatus* (57). We previously noted that the elimination of conidia and fungal material proceeded at a much faster rate in the CCR4^{-/-} mice than in the CCR4^{+/+} mice. The augmented clearance was associated with an increase in a number of cytokines and chemokines normally associated with these cells. Studies previous to ours suggested that the innate immune response in CCR4^{-/-} mice was altered in that these mice were dramatically less susceptible to the development of lipopolysaccharide-induced endotoxic shock (22). More recently, CCR4^{-/-} mice showed prolonged cardiac allograft survival compared with their wild-type counterparts (33). Given that previous reports have suggested that CCL17 can bind and signal through CCR8 (8, 36), the development of invasive pulmonary aspergillosis was also examined in CCR8^{-/-} mice.

Numerous studies have noted the effect of CCR4 ligands on the recruitment of CD4⁺ T cells (3, 20, 24) and NK cells (36). Most recently, CCL22 (via CCR4) has been shown to regulate the recruitment of Foxp3⁺ T regulatory cells to cardiac allografts (42). In the present study, we examined the impact of CCR4 deficiency on the recruitment of T and NK cells into the lungs during invasive aspergillosis because of previous reports highlighting the importance of these two cell populations in mediating protective immune responses against *Aspergillus* (15,

17, 51). We observed that the presence of both cell types was significantly lower in the lungs of CCR4^{-/-} mice than in those of CCR4^{+/+} mice at day 2 after conidial challenge. The diminution in NK cell numbers in the lungs of CCR4^{-/-} mice occurred despite the significantly increased levels of CCL2 in the lungs of these mice. Morrison and colleagues (51) previously reported that CCL2 mediated the recruitment of NK cells to the lungs as a critical early host defense mechanism during invasive pulmonary aspergillosis, but data from the present study suggest that the recruitment signal mediated through CCR4 supersedes that provided by CCL2/CCR2 for the recruitment of NK cells. Thus, these data suggested although CCR4 deficiency impaired the recruitment of T and NK cells into the lung, these cells did not appear to contribute to the protective immune response present in CCR4^{-/-} mice.

At day 2 after conidial challenge, pro-innate immune mediators including TNF- α , CCL2, and CCL3 were significantly increased or altered in the lungs of neutropenic CCR4^{-/-} mice relative to levels of these factors in this group of mice immediately prior to the conidial challenge. In the CCR4^{+/+} group, similar significant increases in these cytokines and chemokines above baseline levels (prior to conidial challenge) were only observed following statistical analysis of whole-lung levels of

CCL3. At day 2 after conidial challenge, whole-lung IL-12 levels were significantly decreased in the CCR4^{+/+} group but unchanged in the CCR4^{-/-} group compared with levels of this cytokine measured at day 0. Most importantly, levels of this cytokine were significantly increased in the CCR4^{-/-} group relative to the CCR4^{+/+} group at day 2 after conidial challenge. Shao and colleagues (60) recently demonstrated that the transfection of dendritic cells with adenovirus encoding IL-12 and the adoptive transfer of these cells pulsed with heat-inactivated *Aspergillus fumigatus* protected naive mice from invasive aspergillosis. Further studies will address the relative importance of IL-12 in the innate immune response present in CCR4^{-/-} mice during invasive aspergillosis.

The source of protective cytokines and chemokines has yet to be determined, but their presence and/or actions may have contributed to the increased recruitment of dendritic (CD11c⁺ CD86⁺) and macrophages (CD11c⁺ F4/80⁺) into the lungs of CCR4^{-/-} mice during invasive pulmonary aspergillosis. Both cell types have been previously shown to play key roles during host resistance to fungi (4, 13, 23, 28). While less is known about the effects of CCL17 on the activation of dendritic cells, CCL17 has a major effect on macrophage activation and in combination with IL-10 this chemokine promotes the alternative activation of macrophages (38). These macrophages have been well characterized and express receptors such as mannose receptor, β -glucan, and scavenger receptors (31). The precise role of alternatively activated macrophages during invasive pulmonary aspergillosis is presently unknown, but it can be assumed that given the inhibitory effect of these cells on the production of IFN- γ and the development of Th1 responses these cells are not positive contributors to the innate immune response against *Aspergillus*. Several studies have shown that an effective Th1 response is required to clear *Aspergillus* from the lungs of immunocompromised mice (10) (11, 50, 54).

Invasive pulmonary aspergillosis indiscriminately afflicts immunocompromised patients, indicating that it is strategically important to determine mechanisms that bolster immune defense mechanisms against this pathogen. Various vaccine- and cell-based therapies have been proposed, but the present study suggests that appropriate protection from invasive pulmonary aspergillosis may be achieved through the selective targeting of a specific chemokine, namely CCL17. Additional experimental studies are required to fully elucidate the effects of CCL17 on the innate immune response directed against this fungal pathogen, and clinical studies are required to determine whether this chemokine is elevated in patients experiencing invasive aspergillosis. Thus, the present studies demonstrate that CCL17 is a critical regulator of host defense against *A. fumigatus*, and this chemokine may be an important therapeutic target during invasive aspergillosis.

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