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Repeated inoculation of killed *Leishmania major* induces durable immune response that protects mice against virulent challenge

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ABSTRACT

It is widely believed that persistence of live parasites at the primary site of infection is important for maintenance of anti-*Leishmania* immunity. However, whether this immunity requires only the presence of antigen and not necessarily live replicating parasites has not been investigated. To determine whether non-replicating antigens could induce and maintain anti-*Leishmania* immunity, we inoculated naïve mice with killed parasites (once or 5 times weekly) either alone or in combination with rIL-12 and challenged them with virulent *Leishmania major* parasites at different times after inoculation. We found that similar to mice that recovered from virulent live *L. major* infection, mice inoculated repeatedly with killed parasites were protected against virulent *L. major* challenge. The protection obtained following 5 weekly inoculations of killed parasites was associated with strong antigen-specific IFN-γ production by cells from the lymph nodes draining the inoculation site. In contrast, mice that received a single or double inoculation of killed parasites either alone or followed with repeated rIL-12 injection were not protected. Repeated antigen inoculation resulted in increased numbers of the IFN-γ-secreting $CD44^{+}CD62L^{-}$ T cells that were comparable in magnitude to that seen in mice with persistent infections. Overall, these results suggest that it is possible to generate and maintain anti-*Leishmania* immunity for a relatively long period of time in the absence of live replicating parasites. However, a certain threshold of effector cells has to be generated in order to achieve this protection.

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1. Introduction

Human leishmaniasis affects more than 12 million people worldwide. According to WHO, close to 1.5–2 million new cases occur annually and 350 million people are considered to be at risk [1,2]. Although, our knowledge of the innate and adaptive immune responses to *Leishmania* parasites continue to grow, there is still no effective vaccine against the disease in humans. It is known that healing from natural or experimental infection with *Leishmania major*, the causative agent of Old World cutaneous leishmaniasis, leads in most cases to long-lasting immunity [3]. This so called infection-induced immunity has been associated with persistence of a small number of parasites at the infection site [4,5]. Manipulations that result in clearance of persistent parasites [4,5] or expansion of regulatory T cells [6] lead to loss of immunity following secondary virulent challenge. For example, IL-10 deficient mice, which are able to achieve sterile cure are highly susceptible to secondary *L. major* challenge [5]. Similarly, infection of the relatively susceptible BALB/c mice with ultra low dose of *L. major* results in resistance that is associated with an exclusive Th1 response, complete parasite clearance and susceptibility to secondary virulent parasite challenge [4]. These results lead to the conclusion that persistent live parasites are required for maintenance of immunity (and hence immunologic memory) in cutaneous leishmaniasis. However, it is unclear whether live replicating parasites or just the persistence of antigen (whether live or killed) is required for this process.

Interleukin 12 (IL-12) promotes the development of naïve $CD4^{+}$ T cells into Th1 cells *in vitro* and *in vivo*. Exposure of naïve $CD4^{+}$ T cells to antigens in the presence of IL-12 results in preferential expansion of IFN-γ-producing $CD4^{+}$ T cells [7]. In line with this, vaccination of BALB/c mice with *Leishmania* antigens in the presence of IL-12 promotes the development of *Leishmania*-specific $CD4^{+}$ Th1 cells and subsequent protection from virulent *L. major* challenge [8]. Treatment of infected Th2-prone highly susceptible BALB/c mice with rIL-12 results in development of Th1 cells and resistance to *L. major* infection [9,10]. In contrast, targeted deletion of IL-12p40 or neutralization of IL-12 by administration of anti-IL-12 mAb results in development of progressive cutaneous lesion and uncontrolled parasite proliferation in *L. major*-infected normally resistance C57BL/6 mice [11–15]. Interestingly, while treatment of IL-12p40 deficient mice with rIL-12 results in resistance, recrudes-

Abbreviations: dLN, draining lymph node; DTH, delayed-type hypersensitivity; SLA, soluble *Leishmania* antigen.

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cence occurs few weeks after cessation of treatment, suggesting that IL-12 is important not only during induction but also for maintenance of anti-*Leishmania* immunity [11–13]. Whether this requirement involves persistent live parasites is not known.

The aim of the present study was to determine whether live parasites are indispensable for induction and/or maintenance of anti-*Leishmania* immunity or whether they simply act to provide a source of antigen depot for continuous restimulation and maintenance of effector cells that mediate immunity. In addition, we also wished to determine whether rIL-12, which has been shown to play critical role in the generation and maintenance of anti-*Leishmania* effector Th1 cells [11–15], is capable of maintaining anti-*Leishmania* immunity in the absence antigen. We show here that it is possible to generate and maintain anti-*Leishmania* effector cells in the absence of live replicating parasites and that IL-12 by itself is not capable of maintaining anti-*Leishmania* immunity. We discuss the implication of these findings in the context of vaccine design and vaccination strategies against cutaneous leishmaniasis.

2. Materials and methods

2.1. Mice

Six to eight week-old female C57BL/6 mice were purchased either from Charles River Laboratory, St. Constante, Quebec or from the University of Manitoba Central Animal Care Services (CACS) breeding facility. All mice were maintained in specific-pathogen free environment at the CACS and were used according to the guidelines stipulated by the Canadian Council for Animal Care.

2.2. Parasites

L. major parasites (MHOM/IL/80/Friedlin), were grown in Graces insect or M199 medium (Invitrogen, Life Technologies, Burlington, Ontario, Canada) supplemented with 20% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 25 mM HEPES (complete parasite medium). All media additives were purchased from Invitrogen. Killed *L. major* parasites were made by 6–8 cycles of freezing (−80 °C) and thawing (56 °C) stationary phase (day 7) promastigotes.

2.3. Vaccination and challenge

Groups of mice were inoculated in the right hind footpad with 5×10^6 live or killed (freeze-thawed) stationary phase (day 7) promastigotes with rIL-12 (0.5 μg, PeproTech, Rocky Hill, NJ) resuspended in 50 μl PBS. Some mice inoculated with killed parasites and rIL-12 further received weekly inoculations of either killed parasites alone for 1 or 4 additional weeks or rIL-12 (0.5 μg/mouse) twice weekly for additional 5 weeks in their footpad. Vaccinated mice were challenged in the left footpads with 5 million virulent *L. major* at 3, 6 or 13 weeks after the last inoculation and delayed-type hypersensitivity (DTH) response and parasite burden were assessed.

2.4. DTH response and determination of parasite burden

DTH response in challenged mice was determined by measuring footpad swelling at 72 h post-challenge using digital calipers (Fisher Scientific, Mississauga, Ontario, Canada). Three weeks after virulent challenge, mice were sacrificed and parasite burden in the challenged footpads was determined by limiting dilution as previously described [16]. Briefly, the footpads were collected and homogenized in 2 ml complete phosphate buffered saline (PBS) using 15 ml tissue grinders (VWR, Edmonton, AB, Canada). The suspension was then plated in 96-well plates in triplicates at 10-fold serial dilution, incubated for 7 days at 27 °C and assessed for parasite growth under a microscope.

2.5. In vitro cell cultures for determination of cytokine response

At sacrifice, the draining lymph nodes were harvested and made into single cell suspensions. Cells were washed, resuspended at a final concentration of 4 million/ml in complete medium (DMEM supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin), and plated at 1 ml/well in 24-well tissue culture plates (Falcon, VWR). Cells were stimulated with soluble *Leishmania* antigen (SLA, 50 μg/ml) for 72 h and the culture supernatant fluids were collected and stored at −20 °C until assayed for cytokines by ELISA.

2.6. Cytokine ELISA

The levels of IFN-γ in 72 h culture supernatant fluids were determined by sandwich ELISA using Ab pairs from BD PharMingen (San Jose, CA) according to manufacturers suggested protocols. The sensitivity of the ELISA was 50 pg/ml.

2.7. Flow cytometric analyses

At the time of sacrifice, single cell suspensions from the lymph nodes draining the vaccination site or spleens were directly stimulated directly *ex vivo* with PMA, ionomycin and brefeldin A (BFA) for 5 h as previously described [17]. The cells were then stained for intracellular IFN-γ expression and surface expression of CD4, CD44 and CD62L, acquired on a 7-color FACSCantor flow cytometer (BD, Mississauga, ON, Canada) and analyzed using Flowjo software (Tree star Inc., Ashland, OR).

2.8. Statistical analysis

Non-parametric one-way analysis of variance (ANOVA) was used to compare mean and standard deviation (SD) of more than two groups. Tukey's test was used where there was significant difference in ANOVA. Differences were considered significant when $p < 0.05$.

3. Results

3.1. Single inoculation of killed L. major with rIL-12 does not induce sustained protection against virulent L. major challenge

We inoculated naïve C57BL/6 mice with killed or live parasites suspended in rIL-12 and assessed their anti-*Leishmania* response and ability to protect against virulent *L. major* challenge at different times post-inoculation. We found that at 3 weeks post-inoculation, killed parasites elicited a significant ($p < 0.01$) increase in the number of cells in the lymph node draining the inoculation site (dLN, Fig. 1A) and these cells produced high amounts of IFN-γ following *in vitro* SLA restimulation (Fig. 1B). This strong IFN-γ recall response was associated with detectable delayed-type hypersensitivity (DTH) response (Fig. 1C) and significant protection (reduction in parasite burden) following virulent *L. major* challenge (Fig. 1D). However, by 6 weeks after inoculation, the number of cells in the dLNs of mice inoculated with killed parasites was strikingly low and not statistically different from naïve mice (Fig. 1E), and these cells failed to produce detectable amounts of IFN-γ in culture supernatant fluids following SLA restimulation (Fig. 1F). In addition, these mice did not display any measurable DTH response and failed to rapidly control parasites following virulent *L. major* challenge

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