IL-17 and IL-22 are associated with protection against human kala azar caused by *Leishmania donovani*

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IL-17 and IL-22 have been shown to increase protection against certain bacteria and fungal pathogens in experimental models. However, no human studies have demonstrated a crucial role of IL-17 and IL-22 in protection against infections. We show here that *Leishmania donovani*, which can cause the lethal visceral disease Kala Azar (KA), stimulates the differentiation of Th17 cells, which produce IL-17, IL-22, and IFN-γ. Analysis of Th1, Th2, and Th17 cytokine responses by cultured PBMCs from individuals in a cohort of subjects who developed KA or were protected against KA during a severe outbreak showed that IL-17 and IL-22 were strongly and independently associated with protection against KA. Our results suggest that, along with Th1 cytokines, IL-17 and IL-22 play complementary roles in human protection against KA, and that a defect in Th17 induction may increase the risk of KA.

**Introduction**

Th17 cells are independently regulated CD4+ T cells initially characterized as producing cytokines in the IL-17 family. They are highly proinflammatory and stimulate the production, by endothelial and epithelial cells and monocytes, of cytokines such as IL-6 and TNF and chemokines such as CXCL8 (also known as IL-8), CXCL10 (also known as IP-10), CXCL1, and CXCL6 (1). Th17 cells carry skin and mucosal homing receptors such as CCR6 and CCR4 (2) and recruit neutrophils and monocytes within tissues (3). IL-17 is mostly produced by Th17 and NKT cells (1). IL-22 is also produced by Th17 cells, and to a lesser extent by Th1 and NK cells (1), and is involved in immunity at the epithelium and mucosal surfaces (4). It also promotes the inflammatory response and participates in tissue repair (5, 6). The functional IL-22 receptor is expressed on hepatocytes, keratinocytes, and fibroblasts, but not on hematopoietic cells (4, 7). Both IL-17 and IL-22 have been shown to increase protection against certain bacteria and fungal pathogens in experimental models (2, 3, 8–13). However, this protective role has yet to be confirmed in humans. We show here that *Leishmania donovani* strongly induces Th17 cell differentiation and that IL-17 and IL-22 are strongly and independently associated with resistance to KA.

*L. donovani* is a protozoan parasite of macrophages responsible for a lethal visceral disease, kala azar (KA). Infected children die due to the multiplication of *L. donovani* in the spleen and liver, associated with splenomegaly, pancytopenia, anemia, and disseminated hemorrhages (14). The protective immune mechanisms operating at early stages of skin and liver infection are not well understood. Most subjects recovering from *L. donovani* infection after drug treatment are protected against repeat infection by Th1-mediated immunity (15). However, one-third of cured patients present subsequent relapses.

Conflict of interest: The authors have declared that no conflict of interest exists.

Nonstandard abbreviations used: KA, kala azar; OR, odds ratio.

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and IL-22 in response to stimulation with concanavalin A, phytohemagglutinin, or ionomycin plus PMA (Figure 1B). Adherent cells produced neither IL-17 nor IL-22 (data not shown). FACS analysis (Figure 1C) showed that stimulation with L. donovani increased the proportion of CD4+IL-17+ cells in cultures by 3- to 70-fold and that 9% to 60% of the induced Th17 cells produced IFN-γ (Table 1). L. donovani induces IL-6, IL-1β, and IL-23 in cultures. Experimental work with human cells has shown that IL-6, TGF-β, IL-1β, IL-23, and possibly IL-21 induce or maintain Th17 cells (1, 24–26). IL-1γ and IL-22 are the cytokines most strongly associated with protection against KA. IL-17 was detectable in 80% of cultures of cells from gp1 subjects, compared with only 30% of cell cultures from gp2 subjects. IL-17 (P < 0.001) and IL-22 (P < 0.001) were higher in cultures of cells from gp1 subjects than in cultures of cells from gp2 subjects (Figure 3A). Furthermore, among the IL-17-positive cultures from gp1 and gp2 subjects, IL-17 levels were higher for subjects in gp1 (P < 0.001) than for subjects in gp2. Almost all cultures produced detectable amounts of IL-22. We carried out a logistic regression analysis to assess the relationship between IL-17 production and the risk of KA. We included IL-17, Th1 cytokines, Th2 cytokines, age, and sex as covariates. The best model for distinguishing between gp1 and gp2 subjects, IL-17 levels were higher for subjects in gp1 (P < 0.001) and IL-17 and IL-22 were high in the cultures. Nonparametric tests showed that subjects in gp1 produced more TNF (P < 0.001), slightly more IL-12p40 (P = 0.03), and more IL-10 (P < 0.001) than did subjects in gp2. No significant differences in IL-5 (P = 0.23), IL-13 (P = 0.053), IFN-γ (P = 0.07), and IL-12p70 (P = 0.11) levels were observed between gp1 and gp2. Multivariate analysis was carried out because correlations between cytokine levels, age, and sex might have confounded the analysis. Analysis I (Table 3) indicated that TNF levels were higher in gp1 cultures (P = 0.003; odds ratio [OR] = 0.23, CI 0.09–0.60), whereas the highest IFN-γ levels were observed in gp2 cultures (P = 0.016; OR = 3, CI 1.22–7.40). Age was a significant confounding variable (P < 0.001), whereas sex was not.

We also compared cytokine levels in cultures of cells from gp1 with those in cultures of cells from gp3. Univariate analysis showed that the subjects in gp3 produced less TNF (P = 0.05) and less IFN-γ (P = 0.03) than did subjects in gp1. However, multivariate analysis revealed no significant associations.

Table 1

<table>
<thead>
<tr>
<th>Experiment % IL-17+CD4+</th>
<th>% IL-17+CD4+ in CD4+</th>
<th>% IL-17+CD4+ in CD4+</th>
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<td></td>
<td>Unstimulated</td>
<td>Ld-stimulated</td>
</tr>
<tr>
<td>1</td>
<td>0.41</td>
<td>2.64</td>
</tr>
<tr>
<td>2</td>
<td>0.13</td>
<td>0.51</td>
</tr>
<tr>
<td>3</td>
<td>0.57</td>
<td>1.72</td>
</tr>
<tr>
<td>4A</td>
<td>0.07</td>
<td>5.14</td>
</tr>
<tr>
<td>5A</td>
<td>0.12</td>
<td>0.24</td>
</tr>
<tr>
<td>6A</td>
<td>0.12</td>
<td>0.93</td>
</tr>
</tbody>
</table>

Six independent experiments on PBMCs primed and boosted with OpsLd, as described in Figure 1C, are presented. * Cultures supplemented with 10 U/ml IL-2. ND, not determined.


Research article
IL-22 class. The proportion of resistant subjects was highest in the classes with the highest levels of IL-17 and IL-22. Conversely, the proportion of subjects with KA increased with decreasing IL-17 and IL-22 levels. We investigated the possibility that this result indicated independent contributions of IL-17 and IL-22 to protection rather than a simple correlation between the levels of these 2 cytokines, by carrying out a regression analysis that included both cytokines and age. IL-17 ($P < 0.001$) and IL-22 ($P = 0.01$) were independently associated with resistance (analysis III, Table 3). An increase of 1,000 pg/ml in IL-22 concentration was associated with a 0.66-fold (CI 0.47–0.91) decrease in the risk of KA, whereas an increase of 100 pg/ml IL-17 concentration was associated with a 0.34-fold (CI 0.22–0.52) decrease in the risk of KA. IL-22 and IL-17 levels in cultures varied from 15 to 10,000 pg/ml and from 15 to 1,000 pg/ml, respectively.

A weak IL-17 response was predictive of KA. The defect in IL-17 and IL-22 in gp2 may be secondary to multiple changes in the immune cytokine network that could have been activated in patients with KA and persisted after clinical cure. We also evaluated IL-17 and IL-22 production in cultures of PBMCs from gp3 subjects. These patients produced significantly less IL-17 than did gp1 subjects ($P = 0.009$), as shown by logistic regression analysis (analysis IV in Table 3). The production of small amounts of IL-17 in culture was associated with a 13-fold increase (OR = 0.084, CI 0.013–0.54) in the risk of KA within 6 months of cytokine evaluation. Once again, IL-17 had the most significant effect, excluding all the Th1 and Th2 cytokines tested from the regression model. We also assessed the effect of IL-22 in the presence of IL-17. We found that both high IL-17 ($P = 0.01$; OR = 0.32, CI 0.13–0.77) and high IL-17 ($P = 0.001$, $P < 0.001$, and IL-1$\beta$ ($P < 0.001$) in cultures of PBMCs and on IL-23 ($P = 0.05$), IL-6 ($P = 0.02$), and IL-1$\beta$ ($P < 0.01$) in cultures of monocytes. Culture duration had a significant effect ($P < 0.01$) in all cultures excepted for IL-1$\beta$ (PBMCs and monocytes) and IL-23 (monocytes). Data are means ± SEM.

**Figure 2**

*L. donovani* induces the production of cytokines that are required for Th17 induction and maintenance. IL-6, IL-23, and IL-1$\beta$ production in IL-17– and IL-22–producing cultures of PBMCs (A) and monocytes (B) from healthy blood donors. PBMCs were primed (day 0) and boosted with OpsLd (day 7), as described in the legend of Figure 1. Monocytes were stimulated once with living *L. donovani* that were opsonized with either HIS or NHS. Cytokine levels were determined at the time points indicated. Results represent the arithmetic means of data from 4 independent experiments with PBMCs and 4 experiments with monocytes. The effects of the stimulation and culture duration were tested by linear regression ($P < 0.05$ was considered significant). Stimulations had a significant effect on IL-17 ($P = 0.003$), IL-22 ($P = 0.05$), IL-6 ($P < 0.001$), and IL-1$\beta$ ($P < 0.001$) in cultures of PBMCs and on IL-23 ($P = 0.05$), IL-6 ($P = 0.02$), and IL-1$\beta$ ($P < 0.01$) in cultures of monocytes. Culture duration had a significant effect ($P < 0.01$) in all cultures excepted for IL-1$\beta$ (PBMCs and monocytes) and IL-23 (monocytes). Data are means ± SEM.
IL-17 and IL-22 levels as a function of IL-23, IL-6, and IL-1β levels in cultures, assuming a similar relationship between the levels of IL-17 or IL-22 and regulatory cytokines in gp1 subjects and gp2 subjects (Figure 4B). Surprisingly, cultured cells from gp2 subjects produced much lower levels of IL-17 for the same amounts of the 3 regulatory cytokines. Cultures of cells from gp1 subjects showed a clear correlation of IL-17 concentration with the concentration of all 3 regulatory cytokines. In contrast, we did not observe such a correlation between IL-17 levels and IL-1β and IL-6 in gp2 cultures. This suggests that a defect in the Th17 differentiation pathway may affect the response to these 2 cytokines in subjects susceptible to KA. The picture was less clear in a similar analysis for IL-22: the IL-22 response increased with IL-23 and IL-6 concentrations. However, this increase was less marked in gp2 subjects than in gp1 subjects.

### Table 2

<table>
<thead>
<tr>
<th>n</th>
<th>Age±</th>
<th>Gender (male/female)</th>
<th>Western blot</th>
<th>IFN-γ±</th>
<th>Selection criteria</th>
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<tbody>
<tr>
<td>gp1</td>
<td>87</td>
<td>32 ± 18</td>
<td>45/42</td>
<td>92%</td>
<td>Positive Western blot, IFN-γ in cultures, and no infection with KA during the entire study</td>
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<tr>
<td>gp2</td>
<td>122</td>
<td>16 ± 9</td>
<td>69/53</td>
<td>99%</td>
<td>KA (Leishmania parasites in lymph nodes, and clinical symptoms). Successfully cured 6–24 months before the study</td>
</tr>
<tr>
<td>gp3</td>
<td>20</td>
<td>16 ± 10</td>
<td>5/15</td>
<td>89%</td>
<td>Positive Western blot and/or IFN-γ, and infection with KA within 6 months following cytokine evaluation</td>
</tr>
</tbody>
</table>

*Data are the arithmetic mean ± SD. ①Stained positive in the 14- to 16-kDa band of a Western blot.*

Discussion

Our studies with PBMCs from healthy individuals and from subjects exposed to *L. donovani* showed that *Leishmania* strongly induced IL-17 and IL-22. This conclusion was further supported by the presence in *L. donovani*-stimulated cultures of IL-1β, IL-23, and IL-6, the key cytokines for the induction and maintenance of the Th17 response (1, 24, 26, 28, 29). No IL-21 was detected, consistent with other reports suggesting that IL-21 may be dispensable for human Th17 induction (30, 31). The heat-killed or parasite extracts used to induce cytokine responses in PBMCs is mostly confined to the presence in *L. donovani*–stimulated cultures of IL-1β and IL-6, the key cytokines that promote the recruitment of Th17 cells by stimulating the release of chemokines, such as CXCL8 (IL-8), CXCL1, and CXCL6 (GCP2), which serve as potent chemoattractants for neutrophils or CXCL10 (IP10), which acts as a chemoattractant for Th1 cells (1, 7). IL-17 also increases the production of IL-6, which has proinflammatory and regulatory effects on the immune response (1). IL-22 increases the production of proinflammatory molecules, such as the S-100A proteins and CXCL5 (6). IL-17 and IL-22 synergistically increase the production of antimicrobial peptides, such as β-defensins, by epithelial cells (37). Finally, IL-22 is involved in epithelial repair (6) and liver protection (38) in chronic infections. Both the increases in epithelial protective barrier function and the recruitment of inflammatory cells, including neutrophils, to the skin and liver, could contribute to protection against *L. donovani*. Work in animals infected with other pathogens has provided support for the view that Th17 cells, IL-17, and IL-22 may be essential for immunity to certain infections (2, 3, 9–13). In these experimental models, neutrophils are important, both as effectors and as modulators of the immune response via secreted molecules such as IL-12. Large numbers of Th17 cells were shown to infiltrate *Mycobacterium tuberculosis* granuloma in vaccinated mice (3), the mucosal sites of *Klebsiella pneumoniae* infection in the lung (39, 40), and the colon of *Citrobacter rodentium*-infected mice (41). IL-23 and IL-22 are essential for the expression of full immunity in these experimental models (3, 39, 41). Finally, Th17 may bridge the gap between innate and adaptive immunity. Th17 cells are required for a recall response of mice vaccinated with *M. tuberculosis* antigen, as they promote the recruitment of Th1 in the lungs by stimulating the release of mediators such as CXCL10 (3). Th17 cells could play the same role in *L. donovani* infections. Naturally resistant subjects with enhanced IL-17 and IL-22 responses would thus react more rapidly to *L. donovani*, attracting strong effectors of innate immunity and recruiting Th1 cells to tissues. These cells would in turn enhance the microbial activity of phagocytes. Thus, Th17 and Th1 cells may play complementary roles in protection against *L. donovani*, with both being required for complete protection. In this association between Th1 and Th17 responses, Th1 cells may play the important role of downregulating the Th17 response after the infection is controlled.
thereby preventing tissue damage resulting from the out-of-control expansion of the Th17 cell population.

We explored the mechanisms that may have accounted for the weak IL-22 and IL-17 responses in KA subjects. The IL-22 response induced by *L. donovani* in our cultures was more dependent on IL-6 than on IL-23, and conversely the IL-17 response was more dependent on IL-23 than on IL-6. Other studies have reported different cytokine requirements for IL-22 and IL-17 production, with IL-22 production dependent on IL-6 but not on TGF-β, and with IL-17 production dependent on TGF-β and IL-6 (42). IL-23 is required to maintain the Th17 response (26, 43). The lower level of production of IL-6 and IL-23 in cultures from subjects with KA may have contributed to the weaker IL-17 and IL-22 responses of these subjects. Nevertheless, IL-6 is also produced by Th17 cells (1), and lower levels of IL-6 production in cultures may be a consequence rather than a cause of the smaller number of Th17 cells. Furthermore, although certain cultures of cells from subjects with KA displayed high levels of IL-6, IL-1β, and IL-23 production, IL-17 levels remained low, suggesting a poor T cell response from KA subjects to IL-6 and IL-1β. This defective response to regulatory cytokines was more marked with IL-17 than with IL-22, probably because IL-22 was also produced by Th1 cells (7). Our observations suggest a defect rendering T cells less responsive to key regulators, such as IL-1β and IL-6, that are crucial for Th17 induction.

In conclusion, we show here that *L. donovani* strongly induces IL-17 and IL-22 responses. We have presented evidence that IL-17 and IL-22 play complementary roles in natural protection against *L. donovani*. This view is consistent with the effects of Th17 on monocytes and neutrophils and the ability of Th17 to recruit Th1 cells to the granuloma. In addition, IL-22 and IL-17 promote tissue repair and strengthen epithelial barriers, thereby contributing to immuno-sur-

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**Figure 3**

Resistance to KA is associated with enhanced IL-17 and IL-22 responses. (A) IL-17, IL-22, and Th1 and Th2 cytokine production by PBMCs from gp1, gp2, and gp3 subjects. Cytokines were quantified by ELISA in supernatants of PBMCs stimulated with 10 μg/ml of *L. donovani* extracts, as indicated in the Methods. IL-4 was undetectable or present in small amounts in all cultures. The data presented are the arithmetic means ± SEM. Cytokine scales (y axes) were set to allow for a 5-fold increase in the mean levels above those of the group with the lowest levels to be represented. (B) Resistance to KA was associated with high levels of both IL-22 and IL-17, whereas KA was associated with low levels of these cytokines. IL-17 and IL-22 levels were each assigned to 3 classes of equal size. The 3 classes for IL-17 were ≤15, 16–120, and 121–1,250 pg/ml. The 3 classes for IL-22 were <190, 190–890, and 900–40,000 pg/ml. Dark bars represent subjects with the lowest IL-17 and IL-22 levels. Data analysis is presented in Table 3.
L. donovani antigens were prepared as described elsewhere (47). The strain MHOM/SD/98/LEM 3566 was isolated from a patient with KA in the village (46) and maintained in promastigote form by in vitro culture at 24°C in RPMI-1640 medium (Invitrogen) supplemented with 10% FBS (Cambrex). Soluble L. donovani antigens were prepared as described elsewhere (47).

Parasite opsonization. Serum samples from healthy white donors from the blood bank in Marseille (Etablissement Français du Sang) (normal human serum [NHS]) and pools of human immune serum (HIS) from subjects living in Babar were used. Parasites were incubated for 45 minutes at 56°C. Parasites were then washed three times in cold RPMI-1640 medium. The parasites that reacted with HIS and NHS will be referred to hereafter as “OpsLd” and “NHSLd,” respectively.

Cell purification and cell cultures. PBMCs were separated from blood by centrifugation over a Ficoll/Hypaque cushion and cultured at 2 × 10^6 cells/ml in RPMI-1640 medium supplemented with 10% FBS and 100 U/ml penicillin/streptomycin. PBMCs from patients (2 × 10^6 cells/ml) were stimulated with 10 μg/ml of L. donovani antigens (referred to as “Ld-ext”). PBMCs from healthy donors were stimulated with 10 μg/ml Ld-ext and again on day 7 with the same stimulations. Monocytes and CD4+ T cells were purified (>98%) by magnetic cell sorting (Miltenyi Biotec) from PBMC cultures. Monocytes (8 × 10^6 cells/500 μl) and purified CD4+ T cells (2 × 10^6 cells/200 μl), purified

### Table 3

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Tested covariates</th>
<th>Covariate in the model</th>
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<td>Th1 and Th2 cytokines</td>
<td>IFN-γ</td>
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### Logistic regression analysis of IL-17, IL-22, and Th1 and Th2 cytokines in the risk of KA

Analysis I compared Th1 and Th2 cytokines in the risk of KA. Analysis IIa added IL-17 to the same analysis and showed the strongest association with protection against KA. Analysis IIb compared IL-22 and Th1 and Th2 cytokines in the risk of KA. Analysis III found that both IL-22 and IL-17 were independently associated with resistance to KA. Analysis IV found that IL-17 was associated with protection against KA that occurred in the months following cytokine evaluation. An OR greater than 1 indicates an increased risk with an increase of the covariate; an OR less than 1 indicates a decreased risk with an increase of the covariate. When the covariates were used as a qualitative variable, the indicated cytokine levels were used to define the covariate class. If IL-17 and IL-22 were treated as quantitative variables, in order to yield a meaningful OR, IL-17 and IL-22 levels were multiplied by 0.01 and 0.001, respectively (e.g., in analysis I, an increase of 100 pg/ml for IL-17 and 1,000 pg/ml for IL-22 corresponded to a reduction of 0.32 and 0.61, respectively, in the risk of KA).

The study was approved by the ethics committee of the University of Khartoum Faculty of Medicine. Informed consent was obtained from all patients. The study was also approved by the village committee.

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on day 15 from PBMC cultures, were stimulated with PMA (100 ng/ml) plus ionomycin (1 μg/ml), concanavalin A (5 μg/ml), or phytohemagglutinin (5 μg/ml) (Sigma-Aldrich). Resting cells were used as controls.

**Intracellular cytokine staining.** Cells were stained for surface markers with PE-Cy7–conjugated anti-CD4 (eBiosciences) antibodies. The labeled cells were pulsed with 100 ng/ml PMA, 1 μg/ml ionomycin and monensin (BD GolgiStop) for 5 hours. Cells were then fixed and permeabilized with BD Cytofix/Cytoperm according to the manufacturer's instructions and incubated with Alexa Fluor 647–conjugated anti–IL-17 and PE-conjugated anti–IFN-γ (eBiosciences) antibodies. Isotype controls were obtained from the corresponding manufacturers. Data were analyzed on a FACSCalibur flow cytometer, using CellQuest software (BD Biosciences).

**Cytokine titration.** Cytokines in the supernatants of cultures were assayed with ELISA kits according to the manufacturer's instructions. In cultures from endemic subjects, IL-6 (Diaclone), IL-17A (R&D Systems), IL-22 (R&D Systems), IL-21 (eBiosciences), IL-5 (BD Biosciences), and IFN-γ (BD Biosciences) levels were measured at 120 hours. IL-1β (R&D Systems), IL-4 (Abcys), IL-23 (eBiosciences), IL-12p40 (BD Biosciences), IL-13 (Diaclone), and IL-10 (BD Biosciences) and TNF (BD Biosciences) were quantified at 48 hours. The supernatants of PBMCs, CD4+ T cells, or monocytes obtained from healthy subjects were collected at the times indicated in the figures. The lower limits of detection for the ELISA analyses were as follows: 0.78 pg/ml for IL-13; 1.56 pg/ml for IL-6; 1.9 pg/ml for IL-1β; 5 pg/ml for IFN-γ; 7.8 pg/ml for IL-23; IL-4, TNF, and IL-10; 15 pg/ml for IL-12p40, IL-17A, and IL-22; and 32.5 pg/ml for IL-21.

**Statistics.** The statistical methods that were used are indicated in the tables and figures. Univariate comparisons were carried out using non-parametric tests (Mann-Whitney tests). P < 0.01 was considered as indi-
cating a significant association and P < 0.05 a suggestive association. The association of cytokines with the risk of KA was also evaluated by logistic regression using clinical binary traits (the covariates were age, sex, and cytokines. Cytokines were assigned to 3 classes (1, 2, and 3) of equal size and treated as binary variables (1 versus 2 + 3, and 1 + 2 versus 3). The cytokine levels defining the classes significantly associated with the phenotype in the regression analysis are indicated in the Table 3 legend. When IL-17 and IL-22 were tested jointly in the model, they were entered as linear variables, making it possible to account for the correlation between the levels of these 2 cytokines. Linear regression was used to evaluate the associations between regulatory cytokines (IL-6, IL-23, and IL-1b) and IL-17 or IL-22 levels.

Results are presented as ORs and CIs, which provide an accurate estimate of the relative risk.


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