The Glycosyl-Insitol-Phosphate and Dimyristoylglycerol Moieties of the Glycosylphosphatidylinositol Anchor of the Trypanosome Variant-Specific Surface Glycoprotein Are Distinct Macrophage-Activating Factors

Stefan Magez, Benoît Stijlemans, Magdalena Radwanska, Etienne Pays, Michael A. J. Ferguson and Patrick De Baetselier


References
This article cites 40 articles, 21 of which you can access for free at: http://www.jimmunol.org/content/160/4/1949.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at: http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
The Glycosyl-Insitol-Phosphate and Dimyristoylglycerol Moieties of the Glycosylphosphatidylinositol Anchor of the Trypanosome Variant-Specific Surface Glycoprotein Are Distinct Macrophage-Activating Factors

Stefan Magez,2,* Benoît Stijlemans,* Magdalena Radwanska,† Etienne Pays,† Michael A. J. Ferguson,‡ and Patrick De Baetselier*

The TNF-α-inducing capacity of different trypanosome components was analyzed in vitro, using as indicator cells a macrophage cell line (2C11/12) or peritoneal exudate cells from LPS-resistant C57/H-HeJ mice and LPS-sensitive C57/H-HeN mice. The variant-specific surface glycoprotein (VSG) was identified as the major TNF-α-inducing component present in trypanosome-soluble extracts. Both soluble (sVSG) and membrane-bound VSG (mfVSG) were shown to manifest similar TNF-α-inducing capacities, indicating that the dimyristoylglycerol (DMG) compound of the mfVSG anchor was not required for TNF-α triggering. Detailed analysis indicated that the glycosyl-insitol-phosphate (GIP) moiety was responsible for the TNF-α-inducing activity of VSG and that the presence of the GIP-associated galactose side chain was essential for optimal TNF-α production. Furthermore, the results showed that the responsiveness of macrophages toward the TNF-α-inducing activity of VSG was strictly dependent on the activation state of the macrophages, since resident macrophages required IFN-γ preactivation to become responsive. Comparative analysis of the ability of both forms of VSG to activate macrophages revealed that mfVSG but not sVSG stimulates macrophages toward IL-1α secretion and acquisition of LPS responsiveness. The priming activity of mfVSG toward LPS responsiveness was also demonstrated in vivo and may be relevant during trypanosome infections, since Trypanosoma brucei-infected mice became gradually LPS-hypersensitive during the course of infection. Collectively, the VSG of trypanosomes encompasses two distinct macrophage-activating components: while the GIP moiety of sVSG mediates TNF-α induction, the DMG compound of the mfVSG anchor contributes to IL-1α induction and LPS sensitization.

based on a regular switch in the expression of VSG variants, jeopardizing the induction of an effective Ab response against the parasite. Interestingly, in response to environmental stress, trypanosomes are capable of liberating their VSG through a VSG lipase (20). This enzyme cleaves the GPI anchor, leaving the dimyristoylgllycerol (DMG) compound of the GPI anchored in the membrane, and releasing the glycosyl-inositol-phosphate (GIP)-VSG part (21). In this report, we compare the macrophage-activating capacity, analyzed in terms of cytokine secretion (TNF-α/IL-1α, IL-6, IL-10, and IL-12) and induction of LPS hyper-responsive-ness, of the released soluble VSG (sVSG) and the membrane-bound form VSG (mVSG). We show that both forms of VSG possess distinct macrophage-activating components.

**Materials and Methods**

**Cell cultures**

2C11/12 macrophages (22) were grown in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.3 mg/ml l-glutamine, all purchased from Life Technologies Laboratories, Grand Island, NY. Adherent cells were selected by a routine 2-day transfer on plastic cell culture plates (Costar, Cambridge, MA). For in vitro TNF-α induction assays, cells were left to adhere for 2 h at 37°C in fresh medium on 96-well culture plates before stimulation with trypanosome components or LPS. Cells were used at a concentration of 4 × 10^5 cells/ml, 100 μl/well. Peritoneal exudate cells (PECs) were harvested from both LPS-sensitive C57BL/6 mice and LPS-resistant C3H/HeN mice (Harlan, Zeist, The Netherlands) by a peritoneal wash with 10 ml of ice-cold sucrose/H2O solution (116 g/L). The collected cells were washed in RPMI 1640 and left to adhere for 2 h at 37°C on 96-well culture plates. Cells were used for TNF-α induction assays at a concentration of 2 × 10^6 cells/ml, 100 μl/well. When indicated, 2C11/12 and PECs were stimulated with recombinant murine IFN-γ (Life Technologies) at a final concentration of 100 U/ml. All cell incubations were done at 37°C in a humid 5% CO2 atmosphere incubator.

**Trypanosomes**

The AnTat 1.1E clone of the EATRO 1125 stock of the pleomorphic bloodstream form was kindly provided by Dr. N. Van Meirvenne (Institute of Tropical Medicine, Antwerp, Belgium). To follow the course of the parasitemia, 6- to 8-wk-old female C57BL/6 mice (Harlan) were injected i.p. with 5000 parasites. At intervals of 2 or 3 days, the number of parasites present in the blood was counted using a light microscope.

**Trypanosome-soluble extract and VSG preparation**

Trypanosomes were harvested from infected blood by DE52 chromatography (23), using sterile PBS (pH 8.0) supplemented with 1% glucose for equilibration and elution. After separation, parasites were washed and resuspended in RPMI 1640 medium at a concentration of 10^6 cells/ml. Crude parasite lysate was obtained by three freeze/thaw cycles in the presence of 1 mM Pefabloc protease inhibitor (Boehringer Mannheim, Mannheim, Germany) and 0.01 mM EDTA (Sigma Chemical Co., St. Louis, MO). Soluble extract was obtained by removing nonsoluble components by 15-min centrifugation at 13,000 × g. vSvSGs were prepared from DE52-purified parasites by osmotic lysis for 5 min at 37°C at 10^7 cells/ml in 10 mM sodium phosphate (pH 8.0) containing 0.1 mM TLCK and 0.1 mM PMSF (both from Boehringer Mannheim). The supernatant was passed through a column of DE52 equilibrated in 10 mM sodium phosphate (pH 8.0), vSvSG was further purified on a column of Sephacryl-S200 (Pharmacia Biotech, Uppsala, Sweden), dialyzed against water overnight at 4°C, and freeze-dried. mVSG was prepared according to the method of Jackson et al. (24). Freeze-dried VSG was resuspended in sterile RPMI 1640 medium just before use. All soluble extract and VSG samples were incubated under gentle shaking for 2 h at room temperature with Pronase-Ret留意us (Bioprocessing, Princeton, NJ) glass beads to remove possible LPS contamination. Afterward, beads were separated by sample filtration over a 22-μm sterile Spin-X centrifuge tube filter (Costar).

Protein concentration of the soluble extract and VSG was estimated by a detergent-compatible protein assay kit (Bio-Rad Laboratories, Hercules, CA) using BSA as a standard.

**Enzyme treatments**

All enzymes used to digest trypanosome-soluble extract or VSG and protease inhibitors were purchased from Boehringer Mannheim. These include recombinant N-glycosidase F from *Flavobacterium meningosepticum*, α-galactosidase from green coffee beans, Pronase from *Streptomyces griseus*, Proteinase K from *Trichirachium album*, and the protease inhibitors Pefabloc SC, PMSF, and TLCK. All enzymes and inhibitors were used according to the manufacturer’s instructions.

N-Glycosidase F digestion was performed in PBS using 5 U/ml enzyme (24 h/37°C).

α-Galactosidase digestion of VSG was performed in 0.1 M sodium acetate buffer, pH 5.0, using 50 U/ml enzyme (2 × 18 h/37°C). Proteinase K digestion was performed in PBS, pH 8.0, using 5 U/ml enzyme (24 h/37°C).

Pefabloc SC was used when indicated (1 mM final concentration) to prevent proteinase activity during lysis preparation by freeze/thaw cycles. Furthermore, it was used to stop Proteinase K digestion of trypanosome-soluble extracts or VSG.

**Preparation of the glycosyl-inositol-phosphate (GIP) fraction**

The GIP fraction, otherwise known as the sVSG COOH-terminal glycopeptide (sCtg-gp), was purified from a Pronase digest of vSvSG (mit 1.4) by ion exchange chromatography on Dowex AG50 and QAE-Sephadex A25, as described earlier (25). This fraction was digested with coffee bean endo-α-mannanase and TNF-α or TNF-α-related proteinase on QAE-Sephadex A25. A negative ion electrospray mass spectrum of final GIP fraction revealed [M-2H]^- pseudo molecular ions at m/z 571.5, 625.5, and 735.5 (in a ratio of 2:1:1) corresponding to the structures Asp-ethanolamine-HPO_4^-6 Man_1-2 Man_1-6 Man_1-4 GlcN_1-6 Man_1-2 Gal_1-3 Man_1-6(Gal_1-3)-Man_1-4-GlcN_1-6(myo-inositol-1-HPO_4^-). (most likely, based on the intact GPI structure of MITat 1.4 VSG) Asp-ethanolamine-HPO_4^-6 Man_1-2 Man_1-6 Man_1-4 GlcN_1-6 Man_1-2 Gal_1-3 Man_1-4-GlcN_1-6(myo-inositol-1-HPO_4^-), respectively (26). The concentration of the GIP fraction was determined by measuring the myo-inositol content by selection ion monitoring mass chromatography-mass spectrometry (27).

**LPS treatment**

For both in vitro and in vivo analyses of LPS sensitization of macrophages, LPS from *Escherichia coli* 055:B5 was used (Difco Laboratories, Detroit, MI). For in vitro experiments, different LPS dilutions were calculated in RPMI 1640 medium and added to 2C11/12 cells or to freshly harvested PECs. After 3 h incubation, culture supernatants were taken and tested for the presence of TNF-α using a TNF-α-specific ELISA. A minimal LPS dose used in sensitization experiments was determined as one-half of the highest concentration that could be added to the cells without observing a reproducible induction of TNF-α production. For in vivo experiments, different LPS dilutions were made in PBS and injected i.p. into LPS-sensitive C57BL/6 mice. Blood was taken from these mice, and serum TNF-α levels were determined by ELISA. A minimal LPS dose was determined as the lowest dose that gave reproducibly measurable TNF-α levels without causing any signs of morbidity.

To test the involvement of TNF-α in LPS-induced mortality of *T. brucei*-infected mice, an i.p. injection of 50 μg of neutralizing anti-TNF-α mAb (PharMingen, San Diego, CA) was used.

**Cytokine detection**

The levels of TNF-α and IL-1α present in culture supernatants or serum were measured using, respectively, a TNF-α-specific and an IL-1α-specific ELISA (Innogenetics, Gent, Belgium). IL-6, IL-10, and IL-12 present in culture supernatants or serum were measured by ELISA using specific Abs purchased from PharMingen. Cytokine levels present in all culture supernatants were measured in triplicates, and mean values were calculated for presentation of the results. SEs never exceeded 15% within a given bioassay. To analyze the significance levels of observation, a Student's *t* test was used and *p* values were indicated in the text. Serum cytokine levels were measured using three mice for every measurement. Mean serum cytokine levels were calculated for presentation of the results.

**Results**

The GIP moiety of sVSG is the main trypanosome-derived TNF-α-inducing component.

We have reported earlier that *T. brucei*-soluble extracts contain soluble components capable of inducing the secretion of TNF-α by a differentiated macrophage cell line (2C11/12) (13). In a first attempt to identify the *T. brucei* components involved in the induction of TNF-α production, efforts were focused on the VSG, since...
This glycoprotein represents approximately 10% of the total protein content of trypanosome lysates. To analyze the possible contribution of VSG in the induction of TNF-α production by 2C11/12 cells, T. brucei-soluble extract was depleted of VSG by immunoprecipitation, and compared with the TNF-α-inducing activity of total trypanosome-soluble extract and purified soluble as well as membrane-bound forms of VSG (sVSG and mfVSG, respectively). As shown in Figure 1, both forms of VSG efficiently trigger TNF-α production by 2C11/12 cells, while depletion of VSG from the trypanosome-soluble extract reduces significantly its TNF-α inducing capacity (p < 0.001 up to a dilution of 1/32). Soluble extracts of procyclic forms that lack the VSG failed to induce detectable levels of TNF-α secretion. Furthermore, at identical protein concentrations, both sVSG and mfVSG are more potent TNF-α inducers than trypanosome-soluble extract. According to these results, VSG is the main trypanosome TNF-α-inducing component, and since sVSG and mfVSG are equally potent TNF-α inducers, the DMG compound of the mfVSG anchor (Fig. 2) is not required for this activity.

To identify the components within sVSG that are crucial for TNF-α induction, sVSG was digested with Proteinase K, N-glycosidase F, and α-galactosidase. As shown in Figure 3a, only treatment with α-galactosidase had a marked effect on the TNF-α-inducing capacity of sVSG (p < 0.001 up to a dilution of 1/32). Neither digestion of the protein moiety nor the removal of N-linked carbohydrates from the VSG preparation resulted in the abolishment of TNF-α induction, indicating that the GIP moiety at the base of the VSG carries the TNF-α-inducing capacity. To confirm this finding, the GIP moiety was purified and checked for its capacity to trigger TNF-α induction before and after α-galactosidase treatment. Indeed, it was found that the GIP moiety of sVSG is fully capable of triggering TNF-α induction and that the α-galactose side chain of this moiety has an amplifying effect on the TNF-α-inducing capacity of the GIP moiety (Fig. 3b).

To further corroborate the involvement of the galactose side chains of GIP in TNF-α induction, the TNF-α-inducing capacity of sVSGs that differ in their galactose side chain composition was compared. AnTat1.1 and MITat1.5 belong to the same VSG class (class I) and have the same galactose side chains, while MITat 1.5 belongs to another class (class III) and is unique, since it lacks the galactose side chains (28, 29). From Figure 4a, it is clear that both of the class I VSG are potent TNF-α inducers, while the class III VSG is significantly less capable of triggering TNF-α production by 2C11/12 cells (p < 0.001 up to a dilution of 1/32). This results confirm the requirement for the galactose side chain for optimal TNF-α-inducing capacity of the GIP moiety of sVSG.

FIGURE 1. Production of TNF-α by 2C11/12 macrophages incubated with total soluble extract (○) from bloodstream form trypanosomes, total soluble extract from procyclic form trypanosomes (×), sVSG (□), mfVSG (■), or total trypanosome extract depleted from VSG by immunoprecipitation (●). Cells were incubated for 24 h with different dilutions of trypanosome-soluble extract or VSG, starting at 500 μg/ml. TNF-α levels in culture supernatants were measured with a TNF-α-specific ELISA.

FIGURE 2. Structure of the GPI anchor of VSG (25). The boxed Asp is the C-terminal residue of the protein. The molecular bound cleaved by the trypanosome GPI-specific phospholipase C is indicated. This cleavage causes sVSG (GIP-VSG) to be released.

FIGURE 3. Production of TNF-α by 2C11/12 macrophages incubated with: a. T. brucei AnTat 1.1 sVSG (□), Proteinase K-treated sVSG (●), N-glycosidase F-treated sVSG (○), or α-galactosidase-treated sVSG (■); cells were incubated for 24 h with different dilutions of sVSG, starting at 500 μg/ml (0.01 mM); or b. purified GIP (○) or α-galactosidase-treated GIP (●). The beginning concentration of the dilution curve was 0.01 mM, as indicated in panel a. TNF-α levels in culture supernatants were measured with a TNF-α-specific ELISA.
Responsiveness of macrophages toward the TNF-α-inducing activity of VSG requires IFN-γ priming

Thus far, the TNF-α-inducing potential of trypanosome-soluble extracts and VSG has been evaluated on 2C11/12 macrophages that are highly differentiated (22). It was therefore of importance to reanalyze the TNF-α-inducing activity of sVSG on resident, non-activated macrophages. To this end, the TNF-α-inducing activity of different VSGs was tested on PECs from LPS-resistant C3H/HeJ mice, and as shown in Figure 4b, these cells barely respond to TNF-α-inducing class I sVSG. The differences in the responses of 2C11/12 cells and PECs toward sVSG might reside in the activation status of the two macrophage cell types. Indeed, preactivation of PECs with IFN-γ renders these macrophages fully responsive toward the TNF-α-inducing activity of class I VSG, but not of class III VSG (Fig. 4d). In the case of 2C11/12 cells, pretreatment with IFN-γ does not significantly increase their responsiveness toward TNF-α-inducing VSGs (compare Fig. 4a and c), indicating that these cells are inherently activated and do not require preactivation.

Since the responsiveness of normal macrophages toward the TNF-α-inducing activity of sVSG requires IFN-γ priming, it was important to reanalyze the involvement of the DMG compound of the VSG anchor in TNF-α induction by normal macrophages, either activated or not. As shown in Figure 5, both mfVSG and sVSG moderately trigger PECs from LPS-resistant C3H/HeJ (Fig. 5a) to produce TNF-α. Although the levels of TNF-α induction are rather low, the TNF-α-inducing activity of mfVSG is consistently higher than that of sVSG. Yet, following activation with IFN-γ, both sVSG and mfVSG are equally capable of triggering high levels of TNF-α production by PECs of LPS-resistant mice (Fig. 5b). In fact, the pattern of responsiveness of IFN-γ-primed PECs toward both types of VSGs is similar to that recorded for 2C11/2 cells (Fig. 1). Collectively, these results show that normal macrophages acquire, upon activation with IFN-γ, a responsiveness toward the GIP moiety of sVSG, and furthermore, that the galactose side chain of this moiety acts as an enhancer for TNF-α induction. Apparently, the DMG compound of mfVSG is not of crucial importance in the triggering of TNF-α production by IFN-γ-activated macrophages. It is important to mention here that the same cytokine induction pattern, with equal levels of secretion, was found when PECs from LPS-sensitive C3H/HeN mice were used in these bioassays (results not shown).

sVSG and mfVSG have a different macrophage-activating capacity

To analyze whether trypanosome components, including sVSG and mfVSG, are involved in a more general way in the induction
of inflammatory cytokine production by macrophages, both 2C11/12 cells and C₅H/HeJ PECs were incubated with trypanosome components and tested for secretion of IL-1α, IL-6, IL-10, and IL-12. As shown in Figure 6a, when 2C11/12 cells were incubated with total soluble trypanosome extract from *T. brucei* AnTat 1.1, high levels of IL-1α and IL-6 were detected in the culture supernatants, but secretion of both IL-10 and IL-12 remained below background levels (i.e., secretion by unstimulated cells). When C₅H/HeJ PECs were used, only IL-1α secretion was recorded (Fig. 6b). Reactivation of 2C11/12 cells or PECs with IFN-γ did not alter the pattern of cytokine secretion. To analyze the role of VSG in the induction of IL-1α and IL-6 secretion, 2C11/12 cells and C₅H/HeJ PECs were incubated with different concentrations of sVSG and mfVSG. Induction of cytokine secretion was compared with secretion induced by total trypanosome extract. While both forms of VSG failed to induce IL-6 secretion, 2C11/12 cells as well as C₅H/HeJ PECs (data not shown), IL-1α induction could be detected only when cells were stimulated with mfVSG or total trypanosome extracts (Fig. 7, a and b). sVSG did not induce detectable IL-1α secretion, and prestimulation of cells with IFN-γ did not alter these induction patterns.

**DMG compound of mVSG anchor sensitizes macrophages toward LPS responsiveness**

Although the VSGs are not potent inducers of TNF-α production by resting macrophages (PECs), such agents might prime macrophages to respond to a secondary triggering agent such as LPS. To test this possibility, 2C11/12 cells and C₅H/HeN PECs were preactivated with parasite-soluble extract, sVSG, and mfVSG, and restimulated with 1 pg/ml LPS. This LPS concentration does not induce a detectable TNF-α production by itself (data not shown). According to Figure 8a, both trypanosome-soluble extract and mfVSG prime 2C11/12 cells to become hypersensitive to LPS, resulting in high TNF-α production. In contrast, preactivation with sVSG does not sensitize the cells to respond to LPS (compare Fig. 8a and Fig. 1).

Similar results were obtained with C₅H/HeN PECs. Indeed, although both the parasite-soluble extract and mfVSG are only moderate TNF-α inducers in the absence of LPS (Fig. 4b), these components are capable of preactivating cells to become high TNF-α producers upon contact with LPS (Fig. 8b). C₅H/HeN PECs produce only low levels of TNF-α upon contact with sVSG, and furthermore, sVSG is not capable of sensitizing macrophages to respond to LPS.

Finally, when 2C11/12 macrophages or PECs were stimulated with 1 pg/ml LPS before stimulation with trypanosome-soluble extract, sVSG, or mfVSG, no prestimulation effects were observed (data not shown).

Collectively, these results indicate that the DMG compound of the mVSG anchor is capable of priming macrophages to become hyper-responsive to LPS.

**The DMG compound of the VSG anchor renders mice hypersensitive to LPS: possible involvement in TNF-α production during trypanosomiasis**

To analyze the in vivo relevance of the above-described observations, C₅H/HeN mice were treated i.p. with sVSG, mfVSG, or parasite-soluble extract and challenged with LPS. The inoculated protein content corresponded to approximately 10⁸ parasites (i.e., 50 μg VSG and 500 μg total trypanosome-soluble extract). Following injection of sVSG, mfVSG, and parasite-soluble extract, serum TNF-α levels remained below detection limit 24 h postinoculation. However, when trypanosome-soluble extract or mfVSG-treated animals were challenged with a minimal dose of LPS (1 μg/mouse) 21 h postinoculation, a significant increase in TNF-α serum levels was recorded within 3 h (Fig. 9). As expected, pretreatment with sVSG did not result into hypersensitivity toward LPS.

The DMG-mediated sensitization of mice to respond to LPS and to produce TNF-α might be relevant during infection with trypanosomes, since serum levels of LPS were reported to increase during trypanosome infection (30). Furthermore, TNF-α levels were documented as correlating with the severity of the disease in
both humans and cattle (9, 10). Therefore, LPS-induced TNF-α levels were measured during the course of the parasitemia (Fig. 10, a and b). As shown in Figure 9b, when a minimal dose of LPS (1 μg/mouse) was injected during the early phase of the infection, a slight increase in the levels of serum TNF-α was measured after 3 h, and the treated mice manifested no signs of morbidity. However, when the first peak of parasitemia was reached and parasite numbers dropped, a dramatic increase in the serum levels of TNF-α was measured upon LPS challenge; 2 h after LPS injection, severe signs of morbidity were recorded, resulting in 100% lethality within 24 h after LPS administration.

The sera from both control-infected mice and LPS-treated infected mice were also screened for the presence of IL-1α, IL-6, IL-10, and IL-12. These measurements indicated that only increased levels of IL-1α and IL-6 could be detected (data not shown), yet these increased levels did not correlate with the occurrence of LPS-induced mortality. Finally, i.p. treatment with a neutralizing anti-TNF-α mAb (50 μg/mouse) 24 h before LPS challenge rescued T. brucei-infected mice from LPS-induced mortality, indicating that TNF-α is a crucial mediator in this event.

These results suggest that during infection the presence of the DMG compound of the VSG anchor may activate macrophages, rendering these cells competent for hyperproduction of TNF-α in the presence of low levels of LPS.

Discussion

TNF-α is an inflammatory cytokine that was originally isolated based on its capacity to induce trypanosomiasis-associated cachexia in rabbits (2, 3). Today, it is known that TNF-α is also involved in trypanosomiasis-associated anemia and in the occurrence of meningoencephalitis signs during late stage infections (9, 10). Although as such, TNF-α can be considered crucially important in the general immunopathology of African trypanosomiasis, it is still unclear which trypanosome factors and which underlying mechanisms contribute to TNF-α induction in the host. Using a macrophage cell line-based bioassay, we report here that sVSG, which is released from the parasite surface through a GPI-specific phospholipase C (20), is the main TNF-α-inducing component of trypanosome-soluble extracts. Furthermore, we report that the GIP moiety at the base of the sVSG is the key to TNF-α induction and that the GIP-associated galactose side chain is crucial for optimal TNF-α induction mediated by sVSG.

TNF-α induction assays with PECs further corroborate the role of both GIP and its galactose side chain in the TNF-α-inducing capacity of sVSG. However, in the case of PECs, sVSG-mediated induction of TNF-α production requires preactivation of macrophages with IFN-γ. This observation does not preclude a role for sVSG during trypanosome infections, since there is ample evidence that African trypanosomes sensitize host T cells, either directly or indirectly, to produce IFN-γ (12, 31–33). Thus, during trypanosome infections, macrophages may be primed with IFN-γ and as such may become responsive toward the TNF-α-inducing
In testing the TNF-α-inducing activity of mfVSG, which differs from sVSG solely by the presence of the DMG moiety, no clear-cut differences were observed between the two forms of VSG. mfVSG may be slightly more potent than sVSG in triggering TNF-α production by resident macrophages, yet when IFN-γ-activated PECs or differentiated 2C11/12 macrophages are used as responder cells, both sVSG and mfVSG exert similar activities. The capacity of mfVSG to stimulate TNF-α production by thioglycollate-elicited macrophages via its GPI anchor was reported earlier by Tachado and Schofield (17). Furthermore, Schofield and Hacket (34) reported that Plasmodium falciparum GPI, free or associated with protein, induces TNF-α production by thioglycollate-elicited macrophages and that removal of the DMG moiety of GPI abolishes the cytokine production. Others, however, have partially disagreed on the functional role of intact malaria GPI, showing that it is the inositol monophosphate of the malaria toxin that is crucial for TNF-α induction (35). As a control, a number of nonparasite-derived GPI-linked membrane proteins were tested for their TNF-α-inducing capacity, and all were found to have a negative score (36). Moreover, it was also shown that Abs against the inositol monophosphate were capable of inhibiting plasmodium-triggered TNF-α induction (37). On the other hand, it was shown that the GPI-anchored m4 surface molecule of Leishmania mexicana was incapable of inducing the secretion of TNF-α although it was capable of inducing intracellular activation of macrophages (38). With respect to still another parasite, it was shown that in the case of Toxoplasma gondii, the major component involved in TNF-α secretion by PECs was the carbohydrate moiety of a glycoprotein present in soluble extracts of the parasite (39). Again, however, for comparative studies of all of these results, it is important to stress that in these experiments as well as in experiments performed with Plasmodium and Leishmania components (17, 39), thioglycollate-elicited PECs and not naïve, non-stimulated cells were used. Our results, obtained with naïve PECs isolated without the use of thioglycollate, are in keeping with those of Schofield and colleagues concerning the TNF-α-inducing capacity of T. brucei mfVSG. However, clear evidence is presented herein that the DMG compound of the GPI of T. brucei is indeed not required for TNF-α induction per se, but is needed to obtain proper macrophages stimulation in the absence of IFN-γ.

Although the DMG moiety of mfVSG is not important for TNF-α induction itself, our results indicate that it plays a crucial role in the stimulation of other macrophage-mediated responses such as IL-1α production and hyper-responsiveness toward LPS. Indeed, both total trypanosome-soluble extract and mfVSG trigger IL-1α secretion by resting PECs and 2C11/12 cells, while sVSG is unable to do so, even when the cells are preactivated with IFN-γ. Similarly, while total trypanosome soluble extract and mfVSG efficiently prime macrophages to respond to suboptimal concentrations of LPS, sVSG completely lacks this priming activity. Hence, the DMG compound of the mfVSG anchor seems to be crucial for its IL-1α-inducing and -priming activity, and similar findings were reported for the glycolipid toxin of P. falciparum (34). Interestingly, however, an early report by Mathias et al. on the induction of IL-1α secretion by macrophages showed that when the macrophage cell line P388D1 was pulsed with sVSG, secretion of IL-1α could be measured (40). This corroborates our results obtained with the 2C11/12 cell line, namely that appropriately activated macrophage cell lines can respond toward sVSG without prior stimulation. In summary, VSG encompasses distinct macrophage-activating components: 1) The GPI moiety, and in particular its galactose side chain, is responsible for TNF-α production; and 2) the GPI moiety, and in particular its DMG anchor, mediates macrophage priming and LPS hyper-responsiveness.

The mechanisms underlying the macrophage-activating capacity of sVSG and mfVSG, it remains to be determined which form of VSG is physiologically relevant during trypanosomiasis. Taking into account that trypanosomes utilize a potent phospholipase C enzyme to release sVSG in stress situations (19) and that sVSG can be detected in the serum during T. brucei infections (45), trypanosome-mediated induction of TNF-α release may be mediated by sVSG. Furthermore, as mentioned above, trypanosome-elicited production of IFN-γ will render macrophages very responsive toward sVSG-mediated TNF-α production. It should be mentioned that a similar situation could occur during natural infections of cattle with Trypanosoma congoense. Indeed, both the sVSG and mfVSG of T. congoense trigger similar TNF-α production by bovine monocytes, provided however that the cells are preactivated with IFN-γ (Dr. M. Sileghem, unpublished observations). The fact that trypanosome infections also sensitize IL-1α production suggests an important role for the DMG compound of the mfVSG anchor. Since IL-1 is described as a TNF-α inducer (46), its release may further fuel TNF-α production. Furthermore, the capacity of mfVSG to prime macrophages to become hyper-responsive toward LPS may be physiologically relevant, since trypanosome infections were reported to increase the serum levels of LPS (30).

In conclusion, the VSG of African trypanosomes exert potent macrophage-activating properties resulting in the production of cytokines such as TNF-α and IL-1α. Given the potentially noxious effects of these cytokines on the host, one might wonder whether their production is beneficial for the parasite. As far as TNF-α is concerned, its parasite-mediated production could make sense because TNF-α has been shown to be involved in trypanosome-elicited immunosuppression (12, 33) and parasite growth regulation (13–16).

Acknowledgments
We thank Drs. A. Beschon and D. Nolan for their interest in this work and their helpful suggestions. We also thank Miss Lea Brys and Miss Martine Gobert for their technical assistance. We thank Angela Mehler for purifying sVSG MITat 1.4 and 1.5 and Dr. S. Cottaz and Prof. John Brimacombe for supplying GlcNa1-6→inositol-1-phosphate. Prof. B. Fraser-Reid for supplying Galα1-3Galβ1-3Manα1-O-(CH2)4-CH3, and Prof. J. van Boon for supplying GlcNa1→6→inositol-1,2 cyclic phosphate.

References


