Microbiota Control of Malaria Transmission

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Abstract

Stable mutualistic interactions between multicellular organisms and microbes are an evolutionarily conserved process with major impact to host physiology and fitness. Humans establish such interactions with a consortium of microorganisms known as the microbiota. Despite the mutualistic nature of these interactions, some bacterial components of the human microbiota express immunogenic glycans that elicit glycan-specific antibody (Ab) responses. The ensuing circulating Ab are protective against infections by pathogens that express those glycans, as demonstrated for *Plasmodium*, the causative agent of malaria. Presumably, a similar protective Ab response acts against other vector-borne diseases.

Microbiota and Natural Antibodies

Humans establish structured and often-mutualistic interactions with their microbiota (see Glossary), which are vertically transmitted and, to some extent, maintained throughout

life via horizontal transmission [1]. This occurs mainly at epithelial interfaces such as the intestinal and urogenital tracts, as well as at the lung and skin, where tightly juxtaposed epithelial cells limit systemic access to potentially damaging microbes and/or their component parts [2]. Resident immune cells at these epithelial barriers sense components of the microbiota via pattern recognition receptors (PRR), eliciting a host response that maintains the functional integrity of epithelial barriers [3]. This involves the production of mucus and anti-microbial peptides as well as IgA and IgM Ab [4, 5] that transverse epithelial barriers and bind to immunogenic components of the microbiota [6], modulating its composition and impact on host physiology [4, 5]. Here, we explore how Ab responses directed against the Galα1-3Gal (α-gal) glycan expressed by bacteria in the gut microbiota, confer protection against malaria [7] and presumably other vector-borne diseases.

Glycan-specific natural Ab

Humans have relatively high levels of circulating anti-glycan Ab [8, 9], including α-gal-specific Ab that account for up to ~1-5% of circulating IgM and IgG and are produced by 1% of the B cell repertoire of healthy adult individuals [10-12]. Anti-glycan Ab, including α-gal-specific Ab, are often referred to as natural Ab (NAb) because they are present in the circulation of healthy individuals in the "absence" of a traceable immunization [13]. While animals maintained under germ-free (GF) conditions can produce relatively low levels of NAb, production of physiologic levels of circulating glycan-specific Ab require the establishment of host microbiota interactions [7, 14]. In keeping with this notion, a significant proportion of circulating NAb recognize glycans expressed by components of the gut microbiota [15], as illustrated for anti-blood group NAb [16], which include α-gal-specific NAb [17].

Based on the immediate glycan recognition at the outer surface of microorganisms, NAb act as a first-line of defense against virus and bacteria [18, 19] and possibly protozoan parasite [20] infections (*reviewed in* [21] and [22]). When present above a certain threshold level at the time of infection, circulating NAb can target pathogens as soon as these breech epithelial barriers [21]. Activation of the classical complement

pathway and Ab-dependent cell-mediated cytotoxicity, limit pathogen expansion and dissemination into vital organs [19](*reviewed in* [21]).

Anti-glycan NAb, including α-gal-specific Ab, are generated in mice by long-lived B cells known as B1 cells [8, 23, 24] as well as by marginal zone B cells in the spleen [25](*reviewed in* [21]). The production of these Ab is triggered upon engagement of PRR and/or the B cell receptor by microbial associated molecular patterns, including glycans such as those in bacterial lipopolysaccharide (LPS). This results in the generation of low affinity (K_d=10^{-4}-10^{-7} M) glycan-specific IgM Ab, via a mechanism that does not require T cell help and does not involve immunoglobulin (Ig) class switch recombination or affinity maturation. However, some glycan-specific Ab responses are associated with the production of high affinity (K_d>10^{-7} M), T cell dependent IgG Ab [13, 26].

Expression of identical or similar glycans by pathogens and their mammalians hosts raises the question as to how Ab responses targeting these glycans are generated. Presumably, glycan-specific Ab responses should only target xeno-glycans that are not expressed as part of self [27], thus avoiding autoimmunity and disease [13]. This constraint was circumvented for some self-glycans such as α-gal through an evolutionarily-based process whereby loss-of-function mutations in genes responsible for the expression of such glycans were selected for and fixed in populations [28] (Box 1, Box 2 and Figure 1).

In contrast to humans, most mammals including mice carry a functional *GGTA1* gene, which encodes a UDP-galactose:β-D-galactosyl-1,4-N-acetyl-D-glucosaminide α-1,3-galactosyltransferase (α1,3GT) that generates the Galα1-3Galβ1-4GlcNAc-R (α-gal) glycan (Box 1, Box 2 and Figure 1). Mammals also express a functional isoglobotriaosylceramide synthase (*iGb3S*) gene, which encodes a UDP-gal:β-D-galactosyl-1,4-glucosyl-ceramide α1,3GT that generates the Galα1-3Galβ1-4Glc-ceramide glycan (Box 1, Box 2 and Figure 1). As a result, α-gal-specific B cells are purged from the B cell repertoire of adult mice, which fail to generate anti-α-gal Ab responses. Deletion of the *Ggta1* gene in mice eliminates the expression of Galα1-3Galβ1-4GlcNAc-R glycan and allows for the production of anti-α-gal Ab [29]. This is possible despite the expression of Galα1-3Galβ1-4Glc-ceramide [30], presumably

because α-gal-specific Ab can discriminate Galα1-3Galβ1-4GlcNAc from Galα1-3Galβ1-4Glc-ceramide, based on GlcNAc or Glc-ceramide recognition, respectively [31, 32]. A similar mechanism is likely to explain how α-gal-specific Ab responses are possible in individuals expressing the Galα1-3Gal(Fucα1-2)β1-3GlcNAc B blood group antigen, suggesting again that anti-α-gal Ab can discriminate Galα1-3Galβ1-4GlcNAc or Galα1-3Galβ1-4Glc-ceramide from Galα1-3Gal(Fucα1-2)β1-3GlcNAc [12] (Box 1).

As argued above for other glycan-specific NAb responses, steady state production of α-gal-specific Ab in humans is sustained most probably by continuous exposure to bacterial components of the microbiota that express α-gal [14, 33]. These are likely to include members of the *Enterobacteriaceae* family of Gram-negative bacteria, such *Klebsiella, Serratia and Escherichia* (*E.*) *coli spp.* as well as Gram-positive bacteria, i.e. *Lactobacillus casei* [14]. In *Enterobacteriaceae*, α-gal is conjugated to LPS at the outer surface of the cell wall, as illustrated for *Salmonella minnesota* and *Klebsiella spp.* [14]. In some cases, α-gal is conjugated to the O-antigen of LPS, as illustrated for *E. coli* O86:B7 [34].

E. coli O86:B7 is a pathobiont of the human microbiota that is likely to contribute to the production of circulating anti-α-gal Ab. *E. coli* O86:B7 expresses the Galα1-3Gal(Fucα1-2)β1-3(or4)GlcNAc glycan conjugated to the to the O-antigen of LPS [34] and feeding live or dead *E. coli* O86:B7 to humans triggers the production of circulating anti-α-gal Ab directed against the Galα1-3Gal(Fucα1-2)β1-3(or4)GlcNAc human B blood group [17]. Moreover, when colonized by *E. coli* O86:B7, GGTA1 deficient (*Ggta1*$^{-/-}$) mice produce anti-α-gal IgM Ab, in the range of adult individuals from a malaria endemic region [7]. Expression of Galα1-3Gal by *E. coli* O86:B7 is under the control of a α1,3 □□□□□□□□□□□□s□□□□□□□(GT) encoded by the *wbnI* gene, which shares high homology with other genes expressed in related *Enterobacteriaceae* such as *E. coli* O55:H7 [34]. To what extent orthologue of the *wbnI* gene control the expression of α-gal glycan in other bacterial species that express α-gal conjugated to LPS, e.g. *Bacteroides ovatus, Helicobacter mustelae, Shigella dysenteriae, Klebsiella*

pneumoniae, *Campylobacter coli*, *Serratia marcescens* or *Salmonella typhimurium* remains to be established [35].

Levels of circulating anti-α-gal IgM Ab in humans are low to undetectable during the first years of post-natal life [7, 10], which is also the case for NAb directed against other glycans [8], including ABO blood group glycans [36]. Levels of circulating anti-α-gal Ab increase over time to reach steady at 3-5 years of age [36, 37]. Maternal transfer of anti-α-gal IgG Ab accounts for the relatively high levels of these Ab in the circulation of newborns, decreasing during the first few months of post-natal life and increasing thereafter to reach a steady state levels within the first 3-5 years [7, 36]. Anti-α-gal IgA NAb are also detected in secretory fluids, such as saliva and colostrum and as such can be vertically transferred by the mother to newborns [38].

One possible explanation for the relative low levels of circulating α-gal-specific IgM NAb of newborns is that the early B cell repertoire lacks glycan-specific B cells, though to arise thereafter either spontaneously or in response to microbiota colonization [8]. Consistent with this notion gut colonization by *E. coli* and *Bifidobacteria* promotes B cell maturation in newborns and infants [39]. Moreover, newborns harbor a simplified microbiota [40], which may be linked to the impact of newborn dietary on microbiota composition [41]. In addition, some components of the newborns' microbiota exert a negative impact over potentially virulent *Enterobacteriaceae* that express α-gal, i.e. colonization resistance [42]. This protective mechanism that prevents enteric infections during early post-natal life, might also avoid exposure to immunogenic bacteria expressing α-gal during a developmental time frame likely inducing a state of immunological tolerance [43].

Expression of α-gal by *Plasmodium* and other related protozoan parasites

Malaria is transmitted to humans through inoculation of a relatively small number of *Plasmodium* sporozoites, upon the bite of an infected female *Anopheles* mosquito [44]. Despite having a reportedly poor glycosylation profile [45], *Plasmodium spp.* express α-gal glycans detected at the surface of sporozoites [7] as well during the blood stage of

infection [7, 46-48]. In *Plasmodium* sporozoites α-gal glycans are conjugated to glycosylphosphatidylinositol (GPI)-anchored proteins [7, 49], other than circumsporozoite protein (CSP) [7], a (GPI)-anchored protein that covers the surface of *Plasmodium* sporozoites [50]. Whether these α-gal glycans are conjugated directly to proteins or lipids and their exact structure remains to be established.

A *α1,3GT* orthologous gene has been characterized in vector-borne protozoan parasites from *Trypanosomes* (*T.*) *spp.* [51], which include *T. brucei*, the causative agent of African human trypanosomiasis, i.e. sleeping sickness and *T. cruzi*, the causative agent of American trypanosomiasis, i.e. Chagas' disease. Both diseases are transmitted to humans via the inoculation of *Trypanosoma* metacyclic trypomastigotes in the skin, either through the bite of a tsetse fly from the *Glossina spp.* or upon deposition of infected feces by blood-sucking *Triatomine* bugs, respectively. *T. brucei* [52] and *T. cruzi* [53, 54] metacyclic trypomastigotes express α-gal glycans, conjugated to GPI-anchored proteins as well to other glycoproteins and glycolipids. Presumably this explains why individuals infected by *T. cruzi* produce high levels of circulating anti-α-gal Ab [54-56]. Whether this is also the case for *T. brucei* infection is not clear.

Leishmania (L.) is another genus of trypanosomatid protozoan parasites that expresses α-gal [55, 57], as illustrated for *L. chagasi*, the causative agent of human visceral leishmaniasis and *L. mexicana* or *braziliensis*, the causative agent of localized cutaneous and mucocutaneous leishmaniasis. Infection is transmitted to humans via inoculation of promastigotes upon the bite of sand flies from the *Phlebotomine spp.* [58]. Leishmaniasis is associated with the production of high levels of circulating anti-α-gal Ab, as illustrated in humans [56] and *Ggta1*$^{-/-}$ mice [59]. Ab directed against α-gal bind to the lipid fraction and associated GPI anchors of *Leishmania* promastigotes [60, 61] and opposite to the flagellar pocket of *Leishmania* amastigotes [55, 62]. This suggests that the α-gal glycans expressed by *Leishmania spp.* are immunogenic and that α-gal-specific Ab might confer to leishmaniasis, which remains to be tested experimentally

Targeting of *Plasmodium* and possibly other vector-borne protozoan parasites by α-gal-specific NAb

Circulating anti-α-gal IgM and IgG Ab target *Plasmodium* sporozoites immediately after inoculation in the skin, conferring sterile protection against malaria transmission [7](Figure 2). Consistent with their well-established cytolytic effect [63], anti-α-gal Ab kill *Plasmodium* sporozoites via a Fc-dependent mechanism involving the activation of the classical complement pathway [7] (Figure 2). Presumably, this explains why in malaria endemic areas, individuals with higher levels of circulating anti-α-gal IgM Ab have decreased risk of *P. falciparum* infection, as compared to infected individuals [7].

Considering that circulating α-gal-specific IgM Ab can be produced in response to α-gal expressing bacteria in the microbiota, gut colonization by those bacteria should confer protection against malaria transmission. In support of this hypothesis *Ggta1*$^{-/-}$ mice are protected from malaria transmission when (mono)colonized by the *E. coli* O86:B7 strain that expresses high levels of α-gal glycans [7] (Figure 2). This protective effect is mediated by the production of circulating α-gal-specific IgM Ab, as demonstrated by loss of protection in *E. coli* O86:B7 colonized *Ggta1*$^{-/-}$*μS*$^{-/-}$ mice, which lack circulating IgM [7]. Whether a similar protective mechanism occurs in humans when colonized by this or other bacterial strains expressing α-gal remains to be established. The recent observation that enteric colonization by *Enterobacteriaceae*, including *E. coli* and *Shigella*, is associated with reduced risk of *P. falciparum* infection in individuals from malaria endemic areas supports this hypothesis [64]. Whether these *Enterobacteriaceae* include α-gal expressing bacteria that trigger the production of circulating α-gal-specific IgM Ab [7, 65] conferring protection against malaria transmission [7], remains to be established (Figure 2).

The protective effect exerted by α-gal-specific IgM Ab against malaria appears to be restricted to the initial pre-hepatic stage of infection, targeting *Plasmodium* sporozoites in the skin but not in blood [7]. Moreover, neither α-gal-specific IgM nor IgG Ab appear to target the later, liver or blood stages of *Plasmodium* infection in mice and as such do not influence parasitemia or the pathogenesis of severe forms of malaria in mice, e.g.,

203 experimental cerebral malaria [7]. The reasons for this are not clear but might relate to
204 specific biologic aspects of IgM Ab, as discussed elsewhere [22].

205 Given the expression of α-gal by trypanosomatid protozoa parasites from
206 *Trypanosoma* and *Leishmania spp.*, it is reasonable to hypothesize that anti-α-gal NAb
207 might exert a similar protective effect against transmission of these vector-borne
208 pathogens. While this has not been formally tested, there is experimental evidence to
209 support this hypothesis. Anti-α-gal Ab can target *T. cruzi* for complement and cell-
210 mediated cytotoxicity *in vitro*, thereby reducing parasite infectivity in mice [66, 67].
211 Whether this is also the case for *T. brucei* was, to the best of our knowledge, not
212 established. Even though α-gal-specific Ab can target *Leishmania spp.* promastigotes
213 and amastigotes, it is not clear whether they confer protection against leishmaniasis.
214 Possibly, these parasites evolved to escape the cytotoxic effect of circulating anti-glycan
215 NAb including α-gal-specific IgM NAb [68, 69]. Supporting this notion, *Trypanosoma*
216 *spp.* can evade Ab cytotoxicity via different strategies, including antigenic variation of
217 α-gal-conjugated GPI-anchored variant surface glycoproteins [51] or shedding of α-gal
218 glycolipids, as demonstrated for *T. brucei* [69]. Moreover rapid establishment of
219 intracellular infection by *T. cruzi* or *Leishmania* might also contribute to escape α-gal-
220 specific IgM cytotoxicity [70].

221

222 **Evolutionary constraints imposed by α-gal specific Ab on vector-borne pathogens**

223 Cytolytic targeting of *Plasmodium* and eventually other protozoan parasites by α-gal-
224 specific IgM Ab would be expected to select loss-of-function mutations in putative
225 parasite *α□□□GT* orthologous genes involved in α-gal expression, a host pathogen
226 antagonistic co-evolution process known as the "Red Queen Hypothesis" [71]. There is
227 indeed evidence that some families of glycosyltransferase genes were purged out of the
228 *Plasmodium* genome [72]. However, this is less likely to occur when the
229 glycosyltransferase is required to support the life cycle of these parasites in their
230 arthropod vectors, which could be the case for *α□□□GT*. Yet another possibility is that

expression α-gal glycans is controlled by putative *α□□□GT* orthologous genes from the parasites as well as their arthropod vectors.

Plasmodium spp. might not express O-glycans, i.e. glycans covalently attached to proteins at serine/threonine (Ser/Thr) residues, but can express short N-glycans, i.e. glycans covalently attached to proteins at asparagine (Asn), as illustrated on the surface of *P. falciparum* trophozoites and schizonts [73]. Moreover *Plasmodium spp*. can synthesize uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) and guanosine diphosphate mannose (GDP-Man), the two α-gal precursors used by α□□□GT [74]. It is possible therefore that N-linked and perhaps O-linked oligosaccharide synthesis occurs in *Plasmodium spp*. via an unconventional and yet non-characterized pathway. However, a *Plasmodium α□□□GT* orthologous gene has so far not been identified [49], which raises the possibility that α□gal detected at the surface of *Plasmodium* sporozoites is produced, at least in part, by the *Anopheles* mosquito vector. That arthropods express α-gal is supported by the finding that the bite of a star tick, *Amblyomma americanum*, can trigger the production of α-gal-specific IgE Ab, eventually associated with the development of red meat allergy [75]. However, there is no clear putative arthropod *α1,3GT* orthologous gene and as such the origin of the α-gal glycans detected in *Plasmodium* sporozoites remains to be established.

If the expression of α-gal at the surface of *Plasmodium* sporozoites is controlled, to at least some extent, by the *Anopheles* mosquito, how would these glycans transfer from the vector into the parasite? The observation that the α-gal is conjugated to GPI-anchored proteins expressed at the surface of *Plasmodium* sporozoites [7], *Trypanosoma* [53] and possibly *Leishmania spp.* [60, 61, 76], opens the possibility that arthropod GPI-anchors may transfer from arthropod vectors to these parasites, via a process known as inter-membrane transfer of GPI-anchored proteins [77, 78]. In support of this notion, *Trypanosoma* GPI-anchored variable surface glycoproteins can transfer to the membrane of mammalian red blood cells [79] and as such it is possible that arthropod GPI-anchored proteins carrying α-gal might also transfer into the membranes of protozoan parasites. This remains however, to be tested experimentally.

Using α□gal glycans as target antigens in vaccines against vector-borne diseases.

Vector-borne diseases account for 17% of all infectious diseases worldwide, corresponding to an estimated one billion infected individuals and one million associated deaths per year (http://www.who.int/mediacentre/factsheets/fs387/en/). While malaria's death toll decreased by 20-30% over the past decade, presumably due to the implementation of global malaria control programs, there are still 219 million infected individuals yearly, of which 660.000 succumb to severe disease (http://www.who.int/mediacentre/factsheets/fs094/en/). Incidence of African human trypanosomiasis has also been steadily decreasing over the past decade, likely due to vector control strategies (http://www.who.int/mediacentre/factsheets/fs259/en/). The situation is somehow different for American trypanosomiasis with an estimated 6-7 million individuals infected by *T. cruzi* and a fraction of those developing Chagas disease (http://www.who.int/mediacentre/factsheets/fs340/en/). There is an estimated 1.3 million individuals infected by *Leishmania spp.* with an associated 20 000 deaths per year (http://www.who.int/mediacentre/factsheets/fs375/en/).

Eradication of vector-borne diseases will require the development of highly efficient vaccines conferring sterile protection against disease transmission. The finding that α-gal in bacterial components of the gut microbiota can trigger a systemic Ab response that confers sterile protection against malaria transmission argues that vaccination against α-gal might contribute to achieve this goal, at least in the context of malaria. Likely, the protective effect conferred by low affinity α-gal-specific IgM NAb produced against enteric bacteria should be enhanced via vaccination approaches eliciting high-affinity α-gal-specific IgG Ab responses [7]. Of note, newborns have only residual levels of circulating anti-α-gal IgM Ab and would benefit particularly from such an approach. Different strategies may be considered, namely glycan-based vaccines [80] or mucosal immunization by bacteria expressing glycans [81].

When conjugated to protein antigen, immunogenic glycans such as α-gal can trigger high-affinity T-cell dependent IgG Ab responses directed against peptide and glycan epitopes [82]. Briefly, glycoconjugates containing α-gal are rapidly captured by circulating anti-α-gal Ab and shuttled, via an Fc-receptor-mediated mechanism, to

dendritic cells that present peptide epitopes to naïve $CD4^+$ T helper (T_H) cells and cross-present to naïve $CD8^+$ T cytotoxic (T_C) cells [25, 83]. These glycoconjugates are also captured by peripheral α-gal-specific B cells, which account for 1% of the peripheral B cell repertoire and can present peptide epitopes to T_H cells [83]. Using such glycoconjugates in a vaccination strategy is particularly well suited when targeting *Plasmodium*, *Trypanosomes* or *Leishmania*, which have an initial extracellular stage of infection in the skin that can be targeted by high affinity glycan-specific IgG Ab. The intracellular stages of infection can be targeted by antigen-specific $CD4^+$ T_H and $CD8^+$ T_C cells, recognizing peptides derived from the glycoconjugate based vaccine.

Oral vaccination by live bacteria expressing specific antigens a widely used approach to obtain protective immunity against pathogens. In one approach, antigens are expressed in attenuated non-pathogenic bacterial strains such as *Salmonella typhi* [84] or *Vibrio cholerae* [85]. Alternatively, antigens can be expressed in live food grade bacteria such as *Lactococcus* [86], probiotic bacteria such as *Bifidobacteria* or gut commensal *Bacteroidetes, Alphaproteobacteria, Actinobacteria, Firmicutes* or *Fusobacteria*. The finding that when expressed in *Lactococcus lactis*, antigenic peptides derived from *Plasmodium* proteins can elicit a protective Ab response against *Plasmodium* infection [86, 87], suggests that this approach might be used in bacteria expressing α-gal to confer immune protection against malaria and possibly other vector-borne diseases.

Concluding remarks

The finding that when expressed by bacterial components of the gut microbiota glycans such as α-gal can elicit a systemic Ab response that confers sterile protection against malaria transmission could have several implications, not only to our current understanding of host microbial interaction but possibly to the eradication of malaria and likely other vector-borne diseases. The realization that mutualistic host-microbiota interactions can exert such protective effects suggests that these might be manipulated to reach therapeutic benefit (see Outstanding Questions box). This may be achieved either by diet manipulation in combination with gut colonization by natural or genetically engineered probiotic bacterial strains expressing α-gal. In order to translate these approaches into clinical practice, it would be important to compare the relative efficiency

of ant-α-gal Ab, as compared to other Ab responses targeting *Plasmodium* sporozoites such as for example those directed against the CSP antigen. Glycoconjugate based vaccines can be generated to combine the immunogenic effect of CSP and achieve robust and long lasting sterile protection against malaria transmission. The uniqueness of the α-gal immunization approach is that it can be used, in combination or not with other antigens, to prevent the transmission of malaria as well other major vector-borne diseases.

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Boxes

Box 1. Family 6 glycosyltransferases

Family□6 α1,3GT are encoded by the *ABO, GGTA1* and *iGbS3* genes and catalyze the formation of 1-3 glycosidic bonds between GalNAc or Gal and a Gal acceptor (EC 2.4.1.87). The human ABO α1,3GT includes α1,3GTA and α1,3GTB, which generate the A and B blood group glycan epitopes, i.e. GalNAcα1-3Gal(Fucα1-2)β1-3GlcNAc and Galα1-3Gal(Fucα1-2)β1-3GlcNAc, respectively. The Fucα1-2β1-3GlcNAc O blood glycan epitope is generated in the absence of α1,3GTA and α1,3GTB activity due to several loss-of-function mutations in these genes, maintained as balanced polymorphisms in human populations [88]. Functional deletions in genes encoding ABO α1,3GT are not associated with overt pathologic outcomes and therefore the physiologic role of the ABO blood group system remains elusive. The glycoprotein α-galactosyltransferase 1 (GGTA1) α1,3GT catalyzes the generation of Galα1-3Galβ1-4GlcNAc-R glycan, bound essentially to proteins. The *GGTA1* gene is functional in nearly all mammals except in Old World monkeys, including humans, which carry a mutated *GGTA1* pseudogene and do not express the Galα1-3Galβ1-4GlcNAc-R glycan [33]. The iGb3S α1,3GT catalyzes the generation of Galα1-3Galβ1-4Glc-ceramide glycan in a subset of isogloboside glycolipids [89]. In a similar manner to the *GGTA1* gene, humans carry a mutated *iGb3S* pseudogene [90] and do not express Galα1-3Galβ1-4Glc-ceramide.

Box 2. Evolutionarily based mechanisms of self vs. non-self glycan discrimination.

Several examples suggest that selection and fixation of loss-of-function in genes encoding glycosyltransferases that generate self-glycans acted as a major driving force in shaping the human anti-glycan Ab repertoire. These include loss-of-function mutations in ancestral anthropoid primates that deleted the *GGTA1* gene, which encodes a α1,3GT generating the Galα1-3Galβ1-4GlcNAc-R glycan [91]. Loss-of-function mutations were also selected and fixed in the human *iGb3S* gene, which encodes a α1,3GT that generates

the Galα1-3Galβ1-4Glc-ceramide glycan [90]. Deletion of these α1,3GT eliminated the expression of α-gal self-glycans and allowed for the emergence of immune reactivity against α-gal glycans, illustrated by the high levels of circulating anti-α-gal Ab detected in healthy humans [10]. Other examples of this evolutionarily based process include loss-of-function of the human cytidine monophosphate-N-acetylneuraminic acid hydroxylase-like (*CMAH*) gene, which suppressed the expression of N-glycolylneuraminic acid (Neu5Gc)[92] and allowed for immune reactivity against this glycan [93]. Loss of function mutations in these glycosyltransferases also altered the ability of some pathogens to bind to host glycans in a manner that supports infection, as proposed for the impact of Neu5Gc elimination in *Plasmodium* infection [94]. It likely that other loss-of-function mutations in glycosyltransferase genes shaped the human anti-glycan Ab repertoire and/or altered host pathogen interactions [9].

Glossary

Antibodies (Ab): The product of *Immunoglobin* (*Ig*) genes that recognize specifically molecular structures known as epitopes, i.e. antigenic determinant. These are part of a larger molecule that can trigger an Ab response, i.e. antigen. Ab are composed of a fragment antigen-binding (Fab) region that binds the epitope and a fragment crystallizable (Fc) region, which endows the Ab with effector function through the engagement of immune-based mechanisms. Different Fc region define IgM, IgA and IgG isotypes and subclasses of IgA (IgA1 and IgA2) or IgG (IgG1, IgG2, IgG3 and IgG4). The main function of Ab is to recognize and neutralize pathogens as well as to avoid microorganism transition into a pathobiont.

Glycosylation: is an evolutionary conserved biologic process defined as an "*enzyme-catalyzed covalent attachment of a carbohydrate to a polypeptide, lipid, polynucleotide, carbohydrate, or other organic compound*" [95]. Glycosylation is catalyzed, in most cases, by glycosyltransferases that use specific sugar nucleotide donors as substrates to generate glycans, that is, "any sugar or assembly of sugars, in free form or attached to another molecule" [95].

Interspecies interactions: include **mutualistic** interactions (i.e. commensalism) when organisms from both species benefit from their interaction, **commensal** interactions (i.e. commensalism) when one of the species benefits from the interaction without detriment to the other and **pathologic** interaction (i.e. parasitism) where one species, i.e. the pathogen, benefits from the interaction in detriment of the other, i.e. the host. In some cases mutualistic or commensal interactions change such that they becomes pathologic. In that case the organism species that benefits from this transition, in detriment of its host, is referred to as a **pathobiont**.

Microbiota: Multicellular organisms establish structured interactions with dynamic communities of microorganisms, including viruses, bacteria and fungi, collectively known as the microbiota. This interspecies relationship can range from mutualistic to commensal or pathogenic, depending on the inherent composition of the microbiota or the immune status of the host. Deregulation of host microbiota interactions impacts on host homeostasis and can have pathogenic effects.

Germ-free: Absence of germs, i.e. microorganisms. Animals or other multicellular organisms can be maintained under experimental germ-free conditions to establish a causal relationship between the microbiota and a given aspect of host physiology. When colonized by a specific microorganism or group thereof, germ-free animals or other multicellular organisms are referred to as gnotobiotic. This experimental approach is often used to determine causal relationship between a given microorganism or group of microorganisms and a given aspect of host physiology.

Red Queen Hypothesis: Living organisms are under a continuous selective pressure of exerted by parasitic relationships so that both host and parasites co-evolve to gain advantage over each other.

Figure Legends:

Figure 1: Evolutionarily based mechanisms of self vs. non-self glycan discrimination. Humans carry loss-of-function mutations that were inherited from ancestral anthropoid primates and hominids, impairing the expression of galactosyltransferase (GT)-encoding genes, such as *GGTA1* and *iGb3S* [90]. These eliminated the expression of Galα1-3Gal (α-gal) glycans as self antigens and allowed for the emergence of anti-α-gal antibody (Ab) responses [10], which confer protection against *Plasmodium* infection [7]. Presumably, this protective effect favored natural selection and fixation of such mutations in modern humans. . Similarly**,** loss-of-function of the human *CMAH* gene (encoding a cytidine monophosphate-N-acetylneuraminic acid hydroxylase-like protein) allowed for immune reactivity against N-glycolylneuraminic acid (Neu5Gc) [93] and likely altered the ability of ancestral forms of *Plasmodium* to infect ancestral hominids. This is thought to have driven *Plasmodium spp.* to co-evolve, which presumably give rise to the modern human pathogen *Plasmodium falciparum* [94]. It is likely that other loss-of-function mutations in glycosyltransferase genes shaped the human anti-glycan Ab repertoire and/or altered host pathogen interactions [9].

Figure 2: Microbiota driven protection against malaria transmission. Gut colonization by the *Enterobacteriaceae E. coli* O86B7, which recapitulates the etiology of anti-Galα1-3Gal (anti-α-gal) IgM antibodies (Ab) production in mice [65] and humans [17], confers protection against malaria transmission in mice [7]. This effect is mediated via the production of circulating anti-α-gal IgM Ab that target α-gal glycan expressed at the surface of *Plasmodium* sporozoites (Spz) [7]. Anti-α-gal IgM Ab trigger the activation of the classical pathway of complement (C1q, C5b9), which kills *Plasmodium* sporozoites in the skin and hence confers sterile protection against malaria [7]. BCR, B cell receptor; PRR, pattern recognition receptor; α3, Galα1-3Gal.

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455 **References**

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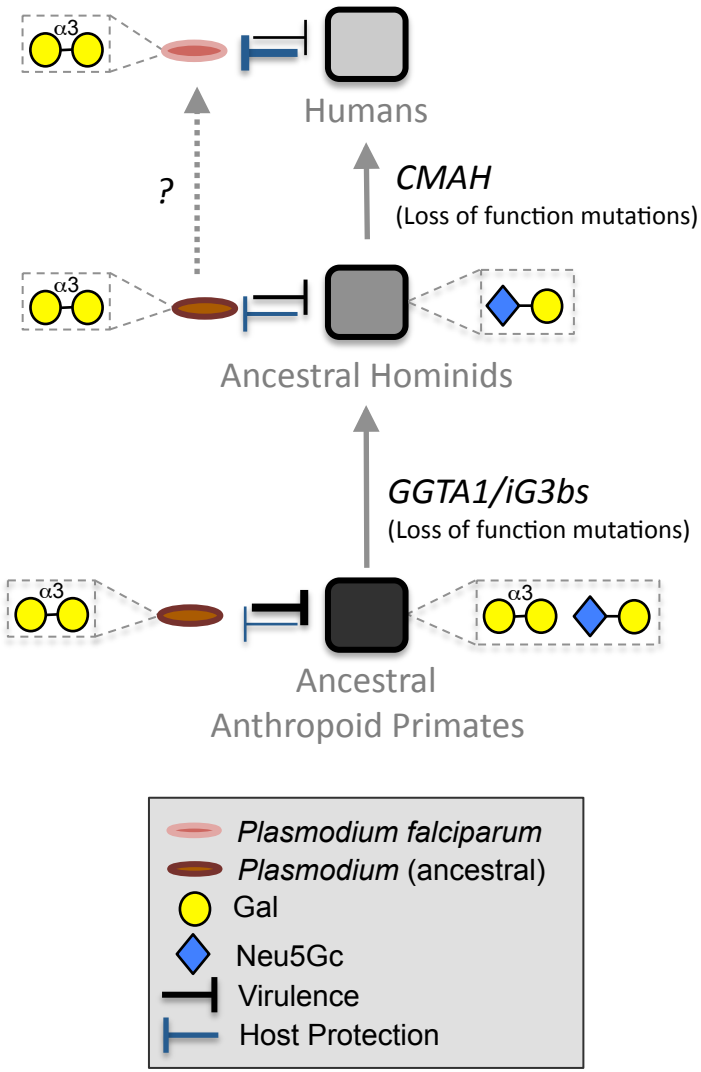
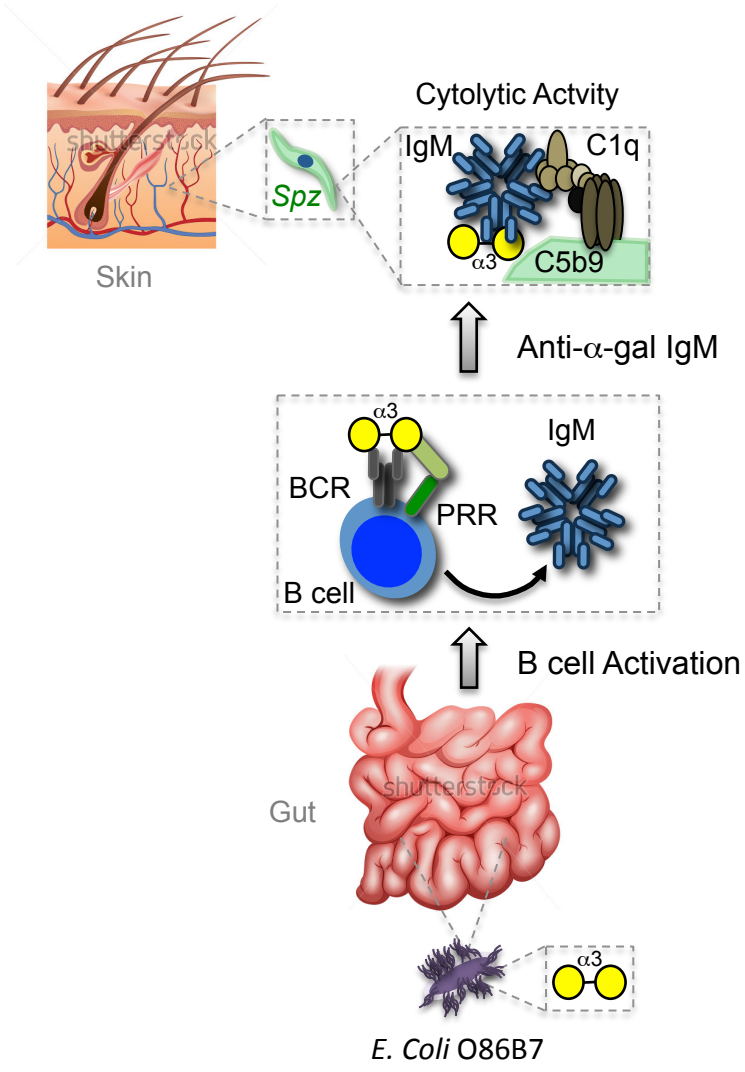
750

OUTSTANDING QUESTIONS BOX

- What are the immunogenic bacteria in the human gut microbiota that drive the production of α-gal-specific antibodies?
- What are the cellular and molecular mechanisms via which immunogenic bacteria in the gut microbiota trigger the production of α-gal-specific antibodies?
- What is the molecular mechanism driving the expression of α-gal glycan in *Plasmodium* spp.
- Do α-gal-specific antibodies confer protection against vector borne pathogens, other than *Plasmodium spp.*?
- Should vaccination against α-gal glycans be considered in the development of malaria vaccines?
- Should vaccination against α-gal glycans be considered in the development of vaccines against vector borne pathogens, other than *Plasmodium spp.*?


α3
Humans
?
CMAH
(Loss of function mutations)
α3
Ancestral Hominids
GGTA1/iG3bs
(Loss of function mutations)
α3
α3
Ancestral
Anthropoid Primates
Plasmodium falciparum
Plasmodium (ancestral)
Gal
Neu5Gc
Virulence
Host Protection


Skin
Spz
Cytolytic Actvity
IgM
C1q
α3
C5b9
Anti-α-gal IgM
α3
IgM
BCR
PRR
B cell
B cell Activation
Gut
α3
E. Coli O86B7