Methods

Egfr, *wg* and *dpp* loss and gain of function

Late third-instar discs were dissected from the *wg* temperature-sensitive mutant, $wg^{l\text{-}12}$, which was shifted to the restrictive temperature (29 °C) for all of the third instar, and from the *dpp* disc-specific mutant, $dpp^{d12/d14}$. $Egfr^{ts}$ refers to the genotype $Egfr^{tsla}/Egfr^{f24}$ (ref. 14). For the initial adult analysis, larvae with this genotype were raised at 18 °C apart from a 24-h shift to the restrictive temperature commencing during the early third instar. For the initial disc analysis, larvae were maintained at the restrictive temperature throughout the third instar and discs were dissected and fixed at the end of this period. For the later timing experiments, 12-h shifts were made at the beginning of the third instar or 24 h later (third instar duration of 4 days at 18 °C) and discs were fixed from late third instars or larvae were allowed to develop to adults.

Ectopic activation of the EGFR pathway was achieved using the UAS/Gal4 system with flies carrying the transgene *UAS-*$Egfr^{ltop4}$, which codes for a constitutively active form[24], and *ptc-GAL*, which is expressed along the anteroposterior compartment boundary (at fairly uniform levels in dorsal and ventral halves); this expression was monitored with UAS–green fluorescent protein (GFP). *UAS-*$Egfr^{ltop4}$ was also expressed ubiquitously using the flp-out technique[27] with an hs-flp; tubulin > CD2 > Gal4 line: larvae were given a 37 °C heat shock for 1 h during the second instar, to induce ubiquitous expression. Misexpression of a secreted form of Spitz, UAS-sSpi, was achieved in a similar manner, but larvae were given a much shorter heat shock of 32.5 °C for 30 min to generate small clones.

Gene expression

Immunostaining was performed using standard techniques with antibodies against the following proteins: Al (rat)[1], Dll (mouse)[2], B (rabbit)[16], Wg (mouse)[25], Dac (mouse)[26], ß-galactosidase (rabbit; Cappell), ß-galactosidase (mouse; Promega). *dpp* and *sty* expression was detected with enhancer traps (staining with an antibody against activated mitogen-activated protein kinase is not reliable in this tissue). *vn*, *rho* and *ru* expression was detected by *in situ* hybridization using standard techniques (unfortunately, antibodies or reliable lacZ lines are not available to test currently whether *vn* and *rho* expression requires autonomous activation of Wg and Dpp signalling pathways).

Clonal analysis

Loss of function clones were generated by flp-mediated mitotic recombination, in conjunction with the minute technique, using the following stocks: *FRT42* arr^{2}/Cyo, *FRT39E* tkv^{7}/Cyo, *FRT42* $Egfr^{tsla}$/Cyo, *y*; $rho^{P\Delta5}$ *FRT80B*/TM6B, *y*; ru^{1} rho^{7M43} *FRT80B*/TM6B, *y*; ru^{1} rho^{7M43} vn^{L6} *FRT2A*/TM6B, which were crossed to one of the following marker stocks: *hs-flp; FRT42 arm-lacZ M(2)60E*/Cyo, *hs-flp; hsGFP M(2)201 FRT39E*/Cyo, *y hs-flp*; y^{+} $M(3)i^{55}$ FRT80B/TM2, *y hs-flp;* y^{+} $M(3)i^{55}$ FRT2A/TM2, *hs-flp*; *hsGFP* $M(3)i^{55}$ *FRT2A*/TM6B. The *arr*, *tkv*, *vn* and *rho* alleles are genetically or molecularly null, ru^{1} is a hypomorph. Clones were induced during the second instar and discs were dissected and fixed from late third instars. Disc clones were marked by the loss of GFP or lacZ transgenes; adult clones were marked by the loss of y^{+}. More details on all these alleles can be found at FlyBase (http://flybase.bio.indiana.edu).

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The author declares that he has no competing financial interests.

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Synthetic GPI as a candidate anti-toxic vaccine in a model of malaria

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The malaria parasite *Plasmodium falciparum* infects 5–10% of the world's population and kills two million people annually[1]. Fatalities are thought to result in part from pathological reactions initiated by a malarial toxin. Glycosylphosphatidylinositol (GPI) originating from the parasite has the properties predicted of a toxin[2–6]; however, a requirement for toxins in general and GPI in particular in malarial pathogenesis and fatality remains unproven. As anti-toxic vaccines can be highly effective public health tools, we sought to determine whether anti-GPI vaccination could prevent pathology and fatalities in the *Plasmodium berghei*/rodent model of severe malaria. The *P. falciparum* GPI glycan of the sequence NH_2-CH_2-CH_2-PO_4-(Manα1-2)6Manα1-2Manα1-6Manα1-4GlcNH_2α1-6*myo*-inositol-1,2-cyclic-phosphate was chemically synthesized, conjugated to carriers, and used to immunize mice. Recipients were substantially protected against malarial acidosis, pulmonary oedema, cerebral syndrome and fatality. Anti-GPI antibodies neutralized pro-inflammatory activity by *P. falciparum in vitro*. Thus, we show that GPI is a significant pro-inflammatory endotoxin of parasitic origin, and that several disease parameters in malarious mice are toxin-dependent. GPI may contribute to pathogenesis and fatalities

in humans. Synthetic GPI is therefore a prototype carbohydrate anti-toxic vaccine against malaria.

Malarial GPI is a candidate toxin that is sufficient to induce cytokine and adhesin expression in macrophages and the vascular endothelium[2,4–6]—both of which are associated with clinically severe malaria[7,8]—and to induce lethality *in vivo*[2–6]. GPIs of *Trypanosoma brucei*[3,9] and *T. cruzi*[10] have similar properties, suggesting that GPIs may act generally as pro-inflammatory agents in eukaryotic parasitism. However, it is not yet established whether GPIs function as toxins in the context of protozoal infections, nor whether intervention against GPIs reduces pathogenesis or fatalities in any disease condition. Indeed the toxic basis of malarial pathogenesis, first conjectured[11] by Camillo Golgi in 1886, remains unproven.

Plasmodium falciparum shows uniquely low levels of *N*- and *O*-linked glycosylation[12,13], and the highly conserved[14] GPI constitutes over 95% of the post-translational carbohydrate modification of parasite proteins[15]. The biological activity of GPI against host tissues requires the contribution of both lipid and carbohydrate domains, and de-acylation of GPIs by enzymatic or chemical hydrolysis renders the carbohydrate moiety non-toxic[2–6]. On the basis of the sequence of the non-toxic *P. falciparum* GPI glycan[16], we chemically synthesized the structure NH_2-CH_2-CH_2-PO_4-(Manα1-2)6Manα1-2Manα1-6Manα1-4GlcNH_2α1-6*myo*-inositol-1,2-cyclic-phosphate (Fig. 1). We confirmed the structure by matrix-assisted laser desorption/ionization–time of flight (MALDI–TOF) mass spectrometry and ^{31}P-NMR (D_2O) (see Supplementary Information). To prepare an immunogen, the synthetic GPI glycan was treated with 2-iminothiolane to introduce a sulphhydryl at the ethanolamine, desalted, and conjugated to maleimide-activated ovalbumin (OVA), in a molar ratio of 3.2:1, or keyhole limpet haemocyanin (KLH), in a molar ratio of 191:1. This material was used to immunize mice.

The synthetic malarial GPI glycan was immunogenic in rodents. Antibodies from animals immunized with KLH-glycan gave positive immunoglobulin-γ (IgG) titres against OVA-glycan but not sham-conjugated OVA-cysteine. No reactivity to GPI glycan was detected in pre-immune sera or in animals receiving sham-conjugated KLH (not shown). Notably, anti-glycan IgG bound to native GPI, as judged by immunofluorescence against intact trophozoites and schizonts (Fig. 2a). Anti-GPI however failed to bind to uninfected erythrocytes, despite these cells expressing endogenous GPIs of host origin (Fig. 2a). In contrast to malarial GPI, mammalian GPIs show amino-sugar or phosphoethanolamine modifications to the core glycan[17], and these epitopic differences may account for the lack of cross-reactivity. These results do not exclude the possibility of serological cross-reactions with other tissues. Unlike controls, in a western blot analysis anti-GPI glycan IgG detected multiple molecular species against *P. falciparum*-infected but not uninfected erythrocytes (Fig. 2b), consistent with the presence in mature schizonts of multiple GPI-modified proteins and their processing products. Thus protein-specific features do not greatly influence the binding of anti-glycan IgG to native GPI anchors.

Tumour-necrosis factor (TNF)-α production by macrophages is widely used as a biochemical marker of malarial endotoxin activity *in vitro*. Purified GPIs are sufficient for TNF production[2–6,9,10], but a predominant role for GPI in parasite pro-inflammatory activity *in vitro* remains unproven. We therefore sought to quantify the contribution of GPI to the total endotoxic activity of malaria. In contrast to control sera, antibodies from mice immunized with KLH-glycan specifically neutralized TNF-α output from macrophages induced by crude total extracts of *P. falciparum* (Fig. 2c). Thus GPI appears sufficient and necessary for the induction by malarial parasites of host pro-inflammatory responses *in vitro*. Naturally, other entities of host or parasite origin may also influence such responses.

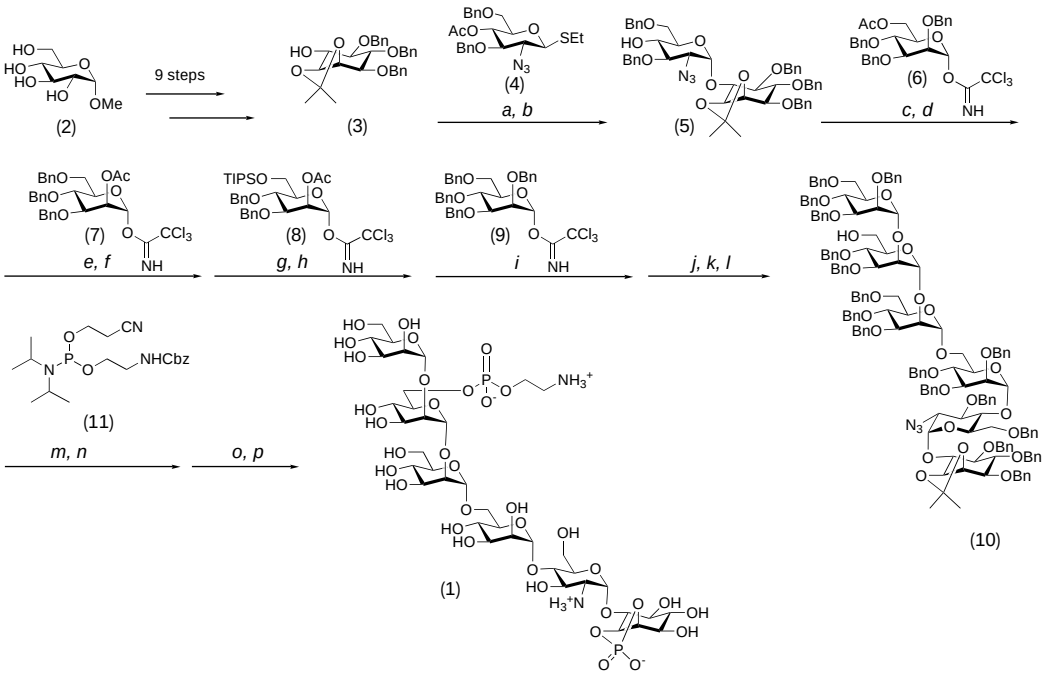

Figure 1 Synthesis of glycan (1). The reagents (*a*–*p*) were added at the indicated intermediates of glycan synthesis (1–10). *a*, (4) AgOTf, NIS, CH_2Cl_2/Et_2O (38%); *b*, NaOMe, CH_2Cl_2/MeOH (83%); *c*, (6) TMSOTf, CH_2Cl_2 (75%); *d*, NaOMe, CH_2Cl_2/MeOH (71%); *e*, (7) TMSOTf, CH_2Cl_2 (92%); *f*, NaOMe (69%); *g*, (8) TBSOTf, CH_2Cl_2 (98%); *h*, NaOMe (83%); *i*, (9) TMSOTf, CH_2Cl_2 (84%); *j*, $(CH_2OH)_2$, CSA, CH_3CN (81%); *k*, $Cl_2P(O)OMe$, pyridine (88%); *l*, TBAF, THF (61%); *m*, (11) tetrazole, CH_3CN; *n*, *t*-BuOOH, CH_3CN (84%, 2 steps); *o*, DBU, CH_2Cl_2; *p*, Na, NH_3, THF (75%, 2 steps). AgOTf, silver trifluoromethanesulphonate; NIS, *N*-iodosuccinimide; CH_2Cl_2, dichloromethane; Et_2O, diethyl ether; NaOMe, sodium methoxide; MeOH, methanol; TMSOTf, trimethylsilyltrifluoromethane sulphonate; TBSOTf, *tert*-butyldimethylsilyl trifluoromethanesulphonate; CSA, camphorsulphonic acid; CH_3CN, acetonitrile; Cbz, carbobenzyloxy; TBAF, tetrabutylammonium fluoride; THF, tetrahydrofuran; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; OBn, O-benzyl.

Humans that are affected by and dying of malaria may suffer systemic, single- or multi-organ involvement, including acute respiratory distress, coagulopathy, shock, acidosis, hypoglycaemia, renal failure, pulmonary oedema and neurological signs[18]. The murine *P. berghei* ANKA severe malaria model has salient features in common with several aspects of the human severe and cerebral malaria syndromes. It manifests a cytokine-dependent encephalopathy associated with upregulation of adhesins on the cerebral microvascular endothelium and attendant neurological complications[19–22]. Pulmonary oedema, lactic acidosis, coagulopathies, shock and renal impairment are also observed[23]. Unlike some, but not all, human cerebral cases of malaria, there is a macrophage infiltrate and compromised blood–brain barrier in the terminal or agonal stages of the murine syndrome. Nonetheless in the proximal or developmental stages the murine disease reflects more accurately the cytokine-dependent inflammatory cascade leading to cerebral and systemic involvement in humans, and thus seems the best available small animal model of clinically severe malaria[24,25]. Validation of GPI as a toxin and a target in this model might therefore allow the development of anti-toxic vaccines and immunotherapeutics that are able to prevent pathogenesis and fatalities in humans.

To this end, C57BL6/J mice primed and boosted twice with 6.5 μg KLH-glycan (0.18 μg glycan) or KLH-cysteine in Freund's adjuvant were challenged with *P. berghei* ANKA. All sham-immunized and naive control mice died with the cerebral syndrome, showing severe neurological signs including loss of reflex, ataxia, and hemiplegia, with hypothermia and occasional haematouria (Fig. 3a). These fatalities were evident early during infection (day 5–8), with relatively low levels of parasitaemia. There were no differences between naive and sham-immunized mice, indicating that exposure to KLH in Freund's adjuvant does not influence the rate of disease. In contrast, mice immunized with chemically synthetic *P. falciparum* GPI glycan coupled to KLH were significantly protected against severe malaria, with clearly reduced death rates (75% survival, $P < 0.02$, Fig. 3a). In four separate additional experiments, results in the range of 58.3–75% survival to day 12 in vaccine recipients ($n = 50$ total) compared with 0–8.7% survival in sham-immunized controls ($n = 85$) were obtained. Parasitaemia levels were not significantly different between test and control groups, demonstrating that prevention of fatality by anti-GPI vaccination does not operate through effects on parasite replication (Fig. 3b). The diagnoses of cerebral malaria, or absence of this condition, were confirmed by histological examination of brains taken 6 days after infection. Sham-immunized mice showed typical pathology including vascular occlusion with both parasitized red blood cells and host leukocytes (Fig. 3c). Immunized animals in contrast showed absent or reduced vascular occlusion despite similar parasite burdens (Fig. 3c).

Severe malaria in both humans[18] and rodents[23] may be associated with additional organ-specific and systemic derangements, including pulmonary oedema and acidosis. Acidosis may be a prime pathophysiological process and is the strongest single prognostic indicator of fatality. The biochemical aetiology of acidosis is unclear, and the relationship of human malarial acidosis to that in the rodent model also remains to be elucidated. Nonetheless, we sought to determine whether anti-GPI vaccination protects against these additional non-cerebral disease syndromes in mice. Both sham-immunized and naive individuals developed pulmonary oedema by day 6 after infection, as measured by lung dry/wet weight ratios, and this was markedly reduced in vaccine recipients (Fig. 3d). Similarly, whereas sham-immunized and naive mice developed significant acidosis as shown by reduced blood pH at day 6, blood pH was maintained at physiological levels in mice that had received the vaccine (Fig. 3e). As parasite burdens were similar in both test and control groups, production of lactic acid by parasite biomass—and any haemolytic anaemia due to parasitaemia at this stage—are not

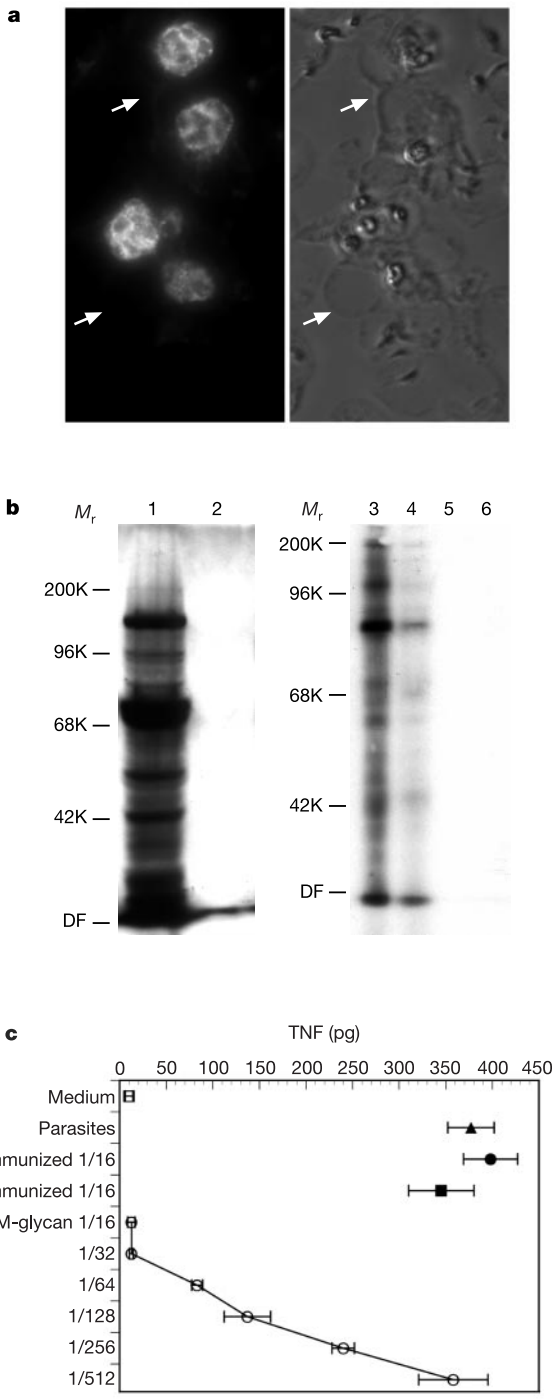

Figure 2 Antibodies raised against synthetic GPI glycan recognize native GPI and neutralize toxin activity *in vitro*. **a**, Reactivity of anti-glycan IgG antibodies with *P. falciparum* trophozoites and schizonts, and lack of reactivity to uninfected erythrocytes detected by immunofluorescence assay (left panel). The right panel shows the same field under white-light illumination. Arrows indicate adjacent uninfected erythrocytes. **b**, Western blot of anti-glycan IgG antibodies (1/200) against parasite-infected (lane 1) and uninfected erythrocytes (lane 2) run in 20-cm slab gel (left panel). The right panel shows a mini-blot comparison of reactivity against parasites by two sera from KLH-glycan-immunized mice (lanes 3, 4), pre-immune serum from lane 3 donor (lane 5), and serum from a mouse immunized with sham KLH (lane 6). All sera were used at 1/400 dilution. The detection antibody was peroxidase-conjugated goat anti-mouse IgG (γ-chain specific). DF, dye front; M_r, relative molecular mass. **c**, Levels (±s.e.m.) of TNF-α in culture supernatants of RAW264.7 cells exposed in triplicate to medium alone (open square), parasites alone (triangle), or parasites in the presence of various dilutions of sera from pre-immune (filled circle), sham-immunized (filled square) or glycan-immunized mice (open circles).

major contributors to acidosis in this model. Clearly, immunizing against GPI prevents the development of pulmonary oedema and acidosis as well as cerebral malaria in *P. berghei* infection.

The aetiology of malarial anaemia in humans is complex and poorly understood. A principal contributory factor is thought to be the failure of stem cells in the bone marrow to repopulate the peripheral erythrocyte compartment, a process known as dyserythropoiesis or erythropoietic suppression. Although *P. berghei* is the best available model for certain aspects of lethal pathogenesis, it is not considered to model adequately these aspects of human malarial anaemia. Indeed, infection models of this condition are not yet fully developed. Unlike malarial infection in humans, *P. berghei* invariably proceeds to a late-end-stage infection characterized by overwhelming parasitaemia associated with profound haemolytic anaemia. Anti-GPI vaccination did not prevent this process, as all immunized animals eventually succumbed to massive parasitaemias by day 15 (mean 64.5% ± 12.1), associated with a 75% reduction in erythrocyte density (data not shown). Although anti-GPI vaccination did not prevent hyperparasitaemia with attendant haemolytic anaemia, the relevance of these observations to human parasite burdens and dyserythropoetic anaemia remains unclear.

This study was designed to test the hypothesis that GPI is causally involved in rodent malarial pathogenesis, including metabolic derangement, and to determine whether vaccination against this target affords clinical protection in the best small animal model available. Mice were primed and boosted with 176 ng glycan per dose, which may be a suboptimal quantity. Systematic optimization with respect to formulation, carrier/hapten ratios, adjuvants, and dosage or timing of the immunization regimen is beyond the scope of this study. Therefore it is possible that the degree of protection against disease observed here may improve further depending on these variables. Similarly, anti-GPI vaccination may conceivably be beneficial in other malarial disease syndromes not sufficiently modelled by acute *P. berghei* ANKA infection, for example, dyserythropoietic anaemia.

After initial susceptibility to severe disease, children in holoendemic regions are thought to develop acquired clinical immunity that protects against life-threatening pathology despite persistent high levels of parasitaemia[26–28]. The validity of this proposition, and whether GPI is a target of clinical immunity, remain to be determined. GPI may be non-self in humans, and antibodies to GPI lipid domains may be associated with protection against disease[29]. The chemical synthesis of GPI fragments reported here should aid in testing these hypotheses and in epitope mapping of human anti-GPI antibodies. In contrast to acquired clinical immunity, anti-parasite immunity takes many more years to develop[28] and is easily lost, reflecting the problems of antigenic diversity, antigenic variation, redundancy in invasion pathways, immune evasion strategies and genetic restriction in the immune response to parasite antigens. Current approaches to anti-malarial vaccines seek nonetheless to induce anti-parasite immunity through parasiticidal mechanisms targeted to parasite protein antigens. The public health potential of alternative anti-disease vaccine strategies is demonstrated by the highly effective tetanus and diptheria toxoid vaccines that protect against the most injurious consequences of infection by targeting bacterial toxins[30]. The findings of this study suggest that GPI is a highly conserved endotoxin of malarial parasite origin. A non-toxic

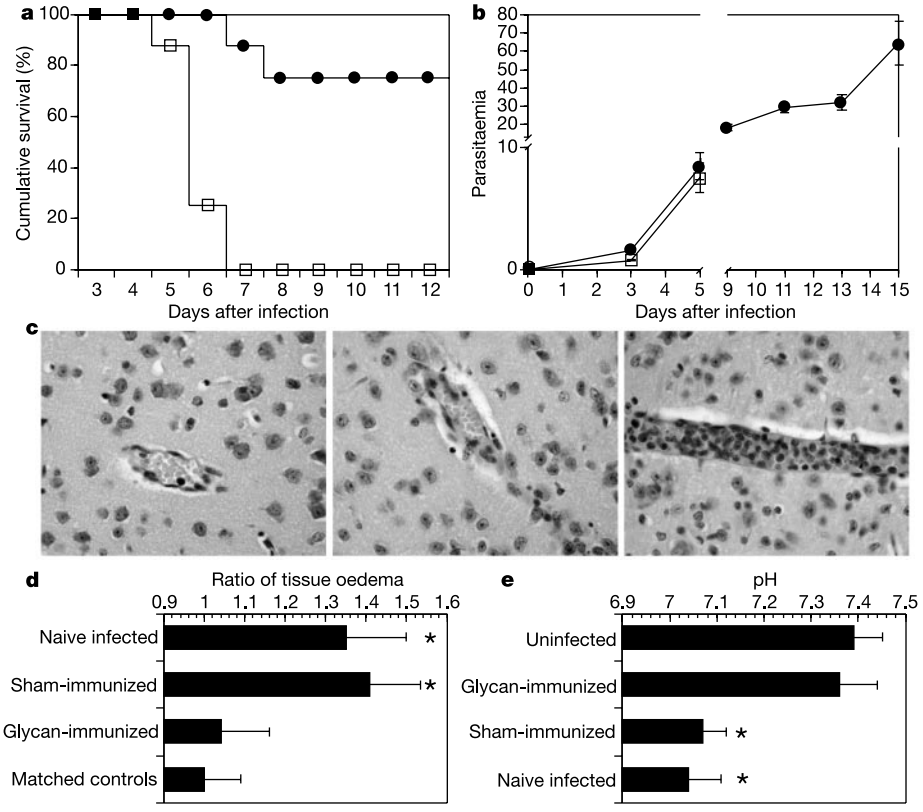

Figure 3 Immunization against the synthetic GPI glycan substantially protects against murine cerebral malaria, pulmonary oedema and acidosis. **a**, **b**, Kaplan–Meier survival plots (**a**) and parasitaemia levels (number of parasites per 100 red blood cells, **b**) of KLH-glycan-immunized (filled circles) and sham-immunized (open squares) mice challenged with *P. berghei* ANKA. **c**, Haemotoxylin and eosin-stained sections of brain tissue showing blood vessels from KLH-glycan-immunized (left and centre panels) and sham-immunized (right panel) mice killed on day 6 after infection. **d**, As an index of pulmonary oedema, the ratio of wet weight to dry weight of lungs from KLH-glycan-immunized ($n = 5$), sham-immunized ($n = 7$) and naive mice ($n = 8$) at day 6 after infection are expressed as a proportion of the lung wet:dry weight ratio of age/sex-matched uninfected ($n = 5$) controls. **e**, pH (±s.e.m.) of serum drawn at day 6 from uninfected mice ($n = 4$) and from naive ($n = 6$), immunized ($n = 5$) and sham-immunized ($n = 6$) donor mice infected with *P. berghei* ANKA. Asterisk, $P < 0.05$.

GPI oligosaccharide coupled to carrier protein is immunogenic and provides significant protection against malarial pathogenesis and fatalities in a preclinical rodent model. GPI may therefore contribute to life-threatening disease in humans. These data suggest that an anti-toxic vaccine against malaria might be feasible and that synthetic fragments of the *P. falciparum* GPI may be developed further to that end. □

Methods

Protein/glycan conjugation

Synthetic GPI glycan 1 was reacted with a tenfold molar excess of Traut's reagent (2-iminothiolane) in 60 mM triethanolamine, 7 mM potassium phosphate, 100 mM NaCl, 1 mM EDTA, pH 8.0 in the cold for 90 min under nitrogen, to introduce a sulphhydryl onto the free primary amine (ethanolamine). The sample was desalted by Biogel P4 filtration in coupling buffer at 4 °C, and the sample added to maleimide-activated KLH or OVA (Pierce) overnight. After exhaustive dialysis against water, conjugation efficiency was estimated by gas chromatography/mass spectroscopy. Samples were hydrolysed in 6 M HCl and the trimethylsilyl derivatives quantified for *myo*-inositol content by selective ion monitoring using *scyllo*-inositol as internal standard. For the generation of sham-conjugated carrier proteins, maleimide-activated KLH or OVA (Pierce) were subjected to identical procedures, except that cysteine was substituted for sulphhydryl-modified glycan.

Infections

All experiments were in accordance with local Animal Ethics Committee regulations. Young adult C57BL6 mice from Jackson Laboratories were pre-bled and inoculated with 6.5 μg KLH-glycan (0.176 μg glycan, $n = 16$) or KLH-cysteine (sham-immunized, $n = 24$) emulsified in Freund's complete adjuvant, and boosted with equal amounts of immunogen in incomplete Freund's adjuvant. After two boosts, mice were rested and injected intraperitoneally with 1×10^6 erythrocytes infected with *P. berghei* ANKA. Naive mice ($n = 12$) served as unimmunized controls. Parasitemia levels were assessed from Giemsa-stained thin films. Mortality was checked twice daily. Mice were judged as developing cerebral malaria if displaying neurological signs such as loss of reflex or ataxia, or dying between days 5 and 12 after infection with relatively low parasitaemia levels. Differences in survival curves of *P. berghei*-infected mice across this time period were assessed by Cox–Mantel log rank transformation on Kaplan–Meier plots. Deaths from day 12 onwards were associated with high parasitaemia, lower rates of cerebral vascular occlusion, and anaemia as determined by haemocytometer counts.

Pathology

For histological analysis of cerebral pathology, brains were taken into 10% neutral-buffered formalin, sectioned (5 μm), and stained with haemotoxylin and eosin. In other experiments, groups of six naive, sham-immunized and KLH-glycan-immunized mice were challenged as above. All mice were killed at day 6, along with age/sex-matched uninfected controls, their serum collected for determination of pH, and lungs removed. The wet weight was determined immediately after removal of the organ, and the dry weight after overnight incubation at 80 °C[23]. Brains were taken for histological examination as above.

TNF output

Mycoplasma-free *P. falciparum* schizonts (3D7 strain) were prepared by gelatin flotation followed either by extraction with sample buffer (for SDS–polyacrylamide gel electrophoresis and western blots) or by saponin lysis and three washes in isotonic buffer. Parasites were taken up by sonication in complete medium, and aliquots of 5×10^6 cell equivalents in 100-μl volumes were pre-incubated for 1 h with the indicated concentration of test or control sera, followed by addition to 4×10^5 target RAW264.7 cells for 16 h in a 96-well plate. Levels of TNF-α in culture supernatants were determined by capture enzyme-linked immunosorbent assay according to manufacturer's protocol (Pharmingen) and quantified by interpolation against recombinant protein standard curves.

Immunofluorescence

Thin films of mature *P. falciparum* cultures at 10% parasitaemia were fixed in acetone at −20 °C and exposed to test and control antisera (1/80) followed, after washing in PBS, by 1/200 dilution of fluorochrome-conjugated goat anti-mouse IgG (γ-chain specific). Slides were photographed under appropriate illumination.

Statistics

For a statistical comparison between test and control groups, we used a Student's *t*-test, except for Kaplan–Meier survival plots, which were tested by Cox–Mantel log rank transformation.

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Competing interests statement

The authors declare that they have no competing financial interests.

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