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Differences in Gamma Interferon Production In Vitro Predict the Pace of the In Vivo Response to *Leishmania amazonensis* in Healthy Volunteers

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The initial encounter of *Leishmania* cells and cells from the immune system is fundamentally important in the outcome of infection and determines disease development or resistance. We evaluated the anti-*Leishmania amazonensis* response of naive volunteers by using an in vitro priming (IVP) system and comparing the responses following in vivo vaccination against the same parasite. In vitro stimulation allowed us to distinguish two groups of individuals, those who produced small amounts of gamma interferon (IFN-γ) ($n = 16$) (low producers) and those who produced large amounts of this cytokine ($n = 16$) (high producers). IFN-γ production was proportional to tumor necrosis factor alpha and interleukin 10 (IL-10) levels but did not correlate with IL-5 production. Volunteers who produced small amounts of IFN-γ in vitro remained low producers 40 days after vaccination, whereas high producers exhibited increased IFN-γ production. However, 6 months after vaccination, all individuals tested produced similarly high levels of IFN-γ upon stimulation of their peripheral blood mononuclear cells with *Leishmania* promastigotes, indicating that low in vitro producers respond slowly in vivo to vaccination. In high IFN-γ producers there was an increased frequency of activated $CD8^+$ T cells both in vitro and in vivo compared to the frequency in low producers, and such cells were positive for IFN-γ as determined by intracellular staining. Such findings suggest that IVP responses can be used to predict the pace of postvaccination responses of test volunteers. Although all vaccinated individuals eventually have a potent anti-*Leishmania* cell-mediated immunity (CMI) response, a delay in mounting the CMI response may influence resistance against leishmaniasis.

T-cell-mediated immunity plays a central role in host responses to intracellular pathogens (18). Cytokines are central elements in the development of an immune response and have received a great deal of attention in both human and experimental leishmaniasis. Cures for leishmaniasis are related to the predominance of a Th1 response, since this leads to the production of gamma interferon (IFN-γ) and activation of parasite-infected macrophages (4). In contrast, a Th2 response with interleukin 4 (IL-4) and IL-10 production often results in disease progression (4). Previous efforts have focused on understanding the early events that influence the development of Th1 or Th2 cells.

The initial steps of human leishmaniasis cannot be examined in vivo due to ethical constraints and the difficulty in estimating the time of infection. Alternative approaches to understanding the key elements implicated in the initial responses to the parasite, which lead to human lymphocyte activity against *Leishmania* cells, include in vitro systems that mimic the initial infection or immunization of normal individuals. Shankar and Titus (32) developed an in vitro system using cells from lymphoid tissues of naive mice and *Leishmania major* promastigotes, which reproduced in vivo responses in murine leishmaniasis. In vitro systems for priming human naive cells against *Leishmania* have been developed for both *Leishmania amazonensis* (30, 31) and *L. major* (9, 19). Predominant development of Th1 or Th0 responses has been observed in these studies (9, 30), which probably reflects the great predominance of these responses in humans. The importance of IL-12 has also been confirmed for both species of *Leishmania* (9, 31). An open question is the relationship of such in vitro systems with human cells to in vivo responses in humans.

Production of IFN-γ by *Leishmania* antigen-stimulated peripheral blood mononuclear cells (PBMC) and expansion of the $CD8^+$ T-cell subset were also reported for individuals who responded to vaccination (23, 26). In anti-*Leishmania* vaccination studies in the New World, dead promastigotes have been used as the antigen, and protection has been induced in approximately 50% of the individuals vaccinated (2, 3). In the present study, we compared the initial human responses to *Leishmania* in in vitro (priming in vitro) and in vivo (response to vaccination) systems. We observed that the human immune response is different in different individuals after the first contact with *Leishmania* and that in vitro differences were similar to in vivo differences following vaccination. Our findings suggest that in vitro priming (IVP) responses can be used to predict the early postvaccination responses of test volunteers and that such a system mimics the initial human in vivo response to *Leishmania*.

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MATERIALS AND METHODS

Volunteers. Thirty-two healthy male volunteers between 18 and 40 years old were included in this study. All individuals participated in the study after informed consent was obtained, and the study was approved by the Committee of Ethics of Centro de Pesquisa Gonçalo Moniz. None of the volunteers had a previous history of leishmaniasis, and each of them had a proliferation index of ≤5 and a negative delayed-type hypersensitivity reaction against *Leishmania* (determined after blood collection). Serology results were negative for leishmaniasis, Chagas' disease, and human immunodeficiency virus. Before vaccination, blood was collected and lymphocytes were primed in vitro with *Leishmania*. After this the volunteers were vaccinated against *Leishmania*, and their immune responses were evaluated 40 days and 6 months after vaccination.

Parasite and antigen. *L. amazonensis* MHOM/BR/87/BA-125 was used for infection and for antigen preparation. Details concerning isolation and characterization of this strain have been reported previously (1). Parasites were cultivated in Schneider's medium (Sigma Aldrich, St. Louis, Mo.) supplemented with 5% fetal calf serum and 50 μg of gentamicin (GIBCO) per ml. In vitro stimulation of PBMC was performed with stationary-phase promastigotes, which were washed three times and resuspended in RPMI 1640 medium at the concentrations indicated below. Soluble *L. amazonensis* antigen (25 μg of protein per ml) was used for an intradermal delayed-type hypersensitivity test (27).

In vitro sensitization of human cells to *Leishmania*. In vitro sensitization to *Leishmania* was performed by using the protocol described by Brodskyn et al. (9), with some modifications. Briefly, PBMC were obtained by using a Ficoll-Hypaque gradient (Sigma Aldrich). The cells were washed three times, and the concentration was adjusted to 5×10^6 viable cells/ml in RPMI 1640 medium (Sigma Aldrich) supplemented with 2 mM L-glutamine (Sigma Aldrich), 10 mM HEPES (Sigma Aldrich), 50 μg of gentamicin (GIBCO) per ml, and 10% AB human serum (Sigma Aldrich). A total of 10^7 cells from this cell suspension were used for the first stimulation. The remaining cells were plated in 24-well plates, and 30 min later the nonadherent cells were removed. The adherent cells were washed and cultivated in complete medium and then allowed to mature into macrophages for 5 days in order to use them as antigen-presenting cells in the second stimulation.

For the first stimulation, a preparation containing 5×10^6 cells/ml (1 ml/well in a 24-well plate) was cultivated with or without live promastigotes (5×10^6 cells/ml) at 37°C in the presence of 5% CO_2 in a humid atmosphere for 6 days. On day 5, mature macrophages were infected with live promastigotes at a ratio of 10:1 for 24 h at 37°C in the presence of 5% CO_2 in a humid atmosphere. Cells recovered from the first stimulation were boosted (1×10^6 cells/ml) with autologous *Leishmania*-infected macrophages in complete RPMI 1640 medium supplemented with 10% supernatant harvested from the first stimulation and cultured for 4 days.

Vaccination. The vaccine used in this study was produced under good manufacturing practice conditions, as prescribed by the World Health Organization, by a licensed Brazilian biotechnology company, BIOBRAS, using a well-defined World Health Organization *L. amazonensis* reference strain (IFLA/BR/67/PH8) as described in detail elsewhere (23). The vaccination protocol consisted of two 1.5-ml doses (1,440 μg/dose) injected intramuscularly with an interval of 21 days between the doses (22).

Cytokine measurement. Cell-free culture supernatants were collected after 96 h of culture (in both stimulation cycles) and were kept frozen at −20°C. Cytokine concentrations were determined by an enzyme-linked immunosorbent assay, using commercially available kits for IFN-γ, tumor necrosis factor alpha (TNF-α) and IL-5 (Duo-set; Genzyme, Cambridge, Mass.) and for IL-10 (Genzyme) according to the manufacturer's instructions.

Flow cytometry. Cells were analyzed for surface expression of CD3, CD4, CD8, and CD25 (Becton Dickinson, Mountain View, Calif.). Cells were prepared for analysis by resuspension in PAB (phosphate-buffered saline, 1% bovine serum albumin, 0.05% sodium azide) and blocked with mouse immunoglobulin (20 μg/ml) and 10% fetal bovine serum for 30 min on ice. The cells were then incubated with labeled antibodies or corresponding controls for an additional 30 min. The cells were fixed with 1% paraformaldelyde in phosphate-buffered saline and analyzed with a FACS flow cytometer and CellQuest software (Becton Dickinson). At least 10,000 events were analyzed per sample. For detection of intracellular IFN-γ, cultured cells were restimulated for 6 h with phorbol myristate acetate (Sigma Aldrich) at a concentration of 200 ng/ml, with ionomycin (Sigma Aldrich) at a concentration of 500 mg/ml, and with brefeldin A (Sigma Aldrich) at a concentration of 10 μg/ml, stained for surface markers (CD4 and CD8), fixed overnight, permeabilized with PAB–0.1% saponin at room temperature, and stained for IFN-γ as described previously (12). The following reagents were used for flow cytometry: fluorescein isothiocyanate (FITC)-labeled anti-human CD3 (clone HIT3a), phycoerythrin (PE)- and FITC-labeled anti-human CD4 (clone RPA-T4), PE- and Cy-labeled anti-human CD8 (clone RPA-T8), FITC- and PE-labeled anti-human CD25 (clone M-A251), and appropriately labeled irrelevant isotype-matched control antibodies from the same suppliers.

Statistical analysis. Comparisons of the cytokine levels in the same individuals were performed by using the Wilcoxon matched pair test. Comparisons between high and low producers were performed by using the Mann-Whitney test. For all statistical analyses we used GraphPad Prism, version 3.00 for Windows (GraphPad Software, San Diego, Calif.).

RESULTS

IFN-γ production during IVP stimulation: defining high and low producers. Using the IVP system, we could discriminate between two types of donors, those whose PBMC produced large amounts of IFN-γ (high producers) and those whose PBMC produced low levels of IFN-γ (low producers). Both groups were defined by the concentration of IFN-γ produced after the second round of stimulation. This definition was based the amount of IFN-γ per 10^6 cells (160 pg), which was the median value obtained for all donors studied and was the cutoff point used. PBMC from high producers secreted IFN-γ at concentrations ranging from 505.6 to 1,099 pg of IFN-γ/10^6 cells. However, low producers continued to secrete small amounts of IFN-γ (34.5 to 253 pg of IFN-γ/10^6 cells) (Fig. 1A) even after the second round of stimulation. All high producers presented an increment from the first round to the second round of stimulation ($P = 0.0005$) (Fig. 1B), whereas a consistent pattern was not observed for low producers (Fig. 1C).

TNF-α, IL-10, and IL-5 production by PBMC from high and low IFN-γ producers. TNF-α and IL-10 have been described as important cytokines in the outcome of *Leishmania* infections, since TNF-α contributes to the clearance of parasites in macrophages (20, 21) and IL-10 downregulates IFN-γ biological activities and secretion (6, 7). We evaluated the levels of these cytokines in supernatants harvested after the first and second rounds of stimulation with live *Leishmania* promastigotes in the priming system. The level of IL-5 was also measured as a marker for Th2 response, since IL-4 could not be consistently detected in cultures of human cells. As shown in Fig. 2A, TNF-α secretion was greater in the group of high producers, and the levels seemed to increase after the second round (22.6 to 81.6 pg of TNFα/10^6 cells). However, the mean levels of TNF-α in low IFN-γ producers were very low, and the levels had a tendency to decrease in supernatants harvested after the second stimulation. Differences between low and high responders were statistically significant in both the first and second rounds of stimulation. Therefore, TNF-α could be considered an important biological marker in our system, because its effect in the initial phase of infection is crucial to killing of parasites, as reported previously (36). We had to consider the hypothesis that the increase in the level of this cytokine observed after priming of PBMC with *L. amazonensis* could reflect a secondary effect caused by production of high levels of IFN-γ, leading to activation of infected macrophages and secretion of this cytokine.

Production of IL-10 (Fig. 2B) was greater in high IFN-γ producers than in low producers ($P = 0.007$). The IL-10 levels in high IFN-γ producers decreased from the first round to the second round of stimulation. On the other hand, IL-10 production increased slightly but not statistically significantly after

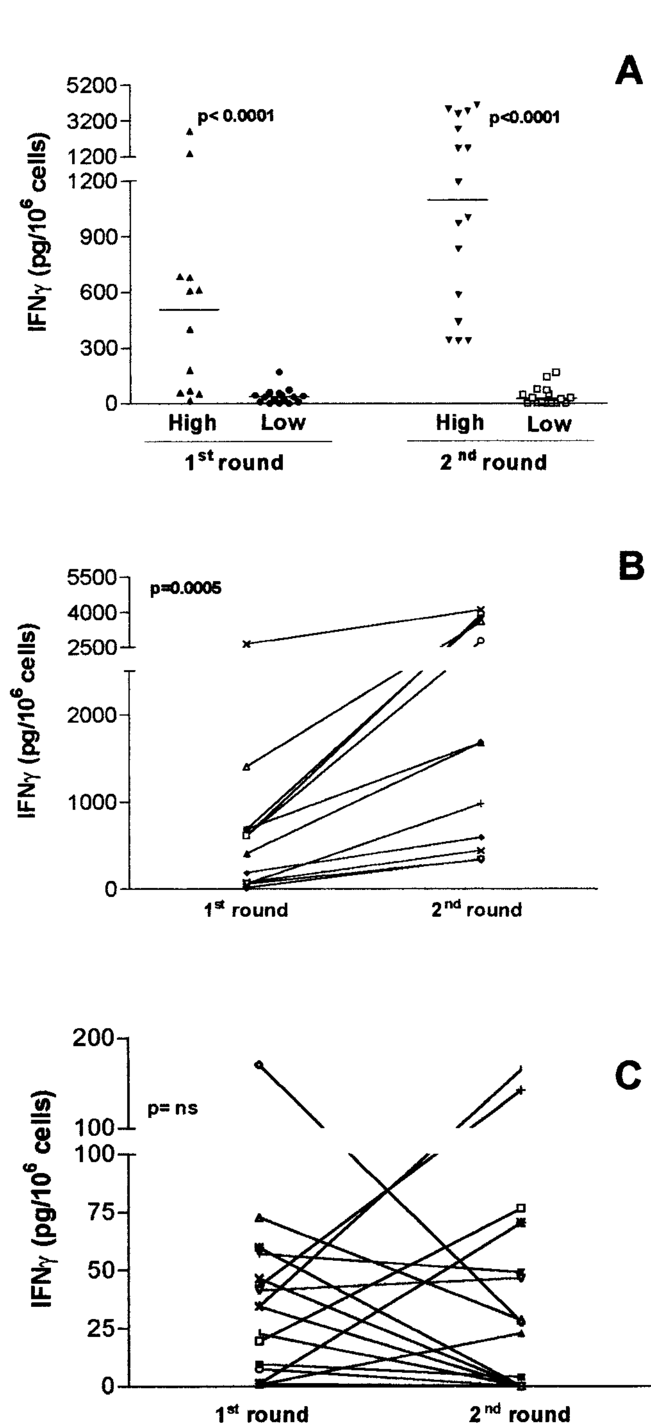

FIG. 1. Levels of IFN-γ obtained from PBMC from high and low responders with the IVP system after the first and second rounds of stimulation. PBMC (5×10^6 cells) from different donors were stimulated with 5×10^6 live *L. amazonensis* promastigotes. After 96 h 100 μl was collected from each well to evaluate IFN-γ production in the supernatants (first stimulation). After 6 days of incubation, cells were harvested and restimulated with infected autologous macrophages for 96 h. After this, supernatants were collected, and the concentrations of IFN-γ were determined (A). (B and C) Variations in the levels of IFN-γ produced by high (B) and low (C) IFN-γ producers. Each line represents a different donor.

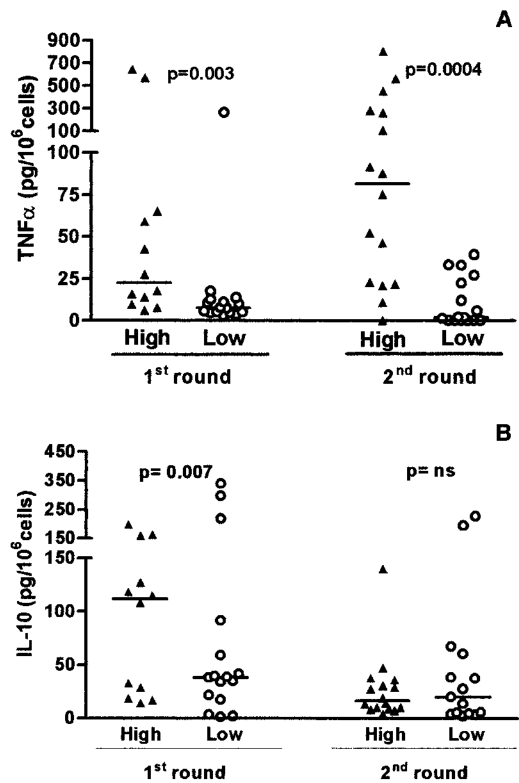

FIG. 2. Levels of TNF-α and IL-10 obtained from PBMC from high and low IFN-γ responders with the IVP system after the first and second rounds of stimulation. PBMC (5×10^6 cells) from different donors were stimulated with 5×10^6 live *L. amazonensis* promastigotes. After 96 h 100 μl was collected from each well to evaluate IFN-γ production in the supernatants (first stimulation). After 6 days of incubation, cells were harvested and restimulated with infected autologous macrophages for 96 h. After this, supernatants were collected, and the concentrations of TNF-α (A) and IL-10 (B) were determined. ns, not significant.

second round of stimulation in low IFN-γ producers. Both groups of donors produced low levels of IL-5, and there was a significant increase in the second stimulation cycle, which was more pronounced in high IFN-γ producers (2.9 to 39.7 pg of IL-5/10^6 cells). Due to the marked dispersion of the data, differences between low and high producers were not statistically significant (data not shown).

Postvaccination evaluation: IFN-γ production. Forty days after vaccination, PBMC from different volunteers classified as high producers after IVP also secreted larger amounts of IFN-γ upon immunization in vivo, whereas low producers after IVP secreted low levels of IFN-γ (Fig. 3A). Again, groups were divided by taking account the median amount of IFN-γ/10^6 cells produced 40 days after vaccination. Most of individuals (81.25%) who were high in vitro producers were also high IFN-γ producers 40 days after vaccination.

Interestingly, 6 months after vaccination, all vaccinated individuals in this study produced similarly high levels of IFN-γ (Fig. 3A). Besides IFN-γ production, we also evaluated proliferative responses and IL-12 production, and no differences were observed between the high and low IFN-γ producers. Actually, these results indicated that eventually all vaccinated

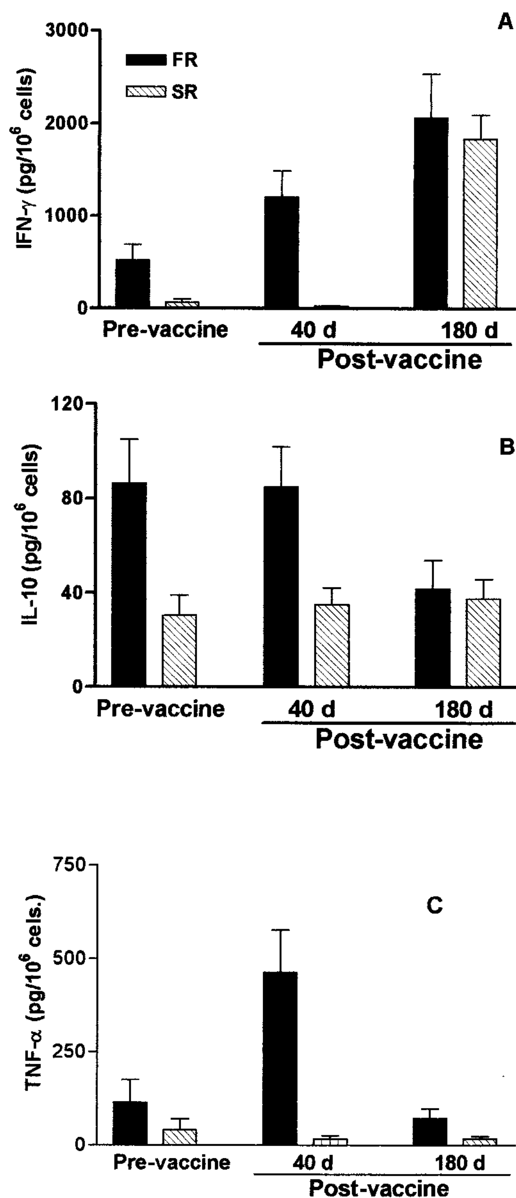

FIG. 3. Levels of IFN-γ and IL-10 obtained from PBMC from healthy donors vaccinated with *L. amazonensis*. Healthy volunteers were vaccinated, and 40 days and 6 months after vaccination their PBMC were obtained and restimulated in vitro with *L. amazonensis* promastigotes for 72 h. Supernatants were harvested, and IFN-γ (A), IL-10 (B), and TNF-α (C) levels were determined by enzyme-linked immunosorbent assays. FR, fast responders; SR, slow responders.

individuals did mount effective cell-mediated immunity responses, but there was a significant delay in individuals who produced low levels of IFN-γ during the early phase of the interaction with live promastigotes. Therefore, it may be more appropriate to refer to fast responders and slow responders, corresponding to the high and low producers observed after IVP.

Postvaccination evaluation: TNF-α, IL-10, and IL-5 production. As described above for IFN-γ production, TNF-α secretion and IL-10 secretion exhibited the same pattern observed after IVP; i.e., fast responders (high producers) produced higher levels of both cytokines, whereas slow responders (low producers) produced lower levels of IL-10 (Fig. 3B) and TNF-α (Fig. 3C) in their supernatants ($P = 0.03$ and $P = 0.002$, respectively). TNF-α production was greater in high responder donors than in low responder donors before vaccination and 40 days after vaccination, but 6 months after vaccination the levels decreased significantly and no differences were observed between high and low producers. These results could reflect an early response to *Leishmania* in vivo with production of inflammatory cytokines, which are downregulated later. At the evaluation 6 months after vaccination there was a significant reduction in IL-10 production in the fast responder group, while the levels remained low in the slow responders (Fig. 3B). Significant amounts of IL-5 were produced by both groups 40 days after vaccination, and there was an important increase 6 months after vaccination. Production was greater in slow responders at both time points studied, although the differences between groups were not significant (data not shown).

Primed in vitro response versus early postvaccination response. As mentioned above, the pattern of responses to IVP was similar to that observed 40 days after vaccination. There were positive correlations between the primed in vitro response and the first postvaccination data for IFN-γ ($P < 0.0001$) (Fig. 4A), TNF-α ($P = 0.0005$) (Fig. 4B), IL-10 ($P = 0.01$) (Fig. 4C), and IL-5 ($P = 0.0005$) (Fig. 4D). These results suggest that IVP response can be used to predict the early postvaccination responses of test volunteers and probably mimic the initial human in vivo responses to *Leishmania*.

$CD8^+$ T cells are activated preferentially by high IFN-γ producers. Since we observed a positive correlation between IVP responses and the first postvaccination data, the next step was to explore the possible mechanisms responsible for the early high levels of IFN-γ production during the responses to *L. amazonensis*. PBMC from high and low producers were primed in vitro with live promastigotes, and cells were harvested after the second stimulation and stained to detect activation markers (CD25) by flow cytometry in $CD4^+$ and $CD8^+$ T cells. As shown in Fig. 5A, there was not a significant difference in the frequency of $CD4^+$ $CD25^+$ T cells between high and low producers. However, we observed a higher frequency of activated $CD8^+$ T cells ($CD25^+$) in the high IFN-γ producers (Fig. 5B). Cells from high-producer donors were harvested after the second round of stimulation and stained to detect intracellular IFN-γ. In fact, the percentage of $CD8^+$ T cells producing IFN-γ was significantly higher than the percentage of $CD4^+$ T cells producing IFN-γ (Fig. 6) (7.5 and 3.5% for $CD8^+$ and $CD4^+$ T cells, respectively), showing that $CD8^+$ T cells are preferentially activated in human immune responses to *L. amazonensis*. Forty days after vaccination, PBMC from fast and slow responders were obtained and stimulated with live promastigotes for 96 h at 37°C in the presence of 5% CO_2, and the cells were then harvested and stained. Although we observed a difference between the fast and slow IFN-γ responders in terms of activation of $CD4^+$ T cells under ex vivo conditions, after in vitro restimulation of the cells the frequencies of $CD4^+$ T cells activated seemed to be the same in the low and high responders. On the other hand, there was a significant increase in the level of activation of $CD8^+$ T cells, represented by the presence of CD25 (Fig. 7B), in donor cells

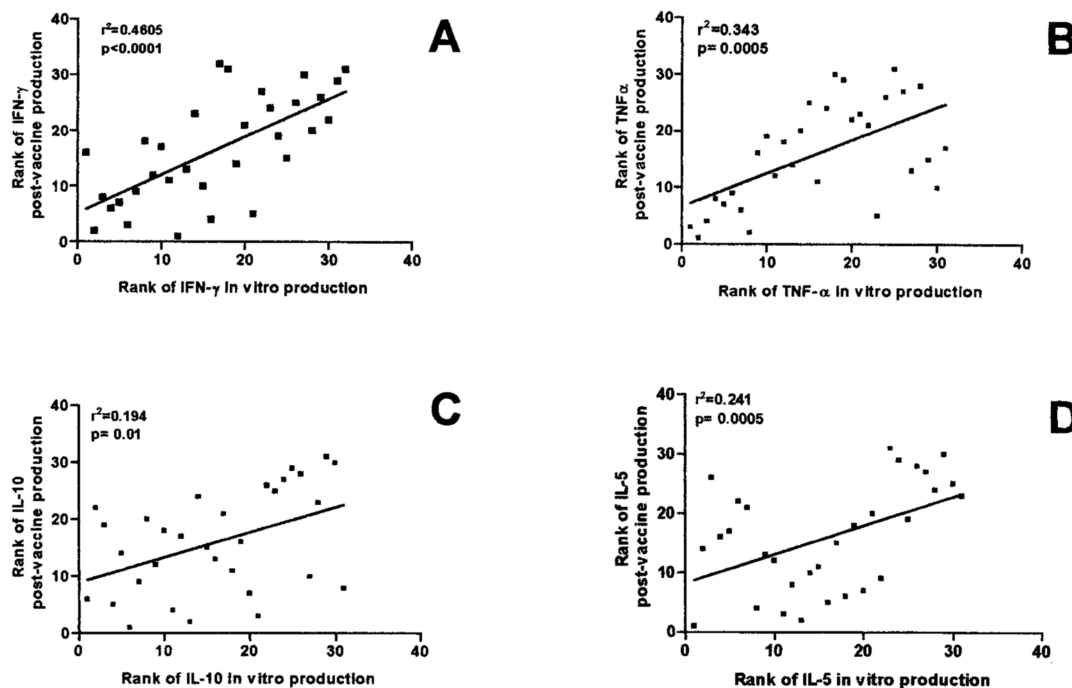

FIG. 4. Positive correlation between the primed in vitro response and the first postvaccination data (40 days) for IFN-γ ($P < 0.0001$) (A), TNF-α ($P = 0.0005$) (B), IL-10 ($P = 0.01$) (C), and IL-5 ($P = 0.0005$) (D).

restimulated in vitro with *Leishmania*. It seemed that fast responders had a greater $CD8^+$ response, as measured by CD25 levels, and that IFN-γ production preferentially occurred in $CD8^+$ cells.

DISCUSSION

In the present study, we showed that IVP of T cells from individuals who have not been exposed to *L. amzonensis* can be used to distinguish volunteers who produce low and high levels of IFN-γ. Additionally, the differences in in vitro IFN-γ production could be used to predict the pace of the in vivo response to *Leishmania* vaccination. It is tempting to speculate that the two types of individuals differ in their responses to natural *Leishmania* infections. If it is assumed that resistance to leishmaniasis is related to fast production of high levels of IFN-γ, reflecting a strong cell-mediated immunity response, low producers get the disease because their cells do not secrete sufficient amounts of IFN-γ to activate macrophages and destroy intracellular parasites. Mice in which IFN-γ production is delayed are more susceptible to *Toxoplasma gondii* than mice which exhibit a faster response (37). Additionally, the proportion of fast and slow responders observed in the present study is similar to the rate of protection against *Leishmania* reported for the vaccine which we employed (22, 23). It was shown previously that 50% of cutaneous leishmaniasis patients who had had the disease for less than 60 days contained low levels of IFN-γ or even no IFN-γ when their PBMC were stimulated with *Leishmania* antigen. However, later during the disease cycle high levels of IFN-γ and TNF-α were produced, suggesting that there was transitory immunosuppression from the PBMC which allowed parasite proliferation (28). These data were somewhat similar to the data obtained in this study for cytokine production from low IFN-γ producers, whose cytokine levels were low 40 days after vaccination but increased substantially after 6 months, reaching levels similar to those in fast responders.

It is interesting that high IFN-γ producers also secreted higher levels of TNF-α and IL-10. It has been shown that TNF-α is directly involved in activation of macrophages and immunoregulation of IFN-γ production (20, 21) and contributes to the control of infection by intracellular pathogens. The correlation between high levels of IFN-γ and high levels of TNF-α could be explained by direct activation of macrophages by the former cytokine, leading to destruction of the parasites (21, 36). A correlation between IFN-γ and IL-10 levels has been observed for leishmaniasis and other diseases (8, 11, 29). Since IL-10 usually exhibits human macrophage-deactivating properties (6, 7), high levels of IL-10 may represent a necessary counterbalance to an extremely polarized immune response, limiting tissue damage. On the other hand, IL-10 may also lead to increased IFN-γ production by NK cells (33).

Low levels of IL-5 production were detected with the IVP system and 40 days after vaccination, but 6 months after vaccination the concentrations of IL-5 in fast and slow IFN-γ producers increased, and the differences between the two groups were not significant. Russo et al. (30), who developed an in vitro system to study early responses of unexposed individuals to *L. amazonensis* infection, and Brodskyn et al. (9), who used an IVP system for *L. major*, also observed low levels of IL-5. Elevated production of Th1 cytokines and low levels of production of Th2 cytokines were also observed in other human T-cell IVP systems (35).

In this study, we had the opportunity to compare results

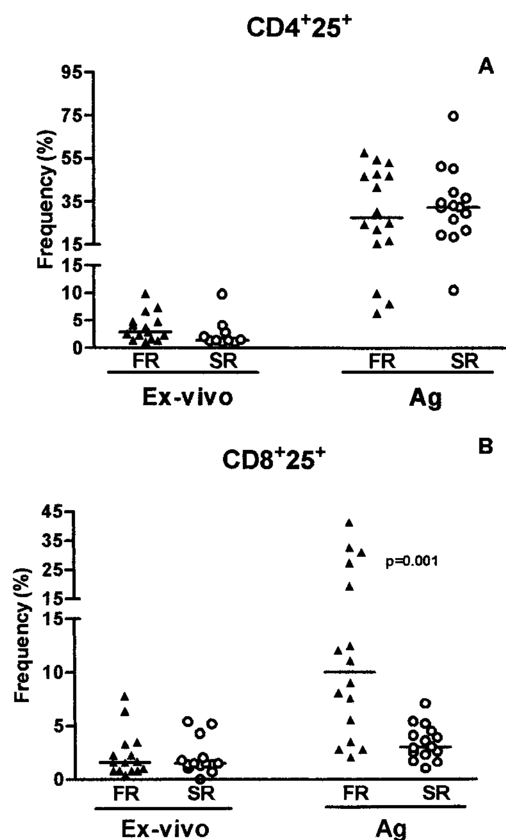

FIG. 5. Frequencies of $CD4^+$ $CD25^+$ and $CD8^+$ $CD25^+$ T cells obtained from PBMC from healthy volunteers ex vivo and after IVP (second round of stimulation). Cells were prepared for analysis by resuspension in PAB and were blocked with mouse immunoglobulin (20 μg/ml) and 10% fetal bovine serum for 30 min on ice. The cells were then incubated with labeled antibodies (anti-CD4, anti-CD8, and anti-CD25) or corresponding controls for an additional 30 min. The cells were fixed with 1% paraformaldeyde in phosphate-buffered saline and analyzed with a FACS flow cytometer and CellQuest software (Becton Dickinson). At least 10,000 events were analyzed per sample. (A) Frequencies of $CD4^+$ $CD25^+$ T cells from fast responders (FR) and slow responders (SR); (B) frequencies of $CD8^+$ $CD25^+$ T cells from fast responders and slow responders. Ag, antigen.

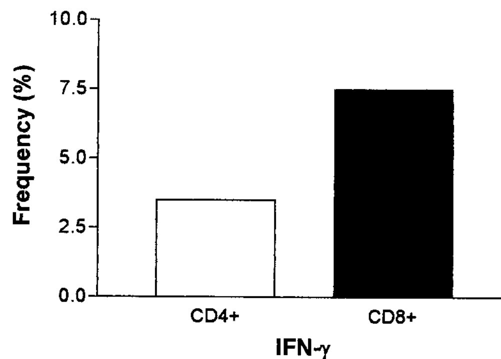

FIG. 6. Detection of intracellular IFN-γ in PBMC obtained after the second round of IVP stimulation. For detection of intracellular IFN-γ and IL-10, cultured cells were restimulated for 6 h with phorbol myristate acetate (200 ng/ml), ionomycin (500 ng/ml; Sigma), and brefeldin A (10 μg/ml), stained for surface markers (CD4 and CD8), fixed overnight, permeabilized with PAB–0.1% saponin at room temperature, and stained for IFN-γ as described in Materials and Methods.

obtained with the IVP system in which PBMC and live *L. amazonensis* were used with results obtained after in vivo immunization of the same donors with a safe vaccine (22). There was a positive correlation between the IVP response and the first postvaccination data (40 days) for all of the cytokines tested. Actually, all vaccinated individuals eventually mounted a potent anti-*Leishmania* cellular immune response 6 months after vaccination. These findings correlate with the in vivo observation that most cutaneous leishmaniasis patients do mount a potent Th1 response, but cutaneous leishmaniasis patients in the initial stages of the disease produce smaller amounts of IFN-γ than patients in the late phase of the disease (28, 29). Upon vaccination normal individuals differ in the pace at which the cellular immune response is mounted rather than in the level of response achieved in the long term, which may have important implications concerning the success of a vaccine.

The data presented here demonstrated that important activation of $CD8^+$ T cells occurred both in vitro and in vivo following exposure to *Leishmania* antigen. Interestingly, the percentage of $CD8^+$ T cells producing IFN-γ was higher than the percentage of $CD4^+$ T cells producing IFN-γ. These results suggest that $CD8^+$ T cells may be responsible for the initial production of Th1 cytokines, which may lead to the dominance of the Th1 response. Natural killer cells may be a very important source of IFN-γ in this situation. While previous reports have shown that $CD8^+$ T cells may not be essential for primary immunity (17), there have been several studies which have shown that $CD8^+$ T cells do have a role in secondary responses (24, 25, 34). LACK DNA induces antigen-specific $CD8^+$ IFN-γ-producing T cells following vaccination of BALB/c mice (14), and depletion of $CD8^+$ T cells at the time of infection abrogated protection (14, 15). Additionally, these mice had diminished frequencies of $CD4^+$ IFN-γ-producing T cells, suggesting that $CD8^+$ T cells have an immunoregulatory function (16). Russo et al. (31), using an in vitro system with soluble *Leishmania* antigen, observed a high frequency of $CD8^+$ T cells, which lysed parasite-infected macrophages, but these cells did not produce IFN-γ. The differences between the results of Russo et al. and our results may be explained by the different protocols that Russo et al. used in their experiments, which generated human T-cell lines with soluble *Leishmania* antigens in the presence of different cytokines. The system used in this study closely mimicked the in vivo situation by infecting autologous macrophages without added cytokines.

On the other hand, low or slow IFN-γ producers could fail to activate CD8 appropriately at the beginning of infection, leading to a delay in IFN-γ production. In addition, secretion of suppressor cytokines, like transforming growth factor β (TGF-β) or IL-4, could affect the pace of cytokine production. In mice, it has been demonstrated that TGF-β inhibits the immune response, allowing parasite growth (5). TGF-β levels differ greatly in different individuals, and the differences in TGF-β levels could be responsible for some of the results observed in our study. Another important aspect concerns the

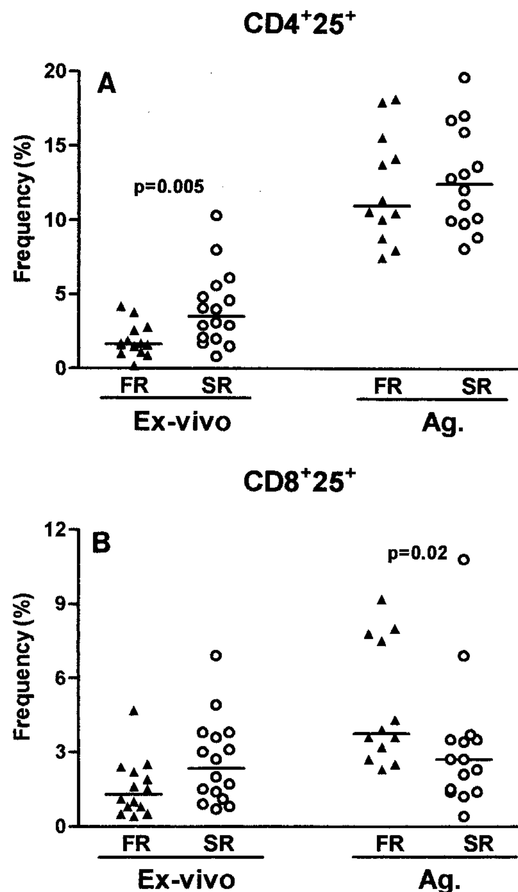

FIG. 7. Frequencies of $CD4^+$ $CD25^+$ and $CD8^+$ $CD25^+$ T cells obtained from PBMC ex vivo and 40 days after vaccination. Using the method described in the legend to Fig. 5, we determined the frequencies of $CD4^+$ $CD25^+$ T cells from fast responders (FR) and slow responders (SR) (A) and of $CD8^+$ $CD25^+$ T cells from fast responders and slow responders (B). PBMC from donors were restimulated with antigen (Ag.) for 72 h, and after this cells were prepared for analysis.

participation of costimulatory molecules in the activation of T-cell responses. It has been shown that these molecules are involved in Th1 and Th2 cytokine production (10, 13). Expression of CD40 or B-7 could have been downregulated in some of the volunteers, delaying activation of their T cells and precluding IFN-γ secretion.

For the first time, we demonstrated that there is a correlation between an in vitro system and in vivo immunization. Therefore, by using this system, we can obtain a detailed understanding of the early response of T cells to *Leishmania* infection, and the information obtained should allow identification of the antigens responsible for triggering early protective responses, which should be crucial for identifying protective *Leishmania* antigens.

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