IL-1-induced inflammation promotes development of leishmaniasis in susceptible BALB/c mice

Elena Voronov1, Shahar Dotan1, Lubov Gayvoronsky1, Rosalyn M. White1, Idan Cohen1, Yakov Krelin1, Fabrice Benchetrit1, Moshe Elkabets1, Monika Huszar2, Joseph El-On1 and Ron N. Apte1

1Shraga Segal Department of Microbiology and Immunology, The Cancer Research Center, Faculty of Health Sciences and Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel
2Pathology Department, Kaplan Medical Center, Rehovot, 76100, Israel

Correspondence to: E. Voronov; E-mail: elena@bgu.ac.il

Transmitting editor: I. Pecht

Received 11 May 2009, accepted 5 January 2010

Abstract

The role of host-derived IL-1 on the course of Leishmania major infection in susceptible BALB/c mice was assessed. Manifestations of the disease were more severe in mice deficient in the physiological inhibitor of IL-1, the IL-1 receptor antagonist (IL-1Ra) in comparison with control mice. In mice lacking one of the IL-1 genes (IL-1α or IL-1β), there was delayed development of the disease and more attenuated systemic inflammatory responses. IL-1α-deficient mice were slightly more resistant to L. major infection compared with IL-1β-knockout mice. During disease progression in IL-1Ra KO and control mice, myeloid-derived suppressor cells invaded the spleen, concomitant to suppression of T cell-mediated immunity and expression of systemic high levels of pro-inflammatory cytokines. In IL-1-deficient mice, Th1 responses were still apparent, even at late stages of the disease. Thus, dose-dependent effects of IL-1 were shown to influence the pathogenesis of murine leishmaniasis in susceptible BALB/c mice. Physiological and supra-physiological levels of IL-1 in the microenvironment promoted an exacerbated form of disease, whereas sub-physiological doses of IL-1 induced a less progressive disease. Thus, manipulation of IL-1 levels in the host, using the IL-1Ra or specific antibodies, has the potential to alleviate symptoms of visceral manifestations of leishmaniasis.

Keywords: interleukin-1 receptor antagonist, interleukin-1α, interleukin-1β, leishmaniasis, pro-inflammatory cytokines

Introduction

Leishmania major is an intracellular protozoan parasite that primarily multiplies within macrophages (1, 2). Symptoms of the disease range from mild skin ulcers to severe mucocutaneous destruction or to visceral symptoms leading to mortality. Disease progression depends on both the strain of Leishmania causing the disease and the host's immunological response. Susceptible BALB/c mice develop a visceral fulminant disease, characterized by a dominant Tₘ2 response, whereas C57BL/6 mice are resistant to L. major and manifest a self-healing local infection, with an IL-12-driven Tₘ1 CD4⁺ T response, elevated IFNγ production, macrophage activation and Leishmania killing (3–5). In most studies, specific immune responses against L. major were assessed. However, inflammatory responses mediated by innate immune cells are also evident in L. major infection, especially in the visceral manifestations of the disease. The relationship between inflammation and specific immunity is complex but important for the outcome of the disease. IL-1 is a major pro-inflammatory cytokine, which, similar to tumor necrosis factor (TNF)α, is considered as an ‘alarm cytokine’ that is secreted by macrophages. IL-1 initiates and propagates inflammation by inducing the expression of adhesion molecules on endothelial cells and leukocytes. The IL-1 family consists of two major agonistic proteins, namely IL-1α and IL-1β, and one physiological antagonist protein, the IL-1 receptor antagonist (IL-1Ra) that binds to the IL-1 receptor type I (IL-1R1) without transmitting an activation signal (reviewed in refs 6–11). IL-1 has been shown to affect the pathogenicity of leishmaniasis by generating an inflammatory response in afflicted tissues and by modulating adaptive T cell-mediated immune responses, which act to limit parasite dissemination (12, 13). Effects of IL-1 on the outcome of leishmaniasis have been described, usually after injection of exogenous recombinant IL-1. However, the role of
IL-1 is an important molecule in murine leishmaniasis

microenvironment-derived IL-1 on the pathogenesis of leishmaniasis has not yet been studied. Here, we have assessed the role of IL-1 in interactions between immunity and inflammation and the differential in vivo involvement of host-derived IL-1α and IL-1β in murine leishmaniasis in susceptible BALB/c mice. Specifically, we have used knockout (KO) mice that selectively lack molecules of the IL-1 family, i.e. IL-1α, IL-1β or IL-1Ra KO mice. IL-1-deficient mice have served as a valuable tool to assess the specific roles of IL-1α and IL-1β in experimental models of infection, autoimmunity and cancer (14, 15). In our previous studies, we have established that microenvironmental IL-1α and IL-1β play different roles in the development of the malignant process and autoimmunity (6,16–20). Our results underscore the key role of IL-1 in controlling inflammatory and immune manifestations in leishmaniasis, as well as influencing the clinical course of the disease. The results also indicate that IL-1 is detrimental in controlling the delicate balance between inflammation and immunity, which is important for the outcome of the disease. Treatment with IL-1Ra is suggested to alleviate the symptoms of the disease.

Methods

Mice

Six-week-old male BALB/c mice were purchased from Harlan Laboratories Limited (Jerusalem, Israel). IL-1 and IL-1Ra KO mice with a BALB/c background were previously described and kindly provided by Prof. Y. Iwakura (21). The IL-1 and IL-1Ra KO mice were bred and kept at the Animal Facilities of the Faculty of Health Sciences, Ben-Gurion University, under aseptic conditions. Mice were treated according to the Animal Care National Institutes of Health guidelines adopted by our Animal Committee.

Leishmania culture and infections

Leishmania major (WHO code: MHOM/IL/80/Friedlin) was used in all experiments. The parasites were maintained at 28°C by biweekly passage in RPMI 1640, supplemented with 10% FBS (Biological Industries, Beit Haemek, Israel), L-glutamine (2 mM), penicillin (100 U ml⁻¹), streptomycin (100 µg ml⁻¹; Biological Industries). The parasites were also maintained as stabiles at −70°C and in vivo in Balb/c mice, as described (22, 23). In initial experiments, we injected different doses (10⁵–10⁷) of L. major promastigotes into IL-1 KO mice, to optimize the dose of parasites. Subsequently, 6-week-old male BALB/c mice were injected s.c. at the base of the tail with the chosen dose of 10⁷ L. major promastigotes per mouse.

Disease development was determined by smears taken from the site of parasite inoculation or from the edge of the lesion using a scalpel. The cellular material thus obtained from the site of parasite inoculation or from the edge of the lesion using a scalpel. The cellular material thus obtained from the site of parasite inoculation or from the edge of the lesion was spread on a slide and examined microscopically after Lesion size was measured in millimeters in two diameters (D, d) taken at right angles and determined according to the formula $S_{mm²} = D \times d/2$. Real-Time PCR detection of L. major in tissues was also performed. Thus, tissue DNA was collected from mice before and 30 days after inoculation of promastigotes. Detection of parasite load in the liver and spleen was performed as described (24).

In vivo treatment of mice with the IL-1Ra

Mice were injected with L. major promastigotes, as indicated above. Recombinant IL-1Ra (100 µg per mouse) was injected i.p. (IL-1Ra was kindly provided by Amgen, Thousand Oaks, CA, USA), starting one day before the injection of Leishmania and then on days 1, 3, 5, 7, 9, 11 and 13 post-injection, as previously described by us (25).

Cell cultures

Spleens were obtained from control, non-treated and Leishmania-infected mice. Freshly prepared cells were used for FACS analysis. Spleen cells (3 × 10⁶ per ml) were cultured in complete RPMI 1640 supplemented with 2% FBS and β-mercaptoethanol (0.05%) at 37°C and supernatants were collected after 48 h for cytokine assays.

Cytokine measurements by ELISA

Murine cytokine levels were measured using commercial ELISA kits (Pharmingen, San Diego, CA, USA), according to the manufacturer’s instructions.

FACS analysis

Cells (10⁶ per sample) were blocked with purified anti-Fcy receptor III/I (CD32/CD16) antibody (clone 93, eBioscience, San Diego, CA, USA) for 30 min, followed by staining with different combinations of the following antibodies: FITC-anti-mouse CD11b (clone M1/70, eBioscience); PE-anti-mouse Gr-1 (clone RB6-8C5, Biolegend, San Diego, CA, USA); PE-anti-B220 (clone RA3-6B2, Pharmingen); FITC-anti-CD3 (clone 145-2C11, Biolegend); FITC-anti-CD4 (clone GK1.5, eBioscience); FITC-anti-CD8 (clone 53-6.7, eBioscience); FITC-anti-F4/80 (clone BM8, eBioscience), PE-anti IL-4Ra (mIL4R-M1, Pharmingen) and APC-anti-Ly6C (AL-21, eBioscience), for 1 h and analyzed by FACS Calibur (Becton-Dickson, San Jose, CA, USA). For the detection of intracellular cytokines, the commercial Cytoperm kit (Pharmingen) was used. Single spleen cell suspensions were permeabilized according to the manufacturer’s instructions. The percent of CD3/IL-10, IL-4 or IFNγ double-positive cells were determined by FACS analysis [PE-anti-IL-10, IL-4 or IFNγ (Pharmingen)]. The data were analyzed using FlowJo software (Tree Star, Inc.). The percentage of stained populations is shown after subtracting background levels obtained with isotype antibodies.

Multiple cytokine analysis by flow cytometry

The FlowCytomix mouse Th1/Th2 10 plex kit (IFNγ, IL-1α, IL-2, IL-4, IL-5, IL-6, IL-10, IL-17, TNFα, granulocyte macrophage colony-stimulating factor; Bender MedSystems GmbH, Vienna, Austria) was used according to the manufacturer’s instructions. Briefly, supernatants from spleen cells or sera obtained from mice injected with L. major promastigotes...
were incubated with fluorescent beads coated with mAbs to the indicated cytokines and biotin-conjugated anti-cytokine antibodies for 2 h. After a further 1 h incubation with streptavidin PE, cytometric analysis was performed.

**Immunostaining procedure**

Histological sections from mice infected with *L. major* promastigotes were taken at different days post-infection and then processed and stained with hematoxylin and eosin using established protocols. For immunohistochemistry, the following antibodies were used: rat anti-mouse F4/80 (SEROTEC, Oxford, UK, 1:20), purified rat anti-mouse Gr-1 (eBioscience, 1:20) and rat anti-human CD3 (SEROTEC, 1:100). The Vectastain Elite ABC Peroxidase kit (Vector Laboratories, Inc, Burlingame, CA, USA) was used for secondary antibody application and detection. Visualization was performed using AEC as a substrate (ZYMED Laboratories Inc, San Francisco, CA, USA). A pathologist examined the slides in a blind manner.

**Reverse transcription–real-time PCR**

For quantitative PCR analysis, RNA was extracted from 20 mg of spleen tissue, using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. cDNA was prepared with the RevertAid™ first strand cDNA Synthesis Kit (Fermentas Inc), using oligo-dT 15mers. Primers for each gene studied were designed with overlapping exon boundaries, using primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The designed amplifiers were <150 bp to ensure high amplification efficiency (26). The primer sequences were: T-bet-forward 5'-TTGCAGCTTGGTGTTGCT-3', reverse 5'-TATGTTCGGCTTCCCATTCT-3'; beta-2-Microglobulin-forward 5'-TGAGCTTCTGGTGCTTGT-3', reverse 5'-CCTTGTTGTTGG-TGGTGCTTGT-3'; beta-2-Microglobulin-forward 5'-TGGTGCTTGT-3', reverse 5'-TATGTTCGGCTTCCCATTCT-3'. Amplification efficiencies were verified by serial dilution standard curve fit. Real-time reverse transcription–PCR was performed with an ABI PRISM 7500 apparatus (Applied Biosystems, Foster City, CA, USA), using 10 ng of the cDNA template. The PCR conditions included a polymerase activation step at 95°C for 10 min followed by cycles of 95°C for 15 s and 60°C for 60 s. Relative quantification was determined by the ΔΔCt method (27), using beta-2-microglobulin as a reference gene.

**Statistical analyses**

Each experiment was repeated 3–5 times with a similar pattern of responses. *In vivo* experiments consisted of 5–10 mice in each experimental group. Shown are results from pooled or single representative experiments, as indicated. Mean values ± standard deviations, are shown. The significance of variations in the results was determined using the two-sided Student's *t*-test; a *P* < 0.05 was considered significant.

**Results**

*Clinical manifestations of L. major infection in IL-1/IL-1Ra-deficient mice*

Mice were injected s.c. into the base of the tail with *L. major* promastigotes (10⁷ per mouse) and the development of nodules/lesions, which are the first manifestation of infection, was assessed at different time intervals. Initial experiments applying different doses of parasites (10⁴–10⁷) have demonstrated similar patterns of infection in IL-1-deficient mice; however, the onset of infection was dose dependent (results not shown). Subsequently, the dose of 10⁷ promastigotes per mouse was chosen and routinely used. As can be seen in Fig. 1(A), on day 14 after infection, no nodules, detected by palpation of swelling at the injection site, were observed in IL-1α or IL-1β KO mice, whereas in control BALB/c mice, there were small nodules in 35% of the mice. In IL-1Ra-deficient mice, there were large palpable nodules in 100% of the mice at this time. On day 14, similar numbers of parasites were observed in injected mice from all experimental groups, as detected in cultures established from samples taken from the injection site (Fig. 1B). On day 40, there were nodules in 100% of control BALB/c mice, whereas in IL-1α or IL-1β KO mice, there were nodules in only 35% of the mice (results not shown). As can be seen in Fig. 1(C), in IL-1-deficient mice, ~10–25% of the mice developed wounds, resulting from ulceration of nodules, on day 50 after the onset of infection. At this time, we observed wounds in 50% of control and 100% of IL-1Ra-deficient mice. On days 75–90, there were wounds in 100% of control and IL-1β-deficient mice, but only in 75% of IL-1α-deficient mice. Differences in the size of the wounds in IL-1 KO mice are shown in Fig. 1(D); they were correlated with patterns of wound development. Large and inflamed wounds were seen at early time intervals in control and IL-1Ra KO mice, whereas only small wounds developed in mice deficient in IL-1, especially IL-1α KO mice. Wound size was measured only during the first 50 days of infection; later, wounds became necrotic and infected and reduced in size in IL-1Ra KO mice and later also in wild type (WT) mice. On day 28, in all groups of mice, we observed a higher parasite load compared with day 14, whereas in IL-1Ra KO mice, this difference was less pronounced, possibly due to a rapid dissemination to internal organs (Fig. 1E). This was, indeed, evident by measuring the parasite load by PCR in the liver and spleen on day 28. A significant increase in the parasite load was observed in IL-1Ra KO mice in both the liver and spleen, compared that in the liver and spleen of control mice (Fig. 1F). No parasites were detected in the liver and spleen of mice deficient in IL-1 genes (results not shown). Similar patterns of pathogenicity were observed when checking mortality rates (Fig. 2A). These results indicate that IL-1 contributes to the pathogenicity of *L. major*; in the absence of one of the IL-1 molecules, the disease is attenuated and when IL-1 is ‘over expressed’ (IL-1Ra-deficient mice), the disease is exacerbated, as compared with control mice.

To further assess the importance of IL-1 in the development of leishmaniasis, IL-1Ra KO mice were treated with recombinant IL-1Ra, as described in Methods. A delay in disease symptoms and mortality was observed compared with non-treated mice. We saw that treatment with IL-1Ra, which attenuates IL-1 activity, decreased mortality and in IL-1Ra KO-treated mice, 100% mortality occurred only on day 110, whereas in untreated mice, on day 85, 100% mortality was already observed. Treatment with IL-1Ra also delayed mortality in WT mice (Fig. 2B).
Visceral manifestations of leishmaniasis in IL-1/IL-1Ra-deficient mice

We next assessed the dissemination of the disease into the reticuloendothelial organs, i.e. the spleen and liver. On day 40, modest, marked and no splenomegaly was observed in control BALB/c, IL-1Ra and IL-1 KO mice, respectively (Fig. 3A). On day 60, splenomegaly was noted in all groups but was most evident in IL-1Ra KO mice.

A histological analysis of spleens was performed 30 days after infection of *L. major* promastigotes. As can be seen in Fig. 3(B), in IL-1Ra KO mice, severe destruction of the white pulps accompanied by an abundance of megakaryocytes, neutrophils and blast-like cells that characterize extramedullary hematopoiesis was observed. These findings were most pronounced at late stages of the disease. Additionally with disease progression, some structural changes were observed in BALB/c mice and to a lesser extent in IL-1 KO mice. Using immunohistochemical stainings, we visualized T cells, macrophages and Gr-1-positive myeloid cells (neutrophils and immature myeloid cells) in sections of spleens
Furthermore, the fraction of Gr-1+ cells (PMN) and a moderate increase in monocytic cells, an increase in the total number of polymorphic nuclear cells, an increase during disease progression. This was especially noted in IL-1Ra KO mice, which manifested the most pronounced inflammatory responses, which contribute to infiltration, expansion and activation of immature myeloid cells. In control and IL-1 KO mice, there was no significant change in the number of T cells or macrophages, but there was a moderate increase in the number of Gr-1+CD11b+ myeloid cells as the disease progressed; however, this increase was much smaller than in IL-1Ra KO mice. An increase in the number of B220-positive B cells was observed during the progression of leishmaniasis in control and IL-1 KO mice, whereas the opposite trend was observed in IL-1Ra KO mice. No difference in the composition of spleen cells was seen in untreated mice of all mouse strains used. Thus, a progressive disease, as observed in IL-1Ra KO mice, is characterized by reduced numbers of T and B cells and an increase in myeloid cells.

Advanced L. major infection of BALB/c mice was associated with lobular hepatitis and an intensive periportal infiltrate and hyperplasia of Kupffer cells. A lobular hepatitis accompanied by the appearance of Leishmania-containing granulomas of histiocytes was further noted in livers of IL-1β KO mice and less pronounced manifestations were observed in IL-1x KO mice. In IL-1Ra KO mice, there was an exacerbated tissue response, accompanied by an intensive infiltration of portal tracts with neutrophils, large granulomas containing parasites and obvious hyperplasia of Kupffer cells (Fig. 4B).

IL-1 is an important molecule in murine leishmaniasis

In order to determine whether IL-1 KO mice are less prone to the development of visceral leishmaniasis, due to early development of protective Th1 responses, we first assessed the expression of T-bet, the major transcription factor that controls Th1 responses. RNA from spleen cells was assessed for T-bet transcription in control and IL-1 family-deficient mice prior to infection and on days 12 and 40, following infection. Higher homeostatic expression levels of T-bet were observed in IL-1-deficient mice, compared with control and IL-1Ra KO mice. During disease progression, on day 12, a moderate elevation in T-bet expression was observed in control, IL-1x and IL-1Ra KO mice, followed by a sharp decrease in its expression on day 40. In IL-1 KO mice with advanced infection, a reduction in expression levels of T-bet was also observed; however, on day 40, the reduction was much less pronounced in IL-1 KO mice compared with IL-1Ra and control mice. These results indicate that during disease progression, suppression of the potential to develop favorable Th1 responses is manifested mainly in control and IL-1Ra KO mice (Fig. 6). This may be due to inhibition of T-bet expression or depletion of populations of T cells that express T-bet.

Cytokine generation in L. major IL-1/IL-Ra-infected mice

As cytokines were shown to play a major role in the pathogenesis of leishmaniasis, it was of interest to evaluate whether endogenous IL-1 affects the cytokine profile of Leishmania-infected mice. Initially, on day 30, we assessed the frequency of CD3-positive cells that express intracellular cytokines, which characterize Th1 (IFNγ) and Th2 (IL-4 and IL-10) polarization. The frequency of IFNγ-producing T cells was similar in all groups of mice. However, in control and IL-1Ra KO mice, a significant increase in T cells producing IL-4 and IL-10 was observed, compared with decreased...
numbers of these cells in IL-1 KO mice (Fig. 7A). As T cell polarization occurs early after antigen exposure to Leishmania, we next assessed the production of Th1-promoting cytokines in spleen cells of mice. On day 12, a significant increase in IL-18, IL-12 and IFN-\(\gamma\) (Th1-type cytokines) was observed in IL-1\(\alpha\) and IL-1\(\beta\) KO mice, compared with control and IL-1Ra KO mice (Fig. 7B–D). IL-12 and IL-18 were secreted spontaneously, without stimulation, whereas IFN-\(\gamma\) secretion required Con A stimulation. Similar cytokine patterns were observed at other time intervals, including at the end-point of the experiment. Naïve spleen cells from the different strains of mice showed no difference in the pattern of cytokine secretion (results not shown). Thus, IL-1 KO mice, which were the most resistant to infection, showed the highest expression levels of Th1-type cytokines throughout disease progression. Only very low levels of Th2 cytokines were observed by ELISA in all \(L.\ majoreinfected groups.

As visceral manifestations of murine leishmaniasis in BALB/c mice include inflammatory responses in reticuloendothelial organs, we assessed the expression of pro-inflammatory
cytokines in spleen cell cultures and sera from infected mice (days 12 and 30), by Cytometrix analysis. Elevated levels of representative pro-inflammatory cytokines tested, i.e. IL-6, TNFα and IL-17, were observed in spleen cell cultures from IL-1Ra KO mice as early as day 12. Levels of IL-6 and TNFα decreased with the progression of the disease in spleen cells, whereas IL-17 levels remained high in this group of mice. These results may be due to the destruction of T cells and the influx of neutrophils to the spleen that we observed in IL-1Ra KO mice. On the contrary, the highest levels of pro-inflammatory cytokines were observed during disease progression in sera from IL-1Ra KO mice (Fig. 8). Only low non-significant levels of pro-inflammatory cytokines were detected in sera from naive and IL-1-deficient mice.

Discussion

In the present study, we are the first, to our knowledge, to assess the role of microenvironment-derived IL-1α and IL-1β in the pathogenesis of murine leishmaniasis. Our results indicate that both IL-1 molecules contribute to the pathogenesis of murine leishmaniasis, as the disease was less severe in mice deficient either in IL-1α or IL-1β, compared with control and IL-1Ra KO mice, which manifest unattenuated
IL-1 activity and more severe disease progression (Fig. 1A, C and D and Fig. 2A). The parasite load at the injection site was similar in all strains of mice and increased with disease progression. In IL-1Ra KO mice, on day 28, the parasite load was lower than in control mice (Fig. 1E). In IL-1Ra KO mice, a significant splenomegaly and severe portal hepatitis were observed from early stages of the disease, whereas in control and IL-1 KO mice, only late, moderate splenomegaly and mild signs of hepatitis were found (Fig. 3A and Fig. 4B). Also, an increased parasite load in spleens and livers of mice deficient in IL-1Ra was detected on day 28 as compared with control mice. Thus, the decrease of the local

Fig. 5. A shift between T cells and MDSC correlated with the progression of murine leishmaniasis and these changes were IL-1 dependent. Spleens were removed from IL-1 KO and control mice on days 12, 30 and 60. Cells were prepared for FACS analysis, as indicated in Methods. (A) Cells were stained with FITC-conjugated anti-CD3 antibodies. (B) PE-conjugated anti-B220-antibodies. (C) Percentage of Gr-1/CD11b-double-positive cells is shown. (D) The total number of monocytic MDSC (CD11b+Ly6G-Ly6Chigh) is shown. (E) The total number of granulocytic MDSC (CD11b+Ly6G+Ly6Clow) is shown. (F) Expression of IL-4Rα on Gr-1/CD11b-double-positive MDSC was assessed; **P < 0.001; *P < 0.05.
IL-1 is an important molecule in murine leishmaniasis

At advanced stages of the disease, on day 30 and onward, splenomegaly accompanied by the accumulation of immature myeloid cells (Gr-1−CD11b+), also termed myeloid-derived suppressor cells (MDSC), was observed mainly in IL-1Ra-deficient mice and later (day 60) in control BALB/c mice, whereas MDSC were not observed in spleens of IL-1 KO mice (Fig. 5C). MDSC were originally described as a population of immune suppressor cells which accumulated in the bone marrow, blood and spleen of mice bearing different pathologies related to chronic inflammation, such as cancer, parasitic diseases and autoimmunity (reviewed in refs 6, 30–35). Recently, heterogeneity of MDSC according to the expression of two different epitopes recognized by Gr-1 antibodies (Ly6G and Ly6C) was suggested (33, 36). Thus, granulocytic MDSC (CD11b+Ly6G+Ly6Clow) and monocytic MDSC (CD11b+Ly6G−Ly6Chigh) were characterized. In Leishmania-infected IL-1Ra KO mice, the dominant population of Gr-1−CD11b+ cells was PMN, rather then monocytic MDSC (Fig. 5D and E). In addition, MDSC with high expression of IL-4Ra were detected in mice deficient in IL-1Ra (Fig. 5F). Increased expression of IL-4Ra on MDSC was shown to correlate with immunosuppressive capacity (37). In IL-1Ra KO mice, accumulation of these cells correlated with a decrease in the total number of T cells. MDSC expansion in the bone marrow is induced by systemic pro-inflammatory cytokines; these cells subsequently migrate to the spleen and peripheral sites, where they may further proliferate and suppress T-cell and NK cell responses concomitantly to induction of Treg and inflammation (reviewed in refs 38–42). We have previously shown that over-expression of IL-1β in tumor cells or in the microenvironment correlated with systemic inflammation, general anergy and increased invasiveness and induces the accumulation of MDSC in reticuloendothelial organs (19). Involvement of IL-1β in Treg induction was recently demonstrated (43, 44). Thus, inflammation induced by host-derived IL-1, possibly, exacerbates leishmaniasis and also suppresses specific immunity.

Protective immunity against L. major depends on the genetically determined ability to mount a Th1 response. Thus, susceptible BALB/c mice develop a dominant Th2 response, whereas in C57BL/6-resistant mice, macrophage and dendritic cell activation by the Th1 product IFN-γ is essential for controlling L. major (reviewed in refs 2–5, 45). IFN-γ secretion, detected in supernatants of spleen cell cultures (Fig. 7D) and in the serum (results not shown), was observed only in IL-1 KO mice, in which secretion of IL-18 and IL-12 was also observed in spleen cell cultures (Fig. 7B and C). T-bet is an essential transcription factor for T-h1 development and function; in T-bet KO mice, naive T cells develop into Th2 cells during L. major infection (reviewed in ref. 46). The differences in IFN-γ secretion are observed early in infected mice, before the significant elevation in MDSC levels; however, MDSC may also contribute to inhibition of T-h1 responses in later stages of the disease. In spleen cells from IL-1 KO mice, enhanced homeostatic expression of T-bet is observed and it is further elevated at early stages of L. major infection (Fig. 6), pointing to the possibility that IL-1 may negatively regulate expression of T-bet and thus inhibit T-h1 and favor T-h2 responses. Indeed, on day 40, almost complete suppression of T-bet is observed in splenocytes of...
control and IL-1Ra KO mice, whereas only a moderate reduction in its expression is observed in IL-1 KO mice. We did not detect significant secretion of IL-4 and IL-10 in spleen cell supernatants or in the serum, possibly due to secretion of low levels, below the detectable threshold of our ELISA kits. However, stainings for intracellular IL-4 and IL-10 in CD3-positive gated T cells demonstrated that in IL-1Ra and in control mice the incidence of IL-4- and IL-10-producing cells is higher than that in IL-1-deficient mice. This suggests that IL-1 may affect the differentiation of function of Th2 effector cells. Patterns of cytokine and T-bet expression, however, indicate that host-derived IL-1 can possibly also inhibit T\(_\text{H}1\) polarization. Numerous studies have reported direct effects of IL-1 on T\(_\text{H}2\) cells, which express IL-1Rs, unlike T\(_\text{H}1\) cells, which do not express IL-1Rs (47). Moreover, IL-1 stimulates the expansion of antigen-stimulated naive and memory CD4 T cells, especially affecting, IL-17- and IL-4-producing cells (48). However, indirect effects of IL-1 on T\(_\text{H}1\) responses can be through induction of cytokine secretion by APC or NK cells. Indeed, it was shown that local application of IL-1 acts in synergy with IL-12 in priming anti-leishmanial T\(_\text{H}1\) responses in BALB/c mice (13). In contrast, the same authors have shown that prolonged treatment of mice with recombinant IL-1 promoted T\(_\text{H}2\) responses and exacerbated disease outcome (12). Conversely, Satoskar et al. have shown in C57BL/6 mice, in which a self-healing leishmaniasis is observed, that IL-1 is not required for mounting an immune response or antigen-dependent proliferation, but it regulates the balance of T\(_\text{H}1\)/T\(_\text{H}2\) responses and may function to negatively regulate IL-4 expression (49). Thus, in Leishmania resistant and sensitive strains of mice, IL-1 may differentially affect immune as well as inflammatory responses.

Elevated expression of pro-inflammatory cytokines, which is characteristic of L. major infection in susceptible BALB/c mice (50–52), was observed in supernatants of spleen cell cultures and sera of mice with progressive disease and was especially accentuated in IL-1Ra mice (Fig. 8). In IL-1\(\beta\) KO mice, higher levels of IL-17 and IL-6 were observed, compared with IL-1\(\alpha\) KO and control mice, which may explain, at least in part, why a more attenuated disease is observed in IL-1\(\alpha\) KO, as compared with IL-1\(\beta\) KO mice.

We observed elevated expression of IL-17 in spleen cell cultures and in the serum (Fig. 8A) of L. major-infected IL-1Ra KO mice. IL-17 is a pro-inflammatory cytokine, which is secreted by T\(_\text{H}17\) cells—a subset of T cells producing IL-17 that develops in the presence of IL-6 and transforming growth factor-\(\beta\)-producing Treg cells (53). IL-17 stimulates a variety of pro-inflammatory cells to produce inflammatory molecules including IL-1, TNF\(\alpha\) and chemokines (54–56). It has also been shown that IL-1 is an upstream cytokine for IL-17 production and for example, in IL-1Ra KO mice, over-expression of IL-1 leads to increased production of IL-17 (57). At inflammatory sites, IL-17 affects neutrophil function,
reduces apoptosis and promotes secretion of pro-inflammatory and tissue damaging molecules (58, 59). Increased IL-17-dependent neutrophil recruitment into lesions of L. major-infected BALB/c mice has previously been shown to significantly contribute to disease outcome (60, 61). In IL-17 KO mice, attenuated leishmaniasis was observed compared with susceptible BALB/c mice (62).

Overall, levels of IL-1 expression positively correlated with local and systemic inflammation and severity of disease. In contrast, protective T\textsubscript{n}1 responses are prevalent in mice with attenuated IL-1 secretion. The net outcome between the effects of IL-1 on inflammation and the development of a favorable anti-leishmanial T\textsubscript{n}1 response, possibly, determines the direction of the disease. Thus, moderate inflammation may potentiate adaptive immunity, whereas vast and systemic inflammation inhibits it. As physiological levels of IL-1 in BALB/c mice induced a progressive disease with a poor outcome, attenuation of IL-1, by the IL-1Ra or by other means, may lead to amelioration of the disease. The IL-1Ra has already been approved by the Food and Drug Administration for the treatment of various inflammatory diseases, such as Rheumatoid arthritis and inflammatory bowel disease, and may have therapeutic potential in leishmaniasis (63).
IL-1 is an important molecule in murine leishmaniasis

Funding
United States-Israel Binational Foundation (BSF 80764401) to R.N.A.; Israel Ministry of Health Chief Scientist’s Office (892933011) to R.N.A.; German–Israeli Project Cooperation (DIP) collaborative program (to R.N.A.); Israel Ministry of Health Chief Scientist’s Office (to E.V. 200816201); German–Israeli DIP collaborative program (to E.V. 89087301).

Acknowledgements
The authors would like to thank their collaborators Prof. Charles A. Dinarello, University of Colorado, Denver; Prof. Yoichiro Iwakura, University of Tokyo, Tokyo, Japan; Prof. Angel Porgador, University Ben-Gurion, Israel; Prof. Mina Fogel, Pathology Department, Kaplan Medical Center, Israel; Mrs Ruth Sneir, University Ben-Gurion, Israel and Dr Elina Bazarsky, University Ben-Gurion, Israel.

References
21. Horai, R., Asano, M., Sudo, K. et al. 1998. Production of mice deficient in genes for interleukin (IL)-1alpha, IL-1beta, IL-1alpha/ beta, and IL-1 receptor antagonist shows that IL-1 beta is crucial in turpentine-induced fever development and glucocorticoid secretion. J. Exp. Med. 187:1463.
colony-stimulating factor-producing vaccines impair the immune response through the recruitment of myeloid suppressor cells. Cancer Res. 64:6357.


Weiss, T., Vitacolonna, M. and Zoller, M. 2009. The efficacy of an IL-1alpha vaccine depends on IL-1R1 availability and concomitant myeloid-derived suppressor cell reduction. J. Immunother. 32:552.


IL-1 is an important molecule in murine leishmaniasis 257


