Comparison of the A2 Gene Locus in Leishmania donovani and Leishmania major and Its Control over Cutaneous Infection*

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In Old World Leishmania infections, Leishmania donovani is responsible for fatal visceral leishmaniasis, and L. major is responsible for non-fatal cutaneous leishmaniasis in humans. The genetic differences between these species which govern the pathology or site of infection are not known. We have therefore carried out detailed analysis of the A2 loci in L. major and L. donovani because A2 is expressed in L. donovani but not L. major, and A2 is required for survival in visceral organs by L. donovani. We demonstrate that although L. major contains A2 gene regulatory sequences, the multiple repeats that exist in L. donovani A2 protein coding regions are absent in L. major, and the remaining corresponding A2 sequences appear to represent non-expressed pseudogenes. It was possible to restore amastigote-specific A2 expression to L. major, confirming that A2 regulatory sequences remain functional in L. major. Although L. major is a cutaneous parasite in rodents and humans, restoring A2 expression to L. major inhibited its ability to establish a cutaneous infection in susceptible BALB/c or resistant C57BL/6 mice, a phenotype typical of L. donovani. There was no detectable cellular immune response against L. major after cutaneous infection with A2-expressing L. major, suggesting that the lack of growth was not attributable to acquired host resistance but to an A2-mediated suppression of parasite survival in skin macrophages. These observations argue that the lack of A2 expression in L. major contributed to its divergence from L. donovani with respect to the pathology of infection.

Leishmania protozoa are the causative agents of human leishmaniasis, which is a serious infectious disease throughout the developing world (1). Leishmaniasis includes a wide range of pathologies ranging from severe lethal visceral infections to self-healing cutaneous lesions, and the specific pathologies are closely related to the species of Leishmania (2). For example, L. donovani causes visceral leishmaniasis, known as Kala Azar in India and the Sudan, which is fatal if not successfully treated and is characterized by irregular bouts of fever, substantial weight loss, swelling of the spleen and liver, and anemia (1, 2). In comparison, L. major infections, common throughout the Middle East, result in cutaneous lesions that usually heal spontaneously (1, 2). The molecular differences between the different species of Leishmania that govern the outcome of infection and pathogenesis in the human host are unknown.

Leishmania protozoa are transmitted to humans through the bite of an infected sandfly. In the sandfly vector, Leishmania exists as flagellated promastigotes, and once they enter the mammalian host after a blood meal, they differentiate into the non-flagellated amastigotes, where they multiply exclusively in the phagolysosomal compartment of macrophages (3, 4). Differentiation from promastigotes to amastigotes is a critical step for the establishment of infection in macrophages, and therefore genes that are specifically expressed in the amastigote stage are likely to be essential for survival in the mammalian host.

The A2 gene family was first identified in L. donovani by virtue of its expression, which is specific to the amastigote stage of the life cycle (5), and it has been established that the A2 protein is among the few widely accepted amastigote-specific molecular markers identified to date (reviewed in Ref. 6). The A2 protein represents a virulence factor, as L. donovani amastigotes deficient in A2 protein through the use of antisense RNA (7) or gene knock-outs (8) are attenuated with respect to survival in visceral organs of infected mice. Moreover, intravenous injection of L. major genetically engineered to express A2 produced higher infection levels in the spleen than control L. major, further supporting the argument that A2 enhances survival in resident macrophages of visceral organs (8).

Multiple copies of the A2 gene are clustered on the 850-kb chromosome of L. donovani, which encode a family of A2 proteins, each containing a secretory leader sequence followed by a 10-amino acid sequence that is repeated from 40 to >90 times, depending on the individual A2 gene (5, 9, 10). Consistent with the A2 gene family structure and expression, a family of A2 proteins ranging from 42 to 100 kDa is specifically expressed in amastigotes but not in promastigotes (7, 10). Anti-A2 antibodies have been detected in >90% of the sera samples from visceral leishmaniasis patients, confirming that the A2 proteins are expressed in the human host (11). Preliminary karyotype analysis by Southern blotting revealed that L. dono-
Leishmania species-specific genes have been identified, and it was therefore possible in the present study to define the genetic basis for the difference in phenotype associated with restored A2 expression on cutaneous infection and the host cellular immune response to A2-expressing L. major. The results of this study suggest that the inability of L. major to express A2 has contributed to its tropism for cutaneous infections.

**EXPERIMENTAL PROCEDURES**

**Mice**—BALB/c mice and C57BL/6 (B/6) were purchased from Charles River Breeding Laboratories or from the Division of Cancer Treatment, National Cancer Institute (Frederick, MD). All mice were maintained in the animal facility under pathogen-free conditions.

**Leishmania Cells and Culture Conditions**—For cutaneous infection in BALB/c mice and Northern blot analysis, L. major Friedlin V9 strain and L. donovani 1S/C2D strains were used. L. major and L. donovani promastigotes were routinely cultured at 27 °C in M199 medium (pH 7.4) supplemented with 10% heat-inactivated fetal bovine serum. The L. donovani 1S/C2D strain can replicate continuously as axenic amastigotes in vitro (12). L. donovani differentiation into amastigotes was performed by shifting the promastigotes to amastigote culture conditions overnight (55 °C in RPMI 1640, pH 5.5), which mimics the host macrophage phagolysosome compartment as described previously (12).

Likewise, L. major promastigote cultures were resuspended in the amastigote culture conditions overnight, and this resulted in viable amastigote-like cells, which were unable to proliferate.

**Plasmid Construction and Transfections**—All plasmid constructs used in this study have been described previously (7, 10). The procedure for transfection was as described (13). The transfected L. major promastigotes were selected and maintained in medium containing 50 μg/ml of G418. The pooled transfectants were used for all subsequent studies (Northern blot, Western blot, and mice infection experiments).

**Southern, Northern, and Western Blot Analysis**—Southern, Northern, and Western blot analysis were performed as described previously (7). Northern blot was used to analyze the size of genomic DNA and RNA after electrophoresis in 0.7% agarose gel. Cells were harvested, resuspended in PBS, and centrifuged to remove cell debris. Total RNA was isolated from the cells using TRIzol reagent (Gibco BRL), and DNA was isolated from the cells using the phenol-chloroform method. The DNA and RNA were fractionated and detected by hybridization with the specific probes.

**Infection of Mice**—For cutaneous infection, female BALB/c mice (Charles River Breeding Laboratories) weighing 18–20 g were infected with 1 × 10^6 cells/well. Metacyclic promastigotes from the different strains were selected, and cells were infected at a ratio of 10 parasites:1 cell. Twenty-four h later, cells were detached from the wells using a syringe pestle, washed, and dried with chlorormethylbenzoylaminotetramethyl rhodamine, Molecular Probes, Eugene, OR. Briefly, cells were resuspended in PBS and dyed for 15 min at room temperature with 5 μg/ml of the red fluorescent dye, washed three times with PBS/0.2% bovine serum albumin, cytospun, and stained with Diff-Quick (Dade Behring, Dudingen, Switzerland) to determine the percentage of infection. In every case the percentage of infected cells was >90%, and it was comparable among the groups. Cells were resuspended at a final concentration of 10^7 infected macrophages/dendritic cells/ml.

**Adoptive Transfer of Infected Cells**—C57BL/6 mice were anesthetized with 50 mg/kg of ketamine (Wyeth Fort Dodge, Iowa) solution. Infected or uninfected cells were inoculated in the ear dermis in a volume of 10 μl. 24 h later ears and draining lymph node cells from infected mice were collected. Ear dorsal and ventral sheets were separated with forceps and deposited dermal side down in RPMI 1640 supplemented with 10% fetal calf serum and antibiotics. Cells were allowed to migrate out of the ear for 16 h. The remaining ear tissue was incubated with Liberase CI, cut in small pieces, and filtered through a 70-μm nylon cell strainer (Becton-Dickinson Mountain View, CA) before being washed twice in RPMI 1640, NaHCO_3, penicillin/streptomycin/gentamicin, 10% fetal calf serum, and 0.05% DNase (Sigma). Cells from draining lymph nodes were mechanically dissociated to single-cell suspensions, and the three cell compartments (cells migrating out of the ear dermis, ear tissue, and lymph node cells) were fixed in 4% paraformaldehyde. For the analysis of the presence of labeled cells, data were collected and analyzed using CELLquest software and a FACScalibur flow cytometer (Becton Dickinson) for the presence of positive cells on the FL-2 channel. The number of cells that spontaneously migrated out of the ear was determined as the number of labeled cells tracked in the media divided by the total number of cells tracked in every compartment (ears, medium, and draining nodes).

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1 The abbreviations used are: UTR, untranslated region; IFN, interferon; IL, interleukin; ORF, open reading frame; neo, neomycin phosphotransferase.

2 For cutaneous infection in C57BL/6 mice and migration experiments, L. major clone V1 (MHOM/TL/80/Friedlin) promastigotes, L. donovani 1S strain, A2, and control cosmids L. major transfecnts were grown at 26 °C in medium 199 supplemented with 20% HI-FCE (Hyclone, Logan, UT), 100 μunits/ml of penicillin, 100 μg/ml of streptomycin, 2 μm t-glutamine, 40 mM Hepes, 0.1 mM adenine (in 50 mM Hepes), 5 μg/ml of hemin (in 50% triethanolamine), and 1 μg/ml of 6-biotin (M199/5S).

3 Infective-stage promastigotes (metacyclics) were isolated from stationary cultures (4–5 days of age) by separation in Ficoll gradient enrich-
A2 in Leishmania Pathogenesis

RESULTS

Comparison of the A2 Gene Structure in L. donovani and L. major—Few genetic differences have been described between L. donovani and L. major, and therefore a comparison of the A2 gene loci provided an opportunity to perform a detailed comparison of a virulent gene family that differs between these Old World Leishmania species. Because A2rel genes neighbor the A2 genes and are conserved between L. donovani and L. major (11), an L. donovani A2rel DNA probe was used to identify A2rel DNA-containing L. major cosmids. Three non-overlapping cosmids termed L5114, L4134, and L610 derived from chromosome 22 were isolated, and their orientation is shown in Fig. 1A. Cosmid L4134 was chosen for DNA sequencing. As also shown in Fig. 1A for comparison, the A2 gene family (white boxes) in L. donovani are clustered in tandem array alternating with the neighboring A2rel gene family (black boxes), and there is more than one cluster per chromo-
some as described previously for L. donovani (8). However, in comparison with L. major, the sequence data revealed that the overall organization of the A2 (white boxes) and A2rel (black boxes) genes differed between L. donovani and L. major. It was, however, of interest that L. major did contain A2 gene sequences and that the flanking regions (shown as A2rel 5' and A2rel 3') of the A2-A2rel gene clusters were very well conserved between L. donovani and L. major (86% identity). Moreover, as with L. donovani, there is more than one cluster of A2 gene-containing sequence per chromosome in L. major. Nevertheless, there are many more copies of the A2 gene sequences (white boxes) in L. donovani than in L. major. The most remarkable difference was that nearly the entire A2 protein coding sequences in the open reading frame (ORF) were absent in the A2 genes in L. major. As a result, the 10-amino acid encoding repeat units of the ORF that are present in all of the L. donovani A2 genes were absent in L. major. Only one 10- amino acid unit remains in the L. major A2 genes preceded by the leader sequence (Fig. 1, B and C). As a result, the predicted corresponding L. major A2 protein would only be 53 amino acids, of which the first 32 N-terminal amino acids are a functional leader sequence. Remarkably, although most of the A2 protein coding sequence was absent in the A2 genes in L. major, the 5' UTR and the 3' UTR of the RNA non-coding region were >90% identical between L. donovani and L. major. This is unusual because protein coding sequences generally remain more conserved than the non-coding sequences, but the opposite is evident when comparing L. major and L. donovani A2 genes.

Southern blot and PCR analysis of L. major genomic DNA and the other A2 cosmids derived from L. major confirmed that L. major has only the truncated version of the A2 genes shown in Fig. 1, B and C, and does not have the multiple copies of the alternating A2-A2rel genes as in L. donovani (data not shown). Taken together, these data revealed significant and unpredicted differences in the A2-A2rel gene clusters in L. major and L. donovani, where L. major has much fewer copies of the A2 genes, and more remarkably, the majority of the protein coding sequences within the remaining A2 genes are absent.

Comparison of A2 Gene Expression in L. donovani and L. major—Northern blot analysis was performed to compare the expression of the A2 gene family in L. donovani and L. major using probes specific to the conserved 3' UTR. As reported previously (5, 8), the A2 genes were abundantly expressed in L. donovani amastigotes and are expressed at much lower levels in promastigotes. As demonstrated in Fig. 2, it was not possible to detect any A2 gene transcripts in L. major by this overexposed Northern blot analysis using a conserved 3' UTR probe, demonstrating these truncated L. major A2 genes are not transcribed or their transcripts are not stable. Taken together, the truncated A2 genes in L. major are not expressed, and this together with the data presented in Fig. 1 strongly suggests that the remaining truncated A2 genes in L. major represent pseudogenes, one of the few such examples in Leishmania.

Conserved A2 3' UTR Function in L. major and the Effect of A2 on L. major Proliferation in Culture and in Macrophages—As detailed above, there are significant differences in the A2 gene structure and expression between L. donovani and L. major. However, it was also evident that the A2 3' UTR regulatory sequence was conserved in L. donovani and L. major. It has been demonstrated previously that the 3' UTR sequence was responsible for mediating amastigote-specific expression in L. donovani (9), and that it was possible to express A2 in L. major (8). It was therefore necessary to determine whether the A2 3' UTR sequence retained the ability to mediate amastigote-specific expression in L. major.
**L. major** promastigotes were transfected with the A2-expressing plasmid pKsneoA2, which contained a complete copy of the *L. donovani* A2 gene including the 5' UTR, A2 protein coding region, and the regulatory 3' UTR as shown in Fig. 3A. Promastigotes were also transfected with a control pKneo plasmid with no A2 coding sequences. Western blot analysis was carried out to determine whether A2 expression could be induced under amastigote culture conditions in the transfected *L. major* culture. As shown in Fig. 3B, it was possible to induce A2 expression by shifting the culture to amastigote-like culture conditions overnight (pH 5.5, 37 °C). As described above, the sequence of *L. major* A2 3' UTR has >90% identity to A2 3' UTR in *L. donovani*, suggesting that the *L. major* A2 3' UTR and associated regulatory factors remained functional in *L. major* with respect to mediating amastigote-specific expression.

Although it has been shown that ectopic expression of A2 enhanced the ability of *L. major* to survive in the spleen of BALB/c mice after intravenous infection (8), it was necessary to study other relevant phenotypes of A2-expressing *L. major*. It was determined whether A2 expression altered the growth properties of *L. major* in axenic culture or in *vitro* cultured macrophages. As shown in Fig. 3C, *L. major* transfected with the pKneoA2 plasmids grew equally well as the control transfected *L. major* under axenic culture conditions. Likewise, A2-expressing and the control *L. major* infected and replicated equally in bone marrow-derived macrophages after infection with metacyclic parasite cultures (Fig. 3, F and E). Taken together, these data demonstrate that it was possible to reconstitute amastigote-specific gene expression of A2 in *L. major*, and this did not alter the ability to replicate in axenic culture or replicate in *vitro* cultured macrophages.

**Subcutaneous Infection of A2-expressing L. major in Genetically Susceptible BALB/c Mice—** *L. major* infections cause cutaneous pathology, and the preceding gene structure and experimental data revealed that *L. major* lacks the ability to express the A2 gene. This therefore provided an excellent opportunity to determine whether ectopic A2 expression would influence the development of cutaneous leishmaniasis by *L. major* in relevant murine infection models. The A2-transfected and control *L. major* promastigotes described above were initially introduced subcutaneously into the footpad of BALB/c mice, and the infection was monitored by measuring the foot-pad thickness for several weeks. Up to the first 8 weeks, the A2-expressing *L. major* did induce swelling of the footpad at levels equal to the control infected mice (Fig. 4A). However, after this initial period, the footpad swelling in the A2 group was significantly reduced in size. Moreover, footpad ulceration typically developed in the mice infected with the control *L. major* on average at 12 weeks after infection, but no ulceration of lesions developed in mice infected with the A2-expressing *L. major*. Isolation of parasites from the infected footpads at 10 weeks after infection revealed that the mice infected with control *L. major* contained six to eight times more parasites than did footpads infected with the A2-expressing *L. major*. These observations argued that expression of A2 protein impaired the ability of *L. major* amastigotes to establish cutaneous infections.

The preceding experiments were carried with *L. major* expressing a single copy of the A2 gene. However, there are at least 11 copies of A2 genes in *L. donovani*-encoding proteins ranging from 45 to 100 kDa, depending on the number of repeat sequences within each gene (8, 10). It was therefore necessary...
Involvement of A2 in Leishmania Pathogenesis

Specifically under amastigote-like culture conditions. This further confirmed the preceding data arguing that the A2 3′ UTR is functional in L. major with respect to mediating amastigote-specific expression. Although the A2 cosmid-containing and control L. major were both less virulent in bone marrow-derived macrophages than the above-described plasmid-transfected cultures, they did however have similar in vitro infection kinetics in macrophages and proliferated equally well in culture (data not shown). We therefore proceeded to infect BALB/c mice subcutaneously with control and A2 cosmid-transfected L. major, and the footpad thickness was followed as described above. As shown in Fig. 4C, the A2 cosmid-transfected L. major failed to induce any footpad swelling and resulted in no detectable footpad infection compared with the control cosmid containing L. major, which was fully virulent with respect to causing cutaneous leishmaniasis. The relative inhibitory effect on cutaneous infection was significantly more dramatic with the A2 cosmid-transfected L. major than with the A2 plasmid-transfected L. major, suggesting that cosmid-derived multiple copies of A2 protein impaired the development of cutaneous infection more effectively than expression of a plasmid-derived single copy of the A2 protein. It is noteworthy that we cannot rule out the possibility that other genes on this cosmid may also contribute to the phenotype. We have, however, determined that the A2rel genes are tandemly repeated with the A2 genes as outlined in Fig. 1, and are also present on this cosmid, could not enhance the A2 phenotype when cotransfecting A2 and A2rel-expressing plasmids into L. major (data not shown).

Cutaneous Infection of A2-expressing L. major in Genetically Resistant C57BL/6 Mice—The above experiments were carried out on the footpads of genetically susceptible BALB/c mice and suggested that restoring A2 expression to L. major resulted in suppression of cutaneous pathology. It was, however, necessary to further investigate this phenotype in an infection model that more closely mimics the pathogenesis of human cutaneous leishmaniasis (16). We therefore examined the effect of A2 expression on the cutaneous pathogenesis by L. major using low-dose infection with 1000 purified metacyclic L. major promastigotes after intradermal inoculation in both ears in genetically resistant C57BL/6 mice. Infection was subsequently monitored by measuring the diameter of the ear lesion over the course of the infection. As shown in Fig. 5A, ears of C57BL/6 mice infected with the control cosmid-transfected L. major developed lesions as early as 2 weeks after infection, peaked at 7 weeks after infection, and healed spontaneously 17–20 weeks after infection, this being the typical L. major infection course resistant C57BL/6 mice (16). However, consistent with what was observed in the footpad infections in susceptible BALB/c mice, the A2 cosmid-transfected L. major failed to induce any lesion in C57BL/6 mice ears. Moreover, parasite loads in ears and local draining lymph nodes were much lower in A2-expressing L. major infected mice than in control L. major-infected mice (Fig. 5, B and C). The amount of parasites (1258 parasites ± 630.9 parasites/ear, Fig. 5B) isolated from mice infected with A2-expressing L. major 6 weeks after infection was similar to that inoculated during original challenge infection, indicating the lack of growth of the A2-expressing L. major in ears of infected C57BL/6 mice. These data confirmed that A2 expression in L. major suppresses cutaneous leishmaniasis.

It was necessary to examine the immune response toward the A2-expressing L. major to determine whether the suppression of survival in the skin was attributable to an enhanced acquired immune response against A2-expressing L. major or attributable to an innate effect of A2 on L. major biology in the skin epithelium. Cells from draining lymph nodes of C57BL/6

Fig. 4. A2 expression in L. major impairs the development of cutaneous infection in the footpad of susceptible BALB/c mice. A. Footpad swelling measurements in mice infected with A2-expressing L. major and control L. major. 5 × 10⁶ stationary-phase promastigotes were subcutaneously inoculated into the hind footpads (four mice/group), and the thickness of footpads was measured weekly for 17 weeks. Data are the mean ± S.D. for the four mice in each group. This experiment was repeated four times with similar results. B. Detection of A2 protein with anti-A2 monoclonal antibody C9 in the A2 cosmid-transfected and control cosmid-transfected (CosTL) L. major cells cultured overnight at 37 °C, pH 5.5. The sizes of the expressed A2 proteins are as indicated. C. Footpad thickness of mice infected with CosA2 or CosTL-transfected L. major promastigotes. Data are the mean ± S.D. for four mice/group. This experiment was repeated three times with similar results.

to confirm the above observation using cosmid-transfected L. major capable of expressing three copies of the A2 genes. As shown in Fig. 4B, Western blot analysis of L. major transfected with the A2 locus containing cosmid revealed the presence of three A2 proteins of 44, 50, and 90 kDa, which were expressed specifically under amastigote-like culture conditions. This further confirmed the preceding data arguing that the A2 3′ UTR is functional in L. major with respect to mediating amastigote-specific expression. Although the A2 cosmid-containing and control L. major were both less virulent in bone marrow-derived macrophages than the above-described plasmid-transfected cultures, they did however have similar in vitro infection kinetics in macrophages and proliferated equally well in culture (data not shown). We therefore proceeded to infect BALB/c mice subcutaneously with control and A2 cosmid-transfected L. major, and the footpad thickness was followed as described above. As shown in Fig. 4C, the A2 cosmid-transfected L. major failed to induce any footpad swelling and resulted in no detectable footpad infection compared with the control cosmid containing L. major, which was fully virulent with respect to causing cutaneous leishmaniasis. The relative inhibitory effect on cutaneous infection was significantly more dramatic with the A2 cosmid-transfected L. major than with the A2 plasmid-transfected L. major, suggesting that cosmid-derived multiple copies of A2 protein impaired the development of cutaneous infection more effectively than expression of a plasmid-derived single copy of the A2 protein. It is noteworthy that we cannot rule out the possibility that other genes on this cosmid may also contribute to the phenotype. We have, however, determined that the A2rel genes are tandemly repeated with the A2 genes as outlined in Fig. 1, and are also present on this cosmid, could not enhance the A2 phenotype when cotransfecting A2 and A2rel-expressing plasmids into L. major (data not shown).

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It was necessary to examine the immune response toward the A2-expressing L. major to determine whether the suppression of survival in the skin was attributable to an enhanced acquired immune response against A2-expressing L. major or attributable to an innate effect of A2 on L. major biology in the skin epithelium. Cells from draining lymph nodes of C57BL/6
mice infected for 6 weeks with control or A2 L. major were pooled and incubated with uninfected or L. major-infected bone marrow-derived dendritic cells. The culture supernatants were collected after 48 h and measured for IFN-γ/H9253 and IL-10 production. As shown in Fig. 6, the control L. major infection in C57BL/6 mice induced high expression of IFN-γ/H9253 and moderate expression of IL-10, indicating a Th1-dominated immune response. However, neither IFN-γ nor IL-10 cytokines were detected in the draining lymph nodes of mice infected with A2-expressing L. major, suggesting that the host immune response was not actively involved in suppressing the infection with A2-expressing L. major. These data argue that the suppression of cutaneous infection was attributable to an innate effect of A2 on L. major survival in the skin.

Macrophages Infected with A2-expressing L. major Migrated Out of the Ear Dermis Better Than Control L. major-infected Macrophages—The inability of A2-expressing L. major to thrive in cutaneous or subcutaneous inoculation sites of mice is characteristic of L. donovani. It was therefore of interest to determine whether A2 expression in L. major induced other phenotypes characteristic of L. donovani. We have recently developed a system to compare the ability of macrophages infected with different species of Leishmania to migrate out of the dermal matrix. Murine macrophages labeled with a fluorescent dye and infected with different strains of L. major or L. donovani are injected in the ears of C57BL/6 mice, and 24 h later, ears are recovered, and the cells sedimenting from the dermis during 16 h are collected and analyzed by fluorescence-activated cell sorter. After these analyses, we found that macrophages infected with visceralizing species migrated out of the dermis in higher levels than macrophages infected with cutaneous species (data not shown). It was therefore possible to determine whether macrophage infected with A2-expressing L. major would behave like L. donovani-infected macrophages...
Involvement of A2 in Leishmania Pathogenesis

in the skin epidermis. The ability to mediate macrophage and dendritic cell migration out of the skin dermis in a higher number than cells infected with the control cosmid, labeled with a fluorescent dye and injected in the ears of C57BL/6 mice. Twenty-four h later, the ears and draining lymph nodes were recovered, and the cells sedimenting from the dermis during 16 h were collected and analyzed by fluorescence-activated cell sorter. The percentage of cells that migrated out of the ear was determined as the number of labeled cells in these migration experiments. As shown in Fig. 7A, the percentage of A2-expressing, L. major-infected macrophages migrating out of the ear dermis was greater than macrophages infected with control L. major. Similarly, dendritic cells infected with A2-expressing L. major also migrated out of the skin dermis in a higher number than cells infected with the control L. major (Fig. 7B). These results reveal that A2-expressing L. major resembled L. donovani with respect to their ability to mediate macrophage and dendritic cell migration from the skin epidermis.

DISCUSSION

A central question in the study of Leishmania is why some species cause cutaneous leishmaniasis at the site of infection, whereas others produce little or no cutaneous pathology but ultimately migrate away from the site of infection and cause fatal visceral infections. One way to address this issue is through defining genotypic differences between phenotypically distinct Leishmania species and to characterize mutants genetically engineered to express species-specific genes. Through this approach, two major observations have been made in the present study. First, a detailed analysis of the DNA sequence, gene expression, and gene regulation of the A2 locus in L. donovani and L. major demonstrate that although L. major retained partial A2 gene sequences, the characteristic A2 protein coding repeated sequences are largely absent in the L. major A2 genes and are no longer expressed. Second, it was possible to restore amastigote-specific A2 expression to L. major, and this severely limited the ability of L. major to establish cutaneous infections in resistant or susceptible mice. L. major is closely related but appears to have diverged earlier and independently from L. donovani (17, 18). Therefore these observations suggest that loss or gain of A2 expression in Old World Leishmania evolution played a significant role in determining the species-specific organ tropism in Leishmania infection.

The inability of A2-expressing L. major to induce cutaneous infection in mice was an unexpected phenotype. This is, however, similar to L. donovani, which expresses high levels of endogenous A2 in amastigotes and likewise is unable to produce cutaneous lesions or to grow well in the skin in mice or humans. We also observed that, similar to macrophages infected with L. donovani, macrophages infected with A2-expressing L. major displayed an increased ability to migrate out of the ear dermis compared with the control L. major-infected macrophages. However, A2-expressing L. major were unable to establish a long-term productive infection in the skin or the local draining lymph node. An inadequate availability of antigen is likely to account for the observation that there was no detectable cellular immune response against the A2-expressing L. major in the C57BL/6 mice, whereas a strong IFN-γ response did develop against the control L. major. Taken together, A2 appeared to mediate an innate suppression of L. major survival in the skin.

It has been shown previously that A2 expression enhanced the ability of L. major to survive in the resident macrophages of the spleen, resulting in splenomegaly in BALB/c mice after direct infection through tail vein injection (8). We have observed similar results in the liver. Consequently, A2 expression in L. major has opposing effects at different sites of infection, enhancing infection in resident macrophages of visceral organs but suppressing infection in inflammatory macrophages in the skin. A2 expression in Leishmania may therefore have diverse effects in different types of macrophages. Despite this, we were unable to detect significant differences between control and A2-expressing L. major in infection of resident peritoneal or bone marrow-derived macrophages in culture.

At the genetic level, it was interesting to discover that although L. major does not express A2 proteins, they do possess what appears to be A2 pseudogenes. The A2 genes in L. major are truncated specifically in the protein coding region and are not expressed into mRNA, yet these genes retained the flanking UTRs that were very well conserved between L. donovani and L. major. Moreover, L. major retained the ability to mediate amastigote-specific expression from the A2 3’ UTR. Therefore, the non-coding regions of the A2 genes in L. donovani and L. major are structurally and functionally more conserved than the coding regions. In evolution, coding sequences are generally better conserved than non-coding regions unless there is a selective pressure against the gene product. Although it is not possible to conclude whether L. donovani gained A2 genes or L. major lost A2 sequences during evolution, we favor the possibility that the A2 was lost from L. major because the A2 regulatory sequences and the A2 protein coding signal sequence are present in the genome of L. major.

The L. donovani type A2 protein coding regions are present in Leishmania species associated with visceral leishmaniasis.

including *L. donovani*, *L. chagasi*, and *L. infantum* but not in cutaneous leishmaniasis species such as *L. major*, *L. tropica*, and *L. braziliensis* (11), and this fits with the overall hypothesis that A2 plays a role in visceral leishmaniasis. However, the A2 coding sequences were also detected by karyotype analyses in *L. mexicana* complex including *L. amazonensis* and *L. mexicana* (11), and the A2 gene products have been detected by Western blot analysis as three larger proteins of >200 kDa in *L. mexicana*. Species from the New World *L. mexicana* complex are associated with diffuse cutaneous infections, and they are phylogenetically farther from the Old World species *L. donovani* and *L. major* (17). There may therefore be significant differences in the rest of the genome that allow *L. mexicana* to survive in the skin despite the presence of the A2 protein. Divergence in gene function between Old World and New World *Leishmania* species would not be without precedent because although lipophosphoglycan is clearly a virulence factor for *L. major* (20), it appears not to be a virulence factor for *L. mexicana* (21). Indeed, such phenotypic differences may also be related to the diversity in the host response to different species of *Leishmania* as discussed previously (22). The potential different roles of A2 proteins may also be associated with the different sizes and abundance of these proteins because A2 proteins are much less abundant in *L. mexicana* than in *L. donovani*. It is also noteworthy that there has been reports of visceral *Leishmania* infections caused by *L. tropica*, a cutaneous *Leishmania* species (23, 24). Although the host physical and immune status can also play important roles in determining the outcome of *Leishmania* infection (24), it is not known what caused the pathogenic change of these *L. tropica* parasites. These observations support the argument that A2 is not the only factor required for visceral infection; however, in the case of *L. donovani*, it does appear to be necessary for visceral infection (7, 8). As the *Leishmania* genome project progresses (25, 26), it will be of great interest to identify additional genomic differences between *Leishmania* species that may provide a better understanding of the tropism and pathogenesis of these pathogens.

REFERENCES
19. Deleted in proof