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SIR2-Deficient *Leishmania infantum* Induces a Defined IFN-γ/IL-10 Pattern That Correlates with Protection[1]

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The ability to manipulate the *Leishmania* genome to create genetically modified parasites by introducing or eliminating genes is considered a powerful alternative for developing a new generation vaccine against leishmaniasis. Previously, we showed that the deletion of one allele of the *Leishmania infantum* silent information regulatory 2 (LiSIR2) locus was sufficient to dramatically affect amastigote axenic proliferation. Furthermore, LiSIR2 single knockout (LiSIR2$^{+/-}$) amastigotes were unable to replicate in vitro inside macrophages. Because this *L. infantum* mutant persisted in BALB/c mice for up to 6 wk but failed to establish an infection, we tested its ability to provide protection toward a virulent *L. infantum* challenge. Strikingly, vaccination with a single i.p. injection of LiSIR2$^{+/-}$ single knockout elicits complete protection. Thus, vaccinated BALB/c mice showed a reversal of T cell anergy with specific anti-*Leishmania* cytotoxic activity and high levels of NO production. Moreover, vaccinated mice simultaneously generated specific anti-*Leishmania* IgG Ab subclasses suggestive of both type 1 and type 2 responses. A strong correlation was found between the elimination of the parasites and an increased *Leishmania*-specific IFN-γ/IL-10 ratio. Therefore, we propose that the polarization to a high IFN-γ/low IL-10 ratio after challenge is a clear indicator of vaccine success. Furthermore these mutants, which presented attenuated virulence, represent a good model to understand the correlatives of protection in visceral leishmaniasis. *The Journal of Immunology,* **2007, 179: 3161–3170.**

Leishmaniasis is a chronic parasitic disease caused by several species of the genus *Leishmania* and is associated with significant morbidity and mortality worldwide. The clinical manifestations of leishmaniasis depend on the interactions between the infecting *Leishmania* species and the host factors and range from mild cutaneous to fatal visceral pathologies (1). In the Northern Mediterranean countries, *Leishmania infantum* is the causative agent for endemic visceral leishmaniasis (2). Chemotherapy has proved to be inadequate and although vaccination is thought to be feasible, as demonstrated by individuals who recover from natural or deliberate infections with *Leishmania* and develop lifelong protection against reinfection, no clinically effective vaccines exist (3, 4).

It has been argued that a protective immune response against both cutaneous and visceral leishmaniasis is dependent on the establishment of Th1 immunity (5). Numerous investigations using a cutaneous leishmaniasis agent, *Leishmania major*, established that the resolution or progression of the cutaneous disease was dependent on the outcome of the development of CD4$^+$ T cell subsets Th1 and Th2, respectively (for review, see Ref. 6). Thus, resistance was associated with IL-12 production by APC, which leads to the differentiation and proliferation of Th1 cells and NK cell activation (7). These cells produce IFN-γ and TNF-α to cause the activation of macrophages that, through a primary production of the NO mechanism, can eliminate the parasites (8). Susceptibility is associated with a Th2 CD4$^+$ lymphocyte expansion and the preferential secretion of IL-4, IL-10, and IL-13 (9, 10). However, this Th1/Th2 dichotomy is lacking in visceral leishmaniasis and fails to explain some nonhealing forms of cutaneous leishmaniasis in mice including *Leishmania mexicana*, *Leishmania amazonensis*, and even some strains of *L. major* (11). Indeed, studies conducted with a *Leishmania* species causing human visceral leishmaniasis both in mice (12) and humans (13) showed that the outcome of the infection does not depend on the differential production of Th1- and Th2-derived cytokines. Although IFN-γ secretion is essential for an optimal parasite clearance, IL-4, which exacerbates susceptibility in cutaneous disease (14), has been proven to have no role in the disease progression of the visceralizing species (15, 16). Consequently, studies indicate that IL-10 is the major immunosuppressor cytokine in visceral leishmaniasis (17) and can be secreted by Th2 cells and T regulatory cells (CD4$^+$ CD25$^+$) present in the lesions (18). Thus, IL-10 is a potent inhibitor of IFN-γ secretion, contributing significantly to the chronic persistence of parasites in the host (19).

In the recent years, much interest has been shown in a vaccination against leishmaniasis. The use of live attenuated organisms as vaccine candidates is attractive because it mimics the natural course of infection leading, therefore, to similar immune responses (20). In addition, higher levels of parasite Ags are delivered at the site of the infection than when using subunit vaccines. Indeed,

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vaccination with avirulent organisms achieved better protection than classical immunizations with selected Ags whatever the adjuvants and delivery systems used (21). Several vaccination strategies that lead to obtaining live attenuated organisms have been attempted, including long-term in vitro cultures (22), selection for temperature sensitivity (23), chemical mutagenesis (24), and parasite culture under drug pressure (25). Genetic manipulation of the *Leishmania* genome has also been used and has allowed the selection of parasites lacking genes essential for long-term survival or responsibility for their virulence, such as the genes encoding for dihydrofolate reductase-thymidylate synthetase (26), cysteine proteinase (27), biopterin transporter BT1 (28), and *LPG2* (29).

The protein encoded by the *SIR2* gene belongs to a highly conserved family of closely related proteins in both prokaryotic and eukaryotic species named Hst proteins (homologous of Sir two) or sirTuins (30). Historically, the biological significance of SIR2-like proteins was attributed to histone deacetylation, leading to chromatin condensation and transcriptional silencing (31). However, diverse cellular localizations were found among the diversity of SIR2 homologues, suggesting that these enzymes have physiological substrates other than histones and, thus, several biological functions inside the different organisms. Indeed, several roles have been attributed to them, including cell cycle progression and chromosome stability (32), DNA damage repair (33), and life span extension in yeast (34) and *Caenorhabditis elegans* (35). We have cloned and sequenced the *L. major* and *L. infantum* genes encoding the SIR2 protein bearing the domain structure characteristic of the SIR2 family (36, 37). The expectation was to find the *Leishmania* SIR2 associated with the nucleus; instead, the protein had a cytoplasmic localization. However, further independent studies revealed the presence of SIR2-related proteins in the cytoplasm of different species (31) and, more recently, in the mitochondria (38).

We have generated *L. infantum* silent information regulatory 2 (LiSIR2)[3] single knockout mutant (LiSIR2$^{+/-}$) by using a gene-targeted disruption strategy (37). This gene proved to be essential for the parasite's survival because the deletion of both alleles required an episomal rescue approach. As a result, the mutant parasites showed a marked reduction of proliferation in the axenic amastigote form and of survival, both in vitro inside macrophages and in vivo in BALB/c mice. Because these mutants failed to establish an infection, we tested their ability to provide protection toward a *L. infantum* infection challenge. In this study, we clearly demonstrate that the LiSIR2$^{+/-}$ is able to invade but not persist in visceral organs and that vaccination with such an attenuated strain significantly protects mice from a virulent challenge. Furthermore, a strong correlation was found between the elimination of parasites and an increased IFN-γ/IL-10 ratio, which permitted the development of both cellular and humoral *Leishmania*-specific responses. Thus, we suggest that the balance of these interleukins after challenge is crucial for the development of a protective cellular and humoral response to visceral leishmaniasis.

Materials and Methods

Animals and parasites

Five- to six-week-old male BALB/c mice obtained from Harlan Iberica and CB-17 SCID mice from Charles River France were maintained in the Instituto de Biologia Molecular e Celular (IBMC; Porto, Portugal) and Institut de Recherche pour le Développement (IRD; Montpellier, France). Mice were kept four per cage and allowed food and water ad libitum. All animals entered into experiments at 8 wk of age. Animal procedures were conducted in IRD-approved facilities (license no. 34-18 to A.O.). The project was approved by the local ethical committees on animal experimentation (project no. CE-LR-0602).

Parasites and infections

A cloned line of virulent *L. infantum* (MHOM/MA/67/ITMAP-263, wild type (WT)) was grown at 26°C by weekly subpassages in complete RPMI 1640 culture medium (RPMIc; Invitrogen Life Technologies) supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM HEPES. The heterozygote LiSIR2$^{+/-}$ and the *SIR2* complemented mutants (*HRN1*) derived from a WT clone were generated as previously described and maintained in their respective selective medium (37). The levels of LiSIR2 synthesis in LiSIR2$^{+/-}$, WT, and *HRN1* parasites were evaluated by Western blotting using an anti-LiSIR2 mAb as described in previous reports (37, 39). The WT and *HRN1* mutant clones were maintained in culture as long as the LiSIR2$^{+/-}$ parasites. All strains are frequently passed through mice to maintain virulence. For infection and immunization of mice, stationary phase promastigotes were collected, washed, and suspended in sterile PBS. A volume of 200 μl of PBS containing 10^8 parasites was injected i.p. For challenge infections, age-matched naive mice and mice previously immunized with LiSIR2$^{+/-}$ parasites (6 wk before) were infected with 200 μl of PBS containing 10^8 WT parasites i.p. Additional experiments were conducted using SCID and BALB/c mice infected with either WT or LiSIR2$^{+/-}$ mutant parasites. Briefly, two groups of SCID and BALB/c mice ($n = 6$ for each) were injected i.p. with 10^8 WT ($n = 6$) or 10^8 LiSIR2$^{+/-}$ mutant parasites ($n = 6$). Three mice from each group were sacrificed at 14 and 56 days postinfection. The parasite load was determined as described below.

In independent experiments, male BALB/c mice ($n = 3$) were inoculated i.p. with 10^8 heat-killed LiSIR2$^{+/-}$ mutant parasite clones. Control groups corresponded to mice ($n = 3$) that received a saline solution (PBS) and mice injected with 10^8 HRN1 clones ($n = 3$). Six weeks after immunization, mice were infected i.p. with 10^8 WT parasites. The parasite load was recorded in the liver and spleen 2 wk after the challenge infection as described below.

Parasite quantification

The parasite burden in the spleen, liver, and draining lymph nodes was determined by limiting dilution as previously described (40). Briefly, the organs were removed, homogenized, and resuspended in RPMIc medium and submitted, in quadruplicate, to the serial 2-fold dilutions in 96 microtitration plates. After 15 days of incubation at 26°C, the presence or absence of motile promastigotes was recorded in each well. The final titer was the last dilution for which the well contained at least one parasite. The number of parasites per gram of organ (parasite burden) was calculated as follows: parasite burden = [(geometric mean of reciprocal titer from each quadruplicate cell culture/weight of homogenized organ) $\times$ reciprocal fraction of the homogenized organ inoculated into the first well].

Proliferation assays by [^{3}H]thymidine incorporation

At different times after infection, mice were euthanized and single cell suspensions from the spleens or draining lymph nodes of all experimental groups were made. Cells (1.25×10^6/ml) were plated in 96-well plates (200 μl) in RPMIc medium for 72 h supplemented or not with Con A (6 μg/ml) or soluble *Leishmania* Ag (SLA; 50 μg/ml). Proliferating activity was evaluated after an 8-h pulse with 1 μCi of [^{3}H]thymidine (Amersham Biosciences), and the incorporation of thymidine was assessed by a scintillation counter.

Cytokine production

Spleens or lymph node cells were stimulated or not with Con A (6 μg/ml) or SLA (50 μg/ml) for 24, 48, and 72 h, depending on the cytokine studied, at 37°C under 5% CO_2. The supernatants were assayed for cytokines by ELISA as described elsewhere (41).

Determination of the Ab responses

Sera from all of the experimental groups of mice were analyzed by ELISA for total Ig quantification and the presence of anti-SLA Abs. Ninety-six-well microtitration plates (microtiter immunoplates; Greiner Bioscience) were coated with unlabeled goat anti-mouse Ig Abs at 5 μg/ml (for total titers) or SLA (10 μg/ml) (for *Leishmania*-specific Ab reactivity) in 0.01 M carbonate/bicarbonate buffer (pH 8.5) overnight at 4°C. The plates were washed with PBS containing 0.1% Tween 20 (PBS-T) and blocked with

[3] Abbreviations used in this paper: LiSIR2, *Leishmania infantum* silent information regulatory 2 parasite; LiSIR2$^{+/-}$, LiSIR2 single knockout parasite; RPMIc, complete RPMI 1640 medium; SLA, soluble *Leishmania* Ag; WT, wild type.

PBS plus 1% gelatin (200 μl/ml) for 1 h at room temperature. The plates were incubated at 37°C with serial dilutions (for total titers) or 1/100 dilutions in triplicate (for *Leishmania*-specific Ab reactivity) for 1 h. After washing with PBS-T, the plates were incubated for 30 min at room temperature with peroxidase-labeled goat anti-mouse Ig at 1/8000 dilution. After washing, the plates were developed with 0.5 mg/ml *o*-phenylenediamine dihydrochloride (Sigma-Aldrich) in citrate buffer. The reactions were stopped by the addition of 50 μl of 3 M HCl to each well. Absorbance values were read at 492 nm in an automatic ELISA reader (Power-Wave XS; Bio-Tek Instruments).

Quantification of NO

Macrophages were collected by peritoneal lavage from the different groups of mice. For the assay, macrophages were cultured in a 96-well tissue culture plate at a concentration of 10^6 cells per milliliter with or without SLA (50 μg/ml). After 48 h, a cell-free culture supernatant was collected and the nitrite level was estimated using a Griess reagent system (Promega) according to the manufacturer's protocol.

In vitro cytotoxicity assays

The in vitro cytotoxicity assay was performed by flow cytometry using a LIVE/DEAD cell-mediated cytotoxicity kit (Molecular Probes,/Invitrogen Life Technologies) according to the manufacturer's protocol. A J774 macrophage cell line incubated in vitro for a period of 10 h with *L. infantum* (the infection was confirmed by Giemsa staining) was used for the target cells (42). Nonadherent splenocytes from the different experimental groups of mice were stimulated with SLA for 7 days, after which they were recovered and used in the cytotoxic assay. Briefly, effector cells were incubated for 4 h with 3,3-dioctadecylloxacarbocyanine ($DiOC_{18}$) target cells in the presence of propidium iodide at different E:T cell ratios. The percentage of corrected specific cytotoxicity was calculated according to the following equation: [(dead target cells/all target cells)$_{+\text{effectors}}$ − (dead target cells/all target cells)$_{-\text{effectors}}$] × 100.

Polymerase chain reaction

PCR was performed using DNA samples isolated from the spleens and livers recovered from WT and LiSIR2$^{+/-}$-immunized and protected mice and two oligonucleotides derived from the *L. infantum* kinetoplast DNA sequence described elsewhere (43).

Statistical analysis

The data were analyzed using Student's *t* test.

Results

LiSIR2$^{+/-}$ is a live attenuated strain that can invade but not persist in visceral organs

We previously reported (37) that the LiSIR2$^{+/-}$ mutant is unable to sustain an infection in the spleen of susceptible BALB/c mice. This incapacity is a direct consequence of *SIR2* gene deficiency, because mice infected with mutants rescued with an episomal *SIR2* gene (*HRN1* clone) reach similar levels of parasite burden as do mice infected with WT parental clone (Fig. 1*A*). To further dissect the organ-specific infectivity of these live attenuated parasites, groups of four BALB/c mice were injected i.p. with 1×10^8 stationary-phase WT, LiSIR2$^{+/-}$, or *HRN1* promastigotes and the parasite burden was measured in the spleen, liver, and draining lymph nodes up to the end of the 8th wk postinfection (Fig. 1). Two weeks after parasite inoculation, similar levels of infection were observed for all strains in both the spleen and liver (Fig. 1, *A* and *B*). Interestingly, the infection of draining lymph nodes is transient in WT-infected mice, whereas it is only detectable before 20 days postinfection in the case of *HRN1* clone-infected mice and virtually not detectable in mice infected with the LiSIR2$^{+/-}$ mutants (Fig. 1*C*). The parasite burden remained highly stable in the spleen and the liver of both WT- and *HRN1*-infected mice up to the 8th week. Mice infected with LiSIR2$^{+/-}$ mutants showed a progressive decline of the parasite burden, and no parasites could be detected in any of the visceral organs from the 8th wk. Taken together, these results suggest that the inactivation of one *SIR2* gene allele does not affect the capacity of *L. infantum* parasites to invade visceral organs but dramatically impairs parasite persistence in these organs.

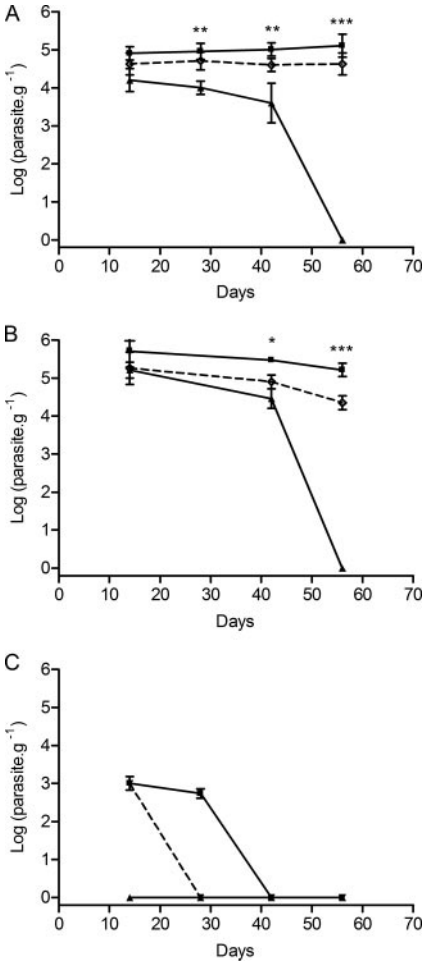

FIGURE 1. Infection with LiSIR2$^{+/-}$ (—▲—), WT (—■—) and *HRN1* (—◇—) parasites. Course of parasite burden progression in the spleen (*A*), liver (*B*), and draining lymph nodes (*C*) of BALB/c mice infected with 1×10^8 stationary phase promastigotes of WT, LiSIR2$^{+/-}$, or *HRN1*. The results are from a representative experiment of three conducted independently. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ between WT and LiSIR2$^{+/-}$ infections.

To further explore whether the clearance of mutant parasites could also occur in the absence of a functional immune system, SCID mice lacking functional T and B cells were infected with WT or LiSIR2$^{+/-}$ mutant parasites. As shown in Fig. 2, at 14 days postinfection SCID mice developed a similar parasite load in the spleen (Fig. 2*A*) and liver (Fig. 2*B*) as the BALB/c mice did whatever the parasite clone used (WT vs LiSIR2$^{+/-}$). However, at 56 days postinfection BALB/c mice were able to clear the LiSIR2$^{+/-}$ mutant from the liver and spleen but not the WT parasites, whereas the SCID mice still developed high LiSIR2$^{+/-}$ and WT parasite loads in the spleen and liver. Thus, these observations suggest that the resolution of LiSIR2$^{+/-}$ is likely to be due to both parasite and host factors.

Vaccination with LiSIR2$^{+/-}$ parasites resulted in an efficient clearance of hepatic and splenic parasite burden

Groups of four age-matched naive and vaccinated BALB/c mice were challenged i.p. 6 wk postimmunization with 1×10^8 virulent *L. infantum* promastigotes. Subsequent assessments of parasite burden in the spleen (Fig. 3*A*) and the liver (Fig. 3*B*) were made in

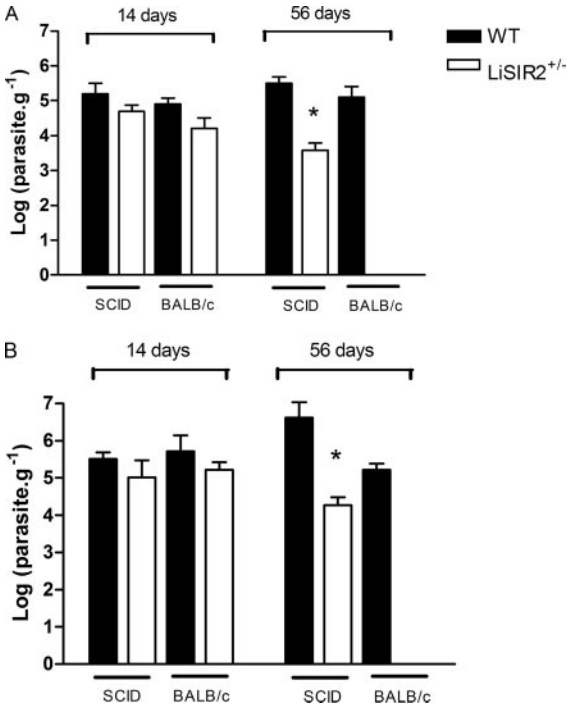

FIGURE 2. Comparative analysis of parasite burdens in the spleen (*A*) and liver (*B*) of CB-17 SCID and BALB/c mice infected with 1×10^8 stationary phase promastigotes of WT or LiSIR2$^{+/-}$ at 14 and 56 days postinfection. *, $p < 0.05$ between WT and LiSIR2$^{+/-}$ infections in CB-17 SCID mice.

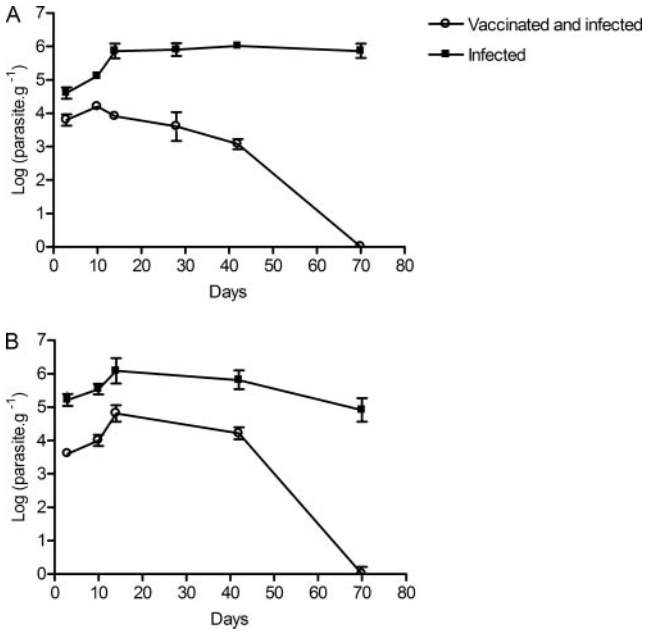

FIGURE 3. Vaccination of BALB/c mice with LiSIR2$^{+/-}$ parasites confers complete protection against a virulent challenge. Age-matched naive and 6-wk LiSIR2$^{+/-}$ mutant-vaccinated mice were challenged with 1×10^8 virulent *L. infantum* promastigotes and the parasite burden was followed in the spleen (*A*) and liver (*B*) of BALB/c mice for 70 days (10 wk). The data represent the means and SD of four animals analyzed individually and are representative of two independent experiments. At all points, $p < 0.01$.

naive and vaccinated mice at different times postchallenge. Immunization with LiSIR2$^{+/-}$ significantly reduces the parasite load as early as 3 days postchallenge as compared with the naive mice group. This immunization can confer a striking resistance to challenge, as observed from the continuous reduction of parasite burden, both in the liver and the spleen, in vaccinated mice after the 15th day postchallenge. Remarkably, at the end of the experiment (70 days postchallenge) a complete absence of parasites was observed in all LiSIR2$^{+/-}$-vaccinated mice. DNA from tissues samples was extracted and subjected to PCR using oligonucleotides derived from a *Leishmania* kinetoplast DNA sequence (43). In contrast to the samples recovered from WT-infected mice in which positive PCR was observed, no positive signals could be evidenced in the case of immunized protected mice (data not shown). Altogether, our results demonstrate that immunization with the LiSIR2$^{+/-}$ protect mice from a *Leishmania* infection.

Complementary experiments were done using three groups of mice ($n = 3$) injected i.p. with either 10^8 heat-killed LiSIR2$^{+/-}$ mutant parasites, *HRN1* clones, or PBS alone. Two weeks later they were challenged with 10^8 WT parental clones. Although a moderate reduction of parasite load was observed in the spleens of LiSIR2$^{+/-}$-immunized mice after 14 days after the challenge infection ($p > 0.05$), no significant difference in the parasite load in the liver could be seen between LiSIR2$^{+/-}$-immunized and infected mice and nonimmunized infected ones or those immunized with an HRN1 clone (Fig. 4). These observations suggest that the in vivo protection requires immunization with live LiSIR2$^{+/-}$ mutant parasites.

Vaccination of mice with the live attenuated LiSIR2$^{+/-}$ strain is associated with a reversion of T cell anergy

Visceral leishmaniasis is characterized by the impairment of cell-mediated responses reflected by a marked T cell anergy to *Leishmania*-specific Ags (44). We investigated the capacity of splenocytes to proliferate in response to SLA or to Con A, a T cell mitogen. During these experiments we observed that the number of spleen cells isolated 14 days after challenge is significantly higher in infected mice ($21.7 \pm 0.7 \times 10^7$) than in protected ($17.5 \pm 1.1 \times 10^7$) or naive ones ($14.4 \pm 1.3 \times 10^7$) ($p < 0.01$). Although cells from infected or vaccinated and infected mice were able to respond to Con A stimulation, only spleen cells from vaccinated mice had the potential to proliferate in response to SLA at 42 and 70 days postchallenge, suggesting therefore that a memory response is maintained over a long period after vaccination with the LiSIR2$^{+/-}$ parasites (Table I).

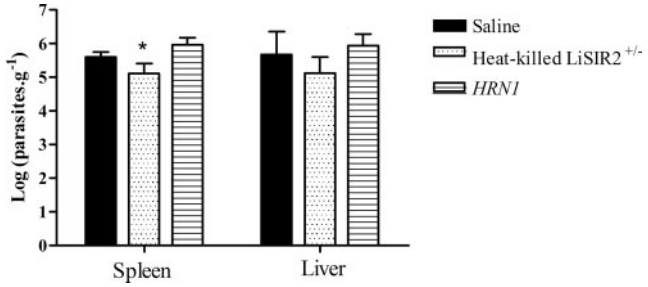

FIGURE 4. Efficient protection requires immunization with live LiSIR2$^{+/-}$ parasites. Mice were previously immunized i.p. with 1×10^8 heat-killed LiSIR2$^{+/-}$ or *HRN1* stationary-phase promastigotes; a group of control mice were injected with saline solution (PBS). Six weeks after immunization, mice were challenged with 1×10^8 virulent WT *L. infantum* promastigotes and the spleen and liver parasite burden was measured at 14 days postchallenge. *, $p < 0.05$.

Table I. *Vaccination of BALB/c mice with LiSIR2$^{+/-}$ parasites induces* Leishmania*-specific proliferative responses and recovery of T cell anergy*[a]

	Cell No. ($\times 10^7 \pm$ SD)	Proliferation (cpm $\pm$ SD)		
		Medium	SLA	Con A
Uninfected	14.4 $\pm$ 1.3	75.7 $\pm$ 3.1	76.97 $\pm$ 2.4	66710.3 $\pm$ 3.2
Vaccinated and infected	17.5 $\pm$ 1.1	325.5 $\pm$ 2.1	1307.9 $\pm$ 49.1	76574.0 $\pm$ 8853.3
Infected	21.7 $\pm$ 0.7	164.7 $\pm$ 25.3	201.5 $\pm$ 18.1	50829.8 $\pm$ 3171.0

[a] Age-matched naive and 6-wk *Li*SIR2$^{+/-}$ mutant-infected mice were challenged with a WT virulent clone ($\times 10^8$) and sacrificed after 14 days postinfection. The total number of spleen cells was enumerated. Cells were stimulated with SLA (50 μg/ml) or ConA (6 μg/ml) for 72 h and pulsed with [^{3}H]thymidine for the last 8 h as described in *Materials and Methods*. Cell proliferation was measured by the amount of thymidine incorporated in the cells. The data represent mean cpm $\pm$ SD from triplicate cultures of four animals analyzed individually and are representative of three independent experiments.

Protection induced by the live attenuated LiSIR2$^{+/-}$ parasites is associated with an increased IFN-γ/IL-10 ratio

IFN-γ and IL-10 are currently thought to be essential cytokines governing the fate of the infection in visceral leishmaniasis. To assess the immune correlations that contribute to the protection conferred by the LiSIR2$^{+/-}$, we focused on the study of secreted IFN-γ, a cytokine associated with the control of the *Leishmania* infection, and IL-10, the major suppressive interleukin in visceral leishmaniasis. For that purpose, we determined the level of IFN-γ and IL-10 produced by spleen cells after an ex vivo restimulation with SLA (50 μg/ml) or Con A (6 μg/ml) over the time course of the experiment. A number of studies have reported suppressed responses toward an ex vivo *Leishmania* stimulus in infected animals, and controversial results were observed when using mitogens such as Con A (45). Indeed, while some studies showed that spleen cells from infected mice maintain intact the responses to Con A (46, 47), others demonstrated an absence or a defective response (48). We observed that the restimulation of spleen cells with ConA was effective whatever the group of mice considered. Analysis of these results suggest that, upon stimulation, as early as

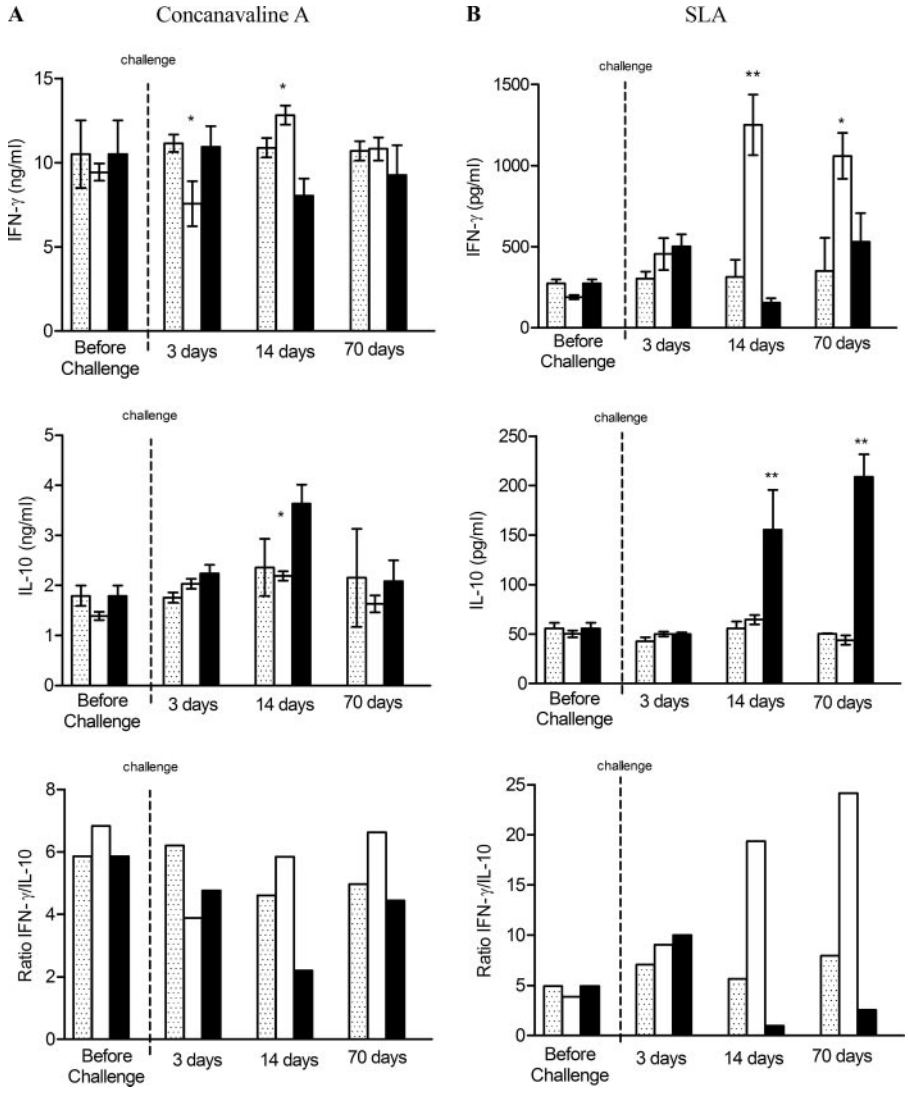

FIGURE 5. Protection induced by LiSIR2$^{+/-}$ parasites is associated with increased production of IFN-γ and down-regulation of IL-10 secretion. At 14 days postchallenge, uninfected, vaccinated and infected, and infected mice were euthanized and spleen cells were restimulated ex vivo with Con A (6 μg/ml) (*A*) or SLA (50 μg/ml) (*B*). Supernatants were removed after 48 and 72 h and the production of IL-10 and IFN-γ was determined by ELISA. For each stimulus a ratio of IFN-γ/IL-10 in culture supernatants is presented. The results are from a representative experiment of three conducted independently. *, $p < 0.05$; **, $p < 0.01$.

Table II. *Spleen cell responses from mice vaccinated with LiSIR2$^{+/-}$ mutants but not challenged with the WT parasites*[a]

	Con A			SLA		
	IFN-γ	IL-10	Ratio	IFN-γ	IL-10	Ratio
Uninfected	10.88 ± 0.58	2.36 ± 0.57	4.61	313.40 ± 106.49	55.65 ± 6.91	5.63
Vaccinated and infected	12.82 ± 0.56	2.19 ± 0.09	5.85	1250.80 ± 186.65	64.6 ± 4.78	19.36
Infected	8.03 ± 1.01	3.63 ± 0.38	2.21	153.50 ± 27.99	155.25 ± 40.34	0.99
Vaccinated but not infected	9.89 ± 0.23	2.15 ± 0.21	4.6	307.7 ± 56.0	67.53 ± 14.99	4.56

[a] Age-matched naive and 6-wk LiSIR2$^{+/-}$ mutant-infected mice were challenged or not with WT virulent clone ($\times 10^8$) and sacrificed after 14 days postinfection. Cells were stimulated with SLA (50 μg/ml) or ConA (6 μg/ml) for 72 h and the levels of secreted IFN-γ and IL-10 were measured by ELISA as described in *Materials and Methods*. The IFN-γ and IL-10 values represented under Con A stimulation are in ng/ml and those represented under SLA stimulation are in pg/ml. The corresponding IFN-γ /IL-10 ratio is shown for each group of mice. The data represent means ± SD from triplicate cultures of four animals analyzed individually and are representative of three independent experiments.

14 days postinfection the spleen cells from infected BALB/c mice secreted significantly lower amounts of IFN-γ and higher levels of IL-10 ($p < 0.05$) than the spleen cells of naive or protected mice. However, during the chronic phase (70 days postinfection) the stimulation of spleen cells with Con A lead to the production of about the same level of IFN-γ or IL-10 regardless of the group of mice considered (Fig. 5*A*). The IFN-γ/IL-10 ratio was in general higher in vaccinated mice than in naive or infected animals. To clarify the status of the IFN-γ/IL-10 ratio in protected animals, restimulation experiments using SLA were performed (Fig. 5*B*). Strikingly, following stimulation the level of IFN-γ production is significantly enhanced in protected mice (Fig. 5*B*). By contrast, in infected animal a large increase in IL-10 production was ascertained (Fig. 5*B*). A nonsignificant increase in the levels of IL-2 and IL-4 was detected in the spleen cell supernatant derived from vaccinated mice (IL-2: 173.8 ± 78.7 pg/ml; IL-4: 58.5 ± 12.4 pg/ml) as compared with control infected ones (IL-2: 120.6 ± 46.2 pg/ml; IL-4: 44.8 ± 8.7 pg/ml). Thus, it is reasonable to assume that the protection observed in mice immunized with LiSIR2$^{+/-}$ parasites could not be attributed to a dominance of a Th1-derived over a Th2-derived cytokine but instead to an efficient production of IFN-γ associated with a down-regulation in the IL-10 secretion. Spleen cell responses from a fourth group of mice vaccinated with LiSIR2$^{+/-}$ mutants but not challenged with the WT parasites upon their stimulation with Con A or SLA ex vivo at 14 days postchallenge infection are shown in Table II. Comparable levels of IFN-γ and IL-10 production as those in cells from normal mice were recorded upon stimulation with ConA or SLA in the spleen cells from mice vaccinated with LiSIR2$^{+/-}$ mutants but not challenged with the WT parasites. In contrast, a challenge infection of LiSIR2$^{+/-}$-immunized mice with WT parasites led to a significant increase of IFN-γ production by spleen cells upon their ex vivo stimulation with SLA, whereas the production of IL-10 was on the same order as that of normal or LiSIR2$^{+/-}$-immunized but not challenged mice. Increased production of IFN-γ could not be evidenced in the case of mice infected with WT parasites. We thus assume that the protection observed after vaccination with the live attenuated LiSIR2$^{+/-}$ strain is associated with an unbalanced IFN-γ/IL-10 ratio due to an IFN-γ secretion 20–25 times greater than that of IL-10.

Protection induced by the live attenuated LiSIR2$^{+/-}$ parasites is associated with the production of reactive nitrogen intermediates

The unbalanced IFN-γ/IL-10 ratio can favor an environment leading to macrophage activation, thus affecting the outcome of the infection in favor of the host. NO is a critical macrophage-derived effector molecule for the control of *Leishmania* infections (49). Remarkably, a significant nitrite production (at 14 and 70 days postchallenge; $p < 0.01$) was observed in protected mice even without SLA stimulation. This increased NO level in protected mice was not observed before the challenge infection, even in the presence of SLA (Fig. 6). Mouse peritoneal macrophages were more prone to produce NO after SLA stimulation, 70 days postchallenge (15.5 vs 9.98 μM after 14 days postinfection; $p < 0.001$). This period appears to be critical, because it corresponds to the clearing of the parasites. Overall, the sustained NO production observed can be attributable to the shift in the secretion of IFN-γ over IL-10, leading to an increased IFN-γ/IL-10 ratio that favors macrophage activation.

*Vaccinated mice present an anti-*Leishmania *cytotoxic response*

Leishmania-specific cell-mediated cytotoxicity is a part of the acquired immune response developed against the parasite (50). This cytotoxic activity can be attributable to Ag-specific CTLs or nonspecific cells such as NK cells and macrophages. Thus, we examined whether, in addition to the unbalanced cytokine response observed, a cytotoxic effector mechanism was generated during LiSIR2$^{+/-}$ immunization and challenge infection. Spleen cells recovered at 14 days postinfection from different groups of mice were enriched in *Leishmania*-specific clones by stimulation ex vivo with SLA for 7 days. Restimulated cells were subsequently tested for their cytotoxic activity against *L. infantum*-infected macrophages (J774, a murine cell line). As shown in Fig. 7, nonadherent spleen cells from protected mice were able to induce a dose-dependent, significant lysis of target cells (34.3 and 44.5% lyses of

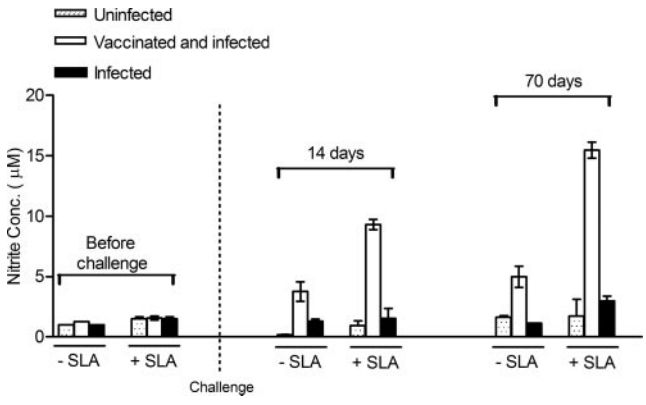

FIGURE 6. Production of reactive nitrogen intermediates in LiSIR2$^{+/-}$ vaccinated mice increases after challenge. Peritoneal macrophages derived from the different experimental groups were recovered before challenge and at 14 and 70 days postchallenge. After ex vivo restimulation with SLA (50 μg/ml) or without SLA for 48 h, nitrite generation was measured as described in *Materials and Methods*. The results are from a representative experiment of two conducted independently.

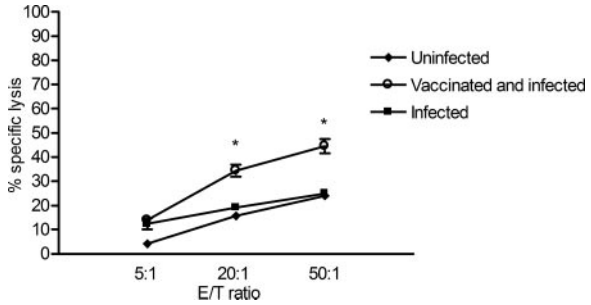

FIGURE 7. LiSIR2$^{+/-}$ vaccination generates anti-*Leishmania*-specific cytotoxic responses. Spleen cells from uninfected, vaccinated and infected, and infected mice were isolated and subjected to an in vitro stimulation with SLA (50 μg/ml). After 7 days, the nonadherent cells were recovered and tested for *Leishmania*-specific cytotoxic activity. A *L. infantum*-J774 macrophage-infected cell line was used for the target cells. Data represent the mean ± SD of a representative experiment from two carried out independently, with four mice per experimental group in each experiment. *, $p < 0.01$ in comparison with uninfected and infected groups.

target cells at 20:1 and 50:1 E:T ratio, respectively). Nonadherent spleen cells recovered in naive or infected control mice induced the lysis of ~20 and 25% of target cells at the higher E:T ratios tested. These results support the notion of a *Leishmania* specificity of the cytotoxic cells. Although the phenotypes of the cytotoxic cells have not been characterized, it is likely that the cytotoxic activity is a part of the mechanisms leading to the parasite clearance.

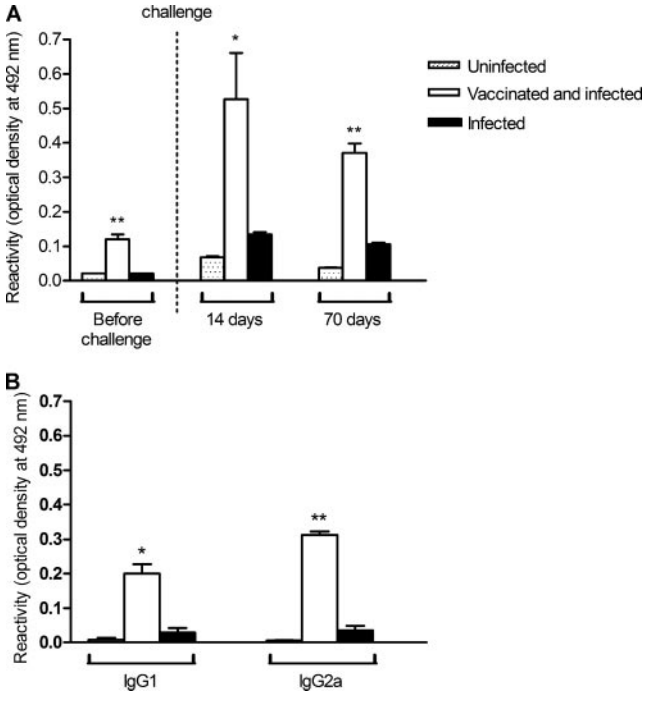

FIGURE 8. Production of specific immunoglobulins in LiSIR2$^{+/-}$-immunized mice. *A*, Sera recovered from uninfected, vaccinated and infected, and infected mice were recovered before challenge and at 14 and 70 days postchallenge were analyzed individually for *Leishmania*-specific IgG reactivity by ELISA. *B*, The anti-*Leishmania* IgG1 and IgG2a isotype analysis was performed at 14 days postchallenge. These results are representative of two experiments and the data represent the mean ± SD. Statistically significant differences between LiSIR2$^{+/-}$ vaccinated and infected and infected values are indicated: *, $p < 0.01$; and **, $p < 0.001$.

LiSIR2$^{+/-}$ live attenuated parasite inoculation into BALB/c mice induced a mixed Th1/Th2 humoral response

Given that there is no clear evidence of an in vivo role for specific Abs in determining the outcome of visceral leishmaniasis (51), it was of interest to investigate whether the vaccination with LiSIR2$^{+/-}$ parasites permitted the development of a *Leishmania*-specific humoral response. Sera collected at various times before and after the infective challenge (14 and 70 days postinfection) were examined for the presence of anti-*Leishmania* Abs. The nature, the isotypes, and the levels of such Abs were determined by ELISA. As shown in Fig. 8*A*, before the challenge infection LiSIR2$^{+/-}$-immunized mice showed a mean OD value for total specific IgG five times higher than that for the control group (infected and naive mice; $p < 0.001$). Fourteen days after the challenge infection, the protected mice developed significant levels of Abs. Indeed, the OD value increased up to five times of that observed before the challenge. In contrast, low OD values were recorded for infected mice before and after the challenge infection, and this is true for the control noninfected mice as well. Moreover, until the end of the experiment the levels of *Leishmania*-specific IgG in vaccinated and infected mice were just slightly decreased but remained significantly higher when compared with those of the control groups. The isotype and subclass analyses showed that both IgG1 and IgG2a were produced in vaccinated mice (Fig. 8*B*).

Discussion

An effective vaccine would be of major importance in the control of leishmaniasis. Its development has been a goal for many years. In some areas, the inoculation of infectious material isolated from cutaneous lesions or cultured virulent promastigotes (a process called leishmanization) in hidden parts of uninfected individuals has been used to prevent further lesions (52). In time, these individuals developed a strong immunity to reinfection, sometimes only after unpleasant clinical episodes. These observations suggest that the use of live attenuated parasite strains may well be considered among the most promising approach for an anti-*Leishmania* vaccine.

The inactivation of one allele of SIR2 in *L. infantum* gave rise to in vitro attenuation of the parasite's virulence. Thus, we decided to test its protective capacity and dissect the mechanism leading to the resistance to reinfection in the BALB/c mice model. Although BALB/c mice are susceptible to visceral leishmaniasis, the development of the infection is self-controlled (53). These mice are considered a better model for subclinical infection than for progressive disease (6). We thus decided to follow the experiment up to the 70th day (10 wk) postchallenge, a period during which parasites are still detectable both in the liver and in the spleen. Also, the i.p. route was chosen because it has been proven to result in a higher homogeneity among infections than the i.v. one (54). In this model, LiSIR2$^{+/-}$ parasites, which were unable to cause disease in susceptible BALB/c mice, did induce protection against infection with virulent *L. infantum* challenge. Indeed, all of the immunized mice subjected to a challenge with virulent *L. infantum* controlled the infection and were able to reduce the parasite burden in visceral organs, achieving elimination of parasites after 70 days after the challenge infection. Strikingly, even after 3 days postchallenge parasites were never recovered in the lymph nodes of the protected mice.

All vaccinated mice developed an active T cell *Leishmania*-specific response after challenge, this response being severely impaired in infected mice. Spleen cells isolated from *L. infantum*-infected animals respond to Con A stimulation but not to

Leishmania Ag stimulus. If previously submitted to LiSIR2$^{+/-}$ vaccination, spleen cells recovered the lymphoproliferative reactivity to SLA stimulation. Thus, immunization with LiSIR2$^{+/-}$ parasites allowed the reestablishment of cellular responses toward *Leishmania* stimulus.

Clearly in the mouse model, despite the presence of cytokines produced by the two Th subsets, the overshadowing of the Th2 response by a Th1 cell-associated response leads to the control of the infection (15, 29). Epidemiologic studies of human visceral leishmaniasis suggested that infected individuals who spontaneously control the infection preferably respond to *Leishmania* Ags with increased production of IFN-γ rather than Th2-derived cytokines (55). These facts prompted us to study the immune response profile after the challenge in mice vaccinated with LiSIR2$^{+/-}$ parasites. Our data showed that in the acute phase (14 days after challenge) where differentiation of the CD4$^+$ subsets had already occurred, although spleen cells from LiSIR2$^{+/-}$-immunized mice that were not challenged with WT parasites were able to produce levels of IFN-γ and IL-10 similar to those of normal spleen cells in response to stimulation ex vivo with parasite Ags, upon challenge infection with WT parasites they were able to down-regulate the IL-10 secretion and significantly increase IFN-γ production in response to *Leishmania* Ags as well as to Con A stimulation. This contrasts with spleen cells from infected BALB/c mice that, when subjected to the same stimuli, secreted large quantities of IL-10 and low amounts of IFN-γ. This response is maintained over the time of the experiment, supporting the notion that an effective cellular immune response is maintained during the chronic phase. The use of IL-10 knockout (17) or transgenic (56) mice has demonstrated an association of IL-10 production with susceptibility to leishmaniasis in experimental murine infections. IL-10 is a pleiotropic Th1 suppressive cytokine able to block macrophage activation, thus preventing Ag-specific T cell stimulation and limiting IFN-γ production (57). But IL-10 seems to be also able to directly promote parasite survival through it influence on the macrophage activation state (58). It is hypothesized that the IFN-γ/IL-10 ratio should be a prechallenge indicator of vaccine success in a cutaneous leishmaniasis (58); this seems also to be the case in a visceral model. Analysis of the cytokine profile showed that the IFN-γ/IL-10 ratio was also increased in comparison to uninfected and infected mice as soon as 3 days postchallenge. Thus, the high IFN-γ/low IL-10 levels observed after challenged in LiSIR2$^{+/-}$ vaccinated mice is necessary for the control of the infection. Although the secretion levels of IL-4, a known Th2 cytokine, in vaccinated mice were not statistically different from those observed in infected mice, the mean values were higher in the former. The importance of IL-4 in controlling visceral leishmaniasis has already been ruled out. Disruption of IL-4 or IL-4Rα did not increase control over visceral leishmaniasis (16, 59). Altogether, we reaffirm the importance of controlling IL-10 secretion but also suggest that the balance between IFN-γ and IL-10 after infection, rather than the Th1 and Th2 cytokines, will define the outcome of the infection.

Peritoneal macrophages from vaccinated and infected mice secrete significant levels of basal NO that extended to higher values when cells were submitted to an ex vivo SLA stimulation. NO production was virtually absent in the case of cells from uninfected and infected groups. NO has a central role in the parasite clearance (60), and the down-regulation of its synthesis was correlated with susceptibility (61). Thus, it might be assumed that in infected mice the increased levels of IL-10 could create an immunosuppressive environment that prevents the production of NO by macrophages. Thus, the shift to IFN-γ production in LiSIR2$^{+/-}$-immunized mice led to the activation of macrophages and contributed to parasite elimination.

The high percentage of specific lysis of the *L. infantum*-infected J774 murine macrophage line suggests that an anti-*Leishmania* cytotoxic response is generated in protected mice. The lysis of parasitized macrophages has been observed in mice (62), hamsters (63), dogs (64), and humans (65) and might be an important mechanism involved in resistance to leishmaniasis. Although the characterization of the cells involved in the cytotoxic activity observed in the present study was not done, several reports have shown that both class I-restricted cytotoxic CD8$^+$ and CD4$^+$ T cells as well as NK cells are potential effector cells. Further studies are needed to determine which of these cells are responsible for the cytotoxic activity in LiSIR2$^{+/-}$-immunized mice.

In experimental leishmaniasis, the specificity of the humoral response and the characterization of the isotype profile after immunization and/or after challenge were determined because they constituted convenient substitute markers of Th1 and Th2 T cell differentiation (66). Our data reaffirmed the absence of an effective *Leishmania*-specific response in infected mice. Immunization with LiSIR2$^{+/-}$ parasites by itself did not induce high levels of specific *Leishmania* Abs; however after challenge a significant increase of *Leishmania*-specific IgG was found in the vaccinated mice when compared with the infected group. Moreover, at 14 days postchallenge the IgG isotype measurements showed equivalent levels of IgG1 and IgG2a Abs. This corroborated the mixed Th1/Th2 cytokine profile observed in vaccinated and infected mice. Although the real contribution of Abs is still under debate, one can speculate that the binding of specific Abs to the parasite could occur when the amastigotes are released from the infected macrophages (51). Subsequent interaction of Ab-opsonized parasites with effector cells could contribute to reduction of the parasite burden.

Our observations demonstrate that LiSIR2$^{+/-}$ parasites were able to colonize but not develop in animals, rendering them unable of causing disease in BALB/c mice. However, when injected into immunodeficient mice, LiSIR2$^{+/-}$ mutants could maintain in vivo for longer period than in immunocompetent mice. Furthermore, our data showed that the immunoprotection induced by immunization required live LiSIR2$^{+/-}$ parasites as immunizing agents. Because the cellular and humoral effector mechanisms were developed only after challenge infection, it is likely that the resolution of LiSIR2$^{+/-}$ infection might be due to combined effects resulting from: 1) the inability of the amastigote form to proliferate; and 2) the action of the host immune system.

Overall, our results demonstrate that vaccination with LiSIR2$^{+/-}$ parasites provided a high degree of protection against virulent *L. infantum* challenge. The unbalanced secretion of IFN-γ and IL-10, which allowed the development of cellular and humoral anti-*Leishmania* responses, led to an optimal elimination of the parasites. Taken together, these results suggest that LiSIR2$^{+/-}$ mutant parasites could not only be among the potential candidates for vaccine development but could also be an interesting biological model for exploring further the immune response correlations to the protective effect against visceral leishmaniasis.

Disclosures

The authors have no financial conflict of interest.

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