Impaired Host Defense, Hematopoiesis, Granulomatous Inflammation and Type 1–Type 2 Cytokine Balance in Mice Lacking CC Chemokine Receptor 1

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Summary

CC chemokine receptor 1 (CCR1) is expressed in neutrophils, monocytes, lymphocytes, and eosinophils, and binds the leukocyte chemoattractant and hematopoiesis regulator macrophage inflammatory protein (MIP)-1α, as well as several related CC chemokines. Four other CCR subtypes are known; their leukocyte and chemokine specificities overlap with, but are not identical to, CCR1, suggesting that CCR1 has both redundant and specific biologic roles. To test this, we have developed CCR1-deficient mice (−/−) by targeted gene disruption. Although the distribution of mature leukocytes was normal, steady state and induced trafficking and proliferation of myeloid progenitor cells were disordered in −/− mice. Moreover, mature neutrophils from −/− mice failed to chemotax in vitro and failed to mobilize into peripheral blood in vivo in response to MIP-1α. Consistent with this, −/− mice had accelerated mortality when challenged with *Aspergillus fumigatus*, a fungus controlled principally by neutrophils. To test the role of CCR1 in granuloma formation, we injected *Schistosoma mansoni* eggs intravenously, and observed a 40% reduction in the size of lung granulomas in −/− mice compared to +/+ littermates. This was associated with increased interferon-γ and decreased interleukin-4 production in −/− versus +/+ lung lymph node cells stimulated with egg-specific antigen, suggesting that CCR1 influences the inflammatory response not only through direct effects on leukocyte chemotaxis, but also through effects on the type 1–type 2 cytokine balance. Thus CCR1 has nonredundant functions in hematopoiesis, host defense, and inflammation.

Chemokines form a large family of secreted proteins ~70–80 amino acids in length that regulate multiple aspects of host defense and inflammation, including angiogenesis, hematopoiesis, and leukocyte chemotaxis (1–6). The ~30 chemokines that are known can be divided roughly evenly into two major groups, CXC and CC, based on the presence (CXC) or absence (CC) of a single amino acid between the first two of four conserved cysteines. CXC chemokines mainly target neutrophils and T cells, whereas CC chemokines target monocytes, eosinophils, basophils, and lymphocytes with variable selectivity, but usually do not target neutrophils.

Chemokines activate leukocytes by binding to seven transmembrane receptors coupled to G_i-type G proteins (7). The twelve receptor subtypes identified so far can be divided into two major groups, given the root names CXCR and CCR, based on specificity for the two major classes of chemokines. Most chemokine receptors bind more than one chemokine, and most chemokines bind to more than one receptor. The receptors are differentially expressed in leukocytes, but the specific patterns of expression overlap considerably.

Gene targeting in mice may be useful for defining specific biological roles for chemokines and chemokine receptors. Using this technique, Cacalano et al. confirmed a role for CXCR2 in neutrophil-mediated acute inflammation (8, 9), which had been suspected based on the preferential expression of CXCR2 in neutrophils (10) and the potent neutrophil chemoattractant activity of CXCR2 ligands (11). They also identified an unexpected role for CXCR2 in negative regulation of hematopoiesis (12).

In the present study, we describe mice lacking CCR1 (13–17). CCR1 is constitutively expressed in neutrophils, monocytes, eosinophils, and T and B lymphocytes (13–19),

and its principal agonists are macrophage inflammatory protein (MIP)-1α, reduced on activation normal T expressed and secreted (RANTES), and monocyte chemoattractant protein 3 (13, 14, 20). CCR2-5 share at least one agonist and at least one leukocyte subset with CCR1 (20–24). MIP-1α has been implicated in both schistosome egg–induced granulomatous inflammation and experimental allergic encephalomyelitis by in vivo neutralization studies in mice, and in virally induced inflammation by challenges of gene-targeted mice (25–27). MIP-1α also has complex effects on hematopoiesis, acting as a negative regulator of stem and immature progenitor cell proliferation (3–6, 28–30), and as a positive regulator of lineage-committed progenitor cell proliferation (30, 31). The in vivo actions of RANTES and monocyte chemoattractant protein 3 have not been studied in as much detail.

Our results indicate that CCR1 deficiency is benign in animals raised undisturbed in specific pathogen-free environments, but that a variety of defects in both innate and acquired immunity can be observed under stressed conditions.

Materials and Methods

Gene Targeting in Embryonic Stem Cells and Generation of Mice. The cloning of the CCR1 gene from a 129/Sv mouse genomic library has been described previously (15). A 6-kb XbaI fragment was cloned into Bluescript. A 352-bp EcoRI–HindIII fragment in the intronless open reading frame (ORF) was replaced by a 1.1-kb neomycin resistance cassette (*neo*^{R})[1] in the same orientation. A 6.2-kb fragment of the resulting insert was excised by digestion of a NotI site in the polylinker at the 3′ end and a KpnI site at the 5′ end from the ORF, and subcloned between the NotI and KpnI sites of the plasmid pPNT, which contains the HSV thymidine kinase gene. The resulting construct contained 1.3 and 3.8 kb of CCR1 sequence 5′ and 3′ from *neo*^{R}, respectively, as shown in Fig. 1 *a*. The construct was linearized with XbaI, and 25 μg DNA was electroporated into 10^7 embryonic stem cells of the J1 cell line. 162 clones resistant to G418, 350 μg/ml of media, and gancyclovir 2 μM were selected and expanded on feeder layer cells in 24-well plates. Two homologous recombinants were identified by Southern blot hybridization of genomic DNA digested with XbaI and hybridized with the probe outside of the construct, indicated in Fig. 1. Nine chimeric mice were produced either by microinjection into or aggregation with C57Bl/6 blastocysts according to standard methods (32, 33). Chimeric mice were mated with wild-type C57BL/6 mice to produce heterozygous mice. Littermates from the mating of heterozygous mice were then analyzed.

Isolation of Mouse Leukocytes. Total leukocytes (80–85% mononuclear cells and 15–20% neutrophils) were isolated from citrated peripheral blood obtained from mouse tails; erythrocytes were lysed in lysis buffer (ACK; BioWhittaker, Walkersville, MD). To obtain enriched populations of neutrophils and macrophages, the peritoneal cavity was washed with PBS 3 or 72 h, respectively, after peritoneal injection with thioglycollate. The purity of both populations was >90% as assessed by light microscopy of Diff-quick–stained cytospin preparations.

Analysis of Progenitor Cells. Unseparated marrow, spleen, and low density blood leukocytes (retrieved after density cut at 1.070 g/cm^3 on Ficoll-Hypaque) were plated at 2×10^4, 4×10^5, and 1×10^5 cells/ml, respectively, in 1% methylcellulose culture medium with 1 U/ml recombinant human Epo, 5% vol/vol pokeweed mitogen mouse spleen cell conditioned medium, 50 ng/ml recombinant murine steel factor, and 0.1 mM hemin. Colonies were scored after 7 d incubation in a humidified environment at 5% CO_2 and 5% O_2, and absolute numbers of progenitors calculated based on nucleated cellularity per organ and colony counts (28, 29). The percentage progenitors in S phase of the cell cycle, an indication of the proliferative status of cells, was determined by the high specific activity tritiated thymidine kill assay described previously (28, 29).

$[Ca^{2+}]_i$ Measurements. Cells (10^7/ml) were loaded with FURA-2 (Molecular Probes, Eugene, OR) as previously described (34). 2×10^6 cells in 2 ml HBSS were placed in a continuously stirred cuvette at 37°C in a fluorimeter (Photon Technology Inc., South Brunswick, NJ). The data are presented as the relative ratio of fluorescence excited alternately at 340 and 380 nm every 0.5 sec, monitored at 510 nm in response to recombinant mouse MIP-1α (Peprotech, Rocky Hill, NJ) or fMet-Leu-Phe (Sigma Chemical Co., St. Louis, MO).

Chemotaxis. Chemotaxis was analyzed using polyvinylpyrrolidone-free polycarbonate membranes with 3-μm pores in 48-well chambers (Neuro Probe, Cabin John, MD). Mouse MIP-1α in HBSS was placed in the lower chamber, and 150,000 peripheral blood leukocytes suspended in HBSS were placed in the upper chamber. After incubation for 45 min at 37°C, the membrane was removed, washed on the upper side with PBS, fixed, and stained. Cells were counted in five randomly selected fields at 1,000-fold magnification. The pore size was chosen to allow migration of neutrophils, but not monocytes. Chemotaxis of thioglycollate-elicited peritoneal macrophages were tested using a filter with 8-μm pores.

Neutrophil Mobilization to Peripheral Blood. Mice were injected subcutaneously with 100 μg/kg of the BB10010 variant of human MIP-1α (a gift of L. Czaplewski, British Biotech Ltd., Oxford, U.K.). BB10010, human MIP-1α, and mouse MIP-1α all have similar selectivity for mouse CCR1 (Gao, J.-L., and P.M. Murphy, unpublished observations). Complete peripheral blood leukocyte counts and differentials were determined for tail vein collections taken before injection and 1 h after injection.

Challenge with Aspergillus fumigatus. *Aspergillus fumigatus* strains were grown on minimal media containing 10 mM $NaNO_3$, 1% glucose, salts, and trace elements for 10 d at 37°C. Conidia were washed off with 0.1 M potassium phosphate buffer (pH 7.0) containing 0.01% Tween 80. This suspension was washed twice with 0.9% NaCl and further diluted with 0.9% NaCl to a final inoculum size of 2.5×10^7 conidia/ml, as determined by cell counting in a hemocytometer. Mice were infected by injecting 0.2 ml of a suspension containing 5×10^6 conidia (the LD_{50} in these experiments) into the lateral tail vein. Animals were then observed for mortality daily. For the preparation of histologic sections, 10^7 spores were injected into CCR1 +/+ mice and CCR1 −/− littermates intravenously, and dying mice were killed 24 and 72 h after injection. Sections were prepared from lung, kidney, liver,

[1]*Abbreviations used in this paper:* BFU-E, erythroid burst-forming unit; CFU-GEMM, granulocyte, erythrocyte, macrophage, megakaryocyte CFU; CFU-GM, granulocyte/macrophage CFU; lps, lipopolysaccharide; MIP, macrophage inflammatory protein; *neo*^{R}, neomycin resistance gene; ORF, open reading frame; RANTES, reduced on activation normal T expressed and secreted; SEA, schistosome egg antigen; TP, thioglycollate-elicited peritoneal.

and brain, and stained with hematoxylin and eosin as well as with Gomori methenamine.

Induction of Pulmonary Granulomas. The induction of synchronous egg-induced granulomas was performed as described (35). In brief, *Schistosoma mansoni* eggs were isolated from the livers of infected mice and enriched for mature eggs (BioMedical Research Instrs., Rockville, MD). To induce granulomas, mice were injected intravenously with 5,000 eggs. Confirmation of embolization of eggs was performed in all mice by histological examination of the lungs. For histopathological morphometry of granulomas, the left lung was inflated with and fixed in Bouin-Hollande solution and processed routinely. The size of the pulmonary granulomas was determined in histological sections stained by a modification of the Dominici stain. The diameters of each reaction containing a single egg were measured in each lung with an ocular micrometer. Approximately 30 granulomas were counted in each lung.

Cytokine Assays. Single cell suspensions of lung-associated lymph nodes were prepared aseptically at various times after injection of schistosome eggs. Lung-associated lymph nodes were pooled from 4 to 5 animals. Cells were plated in 24-well tissue culture plates at a final concentration of 3×10^6 cell/ml in RPMI with 2 mM glutamine, 25 mM Hepes, 10% FBS, 50 μM 2-ME, penicillin, and streptomycin. Cultures were incubated at 37°C in 5% CO_2. Cells were stimulated with schistosome egg antigen at 20 μg/ml. Supernatant fluids were harvested at 72 h and assayed for cytokine activity. IFN-γ was measured by specific two-site ELISA as previously described. IL-4 levels were determined by proliferation of CT4S cells. Cytokine levels were calculated using standard curves constructed using recombinant murine cytokines.

Results

Development of Mice Lacking CCR1. To inactivate CCR1, we replaced a 352-bp HindIII–EcoRI fragment of the ORF with a *neo*^{R} by homologous recombination in 129/Sv embryonic stem cells (Fig. 1 *a*); this corresponds to 33% of the protein from the third to the fifth transmembrane segment. Chimeric mice were made by injecting targeted 129/Sv cell lines into blastocysts from C57Bl/6 mice. The chimeric mice were then mated with C57Bl/6 mice to establish heterozygotes, which were interbred to produce CCR1 +/+, +/−, and −/− litter mates. Genotypes were determined by diagnostic XbaI restriction fragment length polymorphisms of genomic DNA (Fig. 1 *b*). Mutant CCR1 mRNA was detected in neutrophils from +/− and −/−, but not +/+ mice, whereas normal CCR1 mRNA was detected in neutrophils from +/− and +/+, but not −/− mice (Fig. 1 *c*).

CCR1 genotypic frequencies for 423 total progeny of 12 +/− mating pairs were: 28% +/+, 51% +/−, and 21% −/−, which is similar to Mendelian expectation for an autosomal gene. CCR1 −/− mice were viable and fertile, and exhibited normal growth, development, anatomy and behavior compared to +/+ littermates. They have been observed for up to 20 mo of age, and have not exhibited defects in hemostasis or healing of tail wounds, or increased susceptibility to spontaneous infection in a specific pathogen-free environment. No significant differences in the histology of bone marrow, lymph node, spleen, or thymus, or

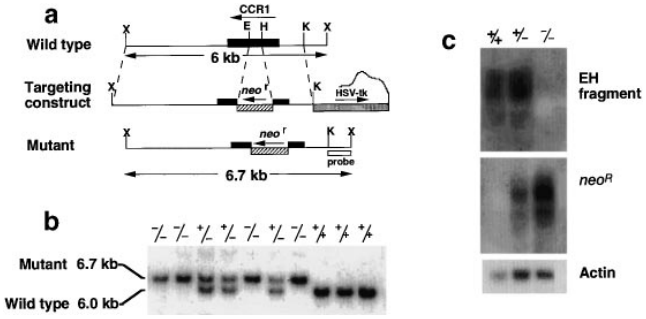

Figure 1. Genetic inactivation of CCR1 in mice. (*a*) Mutagenesis strategy. *neo*^{R} replaced 352 bp of the ORF and was used to select for targeted events. The HSV thymidine kinase gene (*HSV-tk*) was used for counterselection of nontargeted events. (*b*) Genotypic analysis of tail DNA derived from progeny of heterozygous crosses. The mutated allele (6.7-kb XbaI fragment) is distinguished from the endogenous allele (6.0-kb XbaI fragment) using the probe indicated in *a*. *X*, XbaI; *E*, EcoRI; *H*, HindIII; *K*, KpnI. (*c*) Northern blot analysis of total RNA (10 μg/lane) from TP neutrophils from mice with the CCR1 genotype indicated at the top of each lane. The same blot was probed sequentially with an EcoRI–HindIII fragment of the CCR1 ORF (*EH fragment*), *neo*^{R}, and actin, as indicated to the right of each panel.

of the complete blood count and differential were observed between CCR1 −/− mice and CCR1 +/+ littermates. Moreover, FACS® immunophenotyping of leukocytes from spleen, lymph node, and peripheral blood of CCR1 −/− mice using mAbs directed against the neutrophil marker GR-1, the monocyte marker Mac-1, the T cell receptor, and the B cell marker B-220 gave similar patterns for CCR1 −/− and +/+ littermates (at least three animals tested for each genotype for each site; data not shown).

Role of CCR1 in Progenitor Cell Proliferation and Mobilization. Selected chemokines, including MIP-1α, have been implicated in regulation of myeloid stem and progenitor cell proliferation in vitro (3–5, 30, 31) and in vivo (3, 6, 28, 29), and in mobilization of stem cells and progenitor cells from bone marrow to blood (6, 36), but underlying mechanisms, including the receptors involved, are not known. Since CCR1 is one of the receptors that binds MIP-1α, CCR1 −/− mice were assessed in comparison with CCR1 +/+ littermates for absolute numbers of granulocyte-macrophage (granulocyte/macrophage CFU; CFU-GM), erythroid (erythroid burst-forming unit; BFU-E), and multipotential (granulocyte, erythrocyte, macrophage, megakaryocyte CFU; CFU-GEMM) progenitors in the marrow, spleen, and blood (Table 1). Although there were no differences in absolute numbers of progenitors per femur between these mice, significantly decreased numbers of CFU-GM and CFU-GEMM were noted in the spleen and circulating blood of the CCR1 −/−, compared to +/+ mice. BFU-E in the spleen and blood of CCR −/− mice were also decreased, but these differences did not reach statistical significance.

To test this further, we challenged mice with bacterial lipopolysaccharide (lps), which is known to perturb myelopoiesis and to mobilize progenitors from marrow to the spleen and blood of rodents (37). Lps induced a significant

Table 1. *Distribution of Myeloid Progenitor Cells in CCR1 +/+ and −/− Mice*

Tissue Genotype	Nucleated Cells ($\times 10^{-6}$)	No. of Progenitors ($\times 10^{-3}$/femur or spleen, or $\times$ 1/ml blood)		
		CFU-GM	BFU-E	CFU-GEMM
Femur				
+/+	22.8 ± 1.5	60.7 ± 6.1	13.7 ± 1.5	2.8 ± 0.4
−/−	22.0 ± 1.5	50.6 ± 4.6	11.7 ± 0.9	3.0 ± 0.3
Spleen				
+/+	110.8 ± 7.5	7.7 ± 1.1	6.8 ± 1.2	0.9 ± 0.2
−/−	97.0 ± 8.2	4.2 ± 0.6*	4.7 ± 1.0	0.5 ± 0.1‡
Blood				
+/+	1.9 ± 0.5	203 ± 64	109 ± 50	24 ± 10
−/−	1.6 ± 0.3	71 ± 28§	61 ± 25	6 ± 2‖

Results are the mean ± SEM for a total of eight mice from two separate experiments in which each mouse was assessed individually. Statistical significance using Student's *t* test for +/+ versus −/− mice in each category: *$P < 0.005$; ‡$P < 0.02$; §$P < 0.04$; ‖$P < 0.05$. Differences in other categories: $P > 0.05$.

decrease in the numbers of CFU-GM, BFU-E, and CFU-GEMM in marrow from both CCR1 +/+ and −/− mice, suggesting that CCR1 is not required for egress (Table 2). However, the normal complementary increase in progenitor cell numbers observed in spleens of lps-challenged +/+ mice, which was significant and greatest for CFU-GM, was absent in spleens from −/− mice; in fact, BFU-E and CFU-GEMM numbers were actually significantly decreased. This correlated with a greater lps-stimulated increase of progenitors in the blood of −/− mice compared to +/+ mice.

We also assessed the proliferative status of myeloid progenitors in the marrow and spleen of CCR1 −/− and +/+ mice under steady-state and lps-perturbed conditions.

Table 2. *Disordered Myeloid Progenitor Cell Trafficking in CCR1 −/− Mice*

Tissue Genotype	Challenge	No. of Progenitors ($\times 10^{-3}$/femur or spleen, or $\times$ 1/ml blood)		
		CFU-GM	BFU-E	CFU-GEMM
Femur				
+/+	Diluent	70.6 ± 8.6	15.3 ± 2.6	2.9 ± 0.7
+/+	lps	17.8 ± 2.6 (− 75)§	6.4 ± 0.7 (− 58)‡	1.1 ± 0.2 (− 62)*
−/−	Diluent	56.9 ± 6.8	13.0 ± 0.6	3.2 ± 0.6
−/−	lps	14.3 ± 1.8 (− 75)‡	5.7 ± 0.6 (− 56)‡	0.7 ± 0.2 (− 78)‡
Spleen				
+/+	Diluent	8.2 ± 2.2	9.1 ± 1.7	0.9 ± 0.3
+/+	lps	21.6 ± 7.8 (+ 163)*	12.6 ± 4.1 (+ 38)	1.2 ± 0.5 (+ 33)
−/−	Diluent	4.3 ± 1.0	5.6 ± 0.8	0.4 ± 0.1
−/−	lps	4.2 ± 1.1 (− 2)	3.3 ± 0.9 (− 41)*	0.2 ± 0.03 (− 50)‡
Blood				
+/+	Diluent	313 ± 101	198 ± 78	42 ± 15
+/+	lps	705 ± 213 (+ 125)*	203 ± 70 (+ 3)	50 ± 6 (+ 19)
−/−	Diluent	129 ± 39	113 ± 34	10 ± 3
−/−	lps	327 ± 141 (+ 153)*	144 ± 68 (+ 27)	17 ± 11 (+ 70)

Mice were injected intraperitoneally with control diluent or 10 μg *E. coli* lps once daily for 2 d, and killed 24 h after the last injection. Unseparated marrow and spleen cells and low density blood cells were set up in colony assays as described in Materials and Methods, and absolute numbers of progenitors per organ were calculated based on nucleated cellularity and colony counts. Results are mean ± SEM for one experiment with four mice per group in which each mouse was assessed individually. Numbers in parentheses denote percent change in lps versus control diluent-treated mice for either +/+ or −/− mice. Significant differences by Student's *t* test for lps versus control diluent: *$P < 0.05$; ‡$P < 0.01$; §$P < 0.001$.

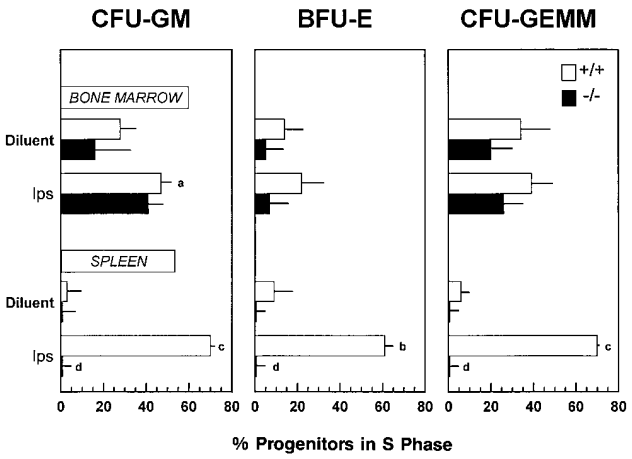

Figure 2. Disordered myeloid progenitor cell proliferation in CCR1 −/− mice. The protocol used and mice evaluated were the same as for Table 2. Significance for −/− compared to +/+ mice given control diluent: *a*, $P < 0.05$; *b*, $P < 0.005$; *c*, $P < 0.001$. Significance for −/− mice given lps compared to +/+ mice given lps: *d*, $P < 0.001$.

Under steady-state conditions (mice given control diluent), progenitors were proliferating in the marrow as determined by the percentage in S phase, whereas progenitors in the spleen were in a slow or noncycling state (compare especially CFU-GM and CFU-GEMM). In CCR1 +/+ and −/− mice not given lps, no significant differences in cycling rates were observed for any progenitor subtype in either marrow or spleen (Fig. 2). However, significant differences were found in progenitor cell proliferation after lps administration to the mice. This was evident in the spleen, but not marrow, where lps induced a rapid proliferation of progenitors in CCR1 +/+, but not in CCR1 −/− mice. Thus, CCR1 may regulate both steady-state and induced movement during adult life of myeloid progenitor cells from marrow to spleen and blood, as well as the resultant proliferative status of these stimulated cells.

Role of CCR1 in Neutrophil-mediated Host Defense. With respect to mature leukocyte function, the results of three separate assays suggested that CCR1 is critical for MIP-1α–induced responses in neutrophils. First, the normal MIP-1α–induced calcium flux in thioglycollate-elicited peritoneal (TP) neutrophils from +/+ mice was absent in cells from −/− mice (Fig. 3 *a*). Also, in four separate experiments the normal MIP-1α–induced calcium flux observed in total peripheral blood-derived leukocytes from +/+ mice was not detectable in −/− mice (data not shown). In contrast, a response to MIP-1α was detected in TP macrophages from −/− mice, suggesting compensation by other MIP-1α receptor subtypes (22, 24). Second, peripheral blood neutrophils from CCR1 −/− mice failed to chemotax in response to MIP-1α in vitro, whereas neutrophils from +/+ mice exhibited a typical bell-shaped dose-response curve (Fig. 3 *b*). Consistent with the calcium flux results, TP macrophages from both CCR1 −/− and +/+ mice responded chemotactically to MIP-1α, and the responses were similar (not shown). Third, neutrophils failed to mobilize to peripheral blood in −/− mice injected subcutaneously with MIP-1α, whereas neutrophil concentrations were increased ~fivefold in +/+ littermates 1 h after injection, consistent with previous reports (6; Fig. 3 *c*).

Since these tests consistently implicated CCR1 in nor-

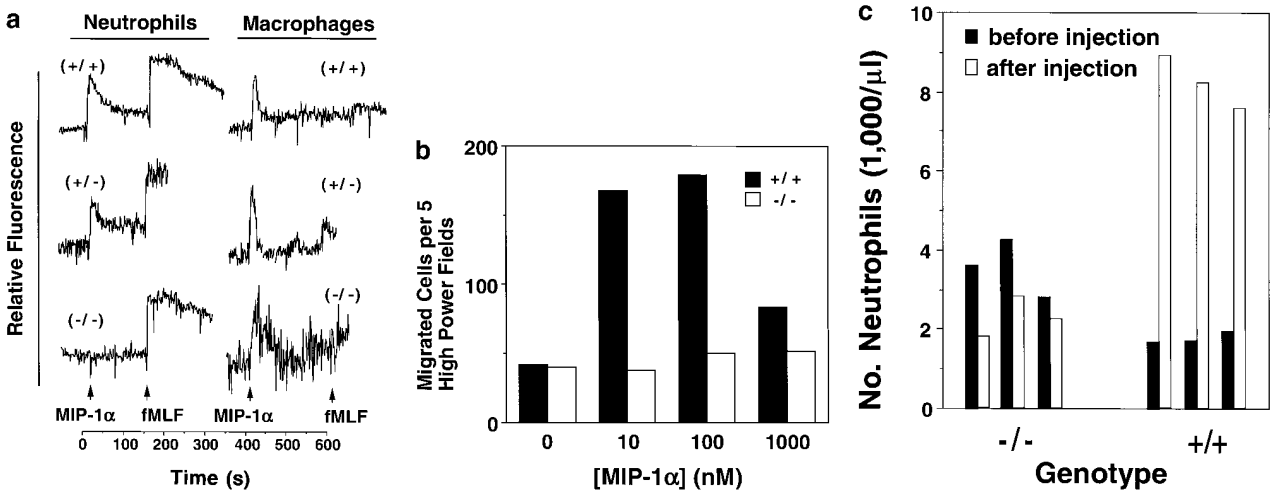

Figure 3. Loss of MIP-1α–dependent functions in mature neutrophils from CCR1 −/− mice. (*a*) Calcium flux response. $[Ca^{2+}]_i$ was monitored in real time in FURA-2–loaded leukocytes stimulated with 100 nM mouse MIP-1α and 5 μM fMet-Leu-Phe. Each tracing represents analysis of 3×10^6 cells from a single mouse. Each tracing corresponds to the TP cell type indicated at the top of the column in which it is found and to the CCR1 genotype indicated at the top left. Agonists were added at the times indicated by the arrows. Results are from a single experiment representative of at least four separate experiments. (*b*) Neutrophil chemotaxis. Results are the mean +/− SEM of triplicate determinations in a single experiment using peripheral blood leukocytes and mouse MIP-1α in vitro, and are representative of three separate experiments. There was no difference in fMet-Leu-Phe–induced chemotaxis between neutrophils from CCR1 −/− and +/+ mice (not shown). (*c*) Neutrophil mobilization to peripheral blood. Each pair of bars represents the peripheral blood neutrophil concentration for a single mouse before and 1 h after subcutaneous injection with 2 μg of the BB10010 variant of MIP-1α. Results are from a single experiment, representative of two separate experiments.

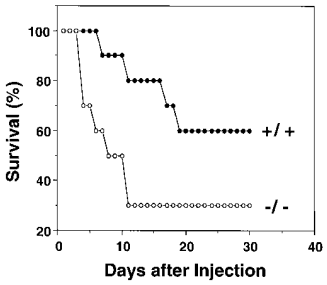

Figure 4. Accelerated lethality in CCR1 −/− mice challenged with *A. fumigatus*. Mice were injected with 5×10^6 spores in the tail vein. Results are from a single experiment with −/− and +/+ sex-matched littermates ($n = 10$ in each group), representative of two separate experiments with a consistent pattern.

mal neutrophil function, we challenged mice intravenously with *A. fumigatus*, an opportunistic fungus in patients with prolonged neutropenia or defects in neutrophil function (38). In this model, CCR1 −/− mice exhibited a markedly higher rate and extent of mortality compared to CCR1 +/+ littermates (Fig. 4). 70% of CCR1 −/− mice injected with 5×10^6 spores died by day 15, in contrast to only 20% in CCR1 +/+ mice during the same period. Only 30% of CCR1 −/− mice survived beyond 30 d of observation, whereas 60% of CCR1 +/+ mice remained healthy. Fatally infected mice from both groups developed severe ataxia associated with fungal invasion of the central nervous system 2–3 d before death. Such symptoms were observed at least 2–3 d earlier in the −/− mice, compared to CCR1 +/+ littermates. The same pattern of accelerated lethality in CCR1 −/− mice was observed when the inoculum was increased to 10^7 spores. Histological sections prepared with the organs of mice injected with 10^7 spores showed robust hyphal growth in kidney, lung, liver, and brain 24 h after injection in CCR1 −/− mice, but were not detected in brain and lungs of CCR1 +/+ mice until 72 h after injection.

At 24 h, kidney sections from CCR1 −/− mice showed multiple foci of fungal growth in both cortex and medulla, but very little inflammation. Only one fungal fragment and no inflammation was detected in CCR1 +/+ kidney at this time point. However, by 72 h, numerous fungal abscesses were observed in both strains of mice. Despite similar histopathology, the lesions were much more advanced with more extensive debris in CCR1 −/− mice, compared to +/+ mice. Extensive karyorexis of the inflammatory cells was observed, which precluded precise identification. At 72 h, both types of mice showed ungerminated, swollen conidia throughout the liver with no inflammation. Host response around brain lesions was also unremarkable. None of the infected organs exhibited hyphal invasion of blood vessels.

Role of CCR1 in Granuloma Formation. In addition to neutrophils, CCR1 is expressed by lymphocytes, macrophages, and eosinophils (14, 17, 19), and we hypothesized that it may regulate migration of all of them in vivo. Therefore, we challenged mice by intravenous injection of *S. mansoni* eggs, which lodge in the lung and induce formation of granulomas composed of lymphocytes, macrophages, eosinophils, and neutrophils (39). A second reason for this challenge was a report that depletion of MIP-1α in mice by injection of neutralizing mAb results in a reduced granulomatous response to schistosome eggs (25). CCR1 −/− mice consistently developed smaller granulomata than +/+ littermates, ~40% decreased 14 d after primary egg injection (Fig. 5 *a*).

This result is consistent with a role for CCR1 in granuloma formation through a direct effect on leukocyte chemotaxis. Moreover, since there were no obvious differences in the cellular composition of the granulomas in −/− and +/+ mice, it suggests a possible effect of CCR1 deficiency on leukocyte extravasation for many leukocyte subtypes in vivo. However, CCR1 could also be acting indirectly through effects on cytokines. Numerous studies have shown that type 2 cytokines (e.g., IL-4 and -10) enhance egg-induced pulmonary granuloma formation in the schistosome model, whereas type 1 cytokines (e.g., IFN-γ and IL-12) are inhibitory (39). To test whether CCR1 influences type 1 and type 2 cytokine expression, we killed mice on days 3, 6, and 14 after egg injection, and analyzed the draining lung lymph node cells for changes in IFN-γ and IL-4 expression in vitro in response to a soluble mix-

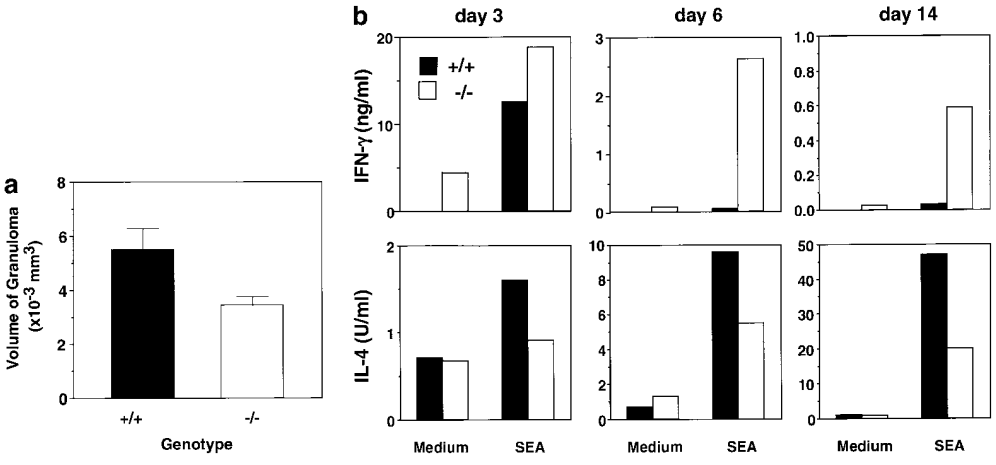

Figure 5. Impaired lung granuloma formation and type 1–type 2 cytokine balance in CCR1 −/− mice. At 2–3 mo of age, sex-matched CCR1 +/+ and −/− littermates were injected intravenously with 5,000 eggs of *S. mansoni*. (*a*) Granuloma formation. Animals were killed 14 d after injection, and granuloma size was determined. Results are the mean +/− SEM of three separate experiments, 4–5 animals/group/experiment, 30 granulomas/animal. $P < 0.01$. (*b*) type 1–type 2 cytokine balance. Mice were killed 3, 6, and 14 d after egg injection. Draining lung lymph node cells were pooled and cultured in vitro with medium alone or medium plus SEA 20 μg/ml for 3 d, and cytokine concentrations were then measured in the supernatants. Each bar represents the mean of 4–5 mice. +/+, *solid bars*; −/−, *open bars*.

ture of schistosome egg antigen (SEA). Consistent with previous reports, the SEA-induced cytokine profile for cells from both −/− and +/+ mice evolved from an early type 1 bias into a later type 2 bias which peaked at a time when granuloma size was maximal. However, we consistently observed elevated constitutive as well as SEA-induced expression of IFN-γ in CCR1 −/− mice compared with +/+ littermates (Fig. 5 *b*). Conversely, cells from −/− mice consistently produced less IL-4 in response to SEA at all time points compared to cells from +/+ mice. Leukocyte subset depletion studies suggested that the increase in constitutive production of IFN-γ was likely attributable to NK cells, which is consistent with previous studies of the origin of IFN-γ in this model (data not shown; 39).

Discussion

Our analysis of CCR1 −/− mice indicates that CCR1 is not required for normal mouse development or for adequate antimicrobial defense when animals are raised undisturbed in specific pathogen-free environments. However, diverse nonredundant roles for CCR1 in immune system function can be observed when the animals are stressed. In particular, our experiments implicate CCR1 in migration of early and later subsets of myeloid progenitor cells from marrow to spleen and blood after lps injection, and the subsequent proliferative stimulation of these cells in the spleen, neutrophil chemotaxis, MIP-1α–induced neutrophil mobilization, neutrophil-mediated antifungal host defense, schistosome egg–induced granulomatous inflammation, and regulation of the type 1–type 2 cytokine balance.

The phenotype of the CCR1 −/− mouse is analogous to that previously described by Cook et al. for MIP-1α −/− mice created by gene targeting (27). Their mice also appear phenotypically normal when raised undisturbed in specific pathogen-free environments, but have impaired inflammatory responses to microbial challenge, specifically with Coxsacckie B and influenza viruses. Humans homozygous for an inherited inactivating mutation of the CCR5 gene (encoding a receptor for MIP-1α, MIP-1β, and RANTES) or the Duffy gene (encoding a receptor for certain CC and CXC chemokines) have been identified, and they have no obvious health problems (40–42). On the contrary, in both cases they appear to be resistant to infectious disease, specifically to HIV-1 in the case of CCR5 deficiency (40, 43–45), and to the malaria-causing protozoan *Plasmodium vivax* in the case of Duffy deficiency (41). In both cases, the chemokine receptor is needed by the pathogen to enter target cells (46, 47). The high tolerance of mice for deficiencies in MIP-1α and CCR1 and of humans for deficiencies in CCR5 and Duffy contrasts with the perinatal lethal phenotype of mice deficient in the CXC chemokine SDF-1 (48) and the disordered hematopoiesis observed in mice deficient in CXCR2 (8, 9, 12), and may be a consequence of the higher degree of redundancy found so far for CC chemokines and their receptors (7). Nevertheless, it is also clear that MIP-1α and CCR1 are not fully compensated under conditions of intense irritation created in the challenge experiments.

Fundamental differences in the structure and function of the chemokine system in mouse and human warrant extra caution in extrapolating to humans from experimental results obtained in the mouse. For example, the mouse has an orthologue of the human IL-8 receptor subtype CXCR2 (49), but counterparts of IL-8 itself and the other known human IL-8 receptor subtype CXCR1 have not been identified. Also, RANTES is a poor agonist for mouse CCR1, but is a potent and effective agonist for human CCR1, even though RANTES binds to both human and mouse CCR1 with similar affinity (13–17). In contrast, MIP-1α (both mouse and human forms) is a high affinity ligand and potent agonist for both human and mouse CCR1. Further, mouse and human neutrophils appear to differ in their use of CCR1. Like mouse neutrophils, human neutrophils express CCR1, and they respond to its agonists with a calcium flux. However, MIP-1α does not chemoattract human neutrophils in vitro (50), whereas our data clearly indicate that it is a potent chemoattractant for mouse neutrophils, consistent with earlier reports of the ability of native MIP-1 to induce neutrophil accumulation at sites of injection (51). CCR1 may have evolved to play a more important role in mouse neutrophils than human neutrophils to compensate for the apparent absence of CXCR1 on mouse neutrophils. Mouse TCA-3 is another example of a CC chemokine that chemoattracts mouse neutrophils. Like MIP-1α, its human homologue, I-309, does not chemoattract human neutrophils (52). The failure of neutrophils from CCR1 −/− mice to respond to MIP-1α suggests that CCR1 is the dominant, and perhaps only, MIP-1α receptor subtype expressed in these cells. Moreover, the data prove that CCR1 is a bona fide chemotactic receptor.

CCR1 appears to be important for mouse neutrophil-mediated host defense, since CCR1 −/− mice are much more susceptible to infection with *A. fumigatus*, a ubiquitous fungus that causes invasive and highly lethal infections in humans and mice mainly when neutrophil number or function are impaired. The neutrophil is most critical in the early clearance of the organism (38). Our data showing large numbers of hyphae in kidney without inflammation at 24 h after challenge suggest that neutrophils block efficient tissue invasion from the blood, and that this is deficient in CCR1 −/− mice. The larger abscesses seen in established kidney lesions in CCR1 −/− mice compared to +/+ littermates indicate that CCR1 is not essential for maturation of the inflammatory response once hyphal invasion of tissue is established.

Loss of CCR1 function is not apparent in macrophages and lymphocytes from CCR1 −/− mice, probably because other MIP-1α receptor subtypes such as CCR4 and CCR5 compensate. We have not directly tested the function of purified eosinophils from CCR1 −/− mice owing to difficulties in obtaining sufficient numbers of cells. However, we have tested the role of CCR1 in eosinophil migration in vivo in the schistosome egg model of granuloma

formation. These granulomas are composed of egg surrounded by a mixed leukocyte infiltrate including neutrophils, eosinophils, macrophages, and T lymphocytes. We consistently observed a marked reduction in granuloma size in CCR1 −/− animals compared to CCR1 +/+ littermates, but found no obvious changes in the cellular composition of the lesions. Detailed immunophenotyping of the granuloma cells will be needed to look for more subtle differences in the cellular composition between CCR1 +/+ and −/− mice. Although this result may be a direct effect of the loss of the chemotactic function of CCR1, our data also suggest a possible indirect mechanism, through effects on the type 1–type 2 cytokine balance. It has been established that granuloma formation in this model is a function of the relative levels of type 1 and type 2 cytokines (25, 35, 39). Type 1 cytokines such as IFN-γ inhibit and type 2 cytokines such as IL-4 promote granuloma formation. Taken together, the results suggest a novel mechanism for leukocyte accumulation at sites of inflammation in vivo, whereby migration is regulated not only by the direct effects of the chemoattractant on cell motility, but also by its effects on type 1–type 2 cytokine balance, both mediated through the same receptor.

Little is known about the movement of hematopoietic stem and progenitor cells between hematopoietic organs during fetal development or adult life. Our results support a role for CCR1 in the trafficking of early and later subsets of myeloid progenitor cells between marrow and spleen. Under steady state conditions, decreased numbers of progenitors were found in the spleen and blood of CCR1 −/−, compared to +/+ mice. Under the stress of lps administration to mice, the enhanced numbers of progenitors seen in the spleen of CCR1 +/+ mice were not detected in the spleen of CCR1 −/− mice; in fact, a decrease in progenitor numbers was seen in CCR1 −/− spleen after lps injection into the mice with increased levels of circulating progenitors in the blood of CCR1 −/−, compared to +/+ mice. In the context of migration of progenitors from organ to organ of adult rodents after stress, it is generally considered that this movement occurs from marrow to spleen via blood (37). However, the biological relevance of this phenomenon is not clear. The data in Table 2 for +/+ mice responding to lps makes it clear that the increase in splenic progenitors in spleen and blood does not completely compensate for the loss of progenitors from the marrow. Thus, there was a loss of 52.8×10^3 CFU-GM, 8.9×10^3 BFU-E, and 1.8×10^3 CFU-GEMM from femurs of +/+ mice treated with lps. However, the increases noted in the spleens of these mice were 13.4×10^3 CFU-GM, 3.5×10^3 BFU-E, and 0.9×10^3 CFU-GEMM. The differences between marrow and spleen could not be accounted for by the progenitors in the blood. What is happening to the unaccounted for progenitors in +/+ mice is not known; however, it is clear that lps-induced events in the spleens of −/− mice are different from those of +/+ mice. MIP-1α, by itself, is a modest mobilizer of myeloid stem and progenitor cells from the marrow to blood (6, 36), but has a synergistic effect when tested with other cytokines such as G-CSF (6). It remains to be determined whether CCR1 is involved in MIP-1α–induced mobilization, alone and in combination with other cytokines.

MIP-1α acts as a negative regulator of hematopoietic stem and immature progenitor cell proliferation in vitro and in vivo (3–5, 28–30). It has also been shown to act as an enhancing cytokine in vitro for the proliferation of more mature progenitors (30, 31). The enhanced proliferation of myeloid progenitor cells in the spleens of CCR1 +/+ but not −/− mice after challenge with lps suggests that under stress, CCR1 acts in a positive regulatory sense for cell proliferation for migration of cycling cells. Further studies will be necessary to clarify whether MIP-1α mediates its myeloid progenitor cell proliferative effects through CCR1 and/or other MIP-1α receptor subtypes.

The finding of nonredundant biological functions for CCR1 is fairly surprising given the large number of chemokines and receptors, and their overlapping in vitro functions. The CCR1 −/− mouse should be useful for analyzing additional roles of this receptor in other types of inflammatory and infectious diseases.

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