Pretreatment of *Leishmania* Homologue of Receptors for Activated C Kinase (LACK) Promotes Disease Progression Caused by *Leishmania amazonensis*

Takahiro OKUNO¹⁾, Makoto TAKEUCHI¹⁾, Yoshitsugu MATSUMOTO²⁾,
Haruki OTSUKA¹⁾, and Yasunobu MATSUMOTO¹⁾

¹⁾Laboratory of Global Animal Resource Science, and ²⁾Department of Molecular Immunology,
Graduate School of Agricultural and Life Sciences, The University of Tokyo, Yayoi
1-1-1, Bunkyo-ku, Tokyo, 113-8657, Japan

Abstract: A cDNA coding *Leishmania* homologue of receptors for activated C kinase (LACK), which was known to play an important role in the early phase of *Leishmania* infection, was molecularly cloned from *Leishmania amazonensis* promastigote by using reverse transcription and nested polymerase chain reaction, and was sequenced. The *L. amazonensis* LACK cDNA showed 97.3 to 99.3% homology and its deduced amino acid sequence showed 98.7 to 99.7% identity in comparison with LACK sequences from five other species. The amino acid sequences in the immunodominant peptide region were completely conserved among *Leishmania* spp. tested. Intravenous pretreatment of the recombinant *L. amazonensis* LACK into BALB/c mice showed progressive lesion development compared to PBS (–) injected control mice, suggesting the important role of LACK in the early phase of *L. amazonensis* infection.

Key words: *Leishmania amazonensis*, *Leishmania* homologue of receptors for activated C kinase, molecular cloning

Introduction

Leishmania is a parasitic protozoan of the family Trypanosomatidae. It has two developmentally distinct stages, the amastigote and promastigote stages. The amastigote is a non-motile form that lives within mammalian host macrophage. The promastigote is a motile, flagellated form that lives within the vector sandfly. Infection of man results in a broad spectrum of diseases ranging from self-healing cutaneous or un-

controlled destructive mucocutaneous forms to fatal visceral disease, in which clinical manifestations are dependent on both parasite strain and the immunologic status of the host [2, 13, 28, 36].

In mouse model, control of *Leishmania* parasite proliferation is T-cell dependent. Especially, CD4⁺ T-cells seem to be required to control growth of the parasite [37]. In studies on *Leishmania major* infection of several mouse strains, resistance and susceptibility have been correlated with the appearance of parasite-specific

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Address corresponding: Y. Matsumoto, Laboratory of Global Animal Resource Science, Department of Molecular Immunology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, 113-8657, Japan

T helper 1 (Th1) and T helper 2 (Th2) cells, respectively [6, 24, 26, 30, 39]. In resistant mice, the preferential induction of IL-12 by macrophage promotes the expansion and activation of Th1 cells leading to IL-12 and IFN- γ production [17, 19], which activate macrophages to produce IFN- γ and large amounts of nitric oxide that mediates the destruction of the parasite [9, 22]. In contrast, in susceptible mice, the preferential induction of IL-4, which inhibits Th1 expansion results in a decrease in the inducible nitric oxide synthesis leading to the multiplication of the parasites [9, 33, 38].

Investigations into the early events of *L. major* infection, which contribute to the development of the Th2 response in susceptible BALB/c mice, have focused on the response to a single leishmania antigen, *Leishmania* homologue of receptors for activated C kinase (LACK) [14, 16, 23], by dominant T cells expressing V α 8-V β 4 TCR [14, 12, 16, 27]. It was suggested that IL-4 production in response to LACK shifts host immune response to Th2-type thus exacerbating the disease [27].

In experimental leishmaniasis, the genus *Leishmania* includes many species, which induce different symptoms such as self-healing cutaneous and visceral leishmaniasis [4, 5, 11], and the different symptoms are influenced by the innate resistance of the host and by the strain of the parasite [25, 32]. *L. amazonensis* is one of the species, which induces severe and complex symptoms of cutaneous leishmaniasis in mouse strains [3, 7, 25]. For example, mouse strains, such as C3H/He, CBA and C57BL/6, are resistant to *L. major* infection, however, these mice are susceptible to *L. amazonensis* infection, exhibiting varied patterns of primary and metastatic lesions [3]. These results indicate that the pathogenicity of *L. amazonensis* is greatly different from that of *L. major*. However, the mechanism which determines the pathogenicity remains poorly understood [1, 20].

In this study, *L. amazonensis* LACK cDNA was cloned and recombinant LACK was produced in bacteria to analyze the *in vivo* role of LACK in *L. amazonensis* infection.

Materials and Methods

Mice: Male BALB/c mice were purchased from Japan SLC Inc. (Tokyo, Japan) and used at 7 weeks of

age.

Parasites: The promastigotes of *L. amazonensis* MPRO/BR/72/M1845, *L. major* RM2, *L. donovani* MHOM/CN/86/Sc-6 were maintained *in vitro* at 25°C in 199 medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Trace scientific LTD., Melbourne, Australia) and 25 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH7.4, ICN, Ohio, USA) and harvested at the stationary phases of growth, as defined by cell concentration and morphology.

Preparation of soluble amastigote antigen: Peritoneal macrophages of BALB/c mice were collected and were placed in 24-well plates and incubated in RPMI 1640-medium (Invitrogen Corp., California, USA) supplemented with 10% FCS at 37°C in 5% CO₂. After 24 h, macrophages were infected with parasites at a multiplicity of three parasites per cell and incubated at 34°C. After three hours, the cultures were rinsed in PBS (-) and re-incubated with fresh medium for a further 24 h. Parasite infected macrophages were washed with PBS (-) three times and collected. Cells were homogenized in microcentrifuge tubes with a homogenization pestle (Scientific Specialities, Inc., California, USA) and passed through a nylon-mesh in order to remove tissue debris. Collected cells were lysed by freezing and thawing ten times. Soluble amastigote antigen was collected by centrifugation at 15,000 r.p.m. for 30 min as the supernatant and stored at -80°C until use.

Cloning and sequencing of LACK cDNA: Total RNA was isolated from the stationary phase of promastigotes by the acid guanidium-phenol-chloroform method [8]. RT-nested PCR was performed with 5 μ g of total RNA using a RT-PCR kit (TAKARA, Tokyo, Japan) according to the manufacturer's protocols. Primers were designed according to the LACK sequence of *L. major* (GeneBank accession number U27568), and were used in PCR to amplify the entire coding region of a 36 KDa protein from *L. amazonensis* cDNA. Primer sequences used were 5'-AACTAACGCTATATAAGTATCA-3' and 5'-TCACTTCTCGCTCGTTCTC-3' for the first PCR and 5'-CAGTTTCTGTACTTTACCGATA-3' and 5'-TTCTCCCTCGCCTACCA-3' for the nested PCR. PCR conditions of each PCR were as follows: initial denaturation at 94°C for 5 min followed by 30 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 1 min, and

a final extension at 72°C for 15 min. The amplified fragment was cloned in pCR2.1 vector (TA cloning kit, Invitrogen Corp., California, USA) and sequenced by an ABI PRISM 310 Genetic Analyzer (PERKIN-ELMER Applied Biosystems, Tokyo, Japan).

Comparison of LACK sequences between *Leishmania* spp.: The nucleotide and deduced amino acid LACK sequences of *L. amazonensis* were compared with those of the five reported LACK sequences of *L. major*, *L. braziliensis*, *L. chagasi*, *L. infantum* and *L. donovani* (GeneBank accession numbers U27568, AF034805, U27569, U49695 and AB075696, respectively).

Preparation of recombinant *L. amazonensis* LACK protein: The LACK protein coding sequence was amplified by PCR using primers of 5'-GGGGGATCCGATGAACTACGAGGGTCAC-3' and 5'-CCCAAGCTTTTACTCGGCGTCGGAGAT-3'. The PCR product was cut off at the *Bam*HI and *Hind*III sites designed in the primer sequences, and was inserted into the same sites of pTrcHis B plasmid vector (Invitrogen Corp., California, USA). The constructed plasmid was transformed into *E. coli*. Top 10 strain (Invitrogen Corp., California, USA), and recombinant protein production was induced by adding Isopropyl-1-thio- β -D-galactopyranoside (IPTG) at a final concentration of 1 mM. The recombinant protein was purified by Chelating Sepharose Fast Flow (Pharmacia Biotech AB, Uppsala, Sweden) according to the manufacturer's instructions.

Preparation of mouse anti-LACK antibody: BALB/c mice were injected intraperitoneally with 50 μ g LACK recombinant protein with Freund's complete adjuvant (DIFCO LABORATORIES, Detroit, USA), followed by injection with 20 μ g LACK recombinant protein with Freund's incomplete adjuvant (DIFCO LABORATORIES) two times at weekly intervals. Anti-LACK antibody in the serum was monitored by ELISA weekly. Collected serum was stored at -20°C until use.

Western Blot Analysis: *L. amazonensis* was harvested at the stationary phase, washed with PBS (-) and centrifuged at 3200 r.p.m. for 5 min. Cells were suspended in PBS (-), lysed, and collected as soluble leishmania antigen (SLA) in the same manner as for the soluble amastigote antigen described above. SLA was also prepared from *L. major* and *L. donovani* promastigotes. All samples were electrophoresed in 12% SDS-polyacrylamide gel, and transferred to Immobilon transfer

membrane (Millipore, Massachusetts, USA) electrophoretically. The blotted membrane was incubated with the anti-LACK antiserum prepared here. Positive signals were visualized using ECL (Amersham Pharmacia Biotech UK Limited, Buckinghamshire, England).

LACK administration and *L. amazonensis* infection: Recombinant LACK protein of 25 μ g was dissolved with 50 μ l of PBS (-) and was injected into BALB/c mice intravenously. The promastigotes of *L. amazonensis* at the late log phase were harvested and washed three times and resuspended with PBS (-). Twelve hours post-injection of recombinant LACK protein, freshly prepared promastigote form parasites (1×10^3 parasites in 50 μ l of PBS (-) per mouse) were infected into the base of the tail (rump) intracutaneously. Lesion development was measured with a dial gauge caliper (KSM-15, KANON, Tokyo, Japan) once a week, and is presented as the area based on average diameter of lesion.

Nucleotide sequence accession number: The nucleotide sequence data reported in this paper will appear in the GeneBank database under the accession number AB075697.

Results

Cloning of *L. amazonensis* LACK cDNA: LACK cDNA was amplified from the total RNA of *L. amazonensis* promastigote by RT-nested PCR. The amplified DNA was cloned into pCR vector and was sequenced. The nucleotide and deduced amino acid sequences of *L. amazonensis* LACK cDNA (Fig. 1) were compared with those of five *Leishmania* species. The nucleotide sequence of *L. amazonensis* LACK in the coding region showed 97.3%, 98.9%, 98.2%, 98.7% and 98.2% homologies with those of *L. major*, *L. braziliensis*, *L. chagasi*, *L. donovani* and *L. infantum* LACK cDNA, respectively. On the amino acid basis, *L. amazonensis* LACK sequence had 98.6%, 99.6%, 99.3%, 99.3% and 99.3% identities to *L. major*, *L. braziliensis*, *L. chagasi*, *L. donovani* and *L. infantum*, respectively. Eighteen amino acid sequences of *L. amazonensis* LACK starting from 156 aa, which was estimated as the immunodominant peptide region, were identical to those of *Leishmania* spp. tested (Table 1).

Detection of LACK protein in promastigote and amastigote forms of *Leishmania* parasites: The anti-

In this study, *L. amazonensis* LACK cDNA was cloned from promastigote form parasite and sequenced.

Fig. 2. Western blot analysis of the LACK protein. Recombinant LACK (Lane 1), promastigote forms of *L. amazonensis* (lane 2), *L. major* (lane 3), *L. donovani* (lane 4) and amastigote form of *L. amazonensis* LACK (lane 5) were subjected to western blot analysis. Authentic LACK proteins in parasites tested were detected at approximately 36 KDa.

Alignment of *L. amazonensis* LACK nucleotide and amino acid sequences revealed significant homology to LACK sequences from several other species including *L. major*, *L. braziliensis*, *L. chagasi*, *L. infantum* and *L. donovani*, implying that LACK may have certain important functions for *Leishmania* parasites [10, 35]. The immunodominant peptide region was considered to be an antigenic region of LACK recognized by V α 8-V β 4 T cells [16]. Extremely high identities of this region indicated that deduced antigenic and immunogenic properties of LACK are closely related among *Leishmania* spp.

From the results of Western blot analysis, the antigenic property of recombinant LACK protein produced in *E. coli* seemed to be the same as that of authentic LACK in *L. amazonensis*. Consistent with a previous report [23], in this study, authentic LACK was detected in amastigotes as well as in promastigotes, suggesting that LACK would function as an aggravating factor in the animal host.

In the experiments using *L. major* and genetically susceptible BALB/c mice, it now appears clear that the balance between the two T-helper cells (Th1/Th2) of CD4⁺ lymphocyte subpopulations determines the final

Fig. 3. Lesion development caused by *L. amazonensis* infection with or without LACK pretreatment. Twelve hours before parasite inoculation, recombinant LACK (25 μ g) was injected intravenously into seven week-old male BALB/c mice. Control mice were injected with PBS (-) the same way. Lesion development was monitored with a dial gauge caliper every week and expressed as the area based on the average diameter of lesions. Asterisks indicate statistically significant differences in the corresponding values between LACK-pretreated mice and PBS (-)-pretreated control ($p < 0.05$).

outcome of the disease [19, 24, 29, 31, 37, 39], and that cytokines play a key role in the differential development of T cell subpopulations. It was reported that a highly restricted subpopulation of CD4⁺ T cells expressing the V α 8-V β 4 T cell receptor was responsible for the early burst of IL-4 transcription following subcutaneous injection of *L. major* LACK protein in BALB/c mice [17, 21, 31]. It was also indicated that induction of IL-4 mRNA by LACK was abrogated by injection of truncated LACK with deletion of the dominant T cell epitope [18] or by injection of LACK into V β 4 CD4-deficient mice [14]. In another report, BALB/c mice made tolerant to LACK by transgenic expression of LACK in the thymus were found to be resistant to *L. major* infection [14]. Furthermore, mucosal immunization of the non-toxic β subunit of cholera toxin (CTB) conjugated LACK allowed the effective tolerization of antigen specific T cells and showed delay of onset of the lesion development [34]. Taken together with these reports, recognition of a single epitope in the LACK antigen drives the early IL-4 response by a restricted population of CD4⁺ T cells responsible for the aberrant

Th2 differentiation that allow progressive disease in *L. major* infection [15, 16, 27].

In this study, progressive development of cutaneous lesions in BALB/c mice caused by *L. amazonensis* was enhanced by recombinant LACK pretreatment. It suggests that LACK protein contributes to the initiation and exacerbation of the disease in murine cutaneous leishmaniasis caused by *L. amazonensis* as well as in *L. major* infection. It is assumed that, in *L. amazonensis* infection, LACK induces IL-4 production by CD4⁺ T cells at the early phase of the parasite infection, leading to disease progression, the same as in *L. major* infection [17, 21, 31].

It should be noted, however, that the pathogenicity of *L. amazonensis* to mouse strains is greatly different from that of *L. major* [1, 3]. For example, *L. major*-resistant C56BL/6 mice are susceptible to *L. amazonensis*, while BALB/c mice are susceptible to both *L. major* and *L. amazonensis* [1]. Further experiments, such as *L. amazonensis* LACK pretreatment to C57BL/6 mice, should be performed in order to clarify the precise point of the difference in pathogenicity between *L. amazonensis* and *L. major*.

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References

1. Afonso, L.C. and Scott, P. 1993. Immune responses associated with susceptibility of C57BL/10 mice to *Leishmania amazonensis*. *Infect. Immun.* 51: 2952–2959.
2. Baily, G. and Nandy, A. 1994. Visceral leishmaniasis: more prevalent and more problematic. *J. Infect.* 29: 241–247.
3. Barral, A., Petersen, E.A., Sacks, D.L., and Neva, F.A. 1983. Late metastatic leishmaniasis in the mouse: a model for mucocutaneous disease. *Am. J. Trop. Med. Hyg.* 32: 277–285.
4. Bradley, D.J. 1974. Genetic control of natural resistance to *Leishmania donovani*. *Nature* 250: 353–354.
5. Bradley, D.J. and Kirkley, J. 1977. Regulation of *Leishmania* population within the host. I. The variable course of *Leishmania donovani* infections in mice. *Clin. Exp. Immunol.* 30: 119–129.
6. Bretscher, P.A., Wei, G., Menon, J.N., and Bielefeldt, O.H. 1992. Establishment of stable, cell-mediated immunity that makes “susceptible” mice resistant to *Leishmania major*. *Science* 257: 539–542.
7. Childs, G.E., Lightner, E.A., McKinney, L., Groves, M.G., Price, E.E., and Hendricks, L.D. 1984. Inbred mice as model hosts for cutaneous leishmaniasis. I. Resistance and susceptibility to infection with *Leishmania braziliensis*, *L. mexicana*, and *L. aethiopica*. *Ann. Trop. Med. Parasitol.* 78: 25–34.
8. Chomczynski, P. and Sacchi, N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162: 156–159.
9. Diefenbach, A., Schindler, H., Donhauser, N., Lorenz, E., Laskay, T., MacMicking, J., Rollinghoff, M., Gresser, I., and Bogdan, C. 1998. Type1 interferon (IFN α/β) and type2 nitric oxide synthase regulate the innate immune response to a protozoan parasite. 1998. *Immunity* 8: 77–87.
10. Gonzalez, A.G., Taladriz, S., Marquet, A., and Larraga, V. 1999. Molecular cloning, cell localization and binding affinity to DNA replication proteins of the p36/LACK protective antigen from *Leishmania infantum*. *Eur. J. Biochem.* 259: 909–916.
11. Grimaldi, G.Jr., Moriearty, P.L., and Hoff, R. 1980. *Leishmania mexicana*: immunology and histopathology in C3H mice. *Exp. Parasitol.* 50:45–56.
12. Haya, H., Pascal, L., Fabienne, T.C., and Jacques, A.L. 1999. Some of the early events underlying Th2 cell maturation and susceptibility to *Leishmania major* infection in BALB/c mice. *Biol. Chem.* 380: 909–914.
13. Herwaldt, B. 1999. Leishmaniasis. *Lancet* 354: 1191–1199.
14. Julia, V., Rassoulzadegan, M., and Glaichenhaus, N. 1996. Resistance to *Leishmania major* induced by tolerance to a single antigen. *Science* 274: 421–423.
15. Launois, P., Ohteki, T., Swihart, K., MacDonald, H.R., and Louis, J.A. 1995. In susceptible mice, *Leishmania major* induce very rapid interleukin-4 production by CD4⁺ T cells which are NK1.1⁺. *Eur. J. Immunol.* 25: 3298–3307.
16. Launois, P., Ohteki, T., Swihart, K., MacDonald, H.R., and Louis, J.A. 1997. IL-4 rapidly produced by V beta 4 V alpha 8 CD4 (+) T cells instructs the development and susceptibility to *Leishmania major* in BALB/c mice. *Immunity* 6: 541–549.
17. Launois, P., Kristin, G.S., Genevieve, M., and Jacques, A.L. 1997. Early production of IL-4 in susceptible mice infected with *Leishmania major* rapidly induces IL-12 unresponsiveness. *J. Immunol.* 158: 3317–3324.
18. Launois, P., Tacchini-Cottier, F., Parra-Lopez, C., and Louis, J.A. 1998. Cytokine in parasitic diseases: the example of cutaneous leishmaniasis. *Int. Rev. Immunol.* 17: 157–180.
19. Luis, C.C.A., Tanya, M.S., Leda, Q.V., Maria, W., Giorgio, T., and Phillip, S. 1994. The adjuvant effect of interleukin-12 in a vaccine against *Leishmania major*. *Science* 14: 235–237.
20. Lynn, S., Cheon, H.C., Jiaren, S., B., Jack, L. Jr., Nancy, H., Ruddle, R., Flavell, A., and Diane, M.P. 1997. Role of CD4⁺ T cells in Pathogenesis Associated with *Leishmania*

- amazonensis* Infection. *J. Immunol.* 158: 5374–5383.
21. Mallard, I., Launois, P., Himmelrich, H., Acha-Orbea, H., Diggelmann, H., Locksley, R.M., and Louis, J.A. 2001. Functional plasticity of the LACK-reactive V β 4-V α 8CD4⁺ T cells normally producing the early IL-4 instruction Th2 cell development and susceptibility to *Leishmania major* in BALB/c mice. *Eur. J. Immunol.* 31: 1288–1296.
 22. Manuel, J. and Ransijin, A. 1997. *Leishmania* spp.: Mechanisms of toxicity of nitrogen oxidation products. *Exp. Parasitol.* 87: 98–111.
 23. Mougneau, E., Altare, F., Wakil, A.E., Zheng, S., Coppola, T., Wang, Z.E., Waldmann, R., Locksley, R.M., and Glaichenhaus, N. 1995. Expression cloning of a protective *Leishmania* antigen. *Science* 268: 563–566.
 24. Muller, I., Pedrazzini, T., Farrel, J.P., and Louis, J. 1989. T-cell responses and immunity to experimental infection with *Leishmania major*. *Ann. Rev. Immunol.* 7: 561–578.
 25. Neal, R.A. and Hale, C. 1983. A comparative study of susceptibility of inbred and outbred mouse strains compared with hamsters to infection with New World cutaneous leishmaniasis. *Parasitol.* 87: 7–13.
 26. Nasser, M. and Modabber, F.Z. 1979. Generalized infection and lack of delayed hypersensitivity in BALB/c mice infected with *Leishmania tropica major*. *Infect. Immun.* 26: 611–614.
 27. Pascal, L., Fatima, C.S., Hayo, H., Carlos, P.L., Fabuene, T.C., and Jaques A.L. 1998. Setting in motion the immune mechanism underlying genetically determined resistance and susceptibility to infection with *Leishmania major*. *Parasite Immunol.* 20: 223–230.
 28. Pearson, R.D. and deQueiroz S.A. 1996. A clinical spectrum of leishmaniasis. *Clin. Infect. Dis.* 22: 1–13.
 29. Reiner, S.L., Wang, Z.E., Hatam, F., Scott, P., and Locksley, R.M. 1993. Th1 and Th2 cell antigen receptors in experimental Leishmaniasis. *Science* 259: 1457–1460.
 30. Reiner, S.L. and Locksley, R.M. 1995. The regulation of immunity to *Leishmania major*. *Ann. Rev. Immunol.* 13: 151–177.
 31. Sanjay, G., Calmen, P., David L.S., and Robert, A.S. 1998. Vaccination requirement for cellular immunity to an intracellular parasitic infection. *Nature Med.* 4: 1409–1415.
 32. Scott, P. A. and Ferrel, J.P. 1982. Experimental cutaneous leishmaniasis: disseminated leishmaniasis in genetically susceptible and resistant mice. *Am. J. Trop. Med. Hyg.* 31: 230–238.
 33. Stenger, S., Donhauser, N., Thurin, H., Rollinghoff, M., and Bogdan, C. 1996. Reactivation of latent leishmaniasis by inhibition of inducible nitric oxide synthase. *J. Exp. Med.* 183: 1501–1514.
 34. Stephen, J.M., Carola, R., Roxanne, P., Valerie, J., Cecil, C., and Nicol, G. 1998. Selective tolerization of Th1-like cells after nasal administration of a cholera toxoid-LACK conjugate. *Eur. J. Immunol.* 28: 424–432.
 35. Taladriz, S., Gonzalez, A.G., Marquet, A., and Larraga, V. 1999. Cloning, molecular analysis and differential cell localization of the p36 RACK analogue antigen from the parasite protozoon *Crithidia fasciculata*. *FEBS Letters* 443: 375–380.
 36. Thirteenth Program Report. 1997. *Special program for Research and Training in Tropical Disease, leishmaniasis*. Geneva: World Health Organization Publications. 100–111.
 37. Titus, R., Theodoe, G., Kimsey, C.M., Shanker, P.B., Hall, A., McGurn, L.M., and Povielli, L. 1992. Roll of T cells in immunity of the intracellular pathogen *Leishmania major*. *Subcell. Biochem.* 18: 99–129.
 38. Wei, Q., Charles, I.G., Smith, A., Feng, G.J., Hang, F.P., Xu, D., Muller, W., Moncada, S., and Liew, F.Y. 1995. Altered immune responses in mice lacking inducible nitric oxide synthase. *Nature* 375: 408–411.
 39. Werner, S. and Tamas, L. 2000. The host response to *Leishmania* infection. *Adv. Immunol.* 74: 275–317.