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Involvement of the Chemokine RANTES (CCL5) in Resistance to Experimental Infection with *Leishmania major*

Helton da Costa Santiago,[1] Carolina Ferreira Oliveira,[1] Luciana Santiago,[1] Fernanda Oliveira Ferraz,[1] Daniele da Glória de Souza,[1] Luiz Antônio Rodrigues De-Freitas,[2] Luís Carlos Crocco Afonso,[3] Mauro Martins Teixeira,[1] Ricardo Tostes Gazzinelli,[1,4] and Leda Quercia Vieira[1]*

Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais,[1] and Centro de Pesquisas René Rachou, FioCruz,[4] Belo Horizonte MG, Centro de Pesquisas Gonçalo Muniz, FioCruz, Salvador, Bahia,[2] and Departamento de Ciências Biológicas and Núcleo de Pesquisa em Ciências Biológicas, Instituto de Ciências Biológicas e Exatas, Universidade Federal de Ouro Preto, Ouro Preto MG,[3] Brazil

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The expression and putative role of chemokines during infection with *Leishmania major* in mice were investigated. CCL5 expression correlates with resistance, and blockade of CCL5 rendered mice more susceptible to infection. CCL5 is part of the cascade of events leading to efficient parasite control in *L. major* infection.

Chemokines are cytokines with important roles in cell migration and activation. *Leishmania* sp. infection induces the expression of various chemokine genes (2, 13, 14). *Leishmania major* induces expression of CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), CXCL2 (MIP-2α), and CXCL10 (γIP-10), along with the receptors CCR5, CCR2, and CCR1, in a time-dependent manner in mice (5, 9). CCR2 gene disruption was associated with increased susceptibility to *L. major* (20); however, CCL2 is important to resistance in humans (15, 16) and mice (22, 25). Here we analyze the kinetics of chemokine expression in resistant and susceptible mice upon infection with *L. major*.

C57BL/6 and BALB/c mice were infected with 10^6 stationary forms of *L. major* (11). The mice were sacrificed 1, 2, 14, and 42 days after infection, and RNA was extracted from lesions for reverse transcription (RT)-PCR analysis (3). The expression of chemokines at the site of infection in resistant (C57BL/6) and susceptible (BALB/c) mice is shown in Fig. 1A and B. Expression levels of CXCL9 (Mig) and CCL5 increased initially in both strains, but expression was further increased after 2 weeks of infection in C57BL/6 mice. BALB/c, but not C57BL/6, mice expressed large amounts of mRNA for CCL2, CCL12 (MCP-5), and CXCL8 (KC). Expression levels of CXCL10 were similar in both strains. As the expression levels of CCL2 and CCL5 diverged between the two mouse strains, these chemokines were investigated further.

The differential expression of CCL2 and CCL5 was confirmed by enzyme-linked immunosorbent assay (ELISA) (Fig. 1C) (21). While BALB/c mice produced CCL2 early at the site of infection, CCL2 was detectable only at week 2 postinfection in C57BL/6 mice. The two strains of mice showed similar levels of CCL2 from week 4 of infection. Similar levels of CCL5 were detected in the early and late stages of infection in both C57BL/6 and BALB/c mice. However, C57BL/6 mice had significantly greater quantities of CCL5 than BALB/c mice at weeks 4 and 6 of infection. The decrease in CCL5 levels observed in C57BL/6 mice coincided with the resolution of infection and inflammation at the site of infection (data not shown).

Further studies were performed to verify whether CCL2 and CCL5 were markers of susceptibility and resistance, as suggested for BALB/c and C57BL/6 mice. Hence, we infected susceptible interleukin-12 knockout (IL-$12^{-/-}$) and gamma interferon knockout (IFN-$\gamma^{-/-}$) C57BL/6 mice and resistant IL-$4^{-/-}$ BALB/c mice (6, 10, 23) with *L. major* (Fig. 2A). CCL5 and CCL2 expression was determined by real-time RT-PCR and ELISA. CCL5 protein and mRNA expression levels correlated well. As shown in Fig. 2, CCL5 expression at week 6 of infection was higher in the resistant (IL-$4^{-/-}$) and lower in the susceptible (IL-$12^{-/-}$ and IFN-$\gamma^{-/-}$) mouse strains. Conversely, there was no correlation between expression of CCL2 protein and susceptibility or resistance to infection (Fig. 2B and C). Moreover, there was no equivalence between CCL2 mRNA and protein expression levels.

The results described above suggest that CCL5 may be relevant to resistance against *L. major* infection. To verify this possibility, C57BL/6 mice were treated daily subcutaneously with Met-RANTES (10 μg/mouse; kindly provided by A. E. Proudfoot, Serono Pharmaceuticals, Geneva, Switzerland), a functional antagonist of CCR1 and CCR5 (1, 7, 12). Treatment started at week 2 of infection, when no significant increase in CCL5 mRNA expression was observed. Treatment with Met-RANTES led to a transitory increase in lesion size (Fig. 3A). Mice were sacrificed at weeks 3 and 5 after treatment. At 3 weeks, there was an increase in IL-4 mRNA but no change in IFN-γ expression in lesions (Fig. 3B). Met-RANTES also promoted an impressive down-regulation of the production of IFN-γ in draining lymph nodes, whereas IL-4 production was unchanged (Table 1). Tissue parasitism in these lesions was evaluated by PCR (19) and was higher in the Met-RANTES-treated group at week 3 of treatment (Fig. 3C), which was further confirmed in another experiment by serial dilution

* Corresponding author. Mailing address: Departamento de Bioquímica e Imunologia, ICB-Universidade Federal de Minas Gerais, CP 486, 30161-970, Belo Horizonte MG, Brazil. Phone: 55-31-34992656. Fax: 55-31-3499-2614. E-mail: lqvieira@icb.ufmg.br.

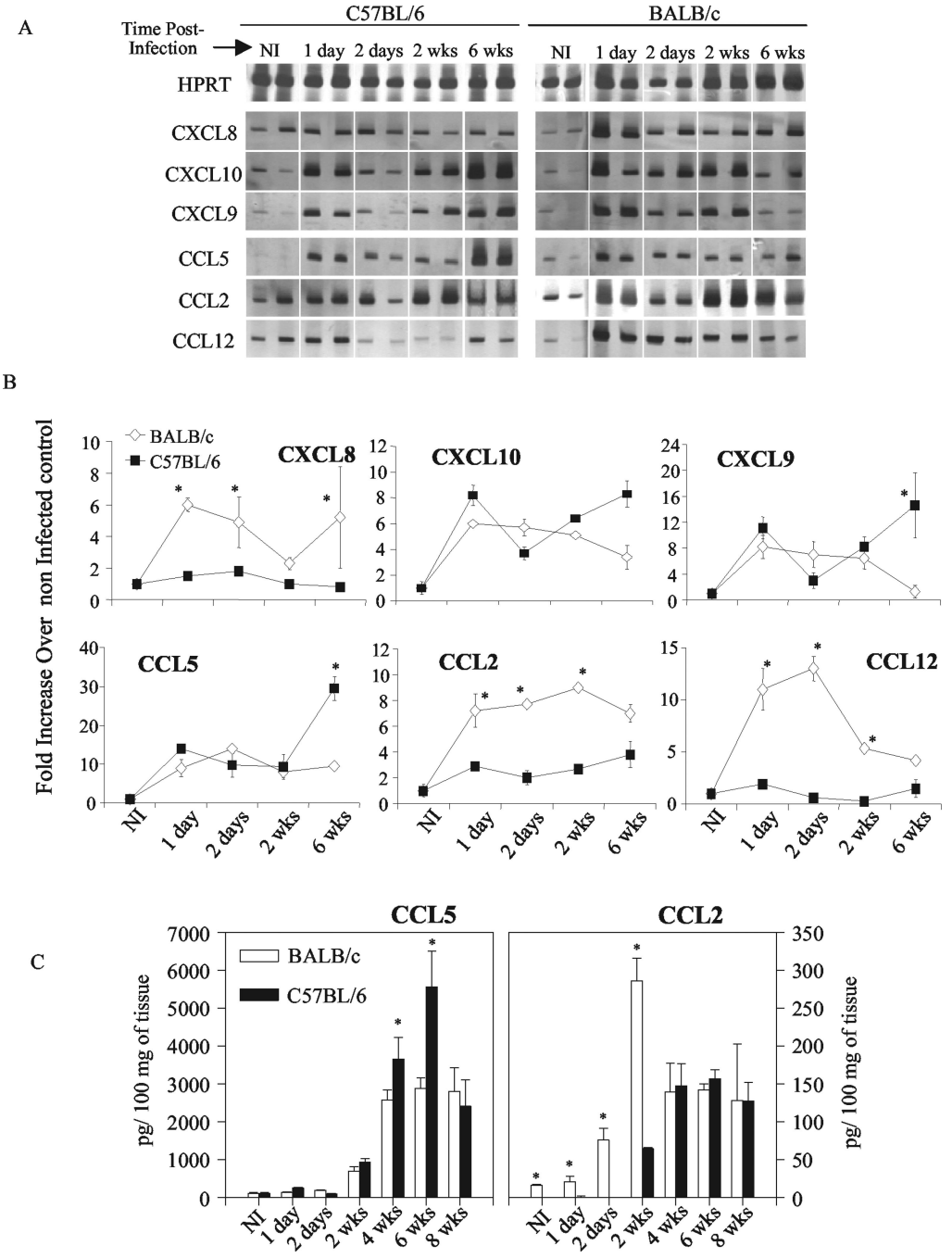

FIG. 1. Kinetics of CXC and CC chemokine mRNA expression in the footpads of C57BL/6 and BALB/c mice infected with *L. major*. (A) Mice were infected with *L. major* and sacrificed at 1 day, 2 days, 2 weeks, and 6 weeks postinfection, and the hind infected footpad was used in assays of mRNA expression by RT-PCR and ELISA. Representative gels are shown. (B) Densitometric analysis was performed, and quantification was normalized to the levels of hypoxanthine phosphoribosyltransferase (HPRT) expression. Results are expressed as *n*-fold increases over results with noninfected (NI) control animals. (C) ELISA for detection of chemokines at the site of infection was performed at 1 and 2 days and at 2, 4, 6, and 8 weeks postinfection. Briefly, footpad proteins were extracted at 50 mg of tissue/100 μl of phosphate-buffered saline to which 0.4 M NaCl, 0.05% Tween 20, and protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzethonium chloride, 10 mM EDTA, and 20 KI aprotinin A/100 ml) were added. The samples were centrifuged for 10 min at 3,000 × *g*, and the supernatant was immediately used for ELISAs. ELISA plates were coated overnight with sheep anti-mouse CCL2 (Pharmingen, San Diego, Calif.) or CCL5 (RD Systems, Minneapolis, Minn.), and ELISAs were performed as recommended by the manufacturer. The anti-CCL2 assay had a sensitivity of 16 pg/ml, and the anti-CCL5 assay had a sensitivity of 32 pg/ml. * and **, statistical differences ($P < 0.05$) between results for C57BL/6 and BALB/c for each point, determined by Student's *t* test. In panel B, each point represents the mean (±standard error [SE]) for at least three mice per time point of one experiment of three performed.

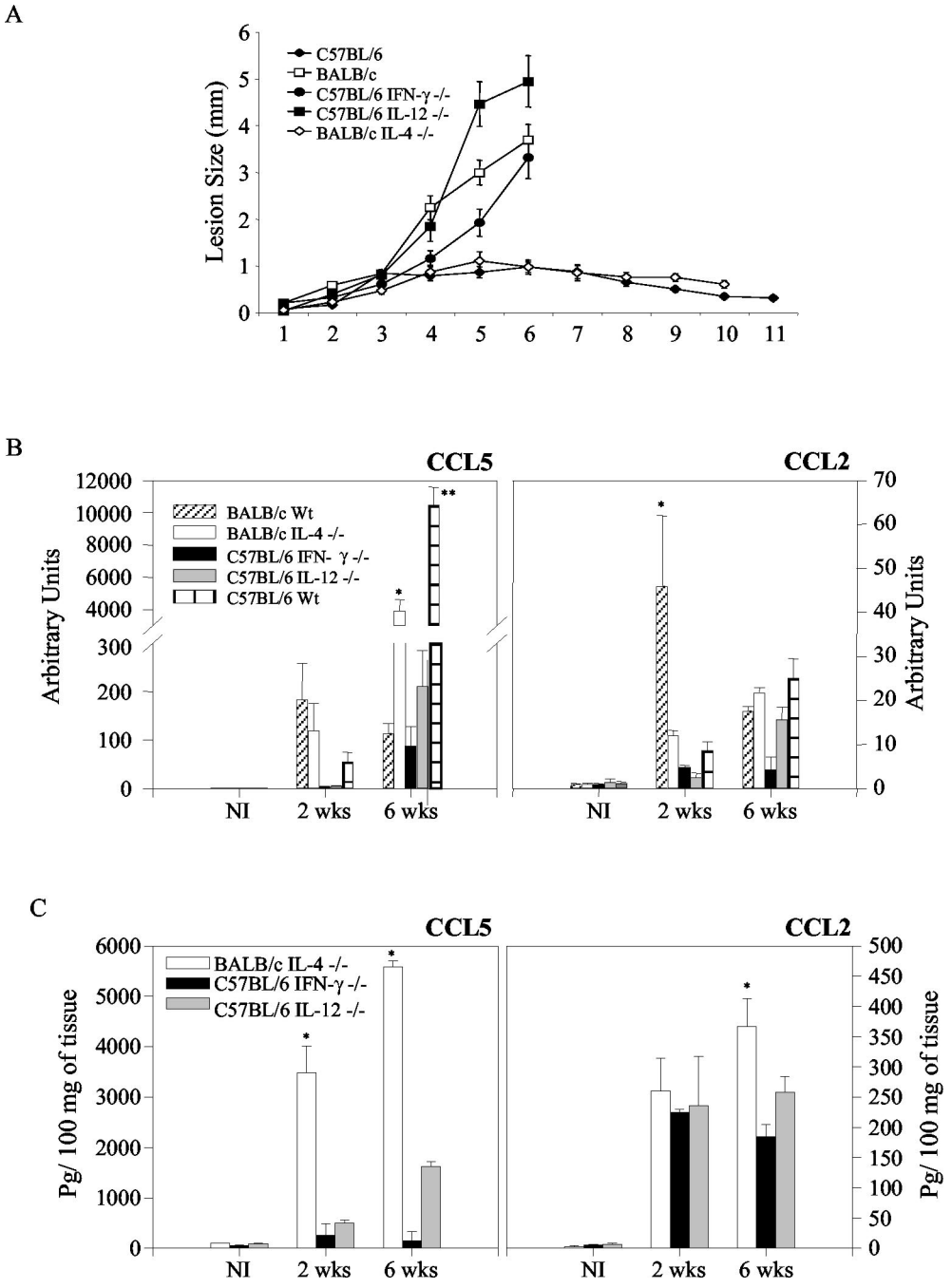

FIG. 2. Course of infection, chemokine expression, and protein production at the site of infection in IL-12, IFN-γ, and IL-4 knockout (−/−) mice and their wild-type control. (A) Mice were infected in both hind footpads with 10^6 stationary-phase *L. major* promastigotes, and lesions were measured weekly. (B) Chemokine expression was determined at 2 and 6 weeks postinfection by semiquantitative real-time RT-PCR and compared with the expression in noninfected controls (NI). Results are expressed in arbitrary units normalized to results for noninfected control animals. (C) ELISA for chemokine production was performed with lesions at 2 and 6 weeks after infection, as described in the legend to Fig. 1. Each point represents the mean (±SE) for three mice per point. Different letters indicate a P value of <0.05 between results for different knockout mice at the same time point, determined by Student's t test. Experiments were repeated, with similar results.

analysis (data not shown). All changes had disappeared by week 5 of treatment. To evaluate why the effects of Met-RANTES were transient, we investigated the presence of anti-Met-RANTES antibodies in serum. Not only were there elevated titers of antibodies in serum (Fig. 3D), but there was also an increase in the expression of CCL5 mRNA following Met-RANTES treatment (data not shown). Hence, it is possible that the effect of Met-RANTES (a competitive antagonist of CCL5 binding to CCR1 and CCR5) is transient due either (i) to the appearance of antibodies (as shown here) which recognize and, possibly, prevent the action of Met-RANTES, or (ii) to the increase in the levels of CCL5 (as suggested by the

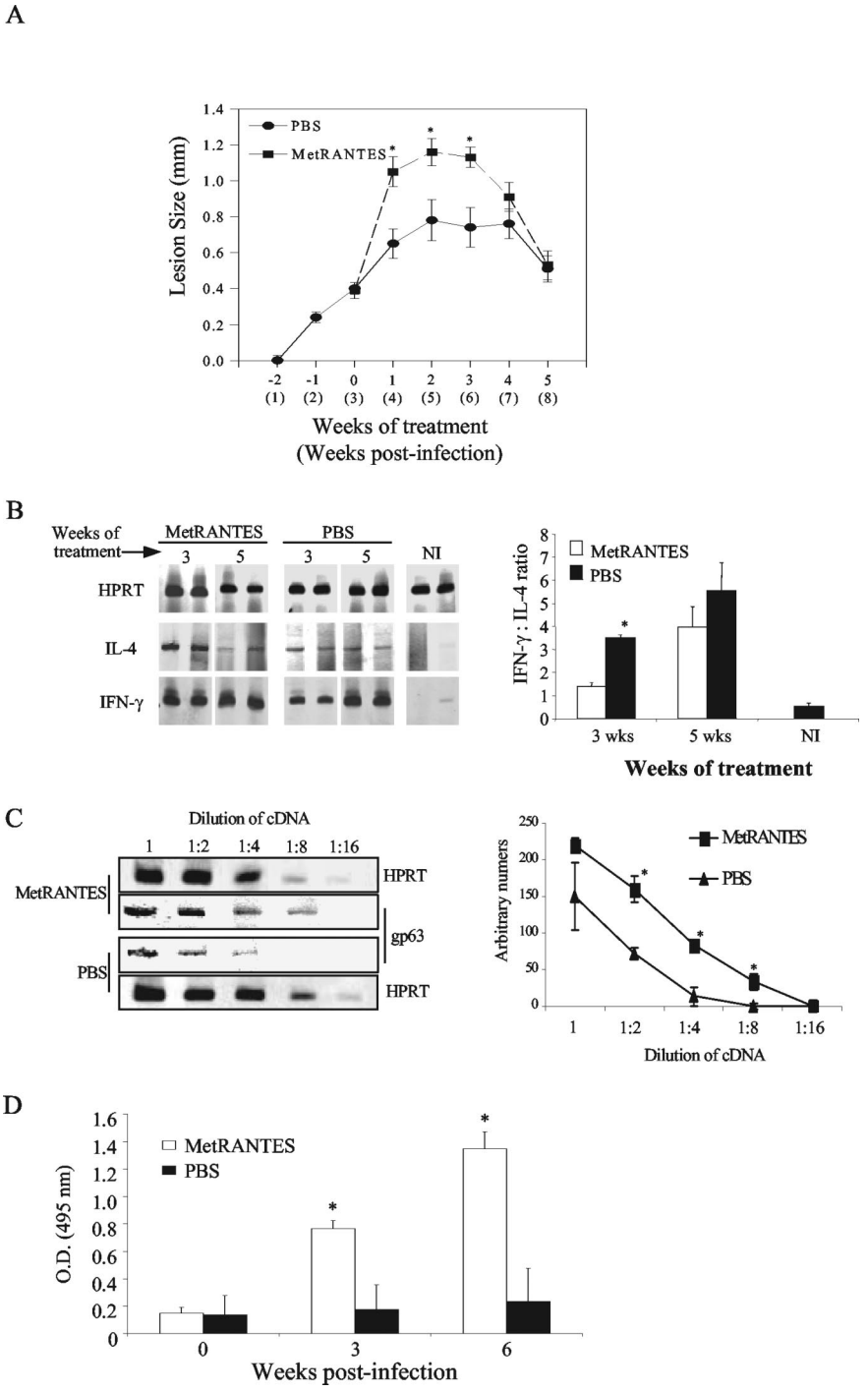

FIG. 3. (A) Effect of Met-RANTES on the course of infection by *L. major*; (B) cytokine mRNA expression by RT-PCR at the site of infection; (C) quantification of *L. major* gp63 mRNA expression at the lesion site; (D) production of anti-MetCCL5 antibody in sera from treated and nontreated animals. (A) C57BL/6 mice were infected with *L. major* in the hind footpads and treated daily from week 3 to 8 postinfection with intralesion injections of Met-RANTES (10 μg/animal) or with phosphate-buffered saline (PBS; vehicle). Lesions were measured weekly, and each point represents the mean result (±SE) for four to eight animals per time point of one experiment of two performed. (B) Animals were sacrificed at weeks 3 and 5 of treatment, and the infected footpads were used to assay mRNA expression by RT-PCR. Representative gels are shown. Densitometric analysis was performed, and the quantification was normalized to the levels of HPRT expression. To determine the Th1/Th2 balance, the densities of the bands were compared by dividing values obtained for IFN-γ by values obtained for IL-4. Each point represents means (±SE) of results for three mice per time point of one of two experiments performed. (C) Parasite load was determined by RT-PCR for *L. major* gp63 using various dilutions of the total lesion cDNA, at 3 weeks of treatment (6 weeks of infection). The densitometric analysis was performed and normalized by HPRT levels at the same dilution factor. Each point represents the mean (±SE) of results for three to four mice. (D) Anti-Met-RANTES antibodies were determined by ELISA of a 1:20 dilution of the serum at the end of the experiment (5 weeks of treatment). Bars represent means (±SE) of results for four to six mice from two different experiments for the absorbance of the serum. *, statistical differences ($P < 0.05$), determined by Student's *t* test. NI, noninfected; O.D., optical density.

TABLE 1. IFN-γ and IL-4 production by culture of lymph node cells from C57BL/6 mice infected with *L. major* and treated with Met-RANTES or not

Lymph node treatment	Time of treatment[b] (wk)	Cytokine production[a]	
		IFN-γ (ng/ml)	IL-4 (pg/ml)
PBS	3	59.9 ± 7.3	39.1 ± 3.0
	5	66.0 ± 0.9	48.0 ± 6.4
Met-RANTES	3	12.1 ± 5.3*	52.5 ± 14.6
	5	47.1 ± 4.6	50.2 ± 15.5

[a] Cytokines were measured by ELISA of culture supernatants. Data represent mean results for three mice per group (±standard error). Levels of cytokine in supernatants from unstimulated cultures were below detection limits. Represented are the data for one experiment of two performed with similar results. An asterisk indicates statistical difference between results for phosphate-buffered saline (PBS)- and Met-RANTES-treated groups at the same time point.

[b] Treatment began at the third week of infection; Met-RANTES was injected subcutaneously at 10 μg/mouse.

mRNA expression) following treatment with the drug. All results were confirmed using anti-CCL5 antiserum (gift from Nicholas Lukacs, University of Michigan Medical School, Ann Arbor), which also rendered mice more susceptible and showing larger lesions, eightfold more parasites/mg of tissue by limiting dilution, and lower IFN-γ production than mice treated with control antibody and no alteration of IL-4 production by draining lymph nodes (data not shown).

Resistance to leishmaniasis is highly dependent on a Th1 response (18). CCL5 up-regulates IL-12 (1), IFN-γ (8), and migration of Th1 cells, particularly memory T cells (24). Treatment with Met-RANTES or anti-CCL5 rendered C57BL/6 animals more susceptible to *L. major* and skewed the immune response from type 1 to type 2 by diminishing IFN-γ production by draining lymph nodes and increasing IL-4 mRNA expression in lesions. Such skewing may explain why the treatment with Met-RANTES or anti-CCL5 promoted increased susceptibility to infection. However, CCR5-deficient mice were not more susceptible to *L. major* than their wild-type counterparts (our unpublished data and reference 20), and CCR1-deficient mice were more resistant than wild-type mice (17). The explanation for such differences is not immediately apparent. Possibly, a blockade of the action of CCL5 on CCR1 and CCR5 simultaneously is necessary for an increase in susceptibility to infection. The expression of CCL2 did not correlate with resistance or susceptibility to infection. This finding is in agreement with those of other studies that found no correlation between CCL2 and susceptibility to leishmaniasis (4, 15, 16, 22, 25). These data challenge the importance of CCL2 in determining susceptibility to *L. major* in models other than that of the wild-type BALB/c mouse. It is likely that the concerted and timely actions of several chemokines and chemokine receptors are necessary to control *Leishmania* infection, and their roles are just beginning to be understood.

Our results demonstrate a correlation between CCL5 expression and resistance to infection. Altogether, these data support a role for CCL5 in the cascade of events leading to parasite control during *L. major* infection.

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