

The Soluble Variant Surface Glycoprotein of African Trypanosomes Is Recognized by a Macrophage Scavenger Receptor and Induces I κ B α Degradation Independently of TRAF6-Mediated TLR Signaling¹

Brian J. Leppert,* John M. Mansfield,[†] and Donna M. Paulnock^{2*}

The GPI residues of soluble variant surface glycoprotein (sVSG) molecules released from the membrane of African trypanosomes during infection induce macrophage activation events. In this study, we demonstrate that the trypanosome sVSG molecule binds to the membrane of murine RAW 264.7 macrophages and activates the NF- κ B cascade independently of a TLR-mediated interaction. The binding of fluorochrome-labeled sVSG molecules to macrophage membranes was saturable, was inhibited by the scavenger receptor-specific ligand maleylated BSA, and was followed by rapid intracellular uptake of the molecules and subsequent internalization to lysosomal compartments. Inhibition of cellular phagocytic and endocytic uptake processes by cytochalasin B and monodansylcadaverine, respectively, revealed that sVSG internalization was necessary for I κ B α degradation and occurred by an actin-dependent, clathrin-independent process. Activation of RAW 264.7 cells by sVSG following treatment of the cells with the TRAF6 inhibitory peptide DIVK resulted in enhanced NF- κ B signaling, suggesting both that TRAF6-dependent TLR activation of the pathway alone is not required for signaling and that TLR pathway components may negatively regulate expression of sVSG-induced signaling. These results demonstrate that stimulation of macrophages by sVSG involves a complex process of receptor-mediated binding and uptake steps, leading to both positive and negative signaling events that ultimately regulate cellular activation. *The Journal of Immunology*, 2007, 179: 548–556.

Macrophages play a key role in the host innate immune system as early responders to microbial infection and as APCs that help coordinate the activation of the adaptive immune system. In this role, macrophages sample the extracellular environments of their hosts for foreign molecules using a variety of receptor-mediated detection and internalization events. One predominant mechanism for detecting microorganisms is through the use of pattern-recognition receptors (PRRs)³ expressed on the surface of the macrophage. TLRs, a type I transmembrane receptor class included in the PRR family, detect pathogen-associated molecular patterns present on a diverse range of microbial ligands comprising both membrane-associated and cytosolic molecules; these include bacterial LPS, single-stranded viral RNA, and unmethylated CpG oligodeoxynucleotide DNA motifs, among others (1). Ligation of PRRs by such molecules

initiates signaling cascades that result in macrophage activation and enhanced APC functions including the activation of NF- κ B, up-regulation of costimulatory molecules, augmented MHC class II expression, increased macrophage endocytosis and phagocytosis, and the production of inflammatory factors that amplify the innate response to infection and stimulate adaptive immunity (2, 3). As a result of activation, macrophages also release reactive nitrogen and oxygen species that have toxic and cytostatic effects on microorganisms (4–6). Ultimately, the initiation of a productive immune response depends on the presence of both early and sustained macrophage responses to microbial challenge.

The causative agents of human African sleeping sickness are the extracellular protozoan parasites *Trypanosoma brucei rhodesiense* and *T. brucei gambiense*. During infection, they express a unique surface coat comprised of GPI-anchored variant surface glycoprotein (VSG) molecules (7, 8). Parasites will escape immune destruction by undergoing antigenic variation that results in expression of new VSG molecules on the trypanosome membrane (9, 10). The large repertoire of VSG genes from which the trypanosome can choose ($\sim 10^3$ VSGs), coupled with allelic exclusion mechanisms preventing multiple VSG genes from being expressed simultaneously, results in the periodic expression of unique antigenic determinants that prevent trypanosomes from being completely eliminated by VSG-specific immune responses. In response to infection, an early polarized type I T cell-mediated immune response is generated that includes production of IFN- γ , a macrophage-activating cytokine linked to host resistance, as well as VSG-specific B cell responses capable of controlling parasites in the blood (11–14). Cleavage of the GPI-anchored VSG molecule from the membrane occurs by a trypanosome GPI-phospholipase C resulting in the release of soluble glycosylinositolphosphate VSG molecules (soluble VSG (sVSG)) with the dimyristoylglycerol moiety remaining embedded in the parasite membrane (15–17).

*Department of Medical Microbiology and Immunology, School of Medicine and Public Health, University of Wisconsin, Madison, WI 53706; and [†]Department of Bacteriology, School of Medicine and Public Health, University of Wisconsin, Madison, WI 53706

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² Address correspondence and reprint requests to Dr. Donna M. Paulnock, Department of Medical Microbiology and Immunology, School of Medicine and Public Health, University of Wisconsin, Madison, WI, 53706. E-mail address: paulnock@wisc.edu

³ Abbreviations used in this paper: PRR, pattern-recognition receptor; VSG, variant surface glycoprotein; sVSG, soluble VSG; TRAF6, TNFR-associated factor 6; OG, Oregon Green; SR, scavenger receptor; mBSA, maleylated BSA; TFA, trifluoroacetic acid; BMM, bone marrow-derived macrophage.

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The precise role of the sVSG molecule in causing macrophage activation is complex and depends on a variety of factors including the concentration and timing of the exposure of the macrophage to host- and parasite-derived factors. Macrophages primed with IFN- γ exhibit an activated phenotype of enhanced gene transcription and release inflammatory mediators including the cytokines TNF, IL-6, IL-12, and NO production following stimulation with sVSG (18, 19); however, reversing the order of exposure results in a down-regulation of IFN- γ -inducible responses (20). Macrophages also appear to respond to sVSG in a MyD88-dependent manner, suggesting a potential role for TLR-mediated signaling in the activation of macrophages by sVSG (21); however, a defined TLR-sVSG interaction has not been identified.

Taken together, these results suggest a highly nuanced system of specific recognition of sVSG by the macrophage. In the experiments presented here, we tested the hypothesis that receptor-mediated macrophage activation by sVSG requires TNFR-associated factor 6 (TRAF6)-dependent TLR signaling using RAW 264.7 macrophages as a model of host macrophages to elucidate early recognition and cellular activation events by sVSG. We show that activation of the NF- κ B cascade by sVSG is not dependent on TRAF6-dependent TLR signaling and that TLR-dependent signaling may negatively regulate I κ B α degradation. Furthermore, Oregon Green (OG)-sVSG binds to the surface of macrophage cells in a saturable manner and can be competitively inhibited by the macrophage scavenger receptor (SR) ligand maleylated BSA (mBSA). Consistent with the general requirements for SR activity, sVSG must be internalized to degrade I κ B α in a process mediated by an actin-dependent, clathrin-independent, acidification-independent phagocytic-like mechanism. These results demonstrate that uptake of the sVSG molecule occurs in a specific, receptor-mediated fashion and suggest that the final activation state of the macrophage may depend upon the coordination of multiple receptor-dependent signaling events.

Materials and Methods

Reagents

The reagents pyruvate, glutamine, penicillin, streptomycin, sodium bicarbonate, cyclophosphamide, glucose, sodium phosphate, zinc acetate, 2-propanol, acetic acid, PMSF, aprotinin, leupeptin, *N*- α -tosyl-L-lysine chloromethyl ketone, pepstatin, DTT, LPS, DEAE, Triton X-100, bafilomycin A, chloroquine, and all lysis buffer components were obtained from Sigma-Aldrich. FBS and RPMI 1640 medium for use in tissue culture were obtained from Invitrogen Life Technologies. Pam₃CSK was from Calbiochem. GpG oligodeoxynucleotide was obtained from Cell Sciences. The anti-LAMP1 Ab was obtained through the University of Iowa Developmental Studies Hybridoma Bank and the anti-I κ B α Ab was obtained from Santa Cruz Biotechnology. All secondary fluorescent Abs and the FluorReporter Oregon Green Protein Labeling kit were obtained from Molecular Probes. Trifluoroacetic acid (TFA) and Western blot HRP luminescent reagents were obtained from Pierce.

Cell culture and mice

The cell line consisting of RAW 264.7 murine peritoneal macrophages (American Type Culture Collection) were cultured *in vitro* using RPMI 1640 medium supplemented with 1 mM pyruvate, 2 mM glutamine, 50 U/ml penicillin, 50 μ g/ml streptomycin, 2 mg/ml sodium bicarbonate, and 10% FBS. Cells were maintained at 37°C in 7% CO₂.

Bone marrow-derived macrophages (BMM) were harvested from the whole marrow of female BALB/c mice obtained from The Jackson Laboratory at ~8 wk of age and incubated in complete RPMI 1640 medium described above at 37°C in 7% CO₂ overnight. After 24 h, nondifferentiated cells were recovered from the supernatant and cultured in conditioned RPMI 1640 medium consisting of RPMI 1640 supplemented with 20% FBS and 20% L-cell-conditioned medium for 7 days. The resulting adherent BMMs were maintained in conditioned RPMI 1640 medium.

Isolation and purification of sVSG

Stabilates of *T. brucei rhodesiense* clone LouTat 1 were grown in Swiss mice and were used to purify LouTat 1 sVSG for *in vitro* study. Mice were immunosuppressed with cyclophosphamide (300 mg/kg) before infection. This treatment suppresses B cell responses to the VSG molecule and prevents immune selection for minor variant antigenic types (22). Trypanosomes were isolated following peritoneal injection at parasitemias approaching 10⁹ parasites/ml blood as previously described (23). Briefly, blood was collected by exsanguinations from the retrobulbar sinus, diluted in sterile heparinized, ice-cold PBS supplemented with 1 mg/ml glucose (PBSG), and passed over a Selectacel DEAE type 40 column equilibrated with PBSG. This technique allows blood components to bind to the column matrix while trypanosomes pass through freely (24). Trypanosomes were collected on ice, washed with PBSG by centrifugation at 1,000 \times g for 10 min at 4°C and counted on a hemacytometer. Washed trypanosomes were resuspended to 10⁹ cells/ml in 0.3 mM zinc acetate containing 0.1 mM *N*- α -tosyl-L-lysine chloromethyl ketone and incubated on ice for 5 min. The treated cell suspension was then centrifuged at 3,000 \times g for 10 min at 4°C. Resulting supernatants were treated with 2 μ g/ml aprotinin, 2 μ g/ml leupeptin, 2 μ g/ml pepstatin, and 1 mM PMSF protease inhibitors and set aside on ice. The remaining cell pellet was resuspended to an equal volume as above in 10 mM sodium phosphate (pH 8), containing the same protease inhibitors and incubated at 37°C for 20 min. The resulting supernatant was chilled to 4°C, centrifuged at 7,000 \times g for 15 min and collected. The zinc acetate and phosphate buffer supernatants were combined and centrifuged at 100,000 \times g for 1 h at 4°C. Supernatant from this centrifugation was reserved and concentrated by centrifugation at 4°C using an Amicon Ultra-30 filter (Millipore). Concentrated supernatants were passed in series over two DEAE-cellulose columns equilibrated with 10 mM phosphate buffer. Fractions containing purified sVSG protein as indicated by A₂₈₀ absorbance were pooled and purity was confirmed by SDS-PAGE under reducing conditions. All sVSG prepared in this manner appeared as a single band of protein with a molecular mass of ~60 kDa. Confirmation of this band as LouTat 1 sVSG was determined by Western blot using anti-LouTat 1 and anti-CRD antisera made as previously described (25).

sVSG obtained using the method described above was subjected to more stringent purification by reverse-phase HPLC based on a previously documented protocol for rapid-scale VSG purification (26). A two-solvent system consisting of 0.1% (v/v) TFA (solvent A) and 2-propanol (solvent B) was used to elute the highly purified sVSG. Before injection, a Supelco Discovery BIO Wide Pore C5 5- μ m semipreparative column (Sigma-Aldrich) was equilibrated to starting conditions (99% solvent A; 1% solvent B). sVSG was solubilized in 0.1% TFA and injected into the column while running under isocratic conditions. Following injection, after A₂₈₀ absorbance returned to baseline levels, an elution gradient program was initiated at a flow rate of 1.5 ml/min. The gradient followed a multiple-step linearly increasing solvent rate: 0–30% 2-propanol over 10 min; hold at 30% 2-propanol for 25 min; 30–80% 2-propanol over 100 min. Under these conditions, elution of LouTat 1 sVSG occurred at ~38% 2-propanol, 51 min after the elution gradient had begun. Fractions were collected every 30 s beginning at 50 min until the A₂₈₀ absorbance returned to baseline levels. Resulting fractions expected to contain sVSG based on the A₂₈₀ trace pattern were pooled and concentrated in RPMI 1640 using Amicon Ultra-30 columns. Purity was assessed by SDS-PAGE and Western blot as indicated above for standard sVSG purification.

Biological activity of sVSG was determined by incubating RAW 264.7 cells with standard or HPLC-purified sVSG and assessing I κ B α degradation as described below. Similar levels of I κ B α degradation were observed with either HPLC purified or our standard sVSG preparations.

I κ B α degradation in macrophages

Macrophages were plated in RPMI 1640 medium supplemented with FBS at a density of 3 \times 10⁵ cells/ml (2 ml) in 12-well tissue culture plates (Costar) and incubated for 24 h at 37°C. Semiconfluent wells were then aspirated, fresh medium was added, and where indicated, 20 U/ml IFN- γ was added to selected cultures to prime macrophages before addition of ligand. Following optional IFN- γ treatment, all cells were incubated an additional 24 h until confluent. After incubation, ligands including 1 μ g/ml CpG oligodeoxynucleotide, 1 ng/ml LPS, 50 ng/ml TNF, or 10 ng/ml Pam₃CSK were added to adherent cultures as described and cells were incubated for 30 min at 37°C. The supernatant fraction was discarded and cells were lysed by addition of lysis buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 0.02% Na₃, 2 mM EDTA, 1% Nonidet P-40) and placed on ice. A total of 50 μ g of total cell lysate was analyzed by SDS-PAGE and Western blot for the presence of I κ B α using an anti-I κ B α Ab. Degradation of the I κ B α protein allows the translocation of NF- κ B to the nucleus to occur and directly correlates to the level of NF- κ B activation in the macrophage (27).

Densitometry was performed on anti-I κ B α -probed blots to quantify the change in I κ B α expression and determine the level of NF- κ B activation relative to untreated controls.

Immunofluorescent microscopy

sVSG prepared as described above was fluorescently labeled with OG using the FluorReporter Oregon Green 488 Protein Labeling kit as directed in the accompanying kit protocol. To examine binding to the membrane of macrophage cells, adherent RAW 264.7 macrophages were grown until confluent in 25-cm² flasks at 37°C. Once confluent, 1×10^6 cells were transferred to microfuge tubes, washed with fresh RPMI 1640, and chilled to 4°C for the remainder of the experiment. OG-sVSG was added to the chilled macrophages at a concentration of 8 μ M and incubated for 30 min to allow binding to occur. Immediately after binding, the cells were washed twice in cold wash buffer (PBS supplemented with 3% FBS) and fixed in 4% formaldehyde for 30 min. Fixed cells were washed twice in wash buffer to remove remaining formaldehyde and optionally stained with anti-LouTat 1 VSG Ab and an Alexa 594-conjugated secondary Ab to identify surface-bound VSG. Stained cells were fixed in 4% formaldehyde, transferred to microscope slides, and imaged on a Zeiss Axioskop microscope running Openlab 4.0 imaging software (Improvision).

To monitor internalization of OG-sVSG, macrophages were grown under adherent conditions in 25-cm² flasks and incubated at 37°C until cells were confluent. Subsequently, 1×10^6 cells were collected, transferred to microfuge tubes, and washed with fresh RPMI 1640. Labeled sVSG was then added at a concentration of 8 μ M and cells were incubated for 30 min at 37°C to allow binding and internalization of the sVSG molecule to occur. Following internalization, cells were washed twice in cold wash buffer and fixed in 4% formaldehyde for 30 min. Fixed cells were washed twice with wash buffer to remove formaldehyde and, where indicated, selected cells were then permeabilized in 0.2% Triton X-100 for 20 min. To differentiate bound sVSG from internalized sVSG, impermeabilized and permeabilized cells were stained with anti-LouTat 1 sVSG Ab and an Alexa 594-conjugated secondary Ab to differentially label membrane-bound sVSG. After staining, cells were washed in wash buffer, fixed in 4% formaldehyde, and imaged as described above.

Compartment colocalization studies were performed as described above for OG-sVSG internalization using a cathepsin D Ab or a LAMP-1 mAb followed by an Alexa 594-conjugated secondary Ab after Triton X-100 permeabilization to stain endosomes and lysosomes, respectively.

Flow cytometry

The kinetics of binding of sVSG to macrophages were determined using flow cytometry in combination with OG-sVSG prepared as described above. A total of 1×10^6 confluent RAW 264.7 cells were washed with fresh RPMI 1640 and transferred to flow cytometry tubes at a concentration of 5×10^6 cells/ml. Transferred cells were chilled to 4°C for the remainder of the experiment. Labeled sVSG was added to aliquotted cells to a concentration of 8 μ M over 1 h. At regular intervals, cells were washed twice in FACS buffer (PBS supplemented with 5% FBS and 0.02% NaN₃) and fixed in 4% formaldehyde. Mean geometric fluorescence of sVSG-treated cells was measured on a FACScalibur (BD Biosciences) running CellQuest software and analyzed using FlowJo (Tree Software). Mean fluorescence intensity was calculated on a gated population of macrophage cells expressing CD11b.

Competitive inhibition of sVSG binding to macrophage membranes by maleylated BSA was assessed by flow cytometry. A total of 1×10^6 RAW 264.7 macrophages were washed in fresh RPMI 1640, transferred to flow cytometry tubes, and chilled to 4°C. Cells were pretreated with a range of concentrations of the mBSA, mannose, or BSA over a range of concentrations from 250 μ g/ml to 5 mg/ml for 30 min. Immediately following pretreatment, labeled sVSG was added to aliquotted cells to a concentration of 8 μ M and allowed to bind for an additional 30 min. Binding of labeled sVSG was measured as described above on a gated population of cells staining positive for the macrophage Ab F4/80.

Inhibition of internalization and lysosomal acidification

Inhibition of endocytic and phagocytic mechanisms in RAW 264.7 macrophages was performed by plating cells at a density of 3×10^5 cells/ml (0.5 ml) in 48-well tissue culture plates using RPMI 1640 medium supplemented with FBS at 37°C until cells were confluent. Wells were then aspirated and washed with fresh medium. Cells were subsequently incubated with cytochalasin B to inhibit actin-mediated membrane rearrangement and phagocytosis, monodansylcadaverine to inhibit clathrin-mediated endocytosis, or bafilomycin A or chloroquine to inhibit lysosomal acidification for 1 h before stimulation with 16 μ M sVSG or other control ligands described above for 30 min at 37°C. The supernatant fraction was dis-

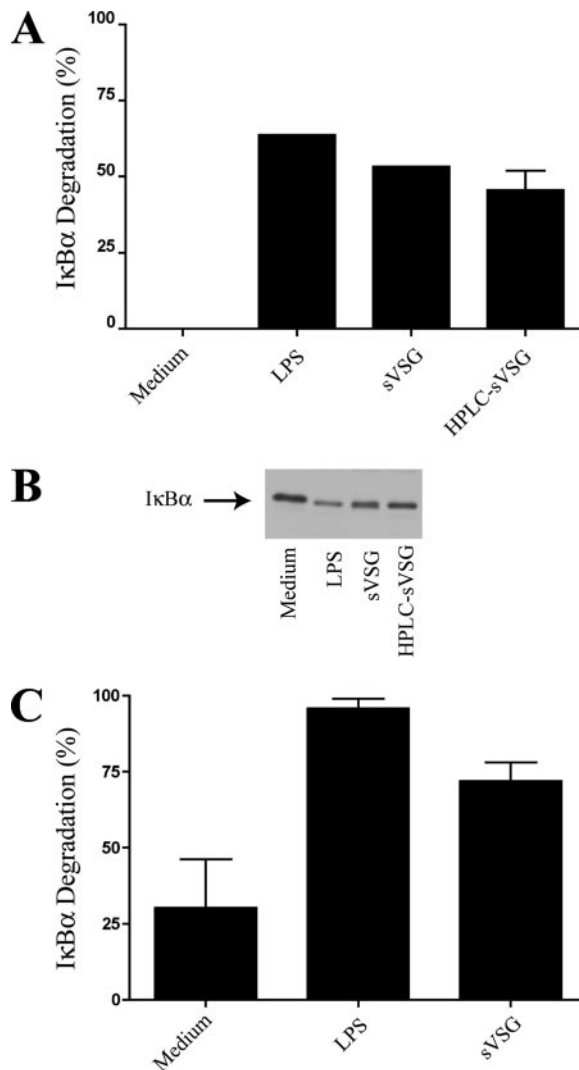


FIGURE 1. sVSG induces I κ B α degradation in macrophages. **A**, RAW 264.7 cells were stimulated with LPS (1 ng/ml), sVSG (16 μ M), or HPLC-purified sVSG (16 μ M) for 30 min and the percentage of I κ B α degradation was determined by densitometry of the resulting Western blots. **B**, Representative Western blot from one experiment showing I κ B α expression. **C**, BMMs were stimulated with CpG (10 μ g/ml), LPS (100 ng/ml), or sVSG (8 μ M) for 30 min and the percentage of I κ B α degradation was determined as above.

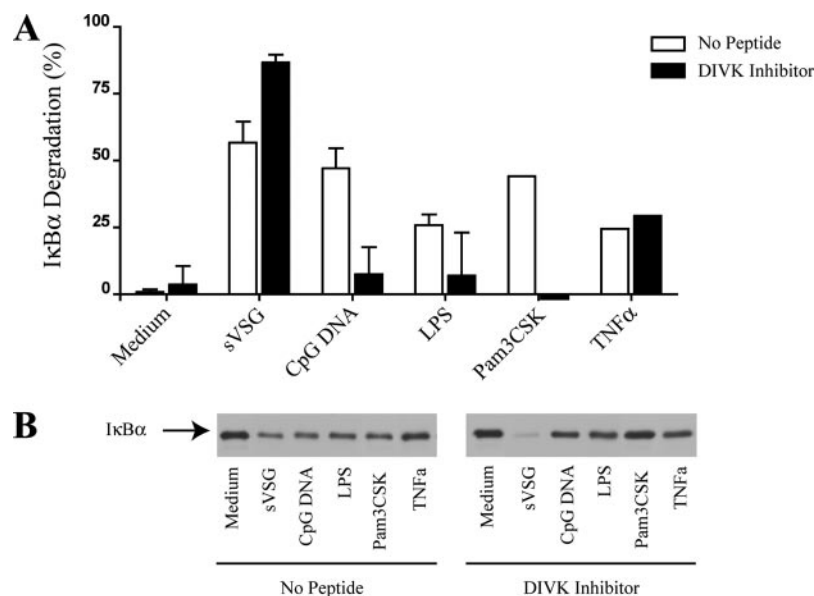
carded and cells were lysed by addition of cold lysis buffer and placed on ice. Total cell lysate (50 μ g) was analyzed by SDS-PAGE and Western blot for the level of I κ B α and quantified by densitometry.

Peptide inhibition of TLR signaling

Down-regulation of TLR receptor signaling by peptide-mediated inhibition of TRAF6 activity has been previously established in RAW 264.7 macrophages using a specific 11-aa sequence derived from the vaccinia A52R protein (28, 29). A 9-aa polyarginine tail was added to the C-terminal end of the A52R sequence to promote cell membrane permeability of the peptide. The 20-residue TRAF6 inhibitory peptide DIVKLTVDLCIRRRRR RRRR (DIVK) and a scrambled control sequence ITCVDVLDLYKRRR RRRRRR (ITCV) were synthesized on an Applied Biosystems Peptide Synthesizer 432A by the University of Wisconsin Peptide Synthesis Core Facility and purified to >95% full-length peptide by HPLC. Resulting peptides were resuspended in 10% acetic acid (short-term DIVK experiments) or 1 mM DTT (>12 h experiments) to maintain conformational structure of the peptide without inducing cellular toxicity.

Degradation of I κ B α in RAW 264.7 macrophages in the presence or absence of inhibitory peptide by sVSG was measured as described above. Briefly, confluent macrophages were pretreated with 1 mM peptide for 15

FIGURE 2. Trypanosome sVSG induces $\text{I}\kappa\text{B}\alpha$ degradation independently of TLR signaling. The role of the TLR in sVSG signaling was tested by inhibition of TLR signaling using the vaccinia DIVK peptide. *A*, Macrophages untreated (\square) or pretreated with DIVK inhibitory peptide (\blacksquare) were stimulated with sVSG (16 μM), CpG DNA (1 $\mu\text{g}/\text{ml}$), LPS (1 ng/ml), Pam₃CSK (10 ng/ml), or TNF- α (50 ng/ml) for 30 min. The percentage of $\text{I}\kappa\text{B}\alpha$ degradation was determined by densitometry analysis of the resulting Western blots. *B*, A representative Western blot from untreated (*left*) and DIVK-treated (*right*) macrophages showing $\text{I}\kappa\text{B}\alpha$ expression.



min at 37°C. Following incubation, cells were treated with 1 mg/ml sVSG and lysed following a 30-min incubation at 37°C. Lysates were then analyzed for expression of $\text{I}\kappa\text{B}\alpha$ as described previously.

Results

sVSG activates macrophages through induction of the NF- κB cascade

To determine whether induction of the transcription factor NF- κB occurs as a result of macrophage exposure to sVSG, we assessed the ability of sVSG to induce the NF- κB cascade by monitoring the degradation of $\text{I}\kappa\text{B}\alpha$ in cells treated with purified sVSG. Activation of NF- κB occurs following the rapid proteolytic degradation of $\text{I}\kappa\text{B}\alpha$ in response to upstream signals (27, 30), freeing NF- κB to translocate to the nucleus and bind to specific transactivating DNA sequences.

RAW 264.7 macrophages or BMMs were incubated with purified LouTat 1 sVSG at concentrations consistent with previously published studies (20) or a control TLR4-dependent NF- κB -activating ligand, LPS, for 30 min and immediately lysed. The percentage of $\text{I}\kappa\text{B}\alpha$ degradation relative to untreated cells was assessed by Western blot to measure the activity of NF- κB (Fig. 1). Rapid degradation of $\text{I}\kappa\text{B}\alpha$ was observed in primary and established cell lines following treatment with both standard-purified and HPLC-purified sVSG. We interpret this result as evidence that NF- κB becomes activated in response to sVSG stimulation of macrophages. The reproducibility of sVSG-induced $\text{I}\kappa\text{B}\alpha$ degradation and its similarity to control TLR ligand-induced degradation at 30 min makes it an ideal time point for assaying sVSG-induced NF- κB signaling.

Inhibition of TLR signaling results in augmented $\text{I}\kappa\text{B}\alpha$ degradation by sVSG

Inhibition of the TRAF6-dependent cascade was used to assess the role of the TLRs in the degradation of $\text{I}\kappa\text{B}\alpha$ by sVSG. Recent studies have indicated that the requisite TRAF6-dependent induction of NF- κB following TLR ligation can be inhibited by the viral vaccinia A52R protein (29, 31). For these experiments, a cell-permeable peptide fragment containing a biologically active 11-aa motif from the A52R protein (DIVK) was engineered as described (28) and used to test the dependence of sVSG signaling on TRAF6 activation.

Degradation of cellular $\text{I}\kappa\text{B}\alpha$ was measured following treatment with sVSG or with control TLR-activating ligands LPS (TLR4) (32), CpG DNA (TLR9) (33), or Pam₃CSK (TLR2) (34) in the presence and absence of peptide to determine the relative change in expression of $\text{I}\kappa\text{B}\alpha$ in response to sVSG. Pretreatment of macrophages with DIVK resulted in the marked reduction of $\text{I}\kappa\text{B}\alpha$ degradation by TLR ligands and reduced activation of NF- κB (Fig. 2). Signaling was not inhibited in cells pretreated with scrambled peptide (data not shown). Specifically, macrophages treated with all control TLR ligands in the absence of peptide were able to activate NF- κB , as demonstrated by degradation of 30–50% of total detectable $\text{I}\kappa\text{B}\alpha$ when compared with unstimulated cells. Macrophages exposed to sVSG in the absence of peptide demonstrated a 60% decrease in the level of cellular $\text{I}\kappa\text{B}\alpha$ compared with cells that did not undergo treatment with ligand, which was consistent with prior results. However, pretreatment of macrophages with the DIVK peptide followed by sVSG resulted in degradation of 90% of cellular $\text{I}\kappa\text{B}\alpha$ and resulted in a 30% enhancement of $\text{I}\kappa\text{B}\alpha$ degradation compared with cells treated with sVSG in the absence of peptide. These results demonstrate that in the absence of TRAF6-dependent TLR signaling, $\text{I}\kappa\text{B}\alpha$ is still degraded in response to sVSG, indicating that TRAF6-mediated signaling by TLRs is not necessary for induction of the NF- κB cascade by sVSG. In addition, the enhanced degradation following TRAF6 inhibition suggests that signaling by TLRs plays a negative role in regulating macrophage activation by this molecule.

Binding of sVSG to macrophage membranes is saturable

The maintenance of NF- κB signaling in the presence of DIVK indicated a mechanism independent of the TLR cascade was responsible for the activation observed in response to sVSG stimulation. To explore the interaction of sVSG with the cell membrane in more detail, the binding properties of sVSG were studied in RAW 264.7 macrophages. Cells were exposed to OG-sVSG at regular intervals to examine binding of the molecule to the cell surface over a 1-h time course. sVSG-treated cells revealed a rapid, saturable binding of the fluorescent sVSG molecule to the membrane (Fig. 3A). Saturation of the signal occurred within 30 min after initial treatment with sVSG and was maintained for the length of the experiment. Macrophages treated with labeled sVSG were imaged by fluorescent microscopy after 30 min of treatment

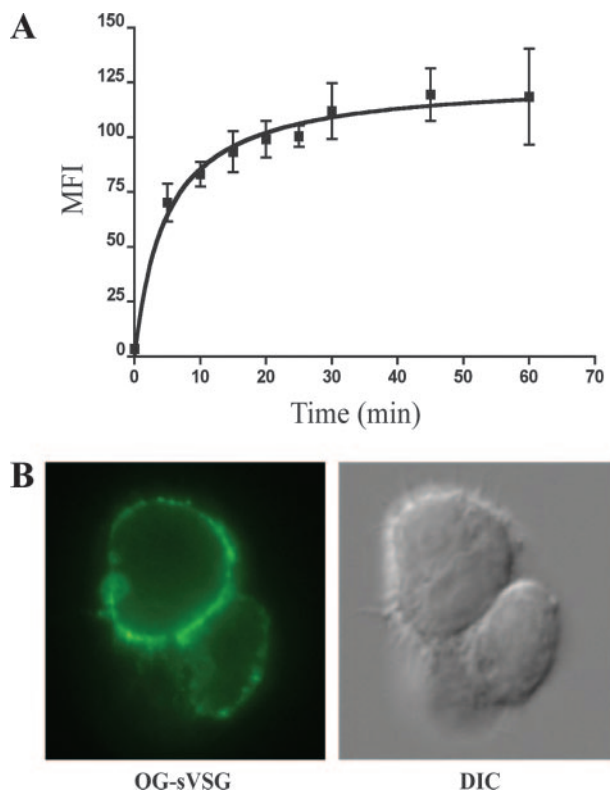


FIGURE 3. sVSG binds to macrophage membranes. *A*, Macrophages were preincubated at 4°C for 15 min and subsequently treated with fluorescent OG-sVSG (8 μ M) at 4°C for 60 min. Samples containing 1×10^6 macrophages were removed at regular intervals and total binding of fluorescent molecules was determined using flow cytometry. Data are represented as the mean fluorescence intensity (MFI) of a gated population of CD11b⁺ cells. *B*, Immunofluorescence micrographs of macrophages incubated with OG-sVSG for 30 min. Images depict the emission from 488 nm excitation (*left*) and the corresponding DIC image (*right*) of a representative field of cells.

(Fig. 3*B*). A robust binding of sVSG was observed across the surface of the macrophage consistent with a specific ligand-receptor interaction. Taken together, these results indicate that sVSG binds specifically to the macrophage membrane, likely through an interaction with a receptor expressed on the macrophage surface.

Maleylated BSA competes for sVSG receptor binding

The pattern of binding and internalization of sVSG by macrophages strongly suggests receptor-specific recognition of this molecule. These data, in combination with previous studies indicating a role for the carbohydrate GPI anchor in the induction of macrophage signaling by sVSG, led us to test receptor candidates that might be involved in the structural recognition of the sVSG molecule, including the macrophage SR and mannose receptor families. Because mBSA and mannose are known ligands of SR and mannose receptor (35, 36), respectively, we performed competitive binding studies by flow cytometry to assess the ability of these cells pretreated with excess mBSA or mannose to bind OG-sVSG. As shown in Fig. 4, there was a 60% reduction in the ability of macrophages to bind OG-sVSG in the presence of 2 mg/ml mBSA, while no reduction in OG-sVSG binding was seen following pretreatment either with mannose or the nonspecific ligand BSA. In addition, we observed attenuated binding of OG-sVSG over a range of mBSA concentrations as low as 250 μ g/ml up to a maximum tested concentration of 5 mg/ml and no change in OG-sVSG binding by mannose or BSA at any tested concentration (data

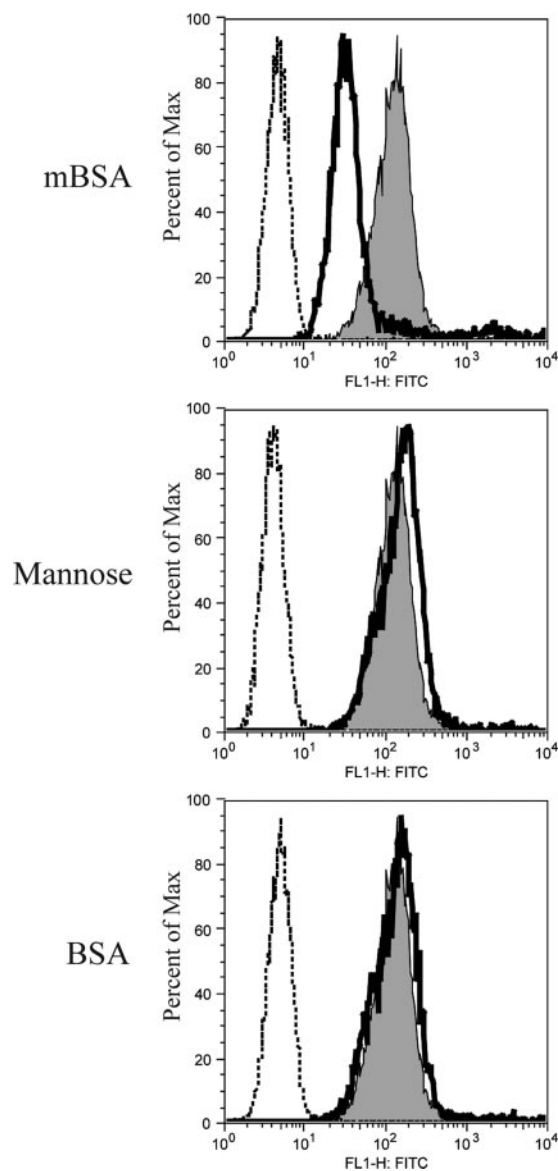


FIGURE 4. Maleylated BSA competes for sVSG binding to macrophage membranes. Macrophages were pretreated with 2 mg/ml of either mBSA, mannose, or BSA for 30 min at 4°C followed by incubation with OG-sVSG for an additional 30 min. Samples containing 1×10^6 macrophages were fixed and analyzed for OG-sVSG fluorescence by flow cytometry. Data are presented as a histogram of OG-sVSG fluorescence of F4/80-positive cells in each ligand-treated population (dotted line: without OG-sVSG treatment but with ligand treatment at left; thin line: OG-sVSG-treated without ligand; thick line: OG-sVSG-treated with ligand).

not shown). Based on these data, we conclude that a receptor in the macrophage SR family is responsible for recognizing trypanosome sVSG.

sVSG is internalized by macrophages

As APCs, macrophages present peptides from parasite ligands in the context of MHC class II molecules that are subsequently recognized by B and T cells of adaptive immunity. Because sVSG is a parasite ligand whose peptides are presented on the surface of APCs in a MHC class II-dependent manner to activate Th cells (12), and because sVSG can bind to the macrophage in a receptor-specific manner, we assessed sVSG internalization by the macrophage after binding. Macrophages were incubated with fluorescent

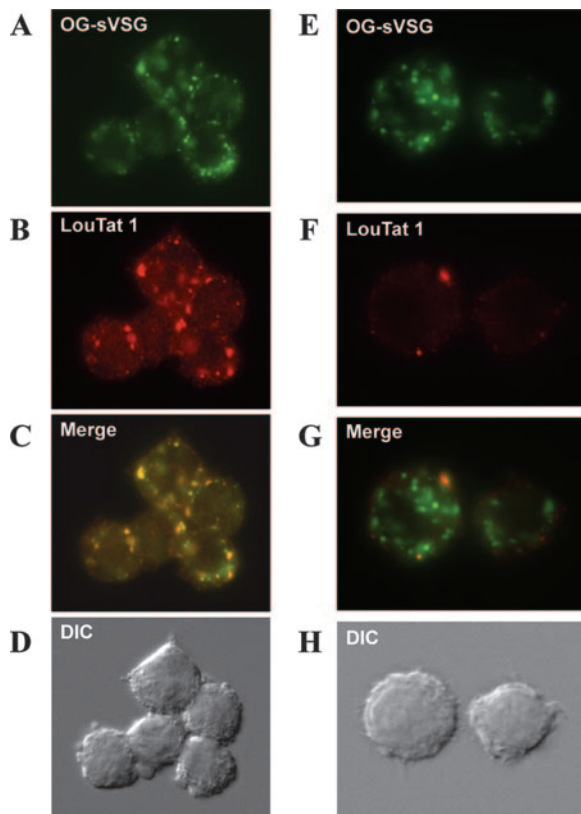


FIGURE 5. sVSG is internalized by macrophages. Immunofluorescence microscopy of macrophages treated at 37°C with OG-sVSG (8 μM) for 30 min and fixed before imaging. Cells were permeabilized (A–C) or left untreated (E–G) following fixation and stained with antiserum specific for LouTat 1 VSG followed by an Alexa 594-conjugated secondary Ab. D and H, Differential interference contrast microscopy image of permeabilized and untreated fields, respectively.

sVSG for 30 min at 37°C to allow the molecule to bind and internalize. Permeabilized and impermeabilized cells were subsequently stained with a LouTat 1 VSG-specific Ab to distinguish internalized from surface-bound sVSG (Fig. 5). Little signal was present on the macrophage surface in both permeabilized and impermeabilized cells, indicating that the majority of sVSG had been internalized in the period after sVSG addition. In permeabilized cells, we observed almost complete and punctate colocalization of both the labeled sVSG and the Ab staining showing that sVSG was rapidly internalized within the 30-min time period. The punctate distribution of the labeled molecule within the cell is consistent with the hypothesis that sVSG is being targeted to a specific compartment within the cell where it could be loaded onto MHC class II molecules and/or promote activation of the NF-κB cascade.

Internalization of sVSG is necessary for IκBα degradation

Because our data demonstrated a rapid movement of sVSG into the cell coincident with changes in IκBα expression, we assessed whether internalization was necessary to initiate IκBα degradation. We used the inhibitors cytochalasin B and monodansylcadaverine to ask whether actin-dependent internalization of sVSG or whether a clathrin-mediated uptake mechanism, respectively, was necessary to induce the NF-κB signaling that occurs in response to this molecule. Macrophages were pretreated with cytochalasin B or monodansylcadaverine and incubated with sVSG for 30 min. IκBα degradation in the resulting cells was measured by Western blot as a readout of NF-κB activation. Macrophages treated with cytocha-

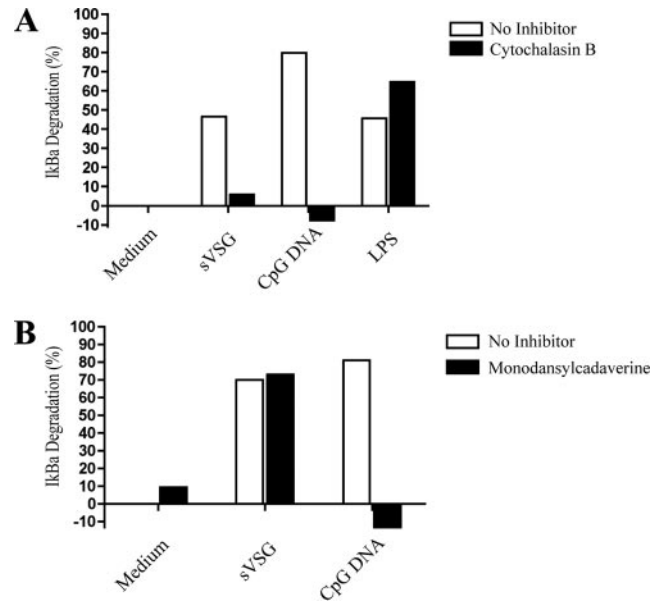


FIGURE 6. sVSG signaling occurs following internalization via an actin-dependent, clathrin-independent mechanism. Macrophages untreated (□), pretreated with the actin polymerization inhibitor cytochalasin B (A, ■) or the endocytic inhibitor monodansylcadaverine (B, ■) were stimulated with sVSG (16 μM), CpG DNA (1 μg/ml), or LPS (1 ng/ml) for 30 min. The percentage of IκBα degradation was measured by Western blot from a representative experiment.

lasin B showed a marked reduction in the ability of sVSG to degrade IκBα, a 90% decrease compared with cells treated with sVSG in the absence of inhibitor (Fig. 6A). As expected, cells treated with CpG DNA also displayed a reduced ability to degrade IκBα in the presence of inhibitor following a pattern similar to that observed with sVSG. Macrophages treated with LPS maintained signaling at equivalent levels in both the presence and absence of inhibitor. Under conditions that inhibit clathrin-mediated endocytosis, there was no change in the level of IκBα degradation in response to sVSG when macrophages were incubated in the presence or absence of monodansylcadaverine; however, NF-κB signaling by CpG DNA, which internalizes in a clathrin-dependent manner, was completely inhibited (Fig. 6B). These results demonstrate that sVSG is internalized in an actin-dependent, clathrin-independent manner and that actin-dependent internalization is necessary for degradation of IκBα by sVSG.

Internalized sVSG localizes to the lysosome

The ability of sVSG to be loaded and presented in a MHC class II-restricted manner, in combination with our results indicating sVSG internalization and a punctate distribution within the macrophage, suggested that sVSG was being localized preferentially to a specific structure following entry. To determine the compartment where sVSG was trafficked, immunofluorescent microscopy was performed on macrophages treated with OG-sVSG for 30 min and subsequently stained with an Ab to the endosomal marker cathepsin D or the lysosomal marker LAMP-1 (Fig. 7). Colocalization of both sVSG and LAMP-1 proteins demonstrates that shortly after internalization, sVSG localizes to the lysosomal compartment. A similar staining pattern was not observed in cells treated with cathepsin D, indicating that sVSG is not found within endosomes. The lack of sVSG colocalization with cathepsin-stained compartments is consistent with our data demonstrating that endosomal-mediated entry of sVSG is not necessary for activation of the NF-κB cascade.

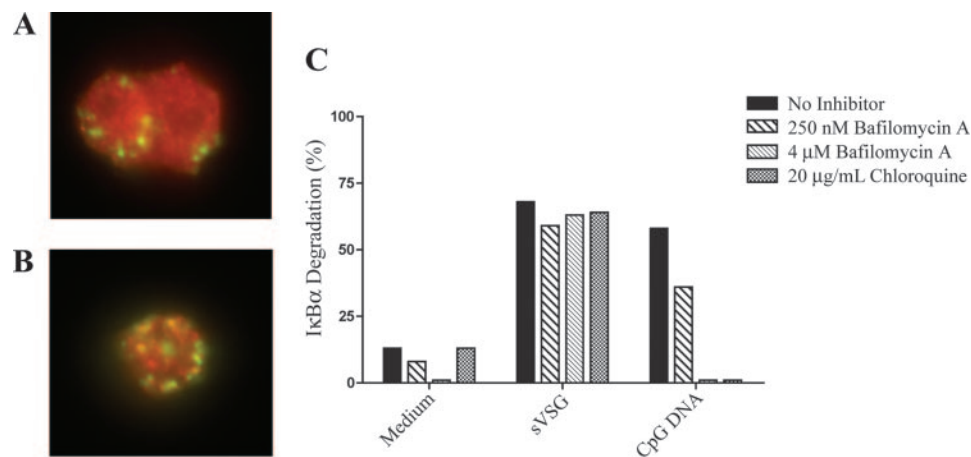


FIGURE 7. Internalized sVSG localizes to the lysosome and does not require acidification to induce I κ B α degradation. *A* and *B*, Macrophages were treated with OG-sVSG for 30 min at 37°C and fixed before staining. Cells were subsequently permeabilized and stained with an Ab against the endosomal marker cathepsin D (*A*) or the lysosome marker LAMP-1 (*B*), followed by an Alexa 594-conjugated secondary Ab. *C*, Macrophages were left untreated (solid bars) or were pretreated with either bafilomycin A (250 nM, thick-lined bars; or 4 μ M, thin-lined bars) or chloroquine (20 μ g/ml, dotted bars) for 1 h followed by incubation with CpG DNA (10 μ g/ml) or sVSG (16 μ M) for 30 min. The percentage of I κ B α was measured by Western blot from a representative experiment.

Acidification of lysosomes before downstream signaling cascade activation occurs following many receptor-mediated internalization events and can play a role in downstream signaling (37). To determine whether lysosome acidification is necessary for induction of NF- κ B signaling by sVSG, bafilomycin A, a specific inhibitor of the vacuolar H⁺-ATPase, or chloroquine, a weak base that is preferentially localized to lysosome and endosome structures, was used to inhibit acidification of the lysosomal compartment. Macrophages were pretreated with bafilomycin A or chloroquine for 1 h followed by sVSG for 30 min, and I κ B α degradation was measured by Western blot. A dose-dependent reduction in I κ B α degradation by CpG DNA was observed in bafilomycin-treated and chloroquine-treated cells, however, there was no effect on I κ B α expression with either inhibitor in macrophages treated with sVSG (Fig. 7C). From these results, we conclude that lysosomal acidification is not necessary to induce I κ B α degradation in response to sVSG. Taken together with the above immunofluorescent binding studies and NF- κ B signaling data, our results provide strong evidence to conclude that sVSG is specifically recognized by a receptor-mediated interaction and trafficked to the lysosome where it could initiate NF- κ B-dependent signaling in the macrophage.

Discussion

The work presented here identifies for the first time the mechanisms regulating recognition of trypanosome sVSG and subsequent induction of signaling in host macrophages. Previous studies have described induction and modulation of signaling by sVSG in the context of enhanced transcription and release of downstream proinflammatory mediators including TNF, NO, IL-6, and IL-12, and the inhibition of IFN- γ -mediated STAT-1 signaling. However, the specific interactions of sVSG with the macrophage membrane, how this interaction results in enhanced activation or modulation of downstream signaling, and whether these effects resulted from receptor-mediated recognition of the sVSG molecule remained unknown. The results of our studies revealed several key aspects of the recognition and phagocytic events necessary for macrophage activation in response to this molecule.

First and foremost, we show here that purified sVSG rapidly enhances I κ B α degradation in primary BMMs and RAW264.7 macrophages that induce the NF- κ B cascade. Though these studies

measure potential NF- κ B activation indirectly through I κ B α , the correlation between I κ B α degradation and NF- κ B activation is well-established. Induction of NF- κ B-dependent transcription is necessary for production of cytokines such as IL-6 and TNF, previously described as primary outcomes of macrophage activation during trypanosome infection (18–20). This result suggests that NF- κ B-dependent transcription events are of particular importance in regulating macrophage activation by African trypanosomes, and ultimately B and T cell functions of adaptive immunity, during the course of disease.

Second, our work addressed specifically whether TRAF6-dependent TLR signaling is required for sVSG-mediated macrophage activation by blocking TRAF6, a key mediator of the downstream activation of NF- κ B by all known TLRs (31, 38). The results of peptide inhibition of TRAF6 activity demonstrate conclusively that TRAF6-dependent TLR recognition of sVSG is not responsible for I κ B α degradation. Moreover, the observation that inhibition of TRAF6 signaling resulted in increased I κ B α degradation strongly suggests a previously unidentified role for negative TLR signaling events that regulate the final activation state of macrophages in response to this trypanosome molecule. Additionally, our results enhance previously published work documenting a role for MyD88 in sVSG-mediated signaling (21). Taken together, sVSG induces a MyD88-dependent, TRAF6-independent mechanism that results in induction of the NF- κ B cascade through degradation of I κ B α . Precedents for receptor-specific, TLR-independent MyD88-mediated signaling leading to NF- κ B activation have been observed previously in ligand recognition by the IL-1R and the IL-18R (39). Current data suggest that sVSG may act in a similar manner. Using alternative approaches to inhibit the TLR cascade independently of MyD88 will make this peptide-inhibited system particularly amenable to further study of the negative regulation of signaling observed here and for subsequent exploration of the role of MyD88-regulated signaling events in the sVSG-induced activation process.

Third, our data demonstrate for the first time the rapid saturable binding of labeled sVSG to the macrophage membrane that correlates with fluorescent micrographs exhibiting distributed staining across the cell surface. Subsequent to binding, but within the time frame of detectable I κ B α degradation, an actin-dependent, clathrin-independent internalization step occurs which is required for

downstream signaling. Blocking this event with monodansylcadaverine completely inhibited NF- κ B signaling as a result of sVSG treatment of macrophages and established a requirement for actin-mediated membrane reorganization characteristic of many phagocytic mechanisms. Internalization is followed by the targeted accumulation of sVSG in lysosomal structures, however, induction of downstream signaling occurs independently of compartmental acidification.

Finally, our results clearly demonstrate a role for a member of the SR family in the recognition and binding of sVSG to the cell surface. The characteristics of the macrophage SR classes are consistent with the structures and functions necessary for binding of the sVSG molecule, the required internalization that is a prerequisite for sVSG-induced I κ B α degradation, and the complex multifunctional activating and inhibiting signaling mechanisms. Previous work has shown that the glycosylinositolphosphate anchor is the sVSG molecule substituent that interacts with a cellular receptor to induce macrophage activation (19). This is consistent with results demonstrating that GPI glycans from a variety of intracellular and extracellular parasites including *Trypanosoma cruzi*, *Plasmodium*, *T. brucei*, and *Leishmania* have previously been shown to induce proinflammatory responses in macrophages (40, 41). These characteristics, along with the inability of mannose to affect sVSG binding, suggest that the SR recognizes a component other than the mannose structures present on the GPI core glycan. The specific identification of the SR responsible for these activities is the subject of ongoing investigation.

Overall, the work presented here details the membrane-localized recognition processes and initial signaling events responsible for NF- κ B-mediated macrophage activation and regulation in response to trypanosome sVSG. The activating effects of sVSG on the macrophage and the coincident modulating effects of this molecule on TLR signaling suggest that multiple receptor-dependent signaling events are responsible for the global activation state of the macrophage. Our results are consistent with a model where one receptor is expressed on the cell surface and mediates binding and internalization while a second receptor expressed in intracellular compartments recognizes internalized sVSG and initiates NF- κ B-dependent signaling. This model is similar to the current paradigm in TLR9 signaling, where in this case, the receptor is localized intracellularly and a second non-CpG-specific receptor mediates entry of the CpG molecule into the cell and directs it to lysosome structures containing TLR9. Efforts currently are underway to test this model.

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Disclosures

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