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Research brief

Leishmania amazonensis: Participation of regulatory T and B cells in the in vitro priming (PIV) of CBA/J spleen cells susceptible response

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Abstract

CBA/J mice are resistant to *Leishmania major* and susceptible to *Leishmania amazonensis*. Early events determine infection outcome. Until now, PIV (in vitro priming) immune response to *L. amazonensis* has not been assessed. Herein, we have shown that compared to *L. major*, *L. amazonensis* induced higher parasite burden associated to similar IL-4, IFN-γ, and TNF-α mRNA expressions and IFN-γ and IL-10 levels. Although similar amounts of IL-10 were detected, the frequency of intracellular IL-10 positive B cells was enhanced in spleen cells stimulated with anti-CD3/anti-CD28, or anti-CD3/anti-CD28 and *L. amazonensis*, compared to *L. major*-stimulation. Interestingly, IL-10- producing B cells were reduced in response to anti-CD3/anti-CD28 stimulation combined with *L. major* compared to the other groups. *L. amazonensis* may favor T regulatory cell development, since 40% of all the $CD4^{+}CD25^{+}$ were $CD25^{high}$ cells. These data suggest that in PIV, susceptibility to *L. amazonensis* is not related to Th cell polarization, but to the presence and activity of regulatory T and B cells.
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Index Descriptors and Abbreviations: CBA/J mice; PIV; *Leishmania amazonensis*; *Leishmania major*; Regulatory T and B cells

1. Introduction

Murine experimental cutaneous leishmaniasis has been widely used to characterize the immune response against *Leishmania*. It is well documented that the ultimate response in resistance to *Leishmania major* infection is associated with differentiation of Th1 CD4+ lymphocytes, leading to parasite destruction by IFN-γ-stimulated macrophages, in TNF-α- and NO-dependent mechanisms (Gomes et al., 2003). Alternatively, susceptibility to *Leishmania amazonensis* was associated with Th2 response, IL-4 production and a failure in IFN-γ-induced macrophage activation (Jones et al., 2000). *L. amazonensis* induces chronically non-healing infections in several mouse strains such as C3H, C57BL/6, and C57BL/10, which are resistant to *L. major*. In addition, BALB/c and CBA/J mice are highly susceptible to *L. amazonensis* (Afonso and Scott, 1993; Lemos de Souza et al., 2000). The immune response to *L. amazonensis* and other species of mexicana complex are quite different from that induced by *L. major*. BALB/c and C57BL/10 mice do not mount a vigorous Th2 response and generate detectable levels of Th1-type cytokines (Afonso and Scott, 1993; Ji et al., 2002).

In this laboratory, we established an interesting murine model to study the response to *Leishmania* infection, the CBA/J mice, which are resistant to *L. major* and susceptible to *L. amazonensis* (Lemos de Souza et al., 2000). Since genetic background differences are overcome in this mouse model, it can be used as a tool to identify the parasite's role in determining infection outcome. Lymph node (LN) cells

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from CBA/J mice infected by *L. major* and re-stimulated in vitro with *Leishmania* promastigotes produced large amounts of IFN-γ and early IL-4, whereas infection by *L. amazonensis* led to stable IL-4 production by LN cells from infected mice (Lemos de Souza et al., 2000). The early cellular events which could explain these differences are unknown.

There is evidence that early events in host–parasite interaction determine the infection outcome (Diefenbach et al., 1998; Laskay et al., 1995; Sadick et al., 1990). PIV stimulated T cells have been used to study selective priming of Th1 and Th2 cells in response to *L. major* stimulation (Shankar and Titus, 1993; Soares et al., 1997). Until now a detailed description of primary immune response of mice to *L. amazonensis* has not been carried out. In the present report, we used the PIV system to further investigate the early events involved in the establishment of the immune response to *L. amazonensis* and *L. major* infection in CBA/J mice.

Spleen cells cultivated in DMEM complete medium supplemented with 0.5% of normal mouse inactivated serum were in vitro stimulated with 1×10^7 stationary phase *L. amazonensis* or *L. major* promastigotes. In all experiments, negative and positive controls were parallel cultures containing no parasites or 10 μg/ml of concanavalin A (ConA), respectively. First, parasite burden was determined at 7 days of parasite stimulation: cultures were washed and incubated in Schneider's medium to liberate intracellular amastigotes to be transformed and multiply into promastigote form. Every day parasite number was checked and only after 7–10 days promastigotes were detected in cell cultures. As expected, at 7 days of infection parasite burden was significantly higher in the *L. amazonensis*-stimulated cultures (*L. amazonensis* $= 1.31 \times 10^7 \pm 1.11 \times 10^6$ promastigotes × *L. major* $= 1.26 \times 10^6 \pm 2.33 \times 10^5$ promastigotes, $n = 3$, Student's t test $p < 0.001$). This result was not due to differences in the ability of parasite in vitro replication, since in our laboratory *L. amazonensis* and *L. major* promastigotes proliferate similarly in axenic cultures.

RT-PCR was performed to analyze cytokine expression at 3 and 7 days of parasite stimulation. Table 1 shows the primer sequences, annealing temperature, cycle numbers for linear phase of amplification and product size (bp). Densitometric analyses were performed for each group: control, *L. amazonensis*- or *L. major*-stimulated cultures. Quantification was normalized to HPRT constitutive expression. Cytokine production was detected in cell supernatants by sandwich ELISA.

It was previously described that production of some cytokines, such as IL-4, often occurs at low levels and is difficult to detect by standard ELISA technique (O'Connor et al., 1999). In the present work, IL-4, IFN-γ, and TNF-α mRNA expression was assessed by RT-PCR in stimulated lymphocytes and these results were not statistically different from control non-stimulated cells, when quantified at 3 and 7 days of parasite stimulation (Figs. 1A–D, ANOVA, $p > 0.05$, $n = 3$). Even though these results displayed high variability, it is noteworthy that in two out of three experiments IL-4 and IFN-γ mRNA expression was higher in *L. amazonensis*- than in *L. major*-stimulated cultures. It is likely that similar TNF-α mRNA expression in *L. major*- and *L. amazonensis*-stimulated cultures is due to the dilution of macrophage mRNAs by mRNAs from the additional T and B cells and also to a late detection of cytokine expression (3 and 7 days of parasite stimulation). We have previously detected a higher expression of this cytokine in *L. major* in comparison to *L. amazonensis*-infected macrophages as early as 4 h of infection (Gomes et al., 2003).

At 3 days of both parasites' stimulation, IFN-γ levels in cell supernatants were low and similar to those produced by control non-stimulated cells (not shown). At 7 days of parasite stimulation, IFN-γ was released in higher amounts in supernatants of *L. amazonensis*-stimulated cells (Fig. 2A, $p < 0.01$, ANOVA, $n = 7$) when compared to control cells, although there was no difference when compared to the production by *L. major*-stimulated cultures. In accordance to our data, some reports have identified the presence of IFN-γ early after *L. amazonensis* or *L. mexicana* infection (Ji et al., 2002). It is possible that, in *L. amazonensis* PIV stimulation, IFN-γ participates in Th2 priming, as previously described (Bocek et al., 2004).

Previously, we demonstrated that upon IFN-γ stimulation, NO was produced in similar amounts by either *L. major*- or *L. amazonensis*-infected macrophages. Yet, parasite burden was only reduced in *L. major* infection (Gomes et al., 2003). In the present work, we observed that NO production was low and/or similar upon *L. amazonensis* or *L. major* PIV stimulation when compared to control non-stimulated cells both at 3 (not shown) and at 7 days of parasite stimulation (Fig. 2B, $p > 0.05$, ANOVA, $n = 3$). Since IFN-γ was detected in stimulated cell supernatants, these data suggest that there are low numbers of NO producing cells in this in vitro system.

Our previous observation showed that both *L. major* and *L. amazonensis* were able to induce IL-10 production

Table 1
Primer sequences and the sizes of PCR product

Oligonucleotide	Sense	Antisense	Annealing temperature (°C)	Cycle number	Product size (bp)
TNF-α	GATCTCAAAGACAACCAACTAGTG	CTCCAGCTGGAAGACTCCTCCCAG	60	30	255
IFN-γ	CATTGAAAGCCTAGAAAGTCTG	CTCATGAATGCATCCTTTTTCG	62	35	267
IL-4	CATCGCCATTTTGAACGAGGTCAC	CTTATCGATGAATCCAGGCATCG	65	35	240
IL-10	AGAAAAGAGAGCTCCATCATGC	AATCACTCTTCACCTGCTCCA	60	35	454
HPRT	AGCTTGCTGGTGAAAAGGAC	TTATAGTCAAGGGCATATCC	62	35	104

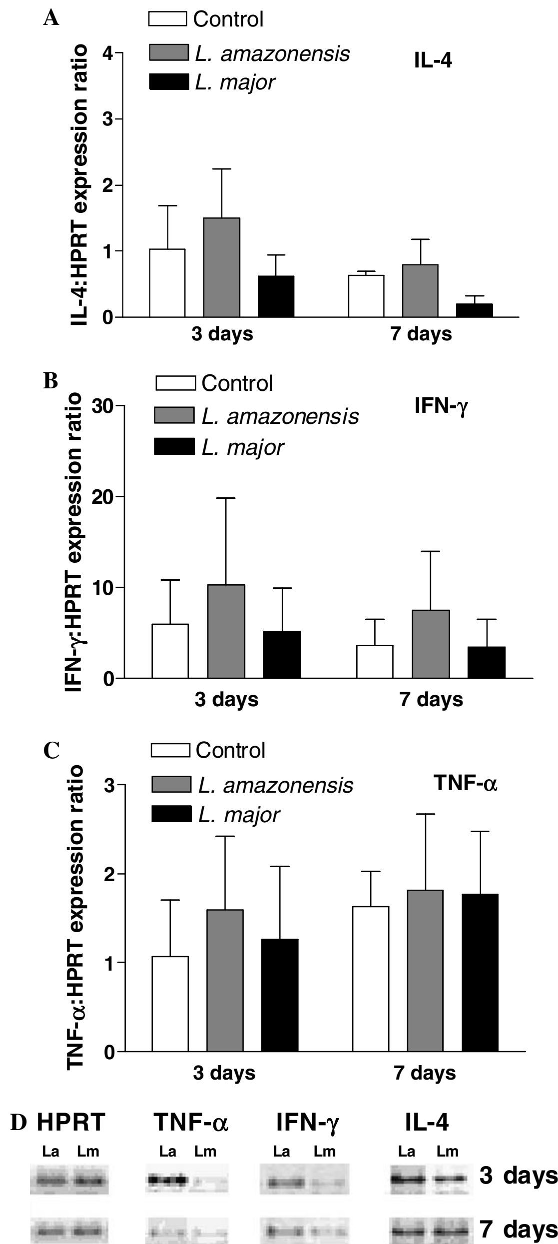

Fig. 1. Cytokine mRNA expression in *Leishmania*-stimulated splenocytes. Splenocytes (1×10^7 cells/ml) cultivated in DMEM complete medium supplemented with 0.5% of normal mouse inactivated serum were in vitro stimulated with 1×10^7 stationary phase *L. amazonensis* or *L. major* promastigotes and total mRNA was extracted for RT-PCR analyses of cytokine expression at 3 and 7 days of parasite stimulation. Densitometric analyses were performed and quantification of IL-4 (A), IFN-γ (B), TNF-α (C) mRNA was normalized to the levels of the constitutive expression of HPRT. Results represent mRNA expression in non-stimulated control cells (white bars), *L. amazonensis*- (gray bars) or *L. major*-stimulated splenocytes (black bars). Lower panels show the expression of cytokines and HPRT at 3 and 7 days of splenocyte stimulation with *L. amazonensis* (La) or *L. major* (Lm) (D). Data represent mean ± SD values of three experiments and no statistical differences were detected by ANOVA.

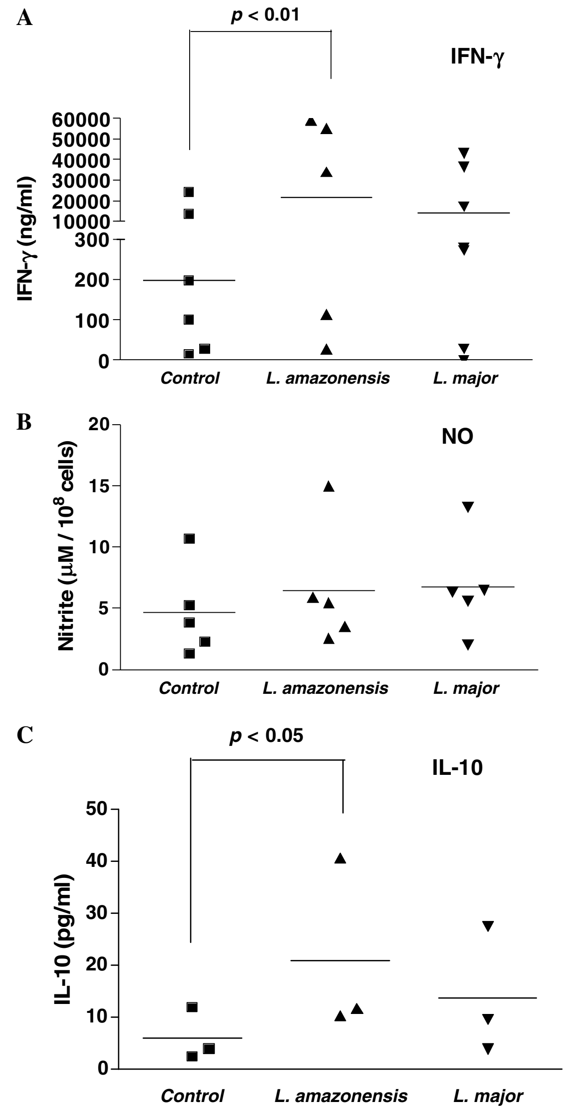

Fig. 2. Spleen cells primary stimulated with *L. amazonensis* and *L. major* induced similar amounts of NO but higher IFN-γ and IL-10 production in comparison to control cells. CBA/J spleen cells were non-stimulated (filled square) either co-cultivated with *L. amazonensis* (filled triangle) or *L. major* (filled inverted triangle) stationary phase promastigotes and culture supernatants were evaluated at 7 days after parasite stimulation by sandwich ELISA for the presence of IFN-γ ($p < 0.05$, $n = 7$) (A), IL-10 ($p < 0.01$, $n = 3$) (C). NO production was determined by Griess reaction and was not different from non-stimulated cells (B). IFN-γ and IL-10 productions by ConA-stimulated cells were higher than the upper limit of the standard curve. Each point represents the mean values of each experiment. Statistical analyses were performed using ANOVA.

upon in vitro recall (Lemos de Souza et al., 2000). IL-10 production during PIV stimulation was then assessed by ELISA. *L. amazonensis* induced release in cell supernatants of 3.3 times more IL-10 than control cells (Fig. 2C, $p < 0.05$, $n = 3$) and 1.5 times more IL-10 than *L. major* stimulation, even though this last difference was not statistically significant. In spite of being similarly produced, IL-10 may differentially contribute to susceptibility and resistance in the *Leishmania* model. Therefore, the role of IL-10 in the early *L. amazonensis*- or *L. major*-induced priming was further evaluated by IL-10 neutralization. Interestingly, control IgG induced decrease in IFN-γ production in *Leishmania*-stimulated cultures. It is possible that as previously described the addition of control IgG to cultures stimulates

an alternative activation of antigen presenting cells, resulting in expression of IL-10 (Afonso and Scott, 1993; Mosser, 2003). As expected, IL-10 neutralization induced enhancement in IFN-γ release in both *L. amazonensis*- and *L. major*-stimulated cultures compared to control IgG-treated cells (Fig. 3A, ANOVA, $p < 0.0001$).

Intracellular cytokine detection by FACS analysis was performed to determine the cell phenotype responsible for IL-10 production in *Leishmania*-stimulated cultures. In these conditions, we observed that $CD4^+$, $CD8^+$, and $CD4^+CD25^+$ T cells did not produce detectable levels of IL-10 after 24, 48, or 72 h (not shown) of stimulation. We also checked for the IL-10 expression in macrophages by RT-PCR and we observed that IL-10 mRNA expression was very low and similar levels were detected in macrophages from *L. amazonensis*- and *L. major*-stimulated cultures (not shown). By flow cytometry, intracellular IL-10 was only detected in B cells after 48 h of spleen cells stimulated by anti-CD3/anti-CD28 (Fig. 3B, 4.7% of total B cells) or by anti-CD3/anti-CD28 combined with *L. amazonensis* (Fig. 3B, 5.3% of the total B cells), where an enhancement in the frequency of IL-10 positive B cells was detected. IL-10 production by B cells participating in Th2 response of *L. major* susceptible BALB/c mice has been previously described (Palanivel et al., 1996). Interestingly, the frequency of IL-10 producing B cells was significantly reduced in response to anti-CD3/anti-CD28 and *L. major* combined stimulation (1.3%) when compared to the two other cell groups. These data indicate that *L. major* may suppress IL-10 production by B cells (Fig. 3B). Although we were unable to detect intracellular IL-10 production by $CD4^+CD25^+$ cells, one may not exclude the possibility that a small amount of IL-10 is produced by $CD4^+$ T cells in our experimental system. Experiments to detect IL-10 and/or foxp3 by real time PCR in *L. amazonensis*-stimulated spleen cells are under way.

To determine whether regulatory T cells contribute to differences in *Leishmania* priming, the frequency of $CD4^+CD25^+$ T lymphocytes was assessed (Figs. 3C–E). We found that in *L. amazonensis*-stimulated cells, $CD4^+CD25^+$ T lymphocytes represented 21.2% of the total $CD4^+$ T cells (Fig. 3C) and in *L. major*-stimulated splenocytes, this

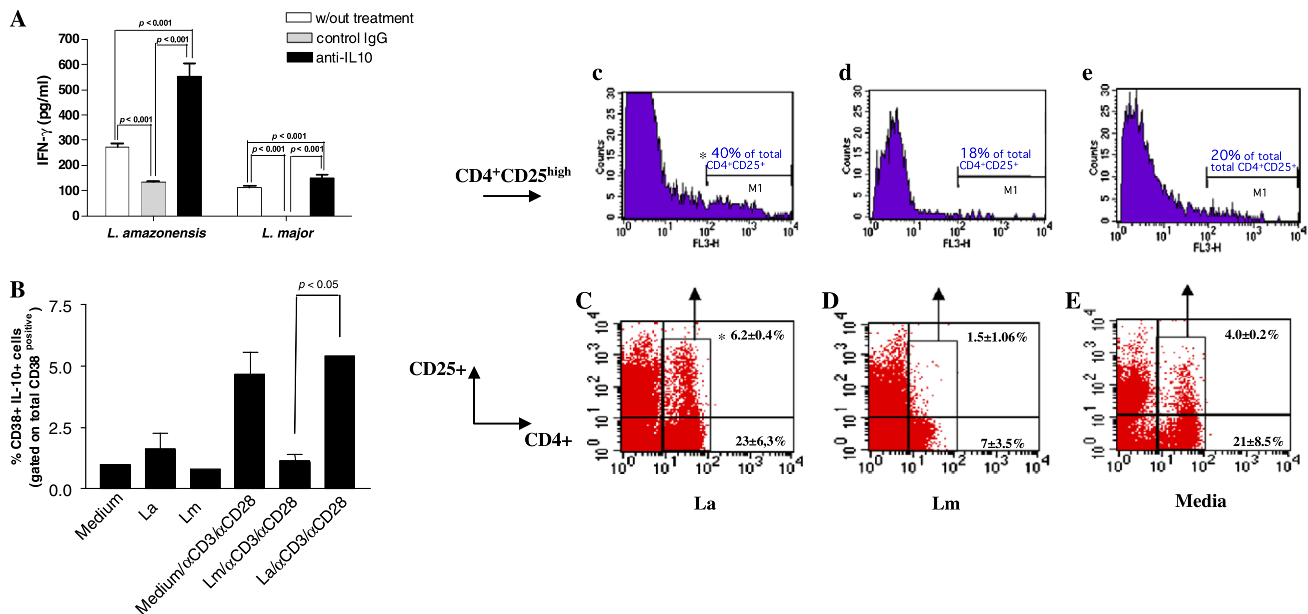

Fig. 3. Analysis of IL10 blocking and regulatory T and B cells in *L. amazonensis*- and *L. major* in vitro priming. (A) CBA/J spleen cells were co-cultivated with *L. amazonensis* or *L. major* in the presence of a blocking monoclonal antibody to IL-10 (5 μg/ml, added every day). Control cells were cultivated in medium alone or in presence of control IgG. Culture supernatants were collected 72 h after parasite stimulation and maintained at −20 °C until tested by sandwich ELISA for the presence of IFN-γ. Bars represent mean values ± SD of one experiment. Statistical analyses were performed using ANOVA ($n = 3$, $p < 0.0001$). (B) For intracytoplasmatic stainings, spleen cells were cultured in 24 well plates pre-coated with 10 μg/well of soluble anti-CD3 monoclonal antibody (clone 2C11) at a cell density of 1×10^7 cells/well in DMEM complete medium supplemented with 5% inactivated fetal calf serum in the presence of 2 μg/well of anti-CD28 (clone PV-1). Brefeldin A was added for the last 6 h of culture at a final concentration of 10 μg/ml. Cells were harvested after 48 h of culture and stained with biotinylated rat anti-mouse CD38 (Southern Biotechnology Associates Inc. Birmingham, AL, USA) plus cychrome–streptavidin. After fixation with 1% paraformaldehyde, spleen cells were continuously exposed to saponin and stained with PE conjugated anti-mouse IL-10 mAb (BD Pharmingen, San Diego, CA) for 30 min at 4 °C. Thirty thousands events were recorded per sample in an appropriate gated region, using a FACScan. Results were analyzed using the CellQuest software. Bars represent the mean of four determinations ± SEM (Mann–Whitney test, $p < 0.05$ was considered statistically significant). (C–E) Flow cytometric analysis was performed to evaluate T cell surface phenotype. Spleen cells were co-cultivated with *L. amazonensis* or *L. major* for 48 h. Stainings were done using antibody-fluorochrome conjugates (Pharmingen, San Diego, CA). FITC-conjugated anti-mouse CD4 and biotinylated rat anti-mouse CD25, followed by the addition of cychrome–streptavidin were used. (C–E) Numbers in the upper right and lower right dot plot quadrants represent the mean ± SEM of $CD4^+CD25^+$ and $CD4^+CD25^-$, respectively. Results represent one out from four determinations. (c–e insets) Histograms represent $CD4^+CD25^+$. M1 represents the percentage of $CD25^{high}$ among total $CD4^+CD25^+$ T cells population.

frequency was significantly lower ($p < 0.05$) representing 17.6% of the total $CD4^+$ T cells (Fig. 3D) and similar to control non-stimulated cells, 16.0% of the total $CD4^+$ T cells (Fig. 3E). $CD4^+CD25^{high+}$ cells with a regulatory phenotype have been recently described (Setoguchi et al., 2005). In our data, it was observed that these T $CD4^+CD25^{high+}$ regulatory cells of *L. amazonensis*-stimulated cells represented 40% from the total $CD4^+CD25^+$ T lymphocytes (Fig. 3C, inset c) compared to 18% in *L. major*-stimulated cultures (Fig. 3D, inset d) and 20% in control non-stimulated cells (Fig. 3E, inset e).

This work was conceived to investigate early events in *L. amazonensis* and *L. major* stimulation by using a PIV system. In summary, our data showed that *L. amazonensis*-induced unefficaceous response is associated with a higher parasite burden and an increase in the percentage of T $CD4^+CD25^{high+}$ regulatory cells. In addition, an enhancement in the frequency of IL-10-producing B cells was observed when cultures were stimulated with the monoclonal antibodies which stimulate T lymphocytes whether combined or not to *L. amazonensis*. On the other hand, the lower parasite burden in *L. major*-stimulated cells was observed to be associated with decrease in the frequency of IL-10 producing B cells.

Taken together, our results suggest that IL-10 produced by B cells contribute to generate these $CD4^+CD25^{high+}$ regulatory cells in the *L. amazonensis* priming, as previously described (reviewed in Curotto de Lafaille and Lafaille, 2002). Finally, these data point to the idea that in the PIV system, susceptibility to *L. amazonensis* is not related to the Th cell polarization, but to the presence of regulatory T and B cells. The virtual absence of IL-4 in this model also favors this hypothesis. In vivo studies will contribute to determine the actual contribution of regulatory T and B cells in the early events and in the infection outcome in CBA/J mouse model.

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References

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

Bocek Jr., P., Foucras, G., Paul, W.E., 2004. Interferon gamma enhances both in vitro and in vivo priming of CD4+ T cells for IL-4 production. The Journal of Experimental Medicine 199, 1619–1630.

Curotto de Lafaille, M.A., Lafaille, J.J., 2002. CD4(+) regulatory T cells in autoimmunity and allergy. Current Opinion of Immunology 14, 771–778.

Diefenbach, A., Schindler, H., Donhauser, N., Lorenz, E., Laskay, T., MacMicking, J., Rollinghoff, M., Gresser, I., Bogdan, C., 1998. Type 1 interferon (IFNalpha/beta) and type 2 nitric oxide synthase regulate the innate immune response to a protozoan parasite. Immunity 8, 77–87.

Gomes, I.N., Calabrich, A.F., Tavares R da, S., Wietzerbin, J., de Freitas, L.A., Veras, P.S.T., 2003. Differential properties of CBA/J mononuclear phagocytes recovered from an inflammatory site and probed with two different species of *Leishmania*. Microbes and Infection 5, 251–260.

Ji, J., Sun, J., Qi, H., Soong, L., 2002. Analysis of T helper cell responses during infection with *Leishmania amazonensis*. American Journal of Tropical Medicine and Hygiene 66, 338–345.

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

Laskay, T., Diefenbach, A., Rollinghoff, M., Solbach, W., 1995. Early parasite containment is decisive for resistance to *Leishmania major* infection. European Journal of Immunology 25, 2220–2227.

Lemos de Souza, V., Ascenção, J.S., Correia, T.M.S., Veras, P.S.T., de-Freitas, L.A.R., 2000. Different *Leishmania* species determine distinct profiles of immune and histopathological responses in CBA mice. Microbes and Infection 2, 1807–1815.

Mosser, D.M., 2003. The many faces of macrophage activation. The Journal of Leukocyte Biology 73, 209–212.

O'Connor, E., Roberts, E.M., Davies, J.D., 1999. Amplification of cytokine-specific ELISAs increases the sensitivity of detection to 5–20 picograms per milliliter. The Journal of Immunological Methods 229, 155–160.

Palanivel, V., Posey, C., Horauf, A.M., Solbach, W., Piessens, W.F., Harn, D.A., 1996. B-cell outgrowth and ligand-specific production of IL-10 correlate with Th2 dominance in certain parasitic diseases. Experimental Parasitology 84, 168–177.

Sadick, M.D., Heinzel, F.P., Holaday, B.J., Pu, R.T., Dawkins, R.S., Locksley, R.M., 1990. Cure of murine leishmaniasis with anti-interleukin 4 monoclonal antibody. Evidence for a T cell-dependent, interferon gamma-independent mechanism. The Journal of Experimental Medicine 171, 115–127.

Setoguchi, R., Hori, S., Takahashi, T., Sakaguchi, S., 2005. Homeostatic maintenance of natural Foxp3(+) CD25(+) CD4(+) regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization. The Journal of Experimental Medicine 201, 723–735.

Shankar, A.H., Titus, R.G., 1993. *Leishmania major*-specific, CD4+, major histocompatibility complex class II-restricted T cells derived in vitro from lymphoid tissues of naive mice. The Journal of Experimental Medicine 178, 101–111.

Soares, M.B., David, J.R., Titus, R.G., 1997. An in vitro model for infection with *Leishmania major* that mimics the immune response in mice. Infection and Immunity 65, 2837–2845.