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Glycosylinositolphosphate Soluble Variant Surface Glycoprotein Inhibits IFN-γ-Induced Nitric Oxide Production Via Reduction in STAT1 Phosphorylation in African Trypanosomiasis

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Macrophages are centrally involved in the host immune response to infection with Trypanosoma brucei rhodesiense, a protozoan parasite responsible for human sleeping sickness in Africa. During trypanosome infections, the host is exposed to parasite-derived molecules that mediate macrophage activation, specifically GPI anchor substituents associated with the shed variant surface glycoprotein (VSG), plus the host-activating agent IFN-γ, which is derived from activated T cells and is essential for resistance to trypanosomes. In this study, we demonstrate that the level and timing of exposure of macrophages to IFN-γ vs GPI ultimately determine the macrophage response at the level of induced gene expression. Treatment of macrophages with IFN-γ followed by GIP-sVSG (the soluble form of VSG containing the glycosylinositolphosphate substituent that is released by parasites) stimulated the induction of gene expression, including transcription of TNF-α, IL-6, GM-CSF, and IL-12p40. In contrast, treatment of macrophages with GPI-sVSG before IFN-γ stimulation resulted in a marked reduction of IFN-γ-induced responses, including transcription of inducible NO synthase and secretion of NO. Additional experiments revealed that the inhibitory activity of GPI-sVSG was associated with reduction in the level of STAT1 phosphorylation, an event required for IFN-γ-induced macrophage activation. These results suggest that modulation of specific aspects of the IFN-γ response may be one mechanism by which trypanosomes overcome host resistance during African trypanosomiasis.

M acrophages comprise the backbone of the host innate immune response, a key element in defense against parasitic infection. In addition to their role as APCs, which allows the adaptive immune system to become responsive to the invading pathogen, macrophages produce products that have both autocrine and paracrine effects that serve to amplify the innate and adaptive immune responses. In addition, macrophages can be stimulated to release reactive oxygen and nitrogen species, which have been demonstrated to have direct pathogen cytostatic and cytotoxic effects (1–5). Thus, a coordinated macrophage response is essential in the initiation and maintenance of a productive host response during microbial infection.

Human African trypanosomiasis is a fatal illness caused by infection with either of two subspecies of the protozoan parasite Trypanosoma brucei (T. b. rhodesiense and T. b. gambiense). The disease is characterized by episodic waves of parasitemia and tissue invasion, in which populations of trypanosomes expressing a variant surface glycoprotein (VSG) grow to high levels in the blood and other tissues. VSG molecules are expressed as an array of homodimers, tethered by GPI anchors to the extracellular surface of the trypanosome plasma membrane, forming a dense glycoprotein coat that protects the membrane of the parasite from the host environment (6, 7). Early during infection, a strong and effective host immune response is mounted against the parasite that includes B cell and Th1 cell stimulation by VSG determinants in addition to macrophage activation, resulting in destruction of trypanosomes expressing the target VSG (8, 9). However, trypanosomes undergo antigenic variation in which they express new VSG genes from a library of up to 10³ different surface Ag genes, effectively ensuring that the host immune response does not fully eliminate the organisms. This cyclical pattern of trypanosome outgrowth and variant specific elimination may continue throughout infection until the animal host succumbs.

Cumulative studies have revealed that both the B cell-mediated Ab response and the Th1 cell responses leading to the production of IFN-γ are required for maximum host resistance to trypanosomes in mice, with IFN-γ acting to induce macrophage trypanolytic and trypanostatic activities (5, 10). Recent studies demonstrated that trypanosome-infected mice with a resistant genetic background, but lacking the IFN-γ gene, were as susceptible as infected scid mice, despite the fact that they made VSG-specific Abs that controlled parasitemia in the blood. These data reveal that IFN-γ is a crucial element of the host response to these parasites (10).

It is well established that the IFN-γ-mediated macrophage activation includes the enhancement of MHC class II expression leading to enhanced Th cell response and the stimulation of microbicidal factors such as NO (11, 12). These two functional activities also appear to play key roles in the host response to trypanosome infection (13, 14). However, during infection with African trypanosomes, parasite-derived molecules also capable of modulating macrophage activation are released. Among these are
the glycosylinositophosphate soluble VSG (GIP-sVSG) molecules, which are cleaved from the trypanosome membrane by the action of GPI-phospholipase C (PLC). In this study, we show that during early trypanosome infection of C57BL/10 mice (days 1–15), a robust IFN-γ response is made, correlating with the rise and fall of the first wave of parasitemia and coincident with a moderate release of GIP-sVSG. In contrast, during late stage infection (days 35–55), IFN-γ production is no longer detectable in mice exhibiting sustained high levels of parasitemia, and abundant levels of GIP-sVSG are present. These in vivo data led us to ask if mice exhibiting sustained high levels of parasitemia, and abundant ability of the host to respond to trypanosome infection.

Experimental infections were established in 6- to 8-wk-old, female C57BL/10 mice by i.p. injection of 1 × 10⁶ trypanosomes suitable for preparation of GIP-sVSG. All animals were housed in University-approved facilities and were handled strictly according to National Institutes of Health and University of Wisconsin-Madison Research Animal Resource Center guidelines.

**Trypanosomes and GIP-sVSG**

Stabulates of *T.b rhodesiense*, clone LouTat 1, used for establishing experimental infections, were first expanded in mice that had been immunosuppressed with cyclophosphamide (300 mg/kg body weight) to permit unrestricted trypanosome growth, as previously described (15, 16). Trypanosomes were subsequently isolated from the blood by cardiac puncture. The blood was diluted with an equal volume of ice-cold buffer containing 500 mM Na₂H₂bis[2-hydroxyethyl] glycine, 50 mM KC₅, 500 mM NaCl, and 1% (v/v) glucose bicarbonate-buffered saline with glucose (BBSG) (all from Sigma-Aldrich, St. Louis, MO) and passed over a DEAE Sephadex column equilibrated with 10 mM phosphate buffer, pH 8.0. All GIP-sVSG samples were assessed for purity by electrophoretic gel analysis; GIP-sVSG purified in this manner appeared as a single band on SDS-polyacrylamide gels run under reducing conditions, with an apparent molecular mass of 62 kDa (data not shown). Confirmation of GIP-sVSG purification and identity was made by Western blot analysis with GIP-sVSG Ab, as previously described (17, 18).

**Reagents**

The following reagents were used for treatment of RAW 264.7 macrophages: murine rIFN-γ (Schering, Bloomfield, NJ; sp. act. 1.7 × 10⁶ U/mg (provided by the American Cancer Society)) and polymyxin B (Sigma-Aldrich). Ligands were resuspended in PBS or medium for cell stimulation, as noted in the text.

**Cells and cell culture**

The RAW 264.7 macrophage cell line, obtained from the American Type Culture Collection (Manassas, VA), was used in all experiments as the target cell for stimulation. Mycoplasma-free cell cultures were maintained in complete medium, consisting of RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with 2 mM glutamine, 1 mM pyruvate, 50 U/ml penicillin, 50 μg/ml streptomycin, and 2 g/L sodium bicarbonate (all from Sigma-Aldrich), plus 10% FBS (Life Technologies). Additional aspects of cell maintenance were as previously described (19, 20).

For cell stimulation, RAW 264.7 monolayers were established in tissue culture dishes (Corning/Costar, Corning, NY) and stimulated with GIP-sVSG and/or IFN-γ for the indicated times at 37°C, 5% CO₂. At completion of the experiment, cells were harvested by scraping with a rubber policeman and processed for protein lysates, RNA, and/or cell-free supernatant fluids. Cell viability in all cases was routinely greater than 95%, as monitored by trypan blue exclusion.

**RNA isolation and RT-PCR**

Isolation of RNA, reverse transcription, and PCR assays were performed, as described previously (19–21). Briefly, total RNA was isolated using RNA STAT-60 (Tel-Test B, Friendswood, TX), according to the manufacturer’s instructions. Synthesis of cDNA from purified RNA was done by priming with oligo(dT) (Roche, Indianapolis, IN), and each cDNA sample was used as a template for gene-specific amplification. PCR amplifications were performed in a 96-well thermocycler (MJR Research, Watertown, MA). Verification of equivalent cDNA loading per PCR was done by assessing amplification of the G3PDH housekeeping gene. G3PDH primers were purchased from Clontech Laboratories (Palo Alto, CA); all other PCR primers were designed in our laboratory using the Oligo 4.0 program (National Biosciences, Plymouth, MN) and have been previously described (19–21). Samples were processed as previously described (19–21). Briefly, amplified cDNA products were separated by electrophoresis in 1.5% agarose gel and visualized by ethidium bromide staining. For RT-PCR experiments involving semiquantitative analysis, reactions were set up as previously described (22); however, agarose gels were stained with SYBR Green I nucleic acid stain (Molecular Probes, Eugene, OR) in TAE buffer for the indicated times at 37°C, 7% CO₂. At completion of the experiment, cells were harvested by scraping with a rubber policeman and processed for protein lysates, RNA, and/or cell-free supernatant fluids. Cell viability in all cases was routinely greater than 95%, as monitored by trypan blue exclusion.
buffer. DNA-associated fluorescence was visualized using the 8806 Typhoon Variable Mode Imager (Molecular Dynamics, Sunnyvale, CA), and data were analyzed using ImageQuant (Molecular Dynamics) and Microsoft Excel software with subtraction of background fluorescent signal.

**Nitrite assays**

The Griess reaction was used to assess the production of NO in culture supernatant fluid by monitoring the level of production of nitrite and nitrate, which are the oxidation products of NO (23). For these experiments, 1.5 x 10⁵ RAW 264.7 cells were plated in 24-well tissue culture plates and grown to confluence before stimulation. At the completion of the experiments, cell-free culture supernatant fluids were obtained by centrifuging the plates at 1200 rpm for 10 min at 4°C. Supernatant fluid (50 μl) was mixed with 50 μl of Griess reagent in 96-well microtiter plates, and the absorbance at 550 nm was quantified on a SpectraMax 250 plate reader (Molecular Devices, Sunnyvale, CA), using the SoftMax Pro 1.1 Software program for the Macintosh (Molecular Devices). NaN₃ in RPMI was used to construct a standard curve for each plate reading. Protein concentrations for each sample were determined using the Bio-Rad protein assay, according to manufacturer’s instructions (Bio-Rad Laboratories, Hercules, CA).

**Statistics and percentage of inhibition**

The statistical significance of the differences observed was assessed by Student’s t test. Differences were considered significant when p values of ≤0.05 were obtained. All experiments were performed at least three times.

Percentage of inhibition was calculated as:

\[
A = 100 \times \left( \frac{(B - D)}{B - C} \right) \%
\]

in which A = percentage of inhibition; B = iNOS transcription and/or NO release after stimulation with IFN-γ; C = iNOS transcription and/or NO release in control treatments; and D = iNOS transcription and/or NO release after IFN-γ stimulation in presence of GIP-sVSG, as described previously (24).

**Western analysis**

RAW 264.7 macrophage cells (1.5 x 10⁵) were lysed in 50 μl ice-cold lysis buffer (150 mM NaCl, 2 mM EDTA, 50 mM Tris, pH 7.4, 1% Non-ident P-40, 0.02% NaN₃, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μM pepstatin, 1 mM PMSE, and 1 mM Na₃VO₄ (all from Sigma-Aldrich)). Protein content was determined as described above. Proteins were resolved by SDS-PAGE (50 μg/lane) under reducing conditions in 10% gels and then transferred to Immobilon polyvinylidene difluoride membrane. Proteins of interest were detected using the following Abs: iNOS (Santa Cruz Biotechnology, Santa Cruz, CA), phospho-STAT1 (Cell Signaling Technology, Beverly, MA), α-actin (Sigma-Aldrich), or STAT1 (Santa Cruz). Immunoreactive proteins were visualized using HRP-labeled secondary Abs and SuperSignal chemiluminescent reagents (Pierce, Rockford, IL).

**Results**

**Relative levels of parasitemia, IFN-γ, and GIP-sVSG vary during the course of trypanosomal infection**

To determine how levels of parasitemia, GIP-sVSG, and IFN-γ change over the course of infection, blood samples were taken from individual C57BL/10 mice every 24 h throughout the duration of infection with *T.b rhodesiense* LouTat 1 (see Materials and Methods). As shown in Fig. 1A, in the first 15 days during infection, IFN-γ levels rise and fall in the serum in a manner concordant with levels of parasitemia. During this period of time, GIP-sVSG also is released into the bloodstream, with peak levels appearing at day 6, when parasitemia is greatest (Fig. 1B). Similar analyses using blood samples obtained during the late stages of infection (day 35 and higher) revealed that the IFN-γ concentration in the serum remained relatively low (<250 pg/ml), while the parasitemia is consistently >1 x 10⁵/ml (Fig. 1A). The sustained increases in the levels of parasites circulating in the blood also result in a concentration of GIP-sVSG during late stage infection that is greater than that found earlier during infection (Fig. 1B).

**IFN-γ priming enhances the response of RAW 264.7 cells to GIP-sVSG**

Our results suggest that macrophages will be exposed in vivo to differing levels of both IFN-γ and GIP-sVSG at various time points of infection. We duplicated some of these conditions in vitro, using doses of 1.6 μM GIP-sVSG and 12 ng/ml (20 U/ml) IFN-γ, applied in various combinations to mimic concentrations occurring at least within the first days after infection. Control cultures of RAW 264.7 macrophages were stimulated with either IFN-γ or GIP-sVSG alone or were stimulated with IFN-γ for 24 h, followed by GIP-sVSG for an additional 24 h, as previously described (25). Cells then were processed for RNA purification, and changes in expression of the above GIP-sVSG-inducible genes...
were monitored by RT-PCR, as described in Materials and Methods. As shown in Fig. 2, stimulation of RAW 264.7 cells with either IFN-γ or GIP-sVSG alone at these concentrations was only marginally effective at gene induction with either ligand, confirming our previous results (25). However, priming of the RAW 264.7 cells with IFN-γ, followed by stimulation with GIP-sVSG, induced readily detectable expression of several genes assessed, including TNF-α, IL-6, and IL-12p40 (Fig. 2). These results demonstrate that IFN-γ priming can enhance the ability of GIP-sVSG to induce RAW 264.7 macrophage gene transcription.

Prior exposure of RAW 264.7 cells to GIP-sVSG decreases IFN-γ-inducible gene expression

Because of the dynamic appearance of IFN-γ vs GIP-sVSG during the course of trypanosome infection, we asked how macrophages would respond if first exposed to GIP-sVSG, followed by stimulation with IFN-γ. RAW 264.7 cells in these experiments were treated for 24 h with GIP-sVSG, followed by an additional 24-h incubation with IFN-γ. Changes in the expression of the IFN-γ-inducible genes previously demonstrated to not be induced by GIP-sVSG (25) were monitored by RT-PCR, as described in Materials and Methods. The results shown in Fig. 3 reveal a subtle, but reproducible decrease in gene transcription of the IFN-γ-inducible genes assessed when the macrophages were first treated with GIP-sVSG, followed by addition of IFN-γ.

To more closely examine the apparent reduction in gene transcription, we used a quantitative RT-PCR method. For these experiments, we elected to focus on the regulation of iNOS, as the product NO is thought to play an antimicrobial role during infection (2). The cDNA prepared from cells to be tested was used as a substrate for PCR that were sampled every three cycles, subjected to gel electrophoresis, and stained with SYBR green dye (see Materials and Methods for details) (Fig. 4A). The relative fluorescence of each cDNA product from IFN-γ-, GIP-sVSG-, and GIP-sVSG + IFN-γ-treated macrophages was compared with that of control-treated cells at each cycle number. These data were used to determine the percentage of gene transcription induced with the various treatments relative to the basal level of transcription found in control-treated macrophages (Fig. 4B). These results show that GIP-sVSG reduces IFN-γ-inducible iNOS gene transcription by greater than 80% relative to macrophages treated with IFN-γ alone. Little or no effect on the induction of gene expression was seen when the cells were treated with the same molecules in the reverse order (data not shown). Transcription of the housekeeping gene G3PDH was not affected by treatment with either GIP-sVSG or IFN-γ individually or in combination (Fig. 4B and data not shown). These results suggest that exposure of RAW 264.7 macrophages to GIP-sVSG modulates at least some aspects of the IFN-γ response in these cells.

Pretreatment of RAW 264.7 cells with GIP-sVSG also inhibits IFN-γ-induced NO production

To further explore the extent of this inhibitory effect of GIP-sVSG, we assessed whether treatment of RAW 264.7 cells with GIP-sVSG could modulate either the IFN-γ-induced expression of iNOS protein (NOSII) or NO production. Using the same induction protocol described above, RAW 264.7 macrophages were treated first with either IFN-γ or GIP-sVSG for 24 h, followed by stimulation for an additional 24 h with the opposing molecule. We then assessed levels of iNOS protein by Western blotting using lysates of treated cells. As shown in Fig. 5A, treatment of RAW 264.7 cells with IFN-γ or GIP-sVSG alone stimulated modest or no increases in iNOS protein production, respectively. However, in a manner similar to that observed for iNOS gene transcription, initial stimulation of RAW 264.7 cells with GIP-sVSG inhibited subsequent IFN-γ-induced iNOS protein production (Fig. 5B).

This same pattern of inhibition vs potentiation was observed when cell-free supernatant fluids, taken from the RAW 264.7 macrophage cultures used for preparation of the protein lysates, were

![Figure 3](Image)

**FIGURE 3.** GIP-sVSG pretreatment reduces IFN-γ-inducible gene transcription. RAW 264.7 cells were stimulated by either control medium (C) or IFN-γ (I) (12 ng/ml), or were treated with GIP-sVSG (24 h), followed by IFN-γ (24 h) (sV+I), as described in Materials and Methods. After stimulation, RNA was isolated and induced gene expression was assessed, as described in the legend of Fig. 2.

![Figure 4](Image)

**FIGURE 4.** Quantitative analysis of GIP-sVSG inhibition of iNOS gene transcription. RAW 264.7 cells were stimulated by either control medium (C), GIP-sVSG (sV) (1.6 μM), or IFN-γ (I) (12 ng/ml), or were treated with GIP-sVSG (24 h), followed by IFN-γ (24 h) (sV+I), as described in Materials and Methods. After stimulation, RNA was isolated and induced gene expression was assessed, as described in the text and Materials and Methods. The results of using iNOS primers for the detection of genespecific cDNA are shown in A, and graphical representations of the relative fluorescence of the PCR products using primers to iNOS (B, upper) and to the constitutively active G3PDH (B, lower) are shown. Results shown are from one representative experiment; three independent experiments were done. *, p < 0.05; 81.5% ± 16% reduction vs IFN-γ alone (I).
IFN-γ of three experiments; were first treated with GIP-sVSG, followed by IFN-γ stimulated by either mediator alone. However, when macrophages were exposed to GIP-sVSG exposure, NO production was increased beyond the level seen with concentrations as low as 0.8 μM. Pretreatment with GIP-sVSG significantly inhibited IFN-γ-induced NO production by close to 100% at the lowest IFN-γ dose used (6 ng/ml) and by ~25% when used at a concentration of 30 ng/ml. In contrast, treatment of RAW 264.7 cells with 1.6 μM GIP-sVSG for 24 h, followed by doses of IFN-γ above 30 ng/ml (60 ng/ml) or 300 ng/ml (500 U/ml), resulted in reduction of IFN-γ-dependent NO production by less than 10%. Thus, high levels of IFN-γ are able to overcome the immunosuppressive activity of GIP-sVSG.

GIP-sVSG inhibits IFN-γ-dependent STAT1 phosphorylation

To begin to understand the mechanism by which GIP-sVSG is able to reduce macrophage responsiveness to IFN-γ stimulation, we examined the effect of this mediator on STAT1 phosphorylation, a well-defined early step in IFN-γ-dependent signaling. For this experiment, macrophages were pretreated with GIP-sVSG for 24 h before stimulation with IFN-γ for 30 min. GIP-sVSG was able to inhibit IFN-γ-dependent STAT1 phosphorylation in a dose-dependent manner, both with respect to the concentration of GIP-sVSG and IFN-γ (Fig. 7). The extent of STAT1 phosphorylation, as monitored by changes in the level of the phosphorylated product, varied widely, from little or no inhibition (1.6 μM GIP-sVSG + 12 ng/ml IFN-γ) to an apparently complete block in appearance of the phosphorylated form (4.0 μM GIP-sVSG + 3 ng/ml IFN-γ). This effect correlates to the ability of IFN-γ to overcome GIP-sVSG inhibition at the level of NO production, as illustrated in Fig. 6. These results suggest that the dose-dependent inhibitory effects of GIP-sVSG are mediated at least in part through regulation of IFN-γ-mediated STAT1 phosphorylation.

Discussion

These experiments provide the first insights into the outcome of coincident exposure of macrophages to IFN-γ and GIP-sVSG during trypanosome infection. Although these two mediators are both released during infection, few studies have looked closely at how they might modulate, individually or together, macrophage activation. Our in vivo assessment reveals that the levels of GIP-sVSG and IFN-γ fluctuate over the course of infection with T. b. rhodesiense 1. In the earliest phase of infection, IFN-γ levels rise and fall rapidly concurrent with the proliferation and subsequent Ab-mediated  

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**FIGURE 5.** GIP-sVSG inhibits IFN-γ-induced iNOS protein translation and NO production. RAW 264.7 cells were stimulated by either control medium (C), GIP-sVSG (sV) (1.6 μM), or IFN-γ (I) (12 ng/ml) alone or in combination (i.e., IFN-γ for 24 h, followed by GIP-sVSG for 24 h or vice versa). After stimulation, cell lysates and cell-free culture supernatant fluids were harvested and assayed for either iNOS protein levels (cell lysates) or nitrite concentration (supernatants) using the Griess reaction, as described in **Materials and Methods**. The protein concentration of each cell lysate and supernatant fluid sample was determined, as described in **Materials and Methods**. Equal amounts of protein (60 μg/lane) were used in the iNOS Western blot, and nitrate concentrations in supernatant fluids were normalized to total sample protein concentrations. Immunoblotting for α-actin was used as a control for protein loading on the Western blot. Values equal the mean ± SEM of a minimum of three experiments; *, p < 0.05 for the decrease in nitrite production relative to levels of nitrite produced by RAW 264.7 cells stimulated with IFN-γ in the presence of medium alone.

**FIGURE 6.** Increasing levels of IFN-γ can overcome GIP-sVSG-mediated inhibition. RAW 264.7 cells were treated for 24 h, 1.6 μM GIP-sVSG before their subsequent activation for 24 h by increasing doses of IFN-γ (6–300 ng/ml). Nitrite concentrations and percentage of inhibition were calculated as described in **Materials and Methods**. Values equal the mean ± SEM of a minimum of three experiments. *, p < 0.05 compared with inhibition of IFN-γ-induced NO in the presence of medium alone.
clearance of parasites in the bloodstream. Also during this period of time, fluctuating levels of GIP-sVSG, the shed form of the trypanosome VSG surface protein coat, are found in the serum. In contrast, during the late phase of infection, the sustained rise in parasitemia, and in the levels of GIP-sVSG associated with parasitemia, is dramatic and high until the host animal ultimately succumbs to infection. IFN-γ levels remain low during this period despite continuing exposure of host T cells to parasite Ags.

With these patterns in mind, we examined the coordinate effects of IFN-γ vs GIP-sVSG on macrophages. GIP-sVSG has been shown to function as an immunostimulatory molecule in other studies (21, 27–29); however, this activity is most clearly observed when the GIP-sVSG acts in combination with the potent macrophage priming agent IFN-γ or is present at very high levels (25). Such results suggest that the modest stimulatory effect of GIP-sVSG is dependent on both the concentration of the ligand as well as the host factors to which the cell has been exposed previously. Our results also demonstrate, however, that GIP-sVSG can inhibit the activation of IFN-γ-inducible macrophage parameters as iNOS gene expression and NO production. This inhibitory effect of GIP-sVSG on the activation of RAW 264.7 macrophages also was shown to be dependent on both the level of the ligand as well as the duration of macrophage exposure to GIP-sVSG before addition of IFN-γ. Taken in sum, these results suggest that the fluctuating levels of host (i.e., IFN-γ) and parasite (i.e., GIP-sVSG) factors during infection act to control macrophage functional activity in a complex and subtle manner, with the outcome determined by the concentration of each mediator, the temporal pattern of its production, and the microenvironment of the target macrophage.

The phenomenon of immune suppression associated with African trypanosomiasis is a well-documented, although not well-understood, aspect of infection. It is clear that infection with trypanosomes is accompanied by the development of increasing immunosuppression that results in a progressive inability of the host to control parasite growth, leading ultimately to death (23–26). For the past two decades, considerable effort has been devoted to elucidating the mechanism of the immunosuppression, focusing primarily on regulation of B and T lymphocyte function. Early studies found that macrophages isolated from parasite-infected mice had profound inhibitory effects on B and T cells both in vitro as well as in vivo, and thus macrophages were identified as the putative mediators of suppressor cell activity (9, 16, 30–32). Subsequent studies showed that treatment of macrophages in vitro with soluble trypanosomal lysate could induce a suppressor cell activity in these cells that mimicked that found in vivo during infection (33). However, the full identity of the trypanosome factor responsible for inducing macrophage suppressor cell activity was not elucidated in this work.

One important component of the soluble trypanosome lysates used in these early studies was in all likelihood parasite-derived GIP-sVSG. Despite the recognition that large amounts of this parasite-derived material are present in the infected host, limited studies to date have directly examined the potential immunomodulatory effects of GIP-sVSG. During infection, the soluble form of the parasite surface coat, GIP-sVSG, is released into the host bloodstream and extravascular tissue spaces as a result of cleavage by the parasite-derived enzyme, GPI-PLC. At times of peak parasitemia in rodent model systems, there can be as many as one billion parasites per milliliter of blood, each expressing ~10^7 molecules of VSG on its surface coat potentially available for cleavage and release (25, 34). Previous work has shown that a single inoculation of GIP-sVSG into the bloodstream can still be detected up to 96 h later, with accumulation apparent in tissues such as the spleen, liver, and lungs (35). These results suggest that the amount of GIP-sVSG identified in the serum in Fig. 1A most likely represents only a fraction of that released by the trypanosomes and potentially available to interact with host immune cells.

Although the studies reported in this work demonstrate that GIP-sVSG inhibits IFN-γ-inducible STAT1 phosphorylation, appreciation of the complete mechanism of macrophage regulation by GIP-sVSG remains to be fully elucidated. Numerous strategies have been described by which other organisms, particularly obligate intracellular microbes resident in macrophages, manipulate macrophage activation with respect to IFN-γ responsiveness and all appear to target points in the receptor-mediated Janus kinase-STAT signaling cascade that is the core of the IFN-γ activation response (28, 29, 36–43), albeit via different mechanisms. Thus, future experiments in this system will examine changes in these signaling pathway components to fully identify the mechanism of inhibition during trypanosomal infection.

In addition to understanding the mechanism of action of this molecule, the means of its interactions with macrophages also remains to be elucidated. Previous studies of the major surface glycosylconjugate of *Leishmania major*, the GPI anchor-related structure glycoinositolphospholipid, have revealed that exogenous addition of this molecule to murine macrophages inhibits the synthesis of IFN-γ-dependent NO in a time- and dose-dependent manner similar to that shown in this work with *T.b rhodesiense*-derived GIP-sVSG (44). The structural similarities between glycoinositolphospholipids and the GPI anchor of GIP-sVSG suggest that this inhibitory activity may be common to molecules with this type of lipid-linked carbohydrate moiety, potentially via their ability to interact with the same (or similar) macrophage receptors and to activate conserved down-regulatory signaling components. Thus, an understanding of the molecular basis of the modifications that take

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**FIGURE 7.** GIP-sVSG inhibits IFN-γ-induced STAT1 phosphorylation. RAW 264.7 cell line macrophages were stimulated with either IFN-γ (6 ng/ml (10 U/ml) or 12 ng/ml (20 U/ml)) or GIP-sVSG (1.6 or 4.0 μM) alone for 30 min or with GIP-sVSG for 24 h before 30-min stimulation with IFN-γ. Cell lysates were prepared, and proteins (50 μg/lane) were resolved by SDS-PAGE for Western blot analysis. STAT1 activation was assessed using a tyrosine phospho-specific anti-STAT1 Ab. To confirm equal protein loading, the same blot was stripped and reprobed with a polyclonal Ab that recognizes multiple sites on the STAT1 proteins.
place during exposure to IFN-γ and GIP-αVSG, of how these mechanisms are altered by changes in the level or time of macrophage exposure to these factors, and of what cell-ligand interactions initiate the inhibitory events are all important for future studies. Full comprehension of these mechanisms could aid in determining how alterations in macrophage function during trypanosome infection act to regulate host resistance vs susceptibility to this disease.

The studies reported in this work have examined critically for the first time macrophage regulation via host-derived IFN-γ and parasite-derived GIP-αVSG, in an effort to understand whether activation vs suppression might predominate during disease and how the macrophage response may be modulated relative to the changing parameters of exposure to these factors. The complex pattern of GIP-αVSG-mediated activation and inhibition of IFN-γ-dependent gene expression revealed by our studies suggests a previously unappreciated regulatory function of this molecule in modulating the macrophage component of the innate immune response and suggests that changes in these two molecules during disease progression could have significant implications for the host.

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