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Up-Regulation of T Helper 2 and Down-Regulation of T Helper 1 Cytokines During Murine Retrovirus-Induced Immunodeficiency Syndrome Enhances Susceptibility of a Resistant Mouse Strain to *Leishmania amazonensis*

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Resistance to and recovery from leishmania infection is dependent on cell-mediated immunity. C57BL/6 mice are resistant to* Leishmania amazonensis *(La) infection but susceptible to LP-BM5 murine leukemia virus (MuLV) infection. MuLV infection leads to a state of immunodeficiency characterized by severe compromise of cell-mediated immunity. When infected with La alone, C57BL/6 mice developed a small transient lesion that evolved to spontaneous healing or a lesion with extremely slow growth. Lesions were predominantly comprised of a lympho-macrophagic infiltrate with few parasitized macrophages. When infected with La and, 4 weeks later, with MuLV (La-MuLV), the mice developed a large uncontrolled nonhealing lesion containing vacuolated and heavily parasitized macrophages. In contrast, mice infected with MuLV first and La 4 weeks later (MuLV-La) developed a small but persistent lesion, characterized histologically by a small number of heavily parasitized macrophages and few lymphocytes. Eight weeks after MuLV infection, both had similar immunological profiles with decreased lymphocyte proliferation, diminished production of interferon-γ, and high production of interleukins 4 and 10. At the time of* L. amazonensis *infection, La-MuLV animals have a normal T cell function whereas in MuLV-La mice this function is already impaired; this may influence the recruitment of macrophages to the site of leishmania injection. (Am J Pathol 1995, 146:635–642)

Leishmaniasis is a parasitic disease that affects 12 million people worldwide, and 80 million people are at risk for disease. Host protective mechanisms in leishmaniasis are largely dependent on cell-mediated immunity (CMI); lymphocytes are important in the resistance to and recovery from infection and most likely act through macrophage activation. Leishmania probably persists in a quiescent state inside the host after clinical cure, as suggested by the occurrence of relapses in patients submitted to immunosuppressive therapy years after clinical cure. In the endemic areas of visceral leishmaniasis the number of subclinical or inapparent infections outnumber the cases of clinical disease, and these individuals have the potential for harboring the parasites for many years. Several cases of concomitant infections by leishmania and human immunodeficiency virus have been reported.[1–7] Relapses of leishmaniasis due to acquired immune deficiency syndrome (AIDS) stresses the potential for a major increase in leishmania infection due to the decreased resistance in new cases and reactivation of quiescent infections in patients. An understanding of how the virus-induced immunosuppression affects the course of leishmaniasis is very important.

Infection of several strains of mice with the LP-BM5 murine leukemia virus (MuLV) induces murine AIDS (MAIDS), which has similarities with human AIDS.[8–18]

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Immunologically, MAIDS is characterized by a severe immunodeficiency with an early functional defect in $CD4^+$ T cells as evidenced by defective production of interleukin (IL)-2 and deficient lymphocyte proliferation to mitogens and soluble antigens.[8,10,17] At later stages there is a defect in the generation of $CD8^+$ T cell activity.[10] Other immunological abnormalities include hypergammaglobulinemia due to polyclonal B cell activation.[8,9,13,18] Susceptible mice develop splenomegaly, lymphadenopathy, and B cell lymphomas.[11] Due to these aspects, MAIDS represents a suitable animal model for investigating the association of immunodeficiency with parasitic diseases and helps in the understanding of the basic mechanisms of such diseases.

Materials and Methods

Experimental Animals

Five- to six-week-old C57BL/6 females were obtained from Jackson Laboratories (Bar Harbor, ME).

Viruses

LP-BM5 MuLVs contain a mixture of replication competent, B-tropic, ecotropic, and mink cell focus viruses and an etiologic replication defective virus.[8,11,13–16,19–21] The virus pools were obtained as cellfree supernatants of chronically infected SC-1 cells (a gift from Herbert C. Morse III, National Institutes of Health, Bethesda, MD) and assayed for their ability to induce disease in C57BL/6 mice as described.[11] Mice were inoculated intraperitoneally with 1.0 ml of an LP-BM5 MuLV virus pool. This inoculum induced MAIDS in 100% of C57BL/6 mice. Development of viral infection was evaluated on the basis of lymphadenopathy (+, visible enlargement of one lymph node; ++, visible enlargement of a lymph node chain; +++, visible enlargement of lymph nodes in more than one body region; ++++, visible and severe enlargement of several peripheral and mediastinal or abdominal lymph nodes) and histological parameters (+, slight enlargement of periarteriolar lymphocyte sheets (PALS); ++, moderate enlargement of PALS with compression of the red pulp; +++, enlargement of PALS and follicles leading to obliteration of the red pulp; ++++, extensive enlargement of PALS with substitution of the small lymphocytes by blast cells and obliteration of the red pulp).

Parasite, Infection, and Antigen Preparation

The BA-32 strain of *Leishmania amazonensis* was used for infection. Details of the isolation, maintenance, and course of disease in mice have been reported elsewhere.[22] Mice were infected subcutaneously into the hind footpad with 5×10^6 amastigotes, either before or after the LP-BM5 infection. Evolution of leishmania infection was monitored by serial measurements of footpad thickness with a dial gauge caliper (Starret Co., Athol, MA). Differences between infected and contralateral uninfected footpads are referred to as lesion size, in millimeters.

Parasite culture for antigen preparation was performed in modified liver infusion tryptose medium[23] supplemented with 10% fetal calf serum. The parasite antigen was used at 10 μg/ml and consisted of freeze-thawed promastigotes centrifuged at 10,000 × g for 30 minutes and filtered through a 0.22-μ filter.

Antibody Detection

Anti-leishmania antibody titers were detected by enzyme-linked immunosorbent assay as described.[22] The plates (Falcon, Lincoln Park, NJ) were sensitized with 1 μg/well of parasite antigen. Sera were diluted 1:20 in phosphate-buffered saline.

Measurement of Cell-Mediated Immunity

Single cell suspensions were obtained from either spleen or lymph nodes (groups of three animals) and used for proliferation and cytokine production assays. Suspensions of 10^6 cells were cultivated in RPMI 1640 (GIBCO BRL, Grand Island, NY) tissue culture medium supplemented with 5% endotoxin-free fetal calf serum (Sellect Gold, Flow Laboratories, McLean, VA), 100 U/ml penicillin, 100 mg/ml streptomycin, and 5 mmol/L 2-mercaptoethanol (all from Sigma Chemical Co., St. Louis, MO), in the presence or absence of either Con A (5 μg/ml) or leishmania antigen (10 μg/ml). The proliferative responses were performed in triplicate cultures of 200 μl with a 4×10^5 cells in round-bottomed microwell tissue culture plates maintained at 37 C in 5% CO_2 in a humidified atmosphere for 3 days for the assays with Con A and 5 days with antigen. In the last 18 hours, the cultures were incubated with 1 μC of [^{3}H]thymidine and then processed for scintillation counting. Cellfree supernatants were obtained 48 hours after stimulation with Con A (5 μg/ml) or leishmania antigen (10 μg/ml) and used for cytokine assays.

Assays for IL-4 were done with the CTLL cell line with anti-IL-2 (S4B6) antibodies diluted in the culture medium.[24,25] IFN-γ production was determined by a sandwich enzyme-linked immunosorbent assay as described.[17]

IL-10 assay was performed with the BD7.7 cell line (Th1), which was maintained by stimulating with irradiated allogeneic mouse (BALB/c) spleen cells and recombinant murine IL-2. Resting cells (2×10^4 cells/well) cultured in the absence of stimulation for 3 days with medium containing 10 ng/ml recombinant human IL-7 (Immunex Corp., Seattle, WA) were incubated for 2 days with supernatants to be tested and then pulsed overnight with [^{3}H]thymidine. IL-10 levels were calculated according to the proliferation inhibition standard curve obtained with recombinant murine IL-10 (Immunex Corp.).

Histological Evaluation

Groups of three animals were sacrificed at different intervals of MAIDS or *L. amazonensis* infection. Infected footpads were fixed in neutral 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin and examined under light microscopy.

Statistical Analysis

Arithmetic or geometric (cytokine levels and antibody titers) means and standard errors of the means were obtained. Comparisons between groups were performed with Mann-Whitney tests. Correlation between the severity of MAIDS and the size of the leishmania lesion was performed by simple regression with linear correlation. All tests were performed with the program GB-Stat version 4.0 (Dynamics Microsystems, Silver Springs, MD). Differences were considered significant if $P < 0.05$.

Results

Course of Infections

The course of *L. amazonensis* infection in C57BL/6 mice was characterized by the development of a small increase in footpad thickness 8 to 12 weeks after infection, with stabilization or slow growth of the lesion (closed circles in upper and lower panels of Figure 1). The course of leishmania infection in mice dually infected with this parasite and MuLV was dependent upon the timing of the virus infection. When *L. amazonensis* infection was initiated 4 weeks before the MuLV infection there was a profound change in the course of the leishmania infection after 6 weeks of viral infection. The leishmania lesion grew progressively and reached larger sizes than those in mice infected with *L. amazonensis* only (Figure 1, upper panel, open circles; a representative experiment of four different experiments). When MuLV infection was performed 4 weeks before *L. amazonensis* infection there was no alteration in leishmania lesion development (Figure 1, lower panel, open circles; one of four different experiments).

All animals infected with MuLV developed splenomegaly, regardless of leishmania infection. Animals at 15 weeks after MuLV infection had a mean spleen weight of 1028 ± 581 mg (mean ± SD, 6.3% of total body weight (BW)) and 1750 ± 849 mg (6.8% of BW) at 19 weeks after infection, whereas uninfected animals of similar age had a mean spleen weight of 79 ± 6.7 mg (0.38% of BW), and leishmania-infected animals had a mean spleen weight of 94 ± 17.9 mg (0.41% of BW). Dually infected animals at 15 weeks after MuLV infection had a mean spleen weight of 1270 ± 536 mg (4.7% of BW) and 2730 ± 1230 mg (9.94% of BW) after 19 weeks.

Development of MuLV infection was variable among animals in each group, with differences in time course and disease severity. When animals were infected initially with MuLV and then with *L. amazonensis*, there was an inverse correlation between MAIDS severity and development of the leishmania lesion. Figure 2 shows that animals with slight MuLV infection had larger lesions than those with severe MAIDS and this correlation was statistically significant.

Lymphocyte Proliferation

MuLV infection either before or after *L. amazonensis* infection abrogated the lymph node cell proliferative responses to both Con A and leishmania antigen as compared with responsive *L. amazonensis*-infected animals (Figure 3, upper panel).

Cytokine Production

Con A stimulation of lymph node cells from *L. amazonensis*-infected C57BL/6 mice resulted in an increase in interferon (IFN)-γ production (1.33 to 2.22 ng/ml; Figure 3, lower panel) and IL-10 (24.8 to 46.8 U/ml) but almost no modification of IL-4 (3.9 to 3.2 U/ml) levels as compared with normal animals (Figure 4). Under the same conditions, MuLV infection leads to a decrease of IFN-γ (13.3 to 2.8 ng/ml; Figure 3, lower panel) and IL-4 production (3.94 to 3.15 U/ml)

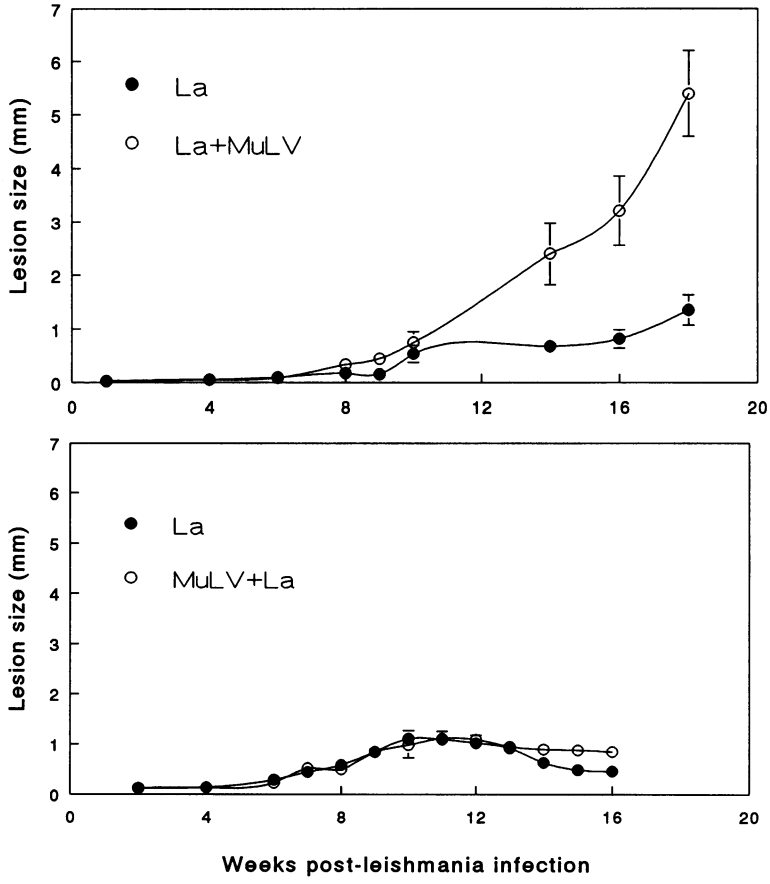


Figure 1. *Effect of MuLV infection on the course of* L. amazonensis *infection in C57BL/6 mice. MuLV infection was performed either after (upper panel) or before (lower panel) leishmania infection. Mice were inoculated with the LP-BM5 MuLV and infected with 5 × 10^6 amastigotes of* L. amazonensis *(BA-32 strain). Measurement of footpad thickness was performed at time points after leishmania infection and differences between infected and contralateral footpad thickness are represented as lesion size (mean ± SEM;* n = *5 mice per group). Differences were significant in the upper panel at 14, 16, and 18 weeks after infection. Results are obtained from a representative of four different experiments.*

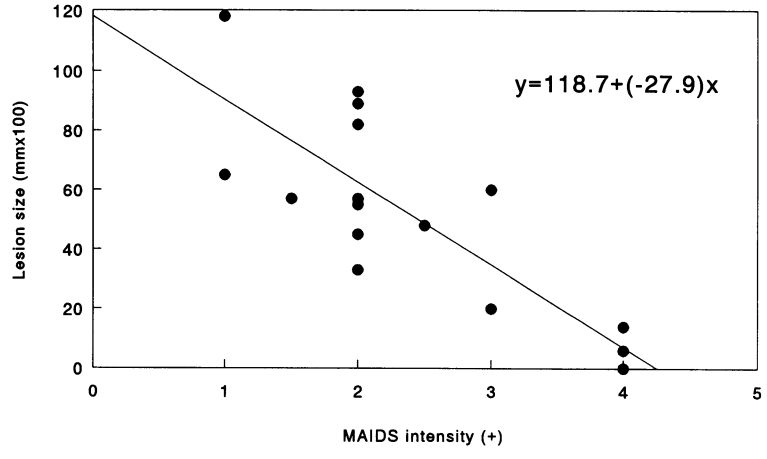
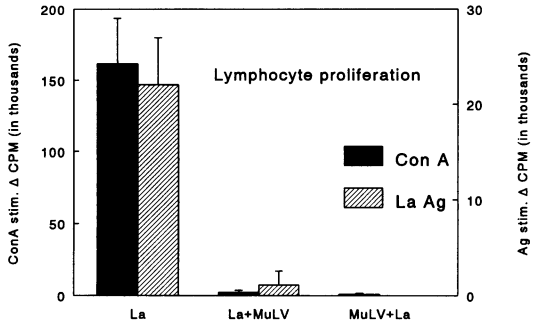


Figure 2. *Inverse correlation between severity of LP-BM5 infection and growth of* L. amazonensis *lesion in C57BL/6 animals. Mice were infected with the LP-BM5 MuLV and 4 weeks later with 5 × 10^6 amastigotes of* L. amazonensis *(BA-32 strain). Severity of LP-BM5 MuLV infection was determined by a clinical scale (see Material and Methods) and lesion size of leishmania infection was determined as in Figure 1. Development of MuLV infection was variable in the group, and animals with slight MuLV infection had larger leishmania lesions, whereas animals with severe MAIDS had smaller leishmania lesions. The inverse correlation between lesion size and MAIDS severity was statistically significant (r^2 = 0.70; correlation coefficient = −0.84; regression analysis of variance* F = *38.37;* P < *0.0001). Results are obtained from two experiments.*

and an increase of IL-10 (24.8 to 77.4 U/ml) levels (Figure 4). Dual infection of MuLV and *L. amazonensis* led to decreased Con A-stimulated IFN-γ production (Figure 3, lower panel) and increased production of IL-4 or IL-10 (Figure 4).

When stimulated by leishmania antigen, lymph node cells of *L. amazonensis*-infected animals produced IFN-γ (Figure 3, lower panel) but not IL-4 or IL-10 at significant levels above those produced by cells from uninfected animals (Figure 4). Leishmania antigen did not stimulate cells from MuLV-infected animals to produce IL-4, IL-10 (Figure 4), or IFN-γ (Figure 3, lower panel) over basal levels produced by normal animals. Concomitant leishmania and MuLV infection resulted in abrogation of leishmania antigen-induced IFN-γ production (Figure 3, lower panel) and



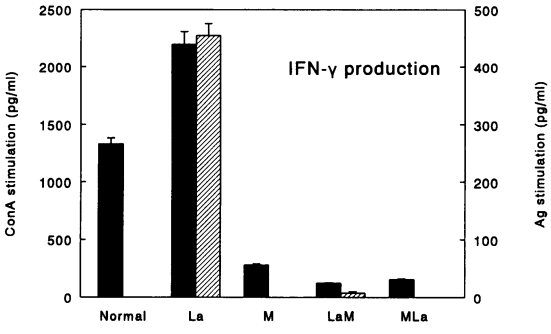
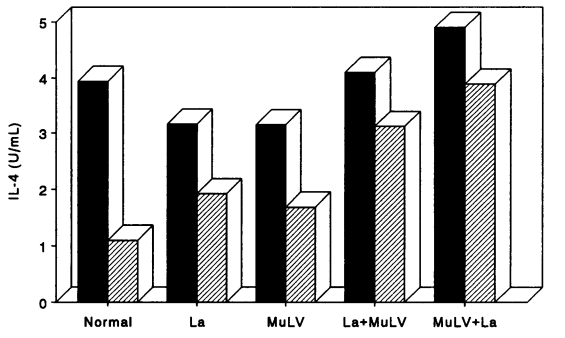


Figure 3. *Lymphocyte proliferation (upper panel) and IFN-γ production (lower panel) by spleen cells from mice* (n = 3) *infected by* L. amazonensis *(La), LP-BM5 (MuLV), or both agents, after either Con A (black bars, left* y *axis) or* L. amazonensis *antigen (hatched bars, right* y *axis) stimulation. La, mice after 8 weeks of infection by* L. amazonensis*; MuLV, mice after 8 weeks of infection by murineleukemia virus; La+MuLV, mice infected with* L. amazonensis *for 8 weeks and with MuLV for 4 weeks; MuLV+La, mice infected with MuLV for 8 weeks and with* L. amazonensis *for 4 weeks.*



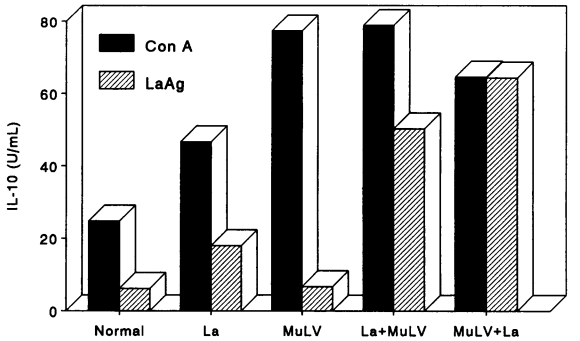


Figure 4. *IL-4 (upper panel) or IL-10 (lower panel) production by spleen cells from mice* (n = 3) *infected by* L. amazonensis *(La), LP-BM5 (MuLV), or both agents, after either Con A (black bars) or* L. amazonensis *antigen (hatched bars) stimulation. La, mice after 8 weeks of infection by* L. amazonensis*; MuLV, mice after 8 weeks of infection by murine leukemia virus; La+MuLV, mice infected with* L. amazonensis *for 8 weeks and with MuLV for 4 weeks; MuLV+La, mice infected with MuLV for 8 weeks and with* L. amazonensis *for 4 weeks.*

an increase in the levels of IL-4 and IL-10 when compared with those produced by animals infected only with *L. amazonensis* (Figure 4).

Anti-Leishmania Antibodies

MuLV infection initiated either before or after *L. amazonensis* infection lead to a decrease of anti-leishmania IgG antibodies from 1/64 or 1/320 (in two different experiments) to undetectable levels (Table I).

Table 1. *Anti-Leishmania Antibody Titers in C57BL/6 Mice Infected with* L. amazonensis, *MuLV, or Both*

Groups	Log mean titer ± SD	Geometric mean
Normal	0	0
La	1.8 ± 0.17	63.5
MuLV	0	0
MuLV+La	0	0
La	2.5 ± 0.42	320
La+MuLV	0.9 ± 0.35	8
MuLV	0	0

La, mice after 8 weeks of infection by *L. amazonensis*; MuLV, mice after 8 weeks of infection by MuLV; MuLV+La, mice infected with MuLV for 8 weeks and with *L. amazonensis* for 4 weeks; La+MuLV, mice infected with *L. amazonensis* for 8 weeks and with MuLV for 4 weeks.

Histopathology

C57BL/6 mice infected by *L. amazonensis* exhibited lesions composed of mononuclear cell infiltration and fibrosis, and the macrophages had small cytoplasm and contained scarce parasites (Figure 5A). Mice infected with *L. amazonensis* and 4 weeks later with MuLV exhibited large lesions that distorted the architecture of the skin and subcutaneous tissue and were composed mainly of vacuolated macrophages mixed with lymphocytes. These lesions resembled those observed in susceptible BALB/c mice (Figure 5B) in which a heavy parasitism of the macrophages was present. Lesions of mice infected initially with MuLV and afterwards with *L. amazonensis* had small lesions that presented as a thin layer contained inside normal

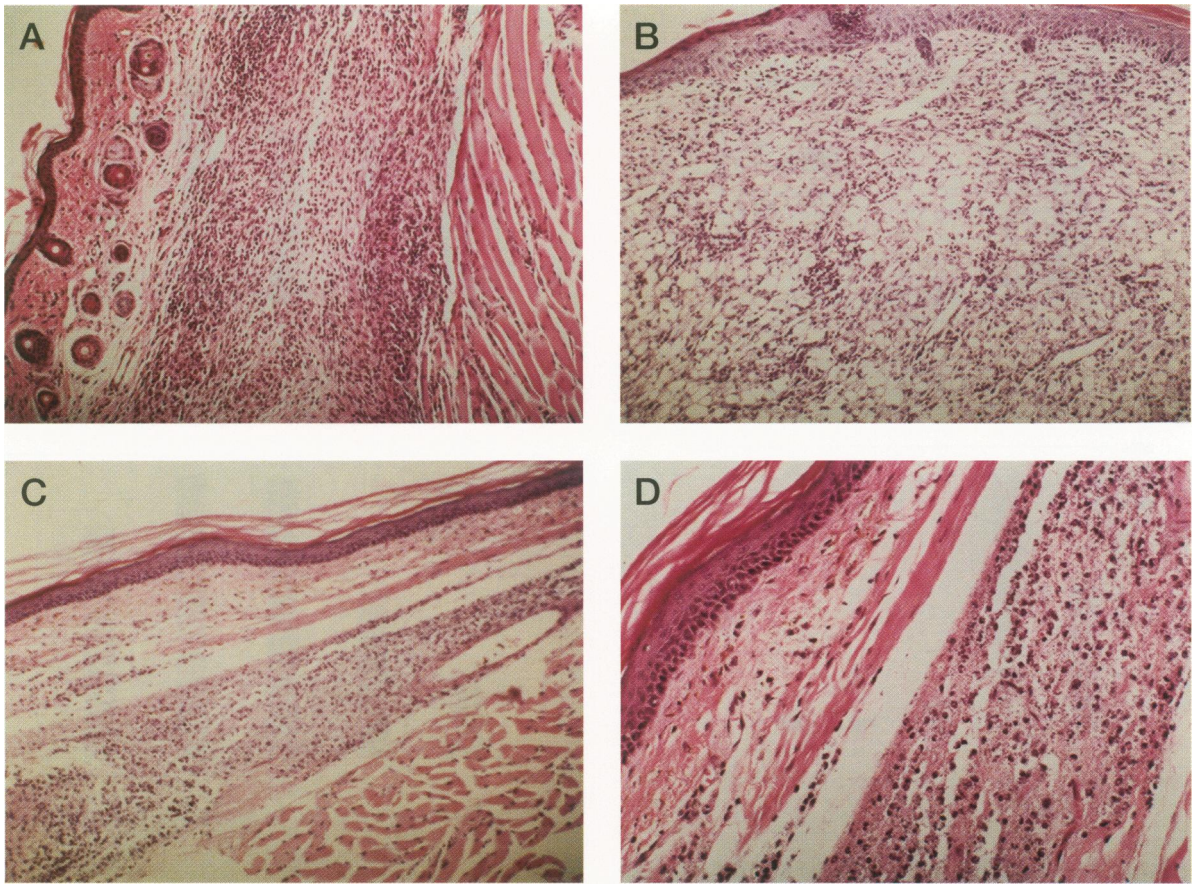


Figure 5. *Histopathological aspects of* L. amazonensis *lesions of C57BL/6 mice. Mice infected solely by* L. amazonensis (**A**) *exhibited mononuclear cell infiltration and fibrosis in the dermis, and macrophages have small cytoplasm and harbor few parasites. Lesions from mice infected with* L. amazonensis *and 4 weeks later with LP-BM5* (**B**) *had extensive lesions and distortion of the dermis architecture by a massive presence of largely vacuolated and heavily parasitized macrophages. Mice infected with LP-BM5 preceding* L. amazonensis (**C** *and* **D**) *infection exhibited well delimited lesions that were characterized by a thin layer of parasitized macrophages surrounded by normal tissue* (**C**). *Higher magnification exhibited few lymphocytes and macrophages with large vacuoles containing numerous parasites* (**D**).

appearing tissues (Figure 5C). Higher magnification revealed the presence of a monotonous macrophage infiltration containing large numbers of parasites and scarce lymphocytes (Figure 5D).

Discussion

The experiments with concomitant MuLV and leishmania interactions confirm previous observations that induction of MAIDS by LP-BM5 MuLV markedly impairs the CMI of susceptible animals[8,10,26] and compromises the resistance to pathogens.[12,27,28] Additionally, we have shown that by changing the timing between the two infections it is possible to dissociate susceptibility and disease progression in leishmania infection. MuLV infection, although always inducing susceptibility, may not lead to disease progression in *L. amazonensis* infection.

The mouse models of leishmaniasis reproduce different aspects of the human disease. When infected with *L. amazonensis*, the highly susceptible BALB/c mice develop a severe disseminated disease with a fatal course, and this severity is paralleled by a lack of CMI responses against leishmania antigen. On the other hand, C57BL/6 mice, when infected by the same parasite, develop a single ulcerated lesion that evolves to an apparent cure. Host resistance against leishmania infection depends on CMI,[29–33] and IFN-γ is considered an important element in this response.[34–39]

As MuLV infection impairs CMI and sharply decreases IFN-γ production, it is expected that this infection would enhance susceptibility to *L. amazonensis* in resistant animals. C57BL/6 infected initially with *L. amazonensis* and 4 weeks later with MuLV actually exhibited a marked increase in leishmania lesion size. Mice infected with *L. amazonensis* and later with MuLV, however, developed only a small lesion although comprised of heavily parasitized macrophages.

After 3 to 4 weeks of MuLV infection, C57BL/6 mice have evident impairment of $CD4^+$ T helper (Th)1 cell function. Production of cytokines characteristic of Th2 clones are produced and lead to a down-regulation of Th1 cytokines.[26] A clear correlation between the time course of viral disease, $CD4^+$ T cell dysfunction and the impairment of host resistance has been shown previously for *Toxoplasma gondii* acute infection.[26] On the other hand, MAIDS infection protected C57BL/6 mice against cerebral malaria by modulating the Th1-type $CD4^+$ cell-mediated pathology through the increased production of IL-10. In this case, protection against cerebral malaria increased with the duration of MAIDS infection.[40]

If Th1 impairment occurs after *L. amazonensis* infection is established, marked parasite growth in a macrophage-rich lesion occurs and leads to production of large lesions. When Th1 impairment occurs before leishmania infection, the parasitized macrophages allow significant parasite growth, but there is no increase in lesion size. Th1 cells may be elemental not only to resistance against leishmania but also to disease progression in leishmaniasis. This is reinforced by the inverse correlation exhibited between MAIDS severity and leishmania lesion development in animals infected with MuLV before *L. amazonensis* infection; animals with more severe MuLV infection and more marked compromise of $CD4^+$ cell function have smaller leishmania lesions.

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