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Synthetic glycosylphosphatidylinositol microarray reveals differential antibody levels and fine specificities in children with mild and severe malaria

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ABSTRACT

Glycosylphosphatidylinositol (GPI) glycolipids abound on the cell surface at the merozoite stage of *Plasmodium falciparum* life cycle are a central toxin in malaria. The contribution of GPI specific humoral immune responses to protection against malaria pathology is not clear, since studies on the correlation between anti-GPI antibody titers and disease severity have yielded contradictory results. Here, we present the application of a carbohydrate microarray based on synthetic *Pf*GPI glycans to assess levels and fine specificities of anti-GPI antibody responses in healthy and malaria diseased individuals. Furthermore, the age dependent development of humoral immune responses against GPI in malaria-exposed children was investigated. Anti-GPI antibodies were only rarely found in children under the age of 18 months. Sera from subjects with severe malaria and healthy children contained antibodies that recognized predominantly synthetic Man_3-GPI and Man_4-GPIs. In contrast, antibodies in sera of children with mild malaria also showed substantial reactivity with truncated glycans comprising glucosamine–inositol moieties without mannose or with only one or two mannose residues.

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1. Introduction

Glycosylphosphatidylinositols are evolutionary conserved glycolipids found in the outer cell membranes of virtually all eukaryotic cells from vertebrates to protozoa and constitute up to 90% of protein glycosylation in protozoan parasites.[1,2] The glycan part of the GPI glycolipid consists of a conserved, linear 6-*O*-(ethanolamine-PO_4)-α-Man-(1-2)-α-Man-(1-6)-α-Man-(1-4)-α-$GlcNH_2$-(1-6)-*myo*-inositol-1-PO_4 core structure. Although evolutionarily conserved, species-dependent branching of the GPI core structure results in unique GPI molecules comprising epitopes that can be recognized by the mammalian immune system.[3] GPIs anchor a diverse range of proteins to the surface of *Plasmodium falciparum*, but may also exist free of protein attachment.[4–6] While Man_4-GPI serves as anchor for surface proteins, Man_3-GPI may primarily exist as the free glycolipid on the parasite cell surface. As for other parasitic diseases, such as trypanosomiasis and toxoplasmosis, certain pathogenic features may be caused by parasite GPI-glycans.[7–9] In vitro and in vivo studies have established plasmodial GPI as potent activator of the host immune system and as inducer of pro-inflammatory cytokines.[10,11] Although inflammatory cytokines are beneficial in controlling and eliminating parasites, excessive production of these cytokines, particularly TNF-α, is thought to play a significant role in aggravating clinical symptoms which may lead to severe malaria.

In populations exposed to *P. falciparum*, the antibody response to purified GPIs is characterized by an age dependent development that correlates with the acquisition of immunity to severe malaria.[12] Therefore it has been postulated that antibodies against plasmodial GPI mediate anti-toxic and anti-disease immunity against malaria and that GPI could be a suitable component of a malaria vaccine. In the present report we show the application of a carbohydrate microarray based on synthetic *Pf*GPI glycans to assess levels and fine specificities of anti-GPI responses in healthy and malaria diseased individuals as well as the age dependent development of anti-GPI responses in children.

2. Results

2.1. Effect of age on the acquisition of anti-GPI IgG responses

Serum samples were collected from children aged 3–60 months (Table 1) in an area of Tanzania with moderate perennial

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transmission of *P. falciparum* malaria. Sera of children with uncomplicated or severe malaria were compared with sera from healthy controls using GPI microarrays spotted with seven synthetic *Pf*GPI analogues (Fig. 1). GPIs **4** and **5** represent Man_3-GPI and GPIs **6** and **7** represent Man_4-GPI species without or with a phosphate ethanolamine group, respectively. GPIs **1–3** are more truncated

Table 1
Anti-GPI IgG responses of Tanzanian children with severe or uncomplicated malaria or healthy controls

Condition	No. of children	Median age in months (range)	Anti-GPI IgG antibodies[a]			
			GPI	Mean	Median	Range
Healthy	50	24 (4–60)	GPI-**1**	2267	2177	0–4742
Mild	87	26 (3–60)	GPI-**1**	8929	2855	0–57,708
Severe	52	29 (6–60)	GPI-**1**	1545	1333	0–13,364
Healthy			GPI-**2**	1770	1743	0–4339
Mild			GPI-**2**	9095	1952	0–48,747
Severe			GPI-**2**	628	427	0–5214
Healthy			GPI-**3**	1944	1345	0–19,379
Mild			GPI-**3**	6706	1920	0–58,944
Severe			GPI-**3**	2741	738	0–43,579
Healthy			GPI-**4**	2591	1506	0–35,195
Mild			GPI-**4**	7924	1676	0–60,086
Severe			GPI-**4**	3052	1046	0–55,193
Healthy			GPI-**5**	5124	1736	0–56,585
Mild			GPI-**5**	3723	458	0–55,705
Severe			GPI-**5**	10,112	1348	0–61,930
Healthy			GPI-**6**	10,219	3739	0–60,994
Mild			GPI-**6**	5480	1690	0–60,553
Severe			GPI-**6**	12,265	3929	0–61,301
Healthy			GPI-**7**	5286	2156	0–39,710
Mild			GPI-**7**	4820	764	0–58,363
Severe			GPI-**7**	13,731	3648	0–58,389

[a] Antibody responses are expressed in fluorescence at 532 nm.

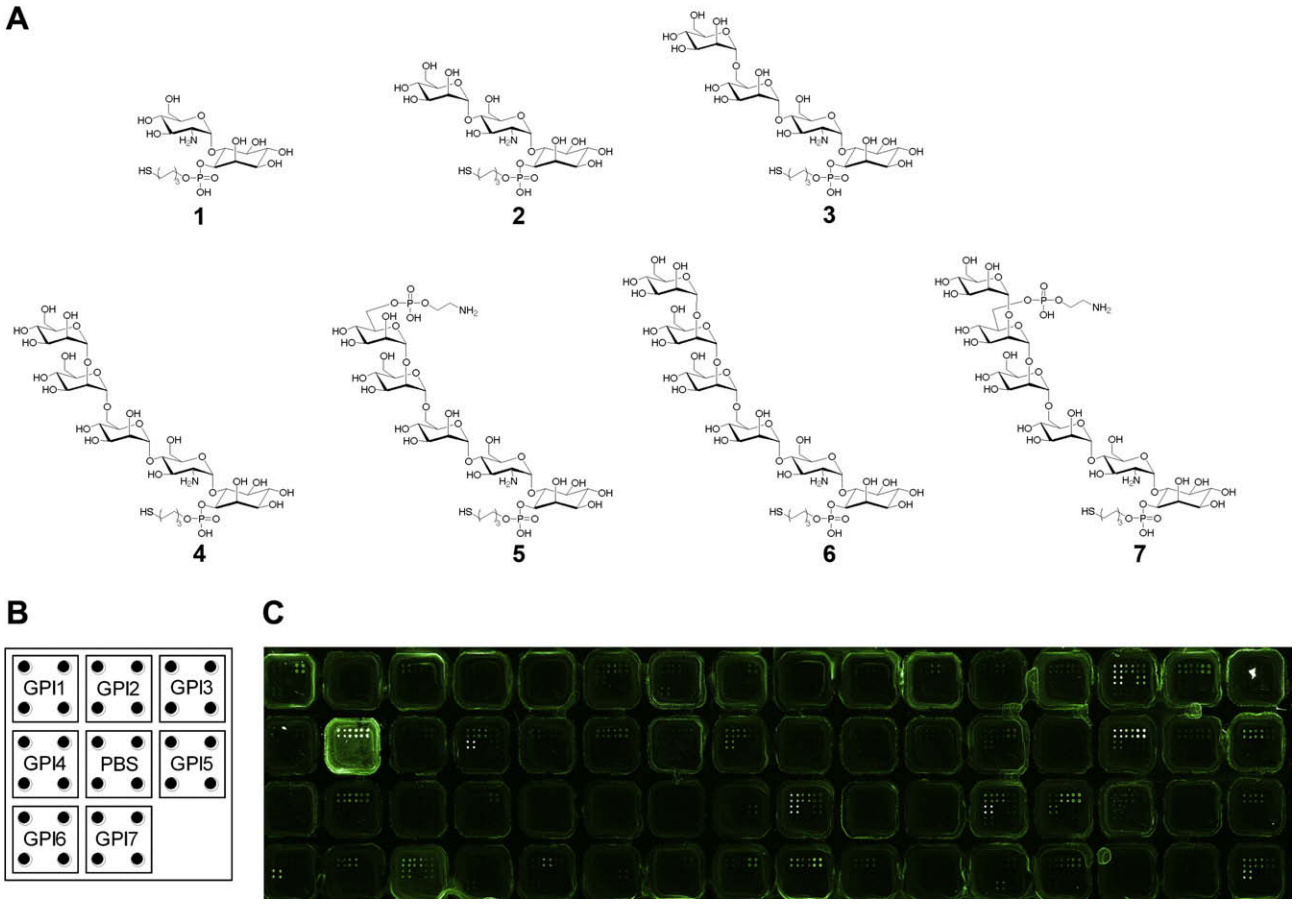

Figure 1. (A) Structures of the seven synthetic GPI-glycans (**1**–**7**) that were printed on a BSA-coated glass slide to build a GPI-glycan microarray. (B) Spotting order of the seven GPI-glycans that were arrayed as quadruplicates in order to ensure reproducibility of the results. In addition, the molecules were arrayed to create 64 identical screening units on a single glass slide. (C) Example microarray image after incubation with 64 different human sera diluted 1:100.

molecules. High anti-GPI IgG titers against the synthetic glycans tested were only rarely found in children under the age of 18 months, even in those with acute malaria infection (Fig. 2).

In accordance with these results, serum samples from healthy Tanzanian infants had no significant anti-GPI IgG antibody levels, while serum IgG from their healthy mothers showed reactivity with Man_3- and Man_4-GPIs (Fig. 3 and data not shown). As expected for healthy adults grown up in a malaria endemic region, continuous exposure to the malaria parasites resulted also in antibody responses against a broad range of protein antigens. This explains, why in immunoblotting experiments with lysates of *P. falciparum* blood stage parasites, serum samples of healthy mothers stained series of protein bands over a broad range of molecular weights (Fig. 4A). Anti-protein antibody levels in the infants were comparatively low (Fig. 4A).

Sera of a GPI seropositive mother and of her GPI seronegative child taken at 2.5 months of age showed a comparable banding pattern in Western blot analysis, reflecting transplacental transfer of maternal IgG specific for parasite proteins (Fig. 4B). Five months later the banding pattern obtained with the serum of the child had changed considerably, demonstrating an active anti-malarial immune response of the child. Taken together these results indicate that sera of children under one year of age may contain anti-malaria protein antibodies, but are largely deficient in anti-GPI antibodies.

2.2. Levels and fine specificities of anti-GPI responses in healthy and malaria diseased children

In microarray analysis sera from both healthy children and from children with severe malaria contained antibodies that recognized predominantly the GPIs **5**, **6**, and **7** (Fig. 5). In contrast, an inverted length-dependent fine specificity of antibodies to *P. falciparum*-related GPI-glycans was observed in children with mild malaria. Sera from these children showed also substantial reactivity with the most truncated GPIs (GPIs **1** to **4**) without a phosphate ethanolamine group. While IgG antibody levels against GPIs **1**, **2**, **3**, and **4** were higher in children with mild malaria than in children of the two other cohorts, the antibody levels against GPIs **5**, **6**, and **7** were higher for samples from children with severe malaria than for samples from subjects with uncomplicated malaria (Table 1).

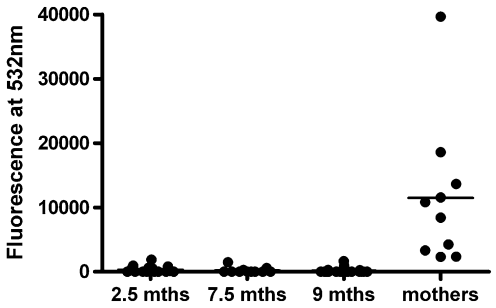

Figure 3. Anti-GPI-**6** responses of healthy Tanzanian infants and mothers. Shown are IgG levels expressed as fluorescence at 532 nm in serial serum samples from infants aged 2.5, 7.5, and 9 months and mothers diluted 1:100. Horizontal lines indicate mean levels of anti-GPI response.

3. Discussion

There is evidence that GPI of *P. falciparum* is involved in the pathogenesis of malaria.[7,8] Attempts to correlate anti-GPI antibody levels and malaria pathology using GPI purified from cultured parasites have yielded contradictory results.[12–14] Assuming that the preparations used were pure and free from contaminants, this inconsistency in results may be related to the fact that GPIs are heterogeneous and difficult to isolate from their natural sources. Therefore, anti-GPI antibody levels measured with extracted GPI critically depend on the ratio of Man_3- and Man_4-GPI in the antigen preparations. Concerns over the purity of GPI preparations derived from *P. falciparum* can be effectively abolished using fully synthetic GPIs for a more accurate analysis of serum samples. We are applying carbohydrate microarrays based on synthetic *P. falciparum* GPI to study antibody responses to different structural elements of this complex antigen. In a previous study, microarray-based serological analyses with synthetic GPIs demonstrated differences in the fine specificity of anti-GPI antibodies in malaria-exposed and non-exposed adult populations, indicating that anti-Man_3 GPI responses are primarily elicited by malaria parasites, while anti-Man_4 GPI antibodies may arise in response to exposure to a variety of pathogens.[3]

In this study we applied the innovative GPI microarray approach to assess levels and fine specificities of anti-GPI antibody

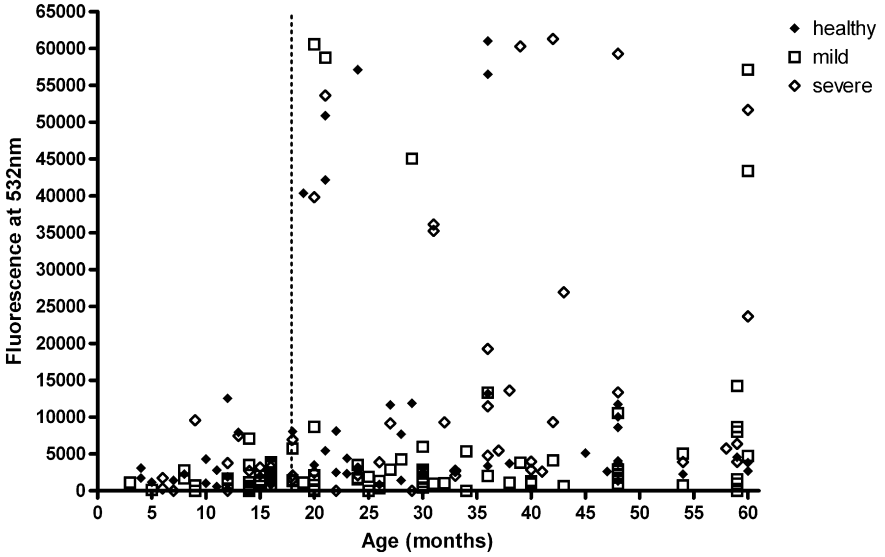

Figure 2. Effect of age on the acquisition of high anti-GPI IgG responses. Shown are anti-GPI-**6** IgG levels from Tanzanian children with severe or mild malaria or healthy controls. Data are expressed as absolute fluorescence at 532 nm for samples at a serum dilution of 1:100.

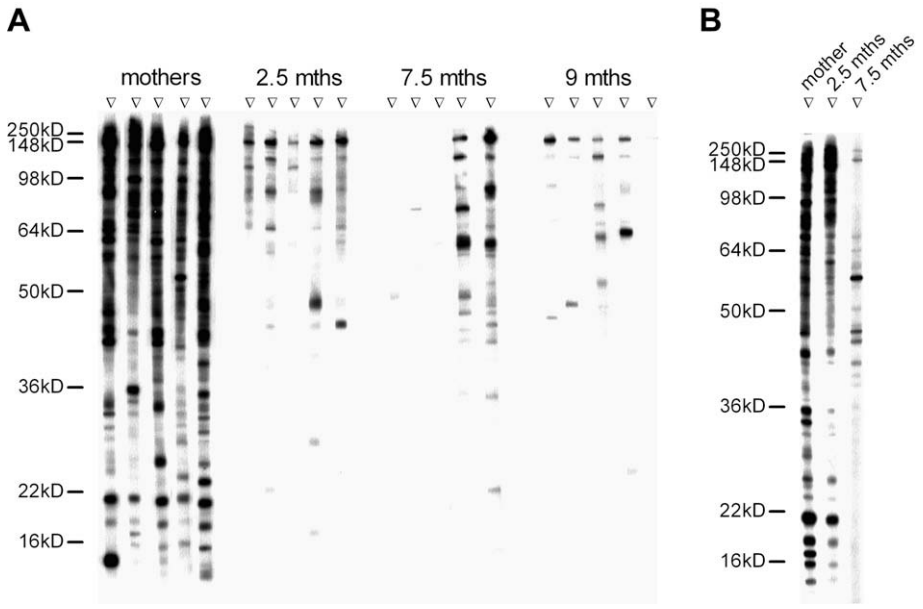

Figure 4. (A) Western blotting analysis with serum samples from healthy Tanzanian infants aged 2.5, 7.5, and 9 months and mothers using parasite lysates of *P. falciparum* blood stage cultures. Each lane represents serum from one individual donor at a dilution of 1:200. (B) Immunoblotting analysis of sera of a mother and her child at 2.5 and 7.5 months of age.

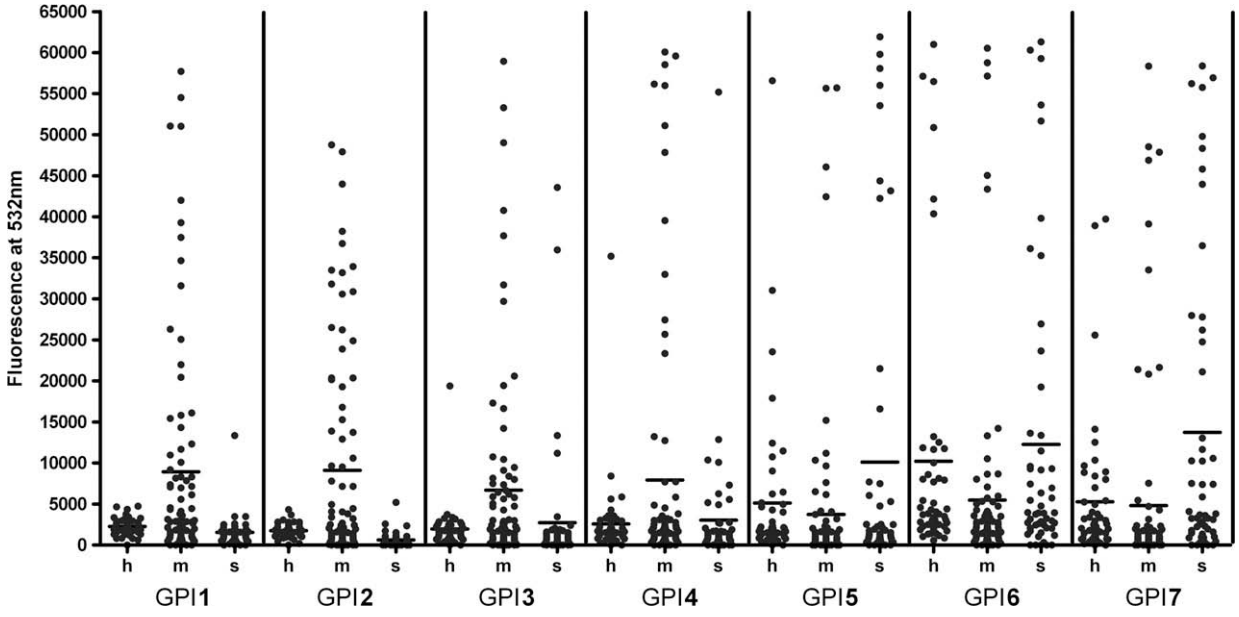

Figure 5. Anti-GPI IgG levels and fine specificities of children with severe or uncomplicated malaria or healthy controls. Shown are IgG responses to each synthetic GPI compound expressed as fluorescence at 532 nm in serum samples diluted 1:100 from Tanzanian children with severe (s) or uncomplicated (m) malaria or healthy (h) controls. Horizontal lines indicate mean levels of anti-GPI response.

responses in healthy and malaria diseased African children. This way a much more differentiated insight into the fine specificity of anti-GPI responses could be obtained than with extracted GPI. Sera from Tanzanian children with severe malaria and from healthy children contained antibodies that recognized predominantly Man_4-GPIs and Man_3-GPI with a phosphate ethanolamine group. Strikingly, a significant different antibody response was found in children with mild malaria. These sera showed substantial reactivity with the most truncated GPIs without a phosphate ethanolamine group. Presence of these antibodies in a proportion of subjects with mild malaria and lack in children with severe disease may suggest that this sub-population of anti-GPI antibodies is involved in protection against the development of severe malaria pathology.

Interestingly, high anti-GPI antibody responses were only rarely found in children under the age of 18 months, even in those with acute malaria infection. It is well known that the immune system of children under two years of age has a reduced capacity to produce antibodies to certain carbohydrate antigens. In humans IgG molecules of all subclasses, but with different efficiencies, cross the placenta and confer passive immunity to the newborn.[15,16] In malaria endemic areas, transplacentally acquired antibodies are thought to be involved in the protection of infants against malaria attacks during the first few weeks of life.[15] Our results indicate that

GPI-specific IgGs are less effective maternally transmitted than anti-protein antibodies.

4. Conclusion

Since it induces the expression of many host genes implicated in malaria pathogenesis, GPI is regarded as a central toxic component of the malaria parasites. However, the contribution of GPI specific antibody responses to protection against clinical malaria is not clear. Studies on the correlation between anti-GPI antibody titers and disease severity using GPI preparations extracted from cultivated parasites as target antigen have yielded contradictory results. Here we have utilized a carbohydrate microarray based on synthetic *Pf*GPI glycans to analyze the fine specificities of anti-GPI antibody responses in healthy and malaria diseased children. Our data argue for a protection-associated role of a sub-population of anti-GPI IgGs cross-reactive with truncated *Pf*GPI glycans. This may have major implications for the design of a GPI-based anti-toxic malaria vaccine candidate. The observed lack of anti-GPI antibody responses in children below 18 months of age may significantly contribute to the vulnerability of infants to severe malaria. Our findings emphasize that for a better understanding of protective anti-toxic and antiparasitic immunity against malaria methods have to be developed that allow analyzing both the strength and the fine specificity of natural immune responses.

5. Experimental part

5.1. Subjects

Sera were collected as described previously from June to September 2003 in Ifakara, Tanzania from children aged 3–60 months presenting with malaria at the hospital.[17] Severe malaria cases were defined according to the World Health Organization criteria for severe malaria.[18] Uncomplicated malaria was defined as the presence of asexual *P. falciparum*, an axillary temperature of >37.5 °C, or symptoms of headache or myalgia but no other signs of severe malaria. Exclusion criteria were confirmed coinfection, malnutrition (mid-upper-arm circumference [MUAC] of <12 cm), or anti-malarial treatment during the last 14 days. Asymptomatic children (presence of *P. falciparum*, axillary temperature of <37.5 °C, and no other symptoms) were age-matched as closely as possible to patients by convenience sampling in the same area in 2006. Ethical clearance was obtained from the Ifakara Health Research and Development Centre's scientific review board and the Medical Research Coordinating Committee of the National Institute for Medical Research in Tanzania.

5.2. Synthetic GPI microarray

Carbohydrates with a terminal sulfhydryl-containing linker were prepared as previously described.[19] The terminal sulfhydryl-containing linker provided a handle for covalent immobilization of the structures to a maleimide-modified surface. Functionalization and printing of slides were performed as previously described.[3] Briefly aldehyde slides (Genetix Ltd, Hampshire, UK) were immersed in PBS containing 1% BSA (w/v) and incubated overnight at room temperature. The slides were rinsed twice with distilled water and twice with 95% ethanol, then dried under a stream of anhydrous argon. Subsequently, the slides were immersed in anhydrous DMF (Aldrich, St. Louis, MO.) containing 1 mg/ml 6-maleimidohexanoic acid *N*-hydosuccinimide ester (Aldrich, St. Louis, MO.) and *N,N*-diisopropylethylamine (100 mM; Fluka, St. Louis, MO.). The slides were incubated in this solution for 24 h at room temperature and washed four times with 95% ethanol. A volume of 3 nL of a 10 μM solution of synthetic GPI-glycan in PBS were deposited on the surface of the slide to form each microarray spot using a Genetix array printer (Genetix Ltd, Hampshire, UK). The slides were subsequently incubated in a humid chamber for 24 h before being quenched with 1 mM β-mercaptoethanol for 2 h at room temperature. The slides were then washed three times with water and stored in a desiccator. Microarrays were validated with FITC-labeled ConA and comparative serum titration experiments in ELISA versus microarray-based analysis, yielding good correspondence of results.[3] Stored slides were stable over two months without alteration in the reproducibility of the experiments.

5.3. Microarray-based immunoassay

Spotted microarray slides were covered with FlexWell-64 (GRACE BIO-LABS, Bend, OR) layers. Microarray wells were blocked with 0.5% milk powder in PBS for 1 h at room temperature followed by three washings with PBS containing 0.01% Tween-20. Afterward, wells were incubated with human serum diluted 1:100 for 2 h at room temperature. After washing, slides were incubated with indocarbocyanine dye-conjugated affinity-pure $F(ab')_2$ fragment goat anti-human IgG (Fc_γ-fragment specific) antibodies (MILAN ANALYTICA AG) for 1 hour at room temperature and washed again. Dried slides were read on a GenePix™ Personal 4100A (Axon Instruments, Sunnyvale, CA) microarray-scanner at a wavelength of 532 nm. The resulting picture was quantitatively analyzed with GenePix™ Pro 6 software. Background fluorescence intensity from spotted buffer without carbohydrates was subtracted to GPI signals for each individual microarray slide.

5.4. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting

Aliquots of lysed *P. falciparum* blood stage parasites were mixed with loading buffer (1.7 ml of 0.5 M Tris–HCl [pH 6.8], 2 ml of glycerol, 4.5 ml of 10% sodium dodecyl sulfate, 1 ml of β-mercaptoethanol, 0.8 ml of bromophenol blue [0.3%, wt/vol]) and heated 10 min at 95 °C before loading on SDS-10% PAGE minigels. As a molecular weight marker, SeeBluePlus (Invitrogen) was used. Separated proteins were transferred electrophoretically to nitrocellulose filter (Protean Nitrocellulose, BA 85; Schleicher & Schuell) by semidry blotting. Blots were blocked with PBS containing 5% milk powder and 0.1% Tween-20 overnight at 4 °C and then incubated with human serum diluted 1:200 for 2 h. After several washing steps, blots were incubated with goat anti-human IgG horseradish peroxidase conjugated Ig (Bio-Rad Laboratories, Hercules, CA.) for 1 h. Blots were developed using the ECL system according to manufacturer's instructions.

References and notes

1. Ferguson, M. A. J.; Williams, A. F. *Annu. Rev. Biochem.* **1988**, *57*, 285.
2. Gowda, D. C.; Davidson, E. A. *Parasitol. Today* **1999**, *15*, 147.
3. Kamena, F.; Tamborrini, M.; Liu, X.; Kwon, Y. U.; Thompson, F.; Pluschke, G.; Seeberger, H. P. *Nat. Chem. Biol.* **2008**, *4*, 238.
4. Sevlever, D.; Humphrey, D. R.; Rosenberry, T. L. *Eur. J. Biochem.* **1995**, *233*, 384.
5. van't Hof, W.; Rodriguez-Boulan, E.; Menon, A. K. *J. Biol. Chem.* **1995**, *270*, 24150.
6. Singh, N.; Liang, L. N.; Tykocinski, M. L.; Tartakoff, A. M. *J. Biol. Chem.* **1996**, *271*, 12879.
7. Almeida, I. C.; Camargo, M. M.; Procópio, D. O.; Silva, L. S.; Mehlert, A.; Travassos, L. R.; Gazzinelli, R. T.; Ferguson, M. A. *EMBO J.* **2000**, *19*, 1476.
8. Debierre-Grockiego, F.; Azzouz, N.; Schmidt, J.; Dubremetz, J. F.; Geyer, H.; Geyer, R.; Weingart, R.; Schmidt, R. R.; Schwarz, R. T. *J. Biol. Chem.* **2003**, *278*, 32987.
9. Schofield, L.; Hewitt, M. C.; Evans, K.; Siomos, M. A.; Seeberger, P. H. *Nature* **2002**, *418*, 785.
10. Schofield, L.; Novakovic, S.; Gerold, P.; Schwarz, R. T.; McConville, M. J.; Tachado, S. D. *J. Immunol.* **1996**, *156*, 1886.

11. Tachado, S. D.; Novakovic, S.; Gerold, P.; McConville, M. J.; Baldwin, T.; Quilici, D.; Schwarz, R. T.; Schofield, L. *J. Immunol.* **1996**, *156*, 1897.
12. Naik, R. S.; Branch, O. H.; Woods, A. S.; Vijaykumar, M.; Perkins, D. J.; Nahlen, B. L.; Lal, A. A.; Cotter, R. J.; Costello, C. E.; Ockenhouse, C. F.; Davidson, E. A.; Gowda, D. C. *J. Exp. Med.* **2000**, *192*, 1563.
13. Boutlis, C. S.; Gowda, D. C.; Naik, R. S.; Maguire, G. P.; Mgone, C. S.; Bockarie, M. J.; Lagog, M.; Ibam, E.; Lorry, K.; Anstey, N. M. *Infect. Immun.* **2002**, *70*, 5052.
14. de Souza, J. B.; Todd, J.; Krishegowda, G.; Gowda, D. C.; Kwiatkowski, D.; Riley, E. M. *Infect. Immun.* **2002**, *70*, 5045.
15. Akum, A. E.; Minang, J. T.; Kuoh, A. J.; Ahmadou, M. J.; Troye-Blomberg, M. *J. Trop. Pediatr.* **2005**, *51*, 182.
16. Englund, J. A. *J. Comp. Pathol.* **2007**, *137*, 16.
17. Rottmann, M.; Lavstsen, T.; Mugasa, J. P.; Kaestli, M.; Jensen, A. T.; Müller, D.; Theander, T.; Beck, H. P. *Infect. Immun.* **2006**, *74*, 3904.
18. World Health Organization, Communicable Diseases Cluster. *Trans. R. Soc. Trop. Med. Hyg.* **2000**, *94*, S1.
19. Kwon, Y. U.; Soucy, R. L.; Snyder, D. A.; Seeberger, P. H. *Chem. Eur. J.* **2005**, *11*, 2493.