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IL-27 and IL-21 Are Associated with T Cell IL-10 Responses in Human Visceral Leishmaniasis

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IL-10 is believed to underlie many of the immunologic defects in human visceral leishmaniasis (VL). We have identified CD4+CD25−Foxp3+ T cells as the major source of IL-10 in the VL spleen. IL-27, a member of the IL-6/IL-12 cytokine family, has been shown to promote development of IL-10–producing T cells, in part by upregulating their production of autocrine IL-21. We investigated whether IL-27 and IL-21 are associated with human VL. IL-27 was elevated in VL plasma, and at pretreatment, spleen cells showed significantly elevated mRNA levels of both IL-27 subunits, IL-27p28 and EBI-3, as well as IL-21, compared with posttreatment biopsies. CD14+ spleen cells were the main source of IL-27 mRNA, whereas CD3+ T cells were the main source of IL-21. IL-27 mRNA could be strongly upregulated in normal donor macrophages with IFN-γ and IL-1β, conditions consistent with those in the VL spleen. Last, a whole-blood assay revealed that most VL patients could produce Ag-specific IFN-γ and IL-10 and that the IL-10 could be augmented with recombinant human IL-21. Thus, proinflammatory cytokines acting on macrophages in the VL spleen have the potential to upregulate IL-27, which in turn can induce IL-21 to expand IL-10–producing T cells as a mechanism of feedback control. The Journal of Immunology, 2011, 186: 000–000.

Visceral leishmaniasis (VL) or kala-azar is a chronic infectious disease caused by the protozoan parasite Leishmania donovani or Leishmania chagasi/infantum. VL is endemic in 62 countries, with a total of 200 million people at risk (1). Ninety percent of the global burden of kala-azar cases occurs in the Indian subcontinent and East Africa where the causative agent is L. donovani. Every year, >100,000 cases of VL occur in India alone, mainly among the poorest population groups living in rural areas (2).

Clinically, the disease is characterized by fever, hepatosplenomegaly, weight loss, anemia, leucopenia, and hypergammaglobulinemia, accompanied by high titers of anti-leishmanial Abs. Following inoculation into the skin by vector sand flies, the parasite infiltrates vital organs and replicates primarily in macrophages of the liver and spleen. Despite the progressive nature of the disease, elevated serum levels of IFN-γ and TNF-α, as well elevated mRNA levels of these cytokines in lesional tissue, are consistent findings in human VL (3–7), suggesting that immunosuppressive mechanisms that inhibit the production of adequate concentrations or functions of effector cytokines may be operating in VL.

On the basis of the extensive experimental and clinical findings, IL-10 is believed to underlie many of the immunologic defects in kala-azar (reviewed in Ref. 8). IL-10 is a regulatory cytokine that has primarily suppressive effects on immune function, targeting multiple activation and Ag presentation pathways of macrophages and dendritic cells (DCs). Although induction of IL-10 by host cells during chronic infection is considered a homeostatic mechanism to limit the tissue damage caused by excessive inflammation, effective clearance of Leishmania can also be compromised. In mice, genetic ablation of IL-10 renders mice highly resistant to L. donovani infection, and treatment with anti–IL-10R Ab promotes clinical cure (9, 10). In humans, patients with an advanced stage of disease have elevated levels of IL-10 in serum as well strongly enhanced IL-10 mRNA levels in spleen, lymph nodes, and bone marrow (3–7). A role for IL-10 in human VL pathology is supported by studies demonstrating the effect of IL-10 inhibition on enhancement of the IFN-γ response by Ag-stimulated PBMC and by the finding that IL-10 neutralization in VL serum inhibited L. donovani replication in macrophages (7, 11, 12).

IL-10 can be produced by many cell types, including innate cells, B cells, and multiple T cell subsets. We have recently addressed the cellular source of IL-10 in human VL and identified CD4+CD25−Foxp3+ T cells as the major source of elevated IL-10 mRNA in the VL spleen (7). The findings suggest that IL-10–producing adaptive regulatory T or Tr1 cells, some of which may coexpress IFN-γ, are important in suppression of anti-leishmanial immunity in human VL. Similar cells have been shown to be an important source of IL-10 in L. donovani–infected mice and in C57BL/6 mice infected with a nonhealing strain of L. major (13–15). There have been recent advances concerning the factors that regulate IL-10 production by Tr1 cells or Th1 cells in autoimmune or infection models in mice. Although originally described as a cofactor with IL-12 for Th1 differentiation, a series of recent reports have implicated IL-27, a member of the IL-6/IL-12 cytokine family, as central to the regulation of T cell IL-10 production (reviewed in Refs. 16 and 17). Furthermore, autocrine IL-21 has
been shown to amplify IL-27–induced T cell IL-10 production in the mouse (18). IL-27 is a heterodimeric cytokine composed of an IL-12p35–related p28 subunit and an IL-12p40–related EBV-induced 3 (EBI-3) subunit and is produced primarily by innate cells such as macrophages and DCs (19). IL-27 signals through a receptor containing the common IL-6 signal transducing subunit gp130 and the unique IL-27Rα subunit. Analysis of the expression patterns of IL-27p28 and EBI-3 has shown that these subunits can be differentially regulated; although some cell types and tissues express both, others express only one, suggesting that the subunits might dimerize extracellularly, or associate with different subunits of the IL-12 family (e.g., IL-35, composed of EBI-3 and p35) (20). Several recent studies indicate that IL-27p28 is regulated at the transcriptional level by TLR ligation and TRIF-mediated activation of IFN regulatory factor (IRF)-3 and/or by types I and II IFN through recruitment of STAT-1/IRF-1 and IRF-8 to the human or mouse IL-27p28 promoter (21–23). In the mouse, EBI-3 is also strongly induced by TLR agonists via activation of transcription factors PU.1 and NF-κB (24).

In murine studies, whereas IL-27 exerts STAT1-dependent Th1–inducing signals in naive cells, it appears to convert activated, inflammatory CD4+ T cells into IL-10–producing Th1 or Tr1 cells in a STAT1- and STAT3-dependent manner (25), presumably as a mechanism of feedback control. Thus, IL-27R–deficient mice can display both a Th1 response defect and exacerbated inflammation, depending on the nature and duration of the inflammatory stimulus. In IL-27R–deficient mice infected with *L. donovani*, the mice displayed enhanced resistance but developed severe liver inflammatory CD4+ T cells into IL-10–producing Th1 or Tr1 cells inducing signals in naive cells, it appears to convert activated, factors PU.1 and NF-

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logy was also associated with *L. major* infection in IL-27R–deficient mice, which in this case was related to reduced fre-

quencies of IL-10–producing Th1 cells and to a striking increase in Ag-specific IL-17–producing CD4+ T cells (27). The ability of IL-27 to suppress Th17 development in other inflammatory set-

tings in the mouse has been reported previously (28, 29).

Apart from a series of in vitro studies demonstrating the role of IL-27 in promoting IL-10 production by human CD4+ T cells and in inhibiting Th17 development (30), only a few studies have investigated the pathophysiological roles of human IL-27, mainly in inflammatory conditions (31, 32). The study of IL-27 in human infection has been confined to the assessment of circulating levels of the cytokine in patients with HIV or hepatitis B virus (33, 34). The aim of the current study was to determine whether IL-27−, IL-21−, and Th17-related cytokines are upregulated in human VL and to understand the cellular source of these cytokines and their possible role in regulation of T cell IL-10 production and in the pathogenesis of this disease.

**Materials and Methods**

**Study subjects**

All patients presented with symptoms of VL at the Kala-azar Research Center (Muzaffarpur, Bihar, India). Their diagnosis was in each case healthy household family members of an active VL case. Aggregate clinical data of patients are presented in Table I. All patients were seronegative for HIV. This work was conducted with ethical approval obtained from institutional review com-

mittees in India and in the United States, and informed consent was obtained from all patients or their guardians.

**Detection of cytokine in plasma**

Plasma levels of IFN-γ, IL-10, and IL-17 were measured by multiplex analysis (Aushon Biosystems, Billerica, MA). IL-27 and IL-21 plasma levels were measured by dual Ab sandwich-ELISA kits, according to the manufacturer’s instruction (R&D Systems, Minneapolis, MN, and eBioscience, San Diego, CA, respectively). The IL-27 ELISA detects the heterodimeric cytokine. Each sample was tested in duplicate, and cytokine concentrations were calculated using a standard curve generated from re-

combiant cytokines. Cytokine values were expressed as picograms per milliliter.

**Real-time PCR with whole and sequentially enriched splenic cell subsets**

Whole splenic needle aspirates (100–150 μl) were directly placed in 1 ml RNAlater (Qiagen, Valencia, CA) and stored at −70°C until use. Total RNA was isolated using the RNasy minikit and QIAshredder homoge-

nizers (Qiagen), according to the manufacturer’s protocol. The quality of RNA was assessed by denaturing agarose gel electrophoresis. cDNA synthesis was performed in 20-μl reactions on 0.5–1 μg RNA using the High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA), which uses random primers and MultiScribe Moloney murine leukemia virus reverse transcriptase enzyme. Incubation conditions for reverse transcription were 10 min at 25°C, followed by 2 h at 37°C, and was performed on a MasterCycler Gradient (Eppendorf North America, Westbury, NY).

RNA was prepared from enriched fractions of splenic aspirate cells, isolated using a modification of the sequential selection protocol described previously (7). Briefly, cell subsets were enriched by sequential positive selection of CD19+ (B cells), followed by CD3+ (T cells) cell selection from the CD19-depleted fraction, CD14+ (monocytes/macrophages) cells were selected from the CD19- and CD3-depleted fraction, followed by CD1+ (DCs) cell selection from the CD19-, CD3-, and CD14-depleted fraction. Finally, CD56+ (NK cells) cells were selected from the CD19-, CD3-, CD14-, and CD16-depleted fraction. All selections were carried out using MACS beads (Miltenyi Biotech, Auburn, CA) and Magnetic LS columns (Miltenyi Biotech), according to the manufacturer’s instructions. Cells remaining after the removal of CD19+, CD3+, CD14+, CD16+, and CD56+ cells were referred to as “depleted.” The positively selected subsets and depleted cells were resuspended in 200–300 μl RNA later and stored at −70°C until use. RNA isolation and cDNA preparation were carried out as described above, and the extent of subset enrichment was validated by PCR amplification of cell-specific markers. Real-time PCR was performed on an ABI Prism 7900HT sequence detection system (Applied Bio-

systems) using cDNA-specific FAM-MGB–labeled primer/probe for IL-10, IFN-γ, IL-27p28, EBI-3, IL-21, IL-12p19, IL-1β, IRF-1, IL-17, ROspy, CD14, CD19, CD1c, and VIC-MGB–labeled 18S mRNA. All reagents were purchased from Applied Biosystems. The relative quantification of products was determined by the number of cycles over 18S mRNA en-

dogenous control required to detect the mRNA of interest.

**In vitro generation of monocyte-derived macrophages**

CD14+ monocytes were obtained by elutriation from normal volunteer blood donors at the National Institutes of Health Clinical Center Department of Transfusion Medicine. Cells were frozen at 1 × 10^6 cells/ml until use. Briefly, monocytes were thawed, and 50 × 10^6 cells were cul-
tured in 6-well tissue culture plates in a volume of 2 ml. All cells were cultured in complete RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 25% heat-inactivated human AB serum (Gemini Bio-

Products, Woodland, CA), 20 mM l-glutamine, 100 U/ml penicillin, and 100μg/ml streptomycin (Invitrogen) at 37°C under 5% CO2. For genera-
tion of macrophages, recombinant human (rh)M-CSF (PeproTech, Rocky Hill, NJ) was added to cultures on days 0, 3, and 5 at 400 ng/ml. Cells were harvested on day 8. Cell numbers and viability were determined by trypan blue exclusion.

**In vitro activation of macrophages and infection**

Harvested macrophages were resuspended at 1–2 × 10^6/ml in complete RPMI 1640 medium, plated in a 48-well tissue culture plate, and stimu-
lated with IFN-γ (10 ng/ml), TNF-α (10 ng/ml), (PeproTech) their combi-
nation, or medium alone for 8 h prior to infection with hamster-derived, tissue-purified amastigotes (4:1 ratio of *L. donovani* strain 9515 (MHOM/
IN/95/9515) isolated from a patient with VL in Bihar, India. Macrophages were cultured for an additional 16 h and either lysed for mRNA expression analysis or harvested for Wright-Giemsa staining of cytospin preparations to monitor infections by light microscopy.

Whole-blood culture assay and cytokine measurement

Heparinized whole blood was collected from active VL patients and ECs. Because plasma from VL cases contain pre-existing levels of cytokines, the plasma was removed, and the blood cells were washed once with saline. The autologous plasma was replaced with an equivalent volume of plasma pooled from healthy individuals living in a nonendemic area for VL. Whole-blood cells were cultured in the absence of Ag or stimulated with *L. donovani*-soluble Ag (SLA; 10 μg/ml) prepared from *L. donovani* stationary-phase promastigotes (prepared as described previously (7)). Where indicated, recombinant human IL-27 (100 ng/ml) (R&D Systems) or human IL-21 (25 ng/ml) (PeproTech) alone or in combination were added to the whole-blood cultures. Cultures were incubated at 37˚C with 5% CO2. After 24 h, culture supernatants were collected and stored at −270˚C until ELISA measurement. The frozen culture supernatant from the whole-blood assay was thawed, and the levels of IFN-γ and IL-10 were measured using Biolegend kits (BioLegend, San Diego, CA), according to the manufacturer’s instruction. Cytokine concentrations in the samples were calculated with a standard curve generated from recombinant cytokines.

Statistics

For statistical analysis, data were analyzed using GraphPad Prism 5 software (GraphPad Software). Differences between groups or paired pre- and posttreatment groups were compared by Mann-Whitney and Wilcoxon signed-rank tests for unpaired and paired analyses.

Results

Active VL is associated with elevated levels of IL-27 in plasma and IL-27 mRNA in spleen

We and others have previously demonstrated elevated plasma levels of IL-10, as well as elevated mRNA levels for IL-10 and IFN-γ in splenic biopsies from VL patients (7, 35). We also demonstrated that T cells are the main source of IL-10 and IFN-γ in the VL spleen. In the mouse, IL-27 was recently found to induce Th1 cell differentiation and T cell IL-10 production and to counterregulate Th17 lineage development. We were interested to know whether a similar pathway might regulate T cell IL-10 production in VL patients. Screening of plasma for IL-27, IL-10, IFN-γ, IL-17, and IL-21 by ELISA and multiplex assays revealed significantly elevated circulating levels of IL-27, IL-10, and IFN-γ in pretreatment samples of VL patients compared to posttreatment samples or ECs.

![FIGURE 1. Cytokines in plasma from VL patients. IFN-γ, IL-10, and IL-17 were measured by a multiplex analysis of plasma obtained from paired VL patients at pre- and posttreatment (*n* = 16) and from ECs (*n* = 10). Dual Ab sandwich ELISA was used to measure plasma IL-27 levels in paired VL patients (*n* = 21) and ECs (*n* = 10). Significant differences are indicated with *p* < 0.05, **p < 0.01, and ***p < 0.001.](http://www.jimmunol.org/)

### Table I. Aggregate clinical data for VL patients and ECs

<table>
<thead>
<tr>
<th></th>
<th>VL (Pretreatment)</th>
<th>VL (Posttreatment)</th>
<th>EC</th>
<th>Control Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>n</em></td>
<td>108</td>
<td>39</td>
<td>29</td>
<td>10</td>
</tr>
<tr>
<td>Age</td>
<td>26.15 ± 15.26 (25)</td>
<td>27 ± 13.42 (25)</td>
<td>33.51 ± 9.23 (35)</td>
<td>ND</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>69/39</td>
<td>25/14</td>
<td>18/11</td>
<td>ND</td>
</tr>
<tr>
<td>Duration of illness (days)</td>
<td>23.71 ± 29.62 (15)</td>
<td>27.2 ± 23.26 (20)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Infection score†</td>
<td>2.07 ± 1.17 (2)</td>
<td>1.92 ± 1.06 (2)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Day 0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day discharge</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spleen size (cm)</td>
<td>4.74 ± 3.9 (3)</td>
<td>4.87 ± 3.34 (4)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Day 0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day discharge</td>
<td>0.53 ± 1.34</td>
<td>0.82 ± 1.63</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>WBC (×10⁸/mm³)</td>
<td>3.18 ± 1.29 (3)</td>
<td>3.09 ± 0.96 (3)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Day 0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day discharge</td>
<td>7.12 ± 2.44 (7)</td>
<td>6.36 ± 2.12 (6.2)</td>
<td>N/D</td>
<td>N/A</td>
</tr>
</tbody>
</table>

*a* Includes 39 donors from whom posttreatment samples were also available.

*b* Mean values ± SD of aggregated data are shown, and median values are in parentheses.

†Scoring of parasite load is on a logarithmic scale from 1 to 6, where 0 is no parasites per 1000 microscopic fields (×1000), 1 is 1–10 parasites per 1000 fields, and 6 is >100 parasites per field.

N/A, not applicable; ND, not done.
VL samples as compared with posttreatment VL or EC (Fig. 1). Circulating IL-27 has not been previously investigated in human VL, and its elevation was reflected in 18 of 21 paired, pre- and posttreatment samples. In the case of IL-17, low levels were detected in only four of the VL plasma and in none of the plasma from posttreatment cases or ECs. Circulating IL-21 was not detectable in any group (data not shown).

Analysis of mRNA by real-time PCR confirmed elevated mRNA for both IFN-γ and IL-10 in pretreatment splenic biopsies compared with posttreatment samples and to levels in splenic cells obtained from HODs (Fig. 2A). The mRNA elevation was reflected in 15 of 16 paired samples for IFN-γ and 14 of 16 for IL-10. Analysis of IL-27 subunits revealed significant elevation of splenic mRNA levels for both IL-27p28 and EBI-3 in pretreatment samples compared with posttreatment or to HODs (Fig. 2A). Furthermore, the difference was reflected in 16 of 18 paired samples for IL-27p28 and 17 of 18 for EBI-3. We also analyzed IRF-1 and IL-1β mRNA, which was involved in transcriptional upregulation of IL-27. Both transcripts were significantly elevated in pretreatment compared with posttreatment samples, which was reflected in 12 of 16 paired samples for IRF-1 and 9 of 10 paired samples for IL-1β (Fig. 2B).

IL-27 has inhibitory effects on mouse Th17 lineage development, and a role for Th17 cells in protection against human VL has been suggested (36). Analysis of mRNA levels of RoRγt and IL-17 revealed no difference between the pre- and posttreatment groups, and in fact both transcripts were below or at the limits of detection in the majority of samples (Fig. 2B). By contrast, mRNA levels of IL-21, which has been proposed as a growth factor for Th17 cells (37, 38), was significantly elevated in pretreatment samples, reflected in 10 of 14 paired samples analyzed separately (Fig. 2C). Because IL-21 has also been shown to promote the IL-27–induced expansion of Tr1 cells, its elevated expression may have relevance to the regulation of T cell IL-10 production in VL patients. Finally, we analyzed the mRNA levels of IL-23, a heterodimeric, IL-12 family cytokine member that is also thought to promote Th17 cell differentiation. Distinct from the other cytokine transcripts analyzed, mRNA levels for the IL-23p19 subunit were significantly reduced in pretreatment as compared with posttreatment samples, which was reflected in 14

**FIGURE 2.** Cytokine mRNA expression in VL spleen. A–C, An ex vivo analysis of relative mRNA levels for indicated genes in splenic aspirates from VL patients before (n = 28) or 21–30 d after treatment (n = 18) and in HOD spleen samples (n = 10). Paired pre- and posttreatment samples (n = 18) are also shown separately. Significant differences are indicated with *p < 0.05, **p < 0.01, and ***p < 0.001.
of 18 paired samples (Fig. 2C). By contrast, the IL-12p40 subunit, which is shared with IL-12, was not differentially expressed. The potential functional relevance of the elevated IL-23 expression is reinforced by the coordinated elevated expression of IL-23R mRNA on the posttreatment cells, which was reflected in 8 of the 14 paired samples analyzed (Fig. 2C).

Cellular source of IL-27 and IL-21 mRNA in VL splenic aspirate cells

Because elevated levels of mRNA for IL-27 subunits and IL-21 were observed in splenic cells from active VL patients, and both cytokines have been implicated in regulating T cell IL-10 secretion, we were interested to know the cellular source of these transcripts. Residual mRNA preparations from the sequentially selected, splenic cell subsets obtained from the patient series examined previously (7), composed of whole spleen, CD19+, CD19-CD25-CD3+, and B and T cells depleted, were taken advantage of to analyze these additional cytokine transcripts by RT-PCR. Ex vivo mRNA analysis clearly showed enhanced IL-27p28 expression in the B and T cell-depleted, macrophage-, and DC-enriched fraction (Fig. 3A). The EBI-3 transcripts were also elevated in these cells relative to the T cell-enriched fraction, although not in comparison with the B cells, for which upregulation of EBI-3 on activated human B cells has been described previously (31). By contrast, CD3+ T cells

![Cellular source of IL-27 and IL-21 mRNA in VL splenic aspirate cells](http://www.jimmunol.org/)

**FIGURE 3.** An ex vivo analysis of cytokine mRNA levels in splenic lymphocytes subsets. A, Relative expression of IL-10, IL-21, IL-27p28, and EBI-3 mRNA levels in sequential, positively selected CD19+, CD3+(CD25−) and in the remaining depleted (innate cells: NK, macrophages, DCs) splenic cell population before treatment (n = 8). B, Relative expression of cell-specific markers (CD19/B cell, CD14/monocytes/macrophages and CD1c/DCs on sequential, positively selected CD19+, CD3+, CD14+, CD1c+, and CD56−) and in the remaining depleted splenic cell population. C, Relative expression of mRNA levels for IL-10, IL-21, IL-27p28, and EBI-3 in positively selected splenic cell fractions from VL patients prior to treatment (n = 8). Significant differences are indicated with *p < 0.05, **p < 0.01, ***p < 0.001, and ns, not significant.
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FIGURE 4. IFN-γ and IL-1β induces IL-27 mRNA in human monocyte-derived macrophages. A, Monocyte-derived macrophages from normal donors (n = 7) were stimulated with rhIFN-γ or rhTNF-α or their combination or medium alone for 8 h prior to infection with L. donovani amastigotes. Macrophages were cultured for another 16 h before being lysed for mRNA expression analysis. B, Monocyte-derived macrophages from normal donors (n = 11) were stimulated with rhIFN-γ or rhIL-1β or their combination or medium alone for 24 h before cells were lysed for mRNA expression analysis. Significant differences are indicated with *p < 0.05, **p < 0.01, and ***p < 0.001.

(Depleted of CD25+ cells) were the main cellular source of IL-21 mRNA in the VL spleen. To support and extend the findings regarding the cellular source of elevated IL-27 and IL-21 mRNA in the VL spleen, splenic aspirate cells from a new series of patients was submitted to a sequential enrichment protocol that included positive selection of CD14+ monocytes/macrophages, CD1c+DCs, and CD56+ NK cells. To confirm the enrichment of the intended subsets, we amplified the cell-specific markers targeted by the MACs beads used for the sequential selection. In each case, the mRNA was expressed at highest levels in the appropriate-enriched fraction, with CD1c also expressed at high levels in the CD19−-enriched cells, although the CD1c−-enriched population will have been depleted of B cells during the sequential enrichment (Fig. 3B). The relative transcript abundance for IL-27 subunits clearly demonstrates CD14+ cells (monocytes/macrophages) to be the main source of both IL-27p28 and EBI-3 mRNA (Fig. 3C). By contrast, CD3+ T cells were the main cellular source of IL-10, and especially IL-21 mRNA, in the spleen of active VL patients (Fig. 3B).

IFN-γ and IL-1β induces IL-27 mRNA in human monocyte-derived macrophages

Having determined that CD14+ cells are an important source of IL-27 mRNA in the VL spleen, we were interested to know whether L. donovani amastigotes, and/or proinflammatory cytokines upregulated in VL (IFN-γ, TNF-α, or IL-1β), have a role in IL-27 induction in human macrophages that may be relevant to its elevated expression in the VL spleen. Monocyte-derived macrophages from normal donors were treated with IFN-γ, TNF-α, IL-1β, their combination, or medium alone for 8 h prior to infection with L. donovani amastigotes. Macrophages were cultured for an additional 16 h before being lysed for mRNA expression analysis. The results demonstrate a strong induction of IL-27p28 mRNA in the presence of IFN-γ and a somewhat weaker induction of EBI-3 (Fig. 4A). TNF-α had no impact on either subunit, either alone or in combination with IFN-γ. Notably, L. donovani amastigotes did not induce IL-27 or enhance IL-27 expression. In an additional set of normal donor macrophages, IL-1β significantly upregulated EBI-3 mRNA expression and produced a synergistic effect on induction of IL-27p28, and especially EBI-3, when used in conjunction with IFN-γ (Fig. 4B). Analysis of IRF-1 and IRF-8 mRNA also revealed enhanced expression of these transcription factors that have each been shown to bind to the p28 promoter to induce IL-27 expression in mouse macrophages (23). Thus, the conditions appropriate for upregulation of both IL-27 subunits in infected or uninfected human macrophages are present in the VL spleen.

IL-21 enhances Ag-specific IL-10 production in active VL cases

Autocrine IL-21 has recently been shown to amplify IL-27–induced T cell IL-10 expression in the mouse. Because we observed elevated mRNA for IL-27, IL-21, and IL-10 in active VL cases, we were interested to determine whether rhIL-27 or rhIL-21, used alone or in combination with soluble Ag prepared from L. donovani promastigotes (SLA), had the potential to stimulate IL-10 production in active VL cases or ECs. We used a whole-blood assay that has recently been shown to detect Ag-specific IFN-γ secretion by peripheral blood cells from the majority of active VL cases (K. Gidwani, O.P. Singh, M. Boelaert, and S. Sundar, submitted for publication) to determine whether Ag-specific IL-10 might also be detected in these patients. ELISA results confirmed the Ag-driven secretion of IFN-γ by peripheral blood cells from VL patients, with 22 of 27 patients producing cytokine above background levels (Fig. 5A), whereas cells from only 4 of 13 healthy ECs produced detectable levels of IFN-γ, reflecting their possible subclinical exposure to infection. Following stimulation with SLA, peripheral blood cells from the majority of active VL cases also produced IL-10, with 23 of 27 patients secreting low but detectable levels of cytokine compared with only 2 of 19 ECs (Fig. 5A). Interestingly, the peripheral blood cells from an independent series of VL patients also produced IL-10 when cultured in the presence of exogenous rhIL-21, whereas no response was detected in the cells from ECs (Fig. 5B). Although a few of
the VL patients also upregulated IL-10 secretion in response to rhIL-27, the majority of VL patients, as well as ECs, did not. Treatment with IL-21 alone, and especially in combination with IL-27, significantly enhanced the Ag-driven IL-10 response. Cells from ECs remained IL-10 unresponsive to all combinations of stimuli, indicating that patient cells had been specifically primed under conditions that made them more responsive to IL-10–inducing signals. The effect of IL-27 in combination with IL-21 to enhance IL-10 production by Ag-stimulated cells was associated with significantly higher levels of IL-21 compared with the Ag-stimulated cells treated with IL-21 alone (Fig. 5). The supernatants from 11 of 13 VL patients contained elevated levels of IL-21 under these conditions, emphasizing the likely role of IL-27 in upregulating autocrine IL-21 by T cells to amplify their IL-10 expression. We were not able to detect IL-21 secreted by the Ag-stimulated cells treated with IL-27 alone (data not shown), perhaps because of rapid consumption of this cytokine in the cultures lacking exogenous IL-21.

**Discussion**

We have previously addressed the cellular source of elevated IL-10 in human VL, and identified CD4+CD25 Foxp3+ T cells as the major producers of IL-10 in the VL spleen (7). The findings suggest that Tr1 cells, or Th subsets that coexpress IL-10, are important in suppression of anti-leishmanial immunity in human VL. In the mouse, various conditions that can promote T cell IL-10 secretion have been recently described that have in common the involvement of IL-12 family members that may self-regulate inflammatory responses via their ability to instruct activated T cells to produce IL-10. Thus, IL-12–induced STAT4 signaling together with a high Ag dose can activate Th1 cells to coexpress IL-10 (39). An alternative IL-12 family member, IL-27, which activates Th1 transcription factors T-bet and STAT1, was shown to upregulate IL-10 expression by Th1 cells (25, 40) and to induce IL-10–producing anti-inflammatory Tr1-like cells (41, 42). Although IL-27 has been shown to promote IL-10 secretion by human CD4+ T cells in vitro (30), its expression in human infection has been confined to the detection of elevated serum levels in individuals infected with HIV or hepatitis B virus (33, 34), and its association with IL-10 regulation in any human infectious disease setting has not been investigated. The current studies were designed to address the possible role of IL-27 in T cell IL-10 regulation in human VL.

We were able to detect elevated circulating levels of IL-27 in VL patients (Fig. 1), as well as significantly elevated mRNA levels for both IL-27p28 and EBI-3 subunits in the VL spleen during active disease. Expression of IL-21 mRNA was also significantly enhanced in pre- as compared with posttreatment splenic aspirate cells and especially in comparison with cells from healthy spleens. The IL-27 transcripts were enriched in CD14+ cells (monocytes/macrophages), whereas the IL-21 and IL-10 transcripts were enriched in CD3+ cells (T cells). The role for IL-21 in amplifying IL-10–producing T cells that have been induced by IL-27 has been recently recognized in mice (43). These data are consistent with the possibility that IL-27 from innate cells induces T cells to produce IL-21 that acts as an autocrine growth factor to expand...
IL-10–producing T cells in the VL spleen (18). As IL-21 is also known to critically regulate Ig production (44), it could be a contributing factor to the high titers of anti-leishmanial Abs in VL patients. The influence of these regulatory cytokines on Ag-specific IL-10 secretion in vitro was investigated. We used a whole-blood assay that has been shown to detect Ag-specific IFN-γ secretion by peripheral blood cells from cured VL and cutaneous leishmaniasis cases (45, 46). More recently, the assays have been found to detect Ag-specific IFN-γ production by cells from active VL cases (K. Gidwani et al., submitted for publication), which is in striking contrast to the extensive experience regarding the lack of this response in VL patients when purified PBMCs have been used. We confirm the IFN-γ responsiveness of VL patients in the whole-blood assay and further report secretion of detectable levels of IL-10 in the majority of patients. So far as we are aware, this is the first demonstration of Ag-driven IL-10 production by cells from VL patients in vitro. Although the findings are consistent with Ag-specific T cell IL-10 expression, it is possible that T cells responding to Ag stimulate IL-10 production from other cells. This might be especially relevant to the regulation of IL-10 production in target organs such as the spleen where macrophages constitute an expanded population of lymphoid cells. We were unable to demonstrate that neutralizing Abs against either IL-27 or IL-21 could inhibit the IL-10 response (data not shown), reflecting either that these cytokines were not involved or that the assay detects peripheral cells already activated to produce IL-10 and are not influenced by the low concentrations of IL-27 or IL-21 produced by APCs or T cells present in the whole-blood assay. By contrast, the peripheral blood cells were IL-10 responsive to exogenous rIL-21, which was able to induce IL-10 from VL patients, even in the absence of Ag. These culture conditions may mimic the high levels of IL-21 plus Ag present in the VL spleen, which might therefore be expected to drive T cell IL-10 secretion during active disease.

The Ag-specific IFN-γ response detected in VL patients is consistent with the elevated levels of IFN-γ in VL plasma as well as elevated IFN-γ mRNA expression in lesional tissue. The coordinate production of Ag-specific IFN-γ and IL-10 might suggest that, although undoubtedly essential for acquired resistance to L. donovani, IFN-γ might also be involved in the regulation of T cell IL-10 expression as a homeostatic mechanism of restrain inflammation. Several recent studies in mice and humans have indicated that IL-27p28 is regulated at the transcriptional level. Given the high expression of p28 mRNA in CD14+–enriched splenic cells from VL patients, we were specifically interested in IFN-γ regulation of IL-27p28 gene expression in human macrophages. In the mouse, IFN-γ signaling in macrophages can contribute to activation of the p28 gene through recruitment of IRF-1 and IRF-8 to an IFN-stimulated regulatory element in the p28 promoter (23, 47). In humans, an effect of IFN-γ on IL-27p28 upregulation in a retinal cell line has been reported (21); however, the effects on human macrophages have not been described previously. Using human monocyte-derived macrophages from a series of healthy donors, we could demonstrate a consistent and powerful induction of p28 mRNA by IFN-γ, accompanied by a more modest upregulation of EBI3. Other proinflammatory mediators, either alone or in combination with IFN-γ, had only a slight effect on p28 expression. By contrast, EBI3 mRNA was substantially upregulated in the presence of both IFN-γ and IL-1β, consistent with the reported effect of IL-1β on EBI-3 expression in a human pre-DC line (48). Thus, the conditions suitable for activation of both IL-27 subunits in human macrophages appear to be present in the VL spleen. Furthermore, the elevated IRF-1 transcripts that we detected in the VL spleen are consistent with the strongly elevated levels of IRF-1 mRNA observed in the donor macrophages following treatment with IFN-γ, reinforcing a possible role for IFN-γ–induced signaling to promote IL-27 production in human VL. Importantly, exposure of the macrophages to L. donovani amastigotes, either alone or in combination with IFN-γ, produced no discernable effects on IL-27p28 or EBI3 gene expression, consistent with the generally low stimulatory potential of these parasites and emphasizing the role of host-derived agonists in upregulating IL-27 as a mechanism of feedback control.

The increased production of IL-27 in human VL might have regulatory consequences beyond the generation of IL-10–producing T cells. Studies in mice have demonstrated that IL-27 can inhibit the differentiation of Th17 cells involved in autoimmunity and pathogenic responses to infection (28, 29). In human CD4+ T cells, IL-27 suppressed IL-17 and IL-22 secretion under Th17-polarizing conditions (i.e., exposure of anti–CD3-stimulated cells to IL-1β and IL-23 (30)). In the VL patients, IL-17 transcripts were expressed in low abundance in both the pre- and posttreatment splenial samples. By contrast, IL-23p19 and IL-23R mRNA were expressed at significantly increased levels in the posttreatment biopsies, unique among the selected transcripts we analyzed in its downregulated expression during active disease. The low or downregulated expression of Th17-associated cytokines in patients with active disease may be particularly significant in light of recent clinical studies in the Sudan showing that L. donovani Ag-stimulated production of IL-17 and IL-22 by PBMCs was strongly and independently associated with protection against VL (36). Although we have no direct evidence that IL-27 inhibited the Th17 response in VL patients, L. major infections in mice have clearly revealed that in the absence of IL-27 signaling, the infection drives a robust Th17 response that in this instance exacerbates cutaneous pathology (27).

In conclusion, IL-27 produced by macrophages, along with IL-21 from T cell sources, are suggested to be disease-promoting cytokines in VL by virtue of their roles in promoting the differentiation and expansion of Ag-specific, IL-10–producing T cells. The studies support the notion that IL-27 is a key instructional cytokine involved in regulating the balance between immunity and pathology in human VL.

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Disclosures

The authors have no financial conflicts of interest.

References


