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Differential Roles of CC Chemokine Ligand 2/Monocyte Chemotactic Protein-1 and CCR2 in the Development of T1 Immunity

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CCR2 and its major ligand, chemokine ligand 2 (CCL2), have been found to influence T1/T2 immune response polarization. Our objective was to directly compare the roles of CCR2 and CCL2 in T1/T2 immune response polarization using a model of pulmonary Cryptococcus neoformans infection. Either deletion of CCR2 or treatment of wild-type mice with CCL2 neutralizing Ab produced significant and comparable reductions in macrophage and T cell recruitment into the lungs following infection. Both CCL2 neutralization and CCR2 deficiency resulted in significantly diminished IFN-γ production, and increased IL-4 and IL-5 production by lung leukocytes (T1 to T2 switch), but only CCR2 deficiency promoted pulmonary eosinophil production and eosinophilia. In the lung-associated lymph nodes (LALN), CCL2-neutralized mice developed Ag-specific and increased IL-4 and IL-5 production by lung leukocytes (T1 to T2 switch), but only CCR2 deficiency promoted pulmonary eosinophil production and eosinophilia. In the lung-associated lymph nodes (LALN), CCL2-neutralized mice developed Ag-specific IFN-γ-producing cells, while CCR2 knockout mice did not. LALN from CCR2 knockout mice also had fewer MHCII+CD11b+ and MHCII+CD11b+ cells, and produced significantly less IL-12p70 and TNF-α when stimulated with heat-killed yeast than LALN from wild-type or CCL2-neutralized mice, consistent with a defect in APC trafficking in CCR2 knockout mice. Neutralization of CCL2 in CCR2 knockout mice did not alter immune response development, demonstrating that the high levels of CCL2 in these mice did not play a role in T2 polarization. Therefore, CCR2 (but not CCL2) is required for afferent T1 development in the lymph nodes. In the absence of CCL2, T1 cells polarize in the LALN, but do not traffic from the lymph nodes to the lungs, resulting in a pulmonary T2 response. The Journal of Immunology, 2002, 168: 4659–4666.

The CC chemokine ligand 2 (CCL2), a chemokine formerly known as monocyte chemotactic protein-1 (MCP-1; Ref. 1) and its receptor, CCR2, have been found to be involved in mediating T1/T2 polarization. CCL2 expression has been associated with T2 (Th2 and Tc2) development in both infectious (2–6) and allergic disease models (7, 8). In addition, CCL2 has been found to enhance IL-4 production by T cells (9–12). In contrast, studies using mice that lack CCR2, the only known functional receptor for CCL2, have generally demonstrated that this receptor promotes T1 (Th1 and Tc1) development in infection models (13–18) and attenuates the pulmonary allergic (T2) response to Aspergillus (19). The reasons for the discrepancy between the pro-T2 actions of CCL2 and the pro-T1 role of CCR2 are not known. However, in the absence of CCR2, CCL2 may promote T2 development via a second, as yet unidentified, receptor (20–22).

CCR2 is clearly important for the resolution of a pulmonary Cryptococcus neoformans infection (16). C. neoformans is an encapsulated yeast that is acquired via the respiratory tract and requires T1-type cell-mediated immunity for clearance of this opportunistic pathogen from the lung (23). T1-type cell-mediated immunity to pulmonary C. neoformans infection is characterized by IFN-γ production, macrophage and lymphocyte infiltration into the lungs, and the development of Ag-specific delayed-type hypersensitivity to C. neoformans (23). The T1-type response requires CD4+ and CD8+ T cells, in addition to the production of the cytokines TNF-α, IL-12, and IFN-γ (23). In contrast to CCR2+/+ mice, CCR2−/− mice produce a strong T2-type immune response to C. neoformans, and cannot clear a pulmonary C. neoformans infection (16). The T2-type response is characterized by reduced macrophage and lymphocyte recruitment, pulmonary eosinophilia, leukocyte production of IL-4 and IL-5 but not IFN-γ, and increased serum IgE. These findings demonstrate that expression of CCR2 is required for the development of a T1-type response to C. neoformans infection and lack of CCR2 results in a switch to a T2-type response.

Previous studies from our laboratory demonstrated that efferent phase (days 5–14 postinfection) production of CCL2 is required for T1-driven mononuclear cell recruitment into the lungs of C. neoformans-infected mice at 2 wk postinfection (24). However,
treatment of mice beginning at day 5 of infection with anti-CCL2 Ab did not produce the pulmonary eosinophilia seen in CCR2−/− mice (16, 24). It is possible that the production of CCL2 before day 5 prevented the development of pulmonary eosinophilia (T2-type response). The objective of this study was to determine the role of CCL2 in the afferent and efferent phase of T1 immunity and compare it to the role of CCR2 in T1 to T2 switching of the pulmonary response.

Materials and Methods

Animals

Wild-type and CCR2−/− mice (25) on an outbred C57BL/6 × 129 genetic background were maintained at the University of Michigan Unit for Laboratory Animal Medicine Facilities (Ann Arbor, MI) under specific pathogen-free conditions in enclosed filter top cages. Clean food and water was given ad libitum. The mice were handled and maintained using microisolation techniques with daily veterinarian monitoring. Bedding from the mice was transferred weekly to cages of uninfected mice to maintain asepsis. Mice were given ad libitum. The mice were handled and maintained using microisolation techniques with daily veterinarian monitoring. Bedding from the mice was transferred weekly to cages of uninfected mice to maintain asepsis.

The needle was removed and the skin closed with cyanoacrylate adhesive.

C. neoformans

C. neoformans strain 52D was obtained from the American Type Culture Collection (24067; Manassas, VA). For infection, yeast were grown to mid-log phase in Sabouraud dextrose broth (1% yeast extract, 2% dextrose, 0.1% NH4Cl) at 37°C–72 h and subsequently bled at weekly intervals and found to be negative for Abs to C. neoformans

Surgical intratracheal inoculation

Mice were anesthetized by i.p. injection of pentobarbital (0.074 mg/g weight of mouse) and restrained on a small surgical board. A small incision was made through the skin over the trachea and the underlying tissue was separated. A 30-gauge needle was used to insert a tuberculin syringe filled with either 300 μl C. neoformans culture. The needle was inserted into the trachea and 30 μl of inoculum (106 CFU) was dispersed into the lungs. The needle was removed and the skin closed with cyanoacrylate adhesive.

Bronchoalveolar lavage

Mice were lavaged after cannulation of the trachea with polyethylene tubing (PE50) that was attached to a 25-gauge needle on a tuberculin syringe. The lungs were lavaged twice with 0.8 ml of PBS containing 5 mM EDTA. The recovered fluid (1.3–1.4 ml total) was spun at 1500 rpm and the supernatant was removed and stored at –20°C until analyzed for estatins (Quantikine M; R&D Systems, Minneapolis, MN) or IFN-γ (OptEIA; BD PharMingen), and IL-5 production by ELISA (OptEIA, BD PharMingen).

Lymph node cell isolation and culture

At 14 days postinfection, LALN (or superior mediastinal nodes) were excised and cells dispersed by mashing in media (RPML, 5% FCS, antibiotics). Isolated LALN cells (2 × 107) were cultured in 24-well plates with 1 ml of compete medium at 37°C and 5% CO2 with no additional stimulus. Supernatants were harvested at 24 h and assayed for IFN-γ, IL-4, and IL-5 production by ELISA (OptEIA, BD PharMingen).

Flow cytometric analysis

Leukocytes (5 × 106) were cultured for 30 min on ice with staining buffer (FA buffer, Dico, 0.1% NaN3, 1% FCS). Each sample was incubated with: 1) 0.12 μg of CyChrome-labeled anti-CD45 (30-F11, BD PharMingen); and either 2) 0.25 μg each of FITC-labeled anti-CD4 (RM4-5) and PE-labeled anti-CD8 (53-6.7); or 3) 0.25 μg of FITC-labeled anti-B220 (RA3-6B2). In other experiments, samples of lung-associated lymph node (LALN) cell suspensions were stained with PE-labeled anti-I-Ab/II-Eβ2 (2G9) and either FITC-labeled anti-CD11b (M1/70) or FITC-labeled anti-CD11c (HL3). The samples were washed in staining buffer and fixed with 2% paraformaldehyde in buffered saline. Stained samples were stored in the dark at 4°C until analyzed by flow cytometry (Coulter Elite ESP; Coulter, Hialeah, FL). Samples were gated for CD45+ cells and then analyzed for staining by the specific FITC- and PE-labeled anti-lymphocyte markers. Recruited lymphocyte numbers in the lungs were calculated by subtracting resident values, as determined in a parallel cohort of uninfected mice (the values for uninfected wild-type and uninfected CCR2−/− mice were not significantly different) from the values measured in infected lungs.

Lung leukocyte culture and cytokine production

Isolated leukocytes (15 × 106) were cultured in 6-well plates with 3 ml of complete medium at 37°C and 5% CO2 with no additional stimulus. Supernatants were harvested at 24 h and assayed for IFN-γ, IL-4, and IL-5 production by ELISA (OptEIA, BD PharMingen).

Statistics

Data (mean ± SEM) for each experimental group were derived from three separate experiments and analyzed by two-way ANOVA. For individual comparisons of multiple groups, post hoc test for simple main effects was used to calculate p values. Means with p < 0.05 were considered statistically significant.

Results

Effects of CCL2 neutralization on pulmonary mononuclear cell recruitment

Our first aim was to compare the effects of CCL2 neutralization (beginning at day 0) to CCR2 deficiency on C. neoformans-induced lung leucocyte recruitment. Total lung leukocytes were isolated from enzymatically digested lungs and pulmonary leucocyte recruitment was determined as outlined in Materials and Methods. Following infection, the lungs of wild-type mice showed a vigorous inflammatory response with 76 million leucocytes recruited at 14 days postinfection (Fig. 1). Leucocyte recruitment was reduced >85% using a hemocytometer. Subsequent flow cytometric analysis (described below) was used to determine the percentage of total leucocytes (CD45+CLA−) within the lung cell suspension for correction of hemocytometer counts. Subsets of isolated leucocytes (neutrophils, eosinophils, macrophages, and total lymphocytes) were determined by Wright-Giemsa staining of samples cytopsin onto slides. As reported previously (16, 27), recruited leucocyte numbers were calculated by subtracting uninfected values, as determined in a parallel cohort of uninfected mice (the values for uninfected wild-type and uninfected CCR2−/− mice were not significantly different), from the values measured in infected lungs.
CCR2/H11001 and CCR2/H11002 and CCR2 pulmonary leukocyte recruitment in resistant wild-type mice. Wild-type CCR2 deficiency resulted in comparable decreases in both CD4 and CD8 T lymphocytes by 14 days postinfection. CCL2 neutralization of pulmonary macrophage recruitment by 54 and 65%, respectively (Fig. 1). Similarly, total lymphocyte recruitment was reduced by 67 and 70% in CCL2-neutralized and CCR2-deficient mice, respectively. Fig. 2 illustrates that a pulmonary C. neoformans infection in wild-type mice induces vigorous recruitment of both T and B lymphocytes by 14 days postinfection. CCL2 neutralization and CCR2 deficiency resulted in comparable decreases in both CD4+ T cell recruitment (reductions of 71 and 82%, respectively) and CD8+ T cell recruitment (reductions of 82 and 90%, respectively). Recruitment of B220+ cells was not significantly affected by either CCL2 neutralization or by deletion of CCR2. These results demonstrate that macrophage and T cell recruitment are similarly reduced by either CCL2 neutralization or deletion of the CCL2 receptor CCR2.

Effects of CCL2 neutralization on pulmonary granulocyte recruitment

The effects of CCL2 neutralization on lung granulocyte recruitment during infection were also assessed, given that eosinophilia is one of the hallmark characteristics of the T2-type immune responses in the lungs of C. neoformans-infected CCR2-deficient mice (16). There were no significant differences in neutrophil recruitment between any of the three treatment groups (5.3 ± 1.6, 2.1 ± 1.2, and 2.6 ± 1.2 million for CCR2+/+, anti-CCR2 treated CCR2+/−, and CCR2−/−, respectively; data not shown). As previously reported, CCR2−/− mice developed a marked eosinophilia at 2 wk postinfection (>90% increase; Fig. 3). However, this eosinophilia was not reproduced by CCL2 neutralization as both control-infected wild-type and anti-CCL2-treated wild-type mice had similar numbers of lung eosinophils at 2 wk postinfection. These results demonstrate that CCL2 neutralization in wild-type mice does not produce the eosinophilia seen in CCR2-deficient mice following C. neoformans infection.

Lung leukocyte IL-5 production was determined based on previous findings demonstrating that this cytokine is required for pulmonary eosinophilia during C. neoformans infection (28). Leukocytes were isolated from infected lungs at 2 wk postinfection and cultured for 24 h with no additional stimulus. Culture supernatants were harvested and assayed for IL-5 production by ELISA. Fig. 3 shows that production of IL-5 by leukocytes from control-infected wild-type mice was similar to that for uninfected mouse leukocytes. CCL2 neutralization in wild-type mice resulted in a 3-fold increase in IL-5 production to a level similar to that of leukocytes from infected CCR2-deficient mice. These results show that either CCL2 neutralization or CCR2 deletion results in equivalent increases in production of IL-5 by lung leukocytes.

Because CCL2 neutralization in wild-type mice fails to produce eosinophilia despite an increase in pulmonary IL-5, pulmonary eotaxin levels were assayed to determine the role of this chemoattractant whose actions are potentiated by IL-5 (29). At 2 wk postinfection, CCR2−/− mice showed significantly elevated bronchoalveolar lavage fluid (BALF) levels of eotaxin compared with infected wild-type mice (Fig. 3), whereas CCL2 neutralization in wild-type mice did not result in increased pulmonary eotaxin levels. These results demonstrate that the development of eosinophilia in Cryptococcus-infected CCR2−/− mice correlates with both elevated eotaxin levels and increased IL-5 production. In contrast, CCL2 neutralization in wild-type mice results in increased IL-5 production without an increase in eotaxin or eosinophil recruitment.
Effects of CCL2 neutralization on pulmonary IL-4 and IFN-γ production

The effects of CCL2 neutralization on production of T1- and T2-type cytokines were determined. Lung leukocytes were isolated from *C. neoformans*-infected mice, cultured without additional stimulus, and assayed for IL-4 and IFN-γ (Fig. 3). Production of IL-4 by lung leukocytes from CCR2−/− mice and CCL2-neutralized mice was significantly greater than for leukocytes from CCR2+/+ mice (Fig. 4). In contrast, IFN-γ production by lung leukocytes from CCR2−/− mice and CCL2-neutralized mice was significantly reduced compared with leukocytes from CCR2+/+ mice. Although IFN-γ production by cultured lung leukocytes from CCL2-neutralized mice was greater than that from CCR2−/− mice, both CCL2 neutralization and CCR2 deletion decreased BALF IFN-γ levels by >90% compared with CCR2+/+ mice (Fig. 4). These data, together with the IL-5 data presented in

Comparison of CCL2 neutralization and CCR2 deletion on LALN expansion

The effect of CCL2 neutralization on LALN expansion was determined by measuring total node cell numbers and by determining the B:T cell ratio (Fig. 5). Analysis of LALN from uninfected mice was not possible because mice maintained under specific pathogen-free conditions in microisolator cages do not have macroscopically visible LALN before infection. Therefore, LALN expand solely in response to inoculation with *C. neoformans*. By 2 wk postinfection, LALN had expanded dramatically and cell suspensions could be made from the harvested nodes (Fig. 5). The total cell yield for LALN isolated from control-infected wild-type mice was 24.0 million/mouse. CCL2 neutralization did not significantly reduce total LALN cell numbers (20.0 million/mouse). However, there were significantly fewer cells in the LALN of CCR2−/− mice compared with LALN of CCR2+/+ mice (14.6 million/mouse, *p* <
CCR2/H11002 pooled for analysis. Plot shown is representative of two experiments. Analyzed by labeled anti-CD11b (M1/70) or FITC-labeled anti-CD11c (HL3) and sessions were stained with PE-labeled anti-I-Ad/I-Ed (2G9) and either FITC-labeled anti-CCL2-treated mice. Phagocyte expansion within the LALN compared with wild-type andicient CCR2 expression also results in altered lym-

Comparison of APC populations of LALN in CCR2 mice or anti-CCL2-treated CCR2 mice. The CD4^+ T cell numbers in the LALN of CCR2 neoformans infection. Flow cytometry was used to determine the numbers of CD4^+, CD8^+, and B220^+ cells within the LALN (Fig. 5). There were no significant differences in LALN B cell numbers between the three groups. The CD4^+ and CD8^+ T cell numbers in the LALN of CCR2^−/− mice were less than in the LALN of either CCR2^+/+ mice or anti-CCL2-treated CCR2^+/+ mice. The reduction in T cells resulted in a greater LALN B:T cell ratio for CCR2^−/− mice (1.82) when compared with B:T ratios of CCR2^+/+ mice (1.36) or anti-CCL2-treated CCR2^+/+ mice (1.33). These results demonstrate that deficient CCR2 expression also results in altered lymphocyte expansion within the LALN compared with wild-type and anti-CCL2-treated mice.

Comparison of CCL2 neutralization and CCR2 deletion on APC numbers and function in the LALN

We also analyzed changes in the expression of MHCII, CD11b, and CD11c on LALN leukocytes to determine whether there was a dichotomy in the roles of CCR2 and CCL2 on the recruitment of potential APCs into the LALN. At 2 wk postinfection, the LALN of CCR2^+/+ mice contained readily detectable numbers of MHCII^+CD11b^+ and MHCII^+CD11c^+ cells (Fig. 6). Neutralization of CCL2 did not alter the percentage or total number of both of these types of cells (Figs. 5 and 6). In contrast, the percentage and total number of both MHCII^+CD11b^+ and MHCII^+CD11c^+ cells in the LALN was lower in CCR2^−/− mice compared with CCR2^+/+ mice (Fig. 6). LALN cells from CCR2^−/− mice also produced significantly less IL-12p70 and TNF-α following stimulation with heat-killed cryptococci or C. albicans than LALN cells from CCR2^+/+ mice or CCL2-neutralized CCR2^−/− mice (Fig. 7). The lack of IL-12 and TNF-α production is consistent with a defect in the number of potential T1-promoting APCs in the LALN of CCR2^−/− mice. Altogether, these results indicate that CCR2, but not CCL2, plays a role in the recruitment of potential T1-promoting APCs to the draining lymph nodes.

Comparison of CCL2 neutralization and CCR2 deletion on T cell polarization within the LALN

Cytokine production by LALN T cells was determined to further assess the roles of CCL2 and CCR2 on T1/T2 polarization. Isolated LALN cells from the three groups of mice were cultured 24 h

FIGURE 5. Comparison of the T and B cell composition of LALN in CCR2^+/+ mice, anti-CCL2-treated CCR2^+/+ mice, and CCR2^−/− mice. Cell suspensions were made from draining lymph nodes harvested from mice at 2 wk postinfection (described in Materials and Methods). Samples of LALN cell suspensions were stained with fluorochrome-labeled Abs specific for lymphocyte subsets (CD4^+, CD8^+, and B220^+) and analyzed by flow cytometry (described in Materials and Methods). Total lymph node cells and lymphocyte subsets were determined on a per mouse basis. Positive staining cells accounted for >90% of the cells in suspension for all groups. *, p < 0.05 in comparison with corresponding CCR2^+/+ group; †, p < 0.05 for CCR2^−/− vs both corresponding CCR2^+/+ and Ab-treated CCR2^+/+ groups; n = 10 for each group; values are the mean ± SEM.

FIGURE 6. Comparison of APC populations of LALN in CCR2^+/+ mice, anti-CCL2-treated CCR2^+/+ mice, and CCR2^−/− mice at day 7 postinfection. Cell suspensions were made from draining lymph nodes harvested from mice at week 1 postinfection. Samples of LALN cell suspensions were stained with PE-labeled anti-I-A^d/E^E^ (2G9) and either FITC-labeled anti-CD11b (M1/70) or FITC-labeled anti-CD11c (HL3) and analyzed by flow cytometry. Lymph nodes from five animals/group were pooled for analysis. Plot shown is representative of two experiments.

FIGURE 7. IL-12p70 and TNF-α production by LALN cells from C. neoformans-infected CCR2^+/+ mice, anti-CCL2-treated CCR2^+/+ mice, and CCR2^−/− mice. Cell suspensions made from LALN of infected mice (week 1 postinfection) were cultured for 24 h with no additional stimulus, HK, or with heat-killed C. albicans. Culture supernatants were then harvested and analyzed for IL-12p70; and b, TNF-α by ELISA. *, p < 0.05 in comparison with corresponding no stimulus group; n = 2 experiments for each group (nodes from five animals were pooled for each experiment); values are the mean + SE.
with either no additional stimulus, \textit{C. neoformans} Ag, or suboptimal concentrations of PMA/ionomycin to stimulate recently activated T cells. Because complement depletion studies have demonstrated that CD4$^+$ and CD8$^+$ T cells are the predominate cell type responsible for cytokine production in our culture system (data not shown), cytokine production was normalized to the number of T cells in culture to eliminate any biasing which may occur due to the reduced number of T cells in cultures of LALN from CCR2$^{-/-}$ mice. Without stimulation, LALN cells from any of the three groups of mice did not produce appreciable amounts of IFN-\(\gamma\) or IL-5 (Fig. 8). LALN T cells from CCR2$^{+/+}$ and anti-CCL2-treated CCR2$^{-/-}$ mice produced significant levels of IFN-\(\gamma\) in response to either \textit{C. neoformans} Ag or low-dose PMA/ionomycin. In contrast, LALN T cells from infected CCR2$^{-/-}$ mice failed to produce significant amounts of IFN-\(\gamma\) following stimulation with either \textit{C. neoformans} Ag or low-dose PMA/ionomycin. The LALN of all groups produced some IL-5 in response to low-dose PMA/ionomycin; however, node cells from CCR2$^{-/-}$ mice tended to produce more of this cytokine (Fig. 8). LALN cell production of IL-4 could not be detected for any of the groups of mice (data not shown). These results demonstrate that expression of CCR2 is required for the development of Ag-specific IFN-\(\gamma\)-producing T cells in LALN. In contrast, CCL2/MCP-1 is not required for the development of Ag-specific IFN-\(\gamma\)-producing T cells in the draining lymph nodes.

\textbf{Effects of CCL2 neutralization on induction of T2-type cytokines in CCR2$^{-/-}$ mice}

In our initial study of pulmonary \textit{C. neoformans} infection in CCR2-deficient mice, one model proposed was that CCL2 may interact with a receptor other than CCR2 to promote T2-type immunity in these mice (16). This hypothesis was tested by neutralizing CCL2 during the first 2 wk of \textit{C. neoformans} infection in CCR2$^{-/-}$ mice. CCL2 neutralization had no effect on lymphocyte recruitment compared with control infected CCR2$^{-/-}$ mice (Fig. 9a). Cytokine production by isolated pulmonary leukocytes was also not affected by in vivo neutralization of CCL2 (Fig. 9b). Furthermore, neither LALN development nor cytokine production by LALN cells from infected CCR2$^{-/-}$ mice was affected by neutralization of CCL2 (data not shown). These results demonstrate that the induction of T2-type cytokine-producing lung leukocytes in infected CCR2$^{-/-}$ mice is not dependent on CCL2.

\textbf{Discussion}

The objective of this study was to determine whether CCL2 is the primary ligand involved in CCR2-dependent development of T1-type immunity. Using a pulmonary \textit{C. neoformans} infection model, neutralization of CCL2 or deletion of CCR2 result in comparable macrophage and T cell recruitment deficits as well as a similar switch from T1- to T2-type cytokine production within the infected lung. However, CCL2 neutralization does not result in pulmonary eosinophilia nor does it produce an IFN-\(\gamma\) defect within the draining lymph nodes, as does CCR2 deletion. These results suggest that the effector phase of cell-mediated immunity (mononuclear cell recruitment) uses the CCL2/CCR2 signaling axis, while the afferent phase (T1 polarization) involves a CCL2-independent, CCR2 signaling pathway.

The results presented in this study demonstrate that the CCR2/CCL2 signaling pathway is required for the effector phase recruitment of macrophages and T cells during infection. Neutralization of CCL2 in wild-type mice produced decreases in macrophage and T cell recruitment comparable to that observed in CCR2-deficient mice (Figs. 1 and 2). These results are also consistent with our previous report that CCL2 neutralization after the development of immunity (post day 5) dramatically reduces macrophage and T cell recruitment during a pulmonary \textit{C. neoformans} infection (24). This is consistent with the fact that both monocytes and activated T cells are known to express CCR2 (30) and CCL2 is chemotactic for these cells.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig8.png}
\caption{IFN-\(\gamma\) and IL-5 production by LALN cells from \textit{C. neoformans}-infected CCR2$^{+/+}$ mice, anti-CCL2-treated CCR2$^{+/+}$ mice, and CCR2$^{-/-}$ mice. Cell suspensions made from LALN of infected mice were cultured for 24 h with no additional stimulus, HKC, or PMA/ionomycin (both at 50 ng/ml). Culture supernatants were then harvested and assayed for, IFN-\(\gamma\) (a) and IL-5 (b) by ELISA. Cytokine production was normalized to 10$^6$ cultured T cells (CD4$^+$ and CD8$^+$) as determined by flow cytometry. * \(p < 0.05\) in comparison with corresponding no stimulus group; \(n = 3\) experiments for each group (nodes from two or four animals were pooled for each experiment); values are means ± SEM.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig9.png}
\caption{Effects of CCL2 neutralization (\(\alpha\)-CCL2) on immune response development in mice lacking CCR2. CCR2$^{-/-}$ mice were infected with \textit{C. neoformans}, treated with CCL2 Ab, and harvested at day 14 as described in Figs. 1–4 for neutralization of CCL2 in wild-type mice. a, Effects of CCL2 neutralization on lymphocyte recruitment in infected CCR2$^{-/-}$ mice. b, Effects of in vivo neutralization of CCL2 on cytokine production by isolated lung leukocytes. \(n = 10\) for each group; values are means ± SEM.}
\end{figure}
for these cells (31, 32). In addition, both CCL2−/− and CCR2-deficient mice have monocye recruitment defects (13, 14, 25). Thus, macrophage and T cell recruitment is largely dependent on the CCR2/CCL2 signaling pathway during the effector phase of the immune response to pulmonary *C. neoformans* infection.

In contrast to the equivalent effects on mononuclear cell recruitment, CCL2 neutralization does not produce the increased eosinophilia seen following deletion of CCR2 (Fig. 3). A similar discrepancy has been observed for CCL2 and CCR2 in producing eosinophilia during allergic pulmonary inflammation in mice. CCL2 neutralization has been shown to abolish eosinophilia associated with the pulmonary allergic response (7), while CCR2 deletion does not diminish (33) or even increases eosinophilia (19, 34). Increased eosinophilia following CCL2 neutralization in *C. neoformans* infection does not occur despite the fact that lung leukocytes isolated from these mice produce high levels of IL-5 (Fig. 3). Eosinophilia in *C. neoformans*-infected mice (16, 27) is a T2-driven process that is dependent on the production of IL-5 (28). The finding that CCR2 deficiency, but not CCL2 neutralization, up-regulated eotaxin levels in the airways (Fig. 3) suggests the reduced eosinophilia in CCL2-neutralized mice results from a trafficking deficit and not to a lack of a pro-eosinophilic environment provided by IL-5 in the lungs of infected mice.

Compared with CCL2 neutralization, CCR2 deletion produced dramatically differing effects within the lymph nodes of infected mice. In contrast to LALN cells from either wild-type or CCL2-neutralized wild-type mice, LALN cells isolated from infected CCR2-deficient mice failed to produce IFN-γ in response to specific Ag stimulation or in response to concentrations of PMA and ionomycin that induce cytokine production only from recently activated T cells (Fig. 6). Consistent with the ELISA data, there also appear to be fewer IFN-γ-producing CD4+ and CD8+ T cells in the lungs of CCR2−/− compared with CCR2+/+ mice (measured by intracellular flow cytometry, data not shown). These findings are similar to previous studies using CCR2-deficient mice which demonstrated a defect in Ag-specific IFN-γ production within the draining lymph nodes following either challenge with purified protein derivative of *Mycobacterium bovis* (13), *Leishmania major* infection (15), *Mycobacterium tuberculosis* (18), or immunization with keyhole limpet hemocyanin (17). The combination of the Ag-specific IFN-γ defect and increased IL-5 production suggest that, unlike either wild-type or CCL2-neutralized wild-type mice, LALN development in *C. neoformans*-infected CCR2−/− mice is characterized by the lack of T1 polarization.

Our studies demonstrate that APC numbers and function are deficient in the LALN of CCR2−/− mice during *C. neoformans* infection (Figs. 5–7). These results are consistent with previous reports of potentially altered APC trafficking in CCR2-deficient mice (15, 17). Different populations of dendritic cells (35–38) and macrophages (39) have been reported to exist which can polarize T cell differentiation to either T1 or T2. Therefore, if APCs promoting T1 differentiation express CCR2, the loss of CCR2 may promote T2 differentiation express CCR2, the loss of CCR2 may influence the manifestation of both T1 and T2 responses (7, 24, 49). Our studies suggest that the T1 or T2 promoting activity of CCL2 in vivo depends on additional factors that may include the timing of CCL2 induction (afferent vs efferent), type of pathogen/Ag, route of inoculation/immunization, and the tissue site. For example, CCL2 is a pro-T1 factor if pulmonary cytokine responses are measured (Figs. 3 and 4), but plays no role in polarization if lymph node responses alone are assayed (Fig. 6). The data in this manuscript also suggest a mechanism to explain the apparent dichotomy between the role of CCL2 and its receptor CCR2 in models of T1/T2 immunity. Thus, the pleiotropic activity of CCL2 (chemokinin, T1, T2, activation, etc.) is influenced by the inflammatory milieu, a function of the type and tissue location of an infectious agent. The pleiotropic activity of mediators such as CCL2 is critical for effective adaptive host defenses.

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**References**

CONTROL OF PULMONARY T1 IMMUNITY BY CCL2 AND CCR2


