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Characterization of the T-Cell Receptor Vβ Repertoire in the Human Immune Response against *Leishmania* Parasites

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In order to explore a possible presence of hyperreactive T-cell clones in human cutaneous leishmaniasis (CL), we have investigated, by flow cytometry, the expression of Vβ chains of T-cell receptors (TCRs) in the following types of cells: (i) peripheral blood mononuclear cells (PBMCs) from CL patients, which were then compared to those from normal volunteers; (ii) unstimulated and soluble *Leishmania* antigen-stimulated draining lymph node cells from CL patients; (iii) PBMCs from volunteers before versus after *Leishmania* immunization; and (iv) PBMCs from healthy volunteers that were primed in vitro with live *Leishmania* parasites. Our results show a modulation in the TCR Vβ repertoire during CL and after antigen stimulation of patients' cells. Vaccination, however, leads to a broad expansion of different Vβ TCRs. We also observed an association between TCR Vβ12 expression, T-cell activation, and gamma interferon production upon in vitro priming with *Leishmania*. Collectively, these results both indicate that infection with live parasites or exposure to parasite antigen can modulate the TCR Vβ repertoire and suggest that TCR Vβ12 may be implicated in the response to *Leishmania*.

Leishmania is an intracellular protozoan parasite that infects humans and causes a wide spectrum of diseases known as leishmaniases. Leishmaniases are endemic in 88 countries, where 350 million people live at risk of infection, and these diseases cause an estimated disease burden of over 2 million disability-adjusted life years (http://www.who.int/tdr/diseases/leish/files/leish-poster.pdf.). In South America, leishmaniasis is caused by a number of different species, among which *Leishmania amazonensis* is an important etiologic agent (19) and may be involved in diverse clinical presentations (4). Cutaneous leishmaniasis (CL) is clinically characterized by the presence of a delimited ulcer with elevated borders and a sharp crater that heals after specific chemotherapy (6). In some cases, satellite lymphadenopathy is observed (2) and may be the first manifestation of the disease (3, 50).

Anti-*Leishmania* cell-mediated immunity (CMI) is present in the majority of CL patients, as evidenced by a positive delayed-type hypersensitivity (DTH) reaction, as well as by gamma interferon (IFN-γ) production by peripheral blood mononuclear cells (PBMCs) after in vitro restimulation with soluble *Leishmania* antigen (SLA). PBMCs of individuals vaccinated with *Leishmania* antigen produce IFN-γ and undergo $CD8^+$ T-cell expansion upon antigen stimulation in vitro (20, 30). Moreover, we have shown that in vitro priming responses can be used to predict the pace of IFN-γ production in individuals vaccinated with *Leishmania* (41). Evaluation of CMI in human leishmaniasis has focused mainly on cytokine production, and few studies have been performed to characterize the antigenic specificity or the functionality of T-cell populations. In an experimental model of CL, expansion of Vα8- and Vβ4-expressing T cells has been related to susceptibility in mice infected with *Leishmania major* (36, 43, 44).

T cells recognize foreign antigens through T-cell antigen receptors (TCRs), and diversity in antigen recognition is generated by α and β genes that rearrange randomly in differentiating T cells. In this sense, the study of the TCR repertoire may contribute to the understanding of disease pathogenesis and, for this reason, has been an important focus of research in several diseases (16, 22, 31, 35, 37, 38, 53). Either PCR-based methods (14, 46, 47) or flow cytometry using TCR Vβ-specific monoclonal antibodies (5, 8, 15, 27) has been employed to analyze the expression of TCR α and β variable genes. Moreover, studies of the TCR Vβ repertoire have also described the role played by microbial toxins or superantigens in activating the human immune system (9, 12, 27, 29). Superantigen stimulation of the immune system or stimulation by dominant antigens leads to the proliferation of specific T-cell populations followed by clonal deletion (18, 52).

Regarding human leishmaniasis, it is known that T cells play an important role in mediating CMI against the parasite, and it has been shown that the predominant T cell in CL lesions

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bears the αβ TCR (33, 34, 40). The TCR Vβ repertoire present in CL patients' lesions caused by *Leishmania braziliensis* was skewed compared to the repertoire detected in the blood (51). It was found that specific T-cell populations localized to CL lesions, and Vβ families 3, 6.6/6.7, and 7 were predominant in the lesions of 50% of patients studied. Since T cells are also involved in the pathogenesis of cutaneous leishmaniasis, it remains important to understand how infection leads to the expansion of the TCR Vβ repertoire and, particularly, which *Leishmania* antigens lead to this expansion. In the present work, we evaluated the TCR Vβ repertoire in CL patients, and we have compared these findings with those obtained following vaccination of healthy individuals and following in vitro priming with *Leishmania* parasites. Our aim was to investigate how T cells bearing the different Vβ families respond in such different settings.

MATERIALS AND METHODS

Study population. Patients ($n = 15$) presenting early signs of CL were recruited in Corte de Pedra (Buenos Aires, Brazil), an area where *L. braziliensis* infection is endemic. All patients were subjected to a complete physical examination as well as to clinical and laboratory evaluations. Infection with *Leishmania* was confirmed by a compatible epidemiological history, parasite isolation after culture in Novy-Nicolle-McNeal medium, positivity by the DTH skin test, or detection of circulating antibodies by immunofluorescence. Clinical characteristics of the patients (13 males and 2 females; age range, 15 to 48 years) included regional lymphadenopathy, considered one of the first clinical signs of infection with *L. braziliensis* (3), and the presence of a cutaneous lesion (mean lesion size of 7.6 mm ± 4.0 mm). Controls from the area of *L. braziliensis* endemicity ($n = 7$; six males and one female; age range, 15 to 48 years) were either CL patients' relatives or people who accompanied them. Controls were in the same age range as CL patients, did not show any sign of infection at the time of the study, and had negative serology for *Leishmania*. Vaccinated individuals ($n = 8$) from Fortaleza (Ceara, Brazil) had no previous history of leishmaniasis, a negative Montenegro skin test, and negative serology for *Leishmania*, Chagas' disease, and human immunodeficiency virus (41). Briefly, blood was collected before and after vaccination with two 1.5-ml doses (1,440 mg/dose; injected intramuscularly, with an interval of 21 days between doses) of a vaccine containing killed promastigotes of a well-defined World Health Organization *L. amazonensis* reference strain (IFLA/BR/67/PH8). The vaccine was produced by BIOBRAS, under good manufacturing practices, as described in reference 30. Healthy individuals ($n = 10$; eight males and two females; age range, 20 to 35 years), from Salvador (Buenos Aires, Brazil) had no previous history of leishmaniasis, a negative DTH skin test, and negative serology for *Leishmania*, Chagas' disease, and human immunodeficiency virus. Informed consent was obtained from all individuals enrolled. This study was approved by the ethics committee from Hospital Universitário Professor Edgar Santos (HUPES-UFBA) and by Hospital Universitário Walter Cantídio (HUWC-UFC).

Isolation of PBMCs. PBMCs from CL patients, controls from the area of *L. braziliensis* endemicity, and vaccinated volunteers were obtained by passage over a Ficoll-Hypaque gradient (Sigma). PBMCs were stained directly for ex vivo evaluation of TCR Vβ, CD4, and CD8 expression by flow cytometry or were used for in vitro priming.

Parasite and antigen. *L. amazonensis* MHOM/BR/87/BA125 was used for in vitro priming and antigen preparation. Details concerning isolation and characterization of this parasite strain have been described elsewhere (1). Parasites were cultivated in Schneider's medium (Sigma) supplemented with 5% fetal calf serum and gentamicin (50 μg/ml) (both from Invitrogen). In vitro stimulation of PBMCs was performed with stationary-phase promastigotes. SLA was prepared as described previously (42).

Lymph node aspiration and cell culture. Lymph nodes from CL patients were aspirated using a 19-gauge needle on a 10-ml syringe as described previously (2). Cells obtained by lymph node aspiration were stained directly for ex vivo evaluation of TCR Vβ, CD4, and CD8 expression by flow cytometry or washed three times and resuspended at a concentration of 3×10^6/ml in RPMI 1640 medium supplemented with 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml) (all from Invitrogen), and 10% human AB serum (Sigma). Cells were plated in 48-well plates (Costar) and incubated at 37°C in 5% CO_2 in the presence or absence of SLA (4 μg/ml) for 5 days. Cells were then collected and stained for evaluation of TCR Vβ, CD4, and CD8 expression by flow cytometry.

In vitro priming with *Leishmania*. In vitro priming was performed as described previously (7), with modifications. PBMCs were obtained as described above, and cells were washed and resuspended in supplemented RPMI 1640 medium at a concentration of 5×10^6 cells/ml. From this suspension, a total of 10^7 cells was used for the first stimulation. The remaining cells were plated in 24-well plates, and after 30 min, nonadherent cells were removed. Adherent cells were washed and cultivated in complete medium for 5 days to allow maturation into macrophages. These macrophages were used as antigen-presenting cells in the second round of stimulation. For the first stimulation, PBMCs (5×10^6 cells/ml) were cultivated in the presence or absence of *L. amazonensis* promastigotes (MHOM/BR/87/BA125) at 37°C in 5% CO_2 for 6 days. On day 5, mature macrophages were infected with live *L. amazonensis* promastigotes at a ratio of 10:1 for 24 h at 37°C in 5% CO_2. Cells recovered from the first stimulation were boosted (1×10^6 cells/ml) with the autologous *Leishmania*-infected mature macrophages in complete RPMI 1640 medium, supplemented with 10% supernatant harvested from the first stimulation, and were cultured for 4 days. Cells were then collected and stained for evaluation of TCR Vβ, CD25, CD4, and CD8 expression by flow cytometry.

Flow cytometry. Cells were stained, simultaneously, with antihuman surface CD4 (RPA-T4) and CD8 (RPA-T8) conjugated to phycoerythrin and peridinin chlorophyll protein, respectively (both from BD Biosciences), and anti-TCR Vβ2 (E22E7-2), Vβ3 (LE-89), Vβ5 (ER-2), Vβ6.1 (CR1034-3), Vβ8 (6C5), Vβ11(C21), Vβ12 (VER2-32), Vβ13 (JU-72), Vβ14 (CAL-1.3), Vβ16 (TAMA-1), Vβ17 (E17.5), Vβ20 (ELL-14), Vβ21.3 (IG-125), and Vβ22 (IMM-546) conjugated to fluorescein isothiocyanate (all from Immunotech). In some cases, cells were stained, simultaneously, with antihuman surface CD4 (RPA-T4) or CD8 (RPA-T8), antihuman interleukin-2R (IL-2R) conjugated to phycoerythrin (FTR-D4) (BD Biosciences), and TCR Vβ12 (VER2-32). Isotype controls were used as appropriate. For each sample, 20,000 events were analyzed using CELLQuest software and a FACSort flow cytometer (Becton Dickinson Immunocytometry).

Statistical analysis. Comparison of levels of TCR Vβ expression among CL patients as well as among controls was performed using the Kruskal-Wallis test, and comparison of TCR Vβ expression levels in the same individuals was performed using the Wilcoxon matched-pair test. Statistical analyses were performed using GraphPad for Windows (Prism Software).

RESULTS

TCR Vβ repertoires in PBMCs from CL patients and controls from the area of *L. braziliensis* endemicity. To begin assessing the TCR Vβ repertoire in CL patients from Brazil, we first examined whether infection with *Leishmania* led to modulation of Vβ expression. CL patients' and control individuals' (same age range and gender) PBMCs were stained ex vivo with a panel of monoclonal antibodies against the different Vβ families as well as for CD4 and CD8. As seen in Fig. 1, infection with *Leishmania* leads to a significant ($P < 0.05$) decrease in the expression of certain TCRs. This decrease is present in both $CD4^+$ (Fig. 1A) (TCRs Vβ5 and Vβ12) and in $CD8^+$ (Fig. 1B) T cells. In the latter, the decrease in TCR Vβ expression is more evident (TCRs Vβ11, Vβ12, Vβ13, and Vβ16).

Comparison of TCR Vβ repertoires in CL patients' PBMCs and draining lymph node cells. Since the frequency of expression of certain Vβ TCRs was significantly decreased in CL patients' PBMCs, we investigated whether this effect also extended to patients' draining lymph node cells. Cells from draining lymph nodes were collected by aspiration and were stained ex vivo as described above. In $CD4^+$ T cells, we observed no differences when comparing the TCR Vβ repertoires from draining lymph node cells and from PBMCs (Fig. 2A). In $CD8^+$ T cells, however, two Vβ families showed an increase in their expression (Vβ12 and Vβ16) and another two (Vβ14 and Vβ17) showed a decrease in their expression (Fig. 2B). There-

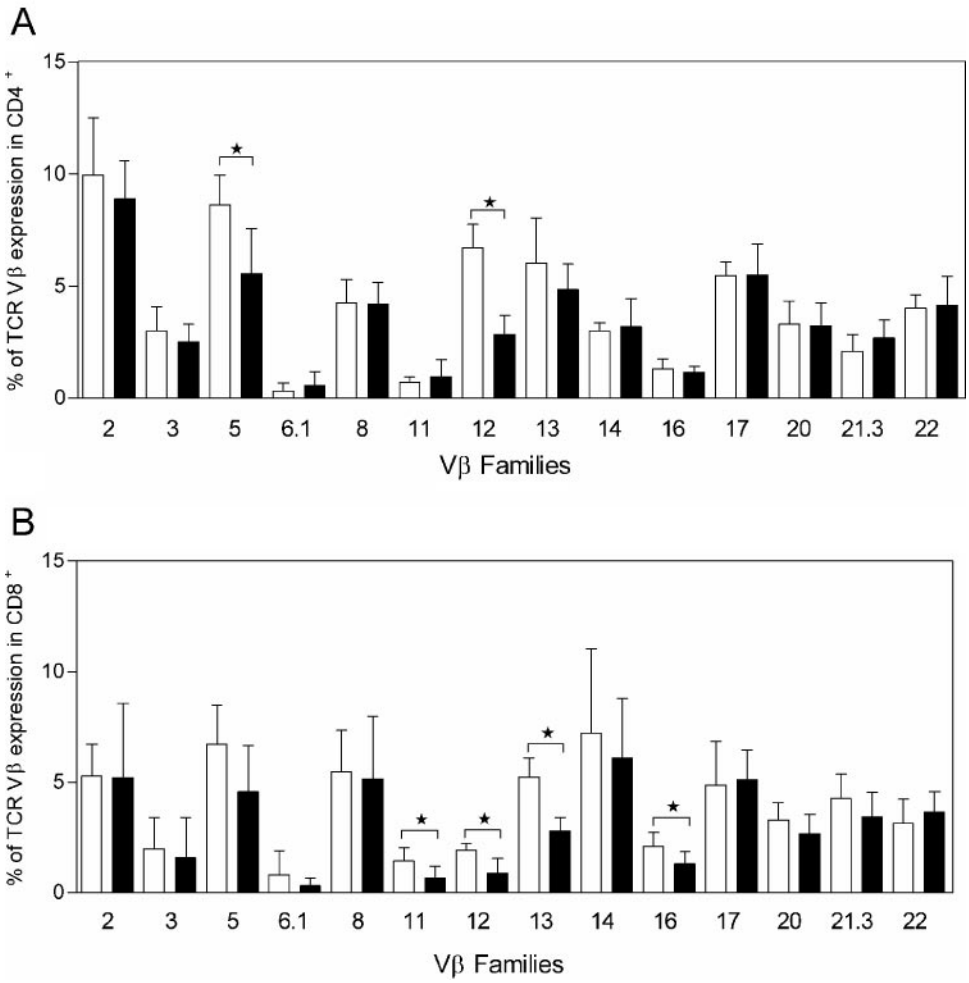

FIG. 1. Ex vivo analysis of the TCR Vβ repertoire in CL patients and controls. PBMCs from CL patients (filled bars) and from controls from the same area of *L. braziliensis* endemicity (open bars) were stained for $CD4^+$ (A) and $CD8^+$ (B) T cells and distinct TCR Vβ families. Data represent the percentages of cells with signals for the particular staining that were greater than the background signals established using isotype controls. The data represent the means and standard deviations of the means of results from 15 CL patients and 7 controls. ⋆, $P < 0.05$.

fore, differences in the TCR Vβ repertoires of CL patients' PBMCs and lymph node cells were restricted to the $CD8^+$ subset.

TCR Vβ repertoire in CL patients' lymph node cells following in vitro stimulation with SLA. Our ex vivo analysis showed that active CL leads to skewing of the TCR Vβ repertoire (Fig. 1). It also showed that the TCR Vβ repertoire of PBMCs differs from that of draining lymph node cells (Fig. 2), particularly for $CD8^+$ T cells. We then investigated whether CL patients' lymph node cells would respond to antigen stimulation and, if so, which Vβ TCRs would expand. Draining lymph node cells from CL patients were cultured in vitro in the presence of SLA and stained for TCR Vβ expression. When comparing the results, we observed that most Vβ TCRs were equally expressed in the presence or absence of antigen stimulation. However, some Vβ TCRs showed an increase in their frequency of expression, following antigen stimulation, in $CD4^+$ (Fig. 3A) and $CD8^+$ (Fig. 3B) T cells, indicating their proliferation in response to the presence of antigen. On the other hand, other Vβ TCRs showed a decrease in frequency of expression following antigen stimulation, in both $CD4^+$ (Fig. 3A) and $CD8^+$ (Fig. 3B) T cells. It is possible that this decrease results from apoptosis following antigen stimulation.

TCR Vβ repertoire following in vivo immunization with *Leishmania* antigen. Next, we examined whether in vivo immunization also led to modulation in TCR Vβ expression. Healthy volunteers were immunized twice over a 2-week interval with a vaccine produced from a reference *L. amazonensis* strain (41). TCR Vβ expression in volunteers' PBMCs was examined ex vivo before and 60 days after the last immunization. As expected, vaccination led to a broad expansion of the TCR Vβ repertoire (Fig. 4). A significant increase in the expression of many Vβ families was observed, both in $CD4^+$ (Fig. 4A) and in $CD8^+$ (Fig. 4B) T cells. However, for $CD4^+$ T cells, repertoire expansion was more evident, as seen by an increase in the expansion of eight different Vβ families (Fig. 4A), especially Vβ6.1, Vβ11, Vβ20, and Vβ21.3. In $CD8^+$ T cells, an increase in the frequency of expression was observed in three Vβ families (Fig. 4B).

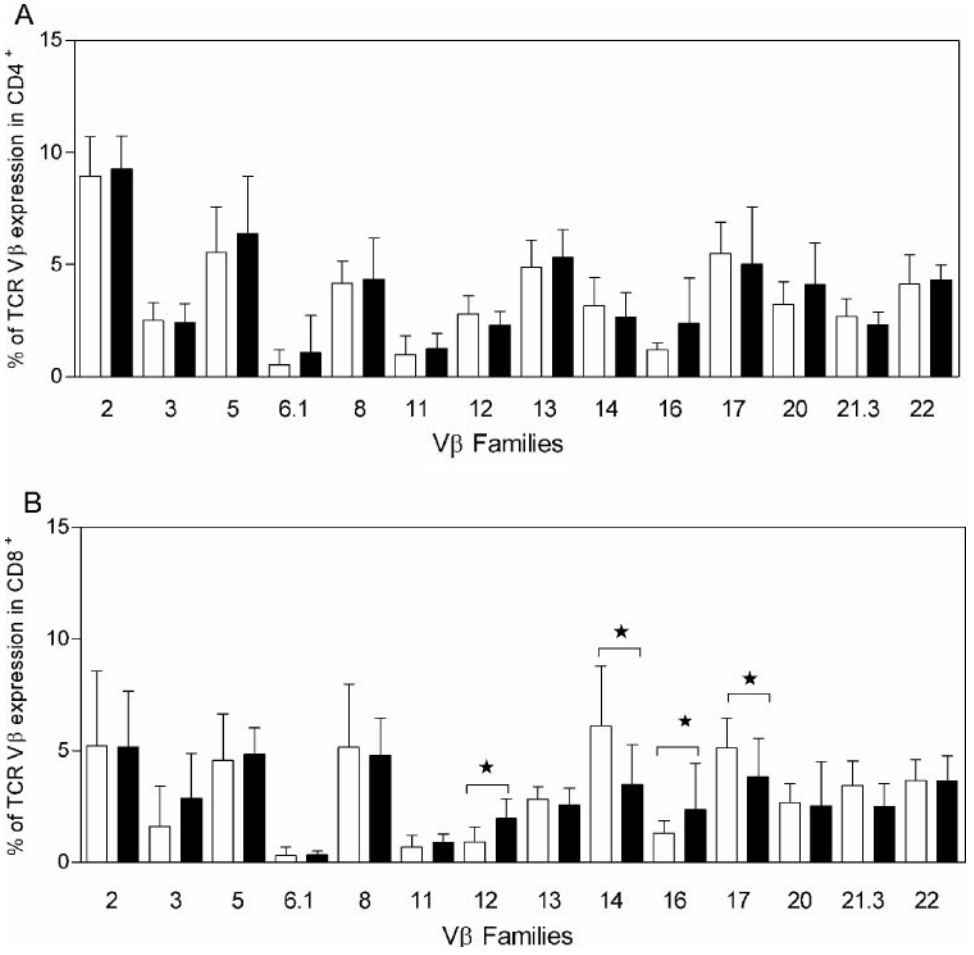

FIG. 2. Ex vivo analysis of the TCR Vβ repertoire in CL patients. PBMCs (open bars) and lymph node cells (filled bars) from CL patients were stained for $CD4^+$ (A) and $CD8^+$ (B) T cells and distinct TCR Vβ families. Data represent the percentages of cells with signals for the particular staining that were greater than the background signals established using isotype controls. The data represent the means and standard deviations of the means of results from 14 CL patients. ⋆, $P < 0.05$.

TCR Vβ12 expression and IFN-γ production following in vivo immunization. We have previously shown that healthy individuals can be categorized as either high IFN-γ producers (high cytokine production by PBMCs) or low IFN-γ producers (low cytokine production by PBMCs) upon in vitro priming of PBMCs with live *Leishmania* parasites (41). In the previous experiments, we observed a consistent modulation in the expression of TCR Vβ12. In fact, among all the Vβ TCRs tested, TCR Vβ12 was the only one for which a significant increase or decrease in expression was detected in all the settings tested. Therefore, taking into account our previous data on the amount of IFN-γ produced by healthy volunteers and the participation of TCR Vβ12 that we had observed so far, we probed for a possible association between TCR Vβ12 expression and IFN-γ production in vaccinated individuals. In order to do so, we assayed IFN-γ production before and after vaccination, and at these same time points, we evaluated TCR Vβ12 expression in PBMCs. Table 1 shows the IFN-γ production for eight vaccinated individuals. Before immunization, IFN-γ production following antigen stimulation was above 300 pg/ml, except in two volunteers (volunteers 1 and 6), who were therefore termed "low" producers. As shown in Fig. 5, TCR Vβ12 expression before vaccination was low, both for $CD4^+$ (Fig. 5A) and $CD8^+$ (Fig. 5B) T cells, for all eight individuals tested. Sixty days postvaccination, we observed that IFN-γ production was above 1,000 pg/ml except in volunteers 1 and 6 (Table 1). When we looked at TCR Vβ12 expression at this same time (Fig. 5), we observed that it increased after vaccination, and this increase was particularly evident in the six individuals for whom IFN-γ production was high (>1,000 pg/ml). This can be observed for both TCR Vβ12 $CD4^+$ (Fig. 5A) and TCR Vβ12 $CD8^+$ (Fig. 5B) cells. Therefore, an association between IFN-γ production and TCR Vβ12 expression exists, and it was not found for other TCRs tested (Vβ13, Vβ14, and Vβ16) (data not shown).

TCR Vβ12 expression following in vitro priming with *Leishmania*. Since we had observed an association between TCR Vβ12 expression and IFN-γ production, we then examined whether Vβ12 expression was also associated with cell activation as measured by IL-2R (CD25) expression. In order to do so, we selected 10 healthy individuals previously categorized as high IFN-γ producers and performed in vitro priming of their cells with live *Leishmania* parasites, as described above. Cells were then stained for the expression of either TCR Vβ12 alone

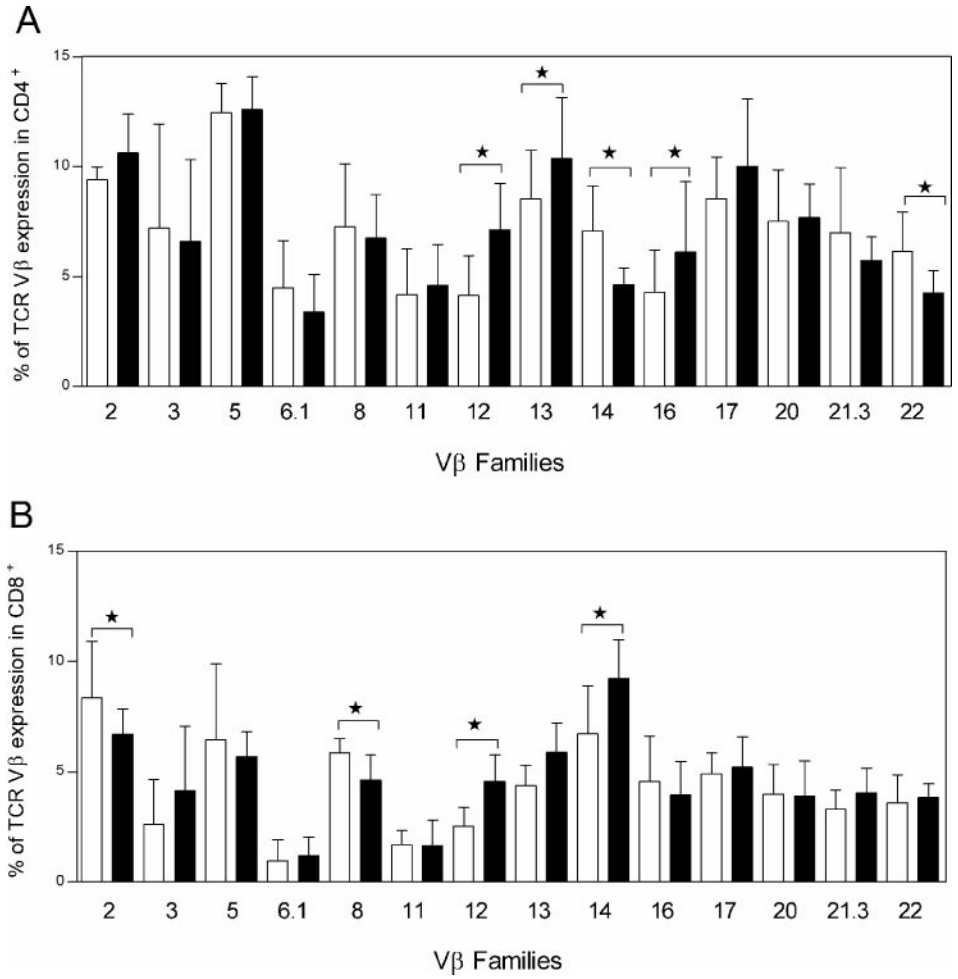

FIG. 3. TCR Vβ repertoire in CL patients after in vitro stimulation with SLA. Lymph node cells from CL patients were cultivated in vitro in the absence (open bars) or in the presence (filled bars) of SLA (4 μg/ml). Cells were stained for $CD4^+$ (A) and $CD8^+$ (B) T cells and distinct TCR Vβ families. Data represent the percentages of cells with signals for the particular staining that were greater than the background signals established using isotype controls. The data represent the means and standard deviations of the means of results from 12 CL patients. ⋆, $P < 0.05$.

or both TCR Vβ12 and IL-2R (CD25). As seen in Fig. 6A, in vitro priming with parasites leads to a significant increase in the expression of both TCR Vβ12 and IL-2R in $CD4^+$ T cells compared to that in unprimed cells. This same effect was also observed in $CD8^+$ T cells (Fig. 6B), indicating that TCR Vβ12-bearing cells expand and are activated in the presence of *L. amazonensis*. We performed this same experiment for the analysis of TCR Vβ2 and TCR Vβ5 expression, and no statistically significant differences were observed among cells primed or not primed with *L. amazonensis* parasites (data not shown).

DISCUSSION

The nature and function of the T-cell subsets clonally expanded as a result of infection have been an important focus of research in terms of disease pathogenesis. In the field of leishmaniasis research, the regulation of T-cell responses is of key importance in balancing disease cure or pathogenesis. Therefore, the identification of specific T-cell populations involved in the immune response continues to be an important goal in studying leishmaniasis. In the present report, we have attempted to define the TCR Vβ repertoire in response to *Leishmania* using flow cytometry and monoclonal antibodies against the different Vβ families of the TCR in both CD4- and CD8-positive lymphocytes. This investigation was conducted for different situations: during active CL with the use of patients' peripheral blood and draining lymph node cells, after vaccination, and after in vivo priming of cells from healthy volunteers. In all cases, we were able to identify specific $CD4^+$ and $CD8^+$ subpopulations, on the basis of TCR Vβ expression, that respond to parasite or antigen stimulation.

We began our study by examining the TCR Vβ repertoire in CL patients from an area in northeastern Brazil where *L. braziliensis* is endemic. Since differences in TCR Vβ repertoires based on DNA polymorphisms of the Vβ region have been reported for African Americans and Caucasians (13), we compared TCR Vβ usage in CL patients with that in healthy control individuals from the same area of endemicity, in order to minimize a possible bias in TCR Vβ usage due to regional differences. We found that active CL did not lead to overexpression or clonal deletion of any Vβ TCR. This finding rules

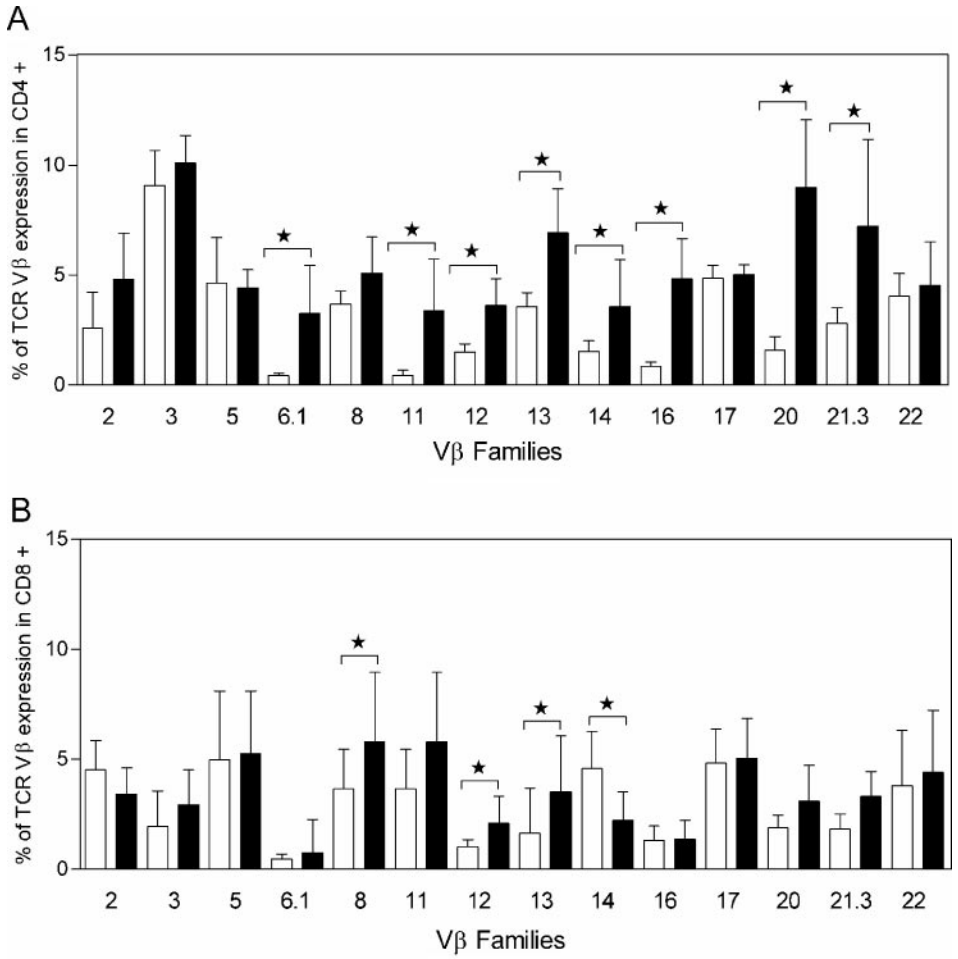

FIG. 4. Ex vivo analysis of the TCR Vβ repertoire in volunteers immunized with *Leishmania* antigen. PBMCs from volunteers were obtained before (open bars) and 60 days after (filled bars) the last immunization. Cells were stained for $CD4^+$ (A) and $CD8^+$ (B) T cells and distinct TCR Vβ families. Data represent the percentages of cells with signals for the particular staining that were greater than the background signals established using isotype controls. The data represent the means and standard deviations of the means of results from eight CL-vaccinated individuals. ⋆, $P < 0.05$.

out the possibility of superantigenic activity during cutaneous leishmaniasis, unlike what has been documented for mice infected with *Plasmodium yoelii* (39) and in humans infected with *Brugia* microfilariae (21). CL patients, however, did display a significant decrease in the expression of certain Vβ families in both $CD4^+$ and $CD8^+$ T cells from peripheral blood.

TABLE 1. In vitro IFN-γ production by PBMCs of healthy vaccinated individuals

Volunteer	Amt of IFN-γ/10^6 cells (pg/ml)[c]	
	Before vaccination	60 days after vaccination
1[a]	47.2	78.98
2[b]	300.56	1,000.25
3[b]	605.52	2,500.98
4[b]	588.65	4,987.25
5[b]	465.21	3,854.68
6[a]	35.36	90.58
7[b]	540.23	5,687.25
8[b]	490.23	4,125.58

[a] Low IFN-γ producer.
[b] High IFN-γ producer.
[c] Cytokine levels were determined by enzyme-linked immunosorbent assay before and 60 days after vaccination.

We then observed whether this decrease in TCR Vβ expression was confined to PBMCs or also extended to draining lymph node cells. Importantly, *L. braziliensis* infection is endemic in the area where patients were recruited, and as such, lymphadenopathy is commonly found as one of the first signs of infection (3). Surprisingly, when we compared cells from CL patients' peripheral blood with cells from draining lymph nodes, we found differences between these two compartments only in the $CD8^+$ subset. Concerning $CD8^+$ T cells, their role was first observed in mouse models of leishmaniasis (32), with which the ability of these cells to transfer DTH was demonstrated. Regarding human leishmaniasis, it was shown that higher proportions of *Leishmania*-reactive $CD8^+$ T cells are present after therapy (11) and in individuals immunized against CL (30). An enrichment of *Leishmania*-reactive $CD8^+$ T cells was observed in the inflammatory infiltrate of CL lesions, suggesting that the recruitment and/or proliferation of such cells could be favored by local immunoregulatory factors (10). $CD8^+$ T cells also play an important role in the initial

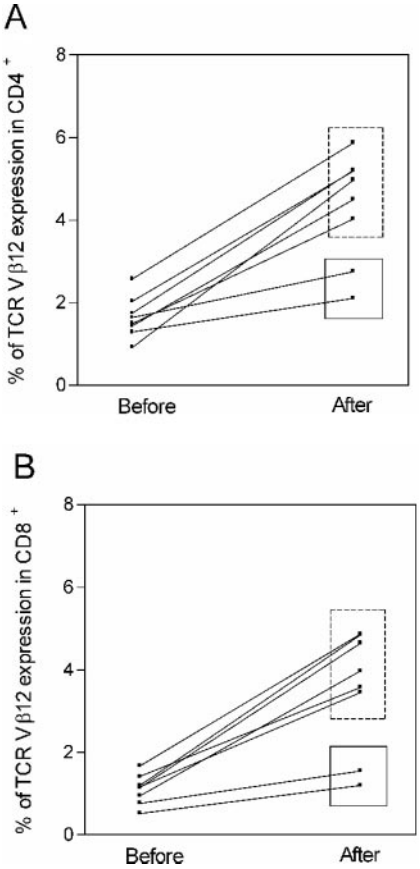

FIG. 5. Association between TCR Vβ12 expression and pace of IFN-γ production in volunteers immunized with *Leishmania* antigen. PBMCs from low producers (solid boxes) and from high producers (dashed boxes) were stained for $CD4^+$ (A) and $CD8^+$ (B) T cells and TCR Vβ12 expression before and after the last immunization. Data represent the percentages of cells with signals for the particular staining that were greater than the background signals established using isotype controls. PBMCs were obtained from eight vaccinated individuals.

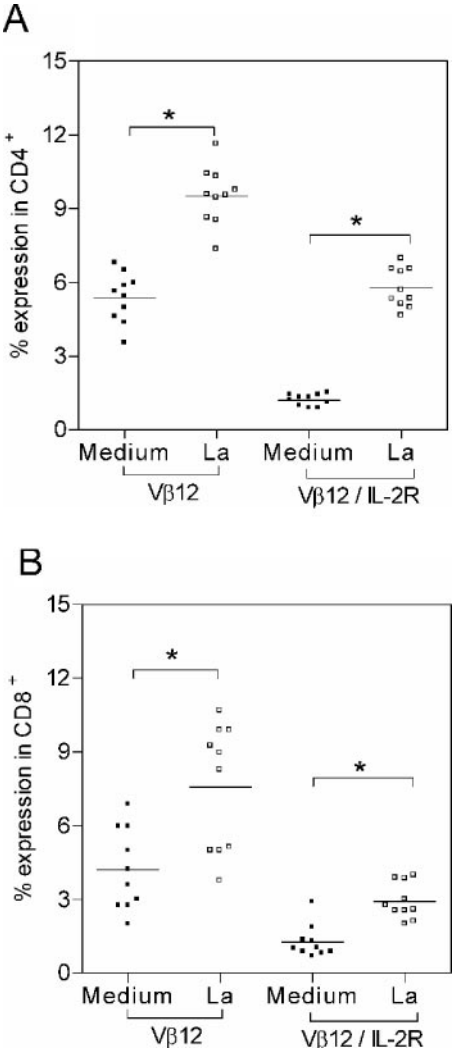

FIG. 6. TCR Vβ repertoire after in vitro priming with *L. amazonensis* promastigotes. PBMCs from healthy volunteers were primed in vitro with *L. amazonensis*. PBMCs were stimulated for 6 days with live parasites. Cells recovered from the first stimulation were boosted with *L. amazonensis*-infected macrophages and cultivated for 4 days. Unstimulated cells (filled squares) or *L. amazonensis*-stimulated cells (open squares) were then stained for $CD4^+$ T cells and TCR Vβ12 or TCR Vβ12 and IL-2R expression (A) or $CD8^+$ T cells and TCR Vβ12 or TCR Vβ12 and IL-2R expression (B). Data represent the percentages of cells with signals for the particular staining that were greater than the background signals established using isotype controls. Data were obtained from 10 healthy individuals. ⋆, $P < 0.001$.

human immune response against *Leishmania* (41). These cells may inhibit the early dissemination of parasites (24) and participate in the immune effector mechanism of parasite elimination through the production of cytokines and through cytotoxic activation (20). In this sense, differences in the TCR Vβ repertoires from $CD8^+$ T cells of draining lymph nodes may indicate which T-cell clones are modulated in response to *Leishmania*: the decrease in Vβ14 and Vβ17 expression in $CD8^+$ lymph node cells may reflect the sequestration of these T-cell clones in the lesion site, leading to their underrepresentation in draining lymph nodes, whereas the increase in Vβ expression (Vβ12 and Vβ16) in $CD8^+$ lymph node cells may reflect their role in the initial immune response to infection.

Since ex vivo staining of $CD8^+$ T cells from draining lymph nodes showed a modulation in Vβ expression, we investigated the effect of in vitro antigen stimulation on the expression of the TCR Vβ repertoire. In these experiments, we chose to use SLA derived from *L. amazonensis*, since the immune responses to *L. braziliensis* and *L. amazonensis* antigens are similar and since *L. amazonensis* is easier to cultivate (45). We observed that most Vβ TCRs were equally expressed in the presence or absence of antigen. The death of unstimulated clones was not pronounced; however, our analysis was based on the percentage of cells that expressed the different Vβ families, precluding any speculation on this finding. The decrease in TCR Vβ expression observed in this setting may be explained by an overt antigen stimulation leading to apoptosis; however, we also did not determine the number of apoptotic or viable cells after antigen stimulation. On the other hand, the increase in TCR Vβ expression also observed in this study indicates that certain T-cell populations are able to selectively proliferate in vitro under specific antigen conditions (23). Oligoclonal expan-

sion of T-cell clones has been documented in filariasis (17), Chagas'disease (31), and leishmaniasis (51). In the last study, it was shown that the Vβ repertoire was skewed in lesions, with an overrepresentation of Vβ3, Vβ6.6/6.7, and Vβ7. In our study, we did not find expansion of any of these particular TCRs after ex vivo analysis or in vitro antigen stimulation. In the work of Uyemura et al. (51), those T-cell clones for which a decrease in Vβ frequency was observed may have resulted from apoptosis due to antigen overstimulation.

We then examined the TCR Vβ repertoire after in vivo immunization with *Leishmania* antigen. The vaccine used in this study contained promastigotes of a reference *L. amazonensis* strain (41). In vivo immunization led to a consistent expansion of T-cell clones bearing different Vβ families, in both $CD4^+$ and $CD8^+$ T cells. It seems likely that this is due to the antigen mixture that was used as a vaccine. It was surprising, however, that the TCR Vβ repertoire was more diverse in $CD4^+$ T cells, with a striking expansion in the TCRs Vβ6.1, Vβ11, and Vβ20. In this vaccination experiment, ex vivo repertoire analysis was conducted 60 days after vaccination. It is possible that a similar expansion occurs during CL. However, since we cannot determine the exact time at which subjects from the area of endemicity are infected with the parasite, we also cannot analyze the TCR Vβ repertoire exactly 60 days after infection.

Upon their first encounter with *Leishmania*, healthy volunteers displayed differences in the amounts of IFN-γ produced following vaccination (41). We then looked for an association between this amount of IFN-γ produced and TCR Vβ expression in immunized volunteers. Interestingly, we found that high IFN-γ producers also showed an increased expression of Vβ12, in both $CD4^+$ and $CD8^+$ T cells, and no such association was found for other Vβ families. We next determined whether this association existed during *Leishmania* infection. To do so, we took advantage of the in vitro priming system (7, 48, 49). Priming of PBMCs from healthy volunteers (previously characterized as high IFN-γ producers) with live parasites led to a significant expansion of TCR Vβ12 T cells which, in turn, also expressed IL-2R. Since we did not evaluate intracellular cytokine production, we can only speculate that *Leishmania* infection leads to TCR Vβ12 T-cell activation and, possibly, IFN-γ production.

In the present study, out of all the Vβ families examined, the frequency of TCR Vβ12 cells was expanded in all situations tested, except in CL patients, in which it was decreased compared to the frequency in controls. Regarding Vβ12 specifically, it has been shown that staphylococcal enterotoxin B preferentially induces the production of Th1 cytokines in TCR Vβ12 T cells from healthy subjects and the production of Th2 cytokines in cells from atopic dermatitis patients (26). Also, an *Actinobacillus actinomycetemcomitans* extracellular preparation activated TCR Vβ12 T cells (54). The identification of similar antigens in *Leishmania* is still deficient, probably due to the complexity of infection. Nonetheless, it has been shown that the early burst of IL-4 associated with susceptibility to leishmaniasis in mice is produced by a restricted population of Vβ4 Vα8 $CD4^+$ T cells after interaction with the LACK antigen (25). Similarly, the glycolipid α-galactosylceramide is able to activate mouse NKT cells that express a TCR composed of an invariant Vα14-Jα18 chain paired, preferentially, with Vβ8.2 or Vβ7 chains (28). At this time we can only speculate that TCR Vβ12 cells play an important role during CL. Nevertheless, it was surprising that this particular TCR was able to respond to in vitro antigen stimulation, vaccination, and in vitro priming. Studies are now in progress to elucidate the role played by TCR Vβ12 cells in terms of the antigen they recognize during infection, whether these cells are present in lesions of CL patients, and, importantly, which cytokines they produce.

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