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Distinct *Leishmania braziliensis* Isolates Induce Different Paces of Chemokine Expression Patterns

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Inflammatory events during *Leishmania braziliensis* infection in mice were investigated. Large lesions were directly correlated with the inflammatory reaction but not with parasite burden. Different *L. braziliensis* strains induce different paces of chemokine expression patterns, leading to diverse cell recruitment and differential inflammatory responses.

Chemokines have been implicated in inflammatory responses against numerous infectious agents, including *Leishmania* (5, 17, 18, 20). *Leishmania braziliensis* is the main agent of cutaneous leishmaniasis (CL) in Brazil; it causes single self-limited cutaneous ulcers and highly destructive mucosal leishmaniasis (10). In this study, using a murine model, we compared *L. braziliensis* strains isolated from two states in Brazil, namely, Ceará and Bahia, located in northeastern Brazil. CL caused by *L. braziliensis* is endemic in both states. In Ceará, the cutaneous lesion is accompanied and sometimes

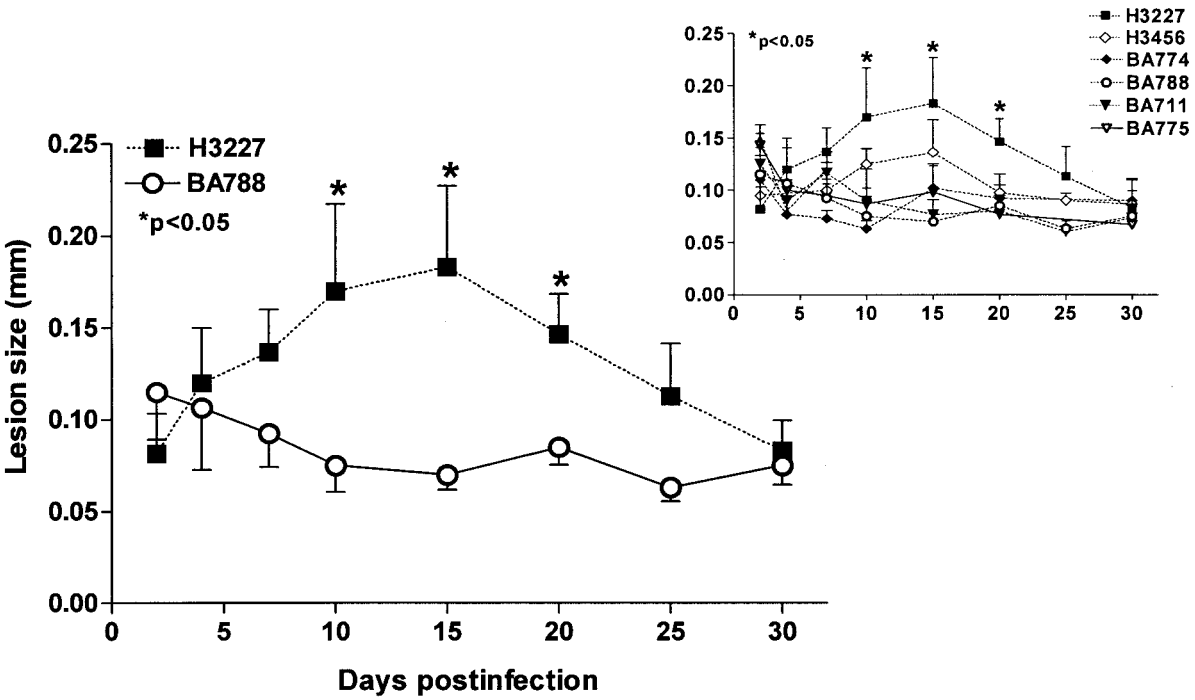

FIG. 1. Course of infection with *L. braziliensis* in BALB/c mice. The inset shows kinetics of lesion development in BALB/c mice during the course of infection with six *L. braziliensis* isolates. The main figure illustrates the time course of infection with the two isolates used here, showing a polar pattern of infection. Mice were inoculated in the hind footpads with 10^6 stationary-phase *L. braziliensis* promastigotes, and lesions were measured weekly for 30 days p.i. The footpads of three to five animals per group were measured. The data shown, reported as the mean and standard error of the mean, are from a single experiment representative of three separate experiments. The asterisk indicates a significant difference between values at the indicated time point, as determined by Student's *t* test ($P < 0.05$). Experiments with all *L. braziliensis* isolates were repeated three times, with similar results.

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preceded by an impressive enlargement of the regional lymph nodes; the term "bubonic leishmaniasis" has been coined to describe this manifestation (23). Bubonic leishmaniasis is restricted to *L. braziliensis* infection in Ceará; however, localized lymphadenopathy has been observed in CL patients from Bahia (2, 3).

Mice were infected with 10^6 stationary-phase forms of *L. braziliensis* (6). In preliminary experiments, the isolates obtained from CL patients from Ceará (MHOM/BR/94/H3227 [H3227]and MHOM/BR/94/H3456) and from Bahia (MHOM/BR/00/BA711, MHOM/BR/00/BA774, MHOM/BR/00/BA775, and MHOM/BR/01/BA788 [BA788]) showed significant differences in pathogenicity (Fig. 1, inset). Further experiments were performed with two of these *L. braziliensis* isolates, H3227 and BA788. The lesions caused by H3227 were larger and persisted longer than those caused by BA788 (Fig. 1). Lesion size differences did not appear to be due to diverse parasite loads, since parasite numbers were not significantly different between H3227- and BA788-infected mice at 15 days postinfection (p.i.) (mean and standard error of the mean, $7.29 \times 10^5 \pm 5.28 \times 10^5$ and $2.64 \times 10^5 \pm 1.20 \times 10^5$, respectively), when lesion sizes were different. Lesions from H3227-infected mice exhibited an inflammatory infiltrate consisting mainly of polymorphonuclear leukocytes and macrophages at 3 days p.i., and these histopathological features persisted at 15 days p.i. Sections from BA788-infected mice showed a less intense and more transient leukocyte infiltrate.

In order to explore the role of the parasite in the histopathological differences observed, we evaluated cell recruitment induced by H3227 and BA788 by using the air pouch model (14, 15). Responses induced by H3227 were three times higher than those induced by BA788 (Fig. 2A); these responses were correlated with a more intense exudate of leukocytes observed in the lesions of H3227-infected mice. H3227 was able to induce more influx of all cell types, attracting mainly more neutrophils and macrophages than BA788 (Fig. 2B). These data reinforce a role of the parasite in the differences observed in the inflammatory processes induced by the two *L. braziliensis* isolates used here.

RNA was extracted from lesions for reverse transcription-PCR analysis of chemokine expression at 6 h, 3 days, and 15 days p.i (12, 16). The sequences of the primers used are shown in Table 1. The expression of CCL2/MCP-1, CCL3/MIP-1α, and CXCL1/KC was upregulated at 6 h p.i. on H3227-induced lesions and only at 3 days p.i. in BA788-infected mice (Fig. 3A). In addition, CCL2/MCP-1, CCL3/MIP-1α, XCL1/lymphotactin-1, CXCL1/KC, and CCL11/eotaxin expression was more strongly induced by H3227 than by BA788. CXCL10/IP-10 was the only chemokine that appeared to be more strongly expressed by BA788 than by H3227. Regarding chemokine receptor expression, H3227 showed significantly higher expression of all chemokine receptors studied here than did BA788 (Fig. 3B). CCR5 was slightly upregulated in BA788-infected mice. Immunohistochemical analysis for the presence of CCL2/MCP-1 and CXCL10/IP-10 proteins in lesions induced by H3227 and BA788 confirmed the results obtained by mRNA expression analysis (Fig. 3C).

Lesions from patients with CL show a significant increase in the expression of CCL2/MCP-1 and CCL3/MIP-1α (20), and in vitro infection with *Leishmania* induces CCL2/MCP-1 and

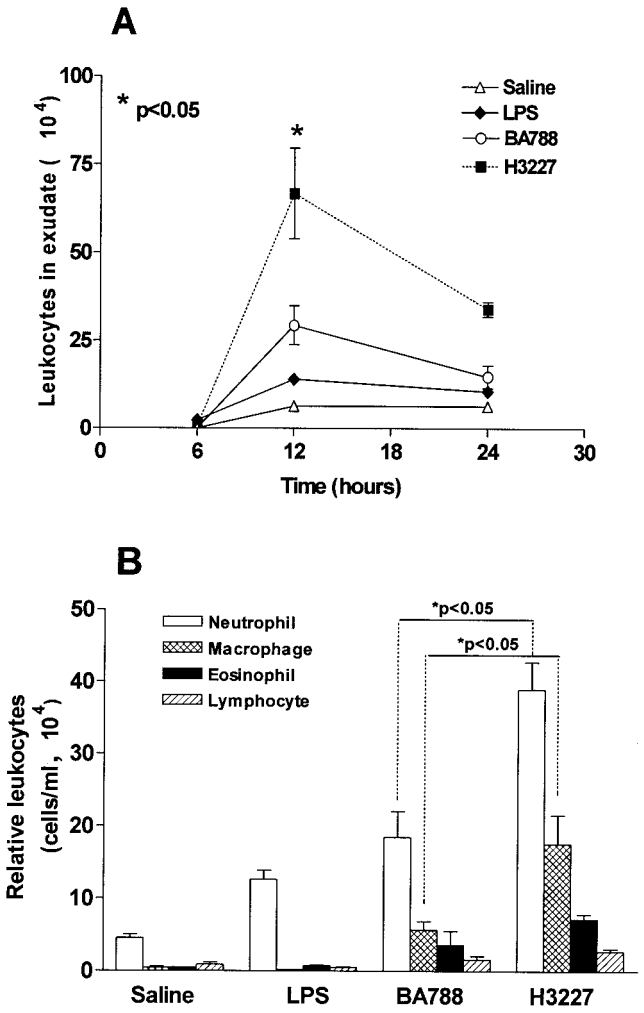
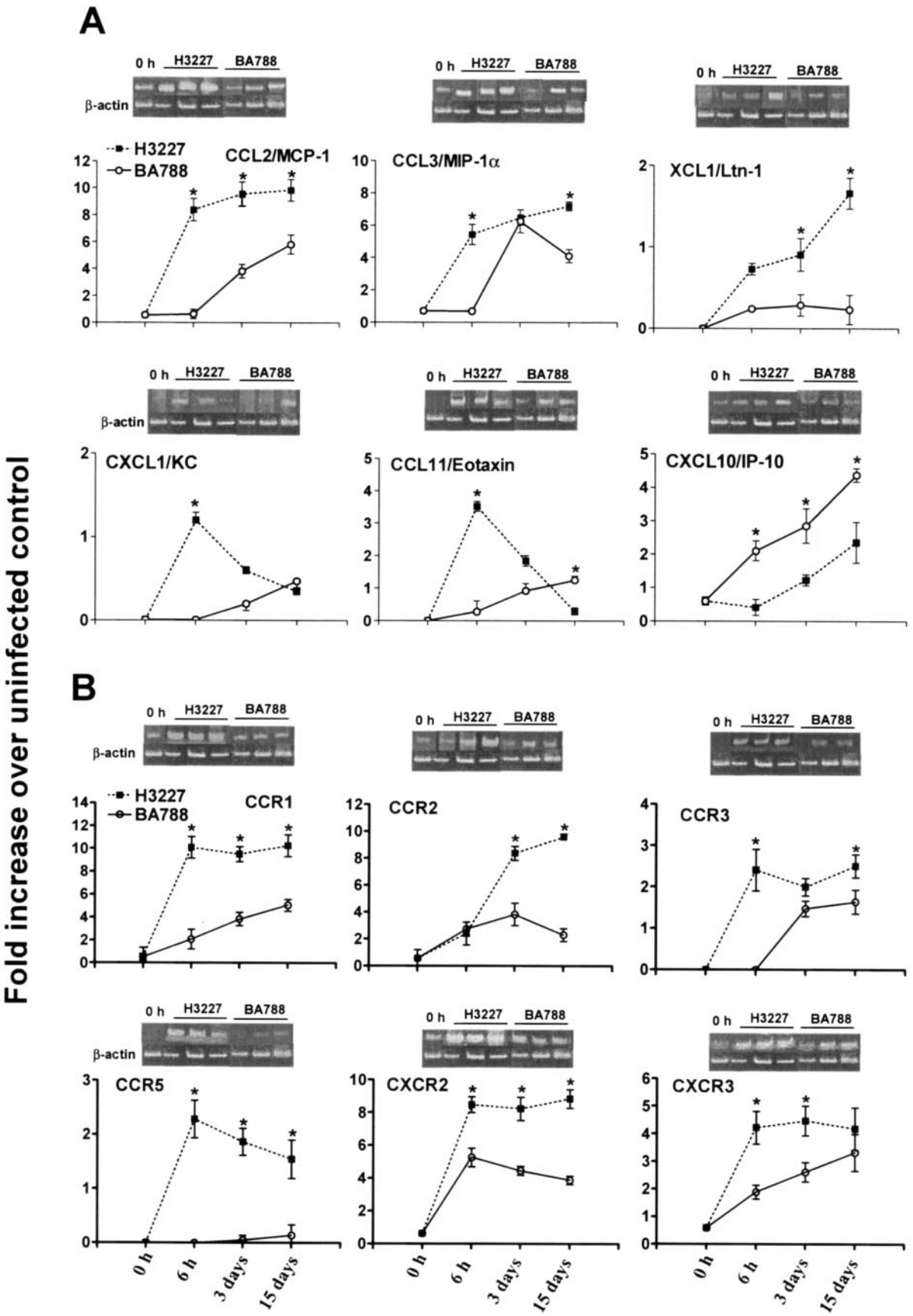


FIG. 2. Kinetics of leukocyte recruitment (A), expressed as total numbers of neutrophils, macrophages, eosinophils, and lymphocytes, in pouch exudates in response to lipopolysaccharide (LPS) or *L. braziliensis* (BA788 and H3227) and comparison of results at 12 h after inoculation (B). Air pouches were prepared by injecting 3 ml of air into the dorsal surface of mice under light anesthesia. Stationary-phase *L. braziliensis* promastigotes (10^7) were injected immediately following the air injection. Control mice were injected with endotoxin-free saline (negative control) and LPS (20 μg/ml; positive control). Mice were killed at the indicated time points, and the pouch contents were washed several time with saline. Exudate cells were centrifuged and stained; proportions of neutrophils, macrophages, eosinophils, and lymphocytes/200 cells were enumerated; and relative cell numbers were calculated from the total number of exudate leukocytes. Data represent the mean and standard error of the mean for three to five mice. The asterisk indicates a significant difference between values at the indicated time point, as determined by Student's *t* test or one-way analysis of variance ($P < 0.05$). Results are representative of two independent experiments.

CXCL1/KC/GRO-α expression in mouse and human macrophages (1, 19). CCL2/MCP-1 and CCL3/MIP-1α are potent chemoattractants for monocytes (9, 13). CXCL1/KC recruits neutrophils and is a dominant chemokine in murine inflammatory responses (4). The earlier expression of CCL2/MCP-1, CCL3/MIP-1α, and CXCL1/KC in more severe lesions may explain the significant and early accumulation of neutrophils and macrophages at the H3227 infection site and suggests that these chemokines can be factors regulating the differential


A
0 h H3227 BA788
β-actin
H3227
BA788
CCL2/MCP-1
CCL3/MIP-1α
XCL1/Ltn-1
CXCL1/KC
CCL11/Eotaxin
CXCL10/IP-10
B
CCR1
CCR2
CCR3
CCR5
CXCR2
CXCR3
Fold increase over uninfected control
0 h
6 h
3 days
15 days

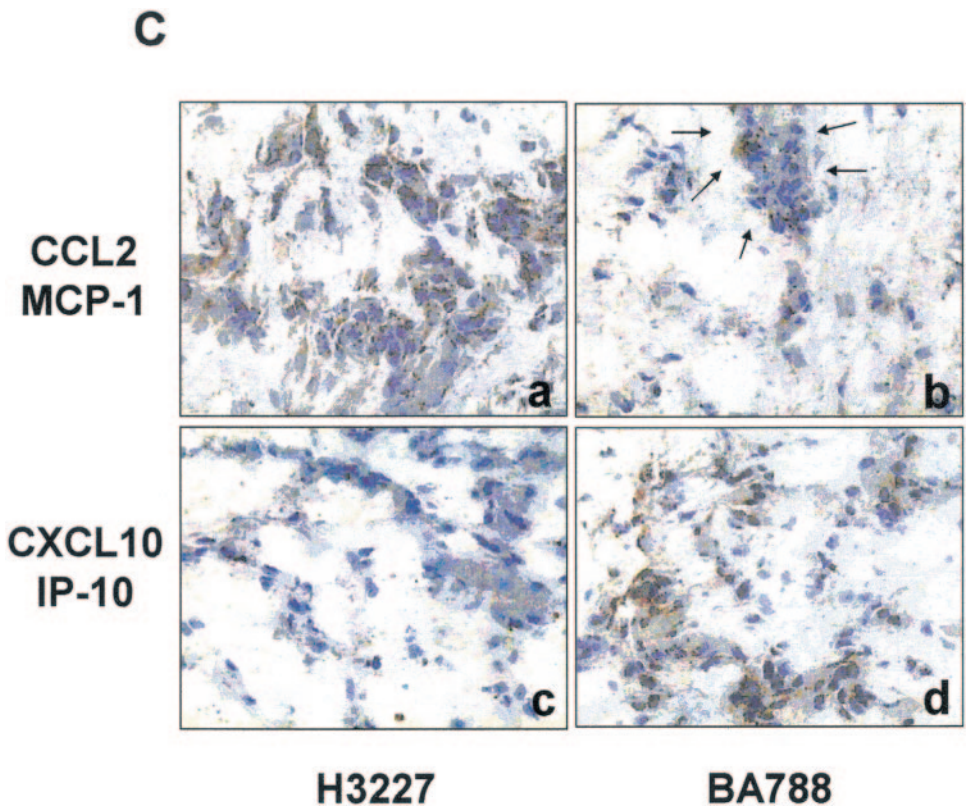


FIG. 3. Chemokine (A) and chemokine receptor (B) mRNA expression and protein production, determined by immunohistochemical analysis (C), in lesions of *L. braziliensis*-infected BALB/c mice. (A and B) Mice were infected with 10^6 H3227 or BA788 promastigotes and killed at 6 h, 3 days, and 15 days p.i. The infected hind footpads were used in assays of mRNA expression by reverse transcription-PCR. Densitometric analysis was performed, and quantification was normalized to the levels of β-actin expression. Results are expressed as *n*-fold increases over results obtained with uninfected control animals (0 h). Upper and lower rows in the gels show the expression of chemokines and β-actin, respectively, at 0 h, 6 h, 3 days, and 15 days p.i. (lanes from left to right). The profiles are representative of at least three independent experiments. In each experiment, mRNA was prepared from pools of three or four mice per time point. Each point in the graphs represents the mean and standard error of the mean for a pool of three or four mice per time point in three experiments. CCL5/RANTES, CXCL9/MIG, and CCL22/MDC expression did not show significant modulation in this model of *L. braziliensis* infection (data not shown). (C) Frozen 5-μm sections of infected and uninfected foot tissues were used to perform immunohistochemical analysis for chemokines. Immunoperoxidase staining clearly showed at 3 days p.i. strong expression of CCL2/MCP-1 in H3227-infected sections (a) and weak expression in BA788-infected sections (b). Strong anti-CXCL10/IP-10 immunoreactivity was seen in sections of BA788-infected mice (d) but not in sections of H3227-infected mice (c). Magnification, ×34.

inflammatory responses which develop upon infection with the two *L. braziliensis* isolates used here.

H3227 induced XCL1/lymphotactin-1 and, to a lesser extent, CXCL10/IP-10. XCL1/lymphotactin-1 is chemotactic for NK, $CD4^+$, and $CD8^+$ T cells in vitro and in vivo (8, 11), and CXCL10/IP-10 activates NK cells in vivo (25). Furthermore, XCL1/lymphotactin-1, CCL3/MIP-1α, CCL4/MIP-1β, and CCL5/RANTES are associated with a Th1 immune re-

TABLE 1. Primer sequences and sizes of PCR products

Oligonucleotide	Sense primer (5′-3′)	Antisense primer (3′-5′)	Product size (bp)
β-Actin	TGG AAT CCT GTG GCA TCC ATG AAA C	TAA AAA GCA GCT CAG TAA CAG TCC G	349
CXCL1/KC/GRO-α	CC TTG ACC CTG AAG CTC CCT TGG TTC	CGT GCG TGT TGA CCA TAC AAT ATG	422
CXCL9/MIG	GAT CAA ACC TGC CTA GAT CC	GGC TGT GTA GAA CAC AGA GT	399
CXCL10/IP-10	TCG CAC CTC CAC ATA GCT TAC AG	TCA GCA GAG ATG TCT GAA TC	310
CCL11/eotaxin	AGT CCT TGG GCG ACT GGT GC	GCA GAG CTC CAC AGC GCT TC	243
CCL2/MCP-1/JE	CTA AGG ACC ACT TGC CAT GGA	CTG GTA GCT CTC TGC CCT GTT T	445
CCL3/MIP-1α	C CGG AAG ATT CCA CGC CAA TTC	T GAG GAA CGT GTC CTG AAG	427
CCL5/RANTES	C CCA CGT CAA GCA GTA TTT C	CTG GTT TCT TGG GTT TGC TGT G	506
CCL22/MDC	GTG GCT CTC GTC CTT CTT GC	GGA CAG TTT ATG GAG TAG CTT	249
XCL1/lymphotactin-1	CAA GAC CTC AGC CAT GAG AC	TGC AAT GGG TTT GGG AAC TG	397
CCR1	TCT CTG ATC TGG TCT TCC TTT T	CCC AGG TGA TAA TAC TGG TGA T	295
CCR2	CTA CGA TGA TGG TGA GCC TTG T	ACC AAT GTG ATA GAG CCC TGT G	368
CCR3	CAA CTT GGC ATT TTC TGA CCT G	TTT CCA GCT GTC TTC TTC ACC T	334
CCR5	CTC TTC CTG CTC ACA CTA CCA T	TGT GTA GAA AAT GAG GAC TGC A	322
CXCR2	GAG AAC CTG GAA ATC AAC AGT T	GTA CTT GTG GCA TGT ACA ATG G	339
CXCR3	ATC TAC CTA TCA GCC AAC TAC G	ACA TCC ACA TTT GCT CTC TGA A	433

sponse (7, 22). Interestingly, CXCL10/IP-10 was the only chemokine that was more strongly expressed in lesions induced by the less pathogenic strain BA788, and its expression was correlated with the earlier production of gamma interferon in the draining lymph nodes and with the larger number of NK cells in the lesions of BA788-infected mice (6). NK cells produce gamma interferon and may contribute to resistance to *L. braziliensis*, as previously shown for *L. major* (21). Therefore, it is possible that XCL1/lymphotactin-1 and CXCL10/IP-10 are involved in resistance to *L. braziliensis* infection in BALB/c mice. Lesions caused by *L. braziliensis* H3227 exhibited a higher level of chemokine receptor expression than did those caused by *L. braziliensis* BA788. BA788 was unable to promote the strong expression of chemokine receptors, a result which was correlated with its reduced capacity to induce leukocyte recruitment. CCR5 was slightly upregulated in BA788-infected mice at 3 days p.i. and was stimulated by CCL3/MIP-1α, which was expressed during the same time period. A low level of expression of CCR5 in lesions was correlated with a lower level of expression of IL-10 in the draining lymph nodes (6), as IL-10 selectively upregulates CCR5 expression in monocytes (24).

Studies with murine macrophages showed that chemokine induction after *Leishmania* infection was dependent on the parasite strain used. Indeed, CCL2/MCP-1 was predominantly induced by avirulent *L. major*. In contrast, virulent parasites induced considerably less CCL2/MCP-1 (19). Therefore, it appears that *Leishmania* virulence is linked to the modulation of chemokine expression by macrophages. The kinetics of chemokine induction seem to be more important than parasite multiplication, and this fact may be related to structural differences between the two isolates used here. Of note, results from an analysis by random amplification of polymorphic DNA showed that strains H3227 and BA788 of *L. braziliensis* are genetically diverse (6).

Collectively, the findings presented here indicate that two *L. braziliensis* isolates, albeit at similar parasite burdens, induced chemokine expression patterns at different paces and/or intensities, leading to diverse cell recruitment and differential inflammatory responses; these features might ultimately be implicated in disease presentations.

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