Experimental Parasitology 130 (2012) 48–57

Contents lists available at SciVerse ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Lack of signaling by IL-4 or by IL-4/IL-13 has more attenuating effects on *Leishmania amazonensis* dorsal skin – than on footpad-infected mice

Tania C. Felizardo [a,*], Maria I.C. Gaspar-Elsas [b], Gloria M.C.A. Lima [a], Ises A. Abrahamsohn [a]

[a] Departamento de Imunologia, Instituto de Ciências Biomédicas IV, Universidade de São Paulo, Av. Prof. Lineu Prestes 1730, 05508-900 São Paulo, SP, Brazil
[b] Departamento de Pediatria, Instituto Fernandes Figueira, Fundação Oswaldo Cruz (FIOCRUZ), Av. Rui Barbosa 716, Flamengo, 22250-020 Rio de Janeiro, RJ, Brazil

ARTICLE INFO

Article history:
Received 20 June 2011
Received in revised form 27 September 2011
Accepted 30 September 2011
Available online 12 October 2011

Keywords:
IL-4
IL-13
Leishmania amazonensis
IL-4 receptor alpha

ABSTRACT

Lesion development in tegumentary leishmaniasis is markedly influenced by the inoculation site and the type and number of injected infective forms. This and the yet unclear contribution of Th2 cytokines as susceptibility factors to *Leishmania amazonensis* infection prompted us to investigate the roles of IL-4, IL-13 and IL-10 on C57BL/6 and BALB/c mice infected in the footpad (paw) or rump with low-dose *L. amazonensis* purified-metacyclics. Wild-type (WT) mice of either strain developed, in the rump, a single large ulcerated lesion whereas paw lesions never ulcerated and were much smaller in C57BL/6 than in BALB/c mice. However, rump-inoculated IL-4-deficient (IL-$4^{-/-}$) C57BL/6 mice did not develop any visible lesions although parasites remained in the dermis and lymph nodes, even after systemic IL-10-receptor blocking. By comparison, all IL-$4^{-/-}$ BALB/c mice developed rump ulcers. Strikingly, only 30% of rump-infected IL-4R$\alpha^{-/-}$ BALB/c mice developed lesions. IL-$4^{-/-}$ mice had higher IFN-γ and lower IL-10 and IL-13 levels than WT mice. Paw-infected IL-4R$\alpha^{-/-}$ BALB/c mice developed minimal paw lesions. While other factors contributing to *L. amazonensis* susceptibility cannot be discounted, our results indicate that absent signalling by IL-4 or by IL-4/IL-13 have more intense attenuating effects on rump than on paw lesions but do not eradicate parasitism.

1. Introduction

In Europe, Africa, and Asia cutaneous leishmaniasis is most commonly caused by the protozoan *Leishmania major*, whereas in the Americas the disease is most often caused by *Leishmania mexicana*, *Leishmania braziliensis* or *Leishmania amazonensis*. In rare cases this last species can also cause diffuse cutaneous leishmaniasis in humans.

Mouse models of *L. major* infection have been extensively studied. Certain mouse strains (e.g. BALB/c, DBA/2, or SWR/J) develop progressively larger lesions with heavy parasite burdens. In contrast to these susceptible strains, mice of resistant strains (e.g. C57BL/6, C3H, CBA) develop small self-curing lesions with few surviving parasites at the inoculation site (Handman, 1999; Sacks and Noben-Trauth, 2002).

However, experimental infections of mice by *L. amazonensis* differ in many aspects from those caused by *L. major*. Most mouse strains develop at the inoculation site of *L. amazonensis* a non-healing lesion, whose size and progression characterize a susceptibility pattern that may be quite distinct from the one observed in *L. major* infection (Osorio y Fortea et al., 2007; Pereira and Alves, 2008; Silveira et al., 2009). Indeed, when *L. amazonensis* is injected in the usual *L. major*-resistant mouse strains, the infection is not self-curing, causes lesions of varying severity, and is not accompanied by polarized Th1 cytokine responses. After *L. amazonensis* infection in the footpad, C57BL/6 mice develop a persistent non-healing lesion, albeit much smaller than observed in similarly infected BALB/c mice. This partial susceptibility of C57BL/6 mice is associated with lower IFN-γ production rather than enhanced IL-4 synthesis (Afonso and Scott, 1993; Jones et al., 2000). Furthermore, dominant Th2-type responses are seldom seen in *L. amazonensis*-susceptible mouse strains (Afonso and Scott, 1993; Lemos de Souza et al., 2000).

In addition, effective control of *L. amazonensis* progression was neither observed in IL-$10^{-/-}$ C57BL/6 mice, in spite of a vigorous Th1 response (Jones et al., 2002), nor in IL-$10^{-/-}$ BALB/c mice (Padigel et al., 2003), although in both models the parasite load was smaller than in their respective wild-type (WT) controls. These results suggest that IL-4 and/or IL-13 would have a major role in susceptibility to *L. amazonensis*.

The relative weight of IL-4 as susceptibility factor to *L. amazonensis* depends on the mouse strain under study: IL-$4^{-/-}$ mice on a C57BL/6 background were no more resistant to footpad infections than the WT controls (Jones et al., 2000), whereas IL-$4^{-/-}$ BALB/c mice developed smaller lesions than their WT counterparts (Guimaraes et al., 2006; Satoskar et al., 1997). The contribution of IL-13 to susceptibility to New World cutaneous leishmaniasis has

* Corresponding author. Present address: 10 Center Drive – CRC, Room 3E-3224, Bethesda, MD 20892, USA. Fax: +55 301 480 3436.
E-mail address: tania.felizardo@nih.gov (T.C. Felizardo).

0014-4894
doi:10.1016/j.exppara.2011.09.015

been primarily investigated in mice infected with *L. mexicana* amastigotes. A comparative study among IL-13$^{-/-}$, IL-4/ IL-13$^{-/-}$, and WT mice (all on a C57Bl/6 X 129sv/EV background) led to the conclusion that IL-13 was not involved in mediating susceptibility to *L. mexicana* (Sosa et al., 2001). However, other research groups have found a distinctive role for IL-13 as compared to IL-4, in determining susceptibility of C57Bl/6 X 129sv/EV mice to *L. mexicana* (Alexander et al., 2002). The contrasting results between these two studies can be ascribed to the longer follow-up of infection in the last one. Reduction of the lesion size in IL-13$^{-/-}$ mice became apparent around 14 weeks after infection with 5×10^6 amastigotes or much earlier when the inoculum was reduced to 2×10^5. A disease exacerbating role for IL-13 as well as IL-4 was also shown when *L. mexicana* infections were compared in IL-4$^{-/-}$, IL-4Rα$^{-/-}$, and WT control mice on a BALB/c background (Alexander et al., 2002).

As for *L. amazonensis* infection, follow-up experiments of 8–12 weeks were reported in comparison with data on *L. mexicana* infections (McMahon-Pratt and Alexander, 2004). Inocula of 10^6 *L. amazonensis* amastigotes were injected into the base of the tail or into the footpads of IL-4$^{-/-}$ and IL-4Rα$^{-/-}$ mice. The results indicate that the role of IL-4 on susceptibility to *L. amazonensis* depends on the inoculation site. Absence of IL-4 had no effect on early lesion progression of paw-infected C57BL/6 WT mice. In contrast, paw-inoculated IL-4$^{-/-}$ and IL-4Rα$^{-/-}$ BALB/c mice both had much smaller lesions than the corresponding WT controls.

Taken together these studies call attention to the importance of the observation period length, the site of infection, and the number of inoculated parasites as variables affecting susceptibility to infection. In addition to these, the developmental stage of the sinjected parasites imparts on the course of infection. In most studies a great number of stationary-phase *L. amazonensis* promastigotes were inoculated in the footpad and paw swelling was chosen as a read-out of lesion development. However, this mouse paw-infection model of *L. amazonensis* has some important drawbacks because of the parasite forms, their number, and the site used for infection. Firstly, the inoculation of small numbers of *L. amazonensis* metacyclic promastigotes in the dermis of the ear results in much slower disease progression (Courret et al., 2003). Secondly, *L. amazonensis* paw lesions generally do not ulcerate, whereas inoculation into the dermis of the rump region evolves as an ulcer (Soong et al., 1997; Terabe et al., 2000), as often is observed in naturally infected humans. We described the course of footpad infection in mice inoculated with smaller numbers of *L. amazonensis* metacyclics in comparison to the classical stationary-phase promastigotes infection model; the patterns of partial resistance of C57BL/6 and susceptibility of BALB/c mice were maintained, but much longer lag phases between metacyclic inoculation and the onset of paw swelling were observed (Felizardo et al., 2007).

Because the several aforementioned factors might influence the role of cytokines on the susceptibility to *L. amazonensis* infection, we carried out the present study. We investigated the effects of IL-4 deprivation and lack of signaling through the IL-4Rα-chain on the resistance to *L. amazonensis* in C57BL/6 and BALB/c mice infected with metacyclic forms in the footpad or in the rump.

2. Materials and methods

2.1. Animals

Interleukin-4-deficient (IL-4$^{-/-}$) and wild-type (WT) female mice (BALB/c and C57BL/6 background), 6–8 weeks old were obtained from the breeding facilities of the Department of Immunology, Institute of Biomedical Sciences, University of São Paulo (ICB/USP). BALB/c IL-4Rα$^{-/-}$ mice were kindly provided by Dr. Maria Ignes C.G. Elsas (Dept. Pesquisa/Instituto Fernandes Figueira/FIOCRUZ-RJ, Rio de Janeiro, Brazil). All procedures involving animals were approved by the ICB/USP Committee on ethical treatment of research animals.

2.2. Parasite

Leishmania (*L.*) *amazonensis* (WHOM/BR/75/Josefa) was kindly provided by Dr. Elvira M. Saraiva (Dept. Immunology/UFRJ/Brazil). Promastigotes forms were cultured *in vitro* at 25 °C in *Grace's Insect Cell Culture Medium* (GIBCO-BRL Life Technologies, Grand Island, NY, USA) supplemented with 20% heat-inactivated-FCS (GIBCO, Argentina, South America), 100 U/ml penicillin (GIBCO-BRL) and 100 μg/ml streptomycin (GIBCO-BRL). Parasite infectivity was maintained by passaging in BALB/c mice.

Purified metacyclic promastigotes were obtained from stationary-phase promastigote cultures by treatment with the 3A1-La monoclonal antibody (hybridoma kindly provided by Dr. Elvira M. Saraiva), which agglutinated the procyclic promastigotes (Courret et al., 1999; Felizardo et al., 2007). Briefly, stationary-phase promastigotes from day 6 of culture were resuspended in PBS (0.01 M phosphate-buffered, 0.15 M NaCl) to a concentration of 1×10^8/ml and incubated with 3A1-La monoclonal antibody (mAb) (1:50) for 20 min at room temperature (RT). PBS was added carefully and the suspension was centrifuged at 40*g* for 5 min at RT. The supernatant containing 85–95% metacyclic forms was collected and washed two times by centrifugation in PBS before injection in the mice.

2.3. Infection of mice with L. amazonensis

Mice were inoculated subcutaneously with 1×10^4 purified metacyclic promastigotes using a 25 Gauge needle into one of the hind footpads or intradermally into the shaved skin of the rump. The paw thickness and the diameter of the lesion in the rump were monitored weekly using a metric calliper. Paw swelling was calculated by subtracting the thickness of the uninfected contralateral paw from that of the infected paw.

2.4. Quantifying parasites in the lesion and in lymph nodes

The parasite burden was estimated according to a previously described limiting dilution method with minor modifications (Lima et al., 1997). The injected paw or the rump skin lesions were aseptically excised, weighed, homogenized and the cell suspensions seeded in 96-well plates. The wells containing parasites were scored using an inverted microscope and the data processed with the ELIDA software program (Taswell, 1986). Differences between two means were considered significant when the means ± the respective 95% confidence intervals did not overlap.

It should be noted that these methods yielded estimates and not the absolute numbers of parasites present in the original cell suspensions. In fact, the serial dilutions of the parasite-containing tissue homogenates and their subsequent culture may originate estimates that significantly exceed the number of parasites present in the original tissue sample in spite of the care taken in performing the assays. In our experimental protocol, mice belonging to the experimental and respective control groups were sacrificed on the same day and the limiting dilution assays for the different organs were run simultaneously, in order to minimize assay variations and enable statistical analysis within the groups of mice on the same day of infection.

2.5. Immunohistochemistry

The tissues were fixed and embedded in paraffin according to a previously published protocol (Beckstead, 1994) with minor modifications. Briefly, the tissues were fixed in JB Fix solution (Tris

buffer containing zinc chloride, zinc acetate and calcium acetate), serially dehydrated to 95% ethanol, incubated in isopropyl alcohol followed by immersion in Clarus EcoK (non-toxic aliphatic hydrocarbon, Clarus Technology, Brazil) and paraffin embedding. The 3 μm-thick sections were unwaxed at 56 °C, immersed in Clarus-EcoK and hydrated. After blocking with Super Block buffer (Pierce Chemical Co., USA) the sections were incubated with anti-*Leishmania* mouse serum, washed and stained with FITC-labelled goat-anti-mouse Immunoglobulin antibody. The nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI, Sigma, St. Louis, MO) and the slides mounted in anti-fading mounting media (Vectra-Shield, USA).

The images were captured using a Nikon fluorescence microscope coupled to Image-Pro Plus software (kindly made available by Dr. Telma Tenorio Zorn, Department of Cell Biology, ICB/USP).

2.6. Treatment in vivo with anti-IL-10 Receptor antibody

Interleukin-4-deficient C57BL/6 mice were treated with anti-IL-10 receptor mAb (1B1.3A) or with the isotype control mAb (GL117.41, anti-β-galactosidase) beginning on the 22nd week of infection. One mg was injected intraperitoneally (i.p.) every three days for two weeks. Anti-IL-10R mAb and the isotype control mAb were purified in our laboratory from hybridomas kindly provided by DNAX Research Laboratories, Palo Alto, CA, USA.

The biologic activity of 1B1.3A mAb was determined by its ability to enhance LPS-induced septic shock. C57BL/6 mice were injected i.p. with 2 mg or 1 mg of 1B1.3A mAb 24 h before receiving i.p. a sub-lethal (500 μg) dose of LPS. All mice treated with 1B1.3A and challenged with LPS died in the next 48 h, while all mice inoculated with isotype control mAb survived.

2.7. Cytokine production by lymph node cells in culture

Cell suspensions from the draining LNs (popliteal or inguinal) were resuspended in RPMI-1640 supplemented with 5% heat-inactivated FCS and antibiotics and seeded at 5×10^6 cells/ml in 24-well-plates. Cells were cultured in the presence of live *L. amazonensis* promastigotes (1×10^6/ml) at 37 °C for 72 h. The supernatants were assayed by two-site sandwich ELISA for IL-10, IL-13, and IFN-γ as previously described (Abrahamsohn and Coffman, 1995). Antibodies and cytokine standards were purchased from Biosciences Pharmingen (Becton Dickinson Co) (IL-10 and IFN-γ) or R&D Systems (IL-13). Streptavidin-horseradish peroxidase was purchased from Southern Biotechnology Associates Inc., AL, USA. The reaction was developed by ABTS reagent and the optical densities were read in an automatic microplate reader (*Versa Max*, Molecular Devices). Cytokine concentrations were determined from standard curves generated from recombinant cytokines.

2.8. Statistical analyses

The data were analyzed by repeated measures analysis of variance (ANOVA) using INSTAT 2 software. Differences were considered significant when $p < 0.05$.

3. Results

3.1. Course of infection by L. amazonensis in wild type and in IL-4$^{-/-}$ BALB/c and C57BL/6 mice inoculated into the footpad or rump

BALB/c or C57BL/6 mice inoculated into the footpad with *L. amazonensis* metacyclic forms developed paw swelling without ulceration (Fig. 1A). Paw swelling in BALB/c mice increased from week 7 of infection, reaching 11 mm (approximately sevenfold thicker than a normal paw (1.5–1.7 mm) by week 24. Paw swelling in relatively resistant C57BL/6 mice also started on week 7 of infection, slowly increasing to a maximum of approximately 2 mm by week 15, followed by sharp decrease around week 17. Thereafter the lesion maintained itself as a persistent indurate swelling of about 0.8–0.9 mm that did not heal (Fig. 1A).

In comparison to BALB/c mice, BALB/c IL-4$^{-/-}$ inoculated in the footpad, had significantly smaller and slower progressing lesions, reaching 4 mm by week 24 (Fig. 1A). However, the difference in paw sizes between C57BL/6 IL-4$^{-/-}$ mice and their WT counterparts was less striking: it was evident between weeks 14 and 17 of infection and became non significant thereafter (Fig. 1A). It is important to mention that even in C57BL/6 IL-4$^{-/-}$ mice the paw lesion did not completely regress and was still present until week 48 of infection (maximum time of observation).

Taken together, these results indicate that for the footpad model of *L. amazonensis* infection: (a) C57BL/6 mice were more resistant than BALB/c mice but were unable to completely heal the paw lesion; (b) IL-4 is an important susceptibility factor for

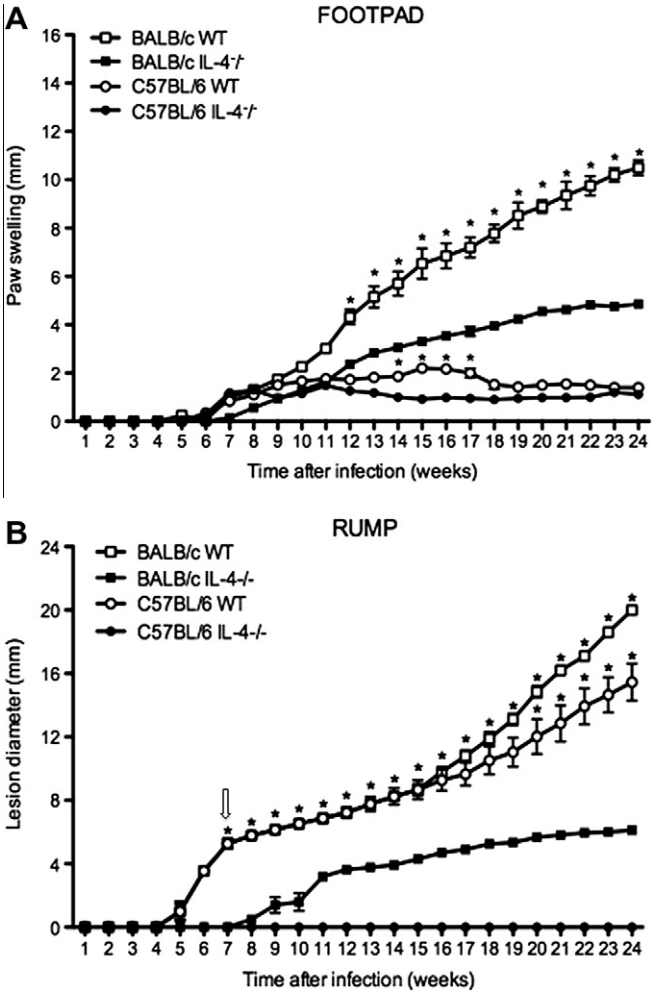

Fig. 1. Lesion development in wild-type (WT) and IL-4$^{-/-}$ BALB/c and C57BL/6 mice inoculated into the footpad or rump in the rump with *L. amazonensis*. The mice were inoculated with 10^4 *L. amazonensis* metacyclics into the dermis of one of the rear footpads (**A**) or the rump (**B**). Paw swelling in **A** is expressed by the difference in thickness between the inoculated and uninoculated paw. Lesion diameter in **B** is the diameter of the ulcer or of the indurated area (when no ulcer exists) at the site of inoculation. Ulceration of rump lesions occurred in BALB/c mice (WT and IL-4$^{-/-}$) and in WT C57BL/6 mice as indicated by the vertical arrow. Rump lesions in IL-4$^{-/-}$ C57BL/6 mice did not ulcerate. The means ± SEM of three independent experiments (6 mice each) are presented. Significant differences between WT BALB/c and IL-4$^{-/-}$ BALB/c occur from day 11 on, in **A** and from day 6 on, in **B**; for WT C57BL/6 vs. IL-4$^{-/-}$ C57BL/6 they are indicated with * in **A** and occur from day 6 on, in **B**.

Table 1
Dissemination *of L. amazonensis* from footpad and rump inoculation sites to distant lymphoid organs in d-type C57 Wild Type and in IL-4$^{-/-}$ BALB/c and C57BL/6 mice.

		BALB/c Organ parasitism after footpad inoculation									C57BL/6 Organ parasitism after footpad inoculation						
		ING-il	*AXI-il*	*POP-cl*	*ING-cl*	*AXI-cl*	*Iliac*	*Spleen*			*ING-il*	*AXI-il*	*POP-cl*	*ING-cl*	*AXI-cl*	*Iliac*	*Spleen*
WT	3 wk	nd	nd	nd	nd	nd	nd	nd	WT	3 wk	+	nd	nd	nd	nd	nd	+
	13 wk	++	nd	++	nd	nd	++	++		13 wk	+	+	+	+	+	++	+
	24 wk	+++	++	+	nd	nd	++	+++		24 wk	nd	+	++	+	+	+	+
IL-4$^{-/-}$	3 wk	nd	nd	nd	nd	nd	nd	nd	IL-4$^{-/-}$	3 wk	nd	nd	nd	nd	nd	nd	nd
	13 wk	nd	nd	+	nd	nd	++	++		13 wk	+	nd	+	nd	nd	+	+
	24 wk	nd	nd	nd	nd	nd	nd	nd		24 wk	+	nd	nd	nd	nd	+	+
		Organ parasitism after rump inoculation									Organ parasitism after rump inoculation						
		AXI-r	*AXI-l*	*POP-r*	*POP-l*	*Iliac*	*Spleen*				*AXI-r*	*AXI-l*	*POP-r*	*POP-l*	*Iliac*	*Spleen*	
WT	3 wk	nd	nd	nd	nd	nd	nd		WT	3 wk	nd	nd	nd	nd	+	nd	
	13 wk	nd	nd	nd	nd	+	+			13 wk	+	+	+	+	++	+	
	24 wk	+++	++	++	+++	+++	+++			24 wk	+	+	+	+	+	+	
IL-4$^{-/-}$	3 wk	nd	nd	nd	nd	nd	nd		IL-4$^{-/-}$	3 wk	nd	nd	nd	nd	nd	nd	
	13 wk	nd	nd	nd	nd	nd	nd			13 wk	nd	nd	nd	nd	+	nd	
	24 wk	nd	nd	nd	nd	nd	nd			24 wk	nd	nd	nd	nd	+	nd	

Organ abbreviations: *ING* (inguinal lymph node), *il* (ipsilateral), *AXI* (axillary lymph node), *r* (right side), *l* (left side), *POP* (popliteal lymph node), *cl* (contralateral), *Iliac* (iliac lymph nodes).
The mice were infected with 10^4 metacyclic forms. The number of parasites in the organs was estimated by a limiting dilution assay (LDA) on the 3rd, 13th and 24th weeks after infection. Data were analyzed by ELIDA software; the mean was calculated from six individual mice. The results are shown as intensity of parasitism: nd (not detected); + ($\leqslant 10^1$ parasites); ++ (10^2–10^3 parasites); +++ ($\geqslant 10^3$ parasites).

BALB/c mice but other cytokines or factors contribute to the maintenance of the lesion; (c) IL-4 is not the factor maintaining the partial susceptibility of C57BL/6 mice, because the lack of IL-4 neither led to complete regression of the paw lesion (cf. Fig. 1A) nor to parasite elimination (cf. Fig. 3 and Table 1).

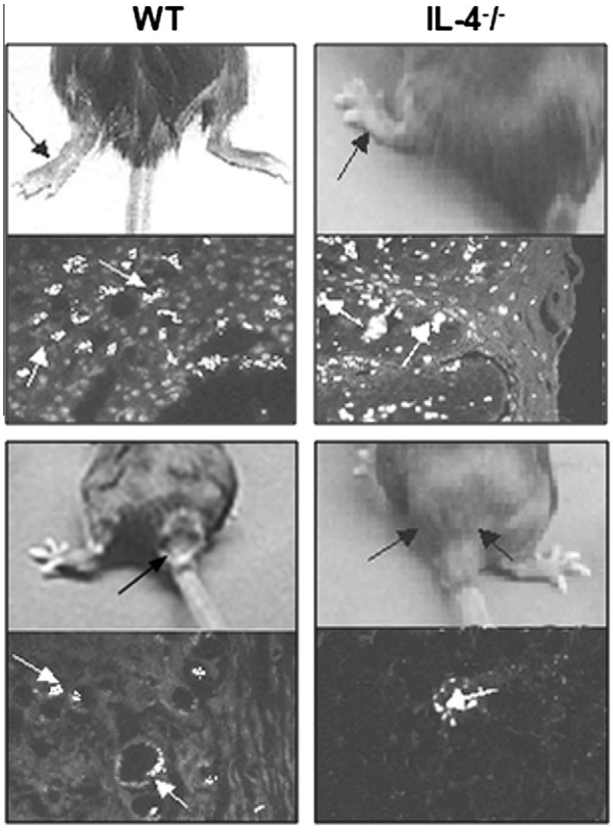

Fig. 2. Lesions and parasite distribution in the skin of WT C57BL/6 and IL-4$^{-/-}$ C57BL/6 mice on the 24th week after inoculation in the footpad or rump with *L. amazonensis*. The minimal lesion in the paw and the absence of macroscopic lesion in the rump of IL-4$^{-/-}$ C57BL/6 mice contrast with the large ulcerated lesion in the rump of a WT C57BL/6 mouse. Photomicrographs (magnification 200×) show that parasites stained by indirect immunofluorescence are present mainly in the dermis underlying the lesions and also in the epidermal layer of the seemingly normal skin of the rump inoculation site in IL-4$^{-/-}$ C57BL/6 mice. Arrows indicate the lesion and point to some of the stained parasites in the tissue sections.

Strikingly different results were obtained when mice were infected in the dermis of the dorsal skin of the animals' rump region with *L. amazonensis* metacyclics. Firstly, the time-lag (6 weeks) for lesion appearance, progression and size were very similar for WT BALB/c and WT C57BL/6 mice until week 16–17. Thereafter, the lesions grew more rapidly in WT BALB/c than in WT C57BL/6 mice, so that by the 24th week of infection they were about 25% larger than those seen in WT C57BL/6 mice. In WT mice of either strain the rump lesion became ulcerated and extended deep into the dermis (Fig. 1B). Secondly, C57BL/6 IL-4$^{-/-}$ mice did not develop any lesion at the rump inoculation site (Fig. 1B). In contrast BALB/c IL-4$^{-/-}$ developed a slowly-growing shallow ulcerated lesion from week 10 of infection, although much smaller than that developing in WT BALB/c mice.

The macroscopic aspect of the lesions in WT C57BL/6 and in C57BL/6 IL-4$^{-/-}$ mice infected in the footpad or in the rump skin and the parasite density in sections of the lesions are shown in Fig. 2. The less intense parasitism of the rump lesions in comparison with the footpad lesions can be seen in the tissue sections stained for parasites by immunofluorescence (Fig. 2). Although rump-inoculated C57BL/6 IL-4$^{-/-}$ mice did not develop any lesion at the inoculation site, the biopsies showed parasites, more often found in the epidermal layer and upper dermis. In comparison, WT C57BL/6 mice had many more parasites located in the deeper dermis of the ulcerated lesion in the rump (Fig. 2).

The overall aspect of the paw and rump lesions in BALB/c mice was similar to those in WT C57BL/6 mice but paw swelling was more pronounced and the dorsal skin ulcers were larger and deeper (data not shown). The infected paws of BALB/c IL-4$^{-/-}$ mice were indurated and swollen to a similar extent as those from C57BL/6 mice, only much thicker (data not shown).

3.2. Parasitic load and dissemination of L. amazonensis in wild-type and in IL-4 deficient BALB/c and C57BL/6 mice

The parasite burden was estimated by LDA in the lesion and the corresponding draining lymph node (LN): popliteal for the

footulated mice and superficial inguinal for the rump-inoculated mice (Fig. 3). Parasite dissemination to distant LNs and to the spleen was also inspected at different times after infection (Table 1).

In general, parasite numbers per 10 mg of tissue were smaller in material taken from the dorsal skin lesions than from footpad tissue, as shown in Fig. 3A for either WT or IL-4$^{-/-}$ C57BL/6 mice, and in Fig. 3C for WT or IL-4$^{-/-}$ BALB/c mice. The photomicrographs of parasite-stained footpad sections in comparison with dorsal skin sections shown in Fig. 2 also illustrate the lesser parasitism of the latter.

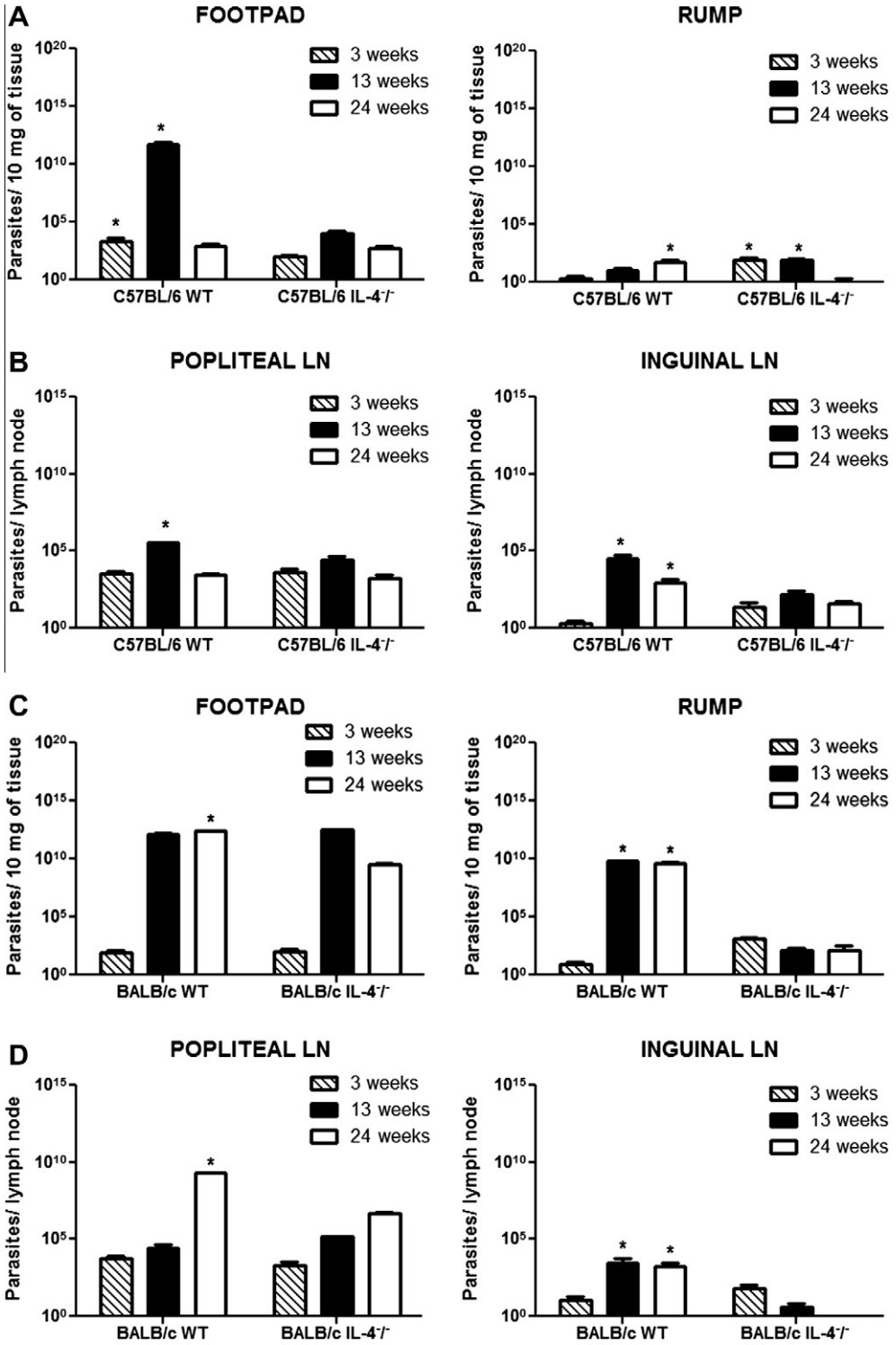

Fig. 3. Parasitic load in the inoculation site and in the draining lymph nodes (LN) from WT and IL-4$^{-/-}$ BALB/c and C57BL/6 mice. C57BL/6 mice (**A** and **B**) and BALB/c mice (**C** and **D**) were inoculated into the footpad or rump with 10^4 *L. amazonensis* metacyclic forms. Quantifying parasites was done by a limiting dilution assay (LDA). The numbers of parasites were estimates obtained by LDA followed by ELIDA analysis for the inoculation sites, footpad or rump lesion (**A** and **C**), and the respectively direct draining LNs (**B** and **D**), popliteal and superficial inguinal. Results are the means of six or seven individual mice and the 95% confidence intervals. Significant differences between WT and IL-4$^{-/-}$ are indicated by*.

A second point that emerges from the data on the lesions' parasite load is that there is no direct correlation between size of lesion and the number of parasites. This point is exemplified by WT BALB/c and BALB/c IL-4$^{-/-}$ mice whose paw swelling data showed a threefold difference (cf. Fig. 1A) despite comparable parasite loads (Fig. 3C). Moreover, there was no correlation of parasite load with the size of the skin lesion of the rump or the extent of ulceration; the size of the lesion and the subsequent ulceration are similar for WT BALB/c and WT C57BL/6 (cf. Fig. 1B). However, the parasite load in the latter is lower by a factor of eight orders of magnitude (cf. Fig. 3A WT C57BL/6 mice vs. Fig. 3C WT BALB/c).

Importantly, C57BL/6 IL-4$^{-/-}$ mice inoculated in the rump, that did not develop any lesions, still harbored a small number of parasites (about 10 or less) at the site of inoculation (Fig. 3A) and also had parasites in the inguinal draining LN throughout the infection (Fig. 3B). At most time points in both mouse strains, the numbers of parasites in the lesion and in the draining LN were smaller for IL-4$^{-/-}$ mice compared to WT controls. However, this was not the case for BALB/c mice inoculated in the footpad until 13 weeks of infection (Fig. 3C-footpad parasite load; 3 and 13 weeks) and the corresponding popliteal LN (Fig. 3D).

Dissemination of parasites from the footpad or rump site occurred in both susceptible and resistant mice. Several lymphoid organs were parasitized throughout the infection and heavier parasitic load was found in WT mice (Table 1). Systemic dissemination of parasites by the blood was observed from the 13th week of infection and occurred in footpad-inoculated WT and BALB/c IL-4$^{-/-}$ mice as well as in rump-inoculated WT BALB/c mice (Table 1). However, for those BALB/c IL-4$^{-/-}$ mice infected in the skin of the rump, parasites were only found in the lesion-draining inguinal LN (Table 1 and Fig. 3). In comparison, WT C57BL/6 mice, whether inoculated in the footpad or in the rump, had disseminated parasites by the blood route to the spleen or iliac LN already on the third week of infection (Table 1). Parasitism of distant organs in C57BL/6 IL-4$^{-/-}$ mice was detected later (13 weeks) than in the WT controls and could be observed in either footpad- or in rump-inoculated mice (Table 1).

Summarizing for the rump skin model of infection by *L. amazonensis*: (a) C57BL/6 mice developed progressive ulcerated lesions as did BALB/c mice but eventually demonstrated better control of parasitism; (b) IL-4 is an important susceptibility factor for BALB/c mice; however, other factors are significant, because BALB/c IL-4$^{-/-}$ mice still developed an ulcer with heavy parasitism; (c) IL-4 is a major susceptibility factor for C57BL/6 mice because C57BL/6 IL-4$^{-/-}$ mice did not develop any detectable lesions at the inoculation site in the dorsal dermis, although parasites were present in low numbers in the skin and draining LNs.

3.3. Production of cytokines in lymph node cell cultures from wild type and IL-4$^{-/-}$ C57BL/6 mice

Cytokines were quantified in supernatants obtained from draining LN cell cultures obtained from WT and IL-4$^{-/-}$ C57BL/6 mice inoculated in the footpad or rump with *L. amazonensis*. The

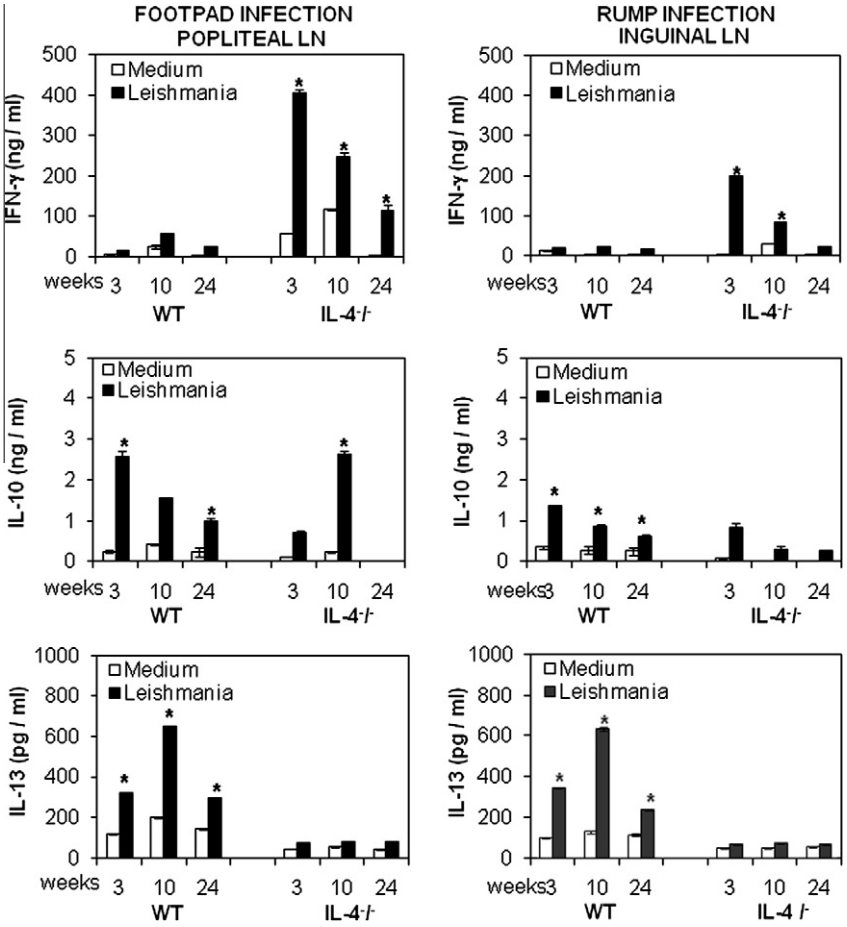

Fig. 4. Cytokine production in WT and IL-4$^{-/-}$ C57BL/6 mice. Mice were infected in the footpad or rump with *L. amazonensis* metacyclics, and cells from the draining LNs were incubated with live promastigotes as a secondary *in vitro* stimulus or maintained only in culture medium for 72 h before supernatant removal for cytokine testing. The amounts of IFN-γ IL-10 and IL-13 were determined by ELISA. Means and SEM of three independent experiments are presented; significant differences between popliteal and inguinal lymph nodes are indicated by *.

comparison of cytokine patterns in these two models of infection in C57BL/6 mice was of interest because IL-4$^{-/-}$ C57BL/6 mice, when inoculated in the rump, harbor parasites but do not develop lesions and when inoculated in the footpad develop small persistent indurated lesions with more parasites in both the lesion and draining LN (cf. Fig. 3A).

As expected, parasite-stimulated LN cultures from IL-4$^{-/-}$ mice, whether infected in the footpad or the rump, had much higher levels of IFN-γ in comparison to WT mice (Fig. 4). Compared to the respective C57BL/6 WT controls, IFN-γ production was higher in popliteal than in inguinal LNs.

Interleukin-10 production levels in LN cultures in general were higher for WT than for IL-4$^{-/-}$ mice, except on week 10 after footpad infection (Fig. 4). Production of IL-10 by the draining LN of WT mice inoculated in the footpad was higher than in the cultures from WT mice inoculated in the rump.

Significant levels of IL-13 were measured in LN cultures from infected WT C57BL/6 mice but there were no significant differences in the amount of IL-13 produced by cultures from footpad-inoculated and rump-inoculated mice. As expected, IL-13 levels were very low in cultures from IL-4$^{-/-}$ mice (Fig. 4). There were no significant differences between IL-12 levels measured in LN cultures from WT and C57BL/6 IL-4$^{-/-}$ mice (data not shown).

In order to test whether the low IL-10 levels observed in the LN of rump-inoculated C57BL/6 IL-4$^{-/-}$ mice were contributing to the maintenance of parasitism, groups of these mice were treated (starting on the 22nd week of infection) systemically with anti-IL-10R or with isotype control antibodies for two weeks. At the end of treatment, parasites were quantified in tissue of the inoculation site and draining LN. No significant differences in the number of parasites were seen between the two groups (data not shown). This result suggests that cytokines other than IL-10 may be involved in the maintenance of residual parasitism in IL-4$^{-/-}$ C57BL/6 mice.

3.4. Course of *L. amazonensis* infection in wild-type and IL-4-receptor-α$^{-/-}$ BALB/c mice

The data so far described indicate that an important share of BALB/c susceptibility to *L. amazonensis* is IL-4-independent. We proceeded to investigate the effects of the lack of signaling by IL-4 and IL-13 on the footpad and rump infection models. Footpad-infected BALB/c IL-4Rα$^{-/-}$ mice had significantly less paw swelling when compared to WT BALB/c (Fig. 5A) and IL-4$^{-/-}$ mice (cf. Fig. 1); mean increase in thickness was about 2 mm and persisted from week 12–24 of infection. However, the parasite burdens in the paw (10^{10}) (Fig. 5B) and the popliteal LN (10^{9}) (Fig. 5C) were still high by the 24th week of infection and similar to those in BALB/c IL-4$^{-/-}$ mice (Fig. 3).

In contrast, when injected in the rump skin, only 30% of BALB/c IL-4Rα$^{-/-}$ mice developed a small indurated lesion (Fig. 5A) that did not ulcerate and was significantly smaller than the lesions in similarly infected BALB/c IL-4$^{-/-}$ mice infected by the same route (cf. Fig. 5 vs. Fig. 1). Only those BALB/c IL-4Rα$^{-/-}$ mice that developed a lesion had parasites at the inoculation site; the numbers were comparable to those found in lesions from BALB/c IL-4$^{-/-}$ mice (Fig. 5B vs. Fig. 3C). Although the directly draining inguinal LN (Fig. 5C) was free of parasites, when other LNs were screened, parasites (estimated as less than 10) were found on the 2nd week of infection in the popliteal LN from the right and left leg indicating spread of the infection; all other distant lymphoid organs examined were free of parasites until the 24th week of infection (data not shown).

4. Discussion

Our results show that the site where *L. amazonensis* is inoculated in the mouse skin determines the type and development of the lesion as well as the extent to which it is affected by the cytokines IL-4 and IL-13.

Inoculation in the footpad resulted in significantly less paw swelling for WT C57BL/6 mice in comparison with WT BALB/c mice, confirming the relative resistance of the former mouse strain to *L. amazonensis* infection. In contrast, after intradermal inoculation of *L. amazonensis* into the skin of the rump, both WT C57BL/6 and WT BALB/c mice developed single progressive ulcerated lesions of comparable size until the 17th week of infection; thereafter the lesion continued to grow at a slower pace in C57BL/6 mice than in BALB/c mice. In spite of the rapidly growing ulcer, C57BL/6 mice controlled the local and systemic parasite load well, whereas BALB/c failed to do so (cf. Figs. 1B, 3 and Table 1). The influence of the inoculation site on pathogenesis had already been shown for some species of *Leishmania* (Nabors and Farrell, 1994; Poulter and Pandolph, 1982). For instance, *L. major* infections of DBA/2 mice, which are highly resistant when infected in the ear pinna but develop progressive lesions when inoculated at the base of the tail (Baldwin et al., 2003). Our results stress the dissociation between size of lesion and parasitic load and the influence of the inoculation site on these two parameters in the infection caused by *L. amazonensis*. Our results showing similar lesion growth in *L. amazonensis* metacyclics-rump-infected WT C57BL/6 and WT BALB/c mice differ from a previous report in which the lesion in WT C57BL/6 was at eight weeks of infection threefold smaller than in BALB/c mice; however, the inoculum used was 10^{6} amastigotes injected in the base of the tail (McMahon-Pratt and Alexander, 2004).

We found that ulcerations occurred only in rump lesions and never in the infected paws, even when they became intensely inflamed as in BALB/c mice. Infection severity and the appearance of ulcerative lesions in the footpad inoculation model of *L. amazonensis* infection correlated with the presence of TCD4^{+} cells (Silva et al., 1994; Soong et al., 1997). In fact, infected SCID C.B-17 mice developed nodular non-ulcerative lesions which ulcerated after transfer of WT TCD4+ spleen cells, confirming the involvement of this cell population in the pathogenesis of the ulcerative process (Terabe et al., 2000). It must be considered that in the footpads the epithelium and the dermis are much thicker than in the hairy skin and that the subcutaneous tissue has denser connective tissue and adipocytes lying in continuity with the muscle layer and bone. In contrast, the subcutaneous tissue of the rump region is thin and loosely attached to the underlying muscle. Most likely, inflammation, collagen synthesis, and wound repair run distinct courses in the paw and in the hairy skin. Recently, differential expression of the gene Fli1 that controls wound healing has been correlated to susceptibility to *L. major* infection (Sakthianandeswaren et al., 2010). Furthermore, Langerhans cells, which do not seem to have an equal distribution in the skin across the body (discussed in Kirkpatrick et al., 1987) also have been associated to *L. major* infection. This subset of dendritic cells promotes an expansion of specific-T regulatory cells which turns into suppression of Th1 immunity (Kautz-Neu et al., 2011).

Besides the structural aspects, cytokine production in *L. major*-infected CB6F1 mice inoculated in the footpad was shown to be predominantly Th1 with high IFN-γ levels, or Th2 with IL-4 and lower IFN-γ when inoculation was performed in the dorsal skin (Nabors et al., 1995). Our results with *L. amazonensis* did not show such differences: in fact, IL-4 production by LN cells draining the paw or the rump lesion was low, whereas IL-10 and IFN-γ production were generally higher in the popliteal LN than in the inguinal LN. Yet, in general, mice infected in the rump were better able to control the parasite load, possibly because of lower IL-10 production.

The lesions of BALB/c mice were significantly smaller in IL-4$^{-/-}$ mice in comparison with WT mice. However, rump-infected IL-4$^{-/-}$ BALB/c efficiently controlled local parasitism but such local

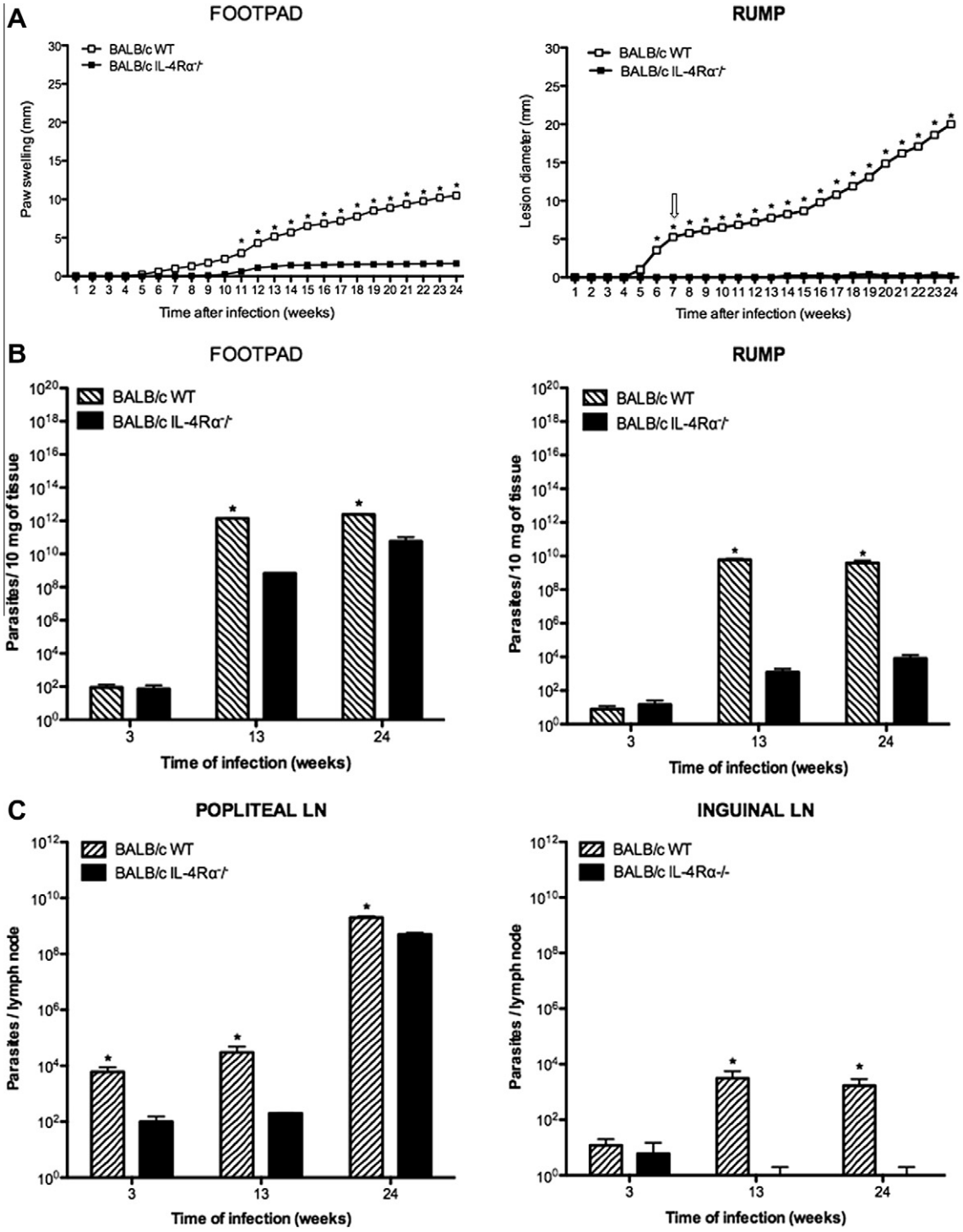

Fig. 5. Lesion development and parasitic load in the inoculation site and regional lymph nodes of WT BALB/c mice and of IL-4R$\alpha^{-/-}$ BALB/c mice infected with *L. amazonensis* in the footpad or in the rump. The mice were inoculated with 10^4 *L. amazonensis* metacyclic forms and the footpad and rump lesions (**A**) were measured as described in the legend to Fig. 1. Ulceration of the lesion in rump-inoculated mice is indicated by vertical arrow. Parasites were quantified (**B**) in the inoculation site and its draining lymph nodes. Popliteal and inguinal lymph nodes (**C**) correspond to the draining organs of footpad and rump, respectively. Parasite quantification was done by limiting dilution assay (LDA) and analyzed by ELIDA software. The values obtained were estimates of parasite numbers. Results represent the means of six or seven individual mice and 95% confidence intervals. Significant differences between WT and IL-4R$\alpha^{-/-}$ are indicated by*.

control was not seen on footpad infected mice. In addition, in C57BL/6 mice IL-4 was also a more important susceptibility factor when inoculation was done in the rump skin than in the footpad because footpad inoculated IL-$4^{-/-}$ C57BL/6 mice still had small, persistent lesions with high parasite load whereas rump-inoculated mice did not have any local lesion and less than 10 parasites in biopsies of the inoculation site. Our findings that in the C57BL/6 paw infection model the lack of IL-4 only partially reduced swelling and parasite load, agree with those obtained by other authors (Jones et al., 2000; McMahon-Pratt and Alexander, 2004). Therefore, the relative importance of IL-4 as a determinant of susceptibility differs according to the inoculation site. Unexpectedly, IFN-γ

production by popliteal LN cells was higher than by inguinal LN cells. Nevertheless, in *L. amazonensis* infection, IFN-γ may either control or favor amastigote multiplication depending on its association with stimuli such as LPS or TNF-α (Qi et al., 2004).

With respect to IL-10, we treated footpad- or rump-infected C57BL/6 IL-4$^{-/-}$ during the chronic phase of infection with anti-IL-10R mAb Asc-1B1 and observed no effect on the lesion or on proximal or distant LN parasitic loads. However, previous work on the role of IL-10 in *L. amazonensis* infections yielded controversial results (Ji et al., 2003; Jones et al., 2002; Padigel et al., 2003). Parasite strain differences are important in this context as was clearly shown for *L. major* strains, whose susceptibility to IFN-γ-activated macrophages is markedly diverse (Noben-Trauth et al., 2003).

Another Th2-type cytokine involved in the susceptibility to *Leishmania* infections is IL-13. The joint participation of IL-13 and IL-4 as susceptibility factors to *L. major* has been documented in several studies which analyzed the role of IL-13 alone (Matthews et al., 2000) or investigated the course of infection in IL-4Rα$^{-/-}$ mice lacking signaling through both IL-4 and IL-13 (Mohrs et al., 1999; Nagase et al., 2007; Noben-Trauth et al., 2003; Radwanska et al., 2007). In order to investigate the role of IL-13 in susceptibility to *L. amazonensis* we compared the course of infection in IL-4Rα$^{-/-}$ BALB/c to IL-4$^{-/-}$ and WT mice of the same strain. In agreement with previous work (Guimaraes et al., 2006), our results (Fig. 1A and B) showed only partial control of infection in *L. amazonensis* footpad-infected IL-4$^{-/-}$ BALB/c mice. However, the lack of signaling by IL-4 and IL-13 had a marked effect at reducing paw swelling in IL-4Rα$^{-/-}$ mice, but did not reduce parasitism in the lesion and the draining LN in comparison with IL-4$^{-/-}$ BALB/c mice (cf. Fig. 5 vs. Fig. 1A and Fig. 3). Most important is that after parasite inoculation in the rump, only 30% of BALB/c IL-4Rα$^{-/-}$ mice became infected. Thus, IL-13 plus IL-4 had a more important role in determining susceptibility to *L. amazonensis* when the infection was performed in the dorsal skin than in the footpad. Comparing these results with those of IL-4$^{-/-}$ on the C57BL/6 background, which also fail to develop a lesion when inoculated in the rump, we can consider that elimination of only IL-4 would be sufficient to avoid lesion and reduce the parasitism in C57BL/6 mice. By contrast, neutralization of both IL-4 and IL-13 would be required to obtain a similar result in susceptible BALB/c mice. However, innate immunity factors such as the wound repair response may also play a pivotal role in the resistance to *L. amazonensis* as described for a group of genes in murine *L. major* infection (Sakthianandeswaren et al., 2005).

These findings indicate that IL-4 and IL-13 have a critical importance in the susceptibility to *L. amazonensis* infection. In addition, the inoculation site and consequently the type of lesion are important factors in the pathogenesis of cutaneous leishmaniasis.

Acknowledgments

We thank Paulo B. Albe for assistance with tissue preparation for immunohistochemistry and Miriam E. Mossoba and James C. Wang for the manuscript correction. This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP). T. C. Felizardo received a fellowship from FAPESP. I. A. A. received a supplementary fellowship from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

References

Abrahamsohn, I.A., Coffman, R.L., 1995. Cytokine and nitric oxide regulation of the immunosuppression in *Trypanosoma cruzi* infection. J. Immunol. 155, 3955–3963.

Afonso, L.C., Scott, P., 1993. Immune responses associated with susceptibility of C57BL/10 mice to *Leishmania amazonensis*. Infect. Immun. 61, 2952–2959.

Alexander, J., Brombacher, F., McGachy, H.A., McKenzie, A.N., Walker, W., Carter, K.C., 2002. An essential role for IL-13 in maintaining a non-healing response following *Leishmania mexicana* infection. Eur. J. Immunol. 32, 2923–2933.

Baldwin, T.M., Elso, C., Curtis, J., Buckingham, L., Handman, E., 2003. The site of *Leishmania major* infection determines disease severity and immune responses. Infect. Immun. 71, 6830–6834.

Beckstead, J.H., 1994. A simple technique for preservation of fixation-sensitive antigens in paraffin-embedded tissues. J. Histochem. Cytochem. 42, 1127–1134.

Courret, N., Lang, T., Milon, G., Antoine, J.C., 2003. Intradermal inoculations of low doses of *Leishmania major* and *Leishmania amazonensis* metacyclic promastigotes induce different immunoparasitic processes and status of protection in BALB/c mice. Int. J. Parasitol. 33, 1373–1383.

Courret, N., Prina, E., Mougneau, E., Saraiva, E.M., Sacks, D.L., Glaichenhaus, N., Antoine, J.C., 1999. Presentation of the *Leishmania* antigen LACK by infected macrophages is dependent upon the virulence of the phagocytosed parasites. Eur. J. Immunol. 29, 762–773.

Felizardo, T.C., Toma, L.S., Borges, N.B., Lima, G.M., Abrahamsohn, I.A., 2007. *Leishmania* (*Leishmania*) *amazonensis* infection and dissemination in mice inoculated with stationary-phase or with purified metacyclic promastigotes. Parasitology 134, 1699–1707.

Guimaraes, E.T., Santos, L.A., Ribeiro dos Santos, R., Teixeira, M.M., dos Santos, W.L., Soares, M.B., 2006. Role of interleukin-4 and prostaglandin E2 in *Leishmania amazonensis* infection of BALB/c mice. Microbes Infect. 8, 1219–1226.

Handman, E., 1999. Cell biology of *Leishmania*. Adv. Parasitol. 44, 1–39.

Ji, J., Sun, J., Soong, L., 2003. Impaired expression of inflammatory cytokines and chemokines at early stages of infection with *Leishmania amazonensis*. Infect. Immun. 71, 4278–4288.

Jones, D.E., Ackermann, M.R., Wille, U., Hunter, C.A., Scott, P., 2002. Early enhanced Th1 response after *Leishmania amazonensis* infection of C57BL/6 interleukin-10-deficient mice does not lead to resolution of infection. Infect. Immun. 70, 2151–2158.

Jones, D.E., Buxbaum, L.U., Scott, P., 2000. IL-4-independent inhibition of IL-12 responsiveness during *Leishmania amazonensis* infection. J. Immunol. 165, 364–372.

Kautz-Neu, K., Noordegraaf, M., Dinges, S., Bennett, C.L., John, D., Clausen, B.E., von Stebut, E., 2011. Langerhans cells are negative regulators of the anti-*Leishmania* response. J. Exp. Med. 208, 885–891.

Kirkpatrick, C.E., Nolan, T.J., Farrell, J.P., 1987. Rate of *Leishmania*-induced skin-lesion development in rodents depends on the site of inoculation. Parasitology 94 (Pt 3), 451–465.

Lemos de Souza, V., Ascencao Souza, J., Correia Silva, T.M., Sampaio Tavares Veras, P., Rodrigues de-Freitas, L.A., 2000. Different *Leishmania* species determine distinct profiles of immune and histopathological responses in CBA mice. Microbes Infect. 2, 1807–1815.

Lima, H.C., Bleyenberg, J.A., Titus, R.G., 1997. A simple method for quantifying *Leishmania* in tissues of infected animals. Parasitol. Today 13, 80–82.

Matthews, D.J., Emson, C.L., McKenzie, G.J., Jolin, H.E., Blackwell, J.M., McKenzie, A.N., 2000. IL-13 is a susceptibility factor for *Leishmania major* infection. J. Immunol. 164, 1458–1462.

McMahon-Pratt, D., Alexander, J., 2004. Does the *Leishmania major* paradigm of pathogenesis and protection hold for New World cutaneous leishmaniases or the visceral disease? Immunol. Rev. 201, 206–224.

Mohrs, M., Ledermann, B., Kohler, G., Dorfmuller, A., Gessner, A., Brombacher, F., 1999. Differences between IL-4- and IL-4 receptor alpha-deficient mice in chronic leishmaniasis reveal a protective role for IL-13 receptor signaling. J. Immunol. 162, 7302–7308.

Nabors, G.S., Farrell, J.P., 1994. Site-specific immunity to Leishmania major in SWR mice. the site of infection influences susceptibility and expression of the antileishmanial immune response. Infect. Immun. 62, 3655–3662.

Nabors, G.S., Nolan, T., Croop, W., Li, J., Farrell, J.P., 1995. The influence of the site of parasite inoculation on the development of Th1 and Th2 type immune responses in (BALB/c × C57BL/6) F1 mice infected with *Leishmania major*. Parasite Immunol. 17, 569–579.

Nagase, H., Jones, K.M., Anderson, C.F., Noben-Trauth, N., 2007. Despite increased CD4+Foxp3+ cells within the infection site, BALB/c IL-4 receptor-deficient mice reveal CD4+Foxp3-negative T cells as a source of IL-10 in *Leishmania major* susceptibility. J. Immunol. 179, 2435–2444.

Noben-Trauth, N., Lira, R., Nagase, H., Paul, W.E., Sacks, D.L., 2003. The relative contribution of IL-4 receptor signaling and IL-10 to susceptibility to *Leishmania major*. J. Immunol. 170, 5152–5158.

Osorio y Fortea, J., Prina, E., de La Llave, E., Lecoeur, H., Lang, T., Milon, G., 2007. Unveiling pathways used by *Leishmania amazonensis* amastigotes to subvert macrophage function. Immunol. Rev. 219, 66–74.

Padigel, U.M., Alexander, J., Farrell, J.P., 2003. The role of interleukin-10 in susceptibility of BALB/c mice to infection with *Leishmania mexicana* and *Leishmania amazonensis*. J. Immunol. 171, 3705–3710.

Pereira, B.A., Alves, C.R., 2008. Immunological characteristics of experimental murine infection with *Leishmania* (*Leishmania*) *amazonensis*. Vet. Parasitol. 158, 239–255.

Poulter, L.W., Pandolph, C.R., 1982. Mechanisms of immunity to leishmaniasis. IV. Significance of lymphatic drainage from the site of infection. Clin. Exp. Immunol. 48, 396–402.

Qi, H., Ji, J., Wanasen, N., Soong, L., 2004. Enhanced replication of *Leishmania amazonensis* amastigotes in gamma interferon-stimulated murine macrophages: implications for the pathogenesis of cutaneous leishmaniasis. Infect. Immun. 72, 988–995.

Radwanska, M., Cutler, A.J., Hoving, J.C., Magez, S., Holscher, C., Bohms, A., Arendse, B., Kirsch, R., Hunig, T., Alexander, J., Kaye, P., Brombacher, F., 2007. Deletion of IL-4Ralpha on CD4 T cells renders BALB/c mice resistant to *Leishmania major* infection. PLoS Pathog. 3, e68.

Sacks, D., Noben-Trauth, N., 2002. The immunology of susceptibility and resistance to Leishmania major in mice. Nat. Rev. Immunol. 2, 845–858.

Sakthianandeswaren, A., Curtis, J.M., Elso, C., Kumar, B., Baldwin, T.M., Lopaticki, S., Kedzierski, L., Smyth, G.K., Foote, S.J., Handman, E., 2010. Fine mapping of *Leishmania major* susceptibility Locus lmr2 and evidence of a role for Fli1 in disease and wound healing. Infect. Immun. 78, 2734–2744.

Sakthianandeswaren, A., Elso, C.M., Simpson, K., Curtis, J.M., Kumar, B., Speed, T.P., Handman, E., Foote, S.J., 2005. The wound repair response controls outcome to cutaneous leishmaniasis. Proc. Natl. Acad. Sci. U S A 102, 15551–15556.

Satoskar, A., Brombacher, F., Dai, W.J., McInnes, I., Liew, F.Y., Alexander, J., Walker, W., 1997. SCID mice reconstituted with IL-4-deficient lymphocytes, but not immunocompetent lymphocytes, are resistant to cutaneous leishmaniasis. J. Immunol. 159, 5005–5013.

Silva, E.M., Bertho, A.L., Mendonca, S.C., 1994. Effect of in vivo depletion of CD4+ T cells on experimental infection of susceptible BALB/c mice with *Leishmania amazonensis*. Acta Trop. 56, 111–120.

Silveira, F.T., Lainson, R., De Castro Gomes, C.M., Laurenti, M.D., Corbett, C.E., 2009. Immunopathogenic competences of *Leishmania* (*V.*) *braziliensis* and *L.* (*L.*) *amazonensis* in American cutaneous leishmaniasis. Parasite Immunol. 31, 423–431.

Soong, L., Chang, C.H., Sun, J., Longley Jr., B.J., Ruddle, N.H., Flavell, R.A., McMahon-Pratt, D., 1997. Role of CD4+ T cells in pathogenesis associated with *Leishmania amazonensis* infection. J. Immunol. 158, 5374–5383.

Sosa, M.R., Rosas, L.E., McKenzie, A.N., Satoskar, A.R., 2001. IL-13 gene-deficient mice are susceptible to cutaneous *L. Mexicana* infection. Eur. J. Immunol. 31, 3255–3260.

Taswell, C., 1986. Limiting dilution assays for the separation, characterization and quantification of biologically active particles and their clonal progeny: statistical methods for LDAs used by ELIDA. In: Pretlow, T.G., Pretlow, T.P. (Eds.), Cell separation: selected methods and applications. Academic Press, New York, pp. 109–145.

Terabe, M., Kuramochi, T., Ito, M., Hatabu, T., Sanjoba, C., Chang, K.P., Onodera, T., Matsumoto, Y., 2000. CD4(+) cells are indispensable for ulcer development in murine cutaneous leishmaniasis. Infect. Immun. 68, 4574–4577.