Cytokine detection at the site of *Leishmania (Leishmania) Amazonensis* subcutaneous inoculation in mice depleted of Natural Killer cells

Márcia Dalastra LAURENTI[1]
Andres ÖRN[2]
Idércio Luiz SINHORINI[3]
Carlos Eduardo Pereira CORBETT[1]

1- Laboratório de Patologia e Doenças Infecciosas do Departamento de Patologia da Faculdade de Medicina da USP, São Paulo - SP
2- Microbiology and Tumorbiology Center, Karolinska Institute, Stockholm, SWEDEN
3- Departamento de Patologia da Faculdade de Medicina Veterinária e Zootecnia da USP, São Paulo - SP

Correspondence to:
MÁRCIA DALASTRA LAURENTI
Departamento de Patologia
Faculdade de Medicina da USP
Av. Dr. Arnaldo, 455 - 1º andar - sl 1209
Cerqueira César
01246-903 - São Paulo – SP
mdlauren@usp.br

Received: 11/02/2003
Accepted: 23/08/2005

Abstract

BALB/c mice depleted and non-depleted of Natural Killer (NK) cells were infected subcutaneously with 10^7 stationary phase promastigotes of *Leishmania (Leishmania) amazonensis* and samples were taken at 24 hours and 7 days after infection. In NK cell-depleted mice, the NK cytotoxic activity of spleen cells decreased at 7 days after infection and more parasites were found in the lesion. The NK cell populations were analyzed by immunohistochemistry in spleen cryosections. An increase of $NK1.1^+$ expression and a decrease of $NK5E6^+$ antigen expression was observed in NK cell-depleted mice compared to non-depleted mice. When the presence of IFN-g, IL-12 and IL-4 at the site of parasite inoculation was analyzed by immunohistochemistry, a large amount of cytokines was detected in NK cell-depleted mice at 24 hours and 7 days after infection. In non-depleted mice, there was a small amount of IL-12 at 24 hours and of IL-4 at 7 days after infection. These data cells suggest that NK cell depletion by ^{90}Sr results in increased parasitism in the lesion. The increase of $NK1.1^+$ expression, which mainly produces IL-4, may take part in the progression of the infection.

Key-words:
Natural Killer cells.
Cytokines.
Cutaneous Leishmaniasis.
Leishmania (Leishmania) amazonensis.
Immunopathology.
$^{90}Strontium$.

Introduction

Leishmaniasis is an infectious disease of chronic evolution caused by the *Leishmania* protozoan, whose transmission involves vector arthroprods from the phlebotomine family. The disease develops when the *Leishmania* parasites survive the attack of the non-specific innate immune response involving cellular and humoral elements, after inoculation. These parasites migrate to target organs and proliferate, evading the specific immune mechanisms[1].

Both innate and specific elements of the immune system contribute to the control or the progression of the disease. At the beginning of the infection, the innate elements have an important role in the outcome of the disease. The direct importance of Natural Killer (NK) cells in the development of visceral leishmaniasis has been shown in mutant beige mice which have low NK cell activity[2]. Concerning cutaneous leishmaniasis, mice with an intermittent suppression or depletion of NK cells by anti-asialo GM-1 or anti-NK1.1 monoclonal antibodies became more susceptible to *Leishmania major* infection[3]. Laurenti et al.[4] showed increased numbers of parasites in the cutaneous lesion of BALB/c mice submitted to NK cell depletion by ^{90}Sr treatment and infected with *Leishmania (Leishmania) amazonensis* promastigotes. It has also been found that non-immune NK cells

are important in *Leishmania* infection as a source of IFN-g with the potential to trigger the Th-1 type of immune response in the early phase of cutaneous leishmaniasis[5].

In view of the importance of NK cells at the beginning of *Leishmania* infection in controlling the number of parasites in the lesion and as a source of IFN-g production, the main objective of the present study was to assess cytokine production at the site of subcutaneous inoculation of *Leishmania (Leishmania) amazonensis* in BALB/c mice submitted to NK cell depletion by ^{90}Sr treatment in the early period of the infection in order to examine some mechanisms involved in the increase of parasitism in this model.

Materials and Methods

Animals: Balb/c mice from the General Colony of the São Paulo University Medical School were maintained in our laboratory during the experiments.

Parasite: Promastigotes of *Leishmania (Leishmania) amazonensis*, HSJD-1 strain, were used throughout the experiments. Promastigotes in the stationary phase from the 3rd culture passage in supplemented RPMI 1640 medium (10% fetal calf serum, 5 mM HEPES, 50 mg/ml gentamicin and 100 U/ml penicillin) were harvested and washed 3 times in sterile saline (3000 rpm/ 10 minutes). The parasite concentration was adjusted to 2 x 10^8 promastigotes/ml and 50 ml was subcutaneously injected into the hind footpads of mice.

NK cell depletion: One dose of 0.6 mCi/g body weight of ^{90}Strontium - ^{90}Sr (AMERSHAM - USA) was injected by the intraperitoneal route in newly weaned mice one month before the parasite infection in order to deplete the mice of NK cells[6].

In vitro spleen NK cell activity from normal (NK+) and NK-depleted (NK-) mice was tested in a 4 hour ^{51}Cr-release microcytotoxicity assay at effector to target ratios of 200:1, 100:1, 50:1 and 25:1 using YAC-1 cells. The specific lysis = [(release (cpm) with effector cells – release in medium alone)/(release in distilled water – release in medium alone)] x 100.

Frozen spleen section from NK+ and NK- mice were submitted to immunohistochemistry using anti-NK cells (5E6) and anti-NK1.1 (PK136) (PharMingen-USA) purified monoclonal antibodies. To evaluate the reaction, semi-quantitative analysis was performed considering negative (-) results for no labeling and positive results according to the following scores: discrete (+) for 1 cell labeled per 10 microscope fields using a 40X objective, moderate (++) for 2-5 cells/field, and intense (+++) for more than 5 cells/ field.

Infection: Three groups of 40 mice, 20 NK- and 20 NK+, were inoculated with 10^7 *Leishmania (Leishmania) amazonensis* promastigotes. One group of 10 mice, not depleted and not infected, was used as control. Fragments from hind footpads were taken at 24 hours and 7 days after inoculation for histopathological study and cytokine detection.

Histopathological study: The lesion and the parasitism at the inoculation site were evaluated by light microscopy on the 7th day of infection in paraffin-embedded sections stained with Hematoxylin & Eosin. The images were obtained using digital photografic system (Leica MPS60) conected to optical microscope (Leica DMR).

In situ cytokine detection: Frozen sections from the site of parasite inoculation were submitted to immunohistochemical reaction for cytokine detection by the method of Sunnemark et al.[7]. Anti-IFN-g (XMG1.2) (ImmonoKontact-DE), anti-IL-12 (C15.6), and anti-IL-4 (BVD4-1D11) purified monoclonal antibodies (PharMingen-USA) were used as primary antibodies for the immunolabeling reaction. To evaluate the reaction, semi-quantitative analysis was performed considering results to be negative (-) in the absence of labeling and positive according to the following scores: discrete (+) for 1-10 cells labeled per microscope

field using a 40X objective, moderate (++) for 10-20 cells/field and intense (+++) for more than 20 cells/field.

Statistical analysis: The One Way Anova multiple range test was applied using the SigmaStat Statistical Software version 1.0. The difference was considered significant when $p<0.05$.

Results

Cytotoxic activity of spleen cells

Samples were taken to evaluate the NK activity of spleen cells before parasite inoculation in a ^{51}Cr release cytotoxic assay of YAC-1 target cells. Severe depletion of NK cytotoxic activity was observed by the lytic activity against YAC-1 when spleen cells from ^{90}Sr-treated animals were used at effector:target cell ratios of 200:1, 100:1 and 50:1 ($p<0.05$) (Figure 1).

Analysis of NK cell populations in spleen tissue

The semi-quantitative analysis of frozen spleen sections from NK- and NK+ BALB/c mice performed by immunohistochemistry using purified anti-NK (5E6) and anti-NK1.1 (PK136) monoclonal antibodies showed discrete expression of both antigens in NK+ mice. On the other hand, NK- mice showed an increase in $NK1.1^+$ expression and absence of $NK5E6^+$ cell antigen expression (Table 1 and Figure 2).

Histopathological analysis and parasitism of the skin lesion

Histopathological analysis of the skin lesion of NK- and NK+ BALB/c mice infected with *Leishmania (Leishmania) amazonensis* did not show significant differences between the two groups concerning inflammation. At the beginning of the infection, the inflammatory infiltrate was characterized by predominance of polymorphonuclear cells, which were gradually replaced by mononuclear cells. The number of parasites in the skin lesion was discrete in NK+ mice at 7 days after infection,

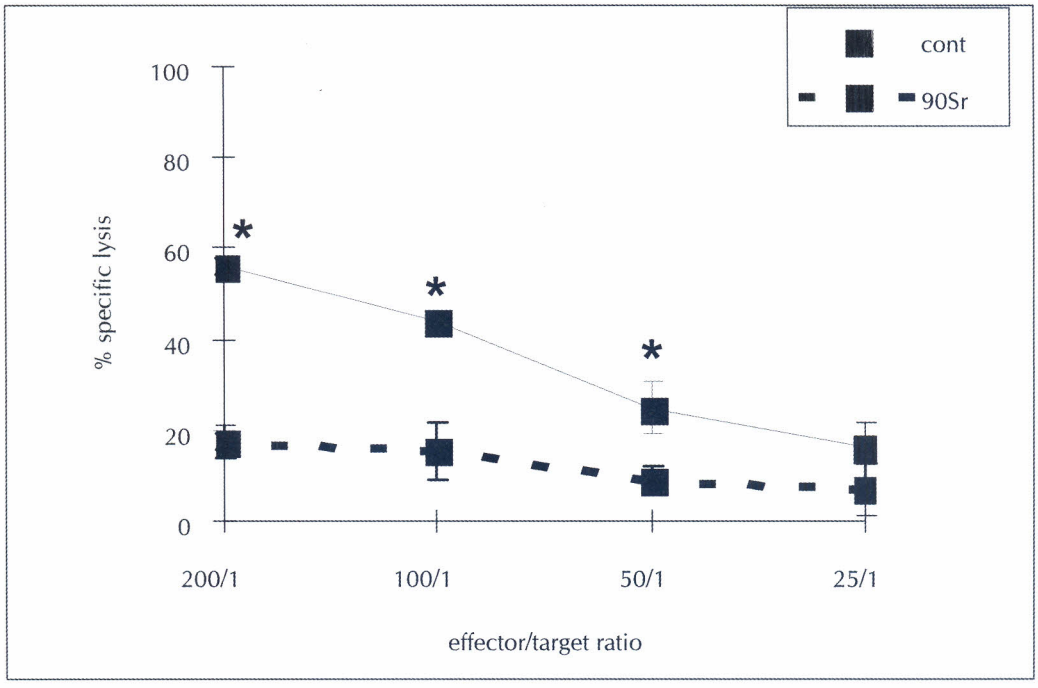

Figure 1 - *In vitro* NK cytotoxic activity of spleen cells from NK- and NK+ BALB/c mice. Data are representative of three separate experiments and show the mean and the standard deviation for 5 animals in each group (* $p<0.05$)

Table 1 - Semi-quantitative analysis of NK cells in spleen tissue from NK- and NK+ BALB/c mice by immunohistochemistry, scored as follows: - negative, + discrete, + + moderate and + + + intense. Data are representative of three separate experiments. São Paulo, 1998

Experimental Animals / Monoclonal Antibody		NK1.1/PK136	NK/5E6
BALB/c	NK-	+ +	-
	NK+	+	+

whereas NK- mice showed an increase of parasitism compared to the control NK+ mice (Figure 3).

Cytokine detection at the inoculation site

The semi-quantitative immunohistochemical analysis of IFN-g in frozen sections from the site of subcutaneous parasite inoculation in BALB/c mice revealed the presence of moderate amounts of IFN-g in NK- mice and its absence in NK+ mice at 24 hours and 7 days after infection. (Table 2 and Figure 4). IL-12 was found to be moderately present in NK- mice at 24 hours and 7 days after infection, although in NK+ mice it was discrete at 24 hours and absent at 7 days of infection (Figure 5). The presence of IL-4 was intense at 24 hours and 7 days after infection in NK- mice, but the cytokine was not detected at 24 hours and was present only in slight amounts at 7 days after infection in NK+ mice (Figure 6).

Discussion

Some pathogenetic aspects during the early period of *Leishmania (Leishmania) amazonensis* infection were studied in BALB/c mice submitted to NK cell depletion by intraperitoneal treatment with ^{90}Strontium (^{90}Sr).

Treatment with ^{90}Sr results in a severe and permanent depletion of NK cell activity without any apparent change in monocyte function or complement activation[6]. Here, a severe depletion of NK cell activity was confirmed by the lytic activity against YAC-1 cells in ^{90}Sr-treated animals.

The importance of NK cells in the resistance to *Leishmania* infection has been described by many authors. Kirkpatrick and Farrel[8] demonstrated the importance of NK cells in the elimination of splenic amastigotes in mice infected with *Leishmania donovani*. Further studies using ^{137}Cs-irradiated mice for NK cells depletion confirmed the role of these cells in the control of visceral leishmaniasis[2]. Later, Laskay, Rollinghoff and Solbach[3] showed an active participation of NK cells in the control of skin parasitism in the early phase of cutaneous leishmaniasis using mice depleted of NK cells by the use of monoclonal antibodies. Laurenti et al.[4] observed increased numbers of *Leishmania* in the skin lesion after ^{90}Sr treatment. The

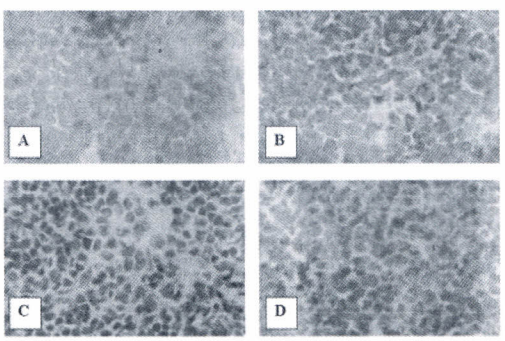

Figure 2 – Immunohistochemistry showing NK cells in frozen section of spleen of BALB/c mice NK- (A, B) and NK+ (C, D). Reaction with anti-NK1.1 (PK136) (A, C) and with anti-NK (5E6) (B, D) monoclonal antibody (ABC - 350x)

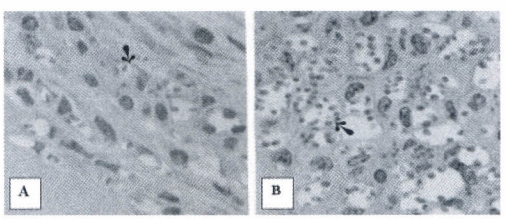

Figure 3 - Histological section from the skin lesion at the *Leishmania* subcutaneous inoculation site of NK+ (A) and NK- (B) BALB/c mice on the 7th day of infection showing predominance of mononuclear cells in the inflammatory infiltrate and tissue parasitism (arrow) (H&E – 675x)

Table 2 - Semi-quantitative cytokine analysis in frozen sections from the site of subcutaneous *Leishmania* inoculation at 24 hours and 7 days of infection by immunohistochemistry, scored as follows: - negative, + discrete, + + moderate, + + + severe. Data are representative of three separate experiments. São Paulo, 1998

Time of infection		24 hours			7 days		
Cytokines / Experimental Animals		IFN-γ	IL-12	IL-4	IFN-γ	IL-12	IL-4
BALB/c	NK -	+ +	+ +	+ + +	+ +	+ +	+ + +
	NK+	-	+	-	-	-	+

present study, which shows increased parasitism in the skin lesion of mice submitted to NK cell depletion with ^{90}Sr and infected with *Leishmania (Leishmania) amazonensis*, reinforces the importance of NK cells in the early period of cutaneous leishmaniasis.

The mechanisms involved in the control of lesion parasitism by NK cells are still partially unknown. However, the importance of NK cells as a source of IFN-g at the beginning of infection is already known[9,10].

Surprisingly, the analysis of IFN-g participation at the site of subcutaneous parasite inoculation showed an increase of IFN-g in NK- animals at 24 hours and 7 days after infection compared to NK+ animals. There are reports showing that IFN-g production is largely dependent on IL-12, whose production is induced at 24 hours after infection[10,11,12]; by the other side, Nylen et al.[13] reported that live *Leishmania* promastigotes can directly induce human peripheral blood NK cells from healthy donors to IFN-g secretion in the absence of IL-12 and the professional antigen presenting cells. Data from the present study show the concomitant and increased presence of IL-12 and IFN-g in the lesion of NK- BALB/c mice at 24 hours and 7 days after infection.

Although a marked decrease in NK cytotoxic activity was observed in the spleen, immunolabeling revealed an *in situ* increase in the expression of NK 1.1^+ cells and a decrease in the expression of NK5E6$^+$ cells after ^{90}Sr treatment. NK cells were described as non-T and non-B cells[14]. However, subsets of T lymphocytes have been reported as NK1.1^+ cells, therefore being processed in the thymus[15]. In this way, it is reasonable to propose that they are saved during the process of depletion by ^{90}Sr injection and are present in the spleen. Once NK5E6$^+$ cells are reduced, it may be

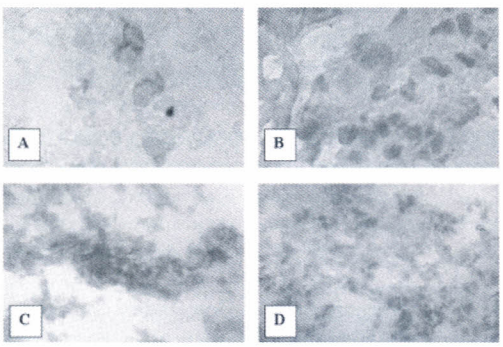

Figure 4 – Immunohistochemistry showing the presence of IFN-g in the subcutaneous inoculation site of parasites in BALB/c mice skin. (A) NK- and (B) NK+, 24 hours of infection (ABC – 675x); (C) NK- and (D) NK+, 7 days of infection (ABC – 350x)

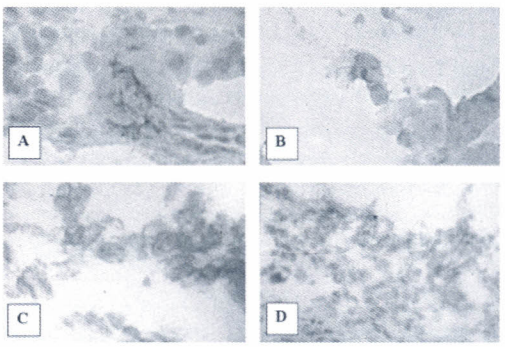

Figure 5 – Immunohistochemistry showing the presence of IL-12 in the subcutaneous inoculation site of parasites in BALB/c mice skin. (A) NK- and (B) NK+, 24 hours of infection (ABC – 675x); (C) NK- and (D) NK+, 7 days of infection (ABC – 675x and 350x, respectively)

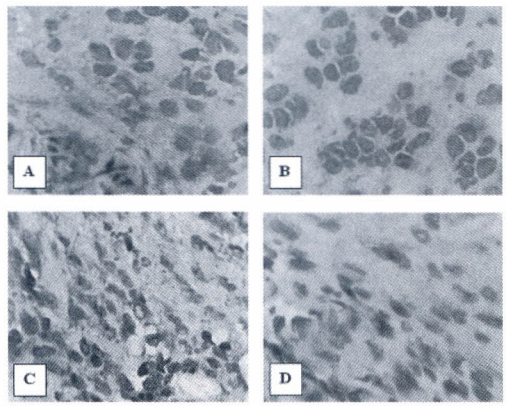

Figure 6 – Immunohistochemistry showing the presence of IL-4 in the subcutaneous inoculation site of parasites in BALB/c mice skin. **(A)** NK- and **(B)** NK+, 24 hours of infection; **(C)** NK- and **(D)** NK+, 7 days of infection (ABC – 675x)

speculated that control of one population over the other may be occurring in NK- animals. So, a decrease in NK5E6$^+$ cells should leading to a higher expression of NK1.1$^+$.

The resistance to *Leishmania* infection is traditionally attributed to the production of IFN-g, which activates the microbicidal mechanisms of macrophages, enabling them to control parasite replication[16,17]. The present data, however, show that in animals treated with ^{90}Sr there is a higher presence of IFN-g and a larger increase of parasitism in the lesion. Recent data have correlated IFN-g to the development of lesions instead of control in leishmaniasis[18,19]. On the other hand, the cytokines which have been traditionally related to susceptibility to infection in cutaneous leishmaniasis are IL-4 and IL-10[17]. Akuffo et al.[20] demonstrated that in innate response to *Leishmania* antigen envolving NK cells, a critical level of IL-12 is required to induce IFN-g secretion below which, IL-10 is released in amounts which apparently inhibit IFN-g secretion and cellular proliferation showing the contribution of NK cells in cross regulation of these two very import immunoregulatory cytokines. Regarding the production of IL-4, it is known that NK 1.1$^+$ cells, along with IFN-g production, result in *in vitro* production of this cytokine[21,22]. Other authors also reported a high production of IL-4 by NK1.1$^+$ cells when mice are stimulated *in vivo*[23]. The present experiments showed a large amount of IL-4 detected by immunohistochemistry at the subcutaneous parasite inoculation site in BALB/c mice depleted of NK cells by ^{90}Sr treatment, probably produced by. NK1.1$^+$ cells, which were increased in NK-depleted mice.

These experiments showed that NK cells are highly important in the initial control of *Leishmania* infection. Data suggest that the regulatory mechanism of different NK cell populations may result in the production of different cytokines at the site of infection, determining the evolution of the disease.

Acknowlegments

To Dr. Dan Sunnemark for the initial steps on standartization of immunohistochemistry reaction for cytokine detection in mouse tissue. This work was supported by LIM-50 HC-FMUSP, CNPq, FAPESP and CAPES - PDEE 0025/95-20.

Detecção de citocinas no sítio de inoculação subcutânea de *Leishmania (Leishmania) amazonensis* em camundongos depletados de células Natural Killer

Resumo

Camundongos BALB/c depletados e não depletados em células *Natural Killer* (NK) foram infectados subcutaneamente com 10^7 promastigotas de *Leishmania (Leishmania) amazonensis* em fase estacionária de crescimento e amostras foram colhidas às 24 horas e 7 dias de infecção. Nos camundongos depletados em células NK, a atividade citotóxica NK de células esplênicas estava diminuída aos 7

Palavras-chave:
Células Natural Killer.
Citocinas.
Leishmaniose cutânea.
Leishmania (Leishmania) amazonensis.
Imunopatologia.
^{90}Estrôncio.

dias de infecção e mais parasitos foram encontrados na lesão. Populações de células NK foram analisadas por imuno-histoquímica em cortes congelados de baço. Foi observado aumento na expressão de células $NK1.1^+$ e diminuição na expressão do antígeno $NK5E6^+$ nos animais depletados em células NK comparados aos camundongos não depletados. A presença de IFN-g, IL-12 e IL-4, analisada por imuno-histoquímica no sítio de inoculação dos parasitos, mostrou que maior quantidade de citocinas foram detectadas nos camundongos depletados em células NK às 24 horas e 7 dias depois da infecção. Nos camundongos não depletados, havia pequenas quantidades de IL-12 às 24 horas e de IL-4 aos 7 dias de infecção. Estes dados sugerem que a depleção de células NK por ^{90}Sr resulta em aumento de parasitismo na lesão. O aumento na expressão de células $NK1.1^+$, as quais produzem principalmente IL-4, pode representar um dos mecanismos que colaborariam para a progressão da infecção.

References

1 BOGDAN, C.; ROLLINGHOFF, M.; SOLBACH, W. Evasion strategies of Leishmania parasites. **Parasitology Today,** v. 14, p. 171-183, 1990.

2 KIRKPATRICK, C. E. et al. Participation of Natural Killer cells in the recovery of mice from visceral leishmaniasis. **Cellular Immunology,** v. 92, p. 163-171, 1985.

3 LASKAY, T.; ROLLINGHOFF, M.; SOLBACH, W. Natural Killer cells participate in the early defense against *Leishmania major* in mice. **European Journal of Immunology**, v. 23, p. 2237-2241, 1993.

4 LAURENTI, M. D. et al. The role of Natural Killer cells in the early period of infection in murine cutaneous leishmaniasis. **Brazilian Journal of Medical and Biological Research**, v. 32, p. 323-325, 1999.

5 SCHARTON, T. M.; SCOTT, P. Natural Killer cells are a source of Interferon g that drives differentiation of CD4+ T cell subsets and induces resistance to *Leishmania major* in mice. **Journal of Experimental Medicine**, v. 178, p. 567-577, 1993.

6 GIDLUND, M. et al. Impact of ^{90}Sr on mouse Natural Killer cells and their regulation by alpha-interferon and interleukin-2. **Scandinavian Journal of Immunology**, v. 31, p. 575-582, 1990.

7 SUNNEMARK, D. et al. Cytokine production in hearts of *Trypanosoma cruzi*-infected mice: Do cytokine patterns in chronic stage reflect the establishment of myocardial pathology? **Scandinavian Journal of Immunology**, v. 44, p. 421-429, 1996.

8 KIRKPATRICK, C. E.; FARREL, J. P. Mechanisms of depression of splenic Natural Killer cell function in C57BL/6 mice infected with *Leishmania donovani*. **Cellular Immunology,** v. 87, p. 601-612, 1984.

9 SCOTT, P. IFN-g modulates the early development of Th1 and Th2 responses in a murine model of cutaneous leishmaniasis. **Journal of Immunology**, v. 147, p. 3149-3155, 1991.

10 SOLBACK, W.; LASKAY, T. *Leishmania major* infection: the overture. **Parasitology Today**, v. 11, p. 394-397, 1995.

11 CHARTON-KERSTEN, T.; SCOTT, P. The role of the immune response in Th1 cell development following *Leishmania major* infection. **Journal of Leukocyte Biology**, v. 57, p. 515-522, 1995.

12 SYPEK, J. P. et al. Resolution of cutaneous leishmaniasis: Interleukin 12 initiates a protective T helper type 1 immune response. **Journal of Experimental Medicine,** v. 177, p. 1797-1802, 1993.

13 NYLÉN, S. et al. Live *Leishmania* promastigotes can directly activate primary human Natural Killer cells to produce Interferon-gamma. **Clinical and Experimental Immunology,** v. 131, p. 457-467, 2003.

14 KIESSLING, R. et al. Killer cells: a functional comparison between natural, immune T-cell and antibody-dependent *in vitro* systems. **Journal of Experimental Medicine,** v. 143, p. 772-780, 1976.

15 VICARI, A. P.; ZLOTNIK, A. Mouse NK1.1 T cells, a new family of T cells. **Immunology Today**, v. 17, p. 71-76, 1996.

16 LOCKSLEY, R. M. et al. Cutaneous host defense in leishmaniasis: interaction of isolated dermal macrophages and epidermal Langerhans cells with the insect-stage promastigote. **Infection and Immunity**, v. 56, p. 336-342, 1988.

17 REINER, S. L.; LOCKSLEY, R. M. The regulation of immunity to *Leishmania major*. **Annual Review of Immunology**, v. 13, p. 151-177, 1995.

18 GUEVARA-MENDOZA, O. et al. Experimental infection of Balb/c mice with *Leishmania panamensis* and *Leishmania mexicana*: induction of early IFN-g but

not IL-4 is associated with the development of cutaneous lesions. **Scandinavian Journal of Immunology**, v. 46, p. 35-40, 1997.

19 SOONG, L. et al. Role of CD4+ T cells in pathogenesis associated with *Leishmania amazonensis* infection. **Journal of Immunology**, v. 158, p. 5374-5383, 1997.

20 AKUFFO, H. et al. Natural Killer cells in cross regulation of IL-12 by IL-10 in Leishmania antigen-stimulated blood donor cells. **Clinical and Experimental Immunology,** v. 117, p. 529-534, 1999.

21 ZLOTNIK, A. et al. Cytokine production by mature and immature CD4-CD8- T cells, ab-T cell receptor+ CD4-CD8- T cells produce IL-4. **Journal of Immunology**, v. 149, p. 1211-1215, 1992.

22 ARASE, H. et al. NK1.1+CD4+CD8- thymocytes with specific lymphokine secretion. **European Journal of Immunology**, v. 23, p. 307-310, 1993.

23 BROWN, R. D. et al. Beta 2-microglobulin-dependent NK1.1+T cells are not essential for T helper cell 2 immune response. **Journal of Experimental Medicine**, v. 184, p. 1295-1304, 1996.