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Antibodies Generated against Streptococci Protect in a Mouse Model of Disseminated Aspergillosis

Rebekah E. Wharton,*†Emily K. Stefanov,* R. Glenn King,* and John F. Kearney*

Invasive aspergillosis (IA) resulting from infection by *Aspergillus fumigatus* is a leading cause of death in immunosuppressed populations. There are limited therapeutic options for this disease and currently no vaccine. There is evidence that some anti-*A. fumigatus* mAbs can provide protection against IA. However, vaccine development has been impeded by a paucity of immunological targets on this organism demonstrated to provide protective responses. Sialylated oligosaccharide epitopes found on a variety of pathogens, including fungi and group B streptococci (GBS), are thought to be major virulence factors of these organisms facilitating pathogen attachment to host cells and modulating complement activation and phagocytosis. Because some of these oligosaccharide structures are conserved across kingdoms, we screened a panel of mAbs raised against GBS serotypes for reactivity to *A. fumigatus*. This approach revealed that SMB19, a GBSIb type–specific mAb, reacts with *A. fumigatus* conidia and hyphae. The presence of this Ab in mice, as a result of passive or active immunization, or by enforced expression of the SMB19 H chain as a transgene, results in significant protection in both i.v. and airway-induced models of IA. This study demonstrates that some Abs generated against bacterial polysaccharides engage fungal pathogens and promote their clearance in vivo and thus provide rationale of alternative strategies for the development of vaccines or therapeutic mAbs against these organisms. *The Journal of Immunology*, 2015, 194: 000–000.
GBS-PS conjugate vaccines may be repurposed to develop successful vaccines against *A. fumigatus* and other clinically important human fungal pathogens.

**Materials and Methods**

### Mice

Eight- to 12-wk-old C57BL/6J, B6.129PF2-Tcrbtm1Mom Tcrdtm1Mom (TCRβ/β−/−), B6.129P2-Tg(β2-m−/−) (β2-m−/−) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred and housed in pathogen-free animal facilities. VH J558 transgenic (Tg) mice were generated using the rearranged VDJ from a J558 IgG3 hybridoma previously described (12). C3Tg (Tg) mice were generated using the rearranged VDJ from a J558 IgG3 hybridoma (13), National Center for Biotechnology Information accession sequence BN000872. Genomic DNA was amplified using the primers 5′-CGCCTGGAGTCGTCGTCGTCGTCGTC-3′ and 5′-GCTTCTAATTTCAGCACACC-3′. The PCR product was cloned into the previously described pCDK construct (14) at NcoI and BamHI restriction sites and VH SMB19 Tg mice were generated as described previously for the VH81x Tg mouse (14).

### Microorganisms and mAbs

*A. fumigatus* (strain 13073) and isolates of *Aspergillus niger* (strain IMI 31274) and *Aspergillus flavus* (strain CBC B5107), all purchased from the American Type Culture Collection, were cultured on potato dextrose agar (Fisher) plates at 37°C for 5 d and then harvested as described (15) in PBS, counted on a hemocytometer, and stored at 4°C. GBS (strain names in parenthesis), including GBSib (H36B), GBSla, GBSII (18RS21), and GBSIII (COH1), used in flow cytometry experiments and vaccinations were gifts from Dr. David Pritchard (University of Alabama at Birmingham). GBS used for vaccines were grown to log phase, washed three times, and fixed overnight in 1% paraformaldehyde at 4°C. mAbs specific for these GBS serotypes, α, 1.3 glucan, and the anti-idiotype Ab SMB26 against SMB19 are described, together with methods of purification and quality control, in Supplemental Table I.

### Biacoare analyses

Biacoare T200 (University of Alabama at Birmingham Multidisciplinary Molecular Interaction Core) was used to assess the binding of SMB19 to GBSib capsular PS. Purified biotinylated GBSib capsular PS was captured on a streptavidin sensor chip for the Biacoare T200. After washing in a running buffer containing 3 mM EDTA to chelate Ca2+, SMB19 was injected over the streptavidin-captured GBSib capsular PS chip surface in the presence of 0.9 mM calcium chloride (red) or 3 mM EDTA (green) (see Fig. 2) in the running buffer at concentrations ranging from 0.68 to 50 μg/ml. Sensorgrams (binding curves) were obtained and displayed simultaneously.

### Treatment of *A. fumigatus* with phospholipase C

*A. fumigatus* conidia were germinated for 18 h in RPMI 1640 in a petri dish at 37°C, harvested, and washed twice in PBS (20 min, 2000 rpm) and once in phosphatidylinositol phospholipase C (PI-PLC) enzyme buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA in dH2O [pH 7.4]).

### Flow cytometry

For *A. fumigatus* flow cytometry staining, *A. fumigatus* conidia were cultured in 50 ml conical tubes for 12 h at 37°C in RPMI 1640 to form hyphae. Then, paraformaldehyde-fixed GBS or live resting *A. fumigatus* conidia or hyphae were blocked at 4°C in 1% BSA and then incubated with purified anti-GBS type-specific IgM mAbs. Polyclonal goat anti-mouse IgM coupled to Cy5 (SouthernBiotech) was used to detect binding by flow cytometry. All GBS type-specific mAbs were gifts from Dr. David Pritchard (University of Alabama at Birmingham). IgM binding to *A. fumigatus* was analyzed on a FACS Calibur (BD Biosciences) and FlowJo software. 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/6 mice. Cells were counted, blocked with anti-FcγRI (Ab93), and stained with the Abs listed below. PE anti-B220 (RA3-6B2), PE-Cy7 anti-B220 (RA3-6B2), PE anti-AA-4.1, FITC anti-CD23 (B3B4), Pacific blue goat anti-IgM, biotin anti-CD23 (B3B4), alkaline phosphatase (AP) anti-idiotype (1D3), FITC anti-CD5 (5D3-7), PE anti-CD11b (M1/70), and Alexa Floures 488 anti-CD21 (7G6) were purchased from BD Pharmingen (San Diego, CA). SMB12, rat anti-SMB19 idiotype hybridoma, was generated using previously described methods (16). Cell frequencies were analyzed on a BD LSR II flow cytometer.

### Intravenous infections with *A. fumigatus*

For experiments involving passive Ab transfer, C57BL/6J, TCRβ−/−, C3−/−, or C5-deficient mice were given 200 μg SMB19, SibD2, or Ab i.p. and then immediately infected i.v. with *A. fumigatus* conidia and monitored for survival. In experiments involving a neutropenic model of infection, these mice were given 200 μg anti-Ly56 (IAB clone hybridoma; a gift from Dr. Thomas Malek) i.p. 1 h prior to infection with *A. fumigatus*, and in some experiments another 1 h post-infection with *A. fumigatus*. On day 0, mice were passively administered 200 μg SMB19 or SibD2 i.p. and then immediately infected i.v. with *A. fumigatus* and monitored for survival. Endpoints for these experiments were gauged as mouse unresponsiveness to touch for >2 h (this includes uncontrolled rolling over in the cage, but not responding when touched). Mice were euthanized with CO2 when they appeared moribund. In experiments involving IA in Tg mice, all SMB19 Tg, J558 Tg, μMT, or C57BL/6J mice were infected with 2 × 102 *A. fumigatus* conidia i.v. and monitored for survival. Mice were euthanized with CO2 when they appeared moribund.

### Intratracheal infections with *A. fumigatus*

In experiments involving a neutropenic model of intratracheal (i.t.) *A. fumigatus* infection, C57BL/6J, SMB19 Tg, and J558 Tg mice were given 200 μg anti-Ly56 (IAB clone hybridoma; a gift from Dr. Thomas Malek) i.p. 2 h prior to i.t. infection with 2 × 102 *A. fumigatus*. Nefβ(−/−)/(p47phox−/−) mice were infected i.t. with 102 *A. fumigatus* and 50 μg SMB19 or control (No Ab) i.t. Intratracheal infections were performed as described in Kin et al. (15). Mice were monitored for survival. Mice were euthanized with CO2 when they appeared moribund.

### Inhibition ELISA

*A. fumigatus* conidia were germinated in a Nunc 96-well MaxiSorp plate in DMEM supplemented with 10% FCS for 8 h at 37°C in a CO2 incubator, then dried overnight at 37°C. Wells were washed with PBS and blocked with 1% BSA. SMB19 (250 mg/ml) in 1% BSA was then added to each well along with 1 mg/ml s-LNT or s-LNT (Carboxybenzyl, Berkshire, UK), 0.5 mg/ml GBSib PS (gift from Dr. Dennis Kasper, Harvard University), or α-1,3 dextran (a gift from Dr. A. Jenea) in Dulbecco’s PBS. After 2 h incubation at 37°C, the wells were washed with PBS and incubated with goat anti-mouse IgM-alkaline phosphatase (AP; SouthernBiotech) for 1 h. Plates were then washed with PBS, developed with AP substrate buffer (pH 9.8), stopped with 5 N NaOH, and the OD was measured at 405 nm on a microplate reader from MBG Labtech (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 min at room temperature. Purified capsular PS from GBSib or GBSII was diluted in Dulbecco’s PBS and added to each well and dried overnight at 37°C. Wells were washed three times with PBS and blocked with 1% BSA. Mouse anti-sera were diluted and added to each well, then incubated for 2 h at 37°C. AP goat anti-mouse IgM (SouthernBiotech) was used as the secondary Ab and plates were developed with AP buffer (pH 9.8). OD was read at 405 nm on a MBG Labtech microplate reader.
ELISA assays for detection of the SMB19 idiotype, α-1,3 dextran, and IgM

Costar 96-well plates were coated with 1 μg/ml (SMB1) anti-idiotype Ab to SMB19, α-1,3 dextran, or anti-IgM (RS3.1) in PBS overnight at 4°C. Plates were then washed with PBS three times and blocked with 1% BSA. Mouse sera were diluted in PBS, added to each well, and plates were incubated for 2 h at 37°C. AP goat anti-mouse IgM (SouthernBiotech) was used as the secondary Ab and plates were developed with AP buffer (pH 9.8). OD was read at 405 nm on a BMG Labtech microplate reader.

**Immunofluorescence tissue section analysis**

Spleens were collected from mice, frozen in OCT, and cryostat sections were fixed in acetone, frozen, and stained with Alexa Fluor 350 anti-IgMa, Alexa Fluor 647 anti-CD11b (M1/70), Alexa Fluor 555 anti-CD107a (clone 1F4, BD Pharmingen), and PE anti-laminin (NB300-144) (Novus Biologicals, Littleton, CO). Biotinylated anti-laminin was visualized with Alexa Fluor 350 streptavidin (BD Pharmingen). Mouse sera were diluted in PBS, added to each well, and plates were incubated with AP buffer (pH 9.8). OD was read at 405 nm on a BMG Labtech microplate reader.

**Quantitative PCR analysis**

Brain and kidney DNA was extracted from SMB19 Tg, J558 Tg, WT C57BL/6 mice using the DNeasy blood and tissue kit (Qiagen, Hilden, Germany). Samples were processed and primers used as described previously (17) prior to analysis on a Bio-Rad iQ5 RT-PCR machine. Results were expressed as A. fumigatus conidial equivalents per gram tissue.

**A. fumigatus microscopy analysis**

A. fumigatus conidia were diluted in RPMI 1640 and seeded on glass slides. Slides contained resting A. fumigatus conidia or A. fumigatus that was germinated for 8 h and fixed in 95% ethanol at 4°C. Slides were washed with PBS, blocked with 1% BSA, stained with Alexa Fluor 555 SMB19 and Alexa Fluor 488 anti-β-1,3 glucan (clone 744, IgM, a gift from Dr. Marta Feldmesser) (1), and mounted with DAPI fluoromount. Slides were analyzed using a Leitz DMRB fluorescent microscope.

**Cytokine analysis**

Sera from SMB19 Tg, J558 Tg, μMT, and C57BL/6J mice were collected at various time points before and after infection with A. fumigatus. 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.

**Statistical analysis**

Statistical comparisons were performed using Prism 4.0 software (GraphPad Software). Data with three or more groups were analyzed by a one-way ANOVA test followed by post hoc analysis, whereas data with two groups were analyzed by a two-tailed unpaired t test to determine whether overall statistically significant differences existed. For survival curves, statistical analysis was performed using the Mantel–Cox Log-rank test. A p value <0.05 was considered statistically significant.

**Results**

**mAbs to the GBSIb capsular PS bind A. fumigatus hyphae and conidia**

We found in recent surveys that certain mAbs, which recognize components from a variety of bacterial species, also bind to A. fumigatus at multiple stages of its life cycle (15). In this analysis, we observed that a previously described mouse mAb, SMB19, bound to A. fumigatus conidia (0 h) and hyphae (12 h) of A. fumigatus as detected by flow cytometry (Fig. 1A) and fluorescence microscopy (15). This mAb has been shown previously to bind to a sialylated oligosaccharide, s-LNT, associated with the capsular PS of GBSIb (13). Other mAbs to members of this streptococcal group, including GBSIa (SIaE7), GBSII (SIIF5C4), GBSIII, (SIIV18), and desialylated GBSIb (SibD2) (described in Supplemental Table I), do not bind either A. fumigatus conidia or hyphae by flow cytometry (Fig. 1A), although all mAbs bind to their respective type-specific PS expressed by the cognate serotype of GBS bacteria (Fig. 1B). The binding of SMB19 was notably brightest at the tip of the germination tube and on outgrown hyphae (Fig. 1C). Similar SMB19 reactivity was observed with A. flavus and A. niger (Supplemental Fig. 1A, 1B).

**Fine specificity and Ca2+-dependent binding of SMB19 to the target oligosaccharide**

The terminal oligosaccharides that define the type-specific capsular PS of GBS are known and are shown in Fig. 2A for types Ia, Ila, and Iib. Sera from SMB19 Tg, J558 Tg, μMT, and C57BL/6J mice that were incubated with the indicated type-specific anti-GBS mAbs (blue) or isotype control (red). Binding was visualized by Cy5-conjugated secondary anti-IgM Ab and analyzed by flow cytometry.

![FIGURE 1.](http://www.jimmunol.org/)

The GBSIb type–specific mAb SMB19 binds to A. fumigatus. (A) Resting conidia (0 h) or A. fumigatus hyphae from cultures (12 h) were incubated with the indicated type-specific anti-GBS mAbs (blue) or isotype control (red). Binding was visualized by Cy5-conjugated secondary anti-IgM Ab and analyzed by flow cytometry. (B) The indicated GBS serotypes were stained with SMB19 (blue) or the GBS type–specific IgM (red) followed by anti-IgM secondary Ab and analyzed by flow cytometry. For the GBSIb serotype, SIaE7 (anti-GBSlia) was used as the control (red). (C) Fluorescence microphotograph (upper panel) and phase contrast (lower panel) of A. fumigatus germlings following 9 h of growth in RPMI 1640 media, EtOH fixation, and staining with SMB19 (red) and anti-α-1,3 glucan mAb A16 (green). Scale bar, 10 μm.
Ib, II, and III. SMB19 binding to its target is highly specific because the capsular PS expressed by GBSIb differs from that of GBSIa and the others by having a \( \beta_1 \rightarrow 3 \) instead of the more common \( \beta_1 \rightarrow 4 \) galactose–N-acetylglucosamine linkage (Fig. 2A). Additionally, SMB19 does not react with the desialylated form of GBSIb, which is associated with the oligosaccharide LNT (13). Inhibition of SMB19 binding to \( A. fumigatus \) with either s-LNT– or GBSIb-purified capsular PS, but not LNT or \( \alpha_1,3 \) dextran, confirms that SMB19 binds a similar epitope to that found on GBSIb (Fig. 2B, 2C). Additionally, surface plasmon resonance analysis was used to confirm previous observations (13) that the binding of SMB19 to the purified GBSIb capsular PS is highly dependent on the presence of calcium (Fig. 2E). Treatment of \( A. fumigatus \) hyphae with PI-PLC to cleave GPI-linked proteins ablated SMB19 binding whereas 1-21 (anti–\( \alpha_1,3 \) glucan) binding was not affected (Fig. 2D).

**FIGURE 2.** SMB19 is highly specific for s-LNT, is calcium-dependent, and recognizes a PI-PLC–sensitive epitope expressed by \( A. fumigatus \). (A) The SMB19-reactive oligosaccharide expressed by GBSIb differs from other GBS capsular PS by a \( \beta_1 \rightarrow 3 \) instead of the more common \( \beta_1 \rightarrow 4 \) linkage. (B) Binding of SMB19 to \( A. fumigatus \) visualized by ELISA assay. \( A. fumigatus \)-coated plates were incubated with SMB19 (black), SMB19 plus s-LNT (green), or SMB19 plus LNT (red) followed by an AP-labeled secondary Ab. Data were analyzed by one-way ANOVA with a Dunnett posttest; overall ANOVA, \( p = 0.0097 \). ** \( p < 0.01 \) by post hoc, pairwise comparisons with a Dunnett test. (C) ELISA plates were coated with \( A. fumigatus \) and incubated with SMB19 (black), SMB19 plus purified GBSIb PS (Ib PS, green), or SMB19 plus \( \alpha_1,3 \) dextran (red) and then with AP-labeled secondary Ab. SMB19 binding was analyzed by measuring the OD at 405 nm following development. Inhibition ELISAs were performed in triplicate and repeated twice with similar results. Data were analyzed by one-way ANOVA; overall ANOVA, \( p = 0.0524 \). * \( p < 0.05 \) by post hoc, pairwise comparisons with a Dunnett test. (D) Flow cytometric histograms of \( A. fumigatus \) hyphae treated with PI-PLC (shaded histograms) or buffer treated (open histograms) were stained with SMB19 (green), SIbD2 (red), or the IgM anti–\( \alpha_1,3 \) glucan mAb 1-21 (blue), followed by secondary anti-mouse IgM-Cy5. (E) Surface plasmon resonance Biacore analysis of SMB19 binding in the presence or absence of \( \text{Ca}^{2+} \). The vertical axis (RU) indicates mAb bound at multiple concentrations during 200 s of flow. Overlaid sensograms obtained with multiple concentration of SMB19 in the presence or absence of calcium are shown. DEX, \( \alpha_1,3 \) dextran; Ib PS, GBSIb-purified capsular PS.

Passive transfer of purified SMB19 mAb and vaccination with GBSIb improves survival in a mouse model of disseminated IA

Because SMB19 binds to \( A. fumigatus \) conidia and hyphae, we determined whether this Ab would protect in a mouse model of disseminated IA. Purified SMB19 (anti-GBSIb), SIbD2 (anti-desialylated GBSIb), or A16 (anti–\( \alpha_1,3 \) glucan) was passively administered i.p. to naive C57BL/6J mice immediately before infection with 2 \( \times \) LD\(_{50}\) \( A. fumigatus \) conidia i.v. Approximately 75% of mice that received SMB19, but only 10–30% of mice that received either SIbD2 or A16, survived 60 d postinfection (Fig. 3A). There were no significant differences in protection between mice that received SIbD2 or A16. Injection i.p. of SMB19 revealed that this IgM mAb has an in vivo half-life of \( \sim \)2 d (Fig. 3B), similar to that found for other IgM Abs. These results indicate that a single treatment with GBSIb PS-specific SMB19, but not SIbD2 specific for the desialylated GBSIb PS.
Passive transfer of SMB19 or vaccination with GBSIb improves survival in a mouse model of disseminated IA. (A) Survival of C57BL/6J 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 2 × 10^3 A. fumigatus conidia. Results were pooled from three separate experiments. Asterisks denote significant differences in average survival compared with mice administered SibD2, A16, or PBS. (B) Serum IgM levels were analyzed at various times before and after passive transfer of 200 μg SMB19 by ELISA. (C) Survival of mice vaccinated i.v. with GBSIb (green, n = 12), GBSII (red, n = 10), or PBS (black, n = 17) 6 d before i.v. infection with A. fumigatus. Results are pooled from two separate experiments with similar results. Asterisks denote significant differences in average survival of GBSIb-vaccinated animals compared with GBSII-vaccinated and PBS control groups. (D-F) Serum GBSIb type–specific IgM (D), SMB19 idiotype+ IgM (E), and GBSII type–specific IgM (F) quantified by ELISA immediately before (day −6) or 6 d after (day 0) vaccination with GBSIb (green) or GBSII (red). Asterisks denote significant differences between serum IgM levels at day −6 and day 0. Data were analyzed by a two-tailed unpaired t test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. In survival studies, statistical significance was determined by a log-rank test.

or A16 specific for A. fumigatus–expressed α-1,3 glucans, improves survival against disseminated IA.

The significant protection provided by passive administration of SMB19 suggested that anti-GBSIb responses, as a result of immunization with GBS-PS, would protect mice from A. fumigatus challenge. To determine whether the Ab response following vaccination is sufficient to replicate the protection observed in passive transfer experiments, and to determine whether a protective response is specific to the GBSIb-PS vaccine, C57BL/6J mice were vaccinated i.v. with GBSIb, GBSII, or administered PBS. Six days following vaccination, at the predetermined peak of the anti-GBS Ab response, mice were infected i.v. with a lethal dose of A. fumigatus conidia. PBS-treated and GBSIb-immunized mice died within ∼15 d, whereas ∼50% of GBSIb-vaccinated mice survived 60 d (at which point the experiment was ended) (Fig. 3C). Thus, GBSIb vaccination significantly prolonged survival compared with PBS sham or GBSII-immunized controls, indicating that the protection induced is specifically associated with GBSIb vaccination. In GBSIb-immunized mice, there was an increase in GBSIb type–specific IgM Ab, in addition to that of the SMB19 idiotype. In contrast, mice vaccinated with GBSII had ∼100 μg/ml GBSIb-reactive serum Ab, but very little GBSIb-specific or SMB19 idiotype+ Ab (Fig. 3D-F). These results indicate that immunization of mice with GBSIb, but not with GBSII, promotes survival in ∼50% of mice for 60 d after A. fumigatus infection. Furthermore, this protection is associated with increased levels of anti-GBSIb Abs and specifically SMB19 idiotype+ Ab.

SMB19 Tg mice are significantly protected against disseminated IA

We have previously observed that passively administered IgM Ab has a limited half-life, and levels of Ab to GBSIb peaks at ∼7 d after immunization and then subsides. To circumvent the transient nature of the anti-PS response in mice, we used a Tg mouse expressing the SMB19 H chain (characterized in Supplemental Fig. 2). In this model, SMB19 idiotype+ Ab is constitutively maintained at ∼10-fold higher levels than in C57BL/6J mice. Additionally, previous studies using μMT mice, which lack functional B cells, suggested that B cells and Abs may increase the susceptibility of mice to A. fumigatus infection (6). To address these issues and further investigate the protective role of SMB19 in IA, we infected SMB19 IgH Tg (SMB19 Tg), μMT, J558 IgH Tg (J558 Tg), or C57BL/6J mice with a lethal dose of A. fumigatus conidia i.v. SMB19 Tg mice were highly protected compared with C57BL/6J, μMT, or J558 Tg mice (Fig. 4A). Interestingly, in contrast to previous reports, μMT mice rapidly succumbed to A. fumigatus infections at similar frequencies to C57BL/6J mice. Additionally, J558 Tg mice, which maintain high levels of Abs specific for α-1,3 glucan (Fig. 4D), a major component of the A. fumigatus cell wall, were not protected compared with C57BL/6J mice. Although C57BL/6J mice and J558 Tg mice both have similar levels of GBSIb PS-binding serum IgM as do SMB19 Tg mice, SMB19 idiotype Abs are significantly lower in these mice. As observed in the GBS vaccination studies described above, the increased levels of SMB19 idiotype-bearing IgM was significantly correlated with protection in this model of IA (Fig. 4B, 4C). In J558 Tg mice, the observed anti-GBSIb Abs likely arose from B cells bearing endogenous Ig gene rearrangements. As expected, μMT mice did not have detectable levels of serum Igs. These results, in conjunction with the protection afforded by purified SMB19 mAb, further indicate that not all polyclonal GBSIb-reactive Abs provide protection in this model of IA (Fig. 4B). Instead, a particular subset of Abs, including those of the SMB19 idiotype, is responsible for the protection observed. Importantly, J558 Tg mice, which express Abs to a major A. fumigatus cell wall

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component, are as susceptible as C57BL/6J and μMT mice to *A. fumigatus* infection.

Because cytokines have been associated with both enhanced susceptibility or resistance to IA, we analyzed cytokine levels in the sera of each of these groups of mice immediately before and at 4 and 7 d after infection with *A. fumigatus*. SMB19 Tg mice had significantly lower levels of granulocyte proliferation and survival cytokines G-CSF and GM-CSF, which have been previously associated with protection against IA. Additionally, none of the groups of mice analyzed had significantly different levels of Th1 or Th2 cytokines previously associated with protection or susceptibility to IA, including IL-4, IL-5, IL-10, IL-2, and IFN-γ (Supplemental Fig. 3). Taken together, these data show that Ab-mediated protection in this model of IA differs mechanistically from protection reported in other systems.

**SMB19 Tg mice survival correlates with decreased *A. fumigatus* fungal burden and neutrophil infiltrates in the brain and kidneys**

Because SMB19 Tg mice demonstrated significantly higher survival rates following challenge with *A. fumigatus* than did WT C57BL/6J, μMT, or J558 Tg mice, we sought to determine whether this protection correlated with decreased fungal burden in the tissues affected during *A. fumigatus* pathogenesis. It has previously been reported that brain and kidneys are the major tissues damaged during this *A. fumigatus* infection model (17); therefore, we analyzed both tissues by immunofluorescence and quantitative PCR following *A. fumigatus* infection. Immunofluorescence analysis revealed that SMB19 Tg mice had significantly fewer *A. fumigatus* lesions in brain (Fig. 5A–E) and kidneys (Fig. 5G–K) compared with C57BL/6J, μMT, or J558 Tg mice 4 d after i.v. infection. There were no detectable *A. fumigatus*-containing lesions in brain or kidney tissue sections from mice that survived 60 d post-infection. Furthermore, immunofluorescence analysis showed that *A. fumigatus*-containing lesions in the brains of C57BL/6J and μMT mice were filled with leukocyte infiltrates, including CD11b+ and Ly6G+ neutrophils. In contrast, *A. fumigatus* lesions were undetectable in tissues from most SMB19 Tg mice 4 d postinfection. The few lesions observed were surrounded by cells weakly expressing CD11b but negative for Ly6G (Fig. 5D, 5J). None of the SMB19 Tg mice had visible lesions of *A. fumigatus* in liver or spleen (data not shown). To more accurately access the global fungal burden within these mice, quantitative PCR for *A. fumigatus* genomic DNA was performed. This analysis confirmed that SMB19 Tg mice had less fungal burden in their brain (Fig. 5F) and kidney (Fig. 5L) than did WT C57BL/6J, μMT, and J558 Tg mice. These data show that the survival of SMB19 Tg mice infected with *A. fumigatus* correlates with a decreased fungal burden and fewer neutrophil infiltrates in tissues known to be damaged as a result of fungal outgrowth following i.v. infection of mice with *A. fumigatus*.

**Neutrophil-depleted SMB19 Tg mice and NcT1[^1]{ij} mice administered SMB19 i.t. are protected after i.t. *A. fumigatus* infection**

Although i.v. infection with *A. fumigatus* conidia may rarely occur, the most common route of disseminated infection in immunodeficient patients is by the inhalation of conidia. Because neutrophil insufficiency as a result of myeloablation is a major risk factor for susceptibility to IA, we used a model of infection that renders...
mice transiently neutropenic. SMB19 Tg, J558 Tg, and WT C57BL/6J mice were given one i.p. injection of neutrophil-depleting anti-Ly6G mAb 2 h before i.t. infection with *A. fumigatus*. This dose of anti-Ly6G is sufficient to maintain neutropenia during the course of 3–4 d after injection. After mice were confirmed to be neutropenic (Fig. 6A), these three groups of mice were infected i.t. with *A. fumigatus* compared with SMB19 Tg at 4 d (D) or 60 d (E). (G–K) Analysis of kidney tissue from C57BL/6J (G), μMT (H), and J558 Tg mice (I) compared with SMB19 Tg mice at 4 (J) and 60 d (K) after i.v. infection with *A. fumigatus*. Sections were 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/6J, μMT, and J558 Tg mice or anti-β-1,3 glucan remnants in SMB19-treated mice. (F and L) Quantitative PCR analysis of fungal burden in brain (F) or kidney (L) of WT C57BL/6J (black), μMT (red), SMB19 Tg (green), and J558 Tg mice (blue) at the indicated times postinfection with *A. fumigatus*. Data represent the means ± SEM from two independent experiments with three mice per group. nd, not detectable. The y-axis represents the number of *A. fumigatus* conidial equivalents per gram of infected tissue extrapolated from cycle threshold calibration curves generated by the inclusion of known numbers of *A. fumigatus* conidia into naive tissue preparations.

FIGURE 5. SMB19 Tg mice have low or undetectable *A. fumigatus* fungal burden in their targeted tissues following *A. fumigatus* challenge. (A–E) Immunofluorescence analysis (original magnification ×30) on frozen sections of cerebrum from C57BL/6J (A), μMT (B), and J558 Tg mice (C) 4 d following i.v. infection with *A. fumigatus* compared with SMB19 Tg at 4 d (D) or 60 d (E). (F and L) Quantitative PCR analysis of fungal burden in brain (F) or kidney (L) of WT C57BL/6J (black), μMT (red), SMB19 Tg (green), and J558 Tg mice (blue) at the indicated times postinfection with *A. fumigatus*. Data represent the means ± SEM from two independent experiments with three mice per group. nd, not detectable. The y-axis represents the number of *A. fumigatus* conidial equivalents per gram of infected tissue extrapolated from cycle threshold calibration curves generated by the inclusion of known numbers of *A. fumigatus* conidia into naive tissue preparations.
observed by passive immunization with SMB19, we passively transferred SMB19 or isotype control mAbs i.p. to C3−/− mice then immediately infected them i.v. with a lethal dose of A. fumigatus conidia. There were no differences in survival of C3−/− mice that received SMB19 or isotype control mAbs (Supplemental Fig. 1D). C5−/− mice treated with SMB19 were as resistant to A. fumigatus infection as were SMB19-treated C57BL/6J mice (data not shown). These data suggest that early complement components are involved in SMB19-mediated protection in mice, likely by promoting opsonophagocytosis of A. fumigatus and not via formation of membrane attack complexes. Because large volumes of data suggest that T cells are necessary for long-term survival of A. fumigatus–infected mice, we next analyzed the role of T cells in SMB19-mediated protection against i.v. infection. SMB19 or isotype control Ab was administered i.p. to TCRβ/−/− mice immediately before i.v. infection with 2 × 10^6 A. fumigatus conidia. TCRβ/−/− mice that received SMB19 mAb are more resistant to infection than are the mice that received isotype control mAb (Supplemental Fig. 1E). The degree of protection observed in these animals is similar to that of C57BL/6J mice that received SMB19 mAb (Fig. 3A). Collectively, these results show that SMB19, when maintained at relatively high levels, endogenously in the Tg mice or passively introduced, provides substantial protection against infection following inhalation of A. fumigatus conidia. These findings contrast with those in the i.v. model, which indicate that SMB19-mediated protection is dependent on neutrophil and complement activity, but not the activity of T cells.

**Discussion**

Abs and B cells historically have not been associated with protection in IA due to the lack of correlation between A. fumigatus Ab titers and IA protection, and partially due to a study demonstrating that μMT mice, which do not make Abs, were less susceptible in comparison with IA than were WT mice (6). However, recent studies on the role of mAbs in protection against A. fumigatus, especially those against β-1,3 glucans (7), have generated new interest in this field. Few studies, however, have identified new epitopes on A. fumigatus that could be targeted therapeutically with mAbs. Our results show that SMB19, an mAb generated against GBSIb, also bound resting A. fumigatus conidia and more strongly to A. fumigatus hyphae. We have shown that this Ab provides striking protection in both the i.v. infection model and, of greater relevance, in an inhalation model of aspergillosis.

This mAb binds an epitope distinct from the sialic acid–containing capsular PS found on other GBS serotypes by its preference for the GBSIb type–specific oligosaccharide containing a β1→3 instead of the more common β1→4 galactose–N-acetylglucosamine linkage characteristic of the other subtypes. The fact that SMB19 bound preferentially to the tips of A. fumigatus hyphae is particularly interesting because the nascent cell wall of growing hyphae is especially vulnerable to external influences and, as such, is the target site for certain antifungal agents. The observation that binding of SMB19 to its oligosaccharide Ag is calcium-dependent may also have some bearing on SMB19 binding to A. fumigatus, because proper outgrowth of fungal hyphal tips relies on high cytoplasmic calcium gradients (18). Dysregulation of these gradients inhibits hyphal growth and disrupts tip morphology. It is possible that SMB19 preferentially binding to A. fumigatus hyphal tips is facilitated by high levels of calcium flux and contributes to the unique protective properties of this Ab.

Passive administration of SMB19 results in significant protection in the i.v. model of infection, in contrast to treatment with A16,
which binds α-1,3 glucan, a major cell wall component of A. fumigatus. Furthermore, C57BL/6J mice vaccinated with GBSb, which induces moderate increases of SMB19 idiotype Ab, were protected to a greater extent than were the mice that received passive Ab. Finally, SMB19 Tg mice, which maintain constitutively high levels of SMB19 idiotype Ab, are significantly resistant to A. fumigatus infection. Furthermore, the highly effective protection shown in the SMB19 Tg mice demonstrates that the protection elicited by immunization with GBSb-PS is not the result of a unique innate immune response primed by that particular organism and not other GBS serotypes, because the SMB19 Tg mice are protected in the absence of immunization. It is also of great interest that J558 Tg mice, which maintain high titers of Abs against α-1,3 glucans, a major component of the A. fumigatus cell wall, are not protected in this model of A. fumigatus. This observation further highlights the unique role of SMB19 idiotype-bearing Abs and its target Ag in mediating the observed protection.

Survival of i.v. infected SMB19 Tg mice correlates with decreased fungal load, neutrophil infiltrates, and abscesses containing actively growing hyphae in the brain and kidneys when compared with infected J558 Tg, C57BL/6J, and µMT mice. Rarely, small areas in the brain of SMB19 Tg mice 4 d postinfection contained what appeared to be A. fumigatus remnants surrounded by MAC1+ and Ly6G+ cells. These cells could be activated resident brain cells such as microglia. This observation suggests that some A. fumigatus conidia penetrated the blood brain barrier and germinated into the hyphal form in SMB19 Tg mice, but their growth was eventually halted by SMB19-mediated killing leaving only nonmetabolic hyphal remnants as shown in Fig. 5. Additionally, C3−/− mice, in contrast to C57BL/6J mice, are not protected from i.v induced IA after passive administration of SMB19. This indicates that complement activation is, at least in part, responsible for the therapeutic effect of SMB19 Ab. Also of interest is that, contrary to previous studies on C5 in IA, our C5-deficient mice were more susceptible to IA than were WT mice. In this study, we used C5-deficient mice on the C57BL/6J background compared with WT C57BL/6J mice, whereas in the previous study DBA/2N mice, which are deficient in C5, were compared with CFW mice (19). Differences in these mouse strains could possibly contribute to the differences in survival of A. fumigatus–infected mice in these studies, which were attributed to the loss of complement components. Many studies have implicated T cell immunity as being particularly important in clearing A. fumigatus following infection. We have shown in the present study that SMB19 Abs do not depend on T cells for the immediate protection observed in this model of acute A. fumigatus infection.

Our studies of A. fumigatus infections during transiently induced neutropenia indicate that neutrophils are important for protection against IA afforded by SMB19 Abs in mice challenged i.v. Interestingly, however, in two models where neutrophils were transiently depleted or were severely impaired in their bactericidal capacity, SMB19 Abs were highly protective against infection when conidia were administered into the lung. In the case of the SMB19 Tg mice, this protection was highly effective and prolonged. SMB19 coadministration with conidia was nearly as protective in Ncf1−/−/J mice, which harbor a defect in NADPH oxidase activity, during the first 11 d following infection. Although many of the SMB19-treated mice eventually succumbed to infection, 20% of these mice survived at least 20 d. These results suggest that if the level of SMB19-like Abs is maintained, it will protect against lethal infection in mice that have severely impaired neutrophil numbers or function, which is commonly associated with aspergillosis in humans. These results also suggest that the protective effects of SMB19-like Abs may be provided by different mechanisms in response to airway or parenteral A. fumigatus infection.

There are several additional strategies to pursue regarding the development of SMB19-like Abs as a potential active or passive therapeutic for patients at risk for IA. Passive Ab could be combined with other antifungal therapies such as amphotericin B, voriconazole, and caspofungin, which are administered as standard treatments for IA patients. Amphotericin B and voriconazole target ergosterol synthesis on the A. fumigatus membrane, whereas caspofungin targets β-1,3-glucan synthase enzymatic activity, which also ultimately affects A. fumigatus membrane synthesis. A recent study showed that mAbs against 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. fumigatus.

In patients with the capacity to mount an Ab response, active immunization is feasible. Additionally, GBS-PS conjugate vaccines against GBS serotypes I–V have already been developed and validated through phase II clinical trials in humans (11). In preliminary studies of sera from humans immunized with these GBS1b conjugate vaccines, we have found that there are both IgM and IgG Abs that bind in a similar pattern to A. fumigatus conidia and hyphal tips as does SMB19. Furthermore, sera from individuals who received the GBSb vaccine inhibit SMB19 binding to A. fumigatus. These results indicate that GBS conjugates could be repurposed for use as antifungal vaccines in at-risk individuals. Our preliminary studies show that SMB19 Ab and these human GBS immune sera also show intense staining of Candida albicans and Rhizopus oryzae during their hyphal growth stages, as well as germinating Fusarium spp. macroconidia, indicating that Abs derived from GBS immunization recognize a common epitope on multiple fungi. Future research will determine whether the induction of SMB19-like Abs of IgM and IgG isotypes induced by GBS conjugate vaccines are “globally” protective against A. fumigatus and other clinically relevant fungi (20, 21).

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Disclosures
The authors have no financial conflicts of interest.

References


Supplementary Table 1: mAb clone names, isotype and antigen specificities

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Supplementary Table 1

IgM antibodies were purified from supernatants of hybridomas grown in Gibco serum free RPMI1640 by affinity chromatography over RS3.1 mouse anti-IgM<sup>a</sup>-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 1. SMB19 binds divergent species of Aspergillus and the protection observed in the i.v. model of I.A. is independent of neutrophil and T cell function, but requires complement activity.

(A) Staining of *Aspergillus flavus* (upper) and *Aspergillus niger* (lower) with SMB19 (red) and calcifluor (chitin binding, green). (B) Corresponding SMB19 (green) binding or isotype control (SIBD2, red) detected by flow cytometry. (C) Survival of transiently neutropenic WT C57BL/6J mice passively administered 200 μg SMB19 (green, n=15) or SlbD2 (red, n=14) i.p. 24 hours before i.v. infection with 2xLD₅₀ (2.5x10⁵) A.f. conidia. (D) Survival of C3⁻/⁻ mice passively transferred i.p. with 200 μg SMB19 (green) or control isotype SlbD2 (red) immediately before i.v. infection with 2xLD₅₀ (2.5x10⁵) A.f. (E) Survival of TCRβ⁻/⁻δ⁻/⁻ mice passively administered SMB19 (green, n=11) or SlbD2 (red, n=12) i.p. immediately before i.v. infection with 2xLD₅₀ (2x10⁶) A.f. conidia. Asterisks denote significant differences in average survival rate of SMB19 BCR Tg mice compared to WT C57BL/6J and J558 BCR Tg mice or mice passively transferred SMB19 compared to isotype control. Statistical significance was determined by log rank test *= p<0.05, **= p<0.01.
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 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 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 SlaE7 (black) binding to SMB19 anti-idiotype mAb, SMBi26. Experiments were performed in triplicate twice with similar results.
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 Th1 and Th2, and neutrophil proliferation-associated 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. Data represent 2 independent experiments with similar results. Data was analyzed using a One-way ANOVA with a Tukey's post test *= 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.