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MAJOR ARTICLE

Granzyme B Produced by Natural Killer Cells Enhances Inflammatory Response and Contributes to the Immunopathology of Cutaneous Leishmaniasis

Taís M. Campos,[1,2] Fernanda O. Novais,[3] Maíra Saldanha,[4] Rúbia Costa,[2] Morgana Lordelo,[5] Daniela Celestino,[2] Camilla Sampaio,[1,2] Natália Tavares,[5] Sérgio Arruda,[4] Paulo Machado,[2,6] Cláudia Brodskyn,[5] Phillip Scott,[3] Edgar M. Carvalho,[1,2,6] and Lucas P. Carvalho[1,2,6]

[1]Laboratório de Pesquisas Clínicas, Instituto Gonçalo Moniz, FIOCRUZ, Salvador, Brazil, [2]Serviço de Imunologia, Complexo Hospitalar Professor Edgard Santos, Universidade Federal da Bahia, Salvador, Brazil, [3]Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, USA, [4]Laboratório Avançado de Saúde Pública, Instituto Gonçalo Moniz, FIOCRUZ, Salvador, Brazil, [5]Laboratório de Interação Parasito-Hospedeiro e Epidemiologia, Instituto Gonçalo Moniz, FIOCRUZ, Salvador, Brazil, [6]Instituto Nacional de Ciências e Tecnologia-Doenças Tropicais, Salvador, Brazil

Background. Skin lesions from patients infected with *Leishmania braziliensis* has been associated with inflammation induced by cytotoxic $CD8^+$ T cells. In addition, $CD8^+$ T cell-mediated cytotoxicity has not been linked to parasite killing. Meanwhile, the cytotoxic role played by natural killer (NK) cells in cutaneous leishmaniasis (CL) remains poorly understood.

Methods. In this study, we observed higher frequencies of NK cells in the peripheral blood of CL patients compared with healthy subjects, and that NK cells expressed more interferon-γ, tumor necrosis factor (TNF), granzyme B, and perforin than $CD8^+$ T cells.

Results. We also found that most of the cytotoxic activity in CL lesions was triggered by NK cells, and that the high levels of granzyme B produced in CL lesions was associated with larger lesion size. Furthermore, an in vitro blockade of granzyme B was observed to decrease TNF production.

Concclusions. Our data, taken together, suggest an important role by NK cells in inducing inflammation in CL, thereby contributing to disease immunopathology.

Keywords. $CD8^+$ T cells; cutaneous leishmaniasis; cytotoxic activity; granzyme B; NK cells.

Cutaneous leishmaniasis (CL) is a neglected tropical disease caused by the protozoan parasite of the genus *Leishmania*, which is transmitted to humans through the bite of the infected sandfly, and it is characterized by the development of ulcerative lesions in the skin [1]. Few parasites are present in CL lesions, and a strong inflammatory response with resultantly high levels of tumor necrosis factor (TNF) and interleukin (IL)-1β production is observed and associated with tissue damage and lesion development [2–4]. In the BALB/c model of CL, the production of interferon (IFN)-γ is the main mechanism of resistance, and both $CD4^+$ T cells and natural killer (NK) cells are considered the main sources of IFN-γ [5].

Previous studies conducted by our group and others have demonstrated an association between inflammation and the cytotoxicity induced by $CD8^+$ T cells in CL [6–11]. Our recent findings suggested that cytotoxic activity by $CD8^+$ T cells leads to inflammasome activation and IL-1β release in CL [10]. In addition, *Leishmania braziliensis* infection was shown to induce degranulation by $CD8^+$ T cells derived from CL lesions, whereas the frequency of $CD8^+$granzyme B^+ T cells was found to positively correlate with disease severity. We were surprised to find that none of these observations indicated that the cytotoxic activity triggered by $CD8^+$ T cell serves as a mechanism for *Leishmania* killing [8, 11, 12].

In contrast to $CD8^+$ T cells, less is known about the role of NK cells in CL. In a recent study, it was observed that NKT cells and double-negative T cells derived from CL lesions displayed a higher percentage of the cytotoxic marker CD107a than $CD8^+$ T cells [13]. In addition, in subjects with subclinical *L braziliensis* infection, defined as individuals without previous or present history of leishmaniasis but with cell-mediated immune response to *Leishmania* antigens, NK cells were determined to be the main source of IFN-γ in blood [14]. Furthermore, the activation of NK cells by *Leishmania* lipophosphoglycan was linked to IFN-γ and TNF production [15, 16]. Because the cytotoxic capacity of NK cells has been poorly studied in human CL, we aimed to assess the contribution of cytotoxic activity by NK cells in the context of *L braziliensis* CL. We found that CL patients presented higher frequencies of NK cells than healthy subjects (HS), and that NK cells were responsible for most of the cytotoxicity activity observed in lesions from CL patients.

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Correspondence: Lucas P. Carvalho, PhD, Rua Waldemar Falcão, 121, Candeal - Salvador/BA CEP: 40296-710, Salvador, Bahia, Brasil (carvalholp76@gmail.com).

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METHODS

Patients

This study was approved by the Institutional Review Board of the Professor Edgard Santos University Hospital Complex (HUPES-UFBA) (protocol no. 25/12) and the National Commission of Ethics in Research (612.907). All subjects provided written informed consent. Forty patients with CL were recruited from an endemic area—Corte de Pedra, Bahia, Brazil. The diagnostic criteria consisted of the presence of an ulcerated lesion on the skin, with no evidence of mucosal involvement, and the detection of *L braziliensis* deoxyribonucleic acid (DNA) by polymerase chain reaction (PCR). The control group consisted of 18 HS living in areas without reported exposure to *Leishmania.* All CL patients were evaluated before beginning treatment.

Soluble *Leishmania* Antigen

An isolate of *L braziliensis* (MHOM/BR/2001) was used to prepare the soluble *Leishmania* antigen (SLA), as previously described [17]. The antigen was tested for endotoxins using the Limulus amebocyte lysate test and applied at a concentration of 5 μg/mL.

Parasites

The *L braziliensis* isolate used for in vitro experimentation was obtained from a skin lesion of a CL patient and characterized as *L braziliensis* after identification of parasite kDNA by PCR, as previously described [18]. Stationary-phase promastigotes were used in infection procedures.

Peripheral Blood Mononuclear Cell Cultures and Tissue Samples Procedures

Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation using a Ficoll-Paque Plus gradient (GE Healthcare), then washed by centrifugation, and resuspended in 0.9% NaCl or Roswell Park Memorial Institute (RPMI) 1640 media (Gibco). The PBMCs were adjusted to a concentration of 1×10^6 cells/mL in 1 mL RPMI 1640 media, supplemented with 10% fetal bovine serum (FBS) (Gibco) and antibiotics. Culturing was performed in the presence or absence of SLA (5 μg/mL) and incubated overnight. Next, Monensin (eBioscience) was added to the cultures, followed by a 4-hour reincubation at 37°C under 5% CO_2. Finally, intracellular cytokines was determined by flow cytometry. Furthermore, granzyme B and IFN-γ was assessed by intracellular staining in NKG2D-positive and -negative subsets. Before the experiments with tissue samples had been performed, healthy skin and lesion fragments were weighted, and results were normalized according to the amount of tissue. Tissue samples from HS skin and CL lesions were incubated without stimuli for 12 hours at 37°C under 5% CO_2. Supernatants were collected, and granzyme B levels were measured by enzyme-linked immunosorbent assay (ELISA) (eBioscience).

Flow Cytometry Analysis

Peripheral blood mononuclear cells (0.5×10^6 cells/mL) were incubated with anti-CD3, anti-CD56, anti-CD16, anti-CD8, and anti-NKG2D monoclonal antibodies (BD Biosciences) for 20 minutes, washed twice by centrifugation, and fixed with 2% paraformaldehyde. For intracellular staining, cells were resuspended in Perm/Wash (BD Biosciences) for 15 minutes, and intracellular labeling was performed using anti-perforin, anti-granzyme B, anti-TNF, and anti-IFN-γ monoclonal antibodies for 30 minutes. For the degranulation assay, tissue fragments were incubated in RPMI (Gibco) with Liberase (Roche) for 60 minutes at 37°C under 5% CO_2. After incubation, 1 mL RPMI containing 10% FBS was added to stop the enzyme reaction. Tissues were dissociated using a cell strainer (40 μm) (BD Pharmingen). Then, cells were ressuspended in complete RPMI (4×10^6/mL) and incubated for 6 hours at 37°C/5% CO_2 with anti-CD107a antibody and Brefeldin A without stimulation, followed by surface staining with anti-CD56 and anti-CD8 antibodies. For analysis of cell viability, before surface staining, cells were washed and stained for live/dead fixable aqua dead cell stain kit (Molecular Probes), according to the manufacturer's instructions.

Functional Assay

For the granzyme inhibition assay, PBMCs were cultured with or without SLA (5 μg/mL) in the presence or absence of a granzyme B inhibitor (3,4-dichloroisocoumarin) (Sigma-Aldrich, St. Louis, MO) at a concentration of 18.4 μM/1×10^6 cells for 72 hours at 37°C under 5% CO_2. Dimethyl sulfoxide (vehicle), 0.4%, was used as a control. Supernatants were collected and cytokines measured by ELISA (R&D Systems).

Immunohistochemistry

Tissue from CL lesions were fixed in buffered formaldehyde and embedded in paraffin. Immunohistochemistry reactions were performed as previously described [2, 19]. In brief, the reactions were performed after peroxidase activity blockade with 3% hydrogen peroxide for 10 minutes and proteins with Protein Block Serum-Free (DAKO, Carpinteria, CA) for 15 minutes. The slides were incubated overnight at 4°C with the antibodies Monoclonal Mouse CD56 (NK cell marker) (Thermo Fisher Scientific, Waltham, MA), dilution 1:200. A mouse and rabbit peroxidase kit/horseradish peroxidase KP500 (Diagnostic BioSystems, Pleasanton, CA) was used to perform the reaction according to the manufacturer's recommendations. Positive cells were defined by the chromogenic substrate, 3,3'-diaminobenzidine ([DAB] eBioscience). In all reactions, a preselected pattern section was used as positive control, and a section that had not been incubated with the primary antibody was used as a negative control.

Statistical Analysis

The Mann-Whitney test was used for comparisons between 2 independent continuous variables, Wilcoxon *U* test was used

for continuous dependent variables, and the Kruskal-Wallis test and Dunn's posttest were used to compare 3 continuous variables. Pearson's or Spearman's rank correlation tests were used for correlation analysis. $P < .05$ was considered statistically significant, and all P values represented are 2-tailed.

RESULTS

Subsets of Cytotoxic Cells in Peripheral Blood

Considering that different cell types can exert cytotoxic activity, subsets were characterized according to CD56, CD16, CD8, and CD3 expression. We performed an ex vivo analysis of PBMC from HS and CL patients by flow cytometry and identified 4 different cytotoxic cell subsets: NK cells ($CD56^+CD3^-CD8^-CD16^{bright}$), $CD8^{dim}$ NK cells ($CD56^+CD3^-CD8^{dim}CD16^+$), NKT-like cells ($CD56^+CD3^+CD8^{bright}CD16^{dim}$), and $CD8^+$ T cells ($CD56^-CD3^+CD8^{bright}CD16^-$) (Figure 1A). We then compared the frequency of each cell type among HS and CL patients. Patients with CL were found to exhibit significantly increased frequencies of NK cells, as well as fewer $CD8^+$ T cells, compared with HS (Figure 1B). These data suggest either the proliferation of NK cells in peripheral blood or the migration of $CD8^+$ T cells to the site of inflammation.

Cytotoxic Cells Expressing Granzyme B, Perforin, Tumor Necrosis Factor, and Interferon-γ in Peripheral Blood

To evaluate whether subsets of cytotoxic cells contribute to the production of cytokines, PBMCs from HS and CL patients were cultured in the presence or absence of SLA for 16 hours. None of the HS cell populations studied produced significant levels of TNF upon stimulation with SLA (Figure 2A). In contrast, higher frequencies of NK ($CD56^+CD8^-CD3^-$), $CD8^{dim}$ NK ($CD56^+CD8^{dim}CD3^-$), and NKT-like ($CD56^+CD8^{bright}CD3^+$) cell subsets expressing TNF were observed in comparison to $CD8^+$ T cells ($CD56^-CD8^{bright}CD3^+$) in CL patients (Figure 2A). Interferon-γ is the main cytokine that activates macrophages to kill *Leishmania* parasites. The frequencies of subsets of NK ($CD56^+CD8^-CD3^-$) and $CD8^{dim}$ NK ($CD56^+CD8^{dim}CD3^-$) cells expressing IFN-γ were higher than that of $CD8^+$ T cells in both HS and CL patients (Figure 2B). When we compared the IFN-γ expression between HS and CL patients, we did not find any difference (Figure 2B). These data indicate that NK cells may participate in the pathogenesis of CL through the production of TNF. In addition, NK cells may be more important than $CD8^+$ T lymphocytes with respect to parasite killing, due to the increased IFN-γ production seen in these cells. Other possible cytotoxic cells were not included in this analysis.

Granzymes and perforin are effector molecules produced by NK cells and $CD8^+$ T lymphocytes [20]. We evaluated the frequency of cytotoxic cell subsets expressing granzyme B and perforin in the peripheral blood of HS and CL patients. In CL patients, 2 subsets of NK cells ($CD56^+CD8^-$ and $CD56^+CD8^{dim}$) were found to express significantly more granzyme B and perforin than $CD8^+$ T cells (Figure 2C and D). In addition, when we compared the expression of granzyme B among HS and CL patients, decreased granzyme B expression was found in $CD8^+$ T cells from CL patients (Figure 2C).

NKG2D$^+$CD4$^+$ T Cells Produce Interferon-γ in Patients With Cutaneous Leishmaniasis

$CD4^+$ T cells can exert cytotoxicity through the expression of NKG2D, an activating receptor of these cells that is also expressed by cytotoxic effector NK and $CD8^+$ T cells. This receptor binds to MICA/B surface receptors on target cells [21]. We first determined the frequencies of NK, $CD8^+$, and $CD4^+$ T cells expressing NKG2D in PBMCs from HS and CL patients. Our results show that the frequency of

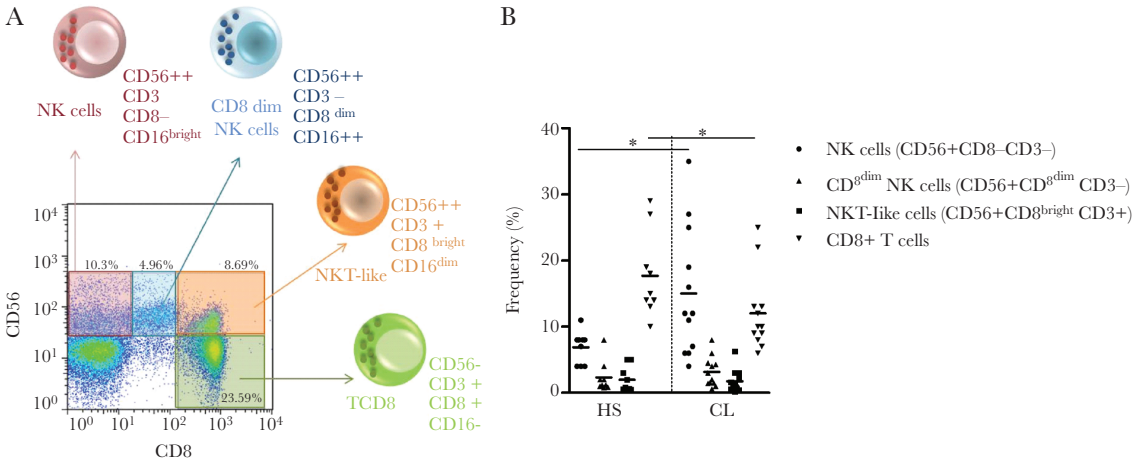

Figure 1. Cytotoxic cell subsets in peripheral blood. (A) Representative plot showing cytotoxic cell subsets in peripheral blood based on expression of CD56, CD8, CD16 and CD3. (B) Frequency of cytotoxic cell subsets in peripheral blood of healthy subjects (HS) (N = 9) and cutaneous leishmaniasis (CL) patients (N = 12). Mann-Whitney test was used to compare cytotoxic cell subsets between HS and CL. *, $P < .05$.

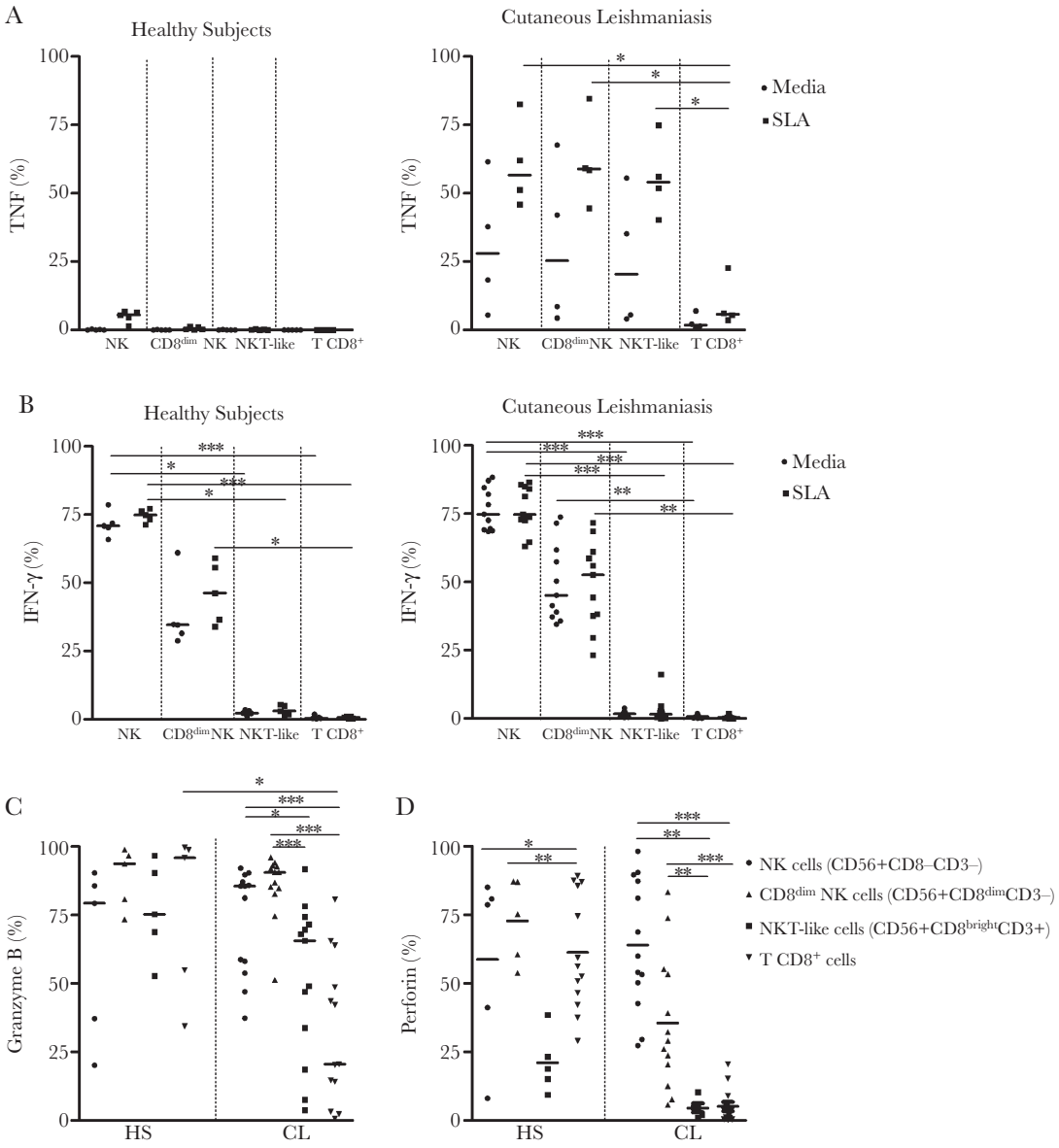

Figure 2. Cytotoxic cells expressing interferon (IFN)-γ, tumor necrosis factor (TNF), granzyme B, and perforin in peripheral blood. Peripheral blood mononuclear cells were cultured in presence or absence of soluble *Leishmania* antigen (SLA) for 16 hours, then TNF and IFN-γ was intracellular stained in cytotoxic cell subsets. Frequency of granzyme B and perforin ex vivo expression was determined by intracellular staining in cytotoxic cell subsets. (A) Frequency of cytotoxic cell subsets expressing TNF in healthy subjects (HS) (N = 5) and cutaneous leishmaniasis (CL) patients (N = 4). (B) Frequency of cytotoxic cell subsets expressing IFN-γ in HS (N = 5) and CL patients (N = 11). (C) Frequency of granzyme B-producing cells in subsets of cytotoxic cells in HS (N = 5) and CL patients (N = 13). (D) Frequency of perforin-producing cells in subsets of cytotoxic cells in HS (N = 5) and CL patients (N = 13). The Wilcoxon test was used to compare results of different conditions in the same subset. The Mann-Whitney test was used to compare cytotoxic cell subsets between HS and CL. The Kruskal-Wallis test was used for comparisons of means between subsets of cytotoxic cells, Dunn's posttest. *, $P < .05$; **, $P < .005$; ***, $P < .0001$.

NK cells expressing NKG2D was significantly higher than that of $CD8^+$ or $CD4^+$ T cells. Moreover, we did not find any difference in the frequency of $CD4^+$ T cells expressing NKG2D between HS and CL patients (Figure 3A). Our current and previous data show a role for $CD8^+$ T and NK cells in cytotoxicity in CL. Since upon SLA stimuli no change in the expression of NKG2D was documented, the data suggest that cytotoxicity mediated by $CD8^+$ T and NK cells may occur in an NKG2D-independent manner. We next investigated whether $CD4^+$ T cells expressing NKG2D produced granzyme B in response to SLA. Low expression of granzyme B was found in the absence or presence of SLA (Figure 3B). Finally, we evaluated the production of IFN-γ by $NKG2D^+CD4^+$ T cells and documented that the addition of SLA-induced $NKG2D^+CD4^+$ T cells to produce significantly more IFN-γ (Figure 3C).

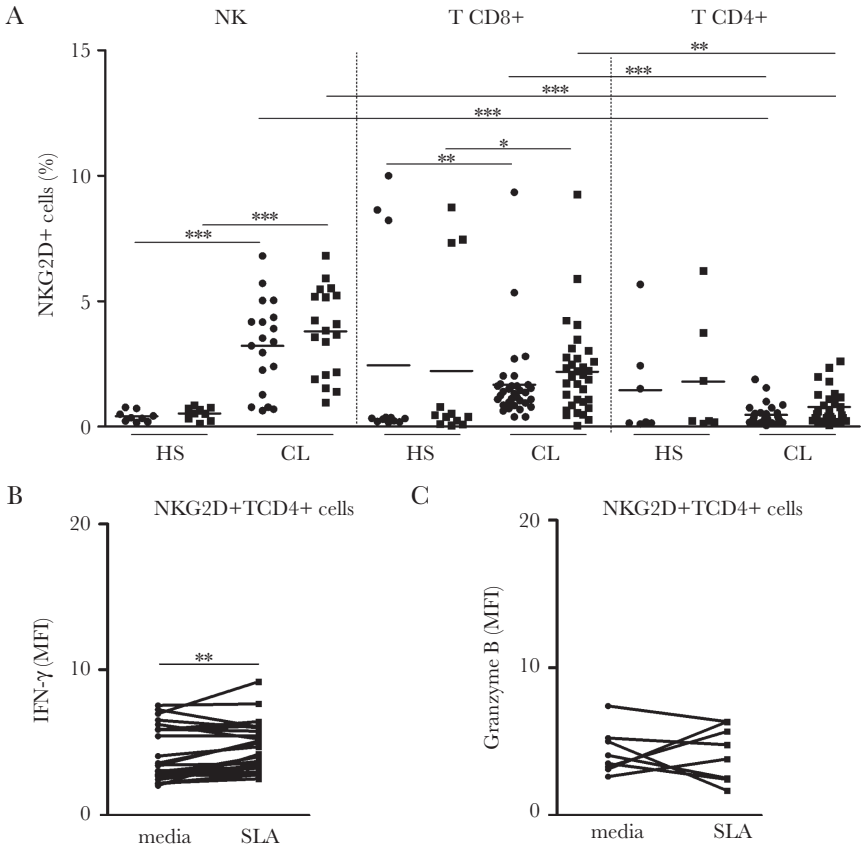

Figure 3. NKG2D$^+$CD4$^+$ T cells produce interferon (IFN)-γ in patients with cutaneous leishmaniasis (CL). Peripheral blood mononuclear cells were cultured in presence or absence of soluble *Leishmania* antigen (SLA) for 16 hours, then granzyme B and IFN-γ were assessed by intracellular staining in NKG2D subsets. (A) Frequency of NKG2D in natural killer (NK) cells (healthy subjects [HS], N = 9; cutaneous leishmaniasis [CL], N = 19), CD8$^+$ T cells (HS, N = 12; CL, N = 33), and CD4$^+$ T cells (HS, N = 7; CL, N = 29) from peripheral blood of HS and CL patients. (B) Mean fluorescence intensity (MFI) of granzyme B in NKG2D$^+$CD4$^+$ T cells from CL patients (N = 9) with and without stimulus with SLA. (C) The MFI of IFN-γ in NKG2D$^+$CD4$^+$ T cells from CL patients (N = 29) with and without stimulus with SLA. Kruskal-Wallis test was used for comparisons of means between NK cells, CD8$^+$ T cells, and CD4$^+$ T cells expressing NKG2D. Wilcoxon test was used for comparisons of means before and after stimulus. *, $P < .05$; **, $P < .005$; ***, $P < .0001$.

Natural Killer Cells Produce Granzyme B and Enhance Cytotoxicity in Cutaneous Leishmaniasis Lesions

Extracellular granzyme B has been found in the extracellular matrix of tissues, plasma, and exudate of chronic wounds [22, 23]. In this study, we evaluated the production of extracellular granzyme B in cultures of normal HS skin biopsies and CL lesions without stimuli. Figure 4A illustrates the high levels of extracellular granzyme B observed in CL patient cultures compared with HS.

CD107a, a lysosomal membrane glycoprotein, is expressed on the surface of cytotoxic cells after activation [24, 25]. Figure 4B details the gate strategy used to assess the expression of CD107a in CD56$^+$ and CD8$^+$ cells. We first gated on lymphocyte population (forward scatter and side scatter), and then on CD56$^+$ or CD8$^+$ cells. To confirm the presence of NK cells in CL lesions, we also performed immunohistochemistry for CD56 (Figure 4C). No differences in the frequency of CD8$^+$ T cells and NK cells expressing CD56 were detected in CL lesions; however, the frequency of degranulating NK cells was significantly higher than the frequency of degranulating CD8$^+$ cells in CL lesions (Figure 4D and E). Thus, although present in similar numbers in CL lesions, NK cells were found to be 7-fold more cytotoxic than CD8$^+$ T cells in CL.

Granzyme B Induces Tumor Necrosis Factor Production and Is Associated With Disease Severity

Extracellular granzymes may induce the production of a variety of cytokines and promote tissue damage, thereby delaying tissue repair [23, 26, 27]. Because the participation of TNF in the immunopathology of *L braziliensis* infection has been well established, we decided to evaluate the contribution of granzyme B with regard to the production of this cytokine. We observed that in vitro inhibition of granzyme B significantly decreased TNF levels in PBMCs from CL patients (Figure 5A). Then, we decided to investigate the contribution of granzyme B to the production of other inflammatory molecules. We found that blockade of granzyme B also decreased the production of IL-6 and CXCL-10 in PBMCs of CL patients (Figure 5A). We also investigated associations between extracellular granzyme B and CL

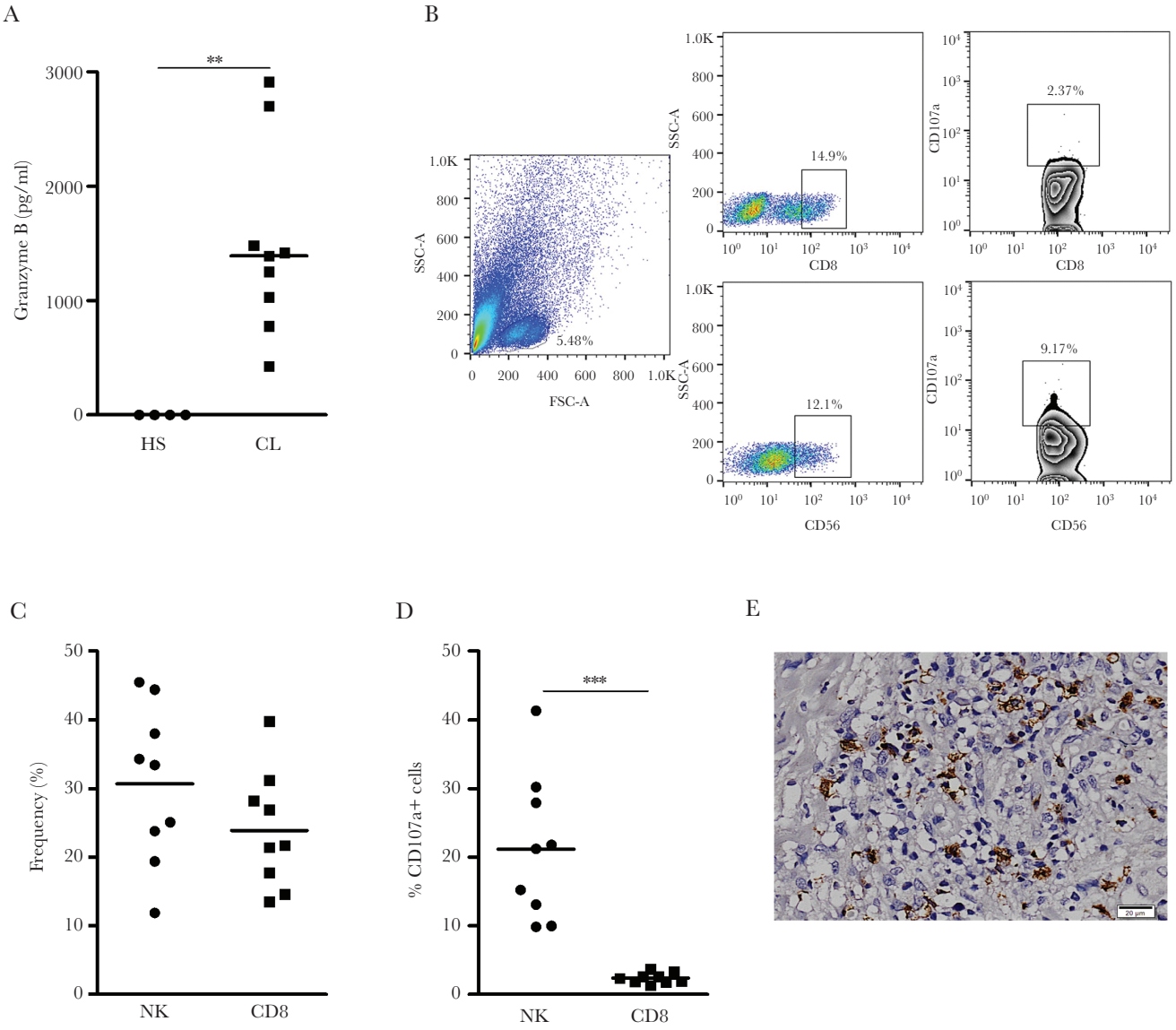

Figure 4. Granzyme B production and natural killer (NK) cell degranulation in lesions of patients with cutaneous leishmaniasis (CL). (A) Biopsies of normal skin from healthy subjects (HS) (N = 4) and of lesions from CL patients (N = 9) were cultured for 12 hours without stimuli, and levels of granzyme B were determinated by enzyme-linked immunosorbent assay. (B) Flow cytometry gate strategy for determination of CD107a expression in NK cells and $CD8^+$ T cells on lesions of CL patients. (C) Frequency of NK cells and $CD8^+$ T cells in lesions of CL patients (N = 9). (D) CD107a expression on NK cells and $CD8^+$ T cells on lesions of CL patients (N = 9). (E) Immunostaining for NK cells in tissue of lesion of CL patients. Slides were photographed (10 randomized fields from each section). Original magnification, ×20. Scale bar = 20 µm. Mann-Whitney test was used to compare different groups. **, $P < .005$; ***, $P < .0001$.

lesion development. A positive correlation was found when analyzing granzyme B levels in cultures of CL lesion biopsies and lesion size (Figure 5B). Moreover, there were no association between granzyme B levels on supernatants of lesions and Leishmanin skin test or days of illness of CL patients. These data, taken together, indicate that extracellular granzyme B is a potent inducer of inflammation and may participate in the pathogenesis of CL.

DISCUSSION

Natural killer cells play an important role in innate immunity and induce apoptosis in infected cells by cytotoxicity [28, 29]. In this study, we investigated the contribution of NK cells in the pathogenesis of *L braziliensis* infection and observed that these cells increase inflammation and, possibly, exacerbate disease in CL patients.

Cytotoxic activity can be triggered by various cell types [20]. Because some NK ($CD3^-$) cells express the CD8 molecule [30, 31], we investigated the frequency of cytotoxic cell subsets in the peripheral blood of CL patients. We found that the $CD8^{dim}$ NK cells ($CD56^+CD16^+CD8^{dim}CD3^-$) subset expresses the CD8 molecule, in addition to the NKT-like cell subset ($CD56^+CD16^{dim}CD8^{bright}CD3^+$). The expression of this molecule in NK cells has

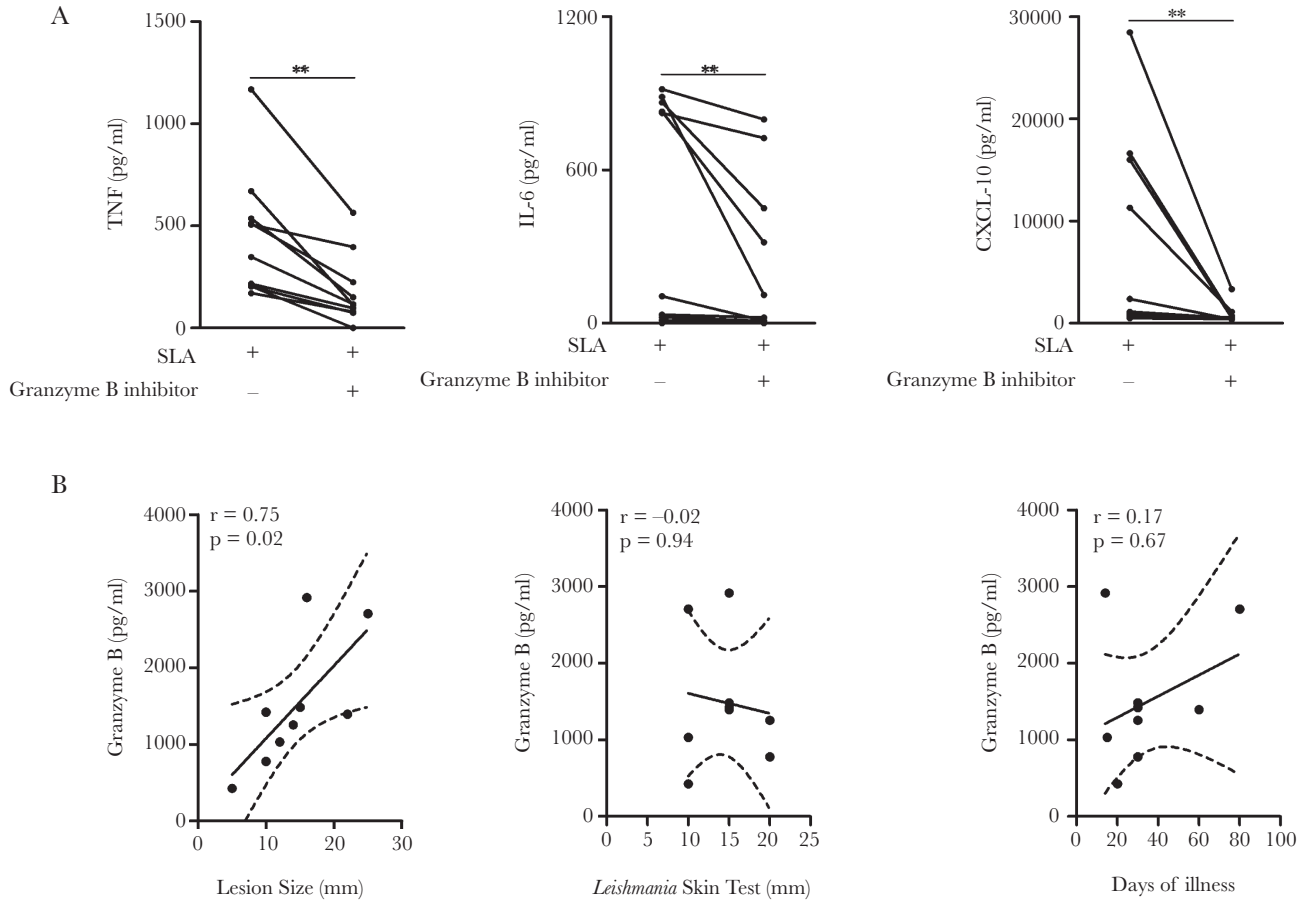

Figure 5. Granzyme B induces tumor necrosis factor (TNF) production and is associated with severity of disease. (A) Peripheral blood mononuclear cells from cutaneous leishmaniasis (CL) patients (N = 10) were stimulated with soluble *Leishmania* antigen (SLA) in the presence or absence of granzyme B inhibitor for 72 hours, and levels of tumor necrosis factor (TNF), interleukin-6 (IL-6), and CXCL-10 were assessed by enzyme-linked immunosorbent assay. (B) Correlation analysis between granzyme B levels on supernatants of lesions, and lesion size, Leishmanin skin test, and days of illness of CL patients (N = 9). Statistical comparison was performed by Spearman's rank correlation test according to nonparametric distribution of samples. Statistical significance, $P < .05$.

been associated with increased cytotoxicity, because it has been demonstrated that NK cells expressing CD8 survive after lysing target cells [30]. Another surface molecule used for the identification of NK cells is CD16, a low-affinity Fc receptor that confers an antibody-dependent cytotoxicity ability to these cells [32]. We observed that 2 subsets of NK cells, $CD8^-$ and $CD8^{dim}$, express CD16. The participation of $CD8^+$ T cells in the pathogenesis of CL has been well studied [6–8, 10, 11, 33, 34], yet little is known about the participation of NK cells in the immune response of CL patients. Because a moderate frequency of NK cells expressing CD8 was observed herein, we believe that, to some extent, the role attributed to $CD8^+$ T cells in the inflammatory response may also be performed by NK cells in CL patients. However, future functional analysis should be performed to determine the contribution of $CD8^+CD56^+$ cells with respect to tissue damage in CL.

Natural killer cells and $CD8^+$ T cells produce proinflammatory cytokines, as well as granzyme and perforin, in response to parasite infection [35]. Our data show that the peripheral blood of CL patients presents an increased frequency of NK cells, and a lower frequency of $CD8^+$ T cells, when compared with HS. One potential explanation for the reduced numbers of $CD8^+$ T cells found is sequestration occurring at the lesion site [8, 11, 36]. An enrichment of CD8 cells in lesions [37] in comparison to the blood [38] were previously shown. The increased frequency of NK cells seen in blood may be associated not only with cytotoxicity, but also with the production of cytokines, such as IFN-γ and TNF, which contribute to macrophage activation [39–41]. In this study, we documented that SLA does not induce IFN-γ production by NK and $CD8^+$ T cells. We have previously shown that, although $CD4^+$ T cells are the main source of IFN-γ, $CD8^+$ T cells contribute to immunopathology in CL patients [11]. It is interesting to note that most NK cells produce IFN-γ with or without stimuli. However, in this case, IFN-γ production may not reflect secretion, because IFN-γ is absent in culture supernatants from HS [42].

Proinflammatory cytokines, such as TNF, are known to induce tissue damage, thereby contributing to the pathology of CL [3, 4, 10]. In this study, we observed that NK cells and NKT-like cells from CL patients produce more TNF compared with $CD8^+$

T cells. Other cell types can also contribute to the production of TNF in CL, because it was previously demonstrated that intermediate monocytes ($CD14^{++}CD16^{+}$) produce TNF in CL patients [43]. We also observed that TNF induces the production of matrix metallopeptidase 9, an enzyme capable of degrading the basement membrane, which contributes to the pathogenesis of CL [44]. Thus, the fact that a high percentage of NK cells produce TNF in CL patients leads us to suggest that these cells may play an important role in ulcer development in CL.

A subset of $CD4^{+}$ T lymphocytes expressing NKG2D possess the ability to trigger cytotoxicity and has been reported as deleterious in autoimmune diseases [45–48]. In this study, we investigated whether this subset of $CD4^{+}$ T cells could contribute to the cytotoxic activity observed in CL patients, and we found that these cells mainly produce IFN-γ. This indicates that although $CD4^{+}$ T cells are able to perform cytotoxic functions, the low expression of NKG2D by these cells, coupled with their ability to produce IFN-γ, suggests that the role played by $CD4^{+}$ T cells is protective rather than pathologic.

The present study observed that 2 NK cell subsets express significantly more granzyme B and perforin than $CD8^{+}$ T cells from CL patients. In addition, $CD8^{+}$ T cells from CL patients also showed decreased granzyme B expression compared with HS. The increased production of granzyme B and perforin in NK cells found herein suggests that these cells not only act rapidly during infection, but they may also exert more cytotoxic activity than $CD8^{+}$ T cells in the context of *L braziliensis* infection.

Several studies have pointed to the nonapoptotic roles of granzyme B, such as the activation of proinflammatory cytokines and extracellular matrix degradation, which leads to inflammation and delayed wound healing [23, 27, 49]. In this study, we found high levels of extracellular granzyme B in CL lesions and a strong positive correlation between extracellular granzyme B and lesion size. Faria et al [7] observed that the frequency of $CD8^{+}$ granzyme B^{+} T cells is associated with the intensity of inflammation in CL patient ulcers. Furthermore, a positive correlation between the frequency of $CD8^{+}$granzyme B^{+} T cells and lesion size in CL patients was also observed [11]. We previously detected the presence of granzyme B in early stage lesions of CL patients, indicating a possible role for granzyme B in the pathogenesis of CL [33].

In this study, our observation of extracellular granzyme B in the lesions of CL patients led us to evaluate the contribution of granzyme B in the inflammatory response. We found that granzyme B influenced the production of proinflammatory cytokines and chemokines, because the neutralization of this protease resulted in downregulated TNF, IL-6, and CXCL-10 production in CL patients. Other authors have similarly shown that proteolytic activity by granzyme B induced the secretion of proinflammatory cytokines IL-18 and IL-1α; however, the mechanism underlying the observed enhancement in TNF secretion by granzyme B remains known [23, 26, 27]. In conclusion, our findings serve to corroborate the available data in the literature and suggest that granzyme B may indeed contribute to an enhanced inflammatory response, which induces lesion development in CL.

CD107a is transported to the surface of degranulating cells when cytotoxic cells interact with target cells, leading to granzyme and perforin release [24, 50]. In the present study, we demonstrated that the cytotoxic activity observed in CL lesions primarily arose from NK cells, which points to an important role played by these cells in the immunopathogenesis of CL. In a study developed in another endemic area for leishmaniasis, Ferraz et al [13] characterized cytotoxic subpopulations in CL lesions and found that NKT cells, $TCD4^{+}$ cells, and double-negative cells presented the highest percentage of CD107a cells in comparison to NK, $CD8^{+}$ T, and double-positive cells. As we only assessed cytotoxicity of NK and $CD8^{+}$ T cells within the lesions of CL patients, it is possible that other cells may also contribute to cytotocic activity in CL patients.

CONCLUSIONS

In summary, we observed that NK cells are present and exert cytotoxicity in CL lesions. These cells were found to not only express more granzyme B, but also produced more TNF than $CD8^{+}$ T cells, which importantly contributes to the immunopathology of CL.

Notes

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