2013

# Anti-Group B Streptococci Monoclonal Antibodies Protect against Invasive Aspergillosis

Rebekah Wharton
*University of Alabama at Birmingham*

# ANTI-GROUP B STREPTOCOCCI MONOCLONAL ANTIBODIES PROTECT AGAINST INVASIVE ASPERGILLOSIS

by

REBEKAH E. WHARTON

ROBINNA G. LORENZ, COMMITTEE CHAIR
SCOTT R. BARNUM
CHARLES O. ELSON III
JOHN F. KEARNEY
MOON H. NAHM

A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

BIRMNGHAM, ALABAMA

2013

ANTI-GROUP B STREPTOCOCCI MONOCLONAL ANTIBODIES PROTECT AGAINST INVASIVE ASPERGILLOSIS

REBEKAH E. WHARTON

MOLECULAR AND CELLULAR PATHOLOGY

## ABSTRACT

*Aspergillus fumigatus* is a ubiquitous saprophytic fungus which can potentially cause life-threatening infections in immunocompromised individuals. Although a serious threat, current treatments are largely ineffective at reducing morbidity and mortality. To this end, we have characterized a monoclonal antibody generated against Group B Streptococci, type Ib, which also recognizes A.f. conidia and hyphae. We show that this antibody appears to bind a similar epitope on both pathogens and provides a significant degree of protection in a murine model of invasive aspergillosis via a mechanism that appears to be dependent on complement. The studies we performed using this antibody should be useful in developing more effective therapies for I.A.

Keywords: *Aspergillus fumigatus*, invasive aspergillosis, Group B Streptococci, monoclonal antibody

DEDICATION

To my family and friends who have supported me throughout this endeavor.

ACKNOWLEDGMENTS

I am truly grateful for the relationships that I have maintained over the course of my graduate training. First, I would like to thank God for giving me the brains and patience to pursue science in the first place. Second, I would like to thank all members of the Kearney lab who have helped spearhead ideas and proofread manuscripts. I would especially like to thank Brian Dizon who introduced me to essentially all the techniques used in the Kearney lab and made me laugh even on the most frustrating days in lab. Additionally, I would like to thank Lisa Jia who grew seemingly endless hybridoma cultures for me to do my experiments.

I also like to thank my mentor, John Kearney for all your help and support over the last 5.5 years. Thank you for (1) teaching me to think critically for myself, (2) purifying the endless hybridoma culture supernatants mentioned above, (3) sending me to conferences and courses to extend my knowledge, and (4) being flexible throughout all the crazy life changes I went through during my time in your lab (having Calvin, moving to Atlanta, etc.)!

Finally, I like to thank my family. First, my parents who put me through my undergraduate studies, which allowed me to be admitted into the Pathology program at UAB and who have been supportive of me and my endeavors as far back as I can remember. My husband, David, who gave up countless job

opportunities to move to Birmingham with me so I could get this degree and who loves me unconditionally! Finally, I thank my son, Calvin, who forfeited some of his playtime with me so that I could finish my experiments and write this dissertation.

TABLE OF CONTENTS

*Page*

LIST OF TABLES

*Table* *Page*

ANTI-GROUP B STREPTOCOCCI MONOCLONAL ANTIBODIES PROTECT AGAINST INVASIVE ASPERGILLOSIS

LIST OF FIGURES

*Figure* *Page*

INTRODUCTION



ANTI-GROUP B STREPTOCOCCI MONOCLONAL ANTIBODIES PROTECT AGAINST INVASIVE ASPERGILLOSIS

## LIST OF ABBREVIATIONS

| | |
|---|---|
| A.f. | *Aspergillus fumigatus* |
| I.A. | Invasive Aspergillosis |
| mAb | monoclonal antibody |
| GBS | Group B Streptococci |
| Neu5Ac | 5-N-acetylneuraminic acid |
| s-LNT | sialyl-lacto-N-tetraose |
| CFU | colony forming unit |
| BMDM | bone marrow derived macrophages |
| LNT | lacto-N-tetraose |
| Ib PS | GBSIb purified capsular polysaccharide |
| DEX | $\alpha$-1,3 dextran |
| i.v. | intravenous |
| i.p. | intraperitoneal |
| PC | phosphorylcholine |
| GAC | Group A carbohydrate |
| WGA | wheat germ agglutinin |
| PFA | paraformaldehyde |
| HK | heat killed |
| HI | heat inactivated |

## INTRODUCTION

*Aspergillus fumigatus* (A.f.) is a ubiquitous, saprophytic fungus that is the leading cause of invasive aspergillosis (I.A.). A.f. naturally colonizes the soil and plays a role in breaking down organic matter. Upon asexual reproduction, conidia are released into the air by conidiophores and are dispersed into the environment via wind currents. A.f. conidia have a relatively small diameter of approximately 2-3 μm, allowing them to be easily inhaled by various hosts and their small size and surface structure allows them to penetrate the alveoli of the lung [1]. It is estimated that humans inhale hundreds of A.f. spores each day, and while causing no adverse effects in most healthy humans, can cause a wide range of diseases in susceptible hosts. These conditons include allergic bronchopulmonary aspergillosis (a hypersensitivity reaction to A.f.), noninvasive aspergillomas (colonization of A.f. in a particular body cavity, such as the lung), and invasive aspergillosis (I.A.) (a life-threatening opportunistic infection) in severely immunocompromised individuals [2]. While considered a relatively harmless and uncommon pathogen for many years, the cases of I.A. are increasing due to the prevalence of immunocompromising diseases such as AIDS and the use of immunosuppressive regimens in haematological disorders, autoimmune diseases, and transplants. Incidences of I.A. in these populations ranging from 0.5%-26% depending on the underlying condition [1].

There are several types of I.A. including pulmonary aspergillosis, tracheobronchitis, acute invasive rhinosinusitis, and disseminated aspergillosis. Of these, the most common form is pulmonary aspergillosis, but the most lethal is disseminated aspergillosis, with a lethality rate exceeding 90% and a median survival time of only 7 days after clinical symptoms occur [3]. The pathology of I.A. is different depending on how the host is immunocompromised. Typically, the patients most at risk for developing I.A. are either neutropenic or are under corticosteroid-induced immunosuppression. Transplant patients or those with hematological diseases are often treated with cyclophosphamide, which depletes peripheral blood leukocytes, including neutrophils. These patients typically get a form of I.A. that is characterized by a hypo-inflammatory response and massive hyphal outgrowth resulting in thrombosis and hemorrhage. Angioinvasion and dissemination occur unless effective neutrophil levels are restored. Alternatively, allogeneic transplant recipients, treated with corticosteroids to prevent graft versus host disease, are typically non-neutropenic. In these patients, disease lethality is often attributed to a hyper-inflammatory response, characterized by minimal hyphal outgrowth, but excessive granulomatous infiltrates and tissue necrosis [4].

Current treatments in the United States for I.A. include antifungal drugs including amphotericin B and voriconazole. Unfortunately, these drugs have a limited success rate of only ~53% of patients treated with voriconazole and ~31% of pulmonary I.A. patients treated with amphotericin B [5]. This efficacy is even less in patients with disseminated I.A. Both of these drugs inhibit growth of A.f.

by targeting the primary sterols involved in A.f. cell wall ergosterol production. Caspofungin, belonging to a new class of antifungal drug, called echinocandins, also inhibits fungal cell wall development by targeting β-1,3-D-glucan synthesis. The efficacy of this drug, however, is similar to that of the other antifungal drugs [6]. The poor efficacy of these drugs is compounded by adverse side effects in the host and emergence of drug-resistant A.f. strains [7]. Clearly, based on these staggering statistics, the development of new therapies to treat I.A. is a necessity.

One attractive way to treat I.A. is to stimulate the host immune system to kill the fungus. Innate immunity plays a critical role in the immunity to A.f. This early host defense includes anatomical barriers, humoral components, and phagocytic cells. Mucociliary clearance by lung epithelial cells is likely the most important anatomical barrier, trapping many A.f. conidia before they can invade the lung. Complement is thought to be an important humoral defense against I.A. since highly virulent strains of Aspergillus bind less C3 on their surface than less virulent strains [8]. Additionally cytokines, including colony stimulating factors and Th1 cytokines have been shown to be important in protection in I.A. [9]. Phagocytic cells including macrophages and neutrophils are particularly important in the immunity to A.f. Macrophages are able to phagocytose and kill a majority of conidia after about 6 hours, coinciding with the time it takes for A.f. to swell [1]. However, it has been shown that some A.f. can still germinate inside macrophages and escape killing [10]. Neutrophils are recruited to the site of infection by cytokines and chemokines produced by macrophages and are

especially important in killing the hyphal form of A.f. Thus, any A.f. that escape from the macrophages or A.f. conidia that germinate outside cells are killed efficiently by neutrophils, via reactive oxygen species and degranulation [11]. Either, or both of these cell types are able to efficiently protect mice from challenge with high doses of A.f. [12].

Adaptive immunity also plays a role in I.A. Several studies have examined the role of T cells and their cytokines in I.A. Disease susceptibility is associated with increases in Th2 cytokines including IL-4, IL-5, and IL-10, whereas protection is associated with increases in Th1 cytokines including, IFN-γ, IL-2, and IL-12. Additionally, administration of IFN-γ, TNF-α, or IL-4 receptor is able to rescue infected mice from disease progression, but interestingly, recombinant IL-12 is not able to rescue mice [1]. Although these cytokines show promising results in mice, their effectiveness at improving disease in humans is largely unstudied, and often reserved for patients that are unresponsive to other antifungal treatments.

The role of antibodies in fungal infections has remained somewhat elusive over the years due to inconclusive results using immune serum. The use of polyclonal serum in these studies fail to take into account important properties of antibody-mediated protection including prozone effects, antibody specificity, and antibody isotype that are likely responsible for the confounding results [13]. However, the development of hybridoma technology, in which studying the protective capacity of antibodies of a single specificity and isotype can be achieved, is beginning to shed more light on the role of specific antibodies in

disease [14]. In fact, due to several recent protection studies with anti-β-1,3 glucan mAbs as well as others, this form of treatment is now beginning to be recognized to have potential in passive or active immunotherapy for fungal infections. Antibodies can elicit protection against microbial infection by a wide range of mechanisms including complement activation, cellular cytotoxicity, opsonization, toxin and viral neutralization, direct killing of microbes, modulation of gene expression, modulation of microbial signal transduction, and modulation of microbial physiology. This versatility of mAbs make them ideal therapeutic tools in I.A., especially in combination with other mAbs or antifungal drugs [15].

In order to rationally identify new targets on A.f. to be used for the development of new therapies for I.A., a general understanding of the structure and molecular interactions of A.f. with the host is needed. The life cycle of A.f. involves conidia, the dormant, metabolically inactive form and invasive, metabolically active hyphae. The conversion of the conidia to hyphae involves four major processes: (1) overcoming spore dormancy, (2) swelling, (3) generation of cell polarity, and (4) germ tube formation and growth. The exit from dormancy occurs at around 2 hours after incubation in a nutritive environment and occurs simultaneously with conidial swelling. The cell polarity is established after ~4 hours incubation in nutritive media and by 8 hours all conidia have formed germ tubes [16]. Polarization, germ tube formation, and growth are highly dynamic processes that involve tight regulation of calcium gradients. Cytoplasmic free calcium levels are maintained at particularly high levels at the growing tip of the hyphae and are likely regulated by a variety of calcium

channels and pumps. Specifically, calcium is thought to enter the hyphae at the growing tip via calcium channels. Homeostasis of the high calcium gradient at the tip is then maintained by pumping out cytoplasmic calcium or its sequestration by proteins or organelles (Fig 1). Disregulation of these calcium gradients by raising or lowering external calcium levels results in inhibition of hyphal growth and changes in branch frequency as well as tip morphology, thus targeting the hyphal tip calcium gradients is an attractive therapeutic target in I.A. [17].

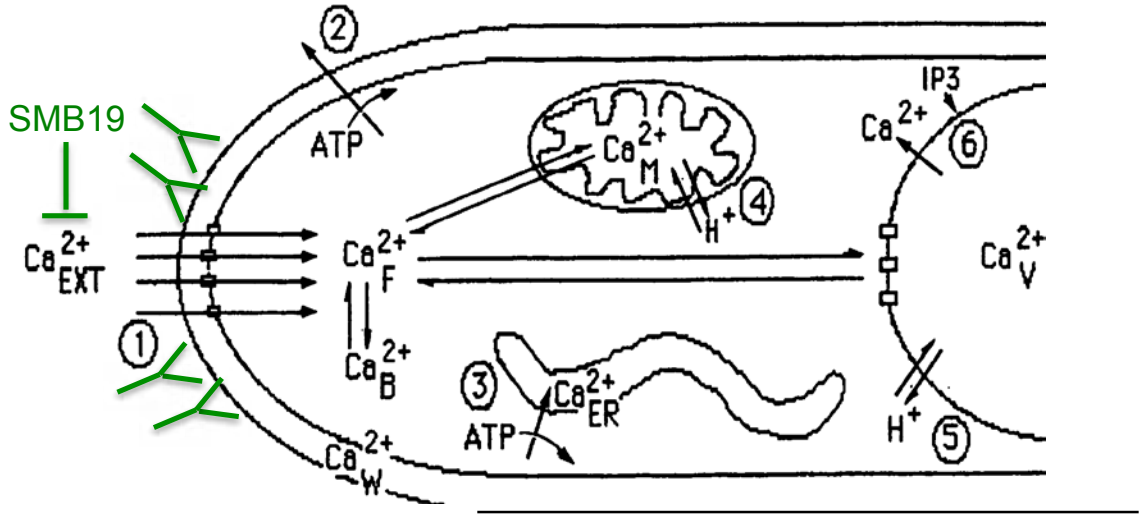



**Fig 1. Mechanisms involved in maintenance of calcium gradients in the growing hyphal tip. Adapted from [17].** High calcium levels are maintained at hyphal tips by (1) membrane calcium channels, (2) membrane Ca2+-ATPases, (3) endoplasmic reticulum Ca2+-ATPases, (4) mitochondria H+/Ca2+ exchanger proteins, (5) vacuole H+/Ca2+ antiport proteins, (6) and IP3-mediated release of calcium from vacuoles. Because the hyphal tip is an area of active growth and remodeling, it may be susceptible to attacks by host defenses such as antibodies and complement.

Although there are continuing wide ranging efforts to study the complex molecular interactions between A.f. and the host, much is already known. Resting A.f. conidia contain a relatively non-immunogenic outer membrane called the rodlet layer, which is comprised largely of the hydrophobic proteins, Rod A and Rod B. Although no receptor for these proteins has been identified, Rod A partially mediates binding to BSA and collagen, likely due to non-specific hydrophobic interactions [18]. Below the rodlet layer is the cell wall, comprised of a complex network of polysaccharides including $\alpha$-1,3 glucans, $\beta$-1,3 and $\beta$-1,6 glucans, chitin, galactomannan, and mannan, which is exposed upon conidial swelling [19]. $\beta$-1,3 glucans have been shown to interact with multiple receptors including dectin-1, complement receptor 3 (mac1), CD5, CD36, and SCARF1 [20-22]. Mannans interact with pentraxin-3, DC-SIGN, langerin, and dectin-2, although there is some redundancy with respect to dectin-2 since it also recognizes $\alpha$-glucans and chitin [19, 23]. These structures exposed after the swelling of conidia are much more immunoreactive than the outer rodlet layer and interact with multiple host cell receptors and ligands, likely leading to complex immune responses which can either be synergistic or antagonistic [23]. In general, polysaccharide cell wall components are poor inducers of antibody and some newly described structures are immunosuppressive [24, 25]. The cell wall has been considered a so-called “achilles heel” for fungi since targeting these components with antibodies can inhibit fungal replication [14]. A relatively unstudied component of A.f. is sialic acid residues which are exposed on the conidial surface and are thought to contribute to conidial dispersal and mediate

interactions with fibronectin [19], laminin, and fibrinogen [26], all of which are upregulated in the context of lung injury (Fig 2).

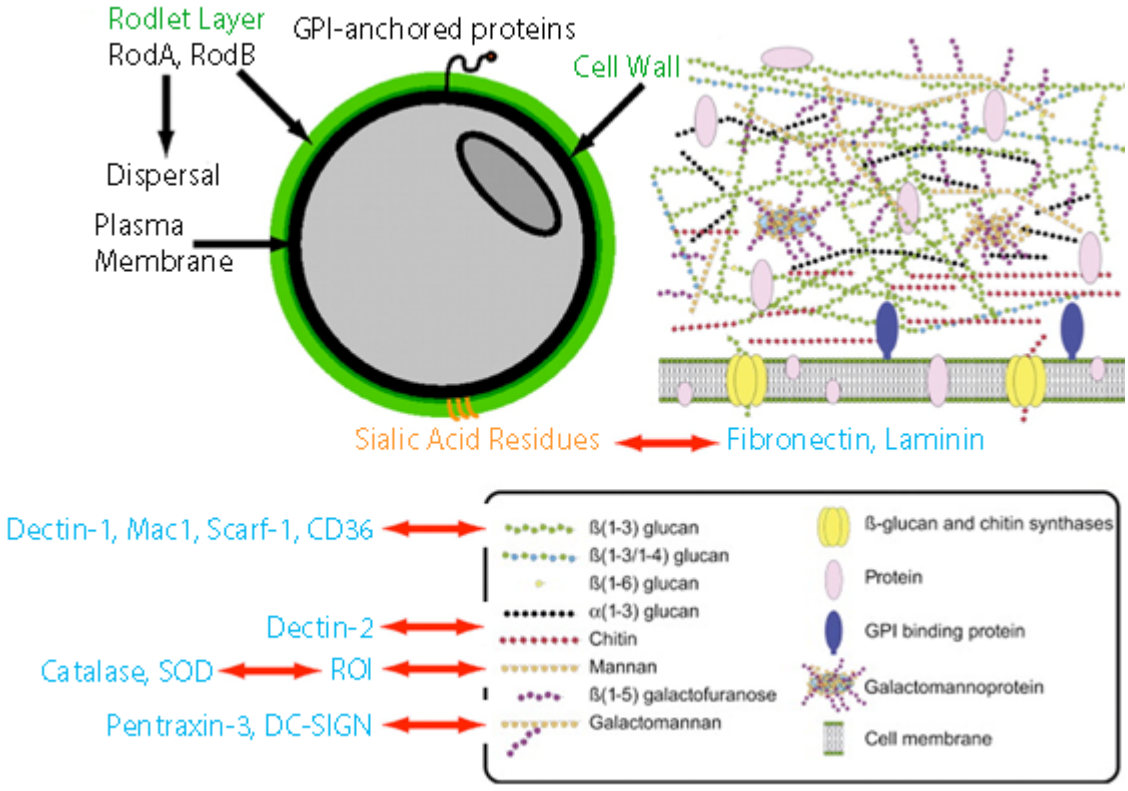



**Fig 2. Structure and molecular interactions of A.f. conidia and hyphae.** Adapted from [19, 27]. Blue fonts indicate known host proteins and receptors that interact with the indicated A.f. components.

Sialic acid residues are attached to polysaccharides, glycoproteins, and glycolipids on a variety of host cells and pathogens, including Group B streptococci (GBS), which express these terminal sialic acid residues on their capsular polysaccharides. Each serotype of GBS contains a unique capsular polysaccharide comprised of glucose, galactose, and N-acetylglucosamine

connected to a terminal sialic acid residue called neuraminic acid (Neu5Ac) [28-30], the same sialic acid found on A.f. [31].

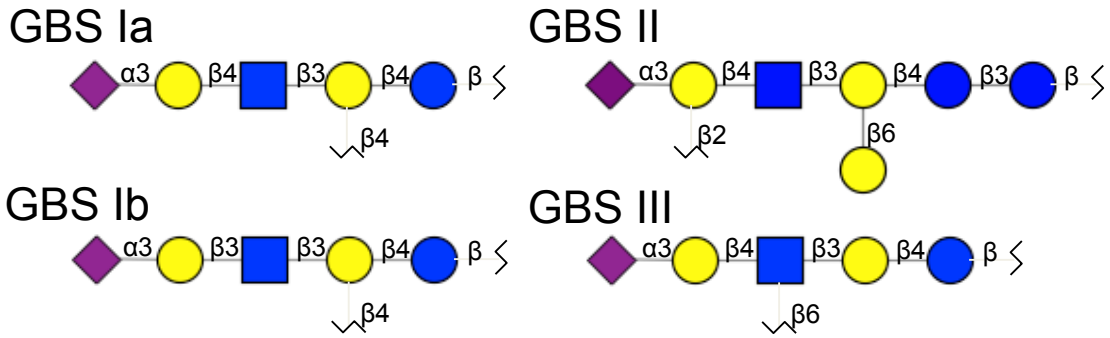



**Fig 3. Structure of the type-specific capsular polysaccharides of several GBS serotypes.** Neu5Ac, red diamonds; galactose, yellow circles; GlcNAc, blue squares; glucose, blue circles.

Neu5Ac is considered a major virulence factor of GBS and it is hypothesized that molecular mimicry of Neu5Ac-associated epitopes on host cells by GBS helps the bacteria evade the host immune system by acting as a non-activating surface for complement. There are some striking correlations between sialic acid density on both GBS and A.f. and complement activation. It has been shown that C3 deposition via the alternative complement pathway is impaired on larger and denser type Ib and type III GBS capsules compared with less dense capsular, desialylated, and acapsular mutant strains of GBS [32]. Likewise, pathogenic A.f. have a greater density of sialic acid on its surface and binds less C3 than lesser or non-pathogenic Aspergillus species [8, 33]. iC3b, the cleaved product of C3 is a ligand for complement receptors on phagocytic cells,

indicating that complement-mediated opsonophagocytosis is likely impaired for A.f. [2]. Additionally, factor H, an inhibitor of the alternative complement pathway is able to bind to both GBS and A.f. [34, 35]. A.f. is also able to produce a soluble complement-inhibitory factor which can inhibit the alternative complement pathway [36]. Taken together, these findings indicate that both highly pathogenic GBS and A.f. have a high density of exposed sialic acid and can effectively evade the alternative complement pathway, likely contributing to their virulence.

High titers of anti-type specific GBS antibodies are thought to overcome this virulence factor and protect the host, by neutralization of this terminal sialic acid residue thus weakening the β1H-C3b interaction leading to deregulation of the C3bBb convertase [37]. Alternatively, antibody may block the sialic acid associated residue in such a way that C3b deposition is allowed [38]. Interestingly, antibodies generated against the desialylated forms of GBS are not protective [39] and desialylated GBS are susceptible to opsonophagocytic killing via the alternative pathway in the absence of GBS type-specific antibody [38].

Our laboratory has generated and extensively studied mAbs against bacterial polysaccharide antigens, so when we realized that other organisms, such as A.f., also expressed similar epitopes, we hypothesized that some of our mAbs may react with these epitopes on A.f. In particular, the realization that A.f and GBS both expressed Neu5Ac, prompted us to determine whether antibodies generated against various serotypes of GBS would also bind to A.f. With the help of Dr. David Pritchard (UAB), we were able to obtain and test type-specific GBS mAbs for reactivity with A.f. One mAb that we tested, SMB19, bound both

GBSIb and A.f. which led us to embark on the studies described in this thesis. In the manuscript to follow we show that SMB19 bound intensely to the tips of A.f. hyphae, an area of rapid growth and remodeling. We also confirmed previous studies showing that binding of SMB19 to sialyl-lacto-N-tetraose, the type-specific polysaccharide on GBSIb is calcium dependent [40]. We also provide evidence that SMB19 reacts with a similar epitope on both GBS and A.f. and that this epitope is apparently GPI-linked.

We also show that passive transfer of SMB19 or vaccination with GBSIb provided a degree of protection in a murine model of I.A. Additionally, SMB19 IgH Tg mice, which maintain high levels of SMB19 idiotype-bearing antibodies, were more resistant to infection with A.f. than μMT mice and WT mice. In addition to showing that SMB19 was protective, we also show that mAbs against α-1,3 glucans, another relatively unstudied component of the A.f. cell wall, were not protective in this model of I.A. as well as J558 Tg mice which express high levels of antibody against α-1,3 glucan.

Besides studying the protective capacity of SMB19 in I.A. in this thesis, we were also eager to determine the mechanism of protection. As mentioned above, antibodies against the intact type-specific polysaccharides of GBS are protective in GBS infections by promoting alternative complement pathway activation and opsonophagocytic killing of GBS. In light of this finding, we hypothesized that SMB19 would be protective in I.A. via a similar mechanism. In the second part of this paper, we show that SMB19's protection in I.A. is dependent on complement components C3 and C5 and that in the presence of

complement SMB19 appears to promote opsonophagocytic killing of A.f. Additionally, we show that SMB19-mediated protection is not dependent on T cells and is only partially dependent on neutrophils, cell types that are typically at low levels in susceptible patient populations. Further studies to elucidate the significance of SMB19's calcium dependent binding and staining pattern on the tips of A.f. hyphae as well as studies to determine SMB19's ability to protect synergistically with antifungal drugs are currently underway.

# ANTI-GROUP B STREPTOCOCCI MONOCLONAL ANTIBODIES PROTECT AGAINST INVASIVE ASPERGILLOSIS

by

REBEKAH E. WHARTON, BRIAN L. DIZON, AND JOHN F. KEARNEY

In preparation for *Immunity*

Format adapted for dissertation

## ABSTRACT

*Aspergillus fumigatus* (A.f.), the cause of invasive Aspergillosis (I.A.), has become a leading cause of death in immunosuppressed populations, due to the lack of effective therapies or vaccines. Studies using anti-A.f. monoclonal antibodies indicate that they can protect against I.A. However, the lack of detailed information on the antigenic targets of protective anti-A.f. antibodies has hampered the development of an effective anti-A.f. vaccine. Sialylated epitopes found on a variety of pathogens including fungi and Group B Streptococci (GBS) are thought to be major virulence factors and are involved in attachment to host cells, uptake by phagocytes, and complement activation. Sialylated epitopes on A.f. may therefore provide a potential target for new therapies. Our preliminary data show that a purified, calcium-dependent GBSIb type-specific antibody, SMB19 (IgM,$\kappa$), also binds to A.f. and prolongs survival in a murine model of I.A. Similarly, immunization of wild type and SMB19 VH bearing immunoglobulin transgenic mice with a GBSIb vaccine also provides protection. Our studies further indicate that this protection is via a mechanism that is dependent on complement, but independent of both T cells and neutrophils. These results suggest that induction of GBSIb specific antibodies, perhaps supplemented with other antifungal therapies, will lead to new vaccines or passive antibody prophylaxis to prevent and treat I.A.

## INTRODUCTION

Fungal infections involving opportunistic pathogens have increased dramatically in the last 20 yrs. This change in status of diseases caused by organisms considered relatively harmless is the result of increased numbers of AIDS patients, and severe immunosuppressive regimens involved in transplantation and chemo-myeloablation. Aspergillus fumigatus (A.f.), which causes invasive Aspergillosis (I.A.), is the most prevalent airborne fungal opportunistic pathogen that causes life-threatening disease in immunosuppressed populations in medical centers worldwide. Mortality rates for I.A. range from 40-80%, and this disease, already a significant health problem, is likely to become more prevalent due to the lack of effective therapies or vaccines [19]. Compounding the serious nature of these infections is the emergence of strains resistant to triazoles, a class of anti-fungal agents.

Most new therapeutic efforts thus far have been directed towards development of adaptive immunity vaccines involving T cells or their cytokines, which are thought to be helpful in clearing fungal infections [41, 42]. However, active vaccination is problematical in the case of immunosuppressed individuals, in particular if they have compromised T cell immunity. Few studies have focused on the development of antibody vaccines or therapies due to the low immunogenicity of cell wall components [24, 25], the lack of serum anti-A.f. antibody correlation with improvement [43] and a study suggesting that μMT mice are resistant to A.f. infections [44]. Protective monoclonal antibodies (mAbs) in A.f. infection models are directed against β-glucans, components of fungal cell

walls [45, 46], and to an undefined glycoprotein [47] however, to our knowledge protection elicited by other antibody-associated A.f. epitopes have not yet been reported. Additionally, passive antibody alone or combined with cell-mediated immunotherapy or antifungal reagents have the potential to provide effective therapy in those with impaired immunity or those about to undergo immunocompromising treatments. Although antibodies to fungal cell wall components, especially polysaccharides, can provide protection, most studies, including our own, have shown that it is difficult to induce these kinds of antibodies [9]. The lack of knowledge of the nature of targets and effector mechanisms involved in protection by anti-A.f. antibodies has hampered the development of an effective anti-A.f. vaccine. Previous attempts to develop vaccines against fungal infections have concentrated on the products made or released by the fungi themselves. There is evidence that cell wall components of these organisms, which have been targeted in the past, are poorly immunogenic (Supplementary Fig 1) either because they have low intrinsic antigenicity or they are able to dampen host responses. [24, 25]

Previous studies have shown that sialic acid associated epitopes are found on pathogens including a variety of fungi [48] and Group B Streptococci (GBS) [49]. Sialylated epitopes on GBS and A.f. are involved in their attachment to host cells, uptake by macrophages and epithelial cells [50], and activation of complement [32, 51]. These findings indicate that A.f. sialylated epitopes may be a potential target for new therapies. In this study we show that a mouse mAb to GBS, type Ib (GBSIb), SMB19 (IgM,κ), specific for the oligosaccharide sialyl-

lacto-N-tetraose (s-LNT) also binds to A.f. conidia and hyphae and is protective in a disseminated mouse model of invasive Aspergillosis.

## MATERIALS AND METHODS

**Mice.** Eight to twelve week old C57BL/6J, B6.129P2-*Tcrb*$^{tm1Mom}$ *Tcrd*$^{tm1Mom}$/J (TCRβ/δ-/-), and B6.129S2-*Igh-6*$^{tm1Cgn}$/J (μMT) mice were purchased from Jackson Laboratories (Bar Harbor, ME) and bred and housed in pathogen-free animal facilities. VH J558 Tg mice were generated using the rearranged VDJ from a J558 IgG3 hybridoma previously described [52]. VH SMB19 Tg mice were generated using the rearranged genomic DNA VDJ from the SMB19 hybridoma [40], NCBI Accession sequence BN000872. Genomic DNA was amplified using the primers 5'-CGCCTGGATGGACTGGGTCCGC-3' and 5'-GCTTCTAATTTCAGCAACCCAC-3'. The PCR product was cloned into the previously described KC9 construct [53] at NcoI and BamHI restriction sites and VH SMB19 Tg mice generated as described previously for the VH81x Tg mouse [54]. C3$^{-/-}$ mice, described previously [55], and C5 deficient mice, generated from backcrossing the DBAJ2 mice onto the C57BL/6 background for over 8 generations, were both gifts from Dr. Scott Barnum, UAB.

**Microorganisms and Monoclonal antibodies.** A.f. 13073 (ATCC) was cultured on potato dextrose agar (Fisher) plates at 37°C for 5 days then harvested in PBS, counted on a hemocytometer, and stored at 4°C. Group B streptococci, (with their cognate mAb clone names in parenthesis), GBS Ib (H36B), GBSIa,

GBSII (18RS21), and GBSIII (COH1) used in flow cytometry experiments and vaccinations were gifts from Dr. David Pritchard (UAB). GBS used for vaccines were grown to log phase, washed 3 times, and fixed overnight in 1% PFA at 4°C. Mabs specific for these GBS serotypes, $\alpha$-1,3 glucan, and the anti-idiotype antibody, SMBi26 against SMB19 are described, together with methods of purification and quality control, in Supplementary Table 1.

**Biacore Analyses.** Biacore T200 (UAB Multidisciplinary Molecular Interaction Core) was used to assess the binding of SMB19 to GBS Ib capsular PS. Purified biotinylated GBSIb capsular PS was captured on a SA (Streptavidin) Sensor Chip for the Biacore T200. After washing in a running buffer containing 3mM EDTA to chelate $Ca^{++}$, SMB19, was injected over the SA-captured GBSIb capsular PS Chip surface in the presence of 0.9mM calcium chloride (red) or 3mM EDTA (green) in the running buffer at concentrations ranging from 0.68 $\mu$g/ml to 50 $\mu$g/ml. Sensorgrams (binding curves) were obtained and displayed simultaneously.

**Flow cytometry.** For A.f. flow cytometry staining, A.f. conidia were cultured in 50 ml conical tubes for 12 hours at 37°C in RPMI to form hyphae. Then, paraformaldehyde (PFA)-fixed GBS or live resting A.f. conidia or hyphae were blocked at 4°C in 1% bovine serum albumin (BSA) then incubated with anti-GBS type-specific IgM mAbs. Polyclonal goat anti-mouse IgM coupled to Cy5 (Southern Biotech, Birmingham, AL) was used to detect binding. All GBS type-

specific mAbs were gifts from Dr. David Pritchard, UAB. IgM binding to A.f. was analyzed on a FACScalibur (Becton-Dickinson, Mountain View, CA).

For the PI-PLC experiments, A.f. conidia were germinated for 18 hours in RPMI in a Petri dish at 37°C. A.f. were harvested and washed 2 times in PBS (20 min, 2000 rpm) and one time in PI-PLC enzyme buffer (50mM Tris HCl, 150mM NaCl, 5mM EDTA in dH20, pH 7.4). A.f. were resuspended in 1 ml enzyme buffer. 0.4 units of Phospholipase C, phosphatidylinositol-specific (MP Biomedicals, Solon, OH) was added to the A.f. and incubated for 60 min at 37°C. A.f. was washed 2 times in PBS and stained with 0.5 μg mAb for 30 min, followed by goat-anti-mouse IgM FITC (Southern Biotech Association) and analyzed on a Becton-Dickenson FACS Calibur. The no PI-PLC samples were treated the same except for the addition of the enzyme and T cells (Thy1) expression was used as a GPI-linked control for the enzyme function.

For SMB19 Tg mouse characterization flow cytometry, spleen, bone marrow, mesenteric lymph nodes, and peritoneal cavity lavage cells were removed from SMB19 Tg or C57BL/6J mice. Cells were counted, blocked with anti-Fcγ (Ab93), and stained with the antibodies listed below. Phycoerythrin (PE) anti-B220 (RA3-6B2), PE-Cy7 anti-B220 (RA3-6B2), PE anti-AA4.1, fluoresceinated (FITC) anti-CD23 (B3B4), pacific blue (PB) goat anti-IgM, biotin anti-CD23 (B3B4), APC-Cy7 anti-CD19 (1D3), FITC anti-CD5 (53-7.3), and PE anti-CD11b (M1/70), and AlexaFluor-488 anti-CD21 (7G6) were purchased from BD Pharmingen (San Diego, CA). SMBi26, rat anti-SMB19 idiotype hybridoma,

was generated using previously described methods [56]. Cell frequencies were analyzed on a Becton Dickinson LSRII flow cytometer.

**Vaccinations.** C57BL/6J mice were vaccinated with either $10^8$ PFA-fixed GBSIb, or GBSII or PBS as controls intravenously (i.v.). Six days after vaccination, mice were infected with a lethal dose of A.f. conidia i.v. and monitored for survival. Mice were euthanized with $CO_2$ when they appeared moribund.

**Passive antibody transfer.** C57BL/6J, TCR$\beta/\delta$-/-, $C3^{-/-}$, or C5 deficient mice were given 200 $\mu$g of SMB19, SIbD2, or A16 intraperitoneally (i.p.) then immediately infected i.v. with A.f. conidia and monitored for survival. Mice were euthanized with $CO_2$ when they appeared moribund. For the neutropenic model of infection, C57BL/6J mice were given 200 $\mu$g of anti-Ly6G (1A8 clone hybridoma a gift from Dr. Thomas Malek) i.p. 1 day prior to infection with A.f. and 2 days post infection with A.f. On day 0, mice were passively administered 200 $\mu$g SMB19 or SIbD2 i.p., then immediately infected with 2LD50 A.f. i.v. and monitored for survival. Mice were euthanized with $CO_2$ when they appeared moribund.

**Transgenic mouse experiments.** SMB19 Tg, J558 Tg, $\mu$MT, or C57BL/6J mice were infected with $2x10^6$ A.f. conidia i.v. and monitored for survival. Mice were euthanized with $CO_2$ when they appeared moribund.

**Inhibition ELISA.** A.f. conidia were germinated in a Nunc 96-well Maxisorp plate in DMEM supplemented with 10% FCS for 8 hours at 37°C in a $CO_2$ incubator, then dried overnight at 37°C. Wells were washed with PBS and blocked with 1% BSA. 250 ng/ml SMB19 in 1% BSA was then added to each well along with 1 mg/ml of s-LNT or LNT (Carbosynth, Berkshire, UK) or 0.5 mg/ml GBSIb polysaccharide (gift from Dr. Dennis Kasper's lab) or $\alpha$-1-3 dextran (a gift from Dr. A. Jeanes) in Dulbecco's PBS (DPBS). After 2 hours incubation at 37°C, the wells were washed with PBS and incubated with goat anti-mouse IgM-AP (SBA, Birmingham, AL) for 1 hour. Plates were then washed with PBS, developed with AP substrate buffer, pH 9.8, and O.D. measured at 405 nm on a BMG Labtech microplate reader (Cary, NC).

**GBSIb type-specific PS and GBSII type-specific PS ELISAs.** Nunc 96-well maxisorp plates were coated with poly-L-lysine for 30 minutes at room temperature. Purified capsular PS from GBSIb or GBSII was diluted in DPBS and added to each well and dried overnight at 37°C. Wells were washed 3 times with PBS, and blocked with 1% BSA. Mouse anti-sera was then diluted and added to each well, then incubated for 2 hours at 37°C. Alkaline phosphatase (AP) goat anti-mouse IgM (Southern Biotech, Birmingham, AL) was used as the secondary antibody and plates were developed with alkaline phosphatase buffer, pH 9.8. O.D. was read at 405 nm on a BMG Labtech microplate reader (Cary, NC).

**SMB19 idiotype, α−1,3 dextran, and IgM$^a$ specific ELISAs.** Costar 96-well plates were coated with 1 µg/ml (SMBi26) anti-idiotype antibody to SMB19, α−1,3 dextran, or anti-IgM$^a$ (RS3.1) in PBS overnight at 4°C. Plates were then washed with PBS 3X and blocked with 1% BSA. Mouse sera were diluted in PBS, added to each well and plates were incubated for 2 hours at 37°C. AP goat anti-mouse IgM (Southern Biotech, Birmingham, AL) was used as the secondary antibody and plates were developed with alkaline phosphatase buffer, pH 9.8. O.D. was read at 405 nm on a BMG Labtech microplate reader (Cary, NC).

**Immunofluorescence Tissue Section Analysis.** Spleen sections from SMB19 Tg mice were fixed in acetone, frozen, and stained with AlexaFluor 350 anti-MOMA-1, AlexaFluor 647 anti-IgM$^a$ (RS3.1), AlexaFluor 488 anti-SMB19 (SMBi26), and PE anti-CD4 (RM4-5, BD Pharmingen) and PE anti-CD8 (53-6.7, BD Pharmingen).

Brain and kidney sections from SMB19 Tg, J558 Tg, µMT, and C57BL/6 mice were frozen and stained with AlexaFluor 647 anti-Ly6G (1A8), AlexaFluor 555 anti-CD11b (M1/70), AlexaFluor 488 anti-β 1-3 glucan (a gift from Dr. M. Feldmesser), and biotin rabbit anti-laminin (NB300-144) (Novus Biologicals, Littleton, CO). Biotin was visualized with AlexaFluor 350 streptavidin (BD Pharmingen, San Diego, CA).

**Quantitative PCR analysis.** Brain and kidney DNA was extracted from SMB19 Tg, J558 Tg, µMT, and WT C57BL/6 mice using the DNeasy Blood and tissue kit

(Qiagen, Hilden, Germany). Samples were processed as described previously [57] and analyzed on a Biorad IQ5 RT-PCR machine. Results are expressed as A.f. conidial equivalents / g tissue.

**A.f. microscopy analysis.** A.f. conidia were diluted in RPMI 1640 and seeded on glass slides. Slides contained resting A.f. conidia or A.f. that were germinated for 8 hours and fixed in 95% ethanol at 4°C. Slides were washed with PBS, blocked with 1% BSA, and stained with AlexaFluor-555 SMB19 and AlexaFluor 488 anti-β−1,3 glucan (Clone 744, IgM,κ gift from Dr. Marta Feldmesser) [58], and mounted with DAPI fluoromount. Slides were analyzed using a Leitz DMRB fluorescent microscope.

**Cytokine analysis.** Antisera from SMB19 Tg, J558 Tg, μMT, and C57BL/6J mice were collected at various time points before and after infection with A.f. Sera were diluted 3 fold and processed using the Milliplex Mouse Cytokine/Chemokine kit (Millipore). Prepared serum samples were run on the Luminex MAGPIX system and results analyzed on Milliplex Analyst software.

**CFU determination.** BMDMs were seeded in 96-well round bottom plates (why did you use round bottom? – they are easier to get off the plate and Brian always said it increased the number of immune complex-macrophage interactions) at 37°C overnight. A.f. were complexed with SMB19 or SIbD2 for 30 minutes at 4°C and incubated with BMDMs at a MOI of 5 A.f. : 1 BMDM in the presence of

1:10 diluted μMT serum containing active complement or inactive complement. After 2 hours, the supernatants from each culture were removed and remaining BMDMs in the plate were lysed with water for 10 minutes. A.f. in the supernatant and associated with the BMDMs were plated on potato dextrose agar slants and incubated overnight at 37°C. Colony forming units (CFUs) were counted after 24 hours.

**Fluorochrome-labeled A.f. uptake by BMDM.** Mouse BMDMs were cultured in complete DMEM supplemented with 10 ng/ml GM-CSF for 8 days as described previously [59]. BMDMs were then seeded onto glass coverslips in complete DMEM and grown overnight at 37°C. AlexaFluor-488 labeled A.f. conidia were complexed with SMB19 or SIbD2 for 30 minutes at 4°C and incubated with BMDMs at a MOI of 5 A.f. : 1 BMDM in the presence of 1:10 diluted μMT serum containing active complement or inactive complement. After 2 hours, the coverslips were placed on ice, washed, and stained with AlexaFluor-647 labeled anti-AlexaFluor-488 and AlexaFluor-555 labeled CD11b (M1/70) to distinguish between internalized and uninternalized A.f. conidia similar to methods previously described [60]. A.f. conidia which were internalized by BMDMs were AlexaFluor 488 positive (green), whereas A.f. that were uninternalized were double positive for AlexaFluor 488 and 647 (yellow). Coverslips were then mounted on slides with DAPI fluoromount and visualized on a Leitz DMRB fluorescent microscope.

## RESULTS

**Monoclonal antibodies to the Group B Streptococci, Type Ib (GBSIb) capsular polysaccharide bind A.f. hyphae and conidia.** We have found in recent surveys that certain mAbs, which recognize components from a variety of bacterial species, also bind to A.f. at multiple stages of its life cycle [61]. In this analysis, we found that a previously described mouse mAb, SMB19, bound to conidia (0 hr) and hyphae (12 hr) of A.f. (Fig 1A) This mAb has been shown previously to bind to a siallylated oligosaccharide, s-LNT associated with the capsular polysaccharide of GBSIb [40]. Other mAbs to members of this streptococcal group including GBSIa (SIaE7), GBSII (SIIF5C4), GBSIII, (SIIIV18), and desialylated GBSIb (SIbD2) do not bind either A.f. conidia or hyphae (Fig 1A). All mAbs bind to the type specific polysaccharides expressed by the cognate serotype of GBS bacteria (Fig 1B). The binding of SMB19 was notable in that it was brightest in intensity at the tip of the germination tube and on outgrown hyphae (Fig 1C). This analysis shows that mAbs isolated by immunizing mice with GBS bind to their type specific GBS isolates, but only SMB19 reacts with both resting conidia and outgrowing hyphae with highest intensity and preferential localization to the hyphal tips. Similar reactivity was observed with *Aspergillus flavus* and *niger* (data not shown).

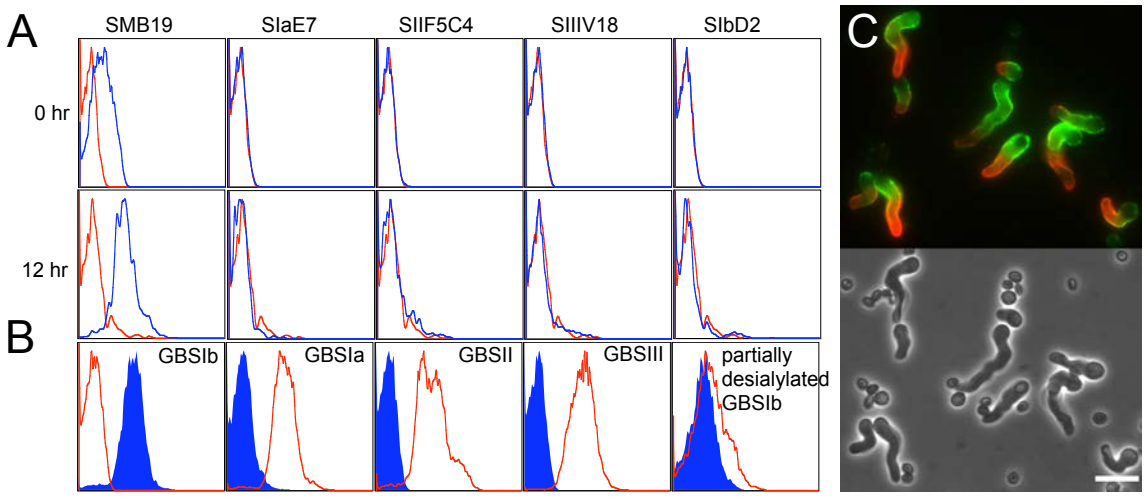



**Fig 1. GBSIb type-specific mAb, SMB19, binds to A.f. conidia (0 hr) and hyphae (12 hr).** (A) A.f. conidia or hyphae were incubated with anti-GBS mAbs SMB19 (anti-GBSIb), SIaE7 (anti-GBSIa), SIIF5C4 (anti-GBSII), SIIIV18 (anti-GBSIII) (blue) or MMA isotype control (red) then with Cy5-conjugated secondary anti IgM antibody. Binding was analyzed by flow cytometry (top two rows). (B) Various GBS serotypes described in bottom row were incubated with SMB19 (blue) or GBS type-specific IgM (red) then with Cy5-conjugated secondary antibody and analyzed for binding to their cognate serotype by flow cytometry. SIaE7 (anti-GBSIa) was used as the control (red) for SMB19 staining of GBSIb. (C) 9 hour A.f. germlings stained with SMB19 (red) and A16 (anti-$\alpha$1-3 glucan IgM, green). Lower panel, corresponding phase contrast field. White bar =10μm.

**Fine Specificity and $Ca^{++}$ dependent binding of SMB19 to the target oligosaccharide.** The terminal oligosaccharides that define the type specific polysaccharides of GBS are known and are shown in Fig 2A for types Ia [28], Ib [28], II [29], and III [30]. SMB19 binding to its target is highly specific since the oligosaccharide expressed by GBSIb differs from GBSIa and the others by having a $\beta$1→3 instead of the more common $\beta$1→4 Gal-GlcNAc link found in GBS capsular polysaccharides (Fig 2A). Additionally, SMB19 does not react with the desialyated form of GBSIb, which is associated with the oligosaccharide lacto-N-tetraose (LNT) [40]. Inhibition of SMB19 binding to A.f. with either s-LNT or GBSIb purified capsular polysaccharide (Ib PS), but not LNT or $\alpha$-1-3-dextran (DEX) confirms that SMB19 binds a similar epitope to that found on GBSIb (Fig 2B, 2C). In addition, Surface Plasmon resonance analysis was used to confirm previous observations [40] that the binding of SMB19 to the purified GBSIb capsular polysaccharide is highly dependent on the presence of calcium (Fig 2D). Treatment of A.f. hyphae with phosphoinositide phospholipase C (PI-PLC) to cleave GPI-linked proteins ablated SMB19 binding whereas 1-21 (anti-$\alpha$-1,3 glucan) binding was unaffected (Fig 2E). Thus, the binding inhibition by oligosaccharide indicates that SMB19 binding to A.f. and GBSIb is to a very similar epitope and this epitope on A.f. is likely associated with a GPI-linked protein. Additionally, SMB19 binding to its oligosaccharide antigen is calcium dependent.

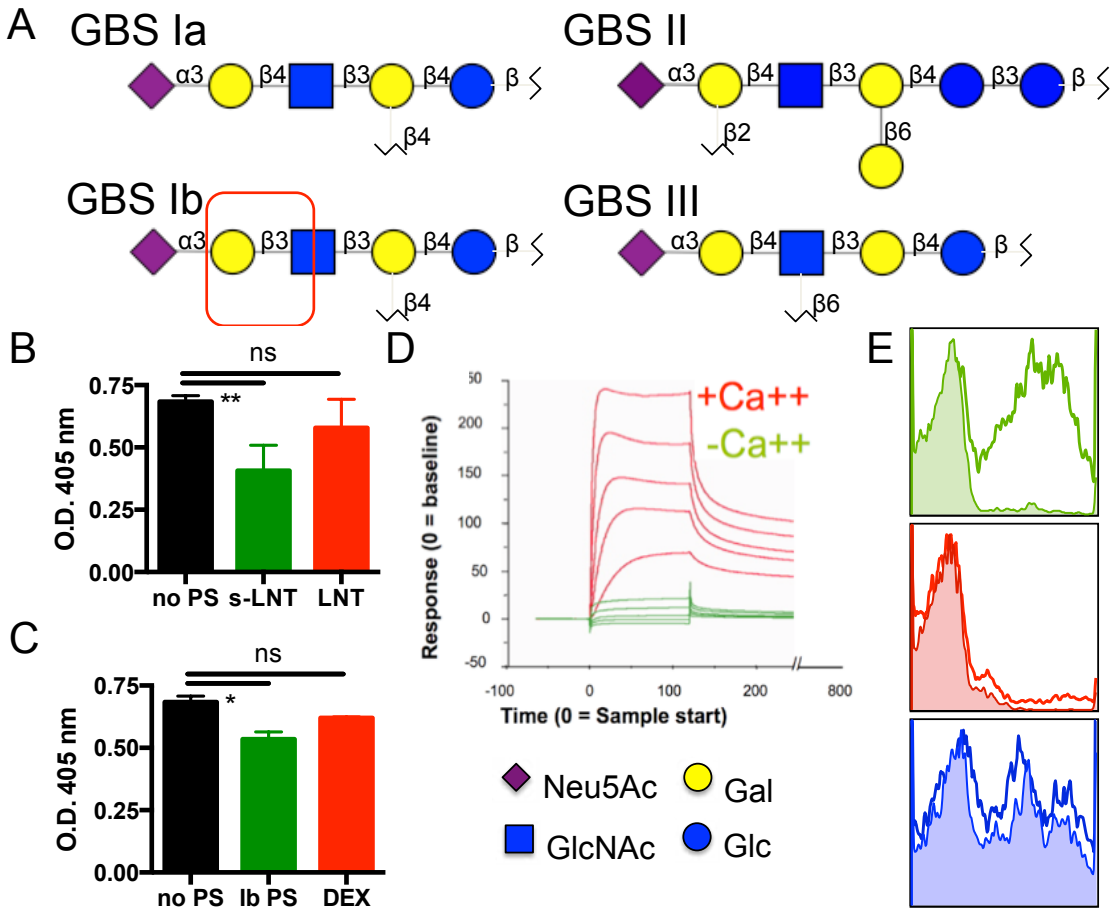



**Fig 2. SMB19 binds a calcium dependent PI-PLC sensitive epitope expressed by A.f.** (A) The SMB19-reactive oligosaccharide expressed by GBSIb differs from other GBS capsular polysaccharides by having a β1→3 instead of the more common β1→4 link. (B) ELISA plates were coated with A.f. and incubated with SMB19 (black), SMB19 plus s-LNT (green), or SMB19 plus LNT (red) then AP-labeled secondary antibody. (C) ELISA plates were coated with A.f. and incubated with SMB19 (black), SMB19 plus purified GBSIb polysaccharide (Ib PS, green), or SMB19 plus α-1,3 dextran (DEX, red) then with AP-labeled secondary antibody. SMB19 binding in both assays was analyzed by measuring the O.D. at 405 nm. Inhibition ELISAs were performed in triplicate and repeated twice with similar results *, $p<0.05$; **, $p<0.01$. (D) Surface Plasmon resonance Biacore analysis of SMB19 binding shows that binding of SMB19 to the purified GBSIb PS is highly dependent on the presence of calcium. The vertical axis (RU) measures the amount of mAb bound at each concentration during 200 sec of flow through. Sensorgrams obtained in the presence or absence of calcium are overlayed. (E) A.f. hyphae treated with PI-PLC (shaded histograms) or buffer treated (open histograms) were stained with SMB19 (green), SIbD2 (red), or 1-21 (blue) followed by secondary anti-mouse IgM-Cy5.

**Passive transfer of purified SMB19 mAb and vaccination with GBSIb improves survival in a mouse model of disseminated I.A.** Since SMB19 binds to A.f. conidia and hyphae, we determined if this antibody would protect in a mouse model of disseminated I.A. Purified SMB19 (anti-GBSIb), SIbD2 (anti-desialylated GBSIb), or A16 (anti-$\alpha$-1,3 glucan) were passively administered intraperitoneally (i.p.) to C57BL/6J mice immediately before i.v. infection with 2LD50 A.f. conidia i.v. ~75% of mice that received SMB19, but only 10-30% of mice that received either SIbD2 or A16 survived 60 days after infection (Fig 3A). There were no significant differences in protection between mice that received SIbD2 or A16. I.p. injection of SMB19 revealed that this IgM mAb has an in vivo half-life of approximately 2 days (Fig 3B), similar to that found for other IgM antibodies. These results indicate that a single treatment with SMB19 reactive with the epitope expressed by intact GBSIb type specific oligosaccharide, but not SIbD2 specific for the desialylated GBSIb oligosaccharide or A16 specific for A.f.-reactive $\alpha$-1,3 glucans improves survival against disseminated I.A. up to 60 days after infection.

Since passive SMB19 antibody gave a substantial degree of protection to i.v. administered A.f. conidia, we tested whether A.f. immunized mouse antisera contained antibody against the GBSIb type specific oligosaccharide. Interestingly, significant amounts of GBSIb type-specific oligosaccharide as well as other cell wall oligosaccharides including $\alpha$-1,3 glucan and Group A carbohydrate were not generated after A.f. infection (Supplementary Fig 1). Because the epitope on A.f. which is recognized by SMB19 did not elicit a

significant GBSIb type-specific antibody response, we performed preliminary survival studies with C57BL/6J mice that were vaccinated i.v. with GBSIb or GBSII and PBS treatment as controls in order to elicit a GBS-specific response. Six days after vaccination, at the predetermined peak of the specific anti-GBS antibody response, mice were infected i.v. with a lethal dose of A.f. conidia. PBS-treated and GBSII-immunized mice died within ~15 days whereas ~50% of GBSIb vaccinated mice survived until 60 days (at which point the experiment was ended) (Fig 3C). Thus, GBSIb vaccination significantly prolonged survival compared to PBS sham or GBSII immunized controls, showing that the protection induced is specifically associated with GBSIb vaccination. In mice that were immunized with GBSIb, there was increased GBSIb type-specific IgM in addition to SMB19 idiotype bearing antibody levels. In contrast, mice vaccinated with GBSII had ~100 μg/ml of serum GBSII reactive antibody but very little GBSIb type-specific or SMB19 idiotype positive antibody (Fig 3D-F). These results indicate that immunization of mice with GBSIb, but not GBSII promoted survival in ~50% of mice for 60 days after A.f. infection. Furthermore, this protection was associated with an increase levels of GBSIb type-specific and idiotype bearing SMB19-like antibody.

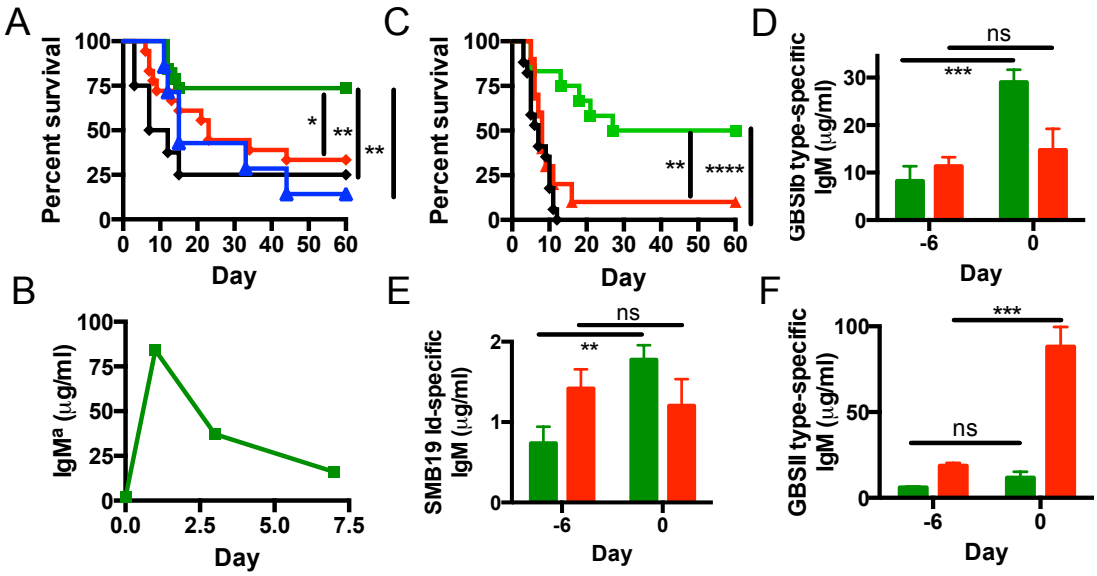



**Fig 3. Passive transfer of SMB19 or vaccination with GBSIb improves survival in a mouse model of disseminated I.A. (**A) Survival of C57BL/6 mice passively transferred with 200 μg SMB19 (green, n=19), SIbD2 (red, n=18), A16 (blue, n=7), or PBS (black, n=8) i.p. immediately before i.v. infection with 2LD50 ($2x10^6$) A.f. Results were pooled from 3 separate experiments. Asterisks denote significant differences in average survival rate compared to SIbD2, A16, and PBS control groups. (B) Serum $IgM^a$ levels were analyzed at various timepoints before and after passive transfer of 200 μg SMB19 into C57BL/6J mice by ELISA. (C) Survival of C57BL/6J mice vaccinated i.v. with GBSIb (green, n=12), GBSII (red, n=10), or PBS (black, n=17) 6 days before i.v. infection with A.f. Results are pooled from 2 separate experiments with similar results. Asterisks denote significant differences in average survival rate compared to GBSII and PBS control groups. (D) Serum GBSIb type-specific IgM, (E) SMB19 idiotype-positive IgM, and (F) GBSII type-specific IgM were quantified by ELISA immediately before (Day -6) or 6 days after (Day 0) vaccination with GBSIb (green) or GBSII (red). Asterisks denote significant differences between serum IgM levels at day -6 and day 0. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

**SMB19 transgenic mice are significantly protected against disseminated I.A.** Since passively administered antibody has a limited half-life and levels of antibody to GBSIb peaks at ~7days after immunization and then subsides we sought to study the effects in a transgenic mouse expressing the SMB19 heavy chain (Supplementary Fig 2). In this case, SMB19 idiotype positive antibody is maintained at an approximately 10 fold higher level than C57BL/6J or J558 Tg mice. Additionally, previous studies using μMT mice suggested that B cells and antibodies were associated with increased susceptibility to A.f. infection [44]. To address these issues and further understand the protective role of SMB19 in I.A., we infected SMB19 IgH transgenic (SMB19 Tg), μMT, J558 IgH transgenic (J558 Tg), or C57BL/6J mice with a lethal dose of A.f. conidia i.v. SMB19 Tg mice were highly protected compared to C57BL/6J, μMT mice, or J558 Tg control mice (Fig 4A). Interestingly, in contrast to previous reports, μMT mice, were not significantly protected from I.A. compared to C57BL/6J mice. Additionally, J558 Tg mice, which maintain high levels of anti-α-1-3 glucan antibodies (Fig 4D), a major component of the A.f. cell wall, were not significantly protected compared to C57BL/6J mice. Protection of SMB19 Tg mice correlated with significantly higher serum levels of SMB19 idiotype bearing IgM compared with J558 Tg, C57BL/6J, or μMT mice, although C57BL/6J mice and J558 Tg mice had similar levels of GBSIb PS binding serum IgM as SMB19 Tg mice. In the case of J558 Tg mice, this antibody likely arose from B cells bearing endogenous Ig gene rearrangements (Fig 4B-C). μMT mice did not have detectable levels of IgM. These results, in conjunction with the protection afforded by purified SMB19

mAb, further indicate that it is not just polyclonal GBSIb-reactive antibody (Figure 4B) but a particular subset of antibodies expressing the SMB19 idiotype that is responsible for the protection observed in this model of IA. Additionally, these results show that J558 Tg mice, even though they express antibodies to a major A.f. cell wall component, are as susceptible as C57BL/6J and μMT mice in this model of A.f. infection.

Since cytokines have been associated with either enhanced susceptibility or resistance to I.A., we further analyzed the sera from each group of mice immediately before and at days 4 and 7 post infection with A.f. for differences in cytokine levels. Interestingly, SMB19 Tg mice had significantly lower levels of granulocyte proliferation and survival cytokines G-CSF and GM-CSF, previously associated with protection in I.A. Additionally, none of the groups of mice analyzed had significantly different levels of Th1 or Th2 cytokines previously associated with protection or susceptibility to I.A., including Il-4, Il-5, Il-10, Il-2, Il-4, and IFN-γ (Supplementary Fig 3). Taken together this data shows that SMB19 Tg mice which are significantly protected from I.A. have lower serum levels of G-CSF and GM-CSF than the other groups of mice which were not protected from I.A., but did not have different levels of Th1 or Th2 associated cytokines.

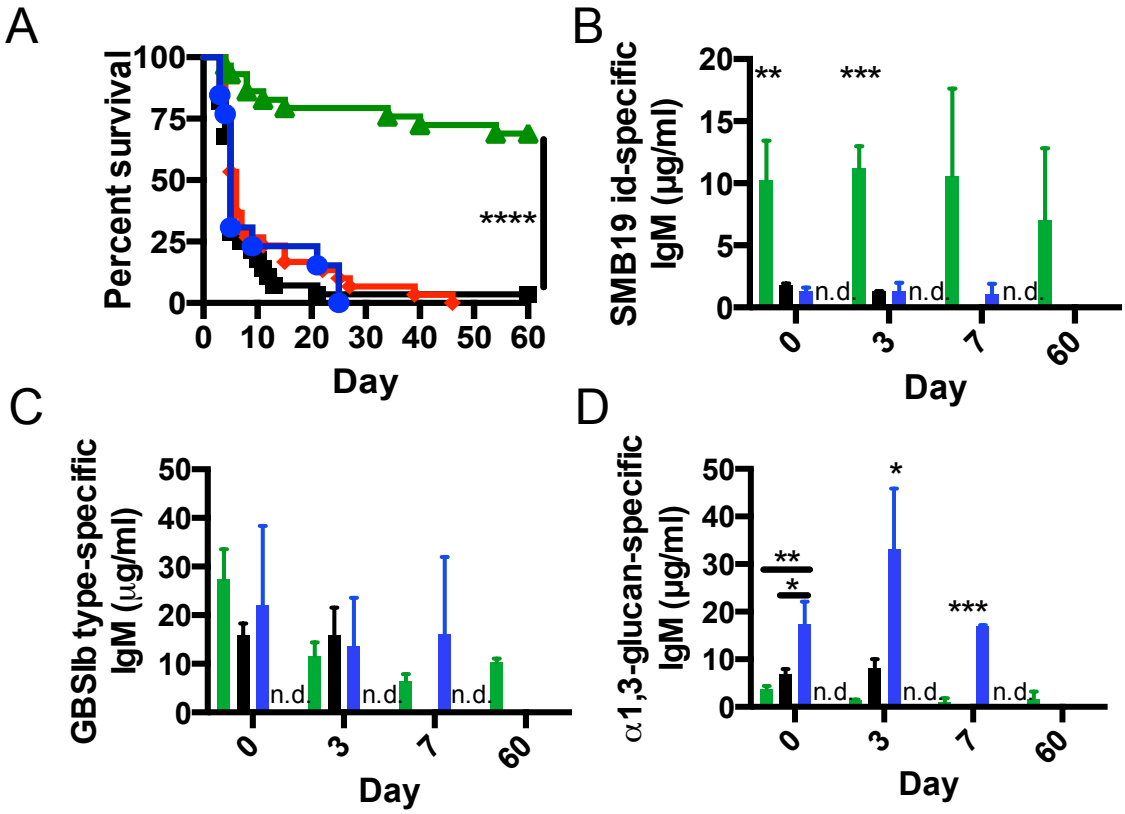



**Fig 4. SMB19 Tg mice, but not C57BL/6J, µMT, or J558 Tg mice are protected in a mouse model of disseminated I.A.** (A) Survival of C57BL/6J (black, n=27), µMT (red, n=30), J558 Tg (blue, n=13) or SMB19 Tg mice (green, n=29) infected with A.f. i.v. Results are pooled from 3 experiments with similar results. Asterisks denote significant differences in average survival rate of SMB19 Tg mice compared to J558 Tg, µMT, and C57BL/6J control groups. (B) Serum SMB19 idiotype-specific IgM, (C) GBSIb type-specific IgM, or (D) α-1,3 glucan specific IgM in C57BL/6J, SMB19 Tg, J558 Tg, or µMT mice at various time points after i.v. infection with A.f. n=3 / group. Bar colors correspond to mouse groups in Fig 5A Results are representative of 2 independent experiments with similar results. Asterisks denote significant differences in serum antibody levels between mouse groups at various time points. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$, ****, $p<0.0001$.

**SMB19 Tg survival correlates with decreased A.f. fungal burden and neutrophil infiltrates in the brain and kidneys.** Because I.A. infected SMB19 Tg mice survived significantly better than infected WT C57BL/6J, μMT, or J558 Tg mice, we determined whether this protection correlated with a decrease in fungal burden in the target tissues. It has previously been reported that brain and kidneys are the major targets of A.f. in this infection model [57], so we analyzed both tissues by immunofluorescence and quantitative PCR, as described previously. Immunofluorescence analysis revealed that SMB19 Tg mice had significantly fewer A.f. lesions in brain (Fig 5A-E) and kidneys (Fig 5G-K) compared to C57BL/6J, μMT, or J558 Tg mice at 4 days post infection. There were no detectable A.f.-containing lesions in the tissue sections analyzed in either brain or kidney by 60 days post infection. Furthermore, immunofluorescence analysis showed that A.f.-containing lesions in the brains of C57BL/6J and μMT mice were filled with leukocyte infiltrates that were CD11b and Ly6G positive, suggesting that these infiltrating cells were neutrophils. SMB19 Tg mice had only sparse or non-detectable lesions in their brains at 4 days post infection. A few of these lesions were surrounded by cells weakly expressing CD11b, but were negative for Ly6G (Fig 5D and 5J). None of these mice had visible lesions of A.f. in liver or spleen (data not shown). Quantitative PCR analysis revealed that SMB19 Tg mice had less fungal burden in their brain (Fig 5F) and kidney (Fig 5L) than WT C57BL/6J, μMT, and J558 Tg mice. These data show that survival of SMB19 Tg mice infected with A.f. correlates with a decrease in fungal burden and neutrophil infiltrates in target tissues.

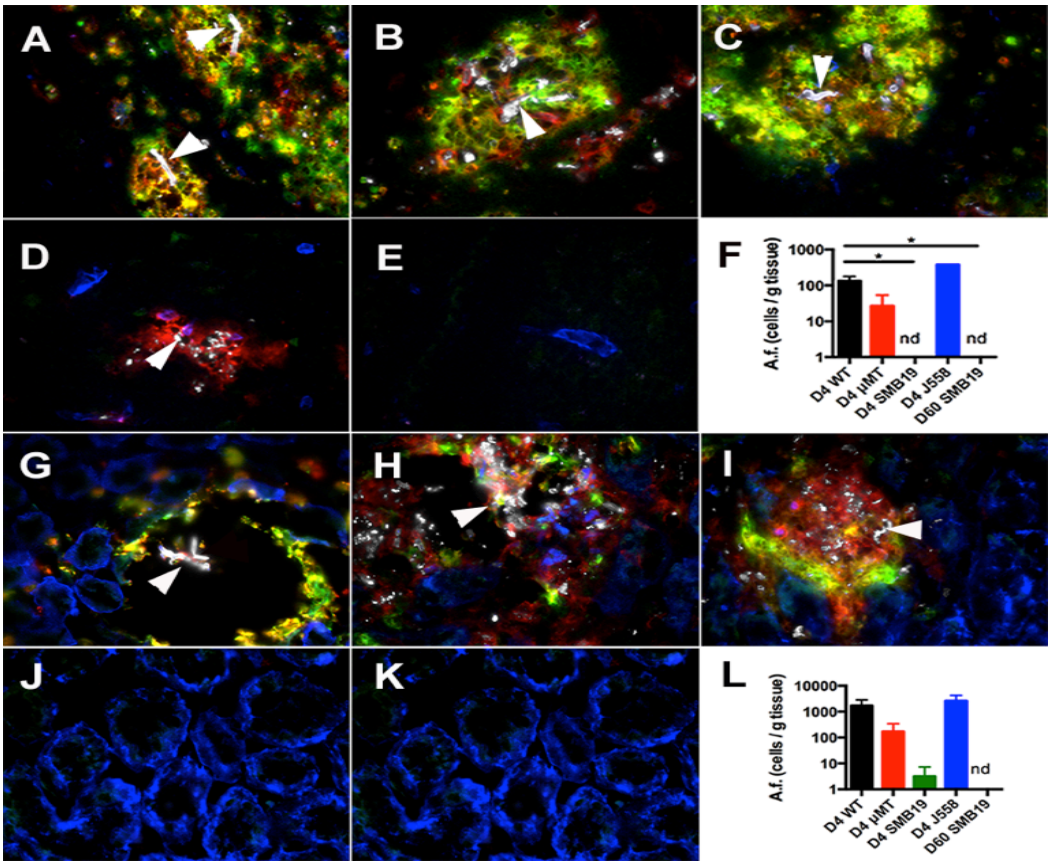



**Fig 5. SMB19 Tg mice have low or undectable A.f. fungal burden in their targeted tissues.** Immunofluorescence analysis of brain from (A) C57BL/6J, (B) μMT mice (C) J558 Tg mice at 4 days after i.v. infection with A.f. compared to SMB19 Tg at (D) 4 days, or (E) at 60 days. A similar analysis of kidney tissue from (G) C57BL/6J, (H) μMT mice (I) J558 Tg (J) compared to SMB19 Tg at (J) 4 days, or (K) 60 days after i.v. infection with A.f. Sections are stained with anti-Mac1 (red), anti-Ly6G (green), anti-β-1-3 glucan (white), and anti-laminin (blue). White arrows indicate morphologically distinct hyphae in tissues from WT C57BL/6, μMT mice and J558 Tg mice but in SMB19 mice only anti-β-1-3 glucan remnants are seen. Quantitative PCR analysis of fungal burden in (F) brain or (L) kidney of WT C57BL/6J (black), μMT (red), SMB19 Tg (green), and J558 Tg (blue) mice 4 or 60 days after infection with A.f. Asterisks denote significant differences in A.f. fungal burden. * ($p<0.05$), n=3/group, nd= not detectable. Results are representative of 2 independent experiments with similar results. The Y axis represents the number of A.f. conidial equivalents / g of infected tissue extrapolated from cycle threshold calibration curves generated by spiking known quantities of A.f. conidia into naïve brain or kidney tissues.

**Protection by SMB19 is complement dependent.** Since GBS type-specific mAbs activated the alternative complement pathway and improved opsono-phagocytosis during GBS infections leading to resistance to infection with virulent GBS [38], we hypothesized that anti-GBSIb mAbs would protect in I.A. via a similar mechanism. We passively transferred SMB19 or isotype control mAbs i.p. to $C3^{-/-}$ (Fig 8A), C5 deficient (Fig 8B) or C57BL/6J mice (Fig 8C), then immediately infected them i.v. with $1x10^6$ A.f. conidia. There were no differences in survival of $C3^{-/-}$ or C5 deficient mice that received SMB19 or isotype control mAbs, but C57BL/6J mice that received SMB19 survived better than those mice that received isotype control, similar to that shown in Fig 3A. Interestingly, in this study, $C3^{-/-}$ mice were much more susceptible to A.f. infection than either C5 deficient or C57BL/6J mice, but C5 deficient and C57BL/6J mice were similarly resistant to A.f. infection. Based on the data we obtained from the $C3^{-/-}$ and C5 deficient mice, we hypothesized that complement may be protecting mice by promoting opsonophagocytosis of A.f.

To test whether complement affected the uptake and killing of A.f. conidia in the presence of SMB19, we incubated bone marrow derived macrophages (BMDMs) from $C3^{-/-}$ mice with AlexaFluor-488 labeled A.f. conidia complexed with SMB19 or isotype control mAb in the presence of serum (containing active complement) or heat inactivated serum (containing no active complement) from μMT mice. Serum from μMT mice was used as a source of complement that was devoid of endogenous antibody. Additionally, BMDMs from $C3^{-/-}$ mice were used to eliminate the possibility that endogenous complement synthesis by the

macrophages would affect the readout. To determine whether SMB19 had an affect on uptake of A.f. conidia in this system, we plated surviving A.f. conidia in the culture supernatants after 2 hours incubation with BMDMs on plates containing potato dextrose agar and counted colony forming units (CFUs). When active complement was present, there were significantly fewer CFUs in the supernatant of cultures containing SMB19 than isotype control mAb. Additionally, there were no significant differences in CFUs from SMB19 or isotype control treated mice when active complement was absent (Fig 6D).

To support this data, we grew BMDMs on glass coverslips overnight, then incubated them for 2 hours with AlexaFluor-488 labeled A.f. conidia complexed with SMB19 or isotype control mAbs and μMT serum containing active or inactive complement at described above. After 2 hours, the coverslips were incubated on ice to prevent further uptake of the A.f by the BMDMs and the cells were stained with CD11b and Alexa Fluor 647 labeled antibody to Alexa Fluor 488, in order to distinguish extracellular A.f. conidia from those that had been engulfed. BMDM cultures that contained both active complement and SMB19 appeared to contain fewer viable and uninternalized A.f. conidia than cultures containing isotype control mAbs or inactive complement. Conidia that were internalized by the BMDMs in cultures containing SMB19 and active complement appeared smaller and in the process of being degraded. BMDMs in cultures containing active complement appeared to take up significantly more A.f. conidia than cultures containing inactive complement (Fig 6E-H). Taken together, these results show that SMB19 together with complement constitutes an important host immune

defense component in combatting I.A. with at least one protection mechanism involving opsonophagocytosis and killing of A.f. conidia by host macrophages.

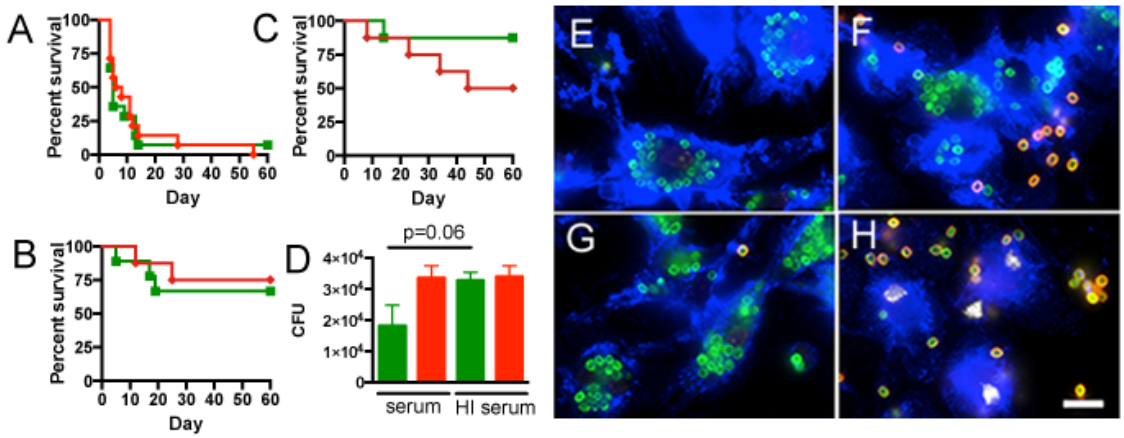



**Fig 6. Protection conferred by SMB19 during I.A. is dependent on complement.** Survival of (A) $C3^{-/-}$ (B) C5 deficient or (C) C57BL/6J mice passively transferred i.p. with 200 μg SMB19 (green) or control isotype SIbD2 (red) immediately before i.v. infection with A.f. Experiments were performed twice with similar results. (D) A.f. CFUs from supernatants of BMDM cultures after 2 hour incubation with A.f. opsonized with SMB19 (green) or SIbD2 (red) in the presence of serum from μMT mice containing active complement (serum) or inactivated complement (HI serum). BMDM uptake after a 2 hour co-culture of AlexaFluor-488 A.f. conidia opsonized with (E) SMB19 in the presence of serum or (F) HI serum. (G) Isotype control SIbD2 with serum or (H) HI serum. Green = Alexa-488 labeled A.f. conidia, red = Alexa 647 labeled Goat anti-Alexa 488 fluorochrome to detect non-phagocytosed conidia, blue = CD11b. White bar =10μm.

**SMB19 protection is partially dependent on neutrophils and independent of T cells.** Since the major human populations susceptible to I.A. are severely immunocompromised or immunosuppressed and have deficits in various immune cells, we sought to determine which cell types were necessary for SMB19-mediated protection in I.A. Since neutrophil insufficiency is a major risk factor for

susceptibility to I.A., we utilized a model of infection that renders mice transiently neutropenic. Mice were given 2 i.p. injections of neutrophil-specific anti-Ly6G mAb, one on day -1 to deplete neutrophils prior to infection, and another on day 2 after infection with A.f. to maintain neutropenia over the course of 5-6 days post infection (Fig 7A). All mice were confirmed to be neutropenic prior to A.f. infection (Fig 7B) and were given SMB19 or isotype control mAbs on day 0 immediately before i.v. infection with 2LD50 ($2.5x10^5$) A.f. SMB19 was still able to afford a degree of protection in the neutropenic mice, however, the degree was somewhat reduced from C57BL/6 mice, suggesting neutrophils are partially necessary for mediating this protection. Reduced survival of both mAb-treated groups of mice suggests, as expected, that neutrophils play an augmenting protective role in long-term survival of mice in this model of I.A. (Fig 7C).

A large amount of data suggests that T cells are necessary for long-term survival of A.f. infected mice. We next analyzed SMB19-mediated protection in I.A. by passively transferring SMB19 or isotype control antibody i.p. to TCRβ/δ-/- mice immediately before i.v. infection with 2LD50 ($2x10^6$) A.f. conidia. TCRβ/δ-/- that received SMB19 mAb were more resistant to infection than the mice that received isotype control mAb (Fig 7D). The degree of protection was similar to that of C57BL/6J mice that received SMB19 mAb vs. isotype control mAb (Fig 3A). Taken together, these results indicate that SMB19-mediated protection in I.A. is partially dependent on neutrophils and independent of T cells.

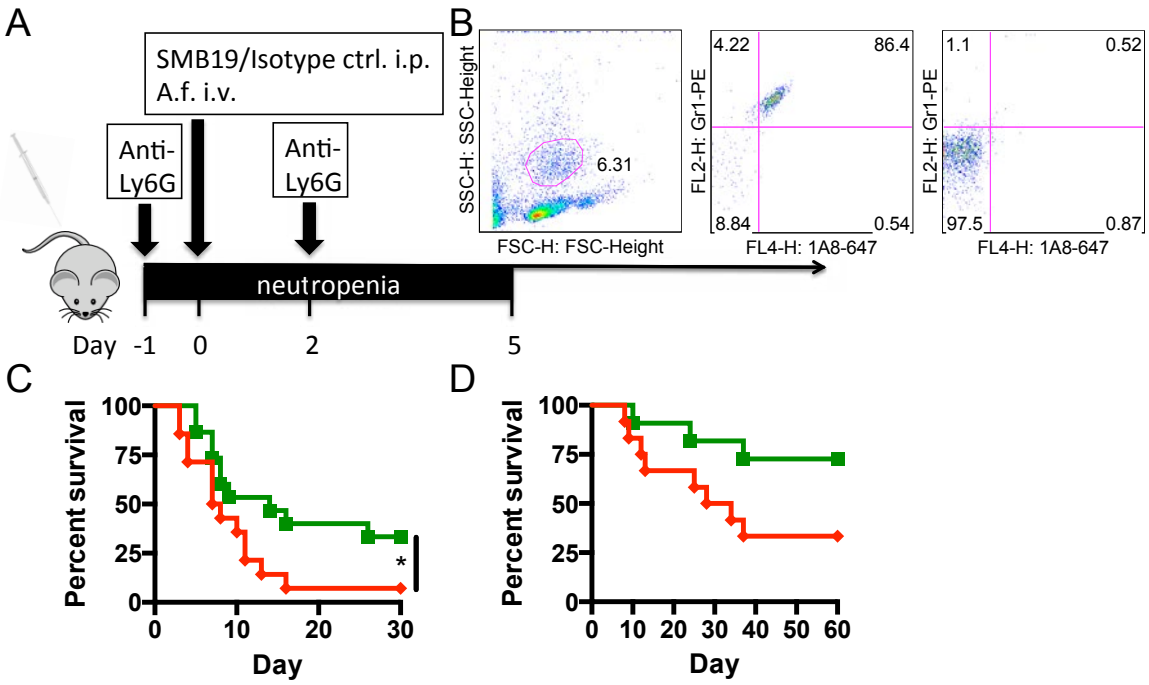



**Fig 7. SMB19 protection in I.A. is only partially impaired in the absence of neutrophils not impaired in the absence of T cells.** (A) Infection scheme in a transient neutropenic model of I.A. infection. Mice were passively transferred 200 μg anti-Ly6G i.p. 1 day prior to A.f. infection and 2 days post-infection with A.f. to deplete peripheral neutrophils. 200 μg SMB19 or isotype control mAb were given i.p. on day 0 immediately before i.v. infection with A.f. (B) Mice were confirmed to be neutropenic at the onset of A.f. infection. FSC-SSC profile of peripheral blood neutrophils in B6 mice (left panel). Peripheral blood from non-neutropenic mouse (middle panel) and anti-Ly6G neutrophil depleted mouse (right panel) were stained with AlexaFluor-647 labeled anti-Ly6G and PE-labeled anti-Gr-1 and analyzed for neutrophil depletion by flow cytometry. (C) Survival of transiently neutropenic WT mice that were passively administered 200 μg SMB19 (green, n=15) or SIbD2 (red, n=14) i.p. immediately before infection with 2LD50 ($2.5x10^5$) A.f. conidia i.v. (D) Survival of TCRβ/δ-/- mice that were passively administered SMB19 (green, n=11) or SIbD2 (red, n=12) i.p. immediately before i.v. infection with 2LD50 ($2x10^6$) A.f. conidia. Asterisks denote significant differences in average survival rate of mice passively transferred SMB19 compared to isotype control. *, $p<0.05$.

## DISCUSSION

Antibodies and B cells historically have not been associated with protection in I.A. due to the lack of an association between A.f. antibody titers and protection in I.A. and partially due to a study showing that μMT mice were less susceptible to I.A. than WT mice [44]. However, recent studies on the role of mAbs in protection against A.f., especially those against β−1,3 glucans [45], have generated new interest in this field. Few studies, however, have identified new epitopes on A.f. that could be targeted therapeutically with mAbs. A few reports in the past have studied the role of sialic acid-associated epitopes on the surface of A.f. in host interactions. Although hypothesized to be major virulence factors of A.f., the only evidence in support of this hypothesis has been the association of higher levels of surface sialic acids on the more virulent strains of Aspergillus [33]. To address in more detail the role of these sialic acid associated epitopes on A.f., we chose to investigate whether mAbs generated against various GBS serotypes, expressing the same sialic acid moiety, neuraminic acid (Neu5Ac) on their capsular polysaccharides as A.f., would bind to A.f. conidia or hyphae. Our results show that SMB19, a mAb generated against GBSIb, also bound to resting A.f. conidia, and more strongly to A.f. hyphae. This mAb binds a sialic acid associated epitope distinct from the sialic acid containing oligosaccharides found on other GBS serotypes by its preference for the GBSIb type specific oligosaccharide containing a β−1-3 instead of β−1-4GalNAc-GlcNAc characteristic of the other subtypes. The fact that SMB19 bound specifically to the tips of A.f. hyphae is particularly interesting since in this part of the growing

hyphae the nascent cell wall is especially vulnerable to external influences and as such is the target site for certain antifungal agents [62, 63] The observation that binding of SMB19 to its oligosaccharide antigen is calcium dependent may also have some bearing on SMB19-binding since proper outgrowth of fungal hyphal tips relies on high cytoplasmic free calcium gradients and disregulation of these calcium gradients results in inhibition of hyphal growth and changes in tip morphology [17]. In light of these studies, it is possible that SMB19 binding is facilitated by high levels of calcium flux and contributes to the unique protective properties of this antibody.

Based on these findings, we sought to determine whether mAbs generated against GBSIb could protect in a murine model of I.A. Our data shows that passive administration of SMB19 protected better in this model of disseminated I.A. than isotype control (anti-desialylated GBSIb polysaccharide) or A16 (anti-$\alpha$-1-3 glucan) mAbs and furthermore C57BL/6J mice vaccinated with GBSIb, which induced moderate increases of SMB19 idiotype positive antibody, which despite relatively short-lived nature of the antibody titers were protected to a greater extent that the mice that received passive antibody. Survival after A.f, infection in SMB19 Tg mice, which maintain constant high levels of SMB19 idiotype positive IgM, was even better. Although there were similar levels of antibodies in the serum of all groups of mice to GBSIb polysaccharide, only SMB19 Tg mice expressed high titers of SMB19 idiotype positive antibody. Thus, it appears that not all GBSIb polysaccharide reactive-antibodies are protective and that it is the SMB19 or SMB19-like antibody portion of the response that is

critical in providing protection against A.f. infection. It is also of great interest that J558 Tg mice, which maintain high titers of antibodies against $\alpha$-1-3 glucans, a major component of the A.f. cell wall, are not protected in this model of A.f.. highlighting the unique role of SMB19 idiotype bearing antibodies and its target antigen in mediating the observed protection. Interestingly, in our study B cell deficient μMT mice on the C57BL/6 background mice were equally susceptible to I.A. compared to WT C57BL/6 mice. This discrepancy could perhaps be due to the use of BALB/c μMT mice in previous studies since these mice, have recently been shown to make both mature B cells and plasma cells and produce normal levels of antibody isotypes, excluding IgM [64]. Survival of SMB19 Tg mice correlated with decreased fungal load in the brain and kidneys compared with J558 Tg, C57BL/6J and μMT mice and their brains and kidneys were devoid of neutrophil infiltrates and abscesses containing actively growing hyphae. Rarely, we found small areas in the brain of SMB19 Tg mice at 4 days post infection that contained what appeared to be A.f. remnants surrounded by mac1+ and Ly6G- cells, which could be activated resident brain cells such as microglia. This suggests that some A.f. may cross the blood brain barrier in SMB19 Tg mice, but not to such an extent that it cannot be cleared within a few days after infection.

Previous studies on the role of sialic acids expressed by GBS in host interactions have suggested that sialic acids are virulence factors because they promote evasion of the host complement system perhaps due to molecular mimicry of sialic acid associated epitopes on host cells. Regardless of the reason, high titers of type-specific GBS antibodies are protective in GBS

infections by activating complement and promoting opsonophagocytosis [38]. Of the few studies directed to the role of sialic acids or complement in I.A., one recently showed that although there is increased complement production in the CNS during disseminated I.A., C3 deposition on A.f. hyphae remains low. In addition, higher complement deposition correlated with enhanced opsonophagocytosis and killing of A.f. [65]. Furthermore, it was recently shown that patients with I.A. had lower serum levels of mannose binding lectin than immunocompromised control patients and mannose binding lectin deficiency was more common among patients with I.A. than controls [66], suggesting that complement is an important defense mechanism in I.A. Our data clearly show that $C3^{-/-}$ and C5 deficient mice, in contrast to WT mice, are not protected from I.A. after passive administration of SMB19, indicating that complement activation is, at least in part, responsible for the therapeutic effect of SMB19 antibody. In addition, in vitro experiments with BMDMs indicate that SMB19 plus active complement promotes uptake of A.f., as indicated by the decrease in supernatant CFU after 2 hours incubation. The enhanced uptake of SMB19 complexed A.f. by macrophages could indicate that these conidia get taken up by different receptors on macrophages, by enhanced interactions with Mac1 (CR3), compared with non-SMB19 complexed conidia. Swelling of conidia has to occur before tethering and uptake by Dectin-1. SMB19 complexed A.f. also appear to be killed quicker than isotype control complexed A.f. as is indicated by a decrease in total culture CFU compared to isotype control mAb (data not shown). Microscopy supported this finding since phagocytosed SMB19 complexed A.f.

conidia appeared smaller and more fragmented than isotype control treated conidia suggesting that they were being degraded after 2 hour incubation. Also of interest is that contrary to previous studies on C5 in I.A., our C5 deficient mice were no more susceptible to I.A. than WT mice. In our study, we used C5 deficient mice on the C57BL/6 background compared to WT C57BL/6 mice, whereas in the previous study, DBA/2N mice, which are deficient in C5 were compared with CFW mice [67]. This could possible contribute to the differences in survival noted between these studies after infection with A.f.

The protection against I.A. afforded by SMB19 antibodies under transiently neutropenic conditions is particularly important since a major risk factor for becoming susceptible to I.A. is severe or prolonged neutropenia. Because SMB19-mediated protection is dependent on C3, it is not surprising that neutrophils play a role in protection since C3 receptor (CR3 / Mac1) is found predominantly on neutrophils. The partial protection observed in the neutropenic mice was likely due to CR3 on macrophages or dendritic cells phagocytosing the SMB19 and C3 opsonized A.f. conidia. Many studies have implicated T cell immunity as particularly important in clearing infections of A.f. That SMB19 antibodies did not depend on T cells for the immediate protection in this model of acute A.f. infection is of interest since this type of immunodeficiency is similar to that found in AIDS patients with CD4 T cell exhaustion. However TCR$\beta/\delta$-/- mice were no more susceptible to A.f. infection that WT mice (data not shown), suggesting that the role of T cells, at least in this model of infection, appeared to

be dispensable, similar to previous human studies where I.A. among AIDS patients is a relatively rare occurrence [68].

The antibody responses against the GBSIb polysaccharide are mostly IgM as usual for these kinds of antigens in mice. These are short-lived and antibody titers begin to subside after 7 days post-vaccination. However identification of the fine details of the SMB19 epitope will facilitate production of a vaccine that will produce longer lasting IgG and memory B cell responses. Additionally, although vaccination with GBSIb is an attractive idea for treatment for I.A., the efficacy of such a treatment, as with any vaccine, could be limited based on the type of underlying condition or immunosuppression undergone by individual I.A. patients. In this case passive immunotherapy could be effective prior to at risk procedures for A.f. infection. However in patients with the capacity to mount an antibody response, active immunization is feasible since GBS polysaccharide conjugate vaccines against GBS serotypes I-V have already been developed and validated through phase II clinical trials in humans [69, 70].

There are several additional strategies to pursue regarding the development of SMB19 like antibodies as a potential active or passive therapy therapeutic for patients at risk for I.A. Passive antibody could be combined with other antifungal therapies such as amphotericin B, voriconazole, and caspofungin which are administered as standard treatments for I.A. patients [71]. Amphotericin B and voriconazole target ergosterol synthesis on the A.f. membrane whereas caspofungin targets β-1,3-D-glucan synthase enzymatic activity, which also ultimately affects A.f. membrane synthesis. A recent study

showed that mAbs against anti-β-1,3 glucans had a modest inhibitory effect on fungal growth in vitro. In limited studies we found that SMB19 did not appear to have a direct effect on in vitro growth of A.f. Recent reports that administration of various cytokines such as G-CSF, GM-CSF, and recombinant IFN-γ which promote neutrophil, macrophage, and dendritic cell proliferation and activation have been used in A.f. infected patients [72]. Additionally, there have been promising results administering Il-12 to promote Th1 responses or neutralizing Il-4 or Il-10 to down-modulate Th2 responses [73, 74]. Because SMB19 antibody protection is not dependent on neutrophils and T cells, combination of SMB19 with these cytokines may also synergistically prolong survival during I.A.

A second therapeutic strategy still left to pursue for SMB19 like antibodies and its target antigens is its potential in protecting against other clinically relevant fungal pathogens which also express Neu5Ac-bearing epitopes on their surface including *Cryptococcus neoformans* and *Candida albicans* [48, 75]. One emerging idea in the fungal field is that antibodies against conserved targets on different fungal pathogens are “globally” protective [14]. Our preliminary studies show that SMB19 antibody also shows intense staining of hyphal tips of Candida albicans during its hyphal growth stage so that SMB19 may follow a similar paradigm in which it may be “globally” protective against its “conserved” target found on GBSIb, A.f., as well as other clinically relevant fungi.

## ACKNOWLEDGMENTS

We thank Lisa Jia, Emily Stefanov, and Jeffrey Sides for helping with several studies in this paper. We also thank David Pritchard, Thomas Malek, and Marta Feldmesser for providing hybridomas, and Dennis Kasper for providing the purified GBSIb and GBSII capsular polysaccharides.

## REFERENCES

1. Hohl, T.M. and M. Feldmesser, *Aspergillus fumigatus: principles of pathogenesis and host defense.* Eukaryot Cell, 2007. **6**(11): p. 1953-63.

2. Cutler, J.E., G.S. Deepe, Jr., and B.S. Klein, *Advances in combating fungal diseases: vaccines on the threshold.* Nat Rev Microbiol, 2007. **5**(1): p. 13-28.

3. Montagnoli, C., et al., *Immunity and tolerance to Aspergillus involve functionally distinct regulatory T cells and tryptophan catabolism.* J Immunol, 2006. **176**(3): p. 1712-23.

4. Fontaine, T., et al., *Galactosaminogalactan, a new immunosuppressive polysaccharide of Aspergillus fumigatus.* PLoS Pathog, 2011. **7**(11): p. e1002372.

5. Latge, J.P., et al., *Chemical and immunological characterization of the extracellular galactomannan of Aspergillus fumigatus.* Infect Immun, 1994. **62**(12): p. 5424-33.

6. de Repentigny, L., et al., *Acquired immunity in experimental murine aspergillosis is mediated by macrophages.* Infect Immun, 1993. **61**(9): p. 3791-802.

7. Montagnoli, C., et al., *A role for antibodies in the generation of memory antifungal immunity.* Eur J Immunol, 2003. **33**(5): p. 1193-204.

8. Torosantucci, A., et al., *Protection by anti-beta-glucan antibodies is associated with restricted beta-1,3 glucan binding specificity and inhibition of fungal growth and adherence.* PLoS One, 2009. **4**(4): p. e5392.

9. Rachini, A., et al., *An anti-beta-glucan monoclonal antibody inhibits growth and capsule formation of Cryptococcus neoformans in vitro and exerts therapeutic, anticryptococcal activity in vivo.* Infect Immun, 2007. **75**(11): p. 5085-94.

10. Chaturvedi, A.K., et al., *Monoclonal immunoglobulin G1 directed against Aspergillus fumigatus cell wall glycoprotein protects against experimental murine aspergillosis.* Clin Diagn Lab Immunol, 2005. **12**(9): p. 1063-8.

11. Blanco, J.L. and M.E. Garcia, *Immune response to fungal infections.* Vet Immunol Immunopathol, 2008. **125**(1-2): p. 47-70.

12. Alviano, C.S., L.R. Travassos, and R. Schauer, *Sialic acids in fungi: a minireview.* Glycoconj J, 1999. **16**(9): p. 545-54.

13. Doran, K.S. and V. Nizet, *Molecular pathogenesis of neonatal group B streptococcal infection: no longer in its infancy.* Mol Microbiol, 2004. **54**(1): p. 23-31.

14. Warwas, M.L., et al., *Structure and role of sialic acids on the surface of Aspergillus fumigatus conidiospores.* Glycobiology, 2007. **17**(4): p. 401-10.

15. Marques, M.B., et al., *Prevention of C3 deposition by capsular polysaccharide is a virulence mechanism of type III group B streptococci.* Infect Immun, 1992. **60**(10): p. 3986-93.

16. Edwards, M.S., M.R. Wessels, and C.J. Baker, *Capsular polysaccharide regulates neutrophil complement receptor interactions with type III group B streptococci.* Infect Immun, 1993. **61**(7): p. 2866-71.

17. Foote, J.B. and J.F. Kearney, *Generation of B cell memory to the bacterial polysaccharide alpha-1,3 dextran.* J Immunol, 2009. **183**(10): p. 6359-68.

18. Pritchard, D.G., B.M. Gray, and M.L. Egan, *Murine monoclonal antibodies to type Ib polysaccharide of group B streptococci bind to human milk oligosaccharides.* Infect Immun, 1992. **60**(4): p. 1598-602.

19. Chen, X., et al., *Evidence for selection of a population of multi-reactive B cells into the splenic marginal zone.* Int Immunol, 1997. **9**(1): p. 27-41.

20. Martin, F., X. Chen, and J.F. Kearney, *Development of VH81X transgene-bearing B cells in fetus and adult: sites for expansion and deletion in conventional and CD5/B1 cells.* Int Immunol, 1997. **9**(4): p. 493-505.

21. Circolo, A., et al., *Genetic disruption of the murine complement C3 promoter region generates deficient mice with extrahepatic expression of C3 mRNA.* Immunopharmacology, 1999. **42**(1-3): p. 135-49.

22. Kearney, J.F., et al., *A new mouse myeloma cell line that has lost immunoglobulin expression but permits the construction of antibody-secreting hybrid cell lines.* J Immunol, 1979. **123**(4): p. 1548-50.

23. Bowman, J.C., et al., *Quantitative PCR assay to measure Aspergillus fumigatus burden in a murine model of disseminated aspergillosis: demonstration of efficacy of caspofungin acetate.* Antimicrob Agents Chemother, 2001. **45**(12): p. 3474-81.

24. Hohl, T.M., et al., *Aspergillus fumigatus triggers inflammatory responses by stage-specific beta-glucan display.* PLoS Pathog, 2005. **1**(3): p. e30.

25. Oliva, C.R., et al., *The integrin Mac-1 (CR3) mediates internalization and directs Bacillus anthracis spores into professional phagocytes.* Proc Natl Acad Sci U S A, 2008. **105**(4): p. 1261-6.

26. Oliva, C., C.L. Turnbough, Jr., and J.F. Kearney, *CD14-Mac-1 interactions in Bacillus anthracis spore internalization by macrophages.* Proc Natl Acad Sci U S A, 2009. **106**(33): p. 13957-62.

27. Kin, N.W., et al., *Antibodies generated against conserved antigens expressed by bacteria and allergen-bearing fungi suppress airway disease.* J Immunol, 2012. **189**(5): p. 2246-56.

28. Jennings, H.J., et al., *Structure of native polysaccharide antigens of type Ia and type Ib group B Streptococcus.* Biochemistry, 1983. **22**(5): p. 1258-64.

29. Jennings, H.J., et al., *Structural determination of the capsular polysaccharide antigen of type II group B Streptococcus.* J Biol Chem, 1983. **258**(3): p. 1793-8.

30. Wessels, M.R., et al., *Structure and immunochemistry of an oligosaccharide repeating unit of the capsular polysaccharide of type III group B Streptococcus. A revised structure for the type III group B streptococcal polysaccharide antigen.* J Biol Chem, 1987. **262**(17): p. 8262-7.

31. Edwards, M.S., et al., *The role of specific antibody in alternative complement pathway-mediated opsonophagocytosis of type III, group B Streptococcus.* J Exp Med, 1980. **151**(5): p. 1275-87.

32. Wasylnka, J.A., M.I. Simmer, and M.M. Moore, *Differences in sialic acid density in pathogenic and non-pathogenic Aspergillus species.* Microbiology, 2001. **147**(Pt 4): p. 869-77.

33. Ansheng, L., et al., *Study on the hyphal responses of Aspergillus fumigatus to the antifungal agent by Bio-Cell Tracer.* Mycopathologia, 1999. **148**(1): p. 17-23.

34. Bowman, J.C., et al., *The antifungal echinocandin caspofungin acetate kills growing cells of Aspergillus fumigatus in vitro.* Antimicrob Agents Chemother, 2002. **46**(9): p. 3001-12.

35. Jackson, S.L. and I.B. Heath, *Roles of calcium ions in hyphal tip growth.* Microbiol Rev, 1993. **57**(2): p. 367-82.

36. Hasan, M., et al., *Incomplete block of B cell development and immunoglobulin production in mice carrying the muMT mutation on the BALB/c background.* Eur J Immunol, 2002. **32**(12): p. 3463-71.

37. Rambach, G., et al., *Complement induction and complement evasion in patients with cerebral aspergillosis.* Microbes Infect, 2008. **10**(14-15): p. 1567-76.

38. Lambourne, J., et al., *Association of mannose-binding lectin deficiency with acute invasive aspergillosis in immunocompromised patients.* Clin Infect Dis, 2009. **49**(10): p. 1486-91.

39. Hector, R.F., E. Yee, and M.S. Collins, *Use of DBA/2N mice in models of systemic candidiasis and pulmonary and systemic aspergillosis.* Infect Immun, 1990. **58**(5): p. 1476-8.

40. Denning, D.W., et al., *Pulmonary aspergillosis in the acquired immunodeficiency syndrome.* N Engl J Med, 1991. **324**(10): p. 654-62.

41. Baker, C.J. and M.S. Edwards, *Group B streptococcal conjugate vaccines.* Arch Dis Child, 2003. **88**(5): p. 375-8.

42. Edwards, M.S., *Group B streptococcal conjugate vaccine: a timely concept for which the time has come.* Hum Vaccin, 2008. **4**(6): p. 444-8.

43. Herbrecht, R., et al., *Voriconazole versus amphotericin B for primary therapy of invasive aspergillosis.* N Engl J Med, 2002. **347**(6): p. 408-15.

44. Segal, B.H. and T.J. Walsh, *Current approaches to diagnosis and treatment of invasive aspergillosis.* Am J Respir Crit Care Med, 2006. **173**(7): p. 707-17.

45. Reichenberger, F., et al., *Diagnosis and treatment of invasive pulmonary aspergillosis in neutropenic patients.* Eur Respir J, 2002. **19**(4): p. 743-55.

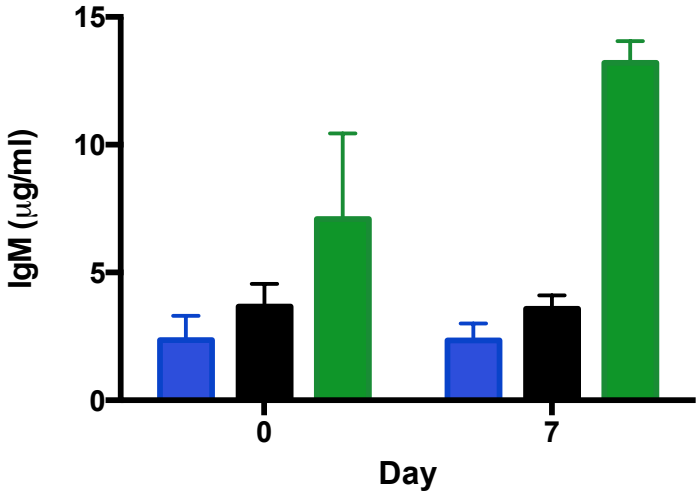
46. Stevens, D.A., *Combination immunotherapy and antifungal chemotherapy.* Clin Infect Dis, 1998. **26**(6): p. 1266-9.

47. Rodrigues, M.L., et al., *Identification of N-acetylneuraminic acid and its 9-O-acetylated derivative on the cell surface of Cryptococcus neoformans: influence on fungal phagocytosis.* Infect Immun, 1997. **65**(12): p. 4937-42.

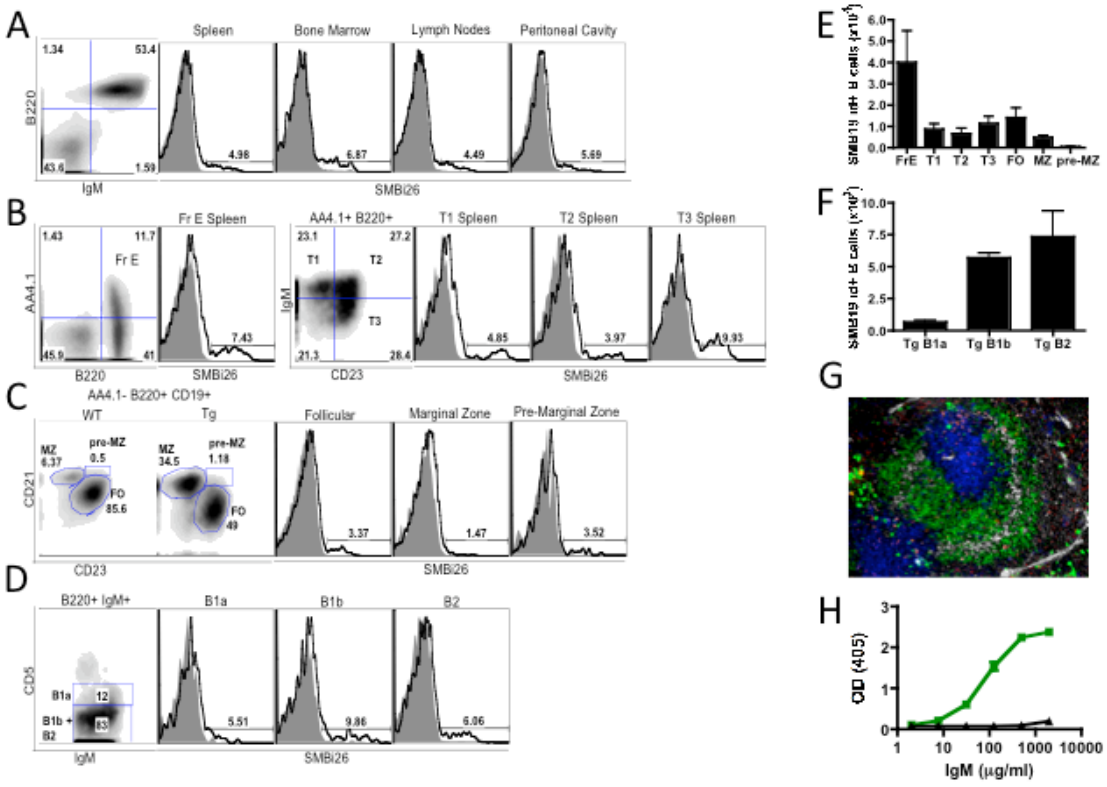
48. Casadevall, A. and L.A. Pirofski, *Antibody-mediated protection through cross-reactivity introduces a fungal heresy into immunological dogma.* Infect Immun, 2007. **75**(11): p. 5074-8.

49. Totolian, A.A., *Pathogenic Streptococci: Present and Future : Proceedings of the XIIth Lancefield International Symposium on Streptococci and Streptococcal Diseases, St.Petersburg, Russia September 6-10, 1993*1994: Lancer Publications.

50. Kearney, J.F., et al., *Induction of germ-line anti-alpha 1-3 dextran antibody responses in mice by members of the Enterobacteriaceae family.* J Immunol, 1985. **135**(5): p. 3468-72.




**Supplementary Fig 1. Infection with A.f. does not elicit significant anti-polysaccharide antibody responses.** Sera from mice pre- and 7 days post-infection with 2LD50 A.f. i.v. were analyzed by ELISA for reactivity with α-1,3 dextran (blue), Group A carbohydrate (black), and GBSIb polysaccharide (green). Experiment was repeated twice with similar results. N=3.

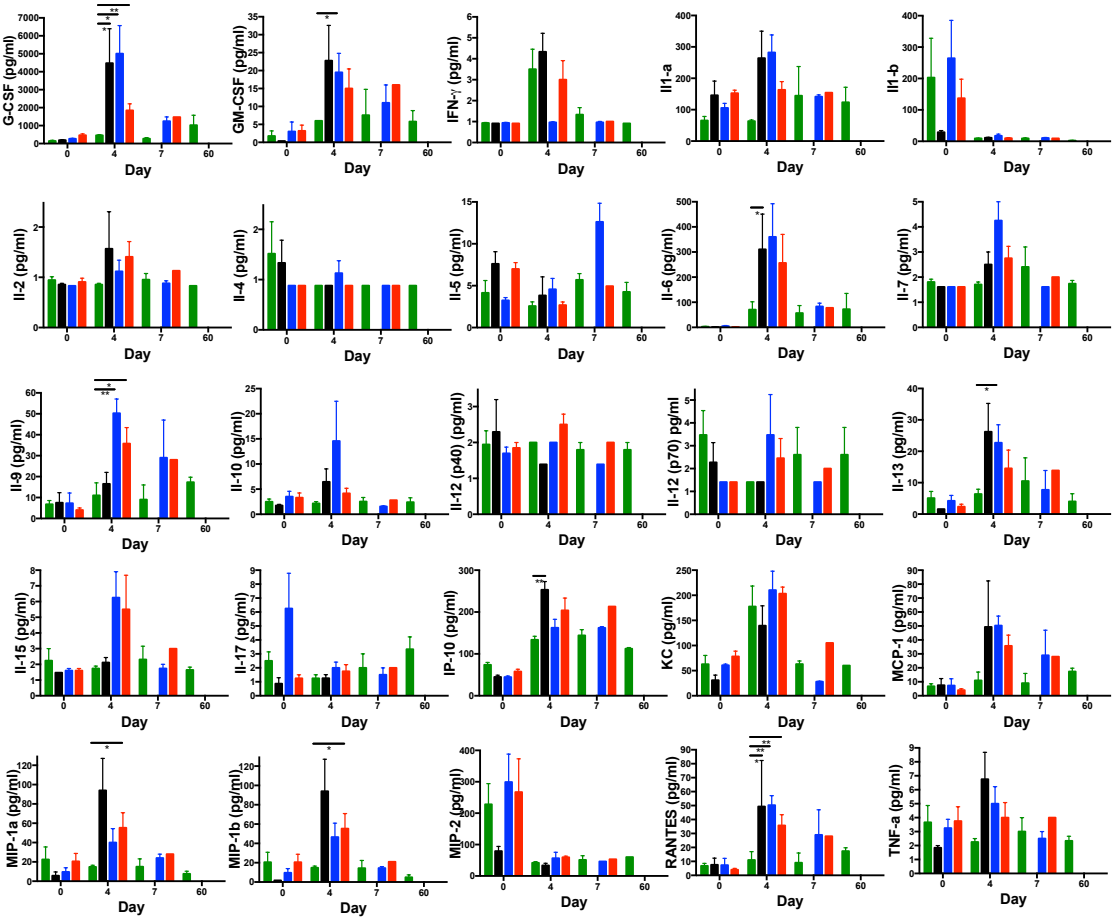



**Supplementary Fig 2. SMB19 Id+ B cells in the peritoneal cavity are enriched in the B1b and B2 subsets, but are not enriched in any subset in the spleen.** A, FACS analysis of SMB19 Id expression on B220+IgM+ cells in representative samples of the spleen, bone marrow, mesenteric lymph nodes, and peritoneal cavity using SMBi26, a rat anti-SMB19 anti-idiotype antibody. B, FACS analysis of SMB19 Id+ enrichment in the immature fraction E, T1, T2, and T3 subsets of the spleen. C, FACS analysis of SMB19 Id+ enrichment in the follicular, marginal zone, and pre-marginal zone mature B cell subsets of the spleen. D, FACS analysis of SMB19 id+ enrichment in the B1a, B1b, and B2 compartments of the peritoneal cavity. For panels A-D, open histograms represent SMB19 Id+ B cells in the VH SMB19 Tg mice and shaded histograms represent SMB19 Id+ B cells in WT littermate control mice. E, Total number of SMB19 Id+ B cells in splenic B cell populations. F, Total number of SMB19 Id+ B cells in peritoneal cavity B cell populations. G, Spleen sections from VH SMB19 Tg mice were stained with anti-IgMa (green), anti-SMB19 Id (red), anti-Moma-1 (white), and anti-CD4+anti-CD8 (blue). H, ELISA analysis of SMB19 (green) or SIaE7 (black) binding to SMB19 anti-idiotype mAb, SMBi26. Experiments were performed in triplicate twice with similar results.

**Supplementary Table 1: Mab clone names, isotype, and antigen specificities**

| Clone name | Specificity | Species | Isotype | References |
|---|---|---|---|---|
| SMB19[1] | Anti-GBSIb | Mouse, BALB/c | IgM,κ | [40] |
| SIaE7 | Anti-GBSIa | Mouse, BALB/c | IgM,κ | [76] |
| SIIF5C4 | Anti-GBSII | Mouse, BALB/c | IgM,κ | [76] |
| SIIIV18 | Anti-GBSIII | Mouse, BALB/c | IgM,κ | [76] |
| SIbD2 | Anti-desialylated GBSIb | Mouse, BALB/c | IgM,κ | [40] |
| A16 | Anti-α-1,3 glucan | Mouse, BALB/c | IgM, λ | [77] |
| 1-21 | Anti-α-1,3 glucan | Mouse, BALB/c | IgM, λ | [77] |
| 744 | Anti-β-1,3 glucan | Mouse, BALB/c | IgM,κ | [58] |
| SMBi26 | Anti-SMB19 | Rat | IgG1 | |

[1] IgM antibodies were purified from supernatants of hybridomas grown in Gibco serum free RPMI1640 by affinity chromatography over RS3.1 mouse anti-IgM$^a$-Sepharose affinity columns. IgG antibodies were purified by passage over Protein G-conjugated Sepharose beads. All antibodies were eluted with 0.1M Glycine-HCL in PBS, concentrated and buffer exchanged into PBS using Centricon Plus-70 centrifugal filters (Millipore). For quality control, purified antibodies were subjected to SDS-PAGE under reducing and non-reducing conditions to ensure correct molecular weight, as well as ELISA for antigen-binding. Purified antibody preparations were tested for endotoxin levels before injection by Limulus assay (Limulus Amebocyte Lysate Pyrogent, Lonza, Walkersville, MD) and were below the limits of detection.




**Supplementary Fig 3. SMB19 Tg mice have lower levels of pro-inflammatory cytokines in their sera after A.f. infection compared to control groups.** Luminex analysis of (A) Th1 and Th2 serum cytokine levels and (B) neutrophil proliferation-asociated and survival serum cytokine levels in SMB19 Tg (green), WT (black), J558 Tg (blue), and μMT (red) mice at various time points after i.v. infection with A.f. *, $p<0.05$, **, $p<0.01$. Asterisks denote significant differences in cytokine levels between SMB19 Tg mice and control groups on day 4 post A.f. infection. Results are representative of 2 independent experiments with similar results.

## SUMMARY DISCUSSION

GBS has been recognized as an important clinically relevant pathogen for several decades now and it is well established that type-specific GBS antibodies are important host immune defenses in the clearance of this pathogen. Although many studies have elucidated that these antibodies are important in activation of the alternative complement pathway and opsonophagocytic clearance of GBS, few studies have addressed many other properties of this class of antibodies including their calcium dependent binding and reactivity with conserved antigens on self components as well as on other pathogens. While our lab has a general interest in studying antibodies to bacterial carbohydrate antigens, we became interested in studying a particular mAb against GBSIb, SMB19, for the reasons mentioned above.

In our study of SMB19, we determined that it specifically reacted with not only with the GBSIb capsular oligosaccharide s-LNT in a calcium dependent manner (A Fig 1), but also with components on host tissues and cells (A Fig 2) and a clinically relevant fungal pathogen, A.f. While not unprecedented since it was previously known that A.f. expressed various sialic acid moieties on its surface, this finding was particularly exciting since little was known about these epitopes and their efficacy as a therapeutic target.

Our work presented in this dissertation shows that SMB19 binds a similar epitope on A.f. and GBSIb, and that its target on A.f. is likely associated with a

GPI-linked protein. Our preliminary studies to identify the SMB19 target protein on A.f. have pointed us to several candidate proteins including glucan endo-1,3-β glucosidase (eglC), conserved serine-rich protein, and a cell wall protein, but futher studies are necessary to further identify the target (Appendix C).

Our studies also show that SMB19 protects in a mouse model of disseminated A.f. Our prior studies on the antibody responses of C57BL/6 mice to various vaccine preparations of GBS revealed that paraformaldehyde fixed GBS would elicit a moderate type-specific IgM antibody response that peaks ~7 days after vaccination similar to that of live GBS (Appendix B). This assisted us in further elucidating that SMB19's protection in I.A. can be mediated through a variety of therapeutic approaches including passive transfer of SMB19 or vaccination with GBSIb. Our development of the SMB19 Tg mouse and an anti-idiotype mAb to SMB19 further helped us to show that this protection is specifically mediated by SMB19 idiotype bearing antibodies and not just any GBSIb type-specific antibodies. Previous studies on the role of GBS type-specific antibodies in the activation of complement pointed us toward studying the role of complement in SMB19's protection during I.A. As hypothesized, SMB19's mechanism of protection in I.A. was dependent on complement as it is in GBS infections, but not dependent on either neutrophils or T cells which are often absent or at low levels in I.A. patients.

Because the currently prescribed antifungal drugs are not very efficacious in clearing A.f., the development of better therapies is necessary. The currently

used antifungals as well as the recently studied protective anti-β-1,3 glucan mAbs have targeted the growth of the fungus instead of eliciting the host immune response for protection, making SMB19-like antibodies an attractive combination therapeutic for I.A. Future studies on SMB19 which are currently underway in our lab will address this topic as well as study the protective capacity of SMB19 in other clinically relevant pathogens including *Candida Albicans*, which SMB19 also binds, hopefully leading to the development of improved I.A. therapeutic strategies.

## GENERAL LIST OF REFERENCES


1. Latge, J.P., *Aspergillus fumigatus and aspergillosis.* Clin Microbiol Rev, 1999. **12**(2): p. 310-50.

2. Dagenais, T.R. and N.P. Keller, *Pathogenesis of Aspergillus fumigatus in Invasive Aspergillosis.* Clin Microbiol Rev, 2009. **22**(3): p. 447-65.

3. Jantunen, E., et al., *Central nervous system aspergillosis in allogeneic stem cell transplant recipients.* Bone Marrow Transplant, 2003. **31**(3): p. 191-6.

4. Stergiopoulou, T., et al., *Host-dependent patterns of tissue injury in invasive pulmonary aspergillosis.* Am J Clin Pathol, 2007. **127**(3): p. 349-55.

5. Herbrecht, R., et al., *Voriconazole versus amphotericin B for primary therapy of invasive aspergillosis.* N Engl J Med, 2002. **347**(6): p. 408-15.

6. McCormack, P.L. and C.M. Perry, *Caspofungin: a review of its use in the treatment of fungal infections.* Drugs, 2005. **65**(14): p. 2049-68.

7. Walsh, T.J., et al., *Treatment of aspergillosis: clinical practice guidelines of the Infectious Diseases Society of America.* Clin Infect Dis, 2008. **46**(3): p. 327-60.

8. Henwick, S., S.V. Hetherington, and C.C. Patrick, *Complement binding to Aspergillus conidia correlates with pathogenicity.* J Lab Clin Med, 1993. **122**(1): p. 27-35.

9. Blanco, J.L. and M.E. Garcia, *Immune response to fungal infections.* Vet Immunol Immunopathol, 2008. **125**(1-2): p. 47-70.

10. Schaffner, A., et al., *Killing of Aspergillus spores depends on the anatomical source of the macrophage.* Infect Immun, 1983. **42**(3): p. 1109-15.

11. Diamond, R.D. and R.A. Clark, *Damage to Aspergillus fumigatus and Rhizopus oryzae hyphae by oxidative and nonoxidative microbicidal products of human neutrophils in vitro.* Infect Immun, 1982. **38**(2): p. 487-95.

12. Schaffner, A., H. Douglas, and A. Braude, *Selective protection against conidia by mononuclear and against mycelia by polymorphonuclear phagocytes in resistance to Aspergillus. Observations on these two lines of defense in vivo and in vitro with human and mouse phagocytes.* J Clin Invest, 1982. **69**(3): p. 617-31.

13. Casadevall, A., *Antibody immunity and invasive fungal infections.* Infect Immun, 1995. **63**(11): p. 4211-8.

14. Casadevall, A. and L.A. Pirofski, *Antibody-mediated protection through cross-reactivity introduces a fungal heresy into immunological dogma.* Infect Immun, 2007. **75**(11): p. 5074-8.

15. Casadevall, A. and L.A. Pirofski, *A new synthesis for antibody-mediated immunity.* Nat Immunol, 2012. **13**(1): p. 21-8.

16. Lamarre, C., et al., *Transcriptomic analysis of the exit from dormancy of Aspergillus fumigatus conidia.* BMC Genomics, 2008. **9**: p. 417.

17. Jackson, S.L. and I.B. Heath, *Roles of calcium ions in hyphal tip growth.* Microbiol Rev, 1993. **57**(2): p. 367-82.

18. Thau, N., et al., *rodletless mutants of Aspergillus fumigatus.* Infect Immun, 1994. **62**(10): p. 4380-8.

19. Hohl, T.M. and M. Feldmesser, *Aspergillus fumigatus: principles of pathogenesis and host defense.* Eukaryot Cell, 2007. **6**(11): p. 1953-63.

20. Netea, M.G., et al., *Immune sensing of Candida albicans requires cooperative recognition of mannans and glucans by lectin and Toll-like receptors.* J Clin Invest, 2006. **116**(6): p. 1642-50.

21. Means, T.K., et al., *Evolutionarily conserved recognition and innate immunity to fungal pathogens by the scavenger receptors SCARF1 and CD36.* J Exp Med, 2009. **206**(3): p. 637-53.

22. Goodridge, H.S., A.J. Wolf, and D.M. Underhill, *Beta-glucan recognition by the innate immune system.* Immunol Rev, 2009. **230**(1): p. 38-50.

23. Levitz, S.M., *Innate recognition of fungal cell walls.* PLoS Pathog, 2010. **6**(4): p. e1000758.

24. Fontaine, T., et al., *Galactosaminogalactan, a new immunosuppressive polysaccharide of Aspergillus fumigatus.* PLoS Pathog, 2011. **7**(11): p. e1002372.

25. Latge, J.P., et al., *Chemical and immunological characterization of the extracellular galactomannan of Aspergillus fumigatus.* Infect Immun, 1994. **62**(12): p. 5424-33.

26. Bouchara, J.P., et al., *Sialic acid-dependent recognition of laminin and fibrinogen by Aspergillus fumigatus conidia.* Infect Immun, 1997. **65**(7): p. 2717-24.

27. Abad, A., et al., *What makes Aspergillus fumigatus a successful pathogen? Genes and molecules involved in invasive aspergillosis.* Rev Iberoam Micol, 2010. **27**(4): p. 155-82.

28. Jennings, H.J., et al., *Structure of native polysaccharide antigens of type Ia and type Ib group B Streptococcus.* Biochemistry, 1983. **22**(5): p. 1258-64.

29. Jennings, H.J., et al., *Structural determination of the capsular polysaccharide antigen of type II group B Streptococcus.* J Biol Chem, 1983. **258**(3): p. 1793-8.

30. Wessels, M.R., et al., *Structure and immunochemistry of an oligosaccharide repeating unit of the capsular polysaccharide of type III group B Streptococcus. A revised structure for the type III group B streptococcal polysaccharide antigen.* J Biol Chem, 1987. **262**(17): p. 8262-7.

31. Carlin, A.F., et al., *Group B streptococcal capsular sialic acids interact with siglecs (immunoglobulin-like lectins) on human leukocytes.* J Bacteriol, 2007. **189**(4): p. 1231-7.

32. Marques, M.B., et al., *Prevention of C3 deposition by capsular polysaccharide is a virulence mechanism of type III group B streptococci.* Infect Immun, 1992. **60**(10): p. 3986-93.

33. Wasylnka, J.A., M.I. Simmer, and M.M. Moore, *Differences in sialic acid density in pathogenic and non-pathogenic Aspergillus species.* Microbiology, 2001. **147**(Pt 4): p. 869-77.

34. Behnsen, J., et al., *The opportunistic human pathogenic fungus Aspergillus fumigatus evades the host complement system.* Infect Immun, 2008. **76**(2): p. 820-7.

35. Jarva, H., et al., *The group B streptococcal beta and pneumococcal Hic proteins are structurally related immune evasion molecules that bind the complement inhibitor factor H in an analogous fashion.* J Immunol, 2004. **172**(5): p. 3111-8.

36. Washburn, R.G., C.H. Hammer, and J.E. Bennett, *Inhibition of complement by culture supernatants of Aspergillus fumigatus.* J Infect Dis, 1986. **154**(6): p. 944-51.

37. Fearon, D.T., *Regulation by membrane sialic acid of beta1H-dependent decay-dissociation of amplification C3 convertase of the alternative complement pathway.* Proc Natl Acad Sci U S A, 1978. **75**(4): p. 1971-5.

38. Edwards, M.S., et al., *The role of specific antibody in alternative complement pathway-mediated opsonophagocytosis of type III, group B Streptococcus.* J Exp Med, 1980. **151**(5): p. 1275-87.

39. Pritchard, D.G., et al., *Immunochemical characterization of the polysaccharide antigens of group B streptococci.* Rev Infect Dis, 1988. **10 Suppl 2**: p. S367-71.

40. Pritchard, D.G., B.M. Gray, and M.L. Egan, *Murine monoclonal antibodies to type Ib polysaccharide of group B streptococci bind to human milk oligosaccharides.* Infect Immun, 1992. **60**(4): p. 1598-602.

41. Cutler, J.E., G.S. Deepe, Jr., and B.S. Klein, *Advances in combating fungal diseases: vaccines on the threshold.* Nat Rev Microbiol, 2007. **5**(1): p. 13-28.

42. Montagnoli, C., et al., *Immunity and tolerance to Aspergillus involve functionally distinct regulatory T cells and tryptophan catabolism.* J Immunol, 2006. **176**(3): p. 1712-23.

43. de Repentigny, L., et al., *Acquired immunity in experimental murine aspergillosis is mediated by macrophages.* Infect Immun, 1993. **61**(9): p. 3791-802.

44. Montagnoli, C., et al., *A role for antibodies in the generation of memory antifungal immunity.* Eur J Immunol, 2003. **33**(5): p. 1193-204.

45. Torosantucci, A., et al., *Protection by anti-beta-glucan antibodies is associated with restricted beta-1,3 glucan binding specificity and inhibition of fungal growth and adherence.* PLoS One, 2009. **4**(4): p. e5392.

46. Rachini, A., et al., *An anti-beta-glucan monoclonal antibody inhibits growth and capsule formation of Cryptococcus neoformans in vitro and exerts therapeutic, anticryptococcal activity in vivo.* Infect Immun, 2007. **75**(11): p. 5085-94.

47. Chaturvedi, A.K., et al., *Monoclonal immunoglobulin G1 directed against Aspergillus fumigatus cell wall glycoprotein protects against experimental murine aspergillosis.* Clin Diagn Lab Immunol, 2005. **12**(9): p. 1063-8.

48. Alviano, C.S., L.R. Travassos, and R. Schauer, *Sialic acids in fungi: a minireview.* Glycoconj J, 1999. **16**(9): p. 545-54.

49. Doran, K.S. and V. Nizet, *Molecular pathogenesis of neonatal group B streptococcal infection: no longer in its infancy.* Mol Microbiol, 2004. **54**(1): p. 23-31.

50. Warwas, M.L., et al., *Structure and role of sialic acids on the surface of Aspergillus fumigatus conidiospores.* Glycobiology, 2007. **17**(4): p. 401-10.

51. Edwards, M.S., M.R. Wessels, and C.J. Baker, *Capsular polysaccharide regulates neutrophil complement receptor interactions with type III group B streptococci.* Infect Immun, 1993. **61**(7): p. 2866-71.

52. Foote, J.B. and J.F. Kearney, *Generation of B cell memory to the bacterial polysaccharide alpha-1,3 dextran.* J Immunol, 2009. **183**(10): p. 6359-68.

53. Chen, X., et al., *Evidence for selection of a population of multi-reactive B cells into the splenic marginal zone.* Int Immunol, 1997. **9**(1): p. 27-41.

54. Martin, F., X. Chen, and J.F. Kearney, *Development of VH81X transgene-bearing B cells in fetus and adult: sites for expansion and deletion in conventional and CD5/B1 cells.* Int Immunol, 1997. **9**(4): p. 493-505.

55. Circolo, A., et al., *Genetic disruption of the murine complement C3 promoter region generates deficient mice with extrahepatic expression of C3 mRNA.* Immunopharmacology, 1999. **42**(1-3): p. 135-49.

56. Kearney, J.F., et al., *A new mouse myeloma cell line that has lost immunoglobulin expression but permits the construction of antibody-secreting hybrid cell lines.* J Immunol, 1979. **123**(4): p. 1548-50.

57. Bowman, J.C., et al., *Quantitative PCR assay to measure Aspergillus fumigatus burden in a murine model of disseminated aspergillosis: demonstration of efficacy of caspofungin acetate.* Antimicrob Agents Chemother, 2001. **45**(12): p. 3474-81.

58. Hohl, T.M., et al., *Aspergillus fumigatus triggers inflammatory responses by stage-specific beta-glucan display.* PLoS Pathog, 2005. **1**(3): p. e30.

59. Oliva, C.R., et al., *The integrin Mac-1 (CR3) mediates internalization and directs Bacillus anthracis spores into professional phagocytes.* Proc Natl Acad Sci U S A, 2008. **105**(4): p. 1261-6.

60. Oliva, C., C.L. Turnbough, Jr., and J.F. Kearney, *CD14-Mac-1 interactions in Bacillus anthracis spore internalization by macrophages.* Proc Natl Acad Sci U S A, 2009. **106**(33): p. 13957-62.

61. Kin, N.W., et al., *Antibodies generated against conserved antigens expressed by bacteria and allergen-bearing fungi suppress airway disease.* J Immunol, 2012. **189**(5): p. 2246-56.

62. Ansheng, L., et al., *Study on the hyphal responses of Aspergillus fumigatus to the antifungal agent by Bio-Cell Tracer.* Mycopathologia, 1999. **148**(1): p. 17-23.

63. Bowman, J.C., et al., *The antifungal echinocandin caspofungin acetate kills growing cells of Aspergillus fumigatus in vitro.* Antimicrob Agents Chemother, 2002. **46**(9): p. 3001-12.

64. Hasan, M., et al., *Incomplete block of B cell development and immunoglobulin production in mice carrying the muMT mutation on the BALB/c background.* Eur J Immunol, 2002. **32**(12): p. 3463-71.

65. Rambach, G., et al., *Complement induction and complement evasion in patients with cerebral aspergillosis.* Microbes Infect, 2008. **10**(14-15): p. 1567-76.

66. Lambourne, J., et al., *Association of mannose-binding lectin deficiency with acute invasive aspergillosis in immunocompromised patients.* Clin Infect Dis, 2009. **49**(10): p. 1486-91.

67. Hector, R.F., E. Yee, and M.S. Collins, *Use of DBA/2N mice in models of systemic candidiasis and pulmonary and systemic aspergillosis.* Infect Immun, 1990. **58**(5): p. 1476-8.

68. Denning, D.W., et al., *Pulmonary aspergillosis in the acquired immunodeficiency syndrome.* N Engl J Med, 1991. **324**(10): p. 654-62.

69. Baker, C.J. and M.S. Edwards, *Group B streptococcal conjugate vaccines.* Arch Dis Child, 2003. **88**(5): p. 375-8.

70. Edwards, M.S., *Group B streptococcal conjugate vaccine: a timely concept for which the time has come.* Hum Vaccin, 2008. **4**(6): p. 444-8.

71. Herbrecht, R., et al., *Voriconazole versus amphotericin B for primary therapy of invasive aspergillosis.* N Engl J Med, 2002. **347**(6): p. 408-15.

72. Segal, B.H. and T.J. Walsh, *Current approaches to diagnosis and treatment of invasive aspergillosis.* Am J Respir Crit Care Med, 2006. **173**(7): p. 707-17.

73. Reichenberger, F., et al., *Diagnosis and treatment of invasive pulmonary aspergillosis in neutropenic patients.* Eur Respir J, 2002. **19**(4): p. 743-55.

74. Stevens, D.A., *Combination immunotherapy and antifungal chemotherapy.* Clin Infect Dis, 1998. **26**(6): p. 1266-9.

75. Rodrigues, M.L., et al., *Identification of N-acetylneuraminic acid and its 9-O-acetylated derivative on the cell surface of Cryptococcus neoformans: influence on fungal phagocytosis.* Infect Immun, 1997. **65**(12): p. 4937-42.

76. Totolian, A.A., *Pathogenic Streptococci: Present and Future : Proceedings of the XIIth Lancefield International Symposium on Streptococci and Streptococcal Diseases, St.Petersburg, Russia September 6-10, 1993*1994: Lancer Publications.

77. Kearney, J.F., et al., *Induction of germ-line anti-alpha 1-3 dextran antibody responses in mice by members of the Enterobacteriaceae family.* J Immunol, 1985. **135**(5): p. 3468-72.

78. Feldman, R.G., et al., *The group B streptococcal capsular carbohydrate: immune response and molecular mimicry.* Adv Exp Med Biol, 1998. **435**: p. 261-9.

79. Charrel-Dennis, M., et al., *TLR-independent type I interferon induction in response to an extracellular bacterial pathogen via intracellular recognition of its DNA.* Cell Host Microbe, 2008. **4**(6): p. 543-54.

# APPENDIX A

# CHARACTERIZATION OF SMB19 BINDING PROPERTIES

**SMB19 binding to sialyl-lacto-N-tetraose associated with the GBSIb capsular polysaccharide is calcium dependent.** The structure of the GBSIb type-specific polysaccharide has previously been described [28] and is shown in Fig 1A. SMB19 is a GBSIb type-specific mAb (IgM, k), which recognizes the oligosaccharide sialyl-lacto-N-tetraose (s-LNT). This oligosaccharide is comprised of galactose, GlcNAc, and glucose sugars linked to a terminal sialic acid residue, called neuraminic acid (Neu5Ac). To confirm that SMB19 binds specifically to sialyl-lacto-N-tetraose (s-LNT), we coated ELISA plates with either s-LNT or lacto-N-tetraose (LNT) as a control and analyzed SMB19 binding by differences in optical density at 405 nm. SMB19 bound in a dose-dependent manner to s-LNT, but did not bind significantly to any concentration of LNT. Additionally, a mAb against the GBSIa type-specific polysaccharide, SIaE7, did not bind to any concentration of s-LNT (Fig 1A). It has also previously been reported that SMB19 binding to s-LNT is calcium dependent [40], so we analyzed the calcium conditions necessary for optimal binding of SMB19 to purified GBSIb polysaccharide by ELISA. ELISA plates were coated with purified GBSIb type-specific polysaccharide, hen egg lysozyme, or SMB19 anti-idiotype antibody. While SMB19 bound slightly to the GBSIb polysaccharide in PBS, binding was enhanced ~2 fold in the presence of additional calcium present in DPBS. Conversely, when 50 mM EGTA was added SMB19 binding was decreased ~2 fold compared with SMB19 binding in PBS. As an isotype control, MD4 was analyzed for binding to hen egg lysozyme under these same conditions, but there were no significant differences in binding despite the different calcium conditions.

Interestingly, SMB19 binding to its anti-idiotype antibody, SMBi26, was also not calcium dependent. To further elucidate which types of antibodies are calcium dependent, we determined whether all anti-GBS antibodies were calcium dependent in their binding to their cognate bacteria. We incubated type-specific anti-GBS mAbs in varying concentrations of EGTA on ELISA plates coated with GBSIa, GBSIb, or GBSII. Anti-GBSIa and anti-GBSIb mAbs appeared to be calcium dependent in their binding to their cognate bacteria, but the anti-GBSII mAb was not, suggesting that not all anti-GBS mAbs are calcium dependent (Fig 1D). Addition of EGTA before anti-GBS mAbs had no affect on binding (Fig 1E). Taken together these data show that SMB19 recognizes the epitope s-LNT on the GBSIb type-specific polysaccharide in a calcium dependent manner and not all anti-GBS mAbs are calcium dependent in their binding to GBS.

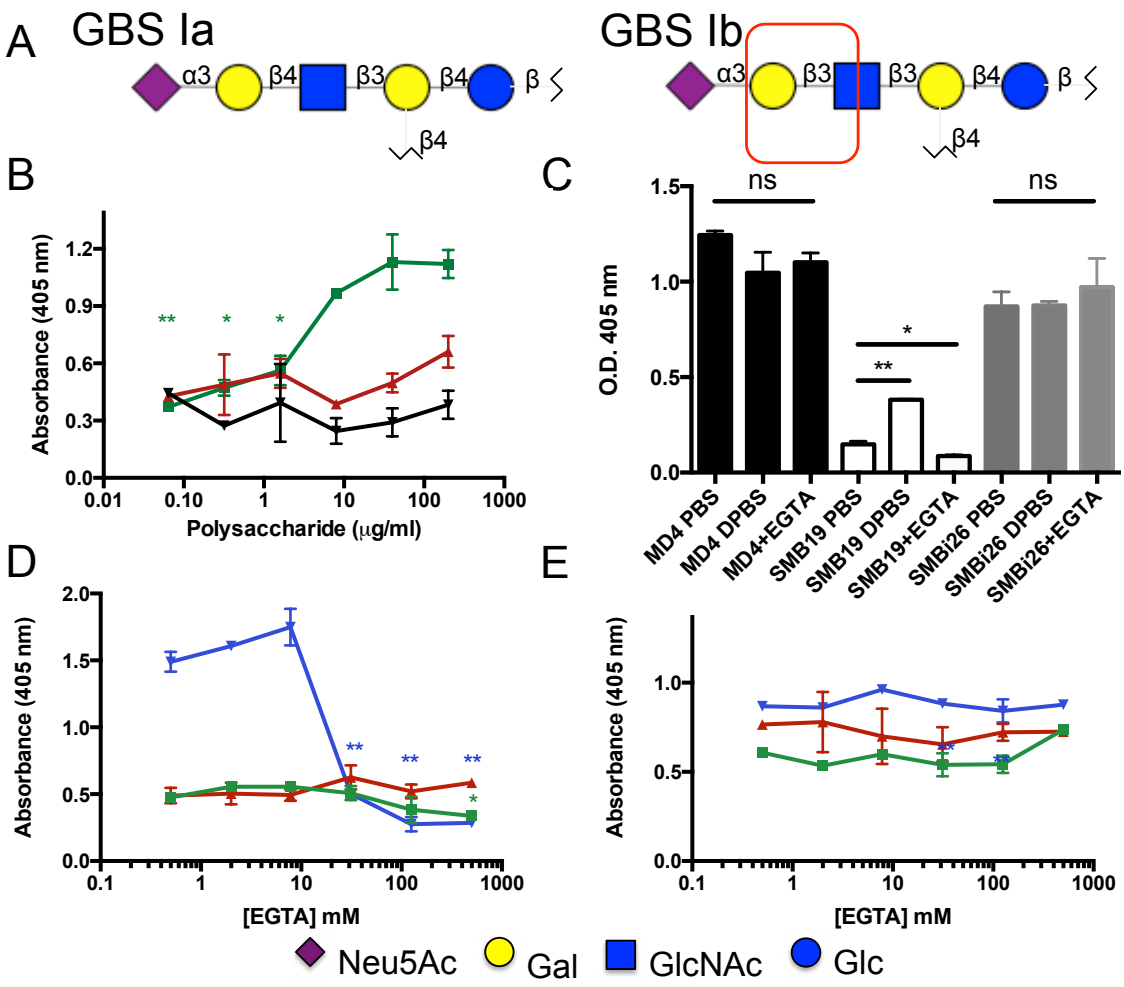
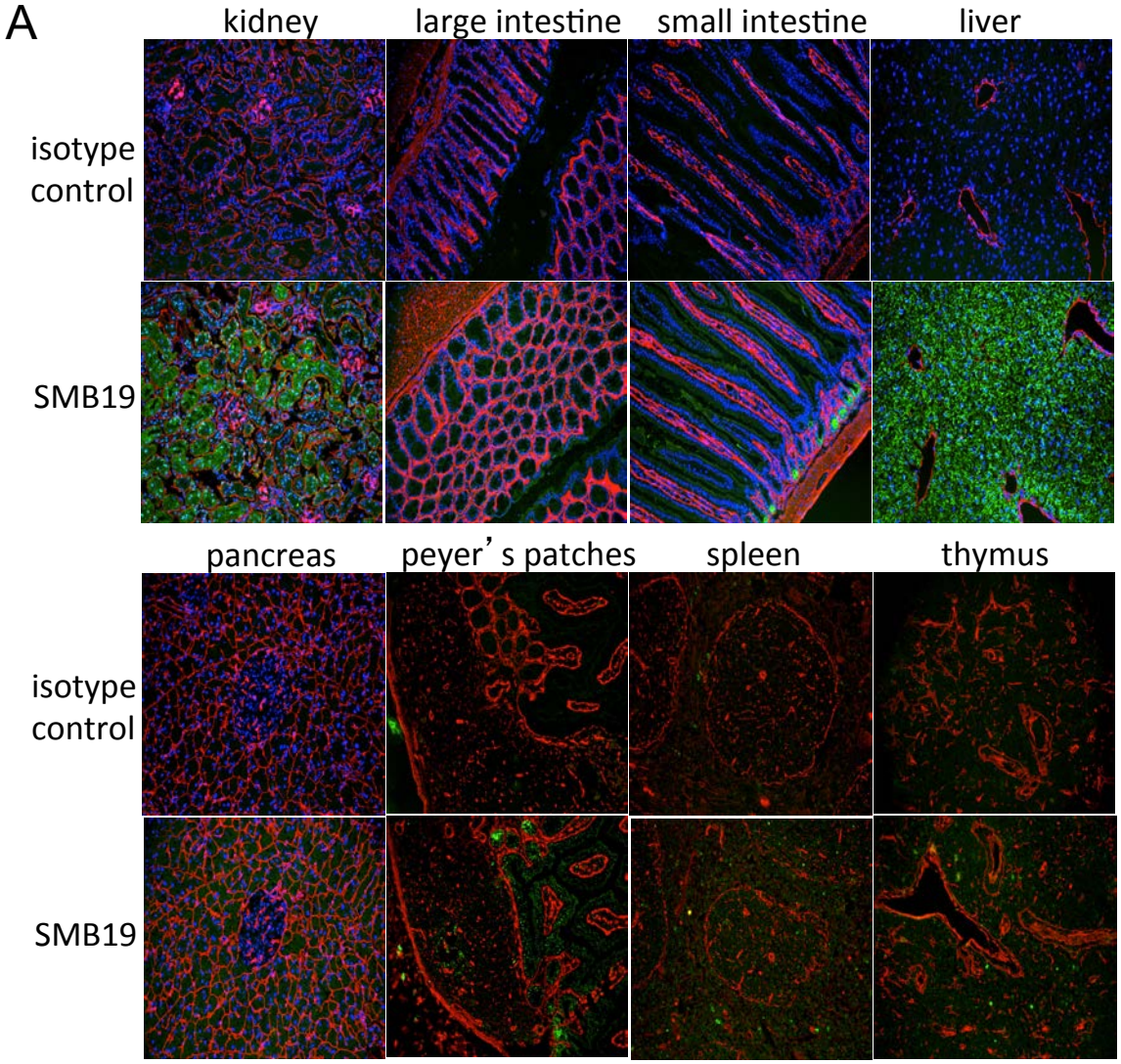



**Fig A1. SMB19 recognizes the polysaccharide s-LNT and its binding is dependent on calcium.** (A) Structure of the GBSIa and GBSIb polysaccharides. (B) ELISA analysis of SMB19 binding to s-LNT (green), LNT (black), and SIaE7 binding to s-LNT (red). (C) ELISA analysis of MD4 binding to HEL (black), SMB19 binding to purified GBSIb polysaccharide (white), or SMB19 binding to SMB19 anti-idiotype antibody, SMBi26 (grey), under various calcium conditions. Experiments were performed in duplicate and repeated two times with similar results. Representative experimental results are shown. ELISA analysis of SMB19 (anti-GBSIb, green), SIaE7 (anti-GBSIa, blue), or SIIF5C4 (anti-GBSII, red) binding to their cognate bacteria under various concentrations of EGTA. EGTA was added (D) after or (E) before mAb. * $p<0.05$, ** $p<0.01$.

**SMB19 binds to mouse autoantigens.** The sialic acid residue, Neu5Ac, is found on a variety of host cells as well as pathogens such as GBS [28, 29]. In fact, it is hypothesized that Neu5Ac on GBS is a major virulence factor due to molecular mimicry of host polysaccharides [78]. To test this hypothesis, we stained mouse adult and neonatal tissue sections with SMB19 to see if there was any cross-reactivity with epitopes on mouse cells. SMB19 stained brightly an as yet unidentified epitope on the kidney tubules, liver, and crypts of the small intestines of adult mice. SMB19 also reacted slightly with pancreas and lymphoid tissues in adult mice (Fig 2A). In the neonate, SMB19 reacted with an epitope in the cerebrum as well as the spleen and thymus (Fig 2B). Thus, SMB19 appears to be autoreactive with murine epitopes. To further identify what SMB19 might be reacting with on mouse cells, we stained RAW cells (data not shown), BMDMs, and BMDCs with SMB19 and analyzed binding by FLOW cytometry. SMB19 appeared to bind surface components of all three cell types when compared to isotype control (Fig 2C). Taken together, this data shows that SMB19 not only recognizes the GBSIb associated oligosaccharide, s-LNT, but also an epitope on mouse tissues and lymphoid cells.


A
kidney
large intestine
small intestine
liver
isotype control
SMB19
pancreas
peyer's patches
spleen
thymus
isotype control
SMB19

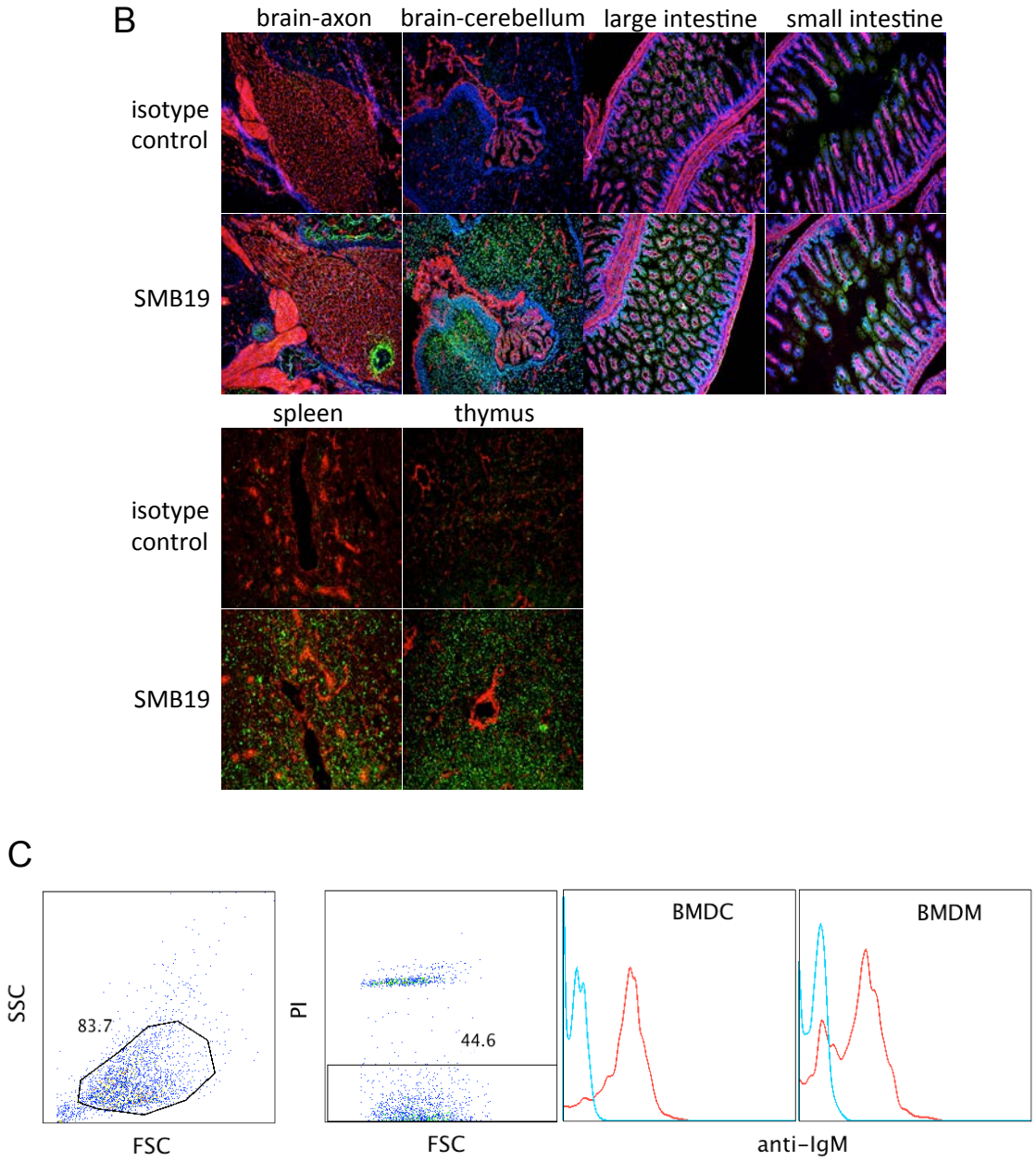



**Fig A2. SMB19 cross reacts with mouse autoantigens.** Immunofluorescence analysis of SMB19 binding to various (A) adult or (B) pup mouse tissues. Anti-pan laminin (red), SMB19 or MD4 isotype control (green), DAPI (blue). DAPI stain not shown for lymphoid tissues. (C) SMB19 (red) or SIbD2 control IgM (blue) were incubated with BMDMs or BMDCs followed by Cy5 conjugated secondary antibody and analyzed for binding by flow cytometry.

# APPENDIX B

# CHARACTERIZATION OF GROUP B STREPTOCOCCI TYPE IB ANTIBODY RESPONSES

**Paraformaldehyde fixed and live GBSIb elicit primary and boosted type-specific immune responses, whereas heat killed GBSIb do not.** It has previously been reported that live, but not heat killed (HK) GBS induce a type 1 interferon response [79]. Thus, we hypothesized that different vaccine preparations would elicit different immune responses. To test how different vaccines affected the primary and boosted type-specific GBSIb antibody response, we vaccinated mice with either $1x10^8$ HK, paraformaldehyde fixed (PFA), or $5x10^4$ live GBSIb. The live dose of bacteria had to be titered down from the vaccine dose due to virulence. The peak of both primary and boosted antibody response occurred ~7 days after vaccination although the primary antibody response was weak in both groups. The PFA GBSIb preparation elicited an antibody response that was similar in trend to that of live GBSIb, whereas the HK preparation did not elicit an antibody response. The PFA vaccine elicited a larger antibody response than the live GBSIb, but this is likely due to the difference in number of bacteria given. The booster vaccine elicited a robust antibody response that was ~10 fold greater than the primary response (Fig 1A). Taken together this shows that PFA fixed GBSIb elicits a similar antibody response to live GBSIb, and HK GBS do not elicit a GBSIb type-specific antibody response.

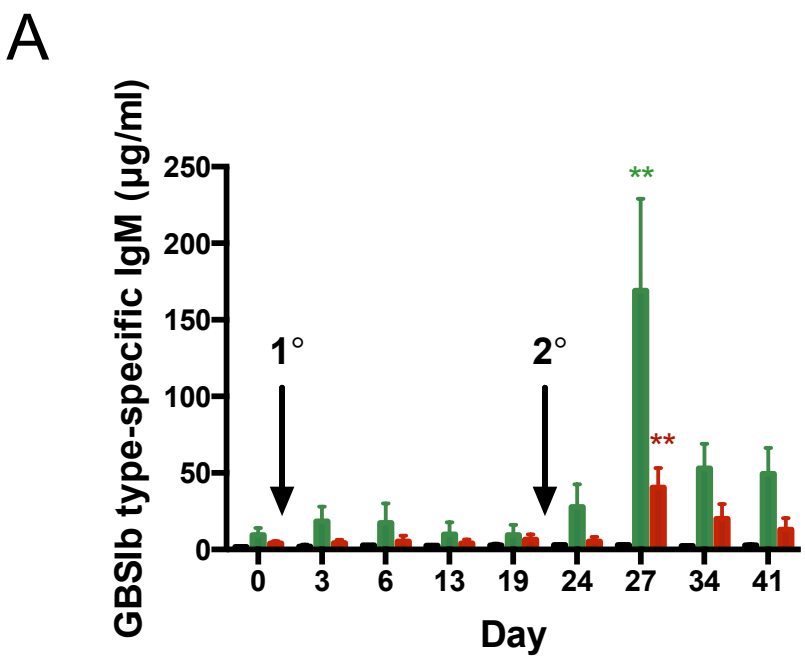



**Fig B1. PFA fixed, but not heat killed GBSIb elicits an antibody response similar to that of live GBSIb.** (A) Mice were vaccinated with $1x10^8$ PFA fixed (green) or heat killed GBSIb (black) or $5x10^4$ live GBSIb (red) on day 0 and day 21. Anti-sera was collected at various timepoints after vaccination and analyzed by ELISA for type-specific GBSIb antibody levels. **, $p<0.01$.

**Memory antibody responses are greater in adult mice vaccinated with GBSIb or GBSIa and rechallenged 8 weeks later with GBSIb than 3 day old pups that were vaccinated with GBSIb or GBSIa and rechallenged with GBSIb 8 weeks later.** To determine how timing of vaccination affects the GBSIb antibody response, we vaccinated 3 day old pups or 8 week old mice with GBSIb or GBSIa, then 8 weeks later rechallenged each group with GBSIb to look for differences in GBSIb-specific antibody responses. Adult mice that received primary vaccination with GBSIb and then were rechallenged with GBSIb after 8 weeks seemed to respond similarly to mice that received primary vaccination with GBSIa, indicating that little to no memory response specific to GBSIb was generated or the antisera generated from each vaccination was cross-reactive (Fig 2A-D). The same trend held true of 3 day old mice that were vaccinated with GBSIb or GBSIa then rechallenged as adults with GBSIb (Fig 2A-D). There were significant differences, however, in both the memory response baseline and peak antibody response levels between mice that were given primary vaccination as pups vs. adults. Mice that received primary vaccination with GBSIb or GBSIa as pups had significantly lower levels of anti-GBS antibodies upon rechallenge 8 weeks later than mice that received primary vaccination with GBSIb or GBSIa as adults. In addition, levels of SMB19 idiotype-bearing antibodies appeared to be significantly lower in mice that received primary vaccination as pups. Taken together these results indicate that timing of primary vaccination with GBS affects the magnitude and clonal response of the long-term anti-GBS memory response.

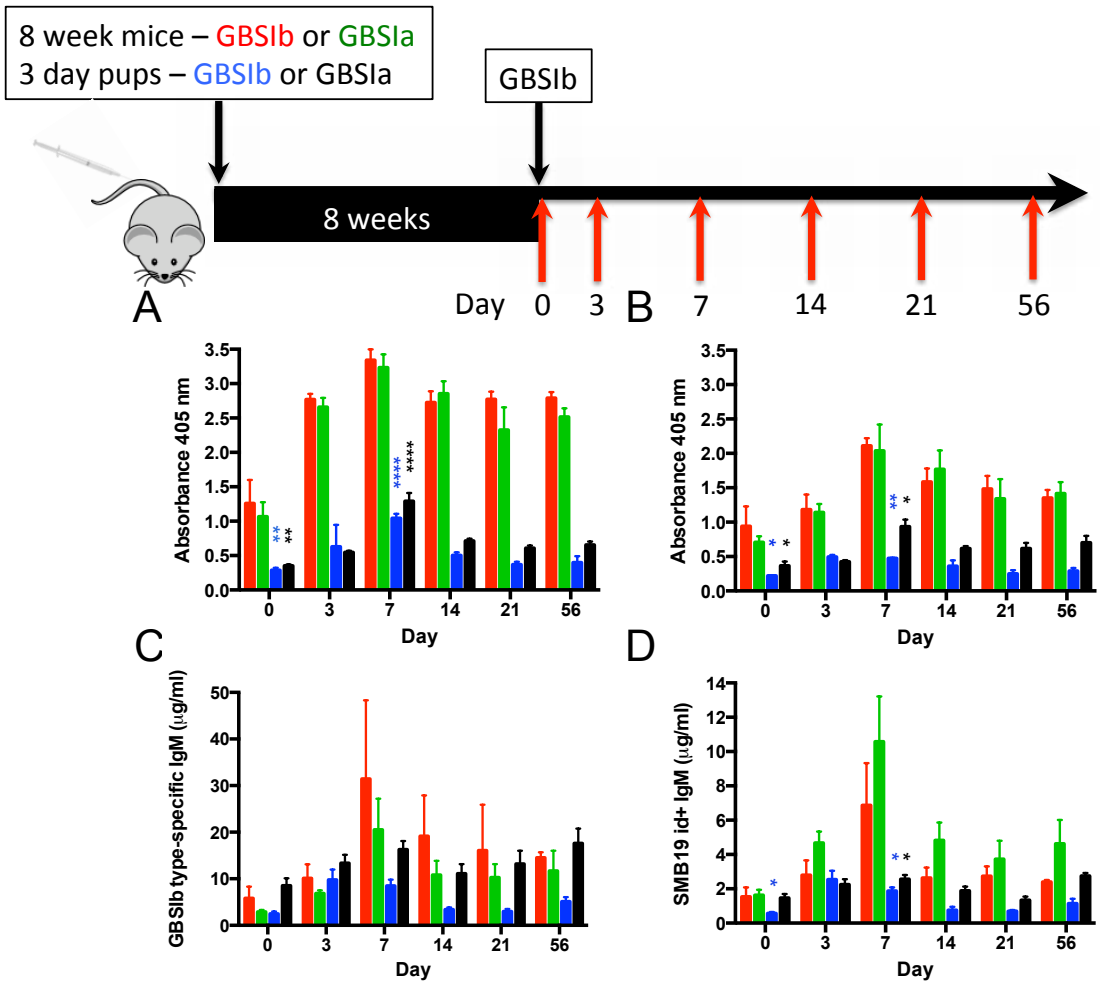



**Fig B2. Mice vaccinated as adults with GBSIb or GBSIa then rechallenged as adults with GBSIb produce higher anti-GBS antibody titers than pups that were vaccinated with GBSIb or GBSIa then rechallenged with GBSIb as adults.** 3 day old pups or 8 week old adult mice were vaccinated with GBSIb or GBSIa, then 8 weeks later rechallenged with GBSIb. Serum antibody IgM titers against whole (A) GBSIb, (B) GBSIa, (C) purified GBSIb type-specific polysaccharide, and (D) SMB19 anti-idiotype were measured by ELISA at various timepoints after rechallenge. *p<0.05, ** p<0.01, **** p,0.0001.

**Mice vaccinated with GBSII have a much larger antibody response than mice vaccinated with GBSIb.** SMB19, as shown in Appendix A Fig 2, binds to an epitope on mouse kidney tubules and liver. To determine if other GBS type-specific antibodies also bound mouse autoantigens, we stained kidney and liver tissue sections with SIIF5C4, anti-GBSII IgM. SIIF5C4 did not bind to either of these tissues (Fig 3D). To determine if the antibody responses between these 2 serotypes were different, mice were vaccinated with $1x10^8$ GBSIb or GBSII on days 0 and 56. Antisera was collected from both groups of mice at various time points before and after primary and secondary vaccination and anti-GBS IgM levels were quantified by ELISA. Mice that received GBSIb mounted a relatively weak GBSIb type-specific antibody response with the primary antibody response concentration peaking around ~30 μg/ml and the memory response peaking around ~100 μg/ml (Fig 3A). Mice vaccinated with GBSII mounted a much stronger antibody response with the primary GBSII type-specific antibody response peaking around ~100 μg/ml and the memory response peaking around ~300 μg/ml (Fig 3B). Antisera from mice vaccinated with GBSIb appeared to cross-react with the GBSII polysaccharide and vice versa (Fig 3A-B). The SMB19 idiotype-positive antibody response was weak in these mice after vaccination with GBSIb with levels peaking around 4 ug/ml in the memory response (Fig 3C). Taken together these results indicate that type-specific GBSII antibodies are not autoreactive with mouse tissues and the GBSII antibody response is approximately 3 fold larger than the GBSIb antibody response.

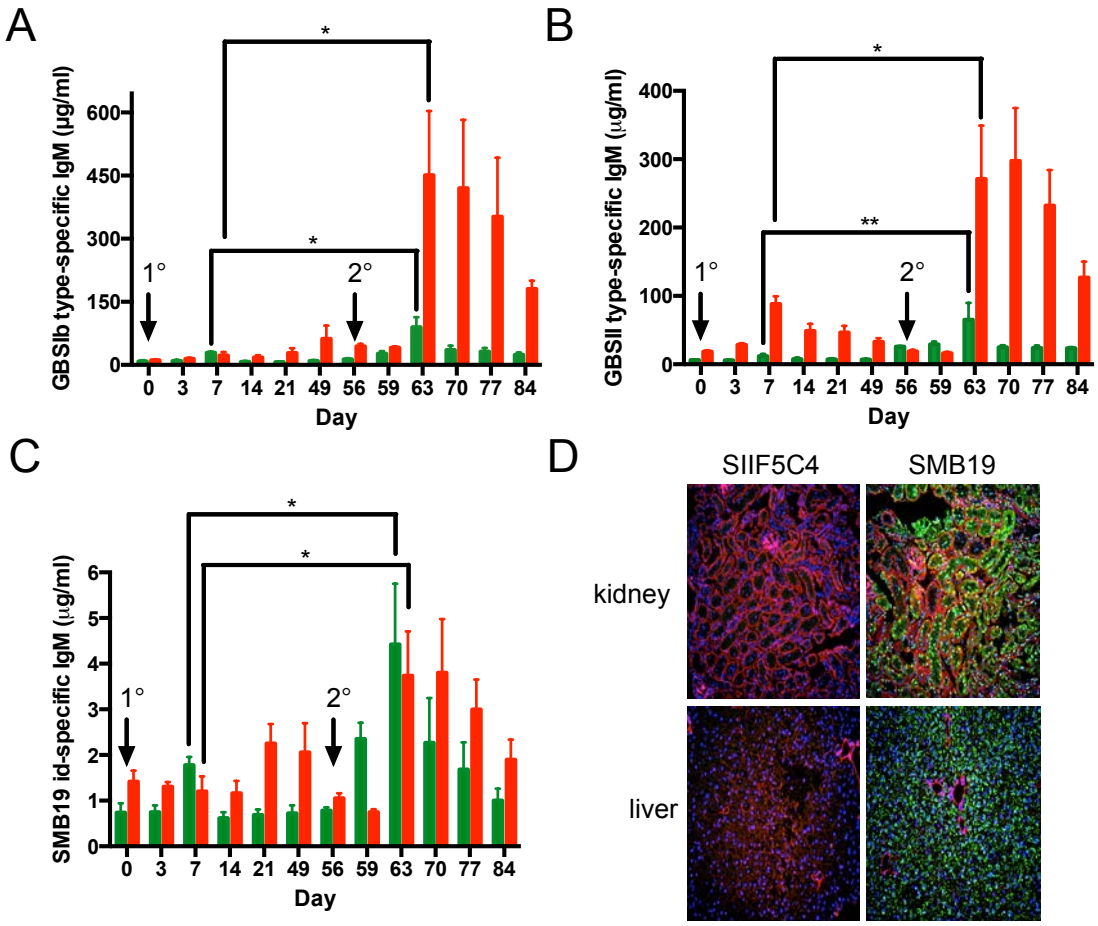



**Fig B3. GBSII vaccination elicits a larger antibody response than GBSIb in adult mice and GBSII type-specific antibodies are not reactive with mouse autoantigens.** C57BL/6 mice were vaccinated with GBSIb (green) or GBSII (red) on days 0 and 56. Antisera from each group was collected at various timepoints before and after vaccinations and analyzed by ELISA for (A) GBSIb type-specific IgM, (B) GBSII type-specific IgM, (C) and SMB19 idiotype-specific IgM. (D) Mouse kidney and liver tissue sections were stained with SIIF5C4 (anti-GBSII IgM) and SMB19 (anti-GBSIb IgM) (green), anti-pan laminin (red), and DAPI (blue).

**Characterization of the GBSIb antibody response in the SMB19 Tg mouse.**

To determine if SMB19 Tg mice responded differently to GBSIb than C57BL/6 mice, we vaccinated both types of mice with GBSIb or GBSIa as a control. Both SMB19 Tg mice and C57BL/6 mice mounted a primary and secondary antibody response which peaked ~7 days post-vaccination, although the SMB19 Tg mouse antibody response was ~3-4 fold larger than C57BL/6 mice. While the SMB19 Tg mouse's GBSIb type-specific antibody response was almost exclusively SMB19 idiotype positive, C57BL/6 mice produced very low levels of SMB19 idiotype positive antibody in response to GBSIb. The boosted antibody response was ~5 fold larger in both WT and SMB19 Tg mice (Fig 4A and 4C). Immunofluorescence analysis revealed that both primary and secondary antibody responses corresponded with plasma cells detected at 3 days. While C57BL/6 mice produced a few plasma cells in response to both primary vaccines (Fig 4D), corresponding to the weak primary antibody response, the SMB19 Tg mice had a much larger plasma cell response corresponding to their 3 fold larger primary response (Fig 4B). The plasma cell response was proportionally larger in both types of mice 3 days after the booster vaccine (day 24) corresponding to the 5 fold increase in antibody levels compared to primary response. Additionally, while SMB19 idiotype-positive specific plasma cells were barely detectable in the C57BL/6 mice vaccinated with GBSIb, they were much more prevalent in the SMB19 Tg mice that were vaccinated with GBSIb (Fig 4B and 4D). Taken together this experiment shows that SMB19 Tg mice make a larger GBSIb

specific antibody response than C57BL/6 mice and the plasma cell responses reflect the antibody responses elicited from vaccination.

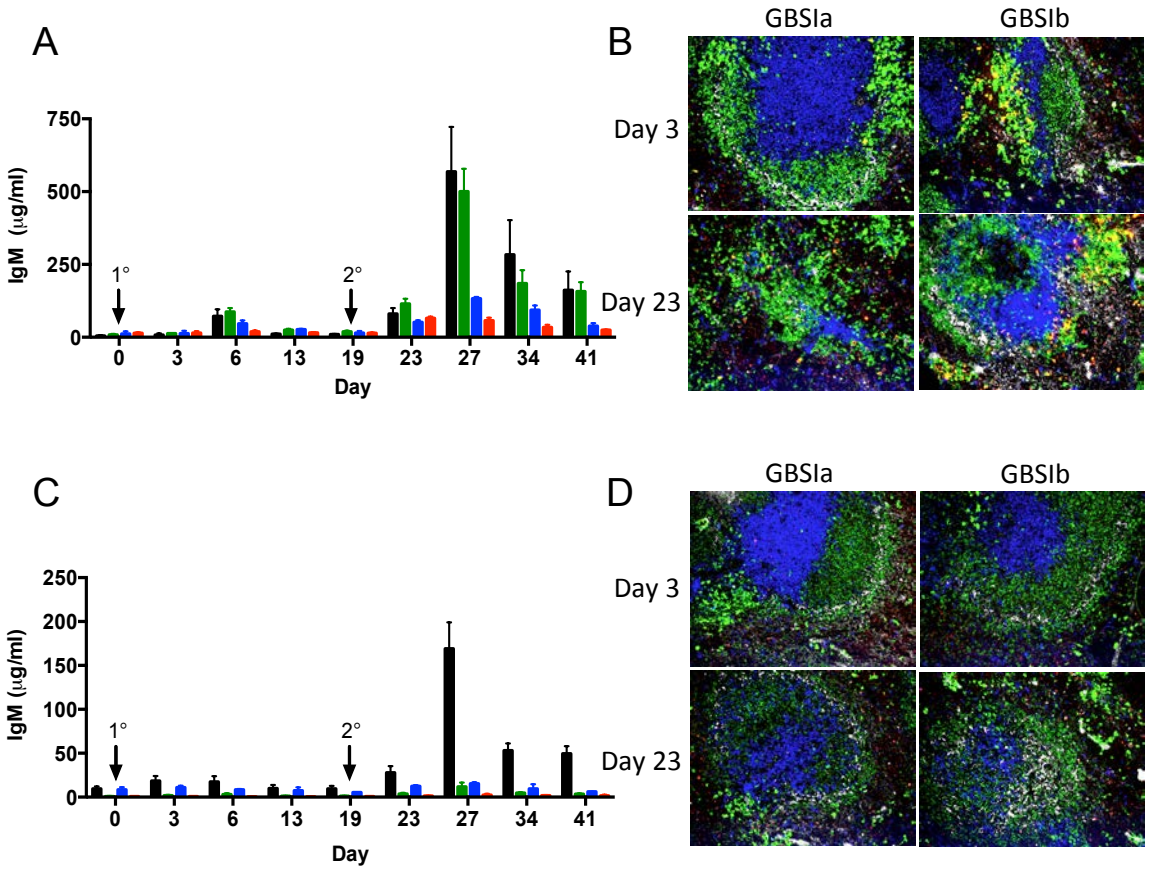



**Fig B4. SMB19 Tg mice produce higher quantities of GBSIb type-specific antibody and SMB19 idiotype positive antibody than C57BL/6 mice.** (A) SMB19 Tg mice and (C) C57BL/6 mice were vaccinated with PFA fixed GBSIb or GBSIa as a control on days 0 and 20. Antisera was analyzed at various timepoints before and after vaccination and analyzed for GBSIb type-specific IgM (black) and SMB19 idiotype positive IgM (green). Blue = GBSIb type-specific IgM from mice vaccinated with GBSIa (blue), SMB19 idiotype positive IgM from mice vaccinated with GBSIa (red). (B) Spleen sections from SMB19 Tg or (D) C57BL/6 mice 3 days after primary or secondary vaccination with GBS. Green = anti-IgM, Blue = anti-CD4+anti-CD8, white = MOMA1, red = anti-idiotype to SMB19. Yellow cells are both IgM and SMB19 idiotype positive.

**Co-immunization with multiple bacteria affects bacteria-specific clonal responses.** Since the timing of vaccination with GBSIb appeared to affect the overall magnitude and clonal memory antibody response, we hypothesized that vaccinating with multiple types of bacteria at the same time might also affect these responses. To test this hypothesis, we vaccinated mice with GBSIb, GAS, R36A, or combinations of these bacteria to determine if peak antibody responses to each antigen was affected. While the total anti-phosphorylcholine (PC) and anti-Group A carbohydrate (GAC) responses appeared to be similar independent of whether multiple bacteria were given at the same time or not (Fig 5A), in some cases the clonal responses appeared to be different. Mice that received R36A or GBSIb in addition to GAS appeared to produce higher levels of IA1 idiotype-specific antibody. Additionally, mice that received GBSIb or GAS in addition to R36A appeared to make higher quantities of TC68 (S107 Vh) compared with mice that received R36A alone (Fig 5B). Taken together, this data suggests that clonal antibody responses are affected by vaccination with multiple bacteria at the same time.

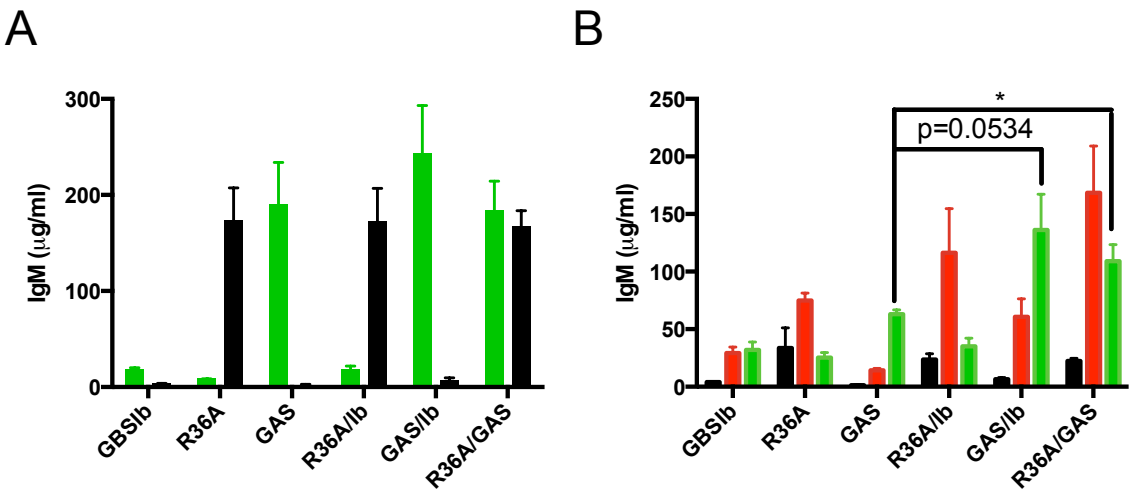



**Fig B5. Co-immunization with R36A or GBSIb with GAS enhances the IA1 clonal response compared to vaccination with GAS alone.** Mice were vaccinated with GBSIb, R36A, GAS, R36A+GBSIb, GAS+GBSIb, or R36A+GAS, then bled seven days later at the peak of the antibody response. Day 7 serum was analyzed by ELISA for PC (black) and GAC (green) specific antibody responses (A) or AB1.2 (black), TC68 (red), and IA1 (green) idiotype positive antibody responses (B). * $p<0.05$.

# APPENDIX C

## IDENTIFICATION OF THE SMB19-REACTIVE EPITOPE ON *ASPERGILLUS FUMIGATUS*

Because SMB19 bound to A.f. conidia and hyphae, we performed preliminary biochemical experiments to identify the epitope on A.f. to which SMB19 binds. A.f. were grown for 18 hours in RPMI at 37°C in a $CO_2$ incubator. Hyphae were harvested by centrifugation and washed in PBS. The hyphal pellet and lysate were collected. Half of the pellet was treated with hot SDS and the other half was left untreated. Both halves were then incubated with hydrofluoric acid to release GPI-linked proteins from the cell wall, precipitated with methanol and dialyzed against water. Half of the SDS-treated pellet was further processed by incubation with wheat germ agglutinin (WGA) agarose beads and eluted with GlcNAc (Fig 1A). Each A.f. fraction was run on a SDS-PAGE gel and western blotted with either SMB19 or isotype control mAb (Fig 1B-D). Several protein bands that were reactive with SMB19 were excised from the SDS-PAGE gel and further analyzed by mass spectrometry for identification. Several interesting proteins came back from this analysis as potential SMB19 targets, including glucan endo-1,3-b-glucosidase, conserved serine-rich protein, and cell wall protein. Characteristics of these proteins are enumerated in Table 1. Further characterization of the WGA reactive protein band was performed by liquid chromatography and revealed that this protein contained glycans with varying amounts of mannose, galactose, glucose, GlcNAc, and Neu5Ac (Fig 2). Interestingly, most of these sugars are components of the GBSIb capsular polysaccharide.

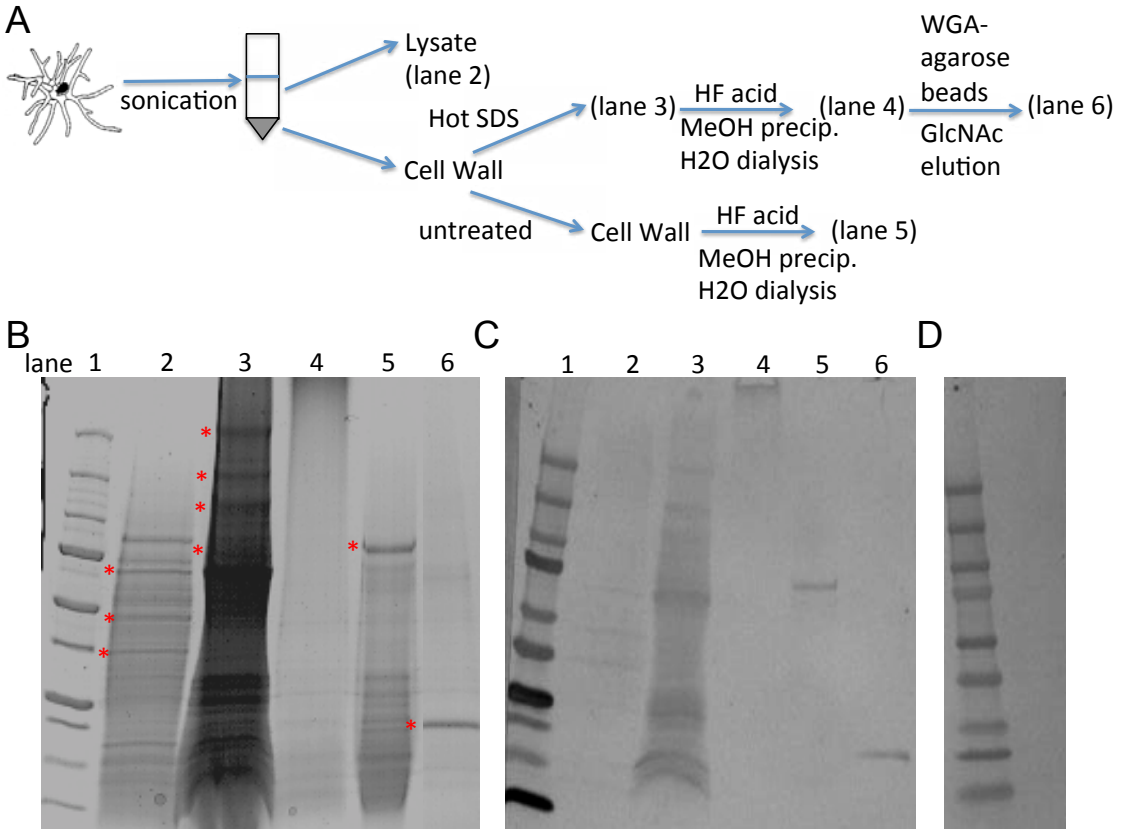



**Fig C1. Isolation of SMB19 reactive GPI-linked A.f. proteins.** (A) Schematic of A.f. GPI-linked protein extraction. A.f. hyphae were sonicated and lysate and pellet collected. Half the pellet was incubated with hot SDS, the other half was left untreated. Both halves were then incubated in HF acid, precipitated with methanol, and dialyzed against water. Half the SDS treated fraction was then incubated with WGA beads and reactive proteins eluted with GlcNAc. (B) SDS-PAGE gel showing protein bands after various A.f. treatments. Western blot of SDS-PAGE gel using (C) SMB19 or (D) SIbD2. Asterisks denote proteins sent for mass spectrometry analysis.

| Protein | Annotation | protein size | comments |
|---|---|---|---|
| Glucan endo-1,3-beta-glucosidase eglC | AFUA_3G00270 | 44.85 KDa | 1.GPI-anchored cell wall protein;<br>2. serine-rich in C-terminal region is potentially heavy O-glycosylated;3.183, 364 and 370 are the predicted N-glycosylation sites. |
| conserved serine-rich protein | AFUA_3G00880 | 21.6 KDa | 1.GPI-anchored cell wall protein;<br>2. serine-rich in C-terminal region;<br>3. a putative adhesin |
| cell wall protein | AFUA_3G10960 | 26.7 KDa | Ser-Thr rich in central region |

**Table C1. Potential SMB19-reactive A.f. target proteins from mass spectrometry analysis.**

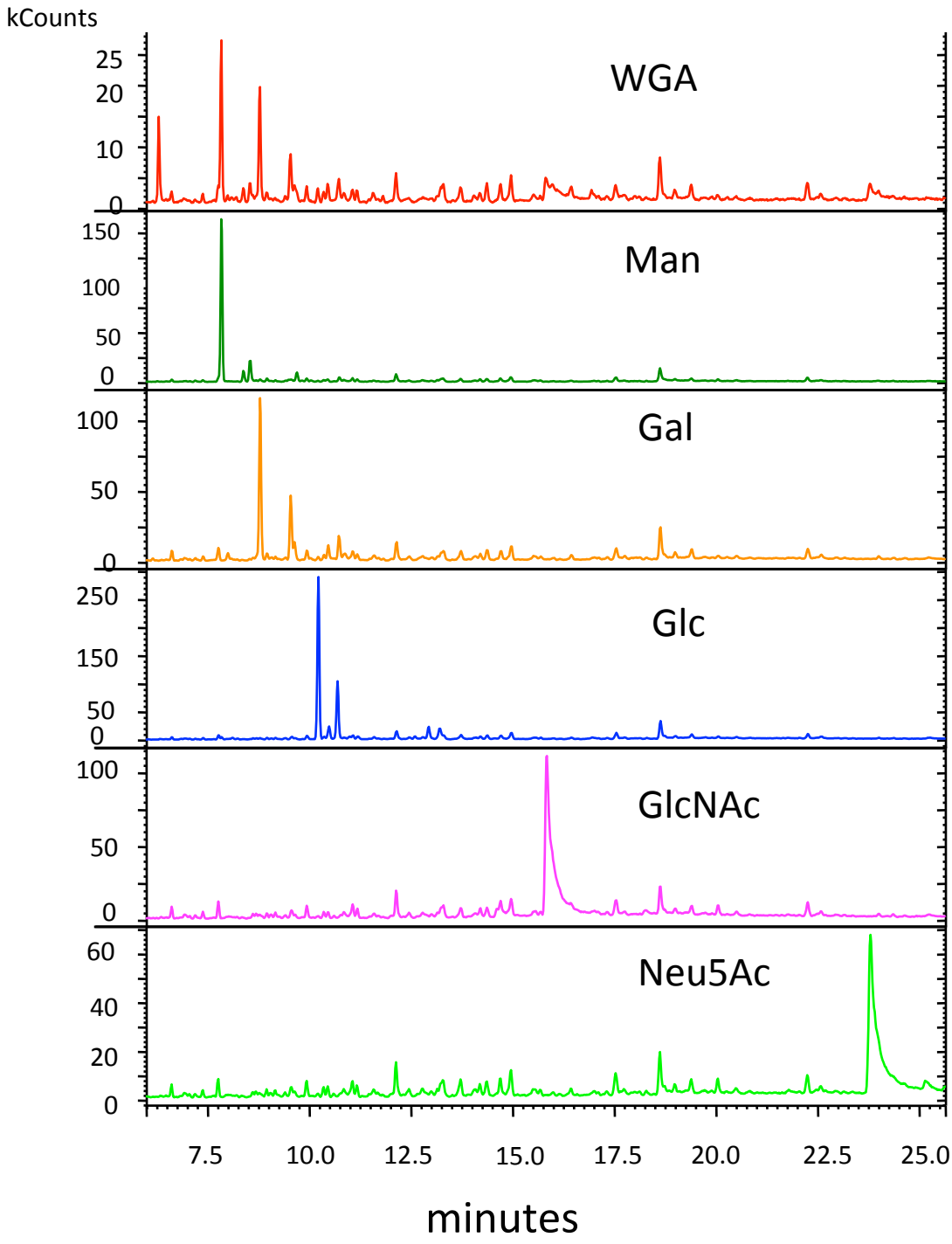



**Fig C2. SMB19 binds to a WGA-reactive glycoprotein.** A.f. cell wall GPI-linked proteins that were reactive with WGA were western blotted and probed with SMB19. The SMB19-reactive protein band was further analyzed by liquid chromatography for common sugar residues.

# APPENDIX D

# IACUC APPROVAL

**THE UNIVERSITY OF ALABAMA AT BIRMINGHAM**

***Institutional Animal Care and Use Committee (IACUC)***

**NOTICE OF RENEWAL**

**DATE:** August 16, 2011

**TO:** JOHN F KEARNEY, Ph.D.
SHEL-410 2182
FAX: (205) 934-1875

**FROM:** Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

**SUBJECT:** Title: Modulation of Allergic Asthma by B Cells and Antibodies
Sponsor: Strategic Program for Asthma Research
Animal Project Number: 110808535

---

As of August 26, 2011, the animal use proposed in the above referenced application is renewed. The University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) approves the use of the following species and numbers of animals:

| Species | Use Category | Number in Category |
|---|---|---|
| Mice | A | 1436 |
| Mice | B | 149 |

Animal use must be renewed by August 25, 2012. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

**Please keep this record for your files, and forward the attached letter to the appropriate granting agency.**

Refer to Animal Protocol Number (APN) 110808535 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at (205) 934-7692.

**Institutional Animal Care and Use Committee**
CH19 Suite 403
933 19th Street South
205.934.7692
FAX 205.934.1188

Mailing Address:
CH19 Suite 403
1530 3RD AVE S
BIRMINGHAM AL 35294-0019

**THE UNIVERSITY OF ALABAMA AT BIRMINGHAM**

***Institutional Animal Care and Use Committee (IACUC)***

**NOTICE OF RENEWAL**

**DATE:** March 16, 2012

**TO:** JOHN F KEARNEY, Ph.D.
SHEL-410 2182
FAX: (205) 934-1875

**FROM:** Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

**SUBJECT:** Title: Regulation of B Cell Clonal Diversity and its Role in Disease
Sponsor: NIH
Animal Project Number: 120409372

---

As of April 15, 2012, the animal use proposed in the above referenced application is renewed. The University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) approves the use of the following species and numbers of animals:

| Species | Use Category | Number in Category |
|---|---|---|
| Mice | A | 1200 |
| Mice | C | 272 |

Animal use must be renewed by April 14, 2013. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

**Please keep this record for your files, and forward the attached letter to the appropriate granting agency.**

Refer to Animal Protocol Number (APN) 120409372 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at (205) 934-7692.

**Institutional Animal Care and Use Committee**
CH19 Suite 403
933 19th Street South
205.934.7692
FAX 205.934.1188

Mailing Address:
CH19 Suite 403
1530 3RD AVE S
BIRMINGHAM AL 35294-0019