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Immunosuppression during acute *Trypanosoma cruzi* infection: involvement of Ly6G (Gr1$^+$)CD11b$^+$ immature myeloid suppressor cells

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Abstract

***Trypanosoma cruzi* infection is associated with a severe unresponsiveness of spleen cells (SC) to antigens and mitogens. A high production of NO by concanavalin A (Con A)-stimulated SC from infected but not from control mice was observed. Neutralization of endogenous IFN-γ production or treatment with NO synthase (NOS) inhibitor, L-*N*-monomethyl-arginine, blocked Con A-induced NO production and greatly restored proliferation by SC from infected mice. This was confirmed by using IFN-γR$^{-/-}$ and inducible NOS (iNOS)$^{-/-}$ knockout mice, since unresponsiveness to mitogens of SC from those infected mice was much less pronounced than in control littermates. Interestingly, SC unresponsiveness was associated with a huge increase in CD11b$^+$ cells that express Ly-6G (Gr1)$^+$ and other immature myeloid markers These cells were absent in infected IFN-γR$^{-/-}$ spleens. Purified immature Gr1$^+$CD11b$^+$ cells produced NO and expressed iNOS upon IFN-γ treatment, and were able to inhibit T cell proliferation. In addition, depletion of myeloid CD11b$^+$ cells abrogated NO production and restored mitogen-induced proliferation, but not IL-2 synthesis, in SC from infected mice. IL-2 production and CD25 cell surface expression by mitogen-activated T cells were greatly depressed in SC from IFN-γR$^{-/-}$ and iNOS$^{-/-}$ mice, confirming that Gr1$^+$CD11b$^+$ cells were not involved in their down-regulation. In contrast, IL-5, tumor necrosis factor and IFN-γ production, and CD69 expression by T cells were not depressed in infected SC. The results indicate the existence of an immunosuppressive mechanism during *T. cruzi* infection, mediated through IFN-γ-dependent NO secretion by immature Ly-6G (Gr1)$^+$CD11b$^+$ myeloid cells.**

Introduction

Trypanosoma cruzi, a protozoan parasite, is the causative agent of Chagas' disease that affects several million people in South and Central America (1). This parasite exists in at least three morphologically distinct forms: infective (metacyclic or blood trypomastigotes), insect borne (epimastigotes) which replicate in the vector, and intracellular replicative (amastigotes) which grow and replicate intracellularly in a variety of mammalian cells (2). From a clinical point of view, *T. cruzi* infections proceed in two phases. In the acute phase, circulating blood trypomastigotes are observed associated with a local inflammation at the sites of infection. During the chronic phase, circulating parasites cannot be observed by inspection of blood, but progressive tissue damage occurs involving the esophagus, colon and heart (1).

Several alterations of the immune response have been described in this disease. Among them, infection with *T. cruzi* is associated in humans, as well as in mice, with a severe unresponsiveness to mitogens and antigens during the acute phase of the disease (3,4). This immunosuppression is thought to facilitate the dissemination and establishment of the parasite in the infected host. This loss of proliferation to mitogens and antigens had been ascribed to many mechanisms. Previous reports have pointed out to T cells (5,6) including γδ T cells (7), as well as adherent cells (3,8) as suppressor cells. Thus, the depletion of adherent cells, partially restored T cell proliferation of spleen cells (SC) (9,10). In addition, inhibition of IL-2 synthesis (5,11) and reduced cell surface expression of IL-2R by activated SC from

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infected mice (12), as well as by activated human peripheral blood cells (13), have been accounted to explain this unresponsiveness. By contrast, activated SC from infected mice produced elevated levels of IFN-γ and tumor necrosis factor (TNF) (14,15).

More recently, it has been proposed that inhibition of T cell proliferation in *T. cruzi*-infected mice takes place through IFN-γ and NO secretion (16). Moreover, another form of suppression involving an enhanced TCR-induced apoptosis of $CD4^+$ T cells from infected mice has been described (16–18). Finally, other soluble substances, including suppressive cytokines as transforming growth factor-β, IL-4, IL-10 and prostaglandins, released upon contact with parasite-antigens, have also been proposed as the cause of *T. cruzi* immunosuppression (3,5,19–21).

On the other hand, cytokines play a key role in regulating both immune response and parasite replication in infected animals, (22,23) Activation of monocytes by cytokines released by T_h1 cells seems to play a major role in controlling infection *in vitro* as well as *in vivo*. Thus, IL-12 produced by macrophages in response to infection mediates resistance to *T. cruzi* (24). *In vitro* studies have identified T_h1 cell-derived TNF and IFN-γ as the most important cytokines involved in the killing of intracellular *T. cruzi* through a NO-mediated L-arginine-dependent killing mechanism (25,26). This was corroborated *in vivo*, since anti IFN-γ administration results in a drastic increase in parasitemia and mortality (27,28). TNF-R1-$FcIgG_3$ transgenic mice are also more susceptible to *T. cruzi* infection, clearly indicating a protective role for TNF (29). Other studies have shown that NO plays a role in host resistance to *T. cruzi* infection in mice (30).

In this manuscript we have tried to clarify the mechanism responsible for the immunosuppression observed in *T. cruzi* infection. Our results have unraveled the existence of a mechanism of immunosuppression that depends in IFN-γ-induced NO secretion by immature myeloid cells (Ly-$6G^+CD11b^+$) that colonize the spleen during the acute phase of infection.

Methods

Mice and parasites

T. cruzi MC and RA strains were a gift from Dr Jorge Alvar (Instituto Carlos III, Madrid). The Tulahuen (T) strain of *T. cruzi* was a gift from Dr John David (Harvard). Specific pathogen-free mice of the 129/SvEv (Sv129) strain and the same strain background with disrupted IFN-γ receptor genes (IFN-$\gamma R^{-/-}$), as well as C57Bl6 and the same strain with disrupted inducible NO synthase genes ($iNOS^{-/-}$; Jackson Laboratories, Bar Harbor, ME), were maintained in the Centro de Biología Molecular animal facilities at the Universidad Autónoma de Madrid. Groups of mice (8–12 weeks old) were infected with different strains of *T. cruzi* by i.p. injection of 10^4 blood trypomastigotes obtained from previously infected mice with frozen trypomastigotes. Parasitemia was measured as previously described (31). The animal research described in this paper complied with national and European Union legislation, and with related codes of practice.

SC cultures

SC suspensions were prepared from infected mice at various days post-infection or from control uninfected mice. SC were depleted of erythrocytes by hypotonic lysis with distilled water, and resuspended in RPMI 1640 complete medium containing 5% FCS, 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 ng /ml) (Gibco, Grand Island, NY). Where indicated, 5 μg/ml concanavalin A (Con A; Sigma, St Louis, MO), 10 ng/ml mouse recombinant granulocyte macrophage colony stimulating factor (GM-CSF; Promega, www.promega.com), 2 mM L-NMMA (N^G-monomethyl-L-arginine; Calbiochem-Behring, La Jolla, CA), 5 μg/ml anti-IFN-γ (rat IgG1 anti-mouse IFN-γ, clone XMG1.2; Endogen, Woburn, MA), 10 ng/ml mouse recombinant IFN-γ (Genzyme, Cambridge, MA) and/or 10 ng/ml mouse recombinant TNF (Genzyme) were added at time 0 without changing the final volume. All experiments described below were performed at least twice to confirm the reproducibility of the results.

Lymphoproliferation assays

SC were cultured in triplicate in flat-bottomed 96-well plates at 2×10^5 cells/well (200 μl/well). After incubation at 37°C and 5% CO_2 for 48 h, 1μCi [^{3}H]thymidine (Amersham, Little Chalfont, UK) was added to each well. The cultures were harvested 18 h later and then processed for measurement of incorporated radioactivity in a liquid scintillation counter.

Measurement of cytokines and NO

SC were cultured in 24-well flat-bottomed plates at 1.2×10^6 cells/well (600 μl/well) with the corresponding stimulus. Cultures were incubated at 37°C in 5% CO_2 for 24 h, and the supernatants were harvested. IL-2, IFN-γ, TNF and IL-5 were detected by a two-site sandwich ELISA (Endogen). NO was measured as nitrite accumulated in the supernatants by using the Griess reaction. Briefly, 100 μl of 0.5% sulfanilamide/0.05% naphtyl-ethylenediamine hydrochloride in 2.5% H_3PO_4 was added to 50 μl of supernatants and incubated for 5 min at room temperature. The absorbance was then measured at 550 nm and nitrite concentrations were extrapolated from a sodium nitrite standard curve.

Flow cytometry

mAb against the following cell surface antigens were used: CD45R/B220 (FITC–rat IgG2a, clone RA3-6B2), CD69 [phycoerythrin (PE)–hamster IgG, clone H1.2F3], Ly-6G (biotin–rat IgG2b, clone RB6-8C5) and CD90.2 (Thy-1.2) (FITC–rat IgG2a, clone 53-2.1) l from PharMingen (San Diego, JA); CD11b (FITC-rat IgG2b, clone M1/70.15) and CD25 (PE–rat IgG1, clone PC61 5.3) from Caltag (Burlingame, CA); CD31 (biotin–rat IgG2a, clone ER-MP12) from BMA (Augst, Switzerland); and CD8a (PE–rat IgG2a, clone 53-6.7), CD11c (FITC–rat IgG1, clone HL3). Biotin-, FITC- and PE-conjugated rat IgG2b (clone R35-38), FITC- and PE-conjugated rat IgG2a (clone R35-95), PE–rat IgG1 (clone R3-34), and PE–conjugated hamster IgG (clone G235–2356) isotype control mAb were from PharMingen. CD59 (Ly-6C) (FITC–rat IgG2a, clone ER-MP20) was generously provided by Dr Jose L. Subiza (Hospital Universitario San Carlos, Madrid). Anti-

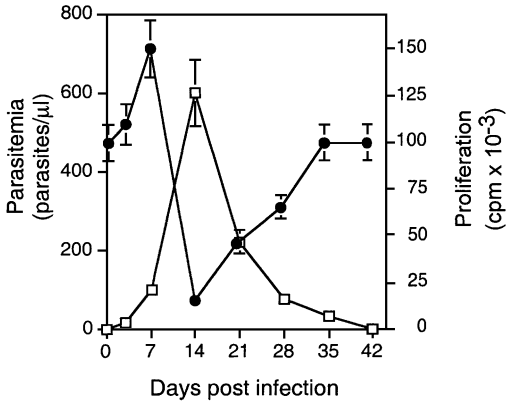

Fig. 1. Time-course of acute *T. cruzi* infection in Sv129 mice. Sv129 mice were infected with 10^4 trypomastigotes of the T strain. At the indicated times mice were sacrificed. Parasitemia levels were measured as the number of parasites/μl in blood. Proliferative responses of SC to Con A (5 μg/ml) were measured as [^{3}H]thymidine incorporation. Results shown are the means ± SD of triplicate samples from three mice in each group (nine independent determinations).

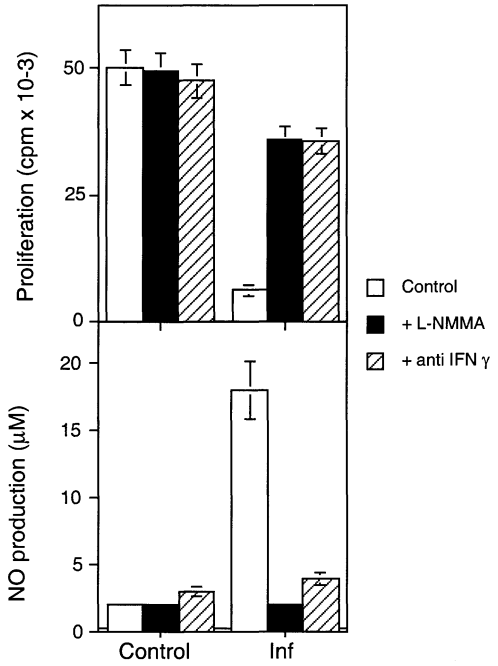

Fig. 2. Role of IFN-γ on NO production and unresponsiveness of SC from *T. cruzi*-infected mice. SC from Sv129 mice, uninfected (control) or 14 days after infection (Inf) with *T. cruzi*, were stimulated with Con A (5 μg/ml) for 72 h in the presence or absence of anti-IFN-γ (5 μg/ml) or L-NMMA (2 mM). Proliferation values are represented as [^{3}H]thymidine incorporation. NO production was measured by the Griess reaction. Results are the means ± SD of three independent experiments.

mouse iNOS mAb (FITC–mouse IgG2a, clone 6) was from Transduction (Lexington, KY).

SC were cultured at 37°C in 24-well flat-bottomed plates at 1.2 × 10^6 cells (600 μl/well) with the corresponding stimulus or maintained at 4°C (unstimulated cells). After stimulation, cells (2 × 10^5) were incubated at 4°C for 20 min with 20% normal rat serum in 50 μl of staining buffer (HBSS/1% FCS/0.05% azide). Subsequently, mAb or their isotypic controls were added at 10 μg/ml and incubated for 30 min at 4°C. After a final wash step in staining buffer, the cells were fixed with 250 μl of 1% PBS paraformaldehyde. At least 2000 viable cells were acquired on the basis of forward/side light scattering and analyzed in a FACScalibur (Becton Dickinson, Mountain View, CA).

Immunomagnetic depletion of SC

In some experiments, SC populations were selectively depleted by immunomagnetic beads (Dynabeads; Dynal, Oslo, Norway) following the manufacturer's instructions. Briefly, cells were incubated with anti-CD11b or Thy-1.2 plus B220 mAb (0.5 μg/10^6 cells) for 30 min, washed and incubated with anti-rat IgG-coated immunomagnetic beads at a 4:1 bead:cell ratio for 1 h at 4°C in a rotor. The efficacy of the process was assessed by flow cytometry. In all cases contamination with remaining positive cells was <3%.

Statistical analysis

Experimental differences over the controls were analyzed by Student's *t*-test. Probability values $P > 0.05$ were considered non-significant. All the experiments described were performed at least twice in order to validate the reproducibility of the results

Results

SC unresponsiveness in the acute phase of T. cruzi infection

Several previous studies have documented the existence of a profound unresponsiveness to mitogens during the acute phase of Chagas' disease in humans as well as in mice. In agreement with that, we found that SC from *T. cruzi* (T strain)-infected Sv129 mice had a strong unresponsiveness to mitogens such as Con A (Fig. 1) or to *T. cruzi* antigens (not shown). Interestingly, the kinetics of suppression in infected animals inversely correlated with parasitemia, being maximal at the peak of parasitemia and then gradually disappearing. Similar results were found in mice infected with other strains of *T. cruzi* such as RA or MC, although the parasitemia levels and the mortality rates observed in the acute phase were higher (not shown).

NO and IFN-γ are involved in the immunosuppression in acute T. cruzi-infected mice

As NO has been suggested to play a role in the unresponsiveness that takes place during *T. cruzi* infection (16), we first assayed NO-derived nitrite production by SC. Unstimulated SC did not produce NO levels detectable by the Griess reaction (data not shown). Upon Con A stimulation, SC from uninfected control Sv129 mice produced very low NO levels. In contrast, SC from *T. cruzi*-infected Sv129 produced very

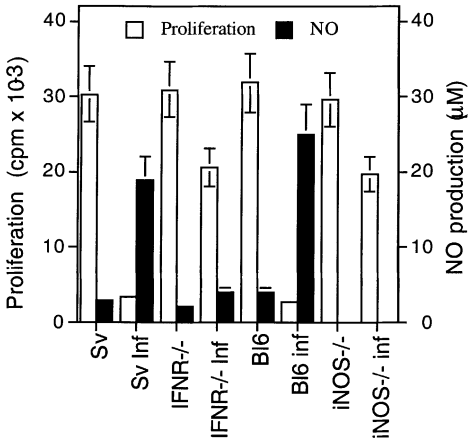

Fig. 3. Proliferation and NO production by SC from *T. cruzi*-infected IFN-γR$^{-/-}$ and iNOS$^{-/-}$ mice. SC from Sv129, IFN-γR$^{-/-}$, C57Bl6 or iNOS$^{-/-}$ mice, uninfected or 14 days after infection with *T. cruzi*, were stimulated with Con A (5 μg/ml) for 72 h. Proliferation values are represented as [^{3}H]thymidine incorporation. NO was measured by the Griess reaction. Results are the means ± SD of three independent experiments.

high levels of NO (Fig. 2). In order to test the possible role of NO in the observed unresponsiveness, we studied the effect of NO neutralization by L-NMMA on the proliferation of SC. As expected, addition of L-NMMA completely inhibited NO production by SC from Sv129-infected mice and partially restored proliferation (~70–80% of the control response) (Fig. 2). This inhibitor did not significantly affect Con A proliferation by SC from control mice. Neutralization of endogenous IFN-γ production with a mAb, which by itself had no effect on Con A-induced proliferation of SC from control mice, restored proliferation of SC from Sv129-infected mice and to the same extent as NO neutralization. In addition, neutralization of endogenously produced IFN-γ prevented NO production by those Con A-stimulated SC.

The above results suggested that NO and IFN-γ were involved in the immunosuppression in the acute phase. To confirm the role of IFN-γ and NO in the immunosuppression, we used mice deficient in IFN-γR or iNOS. SC from uninfected IFN-γR$^{-/-}$ or iNOS$^{-/-}$ mice proliferated almost exactly as did the cells from the control littermates, Sv129 or C57Bl6 respectively, in response to Con A. However, the response of infected IFN-γR$^{-/-}$ or iNOS$^{-/-}$ SC to Con A stimulation was minimally depressed (only 20–30% of inhibition) compared with infected littermates (Fig. 3). NO production was greatly diminished in Con A-activated SC from *T. cruzi*-infected IFN-γR$^{-/-}$ and absent, as expected, from iNOS$^{-/-}$ mice (Fig.3). In agreement with the lack of significant NO production, L-NMMA did not affect proliferation by SC from IFN-γR$^{-/-}$ or iNOS$^{-/-}$ infected mice (not shown).

Presence of suppressive immature myeloid cells in spleens of T. cruzi-infected mice

In order to investigate the nature of the suppressor cells, we first analyzed the cell populations present in the spleen after *T. cruzi* infection. For this, we analyzed the SC populations at day 14 post-infection. As shown in Fig. 4, there was an increase in the cellularity of infected Sv129 mice of ~50–80%. The absolute numbers of (Thy-1.2⁺) T cells were not affected in spleens from *T. cruzi*-infected mice, in agreement with a published report (32), thus discarding a reduction in T cell number as responsible for the decreased proliferative response observed. Cellularity increase was mainly due to CD11b⁺ myeloid cells and B220⁺ B cells. This increase in B220⁺ B cells may be a consequence of the polyclonal activation described in the acute phase (33). The increase in CD11b⁺ cells was remarkable since they represent a minority of the normal SC. Thus, *T. cruzi* infection raised their numbers (10-fold) and percentage (from 5% in uninfected control spleen to sometimes >20% of the total) in infected SC. Similar results were found in infected C57Bl6 (mice not shown). An increase in cells expressing the myeloid Gr1 (Ly6G) and ERMP20 (CD59, Ly6C) markers, parallel to the increase in CD11b, was observed. Interestingly, most of the CD11b⁺ cells in infected spleen also bear the Ly6G (Gr1) marker (Fig. 4B) and the immature marker ERMP20 (not shown) but not CD8a, a putative marker of dendritic cells (<0.5% of cells were Gr1⁺CD8a⁺ or Gr1⁺CD11c⁺) and have a low scatter (not shown), indicating that they are immature cells. In contrast, the total number of mature macrophages as detected by the F4/80 marker was not significantly augmented.

Immature myeloid cells have been described as suppressor cells in other systems of T cell unresponsiveness such as tumor-infiltrated animals (34) or after cytotoxic treatment (35). Thus, we first tested whether the presence of myeloid cells in the spleens correlated with suppression. *T. cruzi*-infected Sv129 mice had an increase in the percent of those immature cells that co-express Ly-6G (Gr1)⁺CD11b⁺ in their spleens 14 days after infection. Interestingly enough, those cells almost returned to basal levels in Sv129-infected mice at day 31 post-infection when the suppression was almost non-existent (Fig. 5). Those myeloid cells were similarly induced by *T. cruzi* infection and with similar kinetics in infected iNOS$^{-/-}$ mice, but strikingly spleens from infected IFN-γR$^{-/-}$ mice lack Gr1⁺CD11b⁺ myeloid cells (Fig. 5). Moreover, treatment with GM-CSF, known to expand these cells, did not increase the numbers of Gr1⁺, CD11b⁺ or ERMP20⁺ cells in SC from IFN-γR$^{-/-}$ obtained 14 days after infection (i.e. Gr1⁺ cells varied from 2.8% of total cells to 2.6% after 48-h culture in presence of GM-CSF). As expected, similar treatment expanded those populations in SC from Sv129 *T. cruzi*-infected mice obtained 14 days after infection (80% increase on average in 48 h cultures in the presence of GM-CSF; not shown). This suggests that IFN-γ signaling was required either to allow migration of those cells from bone marrow or for the expansion of precursors in the spleen.

Next, we tested the suppressive ability of those myeloid cells. First, depletion of CD11b⁺ cells restored proliferation to 70–80% of control levels and drastically reduced NO production by Con A-stimulated SC from *T. cruzi*-infected Sv129 mice, indicating that those cells are the major producers of NO (Fig. 6A). This reversion of the inhibition was similar to that achieved with L-NMMA. In addition, after CD11b⁺ depletion, the ability of L-NMMA to increase cell proliferation disappeared. To further confirm the immunosuppressive nature of Gr1⁺CD11b⁺ cells, those cells were negatively selected from infected SC by depletion of Thy-1⁺ and B220⁺ cells. Those

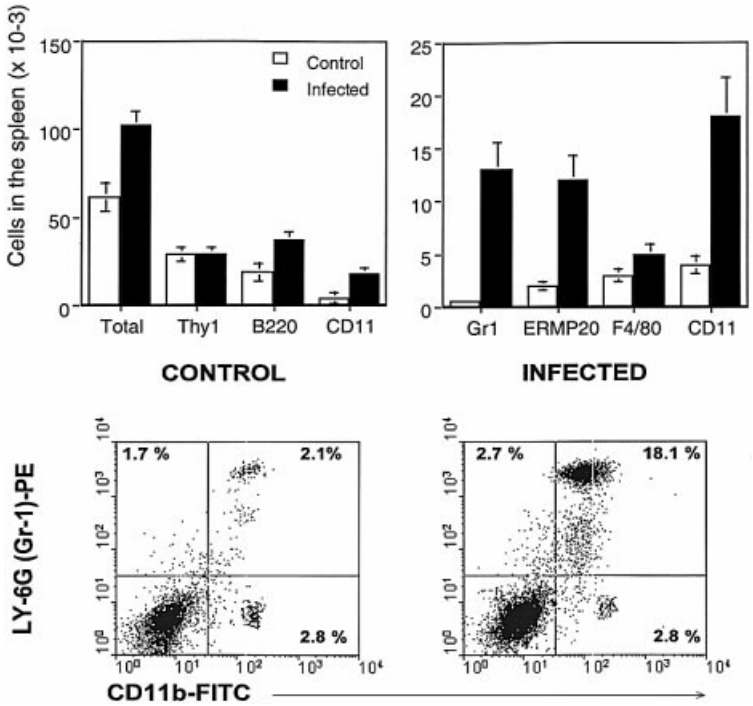

Fig. 4. Cell populations in the spleen of *T. cruzi*-infected mice. (A) Cell populations from uninfected or 14 day infected Sv129 SC were analyzed by flow cytometry and total number of cells evaluated (means ± SD of six mice). (B) SC from Sv129 mice, uninfected (control) or 14 days after infection with *T. cruzi*, were doubly stained with CD11b⁺ and Ly-6G (Gr1)⁺. A representative experiment is shown.

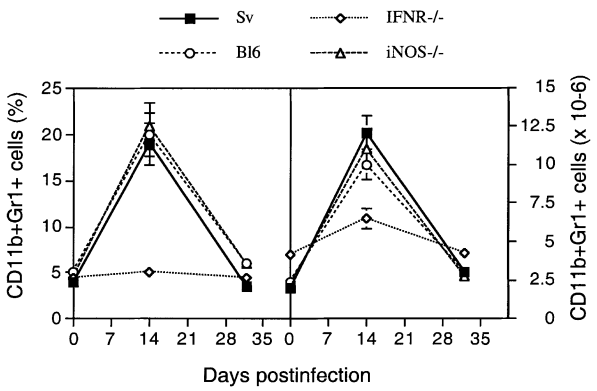

Fig. 5. Colonization of the spleen by Ly-6G (Gr1)⁺CD11b⁺ cells in *T. cruzi*-infected mice. Kinetics of Ly-6G⁺ CD11b⁺ colonization of the spleen in *T. cruzi*-infected mice. SC from Sv129 or IFN-γR$^{-/-}$ infected mice were double stained with CD11b and Ly-6G (Gr1), at 0, 14 and 31 days after infection. Percentages of positive cells are shown. Results shown are the means ± SD of triplicate samples from three mice in each group (nine independent determinations).

cells were >90% Gr1⁺CD11b and did not proliferate to Con A. However, when they were added to T cell cultures from normal mice, they induced a strong dose-dependent suppression of the Con A proliferative response that was totally reverted by addition of the iNOS inhibitor L-NMMA (Fig. 6B). Addition of similar numbers of CD11b⁺ obtained from spleens of control mice had no effect (not shown).

Addition of IFN-γ to unstimulated purified Gr1⁺CD11b⁺ myeloid cells clearly showed that only those cells from infected mice can produce large amounts of NO upon IFN-γ stimulation. This effect was completely blocked by L-NMMA (Fig. 7). By contrast, IFN-γ had only a minor enhancing effect on NO production by myeloid SC from uninfected mice, mostly F4/80 cells. Addition of TNF had a modest effect, but synergized with IFN-γ for NO production by Gr1⁺CD11b⁺ myeloid cells from Sv129-infected mice. Addition of Con A alone to purified Gr1⁺CD11b⁺ myeloid cells did not induce NO production (not shown). Interestingly, Gr1⁺ cells obtained from Con A-stimulated, but not from unstimulated, infected spleen expressed intracellular iNOS, as detected by cytofluorimetry (Fig. 7B). Altogether, the above results indicated that in Sv129-infected mice, IFN-γ (produced after Con A stimulation) induces NO synthesis by a Gr1⁺CD11b⁺ myeloid cell population present in the spleens from infected mice that in turn inhibits cell proliferation.

Immunosuppression by Ly6G (Gr1)⁺CD11b⁺ cells is not related to IL-2–IL2R inhibition

Inhibition of IL-2 and/or IL-2Rα has been proposed as a mechanism to account for *T. cruzi*-mediated immunosuppression synthesis (5,11,12), and it has been related to NO production (36). Therefore, we next investigated if Gr1⁺CD11b⁺ myeloid cells were responsible for those effects. For this, we first analyzed cell surface expression of activation markers and cytokine secretion by SC from various strains of infected mice. The results are summarized in Table 1. In contrast to the sharp reduction in cell proliferation, Con A-activated SC from *T. cruzi* Sv129-infected mice produced much higher levels of TNF and IFN-γ than SC from control mice. In contrast, IL-5 production was about the same in SC

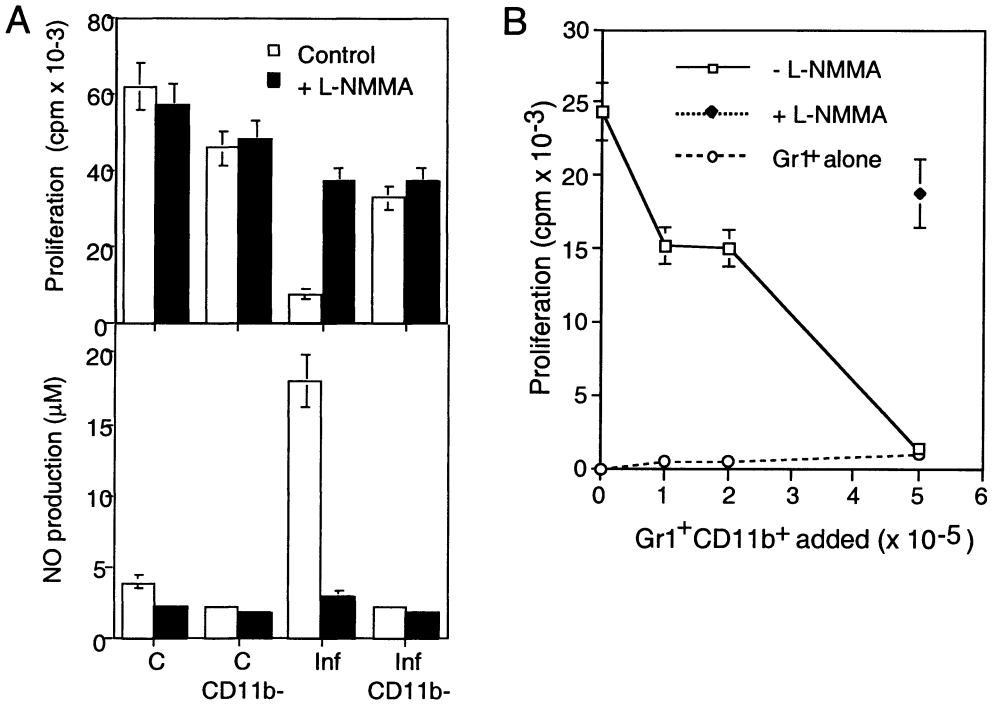

Fig. 6. Involvement of Ly-6G (Gr1)⁺CD11b⁺ cells in the SC unresponsiveness of *T. cruzi*-infected mice. (A) SC from Sv129 uninfected or 14 days after *T. cruzi* infection were depleted or not of CD11b⁺ cells and stimulated with Con A (5 μg/ml) for 72 h in the presence or absence of L-NMMA (2 mM). Proliferation was measured by [³H]thymidine incorporation. NO produced in the supernatants was evaluated by the Griess reaction. Results shown are the means ± SD of duplicate samples from six mice in each group (12 independent determinations). (B) Negatively selected Ly-6G (Gr1)⁺CD11b⁺ cells were added to T cells from SC of uninfected mice in the presence or absence of L-NMMA and proliferation measured by [³H]thymidine incorporation.

from infected as from control mice. Induction of early cell activation markers, such as CD69, in the membrane of T cells upon Con A stimulation was not affected by *T. cruzi* infection. Those results indicate that early activation steps were not affected in SC from infected mice. In addition, no significant differences (except in TNF production) were observed in IFN-γR$^{-/-}$ or iNOS$^{-/-}$ mice, indicating that neither IFN-γ nor NO were involved in those processes.

In contrast, upon Con A stimulation, infected SC from control littermates or from both IFN-γR$^{-/-}$ and iNOS$^{-/-}$ mice had a severe impairment in the induction of IL-2 production (90% inhibition) and in CD25 (IL-2Rα) expression as compared with control cells from uninfected mice (Table 1). CD25 inhibition was observed both at the level of percentage of cells expressing CD25 as well as by the amount of molecules expressed at the cell surface (mean fluorescence intensity, not shown). Since IFN-γR$^{-/-}$ SC have no Gr1⁺CD11b⁺ myeloid cells and in iNOS$^{-/-}$ mice they are not functional, myeloid suppressor cells are unlikely responsible for IL-2 and IL-2R down-regulation in *T. cruzi* infection. This was confirmed by depletion of CD11b⁺ myeloid cells in SC from infected mice, a treatment that did not restore IL-2 production by Con A-stimulated T cells (Fig. 8).

Discussion

Immunosuppression has been extensively documented during *T. cruzi* infection in humans as well as in mice. However, many discrepancies regarding the immune cell type responsible, T cells (5–7) or adherent cells (3,10,11,16), as well as the mechanism responsible, decreased IL-2–IL-2R, increased NO, prostaglandins or apoptosis, still exist (3,5,17,18,20,21). Moreover, the relationship among the different mechanisms and the suppressive cell types was not clear. As previously documented (32,33), we have found that *T. cruzi* infection was associated to an increase in spleen cellularity. We have shown here that this effect was mainly associated to an increase in B cells (B220⁺) as well as in CD11b⁺ cells. The majority of those myeloid cells also bear the Gr1 and ERMP20 surface markers, indicative of being immature cells. However, they do not express CD8a, suggesting that they are not plasmacytoid dendritic cells.

In *T. cruzi*-infected mice, L-NMMA restored Con A-induced T cell proliferation. Noteworthy, this restoration, although never complete (~70–80% of control responses), was clearly associated with down-regulation of NO levels and with neutralization of endogenous IFN-γ. Similar results were found in response to *T. cruzi* antigens (not shown). Our results are in agreement with previous reports showing that addition of IFN-γ suppressed Con A-proliferation by SC from *T. cruzi*-infected mice, but it was without effect in uninfected SC (16). In addition, neutralization of endogenous IFN-γ has been described to partially restore Con A-proliferation by *T. cruzi*-infected SC (16), in perfect agreement with our results.

More interestingly, we have described here for the first time that the IFN-γ-dependent suppressor mechanism is depend-

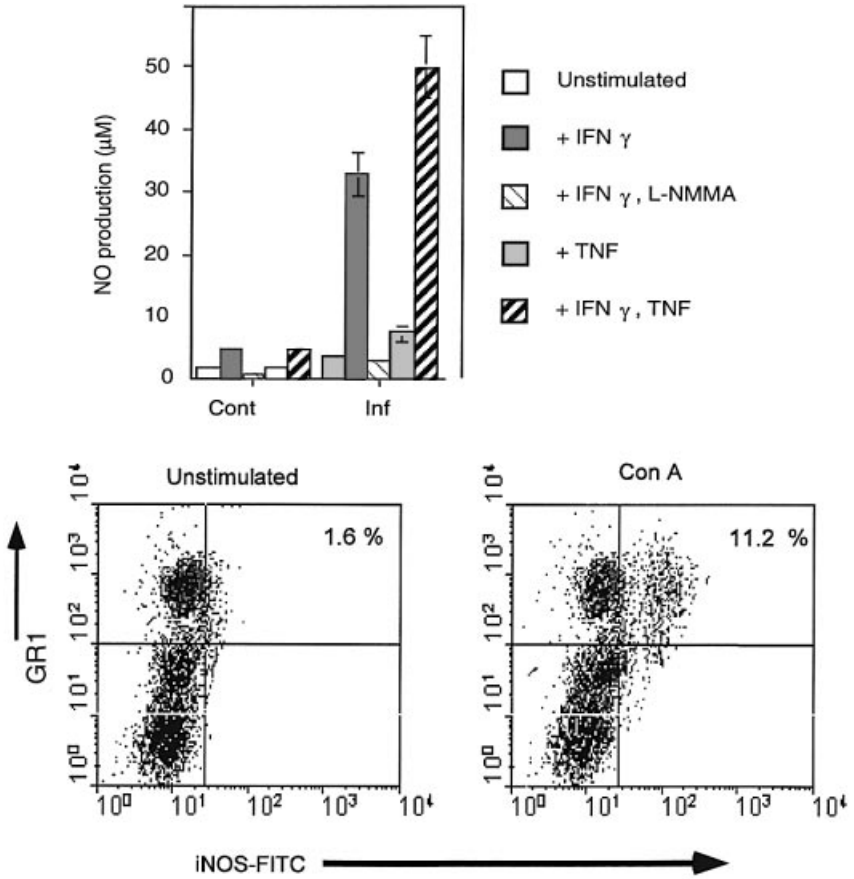

Fig. 7. Production of NO by Ly-6G (Gr1)⁺CD11b⁺ cells. (A) CD11b⁺ cells from Sv129 mice, uninfected or 14 days after infection with *T. cruzi*, were treated for 72 h with IFN-γ (10 ng/ml), TNF (10 ng/ml) or L-NMMA (2 mM) alone or in combination as indicated. NO produced in the supernatants was evaluated by the Griess reaction. Results shown are the means ± SD of triplicate samples from cells obtained from two different animals (six independent determinations). (B) SC from infected Sv129 mice were unstimulated or stimulated with Con A (5 μg/ml). After 48 h, SC were double stained for Gr1 and intracellular iNOS.

Table 1. Cytokine production by Con A-activated SC from *T. cruzi*-infected mice

Mice[a]	CD69 (%)	CD25 (%)	IFN-γ (pg/ml)	TNF (pg/ml)	IL-5 (pg/ml)	IL-2 (pg/ml)
Sv129	60 ± 4	58 ± 6	103 ± 28	130 ± 35	120 ± 21	201 ± 21
Sv129 infected	58 ± 4	21 ± 3[b]	442 ± 121[b]	247 ± 49[b]	128 ± 33	25 ± 12[b]
IFN-γR$^{-/-}$	58 ± 4	55 ± 4	131 ± 42	140 ± 28	60 ± 24	186 ± 31
IFN-γR$^{-/-}$ infected	59 ± 4	20 ± 6[b]	421 ± 134[b]	251 ± 63[b]	71 ± 31	19 ± 14[b]
C57Bl6	ND	18 ± 3	5201 ± 841	870 ± 98	ND	3999 ± 358
C57Bl6 infected	ND	9 ± 1[b]	29162 ± 3041[b]	2415 ± 230[b]	ND	224 ± 38[b]
iNOS$^{-/-}$	ND	23 ± 4	4714 ± 925	305 ± 31	ND	4269 ± 307
iNOS$^{-/-}$ infected	ND	12 ± 4[b]	23203 ± 2409[b]	875 ± 41[b]	ND	266 ± 41[b]

[a]SC from the indicated strains of mice infected (14 days post-infection) or not were activated with Con A (5 μg/ml), and 48 h later the supernatants collected and assayed in duplicate for cytokine content by specific ELISA. Results shown are the means ± SD of three independent experiments performed with SC from two mice in each group (six independent determinations). Unstimulated cells did not produce detectable amounts of cytokines and <2% of them expressed CD25 or CD69.
[b]Significantly different from corresponding uninfected control group, $P < 0.05$.

ent of an immature heterogeneous myeloid population expressing Ly-6G (Gr1) and CD11b. Our results show that a correlation exists between the presence of Ly-6G (Gr1)⁺CD11b⁺ cells, IFN-γ-dependent NO production and restoration of cell proliferation by L-NMMA. Thus, Ly-6G (Gr1)⁺CD11b⁺ were able to produce NO in large quantities upon IFN-γ activation, an effect that was potentiated by TNF. Depletion of CD11b⁺ cells completely eliminated Con A-induced NO production and restored proliferation in SC from infected mice to the same extent as inhibitors of NO synthesis

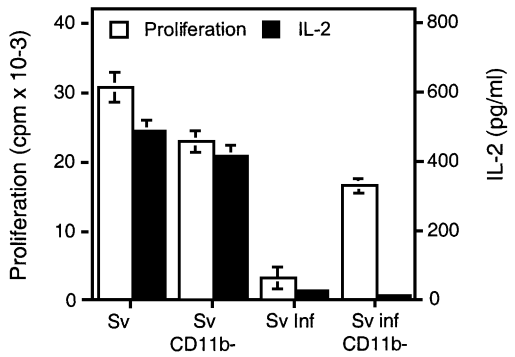

Fig. 8. Effect of depletion of Gr1⁺ cells on IL-2 production by splenic T cells. SC from Sv129 mice, uninfected or 14 days after *T. cruzi* infection, were depleted or not of CD11b⁺ cells and stimulated with Con A (5 μg/ml) for 48 h in the presence or absence of L-NMMA (2 mM). IL-2 production was determined in the supernatants by ELISA. Results shown are the means ± SD of triplicate samples from two mice in each group (six independent determinations).

did. In addition, purified Gr1⁺CD11b⁺ cells inhibited Con A-induced T cell proliferation.

The existence of immature Ly-6G (Gr1)⁺CD11b⁺ suppressor cells by a NO-mediated mechanism has been previously described in other experimental systems. Thus, we and others have recently described that those cells are responsible for immunosuppression that takes place in cyclophosphamide-treated mice (35) or in tumor-bearing animals (34). Moreover, those cells have been described after strong immunization protocols as being able to cause the death by apoptosis of T cells being previously immunosuppressive (37). Those cells have been described as phenotypically heterogeneous, consisting of a mixture of myeloid cells in different stages of differentiation (35,37) that are able to produce high levels of NO upon IFN-γ stimulation (35). Our results indicate that similar cells colonize the spleen in the acute phase of *T. cruzi* infection. In iNOS$^{-/-}$ those cells are similarly induced but cannot synthesize NO, thus preventing then from being suppressive. Surprisingly, in *T. cruzi*-infected IFN-γR$^{-/-}$ mice, those cells are not present in the spleen, indicating that IFN-γ may also play a role either in recruiting them from bone marrow or in expanding a previous population present in the spleen Those populations can be expanded by GM-CSF in SC from infected Sv129 mice but not in IFN-γR$^{-/-}$ mice. This suggests a role of IFN-γ in leukocyte homing and is in concordance with very recent results that have shown that IFN-γ controls homing of B cells in response to antigen (38). However, we cannot exclude that IFN-γ was required for the expansion of a minor population present in the spleen and that this effect cannot be bypassed or substituted by GM-CSF. Thus, IFN-γ not only participates in the mechanism of immunosuppresion by inducing iNOS, but also in the recruitment/expansion of immature cells.

The mechanism by which NO produced by those immature cells suppresses is not completely known. Interestingly enough, those cells are not responsible for the described IL-2 or IL-2Rα down-regulation that takes place during *T. cruzi* infection, since: (i) depletion of those cells did not restore IL-2 production to normal levels, and (ii) IL-2 production or IL-2Rα expression were as severely impaired in IFN-γR$^{-/-}$ mice that do not contain such cells and in iNOS$^{-/-}$ mice that do not produce NO, as in control littermates. This indicates that IL-2 and IL-2Rα inhibition are independent of NO production and IFN-γ signaling, clearly discarding NO as responsible for those activities, as has been proposed (36). Moreover, those results point out to the existence of alternative mechanisms of immunosuppression (3,5,17,18,20,21). We have also found here that T cells from infected spleens have no defects in early T cell activation events such as CD69 expression or cytokine secretion. Therefore, suppression of Ly-6G (Gr1)⁺CD11b⁺-derived NO should be taking place in a late phase of T cell activation. NO is able to induce apoptosis in T cells (39) and phenotypically similar Gr1⁺CD11b⁺ cells generated after strong immunization are able to cause the death by apoptosis of T cells (37). Thus, by analogy with the mechanisms of action of similar suppressor cells, we can tentatively speculate that in *T. cruzi*-activated SC they can cause apoptosis, but at the late stages of activation. Further studies are required to confirm this hypothesis. However, it is somewhat surprising that proliferation of SC was greatly restored when NO production was blocked or Gr1⁺CD11b⁺ cells were eliminated, despite having still >90% reduction in IL-2 and IL-2Rα production. This may indicate that those low amounts of IL-2 and IL-2Rα are sufficient to induce efficient T cell proliferation.

Con A-activated SC from infected animals produced larger amounts of IFN-γ and TNF than Con A-activated SC from control animals, in agreement with previous results (14–16). Those cytokines may contribute to an enhanced immunosuppression by Ly-6G (Gr1)⁺CD11b⁺. Thus, in addition to the widely recognized roles of TNF and IFN-γ in protection (27–29,40) by inducing macrophage activation (25), those cytokines may also contribute to the immunopathology during *T. cruzi* infection by promoting immunosuppression.

Immunosuppression induced by *T. cruzi* has been proposed to help parasite dissemination thorough the body. In IFN-γR$^{-/-}$ or iNOS$^{-/-}$ mice, despite having much higher parasitemias (40), they have very little immunosuppresion. However, in those animals immunosuppression may be not needed to help dissemination, since parasites can grow better due to the lack of protective effector mechanisms. Alternatively, immunosuppresion may not be required for dissemination of the parasite, but rather by the host to avoid an excessive immune response that may also cause damage.

Taken together, our results suggest a model to account for the immunosuppression in acute *T. cruzi* infections. Thus, parasite-derived molecules activate T cells to produce large amounts of IFN-γ that in turn induce the colonization of the spleen by an immature myeloid population Ly-6G (Gr1)⁺CD11b⁺. IFN-γ together with TNF activates those myeloid cells to secrete large amounts of NO. This NO can affect the proliferation of T cells either directly or indirectly, although the exact mechanism of action has not been elucidated. Our results may explain apparently contradictory results on the role of T cells and myeloid cells in this immunosuppression as depletion of either T cells (7) or adherent cells (9,10), as CD11b⁺ cells are, can lead to a decrease of immunosuppression. In summary, we have characterized an immunosuppressive mechanism in *T. cruzi*

infection dependent on iNOS induction on the immature myeloid population Ly-6G (Gr1)⁺CD11b⁺ by IFN-γ.

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Abbreviations

Con A	concanavalin A
GM-CSF	granulocyte macrophage colony stimulating factor
iNOS	inducible nitric oxide synthase
L-NMMA	N^G-monomethyl-L-arginine
SC	spleen cell
TNF	tumor necrosis factor
T	Tulahuen

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