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IL-12 and IFN-γ production, and NK cell activity, in acute and chronic experimental *Trypanosoma cruzi* infections

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Abstract

Resistance to acute *Trypanosoma cruzi* infection is mainly associated with a Th1 immune response, characterized by gamma-interferon (IFN-γ) production and activation of macrophages. The outcome of the Th1 response in the spleen and serum of BALB/c and C3H mice infected with *T. cruzi*, Tulahuén strain was studied. The levels of interleukin-12 p40 (IL-12 p40) and IFN-γ, as well as natural killer (NK) cell cytotoxicity were determined at different time-points during the acute phase, and the production of cytokines was also studied in the chronic infection. At 2 days post-infection (pi), spleen cells from C3H mice increased their NK cell activity and the ex vivo spontaneous release of both IL-12 p40 and IFN-γ. On the other hand, BALB/c mice reached low levels of NK cell cytotoxicity and no IFN-γ production was detected at this time pi, but the cytokine was released at high amounts in the second week of the infection. Seric IL-12 p40 concentrations showed a 3-fold increase in both mouse strains on the second day pi and remained high throughout the acute phase. However, seric IFN-γ levels increased during the late acute infection and were higher in BALB/c than in C3H mice. In chronically infected mice IL-12 p40 was as high as in the acute phase in the serum of both strains, but only BALB/c mice still produced IFN-γ. To the authors' knowledge this is the first report showing the protein levels of IL-12 p40 determined in vivo in acute and chronic *T. cruzi* infections. The results reveal differences between both mouse strains in the mechanisms controlling the onset and fate of the Th1 response triggered by the parasite and a long lasting pro-inflammatory stimuli.

Keywords: *T. cruzi*; IFN-γ; IL-12; NK cell activity; Th1

1. Introduction

The immune response elicited by infections can be predominantly of type Th1 or Th2, depending on the cytokines mainly produced. The former is associated with cell-mediated immunity and macrophage activation, and the latter participates in the humoral response. High levels of interleukin-2 (IL-2), tumor necrosis factor-beta (TNF-β) and gamma-interferon (IFN-γ) characterize a Th1 response, while IL-4, IL-5, IL-6, IL-9, IL-10, IL-13 are mainly secreted during the Th2 type [1]. The predominance of IL-12 or IL-4 during the early stages of the infections, as well as several other factors, skews the immune response toward a Th1 or Th2 type, respectively [1,2]. The Th1 cytokine milieu contributes to the resistance to many intracellular parasites, including *Trypanosoma cruzi*, the etiologic agent of Chagas' disease [1]. Thus the administration of IL-12 has been shown to protect mice, while treatment with antibodies against IL-12 increased their susceptibility to *T. cruzi* infection [3,4]. Nevertheless, there is not such a clear association between Th1 and Th2 cytokine profile with resistance and susceptibility to *T. cruzi* [5,6], as has been described for *Leishmania major* infection [7].

Both $CD4^+$ and $CD8^+$ lymphocyte subsets are involved in the host protection to *T. cruzi* infection, as shown by the increased susceptibility of genomically deleted mice [8] or after the in vivo depletion by treatment with specific antibodies [9,10]. Natural killer (NK) cells also play an important role in the resistance to acute infection [11], mainly due to their early IFN-γ

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production [12]. This cytokine activates phagocytic cells to destroy internalized parasites by increasing H_2O_2 [13], nitric oxide [14,15] and tumor necrosis factor-alpha (TNF-α) production [16]. On the other hand, IL-10 and transforming growth factor-beta (TGF-β) have been shown to downregulate the activation of macrophages in vitro [14] and may be required to prevent the in vivo tissue damage due to the strong T cell activation associated with overproduction of IFN-γ and IL-12 [17].

In humans, the acute stage of the *T. cruzi* infection is followed by an asymptomatic period that can progress to a chronic phase with cardiomyopathy and/or digestive tract manifestations. The course of *T. cruzi* infection in mice, a well-known experimental model for Chagas' disease, depends on the genetic backgrounds of both the parasite and the host, as well as on the parasite dose. BALB/c infected with *T. cruzi*, Tulahuén strain, are more susceptible in the acute phase than C3H mice, reaching higher parasitemias and mortalities [18]. The aim of this work was to study the hallmarks of the Th1 response at different times post-infection (pi), comparing these two experimental models.

The results show differences between the two mouse strains in the kinetics of both IL-12 p40 and IFN-γ production, as well as in the NK cell cytotoxicity, indicating different mechanisms involved in the induction and in the control of the Th1 immune response. The results suggest that the early onset of the Th1 response in the spleen plays an important role in the resistance to the acute infection. On the other hand, the high levels of IFN-γ and IL-12 p40 detected in the sera throughout the infection were not related to resistance, but could be implicated in the inflammatory tissue reaction.

2. Materials and methods

2.1. Mice and parasites

Eight-week-old male mice of the BALB/c (H-2^d) and C3H/He (H-2^k) strains, from the animal facilities at the Instituto Nacional de Parasitología 'Dr. M. Fatala Chabén', ANLIS, were infected intraperitoneally with 150 bloodstream forms of *T. cruzi*, Tulahuén strain. Parasitemias were determined in 5 μl of tail vein blood and mortality was recorded. Sera of mice at different days after infection were fractionated and stored at −70°C. During the chronic phase, mice were studied at 4, 8, 10 and 17 months pi and, as no significant differences were detected among them, data of the 4 independent experiments were pooled. Aged matched non-infected mice were used as controls.

2.2. Spleen cell suspensions

Total spleen cell suspensions were obtained by using a Teflon grinder. Cells were washed twice in RPMI medium (Sigma, St. Louis, MO) containing 5% fetal calf serum (FCS, Gibco BRL, NY), 5×10^{-5} M 2-mercaptoethanol, 100 IU penicillin and 100 μg/ml streptomycin. Red cells were lysed by osmotic shock and viability, assayed by trypan blue exclusion, was >98%. Cell suspensions, devoid of red cells, were adjusted to 10^7 white cells/ml with RPMI containing 10% FCS, 2 mM L-gluthamine (Gibco) and 2 mM HEPES (Sigma).

2.3. NK cell activity

Cells from in vitro maintained A/Sn lymphoma YAC-1 labeled with 100 μCi of $^{51}CrO_4Na_2$ (Dupont-NEN, Boston, MA; specific activity 1 mCi/ml) were used as targets. NK cell cytotoxicity was assayed in a total volume of 200 μl/well in round-bottomed microtitre plates (Microwell, A/S Nunc, Roskilde) at different effector to target ratios, by using 10^4 target cells per well. An effector to target ratio of 50:1 was selected to express the results. After 4 h at 37°C in a CO_2 incubator, the plates were centrifuged and the ^{51}Cr release measured in a gamma-counter (RiaStar Packard, Meriden, CT). Spontaneous (S) and maximum (M) isotope release of target cells were made in cultures with RPMI-10% FCS and 2% Tween-20 (Sigma) in balanced salt solution (PBS), respectively. Percentage of specific cytotoxicity was calculated as: $[(X-S)/(M-S)] \times 100$. Means of triplicates were considered.

2.4. IL-12 and IFN-γ production

Murine spleen cells (10^6) obtained as described above, were cultured in vitro for 48 h in medium alone or in the presence of 1 μg/ml of lipopolysaccharide (LPS, *E. coli* O55:B5, Sigma) or 2 μg/ml of Concanavalin A (Con A, Sigma) with or without the addition of 1000 U/ml of mouse recombinant IL-2 (Sigma) to the cell cultures. The IL-12 p40 and IFN-γ levels in the cell-free supernatants and in the mouse sera were measured by ELISA. Briefly, standards and samples were incubated in microtiter plates (Nunc-Immuno™ plate, MaxiSorp™ surface, Denmark) coated with a capturing rat monoclonal antibody specific for either murine IL-12 p40 (C15.6, Pharmingen, San Diego, CA) or IFN-γ (R46A2, Endogen, Woburn, MA), for 2 h at 37°C. After washing, a rat monoclonal antibody against mouse IL-12 p40 (C17.8, Pharmingen) or IFN-γ (XMG1.2, Endogen) was incubated for 1 h at 28°C. Thereafter horseradish peroxidase-conjugated avidin (Vector, Burlingame, CA) was added to the washed plates. The optical density obtained after the addition of the substrate *o*-phenylenediamine (Sigma) was mea-

sured at 492 nm in an ELISA reader (Packard Spectra Count™). The washing buffer was PBS-0.05% Tween-20 and the reagents were diluted in the same solution containing 1% bovine albumin (Sigma). Optimal concentrations of all reagents were established in preliminary experiments. The standard curves were performed by using either recombinant murine IL-12 or IFN-γ (Pharmingen) diluted in normal mouse serum or in the culture medium, to test sera or supernatants, respectively. The standard curves were lineal up to 4.0 ng/ml. The assay sensitivities were 0.2 and 0.04 ng/ml for IL-12 p40 and IFN-γ, respectively, and these values were indicated in the Figures.

2.5. Statistical analysis

In every case, the differences between groups were examined by Student t-test, using Excel software. Discontinuous variables were normalized by logarithmic transformation. A P-value < 0.05 was considered significant.

3. Results

3.1. The different kinetics of the Th1 response in the spleen were related to the early host resistance

During the acute phase BALB/c infected with *T. cruzi*, Tulahuén strain, were more susceptible than C3H mice, and about a half of them died; parasitemias differed significantly between both strains at all points tested beyond 17 days after infection (Fig. 1). Paralleling the parasitemia, both strains showed a marked splenomegaly starting at 14 days pi, but BALB/c reached about twice the number of cells observed in C3H mice. In the chronic phase, only BALB/c mice still had an increased number of splenocytes.

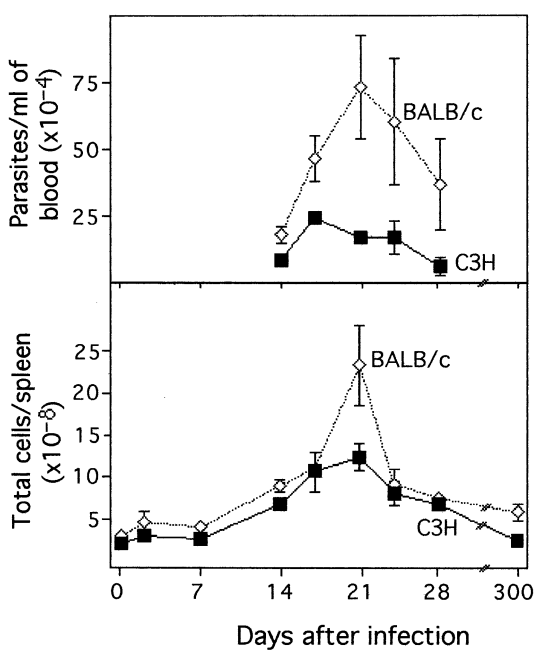

Fig. 1. Parasitemias and number of white cells per spleen in mice inoculated with 150 trypomastigotes of *T. cruzi*, Tulahuén strain. Mean values ± S.E.M. are shown.

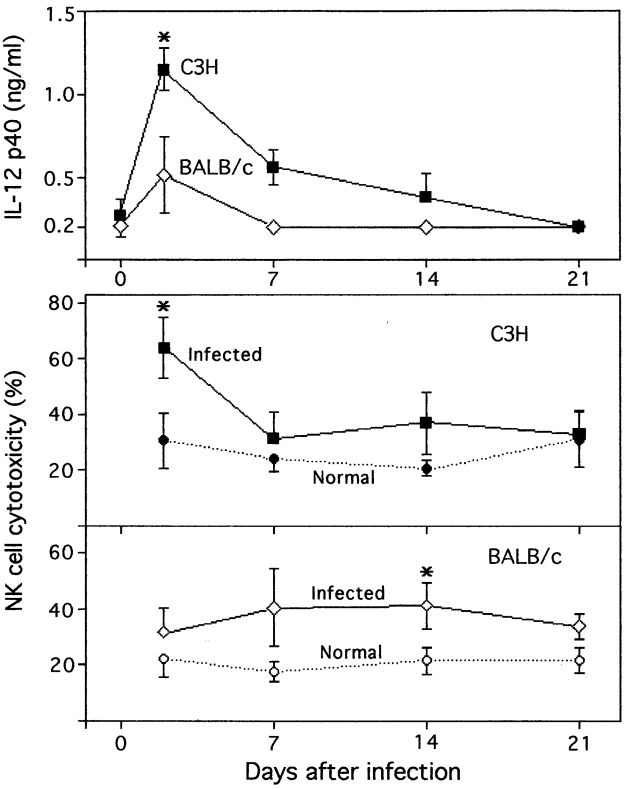

Fig. 2. Kinetic of interelukin (IL)-12 p40 production and natural killer (NK) cell activity in the spleen of mice acutely infected with *T. cruzi*. The spontaneous production of IL-12 p40 by 10^6 total spleen cells and the cytotoxicity against YAC-1 cells from mice at different days after infection and from non infected controls are shown. Preliminary experiments were performed by taking the samples every day during the first week and every 3 days thereafter. No remarkable differences in the pattern of IL-12 p40 and NK cell cytotoxicity were observed at these other time-points. Results of three independent experiments are expressed as means ± S.E.M. of 5–8 mice/group. * Differences with the non-infected controls assayed on the same day are significant.

In order to study the onset of the Th1 immune response, we examined the spleen cells for production of IL-12 p40 and IFN-γ as well as for NK cell cytotoxicity. In C3H mice a significant increase of the spontaneous IL-12 p40 secretion was observed during the first 3 days of the infection, reaching about twice the amounts detected in BALB/c mice (Fig. 2), which did not increase the IL-12 p40 production even when stimulated by LPS (data not shown). The NK cell activity increased significantly at 2 and 14 days pi in C3H and BALB/c mice, respectively (Fig. 2). According to the higher production of IL-12 p40, spleen cells from in-

fected C3H mice showed about two times higher NK cell cytotoxicity than BALB/c mice. The kinetic of IFN-γ production also differed between both mouse strains. At 2 days pi, the spontaneous release of IFN-γ was only observed in C3H mice and it increased about 7 times upon LPS stimulation. On the other hand, BALB/c mice showed high IFN-γ secretion at 14 days pi, and it increased about 2.5 times when cells were stimulated by LPS (Fig. 3).

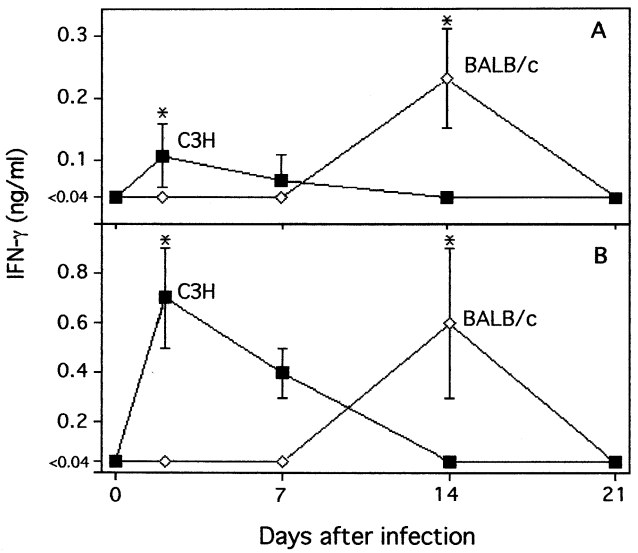

Fig. 3. Kinetic of gamma-interferon (IFN-γ) production by 10^6 unstimulated total spleen cells (A) and after 48 h stimulation with 1 μg/ml lipopolysaccharide (LPS) for (B). Cells from the same mice of Fig. 2 were used. Mean values ± S.E.M. of 5–8 mice/group from three independent experiments are shown. * Differences with non-infected controls are significant.

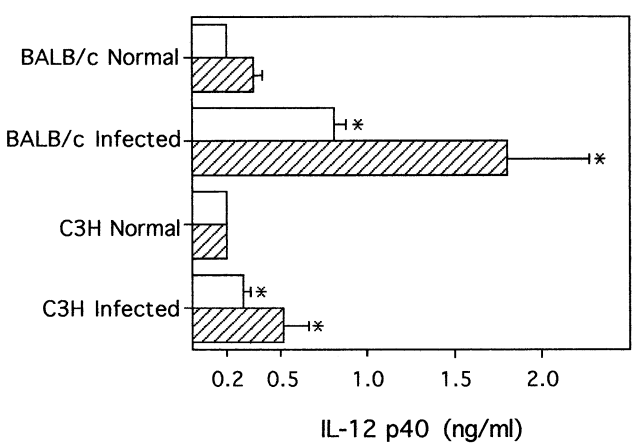

Fig. 4. Levels of interleukin (IL)-12 p40 in the supernatants of 10^6 total spleen cells from chronically infected mice (4–17 months post-infection, pi). Cytokines production by unstimulated cells (open bars) and by cells stimulated for 48 h by lipopolysaccharide (LPS) (1 μg/ml) (dashed bars) were assayed as described in Section 2. Results are expressed as means ± S.E.M. of 4–7 mice/group. * Differences with non-infected controls are significant.

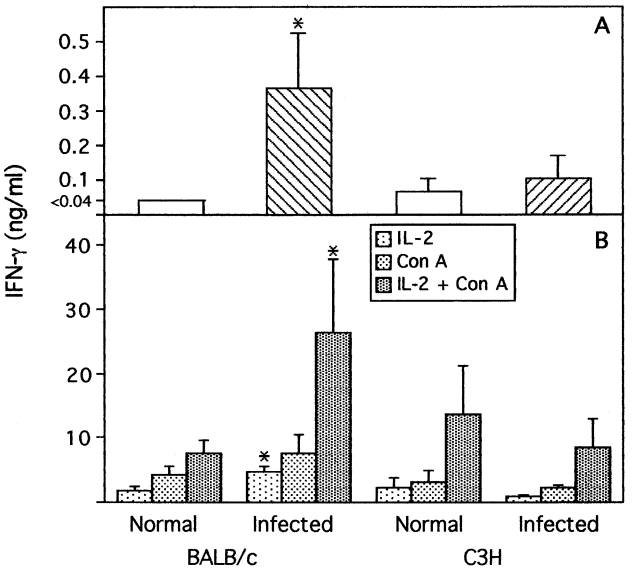

Fig. 5. Gamma-interferon (IFN-γ) produced by 10^6 spleen cells from normal and chronically infected mice (4–17 months post-infection, pi). Cells were incubated 48 h with medium alone (A) or with Con A (2 μg/ml) and/or interleukin (IL)-2 (1000 U/ml) (B). Supernatants were assayed for IFN-γ as described in Section 2. Results are expressed as means ± S.E.M. of 4–7 mice/group. * Differences with non-infected controls are significant.

During the chronic phase, spleen cells from both mouse strains released IL-12 p40 spontaneously, as well as upon LPS stimulation, however, the effect was higher in the BALB/c than in the C3H strain (Fig. 4). On the other hand, splenocytes from chronically infected BALB/c mice produced higher amounts of IFN-γ than the non-infected controls, whereas no differences were observed when spleen cells from infected and control C3H mice were compared, even when stimulated by Con A and IL-2 (Fig. 5).

3.2. The infection also induces a strong systemic Th1 response

The IL-12 p40 and IFN-γ levels were determined in the serum of infected mice, in order to see the overall Th1 response. The kinetic of seric IL-12 p40 was found to be similar among the two mouse strains, with a sharp increase on the second day pi (Fig. 6). Thereafter, the levels remained significantly higher than in control mice and decreased only transiently at 7 days pi in C3H mice.

Although maximal IL-12 p40 levels were found as early as at 2 days pi, IFN-γ started to be detectable in the serum of both mouse strains at 14 days after the infection. At this time, IFN-γ peaked in BALB/c while in C3H mice, maximal concentrations were found during the third and fourth weeks pi, reaching half of the amounts observed in the BALB/c strain (Fig. 6).

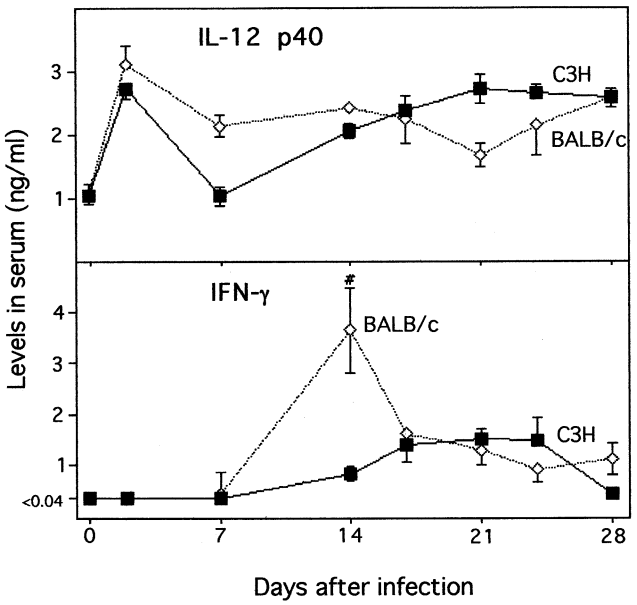

Fig. 6. Kinetic of interleukin (IL)-12 p40 and gamma-interferon (IFN-γ) levels in the serum of mice acutely infected with *T. cruzi*. Sera were collected at days after infection indicated. Preliminary experiments were performed by taking the samples every day during the first week and every 3 days thereafter. No remarkable differences in the pattern of IL-12 p40 and IFN-γ were observed at these other time-points. Results of three independent experiments are expressed as means ± S.E.M. of 4–8 mice/group. IFN-γ levels in infected mice differed significantly with non infected controls at all time points tested beyond 14 days after the infection. # Differences between mouse strains are significant.

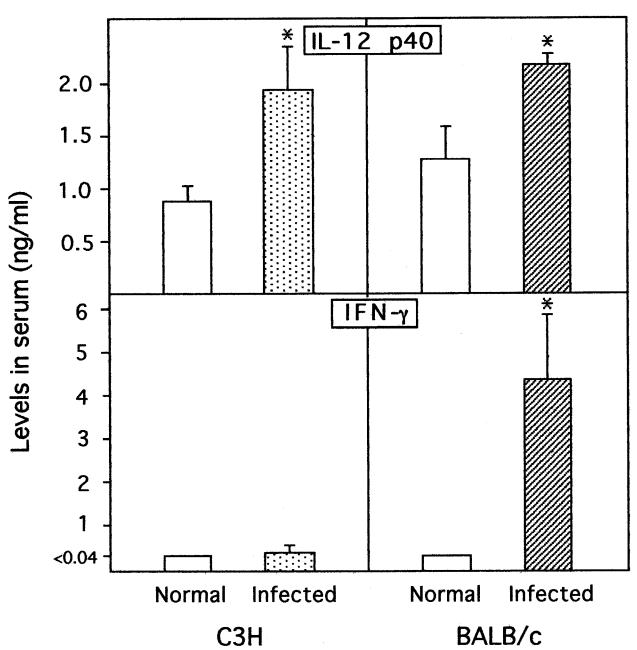

Fig. 7. Levels of interleukin (IL)-12 p40 and gamma-interferon (IFN-γ) in the serum of chronically infected mice (4–17 months post-infection, pi). Results are expressed as means ± S.E.M. of 7–11 mice/group. * Differences with non-infected controls are significant.

Considering that seric IL-12 p40 and IFN-γ levels still remained high at 28 days after the infection, it was investigated whether chronically infected mice still produced the cytokines. Surprisingly, high levels of seric IL-12 p40 were found in all infected mice, with similar amounts to those found in the acute infection (Fig. 7). In spite of the high IL-12 p40 levels found in both mouse strains, seric IFN-γ remained increased only in chronically infected BALB/c mice with concentrations as high as those observed during the acute phase (Fig. 7).

4. Discussion

The immune mechanisms triggered during the first days of *T. cruzi* infections seem to be essential for the control of the early parasite replication, which is associated with host resistance [19]. In this regard, treatment with exogenous IFN-γ or with anti-IFN-γ antibodies showed that this cytokine only plays a central role in the resistance in the early acute phase [20–23]. Accordingly, it was found that the resistance to acute infection, higher in C3H than in BALB/c mice, was related to the early IFN-γ production in the spleen. Both, liver and spleen are central organs engaged in the clearance of the parasite [24]. In the C3H, but not in the BALB/c strain, a very early onset of the Th1 immune response was observed, with a rise of the NK cell activity and IFN-γ release. These results suggest that in C3H mice NK could be the main cell type involved in IFN-γ production in the very early acute infection, as has been previously described in other experimental models [12,25].

On the other hand, splenocytes from BALB/c mice failed to mount an early Th1 response; the NK cell activity and the spontaneous IFN-γ production increased later in the acute phase. Nevertheless, the IFN-γ release was poorly stimulated in the presence of LPS, suggesting that in these mice other cell types, such as those of the phenotype $Thy1^{+}NK1.1^{-}CD4^{-}CD8^{-}$ could be responsible of the production of the cytokine in later phases of the infection [26].

The differences between both mouse strains in the onset of the early Th1 response, and thus in the resistance to the infection, could be related to their genetically different NK cell activity, that is high and low for C3H and BALB/c mice, respectively [27]. The association of early NK cell activation, IFN-γ production and resistance to *T. cruzi* infection has been reported in C57BL/6 mice [12,25], which also have genetically high NK cell activity [27].

In BALB/c mice the low NK cell cytotoxicity could be secondary to the low production of IL-12, the cytokine that triggers the onset of the early Th1 immune response, activating NK cells and thereby in-

creasing IFN-γ production [28]. Comparing both BALB/c and C3H mice in the very early acute infection, it was observed that higher levels of IL-12 p40 correlated with higher NK cell cytotoxicity and IFN-γ secretion. The results may indicate a lower responsiveness for IL-12 p40 production during the acute phase in BALB/c mice that could be also accompanied by a poor response to IL-12 signal. In vitro models to explore the cause of BALB/c susceptibility to *L. major* infection, revealed an early loss of the ability of spleen cells to increase IFN-γ production in response to IL-12, that could precede the inhibitory effect of Th2 cytokines [29]. On the other hand, the late IFN-γ production in *T. cruzi* infected BALB/c mice seems to be triggered by an alternative pathway independent of IL-12, as described in *Toxoplasma gondii* infection [30]. Other cytokines besides IL-12, such as IFN-α/β, IL-15 and/or IL-18, could also be involved in NK cell activation [31–33]. Seric IFN-α/β levels and mRNA transcripts for IL-18 in spleen cells have been found increased in *T. cruzi* infected mice [34,35] but, to the authors' knowledge, the presence of IL-15 has not been explored yet.

In spite of the differences in IL-12 p40 production by spleen cells of BALB/c and C3H mice, it reached very high levels in the serum of both mouse strains on the second day after the infection, and remained high during the whole acute phase. These high amounts of IL-12 p40 could be derived from other sources, besides the spleen, as professional phagocytic cells, located systemically in the host [36]. On the other hand, the increase of seric IFN-γ, observed in the second and third weeks after the infection, had a different pattern in BALB/c and C3H mice, and the levels were not related to resistance, as previously described [26,37].

However, the presence in the serum of the proinflammatory cytokines IL-12 and IFN-γ, may contribute to the onset and/or stimulation of tissue inflammatory reactions. In vivo administration of IL-12 increased the resistance to the infection [3], but high doses resulted in exacerbation of the heart inflammatory infiltrates [38] and earlier mortality [3]. The IFN-γ activation of macrophages for the intracellular killing of the parasite is accompanied by the release of inflammatory products, potentially deleterious to the host; IFN-γ also induces expression of major histocompatibility complex (MHC) class I and II as well as cell adhesion molecules in several cell types [39]. Thus, IL-12 and IFN-γ could act synergistically with IL-1, IL-6 and TNF-α, also associated with resistance mechanisms [16,40,41], in the induction of tissue inflammatory reactions outside the peripheral organs of the immune system, or alternatively, potentiate the already established inflammatory response due to the presence of the parasite in target organs [42,43].

The down regulation of seric levels of IFN-γ in the acute phase, as has been previously described in other experimental models [6,26], could be due to the production of inhibitors of the Th1 response, such as TGF-β, IL-10, nitric oxide or prostaglandins [44,45] that are increased during *T. cruzi* infections [15,23,25,46,47]. TGF-β and/or IL-10 could also account for the failure of spleen cells from BALB/c mice to produce IFN-γ in the very early stages of the infection, since they are spontaneously produced by spleen cells from infected mice [23,46]. Although specific IL-4 release has not been observed in the acute phase of *T. cruzi* infection [48], it can not be ruled out that the presence of IL-13, similarly to IL-4, inhibits the production of proinflammatory cytokines [49]. Thus, Th2 cytokines would have an important role in the control of both the immune response and the inflammatory reaction triggered by the parasite [17,50].

During the chronic phase of the infection, IL-12 p40 levels were still high in the serum of both mouse strains. This finding was surprising, since IL-12 is a cytokine related to the non-specific immune response elicited during the first stages of the infections [39]. However, its presence may reflect the long lasting stimulation of the immune system as a consequence of the persistent parasite load in the chronic infections.

The high levels of IFN-γ in chronically infected BALB/c mice and its absence in C3H mice was also unexpected. According to present knowledge, it is not clear how the IFN-γ production is downregulated in the presence of IL-12, as observed in chronically infected C3H mice. Although in the acute phase the presence of IL-12 p40 is related to experimental evidences of its biological activity [3,38], this is not clear in the chronic stage, since IFN-γ production was induced in BALB/c but not in C3H mice. It must be also taken into account, that the p40 subunit, included in the biologically active cytokine p70, can be overproduced upon stimulation and acts as a specific inhibitor of the p70 [51], making it difficult to predict the biological IL-12 activity that results from the balance p40/p70. These data obtained in the chronic stage, even when unexpected are, to the authors' knowledge, the first report of the protein levels of IL-12 p40 and IFN-γ in *T. cruzi* infections.

Taken together, the results suggest the importance of the innate immune response in the spleen to the initial control of the infection. A Th1 response with optimal production of cytokines that activate macrophages and generate specific antibodies may undoubtedly be associated with resistance mechanisms [14,52]. Nevertheless it is unlikely that susceptibility or resistance to acute *T. cruzi* infection relies on the production of a limited set of cytokines, instead it seems to be related to the balance of several factors which may differ among experimental models. However, the description of the pattern of IL-12/IFN-γ production clearly showed that the Th1 response in the spleen determined the initial

immune control of the infection. On the other hand, the evaluation of Th1 cytokines in serum was not related to resistance but could be considered to predict the risk of tissue damage under the unregulated inflammatory response.

Acknowledgements

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