A Conserved Flagellar Pocket Exposed as a Host Cytokine Binding Molecule

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Trypanosomes use antigenic variation of their variant-specific surface glycoprotein (VSG) coat as defense against the host immune system. However, in order to sustain their growth, they need to expose conserved epitopes, allowing host macromolecule binding and receptor-mediated endocytosis. Here we show that Trypanosoma brucei uses the conserved chitobiose-oligomannose (GlcNAc2-Man5–9) moieties of its VSG as a binding ligand for tumor necrosis factor (TNF), a host cytokine with lectin-like properties. As endocytosis in trypanosomes is restricted to the flagellar pocket, we show that soluble flagellar pocket extracts, and in particular soluble VSG, inhibit the binding of 125I-TNF to trypanosomes. The interaction between TNF and VSG is confirmed by affinity chromatography, biosensor, and dot-blot affinity measurements, and soluble VSG inhibition of TNF-mediated trypanolysis. In all approaches, removal of N-linked carbohydrates abrogates the TNF-VSG interaction. In addition, synthetic high mannose oligosaccharides can block TNF-VSG interactions, and a VSG glycopeptide carrying the GlcNAc2-Man5–9 moiety is shown to inhibit TNF-mediated trypanosome killing in mixed parasite/macrophage cell cultures. Together, these results support the observation that TNF plays a role in growth control of trypanosomes and, moreover, suggest that, by the use of conserved VSG carbohydrates as lectin-binding epitopes, trypanosomes can limit the necessity to express large numbers of invariant surface exposed receptors.

African trypanosomes are extracellular parasitic protozoa transmitted by tsetse flies, causing human sleeping sickness and animal infections. In order to survive, trypanosomes need to evade effective immune recognition and destruction, but also need to bind and endocytose host macromolecules via conserved surface-exposed specific receptors. Although the mechanisms of immune evasion are largely unraveled and mainly rely on antigenic variation of the parasite surface glycoprotein VSG1 (1, 2), until now the information available on the mechanisms by which parasites can selectively take up host macromolecules has been limited (3). Indeed, the only trypanosome receptor identified so far is the ESAG 6/7-encoded transferrin-binding protein (4–6). In contrast, the pathway of endocytosis of host macromolecules itself has been analyzed and documented extensively in case of trypanosomes. Endocytosis was shown only to occur inside the flagellar pocket of the parasite (7). This membrane region is unique in that it lacks the structure of closely arranged microtubules that supports the parasite shape over the rest of the cell surface (8, 9). In bloodstream form trypanosomes, uptake of macromolecules through the flagellar pocket seems to occur via the classical pathway of coated pit formation and transport by coated vesicles to lysozyme-like organelles. This has been described for the uptake of ferritin (7) and transferrin (4), for the trypanolytic factor associated with high density lipoprotein (10), and for the inflammatory host cytokine TNF (11). Interestingly, this pathway is also involved in recycling of VSG (12). As the speed of endocytosis in trypanosomes is extremely high, estimates suggest that the parasite may internalize and recycle as much as 5% of its VSG surface molecules every minute (13). Here it should be mentioned that, during the passage of VSG through the flagellar pocket, epitopes that are shielded on the coat, due to the tight VSG packing, become surface-exposed. For instance, specific lectin labeling of VSG carbohydrate epitopes occurs only in the flagellar pocket (14). In this context, we now show that flagellar pocket-exposed VSG can serve as a receptor for the host cytokine TNF and that binding occurs via a lectin-like interaction involving the chitobiose-oligomannose (GlcNAc2-Man5–9) moiety of the N-linked VSG carbohydrate side chain. Together, the results presented in this work suggest that, although trypanosomes must be able to complete their life cycle in different host species, they can circumvent the necessity to express a wide range of specific receptors by utilizing conserved VSG carbohydrate moieties as lectin-ligands. Furthermore, the results corroborate the direct role of TNF in the control of African trypanosomosis, through parasite lysis described for Trypanosoma brucei (15) and most recently for Trypanosoma gambiense (16).
EXPERIMENTAL PROCEDURES

Binding Assays on Living Trypanosomes—Bloodstream form parasites (T. brucei AnTat 1.1) were isolated at day 6 (peak) of infection, from C3H/HeN mice injected intraperitoneally on day 0 with 1000 trypano- somes/mouse. Trypanosomes were purified on a DEAE 52 column as described (17) and incubated with different concentrations of 125I-TNF (2–80 nM) (Innogenetics) in sterile polyestirone round-bottom tubes (Falcon) at a concentration of 106 trypanosomes/ml in phosphate-buffered saline (pH 8.0) supplemented with 0.1% glucose and 5% fetal calf serum. After a 5-h incubation on ice, cells were washed six times with ice-cold PBS by mild centrifugation (1800 rpm/10 min). The amount of labeled material bound to the cells was measured with a Wizard™ 1470 automatic γ-counter (Wallac). Inhibition of binding was tested by pre-incubating the parasites during 1 h at 4 °C with a 100-fold molar excess of cold TNF. In parallel, inhibition of binding was tested by pre-incubation of the 125I-TNF with flagellar pocket (Fp) extract from T. brucei AnTat 1.1 parasites (18) (100 μg/ml), VSG (50 μg/ml), or N-glycosidase F-treated (Roche Molecular Biochemicals) VSG (50 μg/ml).

For the comparatively inhibitory capacity of VSG, enzyme-digested VSG molecules, VSG carbohydrate moieties, and irrelevant oligosaccharides, parasites were incubated with 40 μM 125I-TNF. Inhibition experiments were performed using VSG (50 μg/ml) or different oligosaccharides (5 μM) obtained from GlycoScience or Sigma (GlcNAc).

Binding Assays on Immobilized VSG—Mini-Leak Sepharose resin (Kem-En-Tec, Biozym) was used to covalently bind VSG or transferrin. The resin (0.5 ml) was washed with distilled water on a suction filter, and 0.5 mg of protein dialyzed against saline was bound overnight at 4 °C in the presence of 7% polyethylene glycol solution. The coupling yield was measured by measuring the optical density at 280 nm before and after coupling. To block excess of activated groups, 2 column volumes of 0.2 M ethanolamine were used and incubated for 5 h at room temperature. Finally, the resin was washed extensively with 0.1 M K2HPO4/NaOH (pH 11), followed by 0.1 M glycine/HCl (pH 2.8). Binding of 125I-TNF to the different columns was analyzed by incubating 1 μg of the labeled cytokine overnight at 4 °C. After washing with 10 column volumes of 1 M HCl to remove weakly or non-covalently bound protein, the columns were washed with 0.1 M glycine/HCl (pH 2.8). Fractions were collected as 100-μl aliquots and analyzed with a Wizard™ 1470 automatic γ-counter (Wallac).

Binding Assays via an Optical Biosensor—Affinity measurements between TNF and VSG from T. brucei AnTat 1.1 parasites were performed using the IAsys instrument with aminosilane cuvettes (Affinity Sensors) as an optical biosensor (11, 19). The immobilization of cytokines to aminosilane was performed as follows. After pre-incubation of the aminosilane cuvettes with 10 mM phosphate buffer (pH 7.7), the homobifunctional cross-linker bis(sulfosuccinimidyl) carbonate (Pierce) was added for 10 min to activate the surface. Following re-equilibration with phosphate buffer, TNF was immobilized for 10 min at 100 μg/ml in phosphate buffer. Remaining active sites were blocked with 1 μM ethanolamine, and the column was washed with 50 mM HCl. Specific binding resulted in a 300 nm reflection shift. All binding reactions were performed at 27 °C.

Binding Assays via Dot Blot—Affinity measurements between TNF and purified soluble VSG were performed using a dot blot technique. VSG or VSG-derived peptides were blotted onto a nitrocellulose membrane at a concentration of 25 μg/spot. The membrane was incubated for 2 h in 3% casein/PBS (pH 7.2), washed twice with PBS supplemented with 0.1% Tween 20 (Sigma), and subsequently incubated overnight with different concentrations of 125I-TNF (2–80 nM) in PBS containing 7.5% bovine serum albumin. Unbound cytokine was removed by extensive washings in PBS supplemented with 1% Tween 20. Specific binding was measured by Molecular Imager (Bio-Rad). Inhibition of specific binding was assayed by pre-incubation of the membranes with a 100-fold excess of cold TNF. The binding data were analyzed using the Prism program (GraphPad Software Inc.). The estimation of KD was obtained via a one-site binding fit (hyperbola).

Deglycosylation and Enzyme Digestion of VSG—N-Glycosidase F from Flavobacterium meningosepticum (Roche Molecular Biochemicals) was used according to the manufacturer’s protocol to remove N-linked carbohydrates from VSG using 5 units/ml enzyme (24 h at 30 °C). As a control, VSG was processed in parallel in the absence of the enzyme. Deglycosylated proteins were isolated with a concanavalin A column (Amersham Pharmacia Biotech) using PBS for equilibration and elution. Fractions of 0.5 ml were collected and tested for carbohydrate content using the silica gel TLC (Filterservice) and orcinol (Sigma) as the developing agent. Further determination of carbohydrate content was obtained by 8% SDS-PAGE and Western blotting. For VSG size determination, 1 μg of VSG was loaded per lane, and a polyclonal rabbit anti-VSG antiserum (1/10,000) was used for detection, in combination with a horseradish peroxidase-coupled goat anti-rabbit polyclonal antibody (1/500) (Sigma). As a control, purified transferrin was used at the same concentration (Sigma).

Deglycosylated and enzyme-digested, the jack bean enzyme (Roche Molecular Biochemicals) was used according to the same protocol as the one used for N-glycosidase F treatment. Purification of the enzyme treated fraction was performed with a concanavalin A column, as described above.

For HF treatment, VSG was dissolved in 10 mM Tris (pH 8.0) by dialysis, and lyophilized. Next, VSG was resuspended in 50% ice-cold HF, incubated on ice for 48 h, and lyophilized twice, resuspending the VSG each time in 10 mM Tris (pH 8.0). A purified VSG fraction, lacking the GIP/GPI core was obtained through a final gel filtration on a S75 (3.2/30) column (Amersham Pharmacia Biotech) equilibrated with 10 mM Tris (pH 8.0).

For Pronase digestion, VSG was dissolved in 100 mM ammonium bicarbonate (pH 8.0) and 20 μl of Pronase (12% w/w) was added. The mixture was incubated at 37 °C for 2.5 h. After an overnight incubation (37 °C), fresh Pronase was added (0.2% w/w) and another 24-h incubation was performed (37 °C). 1 M acetic acid was added to completely neutralize the amion bicarbonate. The digested material was centrifuged to remove insoluble material, and the supernatant was passed through a Dowex AG50X12 cation-exchange column (Bio-Rad) equilibrated with 10 mM acetic acid. The fraction was eluted with 10 mM ammonium hydroxide (pH 12). A 0.1 volume of 100 mM ammonium hydroxide was added to the eluate in order to neutralize the acetic acid. The collected fraction was applied to an anion-exchange column (QAE-A25 Sephadex; Amersham Pharmacia Biotech) equilibrated with 10 mM ammonium acetate. The column was washed with 10 ml of 10 mM ammonium acetate, followed by a linear gradient to 400 mM ammonium acetate. Fractions of 1 ml were collected and lyophilized. Fractions were resuspended in 20 μl of PBS and checked for carbohydrate contents on a silica gel TLC plate (Filterservice) and sprayed with orcinol reagent (Sigma). The flow-through, containing N-linked glycopeptides, was diluted in 0.1% trifluoroacetic acid and loaded onto an analytic microRPC-C2/C18 (ST 4.6/100) reverse phase column (Amersham Pharmacia Biotech) equilibrated against the same buffer. A linear gradient to 0.1% trifluoroacetic acid, 90% acetonitrile was then used to elute bound peptides in 100-μl fractions. Each fraction was lyophilized and resuspended in PBS. The carbohydrate fraction was identified using the TLC plate and orcinol technique. The same protocol was used to isolate the glycopolypeptide fraction from ribonuclease B (Sigma) and transferrin (Sigma).

In Vitro TNF-Related Trypanolysis Assay—The in vitro trypanolytic assay was performed as described previously (11), using a 125I-TNF concentration of 52 ng/ml (1 nM) in order to obtain maximal lysis within 5 h. Inhibition of lysis was analyzed by pre-incubating TNF with (i) VSG, N-glycosidase F-treated VSG, or α-mannosidase-treated VSG (50 μg/ml); (ii) several glycopeptides or non-glycosylated peptides (50 μg/ml); or (iii) different de novo synthesized carbohydrates (5 μM) (Oxford GlycoSciences).
tested over a wide TNF concentration range at 4°C (50 μg/ml) (100-fold molar excess). Inhibition of binding was obtained by (i) preincubation of the TNF with or without Fp extract (100-fold molar excess) or (ii) preincubation of the cells with cold TNF (×100-fold molar excess). Inhibition of 125I-TNF binding to living trypanosomes was also prevented by preincubation of TNF with VSG (50 μg/ml). The inhibitory capacity of VSG was abrogated by N-glycosidase F digestion.

**Ex Vivo TNF-mediated Trypanolysis Assay**—Peritoneal exudate cells (PECs) were harvested from *T. brucei* AnTat 1.1-infected C57Bl/6 WT or C57Bl/6 TNF−/− mice by a peritoneal wash with 10 ml of ice-cold sucrose/H2O solution (116 g/l). The collected cells were washed in RPMI 1640 complemented with 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 0.5 mg/ml L-glutamine (Life Technologies, Inc.) and put into culture at 37°C on 96-well culture plates at a concentration of 2×10^5 cells/ml, 100 μl/well. As the PECs were isolated at the first peak of the parasite infection, harvested cells were contaminated with ~10^5 parasites/ml. The parasite concentration in a 10-μl culture aliquot was followed over a period of 24 h.

**RESULTS**

TNF binding to the surface of African trypanosomes involves a lectin-like event (15) and has previously been demonstrated to occur in the Fp, the only membrane region allowing endocytosis (7, 11). As such we have investigated the involvement of Fp glycoproteins in binding of the cytokine, using a purified Fp fraction that was obtained by a grinding/ultracentrifugation method. Fig. 1A shows that, when 125I-labeled mouse TNF and living *T. brucei* AnTat 1.1 were incubated together at 4°C in the presence of 5% fetal calf serum, the cytokine bound to the parasites in a receptor-mediated fashion. Preincubation of the cytokine with the Fp fraction (100 μg/ml) blocked the binding of 125I-TNF to a similar extent as the inhibition obtained by preincubating parasites with a 100-fold molar excess of cold TNF. Next, as VSG is a major component of the Fp fraction, the inhibitory capacity of purified soluble VSG was tested, as well as the role of the VSG N-linked carbohydrate moiety. Here, it must be emphasized that the AnTat 1.1 VSG is a type I VSG, carrying one single glycosylated site (20). Fig. 1B shows that, when added in excess, purified VSG was equally potent in inhibition of 125I-TNF binding to living trypanosomes as the total Fp fraction, whereas removal of the N-linked GlcNAc2-Man5,6 carbohydrate moiety, a VSG epitope that is only exposed inside the Fp (14), abrogated this inhibition. The complete removal of the carbohydrate chain from the VSG after N-glycosidase F enzyme digestion was guaranteed by using only a non-ConA binding VSG fraction. Furthermore, as shown in Fig. 2, the purity of the soluble VSG preparations and the removal of the N-linked carbohydrate chain were checked by SDS-PAGE in combination with a VSG-specific Western blot revelation, an orcinol-carbohydrate staining on dot blot, and a Coomassie Blue protein staining. As shown in lane A1, VSG appeared as a single band in Western blot, and stained positive in orcinol staining for carbohydrate presence even at 0.5 μg/dot. N-Glycosidase F-digested VSG (lane A2) appeared with a slightly reduced molecular weight, and stained positive in orcinol staining only at a concentration of 5 μg/dot. In addition, a HF treatment of VSG was performed to remove the GIP/GPI carbohydrate core of the soluble molecule. Lane A3 shows that this treatment has only a limited influence on the migration of VSG in SDS-PAGE and the orcinol dot blot staining. Finally, a HF treatment of the N-glycosidase F-digested VSG was performed, in order to obtain completely carbohydrate-free VSG that was used as a negative control for the orcinol staining. The result in lane 4 confirms the removal of all VSG carbohydrates, and furthermore indicates that the minimal orcinol staining of the N-glycosidase F-digested VSG in lane 2 is only due to the presence of the GIP/GPI carbohydrate core. Purity of the VSG preparation is confirmed by the Coomassie Blue protein staining of Fig. 2B.

The direct interaction between TNF and VSG was confirmed by TNF affinity chromatography on immobilized VSG-Sepharose. Here, 1 μg of the labeled cytokine was allowed to bind overnight at 4°C to a column containing 0.5 mg of VSG coupled on 500-μl matrix. Next, the column was washed with 10 volumes of PBS and 10 volumes of NaCl (1 M) in order to remove non-bound and nonspecific bound material. The remaining binding fraction was eluted with glycine/HCl (0.1 M; pH 2.8), collected in 100-μl fractions, quantified in a Wizard® 1470 automatic γ-counter, and analyzed by SDS-PAGE (Fig. 3, panel A). In parallel, two other columns were used with, respectively, Sepharose-N-glycosidase F-treated VSG and a Sepharose-α-mannosidase-treated VSG. Virtually no labeled TNF was found to bind to these columns (Fig. 3, panel B). A fourth column,
used as an extra negative control column, made with Sepharose-transferrin, also failed to bind "I-TNF (results not shown).

In order to further confirm the direct interaction between TNF and VSG and to determine the affinity of the interaction, the binding between the two molecules was studied with the aid of an optical biosensor, using a methodology previously implemented to demonstrate TNF-α binding on total trypanosome-soluble extracts (11). First, using immobilized TNF-a-mannosilane, the association and dissociation was measured for soluble VSG and both N-glycosidase F-treated and α-mannosidase-treated VSG. Fig. 4 (A-F) gives the experimental readouts of conditions in which different concentrations of VSG (from 200 down to 10 nM) were allowed to interact with the immobilized TNF during the binding and dissociation phase of the experiment. Monitoring for each condition both binding and dissociation rates allowed the determination of the $k_{on}$ and $k_{off}$, yielding a $K_D$ value of 73.2 ± 32.3 nM for the interaction between TNF and AnTat 1.1 VSG (Table I), a value in the same range as the previously recorded affinity of TNF for total trypanosome lysate (11). As a negative control, the interaction between TNF and N-glycosidase F-treated VSG (Fig. 4G) and TNF and α-mannosidase-treated VSG (Fig. 4H) was recorded at a ligand concentration of 200 nM. As a negative binding control transferrin was added to the TNF-coated cuvettes at a concentration of 200 nM. No binding was observed here (results not shown).

Although by definition VSG molecules are very heterogeneous in terms of their amino acid sequence and both their glycosylation pattern and number of glycosylation sites, they all share one conserved N-linked GlcNAc₂-Man₉,GlcNAc₂全民新世纪 (21). Therefore, the $K_D$ value for the interaction between TNF and VSGs belonging to different classes (I, AnTat 1.1, MITat 1.4, MITat 1.6; II, MITat 1.1; III: MITat 1.5) was tested. For this purpose a dot blot technique was adopted. VSGs or total trypanosome extracts were blotted on a nitrocellulose filter (25 μg/spot) and incubated in a "I-TNF solution (2–80 nM), preventing nonspecific binding interactions by an overnight membrane blocking step using casein. The presence of 1% bovine serum albumin during the binding assay further ensured specificity of the results. The results were analyzed by spot densitometry on a PhosphorImager (Bio-Rad), and the $K_D$ values were calculated using a one-site binding hyperbola (GraphPad Software Inc.). Compiled results (Table I) show that all VSGs tested were capable of binding TNF. The $K_D$ values were all found to be in the same range, independent of the VSG class. Moreover, the range of $K_D$ values for the TNF/VSG interaction corresponded to the $K_D$ values calculated for the cytokine interaction with total extracts from the different parasite clones.

The fact that different class-specific VSGs bind "I-TNF with similar affinities suggests that most, if not all, VSGs bind TNF via their conserved N-linked high mannose moiety. The question as to whether the presence of a surface exposed GlcNAc₂-Man₉,GlcNAc₂ is the high mannose moiety of the N-linked VSG carbohydrate side chain inside the Fp of trypanosomes contributes to the biological activities of TNF on the parasite was addressed using the previously reported TNF-mediated trypanolysis assay as a readout system. In this bioassay, performed at 30 °C, a concentration of 1 nM recombinant mouse TNF was used for an
TNF Binding on the Surface of African Trypanosomes

Role of the GlcNAc2-polymannose moiety in TNF-VSG interactions

| Inhibitor | Inhibition of TNF binding\(^a\) | Inhibition of trypanolysis\(^b\) |
|-----------|-------------------------------|-------------------------------|
|           | %                             | %                             |
| A.        |                               |                               |
| VSG       | 80 ± 7                        | 75 ± 7                        |
| VSG-NGF   | NIA                           | 3 ± 2                         |
| VSG-\(\alpha\)-mannosidase | NIA                        | 7 ± 3                         |
| B.        |                               |                               |
| VSG glycopeptides\(^c\) | 87 ± 5                     | 71 ± 9                        |
| VSG peptides\(^d\) | NIA                        | 7 ± 6                         |
| C.        |                               |                               |
| GlcNAc2-Man9 | 75 ± 8                     | 78 ± 6                        |
| GlcNAc2-Man6 | 60 ± 5                     | 36 ± 12                       |
| GlcNAc2-Man3 | 40 ± 4                     | 22 ± 6                        |
| GlcNAc2-Man | 38 ± 8                     | 26 ± 8                        |
| GlcNAc2   | 25 ± 3                     | 32 ± 5                        |
| NA\(_b\)   | NIA                        | 9 ± 3                         |
| NA FB      | NIA                        | 4 ± 3                         |
| D.        |                               |                               |
| Ribonuclease B glycopeptides\(^c\) | 62 ± 13                 | 74 ± 10                       |
| Transferrin glycopeptides\(^c\) | NIA                        | 5 ± 2                         |

\(^a\) Inhibition of binding was analyzed at 4°C on living trypanosomes using 40 nM \(^125\)I-TNF and 50 \(\mu\)g/ml VSG, deglycosylated VSG, VSG-derived glycopeptides, VSG-derived non-glycosylated peptides, or glycopeptides derived from ribonuclease B and transferrin (parts A, B, and D). Inhibition of binding was also analyzed using 5 \(\mu\)M oligosaccharides (part C).

\(^b\) Inhibition of TNF-mediated trypanolysis was analyzed at 30°C, using 1 nM TNF and the same concentrations of inhibitor as in Footnote a. All incubations were performed in PBS + 5% FCS and 0.1% glucose.

\(^c\) Peptides were obtained by pronase digestion followed by a cation and anion column purification and carbohydrate detection with orcinol reagent.

\(^d\) Pooled fractions of non-glycosylated peptides.

incubation period of 5 h. In this assay it was shown that preincubation of TNF with soluble VSG inhibited the observed cytokine-mediated lysis (Table II, part A). In all experimental approaches, removal of N-linked carbohydrates from VSG by N-glycosidase F or \(\alpha\)-mannosidase digestion abrogated the TNF-VSG interaction. Next, we analyzed whether degradation of the protein part of the VSG would interfere with the observed interaction between TNF-\(\alpha\) and VSG. To this end, VSG was digested overnight with Pronase (2% w/v) and fractionated using both a cation and an anion exchange column, followed by a reverse phase separation. The glycopeptide fractions were identified by orcinol staining and analyzed for their capacity to inhibit \(^125\)I-TNF and TNF-mediated trypanolysis. The result presented in Table II (part B) show that the glycopeptides inhibited indeed both the binding of \(^125\)I-TNF to living trypanosomes and trypanolysis, whereas pooled non-glycosylated VSG peptides had no inhibitory activity at all (Table II, part B). These results all point to a crucial involvement of the VSG GlcNAc2-Man\(_{n-9}\) moiety in the interaction between TNF and the trypanosome. Accordingly, synthetic GlcNAc2-polymannose oligosaccharides (Fig. 5A) were able to inhibit TNF-trypansomone interactions. Here, an optimal activity for full-length mannose chain (GlcNAc2-Man\(_n\)) was recorded, at a concentration of 5 \(\mu\)M. At this concentration, GlcNAc2-polymannoses with less than 6 mannose molecules showed significantly reduced inhibitory capacity. Irrelevant asialo-triantennary galactosylated (NA\(_b\)) or asialo-biantennary galactosylated and fucose substituted (NA\(_b\)B) oligosaccharides (Fig. 5, B and C) were without effect at a 5 \(\mu\)M concentration (Table II, part C).

Taken together, the trypanosome ligand for TNF appears to be an Fp-exposed, highly branched polylmannose oligosaccharide, which is common to all VSG molecules characterized so far. Matured glycoproteins carrying this specific carbohydrate are extremely rare in or on cells from higher eu-
karyotes, due to post-Golgi remodeling. One exceptional host glycoprotein in this respect is the intracellular protein ribonuclease B (22). As expected, glycopeptides derived from this protein interfered with the TNF-trypansomone interactions to a similar extent as VSG glycopeptides did, while irrelevant bovine transferrin-derived glycopeptides had no inhibitory activity (Table II, part D).

As it has been proposed that activated macrophages play a crucial trypanosomosis control and parasite elimination, PECs were prepared from experimentally infected C57Bl/6 wild type (WT) and TNF\(-/-\) mice on the first parasitemia peak. Cells were placed in RPMI culture medium at a concentration of 2 \(\times\) 10\(^6\) cells/ml. At this cell concentration, the contaminating parasite concentrations for the WT-derived cultures were 1.2 \(\times\) 10\(^6\), 0.75 \(\times\) 10\(^6\), and 0.9 \(\times\) 10\(^6\) parasites/ml in, respectively, three independent experiments. In the TNF\(-/-\)-derived PEC cultures, the parasite contaminations were 3.8 \(\times\) 10\(^6\), 5.2 \(\times\) 10\(^6\) and 4.7 \(\times\) 10\(^6\) parasites/ml in, respectively, three independent experiments. Parasite survival was monitored over a period of 6 h. Fig. 6A shows that, in these \textit{ex vivo} conditions, significant trypanosomes killing was observed in the WT-derived cultures, starting after 3.5 h of incubation, whereas no trypanolysis was observed in the TNF\(-/-\)-derived cultures. Enzyme-linked immunosorbent assay measurements of the TNF content of the WT-derived cell cultures indicated the presence of 738 \(\pm\) 128 pg/ml cytokine. In order to assess the importance of the TNF-VSG/GlcNAc2-Man\(_{n-9}\) interaction in this \textit{ex vivo} lysis assay, the purified VSG glycopeptide fraction, which was shown above to block TNF binding to trypanosomes, was added to the PEC/parasite cocultures at a final concentration of 10 \(\mu\)g/ml. Fig. 6B shows that, indeed, the glycopeptide prevented TNF-mediated trypanosome killing in the WT-derived cultures, but it did not affect parasite survival in TNF\(-/-\)-derived cultures.

Together, these results confirm the importance of the VSG-GlcNAc2-Man\(_{n-9}\) moiety and, in particular, the highly branched mannose chain in the interaction between TNF and the
adding purified VSG glycopeptides to the PEC/parasite cocultures (10 through blocking of the TNF-VSG lectin interaction was assayed by mean percentage of parasite survival from three independent experiments ± standard deviation.

trypanosome, and support the previous finding that the direct action of TNF on the trypanosome can contribute to parasitemia control of African trypanosomes.

DISCUSSION

Specific receptor-mediated endocytosis by African trypanosomes remains poorly understood, mainly due to the fact that so far only a single host macromolecule parasite receptor could be identified, namely the VSG-like GPI-anchored ESAG6/7 transferrin receptor (4–6, 23). However, until now the search for host macromolecule receptors has been mainly based on approaches that ignore the possible involvement of trypanosome-specific glycosylation. Indeed, the screening of prokaryotic or even COS7 expression libraries for recombinant trypanosome molecules does not enable the identification of trypanosome glycoproteins that use unique carbohydrate moieties to bind host macromolecules. Given the facts that (i) trypanosome surface carbohydrate moieties are mainly exposed to macromolecules inside the flagellar pocket (14), and (ii) the flagellar pocket itself is the only membrane region through which trypanosomes can endocytose host material (7), it may be important to consider the possibility of involvement of specific trypanosome carbohydrate moieties in the process of binding and uptake of host macromolecules.

Although in vertebrate immunology protein-protein interactions between cytokines and their receptors are crucial, most cytokines have lectin-like binding domains as well (24). So far, rather limited information is available related to the functions of these domains. Among the proposed possibilities are events such as ensuring the contact between cytokine receptors and their signal transduction partners (25), binding to the extracellular matrix (26), binding to molecules involved in cytokine clearance (27), or even binding of cytokines to microorganisms (28). In the case of TNF, a lectin-like binding domain that was documented to be spatially and functionally distinct from the receptor binding site was shown to be crucial in the interaction with trypanosomes (15). Interesting, combined results, from studies performed both by us on T. brucei and by others on T. gambiense (16), show that this last interaction results in TNF-mediated trypanolysis through the accumulation of the cytokine in acid endocytic vesicles and the subsequent disruption of the vesicle membrane (11). Both the kinetics and the functional mechanisms of this event highly resemble an earlier documented alternative and receptor-independent TNF lysis of mammalian cells (29), a phenomenon that is markedly different from the more classic TNF receptor-mediated cell lysis most often studied in the presence of actinomycin D.

To unravel the process of TNF endocytosis by trypanosomes, the present work focused on the identification of the surface carbohydrate epitope that serves as a ligand for this cytokine. First, the Fp membrane fraction, which is the only trypanosome surface region involved in endocytosis, was shown to inhibit potently TNF-trypanosome interactions. Second, VSG, which is the main component of the Fp fraction, in particular VSG carbohydrate moieties, which are uniquely exposed inside the Fp, inhibited TNF-trypanosome interactions as well. Finally, using purified VSG glycopeptides and de novo synthesized oligosaccharides, the obtained data show that the trypanosome ligand for TNF is a Fp-exposed, highly branched N-linked GlcNAc2-Man9-GlcNAc9 moiety that is common to all VSG molecules characterized so far. Within this carbohydrate branch, the polymannose oligosaccharide is crucial for optimal TNF binding, and although the chitobiose core was shown earlier to inhibit TNF-mediated trypanolysis, the actual results indicate that this carbohydrate contributes only to a minor extent to the interaction between TNF and living trypanosomes. Here, it should be emphasized that glycoproteins carrying VSG-analogous carbohydrates are extremely rare on the surface of higher eukaryotes, due to post-Golgi remodeling (22). Hence, it is likely that the lectin-like domain of TNF enables selective interactions with microorganisms and not with extracellular host glycoproteins. This, together with the possibility of high rate endocytosis through the process of VSG recycling (12), suggested that the process of GlcNAc2-Man9-GlcNAc9-mediated TNF binding may be an effective mechanism for the parasitemia control. In further support of this, we show in this study that peritoneal exudate cells from infected mice can kill trypanosomes ex vivo. This killing activity is largely mediated by TNF, since it was not observed with cells derived from infected TNF−/− mice. Although we have documented previously that recombinant TNF could lyse trypanosomes in vitro (15), the high concentration required for optimal TNF-mediated trypanolysis in this assay raises questions about the relevance of this phenomenon in vivo. The present results, however, combined with the recent observation that macrophage-derived TNF concentrations can also kill T. gambiense parasites in mixed cell cultures (16), suggest that local production of TNF by activated macrophages represents an efficient killing mechanisms in vivo that can explain the impairment of peak parasitemia control observed in TNF−/− mice (30). Furthermore, these results can be linked to reports from others that are highly suggestive for a role of local macrophage activation in early resistance toward both T. brucei (31, 32) and T. congolense (33) infections. As such, local TNF production, rather than systemic serum TNF levels, should be considered.

Fig. 6. A, ex vivo TNF-mediated trypanolysis was analyzed in mixed PEC/parasite cultures derived from both T. brucei AnTat 1.1E infected C57Bl/6 WT mice (□) and C57Bl/6 TNF−/− mice (■). Cells were obtained through a wash of the peritoneal cavity of the mice at the first peak of the infection (day 6). B, inhibition of TNF-mediated trypanolysis through blocking of the TNF-VSG lectin interaction was assayed by adding purified VSG glycopeptides to the PEC/parasite cocultures (10 μg/ml). Abolishment of specific lysis was obtained in WT-derived cultures (○), whereas no effect of the presence of the glycopeptide was observed in the TNF−/−-derived cultures (●). The results are presented as mean percentage of parasite survival from three independent experiments ± standard deviation.

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to play a role in both experimental and human trypanosomosis control.

In conclusion, the results presented here provide the first functional link between a lectin-like cytokine domain, its carbohydrate ligand on a microorganism, and an effector function, in this particular case endocytosis-mediated lysis. Furthermore, from the conceptual point of view and at least of interest in the field of parasite research, our results suggest how trypanosomes may circumvent the necessity to express large arrays of specific protein receptors for host macromolecules required for survival and growth. Indeed, in view of the fact that trypanosomes have to cope with a diversity of effector macromolecule analogues from different potential mammalian host species, the expression of few conserved but parasite-specific lectin ligands would offer a great advantage over the expression of multiple specific protein receptors. The same argument may hold for other protozoan parasites and urges for a broadened view within parasite receptor research. Finally, taking into account the vast majority of cytokines that carry lectin-like binding sites, this work also calls for focused attention to resolve both the biological function and the evolutionary significance of these domains.

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