CCR3 Is Required for Tissue Eosinophilia and Larval Cytotoxicity After Infection with Trichinella spiralis

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*J Immunol* 2002; 168:5730-5736; doi: 10.4049/jimmunol.168.11.5730

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The CCR3 binds at least seven different CC chemokines and is expressed on eosinophils, mast cells (MC), and a subset of Th cells (Th2) that generate cytokines implicated in mucosal immune responses. Using mice with a targeted disruption of CCR3 (CCR3<sup>−/−</sup>) and their +/+ littersmates, we investigated the role of CCR3 in the amplification of tissue eosinophilia and MC hyperplasia in the mouse after infection with *Trichinella spiralis*. In CCR3<sup>−/−</sup> mice, eosinophils are not recruited to the jejunal mucosa after infection and are not present in the skeletal muscle adjacent to encysting larvae. In addition, the number of cysts in the skeletal muscle is increased and the frequency of encysted larvae exhibiting necrosis is reduced. The CCR3<sup>−/−</sup> mice exhibit the expected MC hyperplasia in the jejunum and caecum and reject the adult worms from the small intestine at a normal rate. This study is consistent with distinct functions for MC (adult worm expulsion) and eosinophils (toxicity to larvae) in immunity to *T. spiralis*, and defines the essential requirement for CCR3 in eosinophil, but not MC recruitment to tissues. *The Journal of Immunology*, 2002, 168: 5730–5736.
eosinophils remains incompletely defined. Therefore, we investigated the response by eosinophils and MC in the intestine of Ts-infected mice with a targeted disruption of the CCR3 gene to clarify the role of CCR3 in the trafficking of these cell types and to delineate their respective contributions to the primary anti-helminth response as defined by clearance of the adult worms from the intestines and limitation of larval encystment in skeletal muscle.

Materials and Methods

Animals

All animals were 10–20 wk of age when infected with Ts. CCR3−/− mice were generated as described, and the strain was maintained at the Children’s Hospital Institutional Animal Care and Use Committee-approved facility (Boston, MA) (15). Male mice from the N2 and N3 generations of (BALB/c × 129) × BALB/c were used in all experiments. CCR3+/+ and CCR3−/− mice were age matched within 2 wk of each other. All experiments were conducted with the approval of the Dana-Farber Cancer Institute Animal Care and Use Committee in accordance with Public Health Service Policy and provisions of the Animal Welfare Act.

Infection and enumeration of Ts

Stage 3 infectious larvae were isolated from the skeletal muscle of mice previously infected as described (30, 31). Briefly, the skeletal muscle was obtained by dissection, sliced into small pieces, and digested in 1% HCl/1% peptic (Sigma-Aldrich, St. Louis, MO) for 1–2 h at 37°C with stirring. The larvae were isolated and washed by low speed centrifugation (50 × g for 5 min), counted, and diluted with distilled water to a concentration of ~2000 larvae/ml. Mice were infected with ~400 larvae each by gavage. To minimize variability in infection rates, we constantly alternated gavage. To minimize variability in infection rates, we constantly alternated

Histology

Animals were killed at the indicated time points, and tissue samples were obtained and immediately fixed in 4% paraformaldehyde as described (30). The tissues were embedded in JB4 glycolmethacrylate, sectioned at 2-μm thickness, and placed on glass slides. Eosinophils were counted after the tissues were stained with Congo Red, and MC were counted after the tissues were stained for chloroacetate esterase (32–34). At least 20 high power fields (hpf; ×50 objective) were counted for each mouse. Each data point represents the average of six mice from two separate experiments. Blood eosinophil counts and tissue eosinophil peroxidase content were obtained as described (35, 36). Larval cysts and degenerate cysts contain- ing necrotic larvae were assessed histologically in sections of the tongue after staining with DiffQuick (Dade-Behring, Newark, DE). At least 10 low power fields (lpf; ×10 objective) were counted for each animal, and a mean number of cysts/lpf was calculated. Cysts with necrotic larvae were counted simultaneously and were expressed as the percentage of cysts with necrotic larvae in the tongue for each animal. Values are the mean (±SEM) from 15 animals in two experiments with three animals per group and 2–3 time points per experiment (days 28, 35, and 56).

Results

Role of CCR3 in cell recruitment after infection with Ts

We and others have previously characterized the kinetics of the intestinal eosinophilia (which peaks at about day 11) and MC hyperplasia (which peaks at about day 14) that occur during the induction of a primary Ts infection in BALB/c mice (19–26, 30, 32, 33). In the present study, contrasting the response to Ts infection in CCR3−/− mice and +/+ littermates, the CCR3−/− mice failed to accumulate appreciable numbers of eosinophils in the jejunum, whereas the +/+ mice exhibited jejunal eosinophilia 4 days after infection, with progression to a sharp peak at day 11 before declining to a nadir at day 20 (Fig. 1a). A second increase in jejunal eosinophils in the +/+ mice followed at day 30, and a plateau was maintained through day 56. The jejunal MC hyperplasia peaked at 14 days and was similar in magnitude in both −/− and +/+ mice (Fig. 1b). In contrast to the dramatic difference in jejunal-localized eosinophils, eosinophil counts in the blood of CCR3−/− mice were higher than in the +/+ littersmates on days 4–35 (Fig. 1c). The peripheral blood eosinophilia in the −/− mice increased up to day 21 and then remained fairly constant through day 56, when the experiment was terminated. The eosinophils in the +/+ mice peaked at day 21, declined, and rose again at the end of the experiment at day 56.

The eosinophils found in the jejunum of +/+ mice were localized in the submucosa and lamina propria. In CCR3−/− mice, those few eosinophils observed in the jejunum were associated with the vasculature and did not appear to have extravasated into the connective tissue (Fig. 2, a and b). No differences in the number or localization of the jejunal MCs were observed in the −/− and +/+ mice (Fig. 2, c and d). In the caecum of the CCR3−/− mice, some eosinophils did permeate 7–14 days after infection (Fig. 3, a and c), with the peak value at day 14 being <1/4 of the peak value for the same anatomic region of the +/+ mice at day 11 (Fig. 3c). In the CCR3−/− mice, similar to the jejunum, most of the caecal eosinophils appeared within the venous and lymphatic vasculature (Fig. 3a), whereas in the +/+ mice most of the eosinophils were in the connective tissue of the lamina propria (Fig. 3b). Eosinophils did not intercalate into the intestinal epithelium of the jejunum or the caecum in either the CCR3−/− or the +/+ mice (Figs. 2 and 3). The caecum also demonstrated a mastocytosis from day 7 to the end of the experiment on day 56 that was similar in both CCR3−/− and +/+ mice (data not shown). With resolution of the worm burden, the MCs that remained in the caecum were all

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**FIGURE 1.** Quantitation of the MC and eosinophil response to Ts in the jejunum and blood of CCR3−/− and +/+ littermate control mice. The eosinophils (a) and MC (b) appearing in the jejunum of CCR3−/− mice (solid line) and +/+ mice (dashed line) were enumerated in histological sections taken on the indicated days. Eosinophil concentrations in the blood (c) were obtained in parallel. Each time point represents the average cell number per hpf ± SEM from two experiments with three mice per experiment (a and b) and three to six mice (±SEM) for the eosinophil concentration in the blood (c). The statistically significant differences in blood eosinophil concentrations are indicated by the asterisks (*, p < 0.05; **, p < 0.01).
FIGURE 2. Identification of eosinophils and MC in the small intestine of CCR3−/− and +/+ littermate control mice after infection with T. Eosinophils (arrows, a and b) and MC (c and d) in the jejunum of CCR3−/− mice (a and c) and +/+ mice (b and d) are shown 11 (eosinophils) and 14 (MC) days after infection with T. The 11-day postinfection time point coincides with the time of peak MC numbers appearing in both draining (mesenteric) and nondraining (axillary) lymph nodes. The draining mesenteric lymph nodes of CCR3−/− mice showed greater numbers of eosinophils compared with the +/+ mice early in the infection (Fig. 4e). In addition, the cells are in a different location, being primarily intermixed in the lymphoid follicles in −/− mice (Fig. 4a), and in the peripheral sinusoids of the lymph nodes of +/+ mice (Fig. 4b). These eosinophil locations suggest migration from the blood in the −/− mice and migration from the intestine via the lymph in the +/+ littermates. None of the nondraining lymph nodes exhibited any eosinophilia at any time (Fig. 4, c and d). In both sets of animals, the eosinophils progressed more deeply into the lymphoid follicular areas with time, commonly becoming apoptotic and ingested by resident macrophages (data not shown). The CCR3−/− mice also had more eosinophils in the spleen than the +/+ mice before infection and at all time points examined after infection (data not shown).

Role of CCR3 in worm expulsion and larval cytotoxicity

The CCR3−/− and +/+ mice both had abundant numbers of adult worms in their intestines on day 4 after infection (Fig. 5). The numbers of worms declined progressively thereafter, and worm expulsion was essentially complete by day 14 in both groups of mice. There was a statistically significant delay in worm expulsion in the CCR3−/− mice at day 11 (p < 0.05), but this delay did not alter the final worm expulsion kinetics.

Three to 4 wk after infection, concomitant with the second increase in jejunal eosinophils, T. larvae were observed encysting throughout the skeletal muscle. The larvae that successfully infested muscle cells transformed the nuclei from a pattern of finely dispersed chromatin to an owl-eye architecture, a dark central sphere encircled by a clear halo. The infected muscle cells also responded by the production of an extracellular matrix, which formed the wall of the cyst and stained deeply pink to magenta with DiffQuik (Fig. 6). Inspection revealed no quantitative or kinetic differences in encystment among the intercostal, paravertebral, diaphragmatic, abdominal wall, and tongue skeletal muscles (data not shown); and thus, tongue was used as the reference organ for monitoring larval encystment.

FIGURE 3. Identification of eosinophils in the caecum of CCR3−/− and +/+ littermate control mice after infection with T. Eosinophils in the caecum of CCR3−/− mice (arrows, a) and +/+ mice (arrows, b) are shown 11 days postinfection. c, The variation in eosinophil number (mean number per hpf ± SEM, three mice per group, n = 2) in the caecum over the course of infection for CCR−/− (dashed line) and +/+ (solid line).

Intraepithelial in location. In both the CCR3−/− and +/+ mice, MC accumulated in the spleen concomitantly with the resolution phase of the MC hyperplasia (data not shown) as we have previously described (33). Because eosinophils did not infiltrate the peripheral tissues but did increase in the blood of CCR3−/− mice, we evaluated the numbers appearing in both draining (mesenteric) and nondraining (axillary) lymph nodes. The draining mesenteric lymph nodes of CCR3−/− mice also had more eosinophils in the spleen than the +/+ mice before infection and at all time points examined after infection (data not shown).
Furthermore, the number of cysts in the CCR3/H11002 acysted larvae were surrounded by intact cyst walls (Fig. 6, mice was negligible. Almost no degenerate cysts were observed in number of degenerate cysts containing necrotic larvae in the null increased by 2-fold over their controls (Fig. 7).

Rejection of adult FIGURE 5. Appearance of eosinophils in draining (mesenteric) and nondraining (axillary) lymph nodes of CCR3/H11002 and/H11001 mice after T. infection. Eosinophils (orange) appear in the draining lymph nodes of infected CCR3/H11002 (a) and +/- (b) mice, but not in nondraining lymph nodes (c and d, respectively). Sections were obtained 11 days after infection. e. The average number (±SEM, three mice per group, n = 2) of eosinophils appearing in the draining lymph nodes at various times after infection of CCR3/H11002 (dashed line) and +/- (solid line) mice.

FIGURE 4. Appearance of eosinophils in draining (mesenteric) and nondraining (axillary) lymph nodes of CCR3/H11002 and+/+ littermate control mice. The small intestinal worm burden in CCR3/H11002 mice was in -/− mice (≤1%), whereas ~11% were observed in the +/- controls (Fig. 7b).

Discussion
Eosinophil movement into tissues reflects the composite actions of eosinophil-active chemokines, particularly the CCR3 ligands, adhesion pathways (especially those mediated by P-selectin and α4 integrins), and the eosinophilopoietic cytokine IL-5 (1–5, 12–15, 36–39). IL-5 induces eosinophil maturation and emigration from bone marrow and cytoprotection of the cells in peripheral tissues, while locally produced chemokines such as CCL11 facilitate their activated adhesion to endothelial cells, and direct their movement into the tissues. At the same time, other Th2-derived cytokines provide the mitogenic stimulus for stem cell factor-dependent expansion and maturation of the MC progenitors already localized in peripheral tissues, most notably the jejunum (21, 40–45). CCR3 is found on both MC and eosinophils, effector cells of the Th2 cell-dependent inflammatory response to intestinal nematode parasites. Thus, we compared CCR3/H11002 and congenic BALB/c mice for the appearance and distribution of these cells within select tissues and importantly, for their role in host defense against the intestinal adult worm and skeletal muscle larvae of T. over the course of 8 wk.

CCR3 deficiency did not affect the capacity of mice to mount a peripheral blood eosinophilia in response to T. (Fig. 1c), indicating that CCR3 is not essential to up-regulate the number of eosinophils produced in the bone marrow or to their exit from this tissue. In contrast, the lack of eosinophils in the jejunal submucosa in the infected CCR3/H11002 mice (Figs. 1a and 2a) highlights the critical nature of the CCR3 interaction with its ligands at the vascular interface for adhesion, transendothelial migration, and/or retention of eosinophils in this organ. These data are consistent with the marked effects of CCL11 deficiency in both basal and inflammation-based recruitment of eosinophils to the small intestine (12,
The recruitment of eosinophils to the caecum was partly preserved (Fig. 3), suggesting a secondary specificity for another chemokine-mediated recruitment step, e.g., CCR1, which is expressed and active in eosinophils (46, 47). Eosinophils were found in regional, but not distant, lymph nodes of CCR3−/− animals (Fig. 4), and their location suggests migration out of the vasculature as opposed to the sinusoidal location of eosinophils in the nodes of C57BL/6 mice, indicative of transit via the lymphatics. Therefore, movement of the eosinophils into the draining nodes of the CCR3−/− mice likely reflects a local change in vasculature permeability and adhesion characteristics rather than a transient gastrointestinal residence. These data indicate that eosinophil recruitment into different inflammatory sites is regulated in a tissue-specific manner and that CCR3 is critical for movement into the small intestine, less so for the large intestine, and not at all for regional lymph nodes.

Despite the profound deficit in jejunal eosinophil recruitment in the CCR3−/− mice, the expulsion of the adult worms was affected only transiently on day 11 (Fig. 5), before the establishment of MC hyperplasia was complete (Fig. 1b). This observation is consistent with earlier studies that failed to demonstrate a requirement for eosinophils in the elimination of the adult parasites after a primary infection (19, 21, 23–26, 48, 49). IL-5−/− mice, on a C57BL/6 background, also showed a statistically significant increase in intestinal worm burdens at a single time point, day 16 postinfection, but all the mice had rejected the worms on day 21 (26). The absence of any delay in the overall rejection kinetics for the adult worm (Fig. 5) substantiates the mast cell dependence of the integrated response and indicates that the eosinophil does not have a meaningful role in this function.

The CCR3−/− mice also failed to recruit eosinophils to the skeletal muscle in response to encystment of larvae (Fig. 6), indicating
that eosinophil movement into this tissue also is strictly dependent on CCR3. In the +/+ mice, necrotic larvae were always
surrounded by eosinophils, whereas both eosinophils and necrosis of encysted larvae were lacking in the CCR3−/− mice (Fig. 6). This finding was accompanied by a 2-fold increase in the numbers of larval cysts in the muscle of CCR3−/− mice, as compared with their BALB/c controls (Fig. 7). Although several studies indicated that eosinophils could kill T
s larvae in vitro (27–29), in vivo studies have generated conflicting results on this issue. Grove et al. (23) found that suppression of the eosinophil response in CF1 outbred mice with an anti-eosinophil serum resulted in increased numbers of Ts larvae, counted after digestion of the host tissues. In contrast, Herndon and Hayes (24), using the same mouse strain, prevented eosinophilia by treating the mice with anti-IL-5 and found no difference in adult worm rejection from the intestines or in the number of larvae isolated from the infected animals. Also, C3H/HeN mice overexpressing IL-5 (25) and C57BL/6 IL-5-null mice (26) were similar to their wild-type controls in the number of numbers of the respective life stages of the parasite. Moreover, because the increase in blood-borne eosinophils is due to the production of IL-5, a Th2-derived cytokine (37–39), ample production of this cytokine is occurring. Also, although other investigators have implicated CCR3 in the Th2 response in the lung (7), it appeared most critical in the early stages. In an ongoing response to intestinal helminths that requires >1 wk to develop, any early role of CCR3 in the Th2 response may be compensated by other pathways, such as the CCR4/monocyte chemotactic protein that is important in Th2 responses in the lung (7). Thus, although CCR3 is expressed on both adaptive and innate immunocytes involved in the anti-Ts response, loss of this receptor does not dramatically impair this coordinated effort between the different components.

This study confirms the essential role of CCR3 and its ligands for eosinophil recruitment to the intestines and skeletal muscle in a helminth infection. It also suggests clear distinctions for the roles of MC and eosinophils in the elimination of adult helminths and their larvae, with each cell type playing a direct role in controlling the numbers of the respective life stages of the parasite. Moreover, our study establishes that CCR3 is required neither for MC recruitment to the small intestine nor for the upstream functions of Th2 cells, as evidenced by the MC hyperplasia and adult worm rejection kinetics.

References

FIGURE 7. Quantitation of cysts and necrotic larvae in CCR3−/− and +/+ littermate control mice after Ts infection. The number of cysts per lpf (a) and the percentage of degenerating cysts with necrotic larvae (b) were enumerated in the tongues of CCR3−/− and +/+ mice. The mean cyst number was obtained by counting at least 10 lpf from each animal. Values are the means (±SEM) from 15 animals from 2 experiments composed of 6 animals analyzed on day 28, 6 on day 35, and 3 on day 56. Statistically significant differences between means are indicated by asterisks (*, p < 0.05; **, p < 0.01). The total numbers of cysts counted in the tongues of the 15 animals in each group were 292 for the CCR3−/−, and 252 for the +/+ mice, respectively.


