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Dynamics of immunosuppression in hamsters with experimental visceral leishmaniasis

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Abstract

Immunosuppression has been reported to occur during active visceral leishmaniasis and some factors such as the cytokine profile may be involved in this process. In the mouse model of cutaneous leishmaniasis using *Leishmania* (*Leishmania*) *major*, the Th1 response is related to protection while the Th2 response is related to disease progression. However, in hamsters, which are considered to be an excellent model for the study of visceral leishmaniasis, this dichotomy is not observed. Using outbred 45- to 60-day-old (140 to 150 g) male hamsters infected intraperitoneally with 2 x 10^7 *L.* (*L.*) *chagasi* amastigotes, we evaluated the immune response of spleen cells and the production of cytokines. We used 3 to 7 hamsters per group evaluated. We detected a preserved response to concanavalin A measured by index of proliferation during all periods of infection studied, while a proliferative response to *Leishmania* antigen was detected only at 48 and 72 h post-infection. Messenger RNA from cytokines type 1 (IL-2, TNF-α, IFN-γ) and type 2 (IL-4, IL-10 and TGF-β) detected by reverse transcriptase polymerase chain reaction and produced by spleen cells showed no qualitative difference between control non-infected hamsters and infected hamsters during any period of infection evaluated. Cytokines were measured by the DNA band intensity on agarose gel using the Image Lab 1D L340 software with no differences observed. In conclusion, the present results showed an antigen-dependent immunosuppression in hamsters with active visceral leishmaniasis that was not related to the cytokine profile.

Key words: Immunosuppression; *Leishmania* (*Leishmania*) *chagasi*; Hamster; Cytokine; T-cell proliferation

Introduction

Active visceral leishmaniasis (VL) is accompanied by impairment of the T-cell response and can be fatal if not treated. During VL the suppression of *Leishmania* antigen- or mitogen-induced T cell-mediated immune responses is observed both in humans and in experimental animals (1-3). *Leishmania*-infected hamsters are considered to be an excellent experimental model for the evaluation of immunosuppression during VL since their disease is similar to the human disease with weight loss, hepatosplenomegaly and hypergammaglobulinemia (3-5). In this experimental model, immunosuppression is observed during active disease and a progressive impairment of *Leishmania* antigen- and concanavalin A (Con A)-induced lymphoproliferative response has been demonstrated. Some host-related factors may be involved in the development of immunosuppression, which is partially attributed to *Leishmania* antigen-specific suppression, participation of non-adherent cells, possibly T lymphocytes (4), and reduced nitric oxide production by adherent cells from *Leishmania*-infected hamsters (5,6). Furthermore, an increased production of transforming growth factor-β (TGF-β) by the spleen or peripheral blood mononuclear cells of *Leishmania*-infected hamsters or *Leishmania*-infected mouse T cells has been related to immunosuppression (7-9) and to the impairment of protein kinase C activity or expansion of $CD4^+CD25^+$ cells (8,9). However, using reverse transcriptase (RT)-PCR, dichotomy between the Th1 and Th2 cytokine profiles has not been observed during active VL in *Leishmania donovani*-infected hamsters (10). These results have been obtained during the late and intermediate phases of infection, but suppression is likely to occur during the initial phase of infection. Thus, in the present study, we evaluated the immune response and cytokine profile of hamsters infected with *L.* (*L.*) *chagasi* during the initial, intermediate and late phases of infection.

Material and Methods

Animals

Outbred 45- to 60-day-old male hamsters (*Mesocricetus*

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auratus) from the animal breeding facility of Faculdade de Medicina, Universidade de São Paulo, were maintained at the animal facility of Instituto de Medicina Tropical de São Paulo, Universidade de São Paulo, with free access to water and food throughout experiment.

Parasite

Leishmania (*L.*) *chagasi* (MHOM/BR/72/strain 46) were maintained in hamsters by successive inoculations with an infected spleen homogenate every 3 months. For the experiments, hamsters with VL after 2-3 months of infection were sacrificed under anesthesia (20 mg/kg ketamine and 5 mg/kg xylazine), the spleen was removed aseptically and the amastigotes were purified by the method of Dwyer (11).

Experimental protocol

Three to 7 hamsters per experimental group were inoculated intraperitoneally with 2 x 10^7 purified amastigotes in 1.0 mL 199 medium (Cultilab, Brazil). Four control animals were injected with 1.0 mL 199 medium. The animals were sacrificed and the spleens were weighed for analysis of parasite load on spleen imprints. In addition, *ex vivo* spleen cells were used to measure the proliferative response and to analyze the qualitative and semi-quantitative cytokine profile by RT-PCR.

***In vitro* spleen cell culture**

Erythrocytes from the spleen homogenate were lysed with 2% ammonium chloride in distilled water for 2 min, the osmolarity was immediately adjusted with 10X phosphate-buffered saline and the preparation was washed twice in RPMI 1640 medium. The cell concentration was adjusted to 2 x 10^7 cells/mL in RPMI 1640 medium supplemented with 10 mM HEPES, 100 IU/mL penicillin, 10 µg/mL gentamicin, 2 µM L-glutamine, 10 µM 2-mercaptoethanol (Sigma, USA) and 1% heat-inactivated control hamster serum. The cell suspension obtained from hamsters was used to evaluate the proliferation and detection of mRNA from cytokines.

Cell-proliferation

Spleen cells (2 x 10^7) under culture in 96-well plates were stimulated with 0.5 µg Con A (Amersham Pharmacia, Sweden) or 5 x 10^7 *Leishmania* antigen for 72 h. Six hours before the end of the culture, cells were pulsed with 1 µCi/well ^{3}H-thymidine (specific activity = 5 Ci/mmol; Amersham Life Science, England). They were then harvested with glass fiber filters (Millipore, USA) and counted with a β counter (Packard, USA). Data are reported as proliferation index as count per minute (cpm) in triplicate preparations.

The cells cultured in 24-well plates were processed for the detection of the qualitative and quantitative cytokine profile by RT-PCR.

Qualitative and quantitative cytokine profile

Extraction of total RNA. Spleen cells *ex vivo* and in culture upon receiving the stimuli were suspended in 500 µL Trizol reagent (Invitrogen, USA) and incubated at room temperature for 10 min. Chloroform (200 µL) was added, and the cultures were homogenized vigorously and then centrifuged at 12,000 *g* at 4°C for 15 min. Aqueous phase (500 µL) was separated, incubated with the same volume of isopropanol for 30 min at room temperature and then centrifuged. Absolute ethanol (500 µL) was added to the pellet, which was centrifuged again at 12,000 *g* for 15 min at 4°C. Ethanol was discarded and the pellet was dried for 10 min and suspended in 50 µL RNAse-free distilled water. RNA concentration and quality was determined with a NanoDrop-1000 spectrophotometer (NanoDrop Technologies, USA) and by 0.8% agarose gel electrophoresis and visualized with ethidium bromide.

cDNA preparation. Fifteen microliters of extracted RNA with 0.5 µg/µL of the oligo(dT) 12-18 primer (Invitrogen) and 3 µg/µL of a random primer (Invitrogen) were amplified in a thermocycler (Mastercycler Gradient, Eppendorf, Germany) for 5 min at 75°C and for 5 min at 25°C. Buffer (5X, Invitrogen), 10 mM dNTPs (Invitrogen), 40 U/µL RNAse (Fermentas, UK), 1000 U reverse transcriptase (Fermentas) and autoclaved ultrapure water were added and submitted to an amplification cycle in a thermocycler for 50 min at 37°C, 15 min at 70°C and 10 min at 4°C. The cDNA concentration and quality were analyzed with a NanoDrop-1000 spectrophotometer (NanoDrop Technologies).

Polymerase chain reaction

Cytokine initiator oligonucleotides and hamster HPRT were designed by using the respective gene sequences obtained from Gene Bank and synthesized by Gibco BRL-Life Technologies (Brazil). HPRT: forward, CCACGCC TACTATAGAGTTTG; reverse, CTTGCGATGTCATGG TAGAG; tumor necrosis factor-α (TNF-α): forward, CAC AATCCTCTTCTGCCTGC; reverse, TGTCTTTGAGAG ACATCCCG; TGF-β: forward, GAGAAGAACTGCTGTG TGCG; reverse, ACCCACGTAGTACACGATGG; interleukin-2 (IL-2): forward, AACCCAGCAGCACCTCGAGC; reverse, CAGTTACTGTCTCATCATCG; interferon-γ (IFN-γ): forward, TCATTGAGAGCCAGATCGTC; reverse, GGCTAAGTTTTCGTGACAGG; IL-10: forward, GGACAAC ATACTACTCACTG; reverse, ACAGGGGAGAAATCG ATGAC; IL-4: forward, TCCTATCACTGACGGTAGAG; reverse, TGCAAATGAGGTCTTTCTCC. One microliter cDNA was mixed with 10X PCR buffer (Invitrogen), 10 mM dNTP (Invitrogen), 10 µM specific forward and reverse primers, 0.65 mM magnesium (Fermentas), 0.75 U/µL Taq DNA polymerase (Fermentas), and autoclaved ultrapure water. Samples were submitted to amplification cycles in a thermocycler (Mastercycler Gradient) for 5 min at 95°C, 2 min at 50°C and 2 min at 70°C, and to 35 cycles for 1 min at 95°C, 2 min at 50°C and 2 min at 70°C. The amplified product was analyzed on 2.0% agarose gel and visualized with ethidium bromide. Cytokine mRNA was quantified

using the Image Lab 1D L340 software that measures the DNA band intensity on agarose gel.

Statistical analysis

All experiments were carried out in triplicate and a minimum of four hamsters were used per group according to their infected times or not. Data are reported as means ± SD and were analyzed by ANOVA. Nonparametric data were analyzed by the Kruskal-Wallis test. The level of significance was set at $P < 0.05$ in all analyses.

This study was approved by the Ethics Committee of Instituto de Medicina Tropical, Universidade de São Paulo.

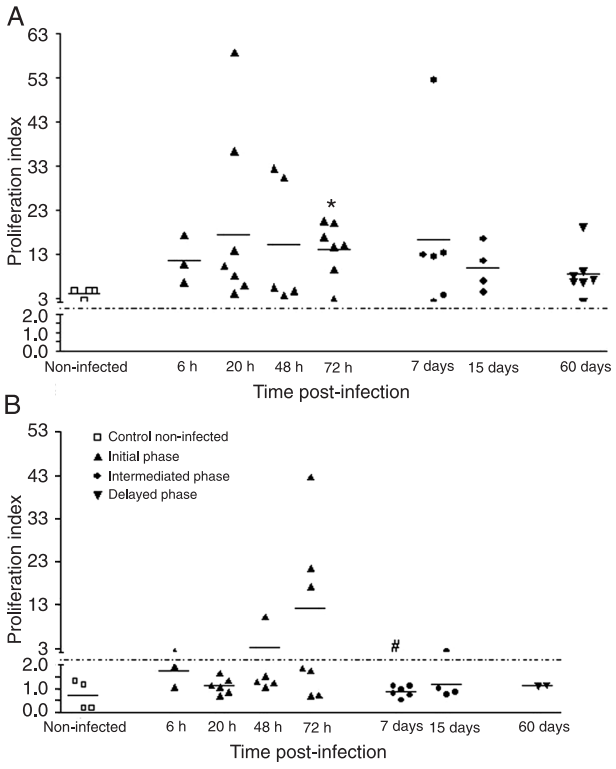

Figure 1. Proliferation of spleen cells of hamsters infected by *Leishmania* (*Leishmania*) *chagasi*. Spleen cells (2 x 10^7 cells/mL) at initial (6, 20, 48, and 72 h), intermediate (7 and 15 days) and late (60 days) time of infection were stimulated with concanavalin A (0.5 µg/mL) (A) or total antigen of LLC (5 x 10^7 parasites/mL) (B). The results are reported as proliferation index (count per min of stimulated cells/count per min of non-stimulated cells) with a cut-off (dashed line) of 2.0. *$P < 0.05$ *vs* non-infected; #$P < 0.05$ *vs* 48 h (Kruskal-Wallis test).

Results

Initially, we observed a progressive increase of parasite load in the spleen of hamsters infected with *L.* (*L.*) *chagasi* (data not shown). To better understand the increase of parasite burden and progression of the disease in this model, we investigated the proliferative response of spleen cells to a mitogen and a *Leishmania* antigen. When the spleen cells in culture were stimulated with Con A, the response was preserved during all periods of infection (Figure 1A), but a specific response to *Leishmania* antigen was only detected at 48 and 72 h post-infection (*pi*) (Figure 1B). No proliferation in response to the *Leishmania* antigen was observed from 7 to 60 days *pi*. When spleen cells from hamsters immunized with total *L.* (*L.*) *chagasi* antigen were stimulated with *Leishmania* antigen and presented a proliferative response, they were used as a positive control for antigen proliferation. Using RT-PCR to detect cytokine mRNA in spleen cells from infected hamsters, we observed the presence of mRNA of all cytokines evaluated, i.e., TNF-α, TGF-β, IL-2, IL-4, IL-10, and IFN-γ during all periods of infection evaluated (Figure 2), as well as in non-infected cells. As a control of RT-PCR, we detected hamster HPRT. No qualitative and quantitative differences in cytokine mRNA produced by spleen cells were observed between infected and non-infected hamsters (Table 1).

Discussion

Hamsters infected with *Leishmania* develop a progressive and severe disease, as is also observed in humans

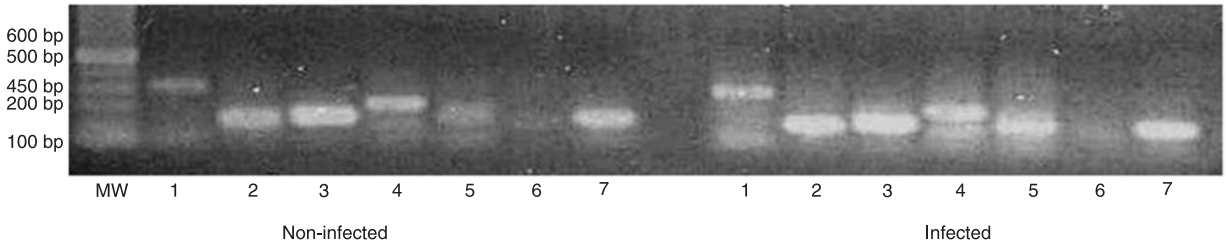

Figure 2. Detection of hamster cytokine mRNA. The mRNA of spleen cells (2 x 10^7 cells/mL) was analyzed by reverse transcriptase polymerase chain reaction (RT-PCR) for *lane 1* = HPRT (420 bp), *lane 2* = TNF-α (260 bp), *lane 3* = TGF-β (270 bp), *lane 4* = IL-2 (400 bp), *lane 5* = IL-4 (290 bp), *lane 6* = IL-10 (280 bp), and *lane 7* = IFN-γ (320 bp) expression both in non-infected and infected-hamsters (15 days post-infection). MW = molecular weight marker.

with active VL (12-15). In the present study, we observed a progressive time-dependent increase of parasite burden in the spleen of hamsters, thus confirming that these animals are good models for the development of active VL and for the study of immunosuppression. For a better understanding of VL progression in hamsters, we first evaluated the proliferative response of T cells to a mitogen and to a *Leishmania* antigen. We observed specific immunosuppression in response to *Leishmania* antigen, which, however, was limited to the initial phase of infection (48 and 72 h *pi*). We have observed a Con A-induced lymphoproliferative response during all experimental periods but a total absence of a *Leishmania* antigen-induced response in the late phase (3). According to the literature, the *Leishmania* antigen-induced response was suppressed (13), but there is a disagreement about the Con A-induced response. Some studies showed a response preserved throughout the experiment (13) while others did not observe it after 42 days of infection (14). Some factors, such as route of inoculation, may affect these results. Intracardiac inoculation produces an important impairment of the T-cell proliferative response to both a mitogen and a *Leishmania* antigen (13), while subcutaneous inoculation induces an immune response to both mitogen and *Leishmania* antigen (15,16). In the present study, we used the intraperitoneal route of inoculation, which promotes progressive infection and development of disease, indicating the occurrence of progressive immunosuppression during active VL similar to that observed in natural infection. Although hamsters are considered to provide a good model of visceral leishmaniasis, the almost complete absence of markers for T cells, macrophages and cytokines impairs a better understanding of the occurrence of the immune response in this model. However, after cytokine cloning from hamsters (6) there was an improvement in the understanding of the immune response in this experimental model. The profile of Th2 cytokines is directly responsible for suppression, but other factors such as adherent cells (17) and macrophage-mediated suppression have been reported to lead to increasing parasite growth and also to be linked to defective antigen presentation (18). The Th1 cytokine IFN-γ plays a key role in the control of infection with many intracellular pathogens, including *Leishmania* spp, and is the cytokine primarily responsible for macrophage activation and killing of intracellular parasites (19,20). In hamsters infected with *L.* (*L.*) *donovani*, adherent spleen cells have been shown to be important lymphoproliferative suppressors and to play a role in defective antigen presentation (13). Furthermore, TGF-β produced by adherent antigen-presenting cells from infected hamsters has been implicated in immunosuppression since a high level of TGF-β was observed in the cell culture supernatant when the *Leishmania* antigen-induced lymphoproliferative response was inhibited (7,8). In the present study, we investigated the cytokine profile produced during the course of VL by spleen cells in culture upon stimulation with Con A and *Leishmania* antigen using RT-PCR and we did not observe a qualitative or quantitative change in cytokine expression by non-infected and infected hamsters during any period of infection studied, in agreement with results obtained by Melby et al. (6). These results are similar to those observed in human VL but contrast with the dominant Th2 responses observed in progressive *L. major* infection in BALB/c mice. The immunosuppression mechanism is not related to the cytokine profile, although the change of a nucleotide in the final portion of sequence of mRNA from IFN-γ has been observed in hamsters (6), possibly contributing to a reduction of the biological effect of this cytokine and indicating the absence of power to control the progression of infection. Interestingly, we observed a strong TGF-β band during all periods of infection evaluated. We emphasize the importance of TGF-β in susceptibility and immunosuppression, a fact that has been demonstrated by the restoration of the proliferative response of non-adherent cells from infected hamsters treated with anti-TGF-β (8). It is attractive to speculate about the possible participation of $CD4^+CD25^+$-regulatory cells in immunosuppression during active VL in hamsters (9). According to our data, the immunosuppression observed in hamsters during active visceral leishmaniasis is an antigen-specific response since the initial phase of infection (after 72 h of infection), as determined by the absence of a lymphoproliferative response to *Leishmania* antigen and a preserved response to Con A. Interestingly, based on our results, the production of cytokines does not contribute to the development of immunosuppression in this experimental model.

Table 1. Quantitation of cytokine mRNA in infected and non-infected control hamsters.

Cytokines	Control	Infected	Relationship between infected and non-infected animals
HPRT	63.708	93.377	1.47
TNF-α	110.038	141.333	1.28
TGF-β	148.550	160.010	1.08
IL-2	90.505	103.582	1.14
IL-4	86.538	124.538	1.44
IL-10	44.007	83.234	1.89
IFN-γ	121.803	105.299	0.89

Cytokine mRNA was measured using the Image Lab 1D L340 software. Data are reported as DNA band intensity and are representative of only 1 animal from each group. The relationship between infected and non-infected animals was measured by the infected band intensity/non-infected band intensity ratio. TNF-α = tumor necrosis factor-α; TGF-β = transforming growth factor-β; IL = interleukin; IFN-γ = interferon-γ.

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