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Leishmania infantum released proteins specifically regulate cytokine expression and production patterns by $CD4^+$ and $CD8^+$ T cells

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Abstract

Specific immune responses by $CD4^+$ and $CD8^+$ T cells, from two infected mice strains (BALB/c and C57BL/6), induced by High, Inter and Low protein fractions released by *Leishmania infantum*, were assessed through the evaluation of IL-12, IFN-γ and IL-10 mRNA by real-time PCR and respective protein production by ELISA. During infection establishment, High and Inter fractions directed both mice strains T cells subsets to increase the production of IFN-γ, associated to IL-12 release. Later on, parasite replication augmented in BALB/c and stabilised in C57BL/6 mice. Inter fraction induced $CD4^+$ T cells to maintain IFN-γ production, with the simultaneous release of IL-12 by both cell subsets in BALB/c mice and by $CD8^+$ T cells in C57BL/6 mice. These observations suggested a prophylactic potential for Inter fraction which was able to induce Th1 response with IL-12 involvement, required for the maintenance of memory cells, in mice strains with different parasitic evolution.

Keywords: *L. infantum* released proteins; $CD4^+$ T cells; $CD8^+$ T cells; Cytokine; Rodent model

1. Introduction

Leishmaniasis are diseases caused by protozoan parasites of the genus *Leishmania* with significant morbidity and mortality that remains a major public health problem in the tropics and subtropics. Zoonotic visceral leishmaniasis due to *Leishmania infantum* is simultaneously a public health and veterinary problem that presents a poor prognosis if not treated.

The parasites of the genus *Leishmania* are deposited in the mammalian skin by the infected sand fly and are phagocytosed by macrophages. Once inside the macrophage's phagolysosome, the parasite replicates and spreads to other cells, tissues and to internal organs depending on the *Leishmania* species. On the other hand, parasite multiplication can also be limited by an efficient host immune response.

To successfully establish an infection, *Leishmania* has developed several strategies of survival, such as preventing host T cell stimulation and specific immune response that would lead to macrophage activation and parasite destruction. The parasite modulates cell-

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mediated immune mechanisms to its favour by interfering with host gene expression patterns involved in immunity and gene products at a post-transcriptional level, or impeding that the products exert any action (Bogdan and Rollinghoff, 1998; Rodriguez et al., 2004). Moreover, it has been demonstrated that resistance to leishmanial infections is associated with the capacity of $CD4^+$ T cells to generate IFN-γ, which activates the parasitised macrophage to kill intracellular *Leishmania* (Liew et al., 1989; Mosmann and Coffman, 1989; Heinzel et al., 1991). Additionally, there are several studies that confirm the role of $CD8^+$ T cells in resistance to *Leishmania* infection (Hill et al., 1989; Muller et al., 1991). These cells have been implicated in the control of visceral leishmaniasis by participating in the specific acquired immune response developed by the host (Mary et al., 1999; Tsagozis et al., 2003; Gomes-Pereira et al., 2004a).

Factors that could interfere with the development of host immune response, leading to an effective reduction of the parasite, include, besides local microenvironment, the nature of parasite antigens that are presented to lymphocytes. To date there is little information on whether isolated *Leishmania* antigens affect lymphocyte functions. On the other hand, it is readily accepted that the outcome of *Leishmania* infection depends both on the parasitic pathogenecity (Gangneux et al., 2000), as well as on the host genetic background (Blackwell, 1996). Therefore, the purpose of this study was to analyse in vitro $CD4^+$ and $CD8^+$ T cells stimulation induced by *L. infantum* released antigens, during the course of visceral leishmaniasis in susceptible BALB/c and in moderately resistant C57BL/6 mice (Monjour et al., 1988; Rosa et al., 2005) through the evaluation of cytokine gene expression and respective protein production.

2. Materials and Methods

2.1. Mice, parasites and infection protocol

C57BL/6 and BALB/c mice, aged 6–8 weeks, were purchased from Harlan Interfauna Ibérica (Spain) and housed at the IHMT animal facilities, fulfilling the EU requirements (86/609/CEE), recognised by Portuguese law (DR DL129/92 and Portaria 1005/92).

L. infantum MON-1 (MCAN/PT/95/IMT 205) was maintained by serial passages in Syrian golden hamsters and amastigotes were isolated from infected spleens. After in vitro transformation, virulent promastigotes collected from the stationary phase of a subculture with less than five passages (Santos-Gomes and Abranches, 1996) was used for mice inoculation, and to obtain released proteins from culture supernatants.

Infection was performed through the intraperitoneal route with 5×10^6 promastigotes per mouse. Two groups of non-infected healthy mice, one of each strain, were maintained as controls. In a previous study (data not show), it was verified that C57BL/6 and BALB/c mice strain showed a similar pattern of *L. infantum* parasitism in the spleen that diverged on day 45 post-infection (pi), presenting an increase parasite replication in BALB/c mice. Therefore, at days 30 and 45 pi, five infected animals and three healthy animals of both mice strains were sacrificed by cervical dislocation and their spleens collected for parasite burden determination and leukocytes isolation. The experiments were repeated three times.

2.2. Isolation of released L. infantum proteins

To isolate *Leishmania* released proteins, supernatants of promastigotes cultures were collected, after 24 h in a protein-free medium. These supernatants were concentrated 10,000× by centrifuging (4000 × *g*, 20 min) using Centricon tubes (Amicom, Millipore, USA). Released proteins were separated by their molecular weight in three groups (<37 kDa—Low group, 37–75 kDa—Inter group and >75 kDa—High group) through SDS-PAGE (Santos-Gomes et al., 2000) and then electroeluted at 100 mA for 30 min using a MiniElectroelutor (Bio-Rad, Milano, Italy). Protein concentration was estimated by the Lowry method.

2.3. Estimation of viable parasites

Spleen parasite burden was calculated by limiting dilution assay, as described by Buffet et al. (1995). Briefly, 0.01 g of spleen from each mouse was removed and homogenised individually in Schneider's medium (Gibco BRL, UK) supplemented with 20% heat-inactivated foetal calf serum (FCS) (Biochrom, Germany). Four-fold serial dilutions of the infected tissues were distributed in 96-multiwell plates (Nunc, Denmark) in which the solid phase of NNN (Novy, McNeal, Nicolle) medium plus Schneider's medium 20% FCS were already set. After 15 days at 24 °C, a sample of each well was examined and defined as positive or negative depending on the presence or absence of promastigotes. The number of parasites per gram of tissue was calculated as follows: (reciprocal titre of the highest dilution which contained at least one parasite/weight of homogenised tissue) × 400.

2.4. $CD4^+$ and $CD8^+$ T cells separation and lymphocyte proliferation assays

Splenic leukocytes were isolated from spleen homogenates by Ficoll (Gibco BRL) density sedimentation, washed with Hank's balanced salt solution (Sigma, UK) and ressuspended in RPMI 1640 medium (Gibco BRL). $CD4^+$ and $CD8^+$ T cells were obtained using the $CD4^+$ and $CD8^+$ (L3T4) microbeads on the MidiMACS system (Miltenyi Biotec, Germany). Briefly, cells were suspended in elution buffer [PBS 0.5% bovine serum albumin (BSA)] at a concentration of 10^7 cells/90 μl to which 10 μl of anti-mouse CD4 and CD8 microbeads were added. After 20 min of incubation at 4 °C, the cells were washed and centrifuged at $300 \times g$ for 10 min at 4 °C, resuspended in 500 μl of elution buffer per 10^8 cells and applied onto a LS column on the magnetic separator. After several washing steps, the column was removed from the separator, and the fractions containing $CD4^+$ (purity of 94.1%) and $CD8^+$ (purity of 91.7%) T cells were collected. Cells were then cultured in RPMI 1640 medium supplemented with 10% FCS and 100 U/ml penicillin, 100 μg ml^{-1} streptomycin, in U-shaped 96-well tissue culture plates (Nunc, Denmark). Assays were performed in triplicate with 2×10^5 cells/well stimulated with 5 μg ml^{-1} of High, Inter and Low protein groups. After 96 h of culture in an atmosphere of 5% CO_2 supernatants were collected for cytokine quantification, while cells were used for RNA extraction.

2.5. DNA extraction and real-time PCR assay

Total RNA was extracted from approximately 5×10^5 cultured spleen leukocytes using the RNeasy Mini kit (Qiagen, Germany) according to the manufacturer's recommendations. The extraction procedure included a DNase treatment using RNase Free-DNase Set Protocol (Qiagen) to prevent DNA contamination. Target RNA was reverse transcribed into cDNA using 200 U M-MLVRT (Life technologies, Gibco BRL), at 37 °C for 60 min in the presence of 3 mM 5× M-MLV RT Buffer (250 mM Tris–HCl, pH 8.3, 375 mM KCl and 15 mM $MgCl_2$) (Gibco BRL), 10 mM BSA (Boehringer, Germany), 1 mM dNTPs (Gibco BRL), 40 U rRNAsin ribonuclease inhibitor and 10× Oligo $(dT)_{15}$ (Promega, USA). The samples were then heated 10 min at 95 °C for RT inactivation and cooled to 4 °C.

Semi-quantitative real-time PCR was performed in the ABI GeneAmp 5700-sequence detection system (Perkin-Elmer/Applied Biosystems, USA). Reaction conditions were powered on a Pentium III Dell Opti Plex GX110, linked directly to the sequence detector. PCR amplifications were performed by using SYBR® Green as a double-strand DNA-specific binding dye with continuous fluorescence monitorisation. Amplification was carried out in a total volume of 20 μl containing 1 μl of cDNA sample, 10 μl of 2× SYBR® Green I dye PCR Master Mix [(containing AmpliTaq Gold® DNA polymerase, dNTP mix with dUTP, passive reference I and optimised buffer components) (Perkin-Elmer/Applied Biosystems)] and primers for IFN-γ, IL-10 and IL-12p40 as described in Rosa et al. (2005). PCR amplification was performed in duplicate wells, using the following conditions: 10 min at 95 °C for AmpliTaq Gold activation, followed by a total of 40 cycles (thermal profile for each cycle: 15 s at 95 °C, 1 min at 60 °C). To quantify the cytokine expression, cDNA plasmid standards for each cytokine and HPRT (Rosa et al., 2005) were used to construct a standard curve in each PCR run.

2.6. Cytokine production

IFN-γ, IL-10 and IL-12(p70) from supernatants of the cultured spleen leukocyte cells were analysed by commercial enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's protocol (Pharmingen, USA). In each cytokine ELISA, a standard curve was generated using a recombinant cytokine.

2.7. Statistical analysis

Data analysis was performed using the SPSS 10.0 statistical program (SPSS Inc., USA). The following tests were used when appropriate, to compare spleen parasite load at both point times within each mice strain and between the two mouse strains, and the relative cytokine levels in stimulated and non-stimulated $CD4^+$ and $CD8^+$ T cells, within each strain of mice: the Kruskal–Wallis test for k independent samples ($k > 2$), the Wilcoxon signed-ranks test for two matched samples and the Mann–Whitney test for two independent samples. In all statistical tests, a 5% level of significance was used.

3. Results

3.1. Parasite burden in the spleen of BALB/c and C57BL/6 mice

As reported in Rosa et al. (2005), the spleen of both mice strains showed similar parasitic loads (6.7×10^3 promastigotes/g) on day 30 pi. On day 45 pi, the levels of parasitism increased significantly in BALB/c

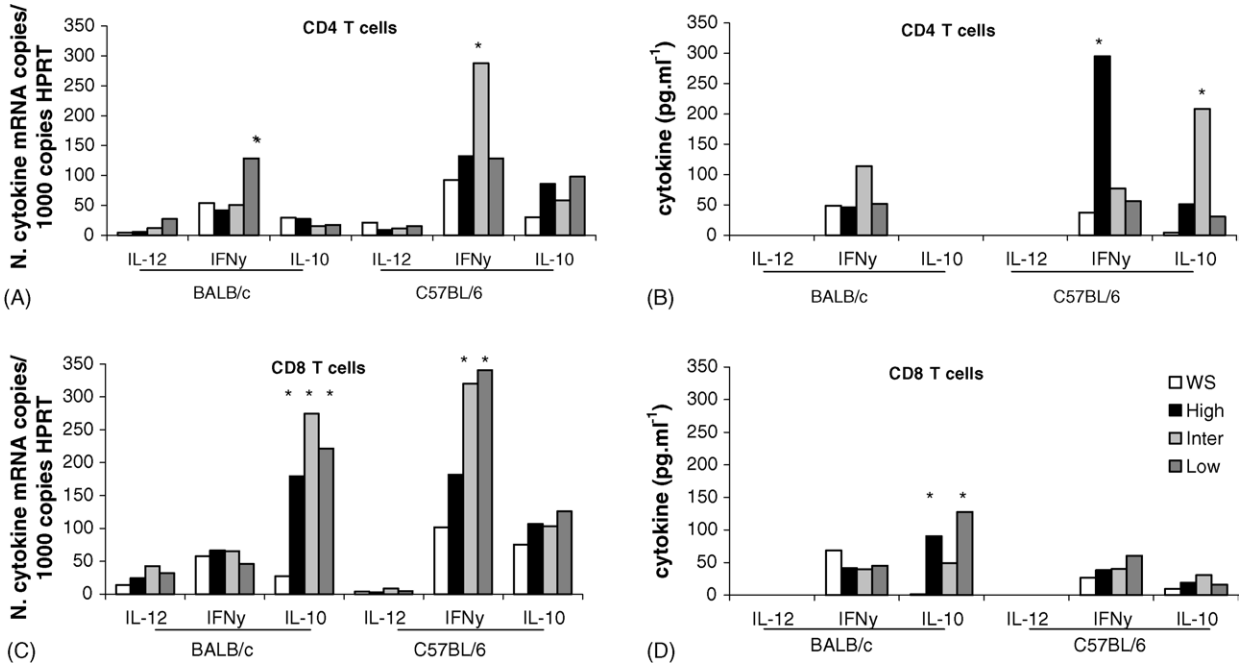

Fig. 1. Expression of IL-12, IFN-γ and IL-10 by $CD4^+$ (A) and $CD8^+$ (C) T cells of BALB/c and C57BL/6 mice and their respective production (B and D). Spleen $CD4^+$ and $CD8^+$ T cells were cultured in the presence of protein fractions (High, Inter and Low) or without exogenous stimulation (WS). Cytokine expression and production were determined by real-time PCR (data are expressed in number of mRNA copies per 1000 copies of HPRT mRNA) and ELISA (data are expressed in pg ml^{-1}), respectively. Values represent mean of three independent experiments. $^*P < 0.05$ stimulated vs. non-stimulated cells.

mice (2.7×10^4 promastigotes/g; $P = 0.005$) whereas in C57BL/6 mice a slight decrease in the parasite load was observed (3.4×10^3 promastigotes/g). At day 45 pi mice strains presented a significant ($P = 0.001$) difference on the levels of viable parasite found in spleen.

3.2. BALB/c spleen $CD4^+$ T cells

$CD4^+$ T cells isolated from spleen of healthy BALB/c mice showed an accentuated increase of IFN-γ expression ($P = 0.049$) when stimulated with Low fraction (Fig. 1A) without correspondent production (Fig. 1B).

$CD4^+$ T cells isolated from spleen of BALB/c mice on day 30 pi and stimulated with protein fractions presented a significant increase in IL-12 mRNA ($P = 0.016$) (Fig. 2A) comparatively to non-stimulated cells. This increment in IL-12 expression was accompanied by important augments of cytokine production by cells stimulated with fractions High ($P = 0.034$) and Inter ($P = 0.014$) (Fig. 2B). Simultaneously, a significant increment ($P = 0.015$) in the IFN-γ expression (Fig. 2C), as well as, equivalent protein production in cells stimulated with fractions High ($P = 0.013$) and Inter ($P = 0.016$) was verified (Fig. 2D).

On day 45 pi, cytokine expressions decreased to baseline levels, except in cells stimulated with Low fraction that maintained elevated IL-12 mRNA values ($P = 0.037$; Fig. 2A). Associated to this expression pattern, significant amounts of IL-12 ($P = 0.037$; Fig. 2B) and IFN-γ ($P = 0.046$; Fig. 2D) were detected in the supernatants of cells stimulated by Inter fraction that also released moderate quantities of IL-10 (Fig. 2F).

3.3. BALB/c spleen $CD8^+$ T cells

The three protein fractions released by *L. infantum* caused a significant increase in IL-10 expression ($P_{High,\ Low} = 0.039$; $P_{Inter} = 0.037$) of $CD8^+$ T cells isolated from spleen of healthy BALB/c (Fig. 1C), but only cells stimulated with High and Low fractions revealed a significant raise ($P = 0.047$) in its production (Fig. 1D).

Stimulated $CD8^+$ T cells from infected mice expressed high levels of IFN-γ mRNA ($P = 0.043$; Fig. 2C) accompanied by significant protein production ($P = 0.047$; Fig. 2D) on day 30 pi.

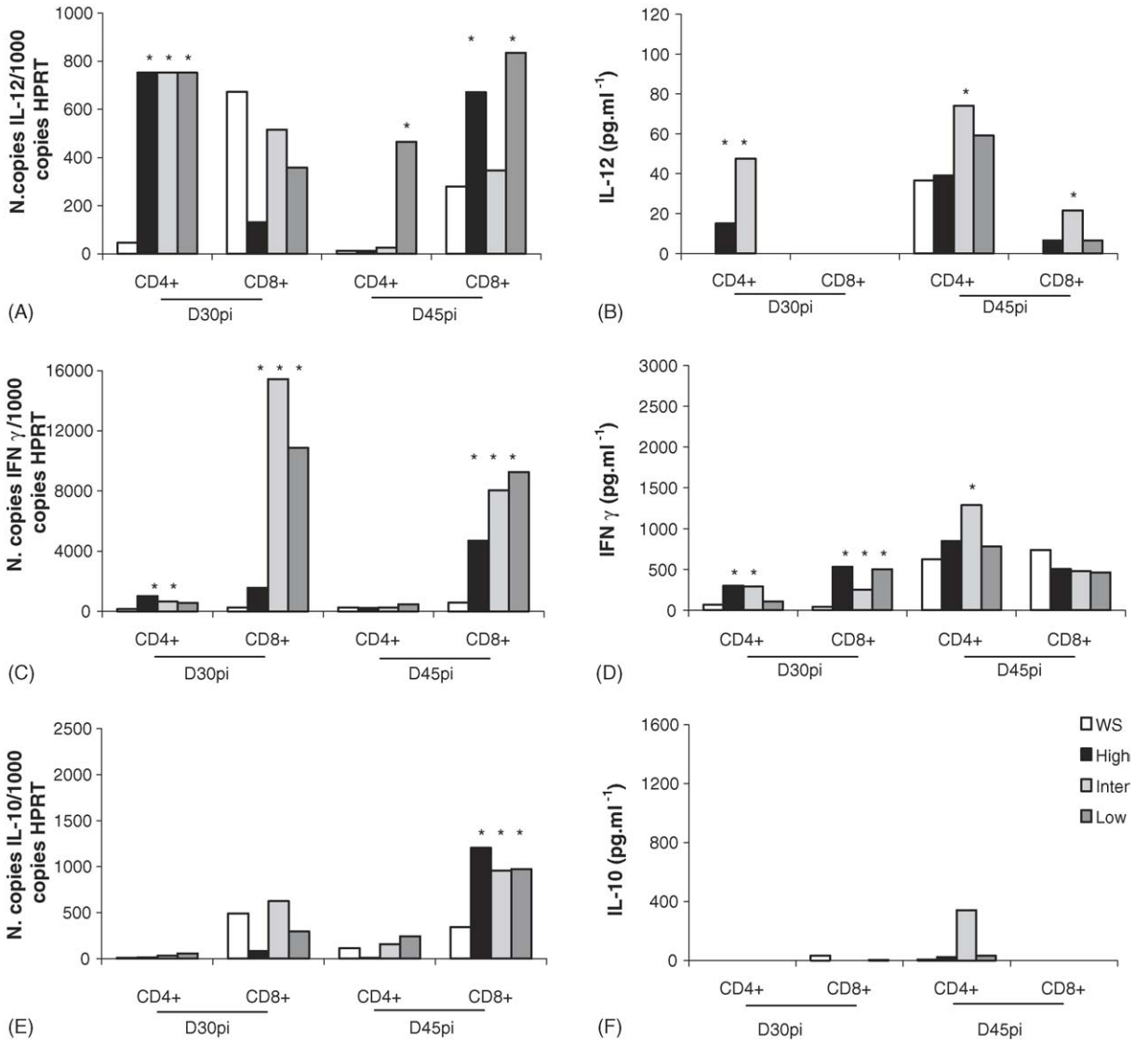

Fig. 2. Expression and production of IL-12 (A and B), IFN-γ (C and D), IL-4 (E and F) and IL-10 (G and H) by $CD4^+$ and $CD8^+$ T cells of BALB/c mice infected with *L. infantum*. At days 30 (D30 pi) and 45 (D45 pi) post-infection spleen $CD4^+$ and $CD8^+$ T cells were cultured in the presence of protein fractions (High, Inter and Low) or without exogenous stimulation (WS). Cytokine expression and production were determined by real-time PCR (data are expressed in number of mRNA copies per 1000 copies of HPRT mRNA) and ELISA (data are expressed in pg ml^{-1}), respectively. Values represent mean of three independent experiments. $^*P < 0.05$ stimulated vs. non-stimulated cells.

On day 45 pi, $CD8^+$ T cells stimulated by High and Low fractions had significant levels of IL-12 ($P_{High} = 0.046$, $P_{Low} = 0.036$; Fig. 2A), IFN-γ ($P_{High} = 0.047$, $P_{Low} = 0.024$; Fig. 2C) and IL-10 mRNA ($P_{High} = 0.041$, $P_{Low} = 0.047$; Fig. 2E). Fraction Inter induced a significant increase of IFN-γ ($P = 0.036$; Fig. 2C) and IL-10 ($P = 0.047$; Fig. 2E). Besides the general increase in mRNA cytokine expressions, only cells stimulated with fraction Inter released significant levels of IL-12 ($P = 0.020$; Fig. 2B).

3.4. C57BL/6 spleen $CD4^+$ T cells

$CD4^+$ T cells isolated from spleen of healthy C57BL/6 mice presented a considerable increase of IFN-γ expression ($P = 0.049$) when stimulated with fraction Inter (Fig. 1A). However, significant production of IFN-γ ($P = 0.037$) and IL-10 ($P = 0.040$) was detected in cells stimulated with fractions High and Inter, respectively (Fig. 1B).

Spleen $CD4^+$ T cells of C57BL/6 mice with 30 days of infection evidenced low levels of cytokine expression

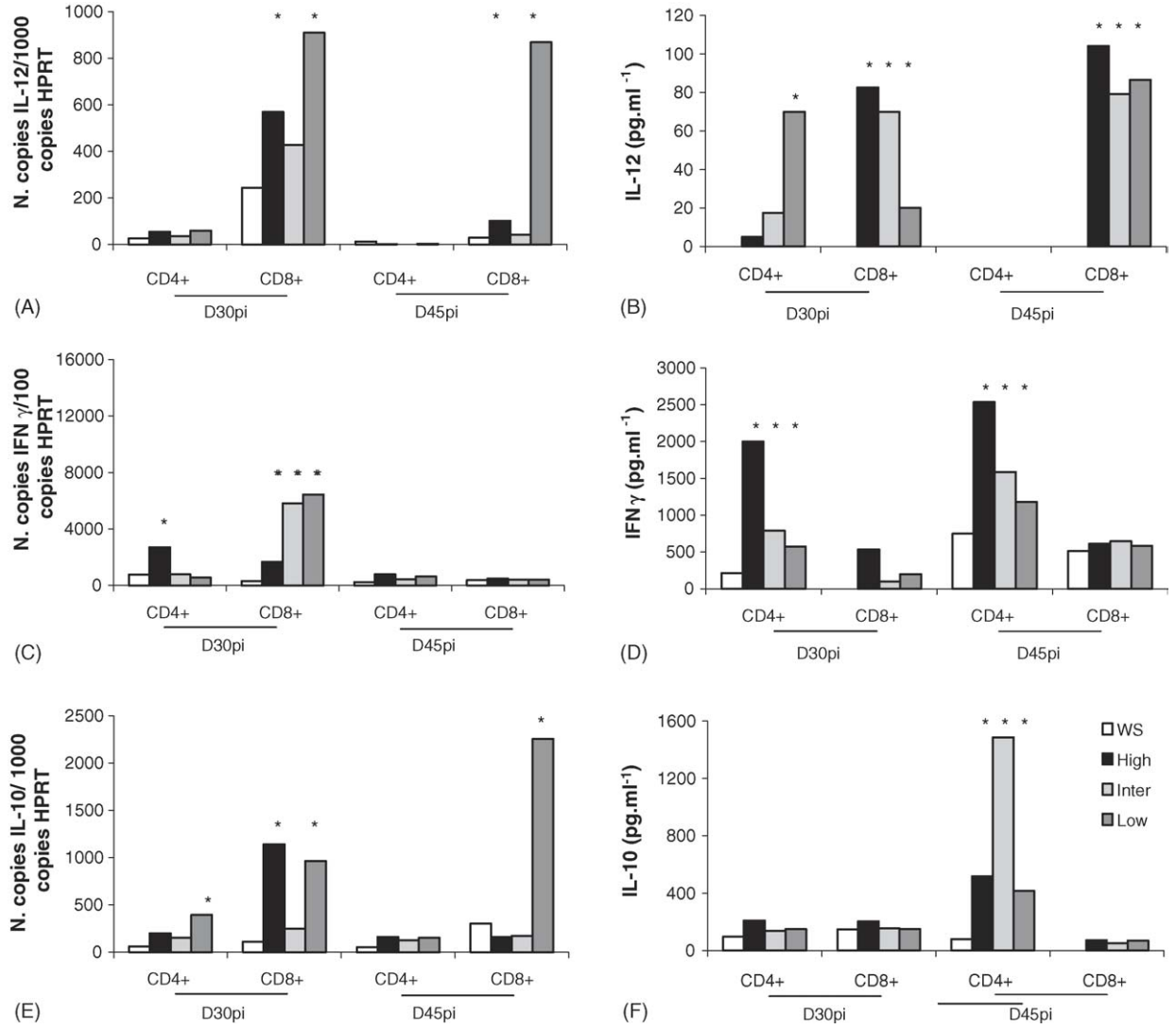

Fig. 3. Expression and production of IL-12 (A and B), IFN-γ (C and D), IL-4 (E and F) and IL-10 (G and H) by $CD4^+$ and $CD8^+$ T cells of C57BL/6 mice infected with *L. infantum*. At days 30 (D30 pi) and 45 (D45 pi) post-infection spleen $CD4^+$ and $CD8^+$ T cells were cultured in the presence of protein fractions (High, Inter and Low) or without exogenous stimulation (WS). Cytokine expression and production were determined by real-time PCR (data are expressed in number of mRNA copies per 1000 copies of HPRT mRNA) and ELISA (data are expressed in pg ml^{-1}), respectively. Values represent mean of three independent experiments. $^*P < 0.05$ stimulated vs. non-stimulated cells.

in the presence of protein fractions. However, High fraction significantly increased IFN-γ mRNA levels ($P = 0.032$; Fig. 3C) and Low fraction induced higher IL-10 expression ($P = 0.047$; Fig. 3E). On other hand, high amounts of IL-12 had been measured in cells stimulated by the Low fraction ($P = 0.049$, Fig. 3B) and of IFN-γ in cells induced by the three protein fractions ($P = 0.028$; Fig. 3D).

On day 45 pi, cells maintained base-line levels of cytokine expression, but protein fractions induced cells to produce significant amounts of IFN-γ ($P = 0.038$; Fig. 3D) and IL-10 ($P = 0.04$; Fig. 3F).

3.5. *C57BL/6 spleen $CD8^+$ T cells*

$CD8^+$ T cells from healthy C57BL/6 mice presented a pronounced increase of IFN-γ expression ($P = 0.049$) when stimulated with Inter and Low fractions (Fig. 1C) without correspondent augment in its production (Fig. 1C).

In C57BL/6 infected mice, IL-12 expression presented a significant increase in $CD8^+$ cells stimulated with High and Low protein fractions, on days 30 ($P = 0.038$) and 45 pi ($P = 0.016$; Fig. 3A), whereas stimulated cells released significant

amounts of this cytokine ($P_{30\,pi} = 0.048$, $P_{45\,pi} = 0.046$; Fig. 3B).

On day 30 pi, $CD8^+$ T cells stimulated with the three protein fractions had a significant number of IFN-γ mRNA copies ($P = 0.048$; Fig. 3C) accompanied by the production of reduced amounts of the respective protein (Fig. 3D). However, on day 45 pi, the expression and production diminished to values similar to those observed in non-stimulated cells.

A significant increase in IL-10 expression was observed in cells stimulated by High fraction on day 30 pi ($P = 0.028$) and by Low fraction on both days of observation ($P_{30\,pi} = 0.032$ and $P_{45\,pi} = 0.012$, Fig. 3E), although followed by the release of small amounts of IL-10 (Fig. 3F).

4. Discussion

The outcome of visceral leishmaniasis is mainly dependent of differential stimulation of T cell subsets and influenced by the cytokines that are being produced. On the other hand, products released by *Leishmania* can be recognised by lymphocytes, functioning as antigenic elements, and intervening in cell activation and differentiation. Moreover, protein fractions released by *L. infantum* exert opposite effects on the specific activation of spleen leukocytes depending on host genetic characteristics and on the magnitude of parasite burden (Rosa et al., 2005). In this study, the effect of protein fractions released by *L. infantum* on the activation of $CD4^+$ and $CD8^+$ T cells from two mice strains with different genetic backgrounds was further analysed.

L. infantum released fractions induced a non-specific cellular immune stimulation in healthy mice. High and Low fractions modulated $CD8^+$ T cells of healthy BALB/c mice to exhibit a Tc2 response, with augmented expression and production of IL-10. Healthy C57BL/6 mice developed a Th1 in presence of High fraction whereas Inter fraction induced a Th2 immune response.

Parasitism followed different patterns of evolution in the spleen of BALB/c and C57BL/6 mice strain. While infection progressed in BALB/c mice, C57BL/6 animals maintained reduced parasite replication levels evidencing different abilities in controlling *L. infantum* infection. During parasite establishment, High and Inter fractions directed BALB/c $CD4^+$ and $CD8^+$ T cells towards a Th1/Tc1 response, evidenced by the increments of IFN-γ mRNA and its respective protein. Additionally, $CD4^+$ T cells also showed high levels of IL-12 mRNA associated to the release of important amounts of this cytokine. This Th1-promoting cytokine plays an important role in the induction of IFN-γ and in the inhibition of IL-4 production (Heinzel et al., 1993; Sypek et al., 1993). Furthermore, the presence of IFN-γ might reinforce the up-regulation of IL-12 expression and production (Launois et al., 1997). These results point to the synergetic modulation of T cells by the two protein fractions towards a protective response. Low fraction also modulates $CD8^+$ T cells towards a Tc1 response at both mRNA and protein levels.

In a similar way, $CD4^+$ T cells of C57BL/6 mice with 1 month of infection also mounted a Th1 response, releasing IFN-γ when induced by protein fractions. Simultaneously, these protein fractions directed $CD8^+$ T cells to produce appreciable quantities of IL-12 evidencing a synergetic regulation of T cells. Moreover, this study confirms that fraction Low is a potent IL-12 inducer, as previously observed in total spleen lymphocytes (Rosa et al., 2005), through the immunomodulation of both T cell subsets.

Taken together these results suggest that at the beginning of infection, when both mice strains presented identical parasite loads, $CD4^+$ T cells were able, upon the appropriate stimulation, to exhibit a protective immune response. In C57Bl/6 mice, High fraction seems to induce non-specific regulation of $CD4^+$ T cells, as healthy mice showed a significant increase in IFN-γ, despite the differences in magnitude of the amounts released by healthy and infected animals.

Forty-five days after infection, spleen parasite load increased in BALB/c mice and protein fractions exhibited different effects on the T cell subsets. Low fraction induced $CD4^+$ T cells to augment IL-12 mRNA levels whereas Inter fraction induced $CD4^+$ T cells to maintain Th1 response and regulated simultaneously both T cells subsets to increase specific IL-12 production. Furthermore, stimulated $CD8^+$ T cells presented a general increase of cytokine mRNAs and exhibited a specific suppression of its products, especially of IFN-γ. Even so, fraction Inter was the only protein fraction that overcame this situation inducing cells to release small amounts of IL-12.

On the contrary, parasite progression in C57BL/6 mice was restricted and the three protein fractions induced similar cell responses. Protein fractions drove $CD4^+$ T cells towards a mixed Th1/Th2 response, with production of IFN-γ and IL-10, whereas $CD8^+$ T cells maintained IL-12 production. The specific production of IL-10 by spleen leukocytes stimulated by *L. infantum* released protein fractions that was demonstrated in a previous study (Rosa et al., 2005) seemed to be a consequence of $CD4^+$ T cell activation.

Therefore, the results obtained in this study suggest that during infection establishment, *L. infantum* released

protein fractions can modulate T cells towards a specific Th1 response with simultaneous production of IL-12 in animals with different genetic backgrounds. Later on, when infection was established, fractions redirected the cytokine production by $CD4^+$ T cells towards a mixed Th1/Th2 response in C57BL/6 mice strain, although the cells had previously been able to develop protective immunity. These protein fractions contributed to the induction of IL-10, which might be associated, in the more resistant mice, to parasite persistence through macrophage deactivation (Belkaid et al., 2001), thus possibly avoiding complete parasite elimination.

In the present study, *L. infantum* released protein fractions induced T cells to augment IFN-γ and IL-10 mRNA expression, although not always followed by an equivalent increment in protein release, pointing to some dissociation between expression and production that could be due to protein degradation, to cell binding, or be a consequence of post-transcriptional regulation. In the past years, it has become evident that post-transcriptional control of gene expression may play an important role, in particular in the regulation of mRNA transcripts encoding cytokines (Butler et al., 2002; Atasoy et al., 2003). Dissociation between IL-12 expression and production was also detected, in the present study. The bioactive IL-12 is a dimer constituted by the subunits p40 and p35 which are the products of different genes independently expressed. In this work, mRNA levels of IL-12 p40 were analysed and the bioactive protein levels (IL-12p70) quantified. It has been seen that the detection of p40 in the absence of bioactive cytokine appears to be a common factor of diverse inflammatory clinical situations (Junghans et al., 1998; Stumbles et al., 1998). Additionally, it is known that the excessive production of p40 originates homodimers that inhibits bioactive IL-12 synthesis (Abdi, 2002; Trinchieri, 2003; Oliveira et al., 2005).

The control of leishmaniasis depends on the recruitment of *Leishmania*-specific T cells to sites of infection (Murray, 2000; Gomes-Pereira et al., 2004b). In this study, Inter fraction revealed a prophylactic potential for *L. infantum* infection, stimulating $CD4^+$ T cells for Th1 response in two mice strains with different genetic backgrounds. Furthermore, Inter fraction in BALB/c and Low fraction in C57BL/6 regulate T cells to produce IL-12. This pro-inflammatory cytokine is required for the induction and predomination of protective immunity (Mattner et al., 1997; Park et al., 2002; Scott, 2003), driving the differentiation of naive $CD4^+$T cells toward the Th1 phenotype (O'Garra and Arai, 2000), and in sustaining memory cells (Stobie et al., 2000). Additionally, some researchers have reported prophylactic advantages on the addition of IL-12 to vaccine candidates (Afonso et al., 1994; Yamakami et al., 2001). However, further studies are needed to better characterise the eventual prophylactic potential of *L. infantum* released Inter and Low protein fractions.

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