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Megan Lee Mickum 4/06/2016

Discovery and synthesis of immunogenic glycan antigens from *Schistosoma mansoni*

By

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Immunology and Molecular Pathogenesis

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Discovery and synthesis of immunogentic glycan antigens from *Schistosoma mansoni*

By

Megan Lee Mickum
B.S., Texas A&M University, 2010

Advisor: Richard D. Cummings, Ph.D.

An abstract of
A dissertation submitted to the Faculty of the
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Abstract

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Glycans are pervasive at the host-pathogen interface, both as stimulators of innate immunity and targets of adaptive immunity for a multitude of pathogens including parasitic helminthes. In the case of schistosomiasis, a disease caused by infection with trematodes of the *Schistosoma* species, glycans play a prominent role the modulation of the host immune system and humoral responses. Glycan structures on schistosome glycoconjugates have gained increasing attention as alternative vaccine and serodiagnostic targets, and a deeper understanding of worm glycans could potentially ignite innovative new strategies for lessening the mortality and morbidity caused by these parasites. A major limitation in the study of the immunogenicity and biological functions of schistosome glycans has been the lack of adequate quantities of the parasite glycans as well as the lack of specific tools needed for identification and study of immune relevant glycans. We sought to generate high affinity and specific IgG monoclonal antibodies to schistosome glycans to facilitate the purification and tracking of immunogenic parasite glycans, and developed a natural N-glycan microarray from *S. mansoni* egg glycoproteins to identify specific antigenic epitopes within the egg glycome which are the targets of adaptive immunity in infected hosts. We then began to "decipher the schistosome glycogenome" by utilizing genomics technologies to develop a semi-synthetic approach for heterologous expression of schistosome glycosyltransferases to generate significant glycan structures. These studies demonstrated the importance of the unique linkages and high levels of fucosylation found in complex schistosome glycans, the success of shotgun glycomics to elucidate host immune targets, and the extremely complex and still enigmatic specificity and functionality of the glycosyltransferase families responsible for the generation of the worm glycome. This work should encourage and support future studies on *Schistosoma's* uniquely fucosylated glycans to elucidate their role in worm biology, explore their feasibility as immune targets, and facilitate the development of novel diagnostic approaches and glycoconjugate vaccine platforms.

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To my family, there are not enough words to express how much your love and support have meant to me during graduate school. Thank you for listening to me when I complain about my

experiments not working or why I couldn't repeat something important. Mom and Dad, since day one you encouraged me, kindled my passion for science, and impressed upon me the value of hard work and education. To my sister Clare, thank you for surviving graduate school and showing me it can be done. Thank you for always knowing how to help after a long day/week/month in the lab, and for always lending me a listening ear after my failures or successes. And to the most important person, my husband Spencer- Your love, support, and encouragement has kept me going, especially through this difficult last year. You may not have always understood exactly what I was working on, but you never failed to tell me that I could accomplish anything. I genuinely don't think I could have done this without you. Thanks for always reminding me what was waiting for us at the end of the tunnel. I love you more than you could know. I like to think that if we can make it through doctorate degrees at the same time, we can do just about anything. Lastly, to our son- It will be many years before you know this, but you were with me as I diligently completed this dissertation. That one kick really helped when I was stuck on part of my discussion, so thanks for that. I can't wait to meet you, kiddo.

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Abbreviation List

AA-MΦ - alternatively activated macrophage

AAL - *Aleuria aurantia* lectin

AcOH - acetic acid

ADCC – antibody-dependent cell-mediated cytotoxicity

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

ALG - altered in glycosylation

αGAb – anti-glycan antibody

APC – antigen presenting cell

asialo-bGP (Na2) – asialolated Bermuda grass pollen (Galβ4GlcNAcβ2Manα3(Galβ4GlcNAcβ2Manα6)Manβ-4GlcNAcβ4GlcNAcβ)

AT - after treatment with praziquantel

BGH - *Biomphalaria glabrata* hemolymph

BMGP – bovine milk glycoprotein

BPL - *Bauhinia purpurea* lectin

BSA – bovine serum albumin fraction V

BS90 - BS-90 resistant *Biomphalaria glabrata* hemolymph

BT – before treatment with praziquantel

BTH- *Bulinus truncatus* hemolymph

CAA – circulating anodic antigen

CCA – circulating cathodic antigen

CDC – Centers for Disease Control

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

CFG – consortium for functional glycomics

CLR – c-type lectin receptor

ConA - concanavalin A

CS - chondroitin sulfate

CT - cholera toxin

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

CV – column volumes

DALYs - disability-adjusted life-years

DC – dendritic cell

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

deoxyHex – deoxy hexose sugar

dF-LDN-dF – Fucα1-2Fucα1-3GalNAcβ1-4(Fucα1-2Fucα1-3)GlcNAc

DHB – 2,5-Dihydroxybenzoic acid

DMEM - Dulbecco's Modified Eagle Medium

DMSO – dimethyl sulfoxide

Dol-P - dolichol phosphate

DSA - Defined schistosome-type glycan microarray

ds-Jurkats - desialylated jurkat cells

DTT – dithiotreitol

EDTA - ethylenediaminetetraacetic acid

EGTA - ethylene glycol tetraacetic acid

ELISA – enzyme-linked immuno assay

ESI - Electrospray Ionization

FBS – fetal bovine albumin

FLDN - Fucα1-3GalNAcβ1-4GlcNAc

FLDNF – Fucα1-3GalNAcβ1-4(Fucα1-3)GlcNAc

FREPs - fibrinogen-related proteins

Fuc- fucose

FuT – fucosyltranserase

GAEAB – glycan AEAB conjugate

GAG – glycosaminoglycan

Gal - galactose

GalT - Galatosyl transferase

GalNAcT - N-acetylgalactosaminyltransferase

GalNAc – N-acetyl galactosamine

GBP – glycan binding protein

GlcNAcT – N-acetylglucosaminyltransferase

GlcA – glucuronic acid

GlcNAc – N-Acetyl glucosamine

GFP – green fluorescent protein

GPI – glycosylphosphatidylinositol

GSI-B4 - *Griffonia simplicifolia* lectin I

GSL-II - *Griffonia simplicifolia* lectin II

HEK - human embryonic kidney cell

Hex - hexose sugar

HexNAc - N-acetylhexoseamine sugar

HPA - *Helix pomatia* agglutinin

HPLC – high pressure liquid chromatography

HRP – horseradish peroxidase

HS - heparin/heparan sulfate

IFN-γ – interferon γ

IL-1 – interleukin 1

IL-10 – interleukin 10

IL-4 – interleukin 4

IL-5 – interleukin 5

IL-6 – interleukin 6

KEGG - Kyoto Encyclopedia of Genes and Genomes

KLH – Keyhole limpet hemocyanin

LDN – LacdiNAc (GalNAcβ1-4GlcNAc)

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

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

LDNFP – lacdiNAc fucopentaose; GalNAcβ1-4[Fucα1-3]GlcNAcβ1-3Galβ1-4Glc

LDNT – LacdiNAc tetratose; GalNAcβ1-4GlcNAcβ1-3Galβ1-4Glc

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

LN – LacNAc, N-acetyllactosamine (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

LTL- *Lotus tetragonolobus* lectin

LTPs – larval transformation products

M3GN2 - $Man_3GlcNAc_2$

MAA - *Maackia amurensis* agglutinin

mAb - monoclonal antibody

Man – mannose

$Man_5GlcNAc_2$ - Manα6(Manα3)Manα6(Manα3)Manβ-4GlcNAcβ4GlcNAcβ

MAGS - metadata-assisted glycan sequencing

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

MDA – mass drug administration

MGL – macrophage galactose-binding lectin

MR – mannose receptor

MS – mass spectrometry

MS^n – multistage mass spectrometry

MW- molecular weight

Na2 - Galβ4GlcNAcβ2Manα3(Galβ4GlcNAcβ2Manα6)Manβ-4GlcNAcβ4GlcNAcβ

NIH – National Institute of Health

NIAID – National Institute of Allergy and Infectious Diseases

NK – natural killer cells

NMR - nuclear magnetic resonance

PAMPs - pathogen-associated molecular patterns

PBS- phosphate buffer saline

PGC - porous graphitized carbon column

PHA-L - *Phaseolus vulgaris* agglutinin L

PHA-E - *Phaseolus vulgaris* agglutinin E

PNA - peanut agglutinin PLA_2- honeybee phospholipase A_2

PNGase A/F – N-Glycosidase A/F

Poly-LN – poly-N-acetyllactosamine

PRRs – as pattern-recognition receptors

PVDF - polyvinylidene difluoride

OPD – o-Phenylenediamine dihydrochloride

RACE PCR - Rapid amplification of cDNA ends polymerase chain reaction

RCA-I - *Ricinus communis* agglutinin I

RFUs - relative fluorescence units

SEA – soluble egg extract

SFM – serum free media

Sjp – *Schistosoma japonicum* protein

Smp – *Schistosoma mansoni* protein

SNA - *Sambucus nigra* agglutinin

SSGM – shotgun schistosome glycan microarray

SWAP – soluble worm antigen

TBS – Tris buffered saline

TFA – trifluoroacetic acid

TGF-α - transforming growth factor α

TLR – Toll-like receptor

TMD – transmembrane domain

Tn – O-linked (Ser/Thr) α-GalNAc

TNF-α – tumor necrosis factor α

UEA-I - *Ulex europaeus* I

WGA - wheat germ agglutinin

WFA - *Wisteria floribunda* agglutinin

WHO – World Health Organization

Xyl - xylose

XYLT - xylosyltransferase

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).

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

1.1 Global state of schistosomiasis

1.1.1 *Burden of helminth disease*

Helminths are multicellular parasitic worms that comprise a major class of human pathogens. 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. Soil-transmitted helminths (gastrointestinal nematodes including *Ascaris*, *Trichuris*, *Necator sp*.) and schistosomes (blood-dwelling trematodes) are the most common (3-6). Although great strides have been made through implementation of chemotherapy and improved sanitation, massive amounts of suffering due to helminth infections persist, and to date, no vaccines for helminths or any human parasite exist.

Schistosomiasis, in particular, is a debilitating vascular disease caused by an infection with parasitic helminthes of the *Schistosoma* species. It is a major public health concern in many developing countries with a wide range of clinical manifestations (5,7-9). More than 240 million people are infected and, in sub-Saharan Africa alone, 280,000 people per year die of schistosomiasis (10,11). Like other helminthic infections, these parasitic worms have a complex life cycle that alternates between an intermediate mollusk host and a definitive vertebrate host resulting in significant morbidity and mortality for the infected human or animal. While the mortality rates are lower than diseases like HIV and malaria, some researchers report that the burden of schistosomiasis rivals HIV and malaria in years of life and productive life lost due to disability and/or death (disability adjusted life years, DALYs) (9,12-15). In light of the

socioeconomic impact of this disease in otherwise poverty-stricken areas in over 70 tropical and subtropical countries, schistosomiasis is considered the third most important parasitic disease in terms of public health impact, has been designated a Neglected Tropical Disease, and is a true "societal poverty trap."(3,5,6,13,16).

1.1.2 *Schistosoma life cycle*

Schistosomes are trematodes of the family *Schistosomatidae* with five strains of clinical importance in humans: *S. mansoni, S. haematobium, S. japonicum, S. mekongi*, and *S. intercalatum* (8,9,16). As depicted in **Figure 1.1**, schistosomes lead a complex life cycle utilizing a mollusk and mammal host. *Schistosoma* eggs are shed in the stool from infected mammals and eventually contaminate fresh water sources in regions with poor sanitation. Eggs in river and lake water hatch, producing miracidia, which infect the intermediate snail host. Within the snails the parasites undergo several rounds of asexual reproduction via mother and daughter sporocysts eventually giving rise to cercariae, the infective stage for humans and animals. Cercariae are shed from the headfoot of a snail as a free-swimming body with a bifurcated tail. Their powerful tail and secreted chemicals allow cercariae to burrow through the skin of exposed humans and animals. During this process they lose their tail and are transformed to schistosomula, the larval stage of the worm. This transformation is accompanied with pronounced changes in shape, metabolism, and expression of antigenic glycoconjugates (17-19). The schistosomula migrate into veins, passing through the lung vasculature, and eventually reside in a strain-specific location in the vasculature, where they mature to adulthood. *S.mansoni* and *S. japonicum* home to the intestines, with *S. mansoni* residing in the portal vein draining the large intestine and *S. japonicum* in the small intestine. *S. haematobium* live in the urinary bladder plexus. Adult male and female worms pair and lay hundreds of eggs per day. Some eggs pass through the wall of the intestine or bladder and are excreted in the stool or urine to complete the life cycle. Other eggs are fated to move through the blood stream and eventually lodge in organs, such as the liver and spleen (8,10,16,20).

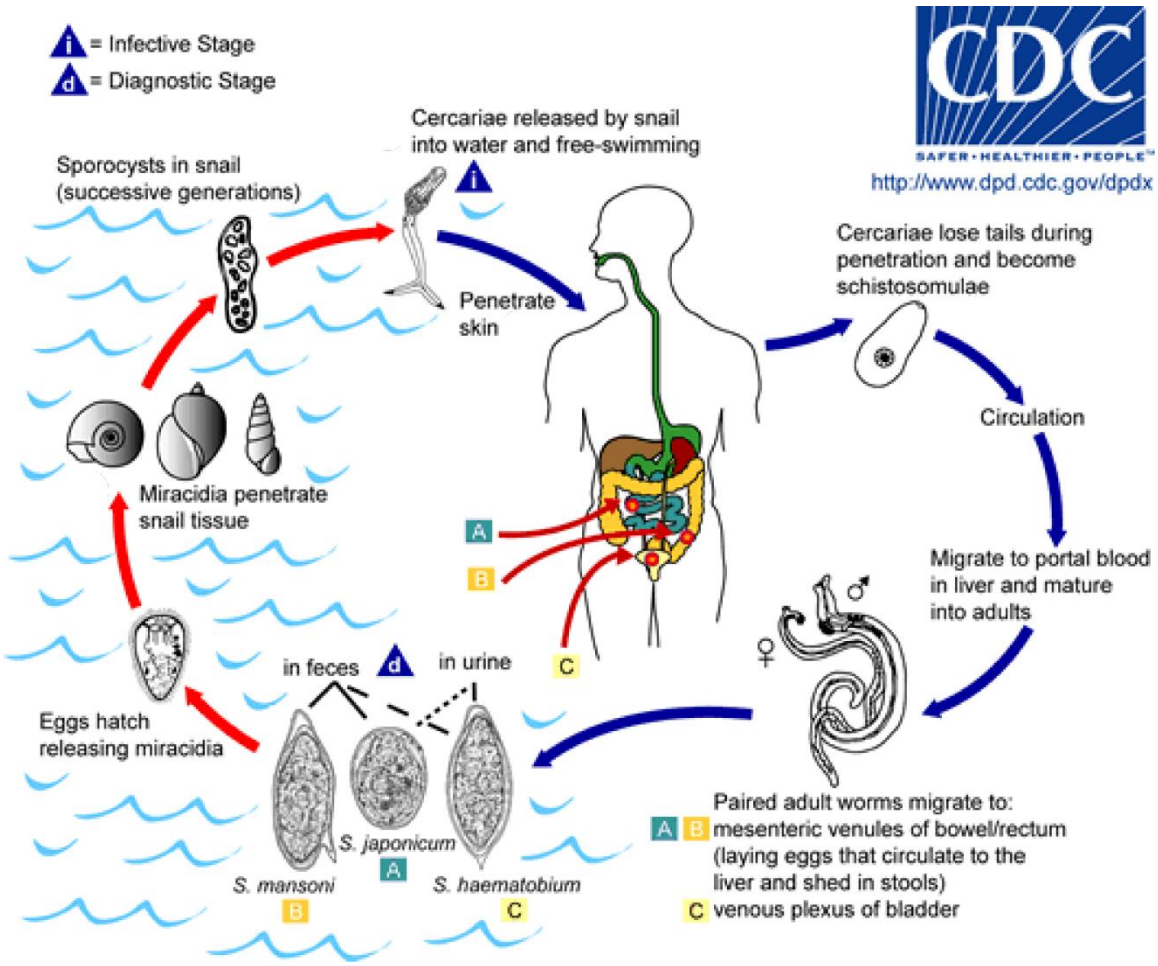


Figure 1.1 The *Schistosoma* life cycle.

(Adapted from the Division of Parasitic Diseases and Malaria, Centers for Disease Control and Prevention and reprinted under Fair Use)

1.1.3 *Clinical manifestations*

The symptoms of schistosomiasis are largely dependent on the infection intensity or egg and worm burden. As a result, the symptoms experienced can range from asymptomatic or mild, to organ damage, cancer, and death in extreme chronic cases. In endemic regions infections manifest with mild symptoms such as anemia, malnutrition, and delayed physical/cognitive development (5,6,21). Non-endemic cases often experience more severe symptoms during the acute phase including fever and chills, termed Katyama fever, accompanied with cercarial dermatitis. Chronic

infection arises slowly and is accompanied by pathological changes in affected organs. Symptoms include portal hypertension, hepatosplenomegaly, inflammation and fibrosis of the liver, as well as organ damage from cell-mediated immune responses to granulomas (16,22-24). Morbidity, measured in disability-adjusted life years (DALYs) or years of life and productive life lost due to disability and/or early death, reflects the debilitating impact of chronic infection. Schistosomiasis is responsible for the loss of more than 1.7 million DALYs per year primarily as the result of sever disease resulting in organ damage and hemorrhage (25,26). However, this is probably a vast underestimation considering DALYs fail to account for the social and economic consequences associated with a more mild infection (5,9,13,14).

1.1.4 *Diagnostics*

Schistosomiasis is routinely diagnosed by the presence of eggs in the stool or urine, depending on the infecting strain. However, eggs are not consistently shed, the severity of infection (worm burden) cannot be accurately determined from egg count, and false negatives are still common. These traditional diagnostic methods are laborious, insufficient to detect low level infection or track variations in worm burden, and eggs of different helminth species endemic in the same area can sometimes be difficult to differentiate (27-30). Commercial ELISA-based detection kits are available for diagnosis of some parasites including malaria (*Plasmodium* species), cryptosporidiosis, and giardia in stool, urine or serum samples. For schistosomiasis and/or infection with *filariasis* and *trichinellosis*, antibody-based tests are available only by special request from the CDC and are not likely to benefit those in endemic areas (31,32). 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 (30,31). Carbohydrates as diagnostic antigens might be a superior alternative. Recent studies have uncovered this new set of potential diagnostic antigens, found in serum and urine, for schistosomiasis and other helminths. A dipstick test for the schistosome

excreted circulating cathodic antigen (CCA) (**Table 1.1**), whose antigenicity is due to Lewis X (Le^X) repeats, is now commercially available (33,34). This test 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 (33,35,36).

1.1.5 *Current treatment and control measures*

Curative drugs, such as prazequantial, do exist, however their effectiveness is dependent on both the life stage of the worm and the presence of the host's humoral immunity (13,37-40). Tissue-dwelling eggs can be excreted for several weeks after treatment. During the same period juvenile worms, which are not affected by the drug treatment, can mature or newly acquired infections can become productive (13,30,37). The current standard is mass drug administration (MDA) campaigns to treat populations, such as community schools, as opposed to targeting infected individuals. Nevertheless, donations of praziquantel are currently meeting only 5-10% global need for schistosomiasis (4,13), and cessation of drug treatment for even a few years can result in recurrence of high levels of schistosome infection, as if the community had never been treated (13,14,41,42). Other control measures include improved water management, monitoring larval shedding in high risk transmission regions, and focal molluscicide application. Implementation of these control measures are a major challenge, and schistosomiasis is still spreading to new regions (14,43-48). Unfortunately, blanket MDA campaigns do not stop the cycle of reinfection. Coupling MDA with sanitation improvements is a great stride for the control and eventual elimination of this disease, however significant helminth infections persist. In addition to the lack of drug availability, a major concern associated with MDA is the development of worm resistance to praziquantel. Resistant schistosomes can be generated in the lab, and reduced susceptibility to praziquantel has been reported in some human schistosomiasis-endemic areas (49-52). Only one new anti-helminthic, tribendimidine, has become available in the last thirty years (53,54). A vaccine is greatly needed due to the high disease burden, high rates of post-treatment

reinfection, and the inability of chemotherapy-based drugs to break the cycle of transmission (55-57).

Development of a vaccine has been a long and fruitless journey. Irradiated, larval schistosomes were the first platform tested, however this approach was impractical for several reasons. There were concerns with radiation dosage, repeated exposure, and vast variation of protection in animal models. Also maintaining a complex life cycle in large scale is incredibly difficult and the danger associated with manufacturing this type of vaccine meant it was not a practical approach (55,58-61). More modern vaccine development has shifted towards recombinant, protein based vaccines. In the 1990s, six *Schistosoma mansoni* proteins were chosen by the WHO to undergo independent laboratory testing. None of these protein vaccines reached the required effectiveness required to move past animal testing (62,63). 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 (64). Several other candidates, including Sm14, may enter clinical trials soon, or are progressing through the pre-clinical pipeline (57). Other studies have focused on the difficulty of identifying effective targets for helminth vaccines and inducing the proper character of immune response (65-67). In regard to schistosomes, the primary protein targets have been membrane-spanning proteins of the tegument, however these proteins may not accessible to the host immune response due to a dense glycocalyx surrounding the adult worms (67-69).

1.2 Importance of schistosome glycoconjugates

It has long been accepted that glycans and glycoconjugates of schistosomes play an essential role in the biology of the parasite, in particular with regard to host-pathogen interactions, however their specific functions remain unclear (7,70-74). Over the past few decades researchers have found that schistosomal glycans are bioactive and can induce innate and adaptive

immunological responses (75-78). Circulating antigens have also proven useful as diagnostics in human and animal hosts (33,35,79,80). A deeper understanding of these glycans and glycoconjugates, and their ability to modulate the immune system, could potentially ignite innovative new strategies for lessening the mortality and morbidity caused by these parasites.

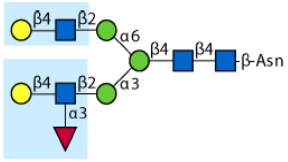
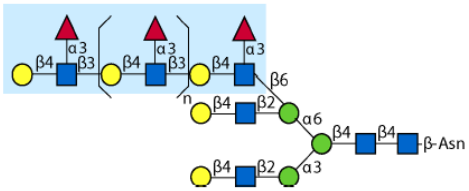
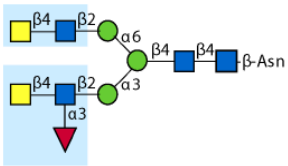
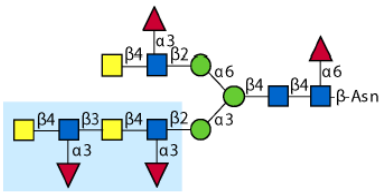
1.2.1 *Structural features of helminthic glycans*

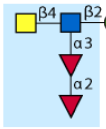
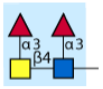
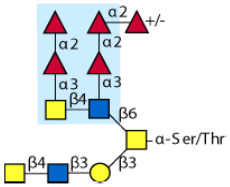
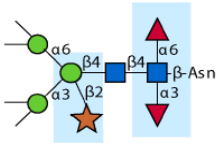
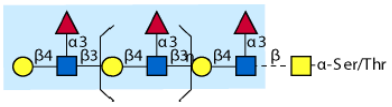
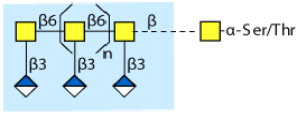
The surface of the schistosome, as well as secreted and excreted products, are rich in glycans and serve as the main site of parasite-host interactions. The schistosome surface is complex and poorly understood and the expression of surface proteins and glycans is highly variable throughout its life stages (69,81,82). Unlike nematodes, which are protected by a cuticle, schistosomes are covered a syncytial layer of cells called the tegument. The tegument is comprised of secreted lipid-rich membranocalyx and glycan-rich glycocalyx, which includes membrane, secreted glycoconjugates, and associated materials. While the glycocalyx is partially lost upon transformation of cercariae to schistosomula it remains clearly prominent in adult worms (83-86).

Within the surfaces and secretions, parasitic helminths produce of many different glycoproteins, containing complex N- and O-glycans, and glycolipids; a mojority of these glycans are unusual and structurally distinct from host glycans (some are depicted in **Table 1.1**). Helminths, such as *S. mansoni*, neither synthesize sialic acid nor acquire it from their hosts, whose glycans typically terminate in sialic acid (70). Helminth glycans commonly terminate with β-linked GalNAc (87-90), often in the sequence GalNAcβ1-4GlcNAc (termed the LacdiNAc motif, LDN), which is not commonly present in vertebrate glycans. In addition, many helminths use unusual sugars, such as tyvelose, found in N-glycans of *T. spiralis* (91-93), which may be useful in both resistance to infection (94) and diagnostics (95,96). Several helminths also generate unusual modifications of sugars, such as the phosphorylcholine modification of glycans of *E. granulosus* (97,98), the free-living *Caenorhabditis elegans* (99,100), and *A. suum* (101). Other examples include the 2-O-methylation of fucose and 4-O-methylation of galactose in highly antigenic glycans

of *T. canis* (102,103). In *S. mansoni* glycans, unique additions of fucose residues are seen on both GlcNAc and GalNAc residues in the LDN motif, giving rise to FLDN, LDNF, poly-LDNF, dF-LDN-dF (89,104-110), as well as unique fucose/xylose modifications of the N-glycan core (70,77,111) (**Table 1.1**). Some nematodes, of which *C. elegans* is best studied, also oddly modify their core fucose residues with galactose (112-114). Interestingly, only the trematode *S. mansoni* (89,115,116) and the cattle lungworm nematode *Dictyocaulus viviparous* (117) have been shown to synthesize glycans containing the terminal motif of the Lewis X (Le^X) antigen, variants of which are also expressed commonly on human cells (116,118). Schistosomes synthesize novel glucuronate-containing glycans on glycoproteins, such as the CAA structure (119). The core structures of the glycolipids in helminths are also unlike those of mammals, such as the presence of the "schisto motif" GalNAcβ1-4Glcβ-Cer (88) of *S. mansoni*, and the "arthro motif" Manβ1-4Glcβ-Cer of *A. suum* (101), instead of the mammalian "lacto motif" Galβ1-4Glcβ-Cer.

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.

(Reprinted from Frontiers in Immunology (1) with permission).

1.2.2 *Parasitic glycan gimmickry*

The unusual nature and antigenicity of parasitic helminth glycans belies the 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" (120-123). However, as previously mentioned, modern studies of schistosomes and other helminth glycoconjugates show that the glycans generated by these organisms are unique and contain features very unlike those of vertebrate hosts (1,77). 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, glycans constitute a major portion of the host's antigenic targets in several helminth infections (124-129). 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 (130,131). Interestingly, few of the antibodies to helminth glycans cross-react with host glycans. The only well-known example of this is Le^X (118). This suggests that even helminth glycans sharing some features with rare mammalian glycoconjugates, such as LDN and LDNF (**Table 1.1**), are presented in a unique fashion on parasites. These observations, as well as the evidence that parasite-derived glycans are bioactive as well as immunogenic, have led to the concept of "glycan gimmickry:, which highlights the key roles of parasite glycans in immunomodulation and evasion of host responses (132).

1.2.3 *Immune modulation*

It has long been recognized that schistosomes (and other helminths) glycans harbor potent immunomodulatory properties and have been found to induce innate and adaptive immune responses in the host (72,73,78,133,134). Understanding this process could translate to improved outcome of disease, co-infections, as well as aid in the development of anti-schistosome vaccines

(55,56,62). Parasite molecules involved in skewing towards a Th2 environment and down-regulation of the immune response are potential treatments for autoimmune or inflammatory conditions. There has already been success in treating animal models of type-1 diabetes, colitis, and multiple sclerosis with therapeutic helminthic infection (135-137). For example, the Le^X trisaccharide, a common glycan motif in schistosome eggs, is a potent inducer of the Th2 responses often via recognition by Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) (78,133,134,138-141). In fact, schistosome egg antigens can suppress TLR-induced dendritic cell (DC) activation when internalized by a combination of DC-SIGN, mannose receptor (MR), and/or macrophage galatose lectin (MGL) (142,143). Le^X can also induce proliferation of B cells, the production of suppressive cytokines IL-10 in peripheral blood mononuclear cells, and function as initiators and/or modulators of granuloma formation (138,144). The mechanisms of the mammalian immune responses to parasitic helminths, and whether it is favorable to the host or the parasite, is incredibly complex.

1.2.4 *Innate immune responses to schistosome glycans*

In some mammalian hosts the adaptive response may help to prevent, limit, or eradicate the infection, while in others it appears ineffectual (145). The ability of adaptive effector mechanisms to limit or clear infection likely depends, in large part, on cues received from the innate response. The innate response functions both to limit the pathology of the infection and directly contributes to destruction and expulsion of worms. However, the parasites have evolved glycan gimmickry approaches to battle the host responses. The balance arrived at in a chronic infection may result in asymptomatic infection even though humans rarely clear all of the infecting organisms (145,146).

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 (147,148) (**Figure 1.2**). Helminths induce effector cell generation consisting of Th2, T

regulatory cells (Tregs) and alternatively activated (AA) MΦ (149-152), which may contribute to the capacity of helminths to counteract inflammation associated with autoimmune disease. 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 (153-156). 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 (156,157). TLRs function as pattern-recognition receptors (PRRs) that can recognize a wide variety of foreign molecular patterns (pathogen-associated molecular patterns or PAMPs), as seen for example where they recognize the many variants of LPS. While CLRs can also function as PRRs, their specificity is often much more restricted, as seen for example with dectin-1, which is a receptor for β-glucan (158). The balance between CLR- and TLR-mediated signals appears crucial to determine the balance between tolerance and immunity (143,153,159,160).

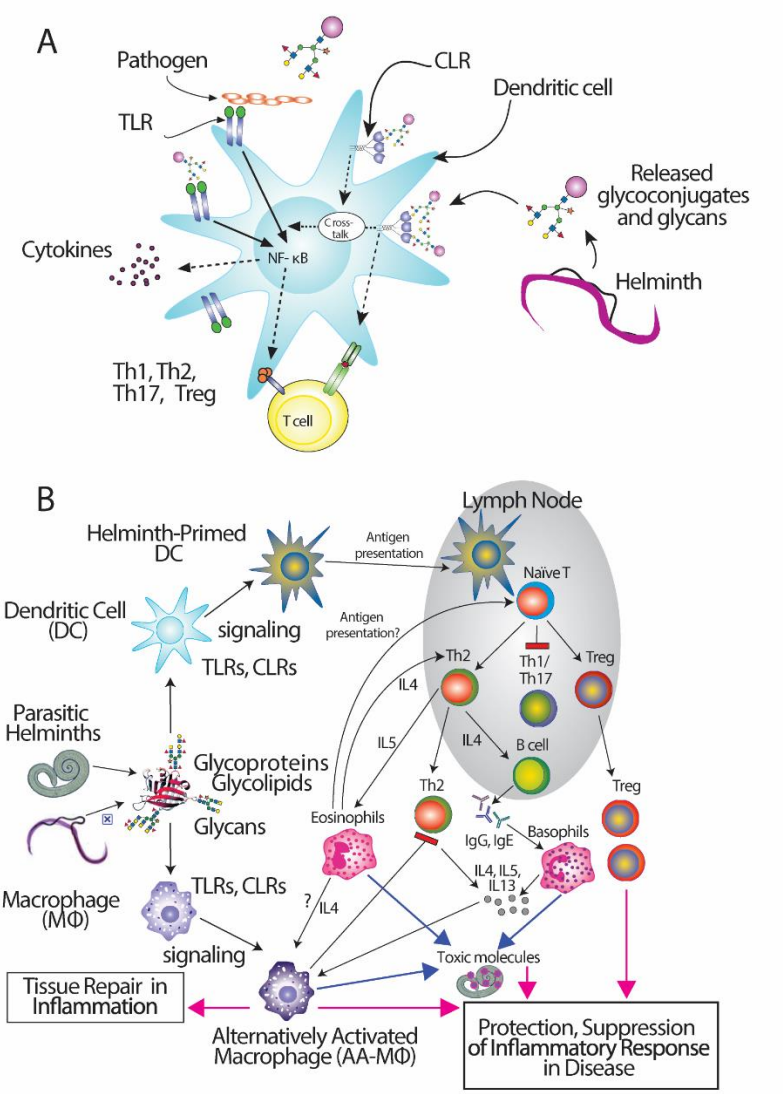


Figure 1.2. Interactions of helminth glycans with immune cells and regulatory pathways.

(A) Glycan-binding proteins such as the C-type lectin receptor (CLR) DC-SIGN in cooperation with Toll-like receptors (TLRs), such as TLR4, regulate dendritic cell responses to parasite glycans. (**B)** Schematic representation of the role of dendritic cells (DC) and macrophages (MΦ) in inducing an anti-inflammatory adaptive immune response upon contact with helminth glycans. (Reprinted from Frontiers in Immunology (1) with permission).

Little has been done on direct effects of intact worms on APCs, and the mechanistic roles of glycans in glycan gimmickry, but several studies using soluble extracts of worms or their eggs have demonstrated the importance of helminth glycans in innate immunomodulation. Early observations showed that egg deposition was responsible for the Th2 character of chronic murine schistosomiasis (161). 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 (144). 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 (134,139). These responses were completely dependent on the presence of intact helminth glycans, since partial oxidation of glycans with periodate abolished the ability of SEA to stimulate these Th2 responses (139). Both SEA and soluble worm proteins from *T. suis* (TSWAP) inhibit LPS-induced secretion of many pro-inflammatory cytokines and chemokines from DCs (139,143,162). This suppressive effect was also periodate-sensitive, while protein denaturation at 80°C, and digestion of the glycoproteins with chymotrypsin had no effect (163). In addition, co-incubation of immature DC with LPS and helminth compounds induced a decrease of CD86 surface expression (143) and a strong upregulation of OX40L expression on the DC surface which was glycan-dependent (162,163). As previously mentioned, one of the glycan determinants which contributes to the Th2-biasing effect of SEA is Le^X, (133,134,139-141), but other schistosome glycans can also induce Th2 biasing, such as core fucosylated/xylosylated N-glycans (164). The unique abilities of helminth glycolipids to drive Th2 bias may involve CD1d-restricted T cells (164). 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 (165).

In regard to the mechanisms of glycan recognition, several CLRs of DC and MΦ, such as DC-SIGN, bind selected glycans, such as Le^X, LDNF and poly-LDNF (78,142) on the defined

glycan microarray from the Consortium for Functional Glycomics (CFG). Human MΦ 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 (166). Related studies using similar microarray approaches have also defined specific interactions of DC-SIGN, MR and MGL with schistosome glycans toward glycans containing Le^X motifs, LDN, LDNF, as well as core α2-Xyl glycans (76). MGL is selectively expressed on APC with elevated levels on tolerogenic DC and AA-MΦ (167), 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 MR and MGL (143). SEA expresses many of the fucosylated glycans used in the microarray studies above. In particular, LDNF and Le^X antigens are expressed on all intra-mammalian stages of the parasite (89,168).

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 (**Figure 1.2**), such as NF-κB signaling downstream of TLRs (169). Interestingly, there is evidence that TLR4 may be involved in responses to *S. mansoni* Le^X-containing glycans (133), indicating interactions and co-signaling via TLR and CLR 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 (76,159,170,171). In contrast, *S. mansoni* fucosylated glycolipids induce a pro-inflammatory response in DCs that is dependent on both DC-SIGN and TLR4 (172). 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 (173), where uptake by MR may contribute to RNAse internalization and impaired protein synthesis through degradation of both ribosomal and messenger RNA (174).

Thus, while much remains to be learned about parasite glycans and their bioactivities, the glycans of parasitic helminths have unique functions in innate immune responses and induce both CLR signaling as well as cross-talk with TLR signaling in the human system. The molecular mechanisms of glycan-dependent innate immune responses are also linked to the adaptive immune responses, as discussed below. Understanding these responses could well lead to the development of novel therapeutic glycans that could be useful in treating human diseases associated with inflammation and autoimmunity.

1.2.5 *Adaptive immune responses to schistosome glycans*

Helminths infections present a dual challenge to immunologists: Firstly, there is an insufficient understanding of the immune effector mechanisms that successfully combat worms. Secondly, the study of adaptive immunity to eukaryotic pathogens has traditionally focused on protein, rather than glycan antigens. As previously mentioned, a substantial portion of the surface-exposed and secreted antigens of helminths consists of glycoconjugates (85,175,176). Thus, crucial insights into immunological control of helminth infection lie at the intersection of these two fields.

Due to the immunomodulatory effects of several glycoconjugates mentioned above, helminths usually elicit a Th2 response (**Figure 1.2B**). Non-endemic individuals newly exposed to *S. mansoni* can suffer from a more Th1-type acute disease known as Katayama fever, in which elevated levels of TNF-α, IL-1 and IL-6, accompany eosinophilia (177,178), but people in endemic regions rarely suffer acute symptoms. Instead, they seem to be pre-disposed to developing a chronic, Th2-type response, the onset of which coincides with egg-laying (178). This may stem from sensitization *in utero* or very early in life (179). The immune response to chronic helminth 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 (67,145). Abundant antibodies of all

subtypes are produced, especially IgE, IgG1 and IgG4 (146,180,181). In schistosomiasis, pathology is primarily due to eosinophilic (type 2) granulomas, consisting of macrophages, CD4+ T cells, eosinophils and collagen that surround eggs trapped in liver, intestinal or bladder tissue, which are eventually converted to fibrotic scars (178,182).

The regulatory response is crucial in control of chronic helminth disease, for the well-being of both host and parasite (**Figure 1.2B**). Schistosomes, hookworms, and filarial nematodes all 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 (145,183,184). 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 (146). AA-MΦ also aid in limiting worm-induced immunopathology. They are induced by Th2 cytokines like IL-4 and IL-13 as well as directly by products of several helminths, including *S. mansoni*, *F. hepatica*, filarial nematodes and tapeworms (151,176,185-187).

1.2.5.1 Correlates of protection from helminth infection

Although the association of Th2-type immunity with helminths has been recognized for decades, we are still unraveling the effector mechanisms through which Th2 components control worm infections. Animal infections with gastrointestinal nematodes provide a model of an effective Th2-mediated response. Immunity to intestinal nematodes depends on Th2 cytokines (IL-4, IL-5, IL-9 and IL-13) and is antagonized by Th1 cytokines. Mast cells and basophils are critical for expulsion of GI worms in some animal models, but are not always necessary (188,189). Th2 cytokines have important protective effects directly on epithelial cells, including goblet cell

hyperplasia, increased smooth muscle contractility, and secretion of molecules that directly target worms (181,189,190).

Animal models of helminth infection have demonstrated that some immunological effector mechanisms are successful in combatting helminth infection. In the brown rat, which eliminates *S. mansoni* before patency, complement fixation, IgG2a and IgE levels, mast cell degranulation and eosinophil-mediated antibody-dependent cellular cytotoxicity (ADCC) have been cited in protection (191-196). In rhesus macaques, another protective model for schistosomiasis where adult worms become attenuated in the weeks after reaching patency, IgG-mediated complement killing of schistosomules and neutralization of adult worm enzymes have been demonstrated (197-199). 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* (200-202).

In human schistosomiasis cohorts, some adults acquire fewer infections and have lower worm burdens compared to children and more susceptible adults (203). Eosinophilia, high IgE levels to heterogeneous antigens, as well as more specific antigens (Sm22), and high IgE/IgG4 ratios are the most well-established correlates of human resistance to schistosomiasis. IgG2, IgG4, and IgM which are, in some cases, against the same molecular species, are negatively correlated (204-208). IgA to the tegumental protein Sm28GST was also correlated with resistance in one study of human subjects (209). Human eosinophils can kill schistosomula *in vitro* via IgG from infection antisera (210,211), 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 (212). IgE also mediates mast cell degranulation, but mastocytosis was found to correlate with susceptibility to reinfection in one occupationally-exposed human cohort (213). 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* (204,214-216). The factors

that 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 the recent observation that $CD23^+$ B cells are associated with resistance in a Kenyan cohort (217). 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 activate cognate parasite-specific B cells. The increasing amount of parasite-specific IgE could thus steadily increase the magnitude of the antibody response over the course of several infections, outweighing the immunosuppressive effects of some worm products (218). 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* cercaria 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 trapping schistosomula as they migrate through the lung (219-222). The protection of these mice is dependent on both antibodies and T cells (223-225). The role of Th1 responses in humans is still unclear. In some populations endemic for schistosomiasis and lymphatic filariasis, a mixed Th1/Th2 profile is associated with an effective immune response, whereas in hookworm infection, only Th2 appears to be correlated with resistance (181,226). In some human populations, resistance to schistosome infection is correlated with increased production of IFN-γ by CD4+ T cells stimulated with recombinant Sm14 protein and other antigens (227,228). Polymorphisms in the IL-4 and IFN-γ genes have also been associated with resistance levels (229). Thus, while many possible *in vitro* and *in vivo* mechanisms against helminths have been described, it is yet unclear

which, if any, of these is implemented by a successful human immune response, and which would be desirable in an anti-helminthic vaccine.

1.2.5.2 Glycans and adaptive immunity

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 are involved in the establishment of such niches, and antibodies to glycans can interfere with this process. In schistosomiasis, 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 (230). 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.

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 schistosomules *in vitro* via eosinophil-mediated ADCC and passively transferred resistance to naïve rats (195). 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 (231,232). Our present knowledge of KLH and schistosome cross-reactive glycans supports the hypothesis that IPLSm1 targeted the FLDNF glycan (109,110). 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 (233). Mice also develop abundant antibodies to glycans, including IgE, IgG1 and IgG3 (but not IgG2) to LDNF, indicative of a skewing toward Th2-type antibody effector mechanisms such as ADCC (234). A murine IgM to

LDN isolated by our group mediates complement killing of schistosomula *in vitro* (168). 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 (116,235).

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 (236,237). 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 (118).

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 multibranched glycans, as diagrammed in **Table 1.1**. The structural presentation of such epitopes as Le^X and LDNF can vary among schistosome life stages, localization and sexes (107,108,238). 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 (115,238). Given that some of these structural variants are somewhat similar to mammalian glycans, this high level of specificity could be crucial to developing an effective 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 (115).

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 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 (208). Levels of IgE to worm glycolipids pre-praziquantel treatment were inversely correlated with egg burden two years after treatment in another population (239). Another investigator found that children had modestly higher titers of IgM and IgG than adults to most glycans (240). 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 glycan preparations that are both pure and accurately mimic the mode of presentation by the parasite, and the differential significance of antibody isotypes and sub-isotypes in human resistance. Further studies are needed to strengthen these correlations and more directly examine the role of αGAbs in protection from schistosomiasis and other human helminth infections.

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-linked glycan on venom allergen Anacylostoma-secreted protein-like antigens (241). Following isolation of a protective IgG2a against *S. mansoni* 38-kDa antigen mentioned above, a second antibody (an IgG2c) that 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 (214). 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 (242). Clearly such counteractive effects of antibodies to glycan antigens should be explored in more detail.

Anti-GAbs are also being considered for diagnostic purposes. Certain defined glycans including LDN, Le^X, FLDN and LDN-dF have different antibody profiles (168,208,243,244). Other

highly fucosylated epitopes, such as FLDNF and dF-LDN-dF are possible diagnostic epitopes due to their unique expression on schistosomes. The monoclonal antibody 114-4D12, which targets dF-LDN-dF, can detect unconjugated oligosaccharides excreted from *S. mansoni* eggs in infection urine. MS/glycan based studies may lead to a new egg-load-related assay helpful in the detection of mild infections (31,245). However, given the differential responses to discrete glycans it is unclear whether the previously mentioned immunodiagnostic tools could differentiate between current and past infection. These 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.

1.2.6 *Glycans interactions with intermediate hosts*

Many of the schistosome glycans have been found to be potent antigens in vertebrate hosts, but their roles in snail infections are poorly understood (7,246,247). Emerging evidence suggests that schistosome glycoconjugates play a pivotal role in both cellular and humoral immune interactions between their molluscan intermediate hosts and the infecting larval stages (247-249).

There appears to be a role for fucosylated carbohydrate epitopes expressed by larval and adult schistosomes in parasite evasion in intermediate and definitive hosts (246,250,251). 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 (252).

It is well known that *S. mansoni* glycan expression is developmentally and stage-specifically regulated, but until recently, the glycan epitopes expressed in miracidia and sporocysts

were largely unknown. 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 (17). 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 (17,246,252). Alpha-1,3-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 (246).

Larval glycans and/or their associated glycoconjugates might also be serving as PAMPs that interact with lectin-like PRRs (253). PRRs, such as toll receptor, C-type lectins, galectins, nucleic acid-sensing receptors, and the intracellular nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) occur both extracellularly and intracellularly, with the galectins being most notable in being both in the cytoplasm and in extracellularly (254,255). In terms of glycoconjugates in helminth infections and their interactions with all types of PRRs, little is known, and the most well studied interactions involve C-type lectins and galectins. There is also evidence that glycans may be important in intermediate hosts innate immunity and PRR recognition and may involve novel PRRs. During their development in the molluscan intermediate host, *S. mansoni* sporocysts release excretory/secretory glycoproteins that bind to lectin PRRs on the surface of the snail host hemocytes and are believed to modulate the ability of the hemocytes to interact with the developing larvae (249). The binding of glycoconjugates to *B. glabrata* hemocyte lectins can trigger the generation of parasite-killing reactive oxygen species, thereby mediating innate immune responses to invading miracidia (246,247,250,251,253,256).

Glycans may also be the targets of humoral immune responses mounted by the molluscan hosts against larval infection. *B. glabrata* snails respond to infection by secreting humoral factors into their hemolymph that bind and precipitate larval excretory/secretory antigens. These factors contain N- and C-terminal domains with similarities to immunoglobulin super-family proteins and fibrinogen, respectively, and are called fibrinogen-related proteins (FREPs) (247,253,257). To counteract FREPs, developing primary sporocysts envelope themselves in a glycan-rich environment comprised mainly of glycoproteins and other glycoconjugates referred to as larval transformation products (LTPs) (253,258). LTP glycoconjugates released during transformation are able to alter patterns of shared glycan epitopes by either binding and blocking, or by exposing them. This is a possible mechanism by which molecules released from early developing larvae may impact initial immune interactions at the host-parasite interface and shows the potent immune modulating effects of LTPs (253,259).

1.3 Glycotechnologies and approaches

Our knowledge of immunorelevant parasitic glycans is limited by our knowledge of the parasite glycan structures themselves. Unlike the sequence of a protein, in which homologous protein sequences between species imply homologous functions, glycan sequences are more complex and seemingly slight changes in structures can profoundly affect biological activities in unpredictable ways. In spite of this complexity, advances in technology for studying glycan structures and defining the specificities of glycan-binding proteins have recently been made.

1.3.1 *Glycan synthesis and glycoconjugate production*

Oligosaccharide enzymatic synthesis is extremely useful and highly specific, however with important limitations. Assuming the enzymes require to generate the glycans structure are known, is difficult and expensive to obtain enough enzyme to produce enough product for functional studies (260). Additionally there are many glycosidic linkages in parasites that appear to crucial

components of several schistosome antigens and do not yet have a designated glycosyltransferase. For some glycan structures 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 (261). Also, recent studies from our lab have shown that the chemical linkers added to reducing end of sugars during the generation of neoglycoconjugates can affect the immune response to those glycans in a species-specific way (262). This is an important caveat to remember utilizing these technologies for a vaccine platform.

1.3.2 *Glycan microarrays*

Our group, in collaboration with the Consortium for Functional Glycomics (CFG), has spearheaded one of the most recent and innovative new glycoltechnologies: the glycan microarray (263). A glycan microarray consists of a library of derivatized glycans or glycopeptides linked to a slide (**Figure 1.3**). This can be a covalent linkage or captured on nitrocellulose-coated slides depending of the type of glycan library. The array can be interrogated with very small samples of glycan binding proteins (GPBs), such as lectins, antibodies or antisera, or even micro-organisms such as viruses and yeast. Samples are incubated on the slides, the unbound washed away, and bound sample detected using fluorescent secondary reagents (**Figure 1.3**). Scanning and quantitation of fluorescent signal for all bound GPBs is reflective of binding affinity within the sample. Glycan microarrays have been validated against traditional methods (Western blot, ELISA) as sensitive and specific serodiagnostics for human infections of *Trichinellosis* and *Salmonellosis*. Additional benefits including use of a very small sample volume, high sensitivity with low background, and detection of binding to distinct epitopes simultaneously (263,264).

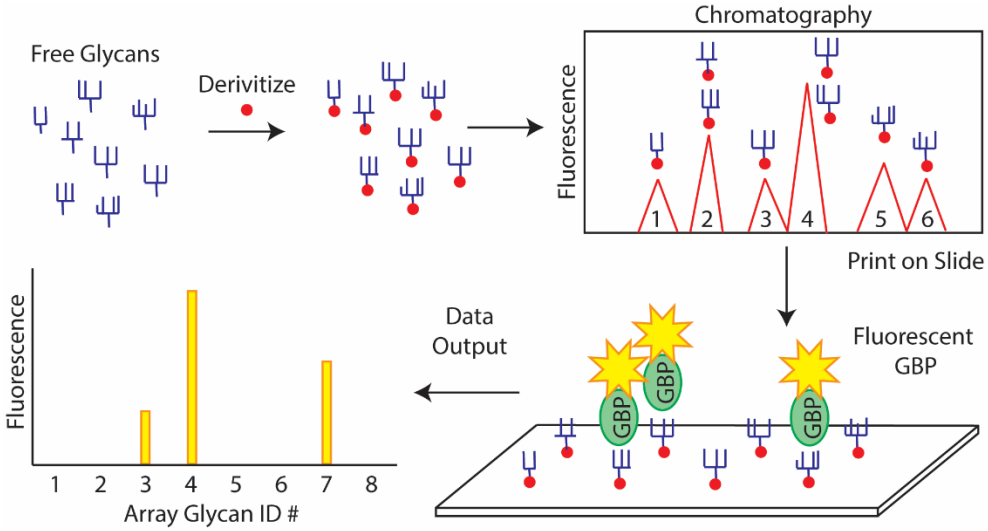


Figure 1.3 Generation of a glycan microarray and data output.

Free reducing end glycans are synthesized or isolated from a natural source (tissue). The glycan library is chemically derivatized with a fluorescent ta or linker which allows for purification for pure samples or separation of multi-glycan mixtures via multidemential HPLC. Each glycan fraction is printed in equal amounts on a NHS-activated glass slide. Arrays are interrogated with fluorescently labeled GBPs, scanned, and analyzed for binding.

Our group use two microarray approaches to study immunoglycomics. One is termed the "shotgun" approach, where glycans are extracted from natural sources such as tissue sample in interest, derivatized with a fluorescent linker, and partially purified before printing (265-268). Using this method, the glycan structures within the library 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) (269,270). More details regarding the application of this technology for schistosomes can be found in Chapter 3.

A second approach in the study of immunorelevant glycan antigens in schistosomes was the development of the defined Schistosomal-type array (DSA). As antigenic glycans are identified

in the literature, we synthesize them via enzymatic or chemical methods and print them on an array. 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, poly-LDN and poly-LDNF, chitin oligosaccharides and several control structures. This tool was very helpful in determining the differential expression of anti-glycan antibodies in schistosome-infected or vaccinated species (271,272), however the DSA is limited by the glycans we can currently generate. It is pivotal that we broaden the range of structures that can be synthesized for this microarray tool.

1.3.3 *Limitations in glycan research and historical approaches*

A major limitation in the study of parasitic glycans is that we are currently unable to chemically synthesize many of the structures in an affordable and facile manner. It is also not feasible to isolate significant quantities of individual glycans from the parasites at each developmental stage. In the past several years, the availability of genomic databases has allowed us and others to take an alternative approach using enzyme technology in a chemo-enzymatic approach to generate glycans and explore their recognition by antibodies and glycan-binding proteins (271-275).

The identification and sequencing of schistosome glycans began in the 1980's with the identification of unusual N- and O-glycans synthesized by short-term cultures of schistosomula and adult worms (87-89,104,276,277). Subsequent studies (105,106,119,278,279) (also see reviews by (1,7,70,72-74)) identified complex types of glycan structures in both membrane associated and circulating antigens. These types of studies, now generally recognized as structural glycomics, involve complex analyses incorporating tandem mass spectrometry (MS), nuclear magnetic resonance (NMR) and compositional and linkage analyses. Unfortunately, while the field has advanced tremendously in identifying many types of glycans synthesized by schistosomes and even glycan structures differences between sexes and schistosome species, it is likely that only a tiny

fraction of the total set of glycans synthesized by any stage of the parasite is known (105,107,234,280,281). Thus, much remains to be learned about the specific sequences and complete structures of schistosome glycans as well as their temporal and spatial expression. One obvious limitation to these studies is that schistosomes are parasites and must be isolated from infected animals, thus limiting their availability as well as creating potential problems in contamination by glycans from the hosts. While structural studies remain important for confirming hypothesized structures and characterizing glycan-protein interactions, a genome approach provides many advantages

1.3.4 *A genomic approach*

While knowledge of schistosome glycans is woefully incomplete, the available evidence indicates that many different glycan linkages and sequences occur. In both simpler organisms, such as *C. elegans*, as well as exceedingly more complex organisms, such as mice and humans, many genes within the genome have been shown to encode enzymes responsible for elaboration of the glycome. These genes, typically referred to as comprising the glycogenome, encode glycosyltransferases, glycosidases, sugar and nucleotide sugar metabolizing enzymes important in glycan biosynthesis, nucleotide sugar transporters, and glycan-binding proteins. It is estimated that mice and humans have over 900 genes involved in elaboration and recognition of their glycomes (282). This background knowledge has set the stage for now exploring the glycogenomes of schistosomes and other parasites and identifying the genes important for elaboration of their glycomes.

In 2009 the nuclear genome of *S. mansoni* was published in *Nature* as a result of a successful international collaboration among multiple institutions (283). The analysis of the 363 megabase genome utilized several gene prediction algorithms, including the extended similarity group (ESG) method, which performs iterative sequence database searches and annotates a query sequence with Gene Ontology terms. At least 11,809 genes were annotated encoding over 13,000

transcripts with unusual intron sizes, distributions, and frequent alternative splicing. The annotated genome sequence was submitted to EMBL (accession numbers FN357292-FN376313) and GeneBD (http://www.genedb.org/Smansoni) (283-285). Shortly after the genome was published, SchistoDB (http://schistoDB.net/) was created to offer researchers a plethora of tools for genomic data mining. SchistoDB incorporates sequences and annotations for *S. mansoni* in a single directory. Several genomic scale analyses are available as well as expressed sequence tags, oligonucleotides, and metabolic pathways. By 2012, the directory was expanded by integrating the data sets from other *Schistosome* species, *S. japonicum* and *S. haematobium* (286-288). Current studies have utilized the genomic data to highlighted transcriptional differences seen throughout lifecycle progression and identify anti-schistosomal candidate molecules including fucosyltransferases via transcriptome analyses and gene microarrays (289,290).

The KEGG (Kyoto Encyclopedia of Genes and Genomes) database uses large-scale molecular datasets generated by genome sequencing and other high-throughput experimental technologies to help scientists understand high-level functions and utilities of various biological systems. With the information generated from the *Schistosoma* genome sequences, KEGG Glycan constructed pathway maps on molecular interactions including glycan biosynthesis and metabolism that are annotated with the specific enzymes/proteins involved and the corresponding genes (http://www.genome.jp/kegg/glycan). The system also characterizes gene/protein functions across organisms, allowing for genes like glycosyltransferases to be finely classified within ortholog groups which may have been overlooked by previous sequence similarity algorithms (291-295).

With the amount of information now available, genomics technologies can be applied to unravel the biology of some of these parasites, including the complexity of glycan biosynthesis (**Figure 1.4, Table 1.2**). Given the vast assortment of glycan epitopes, as well as available databases, it can be predicted that schistosomes express a plethora of glycosyltransferases and other genes required for glycan biosynthesis. A more thorough understanding of the schistosome

glycome could promise faster identification of targets for diagnostics and drug development, as well as a collaborative approach to antigen chemo-enzymatic synthesis and discovery for glycan-based vaccine platform.

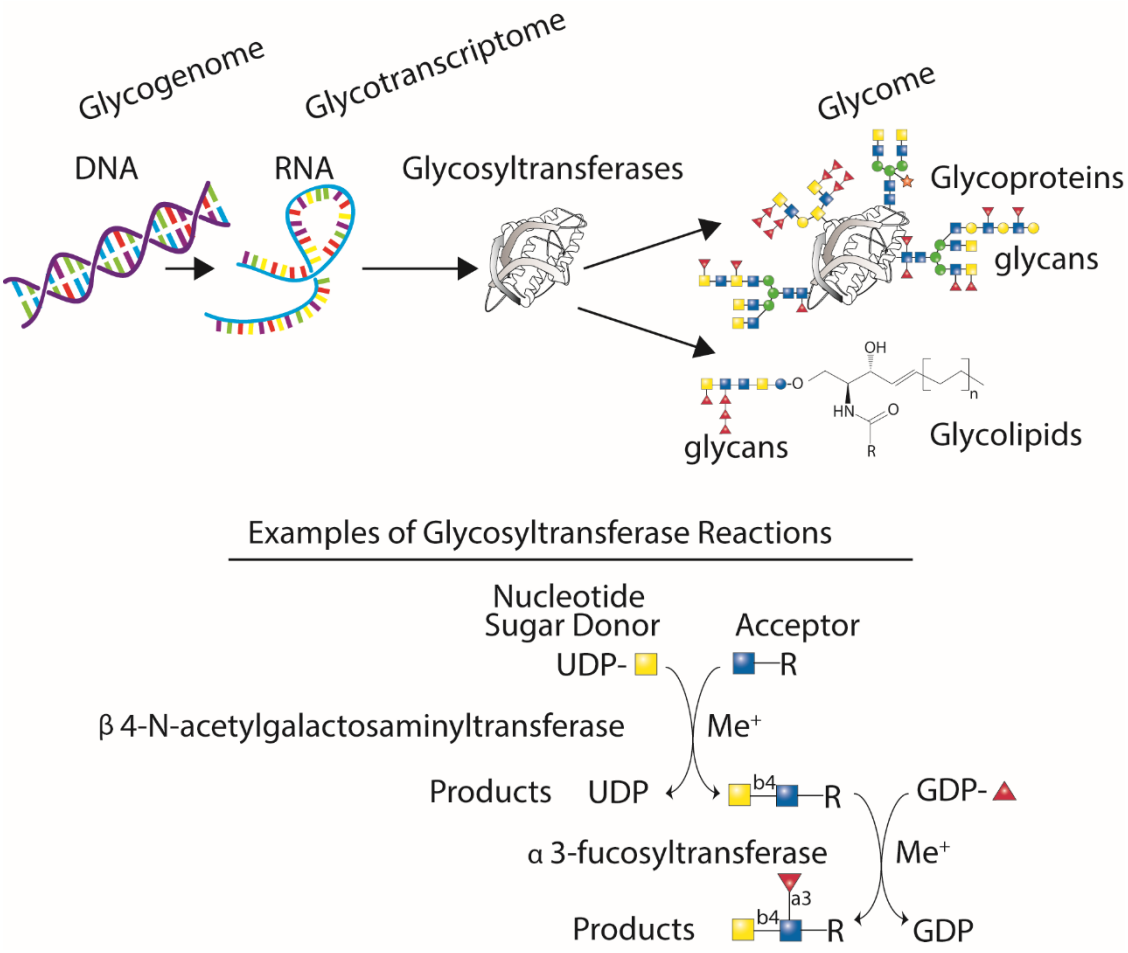


Figure 1.4. Deciphering the glycogenome and enzymatic reactions of glycoslytranferases.

The glycogenome represents the genes encoding the various glycosyltransferases, glycosidases, sugar and nucleotide sugar metabolizing enzymes important in glycan biosynthesis, and nucleotide sugar transporters. The glycosyltransferases generated from the glycotranscriptome in schistosomes represent a large class of predicted enzymes, often requiring metal cofactors (Me^+) that synthesize glycans using donor nucleotide sugars to form glycosidic bonds to acceptors, here represented by a sugar-R, where R=sugar, protein or lipid to which sugars are linked. The products of the biosynthetic reactions have specific glycosidic linkages, e. g. β1,4 or α1,3, and the glycans produced are often acceptors for additional enzymes, thus generating the complex set of glycans representing the glycome of the organism. (Reprinted from Frontiers in Genetics (2) with permission).

1.4 Schistosome glycan biosynthesis pathways

Previous structural studies of schistosome glycoconjugates primarily depend on analytical techniques like mass spectrometry, but are limited due to insufficient quantities of glycans and the need to prepare glycans from for parasites isolated from infected hosts, as well as variation in glycan expression among the life stages, resulting in incomplete glycome profiling (74,107,108,259,296-298). Nevertheless, using the available glycan sequence data and developmentally-regulated expression of glycan antigens, it is predicted that schistosomes contain a multitude of different classes of glycosyltransferases involved in glycan biosynthesis and that their expression is differentially regulated by tissue and life stage (299-301). To date, very few of these enzymes in distinct glycan classes have been studied in detail, however, with the genomic data now available glyco-related genes might be easier to explore in the future (**Figure 1.4, Table 1.2**).

Category	# of Putative Genes	Gene ID		
Glycosyltransferases:				
- Galactosyltransferases and N-acetylglucosaminyltransferases (GalTs and GnTs)[1]	14[2]	Smp 058670 Smp 006930 Smp 210290[3] Smp 146430 Smp 042720	Smp 056260 Smp 024650 Smp 015920 Smp 153110 Smp 149820[3]	Smp 102400 Smp 007950 Smp 151210 Smp 151220
- N-acetylgalactosamine transferases (GalNAcTs)	7[2]	Smp 057620[3] Smp 139230[3] Smp 047240	Smp 159490[3] Smp 211240	Smp 005500[3] Smp 021370
- Fucosyltransferases (FuTs)	22[4]	Smp 175120 Smp 194990 Smp 199790 Smp 030650 Smp 138730 Smp 193870 Smp 054300	Smp 175120 Smp 205640 Smp 154410 Smp 138750 Smp 211180 Smp 193620 Smp 209060	Smp 137740 Smp 028910 Smp 065240 Smp 212520 Smp 137730 Smp 142860 Smp 129750
- Xylosyltransferase	2	Smp 128310	Smp 125150	
Biosynthesis Pathways:				
- N-Glycan	18[5,6]	Smp 051360 Smp 055010 Smp 161590 Smp 035470 Smp 210360 Smp 018760	Smp 045430 Smp 177080 Smp 103930 Smp 055200 Smp 210370 Smp 018750	Smp 082710 Smp 052330 Smp 020770 Smp 105680 Smp 024580 Smp 143430
- O-Glycan	5[5]	Smp 149820[3] Smp 005500[3]	Smp 057620[3] Smp 139230[3]	Smp 015949[3]
- Glycolipid	2[5]	Smp 160210	Smp 157080	
- GPI-anchor	14[5]	Smp 154600 Smp 155890 Smp 046880 Smp 035080 Smp 053460	Smp 136690 Smp 155900 Smp 163640 Smp 128810 Smp 021980	Smp 145290 Smp 017730 Smp 152460 Smp 177040
- GAG	6[5]	Smp 178490 Smp 075450	Smp 083130 Smp 134250	Smp 124020 Smp 210290[3]

[1] Grouped in database, see text for details.
[2] Tally in text references a subset of genes (Ex: 3 β1-4GalNAcTs, 7 total GalNAcTs)
[3] Listed in both glycosyltransferaseas and pathways.
[4] Genes have redundancies, see text reference for details.
[5] Denotes current gene annotations discussed in the text. Not an exhaustive list.
[6] Tally does not account for splice variants.

Table 1.2. Components of the *S. mansoni* glycogenome.

Synthesis pathways and corresponding GeneDB entries for *S. mansoni* genes as of 2014. (Reprinted from Frontiers in Genetics (2) with permission).

1.4.1 *N-glycans*

The N-glycans found in *Schistosoma* glycoproteins feature high mannose and complex-type structures common in eukaryotes and higher organisms (87,277). Thus, it appears that schistosomes follow the conventional pathway for N-glycan core synthesis, where the precursors are synthesized on the cytoplasmic face of the ER membrane beginning with dolichol phosphate (Dol-P) in a step-wise process catalyzed by *ALG* gene enzymes (for *al*tered in *g*lycosylation). Fourteen sugars are sequentially added before en bloc transfer of the entire structure to an Asn-X-Ser/Thr site in a protein. The protein-bound N-glycan is subsequently remodeled in the ER and Golgi by a complex series of reactions catalyzed by membrane-bound glycosidases and glycosyltransferases (302,303).

The genome of *S. mansoni* appears to contain homologs to the ALG genes required for synthesis and remodeling (**Table 1.2**). The splice variant Smp 051360.1 most likely functions as a UDP-N-acetylglucosamine (GlcNAc) dolichylphosphotransferase which forms GlcNAc-P-P-Dol. A second GlcNAc and five mannose (Man) residues are subsequently added by specific glycosyltransferases to generate $Man_5GlcNAc_2$-P-P-Dol on the cytoplasmic side of the ER. Homologs in this pathway include Smp 045430.3 and Smp 082710 as UDP-N acetylglucosaminyltransferase (GlcNAcT) subunits (similar to ALG 14), Smp 055010 as a chitobiosyldiphosphodolichol α-mannosyltransferase, Smp 177080 as an α-1,3-mannosyltransferase (ALG 2), and Smp 52330 probably functions like asparagine-linked glycosylation protein 11 (ALG 11). Other genes responsible in forming the common 14-sugar lipid-linked precursor in animals, $Glc_3Man_9GlcNAc_2$-P-P-Dolichol, are Smp 161590 (simply designated a glycosyltransferase but contains regions similar with an α-1,6-mannosyltransferase), splice variants of Smp 103930 (α1,2-mannosyltransferase, and Smp 096910/Smp 15120 (α1,3-glucosyltransferases) (283,302,303).

The transfer of the 14-sugar glycan in $Glc_3Man_9GlcNAc_2$-P-P-Dolichol to Asn-X-Ser/Thr sequons of a newly synthesized protein is catalyzed by a set of proteins termed the oligosaccharyltransferase (OST) complex. *S. mansoni* genes likely to function as OST subunits are Smp 020770 (α unit), Smp 035470 (β unit), Smp 055200 (γ unit), Smp 105680 (ribophorin I), and Smp 210360/210370 (δ unit) (Chavan et al., 2005; Stanley et al., 2009; Berriman et al., 2009). After covalent attachment of the 14-sugar glycan ($Glc_3Man_9GlcNAc_2$-Asn) a series of processing reactions trim the glycan using α-glucosidases. Smp 024580 and Smp 018760 most likely remove the three Glc residues leaving the high mannose $Man_9GlcNAc_2$-Asn structure. Smp 018750 (α-1,3-mannosidase) and Smp 143430 (α-mannosidase II) remove mannose allowing for the N-glycans to be recognized and further extended/modified by glycosyltransferases, as discussed below, which generate the hybrid or complex-type N-glycans with terminal glycan motifs (87,108,277,283,303).

1.4.2 *O-glycans*

O-glycosylation in schistosomes range from a single sugar residue to large, complex, multi-fucosylated structures fluctuating from 12 to at least 60 glycans in length in the cercarial glycocalyx (106,276,277). Many surface localized schistosome glycoproteins contain a simple O-linked GlcNAc, which probably occurs on intracellular and intranuclear glycoproteins (276,304). Other common structures include Galβ1-3(Galβ1-6)GalNAc (O-glycan shisto core) and mucin-type sequences including GalNAcα1-Ser/Thr (Tn antigen), Galβ1-3GalNAcα1-Ser/Thr (T antigen, core 1), and Galβ1-3(GalNAcβ1-6)GalNAc (core 2) with the core 1 structure being the most common (111,119,277,278). The more complex O-glycans contain unique repeating elements containing GalNAcβ1-4GlcNAcβ1-3Galα1-3 units carrying fucosylated sequences linked to the internal GlcNAc and terminal GalNAc structures (276,305).

In vertebrates, the core 1 O-glycan disaccharide is also the most common of such O-glycan cores and is a precursor to more complex O-glycans such as extended core 1 and core 2 structures. The core 1 structure is synthesized from GalNAcα1-Ser/Thr by the addition of galactose, a reaction

catalyzed by the enzyme core 1 UDP-Gal:GalNAcα1-Ser/Thr β1,3-galactosyltransferase (core 1 β3-Gal-T or T-synthase) (306-308). In *S. mansoni*, Smp 149820 is the only gene designated a glycoprotein-N-acetylgalactosamine β3galactosyltransferase and is considered the ortholog to T-synthase (306), whereas *S. japonicum* has four genes annotated at core 1 β3-Gal-transferase (Sjp 005210, Sjp 0042730, Sjp 0055580, Sjp 0064840, Sjp 0093870) (283,286).

Several UDP-N-Acetylgalactosamine:polypeptide N-acetylgalactosaminyltransferases (GalNAc-transferases, ppGalNAcTs), which generate GalNAcα1-Ser/Thr have been identified and characterized in humans. While the human ppGalNAcTs show similarities in domain structures, sequence motifs, and conserved cysteine residues the overall amino acid sequence similarity of less than 50% suggests changes within this enzyme family during evolution (308). The *S. mansoni* ppGalNAcTs (Smp 005500, Smp 057620, Smp 139230, and Smp 159490) have similar amino acids similarity (approximately 30-50%) among them (283).

1.4.3 *Glycolipids*

Schistosome glycolipids consist of galactosylcermide, glucosylceramides, and glycolipids with extended glycans emanating from the 'schisto core' (GalNAcβ1-4Glc-ceramide). This is in contrast to the human glycolipid core which is lactosylceramide Galβ1-4Glc-ceramide. Schistosomes synthesize glycosphingolipids with a similar method to vertebrates using a glucocerebroside precursor, but instead of using adding the galactose, as in animals, schistosomes instead generates the `schisto-core' structure by the addition of a β1-4GalNAc residue (88,309). The simple schisto-core structure is extensively modified in egg glycosphingolipids of *S. mansoni* and *S. japonicum* with repeating GlcNAc motifs with multiple fucosylation units ((Fucα1-2Fucα1-3)GlcNAcβ1-R) (70,279). *S. mansoni* glycolipids are dominated by fucose. Cercariae often express terminal Le^X and pseudo Lewis Y (Fucα1-3Galβ1-4(Fucα1-3)GlcNAc; pseudoLe^y) structures, while the Fucα1-3GalNAc terminal element was confirmed in *S. mansoni* egg glycolipids (309,310).

Sequencing of the *S. mansoni* genome indicated that schistosomes contain a full complement of genes required for most lipid metabolic processes. In reference to ceramide as major precursor to glycosphingolipids, *S. mansoni* encodes two putative ceramide glucosyltransferase (Smp 160210 and Smp 157080) while *S. japonicum* genome contains four (Sjp 0094210, Sjp 0065630, Sjp 0054080, Sjp 0093880) (283,286). Although not a 'classical' sugar, the genome sequencing of *S. mansoni* also revealed a lipid deficiency where the worms must depend on its host as a source of inositol (283,311).

1.4.4 *GPI-anchored glycoproteins*

It is well known that *S. mansoni* and other schistosome species, produce glycoproteins anchored to membranes through a glycosylphosphatidylinositol lipid anchor (GPI anchor) and thus lacking a transmembrane protein domain. Such GPI anchored glycoproteins have now been found in all animal cells, and were in the parasite world were first extensively studied in trypanosomes (312). Examples of common GPI-anchored proteins previously characterized in schistosomes include alkaline phosphatases and acetylcholinesterase (313-315). Both of *S. mansoni* and *S. japonicum* genomes contain annotations for acetylcholinesterase (Smp 154600, Smp 136690, Sip 0070510, Sjp 0045440, and Sjp 0036280), however only *S. mansoni* appears to have genes currently designated as alkaline phosphatases (Smp 145290, Smp 155890, and Smp 155900) (283,286). *S. mansoni* also expressed and 200 kDa GPI-anchored glycoprotein on its surface which is a target for antibodies which can act synergistically with praziquantal treatment (314,316). According to the database this protein is a product of the gene Smp 017730, however that record has not yet been subjected to final NCBI review (283).

Previously, details about the GPI-anchor biosynthesis pathway in schistosomes were unknown, however several putative proteins from the *S. mansoni* genome are believed to be involved. Phosphatidylinositol N-acetylglucosaminyltransferase catalyzes the first step of glycosylphosphatidylinositol (GPI) anchor formation in all eukaryotes. In mammalian cells, this

enzyme is composed of at least five subunits (PIG-A, PIG-H, PIG-C, GPI1 and PIG-P), with PIG-A functioning as the catalytic subunit (313,317). A splice variant of Smp 046880 (termed Smp 046880.1) has around 50% identity with PIG-A isoforms in a variety of mammals. Smp 163640 and Smp 152460 also show homology with subunits PIG-P and GPI1 respectively. N-acetylglucosaminylphosphatidylinositol deacetylase (PIG-L), the enzyme responsible for the second step in GPI-anchor formation, and PIG-M, which transfers the first mannose to glycosylphosphatidylinositol on the lumenal side of the ER also show homology with the products from genes Smp 035080 and Smp 128810 (283,318,319). Other genes possibly involved in building the common GPI ethanolamine-glycan core include Smp 177040, Smp 053460, and Smp 021980. There is a probability that schistosomes also encode enzymes which allow for heterogeneity within the common core of GPI-anchors, like what is observed in mammals (283,320-322).

1.4.5 *Glycosaminoglycans and proteoglycans*

Little is known about the glycosaminoglycan (GAG) or proteoglycan 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 (323,324). 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 (323).

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 (XYLT1 and XYLT2 in mammals; XYLT or *sqv8* in *C. elegans*). These genes encode enzymes which catalyze the first step in addition of the HS/CS core to proteoglycans, and share the conserved domains Xylosyltransferase C terminal and Core-2/I-

branching enzyme. Other enzymes necessary for construction of the HS/CS core that have been characterized in *C. elegans* include *sqv3* (Gal-transferase I in mammals, encoded by β4GalT7), *sqv8* (GlcA transferase I) and *sqv7* (a UDP-GlcA/GalNAc transporter) (325). The three *Schistosoma* genomes possess genes homologous to each of these, containing the relevant conserved domains (β4GALT7: Smp 210290, Sjp 0062870, Sha 200402; UDP-GlcA/GalNAc transporter: Smp 178490; Sjp 0089300, Sha 103448; GlcA transferase I: Smp 083130, Sjp 0062810, Sha 108192). The enzymes that catalyze polymerization of HS chains in vertebrates are exotosins (EXTs), at least three of which are annotated for *S. mansoni* (Smp 172060, Smp 146320 – two splice variants, Smp 073220). Putative HS 2-O- and 6-O-sulfotransferases and a HS N-deacetylase/N-sulfotransferase are also annotated (Smp 124020, Smp 075450, Smp 134250; Sjp 0060410, Sjp 0082020, Sjp 0094660) (283,286). Interestingly, no homolog of 3-O-sulfotransferase, the activity of which is required for generating the anti-thrombin inhibitory motif of mammalian HS, was found (326).

Circulating anodic antigen (CAA) is another GAG-like, O-linked glycoprotein antigen excreted by schistosomes, which is also under investigation as a diagnostic target (327). CAA is completely unique among all previously identified glycan structures, consisting of the repeating trisaccharide GalNAcβ1,6-(GlcAβ1,3)-GalNAcβ1,6-, although it slightly resembles the backbone sequence of mammalian chondroitin sulfate, a repeating disaccharide containing GalNAc and GlcA (119,328,329). Currently, there are no genes annotated as β1,6-GalNAcT in the *Schistosoma* genomes.

Interestingly, the NCBI gene database contains a second gene annotated as a β3GlcAT (Accession no. CAD98790.1) (286). The conserved residues and domains of β3GlcAT responsible for donor (UDP-GlcA) and acceptor (UDP-Gal) binding, and other critical aspects of the enzyme function, have been characterized (330). The residues associated with donor binding are well-conserved in the schistosome genes. The conserved amino acids associated with acceptor binding are almost completely maintained among the human, mouse, *C. elegans*, *S. mansoni*, and Sjp

0062810 β3GlcAT genes. However, there is a 15-amino acid stretch within the acceptor binding region in which all the sequences are well-conserved except for the second *S. japonicum* β3GlcAT gene. It is tempting to speculate that if the second β3GlcAT indeed represents a distinct gene sequence, then it may be responsible for the addition of GlcA to CAA, a linkage that is otherwise unknown in the animal kingdom. Or, perhaps one of the EXT genes or splice variants could be involved in CAA synthesis.

1.4.6 *Terminal motifs*

It should be noted that sialic acids, common terminal sugars of mammalian glycans, have never been demonstrated as part of schistosome glycan motifs (1,2). In animals and microbes, sialic acid must be activated for use in glycan biosynthesis by conjugation with CTP, a process catalyzed by CMP-Sialic acid synthetase (3). These are encoded by the CMAS gene, which is highly conserved among vertebrates and well-conserved even in other prokaryotes and eukaryotes (4). No genes with significant homology spanning the functional domains of this gene were found in *C. elegans* or *Schistosoma* genomes. Most of the structures of the following glycans are depicted in **Table 1.1**.

1.4.6.1 LN and LDN

LacNAc (LN) and lacdiNAc (LDN) are terminal modifications in *Schistosoma* glycoproteins. LN is more typically found in mammalian glycan structures and is frequently modified through sialylation, fucosylation, sulfation, or other sugars to generate a wide range of glycan epitopes. Glycans containing the LDN motif are commonly expressed by many invertebrates, including schistosome intermediate hosts and human pathogens, but also sometimes occur in vertebrates including several mammalian glycoproteins (5-9). LDN determinants present in parasite glycans has been shown to generate a humoral response by the human immune system, and interestingly both LN and LDN expression can initiate the formation of a granuloma in humans (10,12,331).

Galactosyltransferases (GalTs) and N-acetylgalactosaminyltransferases (GalNAcTs) are crucial to LN and LDN synthesis, respectively. The presence of β1-4GalNAcT and β1-4GalT activity were discovered using extracts created from *S. mansoni* and the bird schistosome *Trichobilharzia ocellata* (9,13,14). Unlike its mammalian homolog, the schistosome β1-4GalT activity is not altered by the presence of α-lactalbumin (15). While a family of human glycosyltransferases responsible for LN synthesis has been reported, the first β1-4GalNAcT cloned and characterized was from *C. elegans* (3,6,16). The Ceβ1-4GalNAcT has been shown to be fully functional with the ability create the LDN antigen on transfected Chinese Hamster Ovary cells (13). An equivalent enzyme that creates the UDP-Gal:β-1,4-GlcNAc linkage necessary for the LN structure has not been identified in *C. elegans*. These advancements in understanding glycosyltransferases are a necessary first step, but research is still far from understanding the complex regulation and glycomics of LN and LDN synthesis.

Currently the schistosome database contains several glycosyltransferases potentially capable of generating these glycans linkages. A search of the database yields three putative β1-4GalNAcT and six β1-4GalT sequences (5). The nucleotide sequences of the β1-4GalNAcTs contain little homology to the *C. elegans* equivalents. However, protein alignments show improved homology among the catalytic domains of the *S. mansoni* and *C. elegans* β1-4GalNAcTs with approximately 30-40% identity. Similar levels of homology are found when comparing the Ceβ1-4GalTs to the putative β1-4GalT sequences. However, the database is far from complete, with many gene sequences lacking exons responsible for transmembrane regions or parts of the catalytic domain.

1.4.6.2 Fucosylated variants

The LN and LDN motifs of schistosomes are also prominently α3-fucosylated on GlcNAc, resulting in Le^X and LDNF, respectively. These trisaccharides function as both immunomodulators and antigens during infection. They are perhaps the best characterized of the C-type lectin ligands

present in schistosomes and targeted by antibodies of many infected hosts, but their exact roles in infection have yet to be elucidated (8,9,16-19).

Both Le^X and LDNF have been documented on glycoproteins and glycolipids of all three major schistosome species (8,10,16,20). Le^X is also a common feature of mammalian glycosylation, although it is often sulfated or sialylated (6). Its expression in schistosomes appears to be limited to the intramammalian stages and is especially prominent in the adult worm gut (5,21-23). Le^X is also one of the major secreted schistosome antigens, with repeats of the antigen making up the polysaccharide portion of CCA found in serum and urine (24). LDNF appears to be expressed by all stages of the parasite, most highly by eggs and the intramolluscan stages (14,16,25,26). In contrast, expression of LDNF is highly restricted in mammals – in humans it has been identified in urokinase and glycodelin (9,13).

Alpha2- 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 FLDN, FLDNF, LDN-dF and dF-LDN-dF variants (105,106,110,332). These structures are not documented in any other parasitic or mammalian host species and induce high antibody responses in humans and primates (110,208,243,244). In fact, FLDNF is believed to be the motif responsible for the serological cross-reactivity with *S. mansoni* glycoconjugates and keyhole limpet hemocyanin (KLH) of the mollusc *Megathura crenulata* (82,109,110,231,333). Additionally, the chitobiose core (-GlcNAcβ1-4GlcNAcβ1-) in complex type N-glycans can contain α6-linked fucose and the non-mammalian α3-linked fucose (105,332). Such core modifications, especially α3- fucosylation, account for the interspecific immunological cross-reactivity observed among plant, insect, and helminth glycoproteins (281,332,334).

Prior to 2013 the fucosyltranferases (FuT) multigene family in *S. mansoni* was essentially unknown and most of the predicted genes not been substantively characterized (335-337). GeneDB designated 22 genes as putative FuTs with various specificities (α3-, α6-, O-). Two genes are further

annotated as functioning on the core (Smp 154410) or generating Lewis structures (Smp 193620), however this activity has not been verified (283). Analysis of the protein products from those genes revealed the database was incomplete, and the genes were fragments of what is expected in a full length FuT protein. Some gene products were prematurely truncated or missing or missing exons in the stem or catalytic domains (300,338,339). Ascertaining this problem with the database, Peterson *et.al*. 2013 (332) published a comprehensive *in silico* study using RACE (Rapid Amplification of cDNA Ends) PCR to determine the full-length transcripts of the FuT genes from a *S. mansoni* cDNA library. Their study identified six α3-FuTs (four new enzymes, one pseudogene, one previously discovered), six α6-FuTs, and two protein O-FuTs. Interestingly, no α2-FuTs were identified. The FuTs identified contain conserved motifs as well as characteristic transmembrane domains, consistent with their putative roles as fucosyltransferases (301,332,340). This new data, when grouped with previous transcript level results, suggest a possible mechanism for differential expression of fucosylated glycans in schistosomes (289,290,332).

1.4.6.3 Polylactosamine and poly-lacdiNAc

S. mansoni, like mammals, generates extended poly-N-acetyllactosamine (Galβ1,4-GlcNAcβ1,3-Galβ1,4-GlcNAc; poly-LN) chains which can be further modified, most notably in the form of poly-Le^X (89). 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 (119,341). Unusually, *S. mansoni* is also able to form extended polymers of lacdiNAc (poly-LDN) and fucosylated lacdiNAc (poly-LDNF) (107,108). 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 (342,343). 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 (344,345). 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 (107), although the regulatory factors that allow extension of LDN in schistosomes but not in mammals are unknown.

Mammalian β3GnTs are part of a family of structurally-related β1,3-glycosyltransferase genes, which includes both GlcNAc- and Gal-transferases (346). The *Schistosoma* genomes contain several genes homologous to this family, some of which are annotated as β3GnTs and others as β3GalTs, which have the conserved Galactosyl-T domain as well as a transmembrane region (283,286,290). The enzymatic activities of the eight known mammalian β3GnT genes have been well-characterized, and each appears to have preferred substrates, such as β3GnT2, which extends poly-LN on 2,6-branches of tri- and tetra-antennary N-glycans, and β3GnT3, which extends poly-LN on O-linked core 1 (346). As most of the *Schistosoma* genes have a similar level of protein sequence similarity to several of the mammalian β3GnT and vica versa, they will need to be cloned and biochemically characterized in order to determine which are responsible for extension of poly-Le^X, poly-LDN(F) on N-glycans and poly-Le^X on O-linked CCA, for example. 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 (34,35,107,238,271,272).

1.4.6.4 Xylose

Core β1,2-xylose linked to the β-mannose of N-glycans was first identified in plants and has since been recognized as a common modification of plant N-glycans and an important epitope of plant glycoprotein allergens. β2-xylosylation was subsequently identified in molluscs and then in *S. mansoni* and *S. japonicum* egg glycoproteins as well as *S. mansoni* cercariae in mass spectrometry studies (105,298). Western blot experiments suggest that several nematode and helminth species carry core β1,2-xylose and that it is variably expressed on glycoproteins of all of the intramammalian life stages of *S. mansoni*, with highest expression in cercaria and eggs

(164,281). Core α3-fucosylated/core β2-xylosylated egg glycoproteins are also drivers of the Th2-immune response in mice and targeted by IgG in *S. mansoni*-infected mice, humans and rhesus monkeys (164,271). However, it is not clear what role such glycoconjugates play in schistosome infection, how they are developmentally regulated, and if antibodies to β1,2-xylose contribute to protection.

There are two core β1,2-xylosyltransferases annotated in the *S. mansoni* genome and three in the *S. japonicum* genome (283,286). Sjp 0055390 shares the greatest protein sequence similarity with other worm, mollusk and plant sequences annotated as β-(1,2)-xylosyltransferases, including the well-characterized β1,2-xylosyltransferase from *Arabadopsis thaliana* (AtXYLT) (286). AtXYLT is a type-II transmembrane protein, similar to other Golgi glycosyltransferases, with a conserved domain of unknown function (DUF563) that also occurs in the *S. japonicum* protein. AtXYLT adds a xylose β-linked to the central mannose of the N-glycan core structure, possibly acting at several points after the addition of GlcNAcβ1,2 to the α1,3-Mannose at the nonreducing end during Golgi N-glycan processing (347-349). Smp 125150 is a shorter sequence which is also annotated as a β1,2-xylosyltransferase (283), but may be a partial sequence as it aligns well with the N-terminal domain of Sjp 0055390 and AtXYLT but ends before the conserved DUF commonly associated with β1,2-XYLTs. Transcriptome analysis (RNA-Seq) of the *S. mansoni* genome suggested that Smp 125150 expression was high in cercaria and decreased through the schistosomula stages to undetectable levels in adult worms (290), which is potentially in agreement with the β2-xylosylation data described above from mass spectrometry and Western blot studies. These two genes therefore represent likely candidates for the *Schistosoma* β1,2-xylosyltransferases, and their improved characterization would benefit the developmental and immunological understanding of these worms.

Armed with this new genetic knowledge, we can now begint to explore a multitude of different classes of glycosyltransferases involved in various pathways of antigenic glycan

biosynthesis and that their expression. This could open up the schistsome glycome to more detailed studies of important antigens and their roles in worms biology and host-pathogen interactions.

1.5 Project aims

Aim I: Identification of immunogenic glycan antigens and reagent development

- To better understand the structural features targeted by infection and develop *S. mansoni* specific glycan reagents and technologies *(Chapters 2-3)*

Aim II: Development of semi-synthetic approach for heterologous expression of glycosyltransferases

- To better understand the biosynthethic pathways of *S. mansoni* glycan production and generate important glycan structures for future studies *(Chapters 4-5)*

While a substantial body of literature on the biology and immunology of schistosome infection exists, the science has yet to translate into more sophisticated solutions for diagnosis, treatment, or prevention. 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. We believe complex carbohydrates of helminths present an exciting opportunity to fill these gaps. Many glycan motifs, within glycoproteins and glycolipids or secreted materials, are unique to helminths or to a particular worm species. They are abundant on worm surfaces and secretions, and humans vigorously target these glycans in the natural immune response. Advances in glyco-technology have steadily increased our ability to understand this often-overlooked area of host-pathogen interactions.

In terms of vaccine development, successful approach may need to target more than one epitope given the rapid turnover of helminth surface antigens and the variation in their expression

among life stages. 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 LDN and LDNF are expressed on the surface of *S. mansoni* cercariae, schistosomules, and adult worms as well as in soluble egg antigens (168), and that LDNF is expressed on all three major schistosome species (234). 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 (82,350). Further studies are needed to better define the antigenic glycan structures of helminths. Furthermore, in order for theses glycans antigens to be explored for their diagnostic or vaccine potential, we need to develop the appropriate reagents and tools.

The identification of novel glycans synthesized by schistosomes, acknowledgment of their unique functions as immunomodulators, and their recognition as antigens has raised awareness of their importance. The complementary elucidation of the genomes of *Schistosoma* species has now opened the way to linking the glycogenome to the glycome, which has important consequences for the future of research in this area. Knowledge of specific genes encoding key parasite enzymes important in glycan synthesis may lead to new drugs targeted block glycan synthesis or metabolism in the parasite. Such a strategy has the potential to target the parasites directly and/or to modulate the host's immune response to the parasite, both of which could have therapeutic value. The availability of identified and functional genes for schistosome glycosyltransferases could lead to their use in semi-synthetic strategies to synthesize glycans that are very difficult to obtain from chemical synthesis. Using chemo-enzymatic approaches it may be possible to generate a wide-variety of schistosome-related glycans and glycan determinants that would be ideal for screening of immune responses to glycan antigens in human and animal (271,272). Finally, knowledge of the schistosome genes could lead to their use in recombinant forms expressed in mammalian or

insect cells to elaborate the schistosome glycome in a heterologous cells for use in immunization and functional studies (272).

Chapter 2. IgG monoclonal antibody F2D2 binds the difucosylated FLDNF glycan epitope

Portions of this chapter were derived from (351) and are the result of a collaboration between R.D.C. and A.K.N. Manuscript is currently under preparation for submission.

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2.1 Introduction

Schistosomiasis is a chronic, debilitating, infectious disease of humans and animals caused by parasitic blood trematodes of the genus *Schistosoma* (5,7-9). The disease is currently managed by treatment with the drug, Praziquantel and by snail intermediate host control measures (13,38,44-46,48,49). Lacking in the disease management regimen are vaccines for the protection of the approximately 700 million people in the endemic regions of the world at risk of contracting infections and for the prevention of the frequent re-infections observed after drug treatments (14,43-45,352). Vaccines and alternative therapies are also needed urgently to stem the emerging threat of the reduced efficacy of Praziquantel reported in sub-Saharan African subpopulations (353-356). Management and control of schistosomiasis is also greatly hampered by the lack of simple, specific and reliable serodiagnostics for detection of infections (30,31). The rather inconvenient

and often unreliable method of examining stool or urine samples for the physical presence of parasite eggs remains the primary methods for diagnosis of infections (27-30).

Many of the current efforts directed at identifying immunogenic schistosome molecules for use as vaccine or serodiagnostic targets have focused primarily on parasite proteins. A variety of different proteins have been identified and proposed as schistosome vaccine candidates based on their immunogenicity in infected hosts (62,63). However, only one of the protein antigens, Bilhvax (Sh28-GST), has been developed to human clinical trial stages at present (64,221,357,358). The lack of success in developing the candidate protein antigens into vaccines may be related to the fact that many of the candidate protein antigens are cytosolic proteins that are not readily accessible to the immune effectors generated against them by vaccination (62,63,65-67).

Glycan structures on schistosome glycoconjugates have gained increasing attention as alternative vaccine and serodiagnostic targets due to observations that major humoral immune responses in infected hosts are directed to the glycan epitopes of the parasites' glycoconjugates (127,129,359). The advantage of using schistosome glycans as vaccine targets is based on the facts that glycan epitopes occur as covalently bonded conjugates on cell surface glycoproteins and glycolipids and thus are accessible for direct interactions with immune effectors released into host body fluids (168). Furthermore, unlike protein targets, a glycan epitope typically occurs on diverse array of glycoconjugates on the parasites' surfaces as observed for mammalian glycoconjugates and thus provide multiple targets for host immune attack (168). Additionally, glycans on secreted schistosome glycoproteins released into host body fluids could provide effective source of glycan antigens for stimulation of host immune responses.

Many immunogenic glycans of schistosomes have now been characterized (7,70-74). These glycans include: Le^X, LDN, LDNF, LDN-dF, FLDNF, dF-LDN-dF (1,7,89,104-110,298,360,361). Knowledge of the glycan structures has allowed the synthesis, testing, and confirmation of the immunogenicity of Le^X, LDN, and LDNF, core xylose/core fucose (CX/CF) and trimannosyl core epitopes in infected hosts (115,271,272,298). The immunogenicity of the

other identified parasite glycans and the protective efficacy of schistosome glycans in general have not been determined. Furthermore, the role of the glycans in schistosome development and overall parasite immunobiology has not been extensively studied. In mammalian systems, glycans on secreted and membrane glycoconjugates play roles in diverse cellular processes including cell-cell interactions, cell development, protein folding and sorting as well as regulation of cellular signal transduction events (362-364). It is conceivable that schistosome glycans play similar roles in many aspects of the parasites' biology including host-schistosome interactions, and that changes in expression of the glycans by the developing parasites could be involved in the evasion tactics employed by the parasites for survival in their permissive hosts (7,17,178,246,252,365). It must be noted that successful development of vaccines against schistosome infections would require an understanding of many aspects of the molecular mechanisms employed by the parasites to survive within the host in the face of the anti-schistosome immune responses, especially anti-glycan responses, mounted by infected hosts (55,57,189,228,361).

A major limitation in the study of the immunogenicity and biological functions of schistosome glycans has been the lack of adequate quantities of the parasite glycans as well as the lack of specific antibodies needed for purification and tracking of the glycans in developing parasites. A small number of schistosome glycan epitopes have now been synthesized by chemi-enzymatic methods in adequate amounts to begin the study of their immunogenicities (240,271,342). We have now initiated research to generate high affinity and specific IgG mAbs to schistosome glycans to facilitate the purification and tracking of immunogenic parasite glycans to promote the elucidation of the biological functions of the glycans. We previously reported the use of splenocytes from Swiss Webster mice infected for 10 wk with *S. mansoni* to generate a specific high affinity IgG mAb to Le^X glycans of the parasites, and showed by the epitope was sparingly expressed on the surface cercariae but was abundantly expressed on the surface of adult schistosomes suggesting a potential role of Le^X glycans in schistosome development (115). We now report the use of the same approach to generate an IgG mAb to difucosylated FLDNF glycan

epitope that is developmentally expressed by the parasites and is also expressed on glycoproteins from keyhole limpet hemocyanin and the hemolymph of susceptible stages of *Biomphalaria glabrata* intermediate snail hosts.

2.2 Materials and Methods

2.2.1 *Materials*

Keyhole limpet hemocyanin (KLH) and cholera toxin (CT) were obtained from Calbiochem (San Diego, CA). Peroxidase conjugated goat anti-mouse IgG (γ chain-specific), peroxidase conjugated goat anti-mouse IgM (μ chain-specific) and ABTS/peroxidase substrate were purchased from Kirkegaard and Perry (Gaithersburg, MD). Peroxidase-conjugated goat anti-mouse IgG isotyping kit was obtained from Southern Biotechnology Associates, Inc. (Birmingham, AL). Precast polyacrylamide gels and SFM media were from Invitrogen (Carlsbad, CA). Silver staining kit, nitrocellulose membrane (22μm pore size), SuperSignal chemiluminescence substrate and spin columns were obtained from Bio-Rad Laboratories (Hercules, CA). BCA protein assay kit, dialysis tubing and Ultralink-hydrazine biosupport were purchased from ThermoFisher Scientific (Rockford, IL). L-fucose, D-glucose, D-galactose, D-N-acetylglucosamine, D-N-acetylgalactosamine, D-mannose, D-xylose, OPI (1 mM oxaloacetate, 0.45 mM pyruvate and 0.2 U/mL Insulin), HAT (5 mM hypoxanthine, 20 μM aminopterin, 1.6 mM thymine) and HT (10 mM hypoxanthine, 1.6 mM thymine) hybridoma media supplements were from Sigma (St Louis, MO). Peptide N-glycosidase F (PNGase F) was obtained from New England Biolabs (Ipswich, MA) and bovine kidney α-fucosidase was from Prozyme (Heyward, CA). Protease inhibitor cocktail tablets were purchased from Roche Applied Science (Indianapolis, IN). MEP HyperCel was from Pall Life Sciences (Ann Arbor, MI). Microtiter ELISA plates (Immulon 4HBX) were from Thermo Electron Corp. (Milford, MA). Iscoves tissue culture media, L-glutamine and fetal bovine serum (FBS) were purchased from MediaTek (Manassas, VA). Tissue culture flasks and plates were from Corning

Life Sciences (Lowell, MA). Cryoprotective medium (15% DMSO in Eagle's medium in Hanks) was obtained from Lonza (Walkersville, MD). Nalge Nunc roller bottles and bovine serum albumin (BSA) was from Fisher Scientific (Pittsburgh, PA)

2.2.2 *Parasites*

Schistosoma mansoni (Puerto Rico strain) life cycle stages were prepared using published protocols (366). Cercariae were shed into snail water from *Biomphalaria glabrata* snails infected for 4 wk with *S. mansoni* miracidia. The released cercariae were chilled at 4°C to immobilize the larvae and centrifuged at 1200 rpm for 5 min at 4°C. The cercariae were recovered in the pellet fractions and used immediately or stored frozen at -80°C. Eggs were isolated from livers of Swiss Webster mice infected for 6 wk with ~250 *S. mansoni* cercariae using protocols described previously (366). The livers were kept overnight at room temperature and then minced to small pieces. The minced livers were suspended in 1.2% NaCl solution and blended for 30 seconds. The liver suspension was applied to a tier of 4 sieves arranged in a decreasing order of mesh sizes from 425, 180, 106, 45μm and sprayed profusely with 1.2% NaCl to push the eggs through the top sieves to the 45 μm sieve where the eggs were retained. The eggs were recovered from the 45μm sieve into a beaker by spraying the mesh with 1.2% NaCl solution. The eggs were washed 3x with 1.2% NaCl solution and 2x with PBS, pH 7.6 and used directly or stored as a wet pellet at -20°C.

2.2.3 *Production of Hybridoma*

Hybridomas were prepared by fusing SP2/O myeloma cells with splenocytes recovered from Swiss Webster mice infected for 10 wk with 30-35 *S. mansoni* cercariae using protocols described previously (367). Briefly, the hybridomas were distributed into 8, 96-well tissue cultures plates and grown in Iscoves media supplemented with 8 mM L-glutamine, 2x HAT and OPI and grown at 37°C and 5% CO_2 atmosphere for 2 wk. Culture supernatants from each well (50 μl) were tested by ELISA using SEA as antigenic target. Hybridomas secreting antibodies to SEA were subsequently analyzed by ELISA against SEA targets that had been pretreated with periodate

($NaIO_4$)/borohydride ($NaBH_4$) to effect the degradation of glycan epitopes and allow selection of hybridomas secreting mAb to glycan epitopes of SEA, KLH, and BGH. Selected hybridomas were selected and cloned by the limiting dilution technique. Single cell clones secreting antibodies to KLH and BGH were selected and designated F2D2. The F2D2 secreting hybridomas were weaned off HAT by first growing them in Iscoves media containing 20 % FBS, HT (100 μM hypoxanthine, 16 μM thymine) and 8 mM glutamine and finally in Iscoves media containing 20% FBS and 8 mM glutamine. The cloning and selection of single cell clones was repeated 3x in Iscoves/20% FBS/8 mM glutamine to ensure clonal purity of the hybridoma. The single cell clones were sequentially expanded into 24-well plates, 12-well plates and T-75 flasks and either expanded into T150 flasks in Iscoves media containing 20% FBS and 8 mM glutamine for production of mAbs or the cells were recovered by centrifugation at 1,200 rpm for 3 min and re-suspended in 50% FBS in cryoprotective media (Lonza, Walkersville, MD) for storage at -150^{o}C.

2.2.4 *Propagation of Hybridoma Cells in Serum Free Media*

Hybridoma clones secreting mAb F2D2 were grown in 4, T-150 tissue culture flasks in Iscoves media containing 20% FBS, 8 mM glutamine to 80% confluence density and the contents of each flask were split equally into two T-150 flasks to obtain eight flasks of cells and equal volumes of SFM/8 mM glutamine were added to each flask to dilute the FBS content of the media in each flask to 10% FBS and the cells were grown to 80% confluence density. The contents of each flask were split equally once again into two T-150 flasks as described above to obtain a total of 16 flasks of cells and equal volumes of SFM/8 mM glutamine were added to make the FBS content of each flask 5% FBS and the cells were grown to 80% confluence density. The contents from all 16, T-150 flasks were recovered into sterile 50 ml tubes and centrifuged at 1,200 rpm for 3 min. The cell pellets were recovered and suspended in 1 L SFM/8 mM glutamine media and transferred into a 2 L tissue culture roller bottle. The cells were grown in a Wheaton roll-in incubator at 37^{o}C in 5% CO_2 atmosphere with a roller motor setting of 20 V over a period of

approximately 1 month till the live cell density dropped to 5%. The contents of the roller bottle were centrifuged at 1,200 rpm for 5 min to remove cells and the culture supernatant was recovered and centrifuged at 15,000 rpm to remove particulate material. The supernatant was recovered and filtered through Whatman No.1 filter paper. Benzamidine was added to the filtrate to a final concentration of 1mM benzamidine and saved for purification of the secreted mAb.

2.2.5 *Purification of Secreted mAb F2D2 by MEP HyperCel*

Monoclonal antibody F2D2 secreted into SFM was purified by hydrophobic charge induction chromatography (HCIC) over columns of MEP HyperCel using instructions provided by the manufacturer (). Briefly, filtered SFM media derived from roller bottles were applied to a 10 ml column of MEP HyperCel (1 x 20 cm column) pre-equilibrated with wash buffer (50 mM Tris-HCl, pH 8.0) and the media was allowed to percolate through the column. Unbound material was washed off the column with wash buffer and 10-ml fractions were collected. The fractions were monitored for protein by UV absorbance measurements at 280 nm to verify complete removal of unbound proteins from the column. Bound mAb was then eluted with 50 mM sodium acetate buffer, pH 4.5, and 5 ml fractions were collected. The eluted column fractions were monitored for protein by UV absorbance at 280 nm as described above and the purity of the eluted protein fractions were determined by SDS-PAGE. Fractions containing mAb were pooled and dialyzed against TBS. The dialysate was centrifuged at 13000 rpm and the supernatant fraction was recovered. The protein content of the fraction was determined by BCA assay and the mAb was aliquoted and stored at 4^{o}C.

2.2.6 *Preparation of Neoglycoproteins*

The human milk oligosaccharides, lacto-N-neo-tetraose (LNnT; Galβ1-4GlcNAcβ1-3Galβ1-4Glc) and lacto-N-fucopentaose III (LNFPIII; Galβ1-4[Fucα1-3]GlcNAcβ1-3Galβ1-4Glc) were purchased from V-Labs (Covington, LA). LacdiNAc tetratose (LDNT; GalNAcβ1-4GlcNAcβ1-3Galβ1-4Glc) and lacdiNAc fucopentaose (LDNFP; GalNAcβ1-4[Fucα1-3]GlcNAcβ1-3Galβ1-4Glc) were synthesized by chemi-enzymatic remodeling of LNnT by a

modification of protocols described previously (Nyame *et al.*, 1999). Briefly, the terminal galactose residue of LNnT was cleaved by treatment with β-galactosidase and replaced with GalNAc in β1-4 linkage using β1-4-N-acetylgalactosaminyltransferase and UDP-GalNAc to generate LDNT. LDNFP was synthesized from LDNT using α1-3-fucosyltransferase and GDP-Fuc. After synthesis of each glycan, the reaction mixtures were passed over columns of QAE-Sephadex to remove proteins, free sugar nucleotides and nucleotides. The unbound fractions which contained the uncharged neutral synthesized oligosaccharides were recovered and dried in a vacuum evaporator. The residue was re-suspended in water and applied to a column BioGel P-2 and the column fractions were monitored for sugar by absorbance at 215 nm. The synthesized LDNT and LDNFP oligosaccharides were recovered in the void fractions and dried down in vacuum evaporator and re-suspended in water. The sugar content was determined by phenol sulfuric assay and the structure of the synthesized oligosaccharides were confirmed by MALDI-TOF MS. The LDNT and LDNFP oligosaccharides were subsequently conjugated chemically to BSA by the reductive amination procedure (262,268).

2.2.7 *Preparation of soluble egg extracts and other detergent extracts*

Soluble egg antigen (SEA) was prepared using protocols described previously (366). *S. mansoni* eggs were suspended in minimal volume of cold PBS, pH 7.6 supplemented with protease inhibitor cocktail at 10x the manufacturer's suggested concentration (1 tablet/1 ml PBS) and sonicated on ice at 55% amplitude with 0.8 sec pulse on/0.2 sec pulse off for 1 min. The homogenate was allowed to cool for 1 min after each round of sonication until no intact eggs could be observed by microscopic examination. The pulverized egg homogenate was centrifuged at 13,000 × g for 30 min at 4 °C and the supernatant fraction containing the soluble egg antigens was recovered. The protein content of the preparation was determined by BCA assay and the extract was aliquoted were stored and frozen at –20 °C.

S. mansoni cercariae were sonicated in cold PBS containing 10x protease inhibitor cocktail (Roche, Indianapolis, IN) essentially as described above for SEA. Triton X-100 was added to the homogenates to a final concentration of 0.5 % detergent and incubated at room temperature for 30 min to solubilize membrane-bound proteins. The parasite homogenates were centrifuged at 13,000 x g to remove insoluble material and the supernatant fractions were recovered as detergent extracts of the parasites. Protein contents of the extracts were determined by the BCA protein assay and the extracts were aliquoted and stored frozen at -20^{o}C.

2.2.8 *Preparation of Cross-Reactive Proteins*

Keyhole limpet hemocyanin (KLH), cholera toxin B-chain (CT), honeybee phospholipase A_2 (PLA_2), and horse radish peroxidase (HRP) were prepared in PBS, pH 7.2 at final concentrations of 5 mg/ml. Bovine milk glycoprotein (BMGP) was purified from nonfat dry milk by chromatography on 10 mg/ml column of concanavalin A-Sepharose (Pharmacia,) as described previously (368). Bound BMGPs were eluted from the column with 500 mM α-methyl-D-mannoside and dialyzed against PBS, pH 7.2. Protein content of the BMGP preparation was determined by BCA assay. All the cross reactive protein samples were aliquoted and stored frozen at -20^{o}C.

2.2.9 *Preparation of Snail Hemolymph*

Hemolymph was derived from young *Biomphalaria glabrata* snails by puncturing the foothead and recovering the released hemolymph with a micropipette. The recovered hemolymph was centrifuged at 16,000 rpm for 30 min to remove particulate material and the supernatant fraction was recovered. The protein content of the hemolymphs were determined by BCA protein assay and the samples were store frozen at -20^{o}C.

2.2.10 *ELISA of Proteins and Nepglycoconjugates*

Soluble egg antigen (SEA; 5 μg/ml), keyhole limpet hemocyanin (KLH; 40 μg/ml), horse radish peroxidase (HRP; 10 μg/ml), bovine milk glycoproteins (BMGP; 10 μg/ml) and honeybee phospholipase A_2 (PLA_2; 10 μg/ml), cholera toxin (CT; 10 μg/ml), *Biomphalaria glabrata* hemolymph (BGH, 1:20 dilution); mutant BS-90 *Biomphalaria glabrata* hemolymph (BS90H, 1:10 dilution) and *Bulinus truncatus* hemolymph (BTH) in phosphate buffer saline (PBS; 6.7 mM KH_2PO_4, pH 7.6, 0.15 M NaCl), pH 7.6 at were used as antigenic targets. Neoglycoconjugate antigens, LDNT-BSA, LDNFP-BSA, and LNFPIII-BSA which bear defined schistosome-type glycan antigens and LNnT-BSA were prepared in PBS at concentrations of 2μg/ml respectively. Microtiter wells (96 wells) were coated with 50 μl of each antigen solution in triplicate and incubated for 30 min at room temperature. The wells were washed and blocked with 250 μl of 3 % BSA solution in PBS for 30 min. The wells were washed incubated with 50 μl of 10 μg/ml solution of mAb F2D2 in dilution solution for 30 min. The wells were washed to remove unbound mAb and incubated for 30 min with 50 μl of 1:10,000 dilution of HRP conjugated goat anti-mouse IgG (γ-chain specific) secondary antibody in dilution solution. The wells were washed to remove unbound secondary antibodies and the bound antibodies were revealed by incubating the wells with 100 μl of ABTS/peroxidase substrate for 5 min followed by absorbance reading of the wells at 405 nm using a Victor3 microtiter plate reader (PerkinElmer, Downers Grove, IL).

Antibody dilutions were carried out in a dilution solution of PBS/1 % BSA/ 0.3 % Tween-20. For ELISA involving cross-reactive antigens, KLH, BGH, HRP, PLA2, BMGP and CT, bound antibodies were detected by incubation with 50 μl of 1:2000 dilution of alkaline phosphatase conjugated goat-anti-mouse IgG (γ-chain specific) secondary antibody followed by incubation with 100 μl of 1 mg/ml solution of *p*-nitrophenyl phosphate substrate in 100 mM Tris-glycine buffer, 1 mM $MgCl_2$, pH 9.0 and absorbance measurements of the wells was conducted at 405 nm. The subclass of mAb F2D2 was determined by coating microtiter wells with SEA and blocking the

wells with BSA followed by incubation with mAb F2D2 as described above. The subclass of the bound mAb was determined by incubations with 50 µl of 1:500 dilutions of alkaline phosphatase conjugated goat anti-mouse IgM, IgG, IgG1, IgG2a, IgG2b or IgG3 and *p*-nitrophenyl phosphate substrate. All the ELISA incubations were performed at room temperature for 30 min/per incubation. The microtiter wells were washed 6x between each incubation using PBS/0.3% Tween-30 buffer and Ultra Wash Plus microtiter plate washer (Dynex Technologies, Chantilly, VA). Each antigen was analyzed in triplicate and the results represent averages of the triplicates.

2.2.11 *ELISA of Periodate/Borohydride Treated Antigens*

Microtiter wells were coated in with SEA, KLH or BGH as described above, and washed 1x with PBS, pH 7.2, followed by 2 washes with 50 mM sodium acetate buffer, pH 4.5. The wells were subsequently incubated with 250 µl of 5 mM solution of sodium periodate (NaIO4) in 50 mM sodium acetate buffer, pH 4.5 at room temperature in the dark for 1 h. Control wells were incubated with 250 µl of 50 mM sodium acetate buffer pH 4.5 without sodium periodate. The wells were washed 1x with 50 mM sodium acetate buffer, pH 4.5, 2x with PBS, pH 7.2 and incubated with 250 µl of 50 mM sodium borohydride (NaBH4) in PBS, pH 7.2 buffer at room temperature for 30 min to convert the reactive aldehydes generated by the periodate treatment to alcohols. The wells were washed 6x with ELISA wash buffer and blocked by incubation with 3% BSA/PBS solution for 1 h. The wells were washed and incubated with 50 µl of 10 µg/ml mAb F2D2 for 30 min followed by incubations with 50 µl of HRP conjugated goat anti-mouse IgG for 30 min and ABTS/H2O2 substrate for 5 min. The absorbance readings of the wells were determined at 405 nm as described above. The antibody incubations and plate washes were carried out as described above.

2.2.12 *ELISA in the Presence of Free Haptenic Sugars*

Microtiter wells were coated in triplicate with 50 µl of either 5 µg/ml SEA, 10 µg/mL of KLH or 1:20 dilution of BGH and blocked with 3% solution of BSA in PBS at room temperature for 30 min as described above. Stock solutions of 1 µg/ml mAb F2D2 in ELISA dilution solution

containing 800 mM of either fucose (Fuc), mannose (Man), xylose (Xyl), galactose (Gal), glucose (Glc), N-acetylgalactosamine (GalNAc) or N-acetylglucosamine (GlcNAc) were prepared and serially diluted 2-fold with 1 μg/ml solution of mAb F2D2 in dilution solution to generate a set of 1 μg/ml solutions of mAb F2D2 containing 800, 400, 200, 100, 50, 25, 12.5, 6.25 mM of the different monosaccharides respectively. A 1 μg/ml F2D2 solution in dilution solution without any monosaccharide was prepared as a control. The antigen coated, BSA blocked microtiter wells were incubated with 50 μl of each dilution of the antibody/monosaccharide solutions in triplicate and bound antibodies were detected by incubations with HRP conjugated goat anti-mouse IgG and ABTS/peroxidase substrate as described above followed by absorbance measurements of the wells at 405 nm. The antibody incubations were carried out at room temperature for 30 min/incubation while substrate incubations were carried out at room temperature for 5 min. The microtiter wells were washed 6x between incubations as described above.

2.2.13 *Glycosidase Treatments*

Approximately 5 μg of SEA was treated with bovine kidney α-fucosidase (Prozyme, San Francisco, CA) at 37°C in a total volume of 20 μl using buffers and instructions provided by the manufacturer. As a control 5 μg of SEA was mock treated by omitting the α-fucosidase from the reaction mixture. SDS-PAGE sample buffer was added to the samples and boiled for 10 min for analysis by SDS-PAGE and Western blot. For the PNGase F treatments, approximately 5 μg of SEA, KLH, or BGH were mixed with denaturing buffer provided by the manufacturer (New England Biolabs, Ipswich, MA) and boiled for 10 min to denature proteins. The samples were cooled to room temperature, mixed with enzyme sample buffer and treated with PNGase F at 37°C according to instructions provided by the manufacturer. SDS-PAGE sample buffer was subsequently added and the enzyme treated samples boiled for 10 min for analysis by SDS-PAGE and Western blot.

2.2.14 *SDS-PAGE and Western blot analysis*

Samples were boiled for 10 minutes in SDS-PAGE sample buffer and separated on 4-20% precast polyacrylamide gels (Invitrogen) at 125 V for 2 h. The gels were either stained with Coomasie blue stain for imaging of protein bands or the separated proteins were transferred from the gels onto nitrocellulose or PVDF membrane at 20 V for 12h at room temperature using a transfer buffer of 48 mM Tris, 39 mM glycine pH 9.2, with 20% methanol. The membrane was blocked by incubation in 5 % BSA solution in Tris-buffer saline (TBS; 50 mM Tris-HCl, pH 7.6, 150 mM NaCl) for 1 h at room temperature with gentle shaking. The membrane was washed 3x in wash buffer (20 mM Tris-HCl, pH 7.6, 0.3 m NaCl, 0.05% Tween-20) by gentle agitation for 10 min per each wash and incubated in 10 μg/ml solution of mAb F2D2 in dilution buffer (TBS, 0.3% Tween-20, 1% BSA) for 1 h at room temperature. The membrane was washed 3x with Western blot wash buffer and incubated in 1: 10,000 dilution of HRP conjugated goat anti-mouse IgG (γ-chain specific) secondary antibody in dilution buffer for 30 min at room temperature. The membrane was washed 3x with wash buffer and antibody bound bands were revealed by incubation in SuperSignal chemiluminescence substrate for 30 to 60 s depending on blot and then imaged on an UVP EC-3 digital imager or exposure to x-ray film.

2.2.15 *Periodate/Borohydride Treatment and Western Blot Analysis*

SEA, KLH and BGH were separated by SDS-PAGE in duplicate and transferred onto nitrocellulose membranes as described above. One membrane was incubated with 100 ml of 5 mM solution of sodium periodate ($NaIO_4$) in 50 mM sodium acetate buffer, pH 4.5 at room temperature in the dark with agitation for 1 h. The other membrane was mock treated by incubation with 50 mM sodium acetate buffer pH 4.5 without sodium periodate. The membranes were washed 1x with 50 mM sodium acetate buffer, pH 4.5, 2x with PBS, pH 7.2 and incubated with 100 ml of 50 mM sodium borohydride ($NaBH_4$) in PBS, pH 7.2 buffer at room temperature for 30 min. The membranes were washed 3x with TBS, blocked with 5% BSA in TBS and incubated with10 μg/ml

mAb F2D2 in Western blot dilution solution. Bound antibodies were revealed by incubation with HRP conjugated goat anti-mouse IgG followed by incubation with SuperSignal chemiluminescence substrate and imaging on a UVP EC-3 imager.

2.2.16 *Beta-Elimination and Western Blotting*

SEA, detergent extracts of cercariae and desialylated Jurkat cells, BGH and KLH were separated by SDS-PAGE in duplicate and the protein bands were transferred on PVDF membrane as described above. One duplicate membrane was subjected to β-elimination by overnight incubation in 55 mM solution of NaOH in water at 40^{o}C with gentle agitation. The other membrane was mock treated in water under the same incubation conditions as the NaOH treated membrane. The treated and mock treated membranes were washed 3x in TBS, pH 7.4, blocked in 5% BSA/TBS for 1 h washed 3x with Western blot wash buffer and incubated for 1 h with 10 μg/ml mAb F2D2 or 2 μg/ml WFA in Western blot dilution buffer. The membranes were washed 3x high salt TTBS and incubated with 1:10,000 dilution of goat anti-mouse IgG HRP conjugate or 1:5000 dilution of HRP conjugated streptavidin in Western blot dilution buffer for 1 h. The membranes were washed 3x with TTBS and reactive bands were revealed by incubation in SuperSignal chemiluminescent and imaging on UVP-EC3 imager as described above.

2.2.17 *Enzymatic Release and Labeling of N-glycans from KLH*

KLH (1 g) was dissolved in 35 ml of deionized-water (di-water) in a glass tube. Methanol (2.67 volumes) and chloroform (1.33 volumes) were added to the KLH solution sequentially with sonication to yield a final mixture of KLH in chloroform: methanol: water in a ratio of 4:8:3. The mixture was centrifuged at 9,000 rpm at ambient temperature for 10 min. The protein pellet was recovered, dried briefly under a jet of nitrogen gas to remove traces of organic solvent and dissolved in 10 ml of 0.2 M Tris-HCl, pH 8.2. Stock solutions of guanidinium chloride and dithiotreitol (DTT) in 0.2 M Tris-HCl, pH 8.2 were added sequentially to a final concentration of 5.33 M guanidinium

chloride and 0.06 M DTT respectively and the mixture was rotated gently for 1 h at room temperature to denature the KLH and reduce disulfide bonds. Thiol groups in KLH were alkylated with iodoacetamide by adjusting the solution to a final concentration of 0.045 M DTT, 6 M guanidinium chloride and 0.045 M iodoacetamide with a stock solution of in 0.2 M Tris-HCl, pH 8.2 and incubating the sample at 37ºC for 30 min. The mixture was transferred into a dialysis tubing (6,000-8,000 molecular weight cut-off) and dialyzed against running di-water overnight at 4 ºC. The dialyzed KLH was lyophilized and dissolved in 15 ml of 50 mM phosphate buffer pH 8.2.

Trypsin (25 mg) was mixed into the KLH solution and incubated overnight at 37 ºC with gentle rotation. The mixture was boiled at 100 ºC for 10 min to inactivate the trypsin and then cooled to room temperature. PNGase F (25 µl of 500,000 U/ml stock) was gently mixed into the solution. Sodium azide (NaN_3) was added to a final concentration of 0.02% NaN_3 and the solution was incubated at 37 ºC for 48 h with gentle rotation. Another 25 µl of PNGase F was added and the mixture was incubated further for 24 h at 37ºC with gentle rotation. Aliquots of the enzyme reaction mixture (200 µl) were sampled sequentially at 0, 24, 48 and 72 h intervals during the PNGase F digestion to determine complete digestion.

After digestion, the solution was sequentially passed through conditioned C18 Sep-Pak cartridge and a Carbograph cartridge. The Carbograph column was washed (6 CVs), and the bound glycans were eluted with 3 CVs of 50% acetonitrile in 0.1% trifluoroacetic acid (TFA) and dried under vacuum before labeling. The AEAB-KLH preparations were labeled with the bifunctional fluorescent linker AEAB (2-amino-N-(2-aminoethyl)-benzamide) as described (268). Dried AEAB-KLH glycans were resuspended in 0.1ml of 0.35M AEAB plus 0.1ml of 1M $NaCNBH_3$ in DMSO:AcOH (7:3 v/v), and incubated at 65ºC for 2h. After cooling, 2ml of acetonitrile was added, the sample incubated at -20ºC for 30min, and centrifuged at 9,000rpm for 10min. The pellet was then dissolved in 0.2ml water for separation by F2D2 immunoaffinity.

2.2.18 *Immobilization of monoclonal antibody F2D2 to Ultralink hydrazine biosupport*

Monoclonal antibody F2D2 was immobilized onto Ultralink hydrazide beads according to the manufacturer's protocol. Briefly, 27 mg of mAb F2D2 in Tris-buffer saline (TBS; 50 mM Tris-HCl, pH 7.6, 0.15 M NaCl) was exchanged into 0.1 M sodium phosphate buffer, pH 7.2, and concentrated to 2 ml using an Amicon ultra-15 centrifugal filter unit (Millipore, Billerica, MA). The antibody solution was mixed with 12 mg of sodium periodate ($NaIO_4$) and incubated for 30 min at room temperature in the dark with periodic swirling. The mixture was passed over a 10 ml desalting column to remove the periodate , and the void fractions were recovered and added to 5 ml of Ultralink hydrazide beads pre-equilibrated in 0.1 M sodium phosphate buffer pH 7.2 in a 5 ml chromatographic column. The Ultralink hydrazide-mAb F2D2 mixture was incubated at RT for 1 h and then overnight at 4 ºC with gentle rotation. Uncoupled mAb was drained from the column and the F2D2-Ultralink matrix was washed 3x alternately with phosphate buffer saline (PBS; 6.7 mM KH_2PO_4, pH 7.6, 0.15 M NaCl, pH 7.2) and 1 M NaCl to remove traces of unconjugated mAb. Approximately 12.2 mg of uncoupled mAb was recovered in the material drained from the column matrix as determined by BCA protein assay. Thus, it was estimated that 14.8 mg of the 27 mg of mAb F2D2 used for coupling was immobilized to the Ultralink matrix. Given that 5 ml of Ultralink matrix was used, the coupling density of the column was calculated to be approximately 2.9 mg of mAb/ml matrix.

2.2.19 *Affinity Purification of PNGase F Released AEAB-labeled KLH Glycans on F2D2-Ultralink Column*

Approximately 2.3 µmols of AEAB-KLH glycans or AEAB-lactose in PBS, pH 7.6 were applied to 2 ml of packed F2D2-Ultralink (2.9 mg antibody/ml Ultralink) in a 4 ml (0.5 x 20 cm) glass columns (Bio Rad, Hercules, CA) and 1 ml fractions were collected. Unbound material was washed from the column with 5 column volumes of PBS, and 1 ml fractions were collected. Bound AEAB-KLH glycans were batch eluted by applying 900 µl of 0.1 M glycine-HCl, pH 2.5 to the

column at a time and allowing the buffer to drain through the column into tubes containing 100 µl of 1 M Tris-base, pH 11. The contents of the tubes were vortexed to mixed and neutralize the eluted material. Ten batch eluted fractions were collected. The fluorescence associated with the unbound and bound column fractions were determined on a Victor3 fluoresecence microtiter plate reader (PerkinElmer) at 355 nm excitation /460 nm emission and the data was plotted on a graph. Peak column fractions from the bound material were pooled separately and desalted using mini carbograph cartridges as previously described. The affinity purified AEAB-KLH glycans were dried in a Speed-vac evaporator and dissolved in 1 ml water mass analysis.

2.2.20 *Mass Spectrometry Analysis of KLH Glycans*

For MS and MS^n analysis, samples were directly infused into a linear ion trap mass spectrometer (LTQ-XL, Thermo Scientific) at a flow rate of 0.4–0.6 µL/min. From the acquired full MS scans, select *m/z* values were selected to undergo MS/MS fragmentation by collision-induced dissociation (CID). The MS/MS fragmentation spectra were generated by applying 35% collision energy and collected over 30 seconds. The acquired MS/MS spectra were processed using XCalibur (Thermo, San Jose).

2.3 Results

2.3.1 *F2D2 is an IgG monoclonal antibody that recognizes a unique glycan epitope in SEA*

Monoclonal antibody F2D2 was produced from a hybridoma clone prepared from splenocytes of Swiss Webster mice infected for 10 wk with *S. mansoni*. Splenocytes from 10 wk old infections were used because previous studies revealed that optimum IgG and minimum IgM responses of *S. mansoni*-infected Swiss Webster mice to glycan antigens of the parasites occur at wk 10 post infection and that splenocytes from mice infected for 10 wk with the trematodes had been used earlier to generate IgG mAb to Le^X antigens (115). The hybridoma secreting mAb F2D2

was identified and selected by ELISA analysis of hybridoma culture supernatants using SEA, defined schistosome glycan antigens and glycoproteins/proteins reported to share cross-reactive epitopes with *S. mansoni* as antigenic targets. This first screening was performed by incubating microtiter wells coated with SEA and neoglycocongugates LNFPIII-BSA, LDN-BSA, LDNFP-BSA which bear the defined Le^X, LDN and LDNF schistosome-type glycan epitopes respectively with hybridoma culture supernatants and probing for bound antibodies (**Figure 2.1A**). The antibodies in the culture supernatant bound to SEA but did not bind to any of the three defined schistosome-type glycans antigens (**Figure 2.1A**).

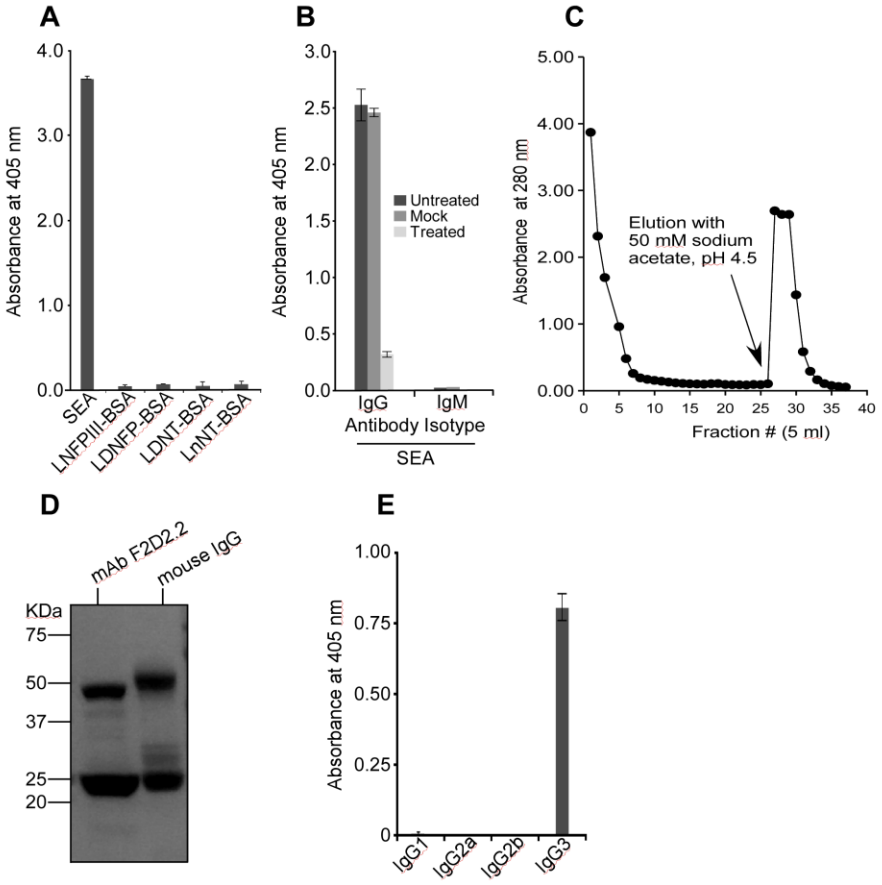


Figure 2.1. Purification and characterization of the binding specificity and isotype of monoclonal antibody F2D2 by ELISA and Western blot.

Microtiter wells were coated with SEA and BSA conjugates of the indicated defined glycan antigens (50 µl of 5 µg/ml solution) and left untreated **(A)**, mock treated, or treated with periodate/sodium borohydride incubated **(B).** Plates were then incubated with F2D2 hybridoma culture supernatant and bound antibodies were probe by peroxidase conjugated goat anti-mouse IgG or IgM and ABTS/peroxidase substrate. **(C)** Serum free media containing secreted mAb F2D2 was applied to a column MEP-Hypercel and bound mAb was eluted with 50 mM acetate buffer, pH 4.5. Fractions containing antibodies as assessed by UV absorbance at 280 nm. **(D)** The purity of the F2D2 preparation was determined by analysis of 10 µg aliquot by SDS-PAGE on 4-20% polyacrylamide gradient gel and stained by Coomassie blue. Mouse IgG was analyzed as a control. **(E)** Micro titer wells were coated with SEA (50 µl of 5 µg/ml solution) and incubated with purified F2D2 (50 µl of 10 µg/ml

solution). Bound antibodies were probed with peroxidase conjugated goat anti-mouse IgG1, IgG2a, IgG2b or IgG3 and ABTS/peroxidase substrate. All the ELISAs were performed in triplicate and the results represent averages of the triplicates.

To control for antibodies reacting to the glycan compenent of SEA, coated microtiter wells were treated with periodate to effect destruction of glycan epitopes by cleavage of vicinal diols within the sugar epitopes followed by treatment with borohydride to convert the reactive aldehydes generated by the periodate reaction to non-reactive alcohols (363). For controls, SEA coated microtiter wells were mock treated by incubating the wells with buffer lacking periodate, followed by incubation with borohydride. The treated and mock treated wells were blocked with BSA, incubated with F2D2 hybridoma culture supernatants and probed for bound antibodies. No antibody binding was observed for ELISA wells treated with periodate/borohydride while the mock treated wells bound the antibody (**Figure 2.1B**) indicating that the antibody in the culture supernatant bound glycan epitopes in SEA. This combination with the neoglyconjugate ELISA suggests F2D2 antibody target is a glycan epitope in SEA distinct from the three defined glycan antigens. We therefore proceeded to clone, purify and characterize the antibody further.

2.3.2 *Purification and Characterization of mAb F2D2*

To further characterize the antigenic specificity of the antibodies secreted by the hybridoma, the cells were cloned by standard cloning protocol described previously (115) to generate a single cell clones secreting antibodies to SEA but not to Le^X, LDN or LDNF epitopes (data not shown) and the mAb was designated F2D2. The clone was weaned for propagation in serum free media as described in "Materials and Methods" and grown in 1 L SFM in roller bottles over a period of 1 month. The culture media was recovered by centrifugation and applied to a column of Mep Hypercel to which the secreted antibodies in the SFM bound. Unbound, non antibody material was washed off the column with PBS and the bound mAb F2D2 was eluted with acetate buffer, pH 4.5 (**Figure 2.1C**) into tubes containing 1 M Tris base to neutralize the eluted

fractions. The bound column fractions were monitored for protein by UV absorbance at 280 nm and fractions containing protein were pooled and the purity of the mAb preparation was determined by SDS-PAGE under reducing conditions using commercially available normal mouse IgG as a control (**Figure 2.1D**).

Two protein bands with apparent MW of 50 kDa and 25kDa representing heavy and light chains of the IgG mAb were observed for both F2D2 and the mouse IgG control (Figure 1D) showing that a pure IgG mAb was derived by the hydrophobic charge induction purification procedure over the Mep Hypercel column. A total of approximately 80-120 mg of purified mAb F2D2 was recovered from 1 l of SFM. The subclass of the purified mAb F2D2 was determined by ELISA using both SEA and KLH as antigens. Microtiter wells were coated with SEA or KLH, blocked with BSA and incubated with the purified mAb F2D2. Bound antibodies were detected by incubations with alkaline phosphatase conjugated goat anti-mouse IgM, IgG, IgG1, IgG2a, IgG2b or IgG3. F2D2 binding was observed in ELISAs probed with anti-mouse IgG3 but not with anti-mouse IgM, IgG1, IgG2a or IgG2b indicating that mAb F2D2 was an IgG3 antibody (**Figure 2.1E**).

2.3.3 *F2D2 binds to glycan epitopes from keyhole limpet hemocyanin (KLH) and Biomphalaria glabrata hemolymph (BGH)*

To characterize the epitope bound by F2D2 further, the mAb was used in ELISA to screen for binding towards a variety of proteins/glycoproteins reported to share cross-binding epitopes with schistosomes. These glycoproteins/proteins included bovine milk glycoproteins (BMGP) which bear LDN epitopes (368), cholera toxin (CT) (369,370), horse radish peroxidase (HRP) which bear core Xyl epitope on N-glycans (371), keyhole limpet hemocyanin (KLH) which bear N-glycans with FLDNF, core Xyl and core α1-3Fuc epitopes on N-glycans (109,110,333), *Biomphalaria glabrata* hemolymph (BGH) which bear FLDNF epitopes (109,333,372,373) and phospholipase A_2 (PLA_2) which bears core α1-3 Fuc epitope (281). Microtiter wells were coated with BMGP, CT, HRP, KLH, BGH and PLA_2 then incubated with mAb F2D2. Since HRP was

being analyzed as antigen, bound antibodies in this ELISA were detected using alkaline phosphatase-conjugated goat-anti mouse IgG and *p*-nitrophenyl phosphate substrate. F2D2 bound with very high affinity to BGH and KLH but not to the any of the other cross-reactive proteins/glycoproteins analyzed **(Figure 2.2A**).

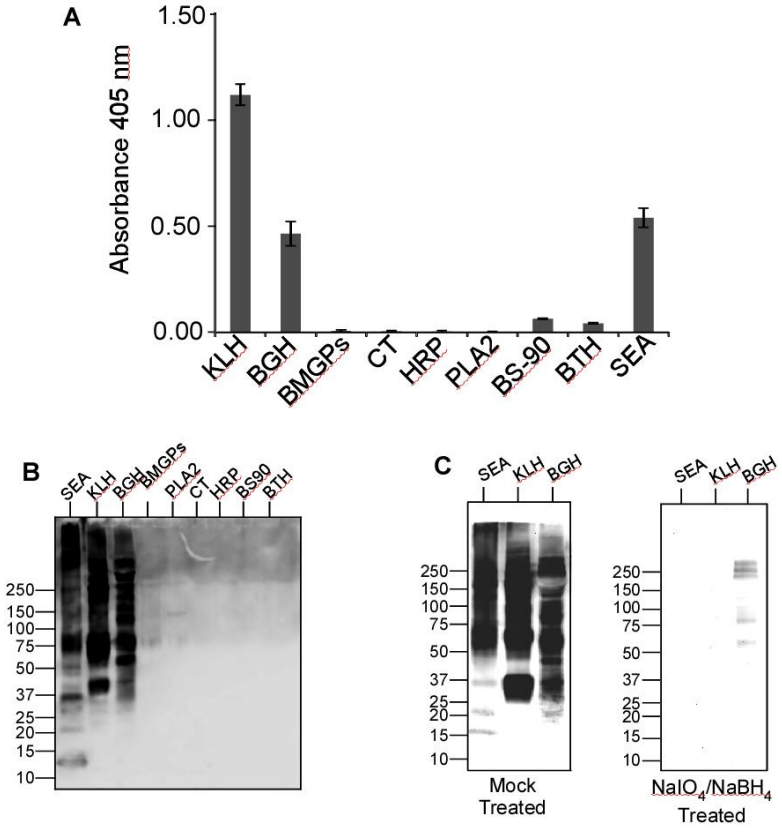


Figure 2.2. Determination of binding specificity of mAb F2D2 towards serologically cross-reactive antigens of schistosomes.

(A) Microtiter wells were coated with keyhole limpet hemocyanin (KLH; 25 μg/ml), *Biomphalaria grabrata* hemolymph (BGH; 1:10 dilution) bovine milk glycoproteins (BMGPs; 40 μg/ml), cholera toxin (CT, 25 μg/ml), horse radish peroxidase (HRP; 25 μg/ml), honeybee phospholipase A2 (PLA2; 25 μg/ml), BS-90 resistant *Biomphalaria glabrata* hemolymph (BS90; 1:10 dilution), *Bulinus truncatus* hemolymph (BTH, 1:10 dilution) and incubated with 10 mg/ml of purified mAb. Bound antibodies were detected with alkaline phosphatase conjugated goat anti-mouse IgG followed by incubation with *p*-nitrophenyl phosphate substrate. Assays were conducted in triplicates and the results

represent averages of the triplicates. SEA (5 μg), KLH, BGH, BMGPs, PLA2, CT, HRP (25 μg each) BS-90 and BTH (30 μl each) were separated by SDS-PAGE and blotted onto nitrocellulose membrane. Membranes were untreated **(B)** or incubated in acetate containing sodium periodate **(C)** with a control mock treated by incubation in acetate buffer without sodium periodate ($NaIO_4$). The membranes were blocked in BSA solution and incubated with 10 mg/ml mAb F2D2. Bound F2D2 was detected by peroxidase conjugated goat anti-mouse IgG and SuperSignal chemiluminescence substrate followed by imaging on a UVP EC-4 digital imager.

The specificity of the binding of F2D2 was confirmed by Western blotting analysis. SEA, KLH, BGH, BMGP, PLA_2, HRP and CT were separated by SDS-PAGE under reducing conditions and blotted on nitrocellulose membrane. The membrane was blocked in BSA and incubated with mAb F2D2. F2D2 bound to a broad range of glycoproteins from KLH and BGH (**Figure 2.2B**) and consistent with the ELISA studies, F2D2 did not bind to any of the other cross-binding antigens. Further, the binding of F2D2 to KLH and BGH was completely sensitive to periodate treatment (**Figure 2.2C**) confirming both the ELISA result and the utility of the antibody in both ELISA and Western blot analyses. Taken together, the ELISA and Western blot results show that mAb F2D2 recognizes a glycan antigen distinct from the schistosome glycan antigens we had defined previously and that glycan epitope bound by the mAb is shared with glycan epitopes on glycoproteins from the hemolymphs of *Biomphalaria* snails and keyhole limpets.

2.3.4 *Fucose is a major determinant of the mAb F2D2-binding epitope*

To elucidate the structural aspects of the glycan epitope bound by mAb F2D2, ELISA of F2D2 against SEA, KLH and BGH targets were carried out in the presence of free monosaccharides commonly found in schistosome glycan structures (70) to assess their potency at inhibiting the binding of the mAb to the glycan epitopes in three antigen targets. The free monosaccharides used included mannose (Man), fucose (Fuc), galactose (Gal), xylose (Xyl), N-acetylglucosamine (GlcNAc) and N-acetylgalactosamine (GalNAc). Glucose (Glc) which is not commonly found in schistosome glycoconjugates was used as a negative control. Microtiter wells coated with SEA,

KLH and BGH and blocked with BSA were incubated with 1 μg/ml solutions of mAb F2D2 prepared in dilution solutions without any monosaccharide or in dilution solutions containing concentrations of the listed monosaccharides starting from 6.25 mM and increasing 2-fold to 12.5 mM, 25 mM, 50 mM, 100 mM, 200 mM, 400 mM and 800 mM. The binding of F2D2 to SEA, KLH and BGH was inhibited by fucose at all the concentrations used (**Figure 2.3A-C**). In contrast, the other monosaccharides caused only modest to no inhibition on mAb F2D2 to the three antigens even at concentrations as high as 800 mM. This result indicates that fucose is a major monosaccharide associated with the glycan epitope on all the three antigenic samples.

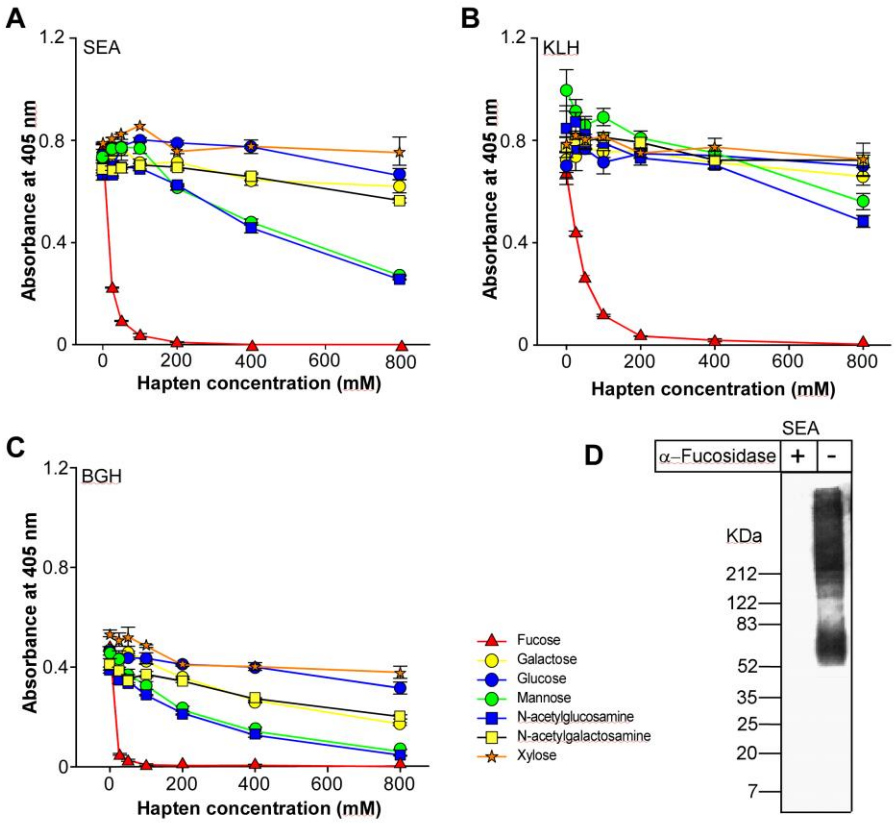


Figure 2.3. Identification of the glycan epitopes recognized by mAb F2D2 by hapten inhibition ELISA and glycosidase treatment.

Microtiter wells were coated with **(A)** SEA (5 μg/ml), **(B)** KLH (25 μg/ml) or **(C)** BGH (1:10 dilution), blocked with BSA and incubated with 1 μg/ml solution of mAb F2D2 containing 0, 50, 100, 200, 400 or 800 mM of either Fucose, Galactose, Glucose, Mannose, N-acetylglucosamine, N-acetylgalactosamine or Xylose. Bound F2D2 was detected by incubations with peroxidase conjugated goat anti-mouse IgG and ABTS/peroxidase substrate. Assays were carried out in triplicates and the results represents averages of the triplicates. **(D)** Fucosidase treated or mock treated SEA (1 μg each) was separated by SDS-PAGE, blotted onto nitrocellulose membranes, blocked in BSA, incubated with 10 μg/ml mAb F2D2 and probed for bound F2D2 by incubations in peroxidase conjugated goat anti-mouse IgG and SuperSignal chemiluminescence substrate.

To confirm fucose as major sugar with the antigenic epitope, SEA was treated with bovine kidney α-fucosidase which is reported to cleave a broad spectrum of terminal α-linked fucose glycosidic bonds (374). As a control, a sample of SEA was mock treated by omitting the α-fucosidase from the digestion mixture. The fucosidase and the mock treated samples were separated by SDS-PAGE, transferred to nitrocellulose membrane and analyzed by Western blot. As expected, a broad range of reactive glycoprotein bands were observed in the mock treated samples but binding was lost in the α-fucosidase treated SEA (**Figure 2.3D**), confirming that fucose is a major monosaccharide with the antigenic epitope bound by F2D2.

2.3.5 *The glycan epitope bound by mAb F2D2 is present on N-glycans of glycoproteins from SEA, KLH AND BGH.*

We next determined the nature of the linkage type of the glycan epitopes bound by mAb F2D2 by determine whether the epitope was present on N-linked or O-linked glycans. To determine whether the epitopes were on N-glycans, SEA, KLH, and BGH were treated with PNGase F to cleave N-linked glycans from their N-glycosidically bonded polypeptides and assessed for loss of mAb F2D2 binding by Western blotting. As controls, another batch of SEA, KLH, and BGH samples were mock treated by omitting PNGase F from the reaction mixture. PNGase F is an endoglycosidase that releases N-glycans from polypeptides by cleaving the N-glycosidic bond between the inner GlcNAc residue of N-glycans from the covalently bonded Asn residue if the GlcNAc residue is not modified α1-3 with a Fuc, a core fucose modification commonly found in plant and invertebrate N-glycans (164,281). The PNGase F treated and untreated samples were separated by SDS-PAGE, blotted onto nitrocellulose membrane, blocked in BSA and incubated with mAb F2D2 (**Figure 2.4**).

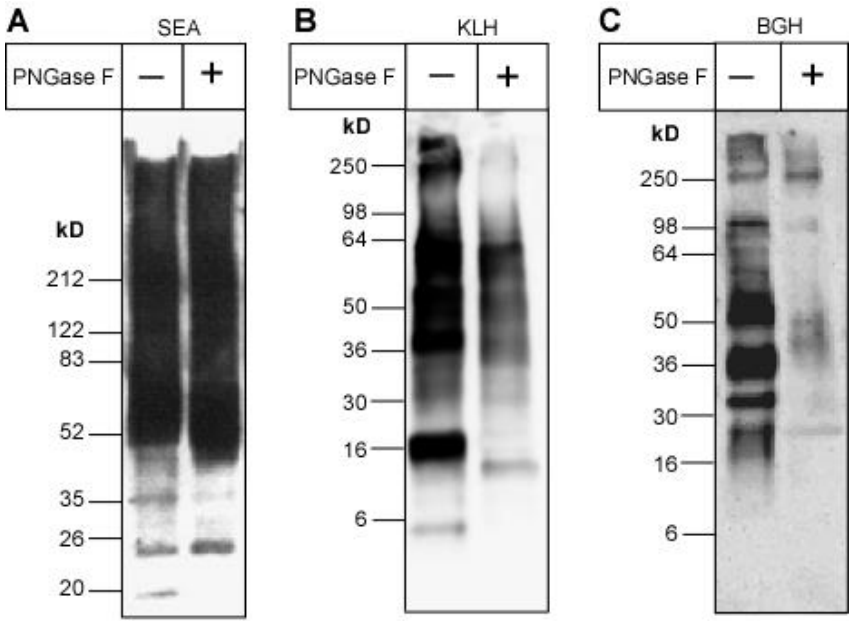


Figure 2.4. Western blot analysis of extracts of *Schistosoma mansoni*, keyhole limpet hemocyanin (KLH) and *Biomphalaria grablata* hemolymph after mock treatment or PNGase F treatment.

(A) SEA (5 μg), **(B)** KLH (25 μg) and **(C)** BGH (20 μl) were either treated with PNGase F (+) or mock treated (-) by omitting PNGase F from the reaction mixture. The samples were separated by SDS-PAGE, blotted onto nitrocellulose membranes, blocked with BSA and incubated with 10 μg/ml of mAb F2D2. Bound antibodies were revealed by incubations with peroxidase conjugate goat anti-mouse IgG and SuperSignal chemiluminescence substrate.

Numerous F2D2-binding bands of diverse MW were observed in the mock treated SEA and only a few of bands, mostly in the low MW range, were lost from the PNGase F treated SEA. Shifts in the MW of the bands from the SEA were apparent in the PNGase F treated sample indicating that the endoglycosidase treatment did release N glycans from glycoproteins suggesting that the glycan structures bearing the F2D2 binding epitopes in SEA are considerably resistant to PNGase F cleavage (**Figure 2.4A**). Numerous glycoproteins from the mock treated KLH and BGH hemolymphs ranging in size from high and low MW bands were bound by mAb F2D2 and majority of the bands were lost after PNGase F treatment (**Figure 2.4B, C**) indicating that the major F2D2-

binding glycan epitopes from KLH and BGH were present on N-glycans. The results from the PNGase F treatment indicated that majority of the F2D2-binding glycan epitopes from adult *S. mansoni*, KLH and BGH were present on N-linked glycans but the majority of the F2D2-binding glycans from SEA extracts PNGase F treatment could not conclusively predict whether the epitopes were N-linked.

2.3.6 *β-elimination of SEA, KLH and BGH*

The observation that the majority of the F2D2 binding glycan epitopes in SEA and a few in KLH and BGH were resistant to PNGase F treatment raised the possibility that some of the F2D2-binding epitopes in the samples were on O-linked glycan structures. To investigate this possibility, we analyzed SEA, cercarial extract as another parasite sample, along with KLH and BGH for release of F2D2-binding glycans from polypeptides in the samples by β-elimination reaction. To carry out the β-elimination reaction, SEA, KLH, and BGH were separated by SDS-PAGE in duplicate and transferred onto PVDF membranes. One membrane was incubated overnight at 40 ^{o}C in 55 mM NaOH to effect in situ release of O-glycans by β-elimination while the other membrane was mock treated by carrying out the incubation in water without NaOH. SEA, KLH, and BGH retained their binding interaction with mAb F2D2 after the mild base treatment and had the same glycoprotein band profile as the mock treated samples (**Figure 2.5A-B**).

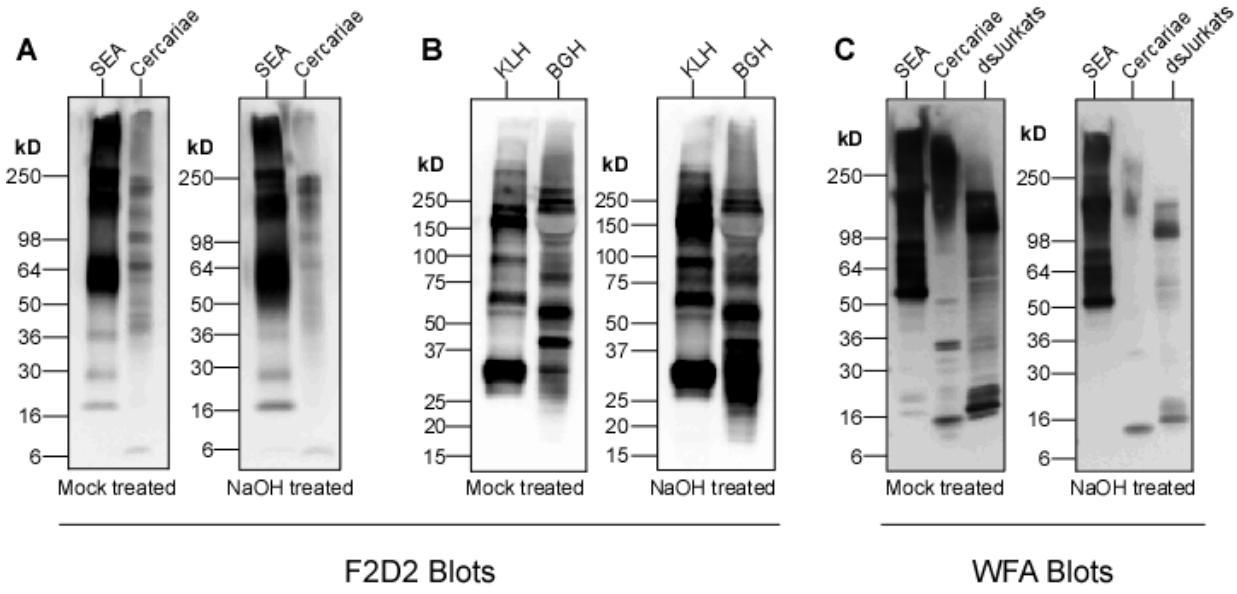


Figure 2.5. F2D2 western blot analysis of extracts after mock treatment or treatment with sodium hydroxide NaOH.

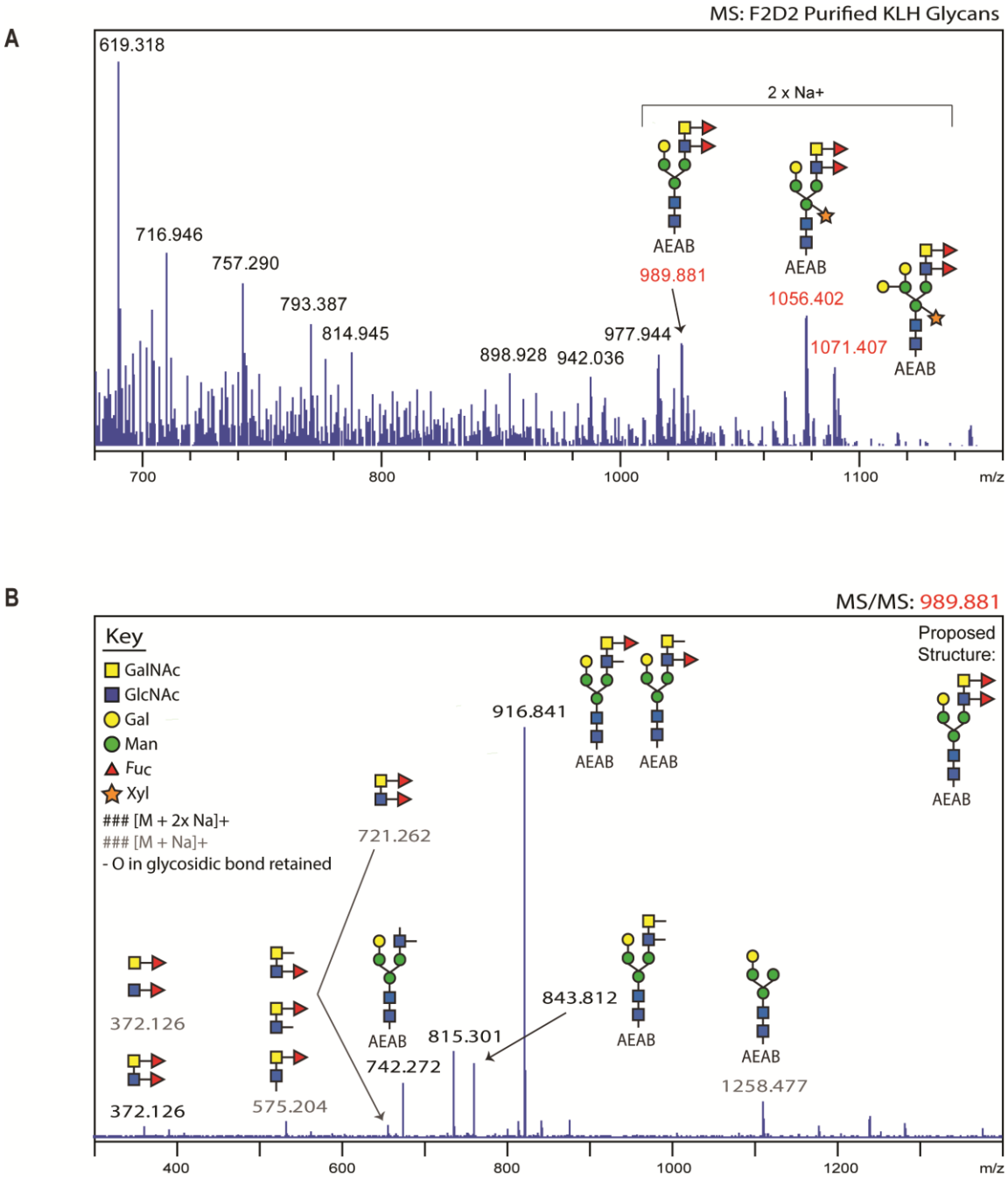
(A) SEA (5 μg) and detergent extract of cercariae (25 μg) and **(B)** KLH (25 μg) and BGH (20 μl) were separated in duplicate by SDS-PAGE and blotted onto PVDF membranes. One set of membranes were incubated overnight at 37°C in 55 mM NaOH and the other set of membranes were mock treated by carrying out the incubations in water. The membranes were washed with water, blocked with BSA and incubated with 10 μg/ml of mAb F2D2. Bound antibodies were probed by incubation with peroxidase conjugated goat anti-mouse IgG followed by incubation with SuperSignal chemiluminescence substrate and imaging on UVP EC-3 digital imager. **(C)** SEA (μg), detergent extract of cercariae (25 μg) and detergent extract of desialylated jurkat cells (ds-Jurkats) were separated by SDS-PAGE in duplicate, blotted onto PVDF membranes and incubated in 55 mM NaOH or water as described. The membranes were washed, blocked in BSA solution and incubated in biotinylated WFA (2μg/ml). Bound lectin was detected by incubation with peroxidase conjugated streptavidin followed by incubation with SuperSignal chemiluminescence substrate.

To control for the process of β-elimination, detergent extracts of Jurkat cells which have a genetic defect in the O-glycosylation pathway that results in the generation of truncated, terminal O-GalNAc and sialylated O-GalNAc were also analyzed (375). The Jurkat cells were treated with neuraminidase to remove sialic acid residues and generate more O-GalNAcs structures for

detection of loss of O-GalNAc epitopes by WFA that binds terminal GalNAc residues (376). Loss of the majority of WFA binding following mild NaOH treatment of Jurkat cell extracts provides a positive control for validation of the *in situ* β-elimination of O-linked glycans on the PVDF membrane (**Figure 2.5C**). Taken together, the PNGase F and mild base treatments show that the fucosylated epitopes bound by mAb F2D2 are present on N-glycans rather than O-glycans and the observed resistance to PNGase F treatment may be due modifications on the chitobiose core of the N-glycans that are known to render N-glycans resistant to cleavage by the endoglycosidase (164,281).

2.3.7 *Identification of the structure of the glycan epitope bound by mAb F2D2 using AEAB-labeled KLH derived glycans*

Due to limited quantities of schistosome material and the resistance of the F2D2 binding glycans in SEA to PNGase F cleavage, KLH was used as a source for the purification and elucidation of the structures of fucosylated N-glycans bound by mAb F2D2. Structural studies of KLH glycans have revealed some unique, heterogeneous glycosylation with the cross reactive epitope, Fucα3GalNAc, existing on less than 5% of the total glycans (109,110,310). To determine whether this is the structure that F2D2 is binding, KLH glycans were isolated, affinity purified using immobilized F2D2, and structurally characterized by MS.


A
MS: F2D2 Purified KLH Glycans
619.318
716.946
757.290
793.387
814.945
898.928
942.036
977.944
989.881
1056.402
1071.407
2 x Na+
AEAB
AEAB
AEAB
700
800
1000
1100
m/z
B
MS/MS: 989.881
Key
GalNAc
GlcNAc
Gal
Man
Fuc
Xyl
[M + 2x Na]+
[M + Na]+
- O in glycosidic bond retained
Proposed Structure:
916.841
721.262
372.126
372.126
575.204
742.272
815.301
843.812
1258.477
AEAB
AEAB
AEAB
AEAB
AEAB
400
600
800
1000
1200
m/z

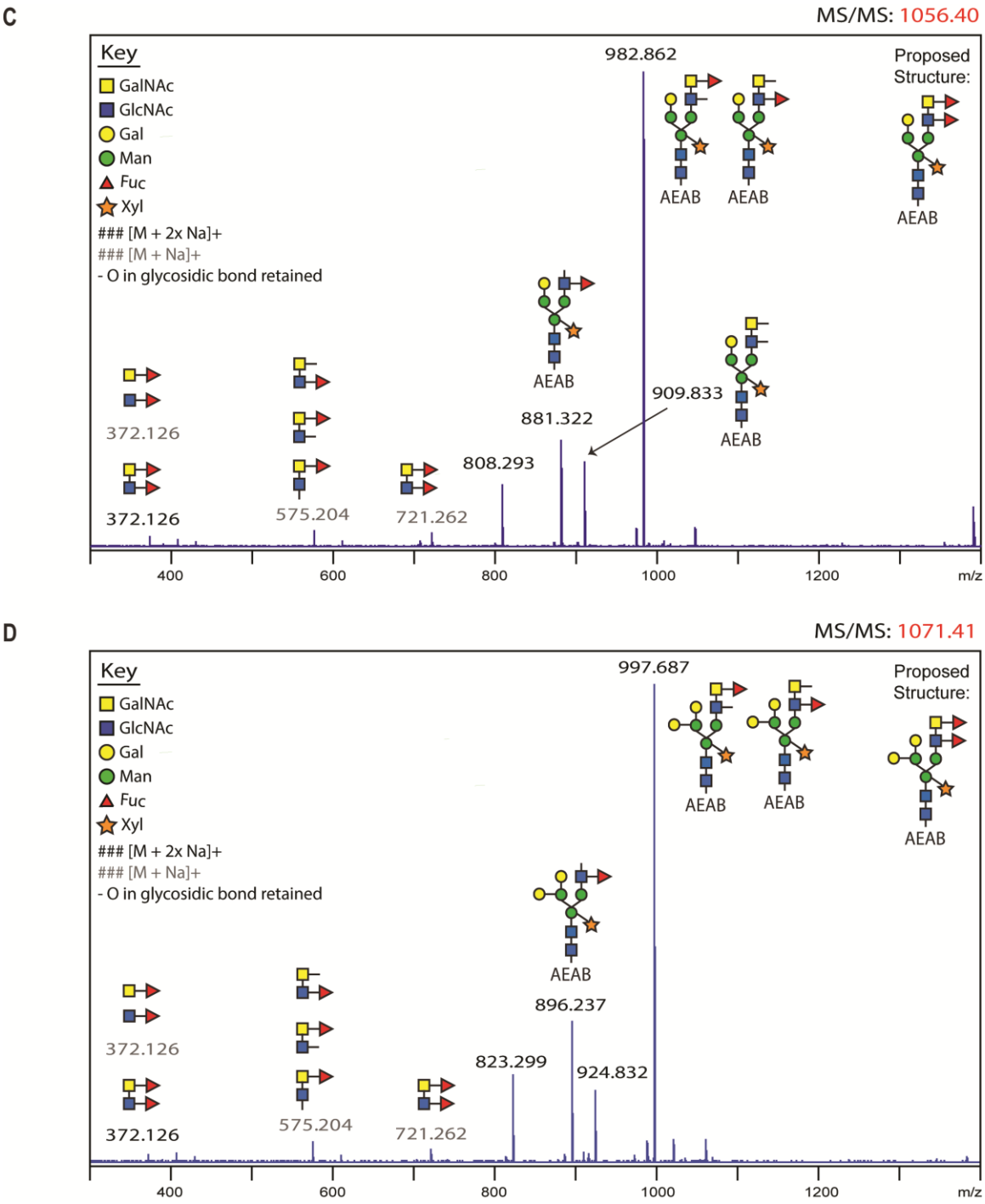


Figure 2.6. Mass spectra for F2D2 purified glycans from KLH containing the FLDNF structure.

Overall mass spectra **(A)** and MS/MS (MS^2) of peaks 989.881 **(B)**, 1056.40 **(C)**, and 1071.41 **(D)** from F2D2 purified KLH glycans, with proposed structure assignments for peaks and major fragments. High charge state fragments (2x Na+) in black, with single

charge in grey (Na+). Reducing end fragments are shown linked to AEAB; non-reducing end fragments are shown as free oligosaccharides.

The KLH glycans were released from denatured, trypsinized proteins by treatment with N-glycosidase PNGase F followed by separation of released glycans from unreleased peptidic material over C18 Sep-Pak and Carbograph columns as described in "Marterials and Methods". The recovered free-reducing N-glycans were conjugated to AEAB, and affinity purified via an F2D2-ultralink column. Mass analysis of the F2D2-bound KLH GAEABs resulted in several samples with peaks suggesting glycan compositions that correlate with documented KLH/*Schistosoma* cross reactive N-glycans **(Figures 2.6**) (109,110,310). These peaks, magnitudes 989.881, 1056.402, and 1071.407, suggest determinants containing 2 deoxyhexose (deoxyHex), 4-5 hexose (Hex), 4 N-acetylhexoseamine (HexNAc) residues, and some glycans contain a pentose, presumably xylose. MS/MS of these peaks, shown in **Figure 2.6B-D**, support the proposed structures consisting of terminal FLDNF on a biantennary N-glycan core, 1-2 Gal moieties, and core β-xylose in higher molecular weight peaks. Our deduction about the glycan composition, linkage, and branching are based on previously published, well characterized KLH glycan structures (109,110,310). From this data we propose that FLDNF is the glycan epitope of F2D2 and an immunodominant target in *S. mansoni* egg glycans that is recognized by antibodies in sera from infected animals.

2.4 Discussion

Monoclonal antibody F2D2 was generated from hybridoma clones prepared from splenocytes of Swiss Webster mice infected for 10 wk with *S. mansoni* cercariae as described previously (115,367) and were selected by ELISA against periodate-sensitive epitopes of SEA **(Figure 2.1A, B)**. The carbohydrate binding specificity was defined by testing the binding of mAb F2D2 towards both defined schistosome-type glycan antigens and to glycoproteins and proteins

reported to contain epitopes that are shared with schistosomes. F2D2 was distinct because it did not bind to any of the defined schistosome glycan antigens, including Le^x, LDN and LDNF. Alternatively, F2D2 bound to glycan epitopes on glycoproteins from the hemolymphs of keyhole limpets and *Biomphalaria glabrata* snail hosts of *S. mansoni* **(Figure 2.2A)**. The binding of F2D2 to SEA, KLH and BGH were all inhibited by free fucose, abolished by α-fucosidase treatments **(Figure 2.3)**, and were also sensitive to PNGase F treatments demonstrating the epitope was a fucosylated N-glycan **(Figure 2.4)**. MS analysis of PNGase F released glycan fraction from KLH affinity purified over columns of immobilized mAb F2D2 gave glycan mass values consistent with N-glycans with a difucosylated LDN outer branch **(Figure 2.6)**.

It has long been known that schistosome share a cross-reactive immunogenic glycan epitope with keyhole limpet hemocyanin (372,377,378), and recent studies show that the cross-reactive epitope is FLDNF epitope (109,110,269). Based on the finding in our study that F2D2 binds to both SEA and KLH, it can be deduced that the epitope in SEA bound by the mAb is FLDNF rather than LDN-dF, considering LDN-dF epitope has not been proven to be present in KLH (109) and antibodies to LDN-dF do not bind to KLH. KLH has been proposed for use as a serodiagnostic antigen for schistosomiasis based on it's reactivity towards sera from infected humans and animals (377,379-381). However, the use of KLH for the diagnosis of schistosome infection has not been feasible thus far, primarily because of the observation that sera from *Trichinella spiralis* infected patients also bind KLH (382). It is possible that the sera from *T. spiralis* patients bind to a distinct set of the complex glycan structures present on KLH glycoproteins, which are separate and distinct from the FLDNF structures (383). The availability of mAb F2D2 that affinity binds the cognate targets in KLH quantitatively should now allow the direct purification of the cross-reactive glycans for assessment of their serodiagnostic potential. At the present time, it is not clear whether there are antigenic glycan epitopes other than FLDNF glycans in KLH. The availability of mAb F2D2 provides a means to affinity deplete FLDNF

epitopes from KLH for testing for the presence of any other antigenic glycans reactive towards sera from *S. mansoni* infected humans and animals. The use of mAb F2D2 for immunoaffinity purification and depletion of FLDNF epitopes is technically feasible because of the high yield of stable antibody from purification processes.

The immunoaffinity purified glycans and glycoproteins can also be used in immunization studies. It was reported previously that immunization of rats with KLH provided up to 70% protection against *S. mansoni* infections and that the cross-reactive epitope was glycan in nature (231). The nature of the immunogenic glycan epitope responsible for the protection was not known. KLH glycoproteins affinity purified over columns of immobilized mAb F2D2 can be used directly for immunization studies. Alternatively, immobilized F2D2 could be utilized to purify FLDNF glycans from released KLH glycans and formulated into conjugates to determine the protective efficacy of the FLDNF glycans themselves. Affinity purified glycans can also be used to study the structure of the cross-reactive epitope in KLH and validate FLDNF glycan as the epitope in SEA bound by mAb F2D2.

Monoclonal antibody F2D2 should also serve as a very useful analytical tool in the study of many aspects of the biological function of the epitope. The antibody should now permit the study of the expression of the epitope during schistosome development in both the vertebrate and snail hosts. Interestingly, Western blot analysis of hemolymph from *S. mansoni* infected and uninfected *B. glabrata* snails reveal several low molecular weight glycoproteins in the hemolymph of the infected snail. It will be interesting to use mAb F2D2 to affinity purify and sequence the proteins from the hemolyph of infected snails to determine whether the proteins are of snail or parasite origin. A recent study reports a correlation between susceptibility and expression of FLDNF epitopes on snail hemolymph glycoproteins (384). The authors report that hemolymph from susceptible Puerto Rican *B. glabrata* snails expressed FLDNF epitopes on numerous glycoproteins while hemolymph from the resistant *B. glabrata* BS-90 strain were negative for FLDNF epitopes as determined by Western blots. Thus, mAb F2D2 will be a very useful reagent in studying snail

susceptibility and resistance particularly in regard to snail age and susceptibility to infection with miracidia. Finally, mAb F2D2 will be useful in assaying for the activity of the fucosyltransferase involved in the biosynthesis of the epitope as well as the expression cloning of the fucosyltransferase.

In conclusion, mAb F2D2 will provide a useful tool for the study of many aspects of the role played by the difucosylated glycan in the development and immunobiology of the parasites in both the vertebrate and molluscan hosts.

2.5 Acknowledgments

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Chapter 3. Identification of antigenic glycans from *Schistosoma mansoni* using a shotgun egg glycan microarray

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3.1 Introduction

Schistosomiasis is a major health problem in endemic tropical and subtropical areas, with more than 200 million people actively infected and 800 million at risk of contracting the disease (11,44,385). Current treatment for disease is limited to the drug praziquantel (38), but cases of drug resistance have been reported (39). Decades of research on schistosomiasis vaccines have yielded only two candidates for clinical trials, and no encouraging results are yet published (57,62,64,386). Thus, there is an urgent need to develop more sensitive diagnostic methods and to identify new vaccine candidates.

Recent studies have shown that a major part of the host immune response to infection is directed against carbohydrate (glycan) antigens in glycoproteins and glycolipids (1,77,80,127,387-390). A wide variety of unusual antigenic determinants include glycans containing the LDN, fucosylated LDN sequences (LDNF, LDN-dF, FLDN, and FLDNF), Le^X, poly-Le^X, core α3 fucose, and core β2 xylose structures (**Table 3.1**) (1,77,80), many of which are expressed by all developmental stages of schistosomes (168). Interestingly, mAbs specific to these glycans recognize these antigens on the surface of 3 hour-old schistosomula and some anti-glycan antibodies can mediate killing *in vitro* in a complement dependent fashion (116,168,231,235). *S. mansoni* infected rhesus monkeys, which are known to self-cure after infection, have IgG to many glycan antigens, including Le^X, LDN, LDNF, core fucose, and core xylose determinants, and their sera are effective in complement-mediated cytolysis of cells expressing Le^X as well as schistosomula larvae *in vitro* (34,271,305). However, it is unknown whether anti-glycan antibodies contribute to parasite killing by animal sera *in vivo*.

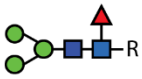
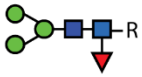
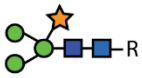
Glycan Sequence	Shorthand	Structure
I. LacNAc termini		
Galβ1-4GlcNAc-R	LN	
Galβ1-4(Fucα1,3)GlcNAc-R	Lewis X (LeX)	
II. LacdiNAc termini		
GalNAcβ1-4GlcNAc-R	LDN	
GalNAcβ1-4(Fucα1-3)GlcNAc-R	LDNF	
GalNAcβ1-4(Fucα1-2Fucα1-3)GlcNAc-R	LDN-dF	
Fucα1-3GalNAcβ1-4GlcNAc R	FLDN	
Fucα1-3GalNAcβ1-4(Fucα1-3)GlcNAc-R	FLDNF	
III. Core modifications		
Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4 GlcNAc-R	Man3GlcNAc2 (M3GN2)	
Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4 (Fucα1-6)GlcNAc-R	Core α6 Fucose (c6F)	
Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4 (Fucα1-3)GlcNAc-R	Core α3 Fucose (c3F)	
Manα1-6(Manα1-3)(Xylβ1-2)Manβ1-4 GlcNAcβ1-4GlcNAc-R	Core Xylose (cX)	
		

Table 3.1. Common glycan structures in *S. mansoni.*

Sequences, shorthand notation, and cartoon depiction of common *Schistosoma* glycans.

The induced humoral responses in infected animals and people are generally proportional to the severity of the disease and consist of many different antibody classes and antibody titers to glycan antigens ((271) and reviewed (77,80)). *S. mansoni* infected patients with either intestinal or hepatosplenic disease generate mainly IgM, but also produce IgG and IgA to LDN, LDNF, and Le^X (168). Antibodies to LDN-dF and FLDN, among other glycan structures both known and unknown,

were also found in infected individuals, with higher titers observed in children than in adults (240,243). Although the role of these glycans and the significance of anti-glycan antibodies in schistosomiasis are not known, glycan antigens may be used as diagnostic tools and/or inform upon new vaccine candidates.

Several studies have focused on defining the schistosome glycome by employing high-resolution liquid chromatography and mass spectrometry techniques (17,74). These global profiling studies have confirmed the major glycan structures previously identified and provided new information on the diversity of glycans and the heterogeneity found among the life stages of the parasite and their secretions (74,111,391,392). Several studies have also demonstrated the feasibility of profiling schistosome glycans using various microarray printing technologies and probing with glycan-binding proteins, antibodies, and antisera, demonstrating that such techniques can be powerful tools for profiling the immune response to this disease (240,265,269,272) and the development or characterization of novel anti-glycan reagents.

The combination of these techniques, i.e. glycomics and/or glycan structural profiling which is guided by the use of immunologically relevant reagents such as antisera and mAbs, has great potential to improve our understanding of the anti-glycan response and uncover novel, immunologically relevant diagnostic and vaccine candidates. We recently found that there were both similarities and substantial differences in the specificity, titers, and isotype composition of anti-glycan antibodies among rhesus monkeys (naturally protected hosts), humans, and mice (chronically infected hosts) based on a small collection of semi-synthetic, schistosome-type biantennary N-glycans (271). In the present study, utilizing methods for fluorescent covalent tagging, separation, and printing of glycans (267,268), we generated a natural N-glycan microarray from *S. mansoni* egg glycoproteins. By interrogating these arrays with sera from different species infected with *S. mansoni*, we identified immunologically relevant fractions for further characterization. Using a combination of mass spectrometry, and lectin and antibody binding studies (metadata-assisted glycan sequencing; MAGS) (266,270), we have identified several glycan

structures that represent the egg N-glycans most prominently recognized by these infected hosts. The shotgun glycan microarray led us to focus on one mAb, F2D2, which targets FLDNF and replicates the binding pattern of infected hosts. This antibody binds all stages of *S. mansoni* and it mediates killing of schistosomula *in vitro*. The results demonstrate the power of incorporating glycomics and immunology to identify disease-relevant glycan antigens.

3.2 Materials and Methods

3.2.1 *Materials*

All chemicals and glycoproteins were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification. HPLC solvents were purchased from Thermo Fisher Scientific (Waltham, MA). PNGase F was purchased from New England Biolabs (Ipswich, MA) and PNGase A was from Sigma-Aldrich. Plant lectins were purchased from Vector Labs (Burlingame, CA) including: PHA-L, *Phaseolus vulgaris* agglutinin L; PHA-E, *Phaseolus vulgaris* agglutinin E; PNA, peanut agglutinin; WFA, *Wisteria floribunda* agglutinin; WGA, wheat germ agglutinin; LTL, *Lotus tetragonolobus* lectin; AAL, *Aleuria aurantia* lectin; SNA, *Sambucus nigra* agglutinin; RCA I, *Ricinus communis* agglutinin I; ConA, concanavalin A; UEA I, *Ulex europaeus* I; MAA, *Maackia amurensis* agglutinin, BPL, *Bauhinia purpurea* lectin, and GSI-B4, *Griffonia simplicifolia* lectin-I. The lectin HPA, *Helix pomatia* agglutinin was purchased from Sigma-Aldrich. An anti-LDN antibody (IgG - clone Y1H5), an anti-LDNF (IgG - clone L6B8), an anti-Le^X (IgM - clone 5F1), two anti-LDN-dF (IgM - clone 290-2D9-A, IgM – clone 290-4A8), an anti-$Man_3GlcNAc_2$ mAb (M3GN2, IgM – clone 100-4G11-A), and an anti-FLNDF antibody (IgG – clone F2D2) were developed as mAbs by production of hybridomas from spleens of mice that had been infected with *S. mansoni*, using methods described previously (244,351,390,393,394). The anti-HRP antibody (anti-peroxidase, polyclonal rabbit IgG, P7899) was purchased from Sigma-Aldrich and used to detect core α3 fucose and core xylose (CF/CX) determinants (395,396). Secondary antibody

conjugates goat anti-human, anti-mouse, and anti-rabbit (Alexa-488 anti-human IgM, Alexa-555 anti-human IgG, Alexa-488 anti-mouse IgM, Alexa-555 anti-mouse IgG, Alexa-633 anti-mouse IgG, Alexa-488 anti-rabbit IgG) and Cy5-streptavidin were from Invitrogen (Carlsbad, CA). Goat anti-mouse HRP-conjugated secondary antibody was purchased from KPL (Gaithersburg, MD). SuperSignal West chemiluminescent substrates were purchased from Thermo Scientific (Rockford, IL). N-hydroxysuccinimide (NHS)-activated slides were purchased from Schott (Elmsford, NY). Printing of glycan arrays was performed using a Piezorray Printer (PerkinElmer, Waltham, MA) and analysis of glycan arrays was accomplished by scanning with a ProScanArray Scanner (Perkin Elmer) equipped with 4 lasers. Percoll was purchased from GE Healthcare (Piscataway, NJ). DMEM was purchased from Cellgro (Manassas, VA). Penicillin/streptomycin was purchased from Gibco (Grand Island, NY). Standard guinea pig complement was purchased from Cedarlane (Burlington, NC). Fetal bovine serum was purchased from Atlanta Biologicals (Lawrenceville, GA). DAPI was purchased from Life Technologies (Foster City, CA). 96-well microplates were purchased from Greiner Bio-One (Frickenhausen, Germany).

3.2.2 *Isolation of Schistosoma mansoni Eggs and Life Stages*

S. mansoni eggs were obtained from livers of infected Swiss Webster female mice (n=30) 7wks after infection with 150 cercariae per mouse. Livers were perfused with perfusion fluid (0.85% NaCl, 0.75% trisodium citrate dihydrate), homogenized in 1.7% NaCl, and the eggs collected by filtration over a series of mesh filters as described (366). Eggs were washed repeatedly by low speed centrifugation to remove any liver tissue and wet pellets of 1-2g were stored frozen at -70^{o}C.

S. mansoni-infected *Biomphalaria glabrata* snails, strain NMRI NR-21962, were provided by the Schistosome Research Reagent Resource Center through BEI Resources, NIAID, NIH. Snail maintenance, collection of cercariae, transformation to schistosomula, and isolation of adult worms from mice was conducted as previously described (272,397). The schistosomula were cultured for

2-3 days in DMEM with 10% FBS, penicillin/streptomycin at a density of 500-1000 organisms/mL in tissue-culture dishes. 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 in compliance with University-approved Biosafety and IACUC protocols.

3.2.3 *Infected Sera*

Mouse serum was obtained and pooled from Swiss Webster mice (n=10) infected with ~60 cercariae of *S. mansoni* Puerto Rican strain after 7wks or 8wks, and 20wks of infection under an approved IACUC protocol at Emory University. Rhesus monkey sera was obtained from 4 monkeys (Chinese rhesus macaques, *M. mulatta*) infected percutaneously with 500 cercariae and sera collected during the course of the infection at various time points from 8wks-78wks post-infection from an IACUC-approved study conducted at the Division of Parasitic Diseases and Malaria at the National Centers for Disease Control and Prevention (CDC). Infections were monitored by examination of eggs in stool samples. Sera from infected humans, four occupationally exposed Kenyan individuals (H-19, H-26, H-42, H-59) who were employed as sand harvesters in the Lake Victoria area and five Peace Corp volunteers in Africa (H1-H5), was provided by the CDC with approval by the ethical review boards of the Kenya Medical Research Institute.

3.2.4 *Schistosome Egg Glycan Isolation and Labeling*

S. mansoni eggs (1.0g pellet) were resuspended in 4ml H_2O, homogenized using a glass homogenizer and sonicated (6 pulses/10sec, Misonix 3000) in an ice bath. The glycolipids were extracted by the method of Folch (398). Methanol was added to 2.7 volumes and the sample was sonicated (2 pulses/10sec). Chloroform was added to 1.33 volumes, bringing the extract to 4:8:3 chloroform:methanol:water, and the extract was sonicated (2 pulses/10sec) and centrifuged at 6,000xg for 10min to separate the aqueous phase containing glycolipids from the pellet containing

the glycoproteins. The glycolipids were stored for other analyses, while the glycoprotein pellet was resuspended in 2ml 0.2M Tris-HCl, pH 8.2 and dried under vacuum to remove residual chloroform. The dried extract was resuspended in 2ml of denaturing buffer (8M guanidinium hydrochloride (GnHCl) in 0.2M Tris-HCl, pH 8.2) and 2ml of reducing buffer (0.18M DTT in 0.2M Tris-HCl, 8M GnHCl, pH 8.2) and incubated for 1h at 25°C. Alkylation was done by addition of 7.75ml of 0.18M iodoacetamide in 0.2M Tris-HCl, 8M GnHCl, pH 8.2 and incubated for 30min at 25°C. The extract was then dialyzed against 8L of water using a 10,000 Da molecular weight limit membrane at 4°C overnight and freeze-dried under vacuum. The dried material was then resuspended in 3ml of 50mM phosphate buffer, pH 8.2, and digested with trypsin (TPCK treated, Sigma), at 100μg/ml for 12h at 37°C.

N-glycans were released from tryptic glycopeptides by treatment with PNGase F. The 3ml glycopeptide mixture was first boiled for 5min to inactivate the trypsin, adjusted to pH 7.5, and 10μl of PNGase F (NEB 500,000 U/ml) plus NaN_3 (0.2%) was added and incubated at 37°C for 24h. After digestion, the solution was boiled for 5min, passed through a C18 Sep-Pak cartridge (2g) with 2 column volumes (CVs) of water, and the flow-through was collected and passed through a 500mg Carbograph cartridge (Grace Discovery Science), to which free glycans bound. The Carbograph column was washed with water (6 CVs), and the bound glycans were eluted with 3 CVs of 50% acetonitrile in 0.1% trifluoroacetic acid (TFA) and dried under vacuum before labeling. To release potential N-glycans that are resistant to PNGase F, as has been seen for N-glycans containing core α3-fucose residues, the glycopeptides retained in the C18 column were eluted with methanol (2 CVs), dried under vacuum, and digested with the glycosidase PNGase A. Digestion was performed by resuspending the dried glycans in 1.0ml of 0.1M citrate-phosphate buffer, pH 5.0, addition of 10μl of PNGase A (from almonds, 60 U/μl) and incubation at 37°C for 24h. The PNGase A released glycans were purified over C18 Sep-Pak and Carbograph columns as before, and dried under vacuum before labeling.

The N-glycan preparations (PNGase A and F digested) were labeled with the bifunctional fluorescent linker 2-amino-N-(2-aminoethyl)-benzamide (AEAB) which was synthesized in the lab and used as described (268). Dried glycans were resuspended in 0.1ml of 0.35M AEAB plus 0.1ml of 1M $NaCNBH_3$ in DMSO:AcOH (7:3 v/v), and incubated at 65^{o}C for 2h. After cooling, 2ml of acetonitrile was added, the sample incubated at -20^{o}C for 30min, and centrifuged at 6,000xg for 10min. The pellets were then dissolved in 0.2ml water for separation by HPLC.

3.2.5 *Separation of AEAB-labeled Glycans (GAEABs) by HPLC and Mass Spectrometry Analysis*

HPLC separations were performed with a Shimadzu HPLC CBM-20A system coupled to a UV detector SPD-20A (set at 330nm) and a fluorescence detector RF-10Axl (set at 330nm/420nm excitation/emission). Both UV absorption and fluorescence intensity were used for the quantification of GAEABs using LNFPIII-AEAB as a standard. The first dimension HPLC separation was done by injection of 199µl of sample into a reverse phase C18 column (250mm x 4.6mm) with mobile phases consisting of acetonitrile and water with 1% TFA. The concentration of acetonitrile increased from 1% to 5% in 40min, and 5% to 20% in 15min. Each peak fraction was collected, quantified by fluorescence, dried under vacuum, and resuspended to a final concentration of 200mM before being used for microarray printing. In addition, each fraction was analyzed by MALDI-TOF mass spectrometry in an Ultraflex II system from Bruker Daltonics (Billerica, MA). GAEAB fractions obtained in the first dimension HPLC, and showing positive results by microarray when probed with lectins and sera, were further separated in a second dimension. The second dimension HPLC was performed using a PGC column (150mm x 4.6mm, Thermo Scientific). The mobile phase was acetonitrile and water with 0.1% TFA, with the acetonitrile gradient increasing from 25% to 55% in 40min. Fractions were collected, quantified by fluorescence, dried under vacuum, and resuspended in water to a final concentration of 100mM before being used for microarray printing.

3.2.6 *Array printing, Binding Assays, and Scanning*

Non-contact printing was performed using a Piezorray Printer. All samples were printed in phosphate buffer (300mM sodium phosphates, pH 8.5). The average spot volume was within 10% variation (intra-tip) of 1/3 nanoliter. Each compound was printed at 100mM and in replicates of six. After printing, slides were placed in a high moisture chamber at 50ºC and incubated for 1h. The slides were washed and blocked with 50mM ethanolamine in 0.1M Tris base, pH 9.0, for 1h, subsequently dried by centrifugation, and stored desiccated at -20ºC. Before assay, the slides were rehydrated for 5min in TSM buffer (20mM Tris-HCl, 150mM NaCl, 2mM $CaCl_2$ and 2mM $MgCl_2$).

Biotinylated lectins and mAbs were used in binding assays to validate each slide for printing efficiency using control GAEABs and for the characterization of schistosome GAEABs. Microarray slides were first assembled in a 16 chamber cassette and washed for 5min with 200µl/well of TSM buffer plus 0.05% Tween-20. Lectins were added at 10µg/ml in TSM binding buffer (TSM buffer plus 0.05% Tween-20, 1% BSA), incubated for 1h at 25ºC, and the slides washed 3 times/5min each in TSM wash buffer (TSM plus 0.05% Tween-20). Slides were then washed 4 times in TSM, and Cy5-SA was added at 5µg/ml, and incubated for 1h at 25ºC in the dark. mAbs and polyclonal antibodies were added at 0.125-20µg/ml in TSM binding buffer or as undiluted hybridoma supernatant, incubated for 1h at 25ºC, and the slides washed 3 times/5min each in TSM wash buffer. Appropriate fluorescently labeled secondary antibodies (Alexa-488 anti-mouse IgM, Alexa-633 anti-mouse IgG, or Alexa-488 anti-rabbit IgG) were added at 2.5-5µg/ml, and incubated for 1h at 25ºC in the dark. After final incubation, the slides were washed 4 times in TSM wash buffer, once in water, dried by centrifugation, and scanned. For serum screening, 200µl of sera diluted in TSM binding buffer (1:100 dilution mouse sera, 1:1000 dilution for rhesus and human sera) was added per well and the slides incubated for 1h at 25ºC. Slides were washed 3 times/5min each in TSM wash buffer, and fluorescently labeled secondary antibodies (Alexa-488 anti-mouse IgM, Alexa-555 anti-mouse IgG, or Alexa-633 anti-mouse IgG) at 5µg/ml in TSM

binding buffer were added for 1h at 25ºC. Following 4 washes in TSM, a rinse in water, and drying by centrifugation, the slides were scanned.

For multi-panel experiments on a single slide, the array layout was designed using Piezorray software according to the dimension of a standard 16-chamber adaptor. The adaptor was affixed to the slide to separate a single slide into 16 chambers sealed from each other during the assay. The slides were scanned with a Perkin Elmer ProScanArray microarray scanner equipped with 4 lasers spanning an excitation range from 488 to 647nm. The scanned images were analyzed with ScanArray Express software and basic statistical methods were applied. Fluorescence scanning for Cy5 was at 649nm/670nm (excitation/emission), and as specified for Alexa-488 and Alexa-555 labeled antibodies.

3.2.7 *Parasite Lysates, SDS-Page, and Western blots*

Cercariae, schistosomula, adult worms, and eggs were isolated and processed for parasite life stage lysates or soluble egg antigen (SEA) as previously described (272,399). For SDS–PAGE and western blotting, 1μg of SEA and 5μg parasite lysates were boiled in 1× NuPAGE SDS sample buffer + 2.5% β-mercaptoethanol for 10min and then run in 10-well Mini-PROTEAN-TGX gels at 200 V for 30min with broad range protein standards (Spectra multicolor). Protein was transferred to a nitrocellulose membrane using Trans-Blot Turbo semi-dry transfer system. Unless noted otherwise, all subsequent incubations and washes were shaking at ambient temperature. Membranes were blocked for 1 hour in 5% (w/v) BSA (bovine serum albumin fraction V) in TBS wash buffer (20 mM Tris, 300 mM NaCl, 0.05% Tween-20). Incubations with F2D2 antibody were overnight at 4ºC at 1μg/ml in BSA diluent (0.5–1% BSA in TBS wash buffer). The membranes were then washed three times for 10 min each in TBS wash buffer. Secondary detection antibody, HRP-conjugated goat anti-mouse-IgG, was added for 1 hour at 1:20,000 in TBS wash buffer. The same wash procedure was repeated, and then SuperSignal West Pico Chemiluminescent Substrate was added for 30s. The membranes were dabbed dry and exposed to film.

3.2.8 *Immunofluorescence Imaging*

Intact cercariae, schistosomula, and adult worms were fixed in 10% neutral buffered formalin for 24h at 4°C. All subsequent incubations and washes were performed at ambient temperatures and gentle shaking. Parasites were washed 4 times with phosphate buffered saline (PBS) and blocked with 3% BSA in PBS for 1h. F2D2 antibody was added at 10μg/mL and incubated for an additional 1h. As controls, a set of the parasites was incubated with an isotype control mouse IgG antibody. Samples were washed four times in PBS and secondary antibody, Alexa-488-labeled goat anti-mouse IgG, diluted 1:100 in 3% BSA in PBS for 1h. The parasites were washed 4 times with PBS to remove unbound excess antibodies and set on glass slides in mounting media. Slides were imaged at 20X magnification.

3.2.9 *Schistosomula Killing Assay*

Schistosomula were isolated as described previously (272,399). After vortex-transformation, separation from tails via Percoll and washing in DMEM, the newly-transformed schistosomula were cultured for 3 hours at 37ºC in 96-well plates, with 50-100 parasites in 40μl of DMEM with penicillin/streptomycin in each well. The specified antibodies were then added, diluted in 12μl DMEM. At 3.5 hours, 15μl of freshly-thawed, active or heat inactivated (1 hour at 55ºC) guinea pig complement was added (final concentration 1:5) followed by 7.5μl fetal bovine serum (final concentration 10%) and penicillin/streptomycin to final concentration 100U/mL. Each antibody and killing condition was assayed in duplicate or triplicate wells within each assay. The total number of live schistosomula were counted at 0 hours. At approximately 22 hours, 1μl of 100μg/mL DAPI stain was added to each well. Dead schistosomula were counted as DAPI positive at 24, 48, and 72 hours.

3.3 Results

3.3.1 *Preparation of N-glycans from Schistosoma mansoni Eggs*

We prepared N-glycans from schistosome eggs for initial HPLC separation (termed 1-dimensional) in order to identify potential immunologically relevant fractions for further study. The overall workflow for the release, conjugation, separation and glycan microarray analysis of N-glycans from *S. mansoni* eggs is shown in **Figure 3.1**, and is described in Materials and Methods. N-glycans were released by consecutive treatments with PNGase F and PNGase A based on previous studies showing the presence of core α3-fucose in schistosome egg glycans (17,74,105,111). PNGase F treatment releases N-glycans that lack α3-fucosylation in the chitobiosyl core, but susceptible glycans may be α6-fucosylated and β-xylosylated in their cores. Successive treatment with PNGase A can efficiently release α3-fucosylated N-glycans (400), and any residual N-glycans if the first digestion is incomplete. The N-glycans released by PNGase F and A were enriched by not binding to C18 Sep-Pak and binding to Carbograph cartridges and then conjugated by reductive amination to the bifunctional fluorescent linker AEAB, which permits direct detection and quantitation of total glycan amounts (267,268). The first dimension separation was performed in a C18 reverse phase column with an acetonitrile gradient and the elution monitored by fluorescence.

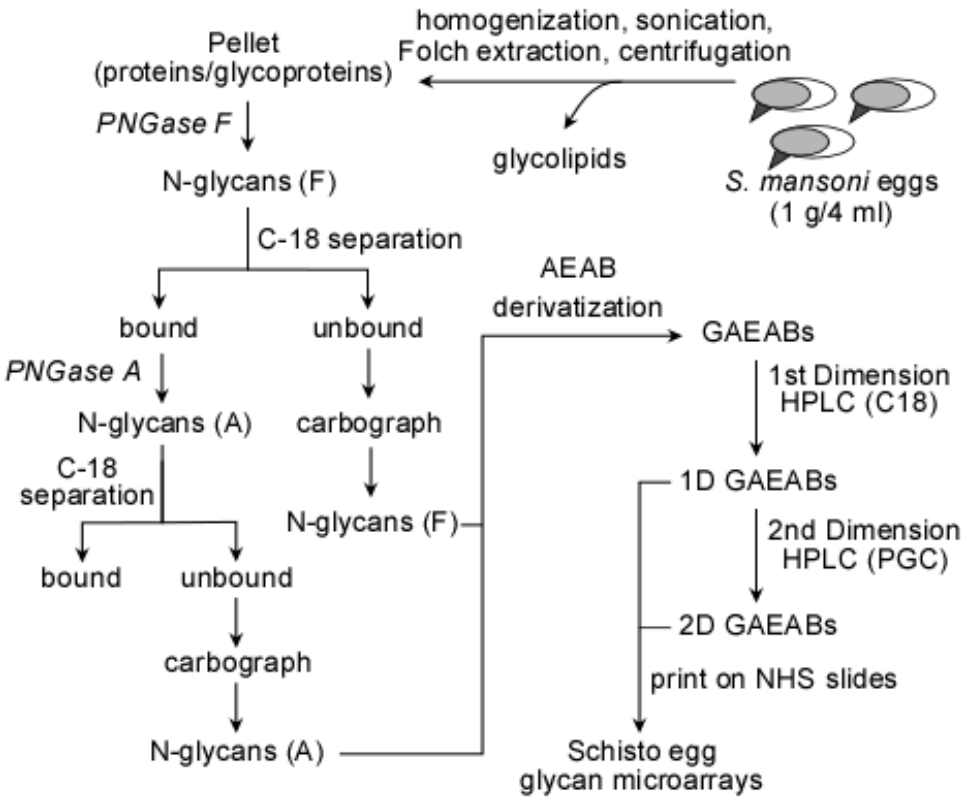


Figure 3.1. Strategy for the preparation of *S. mansoni* egg glycan microarrays.

S. mansoni egg N-glycans are released from total glycoproteins, conjugated to the bifunctional fluorescent linker AEAB, and the derivatized glycans (GAEABs) separated by 2 dimensional HPLC and printed on NHS-activated glass slides for interrogation.

HPLC purification showed 20 major PNGase F-released glycan peaks designated as fractions F1-F20 (**Figure 3.2A),** and 14 PNGase A-released peaks designated as fractions A1-A14 (**Figure 3.2B**). Each collected fraction was quantified by absorbance at 330nm and the area under each peak used for determining the total concentration of glycans released and purified. From 1.1 grams of egg pellet processed, the total amount of N-glycans recovered after separation by HPLC was 321 nmol released by PNGase F and 85 nmol released by PNGase A, on the order of 0.01 – 0.1% of the total material by weight. Each peak fraction was printed onto NHS-activated glass slides at 100μM in the master plate. Since none of the glycans were identified prior to printing on

slides at equal concentrations, the microarray was termed a schistosome shotgun glycan microarray (SSGM) and represents the 1st dimension (1D-SSGM).

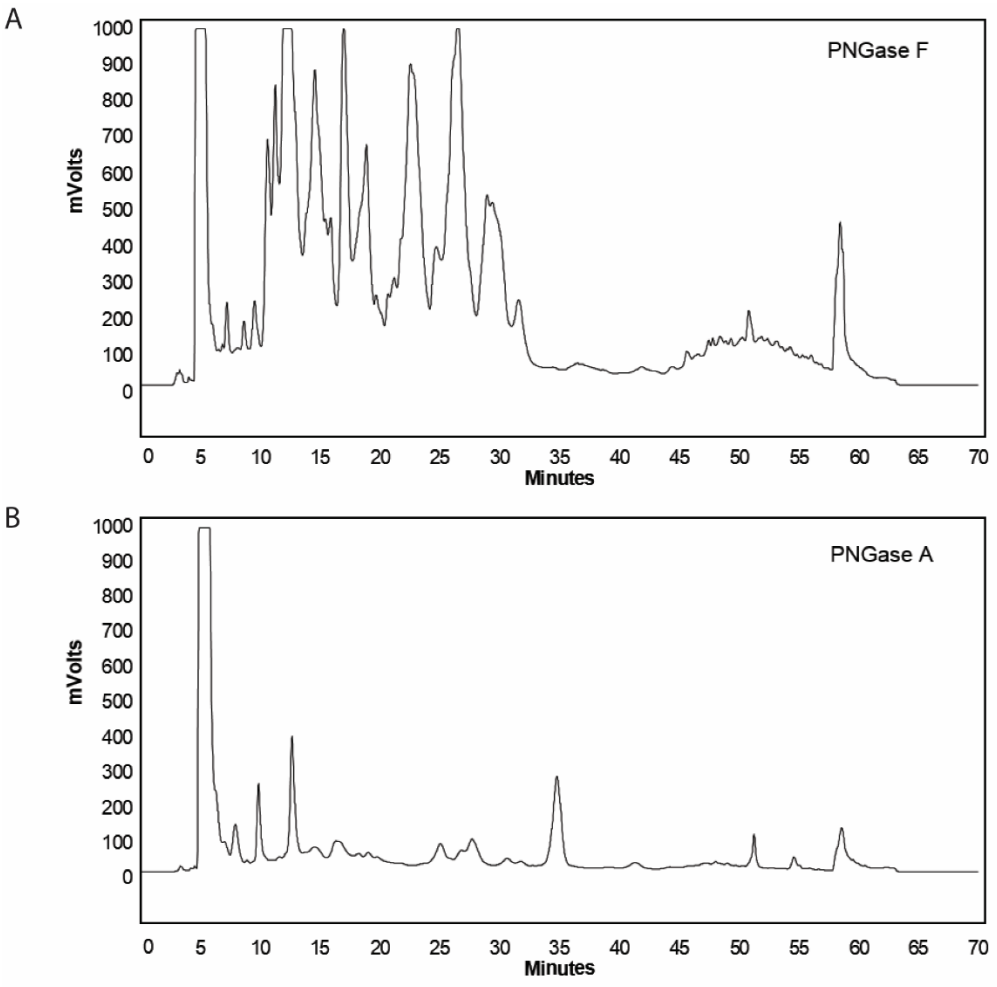


Figure 3.2. First dimension HPLC separation of *S. mansoni* egg GAEABs.

N-glycosidase released glycans from total egg glycoproteins were conjugated to AEAB and the derivatized glycans (GAEABs) separated by reverse phase HPLC on a C18 column. A total of 20 peak fractions were obtained from the **(A)** PNGase F treated material, while the **(B)** PNGase A treated material resulted in 14 peak fractions as detected by 330nm fluorescence.

Microarray slides consisting of the fractions F1-F20, A1-A14, and negative controls (PBS, biotin), were printed with hexa-replicate samples. The 1D-SSGM slides were incubated with a

panel of 13 different lectins of known specificity to establish features of the glycan determinants and validate glycan printing. As shown in **Figure 3.3A**, most PNGase F-released GAEABs were recognized by the fucose-binding lectin AAL (401), with the most prominent binding in fractions F7-F15. Most fractions were also recognized by the mannose-binding lectin ConA, which binds high mannose-, hybrid- and biantennary complex-type N-glycans (402,403), with the strongest binding in fractions F5-F14. Lectins that bound select fractions at lower levels included WGA, which recognizes GlcNAc and N-acetyl-5-neuraminic acid (F1, F3, F11, F15, and F18), WFA, which recognizes terminal α/β-linked GalNAc (F18), and PHA-E and MAA, which recognize bisected, terminal galactosylated N-glycans and α2,3-linked sialic acids, respectively (F14). PNGase A-released GAEABs fractions A3-A9 were also recognized by AAL and fractions A6 and A8 were moderately recognized by Con A (**Figure 3.3B**). Fractions F19, F20, and A14, which are the last fractions eluted from the C18 column, showed low levels of binding to all lectins tested. Aside from F19, F20, and A14, the lectin binding studies showed background levels or only weak binding to egg glycans with the lectins PHA-L, PNA, HPA, SNA, UEA-I, LTL, and RCA-I, which recognize various determinants such as branched and galactosylated N-glycans, blood group H, and terminal sialic acid. This was anticipated, since such epitopes are not characteristic of schistosome glycans but can occur in mouse-derived glycans (266,404), which is a concern since the eggs are prepared from infected mouse organs. Thus, the results indicate that there was not appreciable contamination of the SSGM with mouse-derived glycans and that the schistosome GAEABs separated by the 1D HPLC were primarily recognized by mannose- and fucose-binding lectins. The minimally-purified glycans at this stage represent the PNGase F/A releasable N-glycome and is consistent with previous studies showing the presence of a variety of fucosylated and mannosylated N-glycans in schistosome eggs, miracidia, and egg secretions (17,74,105,111). The development of this array indicates that our methods of isolation and tagging of glycans

generate a glycan library that is representative of the N-glycome of *S. mansoni*, and their interrogation can point to immunologically relevant fractions.

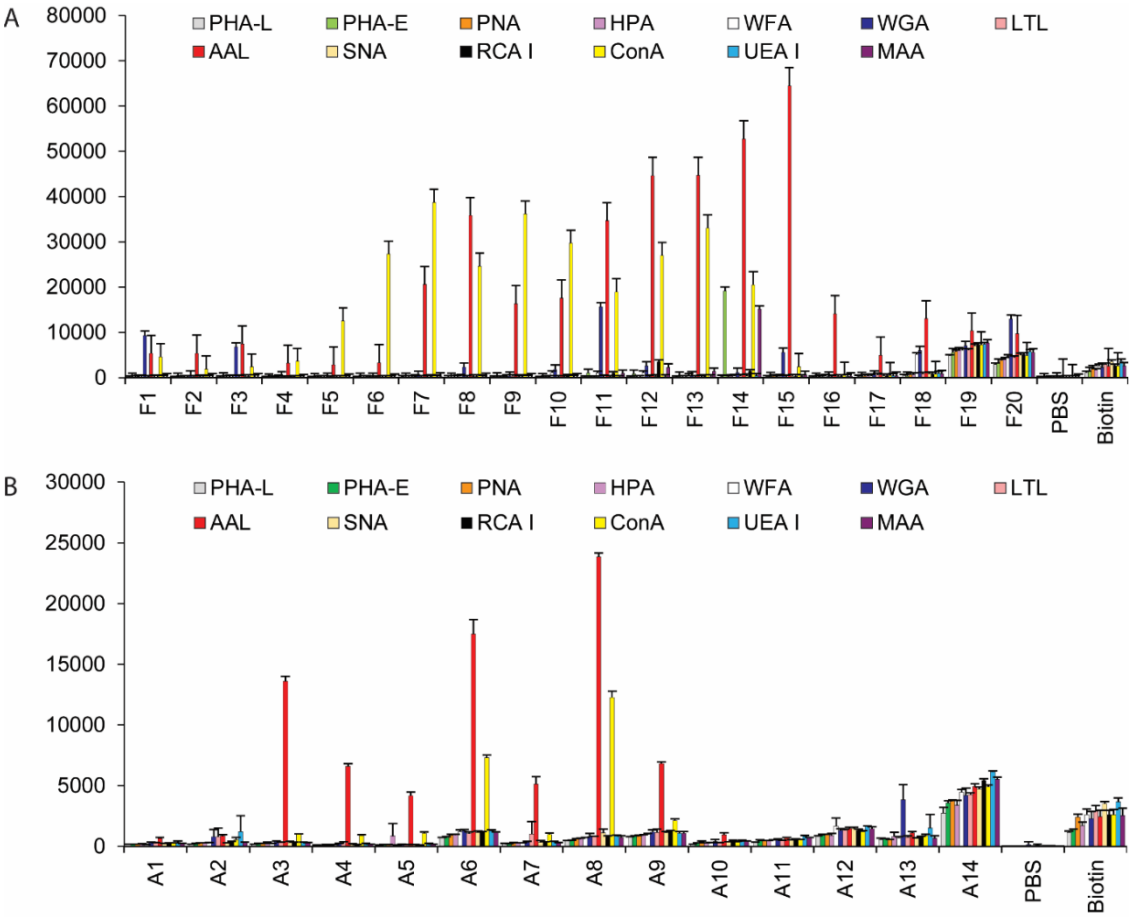


Figure 3.3. *S. mansoni* egg glycans on 1D microarrays are mostly recognized by fucose and mannose binding lectins.

Microarrays containing **(A)** PNGase F obtained GAEAB fractions F1-F20 and **(B)** PNGase A obtained GAEAB fractions A1-A14, when interrogated with a panel of 13 lectins, show that most glycan fractions are recognized by fucose (AAL) and mannose (ConA) binding lectins.

3.3.2 *Identification of Antigenic Egg N-glycan Fractions for Further Purification*

The 1D-SSGM was interrogated with representative sera from acutely and chronically infected mice (pooled), one rhesus monkey, and one human infected with *S. mansoni* to explore whether specific GAEAB fractions might be immunologically relevant (**Figure 3.4**). As shown in **Figure 3.4A**, unexpectedly we found very specific recognition of only a few glycan fractions. For example, only F7-F13 were recognized by IgG in 20wk infected mouse sera, and only F11 was recognized by IgG in 7wk infected mouse sera. F7-F13 were also strongly recognized by IgG in serum from an infected rhesus 8wks post-infection, and binding declined by 78wks after infection (**Figure 3.4B**), at which point the monkeys are considered self-cured, since they showed no detectable eggs in the stool. Serum from an infected human showed a strong IgG response to F7-F13 and F15, with slightly lower levels of reactivity to A13, F1, F3, and F18. IgG binding to most of these fractions was higher before treatment with praziquantel compared to after treatment (**Figure 3.4C**). These results demonstrate that F7-F13 contain natural egg glycan epitopes, predominantly characterized by AAL and ConA binding that are immunologically relevant during infection in mice, rhesus monkeys, and humans. These targets could be significant as antigenic targets for protective antibodies, as immune-modulatory molecules or involved in host-pathogen interactions. Based on this data, we chose these fractions for further purification and analysis.

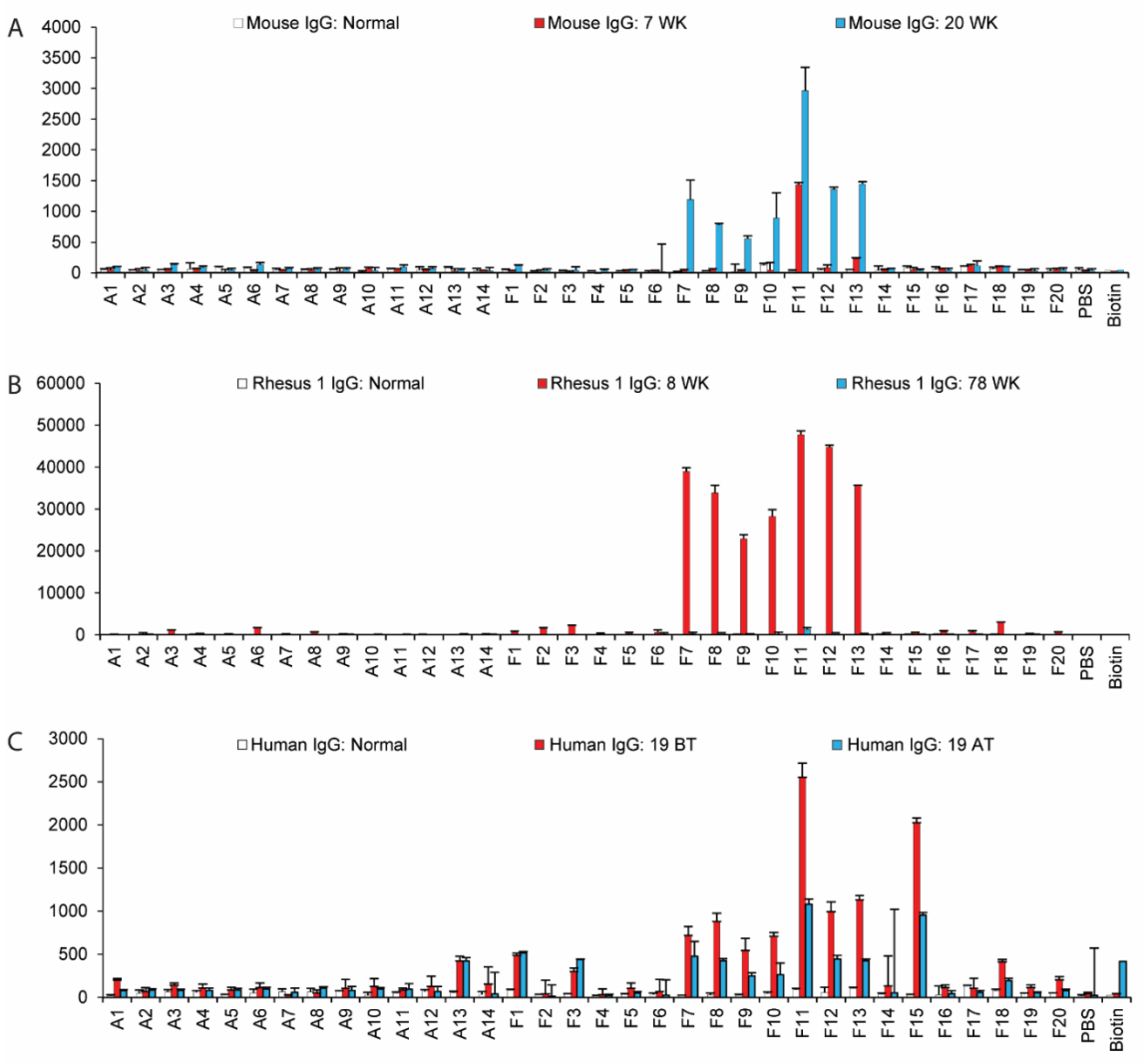


Figure 3.4. A subset of *S. mansoni* egg glycans from the 1D HPLC microarrays are recognized by antibodies in infected sera from mice, rhesus, and human.

Screening of the 1D microarrays with sera from infected hosts shows that GAEAB fractions F7-F13 are recognized by IgG in **(A)** both acute and chronic mice, pooled, **(B)** patent, but not post-patent rhesus, and **(C)** human serum before (BT) and after treatment (AT). Mouse sera were screened at 1:100 while human and rhesus sera were screened at 1:1000.

3.3.3 *Preparation and Characterization of the 2-Dimensional Schistosome Egg Shotgun N-glycan Microarray (2D-SSGM)*

F7-F13 were further separated in a second dimension by HPLC using a reverse phase PGC column and each fraction contained 7-11 peaks for a total of 60 fractions (**Figure 3.5**). Fractions appearing to contain one or a few major GAEABs, or co-eluting GAEABs, were collected and quantified. Fifteen glycan fractions that contained enough material were brought to 100μM and printed on the 2D-SSGM. These fractions, along with parent 1D fractions (F7-0, F10-0, F11-0, F13-0) and 4 controls (LNnT; $Man_5GlcNAc_2$; asialo-bGP (Na2); biotin) were then interrogated with a panel of lectins and several previously characterized mAbs and polyclonal antibodies (**Figure 3.6, Table 3.1**). Most glycan fractions were strongly recognized by ConA and AAL (**Figure 3.6A-B**). Several fractions (F9-4, F11-0, F11-4, F11-5, F11-6) were also recognized by GlcNAc-binding lectin WGA (405,406) and GalNAc-binding lectin BPL (407), while some (F7-4, F7-5, F9-4, F11-4, F11-6) were recognized by the β-Gal-binding lectin RCA-I (402,408). The controls were recognized as predicted by RCA-I, ConA, BPL, and WFA (409-411). The lectins PHA-L, UEA-I, LTL, PNA, GSI-B4, MAA, and HPA showed no significant reactivity of the further separated egg glycans. Therefore, the separation of GAEABs by 2D HPLC allowed the isolation of immunologically relevant glycans with unique lectin binding properties – some of which were not seen in the 1D-separated glycans, probably because in the mixture the relevant glycans were not highly abundant.

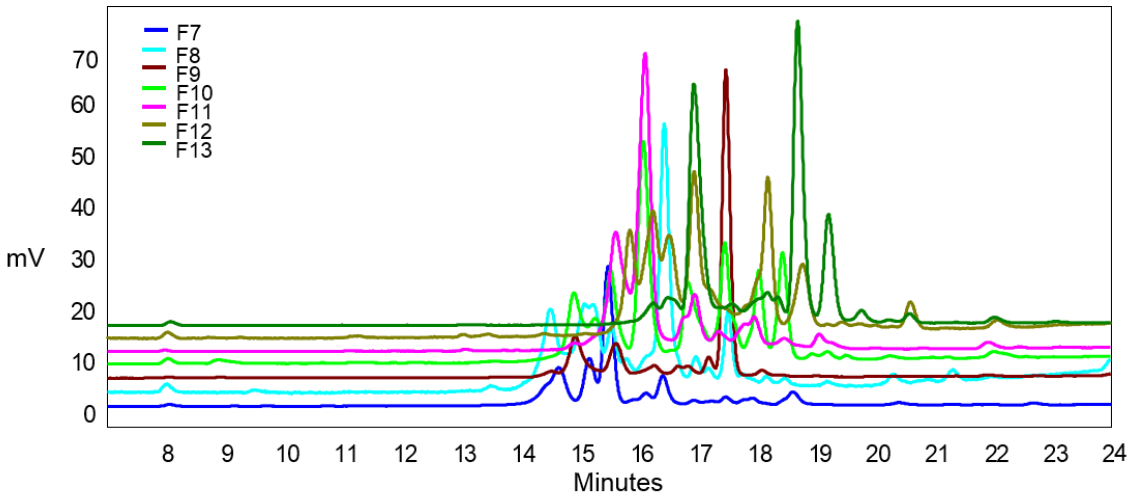


Figure 3.5. Separation of *S. mansoni* egg glycan by 2D HPLC for the 2D-SSGM.

Further separation of GAEAB fractions F7-F13 by reverse phase HPLC utilizing a PGC column reveals a total of 60 peak fractions as detected by 330nm fluorescence.

The array was next interrogated with defined anti-glycan mAbs and polyclonal antibodies (**Figure 3.6C**). We screened with six mouse mAbs derived from schistosome-infected mice, as referenced: anti-LDNF (clone L6B8), anti-LDN (clone Y1H5), anti-Le^X (clone 5F1), anti-M3GN2 (clone 100-4g11-A), and two anti-LDN-dF (clones 290-2D9-A and 290-4A8) (244,272,350,351,394). Anti-LDNF bound fractions F9-4 and F11-4. Interestingly, the anti-LDN, both anti-LDN-dF, and anti-Le^X, the latter of which identifies both terminal and internal Le^X structures, did not bind glycans on this array. Recent glycomics profiling has shown that while PNGase F releasable egg glycans do contain the minimal determinants for LDN, Le^X, and LND-dF determinants (392). However, our studies show that the anti-Le^X, LDN, and LDNF antibodies prefer these determinants on extended chains or non-complex-type N-glycan cores, which may explain the lack of binding to the 2D-SSGM (272,394). In addition, of course, some glycans that may be recognized by these mAb may not occur in significant levels in egg glycoproteins. The anti-M3GN2 antibody (clone 100-4g11-A) recognized fractions F11-0, F11-4, F11-6, and F13-6. In addition to the mAbs generated from infected mice, we also screened with a commercial anti-HRP

antibody produced in rabbits (P7899), which is directed against core β-xylose and core α3-fucose determinants (395,396). The specificity of P7899 for core β-xylose and core α3-fucose determinants was also confirmed in our hands using defined glycan microarrays. On the 2D-SSGM, P7899 bound F10-4, F11-0, F11-5, F13-0, F13-4, and F13-7. This data indicates that the 2D-SSGM contains N-glycans containing terminal fucose, galactose, GalNAc, and GlcNAc, likely forming LDNF, core β-xylose, and truncated Man3 determinants, as well as many yet-undefined fucosylated N-glycan antigens. The array therefore represents a useful tool for discovering novel targets of anti-glycan immunity from a natural library of parasite glycans.

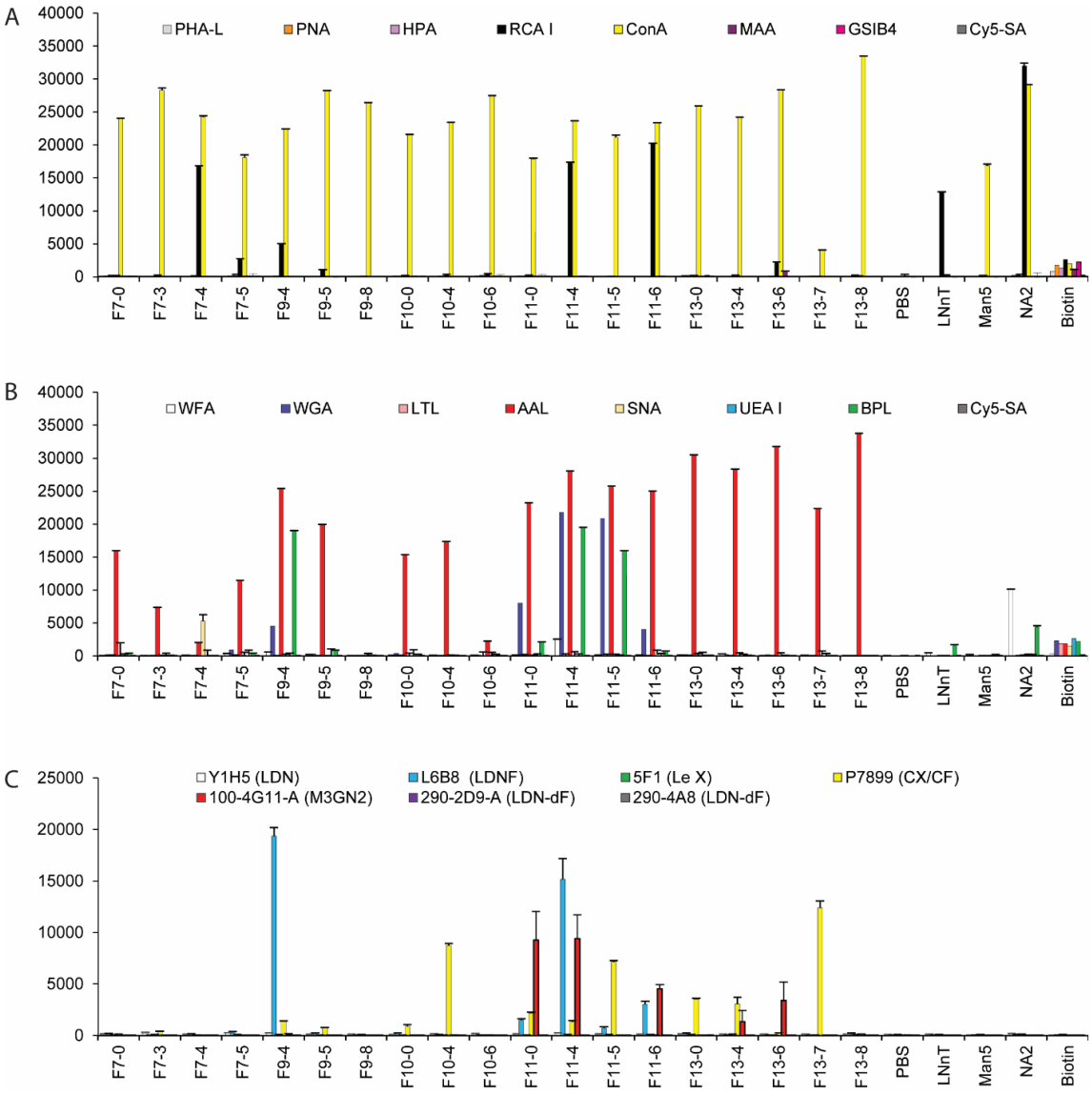


Figure 3.6. Separation of *S. mansoni* egg glycans by 2D HPLC reveals lectin and monoclonal antibody binding specificities.

(A, B) Microarrays containing 19 purified GAEAB fractions tested with a panel of 14 lectins show that while most fractions are highly recognized by fucose (AAL) and mannose (ConA) binding lectins, the separation of fractions uncovered the binding specificities of additional lectins (WGA, BPL, RCA-I). Positive controls LNnT, $Man_5GlcNAc_2$, asialo-bGP (NA2) were typically recognized by lectins RCA-I, ConA, and WFA, respectively. **(C)** Defined and highly specific antibodies also recognized fractions containing LDNF, CX/C3F, and Man3GN2. Antibodies to LDN, LDN-dF, and Le^X did not bind.

3.3.4 *Anti-FLDNF Antibody Shares Common Binding Pattern with Sera from S. mansoni-infected Animals and Humans on 2D-SSGM*

The 2D-SSGM was interrogated with pooled sera from infected mice (acute vs. chronic), sera from 4 infected rhesus monkeys (patent vs. post-patent period), and sera from 9 infected individuals (**Figure 3.7A-C**). Results shown in **Figure 3.7A** demonstrate that infected mice have IgG at 8wk and 20wk post-infection that recognized glycan fractions F9-4, F11-4, and F11-5. Notably, F11-5 was highly recognized by 20wk infected serum. In rhesus monkeys (**Figure 3.7B**), relatively high titer IgGs were found against most glycans (F9-4, F9-5, F10-4, F11-0, F11-4, F11-5, F13-4) with sera obtained during the patent period at 8wks post-infection. However, significant variability was observed among the 4 infected monkeys, with rhesus 1 and 3 always showing the highest response to these glycans. In humans, the 9 individuals tested showed generally lower levels of anti-glycan antibodies in comparison to rhesus monkeys, with two individuals (H-2 and H-4) showing the strongest binding for IgG in sera with fractions F9-4, F11-4, F11-5, and F13-4 (**Figure 3.7C**). These results show that mice, rhesus, and humans differentially recognize the various egg glycans in a pattern that is strikingly similar among the three hosts, but important glycan fractions F9-4, F11-4, and F11-5 were the most highly recognized by IgG antibodies in all three hosts. Notably the F2D2 antibody, which targets FLDNF (412), also bound robustly to fractions F9-4, F11-0, F11-4, F11-5, F13-4 and mildly to F10-4 (**Figure 3.7D**) generating a similar pattern to the mouse, rhesus, and human anti-sera (**Figure 3.7A-C**). The strongest binding occurred with fractions F9-4, F11-4, and F11-5, with F11-5 being the most prominent down to dilutions of 0.125μg/mL (**Figure 3.7D**). We therefore hypothesized that FLDNF may be a major immune target of the anti-glycan response during *S. mansoni* infection.

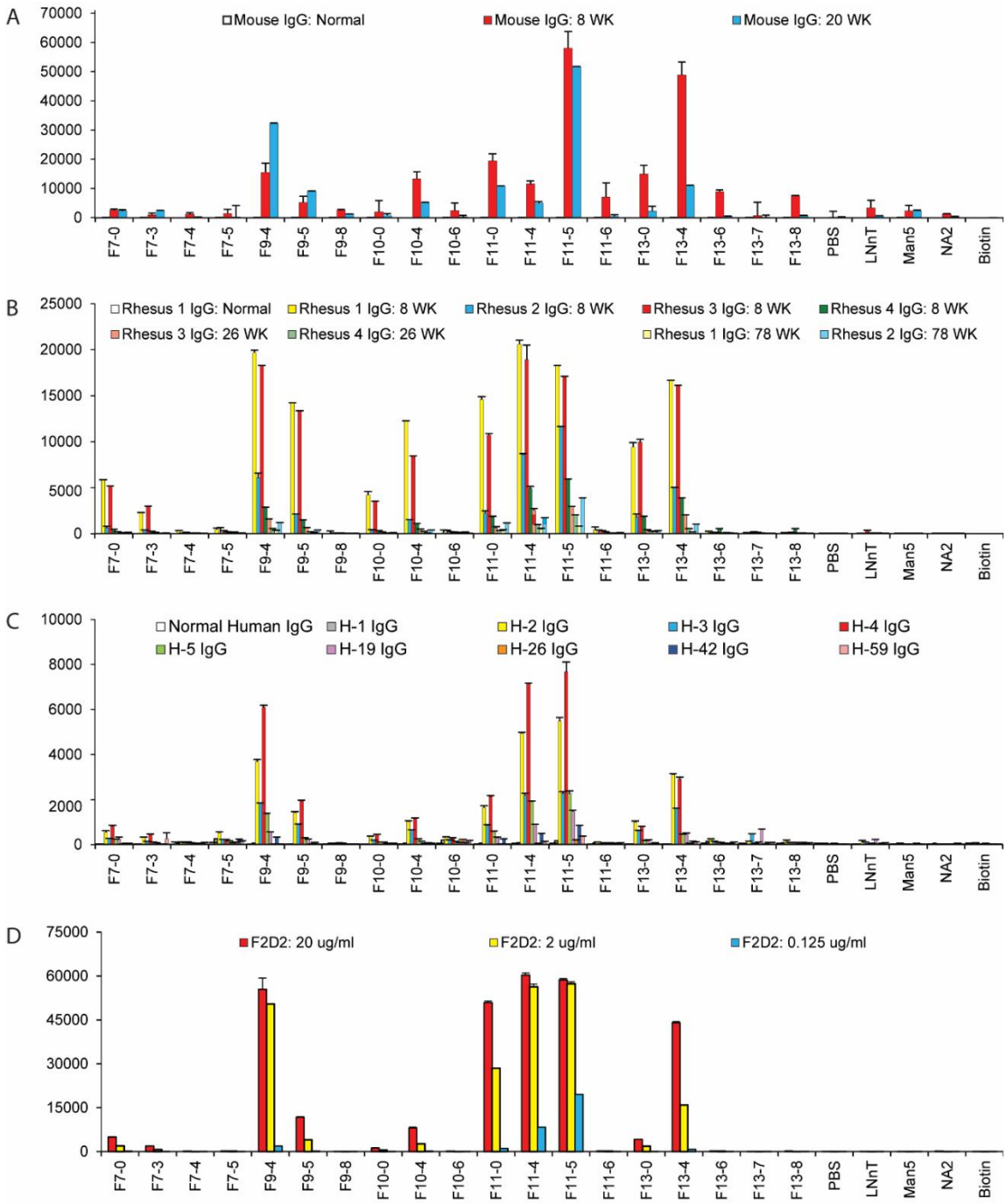


Figure 3.7. Infected sera from mice, rhesus, and humans bind *S. mansoni* egg glycans in a similar pattern to F2D2 antibody.

Microarrays containing 19 purified GAEAB fractions interrogated with sera from infected hosts show that fractions F11-4 and F11-5 are among the most commonly recognized by IgG in sera from (**A**) mice (acute and chronic, pooled), (**B**) rhesus monkeys (patent and post-patent period), and (**C**) humans. Mouse sera were screened at 1:100 while human and

rhesus sera were screened at 1:1000. (**D**) The binding pattern of targeted fractions matches that of the F2D2 antibody which targets FLDNF structures. When titered down, F2D2 retains the most binding to fraction F11-5.

3.3.5 *FLNDF Epitope is Expressed on S. mansoni Intramammalian Life Stages*

Given the similarity of F2D2 specificity with infection antisera on the 2D-SSGM, we hypothesized that this antibody would show binding to intramammalian developmental stages of the parasite. The localization of the FLDNF epitope on *S. mansoni* was investigated by probing whole, fixed parasites, including cercariae, 48 hour old cultured schistosomula, and 8wk old adults. Bound antibody was detected with Alexa-488-conjugated anti-mouse IgG secondary and imaged by fluorescent microscopy. Fluorescence patterns indicated robust expression on the surface of all life stages investigated (**Figure 3.8A**). The FLDNF motif is potently expressed by cercariae, then appears to wane slightly after transformation to schistosomula and subsequent culture. In adults, the expression of FLDNF is markedly different in male versus female worms, with females expressing more glycan antigen.

Immune relevant glycan epitopes have been shown to occur on both glycoproteins and glycolipids. To determine if FLDNF is expressed on glycoproteins, soluble extracts of S. *mansoni* eggs (SEA) and lysates of cercariae, 3 day old schistosomula, and adults were separated by SDS-PAGE probed by western blot with F2D2 (**Figure 3.8B**). Five times less SEA relative to the other extracts was used in the analysis due to the intense expression of the epitope in the parasite eggs. F2D2 bound diverse and unique glycoprotein bands from each of the developmental stages, including glycoproteins from ~35 kDa to >260 kDa in both cercariae and SEA, however the banding pattern is markedly different in these two samples. FLNDF expression appears more restricted in schistosomula and mixed sex adults. In schistosomula, the major glycoprotein is ~40 kDa with several fainter bands ~20 kDa, ~30 kDa, and ~200 kDa. Adults also express the F2D2 epitope on few molecular species, primarily a glycoprotein ~70 kDa with faint expression on proteins of the ~45 kDa and ~230 kDa. Given the robust expression via immunostaining in

schistosomula and adults (**Figure 3.8B**), it is possible that the glycan epitope is also expressed on glycolipids which is consistent with previous reports of lipid antigens (82,110,310,388,413).

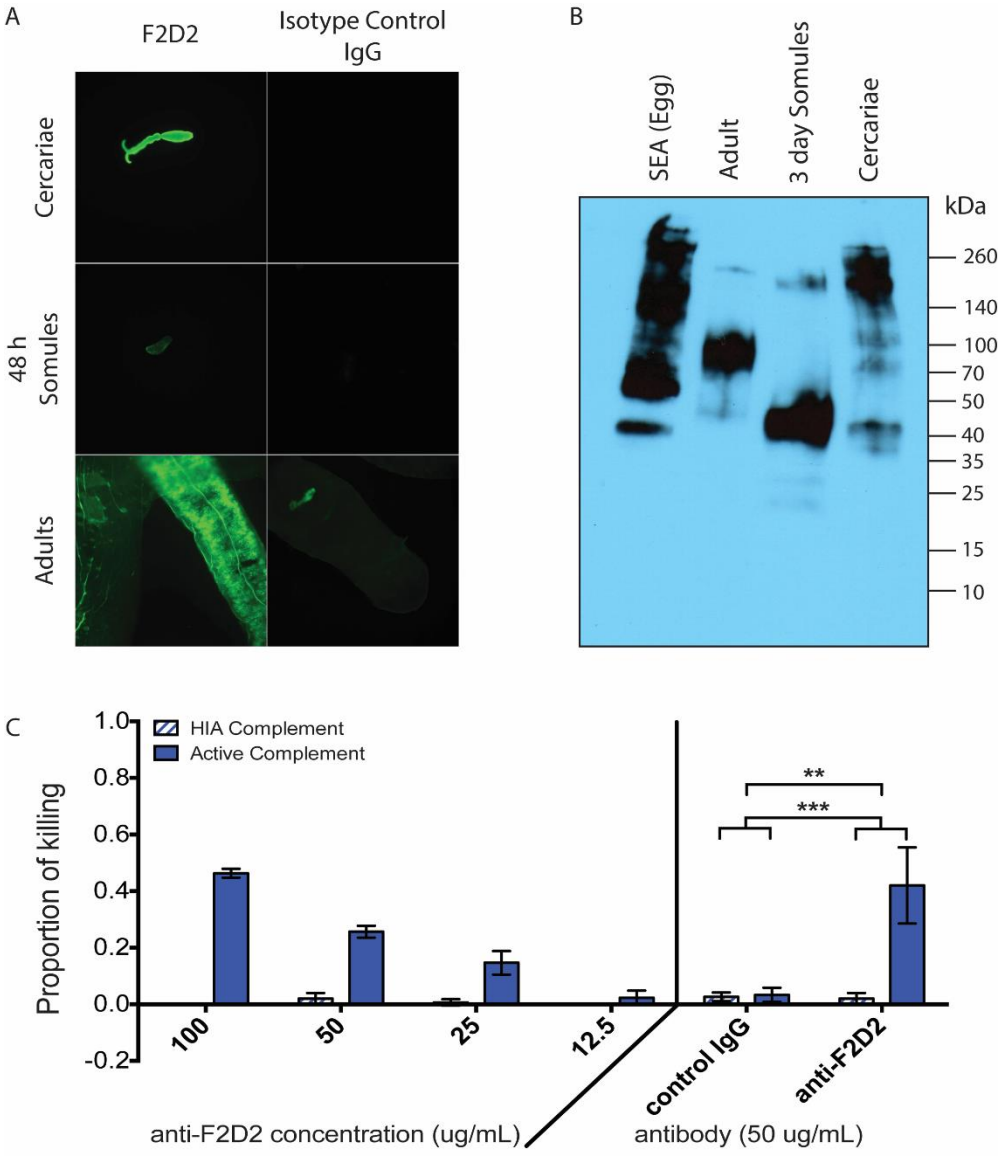


Figure 3.8. The F2D2 antibody target, FLDNF, is expressed on all mammalian life stages and can kill schistosomula *in vitro*.

(A) Immunostaining of fixed parasites with either F2D2 antibody (10μg/ml) or isotype control IgG showing abundant surface expression of FLDNF on intramammalian life stages. Imaged at 20x magnification. **(B)** Western blot of F2D2 binding to parasite life stages showing FLDNF is expressed on glycoproteins in all samples but in a distinct pattern of expression that differs between life stages. SEA at 1μg/ml, all other lysates at 5μg/ml.

(C) *In vitro* killing assays show that F2D2 antibody is lethal to schistosomula, in a complement-dependent manner. A representative dose series (left) and three replicates at 50μg/mL (right) after 48 hours are shown. The killing was significantly dependent on F2D2 antibody compared to control IgG (normal mouse IgG) and on presence of active versus heat-inactivated guinea pig complement by 2-way ANOVA (** $p \leq 0.005$, *** $p \leq 0.001$). Mean ± SD are shown.

3.3.6 *F2D2 Kills* Schistosomula in vitro

Given the evidence that schistosomula express antigens bound by F2D2, which are thought to be the most vulnerable target of host immunity, we tested whether F2D2 was able to kill *in vitro*-transformed schistosomula. Three-hour old schistosomula were cultured for up to 48 hours with F2D2 in the presence of active or heat-inactivated guinea pig complement. The antibody was lethal to schistosomula in a dose-dependent manner at concentrations of 10-100μg/mL (**Figure 3.8C**). At 50μg/mL antibody and at 48 hours, 30-60% of the schistosomula were dead, as indicated by gross morphology and DAPI uptake. F2D2 killing of schistosomula was significantly higher than the baseline toxicity of mouse IgG, and dependent on the presence of active complement (**Figure 3.8C**). These data show that the target of F2D2 could be important to the host immune response.

3.3.7 *Proposed Structures for Antisera Glycan Targets using Metadata-Assisted Glycan Sequencing (MAGS)*

We characterized several of the 2D-GAEAB fractions by MALDI-TOF MS and compiled the compositional mass spectrometry (MS) data with lectin and antibody binding characteristics in order to tentatively predict their composition and propose possible structures, using Metadata-Assisted Glycan Sequencing (MAGS) (266,270). MS profiles for each fraction are shown in **Figures 3.9-3.11**. **Table 3.2** shows the compiled lectin and antibody binding patterns of each 2D fraction along with compositional predictions from MS. The proposed structures, monosaccharide compositions, sequence, and branching were also based on motifs identified in previous literature (269,281,392,413,414).

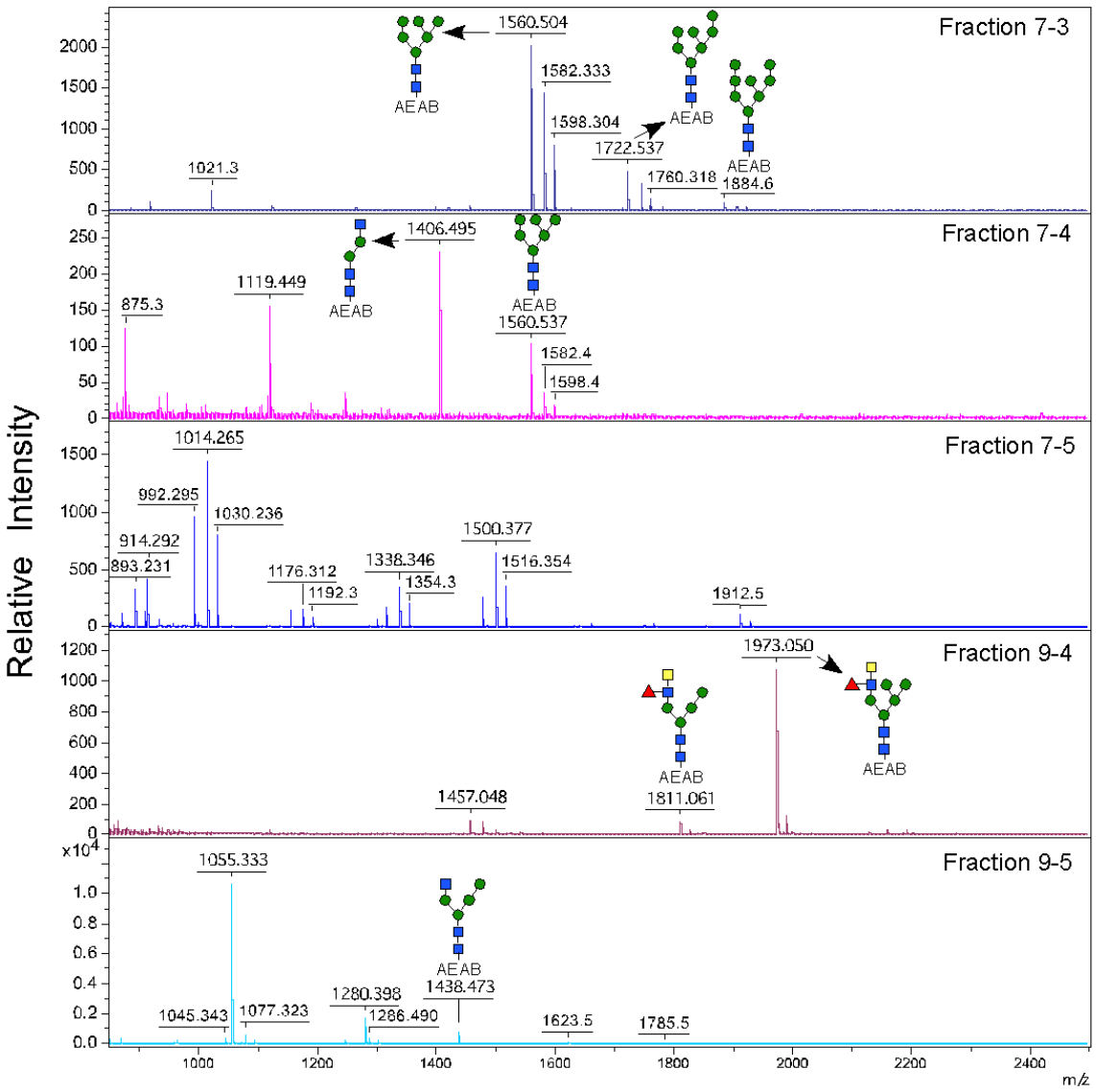


Figure 3.9. MALDI-TOF MS profiles of the 2D-SSGM fractions: 7-3, 7-4, 7-5, 9-4, and 9-5.

AEAB-linked fractions were mixed with DHB and analyzed in negative ion mode. The observed molecular weight is shown above each peak and proposed structures that match glycan compositional data are shown +/- 0.5 Da. Proposed structures reflect molecular weights, MAGS data from the 2D-SSGM, and reported structures in the literature, as well as MS^2 data that was available for select peaks. Where peaks with and without sodium ion were detectable, only one is shown.

For example, F11-5, which was among the most highly reactive with antisera and F2D2 antibody, represents a mixture of primarily core xylosylated glycans with 3-4 Hex, 4-5 HexNAc, and 1-3 deoxyHex residues (**Figure 3.12, Table 3.2**)**.** In addition to robust AAL and ConA binding, F11-5 is also bound by WGA and BPL lectins, and LDNF and anti-HRP antibodies (**Figure 3.6, Table 3.2**), suggesting that it contains determinants with terminal GlcNAc, terminal GalNAc, LDNF, and core β-xylose. F11-5 also contained glycan compositions consistent with a multi-fucosylated truncated N-glycan core and glycans of undetermined composition. MALDI-TOF/TOF of the highest magnitude peak (1926.564) supported the MAGS data, suggesting a core fucose and LDNF (**Figure 3.13**). Although TOF/TOF data was unavailable for some of the smaller peaks of high molecular weight (2072.610, 2275.669), their compositions were consistent with either multi-fucosylated LDN epitopes (FLDNF, LDN-dF) or two singly-fucosylated GlcNAcs on the antennae (**Figure 3.13**). Recognition of F11-5 by anti-LDN-dF and anti-M3GN2 antibodies, however, was low. This MAGS information lead us to hypothesize that antenna in many N-glycans in F11-5 were extended past GlcNAc and that the multi-fucosylated antennae were likely FLDNF rather than LDN-dF.

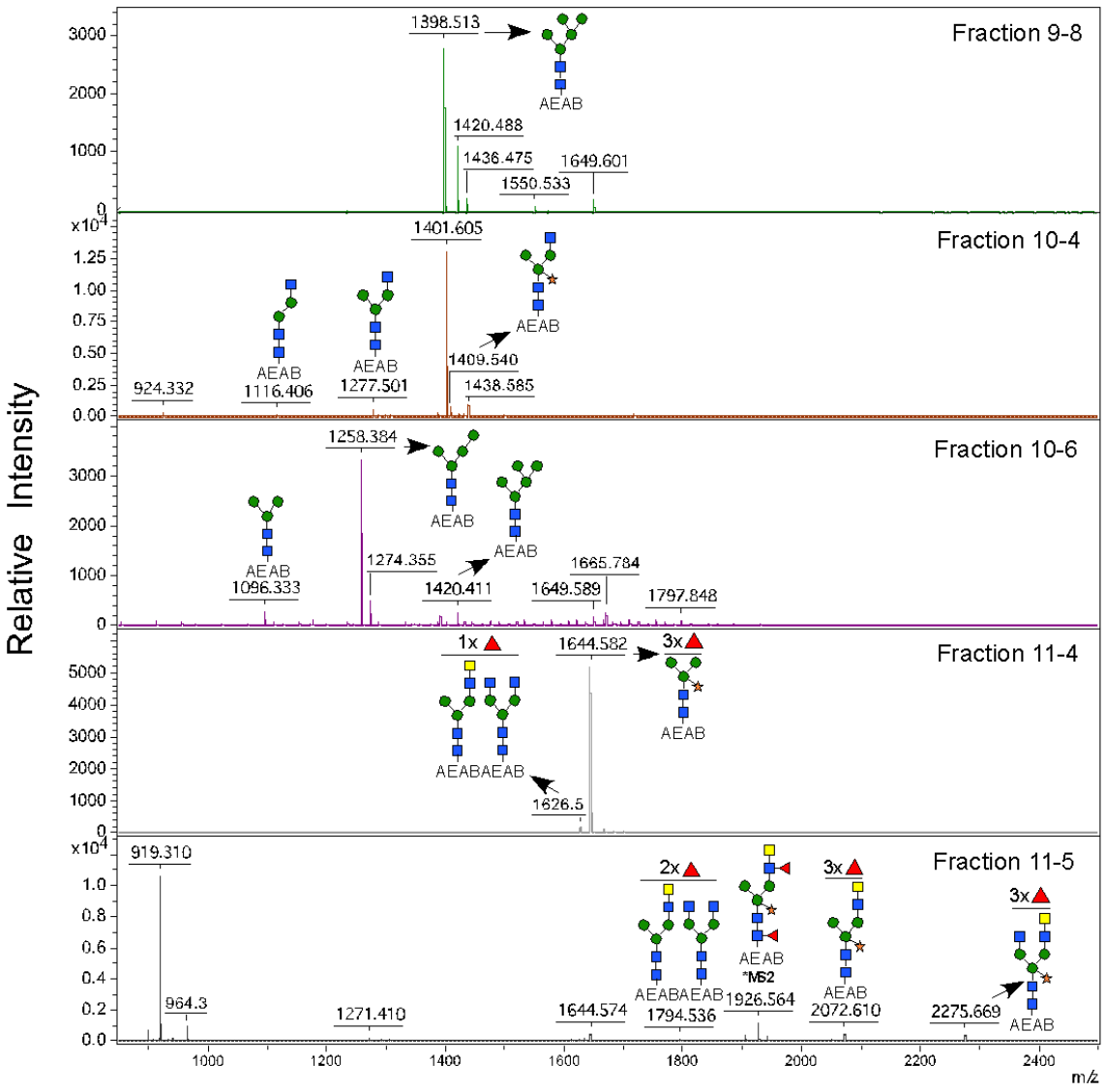


Figure 3.10. MALDI-TOF MS profiles of the 2D-SSGM fractions: 9-8, 10-4, 10-6, 11-4, and 11-5.

AEAB-linked fractions were mixed with DHB and analyzed in negative ion mode. The observed molecular weight is shown above each peak and proposed structures which match oligosaccharide compositional data are shown +/- 0.5 Da. Proposed structures reflect molecular weights, MAGS data from the 2D-SSGM and reported structures in the literature, as well as MS^2 data which was available for select peaks. Where peaks with and without sodium ion were detectable, only one is shown.

Several other fractions were moderately to strongly reactive with F2D2 (F9-4, 11-4, F11-5, F13-4) (**Figure 3.7D, Table 3.2**). These glycan fractions also had compositional and MAGS data consistent with fucosylated LDN determinants (F9-4: 1973, F11-4: 1626.5; F13-4: 1626.5, 2196). In particular, F13-4, which was not reactive with LDNF or LDN-dF antibodies, demonstrated compositions consistent with LDN and 4 Fuc residues, suggesting the presence of fucosylated GalNAc residues (**Figure 3.12**). Serum reactivity with some fractions where F2D2 reactivity was significantly lower or non-existent (F7-3, F9-5, F10-4, F13-0) suggested other immunologically relevant epitopes such as high mannose and core β-xylose containing structures on the glycan array.

These results demonstrate that the most immunodominant egg N-glycans recognized by infected hosts share common determinants containing core β-xylose, 1-4 fucose residues, and are consistent with fucosylated LDN structures. Other epitopes likely contained in the serum immunodominant fractions are high mannose, core β-xylose, core α6-fucose, Le^X, and LDNF. Using both defined reagents and immunologically relevant samples therefore allowed us to partially characterize the egg glycans printed on the array and predict possible immunodominant glycan targets of the response to *S. mansoni*.

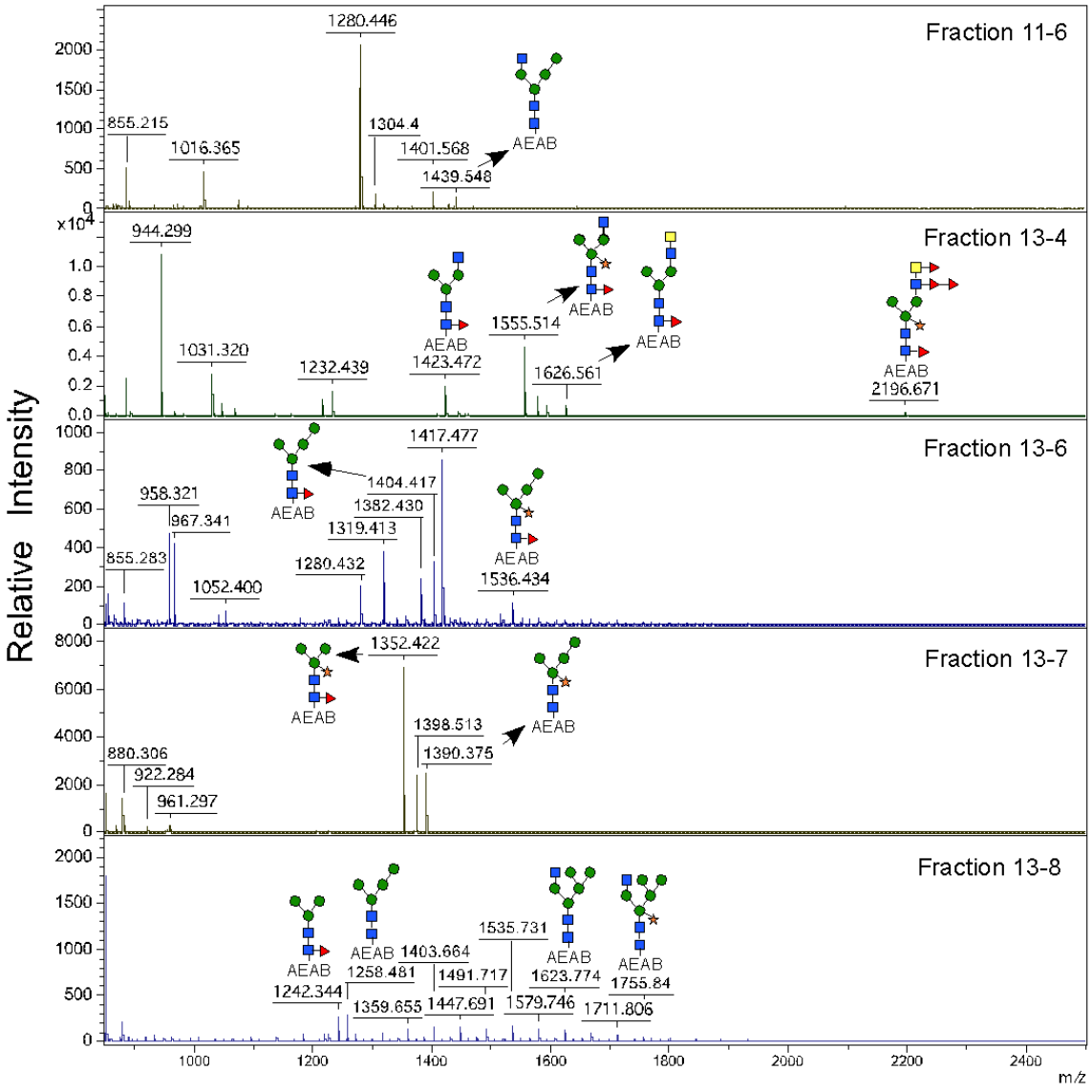
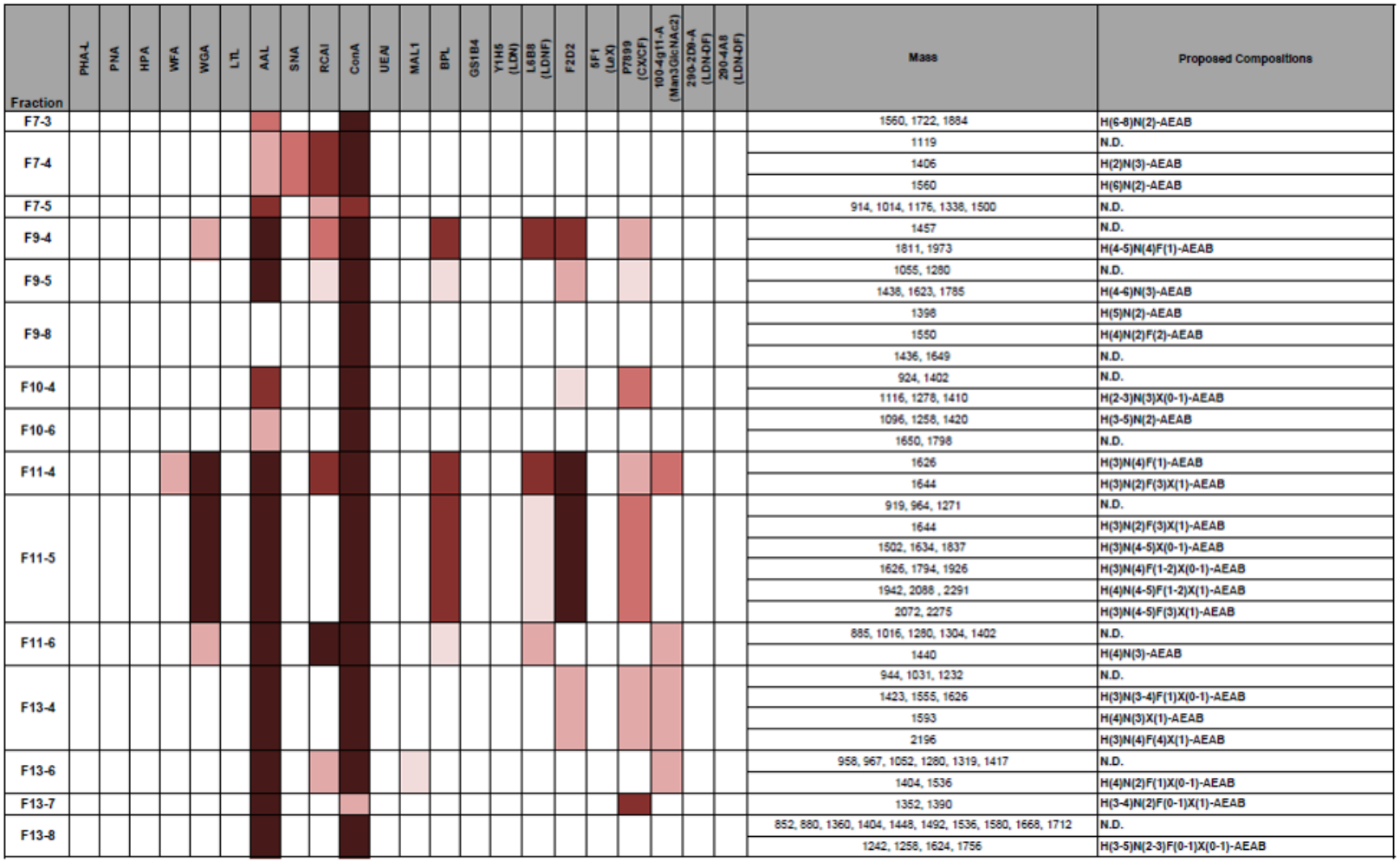


Figure 3.11. MALDI-TOF MS profiles of the 2D-SSGM fractions: 11-6, 13-4, 13-6, 13-7, and 13-8.

AEAB-linked fractions were mixed with DHB and analyzed in negative ion mode. The observed molecular weight is shown above each peak and proposed structures which match oligosaccharide compositional data are shown +/- 0.5 Da. Proposed structures reflect molecular weights, MAGS data from the 2D-SSGM and reported structures in the literature, as well as MS^2 data which was available for select peaks. Where peaks with and without sodium ion were detectable, only one is shown.

3.4 Discussion

Our studies show that specific antigenic epitopes within N-glycans in egg glycoproteins from *S. mansoni* are the targets of adaptive immunity in some animal species and people infected by the parasite. While schistosomes synthesize many types of unusual glycans and both monoclonal and polyclonal antibodies have been found to recognize specific glycan determinants, the overall screening of such responses has been difficult due to the lack of parasite-derived glycan reagents and technologies. The studies here employing total N-glycans from egg glycoproteins, defined glycan microarrays based on schistosome glycan antigens, along with mAbs to glycan antigens, have allowed us to identify key glycan antigens in this parasite, and most notably the recognition of the fucosylated glycan determinant FLDNF. These studies have broad implications for understanding the overall animal and human responses upon infection to glycan antigens from this parasite, and may lead to new diagnostic and vaccine targets.



Fraction	PHA-L	PNA	HPA	WFA	WGA	LTL	AAL	SNA	RCAI	ConA	UEA	MAL1	BPL	GS1B4	Y1H5 (LDN)	L6B8 (LDNF)	F2D2	5F1 (LeX)	P7B9 (CXCF)	100-4g11-A (Man3GlcNAc2)	290-2D9-A (LDN-DF)	290-4A8 (LDN-DF)	Mass	Proposed Compositions
F7-3																							1560, 1722, 1884	H(6-8)N(2)-AEAB
F7-4																							1119	N.D.
																							1406	H(2)N(3)-AEAB
																							1560	H(6)N(2)-AEAB
F7-5																							914, 1014, 1176, 1338, 1500	N.D.
F9-4																							1457	N.D.
																							1811, 1973	H(4-5)N(4)F(1)-AEAB
F9-5																							1055, 1280	N.D.
																							1438, 1623, 1785	H(4-6)N(3)-AEAB
F9-8																							1398	H(5)N(2)-AEAB
																							1550	H(4)N(2)F(2)-AEAB
																							1436, 1649	N.D.
F10-4																							924, 1402	N.D.
																							1116, 1278, 1410	H(2-3)N(3)X(0-1)-AEAB
F10-6																							1096, 1258, 1420	H(3-5)N(2)-AEAB
																							1650, 1798	N.D.
F11-4																							1626	H(3)N(4)F(1)-AEAB
																							1644	H(3)N(2)F(3)X(1)-AEAB
F11-5																							919, 964, 1271	N.D.
																							1644	H(3)N(2)F(3)X(1)-AEAB
																							1502, 1634, 1837	H(3)N(4-5)X(0-1)-AEAB
																							1626, 1794, 1926	H(3)N(4)F(1-2)X(0-1)-AEAB
																							1942, 2088 , 2291	H(4)N(4-5)F(1-2)X(1)-AEAB
																							2072, 2275	H(3)N(4-5)F(3)X(1)-AEAB
F11-6																							885, 1016, 1280, 1304, 1402	N.D.
																							1440	H(4)N(3)-AEAB
F13-4																							944, 1031, 1232	N.D.
																							1423, 1555, 1626	H(3)N(3-4)F(1)X(0-1)-AEAB
																							1593	H(4)N(3)X(1)-AEAB
																							2196	H(3)N(4)F(4)X(1)-AEAB
F13-6																							958, 967, 1052, 1280, 1319, 1417	N.D.
																							1404, 1536	H(4)N(2)F(1)X(0-1)-AEAB
F13-7																							1352, 1390	H(3-4)N(2)F(0-1)X(1)-AEAB
F13-8																							852, 880, 1360, 1404, 1448, 1492, 1536, 1580, 1668, 1712	N.D.
																							1242, 1258, 1624, 1756	H(3-5)N(2-3)F(0-1)X(0-1)-AEAB

RFU Code: >500 Low; >1,000 Moderate; >5,000 Moderate; >10,000 Strong; >20,000 Strong

Table 3.2. Summary of MAGS data for the 2D-SSGM.

Each 2D fraction is shown with lectin and antibody binding magnitudes (**Figure 3.6**), molecular weight peaks obtained from MALDI-TOF MS, and compositional predictions when possible. H, Hexose; N, N-acetylhexosamine; X, xylose; F, fucose; N.D.; no known oligosaccharide mass within 1 Da of the observed molecular weight, or the matching composition does not match structural precedents in the literature. MALDI-TOF MS profiles with predicted cartoon structures are shown in **Figures 3.9-3.11 and 3.13**.

It is well established that schistosome infection of humans, primates, and rodents induces strong humoral responses against parasite antigens, and while antibody titers to glycan antigens are generally proportional to severity of the infection (80), it is not yet clear whether such responses provide protection. Clearly, antibodies to carbohydrate antigens, especially glycoprotein N-glycans, dominate the humoral response in particular during egg deposition (127,415), suggesting that egg N-glycans play an important role in the immunopathogenesis of the disease. Antigenic responses to glycan determinants or epitopes such as Le^X, LDN, LDNF, and LDN-dF give rise to different intensity levels and antibody isotypes (80,127,168,208,238,243,244,371). Recent elegant studies on structural glycomics profiling of schistosomes have shown that schistosome eggs contain a diverse repertoire of high mannose, truncated and complex N-glycans with core motifs such as α3 fucose, α6 fucose, and β2 xylose (17,74,105,111). There is also growing evidence that worm products have immunomodulatory roles and can alter functions and activation of dendritic cells, macrophages, and intestinal epithelial cells (143,163,416-418). However, the precise structures of the molecules that are bioactive in that regard are not yet clear.

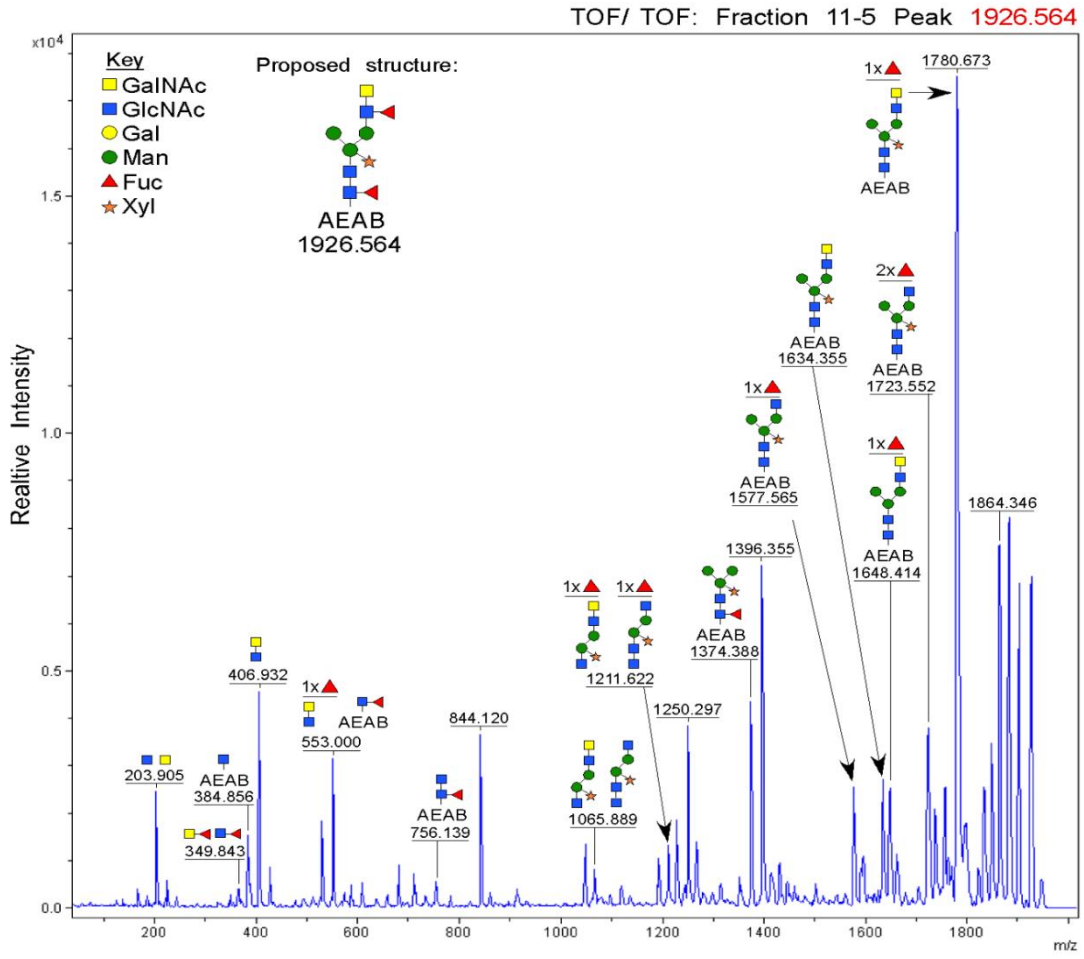


Figure 3.12. MALDI-TOF/TOF profile of peak 1926.564 in Fraction F11-5.

One of the major peaks, 1926.564, of the immunodominant F11-5, with proposed structure assignments for major MS/MS fragments. Reducing end fragments are shown linked to AEAB; non-reducing end fragments are shown as free oligosaccharides.

In targeting the N-glycans of egg glycoproteins, we used both PNGase F and A to release N-glycans from these glycans, and we generated 1D and 2D shotgun glycan microarrays after glycan separation of the fluorescent-labeled glycans by HPLC fractionation. The interactions of glycans on these microarrays with a panel of lectins and antibodies indicated that egg N-glycans are characterized by a high content of α-linked fucose and mannose residues, and are consistent with previous studies showing an abundance of glycans with these modifications in schistosome

eggs (17,74,105,111). To help focus our studies, we interrogated the 1D microarrays with sera from mice, rhesus, and humans, to determine which of these glycan fractions were most relevant to the induction of humoral responses during disease progression in these hosts. We found that fractions F7-F13 were recognized by IgG from all three species (**Figure 3.4**), demonstrating the N-glycans released by PNGase F contain key antigenic determinants. By contrast, we found that PNGase A-released glycans, which were recognized by lectins AAL and ConA, were poorly recognized by infected mouse, rhesus, or human sera. PNGase A can release N-glycans containing unusual core modifications that block PNGase F release. It is possible that cleavage of glycans with PNGase A may result in alteration in the conformation of antigenic features within the glycan cores that are recognized by antibodies, especially in light of results from Luyai *et al* (2014), demonstrating that rhesus and human sera strongly recognized core α3-fucosylated and core β2-xylosylated glycans when presented as glycopeptides on a defined glycan microarray, implying the possible need for a peptide moiety in the recognition (271). The glycan microarrays we prepared here from SEA were developed by reductive amination with AEAB and free glycans, and thus lack a peptide component. Alternatively, the subset of schistosome glycans that receive core α3-fucose modifications may contain fewer terminal immunodominant epitopes. Interestingly, a recent glycomics study demonstrated that there were major differences in the PNGase F- and PNGase A-released N-glycans of both eggs and miracidia. PNGase A-released glycans from eggs were primarily multi-antennary with singly-fucosylated termini (LDN, LDNF, Le^X) while PNGase F-released glycans were primarily biantennary with several multi-fucosylated LDN motifs (392). Our data suggest that the latter type of glycan may be more immunodominant in schistosomiasis-infected hosts.

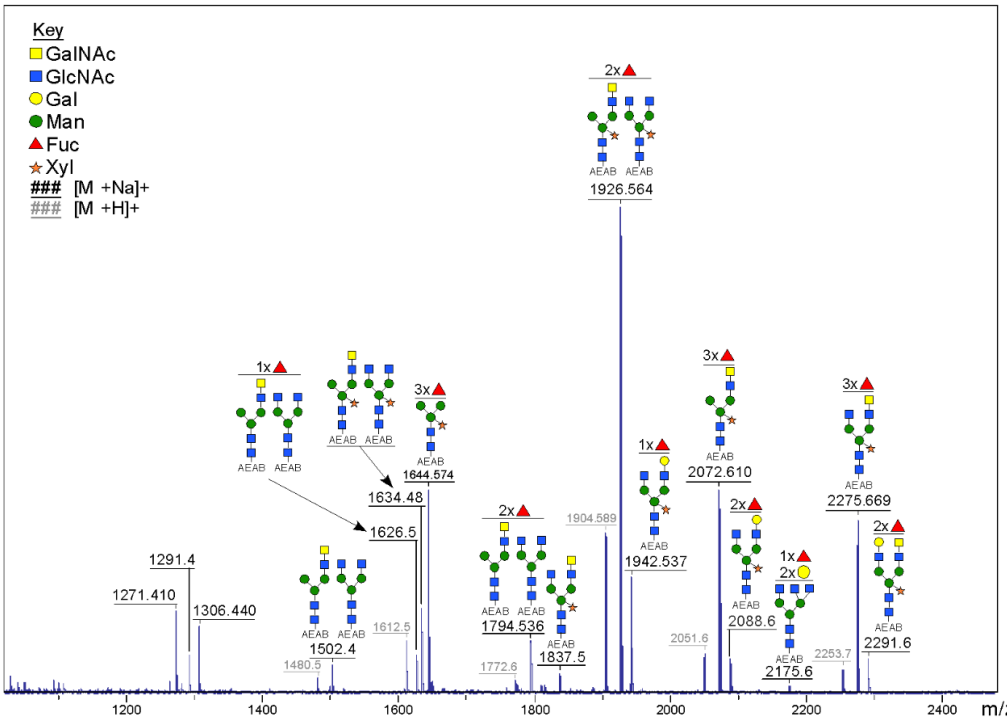


Figure 3.13. MALDI-TOF profile of F2D2 and sera targeted *S. mansoni* egg glycans.

Mass spectrometry analysis of F11-5 predicts glycans with mono and biantennary structures containing LacdiNAc termini, multiple fucose, and core xylose epitopes. Possible fucosylated motifs include LDNF, LDN-dF, FLDN, FLDNF, Le^X, and core α6 fucose (pictured in **Table 3.2**).

Screening of 1D microarrays with serum from infected rhesus macaques showed a very strong IgG response (**Figure 3.4B**), primarily to fractions F7-F13, but only during the patent period at 8wks post-infection. No significant antibody response to these glycans was obtained with the 78wk infected serum in either this study or a recent one from our group (271). The results suggest that anti-glycan responses to some antigens dissipate by this stage of the infection in rhesus. A salient feature of the rhesus is that an infection becomes patent but, above a threshold worm burden, egg output declines over the ensuing weeks to months (419,420) and the rhesus clears the infection and becomes resistant to re-infection (199). Recent studies showed that in rhesus, an early IgG antibody response is associated with the elimination of worms and that peak titers of IgG to core

α3-fucose and core β2-xylose at 8-11 weeks coincide with schistosomula killing by rhesus sera *in vitro* (197). Therefore, it is possible that the strong humoral IgG response to egg glycans induced during the patent phase plays a role in natural worm elimination and acquired resistance in rhesus.

In agreement with these studies, we also observed rhesus serum reactivity against PNGase A-released egg N-glycan fractions A3 and A6, which was of much lower magnitude than we observed toward PNGase F-released glycans. Complex type N-glycans possessing core α3-fucose and core β2-xylose, associated with resistance to PNGase F, but not PNGase A, have been detected in schistosomes (17,74,105,111,244,281,392). Smit *et al* (392) showed that the core α3-fucosylated egg N-glycans were primarily complex, in contrast to the truncated trimannose used on the defined array in Luyai *et al* (271), to which a robust rhesus response was seen. The low reactivity of rhesus antibodies with our PNGase A-released fractions could therefore be explained by a preference for truncated rather than complex core α3 fucosylated N-glycans, which may be expressed earlier than the egg stage. Core α3-fucosylated egg derived glycans are also known to induce a strong Th2 cytokine response in infected mice (164). Thus, both PNGase F- and A-released glycans from the worm appear to be important immunogens and antigens in the response against schistosomiasis.

IgG within one human serum sample recognized fractions F1, F3, F7-F13 and F15, with fractions F11 and F15 showing the highest recognition (**Figure 3.4C**). Compared to the rhesus serum, the human sample lacked the relatively lower reactivity seen to PNGase A-released fractions in the rhesus, but did possess low reactivity to A13 before and after treatment. Reactivity to different PNGase F- and A-released fractions and the presence of IgG at disparate stages of disease progression suggests differences in the immune response to egg glycan antigens among these three mammalian hosts (**Figure 3.4)**. Luyai *et al* (271) also found that there were both similarities and substantial differences in the specificity, titers, and isotype composition of anti-glycan antibodies among humans, rhesus monkeys, and mice (271). An important issue for future studies will be to determine whether the differentially recognized egg glycan epitopes contained in these fractions

represent markers of infection status and/or contribute to productive immune responses in protected hosts.

Further separation of the immunologically relevant GAEAB fractions F7-F13 in a second dimension resulted in a total of 60 purified fractions (**Figure 3.5**), with 15 printed on the 2D microarrays and interrogated with lectins, anti-glycan antibodies, and sera (**Figure 3.6**). We have shown that although many glycans separated by multidimensional HPLC have similar carbohydrate compositions, their separation indicates structural differences that also cause differences in the binding specificity of lectins and antibodies (268). Similar to the 1D parent fractions, most 2D fractions were highly fucosylated and mannose-containing, as demonstrated by their strong binding to the lectins AAL and ConA. Interestingly, further purification by 2D HPLC enriched for N-glycans bound by lectins that only bound weakly to the mixed glycans in the 1D parent fractions, such as the GlcNAc-binding lectin WGA, the GalNAc-binding lectin BPL, and the β-Gal binding lectin RCA-I (**Fig. 3.6A-B, Table 3.2**). Serial purification of heterogeneous glycans is therefore important to define the characteristics of distinct structures. One caveat to our approach may be that using serum to prioritize 1D-separated fractions for further analysis could mask important epitopes in the heterogeneous mixture. In any case, robust analyses of more highly purified fractions is always recommended.

The differential responses of infected animal and human sera to the 2D-SSGM microarrays was striking. When tested with pooled infected mouse serum, sera from 4 infected rhesus, and sera from 9 infected humans, showed that glycan fractions F9-4, F11-0, F11-4, F11-5, and F13-4 were recognized in all species (**Figure 3.7**). Mice responded prominently to fraction F11-5, composed of $Hex_{3\text{-}4}HexNAc_{2\text{-}5}DeoxyHex_{0\text{-}3}Xyl_{0\text{-}1}$ (**Figure 3.7A**), while rhesus (**Figure 3.7B**) and humans (**Figure 3.7C**) showed high reactivity to multiple fractions during the patent phase of disease. In some rhesus monkeys, IgG reactivity against F11-4 and F11-5 was also observed at 26wks and 78wks post-infection, demonstrating that the humoral response to glycans declines after the egg-

laying patent phase but low levels of IgG against particular glycans can remain after egg deposition ceases, worms are eliminated and animals become self-cured.

Due to the striking similarity of the F2D2 mAb binding pattern with antisera (**Figure 3.7**), in particular that of the acutely infected, naturally resistant rhesus monkey, we explored the expression of the FLDNF epitope and the antibody's effector abilities. The F2D2 epitope, FLDNF, is highly expressed on cercarial and egg glycoproteins, and has more restricted expression in cultured schistosomula and adult worms (**Figure 3.8B**). This data, in combination with immunostaining depicting vast surface expression (**Figure 3.8A**), is in agreement with several reports profiling the expression of fucosylated LDN determinants (82,245,350,392), particularly FLDN and FLDNF, which are reported to exist on glycoproteins and glycolipids of the cercarial and egg stage, and predominantly on glycolipids in adults. Importantly, we found that the F2D2 antibody was lethal to schistosomula *in vitro* in a complement-dependent manner. Rhesus serum also has schistosomula-lethal activity from two months post-infection (198,271). It is still unknown whether anti-glycan antibodies contribute to parasite resistance in the context of a host response, but it is tempting to speculate that glycan targets present on schistosomula, such as the F2D2 epitope, could be protective if targeted early in infection.

The predicted structures of glycans contained in F11-5, the fraction most strongly bound by F2D2 and serum samples, suggest they contain a combination of antigenic epitopes such as variably fucosylated LacdiNAc (LDNF, LDN-dF, FLDN, FLDNF), Le^X, and those containing core α3-fucose and β2-xylose (**Figure 3.13, Table 3.2**). The MAGS data for other F2D2 binding fractions suggested the presence of fucosylated LDN motifs (**Figure 3.13, Table 3.2**). Tandem MS data was not available for the highly fucosylated peaks in fractions 11 (2072.6; X1F3H3N4; Na+ ion) and 13 (2196.7; X1F4H3N4, H+ ion), due to the lack of abundance, but the compositions predicted for these peaks matches those of PNGase F-released cercarial and egg glycans recently identified by Smit *et al* (392). In that study they performed exoglycosidase digestions on cercarial

and egg N-glycans, which suggested that FLDNF and FLDN-dF were antigenic determinants, respectively, within these glycans, which was also consistent with the F2D2 epitope.

Several studies in multiple species have attempted to define the various roles that different anti-glycan antibodies to LDN, LDNF, FLDN, FLDNF, LDN-dF, and Le^X may have in disease progression and immunity (127,208,240,243,244,271,272). Chimpanzees vaccinated with radiation-attenuated cercariae or naturally infected developed a strong cellular and humoral immune response predominantly directed against glycans associated with both cercariae and eggs, including LDN, LDNF, Le^x, and undefined glycans present on KLH. Vaccinated chimps showed a 40% reduction in infection intensity compared to infected control chimpanzees (127). Later studies revealed that antibody levels to LDN-dF and FLDN epitopes were strikingly higher than those against LDN, LDNF, and monomeric Le^X in vaccinated or infected chimps and infected human cohorts. Interestingly, the anti-LDN-dF and anti-FLDN antibodies were predominantly IgGs, whereas anti-Le^X, anti-LDN, and anti-LDNF were IgMs, however this was not always consistent in human studies (208,240,243,244). Note that in mice, the acute infection is marked by IgM antibodies to LDNF, LDN, and Le^X (271). Similarly, mice generated predominately IgM and low levels of IgG to LDN and LDNF post immunization with LDN/LDNF expressing cells (272). The differential responses in infected animals raise important questions to address in the future, especially in relation to the specific glycan target, isotype responses, time courses of responses, and whether the specificity and titer of anti-glycan antibodies play a role in disease outcome and overall susceptibility to infection in the host animal.

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Chapter 4. *Schistosoma mansoni* α1,3-Fucosyltransferase-F Generates the Lewis X Antigen

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4.1 Introduction

Schistosomiasis is a debilitating vascular disease caused by an infection with parasitic helminths of the *Schistosoma species*. It causes a wide range of clinical manifestations that dramatically affect quality of life and is a major public health concern in over seventy tropical and subtropical countries. The World Health Organization (WHO) considers schistosomiasis second in socioeconomic importance among diseases worldwide and the third most important parasitic disease in terms of public health impact (7,13,16,422). Despite years of research on schistosome biology, millions are still infected and many more at risk due to insufficient prevention, diagnostics, treatments, and absence of a vaccine.

All life stages of Schistosoma species produce abundant glycoconjugates on their surfaces and in secretions that are exposed to the host immune system. In fact, the *S. mansoni* cercarial glycocalyx is mostly carbohydrate by weight (85,175). The glycome includes many N-glycans and O-glycans linked to proteins, and glycolipids, which are structurally distinct from their definitive host. It has long been accepted that glycans and glycoconjugates may be bioactive, and many of

the schistosome glycans are known to interact with the host immune system in a variety of complex mechanisms, most of which are poorly understood (7,70-72,132,170).

A major constituent of the *Schistosoma* glycome is L-fucose. Fucose is abundant in N- and O-glycans in glycoproteins and in glycolipids of *S. mansoni*, and has been found in α1,2-, α1,3-, α1,4-, and α1,6-linkages (70,423,424). Fucosylated glycoconjugates are prominently involved in the host humoral and cellular immune responses to infection. Antibodies have been identified against a diverse group of immunologically important fucosylated structures on non-reducing ends of protein- and lipid-conjugated oligosaccharides in both mammalian and snail host-associated developmental stages in infected animals and humans. These glycans include LDNF (GalNAcβ1-4(Fucα1-3)GlcNAc), LDN-dF (GalNAcβ1-4(Fucα1-2Fucα1-3)GlcNAc), FLDN (Fucα1-3GalNAcβ1-4GlcNAc), FLDNF (Fucα1-3GalNAcβ1-4(Fucα1-3)GlcNAc), dF-LDN-dF (Fucα1-2Fucα1-3GalNAcβ1-4(Fucα1-2Fucα1-3)GlcNAc), and Lewis X (Le^X; Galβ1-4(Fucα1-3)GlcNAc) (105,110,234,243,246,350,425-427). The genes encoding potential fucosyltransferases have been partly identified recently (2,332,337), but as of yet, there is no information about the enzymatic activities and specificities for these putative smFuTs. The lack of information has created a major limitation in the study of the functions of the fucosylated glycans and how they interact with the host, due to an inability to use enzymatic approaches to synthesize the unusual fucosylated glycans in an affordable and facile manner.

The first phase of sequencing the *S. mansoni* genome in 2009 identified over twenty genes encoding putative fucosyltransferase (FuTs), many of which are predicted to be putative α1,3- or α1,6-FuTs. However, analysis of the corresponding database showed incomplete gene sequences and truncated, possibly non-functional enzymes which required further work to identify the full length gene transcripts (2,283,287). More recent studies have identified the potential breadth of the fucosyltransferase multigene family resulting in six α1,3-FuTs, six α1,6-FuTs, and two O-FuTs. Interestingly, however, no putative α1,2-FuTs were identified (289,332), although such linkages of Fucα1-2Fucα1-2-R are known to occur in *S. mansoni* glycoproteins and glycolipids. This raises a

question as to whether some of the putative α1,3- or α1,6-FuTs might actually have α1,2-FuT activities, or if the α1,2-FuTs have been grouped in another glycosyltransferase family. As previously stated, the functions and biosynthesis of fucosylated oligosaccharides in schistosomes are not yet understood. To learn more about the biosynthesis, regulation, and expression of fucosylated epitopes we focused on the characterization of the *S. mansoni* α1,3-FuT family. In this report, we describe our study of the enzymatic activity of the α1,3-FuT-F activity in S. mansoni (smFuT-F), which we found to be capable of synthesizing the terminal Le^X structures. To our knowledge, this represents the first parasitic worm α1,3-fucosyltransferase heterologously expressed and characterized to date

4.2 Materials and Methods

4.2.1 *Materials*

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification unless otherwise noted. Water and Acetonitrile (HPLC grade) were purchased from Thermo Fisher Scientific (Waltham, MA). Expression vector pGen2 was generated by Dr. Kelley Moremen and the glyco-enzyme repository. Restriction enzymes and ligase were purchased from New England Biolabs, Inc (Beverly, MA). Primers were from Integrated DNA Technologies (Coraville, IA). Lacto-N-neotetraose (LNnT) and 2-amino-N-(2-aminoethyl)benzamide (AEAB) were synthesized (428). Lacto-N-tetraose (LNT) was purchased from V-labs, Inc (Covington, LA). All other glycans were purchased from ELICITYL OligoTech (Grenoble, France). GDP-fucose was purchased from Carbosynth (Berkshire, UK). Polyethylenimine (PEI, 25kDa linear, cat. # 23966) was purchased from Polysciences, Inc and prepared as a stock solution at a concentration of 1mg/mL in a buffer containing 25mM HEPES, pH7.5 and 150mM NaCl. The anti-GFP antibody (clone GT859) was purchased from GeneTex (Irvine, CA) and the secondary antibody HRP conjugate goat anti-mouse was purchased from KPL (Gaithersburg, MD). SuperSignal West

chemiluminescent substrates were purchased from Thermo Scientific (Rockford, IL). Plant lectins were purchased from Vector Labs (Burlingame, CA) including: AAL, Aleuria aurantia lectin and RCA-I, Ricinus communis agglutinin I. An anti-LDNF antibody (IgG - clone L6B8) and an anti-Le^X (IgG - clone F8A1.1) were developed as monoclonal antibodies by production of hybridomas from spleens of mice that had been infected with S. mansoni, as described previously (115,234). Secondary antibody conjugate goat anti-mouse IgG Alexa-633 and Cy5-streptavidin (Cy5-SA) were from Invitrogen (Carlsbad, CA). An Ultraflex-II MALDI-TOF/TOF system from Bruker Daltonics (Billerica, MA) was used for analysis of glycan conjugates. An Orbitrap Fusion Tribrid system from Thermo Fisher Scientific (San Jose, CA) was used for MSn fragmentation analysis of glycans. A FACSCalibur from BD Biosciences (San Jose, CA) with CellQuestPro acquisition software was used for flow cytometry, and FlowJo software was used for data analysis.

4.2.2 *Cloning of* S. mansoni *FuT-F*

The coding region for truncated *S. mansoni* FuT-F, smFuT-F (residues 53-435) was synthesized by Genewiz, INC (South Plainfield, NJ) with an additional NH2-terminal fusion of the 7 amino acid recognition sequence of the tobacco etch virus (TEV) protease. The synthetic DNA contained a flanking 5' EcoRI site and a 3' BamHI site and was subcloned into similarly digested chemically synthesized vector, termed pGen2, containing the following features: promoter, intron, post-regulatory element, termination, and terminal repeat sequences (429,430). The coding region of the resulting fusion protein was comprised of a 25 amino acid NH2-terminal signal sequence from the *T. cruzi* lysosomal α-mannosidase followed by an 8xHis tag, 17 amino acid AviTag, "superfolder" GFP, TEV protease cleavage site, and truncated smFuT-F (431-433). This expression vector was designated smFuT-F-pGEn2 and the recombinant product termed smFuT-F-GFP.

4.2.3 *Expression of smFuT-F-pGen2 in HEK Freestyle Cells*

To determine if smFuT-F-pGen2 encoded a soluble, active α1,3-FuT, smFuT-F-pGen2 was transiently transfected into HEK freestyle cells (HEK293f, Life Technologies, Grand Island, NY). Cells were grown to a density of 2.5x106 cells/mL in Freestyle 293 Expression Medium (Life Technologies) in suspension on a platform shaker in a humidified 37°C incubator. Before transfection, cells were spun down at 300xg for 5min and re-suspended in fresh media. To generate the transfection solutions, smFuT-F-pGen2 was diluted to 4μg/mL in Freestyle 293 Expression Medium and PEI was diluted to 9μg/mL for a final concentration of 0.5μg DNA or PEI/μL media in transfection solution. The DNA solution was added to cells, and the culture allowed to shake in 37°C incubator for 5min. The PEI solution was then added and the cells returned to the shaking incubator. After 24h, the cells were diluted 1:1 with fresh media and supplemented with valproic acid (Sigma-Aldrich, #4543) to a final concentration of 2.2mM. Cells were monitored via flow cytometry by gating on live cells and monitoring GFP positivity at 24h time points. As a control, HEK293f cells were mock transfected under similar conditions with PEI alone (no DNA). After 72h, media was clarified by centrifugation at 3000xg for 10min. Conditioned media and cell pellets were stored at -20oC prior to use.

4.2.4 *SDS-Page and Western Blot of smFuT-F-GFP Expression*

For SDS–PAGE and western blotting, 15μl aliquots of transfection culture or control culture were sampled. Cells were pelleted by centrifugation at 10,000xg for 5min and conditioned media was retained. Cells were suspended in 15μl of fresh media. 1× NuPAGE SDS sample buffer (Invitrogen) with or without 2.5% β-mercaptoethanol (βME) was added to conditioned media or cells boiled for 10min. Samples were run in 10-well Mini-PROTEAN-TGX gels at 110 volts with broad range protein standards (Spectra multicolor). Protein was transferred to a nitrocellulose membrane using Trans-Blot Turbo semi-dry transfer system. Unless noted otherwise, all subsequent incubations and washes were shaking at ambient temperature. Membranes were

blocked for 1h in 5% (w/v) bovine serum albumin fraction V (BSA) in TBS wash buffer (20mM Tris, 300mM NaCl, 0.05% Tween-20). Membrane was washed three times with TBS wash buffer and incubated with anti-GFP antibody 1:10,000 in 1% (w/v) BSA in TBS wash buffer. The membrane was then washed three times in TBS wash buffer. Secondary detection antibody, HRP-conjugated goat anti-mouse-IgG, was added for 1h at 1:20,000 in TBS wash buffer. The same wash procedure was repeated, and then SuperSignal West Pico Chemiluminescent Substrate was added for 30 seconds. The membranes were dabbed dry and exposed to film.

4.2.5 *Purification of Recombinant smFuT-F-GFP*

Cell pellets were re-suspended in a Binding Buffer (50mM NaH2PO4, 150mM NaCl, 10mM imidazole, 2% (v/v) Tween-20, 1X EDTA protease inhibitor, pH8.0) and sonicated over ice to lyse cells. Insoluble debris was removed by centrifugation at 10,000xg for 10min. The pellet was re-suspended in Binding Buffer, sonicated, and spun down two more times. The supernatant was adjusted to 0.5% Tween-20 with detergent free Binding Buffer and loaded onto a column containing 1mL Ni-NTA superflow (Qiagen, Valencia, CA) equilibrated with wash buffer (50mM NaH2PO4, 500mM NaCl, 20 mM imidazole, pH8.0). Following the loading of the sample, the column was washed with 50mL of Wash buffer and eluted in 2mL fractions with Elution buffer (50mM NaH2PO4, 500mM NaCl, 500mM imidazole, pH8.0). Fractions containing GFP were pooled and dialyzed into 25mM HEPES, 300mM NaCl, 50mM imidazole, pH7.5 with a 10 kDa molecular weight cutoff. The dialyzed samples was concentrated using Amicon Ultra 0.5mL Centrifugal Filters with 10kDa molecular weight cutoff (Millipore). Final protein concentration was determined by BCA assay.

4.2.6 *Glycan AEAB Labeling and Purification*

Glycan acceptors were selected and conjugated with AEAB as described previously (394,428,434). Briefly, 1–10 mg of glycan was mixed with 50–250µl of freshly prepared 0.35M AEAB hydrochloride salt and an equal volume of 1M NaCNBH4 in DMSO/AcOH (v/v =

7/3). The conjugation reaction was left to proceed for 2h at 65oC and was stopped by the addition of 10 volumes of cold acetonitrile and allowed to stand for 30min at -20oC. The precipitated glycan-AEAB (GAEAB) derivatives were collected after centrifugation at 10,000xg for 5min. The GAEAB pellet was dried under a vacuum for 10min, reconstituted in water, and centrifuged at 10,000xg for 2min to remove any insoluble material.

A Shimadzu HPLC CBM-20A system coupled with a fluorescence detector RF-10AXL (330nm/420nm excitation/emission) was used for HPLC analysis and separation of GAEABs. The GAEABs were purified by reverse phase HPLC on a porous graphitized carbon column (PGC, 150 × 4.6 mm, Thermo Fisher Scientific) with a gradient of 15-45% acetonitrile (0.1% trifluoroacetic acid) in 40min to separate the GAEABs from any unincorporated label. The effluents were monitored by fluorescence where the GAEAB peak fractions were collected, quantified by fluorescence, and dried under vacuum. GAEABs were reconstituted to 1-5mM stocks in water and stored at -20oC until use.

4.2.7 *Fucosyltransferase Assays*

The fucosyltransferase assays were performed in 50µl reaction mixtures containing 50mM sodium cacodylate, pH7.0, 20mM MnCl2 (or other metal salt), 4mM GDP-fucose, 4µM GAEAB sugar acceptor, and smFuT-F-GFP. No smFuT-F-GFP was added for mock reaction conditions. Reaction mixtures were incubated rotating at room temperature or 37oC for 4d. The enzyme was removed from the reaction mixture via porous graphitized carbon solid phase extraction. Fucosyltransferase GAEAB products were then separated from GAEAB acceptors by reverse phase HPLC on a PGC column. Each peak fraction was collected, quantified by fluorescence, and dried under vacuum. Samples were then processed for either MALDI-TOF MS for molecular mass analysis or Electrospray Ionization Mass spectrometry (porous graphitized carbon column MS) for MS^n fragmentation.

4.2.8 *Mass Spectrometry of FuT Products*

For MALDI-TOF MS analysis, purified fucosyltransferase GAEAB products were acetylated using acetic anhydride in saturated sodium bicarbonate for 30min on ice, followed by room temperature incubation for 1h. Glycans were then desalted via porous graphitized carbon solid phase extraction. Any esters formed during acetylation were hydrolyzed by incubation in 0.01 M sodium hydroxide at room temperature for approximately 3-4h and then lyophilized (112,435). The samples were then permethylated according to reported procedures to increase the sensitivity of MS analysis (436,437). Briefly, a lyophilized sample was treated with 200µL DMSO/NaOH slurry and 200µL methyl iodide for 30min, shaking. The supernatant was then partitioned between 500µL water and 500µL chloroform. The organic layer was washed four times with 500µL water, dried, and re-dissolved in 50% methanol:water for MALDI-TOF MS analysis. Mass spectrometry data were collected using an Ultraflex MALDI TOF/TOF (Bruker Daltonics, Billerica, MA).

For ESI MS and MS^n analysis, AEAB was removed from the fucosyltransferase GAEAB products by incubation in 10mM sodium hypochlorite for 30min at room temperature resulting in a pentose proximal sugar. The reaction was quenched with the addition of formic acid, dried under a vacuum, and then permethylated as described. MS data were collected using an Orbitrap Fusion (Thermo Fisher Scientific). Spray parameter was 3.5 kV. Full MS spectra were acquired with resolution of 60,000 at m/z 200 with an automatic gain control (AGC) target value of 2x105 and maximum ion injection time of 100ms. Ions for MS/MS fragmentation were isolated in the quadrupole with an isolation window of 3 and analyzed in the Orbitrap analyzer. CID fragmentation was performed in the ion trap with normalized collision energy of 35, and rapid scan MS analysis. The raw data was processed using XCalibur (Thermo, San Jose).

4.2.9 *Glycobead Assay*

Purified fucosyltransferase GAEAB products were conjugated to Polybead Carboxylate Microspheres (cat# 17141, 6.0µM, Polysciences, Inc.) using the Polylink protein coupling kit (cat#

24350) with some changes. Briefly, beads were activated by suspension in 160µL of Polylink coupling buffer (50mM MES, 0.05% Proclin-300, pH5.2) with the addition of 20µL of a 200mg/mL 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 200mg/mL N-hydroxysulfosuccinimide (sulfo-NHS) solution. After rotation at room temperature for 30min, beads were washed once with Polylink wash buffer (10mM Tris, 0.05% BSA, 0.05% Proclin-300, pH8.0). Beads were then incubated in 1mM GAEABs (100mM phosphate buffer, pH8.5) rotating, at room temperature for 1h, washed 3x with Polylink wash buffer, and stored at 4oC until use.

Biotinylated lectins and monoclonal antibodies were used to validate the beads for conjugation efficiency and for the characterization of fucosyltransferase GAEAB products. Beads were washed three times in Polylink wash buffer and incubated, rotating for 1h with lections or mAbs (0.05µg/mL AAL, 2.0µg/mL RCA-I, or 1µg/mL antibody) in Binding buffer (20mM Tris-HCl, 150mM NaCl, 2mM MgCl2, 5mM CaCl2, 0.05% Tween-20, 1% BSA, pH7.4). Beads were washed with three times with Polylink wash buffer and incubated for 1h with Cy5-SA or anti-mouse IgG AlexaFluor 633 1:400 in Binding buffer. Beads were washed three times in Polylink wash buffer, re-suspended in water, and analyzed by flow cytometry.

4.2.10 Schistosoma *Life Stages*

S. mansoni-infected *Biomphalaria glabrata* snails, strain NMRI NR-21962, were provided by the Schistosome Research Reagent Resource Center through BEI Resources, NIAID, NIH. Snail maintenance, collection of cercariae, transformation to schistosomula, and isolation of adult worms from mice was conducted as previously described (272,438). Briefly, snails maintained in a dark room were placed into beakers of conditioned water, under a bright light, for 2h. 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 500×g for 10min at 4°C. For transformation to schistosomula, the cercarial pellet was resuspended in 5–10mL of cold DMEM and vortexed on high for 2×45 s periods, with a 3min ice incubation in between; or the pellet was resuspended to

1000 cercaria/mL in cold DMEM and transformed by passage 8 times though a syringe and 22-G needle, in 10mL batches. The suspension of detached schistosomula and tails was allowed to settle and the bottom few milliliters were loaded onto a 60% Percoll gradient in DMEM. The gradient was centrifuged for 15min at 500×g, 4°C. The supernatant was carefully removed, and the schistosomula pellet was washed three times by spinning at 300 × g in cold DMEM. The schistosomula were cultured for 3d in DMEM with 10% FBS, penicillin/streptomycin at a density of 500 organisms/mL in tissue-culture dishes. 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 in compliance with University-approved Biosafety and IACUC protocols.

For adult worms, Swiss-Webster mice (6–8 weeks old) 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 200 (high dose) cercaria 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 7.5wks post-infection for collection of adult worms and eggs. Euthanasia was by intraperitoneal overdose with 300μL of 65mg/mL sodium pentobarbital with 200 U/mL heparin sodium salt, followed by mesenteric perfusion (0.85% NaCl, 0.75% trisodium citrate dihydrate) for worm collection. Worms were washed several times with PBS. All experiments involving mice were approved by the Emory University IACUC.

4.2.11 *Quantitative RT-PCR of FuT Transcription Levels*

Relative transcript abundance in cercariae, newly transformed and 3-day cultured schistosomula, and adults was assessed using the comparative CT (ΔΔCT) method, where β-tubulin was used as an endogenous calibrator gene to normalize the CT values for a gene of interest. The compatibility of the tubulin calibrator and FuT primers under normal reaction conditions was

assessed by plotting ΔCT at various dilutions of cDNA input and determining the slope of the resultant line, and primer efficiencies were deemed compatible if the absolute value of the slope was less than 0.1. Primers used in this study for qPCR are listed in **Table 4.1**. Cercariae, newly transformed schistosomula, 3-day cultured schistosomula, and adult worms were washed 5 times with PBS and total RNA extracted using the RNAeasy kit (Qiagen) according to manufacturer's instructions. RNA was converted to first-strand cDNA using the Superscript III-First-Strand Synthesis System.

Real-time qPCR reactions (20mL/rxn) were prepared in triplicate, comprising 1X SYBR Green PCR Master Mix (Applied Biosystems), 5μM each forward and reverse gene-specific primers, and 15ng RNA input-equivalents parasite cDNA. Reactions were run on a Step One plus Real-time PCR System (Applied Biosystems) with the following cycle profile: initial denaturation at 95ºC/10 min followed by 50 cycles of 95ºC/15 sec, 50ºC/11 sec, and 60ºC/1 min. PCR product accumulation was monitored in real time, and amplification fidelity was assessed by post-cycling thermal dissociation and electrophoretic fractionation. To assess FuT expression by the ΔΔCT method, the geometric mean of tubulin used to normalize FuT CT such that $\Delta CT = CT_{FuT} - CT_{tubulin}$. Student's T-tests were used to compare α1,3-FuT expression in adults versus cercariae and somules across three independent biological replicates, with significance set at $p<\#0.05$.

Name	Sequence
FuT A Forward	5′- TATTCCCTGAACGACCAGAATG
FuT A Reverse	5′- TTAGCCTCCTTCCATGACAAC
FuT B Forward	5′- ACAGCAGATCCAGTTTTGTGTC
FuT B Reverse	5′- GAACTACCCGTACAACTAAATGC
FuT C Forward	5′- GTAAGTGGCAATGGAGACAATATC
FuT C Reverse	5′- CCAAGATTGATTCCTCGGTTTG
FuT D Forward	5′- GAGCTGTCGCTTGGATAGTAAG
FuT D Reverse	5′- GACCACATCGACCATACACATC
FuT E Forward	5′- GTGTCTACTGGCACATACATCTC
FuT E Reverse	5′- CGCAATTTCCGACCATTACATC
FuT F Forward	5′- GTGTCTACTGGCACATACATCTC
FuT F Reverse	5′- CGCAATTTCCGACCATTACATC
β-tubulin Forward	5′- TGATCCAACTGGCACATACC
β-tubulin Reverse	5′- TAGCACGAGGCACATACTTTC

Table 4.1 qPCR Primers for transcription analysis

Sequences of selected primers for FuT-A through -F and β-tubulin used in real-time PCR assays.

4.3 Results

4.3.1 *Identification and cloning of a possible Lewis type fucosyltransferase*

Several recent studies have taken a genomic/transcriptomic approach to identify and explore the fucosyltransferase multigene family in *Schistosoma mansoni*, which has previously been incompletely annotated in the gene database (287,289,332). Peterson *et. al.* (332) recently determined the extent of the FuT family by elucidating the full length gene transcripts for seven putative α1,3-FuT genes (FuT-A-G) resulting in five new full length transcripts (FuT-B-F), one previously identified FuT (FuT-A) (337), and one pseudogene (FuT-G). Phylogenetic and sequence analysis of the six putative FuT genes A-F suggest two lineages, which can be further divided into

three tentative groups based on predicted functionality (**Figure 4.1A**). One lineage consists of a possible core FuT (FuT-C) and the other (FuT-A, -B, -D, -E, and -F) may synthesize more distal glycan motifs (332,340,439,440). However, further dissection of the larger grouping allowed us to tentatively separate out genes that have the highest identity with well-characterized Lewis-type FuTs in other species. Based on these observations, we predicted that FuT-F and FuT-E might generate various Lewis X motifs (Le^X, poly-Le^X) in *Schistosoma* glycoconjugates, whereas the remaining genes may produce other fucosylated motifs (LDNF, FLDN, di-fucose, etc.). Because of these relationships and predictions, we focused on *S. mansoni* FuT-F, also abbreviated smFuT-F.

Typically in animal cells the α1,3-FuTs are Golgi-anchored, type-II transmembrane proteins featuring a single transmembrane domain (TMD), which is flanked by a short cytoplasmic N-terminal tail and a lumenal C-terminus that comprises a globular catalytic domain and a flexible hypervariable stem (301,332,340,423). Sequence analysis of smFuT-F revealed the presence of these well conserved domains found in α1,3-FuTs (**Figure 4.1B**). Importantly, smFuT-F contains the canonical GPD-fucose binding sequence, YxFxL/VxFENSxxxxYxTEK (301,423,441). Analyses of transmembrane topology with TMHMM Server v. 2.0 suggest that smFuT-F is a traditional type-II transmembrane protein containing a single N-terminal TMD. Using NetNGlyc 1.0, there are five predicted N-glycosylation sites at asparagine (N) residues with the sequon NxS/T where x≠P. The presence of a DXD motif is conserved among glycosyltransferase enzymes and believed to play a role in metal ion binding and catalysis (442,443). While markedly different than the motif of α1,3/4-FuTs in humans and other species, VxxHH(W/R)(D/E), Peterson *et. al.* (332) were able to annotate a region in the stem which may contribute to smFuT-F's acceptor specificity (444,445). Thus, the encoded protein of smFuT-F contains all the hallmarks for a Golgi-localized Type II membrane glycosyltransferase, which likely utilizes GDP-fucose as a donor sugar.

To determine whether the smFuT-F gene encodes a functional fucosyltransferase, we generated a truncated version of the gene (residues 53-435) to remove the transmembrane domain (**Figure 4.1B**), and added an N-terminal TEV cleavage sequon flanked by restriction enzyme cut sites. The synthetic gene was then inserted into the mammalian expression vector pGen2 and the resulting plasmid was smFuT-F-pGen2. The subsequent recombinant protein product, smFuT-F-GFP, included a GFP domain, Avi and 8xHis tags, and a *T. cruzi* single peptide targeting smFuT-F-GFP for secretion into the media (**Figure 4.1C**).

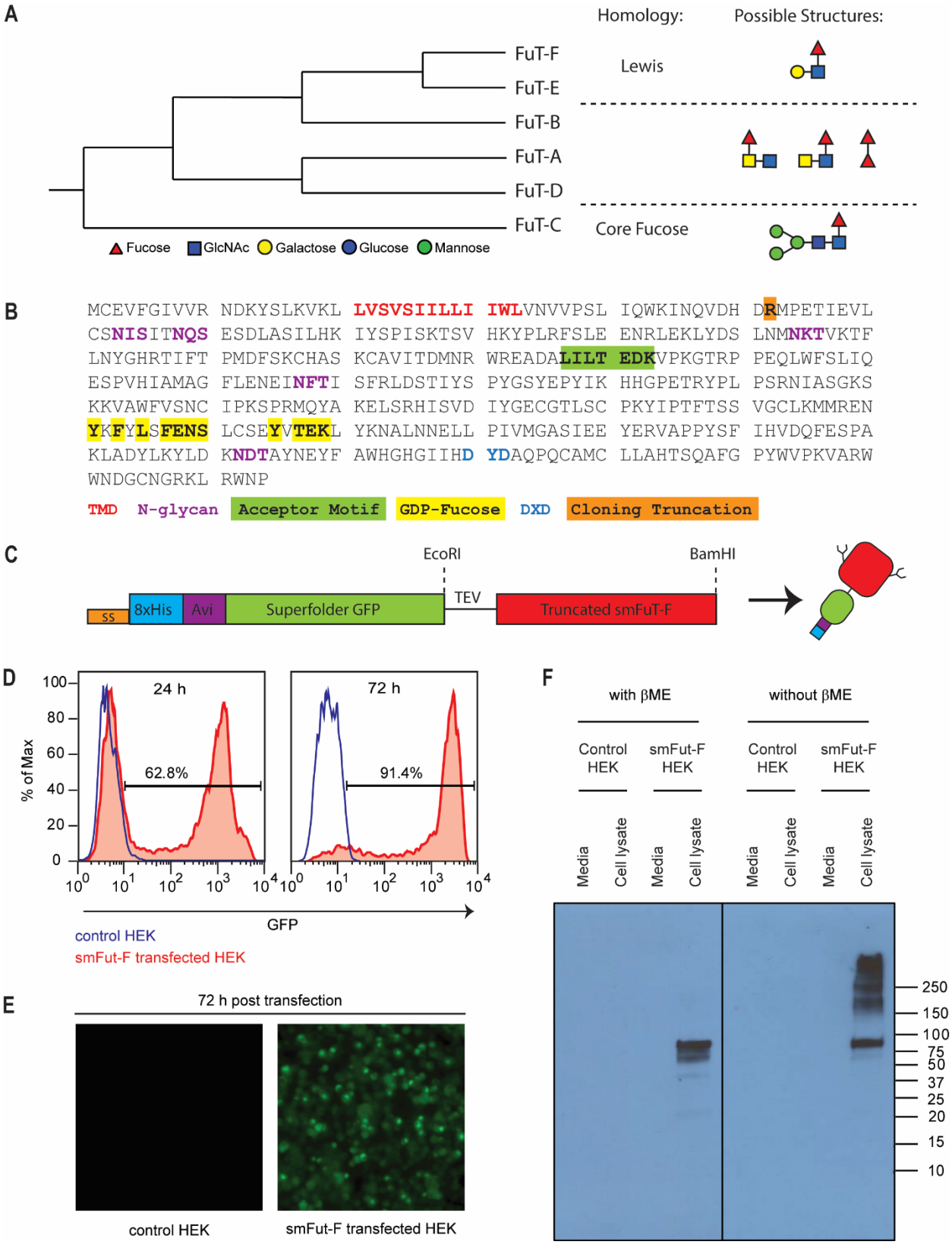
A
Homology:
Possible Structures:
FuT-F
FuT-E
FuT-B
FuT-A
FuT-D
FuT-C
Lewis
Core Fucose
Fucose
GlcNAc
Galactose
Glucose
Mannose
B
MCEVFGIVVR NDKYSLKVKL LVSVSIILLI IWLVNVVPSL IQWKINQVDH DRMPETIEVL
CSNISITNQS ESDLASILHK IYSPISKTSV HKYPLRFSLE ENRLEKLYDS LNMNKTVKTF
LNYGHRTIFT PMDFSKCHAS KCAVITDMNR WREADALILT EDKVPKGTRP PEQLWFSLIQ
ESPVHIAMAG FLENEINFTI SFRLDSTIYS PYGSYEPYIK HHGPETRYPL PSRNIASGKS
KKVAWFVSNC IPKSPRMQYA KELSRHISVD IYGECGTLSC PKYIPTFTSS VGCLKMMREN
YKFYLSFENS LCSEYVTEKL YKNALNNELL PIVMGASIEE YERVAPPYSF IHVDQFESPA
KLADYLKYLD KNDTAYNEYF AWHGHGIIHD YDAQPQCAMC LLAHTSQAFG PYWVPKVARW
WNDGCNGRKL RWNP
TMD
N-glycan
Acceptor Motif
GDP-Fucose
DXD
Cloning Truncation
C
EcoRI
BamHI
TEV
SS
8xHis
Avi
Superfolder GFP
Truncated smFuT-F
D
24 h
72 h
62.8%
91.4%
% of Max
GFP
control HEK
smFut-F transfected HEK
E
72 h post transfection
control HEK
smFut-F transfected HEK
F
with βME
without βME
Control HEK
smFut-F HEK
Media
Cell lysate
250
150
100
75
50
37
25
20
15
10

Figure 4.1. Selection, cloning, and expression of *Schistosoma mansoni* FuT-F.

(A) Sequence analysis of the α1,3-FuT family allows for separation into roughly three groupings with suggested functions and glycan motifs. **(B)** Sequence analysis of smFuT-F revealed the presence of these well conserved domains found in α1,3-FuTs including a transmembrane domain (TMD), five sites of potential N-glycosylation, canonical GDP-fucose biding region, a metal binding DXD motif, and a possible sequence which could affect acceptor specificity. The truncation site for removing the TMD for pGen2 cloning is also annotated. **(C)** Cartoon depiction of the soluble (ss=*T. cruzi* secretion signal), tagged (His tag and Avi Tag), GFP fusion protein (smFuT-F-GFP) that resulted from cloning of truncated smFuT-F into the pGen2 expression vector. GFP positivity of HEK freestyle cells by flow cytometry **(D)** and fluorescent microscopy **(E)**, after transfection with smFuT-F-pGen2or mock transfections (control). By 72h post transfections, over 90% of transfected cells are expressing GFP and can be harvested for smFuT-F-GFP purification. **(F)** Anti-GFP western blot of smFuT-F-pGen2 transfected or control HEK cells and conditioned media 72h post-transfection. smFuT-F-GFP is found in transfected cells but not secreted into the media, possibly due to the higher molecular weight oligomers seen in non-reducing conditions.

4.3.2 *Expression of smFuT-F-pGen2 generates a recombinant GFP fusion protein*

Recombinant protein was generated by transient transfection of the expression construct, smFuT-F-pGen2, into HEK293f cells. Cells were monitored by flow cytometry and microscopy for GFP fluorescence at 24h time points. After 72h, over 90% of transfected cells were strongly GFP positive (**Figure 4.1D, 4.1E**). The expression and localization of the recombinant smFuT-F-GFP protein was confirmed by SDS–PAGE and Western blotting using an antibody directed to the GFP superfolder protein domain. The immunoblot revealed a protein band with apparent MW between 70-100 kDa, which would be expected of a variably glycosylated polypeptide of 74.6 kDa predicted from the full-length GFP fusion protein (**Figure 4.1F**). Surprisingly, smFuT-F-GFP was not secreted into the media as we expected, and Western blotting under non-reducing conditions revealed higher order oligomers (**Figure 4.1F**). Thus, it is possible that smFuT-F-GFP may be oligomerizing with itself or with other Golgi-localized proteins, or glycan acceptors that prevent

its release from the cell. The lack of secretion has not been observed when other, non-schistosomal, glycosyltransferase genes are expressed in the pGen2 vector (data not shown).

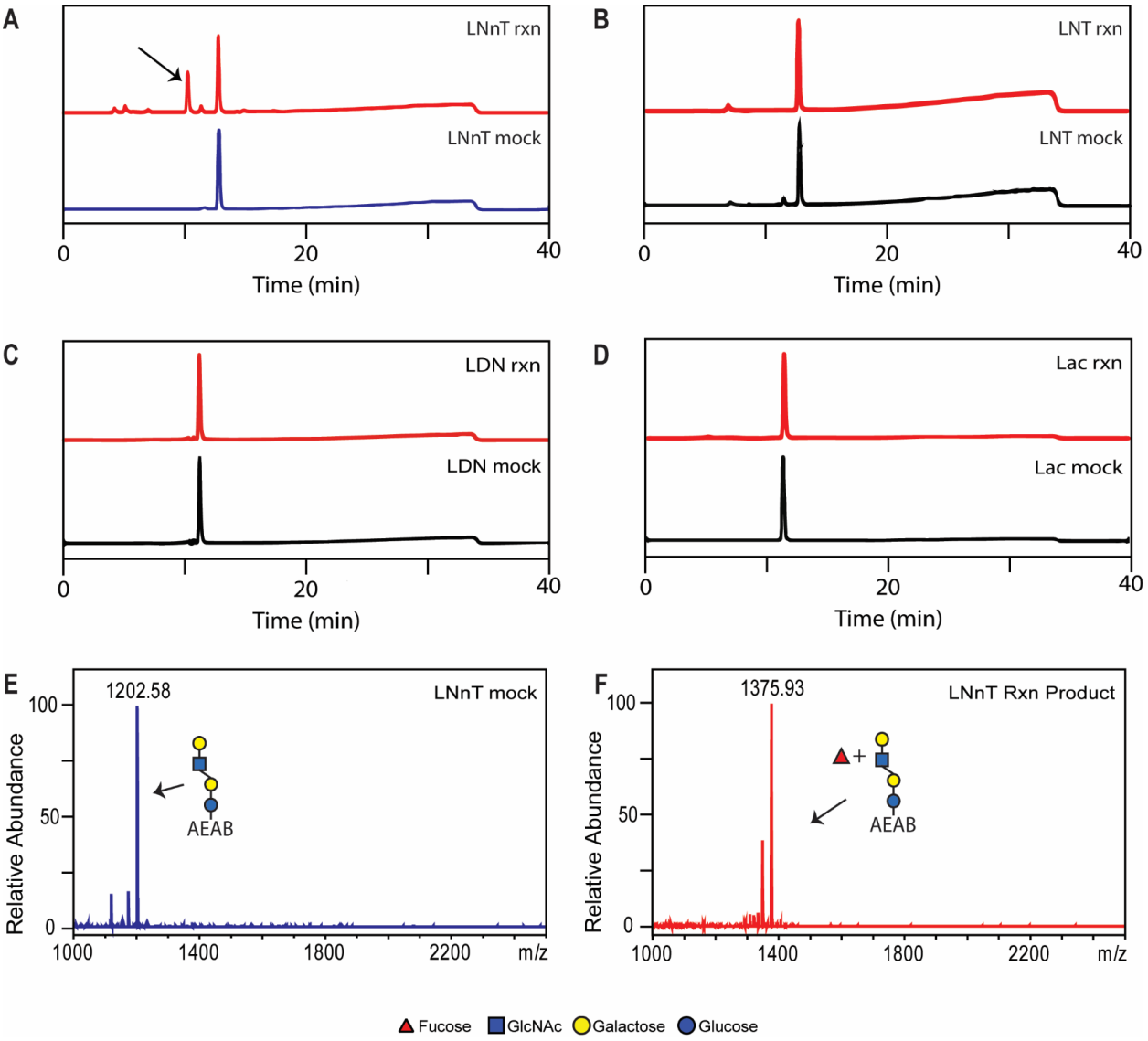

Figure 4.2. Analysis of smFuT-F-GFP activity towards common *Schistosoma* terminal motifs.

HPLC chromatograms of smFuT-F or mock reactions utilizing acceptors LNnT **(A)**, LNT **(B)**, LDN **(C)**, and lactose (lac) as a control **(D)**, and separated over a PGC column. A product peak is seen in the smFuT-F LNnT reaction and noted by the arrow. MALDI-TOF MS spectra of LNnT mock product (m/z=1202.58) **(E)** and the smFuT-F LNnT product (m/z= 1375.93) **(F)** showing the addition of a fucose moiety (LNnT+Fuc). AEAB labeled samples were peracetylated and permethylated prior to MALDI-TOF analysis.

4.3.3 *smFuT-F-GFP adds fucose to type II glycan chains*

To characterize the activity of smFuT-F-GFP, it was purified from the HEF293f cells using Ni-NTA agarose and initially tested with a small panel of glycan acceptors. LacNAc (LN; N-acetyllactosamine), which contains a type II glycan sequence, and LacdiNAc (LDN) are common terminal motifs in *Schistosoma* glycoproteins, however the additional modifications (sialylation, sulfation, fucosylation) often differ between host and parasite (105,132,236,253,446,447), e.g. schistosomes lack sialic acid. Previous work, based on glycan structural analyses, indicated that *Schistosoma* α1,3- or α1,4-FuTs might preferentially utilize type II glycans chain, since type I chains Galβ1-3GlcNAc are not found (423,448,449). To this end, and to compare type I versus type II chains, we selected LNnT, LNT, LDN, and lactose (glycan sequences shown in **Table II**) in our initial screening panel of acceptors (**Figure 4.2A-D**). smFuT-F-GFP generated a product from the type II acceptor LNnT (**Figure 4.2A, Table 4.2**). The smFuT-F product from LNnT was examined by MALDI-TOF MS; the size confirmed that a single fucose had been added - size shift from 1202.575 to 1375.927 m/z (**Figure 4.2E, 4.2F**). It was unclear, however, which residue was modified by fucose; interestingly, the lack of product generated from lactose suggested one of the more distal sugars, GlcNAc or galactose, may be the location of the added fucose. In addition, no detectable product was found for reactions involving the oligosaccharides LNT, LDN, or lactose (**Figure 4.2B-2D, Table 4.2**).

Acceptor substrate	Shorthand	Relative activity (%)
Galβ1-4GlcNAcβ1-3Galβ1-4Glc-AEAB	LNnT	83
Galβ1-3GlcNAcβ1-3Galβ1-4Glc-AEAB	LNT	n.d.
GalNAcβ1-3GlcNAcβ1-3Galβ1-4Glc-AEAB	LDN	n.d.
Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc-AEAB	Para-LNnH (PLNnH)	94
Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc-AEAB	LSTd	6
Galβ1-4GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6)Galβ1-4Glc-AEAB	LNnH	100
Galβ1-3GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6)Galβ1-4Glc-AEAB	LNH	49
Galβ1-4GlcNAcβ1-2Manα1-3(Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAc-AEAB	Na2	76
Galβ1-4Glc-AEAB	Lactose (Lac)	n.d.

- Acceptor demonstrating highest activity set at 100%
- n.d. = no detectable activity

Table 4.2. Activity of smFuT-F-GFP towards selected glycan acceptors

Glycan sequence, shorthand, and relative activity of recombinant smFuT-F-GFP towards each glycan tested. Glycan acceptor demonstrating the highest activity was set at 100%.

To determine linear sequence and branching of the LNnT product from the smFuT-F reaction, a multistage MS^n analysis was used after chemical removal of the AEAB tag and sample methylation. The MS^2 spectrum of a singly charged [M + Na]+ precursor ion (1056.60 m/z) of the pentasaccharide LNnT smFuT-F product is shown in **Fig. 3A**. Major fragmentations are diagrammed, showing the ions represented by cleavage of the glycosidic bonds (**Fig. 3B**) (450-

452). The peak at 660.33 m/z matches the calculated mass of a trisaccharide comprised of Galβ1-4GlcNAc with a fucose of undetermined location. From the MS^2 spectrum (**Fig. 3A**), peak 660.33 m/z was isolated and selected as the precursor ion for MS^3 (**Fig. 3C**). Spectral analysis determined the peak pattern consistent with both glycosidic and cross-ring fragmentation of Galβ1-4(Fuc1-3)GlcNAc (**Fig. 3D**) (450-452). The predicted structure for the LNnT smFuT-F product in illustrated in **Figure 3E**.

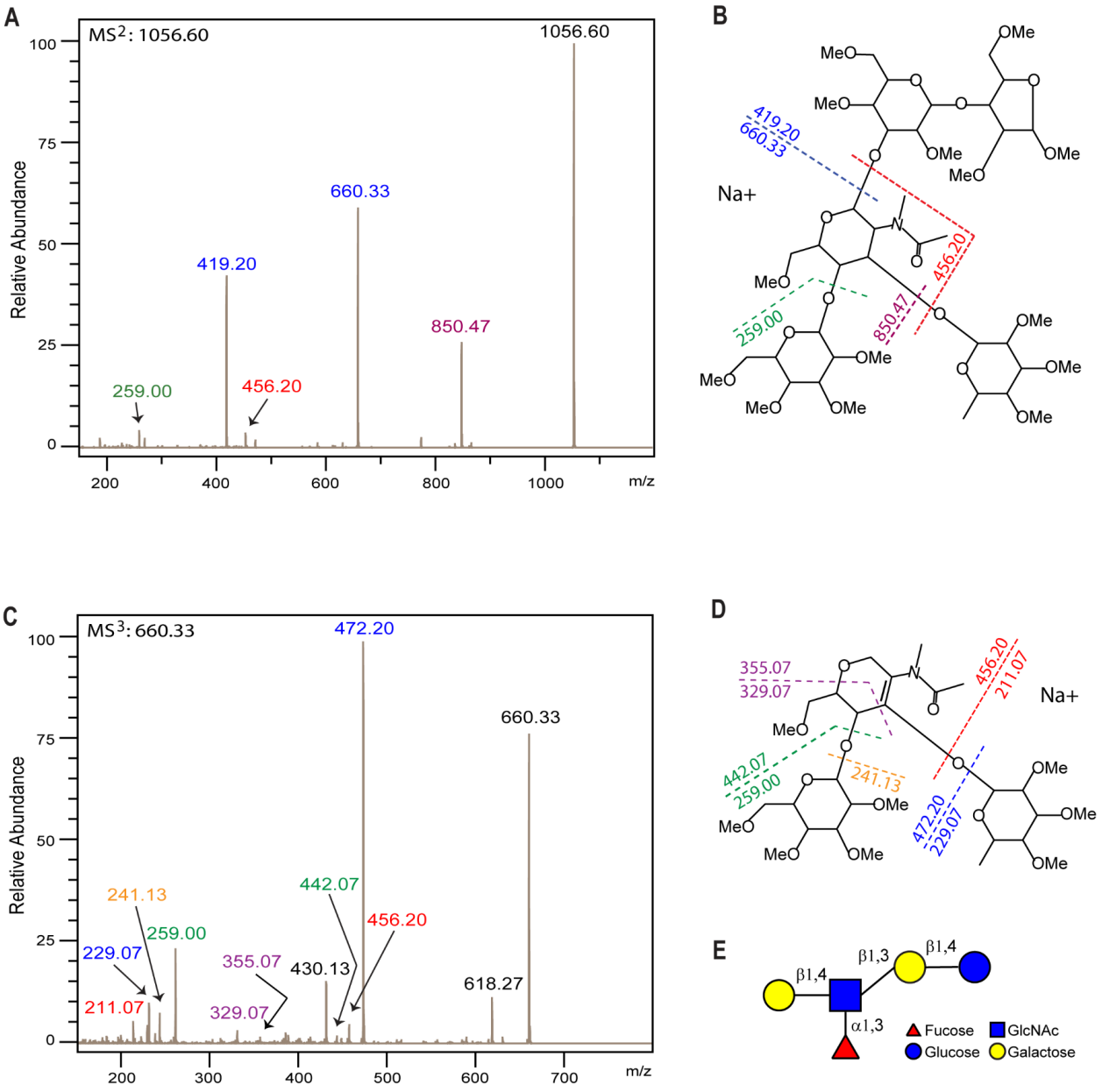

Figure 4.3. MS^n fragmentation of smFuT-F LNnT pentose product.

ESI MS^n spectra of smFuT-F LNnT product after chemical removal of AEAB, resulting in a proximal pentose sugar, and sample permethylation. **(A)** For MS^2 the major product ion (m/z=1056.60) was isolated for fragmentation in the quadrupole and subjected to CID fragmentation in the ion trap with normalized collision energy of 35. The resulting fragments correspond to the glycosidic bond breakages depicted in **(B)**, including a peak at m/z=660.33 which is consistent with the structure Galβ1-3GlcNAc with fucose. **(C)** This ion (m/z=660.33) was selected for MS^3 analysis under conditions similar to the MS^2 spectra. The resulting fragments correspond to the glycosidic bond breakages and cross

ring fragmentation depicted in **(D)**, including a peak at m/z=355.07 which is consistent with the cross ring fragment of GlcNAc with fucose linked to carbon 3. **(E)** Cartoon depiction of the fucose location on smFuT-F LNnT product with predicted linkage.

4.3.4 *smFuT-F generates the Lewis X motif*

To confirm the fucose-GlcNAc bond orientation produced by smFuT-F-GFP, LNnT smFuT-F product or mock reacted LNnT were HPLC purified, conjugated to beads, and stained with highly defined lectins and mAbs. Carboxyl beads were activated with EDC and sulfo-NHS to form amine-reactive sulfo-NHS esters which conjugates to the bifunctional linker AEAB with high efficiency (428). To verify the conjugation of glycans to the beads, biotinylated Gal- and Fuc-specific lectins (RCA-I, AAL) were used to confirm the presence of mock reacted LNnT and a fucosylated LNnT smFuT-F product (**Figure 4.4**). As the presence of fucose on the product of smFuT-F using LNnT as the acceptor was confirmed by both mass spectrometry and lectin binding, we utilized highly specific anti-glycan antibodies to confirm the motif and, by extension, the glycosidic bond orientation. To test for the generation of Le^X we used mAb F8A1.1, which binds terminal motifs, and L6B8, an anti-LDNF antibody, acted as an isotype control (**Figure 4.4**) (115,272). The smFuT-F reaction product beads bound F8A1.1 and did not bind the control antibody, confirming the glycans sequence of the enzyme product is Galβ1-4(Fucα1-3)GlcNAc and the Le^X epitope generating the structure LNFPIII from LNnT and confirming the product predicted in the cartoon **Figure 4.3E**.

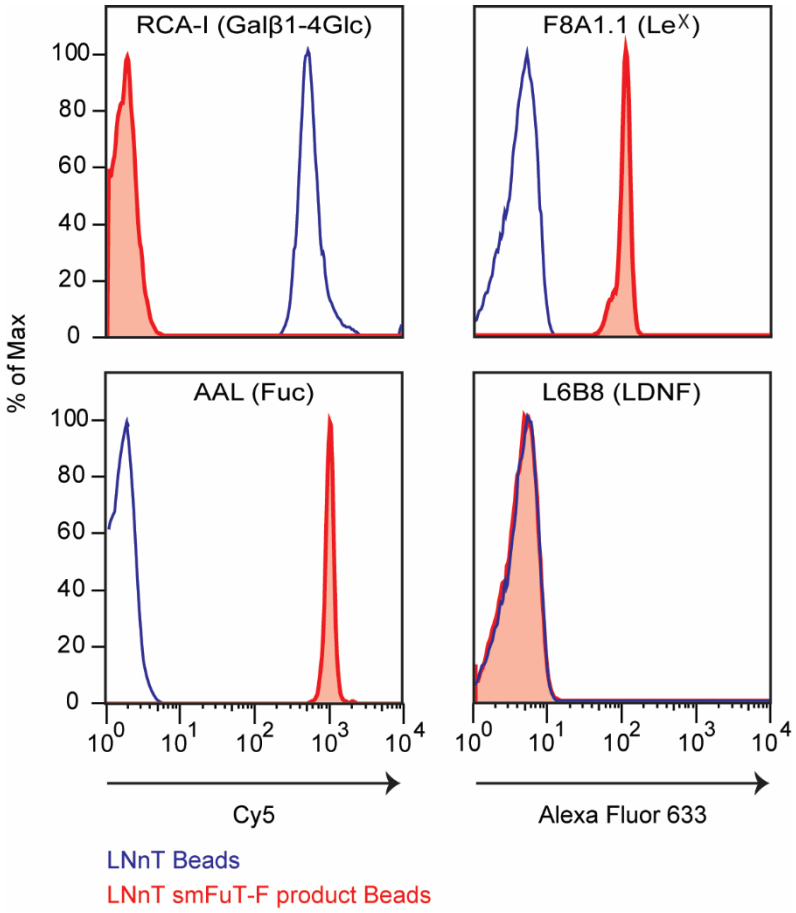

Figure 4.4 smFuT-F-GFP generates Le^X antigen and is bound by monoclonal antibody F8A1.1.

Lectin and anti-glycan antibody staining of mock or smFuT-F reacted LNnT conjugated to carboxybeads and monitored by flow cytometry. Control beads (LNnT mock) were bound by RCA-I (Galβ1-4Glc) but not AAL (Fuc), F8A1.1 (Le^X), or L6B8 (LDNF), which is consistent with the LNnT glycan. SmFuT-F LNnT reacted beads bound AAL (Fuc) and F8A1.1 (Le^X) but not RCA-I (Galβ1-4Glc) or L6B8 (LDNF), which is consistent with the fucose residue added to the 3 carbon of the GlcNAc being in the α-orientation forming the Le^X structure.

4.3.5 *Acceptor specificity of smFuT-F-GFP*

To determine the range of type II glycan acceptors that smFuT-F-GFP might utilize, a panel of five additional acceptors with further modifications on linear chains and branched structures were tested (glycan sequences shown in **Table 4.2**). Despite previous work showing *Schistosoma*

α1,3/4-FuTs are able to utilize α2,3-sialylated type II structures, smFuT-F-GFP did not efficiently fucosylate LSTd, an α2,3-sialylated LNnT structure (**Figure 4.5A, Table 4.2**) (423,448). However, this may not be surprising as *S. mansoni* neither synthesizes sialic acid nor acquires it from their hosts (70,71). Mass spectra of the LSTd product were consistent with LNnT results as MALDI-TOF MS causes loss of sialic acid (**Figure 4.5C**) (453,454).

Schistosoma express Le^X terminally and within poly-Le^X structures comprised both internal and terminal motifs (2,34,115). To determine whether smFuT-F-GFP generate one or both of these structures, we examined its reactivity to poly-N-acetyllactosamine type glycan, PLNnH (**Figure 4.5B**). The minor reaction product 1 had the mass equivalent with PLNnH plus two fucose residues, whereas the major product 2 acquired a single fucose which could have been on the internal or terminal LN (Galβ1-4GlcNAc) sites (**Figure 4.5D, 4.5E**). MS^2 spectrum of a singly charged [M + Na]+ precursor ion (1505.74 m/z) of the PLNnH smFuT-F product 2 (**Figure 4.5F**) and spectral analysis of major peaks from both glycosidic and cross-ring fragmentation concluded that the fucose was located on the terminal LN (**Figure 4.5G**) (450-452). On the basis of this data we propose the primary smFuT-F product generated from PLNnH is Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc depicted in **Figure 4.5H**, and that smFuT-F-GFP preferentially modifies terminal LN sites.

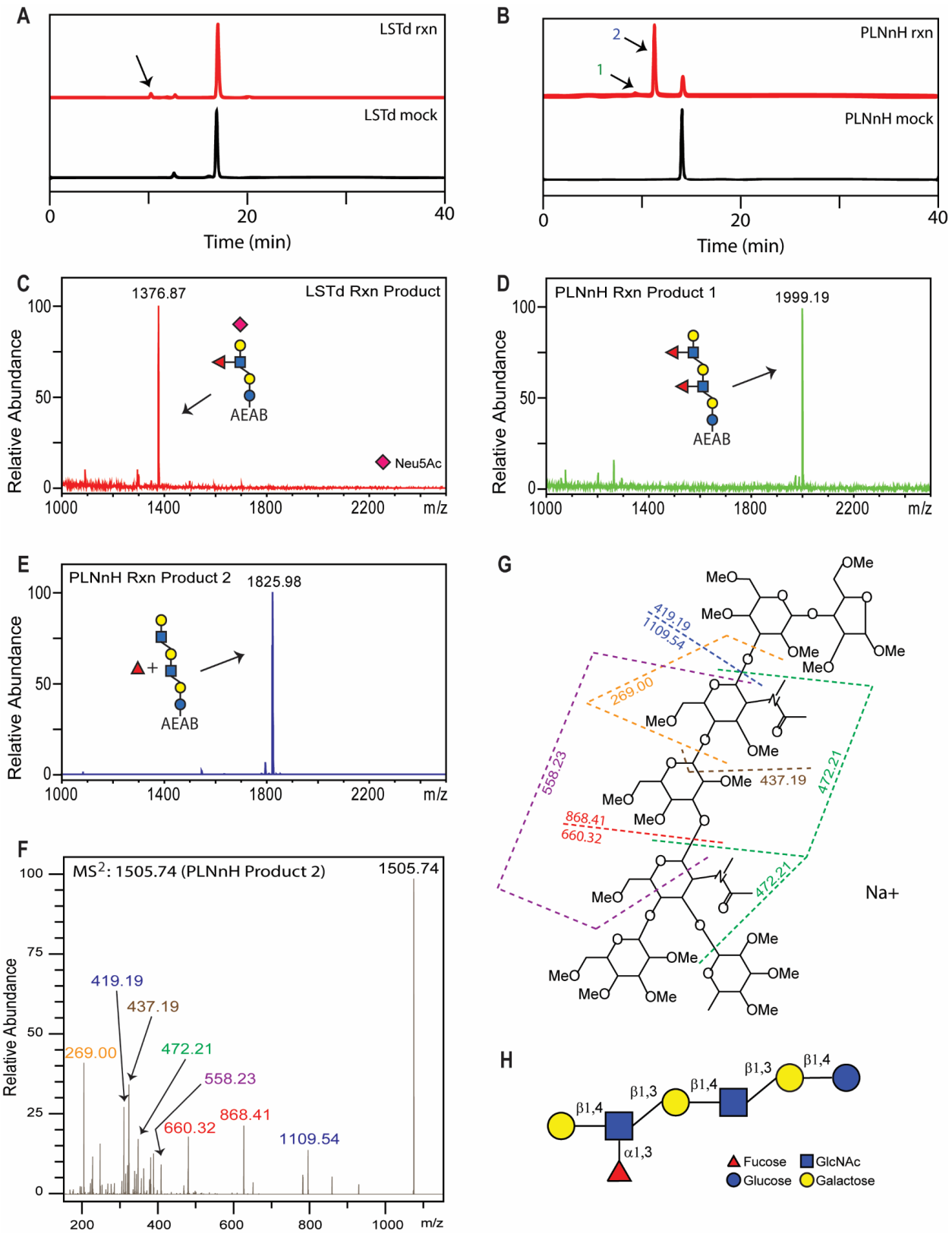
A
LSTd rxn
LSTd mock
0
20
40
Time (min)
B
2
1
PLNnH rxn
PLNnH mock
0
20
40
Time (min)
C
1376.87
LSTd Rxn Product
AEAB
Neu5Ac
Relative Abundance
100
50
0
1000
1400
1800
2200
m/z
D
PLNnH Rxn Product 1
1999.19
AEAB
Relative Abundance
100
50
0
1000
1400
1800
2200
m/z
E
PLNnH Rxn Product 2
1825.98
AEAB
Relative Abundance
100
50
0
1000
1400
1800
2200
m/z
F
MS2: 1505.74 (PLNnH Product 2)
1505.74
419.19
437.19
269.00
472.21
558.23
868.41
660.32
1109.54
Relative Abundance
100
75
50
25
0
200
400
600
800
1000
m/z
G
419.19
1109.54
269.00
558.23
437.19
472.21
868.41
660.32
472.21
Na+
H
β1,4
β1,3
β1,4
β1,3
β1,4
α1,3
Fucose
GlcNAc
Glucose
Galactose

Figure 4.5. Characterization of smFuT-F-GFP activity towards sialylated and poly-N-acetyllactosamine acceptors.

HLPC chromatograms of smFuT-F or mock reactions utilizing acceptors LSTd **(A)** or PLNnH **(B)** and separated over a PGC column. Product peaks are seen in both reactions, with a single product in the case of LSTd and two products for PLNnH and are noted by arrows. MALDI-TOF MS spectrum of smFuT-F LSTd product (m/z=1376.87) **(C)** showing the addition of a fucose moiety and the loss of sialic acid (Neu5Ac) (LSTd+Fuc-Neu5Ac). MALDI-TOF MS spectrum for smFuT-F PLNnH product 1 (m/z= 1999.19) **(D)** shows the accumulation of two fucose residues (PLNnH+2Fuc), and smFuT-F PLNnH product 2 (m/z=1825.98) **(E)** with a single fucose addition on one of the LN motifs (PLNnH+Fuc). AEAB labeled samples were peracetylated and permethylated prior to MALDI-TOF MS analysis. AEAB labeled samples were peracetylated and permethylated prior to MALDI-TOF MS analysis. **(F)** ESI MS^2 spectra of smFuT-F PLNnH product 2 after chemical removal of AEAB, resulting in a proximal pentose sugar, and sample permethylation. The major product ion (m/z= 1505.74) was isolated for MS^2 fragmentation in the quadrupole and subjected to CID fragmentation in the ion trap with normalized collision energy of 35. The resulting fragments correspond to the glycosidic bond breakages and cross-ring fragmentation depicted in **(G)**. **(H)** Cartoon depiction of smFuT-F PLNnH product 2 with the fucose moiety on the terminal LN sequence.

To explore the ability of smFuT-F-GFP to modify branched structures, LNH and LNnH, containing branches with type I and type II chains, were assessed as glycan acceptors. As expected from the LNT and LNnT results, smFuT-F-GFP added fucose to only one branch of LNH, likely the type II chain (**Figure 4.6A, 4.6C**). With LNnH, which has two branches with type II structures, three products were generated (**Figure 4.6B**). From the mass spectrometry results, the first product has two fucose residues, suggesting that both branches were modified by smFuT-F-GFP (**Figure 4.6D**). The second and third products (**Figure 4.6E, 4.6F**) contained a single fucose each on a different branch, which in combination with the orientation of the AEAB tag generated distinctive enough products to allow for the HPLC separation seen in **Figure 4.6B**. Similar results were obtained with the Na2 acceptor, which verified smFuT-F-GFP generation of Le^X on N-glycans (**Figure 4.7**). Overall activity of smFuT-F-GFP to the panel of glycan acceptors, and acceptor sequences, can be seen in **Table 4.2**.

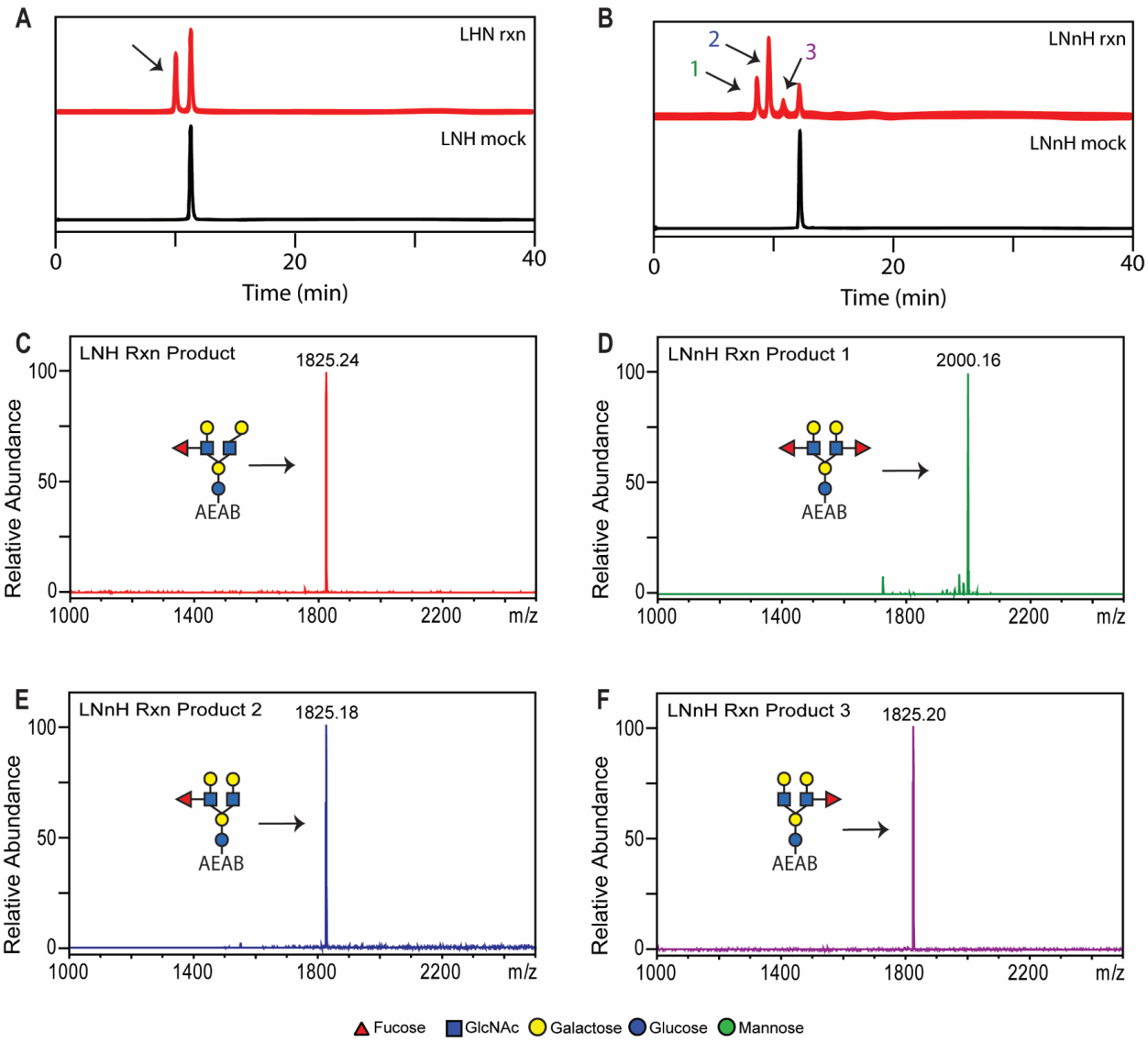

Figure 4.6. Characterization of smFuT-F-GFP activity towards branched type I and type II acceptors.

HLPC chromatograms of smFuT-F or mock reactions utilizing acceptors LNH **(A)** or LNnH **(B)** and separated over a PGC column. Product peaks are seen in both reactions, with a single product in the case of LNH and three products for LNnH and are noted by arrows. MALDI-TOF MS spectrum of smFuT-F LNH product (m/z=1825.24) **(C)** showing the addition of a fucose moiety to the type II branch (LNH+fuc). MALDI-TOF MS spectrum for smFuT-F LNnH product 1 (m/z= 2000.16) **(D)** shows the accumulation of two fucose monosaccharides (LNnH+2Fuc). MALDI-TOF MS for smFuT-F PLNnH product 2 (m/z=1825.18) **(E)** and smFuT-F PLNnH product 3 (m/z=1825.20) **(F)** which each correspond with a single fucose addition, likely on alternate branches. AEAB labeled samples were peracetylated and permethylated prior to MALDI MS analysis.

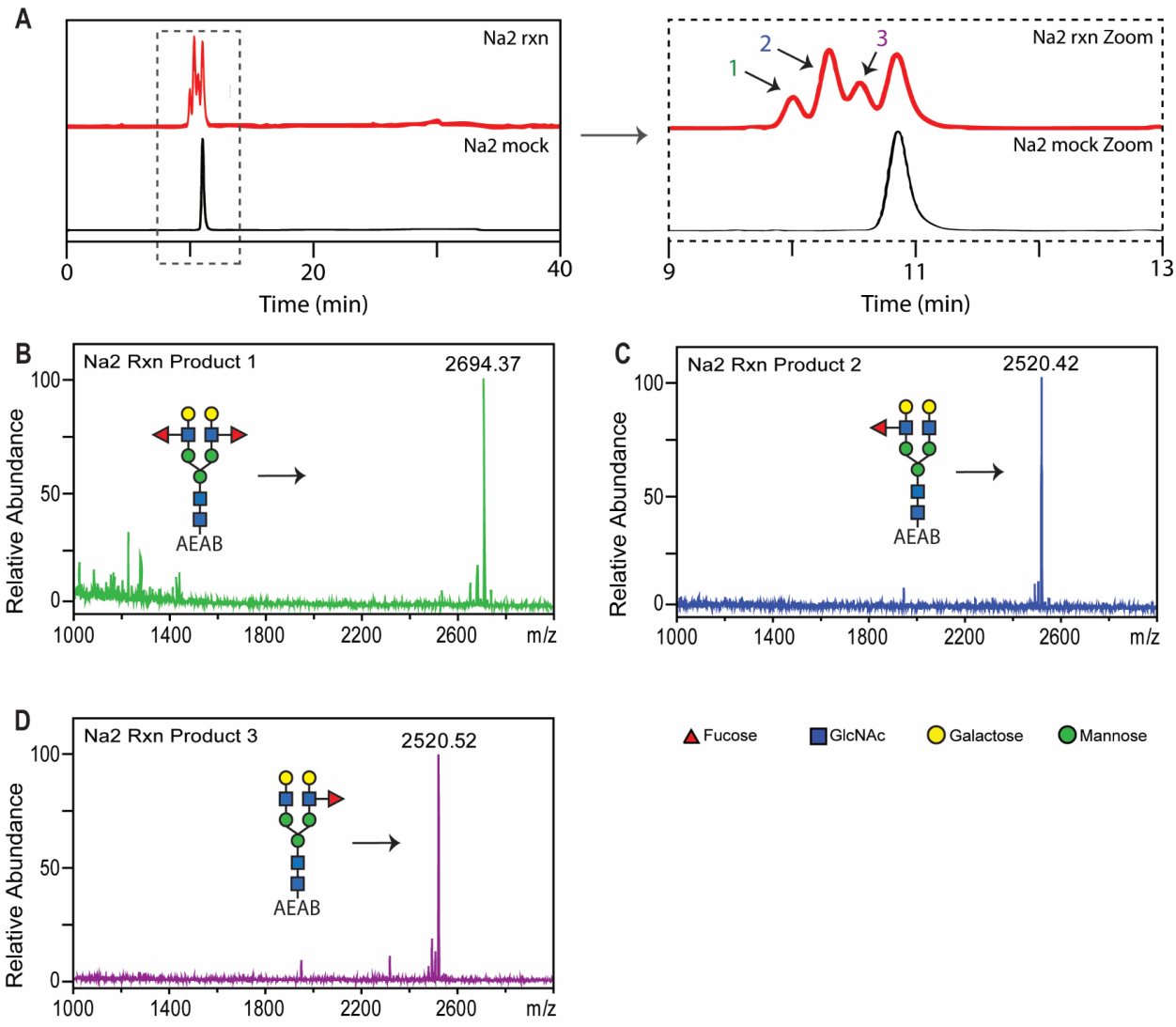

Figure 4.7. Characterization of smFuT-F-GFP activity towards N-glycans acceptors.

HPLC chromatograms of smFuT-F or mock reactions utilizing acceptor Na2 **(A)** separated over a PGC column. Three product peaks are noted by arrows. MALDI-TOF MS spectrum of smFuT-F LNH Na2 product 1 (m/z= 2694.37) **(B)** shows the accumulation of two fucose residues (Na2+2Fuc). MALDI-TOF MS for smFuT-F NA2 product 2 (m/z=2520.42) **(C)** and smFuT-F NA2 product 3 (m/z=2520.52) **(D)** which each correspond with a single fucose addition, likely on alternate branches. AEAB labeled samples were peracetylated and permethylated prior to MALDI-TOF MS analysis.

4.3.6 *Temperature and cation requirement smFuT-F-GFP*

Further analyses were conducted to find the optimum conditions for smFuT-F-GFP including activity assays that were carried out at room temperature or at 37ºC due to the environmental conditions of the worm during a two part (mollusk and mammalian) life cycle. Interestingly, the activity of smFuT-F-GFP was comparable at both temperatures (**Table 4.4**). As smFuT-F-GFP has a DXD motif, cation requirement assays for the smFuT-F-GFP were performed at room temperature (25ºC) using a selection of metals at 20mM (**Table 4.3**). Recombinant enzyme smFuT-F-GFP prefers Mn^{2+} for optimum activity, whereas Ni^{2+} greatly reduced its activity. Other metal ions, Mg^{2+} and Ca^{2+},that might be utilized by glycosyltransferases also promote smFuT-F-GFP activity, however to a lesser degree than seen with Mn^{2+}. Similar levels of activity in the presence of ethylenediaminetetraacetic acid/ethylene glycol tetraacetic acid (EDTA/EGTA) and with no addition of metal chlorides. It is possible that smFuT-F co-purifies with a small amount of metal that cannot be removed by chelation, or that the enzyme mechanism is metal-independent and stimulated by divalent cations through alternative means.

Effect of temperature conditions on smFF activity

	25°C	37°C
Relative activity (%)	100	96

Effect of divalent cations on smFF activity

	Mn^{2+}	Mg^{2+}	Ca^{2+}	Zn^{2+}	EDTA/ EGTA	No addition	mock
Relative activity (%)	100	89	83	4	30	31	n.d.

- Acceptor demonstrating the highest activity was set at 100%
- n.d. = no detectable activity

Table 4.3. Effect of various assay conditions on smFuT-F activity

Effects of temperature and divalent cations on recombinant smFuT-F-GFP activity. Divalent cation assays were conducted at room temperature (25°C). Glycan acceptor demonstrating the highest activity was set at 100%.

4.3.7 *Expression of smFuT-F mRNA in various intra-mammalian life stages*

The available information shows that there is an abundance of Le^X–containing and other fucosylated glycans expressed in intra-mammalian life stages; there is great interest in elucidating their immunomodulatory roles in human and animal hosts (34,78,89,116,118,455). To explore the corresponding α1,3-FuT transcript expression, RT-qPCR of the FuT family was determined in cercariae, newly transformed somules, 3-day *in vitro*-cultured somules, and adult worms (**Figure 4.8**). Real Time qPCR data indicate that FuT-F transcript abundance decreasing during maturation from larvae to adulthood, which inversely correlates with surface expression of Le^X

(115,168,350,456). Similar trends are observed for FuT-E, which is also predicted to generate Lewis type structures (**Figure 4.1A, Figure 4.8**). In contrast, FuT-A appears to increase along with transformation and subsequent culture, reaching its peak in adulthood. For FuT-C, transcript abundance briefly increased after mechanical transformation and then declined once the worms reached adulthood. No significant variations in transcript abundance were observed for FuT-B and FuT-D in these experiments.

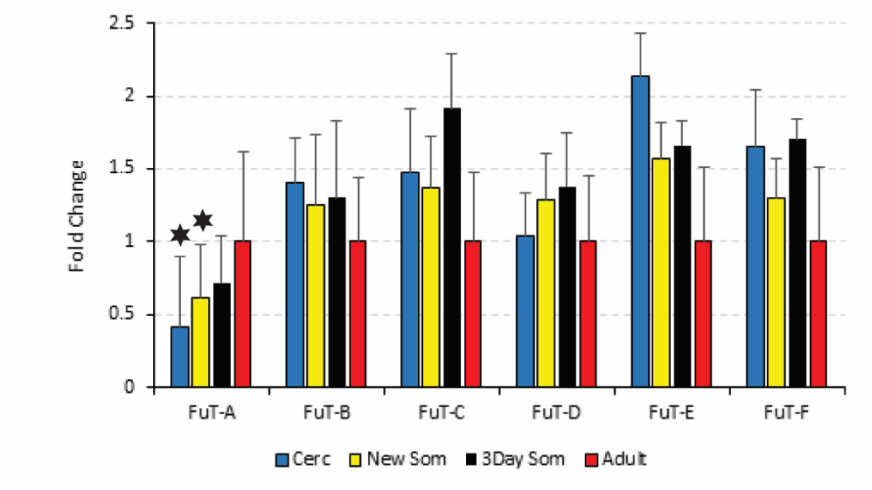

Figure 4.8. α1,3-fucosyltransferase gene transcription in intra-mammalian *Schistosoma mansoni* life stages.

Real-time qPCR was used to examine steady-state levels of α1,3-FuT transcription in cercariae (cerc), newly transformed schistosomula (new som), 3 day *in vitro*-cultured schistosomula (3day som), and adults. Transcript abundance in larval stages was assessed relative to adults, which was arbitrarily set at 1. Data represent the average of three independent biological replicates, and star (★) indicate significantly altered gene transcription ($p<0.05$).

4.4 Discussion

It has long been recognized based on glycan structural analyses that schistosomes contain an α1,3-FuT that fucosylates LN (Ga1β1-4GlcNAc) to generate the Le^X determinant rather than acquiring it from blood, as has been proposed for several blood group antigens (89,116,457,458). In schistosomes, the Le^X antigen was first found on complex-type N-glycans of glycoproteins, but it also exists on lipids and secretions (34,89,110). Some glycans contain the repeating LN disaccharide within the sequence of poly-N-acetyllactosamine, which can have multiple sites of fucosylation on the GlcNAc residues generating the so-called poly-Le^X structures (89). Poly-Le^X has also been reported in O-glycans derived from circulating cathodic antigens (CCA) and has more recently been investigated as an important antigen for diagnostics (34,35,238,341).

Thus, it is important to identify, clone, and biochemical characterize the FuTs that are responsible for biosynthesis of Le^X and the extension of poly-Le^X, on N-glycans and poly-Le^X on O-linked CCA. There is a need for developing a better understanding of the genetic basis of these polymeric antigens, as they are thought to be important antigenic targets, immunomodulators and, in the case of CCA, a validated diagnostic antigen (34,35,238,341). Previous information on FuTs in the gene database has been incompletely annotated; however, more recent studies have taken a glycomics approach to identify and explore the FuT family in *S. mansoni* (287,289,332). In 2013, Peterson *et. al.* (332) published the full length gene transcripts for six α1,3-FuT genes (FuT-A-F) allowing us to finally explore the functionality of schistosome FuTs and more accurately recapitulate portions of the schistosome glycome.

Our study shows that the *S. mansoni* gene FuT-F (smFuT-F) is capable of generating the Le^X antigen on linear and branched glycans (**Figure 4.2-7, Table 4.2**). The sequence of FuT-F contains all the hallmarks of a type II transmembrane FuT (**Figure 4.1B**), but only sequence similarity to other well-characterized enzymes gave insight into its function. For example, amino acids in the hypervariable region of human FuTs determine acceptor specificity between type I and

type II chains, with tryptophan (Trp) common for type I and arginine (Arg) for type II (444,445). Peterson *et. al* (332) was able to annotate a similar region in the *S. mansoni* FuT family stems, with the region in smFuT-F containing lysine (Lys) (**Figure 4.1B**). Two *H. pylori* FuTs also contain Lys, which is charged similarly to Arg, and utilize type II chains like smFuT-F-GFP (**Figure 4.2**) (459,460). Given the differences among the six schistosome FuTs (332), the presence of Lys in the acceptor motif of FuT-F may be serendipitous. However, FuT-E which may also generate Lewis-type structures (**Figure 4.1A**), also contains Lys in the acceptor motif region whereas FuT-A, -B, -C, and -D do not (332).

Recombinant smFuT-F-GFP adds fucose α1,3 to GlcNAc in LN motifs to produce the Le^X structure (**Figure 4.2-4**). A more extensive panel of glycan acceptors allowed us to further characterize the type of Lewis structures generated (**Table 4.2**). The relative amounts of product generated from each acceptor tested may provide some insight into the limitations of smFuT-F in regard to LN motifs it can fucosylate and its preferred acceptors. For example, only 6% of the LSTd acceptor gained a fucose compared to 94% for PLNnH (**Table 4.2, Figure 4.5A, 4.5B**). It is clear that sialylation, a common modification of LN in mammals (132,446), is unfavorable for smFuT-F-GFP activity. PLNnH is a poly-N-acetyllactosamine type glycan with an internal and terminal LN. Over 98% of the product generated contains a single fucose on the terminal LN, illustrating smFuT-F-GFP is unlikely to be responsible for synthesizing poly-Le^X, a common schistosome glycan both on N-glycans and secreted as CCA (**Figure 4.5B, 4.5D-H**) (34,89,341). Thus, it is likely that one of the other FuTs has a complementary function to FuT-F and generates poly-Le^X and/or CCA. smFuT-F-GFP was also able to fucosylate branched glycans as well as linear (**Figure 4.6, 4.7, Table 4.2**). The presence of a type I chain did not prevent fucosylation of an available type II branch, as seen in the LNH reactions (**Figure 4.6A**). When both branches contained type II chains with terminal LN, such as LNnH and Na2, there was a clear preference for one branch compared to the second, but a significant amount of product was double fucosylated (**Figure 4.6B, 4.7A**). In

the future as more of these putative smFuTs are identified, their collective activities to a wide panel of acceptors will need to be explored.

Fucosylated glycoconjugates are observed in all developmental stages of *S. mansoni*; however, previous studies report that much higher levels of FuT expression was found in egg extracts in comparison with cercarial or adult worm extracts, suggesting that FuT activities are differentially regulated during development (423,448). In the case of Le^X expression, adult schistosomes synthesize the Le^X determinant, whereas is appears to be absent or highly restricted in cercariae and freshly transformed schistosomula (115,168,350,456). When comparing mRNA levels in various intramammalian stages there was limited (2-fold maximum) variation between some but not all FuTs (**Figure 4.8**). The observed variation in transcript levels in these life stages is markedly different than the variation seen in snail-associated larvae, where transformation from miracidia to sporocytes correlated with a decrease in FuT expression for all genes (332). However, Le^X expression and localization in the intramolluscan stages are known to developmentally-regulated whereas mother and daughter sporocysts do not express Le^X (246,247). This may be reflected in the FuT-E transcript abundance, if future experiments prove it also functions as a Lewis type FuT, but not for FuT-F where the decrease was minimal (332). This suggests that glycan expression may be regulated in more complicated ways (i.e. tissue specific, internal versus external expression) in which RT-PCR of whole organisms may not be the best methodology. It is also unclear if any significant changes in relative transcript amounts have any observable difference in glycan synthesis or abundance. Also, the above observations in our intramammalian samples and published data for intramolluscan larvae are both incongruous with the results of a preceding transcription study, possibly due to disparities in methodologies (289). Now with the recombinant smFuT-F enzyme, an antibody to the FuT-F enzyme could be generated in order to explore for protein expression as opposed to transcript levels. Future studies are needed on fucosylated glycan structures, antibody specificities for the Le^x antigen, FuT-F protein expression, and transcript

utilization, to address the apparent lack of strict correlation between transcript expression for smFuT-F and expression of Le^x.

It is not well understood what role the regulated expression of Le^X glycan plays in the immunobiology of schistosomes. Some studies suggest that Le^X may be expressed on glycans that contain an immunoregulatory function. The free sugar LNFPIII (Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc), which is the sugar smFuT-F generated from LNnT, is reported to stimulate macrophages *in vitro* to express CD69, secrete IFN-γ, and activate NK cells (141). Van Die *et. al.* (78) reported that Le^X glycans from SEA interact with the human dendritic cell lectin DC-SIGN. There is also evidence that Le^X can also modulate the release of factors that mediate the Th-1 to Th-2 switch observed during chronic *S. mansoni* infections (144). However, infected humans and animals generate IgG and IgM antibodies to Le^X (89,116,118). Le^X is a critical component of the ligands on neutrophils and monocytes recognized by selectins and important in inflammation and leucocyte recruitment (365). Anti-Le^X antibodies were shown to cause complement-dependent cytolysis of leukocytes and possibly other Le^X expressing cells, suggesting that an autoimmune disorder may accompany schistosomiasis (118).

However, Le^X is only a small portion of the glycan repertoire on *S. mansoni* surfaces and glycocalyces and secreted glycoconjugates that are exposed to the host immune system. Other structures including LDNF, FLDN, FLDNF, dF-LDN, etc. are relatively unique to the parasite, yet to be enzymatically or chemically synthesized, and prominently involved in the host humoral and cellular immune responses to infection (105,110,234,240,243,246,350,425,427). Further studies on and function of schistosome α1,3-FuTs-A through E are currently underway to determine their role in fucosylated glycan synthesis and give insights into development of the worm and its possible function in the pathogenesis of the disease. The ability to produce the unique glycans of *S. mansoni* and more accurately recapitulate the fucosylated schistosome glycome will be an invaluable tool in the continued exploration of the roles schistosomal glycans play in both worm biology and their interactions with host species.

4.5 Acknowledgements

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Chapter 5. *Schistosoma mansoni* β1,4-GalNAcTransferase generates LacdiNAc glycans

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5.1 Introduction

Schistosomiasis is a disease in both humans and animals by helminths of the genus *Schistosoma* and results in a multitude of pathologies (7,13,16,70,422). Similar to other parasitic helminths, schistosome produce many complex carbohydrate (glycan) structures linked to proteins and lipids, including N-glycans, O-glycans, and glycolipids, which play an essential role in the biology of the parasite and at a the host-pathogen interface (1,7,17). Recent studies have shown that a major part of the host immune response to infection is directed against glycan antigens in glycoproteins and glycolipids (1,77,80,127,387-390). Within the past 50 years, there has been tremendous progress in understanding the molecular nature of the plethora of *schistosoma* glycoconjugates that interact with the host immune system in a variety of complex mechanisms, and may contribute to its ability to module the host immune system (7,70-72,132,170).

While there are many unique glycans produced by schistosomes, the LacdiNAc (LDN) motif is of particular interest as an antigen, and as a parent structure that is further modified by the addition of one or more fucose (Fuc) residues (234,462,463). While glycans containing the LDN motif are expressed by many invertebrates, the LDN determinants present in parasite glycans have been shown to generate a humoral response by the human immune system and can initiate the

formation of a granuloma in humans (272,464,465). Although it is yet unknown whether they directly contribute to protection *in vivo*, a monoclonal antibody to LDN kills schistosomula *in vitro* (168). Several multifucosylated versions of LDN including α2- and α3-linked multifucosylated glycans with polyfucose elements Fucα1-2Fucα1-3-R and the Fucα1-3GalNAc-motifs (F-LDN, F-LDN-F, LDN-dF, and dF-LDN-dF) appear to be completely unique to schistosomes (105,110,332,462). These structures induce high antibody responses in humans and primates (76,208,240,243,244,466). For these reasons, we believe that the LDN family of antigens includes potential vaccine and diagnostic candidates.

The complete functions and biosynthesis of schistosome glycans are not yet understood, however improving our knowledge of glycan structure has great potential uncover novel, immunologically relevant diagnostic and vaccine candidates and to enhance our understanding of the anti-glycan response. Before the development of a successful vaccines against schistosome infections, understanding of many aspects of the molecular mechanisms employed by the parasites to survive within the host in the face of the anti-schistosome immune responses, especially anti-glycan responses, is required (55,57,189,228,361). A major limitation in the study of the biological functions of schistosome glycans and their immunogenicity has been the lack of adequate quantities of the parasite glycans. However, only a small number of schistosome glycan epitopes have now been synthesized by chemi-enzymatic methods in order to begin the study of their immunogenicities (271,342,343,421). Recently, Mickum *et. al*. (421) has used the genome database in order to recombinant express a schistosome glycosyltransferase that could best recapitulate part of the schistosome glycome. We have now initiated research to generate recombinant glycosyltransferases to facilitate the generation and elucidation of the biological functions of unusual schistosome glycans in an affordable and facile manner. In chapter 4, we reported the characterization of α1,3-Fucosyltransferase F activity in *S. mansoni* (smFuT-F), which we found to be capable of synthesizing the terminal Le^X structures (421). We now report the use of the same

approach to identify the enzymatic activity of a β1-4 N-acetylgalactosaminyltransferases (β1-4GalNAcT) from *S. mansoni* which creates the LDN motif.

5.2 Materials and Methods

5.2.1 *Materials*

All chemicals and enzymes were purchased from Sigma-Aldrich (St. Louis, MO) and used according to manufacturer recommendations and without further purification unless otherwise noted. Water and Acetonitrile (HPLC grade) were purchased from Thermo Fisher Scientific (Waltham, MA). Expression vector pGen2 was kindly provided by Dr. Kelley Moremen and the glycol-enzyme repository. Restriction enzymes and ligase were purchased from New England Biolabs, Inc (Beverly, MA). Primers were from Integrated DNA Technologies (Coraville, IA). Lacto-N-neotetraose (LNnT) and 2-Amino-N-(2-aminoethyl)benzamide (AEAB) were synthesized in the lab (428). Polyethylenimine (PEI, 25 kDa linear, cat. # 23966) was purchased from Polysciences, Inc and prepared as a stock solution at a concentration of 1 mg/ml in a buffer containing 25 mM HEPES, pH 7.5 and 150 mM NaCl. The anti-GFP antibody (clone GT859) was purchased from GeneTex (Irvine, CA) and the secondary antibody HRP conjugate goat anti-mouse was purchased from KPL (Gaithersburg, MD). SuperSignal West chemiluminescent substrates were purchased from Thermo Scientific (Rockford, IL). An Ultraflex-II MALDI-TOF/TOF system from Bruker Daltonics (Billerica, MA) was used for analysis of glycan conjugates. An Orbitrap Fusion Tribrid system from Thermo Fisher Scientific (San Jose, CA) was used for MS^n fragmentation analysis of glycans. A FACSCalibur from BD Biosciences (San Jose, CA) with CellQuestPro acquisition software was used for flow cytometry, and FlowJo software was used for data analysis.

5.2.2 *Cloning of* S. mansoni *βGalNAcT*

The coding region for *S. mansoni* β1-4GalNAcT (smβGalNAcT) was synthesized by Genewiz, INC (South Plainfield, NJ). The synthetic DNA also contained an additional NH2-terminal fusion of the 7 amino acid recognition sequence of the tobacco etch virus (TEV) protease and a flanking 5' EcoRI site and a 3' BamHI site. SmβGalNAcT was subcloned into similarly digested chemically synthesized vector, termed pGen2, as previously described in chapter 4 (421). This expression vector was designated smβGalNAcT-pGEn2 and its protein product, a tagged, soluble, GFP-fusion protein targeted for secretion, was termed smβGalNAcT-GFP.

5.2.3 *Expression of smβGalNAcT-pGen2 in HEK Freestyle Cells*

To determine if smβGalNAcT-pGen2 encoded a soluble, active β1-4GalNAcT, smβGalNAcT-pGen2 was transiently transfected into HEK freestyle cells (HEK293f, Life Technologies, Grand Island, NY) as previously described (421). Using a platform shaker in a humidified 37°C incubator, suspension cells were grown to a density of $1.8x10^6$ cells/ml in Freestyle 293 Expression Medium (Life Technologies). Prior to transfection, cells were prepped by centrifugation at 300xg for 5 min and re-suspension in fresh Freestyle media. To generate the transfection solutions, smβGalNAcT-pGen2 was diluted to 3 μg/mL in Freestyle 293 Expression Medium and PEI was diluted to 9 μg/mL for a final concentration of 0.5 μg DNA or PEI/μL media in transfection solution. The DNA solution was added to cells, and the culture allowed to shake in 37°C incubator for 5 minutes. The PEI solution was then added and the cells returned to the shaking incubator. After 24 hours, the cells were diluted 1:1 with fresh media and supplemented with valproic acid (Sigma, #4543) to a final concentration of 2.2 mM. Cells were monitored by fluorescent microscopy (Olympus IX51, 10x zoom) for GFP positivity at 24 hour time points. As a control, HEK293f cells were mock transfected under similar conditions with PEI alone (no DNA). After 120 hours, media was clarified by centrifugation at 3000xg for 10 minutes. Conditioned media and cell pellets were stored at -20°C prior to use.

5.2.4 *Purification of Recombinant smβGalNAcT-GFP*

To purify smβGalNAcT-GFP, transfected cell pellets were re-suspended in a Binding Buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, 0.25% (v/v) Tween-20, 1X EDTA protease inhibitor, pH 8.0) and sonicated over ice to lyse cells. Centrifugation at 3220xg for 5 minutes was used to remove insoluble debris. Clarified supernatant was loaded onto a column containing 2 ml Ni-NTA superflow (Qiagen, Valencia, CA) equilibrated with wash buffer (50 mM NaH_2PO_4, 500 mM NaCl, 20 mM imidazole, pH 8). After loading the sample, the column was washed with 50 mL of Wash buffer and eluted in 2 mL fractions with Elution buffer (50 mM NaH_2PO_4, 500 mM NaCl, 500 mM imidazole, pH 8.0). Fractions containing GFP were pooled and dialyzed into 25 mM HEPES, 300 mM NaCl, 50 mM imidazole, pH 7.5 with a 10 kDa molecular weight cutoff. The dialyzed samples was concentrated using Amicon Ultra 0.5 mL Centrifugal Filters with 10 kDa molecular weight cutoff (Millipore). Final protein concentration was determined by BCA assay.

5.2.5 *SDS-Page and Western Blot of smβGalNAcT-GFP*

For gels and blots, 1× NuPAGE SDS sample buffer (Invitrogen) with 2.5% β-mercaptoethanol (βME) added to samples and boiled for 10 minutes. Samples were run in 10-well Mini-PROTEAN-TGX gels at 110 volts with protein standards. For western blots, protein was transferred to a nitrocellulose membrane using Trans-Blot Turbo semi-dry transfer system. Unless noted otherwise, all subsequent incubations and washes were shaking at ambient temperature. Membranes were blocked for 1 hour in 5% (w/v) bovine serum albumin fraction V (BSA) in TBS wash buffer (20 mM Tris, 300 mM NaCl, 0.1% Tween-20). Membrane was washed three times with TBS wash buffer and incubated with anti-GFP antibody 1:10,000 in TBS wash buffer. The membrane was then washed three times in TBS wash buffer. Secondary detection antibody, HRP-conjugated goat anti-mouse-IgG, was added for 1 hour at 1:5,000 in TBS wash buffer. The same

wash procedure was repeated, and then SuperSignal West Pico Chemiluminescent Substrate was added for 30 seconds. The membranes were dabbed dry and exposed to film.

5.2.6 *Glycan Digestion, AEAB Labeling, and Purification*

Glycan acceptors were selected and conjugated with AEAB as described previously (394,421,428,434). Prior to AEAB conjugation, Na2 and LNnT were digested with neuraminidase and Aspergillus oryzae β-galactosidase (Sigma) according to manufacturer's recommendations to reveal terminal GlcNAc. These two glycan acceptors and were termed dNa2 and triose respectively. Glycan samples, 1-10 mg, were mixed with 50–250 µl of freshly prepared 0.35 M AEAB hydrochloride salt and an equal volume of 1 M $NaCNBH_4$ in DMSO/AcOH (v/v = 7/3). The conjugation reaction was left to proceed for 2 h at 65°C and then stopped by the addition of 10 volumes of cold acetonitrile. After addition of acetonitrile, the reaction was allowed to stand for 30 min at -20°C. The precipitated glycan-AEAB (GAEAB) derivatives were collected after centrifugation at 10,000x*g* for 5 min. The GAEAB pellet was dried under a vacuum for 10 minutes, reconstituted in water, and centrifuged at 10,000xg for 2 minutes to remove any insoluble material.

A Shimadzu HPLC CBM-20A system coupled with a fluorescence detector RF-10AXL (*330nm/420nm excitation/emission)* was used for HPLC analysis and separation of GAEABs. The GAEABs were purified by reverse phase HPLC on a porous graphitized carbon column (PGC, 150 × 4.6 mm, Thermo Fisher Scientific) with a gradient of 15-45% acetonitrile (0.1% trifluoroacetic acid) in 40 min to separate the GAEABs from any unincorporated label. The effluents were monitored by fluorescence where the GAEAB peak fractions were collected, quantified by fluorescence, and dried under vacuum. GAEABs were reconstituted to 1-5 mM stocks in water and stored at -20°C until use.

5.2.7 *GalNAcT Enzymatic Assay*

The enzymatic assays were performed in 25 µl reaction mixtures containing 50 mM sodium cacodylate, pH 7.0, 10 mM $MnCl_2$, 1 mM UDP-GalNac (or other nucleotide sugar), 40 µM

GAEAB sugar acceptor, and 1-5 μg smβGalNAcT-GFP. No smβGalNAcT-GFP was added for mock reaction conditions. Reaction mixtures were incubated rotating at room temperature or 37°C more than 24 hours. The enzyme was removed from the reaction mixture via porous graphitized carbon solid phase extraction. Enzymatic reaction GAEAB products were then separated from GAEAB acceptors by reverse phase HPLC on a PGC column. Each peak fraction was collected, quantified by fluorescence, and dried under vacuum. Samples were then processed for either MALDI-TOF MS for molecular mass analysis or Electrospray Ionization Mass spectrometry (ESI MS) for MS^n fragmentation.

5.2.8 *Mass Spectrometry of Glycans*

Prior to MALDI-TOF MS analysis, purified GAEAB samples were acetylated and permethylated as previously described in chapter 4 (421). For acetylation, GAEABs were added to acetic anhydride in saturated sodium bicarbonate for 30 minutes on ice and incubated in ambient temperature for 1 hour. Glycans were then desalted via porous graphitized carbon solid phase extraction. Any esters formed during acetylation were hydrolyzed by incubation in 0.01 M sodium hydroxide at ambient temperature for approximately 3-4 hours and then lyophilized (112,435). For permethylation, a lyophilized sample was treated with 200 μL DMSO/NaOH slurry and 200 μL methyl iodide for 30 minutes, shaking. The supernatant was then partitioned between 500 μL water and 500 μL chloroform. The organic layer was washed four times with 500 μL water, dried, and re-dissolved in 50% methanol:water for MALDI-TOF MS analysis. Mass spectrometry data were collected using an Ultraflex MALDI TOF/TOF (Bruker Daltonics, Billerica, MA).

5.3 Results

5.3.1 *Selection of smβ1-4GalNAcT*

The information from the *Schistsoma* gene database represents a great step forward for schistosome research, but its use has revealed highly fragmented data sets which compromised the quality of potential downstream analyses. One such example of this is the incomplete gene sequences listed for various gene families in the glycosylation pathway. Several previous studies have made valuable improvements by utilizing RACE PCR, gene, microarrays, deep DNA sequence data from clonal worms, and RNA sequence data from multiple life stages (17,289,290,332) . As of 2014, there are seven genes are annotated in the *S. mansoni* genome database as probable GalNAcTransferases (SMP genes). For those genes, the coding domain sequences were translated *in silico* and analyzed with Geneious V4.7.6. Using Jukes-Cantor to determine genetic distance with a neighbor joining build, a phylogenetic tree was generated from the translated sequences of the seven SMPs along with a known *C. elegans* GalNAcT and human β4GalNAcT3, which both generate LDN epitopes (**Figure 5.1A**) (343,467,468). Previously published RNA sequencing and transcription data revealed SMP 021370 is highly transcribed in four different points of the worm life cycle (290,469). As a result, SMP 021370 was selected for further computational analysis.

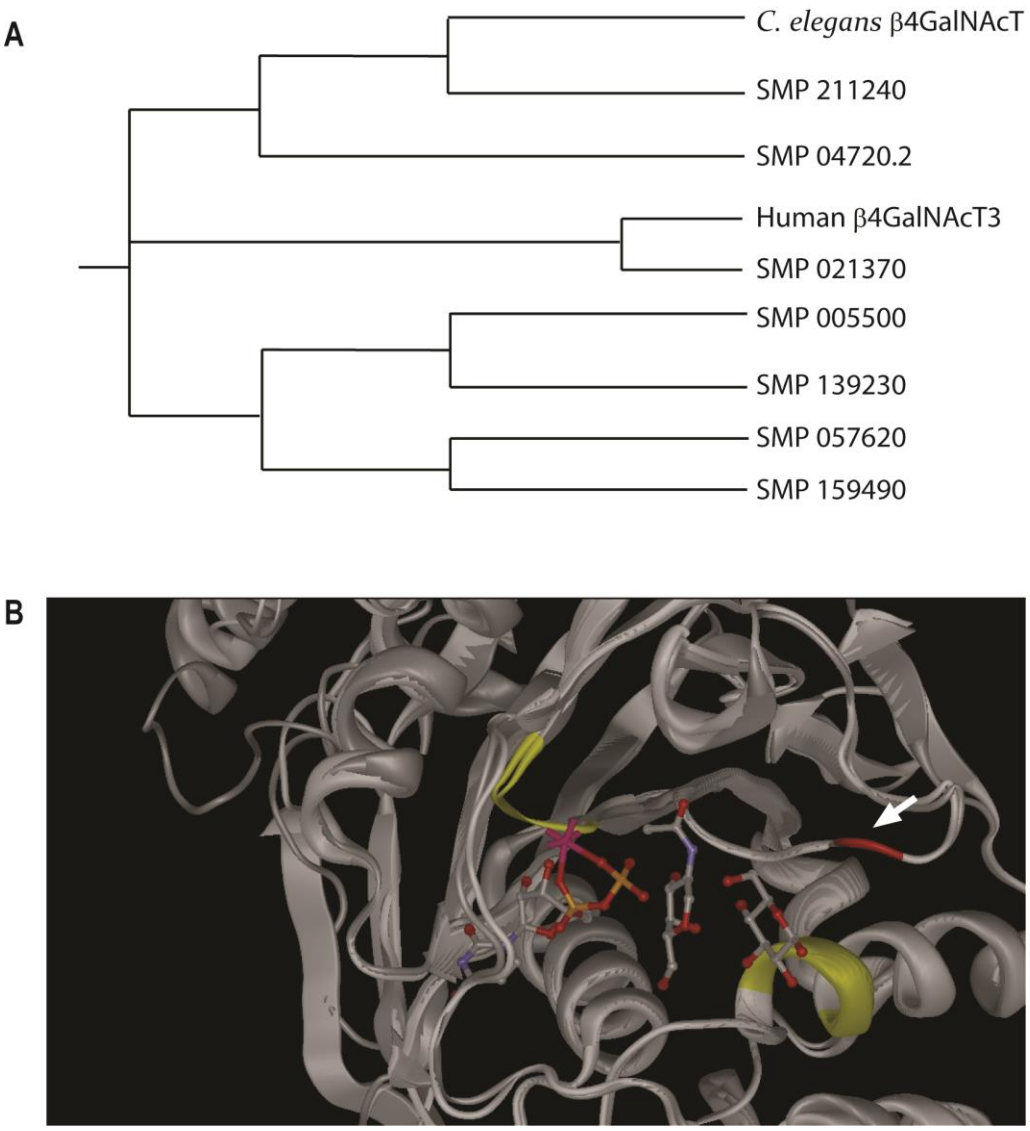


Figure 5.1 Computational analysis of potential β1-4GalNAcTs.

(A) Phylogenetic tree of the seven *S. mansoni* GalNAcTs proteins sequences with the human and *C. elegans* sequences as a reference. SMP 021370 is closely related to a human β1-4GalNAcT3. **(B)** Predicted structure of SMP 021370 aligned to a murine β1,4-GalT crystallized with UDP, GalNAc and Mn^{2+}. The yellow regions correlate with DXD motifs and the red region marked with a white arrow denotes the location of isoleucine, which promotes preference for UDP-GalNAc over UDP-Gal as the donor sugar.

Like most glycosylatranferases, GalNAcTs are Golgi-anchored, type-II transmembrane proteins featuring a single transmembrane domain (TMD), which is flanked by a short cytoplasmic N-terminal tail and a lumenal C-terminus that comprises a globular catalytic domain and a flexible hypervariable stem (301,332,340,423). It is also well established that β1-4GalNAcTs require several motifs in their catalytic domain in order to be functional (299,300,338,343), including binding regions for the nucleotide sugar and metal ion co-factors. NCBI's conserved domain database revealed predicted regions which include binding motifs for a donor-binding site, and metal binding DXD amino acid sequences. However, there was no transmembrane domain in the SMP 021370 sequence predicted by the TMHMM Server v2.0. Furthermore, SMP 021370 contains an isoleucine residue in the predicted acceptor binding pocket. Isoleucine has been shown to have a role in increasing the preference of UDP-GalNAc over UDP-Gal, which is a major factor in differentiating GalNAcTs from GalTs (468,470). For visualization of the acceptor binding regions, the structure predicted by I-TASSER was then aligned to murine β1-4Gal-T1 (j-FATCAT Rigid Body Alignment) which had been co-crystallized with UDP-GalNAc and Mn2+ (Figure 5.1B) (471-473). There is remarkable overlap between the two protein sequences with an root-mean-square deviation (RMSD) of 0.903 Å (474,475). Based on the sequence and domain analysis, RNA expression levels, and predicted structural similarity, the gene transcript SMP 021370 was chosen for synthesize and further experimental characterization. SMP 021370 will further be referred to as smβGalNAcT.

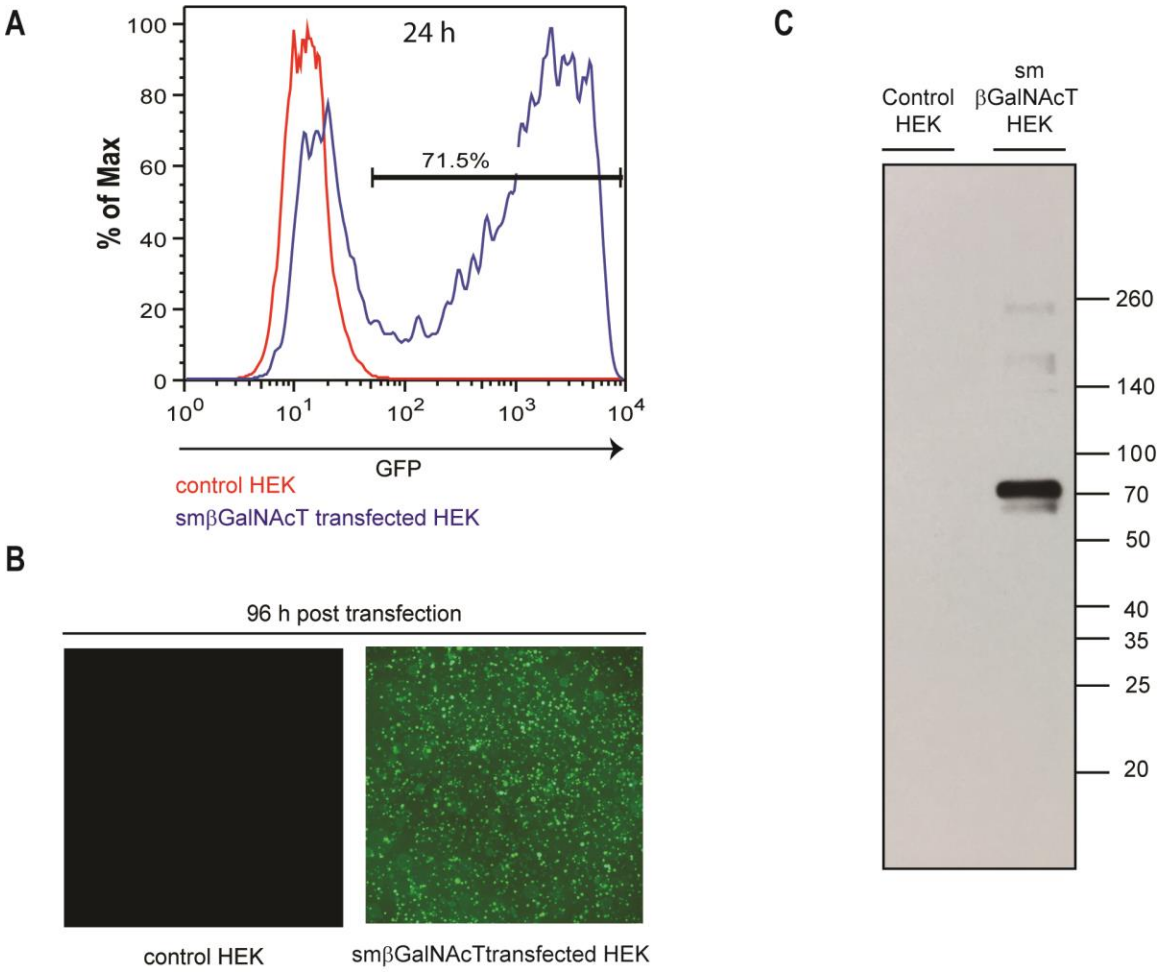


Figure 5.2. Expression of the smβGalNAcT-pGen2 construct in HEK-F cells.

GFP positivity of HEK freestyle cells by flow cytometry **(A)** and fluorescent microscopy **(B)**, after transfection with smβGalNAcT-pGen2 or mock transfections (control). By 72h post transfections, over 90% of transfected cells are expressing GFP and can be harvested for smβGalNAcT-GFP purification. **(C)** Anti-GFP western blot of smβGalNAcT-pGen2 transfected or control HEK cells.

5.3.2 *Expression of recombinant smβGalNAcT-GFP*

Recombinant protein was generated by transient transfection into HEK293f cells of the expression construct, smβGalNAcT-pGen2. Cells were monitored by flow cytometry and microscopy for GFP fluorescence at 24h time points (**Figure 5.2A-B**). After 96h, a majority of transfected cells were strongly GFP positive (**Figure 5.2B**). The expression of the smβGalNAcT-

GFP protein in the cells was confirmed by SDS–PAGE and Western blotting using an antibody directed to the GFP superfolder protein domain. The immunoblot revealed protein bands with apparent MW of 75 kDa, which would be expected of a variably glycosylated polypeptide of around 66.4 kDa predicted from the full-length GFP fusion protein in transfected HEK cells (**Figure 5.2C**).

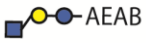
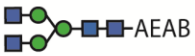
Acceptor substrate	Shorthand	Structure
GlcNAcβ1-3Galβ1-4Glc-AEAB	Triose	
GlcNAcβ1-4GlcNAc-AEAB	Chitobiose	
GlcNAcβ1-2Manα1-3(GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAc-AEAB	dNa2	
Galβ1-4GlcNAcβ1-3Galβ1-4Glc-AEAB	LNnT	

Table 5.1. Glycan acceptors for smβGalNAcT reactions.
Sequence, shorthand name, and cartoon structures of glycan acceptor.

5.3.3 *smβGalNAcT adds GalNAc to terminal GlcNAc in a β-linkage*

To characterize the activity of smβGalNAcT, the GFP fusion protein was purified from the HEF293f cells using Ni-NTA agarose and tested with a small panel of glycan acceptors. AEAB labeled glycan acceptors included a "triose sugar" derived from β1-4galactosidase digested LNnT, chitobiose, digested Na2 (dNa2) which is an N-glycan after treatment with neuraminidase and β1-4galactosidase to reveal a terminal GlcNAc, and LNnT as a control (Acceptors shown in **Table 5.1**). The reactions were monitored for formation of a new glycan product by HPLC (**Figure 5.3**) and MALDI-TOF MS (**Figure 5.4**). New glycan products were observed for all acceptors that terminated in GlcNAc, whereas no product was generated from the LNnT, which contains a terminal Gal (**Figure 5.3**). Additionally, no glycan product was formed when UDP-Gal was utilized as the donor sugar in place of UDP-GalNAc, indicating that there is no overlap in GalT and

GalNAcT activity for smβ1-4GalNAcT (data not shown). The smβ1-4GalNAcT products from the triose and chitobiose reactions were examined by MALDI-TOF MS. The size shift of peaks 997.96 m/z to 1242.97 m/z for triose, and 835.77 m/z to 1080.41 m/z for chitobiose, confirmed the addition of a single GalNAc sugar to each acceptor (**Figure 5.4**).

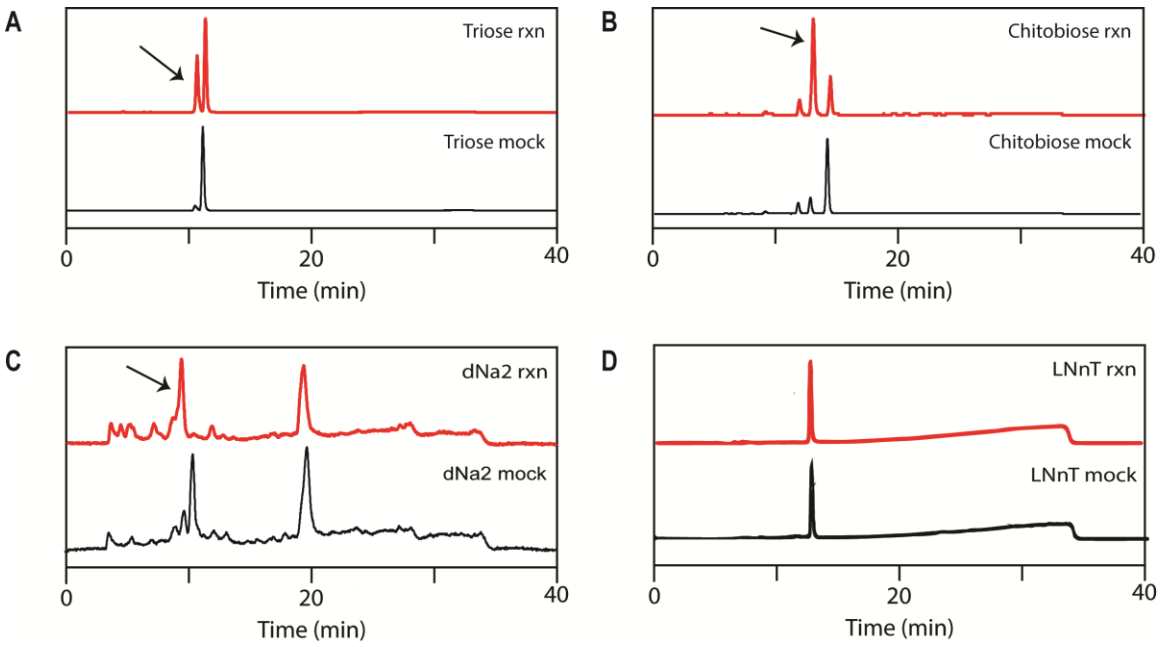


Figure 5.3. Activity of smβ1,4-GalNAcT toward glycans acceptors.

HPLC chromatograms of smβ1,4-GalNAcT or mock reactions utilizing acceptors triose **(A)**, chitobiose **(B)**, dNa2 (digested Na2) **(C)**, and LNnT as a control **(D)**, and separated over a PGC column. Product peaks seen in the smβ1,4-GalNAcT reactions are noted by an arrow.

To confirm that the terminal GlcNAc was added in the β confirmation, enzymatic analysis and MS data was collected. β-N-acetylhexosaminidase is an enzyme which can hydrolyzed β-linked, but not α-linked terminal HexNAc sugars (476-478). The smβ1-4GalNAcT triose product was treated with β-N-acetylhexosaminidase, and monitored by HPLC. Post digestion resulted in a slight peak sift indicating one or more sugar have been removed. MALDI-TOF MS was used to determine the final product was lactose, indicating the terminal GalNac was β-linked the in internal

GlcNAc, which was also removed by β-N-acetylhexosaminidase digestion (**Figure 5.5B**). As schistosome do not link GalNAcβ1-3GlcNAc, the likely product generated is terminal LDN (GalNAcβ1-4GlcNAc) which could be further confirmed by antibody staining or by MS cross ring fragmentation.

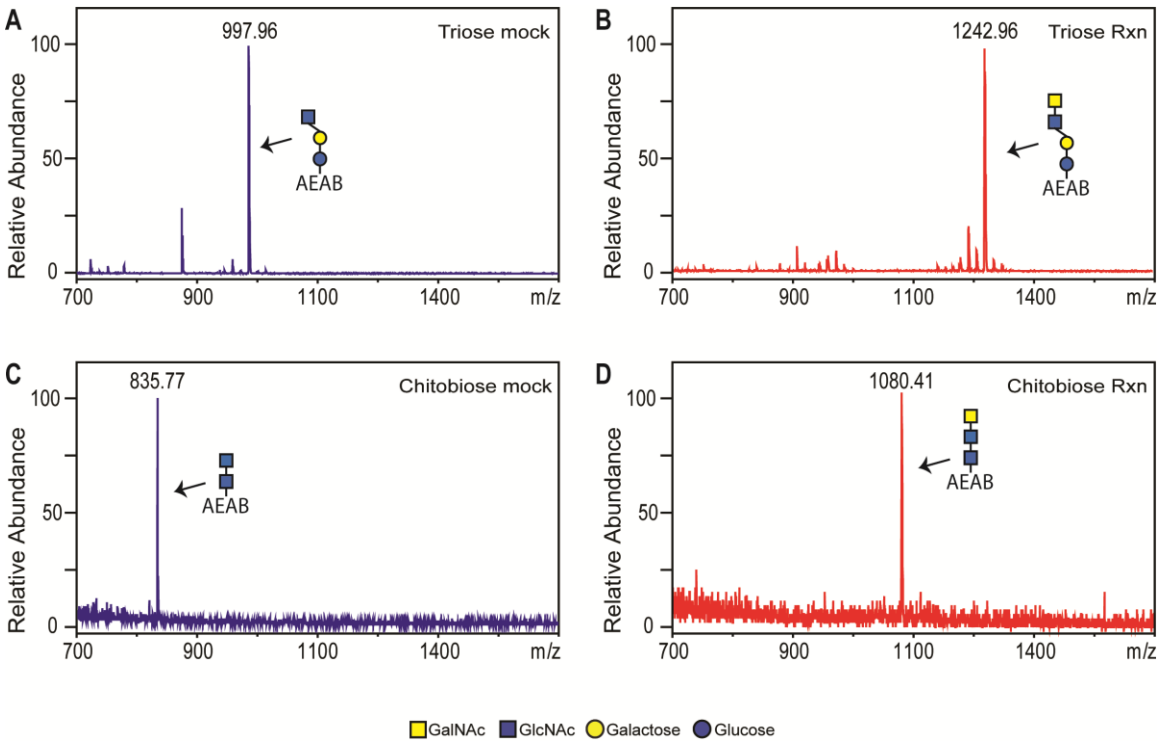


Figure 5.4 smβGalNAcT adds HexNAcs to glycan acceptors.

MALDI-TOF MS spectra of triose mock reacted glycan (m/z=997.96) **(A)** and the smβGalNAcT triose product (m/z= 1242.96) **(B)** showing the addition of a HexNAc moiety. Similar results found for chitobiose mock reacted glycan **(C)** and the smβGalNAcT triose product **(D).** AEAB labeled samples were peracetylated and permethylated prior to MALDI-TOF analysis.

5.4 Discussion

There is a growing appreciation for the role glycans and glycol-conjugates play in modulating a plethora of biological processes, especially in the realm of parasitic infections. A critical liming factor in the study of these glycans in the difficulty in obtaining many of the relevant structures, either via purification or semi-synthetic synthesis approaches. With the advent of next generation sequencing technologies, the ability to sequence the entire genome of any organism becomes an incredible tool in the identification of genes and proteins important in various biological functions, including glycan biosynthesis. The schistosome genome was sequenced in 2009 and much of the data categorized in the GeneDatabase (GeneDB), however the data is incompletely annotated and improvement are still necessary (2,17,283-285). Computational tools available now are sophisticated and crucial in helping identity gene functions when the prediction algorithms fall short or the sequence information is still limited. In the case of parasite glycobiology, these tools have been pivotal in this work to identify the first β1-4GalNAcTransferase from *S. mansoni*.

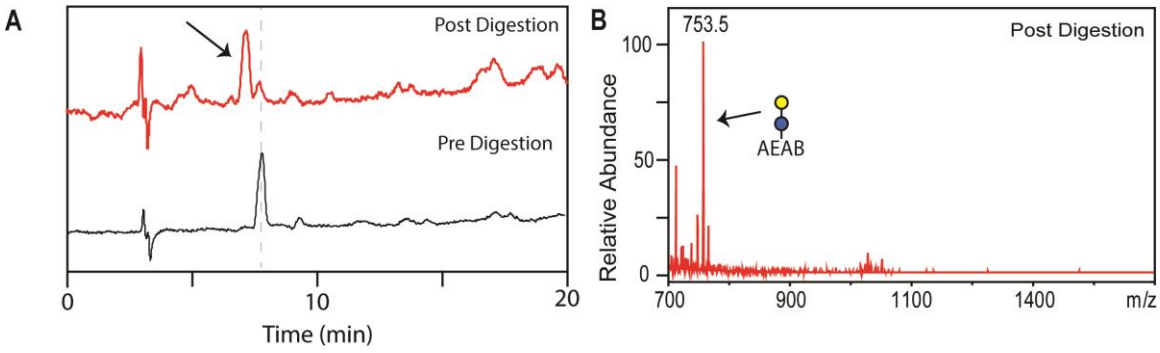


Figure 5.4. Analysis of the triose product synthesized by smβ1,4-GalNAcT.

(A) HPLC profile of the triose product before and after digestion with β-N-acetylhexosaminidase. The shift in glycan peak seen by HPLC confirms the β linkage of both terminal HexNAc sugars. The product generated by the β-N-acetylhexosaminidase digestion (denoted by the arrow in **(A)**) was lactose AEAB and confirmed by MALDI-TOF MS **(B).**

LacdiNAc is the base glycan for many of the multifucosylated schitsome glycans as well as being immunogenically relevant on its own (234,272,462-465). In humans, biosynthesis of LDN can occur by two different β1-4GalNAcTs: β4GalNAcT3, which is expressed in stomach, colon, and testes (468), and βGalNAcT4, which is found in ovaries and the brain (479). The nematode *Caenorhabditis elegans*, has a β4GalNAcT, which when expressed in Chinese hamster ovary (CHO) Lec8 cells has led to the production of N-glycans with LDN repeats (343). Given the inability to purify LDN based glycans direct from the worm, current methods include utilizing these other species enzymes to generate a library of LDN type glycans (342,343). However, this does not accurate reflect the glycome of the schistosome. Thus, it is important to identify, clone, and biochemically characterize the GalNAcTs that are responsible for synthesizing LDN and allows for the extension of further modified LDN based glycan antigens (FLDN, LDNF, FLDNF, dF-LDN, et cetera).

The phylogenetic tree of the seven GalNAcTs annotated on GeneDB showed two distinct groups of sequences when compared to *C. elegans* and human β1-4GalNAcTs, which both generate LDN motifs **(Figure 5.1A)** (343,467,468). The four amino acid sequences that create a monophyletic group in **Figure 5.1A** do not align well with either reference sequence are SMP 057620, SMP 159490, SMP 005500, SMP 139230. Given the difficulty in differentiating between GalTs and GalNAcTs, and the existence of GalNAcTs with other functions, such as those that transfer a GalNAc directly to a peptide, these four genes were passed over for this study (480-483). SMP 021370 was a strong candidate for synthesis because it closely resembles well characterized proteins, both in amino acid alignment and the predicted protein structure (**Figure 5.1**). SMP 021370 also shares an isoleucine residue within the acceptor binding pocket that was correlated with an increased preference in binding UDP-GalNAc **(Figure 5.1B**) (468,470). The I-TASSER predicted structure was also helpful in showing the similarity to a murine β1-4GalNT with a bound Mn^{2+} and UDP-GalNAc, with the metal and donor sugar binding sites found close proximity after alignment (471-473). It is important to note that the SMP 021370 sequence lacks a transmembrane domain, which is unsurprising given in the incomplete nature of the GeneDB, and was of little concern given the goal of generating non-membrane bound protein for rapid purification. Using previously reported transcription data, it appears that SMP 021370 is also the most expressed transcript among four life stages, making it the most biologically relevant transcript as well. Thus, this gene was the most rationale target to focus on for this current study.

Our study showed that the SMP 021370 gene product (referred to as smβGalNAcT-GFP) was able to add GalNAc onto GlcNAc terminating structures, synthesizing LDN motifs **(Figure 5.3-5.5)**. Given the limited number of acceptor tested, it's is unclear how restricted or promiscuous smβGalNAcT-GFP may be with the glycan acceptors. SmβGalNAcT-GFP recognized three different acceptors, and a new glycan product was generated in each reaction when the acceptor contained a terminal GlcNac sugar. This included both short linear chains (triose and chitobiose) and the branched N-glycan (dNa2) (**Figure 5.3**). This may show a proclivity for multiple acceptor

sequences or demonstrate a basic requirement for a terminal GlcNAc with no preference for the preceding sequence. Conversion of dNa2, a bi-antennary N-glycan with a terminal GlcNAc, is important since the majority of *S. mansoni* glycans with LDN are found on N-glycans (104,247,446). Due to similarities between the GalNAcTs and GalTs (468,470), UDP-Gal was also used in several activity assays in place of UDP-GalNAc. There was no synthesis of LacNAc (LN) or any conversion of the acceptor, which supports the conclusion that smβGalNAcT does not utilize UDP-Gal and is selective for the donor UDP-GalNAc (data not shown). Digestions of the smβGalNAcT triose product to lactose by β-N-acetylhexosaminidase indicated the two terminal HexNAc sugars were both linked in β conformations leading us to believe the product generated was LDN **(Figure 5.5)** (476-478). To further confirm the identity of the smβGalNAcT product, α-LDN antibodies or cross-ring MS fragmentation could be done, howveer a GalNAcβ1-3GlcNAc motif has never been found in schitosomes.

LDN is a major building block for a large portion glycan repertoire on *S. mansoni* surfaces and glycocalyces and secreted glycoconjugates that are exposed to the host immune system. Some of these structures including LDNF, FLDN, FLDNF, dF-LDN, dF-LDNF-dF, etc. have not yet been enzymatically or chemically synthesized, and prominently involved in the host humoral and cellular immune responses to infection (105,110,234,240,243,246,350,425,427). Using smβGalNAcT to generate the LDN base unit, further studies on and function of schistosome α1,3-FuTs are currently underway to determine their role in the creation of the fucosylated LDN glycans. Given the current limitations in worm glycan purification, the ability to produce the unique glycans of *S. mansoni* and more accurately recapitulate the schistosome glycome is a huge step forward. This knowledge allows for the continued exploration of the roles schistosomal glycans play in both worm biology and their modualtion of host immune systems, as well as the development of important glycotechnologies including, but not limited to, chemo-enzymatic synthesis, glycogonjugates and vaccine development, and schistosome glycan microarrays.

5.5 Acknowledgments

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Chapter 6. Discussion

The biology and immunology of schistosomes has been a focus in the field of parasitology for many years, however the science has yet to translate into more sophisticated solutions for diagnosis, treatment or prevention. 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. We believe this work supports the idea that the identification of novel glycans synthesized by schistosomes and their recognition as antigens represent an exciting opportunity to fill these gaps for *Schistosoma* species. Elucidation of the schistosome genome has also provided a link between the genome and the worm glycome, which has important consequences for the future of glycan studies.

6.1 Aim I: Identification of immunogenic glycan antigens and reagent development

It has long been recognized that helminths, including schistosomes, possess an abundance of complex and unique glycoconjugates that interact with both the innate and adaptive arms of the host in incredibly complex ways. These glycoconjugates represent a major untapped reservoir of immunomodulatory compounds, which may have the potential to treat autoimmune and inflammatory disorders, and antigenic glycans, which could be exploited as vaccines and diagnostics. Prior to success in a glycan-based vaccine approach, further innovations are necessary. Through the course of an immune response helminth glycans can induce both protective and non-protective antibodies. It is pivotal that we develop technologies to identify particular immunogenic glycan structures and which anti-glycan specificities that may afford protection. Schistosomes synthesize many types of unusual glycans, and both monoclonal and polyclonal antibodies have been found to recognize specific glycan determinants. The overall screening of antibody responses

and their targets, has been difficult due to the lack of parasite-derived glycan reagents and technologies.

An emergent theme from the reagent development studies (Chapter 2 and Chapter 3) is the importance of fucose in schistosome glycans. In Chapter 2 we described the creation of a monoclonal antibody, F2D2 which targets the glycan epitope FLDNF. Previous studies show that FLDNF is the glycan responsible for the cross-reactivity of schistosomes with KLH (109,110,269). While KLH has been proposed for use as a serodiagnostic antigen for schistosomiasis, its use has not been feasible so far because of cross reactivity with sera from *T. spiralis* patients (377,379-382). The availability of mAb F2D2 will now allow the direct purification of the cross-reactive glycans from KLH, causing a depletion of FLDNF epitopes, in order to assess the FLDNF glycan and/or KLH for its serodiagnostic potential and testing for the presence of any other antigenic glycans on KLH. Monoclonal antibody F2D2 will also serve as a very useful analytical tool in the study of many aspects of FLDNF expression. The antibody will also permit the study of the expression of the epitope during schistosome development in both the vertebrate and snail hosts in order to illuminate its biological function. The role of fucosylated glycans in the parasite development and immunobiology is still unclear, however there is some evidence that FLDNF may play a role in mollusk susceptibility to miracidia infection (384). When this mechanism is further understood, it could lead to some additional methods of parasite control.

In Chapter 3 we described the use of shotgun glycomics to create a shotgun array with total N-glycans from egg glycoproteins (2D-SSGM). We used data from a defined glycan microarray based on schistosome glycan antigens, along with anti-glycan antibodies and mass spectrometry results to identify key glycan antigens in this parasite. The most notable was the multi-species recognition of the fucosylated glycan determinant FLDNF, which is highly expressed on multiple life stages (**Figure 3.8**). This discovery has broad implications for understanding the overall animal and human responses upon infection to glycan antigens from this parasite. Additionally, the F2D2 antibody was also found to be lethal to schistosomula *in vitro* which further

suggests this motif as a possible vaccine candidate. With the ability to produce large amounts of stable F2D2 antibody, we can explore F2D2's ability to protect against parasite challenge with a passive transfer study. This could determine if the *in vitro* killing, seen in Chapter 3, by anti-glycan antibodies may contribute to protection. Success would have great significance for the glycol-based vaccine strategies for multi-cellular pathogens.

The conclusion from both of these studies is that fucosylated LDN derivatives are highly targeted by humoral immune responses in multiple infection models and may lead to new diagnostic and vaccine targets. It has been previously determined that humoral responses to glycan determinants or epitopes such as Le^X, LDN, LDNF, and LDN-dF give rise to different intensity levels and antibody isotypes (80,127,168,208,238,243,244,371). It is not yet clear whether such responses provide protection. This could be due to the particular glycan antigen, the antibody isotype, or even the intensity of the humoral response within the timeline of worm maturation. It may be possible that a robust response to multi-fucosylated variants such as FLDNF could contribute to host resistance or even be an early indicator of infection which could lead to better drug administration protocols. Recent glycomics profiling of schistosomes have shown that the parasite produces a diverse repertoire of complex N-glycans with α3 fucose, α6 fucose, and β2 xylose motifs (17,74,105,111) and support the idea that fucosylated glycans should be a focus of future studies.

This works also demonstrates the value of glycan-based technology innovation. The glycan microarrays developed by our group are uniquely poised to identify functional glycans as ligands for glycan-binding proteins (GBP) and expand our knowledge of glycans targeted in infection models. This technology enables us to verify the targets of antisera, define glycan-specific monoclonal antibodies, and GBPs in an unbiased and sensitive manner. One of the overarching questions about the function of anti-glycan antibodies is - what role do they play in protective models? Shotgun glycomics should be applied to multiple life stages of schistosome and using sera from various models to map out the humoral responses during the course of infection and to

highlight differences between the species. Whether a species is permissive or resistant is likely a complicated and multifactor question, however it would be interesting to see if there were major differences between the glycan epitopes targeted, their intensity and isotype, and the maturation of the parasite. Furthermore, the development of hybridomas to interesting glycan antigens should help address the function of anti-glycan antibodies. The role of these antibodies should be assessed in several ways including passive transfer followed by challenged with parasites and by schistosomula killing assays (described in Chapter 3).

6.2 Aim II: Development of semi-synthetic approach for heterologous expression of glycosyltransferases

Anti-glycan antibodies to parasite glycans are present and abundant in all investigated models of schistosomiasis. However, the mechanisms of host immunomodulation by the parasite glycans and the role of glycan epitopes in adaptive immunity of this disease is still poorly understood. The purpose of our studies in Chapters 4 and 5 was to develop novel methods of producing and presenting eukaryotic glycans which could be applied to a several future ventures in glycan library generation and vaccination strategies. Our approach employed heterologous expression of parasite genes in mammalian cells and identified newly generated antigens using specific mono- and polyclonal antibodies to glycan antigens, enzymatic digestion, and mass spectrometry. These studies will promote developments in the future of novel glycoconjugates for diagnostic and vaccine purposes.

S. mansoni, and other helminths, produce of many different complex N- and O-glycans which are unusual and structurally distinct from host glycans (7,70-74). These glycans are of prime importance at the host-pathogen interface, and circulating glycan antigens (CCA, CAA) have proven useful as diagnostics in human and animal hosts (33,35,79,80). In Chapters 2 and 3, we also highlighted the importance of single- and multi-fucosylated LDN motifs for their roles as humoral targets in multispecies infection models. A deeper understanding of these glycans and

glycoconjugates, and their ability to modulate the immune system is pivotal for innovative new strategies for lessening the mortality and morbidity caused by these parasites. Prior to this work, our ability to access these glycan structures has been limited due to the difficulty and labor intensive protocol required to purify a glycan from the worm. Many of the structures are not found in an alternative source and there has been limited success in synthesizing the unique and complex glycan structures.

Our overarching goal from Chapters 4 and 5 was to develop a method which would allow us to identify the glycosyltransferases responsible for creating the interesting fucosylated glycans found in *S. mansoni*. Several considerations had to be made- first, many of the structures seen in schistosome glycans are unique to the parasite and the glycosyltransferases we believe to be responsible for their creation do not have known orthologs in other systems. Additionally there are many glycosidic linkages in parasites that appear as crucial components of several schistosome antigens, such as Fucα1-2Fuc, that have no known glycosyltransferase in the genome database (2,17,283,285,332). This could be due to insufficient sequence information or an overlap with one of the α3/6 FuT's functionality. Thus, when focusing on the FuT multifamily, it was practical to begin with an enzyme function that is well characterized in other species: Le^X.

In Chapter 4, we report the molecular cloning of fucosyltransferase-F (smFuT-F) from *S. mansoni*, as a soluble, GFP fusion protein with its acceptor specificity. The gene smFuT-F was expressed in HEK freestyle cells, purified by affinity chromatography, and analyzed toward a broad panel of glycan acceptors. The enzyme product of smFuT-F effectively utilizes a type II chain acceptor Galβ1-4GlcNAc-R, but notably not the LDN sequence GalNAcβ1-4GlcNAc-R, to generate Le^X type-glycans. To our knowledge, this represents the first α1,3-fucosyltransferase cloned, recombinantly expressed, and enzymatically characterized to date in parasitic worms. This is also a valuable proof-of-concept for our plans to define specific components of the glycome that contribute to immune responses and identify key *S. mansoni* genes involved in glycan synthesis.

Chapter 5 reports the use of the same approach to identify the enzymatic activity of a β1-4 N-acetylgalactosaminyltransferases (β1-4GalNAcT) from *S. mansoni* which creates the LDN motif. This knowledge will synergize with the *S. mansoni* FuT family activities to accurately generate the single- and multi-fucosylated LDN motifs we previously identified as valuable targets for future studies. By exploiting the now available genomic resources, we can employ our approach to develop a more thorough understanding of the schistosome glycome. This could promise faster identification of targets for diagnostics and drug development, as well as a collaborative approach to antigen chemo-enzymatic synthesis and discovery for glycan-based vaccine platform.

6.3 Future directions

A major goal for this project includes cloning the remainder of the schistosome FuT family, FuT A through E, in order to determine their functionality and the repertoire of glycans they produce. Additionally, Chapter 3 highlighted that glycans with core β-xylose and 1-4 fucose residues generating single- and multi-fucosylated LDN structures are potent targets in multiple infection models. Identification of other glycosyltransferases, such as the xylosyltransferase, may be necessary. With the acquired knowledge of the smFuT A-F family, smβGalNAcT enzymes, and others we could develop a library of glycans that accurately represent the schistosome glycome. Such a library would be a resource for future projects including, but not limited to, the generation of a variety of glycan based technologies. For example, glycans conjugated to AEAB could then be linked to protein carriers for studies focused on immune activation and glycoconjugates vaccination strategies. After the synthesis of those glycans, immunization studies in mice could generate a panel of antibodies that focus on various fucosylated glycan motifs that require further exploration. Current methods to generate schistosome-specific monoclonal antibodies via natural infection or from immunization with a proxy glycoprotein has been laborious and has had limited success.

Schistosome glycans could also be linked to NHS activated slides to form new versions of defined glycan microarrays (Chapter 1, section 1.3.2) (271). The development of such arrays will allow us to explore immune responses to glycan antigens in a variety of infected animal species, direct studies focusing on glycans based diagnostics, and define newly developed anti-glycan antibodies in a facile and sensitive manner. These glycans could also be used to facilitate enzymatic studies, aid in antibody characterization, or even be used to absorb out various glycan binding specifies from serum samples. Depletion of particular glycan targets from antisera could help elucidate the role played by anti-glycan antibodies in protective animal models. The generation of a comprehensive schistosome glycan library could be a powerful resource for a variety of experimental strategies.

Previous work from our lab demonstrated that the manner in which glycan epitopes are presented affected the overall immunogenicity of that glycan (262,272,484). This is in agreement with the long held understanding that whole-organism immunogen based vaccines, such as attenuated or inactivated bacteria or viruses, induces a more potent and longer-lasting immunity than purified components or recombinant proteins (485,486). It is possible that the mechanisms which contribute to the success of whole-organism vaccines could lead to triumphs for recombinant whole-cell vaccinations, and could be applied to glycan based vaccination stragteies (272,484). Prasanphanich *et al* (272) validated several attractive features in whole-cell vaccination which is applicable in the development of a potent glycan vaccine towards a multicellular pathogen. Whole-cells provide a dense, membrane-bound presentation of antigens with multivalent epitopes within the same glycan branch as well as along the surface of the cell. In addition, cells contain diverse peptide epitopes for induction of T-dependent immunity and possibly other signals to cue the innate immune response and offer diversity of immune targets.

To explore the feasibility of whole-cell glycan vaccinations, Prasanphanich *et al* (272) immunized mice with whole CHO cells that had been engineered to express a foreign surface glycan and is similar to glycans found on a schistosome (Lec8, Lec8GT, and Lec8GTFT) (**Figure**

6.1A) (342,343). These immunizations induced a polyclonal antibody response that was focused on the major glycan epitope, as opposed to the multiple cellular targets possible with whole-cell vaccination. Lec8 CHO immunization induced a response to Lec8 cells in mice and likely contributed to the immunogenicity of the Lec8GT and Lec8GTFT cells as well. Lec8GT immunizations resulted in antibodies targeting the LDN glycan motif. Similarly, the antibodies made by Lec8GTFT immunized mice were predominantly against LDNF, with very little cross-reactivity to Lec8 cells or to kifunensine-treated cells, which have inhibited glycan production. It was concluded that while the cells likely provided a broad set of T-cell epitopes, the humoral response was clearly focused on the immunodominant LDNF epitopes carried by the variety of CHO N-glycans. However, it is currently unclear if these anti-glycan antibodies could protect mice against parasite challenge.

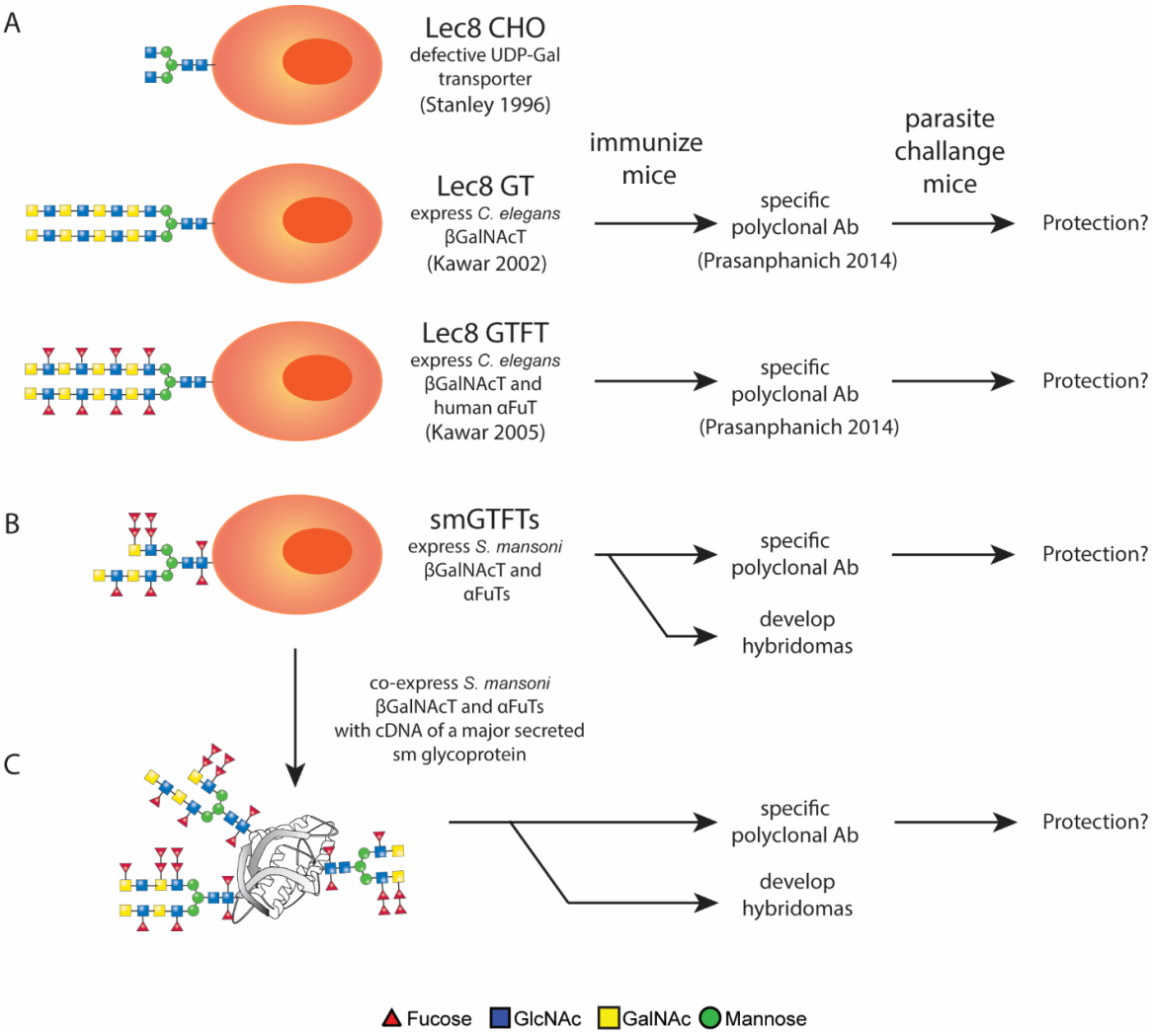

Figure 6.1. Cellular engineering for the production of specific immunity to schistosome glycan determinants.

(Adapted from communications with R.D.C.)

The recombinantly-engineered Lec8GT and LEc8GTFT cells induced a response which was highly focused on the single over-expressed glycan they each expressed, LDN or LDNF. It's important to note that these cells were developed with enzymes from *C. elegans* and from humans which have their own breadth of possible glycan acceptors. This generated a cell glycome that, while processing the motif of interest, does not faithfully mimic what is seen by the host during infection with a schistosome. Previous work from our lab has made it increasingly clear that anti-

glycan antibodies are highly specific for the antigens to which they are generated. Also, slight modifications in the presentation of the glycan can affect the specificity of the response in drastic ways (262,272,484). We commonly refer to tri- and tetra-saccharides as glycan epitopes (or glycotopes), although the presence of the glycan motif does not confirm antibody binding. In the case of Le^X, some antibodies have the capacity to bind internal motifs while others are restricted to the presence of terminal tri-saccharides (115). Another example is the HIV-neutralizing antibody 2G12, which contacts several monosaccharide residues on each of 4 high-mannose glycans of GP120 (487). Simply including a particular glycan structure is not enough to generate a highly successful carbohydrate-based vaccine. A well-designed schistosome cell-based vaccine would benefit from use of the appropriate enzymes to make the glycan target of choice.

The cloning and enzyme characterization studies described in Chapters 4 and 5 begin to demonstrate the breadth of the *Schistosoma mansoni* α1-3FuT multifamily. This knowledge could be used to engineer various eukaryotic cell lines which each produce a particular schistosome glycan of interest. A major limitation the field has previously encountered is that schistosomes cannot be maintained in culture and purification of these glycans from the worm is not feasible. Development of CHO cells using the desired schistosome glycosyltransferases would be useful in several ways. Firstly, these cells could be engineered to produce single- and multi-fucosylated LDN structures which we know to be important immune targets in multiple infection models. The resulting cell glycome would better recapitulate what is created by the worm, which is vital for inducing a robust and effective anti-glycan immune response. These new smGTFT type cells should be a better alternative for whole-cell vaccines and/or used to produce highly targeted hybridomas to each glycan (**Figure 6.1B**). Cells, which can be grown to high density in culture, could be used to generate the glycan libraries should chemo-enzymatic approach produce a less than desired yield. Likely, there would be heterogeneity in the glycans produced, however this may not be a source of concern due to the vast variety seen in the worm glycome.

Another interesting future direction that could be crucial to successful vaccination against multi-cellular pathogens is incorporation of multiple targets into one vaccine. This approach is already successful for coverage of many serotypes by bacterial polysaccharide conjugate vaccines (488,489). Several glycan epitopes could each be incorporated into cell lines and mixed to create a multi-target, multi-stage schistosome vaccine. Yet another possible approach could be to include a major secreted schistosome glycoprotein. With our increased knowledge of the genome, the cDNA of a relevant tegmental or secreted glycoprotein, which would normally carry glycan antigens, could be added to generate a "natural" schistosome glycoconjugate and avoid any complications seen with glycan-protein linkers (**Figure 6.1C**) (262). Including a protein carrier (or even a variety of protein carriers) with the important glycan antigens could serve to generate a robust memory response focused on the glycans.

Given the lack of overlap between mammalian/host glycans and the highly fucosylated LDN structures, the glycosyltransferases responsible (FuT family in particular) could represent an underutilized therapeutic target. With recombinant enzymes we have the opportunity to study the crystallization of enzymes and explore possible inhibitors to the acceptor binding sites. This could lead to targeted drugs and therapies which may aid in parasite clearance. While it is unclear if inhibiting the glycan biosynthesis would be directly involved in worm survival, because of limited knowledge of the role each glycan plays in schistosome biology, inhibiting particular glycosyltransferases could have an effect on the worm's ability to modulate the host's immune response. Interruption to this mechanism could lead to better understanding of worm glycolbiology and better outcomes from infection.

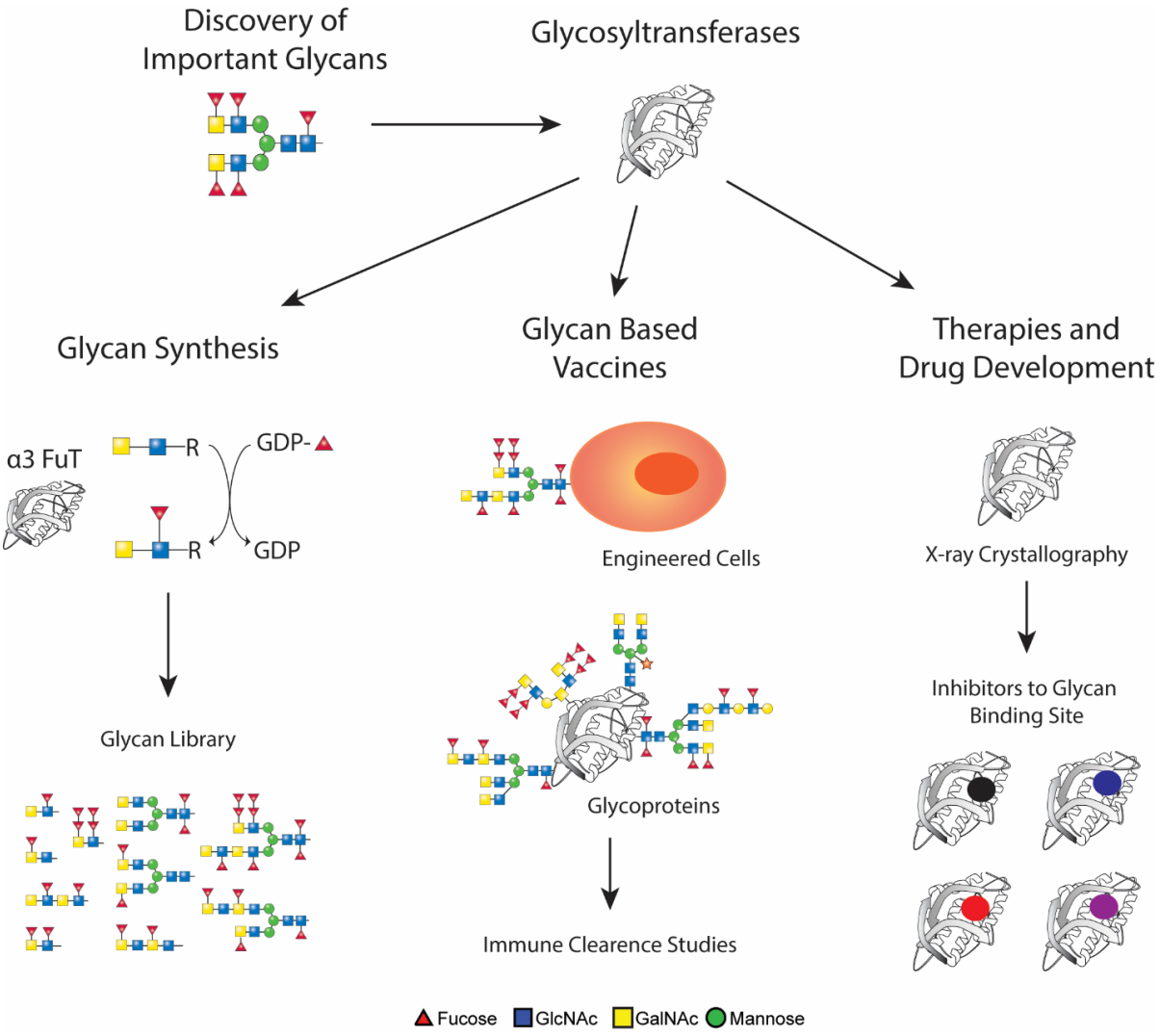

Figure 6.2. Summary of possible future directions.

6.4 Conclusions

With this work, we have shown demonstrated the importance of fucosylation in complex schistosome glycans (particularly single- and multi-fucosylated LDN derivatives), the success of shotgun glycomics to elucidate host immune targets, and the extremely complex and still enigmatic specificity and functionality of the glycosyltransferases responsible for the generation of interesting glycan antigens. Although we still have much to learn about the glycobiology of the schistosome, this work should encourage and support future studies on *Schistosoma's* uniquely fucosylated glycans. It is vital in the development of novel diagnostic approaches and glyco-conjugate vaccine platforms that we reveal glycan biosynthesis pathways and explore their feasibility as immune and drug targets (**Figure 6.2**). We hope this work will inspire the scientific community to devote its valuable time and resources to developing glyco-technologies which have the potential to help with the global challenges posed by schistosomiasis and other helminth infections.

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