Monoclonal Antibody ED1 as a Diagnostic Antibody for Human Invasive Pulmonary Aspergillosis

Submitted by

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Abstract

Invasive fungal infections (IFIs) are a major issue in immunocompromised humans, with *Aspergillus fumigatus* being the second most common cause of IFI after *Candida* species. Due to the rising numbers of solid organ transplant and neutropenic patients, invasive pulmonary aspergillosis (IPA) is a serious concern with mortality rates reaching 90% in certain patient groups. Rapid and accurate diagnosis is essential to improve patient prognosis by allowing early initiation of the correct antifungal treatment. At present, diagnosis of IPA is problematic, with currently available tests relying on pan-fungal detection of the fungal cell wall component β-D-glucan or on detection of *Aspergillus* galactomannan. However, these biomarker tests have inherent limitations, with reports of false-positivity and lack of sensitivity and specificity. Therefore there is a need for an alternative diagnostic assay that can act as adjunct test to the rapid point-of-care lateral-flow assay previously developed for aspergillosis detection which incorporates the *Aspergillus*-specific IgG3 monoclonal antibody (mAb) JF5. This paper describes the characterisation of a newly developed murine mAb ED1, an IgG1, that binds to the epitope β1-5-galactofuranose on the extracellular galactomannoprotein antigen of *Aspergillus fumigatus*. Results show that ED1 can be used in combination with JF5 in a DAS-ELISA immunoassay format to detect IPA in a guinea pig model of disease. The work demonstrates that the laboratory-based DAS-ELISA can be used alongside the LFD to detect the diagnostic galactomannoprotein antigen in serum samples, providing confirmatory tests of invasive infection.

Keywords

Invasive aspergillosis, *Aspergillus*, diagnosis, monoclonal antibody

Abbreviations

mAb, monoclonal antibody; Ig, immunoglobulin; IPA, invasive pulmonary aspergillosis; gal*f*, β1-5-galctofuranose; BALf, bronchoalveolar lavage fluid; GM, galactomannan; BDG,' β-D-glucan; LFD, lateral-flow device; PCR, polymerase chain reaction; ELISA, enzyme-linked-immunosorbent assay; PTA, plate-trapped-antigen; DAS, double-antibody-sandwich; C, competition; NPV, negative

predictive value; PBS, phosphate buffered saline; BSA, bovine serum albumin; PBSA, PBS containing BSA; PDA, potato dextrose agar; SDA, Sabouraud dextrose agar; TCS, tissue culture supernatant.

Declaration

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Introduction

Aspergillus fumigatus and diagnosis of invasive pulmonary aspergillosis

Aspergillus fumigatus, a ubiquitous saprotrophic mould (Latgé 1999), is the second most common cause of invasive fungal infections in immunocompromised patients after the commensal yeast *Candida* (Pappas *et al.* 2010). In the immunocompetent, air-borne spores of the fungus are destroyed by phagocytic cells (alveolar macrophages) of the innate immune system (Latgé 1999), but in individuals with impaired immunity, the fungus is able to evade destruction and the spores germinate to form invasive hyphae (Kwon-Chung and Sugui 2013) capable of causing a range of disseminated infections (Segal 2009) depending on the host's immune status (Stergiopoulou *et al.* 2007).

Invasive pulmonary aspergillosis (IPA), a disseminated infection of which 90% of cases are caused by *A. fumigatus* (Denning 1998; Thompson and Patterson 2008), is one of the leading causes of death in the immunocompromised such as neutropenic, haematological malignancy and hematopoietic stem cell transplant patients, with mortality rates that range between 30 and 95% depending on the nature of the underlying impairment (Brown *et al.* 2012; Patterson *et al.* 2000). The number of cases of IPA has increased in recent years mainly due to rising numbers of patients at risk because of medical intervention, for example aggressive anti-cancer treatment or hematopoietic stem cell or solid organ transplant (Latgé 1999; Brakhage and Langfelder 2002; Kontoyiannis and Bodey 2002). Therefore, rapid diagnosis of *Aspergillus* infections is vital, especially since studies have shown that earlier diagnosis of IPA leads to better patient prognosis (Caillot *et al.* 2001; Michallet and Ito 2009). Early treatment with

antifungal drugs improves patient survival to potentially 80% or greater (Upton *et al.* 2007, Shannon *et al.* 2010).

Despite this, diagnosis of IPA remains a significant challenge. There is currently no single gold standard method of detection, so identification relies on a combination of factors including patient history, clinical symptoms, mycological culture, radiology and antigen testing. Diagnostic procedures such as culture and conventional imaging using computed tomography lack sensitivity (Yeo and Wong 2002) and specificity and may not be achievable due to the invasiveness of the procedures and the nature of the infection (Denning 2000). Many filamentous fungi have similar hyphal morphologies (Guarner and Brandt 2011), thereby making them difficult to identify simply by microscopy and to then treat accordingly. Thus a move has been made towards detection using biomarkers that are characteristic of fungal infections, such as *Aspergillus* galactomannan (GM) and the fungal cell wall component (1-3)-β-D glucan (BDG). Tests that detect these two biomarkers are included in the revised 2008 European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group, National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) guidelines for diagnosis of IPA (De Pauw *et al.* 2008). At present, nucleic acid-based detection systems are not included in the guidelines due to the lack of assay standardisation (De Pauw *et al*. 2008; Bretagne 2010), despite polymerase chain reaction (PCR) having high sensitivity and specificity according to a number of studies (Einsele and Loeffler 2008, Florent *et al.* 2006, Kami *et al.* 2001).

(1-3)-β-D glucan is a cell wall component of many pathogenic fungi excluding *Cryptococcal* and Mucoralean species that lack the sugar in their cell walls (Marty and Koo 2009). The Fungitell test, designed to detect BDG using the Limulus test (Hope *et al.* 2005), is pan-fungal and thus non-specific, and is unable to discriminate between different genera or species of infectious fungi; a main limitation when wanting to diagnose and treat IPA specifically. Notwithstanding this limitation, studies have shown the test to be highly sensitive and to have an excellent negative predictive value (NPV) (Pazos *et al.* 2005; Vlieger *et al.* 2011), although this does vary depending on patient's clinical status (Alexander *et al.* 2010). Due to its high NPV, the BDG Fungitell test could be combined with other methods of detection to improve the accuracy of IPA diagnosis, but insufficient

studies have been conducted to determine whether adjunct tests might improve its specificity for this disease.

In comparison to (1-3)-β-D glucan, galactomannan detection has been shown to be specific and sensitive for IPA detection depending on the patient group being diagnosed and the sample type and threshold used for assay positivity (Maertens *et al.* 2007a; Maertens et al. 2007b). Serial screening using the Bio-Rad Platelia GM ELISA has been used to guide antifungal therapy (Maertens *et al.* 2005; Segal *et al.* 2007). GM is released by *Aspergillus fumigatus* from the cell wall during angioinvasion and is detectable in serum. In clinical settings there is great variability in the way the test is used (Pfeiffer *et al.* 2006), and there are some considerable drawbacks (Verweij and Mennink-Kersten 2006). The GM ELISA is associated with a high number of false positives due to cross-reaction with species such as *Histoplasma* and *Penicillium* (Tortorano *et al.* 2012), and use of β-lactam antibiotics (Boonsarngsuk *et al.* 2010), demonstrating a need for alternative diagnostic tests especially when mixed cultures are present, or during treatment with antimicrobial agents.

Another important consideration is the type of sample used for diagnosis. *Aspergillus* GM has been detected in urine, serum and bronchoalveolar lavage fluid (BAL*f*) (Klont *et al.* 2004) using different immunoassay formats to varying effect depending on the type of test used, the immunocompromised nature of the patient, the absorbance threshold used for test positivity and if the patient is on prophylactic antifungals. Data on the performance of GM detection in urine samples is limited and, although some studies have suggested that it may be a good screen for IPA in humans (Dupont *et al.* 1987), it suffers from variation of GM levels due to dilution within the urine. A recent study has suggested that taking into account the dilution factor increases the utility of the GM test in urine (Reischies *et al.* 2016), however further work is required to understand how this can be transferred to other patient groups.

In contrast, a number of studies across a range of immunocompromised patient groups have demonstrated that BAL*f* testing for GM allows for highly sensitive immunoassays, potentially more so than serum-based immuno-detection (Meersseman *et al.* 2008, Guo *et al.* 2010). However the procedure for collecting BAL*f* samples is highly invasive and so this may not be suitable for certain patient

types. Therefore, serum is a more preferable sample for GM testing in patients where invasive procedures such as bronchoscopy are not suitable, with BAL*f* samples used where possible. Studies have shown that serum testing for GM has varying levels of sensitivity and specificity depending on the detection threshold used and the patient type (Maertens *et al.* 2007a; Maertens *et al*. 2007b; Herbrecht *et al.* 2002). Nevertheless, serum GM levels have also been shown to correlate well with fungal burden in animal models of IPA, suggesting its applicability for human disease detection.

The main limitations of the diagnostic methods described here are that they are time-consuming and require diagnostic laboratory facilities. This can ultimately delay diagnosis and treatment especially in resourced-limited settings where hospital laboratories are not suitably equipped. To overcome these limitations, a non-invasive point-of-care immunodiagnostic test (lateral-flow device (LFD), a product of the University of Exeter spin-out company ISCA Diagnostics) that detects an *Aspergillus* diagnostic antigen using the monoclonal (mAb) IgG3 antibody JF5 has been developed (Thornton 2008a). Lateral-flow devices have been used previously to great effect for simple, rapid diagnosis of bacteria, viruses and toxins (Ngom *et al.* 2010, Kappe and Schulze-Berge 1993) and also in the diagnosis of other fungal infections (Thornton 2008b), and rely on the same technology as the Unilever home pregnancy test.

The JF5 mAb has been shown to bind to the epitope galactofuranose (Gal*f*) present in an *N*-linked galactomannoprotein antigen in the *Aspergillus* hyphal cell wall. Gal*f* is a ringed form of galactose that is found ubiquitously in *Aspergillus* sp. and also is an essential component of many pathogenic fungal cell walls (Barr *et al.* 1984, Vaishnav *et al.* 1998). In the *Aspergillus* cell wall Gal*f* is present in both the alkali soluble and alkali insoluble fractions of the galactomannan (Latge, 2009). However, it is also present in lipophosphogalactomannan and GIPCs that are anchored to the membrane, and can also be found at *N*-glycan ends of many glycoproteins. The frequency of Gal*f*-containing molecules in *Aspergillus* alongside the ubiquity of many other fungal pathogens makes the specificity of galactomannan testing tricky. However several studies have shown the sensitivity and specificity of the JF5 LFD to be comparable with other assays with both BAL*f* and serum samples (Wiederhold *et al.* 2013; Willinger *et al.* 2014; Johnson *et al.* 2015; Hoenigl *et al.* 2012; Miceli *et al.* 2015), with the added benefits of minimal

processing and decreased assay time (10-15 minutes), demonstrating that it can speed up diagnosis and is suitable for monitoring immunocompromised patients at risk of infection. More work is required to further understand the clinical utility of the *Aspergillus* LFD, but it is clear there is a need for a simple non-invasive point-of-care test within the field of IPA diagnostics, of which the LFD has enormous potential for translation to the clinical setting.

Objectives of this work

The objective of this work was to characterise an IgG1 mAb (ED1) complementary to the IgG3 mAb JF5, firstly stabilising the ED1 hybridoma cell line and then determining whether it could be used in combination with JF5 to develop an Enzyme-Linked Immunosorbent Assay (ELISA) complementary to the LFD, for the specific and sensitive diagnosis of IPA. In doing so, I set out to determine the nature of the epitope bound by ED1 by using *A. fumigatus* mutants deficient in UDP-galactopyranose mutase, a key enzyme involved in the biosynthesis of galactofuranose containing glycoconjugates of *A. fumigatus* (Komachi *et al*. 2013; Latgé 2009; Schmalhorst *et al*. 2008). Results show that ED1 binds to the antigenic determinant β1,5-galactofuranose (Gal*f*) present in the diagnostic *Aspergillus* mannoprotein antigen, and that it can be used successfully in a Double-Antibody-Sandwich-ELISA with the IgG3 antibody to detect the diagnostic biomarker in serum from a guinea pig model of IPA. This work demonstrates the utility of the IgG1 mAb ED1 in the development of adjunct tests to complement the JF5 LFD.

Materials and Methods

Ethics statement

All animal work described in this study was conducted under a UK Home Office Project License, and was reviewed by the institution's Animal Welfare Ethical Review Board (AWERB) for approval. The work was carried out in accordance with The Animals (Scientific Procedures) Act 1986 Directive 2010/63/EU, and followed all the Codes of Practice which reinforce this law, including all elements of housing, care, and euthanasia of the animals.

Fungal culture

Fungal cultures were routinely grown on plates and slopes of potato dextrose agar (PDA: Potato Dextrose Broth (PDB: P6685; Sigma), agar 20gL^{-1}) and Sabouraud Dextrose Agar (SDA: Sabouraud Dextrose Broth (SDB: S3306; Sigma), agar 20gL^{-1}), and cultured at 26°C under a 16hour fluorescent light regime. Mutant strains were also grown on Malt Extract Agar (MEA; Malt Extract (ME: 70167; Sigma), agar 20gL^{-1}) or grown in liquid shake cultures of ME, at 60rpm and 30°C. All media was autoclaved at 121°C for 15 minutes before use.

Production of monoclonal antibody ED1 and sensitivity testing

The IgG1-producing hybridoma cell line ED1 developed previously by Dr C.R. Thornton, was stabilised by repeated sub-cloning in tissue culture medium (TCM; 10% (v/v) Foetal Bovine Serum (FBS: FSC-SA; Labtech), 1% (v/v) 200mM L-Glutamine (G7513; Sigma), 0.1% (v/v) penicillin/streptomycin solution (P/S; 60gL^{-1} Penicillin G (P3032; Sigma), 100gL^{-1} Streptomycin (S9137; Sigma), 90% (v/v) RPMI-1640 (R0883; Sigma)) at 37°C and 5% CO_2. Cells were plated out at a dilution of 1 cell/well and screened via Enzyme-Linked Immunosorbent Assay (ELISA). Only wells with both a positive absorbance value and containing a single compact colony were taken forward to eliminate any non-producers present within the cell line. To then ensure monoclonality, cells were sub-cloned by limiting dilution according to the procedure outlined previously (Thornton 2001). Antibody in tissue culture supernatants (TCS) was then tested for *Aspergillus* antigen recognition in Enzyme-Linked Immunosorbent Assay (ELISA) by using microtitre plates coated with *Aspergillus* mannoprotein antigen (Rolle *et al.* 2016). After five rounds of limiting dilution, the stable cell line $ED1^{p}.EC9^{5}$ was generated and cells were stored long-term in liquid N_2 by using the freezing process outlined elsewhere (Thornton 2001).

Enzyme Linked Immunosorbent Assay (ELISA)

The mannoprotein antigen, a product of the University of Exeter spin-out company ISCA Diagnostics and supplied by Dr C.R. Thornton, was dissolved in phosphate buffered saline (PBS: 0.8% NaCl; 0.02% KCl; 0.115% Na_2HPO_4; 0.02% KH_2PO_4; pH7.2) at 1mg/ml and 50µl volumes were used to coat the wells of Maxisorp microtiter plates (442404; Nunc), which were incubated in a sealed

plastic bag at 4°C for 16 hours. Wells were then washed 3 times with PBST (PBS containing 0.05% (v/v) Tween-20), once with PBS and once with dH_2O each for a minimum of 5 minutes before being air-dried in a laminar flow hood and stored for long-term use in sealed plastic bags at 4°C.On use, plates were blocked with 150µl/well PBSA (PBS containing 1% (w/v) bovine serum albumin (BSA: A7906 Sigma)) for 15 minutes and then rinsed once with PBS. Each well was then incubated with 50µl of mAb TCS or TCM for control wells for an hour then washed three times with PBST before incubation with 50µl of goat anti-mouse polyvalent (immunoglobulin classes IgG, IgA and IgM) peroxidase conjugate (A-0412; Sigma Chemical Company, Poole, United Kingdom) diluted 1 in 1000 in PBST for a further hour. After washing three times with PBST and once with PBS, plates were incubated with tetramethyl benzidine solution (TMB substrate: 5ml H_2O, 5ml 0.2M NaOAc, 195µl 0.2M citric acid, 5µl of 30% (v/v) H_2O_2, 100µl of a stock solution (10mg/ml DMSO) of 3,3',5,5'-tetramethyl benzidine (T-2885; Sigma)) for 30 minutes to visualise bound antibody. The reaction was then stopped with 50µl 3M H_2SO_4 per well and absorbance read at 450nm using a microtitre plate reader (Tecan GENios, Tecan Austria GmbH). All incubation steps were carried out in a sealed plastic bag at 23°C, and washes were a minimum of 5 minutes each.

Specificity Screening of ED1 and JF5 antibodies

Tissue culture supernatants containing mAb ED1 or mAb JF5 were tested for specificity against a variety of clinically relevant moulds and yeasts pathogenic to humans. Fungal cultures were grown on plates and slopes of PDA as described above. PBS surface washings of fungal slopes were generated according to the procedure outlined previously (Thornton 2001). In brief, 2 days after inoculation of the slope to ensure active growth 3ml sterile MQ water was used to wash off fungal spores and the liquid collected and centrifuged at 15000rpm for 5 minutes to separate the soluble antigen from the fungal cells. The supernatants were then further diluted 1 in 10 (v:v) in PBS. Soluble antigens were then immobilised to microtitre plates at 50µl per well, with PBS used as a negative control and *Aspergillus* mannoprotein antigen diluted 1 in 1000 in PBS as a positive control. Plates were subsequently incubated, washed and tested via PTA-ELISA with ED1 and JF5 as the primary antibodies and TCM as a control as described above. The threshold for detection was determined from control means (3xTCM abs)

which were consistently in the range of 0.05-0.10, therefore values >0.10 were considered a positive reaction.

Table 1. Details of fungi used in this study.

Organism	Isolate number	Source[a]
Aspergillus fumigatus WT	AF293	SK
Aspergillus fumigatus Δ*glfA*::*hph4.4*	4.4	GD
Aspergillus fumigatus Δ*glfA*::*hph7.4*	7.4	GD
Aspergillus ficuum	555.65	CBS
Aspergillus flavus	91856iii	IMI
Aspergillus nidulans	A4	FGSC
Aspergillus restrictus	116.5	CBS
Aspergillus terreus var. *terreus*	601.65	CBS
Aspergillus wentii	121.32	CBS
Candida albicans	SC5314	SB
Candida albicans	NGY152	CBS
Candida glabrata	4962	CBS
Candida tropicalis var. *tropicalis*	1920	CBS
Cryptococcus neoformans var. *neoformans*	7779	CBS
Filobasidiella bacillospora	10865	CBS
Fusarium oxysporum f.sp. *lycopersici*	167.30	CBS
Fusarium solani	224.34	CBS
Geotricum candidum	115.25	CBS
Lichtheimia corymbifera	TJAFJ713070	CRT
Neosartorya fischeri var. *fischeri*	681.71	CBS
Penicillium cyclopium	132.14	CBS
Penicillium islandicum	338.48	CBS
Rhizopus oryzae	112.09	CBS
Rhizopus stolonifer var. *stolonifer*	389.95	CBS
Rhodotorula sloofiae	Alo-1	CRT
Scedosporium apiospermum	117407	CBS
Scedosporium prolificans	467.74	CBS
Trichosporon asahii var. *asahii*	7632	CBS
Verticillium dahlia	178.66	CBS

a. CBS; Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands. SK; S. Krappman, Institute of Microbiology and Genetics, Department of Molecular Microbiology and Genetics, Georg-August University, Gottingen, Germany. IMI; International Mycological Institute, Egham, England. FGSC; Fungal Genetics Stock Centre, University of Missouri, Kansas City. CRT; C.R.Thornton, University of Exeter, UK. SB; S.Bates, University of Exeter, UK. GD; Genna Davies, University of Exeter, UK.

SDS Page and Western blotting

For sodium-dodecyl-sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), *Aspergillus* antigen dissolved in sterile Milli-Q water (MQ-H_2O) was diluted to 0.5µg/ml in Laemmli buffer (Laemmli, 1970), and then denatured at 100°C for 10 minutes. Proteins were separated on 4-20% gradient polyacrylamide gels at 165V with pre-stained molecular weight ladder (Bio-Rad Laboratories Limited, Hemel Hempstead, UK) for determination of molecular weights. For western blotting, proteins were then transferred electrophoretically onto an activated PVDF membrane for 2 hours at 75V. The membrane was blocked for 16 hours at 4°C with shaking at 15rpm in 1% PBSA, then incubated with mAb JF5 or ED1 TCS diluted 1:2 in 0.5% PBSA solution for 2 hours. The membrane was washed three times in PBS and then incubated with goat anti-mouse IgG (whole molecule) alkaline phosphatase conjugate (A3562; Sigma) diluted 1 in 15,000 in 0.5% PBSA for a further hour. After washing three times in PBS and once in PBST the membrane was equilibrated in APSB buffer (Thornton, 2008a) for 2 minutes, and the reaction visualised with APSB substrate solution for 30 minutes before being stopped with dH2O and dried and stored in between sheets of Whatman filter paper. All incubation steps were carried out at 23°C and 15rpm shaking, and each wash was for a minimum of 5 minutes.

Epitope Characterisation by heat, chemical and enzymatic modification

Heat Treatment Solubilised *Aspergillus* antigen at a concentration of 1mg/ml PBS was tested for heat stability by boiling at 100°C in a water bath. At 10 minute intervals, samples were removed and the antigen immobilised to microtitre plates at a volume of 50µl per well. Non-heat treated *Aspergillus* antigen solution was used at time point 0 as a control. Plates were then incubated overnight, washed and tested via PTA-ELISA as described.

Periodate treatment Microtitre plates coated with *Aspergillus* antigen were incubated with either 50µl of sodium acetate buffer (37% NaOAc (6.8gL^{-1} $CH_3COONa.3H_2O$), 63% HOAc (3gL^{-1} HOAc), pH 4.5) as a control or sodium *m*-periodate solution (20 mM NaIO4 in 50 mM sodium acetate buffer, pH4.5) for 20, 4, 3, 2, 1 or 0hr at 4°C in a sealed plastic bag. Plates were then washed and assayed by PTA-ELISA.

Protease treatment Microtitre plates coated with *Aspergillus* antigen were incubated with 50µl of trypsin solution (T7169 tablets in MQ-H_2O at a 1mg/ml

concentration) and MQ-H_2O as a control or 50µl of pronase solution (0.9mg protease Type XIV from *S. griseus* P5147 in 10ml PBS) and PBS as control. Plates were then incubated at either 37°C or 4°C for four hours in sealed plastic bags and then washed three times in PBST for 5 minutes before being tested via PTA-ELISA.

Dot blot assay of chemical modification of epitope

20 µl Aspergillus antigen diluted to 1mg/ml in Milli-Q water was spotted onto activated PVDF membranes and air-dried. The membranes were then treated with 2ml of proteases (as before) and the respective controls for 4 hours at 37ºC before being washed 3 times in PBS. The membranes were then blocked in 1% in PBSA at 15rpm, 4ºC for 16 hours, then incubated with the respective mAbs diluted in PBSA; ED1 @ 1:2, mJF5 @ 1/1250 and hJF5 @ 1/2500 for 2 hours. After washing three times in PBS, the respective secondary antibodies were then incubated for 1 hour at 1/1000 (anti-mouse polyvalent for ED1 and mJF5, anti-human polyvalent for hJF5). Finally, after washing with three times with PBS and once with PBST the membranes were developed using the Amersham ECL kit. All incubation steps unless stated were carried out at 23ºC and 15rpm shaking. Each was for a minimum of 5 minutes.

Competition ELISA

Carbohydrate binding To investigate the inhibition of mAb binding to the target mannoprotein antigen by sugars, mAbs JF5 and ED1 were first purified using Protein A purification (Pierce). Antibodies were then diluted into PBST solutions containing 400mM of D-(+)-galactose (G0624; Sigma) or D-(+)-mannose (3458; Lancaster) to give starting concentrations of 10µg/ml (ED1) and 0.2µg/ml (JF5) purified antibody protein/ buffer. Plates coated with 1mg/ml *Aspergillus* antigen for mAb ED1 and 15µg/ml for mAb JF5 were then incubated with doubling dilutions of the antibodies in 400mM PBST sugar solutions for 1 hour, with the remaining steps of the C-ELISA conducted as described thereafter. *Aspergillus* antigen at 500µg/ml buffer was used as the positive control and PBST diluent only as the negative control. Working volumes were 50µl per well.

Overlapping epitopes To investigate whether the epitopes of JF5 and ED1 are overlapping mAb ED1, a fully humanised IgG1 version of mouse mAb JF5 (hereafter referred to as hJF5) and mAb HG12 (an unrelated *Scedosporium*-

specific mouse IgG1 mAb (Thornton, 2009)), each purified using Protein A, were used to block binding of Protein A-purified JF5 directly labelled with the enzyme horse radish peroxidase (HRP) at a molar ratio of 1:1 (Thornton, unpublished). The humanised antibody hJF5 has been shown previously to have a higher affinity than mouse JF5 for the target mannoprotein antigen and so was predicted to block binding of the JF5-HRP conjugate. For this reason it was used as a positive control, while the unrelated mAb, HG12 acted as a negative control. An additional negative control consisted of PBST diluent buffer only. A 1 in 2000 dilution of the JF5-HRP conjugate (equivalent to 0.5μg antibody protein/ml) was prepared in PBST containing 10μg/ml of Protein A-purified mAb (ED1, hJF5 or HG12) and then double diluted into 10μg/ml PBST solutions of the respective antibodies in the wells of microtitre plates coated with *Aspergillus* mannoprotein antigen at 1mg/ml. The mixtures were incubated for 1 hour, and the remaining steps of the C-ELISA performed as described thereafter. Working volumes were 50μl per well.

Production of *Aspergillus fumigatus* mutants

Targeted replacement of the *A. fumigatus* UDP-galactopyranose mutase-encoding gene *glfA*, with the hygromycin B phosphotransferase-encoding gene (*hph*), was performed using the split marker recombination method (Catlett et al., 2003; Kershaw & Talbot, 2009; Thornton et al., 2015) and was carried out by G. Davies. The *glfA* gene and flanking sequences were obtained from the *Aspergillus* Genome Database (AspGD, http://www.aspergillusgenome.org/) and used to design primers. Primer pairs glf50.1F/glf50.1R and glf30.1F/glf30.1R were used to amplify the 5' (LF, 1.0-kb) and 3' (RF, 1.0-kb) flanking regions of the *glfA* gene, respectively, from *A. fumigatus* Af293 genomic DNA. Simultaneously, split *hph* templates were amplified to create the 5' amplicon (HY, 1.2-kb) using primers HY split/M13F and 3' amplicon (YG, 0.8-kb) using primers YG split/M13R. Fusion PCR resulted in two products; LFHY (2.1-kb) and RFYG (1.8-kb), using primer pairs glf50.4F/HY split and glf30.1R/nested YG split, respectively. The amplicons were gel-purified and transformed into protoplasts of Af293, replacing *glfA* with the assembled *hph* gene and conferring resistance to hygromycin B. Putative Δ*glfA*::*hph* transformants were selected in the presence

of hygromycin B (600µg/mL) and gene replacement was confirmed by Southern blot (results not shown here).

Aspergillus fumigatus mutants

The two mutants strains of *A. fumigatus* (Δ*glfA*::*hph*4.4 and Δ*glfA*::*hph*7.4) generated as described above, were grown on SDA slopes as described previously. Spores of the mutants and the *A. fumigatus* wild-type strain AF293 were prepared in sterile MQ-H_2O and were used at a final concentration of 10^4 spores/ml, to inoculate 100ml sterile MEB liquid medium. Cultures were incubated as shake cultures at 60rpm and 30°C. Every 24 hours 3 replicate flasks were harvested by filtration of the contents through sterile Miracloth (Calbiochem). The mycelium was dried at 80°C for 48 hours and dry weights determined. The culture filtrate was used to coat the wells of a microtitre plate and PTA-ELISA conducted as described. For western blotting, filtrates were diluted 1:1 (v/v) in Laemmli buffer and denatured by heating at 100°C before SDS-PAGE and western blot processing as described. Antibody binding was determined by using the Amersham ECL Western Blot analysis kit and read on the Syngene G:box using the GeneSys programme.

Immunofluorescence

Spores of *A. fumigatus* AF293 and the mutant strains Δ*glfA*::*hph*4.4 and Δ*glfA*::*hph*7.4, were suspended in MQ-H_2O containing 1% (w/v) D-glucose and 200µl volumes placed on the surface of sterile glass slides. The slides were incubated in a moistened chamber at 37°C to stimulate spore germination and the germlings fixed to the slides according to the method outlined in Thornton (2001). The fixed samples were then incubated with ED1 TCS or TCM only as the negative control for 1 hour before being washed 3 times in PBS. Goat anti-mouse polyvalent FITC conjugate (F1010; Sigma) diluted 1 in 40 in PBS was then applied and the slides incubated in the dark for 30 minutes. Slides were given 3 PBS washes and then coated in a glycerol-PBS mounting medium (Fluoromount F4680; Sigma) before being covered with a coverslip. All washes were for a minimum of 5 minutes whilst rocking at 15 rpm and incubation was carried out in a humid environment at 23°C. Slides were kept in the dark at 4°C before being imaged using an Olympus IX81epifluroescence microscope.

Double-Antibody-Sandwich ELISA of serum and BAL

Wells of microtitre plates were incubated with Protein A-purified ED1 at 20µg/ml PBS overnight at 4ºC and, after washing and air-drying, were blocked for 15 minutes with 150µl of PBS containing 1% BSA and then washed once with PBS prior to DAS-ELISA. Human AB male serum (Labtech) spiked with *Aspergillus* mannoprotein antigen, or serum or bAL from a guinea pig model of IPA (Wiederhold *et al.* 2009; Wiederhold *et al.* 2013), were heat treated at 100°C for 3 minutes with *Aspergillus* sample buffer (PBS containing 0.5% (w/v) ethylenediaminetetraacetic acid (EDTA; D/0700/53, Fisher Scientific), pH6.1), before centrifugation at 13,000 rpm for 5 minutes. The clear supernatant was removed and 50µl volumes transferred to the ED1 coated plates for incubation for 2 hours at 23ºC in a sealed plastic bag. The wells were then washed three times with PBST and subsequently incubated with JF5-HRP conjugate at 32µg/ml in PBST for 1 hour. After washing three times with PBST and once with PBS, bound antibody was visualised with TMB for 30 minutes as described. All washing steps lasted for a minimum of 5 minutes. Guinea pig sera and BAL were tested in a blind study to prevent biased interpretation of DAS-ELISA results; results for GM, β-D-glucan and LFDs tests were withheld until results for the DAS-ELISA samples has been obtained and samples designated as positive or negative for the target antigen using a threshold absorbance value for test positivity. Un-spiked human serum or serum or BAL from control (uninfected) guinea pigs consistently gave absorbance values in DAS-ELISA below 0.225, and so this absorbance value was used as the threshold for assay positivity.

Data Analysis

The difference in means was tested by one-way variance analysis (ANOVA) and statistical significance determined by Tukey-Kramer post-hoc test with a Bonferroni Correction.

Results

Stabilisation of the ED1 cell line

The ED1 cell line was stabilised through five rounds of sub-cloning by limiting dilution. Due to the high proportion of non-producing cells that were outcompeting the antibody-producing hybridomas it was necessary to initially plate cells out at a very low dilution. Only true hybridomas were taken forward and then re-cloned for monoclonality, resulting in $ED1^{p}.EC9^{5}$. This sub-clone was then used in all further experiments to characterise the ED1 mAb.

Specificities of mAbs ED1 and JF5 Monoclonal antibodies JF5 and ED1 were tested in PTA-ELISA for specificity against a range of clinically relevant moulds and yeasts pathogenic to humans (Table 1). Both antibodies reacted strongly with *Aspergillus* species, with the exception of *Aspergillus wentii*, and with the sexual stage of *A. fumigatus*, *Neosartorya fischeri* var. *fischeri* (Fig. 1), showing the mAbs to be genus-specific. Both mAbs also cross-reacted strongly with *Penicillium cyclopium,* as has been reported for mAb JF5 previously (Thornton 2008a), but did not react with the other *Penicillium* species tested. Neither JF5 or ED1 cross-reacted with a range of unrelated fungi including *Candida albicans*, *Cryptococcus neoformans* and *Fusarium solani*.

Western blotting of the ED1 and JF5 antigens

Both mAb JF5 and mAb ED1 gave similar binding patterns in western blots of the *Aspergillus* mannoprotein antigen (Fig. 2A), with characteristic smearing in the molecular weight range 50 to >200kDa. This pattern of binding is consistent with previous studies of JF5 (Thornton, 2008), and with mAbs which bind to other glycosylated antigens of A. fumigatus (Stynen et al. 1992).

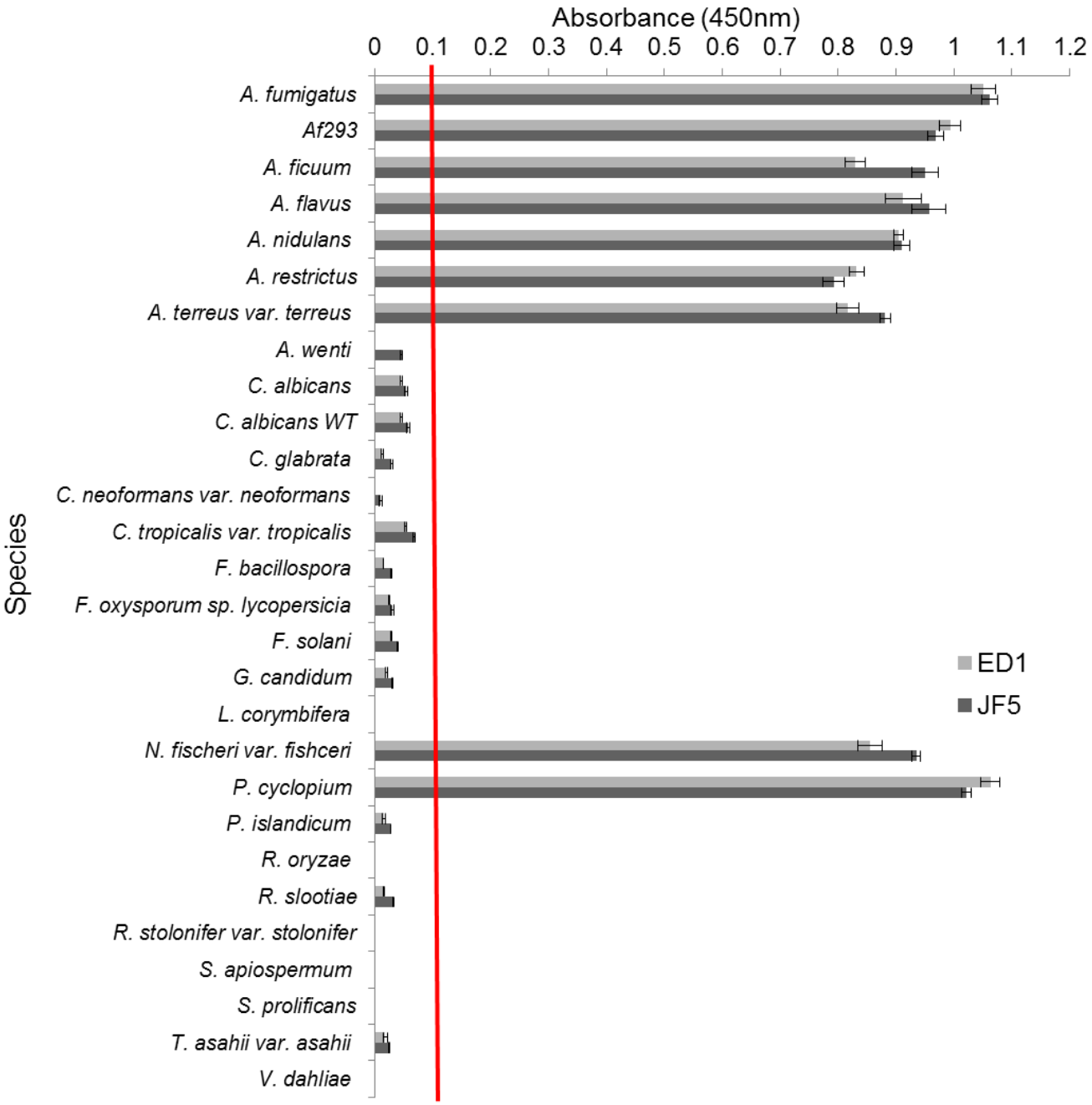

Figure 1 ELISA absorbance values for specificity screening of mAbs ED1 and JF5 against clinically relevant moulds and yeasts. ELISA absorbance values at 450nm against surface washings containing antigens from clinically relevant moulds and yeasts show that both ED1 and JF5 are highly sensitive and also specific to *Aspergillus* species with the exception of recognition of *N. fischeri* var. *fischeri*, a related mould and *P. cyclopium*, which has been previously reported. Wells were coated with 50µl surface washing from slopes inoculated 72 hours previously. Bars represent the mean value of three repeats ± standard error. The red line indicates the threshold value of the ELISA for detection of antigen of 0.1.

Characterisation of epitope by heat, periodate and protease treatment

The *Aspergillus fumigatus* antigen was modified by heat (Fig. 2B), enzymatic digestion (Fig. 2C) and chemical degradation (Fig. 2D and E) to investigate the nature of the epitopes bound by mAbs JF5 and ED1. A reduction in binding of an antibody to a heat-treated antigen would indicate that its epitope is heat labile. Neither of the antibodies showed significant decreases in PTA-ELISA absorbance values over 60 minutes of heating (Fig. 2B), showing that their epitopes are heat stable. Reductions in mAb binding following treatment with pronase shows that its epitope consists of protein, while reductions with trypsin indicate a protein epitope containing positively charged lysine and arginine side chains. Binding of both mAbs was unaffected by both proteases in an ELISA format (Table 2), suggesting that neither ED1 or JF5 bind to protein epitopes. However, in a dot-blot assay ED1 and both mouse and humanised versions of JF5 showed significant reductions in binding after treatment with both pronase and trypsin compared with controls (Fig. 2E). Reductions in mAb binding following chemical digestion of an antigen with periodate shows that its epitope is carbohydrate and contains vicinal hydroxyl groups. The binding of both antibodies was unaffected by periodate (Figs. 2C and 2D). Taken together, these results, and those of the western blots, indicate that both mAbs bind to heat stable, periodate-insensitive carbohydrate epitopes on the mannoprotein antigen.

Table 2 ELISA absorbance values for protease treatment of antigen ± standard error.

	Temp °C	Trypsin	H_2O	Pronase	PBS
JF5	4°C	1.066 ± 0.016	1.102 ± 0.011	1.099 ± 0.014	1.108 ± 0.017
	37°C	1.133 ± 0.017	1.160 ± 0.023	1.106 ± 0.010	1.134 ± 0.020
ED1	4°C	0.882 ± 0.009	0.934 ± 0.015	1.081 ± 0.017	1.044 ± 0.020
	37°C	0.995 ± 0.018	1.006 ± 0.013	1.054 ± 0.025	1.044 ± 0.022

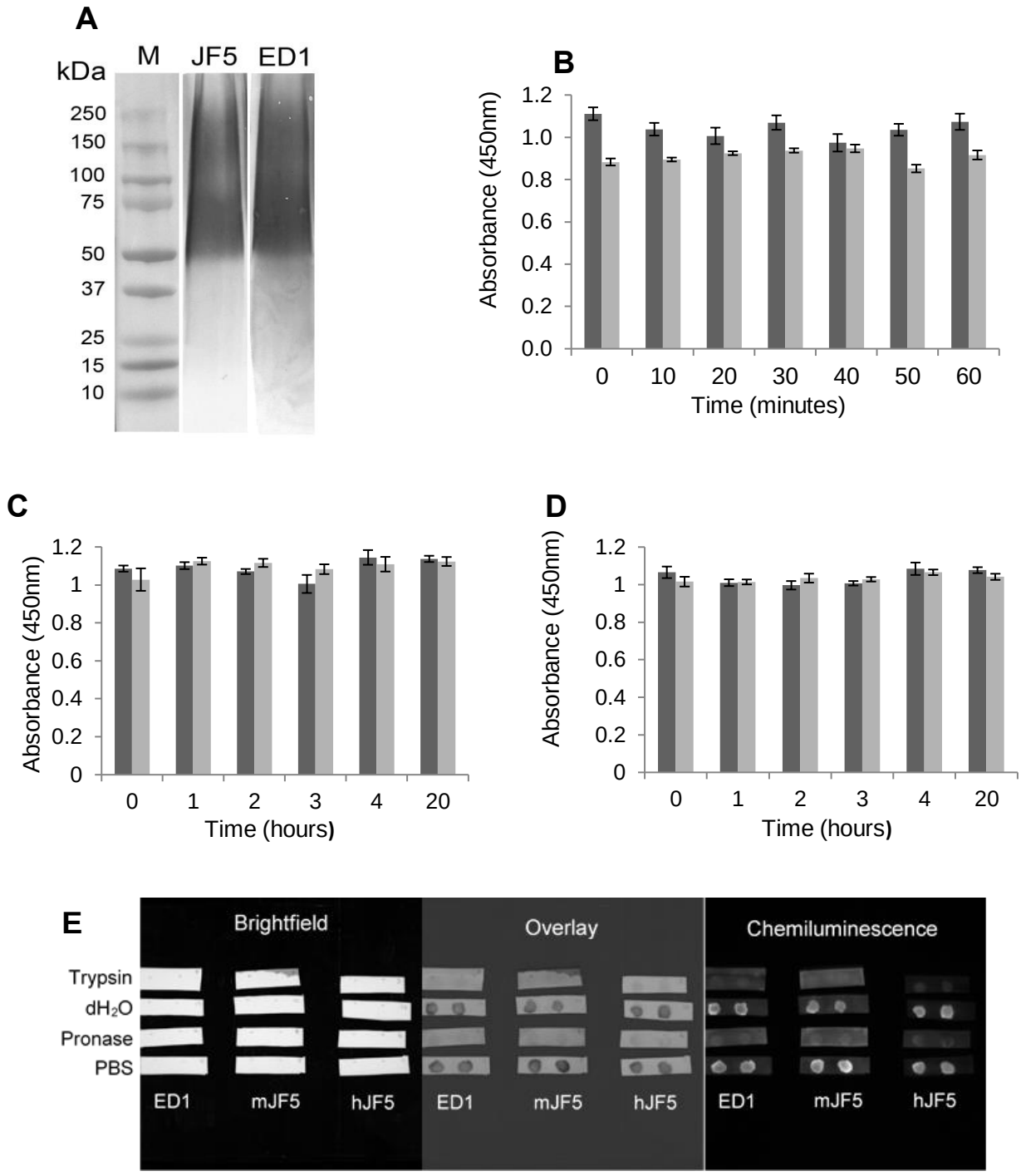

Figure 2 Characterisation of epitope by heat, enzymatically and chemically.
(A) Western immunoblot with mAbs JF5 (lane 2) and ED1 (lane 3) against purified A. fumigatus mannoprotein at 0.5µg/ml under denaturing conditions. Wells were loaded with 40µl sample, and both mAbs show the characteristic glycoprotein smear. Mr (lane1) is broad range molecular weight markers. (B) ELISA absorbance values at 450nm to test for stability of the purified A. fumigatus antigen when boiled at 100ºC for up to 60 minutes. Samples were then immobilised immediately to a microtitre plate and then tested against mAbs JF5 (dark grey) and ED1 (light grey), which show no significant difference over time. (C,D) ELISA absorbance values at 450nm for purified Aspergillus fumigatus antigen immobilised to wells and treated with periodate (dark grey) or acetate only (light grey) at 4ºC over a 20hour period. The wells were then assayed with the mAbs JF5 (C) and ED1 (D) showing no significant different. All bars represent the mean value of three repeats ± standard error. Significance tested by ANOVA and Tukey-Kramer post-hoc test. (E) *Aspergillus* antigen dotted onto PDVF membrane was treated with Trypsin, Pronase or respective controls and then tested for recognition by mAbs ED1, mouse JF5 (mJF5) or humanised form (hJF5). Recognition was visualised using chemiluminescence.

Competition for antigen binding of mAbs with carbohydrates

Monoclonal antibodies ED1 and JF5 were assayed by competition ELISA (C-ELISA) to determine whether the sugars D-galactose and D-mannose at 400mM blocked their binding to the *Aspergillus* mannoprotein antigen (Fig. 3A). Both sugars reduced JF5 binding to the immobilised antigen in the C-ELISA, when compared to the negative control (PBST diluent only). However, the reduction was more pronounced with D-galactose. The *Aspergillus* mannoprotein antigen completely eliminated binding. In contrast, neither sugar had a significant effect on binding of ED1 to the antigen, but its binding was significantly reduced by the mannoprotein antigen.

Competition for antigen binding between mAbs

A competition ELISA (C-ELISA) was conducted with mAbs ED1, hJF5 and HG12 and PBST only to determine whether ED1 and JF5 share similar epitopes. The antibodies were used to compete with mouse JF5 directly conjugated to the enzyme horseradish peroxidase (JF5-HRP). HG12 is a mouse IgG1 mAb specific to the unrelated human pathogenic fungus *Scedosporium* which, along with PBST, acted as a negative control in the experiments. Antibody hJF5 is a fully humanised version of mouse JF5 and has been shown previously to have a higher affinity for the target mannoprotein antigen than mouse JF5. It therefore acted as a positive control in the C-ELISA. Results of the C-ELISA (Fig. 3B) show that mAb ED1 does not significantly reduce binding of the JF5-HRP conjugate in ELISA with absorbance values across conjugate dilution similar to those with HG12 and hJF5. However, ELISA absorbance values were significantly reduced by the higher affinity antibody hJF5. These results show that mAb ED1 and JF5, which bind to the same mannoprotein antigen, either do not share the same epitope, or that sufficient numbers of epitopes exist to prevent saturation of antibody binding.

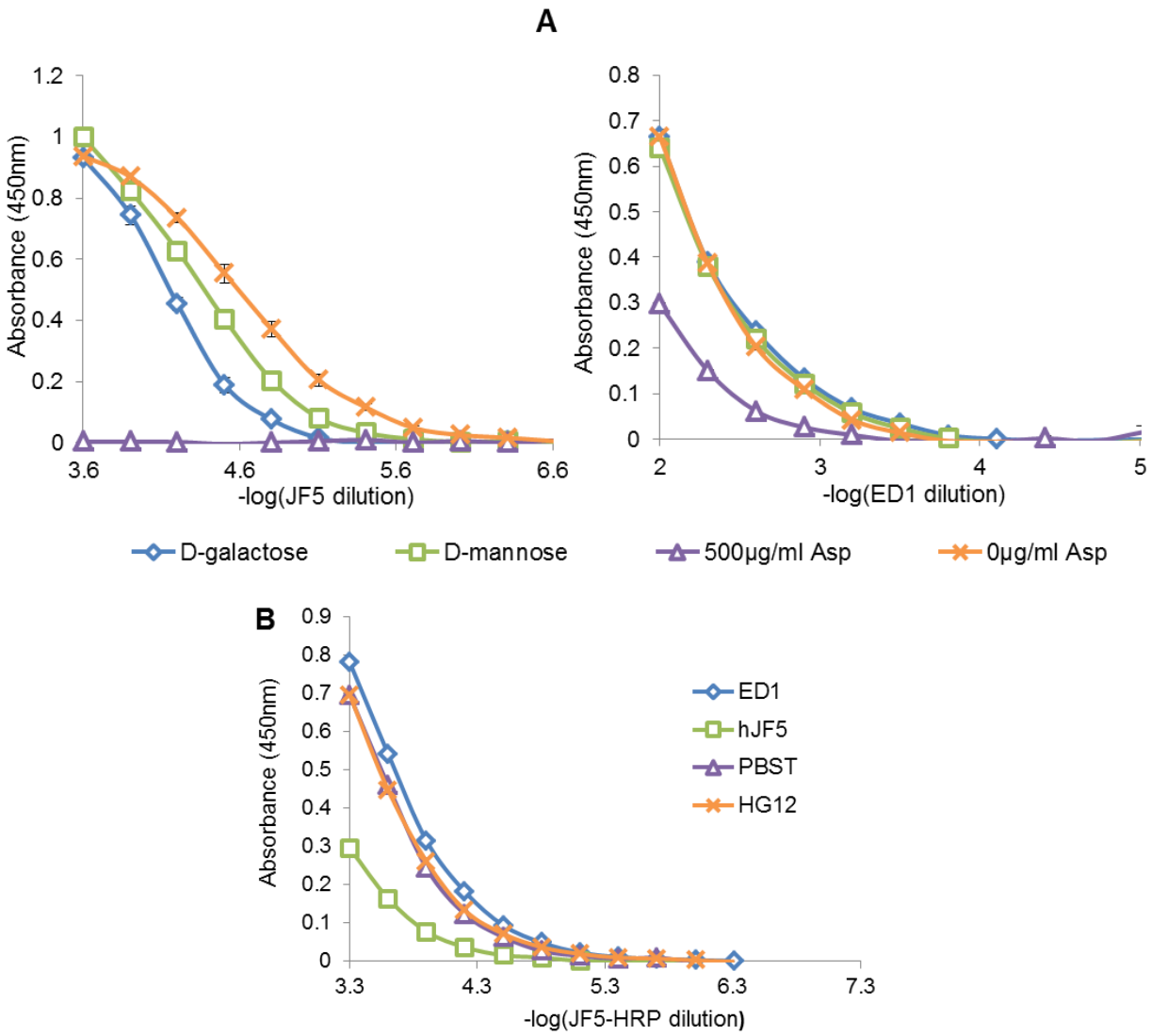

Figure 3 Characterisation of epitope by competition ELISAs for antigen binding. (A) ELISA absorbance values at 450nm of competition for Aspergillus mannoprotein antigen immobilised in microtitre wells. Purified JF5 and ED1 were tested for blocking of binding to the antigen with 400mM D-mannose and D-galactose at starting solutions of 0.2µg/ml and 10µg/ml respectively. Aspergillus antigen at 500µg/ml was used as a positive and PBST diluent as a negative. (B) ELISA absorbance values at 450nm of blocking of binding to the immobilised Aspergillus antigen of the purified JF5-HRP conjugate at a starting concentration of 0.5µg antibody protein/ml by protein A purified mAbs ED1, hJF5 and HG12 (a *Scedosporium* specific IgG1) solutions at 10µg/ml. Bars represent the mean value of three repeats ± standard error.

Characterisation of epitope by using *Aspergillus fumigatus* mutants

Two mutants - Δ*glfA*::*hph*4.4 and Δ*glfA*::*hph*7.4 – that are both deficient in UDP-galactopyranose mutase, a key enzyme involved in the biosynthesis of galactofuranose containing glycoconjugates in *A. fumigatus*, and the wild-type *A. fumigatus* strain AF293, were grown for 4 days in liquid cultures to allow for production of extracellular mannoprotein. The culture supernatants containing the extracellular antigens were then tested by PTA-ELISA for antigen recognition by mAb ED1 (Fig. 4A). Neither 4.4 or 7.4 supernatants were recognised and bound

by ED1, as can be seen in the baseline absorbance values across the 4 days. In comparison, the WT strain's supernatant containing the produced mannoprotein was recognised by ED1, with the signal peaking at Day 3. Using the same culture fluids, a western blot was performed that showed results similar to the ELISA, confirming lack of binding of ED1 to the two enzyme-deficient mutants (Fig. 4B). Immunofluorescence studies using germinated conidia of the mutant and wild-type strains, showed lack of binding of ED1 to the outer cell wall of the mutant cells (Fig. 4C-F) compared with the WT (4G,H,K). It is interesting to note that ED1 binds only to the germinated hyphae and not to the spore itself, as can be seen in (4K) through the lack of fluorescence around the spore. Taken together, these results indicate that ED1 binds to the antigenic determinant galactofuranose (Gal*f*) present in the diagnostic *Aspergillus* mannoprotein antigen, and that this is only produced by the germinated hyphae of the *Aspergillus* WT.

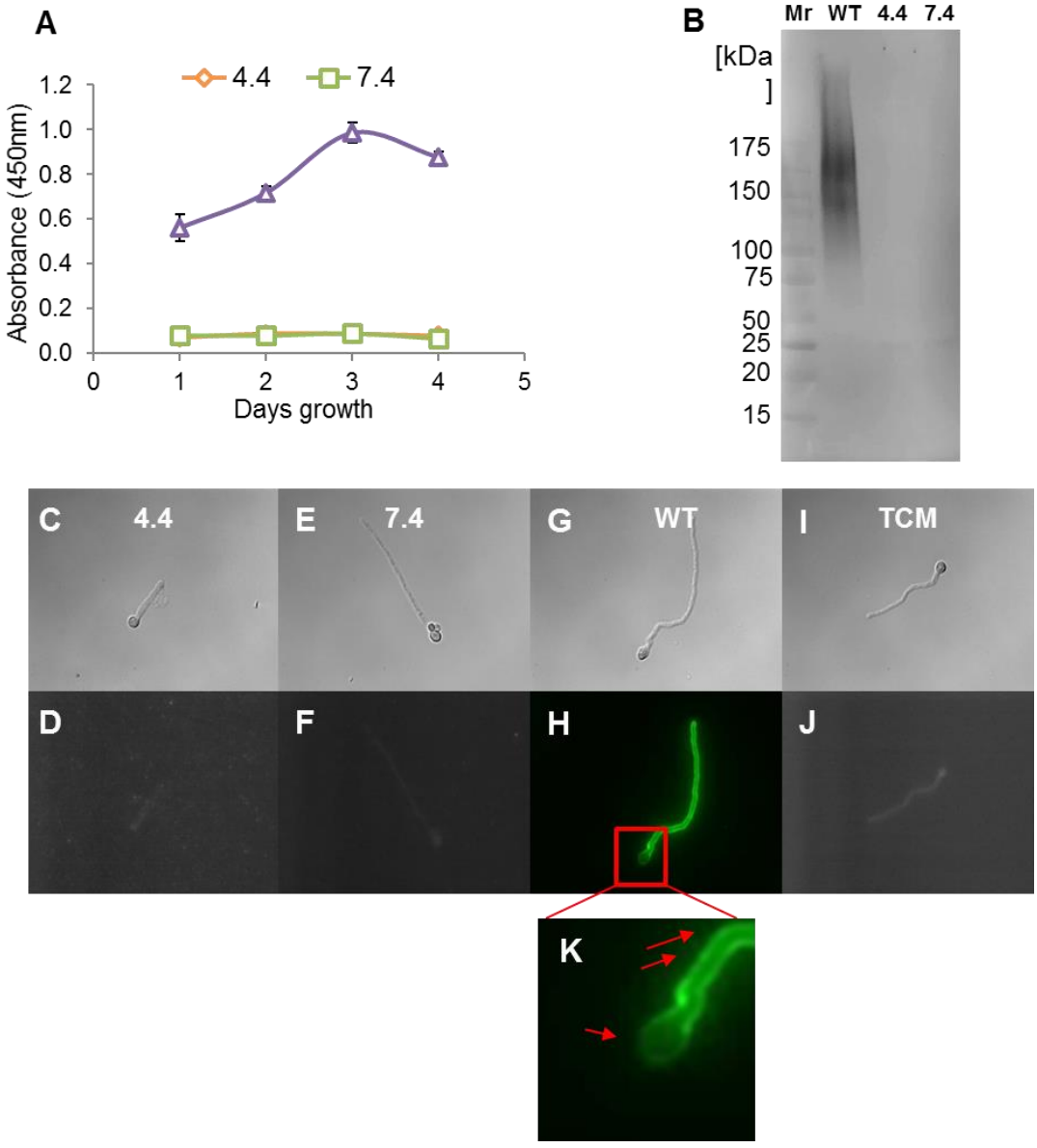

Figure 4 Characterisation of antigen using Aspergillus mutants. Previously generated mutants in UDP-galactopyranose mutase (4.4 and 7.4) and WT strains of Aspergillus fumigatus were tested by ELISA (A), western blot (B) and immunofluorescence (C-K). (A) ELISA absorbance values show a significant difference between the mutant strains and WT. Bars represent the mean value of three repeats ± standard error. (B) Western blot of WT (lane 2), 4.4 (lane 3) and 7.4 (lane 4) strains show the mutants have no protein bands, whereas WT has the characteristic protein smear. (C-J) Photomicrographs of A. fumigatus germinated spore strains 4.4 (C,D), 7.4 (E, F) and WT (G, H) immunostained with ED1 (C-H) or TCM as a control (I,J). (C, E, G and I) Brightfield views of germinated Aspergillus spores. (D, F, H and J) Same field of view but under epifluorescence. (K) is a zoomed in view of the WT spore (1 arrow) and part of the germinated hyphae (2 arrows) highlighting the difference in fluorescence.

Guinea Pig serum and BAL sampling

Monoclonal antibody ED1 was used in combination with JF5 in a DAS-ELISA format to determine whether the two antibodies were compatible for diagnostic antigen detection in serum and BAL samples from a guinea pig model of invasive pulmonary aspergillosis (Wiederhold *et al.* 2009; Wiederhold *et al.* 2013). The samples used had previously been characterised for fungal β-D-glucan and *Aspergillus* galactomannan by using commercial Fungitell and Platelia tests respectively, and also with the *Aspergillus* lateral-flow device (Wiederhold et al., 2008 and 2013). The accuracy of the ED1-JF5 DAS-ELISA was first shown to be able to detect the target antigen by spiking human serum with purified A. *fumigatus* mannoprotein antigen (Thornton, 2008a) at a concentration of 1mg/ml. The DAS-ELISA showed strong recognition of the antigen with a mean absorbance of 1.0495± 0.0208, compared to 0.0120±0.002 for un-spiked control serum. In tests of guinea pig serum and BAL samples there was good concordance between the DAS-ELISA and the three other tests (Table 3). The results show that ED1 and JF5, when used in combination, can be used to accurately detect the diagnostic antigen present in serum and in BAL samples and therefore in detecting IPA in this animal model of infection. As has been reported previously (Wiederhold *et al.* 2013), the β-glucan, GM and LFD assays gave false positive test results (likely due to contamination), with serum samples from three of the Day 5 and Day 7 control animals (samples 32, 44 and 45). The DAS-ELISA was also positive with these 3 samples.

Table 3. Results of guinea pig serum tests using the ED1-JF5 DAS-ELISA, and comparison with β-glucan (BG), galactomannan (GM) and Lateral Flow Device (LFD) tests. The threshold absorbance values for GM and DAS-ELISA test positivities are 0.225 and 0.5 respectively. The threshold value for β-glucan test positivity is 0.5. The LFD test results are recorded as negative (-), weakly positive (+), moderately positive (++) and strongly positive (+++).

GROUP ID	DAS-ELISA (≥0.225)	BG (>80pg/ml)	GM (>0.5)	LFD
1 hour infected				
1	0.2225	0.00	0.32	-
2	0.2315	0.00	0.27	-
3	0.2321		0.19	-
1 hour uninfected				
4	0.2675	28.01	0.19	-
5	0.2125		0.14	-
6	0.2570		0.44	-
Day 3 infected				
7	0.2065	0.00	0.22	-
8	0.2172	6.74	0.60	+
9	0.1990	8.57	0.54	-
10	0.3449	139.97	7.43	++
11	0.2013	0.00	0.54	-
12	0.1779	0.00	0.57	-
13	0.2414	0.00	0.47	-
14	0.1892	0.00	0.42	-
15	0.1999	0.00	0.23	-
16	0.1634	91.43	0.33	-
Day 3 uninfected				
17	0.1901	0.00	0.19	-
18	0.2066	0.00	0.11	-
19	0.2342	18.90	4.18	-
Day 5 infected				
20	0.2583	1.59	1.33	+
21	0.2070	0.00	0.11	-
22	0.2286	22.17	3.61	+
23	0.4157	185.03	8.44	++
24	0.2101	11.11	1.80	+
25	0.5886	0.00	0.29	-
26	0.2014	0.00	0.46	-
27	0.7010	730.55	8.44	+++
28	1.0239	1039.58	8.44	+++
29	0.7549	198.09	8.44	+++
Day 5 uninfected				
30	0.2011	0.00	0.29	-
31	0.1895	2.08	0.19	-
32	0.2697	40.68	7.16	++
Day 7 infected				
33	0.5238	126.85	8.44	++
34	0.1920	0.00	0.25	-
35	0.8276	1025.22	8.44	+++
36	0.5227	131.97	8.44	++
37	1.0518	1059.65	8.44	+++
38	0.9169	1066.91	8.44	+++
39	0.9050	1255.13	8.44	+++
40	0.2266	0.41	0.41	-
41	0.8556	816.68	8.44	+++
42	0.2318	23.36	1.00	+
Day 7 uninfected				
43	0.5321	0.00	2.43	-
44	1.1215	1190.51	8.44	+++
45	0.8456	769.17	8.44	+++

Table 4. Results of guinea pig BAL tests using the ED1-JF5 DAS-ELISA, and comparison with β-glucan (BG), galactomannan (GM) and Lateral Flow Device (LFD) tests. The threshold absorbance values for GM and DAS-ELISA test positivities are 0.225 and 0.5 respectively. The threshold value for β-glucan test positivity is 0.5. The LFD test results are recorded as negative (-), weakly positive (+), moderately positive (++) and strongly positive (+++).

GROUP ID	ELISA	BG (>80pg/ml)	GM (>0.5)	LFD
1 hour infected				
1	0.1447	143.2777	0.36	-
2	0.1449	90.53	0.43	-
3	0.1487		0.22	-
1 hour uninfected				
4	0.1560	0.00	0.20	-
5	0.2777		1.51	-
6	0.1426		0.21	-
Day 3 infected				
7	0.2699	1073.05	2.96	+
8	0.4283	1116.08	5.07	++
9	0.3078	810.69	2.64	+
10	0.4708	1244.13	6.30	+
11	0.4208	1105.50	3.03	+
12	0.4989	860.46	4.66	++
13	0.2082	639.43	1.76	+
14	0.3196	715.53	3.87	+
15	0.2266	427.53	1.42	+
16	0.2925	933.49	1.96	+
Day 3 uninfected				
17	0.1471	42.81	0.19	-
18	0.1580	25.12	0.24	-
19	0.1455	10.30	0.25	-
Day 5 infected				
20	0.4105	1240.95	4.12	++
21	0.2251	1091.37	1.79	+
22	0.5247	1272.48	6.61	++
23	0.5488	1340.97	7.18	++
24	0.6884	1343.79	7.46	++
25	0.3528	1043.25	4.63	++
26	0.4484	1009.25	7.81	++
27	1.0874	1319.56	8.44	+++
28	0.9874	1304.49	8.17	++
29	0.5694	1243.12	7.08	++
Day 5 uninfected				
30	0.1587	45.75	0.38	-
31	0.1427	550.55	0.25	-
32	0.1406	48.46	0.23	-
Day 7 infected				
33	0.9677	1320.55	8.44	++
34	0.5201	1279.74	6.82	++
35	0.7154	1279.02	8.25	++
36	0.3709	1309.42	5.16	+
37	0.6556	1326.05	8.41	++
38	0.7392	1357.50	8.15	++
39	1.1592	1363.26	8.44	+++
40	0.8758	1336.27	8.43	+++
41	1.2670	1275.66	8.44	+++
42	0.4698	1209.52	6.39	+
Day 7 uninfected				
43	0.1668	69.36	0.20	-
44	0.3174	60.33	2.40	-
45	0.2511	91.49	0.55	-

Discussion

In this report, I describe the characterisation of an IgG1 monoclonal antibody ED1, a mAb with similar binding characteristics to the IgG3 mAb JF5 used in the *Aspergillus* lateral-flow device (LFD) (Thornton, 2008a), and show that it similarly recognises the galactofuranose (Gal*f*) epitope present in the diagnostic *Aspergillus* mannoprotein antigen. Although both mAbs appear to recognise the same epitope on the *Aspergillus* antigen, I have shown that ED1 and JF5 can be used in combination, in a double-antibody-sandwich ELISA (DAS-ELISA) format, to successfully detect invasive pulmonary aspergillosis in a guinea pig infection model when used with serum samples. This demonstrates the utility of mAb ED1 as a diagnostic antibody for disease detection.

Specificity tests show that mAb ED1 reacts strongly with antigens from species in the genus *Aspergillus* and also with the closely related fungus *Neosartorya fischeri* var. *fischeri*, the teleomorph or sexual stage of *Aspergillus fischeri*. The exception is *Aspergillus wentii*, a species which JF5 has been previously shown to also not recognise (Thornton 2008a). These results suggest that *A. wentii* lacks the Gal*f* epitope which both mAbs recognise and that the JF5-ED1 DAS-ELISA would not be able to detect an infection by *A. wentii*. Currently this is not a major concern since 90% of invasive aspergillosis infections are caused by *A. fumigatus* (Thompson and Patterson 2008). Nevertheless, *A. wentii* is emerging as an opportunistic pathogen of immunocompromised patients (Halsey *et al.* 2011), and so if the DAS-ELISA were to be used in the clinical setting, lack of *A. wentii* detection would need to be noted.

ED1 was shown to cross-react with *Penicillium cyclopium* as has been reported before for JF5 (Thornton 2008a), but did not recognise *P. islandicum*. This is unsurprising, since a number of studies have shown that mAbs generated against species within the genus *Aspergillus* also cross-react with certain members of the *Penicillium* genus due to similarities between epitopes (Schmechel *et al.* 2005). These studies have also shown that *Aspergillus* mAbs do not recognise members of the *Penicillium* sub-genus *Biverticillium*, of which *P. islandicum* belongs. Further extensive studies of ED1 reactivities with members of the *Penicillium* genus should be undertaken, in line with the cross-reactivity studies conducted previously with mAb JF5 (Thornton 2008a). However, cross-reactivity of ED1 with *Penicillium* species is unlikely to be of concern, since *Penicillium* species are not

indicated in invasive diseases of humans (Walsh and Groll 1999, Lyratzopoulos *et al.* 2002).

Unlike the anti-galactomannan (GM) rat mAb, EB-A2, used in the Bio-Rad Platelia DAS-ELISA test for human IPA detection, ED1 has been shown to not cross-react with several clinically relevant yeasts and moulds including *Cryptococcus*, *Fusarium, Geotrichum* and *Candida* species (Tortorano *et al.* 2012, Giacchino *et al.* 2006, Kappe and Schulze-Berge 1993), which have been identified as possible causes of false-positive results with the commercial GM DAS-ELISA. Therefore, both ED1, and JF5 (Thornton 2008a), have been shown to have greater specificity than EB-A2 and, when used in combination in a DAS-ELISA, could provide improved assay specificity, by reducing false positive detection of non-target pathogens.

The antigen characterisation work conducted here shows that the epitope bound by ED1 is insensitive to heat treatment and periodate oxidation thus indicating that the epitope is a heat-stable carbohydrate moiety that lacks vicinal hydroxyl groups. However, there are contrasting results shown here on the effect proteolytic treatment has on the binding of ED1 to the *Aspergillus* antigen. In the format of an ELISA there appears to be no effect on binding after treatment with both pronase or trypsin, however the same assay in a dot blot form shows significant reduction in binding of the antigen by ED1 and also of binding by both mouse and human forms of JF5. It is possible that the difference seen in mAb binding to the antigen after protease treatment between the ELISA and dot blot formats may be due to how the epitope is presented. Immobilisation to different surfaces, for example plastic microtitre well versus PVDF membrane, may cause the globular antigen to display the epitope in different ways, thus potentially shielding it from treatment with proteases. Previous ELISA studies with JF5 support the findings of the dot blot (Thornton, 2008a), with the JF5 epitope shown previously to be present on *N*-linked mannoprotein antigens within the *Aspergillus* hyphal cell wall, and which are secreted extracellularly during active growth of the fungus (Thornton 2008a).

Work undertaken here using *A. fumigatus* mutants deficient in the enzyme UDP-galactopyranose mutase, a key enzyme involved in the biosynthesis of galactofuranose containing glycoconjugates in *A. fumigatus* (Komachi *et al*. 2013;

Latgé 2009; Schmalhorst *et al*. 2008), has shown that ED1 recognises the epitope β(1,5)-galactofuranose (Gal*f*), the epitope that JF5 has previously been shown to bind to (Thornton, 2008a). The WT immunofluorescence studies show that the galactofuranose recognised by ED1 and JF5 (unpublished data not shown here) is produced only by the germinated hyphae and not by the spore itself. This is unsurprising as Gal*f* production is associated with active growth, and has previously been shown to associate with the hyphal apex (Thornton, 2008).

The epitope Gal*f* is, as discussed earlier, present in GM, an abundant component of the *A. fumigatus* cell wall (Bernard and Latgé 2001), with structural studies showing one component be composed of β1,5-galactofuranose (Gal*f*) side chains, branching from a core mannan structure (Latgé et al. 1994). It is to these external Gal*f* chains that mAbs ED1 and JF5, and also EB-A2, appear to bind. In addition, EB-A2 has been shown, under certain conditions, to recognise Gal*f*(1,2)Man (Latgé 2009). It is unclear at present whether ED1 and JF5 also bind to this structure, and further work is needed to elucidate this. The lack of recognition by either mAb to *Fusarium* species, which also contain galactofuran molecules, indicates that neither JF5 or ED1 recognise internal Gal*f* molecules, as galactofuranosides are not located at terminal positions within this species (Miyazaki and Naoi 1975).

The Gal*f* motif is ubiquitous in nature, and is released during tissue invasion and growth of *A. fumigatus* (Latgé 2009) and other fungal pathogens of humans including *Histoplasma* and *Cryptococcus* (Barr *et al.* 1984, Vaishnav *et al.* 1998). Here we show that galactofuranose is secreted during active growth of hyphae, as WT ELISA values show a peak in absorbance at day 3 which coincided with quiescence of the fungus thereafter. Biosynthesis of Gal*f* is catalysed by UDP-galactopyranose mutase (UGM), a highly conserved enzyme in lower eukaryotes but which is not present in mammals, making it an attractive anti-fungal drug target (Pederson and Turco 2003). In addition, the absence of the epitope Gal*f* in human sugar molecules makes it an ideal candidate for detection of *Aspergillus* lung infections using *in vitro* diagnostic tests and molecular imaging *in vivo*. Recently, Rolle and co-workers (2016) used mAb JF5 in immuno-positron emission tomography magnetic resonance imaging (immuno-PET/MRI) to visualise lung infections by *A. fumigatus in vivo*. The imaging procedure is highly specific, allowing repeated imaging of *A. fumigatus*

lung infections and differentiation of IPA from pulmonary inflammation and from infections caused by bacteria.

As shown here, mutation of the enzyme UGM eliminates Gal*f* production demonstrated by loss of recognition by ED1 and JF5 of the extracellular and cell-wall-bound antigens in ELISA, western blotting and immunofluorescence studies. However, this loss in *A. fumigatus* is non-lethal, unlike in *Mycobacterium smegmatis* (Pan *et al.* 2001), due to the position of the moiety at the terminal ends of Gal*f*-containing molecules in the fungus. Studies have shown that UGM, while non-essential, is required for normal cell wall structure/function, since deletion of the UGM-encoding gene in *Aspergillus nidulans* causes aberrant hyphal morphology (El-Ganiny *et al.* 2008) and, in *A. fumigatus*, causes thinner cell walls, increased susceptibility to drugs, and attenuated virulence (Schmalhorst *et al.* 2008). The importance of Gal*f* as a target epitope for diagnosis of IPA and its function in the *Aspergillus* cell wall, warrants further investigation, furthering our understanding of *A. fumigatus* cell wall recognition by antibodies and providing opportunities for novel treatments for IPA.

The JF5-ED1 DAS-ELISA developed here was accurate in diagnosing IPA in a blind study of serum and BAL samples derived from a guinea pig model of the disease Wiederhold *et al*. 2009; Wiederhold *et al.* 2013). There was good concordance between the DAS-ELISA test results and those obtained using commercial GM and β-D-glucan tests and the *Aspergillus* LFD. That all four of the assays gave positive results for three of the control sera in the sera studies, suggest that these samples were contaminated. False positives due to cross-reactivity with other species of fungi (Tortorano *et al.* 2012), antibiotics (Boonsarngsuk *et al.* 2010) or food (Ansorg *et al.* 1997) are not unusual for the GM and β-glucan tests, but interference from these are unlikely in this case since the guinea infection model was conducted under controlled, sterile conditions. While the DAS-ELISA test results show good promise, further extensive testing of human serum samples is needed to establish the clinical accuracy (specificity and sensitivity) of the test for diagnosing IPA in humans. Such tests should conform with EORTC diagnostic criteria for invasive fungal disease detection (De Pauw *et al.* 2008).

Conclusions and Future Work

The murine IgG1 monoclonal antibody ED1 characterised in this study was shown to bind to the antigenic determinant β1,5-galactofuranose (Gal*f)* present in *A. fumigatus* galactomannan. Despite also recognising the same epitope as JF5, the IgG3 mAb used in the *Aspergillus* lateral flow device, I have shown that ED1 can successfully be incorporated into a double-antibody-sandwich ELISA for diagnostic biomarker detection in serum. Specificity tests show that ED1 is highly specific, recognising clinically important *Aspergillus* species including *A. fumigatu*s, the main cause of invasive pulmonary aspergillosis in humans. It was shown to not react with a number of unrelated, clinically-relevant, moulds, yeasts and yeast-like fungi including *Fusarium* and *Cryptococcus*, which cross-react with the commercial Platelia GM EIA, the *in vitro* diagnostic test currently used for human disease detection. Results presented here suggest that ED1 and JF5, when used in combination, might enable the development of a DAS-ELISA with improved diagnostic accuracy than currently available methods. Extensive testing of the ED1-JF5 DAS-ELISA with human serum and BALf samples is needed to establish the clinical validity of the assay for diagnosing IPA in immunocompromised patients.

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