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Leishmania major: Interleukin-13 increases the infection-induced hyperalgesia and the levels of interleukin-1β and interleukin-12 in rats

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ABSTRACT

Interleukin-13 (IL-13) is a powerful anti-inflammatory cytokine that was previously shown to be a susceptibility factor for *Leishmania major (L. major)* infection. In this study we report a different role for IL-13 in rats infected with *L. major;* rIL-13 stimulates expression of pro-inflammatory cytokines and IL-12 which is a key cytokine in protective immunity against *Leishmania*. Infected rats received daily injections of rIL-13 for eight consecutive days which resulted in increased pain perception for the first week post-infection assessed by thermal pain tests. This hyperalgesia was accompanied by a sustained early up-regulation of interleukin-1β followed by an up-regulation of IL-12p70. Real-time PCR showed no negative impact for rIL-13 upon the clearance of the parasites from the infection sites and blood.

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1. Introduction

Leishmania major (*L. major*) is a protozoan that exists as obligate intracellular parasite. Immunity against *L. major* depends on the secretion of T-helper1 (Th1) cytokines; during the infection macrophages phagocytose the parasites and a rapid secretion of IL-12 from antigen presenting cells (APC), macrophages and dendritic cells, eventually leads to the progress of a resistant immune response (Laskay et al., 1995). IL-12 binds with high-affinity to IL-12R on natural killer (NK) cells, CD4$^+$ T helper 1 cells and cytotoxic T cells. Interferon-gamma (IFN-γ) is then released by NK and T cells and binds to macrophages resulting in a cell-mediated immune response (Rogge et al., 1997). On the other hand, susceptibility to *L. major* occurs when Th2 cytokines predominate accompanied by a state of unresponsiveness to IL-12. It was shown that IL-4 causes a state of insensitivity of CD4$^+$ T cells to IL-12 (Launois et al., 1997) due to a rapid down-regulation of the β2-chain of the IL-12 receptor (Himmelrich et al., 1998). Many factors may play a role in shaping the course of the infection which consists of a complicated network of regulatory/counter-regulatory interactions. These factors will differ according to the genetic background of the host, the early cytokine milieu and the *Leishmania* species being studied (Doherty and Coffman, 1996; Alexander and Bryson, 2005).

After the infection by the parasites, a local inflammatory response is initiated accompanied by an increase in tumor necrosis factor (TNF-α) (Gatti and Bartfai, 1993), which stimulates the production of IL-1β (Kanaan et al., 2000). IL-1β causes further production of TNF-α, and both promote production of IL-6. When IL-6 accumulates, it down-regulates IL-1β and TNF-α production (Schindler et al., 1990). IL-1 produces nociceptive effects and could serve as a mediator that sensitizes nociceptive neurons during inflammation (Jean et al., 1998). The peripheral pro-nociceptive action of IL-1 may be mediated by a complex signaling cascade and secondary production of nitric oxide, bradykinin or prostaglandins (Poole et al., 1999). It enhances the release of substance P in the spinal cord (Malcangio et al., 1996) and induces COX 2 expression (Newton et al., 1997).

IL-13 is a powerful anti-inflammatory cytokine that limits inflammatory hyperalgesia by inhibiting production of TNF-α, IL-1β, and PGE2 (Lorenzetti et al., 2001). IL-13 also enhances the secretion of IL-1 receptor antagonist (IL-1ra) and the release of the decoy IL-1RII, both molecules possess anti-inflammatory properties by antagonizing IL-1 activities (Colotta et al., 1994; Muzio et al., 1994; Vannier et al., 1996). IL-13 was previously shown to be a susceptibility factor for *L. major* infection, overexpression of IL-13 in transgenic animals prevents the onset of a curative Th1 response following infection (Matthews et al., 2000).

When Sprague–Dawley rats in our labs are injected with *L. major* they are able to mount a protective cell-mediated immune response with no visible lesions, redness, or edema, accompanied with four days sustained hyperalgesic state.

This study investigates the effect of the injection of 20 ng/animal of IL-13 for eight consecutive days on the *L. major*-induced hyperalgesia in Sprague–Dawley rats, the levels of IL-1β, IL-12, and IL-4 and the persistence/dissemination of the parasite inside the rat host.

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2. Materials and methods

Adult female rats (Genus: *Rattus norvegicus*, Strain: Sprague–Dawley), eight weeks old and weighing 190–210 g, were used in all experiments. Animals were maintained at an ambient temperature of 20–22 °C and fed a standard rat chow diet (19% protein, 9.6% fat, 4.3% fiber, and 61% carbohydrate) with drinking water *ad libitum*. All procedures involving animals were carried out in accordance with EEC Council Directive 86/609.

Leishmania major (MHOM/SU/73/5 ASKH) parasites were grown at 24 ± 2 °C in a biphasic medium and subcultured every 10 days. The solid medium (underlay) consisted of the Novy-MacNeal-Nicolle medium made of 7 g Bacto-agar, 3 g NaCl, 450 ml distilled water, and 150 ml rabbit blood. The liquid medium (overlay) consisted of Locke's solution made of 8 g NaCl, 0.3 g KH_2PO_4, 0.2 g $CaCl_2{\cdot}2H_2O$, 0.2 g KCl, and 2.5 g glucose in 1 l distilled water, pH 7.2–7.4. The overlay was supplemented with 10% heat-inactivated fetal calf serum (Sigma), 100 IU/ml of penicillin, and 100 IU/ml of streptomycin. At the day of injection, the overlay containing the promastigotes was centrifuged for 10 min at 3000 rpm and the parasites were resuspended in 1 ml Locke's solution. Parasite count was determined using a Neubauer counting chamber and readjusted to have 2×10^6 parasites/100 μl Locke's solution.

2.1. *Leishmania major* quantification

Two groups of rats were used in this part of the study. The first group ($n = 8$) received daily intraperitoneal (i.p.) injections of rIL-13 (20 ng/100 μl) for eight consecutive days, the same group received an injection of *L. major* in the left paw (2×10^6 promastigotes/100 μl Locke's) 1 h after the injection of rIL-13. The second group ($n = 8$) received only an injection of *L. major* at the left paw (2×10^6 promastigotes/100 μl Locke's) without rIL-13 injections. Both groups were sacrificed at day 35 post-infection (p.i.) and the paws, livers and blood subjected to *Leishmania* quantification.

Genomic DNA was extracted from paws, blood, and livers of infected rats. Detection of *Leishmania* DNA was performed using the following primers; (forward, 5′-CCTATTTTACACCAACCCCCAGT-3′ [JW11]; reverse, 5′-GGGTAGGGGCGTTCTGCGAAA-3′ [JW12]) (Nicolas et al., 2000) that amplify a ca. 120-bp fragment of the minicircle kDNA of *L. major*, ca. 10,000 copies of which are present in each parasite. These primers match the conserved sequences of the kinetoplast minicircle but do not match rat nucleic acid sequences.

A real-time hot-start PCR was performed with the LightCycler Fast Start DNA Master SYBR Green I. *L. major* quantification required the generation of a standard curve made of known DNA concentrations extracted from 2.4×10^4, 2.4×10^3, 2.4×10^2, and 24 *L. major* promastigotes. The LightCycler run generated the crossing point (CP) (cycle numbers where fluorescence levels of all samples are the same) of the tested samples (Fig. 1A). The crossing points of the samples were plotted against their log of their concentrations and a linear regression line through the data points was calculated (Fig. 1B). This curve will be used in all subsequent *L. major* quantifications.

2.2. Behavioral measurements

Four groups of rats ($n = 8$) were subjected to different pain tests two days before any treatment, then one group received daily i.p. injections of rIL-13 (20 ng/100 μl) for eight consecutive days, this same group received an injection of *L. major* in the left paw (2×10^6 promastigotes/100 μl Locke's) 1 h after the first injection of rIL-13. The second group received an injection of *L. major* in the left paw (2×10^6 promastigotes/100 μl Locke's). The two remaining groups were used as controls, one with no treatment and the other received i.p. injection of Lock's medium (100 μl/left hind paw).

All groups were subjected to hot plate (HP) tests on a daily basis for eight consecutive days post-infection, then twice weekly for two weeks.

The hot plate pain test measures the thermal pain threshold by placing each rat on a heated pad (temperature: 52 ± 0.5 °C). The latency of paw licking or jumping was taken as an index of pain threshold (Kanaan et al., 1996). Each animal was subjected to this test three times with 30 min interval between consecutive measurements and the average was recorded.

2.3. Immunoassay tests

Nine groups of rats ($n = 8$) were used in this part of the study. The first four groups received an injection of *L. major* in the left paw (2×10^6 promastigotes/100 μl Locke's). Another four groups received daily i.p. injections of rIL-13 (20 ng/100 μl) for a maximum of eight consecutive days, those groups received an injection of *L. major* in the left paw (2×10^6 promastigotes/100 μl Locke's) 1 h after the first injection of rIL-13. The last group was not subjected to any kind of treatments and was used as a control. Animals were sacrificed at days 2, 6, 12, and 20 p.i. Tissues from infected hind paws were collected, weighed, and processed for the quantification of the level of IL-1β, IL-4, and IL-12p70.

The skins were removed under deep anesthesia, the removed parts of the skin were stored at a temperature of −80 °C before processing. Removed paws were individually homogenized for 45 s in 1 ml of phosphate buffered saline (PBS) containing (per 100 ml PBS): 3.155 g NaCl, 0.02 g KCl, 0.02 g KH_2PO_4, 0.115 g Na_2HPO_4, 50 μl Tween-20, 0.5 g bovine serum albumine (BSA), and two tablets of Roche Protease inhibitor cocktail. The supernatants were centrifuged at 13,000 rpm for 60 min at 4 °C and stored at −80 °C until further analysis.

Immunoassay kits (Biosource, International) were used for the quantification of rat IL-1β (sensitivity: <3 pg/mL), IL-4 (sensitivity: <2 pg/mL) and IL-12p70 (sensitivity: <2.5 pg/mL). The kits are solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA). Absorbance was read using SpectraMax Plus microplate reader instrument and concentrations were determined using the SoftMax Pro microplate reader software.

2.4. Statistical analysis

Values are presented as means ± SEM. Comparisons between groups was made using one-way Analysis of Variance (ANOVA) followed by the Bonferoni post hoc test. Statistical significance was assumed at $p < 0.05$.

3. Results

3.1. Effects of rIL-13 on pain threshold

The effect of eight days rIL-13 treatment on pain threshold in rats infected with *L. major* was assessed during a period of 18 days p.i. (Fig. 2). In comparison with the naïve group (non-infected and non-treated), both infected groups showed significant decrease in hot plate latency the second day post-infection, and this persisted for several days. On the other hand, at day 3 p.i., the HP latency of rIL-13 treated rats was significantly lower than that observed in infected group but with no rIL-13 treatment. This difference in HP latency between the rIL-13 treated and untreated rats remained significant till day 6 p.i. Starting day 7 onward, all groups showed similar HP latency. The use of Locke's solution as a transport

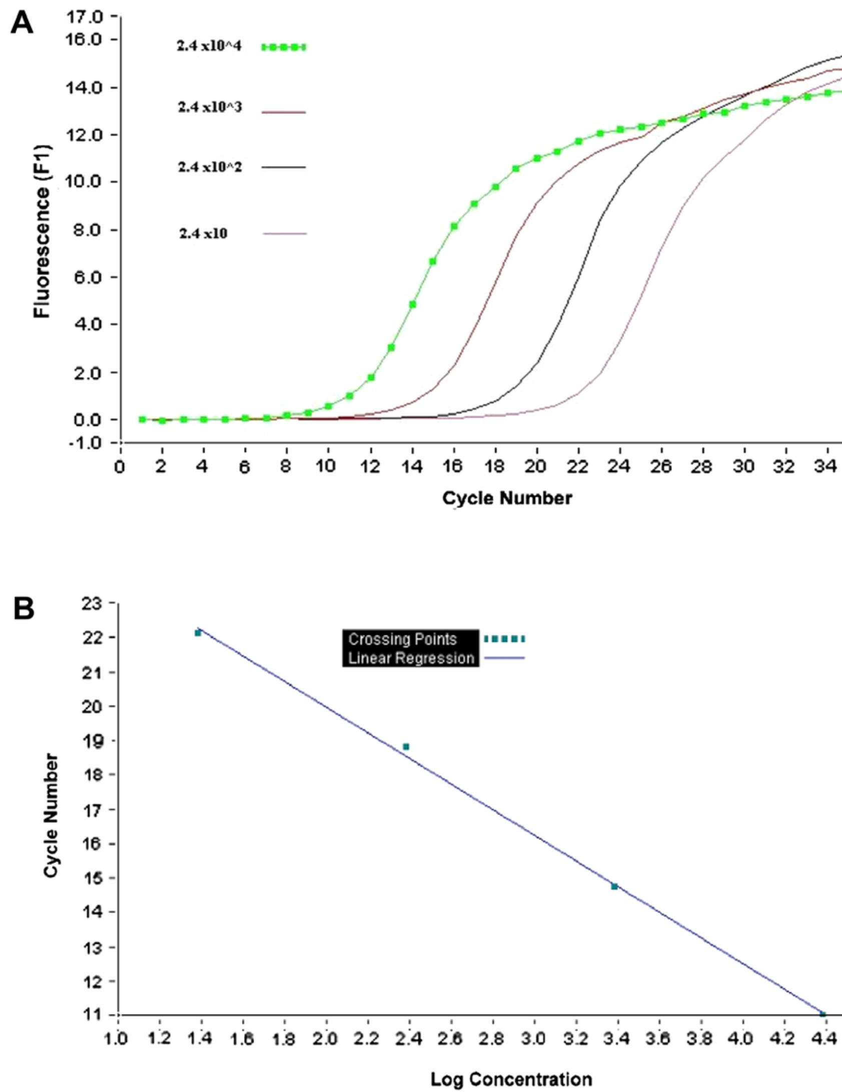

Fig. 1. (A) A real-time hot-start PCR was performed with the LightCycler Fast Start DNA Master SYBR Green I. DNA used corresponded to 2.4×10^4, 2.4×10^3, 2.4×10^2, and 24 *Leishmania major* promastigotes. (B) The standard curve was generated by plotting the crossing cycle number (Crossing Point) versus the log of the concentration for each sample. A linear regression line through the data points was calculated by the LightCycler software.

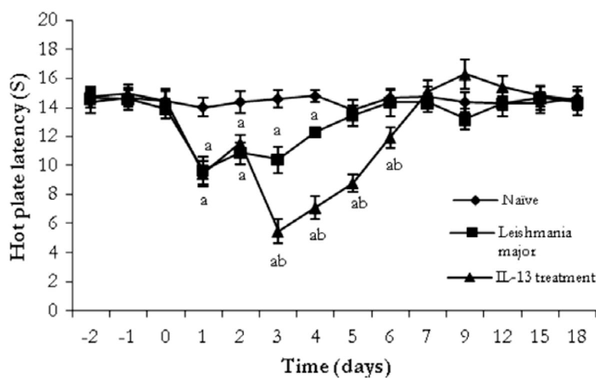

Fig. 2. Time course of the effect of daily intraperitoneal injection of rIL-13 on *Leishmania major*-induced hyperalgesia as assessed by the hot plate test. Rats were injected with 20 ng of rIL-13 for eight consecutive days from day 0, rats were also injected with 2.0×10^6 promastigotes per left hind paw 1 h after initiation of rIL-13 treatment. Each result is the mean of 8 rats +/− standard error mean (SEM). $^{a}p < 0.05$ compared with na. $^{ab}p < 0.05$ compared with rats infected but not treated with rIL-13.

medium did not exhibit any effect since the injection of Locke's solution alone showed similar results with respect to the control (data not shown).

3.2. Effect of rIL-13 on the levels of IL-1β, IL-4, and IL-12 in Leishmania major—infected rats

The effect of eight days of rIL-13 treatment upon levels of IL-1β in the paws of rats infected with *L. major* was determined (Fig. 3A). At days 2 and 6 p.i. both rIL-13-treated and non-treated rats had IL-1β levels significantly higher than the naïve group. However, at day 6 p.i. the levels of IL-1β in treated rats became significantly higher with respect to rIL-13 non-treated rats. At day 12 p.i., the levels of IL-1β remained significantly higher in IL-13-treated rats compared with naïve and non-treated rats, which showed similar tissue concentrations of IL-1β. At day 20 p.i., all groups showed comparable IL-1β levels.

The levels of IL-4 in the paws of rats infected with *Leishmania* following IL-13 treatment is shown in Fig. 3B. Data showed that treatment with rIL-13 had no effect on the levels of IL-4 in animals

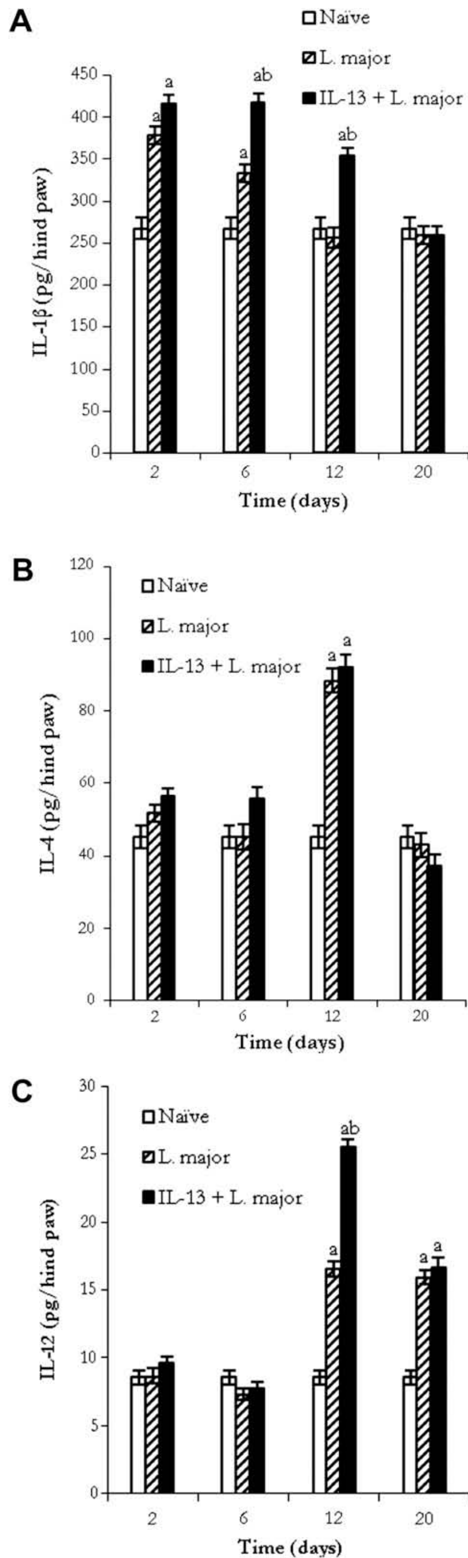

Fig. 3. Effect of daily intraperitoneal injection of rIL-13 on the levels of IL-1β (A), IL-4 (B), and IL-12 (C) in the left paws of rats infected with Leishmania major. Rats were injected with 20 ng of rIL-13 (i.p.) for eight consecutive days and the levels of cytokines were assessed at days 2, 6, 12, and 20 post-infection. Each result is the mean of 8 rats +/− standard error mean (SEM). $^{a}p < 0.05$ compared with na. $^{ab}p < 0.05$ compared with rats infected but not treated with rIL-13.

infected with *L. major*. However, only at day 12 p.i. both infected rat groups exhibited sharp increases in IL-4 levels with respect to the naïve group.

The effect of eight days of rIL-13 treatment upon levels of IL-12 in the paws of rats infected with *L. major* is presented in Fig. 3C. The levels of IL-12 at days 2 and 6 p.i. were similar in all groups. At days 12 and 20 p.i., the levels of IL-12 were significantly higher in both treated and non-treated infected groups with respect to the naïve group. Furthermore, only at day 12 the levels of IL-12 in IL-13-treated rats were significantly higher than those in non-treated rats. However, at day 20, the levels of IL-12 in both latter groups were comparable.

3.3. *Effect of rIL-13 treatment on the persistence and dissemination of Leishmania major parasites in rats*

The effect of rIL-13 treatment upon clearance of the parasite 35 days p.i. from paws, blood, and livers of infected animals is presented in Table 1. Results showed that all infected animals, either treated or non-treated with rIL-13, were able to clear more than 99.9% of the parasites 35 days post-infection and this happened in all tissues studied.

4. Discussion

Inflammation induced by *L. major* has not been previously characterized in rats. Here, for the first time, we characterize *L. major*-induced inflammation in rats, including its hyperalgesic effects and some key inflammatory cytokines produced in this inflammation. We also describe a combinatory effect of *L. major* infection and IL-13 treatment on pain and the levels of expression of key inflammatory cytokines like IL-1β and IL-12. We found that exogenous IL-13 decreases the pain thresholds and stimulates expression of IL-1β and IL-12 in rats infected with *L. major*. To rule out the possibility that IL-13 itself induces those effects, rIL-13 was injected in naive rats. We observed that rIL-13 in naive rats did not affect sensitivity to pain or altered the level of pro-inflammatory cytokines. This observation indicates that rIL-13 induces the reported observations in combination with *L. major* infection.

Infection with *L. major* did not cause any redness, edema, or lesions in the infected paws, but it induced hyperalgesia assessed by the HP suggesting an acute nociceptive-related behavior organized at the supraspinal levels. *L. major* infection also caused an up-regulation in the levels of IL-1β during the first week of infection. This increase in IL-1β correlates well with the findings of the hot plate tests since IL-1β along with other pro-inflammatory cytokines released in the periphery during infection are involved in immune-to-brain communication (Watkins et al., 1995). The communication depends on the pro-inflammatory cytokines activation of certain peripheral nerves like the vagal sensory afferent nerves. When activated, the peripheral nerves signal the brain and stimulate the synthesis of pro-inflammatory cytokines within the central nervous system itself and cause an IL-1-mediated hyperalgesia (Ban et al., 1992; Gatti and Bartfai, 1993; Laye et al., 1995). The decline in the levels of IL-1β and its hyperalgesic effects is a result of the accumulation of IL-6 (Schindler et al., 1990) which was induced by the increase of IL-1β itself (Cunha et al., 1992). IL-6 also induces production of anti-inflammatory proteins: IL-1 receptor antagonist (IL-1ra) and soluble tumor necrosis factor (TNF) receptor (Tilg et al., 1994). IL-1ra will competitively bind the IL-1 receptors and prevents the IL-1-induced hyperalgesia.

Previous studies have demonstrated a negative role for IL-13 in murine cutaneous leishmaniasis caused by *L. major*. IL-13 possesses powerful anti-inflammatory and immunosuppressive properties on macrophages such as down-regulating their anti-leishmanicidal activity (Juttner et al., 1998), and their production of IL-12 (Skeen et al., 1996), and IL-1β (Seitz et al., 1996). In contrast with previous studies we found that rIL-13 injection during *L. major* infection enhanced peripheral hyperalgesia and the levels

Table1
Results of real-time PCR assays for *Leishmania major* DNA in rats' tissues.

Number of rats	IL-13 treatment	Tissue	Parasites injected in left paw	Parasites at day 35 p.i.	Standard deviation	% of parasites cleared
8	No treatment	Left paw	2×10^6	32.81	11.22	99.99
8		Liver	2×10^6	7.24	3.01	99.99
8		Blood	2×10^6	15.64	6.12	99.99
8	20 ng i.p. for eight consecutive days	Left paw	2×10^6	25.92	9.45	99.99
8		Liver	2×10^6	11.73	3.67	99.99
8		Blood	2×10^6	17.27	7.62	99.99

of interleukin-1β and interleukin-12. These observations discard a negative impact for IL-13 on *L. major* infection in rats and may even suggest a protective role since protective immunity against *Leishmania* depends on an IL-12 driven response (Sypek et al., 1993; Scharton-Kersten et al., 1995). In fact, we found no negative impact for rIL-13 upon the clearance of the parasites from the infection sites and blood. In addition, considering that susceptibility to *L. major* depends on an IL-4 driven response (Chatelain et al., 1992; Leal et al., 1993) we found no effect on the levels of IL-4 secretion in infected rats treated with rIL-13.

Although it was previously shown that both IL-4 and IL-12 are present after the initial phase of the infection with *L. major* in resistant hosts (Locksley et al., 1987), we found the increase of both IL-4 and IL-12 at day 12 p.i. to be noteworthy. This suggests that T cells have the potential to develop into Th1 or Th2. This also suggests that the genetically determined resistance to *L. major* is primarily based on the parasite-specific $CD4^+$ T cells maintaining their responsiveness to IL-12. The initial overexpression of IL-4 at day 12 p.i. induced by *L. major* infection declined to reach naïve whereas the overexpression of IL-12 was still detected at day 20 p.i. and real-time PCR showed containment of the parasites suggesting an IL-12 healing driven response.

While it is commonly accepted that IL-13 exerts immunosuppressive and anti-inflammatory actions during infections, it was previously reported to play a protective role during murine listeriosis through enhancing IL-12 production and NK cell cytolytic activity (Flesch et al., 1997). Also, it was found that pretreatment of human peripheral blood mononuclear cells with IL-13 enhanced the production of IL-12 and TNF-α in response to LPS or *Staphylococcus aureus* in these cells (D'Andrea et al., 1995; Bullens et al., 2001). Therefore, we believe that IL-13 possesses both anti and pro-inflammatory capacities regulated by different factors at the infection milieu, and that the parasite and the genetic background of the host may influence the role of IL-13 and give it either immunosuppressive or immunostimulatory functions.

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