Anti-glycan antibody responses during infection with *Schistosoma*

Searching for the sweet spots

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This thesis is dedicated to my parents,
Bernardo Yang and Natalia Lee

Who taught me to finish what I started

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Chapter 1

General introduction

Schistosomiasis

Among the parasitic diseases, schistosomiasis ranks second only to malaria in terms of socio-economic and public health impact. It is estimated that at least 229 million people, spread over 78 countries, required preventive treatment in 2018 [1]. Once a schistosome infection establishes, the parasite can live in the host for decades unless the infection is treated. In general, resistance towards schistosome infection is not acquired rapidly: even partial immunity requires many years of exposure, multiple infections and treatments to develop [2-6]. The main species of *Schistosoma* that infect humans are *S. mansoni*, *S. japonicum* and *S. haematobium*. The former two cause intestinal schistosomiasis, while the latter causes urinary schistosomiasis. Although different species lead to different presentations of the infection, the genome [7-9] and protein coding gene sequences [9] are highly similar, and antigens are strongly recognized by antibodies cross-species [10, 11]. The acute phase of schistosome infection is associated with an innate immune response followed by a Th1 dominant adaptive response. The chronic phase of infection begins as the parasite matures, and egg deposition occurs. During this time, the Th1 pro-inflammatory response becomes down-regulated by the initiation of a more regulatory type Th2 immune response [12], limiting inflammation and immunopathology in order to allow parasite and host-survival. Nevertheless, granuloma formed around tissue-lodged eggs cause debilitating morbidity.

Current treatment of schistosomiasis relies mainly on the use of the chemical agent Praziquantel (PZQ), which is administered in mass treatment programs in endemic areas. The schistosomicidal activity of PZQ is believed to be due to calcium influx into the parasite, paralyzing adult worm muscles and damaging their tegument [13-17]. However, studies have also suggested that the disturbed calcium transport does not completely account for the effects observed after PZQ treatment [18]. Although the mechanism of action of PZQ is not fully established, it ultimately alters the conformation of the surface double lipid bilayer of schistosomes [19], after which exposure of antigens on the damaged worm tegument to the host immune system leads to the killing of the parasite [20, 21]. The host immune system triggered by dying worms alters the antibody and cytokine responses which provides short-term drug-induced resistance to infection [22, 23]. Since the resistance is only temporary and repeated PZQ treatment is required in endemic areas due to rapid reinfection [24], a prophylactic therapy that can prevent schistosomiasis is sought for.

Antibody responses against schistosomes

The high prevalence of schistosomiasis in endemic populations indicates that natural immunity to schistosome infection is rare. Nevertheless, there are studies that showed that individuals in endemic areas can acquire immunity through years of exposure, and multiple rounds of reinfection and treatment [25, 26]. To date, there is still no proof or consensus to how an individual could possibly develop resistance to re-infection. Many studies have explored the host-schistosome interaction in order to reveal the connection between particular immune profiles and disease state. In the lab, radiation-attenuated schistosome cercariae vaccination has demonstrated high levels of protection in different hosts, from rodents [27-31] to primates [32-36]. It is known that in the schistosome infected host, antibodies are raised to numerous antigens of the parasite. The relevance of anti-schistosome antibodies for protection has been demonstrated in passive serum transfer experiments: protection in naive mice can be achieved through passive transfer of serum from radiation-attenuated cercariae vaccinated rabbits and mice [37-39] and serum IgG has been shown to be responsible for this protection [37, 40]. The passively transferable protection has been shown for both *S. mansoni* [37, 40] and *S. japonicum* [39] infections. Additionally, it is proposed that the temporary resistance to infection following PZQ treatment is mediated by antibodies released when the dying worms expose antigens that are not normally encountered by the host immune system [41, 42]. In hosts protected by vaccination, the high anti-parasite antibody titers upon challenge [32-35, 43-45] and the necessity of functional B cells [46] support the hypothesis that antibodies are essential for developing a protective immune response.

These observations have prompted researchers to identify antigenic molecules in schistosome infection/protection models that might form the basis for a practical and effective vaccine. In addition to peptide-directed responses, antibodies towards glycans are of particular interest for schistosomiasis, because antibodies directed towards antigenic glycan motifs on glycoproteins and glycolipids of the parasite are especially abundant [43, 47]. It has been shown that when periodate treatment is performed on schistosome antigenic mixtures, thereby destroying periodate sensitive glycans, infection serum antibody binding to the parasite antigen mixture was greatly decreased [43, 47]. As schistosomes mature through different life-stages in the host, their glycan expression goes through many changes [48]. Unlike proteins that are synthesized by a defined template coded in the corresponding gene, glycan biosynthesis is catalyzed indirectly by glycosyltransferases expressed in cells and tissues [49, 50]. Some glycans are expressed stage specifically in the parasite life-cycle, while many other glycans are found in multiple life-stages [48], either secreted, exposed on the surface [51, 52] or expressed internally [52].

It has been shown that host antibodies elicited by *Schistosoma* antigens from different life stages can cross-react in a carbohydrate dependent manner [47].

Schistosome glycans

Schistosome-derived glycans have structural features that are different from mammalian cell derived glycans [53, 54], such as the lack of terminal sialic acid residues [54] and the abundant presence of terminal GalNAcβ1-4GlcNAc (LDN) branches on N-glycans rather than the Galβ1-4GlcNAc (LN) moiety [55, 56]. Furthermore, the fucosylation of terminal LDN motifs creates various non-mammalian antigenic motifs [48, 51, 56-58]. Common glycan epitopes expressed by schistosomes include terminal Galβ1-4(Fucα1-3)GlcNAc (LeX) [59], previously associated with Th2-inducing properties during schistosome infection [60-63]. The terminal LeX motif is highly expressed in cercariae N-glycans, but as the parasite develops into adult worms, non-fucosylated terminal LDN becomes more dominant [48] (Figure 1). Similarly, changes in the O-glycan and glycolipid repertoire occur during worm development.

Schistosome eggs also express glycoconjugates containing LeX and represent a major source of LeX antigens released in infected individuals [65]. Apart from terminal LeX, multimeric LeX (poly-LeX) is present on glycoproteins of worm excretions [66]. Other antigenic targets of schistosomes include core xylosylated and core fucosylated N-glycans. Core fucosylation can occur either with an α3 or α6 linkage, the former being a very antigenic epitope. Core xylose and core α3-fucose are not specific to schistosomes, but also occur in various other invertebrates [67, 68] and in plants [69-71] In schistosomes, core xylose occurs mainly in cercariae and miracidia [48], while core α3-fucose is exclusively found on N-glycans from miracidia and eggs. Alone or in combination with core xylose, core α3-fucose forms the major cross-reactive carbohydrate epitope that is an IgE target on a variety of plant-derived allergens [72-74]. Another set of antigenic glycan motifs include the (multi-)fucosylated terminal LN and LDN motifs. Multiple-fucosylated motifs are present in all life stages of schistosomes, mainly on O- or lipid-linked glycans and represent highly antigenic and characteristic epitopes in schistosomes [48, 51, 52, 75]. Multiple-fucosylated LDN motifs can be further divided into the (Fucα1-3)GalNAcβ1-4(Fucα1-3)GlcNAc (F-LDN-F) type that contain multiple α3-fucoses; and another type that contain one or more Fucα1-2Fuc motifs, such as (Fucα1-2Fucα1-3)GalNAcβ1-4(Fucα1-2Fucα1-3)GlcNAc (DF-LDN-DF). Both these motifs are highly antigenic. The Fucα1-2Fuc-containing motifs have so far only been found in the human schistosomes and the related bird schistosome *Trichobilharizia ocellata* [76], while F-LDN-F may be more widely occurring in invertebrates as it constitutes the cross-reactive motif with keyhole limpet hemocyanin [52].

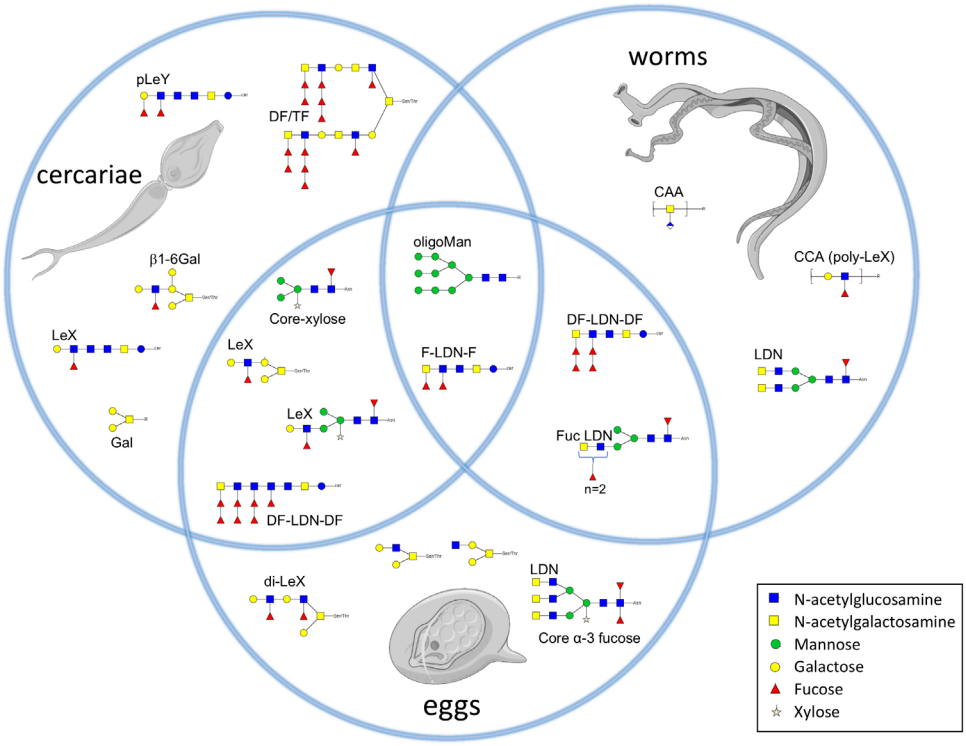


Figure 1. Venn diagram summarizing the expression patterns of glycans and glycan motifs in different life stages of the parasite inside the definite host. This diagram has been adapted from a figure by Hokke and Diepen [64] based on data published by Smit et al. [48].

Anti-glycan antibody responses

Parasite-specific antibodies can be investigated in various hosts in cross-sectional or longitudinal studies. For ethical reasons, human individuals cannot be included in studies without also providing treatment, thereby limiting the possibility of longitudinal studies in endemic areas. Longitudinal studies of experimental animal infections hugely complement cross-sectional human studies by providing the possibility to follow antibody dynamics over the course of infection.

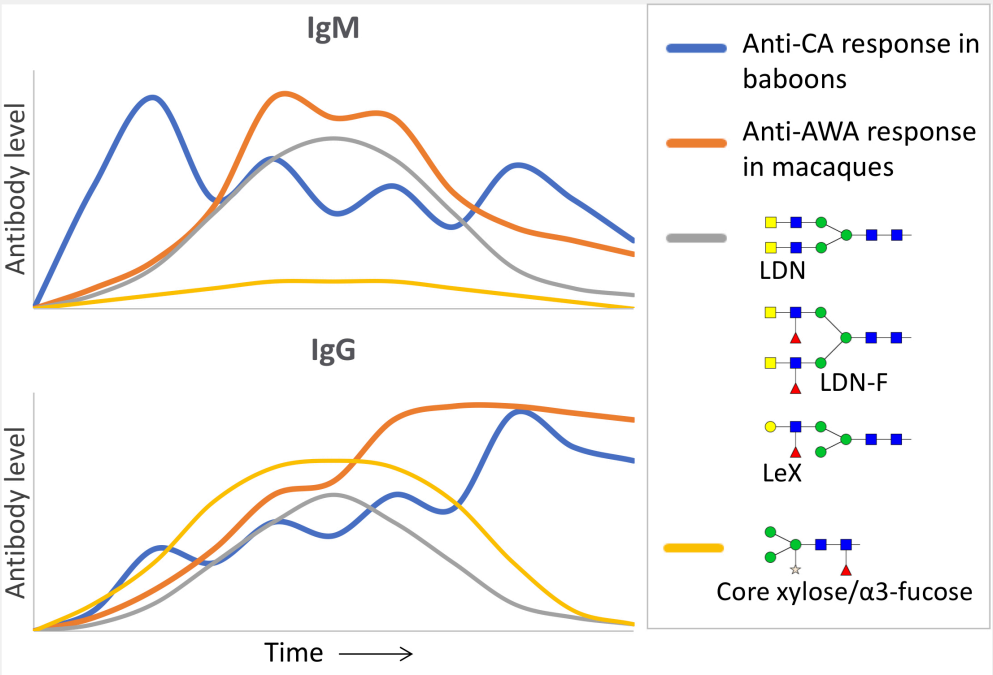
When investigating host antibody responses to the parasite, apart from the antigen specificity, the type of antibodies elicited should be carefully evaluated. During infection, IgM, IgG, IgE as well as IgA towards schistosome antigens are elevated [77]. IgE is commonly recognized as beneficial for the *Schistosoma* infected host by facilitating cytotoxic reactions against schistosome larvae *in vitro* [78-80]. IgE antibody levels to

adult worms have shown to be significantly related to resistance to reinfection, while IgG4 antibodies in the same analysis were related to susceptibility of reinfection [81]. It is suggested that the delay in development of protective immunity against schistosomes is due to the slow build-up of parasite specific IgE in the host [81]. At the same time, IgG4 antibodies are regarded as an IgE blocking antibody that may down-regulate the immune system's response against schistosome antigens [77, 81, 82] and the IgE/IgG4 ratio appears to be an important indicator for susceptibility to reinfection [81, 83, 84]. IgM and IgG2 have also been regarded as blocking antibodies that prevent the binding of antibodies of effective isotypes to the same epitope, thereby inhibiting cytotoxicity mediated towards the parasite by eosinophils or other cells [82, 85-88]. These 'blocking antibodies' have been found to react with glycan epitopes expressed both on schistosome eggs and schistosomula [85]. Moreover, the titers of these blocking antibodies were found to decline with age [86, 87], which may explain why young children are more susceptible to posttreatment reinfection. In contrast, IgG1 and IgG3 against schistosome glycans have been found to be potent inducers of eosinophil mediated cytotoxicity against schistosomula, thus possibly are the protective IgG isotypes.

The association of glycan-specific antibody isotypes and subclasses with protection have not been studied extensively. Nevertheless, it is clear that specific IgG towards antigenic schistosome glycans, including LeX, LDN, GalNAcβ1-4(Fucα1-3)GlcNAc (LDN-F) and core fucose and xylose determinants are elicited by *S. mansoni* infected hosts [89-91]. In particular, infected mice respond to the LDN and LDN-F epitopes with a Th2 type immune response characterized by the presence of IgM, IgG1 and IgG3, but not of the IgG2 subclass [75]. Infected humans also generate IgM, IgG and IgA towards LDN, LDN-F and LeX [92]. An age-related influence towards anti-glycan antibody responses was observed in schistosome infected hosts: children, associated with being less resistant to reinfection than adults, elicit higher titers of antibodies against LDN-DF and F-LDN [93, 94].

It is worth noting that the anti-glycan antibody responses have been suggested in some studies to be a 'smokescreen' response that diverts the host immune system away from attacking vulnerable parasite antigens [43, 47]. On the other hand, studies have also shown that antigenic schistosome glycans could be involved in developing a protective response [75, 89, 95]. These opposing views can result from generalization, disregarding the many types and structures of glycan targets present in schistosomes, and/or the nature of antibodies raised against the target. Unravelling the complex interplay between the antibody responses and the identification of the individual antigenic glycan motifs in schistosomes will help us better understand their role in the host-parasite interactions, thereby unravel their potential as targets for schistosomiasis vaccination and diagnosis.

Box 1. What is currently known about the antibody dynamics in non-human primate protection models for schistosomiasis?



Antibody dynamics has been measured in baboons (vaccination acquired immunity) and rhesus macaques (naturally acquired immunity) during their development of protection in time. In these studies, the probe used for antibody detection were mainly antigenic mixtures such as materials released during cercarial transformation (CA) and soluble adult worm proteins (AWA). Although the majority of antibody responses towards these antigenic mixtures are towards glycans, the variety of glycan motifs present in these mixtures are immense. The antibody responses towards a small selection of synthetic glycan structures have only recently been measured in macaques. Overall, IgM response precedes IgG response by 2-4 weeks. When using antigenic mixtures as probes, IgG responses are found to be sustained in time, while IgM responses decrease gradually. These differences in IgM and IgG dynamics in time is an important consideration for cross-sectional studies that are drawing conclusions from any specific timepoint. When antibody responses towards specific motifs were measured, it was found that the antibody patterns do not always follow that of the antigenic mixtures: different glycan motifs induce different antibody dynamics.
These observations form the bases for one of the main goals of this thesis: to further elucidate antibody dynamics induced by different schistosome glycans.

1

Using glycan microarrays to study antibody-glycan interactions

Recent advances in chromatography and mass spectrometry-based approaches for isolation and characterization of glycans together with specific chemical and enzymatic degradation methods have made it possible to identify many schistosome glycans and glycan motifs. The glycome of *S. mansoni* has been described in detail [48]. Together with immunofluorescent staining of the parasite with anti-glycan monoclonal antibodies [51, 52, 92], there is good understanding of the structure of different glycans, when they are expressed, as well as whether they are present on the immediate host-parasite interface. Although the full glycome of the other two *Schistosoma* species have not been described yet, earlier findings have suggested that they are highly similar to that of *S. mansoni* [96-98].

The separated and characterized parasite glycans can be printed on microarrays, creating 'shotgun glycan-microarrays' [94, 99, 100] that can be interrogated using lectins, monoclonal antibodies or serum/plasma to further characterize the glycan antigens and their binding partners. Shotgun glycan microarrays are valuable for they offer a wider range of glycan elements, including highly complex structures isolated from the organism that are not technologically feasible to synthesize. On the other hand, synthetic glycan arrays [89, 101] can complement the shotgun-glycan microarrays by a) identifying single antigenic motifs in situations where multiple structural motifs exist in the natural glycan and b) identifying the antigenicity of glycans that are not available in sufficient quantities or cannot be purified from the native source. The application of glycan microarray technology has made it possible to study anti-glycan antibody responses to parasite glycans in a high throughput manner.

Scope of this thesis

A better understanding of anti-glycan antibody responses during infection and the development of schistosome resistance could improve current schistosomiasis diagnostic methods as well as identify potential vaccine targets.

In the first part of this thesis, the antibody responses towards schistosome glycans are studied in different models by the use of glycan-microarrays. In **Chapter 2,** the anti-glycan IgG and IgM response in infected rhesus macaques is studied over time while infected rhesus macaques gradually develop immunity towards schistosomiasis. **Chapter 3** describes a vaccine-induced model for protection against schistosome infection, where the anti-glycan IgG and IgM response of radiation-attenuated cercariae vaccinated olive baboons was studied by glycan microarray. These two longitudinal studies reveal the schistosome glycan motifs recognized by a host as well as the anti-glycan antibody dynamics during the development of protection against schistosomiasis. It has been described that the development of anti-glycan IgG response is important for developing protection. Yet, chronically infected individuals also develop IgG towards schistosome antigens. Different IgG subclasses have different immunological properties that may or may not lead to protection. However, the IgG subclass responses towards glycan motifs have not previously been described. Thus, in **Chapter 4**, the IgG subclass responses of human subjects from a schistosome endemic village is investigated.

The last part of this thesis builds on the fact that fucosylated LDN is an antigenic glycan motif recognized by all schistosome infected hosts. However, it's role in protection is unknown. **Chapter 5** describes an immunization plus challenge experiment where rats were immunized with single- and double-fucosylated LDN motifs conjugated to BSA. The worm burden after cercarial challenge of glycoconjugate immunized rats was compared with unconjugated BSA-immunized rats and the anti-glycan IgG and IgM response was measured.

Together, these chapters reveal antigenic schistosome glycan motifs, as well as the corresponding antibody dynamics in the context of infection and the development of a protective immune response towards schistosome infection. This information provides insights into the possibility of using glycans as diagnostic and vaccination candidates which will be succinctly discussed in **Chapter 6**.

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Chapter 2

Specific anti-glycan antibodies are sustained during and after parasite clearance in *Schistosoma japonicum*-infected rhesus macaques

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Abstract

Background: Human immunity to *Schistosoma* infection requires many years of exposure, and multiple infections and treatments to develop. Unlike humans, rhesus macaques clear an established schistosome infection naturally at the same time acquiring immunity towards re-infection. In macaques, schistosome egg production decreases after 8 weeks post-infection and by week 22, physiological impairment of the worm caused by unclarified antibody-mediated processes is observed. Since strong antibody responses have been observed against schistosome glycan antigens in human and animal infections, we here investigate if anti-glycan antibodies are associated with immunity against schistosome infections in macaques.

Methods: We used a microarray containing a large repertoire of glycoprotein- and glycolipid-derived glycans from different schistosome life stages to analyze anti-glycan serum IgG and IgM from *S. japonicum*-infected macaques during the course of infection and self-cure. We also used an *in vitro* schistosomula assay to investigate whether macaque sera containing anti-glycan antibodies can kill schistosomula.

Conclusions/significance: Antibody responses towards schistosome glycans at week 4 post-infection were dominated by IgM while IgG was high at week 8. The profound increase in IgG was observed mainly for antibodies towards a large subset of glycans that contain (multi-)fucosylated terminal GalNAcβ1-4GlcNAc (LDN), and Galβ1-4(Fucα1-3) GlcNAc (LeX) motifs. In general, glycans with a higher degree of fucosylation gave rise to stronger antibody responses than non-fucosylated glycans. Interestingly, even though many IgG and IgM responses had declined by week 22 post-infection, IgG towards O-glycans with highly fucosylated LDN motifs remained. When incubating macaque serum with schistosomula *in vitro*, schistosomula death was positively correlated with the duration of infection of macaques; macaque serum taken 22 weeks post-infection caused most schistosomula to die, suggesting the presence of potentially protective antibodies. We hypothesize that IgGs against highly fucosylated LDN motifs that remain when the worms deteriorate are associated with infection clearance and the resistance to re-infection in macaques.

Introduction

Schistosomiasis is a debilitating parasitic disease caused by members of the helminth genus *Schistosoma (S.),* with *S. mansoni, S. japonicum* and *S. haematobium* being the most prevalent human species. Once *Schistosoma* infection establishes, mature worms can live up to 30 years in the host until treated [1]. Many studies on human *Schistosoma* infection have indicated that resistance to *Schistosoma* infection can be acquired, but this is age-dependent and requires many years of exposure to the parasite, and multiple infections and treatments to develop [2]. Praziquantel (PZQ) is widely used to treat human schistosomiasis by paralyzing adult worm muscles and damaging the tegument [3]. This exposes worm antigens to the host immune system [4] and leads to immune-mediated killing of the parasite. The immune responses triggered by degenerating worms can alter antibody and cytokine responses and provide short-term drug-induced resistance to re-infection [5, 6]. Since this resistance is short-lived, people in endemic areas still require repeated administration of PZQ [7]. An effective elimination strategy would likely require the incorporation of a vaccine to immunize against schistosome (re)infection [8, 9].

Rhesus macaques are permissive hosts for *Schistosoma* infections. In rhesus macaques infected with S. japonicum, oviposition occurs 34-36 days after exposure and the peak egg excretion occurs 7-15 weeks post-infection [10]. However, unlike *Schistosoma* infections in humans where the infection persists with heavy egg shedding for decades, rhesus macaques show various signs of resistance to infection four months after infection [11]. Marked decrease in eggs detected in the feces of macaques is observed 11 weeks post-infection, correlating to the vulnerable health status of the female worms, as seen by the diminished body lengths and size of sexual organs [10]. The rate of adult worm recovery from macaques also greatly decreases to 32% 19 weeks post-infection and 9% by the 42nd week [10]. The same type of worm degeneration and diminished oviposition is observed in *S. mansoni*-infected rhesus macaques [12]. However, the decline in fecal egg output was observed at a slightly earlier time point: 9 weeks post-infection. In addition to eliminating the worms from the body, rhesus macaques were found to be completely resistant to secondary infection 21 weeks after primary infection when challenged with schistosome cercariae, even though the macaques were still in the process of clearing the primary infection [13]. It has been postulated that rhesus macaques clear schistosome infection and become resistant to re-infection through an antibody-mediated process, based on a strong inverse relationship observed between the intensity of IgG response and worm burden [12]. Additionally, when blood feeding worms were cultured *in vitro* with serum of macaques with low worm burden, stunted growth was observed for these worms. Moreover, it has been shown that serum antibodies from infected individuals can

kill schistosomula *in vitro* [14, 15]. Recently, Li et al. have suggested that antibody binding to adult worm esophagus blocks nutrient uptake and eventually lead to starvation of worms [16]. In view of the long time taken for the worms to degenerate, it is likely that the mechanism of clearance does not involve complement fixation [17] but a sustained antibody-mediated process that affects the normal physiology of worms.

An abundance of antibodies is generated in *Schistosoma*-infected hosts that bind to glycans from schistosome glycoproteins and glycolipids [18-24]. Localization studies with glycan-directed monoclonal antibodies [25, 26] and glycomic profiling by mass spectrometry [27] have indicated that *Schistosoma mansoni* glycosylation exhibits stage-specific changes during the life cycle. For example, the structural motifs Fucα1-3GalNAcβ1-4GlcNAc (F-LDN) and Fucα1-3GalNAcβ1-4(Fucα1-3)GlcNAc (F-LDN-F) are abundantly expressed in cercarial and egg glycoproteins but could hardly be detected in adult worm glycoproteins [26]. Nevertheless, multi-fucosylated GalNAcβ1-4GlcNAc (LDN) motifs are present in glycolipids throughout the whole life cycle. Cercarial N-glycans are found to be dominated by the Galβ1-4(Fucα1-3)GlcNAc (LeX) termini [28]. However, the expression of LeX by cercariae is rapidly lost after their transformation into schistosomula, while LDN motifs gradually become predominant in maturing worms [27]. While some glycan types and motifs are expressed in a stage-specific manner, cross-reactive glycans exist between different life stages. It has been shown that many antibodies elicited by egg glycans are cross-reactive with glycans expressed on the surface and secretions of cercariae [29]. Apart from cross-life stage similarities, there are also high cross-species similarities in schistosomes. *S. japonicum* and *S. mansoni* have a 86% homology in protein coding gene sequences [30] and *S. mansoni*-infected human sera recognize *S. japonicum* proteins on a schistosome protein array [31] and vice versa [32]. Although *S. mansoni* glycosylation is better characterized than that of *S. japonicum*, studies have suggested that the glycans expressed by the two species are highly similar [33, 34].

Recently, anti-glycan responses in *Schistosoma* infected humans and animals have been studied using glycan microarray approaches [19, 22-24]. With the aim of identifying possible glycan targets involved in clearance of *Schistosoma* and resistance to reinfection, we here analyzed anti-glycan antibodies in a set of *S. japonicum*-infected rhesus macaque sera collected over a period of 22 weeks, using a microarray containing a large repertoire of N-, O- and glycosphingolipid (GSL) derived glycans isolated from *S. mansoni* larvae, adult worms and eggs [19, 23, 35] complemented with a synthetic microarray containing a set of relevant core-modified N-glycans [35]. After obtaining the antibody profile of schistosome-infected rhesus macaques at different infection stages, we incubated these sera with *in vitro* transformed schistosomula to study the effect of antibodies on parasite survival. Our work provides new insights into glycan motifs that may be the targets of a

protective antibody response to *Schistosoma* infection.

Materials and Methods

Ethics statement

The housing conditions, experimental procedures and animal welfare of the monkeys used in the study were in strict accordance with the national guidelines for the Care and Use of Animals established by the Chinese National Animal Research Authority and applied by the Institutional Animal Care and Use Committee (IACUC) of the Kunming Institute of Zoology, Chinese Academy of Sciences (CAS). The experimental protocol was approved by the Ethics Committee of Kunming Institute of Zoology, CAS (ID SYDW-2011017). The study used six adult male rhesus macaques (*Macaca mulatta*) from the captive-breeding colony at the Kunming Primate Research Center, CAS. They were group-housed prior to the experiment but then singly after infection for fecal sampling purposes. The separate cages were arranged in one large room to allow the monkeys visual, olfactory and auditory interactions with each other. Food and water were available ad libitum and vitamins were provided. The animals were also provided with environmental enrichment, such as toys designed especially for monkeys, to promote psychological well-being. The design and execution of the study complied with the recommendations of the Weatherall report (2006) on "The use of non-human primates in research", which specifically mentions the continuing requirement for their use in schistosome research.

Sera

Rhesus macaques were anaesthetized with ketamine hydrochloride (6 mg/kg body weight. Gutian Pharmaceutical Corporation, Fujian China) and infected percutaneously with 600 cercariae of *S. japonicum* via the shaved abdominal skin for 30 minutes. *S. japonicum* cercariae were obtained from patent *Oncomelania hupensis* snails kept at the Jiangsu Institute of Parasitic Diseases (Wuxi, China) [16]. Blood was obtained by intravenous sampling at 5 time points, week 0, 4, 8, 14 and 22, stood at room temperature for 1 hour (h) to clot, and kept overnight at 4°C to facilitate clot retraction before serum was recovered for storage at -20°C. All animals were individually inspected daily. Those showing signs of diarrhea were given oral dehydration therapy as required

Materials

Cy3 conjugated goat anti-human IgG (Fc-specific) and Alexa Fluor 647 conjugated goat anti-human IgM (μ chain specific) were from Invitrogen (The Netherlands). BSA and ethanolamine were from Sigma (Zwijndrecht, the Netherlands).

Glycan microarray construction and analysis

The shotgun glycan microarray was constructed as described previously [19, 23]. A selection of fractions was analyzed in this study compared to those previously described and contained reverse phase HPLC fractions of glycans isolated from cercariae (82 N-glycan fractions, 114 O-glycan fractions and 21 glycolipid glycans), adult worm (83 N-glycan fractions, 39 O-glycan fractions) and egg (62 egg N-glycan fractions, 110 soluble egg antigen O-glycan fractions and 12 glycolipid glycans). Additionally, 24 blank spots with spot buffer were included for array background control. Each glycan fraction was immobilized on a glass slide in triplicate.

The synthetic array used in this study contained a collection of core-xylosylated and core-α3 and α-6 fucosylated N-glycans with various core extensions [35]. The synthetic N-glycans were immobilized to N-hydroxysuccinimide (NHS)-activated glass slides via a C5 amino linker. Unreacted NHS groups were quenched by blocking with 50 mM ethanolamine in 50 mM sodium borate buffer, pH 9.0 for 1 h. The slides were then washed with PBST, PBS and MilliQ water, dried and then stored at -20°C. The slides were defrosted upon use, followed by serum sample incubation, as described in the following section.

Binding assay

The glycan-microarray binding assay followed the protocol as described by van Diepen et al. [19, 23]. Briefly, the microarray slide was blocked with 2% BSA, 50 mM ethanolamine in PBS. Serum samples were diluted 1:100 in PBS-0.01% Tween20 with 1% BSA. Cy3-labeled anti-human IgG and Alexa Fluor 647-labeled anti-human IgM were diluted 1:1000 in PBS-0.01 Tween20 to detect bound serum antibodies on the slide. All washing steps were performed with successive rinses with PBS-0.05% Tween20 and with PBS. The last washing step was finished by an additional wash with milliQ water and the slides were dried and kept in the dark until scanning.

Scanning and data analysis

A G2565BA scanner (Agilent Technologies, Santa Clara, CA) was used to scan the slides for fluorescence at 10 um resolution using lasers at 532 nm and 633 nm. Total IgG was detected at 532 nm and IgM at 633 nm, the 2-AA label does not fluoresce at these wavelengths. Data and image analysis was performed with GenePix Pro 7.0 software (Molecular Devices, Sunnyvale, CA). Spots were aligned and re-sized using round features with no CPI threshold. Background-subtracted median intensities were averaged per time point and processed as described by Oyelaran et al. [36]. Datasets were $\log_2$ transformed to remove the basic trends of variance. A hierarchical clustering analysis (HCA, complete linkage clustering using Euclidean distance metric) was performed to group associated

glycan fractions using MultiExperiment Viewer v4.5. To identify statistically different IgG and IgM response towards glycan fractions, a paired sample t-test was performed using SPSS. A P value < 0.05 was used to identify glycan fractions that were differentially recognized by serum IgG and IgM antibodies.

Schistosomula transformation

Freshly shed cercariae from snails were centrifuged at 440 x g for 5 min. The buffer was replaced with 37°C M199 medium supplemented with 1:100 1M HEPES pH7.4, 1x antibiotic antimycotic solution (ABAM), 1.5 mM glutamine and 10% FCS. The cercariae were resuspended with the medium and incubated at 37°C for 20 minutes in order to facilitate cercarial transformation into schistosomula. The incubated tube was shaken regularly to avoid sedimentation. Afterwards, the parasites were transferred to a petridish and put on an orbital shaker. During orbital shaking, schistosomula that collect at the center of the petridish were taken out with a pipette, while swimming cercaria and tails that collect at the sides of the petridish were left behind. Isolated schistosomula were resuspended in supplemented M199 medium and cultured in a microtiter plate at 37°C in a humidified atmosphere with 5% CO_2.

Schistosomula killing assay

A total of 400 transformed schistosomula was cultured in each well of a flat bottom 96 well plate containing 100 ul of M199 medium supplemented with HEPES pH7.4, 1x antibiotic ABAM, 1.5mM glutamine and 10% FCS at 37°C in a humidified atmosphere with 5% CO_2. At 3 hours post transformation, 55 ul of medium was carefully taken out of the wells and 5 ul of sera was added to each well to create a 1:10 serum dilution. Where required, sera were treated by incubation at 56°C for 45 minutes to inactivate complement. Each treatment was done in duplicate. Immediately after treatment, the plate was observed under a microscope to detect gross changes, such as schistosomula agglutination. The effect of treatment and induction of schistosomula killing was measured at 24 h and 48 h after treatment. Morphological changes were observed by using brightfield microscopy while schistosomula integrity was determined by fluorescent microscopy (Leica) using propidium iodide (PI) [37] staining at 10 ug/ml. Multiple photographs were taken of each well and the percentage of PI positive schistosomula was counted afterwards.

Results

IgG and IgM responses against schistosome glycans

We incubated sera from six *S. japonicum*-infected rhesus macaques with a schistosome glycan microarray to investigate their anti-glycan antibody responses over a time course of 22 weeks. Figure 1 shows averaged IgG and IgM responses of rhesus macaques towards N-, O- and lipid-glycans isolated from *S. mansoni* cercariae, worms and eggs.

At the onset of infection, weak IgM signals against some glycans present on the schistosome array were detected in macaque serum, probably due to low levels of naturally occurring anti-glycan antibodies [38]. At week 4, strongly increased IgM was found binding to cercarial N- and O-glycans, egg-derived N-glycans and GSL glycans. Noteworthy, even though no eggs are produced 4 weeks post-infection, IgM antibodies were found against egg-derived glycans containing LeX and (fucosylated) LDN motifs; these glycan motifs have previously been shown to be shared with cercariae [26, 33, 34]. At 8 weeks post-infection, when oviposition was highest, maximum anti-glycan IgM titers were observed, especially against GSL-glycans and egg and cercarial O-glycans. These responses decreased at 14 weeks post-infection. At week 22, responses that persisted were very similar to those at week 4, but at a lower intensity. Notably, the IgM response at week 22 post-infection remained positive against a broad range of GSL glycans.

In contrast to IgM, IgG of infected macaques directed towards schistosome glycans was negligible at week 0, while a slight induction of an IgG response against cercarial O-glycans and GSL glycans could be detected at week 4 post-infection. At 8 weeks post-infection, a strong rise of IgG towards egg and cercarial N- and O-glycans was observed. Additionally, IgG against GSL glycans and cercarial O-glycans remained strongly positive at week 14 and week 22, while responses to egg O-glycans were greatly reduced during this period. *Schistosoma* GSL glycans had high IgG and IgM binding at week 22.

IgG and IgM response profiles of *S. japonicum*-infected macaques

Following the IgG and IgM response patterns, we performed a hierarchical clustering analysis to group glycans with similar antibody response profiles. Anti-glycan antibody responses were corrected for baseline (week 0) intensity to obtain the intensity of response induced by infection. Four different glycan clusters were identified, based on IgG dynamics, namely IgG-C1, IgG-C2, IgG-C3 and IgG-C4 (Fig. 2A) and six clusters for IgM dynamics, namely IgM-C1, IgM-C2, IgM-C3, IgM-C4, IgM-C5 and IgM-C6 (Fig. 2B).

IgG-C1 contained glycans that were highly antigenic; antibodies against this cluster

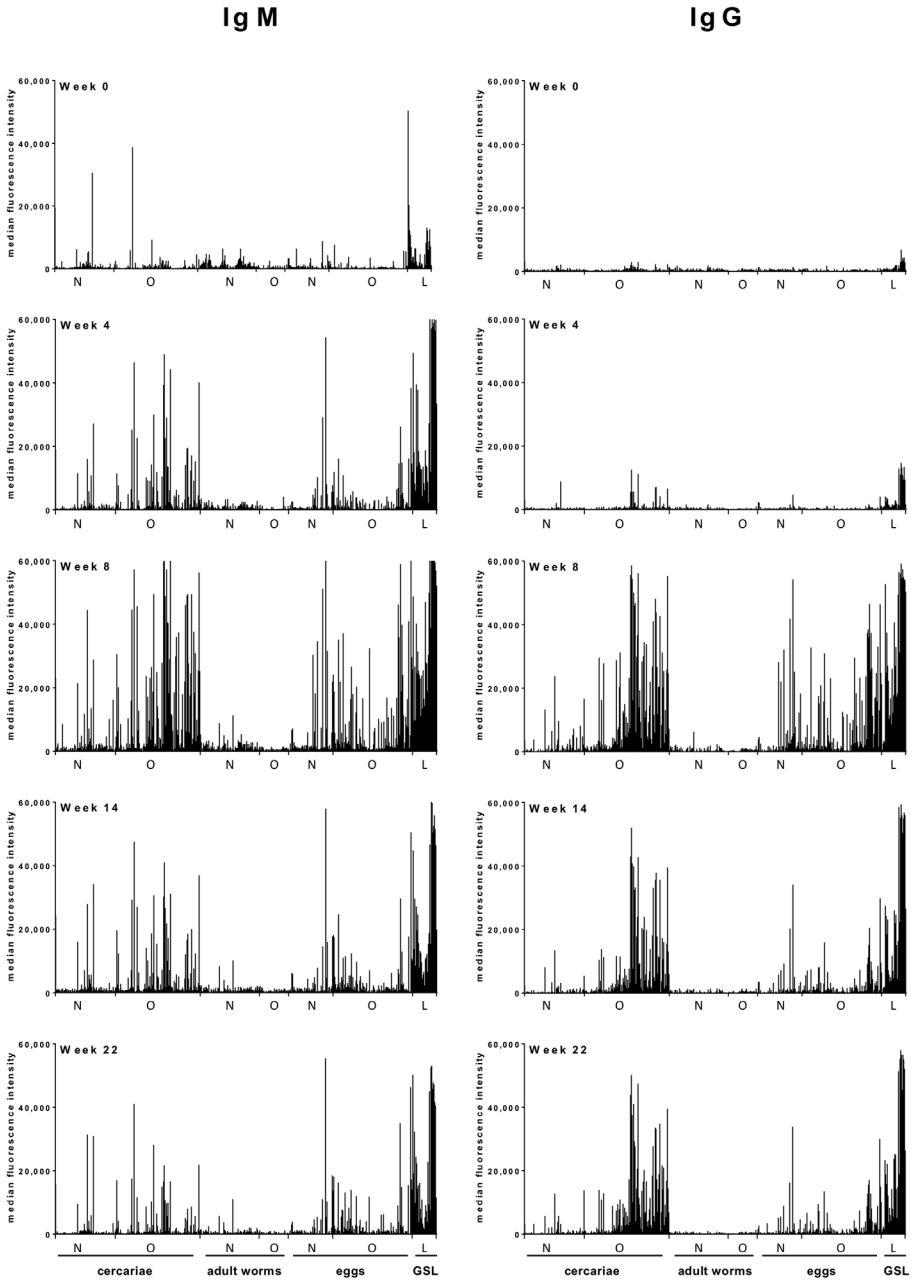

Figure 1. Averaged serum IgG and IgM response from schistosome-infected macaques to glycans isolated from different life stages of schistosomes. The horizontal axis indicates N- and O-glycan fractions from *Schistosoma* cercariae, adult worms, and eggs. Schistosome GSL glycans are shown as a group irrespective of the life stage. Average background-subtracted median fluorescence intensities are shown for IgG and IgM over a time course of 22 weeks. Each bar corresponds to antibody binding to individual glycan fractions printed on the glycan microarray. N: N-glycans. O: O-glycans and L: GSL glycans.

reached a maximum at week 8 post-infection, and remained until week 22 (Fig. 2C). IgG-C2 elicited antibody responses that became positive between weeks 4 and 8, again remaining positive until week 22. In terms of antibody binding based on the detected fluorescence intensity of the array, there was a higher amount of IgG antibodies binding to glycans in IgG-C1 than in IgG-C2. Clusters IgG-C3 and IgG-C4 were smaller in size and glycans in these clusters did not yield high IgG signals. Antibodies against glycans in IgG-C3 peaked at week 8 post-infection and quickly decreased thereafter, while glycans in IgG-C4 did not appear to induce any IgG in macaques during the infection. A closer look at the type of glycans present in each cluster reveals that IgG-C1 and IgG-C2 were dominated by O-glycans and GSL glycans, while IgG-C3 and IgG-C4 contained mostly N-glycans (Fig. 2D and Table S1A).

Six response profiles were identified for IgM binding. There were three clusters that remained positive at week 22, namely IgM-C3, IgM-C4 and IgM-C5 (Fig. 2E). Glycans in IgM-C3, -C4 and -C5 were mainly O-glycans derived from cercariae and eggs and lipid derived glycans (Fig. 2F and Table S3A). On the other hand, clusters IgM-C1, -C2 and -C6, with no sustained antibody binding at week 22 post-infection, contained a majority of N-glycans. IgM-C1, -C2 and -C6 differed in the onset of IgM binding: while glycans in IgM-C2 were bound by macaque serum IgM generated at week 4 and later, glycans in IgM-C6 only had IgM binding at week 8. IgM-C1 had no infection-induced IgM binding at any timepoint.

Previously, the glycan composition of each glycan fraction printed on the array has been determined by mass spectrometry. Based on described structural glycan motifs in the literature [20, 23, 24, 27, 28, 33, 34, 39, 40], the most likely glycan structures for different glycan compositions were deduced for both IgG and IgM. We have summarized the most abundant glycan motifs for each IgG cluster, as well as the representative fractions in each IgG cluster in Table S1B and Table S2. The most common glycan motifs in IgG-C1 and IgG-C2 were the LeX and multi-fucosylated LDN motifs.

O-glycan specific structural elements played an important role in the cluster formation of IgG-C4 and IgG-C2. For example, the *Schistosoma* specific O-glycan core Galβ1-3(Galβ1-6)GalNAc is sometimes found with an additional β1-6Gal on either the Galβ1-3 or the Galβ1-6 [41]. This structural element is found in cercariae-derived O-glycan fractions 3.4 and 6.6, and was one of the representative structures in IgG-C2 (Table S2). Another example of an O-glycan specific motif was the multi-fucosylated Galβ1-4GalNAcβ1-4GlcNAc (Gal-LDN) motif [23, 42]. Compared to IgG-C2, IgG-C1 glycans were more complex and were in general longer in glycan chain length. For example, the di-LeX motif was expressed in both IgG-C1 and IgG-C2, but the tri-LeX motif was uniquely present in IgG-C1. In general, LDN motifs in IgG-C1 contained a higher extent of fucosylation; the

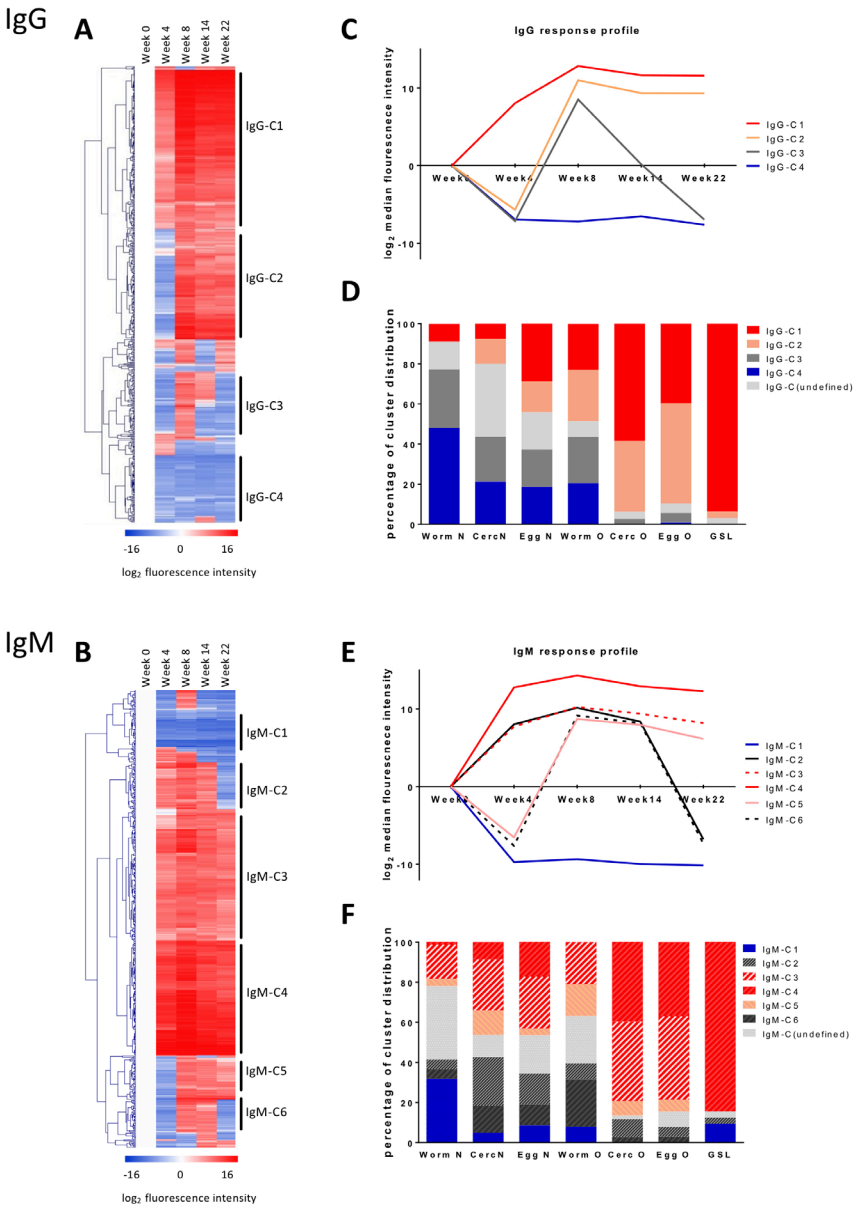

Figure 2. Hierarchical clustering analysis of macaque serum anti-glycan antibody responses over 22 weeks after infection with *Schistosoma japonicum*. Antibody median fluorescence intensity was corrected for baseline and $\log_2$ transformed. The reduction in antibody binding is indicated in blue, while the increase is indicated in red. Four major clusters were identified based on IgG response dynamics over time (A), and six major clusters for IgM (B). The median fluorescence intensity at each time point was averaged to obtain a response profile curve for each IgG (C) and IgM (E) major cluster. Distribution of IgG and IgM cluster profiles based on glycan origin is shown in (D) and (F), respectively. Worm N, worm derived N-glycan; Cerc N, cercaria derived N-glycan; Egg N, egg derived N-glycan; Worm O, worm derived O-glycan; Cerc O, cercaria derived O-glycan; Worm O, worm derived O-glycan; GSL, lipid derived glycan.

previously defined DF-LDN-TF structure in cercarial O-glycan fraction 15.6 [23] was an antigenic motif uniquely found in cluster IgG-C1 (Table S2). In contrast to clusters IgG-C1 and IgG-C2 that were dominated by O–glycans (Table S1A), IgG-C3 and IgG-C4 consisted of 75% and 88% N-glycans, respectively, which were mostly worm-derived (Fig. 2D). Most of the N-glycans in IgG-C4 did not have antigenic elements, but mainly expressed terminal Galβ1-4GlcNAc (LN), mannose and terminal GlcNAc (Gn) motifs (Table S2). Compared to IgG-C4, IgG-C3 contained a higher number of fucosylated motifs, which led to a more antigenic response profile, although these responses were not sustained in time.

The clusters that had the highest amount of and long sustained IgM binding were IgM-C3 and -C4. The glycans in IgM-C3 and –C4 were abundant in antigenic motifs, such as LeX, (F)Gn, multiple fucosylated LDN and O-glycan-specific motifs β1-6 Gal and (fucosylated) Gal-LDN motifs (Table S3B). IgM-C5 was a small cluster that was also IgM positive at week 22, but had less IgM binding than IgM-C3 and IgM-C4. It contained 42% N-glycans, 58% O-glycans and no GSL glycans (Table S3A). IgM-C2 showed a response profile that was not recognized as a significant cluster in IgG. Glycans in IgM-C2 had IgM binding between week 4 and 14 and became undetectable at week 22; this cluster contained 64% N-glycans, 35% O-glycans and 2% lipid derived glycans with many glycans expressing the LeX motif.

When comparing IgG and IgM clusters, it was interesting to see that there was a sharp difference between the IgG antigenicity of N-glycans and O-glycans. A vast majority of N-glycans had no IgG binding at week 22 post-infection and were grouped in low antigenic clusters IgG-C3 and IgG-C4. On the other hand, IgM responses to many N-glycans were positive at week 22. A common pattern in IgG and IgM response profiles was that highly fucosylated glycan motifs led to higher serum antibody levels compared to less fucosylated structures. The more antigenic the cluster was, the more fucosylated motifs it contained. This effect was especially pronounced in IgG profiles.

Infected macaque sera target core xylose/core α3-fucose

In addition to the shotgun array, we tested the same set of macaque sera on a synthetic array previously described by Brzezicka et al.[35]. This array contains a collection of core-xylosylated and core-fucosylated N-glycans. Several of these defined synthetic glycan structures are present in schistosomes, including a number of core α3-fucose modified N-glycans which were not present on the shotgun array. Figure 3 shows IgG responses of macaque sera towards the synthetic glycan array over time (Fig. 3). At week 0 and week 4 post-infection, there were minimal IgG responses against core α3-fucose and terminal LDN structures; the IgG response towards LDN on the α6 branch is stronger than towards LDN on the α3 branch. At week 8 post-infection, there was strongly increased IgG against

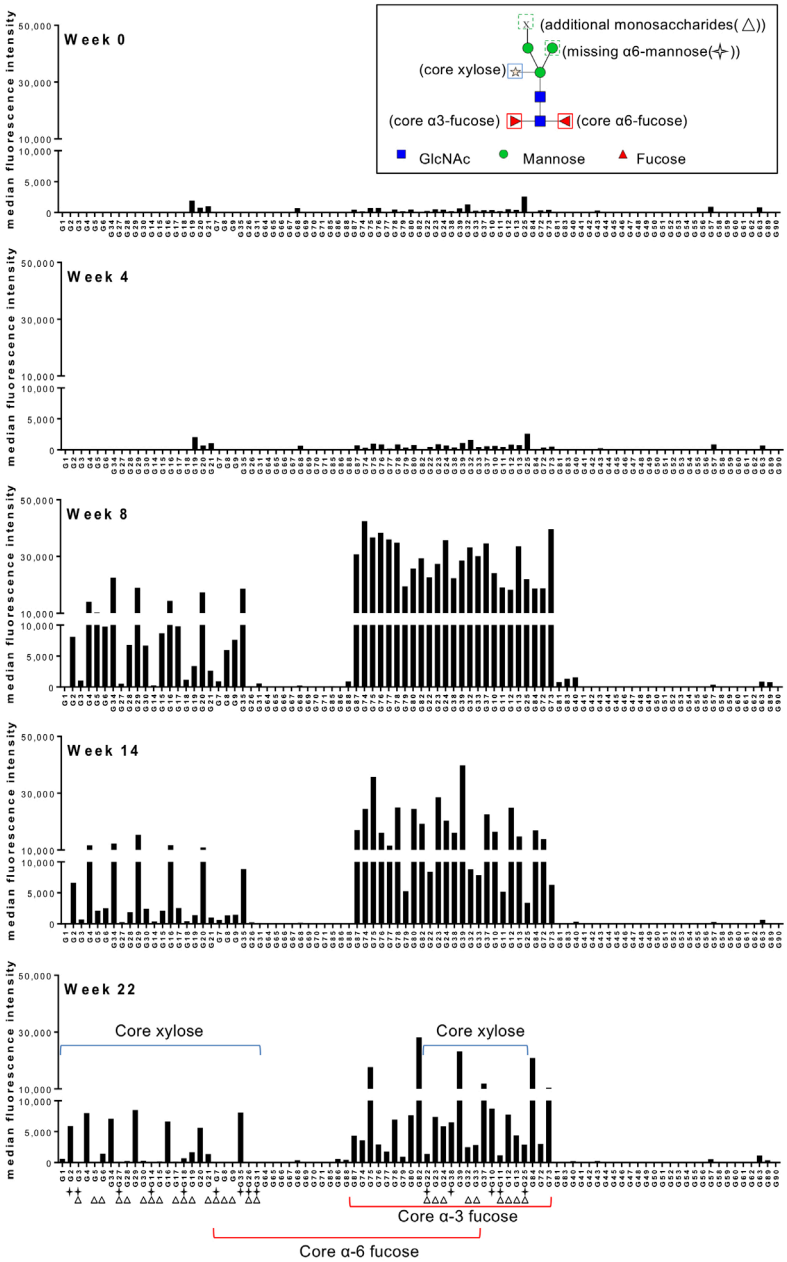

Figure 3. Averaged serum IgG responses from *S. japonicum*-infected rhesus macaques to synthetic core modified N-glycan fractions. The horizontal axis depicts core modified N-glycan fractions that have been synthesized and described by Brzezicka et al. [35]. Average median fluorescence intensities are shown for *S. japonicum*-infected macaque serum IgG over a time course of 22 weeks. Each peak along the vertical axis corresponds to each individual glycan fraction printed on the glycan microarray. Core xylosylated, core α6-fucosylated and core α3-fucosylated structures are indicated. Within core xylosylated structures, those that have additional monosaccharides on the α3-mannose (Δ) and those that miss the core α6-mannose (✧) are indicated.

structures with core xylose and core α3-fucose, either in combination with core α6-fucose, or alone. Interestingly, the absence of α6-mannose on xylosylated structures reduces IgG binding. Additionally, IgG binding was also reduced in xylosylated structures where the α3 branch was occupied by additional monosaccharides. At week 14 post-infection, a general decrease in IgG responses towards antigenic core modified glycans was observed. Those IgGs that remained at week 14 and 22 bound to unhindered core xylose and core α3-fucose-containing glycans. IgG binding to the xylosylated N-glycan core was similarly observed on the shotgun array peaking at week 8 post-infection and then decreasing (Fig. S1). Nevertheless, the amount of IgG binding to xylosylated structures was much lower than to other antigenic motifs on the same array, such as the fucosylated terminal LDN motifs.

An interesting observation on the synthetic array was IgG binding to glycans G82 and G84, which had a Fucα1-3GlcNAcβ1,4(fucα1-3)GlcNAc modified N-glycan core that has been previously identified in *H. contortus* N-glycans [43] but not in schistosomes [27]. IgG towards G82 and G84 remained highly positive at week 22. We tested whether these glycans are recognized by an antibody against the structurally related Fucα1-3GalNAcβ1,4(fucα1-3)GlcNAc (F-LDN-F) motif, as the anti-F-LDN-F response is also sustained at week 22. However, anti-F-LDN-F monoclonal antibody 128-1E7 did not bind to G82 and G84 (Fig. S4), meaning that the Fucα1-3GlcNAcβ1,4 (Fucα1-3)GlcNAc modified N-glycan core is not cross-reactive with the F-LDN-F motif. It is likely that antibodies recognizing glycans G82 and G84 are recognizing core α3-fucose irrespective of the presence or absence of a second α3-fucose.

Macaque sera at later infection time points kill schistosomula *in vitro* with higher effectiveness

To investigate the potential functional involvement of macaque serum antibodies in resistance to infection by promoting schistosomula killing, we incubated sera collected at different infection time points with live *in vitro* transformed *S. mansoni* schistosomula. Schistosomula were treated 3 h post transformation with macaque sera (both heat inactivated and not heat inactivated) and their survival rates at 24 h and 48 h post treatment were determined. The schistosomula were visualized by brightfield microscopy and the viability was assessed by schistosomula integrity with propidium iodide (PI) staining [37]. Macaque infection sera at week 14 and week 22 caused patent agglutination of schistosomula quickly after contact, while week 0 and week 4 sera did not lead to any agglutination. Additionally, we observed a clear time point-dependent effect on gross morphology: schistosomula treated with infection sera at week 0 and week 4 resemble control schistosomula without serum addition after 24 h of incubation (Fig. 4A). Incubation

with sera taken at weeks 8, 14 and 22 caused irregularity of schistosomula surfaces after 24 h of incubation, which became even more pronounced after 48 h, with blebbing of the surface. Similarly, there was an infection time point-dependent relationship with PI positivity, where sera at later infection time points caused most schistosomula death as measured by PI positivity (Fig. 4B). While 3 h transformed schistosomula treated with sera at week 0 resulted in 95% schistosomula survival, week 22 sera reduced survival to 57% after 48 h of incubation.

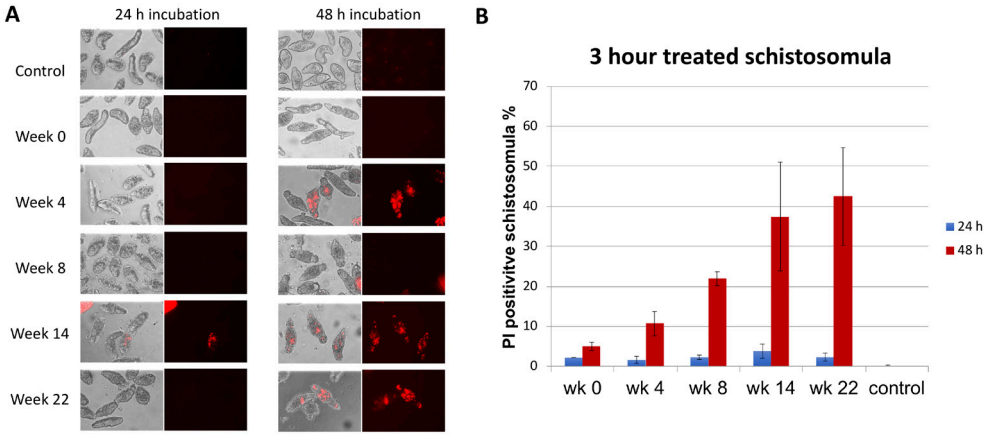

Figure 4. *In vitro* schistosomula incubation with *S. japonicum*-infected rhesus macaque sera collected at different infection time points. Macaque sera were incubated with 3 hour (h) transformed schistosomula. Loss of Schistosomula integrity was visualized by PI positivity. A) Gross morphology of schistosomula after 24 h and 48 h of incubation with macaque sera. B) Percentage of PI-positive schistosomula after 24 h and 48 h of incubation with macaque sera at different infection time points.

Immunofluorescence microscopy has confirmed binding of macaque serum antibodies at late infection time points to the whole surface of schistosomula at 24 h and 48 h post *in vitro* transformation (Fig. S3). Complement factors did not play a role in schistosomula killing as heat-inactivated macaque sera did not lead to increased survival of schistosomula compared to untreated sera taken at the same time point, even though we still observed patent agglutination and blebbing of schistosomula treated with week 8, week 14 and week 22 heat-inactivated serum (Fig. S2).

Glycans with IgG^{high}and IgM^{low} binding at week 22 have multiple fucosylated LDN motifs

Rhesus macaques are protected against a secondary schistosome infection 21 weeks after primary infection [13]. We observed that serum from macaques infected for 22 weeks had

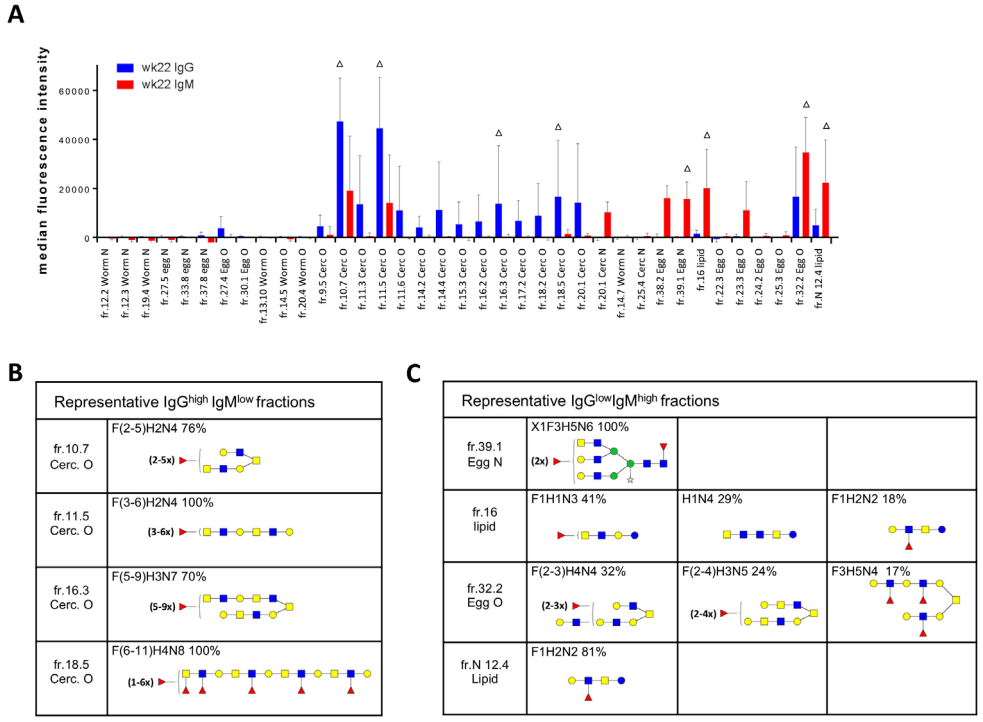

Figure 5. Glycan fractions that have statistically different serum IgG and IgM responses at week 22 post-infection. A) 37 glycan fractions have a significant difference in IgG and IgM binding ($p<0.05$). Represented glycans are marked with Δ. B) The glycan composition and most likely motif of representative fractions of IgG dominant and C) IgM dominant groups are depicted.

superior killing ability on 3 h *in vitro* transformed schistosomula compared to sera taken from earlier infection time points. The glycan array analyses showed that the IgG and IgM balance was changed at week 22 post-infection: while most anti-glycan IgMs have decreased, many anti-glycan IgG responses remained high. IgG is generally considered as the antibody isotype that provides effective protection to infection while IgMs are found to block the activity of protective IgGs by preventing effective antibody-dependent cell-mediated cytotoxicity (ADCC) of schistosomula *in vitro* [14]. We compared IgG and IgM response intensity at week 22 post-infection, to see which glycan fractions would have a difference in IgG and IgM responses, hypothesizing that glycan motifs which are IgG^{high} and IgM^{low} could be targets of protective immunity. We performed a statistical analysis on glycans that had infection-induced antibody binding at week 22 and found 37 glycan fractions that had a significant difference in IgG and IgM binding (Fig. 5A). 12 of these fractions had higher IgM than IgG ($IgG^{low}IgM^{high}$), while 25 fractions had higher IgG than

IgM ($IgG^{high}IgM^{low}$). We saw that the glycan fractions that were $IgG^{high}IgM^{low}$ almost all invariably contained a terminal multi-fucosylated LDN motif (Fig. 5B). In contrast, glycans of the $IgG^{low}IgM^{high}$ group did not have highly fucosylated LDN structures and contained mostly LeX or LDN-F terminating motifs instead (Fig. 5C). Five fractions were selected to represent each group (Fig. 5B and Fig. 5C). Notably, 56% of the glycans in the IgG dominant group were cercarial O-glycans (Table S4).

Discussion

Using a microarray approach, we have followed the anti-glycan IgG and IgM responses in *S. japonicum*-infected rhesus macaques over a time course of 22 weeks, from the time of infection until the macaques have been reported to become resistant to reinfection while eliminating existing worms [13]. The schistosome array used in this study consisted of large repertoire of N-, O- and GSL derived glycans isolated from *S. mansoni* larvae, adult worms and eggs [19, 23]. A similar array has previously been used to study the different anti-glycan responses in adults and children in *S. mansoni* endemic regions [19, 23] and to study antibodies from local lymph node cells during *S. japonicum* infection of rats [24]. This array has been complemented with a previously established focused synthetic array [35] in order to address the antigenic properties of a specific set of xylosyl and fucosyl N-glycan core modifications.

One question addressed in the current study was whether anti-glycan antibodies may be associated with the elimination of mature worms by *S. japonicum*-infected rhesus macaques. Previous studies have indicated that O-glycan expression in schistosome adult worms is limited in comparison to cercariae and eggs, and that antibodies against worm glycans are not very cross-reactive with the highly antigenic egg and cercarial glycans [23]. In line with this observation, we saw minimal antibodies against worm glycoprotein glycans. We have observed that the extent of glycan fucosylation is an important factor for triggering the host immune response. Unlike cercariae and eggs that express many different antigenic highly fucosylated glycans on both glycoprotein and glycolipids, adult worms express antigenic fucosylated glycans such as F-LDN and F-LDN-F predominantly on glycolipids [26, 27] and the less antigenic LDN-F and LeX motifs on specific worm N- and O-glycan subsets [44, 45].

The glycolipid associated F-LDN and F-LDN-F antigen can be found in undefined parenchymal spots or ducts inside the adult worm [26], though the exact function of these parenchymal spots and ducts is not understood. Notably, S*chistosoma* worms isolated from 22 week-infected rhesus macaques have diminished body lengths and reproductive organs [16]. It has previously been suggested that schistosome worm elimination occurs

by macaque IgG attacking gut digestive enzymes, tegument surface hydrolases and antioxidant enzymes, eventually leading to worm death through cessation of blood feeding [12]. Recently, Li et al. have shown that rhesus IgG binds to the esophageal lumen of *S. japonicum* worms and co-localizes with esophageal secreted proteins, MEGs 4.1, 8.2, 9, 11 and VAL-7 [16]. It was suggested that rhesus IgGs block esophageal function making blood difficult to ingest, which eventually leads to the starvation of schistosome worms. It is not known whether glycans are the targets of these IgGs. Interestingly, esophageal located protein MEG-4.1 from *S. mansoni* was found to be heavily O-glycosylated [46] and MEGs 4.1 and 8.2 of *S. japonicum* are predicted to have similar properties [16]. It is noteworthy that three transcripts of glycosyltransferases were highly enriched in the male esophageal region but not in the posterior containing gut and tegument epithelia [47], which might indicate the synthesis of novel esophageal glycans. Unfortunately, esophageal located MEG proteins are expressed and secreted in minute amounts, and their O-glycans are not yet identified. If the esophageal MEG proteins carry unique O-glycans, it is unlikely that these are represented on the array due to their very low relative abundance in the overall worm glycome. Alternatively, MEGs may contain simple mono- and disaccharides such as GalNAc or Galβ1-3GalNAc (T and Tn antigens) that form multivalent O-glycosylated peptide domains. Monosaccharides and disaccharides are not isolated and printed using our glycan array methodology. If feasible, it would be more appropriate to determine binding of antibodies to short O-glycans structured in mucin domains in the context the native O-glycopeptide, or an identical synthetic construct thereof.

As well as eliminating adult worms, rhesus macaques are also found to be resistant towards secondary infection 16 weeks or more after primary exposure to schistosomes [13]. Freshly transformed schistosomula *in vitro* representing 'skin stage' schistosomula *in vivo* have previously been used to study mechanisms that might be related to reinfection [14, 15, 48]. Schistosomula are also susceptible to antibody-mediated damage [8, 49, 50] and may the best stage for the immune system to attack the parasite. Schistosomula-expressed glycans are a subset of glycans expressed by cercaria [27, 40]. We have shown that 3 h transformed schistosomula are killed by macaque sera in an infection time point-dependent manner: sera taken from macaques after 22 weeks of infection were more effective at killing than earlier infection time points, suggesting that the macaques may build up immunity towards the parasite during the infection. Luyai et al. have previously shown that rhesus sera from week 8 and week 11 post-infection were most efficient in killing schistosomula of *S. mansoni in vitro* [22]. However, we observed that macaque sera taken 14 and 22 weeks post-infection were even more effective in killing schistosomula compared to sera taken 8 weeks after infection.

At week 14 and week 22, the most prominent epitopes with high IgG binding were

the highly fucosylated glycan motifs expressed on O-glycans and glycosphingolipid-derived glycans. Our statistical analysis on serum IgG and IgM balance at week 22 post-infection showed that glycans that were statistically $IgG^{high}IgM^{low}$ were mostly cercarial O-glycan fractions. Moreover, these $IgG^{high}IgM^{low}$ cercarial O-glycan fractions all contained highly fucosylated LDN epitopes. IgG is usually considered the protective antibody isotype compared to IgM. Early studies on human resistance to schistosome reinfection found a positive correlation between reinfection intensity and anti-schistosomula and anti-egg IgM antibodies [51, 52]. In addition, a strong inverse relationship is observed between the rapidity and intensity of IgG response and worm burden at 18 weeks in rhesus macaques [12]. We acknowledge that high IgG titers towards a certain glycan epitope do not necessarily lead to resistance to reinfection. Different IgG subtypes have been shown to vary in their potency in inducing eosinophil-mediated killing of schistosomula: human serum IgG1 and IgG3 have been found to be more potent isotypes to induce eosinophil-mediated cytotoxicity, whereas IgG4 antibodies are found to inhibit the cytotoxicity mediated by IgG1 and IgG3 [15]. IgG2 antibodies are only cytotoxic in the presence of activated eosinophils. Additionally, IgG2 and IgG4 were found to correlate with susceptibility to schistosome reinfection [52, 53]. Therefore, a protective response against skin schistosomula may not simply derive from high titers of a certain antibody isotype, but also likely from a balanced selective expression of protective antibodies and an absence of blocking antibodies. In our study, glycans in cluster IgG-C3 possessed IgG binding 8 weeks post-infection, but these IgG responses disappeared after 14 weeks post-infection when the macaques are immune to a secondary infection. The glycans in this profile were mostly N-glycans with motifs such as LN, LeX, LDN, α2-mannose, terminal Gn and terminal fucosylated Gn (Table S1B). We postulate that the disappearance of IgGs against these glycan motifs could be involved in the effective immune protection found in macaques. On the other hand, it is also intriguing to see that antibodies that are sustained after macaque immunity are also found to bind to these motifs. Taking into account the different properties of IgG subtypes, it would be relevant to investigate whether the IgG response against motifs that disappear during infection clearance are of a different subtype than the IgGs that are sustained when macaques are protected.

At week 22, our last serum collection time point, infected macaques are already resistant to secondary infection [10, 16]. We suggest that multi-fucosylated LDN motifs that are $IgG^{high}IgM^{low}$ may be involved in this resistance. However, Luyai et al. showed that macaque sera from week 78 post-infection could not kill freshly transformed schistosomula [22]. It would therefore be very interesting to test whether week 78 infected rhesus macaques lack the high antibody titers against highly fucosylated LDN epitopes. This would further indicate whether antibodies against highly fucosylated LDN epitopes

are involved in resistance to reinfection in rhesus macaques or not. Our results using the synthetic glycan microarray are in accordance with Luyai et al. [22] that schistosome-infected macaques generate high IgG antibody responses to the core xylose and core α3-core fucose and LeX and LDN epitopes of N-glycans. Nevertheless, it is important to realize that core α3-fucose has only been found in mature eggs but not in schistosomula of *S. mansoni* [27] or *S. japonicum* [28]. Therefore, we believe that antibodies against core α3-fucose do not contribute to schistosomula killing. Additionally, unlike the IgG response towards multi-fucosylted LDN motifs that is sustained with time, the IgG response towards core α3-fucose appears during oviposition and decreases when the adult worm stop producing eggs (Fig. 3), further supporting that core α3-fucose is egg-derived.

Our study of *S. japonicum*-infected macaques was performed on a *S. mansoni*-derived glycan microarray. On the basis of high similarity in protein coding gene sequences between the two species [30] and cross species protein recognition by antibodies [31, 32], the glycosylation patterns of the two species are expected to be very similar. Accordingly, we observed extensive binding of *S. mansoni*-derived glycans by serum antibodies from *S. japonicum*-infected rhesus macaques (Fig. 1). Interestingly, although *S. japonicum* glycans were described as less fucosylated than *S. mansoni* [33, 34], our glycan array data shows that antibodies against multi-fucosylated glycans are elicited in *S. japonicum*-infected rhesus macaques, indicating that *S. japonicum* does produce such glycan antigens, including those than contain the Fucα1-2Fucα1- (DF) sequence.

Rhesus macaques generate a plethora of anti-glycan antibodies during *S. japonicum* infection. In this study, we hypothesized that glycan motifs that have high IgG binding could be associated with resistance to *Schistosoma* infection. It appears that the presence of IgG antibodies against multi-fucosylated LDN is correlated with the effectiveness of schistosomula killing *in vitro*. However, it is interesting to see humans that are generally susceptible to re-infection also generate antibodies towards many of these fucosylated glycans, although to a lower extent [19]. Although this observation certainly does not undermine the possible protective effect of anti-glycan antibodies, it indicates that the presence of antibodies towards certain epitopes is not directly related to protection. As discussed earlier, different studies have shown that antibody isotype balance affects resistance to reinfection [15, 51, 52]. Perhaps even in an unprotected infected host, protective antibodies exist, but not in sufficient quantities to overcome the infection, or protective antibodies are overshadowed by the abundance of irrelevant or blocking antibodies. Some researchers have suggested that high antibody responses towards glycans are beneficial not for the host, but for the parasite, by directing the immune system away from epitopes that could provide protective immunity [18, 29]. Mice vaccinated with viable schistosome eggs although eliciting high anti-glycan antibody titers, were not

protected against cercarial challenge *in vivo* [18]. In our case, at least, we have shown that high antibody titers against glycans did not prevent rhesus macaques from eliminating schistosome adult worms while gaining resistance to reinfection. It is imperative to consider that macaques may have intrinsic antibody differences, rather than epitope specificity, that lead to protection. Antibody responses towards schistosome glycans in *S. japonicum*-infected rhesus macaques are very dynamic and worth further detailed study. Deciphering the specificity of antibodies sustained at week 22 post-infection may provide clues to the composition of the antibody pool in a host resistant to schistosome re-infection and may provide valuable information about the glycan targets that could be involved in protection against re-infection.

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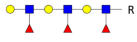
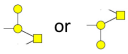
Supplementary information

Table S1

A **Distribution of glycan origin in each IgG glycan cluster**

	IgG-C1		IgG-C2		IgG-C3		IgG-C4	
worm N	4%		0%		33%		51%	
cerc N	3%	17%	8%	15%	26%	75%	23%	88%
egg N	10%		7%		16%		15%	
worm O	5%		8%		13%		11%	
cerc O	37%	66%	33%	84%	4%	25%	0%	12%
egg O	24%		43%		7%		1%	
GSL		17%		1%		0%		0%

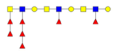
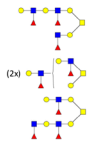
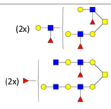
B **Putative glycan motifs present in each IgG glycan cluster**

Putative epitopes		IgG-C1	IgG-C2	IgG-C3	IgG-C4
Size of cluster		177 fractions	123 fractions	69 fractions	75 fractions
Core α6-fucose		6%	7%	32%	41%
Xylose		4%	7%	9%	11%
LN		15%	24%	29%	27%
LeX		33%	34%	23%	13%
Di-LeX		10%	7%	7%	1%
Tri-LeX		2%	0%	0%	0%
LDN		10%	12%	16%	17%
LDN(F=1)		12%	14%	3%	0%
LDN(F≥2)		25%	10%	1%	0%
α2-Mannose		9%	7%	30%	36%
Gn		19%	31%	26%	20%
Gn(F≥1)		20%	30%	13%	4%
β1-6 gal		8%	12%	1%	0%
Gal-LDN		6%	12%	0%	0%
Gal-LDN (F≥1)		6%	13%	0%	0%

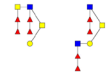
The most likely glycan motifs were depicted for each glycan fraction present in individual clusters. The percentages of each glycan motif present in each glycan cluster is shown. For most of the glycan fraction, more than one possible motif is present.

Table S2

IgG-C1

Name of fraction[a]	Composition of abundant glycans in the fraction[b]	Putative glycan motifs[c]	Likely structure of the most abundant glycan in fraction[d]
*Cerc O fr.12.7	F5H2N5 35%	Gal-LDN(F0-5), LDN(F0-5)	
	F6H2N5 33%	Gal-LDN(F0-6), LDN(F0-6)	
	F8H2N5 23%	Gal-LDN(F2-6), LDN(F2-6)	
+Cerc O fr.15.6	F7H3N6 33%	DF-LDN-TF	
	F9H3N6 29%	DF-LDN-TF	
	F6H3N6 18%	DF-LDN-DF	
	F5H3N6 10%	DF-LDN-F	
Lipid L23.1	F4H1N6 73%	LDN(F0-4)	
Lipid L24.1	F7H1N6 43%	LDN(F2-7)	
	F4H1N6 19%	LDN(0-4)	
	F8H1N6 15%	LDN(F2-8)	
*Egg O fr.32.2	F3H4N4 28%	LeX, Di-LeX, Tri-LeX, FGn	
	F3H5N4 17%	LeX, Di-LeX, Tri-LeX	
	F3H3N5 16%	Gal-LDN(F0-3)	
*Egg O fr.36.2	F4H5N5 15%	LeX, Di-LeX, Tri-LeX, (F)Gn	
	F4H4N6 11%	FGn	

IgG-C2

Name of fraction[a]	Composition of abundant glycans in the fraction[b]	Putative glycan motifs[c]	Likely structure of the most abundant glycan in fraction[d]
Egg O fr.16.3	F2H1N3 100%	LDN(F2), (F0-2)Gn	
Egg O fr.27.2	F3H2N4 54%	(F0-2)Gn, Gal-LDN(F0-3)	
Cerc O fr.8.11	F4H1N3 86%	LDN(F4), (F1-3)Gn	
	F1H5N3 14%	β1-6 Gal, LeX, LN	
Cerc O fr.9.9	H3N5 42%	Gal-LDN	
	H4N2 17%	LN, β1-6 Gal, Gn	
	F4H1N3 16%	LDN(F4), (F1-3)Gn	
	F1H4N2 14%	LeX, β1-6 Gal	
Cerc O fr.3.4	H4N2 100%	β1-6 Gal, LN, Gn	
Cerc O fr.6.6	F1H3N1 100%	β1-6 Gal, LeX	
Egg N fr.38.2	X1F3H5N6 51%	Core α6-fucose, Xylose, LeX, LDN(F0-1), LN	
	X1F1H8N3 49%	Hybrid, Oligomannose, LN	

IgG-C3

Name of fraction[a]	Composition of abundant glycans in the fraction[b]	Putative glycan motifs[c]	Likely structure of the most abundant glycan in fraction[d]
Cerc N fr.25.4	F2H3N4 92%	Core α6-fucose, LDN(F1), (F)Gn	
Egg O fr.26.2	F2H3N3 91%	LeX, diLeX, FGn	
Worm N fr.15.6	F2H4N4 39%	Core α6-fucose, LN, LeX, (F)Gn	
	F1H6N2 35%	Core α6-fucose, oligo-mannose, LDN	
	F1H3N6 11%		
Cerc N fr.32.4	X1F3H5N4 100%	Core α6-fucose, LeX, Core xylose	
egg N fr.26.5	F1H4N4 53%	Core α6-fucose, Gn, LN	
	X1F1H3N5 47%	Core α6-fucose, LDN, Gn, xylose	
egg N fr.32.6	H3N7 67%	LDN, Gn	
	H6N5 33%	LN	
Worm N fr.19.7	H8N2 100%	Oligo-mannose	

IgG-C4

Name of fraction[a]	Composition of abundant glycans in the fraction[b]	Putative glycan motifs[c]	Likely structure of the most abundant glycan in fraction[d]
Egg N fr.23.5	X1F1H3N4 39%	Core α6-fucose, LDN, Gn, xylose	
	F1H4N4 17%	Core α6-fucose, Gn, LN	
	F1H4N3 16%	Core α6-fucose, LN	
Worm N fr.16.7	F1H4N5 100%	Core α6-fucose, LN, LDN	
Cerc N fr.18.6	F1H3N3 100%	Core α6-fucose, Gn	
Worm N fr.10.2	H5N2 58%	Tri-mannosyl	
	H3N4 12%	LDN, Gn	
	F1H3N4 15%	Core α6-fucose, LDN, Gn	
Worm N fr.18.6	H7N2 100%	olligomannose	
Cerc N fr.26.2	F2H4N3 100%	Core α6-fucose, LeX	

[a] Cerc N, cercarial derived N- glycan; Worm N, worm derived N- glycan; egg N-, egg derived N- glycan. Cerc O-, cercarial derived O- glycan; Worm O, worm derived O- glycan; lipid, lipid derived-glycan. Fraction numbers are also shown.
[b] F, fucose; H, hexose; N, N-acetylhexosamine
[c] Putative glycan motifs are proposed on the basis of composition of glycan fractions aided by literature (van Diepen, A., et al., 2015; Jang-Lee, J. et al., 2007)
[d] Most likely structure of the most abundant glycan in the fraction is shown. For the less abundant glycans in each fraction, only proposed motifs are depicted
*fractions previously described by van Diepen, A et al., 2015 including monoclonal anti-glycan antibody results.
+MALDI-TOF-MS/MS spectra of glycans detected in *Schistosoma mansoni* cercarial O- glycan fraction 15.6 shown in paper by van Diepen, A et al., 2015.

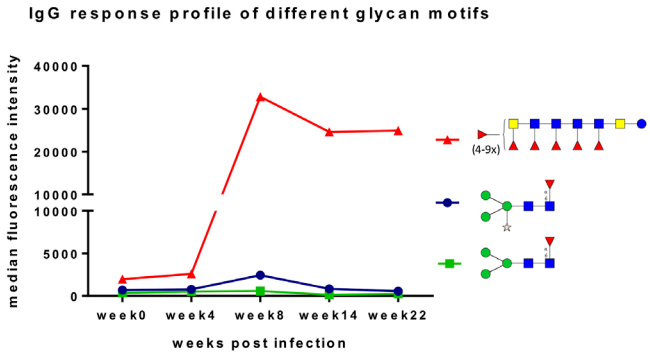


Figure S1. IgG response profile of different glycan motifs. Average median fluorescence intensities are shown for S. japonicum-infected macaque serum IgG over a time course of 22 weeks towards different glycan motifs.

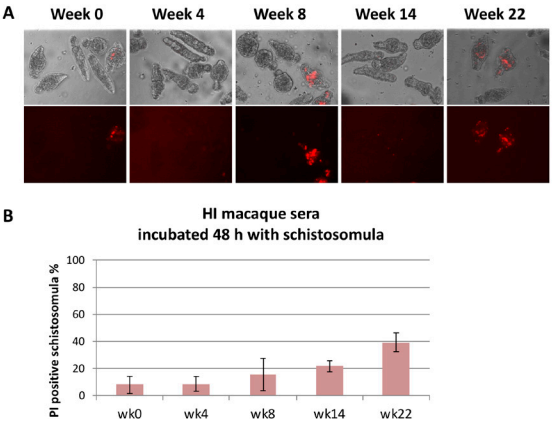


Figure S2. *In vitro* schistosomula incubation with heat inactivated *S. japonicum*-infected macaque sera. *In vitro* schistosomula incubation with heat inactivated (HI) *S. japonicum*-infected rhesus macaque sera collected at different infection time points. HI Macaque sera were incubated with 3 h transformed schistosomula. Loss of Schistosomula integrity was visualized by PI positivity. A) Gross morphology of schistosomula after 48 h of incubation with HI macaque sera. B) Percentage of PI-positive schistosomula after 48 h of incubation with HI macaque sera at different infection time points.

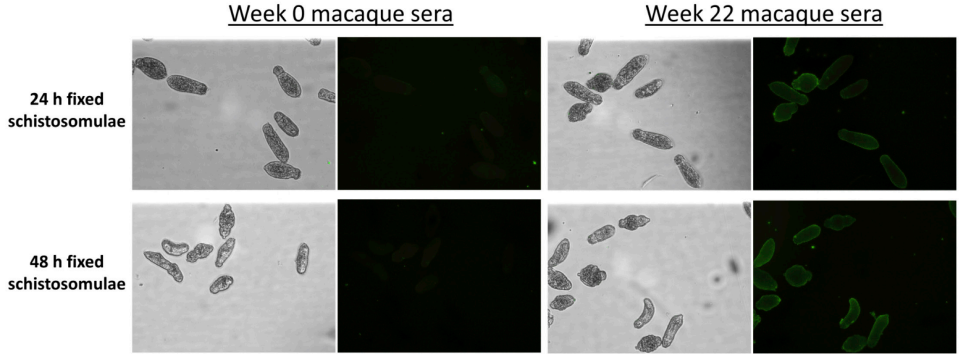


Figure S3. Binding of *S. japonicum*-infected macaque serum antibodies to schistosomula. Schistosomula were fixed 24 h and 48 h after transformation in 2% PFA. Fixed schistosomula were washed twice in 200 µl PBS and then incubated with 0.5 µl of macaque serum (before infection and after 22 weeks of infection) in a suspension of 30 µl for 30 minutes. Parasites were washed 1 time with PBS and then suspended in 25 µl of 50x diluted rabbit-anti-human IgG, IgM IgA antibodies (DAKOPATTS) for 30 minutes. Afterwards, parasites were washed 3 times with PBS and suspended in 25 µl of 200 x diluted Alexa fluor 488-conjugated goat-anti-rabbit IgG (H+L) antibody (invitrogen) for 30 minutes. Finally, the parasites were washed once in PBS and analyzed by fluorescence microscopy.

Table S3

A **Distribution of glycan origin in each IgM glycan cluster**

	IgM-C1		IgM-C2		IgM-C3		IgM-C4		IgM-C5		IgM-C6	
worm N	63%		8%		10%		1%		9%		11%	
cerc N	10%	85%	38%	63%	14%	34%	6%	14%	29%	43%	31%	58%
egg N	12%		17%		10%		8%		6%		17%	
worm O	7%		6%		6%	66%	0%		17%		25%	
cerc O	0%	7%	19%	35%	30%		35%	65%	23%	57%	8%	42%
egg O	0%		10%		30%		30%		17%		8%	
GSL		7%		2%		0%		21%		0%		0%

B **Putative glycan motifs present in each IgM glycan cluster**

Putative epitopes	IgM-C1	IgM-C2	IgM-C3	IgM-C4	IgM-C5	IgM-C6
Size of cluster	41 fractions	52 fractions	145 fractions	127 fractions	35 fractions	36 fractions
Core α6-fucose	34%	29%	15%	9%	23%	17%
Xylose	10%	17%	0%	7%	9%	11%
LN	20%	29%	22%	16%	23%	31%
LeX	5%	29%	34%	28%	31%	22%
Di-LeX	0%	12%	9%	6%	9%	6%
Tri-LeX	0%	2%	0%	2%	0%	0%
LDN	24%	8%	12%	9%	9%	11%
LDN(F=1)	5%	4%	11%	15%	6%	3%
LDN(F≥2)	0%	6%	9%	29%	9%	0%
α2-Mannose	32%	27%	11%	4%	17%	25%
Gn	24%	17%	29%	14%	20%	25%
Gn(F≥1)	0%	8%	26%	19%	20%	14%
β1-6 gal	0%	6%	9%	8%	6%	6%
Gal-LDN	0%	4%	8%	9%	0%	3%
Gal-LDN(F≥1)	0%	2%	9%	10%	0%	3%

The most likely glycan motifs were depicted for each glycan fraction present in individual clusters. The percentages of each glycan motif present in each glycan cluster is shown. For most of the glycan fraction, more than one possible motif is present.

Table S4

A **Glycan origins present in the $IgG^{high}IgM^{low}$ and $IgG^{low}IgM^{high}$ group**

	$IgG^{high}IgM^{low}$		$IgG^{low}IgM^{high}$	
Size of cluster	25 fractions		12 fractions	
worm N	12%		8%	
cerc N	0%	24%	17%	42%
egg N	12%		17%	
worm O	12%		0%	
cerc O	56%	76%	0%	42%
egg O	8%		42%	
lipid		0%		17%

Fractions that had a significant IgG and IgM response difference were grouped into the $IgG^{high}IgM^{low}$ (IgG response>IgM response) or the $IgG^{low}IgM^{high}$ (IgM response>IgG response) group. The $IgG^{high}IgM^{low}$ group was characterized by O- glycans, in particular cercariae derived O- glycans.

B **Putative glycan motifs present in the $IgG^{high}IgM^{low}$ and $IgG^{low}IgM^{high}$ group**

Putative epitopes	$IgG^{high}IgM^{low}$	$IgG^{low}IgM^{high}$
Size of cluster	25 fractions	12 fractions
Core α6-fucose	12%	42%
Xylose	4%	17%
LN	20%	17%
LeX	16%	33%
Di-LeX	12%	17%
Tri-LeX	0%	8%
LDN	36%	25%
LDN(F=1)	32%	33%
LDN(F≥2)	44%	0%
α2-Mannose	8%	0%
Gn	32%	42%
Gn(F≥1)	12%	50%
β1-6 gal	12%	0%
Gal-LDN	12%	25%
Gal-LDN(F≥1)	12%	25%

Glycan Motifs present in the $IgG^{high}IgM^{low}$ and $IgG^{low}IgM^{high}$ group.

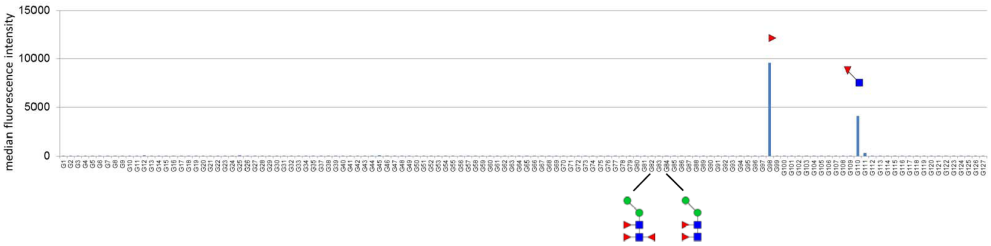


Figure S4. Binding of monoclonal antibody 128-1E7-C to a collection of synthetic glycans. 128-1E7-C is a monoclonal antibody that binds to the Fucα1-3GalNAcβ1,4(fucα1-3)GlcNAc (F-LDN-F)motif (Smit et al., 2015). In this figure, the horizontal axis depicts a collection of other glycans that have previously been synthesized and described by us (Brzezicka et al., 2015). The same binding assay protocol described in the Materials and Methods section was used to detect binding of 128-1E7-C to the glycan microarray. 128-1E7-C was incubated at a concentration of 3.5 µg/ml and Alexa fluor 555-conjugated goat-anti-mouse antibody (Life technologies) diluted 1:1000 was used to detect bound monoclonal antibody on the slide.

Chapter 3

Micro array-assisted analysis of anti-schistosome glycan antibodies elicited by protective vaccination with irradiated cercariae

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Abstract

Baboons vaccinated with radiation-attenuated cercariae develop high levels of protection against schistosome infection, correlating to high antibody titers towards schistosome antigens with unknown molecular identity. Using a microarray consisting of glycans isolated from different life-stages of schistosomes, we studied the anti-glycan immunoglobulin (Ig) G and IgM responses in vaccinated and challenged baboons over a time course of 25 weeks. Anti-glycan IgM responses developed early after vaccination, but did not rise in response to later vaccinations. In contrast, anti-glycan IgG developed more slowly, but was boosted by all five subsequent vaccinations. High IgM and IgG levels against O-glycans and glycosphingolipid glycans of cercariae were observed. At the time of challenge, while most antibody levels decreased in the absence of vaccination, IgG towards a subset of glycans containing multiple-fucosylated motifs remained high until 6 weeks post-challenge during challenge parasite elimination, suggesting a possible role of this IgG in protection.

Background

Schistosomiasis is a chronic and potentially deadly parasitic disease that affects millions of people in (sub)tropical areas [1-4]. Immunity to *Schistosoma* species in humans can be acquired, but is governed by a complex interplay of factors (e.g. frequency of exposure, infections and treatments, and maturation of the immune system) [5-8]. Many efforts have been made to develop a vaccine against schistosomiasis but the goal remains elusive. So far, vaccination with radiation-attenuated (RA) cercariae has given the highest level of protection against infection in several animal models. Optimally attenuated cercariae penetrate the skin and enter the bloodstream but fail to mature as the larvae do not travel beyond the lungs [9-11]. It is hypothesized that the extended time of interaction between parasite and the immune system caused by truncated parasite migration leads to better recruitment of lymphocytes and induction of antibodies [10, 12]. Protection induced by the RA vaccine is effective in naïve animals, and in those receiving drug treatment or chronically infected [13], representing the endemic situation in humans.

Baboons, like humans, are natural hosts for schistosomes [14]. In experimental conditions up to 80% of penetrating cercariae can mature into adult worms [15-17]. Protection induced by the RA cercarial vaccine correlates with the development of parasite specific IgG [15, 18, 19] mostly directed towards the glycan fraction of cercarial and egg secretions [20]. Identifying parasite glycans targeted by the host immune system is crucial to better understand protective and non-protective anti-glycan responses that may be elicited during infection or vaccination. Antigenicity, the dynamics and longevity of antibody responses, are relevant in selecting suitable targets for vaccine development. Our previous studies showed that when rhesus macaques develop “self-cure” resistance against schistosomiasis, they produce an abundance of IgG antibodies against glycans containing multiple fucoses [21]. Such highly fucosylated glycan structures are abundant in cercariae and eggs, two life stages that share many cross-reactive antigens, but not at the surface of adult worms [22]. The resistance to schistosome infection acquired by macaques occurs from 12 weeks post-infection [23] by which time the triggers of antibodies to cross-reactive glycans in cercariae or eggs cannot be distinguished. In contrast, vaccination with RA cercariae provides a unique opportunity to investigate anti-glycan responses induced by cercariae and early schistosomula antigens alone, without the background of a massive anti-egg response.

In this study, we used a glycan microarray to analyse serum from an earlier baboon vaccination study. We investigated the anti-glycan IgG and IgM response over a period of 25 weeks with the aim of revealing glycan targets possibly involved in protection. The array comprises a library of naturally occurring schistosome-derived N-, O- and glycosphingolipid (GSL) glycans isolated from different life stages [24, 25].

Methods

Animal experiment ethics statement

All experimental procedures were approved by independent scientific and ethical committees at the Institute of Primate Research, Nairobi, Kenya. Sera were from experiment 1 of a study in which juvenile olive baboons were exposed to 9,000 irradiated cercariae on 5 occasions at 4-week intervals [18]. Three weeks after the last vaccination, these animals, plus a control untreated group, were challenged with 1,000 cercariae. Blood was sampled every 2 weeks, from vaccinated animals starting at day 0 and from controls at challenge.

Materials

Cy3 conjugated goat anti-human IgG (Fc-specific), BSA and ethanolamine were from Sigma (Zwijndrecht, the Netherlands). Alexafluor 647 conjugated goat anti-human IgM (μ chain specific) was from Invitrogen (Breda, The Netherlands).

Glycan microarray

Previously generated N-, O- and GSL glycan microarrays were used [24, 25]. Printed array slides were incubated with primary sera at 1:100 dilution followed by fluorescently-labeled secondary antibodies at 1:1,000 dilution and scanned using a G2565BA scanner (Agilent Technologies, Santa Clara, CA). Data and image analysis was performed with GenePix Pro 7.0 software (Molecular Devices, Sunnyvale, CA). Background-subtracted median intensities were averaged and processed as previously described [24-26].

Hierarchical clustering analysis

Datasets were $\log_2$ transformed to remove the basic trends of variance. MultiExperiment Viewer v4.5 (https://sourceforge.net/projects/mev-tm4/) was used to perform the hierarchical clustering analysis to group associated glycan fractions. Complete linkage clustering and Euclidean distance metric were used to perform the clustering analysis. The outcome was that glycan fractions inducing similar antibody dynamics were grouped into the same cluster profile.

Schistosomula transformation and binding assays

Cercariae were transformed and antibody binding to schistosomula were determined using previous protocols [21]. Briefly, cercariae were incubated at 37°C in M199 medium supplemented with 1:100 1M HEPES pH7.4, 1x antibiotic antimycotic solution, 1.5 mM glutamine and 10% FCS, for 20 min. Schistosomula were then concentrated in the center of a petri dish on an orbital shaker, resuspended in the above medium and cultured at

37°C in a humidified atmosphere with 5% CO2.

For binding assays, baboon sera were added at 1:10 dilution to duplicate cultures of 250 schistosomula at 3 hours post-transformation. Immediately after treatment, the culture plate was observed to detect changes such as agglutination, and again at 72 hours to determine the morphological effects of treatment and the induction of schistosomula killing [21].

Immunofluorescent microscopy

All steps in the protocol were followed by washing in PBS. Three-hour *in vitro* transformed schistosomula were fixed in 2% paraformaldehyde, then incubated for 30 minutes at 37°C with 20 ul 5x diluted baboon serum, Protein G-purified baboon IgG, or IgG-depleted baboon serum. Antibody binding was detected using at 1:1000 dilution of FITC-conjugated anti-human IgG and AlexaFluor 647-conjugated anti-human IgM antibodies for another 30 minutes at 37°C, followed by transfer to a 96-well plate and analysis by fluorescence microscopy (Leica AF_6000). Where pre-incubations were necessary, they were also performed at 37°C for 30 minutes.

Results

Vaccination with irradiated cercariae induces IgM and IgG responses against various cercarial glycans

We incubated baboon serum with schistosome glycan microarrays to document anti-glycan antibody responses during sequential vaccinations with irradiated cercariae (Figure 1). The IgM response against many glycan targets was detectable by 2 weeks after the first vaccination. It peaked at week 6, two weeks after the second vaccination when most IgM responses were directed against cercarial O-glycans and GSL glycans. Some anti-cercarial glycan responses were cross-reactive with egg-derived N- and O-glycans present on the array, although no eggs were present in the animals. Antibodies to N- and O-glycans derived from adult worms were not detected over the whole time course, but among the GSL glycans several were (co-) expressed by adult worms.

Similar to IgM, cercarial O-glycans and GSL glycans were the main targets of IgG antibodies in the vaccinated baboons but with different dynamics. The induction of the IgG antibodies towards most glycans occurred more slowly than IgM, taking six weeks and two vaccinations to elicit a clear response. Subsequent vaccinations further boosted the IgG response against cercariae-derived O- and GSL glycans. At week 10, a shift of IgM:IgG

ratio occurred (Figure 2) as IgG titers against cercarial O- and GSL glycans continued to rise, while the IgM titers did not, indicative of a heavy-chain class switch. Both IgM and IgG responses towards cercarial O- and GSL glycans followed a sawtooth pattern with a peak two weeks after each vaccination. This pattern was especially consistent for IgG responses against cercarial GSL derived glycans, requiring regular boosting to augment. It is noteworthy that neither IgG nor IgM anti-glycan responses were boosted upon challenge infection at week 19 (Figure S1 and S2). The slight increase in antibody response of vaccinated animals against cercarial O- and GSL glycans at 25 and 23 weeks, respectively, could reflect the few challenge parasites that survived in the vaccinated host. Indeed, in challenge control baboons a steep increase in IgM response against cercarial GSL and O-glycans was observed at 23 weeks (Figure S2B), similar to that seen in week 4 vaccinated baboons.

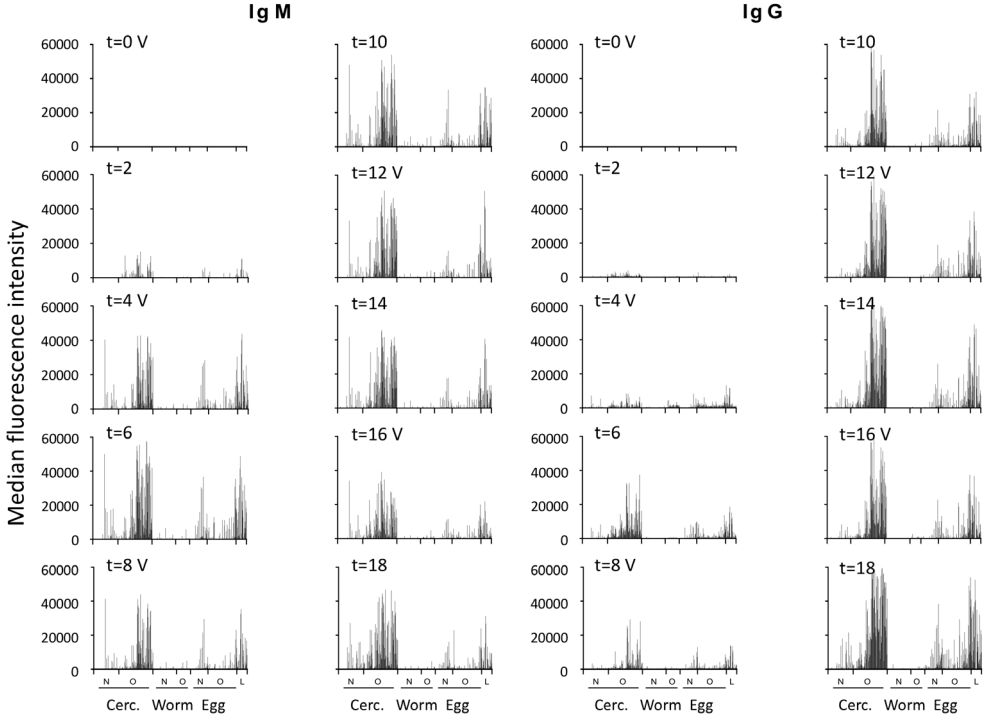

Figure 1. Averaged serum IgG and IgM responses from RA cercaria-vaccinated baboons to glycans isolated from different life stages of schistosomes. The horizontal axis indicates N-, O- and GSL glycan fractions from *S. mansoni* cercariae (Cerc.), adult worms, and eggs. Average background-subtracted median fluorescence intensities are shown for IgG and IgM over a time course of 18 weeks. Each bar corresponds to antibody binding to individual glycan fractions printed on the glycan microarray. N: N-glycans. O: O-glycans and L: glycosphingolipid (GSL) glycans. V: time of vaccination.

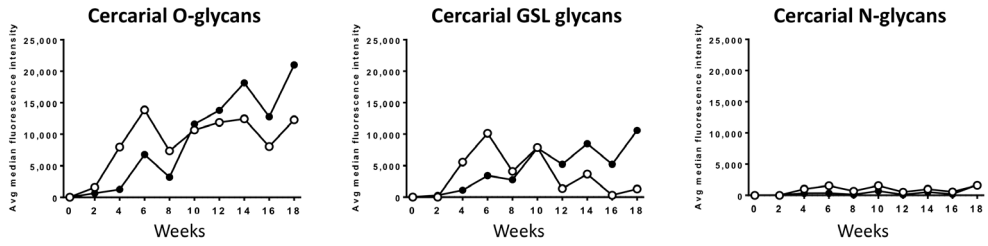

Figure 2. Anti-cercarial-glycan response of RA cercariae vaccinated baboons during vaccination. Averaged IgM (open circle) and IgG (closed circle) response towards cercarial O-, GSL and N-glycans over the 18 weeks of RA cercariae vaccination. GSL: glycosphingolipid glycans.

IgM and IgG response profiles of baboons vaccinated with irradiated cercariae

To better understand the antigenicity of specific cercarial glycan motifs, we performed a hierarchical clustering analysis of the IgM and IgG response patterns, thereby grouping cercarial glycans with similar antigenicity profiles. Anti-glycan antibody responses were corrected for baseline (week 0) intensity. Four IgM response profiles, IgM-C1, IgM-C2, IgM-C3 and IgM-C4, were identified (Figure 3A). IgM-C1 and IgM-C2 followed similar patterns, both reaching their maximum at 6 weeks and remaining relatively stable thereafter, with IgM-C1 containing more potent targets than IgM-C2 (Figure 3B). Substantial changes only occurred in IgM-C1 and IgM-C2 after challenge, where a decline was observed at week 23. The IgM-C1 cluster contained mainly cercarial O-glycans while those in IgM-C3 and IgM-C4 were mostly N- and GSL derived (Figure 3C). The multiple-fucosylated-LDN motif was mainly present in IgM-C1 (Figure 3D), while the Gn motif containing more than one fucose was highly abundant in IgM-C2 but not IgM-C1.

The extent of fucosylation on the Gal-LDN motif expressed on O-glycans did not appear to affect its antigenicity. Four IgG response profiles, IgG-C1, IgG-C2, IgG-C3 and IgG-C4 were also identified (Figure 4A). IgG-C1 contained highly antigenic glycans: IgG against this cluster reached a high titer at 10 weeks post-vaccination with only minor fluctuations thereafter (Figure 4B). Glycans in IgG-C1 bound most antibody and it was the only cluster to which a sustained response was observed at week 19 after cercarial challenge. IgG against glycans in IgG-C2 rose quickly in the first 4 weeks, similar to IgG-C1. Each booster vaccination increased IgG binding to the IgG-C2 glycans, although overall it was lower than to IgG-C1, and unlike in IgG-C1, the IgG response towards glycans in IgG-C2 decreased after week 19. The glycans in both IgG-C3 and IgG-C4 showed very low binding. IgG-C4, the least antigenic cluster of glycans, was dominated by cercarial N-glycans (Figure 4C), while those in IgG-C1 and IgG-C2 were mostly derived from cercarial O-glycans, especially in IgG-C1. Similar to IgM targets, highly fucosylated glycan

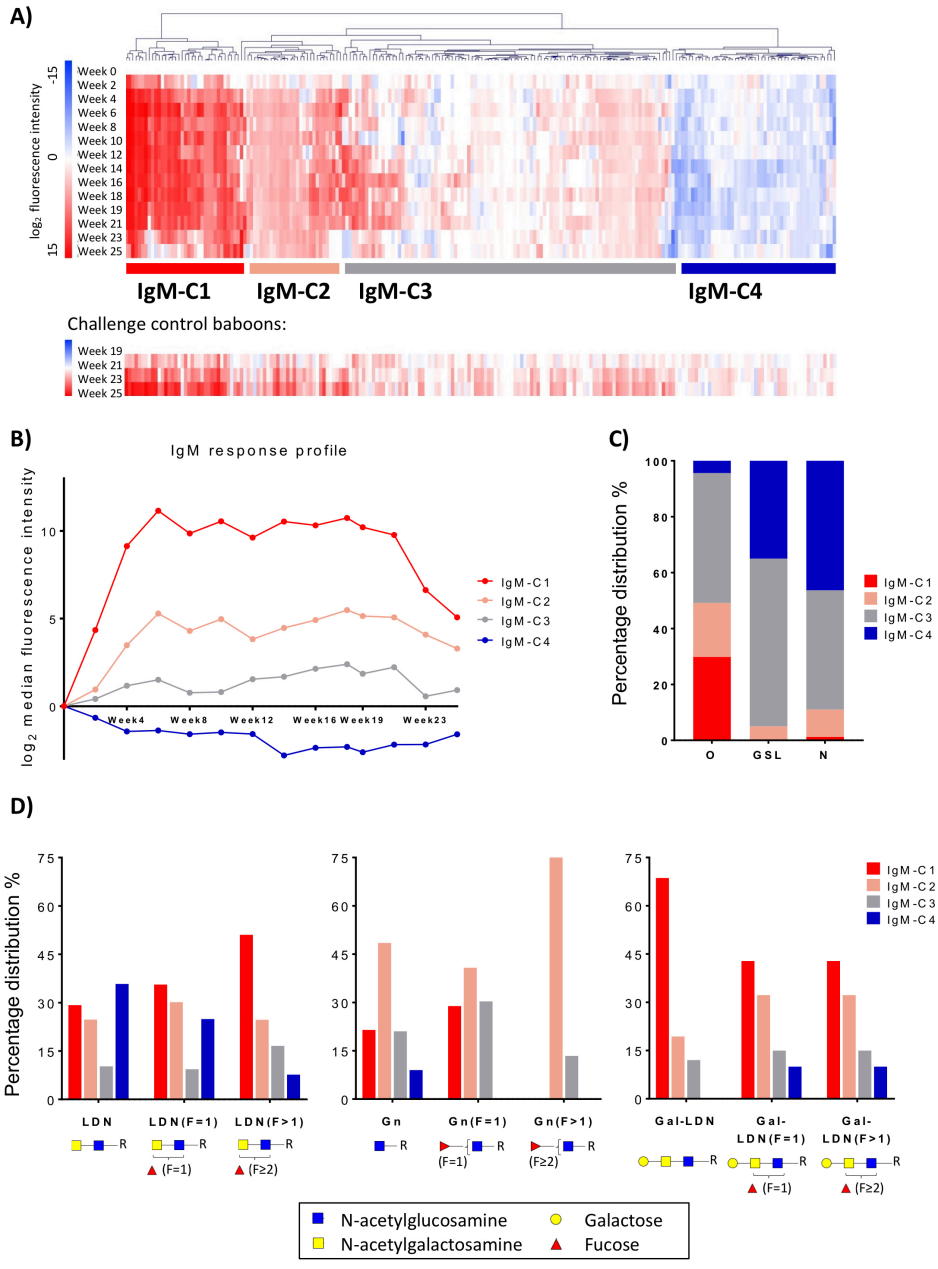

Figure 3. Hierarchical clustering analysis of anti-cercarial glycan IgM response in baboon serum during vaccination and challenge infection. A) Hierarchical cluster of anti-glycan IgM response dynamics. The reduction in antibody binding compared to week 0 is indicated in blue, while the increase is indicated in red. Glycan fractions inducing similar antibody dynamics are grouped into the same cluster profile. B) Response profile curve for each major IgM cluster. C) Distribution of IgM cluster profiles based on glycan origin. D) Percentage distribution of cluster profiles assigned in A) to various glycan motifs. O: O-glycans; GSL: glycosphingolipid glycans; N: N-glycans.

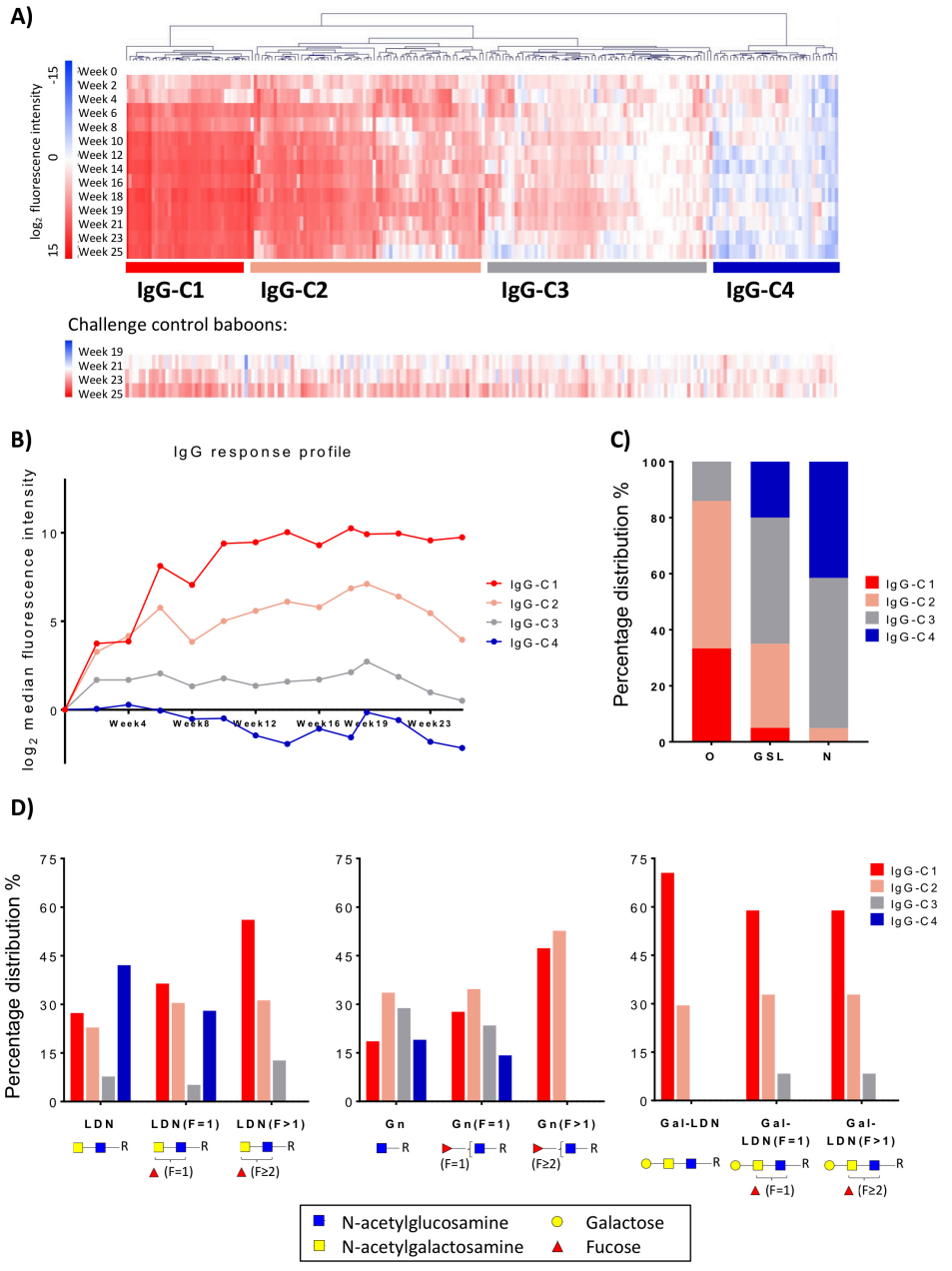

Figure 4. Hierarchical clustering analysis of anti-cercarial glycan IgG response in baboon serum during vaccination and challenge infection. A) Hierarchical cluster of anti-glycan IgG response dynamics. The reduction in antibody binding compared to week 0 is indicated in blue, while the increase is indicated in red. Glycan fractions inducing similar antibody dynamics are grouped into the same cluster profile. B) Response profile curve for each major IgG cluster. C) Distribution of IgG cluster profiles based on glycan origin. D) Percentage distribution of cluster profiles assigned in A) to various glycan motifs. O: O-glycans; GSL: glycosphingolipid glycans; N: N-glycans.

motifs containing e.g. Fucα1-3GalNAcβ1-4(Fucα1-3)GlcNAc- or Fucα1-2Fucα1-3GlcNAc-elements were more abundant in IgG-C1 and IgG-C2 (Figure 4D). Structures with no/low fucose content were more abundant in IgG-C3 and IgG-C4.

Binding of serum IgM and IgG of vaccinated baboons to *in vitro* transformed schistosomula

After identifying antigenic glycan targets of antibodies induced by protective vaccination of baboons, we examined whether the serum would recognize antigens present on the surface of schistosomula, as binding is essential for the action of antibody dependent cellular mechanisms. We found that both IgM and IgG bound to the surface of 3 hour transformed schistosomula fixed in 2% paraformaldehyde (Figure 5A); the binding patterns were in accordance with the presence of anti-glycan IgM and IgG measured by glycan microarray. At week 6 post-vaccination, IgM bound to the surface of schistosomula with high intensity, whereas IgG binding was higher in week 19, correlating with the IgG titers against cercarial O- and GSL glycans shown in figure 2A. In control baboons, upon challenge infection at week 19, IgM and IgG binding to the surface of schistosomula was negative, but by week 25, strong IgM and weak IgG binding to the surface of schistosomula was observed, similar to that seen 6 weeks after vaccination.

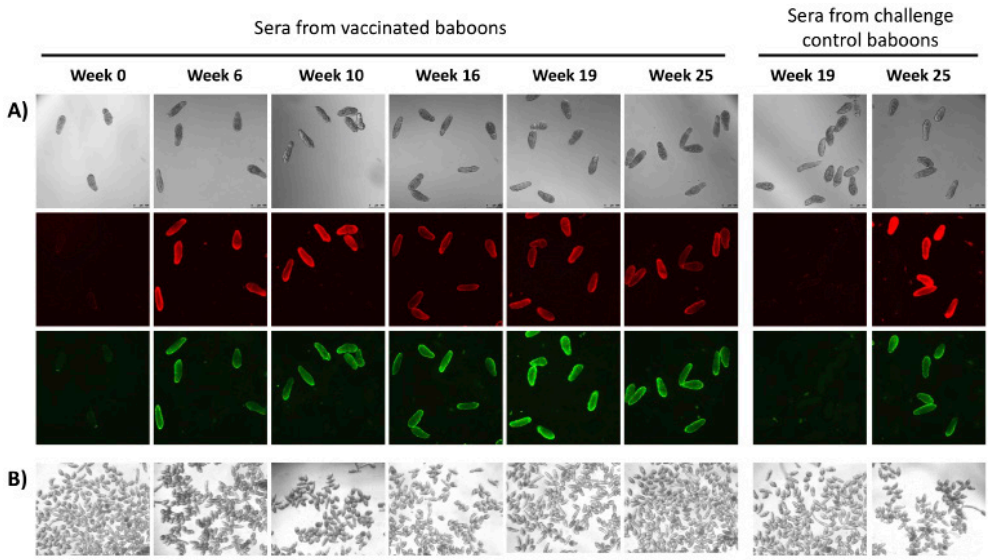

Figure 5. Serum antibody binding to the surface of 3 hour transformed schistosomula. A) anti-human IgM (red) and anti-human IgG (green) antibodies were used to detect vaccinated and challenge control baboon serum IgM and IgG binding on the surface of schistosomula fixed in 2% PFA. B) Agglutination of live schistosomula after 24 hours incubation with baboon sera.

In addition, we also found that vaccinated baboon sera taken at 6 weeks to 19 weeks, as well as week 25 control baboon serum were able to agglutinate live schistosomula *in vitro* (Figure 5B). This agglutination of was not caused by the IgG fraction, as protein G-purified serum IgG did not cause agglutination (Figure S3A), suggesting that other serum components, such as IgM, were responsible. Additionally, IgG-depleted serum from week 6 post-vaccination was able to agglutinate schistosomula more effectively than week 19 vaccination sera, corresponding to the higher IgM titers at week 6, further indicating a role for IgM in the agglutination.

Lastly, since both IgM and IgG bound to the surface of schistosomula, we tested if the presence of one isotype would prevent the binding of the other. In a series of antibody competition experiments, schistosomula were pre-incubated with either purified IgG or IgG-depleted sera before analyzing the binding of IgM and IgG. We found that IgG-depleted sera from week 6, containing high titers of IgM, did not prevent purified IgG binding to the surface of the parasite (Figure S3B). Likewise, pre-incubating with the purified IgG fraction from week 19 vaccinated serum, containing high titers of IgG, did not prevent IgM binding to the surface of schistosomula (Figure S3C).

Discussion

In this study we have explored the glycan-directed antibody response in serum from baboons given protective vaccinations with RA *S. mansoni* cercariae and found that IgM and IgG target highly antigenic glycan structures with multifucosylated GalNAc/GlcNAc motifs. Despite recognizing similar antigenic glycan motifs, only IgG titers were boosted after successive vaccinations, while IgM responses reached a plateau after two vaccinations or decreased to baseline thereafter. Multi-fucosylated glycan motifs are expressed throughout the development of schistosomes, from cercariae to adult worms and eggs, although in different contexts on N-, O- and GSL glycans [22]. As a cercaria transforms into a schistosomulum, it loses its glycocalyx with highly fucosylated glycan motifs on O-glycans, which remains in the epidermis. Thereafter, the schistosomulum still expresses multi-fucosylated glycan motifs on its surface more likely linked to lipids than proteins. Although antigenic O-glycans may no longer be exposed on the surface of 3 day-old schistosomula or on juvenile worms, the glycocalyx residues in the skin may continue to serve as an antigen source eliciting antibodies that can cross react with GSL glycans [22, 27, 28].

The site and mechanism of challenge parasite elimination in hosts protected by the RA vaccine has been controversial. Skin-stage schistosomula are readily killed by

antibody-dependent cellular mechanisms *in vitro* by a combination of murine antibodies and macrophages [29, 30] but *in vivo* evidence is tenuous [29, 30]. Indeed, the developing resistance of maturing schistosomula in killing assays has been attributed to the loss or masking of surface antigens. Thus *ex vivo* isolated lung schistosomula show minimal surface antigenicity when incubated with chronically infected or RA cercaria-vaccinated mouse sera [31], and there is no direct evidence for antibody-mediated killing of lung-stage schistosomula. Surprisingly, therefore, parasite tracking studies have revealed that challenge elimination in RA vaccinated mice occurs at the lung stage of migration [32]. Indeed, some passive immunization experiments in mice have shown that RA cercariae vaccinated mouse serum is most successful when given around 5 days post challenge, when the larvae are in the lungs, rather than at the time of challenge [33, 34], and IgG was the crucial component [34, 35]. The lack of evidence for direct killing led to the proposal that lung-stage schistosomula fail to mature because their intravascular pulmonary migration is blocked by inflammation mediated by activated macrophages [36, 37]. The situation in the vaccinated baboon is unclear because the site and timing of challenge parasite elimination is unknown, which makes evaluation of the potential role of glycans in the process difficult.

Nevertheless, we have previously shown that *in vitro*-transformed *S. mansoni* cercariae still express highly antigenic multi-fucosylated glycan motifs on the schistosomula surface up to one week post-transformation [28], and may thus serve as targets for direct immune attack or initiators of pulmonary inflammation. Any discrepancy could be explained by the faster development of schistosomula *in vivo* compared to *in vitro* transformed counterparts, so losing (or masking) fucose binding sites earlier. To conclude whether the antibodies elicited against multi-fucosylated motifs in RA cercaria-vaccinated baboons can target lung-stage schistosomula would require direct isolation of the parasite developing in the vaccinated baboon.

The lack of information on the site of challenge elimination in baboons leaves open the possibility that skin-stage schistosomula might be the target of immune attack in that host. We found that baboon vaccination serum containing high titers of anti-glycan IgG and IgM bound to the surface of 3-hour schistosomula *in vitro*, indicating the abundance of antigenic targets for interaction with immune effector cells. Unfortunately, due to the limitation of serum, we were unable to purify anti-glycan antibodies to confirm their specific contribution, and whether they could mediate antibody-dependent cellular cytotoxicity. In addition to parasite binding, we observed that the IgG-depleted fraction of baboon serum with high anti-glycan IgM titers was able to agglutinate schistosomula, most likely due to the pentameric IgM structure cross-linking multiple targets. Assuming that IgM was the agglutinating agent and its targets were glycans, then the targets are

most likely lipid-associated because agglutination levels decreased with time in parallel with IgM responses to GSL glycans. We suggest that IgM against GSL glycans is an unlikely participant in protection due to its low titer at week 19 after multiple vaccinations. Furthermore, considering the large size (990kDa) of IgM, it is unlikely to readily leave the bloodstream for the skin tissues [38]. As a result, GSL glycans abundant on the newly transformed schistosomula at the skin stage will remain inaccessible to IgM until they enter the bloodstream about four days after skin penetration.

In several vaccination or resistant primate models, there is a positive correlation with IgG and negative correlation with IgM in relation to protection [15, 18, 21, 39]. Serum IgM has been demonstrated to act as a blocking antibody, preventing cytotoxic attack on schistosomula *in vitro* by effector IgG antibodies [40]. We consistently saw that IgM responses rose rapidly in response to vaccination and challenge, but this increase was short-lived. Ten weeks after vaccination appeared to be the turning point where IgM:IgG ratios changed, with subsequent vaccinations boosting only IgG titers. At the point of challenge, the IgG response against cercarial O- and GSL glycans was at its highest, consistent with the possibility that anti-glycan IgG is important in protection. We have shown *in vitro* via antibody competition experiments that the presence of IgM does not prevent IgG from binding to the surface of schistosomula, but based on our glycan array data, there was no indication that IgM and IgG recognized different glycan targets. This further emphasises that the IgM/IgG balance is relevant in a protection profile, where high IgG titers are essential for protection. Thus the presence of protective IgG could mediate cellular mechanisms necessary for protection in vaccinated baboons. It is worth noting that when schistosomula are grown to the blood-feeding stage *in vitro* in the presence of *S. mansoni*-infected 'self-cure' rhesus macaque serum (versus naïve macaque serum), their growth is inhibited [23]. In addition, incubating 3-hour *in vitro* transformed schistosomula with serum from rhesus macaques resistant to secondary infection caused schistosomula death [21]. In contrast, incubating baboon serum with schistosomula in similar experiments did not cause any observed damage (data not shown), suggesting that the involvement of immune cells is necessary for baboon immunity.

Mature schistosome eggs are a potent source of highly antigenic glycans but vaccination with viable schistosome eggs does not elicit protection in different animal models [20], despite generating high titers of anti-glycan antibodies that react with penetrating schistosomula [41-43]. This observation led to the smokescreen hypothesis suggesting that antigenic glycans deflect the immune response away from critical and susceptible protein epitopes [44]. However, multiple studies have also shown that the presence of eggs does not prevent the host from developing protection. Thus, previous and ongoing schistosome infections do not compromise the efficacy of the RA cercaria

vaccination in baboons [13] and resistance to reinfection occurs after eggs are produced by mature worms in the rhesus macaque self-cure model [21]. In an experimental setting where vaccinated baboons were previously infected with schistosomes, the overwhelming response to parasite egg deposition makes it difficult to discern a contribution from vaccination [13]. The current study protocol gave us the unique opportunity to study anti-glycan responses in the context of protection induced solely by anti-larval immune responses; it confirmed that many larval glycan epitopes are shared with eggs. It is worth noting that each female worm deposits around 300 eggs per day in the blood vessels of the gut wall. Thus a patent infection of around 500 worm pairs in a baboon results in a diurnal production of 150,000 eggs, equivalent to the biomass of five vaccinations with 9000 attenuated cercaria [45]. Given that RA cercaria vaccination can induce protection in infected baboons [13], perhaps the anti-cercarial antibody response may be in some important way different from those induced by eggs. It would be valuable to perform additional experimental immunizations in animal models with cercarial and egg-derived antigen preparations, as well as with defined glycans, to unravel what those differences are.

In this study we analyzed the anti-glycan antibody responses elicited against specific glycan motifs during RA cercaria-vaccination in baboons which eventually developed resistance to challenge infection. IgG against highly antigenic motifs such as multi-fucosylated glycan epitopes were developed at high titers, similar to those previously seen in the 'self-cure' rhesus macaque model. It is notable that the generation of these anti-multi-fucose antibodies does not require the presence of eggs. Nevertheless, multiple vaccinations were necessary to boost the titers of IgG, which resulted in better protection against challenge infection. Such antibodies against highly fucosylated glycans are also generated in humans susceptible to schistosome infections [24]. Future studies to establish the role of antibodies against multi-fucosylated glycan motifs require active vaccination experiments with glycoconjugates containing these glycan epitopes.

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Supplementary information

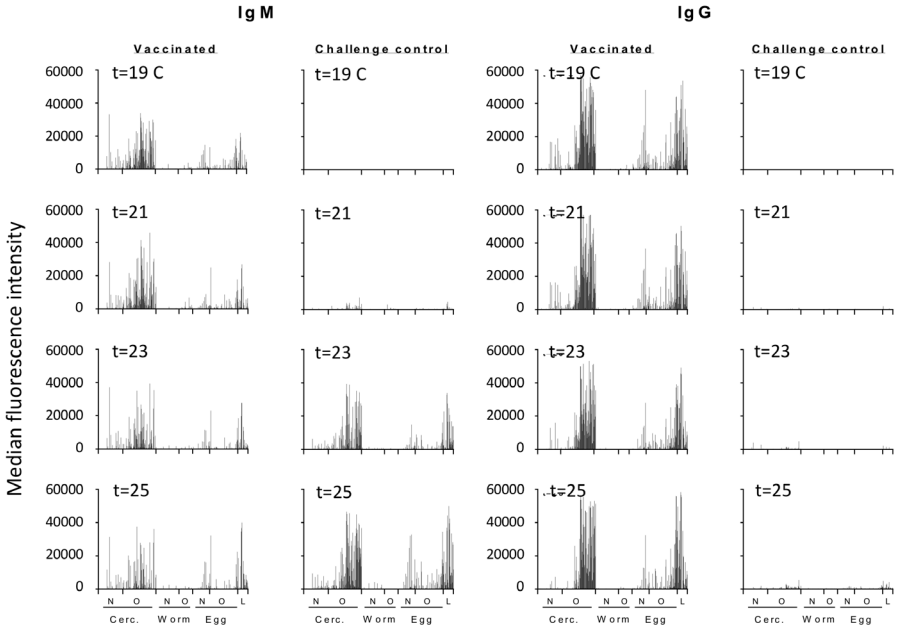

Figure S1. Averaged serum IgM and IgG response from RA cercaria vaccinated and challenge control baboons to glycans isolated from different life stages of schistosomes. The horizontal axis indicates N-, O- and GSL glycan fractions from schistosome cercariae (Cerc.), adult worms, and eggs. Average background-subtracted median fluorescence intensities are shown for IgM and IgG after challenge infection at week 19. Each bar corresponds to antibody binding to individual glycan fractions printed on the glycan microarray. N: N-glycans. O: O-glycans and L: glycosphingolipid (GSL) glycans. C: Challenge infection with live cercaria.

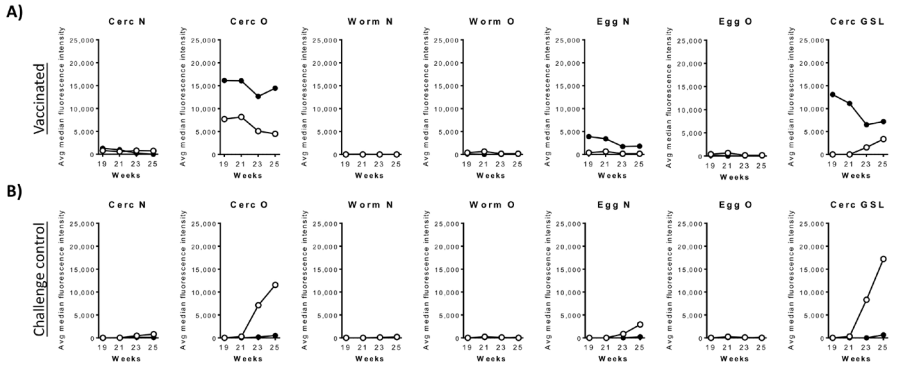

Figure S2. Anti-schistosome glycan response of RA cercaria vaccinated and unvaccinated challenge control baboons after challenge infection. IgG (closed circle) and IgM (open circle) response towards glycan antigens isolated from different lifestages of schistosomes in serum isolated from RA cercaria vaccinated (A) and unvaccinated challenge control (B) baboons. Cerc N: cercaria derived N- glycan. Cerc O: cercaria derived O- glycan. Worm N: worm derived N- glycan. Worm O: worm derived O-glycan. Egg N: egg derived N- glycan. Egg O: Egg derived O- glycan. Cerc GSL: cercaria derived glycosphingolipid linked glycan.

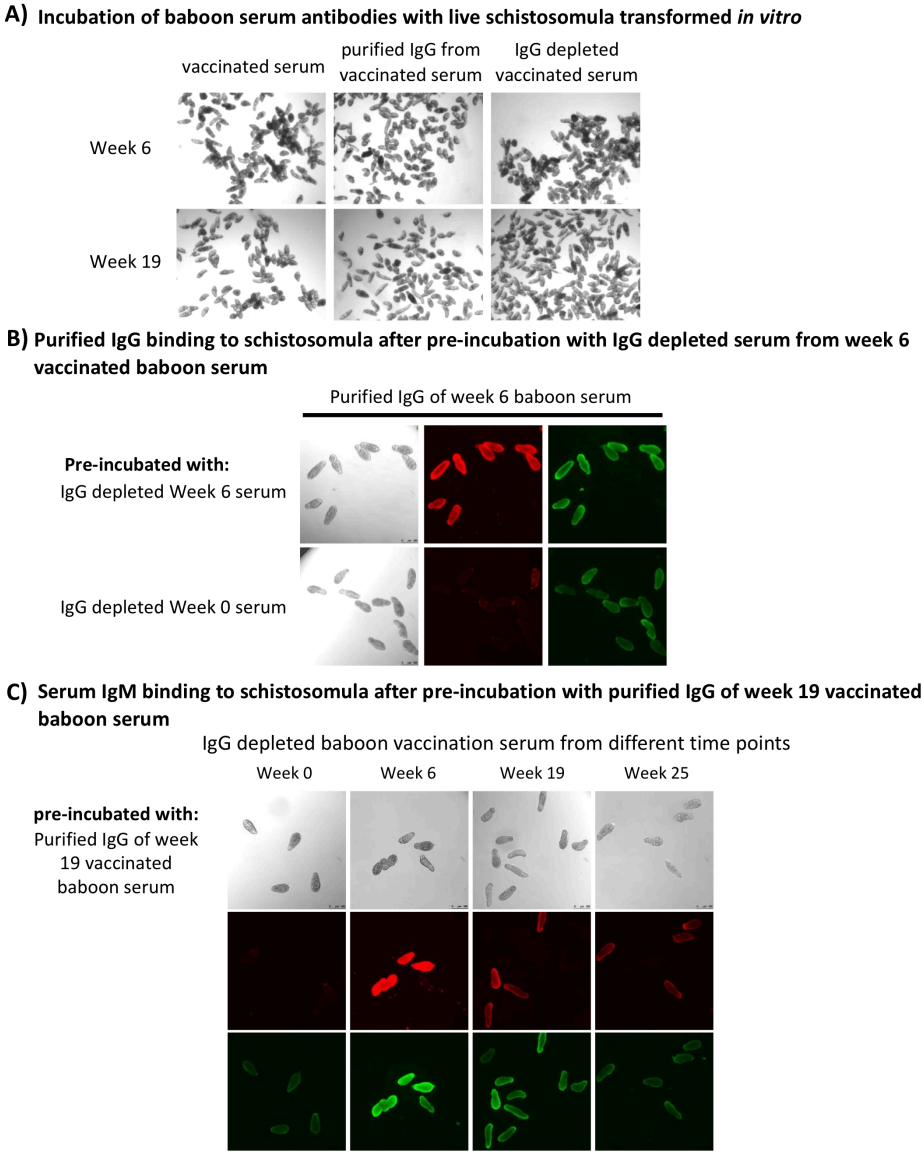

Figure S3. A) Agglutination of live schistosomula *in vitro* by whole serum, purified serum IgG and IgG depleted serum from vaccinated baboons at week 6 and week 19. B) Serum IgM (red) and IgG (green) binding to schistosomula after pre-incubation with IgG depleted serum. The presence of IgM in the pre-incubation did not prevent IgG binding to the surface of schistosomula. C) Serum IgM (red) and IgG (green) binding to schistosomula after pre-incubation with purified IgG of week 19 vaccinated baboon serum. Week 19 vaccinated baboon serum contained high amounts of IgG. Pre-incubation with purified IgG from this time point did not prevent IgM binding to the surface of schistosomula.

Chapter 4

Glycan microarray-assisted identification of IgG subclass targets in schistosomiasis

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Abstract

Infection with schistosomes is accompanied by the induction of antibodies against the parasite. Despite high serum IgG titers against both protein and glycan antigens, infected individuals remain infected until treated and re-infection is common in endemic areas. Parasite specific IgG subclasses may differ in functionality and effectivity with respect to effector functions that contribute to parasite killing and immunity. In this study, we investigated if specific IgG subclass responses target specific antigenic schistosome glycan motifs during human infection. Sera from 41 *S. mansoni* infected individuals from an endemic area in Uganda were incubated on two glycan microarrays, one consisting of a large repertoire of schistosome glycoprotein- and glycolipid- derived glycans and the other consisting of chemically synthesized core xylosylated and fucosylated N-glycans also expressed by schistosomes. Our results show that highly antigenic glycan motifs, such as multi-fucosylated terminal GalNAc(β1-4)GlcNAc (LDN) can be recognized by all IgG subclasses of infection sera, however with highly variable intensities. Detailed analysis of N-glycan core modifications revealed individual antibody responses specific against core-xylosylated and core α3-fucosylated motifs that are life stage specifically expressed by schistosomes. IgG1 and IgG3 were detected against a range of N-glycan core structures, but IgG2 and IgG4, when present, were specific for the core α3-fucose and xylose motifs that were previously found to be IgE targets in schistosomiasis, and in allergies. This study is the first to address IgG subclass responses to defined helminth glycans.

Introduction

Schistosomiasis is a parasitic infection of humans and mammals contracted by exposure to schistosome cercariae shed by infected aquatic snails. Once the parasite establishes itself in a human host, the infection which is associated with debilitating pathology due to tissue-deposition of eggs remains chronic until treated. In the schistosomiasis endemic areas in Africa, South-America and South-East Asia, reinfection after treatment is common. Longitudinal studies have shown that resistance to re-infection with schistosomes develops very slowly. Many years of exposure to schistosomes and multiple treatments are required for the immune response to become effective [1-3]. Various studies have shown that anti-schistosome antibodies are pivotal for anti-parasite immunity. Passive immunization with serum or monoclonal antibodies against schistosome antigens [4-7] were shown to reduce worm burden and egg production in previously unexposed mice upon challenge with schistosomes. Moreover, transfer of sera and of purified IgG from animals immunized with the protective Sm-p80 antigen conferred resistance to challenge infection, and it has been shown that the level of protection induced by immunization with Sm-p80 is reduced in antibody deficient mice [8].

As schistosomes are highly glycosylated organisms that express many glycan motifs different from mammals, it is not surprising that an abundance of antibodies are generated against schistosomal protein- and lipid-linked glycans exposed to the host [9-14]. It is becoming increasingly clear that glycan antigens play an important role in helminth infection immunology, but it remains ambiguous whether anti-glycan antibodies contribute positively or negatively to protection.

Previously we have studied the anti-glycan IgG and IgM responses in schistosome infected rhesus macaques that are able to expel the worms [15], in order to identify glycan targets of antibodies that might be involved in the self-cure mechanism. We showed that serum IgG antibodies against highly fucosylated schistosome glycoproteins and glycolipids formed during infection were sustained when the macaques cleared the infection, indicating that these antibodies are present in a protective context and possibly play a role in the killing of adult worms in the host. Additionally, sera from macaques containing high titers of IgG against highly fucosylated motifs were able to kill schistosomula *in vitro*. [15] and it has been shown that a monoclonal antibody specific for the fucosylated LeX antigen was protective in a mouse model of *S. mansoni* infection [6]. On the other hand, humans that are generally susceptible to reinfection also have high titers of IgG antibodies against highly fucosylated motifs [16], indicating that anti-glycan antibodies are not necessarily protective. Indeed, some studies have regarded schistosome glycans to form a smokescreen that diverts the host immune system from

attacking vulnerable *peptide* epitopes that could mediate protective responses [17-19]. It has however been appreciated that protective *glycan* epitopes may also occur, but these could be under-represented and masked by irrelevant or 'smokescreen' epitopes [20]. As a consequence, protective anti-schistosome glycan responses may be difficult to identify. These observations called for investigations into differential recognition of specific schistosome glycan motifs in different models and cohorts such as reported in several recent glycan microarray-assisted studies [12-16, 21].

Alternatively, research into the IgG subclass-specific response towards defined glycan antigens may shed light on the role of anti-glycan antibodies in infection. Perhaps the key to associate glycan antigens with immunity is not in the antigen itself, but rather by the type of antibody response generated. Particular IgG subclasses have been found to correlate with resistance or susceptibility to schistosome infection: IgG1 against schistosome surface antigens were observed in putatively resistant individuals in a Brazilian cohort [22], while individuals that were chronically infected lacked high IgG1 against the same antigens, but mounted high IgG4 towards various schistosome antigens instead. IgG4, as well as IgG2 have been found to correlate with susceptibility of schistosome reinfection [23, 24]. On the other hand, human serum IgG1 and IgG3, as well as IgE have been found to be potent inducers of eosinophil-mediated cytotoxicity, while IgG4 antibodies were found to be inhibiting cytotoxicity [25] and compete with IgE to prevent antigen cross-linking and IgE mediated effector function [26]. So far, studies on IgG subclass response against schistosome antigens have focused on schistosome surface proteins [22] or complex ill-defined schistosome antigen mixtures such as SEA or AWA [27]. Little is known however, about IgG subclass reactivity to defined schistosome glycan antigens. Interestingly, a recent study has shown that anti-glycan IgE responses are highly restricted towards only a few specific epitopes out of the many glycan antigens expressed by schistosomes, in particular N-glycan core-xylose or core α3-fucose, motifs that are often associated with plant glycans and allergens [28].

In the current study, we address the question whether specific serum IgG subclass responses to defined antigenic glycans occur during human schistosome infection, and whether these might be restricted to certain motifs or not. To this end, we tested the IgG subclass responses in a selection of 41 serum samples from a relatively homogeneous population in terms of exposure and infection intensity [16, 29] towards a large collection of schistosome glycan antigens. Using two glycan microarrays, one comprising a large set of native glycans isolated from different schistosome life-stages [14-16], the other comprising a set of synthetic N-glycans with different core and branch modifications previously described [21] we determined for each of the IgG subclasses which glycan motifs are targeted during infection, and evaluated whether these are different or not for

IgG1-4. Our results indicate that in schistosomiasis sera, each of the IgG subclasses can be directed against a variety of antigenic glycan motifs, but with different patterns for the different individuals tested. To our knowledge, this is the first study that addresses IgG subclass responses to defined glycans and glycan motifs in a helminth infection.

Material and Methods

Sera

Sera used in this study were described in an earlier publication [16] where 41 schistosome infected individuals were selected from an original cohort study in a highly prevalent *S. mansoni* endemic area [29]. The cohort study obtained ethical approval from the Uganda National Council for Science and Technology (UNCST) and was supported by the Cambridge Local Research Ethics Committee. Selected subjects had an age range of 5 to 46 years old, all with patent *S. mansoni* infection. The geometric mean of the intensity of infection measured by egg per gram (epg) feces was 560.65 ($CI_{95\%}$: 343.88, 914.05).

Glycan microarray construction and incubation

A glycan microarray constructed of glycans derived from *S. mansoni* cercariae (75 N-glycan fractions and 102 O-glycan fractions), adult worms (77 N-glycan fractions and 31 O-glycan fractions) and eggs (57 egg N-glycan fractions and 98 soluble egg antigen O-glycan fractions), and 30 glycans derived from glycosphingolipids representing multiple life stage, was described previously [14, 16]. 24 blank spots with spot buffer were included for array background control. Each glycan fraction was immobilized on a glass slide in triplicate. The synthetic glycan microarray containing a collection of core-xylosylated and core-α3 and -α6 fucosylated N-glycans with various core extensions has been described previously [21].

Binding assays of individual or pooled sera on both arrays were conducted following the protocol as described previously [14-16]. Briefly, the microarray slide was blocked with 2% BSA, 50 mM ethanolamine in PBS. Serum samples were diluted 1:100 in PBS-0.01% Tween20 with 1% BSA. All four mouse anti-human isotype antibodies were purchased from Invitrogen. IgG1 and IgG3 were labelled with the Promokine PF-647 labelling kit; IgG2 and IgG4 were labelled with the PF-555 labelling kit following the manufacturer's protocol. All anti-human IgG subclass antibodies were diluted 1:200 in PBS-0.01 Tween20 to detect bound serum antibodies on the slide. All washing steps were performed with successive rinses with PBS-0.05% Tween20 and with PBS. The last washing step was finished by an additional wash with milliQ water and the slides were dried and kept in the dark until scanning.

Scanning and data analysis

A G2565BA scanner (Agilent Technologies, Santa Clara, CA) was used to scan the slides for fluorescence at 10 um resolution using lasers at 532 nm and 633 nm. Anti-IgG2 and anti-IgG4 antibodies were detected at 532 nm and anti-IgG1 and anti-IgG3 antibodies at 633 nm. Data and image analysis was performed with GenePix Pro 7.0 software (Molecular Devices, Sunnyvale, CA). Spots were aligned and re-sized using round features with no CPI threshold. Background-subtracted median intensities were averaged per time point and processed as described by Oyelaran et al. [30]. Datasets were $\log_2$ transformed to remove the basic trends of variance. A hierarchical clustering analysis (HCA, complete linkage clustering using Euclidean distance metric) was performed to group associated glycan fractions using MultiExperiment Viewer v4.5.

Results

Serum IgG subclass response against glycans expressed by schistosomes in infected individuals

To investigate whether IgG subclasses in sera from a schistosome-infected cohort react with specific motifs or subsets of parasite-associated glycans, we determined IgG1, IgG2, IgG3 and IgG4 binding intensities against a large variety of glycans in pooled sera of 41 infected individuals using glycan microarrays. These sera were from a relatively homogeneous population in terms of exposure and infection intensity. All individuals had patent *S. mansoni* infection (geometric mean infection intensity (epg) was 560.65 (CI 95%: 343.88, 914.05)). We found that in the pool IgG of all four subclasses are present against a wide range of schistosome-derived N-, O- and glycosphingolipid glycans printed on the array (Fig. 1A). IgG1 and IgG2 bound to the various glycans with similar high intensities, while anti-glycan IgG4 binding intensities were lowest out of the four subclasses. Glycan targets for each of the IgG subclasses consisted mainly of highly fucosylated LDN epitopes, abundantly expressed on glycolipid-derived glycans and cercarial O-glycans [31]. Interestingly, IgG subclass binding of N-glycan core xylose (abundant on glycans from cercariae and miracidia) and core α3-fucose epitopes (abundant on glycans from eggs and miracidia), as determined by use of the synthetic glycan array, were rather different across the four subclasses (Fig. 1B).

IgG1, IgG3 and IgG4 in pooled infection serum showed higher binding intensities to core α3-fucose containing structures than to structures containing only core xylose, with IgG4 being restricted to different subsets of the α3-fucose containing structures. In contrast, IgG2 response in this pool was mainly directed towards core xylose containing

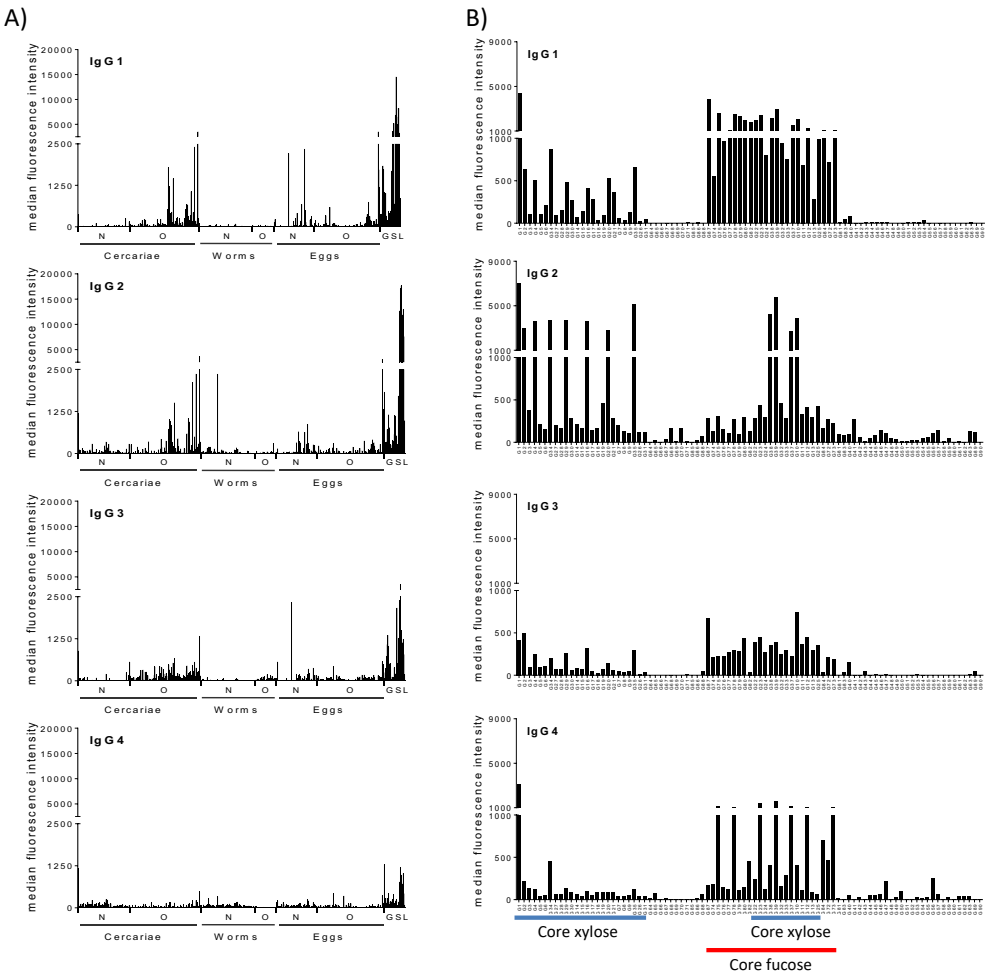

Figure 1. Schistosome infected individuals produce IgG subclass antibodies to schistosome glycans. Averaged serum IgG subclass response from schistosome-infected individuals to A) complex N-, O- and glycosphingolipid glycans isolated from different life stages of schistosomes and B) core modified N-glycans synthesized chemically. The horizontal axis indicates different glycan structures. Each bar corresponds to antibody binding to individual glycan fractions printed on the glycan microarray. Schistosome GSL glycans are shown as a group irrespective of the life stage. N: N-glycans. O: O-glycans and GSL: Glycosphingolipid derived glycans.

N-glycans. Core α3-fucose has been shown to be an antigenic target in schistosome-infected humans, mice and rhesus macaques[12, 15]. Interestingly, alone or in combination with core xylose, core α3-fucose also forms the major cross-reactive carbohydrate epitopes that are IgE targets on a variety of plant-derived allergens [28, 32, 33]. To further analyze the differential reaction of specific IgG subclasses with core-modified N-glycans, we investigated the IgG subclass response in each infected individual on the synthetic glycan array.

Schistosome infected individuals differentially express IgG subclass antibodies towards schistosome glycans

Among the individual sera incubated on the synthetic glycan array a striking variability in IgG subclass response towards the core-modified glycans was observed (Fig. S1). Most sera contained antibodies binding to core α3-fucose and core xylose modified N-glycans. However, this was not a universal response and no clear correlation between IgG subclass and glycan motifs was observed.

IgG subclass response profile of schistosome infected individuals

In view of the variations in IgG subclass responses to the core-modified N-glycans between individuals, we performed a hierarchical clustering analysis and grouped the individuals based on response patterns for each IgG subclass (Fig. 2A). We observed a cluster of individuals that had specific and high IgG1, 2, 3, or 4 binding to core α3-fucose (cluster red). Another cluster of individuals had high IgG binding to core xylose containing structures (cluster blue), while the third cluster, with the highest number of individuals, only had lower amounts of IgG binding to either core α3-fucose or core xylose (cluster yellow). When comparing the IgG subclass distribution for each motif within each serum, we saw that some individuals had high binding to core α3-fucose with all four IgG subclasses (cluster red in all subclasses) (Fig. 2B), while other individuals responded strongly to core α3-fucose structures with only one or two subclasses that were variably of the IgG1, 2, 3, or 4 type. Similarly, two individuals of the 41 individuals responded strongly to core xylose containing N-glycans with all IgG subclasses (cluster blue in all subclasses), while other individuals responded only with one or two subclasses, variably of the IgG2, IgG3 or IgG4 class. These observations indicate that IgG subclass responses towards these glycan antigens are not restricted by the nature of the antigen, but rather appear to be related to each individual response to the infection. One interesting observation was that most of the individuals that had high IgG subclass response towards either core xylose or core α3-fucose were children aged twelve or younger (Fig. 2B).

Structural motifs bound by IgG1 – 4

Next, to gain more insight in the structural determinants that are recognized specifically by the different IgG subclasses, we examined in detail how the binding of IgG1, IgG2, IgG3 and IgG4 to core α3-fucose and core xylose is influenced by adjacent structural elements. Comparison of the signal intensities of four sets of structurally related synthetic glycan structures first revealed that moderate levels of IgG1 and IgG3 reactive with the unsubstituted trimannosyl core glycan ($Man_3GlcNAc_2$) (G42) were detected in most sera

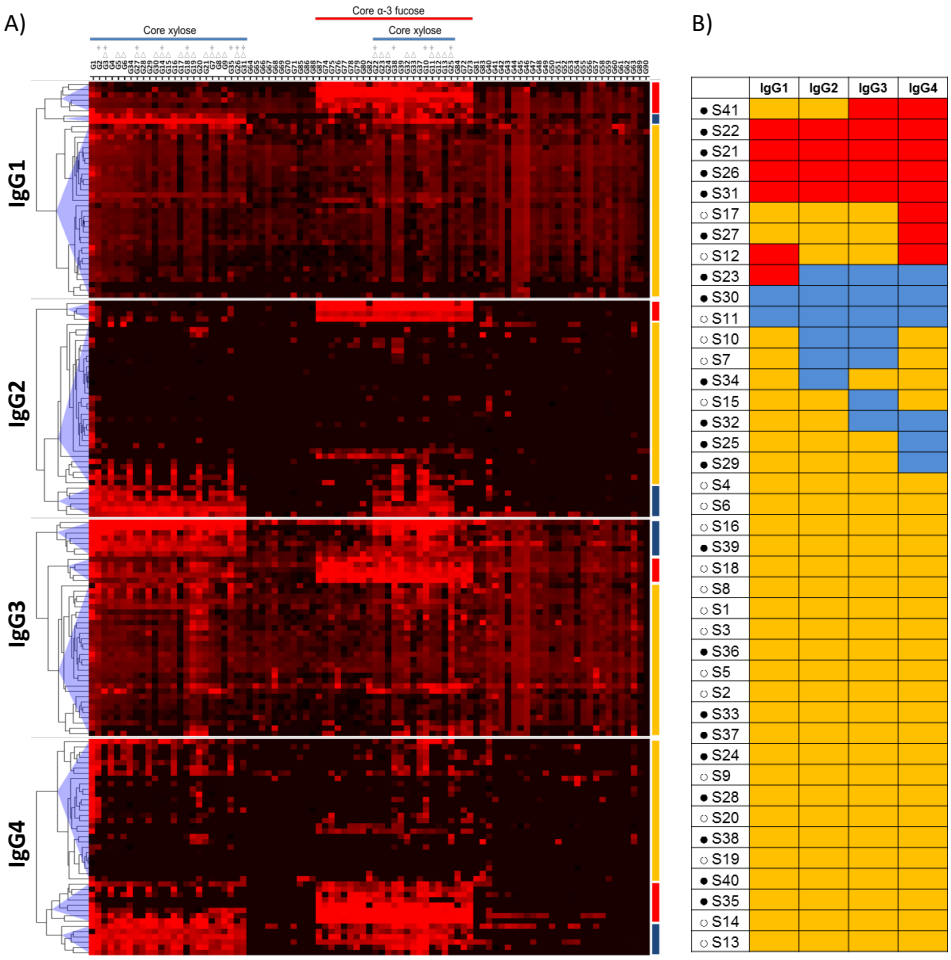

Figure 2. Hierarchical clustering analysis of anti-glycan antibody responses in schistosome infected individuals. A) Heatmap showing IgG subclass response of schistosome infected subjects (columns) to core modified N-glycan fractions (rows) that have been synthesized and described by Brzezicka et al.. Median fluorescence intensity was corrected for baseline and log2 transformed; increase in antibody binding is indicated by the red color intensity. Three major clusters of subjects were identified for each IgG subclass based on antibody binding intensity to glycans: one cluster of individuals had high IgG binding to core α3-fucose (red), another cluster of individuals to core xylose (blue) and one cluster of individuals without specific binding to core α3-fucose or core xylose (yellow). Core xylosylated and core α-3 fucosylated structures are indicated. Within core-xylose containing structures, those that have additional monosaccharides on the α-3mannose (Δ) and those that miss the core α-6mannose (*) are indicated. B) IgG subclass response profile of each schistosome infected individual. Red: high response against core α3-fucose; blue: high response against core xylose; yellow: low response against other glycans. Filled circles: age 12 and under. Open circles: age 20 and older.

tested (Fig. 3A-C). In contrast, IgG2 and IgG4 to the N-glycan core were only detected when either a xylose (G34) or an α3-fucose (G73) residue were present (Fig. 3A, C).

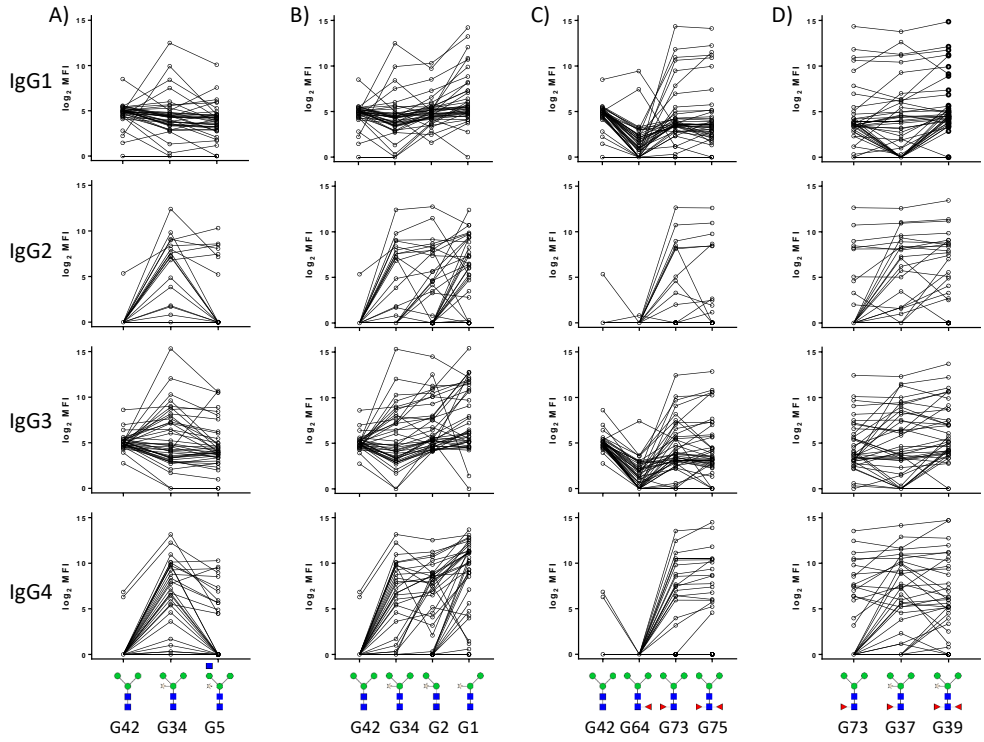

Figure 3. IgG subclass response of schistosome infected individuals to core xylose (A and B) and core α3 fucose (C and D) in the presence or absence of adjacent structural elements. Median fluorescence intensity was corrected for baseline and $\log_2$ transformed. The code for each structure as described in the original publication [21] is indicated.

Interestingly, IgG1 and IgG3 in general did not become more reactive upon addition of core xylose to the trimannosyl core, but signal intensities did increase significantly upon α3-fucosylation while core α6-fucose addition (G64) had the opposite effect of lowering the IgG1 and IgG3 response to the otherwise unsubstituted trimannosyl core (Fig. 3A, C). The addition of α6-fucose had no effect on the reactivity of any of the four IgG subclasses to the α3-fucosylated core however (G64) (Fig. 3C). Regarding the combination of both the antigenic core xylose and core α3-fucose modifications it appears that sera where IgG2 and IgG4 reactivity is negative against core α3-fucose becomes positive upon addition of the xylose (G37) (Fig. 3D). Since reactivity is also observed to core-xylosylated glycans without fucose, it appears that α3-fucose and xylose form independent antigenic motifs. Finally, IgG2 and IgG4, but not IgG1 and IgG3 reactivity against core xylose are hindered by the addition of a GlcNAc branch to the 3-linked Man (G5), but removal of the 3-linked Man (G1) enhances reactivity, indicating that reactivity to xylose depends on its spatial

accessibility (Fig. 3A, B). Together these data suggested that IgG2 and IgG4 reactivity to N-glycan core structures in schistosomiasis sera are either against the core xylose and core α3-fucose motifs, whereas IgG1 and IgG3 are less specific and restricted.

Discussion

In this study we have investigated the IgG subclass responses against defined parasite-derived glycans in schistosome-infected individuals. Detailed glycan microarray analysis indicated that IgG1, 2, 3 and 4 in schistosome infection sera are variably present against a wide range of antigenic parasite glycans. Interestingly, individuals infected with schistosomes respond with particularly high variability to the antigenic α3-fucose and β2-xylose core modifications of N-glycans with respect to the different IgG subclasses. This variability in antibody response was not a reflection of the intensity of infection as all sera included this study were from heavily infected individuals living in a schistosome endemic area. The sera used in this study were selected from a larger study [29], where selected individuals had an age range between 5 to 46 years old, all with patent *S. mansoni* infection, with fecal egg counts between 343.88 and 914.05 epg ($CI_{95\%}$ of geometric mean). Previously, we have used this set of sera to compare the anti-glycan (total) IgG and IgM in schistosome infected children and adults [16] and found that although there were anti-glycan antibody differences between children and adults, other factors apart from age also played a role in shaping anti-glycan antibody response profiles. It is well known that the overall serum IgG subclass distribution is generally different between adults and children. IgG1 and IgG3 development is faster and reaches around 75% of the adult serum levels at the age of five, while IgG2 and IgG4 levels rise much slower, reaching 70% of adult serum levels at the age of 14. Nevertheless, large inter-individual variations were observed previously for IgG subclass development [34], as corroborated by our study. Although most individuals with responses of any subclass towards core modified glycans were children, many children did *not* produce antibodies against core modified N-glycans, raising the question which other factors, possibly cross-reactive antigens, determine the induction of these schistosome-reactive antibodies.

The sample selection used here to study the glycan specificity of IgG subclasses in schistosomiasis infection serum is not suitable for addressing immunoepidemiological questions. Nonetheless, the variable subclass responses to specific glycan antigens observed in this study may have the potential to reflect the immune profile of infected individuals and act as markers for infection with respect to intensity, exposure, chronicity, immunopathology or resistance to infection. High IgG1 titers against a specific set of

schistosome tegument protein antigens for instance have been observed in putatively resistant individuals in a Brazilian cohort but not in chronically infected individuals, where high IgG4 antibodies against the same schistosome antigens were observed instead [22]. In addition, IgG1 and IgG3 against protective schistosome antigens has also been found in naturally resistant individuals but not in chronically infected or unexposed individuals [35]. These studies indicate the importance of IgG1 against protective antigens in developing immunity. In contrast, susceptibility to reinfection in humans has been associated with high levels of IgM, IgG2 and IgG4 [23, 24, 26, 27]. The presence of IgM, IgG2 and IgG4, purified from serum of infected individuals, prevented eosinophil mediated killing of schistosomula by other IgG subclasses present in human infection sera [25]. The IgG4 subclass is usually produced after repeated, long-term antigenic stimulation, and has minimal effector functions. Literature describes highly elevated IgG4 towards schistosome antigens [26] that may compete with IgE and prevent antigen cross-linking and IgE mediated effector functions. Interestingly, although we have not observed very high IgG4 binding intensities against schistosome glycans, precisely the core α3-fucose and core xylose motifs that form the cross-reactive carbohydrate determinants for IgE in plant allergens as well as helminths [28, 33, 36] are also the only IgG4 reactive glycan elements in the tested N-glycans in the current study. It should be noted that expression of core α3-fucose and core xylose motifs during the schistosome life cycles appears to be highly specific. By mass spectrometric glycomics approaches it has been shown that core xylose is abundant on N-glycans of cercariae and miracidia [31], whereas core α3-fucose has been detected in abundance in N-glycans on the secretory egg glycoproteins IPSE/α1 [37] and omega-1 (together with core α6-fucose) [38] and on N-glycans derived from the miracidia [31] and the egg glycoprotein kappa-5 (together with α6-fucose and core xylose) [39]. Both core xylose and core α3-fucose are expressed by plant glycoproteins [33, 40] and in a small subset of other helminths [36]. The specific IgG determined in our study may therefore also have been triggered by other antigen sources other than schistosomes.

It would be interesting to investigate the IgG subclass response of infected individuals towards more complex glycan antigens that are schistosome specific, such as highly fucosylated LDN epitopes on glycoproteins and glycosphingolipids of the parasite and its secretions. Such complex schistosome antigens, induce higher total IgG binding in schistosome infected rhesus macaques compared to core modified epitopes [15]. Given that highly fucosylated motif such as Fucα1-2Fucα1-3 on LDN have not been found in organisms other than schistosomes, it would be interesting to see if different individuals would have a less variable IgG subclass distribution to these complex, more parasite-specific glycan antigens, when these would become available as synthetic, defined antigens.

In this study we have for the first time investigated the IgG subclass response against schistosome glycans in infected individuals. The complex interplay between how antigens trigger IgG subclass response calls for analysis with a larger human cohort with better defined resistant and susceptible immune profiles, to be able to understand whether particular subclasses against particular glycan epitopes are associated with a disease state. Although high inter-individual antibody variation was observed against core modified N-glycans expressed by schistosomes, IgG subclass response against complex antennae schistosome glycans remain to be elucidated.

Acknowledgement

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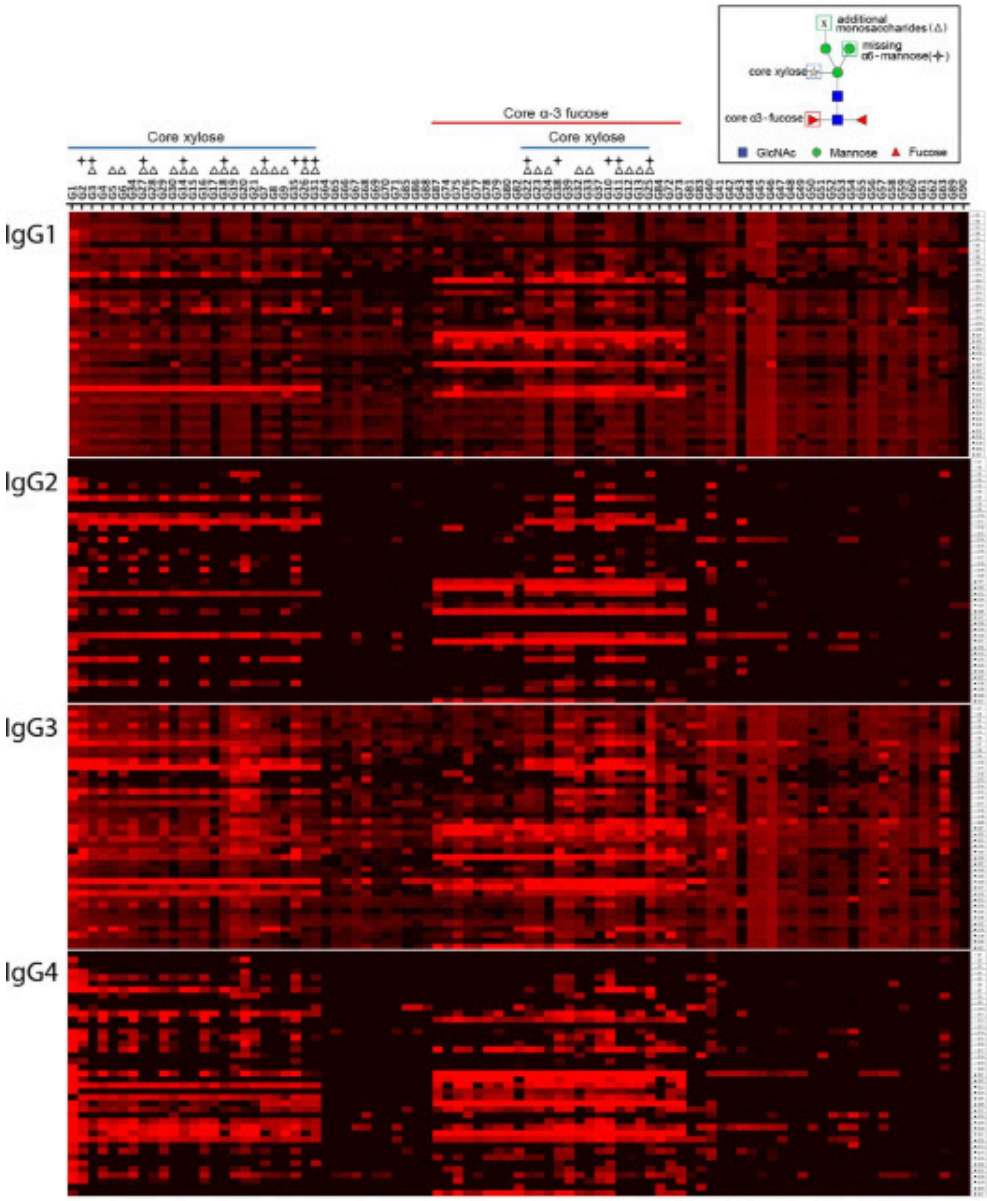

Figure S1. Different schistosome infected individuals respond to glycans with different IgG subclasses. Heatmap showing IgG subclass response of schistosome infected subjects (columns) to core modified N-glycan fractions (rows) that have been synthesized and described by Brzezicka et al.. The position order of each individual along the vertical axis is the same for all four subclasses. Antibody median fluorescence intensity was corrected for baseline and $\log_2$ transformed; increase in antibody binding is indicated by the red color intensity. Core xylosylated and core α-3 fucosylated structures are indicated. Within core xylosylated structures, those that have additional monosaccharides on the α-3 mannose (Δ) and those that miss the core α-6 mannose (✧) are indicated.

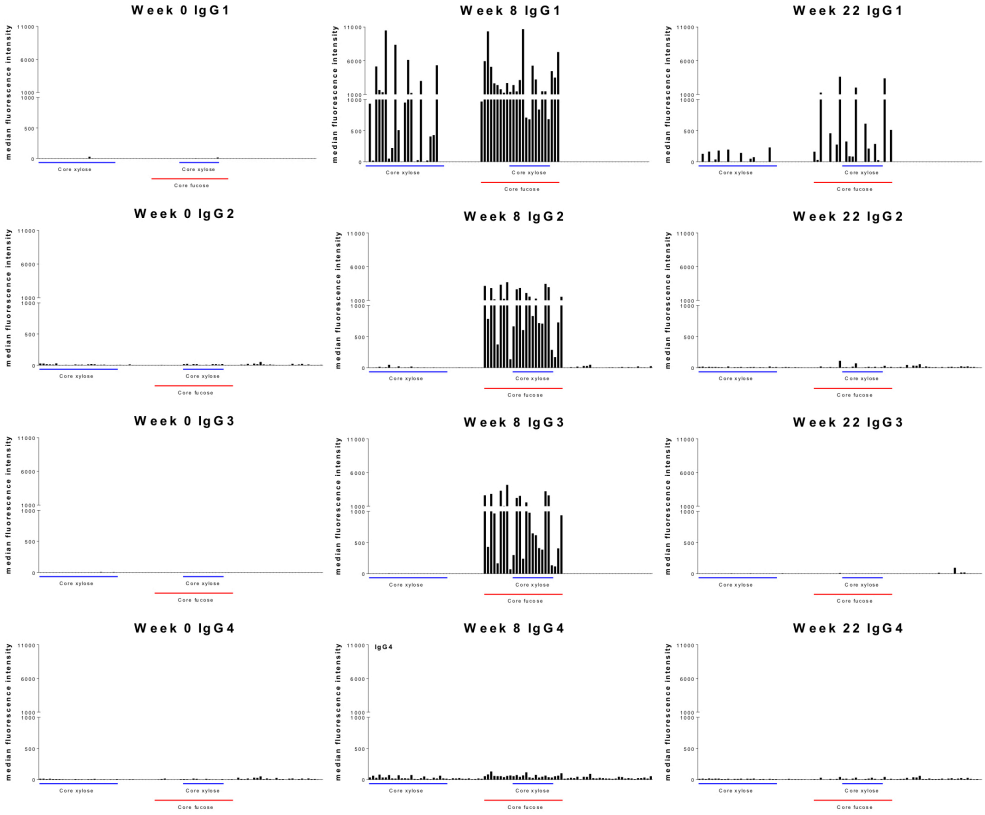

Figure S2. Averaged serum IgG subclass responses from *S. japonicum*-infected rhesus macaques to synthetic core modified N-glycan fractions. The horizontal axis depicts core modified N-glycan fractions that have been synthesized and described by Brzezicka et al.. Average median fluorescence intensities are shown for *S. japonicum*-infected macaque serum IgG over a time course of 22 weeks. Each peak along the vertical axis corresponds to each individual glycan fraction printed on the glycan microarray. Core xylosylated and core α3-fucosylated structures are indicated.

Chapter 5

Vaccination with a fucosylated neoglycoprotein reduces worm burden in *S. mansoni* exposed rats

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Abstract

In the past decades, a substantial number of schistosome proteins have been evaluated as vaccine candidates in rodent models. However, glycan vaccine targets have remained so far unexplored. In this study, rats were immunized with neoglycoproteins Fucα1-3GlcNAcβ1-BSA (FGn-BSA) and Fucα1-2Fucα1-3GlcNAcβ1-BSA (FFGn-BSA) to mimic multi-fucosylated schistosome glycans previously suggested to be associated with protection against schistosomes in non-human primate models. Immunizing rats with FGn-BSA significantly reduced liver worm burden upon *S. mansoni* challenge infection by 38% and 29% in two independent experiments compared to control rats immunized with unconjugated BSA. Rats immunized with FGn-BSA not only generated specific antibodies towards the fucosyl motif of glycoconjugates FGn-BSA and FFGn-BSA, antibodies elicited by FGn-BSA immunization also bound to native glycans isolated from different schistosome life stages, mainly against lipid-derived glycans containing terminal Fucα1-3HexNAc motifs (HexNAc = GalNAc, GlcNAc). Although the mechanism of protection is not clear, we show that FGn-BSA immunized rat serum IgG and IgM antibodies bound to the entire surface of 3 hour transformed schistosomula, confirming the presence of target glycan epitopes on the schistosomulum. This indicates that antibody-dependent mechanisms may be involved in immunity against developing larvae upon challenge infection, which accounts for the protection seen in FGn-BSA immunized rats.

Introduction

Schistosomiasis is a debilitating parasitic disease resulting in high levels of morbidity in areas where schistosome infections are endemic and chronic. Treatment with Praziquantel (PZQ), the only anti-schistosomal drug available, does not prevent reinfection, which limits the success of mass drug administration programs [1]. Moreover, extensive use of PZQ may lead to the emergence of drug-resistant schistosomes, similar to what happened during extensive use of drugs against veterinary helminths [2-5]. Considerable efforts are therefore dedicated to finding and designing an effective therapeutic and/or prophylactic vaccine against schistosomiasis.

Antigens suitable for forming the basis of a vaccine in principle are all immunogenic molecules accessible to the host immune system that can trigger a protective cellular or humoral immune response [6]. For *Schistosoma mansoni*, multiple protein antigens have been explored as vaccine targets, mainly in rodent models, but so far only Sm-TSP-2, rSm14/GLA-SE, and Sm-p80 advanced into clinical development [7-9]. It is well known that in addition to protein antigens, schistosomes express a plethora of antigenic and non-antigenic glycans, mostly conjugated to protein or lipid carriers. Glycans expressed throughout the schistosome life cycle, some life stage-specific, others occurring in all life stages, play an important role in the parasite biology as well as in host-parasite interactions [10, 11]. For example, glycans on egg glycoproteins and -lipids activate innate immune responses and modulate immune cell function in various *in vitro* and *in vivo* systems [12-14], and cercarial secretions have been shown to trigger alternatively activated macrophages via glycans [15, 16]. Moreover, strong anti-glycan antibody responses are induced in schistosome-infected hosts. In particular to secreted and cell-surface expressed glycoproteins, anti-glycan antibody levels are high compared to anti-protein antibodies [17-22]. The relevance of antibodies against schistosome glycans during infection is a subject of debate. Mice vaccinated with schistosome eggs elicit high titers of anti-glycan antibodies, but this vaccination does not lead to protection [17, 23-25]. On the other hand, in animals resistant to schistosome infection after vaccination with irradiated cercariae [26] or in semi-permissive rhesus macaques which 'self-cure' after patent infection, high anti-glycan antibody responses were also observed [27]. These observations raise the question if specific schistosome glycan antigens may be applicable for the development of alternative anti-schistosome vaccine candidates.

Fucosylated glycan antigens containing Fucα1-3GlcNAc and Fucα1-2Fucα1-3GlcNAc elements are expressed by *S. mansoni* throughout the lifecycle, with the latter element displaying a unique Fucα1-2Fuc glycosidic linkage, so far only found in *Schistosoma* and *Trichobilharzia* [28]. We have previously shown that antibodies against these types

of fucosylated glycans are not only elevated during schistosome infection but are also highly abundant in the context of immunity against schistosomes generated by irradiated cercariae vaccination in baboons [26] and by a primary infection in self-curing macaques [27].

To study the possible contribution of anti-fucose antibodies in protective immunity against schistosome infection more directly, we applied a rodent vaccination model utilizing synthetic glycan epitopes. As small glycans alone are not successfully processed and presented by antigen presenting cells to trigger an effective antibody response, affinity maturation and IgG class switching [29, 30], multivalent BSA conjugates of fucosylated di- and trisaccharides were constructed [31]. Rats were immunized with these neoglycoproteins with unconjugated BSA as control, and worm burden after challenge infection with *S. mansoni* cercariae was assessed as end-point read out for the protective effect of immunization. In contrast to humans and mice which are fully permissive, rats are semi-permissive hosts for *S. mansoni* that eliminate most of the parasites around 4 weeks post infection [32-34]. Each early pre-clinical *in vivo* model for schistosome vaccination has its specific limitations and advantage. Although being semi-permissive to *S. mansoni* infection, the rat shares critical decisive humoral effector mechanisms, such as effector cells involved in antibody-dependent cellular-cytotoxicity, and antibody isotype regulatory mechanisms with the human hosts that lack in mice [35]. In particular for prophylactic vaccination, targeting larval and juvenile worms in the skin and lung stage before full maturation, the rat may be a useful experimental vaccination model.

Here we show that immunization with a synthetic Fucα1-3GlcNAcβ1-BSA-conjugate induced IgG and IgM against fucosylated schistosome glycans present on schistosomula and significantly reduced worm burden upon *S. mansoni* challenge.

Materials and methods

Animals and parasites

Seven-week-old Fischer male rats were purchased from Charles River. A Guadeloupean strain of *S. mansoni* used was maintained in *Biomphalaria glabrata* snails and golden hamsters as intermediate and definitive hosts, respectively [36].

Synthesis of glycoconjugates and characterization

The synthesis of fuco-oligosaccharides used for rat immunization has been previously described [31]. Synthetic carbohydrates Fucα1-3GlcNAcβ1-$(CH_2)_6$-NH_2 (FGn) and Fucα1-2Fucα1-3GlcNAcβ1-$(CH_2)_6$-NH_2 (FFGn) were conjugated to BSA using diethyl squarate chemistry as described by van Roon et al. [31], creating multivalent neo-glycoproteins

$(FGn)_n$-BSA and $(FFGn)_n$-BSA with n = 10-12, further denoted FGn-BSA and FFGn-BSA, respectively. Unfucosylated Gn-BSA [31] was used in this study as a control for anti-Fucose serum IgG and IgM in ELISA.

Immunization schedule and challenge

Rats were immunized with 50 µg of FGn-BSA, FFGn-BSA and BSA via sub-cutaneous injection with complete Freund's adjuvant. Two boosts with 30 µg of glycoconjugates were injected 3 weeks and 5 weeks later i.p. in incomplete Freund's adjuvant. Two weeks after the last injection, (7 post-vaccination) rats were first bled and then percutaneously infected with 2000 *S. mansoni* cercariae as described by Smithers and Terry [37]. FGn-BSA immunization was repeated in a second experiment with the same vaccination scheme, except that 60 µg of glycoconjugate was used in the primary immunization. The method of injection, boosting and adjuvant remained the same as the first experiment.

Blood collection and worm burden analysis

Blood was collected from rats before immunization at week 0 (prebleed), before infection at week 7 and upon sacrifice at week 10. At three weeks post infection, rats were euthanized and *Schistosoma* worms were recovered by perfusion. In each case the protection level was calculated by comparing the recovered total worm number from each glycoconjugate group in relation to the control group immunized with BSA.

ELISA

An ELISA was used to detect specific antibodies produced by glycoconjugate-vaccinated rats. Maxisorb Nunc immunoplate (Sigma-Aldrich) were coated overnight with 1 µg/ml of FGn-BSA, FFGn-BSA, Gn-BSA or BSA diluted in pH 9.6 carbonate buffer at 4°C, and blocked with 0.1% Tween diluted in PBS for 1 hr at 37°C. Rat sera collected at 7 weeks post vaccination were pooled for each group and applied as serial dilution starting at 1:200 dilution in 0.05% Tween in PBS; 50 µL rat vaccination sera was applied per well and incubated for 1 hr at 37°C. After washing, the plates were incubated for 1 hr with 10,000x diluted biotinylated goat-anti-rat-IgG (Abcam) and goat anti-rat IgM (Abcam) detection antibody, followed by 10,000x diluted streptavidin-HRP in 0.05% Tween in PBS for 30 minutes at 37°C. 50 µL of TMB was used for detection and the reaction was stopped by 50 µL 1.8 M H_2SO_4 and absorbance was measured at 450 nm.

Glycan microarray

We have previously described the generation and application of microarrays consisting of N-, O- and glycosphingolipid (GSL) glycans isolated from different life stages of *S. mansoni*. [18, 38]. Briefly, printed array slides were incubated with pooled rat vaccination

sera from 7 weeks post vaccination at 1:100 dilution followed by fluorescently labeled secondary antibodies at 1:1,000 dilution and scanned using a G2565BA scanner (Agilent Technologies, Santa Clara, CA). Data and image analysis were performed with GenePix Pro 7.0 software (Molecular Devices, Sunnyvale, CA). Background-subtracted median intensities were averaged and processed as previously described [18, 38, 39].

The structures of a selection of glycan targets printed on the microarray recognized by antibodies in FGn-BSA immunization sera were characterized in detail by MALDI-TOF MS/MS fragmentation as described previously [38].

Schistosomula transformation

Cercariae were transformed to schistosomula as previously described [27]. Briefly, cercariae were incubated at 37°C in M199 medium (Gibco), supplemented with 1:100 1 M HEPES at pH7.4, 1x antibiotic antimycotic solution (ABAM), 1.5 mM glutamine and 10% fetal calf serum for 20 minutes and transferred to a petri dish. Using orbital shaking, schistosomula were concentrated in the center of the petri dish, collected and cultured in the above-mentioned medium for 3 hours.

Immunofluorescent binding assays

Schistosomula collected after the 3 hour incubation period were fixed in 2% paraformaldehyde and incubated with 5x diluted rat vaccination sera for 30 minutes at 37°C. IgG and IgM binding was detected using 1:250 dilution of AlexaFluor 488-conjugated anti-rat IgG and AlexaFluor 647-conjugated anti-rat IgM antibodies for another 30 minutes at 37°C. All steps in the protocol were followed by washing in PBS. Immunofluorescence was analyzed with fluorescence microscopy (Leica AF_6000).

Results

Vaccination with FGn-BSA and FFGn-BSA in rats reduced worm burden after challenge infection

Various types of fucosylated glycans expressed by schistosomes have previously been found to be a target of IgM and IgG in schistosome-infected hosts [18, 21, 26, 27]. To investigate whether responses to these types of antigens may be protective, we performed vaccination experiments in rats with glycoconjugates containing representative fucosylated glycan motifs. Three groups of 8 rats received either FGn-BSA, FFGn-BSA or BSA control in Freund's adjuvant. Three immunizations were given through i.p. injection at week 0, week 3 and week 5 (Figure 1A). At week 7, rats were infected with 2000 cercariae.

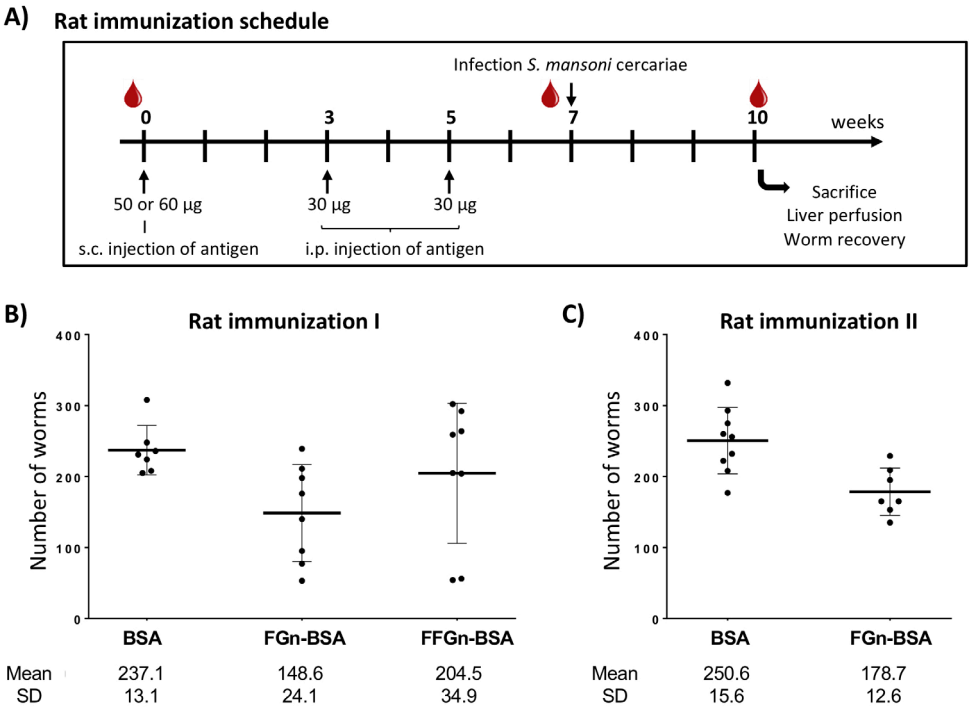

Figure 1. Rat immunization schedule and worm burden following challenge infection in glycoconjugate immunized rats. A) Rats were immunized 3 times with BSA, FGn-BSA, or FFGn-BSA at week 0, week 3 and week 5. In rat immunization I, 50 µg was used in the first injection while 60 µg was used in rat immunization II. At week 7, rats were infected with 2000 *S. mansoni* cercariae percutaneously. Liver perfusion and worm recovery were performed at week 10 when the rats were sacrificed. Blood was sampled at week 0, week 7 and at week 10. B) Worm burden of glycoconjugate immunized rats 3 weeks post challenge infection.

Table 1. The effect of glycoconjugate immunization in rats measured by worm recovery following challenge infection

Glycoconjugates (Adjuvant)	Control group (BSA in Freund)			Vaccinated group			Protection	
	n[1]	Worms recovered[2]		n[1]	Worms recovered[2]		% decrease in worm burden	p (Mann & Whitney)
		mean	sem		mean	sem		
FFGn-BSA (Freund) expt1	8	237	13	8	205	35	14	
FGn-BSA (Freund) expt1				8	149	24	38	<1%
FGn-BSA (Freund) expt2	9	250	16	7	179	13	29	<1%

[1] n, number of rats
[2] rats were infection transcutaneously with 2000 *S. mansoni* cercariae and isolated livers were perfused at day 21 post infection

Three weeks later rats were perfused to recover worms and worm burden was determined. The mean worm count in FGn-BSA immunized rats was 149 worms, while in the control group immunized with BSA on average 237 worms were recovered,

corresponding to a 38% decrease in worm burden ($p<0.01$) in the FGn-BSA immunized group compared with control rats (Figure 1B and Table 1). FFGn-BSA vaccinated rats had a 14% decrease in worm burden, but this reduction was not significant, and the spread was large (sem: 34.9). The protection induced by FGn-BSA immunization in rats was confirmed in a second experiment where we observed a decrease of 29% in worm burden in FGn-BSA vaccinated rats compared with control rats immunized with BSA only (Table 1 and Figure 1C).

Anti-glycan antibodies induced by FGn-BSA and FFGn-BSA immunization

To characterize antibody responses triggered by glycoconjugate immunizations, we measured serum IgM and IgG at week 7 against FGn-BSA and FFGn-BSA, and the Gn-BSA and BSA controls, using ELISA (Figure 2). Rats immunized with FGn-BSA and FFGn-BSA glycoconjugates develop higher IgM response towards the FGn-BSA and FFGn-BSA antigen compared to Gn-BSA and BSA, indicating that antibodies against the fucosyl motifs were induced (Figure 2A). Interestingly, IgM levels against FGn-BSA and FFGn-BSA were similar in FGn-BSA and FFGn-BSA immunized rats, suggesting that a significant cross-reactivity between these antigenic motifs exists. A lower, but clearly detectable reactivity to Gn-BSA by IgM in the sera of FGn-BSA or FFGn-BSA immunized rats was observed, indicative of BSA and/or Gn-linker directed antibodies. Mice immunized with BSA alone also develop an elevated IgM response towards the FFGn-BSA and FGn-BSA conjugates compared to prebleed.

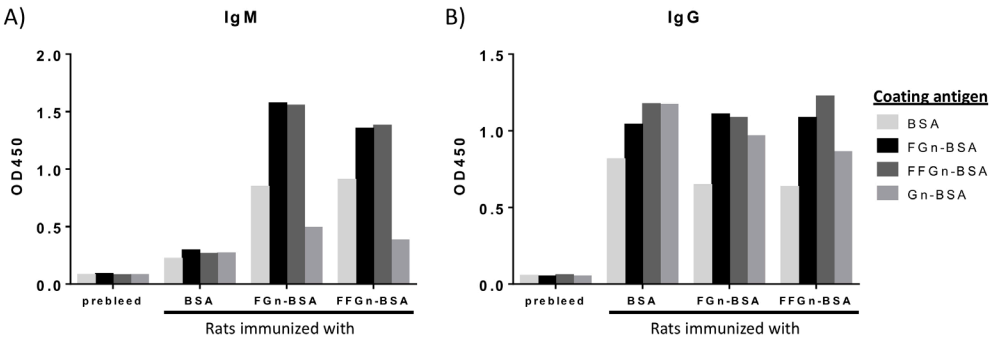

Figure 2. Serum antibody response of immunized rats to defined glycan antigens. BSA, FGn-BSA or FFGn-BSA immunized rat serum were collected 7 weeks post immunization before challenge infection. Serum IgM (A) and IgG (B) antibody response towards BSA controls, FGn-BSA, FFGn-BSA and Gn-BSA were measured by ELISA. Prebleed: pooled rat control serum collected prior to immunization.

The anti-BSA response of BSA immunized rats was lower however than that elicited by FGn-BSA and FFGn-BSA immunized rats, indicating that the presence of fucosylated glycans stimulated the IgM response to BSA. In contrast, IgG responses against BSA where equally high in both BSA immunized and BSA-glycoconjugate immunized rats (Figure 2B). Furthermore, IgG in FGn-BSA and FFGn-BSA immunized rats bound to Gn-BSA with similar intensity as to the matching immunogen. These observations suggest that the IgG detected by the ELISA are mainly directed against the carrier protein BSA.

Next, to find out whether the rat antibodies induced by FGn-BSA and FFGn-BSA vaccination also bound to native schistosome glycans, we performed a schistosome glycan microarray analysis of pooled rat vaccination sera collected at week 7, just before schistosome infection (Figure 3). We found that the FGn-BSA-immunized rats developed IgM that bound to a range of naturally occurring schistosome glycans, most intensely to many glycosphingolipid (GSL)-derived glycans. IgG from FGn-BSA immunized rats bound almost exclusively to GSL-derived glycans on the microarray. Surprisingly, week 7 pre-challenge serum of FFGn-BSA immunized rats on the other hand, did not contain IgM nor IgG significantly recognizing the numerous naturally occurring schistosome glycans present on the microarray.

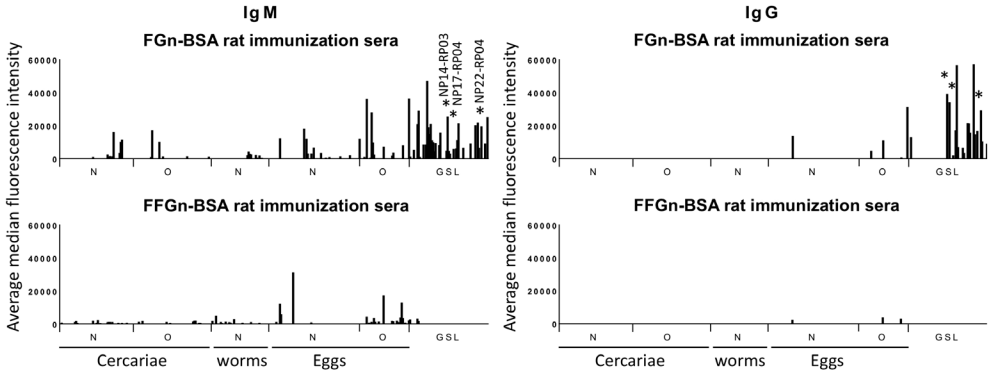

Figure 3. Averaged serum IgM and IgG response from glycoconjugate vaccinated rat sera collected 7 weeks post immunization to glycans isolated from different life stages of schistosomes. The average median fluorescence intensities are shown for FGn-BSA and FFGn-BSA immunized rats after subtraction with that of BSA-immunized rats. Each bar corresponds to antibody binding to individual glycan fractions printed on the glycan microarray. N: N-glycans. O: O-glycans and GSL: glycosphingolipid glycans. *: Glycan fractions described in figure 5.

Whilst FGn-BSA immunization elicited antibodies towards a specific subset of schistosome glycans on the microarray, challenge infection by exposure of immunized rats to *S. mansoni* cercariae induced IgM and IgG to a much wider repertoire of glycans

antigens (Figure 4). At 3 weeks post challenge infection, high IgM levels were measured against a wide range of cercarial N- and O-glycans, GSL-derived glycans isolated of different life stages, and egg-derived N- and O-glycans. As no eggs are produced during schistosome infection in rats, the antibody reactivity towards egg glycans is due to cross-reactivity with a subset of cercarial glycans that contain identical antigenic motifs to eggs [11, 27]. Moreover, anti-glycan IgM and IgG responses mounted upon challenge were in general stronger in FGn-BSA immunized rats than in BSA or FFGn-BSA immunized rats. Compared to IgM, IgG raised following challenge infection was more selective and directed towards a narrower subset of glycan targets.

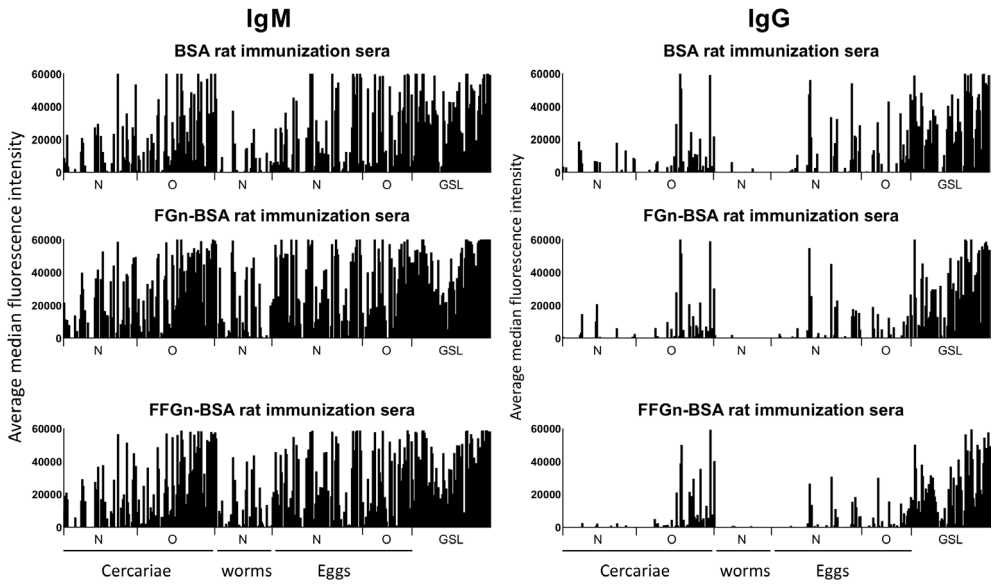

Figure 4. Averaged serum IgM and IgG response from glycoconjugate vaccinated rat sera collected 3 weeks post challenge infection to glycans isolated from different life stages of schistosomes. The average median fluorescence intensities are shown for BSA, FGn-BSA and FFGn-BSA immunized rats. Each bar corresponds to antibody binding to individual glycan fractions printed on the glycan microarray. N: N-glycans. O: O-glycans and GSL: glycosphingolipid glycans.

Hypothesizing that antibodies binding to fucosylated glycans of the schistosome are responsible for the observed protection induced by FGn-BSA immunization, we further investigated which native glycan motifs are recognized by FGn-BSA antisera. MALDI-TOF MS fragmentation analysis was applied to multiple glycan fractions positive for IgM and IgG binding (Figure 5). These MS analyses indicated that glycan antigens recognized by FGn-BSA rat immunization serum all contained Fucα1-3HexNAc motifs

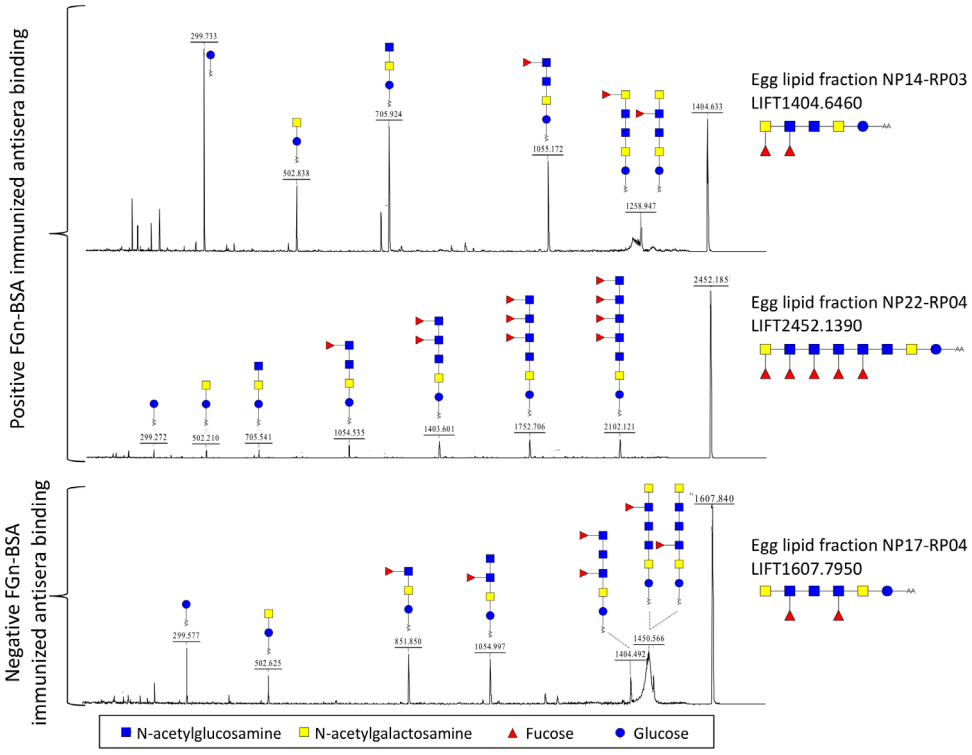

Figure 5. MALDI-TOF MS fragmentation analysis of glycan fractions recognized by serum IgM and IgG of FGN-BSA immunized rats. Representative glycan fractions that had positive binding with FGn-BSA immunized rat antisera are depicted (Egg lipid fraction NP14-RP03 and NP22-RP04); These glycans contained terminal Fucα1-3HexNAc motifs. Glycans lacking terminally fucosylated HexNac residues (Egg lipid fraction NP17-RP04) had no binding with FGn-BSA immunized rat serum.

(HexNAc=GalNAc, GlcNAc), presumably as part of the terminal Fucα1-3GalNAc(Fucα1-3GlcNAcβ1-) tetrasaccharide antigen (F-LDN-F) which is abundant in schistosome glycans [11]. In contrast, to glycans with non-fucosylated terminal GalNAc/GlcNAc residues or with fucosylation at non-terminal positions in the glycan backbone, no binding was observed.

Antisera of glycoconjugate immunized rats bind to the surface of schistosomula 3 h after transformation

After identifying glycans with terminal Fucα1-3HexNAc motifs as antibody targets in FGn-immunized rats, we examined whether the corresponding serum would recognize such antigens present on the surface of schistosomula. Both IgM and IgG of FGn-BSA immunized rat antisera bound to the surface of 3 hour (h) transformed schistosomula (Figure 6). FFGn-BSA immunized and BSA immunized rat serum did not show antibody

binding to the parasite. This is in accordance with the patterns seen in the glycan microarray analysis where only FGn-BSA immunized rats produce antibodies to native schistosome glycans. The presence of antigenic targets on the surface of the parasite recognized by FGn-BSA immunization sera indicates that upon challenge infection antibody-dependent mechanisms are potentially involved in immunity against developing larvae and subsequent reduction of worm burden after challenge in the FGn-BSA immunized group.

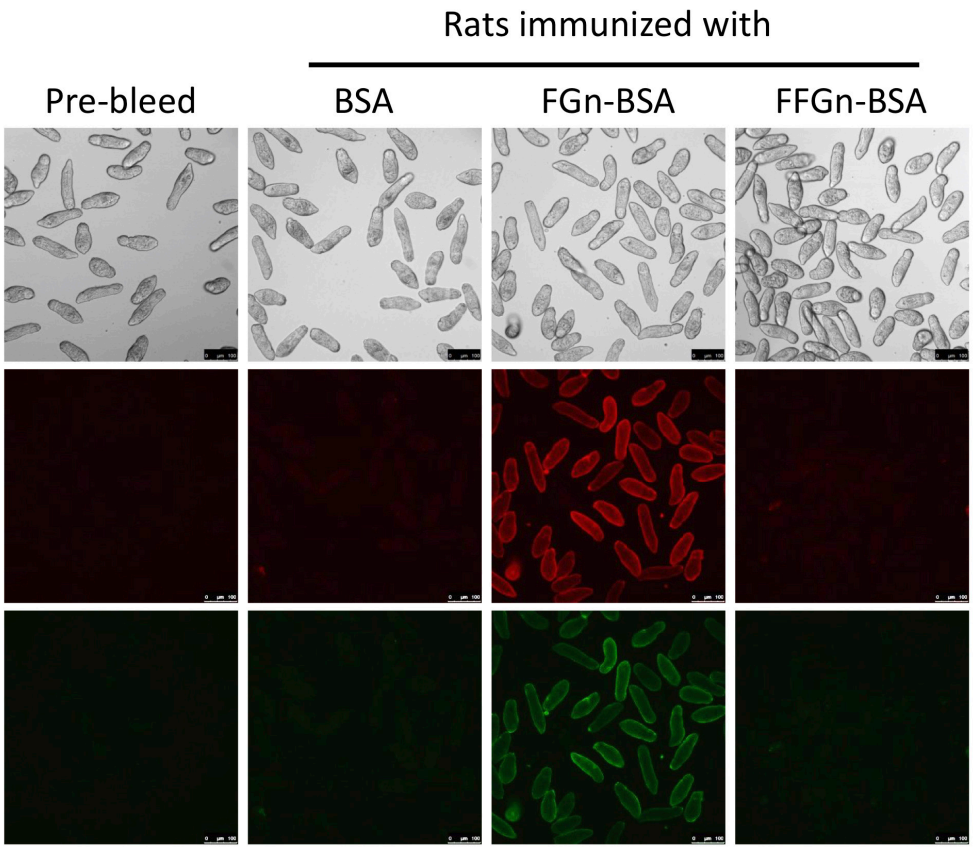

Figure 6. Rat antisera binding to the surface of schistosomula 3 h after transformation. Anti-rat IgM (red) and anti-rat IgG (green) antibodies were used to detect serum IgM and IgG binding on the surface of schistosomula fixed in 2% PFA.

Discussion

In schistosomiasis, antibodies directed to many glycan antigens seem to be unprotective, or perhaps even counter protective by inhibiting immunity or stimulating evasive mechanisms [17]. However, similar to anti-protein antibodies, a subset of anti-glycan antibodies may relate to protection. We here report for the first time that immunization with an antigenic glycan expressed by schistosomes reduces worm burden after challenge infection with *S. mansoni*. In two separate experiments, vaccination of rats with the neoglycoprotein FGn-BSA, a multivalent presentation of the Fucα1-3GlcNAc motif, significantly limited worm burden by 38% and 29%, respectively.

Vaccination with the α3-fucosylated FGn-BSA conjugate induced IgM, and to a lesser extent IgG, antibodies against a wide range of schistosome glycans containing fucosylated motifs as determined by glycan microarray analysis (Figure 3). In addition, FGn-BSA immunization serum IgG and IgM was found to bind to targets over the entire surface of schistosomula 3 hours after transformation (Figure 6), confirming the presence of target glycan epitopes on the schistosomulum surface. Fucosylated glycans are highly abundant on cercariae and schistosomula as shown previously in monoclonal antibody based IFA studies [10]. The current microarray data (Figure 3) suggest that FGn-BSA immune serum IgG binds primarily to GSL glycans. These GSL glycans with fucosylated LDN-motifs have been established to be exposed on the surface of schistosomula for up to two weeks after transformation after they lose their glycocalyx [10].

The serum antibody binding profile of partially protected FGn-BSA immunized rats was very similar to that seen in vaccinated baboons protected from challenge infection [26] and serum from macaques that are resistant against reinfection [27]. This strongly suggests that anti-fucosylated glycan antibody responses in macaques and vaccinated baboons also contributed to the protective immune response in these hosts. Previously, we were not able to directly identify the specific antibody targets associated with protection due to the complexity of the responses induced by irradiated cercariae or full infections. However, in this study we showed that protected rats vaccinated with FGn-BSA developed antibodies that recognized naturally occurring schistosome lipid derived glycans with a fucosylated terminal N-acetylhexosamine. GSL glycans with terminal non-fucosylated N-acetylhexosamine were not bound, despite having fucosylated N-acetylglucosamines in the backbone stretch. This further supports the hypothesis that the protective antibodies generated by FGn-BSA vaccination in rats have a specificity for α3-fucosylated terminal N-acetylhexosamines on schistosome GSL glycans.

In addition to generating fucose specific IgG and IgM antibodies, we observed that FGn-BSA immunized rats raised stronger IgG and especially IgM overall to schistosome

glycans 3 weeks post challenge, when compared to BSA or FFGn-BSA immunized rats. These elevated anti-glycan antibody levels were not specific to certain glycan epitopes only, but FGn-BSA immunization seems to have sensitized the rats to many glycan antigens exposed after the cercarial challenge. Perhaps the elevated antibody response could account for a recall response that prepares the host to resist parasite infection.

Previous studies have shown that effective immunity in the radiation attenuated vaccine model involved a mixed Th1/Th2 immune responses [40, 41], which is why both complete and incomplete Freund's adjuvant were used during vaccination in rats. Perhaps a balanced Th1 and Th2 response is necessary for protection against *S. mansoni* infection. However, Freund's adjuvant cannot be used in humans. On the other hand, Alum, a Th2 response inducer which is considered the safest adjuvant for human vaccines [42, 43], could be useful for extrapolating results. Alternatively, the synthetic TLR4 agonist glucopyranosyl lipid adjuvant formulated in a squalene-in-water emulsion (GLA-SE), a strong Th1 inducer [44] with the ability to increase extrafollicular antibody response and long-lived antibody production [45] that has proved to be a safe and potent clinical stage adjuvant [46], could also be considered to test efficacy under Th1 inducing conditions.

In this study, antibodies against schistosome GSL glycans appear to be crucial for protection. It is known that GSL glycans express highly fucosylated epitopes [11] including terminal Fucα1-3, as well as Fucα1-2Fucα1-3 disaccharide. Interestingly, immunization with FGn-BSA, but not FFGn-BSA appeared to be effective in inducing a protective response. Despite binding to the FFGn-BSA glycoconjugate, serum antibodies of FFGn-BSA immunized rats did not bind to naturally occurring schistosome glycans, indicating that this conjugate fails to induce antibodies that react with native Fucα1-2Fucα1-3- containing glycans on the array. The lack of binding to native Fucα1-2Fucα1-3-containing glycans could possibly be due to the fact that double fucoses often occur in combination with many more fucosylated elements on the same glycan backbone [11], which may hinder the binding of anti-glycan antibodies elicited during FFGn-BSA immunization. Compared to FGn, the FFGn glycan motif is more schistosome specific and highly antigenic during schistosome exposure and infection, and it would be interesting to test whether other synthetic representations of this antigen may induce antibodies more reactive with native glycans.

It is important to realize that in a natural infection, a strong antibody response is directed against many multi-fucosylated motifs on N-, O- as well as GSL glycans. The difference in magnitude between the simple glycoconjugate vaccination and natural infection was clearly observed in the glycan profile of rat antisera 3 weeks post infection (Figure 3 and 4). It is intriguing to see that a small, minimal epitope FGn on BSA, elicited antibodies to singly fucosylated terminal N-acetylhexosamine, and induced partial

protection against *S. mansoni* infection in rats.

In conclusion, we have demonstrated that immunization with a fucosylated glycan, when conjugated to BSA, induce antibodies that bind to schistosomula and reduce worm burden in rats challenged with schistosome infection. Antibodies against fucosylated schistosome GSL glycans appear to be associated with the induced protection. Future studies should further investigate whether vaccinating with more complex glycan antigens and more strongly immunogenic glycan-carrier combinations would generate an antibody profile more similar to that observed in protective contexts, and whether that would lead to stronger protection in a challenged host.

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Chapter 6

Summarizing discussion

Schistosomiasis is the second most socioeconomically devastating parasitic disease after malaria, affecting more than 220 million people in endemic countries. The insufficient knowledge over schistosome antigens and the host immune response against these antigens is a great obstacle towards improving current diagnostic, treatment and prevention methods. The studies presented in this thesis focus on mapping the antibody response against schistosome glycans whilst the host develops immunity towards schistosomiasis. The findings in this thesis provided insight into whether schistosome glycans are worth exploiting in the discovery of novel vaccination candidates; as well as whether glycan motifs or specific anti-glycan antibody responses can serve as good infection markers.

Antigenic schistosome glycan motifs

A major discussion over anti-glycan antibodies in schistosomiasis is whether they are a smokescreen response that directs the host immune response away from recognizing essential protein antigens [1-3], or whether glycans can be potential vaccine candidates because of their high antigenicity and abundant exposure to the host [4-6]. In **chapter 2** and **chapter 3**, we investigated longitudinally the IgG and IgM dynamics during the development of protection in two different animal models. **Chapter 2** described the antibody dynamics to glycans in rhesus macaques during the time they naturally clear an established infection and at the same time develop resistance to secondary infection [7]. **Chapter 3** followed the anti-glycan antibody dynamics in baboons when they developed protection through repeated radiation-attenuated (RA) cercariae vaccination [2]. The use of an elaborate and well defined glycan microarray containing glycans isolated from different life stages of *S. mansoni* to measure antibody responses, instead of crude antigens such as soluble egg antigen (SEA) or adult worm antigen (AWA), greatly improved the specificity of our results. When using the glycan microarrays, we found that in both the baboon and macaque model, IgG response to a small subset of glycans containing multiple-fucosylated terminal GalNAcβ1-4GlcNAc (LDN) and Galβ1-4GlcNAc (LN) motifs remained high throughout infection clearance and the development of resistance. On the other hand, the response to many other non-fucosylated terminal motifs and also to the core xylose and core α3-fucose, previously suggested as potential vaccination candidates [8] ceased after oviposition. Since core xylose and core α3-fucose have not been found on mature worm N-glycans [9], we propose that antibodies against these targets are not involved in worm killing, especially since these antibody responses are short lived. It is worth noting that core α3-fucose, despite being highly antigenic, is found in schistosomes exclusively in eggs, which makes it unsuitable as a vaccine target. Antibodies against N-glycan core modifications may be utilized as stage-specific diagnostic markers, but do

not serve as good vaccination candidates. A suitable vaccine candidate should be based on parasite antigens that are exposed to the host immune system and that are either necessary for parasite survival [10] or can form a handle for an antibody-mediated cellular anti-parasite response. Although specific roles for highly fucosylated LDN and LN motifs in the parasite biology are not known, these antigenic motifs are expressed in all life stages of the parasite in different contexts: cercariae and eggs express these motifs on both glycoproteins and glycolipids, while adult worms express fucosylated LDN predominantly on glycolipids [11, 12]. In all cases these motifs are abundantly present directly at the host-parasite interface (i.e. at the surface or in secretory systems of the parasite).

In **chapter 2** and **chapter 3**, schistosomula killing assays were used as functional assays to assess whether the antibodies generated by macaques and baboons could be protective. One of the limitations of our studies was that we did not have enough macaque and baboon serum to isolate anti-glycan antibodies in sufficient quantities to explore whether anti-glycan antibodies in these sera could be protective in a functional assay. Nevertheless, the antibody binding pattern on schistosomula was shown to be well correlated with the relative abundance of anti-glycan antibodies, likely against highly antigenic multiple-fucosylated LDN motifs. When incubating schistosomula with macaque sera *in vitro*, schistosomula death was positively correlated with the duration of infection in the macaques; suggesting the gradual development of potentially protective antibodies (**chapter 2**). For newly transformed schistosomula, it has been shown that they can be killed by complement, while 24 h transformed schistosomula became insensitive to complement-mediated killing [13]. In our observation, 3 h transformed schistosomula were killed by rhesus macaque serum in a complement-independent manner. Higher killing percentage was positively associated with tight clustering of the parasites by serum antibodies, possibly by hindering physiological mechanisms necessary for parasite survival. Unlike rhesus macaque serum, baboon vaccination serum did not lead to any killing of the parasite (**chapter 3**). It is worth noting that most studies on complement activation and schistosomula death were performed *in vitro*. Thus, the *in vivo* significance of complement on schistosomula has not been asserted and other cellular mediated defense mechanisms may also play an important role during protection. This could be one of the potential reasons why baboon vaccination sera alone was not able to kill schistosomula *in vitro*.

Since highly fucosylated terminal LDN motifs are specific for schistosomes and could induce a long lasting IgG response, we performed a vaccination study to assess the protectivity of this glycan motif. **Chapter 5** describes a vaccination study in rats with synthetic carbohydrates Fucα1-3GlcNAc (FGn) and Fucα1-2Fucα1-3GlcNAc (FFGn) conjugated to BSA. This study is the first to show that immunization with a glycoconjugate

with schistosome glycans can reduce worm burden in an *S. mansoni* infected host. We found that immunizing rats with synthetic FGn-BSA together with complete Freund's adjuvant significantly reduced worm burden upon challenge compared to rats vaccinated with BSA only. In addition, the antibodies generated by FGn-BSA vaccination recognized natural schistosome antigens and were found to bind to the surface of transformed schistosomula. The protection induced in rats by 3 consecutive FGn-BSA vaccinations was found to be 38% and 29% in two independent experiments (**Chapter 5**), showing that partial protection can be achieved, despite being less effective than some other vaccination candidates currently under investigation [14-17]. Future work to improve immunization strategies should consider factors such as the time point of infection, route, as well as vaccine format. Moreover, the library of potential schistosome glycan candidates that can be used in vaccination should be increased. This work however, is challenged by the limited availability of native parasite-derived glycoproteins as well as the difficulty of synthesizing complex glycan structures. An alternative to create recombinant proteins with desired glycosylation is through glycoengineered plants, a maturing technological platform [18-20]. Lewis X, LDN and GalNAcβ1-4(Fucα1-3)GlcNAc (LDN-F) glycan motifs have been successfully produced on recombinant schistosome proteins in plants by fine-tuning the N-glycosylation machinery [19]. Nevertheless, to synthesize multi-fucose containing glycans in plants, the fucosyltransferase responsible for generating the schistosome specific Fucα1-2Fuc linkage, whether it being a specific α2-fucosyltransferase, or another fucosyltransferase with α2-fucosylation activity, needs to be identified [21]. Carbohydrate based vaccines have shown success in bacterial infections, and this wave of interest is extending to helminth vaccination [22]. Helminths are multicellular organisms that go through complex changes both outside and inside the host.

Till this date, there is no consensus over how an individual develops resistance to infection, or what the mechanism of protection is [14, 15]. However, regardless of the mechanisms involved, antibodies are seen as key players in the protective immunity induced by vaccines [14]. Another important issue hindering vaccine development is the lack of clarity in "who is protected" and what the antibody profile of a protected host looks like.

What is a protective antibody profile? lessons learned from longitudinal studies

The rhesus macaque model (**Chapter 2**) and the RA cercariae vaccination baboon model (**Chapter 3**) are well known models of protection. Although controlled human infection (CHI) models are gaining acceptance, and recently a CHI model for *S. mansoni* has been developed and tested on small scale [23-25], most human studies are still cross-sectional

cohort studies that are non-invasive. Longitudinal studies in non-human primates are thus a good complement to human studies by providing the unique opportunity to observe the dynamic interplay between host and parasite**.** We found that upon encounter with schistosome antigens, either through experimental cercariae infection, or by RA cercariae vaccination, IgM was first elicited and followed by IgG approximately 2-4 weeks later. In addition, IgM responses against many glycan epitopes were short lived or lose intensity over time, while IgG responses towards specific glycan subsets remained high. In experimentally infected rhesus macaques, anti-glycan IgG reached maximum titers together with the peak of oviposition at week 8 post infection, while IgM responses towards most glycan epitopes decreased after 8 weeks (**Chapter 2**). This observation in antibody dynamics, especially the increase in IgM titers in relation to oviposition is in accordance with recent studies in mice that suggested anti-worm and anti-egg IgM to be a good measure for acute infection [26]. On the other hand, chronic infections are associated with less oviposition and lower parasite specific IgM titers. That being said, IgM titers are not solely driven by the presence of eggs, as parasite specific IgM appears much earlier than eggs in experimental infections [26, 27], and vaccinating non-human primates with RA cercariae induced IgM and IgG responses not only to cercarial preparations [2], but also to egg and worm preparations [28, 29], most likely via cross-reactive glycans (**Chapter 3**) [9].

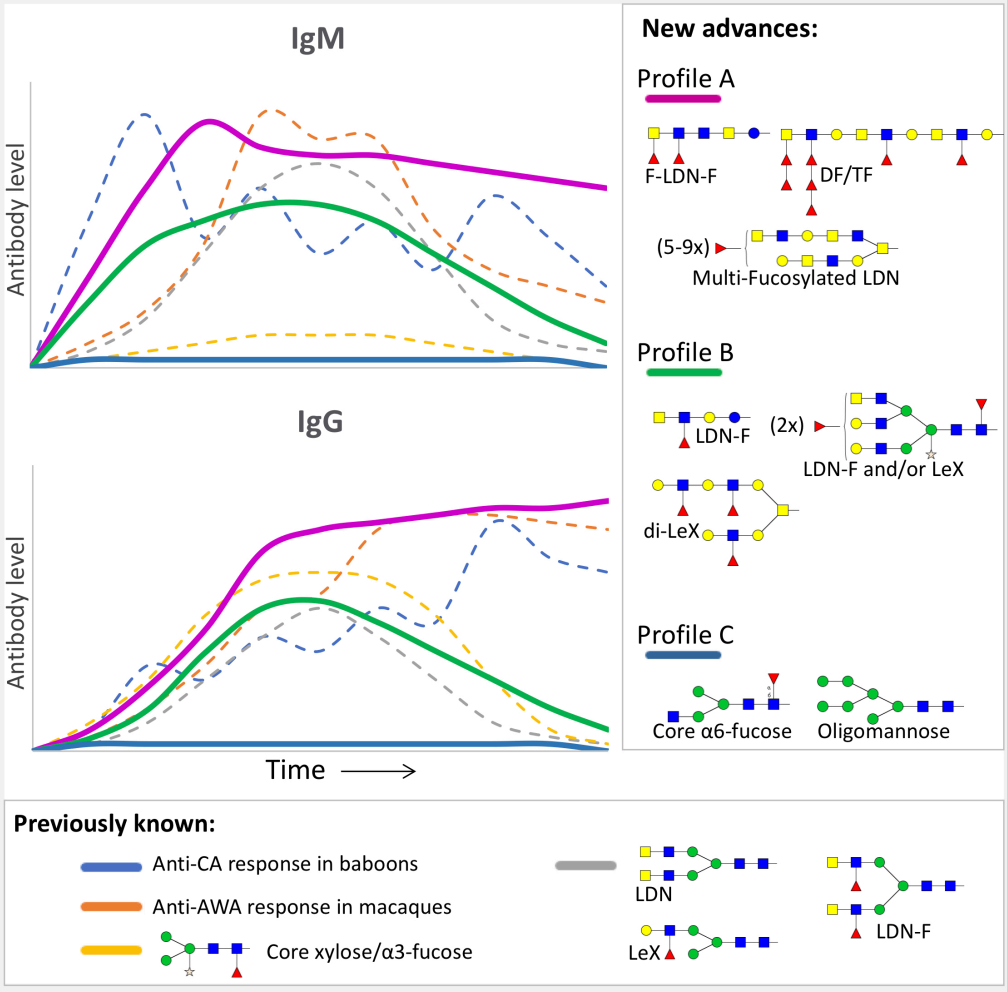
In the vaccination model, high numbers of RA cercariae (in 5 vaccinations) are necessary to boost protective IgG titers in baboons (**Chapter 3**). The anti-cercarial glycan IgG titers continued to increase in a saw tooth pattern until vaccination ceased. In contrast, the anti-glycan IgM titers were highest after 2 vaccinations, but failed to reach beyond this titer with following vaccinations: IgM titers to cercarial glycans either remained constant (towards O-glycans) or decreased (towards GSL glycans), likely due to heavy chain class switch, indicated by a clear shift in IgM:IgG ratio observed at 10 weeks post vaccination. Interestingly, in a different study when baboons were vaccinated 3 times, both IgG and IgM responses are high upon challenge infection [30]. In this situation, the vaccination efficiency is only 30-54% in contrast to vaccinating 5 times accompanied with decreased IgM. Overall, we saw that resistance to reinfection in both animal models, as well as the clearance of primary infection in rhesus macaques was associated with low IgM and high IgG tires against schistosome glycans. Recent studies in mice have proposed that IgG/IgM balances against specific schistosome preparations may be effective for differentiating acute/chronic infections [26], although the authors also recognized the need to characterize and evaluate specific molecules in crude antigens in order to promote increased sensitivity and specificity of the diagnosis. In rhesus macaques, we found that at the timepoint where macaques are resistant towards secondary infection, a statistically different IgG:IgM ratio was observed towards these multiple fucosylated

glycans on cercarial O-glycans specifically. We suggested that high anti-IgG and low anti-IgM ratios against these cercarial glycans could facilitate protection mechanisms that prevent macaques from being re-infected with cercariae (**Chapter 2**).

Previous studies have indicated that IgM is possibly a blocking antibody that prevents eosinophil mediated cytotoxicity by effective IgG subclasses [1]. Moreover, human serum IgM has shown an overall competitive advantage over IgG towards glycans and not peptides [31]. Although we have shown *in vitro* through antibody competition experiments that the presence of IgM does not prevent IgG binding to the surface of schistosomula (**Chapter 3**), there is no indication that IgM and IgG recognize different glycan targets based on glycan array data. Therefore, it is likely that IgM and IgG are competing for the same epitopes, thus emphasizing the importance of IgG/IgM balance in a protection profile.

In addition to IgG and IgM balance, IgG subclass balance and the presence of IgE have also been discussed as indicators of schistosomiasis disease state [32-36]. In **chapter 4** we studied the serum IgG subclass response against a selected panel of core-modified N-glycan targets of 41 *S. mansoni* infected individuals living in an endemic area. We observed that while IgG1 and IgG3 recognized a range of different N-glycan core structures, IgG2 and IgG4, if present, were specific for core α3-fucose and core xylose. It is worth noting that we found large variations between individuals. Previously, sera from the same cohort were separated into children and adults and their averaged total IgG and IgM response was analyzed on the same array as used in this study [37]. Pronounced differences between children and adult anti-glycan IgG were reported: IgG in serum from children, unlike adults, bound to core α3-fucose irrespective of the presence of the core xylose motif. However, when we analyzed the IgG subclass profile of these individuals separately, it appeared that only a minority of individuals had IgG towards core α3-fucose (**Chapter 4**). We also reported that antigenic core xylose and core α3-fucose was not recognized by particular IgG subclasses selectively, but dependent on the individuals. Previous studies trying to attribute the role of IgG subclasses based on cohort studies also faced the same challenge of high individual variability and while some observations are true at a population level, they cannot be seen on the individual level [38, 39]. Therefore, we recommend future studies to consider the size of the cohort as well as defining disease state and/or other infections, knowing that different disease pathologies affect the antibody signatures in patients with schistosomiasis [39] and the occurrence of cross-reaction with other helminths [40, 41]. Another point of consideration is using suitable probes. It has been shown that ELISA-SEA, instead of ELISA-SWAP (Soluble worm preparation), was better at detecting *S. japonicum* infection, irrespective of disease condition [39]. SWAP on the other hand was better than SEA in detecting chronic infections [42].

Box 2. How did our studies advance the knowledge on antibody dynamics in non-human primate protection models for schistosomiasis?



Using glycan-microarrays, we followed the antibody dynamics during which vaccination acquired and naturally acquired immunity developed in baboons and rhesus macaques, respectively. We identified different profile antibody dynamics in these hosts and found that both the antibody profiles of the two hosts, and the glycan motifs that fell under the same profiles, were largely similar (thus are shown as one protective profile). Representative glycan structures are depicted for each profile based on overall occurrence. Glycan motifs in profile A were the most antigenic, and are particularly interesting because in time, the IgG/IgM ratio increases for these motifs, suggesting the possibility of some of them being protective antigens. Profile B consists of antigenic glycans that are short lived, either because they are life stage specific or are not able to elicit long lasting antibody body responses. Not all glycans are antigenic, and these are found in profile C. Glycans in this profile can also occur in the glycome of the host. The consistency of how different host antibody responds, under different conditions, to schistosome glycans confirms that these results form a reliable base for future work within the frame of protective immunity.

Implications for vaccine candidate discovery and diagnostics

The standard method of diagnosing schistosome infection is detection of *Schistosoma* eggs. Both urine filtration [43] (for *S. haematobium* eggs) and Kato-Katz thick smear method (for intestinal schistosome eggs) provide high specificity, being able to distinguish different schistosome species, but lack sensitivity to detect light infections even through repeated sampling [44]. Schistosome antigens such as schistosome circulating cathodic antigen (CCA) [45-51] and circulating anodic antigen (CAA) [52, 53], despite not able to distinguish between schistosome species [54], are much more robust in detecting light-intensity infections in low-endemic areas and are indicators of an active infection. Serological detection of anti-schistosome antibodies can be used to determine whether a person has been exposed to schistosomes, though the inability to distinguish between active and past infection is also one of the biggest limitations of diagnosing with anti-schistosome antibodies [55-57]. The main advantage of using anti-schistosome antibodies as diagnostic markers is that they can be detected very early after infection, even before circulating antigens are released from regurgitating juvenile worms [58] (**Chapters 2** and **3**) and is also highly sensitive in detecting infection in low schistosome burden settings [59, 60]. Whether schistosome glycans and anti-glycan antibodies can serve as better diagnostic markers than existing markers is still under investigation. Cercarial glycans that are highly antigenic and abundantly expressed on the glycocalyx may possibly be an early detection marker for infection. We saw that as early as 2 weeks post RA vaccination (**Chapter 3**), and 4 weeks post infection of rhesus macaque (**Chapter 2**), antibodies were already generated against schistosome specific glycan motifs, such as highly fucosylated LDN/LN motifs (these were timepoints of the first measurement, antibody response could occur earlier than measured). On the other hand, antigenic glycan motifs such as core α3-fucose and core xylose present on schistosomes are not recommended as diagnostic biomarkers due to cross-reaction with glycans present on number of nematodes [61-63], other invertebrates [64] and plants [65-67].

Apart from being diagnostic biomarker candidates, schistosome glycans also have a potential to be vaccination targets. We have already shown that immunizing rats with a minimal epitope of fucosylated BSA glycoconjugate led to protection (Chapter 5). One of the advantages of using schistosome glycan-based vaccines is that many glycan motifs, such as multiple fucosylated motifs, are not only highly antigenic, they are present in multiple life stages and expressed by multiple proteins of the parasite, thereby maximizing the time frame and abundance in which targets of a vaccine are present in the infected or exposed host. The possible danger of immunizing with glycans is that many antigenic

motifs are also present on eggs [9]. If an inflammatory immune response to egg glycan is triggered in patients with patent infection, it may exacerbate immunopathology. With aid of the maturing glycan synthesis technologies, future studies should continue to interrogate more specific, differently fucosylated glycan structures and identify those that give higher levels of protection, but minimal side effects.

Conclusion

The studies in this thesis have clearly demonstrated the diversity and complexity of anti-glycan antibody responses: not all glycan motifs and not all anti-glycan antibody responses are the same. By evaluating schistosome glycan motifs separately, we provided new insights into glycan epitopes in the context of vaccination and infection monitoring. In addition, we have shown that the anti-glycan antibody responses are highly dynamic and versatile: some early, some late, some long-living, others die out fast. Hopefully, the studies presented in this thesis has provided some clarity to the complexity of glycan epitopes, as well as the antibody responses to them.

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Addendum

Summary

Schistosomiasis is a parasitic disease, caused by infection with *Schistosoma* worms, that ranks second only to malaria in terms of socio-economic and public health impact. Once a schistosome infection establishes, the adult worm-pair can reside in the host for decades if not treated. Current treatment of schistosomiasis relies mainly on the use of the chemical drug Praziquantel (PZQ), even though PZQ cannot prevent the rapid re-infections that occur in endemic areas. A prophylactic therapy that can prevent schistosome infection is therefore urgently sought for.

The high prevalence of schistosomiasis in endemic populations indicates that natural immunity is rare. To date, although cases of acquired immunity have been reported, there is no evidence or consensus regarding how these individuals developed resistance, or what the mechanism of resistance is. It is known that in schistosome-infected hosts, antibodies are raised to numerous schistosome antigens, of which a majority are antibodies directed against schistosome glycan motifs. Schistosomes express a plethora of glycans: some glycan structures are expressed in a life-stage specific manner, while others are found in multiple life-stages, either secreted, exposed on the surface or expressed internally. Interestingly, conflicting views exist over the role of antibodies developed against schistosome glycans during infection. While some studies suggest that antibodies against schistosome glycans are a 'smokescreen' response that prevents the host from recognizing vulnerable parasite peptide antigens, others have shown that antigenic schistosome glycans could be involved in developing a protective response. These opposing views can result from generalization of the many types and structures of glycan motifs expressed by schistosomes, and/or the type of antibodies raised against the target. Fortunately, recent advances in technology have made it possible to not only separate and characterize individual naturally occurring schistosome glycans, but also to print these sometimes highly complex glycans isolated from the parasite on microarrays, that can be used further to characterise their binding partners, such as serum antibodies, in a high throughput manner. The possibility to chemically synthesize defined glycan structures further expands the library of glycans that can be analysed with microarray technology, as well as provide the opportunity to vaccinate animals with defined glycoconjugates.

The goal of this thesis is to advance the knowledge on anti-glycan antibody responses during a schistosome infection. In chapter 2 and 3, we used glycan-microarrays to follow the IgG and IgM antibody dynamics; during which naturally acquired and vaccination induced immunity developed in rhesus macaques and baboons, respectively. In chapter

4, we focused on the IgG subclass response against glycans expressed by schistosomes in schistosome infected individuals living in an endemic area. Lastly, chapter 5 describes an immunization and challenge experiment, where rats were immunized with defined glycoconjugates. A better understanding of anti-glycan antibody responses during infection and the development of schistosome resistance could improve current schistosomiasis diagnostic methods as well as identify potential vaccine targets.

Studies described in this thesis

In **Chapter 2**, the antibody response during the development of schistosome resistance was studied in rhesus macaques over a time course of 22 weeks. Unlike humans, rhesus macaques are able to naturally rid themselves of schistosome worms after infection establishes and acquire immunity towards re-infection around 12 weeks post infection. We found that upon experimental infection, macaques responded to schistosome glycans first with IgM antibodies, followed by IgG approximately 2-4 weeks later. Through the course of infection, IgM responses towards many glycan epitopes were short lived, while IgG responses towards specific glycan subsets, namely O-glycans with highly fucosylated LDN motifs, remained high throughout worm clearance. In general, glycans with a higher degree of fucosylation gave rise to stronger antibody responses than non-fucosylated glycans. When incubating macaque serum with schistosomula *in vitro*, schistosomula death was positively correlated with the duration of infection of macaques, suggesting presence of potentially protective antibodies. The findings in this chapter suggest that IgG antibodies against highly fucosylated LDN motifs that remain when the worms deteriorate could be associated with infection clearance and resistance to re-infection in macaques.

In **Chapter 3**, we studied the anti-glycan antibody responses during the development of schistosome resistance in baboons vaccinated with radiation-attenuated (RA) cercariae over a time course of 25 weeks. We found that both IgM and IgG targeted highly antigenic glycan structures with multi-fucosylated GalNAc/GlcNAc motifs expressed on cercarial O-glycans and glycosphingolipids (GSL). However, while IgM responses to these antigenic schistosome glycan motifs plateaued or decreased after 2 vaccinations, each of the 5 consecutive vaccinations boosted the anti-glycan IgG antibody response in baboons against these glycan motifs. At the moment of cercarial challenge, 19 weeks post vaccination, the IgG response against these multi-fucosylated glycan motifs present on cercarial O- and GSL glycans was at its highest, and remained high until 6 weeks post-challenge. These findings support the hypothesis that anti-glycan IgG is important in immunity against challenge infection in RA cercariae vaccinated baboons.

Particular IgG subclasses have been found to correlate with resistance or susceptibility to schistosome infection. In **Chapter 4**, the IgG subclass response of 41 schistosome infected individuals with similar infection intensities was analysed using glycan microarrays. We found that highly antigenic glycan motifs, such as multi-fucosylated LDN motifs can be recognized by all IgG subclasses, with IgG1 and IgG2 being more predominant. When looking at individual response towards core modified N-glycans, IgG2 and IgG4, when present, were specific for core α3-fucose and xylose motifs, while IgG1 and IgG3 were found against a less restricted range of N-glycan modifications. It is worth noting that high inter-individual antibody variation was observed against core modified N-glycans expressed by schistosomes. This study is the first to address IgG subclass responses to defined glycans in a helminth infection.

Finally, to test whether specific antigenic glycan motifs recognized by schistosome infected hosts could be involved in protection against schistosomiasis, a vaccination study was performed, described in **Chapter 5.** We found that immunizing rats with a single fucosylated GlcNAc motif conjugated to BSA (FGn-BSA) significantly reduced worm burden upon cercarial challenge, when compared to rats vaccinated with BSA only or double fucosylated GlcNAc conjugated to BSA (FFGn-BSA). The protection induced in rats by 3 consecutive FGn-BSA vaccinations was 38% and 29% in two independent experiments. Antibodies elicited by FGn-BSA immunization recognized native glycans isolated from schistosomes, mainly against lipid-derived glycans containing terminal fucosylated GalNAc/GlcNAc motifs. Moreover, FGn-BSA immunized rat serum antibodies bound to the entire surface of 3 hour transformed schistosomula, confirming the presence of target glycan epitopes on the schistosomulum. This indicates that antibody-dependent mechanisms may be involved in immunity against developing larvae upon challenge infection which would account for the protection seen in FGn-BSA immunized rats.

Conclusion

The studies described in this thesis demonstrate the diversity and complexity of anti-glycan antibody responses during a schistosome infection. Individual glycan motifs are different in their antigenicity and antibodies towards particular glycans also go through rapid changes throughout infection. By evaluating individual schistosome glycans and corresponding antibodies we provided new and detailed insights into the diverse anti-glycan antibody responses generated in the context of vaccination and infection with schistosomes.

Nederlandse Samenvatting

Schistosomiasis is een ziekte die wordt veroorzaakt door infectie met parasitaire *Schistosoma* wormen. Na malaria is het de parasitaire infectieziekte met de hoogste impact op socio-economisch gebied en de volksgezondheid. Zodra een infectie met *Schistosoma* tot stand is gekomen, kan een volwassen wormpaar jarenlang in de gastheer overleven zolang deze niet wordt behandeld. De huidige behandeling van schistosomiasis is met het geneesmiddel Praziquantel. Dit middel kan echter een snelle herinfectie, zoals in endemische gebieden vaak gebeurt, niet voorkomen. Daarom wordt er naarstig gezocht naar een profylactische behandeling die infectie met *Schistosoma* wél kan voorkomen.

De hoge prevalentie van schistosomiasis in endemische populaties geeft aan dat immuniteit zeldzaam is. Hoewel er voorbeelden zijn van groepen mensen die weerstand tegen infectie hebben ontwikkeld, is het niet duidelijk wat het mechanisme daarvoor is. Het is bekend dat in individuen die met *Schistosoma* geïnfecteerd zijn, antilichamen opgewekt worden tegen een groot aantal schistosoom antigenen en waarvan het grootste deel gericht is tegen glycaanmotieven. *Schistosoma* brengt een scala aan glycanen tot expressie. Sommige glycaan structuren komen in een specifiek levensstadium tot expressie terwijl anderen in meerdere stadia worden aangetroffen, hetzij uitgescheiden, blootgesteld op het oppervlak, of intern. Er zijn tegenstrijdige inzichten over de rol van antilichamen tegen schistosoom glycanen tijdens infectie. Sommige studies suggereren dat antilichamen tegen glycanen een rookgordijn vormen die de gastheer belemmeren om gevoelige peptide antigenen te herkennen, terwijl anderen laten zien dat antigene glycanen betrokken kunnen zijn bij het ontwikkelen van een beschermende reactie. Deze tegenstrijdige inzichten kunnen het resultaat zijn van het generaliseren van de vele types glycanen en de vele verschillende structuren die *Schistosoma* tot expressie brengt en/of het type antilichamen dat daartegen wordt opgewekt. Gelukkig hebben recente technologische vorderingen het mogelijk gemaakt om de verschillende natuurlijk voorkomende complexe schistosoom glycanen van elkaar te scheiden en te analyseren, om deze vervolgens als microarrays te kunnen printen. Deze arrays kunnen vervolgens gebruikt worden om de bindingspartners van glycanen, zoals antilichamen, op een efficiënte en snelle manier te kunnen karakteriseren. De lijst met glycanen die met microarray geanalyseerd kunnen worden kan nog verder worden aangevuld met synthetische glycanen en glycaan deelstructuren. Deze bieden tevens de mogelijkheid tot vaccinatiestudies in dieren.

Het doel van dit proefschrift is om de kennis over anti-glycaan antilichamen tijdens

een *Schistosoma* infectie te vergroten. In hoofdstuk 2 en 3 hebben we glycaan microarrays gebruikt om IgG en IgM tijdens natuurlijk verworven en door vaccinatie geïnduceerde immuniteit te bestuderen in, respectievelijk, resusapen en bavianen. In hoofdstuk 4 hebben we ons gericht op de IgG subklassen tegen schistosoom glycanen in geïnfecteerde individuen die in een endemisch gebied wonen. Tenslotte beschrijft hoofdstuk 5 een immunisatie en infectie experiment waarbij ratten geïmmuniseerd zijn met gedefinieerde synthetische glycoconjugaten. Een beter begrip van anti-glycaan antilichamen tijdens infectie en de ontwikkeling van resistentie tegen schistosomen kan leiden tot betere diagnostische methodes en het identificeren van mogelijke vaccin kandidaten.

Studies beschreven in dit proefschrift

In **hoofdstuk 2** werden serum antilichamen bestudeerd tijdens het ontwikkelen van resistentie tegen schistosomen in resusapen gedurende 22 weken. In tegenstelling tot mensen zijn resusapen in staat om *Schistosoma* wormen op een natuurlijke manier kwijt te raken na infectie. Bovendien ontwikkelen zij na ongeveer 12 weken immuniteit tegen herinfectie met *Schistosoma*. We hebben gevonden dat resusapen na experimentele infectie reageren op *Schistosoma* glycanen met de productie van IgM antilichamen, en dat dit ongeveer 2-4 weken later gevolgd wordt door IgG. Tijdens de infectie bleken de IgM reacties niet lang te duren terwijl IgG reacties tegen specifieke glycanen (O-glycanen met gefucosyleerde LDN motieven) sterk bleven tijdens de klaring van de wormen. In het algemeen geven glycanen met een hogere graad van fucosylering een sterkere antilichaam reactie dan glycanen met minder of geen fucoses. Wanneer serum van geïnfecteerde resusapen *in vitro* wordt toegevoegd aan schistosomula gaat een deel van de schistosomula hierdoor dood. Het aantal schistosomula dat dood gaat is positief geassocieerd met de duur van infectie in de resusapen. Dit suggereert dat er mogelijk beschermende antilichamen aanwezig zijn in deze sera. De bevindingen in dit proefschrift laten zien dat de IgG antilichamen gericht tegen sterk gefucosyleerde LDN motieven, die aanwezig blijven nadat de wormen uiteenvallen, mogelijk geassocieerd zijn met de klaring van de infectie en bescherming tegen herinfectie in resusapen.

In **hoofdstuk 3** hebben we de antilichamen tegen glycanen bestudeerd in bavianen die weerstand tegen schistosomen ontwikkelen na vaccinatie met door bestraling verzwakte cercariën. We hebben gevonden dat zowel IgG als IgM vooral gericht waren tegen glycaan structuren met multi-gefucosyleerde GalNAc/GlcNAc motieven op cercariële O-glycanen en glycosfingolipiden. Terwijl IgM tegen deze antigene glycaan structuren weer afnam na 2 vaccinaties, zorgden de 5 vaccinaties voor een steeds verdere toename van IgG tegen diezelfde glycanen. Op het moment van infectie, 19 weken na de

eerste vaccinatie, was de IgG reactie tegen deze multi-gefucosyleerde glycaanmotieven op O-glycanen en glycosfingolipiden het sterktst, en die bleef sterk tot 6 weken na infectie. Deze bevindingen ondersteunen de hypothese dat anti-glycaan IgG antilichamen belangrijk zijn bij immuniteit tegen *Schistosoma* infectie in bavianen die met bestraalde cercariën zijn gevaccineerd.

Bepaalde IgG subklassen zijn gecorreleerd aan weerstand tegen of juist gevoeligheid voor een *Schistosoma* infectie. In **hoofdstuk 4** zijn de IgG subklassen van 41 geïnfecteerde individuen met vergelijkbare intensiteit van infectie geanalyseerd met behulp van glycaan microarrays. We hebben gevonden dat antigene glycaanmotieven, zoals multi-gefucosyleerd LDN, door alle IgG subklassen herkend kunnen worden en waarbij IgG1 en IgG2 overheersten. Wanneer gekeken werd naar de individuele reacties tegen N-glycanen bleek dat IgG2 en IgG4, indien deze aantoonbaar aanwezig waren, specifiek gericht waren tegen α3-fucose en xylose motieven op de N-glycaan basisstructuur. IgG1 en IgG3, daarentegen, waren gericht tegen een bredere reeks van N-glycaan structuren. Het is de moeite waard om te vermelden dat er grote variatie tussen individuen is gevonden in de antilichamen tegen N-glycaan basisstructuren die schistomen tot expressie brengen. Deze studie is de eerste die IgG subklasse reacties tegen glycanen laat zien gedurende een infectie met een parasitaire worm.

In **hoofdstuk 5** is een vaccinatie studie beschreven waarin wordt getest of antigene glycaanmotieven die herkend worden door het immuunsysteem van de geïnfecteerde gastheer gebruikt kunnen worden om bescherming tegen schistosomiasis te verkrijgen. We hebben gevonden dat het aantal wormen dat zich ontwikkeld na infectie met cercariën significant verlaagd was in ratten die geïmmuniseerd waren met een enkel-gefucosyleerd GlcNAc dat geconjugeerd was aan BSA (FGn-BSA) ten opzichte van ratten die alleen gevaccineerd waren met BSA of met dubbel gefucosyleerd GlcNAc geconjugeerd aan BSA (FFGn-BSA). De bescherming die in ratten geïnduceerd werd door 3 opeenvolgende vaccinaties met FGn-BSA was 38% en 29% in twee onafhankelijk uitgevoerde experimenten. Antilichamen die opgewekt waren door immunisatie met FGn-BSA bleken in staat om natieve glycanen uit schistosomen te herkennen, met name de glycosfingolipide glycanen die een gefucosyleerd GalNAc of GlcNAc motief op het uiteinde bevatten. Bovendien waren antilichamen in sera van met FGn-BSA geïmmuniseerde ratten in staat om aan het gehele oppervlak van 3 uur oude schistosomula te binden, wat de aanwezigheid van glycaan epitopen op schistosomula bevestigt. Dit geeft aan dat antilichaam-afhankelijke mechanismen betrokken kunnen zijn bij de immuniteit tegen zich ontwikkelende larven wat leidt tot bescherming zoals we die gezien hebben in FGn-BSA geïmmuniseerde ratten.

Conclusie

De studies die beschreven staan in dit proefschrift laten de diversiteit en complexiteit van anti-glycaan antilichamen tijdens een *Schistosoma* infectie zien. Individuele glycaanmotieven zijn verschillend in hun antigeniciteit en antilichamen tegen bepaalde glycanen ondergaan snelle veranderingen gedurende infectie. Door individuele schistosoom *Schistosoma* glycanen en bijbehorende antilichamen te evalueren hebben we nieuwe inzichten verkregen in verschillende anti-glycaan antilichaam reacties die gegenereerd worden in de context van vaccinatie en infectie met *Schistosoma*.

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Curriculum vitae

Ya-Yi Michelle Yang was born on 03 February 1988. She is Taiwanese-born Canadian and spent her childhood in Taipei, Taiwan and Mississauga, Canada. At the age of twelve, she moved with her family to Rotterdam, the Netherlands, where she completed her secondary education at Rotterdam International Secondary School. She graduated with a Bachelor of Science (B.Sc.) in Liberal Arts and Sciences from University College Utrecht in 2010, and obtained her Master of Science (M.Sc.) in Biotechnology in 2012 from Wageningen University and Research Centre (WUR). During her masters, she worked as an intern at Genmab B.V., Utrecht, where she was involved in designing novel antibody formats for improving the efficacy of antibody therapy for the treatment of Leukemia. The internship sparked her curiosity for antibodies and in 2013, she started her PhD project on investigating anti-glycan antibody responses in schistosomiasis under the supervision of Prof. Dr. Cornelis Hokke and Dr. Angela van Diepen. The results of the research performed during Michelle's PhD trajectory are described in this thesis.

List of publications

Y.Y. Michelle Yang, R. Alan Wilson, Steffan R.L. Thomas, Thomas M. Kariuki, Angela van Diepen and Cornelis H. Hokke. Micro array-assisted analysis of anti-schistosome glycan antibodies elicited by protective vaccination with irradiated cercariae. J Infect Dis. **2019**; 219(10):1671-1680

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