University of Nevada, Reno

Development of Anti-*Aspergillus fumigatus* Monoclonal Antibodies

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in
Cell and Molecular Biology

by

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May 2013

THE GRADUATE SCHOOL

We recommend that the thesis
prepared under our supervision by

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entitled

Development Of Anti-Aspergillus Fumigatus Monoclonal Antibodies

be accepted in partial fulfillment of the
requirements for the degree of

MASTER OF SCIENCE

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May, 2013

Abstract

Aspergillus fumigatus is an opportunistic fungal pathogen responsible for causing the disease Invasive Aspergillosis (IA). It is difficult to diagnose the disease, given that patients suffering from IA exhibit symptoms similar to many other diseases. Due to rapid dissemination of fungal conidia to deep tissues during the course of infection, a rapid, affordable diagnostic test is needed to increase patient survival. Our lab has previously utilized the novel In Vivo Microbial Antigen Discovery (InMAD) technique to identify several candidate fungal proteins shed into the host as a result of infection. This project focused on the creation of monoclonal antibodies with specificity to these proteins for the ultimate use in lateral flow immunodiagnostics (LFI). Antibodies were generated through an anti-peptide approach and a genetic immunization scheme was evaluated as an alternative. Bioinformatics was used to ensure that all antibodies generated would have specificity for the *A. fumigatus* species and would be able to recognize surface-exposed regions of the protein. Murine immunizations led to the development of hybridoma cell lines for monoclonal antibodies production. Immunoassays and surface plasmon resonance (SPR) studies were carried out to assess the functional characteristics of the antibodies generated through the anti-peptide approach. The genetic workflow resulted in the development of DNA constructs suitable for murine immunization, as well as expressed protein fragments to be used as bi-weekly antigen boosts. These results suggest that the genetic immunization approach is possible and would serve as an alternative to the anti-peptide approach in developing monoclonal antibodies to be used in lateral flow immunodiagnostics.

Acknowledgements

I would like to thank Dr. Thomas Kozel for allowing me the opportunity to work in his laboratory. The experience and knowledge I have obtained under his tutelage have been instrumental in my development as a student. Thanks also to Dr. David AuCoin for guiding me through the world of DNA cloning. His expertise in this area was critical to this research and my understanding of the cloning process. I have gained invaluable experience in the Kozel lab throughout my undergraduate and graduate careers. I would therefore like to thank all of the members of this lab, each of whom has been so vital to the success of this project.

I would particularly like to thank Claire Smalley, Reva Crump, Dana Nuti, Dr. Mark Hubbard, and Breeana Hubbard for their guidance and support. Also, thank you to my friends and family for their support during this time.

Finally, thanks to the National Institutes of Health and the National Institute of Allergy and Infectious Diseases, grant NIH/NIAID R21/R33 AI085548, without which this research would not have been possible.

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Introduction

Aspergillus fumigatus and Invasive Aspergillosis

Aspergillus fumigatus is a saprophytic fungus and is the most common species of the *Aspergillus* genus that causes disease in immunodeficient individuals (Latge, 1999). Typically found in decaying organic matter and soil, *A. fumigatus* is encountered in most regions of the world. It plays a major role in the decomposition of organic materials and is important in the recycling of nitrogenous and carbon-based environmental substances. (Pitt, 1994). Fungal colonies of *A. fumigatus* are produced out of conidiophores, which are made up of conidia, the spore form of the fungus. These conidia are the most often encountered morphology of *A. fumigatus*, and are therefore the most likely disease-causing form of the fungus (Dagenais and Keller, 2009).

Invasive Aspergillosis (IA) is a life-threatening disease in susceptible patients and is therefore associated with high mortality rates (Latge, 1999). IA is one of the most common fungal infections in neutropenic and immunocompromised patients and is most commonly caused by the *A. fumigatus* species (Sheppard, 2011). Spores of *Aspergillus* are inhaled by most humans on a daily basis, but a fully functioning immune system serves as a natural barrier against the invading conidia. However, in immunosuppressed individuals, such as patients undergoing treatment involving immunosuppressive drugs, spores evade detection by the host immune system and are able to deposit on alveolar epithelial tissue (Barnes and Marr, 2006). Because early diagnosis of the infection is critical in order to increase the likelihood of patient survival, a rapid diagnostic test is required (Denning, 1998).

Diagnosis of IA

Current methods used to diagnose IA are quite ineffective, leading to improper treatment of the disease (Ostrosky-Zeichner, 2012). One of the most widely used methods of detection is the galactomannan test. Galactomannan (GM) is a polysaccharide secreted from many different fungi upon infection and can be detected in patient blood or urine samples. This poses a problem, however, because an *Aspergillus*-specific form of the polysaccharide has not been identified. Thus, the GM present in a patient sample could have been derived from a fungal variety not limited to *Aspergillus* (Verdaguer et al., 2007). The immunoassay used to detect GM relies on monoclonal antibodies specific for the polysaccharide, which may yield false-positive results for *Aspergillus* infection when testing a patient sample (Zedek and Miller, 2006). This is because a positive test result could be due to any number of fungal infections, not IA alone. New methods of diagnosing IA are currently being developed and will feature species-level specificity to ensure proper treatment.

Lateral flow immunodiagnostics (LFI) are tools for detecting an antigenic substance within a clinical sample, such as blood or urine. They are rapid, accurate, and affordable dipstick-style devices which are currently being developed for the diagnosis of IA and have the potential to be effective in the diagnosis of other infectious diseases (Kozel and Bauman, 2012). The device is similar to an at-home pregnancy strip and functions at the same high level of effectiveness. The design of the LFI strip is consistent with the World Health Organization ASSURED diagnostics criteria, as it is Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free, and Deliverable to end-users. As such, it is easy to use, requires no training, can be used at a point-of care

institution, and at a cost of less than $1.00 per strip, it is a product which most patient care facilities can afford.

Construction of the LFI testing device is a simple process involving three key features: a conjugate pad, test line, and a control line. The conjugate pad contains an antibody conjugated to colored particles, usually gold nanoparticles, which will bind to the antigen within the sample (Bailes et al., 2012). The test line contains an antibody which is also specific to the target analyte, but targets a different epitope. A downstream control line contains an antibody which will not react with the target antigen in the sample. Once the dipstick is placed into the substrate, antigen will bind the target-specific gold-conjugated antibodies. This complex then progresses along the strip through capillary action until interaction with the test line occurs. The antigen-gold-labeled antibody complex then binds the target-specific antibody on the test line and a color develops via the colored particle from the conjugate pad. Capillary action continues until reaching the control lane, which contains an antibody which will not bind the target analyte. A color change will be produced, serving as a negative control to indicate a successful test. This process takes upwards of several minutes and provides a reliable result to diagnose the disease.

Diagnosis of invasive aspergillosis can be greatly improved through the use of lateral flow tests (Thornton, 2008). Target proteins secreted by the fungus during infection may serve as antigenic targets toward which monoclonal antibodies (mAbs) may be developed for use on the dipstick. In order to discover these targets, a novel technique known as In vivo microbial antigen discovery (InMAD) may be employed (AuCoin, 2012). Through this process, an animal is infected with the pathogen of choice.

Serum samples are taken from that mouse and filtered to remove whole bacteria and leave secreted antigen in the sample. This "InMAD serum" is then used to immunize another animal, and the "InMAD immune serum" is harvested to probe target cell lysates through either a proteome array or immunoblot. Overall, the InMAD strategy will reveal the target candidate antigens to which monoclonal antibodies can be developed for use in the LFI format (Nuti et al., 2011). Although the target antigens may be revealed through this method, these proteins are not always readily available for immunization to develop antibodies, and therefore alternative methods may need to be employed.

Monoclonal Antibody Development

Several strategies exist for generating antigen to use in the development of mAbs for lateral flow diagnostics. Anti-peptide antibodies have been used frequently to generate antibodies with specificity to a particular amino acid sequence (Lee et al., 2010). If the sequence of the target antigen is known, short peptide regions of the protein may be synthetically generated for immunization. Given that the LFI format requires two antibodies, one conjugated to gold particles and another for the test line, epitopes must be chosen which are on opposite geographical "poles" of the protein. Short peptide segments are poorly immunogenic, however, and require conjugation to a carrier protein to induce an immune response in the host organism (Briand et al., 1985). A peptide-conjugate combined with the use of adjuvant for immunization is sufficient to produce a very strong immune response (Jones et al., 1991). Upon immunization, host immune cells known as B-lymphocytes (B-cells) recognize antigen as a foreign particle. The B-cells then produce antigen-specific antibodies on their cell surface for detection of the target molecule. Antibodies produced by B-cells have the ability to bind antigen and serve as a marker for

degradation on the foreign particle (Janeway et al., 2001). This is the general workflow of the immune response in the generation of antibodies and is not unique to anti-peptide antibody development.

Peptide immunization typically generates antibodies reactive to denatured proteins. Genetic immunization has been demonstrated as a means of generating to protein antigens in their native configuration (Brown et al., 2011). In this strategy, a much larger fragment of the antigen of interest is selected based on parameters such as antigenic index, surface probability, and hydrophilicity. This allows for the development of an antibody which will better recognize the three-dimensional structure of the protein than a linear peptide (Kutzler and Weiner, 2008). Other factors must be considered when designing the genetic immunization machinery. Codon usage has been shown to be critically important, as some species preferentially use certain codons over others in translation of DNA into the desired protein sequence (Kurland and Ehrenberg, 1984). Codon optimization for the species in which the protein will eventually be expressed will ensure optimal expression levels and also provide a protein with the expected posttranslational modifications or lack thereof.

With genetic immunization, the sequence of interest is identified at the nucleic acid level and synthetically generated. The gene is inserted into an expression plasmid to be taken up by cells of the host organism. Muscular or dermal cells internalize the plasmid and the translational machinery of the cells allows for expression of the DNA insert, which will then be presented on the cell surface for recognition by host immune cells (Barry and Johnston, 1997). The antibodies generated through this strategy and the

anti-peptide strategy will be polyclonal (pAbs), however, and further experimentation is required to develop a functioning cell line which produces mAbs for use in LFI.

Hybridoma technology is the most useful way to generate mAbs (Kohler and Milstein, 1975). The general workflow of the hybridoma strategy is simple in scope. Once a high titer is observed in the immunized animal's serum, the immune cells from the host are harvested from the spleen and fused with a myeloma cell line to generate a hybridoma cell line (hybrid-myeloma). This immortalized cell line will produce antibody indefinitely in culture. These hybridomas can be subcloned by limiting dilution to isolate a monoclonal cell line which produces mAbs (Staszewski, 1984). These mAbs are highly specific for a single antigenic epitope and once purified from cell supernatant, will serve an important role in creating the desired diagnostic tools.

Antibodies with specificity to a single epitope are important in targeting a protein of interest. In designing synthetic antigens, measures may be taken to ensure that the amino acid sequences to be targeted by the mAb are unique to a particular genus or species. In this case, sequences of interest within a candidate protein may be cross-referenced with all other known proteins in the protein database *in silico* to ensure specificity for the *Aspergillus* genus (Angeletti, 1999). Monoclonal antibodies developed based on these parameters for use in LFI tests would be able to provide an accurate result which would test specifically for IA.

In summary, IA is a serious opportunistic infection which requires early diagnosis to increase the likelihood of patient survival. Current methods of diagnosing IA have several pitfalls, and therefore a more accurate and rapid test is needed. Lateral flow

immunodiagnostics are a logical means of obtaining these results. *Aspergillus*-specific mAbs are the key to developing these diagnostics.

Project Overview

The fungal pathogen *Aspergillus fumigatus* is a target of study in our lab for the creation of lateral flow immunodiagnostics. Previous work employed the use of the InMAD strategy to identify several different antigens secreted in the host as a result of fungal infection by this species. A vast assortment of proteins were recognized via 2D immunoblot and categorized according to their relevance and presence in a variety of animal models. The top 15 targets were ranked based on their prevalence across animal models and the top five were then selected based on their presence across the most common *Aspergillus* species. Each of these five proteins, termed EglC, Ecm33, Catalase, Thioredoxin Reductase, and Peptidyl-prolyl cis-trans isomerase, were found to be secreted in serum and urine in animal models of *A. fumigatus* infection, and were therefore given the highest priority (Chaves, 2012).

EglC, Ecm33, and Catalase, being the top three and the most characterized of the proteins were targeted for this study. Use of the Basic Local Alignment Search Tool (BLAST) revealed that three dimensional structures for the proteins were not yet characterized, but sequence data was readily available. EglC and Ecm33 are GPI-anchored proteins, while Catalase is a secreted protein. These factors were taken into account when designing the constructs to be used in the various immunization schemes. The EglC protein, an endoglucanase found in various species of *Aspergillus*, was observed to perform the best out of these three target proteins, and was therefore studied the most (Hasper et al., 2002).

Bioinformatic analysis of each of these proteins yielded enough information to move forward, including known DNA and amino acid sequences. Through the use of

proteomic analysis software, several peptide sequences of 15 amino acids in length were chosen for the EglC, Ecm33, and Catalase proteins based on several parameters, including hydrophilicity, antigenic index, and surface probability (Angeletti, 1999). Additionally, segments of 100 amino acids in length were chosen for each protein using the same parameters. All sequences chosen for this study were selected based on specificity to the target organism, *Aspergillus fumigatus*. In addition, regions were chosen based on their distance from one another in an attempt to generate antibodies which will target geographically distinct regions of the proteins of interest.

The 15-mer peptides were conjugated to the immunogenic carrier protein Keyhole Limpet Hemocyanin (KLH) for subcutaneous immunization of Balb/c mice with TiterMax Gold Adjuvant. A genetic immunization strategy was employed for the 100 amino acid segments, which were subcloned into the mammalian expression vector pCI for intramuscular or intradermal immunization of Balb/c mice. Another version of the pCI construct, containing a 6x polyhistidine-tag, was created for expression of the protein fragment in HEK 293T cells. This protein fragment could then be purified and the tag cleaved for the immunization boosts and for screening purposes.

From the anti-peptide antibody development process, the EglC protein was found to generate the highest titers in mice and the resulting anti-EglC antibodies produced from a hybridoma cell line consistently demonstrated great binding ability with the target peptide. Monoclonal antibody 4A3, an anti-EglC antibody, was purified from a monoclonal cell line of hybridoma cells. However, immunoassays demonstrated that this antibody only recognized the denatured form of the target protein. This was presumed to be due to the linear structure of a peptide, which may not accurately represent the natural

shape of a peptide within a folded protein. Genetic immunization allows for selection of a larger portion of the protein, thereby serving to better represent the 3D shape of the protein for antibody recognition (Brown et al., 2011).

This research led to the conclusion that anti-peptide antibodies are unfit for the LFI application. The genetic immunization strategy has the potential to generate antibodies which are better tailored to interact with the natural geometry of the native protein. Protein fragments of 100 amino acids in size can be expressed *in vitro* and can be purified for use in screening and immunization boosts. With further study, monoclonal antibodies generated through these means should be analyzed for their worthiness in the LFI format.

Materials and Methods

Bioinformatics and Polypeptide Design. All of the proteins identified through the InMAD study which were of critical importance to this project, namely EglC, Ecm33, and Catalase, were analyzed through several bioinformatics processes. Entrez accession numbers were obtained from the National Center for Biotechnology Information (NCBI) database, a result of the previous study. These accession numbers were input to the Protean software, part of the LaserGene Core Suite by DNASTAR, to choose 15 and 100 amino acid regions unique to each protein. Three parameters were given high priority when selecting each region: hydrophilicity, surface probability, and antigenic index. These parameters, based on different algorithms which are all interrelated to make functional predictions, were used to select the best polypeptide segments possible. In the case of the 15-mer peptides, proline-rich sequences were avoided to eliminate the possibility of secondary structure distortion. Additionally, sequences were chosen to lack charged residues on the carboxy-terminus to avoid unwanted interactions with other molecules. The 100 amino acid segments were chosen based on the same parameters as the 15-mer peptides, and each respective region contained within it one of the corresponding 15-mer peptides. Additionally, codon usage was optimized for *Mus musculus* so as to ensure maximal levels of recombinant protein expression in the mammalian expression system.

Each polypeptide was selected based on criteria that would ensure specificity to the *A. fumigatus* species. BLAST searches were performed on each of the selected 15 and 100 amino acid segments via the Universal Protein Resource (UniProt). Upon confirmation of sequence specificity for the *A. fumigatus* species, 15-mer peptides were

synthesized by ProSci and Peptide2.0. Each peptide was engineered with a cysteine residue added to the amino terminus to facilitate conjugation to KLH via disulfide linkage. The 100 amino acid residues were synthesized by Integrated DNA Technologies (IDT) and delivered in pIDTSMART, a proprietary storage vector supplied to preserve the desired DNA sequence. Only two 100 amino acid residues were selected for the initial stages of the project, both of which were specific to the protein EglC. Gene Inspector and Gene Construction Kit software were used in constructing primers for polymerase chain reaction (PCR) and design of the final construct in the desired pCI plasmid. Each region of the protein, termed Domain 1 and Domain 2 from this point on for simplicity, were subcloned into the pCI vector via PCR, with primers containing a Kozak sequence upstream of the start codon for mammalian expression. Each domain was inserted by restriction enzyme cuts via KpnI on the 5' end and NheI on the 3' end of the DNA. These non-compatible ends would avoid re-ligation of the vector after digestion.

Conjugation of Polypeptides, Immunization, and Screening of Sera. KLH carrier protein was supplied by Thermo Scientific in kit form to facilitate conjugation to the 15-mer peptides. A molar excess of peptide was used in conjugation to KLH to avoid any remaining unbound carrier protein. Unbound peptide was removed through a size-exclusion column, leaving only the peptide-KLH conjugate in solution. Spectroscopic analysis and Beer's Law ($A = \varepsilon cl$) were used to determine the concentration of conjugate created. The molar extinction coefficient (ε) for KLH, 1.4 $Lmol^{-1}cm^{-1}$, was used to evaluate concentration, given a 1:1 conjugation ratio and lack of available extinction coefficient for the 15-mer peptide.

Two different groups of ten female Balb/c mice per peptide were immunized with or without TiterMax Gold adjuvant (TiterMax). Five mice were immunized subcutaneously with 30 µg of peptide-KLH conjugate emulsified with TiterMax Gold per mouse and the other group injected intraperitoneally with the same amount of conjugate lacking adjuvant. Boost injections were performed every two weeks through the same respective injection routes for each group, while limiting the dose of conjugate to 25 µg per mouse. Submandibular bleeds took place every two weeks following the initial immunization. Mouse sera were screened via ELISA and titers evaluated through SoftMax Pro software.

Procedures for screening sera via ELISA were identical for all stages of immunization (every two weeks). 96-well Immulon 1-B plates were coated with 0.01% Poly-L-Lysine and allowed to incubate for one hour at 37 °C. Plates were then washed three times with 300 µl/well PBS and exposed to 5 µg/ml EglC, Ecm33, or Catalase peptide in 0.05% PBST and allowed to incubate overnight. Following a 3x wash with 0.05% PBST, plates were blocked with 0.05% PBST-5% milk for 1.5 hrs at room temperature. This was followed by a 3x wash with 0.05% PBST-5% milk, followed by a serial dilution of mouse serum samples in 0.05% PBST-milk across the plate, starting from a 1:40 dilution. Following a 1.5 hr incubation and a 3x 0.05% PBST-5% milk wash, secondary antibody, goat-anti-mouse IgG-HRP, was added to the plates at a dilution of 1:5000 in 0.05% PBST-5% milk and allowed to incubate for 1.5 hours at room temperature. This was followed by a 3x wash with 0.05% PBST followed by 1:1 mixing of TMB Subsrates A and B. Substrate was added to the plates and allowed to incubate for 30 minutes at room temperature. A phosphoric acid stop solution was added to the plates,

followed by spectroscopic analysis at 450 nm. Pre-immune mouse sera was used as a negative control

Fusion of Immune Mouse Cells with Myeloma Cells. Once mouse serum titers showed a 0.5 O.D. value at a 1/5000 dilution, the top-producing mouse from each group was chosen for fusion. A pre-existing myeloma cell line, P3X63Ag8.653, was maintained in log phase in myeloma-optimized media (RPMI 1640-based) four days prior to the fusion. Three days prior to fusion, the top-producing mouse was given an intravenous tail boost with 20 µg of the appropriate peptide conjugate. 24 hours prior to fusion, five female CD-1 mice per peptide were euthanized by CO_2 narcosis and peritoneal macrophages isolated via post-mortem surgery in a biosafety cabinet. Peritoneal macrophages were seeded onto 48-well plates at a concentration of 4×10^4 cells/0.5 ml/well in Hypoxanthine-Aminopterin-Thymidine (HAT) media and allowed to incubate overnight.

Splenectomy of the top-producing mouse was done in a biosafety cabinet 24 hours after peritoneal macrophage harvest. Mice were euthanized via CO_2 narcosis and an incision made in the abdomen to gain access to the spleen. The spleen was removed, placed into a small amount of RPMI, and a small tear was created on one of the lateral ends of the organ. Cells were massaged out of the spleen until translucency of the organ was observed. Harvested cells were washed three times in RPMI using centrifugation parameters of 300 xg for 7 minutes at 4 °C. Myeloma cells were pelleted with the same protocol. Using a visually-confirmed equal pellet size of myeloma and immune cells, both cell types were combined into a single reaction vessel and washed once more in RPMI. The resulting pellet was placed at 37 °C for the fusion to take place. A 1 mL

volume of polyethylene glycol (PEG) solution, a cell membrane permeability-increasing compound, was added to the cells over a 30 second time course with gentle stirring and allowed to incubate for 30 s. Over the next 60 seconds, 7 mL RPMI was added to the mixture by adding 1 mL over 15 seconds, 2 mL over 30 seconds, and 4 mL over 15 seconds. Over 2 minutes, 43 mL of RPMI was added to the cells. The resulting cell suspension was centrifuged for 5 minutes at 400 xg, 4 °C. Cells were re-suspended in HAT medium and seeded onto 48-well plates containing previously-isolated peritoneal macrophages at 0.5mL/well. Cells were incubated for 10 days at 37 °C while observing colony growth. Media was changed from HAT to Hypoxamnthine-Thymidine (HT) after three weeks, and finally to 15% complete RPMI after another three weeks.

Cloning of Hybridoma Cells. Hybridoma cells having been confirmed visually for colony growth were screened via ELISA to verify binding ability to the appropriate peptide. ELISA procedures were identical to mouse serum screening, with the exception that the 1:40 serum step was replaced with direct, no dilution addition of supernatant from the 48-well hybridoma plate to half of the 96-well Immulon 1-B plate. The highest-producing candidate colonies were sub-cultured on a separate plate, and subsequently screened following the same ELISA process. From this subculture, the best candidate antibody-producing colonies were subject to surface plasmon resonance (SPR) analysis and Western blotting (see below). The best candidate colony was selected for further culture dilutions for mAb production.

Cells from the top candidate colony were seeded onto multiple 96-well plates at a concentration of 1 cell/well to ensure monoclonality. Previously sub-cultured colonies were maintained in a 48-well format as contingent samples. The 1 cell/well colonies were

grown to confluency and monitored via light microscopy to ensure that the colonies were in fact monoclonal. Poisson distribution values were calculated on a per-plate basis at this stage. ELISAs were performed at confluency according to the same procedure as the initial hybridoma clones. From these results, the top-producing, monoclonal colony was seeded onto an additional set of 96-well plates at 1 cell/well. Monoclonal cell colonies were monitored for adherence to the Poisson distribution at this phase. If greater than 37% of wells on a 96-well plate contained no cellular growth, the plate was said to follow a Poisson distribution. Once confluency was reached and ELISA performed, the top-producing, monoclonal colony was chosen for large-scale production of mAb for eventual purification.

The top-producing hybridoma colony from the second 1 cell/well stage of growth was seeded into a larger reaction vessel (T-175 flask, 70 mL culture volume) and grown until confluency. At this point, the cells were pelleted by centrifugation at 1200 RPM for 10 minutes at 4 °C and re-suspended in fresh 10% Complete RPMI 1640. The cellular suspension was added to the cell growth pouch of an Integra Biosciences bioreactor flask and the media chamber filled with fresh RPMI. Cells were allowed to proliferate for one week, following which cells were harvested twice per week and mAb-containing supernatant stored. Supernatant samples were screened biweekly via ELISA using the same parameters as tissue culture plate analysis, with the exception that the supernatant was serial diluted across the plate from a starting dilution of 1/1000. Titers and antibody production concentrations were measured through SoftMax Pro software. Once a sufficient amount of supernatant had been collected, the mAbs were ready for purification through the use of a Protein A Sepharose column.

Western Blot and SPR Analysis. Tissue culture supernatant and purified mAb were subject to Western blot. Supernatant and purified mAb samples to be analyzed through polyacrylamide gel electrophoresis (PAGE) were diluted 1:1 with 2x sodium dodecyl sulfate (SDS), a denaturing agent, and boiled for 10 minutes. Molecular weight marker and the boiled samples were loaded onto a 12% polyacrylamide gel (Bio-Rad Laboratories) and run at 100 V for one hour. The resulting gel was blotted onto a PVDF membrane via tank blot run at 150 V for one hour at 4 °C. The membrane was then placed into a blot box containing 0.05% PBST – 5% milk and incubated overnight while rocking at 4 °C. The membrane was then washed three times with 0.05% TBST for 15 minutes per wash, followed by addition of probing samples diluted in 0.05% PBST – 5% milk and controls at the appropriate concentrations. A one hour incubation was allowed to take place, followed by a 3x wash with 0.05% TBST. The membrane was then exposed to secondary antibody at a 1/5000 dilution in 0.05% PBST-5% milk and allowed to incubate for 30 minutes. This was followed by another 3x wash with 0.05% TBST. SuperSignal West-Femto chemiluminescent substrate was added to the membrane and allowed to develop for one minute, followed by imaging using the Bio-Rad XRS+ imaging system.

Surface plasmon resonance (SPR) data were gathered for several clones of EglC at the 1 cell/well phase using a BIAcore X100 instrument. Running and sample buffers for this experiment was HBS buffer, pH 7.4, containing 10 mM HEPES, 150 mM NaCl, 3 mM EDTA, and 0.05% surfactant P20 (HBS-EP+). For ligand preparation, EglC peptide was coupled to a carboxymethylated dextran (CM5) sensor chip via disulfide linkage by maleimide coupling. A flow cell was left unmodified for reference subtraction. To

evaluate binding, supernatant samples were diluted 1:1, 1:10, and 1:1000 in HBS-EP+ for analysis. At each concentration, supernatant samples from cell culture wells were injected over the modified chip surface at 30 μl/min for 180 s. The chip surface was regenerated between runs with a 1 min pulse of 2 M $MgCl_2$.

Subcloning of DNA and Plasmid Preparation. Polymerase chain reaction (PCR) was performed on the pIDTSMART vector containing the genes encoding the desired 100 amino acid sequences in order to extract the DNA segment of interest. One set of primers was used to generate DNA containing a His-tag, and another set used to generate DNA lacking a His-tag. Parameters for PCR involved 45 cycles of denaturation for 30 s at 94 °C, annealing for 1 min at 58 °C, and extension for 45 s at 72 °C while utilizing PCR Supermix (Invitrogen). The PCR product was purified via gel extraction following 1% agarose gel electrophoresis, then cut alongside pCI plasmid with KpnI and NheI via restriction enzyme double digest. Digestions were carried out at 37 °C for one hour under proper conditions for each enzyme (proper buffer and BSA content) and followed up with a 1% agarose gel electrophoresis and gel extraction to purify the excised fragment. The resulting cut Domains 1 and 2 were ligated with pCI using 1X T4 DNA ligase, 5X ligase buffer, 3 fmol of plasmid DNA and 9 fmol of insert DNA. Ligation was allowed to take place for one hour at room temperature.

The ligation products were transformed into DH5-α *Escherichia coli* cells (Invitrogen). Ligation products for Domains 1 and 2 were added to separate *E. coli* reaction vessels and allowed to incubate for 30 min on ice. The transformation complex was heat shocked for 30 s at 42 °C and then immediately placed back on ice for 2 min. Super Optimal broth with Catabolite repression (SOC) was added to each transformation,

rotated for one hour at 37 °C at 260 RPM, followed by centrifugation of the samples at 4,000 RPM for 5 min. Supernatant was removed and the resulting pellets re-suspended in fresh SOC media. The resulting transformation products were streaked onto plates containing Lysogeny Broth (LB) agar and the antibiotic Ampicillin and grown overnight at 37 °C.

Individual colonies from each transformation were grown in small liquid cultures of LB media until turbidity was observed, at which point the culture volume was scaled-up significantly and allowed to grow overnight at 37 °C. A Maxi-prep was performed to purify the resulting plasmid DNA (QIAGEN), and the final plasmid concentration was determined by spectroscopic analysis. DNA purity was also measured by evaluating the quotient of A260/A280.

Purified plasmids were subjected to restriction enzyme digestion, and genomic sequencing analysis was performed by the UNR Genomics Center. A mass of 1.0 μg DNA per sample was digested by the restriction endonuclease NcoI and run on a 1% agarose gel at 100 V for one hour. The gel was stained using ethidium bromide and imaged via UV illumination. For genomic sequencing of the plasmids, sample amounts of 200 ng of Domain 1 and Domain 2 plasmid were sequenced using several different overlapping primers for each Domain. Sequencing results were analyzed via Gene Inspector software, through which constructs designed on the Gene Construction Kit software were aligned with the sequenced DNA. Following confirmation of pure, correct plasmid DNA, transfection of Human Embryonic Kidney (HEK) 293T cells was possible.

Expression of EglC Protein Domains. HEK 293T cells were transfected with EglC/pCI constructs using the Lipofectamine 2000 transfection reagent. Cells were

grown to confluency in Dulbecco's Modified Eagle Medium and seeded at a cell concentration of 2 x 10^5 cells/mL on a 24-well plate and allowed to adhere. A total of 10 µg DNA per Domain was diluted in Opti-MEM reduced serum media. Four different amounts of Lipofectamine 2000 per Domain were diluted in the same medium, ranging from 2 µl to 5 µl per 50 µl Opti-MEM. A 1:1 ratio of DNA and Lipofectamine were mixed and allowed to incubate for 30 min at room temperature. The total of eight DNA/Lipofectamine samples were then added to each 293T sample well on the 24-well plate and allowed to incubate for 48 hours.

After 48 hours, supernatant was removed, and cell lysates were prepared using lysis buffer (Promega). Preliminary Western blots were performed to monitor protein expression. Blots were probed with either mouse anti-His (1/6000) or rabbit anti-EglC, followed by exposure to goat anti-mouse IgG- HRP (1/5000) or goat anti-rabbit IgG-HRP (1/5000), respectively. Samples containing the greatest protein concentrations were purified over a Ni-NTA column (Pierce) and again subject to the same blotting conditions.

Results

Bioinformatics and Polypeptide Design. Bioinformatic analysis of the *Aspergillus* proteins targeted for this study allowed for prediction of the most immunogenic candidate sequences for the anti-peptide and genetic immunization strategies. Several different regions were targeted for each protein, three for EglC, four for Ecm33, and three for Catalase (figure 1). Of these, EglC eventually showed the strongest performance and was therefore given the highest priority. The anti-EglC mAb was shown to have the strongest apparent binding affinity with the target peptide through SPR, consistently showed high absorbance readings in EIAs, and survived throughout the cell culture phase to the point of antibody purification, whereas Ecm33 and Catalase did not.

Analysis of the EglC protein identified three potential 15 amino acid anti-peptide targets. One of these selected regions overlapped one of the others. The two 100 amino acid targets contained one of these sequences each. The protein itself, which is approximately 30 kD in size, consists of a total of 446 amino acids. Short peptide segments selected for study included amino acid positions 22 – 35, 358 – 371, and 367 – 380. The larger 100 amino acid regions contained amino acids 17 – 116 and 345 – 444. Ecm33 and Catalase yielded four and two targets, respectively, for anti-peptide antibody production and two targets each for genetic immunization. The 15-mer peptide regions selected for the 42 kD, 398 amino acid Ecm33 protein included amino acid positions 27 – 40, 297 – 310, 327 – 340, and 350 – 363. Genetic immunization targets included regions 15 – 114 and 280 – 397. Analysis of the Catalase protein, which is 80 kD in size and 728 amino acids in length, resulted in targeting of amino acid positions 19 – 32 and 546 – 559

for the anti-peptide approach and positions 17 – 116 and 515 – 614 for the genetic immunization approach. EglC sequence 22 – 35 was used in anti-peptide immunization studies, as were sequences 27 – 40 for Ecm33 and 19 – 32 for Catalase. EglC sequences 17 – 116 and 345 – 444 were pursued for genetic immunization.

Analysis of these amino acid sequences via BLAST search confirmed specificity for the *Aspergillus* genus. All selected sequences were conserved across the genus, showing relation to *Aspergillus fumigatus* in every chosen polypeptide. The most favorable peptide sequences would be found exclusively in *A. fumigatus*, so as to ensure specificity to the species. In many cases, however, sequence similarity with other species of *Aspergillus* was unavoidable. Nonetheless, all sequences chosen possessed at least 75% homology with *Aspergillus fumigatus*.

Immunization with Peptide Conjugates. Following conjugation of peptides to KLH and immunization with or without adjuvant, mouse serum titers revealed high antibody production for all protein antigens. Conjugation of peptides with bovine serum albumin (BSA) and subsequent immunization revealed a significantly lowered immune response (data not shown). Immunizations performed with adjuvant yielded slightly higher titers than immunization without adjuvant, thus only titers reported here reflect groups immunized with adjuvant. The EglC peptide gave an average titer value of 274,000 after six weeks, while Ecm33 and Catalase yielded averages of 172,000 and 174,000, respectively (figure 2). Titers were high enough after six weeks to suggest immune cell fusion with myeloma cells in the creation of hybridoma cell lines for each peptide antigen.

Hybridoma Cell Lines. EglC and Ecm33 hybridoma cell lines survived much longer than the Catalase cell line, which failed to produce antibody past the 1 cell/well stage. The Ecm33 cell line showed fair antibody production values through the 1 cell/well phase, but titers reduced to background levels once culture volumes were increased. The EglC hybridoma cell line, however, remained highly productive throughout the cell culture process.

ELISA screenings demonstrated absorbance values above 1.00, where values higher than 0.5 were considered positive. At the second 1 cell/well stage, EglC clone 4A3 revealed Poisson distribution values greater than 60% per plate across five 96-well culture plates (percentages are wells containing no growth on a plate, where values greater than 37% adhere to the Poisson distribution). SPR analysis via BIAcore revealed that this clone outperformed other EglC clones in binding affinity to the target peptide as well. Kinetics data demonstrated that binding of 4A3 to the target peptide yielded a two-fold higher response when compared to three other EglC clones (figure 3). Response units of over 800 were reported for 4A3, whereas other candidates showed 400 or less response units. From these results, clone 4A3 was chosen for mAb purification.

Purified Anti-Peptide EglC mAb. Monoclonal anti-EglC antibody 4A3, having been derived from cell culture plate well 4A3, was purified on a Protein A sepharose column to yield a total of 100 mg mAb. Following purification, the isotype subclass of the antibody was determined to be IgG1 via ELISA. This was expected, given that elution of the antibody from the purification column occurred at a buffer pH value of 8, a common occurrence for this immunoglobulin type. Furthermore, 4A3 was shown to bind the target antigen on a variety of immunoassays.

Since a full-length recombinant EglC protein was not available for study, it was necessary to use *Aspergillus fumigatus* cell lysate and *A. fumigatus*-infected Guinea pig and human serum and urine samples to determine the binding characteristics of 4A3 with target protein. Western blot analysis demonstrated that the antibody bound as a dimer to *Aspergillus fumigatus* lysate at a molecular weight of 30 kD (figure 4). A dot blot utilizing several different infected and uninfected human and guinea pig serum and urine samples revealed binding of mAb 4A3 with both infected and uninfected samples (figure 5).

Molecular Cloning. The two synthetic genes encoding the 100 amino acid segments of interest for EglC were subcloned into the pCI plasmid using primers with and without a 6x His-tag. The restriction enzyme digest carried out to confirm proper insertion of the gene into the vector, performed with an enzyme with two cut sites, yielded two distinct bands per sample (figure 6). Digestion with NcoI for samples with and without the His-tag revealed distinct bands around 550 bp and 3.7 kb . Results were identical for the pCI plasmid containing Domain 1 and for pCI containing Domain 2. Purity analysis and sequencing further characterized the newly constructed plasmids.

Plasmids prepared via Maxi-prep displayed purity levels of 1.68 and 1.72 for Domain 1 and Domain 2, respectively. These values were calculated by evaluating A260/A280, the ratio of DNA to protein in the purified DNA sample. A value of 1.80 is considered perfectly pure. Genomic sequencing data, generated using several vector and insert-specific primers, revealed a lack of mutations. Sequencing results demonstrated that the inserted genes were in-frame and possessed all necessary components for translation to occur, including a start codon, stop codon, and Kozak sequence.

Expression of EglC Protein Domains. HEK 293T cells transfected with 6x His-tagged EglC plasmids were tested for protein expression via Western blot. Blots probed with mouse anti-His antibody revealed a distinct protein band visible in the cell lysate preparation. No bands were present in the supernatant samples. The BipC control, a protein which has been previously confirmed to contain a His-tag, functioned properly as a positive control in this assay. A negative control, in the form of untransfected cell lysate, functioned appropriately as well. Strong bands were shown around the expected 10 kD molecular weight in the lysate samples and were purified for further analysis. Purified samples demonstrated the same strong bands at the 10 kD mark, varying in intensity depending on the column elution (figure 7). An anti-EglC Western blot revealed similar results, with bands appearing at the 10 kD mark (figure 8).

Discussion

Bioinformatics and Polypeptide Design. The lack of a 3D structure increases the level of difficulty in choosing peptides which will lie on opposite poles of the protein (Choi et al., 2013). If the crystal structures of these proteins were known, identification of two geographically distinct epitopes on the surface of the protein would be easily done (Lindskog et al., 2005). This project was carried out under the assumption that sequences chosen at opposite ends of the sequence would lie at physically distinct regions of the protein. However, a folded protein could result in the chosen sequences being located in the same geographic area, thereby obviating this selection process (Alberts et al., 2002). Although the construction of a 3D model of these proteins would be a study in and of itself, the information would be invaluable, leading to selection of the most ideal epitope candidates. The results of this study did demonstrate, however, that the generation of anti-*Aspergillus* antibodies and expression of *Aspergillus* protein fragments are both possible through the processes employed.

Immunization and Hybridomas. High titers observed in mice following immunization allowed for fusion to take place eight weeks from the first injection series, a standard time course for this type of experiment (Shulman et al., 1978). A positive absorbance reading of 0.5 O.D or higher at a serum dilution of 1:5000 is considered optimal for generating hybridoma cell lines, and absorbance values at this dilution were recorded, on average, to be above 1.00. Supernatant from hybridoma cell lines showed good binding ability with the target peptides on ELISAs (absorbance readings > 0.50). The initial post-fusion cell lines produced antibody very well and were stable until reaching the 1 cell/well stage. With the exception of the EglC cell line, an abrupt loss of antibody

production was noted in all cell lines. This loss of production may have been due to mycoplasma infection. Contamination of hybridoma cells with mycoplasma has been noted in other labs and has been demonstrated to cease antibody production (Volgareva et al., 1985). Loss of hybridoma antibody production has occurred in other cell lines in our lab as well, and although a mycoplasma test has not been carried out, this type of contamination could possibly be the cause.

The purified EglC mAb, 4A3, underwent multiple tests to verify its binding characteristics with the native EglC protein. Under denaturing conditions, the mAb bound to an *A. fumigatus* cell lysate preparation as a dimer at the 30 kD range (figure 4). These bands appear at the expected molecular weight, as confirmed by the NCBI protein database. Interestingly, however, the mAb bound unpredictably with *A. fumigatus*-infected serum and urine samples on a non-denaturing dot blot (figure 5). To probe the samples, 1 mg of antibody was required, thus all readings were considered inconclusive.

Because the purified mAb reacted with its target only under denaturing conditions, the use of an anti-peptide mAb was deemed unsuitable for the purposes of an LFI strip. The LFI format requires the use of non-denatured samples, thus the anti-peptide approach was set aside in favor of genetic immunization. Synthetically generated 15-mer peptides have a linear shape, which misrepresents the natural conformation of protein. The genetic immunization approach, which had been shown in previous studies to bind native protein, was undertaken to generate an antibody with a variable region which is more tailored to the structural specifics of the fully natured protein (Brown et al., 2011). Antibodies generated through genetic immunization should outperform anti-peptide antibodies in a number of ways.

DNA Constructs and Protein Expression. Synthetic genes were designed using the same parameters used in selecting the 15-mer peptides. In addition, genes were codon optimized for the *Mus musculus*, the species of animal to be immunized. It has been shown that codon optimization plays a role in obtaining the correct protein of interest by avoiding any unnecessary posttranslational modifications (Angov, 2011). By optimizing the codons for a mammalian species, it was our hope that the expressed protein might be a better model to study than genes which had been codon optimized for expression in *Escherichia coli*, for example.

Genomic sequencing of the final DNA constructs demonstrated that all necessary components for protein expression were present. This included the gene of interest being in-frame, in addition to the presence of the Kozak sequence, start codon, gene of interest, 6x-His tag, and stop codon. Genomic sequencing data was supported by the restriction enzyme digestion of the DNA constructs. The two bands which appeared on the agarose gel following restriction enzyme digestion with NheI is strong evidence supporting the proper insertion of the EglC genes into pCI (Engebrecht and Brent, 2001). Transfection of mammalian cells with these plasmids led to expression of proteins around the expected molecular weight.

The only component not present in the DNA constructs was a signal peptide which allows the transfected cells to transport the protein to the extracellular space. Due to the lack of a signal peptide sequence, it was expected that the expressed protein be found in a lysate of the transfected cells (Graslund et al., 2008). This was the case, as lysate samples from transfected HEK 293T cells contained protein while the supernatant did not (data not shown). Western blot data of purified lysates confirmed the presence of

an approximately 10 kD protein recognized by both anti-His and anti-EglC antibodies, leading to the belief that these are the EglC proteins of interest. Untransfected lysate samples were tested as negative controls in both of these blots, both of which confirmed a lack of protein. Production of these proteins in mass-quantities would allow for their use in screening assays and immunization boosts (Brown et al., 2011).

Further data regarding these proteins may be gathered through mass spectrometric analysis of the resulting bands on an SDS-PAGE. Protein characteristics such as the exact molecular weight and ultimately the amino acid composition may be assessed (Mann et al., 2001). Such data would provide confirmation that the proteins expressed are indeed the proteins of interest, devoid of any unwanted mutation or posttranslational modification.

Immunization studies utilizing these DNA constructs and the expressed protein fragments should lead to the development of a monoclonal antibody with affinity to the native protein. Our lab is also developing antibodies through immunization with full-length recombinant protein. These antibodies may be even more useful than genetic immunization, due to the fact that the full protein is an even better representation of the 3D structure of the protein that one 100 amino acid portion (Brown et al., 2011). The downside, however, is that this method does not allow for selection of particular epitopes. This is problematic in ultimately generating antibodies with specificity for a particular species. Nonetheless, this strategy, along with genetic immunization, provides a means of generating mAbs which should interact with native protein.

In conclusion, anti-peptide monoclonal antibodies are easily obtainable and function well in a variety of denaturing immunoassays. Other approaches, such as genetic

and full-length protein immunization, however, have the potential to be more useful in generating mAbs with the ability to recognize native protein. With the progression of this project, immunization experiments should yield the desired antibodies. These antibodies will eventually be applied to the LFI format with the ultimate goal of being able to diagnose IA in a more rapid, efficient manner.

List of Figures

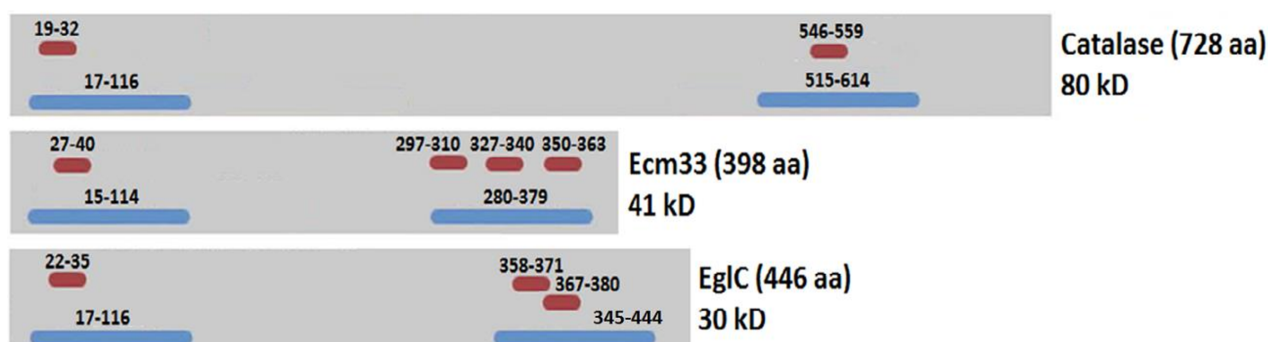

Figure 1. Antigenic peptides chosen as targets for mAb production. Each protein was analyzed using the Protean software by LaserGene to identify potential antigenic 15-mer sequences. Factors considered when designing peptides included antigenic index, surface probability, and hydrophilicity. These sequences were ultimately analyzed via BLAST search to confim specificity to the *Aspergillus* genus. Red areas indicate peptides, blue areas indicate DNA domains.

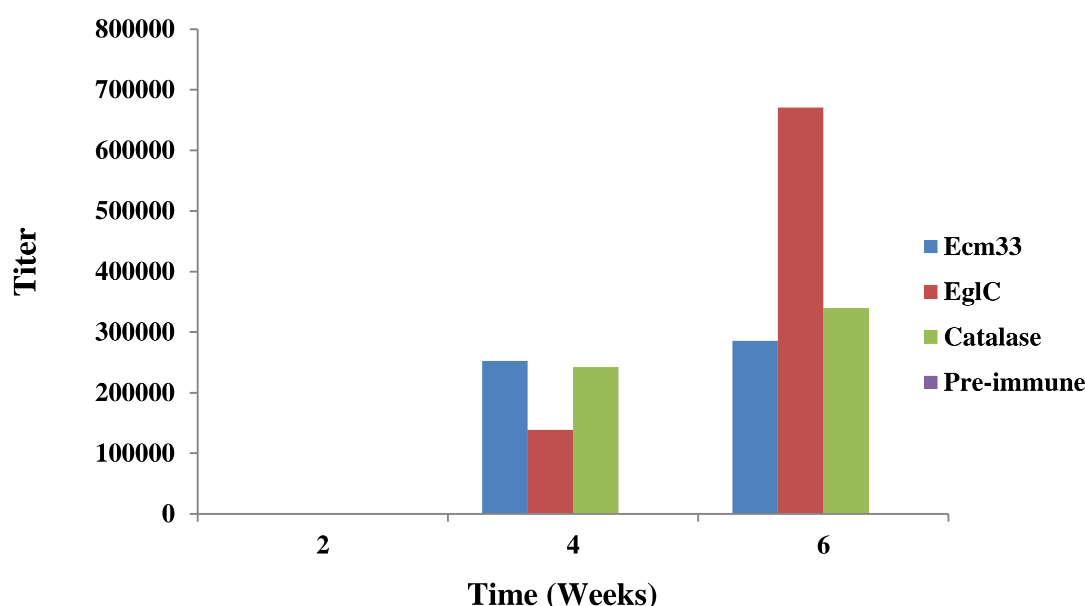

Figure 2. Mouse sera titers for highest-antibody producing animals. Five Balb/c mice per peptide were each immunized subcutaneously with 50 µg of peptide conjugated to KLH and emulsified with TiterMax Gold Adjuvant. 25 µg per mouse boost injections were administered at two-week intervals. Sera was screened via ELISA and titers calculated with SOFTmax Pro software.

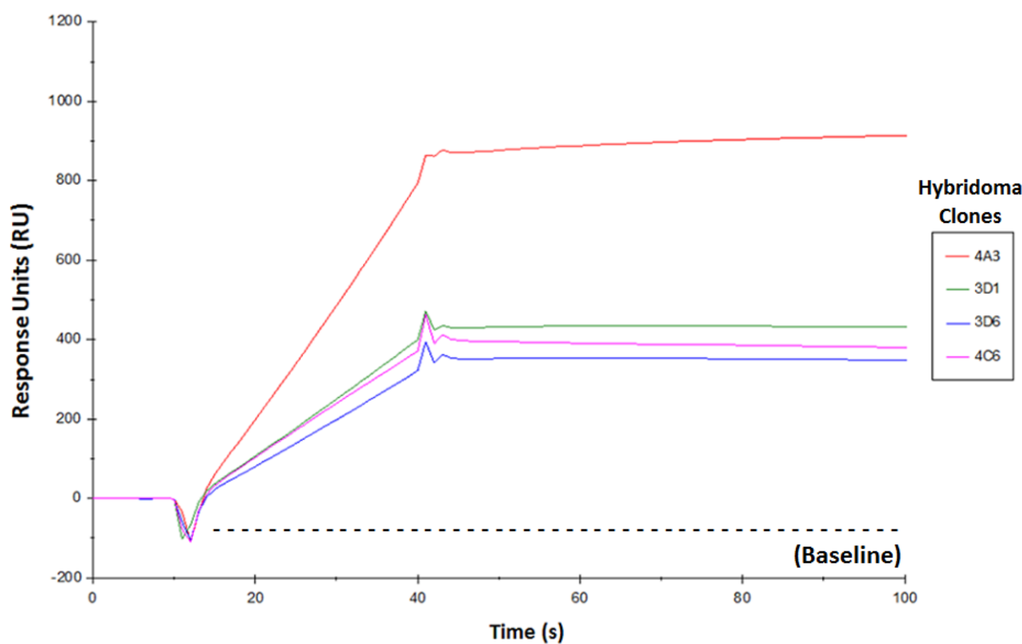

Figure 3. Biacore data for EglC hybridoma supernatant samples. Surface plasmon resonance (SPR) data was obtained using the Biacore X100. Supernatant samples were allowed to flow over a CM5 chip docked with EglC peptide through a disulfide linkage. Kinetics data observed by SPR was used as an estimation of the association and dissociation capabilities between antibody and antigen.

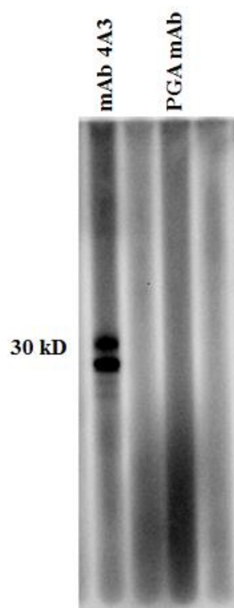

Figure 4. Immunoblot and for purified EglC mAb 4A3. Western blot was performed using 20 µg *A. fumigatus* lysate probed with 5 µg of EglC mAb 4A3. Anti-PGA mAb served as a negative control. Goat-anti-mouse IgG-HRP (1:5000) was used for detection.

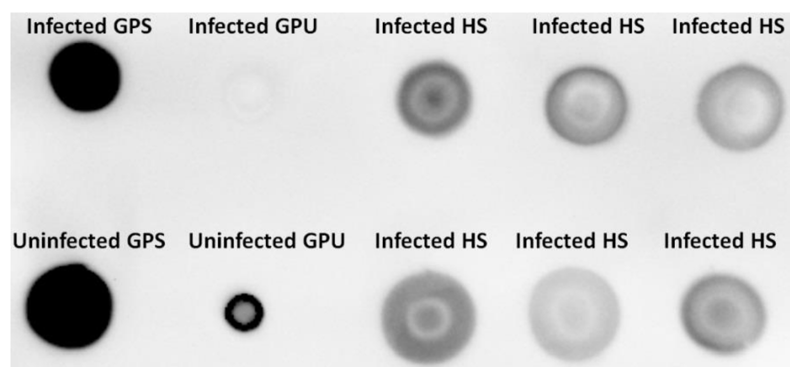

Figure 5. Dot blot for purified EglC mAb 4A3. A dot blot was performed using infected and uninfected guinea pig (GP) and human samples (HS). The mAb was observed to bind both infected and uninfected samples. In this dot blot, 1 mg of antibody was required for detection of samples, which is 1,000-fold higher than the usual amount expected.

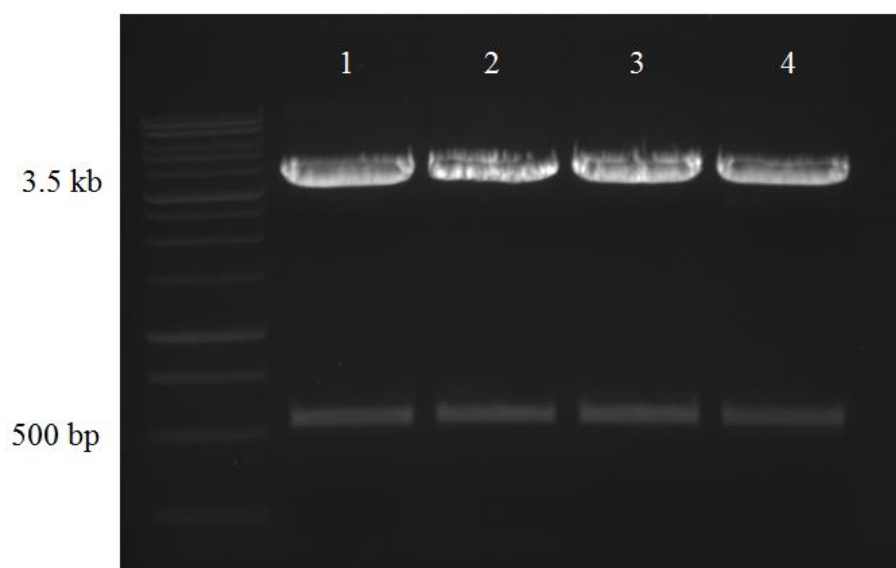

Figure 6. Restriction enzyme digestion of pCI vector containing EglC Domain 1 or 2. Following ligation of vector and insert, the resulting constructs were subject to endonuclease cleavage by restriction enzyme NcoI. Plasmids digested consisted of pCI containing EglC Domain 1 (Lane 1), Domain 1 with His-tag (Lane 2), Domain 2 (Lane 3), and Domain 2 with His-tag (Lane 4). Digested DNA was run on a 1% agarose gel at 100 V for one hour and imaged by ethidium bromide staining under UV illumination.

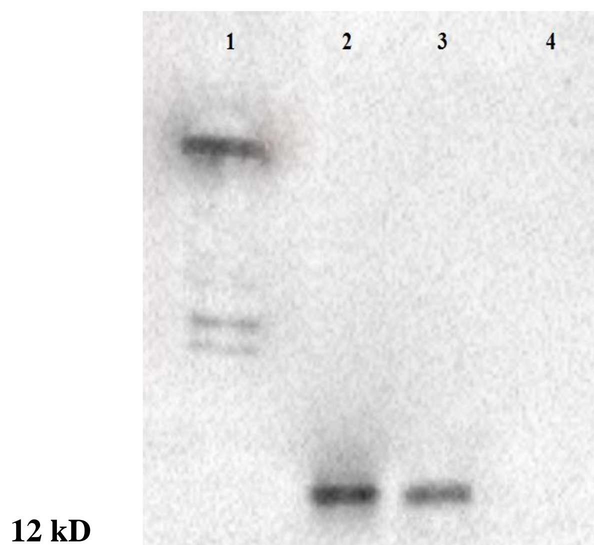

Figure 7. Anti-Polyhistidine Western blot for expressed EglC Domains 1 and 2. Purified proteins from a cell lysate prep of transfected HEK 293T cells were run on a 12% SDS-PAGE at 120 V and blotted onto a nitrocellulose membrane. The resulting blot was probed with 1/6000 Mouse anti-His, followed by exposure to 1/5000 Goat anti-Mouse IgG- HRP. Lanes contained BipC-His positive control (1), EglC Domain 1 (2), EglC Domain 2 (3), and untransfected sample negative control (4).

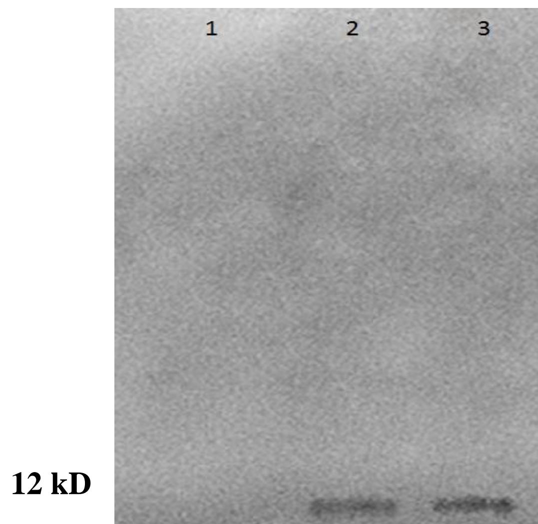

Figure 8. Anti-EglC Western blot for expressed EglC Domain 1. Purified proteins from a cell lysate prep of transfected HEK 293T cells were run on a 12% SDS-PAGE at 120 V and blotted onto a nitrocellulose membrane. The resulting blot was probed with 1/125 Rabbit anti-EglC, followed by exposure to 1/5000 Goat anti-Rabbit IgG- HRP. Lanes contained and untransfected sample negative control (1), EglC Domain 1 Fraction 1 (2), and EglC Domain 1 Fraction 2 (3).

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