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Circulating Immune Complexes (IC) and IC-Induced Levels of GM-CSF Are Increased in Sudanese Patients with Acute Visceral Leishmania donovani Infection Undergoing Sodium Stibogluconate Treatment: Implications for Disease Pathogenesis

Amir Ibrahim Elshafie,²* Erik Åhlin,²* Linda Mathsson,* Gehad ElGhazali,‡§ and Johan Rönnelid³*

Infection with Leishmania donovani is associated with IL-10 as well as with GM-CSF. Immune complexes (IC) exert important functions by stimulation of monocytes/macrophage-mediated production of pro- and anti-inflammatory cytokines in rheumatic diseases. In this investigation, we have explored IC-induced cytokine production during Leishmania infection. Sera from 43 patients with visceral leishmaniasis (VL), 17 patients with post-kala-azar dermal leishmaniasis, and 20 healthy Sudanese controls were precipitated with polyethylene glycol (PEG). The PEG precipitates were added to serum-free PBMC for 20 h, whereupon supernatant levels of IL-1β, IL-6, IL-10, IL-1 receptor antagonist protein, TNF-α, TNF receptor p75, and GM-CSF were investigated using ELISA. Circulating levels of C1q-binding IC were also measured in the serum samples. PEG precipitates from Leishmania-infected patients significantly increased levels of GM-CSF (p = 0.0037) and IL-10 (p < 0.0001), as well as of IL-6 (p < 0.0001) and IL-1 receptor antagonist (p = 0.0238) as compared with PEG precipitates from controls. Patients with acute VL as well as VL patients receiving sodium stibogluconate treatment displayed significantly increased levels of PEG precipitate-induced GM-CSF. The induction of GM-CSF by circulating IC was especially prominent in acute VL patients receiving sodium stibogluconate treatment; ANOVA revealed significant interaction between disease activity and treatment for PEG precipitate-induced levels of GM-CSF (disease activity, p = 0.0006; treatment, p = 0.0005; interaction, p = 0.0046). Parallel associations were determined for C1q-binding immune complexes, but not for any cytokine other than GM-CSF. The importance of IC-induced GM-CSF in leishmaniasis warrants further study. *The Journal of Immunology, 2007, 178: 5383–5389.

Visceral leishmaniasis is a disease caused by protozoa of the Leishmania donovani complex. The parasites, which are introduced into their human hosts by sand flies, rapidly invade macrophages where they multiply inside phagolysosomes. Human infections may be asymptomatic or subclinical or may cause a severe disease called visceral leishmaniasis (VL) or kala-azar (1–3). Clinical manifestations of VL include recurrent fever, hepatosplenomegaly, generalized lymphadenopathy, pancytopenia, and anaemia. Death occurs in the absence of appropriate chemotherapy (4). Major VL outbreaks have occurred in India and the Sudan in recent years and have killed thousands of people (5).

After treatment with sodium stibogluconate, VL patients infected with L. donovani can develop a macular, maculopapular, and nodular rash called post-kala-azar dermal leishmaniasis (PKDL). The incidence of PKDL varies between 50% in the Sudan and 5–10% in India; the reason for this difference is presently unknown (6). Leishmania parasites exhibit an absolute dependency on macrophages (7), within which they multiply. Macrophage activation with ensuing intracellular parasite killing is the host’s primary defense against Leishmania infection. With Leishmania being recognized as an obligate intracellular parasite of macrophages, studies have demonstrated that specific immunity in VL is mediated by CD4+ Th cells and that disease susceptibility is associated with the inability to produce a macrophage-stimulating cytokine profile (Th1 profile) including IFN-γ, IL-2, and IL-12, while conversely an elevated production of immunosuppressive cytokines such as IL-10 and IL-4 (Th2 profile), as well as high levels of TNF-α, may be associated with susceptibility (8, 9).

The hemopoietic growth factor GM-CSF has many stimulatory effects on monocytes/macrophages that are helpful during intracellular infections, such as enhancing phagocytic and metabolic functions and the release of other proinflammatory cytokines (10). Addition of GM-CSF to human monocytes in vitro increases their leishmanicidal effects (11), and, when mice infected with L. donovani were treated with murine GM-CSF, they showed increased leishmanicidal activity, as well as leukocytosis and myelomonocytic accumulation in infected viscera (12). In a small placebo-controlled study, GM-CSF-treated Leishmania chagasi-infected patients had a significantly reduced number of secondary infections, along with increased neutrophil counts (13). The production

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4 Abbreviations used in this paper: VL, visceral leishmaniasis; CIC, circulating immune complex; HSA, human serum albumin; IC, immune complex; IL-1ra, IL-1 receptor antagonist protein; PEG, polyethylene glycol; PKDL, post-kala-azar dermal leishmaniasis; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; TNF-Rp75, TNF receptor p75.
of GM-CSF is increased during infection in the liver of L. donovani-infected BALB/c mice (12) and in mouse bone marrow stromal macrophages (7) findings arguing for a counterregulatory and potentially disease-limiting effect of Leishmania infection-induced GM-CSF production. This hypothesis is also sustained by the fact that Leishmania parasites genetically engineered to produce GM-CSF within their phagosomes showed poor survival within macrophages that had been activated by the GM-CSF to produce an array of proinflammatory cytokines (14). Recently, L. donovani amastigote Ags have been reported to induce GM-CSF production in mouse peritoneal macrophages both in vitro and in vivo (15).

Leishmania-infected patients have increased levels of circulating Clq-binding immune complex (CIC) (16–21), which contain Leishmania Ags (22, 23). Such immune complexes (IC) might affect disease progression and disease outcome through induction of pro- or anti-inflammatory cytokines, in analogy to our earlier findings in rheumatic diseases. We have previously demonstrated that polyethylene glycol (PEG)-precipitated IC from sera of systemic lupus erythematosus (SLE) patients induce IL-6 and IL-10 when added to PBMC (24). Purified cryoglobulins from patients with multiple myeloma and Waldenström’s macroglobulinemia induced TNF-α and IL-10 (25), and IC from rheumatoid arthritis synovial fluids induce TNF-α (26). We have also reported IC-induced cytokine production to depend on monocytes in the PBMC preparations and to require the expression of FcγRIIs on the monocyte surface (24–27).

VL patients exhibit kidney alterations in the form of interstitial nephritis (28) as well as glomerulonephritis (29, 30). Such changes might be mediated through IC-induced cytokines such as IL-10 or GM-CSF. GM-CSF is of importance in T cell/monocyte-mediated forms of kidney damage and experimental glomerulonephritis (31, 32). IC-induced IL-10 was recently demonstrated to be instrumental for disease progression in experimental Leishmania infection in mice (33).

In this article, we have studied the cytokine-inducing effects of IC from clinically well-characterized patients with Leishmania infection. By measuring a panel of pro- and anti-inflammatory cytokines, including naturally occurring cytokine inhibitors, we found increased IC-induced production of pro- and anti-inflammatory cytokines in Leishmania-infected Sudanese patients as compared with healthy controls. Quite unexpectedly, the IC-induced production of GM-CSF was substantially increased, especially from patients with active VL who had recently started sodium stibogluconate treatment. Possible consequences of this finding are discussed.

Materials and Methods

Study area, patients, and establishment of diagnosis

This study was conducted at Tabarakalla rural hospital in Gadarif state, along the lower Abbara river in Gallabat Province, eastern Sudan. The area is located ~70 km southeast from the Gadarif town. It is endemic for L. donovani, and its main vector in that area is Phlebotomus orientalis (34). Patients enrolled in the study mainly came from the Tabarakalla and Barbar Elfogara villages, endemic areas with a high prevalence of both VL and PKDL.

A detailed clinical history was obtained, including tribe, residence, occupation, marital status, medical treatment, abdominal pain, vomiting, nausea, previous history of bleeding tendency, urinary tract infection and insec bites, and family history of VL, hypertension, or diabetes mellitus. Particular emphasis was made regarding any previous form of leishmaniasis. Subjects were questioned about their ethnic and geographic origin and their medical care were classified as acute VL. Patients not severely ill and treated as outpatients with daily injections of sodium stibogluconate were classified as subacute VL. Treated patients received daily i.v. injections of sodium stibogluconate, 20 mg/kg for 30 days, with a mean of 4 days between the start of treatment and blood sampling. PKDL was diagnosed on clinical grounds, on the appearance and distribution of the rash after treatment in previously diagnosed VL patients. There are no laboratory tests for diagnosing PKDL. The interval between VL and the occurrence of PKDL was studied. Additional acute VL patients from the study area who had been treated with sodium stibogluconate were recruited from the treating hospital.

Pallens whole blood was drawn from the Leishmania-infected patients and their healthy controls. Samples were drawn before treatment in newly diagnosed patients. Sera were separated by centrifugation within 2 h of collection. Serum samples were stored and transported frozen in liquid nitrogen until analyzed in Uppsala, Sweden. Sera were obtained from 43 patients with VL (33 males and 10 females; mean age: 21 years) and 17 patients with PKDL (13 males and 4 females; mean age: 10 years), and 20 healthy controls (14 male and 6 females; mean age: 21 years).

Ethical approval for this study was obtained from the Ethical Committee of the Faculty of Medicine, University of Khartoum, from the Ministry of Health at Gadarif State, and from the Ethical Committee at Uppsala University. Informed consent was obtained from all of the adults who participated in the study. For young children, consent was obtained from their parents.

PEG precipitation of IC

Serum samples were thawed and immediately mixed with an equal volume of 5% PEG 6000 with 0.1 M EDTA and left to stand at 4°C overnight before the precipitates were purified and washed in a single-step centrifugation procedure described previously (35). Briefly, 1 ml of PEG containing 5% human serum albumin (HSA; Baxter) and 2.5% PEG 6000 (PBS- HSA-PEG) (10 ml) was added to 1.5 ml of autoclaved Eppendorf cylinders made out of 5-ml autoclaved pipette tips, by cutting 5% human albumin (HSA; Baxter) and 2.5% PEG 6000 (PBS-HSA-PEG). Serum that had been precipitated overnight was diluted 1/3 in RPMI 1640 containing 2.5% PEG 6000 and then placed on top of the PBS-HSA-PEG in the pipette tips. An interface was then formed with the less dense, red RPMI 1640 solution on top. The tubes were centrifuged at 1500 × g at 4°C for 20 min, whereas the precipitates in the lower 2.5% PEG-RPMI 1640 solution were centrifuged down to the bottom of the Eppendorf tube. The remaining PBS-HSA-PEG solution was removed and the pellet containing PEG-precipitated IC was resolved in ice-cold sterile PBS of the original serum volume. The diluted PEG precipitates were placed on ice until used in cell culture experiments.

Preparation of mononuclear cells and cell cultures

Buffy coats were obtained from healthy blood donors and diluted 1/4 in sterile PBS at room temperature, before separation on Ficoll-PAque (GE Health Care). After two washes in PBS, PBMC were diluted to 1 × 10⁶ cells/ml in RPMI 1640 supplemented with 300 mg/ml l-glutamine, 1% HEPES, 1% penicillin-streptomycin, and 4% Ultrosor G (Flow Laboratories). This serum-free system had been optimized for studies of IC-induced cytokine responses. We had found earlier that medium supplemented with 4% Ultrosor G (Flow Laboratories) more efficiently sustains IC-induced cytokine production as compared with other serum substitutes (25, 26, 36). PEG precipitates (10% v/v) were added to the cell cultures directly after PBMC preparation and within 2 h of PEG precipitation. Cell cultures were performed in 300-μl volumes in sterile flat-bottom 96-well plates for 20 h before harvest of the supernatants.

Our experience of different responder cell populations used for IC stimulation showed that PBMC preparations might either be good responders to IC or show generally low or generally activated cytokine production without substantial effects of added IC. Due to such variations, two PBMC donors were used as responder cells in parallel in each experiment except in the
FcγR blockade experiment. The results presented are from the PBMC donor giving the strongest net response following IC stimulation.

Measurement of cytokines in supernatants and serum levels of CIC

Measurement of TNF-α, IL-1β, IL-10, TNF receptor p75 (TNF-Rp75), IL-10, GM-CSF, and IL-1 receptor antagonist (IL-1ra) in supernatants was performed using ELISA. All cytokine ELISAs had been established in the laboratory for the measurement of IC-induced cytokine responses in vitro and are described elsewhere (24, 25, 36) using biotinylated detection Abs, streptavidin-HRP (R&D Systems), and 3,3'-5,5'-tetramethylbenzidine (DakoCytoMation) as substrate. The Abs and recombinant cytokine standards used in the assay were purchased from R&D Systems except for IL-10, TNF-Rp75 (both from BioSource International) and IL-6 (Mabtech). As capture, mouse mAbs MAB610 (2.0 μg/ml, for TNF-α), MAB601 (2.0 μg/ml, for IL-1β), 897C6G9 (4 μg/ml, for TNF-Rp75), MAB615 (2.0 μg/ml, for GM-CSF), and MAB280 (5 μg/ml, for IL-1ra) were used. For detection, biotinylated polyclonal goat Abs BAF210 (0.1 μg/ml for TNF-α), BAF201 (2.5 μg/ml, for IL-1β), BAF280 (25 ng/ml, for IL-1ra), and mouse mAbs 911B3H10 (0.4 μg/ml, for TNF-Rp75) and BAM215 (1.0 μg/ml, for GM-CSF) were used. For IL-10, paired Fab(′)2 Abs (Flexia; BioSource International) were used at concentrations of 1 and 2 μg/ml, respectively.

CIC was measured using a commercial kit (The Binding Site) according to the manufacturer’s instructions.

FcγR blocking experiments

Anti-FcγRII mAb (IV.3 (Fab); Medarex) or anti-FcγRII (3G8 (F(ab′)2); Medarex) were added to the cells and left to stand at 4°C for 30 min before addition of dissolved PEG precipitates. For this specific experiment, frozen and subsequently thawed PEG precipitates were used. The Ab concentration used was 1.5 μg/ml; preliminary experiments had shown an equivalent blocking effect using either 1.5 or 4 μg/ml. Ab IV.3 has earlier been shown to react specifically with FcγRIIα (37, 38).

Statistics

An unpaired  \( t \) test was used for comparisons between groups in unpaired design, a paired  \( t \) test for investigating the effects of FcγR blockade in paired design, and Pearson’s moment product correlation test was used to determine correlations, with Fisher’s R-to-S conversion to determine  \( p \) values. The ratios between pro- and anti-inflammatory cytokines were determined individually for each patient or control, and the distribution of individual ratios were thereafter compared between the groups in unpaired design. Two-way ANOVA was used to investigate the combined effects of disease activity and ongoing sodium stibogluconate treatment on levels of CIC and IC-induced cytokines. Values of  \( p < 0.05 \) were considered to be significant.

Results

PEG-precipitated serum IC from Leishmania-infected patients induce pro- and anti-inflammatory cytokines

PEG precipitates from Leishmania-infected patients induced higher mean levels of all investigated cytokines as compared with healthy Sudanese controls (Table I). This was most evident for IL-6 ( \( p < 0.0001; \) Fig. 1a), IL-10 ( \( p < 0.0001; \) Fig. 1b) and GM-CSF ( \( p = 0.0037; \) Fig. 1c), but did not reach significance for IL-1β ( \( p = 0.052 \) ) and for TNF-α ( \( p = 0.065; \) data not shown).

When ratios between corresponding pro- and anti-inflammatory cytokines were calculated, the PEG IC-induced profile was more proinflammatory among the Leishmania patients as compared with the Sudanese controls ( \( p = 0.0474 \) for TNF-α:TNF-Rp75 and \( p = 0.0008 \) for IL-1β:IL-1ra, respectively; Table I). The same was true for the calculated GM-CSF:IL-10 ratio ( \( p = 0.0014; \) Table I).

Data are presented for the PBMC donor having the strongest net PEG IC-induced cytokine responses. Results did however correlate strongly between the two donors, with  \( r \) values of 0.76, 0.86, and 0.71 for IL-6, IL-10, and GM-CSF, respectively (all  \( p < 0.0001; \) data not shown).

GM-CSF is selectively induced by PEG precipitates from patients with acute VL and VL patients receiving sodium stibogluconate therapy

When all VL and PKDL patients were compared, there was no significant difference in the levels of any of the investigated cytokines induced by PEG-precipitated IC. Subdivision of the VL patients revealed significantly higher GM-CSF levels induced by PEG precipitates from acute VL patients as compared with patients with subacute disease ( \( p = 0.0311 \) ) and among VL patients receiving sodium stibogluconate therapy as compared with untreated

Table I. Differences between subgroups of Leishmania-infected patients

<table>
<thead>
<tr>
<th>Patients (n = 60)</th>
<th>VL (n = 43)/PKDL (n = 17)</th>
<th>Acute VL (n = 18)/Subacute VL (n = 25)</th>
<th>VL Patients on Treatment (n = 12)/VL Patients not on Treatment (n = 31)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β (pg/ml)</td>
<td>451/130</td>
<td>483/369</td>
<td>342/585</td>
</tr>
<tr>
<td>(p = NS)</td>
<td>(p = NS)</td>
<td>(p = NS)</td>
<td>(p = NS)</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>24,739/12,580</td>
<td>24,836/24,494</td>
<td>23,687/25,664</td>
</tr>
<tr>
<td>(p &lt; 0.0001)</td>
<td>(p = NS)</td>
<td>(p = NS)</td>
<td>(p = NS)</td>
</tr>
<tr>
<td>TNF-α (pg/ml)</td>
<td>2,091/659</td>
<td>2,241/1710</td>
<td>1,417/8,35</td>
</tr>
<tr>
<td>(p = NS)</td>
<td>(p = NS)</td>
<td>(p = NS)</td>
<td>(p = NS)</td>
</tr>
<tr>
<td>IL-10 (pg/ml)</td>
<td>126/66</td>
<td>126/127</td>
<td>115/134</td>
</tr>
<tr>
<td>(p &lt; 0.0001)</td>
<td>(p = NS)</td>
<td>(p = NS)</td>
<td>(p = NS)</td>
</tr>
<tr>
<td>TNF-Rp75 (pg/ml)</td>
<td>480/439</td>
<td>479/481</td>
<td>509/458</td>
</tr>
<tr>
<td>(p = NS)</td>
<td>(p = NS)</td>
<td>(p = NS)</td>
<td>(p = NS)</td>
</tr>
<tr>
<td>IL-1ra (pg/ml)</td>
<td>432/211</td>
<td>442/407</td>
<td>366/497</td>
</tr>
<tr>
<td>(p = 0.0238)</td>
<td>(p = NS)</td>
<td>(p = NS)</td>
<td>(p = NS)</td>
</tr>
<tr>
<td>GM-CSF (pg/ml)</td>
<td>780</td>
<td>86/80</td>
<td>134/51</td>
</tr>
<tr>
<td>(p = 0.0037)</td>
<td>(p = NS)</td>
<td>(p = 0.0311)</td>
<td>(p = 0.0060)</td>
</tr>
<tr>
<td>CIC</td>
<td>7.88/4.48</td>
<td>8.59/6.13</td>
<td>14.72/4.00</td>
</tr>
<tr>
<td>(p = NS)</td>
<td>(p = NS)</td>
<td>(p = 0.0077)</td>
<td>(p = 0.0103)</td>
</tr>
<tr>
<td>IL-1β:IL-1ra ratio</td>
<td>0.88/0.60</td>
<td>0.91/0.82</td>
<td>0.86/0.94</td>
</tr>
<tr>
<td>(p = 0.0008)</td>
<td>(p = NS)</td>
<td>(p = NS)</td>
<td>(p = NS)</td>
</tr>
<tr>
<td>TNF:TNF-Rp75 ratio</td>
<td>4.53/1.50</td>
<td>3.88/3.66</td>
<td>3.53/5.85</td>
</tr>
<tr>
<td>(p = 0.0474)</td>
<td>(p = NS)</td>
<td>(p = NS)</td>
<td>(p = NS)</td>
</tr>
<tr>
<td>GM-CSF:IL-10 ratio</td>
<td>0.53/0</td>
<td>0.60/0.36</td>
<td>0.93/0.35</td>
</tr>
<tr>
<td>(p = 0.0014)</td>
<td>(p = NS)</td>
<td>(p = 0.0146)</td>
<td>(p = 0.0131)</td>
</tr>
</tbody>
</table>

*a Differences in levels of cytokines induced in vitro by PEG-precipitated IC, CIC, and ratios between pro- and anti-inflammatory cytokines. Values in bold depict significant differences.
patients (p = 0.0060; Table I). This finding was GM-CSF specific, because none of the other pro- or anti-inflammatory cytokines or cytokine inhibitors exhibited such differences between the VL subgroups (Table I).

High levels of CIC characterize patients with acute VL and VL patients receiving sodium stibogluconate therapy

Patients with acute VL had highly elevated levels of CIC (p = 0.0077; Table I and Fig. 2) as compared with patients with subacute VL. CIC levels were also significantly higher in VL patients receiving sodium stibogluconate therapy than in nontreated VL patients. No such differences were evident in the general comparison between all Leishmania-infected patients and controls, or between VL and PKDL patients (Table I).

Disease severity and treatment interact to induce CIC and PEG-IC-stimulated levels of GM-CSF in VL patients

The comparisons described above had demonstrated higher CIC levels among patients with nontreated VL and VL patients on sodium stibogluconate therapy. The same was true for GM-CSF, but not for any of the other investigated cytokines. We therefore performed two-way ANOVA analyses for VL patients with disease activity and ongoing therapy as independent variables and CIC and cytokine responses as dependent variables. Elevated CIC levels could then be associated with acute VL (p = 0.0004) and with ongoing sodium stibogluconate therapy (p = 0.0025). There was also an interaction between disease activity and ongoing therapy (p = 0.0255), indicating that active disease and ongoing therapy synergized to predispose to high CIC levels (Fig. 3a). Almost identical results emerged when PEG IC-induced levels of GM-CSF instead were used as a dependent variable in ANOVA (disease activity, p = 0.0006; therapy, p = 0.0005; interaction, p = 0.0046; Fig. 3b). None of the other investigated cytokines, including TNF-Rp75 and IL-1ra, exhibited any such association with either disease activity or ongoing treatment.

Strong correlation between CIC and PEG IC-induced levels of GM-CSF in acute VL patients on sodium stibogluconate therapy

There was, as expected, a positive relationship between PEG IC-induced levels of cytokines and CIC levels in general, but the degree of correlation was strongest for GM-CSF. The highest degree of correlation was among acute VL patients and among VL patients receiving sodium stibogluconate therapy (Table II). One exception from this general pattern was the high degree of correlation between CIC and PEG IC-induced levels of IL-10 among subacute VL patients (Table II).

Comparisons of IC-induced cytokine responses in Leishmania-infected patients with different clinical and demographic characteristics

When we compared IC-induced cytokine responses to the dichotomous demographic variables sex, marital status, predominant indoor or outdoor occupation and family history of VL, and to the occurrence of fever, abdominal distension, abdominal pain, vomiting, nausea, bleeding tendency, paleness, jaundice, lymph node enlargement, palpable spleen and palpable liver, only a few weakly significant differences appeared. Patients presenting with abdominal distension and nausea showed higher IC-induced production of IL-6 (p = 0.0400 and 0.0371, respectively; data not shown), whereas IC from patients jaundiced at the time of blood sampling induced higher levels of GM-CSF (p = 0.0211; data not included). Age recorded as a continuous variable only showed a weak negative correlation to IC-induced production of TNF-α (r = −0.257, p = 0.0435; data not shown). No other variables showed any statistical correlations or association to levels of IC or IC-induced cytokines.
Induction of GM-CSF from PBMC by PEG precipitates from L. donovani-infected patients is dependent on FcγRIIa

When responder cells were incubated with Fab or F(ab')2 of Abs blocking FcγRIIa and FcγRIII, we obtained a significant decrease in the production of GM-CSF ($p = 0.0321$) as compared with cell cultures without any FcR blockade. Blockade of FcγRIII, on the other hand, increased GM-CSF production ($p = 0.0088$). The effects of FcγR blockade was smaller on PEG precipitates obtained from four healthy Sudanese controls, especially for FcγRIIa.

**Discussion**

In this investigation, we have demonstrated that PEG-precipitated IC from *Leishmania*-infected patients induce the proinflammatory cytokines GM-CSF and IL-6, as well as the immunosuppressive or anti-inflammatory cytokines IL-10 and IL-1ra. Generally, the effect of *Leishmania*-derived IC was more proinflammatory than anti-inflammatory when the responses between individual cytokines and their natural inhibitors were compared. There was no overt difference in cytokine responses induced by PEG-precipitated IC from VL or PKDL patients. VL patients with severe disease and VL patients with ongoing sodium stibogluconate therapy exhibited significantly increased levels of IC-induced GM-CSF, closely correlated to levels of C1q-binding CIC# measured by conventional techniques. When applying two-way ANOVA, both disease severity and recently initiated sodium stibogluconate therapy were in a synergistic fashion associated with IC-induced levels of GM-CSF, but not with any of the other investigated cytokines. An almost identical association was evident for C1q-binding CIC.

A number of previous studies have reported increased levels of CIC containing parasite Ags in human *Leishmania* infection (16–23). The cytokine GM-CSF induced by *Leishmania* Ags (15) might be either beneficial by activating macrophages to become leishmanicidal (11, 12) or detrimental by stimulating inflammation through a T cell-driven delayed-type hypersensitivity response, thereby facilitating *Leishmania*-associated kidney involvement (31, 32).

Earlier longitudinal studies in treated VL patients have demonstrated decreasing CIC levels after initiation of sodium stibogluconate therapy (18, 39), contrasting with our cross-sectional study. This difference might be due to divergent time intervals between institution of therapy and blood sampling. Whereas the earlier study (18) compared levels of CIC before and 1.5 mo after the start of therapy, our patients were sampled during the very first days (median 4 days) after treatment began. We hypothesize that very early during therapy a massive number of parasites are destroyed, releasing Ag into the circulation and increasing the load of CIC-containing parasite Ags, in analogy with the Jarish-Herxheimer reaction observed after initiation of syphilis therapy (40). We hypothesize that VL patients with active disease have more parasites than do VL patients with subacute disease. Such an hypothesis might account for the differences observed in CIC levels and PEG IC-induced levels of GM-CSF between patients with severe and subacute VL and also for the synergistic effects between IC responses, on the one hand, and disease activity and ongoing therapy on the other, as indicated in our ANOVA analyses.

Our findings of markedly increased levels of CIC and IC-induced GM-CSF very early after sodium stibogluconate treatment also prompt for careful short-term longitudinal studies of IC responses the very first days after treatment begins, aiming to correlate those IC responses to clinical symptoms such as skin rash and signs of transient or permanent kidney involvement.

In a parallel study, we have examined a panel of clinical laboratory measures of kidney and liver function in *Leishmania*-infected patients (41). Our analysis revealed that cystatin C, a marker of kidney function that is superior to creatinine due to its independency of muscle mass, closely correlates with CIC levels and with IC-induced levels of GM-CSF but not with other investigated cytokines. Collectively, these two investigations therefore argue for a role of CIC and IC-induced impairment of kidney function in VL patients with severe disease, possibly in association with newly instiuted sodium stibogluconate treatment, in concordance with the correlation of CIC and creatinine observed in canine leishmaniasis (42). Using a mouse knockout model, GM-CSF has also been demonstrated to be necessary for the development of T cell/macrophage-mediated crescentic glomerulonephritis (32).

A number of earlier studies have implicated IL-10 in the immune suppression associated with *Leishmania* infection, arguing either for Th2 response (43) or regulatory T cells (44) as the main source of immune-suppressive IL-10. In three recent investigations.
of experimental leishmaniasis in mice, IC are implicated in the production of IL-10 (33, 45, 46) in analogy with our own studies of SLE (24) and cryoglobulinemia (25). The idea that IC should have immune-suppressive function in skin biopsies or in the circulation. Did not investigate whether increased amounts of IC were found in parasitic or CIC-containing parasite Ags sequestered in the spleen. Theoretically, the stronger IL-10 response in pre-PKDL patients might reflect IC-induced cytokine production, but Gasim et al. (47) did not investigate whether increased amounts of IC were found in skin biopsies or in the circulation.

In this study, as well as in our previous studies (24–27), IC-induced cytokine production was partially dependent on FcɤRIIa. In the present study, the decrease in GM-CSF production after FcɤRIIa blockade was small (mean, 25.4% decrease). This might be due to the use of frozen and thawed PEG precipitates in this specific experiment. We have found earlier that freshly prepared PEG precipitates from rheumatoid arthritis and SLE patients induce higher levels of cytokines as compared with frozen/thawed PEG precipitates where IC may have been destroyed. Since the FcɤRIIa receptor is primate specific and not appearing in rodents (48), our findings argue that investigations of the effects of IC in Leishmania infection should be performed in human experimental systems. We believe that the stimulatory effect of the FcɤRIII blockade is independent of IC because it is seen also in PEG precipitates from control individuals in this investigation, in healthy control IC (27), as well as with cultures with monomeric IgG (24). On the contrary, we have not ruled out a significant effect of FcɤRII since our PBMC cultures contain monocytes with very low FcɤRIII expression (for discussion, see Ref. 26). The selective importance of FcɤRIII in inflammatory macrophages therefore has to be investigated separately.

We have in this article demonstrated that PEG-precipitated IC from Sudanese patients with leishmaniasis induce pro- and anti-inflammatory cytokines. Notably, IC-induced GM-CSF levels are increased in acute VL patients and in VL patients with ongoing sodium stibogluconate treatment and correlate to a high degree with IC levels. Our findings support recent studies indicating the significance of IC/cytokine-mediated mechanisms in Leishmania infection in mice (33, 45, 46) by also showing a possible impact of IC-mediated cytokine production in human Leishmania infection.

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Disclosures

The authors have no financial conflict of interest.

References


Table II. Correlation between CIC and cytokine responses

<table>
<thead>
<tr>
<th></th>
<th>All Patients and Controls (n = 80)</th>
<th>Controls Only (n = 20)</th>
<th>All Patients (n = 60)</th>
<th>PKDL Patients (n = 17)</th>
<th>VL Patients (n = 43)</th>
<th>Subacute VL Patients (n = 25)</th>
<th>Acute VL Patients (n = 18)</th>
<th>VL Patients on Sodium Stibogluconate Therapy (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIC vs</td>
<td>R = +0.586 (p &lt; 0.0001)</td>
<td>R = +0.598 (p = 0.245)</td>
<td>R = +0.649 (p = 0.477)</td>
<td>R = +0.606 (p = 0.649)</td>
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<td>GM-CSF</td>
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<tr>
<td>CIC vs</td>
<td>R = 0.262 (p = 0.368)</td>
<td>R = +0.319 (p = 0.451)</td>
<td>R = +0.368 (p = 0.499)</td>
<td>R = +0.578 (p = 0.107)</td>
<td>R = +0.499 (p = 0.368)</td>
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<tr>
<td>IL-10</td>
<td>R = 0.0195 (p = 0.0001)</td>
<td>R = 0.241 (p = 0.001)</td>
<td>R = 0.254 (p = 0.0158)</td>
<td>R = 0.008 (p = NS)</td>
<td>R = 0.005 (p = NS)</td>
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<tr>
<td>CIC vs IL-6</td>
<td>R = 0.0287 (p = 0.008)</td>
<td>R = 0.241 (p = 0.001)</td>
<td>R = 0.254 (p = 0.0158)</td>
<td>R = 0.008 (p = NS)</td>
<td>R = 0.005 (p = NS)</td>
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</table>

*Correlation between levels of CIC in serum and levels of IC-induced GM-CSF, IL-10, and IL-6 in vitro. Values in bold depict significant correlations. GM-CSF data are missing for controls due to very low levels of PEG IC-induced levels of GM-CSF (see Table I).


