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Understanding the Immune Response to Glycans of *Schistosoma mansoni*

By

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Graduate Division of Biological and Biomedical Science
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An abstract of
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## Abstract

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Glycans are ubiquitous at the interface between pathogens and host immunity, both as stimulators of innate immunity and targets of adaptive immunity. Schistosomiasis, caused by infection with parasitic helminthes of the *Schistosoma* spp., is a Neglected Tropical Disease that poses an enormous public health burden to the global community, and which features a prominent anti-glycan antibody response. The glycans of this parasite could provide a wealth of possible diagnostic and vaccine targets; however, whether anti-glycan antibodies play a protective role in this infection is not known, and there are no precedents for design of a vaccine targeting eukaryotic glycans. We sought to better understand how glycan antigens similar to those of *S. mansoni* elicit immunity by characterizing the immune response to differentially-linked neo-glycoconjugates (LNnT-BSA) and recombinantly-engineered, poly-LDNF producing cells. We then explored the role of anti-glycan antibodies in schistosomiasis by comparing the anti-glycan reactivity of protected with non-protected animal hosts, children from endemic areas, and resistant and susceptible adults on glycan microarrays. These studies demonstrated the importance of mode of presentation for glycan antigens in immunization, the success of recombinantly-engineered cells as a novel platform for presenting glycan antigens, the exquisite specificity of anti-glycan antibodies, and the extremely complex and still enigmatic relationship of anti-glycan antibodies with schistosomiasis resistance in animals and humans. This work should encourage and inform future studies on methods of incorporating glycans into vaccines and on the role of anti-glycan antibodies in schistosomiasis and other helminth infections.

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**Abbreviations List**

AA MΦ – (alternatively activated) macrophage

AAL - *Aleuria aurantia* lectin

ADCC – antibody-dependent cell-mediated cytotoxicity

Adult - adult worm lysate

AEAB - 2-amino-N-(2-aminoethyl)-benzamide

αGAb – anti-glycan antibody

APC – antigen presenting cell

ASC – antibody-secreting cell

β4-GalT - β4-Galatosyl transferase

B4GALNT – or β4-GalNAcT - β1-4N-acetylgalactosaminyltransferase

CAA – circulating anodic antigen

CCA – circulating cathodic antigen

CDC – Centers for Disease Control

Cer – ceramide

Cerc. – *S. mansoni* cercarial lysate

CF – N-glycan core α3 Fucose (Fucα1-3GlcNAc)

CFG – consortium for functional glycomics

CHO – Chinese Hamster Ovary cells

CLR – c-type lectin receptor

ConA - concanavalin A

CS – chondroitin sulfate

CX - N-glycan core xylose (Xylβ1-2GlcNAc)

DALYs - disability-adjusted life-years

DC – dendritic cell

DC-SIGN - Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin

DHB – 2,5-Dihydroxybenzoic acid

DMSO – dimethyl sulfoxide

DSA - Defined schistosome-type glycan microarray

ELISA – enzyme-linked immuno assay

ESP – egg-secreted proteins

Exp. - film-exposure time

F-LDN - Fucα1-3GalNAcβ1-4GlcNAc

FucT – fucosyltranserase

FUT6 - human α1-3 fucosyltransferase 6

FUT9 - human α1,3-fucosyltransferase 9

GAG – glycosaminoglycan

GalNAc – N-acetyl galactosamine

GlcA – glucuronic acid

GlcNAc – N-Acetyl glucosamine

GSL-II - *Griffonia simplicifolia* lectin II

HRP – horseradish peroxidase

HS – heparin sulfate

IFNγ – Interferon γ

IL-1 – Interleukin 1

IL-10 – interleukin 10

IL-4 – interleukin 4

IL-6 – Interleukin 6

Iso - isotype control antibody

Kif - kifunensine

KLH – Keyhole limpet hemocyanin

L8-GT - Lec8-GalNAcT cells

L8-GTFT- Lec8-GalNAc-FucT cells

LDN – LacdiNAc (GalNAcβ1-4GlcNAc)

LDN-DF - GalNAcβ1-4(Fucα1-2Fucα1-3)GlcNAc

LDNF – fucosylated LacdiNAc (GalNAcβ1-4(Fucα1-3)GlcNAc)

$Le^X$ – Lewis X (Galβ1-4(Fucα1-3)GlcNAc)

LN – LacNAc, *N*-acetyllactosamine (Galβ1-4GlcNAc)

LN – N-acetyl lactosamine, Galβ1-4GlcNAc

LNFPIII – Lacto-N-fucopentaose III (Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc)

LNnT - lacto-N-neo-tetraose (Galβ1-4GlcNAcβ1-3Galβ1-4Glc)

LPS – lipopolysaccharide

MAGS - metadata-assisted glycan sequencing

MALDI-TOF - Matrix-assisted laser desorption/ionization (time of flight)

MDA – mass drug administration

MGL – macrophage galactose-binding lectin

MHC - Major histocompatibility complex

MR – mannose receptor

MS – mass spectrometry

PG – proteoglycan

PNPA - *p*-nitrophenyl anthranilate

POC- point-of-care

Poly-LN – poly-N-acetyllactosamine

PR – putatively resistant

PS – polysaccharide

RFUs - relative fluorescence units

RP – reflectron positive ion mode

SA - streptavidin

SEA – soluble egg extract

Som. - schistosomula lysate

stds - molecular weight standards

SWAP – soluble worm antigen

TACA – tumor-associated carbohydrate antigens

TFA – trifluoroacetic acid

TGF-β - transforming growth factor β

TLR – Toll-like receptor

Tn – O-linked (Ser/Thr) α-GalNAc

TNF – tumor necrosis factor

TSP – tetraspanin

UCP-LF - up-converting phosphor lateral-flow assay

UNDP – United Nations Development Programme

VAL antigens - venom allergen Ancylostoma-secreted protein-like antigens

WFA - *Wisteria floribunda* agglutinin

WHO – World Health Organization

# Chapter 1. Introduction

*Large portions of this chapter have been derived from works originally published in Frontiers in Immunology* [1] *and Frontiers in Genetics* [2]

Nina Salinger Prasanphanich, Megan Mickum, Jamie Heimburg-Molinaro, Kristoffer Leon and Richard D. Cummings

## 1.1 The burden of schistosomiasis and areas of need

### 1.1.1 Global burden of helminthiases and schistosomiasis

Helminths are multicellular parasitic worms that comprise a major class of human pathogens. They rely on a vertebrate host species to complete a portion of their life cycle, which results in significant morbidity for human and animal hosts. The three classes of helminths – nematodes, trematodes and cestodes - account for half of the WHO-designated "Neglected Tropical Diseases," and infect 1-2 billion of the world's poorest people, with soil-transmitted helminths (gastrointestinal nematodes including *Ascaris, Trichuris, Necator sp.*) and schistosomes (blood-dwelling trematodes) being the most common [3]–[5]. Although great strides have been made through implementation of chemotherapy and improved sanitation, significant suffering due to helminth infections persists, and to date, no vaccines for helminths or any human parasite exist.

Schistosomiasis, caused by infection with trematodes of the genus *Schistosoma*, is a major global health burden due to inadequate treatment coverage and lack of a vaccine. More than 240 million people are infected and, in sub-Saharan Africa alone, 280,000 people per year die of schistosomiasis [6], [7]. The symptoms of infection result from the immune response to egg-laying, and therefore depend on infection intensity (i.e. number of worms and/or eggs). Without chemotherapy, 5-10% of those infected develop life-threatening manifestations such as portal hypertension, but much greater proportions, especially among children, display milder symptoms such as bloody stool, anemia, malnutrition and delayed physical/cognitive

development [3], [6], [8]–[11]. With these milder impairments taken into account, some researchers report that the burden of schistosomiasis rivals HIV and malaria in DALYs (years of life and productive life lost due to disability and/or death) [3], [10], [12]–[14]. In light of the socioeconomic impact of this disease in otherwise poverty-stricken areas, and the comparative dearth of funding for research and control, schistosomiasis is designated a Neglected Tropical Disease, and a true "societal poverty trap." [5], [14]–[16].

### 1.1.2 Need for innovation to reduce the burden of schistosomiasis

Treatment of schistosomiasis currently relies on the chemotherapeutic praziquantel [13], [17]. Prevalence in some areas is so high that mass drug administration (MDA) has been implemented for school-age children. Chemotherapy significantly decreases worm burden and morbidity, and is a vital aspect of the schistosomiasis control strategy [18]–[20]. However, donations of praziquantel are currently meeting only 5-10% global need for schistosomiasis [3], [15]. Additionally, the effectiveness of praziquantel varies depending on the worm life-stage. Single-dose cure rates for praziquantel range from 52-95% depending on the study, so this drug does not interrupt the chain of transmission [21], [22]. Due to suboptimal coverage rates, variable efficacy, animal reservoirs of disease, and frequent re-infections in children, global elimination using only current control methods is unlikely [3], [5], [12], [15], [23].

Development of worm resistance to praziquantel, especially in the face of MDA, has long been of concern. Resistant schistosomes can be generated in the lab, and reduced susceptibility to praziquantel has been reported in some human schistosomiasis-endemic areas [24], [25]. Only one new anti-helminthic, tribendimidine, has become available in the last thirty years [26], [27]. However, its mechanism of action is similar to two existing anti-helminthics, and little research is taking place to discover novel mechanisms and drug targets [13], [28]. Clearly, vaccines that expedite the development of immunity are a much-needed intervention in control of schistosomiasis.

Animal models of vaccine-induced immunity have used attenuated cercariae or other complex worm products. Due to the difficulty of maintaining a complex life cycle in large scale, and the danger associated with manufacturing this type of vaccine, these are unlikely to provide a practical vaccine. Thus, modern vaccine development to schistosomes has focused on recombinant proteins. In the mid-1990s, six *Schistosoma mansoni* proteins, studied in various labs, were chosen by the WHO to undergo independent laboratory testing. None of these reached the required 40% effectiveness required to move past animal testing [29], [30]. Two candidates have more recently reached the clinical phase. Bilhvax (Sh28-GST) has progressed through phase I, II and III trials, however, there has been a more than ten-year delay in publishing the results [31]. Another candidate vaccine, Sm14, may enter clinical trials this year, and at least two more candidates are progressing through the pre-clinical pipeline [32]. Other studies have likewise highlighted the difficulty of identifying effective targets and inducing the proper character of immune response for helminth vaccines [14], [33], [34].

The effectiveness of disease control measures is also dependent upon effective measures of disease prevalence, transmission, and morbidity. Traditional diagnostic methods are laborious and insufficient to detect low level infection or track variations in worm burden [35], [36]. The "gold standard" for diagnosis of schistosomiasis continues to be microscopic examination of stool or urine samples for eggs. However, eggs are not consistently shed into feces and urine. Despite recent improvements in the sensitivity and ease-of-use for stool tests, false negatives are still common, the extent of disease in an individual cannot be accurately ascertained just by egg counts, and eggs of different helminth species endemic in the same area can sometimes be difficult to differentiate [17], [23], [35], [36].

### 1.1.3 Why study anti-glycan immune responses to schistosomes?

Clearly, there is an extreme and immediate need for innovations in the control, prevention, and diagnosis of schistosome infection. While a substantial body of literature on the

biology and immunology of helminth infection exists, the science has yet to translate into more sophisticated solutions. This stems from a poor understanding of protective immunological mechanisms, insufficient knowledge of unique molecular structures of helminths, and a lack of innovative vaccine strategies to protect against complex, multicellular pathogens. The complex carbohydrates of helminths present an exciting opportunity to fill these gaps. Helminths synthesize unique glycan structures within glycoproteins and glycolipids, many of which are unique to helminths or to a particular worm species. They are abundant on worm surfaces and secretions, and humans and other animals target these glycans in the natural immune response. Helminth glycans also have potent immunomodulatory effects. Advances in glyco-technology have steadily increased our ability to understand this often-overlooked area of host-pathogen interactions.

The remainder of this chapter will review pertinent aspects of schistosome immunobiology (Section 1.2), describe the glycan structures of the parasite (Section 1.3.1), summarize current knowledge about the role of such structures in the innate (Section 1.3.3) and adaptive (Section 1.3.4) immune response to the parasite, and highlight areas of research in control, diagnostics and prevention which have recently benefitted and will continue to benefit from improved understanding of the immuno-glycobiology of schistosomes (Sections 1.4 and 1.5).

## 1.2 The interface of schistosomes and their hosts

### 1.2.1 *S. mansoni* life cycle and biology

Schistosomes require both a mammalian and a snail host to complete their life cycle (Figure 1.1). *S. mansoni*, the most common cause of schistosomiasis, lives only in fresh water inhabited by the mollusk host *Biomphalaria glabrata*. Its eggs hatch into miracidia, which penetrate susceptible snails. The miracidia circulate in the snail hemolymph and transform into sporocysts, which over the course of about a month generate free-swimming cercariae that exit

the snail [37], [38]. Cercariae penetrate the skin of a human host or other mammals exposed to water harboring infected snails. In the process, they are transformed into schistosomula (larvae), which, after a few days in the dermis, make their way into the venous circulation. Within 1-3 weeks they traverse the narrow pulmonary capillary beds and move to the portal vessels, feeding on blood and growing in size as they move. About five to six weeks after initial infection, male and female worm pairs mate and migrate up the mesenteric vein, where they commence egg laying. Eggs excreted into the stool continue the life cycle if they are deposited back into fresh water, while eggs trapped in the intestinal walls and liver contribute to pathogenesis. [39]. Other mammals, including rodents, non-human primates and bovines, are also infected by schistosomes and can serve as reservoirs of transmission [40]. Adult schistosomes can survive for 6-10 years in a human host, continuously producing eggs [41].

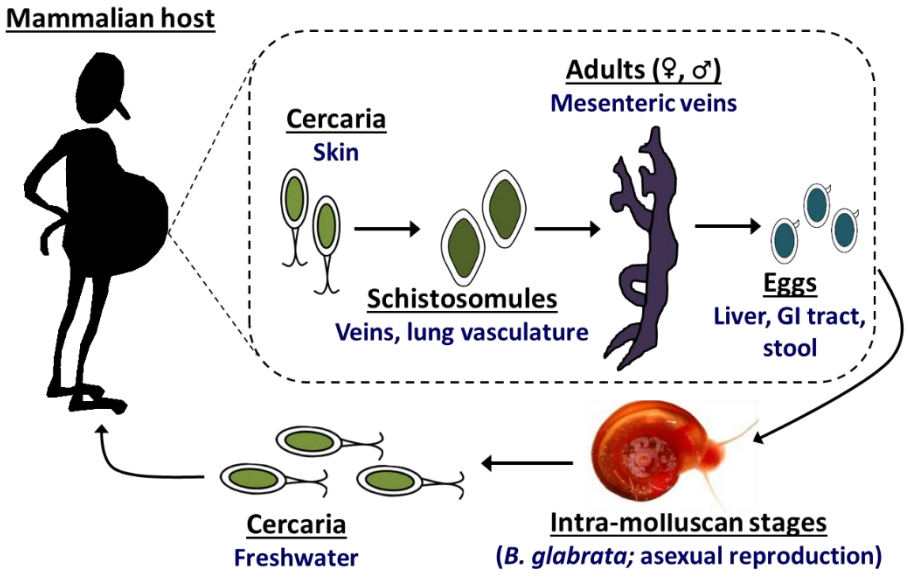



**Figure 1.1 *S. mansoni* life cycle.**

The physical interface of the schistosome and its mammalian host is complex, incompletely understood and variable throughout its life stages. The outer layer consists of a tegument, syncytial layer of cells which are bounded apically by a complex invaginated membrane [42]. The tegument is comprised of secreted lipid-rich “membranocalyx,” as well as

"glycocalyx," the latter of which is partially discarded upon transformation of cercariae to schistosomula, but also appears to be prominent on the surface of adult worms [43]–[46]. The expression of both proteins and glycans (notably, fucose-containing glycans) is regulated from one life stage to the next, and highly variable [47]–[54]. The surface of the worms as well as excreted and secreted products, molted tissue layers, and eggs make up the targets for immune recognition and attack. The GI tract of blood-feeding worms like schistosomes is also exposed to antibodies [55], [56].

### 1.2.2 *S. mansoni* immunology

Mammalian adaptive and innate immune responses to parasitic helminths are incredibly complex. In some mammalian hosts, the adaptive immune response may help to prevent, limit, or eradicate the infection. In others, inflammation may escalate with catastrophic results for the health of the host, all while parasites continue to thrive [57]. The immune response to schistosomiasis could be viewed as having three facets: 1) enabling or directly contributing to the destruction of worms; 2) responding to inflammatory parasite molecules which lead to the pathology of the infection; and 3) responding to immunomodulatory parasite molecules which serve to limit the pathology of the infection. Section 1.2.2.1 will discuss how innate immunity contributes to 2) and 3), while Section 1.2.2.2 will examine the adaptive mechanisms involved in 1).

#### 1.2.2.1 Inflammation and Immunomodulation in Schistosomiasis

The classical picture of the immune response in schistosomiasis emerged from mice, where the early stages of infection induce a Th1-type immune response, which shifts to a Th2-type response upon egg-laying. Travelers from non-endemic regions similarly develop a Th1-type acute syndrome (Katayama fever) characterized by malaise, cough, abdominal pain, eosinophilia and high levels of IL-1, IL-6, IFN-γ, TNF-α and immune complexes [39], [58]. However, people in endemic regions rarely suffer acute symptoms. Instead, they seem to be pre-disposed to

developing a chronic Th2-type response with sub-clinical manifestations [57], [59], [60]. This may stem from sensitization *in utero* or very early in life [61]. Chronic low-level inflammation can lead to anemia, malnutrition and reduced cognitive and physical development in children who are continually re-infected throughout their lives [8], [62]. Without praziquantel treatment, a minority of infected individuals develop chronic granulomatous inflammation so severe it could lead to fatal portal hypertension, but many more suffer the sub-clinical manifestations [10].

The immune response to chronic infection is dominated by a self-reinforcing Th2 feedback loop between cytokines IL-4, IL-5, IL-13 and prominent expansion of eosinophils and mast cells [14], [57]. Abundant antibodies of all subtypes are produced, especially IgE, IgG1 and IgG4 [56], [59], [63]. The immune-pathology centers around eosinophilic (type 2) granulomas, consisting of macrophages, CD4+ T cells, eosinophils and collagen that surround eggs trapped in liver, intestines (*S. mansoni*, *S. japonicum*) or bladder (*S. haematobium*) tissue, which are eventually converted to fibrotic scars [39], [64].

Antigen-presenting cells (APC) including dendritic cells (DC) and macrophages (MΦ) initially encounter invading pathogens and are crucial for regulation of the type of adaptive immune response [65], [66]. Immunomodulatory molecules in schistosome eggs induce effector cell generation consisting of Th2, T regulatory cells (Tregs) and alternatively activated (AA) MΦ [67]–[70]. Pathogen recognition is mediated by two classes of specialized pattern recognition receptors on APC, the Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), which are instrumental in regulation of adaptive immunity [71]–[73]. The balance between CLR- and TLR-mediated signals appears to determine the balance between tolerance and immunity [71], [74], [75].

The regulatory response is crucial in control of chronic disease, for the well-being of both host and parasite. Schistosomes promote the development of Tregs, production of regulatory cytokines like IL-10 and TGF-β from multiple cell types, and IgG4, a non-complement fixing isotype. This type of response, collectively termed “modified Th2,” serves to limit the

immunopathology that would result from an uncontrolled Th2 amplification-loop, and allows the host to remain relatively healthy for the long duration of helminth infection [57], [76], [77]. In concordance with this idea, schistosomiasis patients with chronic liver and spleen inflammation lack the IL-10 response to worm antigens, which is observed in chronic patients with low-level symptoms [59]. AA-MΦ, which can be induced by Th2 cytokines as well as directly by products of schistosomes, also aid in limiting worm-induced immunopathology [69], [78]–[81].

As we will discuss in section 1.3.3, schistosome glycoconjugates are instrumental in the initiation of the Th2 and immunoregulatory responses described above.

1.2.2.2 Mechanisms of parasite destruction and correlates of protection from schistosomiasis

Both animal models as well as human studies have demonstrated that some effector mechanisms of the adaptive immune response are successful in combatting helminths. Brown rats (*Rattus norvegicus*) exposed to *S. mansoni* exhibit no disease pathology and self-cure. A variety of components have been cited to contribute to this phenomenon, including complement fixation, IgG2a and IgE levels, mast cell degranulation and eosinophil-mediated antibody-dependent cellular cytotoxicity (ADCC) (see Section 4.1 for more detail). [82]–[87]. In rhesus macaques (*Macaca mulatta*), another protective model for schistosomiasis, adult worms become attenuated in the weeks after reaching patency. IgG-mediated complement killing of schistosomula and neutralization of adult worm enzymes have been demonstrated in this model [88]–[90]. Other animal models have shown that eosinophils, monocytes/macrophages and neutrophils can mediate *in vitro* ADCC of various helminth larvae including *S. mansoni*, *F. hepatica* and *S. stercoralis* [91]–[93].

In endemic human schistosomiasis cohorts, most adults acquire fewer infections and have lower infection intensity (egg output) compared to children [94]–[96]. Additionally, among adults with frequent exposures, some appear to develop greater resistance to re-infection than others [97], [98]. Natural partial immunity is thought to be developed in response to antigens released by dying worms, and hastened by multiple cycles of drug-treatment and re-infection [98]–[102].

Eosinophilia, high IgE levels to heterogeneous schistosomular and adult antigens, and high IgE/IgG4 ratios, are the most well-established correlates of human resistance to schistosomiasis, while IgG2, IgG4 and IgM (in some cases, against the same molecular species) are negatively correlated [103]–[107]. Antibodies to some specific antigens have also been correlated with resistance, such as IgA to the tegumental protein Sm28GST, IgE to Sm22 and TAL-5, and IgG1 and G3 to TSP-2 [101], [107]–[109].

Human eosinophils can kill schistosomula *in vitro* via IgG from infection antisera [110]–[112], however, there is no direct evidence that ADCC occurs during the course of human infection, and eosinophilia can also be accounted for by the presence of type 2 granulomas [113]. IgE also mediates mast cell degranulation, but mastocytosis was found to correlate with susceptibility to reinfection in one occupationally-exposed human cohort [114]. The negative correlation of IgM, IgG4 and IgG2 with human resistance to schistosomiasis has been attributed to their ability to block IgE and IgG-mediated effector mechanisms *in vitro* [104], [115]–[117]. Thus, the mechanisms that contribute directly to human resistance *in vivo* are poorly understood, and the factors stimulate skewing towards production of either protective or blocking antibodies, sometimes to the same targets, are unknown.

An alternative hypothesis for the association of IgE with protection from schistosomiasis has been formulated based on recent observations that $CD23^+$ B cells are associated with resistance in Kenyan cohorts of both children and occupationally-exposed adults [100], [118]. B cells bind parasite-specific IgE through CD23, the low-affinity IgE receptor, and upon encountering parasite antigen, are activated by IgE crosslinking to endocytose the antigen. This mechanism could enable a large population of B cells to present parasite epitopes to T cells, which would in turn stimulate cognate parasite-specific B cells to produce more antibody. The increasing amount of parasite-specific IgE could thus steadily increase the magnitude of the antibody response over the course of several exposures, outweighing the immunosuppressive effects (discussed in Section 1.2.2.1) of some worm products (to be discussed in Section 1.3.3)

[119]. Such a robust IgE, IgG1, IgG3 and IgA antibody response would perhaps then be capable of destroying larvae and/or adult worms through a combination of the mechanisms, as discussed above.

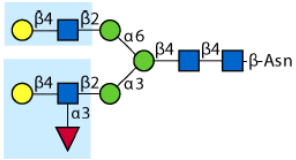
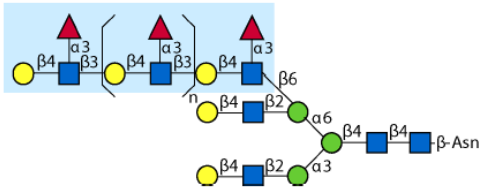
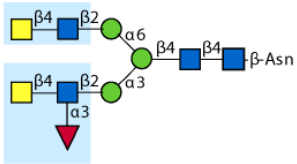
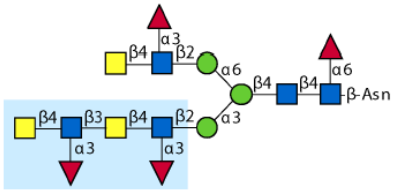
T cell-mediated immunity may also play a role in the defense against helminth infection. Mice repeatedly vaccinated with irradiated *S. mansoni* cercariae develop a high level of protection which has been attributed to both Th1 and Th2 mechanisms, including complement activation, $CD8^+$ T cell cytotoxicity against schistosomula, and T cells and macrophages that trap schistosomula as they migrate through the lung [120]–[123]. The protection of vaccinated mice as well as naturally-protected rats is dependent on both antibodies and T cells (see Section 4.1 for more detail) [124]–[127]. However, the role of Th1 responses in humans is still unclear. In some populations endemic for schistosomiasis, a mixed Th1/Th2 profile is associated with an effective immune response [128]. Resistance to schistosome infection is correlated with increased production of IFNγ by CD4+ T cells stimulated with recombinant Sm14 protein and other antigens [129], [130]. Polymorphisms in the IL-4 and IFNγ genes have also been associated with resistance [131]. Thus, while many possible *in vitro* and *in vivo* mechanisms against helminths have been described, in the absence of functional studies that truly represent the *in vivo* situation (in terms of parasite development and multi-pronged immune responses) it remains unclear whether these markers are contributors to or merely side effects of disease resistance, and which would be desirable in an anti-helminthic vaccine.

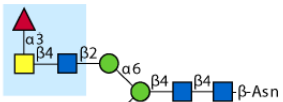
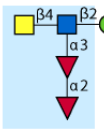
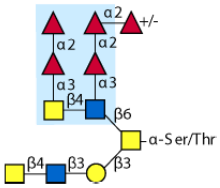
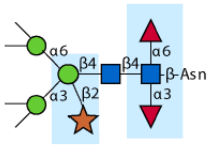
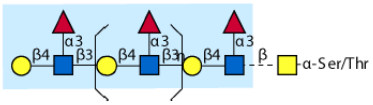
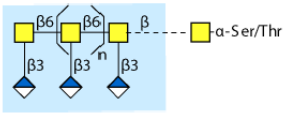
In summary, the immune response to schistosomes is incredibly complex due to the multicellular and highly organized tissue structure of the parasite, variation in glycan and protein antigens throughout its life stages, and remarkable immuno-modulatory abilities. For these reasons, a better understanding of the parasite's glycoconjugates – the major components of the host-parasite interface – is crucial to understanding its immunobiology, and these will be discussed in the next section.

## 1.3 Schistosome glycoconjugates in host-helminth interactions

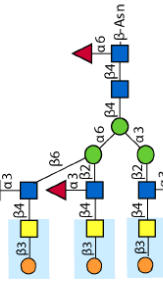
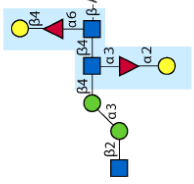
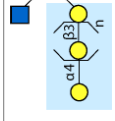
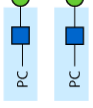
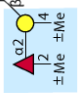
The glycans of schistosomes and other helminthes that have been found to interact with the innate and adaptive immune responses of their hosts are numerous, and beyond the scope of this chapter. They have been reviewed elsewhere [1], [132], [133] and are summarized in Table 1.1. We will now describe the structures characterizing schistosome glycoconjugates and then summarize knowledge on the innate (Section 1.3.3) and adaptive (Section 1.3.4) immune responses to select glycans of interest.

Galactose N-Acetylgalactosamine Glucose N-Acetylglucosamine Mannose Fucose Xylose Glucuronic acid Tyvelose Asn Asparagine Ser/Thr Serine/Threonine PC Phosphorylcholine Me Methyl

| Glycan | Structure | Species | Antigenic? | Receptors | Effects/functions | Reference |
|---|---|---|---|---|---|---|
| LN | | *S. mansoni** | No | | Granuloma induction | van de Vijver et al. (227) |
| Lewis x (Lex) |  | *S. mansoni** | Yes | DC-SIGN, MR (weak) | B cell proliferation, IL-10 production, TH2 bias, immunosuppressive, antibodies can mediate complement lysis of leukocytes | Srivatsan et al. (59), Velupillai and Harn (102), Nyame et al. (63), van Die et al. (117), Meevissen et al. (120) |
| Poly-Lex |  | *S. mansoni** | Yes | | | Srivatsan et al. (59), van Roon et al. (228), Mandalasi et al. (60) |
| LDN |  | *S. mansoni** | Yes | MGL; galectin-3 | Granuloma induction; possible molecular mimicry with snail host; antibodies to LDN lyse schistosomula *in vitro* | Srivatsan et al. (27), Neeleman et al. (28), Nyame et al. (122, 224), van den Berg et al. (100), van Vliet et al. (119), van de Vijver et al. (227), Meevissen et al. (120), Yoshino et al. (284) |
| LDNF | | *Schistosoma* spp.*, *H. contortus*, *T. spiralis* | Yes | DC-SIGN; MGL; MR; CD62E (E-selectin) | DC maturation; antibodies correlate with protection to *H. contortus* | Srivatsan et al. (27), Nyame et al. (223), van Die et al. (117), van Vliet et al. (119), van Liempt et al. (118), Meevissen et al. (120), van Stijn et al. (128) |
| Poly-LDNF |  | *S. mansoni* | Yes | DC-SIGN | | Kawar et al. (289), van Liempt et al. (118), Wuhrer et al. (48, 229) |

| Glycan | Structure | Species | Antigenic? | Receptors | Effects/functions | Reference |
|---|---|---|---|---|---|---|
| FLDN |  | *S. mansoni* | Yes | DC-SIGN | | Naus et al. (234), van Remoortere et al. (233), de Boer et al. (236), Meevissen et al. (120), Frank et al. (51) |
| LDN-DF |  | *S. mansoni* | Yes | | Stimulates IL-10, IL-6, and TNF-α production by PBMC | van der Kleij et al. (116), Naus et al. (234), Frank et al. (51), van Remoortere et al. (233) |
| FLDNF |  | *S. mansoni* | Yes | | Antibodies to FLDNF are protective in rats | Geyer et al. (50), Grzych et al. (171, 221, 222), Wuhrer et al. (46), Kantelhardt et al. (49) |
| DF-LDN-DF |  | *S. mansoni* | Yes | | Diagnostic in urine | Robijn et al. (243, 250) |
| Core β2 Xyl, core α3 Fuc |  | *S. mansoni* | Yes | | Th2 biasing of DCs | Faveeuw et al. (114), Meevissen et al. (120) |
| Circulating cathodic antigen (CCA) |  | *S. mansoni* | Yes | | Diagnostic (urine, sera) | Deelder et al. (65), van Dam et al. (245) |
| Circulating anodic antigen (CAA) |  | *S. mansoni* | Yes | | Diagnostic (urine, sera), forms antibody-antigen complexes | Deelder et al. (65), Vermeer et al. (244) |

*(Continued)*

| Glycan | Structure | Species | Antigenic? | Receptors | Effects/functions | Reference |
|---|---|---|---|---|---|---|
| Tyvelose |  | *T. spiralis* | Yes | | Antibodies to tyvelose are protective and diagnostic | Ellis et al. (30), Reason et al. (31), McVay et al. (215), Bolás-Fernandez and Corral Bezara (72) |
| Galα1-3GalNAc |  | *H. contortus* | Yes | | Antibodies to Galα1-3GalNAc are protective | van Stijn et al. (257) |
| Gal-Fuc |  | *C. elegans*, *A. suum* | Yes | Endogenous and fungal galectins; human Gal-1 | Fungal CGL2 kills *C. elegans* | Yan et al. (54), Butschi et al. (80), Takeuchi et al. (57, 58) |
| Galα1-4Galβ1-3GalNAc |  | *Echinococcus* spp. | Yes | | Diagnostic | Koizumi et al. (254), Diaz et al. (255) |
| PC-glycan |  | Filarial and GI nematodes, *E. granulosus* | Yes | | Anti-inflammatory (both Th1 and Th2); nematode development | Fletcher et al. (41), Peters et al. (217), Paschinger et al. (37), Rzepecka et al. (142), Grabitzki et al. (39) |
| Methylated Fuc/Gal |  | *Toxocara* spp. | Yes | | | Khoo et al. (43), Koizumi et al. (44) |
| PC-glycolipids |  | *Ascaris* spp. | Yes | | Inhibits LPS-induced B cell proliferation and macrophage, IL-12 release; induces PBMC to produce Th1 cytokines | Lochnit et al. (132), Deehan et al. (133), van Riet et al. (134) |

*Defined determinants (common names are indicated) recognized by either antibodies or glycan-binding proteins and lectins are indicated in the blue background boxes, along with the known helminths expressing the determinant. Note that many structures are composite examples, and that some complex and branched glycans may possess one or more of these determinants, such as outer branch LDN-DF and inner core Xyl and Fuc residues. The asterisk indicates glycan motifs that can also be made in mammalian hosts, but are not necessarily cross reactive, due to differences in surrounding structures. Their antigenicity is also noted if they have been confirmed as antibody targets, as well as any known receptors and demonstrated functions, in vitro or in vivo, with selected corresponding references.*

**Table 1.1 A selection of helminth glycans involved in innate and adaptive immunity.**

### 1.3.1 Structural features of schistosome glycoconjugates

To understand the nature of the immune responses to parasite glycoconjugates it is important to identify the unique and common aspects of their complex carbohydrates relative to their vertebrate hosts. Parasitic helminths are characterized by their production of many different glycoproteins and glycolipids, as would be expected from such a complex invertebrate. As eukaryotes, they share some features with their mammalian hosts, such as some similar core structures, but many features of their glycans are structurally and antigenically distinct (Figure 1.2). For example, sialic acids, common terminal sugars of mammalian glycans, have never been demonstrated as part of schistosome glycan motifs [102]–[104]. Helminth glycans commonly terminate with β-linked GalNAc [105]–[108], which is not commonly present in vertebrate glycans. Polyfucose modifications of glycans is observed in schistosomes [134] but not found so far in any vertebrate. In addition, many helminths use unusual sugars and unusual modifications of sugars, which may be useful in both resistance to infection and diagnostics [109]–[121].

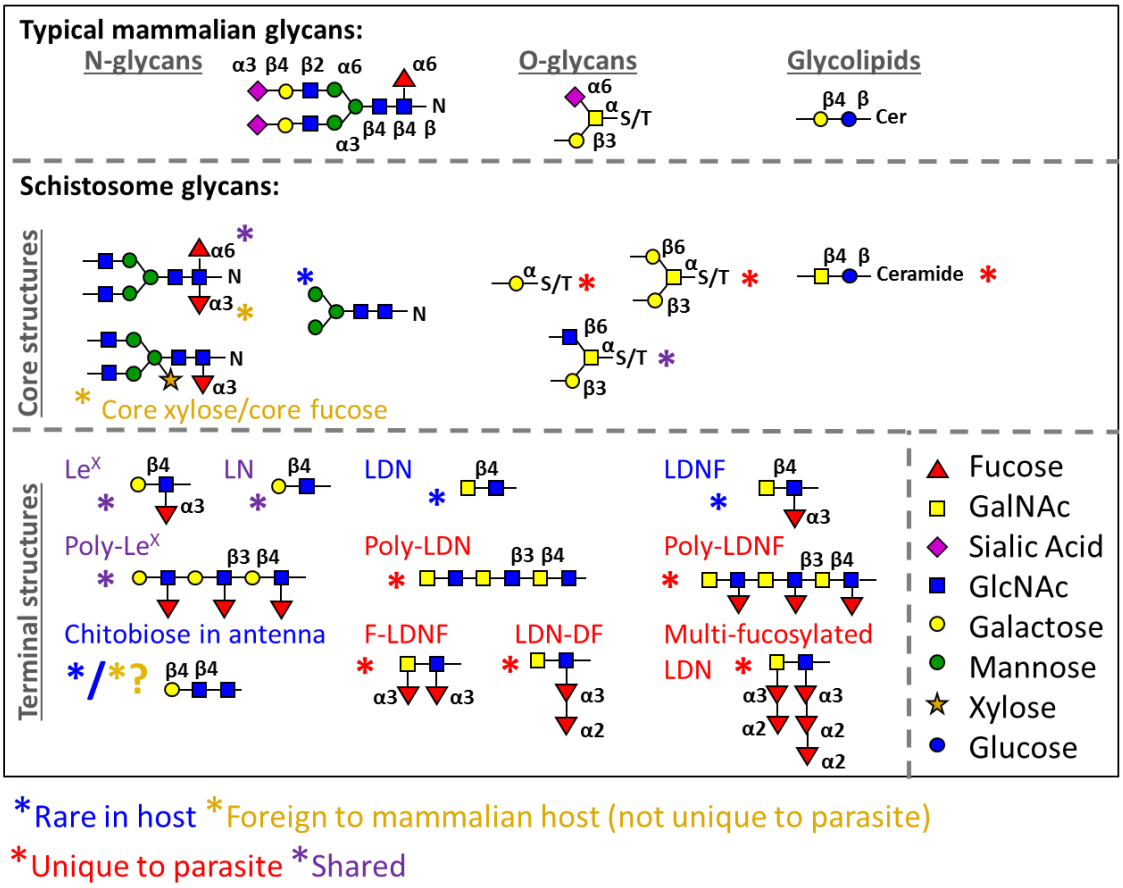



**Figure 1.2 Selected glycans of the glycoproteins and lipids of *S. mansoni***

Selected glycans of the glycoproteins and glycolipids of *S. mansoni* are shown, compared to a few structures commonly made by their mammalian hosts. Core (reducing end) structures are linked to lipid or protein. Terminal (non-reducing end) structures may occur on a variety of underlying core structures. Asterisks denote the level of similarity between the host and parasite structures.

#### 1.3.1.1 Terminal glycan structures

LacNAc (Galβ1-4GlcNAc; LN; *N*-acetyllactosamine) and LacdiNAc (GalNAcβ1-4GlcNAc; LDN) are common terminal modifications in *Schistosoma* glycoproteins. Mammalian glycan structures most frequently terminate in LN, which is usually modified through sialylation, fucosylation, sulfation [135]. Schistosomes more commonly terminate in the LDN motif, which is common in other invertebrates, but only generated on a handful of mammalian proteins [136]–[141]. Schistosomes express both the β4-GalT and β4-GalNAcT to generate LN and LDN,

respectively, which are developmentally regulated [142]. However, the other factors (such as acceptor specificity) that govern the differential expression of these two complementary glycosylation pathways are not known.

When fucose is linked α1-3 to the GlcNAc in the LN or LDN motifs, they are called $Le^x$ and LDNF, respectively [136] (Figure 1.2). Both $Le^X$ and LDNF have been documented on glycoproteins and glycolipids of all three major schistosome species [143]–[145]. $Le^X$ is also a common feature of mammalian glycosylation, although either the Gal or GlcNAc residues are often sulfated and the Gal residue is often sialylated [135]. Its expression in schistosomes appears to be limited to the intramammalian stages and is especially prominent in the adult worm gut [146]–[149]. Interestingly, only the trematode *S. mansoni* [147], [150], [151] and the cattle lungworm nematode *Dictyocaulus viviparous* [152] have been shown to synthesize glycans containing the terminal $Le^x$ motif among helminths. $Le^X$ is also one of the major secreted schistosome antigens, with repeats of the antigen making up the polysaccharide portion of circulating cathodic antigen (CCA) found in serum and urine [153]. LDNF appears to be expressed by all stages of schistosomes, most highly by eggs and the intramolluscan stages, and occurs in other helminthes as well [50], [145], [146], [148], [154], [155]. In contrast, expression of LDNF is highly restricted in mammals – in humans it has been identified in urokinase and glycodelin [156], [157].

Alpha-2- and α3-linked multifucosylated glycans are major constituents of a diverse group of immunologically important LDN derived epitopes. These epitopes contain unique linkages including polyfucose elements Fucα1-2Fucα1-3-R and the Fucα1-3GalNAc-motif generating F-LDN, F-LDN-F, LDN-DF and DF-LDN-DF variants [53], [134], [141], [150], [158]–[162]. These structures are not documented in any other parasitic or mammalian host species and induce high antibody responses in humans and primates [158], [163]–[165].

*S. mansoni,* like mammals, generates extended poly-N-acetyllactosamine (poly-LN) (Galβ1,4-GlcNAcβ1,3-Galβ1,4-GlcNAc; poly-LN) chains which can be further modified, most

notably in the form of poly-Lewis X (poly-$Le^X$) [150]. Poly-$Le^X$ has been demonstrated on N-glycans as well as on the secreted O-linked (possibly core 1 and/or core 2-linked) CCA [153], [166]. Unusually, *S. mansoni* is also able to form extended polymers of LacdiNAc (GalNAcβ1,4-GlcNAcβ1,3-GalNAcβ1,4-GlcNAc; poly-LDN) and fucosylated LacdiNAc (poly-LDNF) [161], [167]. This is the only naturally-occurring example of such a structure; however, cloning of *C. elegans* β1,4-GalNAcT and human α1,3-fucosyltransferase 9 into Chinese Hamster Ovary Lec8 cells resulted in poly-LDN and poly-LDNF on N-glycans [168]. An active β1,3-N-Acetylglucosaminyltransferase (β3GnT) in human serum has also demonstrated useful in chemo-enzymatic generation of both poly-$Le^X$ and poly-LDN extension activities on synthetic acceptors [169], [170]. These data indicate that the β3GnTs which normally generate poly-LN in mammals are likely able to perform the reaction with either β-linked Gal or GalNAc as an acceptor. This is hypothesized to be the case in schistosomes as well [161], although the regulatory factors that allow extension of LDN in schistosomes but not in mammals are unknown. A better understanding of the genetic basis of these polymeric antigens would be helpful as they are thought to be important antigenic targets, immunomodulators and, in the case of CCA, a validated diagnostic antigen [161], [171]–[175].

#### 1.3.1.2 Glycan core structures

The core structures of the glycolipids in helminths may also vary from those of mammals, such as the presence of the "schisto motif" GalNAcβ1-4Glcβ-Cer [176] of *S. mansoni*, instead of the mammalian "lacto motif" Galβ1-4Glcβ-Cer. Additionally, the chitobiose core (-GlcNAcβ1-4GlcNAcβ1-) in complex type N-glycans often contains a fucose residue that is α6-linked to the innermost GlcNAc residue and an additional non-mammalian α3-linked fucose [53], [141]. Such core modifications, especially α3-fucosylation, account for the interspecific immunological cross-reactivity observed among plant, insect, and helminth glycoproteins [53], [177], [178]. Core β1,2-xylose linked to the β-mannose of N-glycans is a common modification

of plant N-glycans and an important epitope of plant glycoprotein allergens [reviewed in 177]. β2-xylosylation was later identified in molluscs and then in *S. mansoni* and *S. japonicum* egg glycoproteins as well as *S. mansoni* cercariae in mass spectrometry studies [141], [180]. Several nematode and helminth species carry core β1,2-xylose, and it is variably expressed on glycoproteins in all of the intramammalian life stages of *S. mansoni*, with highest expression in cercariae and eggs [178], [181]. Data acquired by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) suggested that whereas in *S. japonicum*, core xylose and core α3- and α6- fucose can be contained on the same glycans, *S. mansoni* egg N-glycans contains either core xylose or core α3-fucose but not both [141].

#### 1.3.1.3 Glycosaminoglycans and other polysaccharides

Little is known about the glycosaminoglycan (GAG) or proteoglycan (PG) content of schistosomes. Two studies have isolated GAGs from schistosomes, demonstrating the presence of glycans resembling heparin/heparan sulfate (HS), chondroitin sulfate (CS) and hyaluronic acid [182], [183]. It has been hypothesized that heparin/heparan sulfate in the worm tegument could provide a mechanism of immune evasion by inhibiting the host clotting cascade; however, it has not been verified whether the GAGs isolated are from the parasite or the host and their structures have not been chemically defined [182].

The *Schistosoma* genomes indicate that much of the genetic machinery necessary for synthesizing GAGs is present. *S. mansoni*, *S. japonicum*, and *S. haematobium* all have genes homologous to the xylosyltransferase genes in mammals, mollusks, and nematodes which code for protein-O-xylosylation activity, which catalyzes the first step in addition of the HS/CS core to proteoglycans. The three *Schistosoma* genomes possess genes homologous to each of the other enzymes necessary for construction of the HS/CS core that have been characterized in *C. elegans*, and contain the relevant conserved domains [184].

Circulating anodic antigen (CAA) is another GAG-like, O-linked glycoprotein antigen excreted by schistosomes, which is also under investigation as a diagnostic target [185]. CAA is

completely unique among all previously identified glycan structures, consisting of the repeating trisaccharide (GalNAcβ1,6-(GlcAβ1,3)-GalNAcβ1,6-), although it has the same component monosaccharides also found in mammalian chondroitin sulfate, which is a repeating disaccharide containing GalNAc and GlcA in different linkages [166], [186], [187].

1.3.1.4 "Glycan Gimmickry"

The unusual nature and antigenicity of parasitic helminth glycans belies the apparently commonly held belief among immunologists and parasitologists that parasites do not express antigenic glycans, but rather cloak themselves in parasite-synthesized and/or host-acquired antigens to avoid immune recognition in what has been termed "molecular mimicry" or "antigen sharing" [188], [189]. This concept may no longer be tenable as a general description in regard to parasitic helminths, which synthesize few glycans resembling their vertebrate hosts. In fact, unusual non-mammalian glycans constitute a major portion of the host's antigenic targets in several helminth infections. In non-human primate models of schistosomiasis, they appear to be even more highly targeted than proteins [190]–[195]. When true molecular mimicry by infectious organisms does occur, such as the structural similarity between mammalian ganglioside GM1 and the terminal structure of the lipooligosaccharide from *Campylobacter jejuni*, the mimicry is associated with pathological autoimmunity, as seen in Guillain–Barré syndrome [196], [197]. Interestingly, few of the antibodies to helminth glycans cross-react with host glycans. The only described example of this is $Le^X$ [198], suggesting that even helminth glycans sharing some features with rare mammalian glycoconjugates, such as LDN and LDNF are presented in a unique fashion on parasites. As discussed below, results of multiple studies indicate that parasites instead utilize "glycan gimmickry" [138], in which their glycans can interact with host receptors to modulate host immune responses to the benefit of the parasite.

### 1.3.2 The role of glycans in schistosome biology

Glycans are critical for cell migration and adherence in a multitude of biological processes, from bacteria to humans. There appears to be a role for fucosylated carbohydrate epitopes expressed by larval and adult schistosomes in parasite evasion in intermediate and definitive hosts [149], [199], [200]. During invasion of the snail body, the miracidia penetrate the epithelium allowing for direct interaction of the snail tissues with the miracidial glycocalyx. The carbohydrate epitopes present on the surface of the miracidium during this time may be of prime importance during the invasion process. Recently, it was shown that *B. glabrata* synthesizes a broad battery of N-glycans on multiple glycoproteins comprising at least two carbohydrate determinants that cross-react with glycoconjugates from *S. mansoni* eggs [201]. Using a mass spectrometry approach for glycomic profiling, Hokke *et al.* found evidence for expression of multifucosylated, LDN-terminating di- and triantennary structures, as well as the presence of the truncated trimannosyl and core-xylosylated/core-α1,3-fucosylated N-glycans in miracidia [202]. Lehr *et al.* demonstrated the surface expression of FLDN, FLDNF, LDNF and LDN-DF in miracidia and the presence of these, as well as non-fucosylated LDN, and $Le^x$ glycans in secondary sporocysts [149], [201], [202]. α1,3-fucosylated LDN structures (FLDN, FLDNF, LDNF) are prominently expressed on the larval surface and amongst glycoproteins released during larval transformation and early sporocyst development. This stage-specific expression implies a role for these glycans in snail–schistosome interactions. Also, sharing of specific glycans FLDN and trimannosyl N-glycans with *B. glabrata* suggests an evolutionary convergence of carbohydrate expression between schistosomes and their snail host [149].

Helminths use specialized mechanisms to invade host organisms and establish a niche in their tissues for long-term survival or to enable passage of eggs out of the host. Helminth glycans may be involved in the establishment of such niches, and antibodies to glycans can interfere with this process. *S. mansoni* eggs must traverse the endothelium and intestinal wall in order to exit the

host via stool. Using *in vitro* models of egg attachment to human umbilical vein endothelial cells, antibodies to E-selectin and $Le^X$ were shown to decrease adhesion [203]. Whether the ability of αGAbs to interfere with host tissue interactions in the models is due to blockage of specific glycan-binding interaction or due to other neutralizing or physically damaging effects on the worms, is unclear. However, interference with invasion or adhesion through blocking surface glycans clearly represents an opportunity to induce protection and/or interfere with pathogenesis.

### 1.3.3 The role of glycans in innate immunity to schistosomiasis

Helminth glycoconjugates are intimately involved in the control of inflammatory and regulatory responses to the parasite. This topic has been reviewed elsewhere (reviewed in [57], [204]) and is summarized in Figure 1.3. In this section we will briefly discuss the history of this field as it relates to schistosomes and highlight a few discoveries of interest. A listing of several parasite glycans with known immunomodulatory roles is also presented in Table 1.1.

Little has been done on direct effects of intact worms on antigen presenting cells, but several studies using soluble extracts of worms or their eggs have demonstrated the importance of helminth glycans in immunomodulation. Early observations showed that egg deposition was responsible for the Th2 character of chronic murine schistosomiasis [205]. The Harn group followed up on these observations by showing that LNFPIII, a human milk sugar containing $Le^X$, induced B cell proliferation and IL-10 production by murine spleen cells [206]. They also demonstrated that intranasal administration of *S. mansoni* soluble egg extracts (SEA) to mice promoted IgE and IgG1 production and induced secretion of IL-4, IL-5 and IL-10, but not IFN-γ, by lymphocytes [207], [208]. These responses were completely dependent on the presence of intact helminth glycans, since treatment of glycans with periodate, a chemical treatment that specifically oxidizes monosaccharides with vicinal diols, abolished the ability of SEA to stimulate these Th2 responses [207]. Additionally, SEA inhibits LPS-induced secretion of many pro-inflammatory cytokines and chemokines from DC in a periodate-sensitive manner and

decreases the expression of co-stimulatory molecules during DC maturation [75], [207], [209], [210]. Some of the glycan determinants which contribute to the Th2-biasing effect of SEA are $Le^X$ and core fucosylated/xylosylated N-glycans [181], [207], [211]–[214].

There are over a dozen different C-type lectins expressed in DC and Langerhans cells, and many other glycan-binding proteins, such as selectins, siglecs, and galectins expressed by lymphocytes, all of which have potential to interact with parasite-derived glycans [215], [216]. Whereas TLRs recognize a wide variety of foreign molecular patterns, for example, the many variants of LPS, CLRs, which are still considered as potential receptors for pathogen-associated molecular patterns (PAMPs), interact with a more restricted set of glycans, for example, the dectin-1 receptor for β-glucan [217].

The unique abilities of helminth glycolipids to drive Th2 bias may also involve CD1d-restricted T cells [218]. Treatment of monocytes with *S. mansoni* egg glycolipids, but not adult worm glycolipids, stimulated IL-10, IL-6, and TNF-α production, which was largely dependent on expression of the LDN-DF motif, indicating that helminth glycolipids can induce both pro- and anti-inflammatory cytokine secretion [219]

With regard to the mechanisms of glycan recognition, several CLRs of DC and MΦ, such as DC-SIGN have been shown to bind selected glycans, such as $Le^x$, LDNF and on the defined glycan microarray from the Consortium for Functional Glycomics (CFG) and/or on the surface of recombinantly engineered cells poly-LDNF [220], [221]. Human MΦ galactose-type lectin (MGL), expressed as an Fc fusion protein, binds to a subset of glycans on the CFG microarray, with highest recognition of those containing terminal GalNAc residues [222]. Related studies using similar microarray approaches have also defined specific interactions of DC-SIGN, MR and MGL toward glycans containing $Le^x$ motifs, LDN, LDNF, as well as core β2Xyl [223]. MGL is selectively expressed on APC with elevated levels on tolerogenic DC and AA-MΦ [224], suggesting a role of MGL in the homeostatic control of adaptive immunity. This is consistent with earlier studies showing that DC-SIGN binds components within soluble egg antigens (SEA)

of *S. mansoni*, as do the CLRs mannose receptor (MR) and MGL [75]. SEA expresses many of the fucosylated glycans used in the microarray studies above. In particular, LDN, LDNF and $Le^x$ antigens are expressed on all intra-mammalian stages of the parasite with especially high expression in egg antigens [147], [148], [150], [174].

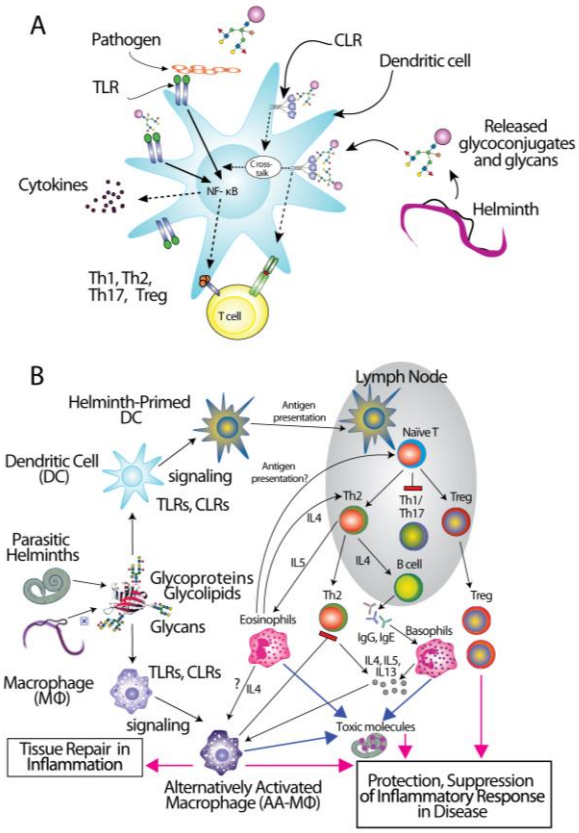



**Figure 1.3. Immunomodulation by helminth glycoconjugates**

The CLRs mentioned above induce endocytosis of bound molecules for antigen presentation but do not induce classical signs of APC activation. They do, however, modulate the gene transcription induced by other receptors, such as NF-κB signaling downstream of Toll-like receptors (TLRs) (Figure 1.3a) [225]. Interestingly, there is evidence that TLR4 may be involved in responses to *S. mansoni* $Le^x$-containing glycans [212], indicating interactions and co-signaling via TLRs and CLRs may contribute to the overall polarization of immunosuppressive responses

to the parasite infections. Recent studies in DC reveal the capacity of some CLRs to induce intracellular signaling cascades upon binding to pathogen-derived glycans, and show that CLR-induced signals intersect with the signaling pathways of several TLRs, including TLR2, TLR4 and TLR8. CLR signaling can "override" the response to a variety of otherwise pro-inflammatory TLR ligands such as LPS, instead inducing secretion of Th2-type or immunoregulatory cytokines, in a TLR-specific manner [210], [226]–[229]. In contrast, *S. mansoni* fucosylated glycolipids induce a pro-inflammatory response in DCs that is dependent on both DC-SIGN and TLR4 [230]. The specific signaling interactions which contribute to this diverse response modulation are still being explored. Novel roles for CLRs interacting with schistosome glycoconjugates have been suggested by a glycoform of RNAse termed omega-1 [231], where uptake by MR may contribute to RNAse internalization and impaired protein synthesis through degradation of both ribosomal and messenger RNA [232], but the mechanism of this effect is controversial.

The immunomodulatory properties of helminth glycans are relevant not only to the outcomes of helminth infections, but are also relevant to the outcomes of vaccinations, co-infections and inflammatory disorders. Helminth infections and their products have a phenomenal ability to ameliorate responses to a variety of inflammatory disorders [233]. For example, *S. mansoni*-infected asthmatics were shown to have lower disease severity compared to asthmatics in non-endemic areas [234]. The pathogenicity of some worms is mild enough that human trials testing the effect of helminth infection on autoimmune conditions have been conducted, and show promising results in some cases [235]. Although this cannot be tested for schistosomes, progress is being made in defining schistosome products that could be exploited for immunomodulation. LNFPIII (containing the $Le^X$ antigen) ameliorates experimental autoimmune encephalitis in mice [236]. Schistosome products have recently been shown to mediate re-activation of latent herpesviruses and modulate the immune response to HTLV-1-associated disease [237], [238]. All of these effects appear to be mediated by modulation of T-cells and/or macrophages to a more Th2 or regulatory phenotype. A better understanding of the specific moieties that mediate such

effects and their immunological mechanisms would allow us to channel the immunomodulatory properties of helminths into purer and more potent immunoregulatory therapies, with great potential for treating multiple inflammatory and infectious diseases.

### 1.3.4 The role of glycans and anti-glycan antibodies in adaptive immunity to schistosomiasis

Helminths produce an abundance of glycoconjugates that are a rich source of antigens for the immune system of their definitive hosts. For example, the *S. mansoni* cercarial glycocalyx, which is shed into the skin during penetration and some of which is retained on the parasite surface, is around 80% carbohydrate by weight [45], [239], [240]. In fact, the majority of the human and animal antibody response to schistosomes is directed to glycan antigens [241], [242]. Anti-glycan antibodies (αGAbs) are a common feature of helminth infections and are abundant in every schistosome-infected host that has been tested [144], [163], [164], [175], [194], [195], [243], [244]. This section will highlight the importance of αGAbs and address the continuing challenges to defining their role in schistosomiasis.

We and others have devoted considerable effort to defining the prominent glycan antigens in *S. mansoni* infection. These include Lewis X, LDN, LDNF, F-LDN, LDNF-DF, core α3 fucose, core xylose, and likely many yet undiscovered epitopes tested (Figure 1.2 and Table 1.1) [144], [163], [164], [175], [194], [195], [243], [244]. A variety of antibody isotypes and sub-isotypes are produced against schistosome glycans, including of IgG1, IgG2, IgG3 and IgM, as well as IgA [148], [163], [245]. Rat and mouse monoclonal antibodies have been developed against several of these epitopes [48], [146]. Anti-glycan IgE has not yet been found in infected humans or mice, thus far, but only a few glycans have been screened, using relatively low-sensitivity assays to probe for this scarce, but potentially crucial, antibody isotype [207], [243].

Most examinations of the anti-glycan response have focused on egg glycans, whereas the anti-glycan response to earlier intra-mammalian life stages may be more important for disease resistance, but is less well understood. A few recent studies have begun to examine this. The

Hokke group has generated multi-life stage shotgun glycan arrays and analyzed the IgG and IgM binding patterns from many infected children and adults [244]. Another group has analyzed the tissue-specific antibody responses of rats to migrating schistosomes on the Consortium for Functional Glycomics Array, showing that the anti-glycan response to lung-stage schistosomula is quite different from what is seen in the serum and spleen [246].

Antibodies generated by mammalian hosts to helminth glycans are not only abundant but highly specific. Schistosomes, for example, present the same glycan epitope in a variety of structural contexts, such as on N-glycans and O-glycans, or as single or multi-branched glycans. The structural presentation of such epitopes as $Le^x$ and LDNF can vary among schistosome life stages, localization and sexes [161], [167], [173]. Data from our lab and others have demonstrated that monoclonal antibodies and sera from infected hosts can discriminate against very similar epitopes, such as the monomeric, biantennary N-glycan, and multimeric forms of the $Le^x$ or LDNF trisaccharide epitopes [147], [173], [174]. Given that some of these structural variants are somewhat similar to mammalian glycans, this high level of specificity could be crucial to developing a parasite-*specific* antibody response. Anti-schistosomal monoclonals with well-defined glycan specificity can be used to isolate parasite glycoconjugates and potentially identify novel vaccine targets including both glycan and protein epitopes [147]. We and others are developing the glycomics tools that will help us to better define the specificity of the αGAbs against helminths, as will be discussed in section 1.5 [244], [247], [248].

The antibody effector mechanisms most well-known to damage or kill schistosomes *in vitro* are ADCC and complement activation, and αGAbs are capable of both. Pioneering work by the Capron group used a semi-permissive rat model to isolate an IgG2a called IPLSm1. The antibody killed schistosomula *in vitro* via eosinophil-mediated ADCC and passively transferred resistance to naïve rats [86]. IPLSm1 targeted a 38-kDa surface glycoprotein which was also recognized by infected monkey and human sera, and was cross reactive with Keyhole Limpet Hemocyanin (KLH) glycans [249], [250]. Our present knowledge of KLH and schistosome cross-

reactive glycans supports the hypothesis that IPLSm1 targeted the FLDNF glycan [158], [162]. The 38-kDa antigen was also used to develop an anti-idiotype vaccine, which conferred 50-80% protection to rats and induced antibodies that mediated ADCC [251]. Mice infected with *S. mansoni* also develop abundant αGAbs, including IgE, IgG1 and IgG3 (but not IgG2) to LDNF, indicative of a skewing toward Th2-type antibody effector mechanisms such as ADCC [144]. A murine IgM to LDN isolated by our group mediates complement killing of schistosomula *in vitro* [148]. The Harn group isolated three murine αGAbs, two of which, an IgM against the $Le^x$ antigen and an IgG2b against an unknown carbohydrate antigen, were protective and mediated *in vitro* complement killing, and an IgG3 that was not [151], [252]. Another group also isolated a mouse IgM, likely against a carbohydrate epitope of schistosomula and adult worms, which was protective *in vivo* and mediated complement-dependent killing *in vitro* [253].

Adaptive immunity to glycans may also be involved in aspects of helminth pathogenesis. LDN and LN-coated beads induce schistosomiasis-like granulomas in murine livers. It is unclear whether this model works through adaptive or innate mechanisms, but fucosylated glycans known to bind C-type lectins did not induce granulomas [254], [255]. The anti-$Le^x$ antibodies induced by schistosomes are cytolytic to human myeloid cell lines. These antibodies could potentially be responsible for mild neutropenia seen in infected humans, or for killing of schistosomula [198].

### 1.3.5 Evidence that αGAbs can mediate destruction of helminthes and protection from infection

Whether human resistance to helminth infection is mediated by αGAbs is a fascinating but complex question, which has only been addressed in a handful of studies examining correlative evidence. One group observed that a schistosomiasis-endemic Kenyan population showed decreases in IgG1 to FLDN and LDN-DF, and increases in IgM to LDN-DF and LDNF, over the course of two years after migrating from a non-endemic to schistosomiasis-endemic area; the same associations were seen with increasing age in the schistosomiasis-endemic resident population [163]. Levels of IgE to worm glycolipids pre-praziquantel treatment were inversely

correlated with egg burden two years after treatment in another population [256]. Using shotgun glycan microarrays made from the intramammalian stages of schistosomes, another research group found that children had modestly higher titers of IgM and IgG than adults to most glycans [244]. Collectively, these studies are difficult to interpret, due to the challenges of identifying human populations that truly show variable resistance and susceptibility (mechanisms of which likely differ among populations), the difficulty of obtaining or generating glycan antigens that are well-defined, pure and accurately mimic the mode of presentation by the parasite, and the differential significance of antibody isotypes and sub-isotypes in human resistance. Different glycan antigens and αGAb isotypes clearly show different patterns. Thus, we are quite a long way from being able to make generalizations about the disease resistance associations of αGAbs as a whole, and even conclusions that have been drawn about particular anti-glycan specificities are tenuous.

Evidence from other helminth infections is supportive of the ability of αGAbs to exert protective effects. The nematode *Trichinella spiralis*, which causes trichinellosis, caps its multi-antennary N-glycans with the unique monosaccharide, tyvelose (3,6-dideoxy-D-mannose). Monoclonal antibodies to tyvelose are a major component of the natural protection conferred on suckling rat pups by infected dams and protect pups when passively transferred. In epithelial cell culture models, antibodies to tyvelose bind surface glycoproteins of the invading L1 larvae, inhibit migration into the cell layer and interfere with molting [257]–[260]. This could be how larvae are prevented from colonizing gastrointestinal epithelium in the protective models. Mucosal antibodies to a carbohydrate antigen of the gastrointestinal nematode *Trichostrongylus colubriformis* also prevent establishment of larvae in the sheep gut. Vaccination experiments have also demonstrated that eukaryotic glycoconjugates are viable vaccine targets. Vaccination of lambs with alhydrogel-adjuvanted excreted/secreted products of the nematode *H. contortus* conferred a high level of protection which was correlated with IgG antibodies to LDNF and Galα1-3GalNAc [154], [261]. Other studies that used natively purified activation-associated

secreted proteins (ASPs) from the cattle nematode *O. ostertagi* also afforded protection, and showed that the recombinantly produced ASPs from *E. coli* were unable to induce protection or any antibodies to native ASPs [262]. Hybrid-type N-glycan structures were characterized on the native protein and, while anti-sera were not directly reactive with the glycan structures, it was hypothesized that they were necessary for proper folding of the native antigen. A vaccine against a *P. falciparum* free lipid anchor glycosylphosphatidylinositol was able to neutralize parasite pathogenesis in mice [263].

Other reports have indicated that antibodies to glycans can be non-protective or even block the development of resistance to helminths. *Heligmosomoides polygyrus*, a well-studied mouse model of gastrointestinal nematode infection, elicits a non-protective immunodominant response to an O-glycan on VAL (venom allergen *Ancylostoma*-secreted protein-like antigens) antigens [264]. Following isolation of a protective IgG2a against *S. mansoni* 38-kDa antigen mentioned above, a second antibody, an IgG2c, which targeted the same glycan, was isolated from infected rats. The IgG2c blocked the protective effect of the IgG2a *in vitro* and *in vivo*, which may be why a response to this epitope was correlated with infection in humans but not with resistance [115]. It had earlier been hypothesized, based on results from a complex series of experiments on chronically-infected and radiation-attenuated cercariae vaccinated mouse sera, that levels of antibody to parasite surface antigens is not simply correlated with protection. Protection may instead depend on a particular balance of blocking and protective antibodies, possibly against the same antigens [265]. Clearly such counteractive effects of antibodies to glycan antigens should be explored in more detail.

Vaccination with irradiated cercariae is still the “gold-standard” protective model for schistosomiasis. Vaccinated non-human primates displaying partial protection from challenge also generate an abundant anti-glycan response [194], [195]. It has been suggested that the anti-glycan component of these responses is a “smokescreen” to the development of protective immunity [195]. However, there is no direct evidence either supporting or discounting this

hypothesis, as the role of αGAbs within antisera or other tissues from a protected host has never been tested in functional studies (see Section 7.2 for a more in-depth discussion of these studies).

The lesson of all these studies is that helminth glycans, like protein epitopes can induce both protective and non-protective antibodies. Rather than viewing glycans as a class of targets and asking, "is their role protective or subversive?" [195] we must develop technologies to identify particular anti-glycan specificities (or combinations of several specificities) and isotypes that may afford protection, and better understand which structural presentations and innate cues are required to incite production of protective versus non-protective antibodies.

## 1.4 The current state of schistosomiasis diagnostics and vaccine development, and opportunities for improvement through glyco-technology

### 1.4.1 Recent progress in diagnostics

Traditional diagnostic methods for schistosomiasis are laborious, require skilled technicians, and are insufficient to detect low level infection or track variations in worm burden [35], [36]. The "gold standard" for diagnosis continues to be microscopic examination of stool or urine samples for eggs. However, eggs are not shed until around 6 weeks after initial infection, and are not consistently shed into feces or urine. Despite improvements in the sensitivity and ease-of-use for stool tests, false negatives are still common, the extent of disease in an individual cannot be accurately ascertained just by egg counts, and eggs of different helminth species endemic in the same area can sometimes be difficult to differentiate [17], [23], [35], [36]. For helminths causing schistosomiasis, filariasis and trichinellosis, antibody-based tests are available, but only by special request from the CDC, and these are not likely to benefit those in endemic areas [37], [266]. Antibody tests are generally sensitive, but they suffer several drawbacks, such as inability to differentiate between active (acute or chronic) and past infections, cross-reactivity among multiple helminth species, and difficulty of performance in the field [23], [266].

Recent studies have uncovered a new set of potential diagnostic antigens, found in serum and urine, for schistosomiasis and other helminths. Carbohydrate-based antigens and αGAbs are promising tools given that they are chemically stable, specific to particular helminth species, vary with stage of infection, and are expressed both on worm surfaces and in secreted products. Several glycan-based detection methods are now in the pipeline for schistosomiasis, which will now be discussed.

#### 1.4.1.1 Glycan-based diagnostics

Evaluation of the success of a schistosomiasis control program – whether based on chemotherapy or vaccination or both – will rely on more sensitive diagnostics than stool exams. A urine dipstick test for the schistosome excreted circulating cathodic antigen (CCA), whose antigenicity is due to $Le^x$ repeats, is now commercially available for point-of-care diagnosis [171], [267]. It is easier to perform in the field, has higher sensitivity than a single Kato-Katz smear, and it can detect prepatent infections in very young children [172], [267], [268]. Another polymeric circulating glycan antigen unique to schistosomes is circulating anodic antigen (CAA). Antibodies to CAA can detect the glycan in serum and urine, and can be used to diagnose infections with many *Schistosoma* species, whereas the CCA-POC assay is primarily useful for *S. mansoni*. Both an ELISA and a dry-reagent lateral flow assay have been developed for CAA detection [269]. The CAA UCP-LF assay recently revealed much higher rates of *S. japonicum* infection than were identified by stool exams in an area of China, where prevalence has declined due to control efforts [270]. Additionally, assays detecting CCA and CAA in serum or urine appear to be more reliable and sensitive diagnostic methods since levels of these antigens fluctuate less than egg counts [271].

Other highly fucosylated epitopes, such as F-LDN-F and DF-LDN-DF are possible diagnostic epitopes due to their unique expression on schistosomes. DF-LDN-DF forms the epitope for the monoclonal antibody 114-4D12. This antibody can be used to isolate free urinary glycans for detection by mass spectrometry, and to identify the DF-LDN-DF on egg

glycoproteins from the blood or urine via ELISA [266], [272], [273]. MS/glycan based studies may lead to a new egg-load-related assay helpful in the detection of mild infections [266], [272]. However, given the differential responses to discrete glycans it is unclear whether immunodiagnostic tools could differentiate between current and past infection. Some studies have shown that keyhole limpet hemocyanin (KLH), which possesses cross-reactive glycans with *S. mansoni*, has the ability to distinguish acute from chronic schistosomiasis, but has low specificity for diagnosing the disease [274], [275].

#### 1.4.1.2 The potential of glycan-based assays for detection of co-endemic infections

Many parasitic helminthes, including schistosomes, are co-endemic with each other and/or other parasitic infections [276], [277]. An exciting prospect inspired by recent advances in glycan array technology is the development of a multi-parasite diagnostic assay based on the "anti-glycome," the collection of antibodies generated to various helminth glycans in body fluids. Modern glycan microarray technology has been used to identify new glycan candidates for diagnosis of helminthes, based on the presence of serum antibodies to foreign epitopes, unusual monosaccharides and/or unique sugar modifications, such as tyvelose in *T. spiralis*, Galα1-3GalNAc in *H. contortus* or phosphorylcholine modifications of nematodes [155], [179], [261]. The glycans of parasitic helminthes thus represent a treasure-trove of diagnostics. The use of shotgun glycomics and screening of reference samples from co-endemic areas could lead to development of powerful, cost-effective tests that would improve control measures for neglected tropical diseases.

### 1.4.2 Recent progress in immune correlates and vaccine development

#### 1.4.2.1 Novel methods of identifying vaccine candidates

In spite of several decades of research, only two vaccine candidates for schistosomiasis have been tested in humans (Sm14 and Sm28GST) [32]. Localization of target proteins may have been a major problem with early vaccine candidates. The worm tegument is a complex and

invaginated syncytial layer overlain by glycan- and lipid-rich membranocalyx layer. Newer strategies use biochemical and proteomic methods to identify protein candidates which are exposed on the worms' surface, accessible to immune effectors, and/or vital for worm functions such as membrane assembly and blood feeding [14], [278], [279]. One of these studies used biotinylation reagents to label accessible adult *S. mansoni* tegument proteins. Only a small subset of proteins was identified, suggesting that many surface proteins are shielded from immune attack by the glycan- and lipid-rich secretions [14], [279]. Six vaccine candidates (Sm14, Sm28GST, IrV-5, paramyosin, Sm23, Sm28-TPI) were compared by the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR/WHO) committee in the mid-1990s [29]. Of the six candidates tested in the 1990s, only two were identifiable in surface or membranocalyx-enriched preparations, one was identifiable in the tegument preparation by mass spectrometry, and none were accessible in biotinylation experiments [42], [279], [280].

Currently, two of the more promising candidates are TSP-2 (tetraspanin-2) and a tegumental protein, Sm29. TSP-2 was originally identified by the signal-sequence trapping method, which uses an expressed sequence tag library to identify membrane and secreted proteins [281]. Sm29 was identified by mining transcriptome data for membrane-bound motifs [282]. They have shown encouraging results in mice both alone and as a chimera, and clinical trials for TSP-2 are planned [109], [283]–[285].

Another issue with vaccine candidates in the past has been stage-specificity. Schistosomula are thought to be the most immunologically vulnerable target in vaccinated mice and rats [286], [287]. However, schistosomula are the most difficult life stage from which to obtain antigens for study, and identification of apparently low-abundancy protective antigens from schistosomula has been a challenge [288]. A recent strategy aiming to overcome these biases is the use of antibody secreting-cell probes (ASC-probes), which are antibody-containing culture supernatants generated from lymph nodes producing a local and stage-specific response to migrating cercariae and lung-stage schistosomula [246]. Using ASCs for antigen identification

has advantages over antiserum, a more traditional tool, which can only identify the most abundant and/or immunodominant antigens, likely to be the result of an immune response to adult worms or eggs [285].

The genomes of *S. mansoni*, *japonicum* and *haematobium* have now been published and various post-genomic methods are being utilized for discovery of vaccine antigens [289]–[291]. The relatively recent wealth of genomic, proteomic and transcriptomic information on schistosomes has led to potentially field-changing advances in schistosome "immunomics," or the characterization of the set of antigens that interact with the immune system [292]. An "immunomic" protein microarray of more than 200 *S. mansoni* and *S. japonicum* proteins has been assembled, based on upregulated expression in schistosomula, known or predicted surface accessibility, high homology among schistosome species and low homology with mammalian proteins [293]. This array has now been used with rat ASC-probes to compare the response of PR (putatively resistant) with chronically-infected humans, resulting in the verification of already known vaccine candidates (Sm29 and SmTSP2) as well as identification of novel candidates (Sj-L6L-1) [292], [294]. The protein microarray has a major disadvantage, however, which is lack of post-translational modifications and potentially mis-folded proteins because it uses a bacterial cell-free expression system. The approach is therefore biased towards protein epitopes which may or may not be exposed in the actual worm. However, combined with other tools like glycan microarray screening and glyco-proteomic approaches, immunomic approaches could be extremely useful in accelerating vaccine development [285].

#### 1.4.2.2 Rationale supporting the development of glycan-based vaccines for schistosomes

Given the rapid turnover of helminth surface antigens, variation in their expression among life stages, a successful vaccine may need to target more than one epitope and/or life-stage. In light of the difficulties faced in developing recombinant protein candidates as schistosome vaccines, we suggest taking advantage of the rich collection of non-protein antigens surrounding vulnerable stages of the worms.

Glycan epitopes offer the advantage of being densely distributed on numerous glycoconjugates on the parasite surface, and expressed throughout multiple life stages. The schistosome is a well-characterized demonstration of this observation. Our group has shown that $Le^X$, LDN and LDNF are expressed on the surface of *S. mansoni* cercariae, schistosomula, and adult worms as well as in soluble egg antigens [148], and that LDNF is expressed on all three major schistosome species [144]. Other fucosylated variants which are not shared by mammalian hosts, such as LDN-DF and FLDNF, have been localized to eggs, cercariae, adult gut and tegument, and appear on numerous distinct glycoproteins and glycolipids as detected by ELISA, western blotting and immunofluorescence of whole parasites and parasite sections [48], [146]. Since there are no vaccines against pathogens as complex as the schistosome, a platform involving broadly-distributed antigenic targets and/or multiple antigens may be necessary.

An additional advantage is that because glycan structure is not linearly encoded in the genome, selective pressure is less likely to result in the escape of glycosylation mutants than is the case for proteins.

Anti-glycan antibodies are a bona-fide correlate of protection for numerous highly successful bacterial-polysaccharide conjugate vaccine. Additionally, as discussed in Section 1.3.4, they have been shown to mediate destruction of schistosome larvae *in vitro*. A recently published work from our lab found that antibodies to several glycan epitopes, including core xylose/core α3 fucose, LDN, LDNF and $Le^X$, are abundant in rhesus monkeys, a naturally-protected host of schistosomiasis. The titer of these antibodies over the course of infection correlated with maximal *in vitro* complement-dependent schistosomula killing ability of the rhesus serum [175]. Similarly to protein antigens in schistosomiasis, αGAbs of subtypes associated with protection can be found in infected hosts [245].

Thus, from our perspective, there is no reason to expect that glycans are any less likely to serve as protective antigens than proteins. Because no precedents exist for a successful vaccine based on eukaryotic glycan antigens, however, more research is needed to exploit helminth

glycan antigens as vaccine candidates. Further studies are needed to better define the glycan antigen structures of helminthes, to develop novel methods of producing and presenting eukaryotic glycans in an immunogenic fashion, and to discover the glycosyltransferases necessary to generate the worm glycan structures that are foreign to mammals. The central role of glycans in adaptive immunity to helminthes and these early studies into their protective capacity indicate that, with further innovation, glycan-based vaccines may be an important intervention in the control of helminth infection.

## 1.5 Recent developments in glyco-technology

Our knowledge of the parasite glycans targeted by the immune system is limited by our knowledge of the parasite glycan structures themselves. Unlike protein sequence, the structure of a single glycan is determined by many different types of genes, which limits the types of genetic approaches that can be used in the study of glycan structure-function relationships. In spite of this complexity, however, great advances in technology for studying parasite glycan structures and defining the specificities of glycan-binding have recently been made.

### 1.5.1 Glycan synthesis and glycoconjugate production

Oligosaccharide synthesis is much more complex than the synthesis of other biomolecules like peptides and DNA. Enzymatic synthesis is extremely useful and highly specific, but it is difficult and expensive to obtain enough enzyme to produce large amounts of product for functional studies [295]. Additionally there are many glycosidic linkages in parasites for which we have not characterized a glycosyltransferase or cannot yet recombinantly produce one, such as the Fucα1-3GalNAc and Fucα1-2Fuc linkages that appear to crucial components of several schistosome antigens. Synthetic methods are also available, but require complicated protocols to achieve the specificity generated by enzymes. Great strides have been made in stream-lining and automating oligosaccharide synthesis, but it is still far from being able to generate the full repertoire of glycans seen in nature [296].

### 1.5.2 Glycan microarray screening

One of the most important recent innovations in the field of glycobiology, which has been spearheaded by our group along with collaborators in the Consortium for Functional Glycomics, has been glycan microarrays [297]. A glycan microarray consists of a collection of derivatized glycans or glycopeptides covalently linked to a glass slide, or in some cases non-covalently captured on nitrocellulose-coated slides. Very small samples of glycan binding proteins (such as lectins, antibodies or antisera) or even micro-organisms (viruses, yeast) can be incubated with the slide, washed and detected using fluorescent secondary reagents. Scanning and quantitation of fluorescent signal for all bound glycans is computerized. Glycan microarrays have been validated against traditional methods (Western blot, ELISA) as sensitive and specific serodiagnostics for human infections such as Trichinellosis and Salmonellosis, and they offer the added benefits of using very small sample volume, being sensitive with low background, and detecting binding to distinct epitopes simultaneously [155], [298].

#### 1.5.2.1 Defined arrays

We use two microarray approaches to schistosome immunoglycomics. The first is termed the defined Schistosomal-type array (DSA) (most current version depicted in Figure 1.4). As antigenic glycans are identified in the literature, we synthesize them via enzymatic or chemical methods and print them on arrays. The DSA currently contains glycans and glycopeptides with LN, LDN, LDNF and $Le^X$ motifs on a variety of underlying core structures, truncated N-glycans with core xylose and/or core α3 fucose, polyLDN and polyLDNF, chitin oligosaccharides and several control structures. The printed structures are validated using ConA (binds tri-mannose of N-glycans), AAL (binds fucose), WFA (binds terminal β-linked GalNAc), GSL-II (binds terminal GlcNAc), as well as antibodies to HRP (bind core xylose/core α3 fucose), anti-LDN and anti-LDNF (Figure 1.4).

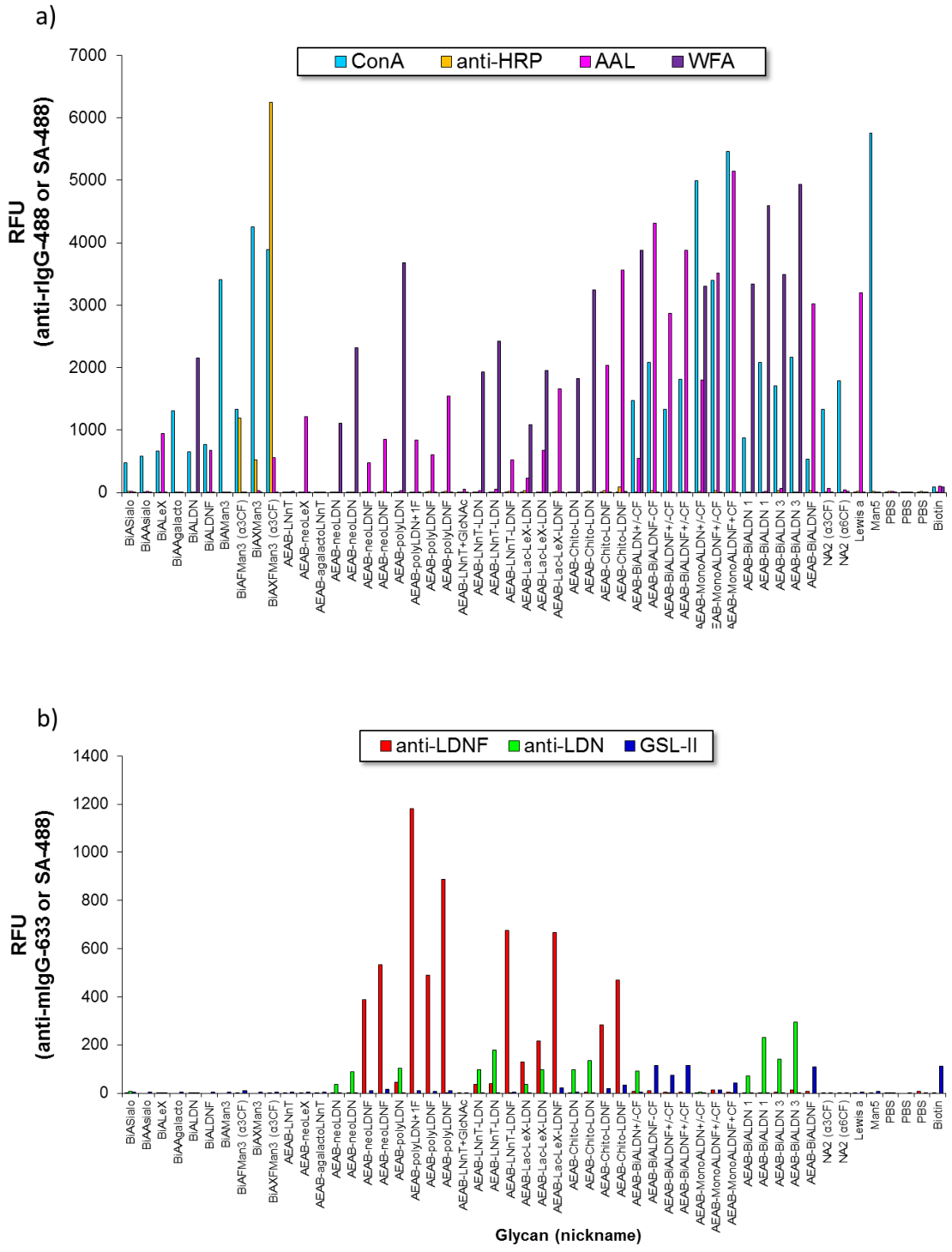



**Figure 1.4 Lectins and monoclonal antibodies are used to validate and quality control the Defined Schistosome-type Array (DSA)**

### 1.5.2.2 Shotgun arrays

The second approach to glycan microarrays we have coined the "shotgun" approach, where glycans are extracted from natural sources such as schistosome eggs, derivatized with a fluorescent linker and partially purified before printing. In this approach, the structures are initially undefined. Binding studies with antibodies or anti-sera identify structures of interest, which can subsequently be characterized using meta-data such as lectin and antibody binding, enzymatic treatments, compositional analysis, mass spectrometry and linkage analysis, a novel approach termed metadata-assisted glycan sequencing (MAGS) [299]. The two-dimensionally purified *S. mansoni* egg N-glycan array (egg array) is depicted in Figure 1.5, bound by some αGAbs and lectins. The egg array has aided the characterization of anti-Schistosomal mouse monoclonal antibodies (anti-F-LDNF) and in discovery of novel immunodominant glycan epitopes in infected rhesus macaque serum (Rivera-Marrero *et al.*, manuscript in preparation).

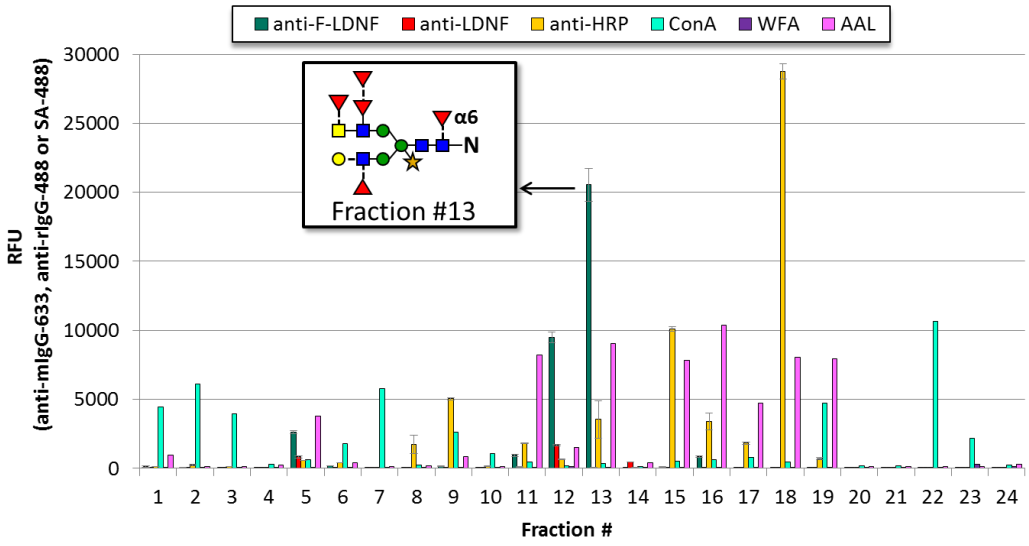



**Figure 1.5 The *S. mansoni* shotgun egg array bound by lectins and antibodies.**

Two antibodies (anti-LDNF and anti-HRP) and three lectins (ConA, WFA, AAL) of known specificity were used to assist in making structural predictions about the glycan antigens in the partially-purified fractions, and to help characterize novel reagents that bind the array (anti-F-LDNF). Composites of the predicted structure for the major component of fraction #13 based on MALDI-TOF, are shown. The major peak of fraction #13 contains di-fucosylated glycans, with

possible positions indicated by the dotted likes. It is predicted to contain F-LDN (Fucα1-3GalNAc) based on reactivity with a recently characterized monoclonal antibody (Nyame *et al.*, manuscript in preparation).

### 1.5.3 Genomics and glycosyltransferase expression, characterization, regulation

The recent availability of genomic information for the three major schistosomes has brought about a new era in understanding the biology of these worms [289]–[291], [300], [301]. This information has greatly improved our ability to profile the life-stage expression of proteins and glycosyltransferases, which has major implications for development of vaccine and diagnostic candidates [52]–[54]. Indeed, schistosomes possess many fucosyltransferases (FucTs), most of which are yet un-characterized and appear to be developmentally regulated [53], [54].

Our group and others are beginning to apply combinations of the above technologies to advance our understanding and utilization of parasite glycobiology. For example, the Wilson group recently used enzymatic reactions on glycan arrays to characterize an unusual N-glycan core-modifying FucT from *C. elegans* [302]. Coupled with improved technologies for recombinant expression of functional schistosome enzymes, this approach should yield a wealth of information about the parasite FucTs that can be applied to antigen synthesis.

## 1.6 Major questions facing the schistosomiasis field

Though several decades of schistosomiasis research has contributed to many advances in the understanding and control of this disease, we are still left with more questions than answers where schistosomiasis immunology is concerned. Table 1.2 lists some of these questions.

| Major Questions in Schistosomiasis Immunology | |
|---|---|
| **General Immunology and Molecular Biology** | |
| | What glycans and proteins contribute to Th2 and regulatory biasing *in vivo*? |
| | What are the genetic and environmental factors leading to immunopathology in humans? |
| | What are the immunodominant targets of T cell and B cell memory in re-infection? |
| | How does infection affect the immune response to other inflammatory conditions, infections and vaccines? |
| | How and why is glycan expression developmentally regulated? |
| | Why do most human hosts "tolerate" rather than eliminate schistosomes? |
| | What role do particular glycans and proteins play in host invasion and tissue homing? |
| **Protective Immunity** | |
| | How do protective mechanisms differ in humans from protected animal models? |
| | Does disease resistance primarily affect cercariae, schistosomula or adult worms? |
| | What effector mechanisms damage/kill/decrease fecundity of cercariae, schistosomula and adults worms *in vivo*? (ADCC, complement, neutralization, T cells . . .?) |
| | What role do eosinophils play in anti-worm immunity? (Direct or indirect? Specific?) |
| ✱ | What immune parameters are associated with human resistance? |
| | Which markers of human resistance are direct contributors to immunity? |
| ✱ | How and through which antibodies is parasite-*specific* immunity effected? |
| | What/do non-specific immune mechanisms contribute to protection? |
| | What are the contributions of tissue-specific versus systemic immunity? |
| | Which type of immune response (Th1, Th2, Treg) is most beneficial for resistance in humans? |
| | What are the genetic and environmental factors leading to the range of disease resistance/tolerance in humans? |
| | What factors determine whether an antibody is "blocking" or protective? |
| | Localization/life stage expression/function of target molecule? |
| | Isotype/sub-isotype? |
| | Affinity? |
| | How does praziquantel expedite the development of resistance? |
| **Vaccines** | |
| | How can we best screen for vaccine candidates likely to be spatially/temporally accessible and which elicit the proper character of immunity? |
| | How can we best screen for vaccine candidates in a way which is unbiased towards proteins, lipids, carbohydrates? |
| | How can multi-stage/multi-target vaccines be designed? |
| ✱ | What aspects of parasite glycan antigens make them antigenic? Immunogenic? |
| **Diagnostics** | |
| ✱ | What markers differentiate recent, acute, chronic, active and past infections? |
| | What markers differentiate schistosomiasis from co-endemic infections? |
| | What markers could be incorporated into a multi-infection testing panel? |
| | How can we best screen for diagnostic candidates in a way which is unbiased towards proteins, lipids, carbohydrates? |

**Table 1.2 Major questions in the field of schistosome immunology.**

Major questions according to the literature as well as from the perspective of these authors. Several of these are being studied by our research program. Asterisks denote the questions which this project was designed to address.

## 1.7 Our project aims

**Part I: Eliciting immunity to parasite glycans: From synthetic glycoconjugates to novel recombinant technologies**

**1)** To better understand the structural factors contributing to glycan-specific immune responses to eukaryotic glycans, and optimize their presentation in vaccines *(Chapters 2-3)*

**Part II: Investigating the role of anti-glycan antibodies in schistosome infection**

**2)** To better understand the role of anti-glycan antibodies in protective models of *S. mansoni*, which could be exploited in human vaccines *(Chapter 4)*

**3)** To better understand the significance of these antibodies in human resistance to *S. mansoni* infections *(Chapters 5-6)*

# Part I: Eliciting immunity to parasite glycans: From synthetic glycoconjugates to novel recombinant technologies

# Chapter 2. An intact reducing-end sugar improves the specificity of the immune response to lacto-N-neotetraose-BSA glycoconjugates

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## 2.1 Introduction

Glycoconjugate vaccines are one of the most important innovations in preventative medicine. Vaccines against *Haemophilus influenza*, pneumococcus and meningococcus have dramatically decreased suffering caused by these organisms (reviewed in Trotter *et al.*, 2008). The licensed glycoconjugate vaccines are limited to those preventing bacterial infections. Pathogens such as fungi and parasites, and tumors, also induce immune responses to non-host-like or altered glycans, but no vaccines targeting eukaryotic glycans have yet been licensed. This gap in the field is likely due to multiple factors, including fundamental differences in structure between relatively large microbial polysaccharides with repeating motifs versus smaller eukaryotic glycans that lack repeating motifs, difficulty in isolating or synthesizing large amounts of eukaryotic glycans, and lack of knowledge about how to design immunogens that induce the desired glycan-specific response.

The traditional method of developing a glycoconjugate vaccine using bacterial capsular polysaccharides (PS), has been largely empirical. The PS is often isolated from bacterial culture, chemically activated and then conjugated to a protein carrier or synthesized to contain a particular linker structure for coupling to protein (Figure 2.1a, b). Some common methods of PS activation

and conjugation, such as periodate oxidation followed by reductive amination, have the disadvantage of destroying carbohydrate epitopes and/or creating neo-epitopes when the sugar rings are broken and then linked to other species [305]. The repeating motifs within bacterial PS provide many modes of antigen presentation and may confer inherent immune-stimulatory properties. Thus, in spite of the crude methods used to conjugate bacterial PS to carrier proteins and the heterogeneity of the final product, conjugate vaccines have been successful at generating protective titers of antibody and long-term memory.

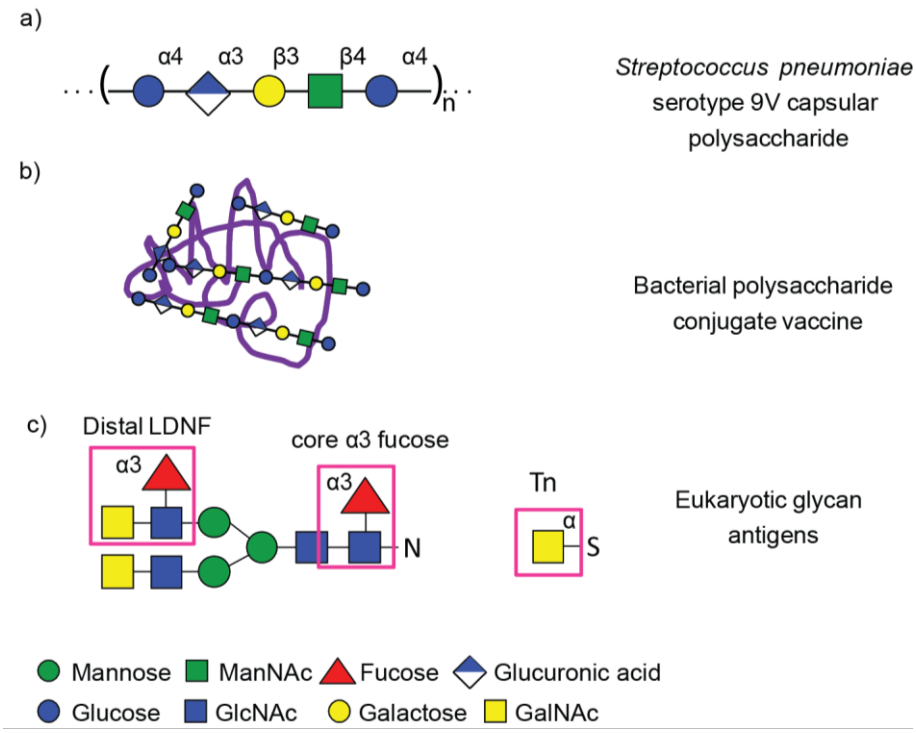



**Figure 2.1 Structural considerations of bacterial polysaccharide-conjugate vaccines versus eukaryotic glycan antigens**

Examples of a typical bacterial polysaccharide, comprised of repeating epitopes, used in preparation of glycoconjugate vaccines (a), schematic representation of a bacterial polysaccharide vaccine prepared using reductive amination, where heterogeneous oligosaccharides are linked in a lattice-like fashion to a protein carrier (b), and a few eukaryotic glycan epitopes targeted in parasitic infection (LDNF, core a3 fucose) and cancer (Tn) (c).

The antigenic glycans of eukaryotes, such as those targeted during schistosome infection and tumor antigens, present unique challenges to glycoconjugate vaccine development. These epitopes are often non-repeating, may occur at the reducing (core) or non-reducing end (distal) of the glycan, and share some features with mammalian glycans (Figure 2.1c) (reviewed in Cummings, 2009; Heimburg-Molinaro, Lum, *et al.*, 2011; Prasanphanich, Mickum, Heimburg-Molinaro, & Cummings, 2013). Previous studies on the specificity of the response in rabbits to human milk glycan-protein conjugates have shown that antisera heavily targets the reducing-end and linker region, and may also possess specificity for the non-reducing end of the sugar, depending on which sugar is used [307], [308]. Our lab has previously attempted mouse immunizations using several different schistosome-like glycopeptide epitopes coupled to carrier proteins, with no glycan-specific antibodies generated (unpublished studies). Since mice are known to make antibodies to the glycan epitopes during schistosome infection, and the carrier proteins used were immunogenic, we hypothesized that the presentation of the glycan epitopes was improper in some way. For example, the epitope may only be antigenic when linked to particular underlying monosaccharides, or when accompanied by certain innate signals provided by the parasite. The type of linker can affect the immunogenicity of the conjugate even with similar levels of glycan:protein loading, for unknown reasons [309]. A variety of strategies incorporating tumor-associated carbohydrate antigens (TACA), from whole-cell immunogens to synthetic conjugates, have shown mixed success in animal and human cancer vaccine trials (reviewed in Heimburg-Molinaro, Lum, *et al.*, 2011). These studies have underscored the complexity of generating glycan-specific immunity and the need for a better understanding of how to package glycan epitopes in vaccines.

Several studies in the last decade have taken more rational approaches to glycoconjugate vaccine development and begun to elucidate structural principles that optimize immune responses to both bacterial and eukaryotic carbohydrates. For example, one group used monoclonal antibodies protective against *C. albicans* to map β-mannan disaccharides as minimal epitopes,

and generated a protective vaccine by linking the disaccharides to a protein via click chemistry [310]. They also demonstrated that diversification of the linker region, by using a mixture of anomers at the chiral carbons, enhanced immunity to the proximal disaccharide portion [311]. Another group found that the length and non-reducing end monosaccharide of synthetic *S. dysenteriae* O-specific polysaccharides in the conjugate vaccine affected vaccine immunogenicity [312]. Other researchers synthesized a vaccine from a Tn-antigen glycopeptide containing a T-cell epitope covalently linked to a TLR ligand, with very promising results in mice [313]. Many vaccine development efforts stand to benefit from such novel approaches and hypothesis-driven studies on the immune response to eukaryotic glycan antigens and glycoconjugates.

Conjugation of polysaccharides to a protein carrier greatly enhances the magnitude and longevity of the anti-glycan response, through interactions between cognate protein-specific T-cells and glycan-specific B-cells [314]–[316]. In a recent dogma-changing study by the Kasper group, it was shown that glycopeptides from processed conjugate vaccines bind MHC, and glycopeptide-specific T-cells could be the main contributor to the vaccine response. The authors took advantage of this feature by evenly spacing peptides along a polysaccharide backbone, resulting in greatly enhanced vaccine efficacy in mice [317]. More studies on the immunological mechanisms of anti-glycan responses could similarly inform structural considerations of glycoconjugate vaccine design.

Our lab has developed novel types of sugar conjugation chemistry in order to immobilize sugar epitopes on microarrays and explore their interactions with glycan binding proteins. One such method uses reductive amination, a process commonly used for sugar-protein conjugation, to tag the glycan with either of the two fluorescent heterobifunctional linkers, 2-amino-N-(2-aminoethyl)-benzamide (AEAB) or *p*-nitrophenyl anthranilate (PNPA) [318], [319]. This process is facile, high-yield, allows for easy quantification and control of sugar:protein conjugation ratios, and results in homogeneous orientation of the glyco-protein epitopes. Such an orientation for eukaryotic glycan epitopes, is preferable to the haphazard way in which bacterial polysaccharides

are usually attached. However, it opens the reducing-end monosaccharide ring, which can create an immunodominant "neo-epitope" from the combination of the opened ring and linker region [307], [308]. We compared the binding properties of commercially-available free reducing glycans which were either reductively aminated ("open-ring") or acryloylated ("closed-ring") to AEAB on glycan microarrays. For most glycan binding proteins (generally those targeting a non-reducing epitope) binding is unaffected by the conjugation method, but antibody recognition of some epitopes was destroyed by reductive amination. Sialyl-Lewis X and type-2 H-antigens for example were recognized by lectins but not monoclonal antibodies when derivatized in this fashion [319]. This study suggests that reductive amination or similarly destructive chemical methods can produce major alterations in glycan antigenicity and are unacceptable for making conjugate vaccines with many eukaryotic glycan epitopes.

We tested this idea by examining the effect of two similar methods of linkage between the reducing end of the glycan, LNnT, and the carrier protein, BSA, where the reducing end sugar ring is either open or closed, on the glycan-specificity of the immune response after immunization in rabbits. This platform was also used to investigate species-specific differences in the anti-glycan response between rabbits and mice, and, in the future, can be used to examine the impact of other structural considerations on immunity.

## 2.2 Materials and Methods

### 2.2.1 Preparation of conjugate vaccines

Derivatization of open-ring (reductively aminated) LNnT (lacto-N-neo-tetraose, Galβ1-4GlcNAcβ1-3Galβ1-4Glc) with the PNPA (*p*-nitrophenyl anthranilate) linker was performed as previously described [318]. Derivatization of closed-ring (acryloylated) LNnT with the PNPA was performed similarly to our previously published work, with some modifications for scale (Figure 2.2) [319]. Briefly, 180mg of free-reducing glycan was dissolved in 3mL of water. An excess of ammonium bicarbonate was added and the mixture was heated at $55^0$C for 1.5 hours.

The reactions were cooled and diluted with water. The reaction mixture was purified in 6 batches, each on a series of two pre-conditioned 1g Carbograph SPE columns and eluted with 50% acetonitrile/10mM ammonium bicarbonate. After removal of organic solvent, the glycosylated amine sugars were snap-frozen and lyophilized. On ice, 600mg of sodium bicarbonate, followed by 2mL of ice cold saturated sodium bicarbonate, followed immediately by 200ul of acryloyl chloride were added to each batch and reactions were vortexed for 10 minutes with venting. 5mL of water was added and the acylated glycosylamides were purified similarly to the previous reaction on Carbograph columns, using 50% acetonitrile/0.1% TFA to elute. The samples were dried and molecular weights verified on MALDI-TOF in RP mode. Samples were reduced with sodium borohydride, followed by acetic acid at $4^0$C to prevent formation of open-ring products, and again desalted as on Carbograph columns. The stabilized glycosylamides were dried and dissolved in 2mL methanol and cooled to $-78^0$C. Ozone was bubbled under the surface until reactions remained blue and they were allowed to remain uncovered in the fume hood to return to room temperature. Methyl sulfide, 300ul, was then added to each batch and incubated overnight at $4^0$C. These activated glycosylamides were dried completely under nitrogen gas and immediately derivatized with PNPA.

To make the open-ring (LNnT-OR-PNPA and Lac-OR-PNPA) and closed ring PNPA (LNnT-CR-PNPA) derivatives, respectively, free-reducing LNnT, lactose and the activated glycosylamide LNnT were each mixed with 0.35M freshly prepared PNPA and 1M sodium cyanoborohydride at a ratio of 10:1 PNPA:glycan, in a solution of 7:3 (v/v) DMSO:acetic acid. The mixtures were heated at $65^0$C for 2hr and quenched by addition of 10 volumes of acetonitrile. They were allowed to precipitate at $-20^0$C for 2hr and then centrifuged at 4000rpm to remove excess PNPA in the supernatant, and the pellet was washed with acetonitrile. They were re-dissolved in water and centrifuged to remove particulate material from the soluble products. The LNnT-OR-PNPA and LNnT-CR-PNPA were stored at $-80^0$C until conjugation with protein.

Aliquots were spotted 1:1 with DHB (2,5-dihydroxybenzoic acid) matrix for MALDI-TOF analysis in RP mode. Theoretical molecular weights of the PNPA conjugates are 949 and 1004g/mol, but observed molecular weights are at 958 and 1013g/mol, respectively.

Lac-OR-PNPA, LNnT-OR-PNPA and LNnT-CR-PNPA were quantified via reverse phase HPLC on a Vydec C18 analytical column to calculate molar amounts of the glycans, and these were added to a 3:2:5 (v/v/v) mixture of water:saturated sodium bicarbonate:BSA 20mg/mL at a glycan:protein molar ratio of about 20:1. These were rotated at $37^0$C for 3 days with 0.05% sodium azide and then dialyzed to water over 24 hrs. The resulting Lac-OR-BSA, LNnT-OR-BSA and LNnT-CR-BSA neoglycoconjugates were spotted in sinapinic acid matrix (20mg/mL in 50% acetonitrile, 0.1%TFA) for MALDI-TOF analysis in order to estimate the resulting conjugation ratio. Purity was assessed by SDS-PAGE. The glycoconjugates were dried and stored at $-20^0$C until use.

### 2.2.2 Immunizations

Glycoconjugates were dissolved at 10mg/mL in sterile PBS. This mixture was diluted 1:5 in PBS and then emulsified at a 1:1 ratio with complete Freund's adjuvant (CFA) for a final antigen concentration of 1mg/mL. The initial immunization of rabbits and mice consisted of 200μg injected subcutaneously per animal, spread out over the neck and hind flank for mice. Boosters of 100μg per animal in incomplete Freund's adjuvant (IFA) were administered at 2, 4, and 6 weeks. Animals were bled via facial vein puncture one day before immunization (pre-bleed), and at weeks 5, 7, and 8. Two rabbits and three mice (one Swiss-Webster, two BL-6, all adult females) were used for these pilot studies.

### 2.2.3 Preparation and analysis of glycan microarrays

The BSA-PNPA microarray (Figure 2.3) was prepared, assayed, and analyzed as described in Heimburg-Molinaro *et al.* [247] with the following modifications. Each glycan or glycoprotein was printed in hexareplicate spots on NHS-activated glass slides using a Piezo

Printer (Piezorray, PerkinElmer). Derivatized glycans were printed with 2 drops (666pL) of 100μM solutions and glycoproteins were printed with 2 drops of 0.5mg/mL solutions, in phosphate buffer, pH 8.0. Binding assays were performed as previously described [247]. Arrays were quality-controlled via binding assays with biotinylated lectins at 1μg/mL and cy5-streptavidin at 0.5μg/mL. For serum binding assays, serum was diluted from 1:50 to 1:500, and wells were washed 3 times with 200μl TSM wash buffer, then 3 times with 200μl TSM buffer with 5 minutes of shaking for each wash, after the primary and secondary incubations. The secondary antibodies, goat anti-mouse IgG-Alexa 568, anti-mouse IgM-Alexa 488, and anti-rabbit IgG-Alexa 488 were used at 5μg/mL. Slides were scanned using the ScanArray Express software on a PerkinElmer Proscanner XL4000. ScanArray Express was used to align spots, remove background, and quantify fluorescence. An excel macro file was then used to average 6 replicate spots for each glycan ID #, determine SEM, SD and %CV.

CFG binding assays were conducted as described [247] with the following modifications: incubating slides with rabbit sera at 1:100 for the primary incubation and goat anti-rabbit IgG-Alexa 488 at 5μg/mL for the secondary incubation.

### 2.2.4 ELISA

Glycoproteins were diluted to 1μg/mL in 0.05M carbonate/bicarbonate buffer pH 9.6 and 50μl were coated in each well of a clear, flat-bottom, non-tissue culture treated 96-well plate overnight at 4°C. Excess coating solution was dumped and the plate was blotted and washed three times with PBS-t (1X PBS with 0.05% tween-20) using a squirt bottle. All remaining incubations were 1 hour at room temperature on a slowly shaking orbital shaker, and all washes were with PBS-t. To each well, 200μl of blocker was added. Because of background issues in mouse serum ELISAs, assays were replicated with two different blockers: 3% BSA in PBS-t (with 1% BSA in serum and antibody dilutions) and non-animal protein NAP blocker (G-Biosciences, St. Louis, MO) 1:2 in PBS-t (with NAP 1:4 in serum and antibody dilutions. The blocker was discarded and

plates were washed three times. The serum was diluted appropriately and 50µl was added to each well. After incubation the serum was discarded and the plate was washed five times. The secondary antibody, goat anti-rabbit IgG or anti-mouse IgG linked to HRP, was diluted 1:1000 and 50µl were added to each well. After incubation the unbound antibody was discarded and the plate was washed five times. The plate was then covered while O-phenylenediamine dihydrochloride was dissolved to 0.5mg/mL in stable peroxide buffer. When the substrate was fully dissolved, 50µl were added to each well. Plates were incubated standing in the dark for 20 minutes. The reaction was quenched with 25µl per well of 3N sulfuric acid. Bubbles were popped and the absorbance of the plate was read at 490nm in a Victor plate reader.

## 2.3 Results

### 2.3.1 Synthesis and characterization of LNnT-BSA glycoconjugate vaccines

In order to generate closed-ring and open-ring sugar-protein conjugates, a commercially available milk glycan, lacto-N-neotetraose (LNnT, Galβ1-4GlcNAcβ1-3Galβ1-4Glc) was derivatized with *p*-nitrophenyl anthranilate (PNPA) via two different methods. Reductive amination alone results in the open-ring derivative, LNnT-OR-PNPA. An open-ring derivatized lactose (Lac, Galβ1-4Glc) was prepared as an additional control. For the preparation of LNnT-CR-PNPA by closed-ring derivatization, LNnT is treated with ammonium bicarbonate to form a glycosylamide, acryloylated with acryloyl chloride, activated with ozone and conjugated with PNPA by reductive amination, sequentially (Figure 2.2, top) [319]. The derivatized LNnT and Lac were then conjugated with bovine serum albumin (BSA), resulting in Lac-OR-BSA, LNnT-BSA and LNnT-CR-BSA (Figure 2.2, bottom). MALDI-TOF studies showed that the conjugates had average sugar:protein ratios of 13:1 (Lac-OR-PNPA), 7:1 (LNnT-OR-PNPA), and 5:1 (LNnT-CR-PNPA).

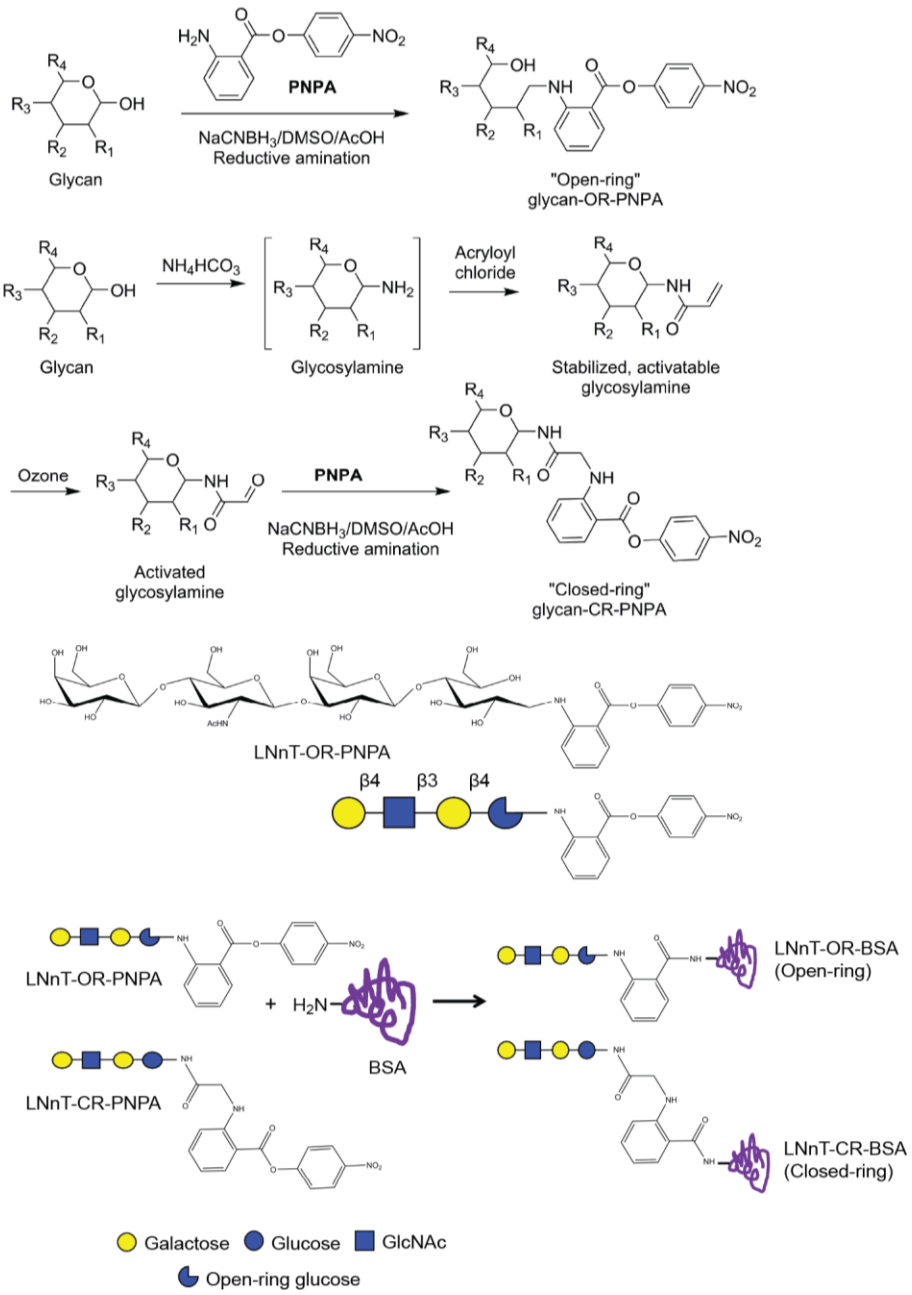



**Figure 2.2 Synthesis of open-ring and closed-ring LNnT-BSA conjugates**

Reaction scheme for synthesis of PNPA-derivatized sugars from free-reducing glycans via the open-ring and closed-ring methods (a), diagram of the open-ring LNnT-PNPA derivative used in this study, with monosaccharide code schematic drawn below (b), and reaction scheme for synthesis of BSA glycoconjugates with the PNPA-derivatized, highlighting the similarities and differences between the open-ring and closed-ring conjugate structures in the linker region (c).

### 2.3.2 Closed-ring linkage induces greater glycan specificity than open-ring linkage in rabbits

Each pair of rabbits was immunized subcutaneously with one of the three glycoconjugates, Lac-OR-BSA, LNnT-BSA or LNnT-CR-BSA, in CFA once and IFA three more times. In order to assess the magnitude and specificity of the immune response, we analyzed the antisera using ELISA plates coated with the immunogens, as well as a custom glycan/glycoprotein microarray (Figure 2.3). The glycan microarray contains 16 different compounds, each printed in hexareplicate spots on NHS slides. Open-ring Lac, LNnT and closed-ring LNnT, as well as several other sugars, including LNT, which is identical to LNnT except for the β3 linkage at the non-reducing end, were printed to demonstrate glycan specificity. Note that AEAB, a linker related to PNPA but with an additional active amino group, was used for efficient printing on NHS-activated glass slides. The ring portion of the molecule attached to the sugar is shared between the two linkers, and when used for protein conjugation, the resulting conjugate is identical. The glycoconjugate immunogens and control glycoproteins were printed on the array as well as the AEAB linker alone.

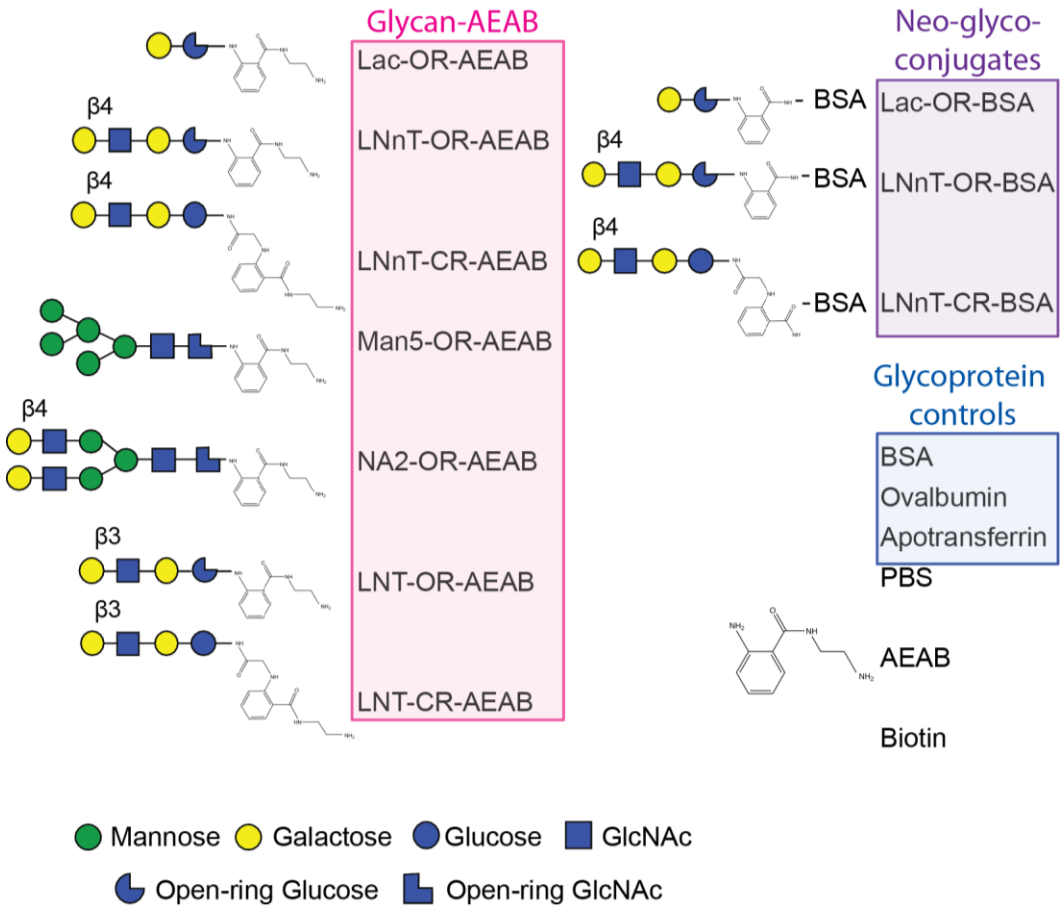



**Figure 2.3 BSA-PNPA Array**

List of glycan and glycoprotein structures printed on the BSA-PNPA array. Numbers correspond to the x-axis labels in Figure 2.4 and Figure 2.7 where immune rabbit and mouse sera were screened.

All of the immunized rabbits produced a robust IgG response by week 5 and in most cases increasing through week 8 (Figure 2.4). The Lac-OR-BSA immunized rabbits primarily targeted a combination of the open-ring derivatized lactose and linker-protein, as evidenced by the binding to Lac-AEAB and Lac-OR-BSA with cross-reactivity to LNnT-BSA, which contains the same open-ring Lac-linker-protein structure internally. Low reactivity with the closed-ring conjugate or BSA alone was observed. Rabbits immunized with the LNnT-OR-BSA conjugate also responded most highly to Lac-OR-BSA and LNnT-OR-BSA, with some reactivity to the open-ring LNnT and a low response to LNnT-CR-BSA. This again indicates that some

combination of the non-reducing end sugar-linker-protein appears to be the primary epitope. The two rabbits also displayed varying levels of antibody to the open-ring LNT sugar indicating that a large portion of the anti-glycan response was not specific for the reducing-end. Rabbits immunized with the closed-ring conjugate (Figure 2.4, panel e), in contrast, targeted closed-ring LNnT as highly or higher than the LNnT-CR-BSA immunogen, with very little cross-reactivity to the other glycoconjugates and a lower response to closed-ring LNT. This response was therefore more specific to the immunogen, and more focused on the tetrasaccharide. Low-level binding of LNT-CR-AEAB suggests that a small portion of the response to the closed-ring conjugate was to an epitope internal to the sugar glycosylamide and/or linker portion rather than to the non-reducing end of the sugar, but the LNnT tetrasaccharide in its closed-ring form was clearly the preferred epitope.

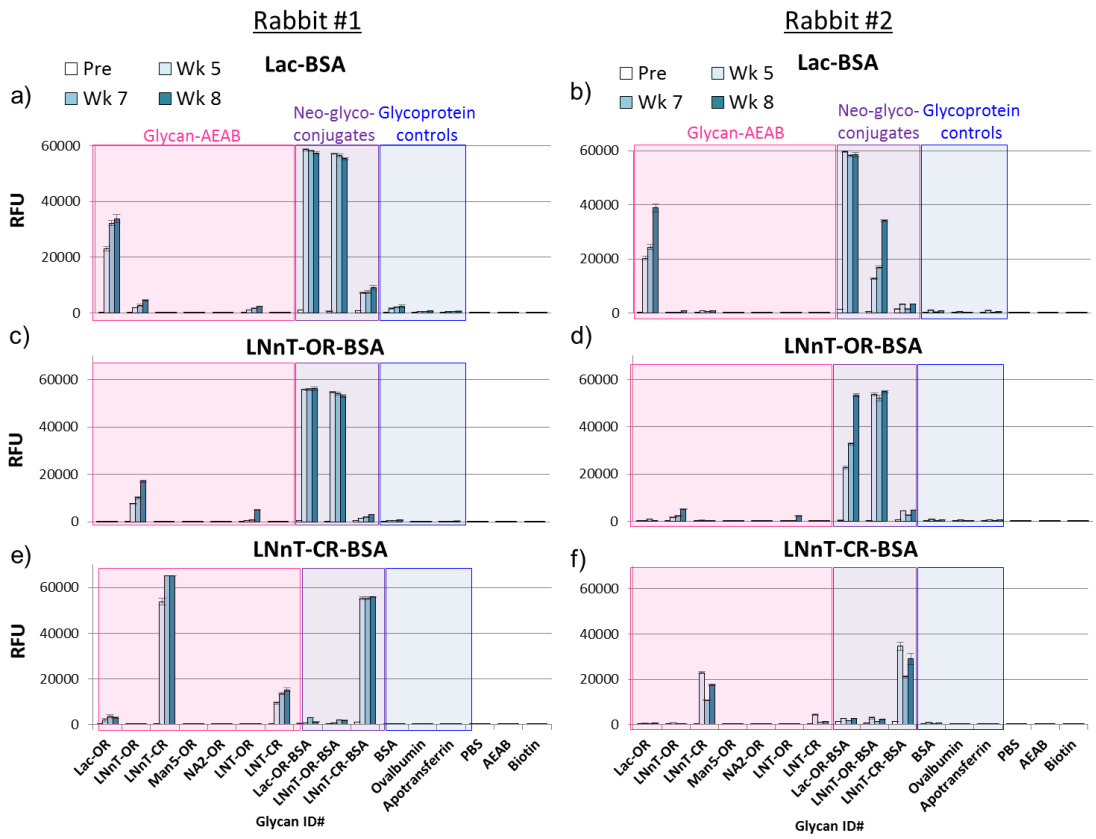


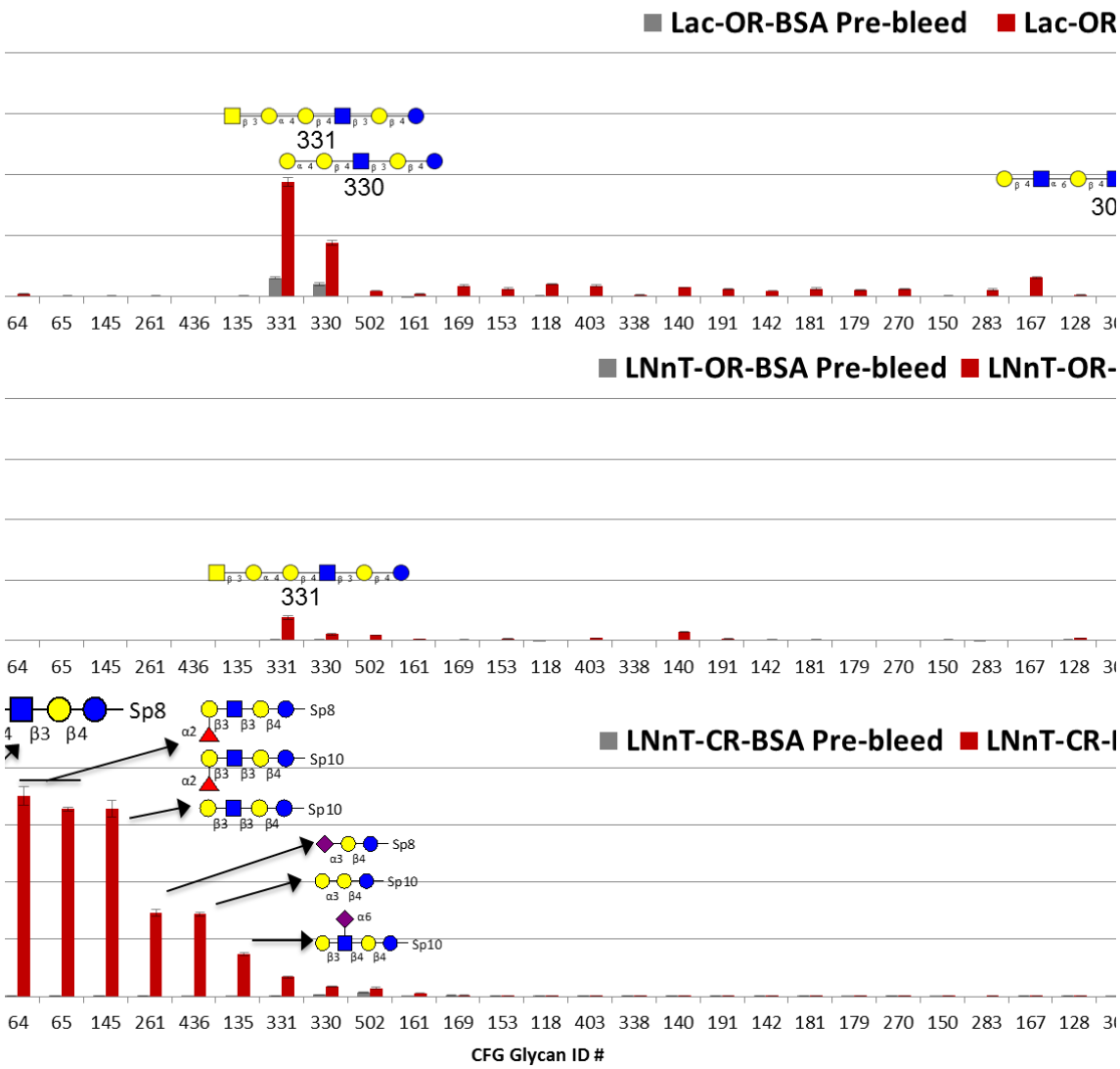

**Figure 2.4 Open- or closed-ring linkage impacts specificity of the rabbit response to LNnT-BSA conjugates**

Serum from two rabbits was diluted at 1:100 and screened on the BSA-PNPA glycan microarray. Serum was detected with anti-rabbit IgG-Alexa 488. Bars correspond to mean of hexareplicate spots +/- standard deviation. RFU, relative fluorescence units; Pre, pre-bleed; Wk 5 – Wk 8, Week 5 – week 8 bleeds. Pink, AEAB-derivatized glycans; purple, BSA neoglycoconjugates; blue, glycoprotein controls. X-axis labels correspond to the structures depicted in Figure 2.3. Note that signals reach the detection maximum near 60,000 RFU.

One rabbit from each group was also analyzed on the Consortium for Functional Glycomics (CFG) array (version 4.2), where hundreds of synthetic glycans are printed using a different closed-ring linkage (unrelated to PNPA) (Figure 2.5 and Table 2.1). For clarity, only relevant (those with LNnT- and Lac-related structures) are shown in Figure 2.5a. Pictoral representations of the structures included are shown in Figure 2.5b. The top 10 binders of each

immunized rabbit screened are listed in Table 2.1, and the full dataset can be found online at http://www.functionalglycomics.org/glycomics/publicdata/primaryscreen.jsp.

The Lac-OR-BSA immunized rabbit displayed many antibody specificities in the week 8 serum, however only a few of the high binders contained Galβ1-4Glc motifs (#41, 42, 86, 330, 331, 243, 404), and most of the structures bound in the week 8 serum were also bound at lower levels in the pre-bleed serum (#400, 330, 331, 304) (Figure 2.5a, top panel and Table 2.1). The LNnT-OR-BSA immunized rabbit displayed a response that was similar in specificity to Lac-OR-BSA but lower in titer (Figure 2.5a, middle panel and Table 2.1). The pre-bleed serum was much lower in this animal, but again most of the binders in the week 8 serum were unrelated to LNnT. Two of the structures on the array containing LNnT, #330 (Galα1-4Galβ1-4 GlcNAcβ1-3Galβ1-4Glcβ-) and #331 (GalNAcβ1-3Galα1-4Galβ1-4 GlcNAcβ1-3Galβ1-4Glcβ-) were found at low levels in one or two of the pre-bleed sera and were boosted in all three immunization groups, thus these antibodies were probably not a result of the specific immunogens. Antibodies to a few unrelated structures (#400, #304, 502) were also boosted in all three groups (Table 2.1); these could be natural antibodies that are boosted non-specifically by the immunization, and some of these specificities (#304; 330 – blood group P1 antigen) and antibodies to related antigens (#400, 331) have been previously reported in serum from healthy human donors [320].


Lac-OR-BSA Pre-bleed
Lac-OR
331
330
64 65 145 261 436 135 331 330 502 161 169 153 118 403 338 140 191 142 181 179 270 150 283 167 128
LNnT-OR-BSA Pre-bleed
LNnT-OR-
331
64 65 145 261 436 135 331 330 502 161 169 153 118 403 338 140 191 142 181 179 270 150 283 167 128
Sp8
β3 β4
α2
Sp10
LNnT-CR-BSA Pre-bleed
LNnT-CR-
α3 β4
α6
64 65 145 261 436 135 331 330 502 161 169 153 118 403 338 140 191 142 181 179 270 150 283 167 128
CFG Glycan ID #

b)

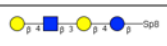
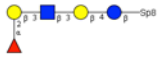
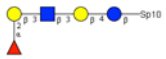
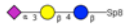
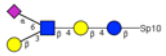
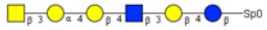
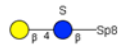
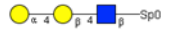
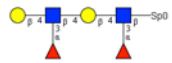
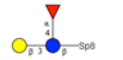
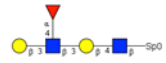
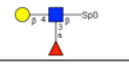
| Chart No. | Glycan Structure | |
|---|---|---|
| **Selected Binders and Non-binders displayed in Figure 2.5:** | | |
| 162 | Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ-Sp8 |  |
| 64 | Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ-Sp8 |  |
| 65 | Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ-Sp10 |  |
| 145 | Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ-Sp10 |  |
| 261 | Neu5Acα2-3Galβ1-4Glcβ-Sp8 |  |
| 436 | Galα1-3Galβ1-4Glc-Sp10 |  |
| 135 | Galβ1-3(Neu5Acα2-6)GlcNAcβ1-4Galβ1-4Glcβ-Sp10 |  |
| 331 | GalNAcβ1-3Galα1-4Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ-Sp0 |  |
| 330 | Galα1-4Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ-Sp0 |  |
| 502 | Galβ1-3GlcNAcα1-3Galβ1-4GlcNAcβ-Sp8 |  |
| 161 | Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ-Sp0 |  |
| 169 | Galβ1-4Glcβ-Sp8 |  |
| 153 | Galβ1-4[6OSO3]Glcβ-Sp8 |  |
| 118 | Galα1-4Galβ1-4GlcNAcβ-Sp0 |  |
| 403 | Galβ1-3GlcNAcα1-6Galβ1-4GlcNAcβ-Sp0 |  |
| 338 | GlcNAcα1-4Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ-Sp0 |  |
| 140 | Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ-Sp0 |  |
| 191 | GlcNAcβ1-6Galβ1-4GlcNAcβ-Sp8 |  |
| 142 | Galβ1-3GalNAcβ1-4Galβ1-4Glcβ-Sp8 |  |
| 181 | GlcNAcβ1-3Galβ1-4Glcβ-Sp0 |  |
| 179 | GlcNAcβ1-3Galβ1-4GlcNAcβ-Sp8 |  |
| 270 | Neu5Acα2-6Galβ1-4Glcβ-Sp8 |  |
| 150 | Galβ1-4(Fucα1-3)GlcNAcβ1-4Galβ1-4(Fucα1-3)GlcNAcβ-Sp0 |  |
| 283 | Neu5Gcα2-6Galβ1-4GlcNAcβ-Sp0 |  |
| 167 | Galβ1-4GlcNAcβ-Sp8 |  |
| 128 | Galβ1-3(Fucα1-4)GlcNAc-Sp8 |  |
| 301 | Galβ1-4GlcNAcα1-6Galβ1-4GlcNAcβ-Sp0 |  |
| 125 | Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4GlcNAcβ-Sp0 |  |
| 171 | GlcNAcα1-6Galβ1-4GlcNAcβ-Sp8 |  |
| 395 | Neu5Acα2-3Galβ1-3GlcNAcβ1-3GalNAcα-Sp14 |  |
| 148 | Galβ1-4(Fucα1-3)GlcNAcβ-Sp0 |  |

**Figure 2.5 Immunized rabbits make a glycan-specific response to closed-ring LNnT-BSA**

Week 8 serum from one rabbit in each immunization group was diluted at 1:100 and screened on the CFG microarray. Selected binders and non-binders are shown here, sorted from high binding to low binding in the LNnT-CR-BSA serum, from left to right on the x-axis. Pictures of the selected structures are listed in b). The glycan ID#s on the X-axis correspond to CFG Chart ID#s. Serum was detected with anti-rabbit IgG-Alexa 488. RFU, relative fluorescence units. (The top binding glycan IDs are listed in table 1; a full listing of glycans on version 4.2 of the CFG can be found at http://www.functionalglycomics.org/static/consortium/resources/resourcecoreh8.shtml

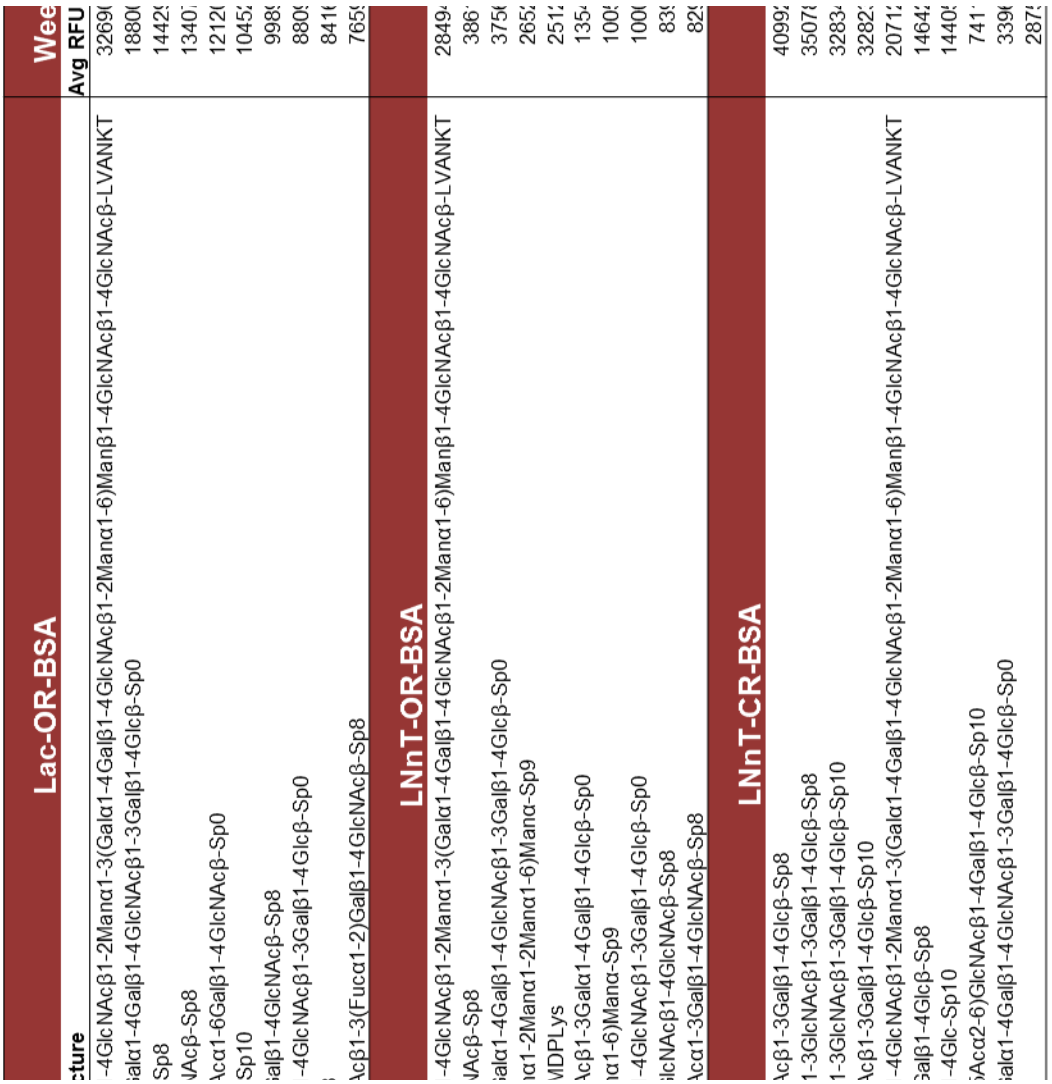



**Table 2.1 Top 10 CFG glycans bound by a rabbit serum from each immunization group**

The top binding glycans detected by CFG array screening of pooled rabbit serum are listed for each immunization, LNnT-CR-BSA (bottom), LNnT-OR-BSA (middle), Lac-OR-BSA (top); a full listing of glycans and linker abbreviations on version 4.2 of the CFG can be found at http://www.functionalglycomics.org/static/consortium/resources/resourcecoreh8.shtml

The LNnT-CR-BSA immunized rabbit was the only sample tested which targeted LNnT (#162) and, with slightly lower magnitude, the isomer LNT (#145) and a fucosylated LNT (#64-65) (Figure 2.5a, bottom panel and Figure 2.5b). These specificities were undetectable in pre-bleed serum. This antisera also targeted three structures (#261, 436, 135) containing internal Galβ1-4Glcβ- moieties just distal to the linker, which were not targeted by the other immunized rabbits. However, the CFG contains many other structures having features in common with LNnT, including GlcNAcα1-4Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ- (#338), GlcNAcβ1-3Galβ1-4Glcβ- (#181) and many Galβ1-4GlcNAc-terminating (ex. #167) and/or Galβ1-4Glc-linked structures (Figure 2.5b), which were negative in the LNnT-CR-BSA antiserum. Interestingly, a version of LNnT attached to a similar but shorter linker (#161) was bound about seventy-fold lower than #162. This could be a result of steric hindrance when the glycan is anchored closer to the slide.

Because glycans on the CFG array are printed in a closed-ring configuration using a linker unrelated to PNPA, this data demonstrates that a portion of the response to LNnT-CR-BSA is indeed specific for LNnT and does not require the linker for binding. The antibodies do appear to require the full tetrasaccharide for binding, but interestingly are permissive to some variations in the non-reducing end (Galβ1-4GlcNAc or Galβ1-3GlcNAc; α1-2 fucosylation) but not others (GlcNAcα1-4).

ELISA studies of the LNnT-CR-BSA-immunized (Figure 2.6a) and LNnT-OR-BSA-immunized (Figure 2.6b) rabbit serum against the various conjugates, where BSA reactivity has been blocked out, supported similar conclusions as the microarray studies. ANOVA with multiple comparisons showed that the week 8 LNnT-CR-BSA immunized rabbit serum was significantly elevated compared to pre-bleed, and was only significantly reactive with LNnT-CR-BSA among the three neo-glycoconjugates coated on ELISA plates. Pooled serum from the LNnT-OR-BSA rabbits was likewise non-cross-reactive with the LNnT-CR-BSA conjugate.

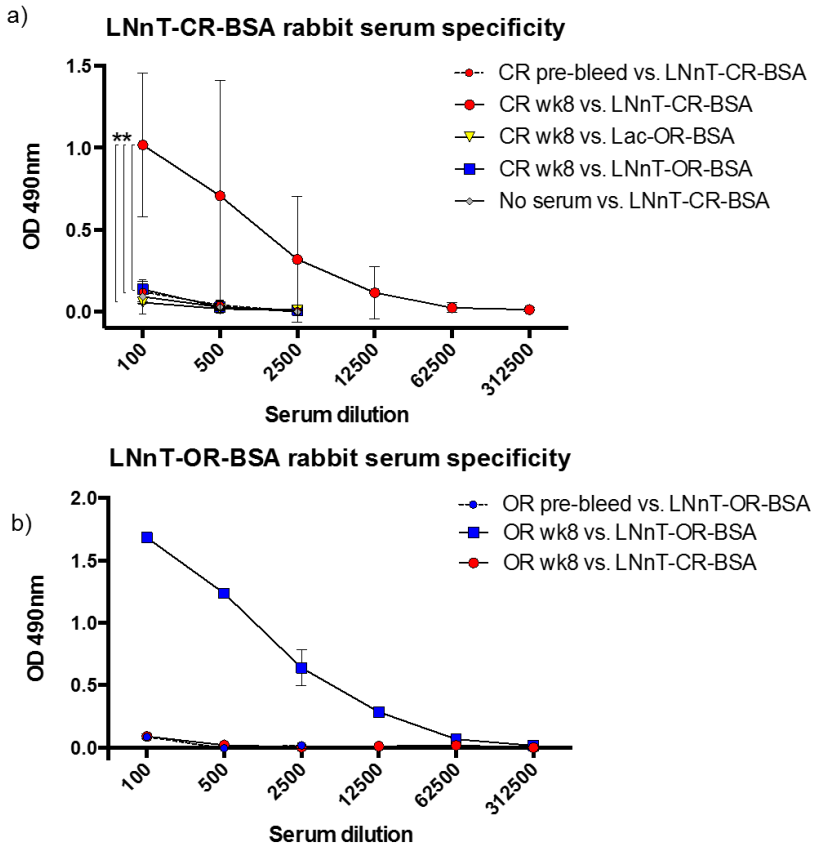



**Figure 2.6 Open- and closed-ring LNnT-BSA rabbit antisera are non-cross reactive with each other.**

Open- and closed-ring LNnT-BSA rabbit antisera are non-cross reactive with each other. ELISA plates were coated with conjugates shown in the legend and interrogated with LNnT-CR-BSA antisera from each rabbit at decreasing concentrations (a). Plates were coated with the conjugates show in the legend and interrogated with LNnT-OR-BSA antisera pooled from two rabbits (b).
2-way ANOVA was performed for the 1:100-1:2500 dilutions, with Dunnett's multiple comparison between the CR wk8 vs. CR pre-bleed, CR wk8 vs. LNnT-OR-BSA and CR wk8 vs. Lac-OR-BSA. ** = P< 0.005, which was significant for the serum vs. coating factor and for the three individual group comparisons shown with brackets. Part a) values are averaged from two rabbits which were each performed in duplicate and it was representative of two experiments. Part b) values are average of duplicates for sera pooled from 2 rabbits. One experiment was performed.

### 2.3.3 Mice immunized with closed-ring LNnT-BSA generate a muted glycan-specific response

As mice are commonly used in vaccine candidate testing, but have in the past been problematic subjects where generation of anti-glycan antibodies is concerned, we next asked

whether mice showed a similar pattern of specificity when immunized with LNnT-BSA. We performed a small pilot study where three mice were immunized in the same fashion as the rabbits. Pooled mouse serum showed a muted IgG response to the sugar-linker compared to the high response made to neo-glycoconjugates when immunized with LNnT-CR-BSA (Figure 2.7a), in contrast to rabbits (Figure 2.7b). A large portion of the IgG appears to be against BSA, with additional responses to the open-ring sugar and/or linker components of Lac-OR-BSA and LNnT-BSA. The IgM response in mice (Figure 2.7c) was similarly composed of antibodies to Lac-OR-BSA and non-specific anti-protein antibodies. While less focused than the IgG response, the rabbit IgM response (Figure 2.7d) also favored LNnT-CR-AEAB and LNnT-CR-BSA, with some IgM binding to the other glycoconjugates and glycoproteins as well.

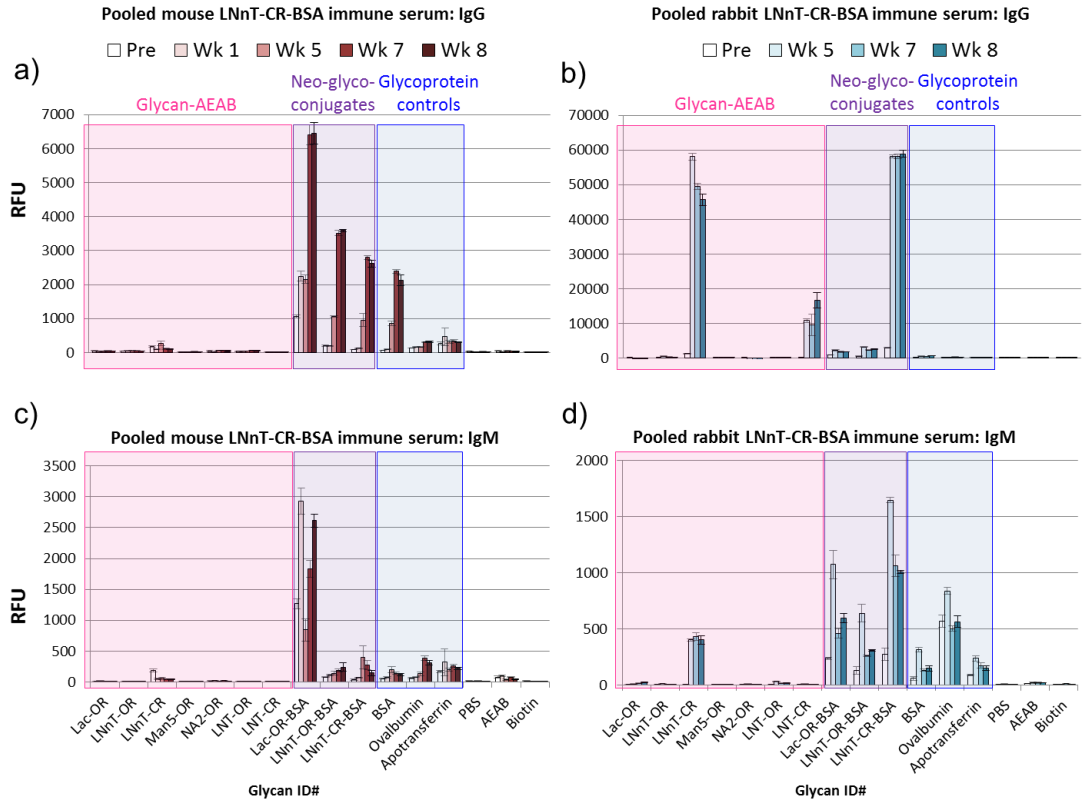



**Figure 2.7 Mice lack glycan-specificity in the response to LNnT-CR-BSA, in contrast to rabbits**

Pooled serum from two rabbits (b,d) and three mice (a,c) was diluted at 1:100 and screened on the BSA-PNPA glycan microarray for IgG (a,b) and IgM (c,d). Serum was detected with goat anti-mouse IgG-Alexa 568, anti-mouse IgM-Alexa 488, and anti-rabbit-Alexa488 at 5μg/mL. Bars correspond to mean of hexareplicate spots +/- standard deviation. RFU, relative fluorescence units; Pre, pre-bleed; Wk 1 – Wk 8, Week 1 – week 8 bleeds. Pink, AEAB-derivatized glycans; purple, BSA neoglycoconjugates; blue, glycoprotein controls. X-axis labels correspond to the structures depicted in figure 2. Note that signals reach the detection maximum near 60,000 RFU.

### 2.4 Discussion and Conclusions

The human immune system responds to eukaryotic glycan antigens during infections, cancer, and autoimmune disorders. However, there is little precedent on how to design vaccines targeting eukaryotic glycan epitopes, which are often non-repeating, may occur at the reducing or non-reducing end (core) of the glycan, and may share some features with mammalian glycans. Epitopes at the non-reducing end of a glycan, for example, might be disrupted by conventional glycoconjugate linkage methods such as reductive amination. The purpose of this study was to compare the effect two different linkage methods, one which opens the ring at the non-reducing end of the glycan and one which creates a longer linker, leaving the ring closed, on the specificity of the vaccine response. We found that these two linkage methods indeed elicited distinct immune responses, indicating that the reducing end of the sugar and linkage type may be crucial for some vaccine epitopes.

Rabbits immunized with open-ring Lac- and LNnT- as well as closed-ring LNnT-CR-BSA conjugates displayed robust IgG responses that were different for each immunogen, as shown by glycan microarray studies. All three immunizations generated a response that was at least in part specific to a “neo-epitope”, created by a combination of the reducing end of the sugar and linker, and probably a portion of the protein carrier as well. However, only the LNnT-CR-BSA immunization resulted in a response that preferred the closed-ring tetrasaccharide and was not cross-reactive with the other sugars or conjugates on the BSA-PNPA array. Low-level binding to LNT on the BSA-PNPA indicated a portion of the response targets the reducing end of

the sugar and linker and was impartial to the non-reducing end linkage. The LNnT-CR-BSA serum also bound LNnT and LNT on the CFG microarray, where a different closed-ring linker was used, indicating that the response was glycan-specific. Agalacto-LNnT was not bound, indicating that the tetrasaccharide backbone was necessary for antibody binding; however, not all modifications of LNnT were bound. The open-ring conjugate immune serum, in contrast, did not bind LNnT or LNT on the CFG array. Thus, LNnT-CR-BSA was most successful at inducing a glycan-specific response to the full tetrasaccharide, which was the desired epitope.

LNnT is a human milk glycan, which contains N-acetyllactosamine (LN), a common determinant of mammalian glycans. The CFG contains many LN-terminating structures, but immunization with either LNnT-CR- or LNnT-OR-BSA did not induce antibodies to LN-containing structures, which could be because it is seen as a self-like antigen in rabbits. Normal human serum and IVIG have previously been shown to contain antibodies to many self-like determinants, including LNnT and LNT [320], [321]. However, the naïve rabbit serum in our study did not bind these glycans, indicating that the immune response seen was likely a result of the LNnT-CR-BSA immunization, and rather than an induction of pre-existing antibody.

Furthermore, ELISA studies indicated that the responses elicited by the open-ring and closed-ring conjugates were not cross-reactive with each other. The reducing end of the sugar and linker must have a profound effect on conjugate antigenicity for two very similar molecules to produce non-overlapping responses. Previous studies on rabbits immunized with open-ring conjugates of sialylated human milk oligosaccharide and albumin showed that antisera often targets the reducing-end and linker region, and sometimes also possessed specificity for the non-reducing end of the sugar, depending on which sugar is used [307], [308]. For example, inhibition of binding of polyclonal anti-sialyllactose sera to its immunogen required a lactose sialylated in the same (either α2-3 or α2-6) linkage. However, immune serum to sialylated LNnT was effectively inhibited by derivatized lactose, which lacks the non-reducing end N-acetyllactosamine and sialylation. Another study which linked closed-ring oligosaccharide

epitopes to a viral scaffold for immunization of chickens showed that the specificity of polyclonal responses was comparable to that of monoclonal antibodies [322]. It could be that closed-ring linkage of the glycan to protein carrier is itself less antigenic, allowing the response to target the glycan epitope. However, some closed-ring conjugation strategies can also create an antigenic neo-epitope which suppresses the response to the glycan [323]. Alternatively, in light of the recently-discovered ability of B-cells to present glyco-peptide species from glycoconjugates to T-cells [317], perhaps open-ring glycopeptides are less apt to be presented by MHC and result in a less robust T-dependent response, which would diminish affinity maturation of anti-glycan antibodies.

In contrast to rabbits, three mice of two different strains did not make a glycan-specific response to the closed-ring conjugate. A large portion of the IgG appears to be against BSA, with the remainder preferring the Lac-OR-BSA conjugate, suggesting that the primary epitopes are the aromatic amine-amide-BSA linkage and contained on the BSA molecule itself. It is possible that the preference for Lac-OR-BSA over LNnT-BSA is due to its higher sugar:protein ratio, or that the antibodies bind preferentially to a terminal lactose. The mice also made IgM, which preferred Lac-OR-BSA and was not sugar-specific, in contrast to rabbits where both IgG and IgM demonstrated specificity for the closed-ring linked tetrasaccharide. Thus, the inability of mice to respond to the sugar is not due to an inability to class-switch, but could be due to an absence of this specificity in the immune repertoire, a difference in presentation of the glycoconjugate, or other yet undiscovered immune factors. This result remains to be confirmed in a more highly-powered experiment and should be investigated in different mouse strains.

Interspecies (goat vs. mouse, rabbit vs. mouse) as well as mouse inter-strain differences in the ability to mount a glycan-specific antibody response to both native glycoproteins and synthetic glycoconjugates have been previously noted in the literature [311], [324]–[329]. The oligosaccharide content of mammalian milk varies drastically among species, with human milk oligosaccharides such as LNnT occurring at 100-1000-fold lower levels in cow’s milk, for

example (reviewed in Bode, 2012). One explanation for the observed differences between rabbits and mice could therefore be a differentially tolerized immune repertoire due to differences in milk oligosaccharide composition. Alternatively, there could be gaps in the murine immune repertoire for other reasons, or mechanistic deficits in the ability to respond to oligosaccharide conjugates. Though we used outbred Swiss Webster mice in our studies, a deficit in the ability to present certain types of glycopeptides could conceivably result from decreased variation at the MHC locus, as is seen in inbred mice, which are more commonly used for glycoconjugate immunization studies. Understanding these strain- and species-specific differences, and how they are similar or different from the human immune response, should be a priority as the choice of animals for testing can determine the success or failure of vaccine candidates.

In summary, we have demonstrated that the type of linkage between a glycan epitope and its carrier protein can profoundly impact the specificity of the immune response. Particular attention should be paid when the glycan epitope is small and opening of the non-reducing end ring could result in destruction or alteration of the desired epitope. Closed-ring linkage was preferable for preservation of the antigenicity of the LNnT tetrasaccharide, and further research studies should address whether this is more generally applicable to eukaryotic glycans in the context of other glycoconjugate vaccines.

## 2.5 Acknowledgements

The authors would like to thank the Consortium for Functional Glycomics for use of the mammalian glycan array.

# Chapter 3. Immunization with recombinantly expressed glycan antigens from *Schistosoma mansoni* induces glycan-specific antibodies against the parasite

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## 3.1 Introduction

Human helminthiases, infections caused by multicellular parasitic worms, affect 1-2 billion people and may account for up to 100 million disability-adjusted life years [3], [4], [14]. The most common are hookworm and schistosomiasis, with *Schistosoma mansoni* being one of the most widespread schistosomes [3], [23]. With the poorest 20% of the world's population infected by helminths, treatment needs are far from being met [5], [15]. To date, no vaccines against helminths or any human parasites are in use. Attenuated or killed organisms are impractical as vaccine solutions because of complex helminth life cycles, and several recombinant protein candidates have been tried without success [29], [34]. Thus, we are in need of novel scientific approaches to developing vaccines for these complex pathogens.

Helminth glycans represent a major untapped reservoir of vaccine candidates. Larvae, adult worms and eggs produce an abundance of glycoconjugates on their surfaces and in secretions that are exposed to the host immune system. The *S. mansoni* cercarial glycocalyx is roughly 80% carbohydrate by weight [45], [239]. Glycans are effective vaccine targets for many encapsulated bacterial pathogens [331], [332], however, development of such vaccines has been

largely empirical and no precedent exists for how to design vaccines targeting eukaryotic glycoconjugates. Parasite glycans contain many structures and/or modifications which are foreign to the host's immune system (recently reviewed in [1]). In contrast to bacterial polysaccharides, however, they may have core structures or other structural features in common with their mammalian hosts. We currently lack a thorough understanding of the structural basis for antigenicity and immunogenicity of eukaryotic glycans.

In schistosomiasis, glycans behave both as immunomodulators and antigens. The infection is initiated when water-born cercariae penetrate mammalian skin, transform into schistosomula (larvae) and migrate into the vasculature. Over the course of about six weeks, they grow into adult worms which lay eggs in the mesenteries. Schistosome egg glycoconjugates are instrumental in biasing the immune response towards a Th2 phenotype and inducing the eosinophilic granulomas which characterize the pathology of this disease [181], [207], [211], [254], [255], [333]. In *S. mansoni*-infected humans, primates and mice, the majority of the antibody response is directed against glycans [194], [195], [241], [242]. Anti-glycan antibodies are able to kill helminths *in vitro* and/or protect against infection in several models of helminth infection when passively transferred [86], [148], [151], [251], [252], [259], [261]. However, the protective ability of anti-glycan antibodies appears to be highly dependent on the particular molecular target and the isotype/subisotype of antibody [115], [151], [334]. In order to exploit helminth glycans as vaccine targets, we must identify those associated with protection and learn how to mimic the parasite's immunogenic presentation of such glycans so as to elicit protective antibody isotypes.

The LacdiNAc (GalNAcβ1,4GlcNAc; LDN) family of glycans, especially LDNF (GalNAcβ1,4(Fucα1,3)GlcNAc) and several multiply-fucosylated variants, are highly targeted in anti-schistosomal responses [48], [132], [144], [163], [335]. Antibodies to LDNF have been identified in the sera of *S. mansoni*-infected humans, mice and importantly, brown rats and rhesus monkeys, which are models of natural protective immunity ([144], [148], [163], [175] and

unpublished data). Although it is yet unknown whether they directly contribute to protection *in vivo*, a monoclonal antibody to LDN kills schistosomula *in vitro*, and antibodies to LDNF are found in high titer in rhesus and mouse antisera that are lethal to schistosomula [148], [175]. Several multi-fucosylated versions of LDN(F) appear to be completely unique to schistosomes [106], [158], [335]. For these reasons, we believe that the LDN(F) family of antigens includes potential vaccine and diagnostic candidates.

Though the antigenicity of the LDNF is well-established, it is yet unclear which particular glycoconjugates and/or structural variations of the epitope provoke this abundant antibody response. LDNF is expressed by intramammalian and intramolluscan schistosome stages and, importantly, is found on the surface of larvae, the most immunologically vulnerable stage, and adult worms, which are cleared in some animal models [48], [50], [88], [144], [146], [148], [243]. Both glycoproteins and glycolipids in adult worms and eggs express LDNF, and importantly, male worms express polymeric repeats of LDN and LDNF on their N-glycan antennae [48], [145], [161], [167]. In contrast, the expression of LDNF in mammals is rare, occurring strictly as a monomer on N-glycans of only a few known proteins [136], [156], [336]. Determining the optimal mode of presentation of parasite glycans in vaccines has been virtually impossible due the current limitations of synthetic technology to replicate certain aspects of natural glycan presentation. Additionally, many antigenic schistosome glycans, such as poly-LDNF, are the result of glycosyltransferase activities and target specificities unique to the parasite, which are ill-defined and cannot be replicated *in vitro*. Thus, if anti-glycan vaccines are to be useful, it will be necessary to develop a vaccine platform that allows “native-like” expression and presentation of pathogen glycoconjugates, where well-defined variations in glycan structure and presentation can be made for the purposes of studying glycoconjugate immunogenicity and glycan antigen discovery.

We hypothesized that expression of schistosome glycan epitopes in a mammalian expression system would recapitulate the immunogenicity and antigenicity of these epitopes

generated by the parasite itself. Whole cells and cell membrane preparations have often been used as immunogens for the production of polyclonal and monoclonal antibodies to specific surface glycans [337]–[340]. Whole cell vaccination has more recently been pursued as a tumor vaccination strategy, with both autologous and allogeneic cancer cells as well as dendritic cells (DC) and malignant cell-DC fusions all under clinical investigation (reviewed in de Gruijl *et al.* 2008; Milani *et al.* 2013; Palucka and Banchereau 2013; Srivatsan *et al.* 2013). Chinese Hamster Ovary (CHO) cells are a model system for glycosylation pathways and can be readily manipulated to produce a variety of glycans that are very similar to the native parasite antigens, in a high-density, membrane-bound presentation. In previous work by our lab, *C. elegans* β1,4-N-acetylgalactosaminyltransferase (B4GALNT1) and human α1,3-fucosyltransferase 9 (FUT9) were transfected into the stable mutant CHO cell line, Lec8, to generate Lec8-GalNAcT (L8-GT) and Lec8-GalNAcT-FucT (L8-GTFT) cells (Figure 3.1) [168], [345]. These cell lines produce polymers of LDN and LDNF on N-glycans as terminal extensions on complex-type structures, which we will refer to as poly-LDN and poly-LDNF. In this study, we have shown that immunizing mice with these cells elicits abundant and highly specific antibodies to LDN and LDNF, the latter of which are sustained for at least 20 weeks after a booster immunization, and bind to parasite glycans. We have thus validated the approach of using recombinant cellular expression of parasite glycans for future use in vaccine development for this very important class of pathogens.

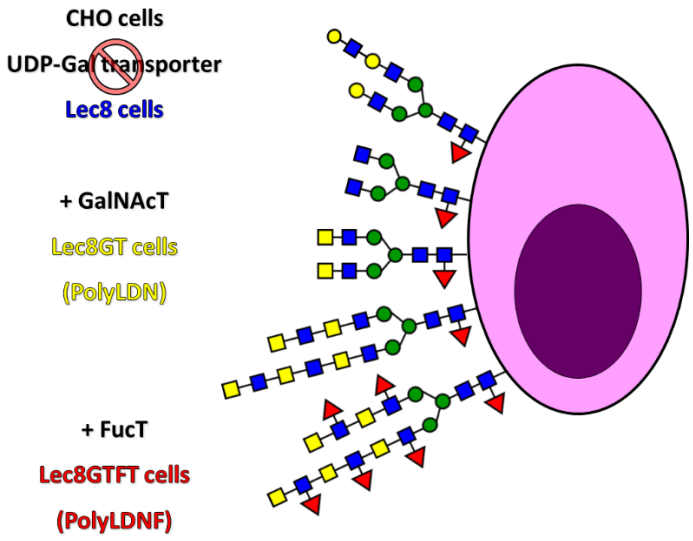


**Figure 3.1 Engineering of Chinese Hamster Ovary cells to produce poly-LDNF.**

The Lec8 mutant of CHO cells was engineered with *C. elegans* β1,4-N-acetylgalactosaminyltransferase (B4GALNT1) and human α1,3-fucosyltransferase 9 (FUT9), to generate Lec8-GalNAcT (L8-GT) and Lec8-GalNAcT-FucT (L8-GTFT) cells

## 3.2 Materials and Methods

### 3.2.1 Materials.

General chemicals and glycosyltransferase enzymes were purchased from Sigma (St. Louis, MO) or Fisher Scientific (Pittsburg, PA) unless otherwise noted.

#### 3.2.1.1 Cell culture reagents

DMEM, G418 sulfate and sterile PBS without calcium or magnesium were purchased from Cellgro (Manassas, VA). Fetal bovine serum was purchased from Atlanta Biologicals (Lawrenceville, GA). Glutamine, penicillin/streptomycin and 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA) were purchased from Gibco (Grand Island, NY). Zeocin was purchased from Invivogen (San Diego, CA). Tissue-culture treated flasks were purchased from Corning (Corning, NY). Kifunensine was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). Petri dishes for schistosomula culture were obtained from Falcon (Franklin Lakes, NJ).

3.2.1.2 Animal work

Luer-Lok syringes were obtained from Becton Dickinson & Co. (Franklin Lakes, NJ). Precision Glide needles were obtained from Fisher Scientific (Suwanee, GA). Sterile Goldenrod lancets were obtained from Medipoint (Mineola, NY, USA). Microtainer polymer gel serum collection tubes were obtained from Becton Dickinson & Co. (Franklin Lakes, NJ).

3.2.1.3 Antibodies and flow cytometry

Peroxidase-conjugated goat anti-mouse IgG (γ) and IgM (μ) were purchased from KPL (Gaithersburg, MD) and Sigma (St. Louis, MO). AlexaFluor-488- and AlexaFluor-633-conjugated secondary goat anti-mouse IgG (H+L) and IgM (μ) were purchased from Invitrogen/MP (Eugene, OR). Mouse $IgG_1$ isotype control was purchased from R&D Systems (Minneapolis, MN). Polystyrene tubes for flow cytometry were purchased from BD Falcon (Franklin Lakes, NJ).

3.2.1.4 Parasite isolation, lysates and Western blots

Complete MINI protease inhibitor tablets (EDTA free) were purchased from Roche Diagnostics (Mannheim, Germany). 70μm nylon cell strainers were purchased from BD Falcon (Franklin Lakes, NJ). Percoll was purchased from GE healthcare. Potassium chloride was obtained from J.T. Baker Inc. (Phillipsburg, NJ). Amicon Ultra-4 centrifugal filter devices were purchased from Millipore (Tullagreen, Carrigtwohill CO, CORK, Ireland). The PNGaseF kit was purchased from New England BioLabs (P0704L). Mini-PROTEAN-TGX 4-20% gels for SDS-PAGE, Kaleidoscope Precision-Plus Protein standards, the Trans-Blot Turbo system, and accompanying nitrocellulose membranes, filter paper stacks and transfer buffer were purchased from Bio-Rad (Hercules, CA, USA and membranes, Munich, Germany). Non-fat dried milk was obtained from the grocery store (Publix brand). Pierce BCA protein assay kit, Pierce 660nm protein assay kit, and SuperSignal West Pico and Femto Chemiluminescent substrates were purchased from Thermo Scientific (Rockford, IL). 4x NuPAGE SDS sample buffer was

purchased from Invitrogen (Carlsbad, Germany). Metal sieves (425µm, 180µm, 106µm, 45µm, 20µm) were obtained from VWR Scientific (West Chester, PA).

3.2.1.5 Preparation of glycans and microarrays

2-amino(N-aminoethyl) benzamide (AEAB) was synthesized from as previously published [346]. Chicken glycopeptides (NA2) was prepared from eggs as previously published [347]. Chitin hydrolysate, E-PHA agarose and biotinylated lectins (ConA, AAL, WFA, GSL-II) were purchased from Vector Labs (Burlingame, CA). Chitin oligosaccharides were then fractionated by normal phase HPLC. GDP-Fucose (GDP-Fuc), UDP N-Acetylgalactosamine (UDP-GalNAc) and UDP-N-Acetylglucosamine (UDP-GlcNAc) were purchased from Kyowa Hakko Kogyo Co. (Tokyo, Japan). Affigel 10 was purchased from Bio-Rad (Hercules, CA). IgG to the HPC4 epitope tag was purified from supernatant, kind gift from Dr. Chuck Esmon (University of Oklahoma Health Sciences Center). Neuraminidase, Protein A agarose, and shrimp alkaline phosphatase (SAP) were was purchased from Roche Diagnostics (Mannheim, Germany). Recombinant *H. pylori* β1,3-N-acetylglucosaminyltransferase was a kind gift from Warren Wakarchuk [348]. Guanidine hydrochloride and iodoacetamide were purchased from Acros Organics (New Jersey, USA). TPCK-treated trypsin was purchased from Worthington Biochemical Corp (Lakewood, NJ). Dialysis tubing was obtained from Spectrum (Rancho Dominguez, CA). Sep-Pak cartridges (reverse phase $C_{18}$) were purchased from Waters (Milford, MA, USA). Carbograph columns and slide separation chambers were purchased from Grace Davison Discovery Sciences (Deerfield, IL). NHS slides were purchased from Schott (Elmsford, NY). Tris-HCl was obtained from ProMega (Madison, WI).

3.2.2 Methods

*3.2.2.1* Animals and Infection with *S. mansoni*

Female Swiss-Webster mice (6-8 weeks old) were used for immunization. *S. mansoni*-infected mice were obtained from the Schistosomiasis Resource Center of the Biomedical

Research Institute in Rockville, MD. Female Swiss-Webster mice (4-6 weeks old) from Taconic Farms were infected with an average of 30 (low dose) or 200 (high dose) cercariae per mouse, and shipped to our facility. Infected mice were monitored for abdominal distention and piloerection and sacrificed if experiencing excessive stress. High dose-infected mice were sacrificed at 7.5 weeks post-infection for collection of adult worms and eggs, and low-dose infected mice were sacrificed at 20 weeks post infection, after obtaining acute infection antisera (8 weeks post-infection) and chronic infection antisera (20 weeks post-infection). All sera were obtained by facial vein puncture except the chronic infection sera, which was obtained by cardiac puncture immediately following euthanasia. At the conclusion of our experiments, all immunized mice were euthanized by carbon dioxide inhalation followed by cervical dislocation, and all infected mice were euthanized by intraperitoneal overdose with 300µl of 65mg/ml sodium pentobarbital with 200 U/ml heparin sodium salt. All experiments involving mice were approved by the Emory University IACUC.

3.2.2.2 Isolation of *S. mansoni* life stages

*Schistosoma mansoni*-infected *Biomphalaria glabrata* snails were obtained from the Schistosomiasis Resource Center of the Biomedical Research Institute in Rockville, MD and maintained under conditions specified in unit 19.1 of Current Protocols in Immunology, "Schistosomiasis" [349]. *S. mansoni* cercariae and schistosomula were obtained from infected snails as per the above literature with some modifications. Briefly, snails maintained in a dark room were placed into beakers of conditioned water, under a bright light, for two hours. The water was filtered through 70µm nylon cell strainers to collect cercariae. After counting, the cercariae were incubated on ice for 30 min, and centrifuged at 500xg for 10 minutes at $4^0$C. For transformation to schistosomula, the cercarial pellet was resuspended in 5-10mL of cold DMEM and vortexed on high for 2x 45 second periods, with a 3-minute ice incubation in between; or the pellet was resuspended to 1000 cercariae/mL in cold DMEM and transformed by passage 8 times though a syringe and 22-G needle, in 10 mL batches. The suspension of detached schistosomula

and tails was allowed to settle and the bottom few mL were loaded onto a 40 or 60% Percoll gradient in DMEM. The gradient was centrifuged for 15 minutes at 500xg, $4^0$C. The supernatant was carefully removed down to the bottom 1-2 mL, and the schistosomula pellet was washed 3 times by spinning at 300xg in cold DMEM. The schistosomula were cultured for 3 days in DMEM with 10% FBS, penicillin/streptomycin at a density of 500-1000 organisms/mL in tissue-culture dishes. For preparation of adult worms and eggs, 50 high-dose infected Swiss-Webster mice were sacrificed after 7.5 weeks of infection. The mesenteric veins were perfused with saline (0.85% sodium chloride, 0.75% sodium citrate) as described previously [349]. Adult worm pairs were centrifuged twice at 1000 rpm in ultracentrifuge bottles, and three times in 15mL conical tubes in a table-top centrifuge at 200 rpm, washing with perfusion fluid in between spins, at $4^0$C, until the worms were well washed. The loosely pelleted worms were then snap-frozen in a minimal amount of perfusion fluid and stored at $-80^0$C. The livers were removed from each mouse immediately following perfusion and stored in cold 1.2% NaCl solution overnight to prevent hatching of eggs. The livers were cut into 1-2 cm sections, processed in a Waring blender, and then loaded onto a series of 4 sieves, as described in the above literature. Eggs were washed through to the bottom sieve, and then swirled in petri dishes to further purify them from debris. All work with *B. glabrata* and *S. mansoni* was approved by the Emory University Office of Occupational Health and Safety, and conducted in BSL-II animal surgery facilities and laboratories.

3.2.2.3 Cell lines and preparation of immunogens

Chinese Hamster Ovary (CHO) (Pro-5) cells were obtained from ATCC (CRL-1781). CHO Lec8 cells were obtained from ATCC (CRL-1737). Transfections with glycosyltransferases, selection of stable, clonal cell lines and confirmation of glycosyltransferase activity and N-glycan expression were performed as detailed in Kawar *et. al* [168]. Cells were thawed from $-135^0$C and grown adherently in tissue culture-treated 75cm$^2$ flasks with complete DMEM (10% heat-

inactivated FBS/2mM glutamine/100U/mL penicillin/streptomycin). Lec8-GT cells were additionally cultured with 0.6mg/ml G418 sulfate, and Lec8-GTFT cells, with G418 and 0.4mg/ml zeocin. For routine maintenance they were grown to 1-2x$10^7$ cells/flask and detached for 2 minutes in 0.25% trypsin/EDTA, which was quenched with complete DMEM and split 1:10-1:20, twice per week. To prepare cells for immunization, they were grown in 225$cm^2$ flasks to 90% confluence, washed extensively with cold PBS, and then detached by scraping in a minimal volume of cold PBS. The volume was then brought to 50mL and cells were centrifuged for 5 minutes at 1000rpm. The cell pellet was resuspended in 10mL cold PBS, counted, and density was adjusted to 2.5 x $10^6$cells per 200µl immunization dose. They were frozen at -$20^0$C until use. Kifunensine was dissolved in DMSO to 1mg/mL and stored at -$20^0$C. Cells were grown for 8 days in complete DMEM with kifunensine at 10ug/mL, with two splits into fresh kifunensine-containing medium during that period, and then prepared for flow cytometry or lysate as described below. The lack of complex N-glycan expression was confirmed by staining with Concanavalin A (ConA) and anti-glycan monoclonals on flow cytometry, before staining with antisera.

3.2.2.4 Antibodies

Mouse monoclonal IgGs anti-LDN and anti-LDNF were produced from *S. mansoni*-infected mice and purified as described [144], [147], [350].

3.2.2.5 Immunization and serum collection

Cellular immunogen preparations in PBS were thawed 30 minutes before use and gently vortexed to resuspend. 200µl containing 2.5 x $10^6$ freshly-thawed cells in PBS was delivered to each mouse via intraperitoneal injection. Mice were monitored for 30 minutes after injections and the following day for signs of distress and sacrificed if necessary. Primary cellular immunizations **(Fig. 3a)** were delivered at 0, 2 and 4 weeks. At week 19, secondary (or primary, as a control) immunizations were delivered. 50-100µl of blood was collected from the facial vein of each

mouse using sterile lancets and centrifuged in Microtainer serum collection tubes for 6 minutes at 6000rpm to isolate serum. For immunized mice, bleeds were taken the day before each primary immunization and 2 weeks after the last, the day before the secondary immunization, and 1 and 2 weeks after, and finally, at 40 weeks, after which all of the mice were euthanized. Serum was stored at -20$^0$C either as individual mouse aliquots or in aliquots pooled from 5-10 mice per group. For Western blotting, flow cytometry and glycan array experiments, serum was thawed at 4$^0$C and used within 2 weeks of thawing.

3.2.2.6 Flow cytometry

Batches of 5-10x10$^7$ cells were detached with trypsin as described above, quenched and then washed 3 times by centrifugation at 600xg for 7 minutes, followed by resuspension in 50mL of ice-cold, sterile PBS. After each wash, a 25mL serological pipette was used to bring the cells to single-cell suspension. Cell count and viability were recorded before the last spin and then cells were resuspended in 37.5mL cold PBS, and 12.5mL of 8% paraformaldehyde was added, for a final concentration of 2%. They were rotated in the dark, overnight at 4$^0$C. Fixed cells were then washed 3x with 50mL cold PBS as above. They were stored at 4$^0$C, with 10$^7$ cells/mL, in PBS with sodium azide 0.1%, for up to 4 months without any changes noted on flow cytometry. For flow cytometry experiments, 150µl of cold PBS containing appropriately diluted antibody, lectin or serum sample (1:100) was added to each 5mL polystyrene tube on ice and then gently mixed with 50µl containing 5x10$^5$ fixed cells. Incubations were for 45 minutes, in the dark, on ice. The samples were brought up with 1mL cold PBS before centrifuging for 5 min. at 1000rpm, 4$^0$C, with brakes on 5 out of 10. Supernatants were decanted and the cell pellet resuspended in 200µl of the appropriate secondary detection reagent diluted at 1:1000 – goat α-mouse IgG-488 or 633, gαmIgM-488, or a mixture of the two for serum and monoclonal antibodies, or streptavidin-488 for biotinylated lectins. After the secondary incubation and centrifugation as above, cells were resuspended in 500µl cold PBS for analysis on the FACSCalibur using CellQuestPro acquisition

software. Each stain was performed in duplicate and 10,000 events were collected per sample using FL-1 for -488 and FL-4 for -633 with no compensation. Data was analyzed using FlowJo software by gating on the live cell population in SSC vs. FSC and recording geometric mean fluorescence intensity of FL-1 or FL-4 for each live cell population. Statistical analysis was performed using GraphPad Prism using 1-way or 2-way ANOVA, with or without repeated measures, and using Tukey's or Dunnett's post-test multiple comparisons, as indicated in each figure legend. Alpha of 0.05 was used as the cutoff for significance and levels of significance are specified in the figure legends.

3.2.2.7 Preparation of cell and parasite lysates

Lysates of Lec8, L8-GT and L8-GTFT cells were made by detaching two 75cm$^2$ flasks of 90% confluent cells as detailed above, quenching, and washing 3 times with 5mL room-temperature sterile PBS. After removing supernatant, cells were resuspended in approximately 100µl per 4x10$^6$ cells of freshly thawed lysis buffer (100mM sodium cacodylate pH7.0/1.5% triton X-100/1 tablet complete MINI protease inhibitor per 10mL buffer in ddH20), vortexed vigorously for 30 seconds, and incubated for 40 minutes on ice, vortexing once in the middle. The lysate was centrifuged for 10 minutes at 4000rpm at 4$^0$C to remove cell debris. The supernatant was aliquotted for storage at -20$^0$C, quantified via BCA assay, and used for Western blotting within 2 weeks of thawing. N-glycan removal from the cell lysate was performed according to the manufacturer's protocol, using 2.5µl PNGaseF enzyme for 50ug of lysate. After inactivating the enzyme, lysates were stored at -20$^0$C until use. For preparation of cercarial lysate, after spinning the chilled cercariae as describe above, the supernatant was removed and the pellet was transferred to 1.5 mL Eppendorf tubes in at most 50 µl of parasite lysis buffer (50mM Tris buffer pH 8.0, 2.5% 2-mercaptoethanol, 1% Triton-X-100, 1mM EDTA and 1 tablet of Complete Mini Protease Inhibitor per 10mL of lysis buffer) per 10,000 cercariae. This was vortexed, boiled for 15 minutes (vortexing once during the boiling incubation), and centrifuged at 20,000xg for 2

minutes. The supernatant was removed to clean tube, and a small amount of lysis buffer was added to the pellet for another 10 minutes boiling incubation, after which the spin was repeated and the supernatants were pooled. After culture, schistosomula were washed three times in cold PBS and then lysed as described above for cercariae, using at most 25 µl of parasite lysis buffer per 10,000 schistosomula. Adult worm lysate was made by bringing a freshly-thawed adult worm pellet of approximately 0.5 mL up in 5mL of PBS, spinning at 500xg for 10 min at $4^0$C, and adding 3 mL of lysis buffer to the pellet. The worm lysate was made as described above except 1% SDS and 1mM phenylmethylsulfonylfluoride (PMSF) were used instead of Triton-X-100 and the Complete Mini tablet, respectively, and the SDS was salted out using 100mM KCl so the lysates could be concentrated in Amicon spin filter tubes (3000 Da MWCO). Triton X-100 was then added back to 1% to the concentrated adult worm lysates. Soluble egg antigen was prepared as per unit 19.1, "Schistosomiasis," of Current Protocols in Immunology[349]. Parasite lysates were quantified by the Pierce 660nm protein assay and stored in aliquots at $-80^0$C. For experiments where both cell and parasite lysates were compared, their concentrations were standardized by including SEA in both the BCA and Pierce 660 assays.

3.2.2.8 Western blots

For SDS-PAGE and Western blotting, 5-12µg of cell or parasite lysates (for each experiment the gel was equally loaded) were boiled in 1x NuPAGE SDS sample buffer + 2.5% β-mercaptoethanol for 10 minutes and then run in 10- or 12-well Mini-PROTEAN-TGX gels at 200V for 30 minutes, with 7µl of protein standards. Protein was transferred to a nitrocellulose membrane using the 10-minute High Molecular Weight program in the Trans-Blot Turbo semi-dry transfer system. All subsequent incubations were shaking at ambient temperature. Membranes were stained with 0.1% Ponceau S in 5% acetic acid to check for equal loading and transfer, and destained with TBS wash buffer (20mM Tris, 300mM NaCl, 0.1% Tween-20). For staining with serum, membranes were blocked for 2h or overnight in 2-3% (w/v) milk (de-fatted dried milk in

20mM Tris, 300mM NaCl). Incubations with serum (1:500 to 1:1000 dilutions; same dilution is used where multiple serum samples are compared on the same date) were 1hr in milk diluent (0.5-1% milk in 10mM Tris, 150mM NaCl, 0.1% Tween-20). The membranes were then washed 3 times quickly and 3 times for 10 minutes each in TBS wash buffer. Secondary detection antibodies (HRP-conjugated goat anti-mouse-IgG or –IgM) were added for one hour at 1:3000-5000 in milk diluent. The same wash procedure was repeated, and then SuperSignal West Pico or Femto Chemiluminescent Substrate was added for 30 seconds. The membranes were dabbed dry and exposed to film for 1s-3min (panes shown from the same date used the same substrate and exposure time). When blotting was performed only with monoclonal antibodies and not with serum, a similar protocol was followed except membranes were blocked for 1hr or overnight in 5% bovine serum albumin fraction V (BSA), primary and secondary incubations were in 5% BSA diluents with the secondary antibody at 1:10000, and three washes were performed for five minutes each after each incubation.

3.2.2.9 Chemo-enzymatic synthesis of array glycans

Biantennary N-glycopeptides were generated as described in Luyai *et al.* [175]. All other glycan starting products (LNnT, NA2, chitotriose, released IgG N-glycans) were conjugated to 2-amino(N-aminoethyl) benzamide (AEAB) at the reducing end, as described previously [247], before further modification with glycosyltransferases. Recombinant HPC4-tagged β1-4N-acetylgalactosaminyltransferase (B4GALNT) and human α1-3 fucosyltransferase 6 (FUT6) were cloned and expressed in SF9 cells as previously described [345], [351]. Anti-HPC4-linked Affigel 10 beads were prepared according to the manufacturer's instructions, by incubating for 4 hours with 1mL of a 2 mg/ml αHPC4 IgG solution in 100mM 3-(N-morpholino)propanesulfonic acid (MOPS) buffer pH7.5, at $4^0$C, and then washed and stored at $4^0$C in 50mM sodium cacodylate, pH7, with 0.2% sodium azide. The beads were washed three times with 100mM sodium cacodylate, pH7 with 2mM calcium chloride and 0.1% sodium azide and then rotated for 2 hours, slowly, at ambient temperature with freshly-thawed supernatant containing the recombinant

B4GALNT or FUT6 from clarified SF9 cell medium. A ratio of approximately 60:1 (v/v) supernatant to beads was used. The supernatant was then removed and beads washed three times as above before combining with the glycan acceptor in the reaction mix as specified below.

To synthesize LDN, galactose-terminating AEAB-conjugated glycans were digested with β-galactosidase (β-gal) in 30mM sodium acetate buffer, pH 5.2. (β-gal was previously dialyzed into 30mM sodium acetate pH 5.2), with 1mg glycan per 100ul reaction, at $37^0$C. The reaction was boiled and then either ethanol-precipitated or brought up directly in B4GALNT reaction. The volume was increased three-fold to a final concentration of 50mM sodium cacodylate, pH 7, 0.1% sodium azide, 25mM manganese chloride, 1µl shrimp alkaline phosphatase, 2mM UDP-GalNAc and mixed with a slurry of Affigel beads bound to B4GALNT at a ratio of 5:1 (reaction volume:slurry volume). When GalNAc addition was complete, the supernatant was removed to a clean tube and the reaction volume was increased slightly, maintaining the same buffer conditions, except that no additional manganese chloride was added, and GDP-fucose was added instead of UDP-GalNAc. This reaction mix was added to beads carrying FUT6 enzyme, prepared by the same method described above. All reactions were monitored by mixing 0.5µl of the reaction with 2,5-Dihydroxybenzoic acid (DHB) matrix on a target plate and analyzing sample on a MALDI-TOF (Bruker Daltonics Ultraflex II TOF/TOF) in reflectron positive (RP) mode. Fresh UDP-GalNAc or GDP-Fucose were added as needed and, if reaction was not complete within 36 hours, fresh enzyme/beads were prepared for the reaction mix. Poly-LDN and poly-LDNF were prepared using AEAB-LNnT as a starting product and following the LDN chemo-enzymatic synthesis method described above. Fresh human serum was used to catalyze the addition of GlcNAc to LDN [169], [352]. Briefly, 200nmol of acceptor was added to 5.6µmol UDP-GlcNAc, 1.6µmol manganese chloride, approximately 7mg of fresh human serum concentrate (or the 30-65% fraction of ammonium sulfate precipitate from the serum), and this was brought up to 200µl in 50mM Tris-HCl, pH7.4 with 0.04% sodium azide. The reaction was run for 6-9 days at $37^0$C and then purified using ethanol precipitation, C18 SepPak and Carbograph columns and HPLC as

described previously [247]. The LDN/LDNF chemo-enzymatic synthesis was then repeated to obtain two repeats of LDN and addition of 1-2 fucose residues. These were also HPLC purified as described above and the mass of each purified peak as well as position of the fucose was verified by tandem mass spectrometry. Human IgG N-glycans were obtained by purifying IgG from 10mL of fresh human serum using a protein A agarose column at $4^0$C, according to the manufacturer's instructions. The serum was centrifuged at 10,000xg and 2mL batches of supernatant were adjusted to pH8 using Tris HCl and loaded onto 2mL of resin. Tris-HCl was used as wash buffer, and 100mM glycine for elution into 1M Tris HCl pH 8.0, and the column was regenerated between each batch. The purity was checked on SDS-PAGE and then purified IgG was dialyzed into PBS. The IgG was frozen, lyophilized, and then denatured in 8M Guanidine HCl/90mM DL-dithiothreitol (DTT)/200mM Tris-HCl pH8.2 for 1 hour at ambient temperature on an orbital shaker. It was then alkylated using 120mM iodoacetamide in the same buffer for 30 min. The denatured IgG was dialyzed into water at $4^0$C overnight in 6-8kDa MWCO tubing, frozen and lyophilized. 222mg of denatured IgG were obtained from the 10mL of serum. This was dissolved in 50mM phosphate buffer pH 8.2 and TPCK-treated trypsin was added to 0.33 mg/mL, and rotated at $37^0$C for 12 hours. The sample was boiled for 5 min. to deactivate trypsin and digestion was checked via SDS-PAGE. The pH was adjusted to 7.5 with HCl and sodium azide 0.02% and 50µl PNGaseF were added. The reaction was incubated at $37^0$C for 72 hours and then boiled for 5 min. The glycans were purified by flowing through a 10g C18 SepPak column, onto a 1g Carbograph column, and eluted from the Carbograph with 50% acetonitrile/0.1% trifluoroacetic acid (TFA). The free IgG N-glycans (23mg) were frozen & lyophilized. Neutral and monosialyl glycan fractions were isolated by purification on DEAE cellulose resin and elution in 2mM and 20mM pyridyl acetate buffer, respectively. The eluted fractions were monitored by phenol sulfuric acid assay for hexose content, and peaks were frozen and lyophilized. A small amount of the neutral and monosialyl fractions was permethylated for MALDI-TOF, and the remainder was conjugated to AEAB. The labeled glycan pools were quantified using a Shimadzu

spectrofluorophotometer RF-5301PC against AEAB-lactose standards. 1.6μmol of monosialyl and 1.1μmol of neutral glycans were obtained. Both the neutral and monosialyl glycan mixtures were enriched for non-bisected glycans by column purification over 2mL of E-PHA agarose in PBS. A mixture of non-bisected, +/- core fucosylated glycans were collected between 2-6 mL of eluent. These were purified on Carbograph columns. Batches of 200nmol of the mixture of AEAB-labeled, non-bisected, monosialyl N-glycans were digested with 25μl β-gal and 25μl β-N-Acetylglucosaminidase from *Canavalia ensiformis* in 1mL of 50mM sodium acetate buffer pH5.0 at $37^0$C for 24 hours. Digestion with α-mannosidase was attempted but we found that mannose could not be removed when the other branch of the glycan was intact. Enzymes were removed by ethanol precipitation and glycans were dried. These were digested with 5μl neuraminidase and 5μl β-gal in 300μl of 50mM sodium acetate buffer with 4mM calcium chloride at pH5.5 for 4 hours at $37^0$C and then ethanol precipitated and Carbograph purified. The non-bisected neutral glycan mixture was simply digested with β-gal as above. The mixture of +/-core fucosylated GlcNAc-terminating biantennary N-glycans (from neutral fraction), and the mixture of +/-core fucosylated asymmetrical Man-/GlcNAc-terminating N-glycans (from monosialyl fraction) were then converted to biantennary and monoantennary, respectively, LDN and LDNF, using the chemo-enzymatic synthesis method described above. The products were HPLC-purified using an analytical Porous Graphite Carbon (PGC) column, 25 minute gradient of 15-40% acetonitrile in water with 0.1% TFA, and then re-purified using a 15-25% gradient. Purified mono- or biantennary LDN or LDNF of the expected molecular weight were successfully isolated from other contaminating N-glycans via this method, with the +/- core-fucosylated glycans co-eluting in most cases, so they were printed as a mixture. All other AEAB-labeled glycans were HPLC-purified using a PGC column, 15-40% acetonitrile gradient, using fluorescence detection at 330nm (Shimadzu Scientific Instruments). All molecular weights of purified HPLC fractions were verified by MALDI-TOF in RP mode, and, if necessary, fragmented by MALDI-TOF-TOF.

The glycans were quantified by fluorescence as described above and adjusted to 10ul of 100uM glycan, or, if not enough was available, 50uM, before printing.

3.2.2.10 Preparation and analysis of glycan microarrays

Glycan microarrays were prepared, assayed and analyzed as described in Heimburg-Molinaro *et al.* [247] with some modifications. 10µl of each glycan at 50µM or 100µM was mixed with 1µl of 10x phosphate buffer in 384-well plates and printed on NHS-activated glass slides using a Piezo Printer (Piezorray, PerkinElmer). Slides were stored at -20$^0$C until use. Binding assays were performed as per Heimburg-Molinaro *et al.* for experiments with monoclonal antibodies and biotinylated lectins, at the indicated concentrations. For experiments with serum, serum was diluted 1:50, and wells were washed 3 times with 200µl TSM wash buffer, then 3 times with 200µl TSM buffer with 5 minutes of shaking for each wash, after the primary and secondary incubations. The secondary antibodies, goat anti-mouse IgG or IgM, -Alexa488 or –Alexa-633, and streptavidin-Alexa488 were used at 5µg/mL. Slides were scanned using the ScanArray Express software on a PerkinElmer Proscanner XL4000. ScanArray Express was used to align spots, remove background and quantify fluorescence. An excel macro file was then used to average 4-6 replicate spots for each glycan ID #, determine SEM, SD and %CV, and generate bar graphs.

## 3.3 Results

### 3.3.1 Recombinantly engineered Lec8 cells express surface-bound poly-LDN and poly–LDNF

We developed a recombinant expression system for the LDN(F) family of schistosome glycan antigens using the Lec8 cell line, a stable glycosylation mutant of CHO cells, which have a non-functional UDP-galactose transporter. All of the N-glycan branches homogeneously truncate in N-acetylglucosamine (GlcNAc) and O-glycans truncate in N-acetylgalactosamine (GalNAc or Tn antigen) [353], [354]. We serially transfected Lec8 cells with *C. elegans* B4GALNT1 and human FUT9 [168]. The resulting stable, clonal cell lines are referred to as L8-

GT and L8-GTFT. Activity of an endogenous mammalian β1,3-N-Acetylglucosaminyltransferase, which normally allows extension of poly-N-acetyllactosamine (LN) and poly-Lewis$^X$ (Le$^X$) branches in wild-type cells, most likely catalyzed extension of the corresponding engineered antigens, poly-LDN and poly-LDNF, in our cell lines. MALDI-TOF mass spectrometry indicated that 2-6 linear repeats of LDN and 2-4 linear repeats of LDNF were present on each N-glycan branch of the L8-GT and L8-GTFT cells, respectively, depending on the number of branches, indicating that the activity of the enzymes was sufficient to alter the glycome [168]. The O-glycans and glycolipids of these cells do not contain the antigens as the B4GALNT1 can only use β-linked GlcNAc as acceptor [168], [345], [354], [355].

We first examined surface expression of the engineered glycans by performing flow cytometry on paraformaldehyde-fixed cells stained with lectins (Figure 3.2a) and monoclonal antibodies (Figure 3.2b-c). The lectins Concanavalin A (ConA), *Griffonia simplicifolia* lectin II (GSL-II), *Wisteria floribunda* agglutinin (WFA), and *Aleuria aurantia* lectin (AAL) bind tri-mannose, terminal GlcNAc, terminal GalNAc, and fucose, respectively [356]. ConA binding indicated that the cell lines expressed similar amounts of N-glycans on their surface. WFA, and AAL binding were significantly higher in the L8-GT and L8-GTFT cells, respectively, indicating a successful transformation from mostly GlcNAc-terminating glycans in Lec8, to mostly GalNAc-terminating glycans in L8-GT and fucosylated glycans in L8-GTFT (Figure 3.2a). We stained the cells with monoclonal antibodies to the LDN and LDNF epitopes, generated from hybridomas derived from spleen cells of *S. mansoni*-infected mice by methods described previously [144], [147], [350]. Anti-LDN bound significantly higher to L8-GT cells compared with the other two lines (Figure 3.2b, 3.2c - left). Anti-LDN reactivity with L8-GTFT cells was reduced to a level not significantly different from Lec8, indicating a robust loss of LDN epitopes as they are converted to LDNF epitopes. Anti-LDNF bound significantly higher to L8-GTFT cells compared with the other two lines, and only slightly higher to L8-GT than to Lec8 (Figure 3.2b, 3.2c - right), which could indicate low-level cross-reactivity with LDN epitopes. The binding of

both anti-LDN and –LDNF was significantly abrogated by growing cells in the presence of kifunensine [357], an α-mannosidase inhibitor of complex N-glycan processing, verifying that the expression of LDN and LDNF occurs on N-glycans (Figure 3.2c).

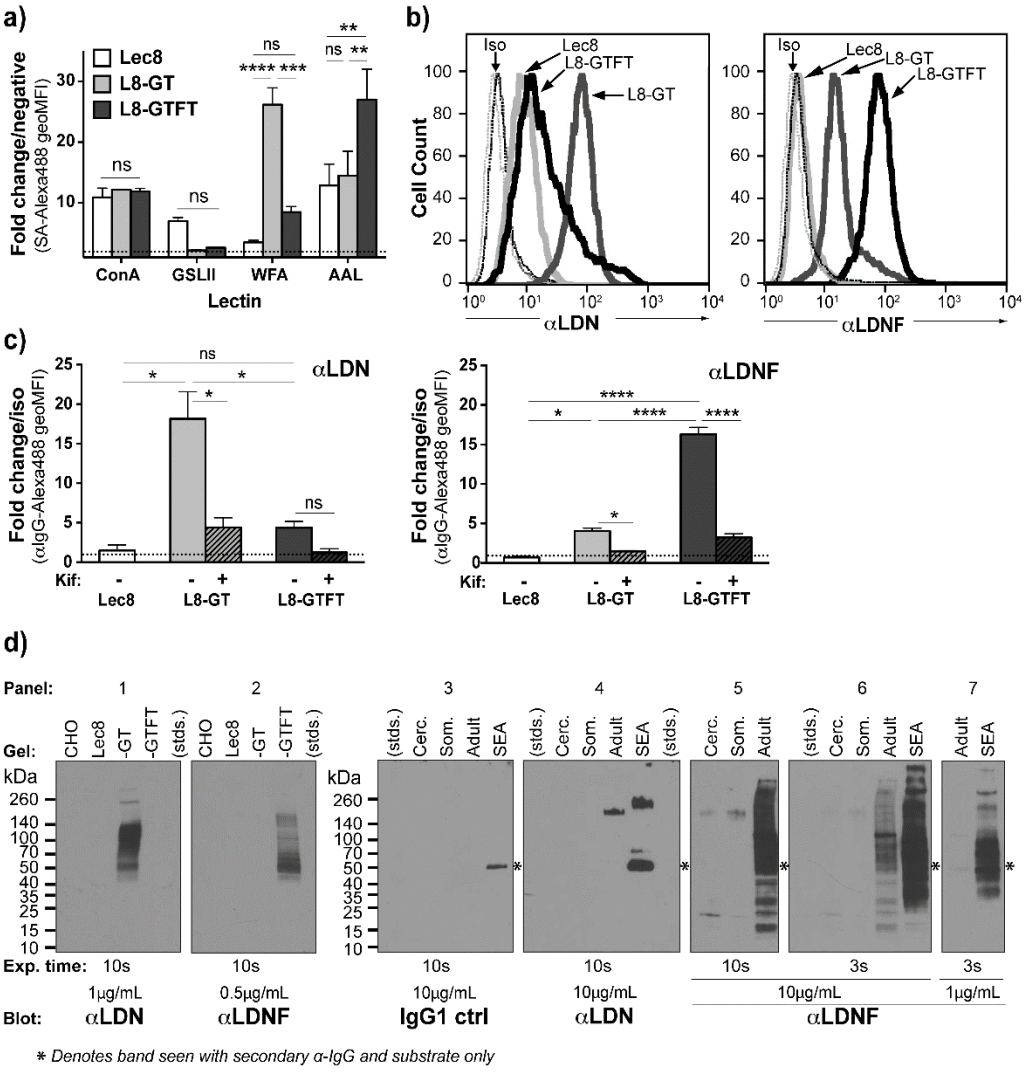


**Figure 3.2 Recombinantly engineered Lec8 cells express surface-bound LDN and LDNF.**

**(a-c)** Lec8 (white bars), Lec8-GalNAcT (L8-GT; gray bars) and Lec8-GalNAcT-FucT (L8-GTFT; black bars) were fixed and stained with biotinylated lectins **(a)** and mouse monoclonal antibodies **(b, c)** for flow cytometry analysis. **(a)** Data are expressed as fold change in geoMFI over the negative control, SA-488, represented on the graph as a dotted line. Mean +/- SEM of two independent experiments are shown and two-way ANOVA with Tukey's multiple

comparisons test was used to determine significance. **(b)** A representative shift with each monoclonal antibody is shown. **(c)** Cells were treated with (hashed bars) or without kifunensine for 8 days to disrupt complex N-glycan synthesis before fixation. Data are expressed as fold change in geoMFI over the negative control - mouse IgG1 isotype control for **(b)**, and IgG1 isotype control or normal mouse IgG for **(c)**. Error bars represent SEM of two **(c - left)** or three **(c - right)** independent experiments and one-way ANOVA with Tukey's test were used. **(d)** SDS-PAGE and Western blotting were performed on cell and *Schistosoma mansoni* life stage lysates and using the monoclonal antibodies, with detection by HRP-conjugated secondary antibodies and chemiluminescent substrate on film. Primary antibody concentrations and exposure times are specified for each pane. The six-point star denotes bands that are not antibody-specific, since they appear with secondary antibody and substrate only when SEA and adult worm lysates are stained, likely the result of trace amounts of mouse immunoglobulins in the parasite preparations. **SA** streptavidin; **ConA** concanavalin A; **GSL-II** *Griffonia simplicifolia* lectin II; **WFA** *Wisteria floribunda* agglutinin; **AAL** *Aleuria aurantia* lectin; **Kif** kifunensine; **Iso** isotype control antibody or normal mouse IgG; **stds** molecular weight standards; **Cerc.** cercarial lysate; **Som.** schistosomula lysate; **Adult** adult worm lysate; **SEA** soluble egg antigen; **ns** $p \geq 0.05$; ***** $p = 0.01$-$0.05$; ****** $p = 0.001$-$0.01$; ******* $p = 0.0001$-$0.001$; ******** $p < 0.0001$.

To examine the LDN/LDNF content of the cells relative to schistosome life stages and characterize the molecular species carrying these glycans, we prepared lysates of *S. mansoni* cercariae, 3-day cultured schistosomula, adult worms and soluble egg antigen (SEA) (the latter two prepared from infected mice). We performed SDS-PAGE of protein concentration-standardized cell and parasite life stage lysates and Western blotted with anti-LDN and anti-LDNF (Figure 3.2d). The anti-LDN and –LDNF were highly specific for L8-GT and L8-GTFT cells, respectively, and stained a range of glycoproteins between 40-260kDa (Figure 3.2d – panels 1-2). Anti-LDN stained only a few distinct species of SEA and adult worm lysate (Figure 3.2d – panels 3-4). In contrast, anti-LDNF showed broad-range staining of SEA glycoproteins as well as less intense reactivity with several species in adult worm lysate, and a few distinct species in schistosomula and cercarial lysates, as demonstrated by using different concentrations of the antibody and exposure times (Figure 3.2d – panels 5-7). [Note: The non-specific band at 50kDa marked with a six-point star (Figure 3.2d – panels 3-7). is likely due to cross-reactivity of the HRP-conjugated anti-mouse-IgG secondary detection reagent with the parasite lysates prepared

from mice, and stains when only secondary antibody is used.] Taken together, these results demonstrate that LDN and LDNF antigens are abundantly expressed on a variety of cell-surface L8-GT and L8-GTFT cells glycoproteins, respectively, at levels greater than in the early stages of schistosome infection and comparable to LDNF expression of adult worms and eggs in robustness and molecular complexity. Additionally, the cells present LDN and LDNF on N-glycans in such a way that they can be recognized by monoclonal antibodies originally generated by mice in response to parasite infection.

### 3.3.2 Recombinantly expressed poly-LDN and -LDNF are cross-reactive with schistosome antigens.

We next asked whether the glycan antigens on the CHO cell derivatives were recognized by antisera from experimental murine *S. mansoni* infection, a common laboratory model for schistosomiasis. Chronically-infected mouse antisera (20-week, low cercarial dose) had significantly more IgM (Figure 3.3a – left) and IgG (Figure 3.3a – right) specific for L8-GTFT cells compared to Lec8 and L8-GT cells. Antiserum from both acutely and chronically *S. mansoni*-infected (8-week and 20-week, low cercarial dose) contained IgM specific for N-glycans on L8-GT and L8-GTFT cells (Figure 3.3b – left), and IgG specific for N-glycans on L8-GTFT cells (Figure 3.3b – right). Western blotting also showed that, whereas naïve serum had no specific staining of the cell lysates (Figure 3.3c – Panel 1), 8-week infection antisera contained IgG that bound L8-GTFT cell lysate (Figure 3.3c – Panel 2). The infection antisera stained some higher molecular weight species of L8-GTFT cells that were less prominent in the anti-LDNF stain **(**Figure 3.2d – panel 2), indicating that the anti-LDNF response during infection may differ in breadth from the monoclonal. Staining of *S. mansoni* cercariae, schistosomula, worm and egg antigens are shown as a positive control (Figure 3.3c – panels 2, 4). These results indicate that by over-expressing parasite glycan epitopes in mammalian cells, we were able to generate an immunogen which mimics the antigenicity presented by *S. mansoni* during infection.

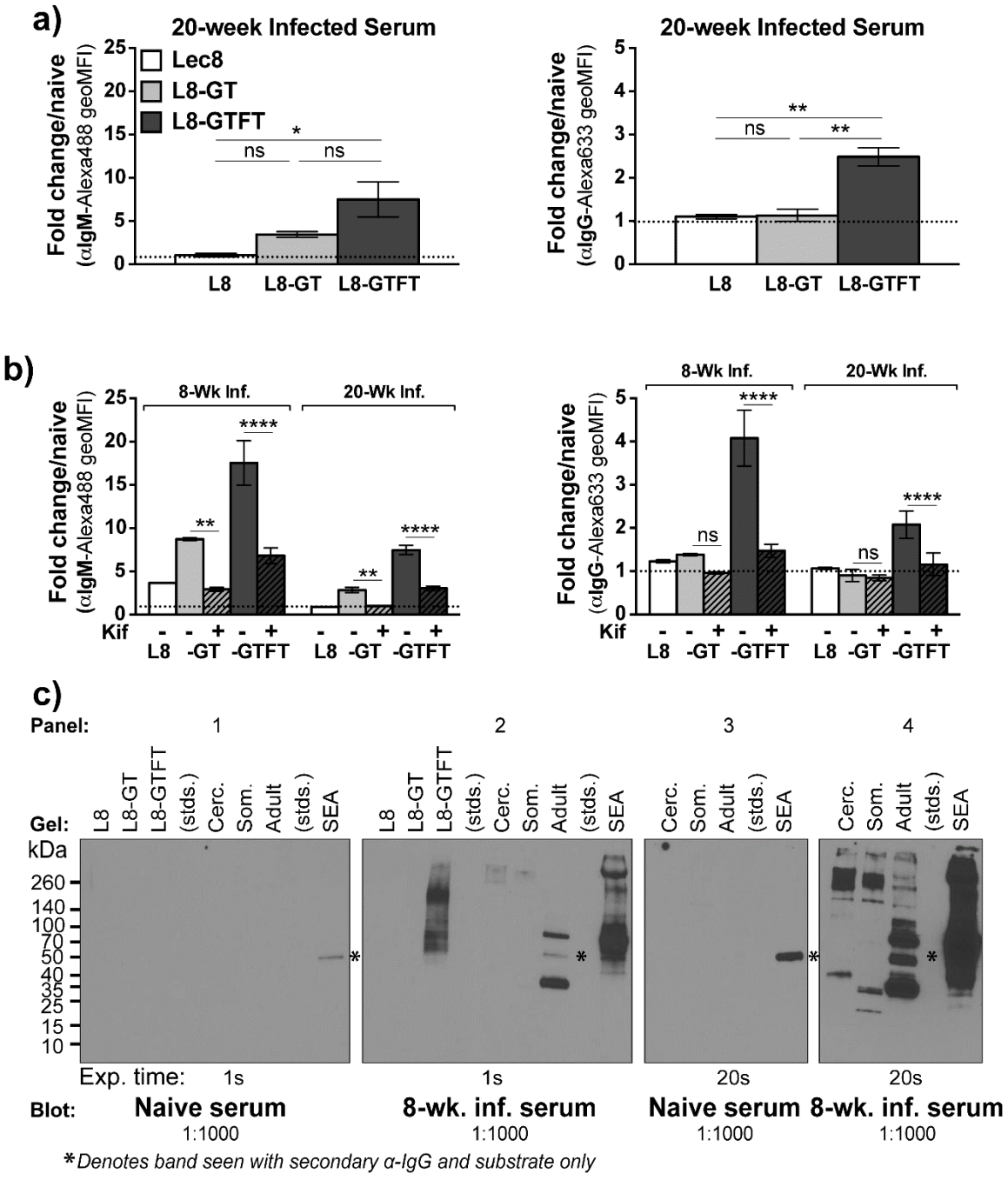


**Figure 3.3 Recombinantly expressed LDN and LDNF are cross-reactive with schistosome antigens.**

Cells were fixed and stained with pooled antisera from naïve, 8-week or 20-week low-dose *S. mansoni*-infected mice on flow cytometry. Bound IgM (left) or IgG (right) was detected with Alexa-488 or Alexa-633-conjugated secondary antibodies. White bars, Lec8 cells; gray bars, L8-GT cells; black bars, L8-GTFT cells. **(a)** Mean +/- SEM of the geoMFI fold change of 20-week infected serum over naïve serum for three experiments is shown and one-way ANOVA with

Tukey's multiple comparisons test was used. **(b)** Mean +/- SD of the geoMFI fold change of 8-week and 20-week infected serum over naïve serum for two independent experiments with kifunensine- (hashed bars) or mock-treated cells is shown and two-way ANOVA with Tukey's test used to compare the effect of kifunensine treatment independent of the serum used. **(c)** Cell lysates were Western blotted with infected antisera, using parasite life stages as a positive control, and detected with HRP-conjugated anti-mouse IgG at 1:5000. The six-point star denotes non-specific bands that appear with secondary antibody and substrate only when SEA and adult worm lysates are stained. **Kif** kifunensine; **stds** molecular weight standards; **Cerc.** *S. mansoni* cercarial lysate; **Som.** schistosomula lysate; **Adult** adult worm lysate; **SEA** soluble egg antigen; **Exp.** exposure time; **ns** p ≥0.05; ***** p =0.01-0.05; ****** p =0.001-0.01; ******* p =0.0001-0.001; ******** p <0.0001.Whole cell vaccines are immunogenic and induce a sustained anamnestic response.

To determine the effectiveness of the engineered cells at inducing an anti-glycan response, we immunized 10 female Swiss-Webster mice with each of the cell lines, or a PBS control. We delivered $10^6$-$10^7$ cells (stored frozen and thawed immediately before use) intraperitoneally, without adjuvant, to each mouse (Figure 3.4a). Three primary immunizations were given at the onset of weeks 0, 2 and 4, followed by a waiting period, and then a secondary immunization at week 19. Facial vein bleeds were taken to obtain serum from each animal at weeks 0 (pre-bleed), 2, 4, 6, 19, 20, 21 and 40, at which point the animals were sacrificed. Bleeds were taken the day before an injection. At 19 weeks, 3-4 PBS-immune mice were given primary immunizations of each cell type to directly compare to the secondary immunizations given at the same time.

All three of the cell lines were immunogenic based on flow cytometry of pooled immune serum binding to the surface of homologous fixed cells. Representative shifts from weeks 0 (naïve), to weeks 6 (two weeks after the third immunization) and 20 (one week after the secondary immunization) are shown (Figure 3.4b). L8-GT cells induced the greatest IgM shift at week 20 compared to naïve (Figure 3.4b – left), whereas for IgG, the Lec8 cell immunization induced no perceptible shift and L8-GTFT cells induced the most robust shift at 20 weeks (Figure 3.4b – right). [Note that for all flow cytometry experiments, the relative levels of IgG and IgM in a sample cannot be quantitatively compared, because different secondary antibodies are used.] To

determine whether the cells induced a memory response, we compared pooled immune sera from before (week 19) and one week after (week 20) the simultaneous primary and secondary immunization groups (Figure 3.4c). Both L8-GT and L8-GTFT cells induced an anamnestic response, indicated by comparing the shifts of secondary with primary immunization at 20 weeks. The L8-GT cells appeared to induce more IgM memory than L8-GTFT cells (Figure 3.4c – left), whereas the reverse was true for IgG memory (Figure 3.4c - right).

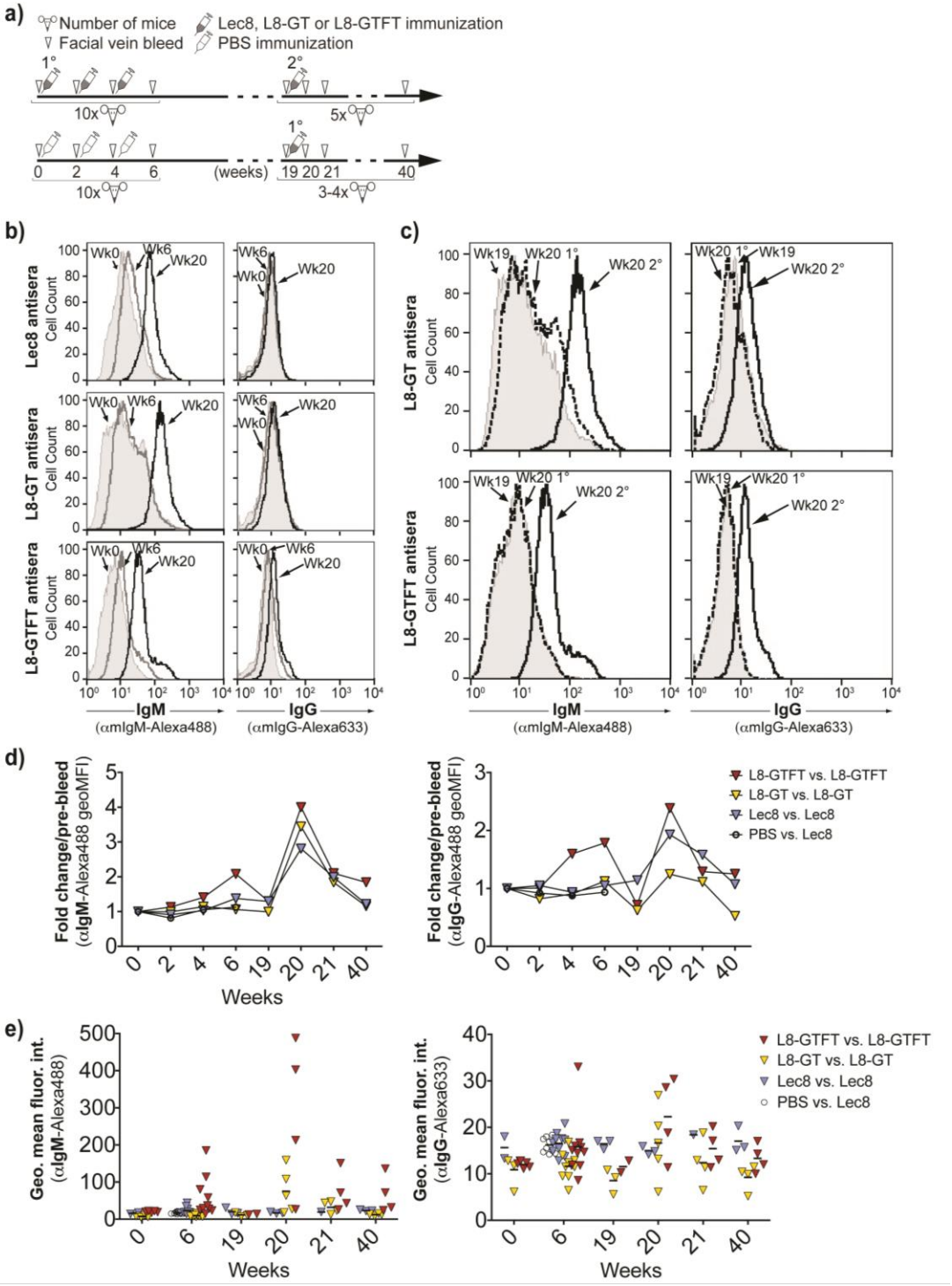


**Figure 3.4 Whole-cell poly-LDN(F) vaccines are immunogenic and induce a sustained anamnestic response.**

**a)** Freshly-thawed aliquots of $10^6$-$10^7$ cells, or PBS, were delivered without adjuvant, intra-peritoneally to female Swiss-Webster mice and facial vein bleeds were collected according to the

immunization scheme depicted. Three immunizations were given at 0, 2 and 4 weeks ($1^0$), and one secondary ($2^0$) boost was given at week 19, to the number of mice indicated under each timeline. The mice given PBS immunizations at weeks 0-4 made no detectable response and were therefore used for $1^0$ immunization at 19 weeks. **b-e)** To measure immunogenicity of each cell type, antisera from immunized mice was run on flow cytometry against fixed cells of the same type to assess IgM and IgG binding to surface antigens. **b)** Histograms representative of 2-3 experiments are shown for the pooled antisera at week 0 (solid gray), week 6 (thick gray line) and week 20 (thick black line) post-immunization for IgM (left) and IgG (right). **c)** The memory response was assessed by comparing pooled week 19 sera (pre-$2^0$; solid gray) with shifts from week 20 sera from $2^0$ immunized mice (thick black line) and $1^0$ immunized mice (which had previously received PBS as mock immunization; dashed black line), representative of 2-3 experiments. **d)** The increase in antibody binding to homologous cell types, expressed as fold change in average MFI relative to week 0, was measured for antisera pooled from each group of mice over time, representative of 3 experiments. Yellow triangles, Lec8-immunized vs. Lec8 cells; blue triangles, L8-GT-immunized vs. L8-GT cells; red triangles; L8-GTFT-immunized vs. L8-GTFT cells; open circles, PBS-immunized vs. Lec8 cells (only shown through week 6); black dash, mean for each group. **e)** Flow cytometry was also used to assess the range of magnitudes in IgM (left) and IgG (right) response for individual mice, where enough serum was available. Two-way ANOVA with repeated measures was performed on 3-4 mice from each of the L8, L8-GT and L8-GTFT which had complete data at weeks 0, 6, 20 and 40. Within each group, time points were compared with week 0 using Dunnett's multiple comparisons test.

We compared the fold change in mean fluorescence intensity (MFI) relative to week 0 of each of the pooled antisera binding to homologous cells over the course of the experiment (Figure 3.4d). The Lec8 and L8-GT immunizations induced an increase in IgG and IgM binding that was only detectable by flow cytometry at week 20, whereas L8-GTFT cells induced more robust primary IgG and IgM responses that were detectable at weeks 4 and 6, and peaked at week 20. Additionally, the L8-GTFT IgM and IgG responses were still elevated at week 40 whereas the response to other cell types had declined to levels seen at week 0.

The magnitude of response to cellular immunogens was highly variable among individual mice (Figure 3.4e). Not all mice were included in the individual analysis because of serum shortage, which accounts for the slight discrepancy between flow cytometry results of pooled and individual sera. However, it was evident that in each group, some mice responded very highly

while some hardly responded at all, which could stem from individual differences in ability to respond to the immunogens, or variability in the efficiency of delivery. To assess the relative immunogenicity of the cellular immunogens, we performed 2-way ANOVA with repeated measures on the individual mice which had complete longitudinal data at weeks 0, 6, 20 and 40 (n=3-4 per group). Within each group, time points were compared with week 0 using Dunnett's multiple comparisons test. The IgM and IgG responses to L8-GTFT cells, and the IgG response to L8-GT cells were significantly elevated ($p < 0.05$) compared to the pre-bleed sera. Mice immunized with PBS did not show any detectable response to Lec8 cells through 6 weeks (Figure 3.4d-e).

Taken together, flow cytometry data demonstrate that all three cell lines were immunogenic without adjuvant and induced a memory response. L8-GTFT cells, simply by virtue of their engineered glycans, appear to be the most potent immunogen in terms of inducing the highest response to primary immunization, and generating a more sustained IgG memory response than the other cell types.

### 3.3.3 Lec8-GT and -GTFT cells induce IgM and IgG antibodies specific for LDN and LDNF glycans.

In order to examine the specificity of the immune response, we first looked at the binding of immune sera from several high-responding individual mice to homologous cell types, heterologous cell types and kifunensine-treated cells on flow cytometry (Figure 3.5a). Each pie chart represents the sum of raw MFI values for the indicated antisera binding to different cell types. In all L8-GT (top) and –GTFT (bottom)-immunized animals tested, we found that the largest portion of binding seen was to the homologous cell type (dark gray for the binding to L8-GT cells, and black for binding to L8-GTFT cells.) In one mouse (#046) that was tested for specificity at weeks 6 and 20, the portion of cell-specific response was amplified at the later time point. This indicates that antibody responses were specific to the cells used for immunization and specific for cell-surface N-glycans.

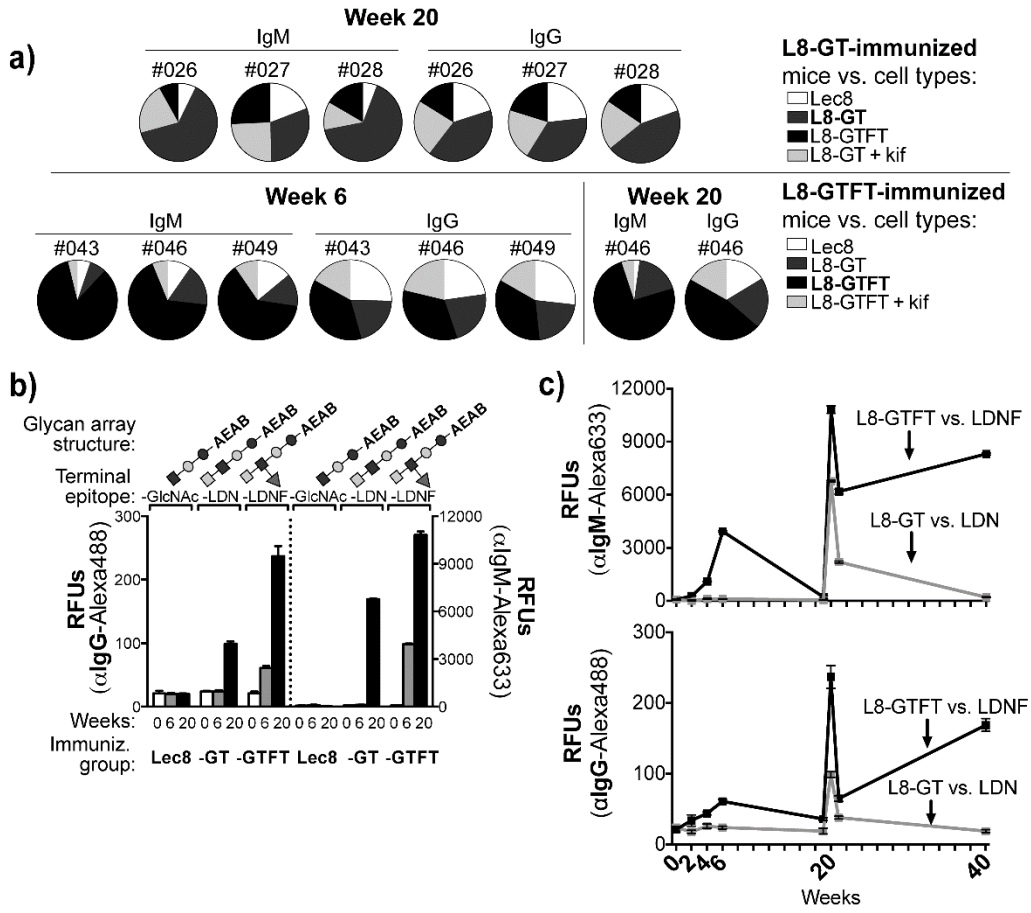


**Figure 3.5 L8-GTFT cells induce long-lasting IgM and IgG antibodies specific for LDNF.**

**a)** Sera from individual L8-GT- (top) and L8-GTFT-immunized (bottom) mice was run on flow cytometry against heterologous cell types (Lec8, white; L8-GT, dark gray; L8-GTFT, black; L8-GT or L8-GTFT treated with kifunensine, light gray) to assess specificity of the response. Individual mouse numbers, isotype and week of the serum sample are indicated above each pie chart, in which the relative binding to each cell type (each slice) is represented as a portion of the sum of geoMFIs of binding for all the cell types (whole pie). Note that naïve serum was also run on each cell type as a negative control, with similar binding. **b)** The pooled antisera from weeks 0 (white bars), 6 (gray bars) and 20 (black bars) was run on glycan microarrays to assess glycan-specific binding of serum IgG (left y-axis) an IgM (right y-axis). Glycan array structures derived from LNnT, terminating in GlcNAc (glycan ID #13) or with a single unit of LDN (ID #14) or LDNF (ID #16) were printed at 100uM and are shown above each group of bars. Glycan ID#s and monosaccharide symbols correspond to **Fig. 5a**, which depicts the entire glycan array with controls – only three selected structures are shown in this figure. Binding is representative of two experiments. **c)** Pooled sera from L8-GT (gray) and L8-GTFT-immunized (black) mice at all time points and binding to LDN and LDNF is shown, representative of 2 experiments. The mean RFUs +/- SD are plotted for binding to six replicate spots of each glycan. **Kif,** kifunensine; **RFUs**,

relative fluorescence units; **-GlcNAc**; agalacto-Lacto-n-neo-tetraose (LNnT); **-LDN**, LacdiNAc made by adding GalNAc to agalacto-LNnT; **-LDNF**, fucosylated LacdiNAc made by adding fucose to –LDN.

To more directly examine glycan specificity of the immune response to the cells, we probed glycan microarrays with pooled immune sera. Glycan microarrays consist of a collection of glycans that have been functionalized and printed on glass slides (as described in Materials and Methods section and our previous publications) [247], [358]. Slides are then incubated with antibodies or lectins and detected by appropriate fluorescent secondary reagents as relative fluorescent units (RFUs). This yields an extremely sensitive and specific read-out of glycan-binding patterns. [Note that for all glycan array experiments, the relative levels of IgG and IgM in a sample cannot be quantitatively compared, since different secondary antibodies are used.]

We first examined reactivity to three defined glycan structures, chemo-enzymatically synthesized by remodeling Lacto-N-neotetraose (LNnT; Galβ1-4GlcNAcβ1-3Galβ1-4Glc) to contain one unit of either LDN or LDNF. Agalacto-LNnT (terminating in GlcNAc) was generated by digestion with β-galactosidase. LDN was generated using recombinant *C. elegans* β1-4N-acetylgalactosaminyltransferase (B4GALNT) to add GalNAc to the non-reducing end, and LDNF was generated using recombinant human α1-3Fucosyltranserase 6 (FUT6) to add fucose to the GlcNAc (Figure 3.5b; the monosaccharide key can be found at the bottom of Figure 3.6a).

When we compared pooled serum from each immunization group at weeks 0, 6 and 20, we found that, consistent with flow cytometry data, the L8-GT cells induced detectable LDN-specific antibody only at 20 weeks, whereas the L8-GTFT-immunized mice expressed the highest titer of glycan-specific IgG and IgM to LDNF at both time points (Figure 3.5b). Lec8-immunized mice made no detectable antibodies to agalacto-LNnT. We examined the IgM (Figure 3.5c - top) and IgG (Figure 3.5c - bottom) responses to LDN and LDNF in pooled serum over the course of the whole experiment by plotting the glycan array RFUs at each time point. In the L8-GTFT group, glycan-specific IgG and IgM were detectable beginning at week 2 and steadily increased

with repeated immunizations. They peaked at week 20 after the booster immunization, declined at week 21, and were sustained through week 40 when the mice were sacrificed. In contrast, glycan-specific antibodies to LDN were detectable in L8-GT-immunized mice only at weeks 20 and 21, and declined again to undetectable levels by week 40. Based on the glycan array data, we conclude that expression of polymeric LDN and LDNF on the surface of Lec8 cells allowed for production of glycan-specific antibodies in mice, and that L8-GTFT cells induced a robust LDNF-specific primary and secondary response, which was sustained for at least 20 weeks after the last immunization.

### 3.3.4 Antibodies to parasite glycans discriminate among very similar presentations of the epitopes.

Our Defined Schistosome-type Array (DSA) is a collection of chemically defined glycans and glycopeptides containing schistosome-like glycan epitopes, such as LDN, LDNF, Lewis-X ($Le^X$) and core xylose/core α3-fucose (CX/CF), as well as control structures, where each structure is represented by a unique glycan ID # on the structure list (Figure 3.6a) and on the x-axis (Figure 3.6b-e). A previous version of the DSA consisted of a collection of biantennary N-glycopeptides (glycan ID #1-10) and straight-chain glycans derived from LNnT (#11-16). The glycopeptides were isolated from natural glycoproteins and chemoenzymatically modified to contain biantennary LDN, LDNF or $Le^X$ using recombinant *C. elegans* B4GALNT1 and FUT6 [175]. LNnT was similarly modified to contain a single unit of LDN, LDNF or $Le^X$. While examining the specificity of the anti-schistosomal immune response on that version of the array, we observed that antibodies to LDNF bound well to LNnT-derived-LDNF (#15-16) but not at all to a biantennary N-glycopeptide bearing LDNF (#6). This unprecedented finding prompted us to better define the specificity of anti-schistosomal antibodies by expanding the DSA to include multiple variants of LDN and LDNF which represent the variety of "structural contexts" in which LDN(F) might be presented on the parasite (Figure 3.6a).

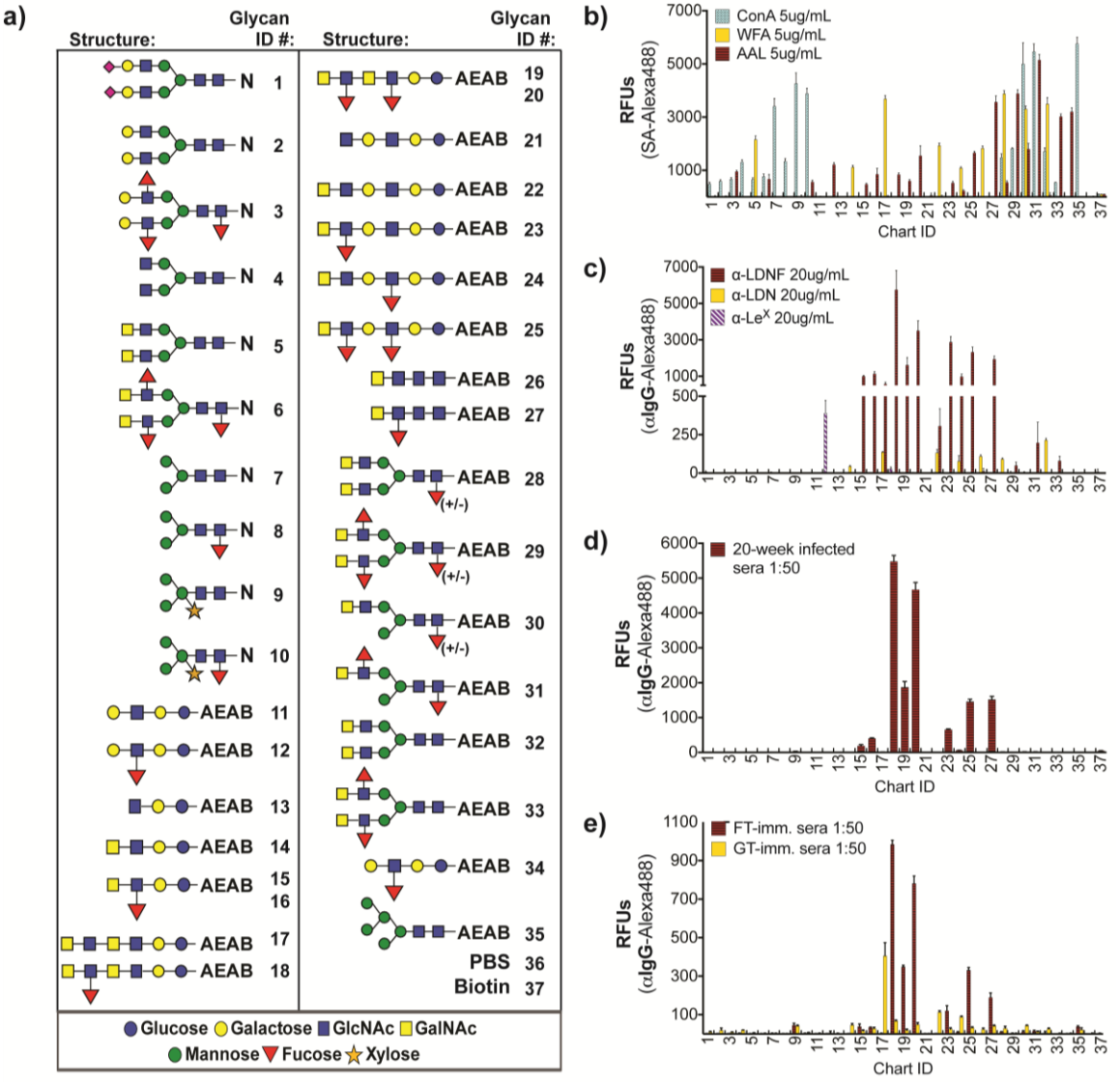


**Figure 3.6 Antibodies to schistosome glycans discriminate among very similar presentations of the epitope.**

**a)** N-linked glycopeptides and AEAB-linked glycans were modified to include several variants of LDN, LDNF and other schistosome antigens and printed on glass slides, called the Defined Schistosome-type Microarray (DSA). LDN-terminating glycans are printed at 50µM and all other glycans are printed at 100µM due to variation in reaction yields. Some LDNF-terminating glycans are printed at 50µM and 100µM, in that order, for comparison with LDN. **b)** Biotinylated lectins binding tri-mannose (ConA), terminal GalNAc (WFA) and fucose (AAL) were used to quality-control printing of the slides. **c)** Anti-schistosomal monoclonal antibodies, **d)** antisera from chronically *S. mansoni*-infected mice and **e)** week 20 antisera from L8-GT and L8-GTFT (FT) immunization were tested on the DSA. Streptavidin-Alexa488 or goat anti-mouse-IgG-Alexa488 were used to detect biotinylated lectins and antibodies bound to the slides, respectively.

Mean RFUs +/- SD of tetra-replicate spots for each glycan ID# are shown. **N**, asparagine; **AEAB**, 2-amino-N- (2-aminoethyl)-benzamide; **RFUs** relative fluorescence units; **SA** streptavidin; **ConA** concanavalin A; **WFA** *Wisteria floribunda* agglutinin; **AAL** *Aleuria aurantia* lectin.

To this end, the LNnT-derived-LDN structure (#14) was used as starting material for extension catalyzed by the β1-3-N-acetylglucosaminyltransferase activity (B3GnT) in normal human serum, and then further modified as above to contain two repeats of LDN (#17), with one or two fucose residues added (glycan ID #18-20). LNnT (#11) was also extended into LDN and LDNF using a recombinant *H. pylori* β1,3-N-acetylglucosaminyltransferase [348] (#21-25). LDN and LDNF were also synthesized from chitotriose (#26-27) and agalacto-NA2 (biantennary N-glycan from chicken glycopeptide) (#32-33). To obtain biantennary or mono-antennary LDN and LDNF, human IgG N-glycans were fractionated by charge, and asialo- and mono-sialyl N-glycans were used to either symmetrically or asymmetrically digest their antennae (#28-31). All glycans were labeled at the reducing end with a fluorescent tag, purified, confirmed for correct molecular weight on MALDI-TOF. All structures were printed at 100uM, except for the new LDN-containing structures, which were printed at 50uM due to the low efficiency of the serum B3GnT reaction in generating poly-LDN. LNnT-derived-LDNF (#15-16) and poly-LDNF (19-20) were therefore printed at both 50μM and 100μM for the purpose of comparing immune response among immunization groups **(**Figure 3.6**)**.

We tested a panel of lectins on the DSA as controls, and their binding patterns verified the successful printing of all desired structures **(**Figure 3.6**b)**. For example, ConA bound to all of the N-glycans as expected, since it recognizes a wide variety of biantennary complex-type N-glycans, WFA bound to all of the GalNAc-terminating glycans, and AAL bound to all of the fucose-containing glycans. In contrast to the lectin binding patterns, monoclonal antibodies generated during parasite infection and polyclonal sera from infected mice can differentiate acutely among very similar structures on the array (Figure 3.6-d). The anti-LDNF antibody (Figure 3.6c) and chronically-infected mouse serum (Figure 3.6d) had highest reactivity with the

poly LDN(F) chains terminating in LDNF (#18-20), and also bound to LDNF linked to lactose, N-acetyllactosamine, $Le^X$ and chitobiose (#15-16, 23, 25, 27). The antibody had little reactivity with the monoantennary or biantennary versions of LDNF (#6, 29, 31, 33), and chronically-infected mouse serum had none. In contrast, anti-LDN (Figure 3.6c) reacted more similarly with all of the straight-chain versions of LDN as well as the synthetic biantennary N-glycans (#14, 17, 22, 24, 26, 28, 32). Curiously, it did not react with the monoantennary or biantennary glycopeptide versions of LDN (#5, 30). We have observed that another anti-schistosomal monoclonal antibody, targeting the $Le^X$ glycan, which was recently characterized by Mandalasi *et al.* [147] (Figure 3.6c), is also specific for an LNnT-derived version of $Le^X$ (#12) over a biantennary glycopeptide (#3).

The array profiles of L8-GTFT week 20 secondary immunized antisera (Figure 3.6e) showed similar specificity to the chronically infected mouse antisera (Figure 3.6d). For IgG, both immune and infected serum samples showed a clear preference for extended straight-chain versus biantennary versions of LDNF, with a difference of roughly 100-fold between the highest binders (#18, 20) and the N-linked versions (#29, 31, 33). The chronically infected serum had about 5-fold higher activity than the L8-GTFT-immune serum, but showed exactly the same pattern of specificity. The IgM response of L8-GTFT-immune serum was identical to IgG in specificity on the DSA, whereas infected mouse IgM bound most highly to extended versions of LDNF but also had high reactivity with $Le^X$, straight-chain and biantennary LDN, and biantennary LDNF (data not shown). In comparison to infection antisera reactivity on the DSA, L8-GTFT immunization appears to have exactly replicated the IgG binding pattern and biased the IgM response towards extended versions of LDNF versus biantennary versions.

The L8-GT-immunized group also generated IgG that preferred straight-chain versions of the epitope, and titers of IgG to poly-LDN (#17) were similar to the IgG generated to poly-LDNF (#19) by L8-GTFT immunization at the week 20 time point (Figure 3.6). The anti-LDN IgG and IgM had relatively more reactivity to N-glycan versions of the epitope when compared with the

anti-LDNF response, binding about 10-fold less to these (#28, 30, 32) than to poly-LDN (#17). In comparison to the anti-LDN monoclonal (Figure 3.6c) and infection antisera IgM (data not shown), the L8-GT cellular immunization seems to have skewed the specificity of the response more towards extended versions of LDN versus LDN on N-glycans, in a similar fashion to L8-GTFT immunization.

Taken together, the glycan microarray data demonstrates that antibodies generated to parasite glycans, both during infection and immunization, are highly specific for particular presentations of glycan epitopes, and that L8-GTFT cells mimicked the pattern of anti-LDNF IgG reactivity displayed by infection antisera.

### 3.3.5 Lec8-GTFT cell antiserum binds to schistosome glycans.

Conceivably, a vaccine to protect against schistosome infection or mitigate its pathology by reducing worm and/or egg burden would need to target the intra-mammalian life stages of the parasite. We used serum from several high-responding individual L8-GTFT-immune mice at the 6 and 20 week time points to explore reactivity with parasite life stages by performing Western blots against concentration-standardized cell lysates (Figure 3.7a) and *S. mansoni* lysates (Figure 3.7b). As negative controls, we found that the pre-bleed serum had no specific staining of cells or parasites (Figure 3.7a,b – panels 1, 3, 5, 7, 9, 11, 14). As a positive control, the L8-GTFT-immune serum reacted with L8-GTFT cell lysate, but not with Lec8 cell lysate or with L8-GTFT cell lysate where N-glycans have been removed with PNGaseF (Figure 3.7a – panels 2, 4). In all cases, the immune sera were most highly reactive to SEA when compared with the earlier life stages (Figure 3.7b – panels 6, 8, 10, 12), and in at least one mouse there was reactivity to cercarial lysate (Figure 3.7b – panel 15). This was not surprising given that SEA also demonstrated the most LDNF staining when blotted with anti-LDNF (Figure 3.7d). In the immune serum samples, IgM binding to SEA was most readily detectable, staining many molecular species from 40 to >260 kDa (Figure 3.7b – panel 6). A molecular species around

70kDa in SEA was stained by IgG from at least three different L8-GTFT-immunized mice (Figure 3.7b – panels 8, 10, 12). This species may correspond to a ~70kDa antigen that was also one of the most highly reactive bands when staining with anti-LDNF and infected mouse serum (Figure 3.7c – panels 17, 18). 8-week and 20-week-infected mouse sera are shown staining SEA and cercarial lysate in two of the experiments for comparison (Figure 3.7b – panels 13, 16), demonstrating that the anti-parasite titer generated by L8-GTFT immunization was much lower than what is characteristic of infected mice.

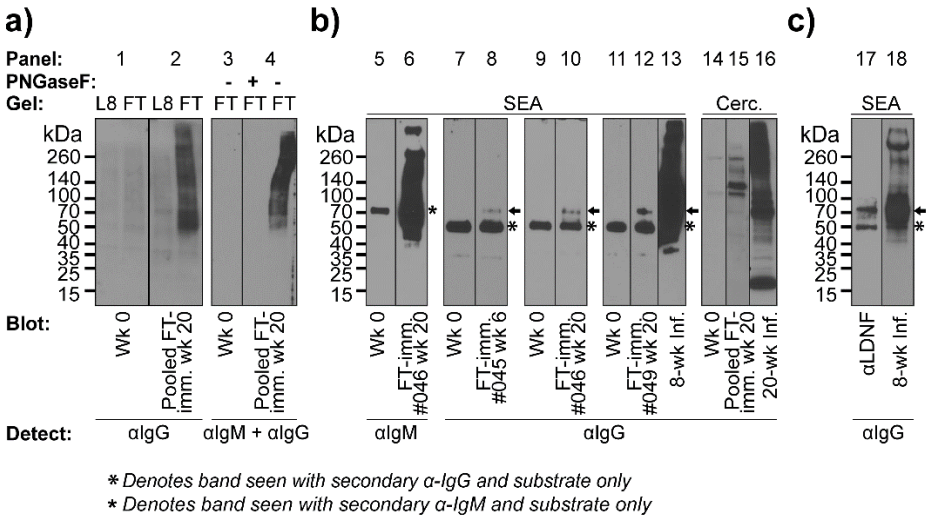


**Figure 3.7 L8-GTFT cell antisera binds to schistosome glycoproteins.**

**a)** Binding to cell N-glycans was confirmed by Western blotting of pre-bleed and week 20 L8-GTFT (FT) antisera against Lec8 and L8-GTFT (FT) cell lysate, mock or PNGaseF-treated to remove N-glycans. **b)** Pre-bleed sera and antisera from individual L8-GTFT-immunized mice that responded highly in flow cytometry experiments were used to stain parasite life stage lysates, with infected mouse sera shown as a positive control in some experiments. **c)** Low-exposure stains of SEA with anti-LDNF and infected sera are shown for comparison with the molecular weights stained by immune sera in **(b)**. Panes separated by a line were run in the same experiment and membranes were cut to allow for incubation with naïve, immune, or infected sera. Membranes separated by space were run on different days under similar conditions. **SEA** Soluble egg antigen; **Cerc.** cercarial lysate **(b)**. Blots were detected with HRP-labeled goat anti-mouse IgG or IgM.

These data demonstrate that vaccination of mice with L8-GTFT cells induced a response that is cross-reactive with *S. mansoni* egg glycoproteins. While the magnitude of the response induced by this vaccination regimen may not be sufficient to protect against schistosomiasis, our studies show that the unadjuvanted cells were immunogenic, successfully mimicked the glycan antigenicity induced by *S. mansoni* infection and induced antibodies that bound to parasite antigens.

### 3.4 Discussion

Pathogenic helminths possess a wealth of glycans that are antigenic to their mammalian hosts. Translation of these antigenic glycans into vaccine candidates is contingent upon our ability to package them in a way that 1) is immunogenic, and 2) induces an immune response specific for parasite glycans. In this study, we have developed a recombinant cellular engineering platform with which to achieve these ends, and demonstrated their utility in manipulating the anti-glycan immune response and as potential vaccine antigens.

Immunization with cells or cell membrane preparations is an established technique for development of monoclonal antibodies to cell surface antigens, such as cancer antigens, blood group antigens and glycosphingolipids [337]–[339], [359]–[361]. However, there are few examples in the literature of engineering cell lines to generate immunogens with particular glycosylation patterns [340], [362], and, to our knowledge, this is the first application of such a strategy for generation of antibodies to the glycan antigens of a eukaryotic pathogen. Glycans are prominent antigens in many pathogenic infections, such as other helminthiases, leishmaniasis, and HIV, as well as several autoimmune diseases. Manipulation of mammalian cell lines with well-characterized glycosylation patterns, such as the Lec series mutants, will enable us to better characterize the function of glycosyltransferases from such pathogens, to determine the precise glycoforms targeted by parasite- or virus-neutralizing antibodies and autoantibodies to glycoproteins, and to test the immunogenicity of vaccine candidates in their appropriately

glycosylated state. The contributions of this strategy, coupled with glycan microarray technology, to glyco-immunology and vaccinology could be far-reaching.

The cells tested in this study were immunogenic without adjuvant and induced an anamnestic response consisting of IgM and IgG, suggesting that many molecular species in the Lec8 cells were immunogenic. The addition of the polymeric LDNF on cell surface N-glycans improved the cells' immunogenicity, as evidenced by higher magnitude IgG responses and longer-lasting antibodies than the parent cell lines. This raises interesting questions about the role of LDNF motifs in innate immunity. It is possible that repeating LDNF units, or fucose-containing antigens in general, specifically contribute to a robust, T-cell dependent anti-glycan response. LDNF is a ligand for DC-SIGN [220], [221], an internalizing C-type lectin receptor found on DC. LDNF was recently shown to be among the fucosylated glycans which promote DC-SIGN/TLR-4-mediated activation of human DC to a pro-inflammatory phenotype by worm glycolipids [230]. These cells could be used to determine the effect of fucosylated cell-surface motifs on parameters of immunogenicity such as internalization by antigen presenting cells (APC), T cell proliferation and generation of memory.

In spite of the impressive natural immunogenicity of the cells, Western blot and glycan array data showed that both the titer of glycan-specific antibodies and the anti-parasite titer generated by the cells were lower than that of chronically- and acutely-infected mouse serum. The variability of the response among mice was also high. This could be due to the relatively low dosage used, especially since membrane glycoproteins comprise a small percentage of total cellular material. Future studies will adjust the immunization regimen, including increasing the dosage above $10^7$ cells per animal, fixing cells and using adjuvants, with the goal of increasing the magnitude of anti-parasite response.

Immunization with Lec8 cells induced sizable shifts in IgG and IgM on flow cytometry but failed to induce GlcNAc-specific antibodies. The L8-GTFT cells, in contrast, induced glycan-specific antibodies, and when lysates were treated with N-glycanase, the immune serum reactivity

was completely lost. This indicates that addition of LDNF created an immunodominant antigen. The immunodominance of LDNF could be due to its carriage on many distinct molecular species and thus linked to many distinct T-cell epitopes, or due to special immunogenic properties of the poly-LDNF itself, as suggested above. Membrane-bound antigen may be a more efficient and more physiological method of stimulating B cell receptors [363], [364], however it is unclear whether this phenomenon is limited to antigens on the surface of APC or whether antigens on other cell membranes have the same effect. The robust IgM anamnestic response we observed for L8-GT and L8-GTFT cells was unexpected. We suspect that LDN(F) glycoconjugates are behaving as T-dependent antigens, but it is possible that poly-LDN(F) could cross-link B-cell receptors, act as a ligand for pattern recognition receptors and/or stimulate T-dependent or T-independent memory in non-classical B cells (reviewed in [365], [366]). More work is needed to understand the nature of adaptive immunity to eukaryotic glycan antigens in both vaccination and parasitic infections.

The glycan array studies of L8-GTFT immune serum, infected mouse serum and monoclonal α-LDNF antibody presented here demonstrated two extremely interesting findings. First, that antibodies generated to parasite glycans are exquisitely specific for particular forms of their epitope. In the case of LDNF, for example, IgG antibodies generated during chronic infection have a clear preference for LDNF when the trisaccharide is not directly linked to mannose. We observed the same pattern of selectivity with an $\alpha Le^X$ antibody, whereas the αLDN antibodies examined in this study had varying levels of binding to mannose-linked LDN, suggesting that such fine specificities are a common feature of anti-glycan antibodies. In agreement with our results, it has previously been noted that most antibodies to N-glycans are IgM, whereas all of the monoclonals used in this study were IgG [340]. In the case of LDNF, the antibody selectivity could be due to a particular structural presentation of LDNF, such as poly-LDNF, being immunodominant and leading to an abundance of affinity-matured antibodies that are highly specific for that presentation, or because mammals are tolerized to mannose-linked

LDNF, which may resemble a select few host glycoproteins [136], [156], [336]. The Stanley group found that when immunizing with Lec10 cells producing bisected N-glycans, only mutant mice lacking endogenous bisecting GlcNAc could produce antibodies to the structure [340]. We hypothesize that the "antigenic" form of LDNF in *S. mansoni* occurs on the non-reducing termini of glycolipids, O-glycans, and/or of extended (non-mannose-linked) N-glycan chains. Preliminary evidence suggests cercarial LDNF is carried on species other than PNGaseF-sensitive N-glycans, and studies are ongoing to answer this question.

Secondly, the poly-LDNF-expressing cells exactly recapitulated the profile of αLDNF specificities induced during *S. mansoni* infection of mice. This collection of glycans is by no means exhaustive; nevertheless, among the glycans assayed on our microarray, the immune serum and the infection serum shared the same immunodominant epitope, poly-LDNF. Our group first identified the LDNF on the biantennary N-glycans of *S. mansoni*, and, at the time, it was noted that glycopeptides with that structure were not reactive with sera from infected hamsters [160]. Furthermore, previous experiments in which biantennary LDN and LDNF were synthetically linked to carrier proteins failed to elicit a glycan-specific response, even though the conjugates were immunogenic. Cellular immunogens, in contrast, possess a high density of LDNF carried as polymeric chains. Array results suggest that, unlike synthetic glycoconjugates of biantennary LDNF, this mode of presenting LDNF provides sufficient immunogenicity, and the correct antigenicity, to induce an LDNF-specific antibody response that mimics the murine response to this antigen during parasite infection. Thus, well-defined glycan microarray structures have identified features of anti-glycan antibodies that could be helpful in design of glycan-based vaccines or diagnostics. The addition of more schistosome glycans to the array and screening of stage-specific antisera, which is currently being done by our lab and others [244] will yield additional insights.

We chose to express LDN and LDNF because they are building blocks for several unique schistosome antigens and because antibodies to these epitopes are found in schistosomula-lethal

sera of naturally protected hosts [175]. Antibodies to LDNF are also implicated as a correlate of protection in vaccination of sheep against *Haemonchus contortus*, and a potential serodiagnostic for trichinellosis [154], [155]. The L8-GTFT immunization group generated both IgM and IgG that bound to *S. mansoni* life stages, mainly to SEA. This result supports our hypothesis that expression of schistosome glycan epitopes in mammalian cells can recapitulate the immunity generated by the parasite to these epitopes. The finding that immune serum primarily targeted the egg stage was unsurprising, given that LDNF is most abundantly expressed on eggs, which was confirmed in this and previous studies by our lab and others using different clones of αLDNF [48], [50], [148]. An ideal schistosomiasis vaccine, however, would likely target the earlier intra-mammalian life stages. There are other antigenic glycans that are expressed more highly on larval stages [48], and the cellular expression system could be adapted to target such antigens. A vaccine targeting one glycan antigen that is highly expressed on the larval or adult worm surface, or a few glycan and protein antigens that are differentially localized and/or expressed on multiple intra-mammalian life stages might be effective against helminths. Many of the glycan epitopes targeted in schistosome infection have been discovered [134], [146], [150], [160], [272], [367], but few have been characterized in terms of protective ability. The recombinant engineering strategy could be utilized to assess candidate glycoconjugates by co-expressing parasite glycans and proteins, either in a whole-cell platform or as secreted, soluble, parasite-like glycoproteins.

This study serves as a proof-of-concept that glyco-engineered cells, in contrast to synthetic glycoconjugates carrying similar epitopes, induce a long-lived and highly specific antibody response towards an immunodominant surface-expressed glycan. Our work has highlighted the complex epitope specificity of anti-glycan antibodies. This type of information, along with further advances in helminth glycomics and glycan microarray technology, will shape the structural guidelines for design of glycan-based vaccines in the future. In addition to providing new directions for helminth vaccine development, these cells should facilitate the

discovery of novel glycosyltransferase activities in a variety of systems, and help us better understand the role of glycan antigens in infectious and autoimmune diseases.

### 3.5 Acknowledgements

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# Part II: Investigating the role of anti-glycan antibodies in schistosome infection

# Chapter 4. *Schistosoma mansoni*-infected Brown Rats Target Core Xylose/Core α3 Fucose Epitopes during a Protective Response

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## 4.1 Introduction

Schistosomiasis, caused by infection with parasitic helminths of the genus *Schistosoma*, infects over two hundred million people and causes up to seventy million disability-adjusted-life years [5], [14]. Many mammals can be infected, including rodents, non-human primates and bovines. Some species, like humans, are susceptible to chronic infection which can only be cured with chemotherapeutic treatment, while other species can clear the worms before or after they reach fecundity [reviewed in 3]. These species provide a natural example of protective mechanisms that could potentially be exploited to bolster human resistance.

Rodents, for example, display varying levels of susceptibility to *Schistosoma mansoni* infection, even though the percentage of cercariae that penetrate skin is similar [368]. Between eight and twenty weeks post infection, mice harbor roughly half of the number of worms they were exposed to, which lay thousands of eggs, and exhibit hepatosplenomegaly [368], [369]. Although they develop partial immunity (up to 90%) after vaccination with irradiated cercariae, mice can become chronically infected multiple times [370]. Laboratory rats (*R. norvegicus*), by contrast, are considered a semi-permissive host since they are highly resistant to a large dose (ex. 500 cercariae) but relatively tolerant to lower doses (10-50 cercariae) [127]. At any dose, lab rats have reduced the number of worms to 0-10% by eight weeks post infection, and although there is

some egg exposure, eggs are infertile and rats do not become sick [127], [368], [371]–[374]. Furthermore, rats will clear a second infection even more rapidly than the first [375]. Review of this literature led us to wonder what mechanisms contribute to protection in rats which are ineffective in mice during the first 8 weeks of infection.

It has been suggested that both immunological and physiological factors could play a role in the differing permissiveness of rodents to *S. mansoni* infection. The overwhelming body of evidence demonstrates that humoral responses strongly contribute to rejection of worms in primary and secondary rat infections and in vaccination with radiation-attenuated cercariae [126], [127], [374]–[377]. Rat serum develops its protective capacity between 2-7 weeks after infection, and when transferred from exposed rats to naïve rats, it is only protective until the first few days after challenge, suggesting that the critical targets of immunity during rat rejection are the early migrating schistosomulum [127], [375], [378]. Adsorption of IgG2a and IgE from infected rat serum abrogated its protective ability [82]. Partial resistance in vaccinated mice has been attributed to IgG and IgM in several studies, with passive transfer of immune sera to other mice resulting in 20-62% protection to re-infection [120], [126], [378], [379]. Passive transfer of rat hyper-immune serum to mice, on the other hand, confers up to 88% protection from challenge [378]. Various antibody-mediated killing mechanisms have been observed *in vitro* using rat serum, including IgG and IgE-mediated schistosomula killing by eosinophils and both innate and adaptive complement activation [84], [85], [87], [120], [126].

All *S. mansoni*-infected hosts examined to date also display an abundant humoral response to glycan antigens of the parasite [144], [163], [164], [175], [194], [195], [243], [244]. These include Lewis X ($Le^X$), LacdiNAc (LDN), Fucosylated LacdiNAc (LDNF), F-LDN, LDNF-DF, core α3 fucose (CF), core xylose (CX) (Figure 1.2), and likely many yet undiscovered epitopes tested [144], [163], [164], [175], [194], [195], [243], [244]. Anti-glycan antibodies capable of killing schistosomula *in vitro* and protecting mice or rats by passive transfer have been isolated by several groups in the past, raising hopes that a well-timed anti-glycan response of the

proper specificity and isotype composition could be protective in humans [86], [148], [151], [249]–[253]. However, the role of the anti-glycan component of the immune response in either in partially resistant human populations or in protective animal models has never been directly tested.

The goal of our study was to identify differences in the anti-glycan titer, specificity or life-stage targeting between rats and mice that might contribute to the protective immune response in one and not the other. We observed some striking differences between the two species including a greater breadth of anti-glycan targeting, a prominent anti-core xylose/core α3-fucose response and periodate-sensitive targeting of small molecular weight species in larval and adult parasite life stages by the rat, which were not observed in mice. Further investigation into the functional significance of these differential anti-glycan responses is warranted.

## 4.2 Materials and Methods

### 4.2.1 Antibodies

Secondary antibodies used for array screening were purchased from Invitrogen. Goat α-mIgG-Alexa 488, α-mIgM-Alexa 633 (for DSA); goat α-rat IgG-Alexa 546 (A11081), goat α-rat IgM-Alexa 488 (A21212) (for DSA and CFG); α-mouse IgM-488 (A21042), α-mouse IgG-568 (A11031) (for CFG); goat anti-rabbit IgG-Alexa 488 (DSA). HRP-conjugated secondary anti-mouse and anti-rabbit antibodies and streptavidin-HRP used for Western blotting and ELISA were purchased from KPL. Rabbit anti-rat IgG-HRP was purchased from Dako (P0450). Anti-horseradish peroxidase antibody produced in rabbit was purchased from Sigma (P7899).

### 4.2.2 Animal infections

Male Fischer rats were infected with 1000 *S. mansoni* cercariae (high dose) and re-infected at 4 weeks, at which time point it had previously been demonstrated that most adult worms were cleared [380]. Pooled rat sera was obtained at 4 weeks post-secondary infection from 5 animals.

*S. mansoni*-infected mice were kindly provided by the Schistosome Research Reagent Resource Center (Rockville, MD) for distribution by BEI Resources, NIAID, NIH. Female Swiss-Webster mice (4-6 weeks old) from Taconic Farms were infected with an average of 30 (low dose) or 200 (high dose) cercariae per mouse, and shipped to our facility. Infected mice were monitored for abdominal distention and piloerection and sacrificed if experiencing excessive stress. High dose-infected mice were sacrificed at 7.5 weeks post-infection for collection of adult worms and eggs, and low-dose infected mice were sacrificed at 20 weeks post infection. Sera were pooled from 5 animals in each group at 2 weeks, 4 weeks and 6 weeks; 8 week and 20 week sera were obtained from low dose infected animals only. All sera were obtained by facial vein puncture except the chronic infection sera, which was obtained by cardiac puncture following euthanasia. At the conclusion of our experiments, all infected mice were euthanized by intraperitoneal overdose with 300µl of 65mg/ml sodium pentobarbital with 200 U/ml heparin sodium salt. All experiments involving mice were approved by the Emory University IACUC.

### 4.2.3 Array binding assays

The Defined Schistosome-type Array was prepared and validated as previously described [174], [175], [247]. Binding assays were performed as per Heimburg-Molinaro *et al.* for experiments with rabbit anti-HRP [247]. For binding assays with animal sera, rat and mouse serum was diluted 1:50 in TSM binding buffer (20mM Tris-HCl pH 7.4, 150mM sodium chloride, 2mM calcium chloride, 2mM magnesium chloride, 0.05% tween-20, 1% BSA) for a 1-hour incubation with rocking, and wells were washed 3 times with 200µl TSM wash buffer (20mM Tris-HCl pH 7.4, 150mM sodium chloride, 2mM calcium chloride, 2mM magnesium chloride, 0.05% tween-20), then 3 times with 200µl TSM buffer (20mM Tris-HCl pH 7.4, 150mM sodium chloride, 2mM calcium chloride, 2mM magnesium chloride) with 5 minutes of shaking for each wash, after the primary and secondary incubations. Secondary antibodies were used at 5µg/mL.

For HRP blocking experiments on the DSA, 100ug of HRP that had previously been boiled was added to the naïve or infected sera diluted in array binding buffer and rocked for 10 minutes at room temperature before adding to the array chambers.

Binding assays on the CFG array version 5.1 were conducted as described [247] with the following modifications: Mouse and rat sera were used at 1:50 in 70ul of binding buffer for the primary incubation. After each incubation, the slide was dipped three times and rocked for 3 5-minute incubations in wash buffer with tween, and the same procedure was then repeated in wash buffer without tween. Naïve mouse serum and naïve rat serum were run first and, once scanned and verified to be negative, slides were re-used for infection anti-sera.

Slides were scanned using the ScanArray Express software on a PerkinElmer Proscanner XL4000. ScanArray Express was used to align spots, remove background and quantify fluorescence. An excel macro file was then used to average 4 replicate spots for each glycan ID # and determine SEM, SD and %CV. Glycopattern website (https://glycopattern.emory.edu/) was used to visualize top binders and compare heat map results among samples on the CFG [381].

### 4.2.4 Isolation of *Schistosoma mansoni* life stages and preparation of parasite lysates

*S. mansoni*-infected *Biomphalaria glabrata* snails were provided by the Schistosome Research Reagent Resource Center for distribution by BEI Resources, NIAID, NIH: *S. mansoni*, strain NMRI NR-21962. Snail maintenance and collection of cercariae was as per unit 19.1 of Current Protocols in Immunology, "Schistosomiasis" [349] with modifications described previously [174]. For transformation to schistosomula, the cercarial pellet was resuspended in 5-10mL of cold DMEM and vortexed on high for 2x 45 second periods, with a 3-minute ice incubation in between; or the pellet was resuspended to 1000 cercariae/mL in cold DMEM and transformed by passage 8 times though a syringe and 22-G needle, in 10 mL batches. The suspension of detached schistosomula and tails was allowed to settle and the bottom few mL were loaded onto a 40 or 60% Percoll gradient in DMEM. The gradient was centrifuged for 15 minutes

at 500xg, $4^0$C. The supernatant was carefully removed down to the bottom 1-2 mL, and the schistosomula pellet was washed 3 times by spinning at 300xg in cold DMEM. The schistosomula were cultured for 3 days in DMEM with 10% FBS, penicillin/streptomycin at a density of 500-1000 organisms/mL in tissue-culture dishes. Preparation of adult worms and eggs, and soluble egg antigen was as per Lewis et al (1998) and with modifications described previously [174]. All work with *B. glabrata* and *S. mansoni* was approved by the Emory University Office of Occupational Health and Safety, and conducted in BSL-II animal surgery facilities and laboratories.

Schistosomula were prepared by vortexing cercariae for 45 seconds, icing 3 minutes, and vortexing another 45 seconds to remove tails. Schistosomula were isolated by spinning at 1500 rpm for 10 minutes through a 60% Percoll solution in PBS and washing the schistosomula pellet twice in cold DMEM with Penn/Strep, with 1000 rpm spins. They were cultured for 3 days at 37°C with 5% carbon dioxide in DMEM with 10% fetal bovine serum and Penn/Strep. After culture they were spun at 1000 rpm at 4°C for 10 minutes and washed twice in cold PBS before adding lysis buffer.

For preparation of parasite lysates, chilled pelleted cercariae or schistosomula were transferred to 1.5 mL Eppendorf tubes in at most 50 µl of parasite lysis buffer (50mM Tris buffer pH 8.0, 2.5% 2-mercaptoethanol, 1% Triton-X-100, 1mM EDTA and 1 tablet of Complete Mini Protease Inhibitor per 10mL of lysis buffer) per 10,000 cercariae or 25 µl of parasite lysis buffer per 10,000 schistosomula. This was vortexed, boiled for 15 minutes (vortexing once during the boiling incubation), and centrifuged at 20,000xg for 2 minutes. The supernatant was removed to a clean tube, and a small amount of lysis buffer was added to the pellet for another 10 minute boiling incubation, after which the spin was repeated and the supernatants were pooled. Adult worm lysate was made by bringing a freshly-thawed adult worm pellet of approximately 0.5 mL up in 5mL of PBS, spinning at 500xg for 10 min at $4^0$C, and adding 3 mL of lysis buffer to the

pellet. The worm lysate was made as described above except 1% SDS and 1mM phenylmethylsulfonylfluoride (PMSF) were used instead of Triton-X-100 and the Complete Mini tablet, respectively, and the SDS was salted out using 100mM KCl so the lysates could be concentrated in Amicon spin filter tubes (3000 Da MWCO). Triton X-100 was then added back to 1% to the concentrated adult worm lysates. Soluble egg antigen was prepared as per unit 19.1, "Schistosomiasis," of Current Protocols in Immunology [349]. Parasite lysates were quantified by the Pierce 660nm and BCA protein assays and stored in aliquots at -80$^0$C.

Periodate treatment of parasite lysates in solution was accomplished by dialyzing cercarial lysate into water overnight to remove buffer and reducing agents and then adjusting the buffer to 0.1M sodium acetate, 0.15M NaCl, pH5.5. Sodium m-periodate was added to 20mM and the mixture was rotated in the dark at 4ºC overnight. The reaction was quenched with a molar excess of sodium borohydride. The reactions were again dialyzed to water and concentrated.

### 4.2.5 SDS-PAGE and Western blots

For SDS-PAGE, 5-15µg per well of parasite lysates (lanes in the same figure were equally loaded unless specified) or 2ug/well of glycoproteins (BSA and HRP) were boiled in 1x NuPAGE SDS sample buffer + 2.5% β-mercaptoethanol for 10 minutes and then run in Mini-PROTEAN-TGX gels at 200V for 30 minutes, with 7µl of protein standards. Protein was transferred to a nitrocellulose membrane using the 10-minute High Molecular Weight program, 7-minute mixed molecular weight program or 30-minute standard program in the Trans-Blot Turbo semi-dry transfer system. All subsequent incubations were shaking at ambient temperature. Membranes were stained with 0.1% Ponceau S in 5% acetic acid to check for equal loading and transfer, and destained with 1X TBS-t wash buffer (20mM Tris, 300mM NaCl, 0.1% Tween-20). At this point the membrane was sometimes stored overnight at 4ºC in TBS.

To silver stain, SDS-PAGE gels were placed in destaining solution (50% ethanol, 10% acetic acid) for 1 hour and then washed in 50% ethanol three times, for 20 minutes each. All

incubations were with shaking. The gel was then placed in pre-treatment solution (0.02% sodium thiosulfate) for one minute and washed three times, 20 seconds each in distilled water. It was then placed in impregnating solution (0.2% silver nitrate with 0.03% formaldehyde) for 20 minutes. The gel was then washed three times in distilled water for 20 seconds each and placed in developer solution (6% sodium carbonate, 0.02% formaldehyde, 0.0004% sodium thiosulfate) until bands appeared and destained.

For staining with serum, membranes were blocked for 2h in 2% (w/v) de-fatted dried milk in 0.5X TBS-t (10mM Tris, 150mM NaCl, 0.05% tween), followed by one 10-minute wash in 1X TBS-t. Sera were diluted 1:1000 in milk diluent (0.5% milk in 0.5X TBS-t) and incubated with the membranes for 1 hour. The membranes were then washed 3 times quickly and 3 times for 5 minutes each in 1X TBS-t. Secondary detection antibodies (HRP-conjugated goat anti-mouse-IgG or –IgM) were added for one hour at 1:5000 (mouse) or 1:3000 (rat) in milk diluent. The same wash procedure was repeated, and then Super Signal West Pico Chemiluminescent Substrate was added for 30 seconds. The membranes were dabbed dry and exposed to film for 1s-3min (exposure times are indicated when this varied for panes shown in the same figure).

When blotting was performed with rabbit anti-HRP, a similar protocol was followed except blocking and antibody incubations were performed in 5% bovine serum albumin fraction V (BSA) diluent, the primary antibody was used at 1ug/mL, the secondary anti-rabbit antibody was used at 1:5000, and three five-minute washes were performed after each incubation.

When HRP was used to block reactivities in Western blots, the protein was added to the sera/primary antibody in diluent for 5 minutes, rocking, before adding to the membrane.

When periodate-treated lysates were stained with sera, anti-rat and anti-mouse secondaries were both used at 1:3000.

### 4.2.6 ELISA

Horseradish peroxidase type VI (HRP) (Sigma, P8375) was permanently denatured to destroy endogenous peroxidase activity before use in ELISA, or boiled before SDS-PAGE/Western blots. HRP was dissolved in 0.2M Tris-HCl with 8M guanidine HCl and 60mM dithiothreitol and rolled for 1 hour at room temperature. Iodoacetamide was added to 100mM and incubated for another 30 minutes, followed by dialysis of the HRP into water. It was verified that the denatured HRP gave no background signal from endogenous enzyme activity at the coating concentrations used.

HRP was coated on ELISA plates at 0.5ug/mL in 0.05M carbonate/bicarbonate buffer pH 9.6, 50µl per well, in a clear, flat-bottom, non-tissue culture treated 96-well plate (Greiner, 655101) overnight at 4°C. Excess solution was discarded and wells were washed three times in PBS-t (0.05% tween-20). Sodium acetate 0.1M, pH 5.5 was added, 100µl per well, for 30 minutes, and then 20mM sodium-m-periodate in sodium acetate buffer, or buffer alone for mock-treatment, 100µl per well, was added for an overnight incubation at 4°C in the dark. The periodate solution was then discarded and all wells were quenched with 150µl of 0.1M sodium borohydride solution in PBS-t for 30 minutes at room temperature. Wells were then washed three times with PBS-t using a squirt bottle and the ELISA was performed.

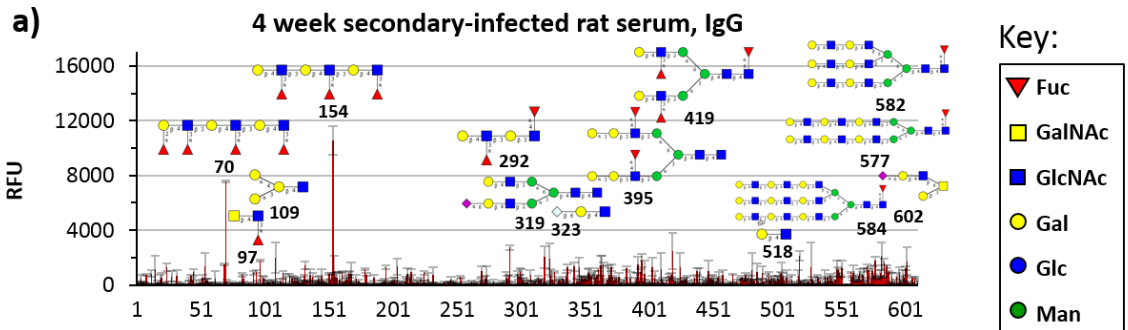
Plates were blocked with 200µl per well of 3% BSA in PBS-t. All incubations were for 1 hour at room temperature with rocking. Primary and secondary antibodies were made up in PBS-t with 1% BSA (1-10ug/mL for rabbit anti-HRP; 1:1000 for HRP-conjugated goat anti-rabbit IgG and 50µl was added to each well. For detection, 1 tablet of O-phenylenediamine dihydrochloride was dissolved to 0.5mg/mL in stable peroxide buffer (Thermo) and 50µl was added to each well, and incubated, standing, in the dark for 10 minutes. To quench the reaction, 25µl of sulfuric acid was added. The plate was read at 490nm on a Victor plate reader.

## 4.3 Results

### 4.3.1 Secondary-infected rat sera has broad anti-glycan targeting of Lewis X and poly-N-acetyllactosamines (poly-LN) on the Consortium for Functional Glycomics Array

We first asked what differences in glycan specificity were seen between rats and mice on the Consortium for Functional Glycomics (CFG) Array, a large collection of synthetic, predominantly mammalian-like glycan structures. Pooled sera was obtained from Fischer rats at 4 weeks post-secondary infection with 1000 *S. mansoni* cercariae, when they are actively rejecting a secondary infection, and compared with pooled *S. mansoni*-infected Swiss-Webster mouse serum from various time points during acute and chronic infection. Sera from 4-6 weeks post-high dose (200 cercariae) infection is shown for comparison with that of rats, as well as sera from low dose (30 cercariae) chronic infections at 8-20 weeks (Figure 4.1 and Figure 4.2). (Mice infected with a high dose of cercariae succumb to infection and must be sacrificed by around 8 weeks.) The CFG structures discussed below are depicted in Figure 4.1 and Figure 4.2, and a full listing of printed structures on the CFG version 5.1 can be found at http://www.functionalglycomics.org/static/consortium/resources/resourcecoreh8.shtml.

Infected rat serum contains a great breadth of IgG binding to glycans on the CFG array (Figure 4.1a-b). The primary targets were glycans containing the $Le^X$ and lactosamine-type structures, including polymeric forms of these antigens ($Le^X$: ID#s 70, 154, 292, 419; poly-N-acetyllactosamine: #571-584). Naïve rat serum demonstrated virtually no binding to the CFG array (data not shown). The top thirty structures bound by rat IgG are sorted from highest (red) to lowest (black) relative fluorescent units (RFUs) in Figure 4.1b, and the adjacent columns provide a comparison of how strongly the same glycan IDs were bound at various time points in mouse infection. The heatmap demonstrates the large number of low-to-moderate level binders in rat serum. As mouse infection progresses from acute to chronic, more of the structures bound by rat serum are also targeted in mouse serum (Figure 4.1b).



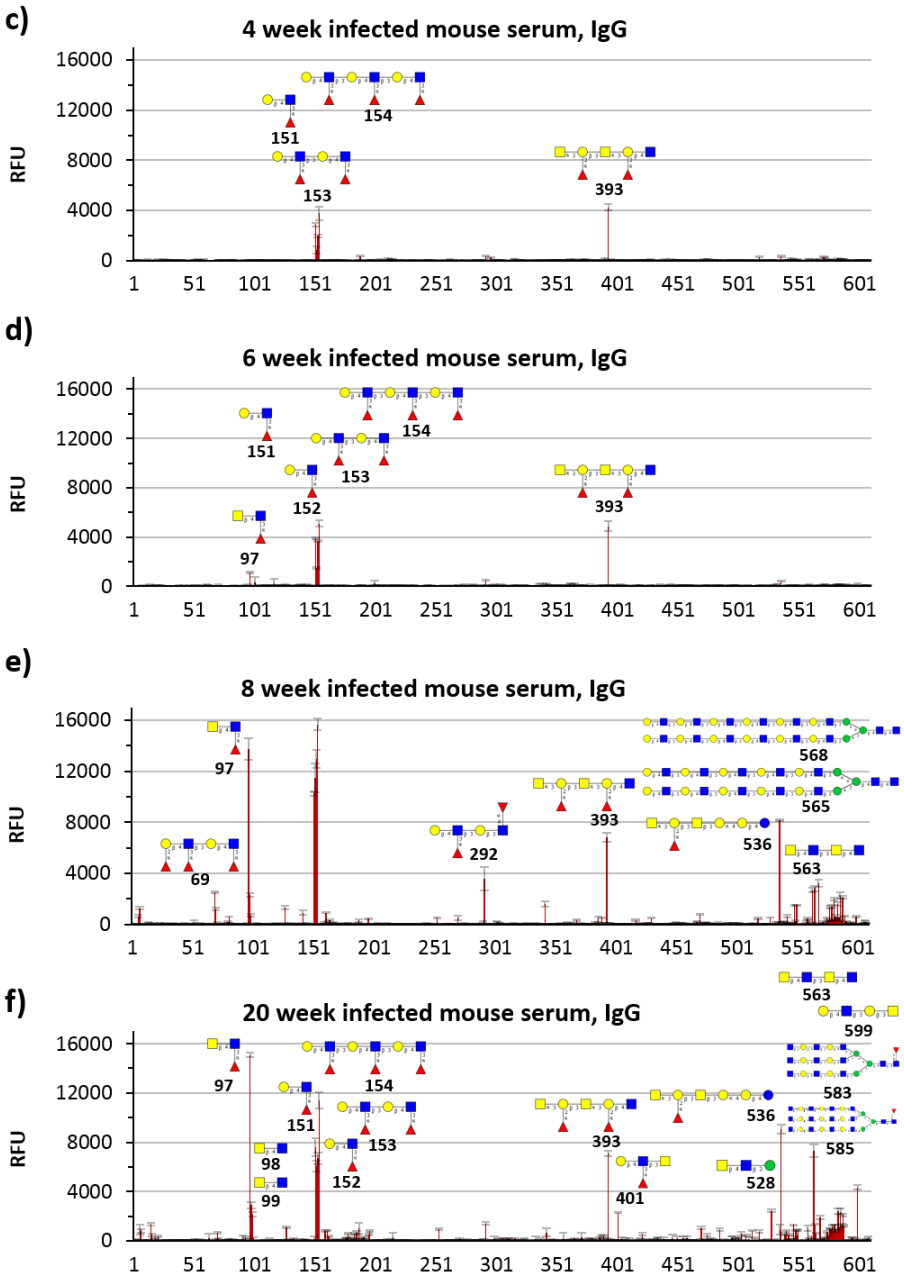

**b) CFG Binders of Infected Rat and Mouse Serum: Sorted high to low by rat serum**

| | 2° Inf. Rat Week | 1° Inf. Mouse Week | | | |
|---|---|---|---|---|---|
| Chart No. | 4 | 4 | 6 | 8 | 20 |
| 154 | 10561 | 3770 | 5075 | 15682 | 11406 |
| 70 | 7560 | 6 | 31 | 1189 | 653 |
| 292 | 2734 | 361 | 470 | 3566 | 1400 |
| 419 | 2508 | 18 | 60 | 40 | 100 |
| 518 | 2136 | 293 | 22 | 449 | 740 |
| 582 | 2111 | 39 | 130 | 595 | 693 |
| 319 | 2040 | 24 | 30 | 43 | 21 |
| 109 | 1987 | 20 | 52 | 38 | 40 |
| 323 | 1897 | 9 | 31 | 36 | 78 |
| 395 | 1785 | 80 | 105 | 75 | 151 |
| 577 | 1780 | 67 | 69 | 1353 | 961 |
| 97 | 1771 | 29 | 1089 | 13762 | 15092 |
| 602 | 1745 | 23 | 29 | 25 | 43 |
| 584 | 1668 | 44 | 105 | 1613 | 1078 |
| 365 | 1533 | 114 | 178 | 114 | 193 |
| 69 | 1505 | -2 | 21 | 2546 | 112 |
| 392 | 1499 | 36 | 87 | 71 | 106 |
| 132 | 1444 | 21 | 43 | 61 | 62 |
| 363 | 1410 | 17 | 123 | 40 | 54 |
| 571 | 1394 | 280 | 134 | 144 | 179 |
| 586 | 1359 | 76 | 143 | 2246 | 1228 |
| 342 | 1356 | 39 | 205 | 1611 | 680 |
| 204 | 1355 | 106 | 40 | 64 | 68 |
| 54 | 1349 | 22 | 38 | -14 | 33 |
| 403 | 1333 | 25 | 87 | 36 | 63 |
| 527 | 1331 | 24 | 61 | 40 | 41 |
| 24 | 1304 | 39 | 82 | 57 | 50 |
| 195 | 1301 | 5 | 35 | 32 | 512 |
| 425 | 1297 | 9 | 17 | 35 | 54 |
| 337 | 1296 | 45 | 102 | 62 | 110 |

**Figure 4.1 Secondary-infected rat IgG has broad anti-glycan targeting, including antibodies to Le$^X$ and poly-LN.**

Rat serum from 4 weeks post *S. mansoni* re-infection (a) and mouse serum from 4-20 weeks (c-f) weeks post infection binding to the Consortium for Functional Glycomics (CFG) v5.1 array. Anti-mouse IgG-Alexa 568 and anti-rat IgG Alexa 546 were used for detection of IgG (a-f). Each bar represents an average of binding intensity (relative fluorescence units, RFU) to hexareplicate spots for each glycan ID number (listed on the X-axis and above selected bars) +/- standard

deviation. The top binding structures are depicted, according to the monosaccharide key. Heatmaps of the binding RFU, sorted by highest binders (red) to non-binders (black) in infected rat serum (b), 6- and 8-week-infected mouse serum (g-h) are shown. Only the top 20 binders are shown. Full listing of binders and non-binders can be found at the *Consortium for Functional Glycomics* website. The longitudinal binding of mouse infection antisera, color-coded by groups of glycans with similar terminal epitopes, is displayed in (i). ($Le^X$, blue; LDNF, red; LDN, green; poly-LN, orange).


c)
4 week infected mouse serum, IgG
RFU
16000
12000
8000
4000
0
1 51 101 151 201 251 301 351 401 451 501 551 601
151
154
153
393
d)
6 week infected mouse serum, IgG
RFU
151
154
153
152
97
393
e)
8 week infected mouse serum, IgG
RFU
97
69
292
393
568
565
536
563
f)
20 week infected mouse serum, IgG
RFU
97
154
151
153
98
152
99
393
401
563
599
583
536
585
528

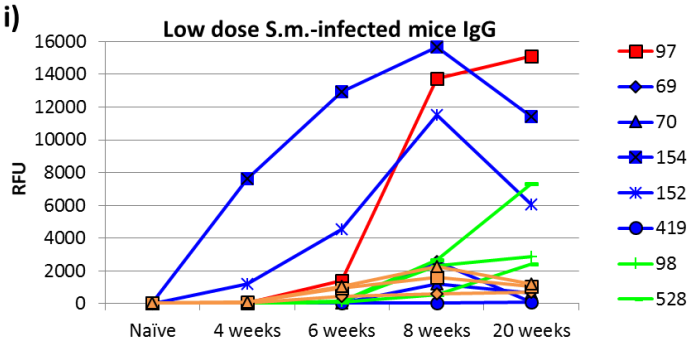
**g) Sorted by 6-wk Inf. Mouse**

| Chart No. | 2° Inf. Rat Week: 4 | 1° Inf. Mouse Week: 4 | 6 | 8 | 20 |
|---|---|---|---|---|---|
| 154 | 10561 | 3770 | 5075 | 15682 | 11406 |
| 393 | 335 | 4243 | 4874 | 6834 | 7096 |
| 151 | 812 | 2873 | 3902 | 10259 | 7606 |
| 153 | 835 | 1974 | 3677 | 12977 | 6688 |
| 152 | 391 | 839 | 1500 | 11517 | 6058 |
| 97 | 1771 | 29 | 1089 | 13762 | 15092 |
| 292 | 2734 | 361 | 470 | 3566 | 1400 |
| 536 | 14 | 339 | 400 | 8164 | 9048 |
| 101 | 90 | 8 | 274 | 15 | 10 |
| 117 | 479 | 26 | 250 | 31 | 50 |
| 200 | 229 | 5 | 222 | 33 | 25 |
| 342 | 1356 | 39 | 205 | 1611 | 680 |
| 365 | 1533 | 114 | 178 | 114 | 193 |
| 572 | 1094 | 207 | 174 | 174 | 266 |
| 361 | 1160 | 81 | 146 | 51 | 66 |
| 586 | 1359 | 76 | 143 | 2246 | 1228 |
| 571 | 1394 | 280 | 134 | 144 | 179 |
| 582 | 2111 | 39 | 130 | 595 | 693 |
| 362 | 344 | 36 | 124 | 60 | 96 |
| 363 | 1410 | 17 | 123 | 40 | 54 |
| 533 | 469 | 28 | 116 | 144 | 92 |
| 14 | 58 | 9 | 109 | 96 | 16 |
| 559 | 606 | 72 | 107 | 483 | 188 |
| 274 | 760 | 16 | 106 | 20 | 32 |
| 584 | 1668 | 44 | 105 | 1613 | 1078 |
| 590 | 616 | 70 | 105 | 656 | 82 |
| 395 | 1785 | 80 | 105 | 75 | 151 |
| 445 | 445 | 75 | 104 | 43 | 57 |
| 337 | 1296 | 45 | 102 | 62 | 110 |
| 588 | 997 | 98 | 101 | 2119 | 1251 |

**h) Sorted by 8-wk Inf. Mouse**

| Chart No. | 2° Inf. Rat Week: 4 | 1° Inf. Mouse Week: 4 | 6 | 8 | 20 |
|---|---|---|---|---|---|
| 154 | 10561 | 3770 | 5075 | 15682 | 11406 |
| 97 | 1771 | 29 | 1089 | 13762 | 15092 |
| 153 | 835 | 1974 | 3677 | 12977 | 6688 |
| 152 | 391 | 839 | 1500 | 11517 | 6058 |
| 151 | 812 | 2873 | 3902 | 10259 | 7606 |
| 536 | 14 | 339 | 400 | 8164 | 9048 |
| 393 | 335 | 4243 | 4874 | 6834 | 7096 |
| 292 | 2734 | 361 | 470 | 3566 | 1400 |
| 568 | 729 | 83 | 80 | 3222 | 1919 |
| 565 | 877 | 24 | 67 | 2788 | 611 |
| 563 | 316 | 8 | 12 | 2686 | 7317 |
| 69 | 1505 | -2 | 21 | 2546 | 112 |
| 98 | 96 | 21 | 0 | 2298 | 2884 |
| 586 | 1359 | 76 | 143 | 2246 | 1228 |
| 588 | 997 | 98 | 101 | 2119 | 1251 |
| 581 | 863 | 28 | 70 | 1999 | 954 |
| 584 | 1668 | 44 | 105 | 1613 | 1078 |
| 342 | 1356 | 39 | 205 | 1611 | 680 |
| 548 | 1096 | 40 | 71 | 1527 | 846 |
| 550 | 656 | 73 | 53 | 1501 | 846 |
| 579 | 514 | 40 | 81 | 1403 | 788 |
| 577 | 1780 | 67 | 69 | 1353 | 961 |
| 127 | 236 | 18 | 43 | 1318 | 1061 |
| 7 | 118 | -3 | 16 | 1276 | 788 |
| 70 | 7560 | 6 | 31 | 1189 | 653 |
| 161 | 981 | 30 | 41 | 926 | 822 |
| 142 | 359 | 10 | 12 | 924 | 130 |
| 470 | 43 | -5 | 16 | 766 | 1043 |
| 99 | 520 | 19 | 22 | 689 | 2140 |
| 590 | 616 | 70 | 105 | 656 | 82 |

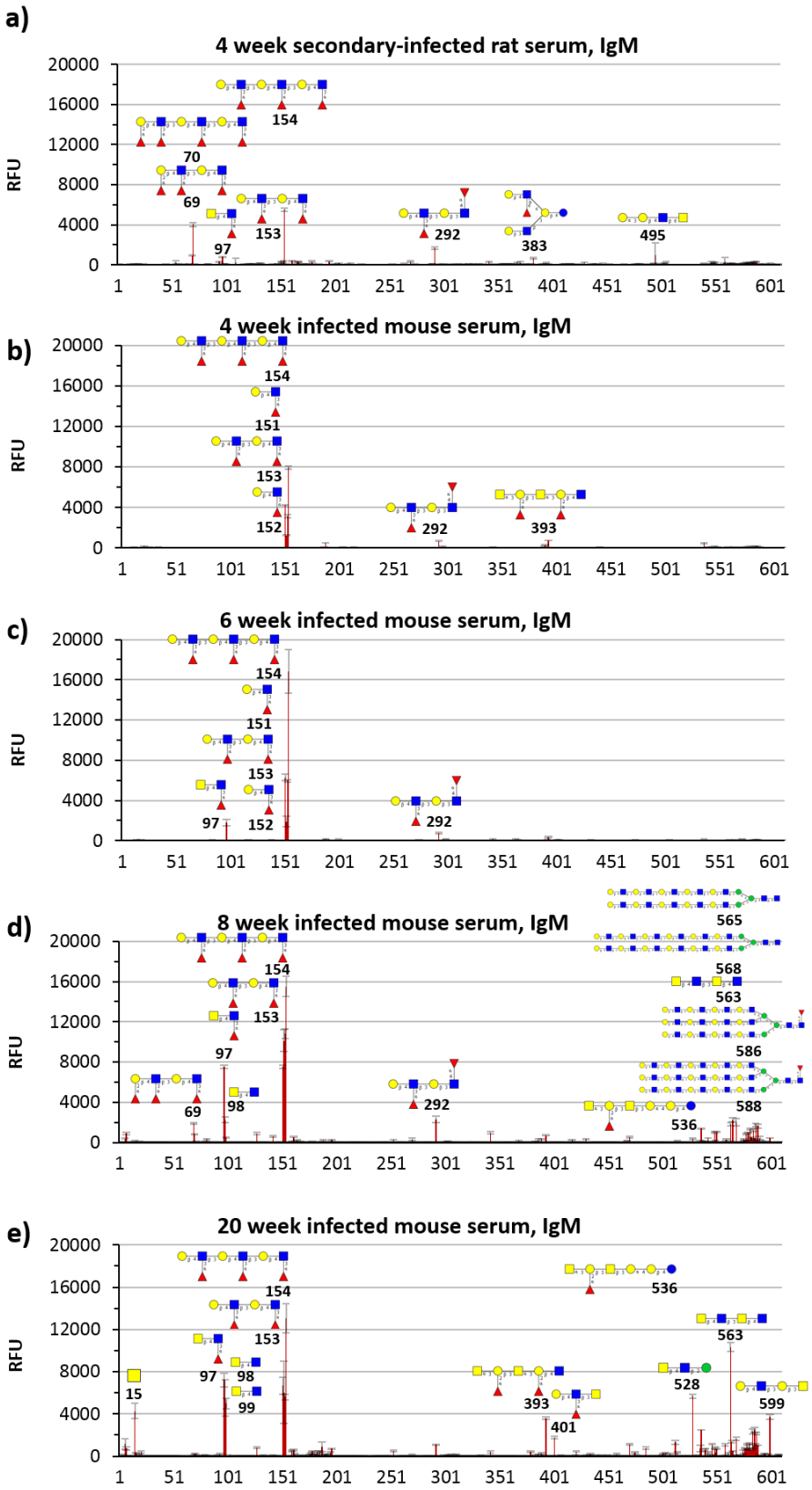
**i)**




Infected mice also bound poly-Le$^X$ at the 4-week (before egg-laying has commenced) and 6-week time points (Figure 4.1c-d) and showed a marked broadening of the response by 8 weeks to include several Le$^X$ variants, LDN, LDNF and lactosamine-containing structures (Figure 4.1e-f). A less robust response to poly-LN was also seen at 6 weeks in the low dose infection (data not shown). Heatmaps of the top binders, this time sorted by highest binders in 6-week and 8-week infected mouse sera, are shown in Figure 4.1g-h. The mouse IgG response during early infection

(4- and 6- week time points) is focused on $Le^X$ variants (ID#s 151-154) and LDNF (ID# 97) with few other significant binders (Figure 4.1g). The later infection time points, which occur after egg-laying is well-established, display a marked broadening in the IgG response, with many specificities similar to the rat IgG response (Figure 4.1h). The chronic mouse serum is also notable for prominent targeting of LDN (ID# 98-99, 528, 563) and LDNF (#97), which are bound only at a low level by rat serum IgG. Plotting binding to several of the aforementioned structures over the course of a low-dose mouse infection highlighted the fact that antibodies to $Le^X$ are among the earliest to be detected, followed by LDNF and lactosamine antigens, and finally from 8 weeks onwards, IgG to LDN appears (Figure 4.1i).

The IgM binding profile during rat infection was also primarily focused on Lewis-X containing structures (ID #70, 153, 154, 161, 292, 383) but did not show the preponderance of antibodies to poly-LN-containing structures that was bound by IgG (Figure 4.2a). The mouse IgM binding profile was very similar to what was bound by IgG, focusing on $Le^X$ structures at the early time points, then expanding to LDNF, and, at 8 weeks and later, LDN and poly-LN (Figure 4.2b-f).


a)
4 week secondary-infected rat serum, IgM
RFU
154
70
69
153
97
292
383
495
b)
4 week infected mouse serum, IgM
154
151
153
152
292
393
c)
6 week infected mouse serum, IgM
154
151
153
97
152
292
d)
8 week infected mouse serum, IgM
565
568
563
154
153
586
97
69
98
292
588
536
e)
20 week infected mouse serum, IgM
536
154
563
153
97
98
528
15
393
599
99
401
0
4000
8000
12000
16000
20000
1
51
101
151
201
251
301
351
401
451
501
551
601

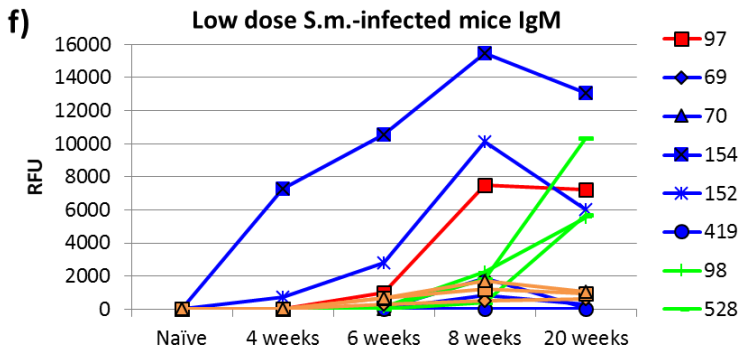
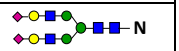
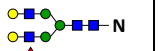
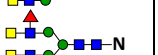
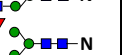
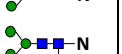
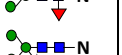
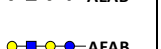
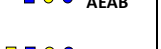
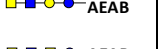



**Figure 4.2 Secondary-infected rat IgM targets Lewis X and LDNF.**

Rat serum from 4 weeks post *S. mansoni* re-infection (a) and mouse serum from 4-20 weeks (b-e) weeks post infection binding to the Consortium for Functional Glycomics (CFG) v5.1 array. Anti-mouse IgM-Alexa 488 and anti-rat IgM-Alexa 488 were used for detection of IgM (a-e). Each bar represents an average of binding intensity (relative fluorescence units, RFU) to hexareplicate spots for each glycan ID number (listed on the X-axis and above selected bars) +/- standard deviation. The top binding structures are depicted, according to the monosaccharide key. Full listing of binders and non-binders can be found at the *Consortium for Functional Glycomics* website. The longitudinal binding of mouse infection antisera, color-coded by groups of glycans with similar terminal epitopes, is displayed in (f). (Lewis X, blue; LDNF, red; LDN, green; poly-LN, orange).

### 4.3.2 Rats, but not mice, target core xylose/core α3 fucose on the Defined Schistosome-Type Array

Given the prominent anti-glycan response observed in both schistosome-infected rodent models, we wanted to compare their antibody responses using a defined schistosome-type glycan array (DSA). The DSA is a collection of structurally defined, semi-synthetic variants of LDN, LDNF, $Le^X$, core xylosylated/core α3-fucosylated N-glycans (CX/CF) and control glycans, which has been validated using lectins, monoclonal antibodies and infection antisera [174], [175]. A list of structures found on the DSA is provided below (Figure 4.3).

| Glycan ID # | Nickname | Conc. | Pictoral Structure |
|---|---|---|---|
| 1 | BiASialo | 100uM |  |
| 2 | BiAAsialo | 100uM |  |
| 3 | BiALe$^{x}$ | 100uM |  |
| 4 | BiAAgalacto | 100uM |  |
| 5 | BiALDN | 100uM |  |
| 6 | BiALDNF | 100uM |  |
| 7 | BiAMan3 | 100uM |  |
| 8 | BiAFMan3 (α3CF) | 100uM |  |
| 9 | BiAXMan3 | 100uM |  |
| 10 | BiAXFMan3 (α3CF) | 100uM |  |
| 11 | AEAB-LNnT | 100uM |  |
| 12 | AEAB-neoLe$^{x}$ | 100uM |  |
| 13 | AEAB-agalactoLNnT | 100uM |  |
| 14 | AEAB-neoLDN | 100uM |  |
| 15 | AEAB-neoLDNF | 100uM |  |
| 16 | AEAB-polyLDN | 50uM |  |
| 17 | AEAB-polyLDN+1F | 50uM |  |
| 18 | AEAB-polyLDNF | 50uM |  |

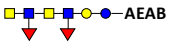
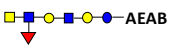
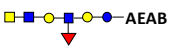
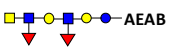
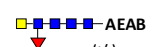
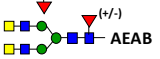
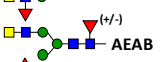
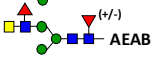
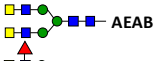
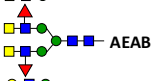
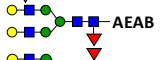
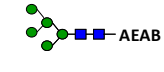
| Glycan ID # | Nickname | Conc. | Pictoral Structure |
|---|---|---|---|
| 19 | AEAB-polyLDNF | 100uM |  |
| 20 | AEAB-LNnT+GlcNAc | 100uM |  |
| 21 | AEAB-LNnT-LDN | 100uM |  |
| 22 | AEAB-LNnT-LDNF | 100uM |  |
| 23 | AEAB-Lac-LeX-LDN | 100uM |  |
| 24 | AEAB-Lac-LeX-LDNF | 100uM |  |
| 25 | AEAB-Chito-LDN | 100uM |  |
| 26 | AEAB-Chito-LDNF | 100uM |  |
| 27 | AEAB-BiALDN (+/-α6CF) | 50uM |  |
| 28 | AEAB-BiALDNF (+/-α6CF) | 100uM |  |
| 29 | AEAB-MonoALDN (+/α6 CF) | 50uM |  |
| 30 | AEAB-MonoALDNF (+/-α6 CF) | 100uM |  |
| 31 | AEAB-BiALDN 3 | 100uM |  |
| 32 | AEAB-BiALDNF | 100uM |  |
| 33 | NA2 (α3CF) | 50uM |  |
| 34 | NA2 (α6CF) | 50uM |  |
| 35 | *Man5* | 100uM |  |
| 36 | *PBS* | | |

**Figure 4.3 List of structures on the Defined Schistosome-type Array (DSA)**

Rat antisera was reactive to LDNF and, to a lesser extent, LDN variants on the DSA, with the highest IgG binder being a single unit of LDNF, and lower binding to repeating and biantennary versions of the antigen (Figure 4.4a). The IgM response was similar with increased LDN reactivity (Figure 4.4c). Interestingly, rat IgG bound highly to CX/CF (ID #10), and preferred the combination epitope to either core xylose (ID #9) or core α3 fucose (ID #8) alone. No IgM to this epitope was detectable.

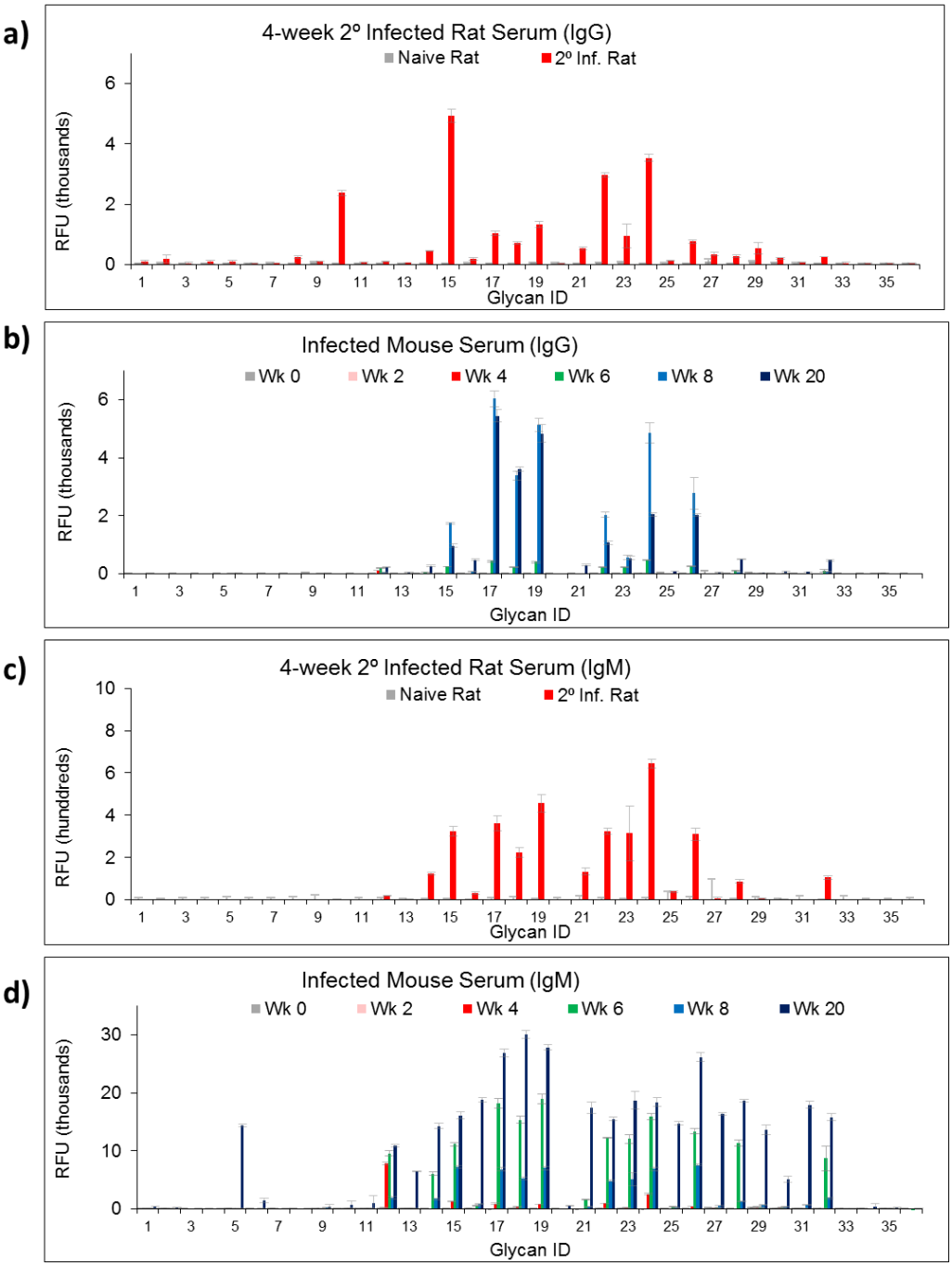



**Figure 4.4 Rats, but not mice, generate IgG to core xylose/core α3 fucose epitope.**

*S. mansoni* twice-infected rat serum, and mouse sera from various infection time points, were diluted 1:50 and screened for IgG (a-b) and IgM (c-d) binding to the DSA. Goat α-mouse IgG-Alexa 488, α-mouse IgM-Alexa 633, goat α-rat IgG-Alexa 546 and goat α-rat IgM-Alexa 488 were used for detection. Each bar represents an average of binding intensity (relative fluorescence units, RFU) to hexareplicate spots for each glycan ID number (listed on the x-axis and above

selected bars) +/- standard deviation. Glycan ID numbers (x-axis) correspond to those listed in Figure 4.3.

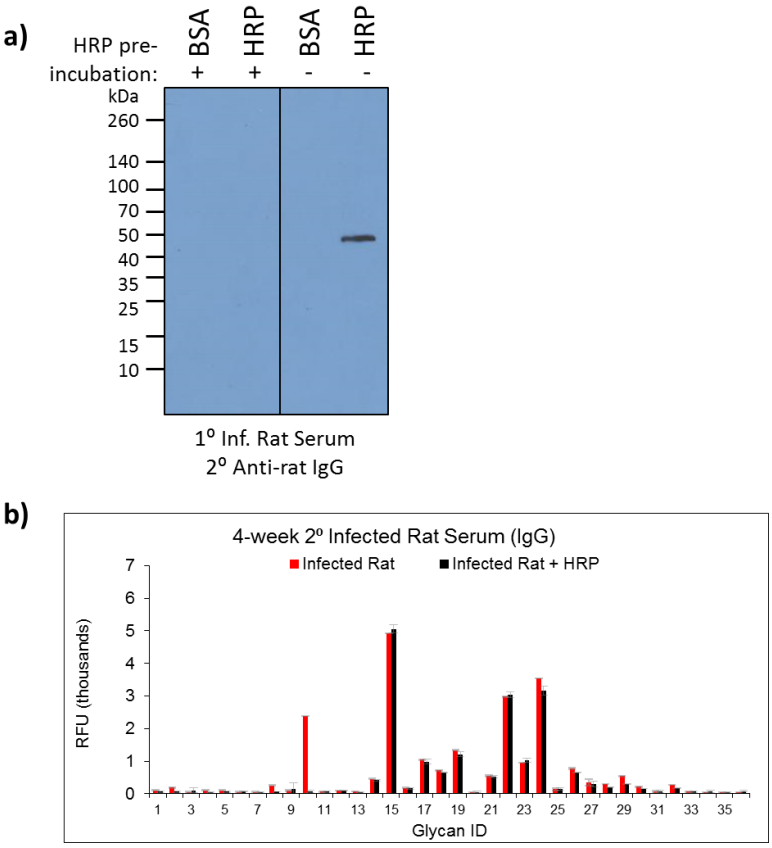
The mouse IgG response was low until 8 weeks, and at 8 and 20 weeks, extended chain variants of LDNF were the primary target on the DSA (ID #17, 19, 24) (Figure 4.4b). Low-level IgG responses to LDN and $Le^X$ were also observed (ID #16, 21). A similar broadening of the IgM response to DSA glycans occurred as was seen on the CFG array, where after an early response to $Le^X$ (ID #12), several LDN and LDNF variants, both biantennary and extended chains, were increasingly bound from weeks 6 through 20 (Figure 4.4d). (The large difference in magnitude of IgM bound to the DSA between rats and mice is likely not significant, since control studies show that the anti-mouse secondary reagent recognizes mouse IgM more strongly on arrays compared with anti-rat secondary antibody recognition of rat IgM. No response to CX/CF was seen in the mouse antisera from this infection, or during any other mouse infection previously conducted in our lab.

Taken together, the glycan microarray data show that antisera from rats which are actively rejecting worms targets a great breadth of glycan antigens, whereas the mouse anti-glycan response is more limited during acute infection and increases in breadth once the chronic immune response is established. Mouse sera also has increased focus on LDN and LDNF antigens and a notable lack of binding to the CX/CF, the latter of which is a major target of rat IgG.

### 4.3.3 Infected rat sera binds CX/CF on HRP

We wanted to know whether *S. mansoni*-infected rat serum was also reactive with the plant glycoprotein horseradish peroxidase (HRP), which possesses N-glycans that are about 80% core xylosylated and core α3-fucosylated, as the glycoprotein could be a helpful tool in better understanding the molecular nature and function of rat anti-CX/CF antibodies. Western blotting of HRP, and as a negative control, the mammalian protein BSA, with infected rat serum showed

that sera was indeed reactive with the plant glycoprotein, and pre-incubation of the serum with




HRP blocked binding (Figure 4.5a). To verify that this cross-reactivity was glycan specific, rat serum was pre-incubated with HRP before binding to the DSA, resulting in a total loss of binding to CX/CF (ID #10) while other anti-glycan reactivities in the rat serum remained intact (Figure 4.5b).

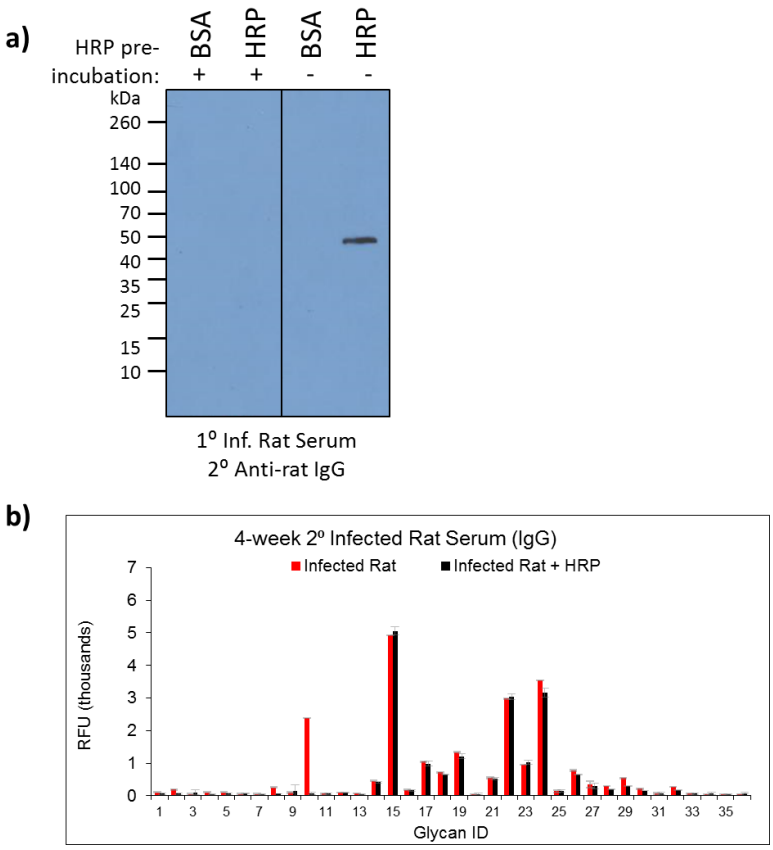



**Figure 4.5 Infected rat serum binds core xylose/core α3 fucose on HRP.**

Twice-infected rat serum, diluted to 1:1000, was used to stain Western blots of bovine serum albumin (BSA) and horseradish peroxidase (HRP), with or without pre-incubation of the serum with soluble HRP in the binding buffer (a). A similar experiment was performed by pre-incubating the rat serum with HRP before analyzing binding to the DSA (b), otherwise as described for Figure 4.4.

Knowing that the anti-CX/CF component of rat antiserum was cross-reactive with CX/CF on HRP, we made use of polyclonal anti-HRP rabbit serum (rabαHRP). RabαHRP is commercially available, and is predominantly specific for the CX/CF glycans of the glycoprotein, as previously demonstrated by cross-reactivity with other plant glycoproteins and ELISA inhibition studies with neo-glycoconjugates [181], [382]. We verified this by ELISA of rabαHRP binding to HRP-coated plates with or without periodate, which oxidizes vicinal diols of many carbohydrate epitopes (Figure 4.6a), and screening of rabαHRP on the DSA (Figure 4.6b).

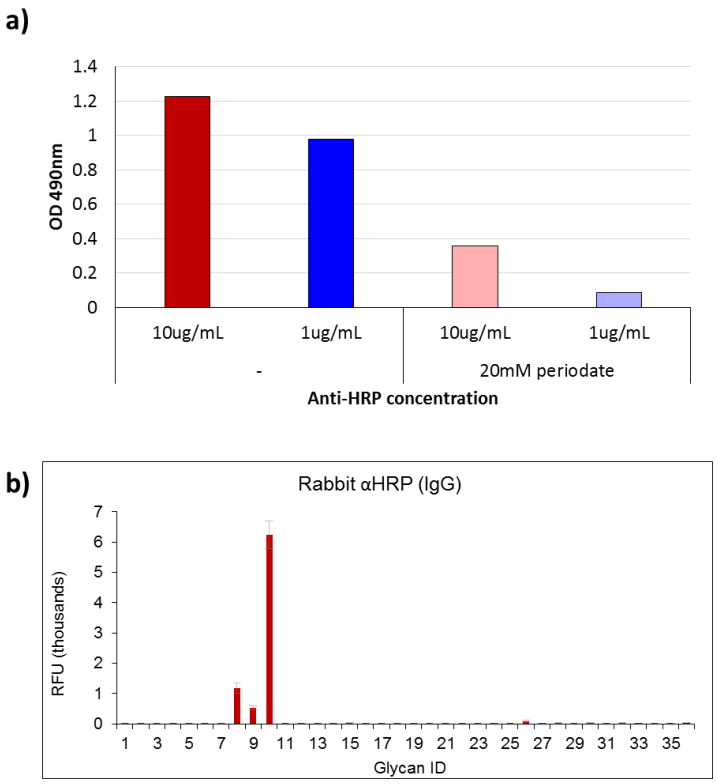



**Figure 4.6. Rabbit anti-HRP also binds CX/CF glycans on HRP.**

Polyclonal rabbit anti-HRP IgG, at high (red) and lower (blue) concentrations, was analyzed for binding to HRP-coated ELISA plates, treated with (light bars) or without (dark bars) periodate (a). RabαHRP was also screened on the DSA, with similar binding pattern to CX/CF epitopes (glycan ID#s 8-10) as rat antisera (b).

### 4.3.4 Rat antisera, mouse antisera and anti-HRP target several overlapping molecular species on Western blot

We then asked which molecular species on the intramammalian life stages of *S. mansoni* carry CX/CF epitopes, and whether these species were also differentially targeted by rats and mice. Western blotting of rabαHRP against lysates of cercariae (C), 3-day schistosomula (S), adult worms (A) and soluble egg antigen (E) demonstrated that the CX/CF epitope is found to varying extents on all intramammalian life stages (Figure 4.7a). SEA was the strongest and most broadly stained lysate, and high molecular weight species in both SEA and cercariae were heavily stained. Adult worm and 3-day schistosomula also have several molecular species which stain at moderate and low levels, respectively.

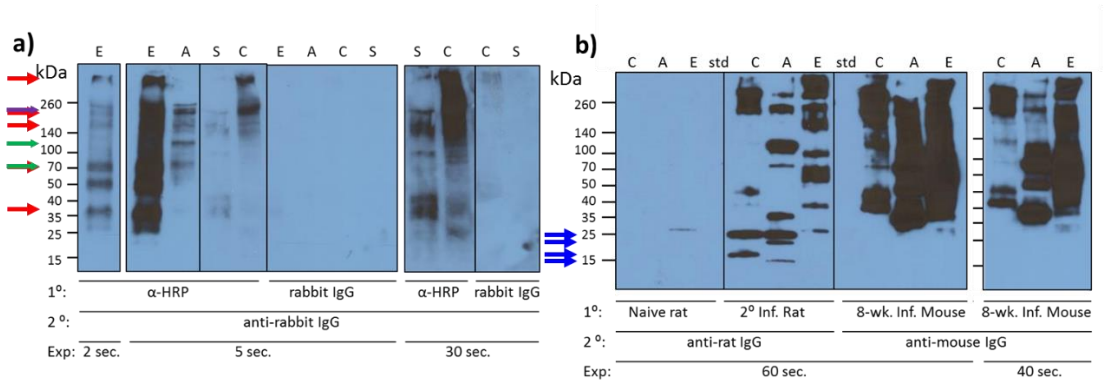



**Figure 4.7 Rat antisera, mouse antisera and anti-HRP target several overlapping molecular species in *S. mansoni* lysates.**

*S. mansoni* life stage lysates, normalized to total protein content, were run on SDS-PAGE and Western blots were stained with rabαHRP at 1ug/mL (a). SEA, E; adult worms, A; 3-day schistosomula, S; cercariae, C. Multiple exposures of the same blot are shown to visualize molecular species stained with differing strengths. Rabbit IgG was used a negative control. Infected rat serum (left) and mouse serum (right) were used to stain *S. mansoni* life stages, with naïve rat sera as a negative control (b). Sera were used at 1:1000. Red arrows, Possible common targets of rabαHRP and rat serum in SEA; Green arrows, Possible common targets of rabαHRP and rat serum in adult worm lysate; Purple arrow, Possible common targets of rabαHRP and rat serum in adult worm and cercarial lysates; Blue arrows; targets of rat serum not appearing in mouse serum blots.

*S. mansoni* lysates were also blotted with infected rat and mouse sera (Figure 4.7b). A notable difference between the rat and mouse sera was that rat sera bound as strongly to several molecular species on cercariae and adults as compared to SEA, whereas 8-week infected mouse sera targeted SEA substantially more than the earlier life stages. This could be due to rats being relatively intolerant to the infectious dose used, with little to no egg-laying taking place, whereas 8-week infected mice are exposed to heavy egg-laying. Comparison of the cercarial and adult staining patterns between rats and mice, which have a very similar banding pattern on glycoproteins 40kDa and larger, drew our attention to a few bands located at approximately 14kDa (adults), 15kDa (cercariae), 22kDa (adults) and 25 kDa (cercariae and adults), which are stained by rat sera and not mouse sera, even when the mouse sera blot is overexposed (Figure 4.7b)

Comparison of the anti-HRP blot with infected rat antisera blots pointed to some potentially common bands: Slightly below 260kDa in cercariae and adults; between 100-140 and just above 70 in adults; around 70, 50, between 35-40 and several species between 140-260, and a species much higher than 260 in SEA. This suggests that some major molecular species targeted by the rat immune response could be due to CX/CF binding, although whether these common bands are actually the same molecular species cannot be determined from this experiment. Most of these species in cercariae and adult worms appear to be targeted by mouse as well as rat, which led us to question whether this is due to the same anti-protein reactivities, the same anti-glycan reactivities, or different anti-glycan reactivities on the same proteins. Additionally, the small molecular weight species of cercariae and adult worms bound by rat and not mouse antisera, which did not react with rabαHRP but could possess other antigenic protein or glycan epitopes, were of particular interest to us.

### 4.3.5 HRP blocks reactivity of rat antisera with select parasite moieties

We wanted to know if any of the bands stained by both rat antisera and rabαHRP were generated by antibodies in rat serum binding CX/CF on parasite glycoproteins, so we set up blocking experiments where infected rat sera was pre-incubated with HRP and observed for loss of binding to parasite life stages. As a control, pre-incubation of rabαHRP with HRP abrogated binding to SEA (Figure 4.8a). Bands of parasite lysates that appear to be inhibited by HRP include those between 35-40kDa (cercariae), bands straddling 140 (SEA), bands above 260 and around 25-35 (adults) (Figure 4.8b-c). The inhibition studies therefore supported the idea that CX/CF could be responsible for a portion of the reactivity of infected rat sera with intramammalian schistosome life stages.

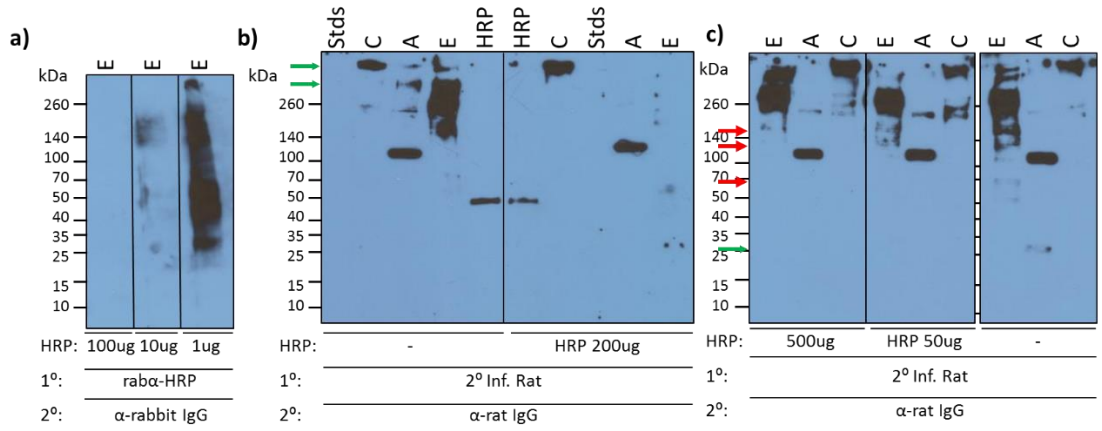



**Figure 4.8 HRP blocks reactivity of rat antisera with select parasite moieties.**

RabαHRP was pre-incubated with various concentrations of HRP and used to stain *S. mansoni* soluble egg extract (a). Rat serum was then likewise pre-incubated with the indicated concentrations of HRP before staining *S. mansoni* lysates (b) and (c). Soluble egg extract, E; Adult worm lysate, A; Cercarial lysate, C; Protein size standards (stds). Red arrows, Possible HRP-inhibited species targeted by rat serum in SEA; Green arrows, Possible HRP-inhibited species targeted by rat serum in adult worm lysate.

### 4.3.6 Rat and mouse IgG against larval and adult lysates is predominantly to periodate-sensitive epitopes

Finally, we asked whether most of the major reactivity of rat and mouse antisera, which was not blocked by HRP, is due to anti-glycan antibodies besides anti-HRP. We periodate- or mock-treated cercarial lysates and blotted with rat and mouse antisera. Periodate treatment left glycoproteins intact (Figure 4.9a) while destroying the glycan reactivity of AAL, a fucose-binding lectin and rabαHRP with cercarial lysate (Figure 4.9b). Most of the bands stained by both rat and mouse antisera, including the 15kDa and 25kDa bands bound by rat serum only, were lost after periodate treatment (Figure 4.9b).

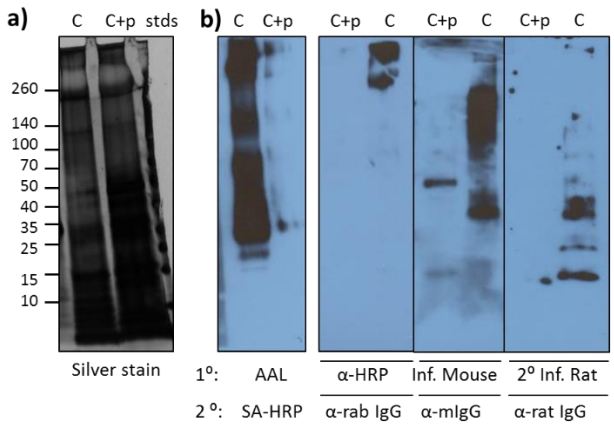



**Figure 4.9 Rat and mouse IgG is predominantly against periodate-sensitive epitopes of larval and adult lysates.**

Cercarial lysate was treated with (C+p) or without (C) periodate and then run on SDS-PAGE and silver stained to verify proteins were not degraded (a). Treated cercarial lysates were blotted with AAL and rabαHRP to verify loss of glycan reactivity (b – left), and infected rat and mouse sera (b- right).

Taken together, the Western blotting studies support the idea that CX/CF, a glycan epitope targeted by rats and not mice, accounts for some of the targeting of early schistosome life stages by the rat antibody response, and many of the other antibodies made against larvae and adult worms, including those made by rats and not mice, are likely anti-glycan antibodies.

## 4.4 Discussion

Schistosomiasis is a globally prevalent, debilitating disease that is poorly controlled by chemotherapy and for which no vaccine exists. Although some humans develop partial resistance and certain animal models, such as the Brown rat, are naturally protected, these examples of successful immunity have yet to translate into clear correlates of protection which could be exploited in vaccine development. The aim of this study was to compare a key aspect of the humoral immune response to *S. mansoni*, the anti-glycan response, of Brown rats with mice, a chronic infection model. We identified several differences in their anti-glycan responses, which could contribute to the difference in susceptibility between these two hosts.

The most interesting observation was that rats produce IgG to the xylose/core α3 fucose epitope on the N-glycan core, whereas sera pooled from infected mice showed no response to this epitope. Our studies suggest that, based on the specificity of the rat antisera, the *S. mansoni* core epitope is cross-reactive with CX/CF moieties on HRP glycoprotein. Therefore we used HRP, as well as a commercial polyclonal antibody to HRP, to examine the distribution of the epitope on *S. mansoni* life stages and identify glycoprotein candidates that are targeted by the rat antibodies. Western blots of rat serum and anti-HRP on parasite lysates had several shared bands, suggesting, although not proving, that rats may target some of the same glycoprotein species bound by anti-HRP, which should carry CX/CF.

Core xylose and core α3 fucose moieties are common N-glycan core modifications in plants that also occur in schistosomes [141]. Immunization of mammals with plant glycoproteins carrying the dual epitope tends to produce an antibody response which can be fractionated into those reactive with core xylose and those reactive core fucose, suggesting they form two distinct epitopes [382]. Additionally, the cross-reactivity of *C. elegans* with HRP was shown to be due to core α3 fucose modification [177]. We observed that polyclonal rat anti-sera was reactive with glycan ID #10, containing both modifications, and possessed little to no cross-reactivity with the two glycans containing core fucose or core xylose alone (Figure 4.4; ID #8-9), suggesting that the preferred epitope to which rats respond is the dual modified core. However, MALDI-TOF-MS data suggested that *S. japonicum* contained CX and core α3- and α6- fucose on the same glycans *while S. mansoni* egg N-glycans contained either CX or core α3-fucose but not both [141]. Perhaps *S. mansoni* does contain small but highly antigenic amounts of the dual epitope, or, perhaps there is a dual epitope contained in the earlier life stages which incites such a response in rats.

Why rats target CX and core CF in response to *S. mansoni* infection while mice do not is another interesting question. This could related to differences in the character of immunity generated by the two different hosts to the parasite, genetic differences or tolerization of the

mouse to certain epitopes. In support of the latter hypothesis, a few investigators have previously noted that immunization of certain strains of mice with HRP fails to produce glycan-specific antibodies to the core epitopes, while immunization of rats and rabbits with HRP does induce a glycan-specific response [324], [325], [327]. Faveeuw *et al.* observed that *S. mansoni*-infected C57BL/6 mice had weak but detectable IgG1 responses to plant glycoproteins carrying core xylose and core α3 fucose, and that this strain also was able to produce glycan-specific antibodies in response to HRP immunization, whereas BALB/c mice were not [181], [327]. Given that Swiss-Webster mice are outbred, it seems unlikely that genetic differences would be the reason for their lack of response in our study, but the mechanism remains to be elucidated.

The identities of the CX/CF glycoprotein species are of great interest to us, given our observations that rats target this glycan as well as recent publications showing that rhesus monkeys and, to a lesser extent, humans, also generate IgG to CX/CF during *S. mansoni* infection [175]. Antibodies to this glycan could be involved in protective mechanisms against the parasite, such as complement-mediated lysis of schistosomula or antibody-dependent cellular cytotoxicity, both of which have been demonstrated using rat serum or rat monoclonal antibodies *in vitro* and appear to be important *in vivo* [85]–[87], [127], [383]. In agreement with our data, expression of this epitope has previously been shown on intramammalian stages of *S. mansoni* and *S. japonicum*, as well as on the parasite surface, which makes it a potentially attractive vaccine candidate [181].

Core xylose and core α3 fucose are known plant allergens, but the clinical significance of IgE to these glycans in patients is debated [179], [384]. Additionally, schistosomiasis-infected individuals have a down-regulated allergic response [reviewed in 48]; thus, presented in certain contexts or in some (ex. schistosomiasis endemic) individuals, the epitope may not elicit an allergic response and should not be automatically discounted as a possible vaccine antigen. Indeed, it is possible that the protein carrier could affect the type of immunity generated, be a part of the epitope or even block the epitope, since CX and CF are contained in the reducing end of

the glycan, proximal to the protein [384]. Other innate immune cues provided by the parasite could also modulate the character of immunity generated to this epitope.

Although the Western blot studies with HRP blocking do not definitively identify molecular species carrying this epitope, they suggest that some of the rat antisera targets are CX/CF. We plan to follow up this observation by immunoprecipitating the CX/CF-containing glycoproteins from larval and adult worm lysates, which can be used for Western blots that will verify if serum targeting of these species is seen and glycoproteomic studies to identify the bands. This information, coupled with several novel approaches that have recently pointed to several new vaccine candidates based on surface accessibility and stage-specific local immune response, could form a basis for selection of glycoprotein vaccine candidates. Thus, discovery of the proteins carrying this epitope in parasites would not only be helpful in understanding the immune response to it, but might additionally offer peptide epitopes to be incorporated into a vaccine eliciting robust, multi-pronged immunity against this complex parasite.

A few small glycoproteins were stained by rat and not mouse serum in cercarial and adult worm lysates. Interestingly, these size of two of these bands correspond closely to bands stained by rat scFvs which have been tentatively identified by our collaborator as Ly6 (~13kDa) and Sm29 (~35kDa) [39 and C. B. Shoemaker, personal communication]. Sm29 is a promising vaccine candidate currently in pre-clinical testing [283], [284], which has probable glycosylation sites [282], [385]. Ly6 is part of a family of *S. mansoni* homologs of the human complement inhibitory factor CD59, the *S. japonicum* ortholog of which was also recently implicated as a vaccine candidate in immunomic studies [294], [386]. Given that reactivity to both of these species in rat serum was abolished by periodate treatment in our preliminary studies, characterization of the native glycosylation of these two species could be germane to their development as vaccine candidates. However, periodate oxidation does not destroy all glycan epitopes, and can also alter peptide epitopes, so other methods must be used to clarify these findings.

Another interesting observation was the great breadth of the anti-glycan response observed in rats, including $Le^X$-containing glycans among the highest binders on the CFG, and many poly-LN containing structures that were bound at a lower level. Another group recently published a report of the glycan binding of low- to medium-dose infected rat antibody-secreting cell probes on the CFG Array, and also identified $Le^X$ and Poly-LNs as two of the major motifs recognized, along with β3-GlcNAc terminating structures [246]. Mice also focused on $Le^X$ glycans early in infection, but it appears that they do not develop a breadth comparable to rats until after egg-laying has commenced. It is difficult to interpret these observations based solely on the data presented here. $Le^X$, LN and polymeric variants thereof are not uncommonly found in the mammalian glycome [reviewed in 15]. However, this does not rule out the possibility that they could be effective targets of anti-parasite immunity and/or presented on the parasite in a fashion that is unique from the mammalian antigens. One hypothesis is that having anti-glycan antibodies of many specificities, or many anti-glycan antibodies that are of relatively low affinity/specificity could be advantageous for binding common determinants that are densely distributed on the parasite. Why twice-infected rats produce a broader response than mice do, at least during a primary infection, is unclear, but could be related to differences in amount or duration of exposure to egg antigens, or differences in tolerization to antigenic determinants similar to self-antigens.

Another hypothesis is, given that many of the glycan antigens targeted by rats and mice are the same ($Le^X$, LDN, LDNF), perhaps the mouse possesses some inhibitory factor, such as blocking antibodies, which are not present in rats. Blocking antibodies have been observed in rats, mice and hypothesized to exist in endemic humans [115], [241], [387]–[389]. Other immune factors such as IL-10 have also been observed to block the development of resistance to re-infection in mice [390].

Twice-infected rats at the high doses used in this study should have had minimal, if any, exposure to *S. mansoni* egg antigens, but have had been boosted against larval and adult antigens,

and actively reject the second infection. The mouse serum used in this study, on the other hand, is from primary infection. Since mice, like humans, can be cured and re-infected while acquiring only partial resistance upon re-infection, it would be more informative to compare a secondary mouse infection, where, as in rat infection, exposure to egg antigens has been limited, with the response of twice-infected rats. These studies are planned for the future.

A shortcoming of this study is the use of pooled serum for array studies. It is unclear, for example, whether all infected rats target CX/CF or just one targets the epitope at a very high level. In fact, significant variation in individual targeting of this epitope has been seen in our previously published [175] and unpublished results (Figure 5.6 and Figure 6.3) in rhesus monkeys and in humans, respectively. However, glycan arrays in this context are primarily employed as a tool for generating hypotheses which can be followed up on using individual sera and other techniques which will elucidate the functional significance of the antibodies we observed. To assess the prospect of CX/CF as a protective epitope, we will examine whether antibodies to this epitope can mediate schistosomula killing through immune mechanisms *in vitro* and/or can transfer protection to challenge infection, and whether blocking of such antibodies in rat infection antiserum abolishes the *in vitro* killing ability of the antiserum. We are currently developing the techniques to perform these experiments and ask similar questions about other anti-glycan antibodies.

In conclusion, we identified several differences, notably the targeting of CX/CF in the anti-glycan responses of rats and mice, which could contribute to the difference in susceptibility between these two hosts. Future studies should elucidate the functional significance of these anti-glycan antibodies and assess their contribution to disease resistance in rats.

# Chapter 5. Anti-glycan antibodies in a population of occupationally *S. mansoni*-exposed resistant and susceptible humans

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## 5.1 Introduction

Schistosomiasis is a parasitic infection caused by helminths (worms) of the *Schistosoma sp*. In spite of decades of research on the immunology of schistosomiasis and protective mechanisms in both humans and animal models, clear correlates of protection to this disease have yet to emerge. Humans are unable to clear the infection without assistance from chemotherapy, but some groups of people in endemic areas are thought to exhibit partial resistance to infection.

One such group consists of putatively "endemic normal" (EN) individuals in Brazil. These individuals are egg-negative despite frequent exposure to contaminated water [97]. Compared to age- and exposure-matched individuals, these people exhibit higher IL-5, IL-13 and IFN-g secretion from mononuclear cells in response to egg antigens, similar levels of IL-10 and lower levels of IgE and IgG4 to worm and egg antigens [391]. Resistance in this group therefore seems to be associated with a mix of Th1 and Th2 responses to parasite antigens. Human resistance to schistosomes has also been studied as a function of age and exposure history, where numerous lines of evidence suggest that as exposures continue over time, endemic individuals acquire partial resistance [392] and that such resistance is immunologically mediated [393]–[395]. Intensity of infection and prevalence peak during adolescence and then decrease to lower levels by adulthood [23], [96], [106], [396]–[399]. Multiple studies have shown that eosinophilia,

antibodies to adult worm antigen (especially IgE), and PBMC proliferation and/or Th2 cytokine secretion are associated with resistance to re-infection and that these parameters increase with age [116], [395], [400]–[402]. The studies discussed above, however, suffer from some shortcomings. The EN studies are not prospective, although they are matched for age and water exposure, so it is unclear whether the phenotype is a cause or result of lower level infections. In the age-related resistance studies, it is hard to dissociate immune correlates from other age-dependent effects.

A more optimal setting in which to probe for age-independent correlates of resistance is a long-term study in which participants are of similar age, have similar levels of water exposure, and frequent re-infection occurs. This has been done through a collaboration between the Centers for Disease Control and the Kenya Medical Research Institute, which followed occupationally-exposed car washers in Kisumu, Kenya over periods of 1-10 years. The men were treated with praziquantel and after each cure, the time to re-infection was measured. When water exposure and HIV status were controlled for, the participants could be stratified into: 1) Resistant individuals, who acquired few or no re-infections and had the longest average time to re-infection; 2) Susceptible individuals, who had the greatest number of re-infections and consistently had a short time to re-infection; and 3) Changing individuals, those who had a moderate number of re-infections but whose average time to re-infection increased as the study progressed (Figure 5.1) [98]. Investigations into the immune responses of these groups subsequently demonstrated higher anti-SWAP IgE levels over time in group C, positive correlation of circulating CD23+ (low-affinity IgE receptor) B cells with resistance, positive correlation of eosinophilia with resistance, and associations of polymorphisms in the IL-4, IL-13 and IFN-γ genes with resistance [99], [118], [131], [403]. In this population it therefore appears that partial immunity to re-infection does develop in some individuals, independent of age-related factors, and several aspects of the Th2-type response are correlated with resistance.

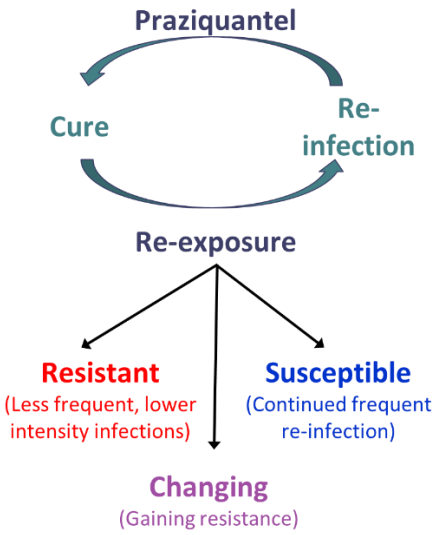



**Figure 5.1 Re-infection cure cycles induce partial resistance in some humans**

Anti-glycan antibodies (αGAbs) are capable of killing schistosomula *in vitro*, and have been correlated with or shown to be directly responsible for protection in different animal models of helminth infections [86], [148], [151], [175], [252], [257], [259], [260], [404], [405]. However, their role in human schistosomiasis is still unclear. Several investigators have examined the association of αGAbs of varying specificities and isotypes with age-related resistance or resistance to re-infection, with contrasting results [104], [148], [163], [244], [245], [256], [335], [387]. For example, one group demonstrated that IgG1 to certain glycan epitopes (LDN-DF and F-LDN) declined with age and exposure history, while IgM to other glycan epitopes (LDN-DF and LDNF) increased (a review of pertinent glycan structures and diagrams can be found in Section 1.3.1) [163], [335]. Another study showed that IgE to worm glycolipids was negatively associated with re-infection intensity [256]. These studies have made it clear that the relationship of αGAbs with resistance to schistosomiasis is complex.

Our group has developed multiple glycan microarrays which allow for sensitive and simultaneous analysis of antibodies to many different glycan antigens, including several of the glycans which are found to be targeted in numerous schistosome-infected hosts. We therefore

wanted to combine this powerful technology with the unique cohort of resistant, susceptible and changing individuals identified in the KEMRI/CDC studies to see if any meaningful patterns of anti-glycan reactivity emerged.

## 5.2 Materials and Methods

### 5.2.1 Study population and plasma samples.

Human plasmas were obtained from occupationally exposed adult men employed as car washers in Kisumu, Kenya, between 1995 and 2009. Upon enrollment, stool exams were performed to diagnose schistosomiasis (almost exclusively *S. mansoni*) and praziquantel was administered once or twice until stools were egg negative one month later. Repeat stool exams were the performed every two months, and praziquantel was administered when infections were found. Full description of the study and follow up methods has been described previously [98]. Classification as resistant, susceptible or changing was made on the basis of number re-infections per follow up time, the time to re-infection over the course of the study, and normalized to number of cars washed. Samples had been previously thawed and re-frozen over the course of many years and equal sample integrity could not be guaranteed. The first 14 samples (4 resistant, 10 susceptible) were baseline sera which had been stored at the University of Georgia for the last several years. The remaining samples, taken over a range of time periods from 24 individuals (7 resistant, 10 susceptible, 7 changing) had been stored frozen at KEMRI. Glycan arrays used for the second set of 24 individuals were carried, dessicated, at ambient temperature, by air from the USA to Kenya and back. Previous tests had demonstrated that storage at ambient temperature for this amount of time resulted in an identical binding pattern with a slight (10%) reduction in signal.

### 5.2.2 Consortium for Functional Glycomics Array (CFG).

Pooled susceptible and resistant sera were assayed and analyzed on the CFG (version 5.1) as previously described [247] for “Alternate protocol 1: The glycan epitopes for mouse

monoclonal antibodies are detected using fluorescent anti-mouse IgG," with the following modifications: Particular matter was removed before use by spinning on a table-top microcentrifuge before assay on arrays. Sera were diluted to 1:250 and detected with goat anti-human IgM-Alexa 488 and anti-human IgG-Alexa 633 at 5ug/mL each. After each incubation, 6 dip washes in each buffer, TSM wash buffer and TSM, were performed.

5.2.3 Defined Schistosomal-type Array (DSA) and Natural Egg Array.

Pooled susceptible and resistant sera were assayed and analyzed on the DSA as previously described (section 3.2.2.10) for mouse and rat sera, except they were diluted to 1:100 and detected with goat anti-human IgM-Alexa 488 and anti-human IgG-Alexa 633 at 2.5ug/mL each. The list of structures is found below (Figure 4.3). Sera were assayed on the egg array using the same protocol as for the DSA when IgG and IgM were detected. A similar protocol was used to detect IgG1, IgG2 and IgM in individual human sera except the secondary incubation was with mouse anti-human IgG1 or mouse anti-human IgG2 at 5ug/mL, and the tertiary incubation was with goat anti-mouse IgG-Alexa 633 or goat anti-human IgM-Alexa 488 at 5ug/mL. Pictoral representations of the structures included on the DSA are found in Figure 4.3 (note that the order of structures and number of structures included varies from Figure 4.3 and varies between Figure 5.4 and Figure 5.6, so structure nicknames have been included on the x-axis for clarity).

5.2.4 SEA ELISA with/without periodate treatment.

Soluble egg antigen was prepared as described in sections 3.2.2.2 and 3.2.2.7. SEA was diluted to 10ug/mL in 0.05M carbonate/bicarbonate buffer pH 9.6 and 50µl were coated in each well of a clear, flat-bottom, non-tissue culture treated 96-well plate overnight at $4^0$C. Excess coating solution was dumped and the plate was blotted and washed three times with PBS-t (1X PBS with 0.05% tween-20) using a squirt bottle. To each well, 300µl of blocker (PBS-0.3% tween) was added and incubated covered in parafilm, standing, at room temperature for 1 hour. All remaining incubations were 1 hour at room temperature on a slowly shaking orbital shaker,

and all washes were with PBS-t. The blocker was discarded and plates were washed three times. The serum was diluted to 1:500 in PBS-0.3% tween and was added to each well, each sample in quadruplicate. After incubation the serum was discarded and the plate was washed five times. The secondary antibody, goat anti-human IgG linked to HRP, was diluted 1:1000 in PBS-0.3% tween and 50µl were added to each well. After incubation the unbound antibody was discarded and the plate was washed three times. The plate was then covered while O-phenylenediamine dihydrochloride was dissolved to 0.5mg/mL in stable peroxide buffer. When the substrate was fully dissolved, 50µl were added to each well. Plates were incubated standing in the dark for 20 minutes. The reaction was quenched with 25µl per well of 3N sulfuric acid. Bubbles were popped and the absorbance of the plate was read at 490nm in a Victor plate reader. Half of the quadruplicate wells were periodate-treated after coating and half were mock-treated. In-plate periodate treatment as performed as described on page 121.

## 5.3 Results

### 5.3.1 Reactivity of resistant and susceptible plasmas to egg glycans

We first obtained plasma samples from resistant (R, n=4) and susceptible (S, n=10) individuals. We asked whether the two groups significantly differed in the total proportion of anti-glycan reactivity in their sera. Multi-well plates were coated with soluble egg antigen (SEA) and half of the wells were periodate-treated, which destroys most glycan epitopes, and the plasmas were used for ELISA with IgG detection. All the plasmas displayed some loss of binding to periodate-treated SEA compared with mock-treated, but there was a wide degree of variation in the periodate-sensitive proportion of IgG among samples in both groups (Figure 5.2).

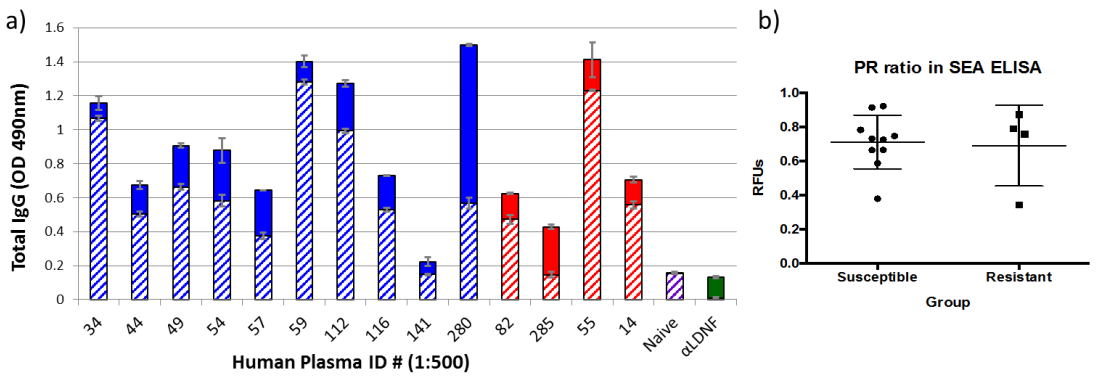



**Figure 5.2 Resistant and susceptible humans display a wide range of reactivities to periodate-sensitive glycans of SEA**

Plasma IgG was screened for binding to mock- or periodate-treated SEA-coated ELISA plates (a). αLDNF monoclonal antibody was used as control for the effect of periodate treatment. Blue, susceptible; Red, resistant; Purple, naïve; Solid bars, periodate-sensitive; Hashed bars, periodate-resistant. The ratios of reactivity remaining after periodate treatment to total reactivity to mock-treated SEA (PR ratios) are plotted in (b).

### 5.3.2 Pooled resistant and susceptible plasma on the Consortium for Functional Glycomics (CFG) array.

To begin exploring the anti-glycan reactivity of the plasmas, we screened a pool of the R samples and a pool of the S samples, as well as a naïve human plasma, on the CFG array, a collection of over 600 synthetic mammalian-type glycans (Figure 5.3, IgG is shown). Normal human plasma primarily bound to blood group antigens including several type 1 and type 2 A (Glycan ID #s 381, 373, 451, 523, 82) and B antigens (#450, 374), $Le^a$ (#365, 533) and Forssman antigen (#415). The top IgG binders in the pooled susceptible group were several LDN variants (#563, 528, 98, 99, 512) and LDNF variants (#97, 503). Both the resistant and susceptible plasma pools displayed more prominent targeting of chitin oligosaccharides (#189-191, 310,311), and higher binding to a β3-linked glucuronic acid (#307) than the naïve plasma. The specificities bound by IgM were similar with LDN and LDNF among the highest binders, although the susceptible pool had higher IgM reactivity than the resistant pool.

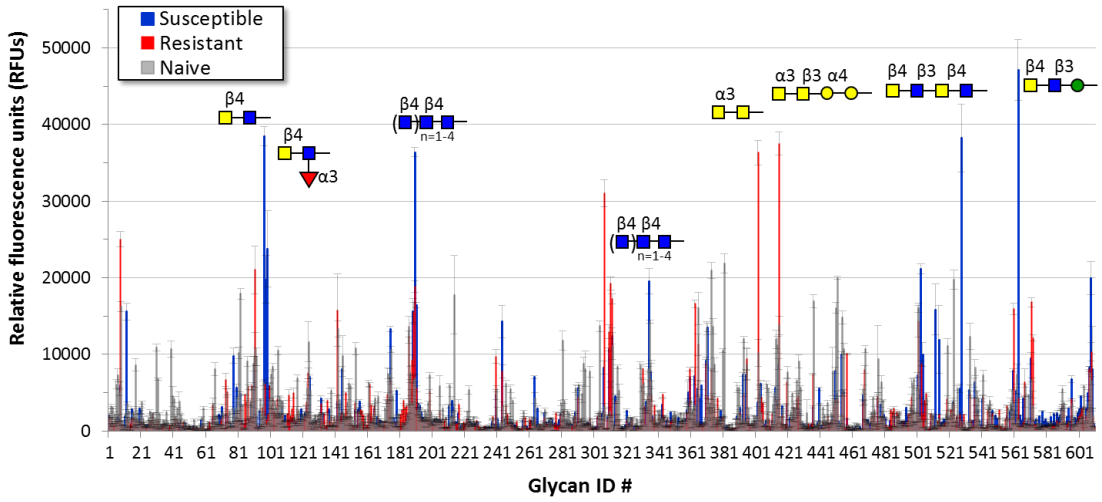



**Figure 5.3 Pooled resistant and susceptible plasma on the Consortium for Functional Glycomics (CFG) array**

5.3.3 Pooled resistant and susceptible plasma on schistosome glycan arrays.

Given the prominent anti-glycan response observed in both schistosome-infected rodent models, we wanted to compare their antibody responses using glycan arrays with schistosome-type glycans, the defined schistosome-type glycan array (DSA), and the natural egg glycan array (egg array). The DSA is a collection of structurally defined, semi-synthetic variants of LDN, LDNF, $Le^X$, core xylosylated/core α3-fucosylated N-glycans (CX/CF) and control glycans, which has been validated using lectins, monoclonal antibodies and infection antisera [174], [175]. The egg array is a shotgun array of 2-dimensionally separated N-glycans prepared from *S. mansoni* eggs, the immunodominant glycan of which has been identified at F-LDNF (Rivera-Marrero *et al.*, manuscript in preparation) (major component of fraction #13).

Pooled susceptible plasma displayed robust IgG and IgM targeting of most LDN and LDNF-containing glycans on the DSA as well as core α3 fucose and core xylose/core α3 fucose (Figure 5.4a). Resistant plasma showed a similar pattern except titers of both IgG and IgM were about 4-10-fold lower than in the susceptible plasma. (Note that the order of structures and number of structures included varies from Figure 4.3 and varies between Figure 5.4 and Figure

5.6, so structure nicknames have been included on the x-axis for clarity. Pictoral representations of the structures included on the DSA are found in Figure 4.3 and Figure 3.6).

On the egg array, both samples showed a pattern of binding extremely similar to a mouse monoclonal antibody against F-LDNF, with fraction #13 as the top binder (Figure 5.4b). However, the susceptible plasma again showed a much greater amount of IgG reactivity, and also had a greater breadth of reactivity to other fractions (which are also bound by α-F-LDNF, but not as highly relative to fraction #13), than the resistant plasma, which only showed substantial reactivity with fraction #13.

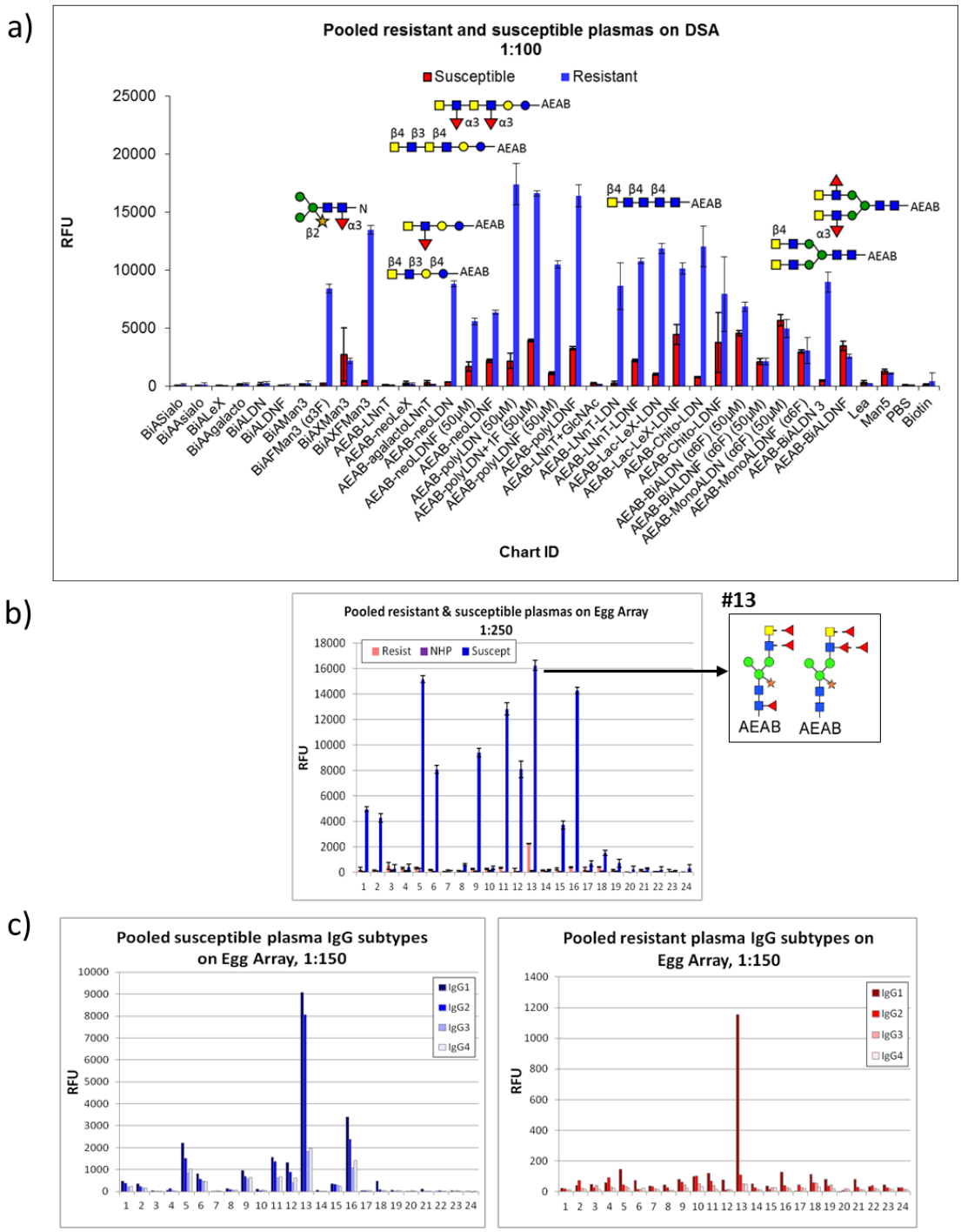



**Figure 5.4 Pooled resistant and susceptible plasmas target LDNF, LDN and CX/CF on the DSA, and F-LDN(F) on the Egg Array**

Pooled resistant (red), susceptible (blue) and uninfected (purple) human plasmas were screened for IgG binding to the Defined Schistosomal-type Array (DSA) (a), and shotgun Egg N-glycan Array (b). Each bar represents an average of binding intensity (relative fluorescence units, RFU) to hexareplicate spots for each glycan ID number (listed on the X-axis and above selected bars)

+/- standard deviation. Glycan ID numbers (x-axis) correspond to 2-dimensionally purified HPLC fractions as described in (Rivera-Marrero *et al.*, manuscript in preparation). Composites of the predicted structure for the major component of fraction #13 based on MALDI-TOF, are shown. The structure is predicted to contain F-LDN based on reactivity with a recently characterized monoclonal antibody (Nyame *et al.*, manuscript in preparation). IgG subtypes were then detected within the R and S plasma pools (c).

Given the correlations made in some human studies of IgE/Ig4 ratio with resistance, we wanted to investigate the anti-glycan sub-isotype composition of the resistant and susceptible plasma pools (Figure 5.4c). When we detected the bound plasma with secondary antibodies to each IgG subtype, we found that the susceptible plasma contained nearly equal amounts of IgG1 and IgG2, and 2-4-fold lower amounts of IgG3 and IgG4. The pooled resistant plasma, by contrast, was almost exclusively IgG1, with about 10-fold lessIgG2 and more than 10-fold less IgG3 and IgG4. The relative RFUs do not represent a quantitative comparison among the different subtypes since the secondary reagents are different, but the difference in ratios of the subtypes within each plasma sample was apparent.

### 5.3.4 Isotype composition of individual resistant and susceptible plasmas on the egg array.

To determine if there was a correlation between the ratio of isotypes to egg glycans and disease resistance, we ran each individual resistant and susceptible plasma on the egg array and detected IgG1, IgG2 and IgM. We found that there was quite a variety of isotype ratios within both the R and S groups (Figure 5.5a). Some individuals had very high levels of IgG2 and IgM in addition to IgG1 (individual S280), while others had barely detectable amounts of IgG2 and IgM (R055, S044). We explored whether differences between the R and S groups were significantly associated with differences in IgG1, IgG2, IgM or ratios of these to fraction 13, but the variability among the samples was too high and the sample number too low to determine if any significant differences existed (Figure 5.5c). We additionally explored whether the isotype levels were correlated with data such as the number of infections per month of average time to next infection

over the whole study period, and found that using this small number of individuals (n=14), there were weakly positive correlations between the number of infections per month and IgG2, and average time to next infection and IgG1/IgG2 to fraction #13 (Figure 5.5b). (Note that the frequency of re-infection statistics were not normalized to number of cars washed for this analysis.)

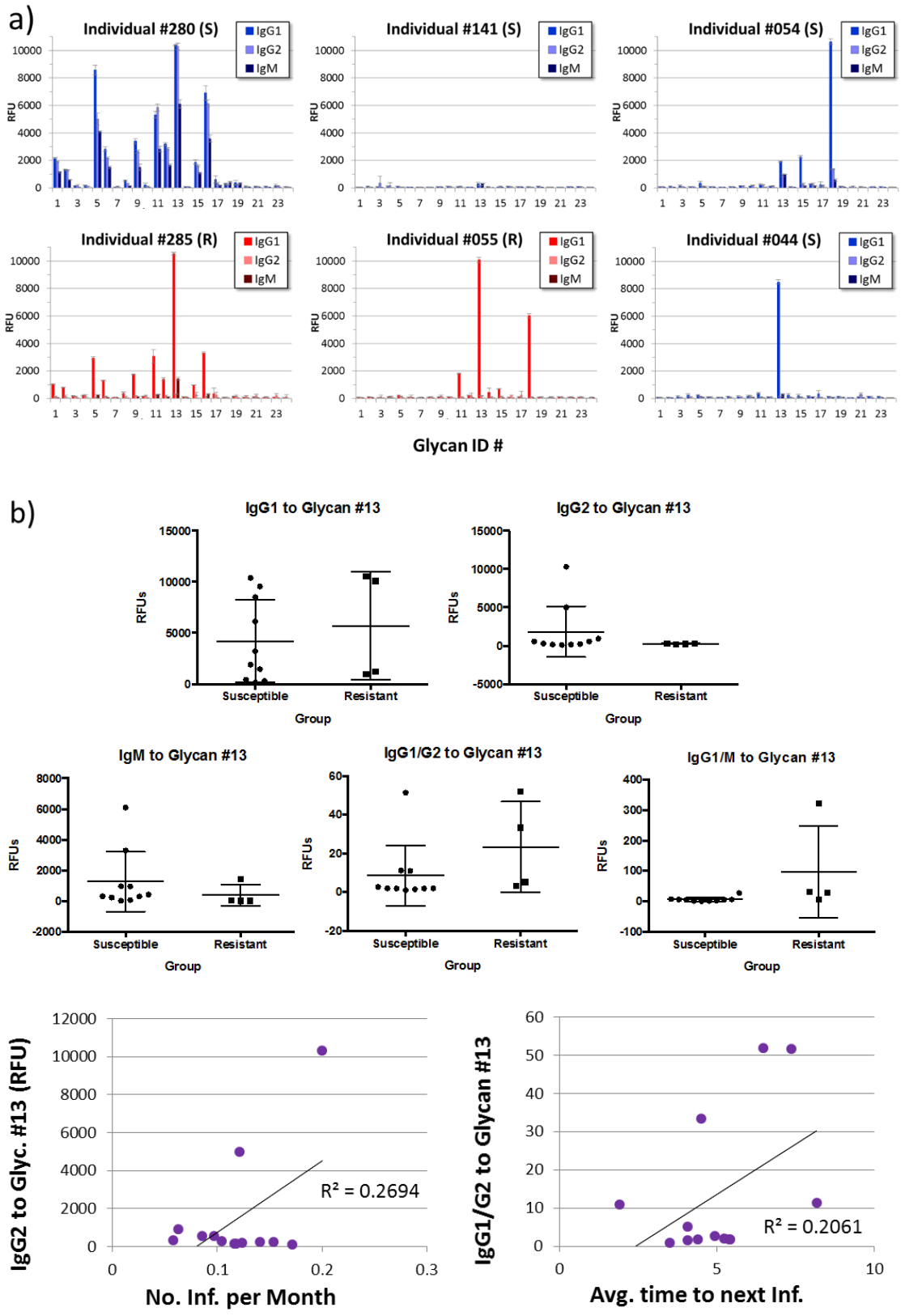



**Figure 5.5 IgG1, IgG2, IgM compositions of individual R and S plasmas on the Egg Array.**

Individual resistant (red), susceptible (blue) and uninfected (purple) human plasmas were screened for IgG1, IgG2 and IgM binding to the shotgun Egg N-glycan Array, and 6 representative individuals are shown (a). Each bar represents an average of binding intensity (relative fluorescence units, RFU) to hexareplicate spots for each glycan ID number (listed on the X-axis and above selected bars) +/- standard deviation. Comparisons of the level of various isotypes or ratios of isotypes to fraction #13 between the R and S groups were made in (b), and levels of the isotypes were also plotted against the number of infections per month and average time to next infection throughout the study period (not normalized to number of cars washed).

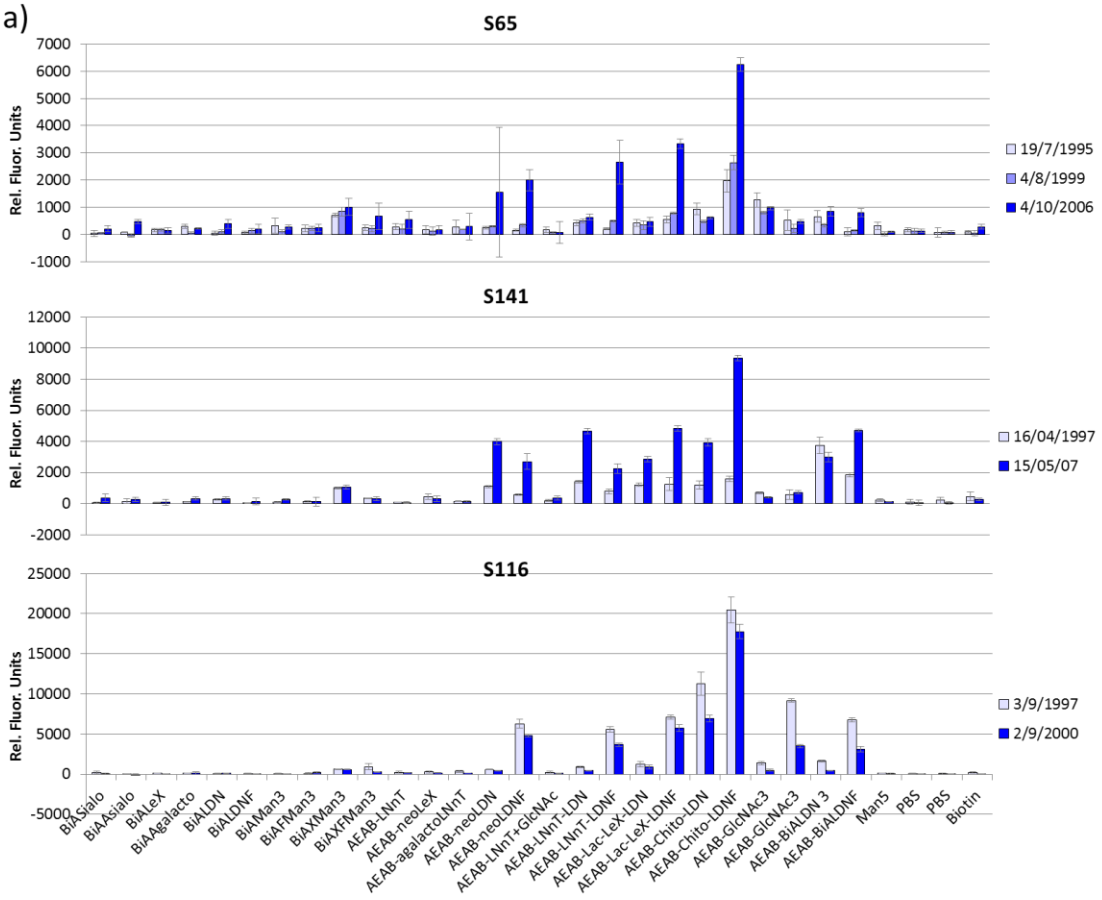
We also examined the IgG3 and IgG4 compositions of the individual plasmas on the DSA (data not shown). Most of the data was of poor quality and only one individual showed a high response in both IgG3 and IgG4 (S280). In this individual, interestingly, IgG4 bound to core xylose/core α3 fucose epitopes as well as LDN and LDNF, while IgG3 bound LDN and LDNF glycans with no detectable reactivity to core xylose/core α3 fucose.

### 5.3.5 Patterns of susceptible, resistant and changing individuals on the DSA over time.

Because the plasma samples showed interesting differences in anti-glycan reactivity but the initial sample size was not large enough to see statistically significant differences, we obtained approximately 130 plasmas taken from 24 individuals (7 resistant, 10 susceptible, 7 changing) at various time points during the course of the study (we were not able to obtain full records for the study participants, thus it is now known whether they were actively infected at the time the plasma samples were taken.) We screened the samples on the DSA for IgG and IgM, and asked whether the anti-glycan reactivity in the various groups remained stable or changed over time in any common pattern. Due to experimental miscommunications, the detection protocol was completed for only 54 plasma samples. Of these, only 40 resulted in interpretable data due to high levels of patchy background on the arrays. This could be a result of the age and/or storage history of the samples, contamination, and may have been worsened by transport of the slides at room temperature before scanning.

A few of the S, R, and C individual staining patterns on the DSA are shown in Figure 5.6 (a, S, IgG; b, R, IgG; c, C, IgG; d, C, IgM). We used this data to plot reactivities to several individual glycans over time in the three groups (Figure 5.7). Most of the S plasmas remained stable or increased in anti-glycan titer, whereas most R and C plasmas remained stable or decreased in anti-glycan titer over time. We analyzed the data using two-way repeated measures ANOVA for all R, S and C sera that had interpretable results for at least two time points, or the first and last time points, for a few of the glycan structures: Chito-LDNF (IgM), neoLDNF (IgG) and biantennary LDNF (IgG). No significant differences were seen between immune status groups or due to the interaction between immune status and time. The most significant source of variation was within subjects ($p < 0.05$), and time was a somewhat significant factor ($0.1 > p > 0.05$), indicating that there is a large degree of variation among individuals, and that individuals' antibody responses may be trending over time, but not necessarily based on their R, S and C classification.

a)


S65
Rel. Fluor. Units
7000
6000
5000
4000
3000
2000
1000
0
-1000
19/7/1995
4/8/1999
4/10/2006
S141
Rel. Fluor. Units
12000
10000
8000
6000
4000
2000
0
-2000
16/04/1997
15/05/07
S116
Rel. Fluor. Units
25000
20000
15000
10000
5000
0
-5000
3/9/1997
2/9/2000
BiASialo
BiAASialo
BiALeX
BiAAgalacto
BiALDN
BiALDNF
BiAMan3
BiAFMan3
BiAXMan3
BiAXFMan3
AEAB-LNnT
AEAB-neoLeX
AEAB-agalactoLNnT
AEAB-neoLDN
AEAB-neoLDNF
AEAB-LNnT+GlcNAc
AEAB-LNnT-LDN
AEAB-LNnT-LDNF
AEAB-Lac-LeX-LDN
AEAB-Lac-LeX-LDNF
AEAB-Chito-LDN
AEAB-Chito-LDNF
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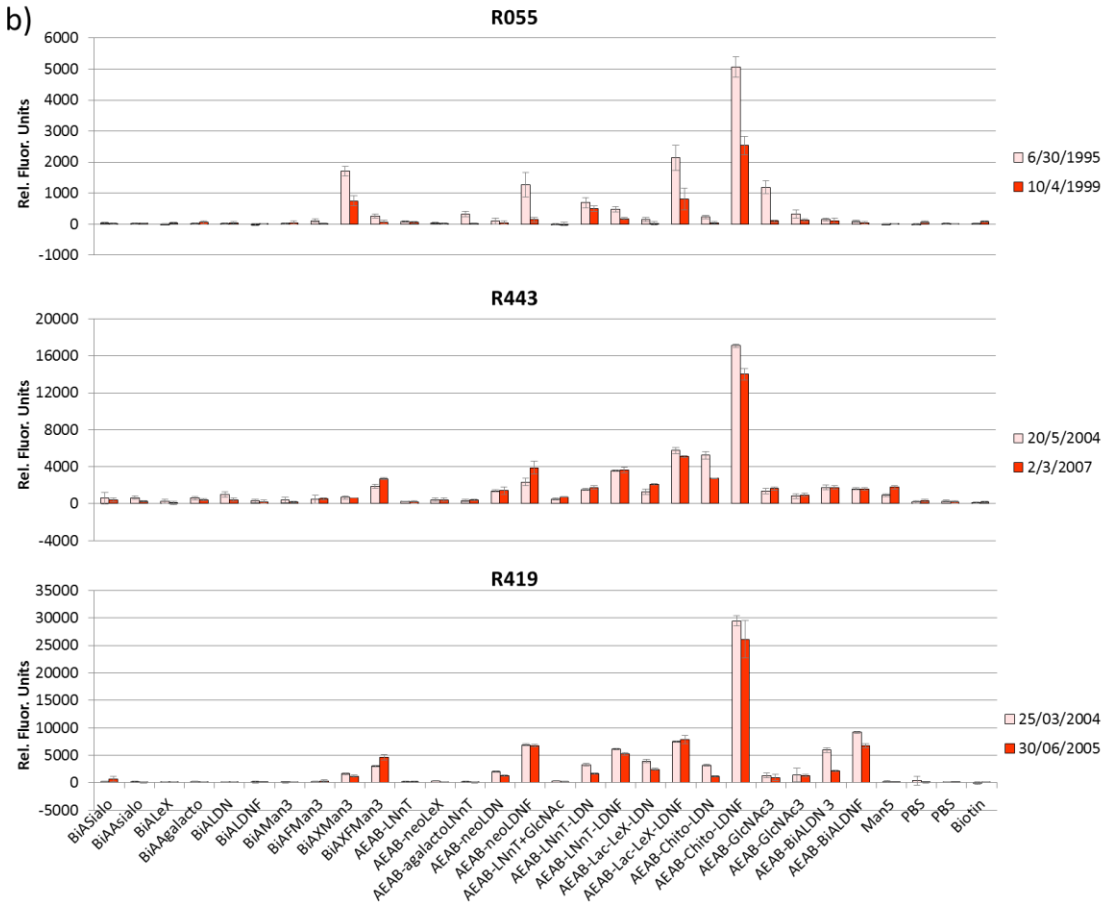
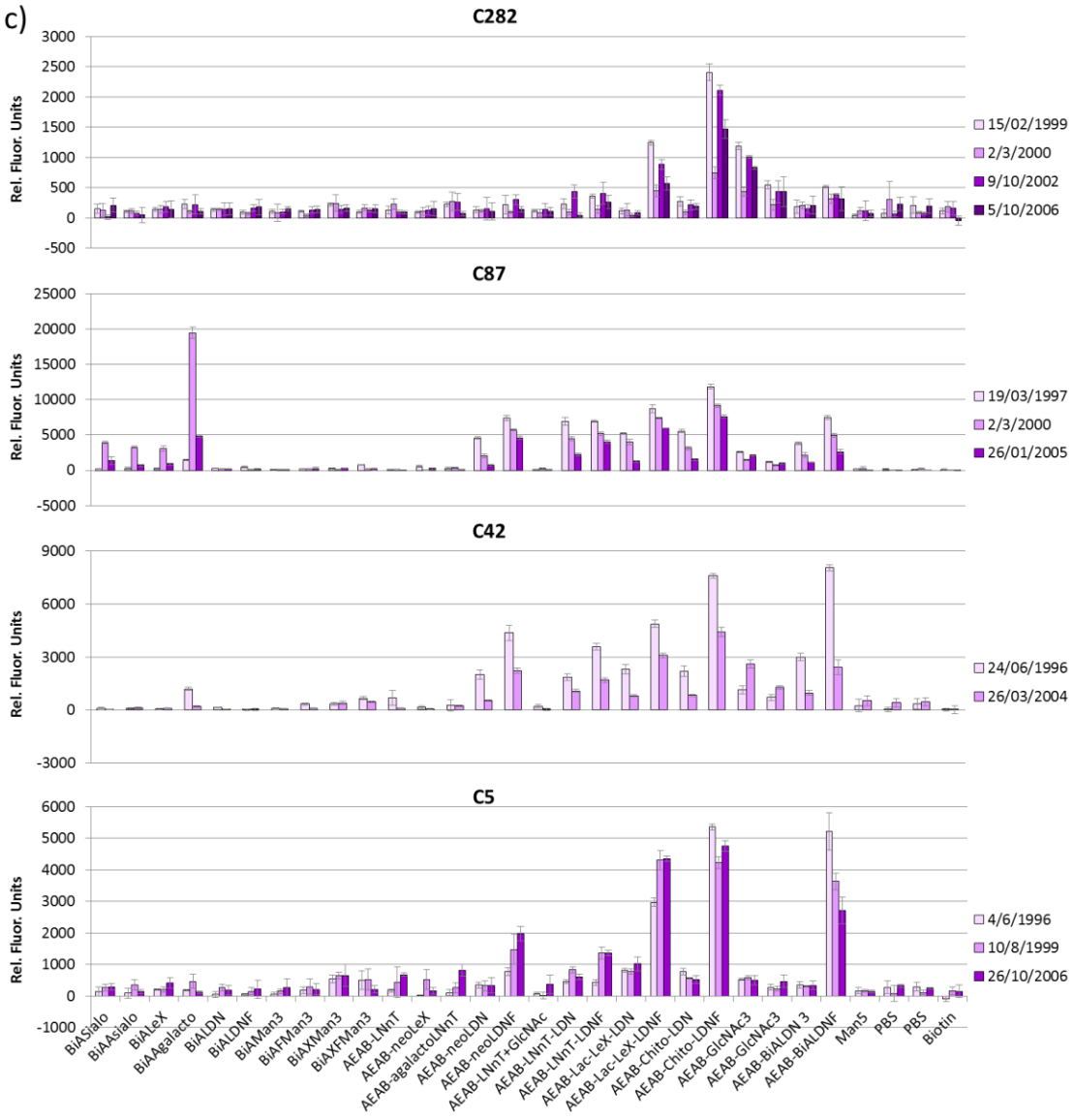
Man5
PBS
PBS
Biotin

b)


R055
Rel. Fluor. Units
6000
5000
4000
3000
2000
1000
0
-1000
6/30/1995
10/4/1999
R443
Rel. Fluor. Units
20000
16000
12000
8000
4000
0
-4000
20/5/2004
2/3/2007
R419
Rel. Fluor. Units
35000
30000
25000
20000
15000
10000
5000
0
-5000
25/03/2004
30/06/2005
BiASialo
BiAAsialo
BiALeX
BiAAgalacto
BiALDN
BiALDNF
BiAMan3
BiAFMan3
BiAXMan3
BiAXFMan3
AEAB-LNnT
AEAB-neoLeX
AEAB-agalactoLNnT
AEAB-neoLDN
AEAB-neoLDNF
AEAB-LNnT+GlcNAc
AEAB-LNnT-LDN
AEAB-LNnT-LDNF
AEAB-Lac-LeX-LDN
AEAB-Lac-LeX-LDNF
AEAB-Chito-LDN
AEAB-Chito-LDNF
AEAB-GlcNAc3
AEAB-GlcNAc3
AEAB-BiALDN 3
AEAB-BiALDNF
Man5
PBS
PBS
Biotin

c)


C282
Rel. Fluor. Units
3000
2500
2000
1500
1000
500
0
-500
15/02/1999
2/3/2000
9/10/2002
5/10/2006
C87
Rel. Fluor. Units
25000
20000
15000
10000
5000
0
-5000
19/03/1997
2/3/2000
26/01/2005
C42
Rel. Fluor. Units
9000
6000
3000
0
-3000
24/06/1996
26/03/2004
C5
Rel. Fluor. Units
6000
5000
4000
3000
2000
1000
0
-1000
4/6/1996
10/8/1999
26/10/2006
BiASialo
BiAASialo
BiALeX
BiAAgalacto
BiALDN
BiALDNF
BiAMan3
BiAFMan3
BiAXMan3
BiAXFMan3
AEAB-LNnT
AEAB-neoLeX
AEAB-agalactoLNnT
AEAB-neoLDN
AEAB-neoLDNF
AEAB-LNnT+GlcNAc
AEAB-LNnT-LDN
AEAB-LNnT-LDNF
AEAB-Lac-LeX-LDN
AEAB-Lac-LeX-LDNF
AEAB-Chito-LDN
AEAB-Chito-LDNF
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AEAB-BiALDN 3
AEAB-BiALDNF
Man5
PBS
PBS
Biotin

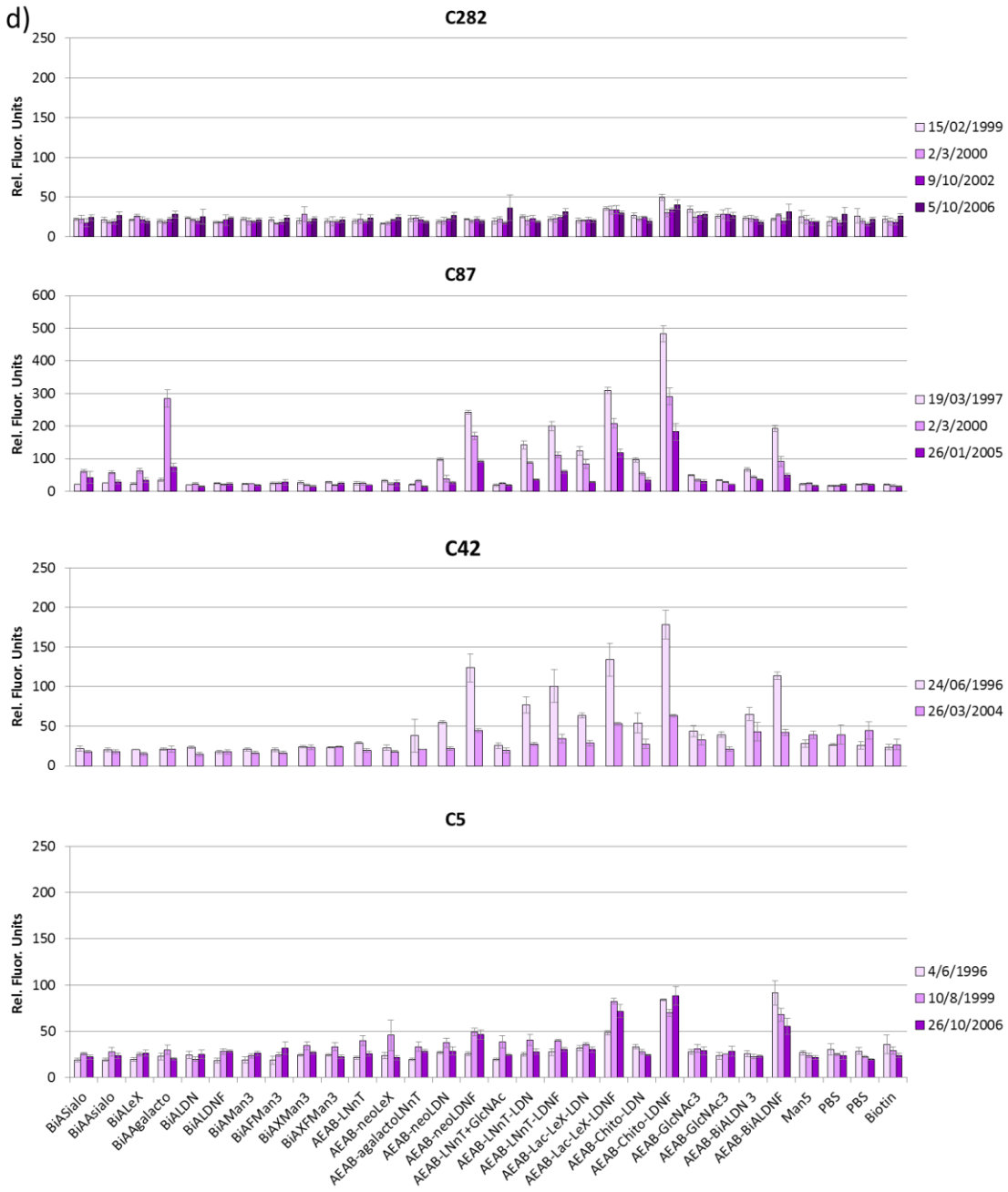


**Figure 5.6 Profiles of representative resistant, susceptible and changing individuals over the course of the study on DSA.**

Susceptible (S), IgG (a); Resistant (R), IgG (b); Changing (C), IgG (c); Changing (C), IgG (d). Dates (DD/MM/YYYY) indicate the dates plasmas were collected.

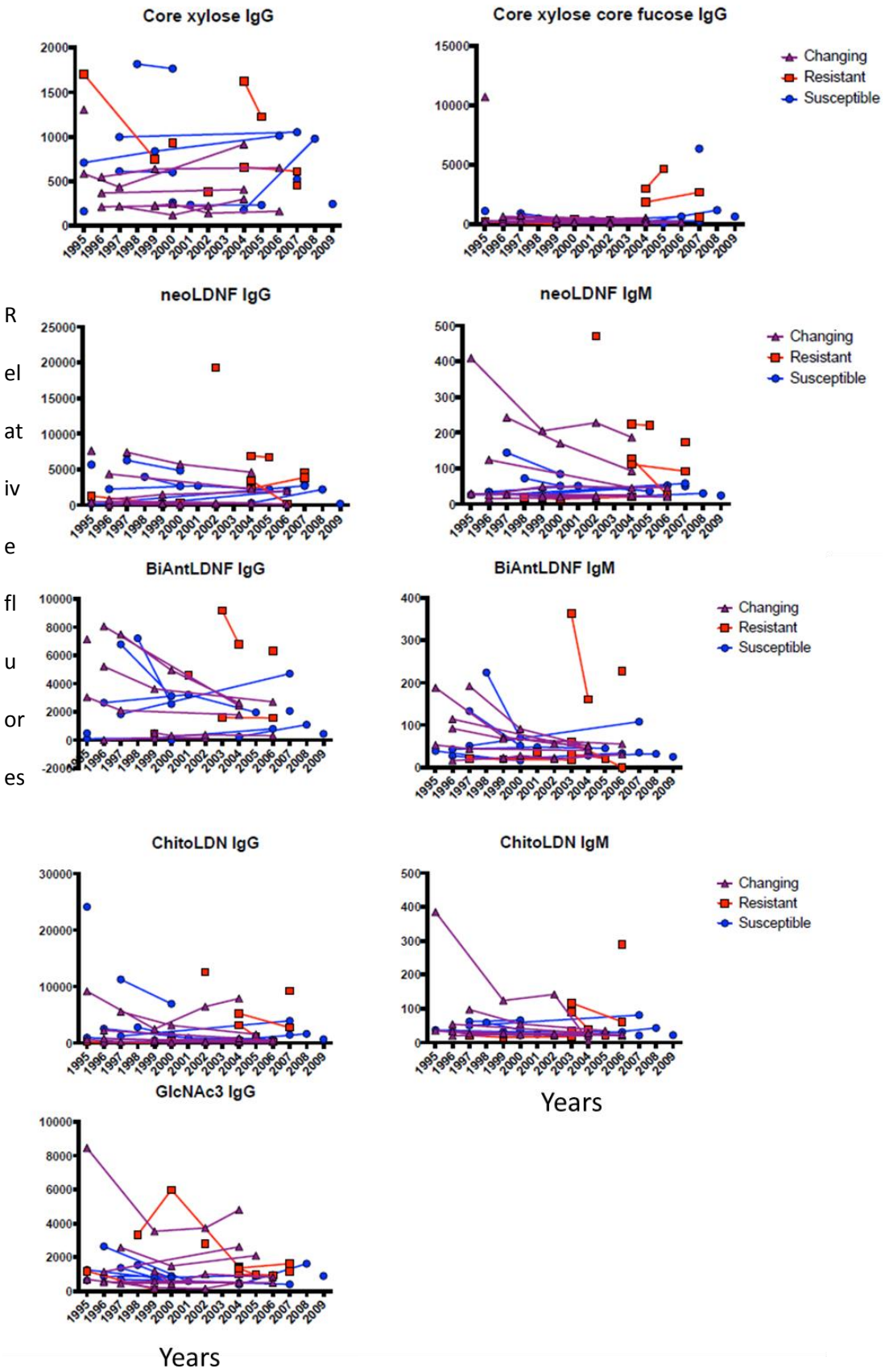
Core xylose IgG
Core xylose core fucose IgG
Changing
Resistant
Susceptible
neoLDNF IgG
neoLDNF IgM
BiAntLDNF IgG
BiAntLDNF IgM
ChitoLDN IgG
ChitoLDN IgM
GlcNAc3 IgG
Relative fluorores
Years

**Figure 5.7 Reactivity of resistant, susceptible and changing individual plasmas over time to selected glycans of the DSA.**

Individual plasmas were screened on the DSA and reactivities to select glycans (graph titles) were plotted over time (years). Plasmas from the same individual are connected by a line.

## 5.4 Discussion

Humans living in schistosomiasis-endemic areas exhibit varying levels of partial resistance to infection. A large portion of the antibodies against schistosomes are anti-glycan antibodies; however, it is unknown whether these anti-glycan antibodies correlate with resistance to infection and/or what role they play during infection. We examined anti-glycan reactivity in about 55 human plasma samples from a total of 29 occupationally-exposed individuals previously characterized as having an immune status of "susceptible," "resistant" or "changing" to *Schistosoma mansoni* [98]. We found that these individuals express a variety of glycan-specific antibodies not found at high levels in samples from infection-naïve individuals, including antibodies to chitin, LacdiNAc (LDN), fucosylated lacdiNAc (LDNF), core xylose/core α1,3-fucose (CX/CF), doubly-fucosylated LDN (F-LDNF), as well as other undefined fucosylated N-glycans. There was a wide degree of variation in the portion of anti-glycan response to SEA, the anti-glycan titer, specificity, immunoglobulin subtype composition and changes in titers over time among the individual patients we characterized. Taken together, these results suggest that schistosomiasis-infected humans may generate an anti-glycan response to the parasite both in the context of natural susceptibility and natural resistance.

The power of our study was limited by several factors. The quality of array data was highly variable, with some samples having high patchy background which made analysis of array binding patterns difficult or sometimes impossible. This was likely due to the age and poor storage conditions of the samples. Because of the difficulty of finding samples, experimental miscommunications, and time/geographical limitations, we were not able to screen as many samples as we had hoped. And because of the natural variability seen among the human samples

from which we did obtain interpretable data, and the small numbers, no statistically significant conclusions could be drawn.

However, in spite of these limitations, we did observe some potentially interesting differences in the titers and isotype compositions of the anti-glycan response which could be related to differences in schistosomiasis susceptibility. We observed a slight trend toward higher ratios of IgG1/IgG2 to egg array fraction 13 (dominant epitope, F-LDNF) in patients who got fewer re-infections and/or took longer to become re-infected. The lack of IgG4 to F-LDNF in the pooled resistant plasma compared to pooled susceptible plasma is also interesting and should be examined in individual sera, given the negative association of this subtype with resistance in multiple studies [103], [406], [407].

When IgG and IgM against the DSA (mostly LDN, LDNF, CX/CF) were examined longitudinally, most resistant and changing individuals appeared to remained stable or decrease in anti-glycan reactivity, whereas most susceptible individuals remained stable or increased. We expected that most individuals in the R and S categories would demonstrate a relatively stable response over time and that C individuals would change, since in the former case, the time to re-infection remained stable over the course of the study and in the latter case, it decreased. However, there are likely several components of the immune response which could change over time to contribute to the overall R or S phenotype, and changes in antibody levels to the glycans we examined probably only represent one aspect of the many changes that are occurring. Additionally, for the longitudinal samples, we do not know whether the individuals were infected when the samples were drawn, and many other parameters which could have contributed to variability in antibody levels of the samples are also unknown. The ANOVA suggested that individuals' responses to the DSA significantly differed from each other and, somewhat significantly, over time, but that S, R or C classification was not a significant factor in these differences. Thus, it would be interesting to follow up on with additional samples and assess

whether other factors besides the S, R and C classifications used here are associated with the individual differences in anti-glycan titers we observed.

Taken together, these results suggest that schistosomiasis-infected humans may generate an anti-glycan response to the worm both in the context of natural susceptibility and natural resistance, and that individuals displayed a wide range of differences in the titer, specificity and isotype composition in their anti-glycan response. These individual; differences could be related to differences in susceptibility or other factors. While certain antibody isotypes to some glycans could negatively contribute to the development of resistance, a high-titer anti-glycan response is clearly not inhibitory to the development of resistance, as was demonstrated by several individuals we examined who were categorized as highly resistant. These observations warrant studies on a larger number of human samples as well as functional studies to better understand the role of anti-glycan antibodies in resistance or susceptibility to schistosomiasis.

## 5.5 Acknowledgements

The authors would like to acknowledge the kind help of Jen Carter for organizing plasma samples at the University of Georgia and explaining patient data, and Harrison and other Karanja Lab members for assistance in locating samples at the Kenya Medical Research Institute.

# Chapter 6. Patterns of reactivity to *Schistosoma mansoni* egg glycan antigens in a population of treatment-naïve Kenyan school children

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## 6.1 Introduction

Schistosomiasis, caused by infection with parasitic helminths of the *Schistosoma spp*., affects more than 200 million people world-wide [408]. A substantial portion of the disease burden in endemic areas falls on children. When the chronic, sub-clinical symptoms often seen in children are taken into account, schistosomiasis causes as many DALYs (disability-adjusted life-years) as malaria [8], [10], [11], [14]. In *S. mansoni*-endemic communities, the prevalence and intensity of infection peak between adolescence and young adulthood, followed by a decline throughout adulthood, even as exposure to the parasite continues [23], [96], [106], [396]–[399]. There is a growing need to understand the factors that underlie naturally-acquired partial resistance in humans as we work towards rational design of a vaccine for schistosomiasis.

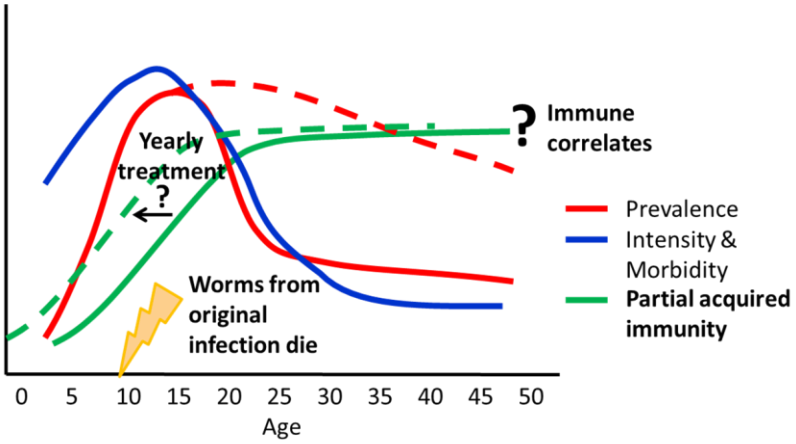



**Figure 6.1 Age-related partial resistance schistosomiasis.**

Both disease prevalence and intensity peak in young adolescents and then decline (red and blue lines). Age-related resistance is related to exposure history, and possibly other age-related factors, and is thought to result from exposure to antigens of dying worms (yellow bolt). Several correlates of resistance have been identified as related to increasing age or resistance to re-infection following treatment (green line), but it is unclear which directly contribute to cure. Praziquantel treatment expedites worm death and is thus thought to expedite the development of resistance (dotted green line).

Age-related resistance to schistosomiasis been attributed to both exposure history as well as exposure-independent age-related factors [392], [399], [409]. Whatever the etiology, this partial resistance is associated with numerous immunological changes [388], [392], [393], [395]. Several parameters have emerged as potential correlates of human resistance. Peripheral blood eosinophilia, IgE, IgG and IgE/IgG4 ratios to adult worm and larval antigens are associated with increased age and/or resistance to re-infection after praziquantel treatment [94], [100], [101], [103], [105], [106], [388], [393], [403], [410]–[413]. In contrast, most studies have found that IgG and IgE to soluble egg antigen (SEA) decrease with age, while IgM remains constant or decreases, and IgG2 to larval and/or adult antigen negatively correlates with resistance to reinfection and age [103], [105], [106], [412], [413]. Eosinophils and other immune cells can mediate destruction of larvae *in vitro* through human IgG and IgE [87], [111], [113], [383], [414]

but it is yet unclear if these and other *in vitro* killing mechanisms contribute to human protection *in vivo*.

Anti-glycan antibodies (αGAbs) are capable of killing schistosomula *in vitro*, and have been correlated with or shown to be directly responsible for protection in different animal models of helminth infections [86], [148], [151], [175], [252], [257], [259], [260], [404], [405]. However, their role in human schistosomiasis is still unclear. One method of examining anti-glycan reactivity is through the use of periodate, which destroys many, but not all, carbohydrate epitopes [259], [415]. Early studies showed that in infected individuals, IgG2, which tends to be restricted to periodate-sensitive epitopes, and IgM can block ADCC killing of schistosomula *in vitro* and are associated with susceptibility to re-infection after treatment, whereas total IgG to schistosomula antigen, including IgG1 and IgG3, which target a mix of periodate-sensitive and periodate resistant epitopes, correlated with resistance to re-infection [104], [245], [387]. It was hypothesized that as children gain exposure and resistance over time, their isotype composition may shift from "blocking" to "protective" antibodies, possibly through a combination of isotype switching and altered antibody targeting. Subsequent investigators, however, demonstrated that IgG1 to certain glycan epitopes (LDN-DF and F-LDN) declined with age and exposure history, while IgM to other glycan epitopes (LDN-DF and LDNF) increased, indicating that the previous hypothesis may not be true for all epitopes (a review of pertinent glycan structures and diagrams can be found in Section 1.3.1) [163], [335]. Another study showed that IgE to worm glycolipids was negatively associated with re-infection intensity [256]. Some studies have also demonstrated that IgG4 to some parasite carbohydrates correlates with infection intensity and that IgG to lacdiNAc (LDN), fucosylated lacdiNAc (LDNF) and Lewis X ($Le^X$) were higher in patients with higher disease morbidity [104], [148]. A recent study compared the anti-glycan responses of children and adults to a wide range of un-defined glycans from various parasite life stages and found that overall, children had modestly higher responses than adults to most structures for both

IgG and IgM [244]. Further structural characterization of the glycans used in that study may yield more valuable information.

In summary, αGAbs as a whole or broadly characterized into subtype groups seem to have a complex relationship with age-related resistance, and more work is needed to discern which particular subtypes to particular glycan epitopes may be contributing to protection. The major goal of this study was to explore patterns of αGAbs throughout adolescence, the time period which seems to be most crucial for gaining resistance [399].

We studied a population of *S. mansoni*-infected children residing in a high-prevalence area of Kenya. The school children, aged 6-22, are enrolled in a mass drug administration (MDA) study which involves follow up on various disease and immunological parameters over a 5-year period with yearly stool exams and treatment. Thus, the study presents the opportunity not only to look at how the disease response changes with increased age and length of exposure in a treatment-naïve population, but also to examine how immune responses change with MDA introduction and maintenance, which is thought to hasten development of natural immunity [99]. In this preliminary study, we have analyzed a cross-section of the baseline plasma for antibodies to periodate- and mock-treated *S. mansoni* SEA, as well as two parasite-cross-reactive glycoproteins, keyhole limpet hemocyanin (KLH) and horseradish peroxidase (HRP). The major glycan responsible for KLH cross-reactivity is F-LDNF, which is foreign to the mammalian host [158], [162]. HRP possesses core-xylose/core-α3-fucose (CX/CF) on truncated N-glycans, an epitope which is also foreign to the host but is expressed by some plants and insects, as well as parasites, and is associated with allergic responses [384], [416]. These two glycan epitopes are targeted by *S. mansoni*-infected humans as well as a protected animal host, the rhesus monkey (see Chapter 5 and [175]).

Given that the children in this study can be considered chronically infected, one would expect certain aspects of immunity which contribute to disease resistance, such as parasite-

specific antibodies, to either increase over time or change qualitatively to become more effective. Two possible null hypotheses would be that if anti-glycan antibodies are not contributing to disease resistance, they would show no correlation with age, or they would follow the pattern of infection intensity. In fact, we found that the anti-glycan responses examined in this study showed neither trend; instead, they were found to either increase or decrease with age, suggesting that immunity to some but not all egg glycans may be related to the development of age-related partial resistance.

## 6.2 Materials and Methods

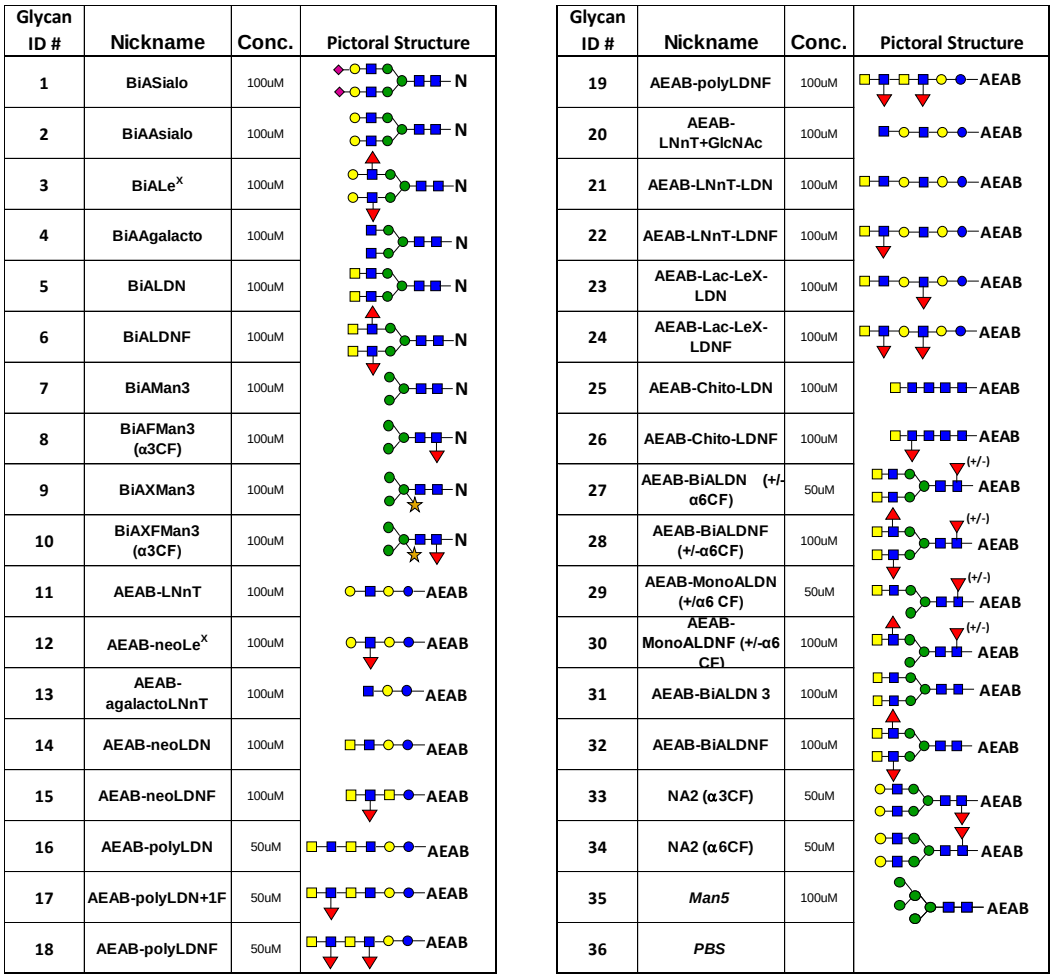
### 6.2.1 Study Population.

School children in a high prevalence *S. mansoni*-endemic area of Western Kenya, Asembo Bay, were enrolled in a 5-year study on village-wide annual Mass Drug Administration (MDA). Stool, blood and growth & morbidity data were collected yearly from a cross section of the children, aged 6-22, for monitoring of *S. mansoni* infection status, other helminth and malarial infections, as well as several cellular and humoral parameters thought to correlate with protection from schistosome infection. Praziquantel was then administered to all children, as well as other medications if necessary. For the ELISA studies, the baseline plasma samples (before treatment) from 146-153 of the children in the cross-section were tested for each data set. For glycan array studies, plasmas were from malaria positive, *S. mansoni*-egg positive children from the nearby high-prevalence area, Usoma, Kenya, who were also predominantly naïve to treatment [417].

### 6.2.2 Glycan microarrays

Synthesis and validation of the Defined Schistosome-type array and the Natural Egg N-glycan Array have been described in works previously published and currently in preparation, and are summarized in Sections 1.5.2.1 and 1.5.2.2 [174], [175] (and Rivera-Marrero, *et al.*, manuscript in preparation). Lectins and monoclonal antibodies were run concurrently with these

studies to ensure quality control of the slides. Glycan microarrays were printed, assayed and analyzed as described in Heimburg-Molinaro *et al.* (2011) with some modifications [247]. Ten microliters of each glycan at 50 or 100 μM was mixed with 1 μL of 10× phosphate buffer in 384-well plates and printed on NHS-activated glass slides using a Piezo Printer (Piezorray, PerkinElmer). Slides were stored at −20°C until use. Binding assays were performed as per Heimburg-Molinaro *et al.* [247] with the following modifications: Plasma was diluted 1:50, and wells were washed three times with 200 μL of TSM wash buffer, then three times with 200 μL TSM buffer with 5 min of shaking for each wash, after the primary and secondary incubations. Secondary antibodies, goat anti-human IgG-Alexa633 (Invitrogen Cat #A21091) goat anti-human IgM-Alexa488 (Invitrogen Cat #A21215), and streptavidin-Alexa488 were used at 5 μg/mL. Slides were scanned using the ScanArray Express software on a PerkinElmer Proscanner XL4000. ScanArray Express was used to align spots, remove background and quantify fluorescence. An excel macro file was then used to average 4–6 replicate spots for each glycan ID, and determine SEM, SD and %CV.



| Glycan ID # | Nickname | Conc. | Pictoral Structure |
|---|---|---|---|
| 1 | BiASialo | 100uM | |
| 2 | BiAAsialo | 100uM | |
| 3 | BiALe$^{x}$ | 100uM | |
| 4 | BiAAgalacto | 100uM | |
| 5 | BiALDN | 100uM | |
| 6 | BiALDNF | 100uM | |
| 7 | BiAMan3 | 100uM | |
| 8 | BiAFMan3 (α3CF) | 100uM | |
| 9 | BiAXMan3 | 100uM | |
| 10 | BiAXFMan3 (α3CF) | 100uM | |
| 11 | AEAB-LNnT | 100uM | |
| 12 | AEAB-neoLe$^{x}$ | 100uM | |
| 13 | AEAB-agalactoLNnT | 100uM | |
| 14 | AEAB-neoLDN | 100uM | |
| 15 | AEAB-neoLDNF | 100uM | |
| 16 | AEAB-polyLDN | 50uM | |
| 17 | AEAB-polyLDN+1F | 50uM | |
| 18 | AEAB-polyLDNF | 50uM | |

| Glycan ID # | Nickname | Conc. | Pictoral Structure |
|---|---|---|---|
| 19 | AEAB-polyLDNF | 100uM | |
| 20 | AEAB-LNnT+GlcNAc | 100uM | |
| 21 | AEAB-LNnT-LDN | 100uM | |
| 22 | AEAB-LNnT-LDNF | 100uM | |
| 23 | AEAB-Lac-LeX-LDN | 100uM | |
| 24 | AEAB-Lac-LeX-LDNF | 100uM | |
| 25 | AEAB-Chito-LDN | 100uM | |
| 26 | AEAB-Chito-LDNF | 100uM | |
| 27 | AEAB-BiALDN (+/-α6CF) | 50uM | |
| 28 | AEAB-BiALDNF (+/-α6CF) | 100uM | |
| 29 | AEAB-MonoALDN (+/α6 CF) | 50uM | |
| 30 | AEAB-MonoALDNF (+/-α6 CF) | 100uM | |
| 31 | AEAB-BiALDN 3 | 100uM | |
| 32 | AEAB-BiALDNF | 100uM | |
| 33 | NA2 (α3CF) | 50uM | |
| 34 | NA2 (α6CF) | 50uM | |
| 35 | *Man5* | 100uM | |
| 36 | *PBS* | | |

**Figure 6.2 List of structures on the Defined Schistosome-type Array (DSA)**

### 6.2.3 Preparation of ELISA antigens

*S. mansoni* soluble egg antigen (SEA) was prepared as previously described [349]. Horseradish peroxidase type VI (HRP; Sigma) was oxidized via periodate-treatment (or mock-treated) in solution and reduced/alkylated prior to coating on ELISA plates. The HRP was dissolved at 1mg/mL in 0.1M sodium acetate pH5.5 and sodium meta-periodate was added to 20mM, or buffer for mock-treated antigen. The reaction was rolled for 8hr in the dark at 4°C, and then quenched with 0.1M sodium borohydride, rolling at room temperature, for 30 min. The oxidized proteins were dialyzed into 0.2M Tris HCl pH8.2 and then denatured by incubation in 8M guanidine HCl and 0.03M DTT in a buffer of 0.2M Tris HCl, 0.15M NaCl, pH 8.2 for 1 hour,

rolling, at room temperature. Iodoacetamide was then added to 0.1M for 30 minutes. The reduced, alkylated proteins were dialyzed to water and lyophilized. It was confirmed that the treated HRP had very little enzyme activity remaining and that at the coating concentrations used, background signal from the HRP coating and peroxidase substrate was negligible. Keyhole limpet hemocyanin (KLH; Calbiochem), BSA and asialofetuin were used as provided by the manufacturer. All coating antigens were quantified by BCA assay (Thermo-Pierce).

### 6.3 ELISA antigen coating and in-plate periodate treatment

*S. mansoni* soluble egg antigen (SEA) was obtained as previously described. 96-well microplates (Greiner Bio-One, Frickenhausen, Germany) were coated with 10 μg/mL SEA, KLH or HRP overnight at $4^0$C in 50mM carbonate/bicarbonate buffer, pH 9.6, 50 μl per well. The excess antigen was discarded and plates were washed 3x with PBS-t wash (PBS/0.05% tween-20), 250 μl per well. The KLH and HRP plates were sealed with parafilm and stored at $4^0$C in PBS wash overnight or up to 3 days until use.

In-plate periodate treatment of SEA for ELISA studies was performed after coating overnight. Excess coating antigen was discarded and washed 3x with PBS wash. The plates were incubated overnight in the dark at $4^0$C with freshly-made 20mM sodium meta-periodate in 0.1M sodium acetate pH5.5, 100 μl per well. On each plate duplicate columns were alternately mock-(buffer alone) or periodate-treated. The solution was discarded and wells were washed 3x with sodium acetate buffer. All wells were then quenched with 0.1M sodium borohydride in PBS wash for 30 min. at ambient temperature, 150 μl per well, and then washed 3x with PBS wash. Treated SEA plates were stored in PBS wash covered with parafilm at ambient temperature for three days and then at $4^0$C for up to 8 weeks until use, and re-hydrated with PBS-t as necessary. This was followed with blocking in PBS/0.3% tween-20 and the ELISA protocol detailed below.

### 6.4 ELISA

SEA-coated plates were blocked with 250 µl of high-tween PBS (PBS/0.3% tween-20) for 2hr or overnight at $4^0$C, standing. They were washed once with PBS wash and then human plasma samples (1:150 for IgG detection, 1:300 for IgM detection), mouse monoclonal antibody control (1µg/mL) and plasma standards were diluted in high-tween PBS and added for a 2-hr primary incubation, 50 µl per well, with shaking. After primary incubation the wells were washed 5x. HRP-conjugated secondary antibodies diluted in high-tween PBS were added (anti-human IgG, 1:5000; anti-human IgM, 1:2000; anti-mouse IgG, 1:1000) for a 1-hr incubation, 50 µl per well, with shaking. The plates were washed 3x and then Sigmafast OPD (1 tablet per 20 mL buffer) was added at 100 µl per well. Plates were allowed to develop in the dark for 8 minutes (IgM) and 10 minutes (IgG) and 25 µl of sulfuric acid was added to each well to stop the development. Large bubbles were popped and the plates were read at 490 nm. For KLH and HRP ELISAs, the protocol was performed similarly except all blocking, primary and secondary incubations were 1hr. Human plasma was diluted to 1:100 for detection of HRP IgG and 1:200 for KLH IgG and IgM. OPD development was 14 min. for HRP and KLH IgM, and 7 min. for KLH IgG.

All samples were assayed in duplicate and all plates included a blank (no primary serum or antibody), a mouse or rabbit monoclonal antibody as positive control, commercial normal human plasma as the minimum threshold for disease-specific antibodies, and a standard curve of plasma pooled from *S. mansoni*-positive adult donors, serially diluted from 1:50 to 1:6400 for glycoproteins, 1:33 to 1:8100 for SEA IgM, and 1:100 to 1:8100 for SEA IgG. Plasmas that registered higher or lower than the standard curve were re-assayed at appropriate dilutions and multiplied appropriately during the analysis. All values were blanked against wells with secondary antibody and OPD only on the same antigen.

For KLH glycoprotein specificity ELISAs, KLH, BSA and asialofetuin were coated at 10ug/mL in duplicate columns of the same plate. Plasmas representative of the range of ages and KLH IgG/IgM reactivities in the sample were selected and run at concentrations ranging from 1:200-1:600, based on their potency in the KLH ELISA, as well as commercial normal human plasma. HRP specificity ELISAs were run in a similar manner and coated with 10 μg/ml mock- or periodate-treated HRP, BSA and asialofetuin. Plasmas were run between 1:100 and 1:1000.

IgG subtype ELISAs on SEA mock/periodate coated plates were performed using the same protocol detailed above, except secondary anti-human antibodies were diluted as follows: IgG 1:5000, IgG1 and IgG4 1:1000, IgG2 1:500, IgG3 1:800 and IgM 1:2000. Plasmas representative of the range of ages and potencies of IgG/IgM to SEA in the sample were selected and run at 1:200 for detection of IgG and IgM, and 1:400 for detection of IgG subtypes.

### 6.4.1 Data analysis

For HRP and KLH IgG/IgM ELISAs, the standard curve ODs were assigned values, with the 1:50 dilution set arbitrarily at 1000 and each subsequent dilution as a fraction of 1000. Each curve was fit with a quadratic function in order to assign a value, representative of antibody concentration, to the OD of each unknown. On each ELISA plate, the standard curve was used to define an upper and lower limit of detection. For curves with a concave function, the upper limit of detection (LOD) was set at the maximum value (when the first derivative equals zero). A minority of curves possessed a convex shape, but the minimum value was less than zero in all curves. Hence, the standard curve was a non-decreasing function in the data range and no upper LOD exists. The lower LOD was defined as concentration corresponding to the upper limit of the 95% confidence interval when the concentration equals zero [418]. When the unknown plasma samples were transformed using this curve, any values above the upper LOD or below the lower LOD were treated as censored. The rates of censoring were 13-14% (KLH), 13-32% (SEA) and 50% (HRP). Concentrations for unknown samples were adjusted for dilution and $\log_{10}$-

transformed. All technical and biological replicate values for antibody concentrations of each individual plasma (2-8 values/individual) were then fit versus age using a modification of a linear mixed model to account for both upper and lower detection limits [419]. Models were fit in SAS Software version 9.3 (SAS Institute, Inc., Cary, NC) and the 5% level of significance was used to determine statistically significant associations between age and antibody concentration. Higher order relationships between age and antibody concentration were explored, but linear associations provided the best fit according to Schwarz's Criterion [420].

For SEA ELISAs, regressions were performed similarly to the method described above, except concentrations of antibody to mock-treated SEA were standardized against a positive plasma curve on mock-treated SEA and antibody to periodate-treated SEA was standardized against a periodate-treated SEA standard curve. In the PR ratio analysis, censored values were assigned a value twice the upper detection limit or half of the lower detection limit for each assay as appropriate. The PR ratios for each replicate of each plasma tested were then taken, log transformed and analyzed with a linear mixed model [421].

The percent of positive samples for antibodies to glycoproteins was determined as the total number of individuals tested minus the number of individuals who got at least one negative reading compared to normal human serum divided by the total number of individuals using raw ODs.

## 6.5 Results

### 6.5.1 Children in *S. mansoni*-endemic areas respond with highly variable patterns of IgG and IgM to parasite-associated glycan antigens

We first asked, what are some of the major glycan epitopes targeted in a small sample of schistosomiasis-exposed children? The infection intensity vs. age distribution in this population is relatively consistent with literature for this area, with eggs per gram of stool peaking between ages 10-12. We reasoned that the epitopes displaying a greater amount variability in antibody

targeting might show interesting and potentially significant relationships with parameters related to immune status such as age and re-infection frequency or intensity when examined in a larger population. We used two glycan microarrays containing schistosome-associated glycan epitopes, the defined schistosome-type microarray (DSA) and the natural egg N-glycan array (egg array). These have been previously validated using lectins, monoclonal antibodies and infection antisera from animal models [174], [175], (Rivera-Marrero, *et al.*, manuscript in preparation). The DSA is a collection of defined, semi-synthetic variants of LDN, LDNF, $Le^X$, and CX/CF and control glycans (a full listing and pictoral representations of structures on the DSA is found in Figure 6.2). The egg array is a collection of partially-purified N-glycans released from *S. mansoni* eggs, where several of the major glycan compositions are inferred based on metadata from mass spectrometry and antibody and lectin binding. The immunodominant antigen on this array is thought to be F-LDNF, and other epitopes present include LDNF, CX/CF and other multiply-fucosylated N-glycan branches.

We screened 10 plasmas from *S. mansoni* egg negative and 11 egg positive children, aged 7-15, on each microarray and detected bound human IgG and IgM. Naïve plasma from a commercial source is undetectable on both arrays (data not shown), whereas the majority of the schistosomiasis-exposed children were reactive to the arrays examined (13/21 on the egg array, 21/21 on the DSA). To our surprise, both the egg negative and positive groups displayed highly variable titers and patterns of epitope reactivity. Figure 6.3a and 6.1b each show four egg positive and four egg negative individuals representative of the IgG and IgM binding patterns seen on the DSA (Figure 6.3a) and egg array (Figure 6.3b). On the DSA, individuals responded most highly with IgG and IgM to LDNF variants, especially poly-LDNF and other extended chains terminating in LDNF. Several individuals also responded equally or to a lesser degree with IgG and IgM to straight chains and N-glycans terminating in LDN. About half of the individuals also had intermediate to high titers of IgG to CX/CF. On the egg array, those individuals that responded with IgG and IgM showed a pattern consistent with F-LDNF binding as defined by

monoclonal antibody binding, where fraction 13 is the highest binder and other fractions including 11, 12, 5, 1 and 16 are also bound. Fraction 9 is known to contain CX/CF and was also bound by individuals who bound this epitope on the defined array.

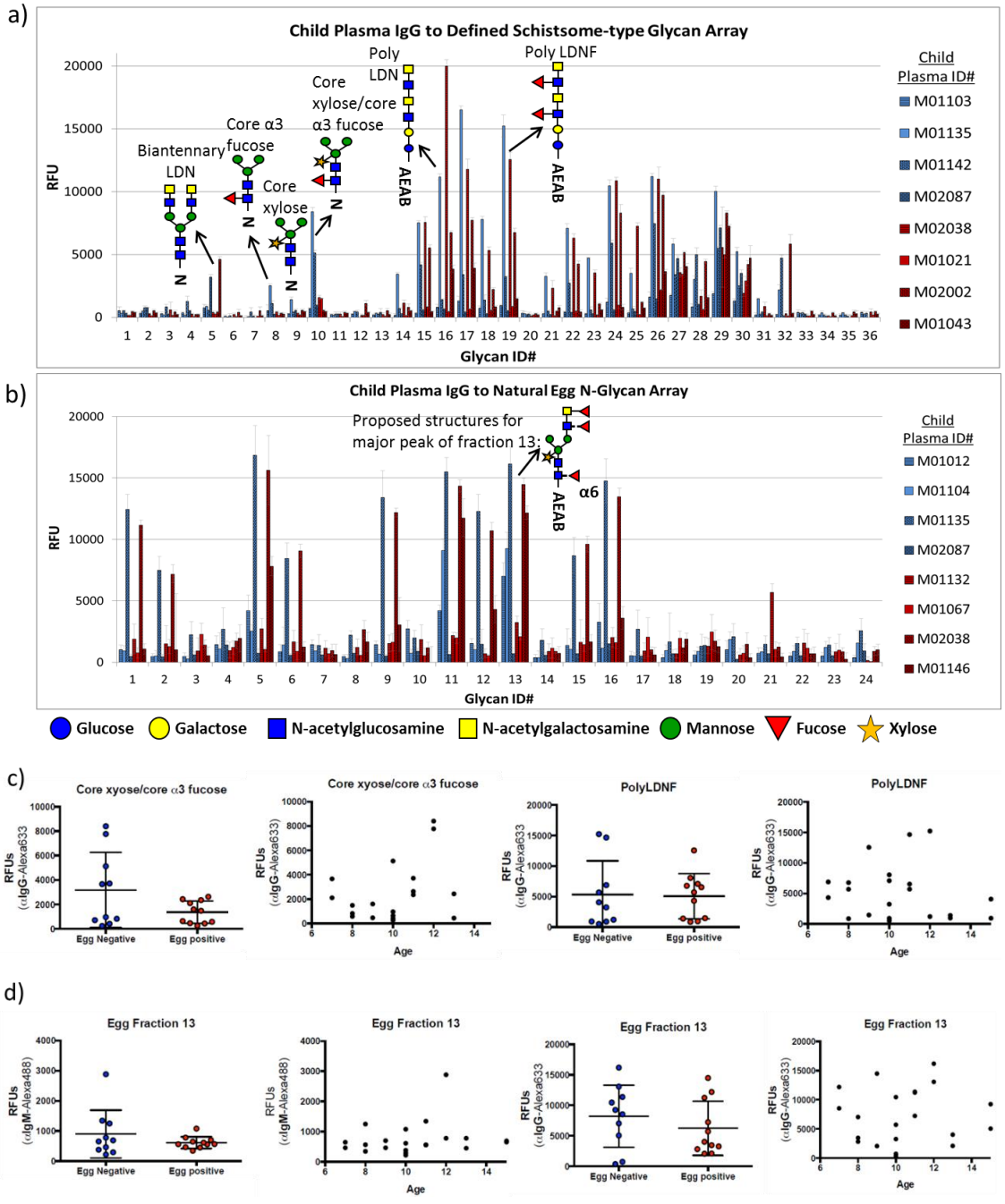



**Figure 6.3 *S. mansoni*-exposed child plasmas bind parasite glycan epitopes on a synthetic (a) and natural (b) glycan array.**

*S. mansoni*-exposed child plasmas bind parasite glycan epitopes on a synthetic (a) and natural (b) glycan array. Four plasmas representative of egg-positive (n – 11; red) and egg-negative (n = 10; blue) children are shown. Bars represent the mean of IgG binding to hexareplicate glycan spots, +/- the standard deviation. Pictoral representations of some of the major glycan structures targeted and discussed in this paper are shown, where each shape represents a monosaccharide. The glycan ID numbers in (a) each represent a defined, purified glycopeptide or AEAB-tagged glycan. The glycan ID numbers in (b) each represent a partially-purified fraction of tagged schistosome egg glycans which has been characterized via antibody and lectin binding and mass spectrometry; the structure shown is the major peak of fraction 13, with dotted lines meaning "either/or". A full listing of the structures on the array is found in Supplementary Figure 1. The mean IgG binding for each sample to glycan ID #10 and glycan ID #19 on the Defined Array is plotted in (c) as a function of infection status and age. The mean IgG and IgM binding for each sample to glycan ID #13 on the Natural Array is plotted in (d) as a function of infection status and age. **RFU** relative fluorescence units.

The distribution of responses among individuals for a few epitopes of interest is shown in Figure 6.3c (DSA) and Figure 6.3d (egg array.) No significant differences were seen between the egg negative and egg positive children for LDNF variants, CX/CF and Egg Array fraction 13 glycans, indicating that antibodies to these epitopes may be related to exposure rather than active infection, and may be maintained after infection is cleared. However, it is also likely that the egg-negative children were lightly infected, as the prevalence of infection by SWAP ELISA was considerably higher than that of Kato-Katz stool exams [417]. This led us to wonder what other factors might be related to the varied presence of such anti-glycan antibodies in infected children, and whether anti-glycan antibodies could be involved in clearance of worms and/or the partial resistance to re-infection that is seen in endemic populations as children age with continued exposure. Alternatively, the persistence of these anti-glycan antibodies could be a result of frequently occurring infections and unrelated to protection. In the former scenario we would expect protective antibodies to increase as children age, while in the latter scenario we would expect the antibodies to remain unchanged or decrease as an antibody response to other protective epitopes mounts. We therefore sought to define the relationship of some of the above anti-glycan antibodies with age in a larger population of egg positive Kenyan schoolchildren.

### 6.5.2 The response to periodate-resistant soluble egg antigens becomes more dominant as children age

We assayed plasmas from approximately 150 *S. mansoni* egg-positive, treatment-naive school children in a high prevalence region of Western Kenya. The infection intensity profile of this population was similar to others reported in the literature, with eggs per gram of stool peaking from ages 10-12. In order to examine the relationship of αGAbs with age, we began with a crude parasite preparation, *S. mansoni* SEA. SEA glycan antigens include LDN, $Le^X$, LDNF, multifucosylated LDNs, and many other glycans, based on monoclonal antibody and lectin binding assays. We performed ELISAs using plates coated with SEA where duplicate columns were alternately mock- or periodate-treated, which destroys the reactivity of most glycan structures. It was therefore possible to determine a "periodate-resistant ratio" (PR ratio) of the SEA-reactive IgG and IgM for each plasma. A standard curve of pooled highly *S. mansoni*-reactive adult plasmas was performed on each plate and used to assign antibody concentration values to each individual child's mock-treated and periodate-treated SEA response. Standard curves were also used to define upper and lower limits of detection, outside of which the data was treated as censored, as described in materials and methods. For the PR ratio analysis, censored were assigned a value twice the upper detection limit or half of the lower detection limit for each assay as appropriate. The PR ratios for each replicate of each plasma tested were then taken, log transformed and fit to a linear mixed model.

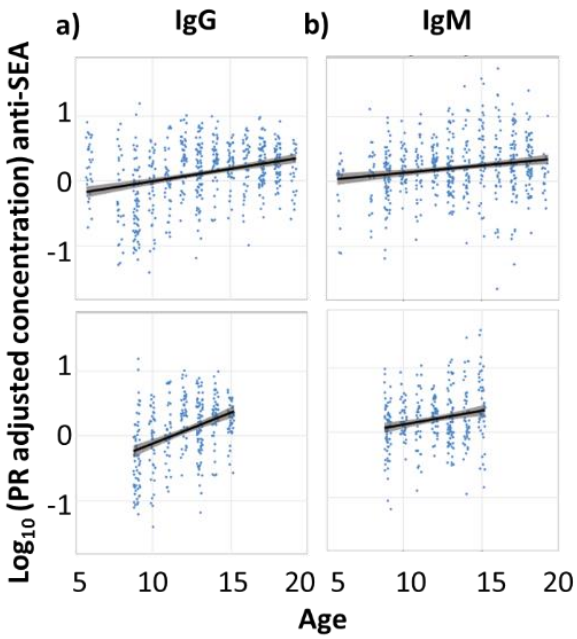



**Figure 6.4 PR ratios of IgG (a) and IgM (b) to SEA are positively correlated with age in linear regression models.**

PR ratios are derived from the log-transformed ratio of antibody binding values to periodate-treated SEA on ELISA, divided by mock-treated SEA. The fit for all ages tested is shown (top) and for only the age 9-15 subjects (bottom). Each point represents one observation (IgG: n=604; IgM: n=576 for ages 6-19) made on individual plasmas (IgG: n=146 for ages 6-19). Shaded gray areas represent 95% confidence bands of the model.

Figure 6.4 shows that the proportions of periodate-resistant IgG (Figure 6.4a) and IgM (Figure 6.4b) to SEA were positively correlated with age. Table 6.1 shows parameters obtained from regressions performed on the entire population and also just for the adolescent group (ages 9-15), showing that the upward trend in PR ratio was most pronounced in this middle age group for both IgG and IgM. Antibody levels seemed to stabilize in the older children.

| Antigen | Dependent variable | Independent variable | Slope | 95% Confidence Interval | P-value |
|---|---|---|---|---|---|
| **SEA** | Log(**IgG PR Ratio**) | Age 6-19 | **0.034** | (0.0158, 0.0527) | **0.0003** |
| | | Age 9-15 | **0.078** | (-0.0363, 0.1190) | **0.0003** |
| **SEA** | Log(**IgM PR Ratio**) | Age 6-19 | **0.020** | (0.0024, 0.0375) | **0.0257** |
| | | Age 9-15 | **0.040** | (0.0043, 0.0763) | **0.0285** |
| **SEA (M)** | Log(**IgG**) | Age 6-19 | **-0.024** | (-0.0449, -0.0024) | **0.0297** |
| | | Age 9-15 | **-0.055** | (-0.1039, -0.007) | **0.0254** |
| **SEA (P)** | Log(**IgG**) | Age 6-19 | **0.020** | (0.0036, 0.0366) | **0.0172** |
| | | Age 9-15 | 0.033 | (-0.0016, 0.0685) | 0.0610 |
| **SEA (M)** | Log(**IgM**) | Age 6-19 | **0.025** | (0.0000, 0.0502) | **0.0496** |
| | | Age 9-15 | **-0.050** | (-0.0902, -0.0102) | **0.0144** |
| **SEA (P)** | Log(**IgM**) | Age 6-19 | -0.008 | (-0.0233, -0.0082) | 0.3435 |
| | | Age 9-15 | -0.011 | (-0.0432, 0.0215) | 0.5078 |

**Table 6.1 Linear regression parameters for the association between anti-SEA responses and age.**

The periodate-resistant portion of the anti-SEA response is positively correlated with age as shown by fitting ELISA data for both the PR ratios, and the mock-treated SEA and periodate-treated SEA reactivities independently, with mixed models of linear regression. PR ratio, ratio of periodate-treated over mock-treated SEA reactivity; M, mock-treated; P, periodate-treated. Values in bold are those that were significant based on an alpha of 0.05.

This led us to wonder whether the increase seen in PR ratios with age was the result of a true increase in the titer of periodate-resistant antibodies, or a decrease in the overall anti-SEA titer, or both. Linear regressions of the titers to mock-treated and periodate-treated SEA for all individuals showed that IgG to periodate-resistant epitopes increased, while IgG to mock-treated SEA decreased (Figure 6.5a, Table 6.1). IgM to mock-treated SEA increased, while IgM to PR-SEA epitopes did not show a significant trend (Figure 6.5b, Table 6.1).

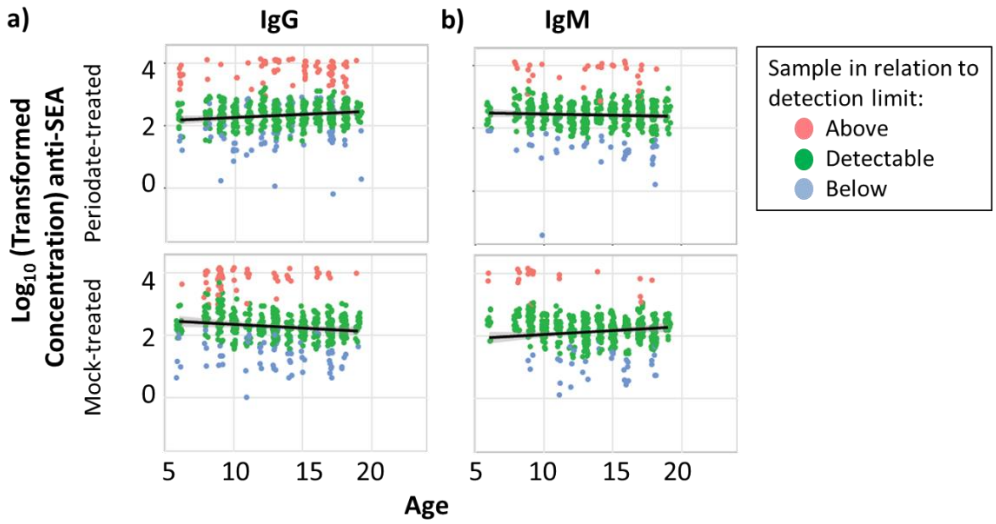



**Figure 6.5 The IgG response to SEA periodate-resistant epitopes increases with age.**

IgG (a) and IgM (b) to periodate-treated (top) and mock-treated (bottom) SEA show distinctive trends when fit to linear regression models. Each observation for IgG (n=604) and IgM (n=576) made on each individual plasma (n=146) is shown. Observations are color-coded based on whether they fell within the detection limits (green), above the detection limit (red) or below the detection limit (blue) defined by the standard curve for each assay. Blue and red data points were treated as censored in the regression analysis. Shaded gray areas represent 95% confidence bands of the model.

Taken together, the SEA ELISA data indicates that, whereas the total anti-SEA IgG response is decreasing, both the proportion and the overall amount of the anti-SEA IgG response to periodate-resistant epitopes (primarily non-glycan in nature) increase as children age. Conversely, one would expect that IgG levels to immunodominant SEA glycan antigens are inversely correlated with age, most obviously over the adolescent period, during which partial disease resistance is believed to be acquired.

### 6.5.3 Antibodies to select glycan antigens decrease as children age

In order to assess whether the downward trend in antibodies to SEA glycans was replicated for individual glycan epitopes, we chose two glycans of interest: Core xylose/core α3-fucose (glycan ID#s 8-10 in Figure 6.3a) and F-LDNF (a dominant epitope in fraction #13 of

Figure 6.3b), both of which were targeted by *S. mansoni*-exposed child plasmas at quite varied levels in our initial microarray studies, and both of which are targeted in protective animal models of the infection [175]. Due to the lack of high-throughput platforms for assaying glycan-specific antibody titers, we used glycoproteins that are cross-reactive with the parasite due to well-characterized N-glycan moieties: Horseradish peroxidase (HRP) and Keyhole Limpet Hemocyanin (KLH) (Figure 6.6). Core xylosylated/core α3-fucosylated tri-mannose makes up approximately 80% of the N-glycan content of HRP [416]. F-LDNF is a minor component of the heavily glycosylated KLH glycoprotein but has been demonstrated to be responsible for the cross-reactivity of the protein with *S. mansoni* extracts [158], [162]. These glycoproteins were used as proxies for the glycan epitopes in ELISA studies.

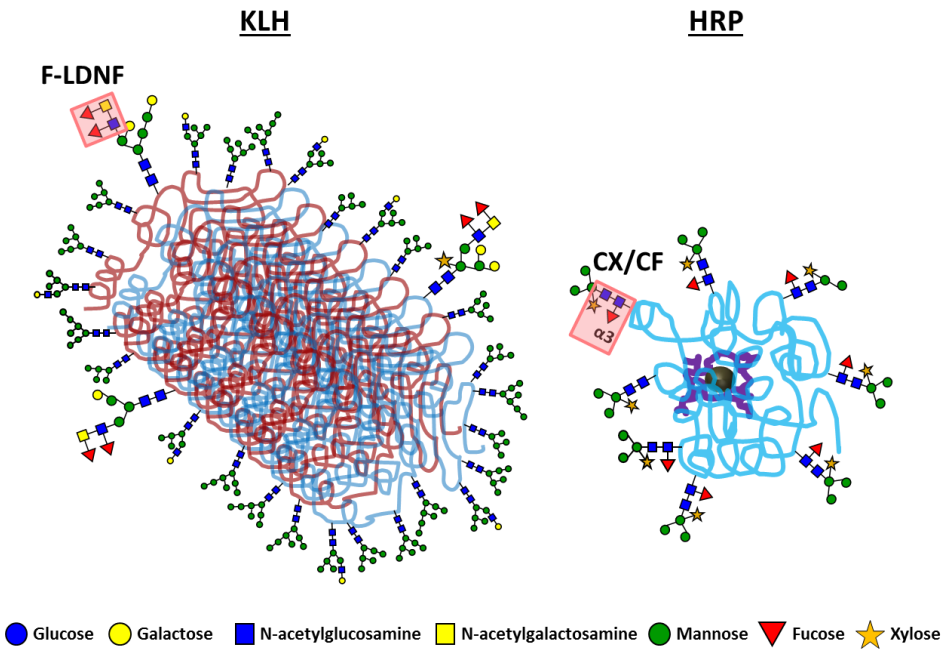



**Figure 6.6 HRP and KLH possess glycan epitopes which are cross-reactive with *S. mansoni***

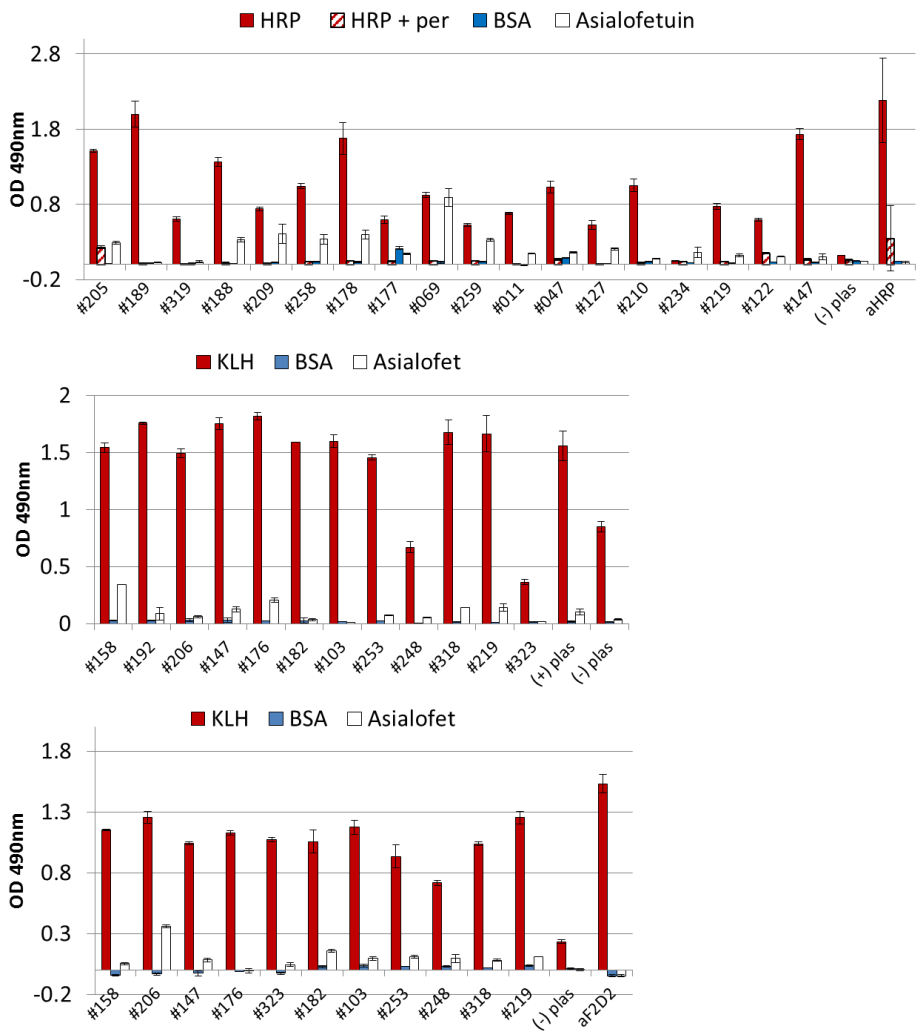



**Figure 6.7 The antibody reactivity seen in *S. mansoni*-infected children is glycoprotein-specific.**

To assess the specificity of the responses seen in ELISA studies, a cross-section of children with moderate to high responses were re-assayed against panels of glycoproteins. IgG responses to HRP, periodate-treated HRP, BSA and asialofetuin were compared in (a). IgG (top) and IgM (bottom) responses to KLH, BSA and asialofetuin were compared in (b). (-) plas, commercial normal human plasma; aHRP, polyclonal rabbit anti-HRP; (+) plas, adult *S. mansoni* positive plasma used for standard curves; aF2D2, mouse monoclonal targeting immunodominant F-LDNF epitope of KLH.

To verify that the anti-HRP and anti-KLH antibodies observed in this cohort were glycan-specific, we chose a cross-section of individual plasmas that displayed moderate to high titers against the glycoproteins for specificity testing (Figure 6.7). Plasmas with anti-HRP activity

had little to no reactivity to the periodate-treated form of the protein, and only a few individuals displayed detectable cross-reactivity with a non-glycosylated model protein, BSA. Some individuals had detectable reactivity with asialofetuin, a prototypical mammalian glycoprotein, which was in all cases but one, much lower than the reactivity with HRP, indicating that some common glycan motifs could be responsible for a portion of the anti-HRP reactivity observed in ELISAs. Similar results were obtained from plasmas tested for IgG and IgM specificity to KLH, BSA and asialofetuin. These results suggest that the great majority of the antibody reactivity observed in the glycoprotein ELISAs of infected plasmas is indeed directed toward to the glycans of these proteins.

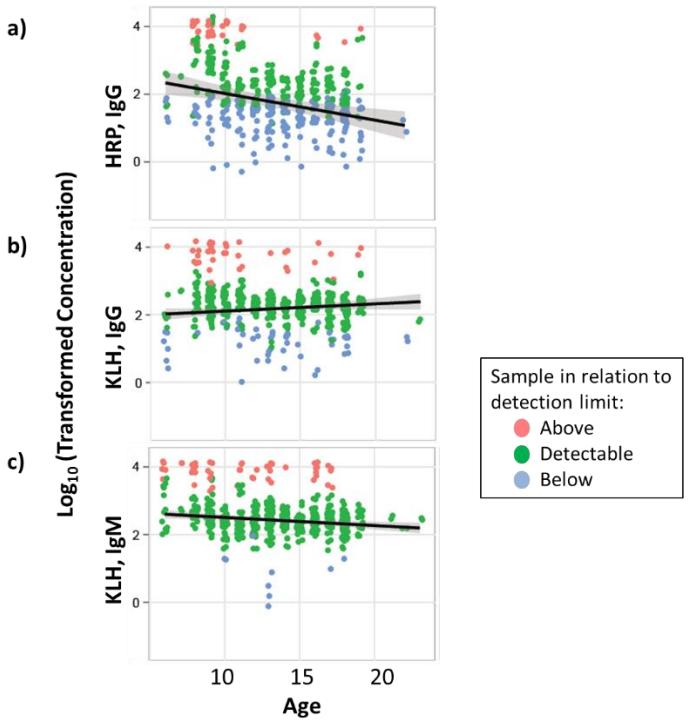



**Figure 6.8 Responses to glycoproteins may be positively or negatively correlated with age.**

IgG (a,b) and IgM (c) to glycoproteins HRP (top) and HRP (middle, bottom) show distinctive trends when fit to linear regression models. Each observation (n=149-153) made on each individual plasma (n=518-552) is shown. Observations are color-coded based on whether they fell within the detection limits (green), above the detection limit (red) or below the detection limit (blue) defined by the standard curve for each assay. Blue and red data points were treated as

censored in the regression analysis. Shaded gray areas represent 95% confidence bands of the model.

Similarly to the SEA studies, standard curves on each plate were used to assign antibody concentration values to each individual plasma tested for IgG to HRP (Figure 6.8a), and IgG and IgM to KLH (Figure 6.8b-c). Standard curves were also used to define upper and lower limits of detection, outside of which the data was treated as censored, as described in materials and methods. All the antibody concentrations generated from the child plasmas tested were then log transformed and fit to mixed models accounting for censored values. Figure 6.8 and Table 6.2 show that IgG to HRP and IgM to KLH decreased significantly as children aged. In contrast, IgG to KLH increased slightly but not significantly for all ages, whereas the increase was pronounced within the 9-15 year old age group. The data suggest that antibodies to individual glycan epitopes follow specific trends as children age which may or may not be correlated with those of crude antigen preparations such as SEA glycans.

| Antigen | Dependent variable | Independent variable | Slope | 95% Confidence Interval | P-value | Percent positive |
|---|---|---|---|---|---|---|
| **HRP** | Log(**IgG**) | Age 6-19 | **-0.078** | (-0.1206, -0.0359) | **0.0004** | 52% |
| | | Age 9-15 | **-0.192** | (-0.2855, -0.0985) | **0.0001** | |
| **KLH** | Log(**IgG**) | Age 6-19 | 0.021 | (-0.0014, 0.0439) | 0.0654 | 91% |
| | | Age 9-15 | **0.150** | (0.1504, 0.1505) | **0.0000** | |
| **KLH** | Log(**IgM**) | Age 6-19 | **-0.024** | (-0.0387, -0.0091) | **0.0018** | 96% |
| | | Age 9-15 | -0.026 | (-0.0608, 0.0097) | 0.1536 | |

**Table 6.2 Responses to glycoproteins may be positively or negatively correlated with age.** ELISA data for IgG to HRP and IgG and IgM to KLH has been fit with mixed models of linear regression. Values in bold are those which were significant based on an alpha of 0.05.

### 6.5.4 Anti-glycan antibodies in infected children belong to a variety of subclasses

A subset of individual plasmas which showed moderate to high anti-SEA IgG titers were used to determine the IgG subtype composition of total and periodate-resistant antibodies to SEA.

Representative data is included in Figure 6.9a. Of the individuals with low PR ratios, 5/10 were IgG4 dominant, 1/10 was IgG1 dominant, and the remainder displayed a mix of G1and G3 or G1, G3 and G4. Where there was a high enough titer of IgG subtypes to determine which displayed the most periodate sensitivity (which would correlate with anti-glycan portion), most individuals had periodate-sensitive IgG1, in some cases accompanied by periodate-sensitive IgG3 or IgG4. In individuals with high PR ratios (a low anti-glycan proportion), IgG4 dominance or a mix of G1, G3 and G4 was most commonly seen. Individuals with moderate to high titers of IgG to KLH and HRP were also tested for isotype balance, displaying a mix of IgG1, IgG3 and IgG4 to these glycoproteins (Figure 6.9b-c). The data suggest that schistosomiasis-infected children tend to produce αGAbs with varied (sub)isotype composition, including resistance-associated and susceptibility-associated antibody subtypes.

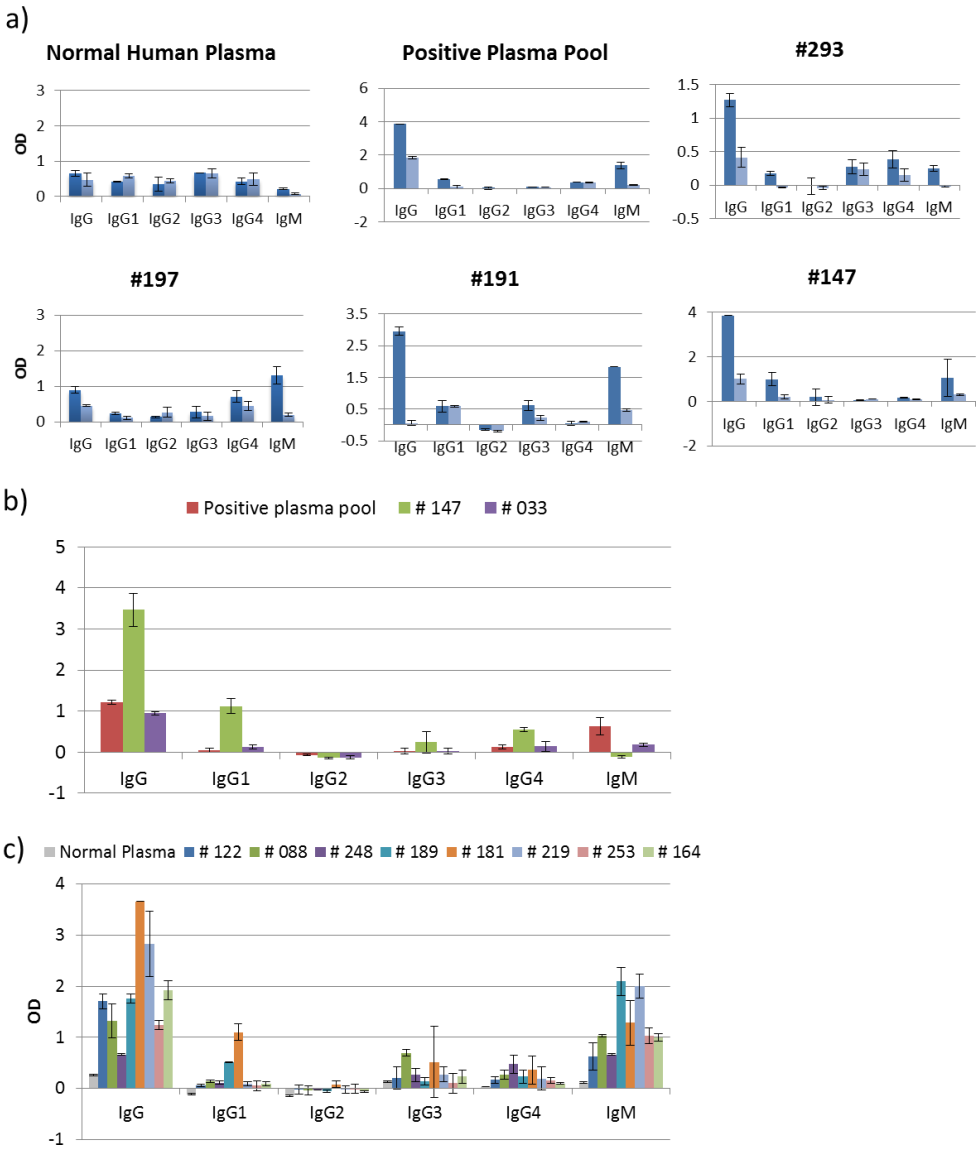



**Figure 6.9 Anti-glycan antibodies of *S. mansoni*-infected children include various IgG sub-isotypes.**

Children with moderate to high anti-SEA reactivity and a range of periodate-resistances to SEA were re-assayed using subtype-specific secondary reagents on mock-treated (dark bars) and periodate-treated (light bars) SEA (a). Each plasma was diluted appropriately to be within the standard curve and secondary reagent dilutions were optimized to obtain roughly equal signal using normal human plasma. Normal human plasma, the infected adult pool used for standard curves, and four representative children (#293, #197, #191, and #147) are shown. The subtype composition of the adult plasma pool and two representative children on HRP-coated plates is shown in (b). The subtype composition of normal human plasma and eight representative children on KLH-coated plates is shown in (b). Mean of two to three replicate wells, +/- SD are shown.

### 6.5.5 Rates of positive reaction to glycoprotein antigens

We also used the ELISA data to calculate rates of positive reaction to the glycoproteins studied among egg positive children, relative to a commercial unexposed human plasma sample (Table 6.2). Only about half of the population was reactive to HRP, while 91% and 96% of the population were positive for IgG and IgM, respectively, to KLH. Anti-KLH IgM was therefore the most sensitive indicator of schistosome infection included in this study.

### 6.5.6 Relationship with infection intensity

None of the anti-glycan antibodies or SEA responses tested showed a significant relationship eggs per gram of stool, an indicator of infection intensity which rises, peaks around age 10-12 in this population, and then falls. This indicated that the responses examined are not simply related to the antigen load.

## 6.6 Discussion

The factors that underlie naturally-acquired partial resistance to schistosomiasis in humans are poorly understood. Anti-glycan antibodies (αGAbs) are abundant in all infected hosts, can kill parasite larvae *in vitro*, and mediate resistance in some animal models of helminth infection but, like most other immunologic parameters, their contribution to schistosomiasis resistance *in vivo* is uncertain. In endemic areas, increased age is associated with partial resistance as exposures to worms continue and the immune response becomes more effective. The aim of this study was to identify glycan epitopes targeted by children in endemic areas, and examine trends of reactivity to such epitopes with age in a pediatric population.

By screening plasmas from 21 children on glycan microarrays, we identified several epitopes of interest. The glycan epitopes targeted include LDN and LDNF in various presentations, CX/CF and F-LDNF. Reactivity to each these epitopes in *S. mansoni*-infected humans has been demonstrated [148], [163], [175], [244], [422], but this is the first report of such antibodies occurring in both egg-positive and egg-negative individuals, and, for CX/CF

antibodies, in children. All of these glycans are expressed by the early intramammalian and/or adult stages of the worm, generally occurring at lower levels than on eggs. They are located on immunologically accessible areas such as the cercarial oral sucker, worm tegument and/or excretory/secretory system [48], [146], [148], [174], [181], indicating they are potential targets of immune attack. Recent studies by our lab demonstrate that Rhesus monkeys, which are naturally protected hosts, generate high antibody responses to all four of these glycans at time points when their sera are lethal to schistosomula *in vitro* [175].

The small number of children included in the array studies displayed a wide range of IgM and IgG levels, but, interestingly, egg positive and negative children displayed similar patterns. These samples were from a highly endemic area, so is likely that some of the egg-negative children were lightly infected, and a near-certainty that they had been exposed [417]. Still, the results are in agreement with previous studies showing that IgG and IgM to KLH persisted for 2 months and 2 years, respectively, after treatment, and that antibodies to CX/CF, LDN and LDNF declined but were still detectable after treatment in 4 of 5 humans [175], [423]. The KLH response was demonstrated to differentiate acute schistosomiasis in travelers from those chronically infected, but the responses among endemic individuals, whether infected or uninfected were similar [274]. Neither glycan epitope is unique to schistosomes among helminths, but rates of positivity for F-LDNF and KLH among infected patients are greater than 90% [178], [423]–[425]. Our KLH ELISA studies demonstrated very high rates (91% and 96%) of IgG and IgM positives, respectively, among egg positive children, when compared with a commercial plasma. Thus, antibodies to KLH and F-LDNF appear to be sensitive but non-specific markers of exposure, rather than active infection, which could be attractive properties for a diagnostic at the later stages of schistosomiasis elimination. The potential persistence of antibodies to both F-LDNF and CX/CF after clearance of infection makes them interesting candidates for a role in disease resistance. In particular, the long-term persistence of antibodies to KLH and F-LDNF in the absence of infection suggests either the continued presence of antigen,

or immunological memory, or both. We would need to compare these samples to truly infection-naïve children, cured children, and those with greater resistance to re-infection in order to assess the above hypotheses.

We then tested a larger number of samples spanning the age range of schoolchildren via ELISA to identify trends of anti-glycan reactivity to SEA, HRP and KLH. Periodate oxidation, which destroys most, but not all (including some uncommon monosaccharides and epitopes that might rely heavily on the N-glycan core tri-mannose) carbohydrate reactivity, making it a useful but imperfect tool [259], [415]. In egg positive children, age was positively correlated with the ratio of periodate-resistant to total SEA reactivity, for both IgG and IgM. The increase was most pronounced over the adolescent years and appeared to stabilize over the late teenage years. This finding is especially interesting, given that in endemic populations, re-infection intensity profiles peak at ages 9-12 and decline precipitously after that [399]. The intensity in our population also peaked around ages 10-12. Thus, it seems likely that the trend of increasing PR ratio in response to SEA is associated with age-related resistance.

Regression models of the total mock-treated or periodate-treated SEA responses revealed that the biasing towards a periodate-resistant response over the adolescent years takes place in the context of a decreasing anti-SEA response, especially for IgG, where the total anti-SEA response appears to be decreasing as the periodate-resistant response increases. Our results using mock-treated SEA are consistent with the literature showing a reduction in IgG and a reduction or no change in IgM with increasing age [103], [105], [106], [412], [413]. From these trends it can be inferred that the both the proportion and total amount of IgG to periodate-sensitive SEA epitopes decrease with age. Neither SEA IgG, IgM or periodate-resistant ratios were significantly correlated with eggs per gram of stool, indicating that the trends are not directly related to infection intensity. One way to interpret these correlations is that as parasites die *in vivo*, and as new parasite exposures mount, innate or other immune factors direct a shift in the immunodominance from some periodate-sensitive to some periodate-resistant epitopes, and

mounting titers of the latter contribute to a more effective immune response against surviving worms and/or new infections. This generalization would not necessarily apply to all glycan epitopes, nor would it rule out a productive role for αGAbs in when they are present at sufficient titer.

The range of human responses seen to CX/CF and F-LDNF on glycan microarrays, as well as the previous identification of antibodies to these epitopes in protective models, led us to more closely examine reactivity to these two epitopes, using the glycoproteins HRP and KLH as proxies. Our results show that IgG to HRP decreases as children age, corresponding to a roughly 10-fold decrease in average OD over the age range tested. HRP glycans consist of 75-80% trimannosylated N-glycans bearing the core xylose/core a3-fucose moiety, and the cross reactivity of *S. mansoni* anti-sera with this glycan moiety of HRP has been characterized [178], [416]. Mammals appear to generate a polyclonal response to the glycan that can be fractionated into xylose-reactive and fucose-reactive components [178], [382]. Our array studies indicate that the highest reactivity is to the dual epitope in all sera tested, but whether this is due to a preference of individual antibodies, or a cumulative effect of polyclonal specificities, is unknown. It should be noted that this glycan is also a common plant allergen, and both allergic and non-allergic individuals without parasite infection may be reactive [415], [426]. Though the positive responses seen in our study are not definitively parasite-specific, a previous study from our lab strongly suggests that in both rhesus monkeys and humans, levels of antibody to CX/CF are associated with *S. mansoni* infection status [175]. The negative correlation of these antibodies with age in children suggests that they are not contributing to the development of natural resistance; however, their temporal association with *in vitro* schistosomula killing in rhesus monkeys [175] and their presence in other protective animal models (unpublished data) warrants further study in human.

Given that the magnitude of the PR response to SEA increased as levels of total anti-SEA IgG decreased, the magnitude of the periodate-sensitive IgG response should simultaneously decrease. Interestingly, anti-HRP IgG, which is just one component of a very complex mixture of

antibodies to SEA glycans, followed the same trend as total anti-SEA IgG, whereas anti-KLH IgG showed an upward trend, with a roughly 2-fold increase in average OD. Both of these trends became more pronounced when restricted to the adolescent age range, similarly to the patterns seen with anti-SEA. This may mean that while the bulk of anti-egg glycan antibodies are not contributing to resistance, antibodies to certain epitopes, such as KLH glycans, are contributing to a productive immune response. In order to test this hypothesis, anti-KLH response could be studied in relation to better readouts of resistance, such as exposure-normalized re-infection rates.

Previous studies have also implicated KLH glycans as potential protective epitopes. A monoclonal antibody to a 38-kDa schistosomular surface antigen, which was later shown to be cross-reactive with KLH, has protective effects *in vitro* and in rats [250], [427]. KLH immunization produced a similar effect [250]. The major cross-reactive epitope was later shown to be F-LDNF [158], [162]. Although F-LDNF bearing glycans constitute less than 5% of KLH glycans, the glycoprotein has a potent inhibitory effect on *S. mansoni* antisera [158], possibly owing to its extremely high oligomerization state. This suggests that KLH cross-reactive glycans may be immunodominant epitopes in schistosome antisera. Other investigators have observed a positive correlation between IgG1 to F-LDN and KLH in humans, and suggested that additional cross-reactive epitopes were responsible for anti-KLH reactivity in patients [163] A smaller fraction of the cross-reactive glycans of KLH are also core xylosylated, representing a second cross-reactive epitope shared by the glycoprotein and the parasite, however, whether this glycan also contributes to the observed immunological cross reactivity, has not been studied [162].

Langley *et al.* found that in *S. mansoni* antisera, IgG2 targeted periodate-sensitive SEA epitopes, IgG4 bound periodate-resistant epitopes, and IgG1 and G3 bound a mixture [245]. We similarly found that children responded to SEA periodate-sensitive epitopes, KLH and HRP with IgG1 and IgG3, but we also observed IgG4 in some patients. IgG2 and IgG4 have been negatively associated with disease resistance in several studies [103], [104]. Human IgG1 and IgG3 are cytophilic antibodies and levels of these antibodies to at least one worm antigen (tetraspanin 2)

are elevated in schistosomiasis- resistant individuals [109], [292]; most disease-resistance studies have not addressed the role of these isotypes in the anti-parasite response. IgG1 and IgG2 to SEA have been shown to decrease with age and IgG3 and G4, to remain stable and increase, respectively [106], [413]. Given the positive trend of IgG to KLH seen in our study, it would be interesting to see whether KLH glycan-specific IgG1 and/or IgG3 also trends upward with age and/or is elevated in resistant individuals.

In conclusion, we found that age is positively correlated with a bias toward periodate-resistant SEA epitopes in a *S. mansoni*-infected pediatric population. Within the context of this trend, antibodies to some individual egg glycan epitopes are negatively correlated while others – such as IgG to KLH are positively correlated with age, warranting further examination of their relationship with disease resistance. Important future directions to better understand the relationship of anti-glycan antibodies with disease resistance should focus on the response to glycan epitopes of larval and worm antigens, which are more closely correlated with disease resistance, the presence of other glycan-specific isotypes associated with protection such as IgE, and the relationship of these antibodies with measures of disease resistance such as intensity and frequency of re-infection after treatment. MDA studies such as this one, in which age-related immunological parameters are measured before and then after multiple years of MDA in a community, may provide a good platform for examining such correlations.

## 6.7 Acknowledgements

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# Chapter 7. Discussion

## 7.1 Part I: Eliciting immunity to parasite glycans: From synthetic glycoconjugates to novel recombinant technologies

Eukaryotic glycans are crucial in the interactions between parasites and their hosts, and represent a major class of antigens in parasite infection as well as other disorders such as cancer and autoimmune diseases. In contrast to the highly successful bacterial polysaccharide conjugate vaccines, however, no precedent exists for how to design a vaccine targeting eukaryotic glycans. Vaccine development has historically been empirical – with many important successes – but when vaccine design is not rationally guided by a foundation of basic principles, trial-and-error can be an endless cycle. This work has contributed a few insights which could be important in informing the design and testing of glyco-conjugates and other carbohydrate-based vaccines.

One theme that has emerged from both immunization studies (Chapter 2 and Chapter 3) is that small glycan epitopes – tri- or tetra-saccharides – for example, come within a larger structural context that is crucial for their antigenicity. Or, looking at it from a different perspective, the minimal glycan epitope of an anti-glycan antibody (αGAb) is larger than one might think. For many years, our lab experimented with biantennary LDN and LDNF glycopeptides conjugated to proteins as anti-schistosomal vaccines in mice. This was based on literature demonstrating the presence of LDN and LDNF on N-glycans of schistosomes [160]. Although the conjugates were immunogenic, glycan-specific antibodies were never generated (unpublished data). In Chapter 3, we described how immunization of mice with cells expressing poly-LDN and –LDNF resulted in glycan-specific antibodies. When the immunization antisera were analyzed on a microarray containing many different variants of LDN and LDNF, it was found that the antisera bound LDN or LDNF linked to GalNAc, GlcNAc or Gal on a straight chain, but could not bind to LDN or LDNF linked to tri-mannose. The cellular immunogens, of course, are not equivalent to the glyco-conjugate vaccines, besides having different presentations

of the LDNF antigen, so it is impossible to say whether the difference in glycan presentation was the major factor in generating glycan-specific antibodies. But furthermore, we found that mouse infection antisera and monoclonal antibodies to LDNF made from infected mice also preferred the same LDNF variants. It appears that both the context of recombinant cell vaccination or schistosome infection, mice fail to generate an antibody to LDNF linked to tri-mannose, while they make antibodies to a variety of other LDNF-containing glycans. We therefore conclude that the inciting antigen in both situations is not LDNF linked to tri-mannose, but some other LDNF. Either mice are unable to make an antibody to LDNF presented in exactly this manner (due to steric hindrance, an improper secondary structure of the glycan, or tolerization to a self-like antigen), even though it is presented in such a manner by the parasite, or the inciting antigens are other versions of LDNF – perhaps poly LDNF or LDNF on a glycolipid (both linked to GalNAc) – and part of this underlying linkage constitutes the minimal epitope.

Similarly, when we immunized mice with LNnT linked to BSA, opening of the reducing-end sugar ring proximal to the protein prevented the generation of antibodies to LNnT, whereas when the ring was closed, antibodies to the desired glycan structure were generated (Chapter 2). It appears that the intact reducing-end ring is part of the minimal epitope for antibodies to this structure, as antibodies to LN (the distal disaccharide) and agalacto-LNnT (the proximal trisaccharide) were not generated. Additionally, the antibodies generated were permissive to some modifications of the LNnT tetrasaccharide but not others. One might think the open-ring version would simply generate antibodies to the tri-saccharide that might possess lower specificity or affinity for LNnT on the glycan array, or to other LN-containing structures on the CFG array (of which there are plenty), but this was not the case. The antibodies generated were to the open-ring LNnT-linker-protein combination. Thus, opening or closing of the sugar ring proximal to the protein dictated whether a glycan-focused response was made, or not.

The uniting conclusion from both of these studies is that anti-glycan antibodies are highly specific for the antigens to which they are generated, and slight modifications can affect the

specificity of the response in drastic ways. Although tri- and tetra-saccharides, such as $Le^x$ or other blood-group antigens, are commonly discussed as antibody "epitopes", in fact, αGAbs may have footprints larger than this. An example is the HIV-neutralizing antibody 2G12, which contacts several monosaccharide residues on each of 4 high-mannose glycans of GP120 [428]. In terms of designing carbohydrate-based vaccines, this means that it is not enough to know that schistosomes make LDNF and that it occurs on N-glycans or O-glycans and to make a vaccine containing that glycans. We must know precisely what the structure inducing the immune response is, and the precise specificities of the antibodies that result, in order to make a vaccine that will elicit αGAbs in a predictable manner. Combining technologies like shotgun glycan microarrays and/or glycopeptide microarrays, glycoproteomics and crystal structure of binding complexes could yield this type of information about an antibody thought to have protective potential (and its epitope).

This work also demonstrates that aspects of how glycan epitopes are presented within the context of the whole immunogen affect immunogenicity and the focus of the response. It has long been known that vaccination with "whole" immunogens such as attenuated or killed bacteria, viruses and parasites induces a more robust, longer-lasting immunity than purified components or recombinant proteins [reviewed in 418]. The same mechanisms that contribute to the success of whole-organism vaccines could be in part responsible for the success of our recombinant whole-cell vaccinations. The whole-cell platform has several attractive features: Dense, membrane-bound presentation of antigens, epitopes that are multivalent both within the same glycan branch as well as along the surface of the cell, many and diverse peptide epitopes for induction of T-dependent immunity, and possibly other "danger" signals to cue the innate immune response.

An additional reason that whole immunogens work so well is probably that a response is induced against a diversity of targets, many of which are then available for immune attack on the organism. One might expect that vaccinating mice with whole hamster cells possessing a foreign surface glycan epitope would induce an antibody response with great breadth to multiple cellular

targets, including the glycan epitope. In fact, our results showed that this was not the case. Lec8 immunization induced an immune response to Lec8 cells in mice and likely contributed to the immunogenicity of the Lec8GT and Lec8GTFT cells as well. But the antibodies made by Lec8-GTFT immunized mice were predominantly against LDNF, with very little cross-reactivity to Lec8 cells or to kifunensine-treated Lec8-GTFT cells, when the secondary immune response was examined. Thus, while the cells likely provided a broad set of T-cell epitopes, the antibody response was clearly focused on the immunodominant LDNF epitopes carried by the N-glycans. Including a variety of protein carriers with the same glycan antigen could serve to generate a robust memory response focused on the glycan in a similar way to the cellular immunogens.

The recombinantly-engineered cells in this study induced a response which was highly focused on one over-expressed glycan, LDNF. An interesting future direction that could be crucial to successful vaccination against multi-cellular pathogens is incorporation of multiple targets into one vaccine. Indeed, this is an increasingly population approach used for coverage of many serotypes by bacterial polysaccharide conjugate vaccines [430]. Several glycan and protein epitopes could each be incorporated into cell lines and mixed to create a multi-target, multi-stage schistosome vaccine.

Whether the cellular immunogens possessed “danger” signals stimulating the innate immune system is also an interesting question to pursue. The Lec8-GT and Lec8-GTFT cells appeared to be more immunogenic than unmodified Lec8 cells. A high amount of fucosylation is a common feature of parasite glycans. Just as the prevalence of GalNAc, fucose and mannose-terminating glycans, and lack of sialylated terminals in schistosomes contribute to the recognition of the parasites as foreign by innate pattern recognition receptors of the host, a global change in the cell surface monosaccharide composition could mediate a similar phenomenon in the cellular immunization. The polymeric presentation of fucosylated epitopes could form a pattern recognized by C-type lectins or other innate receptors [221], [431], [432]. Indeed, glycoproteins with related fucosylated epitopes have been shown to possess greater immunogenicity and in

some cases, induce a completely distinct character of immunity than their recombinant or non-glycosylated counterparts [433], [434].

Another aspect of glycan presentation in the whole-cell platform is its surface display. In many infections and other biological interactions, glycans are encountered as part of the dense glycocalyx surrounding a cell membrane [45], [435], [436]. In fact, both parasite-bound as well as secreted antigens induce robust immunity in schistosomes [194]. Studies have suggested that activation of B-cells by cell membrane-bound antigens and/or particular antigens on the surface of APC may be a critical factor in eliciting immune responses *in vivo* [364], [437]–[439]. Whether whole-cell vaccines, membranous portions of whole-cell vaccines or even parasites could elicit similar responses from B cells in addition to APC presenting such antigens, is a fascinating possibility that remains to be explored.

Interestingly, the rules governing immune responses to glycan antigens appear to vary between and within species. Rabbits immunized with LNnT-BSA conjugates – either open or closed-ring – responded to the sugar-linker portion with little cross-reactivity to BSA, while a significant portion of the mouse response was to BSA alone and glycan-specific antibodies were hardly detectable. Although mice developed a glycan-specific response to the cellular immunogens, we did not compare them to rabbits. Many groups have noted difficulties in inducing some strains of mice to produce glycan-specific antibodies that are easily generated in rats or rabbits to a variety of immunogens [181], [324], [325], [327]. Given that mice are one of the primary species used in pre-clinical development of vaccines, not only for schistosomiasis but for most diseases, perhaps they should be used with caution in the development of glyco-conjugate vaccines. Further studies should be dedicated to assessing which animal models and strains are most similar to the human anti-glycan response in this sense and understanding the mechanisms that account for these inter-species differences.

In conclusion, this work has highlighted a few potentially important aspects of carbohydrate-based vaccine development:

1) Glycan epitopes come within a larger structural context that is crucial for their antigenicity.
2) Use of recombinantly-engineered cells for production of glycoprotein vaccines, and/or use of these cells as vaccines, has many attractive features which should not be overlooked
3) Inter-species and inter-strain differences in the response to glycan antigens should be taken into account during pre-clinical development of vaccine candidates

## 7.2 Part II: Investigating the role of anti-glycan antibodies in schistosome infection

Anti-glycan antibodies (αGAbs) to parasite glycans are abundant in every model of schistosomiasis investigated thus far. While the effects of the parasite glycans on host immunomodulation are well-studied, the role of glycan epitopes in adaptive immunity of this disease is still poorly understood. The purpose of our studies was to examine the role of αGAbs in humans and other protected models of *S. mansoni* through correlations with disease susceptibility and functional studies. Taken together, our results support a few general themes concerning the role of αGAbs in schistosomiasis.

The first theme that emerges from this work is that αGAbs are even more universal in schistosome-infected hosts than was once thought. Chapter 4 demonstrated the first published report of the anti-glycan specificity of serum from high-dose infected rats, a protective model, which share many features of their anti-glycan response with mice, a permissive model. Chapter 5 demonstrated that occupationally-exposed adults, both those who develop a high level of disease resistance and those who remain susceptible over multiple cure/re-infection cycles, also have a variety of anti-glycan titers and specificities. To our knowledge, this was the first study of the anti-glycan response in a resistant human population. Finally, Chapter 6 showed that children of all ages in a *S. mansoni*-endemic area generate αGAbs, even putatively egg-negative children (likely having low-level infection and/or previously exposed). Thus, in both protected and

permissive hosts, and susceptible and resistant humans, αGAbs are not only present but often comparable in titer and specificity.

We have demonstrated that αGAbs are present in hosts who develop resistance at comparable titers to those who do not, thus, αGAbs do not necessarily obstruct the development of protective immunity, as some researchers have suggested. Whether they contribute positively to protective immunity, are irrelevant, or contribute negatively while other immune mechanisms are somehow able to overcome the negative effects of αGAbs, is still unknown. We have yet to conduct functional studies as to the role of some of the observed αGAbs in the rat and mouse models, but are developing the technologies to do so in the future.

The role of these antibodies can be addressed in several ways: 1) Passive transfer of αGAbs into animals that will be challenged with parasites; 2) Schistosomula killing can be tested *in vitro*, with complement and cytotoxic cells, using monoclonal αGAbs, polyclonal αGAbs and lethal infection anti-serum which has been either blocked or depleted of αGAbs. This could help elucidate the role played by αGAbs – to particular life stages or particular glycan epitopes – in protective animal models like the lab rat and rhesus monkey. Both of these techniques have already been used to show the αGAbs CAN mediate parasite destruction *in vitro* or protection from infection. However, a distinct question is: DO αGAbs contribute significantly to the parasite destruction observed *in vitro* with whole serum, or the protection observed *in vivo*, when the entire immune response is engaged? We hope that studies using selective depletion of αGAbs can help answer the latter question, and that similar techniques might then be applied to understanding the mechanisms at work in parasite killing by human serum, as well as implicate potential vaccine candidates.

In the absence of functional studies, however, our results have suggested several possible hypotheses for the contributions of αGAbs to immunity:

1) The isotype balance of αGAbs, similar to what has been found for the overall isotype balance to worm antigens in the literature [103], [104], [406], [407], could affect resistance. This was suggested by the lack of IgG2, 3 and 4 seen in pooled plasma from resistant individuals in Chapter 5, as well as the trend toward decreasing total IgG and IgM in several resistant and changing individuals over time, although we were not able to examine enough individual samples with all the appropriate secondary reagents to generate statistically significant results.
2) αGAbs of some specificities could be positive contributors while others are negative contributors to immunity. This was supported by observations in Chapter 6, where opposite trends with age were seen in antibodies to two glycoproteins, and the periodate-resistant portion of the anti-SEA response trended upwards with age. Given the developmental regulation of glycan antigens during the life cycle of the worm, this hypothesis makes sense [51], [53], [146], [440].
3) The mere presence, type or amount of αGAbs could be less important than aspects of the effector mechanisms that use these antibodies to target parasites – i.e. the αGAbs remain the same whether the host is resistant or susceptible, but other aspects of immunity, such as levels of complement or eosinophils, localization of innate cells mediating ADCC, expression of receptors involved in ADCC, or cytokines that support/repress certain antibody-dependent immune mechanisms, etc., change over time or differ between susceptible and resistant hosts. The literature supports such a hypothesis, with immunological factors such as eosinophils, CD23+ B cells, mast cells, IL-10 and genetic polymorphisms in cytokines all having been recently correlated with susceptibility or resistance in humans [118], [131], [390], [403]. These interesting findings now await a mechanistic understanding of how they exert their

effects on worms, and whether or how much the targets and types of antibodies they work through matter.

4) Particular molecular species, targeted by antibodies to specific proteins and/or glycans, could be crucial for anti-parasite immune mechanisms, and "vulnerable" species might be targeted only by αGAbs with very particular specificities or αGAbs to yet unknown glycan antigens. The differential staining of molecular species by rat and mouse sera seen in Chapter 4, which appears to be periodate-sensitive, begs further investigation of this "Achilles' Heel" hypothesis. There is also literature supporting the idea that antibodies to very specific targets that are crucial to worm functions could be important in parasite destruction [88].

5) The presence, type or amount of αGAbs is completely unrelated in any way to disease immunity. The fact that we observed high amount of variation in αGabs among the individuals we screened, but that any differences we observed between resistant and susceptible humans as a group, or young children versus old, were subtle, supports this hypothesis. If this hypothesis is true, then it would be fascinating to discover what genetic or environmental factors are responsible for such large differences in the individual anti-glycan responses.

A final possibility worthy of discussion is that 6) αGAbs block the development of resistance. For decades, researchers in the field have debated the role of αGAbs, with some dismissing them as un-helpful, based on indirect and/or over-interpreted evidence [123], [194], [195]. However, a close reading of the literature reveals that it does not support such a sweeping conclusion. An often-cited early study showed that infection with either all female or all male cercariae, which will fail to become sexually mature and lay eggs, induced partial resistance in rhesus macaques, while immunization with eggs did not, even though *in vitro* antibody responses were detected [441]. It was concluded that antibodies made in response to the eggs were not

sufficient for protection. Subsequently it was demonstrated that egg immunization also failed to confer resistance on mice, even though the sera mediated eosinophil-dependent killing of schistosomula *in vitro*. However, in the mouse model, irradiated cercariae did confer resistance, while single-sex infection did not [442]. It became evident from this study that levels of *in vitro* eosinophil-dependent killing of schistosomula in sera did not correlate with the ability of the sera to protect *in vivo*.

Over the next few years, three separate groups characterized mouse and rat monoclonal antibodies of various isotypes that were against carbohydrate antigens cross-reactive with schistosomula. In the first set of studies, a rat IgG2a and a rat IgG2c against carbohydrates of the same schistosomular 38-kDa antigen were characterized. The former was protective in passive transfer studies and the latter could block activity of the former *in vitro* as well as block its protection *in vivo* [86], [115], [250], [427]. The second group isolated a variety of mouse monoclonals against distinct carbohydrates, some of which (an IgG2b and an IgM) were protective and some of which were not. The protective mouse IgG2b was later shown to be directed against $Le^X$ [151], [252], [443]. The third group isolated a mouse IgG1 and a mouse IgM against a non-carbohydrate epitope of schistosomular 20-kDa antigen and a carbohydrate epitope of a schitsosomular 200-kDa antigen, respectively. The latter blocked *in vitro* killing activity of the former as well as killing activity of vaccinated mouse serum. Neither of these antibodies demonstrated protection *in vivo*, however. Interestingly, they also showed that adsorption of chronically-infected mouse sera with cercariae increased its protective ability, and that at least one of the specificities removed from this sera was the 200-kDa antigen [444], [445]. Although most of the antigenicity of the schistosomular surface was shown to be periodate-sensitive, they demonstrated that the bulk of the antibodies in vaccinated mouse serum were to periodate-resistant schistosomular epitopes [241].

A series of cure/re-infection studies in children subsequently demonstrated correlation of total IgG, IgG2 and IgM to egg antigens with susceptibility to re-infection [104], [387]. The IgG2

fraction of the anti-egg response was most closely associated with a schistosomular polysaccharide preparation. However, the anti-egg IgG binding to periodate-treated schistosomular antigens was not correlated with re-infection intensity, and anti-egg IgG binding to shed schistosomular antigen, which has high carbohydrate content, was correlated with resistance, especially in patients who had only low levels of the presumably blocking IgG2 [104].

We can conclude from these studies that antibodies to carbohydrates are able to mediate schistosomula killing *in vitro* as well as passively transfer protection *in vivo*, and that other anti-glycan antibodies can block these activities. It is also clear that blocking antibodies exist in the context of both rodent and human infection, some of which are anti-glycan, but there is no evidence that blocking antibodies are primarily anti-glycan, and the same scrutiny has not been applied to antibodies to particular protein antigens or protein portions of crude antigens. Some isotypes (IgM, IgG2) are associated with blocking activity in humans, and blocking isotypes are associated with some carbohydrate reactivities. Non-blocking isotypes IgG1 and IgG3, however, are directed towards both carbohydrate and non-carbohydrate epitopes [104], [245], and the nature of IgE targets is still not well-studied enough to make a conclusion about carbohydrate targeting of this clearly protection-associated isotype.

The investigators conducting these early studies rightfully acknowledged the complex relationship emerging among the *in vitro* activity of protective and blocking antibodies, passive protection conferred by antibodies in animal models, and the true *in vivo* situation in human resistance. They therefore urge further studies to elucidate antigens (either protein or carbohydrate) that elicit strictly protective responses, and to better understand the mechanisms of generating blocking and protective antibodies [151], [241], [445], [446]. However, comments in some more recent works seem to overlook the complexity of the situation and instead make generalizations about the un-protective nature of anti-glycan antibodies. The antibodies made by chimpanzees vaccinated with irradiated cercariae, which induces partial protection, were shown to be overwhelmingly against periodate-sensitive antigens when responses to KLH, soluble worm

antigens (SWAP), SEA, and secretions of eggs (ESP) and schistosomula were examined – both during the vaccination as well as during the acute phase of challenge infection – which is when the vaccine would have to work in order to have an effect on the disease burden. IgM to LDN and LDNF, as well as IgG to periodate-sensitive and periodate-resistant SWAP and egg-secreted proteins (ESP) were boosted by the challenge. The vaccinated animals also had somewhat higher reactivity to periodate-resistant SWAP and ESP (the same comparison was not made for the periodate-sensitive portions) and variable but higher titers of antibodies to KLH, LDN and LDNF compared to control animals during the acute challenge. Thus, all the data presented in the paper is consistent with the hypothesis that glycan epitopes – including but likely not limited to the specific antigens tested in the paper - of SWAP and ESP are involved in protection of the vaccinated chimps. Yet, quizzically, the authors discuss the anti-glycan responses only in terms of how they could be contributing to the "smoke screen" which subverts protective immunity [195]. The authors seem to be seeking an explanation for their favored hypothesis instead of interpreting the data presented in their own study. Collaborators who examined the chimpanzees' response to two more schistosome glycan antigens, F-LDNF and LDN-DF, found that these were similarly induced at higher levels by vaccination than the more host-like glycans, and they provided a more open-minded interpretation [164].

A similar study on baboons followed, in which, again, the response to infection and vaccination was primarily against glycan epitopes (more so for somatic than secreted antigen preparations), and again, no evidence was provided on the role of either portion of the response. They conclude by immunizing mice with eggs, which also elicits an anti-glycan response but is not protective, and offer this as evidence for the smokescreen hypothesis [194]. This interpretation is flawed for a few reasons: Eggs also contain a host of immunomodulatory components which affect the character of the immune response to any antigens, glycan or protein. Eggs may not contain or properly present the glycan or protein antigens that are necessary for targeting the earlier parasite life stages. Additionally, the larval and adult stages (as well as

attenuated cercariae) are also rich in antigenic carbohydrates, and are more appropriate targets for disease resistance, yet the authors did not attempt vaccination of mice with such products. In our opinion, more attention should be devoted to identifying glycan antigens of cercariae, schistosomula and adult worms, and understanding the effect of αGAbs on these life stages *in vitro* and *in vivo*, challenging as it might be to work with these life stages.

### 7.3 Conclusions and future directions

We have shown with this work that there are important structural considerations to take into account when eliciting immunity to glycans, that recombinant cells provide an encouraging platform for the development of carbohydrate-based vaccines against parasite antigens, and that anti-glycan antibodies are a ubiquitous, yet still enigmatic, feature of the immune response in all *S. mansoni*-infected hosts and multiple groups of humans. Although we still have much to learn about the glyco-immunology of vaccines and parasite infection, our studies have made it clear that glycan antigens should not be overlooked in the pursuit of novel diagnostics and vaccines, for schistosomes or for any other pathogen. Technology for screening and testing vaccine and diagnostic candidates must be inclusive of post-translational modification in order to maximize the opportunities of developing effective vaccines against multi-cellular parasites. We hope this work will inspire the scientific community to devote careful consideration, time and resources to developing glyco-inclusive methods of approaching the global challenges posed by schistosomiasis and other helminth infections.

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