Neutralization of Malaria Glycosylphosphatidylinositol *In Vitro* by Serum IgG from Malaria-Exposed Individuals[▽]

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Parasite-derived glycosylphosphatidylinositol (GPI) is believed to be a major inducer of the pathways leading to pathology and morbidity during *Plasmodium falciparum* infection and has been termed a malaria “toxin.” The generation of neutralizing anti-GPI (“antitoxic”) antibodies has therefore been hypothesized to be an important step in the acquisition of antidiisease immunity to malaria; however, to date the GPI-neutralizing capacity of antibodies induced during natural *Plasmodium falciparum* infection has not been evaluated. Here we describe the development of an *in vitro* macrophage-based assay to assess the neutralizing capacity of malarial GPI-specific IgG. We demonstrate that IgG from *Plasmodium falciparum*-exposed individuals can significantly inhibit the GPI-induced activation of macrophages *in vitro*, as shown by reduced levels of tumor necrosis factor production and attenuation of CD40 expression. The GPI-neutralizing capacity of individual IgG samples was directly correlated with the anti-GPI antibody titer. IgG from malaria-exposed individuals also neutralized the macrophage-activating effects of *P. falciparum* schizont extract (PfSE), but there was only a poor correlation between PfSE-neutralizing activity and the anti-GPI antibody titer, suggesting that PfSE contains other macrophage-activating moieties, in addition to GPI. In conclusion, we have established an *in vitro* assay to test the toxin-neutralizing activities of antimalarial antibodies and have shown that anti-GPI antibodies from malaria-immune individuals are able to neutralize GPI-induced macrophage activation; however, the clinical relevance of anti-GPI antibodies remains to be proven, given that malarial schizonts contain other proinflammatory moieties, in addition to GPI.

Human *Plasmodium falciparum* infection presents as a spectrum ranging from mild febrile disease to severe disease and death. Many of the severe symptoms of malaria, including severe anemia and cerebral malaria, are believed to be either induced or exacerbated by proinflammatory cytokines released as part of the antiparasitic immune response (24, 25; reviewed in references 9 and 38). One of the best-characterized triggers of the innate immune system during *P. falciparum* infection is the exoantigen glycosylphosphatidylinositol (GPI), the membrane anchor of multiple parasite surface proteins (reviewed in references 6 and 18). GPI is released from rupturing, infected red blood cells at schizogony (3, 23, 30); activates CD36- and Toll-like receptor 2 (TLR2)- and TLR4-dependent signaling cascades; and induces tumor necrosis factor (TNF), interleukin-12 (IL-12), IL-6, and NO production from macrophages *in vitro* (reviewed in reference 6; 22, 34, 49). Injection of GPI into rodents induces pathologies comparable to those observed during acute *P. falciparum* infection, including pyrexia and hypoglycemia (15, 39), and immunization with GPI reduces the inflammation associated with acute *P. berghei* infection in mice (40). Accordingly, it has been suggested that GPI is an impor-

tant inflammatory mediator during *P. falciparum* infection and that the immune responses induced specifically by *P. falciparum* GPI may be involved in the development of severe malarial disease (7). As a consequence, the acquisition of antibodies that dampen the response to GPI may represent an important element of malarial antidiisease immunity.

In agreement with this hypothesis, high levels of anti-GPI antibodies correlated with resistance to anemia and fever in Kenyan children (28), and in Senegal, lower levels of anti-GPI antibodies were found in individuals with cerebral malaria than in individuals with uncomplicated malaria (35). However, no significant differences in anti-GPI antibody responses were observed between Gambian children with severe (mainly cerebral) malaria or mild malaria (14), high levels of anti-GPI antibodies were reported in Malian children with cerebral malaria (8), and no association was observed between anti-GPI antibodies and the ability of Papua New Guinean children to tolerate high-density parasitemia (5).

These conflicting data suggest that there is no simple relationship between the titers of circulating antibodies to GPI and clinical immunity to malaria. We considered two possible explanations for this: that GPI may not be the only—or even the major—malaria toxin and/or that antibodies which bind to GPI in an enzyme-linked immunosorbent assay (ELISA; as used in all the clinical studies to date) may not necessarily neutralize its activity. In support of the former hypothesis, at least four other proinflammatory components of *P. falciparum* schizonts

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have been described, namely, hemozoin (the insoluble breakdown product of hemoglobin which accumulates inside parasitized red blood cells) (11, 41), parasite membrane-derived microparticles (12), protein-DNA complexes (48), and uric acid (31). In support of the latter argument, the 4th mannose moiety of the glycan region has been shown to be critical for the activation of macrophages (46), suggesting that antibodies that target different epitopes of the GPI molecule may have different neutralizing capacities (reviewed in reference 18; 29).

To begin to distinguish between these two possibilities, we have developed an *in vitro* neutralization assay to determine whether purified IgG from *P. falciparum*-exposed individuals can inhibit GPI- or *P. falciparum* schizont extract (PfSE)-induced macrophage activation. We find that ELISA-based anti-GPI antibody titers correlate strongly with the ability of the IgG to neutralize GPI-induced TNF production and CD40 upregulation on macrophages, suggesting that the failure of some anti-GPI antibodies to neutralize GPI is an unlikely explanation for the disparate clinical findings. Interestingly, the anti-PfSE antibody titers measured by ELISA correlated strongly with the neutralization of PfSE-induced macrophage activation; but anti-PfSE and anti-GPI ELISA titers were poorly correlated, as were anti-GPI and anti-PfSE neutralizing capacities, indicating that GPI may not be either the primary immunogenic component of PfSE or the primary trigger for macrophage activation. These data support the notion that the malaria "toxin" is a complex mixture of proinflammatory molecules; understanding the relative activities of known—and possibly novel—toxic parasite products should facilitate the development of antidisease/antitoxic vaccines and adjunct therapies.

MATERIALS AND METHODS

Human sera and study subject description. Serum samples were obtained during the long dry (low malaria transmission) season from 33 putatively immune, healthy, *P. falciparum*-uninfected (i.e., blood film-negative) adult blood donors from a rural village in The Gambia, West Africa (37). All of these individuals were adults who had lived their entire lives in this community, where malaria is endemic. None of the individuals had experienced clinical malaria for a number of years, indicating that they had developed clinical and/or antiparasitic immunity to malaria. Control sera were obtained from six malaria-nonexposed volunteers from the United Kingdom. The study was approved by the MRC/Gambia Government Ethical committee and by the ethical review committee of LSHTM.

Testing of serum samples for endotoxin. Endotoxin contamination of serum samples was assessed by the *Limulus* amoebocyte lysate (LAL) gel formation assay, performed according to the manufacturer's instructions (National Institute for Biological Standards, United Kingdom). Briefly, 10 μ l of LAL was added to 10 μ l test serum diluted in endotoxin-free water and spotted onto a sterile, template-marked, 9-cm petri dish. After incubation for 1 h at 37°C, 1 μ l of 0.2% new methylene blue was added to each droplet (lysate plus test serum). A positive result was indicated by an immiscible area of blue dye on the droplet, indicative of gel formation. The level of contamination was determined by comparison of the results with those of a parallel assay using endotoxin standards ranging from 0.008 to 10 international units (IU)/ml. The sensitivity of the assay was 0.03 IU endotoxin per ml.

Endotoxin removal and purification of IgG by protein G chromatography. Sera were diluted 1:5 in phosphate-buffered saline (PBS) and incubated with polymyxin B-coated agarose beads at a concentration of 100 to 200 μ l beads/ml serum (Sigma, United Kingdom). Sera were then filtered (pore size, 0.2 μ m; Millipore) and checked for endotoxin contamination (by LAL assay) before application to a HiTrap protein G column (Amersham Biosciences, United Kingdom). The eluted IgG fractions were pooled and dialyzed extensively against PBS, before and after filtration through an Amicon filter (Fisher Scientific, United Kingdom) to concentrate IgG into a volume equivalent to the starting

serum volume. IgG protein concentrations were estimated by spectrometry (A_{280}).

***P. falciparum* schizont preparations.** *P. falciparum* parasites of the 3D7 strain were grown as described previously (1). Mature schizonts were harvested by centrifugation through 60% Percoll (Sigma) and freeze-thawed three times in liquid nitrogen, followed by ultrasonication to produce PfSE, which was stored at -70°C until it was used. Control extracts were prepared identically using uninfected red blood cells. Cultures were routinely shown to be *Mycoplasma* free by PCR (Stratagene, United Kingdom).

Isolation, purification, and quantification of GPIs. GPIs were isolated and purified by high-performance liquid chromatography (HPLC), as described previously (28). Briefly, *P. falciparum* cultures at 20 to 30% parasitemia were harvested, and the parasites were released by treatment with 0.05% saponin in Trager's buffer (56 mM NaCl, 59 mM KCl, 1 mM NaH₂PO₄, 10 mM K₂HPO₄, 11 mM NaHCO₃, 14 mM glucose, pH 7.4). The erythrocyte membrane fragments were removed from the parasites by density centrifugation on cushions of 5% bovine serum albumin in Trager's buffer, and the purified parasites were lyophilized. To monitor GPIs during the isolation and purification steps, the parasite preparation was mixed with an aliquot of parasites metabolically labeled with [³H]glucosamine and lyophilized. The dried parasites were extracted with chloroform-methanol (2:1, vol/vol), followed by chloroform-methanol-water (10:10:3, vol/vol/vol). The latter organic extract, which contained the GPIs, was dried and the residue was partitioned between water and water-saturated 1-butanol. The 1-butanol layer was dried and the GPIs were purified by reversed-phase HPLC using a C₄ Supelcosil column (4.6 by 250 mm, 5- μ m particle size). The purities of several batches of GPIs prepared by this procedure were >95%, as judged by mass spectrometry (28). The purified GPIs were quantitated by determining their mannose and glucosamine contents by high-pH anion-exchange HPLC of the aliquots of samples hydrolyzed with 2.5 M trifluoroacetic acid (TFA) at 100°C for 4 h or 3 M HCl at 100°C for 3 h (19). Data from this analysis confirmed the presence of mannose and glucosamine (both diagnostic of GPIs) as the main constituents following TFA and HCl hydrolysis, respectively (22). For use in neutralization assays, *P. falciparum* GPI was reconstituted with ethanol-endotoxin-free water-1-propanol at a ratio of 78:20:2 (vol/vol/vol).

Anti-GPI and anti-PfSE antibody assay. Anti-GPI and anti-PfSE IgG antibodies were detected by ELISA on *P. falciparum* GPI-coated or PfSE-coated Maxisorp (Nunc, Denmark) microtiter plates, respectively, as described previously (14, 27). The anti-GPI and anti-PfSE titers in the purified IgG preparations were measured (see above).

Briefly, plates were coated with 1 ng GPI or the PfSE equivalent of 5×10^3 schizont-infected red blood cells per well. IgG samples were diluted in TBS-casein with 0.05% Tween 20 (for anti-GPI assays) or PBS-2% dried skimmed milk with 0.05% Tween 20 (for anti-PfSE assays) before addition to plates. Specific IgG binding was detected with horseradish peroxidase (HRP)-conjugated rabbit anti-human IgG with H₂O₂ as the substrate and *o*-phenylenediamine as the chromogen. All samples were tested in triplicate, and all assays were repeated at least once. Detection of IgG isotypes was carried out using murine antibodies to human IgG1 (Skybio, United Kingdom) or IgG3 (Serotec, United Kingdom) and antimouse-HRP conjugate. The optical density values at a 1:100 dilution were converted to midpoint titers using fitted sigmoid curves derived from hyperimmune sera.

GPI and PfSE neutralization assays. The RAW264.7 murine macrophage cell line (European Collection of Cell Cultures [ECACC]; Sigma), which secretes TNF in the presence of *Plasmodium falciparum* schizont extracts or GPI (40), was used for the GPI and PfSE neutralization assays. This cell line has previously been used to assess neutralization of toxic shock syndrome toxin 1 (TSS-1) by anti-human TSS-1 antibodies *in vitro* (20). Purified IgG (100 μ l at 0.1 mg/ml) was incubated with 2 μ l of GPI (1 to 5 μ g/ml, depending on the experiment) or PfSE (50 μ l at 10⁷/ml schizont equivalents) in triplicate in a sterile 96-well tissue culture plate (Nunc) for 1 h at 37°C with gentle mixing. Following the 1-h incubation, 5×10^4 RAW264.7 cells were added to each well, and after incubation for 24 h (37°C, 5% CO₂) the supernatants were collected and assayed for TNF production. Positive-control cultures contained 100 μ l of RPMI rather than IgG; negative-control cultures contained IgG but no GPI.

Macrophage culture supernatants were assayed by standard capture ELISA using Immunolon 4 HBX plates (ThermoLabsystems United Kingdom) coated with monoclonal hamster antibody against murine TNF (TN3; a gift from Celltech, Slough, United Kingdom). Bound TNF was detected with a biotinylated goat anti-mouse TNF antibody (R&D, United Kingdom), streptavidin peroxidase (Sigma), and *o*-phenylenediamine (Sigma). Recombinant murine TNF (R&D) was used as a standard. Each supernatant was tested in duplicate, and all assays were repeated at least twice. Specific TNF release from each culture well was calculated by subtracting the appropriate background values, and TNF

values were calculated as a percentage of that released by the positive (PfSE or GPI) control.

TNF neutralization was calculated according to the following formula: % neutralization = $100 - [(A/B) \times 100]$, where A is the specific amount of TNF released after preincubation with test IgG [(mean amount of TNF released by RAW264.7 cells incubated with IgG plus GPI or PfSE) - (mean amount of TNF released by RAW264.7 cells incubated with IgG alone)], and B is the specific amount of GPI-induced or PfSE-induced TNF released for the positive control [(mean amount of TNF released by RAW264.7 cells incubated with either GPI or PfSE) - (mean amount of TNF released by RAW264.7 cells incubated in medium alone)].

Flow cytometry. At the end of some neutralization experiments, the macrophage monolayer was harvested by gentle scraping for analysis of surface marker characteristics. Cells were stained with phycoerythrin-conjugated anti-mouse F4-80 (clone BM8; E-Bioscience, San Diego, CA), to assess the proportion of cultured cells that were *bona fide* macrophages (routinely, >90%), and antigen-presenting cell-conjugated anti-CD40 (clone 1C10; E-Bioscience) and analyzed by flow cytometry (FACS Calibur instrument; BD).

Statistical analysis. Neutralization of the GPI-induced or PfSE-induced TNF induction by individual IgG preparations was regarded to be specific if it exceeded the mean plus 2 standard deviations (SDs) of the values for the European controls (for GPI, 13.1 ± 8.0 and cutoff = 21.1%; for PfSE, 13.9 ± 8.7 and cutoff = 23.6%). Samples giving values below these cutoff values were classified as "nonneutralizers." Associations between the neutralization of GPI-induced TNF production and the anti-GPI or anti-PfSE titer and the neutralization of PfSE-induced TNF production and the anti-PfSE or anti-GPI titer were analyzed by linear regression of the individual neutralization measurements, allowing repeated measures per person by normally distributed random effects. Regression analyses were carried out with the SAS program (version 8.1; SAS Institute Inc.).

RESULTS

Anti-GPI antibody titers in malaria-exposed individuals.

Sera from 33 healthy, uninfected malaria-exposed immune adults from The Gambia and 6 nonexposed controls from the United Kingdom were assayed for anti-GPI IgG (total), IgG1, and IgG3 (Fig. 1). The midpoint anti-GPI antibody (total IgG) titers in the Gambian IgG samples ranged from 1/4 to 1/125 (mean titer $\pm$ standard error of the mean [SEM], 20.5 ± 4.5), whereas the anti-GPI titers in the control IgG samples from the United Kingdom were no higher than the values in the PBS-treated samples (background) (midpoint titer range, 1/2 or 1/4; mean titer $\pm$ SEM, 2.7 ± 0.2) (Fig. 1a). These data show that anti-GPI antibodies are found in malaria-exposed individuals and that minimal levels of natural or cross-reactive GPI-binding IgG are found in non-malaria-exposed individuals. Consistent with previous findings (4), anti-GPI antibodies were a mixed population of both IgG1 and IgG3 subclasses (Fig. 1b and c).

Serum samples must be treated for endotoxin contamination prior to use in functional assays. Since endotoxin (LPS) is a potent activator of macrophages, we checked all serum samples for possible endotoxin contamination prior to use in bioassays. Endotoxin contamination of samples may reflect recent bacterial infection of the donor, leakage of endotoxin across the gut wall following an acute infection (2, 45), or inappropriate sample collection or storage. Three randomly selected Gambian serum samples contained significant levels of endotoxin, ranging from 0.45 to 0.61 IU/ml (mean, 0.53 ± 0.08 IU/ml) (Fig. 2a). Not surprisingly, given their high endotoxin concentrations, these Gambian serum samples were able to directly activate macrophages *in vitro*: when serum (diluted 1:5) was added to macrophages, the concentration of TNF in supernatants collected 24 h later appeared to correlate with

FIG. 1. Anti-GPI IgG and IgG subclass antibody titers in control and malaria-immune purified IgG samples. The midpoint titers of total anti-GPI IgG (a) and of GPI-specific IgG1 (b), and IgG3 (c) in European control (Eu con; open symbols) and immune Gambian individuals (solid symbols) are shown. Data represent the mean (solid horizontal bar) of the total antibody titers for 6 control and 33 immune subjects. Significant differences were noted between immune and European controls: ***, $P < 0.0001$; **, $P < 0.001$.

the level of endotoxin contamination in the sample (compare Fig. 2a with b). In contrast, control samples from United Kingdom donors, processed under strict aseptic conditions, were free from endotoxin contamination (0.06 to 0.08 IU/ml; mean, 0.07 ± 0.01 IU/ml) and failed to activate macrophages (Fig. 2a and b). To see if removal of endotoxin prevented macrophage activation, all test serum samples, including control samples from United Kingdom donors, were pretreated with polymyxin B. Polymyxin B treatment effectively removed endotoxin from the samples (Fig. 2a), and these absorbed endotoxin-free sera no longer induced TNF production from macrophages (Fig. 2b). Overall, sera with endotoxin concentrations of <0.07 IU/ml did not elicit TNF release from macrophages *in vitro*. Subsequently, all samples were absorbed with polymyxin B prior to use in bioassays, and the background levels of TNF

FIG. 2. Endotoxin contamination of human serum samples invalidates bioassay but can be overcome by endotoxin absorption. The endotoxin (a) and TNF (b) levels in the supernatants of RAW264.7 cells cultured for 24 h with control serum samples (samples 1 to 3) or immune serum samples (samples 4 to 6) before (solid bar) and after (open bar) endotoxin removal by polymyxin B absorption were determined. Each bar represents the mean $\pm$ SD for one serum sample tested on two separate occasions. Eu, European.

(macrophages plus IgG, without GPI or PfSE) were low in all cases (0.02 to 0.09 ng/ml).

GPI-mediated macrophage activation can be neutralized by immune IgG. In accordance with previous studies showing that GPI is highly proinflammatory both *in vitro* and *in vivo* (22, 39, 40, 43), we found that purified GPI activates macrophages, in a dose-dependent manner, to produce TNF (Fig. 3a). GPI at a concentration of 5.0 μ g/ml reproducibly induced significant TNF secretion, and this concentration was used for all subsequent experiments.

To establish the optimal concentration of IgG for use in the GPI neutralization assay, we purified five IgG samples (three from immune individuals with high anti-GPI antibody titers and two controls), confirmed that they were free of endotoxin, incubated them (at concentrations of 0.001, 0.01, 0.1, and 1 mg/ml IgG) with 5.0 μ g/ml GPI, and added them to RAW264.7 cells. The level of background TNF release (by the cells alone) ranged from 0.05 ng/ml to 0.10 ng/ml; GPI solvent and IgG alone induced only background levels of TNF (results not shown). The reductions in the levels of TNF secretion in the presence of IgG were calculated (as described in Materials and Methods) as a percentage of the TNF secretion in the positive-control wells (GPI without IgG). The two European control IgG samples had no effect on GPI-induced TNF secretion, whereas GPI-induced TNF production was inhibited, in a dose-dependent manner, by prior incubation with the immune IgG preparations, with maximal inhibition (70 to 80%) occurring at IgG concentrations of 0.1 mg/ml. On the basis of the results of these experiments, samples were used at a total IgG concentration of 0.1 mg/ml for all subsequent experiments.

FIG. 3. Optimization of GPI neutralization assay. (a) RAW264.7 macrophages were cultured with increasing concentrations of *P. falciparum* GPI for 24 h, and the amount of TNF in the supernatant was measured by ELISA. Each point represents the mean $\pm$ SD from three separate experiments. (b) GPI (at a concentration of 5 μ g/ml) was incubated with increasing concentrations of purified, endotoxin-free IgG from European (Eu) control serum samples (open symbols) or immune serum samples (solid symbol) before addition to RAW264.7 macrophages; the TNF concentration in the culture supernatants was assessed after 24 h incubation. Representative data from two control and three immune samples are shown. Each point represents the mean from a representative of two separate experiments. (c) GPI (at a concentration of 5 μ g/ml) was incubated with 0.1 mg/ml purified, endotoxin-free IgG (open bars) or with IgG from untreated samples (solid bars) from control (European) or immune (Imm) individuals before addition to RAW264.7 macrophages; the TNF concentration in the culture supernatants was assessed after 24 h incubation and is expressed as a percentage of that for the GPI control (i.e., GPI alone without IgG). Representative data from two control and three immune samples are shown. Each bar represents the mean $\pm$ SD from two separate experiments.

It is important to note, however, that although GPI was neutralized by the IgG samples from immune individuals, this neutralizing effect was observed only after treatment of sera to remove endotoxin, as shown in Fig. 3c. Thus, the effect of endotoxin contamination masked the neutralizing capacity of the IgG if prior endotoxin removal was not carried out, demonstrating the importance of appropriate endotoxin testing and absorption of samples prior to their use in cell-based bioassays.

We also tested the GPI-neutralizing capacity of IgG purified from individuals with acute non-*P. falciparum* infections (bacterial or viral), which may contain antibodies to LPS. The anti-GPI antibody titers were 1:2 to 1:4 in these nonmalaria acute-phase samples, and none of these IgG samples (at a normalized IgG concentration of 0.1 mg/ml) were able to block GPI-induced macrophage activation. Furthermore, three GPI-neutralizing immune IgG samples with anti-GPI antibody titers of >1:40 failed to block LPS-induced TNF production *in vitro*, demonstrating the specificity of the anti-GPI antibody-mediated neutralization (data not shown).

GPI neutralization capacity is directly correlated with anti-GPI antibody titer. To examine the relationship between the anti-GPI antibody titer and neutralization of GPI-induced TNF production by macrophages, we purified—after treatment to remove endotoxin contamination—IgG from 33 immune and 6 control serum samples and tested them by ELISA and an *in vitro* assay (Fig. 4). The levels of reduction of GPI-induced TNF by control IgG ranged from 4.1% to 19.3%, giving a cutoff (mean + 2 SDs) of 21.2% (shown as a horizontal dashed line in Fig. 4a); immune IgG samples giving less than 21.2% reduction in TNF secretion were classified as nonneutralizers. We observed a strong and highly significant correlation ($R^2 = 0.82$; $P < 0.0001$) between the anti-GPI antibody titer of the IgG samples and the neutralization of GPI-induced TNF release (Fig. 4a); samples with a midpoint anti-GPI titer greater than 1:10 were particularly efficient at neutralization.

It has been suggested that acquisition of antidiarrheal immunity may depend on the IgG subclass of the anti-GPI antibodies (4). As we found IgG1 and IgG3 anti-GPI antibodies in malaria-immune individuals (Fig. 1b and c), we next examined the association between GPI neutralization and anti-GPI IgG subclass. The anti-GPI IgG1 titers were highly significantly associated with GPI neutralization ($R^2 = 0.38$; $P < 0.002$) (Fig. 4b), but this association was less apparent for IgG3 ($R^2 = 0.15$; $P = 0.08$) (Fig. 4c).

Antibody-mediated neutralization of GPI-induced macrophage activation also blocks expression of cell surface CD40. High-titer anti-GPI antibodies are clearly able to inhibit GPI-induced TNF production from macrophages. While this regulatory pathway may have direct *in vivo* consequences in terms of inflammation-associated pathologies during *P. falciparum* infection, it was of interest to assess whether anti-GPI antibodies suppressed other aspects of macrophage activation, specifically, whether anti-GPI antibodies also suppressed GPI-induced upregulation of surface CD40. Representative plots showing the gating of F4-80-positive macrophages are shown in Fig. 5a. Importantly, endotoxin-absorbed IgG samples did not affect CD40 expression (data not shown), whereas expression of surface CD40 was significantly upregulated following GPI stimulation (Fig. 5b). Prior incubation of GPI with control IgG

FIG. 4. IgG from malaria-immune individuals neutralizes GPI-induced TNF production. (a) Neutralization of GPI-induced TNF release from macrophages after incubation with endotoxin-free IgG. One hundred microliters IgG (0.1 mg/ml) was incubated with 2 μ l GPI (5 μ g/ml) for 1 h at 37°C before addition to 100 μ l of RAW264.7 cells (5×10^4); after a further 24 h incubation, the culture supernatants were assayed for TNF release. Each point represents the mean percent TNF reduction ($\pm$ SD) of IgG samples from control subjects (open symbols) and immune subjects (solid symbols) from at least three separate experiments. The horizontal dashed line shows the cutoff level for neutralizing activity; samples with values below this cutoff are classified as nonneutralizers. The regression line shows a significant ($P < 0.0001$) association between neutralization and the anti-GPI antibody titer. Correlation between TNF reduction and anti-GPI IgG1 (b) and IgG3 (c) titers. The associations were significant for IgG1 ($P = 0.002$) but not for IgG3 ($P = 0.08$).

had no significant effect on CD40 expression (Fig. 5c). In contrast, the GPI-induced upregulation of CD40 was completely inhibited by prior incubation of GPI with immune IgG (Fig. 5d). These data demonstrate that anti-GPI IgG can suppress multiple GPI-induced macrophage inflammatory processes.

FIG. 5. Attenuation of GPI-induced upregulation of CD40 expression on macrophages by purified IgG from malaria-immune individuals. One hundred microliters IgG (0.1 mg/ml) was incubated with 2 μ l GPI (5 μ g/ml) for 1 h at 37°C, before addition to 100 μ l of RAW264.7 cells (5×10^4); after a further 24 h incubation, the cells were examined for CD40 expression. (a) Representative dot plots demonstrating the homogeneity of RAW264.7 macrophage cultures (left, side and forward scatters; right, forward scatter versus F4-80 staining). (b to d) Histograms (left) and bar charts (right) showing the mean fluorescence intensity (MFI) of CD40 expression on macrophages following no stimulation (Stim) (filled area) or stimulation with GPI (gray solid line) or LPS (black solid line) (b), stimulation with GPI (filled area) or with GPI previously incubated with the European (EU) control IgG (black solid line) (c), and stimulation with GPI (filled area) or with GPI previously incubated with neutralizing immune IgG (black solid line, left) or nonneutralizing IgG (black solid line, right). The results in the bar graphs are the mean fluorescence intensity of CD40 expression $\pm$ SEM. The results are representative of those from two replicate experiments, with three or four IgG samples being tested in each experiment. APC, antigen-presenting cells; Neut, neutralization titer; Non-Neut, nonneutralization titer; N/A, not applicable.

Antibody-mediated neutralization of PfSE-induced macrophage activation. Since GPI is only one of several parasite moieties released at schizont rupture, we wanted to assess the extent to which anti-GPI antibodies contributed to the overall antitoxic activity of immune IgG.

To address this, we first examined the ability of PfSE to stimulate TNF production by macrophages and determined the titers and neutralizing capacities of anti-PfSE antibodies in IgG samples from the malaria-immune donors (Fig. 6). PfSE induced TNF production in a dose-dependent manner, with high levels of TNF (0.65 to 1.1 ng/ml) being produced at PfSE concentrations equivalent to 2.5×10^6 schizonts/well (an approximately 20:1 schizont-to-macrophage ratio) (Fig. 6a). For subsequent neutralization assays, 3×10^6 schizonts/well were used. As expected, high levels of anti-PfSE IgG antibodies were observed in purified IgG samples from malaria-immune individuals (midpoint titers, 1:16 to 1:150), whereas very low levels of antibody binding were observed for the control samples (Fig. 6b). Importantly, preincubation of PfSE with IgG from immune individuals neutralized PfSE-induced TNF secretion from macrophages (Fig. 6c), and there was a good correlation between the anti-PfSE ELISA titer and neutralization capacity ($R^2 = 0.43$; $P = 0.008$), with samples with an anti-PfSE titer of $>1:70$ being particularly effective at neutralization. Of note, the correlation between PfSE neutralization and the anti-PfSE antibody titer was weaker than that between GPI neutralization and the anti-GPI antibody titer (Fig. 4a), which likely reflects the greater complexity of PfSE.

To begin to assess the contribution of anti-GPI antibodies to the overall neutralizing activity of the IgG samples, we first compared the titers of antibodies to PfSE with those to GPI in individual samples. Although there was a highly significant association between the anti-PfSE and anti-GPI antibody titers ($R^2 = 0.41$; $P = 0.009$), samples with similar anti-PfSE titers varied as much as 10-fold in their anti-GPI titers, and some samples with similar anti-GPI titers varied by up to 5-fold in their anti-PfSE titers (Fig. 7a). We next looked for associations between the anti-GPI titer and the ability to neutralize PfSE (Fig. 7b) and, vice versa, the anti-PfSE titer and the ability to neutralize GPI (Fig. 7c). Although these parameters were significantly correlated in both cases ($R^2 = 0.31$ and $P = 0.032$ and $R^2 = 0.36$ and $P = 0.034$, respectively), these heterologous correlations were noticeably weaker than the homologous comparison for GPI ($R^2 = 0.82$; $P < 0.0001$) and somewhat weaker than that for PfSE ($R^2 = 0.43$; $P = 0.008$). Together, the results of these analyses suggest that the macrophage-activating capacity of PfSE may not be solely due to GPI and that anti-GPI antibodies may represent only a fraction of the total antitoxic antibody pool in immune serum.

DISCUSSION

Despite suggestions that much of the morbidity and pathology of *P. falciparum* infection is due to the strong proinflammatory immune response generated against the parasite (reviewed in references 6, 10, and 38), the nature of the molecules that induce these inflammatory responses is hotly debated (reviewed in reference 16). Thus, malaria-induced inflammation may result from the activities of a small number of dominant antigens (including GPI), or conversely, it may be the accumu-

FIG. 6. IgG from malaria-immune individuals neutralizes PfSE-induced TNF production. (a) Dose-response curve for TNF production from RAW264.7 macrophages after 24 h stimulation with increasing concentrations of PfSE; each point represents the mean $\pm$ SD of data from two separate experiments. (b) Anti-PfSE IgG antibody titers for 6 European (Eu) control subjects (open symbols) and 23 immune subjects (solid symbols); the solid horizontal bar depicts the mean for each group. IgG titers were significantly higher among immune samples than control samples (***, $P < 0.0001$). (c) Neutralization of PfSE-induced TNF production. PfSE was incubated with IgG samples before addition to RAW264.7 cells, and the level of TNF in the culture supernatants was measured 24 h later. Each point represents the mean percent TNF reduction ($\pm$ SD) relative to the level of stimulation achieved with PfSE alone from at least three separate experiments for each sample; IgG samples were from 6 European control subjects (open symbols) and 23 immune subjects (solid symbols). The horizontal dashed line shows the cutoff level for neutralizing activity; samples with values below this cutoff are classified as nonneutralizers. The regression line shows a significant ($R^2 = 0.43$; $P = 0.008$) association between neutralization and the anti-PfSE titer.

lation of numerous responses to a myriad of parasite antigens (reviewed in references 16, 26, and 31). Correlative studies have reported that the prevalence, concentration, and persistence of anti-GPI antibodies increase with age in malaria-

FIG. 7. Correlation between anti-GPI antibody titer and neutralization of PfSE-induced macrophage activation. Correlation between anti-PfSE IgG titer (ELISA) and anti-GPI IgG titer (ELISA) in immune samples (a); PfSE-neutralizing activity (percent TNF reduction) and anti-GPI antibody titer (ELISA) (b); and GPI-neutralizing activity (percent TNF reduction) and anti-PfSE titer (ELISA) (c). All associations were statistically significant: $P = 0.009$ (a); $P = 0.032$ (b); $P = 0.034$ (c).

exposed populations as they become immune to severe malarial disease (reviewed in reference 6), suggesting that anti-GPI antibodies may be an important component of antidiisease immunity; however, to date, there has been a lack of functional evidence that these antibodies can block parasite-mediated inflammation, and no study has examined the relative importance of GPI in the overall parasite-induced inflammatory response.

In the present study, we have shown that antibodies purified from the sera of individuals exposed to *P. falciparum* are able to specifically neutralize the GPI-induced activation of macrophages *in vitro*, as measured by the attenuation of TNF production and suppression of surface CD40 expression. This anti-inflammatory response is likely to have important consequences *in vivo* during infection: CD40 is rapidly upregu-

lated following macrophage activation, and its ligation by CD40L on activated T cells augments effector function (NO, reactive oxygen intermediate, and cytokine secretion) (36, 42). Although macrophages play an essential role in clearance of *P. falciparum*-infected red blood cells (13), activated macrophages are also implicated in the pathogenesis of cerebral malaria (32). Conceivably, therefore, high levels of antibodies against the triggers of macrophage activation may dampen the inflammatory response to malaria, reducing the risk of severe pathology. Moreover, we have shown that the extent of GPI neutralization was directly correlated with the titer of anti-GPI IgG antibody, suggesting that neutralizing capacity is simply a function of the antibody titer. However, all of the immune samples used in the present study were from adults; whether antibodies from children would be similarly effective at GPI neutralization remains to be determined, but there is evidence to suggest qualitative maturation of the anti-GPI response both with increasing age and with increasing malaria exposure. For example, it has been reported from Papua New Guinea that although IgG3 is the predominant anti-GPI IgG isotype, IgG1 levels increase steadily with increasing age (4). However, in Tanzania anti-GPI antibodies became increasingly polarized toward the IgG3 subclass with increasing cumulative exposure to malaria (44). In the current study, anti-GPI IgG1 titers correlated more closely than IgG3 titers with GPI neutralization, suggesting either that IgG1 antibodies are more effective at neutralizing or that they are in fact present at a higher concentration. Unfortunately, it is not possible to directly compare IgG subclass concentrations using ELISA techniques, since the secondary antibodies used to detect the two subclasses may differ in their avidities. Further work is thus required to clarify this point and to determine whether maturation of the anti-GPI response in terms of class switching is beneficial in terms of antitoxic function. In addition, the avidity of the anti-GPI antibodies may increase following repeated parasite exposure, significantly augmenting the neutralizing capacity of the antibodies.

The titers of anti-GPI antibodies varied significantly among the IgG samples from malaria-exposed individuals. Two clear subgroups were evident in the immune group: one with high anti-GPI titers and high neutralizing activity and one with low anti-GPI titers and low neutralizing activity. As repeated boosting through *P. falciparum* infection is believed to be required for the maintenance of anti-GPI antibodies (reviewed in reference 6), the anti-GPI antibody titer may reflect the time since last exposure to the parasite (14, 21). Nonetheless, as all the Gambian samples were from individuals perceived to be immune to the severe complications of malaria, the results raise important questions regarding the overall importance of anti-GPI antibodies in immunity to malaria. Similarly, previous studies have generated conflicting results regarding the association between anti-GPI antibodies and protection from severe malarial disease (5, 14, 21). While these earlier studies were based entirely on the quantification of anti-GPI antibodies by static binding assays—which fail to provide any information on the function of the anti-GPI antibodies—the results of our study bring into question the role of functional, neutralizing anti-GPI antibodies in protection from severe malarial disease. It is possible that immune individuals with low anti-GPI titers are protected by other mechanisms (for exam-

ple, antibodies that agglutinate or opsonize merozoites or block sporozoite invasion of the liver), but it is also possible that anti-GPI antibodies play only a minor role in clinical immunity.

Although IgG samples containing high-titer anti-GPI antibodies were effective at neutralizing GPI-induced macrophage activation, they appeared to be less effective at reducing PfSE-induced macrophage activation. The most obvious interpretation of these data, although it is perhaps unsurprising, is that PfSE contains other proinflammatory parasite molecules. Thus, effective antidiarrheal immunity will probably be composed of strong neutralizing antibody responses to multiple parasite molecules as well as appropriately timed regulatory responses that moderate proinflammatory cytokine production by macrophages and other cell populations (17, 26). Other parasite-derived molecules involved in the generation of proinflammatory immune responses during *P. falciparum* infection may include protein-DNA complexes (48) and hemozoin (11, 41), which has been shown to activate the nucleotide-binding domain, leucine-rich repeat-, and Pyd-containing protein 3 (NALP3) inflammasome (41), trigger TLR9 signaling, and be an endogenous adjuvant in malaria-induced vaccination (47). Nevertheless, hemozoin has also been shown to inhibit macrophage and dendritic cell activation, and the importance of the inflammasome and TLR9 during *P. falciparum* infection is unclear (33, 47). Recently, parasitized red blood cell (pRBC)-derived microparticles have been shown to stimulate strong proinflammatory immune responses through MyD88 and TLR4 (12), but the nature of the antigens incorporated in these microparticles requires further investigation. Thus, further studies will be required to determine the importance of GPI in the overall PfSE-induced inflammatory response. Examining the composition of PfSE to identify the primary inflammatory moieties is a complex task; however, it is only by identifying the most potent and inflammatory parasite-derived moieties that we can begin to unravel the basis of antidiarrheal immunity and protection against severe malarial disease.

In conclusion, we have developed an assay that enables the direct investigation of the neutralizing function of antitoxic antibodies purified from human sera. We have demonstrated that human serum samples may be contaminated by endotoxin and that removal of endotoxin is essential before field samples may be used in bioassays. We have shown that PfSE- and GPI-neutralizing activities are highly correlated with antibody ELISA titers, but our data also question the relative importance of anti-GPI antibodies in the overall antitoxic activity of human IgG. In the future, this assay will allow detailed examination of the neutralizing activities of antibodies to a variety of malarial molecules and will help define the kinetics of functional antibody generation and maintenance over time, which may then be correlated with levels of parasite exposure and immunity to severe malarial disease. This study forms the basis for future large-scale studies on the role of toxin-neutralizing antibodies during *P. falciparum* infection.

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