Leishmania Specific CD4 T Cells Release IFNγ That Limits Parasite Replication in Patients with Visceral Leishmaniasis

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Abstract

Visceral leishmaniasis (VL) is associated with increased circulating levels of multiple pro-inflammatory cytokines and chemokines, including IL-12, IFNγ, and TNFα, and elevated expression of IFNγ mRNA in lesional tissue such as the spleen and bone marrow. However, an immunological feature of VL patients is that their peripheral blood mononuclear cells (PBMCs) typically fail to respond to stimulation with leishmanial antigen. Unexpectedly, it was recently shown that Leishmania specific IFNγ can readily be detected when a whole blood stimulation assay (WBA) is used. We sought to define the conditions that permit whole blood cells to respond to antigen stimulation, and clarify the biological role of the IFNγ found to be released by cells from VL patients. CD4+ T cells were found to be crucial for and the main source of the IFNγ production in Leishmania stimulated whole blood (WB) cultures. Complement, antibodies and red blood cells present in whole blood do not play a significant role in the IFNγ response. The IFNγ production was reduced by blockade of human leukocyte antigen (HLA)-DR, indicating that the response to leishmanial antigens observed in WB of active VL patients is a classical HLA- T cell receptor (TCR) driven reaction. Most importantly, blockade of IFNγ in ex-vivo splenic aspirate cultures demonstrated that despite the progressive nature of their disease, the endogenous IFNγ produced in patients with active VL serves to limit parasite growth.

Introduction

Visceral leishmaniasis is a chronic disease caused by the protozoan parasites Leishmania donovani and Leishmania infantum/chagasi. Leishmania are transmitted by the bite of phlebotomine sand flies, and replicate within macrophages of their mammalian hosts. In VL, the target organs are chiefly the liver and the spleen. The disease is characterized by prolonged fever, spleno-hepatomegaly, wasting, hypergammaglobulinemia, pancytopenia and almost always leads to death if left untreated. Paradoxically, the acute phase of VL is associated with elevated expression of IFNγ mRNA in lesional tissue, such as the spleen and bone marrow, as well as increased circulating levels of multiple pro-inflammatory cytokines and chemokines, including IL-12, IFNγ and TNFα [4,6]. These results imply that the failure to respond to Leishmania antigen stimulation observed in VL patients is not due to a defect in the ability to mount protective Th1 responses per se, but rather to induction of suppressive factors, e.g. IL-10, resulting in unresponsiveness of infected macrophages to activation signals [7].
Author Summary

Our research aims to understand the immune failure underlying progression of human visceral leishmaniasis (VL). A key immunological feature of VL patients is that their peripheral blood mononuclear cells (PBMCs) do not respond to stimulation with leishmanial antigen. Surprisingly, when employing a whole blood assay we discovered significant levels of IFNγ in response to soluble *Leishmania donovani* antigen (WBA) in VL patients. We were interested to understand the relevance of the IFNγ to the antiparasitic response. Animal models and in vitro studies have shown that IFNγ is a key effector cytokine required for control of the infection, however, the role of endogenous IFNγ in control of parasites in VL patients, has not been demonstrated. Our results show that CD4 cells were required for and were the source of *Leishmania* specific IFNγ in WBA of VL patients. Optimal IFNγ response required interaction with HLA-DR, supporting that VL is not due to an intrinsic Th1 response defect per se. The *Leishmania* driven IFNγ appears to limit parasite growth in patients with active VL since blockade of IFNγ ex-vivo in splenic aspirate cultures enhanced parasite survival. This suggests that IFNγ may have been prematurely dismissed as an adjunct therapy in treatment of VL.

Studying immunological aspects of human VL has been severely hampered by the inability to measure antigen specific responses, including IL-10, using PBMC. The discovery of antigen specific cytokine responses following stimulation of whole blood (WB) [9] showed that VL patients are not void of *Leishmania* specific IFNγ responses, findings that could be reconciled with the elevated levels of IFNγ mRNA and circulating cytokines detected in active VL patients. Subsequent studies reported that the whole blood assay (WBA) could also be used to detect antigen-specific IL-10 responses [9,10]. Thus, the WBA has opened up new possibilities for research aimed at understanding immunological determinants of the disease [8,9,10,11].

We sought to define the requirements for IFNγ production seen using the WBA, and determine if the IFNγ had a biological function in patients with active VL. We show that CD4+ T cells produce *Leishmania* specific IFNγ in WB cultures. The responses to stimulation with *Leishmania* antigen observed in WB cultures of active VL patients occurred in the absence of complement, antibodies or cytokines present in serum of VL patients. Employing a splenic aspirate (SA) culture technique, as complement, antibodies or cytokines present in serum of VL WB cultures of active VL patients occurred in the absence of responses to stimulation with *Leishmania*. We show that CD4+ T cells release IFNγ that control parasite replication in human VL.

Materials and Methods

Study groups

All VL patients presented with clinical symptoms of kala-azar at the Kala-azar Medical Research Center (KAMRC), Muzaffarpur, Bihar, India, and were confirmed to be VL positive by detection of amastigotes in splenic aspirates and/or by detection of antibodies against the recombinant antigen, K39. Venous blood and/or splenic aspirates (SA) samples collected from 84 (33 female and 51 male) patients with active VL were included in this study. All patients were treated with Amphotericin B and eventually cured.

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smears for diagnostic purpose, the residual cells were placed was obtained by fine needle biopsy, following preparation of nature of 4–8 transported to the laboratory at BHU maintaining a temper- ml penicillin (C-RPMI) and 5 U/ml heparin. Samples were directly in 1 ml RPMI supplemented with 10% heat-inactivated blood agar overlaid by 100 m

150 diluted by transfer of 50 ml SA suspension was directly plated in a 96-well and serially SA onto biphasic medium of 50 l/well). Monoclonal antibody against human IFN-γ, clone 25723 (R&D Systems) or control IgG2b clone 20116 (R&D Systems) were each added to a final concentration of 20 μg/ml. The SA was incubated for 3 days at 37°C (R&D Biosciences), cells were surface stained using combinations of FITC, PE and PerCP/PE-Cy5 conjugated antibodies directed to CD3 (Clone UCHT1), CD4 or CD8 (all from BD Biosciences). Surface stained cells were fixed and permeabilized using BD Cytofix/Cytoperm, as per manufactures instruction, washed in permeabilization buffer (BD) and stained for presence of intracellular IFNγ and IL-10 using APC and PE conjugated antibodies (both from Pharmingen) respectively. Following intra cellular staining (ICS), samples were acquired on FACSort (BD Biosciences) and analyzed using CellQuest Pro (BD) or Flowjo (Treestar) software. Analysis was done on cells gated as viable lymphocytes based on their forward–side scatter. SEB (10 μg/ml) stimulated samples were used as positive control for ICS (not shown).

Statistical analyses

Statistical analyses were done using PRISM5 (GraphPad Software). Different treatments using the same donor samples were compared by the Wilcoxon signed rank test for paired samples. Correlation between results was determined using Spearman-test for non-parametric correlations. Differences with P-values<0.05 were considered as significant. Outliers (donors with extreme values in one or more of the test conditions) were removed from data sets after being defined as outlier using GraphPad on-line Grubb’s test for outliers.

Results

Kinetics of Leishmania specific IFNγ secretion in whole blood cultures of patients with active VL

The whole blood Quantiferon assay (WBA) was originally designed as a tool for diagnosis of tuberculosis, and detects cytokine (IFNγ) concentrations in plasma supernatants after

| Aggregate clinical data for VL patients. |
|------------------------------------------|
| N            | 84          |
| Age (years)  | 29.45±17.6  |
| Sex (M/F)    | 51/33       |
| Duration of illness (days)               | 37.31±35.29 (30) |
| WBC counts, pre treatment                | 3332±1183 (3100) |
| WBC counts, post treatment*              | 6795±2887 (6300) |
| Spleen Size (cm), pre treatment*         | 4.43±2.82 (4)   |
| Spleen Size (cm), post treatment*        | 0.81±1.42 (0)   |
| Splenic Scoreb  | 2.37±1.11c |

Median values are given within brackets.
* Post treatment values are from day 15 or day 30 post treatment.
^ Scoring of parasite load is on a logarithmic scale from 1 to 6, were 0 is no parasites per 1000 microscopic fields (1000×), 1 is 1–10 parasites per 1000 fields, and 6 is > 100 parasites per field. ND = not done; NA = not applicable.
^ Splenic scores presented are only based on patients used to assess the ex-vivo effect of IFNγ blockade.

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16–24 hours of incubation with antigen. To determine the kinetics of the WB responses in VL patients we measured secreted cytokines in supernatants after 6 hours to five days of stimulation with soluble *Leishmania* antigen (SLA). The induction of IFNγ was rapid and observed in supernatants already 6 hours after stimulation, reaching a plateau at 18–24 hours (Figure 1a, b). Antigen-induced IFNγ was not detected in WB cultures following 72 hours culture or more (Figure 1b). We conclude that the IFNγ response seen in the WB cultures is rapid and short lived. For practical reasons stimulation times of 24 hours were used in subsequent assays if not otherwise indicated.

We further tested if antigens specific responses could be detected in short-term (24 hr) splenic aspirate (SA) cultures. In line with the observations made using the WBA, an increase in IFNγ was observed in supernatants of 73% of SA cultures following stimulation with SLA, indicating that antigen specific cells are present at the site of infection (Figure 1c). In contrast to the SLA stimulated WB cultures where IL-10 tended to be induced [9,10], IL-10 levels dropped in SA cultures following SLA stimulation (Figure 1d).

The WB response was not affected by serum complement inactivation, replacement of autologous plasma, or red blood cell lysis.

The immune system of patients with VL is highly activated. We considered the possibility that other blood cell or serum components that are removed in the process of PBMC purification could be required for the *Leishmania* specific WB response. To address the effect of plasma components we replaced the plasma with i) autologous heat-inactivated plasma, to determine the role of complement, or ii) heat inactivated fetal calf serum (HI-FCS), to remove antibodies, complement, or other serum factors such as cytokines that may be elevated in VL. To address if RBC were important, we lysed the RBC using hypotonic treatment. None of

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**Figure 1. Induction of IFNγ in SLA stimulated whole blood and splenic aspirates from patients with active VL.** a) IFNγ in SLA stimulated (black bars) and unstimulated (white bars) WB during the first 24 hours of culture and b) IFNγ production (measured in units by QuantiFeron kit) in culture supernatants from SLA stimulated WB after 24 hours, 72 hours and 5 days incubation at 37°C, 5%CO2. c) IFNγ in 24 hour cultures of SLA stimulated and unstimulated SA from patients with active VL (n = 15) and d) IL-10 in 24-hour SLA stimulated and unstimulated SA (left) and WB (right), n = 8. Results show in a mean OD±SD of the samples tested, in b–d one dot represents one sample. Stimulated and unstimulated samples were compared by Wilcoxon matched paired test and statistical significances are indicated with *** p<0.001, ** p<0.01 and * p<0.05.

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Figure 2. SLA induced IFNγ secretion in WB of active VL patients is not dependent on plasma proteins or RBC. IFNγ in SLA stimulated WB from active VL patients (n = 8/9) following replacement of plasma or lysis or RBC as indicated (WB = whole blood untouched; HI-Auto = replacement with heat inactivated [12] autologous plasma; HI-FCS = replacement with HI-FCS). Net responses (stimulated minus unstimulated)-SD are shown. The stimulation indices (stimulated/unstimulated) are indicated above each bar to as an additional comparison. Comparisons between treatments were made using Wilcoxon matched paired test; there were no significant differences between the different culture conditions. doi:10.1371/journal.pntd.0003198.g002

these treatments affected the net production of IFNγ measured using the WBA (figure 2), indicating that complement, antibodies, cytokines, or RBC are not important for the observed SLA induced IFNγ production in WB. Indeed, removal of autologous plasma with HI-FCS potentiated the SLA induced response (figure 2). The replacement of plasma with FCS was subsequently employed in some of the assays that followed.

CD4 T cells are the source of IFNγ in the WB response to SLA in patients with active VL

Understanding the cellular source/s of IFNγ in the WB is critical to our reinterpretation of the immunologic defects in kala-azar. To determine the cellular requirements for IFNγ production we removed various subsets from whole blood of VL patients prior to stimulation with SLA. Removal of CD4 cells caused a substantial loss of SLA induced IFNγ in WB cultures, while removal of CD8 cells had no effect (figure 3a). Blockade of HLA using a pan-HLA-DR antibody caused a significant loss of SLA induced IFNγ (figure 3b). This suggests that the IFNγ response induced by SLA stimulation depends on HLA-TCR interaction. Three out of the 12 patient samples in which the effect of HLA-DR blockade was evaluated had low IFNγ responses to SLA [< 100 pg/ml]. To confirm CD4 T cells as the source of IFNγ in WB, we assessed intracellular IFNγ by FACS. SLA induced IFNγ was only observed in the CD3+ population (all events considered). Figure 3c shows that the IFNγ is produced by CD3+CD4+ cells, while figure 3d shows that there is a strong correlation between the frequency of IFNγ positive T cells (CD3+) and the IFNγ measured in WB culture supernatants by ELISA. IFNγ was not detected in the CD3+CD8+ population following SLA stimulation and almost all cells producing IFNγ following SLA stimulation were CD3+CD8- (not shown).

To test if neutrophils contributed to the IFNγ responses CD15+ cells were removed using depletion beads, this caused a partial though significant loss of SLA induced IFNγ (figure S1), which may indicate an involvement of neutrophils in the observed SLA response, but since CD15 can be expressed on other cells, i.e. monocyte, we cannot exclude that the effect seen is due to removal of these cells.

IL-10 can be induced in stimulated WB from VL patients, albeit at low levels. Removal of CD4 cells caused a small but significant reduction of the amount of detectable IL-10 in SLA stimulated WB (figure 3e), indicating that CD4+ and other cells are sources of antigen-specific IL-10 in VL patients. CD6 cells do not appear to contribute to SLA induced IL-10 response, and their removal caused a slight enhancement of this response (Figure 3e). The source of SLA induced IL-10 could not be confirmed by intracellular staining as the number IL-10 positive cells was below the limit of reliable detection.

Endogenous IFNγ limit parasite replication in the spleen of visceral leishmaniasis patients

In experimental models it is well established that IFNγ mediates control of parasite replication [16] and that lack of IFNγ signalling causes disease progression [17,18]. The same protective function is assumed in humans, but the direct proof that IFNγ controls parasite replication in human VL is lacking. To test if the endogenous IFNγ, which we now know to be elevated during active disease, plays a role in parasite control, we treated ex vivo SA cultures with neutralizing antibodies against human IFNγ followed by assessment of parasite growth, as previously described in assays designed to test the function of endogenous IL-10 [11]. Following neutralization of IFNγ, the parasite load in SA increased in 19/31 (61%), was unchanged in 8/31 (26%) and decreased in 4/31 (13%) samples (figure 4a). The IL-10 levels in the SA supernatants were not affected by neutralization of IFNγ (figure 4b), suggesting that the inhibitory effect of IL-10 on parasite killing does not completely abolish the parasite-controlling effects of endogenous IFNγ. The background levels of IFNγ detectable in ex vivo SA cultures were significantly reduced when CD4 cells were removed (figure 4c), indicating that CD4 cells are needed for the splenic IFNγ production.

Discussion

In the search for markers of L. donovani infection, epidemiological studies utilising a WBA revealed Leishmania specific IFNγ responses, long considered absent, in patients with active VL [8]. The goals of the current study were to validate the prior WBA results, to reveal the conditions required for SLA induced IFNγ secretion by WB and to determine if the IFNγ seen in patients with active disease functions to limit the infection.

Whole blood contains cell populations, proteins, lipids and sugars that are largely removed when PBMC are purified. To test if such components were required for the antigen specific response we deprived WB cultures of RBC, plasma and complement. We found that replacement of autologous plasma and RBC lysis had no effect on the SLA induced IFNγ response. By contrast, removal of CD14+ cells revealed these cells to be the main source of antigen specific IFNγ secretion in the WB cultures, a finding that was substantiated by direct intracellular staining. In line with previous observation CD8 T cells were not found to contribute to SLA responses in patients with active VL [19].

Removal of CD15+ cells also reduced the IFNγ levels detectable in the SLA stimulated WB. CD15 (Lewis X) is a carbohydrate adhesion molecule primarily expressed on mature neutrophils in blood, but is also present on a subset of monocytes [20]. The decline in IFNγ levels following CD15 depletion may thus be explained by a reduction of APCs required for the T cell response, but could also imply that neutrophils contribute to the response. By contrast, Abebe et al. have proposed, based on the observation...
that VL patients have more CD15+ and higher content of arginase expressing CD15+ cells pre compared to post treatment patients or endemic controls, that neutrophils contribute to the unresponsiveness of VL PBMC [12]. Neutrophil inhibition of the antigen-specific IFNγ response in VL patients is not supported by the data presented here, where a reduction in IFNγ secretion by WB cells was observed following CD15 depletion.

The detection of IFNγ responses in stimulated splenic aspirate cells (figure 1c) indicates that antigen specific and responsive cells are present at the site of infection. Depletion of CD4 cells from \textit{ex vivo} SA cultures support these cells as the source of IFNγ at the site of infection. In contrast to the WB, where IL-10 was also induced following SLA stimulation, IL-10 levels decreased in SA following SLA stimulation (figure 1d). More critically, we show that the endogenous IFNγ produced by splenic cells is biologically active and served to limit parasite growth in the SA cultures from the majority of VL patients, as shown by the increase in parasite numbers after IFNγ neutralization \textit{ex-vivo}. The lack of effect of the IFNγ neutralization on parasite growth observed in some samples can be attributed to the nature of the SA. The sampling is done blind and the aspirates may vary in red and white blood cell content as well as the extent of disruption of infected cells, resulting in extracellular amastigotes that will be unaffected by the level of IFNγ released. The treatment with anti-IFNγ-antibodies did not

**Figure 3.** CD4 cells are necessary for and the source of IFNγ in SLA stimulated WB from patients with active VL. IFNγ production in SLA stimulated WB cultures of active VL patients following depletion of a) CD4 and CD8 positive cells. b) Effect of pan-HLA-DR blockade on SLA induced IFNγ, compared to IgG2a isotype control treatment c) Intra cellular IFNγ lymphocytes, following SLA stimulation of WB culture. The dot-plots to the left (red) show IFNγ production in total gated lymphocytes and the dot-plots to the right (purple) show SLA induced IFNγ in gated CD3+ cells. In the graph the frequencies of IFNγ+ CD4+ T cells are shown; combined results of three different experiments. d) Correlation between percentages of IFNγ positive cells in gated CD3+ lymphocytes as determined by ICS and IFNγ levels in WB supernatants following 24 hours of SLA stimulation. e) IL-10 levels in SLA stimulated WB supernatants following removal of CD4 and CD8 positive cells. Plasma was replaced with HI-FCS prior to incubation with MicroBeads or HLA-DR blockade, MB = mock treatment with magnetic beads directed against FITC. Each dot represents one sample. Net SLA response (e.g. stimulated minus unstimulated) samples are shown, if not otherwise indicated. Comparison between treatments was made using Wilcoxon matched paired test and statistical significances are indicated with *** \( p<0.001 \), ** \( p<0.01 \) and * \( p<0.05 \).

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The SLA induced IFN-γ also underlie the lack of response in some patients. We found that antigen specific T cells in the curative response to pentavalent antimony [22]. Our findings reinforce the rationale for the development of VL were recently identified within in the MHC class II region [21]. The influence of allelic differences and role of different MHC molecules on WB SLA responses are of interest since risk alleles for VL progression would be far worse in the absence of the endogenous IFN-γ that they produce. Notably, there are patients whose cellular responses cannot be detected even when using the WBA. While not directly reflected in the clinical parameters (i.e. blood chemistry), these patients may have progressed further in the disease and lost the responding population. It may be noted that there was a negative correlation between SLA induced IFN-γ response in WBA and parasite load in blood (Spearman r = −0.66; p = 0.004, n = 17), which indicates that the WB SLA response to a degree may reflect the severity of disease. Genetic or acquired defects in their ability to mount Th1 responses to Leishmania may also underlie the lack of response in some patients. We found that the SLA induced IFN-γ response involved HLA-DR interaction as treatment with HLA-DR blocking antibody reduced the IFN-γ levels in all donors tested (figure 3b), with an average decrease of 70% compared to control antibody treatment. The partial effect observed may be explained by utilization of HLA-DQ in the presentation of leishmanial antigens to T cells. While HLA-DR together with its peptide is the classical ligand for T cells recognizing foreign antigens, HLA-DQ may also present peptides from pathogens and initiate T cells responses. The role of HLA molecules on WB SLA responses are of interest since risk alleles for development of VL were recently identified within in the MHC class II region [21]. The influence of allelic differences and role of different MHC molecules in the ability to drive Leishmania specific responses in the WB culture are under current investigation.

The functional Th1 response in active VL patients may also be highly relevant to their response to treatment. L. donovani infection in T cell deficient mice revealed a clear role for antigen specific T cells in the curative response to pentavalent antimony [22]. Our findings reinforce the rationale for the prior VL treatment trials carried out in the 1990s involving recombinant IFN-γ, indicating that monotherapy could be beneficial [23,24]. The lack of response to monotherapy in some patients and the absence of a long-lasting therapeutic effect, as well as the limited success as adjunct therapy with sodium stibogluconate [25], discouraged further trials. Our present and more recent studies suggest that antigen-specific IFN-γ production may in some patients not be the limiting factor in their non-curative response.

In summary, our data support the notion that disease progression in VL is not due to a complete failure in Th1 development. Our findings make clear that WB cultures may allow detection of functionally relevant immune responses not seen using PBMC. Most patients with VL have antigen specific CD4 T cells capable of secreting IFN-γ both in the blood and at the site of infection - the spleen. We further show that the IFN-γ produced by VL patients play a role in limiting parasite growth.

Supporting Information

**Figure S1** CD15 cells contribute to SLA responses in WB cultures of VL patients. Effect of CD15 depletion (MACS, Miltenyi) on SLA driven IFN-γ response in WB cultures from VL patients. Net responses (SLA stimulated minus unstimulated are shown). Comparison between treatments (CD15 beads or control beads = MB) was made using Wilcoxon matched paired test, and statistical significance is indicated with P-value. (TIF)

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Author Contributions

Conceived and designed the experiments: RK DS SN. Performed the experiments: RK DS SN. Analyzed the data: RK NS SG OPS KG SN. Contributed reagents/materials/analysis tools: MR SS SN. Contributed to the writing of the manuscript: RK DS SS SN. Organization of sample collection from patients: RK MR SS. doi:10.1371/journal.pntd.0003198.g001

Figure 4. Endogenous IFN-γ limit parasite replication. a) Parasite load in ex-vivo SA culture of VL patients (n = 31) treated with neutralizing anti-human-IFNγ antibodies or isotype control IgG2b antibodies for three days. Parasite load was determined by limiting dilution on NNN-blood agar medium. b) IL-10 levels in SA culture supernatants (n = 10), treated as described in a. c) Effect of CD4 depletion on spontaneous IFN-γ production in SA-biopsy cultures. Each dot represents one sample, paired samples connected by a line. Comparison between treatments was made using Wilcoxon matched paired test and statistical significances are indicated in the graphs when P<0.05.

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