Efficacy of Antibody to PNAG Against Keratitis Caused by Fungal Pathogens

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PURPOSE. Developing immunotherapies for fungal eye infections is a high priority. We analyzed fungal pathogens for expression of the surface polysaccharide, poly-N-acetyl glucosamine (PNAG), and used a mouse model of ocular keratitis caused by *Aspergillus flavus*, *A. fumigatus*, or *Fusarium solani* to determine if PNAG was an immunotherapy target and requirements for ancillary cellular and molecular immune effectors.

METHODS. Enzyme-linked immunosorbent assay (ELISA) or immunofluorescence was used to detect PNAG on fungal cells. Keratitis was induced by scratching corneas of C57BL/6, IL-17R KO, RAG-1 KO, or IL-22 KO mice followed by inoculation with fungal pathogens. Goat antibodies to PNAG, a PNAG-specific human IgG1 monoclonal antibody, or control antibodies were injected either prophylactically plus therapeutically or therapeutically only, and corneal pathology and fungal levels determined in infected eyes at 24 or 48 hours after infection.

RESULTS. All tested fungal species produced PNAG. Prophylactic or therapeutic treatment by intraperitoneal (IP) injection of antibody to PNAG combined with post-infection topical application of antibody, the latter also used for *A. fumigatus*, led to reduced fungal levels, corneal pathology, and cytokine expression. Topical administration only of the PNAG monoclonal antibodies (MAb) reduced fungal loads and corneal pathology. There was no antibody protection in IL-17R KO, RAG-1 KO, or IL-22 KO mice.

CONCLUSIONS. Poly-N-acetyl glucosamine is produced by clinically important fungal ocular pathogens. Antibody to PNAG demonstrated protection against Aspergillus and Fusarium keratitis, requiring T cells producing IL-17 and IL-22. These findings indicate the potential to prevent or treat fungal infections by vaccines and immunotherapeutics to PNAG.

Keywords: fungal keratitis, PNAG, vaccine, MAb, immunotherapy

Infectious keratitis is a leading cause of monocular blindness worldwide[1] and up to 65% of the corneal ulcers are caused by fungal pathogens.[2] The main risk factors for fungal keratitis are immunosuppression and ocular trauma from implants or contact lenses. The most prevalent genera are Fusarium, Aspergillus, and Candida. Once the Aspergillus and Fusarium conidia germinate in the corneal stroma, the hyphae can penetrate into this tissue to cause ulceration, severe pain, and visual impairment.[3-5] Currently, the number of effective antifungal drugs is small and they are less tissue-permeable compared to antibacterial drugs.[1] More than 60% of fungal keratitis cases require surgical intervention.[5] Therefore, there is an urgent need to develop new treatment strategies for effective therapy of this disease.

Several prior studies have indicated immune mechanisms can control fungal keratitis. In two studies, Taylor et al.[6,7] showed an important role for IL-17–producing polymorphonuclear neutrophils (PMN) for protection against Aspergillus and Fusarium corneal infections in mice. Additionally, Zhang et al.[8] demonstrated that a CD4+-T cell TH1-type adaptive immune response and immunologic memory were induced by *Candida albicans* keratitis or by immunization with killed spores, implicating antibody and cellular responses in the response against fungal keratitis. These studies hinted that immunotherapy for fungal keratitis is possible, but to date, there are no clinically applicable vaccines or antibody-based immunotherapies for these sight-destroying infections.

To address this major medical need, we determined if the surface antigen, poly-*N*-acetyl-β-(1-6)-glucosamine (PNAG), that is expressed by a broad range of microbial pathogens[9] could be a target for prevention of keratitis caused by Aspergillus spp. or Fusarium spp. Antibodies to PNAG have shown bactericidal and opsonic killing activities along with protective efficacy in mice against infectious keratitis caused by several human pathogens,[9-12] including methicillin-resistant *Staphylococcus aureus* (MRSA).[13] A fully human IgG1 monoclonal antibody (MAb) to PNAG[14] detects the antigen on the surface of prokaryotic and eukaryotic microbial organisms, including fungi, and does not cross-react with fungal glucans.[9] Protective efficacy of the MAb to PNAG against *C. albicans* keratitis in mice has been demonstrated,[9] but the efficacy of targeting PNAG on other major corneal fungal pathogens has

not been determined to our knowledge. In the current study, we evaluated whether antibodies to PNAG-mediated killing of *A. flavus* and *F. solani* in opsonophagocytic assays and were protective following either prophylactic or therapeutic administration in an experimental keratitis model. We additionally evaluated protection against *A. fumigatus* keratitis. The pattern of antibody administration was designed to mimic potential uses for human clinical settings, including prophylactic administration that might be useful for those at high risk for infection, such as following corneal injury, as well as therapeutic administration after infection is established, a potential component of therapeutic treatment modalities. Antibody to PNAG provided reduced fungal burdens in infected corneas and lower median pathology against all the fungal pathogens in all settings tested, indicative of a potential broad efficacy targeting these therapeutically challenging infectious agents.

MATERIALS AND METHODS

Fungal Strains, Cells, and Mice

Aspergillus flavus strain (BP09-1), *A. fumigatus* and *F. solani* (B1-11) are clinical isolates kindly provided by Darlene Miller, Bascom-Palmer Eye Institute. *Aspergillus flavus* and *A. fumigatus* were cultured on Sabouraud Dextrose Agar (SDA) at 28°C for 3 days. For use, the conidia were scraped from the SDA plate into PBS, placed into a tube, conidia counted with a hemocytometer, and then adjusted to a concentration of approximately 10^9 conidia/ml. *Fusarium solani* was grown in Sabouraud Dextrose broth (SDB) 30°C with shaking at 225 rpm overnight and then adjusted to approximately 2×10^9 CFU/mL after counting. C57BL/6 mice (6–8 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Mice deficient in *recombinase activating gene-1* (*RAG-1* KO), IL-17 receptor (IL-17R KO), and IL-22 (IL-22 KO) were bred in our animal facility.

Antibody to PNAG

Polyclonal antibody to PNAG was raised in goats using a synthetic oligosaccharide of polyglucosamine, 9GlcNH$_2$ conjugated to the carrier protein tetanus toxoid (9GlcNH$_2$-TT).[15] Monoclonal antibody to PNAG also was used in this study, which is a fully human IgG1 MAb F598.[14] Controls were normal goat serum or human IgG1 MAb F429 specific to *Pseudomonas aeruginosa* alginate,[16] respectively.

ELISA Assay

To coat wells for ELISA detection of PNAG expression, *A. flavus* (10 μL of a frozen stock) was inoculated into 100 μL of SDB per well of 96-well sterile tissue culture microplates, and grown for 3 days at room temperature. *Fusarium solani* was grown on SDA plates and suspended in 0.04 M sodium phosphate buffer, pH 7.2 to an OD$_{650}$ nm of 1.0 (approximately 2×10^9 CFU/ml). Then, 100 μl of this suspension used to coat 96 well ELISA plates (Immulon 4; Thermo Fisher Scientific, Waltham, MA, USA). Next, the plates were incubated at 37°C for 1 hour to bind *F. solani* to the wells. After sensitization with both strains, wells were aspirated and washed with 400 μL of PBS with 0.05%Tween-20 (PBS-T), then each well blocked with PBS containing 1% BSA at 37°C for 2 hours. After washing, 100 μl of MAb F598 or control MAb F429 (20 μg/mL) was added, the MAb F598 to PNAG serially diluted in PBS-T and plates incubated at 37°C for 2 hours. Next, plates were washed then MAb binding detected with an anti-human IgG-alkaline phosphatase conjugated secondary

antibody (Sigma-Aldrich Corp., St. Louis, MO, USA). After 1 hour at room temperature, wells were washed and then 100 μl of 1 mg/ml p-nitrophenyl phosphate (3.8 mM) added to generate the indicator dye and intensity of the color measured at OD$_{405}$ nm.

Opsonophagocytic Killing Assay

In vitro killing of *A. flavus* and *F. solani* followed published protocols used with *S. aureus*.[17] Briefly, the fungal strains were grown as described above, adjusted via counting in a hemocytometer to approximately 2×10^8 CFU/ml, and a 1:100 dilution in RPMI-FBS made for use in the killing assay. White blood cells (WBC) were prepared from fresh human blood collected from healthy adult volunteers under an Institutional Review Board approved protocol using dextran-heparin buffer layering for separation. Human subjects research adhered to the tenets of the Declaration of Helsinki. White blood cells were adjusted to 5×10^6 per ml. Baby rabbit serum (Accurate Chemical, Westbury, NY, USA) diluted 1:15 in RPMI-FBS was used as the complement source, adsorbed at 4°C for 30 minutes with the target fungal cells resuspended from a pellet containing approximately 10^9 CFU/ml and with continual mixing, then the complement centrifuged and filter sterilized. Either MAb or a polyclonal antiserum to PNAG, or normal rabbit serum (NRS) or MAb F429 as a control were used at concentrations indicated in the Figures.

The phagocytic killing assay consisted of 100 μl (each) of the WBC suspension, target fungal strain, dilutions of test sera, and the absorbed complement source. The reaction mixture was incubated on a rotor rack at 37°C for 90 minutes; samples were taken at time zero and after 90 minutes, aliquots diluted in tryptic soy broth with 0.5% Tween to inhibit cellular aggregation, and samples were plated onto SDA plates. Controls consisted of tubes lacking any serum, tubes with NRS or control MAb F429, tubes containing antibody to PNAG and complement but lacking WBC. Killing of fungal strains was only obtained when antibody to PNAG, complement, and WBC all were present. The percentage of killing was calculated by determining the ratio of the number of CFU surviving in the tubes with bacteria, leukocytes, complement, and sera to the number of CFU surviving in tubes lacking sera but containing bacteria, complement, and leukocytes. Killing rates of >30% are considered biologically significant, as this level of killing is associated with antisera that provide in vivo protection in animal studies.[8,17]

Corneal Infections in Mice

The experimental protocols were approved by the Institutional Animal Care and Use Committee of the Harvard Medical Area, Office for Research Subject Protection and were consistent with the ARVO Animal Statement for the Use of Animals in Ophthalmic and Vision Research. We adapted the mouse model of ulcerative keratitis[18] to evaluate protective efficacy of antibody to PNAG against fungal infections and associated corneal pathology. Briefly, mice were anesthetized by intraperitoneal (IP) injection with ketamine (100 mg/Kg) and xylazine (10 mg/Kg), and when there was no response to corneal touching, three scratches (1 cm) were made on one cornea with a 26-gauge needle, Next, 5 μl containing the infectious inoculum (1×10^7 cells/eye) was placed on the injured cornea. Mice were left recumbent and observed until awake and mobile. For evaluation of the prophylactic protective efficacy of antibody to PNAG, mice were injected IP 16 hours before infection with 300 μg of MAb to PNAG,[14] or control MAb.[16] At 4 hours after infection, an additional 300 μg of MAb to PNAG or control MAb were injected IP, and then
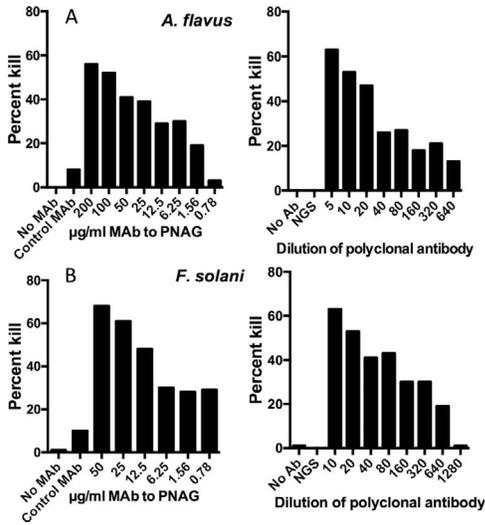
FIGURE 1. Opsonic killing of *A. flavus* and *F. solani* by antibody to PNAG. Aspergillus flavus (**A**) and *F. solani* (**B**) are effectively killed by human IgG1 MAb (*left*) or goat polyclonal antibody to PNAG (*right*) in the presence of polymorphonuclear leukocytes and complement. *Scale bars*: Mean of duplicate determinations.

10 µg/eye applied topically at 4, 16 or 24, and 32 hours after infection. Some experiments with *A. flavus* were terminated at 24 hours if there was severe corneal pathology in ≥25% of the controls (grade 4) at this time. For evaluation of therapeutic efficacy, normal and immune polyclonal goat antibody to PNAG raised to the synthetic oligosaccharide, 9GlcNH$_2$ conjugated to tetanus toxoid,[15] or the PNAG-specific or control human IgG1 MAbs were used. Sera or MAbs were injected IP 4 hours after infection and an additional 10 µl serum or 10 µg antibody in 5 µl of PBS applied topically 4, 24,
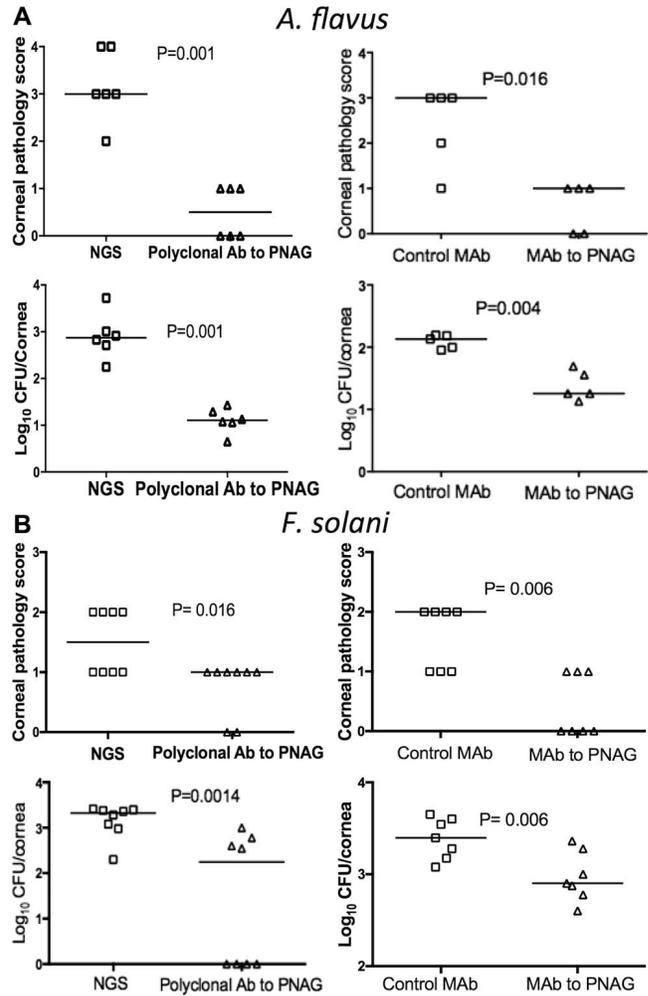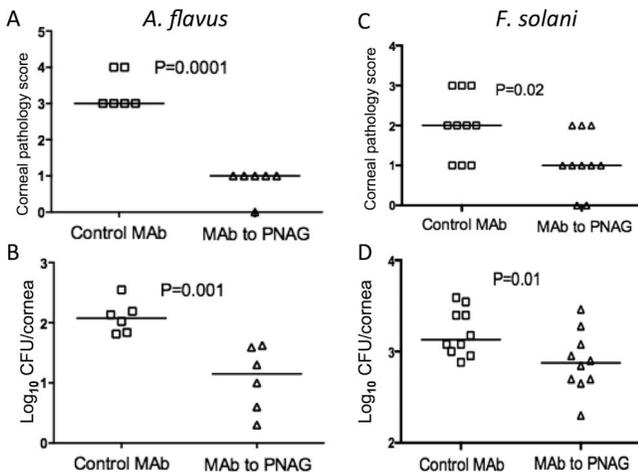

FIGURE 2. Combination of pre- and post-infection administration of MAb to PNAG reduces fungal burdens and corneal pathology due to *A. flavus* (*N* = 6 mice/group) after 24 hours of infection or *F. solani* (*N* = 10 mice per group) after 48 hours of infection; 300 µg of control IgG MAb (□) or MAb to PNAG (△) were administered IP 16 hours before and 4 hours after infection along with topical application of (**A, B**) *A. flavus*-infected mice, 10 µg doses given 4 and 16 hours after infection or (**C, D**) *F. solani*-infected mice, 10 µg doses given 4, 24, and 32 hours after infection. Mice were killed either at 24 hours (**A, B**) or 48 hours (**C, D**) after infection and pathology scores (**A, C**) and CFU/cornea (**B, D**) determined. *Symbols* represent individual animals. *Scale bars*: Medians and *P* values determined by nonparametric *t* tests.

FIGURE 3. Effect of systemic plus topical therapeutic administration of polyclonal antibody or MAb to PNAG on corneal disease after 24 hours of infection with *A. flavus* (*N* = 6 mice/group with polyclonal antibody, 5 mice/group with MAb) or *F. solani* (*N* = 8 mice/group with polyclonal Ab; *N* = 7 mice/group with MAb) after 48 hours of infection. 300 µl of NGS (□) or polyclonal antibody to PNAG (△), or 300 µg of control MAb (□) or MAb to PNAG (△) were injected IP 4 hours after infection. For *A. flavus* (**A**), 10 µl of polyclonal antibody or 10 µg of MAb were applied topically 4 and 16 hours after infection and pathology scores and CFU/cornea determined 24 hours after infection. For *F. solani* (**B**), 10 µl of polyclonal antibody or 10 µg of MAb were applied 4, 24, and 32 hours after infection and pathology scores and CFU/cornea determined 48 hours after infection. *Symbols* represent individual animals. *Scale bars*: Medians and *P* values determined by nonparametric *t* tests.

and 32 hours after infection. At the time of euthanasia, corneas were scored for pathology on a scale of 0 to 4, then excised, and fungal CFU determined. Eyes were assigned a pathology score at the end of the experiment using the following scheme: 0, eye macroscopically identical to the uninfected contralateral control eye; 1, faint opacity partially covering the pupil; 2, dense opacity covering the pupil; 3, dense opacity covering the entire anterior segment; and 4, perforation of the cornea and/or phthisis bulbi (shrinkage of the globe after inflammatory disease). Pathology scores were determined by two independent observers unaware of the experimental conditions. Scoring was congruent >95% of the time, in cases where there were discrepancies, the lower score was used. To determine the efficacy of topical
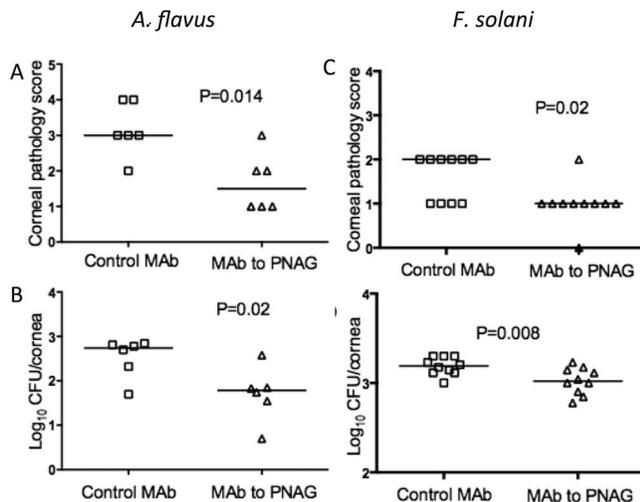
FIGURE 4. Therapeutic effect of topical administration only of MAb to PNAG on corneal disease and infection with *A. flavus* ($N = 6$ mice/group) after 24 hours of infection or *F. solani* ($N = 10$ mice/group) after 48 hours of infection; 10 μg of MAb were applied topically at (**A, B**) 4, 16, and 20 hours after infection with *A. flavus* or (**C, D**) at 4, 24, and 32 hours after infection with *F. solani*. *Symbols* represent individual animals. *Scale bars*: Medians and *P* values determined by nonparametric *t* tests.

administration only of MAb to PNAG on corneal disease due to infection with *A. flavus* and *F. solani*, 10 μg of MAb in 5 μl of PBS were applied topically at 4, 16, 24, and 32 hours after infection. Post-infection pathology scores and CFU/cornea were determined at 24 or 48 hours. The earlier time was used when controls showed a pathology score of 4 after 24 hours. Each experiment depicted in the Figures is the result of one determination, reproducibility of the results were obtained by conducting experiments with multiple antibodies and multiple fungal strains.

Histopathology Examinations

Infected eyes treated with either MAb to PNAG or the isotype control MAb were enucleated from euthanized mice and fixed in 4% paraformaldehyde then embedded in paraffin. Sections (4 μm) were cut, and stained with Hematoxylin-Eosin (HE) or Gömöri's methenamine silver (GMS) to visualize tissue morphology and fungal hyphae.

Analysis of PMN Infiltration Into Corneas

Following 48 hours of corneal infection as described above using *F. solani* or *A. flavus* and IP administration of MAbs initiated 4 hours after infection (300 μg/mouse) along with topical post-infection treatment at 4, 24, and 32 hours, corneas were excised from enucleated eyes, homogenized in PBS containing 0.5% hexadecyltrimethylammonium bromide and a protease inhibitor cocktail. Samples were freeze-thawed three times, sonicated on ice, and centrifuged at 14,000g for 10 minutes at 4°C. Myeloperoxidase (MPO) levels were evaluated in supernatants using an ELISA kit (Abcam, Cambridge, United Kingdom) following the instructions in the user's manual.

Cytokine Analysis

Mouse corneas were excised 48 hours after infection by *A. flavus* or *F. solani*, respectively, and homogenized in 200 μL of PBS-0.1%Triton X-100. The cellular particles were removed by
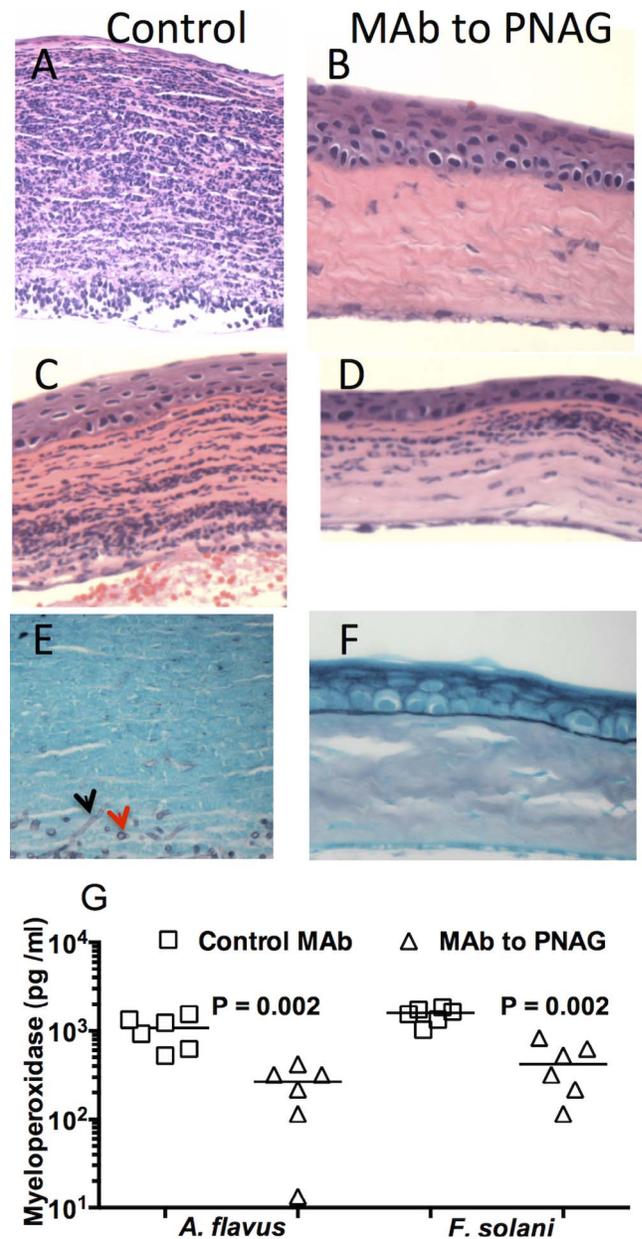

FIGURE 5. Histologic appearance, inflammatory cell infiltration, and quantitative myeloperoxidase analysis of mouse corneas infected with *A. flavus* (**A, B, E, F**) or *F. solani* (**C, D**). (**A, C, E**) Corneas from mice treated with control MAb. (**B, D, F**) Corneas from mice treated with MAb to PNAG. Magnification of (**A, B, E, F**) ×40, and (**C, D**) ×60. (**A–D**) H & E stains. (**E–F**) Gomori's methenamine silver stain. *Black arrow* shows hyphae, *red arrow* conidia. (**G**) Myeloperoxidase levels (pg/mL) in corneas of mice 48 hours after infection with *A. flavus* ($N = 6$ mice/group) or *F. solani* (6 mice/group) in animals treated with an IP injection of 300 μg/mouse of the control MAb or MAb to PNAG 4 hours after infection followed by topical application with 10 μg MAb/eye at 4, 24, and 32 hours after infection.

centrifugation and the supernatants were used for cytokine analysis. Cytokines were measured using multiplex assay technology by Luminex (Austin, TX, USA). The cytokines measured included TNF-α, INF-γ, IL-1α, IL-1β, IL-6, IL-10, monocyte chemoattractant protein-1 (MCP-1), IL-12p40, IL-12p70, IL-13, IL-17, IL-23, IL-5, IL-4, IL-2. The plate was read on a Luminex MAGPIX instrument (Luminex). Data acquisition
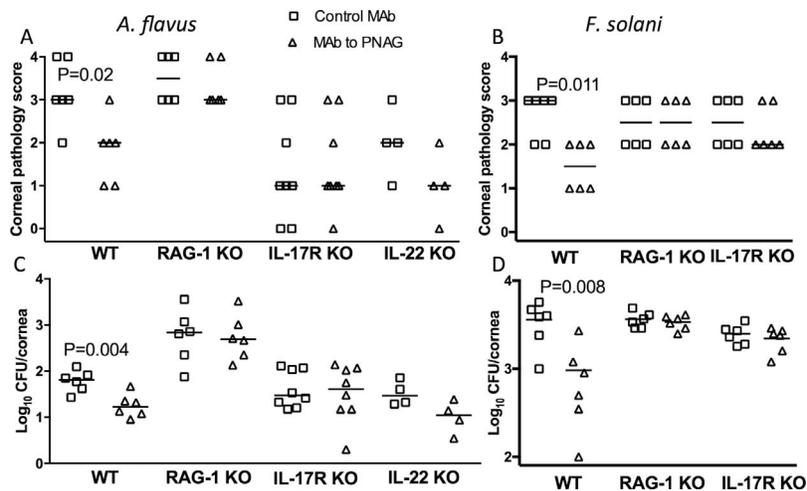
FIGURE 6. Effect of loss of IL-17 receptor (IL-17R KO), mature T and B cells (RAG-1 KO), and IL-22 (IL-22 KO) on protective efficacy of MAb F598 to PNAG against corneal pathology and infection with *A. flavus* ($N = 6$ WT or RAG KO mice/group, $N = 8$ IL-17R KO mice/group, $N = 4$ IL-22 KO mice/group, *F. solani* $N = 6$ mice/group for all mouse strains). A total of 300 µg of MAb were injected IP 4 hours after infection along with 10 µg of MAb applied topically at this time as well as at 16 hours after infection with *A. flavus* (**A**, **C**) or 300 µg of MAb injected IP 4 hours after infection and 10 µg of MAb applied topically 4, 24, and 32 hours after infection with *F. solani* (**B**, **D**). After infection pathology scores and CFU/cornea were determined at 24 hours for *A. flavus* or 48 hours for *F. solani*. *Symbols* represent individual animals. *Scale bars*: Medians and *P* values determined by nonparametric *t* tests.

and analysis was conducted using the Luminex *x*PONENT software.

Statistical Analysis

For pairwise comparisons, the nonparametric Mann Whitney *U* test was used. For multigroup analysis a nonparametric 1-way ANOVA followed by Dunn's procedure for pairwise comparisons was performed. The PRISM statistical software was used. *P* values <0.05 were considered significant.

RESULTS

Expression of PNAG by *A. flavus* and *F. solani*

Poly-N-acetyl glucosamine is a conserved polysaccharide that has been detected on the surface of many kinds of microbial pathogens by confocal immunofluorescent microscopy using the antigen-specific human IgG1 MAb F598. Poly-N-acetyl glucosamine has been shown previously to be present on the surface of yeast and hyphal forms of *A. flavus* and *F. solani*.[9] We further examined the expression of PNAG on the surface of the specific *A. flavus* and *F. solani* used in these experiments by a semiquantitative ELISA using different concentrations of MAb F598 to PNAG, which bound to *A. flavus* and *F. solani* cells in a dose-dependent manner (Supplementary Fig. S1).

PNAG on *A. flavus* and *F. solani* is a Target of Opsonic Antibodies

Opsonophagocytic killing assays are well-established in vitro tests that often correlate with an antibody's protective activity.[17] We used this assay to investigate the ability of MAb F598 to PNAG and polyclonal antibody raised to 9GlcNH2-TT vaccine to promote the killing of *A. flavus* or *F. solani* in the presence of rabbit complement and human PMNs. As presented in Figure 1, the human IgG1 MAb F598 and goat antisera to PNAG mediated killing of *A. flavus* and *F. solani*

when compared to the controls. There was no fungal killing without PMNs or complement.

Protective and Therapeutic Efficacy of Antibodies to PNAG in Mouse Keratitis Caused by *A. flavus* and *F. solani*

Since antibodies to PNAG have significant prophylactic or therapeutic protective efficacy in mice against various infections caused by PNAG-producing bacteria, such as *Acinetobacter baumannii*,[10] *S. aureus*, and *Escherichia coli*,[19] we tested whether antibodies to PNAG also have protective efficacy against fungal corneal infections with *A. flavus* in mouse corneas. We first evaluated the efficacy of the MAb to PNAG, and found it significantly reduced the corneal pathology and fungal loads after 24 hours of infection with *A. flavus* when compared to a control MAb (Figs. 2A, 2B). Following *F. solani* infection, the MAb to PNAG also reduced fungal burdens and pathology at 48 hours after infection (Figs. 2C, 2D).

As therapeutic and aggressive administration of antibody to an individual with an already-infected cornea could comprise a likely use of such reagents, we evaluated the impact on disease from the polyclonal goat antibody or the MAb to PNAG by administering doses systemically (IP) and topically starting 4 hours after infection. As shown in Figure 3, both polyclonal goat antibody raised to 9GlcNH2-TT and the MAb to PNAG significantly reduced the corneal pathology and fungal loads in *A. flavus* and *F. solani*-infected corneas when compared to NGS or control MAb. To further validate the therapeutic efficacy of the MAb to PNAG, we tested *A. fumigatus* for PNAG production and the MAb for protective efficacy and found this additional fungal pathogen was susceptible to the protective activities of MAb to PNAG (Supplementary Fig. S2.

As systemic administration of antibody therapeutics might not reach an infected cornea rapidly,[20] we lastly evaluated whether topical administration alone with 10 µg of MAb to PNAG applied at 4, 16, and 20 hours after infection for *A. flavus*, or 4, 24, and 32 hours after infection for *F. solani*, could
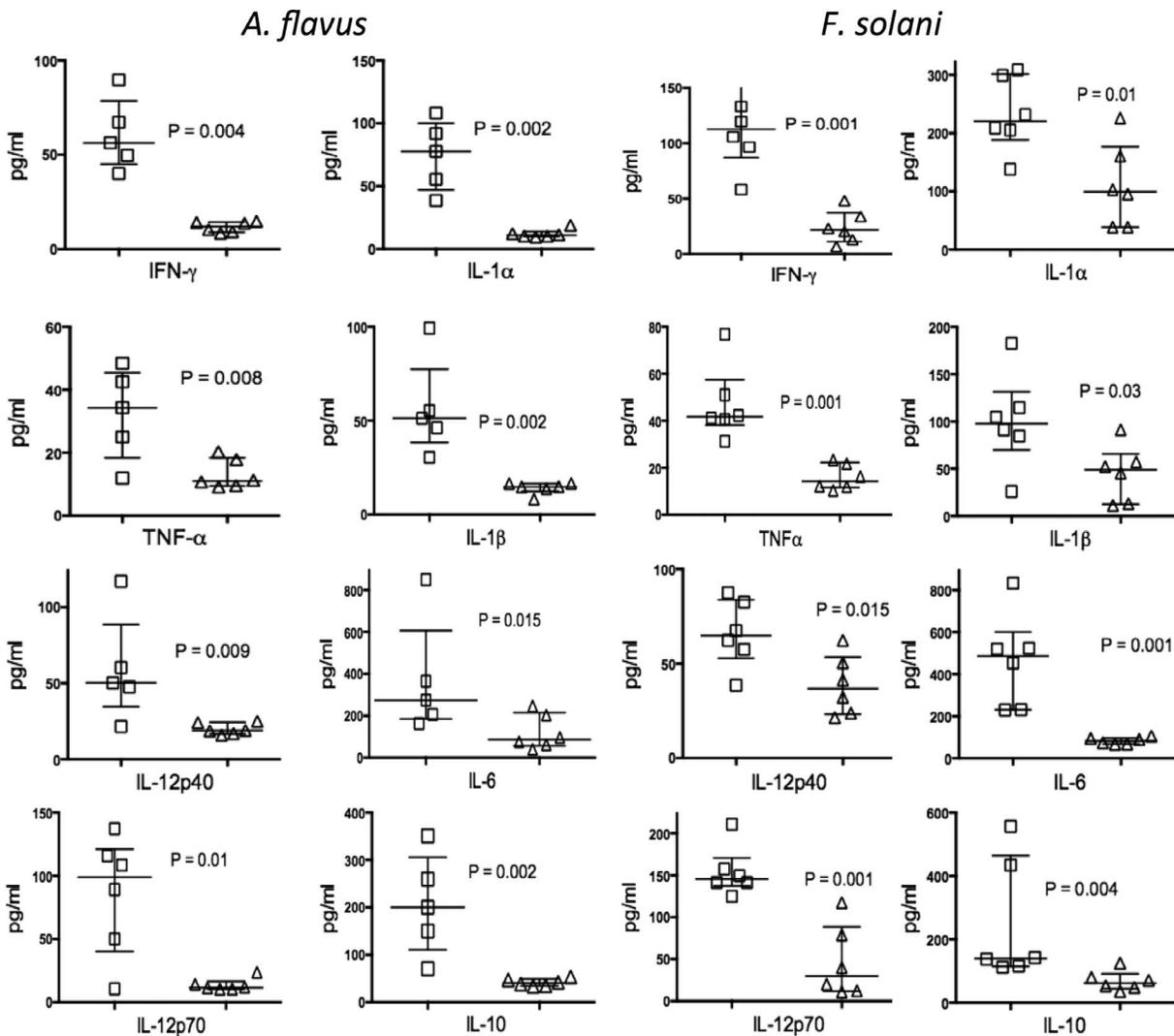
FIGURE 7. Effect of systemic (IP) and topical administration of control IgG MAb (□) or MAb to PNAG (△) on cytokines in corneas following infection with *F. solani* (*N* = 5 or 6 mice/group) or *A. flavus* (*N* = 6 mice/group). A total of 300 μg of control IgG MAb or MAb to PNAG were injected IP and applied topically. *Symbols* represent individual animals. *Scale bars*: Medians. *Error bars*: 25th and 75th percentiles, and *P* values determined by nonparametric *t* tests.

reduce the pathology score and lower fungal loads in the corneas of infected mice. This approach also was successful (Fig. 4), indicating topical administration only of antibody to PNAG has therapeutic efficacy.

Histopathologic and Myeloperoxidase Evaluations of Effects of Immunity to PNAG on Corneal Pathology

Histopathologic studies of corneas following 24 hours of infection with *A. flavus* or 48 hours of infection with *F. solani* showed a large infiltrate of inflammatory cells and obvious edema in control MAb treated corneas, whereas there was much less inflammation and edema in corneas of mice treated with MAb to PNAG (Figs. 5A–D). Gomori's methenamine silver staining of a corneal section from an *A. flavus*–infected mouse showed many hyphae in an edematous cornea, while no hyphae could be found in MAb-treated cornea (Figs. 5E, 5F). Consistent with these visual findings, quantitative analysis of MPO levels in mouse corneas 48 hours after infection showed significantly less MPO in tissues from animals given the PNAG

MAb, indicative of reduced PMN infiltration in these corneas (Fig. 5G).

Effect of Systemic (IP) or Topical Therapeutic Administration of MAb to PNAG on Corneal Infection With *A. flavus* and *F. solani* in WT, IL-17 R KO, RAG-1 KO or IL-22 KO Mice

Although antibody-mediated protection is known to primarily involve complement and phagocytes, recent studies also implicate T-cell based effectors as critical components of in vivo immunity, usually due to IL-17–mediated PMN activation. We evaluated whether this was the case for protection against fungal keratitis by determining the effect on protection from lack of the IL-17 receptor (IL17R KO mice) or T- and B-cells (RAG-1 KO mice). Loss of both of these components of the immune system abolished the protective efficacy of MAb to PNAG in corneal infections caused by *A. flavus* or *F. solani* (Fig. 6). Additionally, IL-22 impacts this system as well, and we further found IL-22 KO mice could not use the MAb to

PNAG to promote *A. flavus* clearance from the abraded cornea (Fig. 6).

Impact of Antibody to PNAG on Cytokine Expression During *A. flavus* and *F. solani* Corneal Infections

Since antibody to PNAG could attenuate corneal pathology caused by fungal infection, we next examined the expression of a panel of cytokines in the corneas in association with treatment with control or MAb to PNAG during *A. flavus* or *F. solani* infection. As shown in Figure 7, essentially all of the measured cytokines were significantly decreased by MAb F598 treatment of *A. flavus* or *F. solani* infections, indicating that control of fungal burdens is associated with reduced cytokine expression and better outcomes in terms of corneal clarity due to treatment with antibody to PNAG.

DISCUSSION

Fungal infection of the cornea is one of the main causes of blindness in many parts of the world. Further impacting treatment of these infections are the toxicities and poor effectiveness of current antifungal therapies.[21] Therefore, there is a pressing need to develop other new therapeutic strategies. Susceptibility to fungal infections is associated with loss of phagocytic function and cellular immunity,[22] such as occurs in HIV-infected individuals. In particular, T cells have been confirmed to have a key role in antifungal host defense,[23,24] whereas antibody-based vaccinations or passive immunotherapies have had a variable effect in experimental animal models.[25] In this study, we evaluated the efficacy of antibodies to PNAG, a conserved antigenic target on the surface of multiple microbial pathogens, against fungal keratitis in mice caused by *A. flavus* or *F. solani*. We found polyclonal antibody and a human IgG1 MAb to PNAG mediated high levels of opsonic killing of *A. flavus and F. solani* and significantly reduced corneal pathology and fungal loads in corneas of mice challenged with these two organisms. The protective activity was associated with opsonic killing activity of antibody to PNAG as well as in vivo factors produced by T cells and dependent on IL-17 and IL-22.

Humoral immunity to surface carbohydrate antigens of microbes is thought primarily to involve antibody, complement, and, for some pathogens, such as gram-positive organisms, PMN or other phagocytes.[26,27] Recent data, including those testing the efficacy of MAb F598 to PNAG in *S. aureus* infections, also have shown a role for cellular effectors including T cells, IL-17, and IL-22.[28–31] Prior studies from Taylor et al.[6,7] demonstrated an important role for IL-17–producing neutrophils in host immune responses to fungal keratitis. In settings where either T-cells (RAG-1 KO mice), IL-17 activity (IL-17R KO mice), or IL-22 activity (IL-22 KO mice) was not operative, we found the MAb F598 showed no significant therapeutic efficacy. Thus, these components of the cellular immune system are needed for antibody-mediated adaptive immunity and effective clearance of *A. flavus, A. fumigatus*, and *F. solani* from the cornea. Our results are comparable to those of another group that evaluated the efficacy against lethal sepsis of antibody to the *S. aureus* iron-surface determinant B (IsdB) in severe-combined immunodeficient mice (SCID). They found no protection against lethal challenge in the SCID mice, whereas WT mice were highly protected.[32] These results are of note when considering that the cornea is an immune-privileged tissue, wherein many immune cellular effectors are excluded or inactivated to prevent pathologic tissue damage. Clearly some subsets of T

cells are needed during fungal keratitis to provide important immune effectors, as well as sources of IL-17 and IL-22 for antibody to PNAG to protect against fungal keratitis. Major sources of IL-17 and IL-22 cytokines are the lymphoid-tissue inducer-like cells with the surface marker phenotype CD4$^+$ CD3$^-$ NK1.1$^-$ CD11b$^-$ Gr1$^-$ CD11c$^-$ B220$^-$.[33] Notably, these cells are present in RAG-2 KO mice and produce IL-17 and IL-22 in response to zymosan injection,[33] but the lack of effectiveness of antibody to PNAG in the RAG-1 KO mice suggests that these cytokines are necessary but not sufficient by themselves to mediate antibody-based protective immunity in the eye.

The analysis of the cytokine secretion patterns in mice given effective immunotherapies to PNAG showed that responses were reduced across the board. Likely this reflects the reduction in fungal burden due to antibody-mediated clearance. The cytokine results highlight the caution that must be used when trying to associate a specific type of immune response with antibody-mediated protection in that most of the cytokine production is driven by the microbial load in a tissue.

In conclusion, we have found administering antibody to PNAG before infection followed by topical application provided protection against *A. flavus, A. fumigatus*, and *F. solani* keratitis. This situation might mimic that of a human clinical presentation wherein a patient presents with an injured eye at risk for infection and an antibody infusion would be administered followed by topical treatment if infection develops. Similarly, therapeutic administration of systemic and topical antibody after infection was efficacious, a situation mimicking that of a clinical presentation of an already infected cornea. In addition to antibody, the lowering of fungal burdens and pathology required T cells, IL-17, and IL-22 in these mice, and was associated with in vitro opsonic killing dependent upon PMN and complement. These results support the potential use of active or passive vaccination targeting PNAG in protecting or treating fungal keratitis.

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