

Gut Microbiota Elicits a Protective Immune Response against Malaria Transmission

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SUMMARY

Glycosylation processes are under high natural selection pressure, presumably because these can modulate resistance to infection. Here, we asked whether inactivation of the UDP-galactose:β-galactoside-α1-3-galactosyltransferase (*α1,3GT*) gene, which ablated the expression of the Galα1-3Galβ1-4GlcNAc-R (α-gal) glycan and allowed for the production of anti-α-gal antibodies (Abs) in humans, confers protection against *Plasmodium spp.* infection, the causative agent of malaria and a major driving force in human evolution. We demonstrate that both *Plasmodium spp.* and the human gut pathobiont *E. coli* O86:B7 express α-gal and that anti-α-gal Abs are associated with protection against malaria transmission in humans as well as in α1,3GT-deficient mice, which produce protective anti-α-gal Abs when colonized by *E. coli* O86:B7. Anti-α-gal Abs target *Plasmodium* sporozoites for complement-mediated cytotoxicity in the skin, immediately after inoculation by *Anopheles* mosquitoes. Vaccination against α-gal confers sterile protection against malaria in mice, suggesting that a similar approach may reduce malaria transmission in humans.

INTRODUCTION

Humans have relatively high levels of circulating antibodies (Abs) recognizing xeno-glycans expressed by pathogens (Oyeran et al., 2009). As for other antigens, xeno-glycans cannot be targeted by the immune system when also expressed as

self-glycans. This limitation can be bypassed by natural selection of mutations that inactivate the expression of self-glycans (Bishop and Gagneux, 2007). Presumably, natural selection of such loss-of-function mutations tailored the human anti-glycan immune repertoire through evolution (Bishop and Gagneux, 2007). This notion is supported by the inactivation of the cytidine monophosphate-N-acetylneuraminic acid hydroxylase-like (*CMAH*) gene in humans, which suppressed the expression of N-glycolylneuraminic acid (Neu5Gc) (Hayakawa et al., 2001) and allowed for immune reactivity against Neu5Gc (Tangvoranuntakul et al., 2003). In a similar manner, inactivation of the α1,3GT gene, which suppressed the expression of the Galα1-3Galβ1-4GlcNAc-R (α-gal) carbohydrate in ancestral anthropoid primates that gave rise to humans (Galili and Swanson, 1991), also allowed for immune reactivity against α-gal (Galili et al., 1984). While it has been argued that this evolutionary process is driven to a large extent by the acquisition of immune-resistance against pathogens expressing such glycans (Bishop and Gagneux, 2007; Cywes-Bentley et al., 2013), this was never tested experimentally.

Humans do not express α-gal and up to 1%–5% of the repertoire of circulating immunoglobulin M (IgM) and immunoglobulin G (IgG) in healthy adults is directed against this glycan (Macher and Galili, 2008). Production of α-gal-specific Abs is thought to be driven by exposure to bacterial components of the microbiota expressing α-gal (Macher and Galili, 2008), including specific members of the *Klebsiella spp.*, *Serratia spp.*, and *Escherichia coli spp.* (Galili et al., 1988). Expression of α-gal by these *Enterobacteriaceae* is associated with the bacterial capsule and cell wall glycoproteins, as well as with lipopolysaccharide (LPS) (Galili et al., 1988). Gut colonization by the human pathobiont *E. coli* O86:B7 (Pal et al., 1969) recapitulates the etiology of anti-α-gal Ab production in mice (Posekany et al., 2002) and in primates (Mañez et al., 2001), as well as the production of Abs directed against the α-gal-related anti-B blood group glycan in chickens

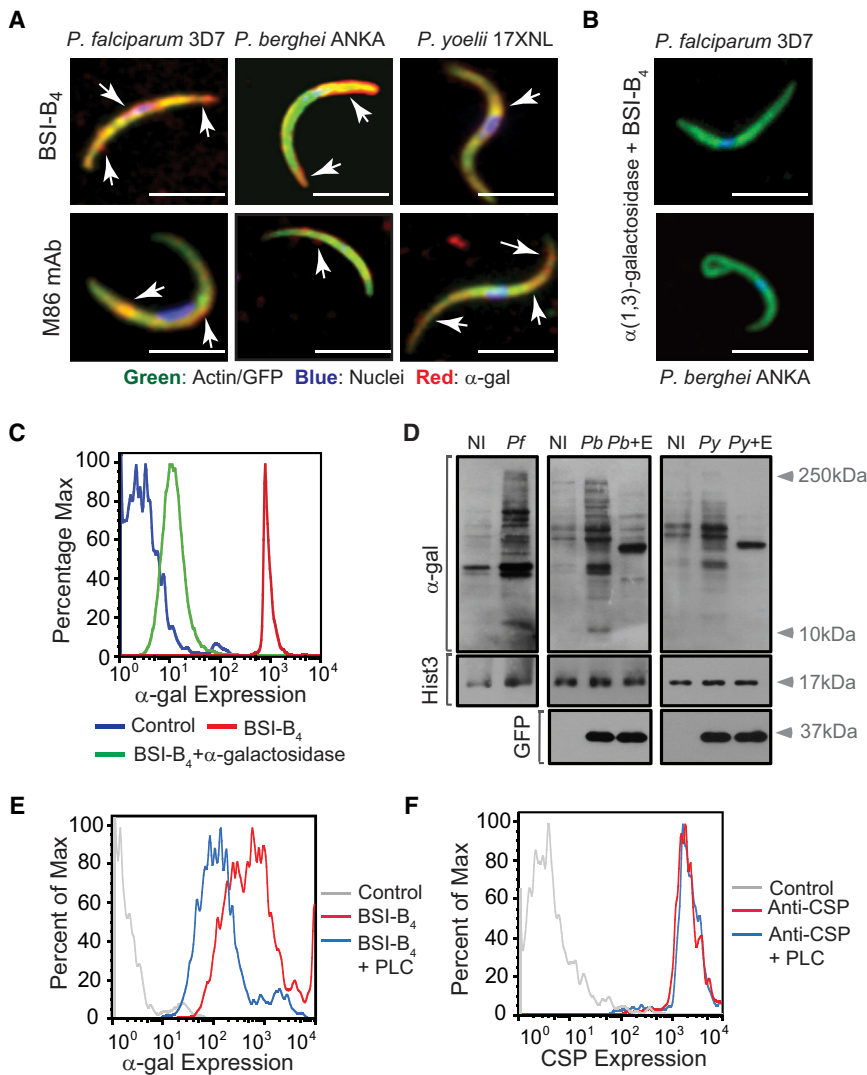


Figure 1. Detection of α -Gal in *Plasmodium* Sporozoites

(A) Composite images of GFP/actin (green), α -gal (red; white arrows), and DNA (blue) in *Plasmodium* sporozoites.

(B) Same staining as (A), after removal of α -gal by α -galactosidase. Images are representative of 2–3 independent experiments. Scale bar, 5 μ m.

(C) Detection of α -gal in *PbA*^{Hsp70-GFP} sporozoites by flow cytometry, representative of three independent experiments.

(D) Detection of α -gal in proteins extracted from salivary glands of noninfected (NI), *P. falciparum* 3D7 (Pf), *PbA*^{Hsp70-GFP} (Pb), or *P. yoelii* 17XNL (Py)-infected *A. mosquitoes*. Histone H3 (Hist3) and GFP were detected as loading controls. When indicated, α -gal was digested using α -galactosidase (E).

(E and F) Detection of α -gal (E) and CSP (F) in *PbA*^{Hsp70-GFP} sporozoites treated or not with phospholipase C (+PLC). Control is not stained. Data representative of 2–4 independent experiments.

See also Figure S1.

Malaria is transmitted to humans by the inoculation of *Plasmodium* sporozoites via the bite of female *Anopheles* (A.) mosquitoes (Ménard et al., 2013). While transmission may be rather efficient, only a fraction of the inoculated parasites manage to progress toward the establishment of infection (Rickman et al., 1990; Sauerwein et al., 2011; Verhage et al., 2005), hinting at a natural mechanism of protection that presumably targets the initial phases of the *Plasmodium* life cycle. Here, we demonstrate that production of anti- α -gal Abs in response to the gut *E. coli* O86:B7 pathobiont contributes

critically to this natural defense mechanism, reducing malaria transmission by *A. mosquitoes*.

RESULTS

Plasmodium spp. Express the α -Gal Glycan

The α -gal glycan was detected on the surface of *Plasmodium* sporozoites, as assessed by immunofluorescence for the human pathogen *Plasmodium falciparum* 3D7, as well as for the transgenic GFP-expressing strains of the rodent pathogens *Plasmodium berghei* ANKA (*PbA*) or *Plasmodium yoelii* 17XNL, using the lectin *Bandeiraea (Griffonia) simplicifolia*-I isolectin IB₄ (BSI-B₄) (Galili et al., 1985) or an anti- α -gal monoclonal antibody (M86 mAb) (Galili et al., 1998) (Figure 1A; Figures S1A and S1B available online). Specificity of α -gal detection was confirmed by its enzymatic removal using α -galactosidase (Figures 1B and 1C). Expression of α -gal was associated with proteins, as assessed by western blot in whole-cell extracts from *P. falciparum* 3D7, *PbA*, or *P. yoelii* 17XNL sporozoites

(Springer et al., 1959) and humans (Springer and Horton, 1969). This argues that gut colonization by *E. coli* O86:B7 may be particularly relevant in triggering the production of α -gal-specific Abs, presumably contributing to the high titers of these circulating Abs in healthy adult humans (Galili et al., 1988). Moreover, anti- α -gal Abs may also be produced in response to infection by pathogens expressing α -gal, such illustrated for gram-negative bacteria from *Salmonella* spp. or for protozoan parasites from *Trypanosoma* spp. (Avila et al., 1989).

Anti- α -gal Abs are cytotoxic toward α -gal-expressing pathogens, as demonstrated in vitro for bacteria (Galili et al., 1988), protozoan parasites (Avila et al., 1989), and viruses enveloped by xenogeneic α -gal-expressing cell membranes (Takeuchi et al., 1996). Whether anti- α -gal Abs confer resistance to these and/or other pathogens in vivo has, to the best of our knowledge, not been established. Here, we tested this hypothesis specifically for *Plasmodium* spp. infection, the causative agent of malaria and a major driving force that shaped the evolution of anthropoid primates, including humans.

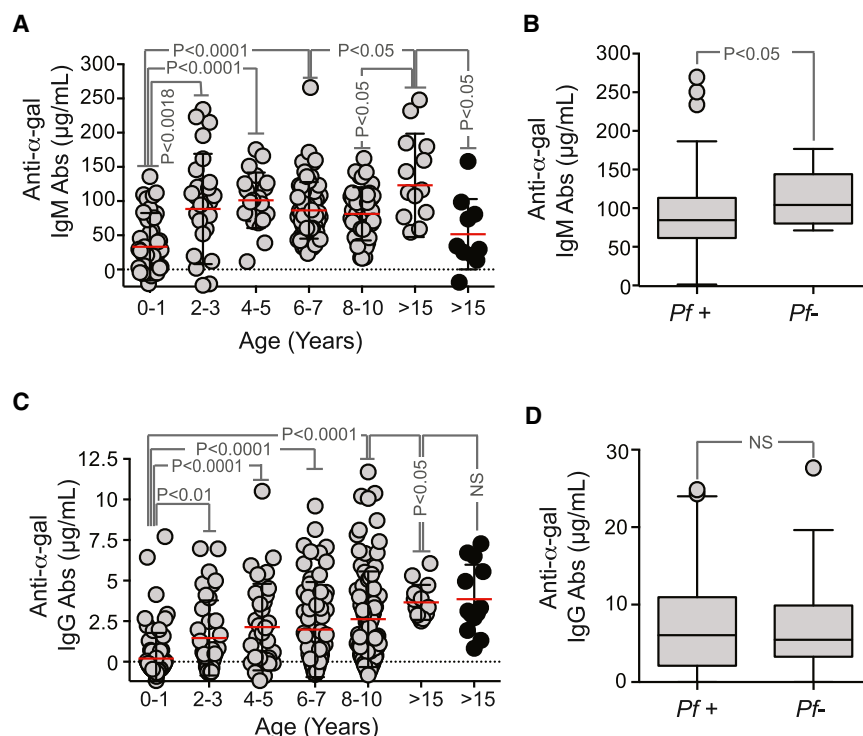


Figure 2. Anti- α -Gal IgM Abs Are Associated with Protection against Malaria Transmission in Individuals from a Malaria Endemic Region

(A) Anti- α -gal IgM Abs in individuals from a malaria endemic region in Mali (gray dots) or from the United States (black dots). Mean (red bars) \pm SD. (B) Levels of anti- α -gal IgM Abs in *P. falciparum*-infected (Pf+) versus noninfected (Pf-) children >4 years of age are shown as box plots in the same population as in (A). (C) Anti- α -gal IgG Abs in individuals from a malaria endemic region in Mali (gray dots) or from the United States (black dots). Mean (red bars) \pm SD. (D) Levels of anti- α -gal IgG Abs in *P. falciparum*-infected (Pf+) versus noninfected (Pf-) children >4 years of age are shown as box plots in the same population as in (C).

increased with age, reaching an average of 123.03 μ g/ml (95% CI: 79.3–166.7 μ g/ml) in adults—more than twice the level reported in adults with no malaria exposure, i.e., 51.6 μ g/ml (95% CI: 14.9–88.3 μ g/ml) (Figure 2A) (Avila et al., 1992; Doenz et al., 2000; Galili et al., 1984; Parker et al., 1999). The average level of

(Figure 1D) and confirmed by enzymatic removal of α -gal (Figure 1D). Residual levels of α -gal were detected in the salivary glands of noninfected mosquitoes, suggesting that this glycan may be generated, at least partially, by *A. mosquitoes* (Figure 1D).

Expression of α -gal by *PbA* sporozoites was reduced by \sim 4-fold when the glycosylphosphatidylinositol (GPI) anchor was cleaved by phospholipase C (PLC), as assessed by flow cytometry (Figure 1E). In contrast, GPI cleavage failed to reduce the expression of circumsporozoite protein (CSP), the main protein expressed at the surface of *Plasmodium* sporozoites (Figure 1F). This suggests that α -gal is bound to GPI-anchored surface proteins, including or not CSP, which despite being GPI-anchored (Moran and Caras, 1994) is resistant to PLC cleavage (Kimmel et al., 2003) (Figure 1F).

α -Gal-Specific IgM Abs Are Associated with Protection from *P. falciparum* Infection in Humans

We investigated whether a correlation exists between the levels of anti- α -gal Abs in healthy uninfected children and adults before the malaria season ($n = 330$ for IgG; $n = 229$ for IgM) and subsequent risk of *P. falciparum* infection (determined by biweekly PCR analysis of fingerprick blood samples) and febrile malaria (determined by weekly physical examination), during the ensuing 6 month malaria season in a cohort study in Mali, where this season is predictable and intense (Tran et al., 2014). In children <2 years, the average level of anti- α -gal IgM Abs was 33.4 μ g/ml (95% confidence interval [CI]: 18.4–48.3 μ g/ml) (Figure 2A), similar to that reported in children with no history of malaria exposure (Avila et al., 1992; Doenz et al., 2000; Galili et al., 1984; Parker et al., 1999). However, anti- α -gal IgM Abs

anti- α -gal IgM Abs in children >4 years of age who had no *P. falciparum* infections detected during the 6-month malaria season ($n = 13$) was higher than those who became infected ($n = 141$) (Figure 2B). This suggests that there is a positive correlation between the levels of anti- α -gal IgM Abs and incidence of *P. falciparum* infection.

The average level of anti- α -gal IgG Abs in children <2 years was 1.46 μ g/ml (95% CI: 0.22–0.69 μ g/ml) and increased in adults to 3.66 μ g/ml (95% CI: 3.04–4.28 μ g/ml) (Figure 2C). In contrast to IgM, the levels of circulating α -gal-specific IgG were similar between malaria-exposed and nonexposed adults, suggesting that *P. falciparum* infection fails to drive an IgG response directed against α -gal (Figure 2D). This also suggests that there is no correlation between anti- α -gal IgG Abs and incidence of *P. falciparum* infection. Time-to-event analysis did not show a correlation between α -gal-specific IgM and IgG levels before the malaria season and subsequent risk of *P. falciparum* infection ($p = 0.76$ and $p = 0.08$, respectively) or febrile malaria ($p = 0.35$ and $p = 0.18$, respectively).

Gut Colonization by *E. coli* O86:B7 Elicits a Protective α -Gal-Specific IgM Ab Response against Malaria Transmission

To test whether anti- α -gal IgM Abs are protective against malaria transmission, we took advantage of “human-like” α 1,3Gt-deficient mice. Unlike humans, wild-type mice have a functional α 1,3Gt gene and express α -gal on secreted and cell-surface glycoconjugates, suppressing the development of anti- α -gal immunity (Yang et al., 1998). Deletion of α 1,3Gt gene eliminates α -gal (Tearle et al., 1996), allowing for anti- α -gal Ab production in α 1,3Gt $^{-/-}$ mice (Chiang et al., 2000; Tearle et al., 1996; Yang

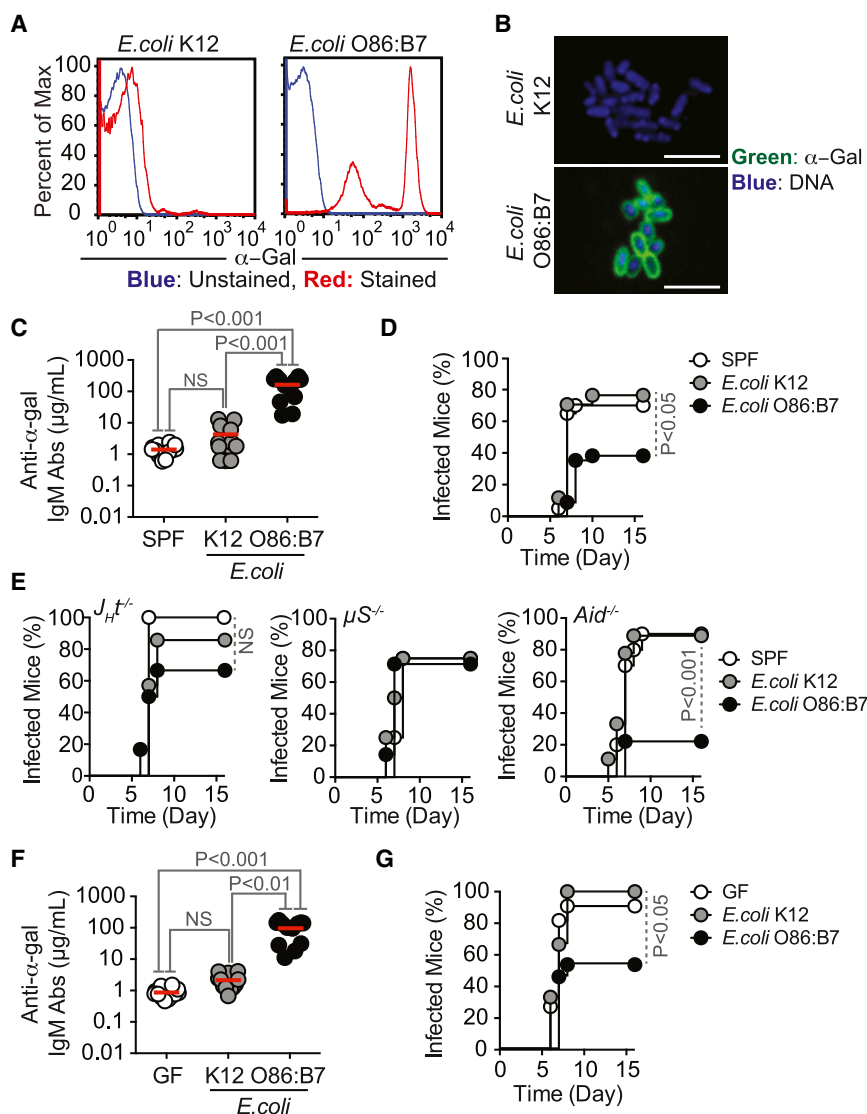


Figure 3. Gut Colonization by *E. coli* Expressing α -Gal Protects against *Plasmodium* Infection

(A and B) Detection of α -gal in *E. coli* strains by (A) flow cytometry and (B) immunofluorescence. Representative of 2–3 independent experiments. Composite images in (B), i.e., α -gal (green) and DNA (blue) at 100 \times magnification. Scale bar, 10 μ m.

(C and D) $\alpha 1,3Gt^{-/-}$ mice maintained under SPF were treated with streptomycin for 7 days. (C) Anti- α -gal IgM Abs levels were measured in $\alpha 1,3Gt^{-/-}$ mice not colonized (SPF), colonized with *E. coli* K12, or colonized with O86:B7 strains (2–3 experiments; n = 12). (D) Incidence of blood stage of *Plasmodium* infection (%) in mice colonized as in (C) and exposed to *PbA*^{EEF1a-GFP}-infected *A. stephensi* mosquitoes (four experiments; n = 17–34).

(E) Incidence of blood stage of *Plasmodium* infection (%) in $\alpha 1,3Gt^{-/-}$ *J_HT^{-/-}*, $\alpha 1,3Gt^{-/-}$ *Aid^{-/-}*, and $\alpha 1,3Gt^{-/-}$ μ S^{-/-} mice colonized as in (C) and exposed to *PbA*^{Hsp70-GFP}-infected *A. stephensi* mosquitoes (1–2 experiments; n = 4–10).

(F) Anti- α -gal IgM Abs were measured in GF $\alpha 1,3Gt^{-/-}$ mice not colonized (GF), colonized with *E. coli* K12, or colonized with O86:B7 strains (2–3 experiments; n = 12). (G) Incidence of blood stage of *Plasmodium* infection (%) in mice colonized as in (F) and exposed to *PbA*^{EEF1a-GFP}-infected *A. stephensi* mosquitoes (four experiments; n = 9–13).

Mean (red bars).

See also Figure S2.

et al., 1998). However, $\alpha 1,3Gt^{-/-}$ mice are known to produce only residual levels of circulating anti- α -gal Abs when maintained under specific pathogen-free (SPF) conditions (Chiang et al., 2000). Production of anti- α -gal Abs can be enhanced upon enteric exposure to *E. coli* O86:B7 (Posekany et al., 2002). We confirmed that *E. coli* O86:B7 expresses high levels of α -gal (Yi et al., 2006), which is not the case for the *E. coli* K12 strain (Figures 3A and 3B). Colonization of $\alpha 1,3Gt^{-/-}$ mice by *E. coli* O86:B7 after antibiotic treatment (streptomycin sulfate; 5 g/l in drinking water for 7 days prior to colonization) increased the levels of circulating anti- α -gal IgM Abs from 1.4 μ g/ml (95% CI: 1.1–1.8 μ g/ml) to 162.9 μ g/ml (95% CI: 95.89–230.1 μ g/ml) before and after colonization, respectively (Figure 3C). Levels of anti- α -gal IgM Abs in colonized $\alpha 1,3Gt^{-/-}$ mice were in the range of adult individuals from a malaria endemic region (Figure 2A). In contrast, the levels of circulating anti- α -gal IgG Abs remained at residual levels, i.e., <1 μ g/ml (Figure S2A), again in the range of adult individuals from a malaria endemic region (Fig-

ure 2C). Colonization by *E. coli* K12 did not induce the production of circulating anti- α -gal Abs (Figure 3C). Gut colonization by *E. coli* O86:B7 was associated with protection of $\alpha 1,3Gt^{-/-}$ mice from *PbA* transmission by infected *Anopheles stephensi* mosquitoes (Figure 3D). This

was not the case when $\alpha 1,3Gt^{-/-}$ mice were or were not colonized by *E. coli* K12 (Figure 3D). To determine whether the protective effect associated with gut colonization by *E. coli* O86:B7 is mediated by anti- α -gal Abs, we performed similar colonization experiments in $\alpha 1,3Gt^{-/-}$ *J_HT^{-/-}* lacking B cells (Gu et al., 1993), $\alpha 1,3Gt^{-/-}$ μ S^{-/-} mice lacking circulating IgM (Ehrenstein et al., 1998) or $\alpha 1,3Gt^{-/-}$ *Aid^{-/-}* mice that fail to undergo Ig class switch recombination or somatic hypermutation (Muramatsu et al., 2000). Gut colonization by *E. coli* O86:B7 failed to protect $\alpha 1,3Gt^{-/-}$ *J_HT^{-/-}* and $\alpha 1,3Gt^{-/-}$ μ S^{-/-}, but not $\alpha 1,3Gt^{-/-}$ *Aid^{-/-}* mice from *PbA*-infected mosquitoes, as compared to genetic-matched control mice colonized or not by *E. coli* K12 (Figure 3E). This shows that the protective effect of gut colonization by *E. coli* O86:B7 acts via a mechanism mediated by anti- α -gal IgM Abs that do not undergo somatic hypermutation.

Germ-free (GF) $\alpha 1,3Gt^{-/-}$ mice had low but detectable levels of anti- α -gal IgM Abs, i.e., 0.87 μ g/ml (95% CI: 0.66–1.1 μ g/ml),

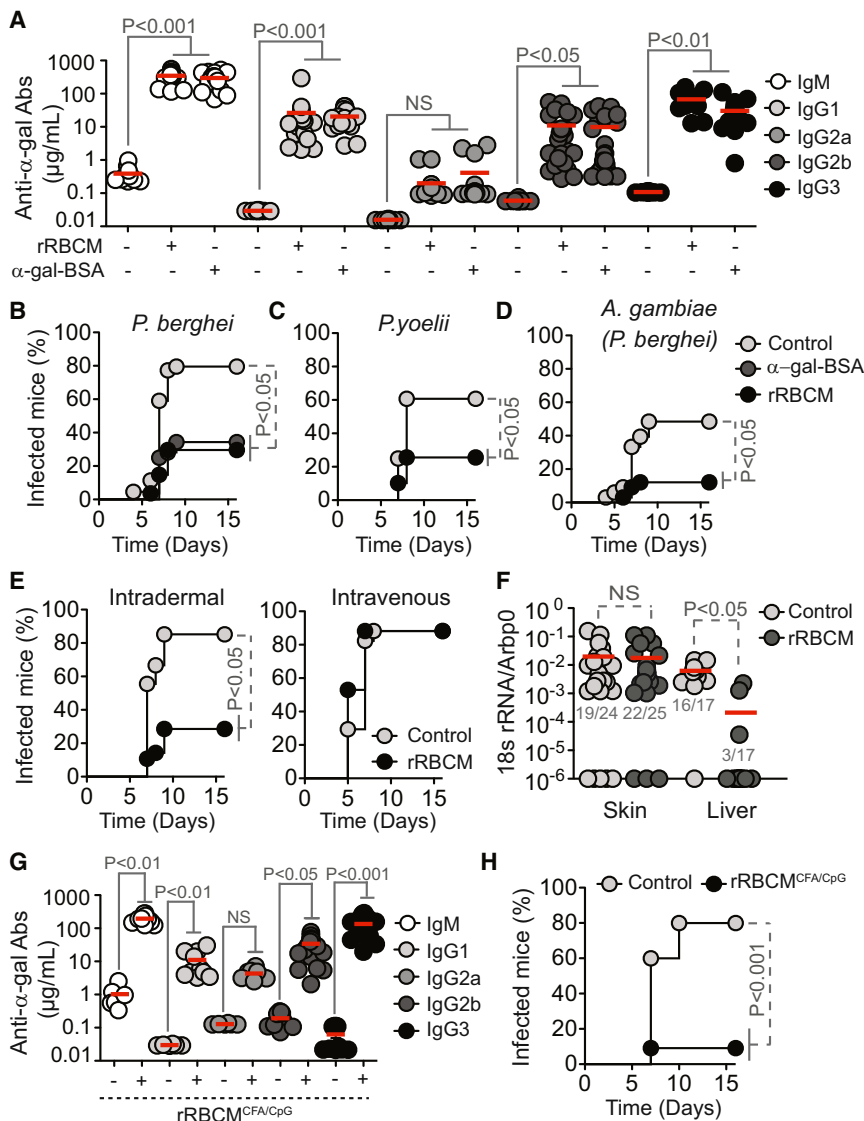


Figure 4. Protective Effect of α -Gal Immunization

(A) Anti- α -gal Abs in the serum of control (-) versus rRBCM (+) or α -gal-BSA (+) immunized $\alpha 1,3\text{Gt}^{-/-}$ mice (2–3 experiments; $n = 12$ –29).

(B–D) Incidence of blood stage of infection (%) in $\alpha 1,3\text{Gt}^{-/-}$ mice treated as in (A) and exposed to (B) $PbA^{\text{EEF1a-GFP}}$ -infected *A. stephensi* mosquitoes (seven experiments; $n = 27$ –44), (C) *P. yoelii* 17XNL-infected *A. stephensi* mosquitoes (five experiments; $n = 28$ –39), or (D) $PbA^{\text{EEF1a-GFP}}$ -infected *A. gambiae* mosquitoes (four experiments; $n = 27$ –34).

(E) Incidence of blood stage of infection (%) in nonimmunized (control) versus immunized (rRBCM) $\alpha 1,3\text{Gt}^{-/-}$ mice receiving $PbA^{\text{EEF1a-GFP}}$ sporozoites (3–4 experiments; $n = 17$ –28).

(F) *Plasmodium* 18S rRNA/Arbp0 mRNA in skin and liver of nonimmunized (control) versus immunized (rRBCM) $\alpha 1,3\text{Gt}^{-/-}$ mice exposed to $PbA^{\text{EEF1a-GFP}}$ -infected *A. stephensi* mosquitoes (3–5 experiments). Infected/total mice (gray nbs).

(G) Same as (A) in control (-) versus immunized (+; rRBCM emulsified in CFA+CPG) $\alpha 1,3\text{Gt}^{-/-}$ mice (two experiments; $n = 6$ –23).

(H) Incidence of blood stage of infection (%) in $\alpha 1,3\text{Gt}^{-/-}$ mice treated as in (G) and infected as in (B) (three experiments; $n = 16$ –19). In (A), (F), and (G), dots are individual mice and mean (red bars). See also Figures S3 and S4.

suggesting that these are natural Abs (Figure 3F). The production of anti- α -gal IgM Abs in GF $\alpha 1,3\text{Gt}^{-/-}$ mice being driven by expression of these glycans in food components is possible, but this has not been tested. GF $\alpha 1,3\text{Gt}^{-/-}$ mice did not produce anti- α -gal IgG Abs (Figure S2C). Susceptibility to *PbA* transmission by infected *A. mosquitoes* was similar in SPF versus GF $\alpha 1,3\text{Gt}^{-/-}$ mice (Figures 3D and 3G). When GF $\alpha 1,3\text{Gt}^{-/-}$ mice were monocolonized by *E. coli* O86:B7, the levels of circulating anti- α -gal IgM Abs increased to 96.62 $\mu\text{g/mL}$ (95% CI: 59.32–133.9 $\mu\text{g/mL}$) (Figure 3F), which is the range in which adult individuals from a malaria endemic region (Figure 2A), without concomitant induction of anti- α -gal IgG Abs (Figure S2C). Monocolonization by *E. coli* O86:B7, but not by *E. coli* K12, protected $\alpha 1,3\text{Gt}^{-/-}$ mice from *PbA* transmission by *A. mosquitoes* (Figure 3F). This suggests that gut colonization by a specific pathobiont expressing α -gal recapitulates to a large extent the normal etiology of the human anti- α -gal Ab response (Figure 2) and in-

duces protection against *Plasmodium* infection, such as observed in a malaria endemic region (Figure 2).

It should be noted that the percentage of infected red blood cell (RBC), i.e., parasitemia, and incidence of mortality were similar among those $\alpha 1,3\text{Gt}^{-/-}$ mice that were infected by *PbA* regardless of colonization (Figures S2B and S2D). This suggests that gut colonization by *E. coli* O86:B7 protects against *Plasmodium*

Immunization against α -Gal Protects from *Plasmodium* Transmission

Immunization of $\alpha 1,3\text{Gt}^{-/-}$ mice against α -gal, using rabbit RBC membranes (rRBCM) expressing high levels of α -gal or synthetic α -gal conjugated to BSA (α -gal-BSA) elicited the production of circulating anti- α -gal IgM and IgG Abs (Figure 4A). Control $\alpha 1,3\text{Gt}^{+/+}$ mice failed to produce anti- α -gal Abs (Chiang et al., 2000; Tearle et al., 1996; Yang et al., 1998) (Figure S3A). Circulating anti- α -gal immunoglobulin E (IgE) Abs were undetectable in control or immunized $\alpha 1,3\text{Gt}^{-/-}$ and $\alpha 1,3\text{Gt}^{+/+}$ mice (data not shown). The concentration of anti- α -gal IgM Abs in the plasma of immunized $\alpha 1,3\text{Gt}^{-/-}$ mice was in the range of adult individuals from malaria endemic regions (Figure 2A). Circulating anti- α -gal IgG Abs in immunized

$\alpha 1,3\text{Gt}^{-/-}$ mice, predominantly from IgG1, IgG2b, and IgG3 subclasses, were present at higher concentrations, as compared to total IgG in adult individuals from malaria endemic regions. Little or no circulating IgG2a (Figure 4A) or IgG2c (data not shown) were detected in immunized $\alpha 1,3\text{Gt}^{-/-}$ mice.

Immunization against α -gal protected $\alpha 1,3\text{Gt}^{-/-}$ mice from *PbA* (Figure 4B) and *P. yoelli* 17XNL (Figure 4C) transmission by infected *A. stephensi* mosquitoes, as well as from *PbA* transmission by *A. gambiae* mosquitoes (Figure 4D) versus control nonimmunized $\alpha 1,3\text{Gt}^{-/-}$ mice. Control immunized $\alpha 1,3\text{Gt}^{+/+}$ mice were neither protected from *PbA* (Figure S3B) nor *P. yoelli* 17XNL (Figure S3C) transmission by *A. stephensi* mosquitoes nor against *PbA* transmission by *A. gambiae* mosquitoes (Figure S3D) versus naive $\alpha 1,3\text{Gt}^{+/+}$ mice.

Immunized $\alpha 1,3\text{Gt}^{-/-}$ mice were protected from artificial transmission of *PbA* sporozoites via intradermal inoculation versus control nonimmunized $\alpha 1,3\text{Gt}^{-/-}$ mice (Figure 4E) or control immunized or nonimmunized $\alpha 1,3\text{Gt}^{+/+}$ mice (Figure S3E). Protection was no longer observed when sporozoites were inoculated intravenously (Figures 4E and S3E). This suggests that the protective effect of α -gal immunization is exerted in the dermis, presumably via an anti- α -gal Ab driven mechanism that is no longer effective once sporozoites reach the blood.

PbA transmission was associated with accumulation of *Plasmodium* 18S rRNA at the site of inoculation, as quantified in the ear pinna by qRT-PCR (Figure 4F). The relative amount of *Plasmodium* 18S rRNA was similar in immunized versus control nonimmunized $\alpha 1,3\text{Gt}^{-/-}$ mice (Figure 4F) or control immunized or nonimmunized $\alpha 1,3\text{Gt}^{+/+}$ mice (Figure S3F). Immunized $\alpha 1,3\text{Gt}^{-/-}$ mice did not accumulate *Plasmodium* 18S rRNA in the liver, when compared to control nonimmunized $\alpha 1,3\text{Gt}^{-/-}$ mice (Figure 4F) or control nonimmunized or immunized $\alpha 1,3\text{Gt}^{+/+}$ mice (Figure S3F). This suggests that α -gal immunization arrests the transit of inoculated sporozoites from the skin into the liver, without interfering with sporozoite inoculation by *A. mosquitoes*.

TLR9 Agonist Adjuvant Enhances the Protective Effect of α -Gal Immunization

Immunization of $\alpha 1,3\text{Gt}^{-/-}$ mice with rRBCM emulsified in complete Freund's adjuvant (CFA), supplemented with toll-like receptor 9 agonist CpG, enhanced anti- α -gal IgG2b and IgG3 Ab response by 2- to 3-fold (Figure 4G) versus immunization without adjuvant (Figure 4A). This was associated with 88% reduction in the relative risk of transmission of *PbA* infection by *A. mosquitoes* (95% CI: 0.032–0.452) versus 61% reduction upon immunization without adjuvant (95% CI: 0.209–0.726) (Figures 4B and 4H). This protective effect was not observed in control $\alpha 1,3\text{Gt}^{+/+}$ mice (Figures S3G and S3H).

Parasitemias were similar in immunized $\alpha 1,3\text{Gt}^{-/-}$ mice not protected from *PbA* infection versus control nonimmunized $\alpha 1,3\text{Gt}^{-/-}$ mice as well as control nonimmunized or immunized $\alpha 1,3\text{Gt}^{+/+}$ mice (data not shown). Moreover, when infected, all mice succumbed to experimental cerebral malaria. This suggests that while protective against malaria transmission, α -gal immunization is not protective against the development of severe disease if *Plasmodium* manages to establish infection. In

keeping with this notion, when inoculated with *PbA*-infected RBC, immunized $\alpha 1,3\text{Gt}^{-/-}$ mice developed similar levels of parasitemia and disease severity, as compared to control nonimmunized $\alpha 1,3\text{Gt}^{-/-}$ mice as well as to control nonimmunized or immunized $\alpha 1,3\text{Gt}^{+/+}$ mice (Figure S4A).

We tested further whether the protective effect conferred by α -gal immunization is associated with sterile protection, i.e., inability of *Plasmodium* to establish blood stage of infection. Passive transfer of RBCs harvested from protected immunized $\alpha 1,3\text{Gt}^{-/-}$ mice at day 8–9 post-*PbA* transmission by *A. mosquitoes* failed to transmit disease to naive $\alpha 1,3\text{Gt}^{-/-}$ mice (Figure S4B). In contrast, passive transfer of RBC harvested from nonprotected immunized $\alpha 1,3\text{Gt}^{-/-}$ mice, readily transmitted disease to naive $\alpha 1,3\text{Gt}^{-/-}$ mice (Figure S4B). This demonstrates that the protective effect of immunization against α -gal is associated with sterile protection against malaria.

Anti- α -Gal IgM and IgG Abs Produced in Response to α -Gal Immunization Confer Protection against Malaria Transmission

We asked whether the protective effect of α -gal immunization is mediated by anti- α -gal IgM and/or IgG Abs. Immunized $\alpha 1,3\text{Gt}^{-/-}\text{J}_\text{H}\text{T}^{-/-}$ mice failed to produce anti- α -gal IgM or IgG Abs versus naive $\alpha 1,3\text{Gt}^{-/-}\text{J}_\text{H}\text{T}^{-/-}$ mice or immunized $\alpha 1,3\text{Gt}^{-/-}$ mice (Figure 5A). Moreover, immunized $\alpha 1,3\text{Gt}^{-/-}\text{J}_\text{H}\text{T}^{-/-}$ mice were not protected against *PbA* transmission by *A. mosquitoes* versus control nonimmunized $\alpha 1,3\text{Gt}^{-/-}\text{J}_\text{H}\text{T}^{-/-}$ mice (Figure 5B). This shows that the protective effect of α -gal immunization is mediated via a B cell-dependent mechanism.

Immunization of $\alpha 1,3\text{Gt}^{-/-}\text{Aid}^{-/-}$ mice failed to induce the production of anti- α -gal IgG, but not IgM Abs, versus naive $\alpha 1,3\text{Gt}^{-/-}\text{Aid}^{-/-}$ or immunized $\alpha 1,3\text{Gt}^{-/-}$ mice (Figure 5A). Immunized $\alpha 1,3\text{Gt}^{-/-}\text{Aid}^{-/-}$ mice were nevertheless protected against *PbA* transmission by *A. mosquitoes* versus nonimmunized $\alpha 1,3\text{Gt}^{-/-}\text{Aid}^{-/-}$ mice (Figure 5B). This confirms that anti- α -gal IgM Abs can confer protection against malaria transmission (Figure 2B) and that the protective effect of α -gal-specific IgM Abs does not require somatic hypermutation.

Immunization of $\alpha 1,3\text{Gt}^{-/-}\mu\text{S}^{-/-}$ mice failed to induce anti- α -gal IgM Abs, without interfering with anti- α -gal IgG Ab response versus naive $\alpha 1,3\text{Gt}^{-/-}\mu\text{S}^{-/-}$ mice or immunized $\alpha 1,3\text{Gt}^{-/-}$ mice (Figure 5A). Immunized $\alpha 1,3\text{Gt}^{-/-}\mu\text{S}^{-/-}$ mice were nevertheless protected from *PbA* transmission by *A. mosquitoes* versus control naive $\alpha 1,3\text{Gt}^{-/-}\mu\text{S}^{-/-}$ mice (Figure 5B). Immunized $\alpha 1,3\text{Gt}^{-/-}$ mice did not produce circulating anti- α -gal IgA or IgE Abs (data not shown) and a putative protective effect for these Ig isotypes was excluded. This demonstrates that anti- α -gal IgG Abs produced in response to immunization confer protection against malaria transmission.

Immunization of $\alpha 1,3\text{Gt}^{-/-}\text{Tcr}\beta^{-/-}$ mice lacking mature $\alpha\beta$ T cells (Mombaerts et al., 1992) compromised anti- α -gal IgM and IgG response versus control immunized $\alpha 1,3\text{Gt}^{-/-}$ mice (Figure 5A). Immunized $\alpha 1,3\text{Gt}^{-/-}\text{Tcr}\beta^{-/-}$ mice were not protected from *PbA* transmission by *A. mosquitoes* versus control naive $\alpha 1,3\text{Gt}^{-/-}\text{Tcr}\beta^{-/-}$ mice (Figure 5B). This shows that anti- α -gal Abs produced in response to immunization are T cell-dependent (Cretin et al., 2002) and so is their protective effect.

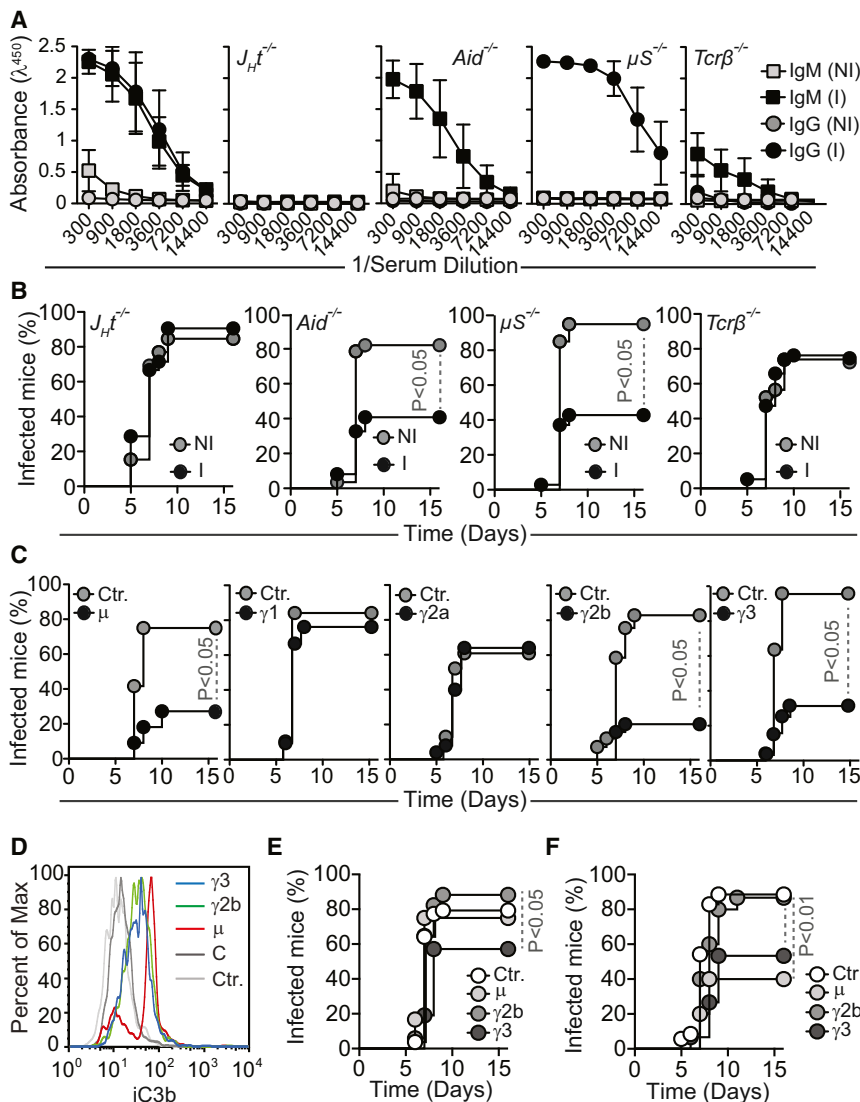


Figure 5. Protective Effect of Anti- α -Gal Abs

(A) Relative absorbance of anti- α -gal Abs (Mean \pm SD) in serial serum dilutions from nonimmunized (NI) or rRBCM-immunized (I) $\alpha 1,3Gt^{-/-}$ mice (two experiments; $n = 10$).

(B) Incidence of blood stage infection (%) in specific immune component-deleted $\alpha 1,3Gt^{-/-}$ mice immunized (I) or not (NI) as in (A) and exposed to $PbA^{EEF1a-GFP}$ -infected mosquitoes (3–7 experiments; $n = 13$ –41).

(C) Incidence of blood stage of infection (%) in $\alpha 1,3Gt^{-/-}$ mice after passive transfer of anti- α -gal Abs versus controls (no passive transfer; ctr.) exposed to $PbA^{EEF1a-GFP}$ -infected mosquitoes (4–7 experiments; $n = 19$ –32).

(D) C3 deposition in $PbA^{Hsp70-GFP}$ sporozoites not exposed (ctr.) or exposed to anti- α -gal Abs plus mouse complement (C). Representative of three independent experiments.

(E) Incidence of blood stage of infection (%) in $\alpha 1,3Gt^{-/-} C3^{-/-}$ mice after passive transfer of anti- α -gal IgM (μ), IgG2b ($\gamma 2b$), or IgG3 ($\gamma 3$) Abs versus controls (ctr.; no passive transfer) not receiving Abs, exposed to $PbA^{EEF1a-GFP}$ -infected mosquitoes (four experiments; $n = 21$ –37).

(F) Same as (E) in PMN-depleted $\alpha 1,3Gt^{-/-}$ mice (four experiments; $n = 15$ –25). See also Figures S5 and S6.

Naive and immunized $\alpha 1,3Gt^{-/-} J_H T^{-/-}$ (Figure S5A), $\alpha 1,3Gt^{-/-} Aid^{-/-}$, (Figure S5B) and $\alpha 1,3Gt^{-/-} \mu S^{-/-}$ (Figure S5C) mice, not protected from PbA transmission, developed similar levels of parasitemia and succumbed to experimental cerebral malaria. This was not the case for $\alpha 1,3Gt^{-/-} Tcr\beta^{-/-}$ mice (Figure S5D), consistent with the involvement of T cells in the pathogenesis of experimental cerebral malaria (Belnoue et al., 2002).

Passive transfer of anti- α -gal IgM to naive $\alpha 1,3Gt^{-/-}$ mice conferred protection against PbA transmission by *A. mosquitoes* (Figure 5C). This was also the case for passive transfer of anti- α -gal Abs from specific IgG subclasses, namely, IgG2b and IgG3 (Figure 5C), but not IgG1 or IgG2a (Figure 5C). Relative binding to α -gal was similar for all mAbs tested, as assessed by ELISA using α -gal-BSA as a solid-phase antigen (Figure S6A) or by immunofluorescence using PbA sporozoites (Figure S6B). Specificity of anti- α -gal binding to *Plasmodium* sporozoites was assessed by enzymatic removal of α -gal, confirming that these mAbs recognize specifically and only the α -gal glycan on the sur-

face of *Plasmodium* sporozoites (Figure S6C). IgG2a, IgG2b, and IgG3 mAbs are class-switched mutants derived from the original anti- α -gal IgG1 clone and as such have similar affinities for α -gal (Ding et al., 2008). These data reveal that while IgM anti- α -gal Abs are sufficient per se to confer protection against malaria transmission, this protective effect can be enhanced when specific subclasses anti- α -gal IgG Abs are present at sufficient high levels.

Once bound to the surface of *Plasmodium* sporozoites, anti- α -gal IgM, IgG2b, and IgG3 mAbs activated the classical pathway of complement, as assessed by C3 deposition (Figure 5D). Anti- α -gal IgG1 or IgG2a mAbs failed to activate complement (data not shown), and complement activation was also not observed in the absence of anti- α -gal Abs (Figures 5D and S7), showing that the alternative and lectin pathways of complement are not activated by *Plasmodium* sporozoites.

We then asked whether the protective effect exerted by anti- α -gal Abs is mediated via a mechanism involving the activation of the complement cascade (Figure 5D) (Ding et al., 2008; Miyatake et al., 1998). Passive transfer of anti- α -gal IgM Abs or anti- α -gal IgG2b mAb to $\alpha 1,3Gt^{-/-} C3^{-/-}$ mice, which lack C3 and cannot activate the complement cascade, failed to confer protection against PbA transmission versus control $\alpha 1,3Gt^{-/-} C3^{-/-}$ mice (Figure 5E). Passive transfer of anti- α -gal IgG3 mAb to $\alpha 1,3Gt^{-/-} C3^{-/-}$ mice conferred residual but significant protection versus control $\alpha 1,3Gt^{-/-} C3^{-/-}$ mice (Figure 5E). This

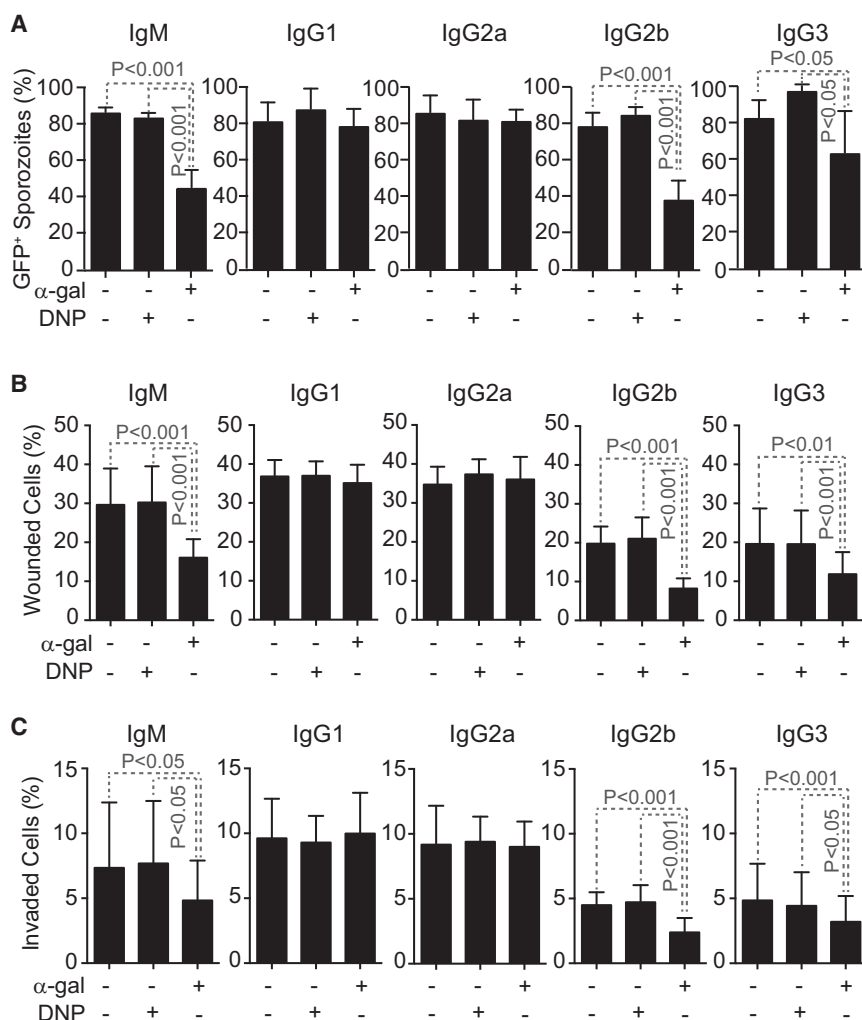


Figure 6. Protective Effect of Anti- α -Gal Abs against Hepatocyte Infection

(A) Mean percentage (%) of viable GFP⁺ *PbA*^{Hsp70-GFP} sporozoites ± STD (3–4 experiments) after exposure in vitro to anti- α -gal or control anti-DNP mAbs in the presence of mouse complement. (B and C) Mean percentage (%) of HepG2 cells (B) wounded (Dextran-Red⁺) or (C) invaded (GFP⁺) by *PbA*^{Hsp70-GFP} sporozoites treated as in (A) ± SD (six experiments).

while preventing the onset of cerebral malaria (data not shown), consistent with previous findings (Chen et al., 2000).

Anti- α -Gal Abs Are Cytotoxic to *Plasmodium* Sporozoites

Complement activation by anti- α -gal IgM, IgG2b, or IgG3 mAb was cytotoxic to *PbA* sporozoites in vitro, as assessed by sporozoite GFP expression (Figure 6A). Anti- α -gal IgG1 and IgG2a mAbs, which did not activate complement when bound to *Plasmodium* sporozoites (data not shown), were not cytotoxic (Figure 6A). The cytotoxic effect of anti- α -gal IgM, IgG2b, and IgG3 was similar when using mouse (Figure 6A) or rabbit (Figure S7A) complement but was strictly dependent on the presence of complement (Figure S7B). A similar cytotoxic effect was observed when quantifying viable “crescent-shaped” sporozoites (Figure S7C), an independent readout for sporozoite viability (Hegge et al., 2010). Isotype-matched control anti-dinitrophenyl (DNP)

Abs were not cytotoxic to *PbA* sporozoites in vitro (Figures 6A and S7A–S7C).

Anti- α -Gal Abs Inhibit Hepatocyte Invasion by *Plasmodium* Sporozoites

We asked whether anti- α -gal Abs inhibit hepatocyte transmigration (wounding) and/or hepatocyte invasion by *Plasmodium* sporozoites (Mota et al., 2001). Complement activation by anti- α -gal IgM, IgG2b, and IgG3 Abs inhibited hepatocyte transmigration (Figure 6B) and invasion (Figure 6C), as assessed in vitro for *PbA* sporozoites. This inhibitory effect was not observed when using anti- α -gal IgG1 or IgG2a Abs or isotype/subclass-matched control anti-DNP Abs (Figures 6B and 6C).

We then assessed whether anti- α -gal Abs inhibit the development of exoerythrocytic forms (EEF) of *Plasmodium*. Complement activation by anti- α -gal IgM, IgG2b, and IgG3 Abs reduced the number of EEF (Figure 7A), as well as the average EEF size (Figures 7B and 7C) formed in vitro by *PbA* sporozoites. Anti- α -gal IgG1 Abs did not show this inhibitory effect, while anti- α -gal IgG2a Abs did not reduce the number of EEF (Figure 7A) but had a residual inhibitory effect on EEF size (Figures 7B and

suggests that the protective effect exerted by anti- α -gal IgM and IgG2b Abs acts via a mechanism that is strictly complement dependent, whereas the protective effect of anti- α -gal IgG3 Abs is partially but probably not strictly dependent on complement activation. Infection incidence was similar in control $\alpha 1,3Gt^{-/-}$ C3^{-/-} versus $\alpha 1,3Gt^{-/-}$ C3^{+/+} mice (Figures 5C and 5E).

Complement activation generates C3a and C5a chemoattractants that promote IgG-dependent polymorphonuclear (PMN) cell cytotoxicity (Ding et al., 2008; Nimmerjahn and Ravetch, 2008; Yin et al., 2004). Therefore, we asked whether the protective effect of anti- α -gal Abs involves PMN cells. Passive transfer of anti- α -gal IgG2b Abs to $\alpha 1,3Gt^{-/-}$ mice, depleted from PMN cells by the administration of anti-Ly-6G (Gr-1) (Porcherie et al., 2011), failed to confer protection against *PbA* transmission by *A. mosquitoes*, whereas passive transfer of anti- α -gal IgM or IgG3 Abs conferred protection (Figure 5F). This suggests that the protective effect exerted by anti- α -gal IgM and IgG2b Abs acts via a mechanism strictly dependent on PMN cells, whereas the protective effect of anti- α -gal IgG3 Abs is partially but probably not strictly dependent on PMN cells. Depletion of PMN cells per se did not interfere with *Plasmodium* infection (Figure 5F)

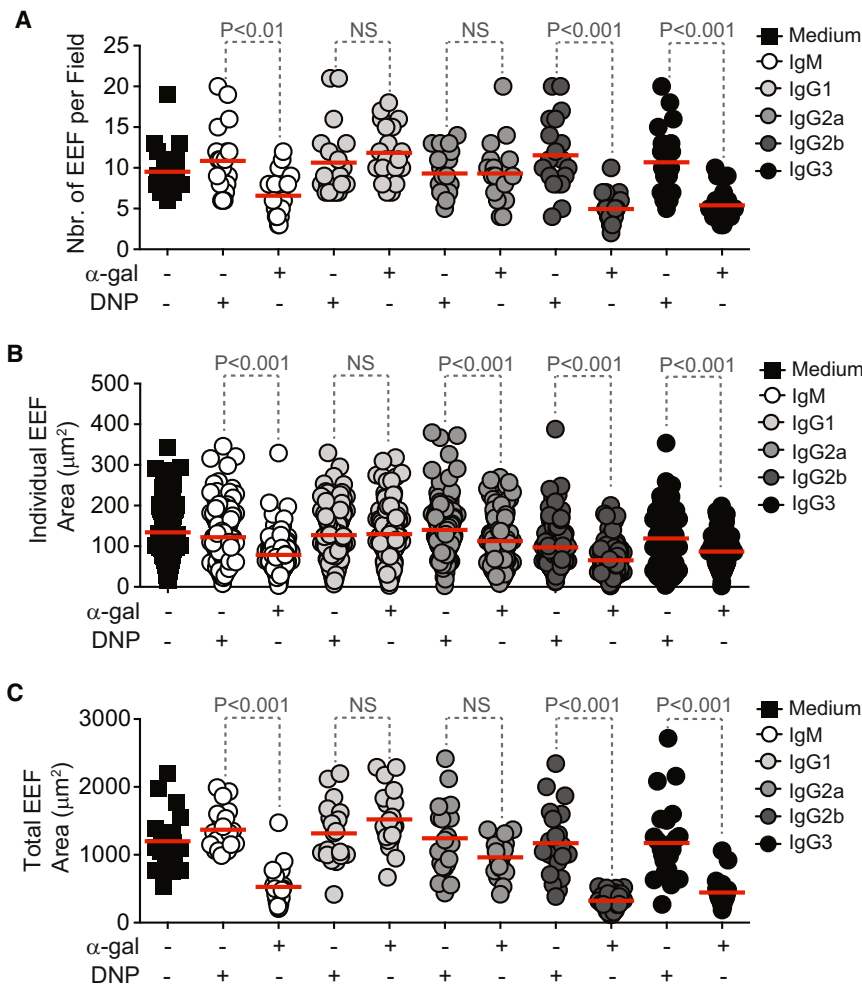


Figure 7. Protective Effect of Anti- α -Gal Abs against *Plasmodium* Maturation in Hepatocytes

(A) Number of EEF per field (dots; 20–23 fields). (B) Area of individual EEF (dots) ($n = 111$ –256 EEFs counted in 20–23 fields). (C) Total area of EEF (dots) per field (20–23 fields). HepG2 cells were incubated with *PbA*^{Hsp70-GFP} sporozoites, previously exposed to anti- α -gal or control anti-DNP mAbs in the presence of complement (A–C). See also Figure S7.

The protective effect exerted by anti- α -gal IgM Abs should be relevant to understand why malaria incidence is higher in children versus adults from malaria endemic regions (Modiano et al., 1996). Relative absence of these antibodies in children under the age of 2–3 years should favor malaria transmission, as compared to adults that have higher levels of circulating anti- α -gal IgM Abs (Figure 2A). This relative absence of anti- α -gal IgM in children may be explained by the (1) kinetics of establishment of an adult-like gut microbiota (Ringel-Kulka et al., 2013), (2) requirement of environmental and dietary exposure in the establishment of an adult-like gut microbiota, and/or (3) the kinetics of the establishment of adult-like B cell repertoire, including anti- α -gal B cells.

The protective effect exerted by anti- α -gal IgM Abs might also contribute to

7C). Isotype/subclass-matched control anti-DNP Abs did not modulate EEF numbers (Figure 7A) or average size (Figures 7B and 7C).

DISCUSSION

When inoculated in humans through the bite of an *A. mosquito*, *Plasmodium* sporozoites are confronted with relatively high levels of cytotoxic anti- α -gal IgM Abs (Figure 2A). That these Abs are protective against malaria transmission is supported by three independent lines of evidence. First, individuals from a malaria endemic region that show evidence of decreased *P. falciparum* infection risk have higher levels of circulating α -gal-specific IgM Abs, as compared to individuals who are susceptible to *P. falciparum* infection (Figure 2B). Second, when present at levels similar to those observed in individuals from a malaria endemic region—in $\alpha 1,3\text{Gt}^{-/-}$ mice colonized by human gut pathobiont *E. coli* O86:B7 expressing α -gal (Figure 3) or in immunized $\alpha 1,3\text{Gt}^{-/-}$ mice (Figures 4A and 4B)—anti- α -gal IgM Abs confer protection against malaria transmission. Third, passive transfer of anti- α -gal IgM Abs is sufficient per se to protect $\alpha 1,3\text{Gt}^{-/-}$ mice from malaria transmission (Figure 5C).

explain why only a small fraction of *Plasmodium* sporozoites inoculated by mosquitoes manage to progress toward the establishment of infection in humans. This is true even when *Plasmodium* sporozoites are inoculated under controlled experimental conditions in adults (Rickman et al., 1990; Sauerwein et al., 2011; Verhage et al., 2005). Presumably, when present at sufficient high levels in adults, circulating anti- α -gal IgM Abs prevent the large majority of *Plasmodium* sporozoites from establishing a successful infection. However, infection is established if as few as a couple of *Plasmodium* sporozoites manage to escape this natural mechanism of protection.

Whether α -gal detected at the surface of *Plasmodium* sporozoites (Figure 1) is produced by *Plasmodium* and/or by the mosquito is not clear. The salivary glands of noninfected mosquitoes express low levels of α -gal, as detected by western blot (Figure 1) and immunostaining (data not shown). *Plasmodium* sporozoites are masked by mosquito laminin (Warburg et al., 2007), an evolutionary conserved glycoprotein that in other species contains α -gal (Takahashi et al., 2014). It is possible therefore that anti- α -gal Abs recognize laminin or another mosquito-derived protein expressing α -gal, masking *Plasmodium* sporozoites (Warburg et al., 2007).

It is now well established that specific components of the gut microbiota can modulate immunity and resistance to infection (Belkaid and Hand, 2014; Honda and Littman, 2012). In support of this notion, resistance to viral and bacterial (Fagundes et al., 2012) infections is impaired in GF mice (Dolowy and Muldoon, 1964) or mice subjected to antibiotic-driven dysbiosis (Ichinohe et al., 2011). We reasoned that xeno-glycans expressed by specific components of the gut microbiota might trigger a protective immune response against pathogens expressing the same xeno-glycans. We show that this is the case for α -gal, a xeno-glycan expressed by the human gut pathobiont *E. coli* O86:B7, as well by *Plasmodium* spp. (Figures 1, 3A, and 3B). When colonized by *E. coli* O86:B7, $\alpha 1,3Gt^{-/-}$ mice produce an anti- α -gal IgM Ab response (Figures 3C and 3F) that confer protection against *Plasmodium* infection (Figures 3D, 3E, 3G, and 5C) via a lytic mechanism mediated by complement activation (Figures 5E and 6A). It is worth noticing that in a similar manner to other microbiota-driven resistance mechanisms, the protective effect exerted by *E. coli* O86:B7 acts at the level of a tissue barrier, i.e., the skin, to prevent *Plasmodium* transmission (Figures 4E and 4F).

Levels of circulating anti- α -gal IgG Abs in individuals from a malaria endemic region (Figure 2C), as well as in $\alpha 1,3Gt^{-/-}$ mice colonized with *E. coli* O86:B7 (Figures S2A and S2C), are ~ 30 -fold and ~ 40 to 70-fold, respectively, lower than levels of IgM anti- α -gal Abs. This may explain why basal levels anti- α -gal IgG Abs in individuals from a malaria endemic region are not associated with decreased risk of *P. falciparum* infection (Figure 2D). This also suggests that *P. falciparum* infection fails to induce class switch of the anti- α -gal Ig Ab response in those individuals. It is possible therefore that *P. falciparum* represses Ig class-switch recombination, explaining the residual levels of circulating anti- α -gal IgG Abs (Figure 2C).

While anti- α -gal Abs can provide sterile protection against malaria in mice (Figures 5B, 5C, and S4B), this is not commonly observed in malaria endemic regions in which adult individuals have circulating anti- α -gal IgM Abs, possibly because the levels of these Abs are below a threshold level required to provide sterile protection (Figure 2). However, we show that this natural mechanism of protection can be enhanced via immunization using adjuvants that favor the production of T cell-dependent complement fixing anti- α -gal IgG Abs (Figures 4G and 4H). Moreover, when coupled to *Plasmodium* antigens, this approach should enhance the immunogenicity of such antigens (Benatui et al., 2005) and boost the protective efficacy of candidate malaria vaccines based on such antigens (Olotu et al., 2013). This approach should be useful in preventing not only individual infections but also disease transmission given the protective effect of anti- α -gal Abs.

It is possible that the protective effect of “attenuated” sporozoite vaccine trials against malaria (Seder et al., 2013) is driven to some extent by an anti- α -gal Ab response, given the expression of α -gal by *Plasmodium falciparum* sporozoites (Figure 1). Whether a correlation can be established between the effectiveness of such candidate vaccines and a putative anti- α -gal IgG Ab response has not been established but may be useful to consider as a retrospective analyses.

As a final note, we predict that in a similar manner to anti- α -gal Abs other anti-glycan Abs may confer protection against malaria

as well as other vector-borne protozoan parasites (Huflejt et al., 2009; Lacroix-Desmazes et al., 1995; Nagele et al., 2013). Moreover, anti- α -gal Abs may also target other vector-borne protozoan parasites expressing α -gal, such as *Leishmania* spp. and *Trypanosoma* spp., the causative agents of Leishmaniasis and Trypanosomiasis, respectively (Avila et al., 1989). As such, vaccination approaches similar to the one proposed here for malaria may be considered against these diseases as well.

EXPERIMENTAL PROCEDURES

Cohort Study

For detailed analysis, see the Extended Experimental Procedures.

Immunization against α -Gal

Eight- to ten-week-old mice received 3×10^8 rabbit rRBCM equivalents (100 μ l; PBS; intraperitoneal [i.p.]). Adjuvants are described in the Extended Experimental Procedures. Mouse serum was collected 2 weeks after last immunization, and circulating anti- α -gal Abs were quantified by ELISA. See the Extended Experimental Procedures for details on anti- α -gal ELISA.

Passive Transfer of Anti- α -Gal mAbs

$\alpha 1,3Gt^{-/-}$ mice received anti- α -gal IgG1, IgG2a, IgG2b, and IgG3 mAbs (Ding et al., 2008; Yin et al., 2004) (150 μ g; 100 μ l per mouse) or polyclonal IgM (150 μ g; 300–400 μ l per mouse) via a single intravenous (i.v.) injection 24 hr prior to mosquito exposure.

Plasmodium Strains

Transgenic *P. berghei* ANKA (*PbA*) strains expressing GFP under the *eef1 α* promoter, i.e., *PbA^{Eef1 α -GFP}* (259cl1; MR4; MRA-865) (Franke-Fayard et al., 2004), or under the *hsp70* promoter (Ishino et al., 2006), i.e., *PbA^{Hsp70-GFP}* (kindly provided by Robert Menard, Institut Pasteur), transgenic *P. yoelii* 17XNL strain expressing GFP under the *PbA eef1 α* promoter (MR4; MRA-817; kindly provided by Robert Menard, Institut Pasteur) (Weiss et al., 1989). For sporozoite production, see the Extended Experimental Procedures.

Plasmodium Transmission

A. stephensi or *gambiae* mosquitoes were allowed to feed on anesthetized mice (i.p.; 125 mg/kg ketamine; 12.5 mg/kg xylazine) placed on a warming tray. Two mosquitoes were allowed to probe and feed independently (90–100 s) on restricted to the edge of the mouse ear (10–12/3–4 mm) and dissected thereafter for confirmation of sporozoites in salivary glands. If negative, infection was repeated.

Sporozoites Inoculation

PbA^{Eef1 α -GFP} sporozoites were inoculated (i.d.) in the ear pinna (750 sporozoites in 20–30 μ l; 1% BSA in PBS) or i.v. in the retro-orbital vein (150 sporozoites in 50 μ l; 1% BSA in PBS) using a microsyringe (Nanofil 100 μ l; 33G beveled needle; World Precision Instruments).

Detection of α -Gal in Plasmodium Sporozoites

Sporozoites were stained with Alexa Fluor 647-conjugated BSI-IB₄ or Alexa Fluor 647-conjugated anti- α -gal mAbs and detected by confocal microscope or flow cytometer. For detection of α -gal in *PbA^{Hsp70-GFP}* by western blotting, see the Extended Experimental Procedures. Green coffee bean α -galactosidase (50–200 μ l; 5 U/ml; 60–90 min; 25°C; Sigma Chemical) was used to hydrolyze terminal α -galactosyl moieties from glycolipids and glycoproteins (Luo et al., 1999).

Statistical Analysis

All tests (except human cohort studies) were performed using the GraphPad Prism (v. 6.0) (GraphPad Software). Human analyses were performed in R (v. 3.0.2). Detailed analyses are described in the Extended Experimental Procedures.

Mice

Experiments in mice were performed in accordance with protocols approved by the Ethics Committee of the Instituto Gulbenkian de Ciência and the Portuguese National Entity (DGAV-Direção Geral de Alimentação e Veterinária). Experiments in mice were performed in accordance with the Portuguese (Decreto-Lei no. 113/2013) and European (directive 2010/63/EU) legislation related to housing, husbandry, and animal welfare. C57BL/6 $J_H T^{-/-}$ (Gu et al., 1993), $Tcr\beta^{-/-}$ (Mombaerts et al., 1992), $Aid^{-/-}$ (Muramatsu et al., 2000), $\mu S^{-/-}$ (Ehrenstein et al., 1998), and $C3^{-/-}$ (Circolo et al., 1999) mice were crossed with C57BL/6 $\alpha 1,3Gt^{-/-}$ mice (Shinkel et al., 1997; Tearle et al., 1996). For the details on genotyping, see the [Extended Experimental Procedures](#).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and seven figures and can be found with this article online at <http://dx.doi.org/10.1016/j.cell.2014.10.053>.

AUTHOR CONTRIBUTIONS

B.Y. contributed to study design, performed, and/or contributed critically to all experiments, analyzed data, and contributed to writing of the manuscript. In some experiments, B.Y. was assisted by S.R., S.P., T.M.T., and P.D.C. designed, performed, and analyzed the human studies. R.G. performed the western blot experiments. H.S. supervised J.G. in the establishment and maintenance of *Plasmodium*-infected A. mosquitoes. A.R. produced and trouble-shooted all mAb production. P.J.C. and A.J.F.A. generated $\alpha 1,3Gt^{-/-}$ mice. A.S.C. provided anti- α -gal hybridomas. O.K.D. and B.T. organized the human studies and provided the human serum samples. M.P.S. formulated the original hypothesis, drove the study design, and wrote the manuscript with B.Y. All authors read and approved the manuscript.

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