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*J Immunol* 2007; 179:4003-4014; doi: 10.4049/jimmunol.179.6.4003
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A Glycosylphosphatidylinositol-Based Treatment Alleviates Trypanosomiasis-Associated Immunopathology

Benoît Stijlemans,† Toya Nath Baral,*, Martin Guilliams,† Lea Brys,† Johanna Korf,* Michael Drennan,‡ Jan Van Den Abbeele,‡ Patrick De Baetselier,* and Stefan Magez*

The GPI-anchored trypanosome variant surface glycoprotein (VSG) triggers macrophages to produce TNF, involved in trypanosomiasis-associated inflammation and the clinical manifestation of sleeping sickness. Aiming at inhibiting immunopathology during experimental Trypanosoma brucei infections, a VSG-derived GPI-based treatment approach was developed. To achieve this, mice were exposed to the GPI before an infectious trypanosome challenge. This GPI-based strategy resulted in a significant prolonged survival and a substantial protection against infection-associated weight loss, liver damage, acidosis, and anemia; the latter was shown to be Ab-independent and correlated with reduced macrophage-mediated RBC clearance. In addition, GPI-based treatment resulted in reduced circulating serum levels of the inflammatory cytokines TNF and IL-6, abrogation of infection-induced LPS hypersensitivity, and an increase in circulating IL-10. At the level of trypanosomiasis-associated macrophage activation, the GPI-based treatment resulted in an impaired secretion of TNF by VSG and LPS pulsed macrophages, a reduced expression of the inflammatory cytokine genes TNF, IL-6, and IL-12, and an increased expression of the anti-inflammatory cytokine gene IL-10. In addition, this change in cytokine pattern upon GPI-based treatment was associated with the expression of alternatively activated macrophage markers. Finally, the GPI-based treatment also reduced the infection-associated pathology in Trypanosoma congolense and Trypanosoma evansi model systems as well as in tsetse fly challenge experiments, indicating potential field applicability for this intervention strategy. The Journal of Immunology, 2007, 179: 4003–4014.

Trypanosomiasis is a lethal disease affecting both humans and livestock. Although human African trypanosomiasis or sleeping sickness is caused by Trypanosoma brucei rhodesiense and Trypanosoma brucei gambiense, animal trypanosomiasis is caused by Trypanosoma congolense, Trypanosoma vivax, T. brucei brucei and Trypanosoma evansi (1, 2). Conventional anti-trypanosome treatment of infected hosts faces several problems including high drug toxicity and increasing drug resistance (2–4). In addition, the wide distribution of trypanosomes throughout the African wildlife reservoir confronts the region with an extensive problem of reinfection (5). Although vaccination against trypanosomes offers the most sustainable long-term solution, so far this strategy is beyond reach. Indeed trypanosomes are extracellular parasites that are adapted to immune confrontation through a mechanism of antigenic variation of GPI-anchored variant surface glycoproteins (VSG) (6, 7). During infection, released VSG interacts with various immune cells triggering an effective host anti-parasite immune response, including macrophage and B cell activation and Th1 cell stimulation (8, 9). Although this response efficiently eliminates trypanosomes expressing the target VSG, VSG switching allows immune escape and development of a new parasitemia wave. Consequently the immune system is chronically exposed to damaged trypanosomes and thereof released components that may elicit infection-associated immunopathology. Hereby, massive release of VSG-associated GPI moieties could play a major role. Indeed we, and others, have shown that the GPI moiety of VSG triggers macrophages to release proinflammatory cytokines in particular TNF (10–12). Though the proinflammatory potential of GPI molecules was amply documented in different parasitic infections including Chagas’ disease (13), leishmaniasis (14), trypanosomiasis (15), and malaria (16), so far compelling evidence for a relationship between the proinflammatory properties and disease-inducing potential of parasite-derived GPs was provided only for malaria. In the first instance, adults who acquired resistance to clinical malaria manifested persistently increased levels of serum anti-GPI Abs, whereas susceptible patients that suffered from malaria-associated anemia and fever, lacked an anti-GPI Ab response (17, 18) suggesting a protective role of anti-GPI Abs against clinical malaria. Second, a GPI carbohydrate core-based vaccine that conferred partial protection to malaria-associated pathology was designed (19). In view of these findings and based on the documented role of the GPI moiety of VSG in trypanosomiasis-associated pathology (12), an anti-trypanosome GPI intervention strategy was envisaged to protect against sleeping sickness. However, during trypanosomiasis, it...
should be emphasized that both the lipid (dimeristoylglycerol compound: DMG) and carbohydrate moiety of the GPI play distinct roles in macrophage activation and TNF-mediated immunopathology (20). Although the galactose-modified glycosylinositol phosphate moiety (GIP) of VSG is the minimal moiety needed for the optimal induction of TNF production by IFN-γ-activated macrophages, the DMG compound contributes to macrophage overactivation (TNF and IL-1 secretion) and LPS hypersensitivity during chronic trypanosome infections (12). Thus in the present work, animals were treated with intact GPI (encompassing both the lipid and the carbohydrate moiety) before infection with trypanosomes. Upon subsequent trypanosome challenge, the GPI-based treatment was found to 1) alleviate infection-associated inflammation and pathology, 2) prolong the host survival, and 3) modulate the macrophage activation state. Interestingly, in contrast to what could be expected based upon the malaria GPI vaccination studies, the protective effect of GPI-based treatment on pathology is B cell independent, and probably mediated by the alteration of the macrophage activation stage.

Materials and Methods

VSG purification

Trypanosoma brucei beelli AntTat 1.1, T. congolense Tc13 and T. evansi (ITMAS 110297, code KETRI 2480) were used to isolate VSG. Soluble VSG was produced as described by Stijlemans et al. (21) with 45 μl of a 0.8% solution in SDS-PAGE for purity (>95%). Membrane form VSG (mVSG) was obtained through a combination of previously described protocols (22, 23), followed by an additional gel filtration step. In short, 3–10⁹ parasites were resuspended in (8 ml each) ice-cold PBS and protease inhibitor solution (2 mM N-α-p-tosyl-l-lysine chloromethyl ketone (TLCK), 1 ml of complete protease inhibitor (Roche) supplemented with 20 mM p-chloromercuribenzenesulfonic acid (PCMBS; Sigma-Aldrich) as inhibitor of the PLC, and incubated for 30 min on ice followed by a three times alteration from liquid nitrogen/37°C. Parasites were pelleted and resuspended in solution A (100 mM HEPES NaOH (pH 6.9) 10 mM PCMBs, 1 mM TLCK, 0.1 mM PMSF). The soluble part was removed by ultra centrifugation (Centrikon T-2070; BRS Belgium) (100,000 g, 30 min at 4°C), and the pellet was resuspended in solution A containing 2% n-octylglucopyranoside (Roche) for 15 min on ice. Following a final ultracentrifugation step (100,000 × g for 60 min at 4°C), resulting mVSG was concentrated, passed over a size-exclusion column (Superdex 75; Pharmacia) equilibrated against 10 mM Tris, 0.05% n-octylglucopyranoside (pH 7.5) and checked in SDS-PAGE for purity (>95%). Western blot analysis, using rabbit polyclonal anti-VSG and anti-cross-reacting determinant Abs confirmed the presence of the GPI anchor on mVSG (24).

HPLC purification of the GPI moiety

A modification of the method described by Ferguson et al. (25) was used to isolate the GPI moiety. In brief, purified mVSG (1–5 mg) was supplemented with 1 mM CaCl₂ after digestion with 2% by weight of Pronase (Roche) for 12 h at 37°C, followed by an additional 12 h digestion with 0.2% Pronase. The released GPI was extracted with water-saturated Bu-tanol, washed with water, dried, and further separated from contaminating peptides by reverse-phase chromatography using a C₂/C₁₈ column (Phar- macia) equilibrated with 0.1% trifluoroacetic acid. Fractions collected by applying a linear gradient of 10–60% isopropanol containing 0.1% trifluoroacetic acid were lyophilized and resolved in water-saturated 1-butanol. Identification of the GPI-containing fraction was achieved by a combination of thin-layer chromatography (Polygram Sil-60; Macherey Nagel) and a phosphate detection method recommended by the suppliers.

Preparation of liposomes containing GPI

The method of Bangham et al. (26) for the preparation of liposomes was followed. In brief, 1–5 μg of purified GPI, determined according to a phosphorus determination kit, was lyophilized and mixed with 0.15 ml of phosphatidylcholine (Sigma-Aldrich; 100 μg/ml in chloroform) in 1.5 ml of Eppendorfs. Chloroform was evaporated under a stream of N₂ gas, and 1 ml of sterile PBS was added. The suspension was processed in an ultrasonic bath at 60°C for 20 min, followed by a vortex step (10 min). Non-incorporated material was removed by washing three times with PBS. The obtained GPI liposomes were resuspended in 1 ml of PBS. Control liposome suspensions were prepared similarly without the addition of GPI.

Immunization

Groups of 5–10 male C57BL/6 or BALB/c, 8-wk-old mice (Harlan) or B cell-deficient and C1d-deficient mice (a gift from Dr. B. Ryffel, CNRS, France) or C3H/HeN, TLR4–/– and TLR2/4–/– mice (obtained from Tulipar) were used. Taking into account that the GPI moiety lacks conventional T cell epitopes (27), 8-mercaptothiogosamine (8-MG) (Sigma-Aldrich) was used to increase immune responses against GPI liposomes. This biochemical response modifier triggers T cell independent B cell activation, including Abs isotype class switch to IgG (28, 29). 1) GPI-based pre-exposure: mice were injected i.p. with 200 μl of 8-MG (30 mg/ml) 2 h before the injection of 100 μl GPI liposomes (corresponding to 0.1–0.5 μg GPI). Three weeks later mice were again injected with 8-MG and GPI liposome boosted. 2) Mock-treated mice were treated as above with empty liposomes. As additional groups of mice, 8-MG treatment was omitted before injection of empty liposomes or GPI liposomes. Mice were bled 2 wk later and sera were tested for their anti-GPI Ab titer on both mVSG and purified GPI.

Experimental infections

Clonal infections were performed by using frozen stabile stocks of An-Tat1.1, Tc13, or KETRI2480 parasites. Mice were infected by i.p. injection with 5 × 10⁹ trypanosomes (diluted in PBS) per mouse, 3 wk after the boost. 

Tsetse fly transmitted trypanosome infections were performed at the same time point after the boost. In brief, freshly emerged tsetse flies were infected by feeding on AnTAR1 infected mice at the peak of parasitemia. To obtain a pleomorphic trypanosome population at high titer, these mice were immune suppressed with cyclophosphamide (20 mg/kg; Sigma-Aldrich). Flies were screened for a mature salivary gland infection 28 days after the infected bloodmeal by induced probing on prewarmed glass slides followed by a microscopic analysis for the presence of metacyclic trypanosomes in the saliv. Infection of tsetse flies with T. brucei parasites was performed in compliance with the regulations for bio-safety and under approval from the Environmental administration of the Flemish government. To initiate a natural infection, one individual tsetse fly with a mature salivary gland infection was allowed to feed per mouse. To avoid interrupted tsetse feeding, mice were anesthetized before the tsetse exposure.

Solid-phase binding ELISA

Purified VSGs (AnTat 1.1, Tc13, or KETRI 2480) were coated onto 96-wells plates (Nunc) (100 μl of 1 μg/ml in 0.1 M NaHCO₃ (pH 8.2)). After blocking (2 h, room temperature, 10% FCS in PBS) plates were incubated with serial dilutions of serum and bound IgG were detected with an anti-mouse-HRP Ab (Sigma-Aldrich) using peroxidase substrate. Plates were washed three times in between each step with PBS/0.1% Tween 20. Detection of anti-GPI Abs was also performed on purified GPI coated on 96-well Immunolon-2HB plates (Thermo Labsystems) as described for VSG-coated plates with the exception that washings and Ab dilutions were performed in PBS without Tween 20.

Cytokine and RBC analysis

Concentrations of TNF (R&D Systems), IL-6, and IL-10 (Pharmingen) in serum and cell supernatants were determined by sandwich ELISA as recommended by the suppliers.

RBC counts in blood taken by tail-cut were performed via hematocytometer.

Serum pH and aspartate transaminase level measurement

Serum pH levels were measured using the UriCheck-9 test (RapiMed Diagnostics) according to the manufacturers’ instructions. Serum levels of AST, as indicator of hepatocellular damage, were determined by an AST/ GOT kit (Sigma-Aldrich).

Locomotor activity measurement

This was measured as the total time per hour spent by mice on running in their cage, eating, drinking, and cleaning their fur and nest. Mice were kept in a 12-h light/dark regimen, and locomotor activity was recorded during the first 2 h of the light period, 8 days postinfection (p.i.).
Cell cultures

Peritoneal cells from GPI-based treated or mock-treated infected mice (10 days p.i.) were cultured in 48-well plates (Nunc; 5 \times 10^5 cells/ml) in complete RPMI 1640 supplemented with 2 mM glutamine, 1 mM pyruvate, 50 U/ml penicillin, 50 µg/ml streptomycin and 2 µL sodium bicarbonate, plus 10% FCS (Invitrogen Life Technologies). Cells were allowed to adhere for 3 h after which the plates were washed 2 times with RPMI 1640. CD11b expression analysis on these adherent cells indicated similar percentages of CD11b+ myeloid cells in GPI-based treated/infected (97.3%) versus mock-treated/infected (96.2%) mice. Cells were then stimulated with sVSG (10 µg/ml) or LPS (100 ng/ml) and incubated at 37°C, 5% CO_2 for 24 h. Cell supernatants were collected and analyzed for cytokine content.

LPS hypersensitivity

Based on previous data (30), T. brucei brucei infected GPI-pre-exposed mice were injected i.p. at 6 days p.i. with a range between 0.1 µg and 50 µg of LPS used for the determination of the LD_50 LPS dose by peritoneal administration. As such, the LD_50 LPS dose was determined at 0.5 µg/mouse. Next, infected mock-treated mice were inoculated with 0.5 µg of LPS/mouse and mortality was recorded. Five mice were used per experimental group.

Real-time quantitative PCR (RT-QPCR) analysis

RNA isolation was performed using RNeasy kit (Qiagen). From each sample 1 µg of RNA was converted into cDNA using a Superscript II reverse transcription reagent kit (Roche Molecular Systems). RT-QPCR was performed on an iCycler apparatus using iQ SYBR Green Supermix (Bio-Rad). PCR conditions were as described (31). Primers used were: TNF-F: 5’-CTCTTACACAGGAACTTACT-3’, TNF-R: 5’-GTCTAC TCCCAAGTCTCTTC-3’, IL-12 p40-F: 5’-GAAAGAAGCTCGGCACT CAC-3’, IL-12 p40-R: 5’-CTCTTCTCTGACAGACAGACAC-3’, IL-6-F: 5’-GTCTTCTTGGATGTTACATAG-3’, IL-6-R: 5’-GTCAGATA CCTGCAAGACAG-3’, IL-10-F: 5’-ACTCAATACACACTTGAG TG-3’, IL-10-R: 5’-GGACCTTTAAGGGTTACTTGG-3’, Sepp1-F: 5’-TT CTGCGGCCATCCAGATTG-3’, Sepp1-R: 5’-CACAAGAGCCCA CATCCTG-3’, F13a1-F: 5’-CAGCAAGATAAACGAACATC-3’, F13a1-R: 5’-TGCCCTACATTCTCTGTT-3’, F13b-F: 5’-AGTTG ATCAGCACGAGAG-3’, F13b-R: 5’-GTCATGAGGTTTTGTTT G-3’, IL-10-IFN-γ-F: 5’-GCAAATGGAGCCGTCTGTGC-3’, IL-10-IFN-γ-R: 5’-GTCTTCTTGGATGTTACATAG-3’, TNF-R: 5’-CTGCAGATA CCTGCAAGACAG-3’, Sepp1-F: 5’-ACTCAATACACACTTGAG TG-3’, IL-10-R: 5’-GGACCTTTAAGGGTTACTTGG-3’.

RBC clearance assay

Livers from T. brucei brucei-infected treated mice were isolated on day 8 p.i., cut into pieces, and incubated in RPMI 16400.01% collagenase (Boehringer-Mannheim) for 1 h. After RBC lysis, cells were washed 3 times in complete RPMI 1640 and cultured at 5 \times 10^5/ml in a 96-well culture plate (Nunc). Following 3 h of adherence, cells were washed 3 times with RPMI 1640 supplemented with 2 mM glutamine, 1 mM pyruvate, 50 U/ml penicillin, 50 µg/ml streptomycin and 2 µL sodium bicarbonate, plus 10% FCS (Invitrogen Life Technologies) and overlayed on 10 ml of Lymphoprep (Lucron Bioproducts). After centrifugation (430 \times g, 30 min, 17°C), the layer of low-density cells at the interface containing nonparenchymal cells was harvested.

MACS sorting

CD11b+ cells were isolated from the nonparenchymal cells of the liver by positive CD11b selection on magnetic separation columns according to the manufacturer’s protocol (Miltenyi Biotec) with a purity ranging from 85 to 95%. Cells were counted and used for further analysis by FACS or used for RT-QPCR analysis (Trizol pellets of 3 \times 10^6 cells, stored at −80°C).

Flow cytometry

MACS sorted liver cells from control, GPI-based treated and mock-treated C57BL/6 mice before and at day 8–10 after T. brucei infection, were further tested for purity via FACS. Briefly, starting from a stock solution of 5 \times 10^6 cells/ml, 100 µl was used for labeling. The cells were incubated for 20 min at 4°C with Fc blocking Ab (2.4G2, BD Biosciences) to block nonspecific binding and further surface stained with FITC conjugated rat anti-CD11b (Mac-1) Ab (BD Pharmingen) or FITC conjugated matching control Ab. The cells were washed twice with PBS before analyzing them on FACSCanto II (BD Biosciences), using FACS Diva software (BD Biosciences).

Statistical analysis

The GraphPad Prism software was used for statistical analyses (Student’s t test). Values of p ≤ 0.05 are considered statistically significant.

Results

GPI-based treatment reduces T. brucei brucei-elicited clinical disease manifestations during infection

Clinical manifestation of African trypanosome infections is marked by the occurrence of severe pathology, involving severe weight loss, reduced locomotor activity, serum acidosis and anemia. However, the most prominent feature of pathology is anemia, the major cause of death of infected cattle (32). Studies in malaria and other infectious diseases have shown that TNF plays a crucial role in anemia development (33–35). This pathological symptom was linked to the TNF-inducing capacity of parasite-released GPI molecules (15). Important to mention is that trypanotolerance is comprised of two parameters, 1) the capacity to limit parasite growth and 2) the capacity to reduce anemia development that occurs independent of parasite control (32). Hence, here the influence of GPI-based treatment on the induction of clinical disease severity was analyzed in T. brucei brucei infected mice.

Starting from purified mfVSG of AnTat1.1 trypanosomes, the GPI moiety was isolated. Conventional immunization with this GPI in complete Freund’s adjuvant (CFA), followed by boosting with GPI in incomplete Freund’s adjuvant failed to induce anti-GPI Abs (data not shown) and did not affect the induction of anemia in T. brucei brucei AnTat1.1 infected mice (Table I). Hence, alternative GPI immunization procedures were evaluated by 1) incorporating the GPI moiety into liposomes to increase its delivery and retention, and 2) administrating 8-mercaptopurine (8-MG), a biological response modifier that triggers T cell independent B cell activation, including Ab isotype class switch to IgG (28, 36, 37), to improve the immunogenicity of the GPI at the B cell level. The adopted intraperitoneal (i.p.) immunization schedule included treatment with 8-MG 2 h before administration of GPI liposomes, repeated twice at 3 wk intervals, and was referred to as “GPI-based” treatment (Fig. 1). Control groups included GPI liposome treatment without 8-MG priming (GPI liposome) and empty-liposome treatment with 8-MG (‘mock’)-treated or without 8-MG priming (Liposome).

Two weeks after the second GPI treatment, sera were evaluated for the presence of anti-GPI Abs. Only GPI-based treated mice developed low yet detectable levels of anti-mfVSG and anti-GPI IgG Abs (Fig. 1, a and b, respectively).
Three weeks after the second GPI-based treatment, mice were challenged with *T. brucei brucei* AnTat1.1 and parasitemia, anemia and survival were monitored. As shown in Fig. 2a (one representative of eight individual experiments), there was no significant difference in initial parasite development (first waves of parasitemia) between GPI-based treated and mock-treated infected C57BL/6 mice. Regarding anemia development, Fig. 2, b and c (one representative of eight individual experiments, summarized in Table I) clearly show that the GPI-based treatment resulted in a significant reduction in anemia as compared with mock-treated mice. Hereby it should be emphasized that treatment with 8-MG alone or pretreatment with GPI in the absence of 8-MG had no effect on the anemia development (Table I). In addition GPI-based treatment significantly prolonged the survival of infected mice as compared with mock-treated/infected mice (Fig. 2, d and e, respectively).

As mentioned above, in addition to anemia, experimental trypanosome infections elicit other clinical disease symptoms (30, 38). Fig. 2f shows that while mock-treated mice exhibit serum acidosis during the chronic anemic stage of infection, GPI-based treated mice are rescued from this disease complication. Furthermore, trypanosome infections have been reported to result in severe liver pathology (38), that can be monitored by measuring the levels of serum AST. Indeed, as shown in Fig. 2g, the levels of AST increase dramatically during the chronic stage of infection and GPI-based treatment reduces AST levels to normal levels. Finally, *T. brucei brucei* infections were also documented to result in severe weight loss and impairment of locomotor activity (30). Here again GPI-based treatment significantly reduced these pathological features (Fig. 2, h and i), and as such alleviates infection-associated morbidity throughout the chronic stage of infection.

### Table I. Overview of evaluated GPI and mock treatments in different mouse strains

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Treatment</th>
<th>No. of Expts.</th>
<th>No. of Mice/Expt.</th>
<th>%RBC Reduction (day 18 p.i.)</th>
<th>p Valueb</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57B1/6</td>
<td>CFA/ICFA</td>
<td>2</td>
<td>8</td>
<td>55.0 ± 3.4</td>
<td>&gt;0.5</td>
</tr>
<tr>
<td>C57B1/6</td>
<td>CFA/ICFA/GPI</td>
<td>2</td>
<td>8</td>
<td>56.0 ± 2.9</td>
<td>&gt;0.5</td>
</tr>
<tr>
<td>C57B1/6</td>
<td>Mock</td>
<td>8</td>
<td>32</td>
<td>55.2 ± 3.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>C57B1/6</td>
<td>Lipo</td>
<td>8</td>
<td>32</td>
<td>34.4 ± 8.4</td>
<td>&gt;0.5</td>
</tr>
<tr>
<td>C57B1/6</td>
<td>Lipo/GPI</td>
<td>2</td>
<td>10</td>
<td>54.7 ± 2.5</td>
<td>&gt;0.5</td>
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<tr>
<td>C57B1/6</td>
<td>8-MG</td>
<td>8</td>
<td>32</td>
<td>51.7 ± 1.5</td>
<td>&gt;0.5</td>
</tr>
<tr>
<td>C57B1/6</td>
<td>Mock</td>
<td>8</td>
<td>32</td>
<td>60.3 ± 3.5</td>
<td>&gt;0.5</td>
</tr>
<tr>
<td>B cell−/−</td>
<td>GPI-based</td>
<td>2</td>
<td>6</td>
<td>57.8 ± 2.6</td>
<td>&gt;0.5</td>
</tr>
<tr>
<td>C3H/N</td>
<td>Mock</td>
<td>1</td>
<td>5</td>
<td>35.4 ± 3.3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>C3H/N</td>
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<td>1</td>
<td>5</td>
<td>18.1 ± 2.9</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>C3H</td>
<td>Mock</td>
<td>1</td>
<td>7</td>
<td>28.5 ± 2.3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>C3H</td>
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<td>7</td>
<td>12.1 ± 2.1</td>
<td>&gt;0.5</td>
</tr>
<tr>
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<td>Mock</td>
<td>1</td>
<td>5</td>
<td>33.3 ± 4.2</td>
<td>&gt;0.5</td>
</tr>
<tr>
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<td>5</td>
<td>19.8 ± 3.2</td>
<td>&gt;0.5</td>
</tr>
<tr>
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<td>Mock</td>
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<td>8</td>
<td>40.0 ± 1.7</td>
<td>&gt;0.5</td>
</tr>
<tr>
<td>CD1d−/−</td>
<td>GPI-based</td>
<td>2</td>
<td>8</td>
<td>37.9 ± 2.5</td>
<td>&gt;0.5</td>
</tr>
</tbody>
</table>

a %RBC reduction values are calculated relative to naïve non-infected animals. Results are represented as mean values ± SD.

b p values of results obtained in GPI-treated mice are calculated as compared to mock-treated mice.

Three weeks after the second GPI-based treatment, mice were challenged with *T. brucei brucei* AnTat1.1 and parasitemia, anemia and survival were monitored. As shown in Fig. 2a (one representative of eight individual experiments), there was no significant difference in initial parasite development (first waves of parasitemia) between GPI-based treated and mock-treated infected C57BL/6 mice. Regarding anemia development, Fig. 2, b and c (one representative of eight individual experiments, summarized in Table I) clearly show that the GPI-based treatment resulted in a significant reduction in anemia as compared with mock-treated mice. Hereby it should be emphasized that treatment with 8-MG alone or pretreatment with GPI in the absence of 8-MG had no effect on the anemia development (Table I). In addition GPI-based treatment significantly prolonged the survival of infected mice as compared with mock-treated/infected mice (Fig. 2, d and e, respectively).

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### Efficacy of the GPI-based treatment strategy in other models of African trypanosomiasis

To evaluate the general applicability of the GPI-based treatment in conferring protection against trypanosomiasis-associated immunopathology, similar experiments were performed in different (murine) models of trypanosomiasis. Because *T. congolense* represents a major cause of cattle trypanosomiasis, the protective effect of the...
**FIGURE 2.** GPI-based treatment confers protection during *T. brucei brucei* infection. Parasitemia levels of GPI-based treated (■) and mock-treated (▲) mice (a). Alterations in RBC counts (b) and survival times (d) of GPI-based treated/infected C57BL/6 mice in the presence (■) or absence (▲) of 8-MG are shown. Control data include: alterations in RBC counts (c) and survival times of mock-treated/infected mice (e) in the presence (▲) or absence (▲) of 8-MG. Results are presented as mean % values of 10 individual mice ± SD compared with preinfection data. Alterations in serum pH (f) and AST levels (g) as well as changes in total body mass (h) and locomotor activity (i) were recorded for GPI-based treated (black bars) and mock-treated (white bars) *T. brucei brucei*-infected C57BL/6 mice, as well as non-treated/infected mice (gray bars). Three mice per experimental group were used, and the results are expressed as means ± SD.

*T. congolense* GPI-based treatment was evaluated in infections with the well documented (39) Tc13 *T. congolense* parasites. To this end protection experiments in *T. congolense* infected C57BL/6 mice were conducted with *T. congolense* derived GPI. As shown in Fig. 3a, the *T. congolense* GPI-based treatment reduced drastically the level of anemia and caused a significant prolongation of the mean survival time (MS: 155 ± 12 days for GPI-based treated vs 126 ± 5 days for mock-treated, *p* value: 0.014). A similar
treatment was tested for another unrelated trypanosome species namely *T. evansi* (ITMAS 110297, code KETRI 2480) and here also a *T. evansi* GPI-based treatment impaired the development of anemia and significantly prolonged the lifespan of *T. evansi*-infected mice (Fig. 3b) (MS: 48 ± 5 days for GPI-based treated vs 22 ± 5 days for mock-treated, *p* value: 0.001). Finally, also in a tsetse fly transmitted trypanosome infection (AnTAR1) GPI-based treatment protected mice against the development of anemia and resulted in a significant prolongation in survival time (Fig. 3c) (MS: 33 ± 3 days for GPI-based treated vs 24 ± 3 days for mock-treated, *p* value: 0.02).

Collectively these results show that pretreatment with the VSG-derived GPI moiety alleviates the development of anemia in different experimental models of African trypanosomiasis.

**FIGURE 3.** GPI-based treatment confers protection in different trypanosome infection models. Alterations in RBC counts and survival times of GPI-based treated (■) or mock-treated (□) infected C57BL/6 mice are shown. *a*, *T. congolense* GPI-based treatment and challenge; *b*, *T. evansi* KETRI 2480 GPI-based treatment and challenge; *c*, AnTat1.1 GPI-based treatment followed by tsetse-transmitted *T. brucei* AnTAR1 challenge. Results are presented as mean % values of five individual mice ± SD compared with preinfection data.

**FIGURE 4.** RBC clearance by AdLC of *T. brucei brucei* infected C57BL/6 mice. *a*, RBC clearance by AdLC isolated from GPI-based treated or mock-treated mice, before (white bars) or during infection (black bars) with *T. brucei brucei* (day 8). Liver cells were incubated overnight with RBC isolated from *T. brucei brucei* infected mice (RBCi) in a ratio of 1:10. RBC clearance was expressed as mean % clearance (± SD) of three independent experiments compared with the initial RBC number. In an alternative experimental setup, a RBCi monolayer was cultured overnight on AdLC from mock-treated/infected mice (*b*), GPI-based treated/infected mice (*c*), or noninfected mice (*d*). RBCi clearance was visualized as plague formation under a light microscope.

The *T. brucei brucei* GPI-based treatment reduces macrophage-mediated RBC destruction

As extravascular RBC destruction and liver macrophage-mediated phagocytosis of RBCs have both been proposed as mechanisms underlying anemia (40), RBC clearance was analyzed in an adherent liver cell coculture system. As shown in Fig. 4a, when RBC isolated from *T. brucei brucei* infected mice (RBCi) were exposed to the adherent liver cell fraction isolated from infected mice at day 8 of infection, i.e., at the time point whereupon anemia appears in vivo, they were efficiently cleared by adherent liver cells from mock-treated/infected mice. In contrast, adherent liver cells from GPI-based treated/infected mice had significantly reduced RBCi clearance activity. In an alternative experimental setup, the observed differences in RBC clearance activity were visualized by light microscopy analysis of the integrity of an RBCi monolayer.
after 24 h incubation in the presence of adherent liver cells. Here, adherent cells from mock-treated and infected mice destroyed the RBCi monolayer, resulting in clearance plagues throughout all microscopy fields analyzed (Fig. 4b). In contrast, the number of plagues caused by adherent liver cells from GPI-based treated/infected mice was clearly reduced (Fig. 4c). Incubation of adherent liver cells from noninfected mice with an RBCi monolayer did not result in plague formation (Fig. 4d).

GPI-based treatment alleviates anemia in a B cell independent manner

To assess whether the alleviation of anemia development by the GPI-based treatment is Ab dependent, B cell deficient C57BL/6 mice were treated as described above. According to the results (Table I) B cell deficient mice also develop anemia, indicating that Abs themselves play no significant role in anemia development. Furthermore

<table>
<thead>
<tr>
<th>Vaccination Scheme</th>
<th>Postpeak (day 8) (pg/ml)</th>
<th>Reduction/Increase (%)</th>
<th>p Value</th>
<th>30 Days p.i. (pg/ml)</th>
<th>Reduction/Increase (%)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TFN</td>
<td>No vacc.</td>
<td>270 ± 20</td>
<td>−15.1</td>
<td>50 ± 12.5</td>
<td>−15.4</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td></td>
<td>Mock</td>
<td>229.2 ± 15</td>
<td>−73.7</td>
<td>42.3 ± 21.6</td>
<td>−58.4</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td></td>
<td>GPI-based</td>
<td>63.1 ± 9.8</td>
<td>0.0001</td>
<td>20.8 ± 7.91</td>
<td>−76.3</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>IL-6</td>
<td>No vacc.</td>
<td>1830 ± 125</td>
<td>0.125</td>
<td>1560 ± 125</td>
<td>0.125</td>
<td>0.125</td>
</tr>
<tr>
<td></td>
<td>Mock</td>
<td>1292 ± 170</td>
<td>−29.4</td>
<td>1107.6 ± 22</td>
<td>−29.0</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td></td>
<td>GPI-based</td>
<td>375.6 ± 50.6</td>
<td>&lt;0.001</td>
<td>370.23 ± 50</td>
<td>&lt;0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>IL-10</td>
<td>No vacc.</td>
<td>200 ± 20</td>
<td>72 ± 9.8</td>
<td>72 ± 9.8</td>
<td>72 ± 9.8</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td></td>
<td>Mock</td>
<td>188.53 ± 20</td>
<td>−57.7</td>
<td>670.6 ± 6.0</td>
<td>−6.9</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td></td>
<td>GPI-based</td>
<td>270.3 ± 12</td>
<td>−35.7</td>
<td>117 ± 7.69</td>
<td>6.25</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

For all cytokines measured, 5 individual mice were used. Results are represented as mean values ± SD. Non-infected animals do not have detectable levels of any cytokine tested. 

p values are calculated as compared to mock-treated mice.

FIGURE 5. Modulation of T. brucei brucei infection-induced inflammation after GPI-based treatment. a, TNF secretion by adherent peritoneal cells from GPI-based treated (black bars) or mock-treated (white bars) T. brucei brucei infected C57BL/6 (left panel) and TLR4−/− (right panel) mice restimulated in vitro at day 10 p.i. with sVSG or LPS. TNF secretion by adherent peritoneal cells from naive C57BL/6 mice is shown in gray bars. Five mice were used per experimental group. b, Survival of GPI-based treated (●) or mock-treated T. brucei brucei infected C57BL/6 mice (□) injected i.p. at day 6 p.i. with 0.5 μg of LPS, the LD₉₀ dose for infected GPI-based treated mice. Control mice injected with the same LPS LD₉₀ dose are represented by a gray box. Five mice were used per experimental group, and results are representative of one of two similar experiments. c, TNF, IL-6, IL-12p40, and IL-10 gene expression levels were quantified by real-time PCR on adherent peritoneal cells from GPI-based treated (black bars), mock-treated (white bars) T. brucei brucei infected C57BL/6 mice at day 10 p.i. Gene expression levels of GPI-based treated and mock-treated noninfected C57BL/6 mice are represented as gray bars. Fold induction gene expression was normalized using s12, relative to noninfected mice. Five mice were used per experimental group, and the results are expressed ± SD.
GPI-based treatment alleviates anemia in B cell deficient mice. Hence, although anti-GPI Abs were raised by this treatment, the effect of GPI-based treatment on anemia is Ab-independent, indicating that most likely cellular immune components are involved.

**GPI-based treatment reduces** *T. brucei* brucei-elicited type I inflammation mediated by macrophages

Although it is well accepted that African trypanosome infections results in macrophage activation, the status of macrophage activation however may influence the outcome of the disease. Indeed, the sequential activation of classically activated macrophages (caMφ) in a type I cytokine environment at the beginning of infection and important to control the infection, followed by activation of alternatively activated macrophages (aaMφ) in a type II cytokine environment during the late/chronic stage of infection results in the increased resistance against *T. brucei brucei* infections (41). However, persistence of a type I cytokine environment by caMφ during chronic phase of infection may lead to pathology. This could be attributed to the enhanced macrophage activation resulting in an enhanced erythrophagocytosis (destruction of RBC) and finally leading to anemia development.

To assess the influence of GPI-based treatment on the inflammatory/anti-inflammatory cytokine balance of *T. brucei* infected mice serum levels of TNF, IL-6 and IL-10 were analyzed on day 8 (early) and 30 (late) of infection. As shown in Table II, GPI-based treatment reduced the circulating levels of the inflammatory cytokine TNF during both the early and chronic late stages of infection, as compared with mock-treated mice. The same tendency was observed for serum levels of IL-6. In contrast, GPI-based treatment significantly increased serum levels of the anti-inflammatory cytokine IL-10, during both the early and late stages of infection. These results suggest that the GPI-based treatment tempers infection-elicited inflammatory type I response possibly by interfering with macrophage activation.
To analyze the effect of the GPI-based treatment on infection-associated TNF-secretion by macrophages, adherent peritoneal cells, isolated at day 10 p.i., were stimulated in vitro with either sVSG or LPS. As shown in Fig. 5a (left panel), GPI-based treatment induced a significant down-regulation of TNF secretion by sVSG or LPS-pulsed adherent peritoneal cells suggesting that this treatment desensitizes the responsiveness of infection-associated macrophages toward inflammatory components. This macrophage desensitization was not attributed to the contamination of the GPI liposome preparation with LPS, because both in TLR4−/− as well as TLR2/4−/− mice this GPI-based treatment followed by a T. brucei brucei challenge resulted in a significant impairment of anemia development (Table I). Furthermore, in vitro restimulation of adherent peritoneal cells from TLR4−/− mice at day 10 p.i. with sVSG confirmed that the macrophage desensitization by GPI-based treatment did not rely on potential LPS contamination (Fig. 5a, right panel). Another connotation of this observation is that the desensitization by the GPI-based treatment does not involve a TLR4 signaling pathway. To test whether this state of desensitization was of any in vivo relevance, LPS-induced mortality, a read-out for trypanosome elicited macrophage activation in vivo (30), was compared in GPI-based treated and mock-treated infected mice. According to Fig. 5b GPI-based treated mice exhibited a LD50 LPS-dose of 0.5 μg, when injected i.p. at day 6 of T. brucei brucei infection. In contrast, in mock-treated infected mice this LPS-dose caused 100% mortality within 24 h after injection. Together these data show that GPI-based treatment resulted in a desensitization of macrophages toward sVSG and LPS during infection, in vitro as well as in vivo.

To further analyze the effect of GPI-based treatment on infection-associated macrophage activation, mRNA levels of inflammatory cytokines (TNF, IL-6, IL-12p40) and the anti-inflammatory cytokine IL-10 were analyzed in different adherent peritoneal cell populations (see Fig. 5). As shown in Fig. 5c, adherent peritoneal cells from GPI-based treated/infected mice showed a reduced TNF, IL-6, IL-12p40 expression and a clear up-regulation of IL-10 expression as compared with mock-treated/infected mice. In this context, it is important to mention that the GPI-based treatment or mock-treatment itself did not induce any significant alteration in the expression of the genes tested in the absence of a trypanosome challenge (Fig. 5c).

**GPI-based treatment modulates the macrophage activation state during T. brucei infection**

Given that 1) the liver is the major organ of parasite clearance and RBC-destruction, 2) liver injury is a very important pathological complication during infection (42), and 3) GPI-based treated/infected mice exhibit reduced anemia and liver damage (AST levels), the macrophage activation state in this organ was investigated. First we evaluated gene-expression and protein secretion of liver-derived CD11b+ cells to confirm the cytokine profiles observed in the adherent peritoneal cell populations (see Fig. 5). As shown in Fig. 6, a and b, the GPI-based treatment results in decreased TNF and increased IL-10 production, both at gene-expression and protein secretion level. Next, purified liver-derived CD11b+ cells were thoroughly analyzed in terms of gene expression of aM0 markers (43). RT-QPCR analysis revealed higher expression levels of genes associated with protection against oxidative burst (Sepp (44)), tissue repair and wound healing (F13a1, (45)), prevention of apoptosis (Psap, (46)) and down-regulation of inflammation (Mrc1, (47) in GPI-based treated/infected mice as compared with mock-treated/infected mice (Fig. 6c). Collectively, these data indicate that GPI-based treatment triggers the development of aM0.

The protective effect of the GPI-based treatment during T. brucei brucei infection involves a CD1d-mediated pathway

It is well documented that glycolipids, including GPI’s from T. brucei, can be presented in the context of CD1d, expressed on APCs, resulting in the activation of CD1d-restricted T cells (27, 48, 49). Therefore, the possible involvement of the CD1d-mediated pathways in the protective effects observed in GPI-based treated/infected animals was envisaged and evaluated using CD1d−/− mice. As shown in Table I, the protective effect of the GPI-based treatment, with respect to alleviation of anemia, was abrogated (%RBC reduction at day 18 p.i. in GPI-based treated 37.9 ± 2.5% and mock-treated 40.0 ± 1.7%), confirming a possible involvement of the CD1d-pathway.

**Discussion**

During parasitic infections, we and others have shown that pathogen derived GPI is involved in TNF induction (10–12), hereby playing a major role in induction of pathology and the clinical manifestation of infection (50, 51). Early on after the discovery of the structure of the GPI from trypanosome VSG, GPI anchors were described in plasmodium (52, 53) and identified as the malarialxin capable of inducing TNF production when added to macrophage cultures (54). Detailed analysis of the functional structure of plasmodium GPI, in the context of macrophage activation, lead to the identification of two distinct structural elements, the carboxylate and lipid moieties respectively (20). Although the glycan core was found to activate protein tyrosine kinase, the diacylglycerol moiety was proposed to function as a second messenger for protein kinase C activation (20). Together, these GPI-triggered signal pathways induce the secretion of proinflammatory cytokines including TNF as well as IL-1 and IL-6 (55, 56). Hereby the induction of TNF by plasmodium GPI in macrophages required recognition of the distal fourth mannose residue of the glycan core (57). Accordingly, interfering with this recognition can inhibit the macrophage-activating potential of the GPI moiety. In this vein a successful vaccination strategy was developed by Schofield et al. (19) based on a synthetic carbohydrate moiety that mimics the plasmodium GPI core. Indeed, conventional immunizations using CFA with this synthetic component resulted in the production of neutralizing anti-GPI Abs and those Abs were shown to be able to neutralize the TNF induction by macrophages exposed to plasmodium extracts. Furthermore in challenge experiments, this vaccination conferred partial protection against malarial pathogenesis.

Based on the documented role of the GPI moiety of VSG in trypanosomiasis-associated pathology (12), an anti-trypanosome GPI intervention strategy was envisaged to protect against sleeping sickness. In contrast to plasmodium GPI, trypanosome GPI lacks a fourth α-mannose but has a galactose modification linked to the first mannose which is essential for TNF induction (12). However, the presence of an inducible endogenous trypanosome phospholipase C (PLC) enzyme hampers an intervention approach based solely on the carbohydrate core. Indeed, upon external stress, trypanosomes cleave most of their VSG by using their PLC, releasing sVSG that harbors the GPI carbohydrate moiety and leaving the GPI dimeristoylglycerol (DMG) lipid anchor in the membrane (58). Hence, while inhibition of plasmodium GPI could be achieved through the generation of neutralizing Abs recognizing the carbohydrate core, such approach will not affect or neutralize the DMG lipid-moiety that accounts for induction of LPS-hypersensitivity during trypanosome infections (12). Therefore in this study uncleaved VSG-derived GPI encompassing the glycan and lipid moieties, obtained through the chemical inhibition of the trypanosome PLC was used. To increase the retention, delivery and
immunogenicity of the GPI molecule a new intervention strategy based on liposome encapsulation combined with the immunomodulator 8-mercaptopoguanosine was developed. This GPI-based treatment resulted in a significant protection against host clinical manifestations of T. brucei-induced pathology like anemia, acidosis, serum AST levels, weight loss and locomotor activity, without influencing initial parasite development.

In addition, this GPI-based treatment was found to be successful in preventing the development of anemia in other more relevant infectious trypanosome systems, like T. congolense and T. evansi. Moreover even in a more natural system, using tsetse fly transmission, the GPI-based treatment resulted in significant protection against anemia development.

Surprisingly, although anti-GPI Abs were raised by the GPI-based treatment strategy, the efficacy of GPI-based treatment at the level of anemia did not rely on the humoral immune responses as GPI-based treated, infected B cell deficient mice also exhibited similar protection as compared with GPI pre-exposed and infected wild-type C57BL/6 mice. Therefore, the protective effects observed in our GPI-based treatment intervention strategy might rely on a different mechanism than that one proposed by Schofield et al. (19) in the malaria GPI vaccination model, where Abs were suggested to be crucial for the protective effects. This could be due to a difference in the Ag used. Indeed, while in the case of malaria a potential involvement is suggested, in our studies the whole GPI was administered including its macromodulator 8-mercaptoguanosine was used, in the present studies the whole GPI was administered including its macrophage modulating components.

Hence, we propose that the success of the GPI-based treatment intervention strategy relies on an attenuation of parasite-elicited type I inflammation, in particular at the level of macrophage activation. Collectively our results are highly supportive for this assumption. First, GPI-based treatment shifted the balance of (pro-)inflammatory cytokines TNF and IL-6 toward IL-10 in the serum during infection. Second, GPI-based treatment influenced the macrophage activation state elicited during the early phase of African trypanosome infections. At this stage macrophages are primed by IFN-γ, and further triggered by trypanosome released sVSG/mfVSG (12, 59) and possibly by LPS. Indeed, LPS is detectable in the serum of trypanosome infected animals, presumably due to ongoing gut-inflammation and bacterial leakage (60). Together, this leads toward a hyperactive classical activation state of macrophages (caMφ or M1), prone to produce massive amounts of TNF (12, 61). Such caMφ were found herein to be desensitized by the GPI-based treatment as evidenced by their impaired capacity to produce TNF upon triggering with sVSG or LPS. Moreover, GPI-based treated T. brucei brucei infected mice were found to be more resistant to LPS-mediated lethality. This desensitization, or as some may refer to as tolerance, was not due to a potential LPS contamination of the GPI liposome preparation because GPI-based treated, T. brucei brucei infected TLR4−/− as well as TLR2/4−/− mice were found to be protected against anemia development (see Table I).

Furthermore, at the mRNA level, macrophages from GPI-based treated/infected animals exhibited a reduced TNF, IL-6 and IL-12p40 during the course of infection, because dendritic cells as well as adipocytes were reported to be potent producers of proinflammatory cytokines in response to TLR ligands (63, 64).

Though the mechanisms underlying this macrophage reprogramming are so far not determined, at least one possibility is worthwhile to consider. As demonstrated by Coller et al. (59) the timing of macrophage exposure to the GPI moiety of VSG may determine the inflammatory response to trypanosome infections. Indeed while macrophage activation by IFN-γ, mimicking early priming during trypanosome infections, favors the expression of TNF, IL-1β and IL-12p40 in response to GIPs, i.e., the GPI anchor substituent associated with sVSG (sVSG/GIP), treatment with sVSG/GIP before IFN-γ stimulation resulted in a marked reduction of IFN-γ-induced responses such as iNOS. The inhibitory activity of GPI-related molecules on macrophage activation was also reported for Leishmania major, where a glycoinositol-phospholipid was found to inhibit the synthesis of IFN-γ-dependant NO production (65). According to our results, macrophage reprogramming through GPI pre-exposure, impacts on macrophage- and TNF-mediated clinical disease symptoms such as anemia, serum acidosis, liver damage, and weight loss. As severe anemia is considered the most important cause of death in natural T. brucei brucei as well as T. congolense infections, this parameter of pathology was analyzed in more detail. Because erythrophagocytosis was documented to contribute to extensive extra-vascular destruction of RBCi during African trypanosomiasis (66), the protective effect of GPI pre-exposure on infection-associated anemia might again reflect a modulation of the macrophage activation state. Indeed, while adherent liver cells from infected mice destroy RBCi in vitro, GPI-based treatment greatly reduced this activity. Therefore, switching from a caMφ state during early state of infection to a more aaMφ state during the course of infection, via the GPI-based treatment, could play a role in reduced development of anemia.

In conclusion, the results presented herein show that GPI-based treatment alleviates infection-associated pathology during African trypanosomiasis, resulting in an increased lifespan of the infected host. This intervention strategy reduced the inflammatory immune response during infection, resulting in a desensitization and alternative activation of macrophages. These aaMφ could play a cardinal role in the GPI-based treatment, as these cells produce high levels of IL-10 and express genes potentially involved in anti-inflammation and tissue repair. Finally, the CD1d signaling pathway was found to be crucial for the beneficial effects of the GPI-based treatment, suggesting a link between CD1d signaling and development of aaMφ.

Acknowledgments
We thank Dr. G. Raes, Dr. A. Beschin, and Prof. Dr. F. Brombacher for their helpful advice and comments on the manuscript. This work was supported by the kind technical assistance of Ella Omasta, Marie-Thérèse Detobel, and Els Maes.

Disclosures
The authors have no financial conflict of interest.

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