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9-O-Acetylated Sialoglycoproteins Are Important Immunomodulators in Indian Visceral Leishmaniasis▽

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Overexpression of disease-associated 9-O-acetylated sialoglycoproteins (9-O-AcSGPs) on peripheral blood mononuclear cells (PBMC) of visceral leishmaniasis (VL) patients ($PBMC_{VL}$) compared to their levels of expression in healthy individuals has been demonstrated using a lectin, achatinin-H, with specificity toward 9-O-acetylated sialic acid derivatives α2-6 linkage with subterminal *N*-acetylgalactosamine (9-O-AcSAα2-6GalNAc). The decreased presence of disease-associated 9-O-AcSGPs on different immune cells of parasitologically cured individuals after successful treatment relative to the levels in patients with active VL prior to treatment was demonstrated. However, their contributory role as immunomodulatory determinants on $PBMC_{VL}$ remained unexplored. Accordingly, 9-O-AcSGPs on $PBMC_{VL}$ were sensitized with achatinin-H, leading to their enhanced proliferation compared to that observed with different known mitogens or parasite antigen. This lymphoproliferative response was characterized by evaluation of the TH1/TH2 response by intracellular staining and enzyme-linked immunosorbent assay for secreted cytokines, and the results were corroborated by their genetic expression. Sensitized $PBMC_{VL}$ evidenced a mixed TH1/TH2 cellular response with a predominance of the TH1 response, indicating the ability of 9-O-AcSGPs to modulate the host cell toward a favorable response. Interestingly, the humoral and cellular responses showed a good correlation. Further, high levels of anti-9-O-AcSGP antibodies with an order of distribution of immunoglobulin M (IgM) > IgG1 = IgG3 > IgG4 > IgG2 > IgE could be explained by a mixed TH1/TH2 response. A good correlation of enhanced 9-O-AcSGPs with both the cell-mediated ($r = 0.98$) and humoral ($r = 0.99$) response was observed. In summary, it may be concluded that sensitization of 9-O-AcSGPs on $PBMC_{VL}$ may provide a basis for the modulation of the host's immune response by their controlled expression, leading to a beneficial immune response and influencing the disease pathology.

Sialic acids comprise a family of 9-carbon carboxylated acidic monosaccharides that exist as either N- or O-substituted forms (35, 36, 47–49). Sialic acid is commonly referred to as *N*-acetyl neuraminic acid, or Neu5Ac (36). A diverse range of almost 50 known derivatives of sialic acids has been documented. Among them, 7-, 8-, and 9-O-acetylated derivatives (O-AcSA) are essential constituents of the cell membrane well noted for their involvement in different physiological and pathological processes (12, 19, 36).

Human visceral leishmaniasis (VL), or kala-azar, caused by the intracellular protozoan parasite *Leishmania donovani*, is endemic in 62 countries and is a major public health threat, with an estimated 200 million people worldwide at risk (13, 16). The severity of VL pathogenesis is predominantly marked by immunosuppression that is manifested by decreased levels of gamma interferon (IFN-γ) and interleukin-12 (IL-12), along with an increased TH2 response evidenced by elevated levels of IL-4 and IL-10 (3, 18, 33, 52). The active disease state also induces the production of high levels of specific antibody subclasses whose involvement in conferring host protection has yet to be proved (34). More importantly, increasing drug resistance of the leishmanial parasites has worsened the scenario (45). Therefore, an urgent need exists for the search for newer determinants which would in turn affect the disease's pathology through balanced beneficial immunomodulation of the host.

In this regard, we have demonstrated the presence of disease-associated 9-O-acetylated sialoglycoproteins (9-O-AcSGPs) on different immune cells of peripheral blood mononuclear cells (PBMC) of patients with active VL ($PBMC_{VL}$) using a lectin, achatinin-H, having preferential affinity toward terminal 9-O-AcSA derivatives α2-6 linked to subterminal *N*-acetylgalactosamine, 9-O-AcSAα2-6GalNAc (4, 22, 37, 38). However, their role in modulating the immune response and thereby regulating the clinical course of the disease remained unaddressed. Accordingly, the present investigation focused on (i) the differential expression of 9-O-AcSGPs on immune cells of patients compared to their expression on PBMC of patients after successful treatment (posttreatment; $PBMC_{PT}$); (ii) the lymphoproliferative response upon sensitization of disease-associated 9-O-AcSGPs on $PBMC_{VL}$; and subsequently, (iii) the characterization of a mixed TH1/TH2 response upon sensitization of 9-O-AcSGPs. Since cytokines regulate antibody isotype switching during B-cell development (17, 24, 32, 34, 43), a study of the subclass distribution of the antibodies was performed to unravel the processes involved in the divergence of the immune responses during disease. In this regard, we also provide evidence for a good correlation between TH1-

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cell-mediated and humoral responses as evaluated by measuring anti-O-AcSGP-specific antibody subclass expression. Taken together, the results of this in vitro study unravel the immunomodulatory role of 9-O-AcSGPs and their influence on the disease's pathology, which may provide a basis for triggering a beneficial immune response by their controlled expression in the active disease state.

MATERIALS AND METHODS

Study subjects. The study involved patients with clinically confirmed VL (n = 25) at the time of diagnosis at the School of Tropical Medicine, Kolkata, India. Diagnosis of VL was based on microscopic demonstration of *Leishmania* amastigotes in splenic aspirates, according to WHO recommendations (51). The diagnosis was further confirmed by three in-house techniques, namely, (i) parasite antigen enzyme-linked immunosorbent assay (ELISA) (9) for estimation of antileishmanial serology, (ii) erythrocyte binding (11) and hemagglutination assays for quantification of the increased presence of linkage-specific 9-O-AcSGPs, and (iii) ELISA for detection of anti-9-O-AcSGP antibodies (10). The healthy volunteers (n = 25) were individuals with negative results for antileishmanial serology, erythrocyte binding assay, and anti-9-O-AcSGP antibody titer. Patients with active VL were treated with sodium antimony gluconate (20 mg/kg of body weight/day for 3 months) or amphotericin B (1 mg/kg/per day for 1 month), respectively. After completing chemotherapy, the patients were monitored for the disappearance of the clinical symptoms and were also evaluated by the described in-house techniques. The institutional ethical committee approved the study. Blood was collected after obtaining informed consent of the donors, patients, or in the case of minors, from the parent or guardian.

Isolation of PBMC. PBMC were separated using density gradient centrifugation at 400 × g for 30 min by layering peripheral blood over Ficoll-Hypaque (1:1; Amersham Pharmacia, Uppsala, Sweden). The layer of $PBMC_{VL}$ was washed twice in phosphate-buffered saline (0.02 M, pH 7.2) and resuspended in RPMI 1640 medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% heat-inactivated fetal calf serum (medium A). Prior to the assays, the cellular viability was checked by using trypan blue exclusion, which revealed >95% viability. In parallel, $PBMC_{PT}$ and PBMC from healthy donors ($PBMC_H$) were isolated similarly.

Probe. The lectin achatinin-H was affinity purified using bovine submandibular mucin (BSM), known to contain a high percentage of 9-O-AcSAs, as an affinity matrix (27, 37–38). The carbohydrate binding specificity of achatinin-H was checked by hemagglutination and hemagglutination inhibition assays using several mono- and disaccharides, as well as several sialoglycoproteins, as inhibitory reagents (30, 38). Achatinin-H was conjugated with fluorescein isothiocyanate (FITC) and used for flow cytometry (8).

Detection of 9-O-AcSGPs on $PBMC_{VL}$ subsets by flow cytometry. Different monoclonal antibodies such as anti-CD3, CD13, CD16, and CD19 antibodies used for the assay were from Pharmingen (San Diego, CA). PBMC (1×10^6/100 μl) were suspended in medium A and stained on ice for 1 h with FITC–achatinin-H and phycoerythrin (PE)–anti-CD monoclonal antibodies along with appropriate isotype controls. The cells were then washed, fixed in paraformaldehyde (1%), acquired on a FACSCalibur flow cytometer, and analyzed using CellQuest software (Becton, Dickinson, and Co., Mountain View, CA). The specificity of achatinin-H interaction with 9-O-AcSGPs on $PBMC_{VL}$ was confirmed after incubation of the cells with 9-*O*-acetyl hemagglutinin esterase of influenza C virus for 1 h at 37°C, as described elsewhere (8, 50). In parallel, $PBMC_{PT}$ and $PBMC_H$ were similarly processed.

Determination of the specificity of 9-O-AcSGPs by inhibition ELISA. 9-O-AcSGPs on $PBMC_{VL}$ were affinity purified using achatinin-H as an affinity matrix as described elsewhere (22). The binding of 9-O-AcSGPs to achatinin-H was detected by incubation with anti-9-O-AcSGP antibodies (1 μg) that were affinity purified from sera of patients with active VL overnight at 4°C (10). The binding specificity of purified 9-O-AcSGPs was confirmed by inhibition ELISA (30). Several inhibitors, including synthetic Me-α-Neu5,9Ac_2, BSM, which has a large amount of linkage-specific 9-O-AcSA (38), de-O-acetylated BSM, desialylated BSM, sheep submandibular mucin, human chorionic gonadotrophin, fetuin, and α_1-acid glycoprotein having no O-AcSAs, were used. The binding of 9-O-AcSGPs to immobilized achatinin-H (1 μg/100 μl/well) in Tris-buffered saline (TBS; 0.05 M, pH 7.2) at 4°C was performed in the presence of these inhibitors to compare their capacity to inhibit this interaction. Affinity-purified disease-associated 9-O-AcSGPs (1 μg) in 50 μl of TBS containing 30 mM Ca^{2+} (TBS-Ca) and bovine serum albumin (2%) were initially incubated for 30 min at 4°C with each inhibitor (50 μl, 10 to 60 mM) separately. After subsequent blocking and washing, the mixture (100 μl) was added to achatinin-H-coated wells and incubated overnight at 4°C, and the antigen–anti-O-AcSGP antibody complex was subsequently measured colorimetrically using horseradish peroxidase (HRP)-conjugated protein A (diluted 1:10,000; Cappel, St. Louis, MO) and azino-bis-thio-sulfonic acid (Sigma, St. Louis, MO) with recording at 405 nm in an ELISA reader.

In vitro lymphoproliferation assay. The lymphoproliferation assay was performed with achatinin-H (0.1 to 10 μg) as described elsewhere (40). In brief, $PBMC_{VL}$ (1×10^5 cells/ml) from patients before and after treatment were cultured in triplicate in medium A, in the presence or absence of achatinin-H, in 96-well microtiter plates for 0 to 96 h at 37°C in an atmosphere of 5% CO_2. All the parameters of the assay, such as cell concentration and time kinetics, were varied while keeping others constant to optimize the culture conditions. In parallel, a lymphoproliferation assay was carried out using known T- and B-cell mitogens, such as concanavalin A (ConA) and phytohemagglutinin (PHA). Additionally, crude parasite antigen (9) was used under identical conditions.

Cultures were pulsed with [^{3}H]thymidine (1 μCi/well) for 18 h, and [^{3}H]thymidine uptake was measured. The dose for maximal proliferation of each sample was determined from the graph obtained by plotting the incorporated radioactivity against the dose of mitogen. Results were also expressed as the stimulation index (SI), which was calculated as follows: SI = mean counts per minute in stimulated culture/mean counts per minute in unstimulated culture.

The lymphoproliferation assay of $PBMC_{VL}$ upon sensitization of 9-O-AcSGPs with achatinin-H was also performed by incubating the lectin (0.10 μg) with BSM at the optimal inhibitory concentration (0.05 μmol/liter) for 1 h at 37°C, and the cells were subsequently cultured for 72 h to assess the specificity. Cells were de-O-acetylated by esterase treatment (8, 50). In parallel, de-O-acetylated cells in the presence and absence of achatinin-H and BSM served as different controls. $PBMC_H$ were processed similarly for comparison.

Additionally, viable cells after lymphoproliferation were assessed by methylthiazoltetrazolium (MTT) assay. In brief, MTT solution (100 μg/50 μl) was added to each well separately and further incubated for 4 h at 37°C. The mitochondrial enzyme succinate dehydrogenase cleaves the tetrazolium salt, MTT, into a blue-colored product (formazan) that occurs as crystals (15). The crystals of formazan were dissolved in dimethyl sulfoxide (450 μl), and the absorbance at 570 nm was recorded in a spectrophotometer as a quantitative measure of cell proliferation (or viability). In parallel, under identical conditions, viable cells were assessed by the conventional trypan blue dye exclusion assay.

Cytokine ELISA. Following the culture of PBMC in the presence or absence of achatinin-H, the cell-free culture supernatants were collected at the indicated time points (0 to 96 h) used in the lymphoproliferation assay and stored at −70°C for the detection of released cytokines, namely, IFN-γ, IL-10, IL-12, IL-2, and tumor necrosis factor (TNF-α). The respective cytokines were quantified using commercially available ELISA kits according to the manufacturer's instructions (OptEIA kit for humans; Pharmingen, San Diego, CA). Briefly, microtiter wells were coated with individual cytokine antibodies, and after subsequent nonspecific blocking with phosphate-buffered saline–bovine serum albumin, cytokine standards or culture supernatants were added to designated wells and incubated for 1 h at 25°C. To determine the concentration of cytokines in culture supernatants, standard curves were constructed using recombinant cytokines, and cytokine concentrations in test samples were determined from the corresponding concentration values from the standard curve.

Intracellular cytokine analysis by flow cytometry. Intracellular cytokines of the $PBMC_{VL}$ resulting from sensitization of 9-O-AcSGPs by achatinin-H were detected. Cells were cultured with or without achatinin-H for 72 h, washed, and incubated with brefeldin A (10 μg/ml) for a further 4 h at 37°C. Washed cells were permeabilized using fluorescence-activated cell sorter permeabilizing solution (500 μl) for 6 to 8 min, stained with PE-conjugated cytokine-specific antibodies for 30 min at 4°C in the dark, processed, and analyzed by flow cytometry. The cells were initially gated for the presence of cell surface 9-O-AcSGPs by using FITC–achatinin-H and analyzed for intracellular cytokines. In parallel, $PBMC_{PT}$ and $PBMC_H$ were processed similarly. Unsensitized PBMC served as negative controls.

Reverse transcription-PCR studies. RNA (1 μg) isolated from $PBMC_{VL}$ after subsequent sensitization with achatinin-H (0.1 μg) by Trizol was reverse transcribed using a poly(dT) oligonucleotide and SuperScript II reverse transcriptase (20). The cDNA was amplified using specific primers as follows: IFN-γ forward, 5′-TCTGCATCGTTTTGGGTTCT-3′, and reverse, 5′-CAGCTTTTCGAAGTCATCTC-3′ (product size, 300 bp); IL-12p40 forward, 5′-CCAAGAACTTGCAGCAGCTGAAG-3′, and reverse, 5′-TGGGTCTATTCCGTTGTGTC-3′ (product size, 360 bp); IL-10 forward, 5′-AGATCTCCGAGATGCCTTTCA-3′, and reverse, 5′-TTTCGTATCTTCATTGTCATGTA-3′ (product size, 408 bp);

IL-4 forward, 5′-CGGCAACTTTGACCACGGACACAAGTGCG-3′, and reverse, 5′-AGGACACTTCCTTCGGTTGGTCTCATGCA-3′ (product size, 244 bp); and TNF-α forward, 5′-GAGTGACAAGCCTGTAGCCCATGTTGTAG CA-3′, and reverse, 5′-GCAATGATCCCAAAGTAGACCTGCCCAGACT-3′ (product size, 444 bp). Each PCR cycle consisted of denaturation at 94°C for 30 s, annealing at 55°C for 45 s, and extension at 72°C for 1 min using specific primers for cytokines in a Perkin-Elmer DNA thermal cycler. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), used as control, was PCR amplified using 5′-ATGGGGAAGGTGAAGGTCGG-3′ for the forward primer and 5′-GGGT GCTAAGCAGTTGGT-3′ for the reverse primer (product size, 540 bp). An agarose gel (1%) stained with ethidium bromide was used to analyze the products of PCR (10 μl) after visualization under UV light. Quantification of the comparative intensities of the bands for the respective cytokines were scored by densitometry using Quantity One software.

Subclass ELISA. The levels of different anti-O-AcSGP-specific antibody subclasses in VL patients before and after treatment were determined by subclass ELISA. BSM in Tris buffer (0.05 M, pH 7.2) served as the coating antigen (1 μg/100 μl/well). After nonspecific blocking, serum samples (diluted 1:50, 100 μl/well) were added and incubated overnight at 4°C. The wells were incubated separately with HRP-conjugated mouse anti-human immunoglobulin G1 (IgG1), IgG2, IgG3, IgG4, and IgM (1:5,000), respectively, to determine the levels of subclasses of anti-O-AcSGP-specific antibodies. Next, the wells were washed with TBS (0.05 M, pH 7.2) containing 0.1% Tween 20 and incubated with HRP-conjugated anti-mouse IgG, and the antigen-antibody complex was colorimetrically measured on an ELISA reader at 405 nm, using azino-bis-thiosulfonic acid as the substrate.

For determination of levels of anti-O-AcSGP-specific IgE, patients' sera (1:10, 100 μl/well) were preincubated with Sepharose-protein G beads (25% vol/vol) for 18 h at 4°C (2, 41). The mixture was centrifuged at 2,000 × *g* for 5 min at 4°C, and the supernatant (100 μl) was added to the wells and incubated. After washing, HRP–anti-human IgE (1:2,500; Calbiochem, CA) was added and the antigen-antibody complex was measured as described previously.

Statistical analysis. Results are expressed as the means ± standard deviations (SD) for individual sets of experiments. Statistical analysis was performed using Graph-Pad Prism statistics software (Graph-Pad Software, San Diego, CA). Student's unpaired or paired *t* tests were used. Reported values are two tailed, and *P* values lower than 0.05 were considered statistically significant. The Spearman correlation test was used for the comparison of independent variables.

RESULTS

Study subjects. Among patients with VL, 18 of 25 were male. The average and the median ages were comparable for patients with VL and healthy controls. The clinical and laboratory features of the patients on admission and after treatment are summarized in Table 1. The response to sodium antimony gluconate or amphotericin B therapy was timely. Leucopenia and decreased hemoglobin were observed in patients with active VL who were subsequently monitored during the course of treatment. The splenic aspirate smears of all the patients after treatment showed an absence of parasites (i.e., *L. donovani* bodies), and they were therefore clinically defined as parasitologically cured.

At the time of diagnosis, the sera of patients with active VL had high levels of parasite-specific antibodies compared to the levels in individuals after treatment (means ± SD at an optical density of 405 nm [OD_{405}], 1.15 ± 0.65 versus 0.202 ± 0.71), as determined by parasite-specific ELISA using immobilized crude parasite antigen (9). The 9-O-AcSGPs were exclusively detected on erythrocytes of these patients, as determined by flow cytometry using FITC–achatinin-H (68% ± 5.15%), erythrocyte binding ELISA (mean ± SD at OD_{405}, 1.18 ± 0.61), and hemagglutination assay (mean ± standard error of the mean, 145.70 ± 42.21) using achatinin-H as an analytical probe (11, 27, 38). The erythrocytes from patients after treatment and from healthy individuals completely lacked these sialoglycotopes.

TABLE 1. Clinical features of the study population[a]

Feature	Value for group		
	VL patients		Healthy controls
	With active disease	Posttreatment	
Age (yr)	25.2 ± 10.75	26 ± 11.05	30.5 ± 8.65
Median (range)	22 (6–46)	19 (7–41)	31 (25–52)
Sex ratio (male:female)	18:7	18:7	16:9
Body wt (kg)	39.52 ± 11.36	41.00 ± 10.18	60 ± 6.25
BMI[b]	17 ± 0.65	20.5 ± 1.05	22 ± 1.15
Duration of illness (mo)	4.25 ± 2.25	NA	NA
Karnofsky score[c]	73.15 ± 2.25	83.50 ± 3.16	95.02 ± 5.72
Splenic amastigote score[d]	4.25 ± 0.27	0	0
Spleen size (cm)	8.15 ± 1.12	4.01 ± 1.02	Not palpable
White blood cell count ($10^3/mm^3$)	3.53 ± 0.81	4.55 ± 1.42	8.2 ± 2.11
Red blood cell count ($10^6/\mu l$)	1.75 ± 1.21	3.15 ± 0.88	5.25 ± 1.71
Hemoglobin concn (g/dl)	5.22 ± 0.42	9.44 ± 0.15	11.5 ± 2.21
Mean cell hemoglobin concn (g/dl)	30 ± 1.22	32 ± 1.12	35 ± 1.86
Hematocrit (%)	33 ± 1.17	39 ± 1.15	52 ± 3.05
Parasite antigen ELISA[e]	1.15 ± 0.65	0.202 ± 0.71	0.121 ± 0.31
Erythrocyte binding assay[f]	1.18 ± 0.61	0.185 ± 0.81	0.102 ± 0.43
9-O-AcSA-positive erythrocytes (%)[f]	68 ± 5.15	6.50 ± 1.25	ND

[a] Data are given as the mean ± SD unless otherwise indicated. NA, not applicable; ND, not detectable.

[b] Body mass index (BMI) is weight in kilograms divided by square of height in meters; normal BMI is 18.5 to 24.9.

[c] The Karnofsky performance scale is as follows: 100, able to carry on normal activity and no special care is needed, with no complaints and no evidence of disease; 90, able to carry on normal activity, with minor signs or symptoms of disease; 80, normal activity with effort, with some signs or symptoms of disease; 70, unable to work but able to live at home and care for most personal needs, with various amounts of assistance needed.

[d] 5, >10 to 100 parasites/field; 4, >1 to 10 parasites/field; 3, >1 to 10 parasites/10 fields; 2, >1 to 10 parasites/100 fields; 1, >1 to 10 parasites/1,000 fields; 0, no parasites or >1 to 10 parasites/>1,000 fields.

[e] Antileishmanial serology was estimated using immobilized crude parasite antigen as described elsewhere (20).

[f] Increased presence of linkage-specific 9-O-AcSGPs on erythrocytes of patients with active VL was quantified by erythrocyte-binding assay and flow cytometry (21).

Status of 9-O-AcSGPs on immune cells of VL patients before and after successful chemotherapy. To determine the contributory role of 9-O-AcSGPs on $PBMC_{VL}$ in immunomodulation, their status on different immune cells was monitored before and after chemotherapy. Interestingly, two-color flow cytometry demonstrated decreased FITC–achatinin-H binding to the surface of T, B, and NK cells and monocytes of $PBMC_{PT}$, reflecting low levels of 9-O-AcSGPs, comparable to the levels in $PBMC_{H,}$ and indicating their restricted distribution in the active disease state that could be potentially important for immunomodulation (Table 2).

The binding specificity of 9-O-AcSGPs on $PBMC_{VL}$ was determined by an inhibition ELISA. Initially, achatinin-H was used as the coating antigen. The binding of purified 9-O-AcSGPs by achatinin-H was inhibited with increasing concentrations of synthetic Me-α-Neu5,9Ac_2. This binding was maxi-

TABLE 2. Comparative profiles of 9-O-AcSA in patients with active VL and posttreatment

Cell type	% of 9-O-AcSA-positive cells from[a]		
	VL patients		Healthy controls
	With active disease[c]	Posttreatment	
PBMC	84.82 ± 3.55[b]	7 ± 3.15	6.5 ± 2.5
T	60.23 ± 4.68[b]	10.01 ± 5.01	8.22 ± 3.55
B	19.14 ± 0.35	6.34 ± 0.76	5.14 ± 1.11
NK	11.26 ± 3.23	4.06 ± 2.13	4.16 ± 1.32
Monocyte	47.72 ± 0.76[b]	7.52 ± 2.16	6.02 ± 1.46

[a] Data are given as the mean ± SD. To determine the distribution of 9-O-AcSA on cells of different lineages in patients with active VL and posttreatment ($n = 25$), double-color flow cytometric analysis was performed. PBMC were incubated with FITC-achatinin-H and respective PE-conjugated lineage-specific monoclonal antibodies and analyzed as described in Materials and Methods.

[b] Significantly different from healthy control subjects ($P < 0.05$).

[c] The experiment was performed with the same group of active VL patients used for the previous study, as described elsewhere (22). After successful chemotherapy, paired samples from this group of patients were used for comparison.

mally inhibited at 60 mM of Me-α-Neu5,9Ac$_2$. In parallel, BSM, an O-acetylated sialoglycoprotein with high 9-O-AcSA content, showed the greatest inhibition of binding of purified 9-O-AcSGPs with achatinin-H. Several sialoglycoproteins with no O-AcSA, namely de-O-acetylated BSM, sheep submandibular mucin, fetuin, human chorionic gonadotrophin, and α_1-acid glycoprotein, showed no binding.

Sensitization of 9-O-AcSGPs on PBMC$_{VL}$ induces their enhanced proliferation. With a view to addressing the role of 9-O-AcSGPs in immunomodulation, their involvement in lymphoproliferation of PBMC$_{VL}$ after sensitization in the presence of different doses of achatinin-H for 72 h or without sensitization was measured. Maximal lymphoproliferation of PBMC$_{VL}$ was achieved with 0.1 μg of achatinin-H at 72 h as determined by both the radiometric (Fig. 1A) and colorimetric MTT (Fig. 1B) assays. VL is typically associated with leucopenia accompanied by immunosuppression; the response of PBMC$_{VL}$ to known T- and B-cell mitogens (ConA and PHA) and parasite antigen was, as expected, much lower than that evidenced with achatinin-H (Fig. 1C). Significantly, the SI of PBMC$_{VL}$ was 28 after sensitization of 9-O-AcSGPs for 72 h (Fig. 1D). Enhanced proliferation provided the first indication of 9-O-AcSGPs as one of the important determinants on PBMC$_{VL}$ responsible for triggering immune modulation.

PBMC$_{VL}$ preincubated with the optimum inhibitory concentration of BSM and cultured in the presence of achatinin-H (0.1 μg) demonstrated an absence of lymphoproliferation (Fig. 1E). In parallel, PBMC$_{VL}$ treated with *O*-acetyl esterase and cultured similarly in the presence of achatinin-H demonstrated an absence of proliferation (Fig. 1E). BSM alone, in the absence of achatinin-H and under similar conditions, demonstrated no proliferative response, indicating that the lymphoproliferation was through 9-O-AcSGPs. In contrast, PBMC$_{PT}$ and PBMC$_{H}$ showed negligible proliferation even at a very high dose of achatinin-H (10 μg), due to a minimal presence of 9-O-AcSGPs and their lesser affinity toward achatinin-H, indicating that this response was specifically associated with the clinical status of the disease.

Lymphoproliferative response on sensitization of 9-O-AcSGPs on PBMC$_{VL}$ induces enhanced cytokine secretion. As immunity to leishmania infection is cell mediated and mainly controlled by parasite killing due to activation of host cells, we analyzed the cytokines secreted by the PBMC$_{VL}$ following sensitization of disease-associated 9-O-AcSGPs at various time points as an index of activation and regulation of the immune response.

The secretion of both TH1 and TH2 cytokines in the supernatants of proliferated PBMC$_{VL}$ showed a time-dependent response that was maximal at 72 h. Interestingly, sensitization of 9-O-AcSGPs demonstrated a mixed TH1/TH2 type with a predominance of the TH1 response (Fig. 2, Table 3).

Specifically, in the 9-O-AcSGP-sensitized PBMC$_{VL}$ culture supernatants, the levels of the signature TH1 cytokine IFN-γ, which is instrumental in activating the host's leishmanicidal activity (28), were considerably higher than in unsensitized PBMC$_{VL}$, with a mean ± SD of 162 ± 4.21 pg/ml versus 35 ± 3.51 pg/ml (Fig. 2A). Another cytokine, IL-12, also plays an important role in regulating the onset and continuation of the TH1 response (46) that confers protection against leishmaniasis. Sensitized PBMC$_{VL}$ demonstrated a 12.67-fold-higher level of IL-12 than unsensitized PBMC$_{VL}$ (Fig. 2B). Similarly, the level of IL-2 was also higher (Fig. 2C), indicating the predominance of the TH1 response mediated by sensitization of 9-O-AcSGPs in the active disease condition of VL.

The level of the TH2 cytokine IL-10, an important regulator of immunosuppression (14, 34), was slightly higher in 9-O-AcSGP-sensitized PBMC$_{VL}$ than in the unsensitized controls, the mean ± SD being 116 ± 6.50 pg/ml versus 95 ± 5.6 pg/ml (Fig. 2D). IL-4, a cytokine of the TH2 response, also evidenced a small increase after sensitization of PBMC$_{VL}$ (Fig. 2E), indicating that although the response was mixed, it predominantly favored the increase of TH1-specific cytokines.

More importantly, TNF-α, one of the essential fundamental cytokines involved in the host response in confronting microbial infection (31), was drastically elevated in the culture supernatants of sensitized PBMC$_{VL}$ compared to its level in unsensitized controls (Fig. 2F), further indicating that proper balanced modulation of this sialoglycotope leads to a beneficial host response.

The predominance of TH1 cytokines after sensitization of 9-O-AcSGPs on PBMC$_{VL}$ was further confirmed by the results of intracellular staining of cytokines using flow cytometry (Fig. 3), which were consistent with the pattern of secreted cytokines. After sensitization, the percentages of 9-O-AcSA-positive cells that stained for IFN-γ and IL-12 were two- and fivefold higher, respectively, than in unsensitized PBMC$_{VL}$ (Fig. 3). On the other hand, in the 9-O-AcSA-positive cells stained for TH2 cytokines, the levels of IL-10 and IL-4 were lower, suggesting that these disease-associated 9-O-AcSGPs could be successfully modulated toward a host-protective beneficial response.

Under similar conditions, PBMC$_{PT}$ and PBMC$_{H}$ with negligible levels of 9-O-AcSGPs demonstrated insignificant levels of secreted cytokines and an almost complete absence of double-positive cells, suggesting that these unique 9-O-acetylated sialoglycoproteins are one of the potential determinants responsible for disease-specific host immune modulation (Table 3).

To further confirm our results, we studied the genetic ex-

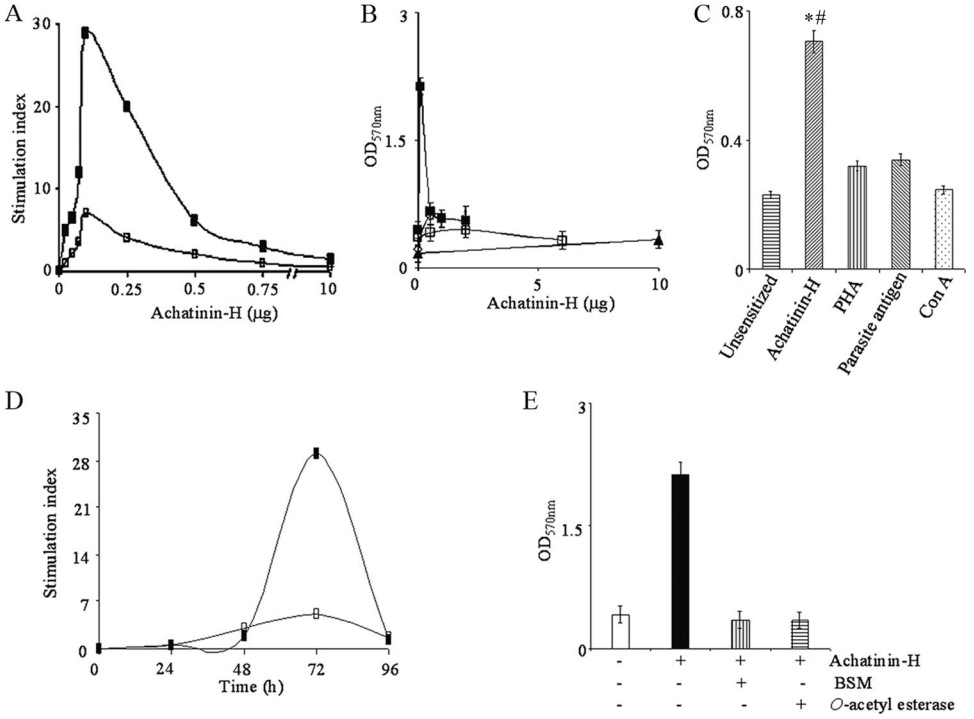

FIG. 1. Enhanced lymphoproliferation of $PBMC_{VL}$ upon sensitization of disease-associated 9-O-AcSGPs by achatinin-H. (A) SI as demonstrated by the radiometric method. $PBMC_{VL}$ (1×10^5 cells/ml) before (■) and after (□) sensitization were cultured separately with achatinin-H (0.1 to 12 μg) for 72 h at 37°C in an atmosphere of 5% CO_2. Cultures were pulsed with [^{3}H]thymidine (1 μCi/well) for 18 h, and its uptake was measured. Results are expressed as the SI as described in Materials and Methods. The data show a profile representative of the results for 25 patients. The points represent the means ± SD of the results for triplicate determinations. (B) Maximal lymphoproliferation demonstrated by colorimetric MTT assay. $PBMC_{VL}$ ($n = 25$) before (■) and after (□) treatment were sensitized through 9-O-AcSGPs by using different concentrations of achatinin-H (0.1 to 10 μg) for 72 h at 37°C in an atmosphere of 5% CO_2 and assessed by MTT assay. The formazan crystals that formed were dissolved in dimethyl sulfoxide (450 μl), and the absorbance at 570 nm was recorded with a spectrophotometer as a quantitative measure of cell proliferation (or viability). In parallel, $PBMC_H$ ($n = 25$) (▲) were processed similarly. The points represent the means ± SD of the results for triplicate determinations. (C) Comparison of levels of lymphoproliferation induced by different mitogens versus the level induced by achatinin-H. $PBMC_{VL}$ (1×10^5 cells/ml; $n = 25$) were sensitized with two well-known mitogens, namely, PHA and ConA, and with crude parasite antigen and achatinin-H (0.1 μg) under identical assay conditions. Subsequently, the viability of the cells was assessed by MTT as described for panel B. Unsensitized $PBMC_{VL}$ served as controls for the assay. The points represent the means ± SD the results for triplicate determinations. *, P value of <0.01 for the observed lymphoproliferation with achatinin-H versus that in unsensitized controls; #, P value of <0.05 versus the lymphoproliferation with other mitogens (PHA and ConA). (D) SI of $PBMC_{VL}$ sensitized with achatinin-H (0.1 μg) before (■) and after (□) treatment plotted against different culture time points (0 to 96 h). The data show a profile representative of the results for 25 patients. Each point represents the mean ± SD of the results for triplicate determinations. (E) 9-O-AcSGPs serve as an important glycotope for lymphoproliferation after sensitization with achatinin-H. $PBMC_{VL}$ were cultured in the absence (□) or presence of achatinin-H (0.1 μg) before (■) and after preincubation with BSM (+) and were de-O-acetylated (+ *O*-acetyl esterase), and their viability was assessed by MTT assay as described in Materials and Methods. The data show a profile representative of the results for 25 patients. The points represent the means ± SD of the results for triplicate determinations. +, present; −, absent.

pression of the cytokines after subsequent sensitization of 9-O-AcSGPs on $PBMC_{VL}$ for 0 to 96 h (Fig. 4). Cytokine transcripts of both IFN-γ and IL-12 demonstrated maximal overexpression at 72 h as shown by densitometry scanning (Fig. 4). In contrast, the levels of of TH2 cytokines IL-10 and IL-4 released after sensitization of $PBMC_{VL}$ were lower than the levels of TH1 cytokines. As expected, unsensitized cells evidenced basal levels of cytokine synthesis.

Enhanced levels of anti-O-AcSGP antibodies in patients with active VL and their reduction after successful chemotherapy. In order to correlate the induced cellular responses with the humoral, serum samples from patients with active VL before and after treatment and from healthy control subjects were tested for the distribution of anti-O-AcSGP-specific antibody subclasses.

All the anti-O-AcSGP antibody subclasses were elevated in the sera of VL patients (Fig. 5). The level of anti-O-AcSGP IgM (Fig. 5A) was maximal in patients with active VL (mean ± SD at OD_{405}, 1.25 ± 0.07). The levels of anti-O-AcSGP IgG1 (Fig. 5B) and IgG3 (Fig. 5D) were also significantly higher, with the means ± SD at OD_{405} being 1.05 ± 0.06 and 1.03 ± 0.07, respectively, possibly due to the predominance of the induced TH1 response in the milieu. The levels of anti-O-AcSGP IgG2 (Fig. 5C), IgG4 (Fig. 5E), and IgE (Fig. 5F) were also increased. Taken together, our data indicate that the subclass order of levels of anti-O-AcSGP antibodies in VL patients at the time of diagnosis is IgM > IgG1 = IgG3 > IgG4 > IgG2 > IgE, revealing a mixed TH1/TH2 response. The enhanced anti-O-AcSGP antibodies showed a good correlation ($r = 0.99$) with the overexpression of 9-O-AcSGPs on $PBMC_{VL}$.

The levels of all the anti-O-AcSGP antibody subclasses declined in the sera of successfully treated individuals and

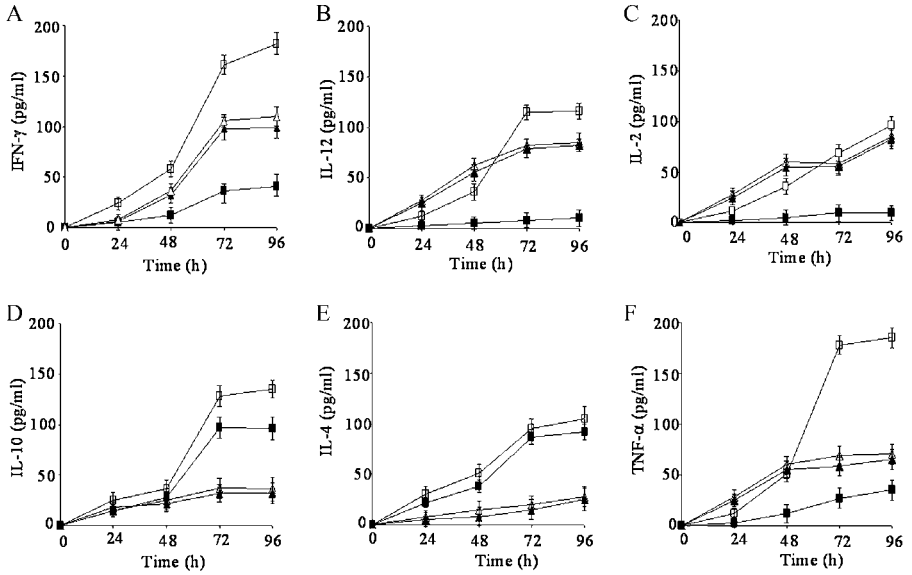

FIG. 2. Enhanced secretion of TH1 cytokines by 9-O-AcSGP-sensitized $PBMC_{VL}$. $PBMC_{VL}$ were cultured after sensitization in the absence (■) or presence (□) of achatinin-H (0.1 μg) for 0 to 96 h. The concentration of the released cytokines, namely, IFN-γ (A), IL-12 (B), IL-2 (C), IL-10 (D), IL-4 (E), and TNF-α (F), in the culture supernatant was detected by ELISA as described in Materials and Methods. In parallel, $PBMC_{PT}$ were sensitized in the absence (▲) or presence (△) of achatinin-H under identical conditions, where unsensitized cells served as controls. The data show a profile representative of the results for VL patients ($n = 25$). The points represent the means ± SD of the results for triplicate sets.

were comparable to the levels evidenced in healthy donors (Fig. 5).

DISCUSSION

Immunosuppression is a predominant feature associated with VL (29, 33), which presents as a decreased TH1 response along with a simultaneous elevation of the TH2 response. The considerable decline in the expression of disease-associated 9-O-AcSGPs on $PBMC_{PT}$ compared to the level on $PBMC_{VL}$ instigated us to explore their contribution as immunomodulatory determinants. Importantly, sensitization of these molecules resulted in enhanced lymphoproliferation of $PBMC_{VL}$ that led to the production of a mixed TH1/TH2 response with a predominance of TH1 cytokines. A good correlation of this cellular response with the humoral response suggested the probable beneficial role of 9-O-AcSGPs in modulating the host immune response, depending upon the clinical status.

The search for immunomodulatory determinants as effective vaccine candidates is an ever-expanding area of research. A number of parasite antigens have been shown to possess the capacity to modulate the immune response of the host. The crude parasite antigen has been documented to produce a mixed TH1/TH2 response in $PBMC_{VL}$ (16, 23, 34). Notable among them is kinetoplast membrane protein 11 of *L. donovani*, which stimulated both the TH1/TH2 response and the production of recombinant papLe22 of *L. infantum* that triggered $PBMC_{VL}$ to liberate IL-10 (7, 44). Although the induction of a mixed TH1/TH2 response has been associated with therapy in patients with active VL, the elicitation of a response with a predominance of TH1 by immunomodulatory determinants from either parasite or host is certainly preferable (34).

TABLE 3. Comparison of levels of TH1/TH2 cytokines in culture supernatants of 9-O-AcSGP-sensitized and unsensitized PBMC[a]

Cytokine	Amt (pg/ml) of cytokine released by:					
	$PBMC_{VL}$		$PBMC_{PT}$		$PBMC_{H}$	
	Unsensitized	Sensitized[b]	Unsensitized	Sensitized	Unsensitized	Sensitized
IL-12	9 ± 3.20	114 ± 4.25	77.55 ± 6.24	80 ± 2.64	43 ± 6.20	44 ± 5.55
IFN-γ	35 ± 3.51	162 ± 4.21	96 ± 5.51	99 ± 2.51	32 ± 1.75	36 ± 5.4
IL-2	6.22 ± 2.45	67.50 ± 2.81	55 ± 3.5	57.51 ± 3	65 ± 4.30	64.45 ± 4
IL-10	95 ± 5.6	116 ± 6.50	34.55 ± 8.25	37.85 ± 3	55 ± 5	59.25 ± 3
IL-4	85 ± 3.15	96 ± 3.51	15.20 ± 2.16	19 ± 1.21	10.15 ± 0.91	9.33 ± 2
TNF-α	25.25 ± 3.21	175 ± 4.53	56.55 ± 4.12	67 ± 3.81	70 ± 2.81	72 ± 3.65

[a] Data are expressed as the means ± SD of triplicate sets and are representative of the results for 25 patients and healthy individuals. The detection of released cytokines in the cell-free culture supernatants following culture before and after 72 h of sensitization was performed using commercially available ELISA kits according to the manufacturer's instructions. Briefly, ELISA plates were coated with individual cytokine antibodies and blocked, after which cytokine standards or culture supernatants were added to the wells for quantification as described in Materials and Methods.

[b] All values were significantly different from those for healthy control subjects ($P < 0.05$) and from those for VL patients posttreatment ($P < 0.01$).

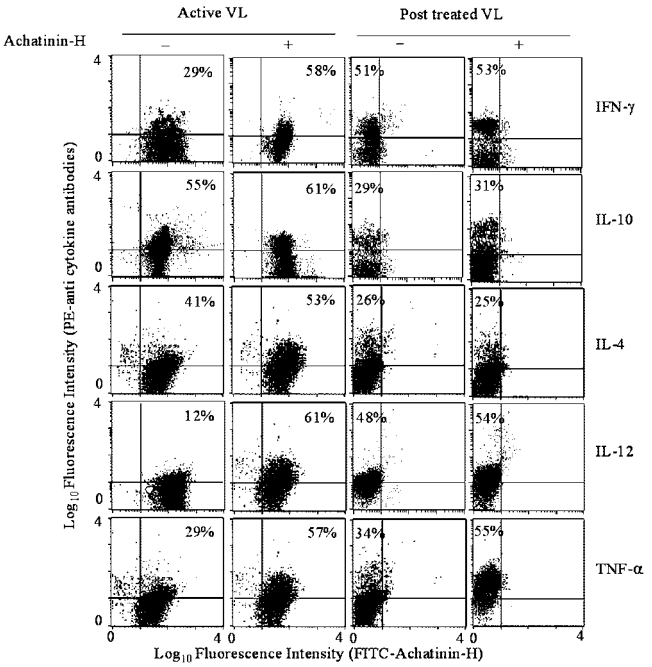

FIG. 3. Representative profiles of cell-mediated responses upon sensitization of 9-O-AcSGPs on $PBMC_{VL}$. 9-O-AcSGPs of $PBMC_{VL}$ (n = 15) were sensitized with achatinin-H (0.1 μg) for 72 h and cultured at 37°C in an atmosphere of 5% CO_2. Subsequently, the cells were stained for intracellular cytokines with PE-conjugated, cytokine-specific antibodies for 30 min at 4°C in the dark, processed, and analyzed by flow cytometry using CellQuest software. Lymphocytes were gated on the basis of their light scatter properties and were checked for the presence of cell surface 9-O-AcSGPs using FITC–achatinin-H to determine the percentages of 9-O-AcSA-positive cells that were stained for the respective intracellular cytokines. In parallel, $PBMC_{PT}$ were processed similarly. Unsensitized PBMC served as negative controls.

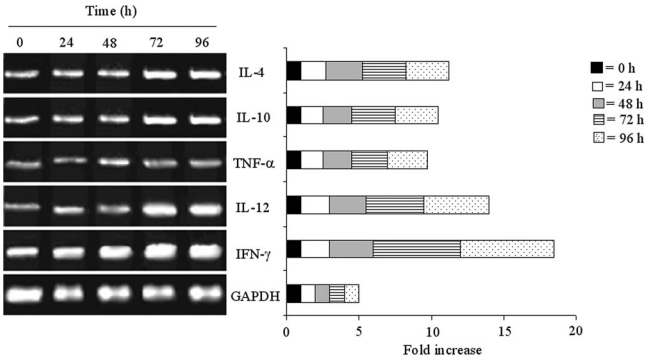

FIG. 4. Enhanced mRNA expression of the TH1 cytokines in sensitized $PBMC_{VL}$. The levels of different cytokines along with GAPDH (housekeeping gene) were evaluated by reverse transcription of RNA (0.1 μg) extracted from achatinin-H (0.1 μg) sensitized $PBMC_{VL}$ (n = 10) at different time points (0 to 96 h) as described in Materials and Methods. The mRNA expression of unsensitized cells served as the control. The values were compared by densitometric scores and are represented as the increase in band intensity compared with the expression of GAPDH at the respective time points.

However, the role of O-AcSGPs in immunomodulation of the host's immune response is a relatively unexplored area in the disease biology of VL. Previous studies by our group, using the preferential binding specificity of achatinin-H, have shown the expression on the cell surface of promastigotes of two specific 9-O-AcSGPs with molecular masses of 123 and 109 kDa (8, 27). Concomitantly, enhanced anti-9-O-AcSGP-specific antibody responses against these parasite-specific sialoglycoproteins have been witnessed as a part of the host defense mechanism (5, 6, 10, 39). Furthermore, these antibodies, as reported earlier, are leishmanicidal in activity; that is, they are capable of triggering the classical complement pathway, thereby causing *Leishmania* cell death (5). Alternatively, several other 9-O-AcSGPs have been documented on immune cells (65, 56, and 19 kDa) and erythrocytes (112, 107, 103, 57, 51, and 48 kDa) of VL patients (4, 11, 22). Since the alteration of sialic acid expression (via the alteration of enzymes responsible for sialic acid metabolism) has been well documented as a disease-induced phenomenon (26, 48, 49), the induction of these 9-O-AcSGPs on the host cells of patients with active VL is possibly a result of parasitic infection. Importantly, previous studies have demonstrated that each of these 9-O-AcSGP molecules is different from the others at the protein level, as revealed in isoelectric-focusing studies by the presence of discrete bands indicating their molecular heterogeneity, even though they are all alike in having 9-O-AcSA on their terminal surface (22). Thus, anti-9-O-AcSGP antibodies driven by the 9-O-AcSGPs present on the parasites recognize the disease-associated 9-O-AcSGPs on $PBMC_{VL}$ via recognition of the terminal 9-O-AcSA. Taking into consideration the importance of 9-O-AcSGPs in VL, their role as determinants promoting immunomodulation by pronounced lymphoproliferation has been investigated.

Our results, therefore, provide a clue to the involvement of enhanced sialylation on the immune cells of active VL patients (Table 2). The decline of 9-O-AcSGPs on T and B lymphocytes, as well as monocytes, in patients after successful chemotherapy was comparable to the levels in healthy donors and definitely suggested a probable link between this sialoglycotope and the course of the disease. The occurrence of enhanced sialylation on the surface of immune cells of VL patients hints at the presence of differential levels of four main enzymes, O-acetyltransferase, sialyltransferases, sialidase, and O-acetylesterases, that are responsible for regulating the metabolism of sialoglycoproteins or, more precisely, sialic acids (26, 42). Although limited molecular information about these enzymes during disease conditions has decelerated their exploration, studies pertaining to the causes of the altered expression of sialic acids are in progress.

The elevated levels of 9-O-AcSGP on all cells of $PBMC_{VL}$ indicate their probable involvement in enhanced proliferation. This was clearly demonstrated by sensitization of the newly induced 9-O-AcSAα2-6GalNAc sialoglycotopes on $PBMC_{VL}$ (Fig. 1). The increase in the lymphoproliferative response of $PBMC_{VL}$ through disease-associated 9-O-AcSGPs reflected their biologically active nature during disease presentation. The sensitization of $PBMC_{VL}$ after preincubation with BSM or de-O-acetylation demonstrated an absence of lymphoproliferation, reconfirming the inevitable involvement of O-AcSAs in this interaction (Fig. 1E). In contrast, the lack of proliferation of $PBMC_{PT}$ and $PBMC_{H}$ under similar conditions further confirmed the disease-associated sensitization of 9-O-AcSGPs,

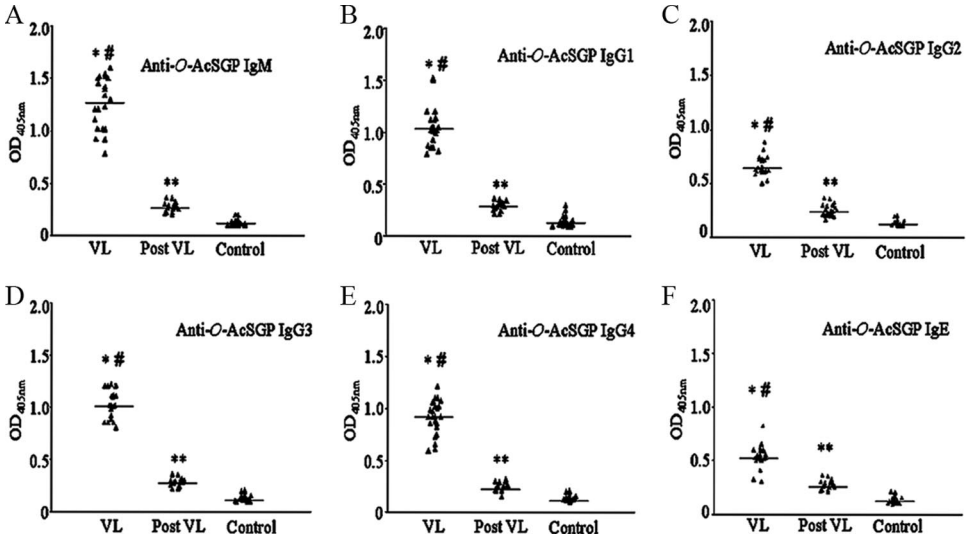

FIG. 5. Distribution of anti-O-AcSGP-specific antibody subclasses in VL patients before and after treatment. The levels of anti-O-AcSGP-specific IgM (A), IgG1 (B), IgG2 (C), IgG3 (D), IgG4 (E), and IgE (F) in VL patients before treatment ($n = 25$) were determined by subclass ELISA as described in Materials and Methods. The sera of individuals after treatment ($n = 25$) were tested similarly. Horizontal lines indicate mean values. *, P value of <0.01 for difference from results for successfully treated individuals; #, P value of <0.05, and **, P value of <0.01 for difference from results for healthy controls.

indicating their ability to generate an immunomodulatory response that contributes to VL pathogenesis. Interestingly, the much-lower lymphoproliferative response with known T- and B-cell mitogens and parasite antigen further confirmed the importance of 9-O-AcSGPs as potent immunomodulatory molecules influencing the pathology of VL.

Cytokines are the most important determinants that play a role in the defense mechanism of the host against infection. Because the pathology of VL is associated with the suppression of cell-mediated immunity (23, 33), we analyzed at the extracellular, intracellular, and genetic levels the pattern of cytokines resulting from the lymphoproliferative response upon sensitization of disease-associated 9-O-AcSGPs on $PBMC_{VL}$. The IFN-γ-mediated innate and cellular responses that are instrumental in triggering leishmanicidal responses by activated macrophages determine the disease status and are also prerequisites for protection (28, 29, 31, 34). Elevated levels of IL-4 and IL-10, the signature TH2 cytokines, correlate with the active disease state, whereas the level of IFN-γ, along with the level of IL-12, increases in patients after successful chemotherapy, signifying the restoration of the suppressed TH1 response (34). A 12.67-fold increase in the amount of IL-12 secreted by sensitized $PBMC_{VL}$ was probably sufficient to modulate a level of production of IFN-γ that was 4.6-fold-higher than the level in unsensitized controls (Fig. 2). Therefore, sensitization of 9-O-AcSGPs appears to play an important role in modulating the immune response toward a TH1 bias. Moreover, the fact that the amount of IL-2 in the culture supernatant of sensitized $PBMC_{VL}$ was larger than the amount in the unsensitized controls reconfirmed the predominance of the TH1 response as a result of the proliferation induced through sensitization of 9-O-AcSGPs (Table 3). Additionally, the increased secretion of both IL-10 and IL-4 by sensitized $PBMC_{VL}$ suggests that this interaction was also capable of generating a TH2 response that was essentially lower than the TH1 response. This trend of cytokine secretion was evidenced at the intracellular level, at which assays demonstrated an increased percentage of 9-O-AcSA-positive cells that stained for the TH1 cytokines after sensitization (Fig. 3). The results were consistent with the increased mRNA expression of IFN-γ, IL-12, and TNF-α (Fig. 4), indicating the potential of 9-O-AcSGPs in driving $PBMC_{VL}$ toward a mixed TH1/TH2 response, with a bias toward the TH1 response, through successful modulation. Importantly, increased levels of TNF-α in sensitized $PBMC_{VL}$ reflected the increased expression of proinflammatory cytokines upon sensitization, revealing the importance of 9-O-AcSGPs in VL pathogenesis. Taken together, these findings indicate that 9-O-AcSGPs appear to be important molecules contributing to the pathogenesis of VL by regulation of the immune responses in favor of the host.

The overall profile of the cellular responses is indispensable in defining the disease status of VL patients before and after chemotherapy (23). However, the disease also presents marked levels of parasite-specific antibodies in the sera of VL patients, which have diagnostic relevance as they happen to be the first signs of the infection (9, 25, 39, 41). Hence, a proper correlation of the cellular and humoral responses is key to understanding the disease pathogenesis. Therefore, the observed good correlation of the cell-mediated and humoral responses evidenced by increased subclass distribution of anti-O-AcSGP-specific antibodies confirmed the induced mixed cytokine response (Fig. 5) and reflected the highly immunogenic nature of 9-O-AcSGPs. It is known that IFN-γ probably upregulates isotypes IgG1 and IgG3 (1, 32) and that the TH2 cytokines stimulate the production of IgG4 and IgE (17, 24). The increased levels of anti-9-O-AcSGP IgG1 and IgG3 showed the predominant TH1 response elicited upon sensitization of 9-O-AcSGPs. Furthermore, the increase in IgE and IgG4 levels in patients with active VL in comparison to the levels in individuals after treatment could be explained by the

activation of the TH2 response, supporting the mixed response generated subsequent to sensitization. Hence, a good correlation of enhanced 9-O-AcSGPs with both the cell-mediated (r = 0.98) and humoral (r = 0.99) response was observed, further signifying their importance as immunomodulatory determinants.

Taken together, our results conclusively prove the considerable reduction in expression of 9-O-AcSGPs on different immune cells and of their specific antibody isotypes in the sera of parasitologically cured VL patients compared to the levels in patients with active VL. Additionally, we have demonstrated that these molecules can be sensitized, resulting in a cascade of immunomodulatory effects generating an elevated immune response with a TH1 bias. This kind of sensitization of O-acetylated sialoglycotopes via specific ligands hints at their biological role in determining immune responses that decided the course of the disease. In agreement with the observed finding, the anti-9-O-AcSGPs present in VL patients' sera may act as ligands for 9-O-AcSGPs on $PBMC_{VL}$, which could possibly be instrumental in causing such effects in the host. In-depth studies to reveal the causative molecules responsible for such immunomodulation are ongoing. With the development of newer sialoglycotherapeutic strategies (21), the controlled expression of these molecules may be exploited to pave the way for generating beneficial effects in VL patients.

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