

Immunological Evaluation of Synthetic Glycosylphosphatidylinositol Glycoconjugates as Vaccine Candidates against Malaria

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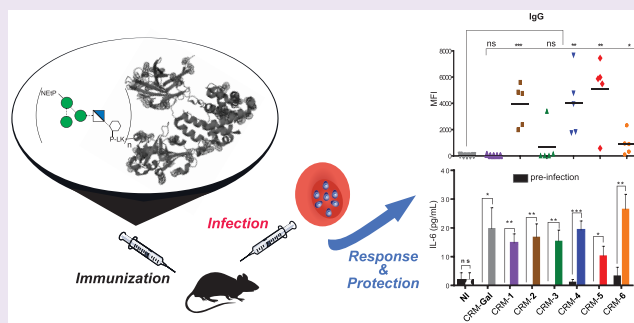
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S Supporting Information

ABSTRACT: Glycosylphosphatidylinositols (GPIs) are complex glycolipids present on the surfaces of *Plasmodium* parasites that may act as toxins during the progression of malaria. GPIs can activate the immune system during infection and induce the formation of anti-GPI antibodies that neutralize their activity. Therefore, an antitoxic vaccine based on GPI glycoconjugates may prevent malaria pathogenesis. To evaluate the role of three key modifications on *Plasmodium* GPI glycan in the activity of these glycolipids, we synthesized and investigated six structurally distinct GPI fragments from *Plasmodium falciparum*. The synthetic glycans were conjugated to the CRM197 carrier protein and were tested for immunogenicity and efficacy as antimalarial vaccine candidates in an experimental cerebral malaria model using C57BL/6J mice. Protection may be dependent on both the antibody and the cellular immune response to GPIs, and the elicited immune response depends on the orientation of the glycan, the number of mannoses in the structure, and the presence of the phosphoethanolamine and inositol units. This study provides insights into the epitopes in GPIs and contributes to the development of GPI-based antitoxin vaccine candidates against cerebral malaria.



Malaria is a serious and fatal disease in humans caused by protozoan parasites of the genus *Plasmodium* and a major health threat in developing countries. Nearly half of the world's population is at risk of malaria infection, especially children and pregnant women in sub-Saharan Africa.¹ In 2017, 219 million cases of malaria and 435000 deaths were reported.² Young children, elderly persons, and immunosuppressed persons are at higher risk of cerebral malaria (CM), the most severe form of the disease, which is caused by *Plasmodium falciparum* and can lead to long-term neurologic sequelae or death.³ The treatment of *P. falciparum* is becoming difficult due to the appearance of drug-resistant parasite strains.⁴

Various measures for controlling malaria have reduced the number of yearly infections,⁵ but mortality and morbidity remain high. The development of a malaria vaccine remains a global priority. Most of the tested vaccine candidates contain attenuated or killed parasites or protein subunits that target the pre-erythrocytic stage, blood stage, or transmission stage of the parasite.⁵ However, these vaccine candidates have failed, and a vaccine with high efficacy is still required to combat and control this disease.

An antitoxin vaccine represents an alternative approach to target malarial pathogenesis in humans.⁶ Glycosylphosphati-

dylinositol (GPI), a glycolipid ubiquitous in eukaryotes, has been proposed as a prominent toxin in malaria that can be used for the development of a vaccine.⁷ The glycan of GPIs exists in two forms, with and without the Man-IV unit, and is highly conserved across the different species of *Plasmodium*. Whereas GPIs lacking the Man-IV are mostly found as free glycolipids on the parasite membrane, GPIs containing this unit anchor proteins to the cell membrane. *P. falciparum* GPIs (*Pf*GPIs) constitute $\leq 90\%$ of the protein glycosylation on the parasite membrane^{8,9} and are synthesized during the entire life cycle, and their biosynthesis is an important process for the development and survival of the parasites.¹⁰

During malaria infection, *Pf*GPIs activate and induce the production of the pro-inflammatory cytokines TNF- α and IL-1 in macrophages.¹¹ Furthermore, anti-GPI antibodies are present in sera of malaria patients from endemic areas,^{12,13} and the toxic activity of GPIs released into the bloodstream during infection can be blocked using GPI-mediated signaling

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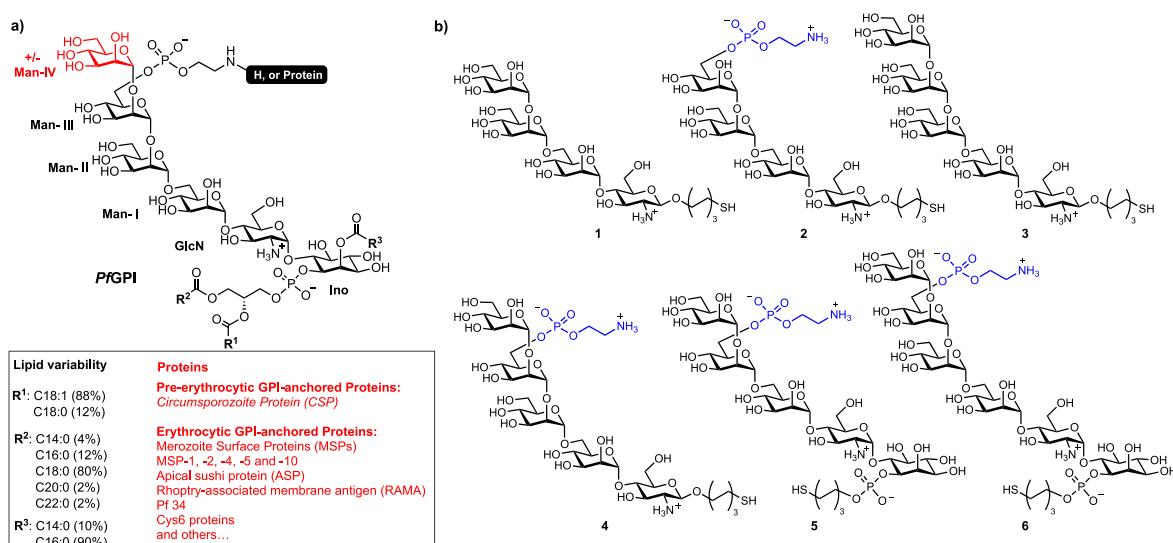


Figure 1. Glycosylphosphatidylinositol (GPI) of *P. falciparum*. (a) Structure of the natural GPIs, where Man-IV is found in combination with a protein attached. (b) Synthetic *Pf*GPI glycan fragments for conjugation to the CRM carrier protein and for immunization studies.

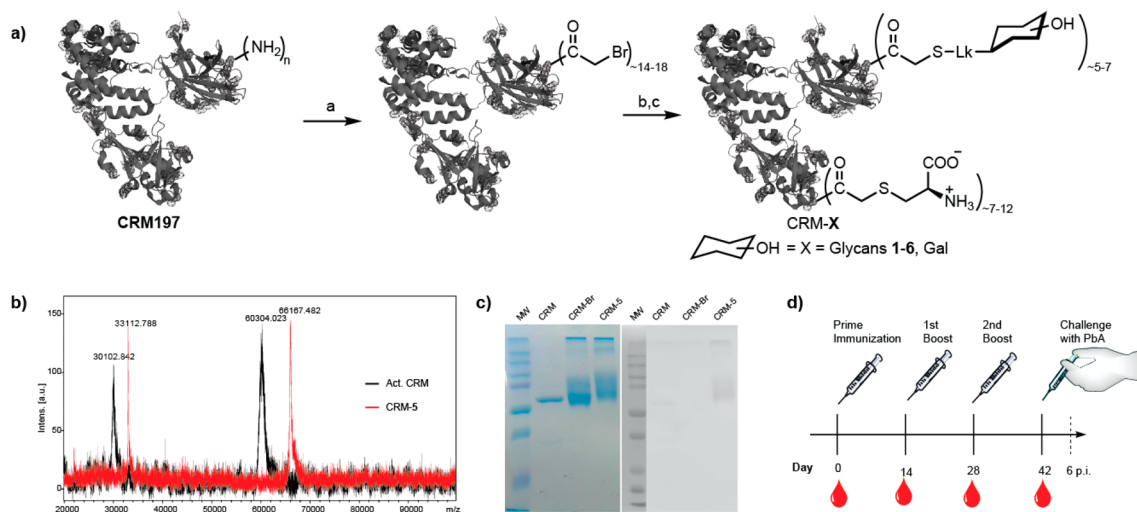


Figure 2. Synthesis and characterization of CRM glycoconjugates. (a) Reagents and conditions: (a) Succinimidyl 3-(bromoacetamido)propionate (SBAP), 0.1 M NaP_i, pH 7.4, room temperature; (b) GPIX, 0.1 M NaP_i, pH 8.0, 1 mM EDTA, room temperature; (c) L-cysteine, 0.1 M NaP_i, pH 8.0, 1 mM EDTA, room temperature. (b) MALDI-TOF analysis of CRM-5. (c) SDS-PAGE and Western blot analysis of CRM-5. (d) Immunization protocol used in the murine *P. berghei* ANKA model.

and monoclonal antibodies.¹⁴ Thus, there is a growing interest in understanding the role of *Pf*GPIs and their modifications as a basis to develop GPI derivatives as vaccine candidates against malaria.

Isolated GPIs have been used to study the anti-GPI antibody response in malaria, but the results from these studies cannot be used to establish a relationship among GPI structure, host anti-GPI IgG response, and infection.^{12,13,15–19} Some reports describe the GPI glycan as the dominant glycolipid epitope; others propose the lipid part as the dominant epitope in the immunogenic activity of the glycolipid.^{12,18,19} These discrepancies can be attributed to the difference in the composition and purity of the isolated glycolipids. To investigate the role of the GPI glycan, a series of GPI glycan fragments was synthesized, printed on microarrays, and used to analyze the specificity of anti-GPI antibodies recognizing the synthetic glycans.²⁰ This study showed differences in antibody recognition depending on glycan size and composition.

However, there is still limited insight into the role of the GPI glycan modifications and the immunogenicity of the *Pf*GPI glycans.

Here, a series of GPI fragments resembling the two main *Pf*GPIs was synthesized to evaluate the role of *myo*-inositol, phosphoethanolamine, and Man-IV elongation in immunogenicity and protection from CM. Glycoconjugates were obtained by coupling synthetic GPI glycans to the commonly used carrier protein CRM197 and used to evaluate the production of anti-glycan antibodies, T cell activation, and protection of mice from challenge with *Plasmodium berghei* ANKA (PbA), an experimental cerebral malaria (ECM) model.

RESULTS

Design of GPI Glycan Fragments. Six GPI fragments (1–6) were designed to evaluate the immunogenicity of the natural *Pf*GPI glycan-specific modifications, a PEtN unit at Man-III, a *myo*-inositol, and an α 1–2-mannose (Man-IV)

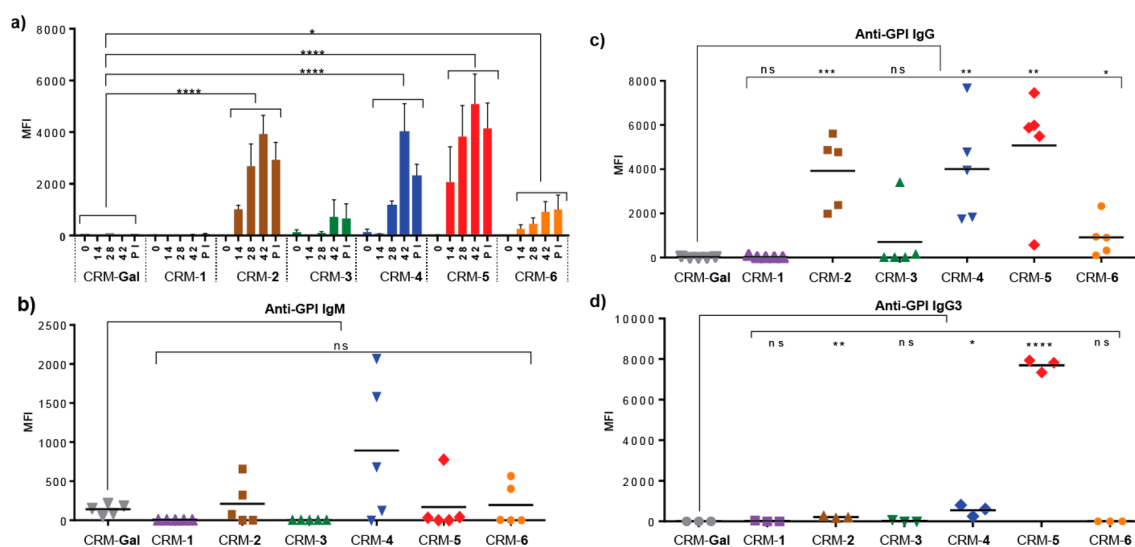


Figure 3. Anti-GPI antibody responses for mice immunized with the glycoconjugates. (a) Statistical significance determined using two-way analysis of variance to compare anti-GPI antibody levels between groups. PI = post-infection. End point anti-GPI serum IgM (b) and IgG (c) antibodies after the second boost (day 42) against CRM and glycans 1–6. (d) IgG3 antibody levels. Data shown as triplicates with the mean of 1:50 diluted and pooled sera from five mice per group. Statistical significance was determined using Student's *t* test. Statistical significance is shown as follows: ns, not significant; **p* < 0.05; ***p* < 0.01; ****p* < 0.001; *****p* < 0.0001. MFI = mean fluorescence intensity.

attached to Man-III (Figure 1a). All fragments were designed with a thiol linker for conjugation to the CRM197 carrier protein, keeping the amine group of the PETN moiety unmodified.^{21–23} The linker was attached either at the reducing end of the glycan or as part of a phosphodiester linked at the 1-O position of *myo*-inositol to maintain the natural orientation of the GPI. Fragments 1–4 all lack the *myo*-inositol unit, while fragments 1 and 3 in addition lack the PETN unit at Man-III. Fragment 5 carries a PETN attached at Man-II to evaluate the role of phosphorylation, and glycan 6 containing the complete GPI structure was used as a reference (Figure 1b).

Synthesis and Conjugation of GPI Glycans. GPI glycan fragments 1–6 were synthesized by a convergent strategy using an [$x + 2$, $x = 2, 3$, or 4] glycosylation strategy and a set of building blocks and protocols that we had established previously (Schemes S1–S5).^{24,25} Upon assembly of the fully protected glycan, fragments 2 and 4–6 were phosphorylated using phosphitylation with H-phosphonates followed by oxidation. The final deprotection of all glycans was achieved by Birch reduction. The products were obtained after size exclusion chromatography purification in 57–75% yield (for further details, see the Supporting Information).

GPI fragments 1–6 were conjugated to the CRM197 carrier protein, a nontoxic variant of diphtheria toxin,²⁶ to obtain the corresponding glycoconjugates (CRM-GPIs) CRM-1–CRM-6. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis of the glycoconjugates showed that between five and nine glycan units were coupled per protein, corresponding to a glycan content of 6–14% (Figure S3). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot analysis using biotinylated concanavalin A for detection confirmed glycoconjugate formation. In addition to a main band, corresponding to the monomeric form of the CRM, a glycoconjugate dimer and a trimer were also detected (Figure 2c and Supporting Information). A galactose-CRM glycoconjugate (CRM-Gal) was prepared as a negative control for

the immunization experiments. Circular dichroism (CD) analysis of conjugates CRM-5 and CRM-Gal and a comparison with the CD spectrum of CRM²⁷ showed no effect of the conjugation process on the global structure of the protein (Figure S4).

Anti-GPI Antibody Response and Cross-Reactivity. CRM-GPI glycoconjugates CRM-2 and CRM-4–CRM-6 were highly immunogenic in mice and induced a significant titer of anti-GPI IgG antibodies compared to control mice (Figure 3). In contrast, no induction or a very weak antibody response was observed against glycoconjugates lacking PETN and *myo*-inositol, CRM-1 and CRM-3, respectively (Figure 3a). The glycoconjugates of glycans containing trimannose and PETN (CRM-2 and CRM-5) induced a marked increase in the level of anti-glycan antibodies after prime immunization (day 14), whereas glycoconjugates having a tetramannose, CRM-4 and CRM-6, induced significant anti-GPI antibody levels only after the first boost on day 28. The highest antibody titer after the second boost was observed in mice immunized with CRM-5. Interestingly, the antibody levels dropped for all groups after infection, except for the group that had received CRM-6. This reduction of antibody levels is a result of parasite-induced immune suppression or binding of anti-GPI antibodies to naturally occurring GPIs after PbA infection.

The anti-glycan IgG antibody levels after the second boost on day 42 revealed a significant increase in antibody levels against glycoconjugates CRM-2 and CRM-4–CRM-6 compared to the CRM-Gal control (Figure 3c). However, on day 42 no significant difference in the anti-GPI IgM levels was detected between mice immunized with the glycoconjugates and the controls (Figure 3b), showing a complete isotype switch from early IgM to IgG antibodies. Antibody isotype analysis of subclasses IgG1–IgG3 was performed using a pool of sera from five mice per group. Binding of anti-glycan IgG1 and IgG2 antibodies to respective fragments revealed similar patterns as described above. In contrast, sera of mice immunized with CRM-3 displayed a significant increase in the level of antibody binding compared to that of control mice

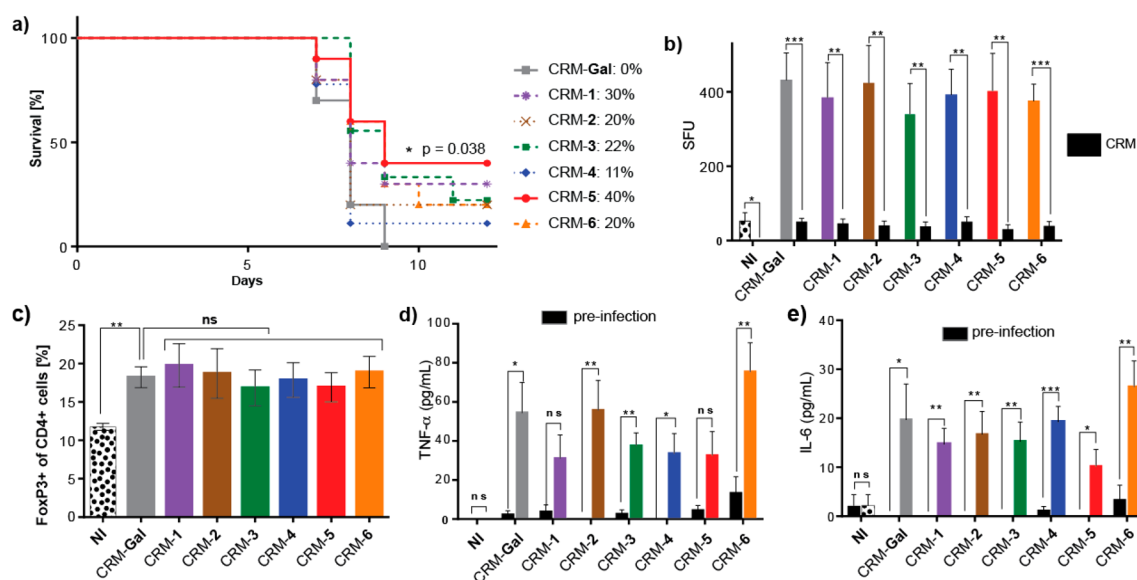


Figure 4. Protection from ECM and cellular immune response in immunized mice. (a) Mice immunized with GPI conjugates exhibited a decreased ECM incidence immunized with CRM-Gal. Mice were euthanized at an early stage of ECM to minimize suffering. (b) IFN- γ production by T cells upon restimulation of spleen cells by their immunized CRM-GPI conjugate or CRM alone. (c) Frequencies of regulatory T cells in all challenged groups were compared to those of the non-infected control group. Serum levels of pro-inflammatory cytokines TNF- α (d) and IL-6 (e) determined pre- and post-PbA infection (days 42 and 48) using the cytometric bead array. Data are shown as means \pm the standard error of the mean. Statistical significance was determined using Student's *t* test. Statistical significance is shown as follows: **p* < 0.05; ***p* < 0.01; ****p* < 0.001. SFU = spot forming unit.

(Figure S8). Anti-glycan IgG3 antibody levels that play an important role in anti-polysaccharide antibody responses in mice^{28,29} were drastically increased for CRM-5 and only marginally increased for CRM-2 and CRM-4 (Figure S8). Cross-reactivity of the anti-GPI antibodies against fragments 1–6 was observed across all compounds with distinct characteristics (Figures S5 and S6). Control mice immunized with CRM-Gal did not develop cross-reacting antibodies against GPI fragments but did against galactose (Figure S6). Glycoconjugates CRM-1 and CRM-3 (without PEtN and inositol) induced cross-reacting antibodies that exhibited binding to fragments 2–4, whereas no reactivity was detected against inositol-containing fragments 5 and 6. Mice immunized with CRM-2 and CRM-4 developed predominantly cross-reacting antibodies against PEtN-containing glycans 2 and 4, respectively, but notably less against inositol-containing fragments 5 and 6, which also contain PEtN. By contrast, glycoconjugates CRM-5 and CRM-6, containing both PEtN and inositol, induced cross-reacting antibodies with high levels of binding of fragments 5 and 6, moderate binding PEtN containing fragments 2 and 4, and no detectable reactivity against 1 and 3. To test the specificity of the antibodies against PEtN, we synthesized Gal-PEtN and evaluated the binding of antibodies to this structure. Sera of CRM-1 groups did not show binding. However, antibodies from CRM-Gal bound moderately, and the antibodies of CRM-2, -4, and -5 showed high levels of binding (Figure S9).

Protection against ECM. All CRM-GPI-immunized mice except for the CRM-5 group displayed a CM incidence that was not significantly different from that of the control group. However, CRM-5 induced significant protection from cerebral malaria with a survival rate of 40%. Mice immunized with CRM-2 and CRM-4–CRM-6 displayed increased levels of anti-GPI antibodies compared to the control group (Figure 4a). Protection in 30% of the animals was observed in the

group immunized with CRM-1, having the smaller glycan load and weaker antibody response; however, values did not reach statistical significance. CRM-5-immunized mice displayed a lower level of serum IFN- γ and did not develop a significant increase in the level of TNF- α post-infection, in contrast to mice immunized with other GPI glycoconjugates (see below). This reduction in pro-inflammatory cytokine production can explain the ameliorated clinical symptoms of mice immunized with CRM-5.

Vaccine-Specific T Cell Response. An immunological characterization of five mice per group, sacrificed on day 6 post-infection, was performed to determine spleen cell populations, brain T cell sequestration, and serum cytokine levels. The CRM- and CRM-GPI-specific T cell response was analyzed by determining the production of IFN- γ by isolated splenocytes upon restimulation with the corresponding glycoconjugate used for immunization. ELISpot analysis showed baseline frequencies of CRM-specific IFN- γ + T cells in all immunized animals. A significant increase in the level of T cell activation of CRM-GPI-restimulated splenocytes was observed in all groups. Interestingly, this increase was also observed in cells of CRM-Gal-immunized mice, suggesting more efficient uptake of CRM-GPIs and CRM-Gal into antigen-presenting cells than of CRM alone and an enhanced subsequent MHC-II presentation of CRM-derived peptides (Figure 4b).³⁰ No difference in the number of IFN- γ spot forming units (SFU) was detected between mice immunized with either CRM-Gal or CRM-GPIs (Figure 4b).

Spleen Cell Composition. Spleen cells were isolated on day 6 post-infection. Analysis of T cell frequency in spleen and T cell activation in CRM-GPI-immunized and PbA-challenged mice revealed a lymphopenia for CD4+ but not for CD8+ T cells (Figure S10). Early leukopenia, particularly for CD4+ T cells, is a well-known phenomenon during malaria. The level of T cell activation, as defined by expression of the early

activation marker CD69, was however higher in both CD4+ and CD8+ T cells than in non-infected controls. Interestingly, the level of CD4+ and CD8+ T cell activation was slightly decreased in CRM-5- and CRM-3-immunized mice, as would be expected considering the lower level of pro-inflammatory serum cytokines detected in these groups.

The proportion of regulatory T cells (Treg), measured by intracellular staining of the Treg-specific transcription factor FoxP3, was significantly increased in all PbA-challenged groups compared to the non-infected control group (Figure 4c). No significant difference was observed between CRM-Gal- and CRM-GPI-immunized mice, suggesting that glycan antitoxic vaccination does not markedly impact regulatory T cell frequency.

P. berghei ANKA challenge was accompanied by an activation of macrophages and dendritic cells in all groups as indicated by an increased level of expression of the costimulatory molecule CD80 on CD11b+ cells (mainly macrophages) and CD11c+ cells (mainly dendritic cells) (data not shown).

Serum Cytokine Levels. CRM-GPI-immunized and control mice displayed low or only background levels of IFN- γ , TNF- α , and IL-6 after the second boost vaccination (Figure 4 and Figure S6). To investigate whether the glycoconjugates' immunizations modified the pro-inflammatory cytokine responses during PbA infection, serum cytokines of mice were further analyzed on day 48 (day 6 post-infection). Generally, PbA-infected groups displayed significantly increased levels of IFN- γ , TNF- α , and IL-6 after challenge (Figure 4d,e). The level of TNF- α was not significantly increased in CRM-1- and CRM-5-immunized mice, and the level was similar to that of conjugates CRM-3 and CRM-4. The level of pro-inflammatory cytokines post-infection varied among CRM-GPI-vaccinated mice. While mice immunized with CRM-6 showed consistently increased levels of IFN- γ , TNF- α , and IL-6, those were considerably reduced for mice immunized with CRM-5. However, no significant difference in cytokine levels was seen in CRM-GPI-immunized versus CRM-Gal control mice. Analysis of serum cytokines revealed that CRM-GPIs did not induce an unspecific inflammatory response. Serum TNF- α and IFN- γ levels were correlated with ECM and CM susceptibility. Interestingly, CRM-5 immunized mice did not develop a significant increase in TNF- α level and displayed a lower level of serum IFN- γ , which is in accordance with the reduced incidence of CM in this group.

DISCUSSION

On the basis of previous reports describing the toxicity of GPIs, their role in CM, and the interaction of isolated and synthetic GPIs with antibodies in sera of infected patients from endemic areas,^{15,20} a library of six GPI glycan fragments was used to evaluate the role of the phosphoethanolamine (PEtN), the *myo*-inositol, and the mannose at the nonreducing end (Man-IV) of the GPI glycan in the immune protection of mice in a murine malaria model. Fragments 1–6 were synthesized and conjugated to the carrier protein CRM197. These CRM glycoconjugates were used to immunize mice in the presence of Alum as the adjuvant, commonly used in glycoconjugate vaccines,^{31,32} before mice were challenged with *P. berghei* ANKA.

Determination of the anti-glycan antibodies using microarrays showed that all CRM-GPI glycoconjugates in this study were immunogenic and induced the production of anti-glycan

antibodies. Conjugates of glycans having only mannose and glucosamine, CRM-1 and CRM-3, were less immunogenic than glycoconjugates containing PEtN and *myo*-inositol. A cross-reactivity microarray analysis revealed that the length of the mannose backbone is important for immune recognition. While the GPI-1 having only Man3 was not detected by antibodies, CRM-3 carrying glycans with the Man-IV unit was more immunogenic and was detected by cross-reacting antibodies from CRM-1-immunized mice. This observation is in line with previous findings for human anti-GPI antibody binding, where glycan fragments containing fewer than five carbohydrate units were not recognized.²⁰ In contrast to human sera that showed a slight difference in antibody recognition toward GPIs with or without a PEtN unit,²⁰ PEtN was also detected as an important immunogenic epitope in mice. Substantial differences were observed in this study between glycoconjugates having identical glycans with or without PEtN [CRM-1 vs CRM-2 and CRM-3 vs CRM-4 (Figure 3)]. This observation was confirmed by binding of antibodies from CRM-2, -4, and -5 to Gal-PEtN, which was not observed for antibodies from CRM-1. It is noteworthy, and in agreement with previous reports,³³ that inositol was also an immunogenic epitope in the glycans, which was observed in the immune response to CRM-5 and CRM-6.

IgG subclass analysis further revealed unique findings for the most protective glycoconjugate vaccine in this study. In agreement with previous studies of immunoglobulin subclasses against naturally occurring *Plasmodium* GPIs,³⁴ the IgG3 subclass was highly abundant in mice immunized with CRM-5 but not in mice immunized with other glycoconjugates (Figure 3d). Because IgG1 and IgG3 share the highest affinity for Fc receptors on phagocytic cells, high titers of these IgG subclasses might have facilitated efficient GPI clearance post-infection. However, the reason for the sharp increase in IgG3 for CRM-5 needs to be further elucidated.

Mice immunized with conjugates CRM-1, CRM-2, and CRM-5, containing the Man₃-GlcN moiety, revealed higher resistance to ECM compared to the fragments containing the Man₄-GlcN moiety, CRM-3, CRM-4, and CRM-6, which is the main structure found on the structures of GPI-modified proteins attached to the surface of *P. falciparum*.³⁵ GPIs containing only three mannoses exist mostly in free GPIs that are five times more abundant than GPI anchoring proteins.^{33,36} All GPI fragments were conjugated to CRM via the glucosamine or *myo*-inositol unit to retain the GPIs' natural orientation within the cell membrane. This type of conjugation was selected to improve the presentation of the PEtN unit, which plays an important role in the production of antibodies and protection against ECM. The higher protective potential of NEtP-Man₃-GlcN-containing fragments compared to that of NEtP-Man₄-GlcN-containing fragments could have been influenced by a lack of protein attached to the PEtN unit, which may cause a different presentation of the four mannoses, thus reducing the immunogenicity of the Man₄-GPI. This observation is confirmed by the results for CRM-6 and previous studies using a similar glycan conjugated to KLH via the PEtN on Man-III that protected mice from cerebral pathology.⁷ Although the change from KLH to CRM for conjugation and from Freund's adjuvant to Alum may have contributed to the generally lower level of protection observed in this study, our results provide evidence that the site of conjugation and the GPI-glycan structure should be carefully considered. Accordingly, structures containing the free NEtP-

Man₃-GlcN epitope or a protein-bound NEtP-Man₄-GlcN may influence vaccine efficacy.

Furthermore, this study demonstrates that immunogenicity varies according to the composition of GPI structures. Mice immunized with CRM-5 exhibited a slightly reduced level of activation of CD4⁺ and CD8⁺ T cells, accompanied by decreased levels of the pro-inflammatory cytokines IFN- γ and TNF- α . Immunization with other CRM-GPI conjugates did not affect the cellular or humoral immune response in a similar manner. Cytokine levels in fact may have influenced CD8⁺ T cell sequestration in the brain, thus impacting ECM development. These observations correlate with the enhanced rate of survival of mice immunized with CRM-5.

CONCLUSION

A series of P_fGPI fragments were synthesized, conjugated to the CRM-197 carrier protein, and used to perform a structure–activity relationship study with regard to immunogenicity and protection against experimental cerebral malaria (ECM) in mice. Moderate differences in ECM incidence between mice immunized with the different CRM-GPI glycoconjugates were observed that depended on the number of mannoses (Man₃ > Man₄) and the presence of PEtN and *myo*-inositol units on the glycans. The highest level of protection (40%) was observed in mice immunized with CRM-5, a glycoconjugate containing the GPI glycan core with the PEtN at a non-natural position. Although protection can be partially attributed to antibody responses against CRM-5, alterations in T cell activation as well as the level of pro-inflammatory cytokines may also have affected ECM susceptibility.

METHODS

Chemical Synthesis of GPI Fragments. All glycans were synthesized from protected building blocks using established solution-phase protocols, as described in the [Supporting Information](#).

Conjugation of Fragments 1–6 to CRM-197. CRM carrier protein was purchased from Pfenex Inc. A 1 M solution of resin-bound TCEP was purchased from Thermo Scientific, and autoclaved sterile water was used for the conjugation. First, 250 μ L of a TCEP resin solution was centrifuged for 3 min, and excess water was removed. TCEP resin was resuspended in 150 μ L of 0.1 M sodium phosphate buffer (pH 8.0) and centrifuged for 3 min. The excess buffer was removed, and autoclaved water was added. The GPI fragment was dissolved in 120 μ L of water, transferred to the TCEP solution, and incubated at room temperature. After 1 h, TCEP resin was filtered off using a syringe and washed with autoclaved water (5 \times 50 μ L). All water fractions were then combined and lyophilized. The reduction of the disulfide was confirmed by liquid chromatography and mass spectrometry.

A solution of SBAP (1.58 mg) in DMF (60 μ L) was added to a stirred solution of CRM (3 mg) in 0.1 M sodium phosphate buffer (pH 7.4) at room temperature. The reaction mixture was stirred for 1 h, and the solution was concentrated to 250 μ L using an Amicon Ultra-4 centrifugal filter tube (10 kDa cutoff, Millipore) and washed with water (4 \times 1 mL) and 0.1 M sodium phosphate buffer (pH 8.0) (1 \times 1 mL). Finally, the activated CRM was concentrated to obtain \sim 100 μ L of a solution. The solution of activated CRM was added to the reduced fragment, and the mixture was stirred overnight at room temperature. Then, the solution was concentrated to a volume of 250 μ L using an Amicon Ultra-4 Centrifugal tube (10 kDa cutoff, Millipore) and washed with water (4 \times 1 mL) and 0.1 M sodium phosphate buffer (pH 8.0) to obtain 250 μ L of the CRM-GPI glycoconjugate solution. Cysteine (0.94 mg) was added directly to the solution in the centricon, and the mixture incubated for 1 h at room

temperature. Then, the mixture was washed with water (4 \times 1 mL) and PBS buffer to obtain the desired CRM-GPI glycoconjugate.

Characterization of Conjugates. All protein intermediates and the final CRM-GPI glycoconjugates were characterized by MALDI-TOF mass spectrometry and by gel electrophoresis. SDS–PAGE gels were directly stained with Coomassie Brilliant Blue R250 (CBB) (Sigma-Aldrich, Munich, Germany, 6104-59-2) or used for Western blotting. For gel electrophoresis, glycoconjugates were diluted 1:10 in sterile water, loading buffer was added, and the mixture was boiled for 5 min and loaded onto a 12% polyacrylamide gel. Gel electrophoresis was performed at 150 V and 150 mA for 60 min. Western blot transfer was accomplished at 100 V and 35 mA. The membrane was routinely tested for protein transfer with Ponceau S (Sigma-Aldrich, 6226-79-5) and subsequently blocked for 2 h with 5% BSA in PBS-T at room temperature. Biotinylated concanavalin-A (Vector Laboratories, Burlingame, CA, B-1005) was diluted 1:500 in 1 \times PBS containing 5% BSA, 0.01 mM Mn²⁺, and 0.1 mM Ca²⁺ and incubated at room temperature for 2 h while being shaken. The membrane was subsequently washed, streptavidin HRP (BD Pharmingen, Heidelberg, Germany, 557630) added at a 1:500 ratio in 1 \times PBS, 5% BSA, 0.01 mM Mn²⁺, and 0.1 mM Ca²⁺, and the mixture incubated for 1 h at room temperature. Finally, the membrane was washed again in 1 \times PBS, 0.01 mM Mn²⁺, and 0.1 mM Ca²⁺ and developed by enhanced luminol-based chemiluminescence according to the manufacturer's instructions (Thermo Fisher Scientific, Darmstadt, Germany, 32109). A dual-color precision protein standard (Bio-Rad Laboratories, Munich, Germany, 161-0374) was used as the protein standard. CD spectra of CRM-5 and CRM-Gal were recorded on a Applied Photophysics Chirascan spectrophotometer using a 0.3–0.5 mg mL⁻¹ solution in PBS buffer. Spectra were recorded between 190 and 260 nm at 25 $^{\circ}$ C using PBS buffer as a reference.

Animal Experiments. Animals were treated strictly according to German and European Law (Directive 2010/63/EU). Recommendations of the Society for Laboratory Animal Science (GV-SOLAS) and of the Federation of European Laboratory Animal Science Associations (FELASA) were followed. The Office for Health and Social Affairs Berlin (LAGeSo) approved the experiment (Permit G0239/14). All efforts were made to minimize suffering.

All C57BL/6Jrj mice used in this study were obtained from Janvier Laboratories (Saint-Berthevin, France). Mice were housed in individually ventilated cages (IVCs) under specific pathogen free (SPF) conditions in the animal facility of the Federal Institute for Risk Assessment (BfR, Berlin, Germany). Mice were provided food and water *ad libitum*. Upon delivery (day -7), mice were allowed to rest for 1 week before experiments were started.

Immunization and Challenge Experiment in C57BL/6Jrj Mice. C57BL/6Jrj mice were divided into groups of 15 animals. Mice were vaccinated intraperitoneally (ip) on days 0, 14, and 28. Study groups were immunized with CRM-GPIs, and control mice were immunized with CRM-Gal. Galactose-conjugated CRM was used as a control to account for possible conformational changes in the glycan-loaded carrier protein that has been described previously.³⁷ Serum was obtained at 14-day intervals: prior prime, first and second boost vaccination, and post-infection. All mice were challenged on day 42 with 1 \times 10⁶ PbA infected erythrocytes ip. On day 6 post-infection, five mice per group were sacrificed for the immunological characterization, consisting of spleen cell composition, brain T cell sequestration, and carrier protein-specific T cell restimulation. The 10 remaining mice per group were used to assess ECM incidence as a readout of vaccine efficacy. Group sizes of 10 mice for vaccine efficacy studies were based on the expected incidence of ECM in C57BL/6 mice between 80% and 100%. The dependent variable (survival) in this group was analyzed using a log-rank test.

Immunization. On day 0, 5-week-old female C57BL/6Jrj mice were prime-immunized ip with CRM-GPIs glycoconjugates or CRM-Gal as a control. Two boost immunizations were performed at 14-day intervals (days 14 and 28). Each mouse was injected with a total of 100 μ L ip of either CRM-GPI glycoconjugate or CRM-Gal formulated with aluminum hydroxide (Alum). Immunizations were performed using 5 μ g of glycan per vaccination. Due to diverging

loadings of glycans on CRM, conjugates were diluted in sterile PBS accordingly. CRM-GPI glycoconjugates were formulated with aluminum hydroxide in a 1:2 ratio (Alhydrogel, Brenntag, Denmark) and rotated overnight at 4 °C before immunization.

Serum Collection. Blood was taken from each mouse before prime immunization, first and second boost, and PbA infection (days 0, 14, 28, and 42, respectively). Blood was allowed to coagulate for at least 30 min at room temperature and then centrifuged at 2000g for 15 min. Isolated serum was stored at −80 °C until further use.

***P. berghei* ANKA Infection.** Mice were infected with *P. berghei* ANKA MRA-671 stabilates (stocks obtained from the Malaria Research and Reference Reagent Resource Center, Manassas, VA). Frozen stabilates [2×10^7 infected red blood cells (RBCs), stored in liquid nitrogen in a solution of 0.9% NaCl, 4.6% sorbitol, and 35% glycerol] were quickly thawed by hand and carefully resuspended in sterile PBS. Ten mice were infected intraperitoneally with one stabilate in 100 μ L of an RBC suspension, corresponding to 1×10^6 infected RBCs. Mice were randomized prior to infection. It has consistently been shown that C57BL/6J mice infected with *P. berghei* ANKA develop neurological symptoms from day 6 on. Hence, all mice were monitored repeatedly daily for early signs of ECM according to a predefined scoring system. In accordance with the animal study proposal, mice scoring ≥ 3 on this predefined system or exhibiting a weight loss of $\geq 20\%$ were humanely euthanized. All mice were euthanized 12 days post-infection (day 54).

Glycan Array Printing and Development. Solutions of GPI fragments 1–6 were diluted to 1 and 0.2 mM using 0.1 \times PBS (pH 7.4). Galactose, CRM, BSA spacer, buffer, and *Streptococcus pneumoniae* polysaccharide were further added as controls. Then, 25 and 5 mM TCEP (pH 8) were added to the glycan and protein solutions, respectively, 20 min prior to printing. Glycans and proteins were printed on maleimide slides (PolyAn, Berlin, Germany) using a SciFlexarray microarray printer (Sciencion, Berlin, Germany) in triplicate in a 64-well format. Following a 24 h incubation in a wet chamber at room temperature, slides were quenched for 1 h with 0.1% (v/v) 2-mercaptoethanol in PBS at room temperature. Slides were washed with water, dried, and stored in a desiccator until they were used.

Anti-GPI antibody levels in CRM-GPI-immunized and control mice were measured on days 0, 14, 28, and 42 and on day 6 post-infection. Printed slides were blocked with 1% BSA in 1 \times PBS for 1 h at room temperature, washed with water, and dried. Serum dilutions (1:50) were added to the corresponding well, and the slides were incubated at 4 °C in a wet chamber overnight. Following incubation, serum was discarded and wells were washed three times with 1 \times PBS and 0.1% Tween. Then, 23 μ L of one of the following anti-mouse IgG or IgM antibody dilutions was added and incubated 1 h at room temperature in a dark wet chamber: rabbit anti-mouse IgG-FITC (diluted 1:400) (Sigma-Aldrich, F9137), donkey anti-mouse IgM-Alexa Fluor 594 (1:200) (Dianova, Hamburg, Germany, 715-585-020), goat anti-mouse IgG1-AlexaFluor 594 (1:400) (Thermo Fischer Scientific, Darmstadt, Germany, A21125), goat anti-mouse IgG2-AlexaFluor 647 (1:200) (Thermo Fischer Scientific A21241), goat anti-mouse IgG3-AlexaFluor 488 (1:200) (Thermo Fischer Scientific, A21151), and goat anti-rabbit IgG-FITC (1:400) (Dianova, ab6717). Slides were washed three times with PBS and 0.1% Tween, rinsed carefully with water, dried, and measured by an Axon GenePix 4300A fluorescent scanner (Molecular Devices, Sunnyvale, CA). The values used for analysis correspond to the average of the mean fluorescent intensities after background subtraction of triplicates.

Statistical Analysis. Statistical analysis was performed using GraphPad Prism (GraphPad Software Inc., La Jolla, CA). Unpaired Student's *t* test was used to compare different sets of data, whereas two-way analysis of variance was used to compare anti-glycan antibody levels over time between immunized and non-immunized groups. The log-rank test was employed for analysis of survival between different groups. Statistical significance within figures is shown as follows: **p* < 0.05; ***p* < 0.01; ****p* < 0.001; *****p* < 0.0001.

■ ASSOCIATED CONTENT

§ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscchembio.9b00739.

Full experimental details, characterization and spectra of the compounds and conjugates, microarray data, and immunological characterization of the sera of mice (PDF)

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Notes

The authors declare no competing financial interest.

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