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Development of Glycoconjugate Vaccines for Schistosomiasis

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Development of Glycoconjugate Vaccines for Schistosomiasis

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An abstract of
a dissertation submitted to the Faculty of the Graduate School of Emory University
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2010

Abstract

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By Anthony Esheminye Luyai

Schistosomiasis is a debilitating parasitic disease of humans and endemic in the tropics. Although the mortality attributed to schistosomiasis is second only to malaria, surprisingly, it remains largely neglected and research efforts towards developing a protective vaccine are minimal. In my studies, I have sought to dissect the humoral immune responses to defined schistosome glycan epitopes in humans, rhesus monkeys, and mice. Using glycopeptides terminating with schistosome specific glycan epitopes, I have helped to develop a defined schistosome glycan microarray to show that while rhesus monkeys generate predominantly high titer anti-core xylose/core fucose IgG antibodies, humans generate low titer antibodies to the same epitope. Interestingly, the peak of anti-core xylose/core fucose IgG generation in the rhesus monkeys coincides with sera from these animals having the highest schistosomula killing effect observed *in vitro*. Mice chronically infected with schistosomiasis generate high titer anti-LDNF IgM antibodies that are cytolytic to schistosomula *in vitro.* Additionally, mice immunized with BSA conjugates of LDN and Man3 generated antibodies that are cytolytic to 3h-old schistosomula *in vitro*. To facilitate the development of glycoconjugate candidate vaccines for schistosomiasis, I have used a commercially available chemical, *p*-nitrophenyl anthranilate (PNPA), as a heterobifunctional linker and helped to develop an efficient and quantitative way of conjugating glycans with a free reducing end via reductive amination to carrier proteins. Our studies support the concept of developing glycoconjugates as potential protective candidate vaccines and for generating new serodiagnostic tools for schistosomiasis. In my thesis work I show that core xylose and/or core fucose epitopes have a potential.

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First and foremost I would like to thank God for giving me life and his son Jesus Christ for salvation. I immensely want to thank my mentor Dr. Richard Cummings for accepting me in his lab and teaching me the best ways to carry out experiments. I will always remember his remarks; “Anthony, you can never have too many controls”. Many thanks too to the awesome lab members in the Cummings lab. To my committee members, thanks for your help and shaping me into a researcher.

Special thanks go to my family for their relentless encouragement and understanding whenever I took long hours away from them to be in the lab. To my dear wife Susan, you are my shining star and without you I could not have attained this PhD. I dedicate this achievement to my late mother Peninnah Nyamatta Luyai and my late father Henry Ford Luyai for giving the gift of life.

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Chapter 1: Introduction

1.1 Introduction to Dissertation

Schistosomiasis

Schistosomiasis is one of the most prevalent parasitic diseases in the world, first described by a German pathologist Theodor Maximilian Bilharz in 1851 in Egypt [1,2,3]. The disease is commonly referred to as Bilharzia or Bilharziosis. In Sub-Saharan Africa, schistosomiasis is second only to malaria as a disease of great economic importance. More than 5 million people exhibit schistosome-specific symptoms, especially worm burden, and upwards of 200 million people stand the risk of exposure [1]. Although the development of schistosomacidal therapeutic agents like Praziquantel has played a big role in the management of schistosomiasis, re-infection and emergence of drug resistant strains has been a major draw-back in fighting this malady [2]. So far, no protective vaccine has been developed against schistosomiasis or any other parasitic infections despite extensive research efforts in this area [3]. Failure in producing an efficacious vaccine is attributed to the fact that research has been focused on the development of protein-based vaccines with minimal success in their trials experiments. Some of the failed protein-based vaccine candidates include the 63 kDa parasite myosin, the 97 kDa paramyosin, the 28 kDa triose phosphate isomerase (TPI), the 23 kDa integral membrane protein (Sm23), the 26 and 28 kDa glutathione-S-transferases (GSTs), and the 28 kDa *S. haematobium* GST (Sh28GST) [4]. Currently, Sm14, a 14 kDa fatty acid-binding *S. mansoni* protein has shown some promising result in the mouse model but studies in humans have yet to be performed [5]. However, recent advances in schistosome glycobiology pioneered by our laboratory and others have demonstrated that immune responses against schistosomes are mostly directed towards glycan epitopes[6-10]. Glycans decorate the outer surface membranes of all

stages of the schistosome, making a dense coat of sugars referred to as the glycocalyx. The host immune system responds to some of the schistosome specific glycans embedded in the glycocalyx.

1.2 The general biology of schistosomes

Schistosomes are digenian (have an alteration of generations) trematode blood flukes that parasitize man and other mammals. *Schistosoma haematobium, S. mansoni,* and *S. japonicum* account for the majority of human schistosome infections [2]. Humans (definitive hosts) are infected by exposure to water contaminated with infective free-swimming aerobic larvae called cercariae. Upon entry via skin penetration, the cercariae transform to anaerobic schistosomula and move through the lymphatic system, heart, and lungs, and settle in the hepatic portal vein and the mesenteric veins of the large intestine, where they mature and male/female pairs are established. The dioecious adults (having male and female reproductive organs in separate individuals) do not multiply in the body but live there for many years where sexual reproduction and egg production occur. In infected humans, the layed eggs escape the vasculature and are trapped in the neighboring tissues. Most of the eggs in the vasculature are swept back to the liver where they escape the vasculature and lodge in the liver tissue. The outer membrane covering of eggs are leaky and the antigens exuded from them elicit delayed-type hypersensitivity leading to formation of granulomas in the urogenital system (*S. haematobium*) or in the liver and intestines (*S. mansoni* and *S. japonicum*). The granulomas sequester the eggs, ameliorating the disease conditions, but formation of too many granulomas leads to hepatosplenomegaly and ultimately to death [11]. Hence the deposition of eggs in the liver and other tissues causes the pathology. Eggs leave the body in the urine or feces. Upon contact with fresh water, the eggs hatch to liberate the miracidium larva, which infects specific types of fresh water snails

(intermediate hosts). The types of snails the miracidia infects are determined by the species of the schistosome. Within the snail, the parasites multiply asexually, first forming sporocysts which transform to free-swimming infective cercariae. Humans wading in the contaminated water are infected when cercariae burrow into their skin, transform into schistosomula, and enter the peripheral blood vessels and migrate to the portal system commencing another cycle (Fig.1).

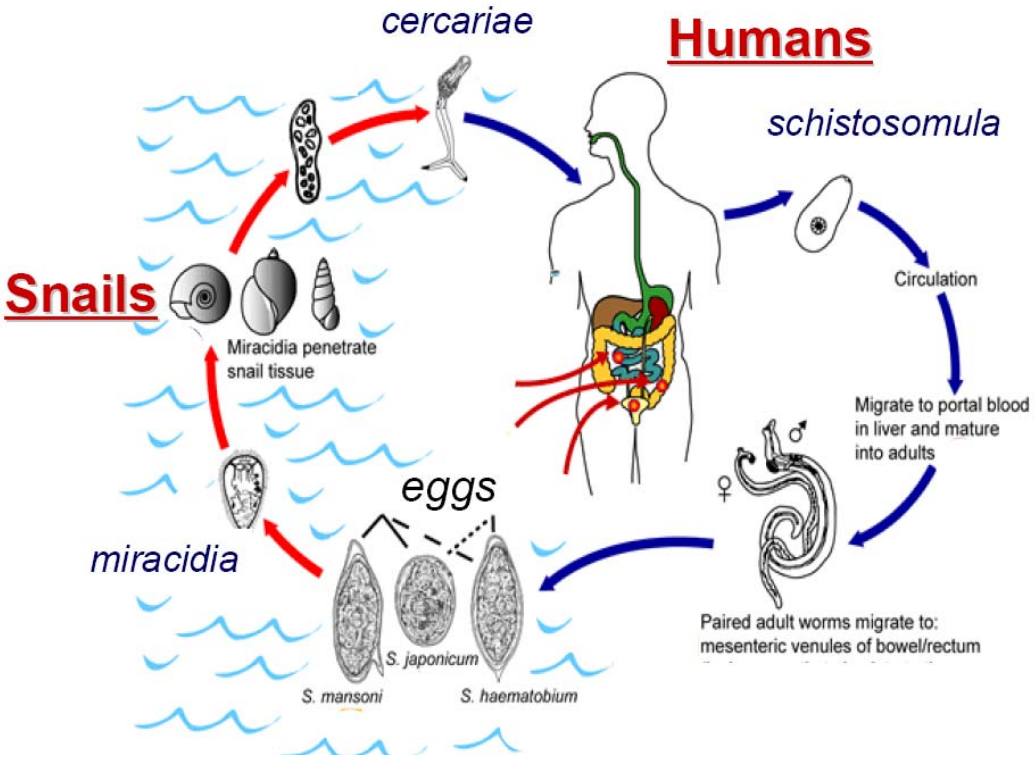

Fig.1-1 The schistosome life cycle

Schistosome specific immune responses

Animals infected by helminthes usually mount both cell-mediated and humoral immune responses which are of polarized CD4+ T_H2-type against products from these parasites [13]. Immediately after infection with *S. mansoni*, the cytokine profile observed in mice is that of T_H1 related response. After egg laying commences which occurs about 6 weeks after infection, T_H1 responses are down regulated and T_H2 responses start rising coinciding with the acute phase of the disease. T_H2 responses reach their maximal levels 8 week after infection then start decreasing. In the chronic phase T_H2 responses are always maintained at a higher level than T_H1 responses (Fig. 2).

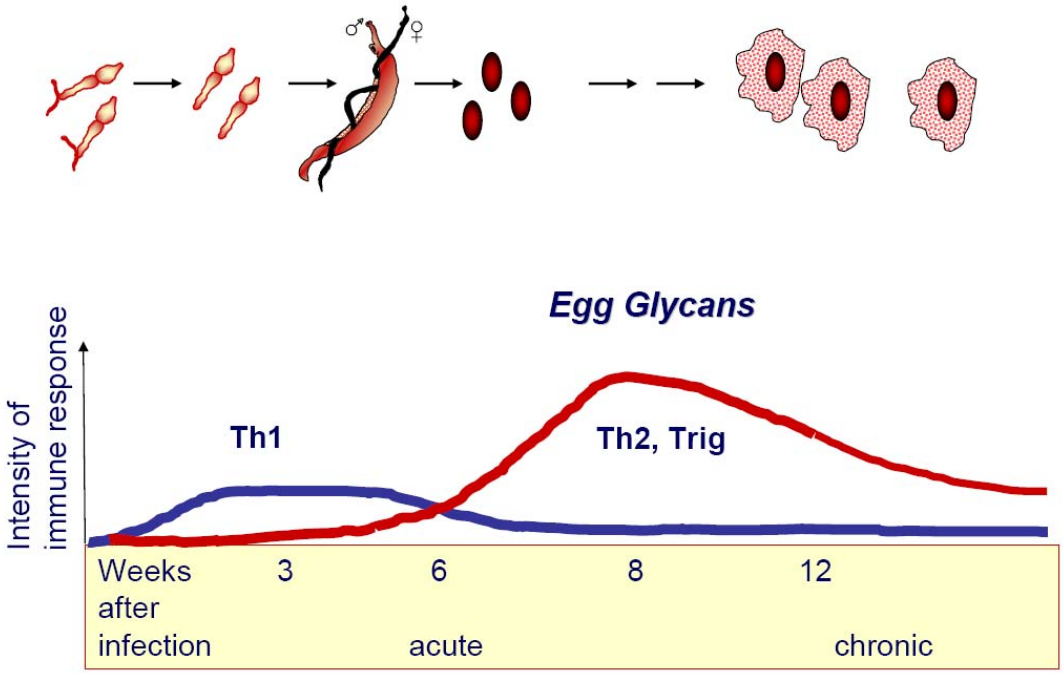

Fig.1- 2 Immune responses in mice during an infection with *S. mansoni*

The acute phase culminates in granuloma formation in areas where eggs are entrapped [13, 14]. The cytokine profiles in humans during infection with *S. mansoni* mirror those observed in mice; both species are permissive hosts of schistosomes. Eosinophilia is also observed during both the acute and chronic phases of schistosomiasis. Eosinophilia and IgE production play a major role in management of the disease through antibody dependent cell cytoxicity (ADCC) [15].

In schistosomiasis, as mentioned above, the immune responses are directed to glycans which decorate the surfaces of schistosomes in all stages of the schistosomes and to secreted glycoproteins from their eggs. In the murine system and to some extent in primates, egg laying, which commences at 5-6 weeks after infection, coincides with generation of anti-glycan antibodies and T_H2-related cytokines [7, 14, 16]. By contrast, protein epitopes are less recognized. Studies in our laboratory and others have shown that the glycosylation pattern of schistosomes consist of both N- and O-linked glycans with similar core structures to those found in mammalian glycoproteins but with further schistosome-specific modifications (Fig.3). It has been shown that infected humans, rodents, and primates mount IgG, IgA, and IgM humoral immune responses to these glycan epitopes [7, 9] and that antibody titers to these glycan antigens are generally proportional to severity of the disease [17, 18].

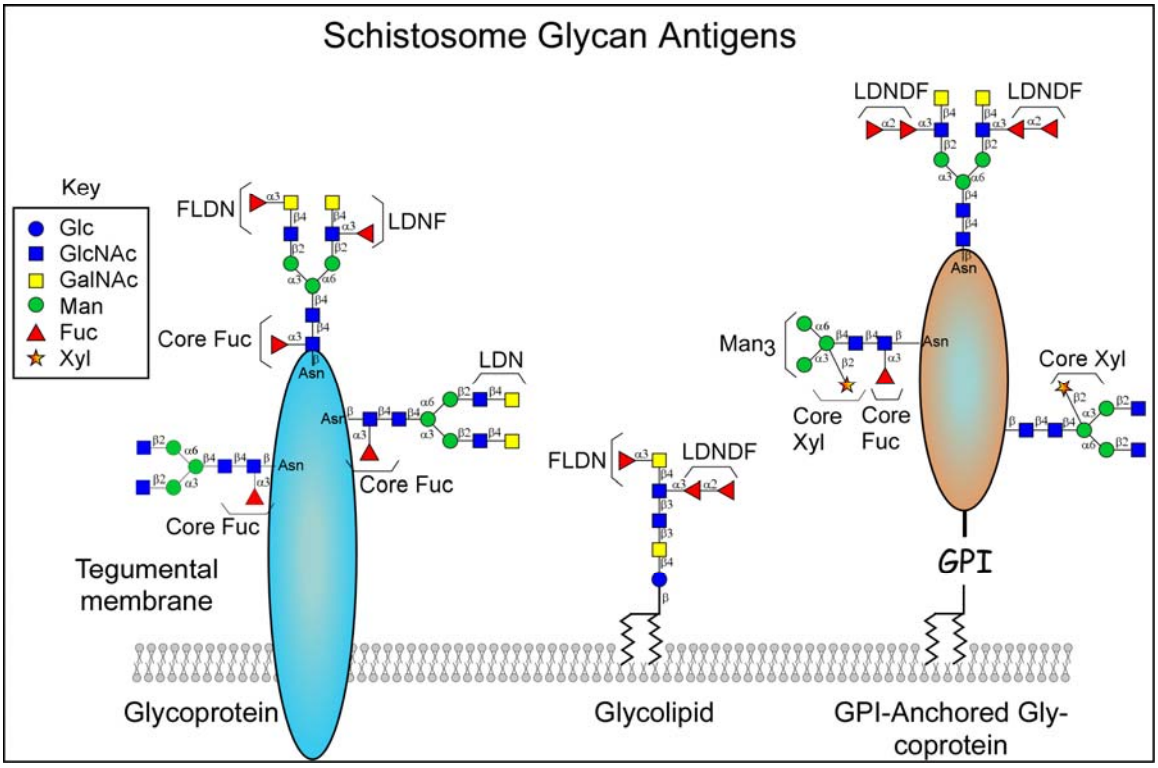

Fig. 1-3 Schistosome-specific glycan epitopes.

Fig. 1-3 Schistosome-specific glycan epitopes.

Nine different terminating glycan epitopes have been identified in all stages of the schistosome life cycle. LDN: GalNAc(β1−4)[Fuc(α1−3)]GlcNAc-

.FLDNF:Fuc(α1−3)GalNAc(β1−4)[Fuc(α1−3)]GlcNAc-R, FLDN:

(Fuc(α1−3)GalNAc(β1−4)GlcNAcβ1-R, Man3: Xyl, xylose., Fuc: fucose Gal, galactose; GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylglucosamine LDN-DF:

GalNAc(β1−4)[Fuc(α1−2)Fuc(α1−3)]GlcNAc-R, GPI: Glycosylphosphatidylinositol.

1.3 Killing of schistosomula by anti-glycan antibodies

Schistosomula, the infective larvae, are effectively killed by schistosome-specific anti-glycan antibodies both *in vivo* and *in vitro* [7, 19]. Fig. 4 shows confocal images of binding of monoclonal antibodies specific for LDN, LDNF, and Lewis-X (Le^x) glycan epitopes on the schistosomula surface.

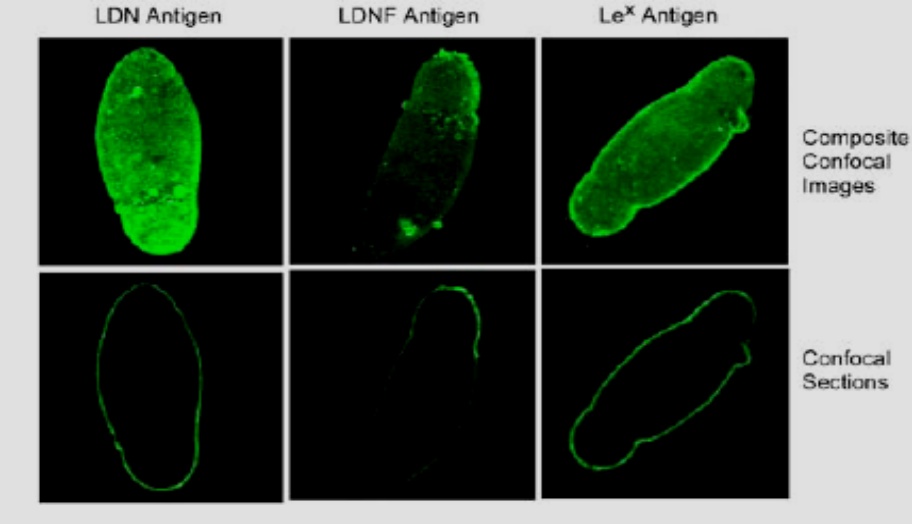

Fig. 1-4 Glycans on surfaces of schistosomula.

Fig. 1-4 Glycans on surfaces of schistosomula.

Surfaces of 3 hr-old schistosomula are covered with glycoproteins expressing LDN, LDNF, and Le^x antigens [7].

Experiments conducted in our lab have shown that 3 hour-old schistosomula are killed by monoclonal antibodies specific to LDN antigen in a complement dependent manner (Fig.5). The killing of schistosomula *in vitro* can also be quantified by Flow cytometric method (Fig.6). These two observation are in support of the fact that schistosomula express the above mentioned glycotopes on their surfaces [7].

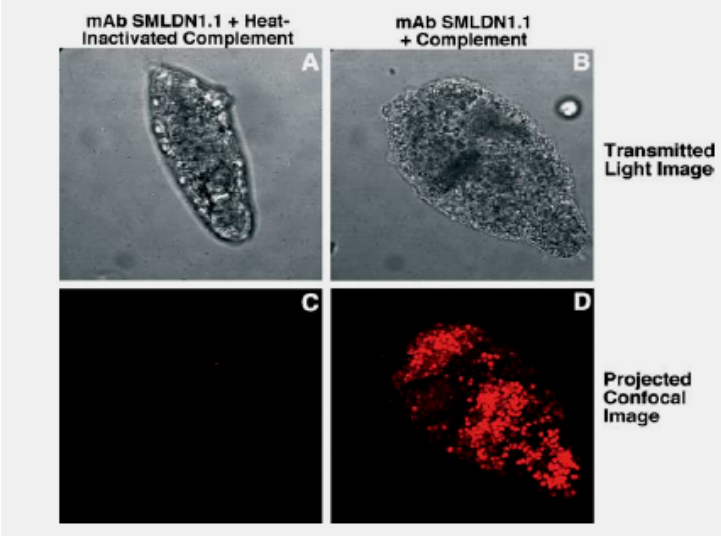

Fig.1-5 Analysis of schistosomula lysis by laser confocal microscopy after treatment with complement and antibodies.

Fig.1-5 Analysis of schistosomula lysis by laser confocal microscopy after treatment with complement and antibodies.

3hr-old schistosumula were incubated in 1mg/ml solution of mAb SmLDN.1 in either heat inactivated rabbit complement (A and C) or fresh complement (B and D) for 48 hr. Propidium iodide was added to a final concentration of 10mg/ml and then parasites were imaged under a confocal microscope [7].

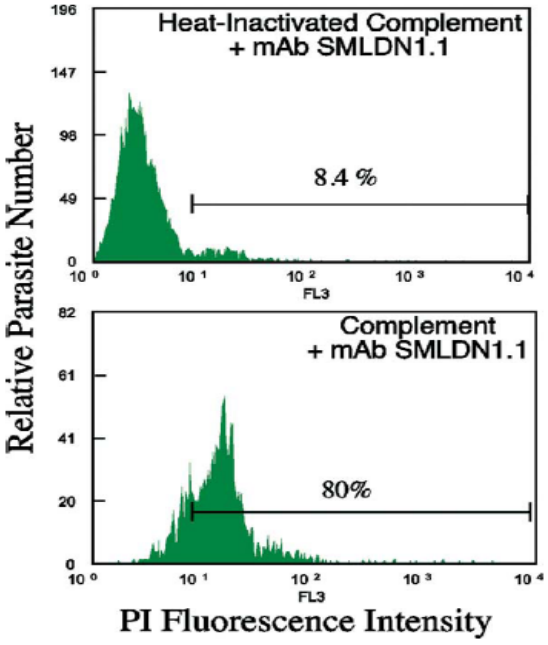

Fig.1-6 Quantitative Flow cytometric analysis of antibody and complement mediated lysis of schistosomula.

Fig.1-6 Quantitative Flow cytometric analysis of antibody and complement mediated lysis of schistosomula.

3hr-old schistosomula (about 5000) were treated with mAb to LDN in the presence of either heat-inactivated or fresh complement as described in Fig. 2. Propidium iodide was added and the parasites analyzed by flow cytometry. Results are representative of experiments performed in triplicates with a standard error of <5% [7].

Antigen presenting cells (APCs) are not able to phagocytose, process, and present whole cercariae or schistosomula (early parasitic stage during infection) to the host immune system [20]. The schistosomula stage is the only immune vulnerable stage known of schistosomes[9]. One reason why processing of schistosomula by APCs is difficult is that it is many thousand-fold larger than any APC. Hence, no significant humoral responses to parasite antigens are detected early during a schistosome infection, whereas anti-glycan responses peak approximately 5 weeks following infection and coinciding with the onset of egg laying (Fig.7). Schistosome eggs secrete large amounts of antigens, mainly glycoproteins and glycolipids that are processed by APCs. Earlier studies have shown that antibody titers to the above glycan antigens increase during infection soon after egg-laying commences[21].

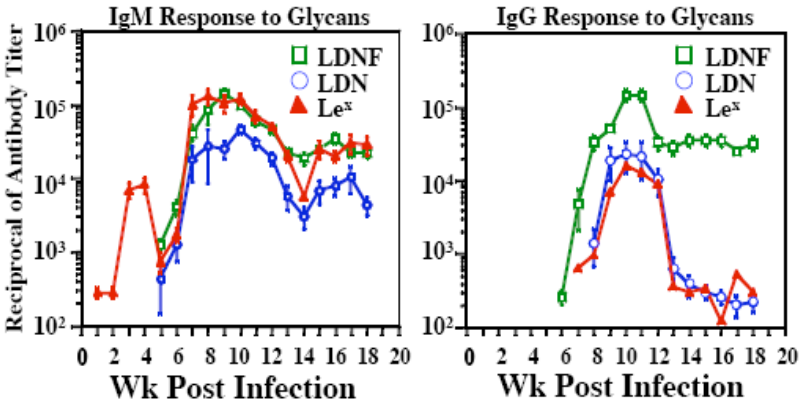

Fig.1-7 Peak IgM and IgG responses of *S. mansoni*-infected mice to various glycotopes.

Fig.1-7 Peak IgM and IgG responses of *S. mansoni*-infected mice to various glycotopes.

Mice infected with 50 cercariae were bled weekly by a tail snip to obtain serum which was serially diluted and used in ELISA against neoglycoconjugates terminating in LDN, LDNF, and Le^x[7].

Since strong immune responses are mounted late during a chronic schistosome infection, parasites continue to thrive in the host during early infection phases. When the strong immune responses to the parasite commence, it is after the schistosomula have developed into adult worms, which are refractory to any immune insult. Interestingly, this may be related to the remarkable evidence that existing infections are correlated with reduced chances of re-infection by the same parasite or even by other helminthes parasites [22], a phenomenon referred to as concomitant immunity [23].

1.4 Protective immunity to helminthes induced by partially defined glycan antigens

Vaccination of animals with glycoprotein-enriched materials has been shown to confer partial protection against various helminth infections [22, 24, 25]. Results from these studies, and preliminary data from our laboratory show that mice immunized with schistosome-specific glycans generate antibodies that kill schistosomula *in vitro.* The killing of schistosomula is complement-dependent [24]. In parallel nematode vaccination studies, immunization of goats with *Haemonchus contortus*-derived glycoproteins (so-called excretory/secretory or ES glycoproteins) provided protection against the parasite [26]. Studies in both van Die's laboratory and ours showed that these parasites express various glycoprotein epitopes including LDN and LDNF found within the ES glycoproteins as well as their L3 larval stage. Lambs immunized with ES glycoproteins and subsequently challenged with the infective L3 larvae showed 54% reduction in worm burden and concomitant 89% reduction in parasite egg output [27]. Serum from immunized lambs contained high anti-LDNF antibody levels, which switched over time from IgM to IgG class, and provided significant protection against infection. Such studies continue to support the development of glycan based anti-helminth vaccines suggesting that a similar approach can be assumed for schistosomiasis.

1.5 Vaccination against schistosomiasis with irradiated cercariae

Mice infected with live, attenuated *S. mansoni* cercariae (by exposure to ionizing radiation) are somewhat protected against a challenge infection [28, 29]. In chimpanzees, repeated vaccinations with irradiated cercariae showed specific humoral immune responses, mainly involving the production of IgG and IgM antibodies directed to glycans on the cercariae [26]. These antibodies also cross-react with glycans on all stages of schistosomes.

1.6 Synthesis of schistosome-specific glycopeptides

Schistosome-specific glycan epitopes were synthesized from commercial bovine fibrinogen and peroxidase from horseradish by chemical and enzymatic methods. These glycoproteins were digested by pronase and subsequently treated with various glycosidases and glycosyltransferases to make schistosome-specific glycans (Fig. 3). Schistosomes synthesize biantennary N-glycans terminating with LDN, LDNF, core xylose, core fucose, and Man3, among other glycan epitopes. These **glycopeptides** were then purified to homogeneity. A general scheme for synthesis of schistosome-specific glycopeptides in shown in Fig. 8.

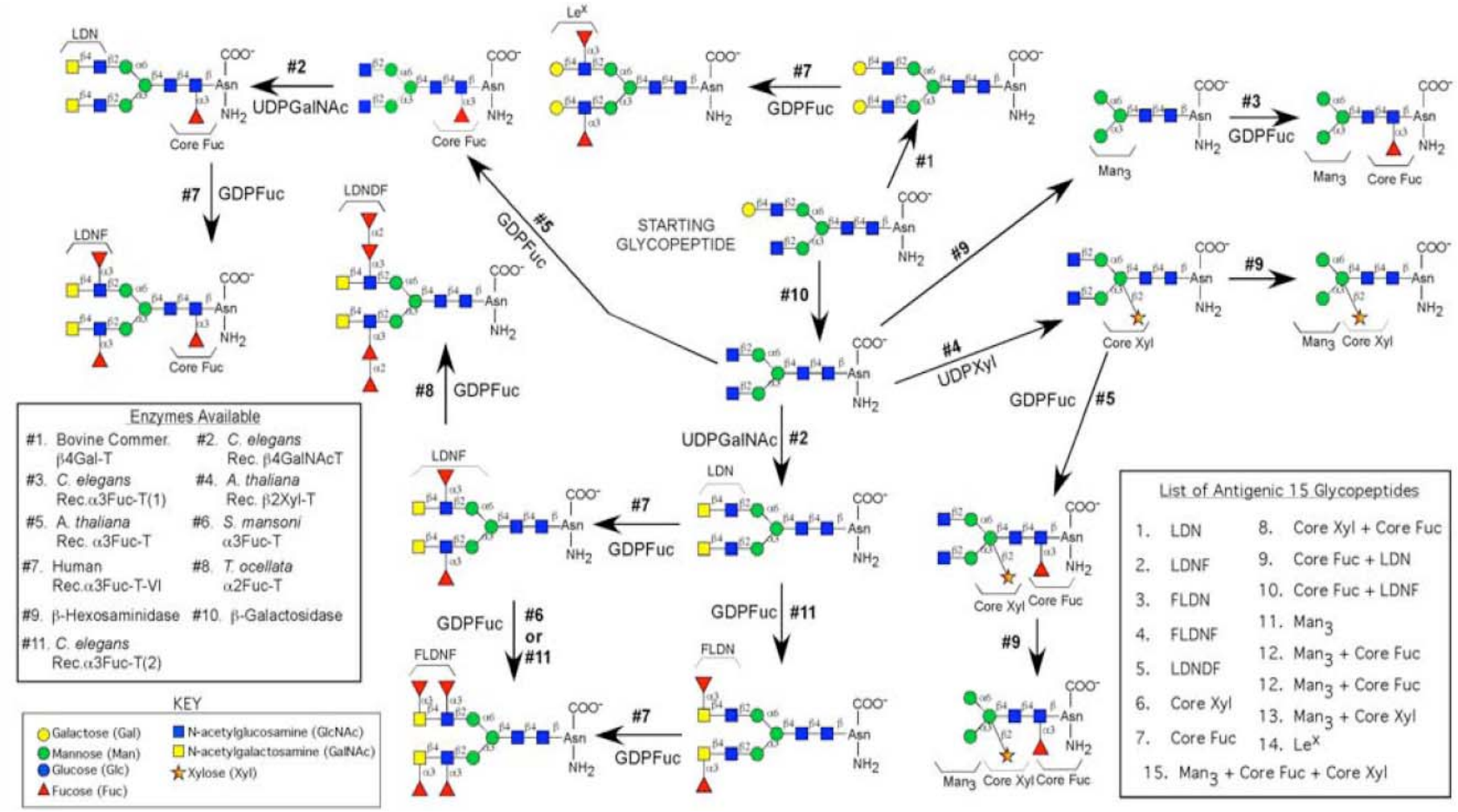

Fig.1-8 Semi-synthetic pathways utilizing a core glycopeptides for remodeling to generate various glycopeptides containing individual and mixed *Schistosoma mansoni*-specific antigens.

1.7 Conjugation of glycan epitopes to protein carriers

The chemistry of conjugation exploited the reactivity of the amino terminal group present on N-linked glycopeptides after pronase digestion. These glycopepetides were purified by affinity chromatography on a concanavalin A sepharose column. Using novel chemistry it is possible to exploit the amino termini of the glycopeptides and functionalize them by the introduction of reactive thiol groups, which have the potential to react with maleimidyl groups conjugated to a carrier protein (Fig. 9). This chemistry is efficient with all the steps in the synthesis going to 95% completion. In this work, the same chemistry was used to conjugate schistosome specific glycans to BSA carrier protein.

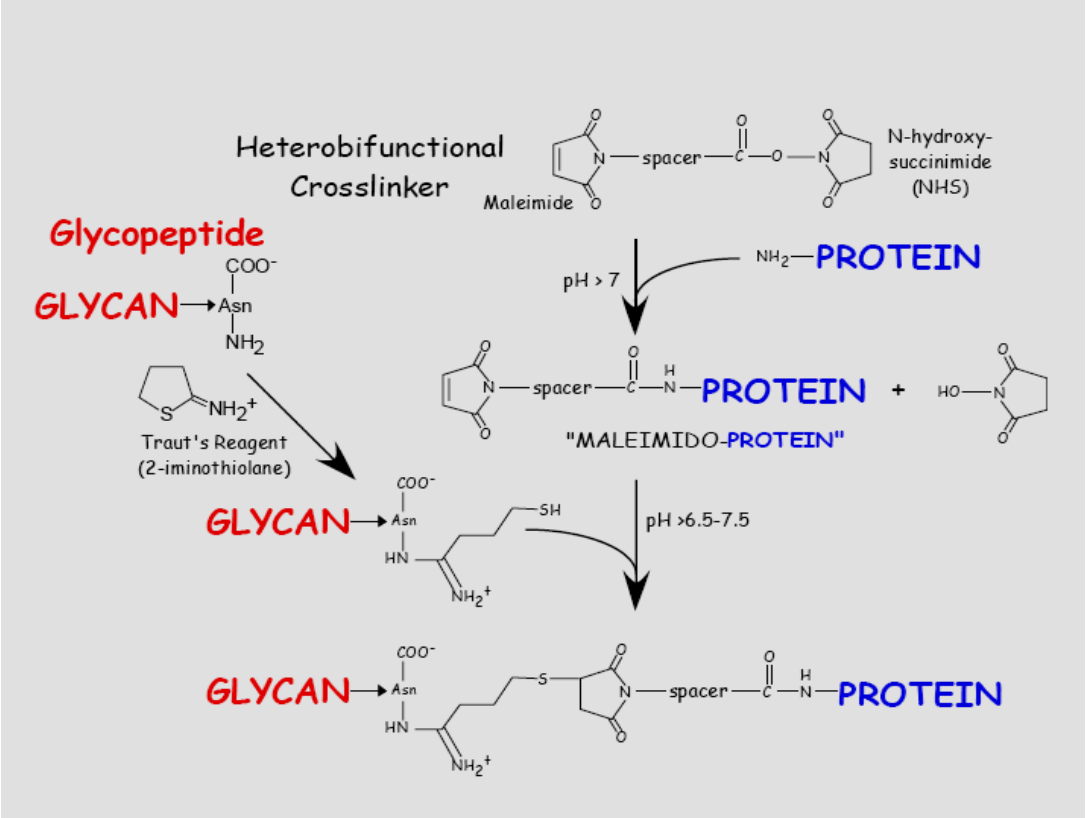

Fig.1-9 A general conjugation reaction scheme.

1.8 Significance of the study

Schistosomiasis is a debilitating parasitic disease affecting millions of people. There is no vaccine available to prevent schistosomiasis (or any other major human parasite). Many schistosome specific protein antigens have been tried as possible vaccine candidates with very poor results. Although Praziqauntel is an effective one-dose therapeutic against this disease, re-infection in endemic areas is common and drug resistant strains are on the uprise [22, 24, 25, 30]. Recent studies have shown that the primary antigens of schistosomes are glycoconjugates. However, very little attention has been given to the development of a schistosome glycan-based vaccine. In this thesis, we provide evidence to support the design and testing of a glycan based anti-schistosome vaccine. We have identified common schistosome specific glycan structures in all stages of the parasite. We have used chemical and enzymatic methods to synthesize these glycans from natural animal and plant products. We have devised a novel way of conjugating these glycans to relevant carrier proteins known to elicit strong immune responses. Our immunization and profiling of humoral responses in schistosome infected animal species will contribute immensely to understanding the schistosome immunobiology and offer novel ways of developing anti-schistosome vaccines.

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Chapter 2: Construction of a defined *Schistosoma mansoni* glycan array and elucidation of humoral immune responses in schistosome-infected rhesus monkeys, humans, and mice to schistosome-specific glycan epitopes

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2.1 Abstract

Schistosomiasis is a debilitating parasitic disease of humans, endemic in the tropics, particularly Sub-Saharan Africa, Asia, and Latin America. Although the mortality attributed to schistosomiasis is second only to malaria among parasitic diseases, surprisingly, it remains largely neglected and research efforts towards developing a protective vaccine are minimal. In this study, we sought to dissect the humoral immune responses to defined schistosome glycan epitopes in schistosome-infected humans, rhesus monkeys, and mice. Using glycopeptides terminating with schistosome-specific glycan epitopes synthesized in our laboratory, we have developed a defined schistosome glycan array. We have used this array to chart the antibody responses over the course of infection with schistosomiasis. While rhesus monkeys generate predominantly high titer anti-core xylose/core fucose IgG antibodies, humans generate low titer antibodies to these epitopes. The peak of anti-core xylose/core fucose IgG generation in the monkeys coincides with sera having the highest schistosomula killing effect observed *in vitro*. Mice chronically infected with schistosomiasis generate high titer anti-LDNF IgM antibodies that are cytolytic to schistosomula *in vitro*. These studies support the concept of developing glycoconjugates as potential protective candidate vaccines and for generating new serodiagnostic tools for schistosomiasis. We show that core xylose and/or core fucose epitopes may have such potential. In addition, we postulate that the high titer antibodies to schistosome-specific glycans generated by rhesus monkeys during the course of infection with schistosomiasis afford them spontaneous clearance of the infection.

Abbreviations: LDN—LacdiNAc, GalNAcβ1 → 4GlcNAc; LDNF, fucosylated LacdiNAc, GalNAcβ1 → 4(Fucα1 → 3)GlcNAc; Le^x—Lewis X, Galβ1 → 4(Fucα1 → 3)GlcNAc;

PBS, phosphate-buffered saline; BSA, bovine serum albumin; CFG, Consortium for Functional Glycomics.

2.2 Introduction

Schistosomiasis is one of the most prevalent parasitic diseases in the world[1-3]. The vasculature dwelling trematodes of the *Schistosoma* genus infect humans, non-human primates, and domesticated animals such as cattle[4]. In Sub-Saharan Africa, schistosomiasis is only second to malaria as a disease of great economic importance. More than 5 million people exhibit schistosome-specific symptoms, especially worm burden, and upwards of 200 million people are at risk of exposure[5]. The impact of the disease is mirrored in its high morbidity during the chronic phase which can last longer than 30 years[4]. Although the development of chemotherapeutic agents like Praziquantel has played a big role in management of schistosomiasis, re-infection and emergence of drug resistant strains has been a major draw-back in fighting this malady[6]. These factors make the global control of schistosomiasis a distant reality[5]. Thus far, no protective vaccine has been developed against schistosomiasis or against other parasitic infections despite much research[7]**.** There has been no success in obtaining an efficacious vaccine for schistosomiasis due to researchers focusing on development of protein-based vaccines and ignoring the candid fact that immune responses are directed towards glycan epitopes displayed on glycoproteins, glycolipids, and glycosylphosphatidylinositol (GPI) anchored proteins on this parasite's glycocalyx[8].

There is a need to understand glycan-based immune responses to schistosomes in both model animals and humans if rapid progress in vaccine development is to be realized[9]. Since the design and synthesis of glycan antigens in large quantities is a daunting task, no study has tested the ability of schistosome-specific glycan antigens to

provide protective immunity against schistosomiasis[10]. In order to alleviate this situation and facilitate study of humoral responses directed towards schistosome glycan epitopes, high-throughput methods of glycan preparation, characterization, and immobilization on solid support at microscale levels for analysis is necessary[11]. To this end, versatile array formats that can be utilized for further interrogation with sera from animal models and humans with schistosome infections are needed. An additional attractive feature of the array approach is in the use of minute amounts of sera samples for the assays allowing many binding experiments to be performed.

In this paper we report the generation of a defined schistosome glycan array and show its usefulness in studying the humoral immune responses directed towards schistosome-specific glycan epitopes in mice, monkeys, and humans. The array slides, printed with N-linked biantennary glycopeptides terminating with schistosome specific glycan epitopes, were interrogated with sera from infected mice, monkeys, and humans at various time points of infection. We compared the results we obtained on our defined schistosome array with results obtained from the available Consortium for Functional Glycomics (CFG) glycan array. We show that when infected with *S. mansoni,* these animals mount humoral immune responses directed towards different schistosome glycan epitopes. Remarkably, the glycan epitopes that infected animal sera binds to are expressed in all developmental stages of schistosomes[8, 12]. We showed that during the chronic phase of a infection, mice react strongly to LDNF, in agreement with the findings of Nyame *et al*[13]. We also report that infected monkeys show strong reactions against the core xylose/core fucose epitope whereas humans show a range of low responses against LDN, LDNF, core xylose/core fucose, and core xylose epitopes. Although most of these responses are generally subtle in infected humans, we observed a notable anti-core xylose/core fucose response. In conclusion, the construction of a defined schistosome glycan array and its subsequent use in profiling

the humoral immune response in infected humans and model animals is an important step in elucidating key glycan antigens in schistosomes that could be of primary importance in not only developing anti-schistosome model vaccines but also in the generation of potentially sensitive serodiagnostic tools.

2.3 Materials and methods

Materials

Bovine fibrinogen and peroxidase, Type VI from horseradish were purchased from Sigma (St. Louis, MO) and were used without further purification. Defatted Bermuda grass pollen was obtained from Greer (Lenoir, NC). ConA-Sepharose was obtained from GE Healthcare Bio-Sciences (Uppsala, Sweden). Pronase, a mixture of proteinases isolated from the extracellular fluid of *Streptomyces griseus,* research-grade quality was purchased from Calbiochem (San Diego, CA). *Aspergillus oryzae* β-galactosidase and Jack bean β-N-acetylglucosaminidase (hexosaminidase), both research-grade quality, were purchased from Sigma. One thousand MW-cutoff Float-A-lyzer dialysis tubing was purchased from Spectrum Laboratories, Inc. (Rancho Dominguez, CA). Graphitized carbon cartridges were purchased from Alltech (Deerfield, IL) while reverse phase (RP) Sep-Pak C18 columns were obtained from Waters (Ireland). Infected mice, monkey, and human sera were a kind gift from Dr. Evan Secor (CDC, Atlanta, GA). *N*-hydroxysuccinamide (NHS)-activated slides were purchased from Schott Microarray solutions, Inc., (Louisville, KY). Anti-LDN IgM monoclonal antibody was a kind gift from Dr. Kwame Nyame (University of Maryland Eastern Shore, MD). All other chemicals were purchased from Sigma and used without further purification.

Glycopeptide preparation

Glycopeptides were synthesized from commonly available natural sources. Sialylated, asialo-, agalacto-, LDN, LDNF, and Man3 N-linked biantennary glycopeptides were synthesized from bovine fibrinogen. This glycoprotein exclusively contains biantennary N-glycans lacking fucose and containing 1 or 2 outer sialic acid residues[14]. To make sialylated glycopeptides, bovine fibrinogen (1g) was treated with 100mg of pronase in 200ml of 1X pronase buffer (10X composition includes I.0M Tris-HCl, pH 8, 10mM $MgCl_2$, 10mM $CaCl_2$, and 1% NaN_3) overnight at room temperature. To remove undigested protein, the resultant digest was passed over a RP C18 Sep-Pak column and the flow-through collected. To purify the glycopeptide product, the sample was applied to a 100ml column of ConA-Sepharose. ConA is a plant lectin (*Canavalia ensiformis*) that binds oligomannose-, hybrid-, and complex-type biantennary N-glycans[15-17]. The column was washed 3 times with 1X pronase buffer and bound glycopeptides were then eluted using 1500ml of 100mM α-methyl mannoside. The collected glycopeptides were desalted over a graphitized carbon cartridge, to which the glycopeptides bound. After washing the cartridge several times with deionized water, the bound glycopeptides were eluted with 3 column volumes of 30% acetonitrile with 0.01% TFA and dried in a speed-vac. The dry glycopeptides were reconstituted with deionized water and their concentration estimated by the standard phenol-sulfuric acid method and their weights determined by drying and weighing [18]. To make asialo-glycopeptide, the reconstituted sialo-glycopeptides were desialylated by treatment with 100mM HCL at 80ºC for 30min. The sample was neutralized with 100mM NaOH, desalted over graphitized carbon cartridge, and eluted with 3 column volumes of 30% acetonitrile with 0.01% TFA to isolate the asialo-glycopeptides. Complete desialylation was confirmed by matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) profiling. For mass spectrometry analysis, 0.5µl of

serially diluted glycopeptides in water was mixed with 0.5µl of 2,5-Dihydroxy benzoic acid (DHB) matrix, air-dried, and analyzed in a MALDI-TOF mass spectrometer. Agalacto-glycopeptides were generated by digesting 24mg of asialo-glycopeptides in 10ml of sodium acetate (NaOAc) buffer pH 5.2 with 20mg of *β*-galactosidase at 37ºC for 12h. The *β*-galactosidase was previously dialyzed twice in 2L of 30mM NaOAc, pH 5.2 for 12h at 4ºC. The digested sample was passed sequentially over a RP C18 column and a graphitized carbon cartridge before elution with 30% acetonitrile with 0.01% TFA and dried in a speed-vac to generate degalactosylated (agalacto)-glycopeptides. Complete degalactosylation was confirmed by MALDI-TOF-MS profiling as previously described above. From these agalacto-glycopeptides, the LDN-glycopeptides were generated using recombinant *β*1-4 N-acetylgalactosaminyltransferase (β1-4GalNAcT) from *Caenorhabditis elegans*, based on previous methods[19]. Briefly, 50ml medium containing *β*1-4GalNAcT was centrifuged at 1,500xg for 5min to remove cellular debris and incubated with HPC4-UltraLink beads (5mg of HPC4 antibody/ml of beads; 0.1µl of beads/ml of medium) for 1h at room temperature on a rotating platform. The beads were collected by centrifugation at 600xg for 3min and washed three times with 10ml of 100mM sodium cacodylate buffer, pH 7.0 with addition of 2mM $CaCl_2$. The beads were resuspended in the same buffer with the addition of 20mM $MnCl_2$ and used as the enzyme source. Ten mg of agalacto-glycopeptide was incubated with the beads in 100mM sodium cacodylate buffer, pH 7.0 containing 2mM of UDP-GalNAc, 20mM $MnCl_2$. and 3 units of calf intestinal alkaline phosphatase in a total volume of 10ml. The mixture was incubated for 48h at room temperature with slow rotation. After incubation, supernatant was recovered by centrifugation at 600xg for 3min and passed sequentially over a RP C18 column and a graphitized carbon cartridge before elution with 30% acetonitrile with 0.01% TFA and dried in a speed-vac to generate LDN-glycopeptide. LDNF was generated from LDN by the addition of fucose using GDP-Fucose as sugar

donor and recombinant human α1-3 Fucosyltransferase VI (FucTVI) as the enzyme based on previous method[20]. The preparation of FucTVI from medium containing the enzyme and its subsequent immobilization on beads followed the procedure used to prepare β1-4GalNAcT except that no $MnCl_2$ was added. Ten mg of LDN-glycopeptides were then incubated with the beads in 100mM sodium cacodylate buffer, pH 7.0 containing 50mM of GDP-Fucose, and 3 units of calf intestinal alkaline phosphatase. The mixture was incubated for 48h at room temperature with slow rotation. Every 12h, fresh 50mM GDP-Fucose was added and incubation continued. After incubation, LDNF-glycopeptides were purified from the supernatant in a similar way as LDN-glycopeptides. To generate Man3-glycopeptides, 15mg of agalacto-glycopeptides were treated with 150µl (10 units) hexosaminidase in 5ml of 30mM NaOAc, pH 5.2. The digest was transferred into 500 MW-cutoff Float-A-lyzer dialysis tubing and digestion continued for 12h while dialyzing against 30mM NaOAc buffer, pH 5.2 at room temperature. The Man3-glycopepetide product was purified in the same way as the other glycopeptides above. Core xylose/core fucose and core xylose N-linked biantennary glycopeptides were synthesized from horseradish peroxidase, Type VI. This glycoprotein exclusively contains biantennary N-glycans with an α1-3 core fucose and a β1-2 xylose[21, 22]. Briefly, 100mg of Pronase was used to digest 1g horseradish peroxidase, Type VI, in 100ml of 1X pronase buffer. The glycopeptides were then sequentially purified in the same way as the sialylated or asialo-glycopeptides above. Core xylose-glycopeptides were chemically synthesized from core xylose/core fucose glycopeptides by incubation with 25mM sulfuric acid at 80ºC for 30min. The sample was neutralized with 50mM NaOH, desalted over graphitized carbon cartridge, and eluted with 3 column volumes of 30% acetonitrile with 0.01% TFA and dried in a speed vac to isolate the core xylose-glycopeptides. Fucosylated Man3 was prepared from Bermuda grass pollen which contains IgE-binding proteins carrying N-linked glycans

with *α*1-3 core fucose[23]. Briefly, crude extract was prepared by extracting 5g defatted pollen with 1X PBS for 16h at 4ºC with constant stirring. The extract was then filtered through a Whatman No. 1 filter and the eluent centrifuged at 800xg for 45 minutes at 4ºC. The supernatant was then dialyzed at 4ºC against 1X pronase buffer. The dialyzed sample was then lyophilized, dissolved in 100ml of 1X pronase buffer and digested with 100mg of Pronase overnight at room temperature. The fucosylated Man3 glycopeptides were then sequentially purified in the same way as the sialylated or asialo-glycopeptides above.

Defined schistosome glycan array preparation and analysis

The printing of glycopeptides to generate a defined schistosome glycan array on *N*-hydroxysuccinamide (NHS)-activated slides purchased from Schott was carried out at two different concentrations, 0.4mg/ml and 0.2mg/ml using a Piezorray printer from Perkin Elmer following a previous procedure[24]. Briefly, all the samples were printed in phosphate buffer (300mM sodium phosphates, pH 8.5). The average spot volume was within 10% variation of 1/3nl. After printing, the slides were boxed loosely and put in a high moisture chamber at 50^{o}C and incubated for 1h and then washed and blocked with 50mM ethanolamine in 0.1M Tris buffer (pH 9.0) for 1h. The slides were then dried by centrifugation and used immediately or stored desiccated at -20^{o}C. Before using, the slides were rehydrated for 5 minutes in TSM buffer (20mM Tris-HCL, 150mM sodium chloride (NaCl), 0.2mM calcium chloride ($CaCl_2$), and 0.2mM magnesium chloride ($MgCl_2$)). To validate the printing, biotinylated lectins were used in a binding assay. 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 applied on 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 covering an excitation range from 488nm to 637nm. The scanned images were analyzed with the ScanArray Express software. Detection of bound biotinylated lectins were carried out by incubation with cyanine5-streptavidin. To investigate specific humoral responses, all sera were analysed at 1:100 and/or 1:000 dilutions. Detection of bound mouse sera antibodies was carried out by incubation with Alexa568 labeled goat anti-mouse IgG and Alexa488 labeled goat anti-mouse IgM. For cyanine-5 fluorescence, 649nm (Ex) and 670nm (Em) were used. For Alexa488, 495nm (Ex) and 519nm (Em) were used. For Alexa568, 579nm (Ex) and 604nm (Em) were used. All images obtained from the scanner were in grayscale and colored for easy discrimination.

CFG Glycan array preparation and analysis

Glycan microarrays were prepared as described previously[25]. Briefly, aminoalkyl glycosides were covalently coupled to N-succinimidyl-activated glass slides (1 inch by 3 inch) in a sodium phosphate buffer, pH 8.5, 0.005% Tween 20. Slides were immersed in 50mM ethanolamine, 50mM sodium borate, pH 9.0, for 1h, rinsed with water, dried under a stream of microfiltered air and stored in a desiccator until use. For sera recognition of glycans on the printed glycan microarray, dilutions of 1:100 for mice and 1:1000 for monkeys and humans were performed in TSM Binding Buffer containing 1% BSA and 0.05% Tween 20 for 1h at room temperature in a dark humid chamber. The slide was washed by successive immersion in TSM containing 0.05% Tween 20 (4 times), TSM (4 times), and then incubated with fluorescently-labeled appropriate secondary antibody (anti-mouse IgG or IgM, or anti-human IgG or IgM). After 1h at room temperature in a dark humid chamber, the slide was washed by successive immersion in TSM containing 0.05% Tween 20 (4 times), TSM (4 times), and water (4 times). The slide was dried by microcentrifugation. An image of bound fluorescence was obtained

using a microarray scanner (ScanArray Express, PerkinElmer Lifer Sciences). The integrated spot intensities were determined using Imagene software (BioDiscovery).

In vitro killing of schistosomula

Schistosoma mansoni (Puerto Rican strain) cercariae were generated according to published protocol with slight alteration[26]. Briefly, schistosome-infected *Biomphalaria glabrata* snails were kept in the dark for two days, and then exposed to light for 2h to cause shedding of cercariae. The free swimming cercariae were filtered through a 100µm sieve to remove snail and aquarium debris, chilled on ice for 30 min, and centrifuged at 500xg (swinging bucket) for 10min at 4°C. The cercarial pellet was suspended in DMEM buffer containing penicillin and streptomycin to prepare *in vitro* transformed schistosomula. Transformation to schistosomula was carried out by vortexing at top speed to effect the dislocation of cercarial tails. The schistosomula bodies were separated from cercarial tails by centrifugation on a 70% Percoll gradient at 1100xg. Schistosomula were recovered from the pellet fraction and washed twice with DMEM containing penicillin and streptomycin. The schistosomula were subsequently cultured in flat-bottomed microtiter plates (200-250 schistosomula/well) in 90µL DMEM containing 10% fetal bovine serum, penicillin, and streptomycin for 3h at 37 °C in a humidified atmosphere with 5% CO_2. To these 3h transformed schistosomula, 10µL of rhesus monkey, human or mice sera was added and the plates reincubated at the same conditions above. After 48h of incubation, schistosomula killing was assessed by two independent methods. Firstly, gross anatomy observation of the schistosomula bodies including rotund appearance, outer membrane blebbing, and protrusion of the acetabular gland, and lack of movement were used as indicators of death. Secondly, uptake of fluorescent propidium iodide (PI), at 10mg/ml was used to confirm death of schistosomula.

2.4 Results

Generation of the defined schistosome array and validation of binding specificities

We have successfully generated a defined schistosome glycan array using glycopeptides synthesized in the laboratory and printed them on NHS-activated microarray slides (**Fig.2-1**).

Fig.1

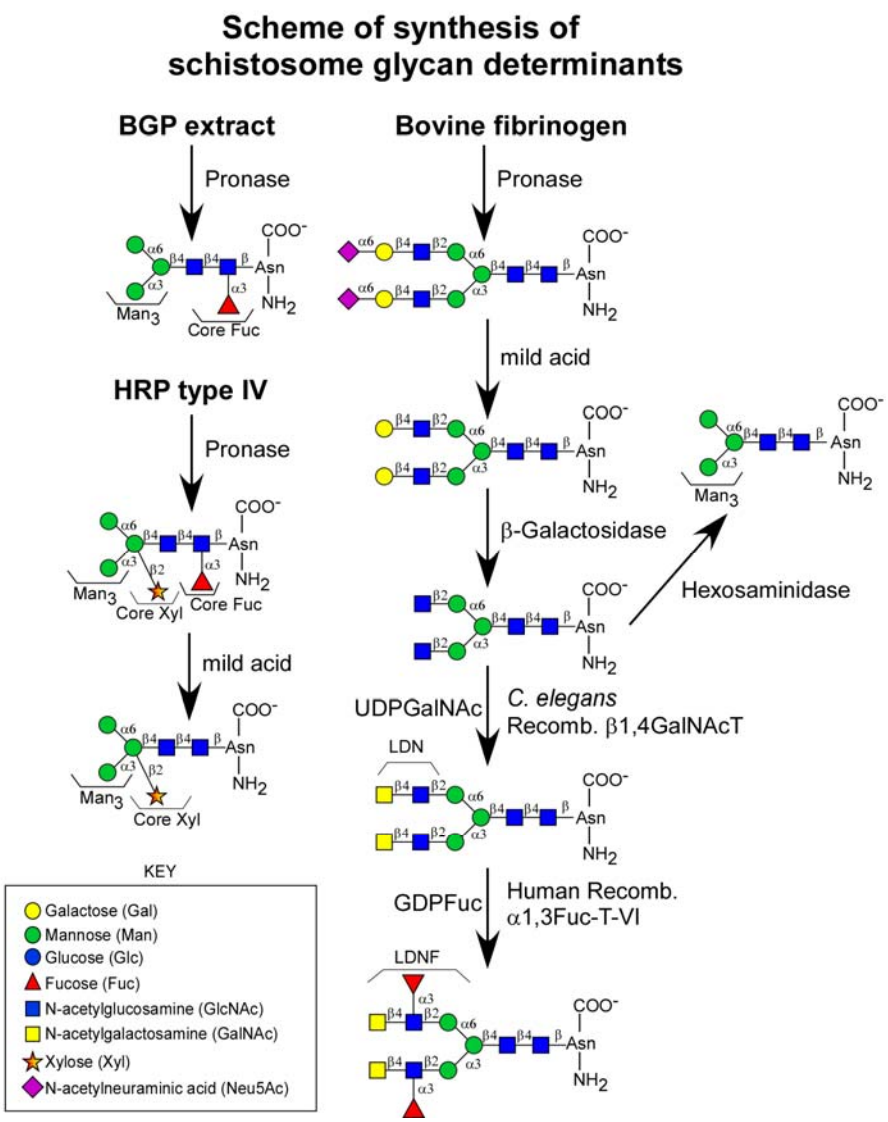


Fig.2-1. Synthesis of glycan epitopes

All the glycopeptides printed on the defined schistosome array were synthesized by a combination of chemical and enzymatic methods using natural starting products as shown in the schematic. The schistosome specific epitopes are marked by a 'boat' shaped bracket and named, e.g Man3, LDN, etc. MALDI-TOF-MS analysis was performed on the 9 glycopeptides that were generated, including 6 schistosome specific glycan epitopes and 3 control glycopeptides. The masses of the glycopeptide constructs were as expected (**Fig.2-2**). The glycans/glycopeptides that were printed on the defined schistosome array are listed in **Table 2-1**.

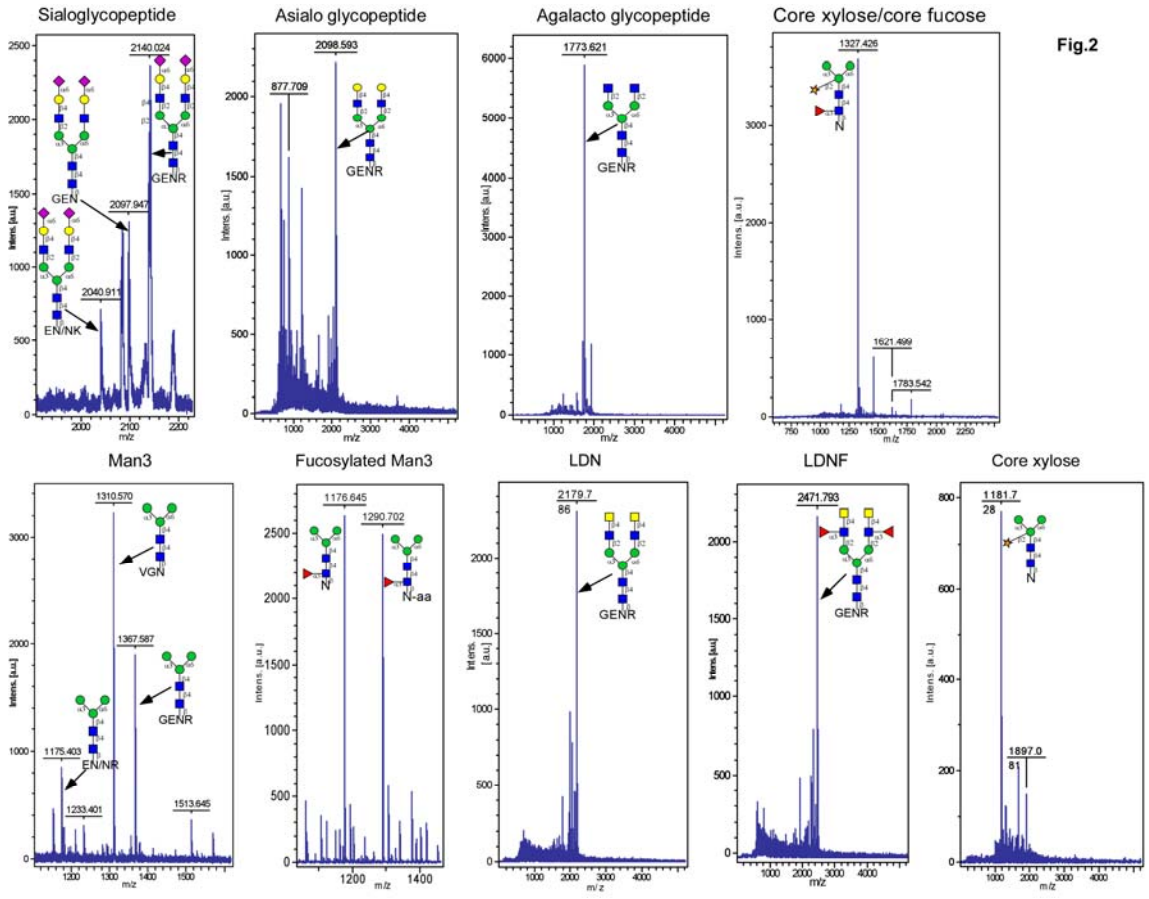


Fig.2-2. MALDI-TOF-MS Profiles of glycopeptides.

Chart ID	Detail
1	Agalacto Biantennary glycopeptide-0.4 mg/ml
2	Agalacto Biantennary glycopeptide-0.2 mg/ml
3	Asialo biantennary glycopeptide-0.4 mg/ml
4	Asialo biantennary glycopeptide-0.2 mg/ml
5	Sialylated biantennary glycopeptide-0.4 mg/ml
6	Sialylated biantennary glycopeptide-0.2 mg/ml
7	Man3 biantennary glycopeptide-0.4 mg/ml
8	Man3 biantennary glycopeptide-0.2 mg/ml
9	Core-Xylose/core-fucose biantennary glycopeptide-0.4 mg/ml
10	Core-Xylose/core-fucose biantennary glycopeptide-0.2 mg/ml
11	Core-Xylose biantennary glycopeptide-0.4 mg/ml
12	Core-Xylose biantennary glycopeptide-0.2 mg/ml
13	LDN biantennary glycopeptide-0.4 mg/ml
14	LDN biantennary glycopeptide-0.2 mg/ml
15	LDN biantennary glycopeptide-0.4 mg/ml
16	LDN biantennary glycopeptide-0.2 mg/ml
17	Lewis a- 100μM
18	Lewis x- 100μM
19	PBS
20	Biotin
21	Fucosylated Man3-0.4 mg/ml
22	Fucosylated Man3-0.2 mg/ml
23	NA2-100μM PBS
24	Man5-100μM Biotin

Table 2-1. List of glycans/glycopeptides on the defined schistosome array.

To validate the printing efficiency and specificity, lectins of varying binding specificities were used in a binding assay. The lectins bound to the different glycopeptides with the expected specificities (**Fig.3A**). For further validation, a monoclonal antibody to LDN glycopeptide showed specific binding to LDN printed spots on the array (**Fig.3B**).

Fig.3A

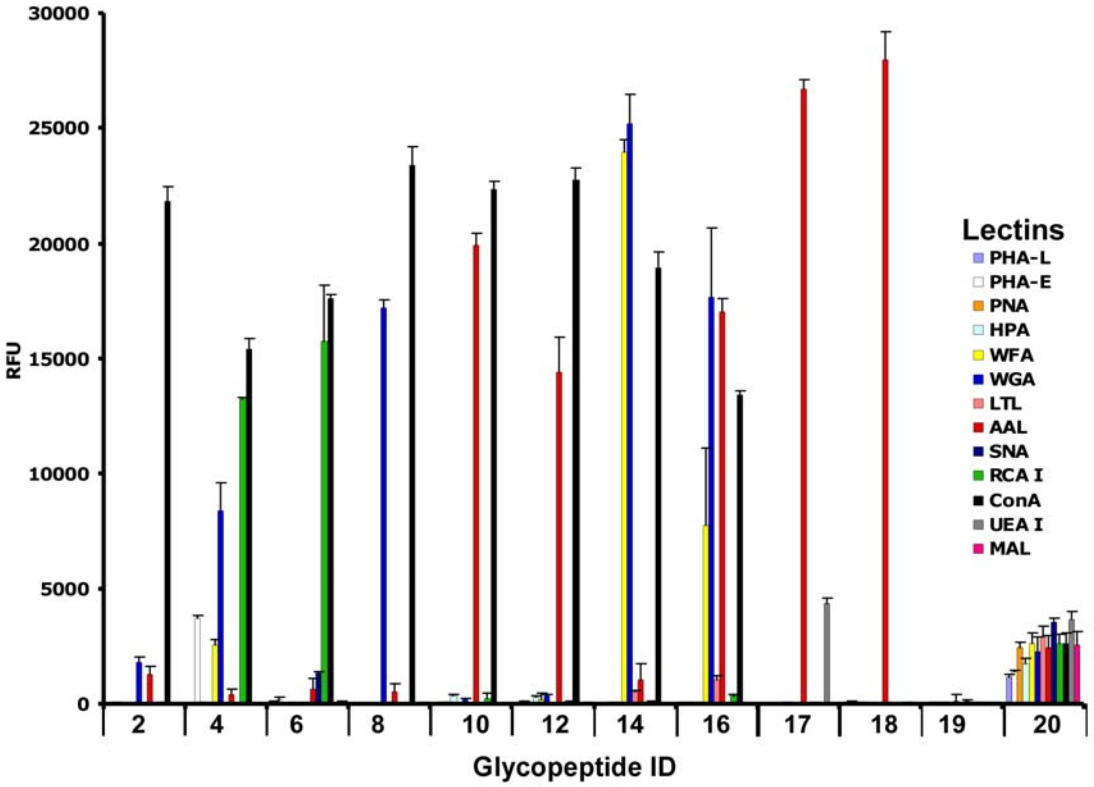


Fig.3B

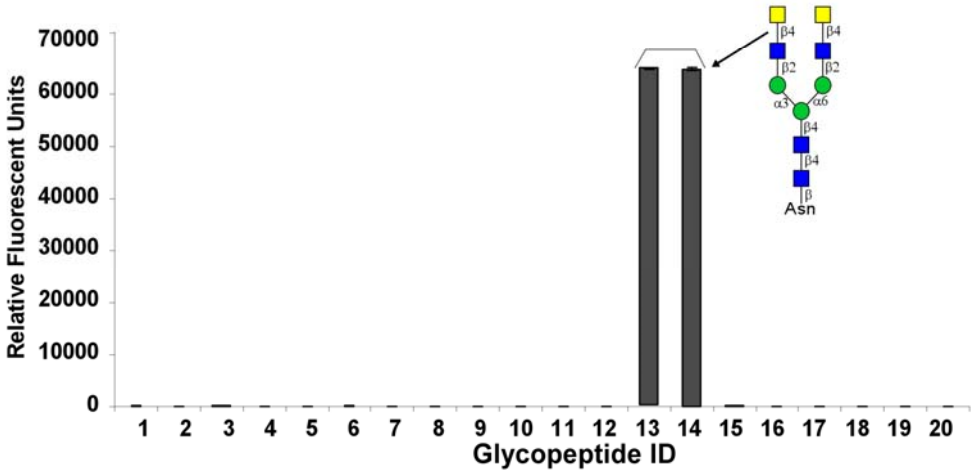


Fig.2-3. Glycan binding specificity on the defined schistosome array.

Fig.2-3. Glycan binding specificity on the defined schistosome array

Biotinylated Lectins (0.1 µg/ml) (**A**) and IgM monoclonal antibody to LDN (**B**) were used to interrogate defined schistosome glycopeptides binding specificities. After incubation with the glycan microarray slides, detection of bound biotinylated lectins was performed with cyanine5-streptavidin (**A).** PHA-L, *Phaseolus vulgaris* agglutinin L; PHA-E, *Phaseolus vulgaris* agglutinin E; PNA, Peanut agglutinin; HPA, *Helix pomatia* 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; UEA I, *Ulex europaeus* I; MAL, *Maackia amurensis* lectin. (**B)** Detection of bound IgM monoclonal antibody to LDN was detected with Alexa488-labeled goat anti-mouse IgM secondary antibody. ID numbers on x-axes correspond to glycopeptides listed in **Table 2-1**.

Analysis of infected monkey sera

We investigated humoral immune response to schistosome glycan antigens in four rhesus monkeys chronically infected with *S. mansoni*, a trematode parasite that causes schistosomiasis in permissive hosts. The results obtained show that monkeys chronically infected with this parasite responded strongly to core xylose/core fucose glycan antigen on the defined schistosome array when their sera was diluted 1:1000 (**Fig.4A-E**). When the core xylose/core fucose epitope was defucosylated by mild acid hydrolysis, anti-IgG responses to this new epitope (core xylose) were abrogated (**Fig.4C-E,** glycopeptides #s 9 and 10 versus 11 and 12). Interestingly, for all the four monkeys, these anti-core xylose/core fucose responses disappeared at 1½ years post infection at which point all the monkeys were recorded to be free of the infection (**Fig.4F**) From 6 to 11 weeks after infection, sera from all the four monkey showed very high levels of anti-core xylose/core fucose IgGs on this array (**Fig.4G**). On a different version of the array pooled sera used at 1:1000 from all the 4 monkeys, responded to fucosylated man3, a glycopeptide lacking core xylose (**Fig.4H**).

Fig.4

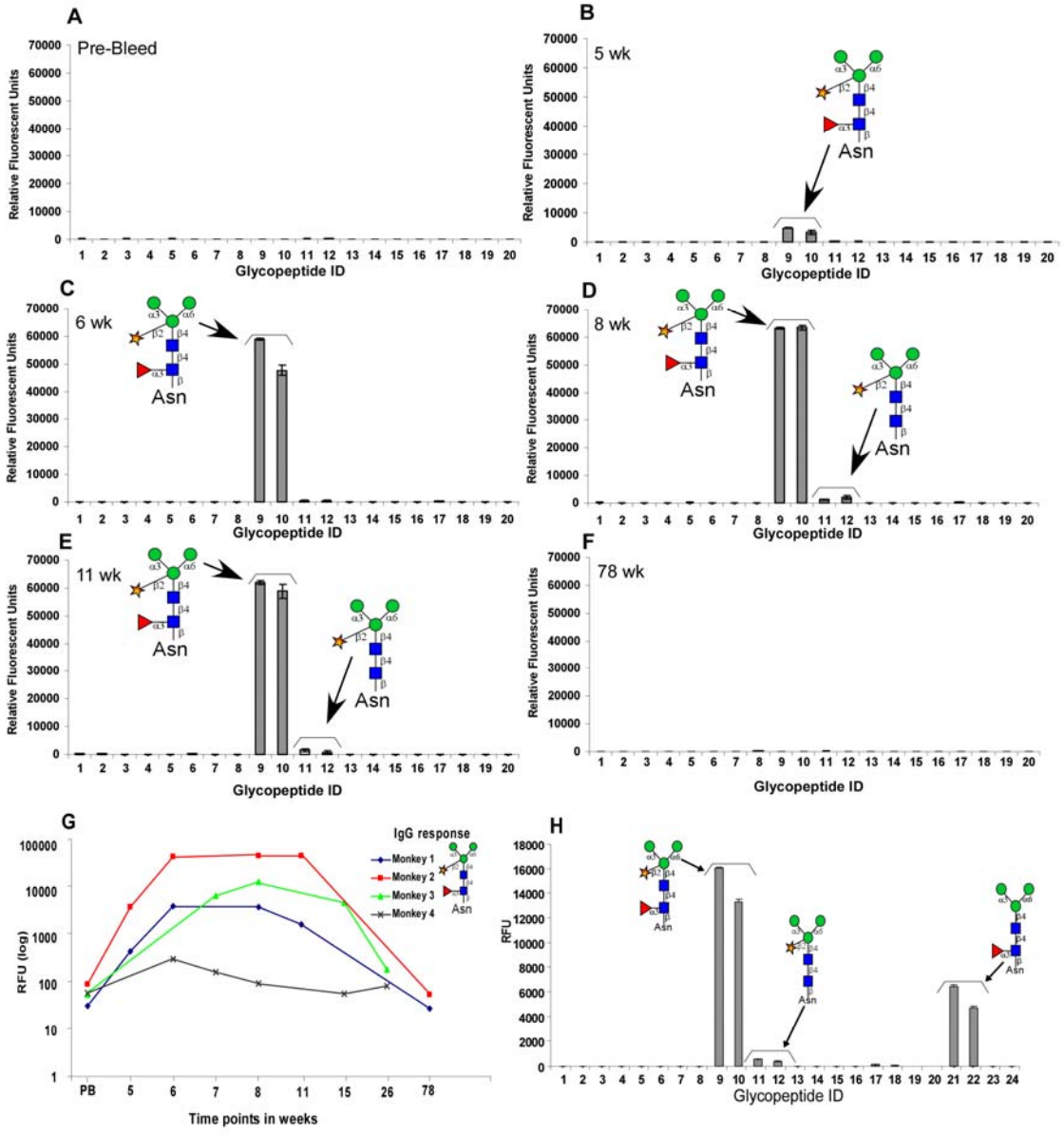


Fig.2-4. Infected Rhesus monkeys predominantly generate IgG to core xylose/core fucose epitope on the defined schistosome array.

Fig.2-4. Infected Rhesus monkeys predominantly generate IgG to core xylose/core fucose epitope on the defined schistosome array. Pre-bleed (**A**) and schistosome infected rhesus monkey sera at various time points (**B-F**) were used to probe the printed defined schistosome array. The result shows data from one monkey sera serving as representative of the four rhesus monkeys sera tested. Binding of infected monkey sera was specifically directed to core xylose/core fucose glycan epitope (**B-E**, ID# 9,10). Binding was abrogated when this glycopeptide was defucosylated by mild acid hydrolysis (**B-E**, ID# 11,12). (**G**) Binding of pre-bleed and schistosome infected rhesus monkey sera at various time points (weeks) from four different monkeys to the core xylose/core fucose epitope (ID# 9,10) plotted on a log scale. (**H**) Binding of 8-week infected monkey sera on the defined schistosome array bearing an additional fucosylated Man3 epitope (ID# 21,22). Alexa568-labeled goat anti-mouse IgG was used to detect antibody binding. ID numbers on x-axes correspond to glycopeptides listed in **Table 2-1**.

Although anti-core xylose/core fucose IgM responses were also observed, their titers were much lower compared to IgG titers (data not shown). This trend was observed in all monkeys. Pooled sera from these infected monkeys used at 1:10 dilution was shown to be cytolytic to 3h old mechanically transformed schistosomula *in vitro* after 48h incubation at 37°C. Sera from 8- and 11-week infected monkeys, which were shown to have the highest anti-core xylose/core fucose responses on the defined schistosome array had the highest schistosomula killing effects (**Fig.5E-H**). The schistosomula killing effect observed with 5 week infected sera mirrored that observed with 1½ year infected monkey sera as both had minimal killing effects (**Fig.5D,J**) as revealed by lack of PI staining of the schistosomula.

Fig. 5

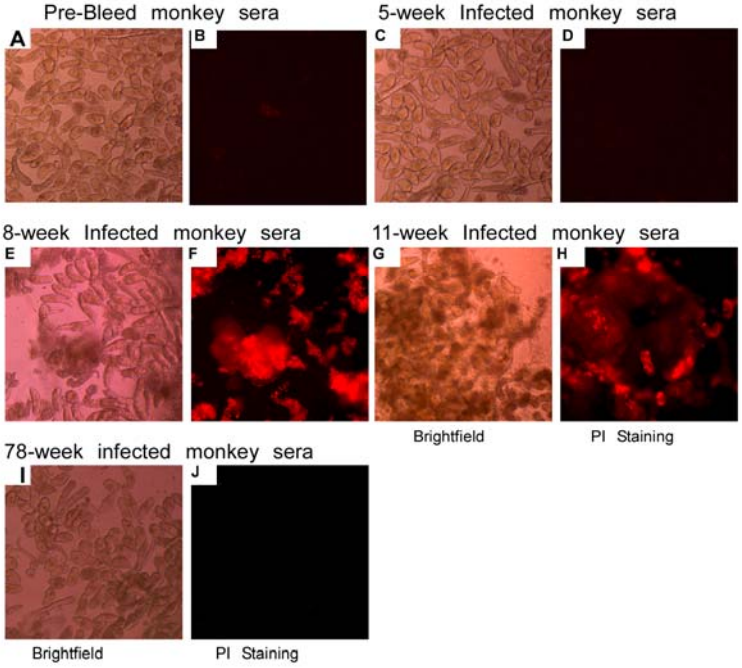


Fig.6

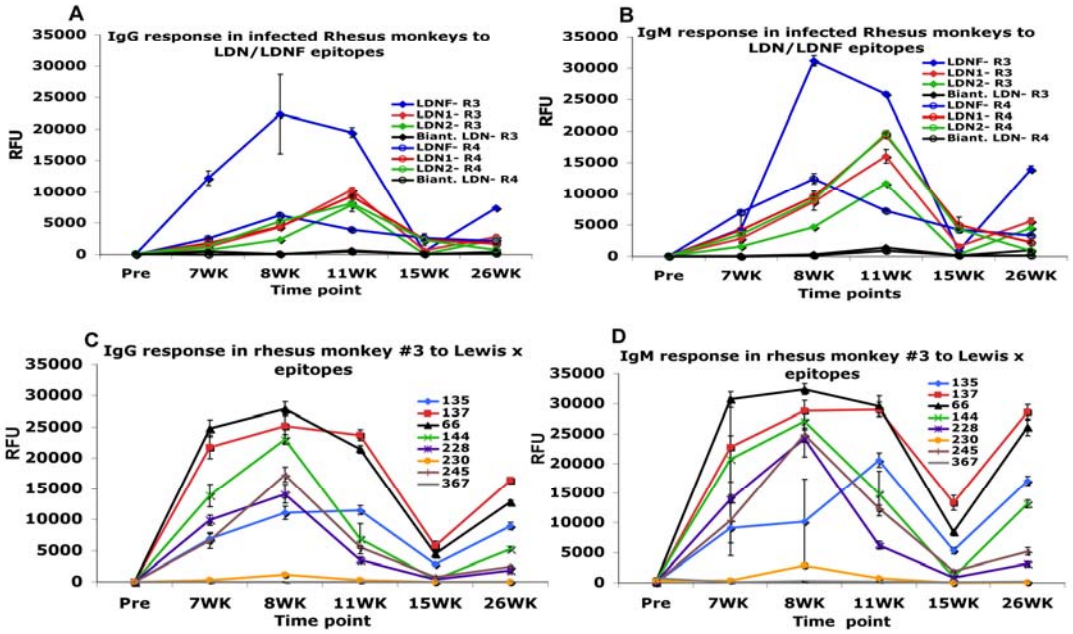


Fig.2-5. Infected rhesus monkey sera is cytolytic to 3h-old schistosomula *in vitro.*

Fig.2-6. Rhesus monkeys generate IgG and IgM antibodies to LDN, LDNF, and Le^x during the course of infection with *S. mansoni.*

Fig.2-5. Infected rhesus monkey sera is cytolytic to 3h-old schistosomula *in vitro.* Mechanically transformed schistosomula were treated with pooled sera from *S. mansoni* infected rhesus monkeys at various time points (**A-J)**. Death of schistosomula was recorded by gross anatomy observation (Brightfield) and fluorescent propidium iodide (PI) staining. Killing was observed as clumping, rotund body shape, protrusions of acetabular gland, and PI uptake in 8-week (**E, F**) and 11-week (**G, H**) infected sera.

Fig.2-6. Rhesus monkeys generate IgG and IgM antibodies to LDN, LDNF, and Le^x during the course of infection with *S. mansoni.* Four rhesus monkeys were infected with *S. mansoni* and their humoral immune responses analyzed over a period of 26 weeks on the CFG glycan array. Data shown is from two representative monkeys (R3 and R4). The monkeys generated anti-LDN and -LDNF IgGs (**A**)and IgMs (**B**) with maximum levels observed at 11 weeks after infection. Responses to various Le^x antigens were highest at 8 week after infection (**C**) IgG anf (**D**) IgM.

	Propidium Iodide (PI) staining			Gross Anatomy observation		
Treatment	**Total # somules (48 hrs)**	**Total # dead somules**	**% dead**	**Total # somules (48 hrs)**	**Total # dead somules**	**% dead**
Pre-bleed	150	2	**1.3**	150	1	**0.6**
5-week	120	1	**0.8**	120	2	**1.7**
8-week	139	130	**93.5**	139	132	**95**
11-week	135	135	**100**	135	135	**100**
78-week	125	1	**0.8**	125	2	**1.6**

Table 2-2. Quantitation of cytolysis by monkey sera.

Table 2-2. Quantitation of cytolysis by monkey sera. Data represents the average of 3 different fields measured in one experiment, and experiment is representative of 2 replicates.

In a parallel study, sera from these four monkeys were used to interrogate anti-glycan responses to various glycan epitopes printed on the CFG array. This array does not have glycans with core xylose/core fucose nor core fucose epitopes. On this array, and all printed glycan targets are straight-chain saccharides with varying -CH_2 spacer arms. Data from monkey #3 sera is shown as representative of the four monkeys sera. Results obtained on this array show that in the four monkeys, anti-LDN and -LDNF antibodies (IgG and IgM) levels were highest at 11 weeks after infection. These anti-LDNF and anti-LDN antibody levels started tapering off at the 11th week and were lowest after the 15th week after infection. At the 26th week, the anti-LDN and -LDNF responses were similar to those observed at the 7th week after infection (**Fig.6A,B**). Additionally, during the course of infection, monkeys responded actively to glycans terminating with Lewis X (Le^x) and poly-Le^x antigens. Representative data from monkeys 3 and 4 are shown. The anti-Le^x responses are highest at 8 weeks after infection in all monkeys. These anti-Le^x responses start increasing again 15 weeks after infection. In the four monkeys, the anti-Le^x antibody levels at 26 weeks are just below those observed at 11 weeks after infection (**Fig.6C,D**). This increase in anti-Le^x antibodies also coincides with spontaneous cure of the infection.

Analysis of infected human sera

Analysis of infected human sera on our defined schistosome array showed predominantly IgG responses to both core xylose/core fucose and core xylose epitopes, with the responses being higher before than after treatment with Praziquantel (**Fig.7A,B**). The data shown is from one human subject (# 42) and is representative of the five patients' sera samples analyzed used at a 1:100 dilution. Although the times in which these human subjects were infected are not known, the trends of their anti-glycan responses are consistent. Results from the CFG array shows that infected humans

have relatively higher levels of anti-LDNF and -LDN antibodies (IgG and IgM) before treatment. After treatment, these antibody responses generally dropped (**Fig.7C,D**). This trend is seen in all of the human sera samples analyzed. The levels of anti-Le^x antibodies (IgG and IgM) are low in all infected humans before and after treatment (**Fig.7E,F**). Compared to normal human sera, pooled sera from newly infected humans (1 to 2 years of living in schistosome endemic regions in Africa) used at a 1:100 dilution showed anti-glycan responses to core xylose/core fucose, core xylose, and to fucosylated Man3 which were predominantly of the IgG class (**Fig.7G,H**). Pooled sera from infected humans before and after treatment did not have a schistosomula killing effect *in vitro* (**Fig.8A-D**). Likewise, pooled sera from newly infected humans did not have any cytolytic effect to schistosomula (**Fig 8E-H**).

Fig.7

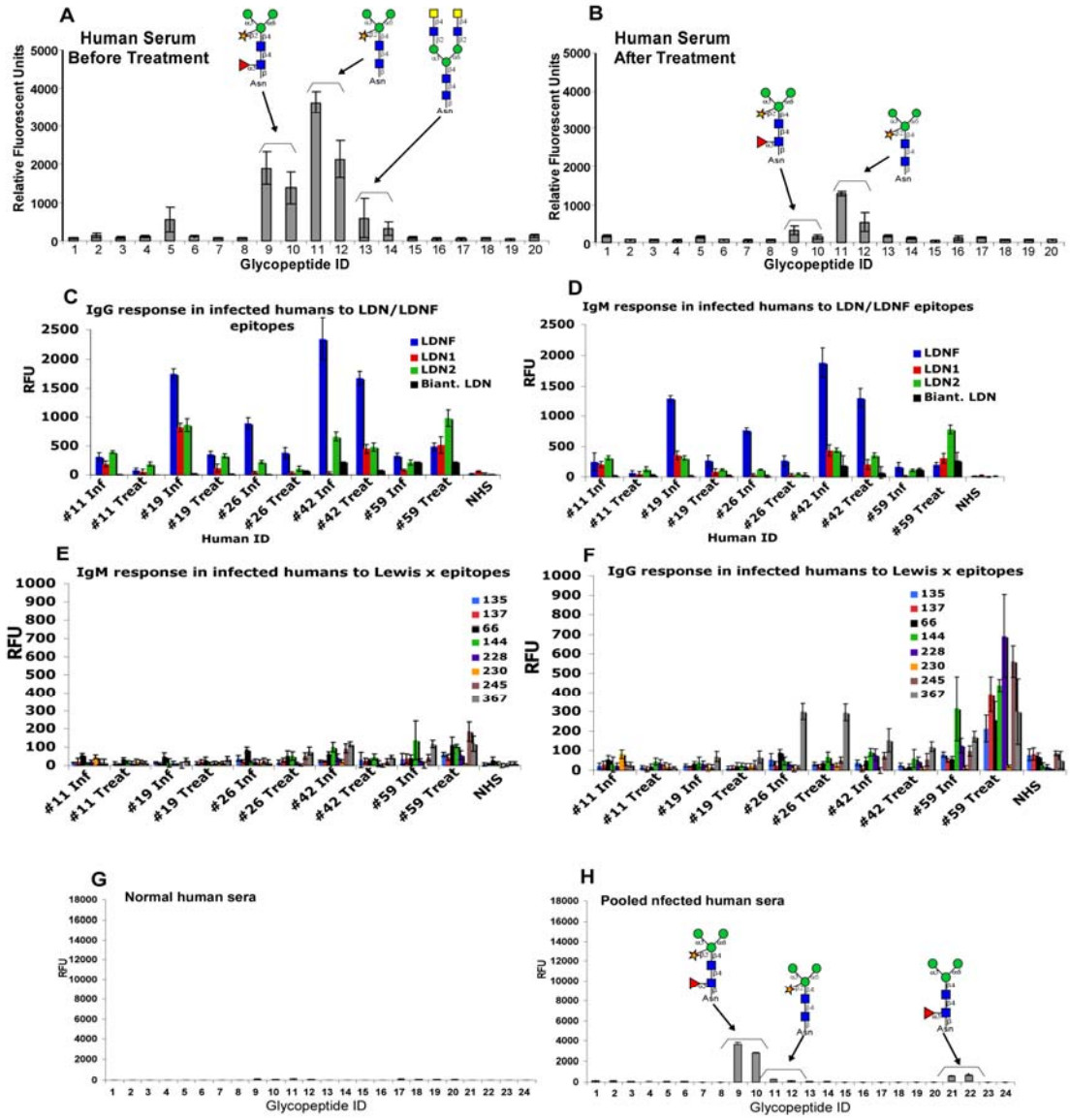


Fig.2-7. Antibody responses in infected humans.

Fig.2-7. Antibody responses in infected humans. Sera from infected humans before (**A**) and after (**B**) treatment with Praziquantel were used to probe the printed defined schistosome glycan array. The data is from human # 42 sera serving as representative of all 5 human sera samples. Five Human sera samples before and after treatment were used to probe a CFG glycan array. IgG (**C**) and IgM (**D**) responses to LDN and LDNF. IgG (**E**) and IgM (**E**) responses to various Le^x antigens. Binding of normal (**G**) and pooled newly infected (**H**) human sera on the defined schistosome array bearing an additional fucosylated Man3 epitope (ID# 21,22).

Fig.8

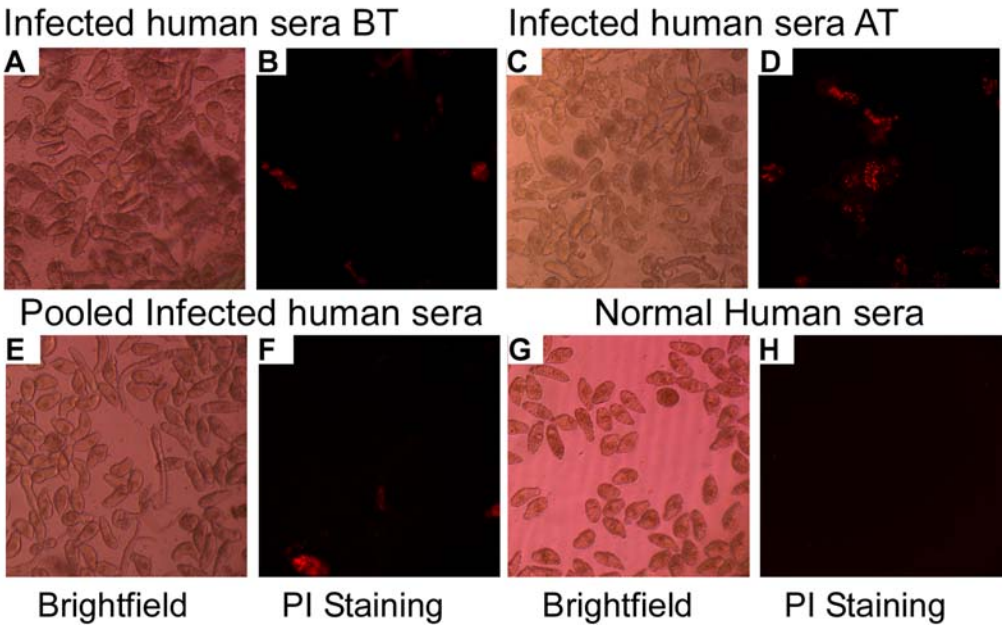


Fig.2-8. Infected human sera is not cytolytic to schistosomula *in vitro.*

Fig.2-8. Infected human sera is not cytolytic to schistosomula *in vitro.* Mechanically transformed schistosomula were treated with pooled sera from *S. mansoni* infected humans before and after treatment. Death of schistosomula was recorded by gross anatomy observation (Brightfield) and fluorescent propidium iodide (PI) staining. (**A – H)** No killing was observed with any of the sera.

	Propidium Iodide (PI) staining			Gross Anatomy observation		
Treatment	**Total # somules (48 hrs)**	**Total # dead somules**	**% dead**	**Total # somules (48 hrs)**	**Total # dead somules**	**% dead**
NHS	120	0	**0**	120	1	**0.8**
Inf. HS BT	122	3	**2.5**	122	5	**4.0**
Inf. HS AT	125	3	**2.4**	125	4	**3.2**
Pooled Inf. HS	120	1	**0.8**	120	4	**3.3**
NHS	120	0	**0**	120	1	**0.8**

Table 2-3. Quantitation of cytolysis by human sera.

Table 2-3. Quantitation of cytolysis by human sera. NHS = normal human sera, Inf. = infected, BT = before treatment, AT = after treatment. Data represents the average of 3 different fields measured in one experiment, and experiment is representative of 2 replicates.

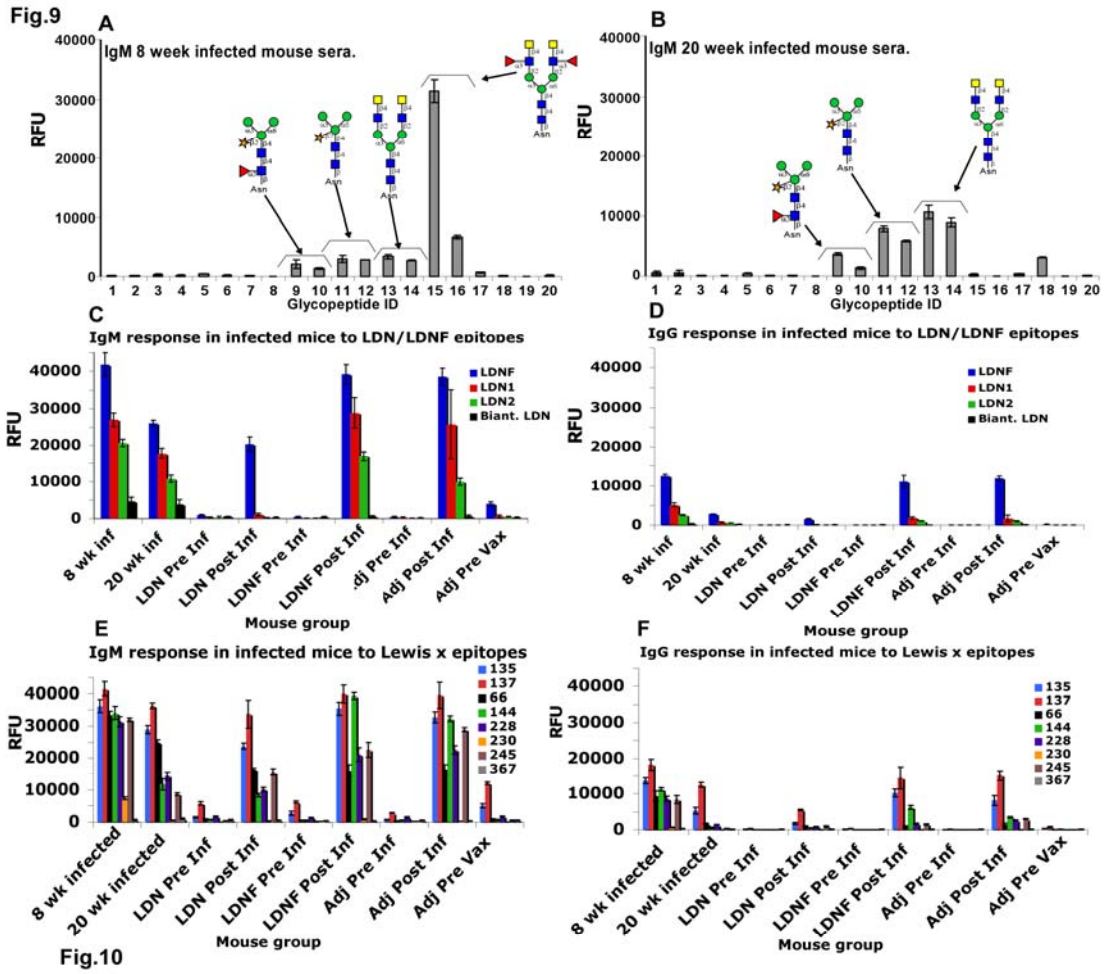
Glycan #	
135	**Galb1-4(Fuca1-3)GlcNAc**b-Sp8
137	**Galb1-4(Fuca1-3)GlcNAc**b1-4**Galb1-4(Fuca1-3)GlcNAc**b1-4**Galb1-4(Fuca1-3)GlcNAc**b-Sp0
66	Fuca1-2**Galb1-4(Fuca1-3)GlcNAc**b1-3**Galb1-4(Fuca1-3)GlcNAc**b1-3**Galb1-4(Fuca1-3)GlcNAc**b-Sp0
144	Galb1-4GlcNAcb1-3**Galb1-4(Fuca1-3)GlcNAc**b1-3**Galb1-4(Fuca1-3)GlcNAc**b-Sp0
228	Neu5Aca2-3**Galb1-4(Fuca1-3)GlcNAc**b1-3**Galb1-4(Fuca1-3)GlcNAc**b1-3**Galb1-4(Fuca1-3)GlcNAc**b-Sp0
230	Neu5Aca2-3**Galb1-4(Fuca1-3)GlcNAc**b-Sp8
245	Neu5Aca2-6Galb1-4GlcNAcb1-3**Galb1-4(Fuca1-3)GlcNAc**b1-3**Galb1-4(Fuca1-3)GlcNAc**b-Sp0
367	Gala1-3**Galb1-4(Fuca1-3)GlcNAc**b1-2Mana1-3(Gala1-3**Galb1-4(Fuca1-3)GlcNAc**b1-2Mana1-6)Manb1-4GlcNAcb1-4GlcNAcb-Sp20

Table 2-4. Glycans containing Lewis X epitopes.

Table 2-4. Glycans containing Lewis X epitopes. Glycan #135-terminal Lewis X, #137- terminal and poly-Lewis X, #66 and #144- internal poly-Lewis X, #228 and #245-sialylated poly-Lewis X, #230- sialylated Lewis X, #367- Lewis X on biantennary glycan structure.

Analysis of infected mice sera

Mice infected with schistosomiasis predominantly generated IgM antibodies to LDNF glycopeptides 8 weeks after infection on the defined schistosome array (**Fig.9A**). Sera from 20 week infected mice showed no response to this epitope (**Fig.9B**). Similarly, on the CFG array, 8 week infected mice sera had higher IgM antibody titer to LDNF glycan epitope than 20 week infected sera. In these mice, IgG responses to the LDNF epitope were low. All infected mice generally generated higher anti-LDNF than anti-LDN IgM antibodies (**Fig.9C,D**). The anti-LDN IgM responses towards straight chain LDN were many fold higher than those towards biantennary LDN on the CFG array (**Fig.9C,D**). In infected mice, anti-Le^x responses (terminating Le^x and poly-Le^x) were higher at 8 weeks than 20 weeks after infection. Anti-Le^x IgM responses were generally two or more fold higher than IgG responses. The humoral immune response in mice towards terminating Le^x epitopes was just as high as those towards poly-Le^x epitopes as portrayed by both anti-Le^x IgM and IgG levels (**Fig.9E,F)**. Neither normal nor culture media alone had any cytolytic effect to 3h-old mechanically transformed schistosomula *in vitro* (Fig. 10 A-D). However, 20 week infected mouse sera had a higher killing effect than 8 week sera (**Fig.10E-H**).



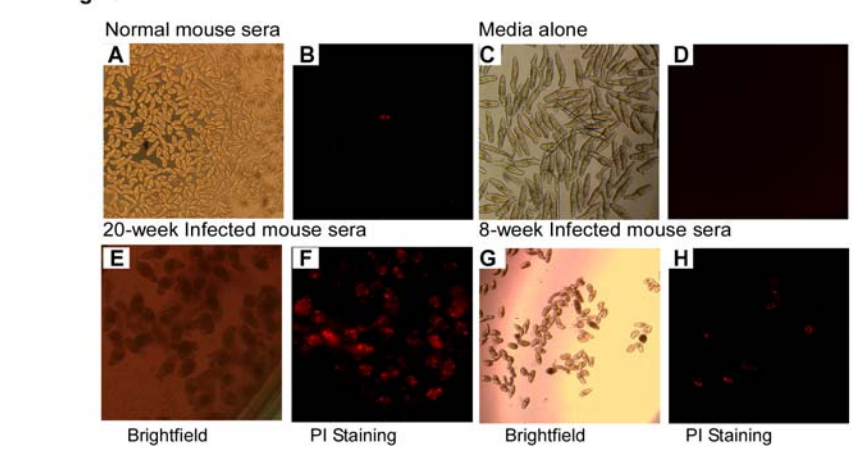


Fig.2-9. Antibody responses in infected mice.

Fig.2-10. Infected mice sera is cytolytic to schistosomula *in vitro.*

Fig.2-9. Antibody responses in infected mice. Sera from infected mice was used to probe the defined schistosome glycan array Alexa488-labeled goat anti-mouse IgM or IgG was used to detect antibody binding. (**A**) Humoral responses in 8-week infected mice sera were predominantly IgM to LDNF epitope (ID #s 15, 16). (**B**) Response in 20-week infected sera. The same infected mice sera were used to probe a CFG array. Other two groups of mice were vaccinated with BSA- LDN or BSA-LDNF and bled before and 8 weeks after infection. Another group of mice received only the adjuvant used in the immunization and served as a control. Sera from these mice were used to probe a CFG array. Alexa488-labeled goat anti-mouse IgM or IgG was used to detect antibody binding to the glycan epitopes. IgM (**C**) and IgG (**D**) responses to LDNF and various LDN epitopes. IgM (**E**) and IgG (**F**) responses to various Le^x antigens.

Fig.2-10. Infected mice sera is cytolytic to schistosomula *in vitro.* Mechanically transformed schistosomula were treated with sera from *S. mansoni* infected mice collected 8 weeks or 20 weeks after infection. Death of schistosomula was recorded by gross anatomy observation (Brightfield) and fluorescent propidium iodide (PI) staining. (**A -D)** No killing was observed with normal mice sera or culture media. Most Killing was observed with 20-week (**E,F**) than with 8-week (**G-H**) infected sera.

	Propidium Iodide (PI) staining			Gross Anatomy observation		
Treatment	**Total # somules (48 hrs)**	**Total # dead somules**	**% dead**	**Total # somules (48 hrs)**	**Total # dead somules**	**% dead**
NMS	159	1	**0.6**	159	2	**1.3**
Media	150	0	**0**	150	1	**0.7**
20-week	120	119	**99.2**	120	101	**84.2**
8-week	109	79	**72.5**	109	70	**64.2**

Table 2-5. Quantitation of cytolysis by mouse sera.

Table 2-5. Quantitation of cytolysis by mouse sera. NMS = normal mouse sera. Data represents the average of 3 different fields measured in one experiment, and experiment is representative of 2 replicates.

2.5 Discussion

In this paper, we report for the first time the construction of a defined schistosome glycan array using glycopeptides terminating with schistosome specific glycan epitopes as shown in the scheme of synthesis flow diagram (**Fig.1**). These glycan epitopes were synthesized from natural plant and animal starting materials using chemical and enzymatic methods developed in our laboratory. Their masses were determined by MALDI-TOF-MS to validate their identity before printing on NHS activated glass slides to generate the defined schistosome array (**Fig.2)**. The printed array was validated by lectin binding assays, in which lectins bound specific glycans as expected depending on their binding specificities (**Fig.3A**). However, AAL, a core fucose binding lectin also bound to defucosylated core xylose epitope. This binding may be due to either incomplete defucosylation of core xylose/core fucose with mild acid, leaving residual amounts of intact core fucose, or to the concentration of AAL having been high enough to generate non-specific binding to this epitope. One attractive feature of the defined schistosome glycan array is the ability to use very minute amounts of sera samples per single analysis. In addition, the sensitivity on these arrays is much higher than that of a standard ELISA, enabling the detection of even subtle antibody changes in samples under investigation. Combined, these features permit the analyses of many different serum samples without compromising the degree of specificity, as seen in a binding experiment when an IgM monoclonal antibody to LDN was used to interrogate the array (**Fig.3B**). The monoclonal antibody bound specifically to LDN glycopeptides as expected and did not bind to closely related glycopeptides like LDNF. In this study we have used this defined schistosome array to investigate the humoral immune response in rhesus monkeys, humans, and mice during the course of infection with *S. mansoni* to glycan antigens. We chose to study humoral responses to glycans in these animal species because unlike humans, rhesus monkeys are non permissive hosts to

schistosomes. Swiss Webster mice are a good model for studying the immune responses during infection because they are not only affordable but are permissive hosts too. Additionally apart from being an outbred strain, these mice are easy to house and handle. However, mice are not natural hosts of schistosomes. Here, we show that during a schistosomiasis infection, rhesus monkeys generate very high anti-glycan IgG antibodies to specific glycans. When schistosome infected rhesus monkey sera were screened on our defined schistosome array, we observed that most of the IgG responses were directed specifically towards core xylose/core fucose epitope (**Fig.4C-E, and G)**. Core xylose/core fucose is a common plant epitope associated with IgE production in humans and subsequent allergic reactions. Most of the antibody responses observed against this epitope (Glycopeptide #s 9 and 10) were abrogated when it was defucosylated and converted to core xylose (Glycopeptide #s 11 and 12) by mild acid hydrolysis. These results suggest that the anti-glycan IgG response observed were strongly associated with the α1-3 core fucose part of the core xylose/core fucose epitope. The very low response to the core xylose epitope suggests that the defucosylation step was near completion. Binding of pooled infected monkey sera to a different version of the defined schistosome array which also had fucosylated Man3, an epitope lacking core xylose but has α1-3 core fucose (Glycopeptide #s 19 and 20) was tested. The infected monkey sera bound to this epitope suggesting that the α1-3 core fucose is necessary for the sera to bind (**Fig.4H**). This result suggests that α1-3core fucose is a major part of the core xylose/ core fucose glycan determinant in schistosome. Since no binding to core/xylose core fucose epitope was observed with monkey sera before infection (pre-bleed), response to this epitope in infected monkey sera may not be from any other source except from the deliberate *S. mansoni* infection. We were curious to find out whether these schistosome infected monkey sera had any effect on the anatomy and physiology of live schistosomula, a stage of schistosome life

cycle vulnerable to immune lysis[27]. This life cycle stage is covered with a glycocalyx entirely made up of antigenic glycans, one of them being core xylose/core fucose[21]. To this end, we developed an *in vitro* schistosomula killing assay. Pooled infected monkey sera diluted at 1:10 in culture medium were observed to kill mechanically transformed schistosomula *in vitro* when incubated together for 48h. Pre-bleed and 5-week infected sera were not cytolytic to schistosomula (Fig.4A-D). Interestingly, maximal killing effect was observed with 8- and 11-week infected sera (**Fig.5E-H**). These sera agglutinated the schistosomula during lysis (**Fig.5E,G**). The 8- and 11-week infected monkey sera also showed maximal anti-glycan antibody levels not only on the defined schistosome array (to core xylose/core fucose) but also on the CFG array (to LDN, LDNF, and Le^x) (**Fig.6A-D**). In a previous study, monoclonal antibodies to these 3 glycan epitopes were shown to cause lysis of 3h old schistosomula *in vitro* in a complement-mediated manner[28]. The high anti-glycan IgG antibodies in infected monkey sera and its ability to agglutinate and kill schistosomula *in vitro* could be a reason why rhesus monkeys have the ability of clearing schistosome infections spontaneously. It is remarkable that infected monkey sera bound to some glycans on the CFG array but not on the defined schistosome array although both arrays have some similar glycan determinants like LDN, LDNF, and Le^x. Several reasons account for these differences. On one hand, the two LDN and one LDNF epitopes on the CFG were tethered to the slides via varying $-CH_2$ spacer arms of while the ones on our defined schistosome array were all biantennary glycopeptides and tethered to the slides via their amino termini. On the other hand, the chemistries involved in the printing of the CGF (epoxy-activated slides) differed significantly from the one used to print the biantennary glycopeptides on our defined schistosome array (NHS-activated slides). Most importantly, glycans on the CFG array are synthetically derived straight chain glycans, while the glycans on our defined schistosome array are naturally derived

biantennary glycopeptides. These differences may play important roles in the presentation of the different terminating glycan epitopes leading to varying immune sera binding results. One common feature on both arrays is the presence of biantennary LDN glycan. Infected rhesus monkey sera responses to this glycan on the CFG array mirrored that observed on our defined schistosome array; both were low (**Fig.4**, **6A,B**). Most importantly, glycopeptides on our defined schistosome array most likely resemble those in schistosomes and antibody binding to them is representative of the binding that would be expected *in vivo*.

Results obtained from our studies show that humans infected with *Schistosoma mansoni* respond more to core xylose/core fucose and core xylose epitopes than to any other epitope on the defined schistosome glycan array. The IgG responses to these glycan epitopes were significantly high before and then dwindled after treatment (Glycopeptide #s 11 and 12) (**Fig.7A,B**). The IgM responses follow the same trend but their levels are much lower. The times when these humans were infected are not established and hence their infections may have been chronic. On the CFG array, although the responses to LDNF, biantennary LDN, and both LDN epitopes are relatively low in humans, it is observed that these anti-glycan responses are IgMs and IgGs and are of similar levels. Importantly, both IgG and IgM levels to these glycan epitopes in these humans are higher before than after treatment with responses to LDNF being the highest in four out of the five humans (**Fig.7C,D**). One parallel observation is that on both arrays, the responses to biantennary LDN were very low. Responses to Le^x epitopes were all very low on the CFG array suggesting that these epitopes may not be good antigen targets for vaccine development or diagnosis of human schistosomiasis (**Fig.7E,F**).

Sera from these infected humans had minimal schistosomula killing effect both before and after treatment (**Fig.8A-D**). These low levels of anti-glycan antibodies may

be the reason why schistosomula survive in the human vasculature during episodes of reinfection. The inability of *S. mansoni* infected human sera to kill schistosomula *in vitro* as shown in our experiments further confirms this proposition. It is well documented that some adult humans living in schistosome-endemic areas have somewhat high titers of anti-glycan antibodies to schistosome specific glycans, an attribute that possibly affords them resistance to re-infection compared to children living in the same area. Children below puberty ages are more susceptible to infection and reinfection[28]. The occurrence of relatively higher anti-glycan IgGs than IgMs in humans is due antibody isotype class switching. Pooled sera from newly infected humans had no schistosomula killing effect (**Fig.8E,F**). In these humans, although the anti-glycan IgG responses mirrored those observed in rhesus monkeys, their levels were many fold lower even though their sera was used at higher dilution. This may be the reason why humans remain vulnerable to schistosome reinfection even after treatment, unlike rhesus monkeys.

The humoral immune responses to schistosome glycan epitopes observed in mice infected with *S. mansoni* on the defined schistosome glycan array were predominantly directed towards the LDNF epitope and the antibodies generated against it were entirely of IgMs class. This observation was more remarkable in 8-week infected mice sera which showed anti-LDNF IgM responses exceeding those towards any other epitope by more than 3 fold (**Fig.9A**). At 20 weeks after infection, this anti-LDNF response diminished (**Fig.9B**). Response to LDNF in infected mice was also observed on the CFG array suggesting that this epitope is important as an antigen target. Responses to two other LDN epitopes on the CFG array were also observed but responses to biantennary LDN were minimal (**Fig.9C)**. Remarkably, like infected monkeys but unlike infected humans, infected mice responded strongly to all the Le^x epitopes (**Fig.9E**). These responses were largely of the IgM class. Since infected mice, unlike infected

rhesus monkeys, remain infected even with these high anti-Le^x responses, these results suggest that anti-Le^x antibodies do not offer protection against *S. mansoni* infection but may have potential for diagnosis in these model animals. In an *in vitro* killing assay, both 8-and 20-week infected mice sera had the ability to kill mechanically transformed schistosomula (**Fig.10E-H**). Notably, although 20-week infected sera had lower anti-glycan antibody titers compared to 8-week, it had a remarkable schistosomula killing effect. The killing could be attributed to the many types of anti-schistosome IgGs generated over time during a chronic infection as shown in a previous study by *Dean et al*[30]. The high anti-glycan antibodies 8 weeks after infection observed on both arrays in rhesus monkey and mice is not unusual because in the schistosome life cycle such periods of infection coincides with maximal egg laying by the established worm pairs in host vasculature[13]. The leaky schistosome eggs "shell" allows for glycoproteins and glycolipids synthesized inside to leach out and become processed and presented to the host immune system by antigen presenting cells. The above mentioned events culminate in high antibody production to terminating glycans on the glycopeptides and glycolipids. Additionally, eggs have high concentrations of glycosyltransferases including fucosyltransferases which catalyze the formation of glycans with fucose[12, 29].

In our study, it is apparent that while rhesus monkeys respond strongly to core xylose/core fucose epitope, humans respond weakly to this epitope during infection. Hence we can draw similarities between humans and rhesus monkeys in the way they respond to schistosome-specific glycans during an infection with schistosomiasis. This makes the rhesus monkey a good model for studying glycan response during *S. mansoni* infection. It is an established fact that although this species of monkeys in our study get infected with schistosomes, they mount strong immune responses to the parasites and subsequently get rid of the infection and acquire active immunity.

Humans, who are permissive hosts, get infected, mount weak immune responses to the parasites, and conversely the parasites establish in their vasculature. Infected human adults in schistosome endemic regions cannot be super-infected with the same schistosome strain. These humans have some form of immunity to new schistosome infection episodes, a phenomenon called concomitant immunity[31]. This immunity could be a result of increasing anti-glycan antibodies generated during a chronic infection. Surprisingly, children in schistosome endemic areas do not acquire concomitant immunity even after several exposures to schistosome infection as their immune systems are usually under developed[32]. Surprisingly, some individuals in these regions, although under constant exposure, never get infected and hence are "endemic normals"[33].

Therefore, although rhesus monkeys and adult humans respond differently during the course of infection with schistosomiasis, they both have the capacity to fend off re-infection fully (monkeys) or at least to a certain significant degree (humans). In rhesus monkeys, the strong humoral responses to core xylose/core fucose seem to play an important role in the way they respond to both existing and new schistosome infections after an initial exposure. In these animals, the generations of high titer anti-glycan responses coincide afford them spontaneous clearance of infection and resistance to re-infection. Humans generate low titer anti-glycan antibodies and become chronically infected. We have also established that while α1-3 core fucose is necessary for potentiating maximal immune response to core xylose/core fucose both in rhesus monkeys and humans. Remarkably in humans responses to core xylose are synergistic in attaining full response to core xylose/core fucose epitope. We speculate that this glycan epitope has potential for development of future diagnostic tools for schistosomiasis and could also be used to generate potential candidate glycoconjugate vaccines against this parasite.

In conclusion, we have for the first time developed a defined schistosome specific glycan array that can be used to profile the immune responses of humans and model animals infected with schistosomiasis. Using this array, we have shown that rhesus monkeys and humans have similarities in their humoral immune responses to specific glycan epitopes from various stages of the schistosome life cycle. We report that anti-glycan antibody titers in infected rhesus monkeys are much higher than in infected humans presumably contributing to the monkeys' ability to get rid of the infection. We have shown that the core xylose and core fucose or their combinations are important antigens for potential use in diagnosis and vaccine development for schistosomiasis. Lastly, we have shown that although mice are a permissive host to schistosomes, they widely differ from rhesus monkeys and humans in their immune responses to schistosome glycans. We therefore speculate that a candidate glycoconjugate vaccine that can protect mice against schistosomiasis will not necessary be successful in protecting humans. We therefore suggest the use of primate model animals in studying immune responses during *S. mansoni* infection.

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Chapter 3: Mice Immunized with Glycan-Protein Conjugates of LDN and Man3 Generate Cytolytic IgG and IgM Against *Schistosoma mansoni* Schistosomula *in vitro*

Manuscript completed

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3.1 Abstract

Schistosomiasis is among the neglected parasitic diseases affecting people in the tropics, particularly sub-Saharan Africa and parts of Asia and is caused by the helminth trematode *Schistosoma sp*. After infection through animal skin, the worm develops within 5-6 weeks from an immature larval (schistosomulum) stage to female/male worm pairs that begin egg laying. Eggs produce an abundant array of glycoconjugates that are highly immunogenic and may be associated with partial protection against new infection, which may be effective against the schistosomula. Two of the more unusual glycan antigens occur on N-glycans as LDN (GalNAcβ1-4GlcNAc-R) and Man3 (Manα1-3(Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAcβ1-Asn). To test whether antibodies associated with parasite killing can be generated by immunization, we synthesized LDN-glycopeptides and Man3-glycopeptides and conjugated them by a novel strategy to the protein carrier bovine serum albumin. We show that sera from mice immunized with these constructs generate high titer IgG toward LDN-glycopeptide and Man3-glycopeptide. These anti-sera are effective in killing mechanically transformed schistosomula *in vitro* in a complement-dependent manner. Thus, immunization with protein-glycan conjugates can result in killing levels of antibodies toward the immune-sensitive schistosomula and provides a new direction to pursue in terms of vaccine development against this disease. [196 words]

Abbreviations: LDN- LacdiNAc, GalNAcβ1-4GlcNAc; Man3- Manα1-3(Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAcβ1-Asn; PBS- phosphate-buffered saline; BSA- bovine serum albumin; NaOAc- sodium acetate; RP- reverse phase; DHB- 2,5-Dihydroxy benzoic acid; MALDI-TOF-MS matrix-assisted laser-desorption ionization time-of-flight mass spectrometry.

3.2 Introduction

Schistosomiasis is a parasitic disease of the tropics caused by trematode flatworms (blood flukes) of the genus *Schistosoma.* This disease is one of the most prevalent worldwide, especially in sub-Saharan Africa, and it has been estimated that approximately 800 million people were at risk of schistosomiasis and over 200 million people were infected as of 2003 (1-3). Schistosomes have a complex life cycle that includes the free-swimming, fresh water larvae (cercariae) and the parasitic adult worm male/female pairs which reside in humans and other animals that serve as their obligate hosts. Upon skin penetration, the cercariae transform into the larval stage of schistosomula and begin a remarkable transformation to adult male/female worm pairs that lay thousands of eggs daily and reside in mesenteric veins for decades. While there are strong immune responses to the parasites upon sexual maturity and egg laying, the adult worms are refractile to immune killing in human hosts. Infected human hosts, while unable to clear the infection, appear resistant to further infections, a process termed concomitant immunity (4). Strong immunological responses to infections, especially in the case of *Schistosoma mansoni* and *S. japonicum*, lead to hepatosplenic disease and morbidity/mortality (5). Interestingly, some primates, such as *Macaca mulatta* (rhesus monkey), spontaneously clear the infection within a few months after infection and acquire resistance to infection (6,7). Thus, there is hope that it is possible, with a better understanding of immune responses to the worms, to develop effective vaccines against these parasites.

Immune responses to schistosomes vary depending on the animals and species, but in general a strong T_H2 immunity is induced (3,8). Much of the antibody response during infection is against glycan antigens (9-12). Asn-linked oligosaccharides (N-glycans) in egg glycoproteins and those on schistosomula and adult worms contain many of the known glycan antigens in *S. mansoni*. Among these

are the antigens LDN (GalNAcβ1-4GlcNAc β1-Asn) and Man3 (Manα1-3(Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAcβ1-Asn) (9,13). LDN is a common determinant among helminths and antibodies to LDN are present in humans with schistosomiasis and in infected rodents (8,11,14). Man3 is an uncommon truncated N-glycan also commonly found in many worms, including nematodes and trematodes (15). However, to explore the potential of these antigens as protective antigens for the disease, we sought to develop chemical/enzymatic strategies for generating LDN and Man3 and couple the glycans to proteins for immunizations and antibody screening. We reported previously that mice infected with *S. mansoni* generated IgG and IgM antibodies against the LDN glycan epitope (16).

Free glycans alone usually elicit minimal T cell-independent immune responses (17,18), but glycan-protein conjugates induce T cell-dependent responses (18). We have explored this approach by generating purified LDN and Man3 antigens on glycopeptides and conjugating these to the carrier protein bovine serum albumin (BSA). Here we show that immunization of mice with LDN- and Man3-protein conjugates cause generation of relatively high titer IgG that is effective in killing mechanically transformed schistosomula *in vitro*, and suggest that anti-glycan responses should be a component of future vaccine studies against schistosomiasis.

3.3 Materials and Methods

Materials

Bovine fibrinogen (type VI) was purchased from Sigma (St. Louis, MO) and used without further purification. ConA-Sepharose was obtained from GE Healthcare Bio-Sciences (Uppsala, Sweden). Pronase, a mixture of proteinases isolated from the extracellular fluid of *Streptomyces griseus,* research grade quality was purchased from

Calbiochem (San Diego, CA). *Aspergillus oryzae* β-galactosidase and Jack bean β-N-acetylglucosaminidase (hexosaminidase), both research-grade quality, were purchased from Sigma. The β-galactosidase was dialyzed in 1000 MW-cutoff Float-A-lyzer dialysis tubing purchased from Spectrum Laboratories, Inc. (Rancho Dominguez, CA) against 4L of 50mM sodium acetate (NaOAc) in deionized water, pH5.2, before use, to remove endogenous small-sized contaminants. Graphitized carbon cartridges were purchased from Alltech (Deerfield, IL) while reverse phase Sep-Pak C18 columns were obtained from Waters (Ireland). NHS-LC-LC-Biotin, streptavidin coated ELISA plates, and maleimide-activated BSA conjugation kits were purchased from Pierce (Rockford, IL). Tissue culture plates, 48 well, flat bottomed were purchased from Becton Dickson labware (Franklin Lakes, NJ). Goat anti-mouse IgG isotype-specific antibodies were purchased from Southern Biotechnologies (Birmingham, AL). Aluminum hydroxide gel (alhydrogel) was purchased from Sigma. All other chemicals used in this study were purchased from Sigma and used without further purification unless stated otherwise. Swiss Webster out-bred mice were purchased from Charles River Laboratories International, Inc. (Wilmington, MA).

Synthesis of LDN-glycopeptide and Man3-glycopeptide

LDN-glycopeptides were synthesized from bovine fibrinogen type VI. This glycoprotein exclusively contains biantennary N-glycans lacking fucose and containing 1 or 2 outer sialic acid residues (19). Briefly, bovine fibrinogen (1g) was treated with 200mg of pronase in 200ml of 1X pronase buffer (10X, IM Tris-HCl, pH8, 10mM $MgCl_2$, 10mM $CaCl_2$, and 1% NaN_3) at room temperature overnight. To remove undigested protein, the resultant digest was passed over a reverse phase (RP) C18 Sep-Pak column and the flow-through collected. To purify the glycopeptide product, the sample was applied to a 100ml column of ConA-Sepharose. ConA is a plant lectin (*Canavalia ensiformis*) that

binds oligomannose-type and complex-type biantennary N-glycans (20-22). The column was washed 3 times with 1X pronase buffer and bound glycopeptides were then eluted using 1500ml of 100mM α-methyl mannoside. The collected glycopeptides were desalted over a graphitized carbon cartridge, to which the glycopeptides bind. After washing the cartridge several times with deionized water, the bound glycopeptides were eluted with 3 column volumes of 30% acetonitrile with 0.01% TFA and dried in a speed-vac. The dry glycopeptides were then reconstituted with deionized water and their concentration determined by the standard phenol-sulfuric acid method (23) and subsequently by drying and weighing. The reconstituted glycopeptides were desialylated by treatment with 100mM HCL at 80ºC for 30min. The sample was neutralized with 100mM NaOH, desalted over graphitized carbon cartridge, and eluted with 3 column volumes of 30% acetonitrile with 0.01% TFA to isolate the desialylated (asialo)-glycopeptides. Complete desialylation was confirmed by matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) profiling. For mass spectrometry analysis, 0.5µl of glycan was mixed with 0.5µl of 2,5-Dihydroxy benzoic acid (DHB) matrix, air-dried, and analyzed in a MALDI-TOF mass spectrometer. Agalacto-glycopeptides were generated by digesting 24mg of asialo-glycopeptides in 10ml of NaOAc, pH 5.2 buffer with 20mg of *β*-galactosidase at 37ºC for 12h. The *β*-galactosidase was previously dialyzed twice in 2L of in 30mM NaOAc, pH 5.2 for 12h at 4ºC. The digested sample was passed sequentially over a RP C18 column and a graphitized carbon cartridge before elution with 30% acetonitrile with 0.01% TFA and dried in a speed-vac to generate degalactosylated (agalacto)-glycopeptides. From these agalacto-glycopeptides, the LDN-glycopeptide was generated using recombinant β1-4 N-acetylgalactosaminyltransferase (β1-4GalNAcT) from *Caenorhabditis elegans*, based on previous methods (24). Briefly, 50ml medium containing β1-4GalNAcT was centrifuged at 1,500xg for 5min to remove cellular debris and incubated with HPC4-

UltraLink beads (5mg of HPC4 antibody/ml of beads; 0.1µl of beads/ml of medium) for 1h at room temperature on a rotating platform. The beads were collected by centrifugation at 600xg for 3min and washed three times with 10ml of 100mM sodium cacodylate, pH7.0 and 2mM $CaCl_2$. The beads were resuspended in the same buffer with the addition of 20mM $MnCl_2$ and used as the enzyme source. Ten mg of agalacto-glycopeptide was incubated with the beads in 100mM sodium cacodylate buffer, pH7.0 containing 2mM of UDP-GalNAc, 20mM $MnCl_2$. and 3 units of calf intestinal alkaline phosphatase. The mixture was incubated for 48h at room temperature with slow rotation. After incubation, supernatant was recovered by centrifugation at 600xg for 3min and passed sequentially over a RP C18 column and a graphitized carbon cartridge before elution with 30% acetonitrile with 0.01% TFA and dried in a speed-vac to generate LDN-glycopeptide. To generate Man3-glycopeptide, 15mg of agalacto-glycopeptide was treated with 150µl (10 units) hexosaminidase in 5ml of 30mM NaOAc, pH5.2. The digest was transferred into 500 MW-cutoff Float-A-lyzer dialysis tubing and digestion continued for 12h while dialyzing against 30mM NaOAc, pH5.2 at room temperature. The Man3-glycopepetide product was purified in the same way as LDN-glycopeptide above.

Generation of glycan-BSA conjugates by covalent attachment of LDN-glycopeptide and Man3-glycopeptide to maleimide-activated BSA

LDN-glycopeptide and Man3-glycopeptide (3mg each) were weighed out separately in 1500µl microfuge tubes and treated with 5mg of Traut's reagent (2-iminothiolane) (25) in 500µl of IX PBS for not more than five minutes at room temperature. This amidation reagent reacts with the single primary amine on the glycopeptide Asn residue to introduce a free sulfhydryl. Maleimide-activated BSA (2mg) was added directly to the reaction mixtures and swirled at room temperature for at least 1h and purified by gel

filtration following manufacturer's instructions. Fractions of 500µl were collected separately and their absorbance determined at 280nm. The first fractions to elute with optical densities >0.4 were pooled, and represented the glycan-maleimide-BSA conjugates. The concentrations of the pooled fractions were determined by sample lyophilization and direct weighing of the dry powder. MALDI-TOF-MS was used to determine the molecular weights of the glycan-BSA conjugates and subsequently estimate the molar ratio of glycans to BSA in the conjugate.

Biotinylation of LDN-glycopeptide and Man3-glycopeptide

One mg of LDN-glycopeptide and Man3-glycopeptide were independently biotinylated using excess NHS-LC-LC-Biotin (5mg) in 1X PBS buffer at room temperature. The biotinylated glycans were dialyzed against water to remove excess biotin. Glycan-biotin conjugates were dried in a speed-vac and their weights measured. The phenol/sulfuric acid assay was used to estimate the amount of hexoses in the glycan-biotin conjugates (23). Biotinylation was confirmed by MALDI-TOF-MS. The biotinylated glycans were then resuspended in carbonate buffer at 1mg/ml.

Immunization of mice

The concentrations of glycan-BSA conjugates or control BSA were 3mg/ml in 1X PBS. For vaccination, enough glycan conjugate for vaccinating 10 mice at 100µg each was aliquoted and mixed with equal volume of alhydrogel adjuvant. The volumes were calculated such that each mouse received 100µg of immunogen in a total volume of 100µl of immunogen/alhydrogel mixture. The immunogens were allowed to adsorb to alhydrogel adjuvant for 1h at room temperature with slow mixing. Ten female 8-week old Swiss Webster mice were immunized with a total of 100µg of LDN-BSA or Man3-BSA at 4 different subcutaneous locations on the back using a 25G hypodermic needle.

Immunization was repeated twice at two-week intervals and each time mice were bled via the retro-orbital route into a serum separation tube before immunization. As controls, 10 mice were immunized with adjuvant alone, 10 mice with BSA alone, and 10 mice were not immunized. Two weeks after the 3rd immunization, a final bleed from all mice was obtained. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Emory University.

Antibody ELISA

ELISAs were performed using streptavidin-coated plates. The plates were coated with 50µl of 0.2µg/ml biotinylated LDN-glycopeptide or Man3-glycopeptide overnight and processed as described (26). Briefly, immune sera were added in the wells at a 1:100 dilution. Reactivity of the sera to wells containing immobilized biotinylated LDN-glycopeptide or Man3-glycopeptide was detected by incubation with HRP conjugated goat anti-mouse IgG or IgM and ABTS/peroxidase substrate. Absorbance of each well was determined at 492nm on a microtiter plate reader (Molecular Devices, Sunnydale, CA).

IgG isotyping ELISA

Pooled immune sera was diluted 1:100 and analyzed against biotinylated LDN-glycopeptide or Man3-glycopeptide bound on streptavidin coated ELISA plates and processed in the same way as described above. The isotypes of bound antibodies were determined by incubation with a 1:100 dilution of HRP-conjugated anti-mouse immunoglobulin isotype-specific antibodies following manufacturer's instructions.

Killing of schistosomula in vitro

S. mansoni (Puerto Rican strain) cercariae were generated according to a published protocol with slight alteration (27). Briefly, schistosome-infected *Biomphalaria glabrata* snails were kept in the dark for two days, and then exposed to light for 2h to cause shedding of cercariae. The free swimming cercariae were filtered through a 100μm sieve to remove snail and aquarium debris, chilled on ice for 30 min, and centrifuged at 500xg (swinging bucket) for 10min at 4°C. The cercarial pellet was suspended in DMEM buffer containing penicillin and streptomycin to prepare *in vitro* transformed schistosomula. Transformation to schistosomula was carried out by vortexing at top speed to effect the dislocation of cercarial tails. The schistosomula bodies were separated from cercarial tails by centrifugation on a 70% Percoll gradient. Schistosomula were recovered from the pellet fraction and washed twice with DMEM containing penicillin and streptomycin. The schistosomula were subsequently cultured in 48-well flat-bottomed microtiter plates (200-250 schistosomula/well) in 90μL DMEM containing 10% fetal bovine serum, penicillin, and streptomycin for 3h at 37 °C in a humidified atmosphere with 5% CO_2. To these 3h transformed schistosomula, 10μL of pooled immune sera from either LDN-BSA or Man3-BSA vaccinated mice were added and the plates incubated. After 48h of incubation, schistosomulum killing was assessed by two independent methods using microscopic examination (model: 1× 51S8F-3; Olympus). First, gross anatomy of the schistosomula bodies including opaque granular appearance, outer membrane blebbing, protrusion of the acetabular gland, and lack of movement were used as indicators of death. Second, uptake of fluorescent propidium iodide (PI) dye at 10μg/ml, which is a cell-impermeable, nucleic acid-binding dye, was used to confirm death of schistosomula and loss of cellular integrity (28), as described previously (29).

3.4 Results

Synthesis of LDN-glycopeptide and Man3-glycopeptide

Both LDN-glycopeptide and Man3-glycopeptide were synthesized from bovine fibrinogen (type VI) by chemo-enzymatic methods and by ConA-Sepharose column chromatography purification as illustrated (**Fig.1A**). Bovine fibrinogen represents a convenient starting material for remodeling N-glycans, since it contains primarily a biantennary N-glycan lacking fucose and is mono- or disialylated (19). In this strategy, pronase digestion resulted in glycopeptides containing a single Asn residue or a small residual peptide of two or three amino acids. MALDI-TOF-MS-TOF-MS data showed different mass profiles of both LDN and Man3 glycopeptides (**Fig.1B,C**). Significant mass peaks were matched to proposed glycopeptide structures, based on the known sequence of bovine fibrinogen. The different mass peaks for both LDN and Man3 were primarily due to the varying amino acids in the peptide portion of the glycopeptides, and the glycan structures were relatively homogeneous. The yields of both LDN-glycopeptide and Man3-glycopeptide were estimated by the phenol/sulfuric acid method and by drying and weighing. Approximately 10mg of both LDN-glycopeptide and Man3-glycopeptide were conveniently generated by this strategy from 1g of bovine fibrinogen.

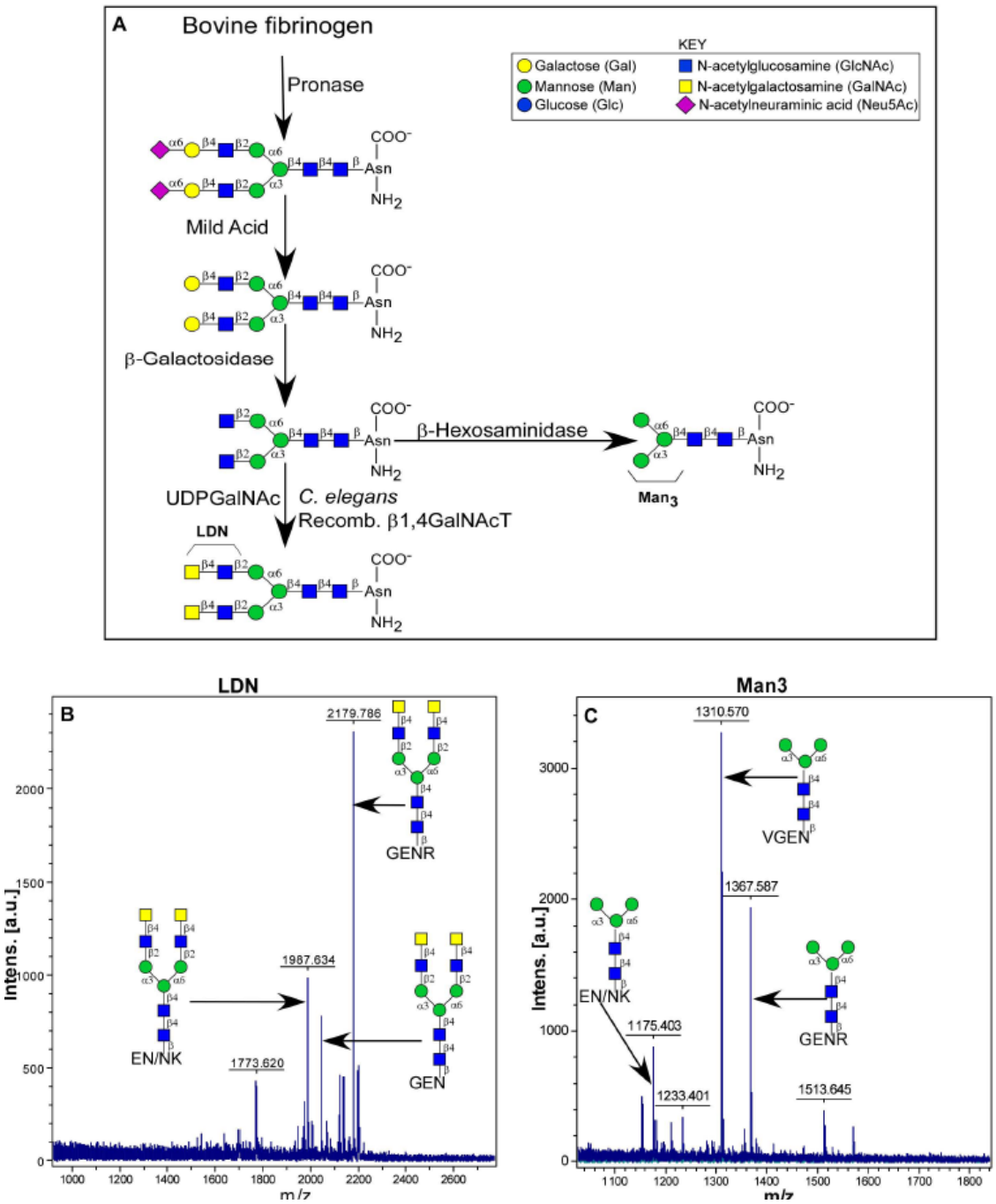

Fig.3-1. Generation of LDN-glycopeptides and Man3-glycopeptides.

Fig.3-1. Generation of LDN-glycopeptides and Man3-glycopeptides. A: Flow chart showing the synthesis of LDN-glycopeptide and Man3-glycopeptide. **B and C:** MALDI-TOF-MS profiles for LDN-glycopeptides and Man3-glycopeptides, respectively. The differences in glycopeptide masses in each profile are due to the presence of different amino acids in the peptide portion of the glycopeptides.

Conjugation of LDN- and Man3-glycopeptides to BSA

LDN-glycopeptide and Man3-glycopeptide were activated by Traut's reagent (2-iminothiolane) (**Fig.2A**) to generate a free sulfhydryl, and then conjugated to maleimide-activated BSA. Approximately 100ng of the glycan-protein conjugates were analyzed by SDS–PAGE and silver staining. Maleimide-BSA reacted with free cysteine alone and underivatized BSA served as conjugation controls. Clear mass shifts were observed after conjugation when the conjugates were analyzed by silver stained SDS-PAGE (**Fig.2B**). To confirm the mass shifts, MALDI-TOF-MS mass spectrometry was carried out on the samples and all glycan-protein conjugates showed increased size (**Fig.2C-E)**. The difference in masses between maleimide-activated BSA and glycan-BSA conjugates were used to estimate the degree of conjugation. The results indicate that the ratio of LDN:BSA and Man3:BSA was 15:1 and 12:1, respectively. The relatively high level of conjugation by this strategy is superior to prior studies where we achieved on average only 2-10:1 ratios of glycans conjugated to BSA using reductive amination with glycans larger than disaccharides (30).

Figure 2. Luyai *et al*

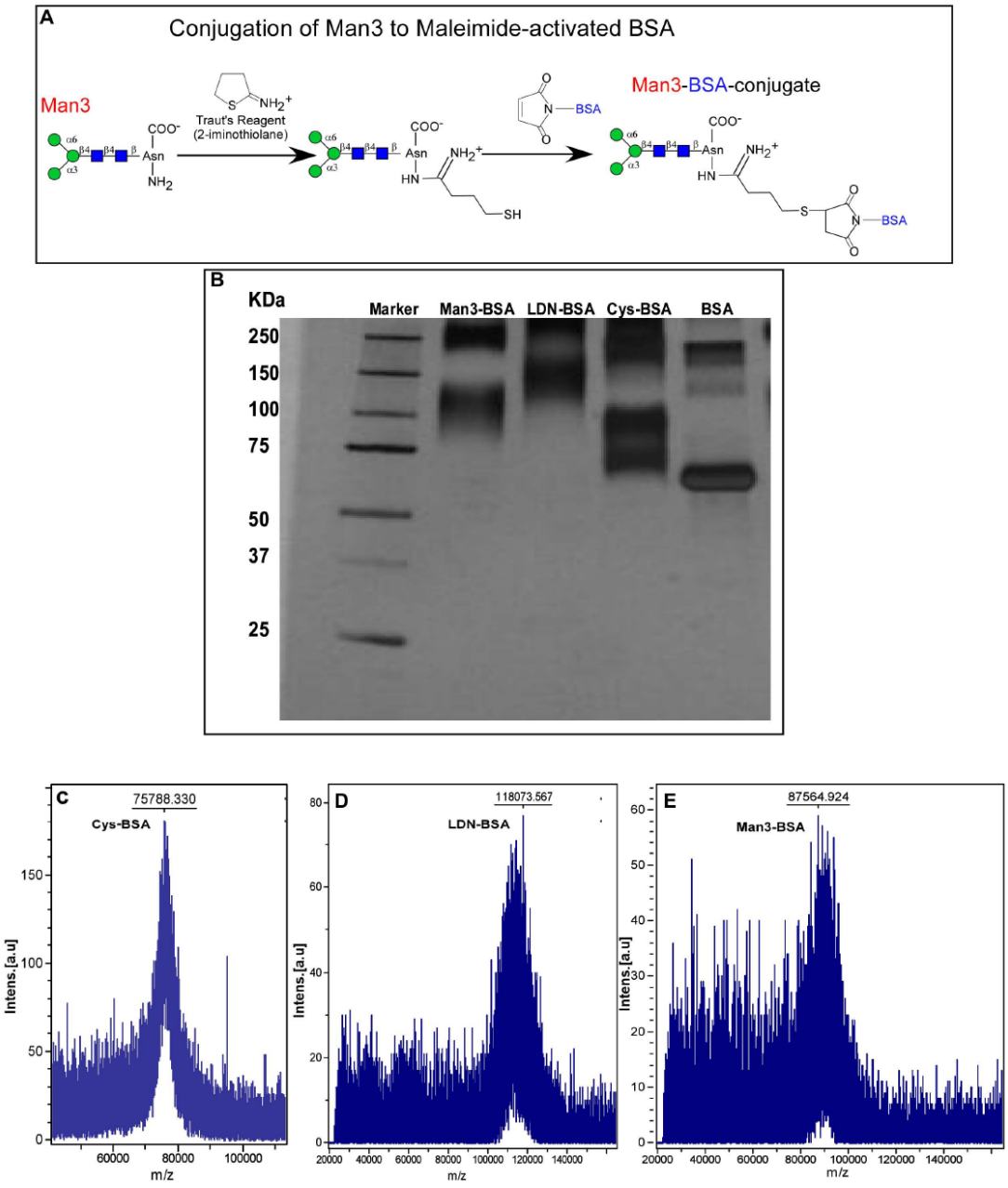

Fig.3-2. Conjugation of LDN-glycopeptides and Man3-glycopeptides to maleimide-activated BSA.

Fig.3-2. Conjugation of LDN-glycopeptides and Man3-glycopeptides to maleimide-activated BSA.A: Flow chart showing the derivatization of Man3-glycopeptide with maleimide reactive –SH group and subsequent conjugation to maleimide-activated BSA. LDN-glycopeptides was conjugated by the same method. **B:** Man3-BSA, LDN-BSA, Cys-BSA and unconjugated BSA analysis by SDS-PAGE and silver staining. **C:** MALDI-TOF-MS profiles of Cys-BSA, LDN-BSA, and Man3-BSA. In both **B** and **C**, defined mass shifts are seen for all glycopeptide-BSA conjugates compared to Cys-BSA and underivatized BSA.

Biotinylation of LDN-glycopeptide and Man3-glycopeptide

To allow ELISA-based testing for antibodies to glycans following immunization with glycan-BSA conjugates, we biotinylated both LDN-glycopeptide and Man3-glycopeptide. Both LDN-glycopeptide and Man3-glycopeptide were efficiently biotinylated with NHS-LC-LC-biotin, as seen by the mass shift difference between free glycans and their biotinylated versions (**Fig.3A,B**). No parent glycopeptide mass profile is identifiable, suggesting that all the available glycopeptides were biotinylated. The weight of glycans in the biotinylated glycan preparations estimated by phenol/sulfuric method was 0.7mg of biotinylated LDN-glycopeptide and 0.68mg of Man3-glycopeptide.

Figure 3. Luyai *et al*

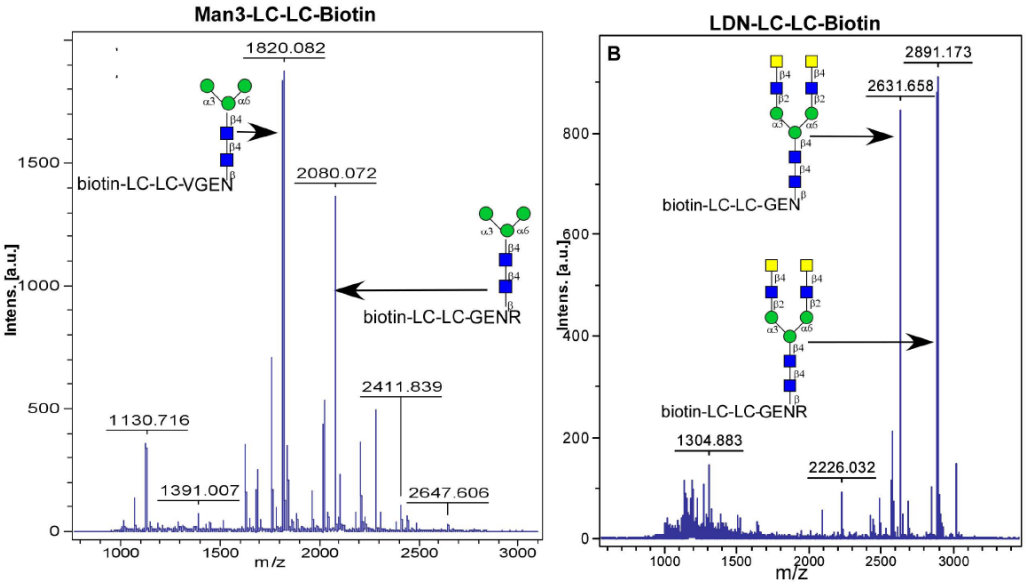

Fig.3-3. MALDI-TOF-MS profiles of Man3-LC-LC-biotin and LDN-LC-LC-biotin.

Fig.3-3. MALDI-TOF-MS profiles of Man3-LC-LC-biotin and LDN-LC-LC-biotin. Man3-glycopeptides or LDN-glycopeptides were reacted with excess NHS-LC-LC-biotin and purified following the procedure in Materials and Methods. Biotinylated glycopeptides were analyzed by MALDI-TOF-MS. **A** and **B** show MALDI-TOF-MS analyses of Man3-glycopeptides and LDN-glycopeptides respectively.

Immunization study

To test immune responses to the glycan conjugates, we used out-bred Swiss-Webster mice that were immunized subcutaneously over four sites with a total of 100μg of vaccine conjugate adsorbed to alhydrogel adjuvant. Vaccination groups included 10 mice per group for LDN-BSA, Man3-BSA, BSA control, and adjuvant alone. Before vaccination, the mice were pre-bled (Pb) and normal sera isolated from peripheral blood. The mice were vaccinated and bled after two weeks to obtain sera (Vac1). Vaccination was repeated twice and sera collected two weeks after each vaccination to obtain Vac2 and Vac3 sera, which was stored at -20°C until use. ELISAs were performed on these individual sera samples using biotinylated LDN-glycopeptides and Man3-glycopeptides captured on streptavidin-coated plates. This was necessary to allow measurements of anti-glycan responses independently of the carrier BSA antigen. ELISA analyses showed that immunized mice generated specific anti-LDN and anti-Man3 antibodies. IgG antibody levels to both LDN and Man3 were highest at the Vac3 time point tested at a 1:100 dilution (**Fig.4A-D**). Individual mouse values are shown (**Fig.4A,C**) as well as the average of all mice per group (**Fig.4B,D**). At these time points, IgM levels were not as high as IgG (data not shown).Our results are consistent with results obtained earlier in our laboratory which showed that mice infected with *S. mansoni* generated antibodies to LDN and that sera of infected mice, but not uninfected mice, contained primarily IgM and low levels of IgG toward this glycan determinant (16).

Figure 4. Luyai *et al*

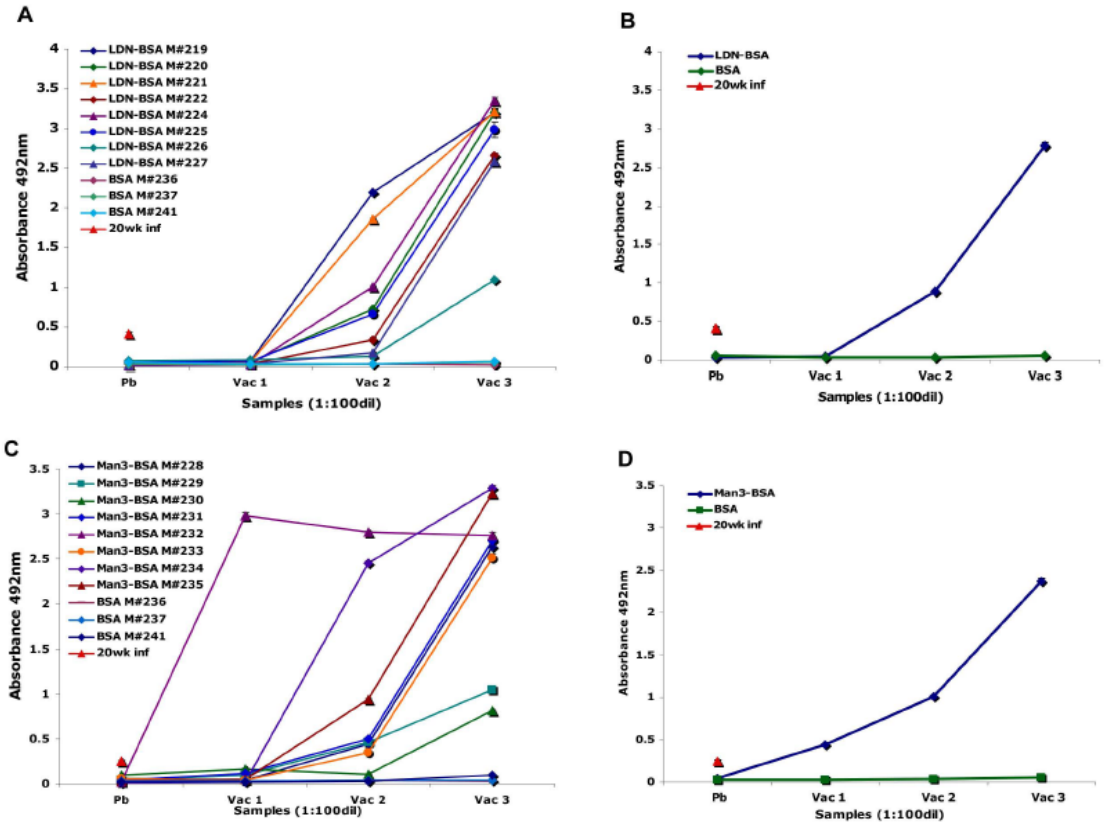

Fig.3-4. Mice immunized with LDN-BSA and Man3-BSA generate IgG antibodies to LDN and Man3, respectively.

Fig.3-4. Mice immunized with LDN-BSA and Man3-BSA generate IgG antibodies to LDN and Man3, respectively. The presence of IgG to LDN or Man3 was determined by ELISA using biotinylated LDN-glycopeptides or Man3-glycopeptides as the antigenic targets following procedures in Materials and Methods. .**A**: ELISA results from 8 LDN-BSA immunized mice and 3 control mice immunized with Cys-BSA. **B**: Average of responses observed in A. **C**: ELISA results from 8 Man3-BSA immunized mice and 3 control mice immunized with Cys-BSA. **D**: Average of responses observed in C. Pooled sera from 20 week infected mice (20wk inf) served as a positive control for ELISAs. Results represent averages of triplicate ELISA determinations, error bars = M#: mouse number.

Four randomly chosen Vac3 sera samples from each group were assayed for both their IgG and IgM antibody titers. **Fig.5A,C** shows that sera from LDN-BSA and Man3-BSA immunized mice had high IgG titers (Absorbance greater OD 0.12 (492nm) at 1:3200 dilution). IgM antibody titers for both LDN-BSA and Man3-BSA sera were low (Absorbance greater OD 0.12 (492nm) at1:200) (**Fig.5B,D**). The IgG antibody isotypes were then determined by specific LDN or Man3 ELISA. As shown in **Fig.6A,B**, IgG_1 was the prevalent isotype in these sera followed by IgG_{2b} and IgG_{2a}. Only Man3-BSA sera showed any signal for IgG_3 (**Fig.6B**).

Figure 5. Luyai *et al*

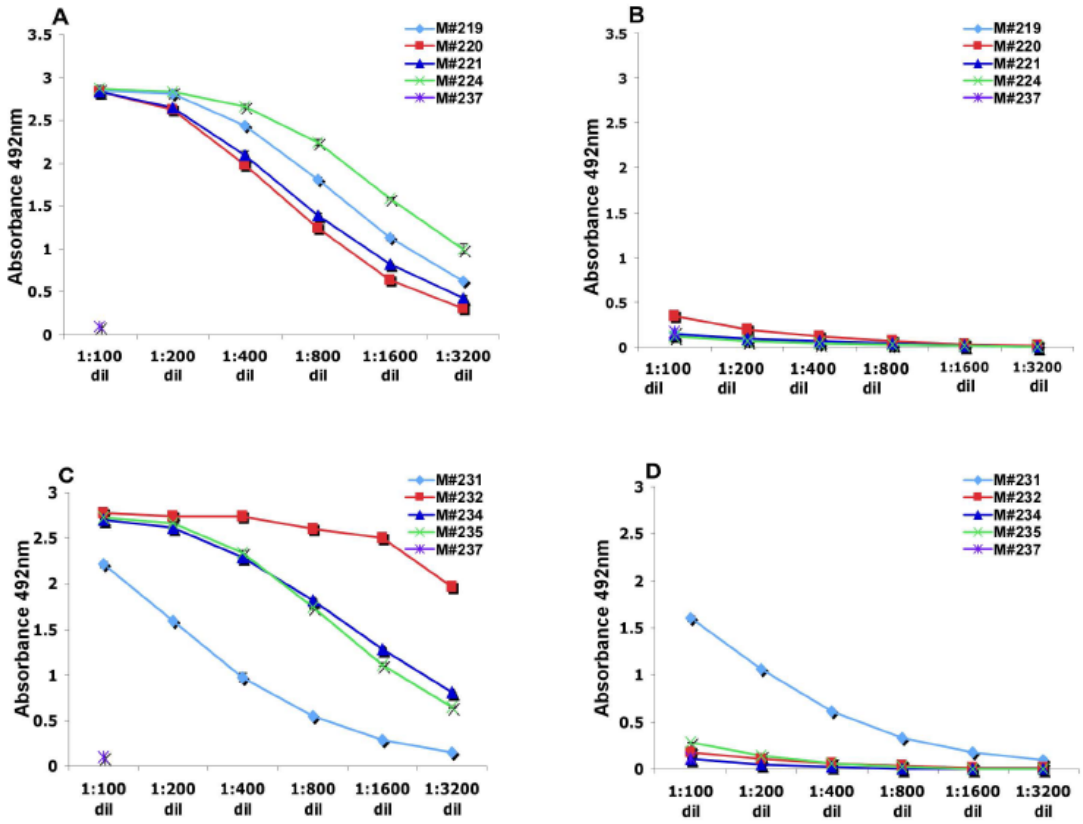

Fig.3-5. Mice immunized with LDN-BSA and Man3-BSA generate high titer IgG antibodies to LDN and Man3 glycans respectively.

Fig.3-5. Mice immunized with LDN-BSA and Man3-BSA generate high titer IgG antibodies to LDN and Man3 glycans respectively Anti-LDN or anti-Man3 antibody titers were determined by ELISA using biotinylated LDN-glycopeptides or Man3-glycopeptides as the antigenic targets following procedures in Materials and Methods..**A**: Anti-LDN IgG titers of four different mice immunized with LDN-BSA. **B**: Anti-LDN IgM titers of four different mice immunized with LDN-BSA. **C**: Anti-Man3 IgG titers of four different mice immunized with Man3-BSA. **D**: Anti-Man3 IgM titers of four different mice immunized with Man3-BSA. The results represent averages of triplicate ELISA determinations for four randomly selected mice from each group, error bars = . Mouse 237 (M#237) was immunized with Cys-BSA and served as a control. M#: mouse number. Error bars =+/- 1SD from the average.

Figure 6. Luyai *et al*

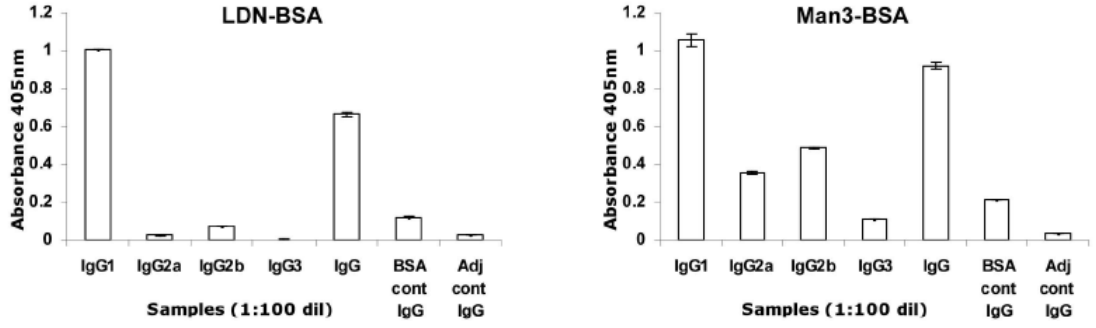

Fig.3-6. Analysis of the isotypes of the anti-LDN and anti-Man3 IgG antibodies in sera from LDN-BSA and Man3-BSA immunized mice.

Fig.3-6. Analysis of the isotypes of the anti-LDN and anti-Man3 IgG antibodies in sera from LDN-BSA and Man3-BSA immunized mice. Pooled immune sera was diluted and analyzed against biotinylated LDN-glycopeptides or Man3-glycopeptides following ELISA procedures described in Materials and Methods. **A**: IgG isotypes in pooled LDN-BSA immune sera. **B**: IgG isotypes in pooled Man3-BSA immune sera. Sera from mice immunized with Cys-BSA or Adjuvant (Adj) served as controls (cont). The results represent averages of triplicate ELISA determinations, error bars =+/- 1SD from the average.

In vitro schistosomula killing

Pooled Vac3 sera from either LDN-BSA or Man3-BSA immunized mice, diluted 1:10 had the capacity to kill mechanically transformed schistosomula *in vitro* after 36h of incubation as determined by gross anatomy or by PI staining (**Fig.7A-D**). Heating the LDN-BSA sera for 30min at 56°C significantly reduced its killing effect **(Fig.7E,F**). Neither BSA immunized mouse sera nor normal mouse sera had any significant killing effect as seen by gross anatomy or by PI staining (**Fig.7G-J**). All schistosomula not exposed to sera looked healthy (**Fig.K,L**). To quantify the killing effect of each serum sample, the two methods of determining schistosomula death were employed and percentages of death were calculated and compared. **Table 3-1** shows that LDN-BSA immune sera had a higher percentage killing effect (58.8%) than Man3-BSA (24.8%) immune sera by the PI uptake method, and the gross anatomy observations corroborated these results. Immune sera from BSA immunized mice and normal sera had comparable schistosomula killing effect, and were assumed to be the baseline schistosomula killing levels. These data indicate that mice immunized with glycan conjugates generate antibodies that have the ability to specifically kill schistosomula *in vitro*.

	Propidium Iodide (PI) staining			Gross Anatomy observation		
Treatment	**Total # somules (36 hrs)**	**Total # dead somules**	**% dead**	**Total # somules (36 hrs)**	**Total # dead somules**	**% dead**
NMS	198	4	**2.0**	198	5	**2.5**
BSA	80	3	**3.8**	80	4	**5**
LDN-BSA	136	80	**58.8**	136	93	**68.3**
HI LDN-BSA	78	24	**30.8**	78	31	**39.7**
Man3-BSA	161	40	**24.8**	161	46	**28.5**

Table 3-1. Quantitation of cytolysis by mouse sera.

Table 3-1. Quantitation of cytolysis by mouse sera. Results show the percentage of schistosomula killing as recorded by gross anatomy or propidium iodide uptake in two different fields of view in an inverted light microscope at 100X magnification. NMS = normal mouse sera, HI = heat inactivated. Data represents the average of 3 different fields measured in one experiment, and experiment is representative of 2 replicates.

Figure 7. Luyai *et al*

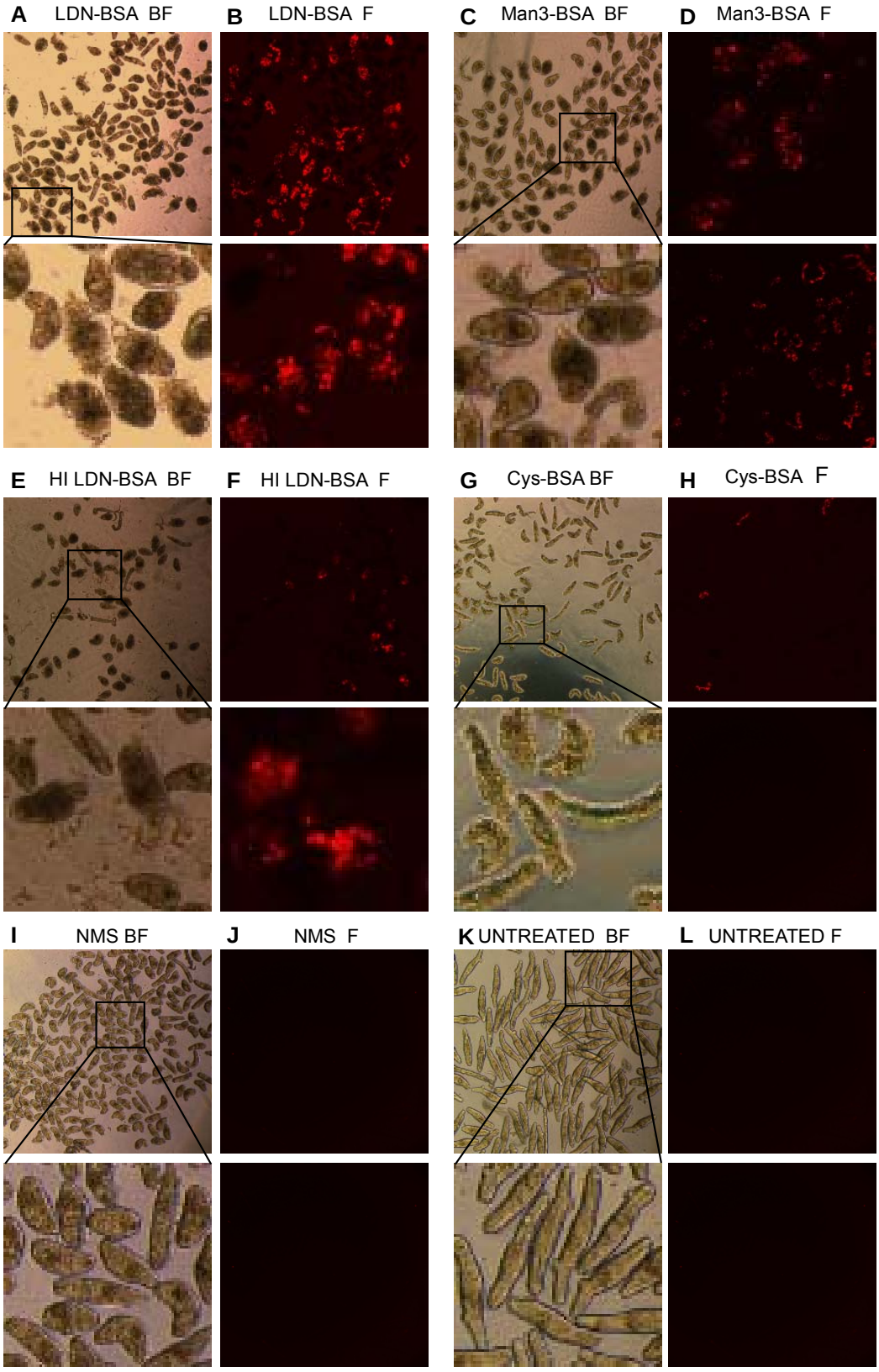

Fig.3-7. *In vitro* schistosomula killing. Mechanically transformed schistosomula were exposed to pooled immune Vac3 sera as described in Materials and Methods. After 36h of incubation at 37ºC, death of schistosomula was recorded by gross anatomy observation or fluorescent propidium iodide (PI) intake. Killing was observed when LDN-BSA and Man3-BSA sera were used in the killing assay. In these sera, schistosomula were observed to assume rotund body shapes, show protrusions of acetabular gland, and concomitantly take in PI. The images are one representative of three different fields from two replicate experiments at 100X magnification. (BF: Bright Field, F: Fluorescence, HI: Heat inactivated, NMS: normal mouse sera.)

3.5 Discussion

In an earlier publication, our laboratory showed that mice infected with *S. mansoni* generated anti-carbohydrate antibodies (16). One of the glycan epitopes is LDN, among others. Here, we have investigated the immune potential of LDN and Man3 in mice when used as exogenous antigens. First we sought to generate the glycans using chemo-enzymatic methods (**Fig.1A**) in high enough amounts to be conjugated to carrier proteins and to allow for an immunization study. We generated LDN-glycopeptide and Man3-glycopeptide, which by MALDI-TOF-MS examination were homogeneous in their glycan portion (**Fig.1B,C**). The heterogeneity observed in MS data was due to varying numbers of amino acids left on the peptide portion of the glycopeptide after pronase digestion of their parent glycoprotein, bovine fibrinogen. Since the glycopeptides were to be conjugated to BSA, a large protein, we speculated that the effect of the peptides on the overall efficacy of the conjugate would not be significant. LDN-glycopeptide and Man3-glycopeptide were conjugated to maleimide-activated BSA following the scheme shown in **Fig.2A**. BSA is a 67 kDa non-glycosylated protein. The integrity of the glycopeptide-BSA conjugates were investigated by SDS-PAGE gel electrophoresis. Compared to BSA reacted with cysteine, a preparation that served as a conjugation control, all of the other glycopeptide-BSA conjugates had clear mass shifts. Native BSA was included on the gel as a control for derivatization. (**Fig.2B)**. These results suggested that LDN-glycopeptide and Man3-glycopeptide had been successfully conjugated to maleimide-activated BSA. Maleimide-activated BSA has 59 lysines present in the protein portion, and 30-35 of these amino acids are activated by attachment to the sulfhydryl reactive maleimide moieties (product information from Pierce, product # 77607). We wanted to know the molar ratios of both LDN-glycopeptide and Man3-glycopeptide to maleimide-BSA after conjugation. The MALDI-TOF-MS data

obtained showed clear mass shifts of LDN-BSA and Man3-BSA conjugates compared to Cys-BSA. The observed mass shifts translated to 15 moles of LDN-glycopeptide and 12 moles of Man3-glycopeptide per mole of BSA (**Fig.2C-E)**. With these conjugates in hand, we immunized Swiss Webster out-bred mice with the glycopeptide conjugates as described in materials and methods. We biotinylated 1mg of both LDN-glycopeptide and Man3-glycopeptide to be used for glycan specific ELISAs. MALDI-TOF-MS analysis of the biotinylated glycopeptides confirmed that the biotinylation reaction had gone to completion (**Fig.3A,B**). We subsequently developed glycan specific ELISAs by using streptavidin coated ELISA plates and recoating them with biotinylated LDN-glycopeptide or Man3-glycopeptide. Building on the fact that streptavidin binds biotin with a very low dissociation constant (K_d), the addition of glycopeptide-biotin conjugates to the plates left the glycan portion of the conjugate exposed for binding complementary antibodies. This scheme enabled us develop ELISAs specific for detection of anti-LDN or anti-Man3 antibodies present in immune mice sera. Immunization results obtained using these glycan-specific ELISAs showed that mice vaccinated with LDN-BSA or Man3-BSA generated high anti-LDN and anti-Man3 IgG antibodies two weeks after the third vaccination (**Fig.5A-D**). In addition, sera from four randomly chosen mice in each group were assessed for antibody titers. IgG titers for both LDN-BSA and Man3-BSA were greater than OD 0.12 (492nm) at 1:3200 dilution of individual sera (**Fig.5A,C**). IgM antibody titers in both groups were low (**Fig.5B,C**). Previous data from our laboratory showed that mice chronically infected with *S. mansoni* generated higher IgM antibodies to glycans, particularly LDNF (GalNAcβ1-4(Fucα1-3)GlcNAc-R) and LDN. IgG responses to these glycans were low (16,31). In this study, we show that when LDN and Man3 are conjugated to BSA carrier protein to potentiate a T cell-dependent immune response, high titer anti-glycan IgG antibodies were generated (**Fig.4A-D** and **5A,C**). These high titer anti-glycan IgG antibodies are a result of antibody class switching which

is important in the development of active humoral immunity and memory. Further, we determined the IgG isotypes in the immune sera and observed that in both LDN-BSA and Man3-BSA immunized mice, IgG_1 levels were highest (**Fig.6A,B**). Compared to LDN-BSA, Man3-BSA immune sera also had significant levels of complement fixing IgG_{2b} (**Fig.6B**). We then determined whether the high anti-glycan antibody titers observed in this study had any significant effect on schistosomula killing *in vitro*. Schistosomula are immune vulnerable and have been shown to be killed by sera from *S. mansoni* infected rodents (26).

In an earlier study, Nyame *et al* showed that a monoclonal antibody to LDN had the capacity to kill schistosomula *in vitro* in a complement-dependent manner (29). We therefore conducted schistosomula killing assays using sera from LDN-BSA and Man3-BSA vaccinated mice. In addition, in previous schistosomula killing assays, investigators added exogenous complement to the schistosomula/media/sera mixture to augment killing. We therefore set our schistosomula killing assay without the addition of exogenous complement. In our assays, 10µl of pooled immune sera was added to schistosomula in a total volume of 100µl in flat-bottomed tissue culture plates. Each well contained between 200-250 schistosomula. Since no exogenous complement was added in the killing assays, we speculated that the endogenous complement was sufficient to promote schistosomula killing in conjunction with the anti-glycan antibodies. All sera samples had been aliqouted in small volumes to avoid frequent freeze/thaw cycles which compromise the integrity of complement. The results obtained showed that at a 1:10 dilution, sera from LDN-BSA and Man3-BSA immunized mice had the capacity to kill mechanically transformed schistosomula as determined by gross anatomy observation **(Fig.7A,C**) or by PI uptake analysis (**Fig.7B,D**). BSA and normal sera did not have any killing effect (**Fig.7G,H** and **7I,J**). The few dead schistosomula (2 to 3) in these two assays could have been from injury during schistosomula preparation as

similar numbers of deaths were seen even with no sera treatment (**Fig.7K**). Inactivation of complement in LDN-BSA sera by heating at 56°C for 30min drastically reduced its schistosomula killing effect (from 59.1% to 32.2% by Pi uptake) suggesting that the killing observed was complement mediated due to endogenous complement (**Fig.7E,F).**

Sera from LDN-BSA immunized mice had 59.1% schistosomula killing effect compared to 32.9% for Man3-BSA immune sera after 36h as determined by PI uptake (**Table 3-1**) suggesting that LDN is more immunogenic than Man3 determinant. Gross anatomy observation showed an increase in schistosomula killing of about 5% over the calculations for PI killing for both LDN-BSA and Man3-BSA immune sera (**Table 3-1**). Determination of death by gross anatomy observation included scoring as dead moribund schistosomula, which looked rotund with protrusion of the acetabular gland and showed no movement. Some of these schistosomula may not have had compromised membrane integrity yet and therefore had not taken in PI at the time of counting after PI exposure.

From this study, we have shown that the generation of glycoconjugate candidate vaccines for schistosomiasis can be achieved. For the first time, we have synthesized biantennary glycopeptides terminating in LDN and Man3 glycan determinants from cheaply available natural sources such as bovine fibrinogen, conjugated them to BSA using novel chemistry, and conducted an immunization study in out-bred mice. To the best of our knowledge, no immunization study has been done using any biantennary N-linked glycopeptides terminating in any known schistosome glycan epitope. Although some binding studies have been done with LDN epitope, this glycan was a tetrasaccharide (16). No study has featured Man3 glycan epitope in any form. Our key finding in this study is that mice immunized with LDN-BSA and Man3-BSA conjugates

generated antibodies that were cytolytic to schistosomula *in vitro*. IgG antibodies comprised the major percentage of these antibodies, suggesting that there was antibody class switching and hence the involvement of T cell-dependent humoral immune response. The *in vitro* schistosomula killing effects observed with both LDN-BSA and Man3-BSA immune sera continue to gather support for the generation of a glycoconjugate vaccine against schistosomiasis. From our data, we speculate that a vaccine candidate containing LDN- and Man3-protein conjugates can afford mice protection against a live challenge infection with *S. mansoni*. We propose more research efforts be directed towards the identification of other schistosome-specific glycan epitopes, potent immunogenic protein carriers, efficient adjuvants, and improved methods of conjugation to produce a more efficacious vaccine.

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Chapter 4: Facile preparation of fluorescent neo-glycoproteins using *p*-nitrophenyl anthranilate as a heterobifunctional linker

Paper published 2009 in Bioconjugate Chemistry 20(8): 1618–1624.

Authors: Anthony Luyai, Yi Lasanajak, David F. Smith, Richard D. Cummings, and Xuezheng Song

4.1 Abstract

A facile preparation of neoglycoconjugates has been developed with a commercially available chemical, *p*-nitrophenyl anthranilate (PNPA), as a heterobifunctional linker. The two functional groups of PNPA, the aromatic amine and the *p*-nitrophenyl ester, are fully differentiated to selectively conjugate with glycans and other biomolecules containing nucleophiles. PNPA is efficiently conjugated with free reducing glycans via reductive amination. The glycan-PNPA conjugates (GPNPAs) can be easily purified and quantified by UV absorption. The active *p*-nitrophenyl ester in the GPNPA conjugates readily reacts with amines under mild conditions, and the resulting conjugates acquire strong fluorescence. This approach was used to prepare several fluorescent neoglycoproteins. The neoglycoproteins were covalently printed on activated glass slides and were bound by appropriate lectins recognizing the glycans.

4.2 Introduction

Glycoconjugates including glycoproteins, glycolipids, and proteoglycans play important roles in many biological systems (*1*). Neoglycoconjugates, including neoglycolipids and neoglycoproteins as artificial mimics of natural glycoconjugates (*2*-*6*), have found many applications in various areas. One of the major applications is the development of carbohydrate-based vaccines and drugs (*7*-*11*).

To prepare neoglycoconjugates, and specifically neoglycoproteins, usually one or both of the molecules (glycan and protein) are derivatized with an appropriate functional group and then covalently linked. Theoretically the linkages could be built through many different chemical reactions. Practically, however, derivatization is limited by several critical requirements. First, since glycans from natural sources or expensive synthetic approaches are often available only in small quantities, the glycan incorporation reaction

should be highly efficient. Second, the reaction conditions should ideally be in aqueous buffer under physiological pH to avoid altering the glycan or denaturation of proteins, which might affect the solubility and immunogenicity of the conjugate. Third, the linkage itself should not decrease the immunogenicity of the conjugate and should be of minimal toxicity and antigenicity. There have been numerous methods developed for protein-carbohydrate conjugation, or neoglycoprotein synthesis, based on heterobifunctional or homobifunctional crosslinkers (*12, 13*). Stowell et al. used 2-imino-2-methoxyethyl 1-thioglycosides to link glycans to proteins (*14, 15*). Diethyl squarate has been used for efficient conjugation of proteins and carbohydrates; however, the potential immune response of the linker itself has impeded its application in vaccine development (*16*). Click chemistry (*17*) as well as a number of other protein- or peptide-carbohydrate conjugation methods have been employed for carbohydrate bioconjugation (*18-21*). Activated dicarboxylic acids can be used as homobifunctional linkers but usually no intermediate glycan derivatives can be separated. Wu et al. utilized bis-*p*-nitrophenylesters (*22*) to conjugate glycans and proteins. The intermediates can be separated and purified; however, the glycans need to be pre-modified to possess an active amine. Reductive amination has also been used in the conjugation of free reducing glycans (*23*) or ninhydrin treated glycopeptides (*24*) to proteins, however, the reaction conditions are relatively harsh, they require large quantities of glycan and often the yields are very low.

Here we describe a simple and efficient method to conjugate free reducing glycans with proteins using a commercially available chemical linker **(Fig.4.1**). The linker, *p*-nitrophenyl anthranilate (PNPA), has two different functional groups; an aryl amine that can react with free reducing glycans by reductive amination and an active *p*-nitrophenyl ester that can react with nucleophiles. We show that this bifunctional linker is a useful

reagent for preparing fluorescent neoglycoproteins from naturally occurring complex glycans. Moreover, the neoglycoconjugates acquire fluorescence upon linking, thus providing a ready means of following the reaction and quantifying the products.

Fig. 1 - Luyai, et al

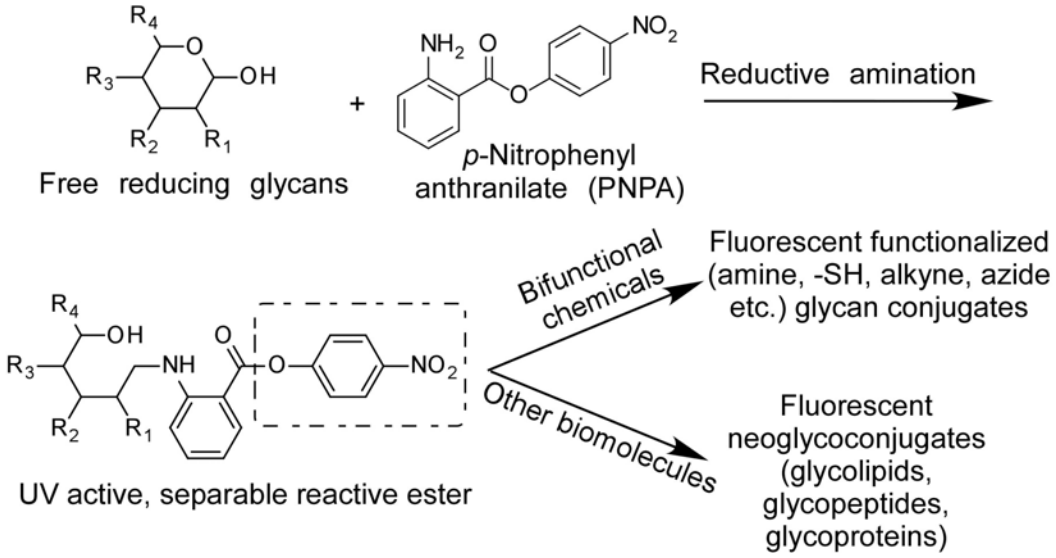

Fig.4-1. General strategy for using *p*-nitrophenyl anthranilate (PNPA) as a heterobifunctional linker to make neoglycoconjugates and other potential applications.

4.3 Experimental Procedures

Free reducing glycans were purchased from V-labs and stored at -20 °C until use. All chemicals were purchased from Sigma-Aldrich and used without further purification. HPLC solvents were purchased from Fisher Scientific. An Ultraflex-II TOF/TOF system from Bruker Daltonics was used for MALDI-TOF-MS analysis of glycan conjugates.

Glycan-PNPA conjugation

The conjugation of glycan with PNPA was carried out using the common reductive amination procedure for free glycan labeling with modifications on sample purification. Briefly, to a free reducing glycan (0.1 to 1 mg), freshly prepared PNPA solution (0.35 M in DMSO/AcOH=7:3 (v/v), 25-50 μL) and an equal volume $NaCNBH_3$ solution (1 M in DMSO/AcOH=7:3 (v/v)) were added. The mixture was heated at 65^{o}C for 2 h. The reaction mixture was quenched by addition of acetonitrile (0.5 to 1 mL). The mixture was cooled at -20^{o}C for 2 h and centrifuged at 10,000 g for 5 min. The supernatant was discarded and the pellet subjected to either C18 Sep-pak for desalting or C18 HPLC for direct purification. For reaction with ethylenediamine, LNnT-PNPA (0.1 to 1 mg) was dissolved in 20-200 μL 10% ethylenediamine and the solution was kept at room temperature for 30 min and subjected to HPLC analysis.

HPAEC-PAD and High performance liquid chromatography (HPLC)

High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) analysis was carried out with a Dionex ICS-3000 system with a Carbpac PA-100 column. The eluent gradient was set to 0-125 mM sodium acetate over 50 min in sodium hydroxide (100 mM).

A Shimadzu HPLC CBM-20A system was used for HPLC analysis and separation of glycan-PNPA conjugates. For reverse phase HPLC, it was coupled with a Vydec C18 HPLC column and a UV detector SPD-20A. UV absorptions at 330 nm and 280 nm were used to detect and quantify GPNPAs and BSA-GPNPA conjugates. The mobile phase was acetonitrile and water with 0.1% trifluoroacetic acid (TFA). The concentration of acetonitrile increased from 1% to 90% in 30 min with a linear gradient. For SEC-HPLC, a Biobasic-60 SEC column was used. The eluent was ammonium acetate (10 mM) at pH 4.5. The flow rate was set at 1mL/min for all HPLC runs.

Conjugation of proteins with GPNPAs

Protein conjugation with GPNPAs was carried out in various buffers as described above. Protein concentration was kept at 5 mg/mL. Aqueous buffer with 1% DMSO was used. The protein conjugates were purified by SEC by collecting the eluent with strong absorption at UV 280 nm. Lyophilized fractions were dissolved in water or suitable buffer for MALDI-TOF-MS characterization and printing.

Printing, binding assay, and scanning

The printing of protein-glycan conjugates on NHS-activated slides and Epoxy slides was carried out according to previous procedure (*25*). Biotinylated lectins were used in the binding assay and the bound lectins were detected by a secondary incubation with cyanine 5-streptavidin. The slides were scanned with a Perkin Elmer ProScanArray microarray scanner equipped with 4 lasers covering an excitation range from 488 nm to 633 nm. The scanned images were analyzed with the ScanArray Express software. For cyanine 5 fluorescence, 649 nm (Ex) and 670 nm (Em) were used. All the images obtained from the scanner were in grayscale and colored for easy discrimination.

4.4 Results

Derivatization of free reducing sugars with PNPA by reductive amination

The preliminary study of PNPA conjugation with free reducing glycans was carried out using lactoneotetraose (LNnT) (Galβ1-4GlcNAcβ1-3Galβ1-4Glc) as the model compound. The conjugation reaction is shown in Figure 2a. The reaction time was optimized by analyzing free LNnT and the product mixture after various reaction times using HPAEC-PAD (Figure 2b). The conversion of LNnT did not significantly increase after 2 h of heating at 65^{o}C, when >80% yield was obtained. The reaction mixture was precipitated in 10 volumes of acetonitrile and the supernatant and precipitate were analyzed using RP-HPLC (Figure 2c). Most of the excess PNPA remained in the supernatant and the LNnT-PNPA conjugate was precipitated in high yield (>90%). The major product peak was collected from RP-HPLC and analyzed by ESI-MS (Figure 2d), which showed an expected mass at 950.3237 [M+H^{+}] (Calc. 950.3248) and confirmed the successful derivatization of the reducing glycan. Interestingly, the behavior of the LNnT-PNPA conjugate on MALDI-TOF-MS was quite different from ESI-MS. Although the expected molecular ion peak at 972.61 [M+Na^{+}] (Calc. 972.3068) is evident in the spectrum, the most intense peak occurs at 958.63, along with other fragmentation peaks at 956.62 and 942.63 (Figure 2e). The fragmentation ions of -14, -16, and -30 Da caused by radical reactions on the nitro-group (*26*) further confirmed the installation of PNPA. The pattern is highly reproducible and serves as an indicator for all glycan-PNPA conjugates in mass spectrometry analysis (**Fig.4- 2e**).

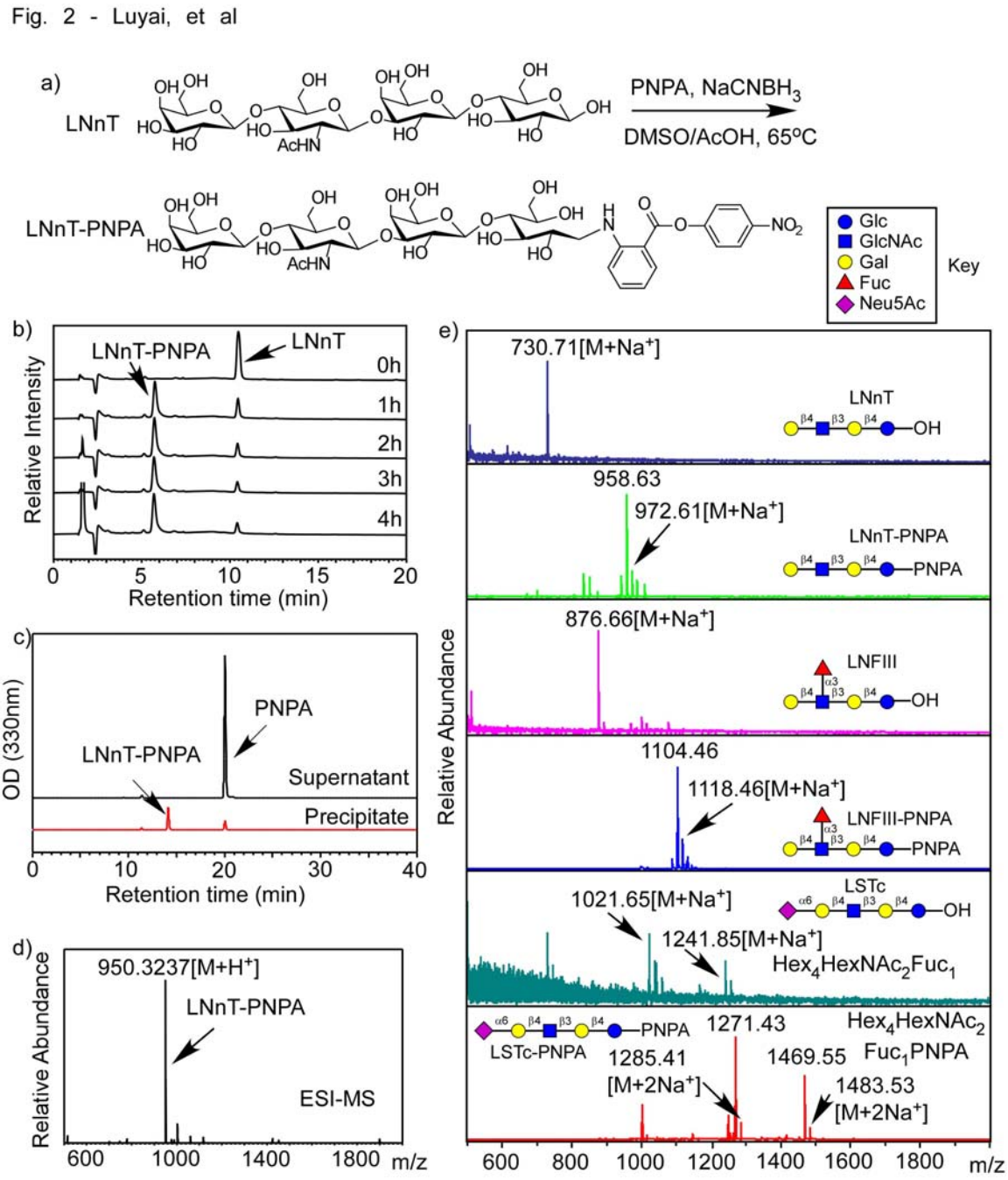

Fig.4-2. **The PNPA derivatization of LNnT.**

Fig.4-2. **The PNPA derivatization of LNnT.a)** The chemical reaction equation; **b)** Dionex profiles of LNnT PNPA conjugation over a time course; c) RP-HPLC profiles of supernatant and precipitate after precipitation of the LNnT-PNPA conjugation with acetonitrile; **d)** ESI-MS of the HPLC purified LNnT-PNPA showing the expected mass; **e)** MALDI-TOF-MS of the starting material and the product of several compounds, showing the expected masses and fragmented major peaks.

Reaction of glycan-PNPA (GPNPA) conjugates with nucleophiles

The reactivity of the GPNPAs towards nucleophiles was tested with ethylenediamine. This mild reaction at room temperature transforms the electrophilic *p*-nitrophenyl ester into a nucleophilic amino group (**Fig.4-3a**). Addition of 10% ethylenediamine at room temperature for 30 min quantitatively converted LNnT-PNPA to the expected LNnT-AEAB as shown by RP-HPLC (**Fig.4-3b**). The major product was purified by HPLC and characterized by MALDI-TOF-MS (**Fig.4-3c**). This confirmed the formation of LNnT-AEAB conjugate, and therefore demonstrated the general reactivity of *p*-nitrophenyl ester towards alkylamines. The minor peak that eluted at 11.2 min with the reaction products matched *p*-nitrophenol standard, confirming the PNPA conjugation with glycans and its reactivity towards amines (nucleophiles) as an active ester.

Fig. 3 - Luyai, et al

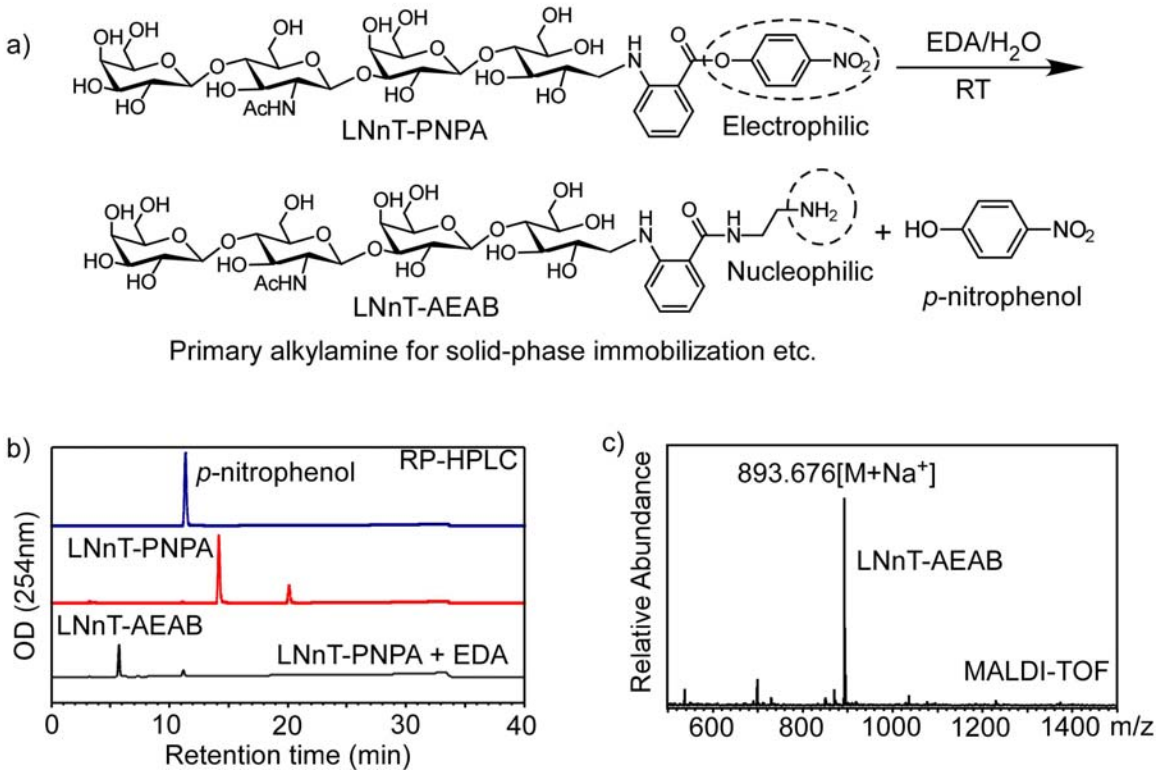

Fig.4-3. a) The reaction of LNnT-PNPA with ethylenediamine

Fig.4-3. a) The reaction of LNnT-PNPA with ethylenediamine; b) HPLC profile of *p*-nitrophenol, LNnT-PNPA and its product after reaction with ethylenediamine; **c)** MALDI-TOF-MS of HPLC purified product of LNnT-PNPA and ethylenediamine.

Conjugation of proteins with GPNPAs

The conjugation of GPNPA with proteins was evaluated with BSA and LNnT-PNPA. To optimize the conjugation, various buffers and temperatures were tested (**Fig.4-4 and Table 4-1**). Figure 4a is a schematic representation of the reaction between GPNPA and proteins. Lysine residues in proteins can attack the active *p*-nitrophenol ester to form a stable amide bond. In this reaction, glycan and protein are thus linked through small fluorescent anthranilamide moiety. **Fig.4-4b** shows the MALDI-TOF-MS analyses that compare the conjugation of LNnT-PNPA with BSA in various buffers at 37^{o}C. The starting molar ratio of LNnT-PNPA and BSA was 10:1. While significant conjugation occurred in all buffers, reactions were faster in more basic conditions (pH 8.5 over pH 7.5). There was no significant conjugation difference seen in phosphate, MOPS, and carbonate buffers based on analysis by mass spectrometry. The conjugation efficiencies were estimated to be 2-3.5 glycans/protein molecule based on the MALDI-TOF-MS peak values, indicating a yield of 20-35%. Figure 4c shows the fluorescent SEC-HPLC profiles of the conjugations in different buffers. The protein-glycan conjugates are fluorescent as expected (Ex 330 nm, Em 420 nm), and the intensity of the fluorescence can be directly correlated to the conjugation efficiency. Apparently, a relatively higher temperature (37^{o}C over 22^{o}C) and pH (8.5 over 7.5) significantly increased the conjugation efficiency. More interestingly, the fluorescence of the LNnT-BSA conjugate generated in carbonate buffer is stronger than those in PBS or MOPS buffer, suggesting a faster reaction. It is worthwhile to note that another fluorescent peak eluting at the same time as LNnT-AEAB is observed. This is presumably the hydrolysis product of LNnT-PNPA (LNnT-anthranilic acid, M.W. 828.77), which has a similar size with LNnT-AEAB (870.85). This peak is of significantly lower intensity than the protein peak, indicating that hydrolysis of GPNPA under these conditions is not seriously affecting the protein-glycan conjugation. To further evaluate the conjugation of GPNPAs with proteins, we tested the conjugation of

lactose-PNPA (Galβ1-4Glc-PNPA) with BSA at different molar ratios (**Fig.4-4d and Table 4-1).** The increased amount of lactose-PNPA, as expected, increased the average number of lactose-PNPA conjugated per BSA molecule. At 100:1 ratio, ~17-18 lactose/BSA were conjugated.

Fig. 4 - Luyai, et al

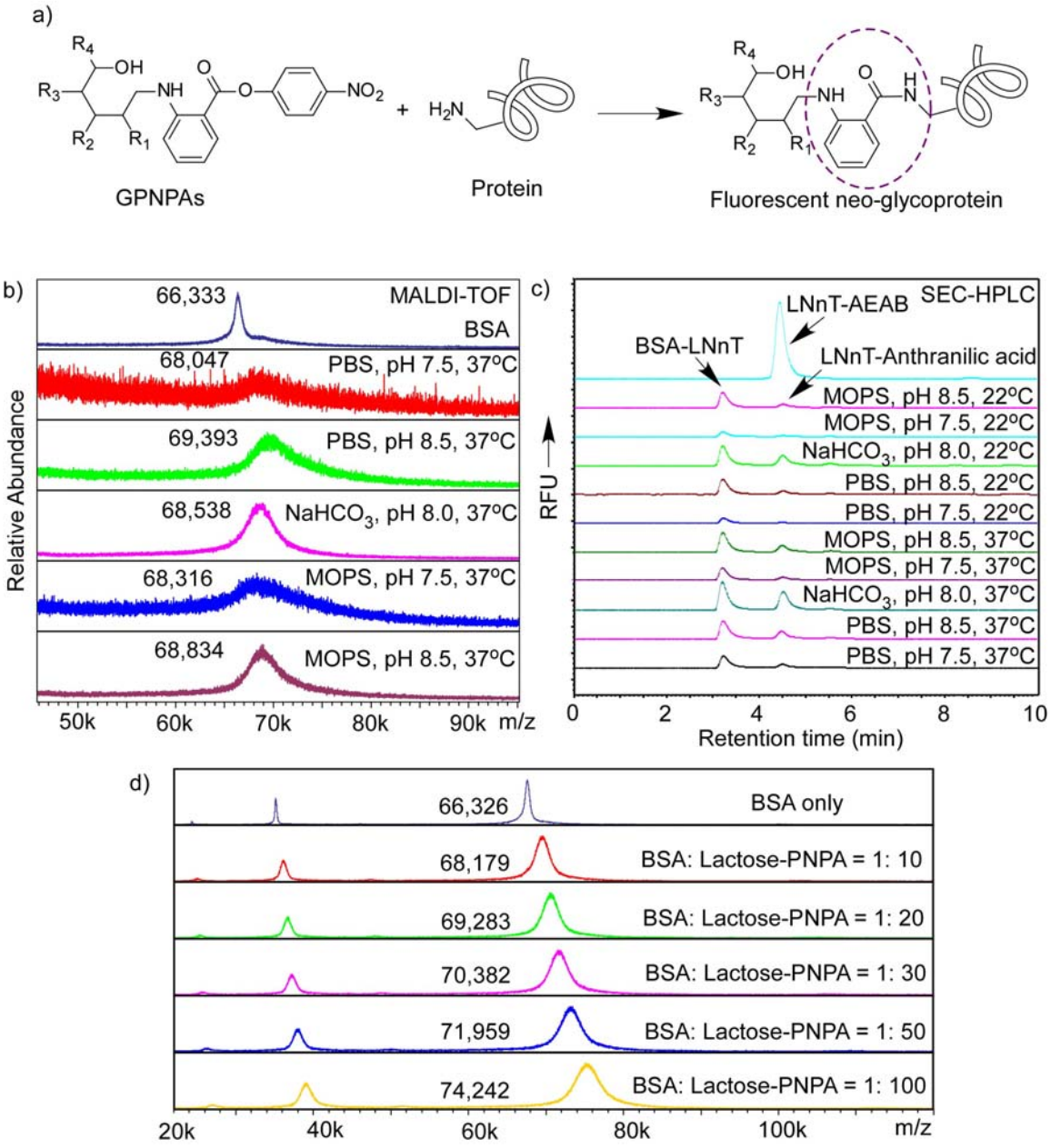

Fig.4-4. BSA conjugation with LNnT-PNPA.

Fig.4-4. BSA conjugation with LNnT-PNPA. a) The general equation of protein conjugation with GPNPAs; **b)** Overlay of MALDI-TOF-MS profiles of BSA conjugation with LNnT-PNPA at 37^{o}C in various buffers; **c)** Fluorescent SEC-HPLC profiles of BSA conjugation with LNnT-PNPA at different temperatures and buffers; **d)** Overlay of MALDI-TOF-MS profiles of BSA conjugation with Lactose-PNPA at different ratios.

Glycan PNPA	Temp (°C)	Buffer	pH	Starting Molar Ratio (Glycan:BSA)	Product Molar Ratio (Glycan:BSA)
LNnT	37	PBS	7.5	10	2.1
LNnT	37	PBS	8.5	10	3.8
LNnT	37	$NaHCO_3$	8.0	10	2.7
LNnT	37	MOPS	7.5	10	2.4
LNnT	37	MOPS	8.5	10	3.1
LNnT	22	PBS	7.5	10	0.5
LNnT	22	PBS	8.5	10	2.2
LNnT	22	$NaHCO_3$	8.0	10	2.0
LNnT	22	MOPS	7.5	10	0.0
LNnT	22	MOPS	8.5	10	1.5
Lactose	37	$NaHCO_3$	8.0	10	4.1
Lactose	37	$NaHCO_3$	8.0	20	6.6
Lactose	37	$NaHCO_3$	8.0	30	9.1
Lactose	37	$NaHCO_3$	8.0	50	12.6
Lactose	37	$NaHCO_3$	8.0	100	17.7

Table 4-1. The conjugation of BSA with glycan-PNPA conjugates in different buffers, pH, and molar ratios.

Printing and recognition of glycan-BSA conjugates on microarray slides

To validate the structural integrity of glycans after conjugation with proteins, we conjugated several GPNPAs (LNnT, LNFIII, and LSTc) with BSA using a 5:1 molar ratio of glycan/protein. The conjugates were purified by SEC-HPLC. The MALDI-TOF-MS profiles in **Fig.4-5a** show the mass shifts compared to BSA alone, indicating that 1.5-2 glycans/protein molecule were conjugated. The purified BSA-glycan conjugates were printed on epoxy and NHS-activated glass slides and interrogated with several plant lectins. All three lectins showed expected bindings on both epoxy and NHS-activated slides (**Fig.4-5b**). AAL, a fucose binding lectin (*27*), bound to BSA-LNFIII conjugates. RCA I, a plant lectin that recognizes β1,4-linked galactose residues (*28*) bound to BSA-LNnT conjugates and also to BSA-LSTc conjugates, weakly since α2,6-sialylation does not fully abolish its binding to Galβ1,4-R moieties. SNA, a plant lectin that binds α2,6-linked sialic acid residues (*29*), bound to BSA-LSTc conjugates, as expected.

Fig. 5 - Luyai, et al

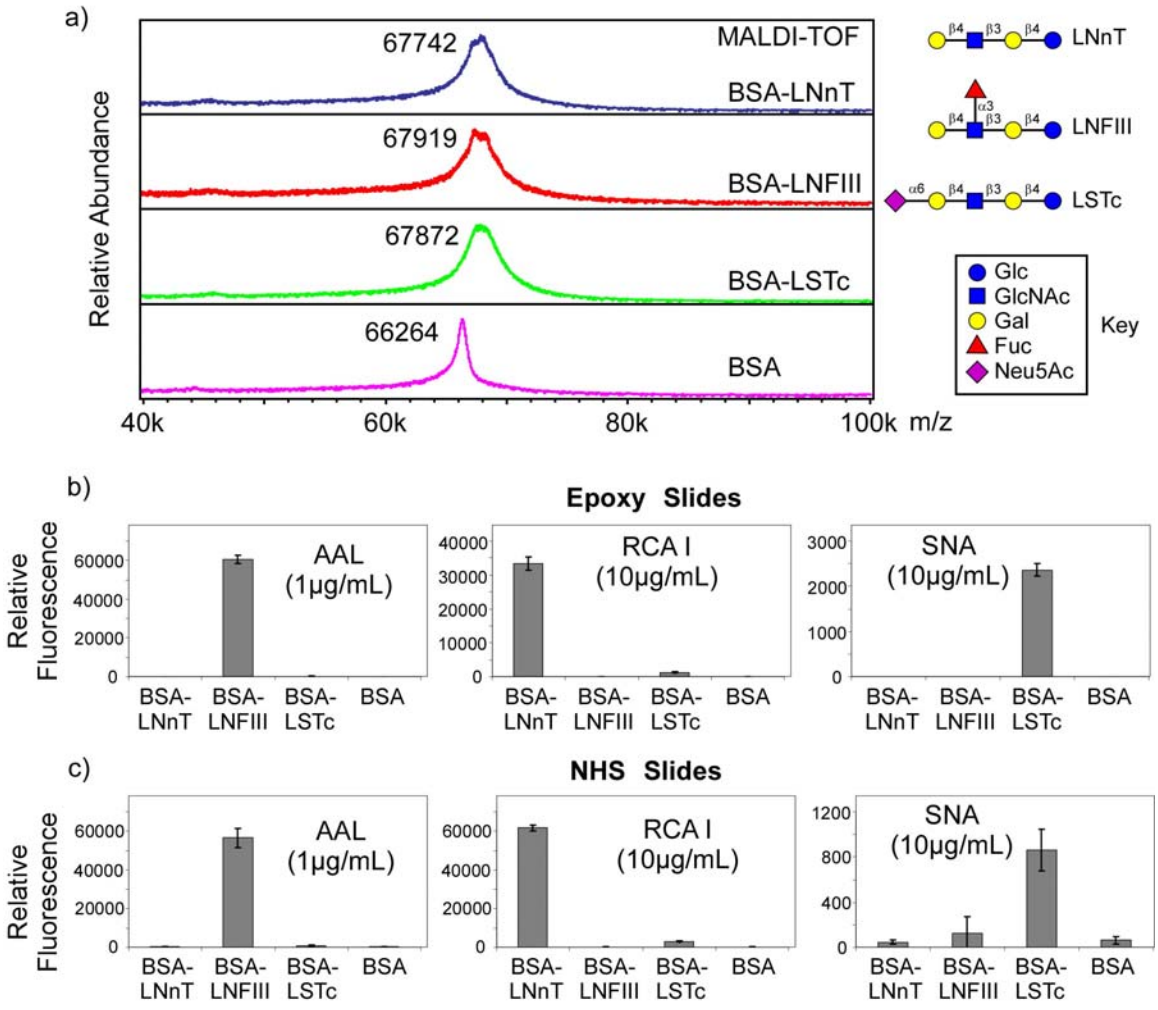

Fig.4-5. a) MALDI-TOF-MS of conjugates of BSA with LNnT-PNPA, LNFIII-PNPA, and LSTc-PNPA

Fig.4-5. a) MALDI-TOF-MS of conjugates of BSA with LNnT-PNPA, LNFIII-PNPA, and LSTc-PNPA; **b)** and c) The conjugates of BSA-LNnT, -LNFIII, and -LSTc, and BSA alone printed on epoxy slides **(b)** and NHS slides **(c)** and interrogated with the lectins AAL, RCA I, and SNA. The glycan structures are indicated along with a key to the symbols.

4.5 Discussion

p-Nitrophenyl ester is known to be an active ester due to the strong electronegativity of the nitro group. This active ester, however, is well-tolerated by the aromatic amine group in PNPA, which is a nucleophile. The existence of two opposing but tolerant functional groups makes PNPA a promising heterobifunctional linker under finely tuned conjugation conditions. Reductive amination of PNPA with free reducing glycans does not interfere with the *p*-nitrophenyl ester, as shown by HPAEC, HPLC, and MALDI-TOF-MS (**Fig.4-2b-d**). Although this reaction does not go to completion under the commonly used conditions, it is still highly efficient (>80% yield) and non-selective towards many glycans, as shown by LNnT, LNFIII, and LSTc conjugation profiles (**Fig.4-2e**). Furthermore, GPNPAs can be easily separated from unconjugated free glycans by C18 Sep-pak due to the strong hydrophobicity of the linker. The high conjugation efficiency, non-selectivity, and easy separation make this approach appropriate for extracting natural glycans as active esters for neoglycoconjugate preparation. We have noticed that by direct reductive amination with glycans, the reducing end ring structure is opened. This is of concern in situations where the whole glycan structures are considered epitopes, such as conjugation of mono- and disaccharides or structures with epitopes close to the reducing end. In most cases, we are more interested in the terminal moieties as epitopes, which should not be affected by this strategy. Under these circumstances, the ability to utilize complex natural glycans directly without laborious synthesis is of more importance than the reducing end structural variation. On the other hand, this strategy could also be applied to any biomolecules with an aldehyde group, which could be installed at the glycan reducing end without breaking the ring structures (*30*).

It is worthwhile to note the interesting behavior of GPNPAs on MALDI-TOF-MS. While ESI-MS clearly showed the molecular ion, MALDI-TOF of GPNPAs showed a reproducible fragmentation pattern besides the molecular ion. This radical reaction related pattern is specific to the nitro-group. While it might complicate mass spectra to a certain extent, it could also serve as a fingerprint for confirming glycan-PNPA conjugates in mass spectrometry analysis. Nevertheless, this does not interfere with the reactivity of GPNPAs for conjugation with other molecules, as shown in **Fig.4-3**. LNnT-PNPA quickly reacts with ethylenediamine to form LNnT-AEAB. The electrophile (ester) of LNnT-PNPA is easily transformed to a nucleophile (amine), enabling its direct conjugation with electrophilic molecules such as epoxy and NHS esters. We have demonstrated the use of AEAB conjugation with glycans for generation of fluorescent natural glycan arrays (*31*), providing an alternative for this application. Although AEAB and PNPA are all heterobifunctional linkers that can be used to conjugate free reducing glycans with other biomolecules or solid surfaces, they are complementary in terms of the reactivity. GAEABs have a nucleophilic alkylamine while GPNPAs have a good leaving group that is reactive towards nucleophiles. Application of both conjugations offers more flexibility in the design and preparation of bioconjugates.

Protein-carbohydrate conjugation has become an important route for generating glycan-related antibodies. Many methods have been developed, most of which focus on conjugating synthetic glycans through bifunctional linkers. In our effort to develop carbohydrate-based vaccines against schistosomiasis and other pathogens (*32*), we sought to conjugate complex glycans extracted from natural sources, which are usually very complicated to synthesize with appropriate linkers attached. Therefore we needed an effective method to efficiently conjugate free reducing glycans with protein carriers. Although there are a number of methods developed for protein-carbohydrate

conjugations, most of them are not targeting naturally occurring complex glycans, which are difficult to obtain through synthesis. Although direct coupling of glycans with proteins by reductive amination has been used, the yields are often disappointing. To obtain homogeneous natural glycans is also a major challenge. PNPA, as a heterobifunctional linker, can react efficiently and selectively with glycans and proteins. GPNPAs can be easily prepared and purified from natural sources based on their UV absorbance. The GPNPAs can be efficiently conjugated with BSA, as shown by MALDI-TOF-MS and HPLC (**Fig.4-4**). MALDI-TOF is the most common method used to determine the protein-carbohydrate conjugation efficiency based on the mass shift before and after conjugation. However, with more glycans and linkers added, the MALDI-TOF-MS peak broadens quickly so that the peak value does not accurately represent the average molecular weight of protein conjugates. The protein-glycan conjugates prepared with the GPNPA strategy are fluorescent, which can greatly facilitate the quantification of conjugated glycans even at a minimal level (**Fig.4-4c**). Furthermore, with appropriate UV range fluorescence detection methods, this fluorescence could be used to detect small amounts of protein in purification steps and bioassays. Based on the mass and fluorescence of the conjugates, we confirmed that various glycans such as lactose, LNnT, and LSTc can be reproducibly conjugated to proteins. The molar ratio of glycans versus glycan-protein conjugates can be driven by increasing the molar ratio of glycans to proteins, reaching 17-18 glycans/conjugate at 100:1 glycan/protein molar ratio.

Upon conjugation of glycans to proteins, it is difficult to validate that the structures of glycans are not affected during the glycan-protein conjugation process. Therefore, we conjugated and printed several glycan-protein conjugates on activated glass slides and interrogated them with various lectins. The conjugation of LNnT, LNFIII, and LSTc GPNPAs to BSA showed similar mass shifts (**Fig.4-5a**), indicating the general

applicability of this approach to different glycans. This is especially useful for sialylated structures, as the sialic acid is not compatible with many conjugation approaches relying on carboxylic acid activation. With *p*-nitrophenyl ester incorporated onto the glycan reducing end by reductive amination, sialylated glycans can be conjugated to proteins as easily as other glycans. When the BSA conjugates of LNnT, LNFIII, and LSTc were printed on activated glass slides (epoxy and NHS) and assayed with three lectins, AAL, RCA I, and SNA, expected specific binding was observed for each lectin. These results show that the glycan structures are presented after the conjugation process in a manner that is consistent with their predicted recognition, which implies that such conjugates can be used to explore biological functions of glycans. It is also worth noting that this approach provides an alternative platform for natural glycan microarray printing. While suitably derivatized glycans (*25, 31, 33, 34*) can be directly printed on NHS or epoxy slides for successful carbohydrate binding protein screening, printing of protein-glycan conjugates presents the glycan structures in the context of protein. This somewhat addresses concerns about nonspecific surface interactions related to microarray slide presentation and may provide a desirable alternative presentation strategy under certain circumstances. The fact that the protein-glycan conjugates are fluorescent is also a major advantage when quantifiable microscale material is used in microarray printing.

One general issue in using protein-carbohydrate conjugates as potential vaccines is that the linker itself is sometimes immunogenic or alters immunogenic potential of the glycan. While no testing is yet available on the immunogenicity of anthranilamide derivatives, the fluorescent anthranilamide is generally considered safe as a chemical and the closely related anthranilic acid is the precursor of the amino acid tryptophan. Thus, while not yet tested *in vivo*, we anticipate that this linker will have minimal immunogenicity and toxicity.

In conclusion, we have developed a novel approach utilizing a commercially available chemical as a convenient fluorescent linker for protein-carbohydrate conjugation. The ease and efficiency, the ability to utilize natural glycans, and the acquired fluorescence of the final conjugates, make this approach very promising in the development of carbohydrate-directed antibodies and carbohydrate-based vaccines.

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Footnotes

Abbreviations: AAL- *Aleuria aurantia* lectin*;* AEAB- 2-amino-N-(2-aminoethyl)-benzamide; GBP- glycan binding protein; GPNPA- Glycan-PNPA conjugate; HPLC- high performance liquid chromatography; LNFIII- lacto-N-fucopentaose III; LNnT- lacto-N-neotetraose; LSTc- lacto-N-sialyltetraose c; NHS- N-hydroxysuccinimide; PNPA- *p*-

Nitrophenyl anthranilate; RCA I- *Ricinus communis* Agglutinin I; RFU- relative fluorescence unit; SNA- *Sambucus nigra* lectin.

Chapter 5: Conclusion and Future Directions

5.1 Conclusion

In the first chapter of this thesis, I described our intent to investigate the immune responses in mice, rhesus monkeys, and humans during infection with *S. mansoni.* Using the defined schistosome glycan array that I prepared, we showed that rhesus monkeys mount a formidable humoral immune response to the core xylose/core fucose glycan epitope. Evidence shows that this antigen decorates all developmental stages of the schistosomes [1,2,4]. It is noteworthy to point out that core xylose/core fucose antigen is also associated with IgE production and allergic reactions in humans [3]. A key finding in our study is rhesus monkeys infected with *S. mansoni* generated high titer anti-core xylose/core fucose IgGs. Maximal antibody generation was observed between 8 and 11 weeks after infection, a time frame, which coincides with egg laying. Sera from these time points had the ability to agglutinate and kill mechanically transformed schistosomula *in vitro*. Remarkably, sera from the 1½ year (78 weeks) time point did not have appreciable schistosomula killing effect, mirroring the effect seen with 5-week infected sera. Schistosomes pair up and start laying eggs in their host vasculature after week 5. Hence, sera from this time point and before do not contain much anti-glycan antibodies, unlike sera drawn 8 and 11 weeks after infection.

Much of the anti-glycan response observed at these time points is due the processing and presentation of glycan antigens from the leaky schistosome eggs. At 1½ years, the monkeys are free of worms and show no symptoms of infection, hence the reason for minimal anti-glycan antibodies detected. These animals do not only cure the schistosome infection but also acquire immunity to further reinfection, an inherent phenomenon in these primates [5]. We also showed that sera from these animals had

the capacity to kill mechanically transformed schistosomula *in vitro* as earlier observed by Clegg *et al* and others [5]. Humans on the other hand only generate low titer anti-core xylose/core fucose IgGs and are always vulnerable to reinfection, particularly children [6, 7]. When compared, the anti-glycan antibody levels in rhesus monkeys were about 60 fold higher than those in infected humans. We hence speculate that a vaccine candidate able to augment human humoral immune response to the anti-core xylose/core fucose epitope would protective them against *S. mansoni* infection.

The reaction of human sera to core xylose/core fucose epitope on our defined schistosome glycan arrays could also be used as a platform for generation of low cost and fast sero-diagnostics for schistosomiasis. Using this platform, depending on antibody response registered, a subject's level of exposure to schistosomiasis can be assessed. Although mice have been used as a model for studying schistosomiasis, our study has shown that they do not directly mimic the immune responses seen in primates. Data from our defined schistosome array showed that infected mice generated mostly IgM antibodies to the LDNF epitope. Although the infected mice generated antibodies to the LDN epitope too, the titers were much lower than those for LDNF. Furthermore, the IgG levels in these mice were lower than IgM levels. In similar experiments, Nyame *et al* showed that there was no differences in antibody type generated during a *S. mansoni* infection between outbreed Swiss Webster mice and inbred BalbC mice [8, 10]. In a parallel experiment, we showed that sera from 20-week *S. mansoni* infected mice can kill mechanically transformed schistosomula *in vitro*.

In this chapter we showed that mice generated preferentially different anti-glycan antibodies depending on whether they were infected or immunized. During an infection with schistosomiasis, more glycan antigens are prevalent for processing and

presentation to the immune system only after egg laying commences, hence sera from schistosome infected mice start showing an *in vitro* schistosomula killing effect only after the 5th week of infection, a time which coincides with egg laying. Additionally, we have shown that sera from 20-week sera have a higher schistosomula killing effect than 8-week infected mice sera, a fact attributed to having more egg antigens at 20 week hence heightened humoral immune at this time point. LDN and Man3 are not as good as LDNF in stimulating the mouse immune system during an infection with *S. masoni*. The reason behind this fact is unknown but may be due to the availability of the antigenic α1-3 Fucose on LDNF, a glycan linkage lacking from animal glycoproteins. *S. mansoni* infected mice mount strong anti-LDNF humoral responses which culminate in the generation of high titer IgM towards this antigen. The high titer antibodies are involved in complement mediated lysis of mechanically transformed schistosomula *in vitro* when either 8- or 20- week infected mice sera are used in a killing assay.

Therefore, while mice are an excellent model for studying schistosomiasis they may not be the best model for studying glycan-related response against this parasite if parallels to human infection are to be drawn. From the above observations it is rational to speculate that a glycan based vaccine that can protect mice against schistosomiasis may not necessarily afford humans protection of a similar magnitude. However, research in mice should not be underestimated because it continues to gather evidence towards generation of carbohydrate based vaccine against *S. mansoni* and other helminthes as well. It is a well established fact that many nematodes and trematodes have similar glycan structures on their tegument and these glycans play an important role in parasite/host interaction and evasion of the host immune insults [9]. Elucidation of these glycan structures is key in developing glycan based vaccines and therapies against such parasites.

In the second chapter we showed that mice immunized with LDN- or Man3-BSA conjugates generated high titer antibodies against the glycan portion of the conjugate and that sera from these mice are cytolytic to schistosomula *in vitro.* These antibodies are both of the IgG and IgM serotype with titers to IgG being higher. Although mice infected with schistosomiasis generate higher titer IgM than IgG antibodies to glycan antigens unlike primates, our immunization results show that when conjugated to a suitable protein-carrier like BSA, LDN and Man3 invoke a T-cell dependent immune response leading to the generation of cytolytic IgGs. IgM responses to these glycan epitopes in an immunization study are very low.

In the third chapter, we have used a commercially available chemical, *p*-nitrophenyl anthranilate (PNPA), as a heterobifunctional linker and developed a new method for preparing neoglycoconjugates. This approach can be applied to protein-carbohydrate conjugation to prepare fluorescent neoglycoproteins which can be used as candidate vaccine for schistosomiasis. The attractive features of this cross linker include UV absorbance and fluorescence capabilities of the glycan-cross linker-protein conjugate. Using these features, purification and quantification of the glycoconjugates is easily tenable.

5.2 Future Directions

It is my intention to continue improving the defined schistosome glycan array by the addition of more schistosome specific glycan epitopes as our lab and others continue discovering them. Using our current version of the array we have shown that monkeys respond strongly to core xylose/core fucose epitope while humans only show a subtle response. Importantly, rhesus monkeys spontaneously clear the *S. mansoni* infection while humans do not. I would like to test *S. mansoni* infected sera from another model

permissive host like baboons which get infected but do clear the infection. I speculate that, like in humans, response to core xylose/core fucose in infected baboon will be subtle. To augment the anti-core xylose/core fucose response in humans, a candidate vaccine bearing this epitope conjugated to a suitable immunogenic carrier protein has to be constructed. Since we have shown that mice respond subtly to core xylose/core fucose epitope during an infection with *S. mansoni,* they are an ideal model to use. Using different protein carriers conjugated to core xylose/core fucose and used in an immunization, we hope to augment humoral immune responses to the glycan portion of the immunogen. In doing so, we will have identified the best protein carrier to be used for conjugation for construction of an anti-schistosome candidate vaccine. Since core xylose/core fucose epitope can be readily prepared from horseradish peroxidase, it is my intent to generate enough amounts of the glycan to be used in a well controlled immunization study. At the same time I will continue evaluating the glycans we already have including LDN and LDNF among others in immunization studies. As we continue updating our defined schistosome array with new schistosome specific glycan determinants, we hope to use the ones which infected animals respond to early in *S. mansoni* infection to develop new specific and faster serodiagnostic tools.

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