



Research review paper

Aspergillus-specific antibodies – Targets and applicationsMax Schubert^a, Holger Spiegel^a, Stefan Schillberg^{a,b,*}, Greta Nölke^a^a Fraunhofer Institute for Molecular Biology and Applied Ecology IME, Forckenbeckstrasse 6, 52074 Aachen, Germany^b Justus-Liebig University Giessen, Institute for Phytopathology and Applied Zoology, Phytopathology Department, Heinrich-Buff-Ring 26-32, 35392 Giessen, Germany

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ABSTRACT

Aspergillus is a fungal genus comprising several hundred species, many of which can damage the health of plants, animals and humans by direct infection and/or due to the production of toxic secondary metabolites known as mycotoxins. *Aspergillus*-specific antibodies have been generated against polypeptides, polysaccharides and secondary metabolites found in the cell wall or secretions, and these can be used to detect and monitor infections or to quantify mycotoxin contamination in food and feed. However, most *Aspergillus*-specific antibodies are generated against heterogeneous antigen preparations and the specific target remains unknown. Target identification is important because this can help to characterize fungal morphology, confirm host penetration by opportunistic pathogens, detect specific disease-related biomarkers, identify new candidate targets for antifungal drug design, and qualify antibodies for diagnostic and therapeutic applications. In this review, we discuss how antibodies are raised against heterogeneous *Aspergillus* antigen preparations and how they can be characterized, focusing on strategies to identify their specific antigens and epitopes. We also discuss the therapeutic, diagnostic and biotechnological applications of *Aspergillus*-specific antibodies.

1. Introduction

The genus *Aspergillus* comprises 339 filamentous fungi that are known for both their beneficial and harmful characteristics (Samson et al., 2014). Some species (including *A. niger*, *A. sojae* and *A. oryzae*) are used in the food and chemical industries because they produce valuable metabolites and enzymes (Ichishima, 2016; Schuster et al., 2002). Similarly, *A. terreus* produces the polyketide drug lovastatin, which is used to lower cholesterol levels and prevent cardiovascular disease (Hendrickson et al., 1999). In contrast, certain soil-borne *Aspergillus* species are plant pathogens that also produce harmful metabolites known as mycotoxins.

For example, *A. flavus*, *A. parasiticus* and *A. nominus* infect oil-rich staple crops such as maize (*Zea mays*), and produce > 20 highly toxic aflatoxins that damage the liver and immune system and also act as teratogens (Klich, 2007). Despite the strict regulation of aflatoxin levels in food and feed, aflatoxin-producing *Aspergillus* species cause billions of dollars annually due to crop damage, contamination with mycotoxins, and their impact on public health (Mitchell et al., 2016a, 2016b; Otsuki et al., 2001; Rodrigues et al., 2011). Interestingly, some *Aspergillus* species have both positive and negative effects. For example, *Aspergillus* black rot is caused by *A. niger*, which infects a wide range of crops (Gautam et al., 2011), whereas *A. terreus* was recently shown to cause foliar blight disease in potato (*Solanum tuberosum*) (Louis et al., 2014).

A. fumigatus, *A. flavus*, *A. niger*, *A. ustus* and *A. terreus* are opportunistic pathogens in humans, generally causing mild infections but also life-threatening diseases in immunocompromised individuals (Latgé, 1999; Seyedmousavi et al., 2015). Furthermore, *A. versicolor* can trigger asthma in indoor environments (Benndorf et al., 2008). *A. fumigatus*, the most important human pathogen in the *Aspergillus* genus, enters the human body via the respiratory tract and causes allergic bronchopulmonary aspergillosis (ABPA), chronic pulmonary aspergillosis (CPA) and invasive aspergillosis (IA) in immunocompromised patients suffering from cystic fibrosis, infected with the Human immunodeficiency virus (HIV) or recovering from stem cell/organ transplants (Singh and Paterson, 2005). Data from 2015 to 2017 (individual country burden estimates) indicate that the incidence of *Aspergillus*-related diseases per 100,000 people in 29 industrial, transition and developing countries is 0.05–15.99 for IA, 1.38–126.19 for CPA and 26–372 for ABPA (Denning, 2017).

Aspergillus-related diseases differ not only in terms of incidence, progression and clinical profile, but also their specific antibody responses in the human host (Hedayati et al., 2007; Page et al., 2015). ABPA is caused by chronic exposure to aerial conidia, inducing a hypersensitivity reaction resembling asthma. Triggers for ABPA include 23 distinct *A. fumigatus* allergens, most of which are secreted. These have been produced as recombinant proteins in bacteria, and most contain immunodominant peptides that bind human IgE (Bhetariya

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et al., 2011). CPA is accompanied by both IgG and IgE responses (Denning et al., 2006), whereas the principal diagnostic biomarker of IA is the fungal polysaccharide galactomannan. Diagnostic platforms for CPA and ABPA have been developed to detect humoral *Aspergillus*-specific IgG levels (Richardson and Page, 2017) and have been validated in large test groups (Dumollard et al., 2016; Page et al., 2016). These serological platforms are a significant component in the differential diagnosis and management of Aspergillosis (Patterson et al., 2016).

2. Targets and applications of *Aspergillus*-specific antibodies

Numerous *Aspergillus*-specific polyclonal, monoclonal and recombinant antibodies have been generated against cell wall components, secreted antigens (exoantigens) including extracellular polysaccharides and secondary metabolites such as aflatoxins. Whereas polyclonal antisera comprise diverse pools of antibodies recognizing multiple antigens and/or epitopes, monoclonal and recombinant antibodies are unique molecules with defined physical, biochemical and immunological properties, and they detect a specific epitope on a single antigen. *Aspergillus*-specific antibodies have been used for detection and quantification of pathogenic *Aspergillus* spp. and their produced secondary metabolites in plant pathology, human and animal health, and food and feed safety (Fig. 1). In particular, *Aspergillus*-specific antibodies have been used for in situ staining (Choi et al., 2004) and in vivo imaging (Rolle et al., 2016) to detect *Aspergillus* infections. This review focuses on the identification of antigens/epitopes recognized by monoclonal and recombinant antibodies generated using culture-derived complex antigen preparations. Since most of the *Aspergillus*-specific antibodies recognize *exo*- and cell wall antigens this review focusses on these two groups but also information about the small group of antibodies binding to intracellular antigens can be found e.g. in Section 5.1 and in Table 3.

2.1. Exoantigen-specific antibodies

The detection of aflatoxins, the toxic secondary metabolites secreted by certain *Aspergillus* spp., is the most important application of *Aspergillus*-specific antibodies in food and feed safety. In this review, we have summarized the most important monoclonal and recombinant antibodies that have been generated against the main aflatoxins (AflB1, AflB2, AflG1 and AflG2) and the aflatoxin metabolite AflM1 in milk (Table 1). Aflatoxin-specific monoclonal antibodies (mAbs) are used commercially to detect and quantify aflatoxins in agricultural products (Jiang et al., 2013; Thornton, 2010; Wacoo et al., 2014). Additional mAbs have been generated against other *Aspergillus* secondary metabolites, such as ochratoxin A, sterigmatocystin and gliotoxin (Table 1).

The second most important application of *Aspergillus*-specific

antibodies is the detection of galactomannan which is secreted by human pathogenic *Aspergillus* spp. Because galactomannan/galactofuranose (Galf) are the main biomarker for IA, eight Galf-specific mAbs have been generated for the immunological detection of *A. fumigatus* in medical samples (Thornton, 2010) (Table 2).

2.2. Cell wall-specific antibodies

Aspergillus cell wall-specific antibodies (~80%) are generated from crude antigens prepared from *Aspergillus* cultures, whereas ~20% are prepared from recombinant or defined antigens (Tables 2 & 3). However, the precise target antigen has been identified for only ~30% of the generated *Aspergillus*-specific mAbs (Tables 2 & 3). The identification of epitopes recognized by *Aspergillus*-specific antibodies allows the detailed analysis of cell wall composition and dynamic fungal morphology, whereas only limited interpretation is possible when using uncharacterized antibodies. Moreover antibodies to cellular or cell wall antigens facilitate the detection and quantification of infections, whereas epitope mapping can help to determine the mechanism of a biological process (mode of action), the structure of an antigen, or the potential of an antibody for diagnostic or therapeutic use (Ladner, 2007).

Recently, the target of an antibody raised against a heterogeneous *Fusarium* extract (Peschen et al., 2004) which was used for antibody-mediated resistance strategies (Safarnejad et al., 2011; Schillberg et al., 2001) to generate *Fusarium*-resistant wheat (Li et al., 2008a) was identified as the membrane-bound glyoxal oxidase (Song et al., 2015). Target identification may reveal unanticipated weaknesses in pathogens, particularly if the antigens are presented during particular phases of development or are required for key stages of infection such as host penetration. In the context of agriculture, this facilitates the design of new control strategies that can be applied in the field.

When such vulnerable targets are found in human pathogens, the antibody may be valuable in a therapeutic capacity: for example, mAb Ak-14 inhibits the adhesion of *A. fumigatus* conidia to host tissue in vitro (Kumar and Shukla, 2015) and mAb A9 can kill *A. fumigatus* conidia (Chaturvedi et al., 2005). Pathogen neutralization by cell wall-specific antibodies has been reported for *Candida albicans* (Casadevall and Pirofski, 2007) and *Cryptococcus neoformans* (Rachini et al., 2007) but not yet for *Aspergillus* species.

3. Preparation of *Aspergillus* antigens

The principal source of *Aspergillus* antigens to generate specific antibodies is in vitro fungal cultures prepared using liquid or solid media, to generate large amounts of defined starting material. Especially because, the proteome and metabolome of *Aspergillus* spp. are highly dependent on the growth conditions (Imanaka et al., 2010; Meletiadis

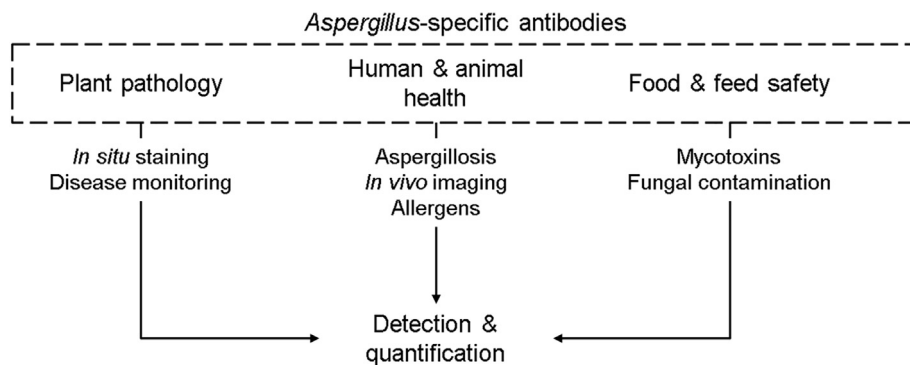


Fig. 1. Applications of *Aspergillus*-specific antibodies.

Aspergillus-specific antibodies are used in plant pathology, human and animal health and food and feed safety to detect fungal structures, *Aspergillus* allergens and toxic secondary metabolites in different fields of application.

Table 1
Monoclonal *Aspergillus*-specific antibodies against secondary metabolites.
Ab = antibody, Af = aflatoxin, Bg = bovine serum albumin, BSA = bovine serum albumin, cRIA = competitive radioimmunoassay, dcELISA = direct competitive ELISA, ELISA = enzyme-linked immunosorbent assay, hTF = human apo transferrin, HSA = human serum albumin, IC₅₀ = half maximal inhibitory concentration, icELISA = indirect competitive ELISA, KLH = keyhole limpet hemocyanin, scFv = single chain fragment variable, LFA = lateral flow assay, NA = not available, OTA = ochratoxin A, STC = sterigmatocystin, SPR = surface plasmon resonance, USERIA = ultrasensitive radioimmunoassay, VHH = nanobody, a = pmol, b = pg per assay.

Antibody	Antibody generation			Antibody characterization					Reference
	Abs	Ab class	Ab source	Host	Antigen species	Immunogen	IC ₅₀ (ng/ml)	Compound	
1-A-2	2	IgG1	Hybridoma	Mouse	<i>Aspergillus</i> spp.	AB1-modified DNA	1 ^a	AB1-DNA	(Haugen et al., 1981)
2B11	1	IgM	Hybridoma	Mouse	<i>Aspergillus</i> spp.	AB1-BGG	3 ^a	AB1, AB2, AfM1	(Groopman et al., 1984)
AMW-1	3	IgG2b	Hybridoma	Mouse	<i>Aspergillus</i> spp.	AM1-BSA	25	dcELISA	(Woychik et al., 1984)
4E1	1	IgG2a	Hybridoma	Mouse	<i>Aspergillus</i> spp.	AB1-BSA	3	dcELISA	(Candlish et al., 1985)
Af2	5	IgG1	Hybridoma	Mouse	<i>Aspergillus</i> spp.	AB1-BSA	20 ^b	icELISA	(Kawamura et al., 1988)
IOE2	1	IgG1	Hybridoma	Mouse	<i>Aspergillus</i> spp.	OTA-BSA	NA	OTA	(Candlish et al., 1988)
575B8F12	6	IgG1	Hybridoma	Mouse	<i>Aspergillus</i> spp.	AB3-BSA	141 ^b	cRIA	(Hefle and Chu, 1990)
3	7	IgG2a	Hybridoma	Rat	<i>Aspergillus</i> spp.	AB1-BSA	NA	icELISA	(Ward et al., 1990)
2F1	2	NA	Hybridoma	Mouse	<i>Aspergillus</i> spp.	AB1-HSA	2.4	ELISA	(Wang et al., 1995)
10D5-1A11	12	IgG1	Hybridoma	Mouse	<i>Aspergillus</i> spp.	AB1-BSA	0.06	icELISA	(Devi et al., 1999)
Afa-2	6	scFv	Phage display	Semi synthetic	<i>Aspergillus</i> spp.	AB1-BSA/AB1	NA	icELISA, SPR	(Moghaddam et al., 2001)
C4	6	scFv	Phage display	Mouse	<i>Aspergillus</i> spp.	AB1-BSA	NA	icELISA, SPR	(Daly et al., 2002)
34	2	NA	Hybridoma	Mouse	<i>Aspergillus</i> spp.	AB1-BSA	0.196	dcELISA	(Kolossova et al., 2006)
10C9	3	IgG1	Hybridoma	Mouse	<i>Aspergillus</i> spp.	AB1-BSA	1.25	icELISA	(Li et al., 2008b)
IC11	5	IgG2a	Hybridoma	Mouse	<i>Aspergillus</i> spp.	AB1-BSA	0.001	icELISA	(Zhang et al., 2009a)
IC8	3	mAbs	Hybridoma	Mouse	<i>Aspergillus</i> spp.	AF1-BSA	13.92	icELISA	(Zhang et al., 2009b)
3B1	1	Ig2a	Hybridoma	Mouse	<i>Aspergillus</i> spp.	AM1-BSA	0.62	icELISA	(Pei et al., 2009)
C3	3	scFv	Phage display	Human naïve library	<i>Aspergillus</i> spp.	AB1-BSA	NA	icELISA	(Pansri et al., 2009)
H4	13	scFv	Phage display	Human library	<i>Aspergillus</i> spp.	AB1-BSA	0.4	ELISA	(Yang et al., 2009)
2C9	1	IgG1	Hybridoma	Mouse	<i>Aspergillus</i> spp.	AM1-BSA	67	icELISA	(Guan et al., 2011)
kj-AFB1	9	IgG1	Hybridoma	Mouse	<i>Aspergillus</i> spp.	AB1-BSA	4.36	dcELISA	(Kim et al., 2011)
2C12	1	mAb	Hybridoma	Mouse	<i>Aspergillus</i> spp.	AB1-BSA	8	dcELISA	(Min et al., 2011)
2C12	1	scFv	Hybridoma 2C12	<i>E. coli</i>	<i>Aspergillus</i> spp.	AB1-BSA	NA	icELISA	(Rangnoi et al., 2011)
YM1-C3	2	scFv	Phage display	Human library	<i>Aspergillus</i> spp.	AB1-BSA	35	icELISA	(Li et al., 2012)
IC11	7	NA	Hybridoma	Mouse	<i>Aspergillus</i> spp.	AB1-BSA	NA	icELISA	(Jiang et al., 2013)
3C10	1	IgG1	Hybridoma	Mouse	<i>Aspergillus</i> spp.	AF1-BSA	0.081	icELISA	(Wang et al., 2013)
VHH 2-5	11	VHH	Phage display	Alpaca library	<i>Aspergillus</i> spp.	Anti-Af mAb 1C11	0.16	icELISA	(Zhang et al., 2013a)
7C6-H1	1	IgG	Hybridoma	Mouse	<i>Aspergillus</i> spp.	AM1-KLH	0.048	icELISA, LFA	(Zhang et al., 2013b)
11A9	1	IgG	Hybridoma	Mouse	<i>Aspergillus</i> spp.	AB1-KLH	0.059	icELISA	(Zhang et al., 2013a)
Nb26	2	VHH	Phage display	Alpaca	<i>Aspergillus</i> spp.	AB1-BSA	0.754	NA	(He et al., 2014)
VerA 3	3	IgG1	Hybridoma	Mouse	<i>Aspergillus</i> spp.	STC-BSA	0.36	icELISA	(Li et al., 2014)
A218	2	IgG2a	Hybridoma	Mouse	<i>Aspergillus</i> spp.	AB1-CMO-BSA	1.53	icELISA	(Soukhanloo et al., 2014)
2C10	4	mAb	Hybridoma	Mouse	<i>Aspergillus</i> spp.	AB1-BSA	9.54	icELISA	(Liu et al., 2015)
scFv-M37	5	scFv	Yeast display	Mouse	<i>Aspergillus</i> spp.	AB1-BSA	ca. 100	dcELISA	(Min et al., 2015)
2D8	2	IgG1	Hybridoma	Mouse	<i>Aspergillus</i> spp.	OTA-BSA	0.38	icELISA	(Zhang et al., 2015b)
D12E2	1	IgA	Hybridoma	Mouse	<i>Aspergillus</i> spp.	AB1-HTF	54.21	icELISA	(Ertekin et al., 2016)
AFM1-9	5	IgG1	Hybridoma	Mouse	<i>Aspergillus</i> spp.	AM1-BSA	0.002	icELISA	(Chadseesuwat et al., 2016)
2G6	3	NA	Hybridoma	Mouse	<i>Aspergillus</i> spp.	AF1-BSA	17.18	icELISA	(Li et al., 2016)
10F3C10	1	IgG1	Hybridoma	Mouse	<i>Aspergillus</i> spp.	AM1-BSA	0.022	dcELISA, LFA	(Liu et al., 2016a)
3F6G11	2	IgG1	Hybridoma	Mouse	<i>Aspergillus</i> spp.	AB1-BSA	0.051	icELISA	(Liu et al., 2016b)
3D8	4	IgG1	Hybridoma	Mouse	<i>Aspergillus</i> spp.	AM1-KLH	64.75	icELISA	(Peng et al., 2016)
1D3	2	IgG2a	Hybridoma	Mouse	<i>Aspergillus</i> spp.	AM1-KLH	70 ^a	icELISA	(Zhang et al., 2016)
5H3	2	NA	Hybridoma	Mouse	<i>Aspergillus</i> spp.	AB1-KLH	0.04	dcELISA	(Zhang et al., 2017)

Table 2
 Monoclonal *Aspergillus*-specific antibodies against carbohydrates.
 Ab = antibody, ELISA = enzyme linked immunosorbent assay, CWF = cell wall fragments, GalF = galactosaminogalactan, GM-1 = synthetic pentasaccharide galactomannan, ID = immunodiffusion, IFA = immunofluorescence analysis, LAA = latex agglutination assay, LFA = lateral flow assay, NA = not available, SPR = surface plasmon resonance spectroscopy.

Antibody		Antibody generation			Antibody characterization				Reference		
Name	Abs	Ab class	Ab source	Host	Antigen species	Immunogen	Immunoblot (kDa)	Antigen localization	Immunoassay	Epitope/antigen	Reference
EB-A2	7	IgM	Hybridoma	Rat	<i>A. flavus</i>	Inactivated conidia	41–90	Hyphae and exoantigen	ELISA, LAA	Galf	(Stynen et al., 1992; Van Cutsem et al., 1990)
1	2	IgM	Hybridoma	Mouse	<i>A. fumigatus</i>	Alkali-soluble proteins	49, 62, 80	Hyphae	ELISA, ID	Carbohydrate (unknown)	(Ste-Marie et al., 1990)
40		IgM	Hybridoma	Mouse	<i>A. fumigatus</i>	Alkali-soluble proteins	–	Hyphae	ELISA, ID	Carbohydrate (unknown)	
AF-BJE6	1	NA	Hybridoma	Mouse	<i>A. flavus</i>	Soluble fraction	NA	NA	ELISA	Carbohydrate epitope of catalase	(Dewey et al., 1993)
AF-CG8	6	IgM	Hybridoma	Mouse	<i>A. flavus</i>	Soluble fraction	NA	Hyphae	ELISA, IFA	Carbohydrate (unknown)	(Gurr et al., 1997)
AF-CA2	2	NA	Hybridoma	Mouse	<i>A. flavus</i>	Soluble fraction	NA	Hyphae	ELISA	Carbohydrate (unknown)	(Dewey and Meyer, 2004)
L10–1	1	IgM	Hybridoma	Mouse	<i>A. fumigatus</i>	Exoantigens	> 75	Hyphae and exoantigen	ELISA	Galf	(Heesemann et al., 2011; McCormick et al., 2010)
L99–13	1	IgM	Hybridoma	Mouse	<i>A. fumigatus</i>	Exoantigens	> 75	Hyphae and exoantigen	ELISA	Galf	(Heesemann et al., 2011)
anti-GG	1	IgG	Hybridoma	Mouse	<i>A. fumigatus</i>	Whole cell extract	–	Hyphae and exoantigen	ELISA	GAG	(Fontaine et al., 2011)
476	1	IgM	Hybridoma	Mouse	<i>A. fumigatus</i>	Inactivated germ tubes	NA	Exoantigen	ELISA	Galf	(Dufresne et al., 2012)
AK-14	1	IgM	Hybridoma	Mouse	<i>A. fumigatus</i>	Exoantigens	> 43	Hyphae and exoantigen	ELISA	Carbohydrate (unknown)	(Kumar and Shukla, 2015)
AB135–8	1	IgM	Hybridoma	Mouse	<i>A. fumigatus</i>	Inactivated germ tubes	NA	Hyphae and exoantigen	ELISA	Galf	(Wiedemann et al., 2016)
7B8	9	IgG	Hybridoma	Mouse	synthetic	GM-1-BSA	–	Hyphae and exoantigen	ELISA, SPR	Galf trisaccharide	(Matveev et al., 2018)
8G4		IgG	Hybridoma	Mouse	synthetic	GM-1-BSA	–	Hyphae and exoantigen	ELISA, SPR	Galf pentasaccharide	
AP3	8	IgG1	Hybridoma	Mouse	<i>A. parasiticus</i>	CWF	> 35	Hyphae and exoantigen	ELISA, LFA	Galf	(Schubert et al., unpublished; Rettcher, et al., unpublished)

Table 3

Antibody	Antibody generation			Antibody characterization				Reference					
	Name	Abs	Ab class	Ab source	Host	Antigen species	Immunogen		Immunoblot (kDa)	Antigen localization	Immunoassay	Epitope/antigen	
7H10 C-5 F6G5	1	IgG	Hybridoma	Mouse		<i>A. ficum</i>	Phyrase ⁺	85	Exoantigen	Blot	Phyrase	(Ullah and Gibson, 1987)	
	3	NA	Hybridoma	Mouse		<i>A. nidulans</i>	Whole cell extract*	90	Intracellular	Blot, EA	Nitrate reductase	(Downey and South, 1988)	
	6	IgM	Hybridoma	Mouse		<i>A. fumigatus</i>	Exoantigen	12-50	Exoantigen	Blot	Glycoprotein (unknown)	(Kurup, 1989; Reijula et al., 1992)	
	1	IgG	Hybridoma	Mouse		<i>A. fumigatus</i>	Exoantigen*	NA	Exoantigen and hyphae	ELISA	Glycoprotein (unknown)	(Kurup et al., 1991)	
	2-2-9	IgG1	Ascites	Mouse		<i>A. fumigatus</i>	Whole cell extract ⁺	58	Hyphae	IFA	Glycoprotein (unknown)	(Pratamico and Buckley, 1991)	
	4A6	IgG1	Hybridoma	Mouse		<i>A. fumigatus</i>	Soluble fraction	18	Exoantigen	Blot	Mitogillin (<i>Asp f</i> 1)	(Arruda et al., 1992)	
10G7 Asp H10 IL-B8	7	IgG1	Hybridoma	Mouse		<i>A. fumigatus</i>	Soluble fraction	16	Exoantigen	Blot	Protein (unknown)		
	10G7	IgG1	Hybridoma	Mouse		<i>A. fumigatus</i>	Soluble fraction	16	Exoantigen	Blot	Protein (unknown)		
	Asp H10	NA	Hybridoma	Mouse		<i>A. fumigatus</i>	Exoantigen*	NA	NA	NA	Glycoprotein (unknown)	(Kumar et al., 1993)	
	IL-B8	6	NA	Hybridoma	Mouse		<i>A. fumigatus</i>	Exoantigen ⁺	35-37	Exoantigen	ELISA, Blot	<i>Asp f</i> 2	(Kurup et al., 1991; Tang et al., 2000)
	4B10	5	IgG1	Hybridoma	Mouse		<i>A. flavus</i>	Exoantigen	97	Conidia and hyphae	IHC	Glycoprotein (unknown)	(Hetherington et al., 1994)
	6E4	IgG1	Hybridoma	Mouse		<i>A. flavus</i>	Exoantigen	21, 42, 97	Conidia and hyphae	IHC	Glycoprotein (unknown)		
2G6 WF-AF-1 WSSA-AF-4 IIIb4 D6.2.2 NA 10D2 164G	2G6	IgG3	Hybridoma	Mouse		<i>A. flavus</i>	Inactivated conidia	97	Conidia and hyphae	IHC	Glycoprotein (unknown)		
	WF-AF-1	8	IgM	Hybridoma	Mouse		<i>A. fumigatus</i>	CWF	106	Hyphae	IHC, ELISA	Glycoprotein (unknown)	(Jensen et al., 1996)
	WSSA-AF-4	IgM	Hybridoma	Mouse		<i>A. fumigatus</i>	Whole cell extract	35-51	NA	IHC, ELISA	Glycoprotein (unknown)		
	IIIb4	12	IgG1	Hybridoma	Mouse		<i>A. oryzae</i>	Whole cell extract	NA	Exoantigen	ELISA	α-amylase (<i>Asp o</i> 2)	(Sander et al., 1997)
	D6.2.2	5	IgG2a	Hybridoma	Mouse		<i>A. flavus</i>	Whole cell extract	NA	NA	ELISA	Protein (unknown)	(Candler et al., 1997)
	NA	NA	NA	Hybridoma	Mouse		<i>A. fumigatus</i>	<i>Asp f</i> 4 (<i>E. coli</i>)	30	Intracellular	ELISA, Blot	<i>Asp f</i> 4	(Cramer, 1998)
611F 16C4 scFv C10	10D2	12	IgG1	Hybridoma	Mouse		<i>A. parasiticus</i>	Whole cell extract*	43	Intracellular	Blot, EA	Norsolorinic acid reductase	(Lee, 1999)
	164G	2	IgG1	Hybridoma	Mouse		<i>A. fumigatus/A. flavus</i>	Whole cell extract	NA	Hyphae	IHC	Protein (unknown)	(Fenelon et al., 1999)
	611F	IgG3	Hybridoma	Mouse		<i>A. fumigatus/A. flavus</i>	Whole cell extract	NA	Hyphae	IHC	Protein (unknown)		
	16C4	15	IgM	Hybridoma	Mouse		<i>A. fumigatus</i>	CWF	70	Hyphae	ELISA, IFA	<i>Aspergillus</i> catalase 1	(Momany et al., 2004a)
	scFv C10	19	scFv	Phage display	Human library		<i>A. niger</i>	Glucose oxidase (<i>E. coli</i>)	75	NA	ELISA, SPR	Glucose oxidase	(Ascione et al., 2004)
	A9	11	IgG1	Hybridoma	Mouse		<i>A. fumigatus</i>	CWF	> 95	Conidia and hyphae	FACS	Protein (unknown)	(Chaturvedi et al., 2005a)
B-M1-A12 B8-C4 H73 20C5	B-M1-A12	2	IgG1	Hybridoma	Mouse		<i>A. fumigatus</i>	Whole cell extract	23	Intracellular	Blot	MnSD (<i>Asp f</i> 6)	(Schwiebacher et al., 2005a)
	B8-C4	NA	Hybridoma	Mouse		<i>A. fumigatus</i>	Whole cell extract	11	Intracellular	Blot	P2 protein (<i>Asp f</i> 8)		
	H73	1	NA	Hybridoma	Mouse		<i>A. fumigatus</i>	Mitogillin (<i>E. coli</i>)	18	Exoantigen	Blot	Mitogillin (<i>Asp f</i> 1)	(Schwiebacher et al., 2005b)
	20C5	46	IgM	Hybridoma	Mouse		<i>A. versicolor</i>	Intact and homogenized conidia	NA	Hyphae	ELISA	Protein and carbohydrate (unknown)	(Schmechel et al., 2005)
	24A6	1	IgG1	Hybridoma	Mouse		<i>A. fumigatus</i>	Inactivated conidia	33	NA	Blot	Protein (unknown)	(Hoselton et al., 2007)
	EA2	8	IgG1	Hybridoma	Mouse		<i>A. fumigatus</i>	Exoantigen ⁺	25, 50, 75	Conidia and hyphae	ELISA	Mannoproteins (unknown)	(Hao et al., 2008)
19B2 scFvAF1 P2C2 15B5	Con1	2	IgM	Hybridoma	Mouse		<i>A. fumigatus</i>	Inactivated conidia	-	Conidia and hyphae	ELISA	Mannoproteins (unknown)	
	MA6	7	IgG1	Hybridoma	Mouse		<i>A. fumigatus</i>	CWF	25, 50, 75	Conidia and hyphae	ELISA	Mannoproteins (unknown)	
	JF5	1	IgG3	Hybridoma	Mouse		<i>A. fumigatus</i>	CWF	> 90	Hyphae and exoantigen	ELISA, LFA	Protein (unknown)	(Thornton, 2008)
	FUM 20	5	IgG1	Hybridoma	Mouse		<i>A. fumigatus</i>	Whole cell extract	33, 34	Exoantigen	ELISA	Serine proteinases	(Lin et al., 2000)
	13E12	23	IgG1	Hybridoma	Mouse		<i>A. terreus</i>	Exoantigen	18, 45, 70	Exoantigen and extracellular matrix	ELISA	Leucine aminopeptidase	(Nayak et al., 2011)
	19B2	IgG1	Hybridoma	Mouse		<i>A. terreus</i>	Exoantigen	10, 45, 100, 150	Exoantigen, hyphae, cytoplasm	ELISA	Dipeptidyl-peptidase V		
scFvAF1 P2C2 15B5	scFvAF1	4	scFv	Phage display	<i>E. coli</i>		Membrane fraction	NA	Membrane	ELISA, SPR	Protein (unknown)	(Krishnaswamy et al., 2011)	
	P2C2	5	IgG1	Hybridoma	Mouse		Xylanase	ca. 28	Exoantigen	ELISA, Blot, SPR	Xylanase	(Sega et al., 2012)	
	15B5	32	IgG1	Hybridoma	Mouse		Terrelysin (<i>E. coli</i>)	16.5	Hyphae	ELISA, Blot	Terrelysin	(Navak et al., 2012)	

(continued on next page)

Table 3 (continued)

Antibody	Antibody generation			Antigen species			Antibody characterization			Reference
	Abs	Ab class	Ab source	Host	Antigen species	Immunogen	Immunoblot (kDa)	Antigen localization	Immunoassay	
Afmp1-M5	20	IgG1	Hybridoma	Mouse	<i>A. fumigatus</i>	Afmp1p (<i>P. pastoris</i>)	40	Hyphae	ELISA, IFA	(Wang et al., 2012)
Afmp4-M1	13	IgG1	Hybridoma	Mouse	<i>A. fumigatus</i>	Afmp4p (<i>P. pastoris</i>)	19	Hyphae	ELISA, IFA	(Xue et al., 2013)
AfSA4	35	scFv	Phage display	Chicken	<i>A. flavus</i>	CWP	> 55	Conidia and hyphae	ELISA	(Beck et al., 2014)
2A8	1	IgG	Hybridoma	Mouse	<i>A. flavus</i>	CWP	> 55	Conidia and hyphae	ELISA	This review
F45-2	5	NA	Hybridoma	Mouse	<i>A. fumigatus</i>	Chitosanase (<i>E. coli</i>)	25	Exoantigen	ELISA, IFA	
AP10	8	IgG1	Hybridoma	Mouse	<i>A. parvaticus</i>	CWF	37	Intracellular and conidia	ELISA	
PO8-VHH	3	VHH	Phage display	Alpaca	<i>Aspergillus</i> spp.	Whole cell extract	ND	Intracellular and extracellular	ELISA	(Wang et al., 2017)

et al., 2001; Pechanova and Pechan, 2015; Zhang et al., 2015a), particularly stress-induced changes that affect protein expression, cell wall composition and the production of secondary metabolites have to be considered (Champer et al., 2016; Madrenys-Brunet and Torres-Rodriguez, 1995; Suh et al., 2012). Conidial and mycelial cell wall and cellular antigens, as well as exoantigens, can be extracted from *Aspergillus* cultures and used separately for antibody generation (Fig. 2).

3.1. Exoantigens

Aspergillus exoantigens can be recovered from spent media without disrupting the cells, and are therefore easy to separate from cell wall antigens. The exoantigen profile differs according to the *Aspergillus* species and tends to comprise a mixture of exopolysaccharides (EPS), hydrolytic enzymes, glycoproteins, and a wide range of toxic and non-toxic secondary metabolites (Drouhet et al., 1988; Frisvad et al., 2007; Nielsen et al., 2009). Exoantigens can be enriched by diafiltration or freeze-drying, whereas selective precipitation methods facilitate fractionation of different filtrate compounds which can be further concentrated by chromatography to produce concentrated exoantigen preparations for immunization (Fig. 2).

The most common *Aspergillus* EPS are galactomannans, comprising a galacto- α -1,2/ α -1,6-mannan core with terminal β -1,5-Galf residues, and galactosaminogalactan (GAG), comprising α -1,4-linked galactose (15–60%) and α -1,4-linked *N*-acetylgalactosamine. Neither of these heteropolysaccharides is present in resting conidia but they accumulate in the hyphal cell wall (Fontaine et al., 2011) and act as virulence factors that promote infection (Bozza et al., 2009; Schmalhorst et al., 2008). In *A. fumigatus*, Galf-containing molecules are found (i) in the filtrate in the form of secreted glycoproteins, (ii) in the alkali-soluble and insoluble fractions of the cell wall, (iii) on plasma membranes as glycosylphosphatidylinositol (GPI)-anchored lipophosphogalactomannans, and (iv) in several sphingolipids anchored to the membrane by inositol phosphoceramide (Latge, 2009). Galactomannan can usually be precipitated with ethanol, whereas GAG requires a further extraction step using 8 M urea (Fontaine et al., 2011).

Proteins from the exoantigen fraction can be enriched by precipitation with ammonium sulfate, trichloroacetic acid or acetone. Highly *O*-mannosylated glycoproteins with α -1,3-mannose and α -1,6-mannose linkages can be purified directly from the culture medium (Hao et al., 2008) or from total protein extracts (da Silva Bahia et al., 2003; Fratamico and Buckley, 1991) by affinity chromatography using the lectin concanavalin A (ConA). Dialysis followed by ion exchange chromatography and size exclusion chromatography can yield even purer fractions of the desired antigen for immunization.

Aspergillus exoantigens also include small molecules such as aflatoxins, sterigmatocystin, gliotoxin, helveolic acid, ochratoxins, termitrem B, fumigaclavine A, patulin, cyclopiazonic acid and many more. These toxic compounds are only sparingly soluble in water and must be extracted with different organic solvents (chloroform, methanol or acetonitrile). Besides separation by thin layer chromatography (TLC), secondary metabolites can be purified by high performance liquid chromatography (HPLC).

3.2. Cell wall and cellular antigens

Over the last 50 years, detailed characterization of the *Aspergillus* cell wall has revealed that it is predominantly composed of glucans, mannans, chitin, lipids and glycoproteins (Bartnicki-Garcia, 1968; Johnston, 1965; Ruiz-Herrera, 1967). Its highly complex three dimensional structure has also been investigated (Latge, 2017). Much research has focused on the human pathogen *A. fumigatus*, and previous reviews have covered cell wall synthesis (Bernard and Latgé, 2001; Gastebois et al., 2009; Henry et al., 2016; Latge et al., 2005), biofilm formation (Beauvais et al., 2014; Loussert et al., 2010; Mitchell et al., 2016a, 2016b), cell wall structure (Latgé, 2007; Latge and Beauvais,

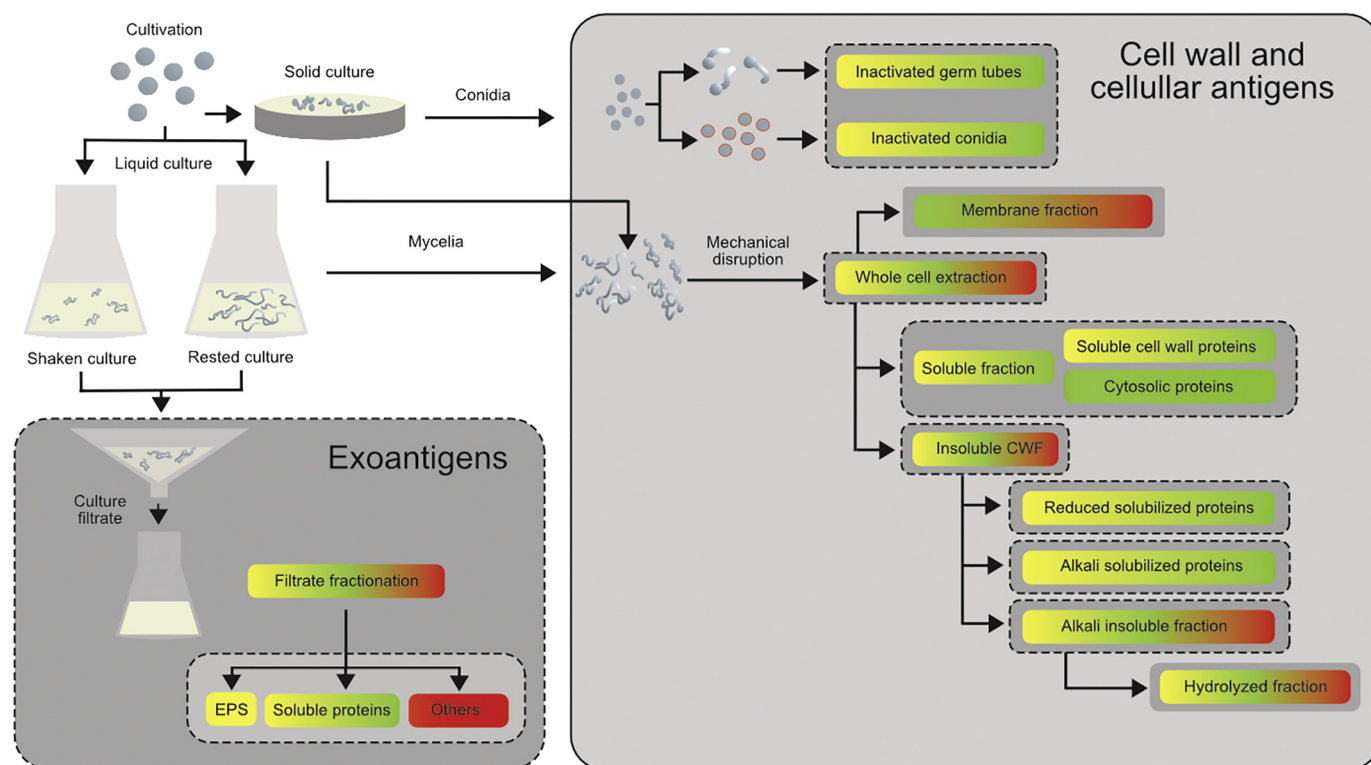


Fig. 2. Preparation of complex *Aspergillus* antigen mixtures for immunization and antigen localization.

Aspergillus spp. are cultivated on solid agar or liquid broth to obtain cell wall antigens and exoantigens. Conidia are isolated from the surface of solid cultures. Inactivated conidia or germ tubes can be used for immunization. *Aspergillus* mycelia and mycelial aggregates are obtained by filtration from liquid cultures which are used to isolate cell wall antigens, whereas the culture filtrate is used to isolate exoantigens. *Aspergillus* mycelia are crushed by mechanical disruption to obtain the whole cell extract that contains soluble cell wall proteins (CWPs), cytosolic proteins and insoluble cell wall fragments (CWF). *Aspergillus* membrane fractions are isolated by ultracentrifugation to extract membrane proteins and other compounds such as lipids. The insoluble CWF are washed extensively to remove intracellular antigens. Reduced solubilized CWPs can be released by reducing agents, whereas mild alkali treatment release alkali-soluble proteins from the CWF. The alkali-insoluble fraction can be processed by further chemical and enzymatic hydrolysis that release proteins that are linked by stronger binding forces. The *Aspergillus* culture filtrate can also be precipitated to concentrate exopolysaccharides (EPS), proteins or others (e.g. secondary metabolites). Further chromatography steps yield purer antigen fractions. Fractions used for immunization are shown dashed. Antigen color code: protein (■), carbohydrate (■), others (■).

2014; Lee and Sheppard, 2016) and virulence (Valiante et al., 2015) in this species.

The most abundant carbohydrate in the upper layer of the *Aspergillus* cell wall is β -1,3-glucan. The linear chains are branched and cross-linked via β -1,6-linkages. Cross-links to chitin maintain the structural integrity of the cell wall and chitin therefore accounts for 10–20% of the total dry weight of *Aspergillus* and other filamentous fungi, compared to only 2% in yeasts (Bowman and Free, 2006; Klis, 1994). Although β -1,3-glucan is common in many fungi, α -1,3-glucan and β -1,3-glucan linked to β -1,4-glucan, β -1,5-Galf, galactomannan and GAG are characteristic carbohydrates found in *Aspergillus* (Latge et al., 2005).

The *Aspergillus* cell wall antigens used for immunization can be described as either conidial or mycelial antigens (Fig. 2). *Aspergillus* cultures on solid agar or in liquid medium without agitation can produce sexual ascospores in asocarps (circular fruiting bodies) or asexual conidiospores (conidia) in conidiophores, depending on the precise environmental conditions (Fig. 2). The conidia can be separated from the mycelia by filtration, and formaldehyde-inactivated spores can be used as an immunogen. Moreover, conidia can be cultured for a short time to yield germ tubes, containing further unique antigens (Asif et al., 2006; Paris et al., 2003a). The *Aspergillus* conidial cell wall, germinating conidia and hyphae differ significantly in their structure and chemical composition (Girardin et al., 1999; Jensen et al., 2010; Kniemeyer et al., 2016; Kniemeyer et al., 2009; Suh et al., 2012). *A. fumigatus* conidia comprise an outer layer of immunologically inert hydrophobic rodlets (RodA fibrils) and an inner melanin layer which together promote

virulence by helping to evade the vertebrate immune system (Paris et al., 2003a; Pihet et al., 2009).

The cell wall of *Aspergillus* mycelium is adorned with heavily glycosylated cell wall proteins (CWPs) that form part of the structure and also provide enzymatic functions or act as receptors for signaling molecules. These cellular antigens are linked by different linkages to the fungal cell wall or can be attached to the plasma membrane via GPI anchors and fulfill important functions in morphogenesis, cell wall organization and virulence (Bruneau et al., 2001; Rast et al., 2003). CWPs are often species-specific and therefore ideal targets to generate antibodies that only recognize particular fungal species. In addition to GPI anchors, the interaction between CWPs and the multilayered *Aspergillus* cell wall features diverse linkages ranging from weak non-covalent bonds to strong covalent bonds that form cross-links with the carbohydrate scaffold, allowing the sequential extraction of CWPs from whole-cell extracts using reducing agents, mild alkali buffer, chemical or enzymatic hydrolysis (Fig. 2).

Further purification and chromatography are recommended to concentrate the extracted CWPs. However, the sequential extraction of cell wall and membrane associated CWPs is more often used to characterize linkages and determine the abundance of cell wall antigens than to prepare protein antigens for immunization.

Unlike the conidial preparations, *Aspergillus* mycelial cultures are mechanically disrupted under liquid nitrogen or in a bead mill, using a buffer containing protease inhibitors such as phenylmethylsulfonyl fluoride to inhibit endogenous or exogenous proteases. The resulting whole cell extract (Fig. 2) contains a mixture of soluble CWPs, cytosolic

proteins, and insoluble cell wall fragments (CWFs) that include chitin, β -glucan, membrane fragments and linked CWP. *Aspergillus* membranes and membrane-associated proteins can be isolated by ultracentrifugation. Moreover, soluble CWPs present in the soluble fraction can be associated with the cell or secreted as exoantigens (Haynes et al., 1990; Latge et al., 1993; Latge et al., 1991; Longbottom, 1983). Many cytosolic proteins and exoantigens in *A. fumigatus* have been identified as allergens that induce classic IgE-binding responses in humans or mammals, and are associated with asthma in the context of ABPA and cystic fibrosis (Kurup, 2005; Singh et al., 2010). In contrast, CWPs that are bound to the insoluble CWF by non-covalent bonds or disulfide bridges, such as the cell wall acid phosphatase PhoAp (Bernard et al., 2002), are extracted using reducing detergents to separate these proteins from insoluble structural cell wall components.

Sodium hydroxide is used to extract alkali-sensitive proteins, e.g. highly O-mannosylated proteins that are covalently linked to β -1,3-glucan and carbohydrates. Besides alkali-sensitive proteins, the alkali-soluble fraction mainly comprises linear α -1,3-glucans with intra-chain α -1,4-linked glucose units (40% dry weight), galactomannan and GAG (Fontaine et al., 2000). Further treatment involving a transition from mild alkali to hot alkali releases pseudonigeran (α -1,3-linked α -D-glucan) from the *A. niger* cell wall (Horisberger et al., 1972), as well as O-linked oligosaccharides featuring β -GalF, peptidoglycan (Leitão et al., 2003) and N-linked and O-linked peptidogalactomannans (Haido et al., 1998).

The remaining rigid cell wall skeleton is the alkali-insoluble fraction and contains branched β -1,3-glucans (4% of β -1,6 branch points) linked to a fibrillar core of chitin, linear β -1,3/ β -1,4-glucan, insoluble α -1,4-glucan and β -1,5-linked galactomannans (Fontaine et al., 2000). Covalently-linked CWPs in the alkali-insoluble fraction are often double anchored to chitin and β -1,3-glucan, and can only be released by chemical or enzymatic hydrolysis (Fig. 2). Quantazyme (β -1,3-glucanase plus β -1,6-glucanase) or laminarinase (β -1,3(4)-glucanase) and exochitinases degrade this alkali-insoluble fraction into smaller oligosaccharides and release CWPs that are linked indirectly to β -glucan via β -1,3-linkages and to chitin via β -1,6-linkages.

GPI-anchored proteins present in the isolated membrane fraction and alkali-insoluble fraction are solely anchored to the plasma membrane in *Aspergillus* spp. and, in contrast to yeasts, are not also covalently attached to cell wall polysaccharides (Latge et al., 2005). Only a few *Aspergillus* GPI-anchored proteins have been experimentally characterized thus far (Bruneau et al., 2001; Li et al., 2007; Mouyna et al., 2000; Vaknin et al., 2014). Recently, in silico genome mining in *A. fumigatus*, *A. nidulans* and *A. oryzae* revealed 115, 129 and 136 putative GPI-anchored proteins, respectively, representing ~1.2% of the total proteome (Cao et al., 2009). The unique structure of GPI anchors allows their specific cleavage to release the attached protein moiety. Furthermore, aqueous hydrofluoric acid can release CWPs linked by β -1,6-glycosidic bonds, double anchors and hitherto uncharacterized linkages (Damveld et al., 2005). CwpA, a GPI-anchored *A. niger* mannoprotein (Damveld et al., 2005) and the *A. fumigatus* cell surface protein A (CspA) (Levdansky et al., 2010) have been isolated in this manner. Alternatively, GPI-anchored proteins can be isolated from *Aspergillus* membranes using β -N-octylglucopyranoside (Bruneau et al., 2001) or phospholipase C, which specifically hydrolyze the phosphatidylinositol phosphodiester bond.

3.3. Recombinant antigens and hapten fusions

If a purified *Aspergillus* antigen is available, it can be used to generate mAbs against a known target (Table 3). The most straightforward way to prepare pure proteinaceous antigens is to express them as recombinant proteins, including epitope tags that facilitate purification. Cloned *Aspergillus* genes can be expressed in bacteria, yeast, animal cells, cell-free systems or plants. The use of recombinant proteins as immunogens allows the generation of specific antibodies against several

immunodominant epitopes in a single protein. *Aspergillus* antigens that are difficult to express in their native state can be produced as fusion proteins with partners such as glutathione-S-transferase, or individual immunodominant epitopes can be chemically fused to haptens for immunization.

The WHO/IUIS Allergen Nomenclature Sub-committee database lists 30 *Aspergillus* allergens, most (23) from *A. fumigatus* as well as one each from *A. flavus* and *A. versicolor*, two from *A. oryzae* and three from *A. niger* (<http://www.allergen.org/>). Because these allergens induce strong IgE-binding responses, some have been expressed as recombinant proteins for evaluation as diagnostic markers for CPA and ABPA (Bhetariya et al., 2011; Kurup, 2005; Kurup and Banerjee, 2000; Kurup et al., 2006). Several allergen-specific antibodies have been generated against such allergens, including mAbs recognizing Asp f 4 (Cramer, 1998), Asp f 1 (Schwienbacher et al., 2005b) and Asp v 13 (Liang et al., 2011), and polyclonal Asp f 13 antisera (Chow et al., 2000). Further antibodies have been generated against recombinant glucose oxidase (Ascione et al., 2004), terrelysin (Nayak et al., 2012), chitosanase (Beck et al., 2014) and the galactomannoproteins Afmp1p and Afmp4p (Wang et al., 2012), as well as polyclonal antisera against the *A. flavus* galactomannoprotein Afmp1p (Woo et al., 2003) and the *A. fumigatus* galactomannoproteins Afmp1p (Chan et al., 2002; Yuen et al., 2001) and Afmp2p (Chong et al., 2004).

The first antibodies against aflatoxins were produced in the late 1970s (Chu and Ueno, 1977) and the number of antibodies recognizing the most important classes (Afb1, Afb2, AFG1 and AFG2), the aflatoxin metabolite AfM1, and aflatoxin adducts, is still growing (Table 1). Purified secondary metabolites such as aflatoxins do not elicit an immune response and must be coupled to protein carriers (haptens) such as bovine serum albumin or keyhole limpet hemocyanin to elicit an immune response in immunized animals. Most aflatoxin-hapten conjugates are prepared by the synthesis of a carboxymethylloxime spacer at ring position 1 of the aflatoxin molecule, which yields antibodies that recognize epitopes at positions 8 and 9, with selective specificity for Afb1 > AFG1 > Afb2 > AFG2 (Chu and Ueno, 1977). Antibodies recognizing epitopes at ring position 1 of the aflatoxin molecule are prepared using haptens synthesized by N-hydroxysuccinimide chemistry, specifically by esterification at position 8 in the presence of glycolic acid (Cervino et al., 2007). Alternatively, aflatoxins can be coupled by 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide to cationized bovine serum albumin via a Mannich-type reaction (Zhou et al., 2007).

Aflatoxin-specific antibodies are selected using either another aflatoxin-hapten conjugate in a direct enzyme-linked immunosorbent assay (ELISA) or free aflatoxin in an indirect competitive ELISA. It is unnecessary to identify the epitope recognized by aflatoxin-specific antibodies because the most important characteristics are the cross-reactivity to related aflatoxins and the half-maximal inhibitory concentration (IC₅₀). Numerous mAbs have been generated that recognize Afb1 alone, AFG1 and AFG2, AfM1 alone, or all five main classes (Afb1, Afb2, AFG1, AFG2 and AfM1), and IC₅₀ values in the μ g/ml to pg/ml-range have been reported (Table 1). Moreover, recombinant single-chain fragment variable (scFv) antibodies and nanobodies (VHH) generated from phage display libraries are used in aflatoxin detection assays. However the majority of these antibodies were characterized and validated using different immunoassays and validation guidelines. Therefore it is almost impossible to recommend any procedure for generating an antibody with high sensitivity and specificity (Table 1).

4. Antibody generation and characterization

More than 420 *Aspergillus*-specific antibodies have been generated using antigens prepared from *Aspergillus* cultures (Tables 1–3). Heterogeneous antigen mixtures are recommended (i) if pathogen-specific antibodies need to be generated against a portfolio of different targets, (ii) if the target is unknown, and (iii) if glycosylation is important for pathogen detection.

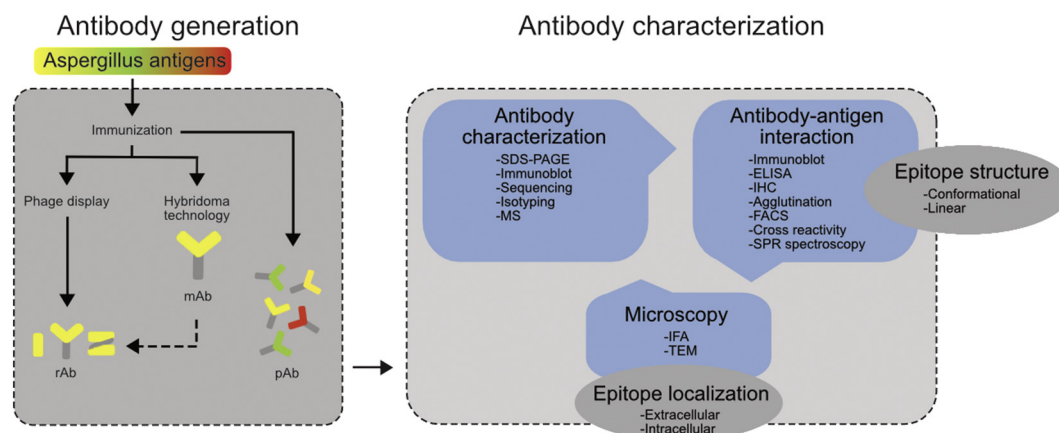


Fig. 3. Workflow for the characterization of *Aspergillus*-specific antibodies. Prepared *Aspergillus* antigens (Fig. 2) are used to immunize animals. Polyclonal antisera (pAb) can be isolated from the serum of immunized animals, whereas hybridoma technology can be used to generate monoclonal antibodies (mAbs). Alternatively, recombinant antibodies (rAbs) can be generated from mAb sequences or antibodies can be derived by phage display from naïve libraries or hyperimmunized animals. The specific antigens are used in enzyme-linked immunosorbent assay (ELISA) to screen *Aspergillus*-specific antibodies. The *Aspergillus*-specific antibodies and the antibody–antigen interaction are further characterized by the various methods as shown. Microscopic analysis of *Aspergillus* cells and *Aspergillus*-specific antibodies can reveal the localization of the antigen. Chemical analysis of the antigen is required to determine the chemical nature of the epitope. Further abbreviations: FACS = fluorescence activated cell sorting, IFA = immunofluorescence analysis, IHC = immunohistochemistry, MS = mass spectrometry, SDS-PAGE = sodium dodecylsulfate polyacrylamide gel electrophoresis, SPR = surface plasmon resonance spectroscopy, TEM = transmission electron microscopy. Antigen color code: protein (green), carbohydrate (yellow), others (red).

Rodents, chickens or rabbits are immunized with prepared *Aspergillus* antigens and an appropriate adjuvant to elicit an immune response that results in the generation of antibody-producing cells (Fig. 3). Polyclonal antibodies that detect several different antigens can be easily purified from the sera of hyperimmunized animals, and can be used to detect *Aspergillus* exoantigens (Chen et al., 2000; Lu et al., 1995; Shapira et al., 1997) and mycelial antigens (Anand and Rati, 2006; da Silva Bahia et al., 2003; Haido et al., 1998; Kaufman et al., 1997; Sun et al., 2012; Tsai and Yu, 1997) without further antigen identification.

Isolated splenic lymphocytes from hyperimmunized mice or rats are used to generate immortal hybridoma cell lines that produce mAbs (Tables 1–3). In contrast to polyclonal antisera, mAbs recognize a unique epitope and are ideal candidates for the identification and characterization of defined target antigens. In case of mAbs produced by hybridoma clones, the sequence information from the mAb heavy and light chains can be easily be retrieved and used to generate recombinant antibody formats like scFvs or the fragment antigen binding (Fab) format, which retain the same specificity as the parental mAb but provide different structural and functional features (Fig. 3). Alternatively, specific scFv or Fab libraries can be generated from peripheral blood lymphocytes isolated from animals immunized with *Aspergillus* antigens and screened by phage display (Fig. 3). A phage display library prepared from a chicken immunized with *A. flavus* CWP yielded the specific antibody scFvAfSA4, with a binding affinity (K_D) of 2.8×10^{-7} M (Xue et al., 2013). Similarly, the specific antibody scFv AF1 (5.22×10^{-11} M) was raised against *A. fumigatus* membrane proteins (Krishnaswamy et al., 2011). The newest development in recombinant antibody technology are single domain antibodies (nanobodies) which showed an improved binding affinity and stability compared to conventional recombinant antibodies (He et al., 2014; Wang et al., 2017; Wang et al., 2013).

Antibody–antigen interactions can be characterized by immunological methods such as ELISA, immunohistochemistry and agglutination assays to determine the reactivity towards a given antigen, whereas cross-reactivity tests can determine if an antibody detects epitopes shared with related fungi or other pathogens (Fig. 3). Immunoblot detection using *Aspergillus*-specific antibodies can not only estimate the molecular weight of the corresponding antigen but can also determine if the epitope persists under denaturing conditions (linear epitope) or is denatured (conformational epitope). The latter are

unsuitable for in vitro detection under denaturing conditions but can recognize the native antigen in situ in the *Aspergillus* cell wall. Fluorescence activated cell sorting (FACS) using *Aspergillus*-specific antibodies can be applied to distinguish between populations of swollen conidia, resting conidia and young hyphae (Hetherington et al., 1994) and to characterize the inhibitory potential of *Aspergillus*-specific antibodies (Chaturvedi et al., 2005).

The binding affinity of *Aspergillus*-specific antibodies can be determined by ELISA or surface plasmon resonance (SPR) spectroscopy, and these approaches are often used to compare panels of mAbs to select those with the best binding characteristics with respect to the desired application. For the in vivo application of therapeutic antibodies, serum clearance assays are used to determine pharmacokinetic properties. For example, a scFv panel targeting *A. fumigatus* glycosylhydrolase (*Asp f 9*) variant Crf2, which is displayed on the surface of growing hyphae, was generated by phage display (Schutte et al., 2009). The serum stability of the Crf2-specific scFv MS130h-IIIC2 (K_D 1.4×10^{-8} M, serum half life > 27 days) was found to be greater than that of MS130i-IIIC3 (K_D 9.6×10^{-10} M, serum half life < 1 day) making the former more suitable for therapeutic applications (Schutte et al., 2009).

A potential drawback of heterogeneous *Aspergillus* antigen preparations is that immunization may also generate mAbs against intracellular targets, which are not suitable for in situ detection. Immunofluorescence microscopy, transmission electron microscopy and topological mapping by cell wall-degrading enzymes are important tools to distinguish between antibodies that recognize epitopes on the outer cell wall at different stages of the fungal growth and those that recognize intracellular epitopes.

Aspergillus-specific mAbs can be characterized further by isotyping, which provides more information about the nature of an antigen. Antibodies recognizing carbohydrate antigens are typically class IgM because carbohydrates are not efficiently presented to T-cells and fail to undergo somatic hypermutation (Cobb and Kasper, 2005). In contrast, *Aspergillus*-specific antibodies detecting proteinaceous antigens are usually class IgG (Table 3). The variable genes (V-genes) of a hybridoma clone that produces *Aspergillus*-specific mAbs can be identified by amplifying the V-gene sequences from hybridoma RNA, or by the direct analysis of heavy and light chain peptides by mass spectrometry. If the sequence of the complementarity determining regions matches a

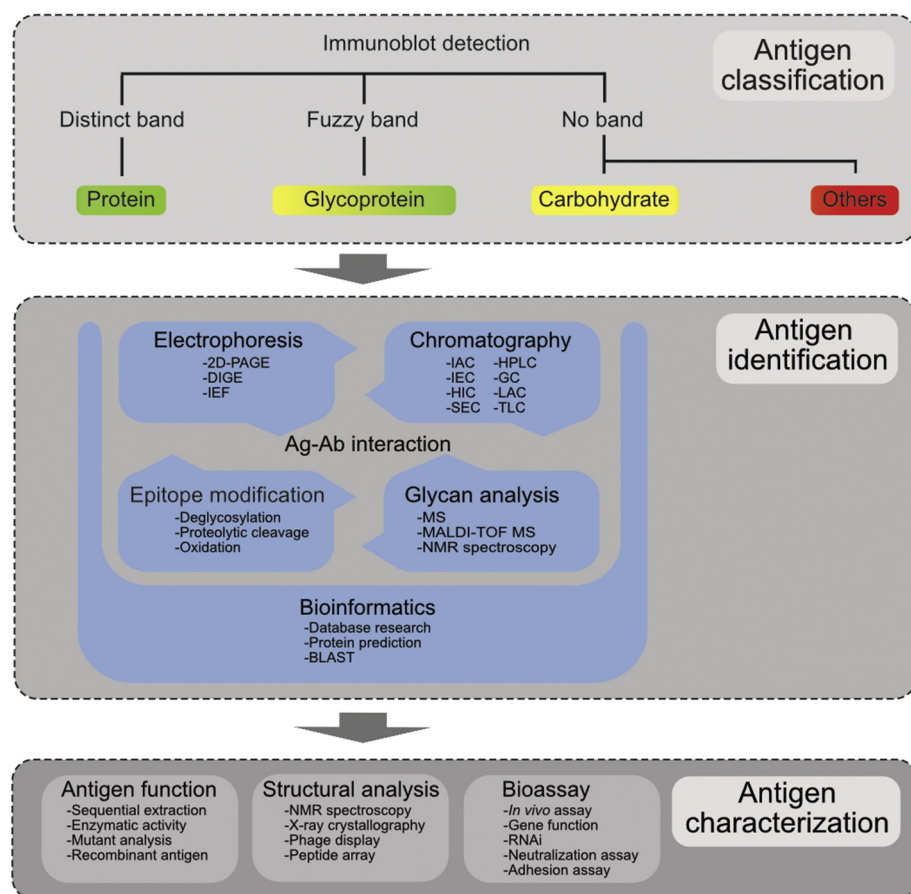


Fig. 4. Strategies for the identification of epitopes recognized by *Aspergillus*-specific antibodies.

Abbreviations: 2D-PAGE = two-dimensional polyacrylamide gel electrophoresis, Ab = antibody, Ag = antigen, BLAST = basic local alignment search tool, DIGE = difference in-gel electrophoresis, GC = gas chromatography, HIC = hydrophobic interaction chromatography, HPLC = high performance liquid chromatography, IAC = immunoaffinity chromatography, IEF = isoelectric focusing, IEC = ion exchange chromatography, LAC = lectin affinity chromatography, MALDI-TOF = matrix-assisted laser desorption ionization – time of flight, MS = mass spectrometry, NMR = nuclear magnetic resonance, RNAi = RNA interference, SEC = size exclusion chromatography, TLC = thin layer chromatography. Antigen color code: protein (green), carbohydrate (yellow), others (red).

sequence already found in the ImMunoGeneTics (IMGT) database (<http://www.imgt.org>) this will help to identify the specific antigen.

5. Identification of the *Aspergillus* antigens

The characterization of *Aspergillus*-specific antibodies (Fig. 3) is the first step towards epitope mapping and this provides initial insight into the nature of the corresponding antigen. Several methods are then combined to isolate single antigens from complex mixtures for further analysis of antibody–antigen binding, which is the central component of epitope mapping. Fig. 4 summarizes the various procedures that can be used for the isolation, identification and characterization of antigens, reflecting the strategies applied in the case of existing *Aspergillus*-specific antibodies (Tables 1–3). Immunoblot analysis of fractionated antigen mixtures can indicate whether the antigen is a protein (distinct band), a glycoprotein (fuzzy band), or another type of molecule, such as a carbohydrate or metabolite (no band) (Fig. 4). However, CWPs are often glycosylated and crosslinked to the carbohydrate backbone of the fungal cell wall, which makes epitope mapping more challenging. Antigen modification may be necessary to determine whether the epitope of a glycoprotein is a protein or carbohydrate. Chromatography and/or electrophoresis can be used to separate proteinaceous or charged carbohydrate antigens, but more complex analytical methods are required to resolve the glycan structure of isolated carbohydrate or lipid antigens. Finally, the antibody–antigen complex including the precise binding site, the binding forces and atoms involved can be characterized in detail.

5.1. Protein antigens

Aspergillus proteins are located in different cellular compartments of the fungal cell, bound to the cell wall or secreted as allergens and/or

virulence factors into the environment. Ammoniumsulfate-precipitated soluble CWP of *A. fumigatus* have been partially purified by size-exclusion chromatography, yielding a crude preparation with a protein-to-carbohydrate ratio of 18:1 (Arruda et al., 1992). The immunization of mice with this preparation generated mAb 4A6, which was subsequently found to recognize mitogillin, an 18 kDa ribotoxin which is detected by sera from ABPA patients (Arruda et al., 1990). This was the first mAb that specifically recognized the major *A. fumigatus* allergen *Asp f 1*. Using the same approach, mAb IL-B8 was shown to recognize the 37 kDa fibrinogen-binding β -glucanase *Asp f 2* (Kumar and Kurup, 1993; Tang et al., 2000). Antibodies raised against *A. fumigatus* whole cell extracts have been characterized in the same manner (Schwienbacher et al., 2005a). Accordingly, B-M1-A12 was shown to detect the hypha-specific 23 kDa allergen *Asp f 6* (manganese-dependent superoxide dismutase), whereas B8-C4 was shown to detect *Asp f 8* (P2 protein), an 11 kDa intracellular protein which is present in conidia and hyphae but not restricted to ABPA patients (Schwienbacher et al., 2005a). The mAb Fum20 was raised against *A. fumigatus* water-soluble whole cell extracts and was shown to recognize a 34 kDa protein in the sera of asthma patients (Lin et al., 2000). N-terminal amino acid sequencing revealed that FUM20 detects an epitope shared by an *A. fumigatus* alkaline serine protease and vacuolar serine protease, which could not be assigned clearly to *A. fumigatus* allergens *Asp f 13* and *Asp f 18* (Lin et al., 2000). Further proteomic analysis of whole cell antigen mixtures from *A. fumigatus* (Asif et al., 2006; Gautam et al., 2007; Kniemeyer, 2011; Kniemeyer et al., 2016; Kniemeyer et al., 2009; Vodisch et al., 2009), *A. versicolor* (Benndorf et al., 2008) and *A. flavus* (Pechanova et al., 2013) have identified potential new allergens which could be used as diagnostic markers.

We have generated a portfolio of specific antibodies against aflatoxigenic *A. flavus* and *A. parasiticus* using heterogeneous CWFs for immunization. The same CWFs yielded antibodies specific for several

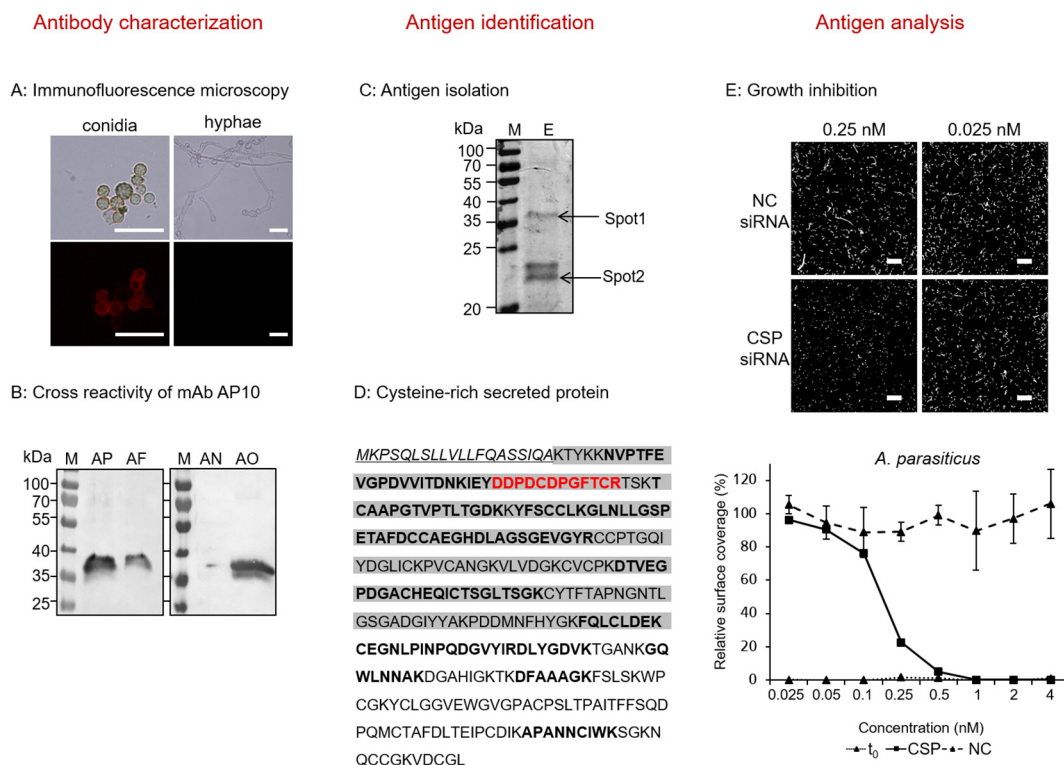


Fig. 5. Identification of the antigen recognized by mAb AP10.

A: Immunofluorescence microscopy showing mAb AP10 binding to *A. flavus* conidia and hyphae. Immobilized samples were incubated sequentially with the *Aspergillus*-specific mAb AP10 and a fluorophore-conjugated goat anti-mouse antibody. Samples were analyzed by confocal microscopy (594 nm). Scale bars = 50 μ m. **B:** Cross reactivity of mAb AP10 to *Aspergillus* spp. *Aspergillus* cell wall proteins were separated by SDS-PAGE and probed with mAb AP10. M = pre-stained protein marker (Fermentas), AP = *A. parasiticus*, AF = *A. flavus*, AN = *A. nidulans*, AO = *A. oryzae*. **C:** Immunoaffinity chromatography. NHS-coupled mAb AP10 and dialyzed CWP were used to identify the antigen of mAb AP10. Bound proteins were eluted in a reducing SDS-containing buffer and analyzed by SDS-PAGE. Detected proteins at 37 kDa (spot 1) and 22 kDa (spot 2) were also detected by immunoblotting using mAb AP10. **D:** Amino acid sequence of cysteine-rich secreted protein (CSP) and mAb AP10 epitope identification. Full amino acid sequence of *A. flavus* CSP (XP_002374495.1). Highlights show the signal peptide (italic and underlined), empirical peptide sequences (in bold) from protein spots 1 and 2, homologies in spots 1 and spot 2 (gray), and the epitope sequence determined by Pepscan-ELISA (red). **E:** Qualitative and quantitative effect of CSP-specific siRNA to the growth of *A. parasiticus*. We incubated 400 *A. parasiticus* conidia with CSP-specific siRNA (0.025–4 nM) diluted in 1:5 RPMI medium for 12 h at 28 °C in the dark before adding calcofluor white (diluted 1:20 in water) and observing the stained mycelia using the Opera® High Content Screening confocal microscope. Relative surface coverage (%) was calculated by ImageJ (NIH, Bethesda, USA). CSP = CSP-specific siRNA, NC = water control after 12 h, t₀ = water control before incubation. Scale bars = 100 μ m.

different intracellular and extracellular antigens: mAb AP3 was shown to bind GalF (Schubert et al., unpublished), whereas mAb AP10 did not bind *A. flavus* hyphae but bound strongly to the resting conidia (Fig. 5A). These data suggest that the antigen is displayed by conidia but is either absent or inaccessible during hyphal growth. Interestingly, mAb AP10 recognizes a single 37 kDa protein in whole cell protein extracts of *A. flavus*, *A. parasiticus* and *A. oryzae* (Fig. 5B) but not other *Aspergillus* spp., nor fungi from other genera (data not shown) indicating that the antigen is conserved among the closely-related species of *Aspergillus* section *Flavi*.

Because the 37 kDa protein recognized by mAb AP10 could not be clearly assigned to a single protein by mass spectrometry, dialyzed *A. parasiticus* CWP extracts were used to isolate the antigen by immunoaffinity chromatography (Fig. 5C). Mass spectrometry identified the isolated protein as the cysteine-rich secreted protein (CSP) (Fig. 5D). This protein is 338 amino acids in length with an acidic pI of 5.46, and its function is unknown (Payne et al., 2006). It shares 99% sequence coverage with a hypothetical protein (AOR_1_986114) from *A. oryzae* (gi317149420) explaining the cross reactivity observed in immunoblot experiments (Fig. 5B). The *A. parasiticus* CSP shows 65% identity to *A. fumigatus* Af293 (AFUA_7601060) and 49% identity to *A. nidulans* AN7836.2, confirming it is found exclusively in section *Flavi*. Sequence analysis with SignalP 4.1 predicted that the pro-protein would include a 21-residue signal peptide which we assume was cleaved off during secretion. Further analysis revealed the lack of a

transmembrane domain and the absence of a GPI anchor, the absence of N-linked glycans but the presence of numerous potential O-glycosylation sites. Recombinant CSP expressed in *Escherichia coli* BL21 and subsequent detection of histidine-tagged CSP using mAb AP10 confirmed the mass spectrometry data (not shown) and a Pepscan-ELISA confirmed that the mAb AP10 epitope is continuous and located near the N-terminus, probably comprising the 12 amino acids DDPDCDPGFTCR (Fig. 5D).

The biological function of CSP was investigated by RNA interference (RNAi) using chemically synthesized CSP-specific 27-mer RNA duplexes as substrates for the Dicer complex. The incubation of these substrates with *A. flavus* and *A. parasiticus* conidia achieved growth inhibition at low concentrations (*A. flavus* IC₅₀ = 0.2 nM, *A. parasiticus* IC₅₀ = 0.175 nM) and germination failure at higher concentrations (Fig. 5E).

Phylogenetic analysis indicated that CSP is exclusively present in *Aspergillus* section *Flavi* such as *A. flavus* and *A. parasiticus*, making it an excellent candidate for the RNAi-based control of aflatoxigenic fungi in the field (Nölke et al., 2016). Nevertheless, more detailed investigations are required to determine the localization and function of CSP in the lifecycle of *Aspergillus* spp. section *Flavi*.

5.2. Glycoproteins

Aspergillus-specific antibodies generated against cell wall antigens often detect single proteins and protein fragments of different size that

share the same epitope or fuzzy bands representing glycoproteins with heterogeneous glycan structures by immunoblotting (Klis et al., 2007). The IgM mAb 16C4 generated against *A. fumigatus* CWF preparations is of particular interest because it recognizes a specific antigen present at the apical region when the polarity of the fungal tip is established (Momany et al., 2004). Fractionation of the crude extract by hydrophobic interaction chromatography released four proteins (4, 35, 38 and 70 kDa) that were detected by mAb 16C4, including the mycelial catalase Cat1p (GenBank U97574.1). This is a tetrameric protein in which each 90 kDa subunit contains three potential *N*-glycosylation sites and 19 potential *O*-glycosylation sites (Calera et al., 1997). Cat1p functions as a virulence factor and protects the fungi against reactive oxygen species released by the host (Paris et al., 2003b), but it remains unclear whether Cat1p or a co-purified Cat1p-associated protein is the real mAb 16C4 target (Momany et al., 2004).

Two IgGs raised against *A. terreus* exoantigens have also been used to purify the corresponding antigens by immunoaffinity chromatography (Nayak et al., 2011). Immunoblotting of the culture supernatants revealed that mAb 19B2 detected two distinct protein bands (100 and 150 kDa), whereas mAb 13E11 detected a fuzzy band spanning the range 50–75 kDa. The antibodies were coupled to protein G sepharose and used to immunoprecipitate the antigens. Subsequent mass spectrometry revealed that mAb 13E11 recognizes a putative uncharacterized leucine aminopeptidase (Q0CAZ7) associated with extracellular matrix structures containing DNA, whereas mAb 19B2 recognizes a putative dipeptidyl-peptidase V (Q0C8V9) primarily localized in the cytosol (Nayak et al., 2011). Both proteins feature a signal peptide for secretion and multiple sites for *N*-linked and *O*-linked glycans. Carbohydrates were found to account for 10% of the dipeptidyl-peptidase antigen (Beauvais et al., 1997). Although its function is unknown, the dipeptidyl-peptidase is likely to provide nutrition by cleaving *N*-terminal residues from polypeptide chains and may also be involved in cell-mediated immunity (Beauvais et al., 1997).

Potential glycoprotein antigens should be investigated in detail because the epitope may consist of a peptide, glycan or a combination. Incubation with periodate can be used to determine whether an *Aspergillus*-specific antibody recognizes a carbohydrate or peptide epitope because periodate cleaves the 1,2-diol linkage of glycans and oxidizes the exposed sugar residues (Fig. 4). For example, mAb A9 (IgG1) detects an *A. fumigatus* 97 kDa glycoprotein before and after treatment with periodate, indicating the epitope is a peptide (Chaturvedi et al., 2005). Similarly, the partial enzymatic removal of *N*-linked glycans can also distinguish between peptide and carbohydrate epitopes. For example, mAb JF5 (IgG3) detects a > 90 kDa glycoprotein (fuzzy band) in immunoblots of crude extracts but a sharp 40 kDa band after treatment with PNGase, suggesting that the epitope is a peptide (Thornton, 2008). Accordingly, proteolytic degradation of the glycoprotein with pronase and trypsin abolished JF5 binding, confirming these earlier results. Recently, JF5 has been used for non-invasive detection of *A. fumigatus* lung infection (Rolle et al., 2016). Although the nature of the JF5 antigen is known, it has not yet been identified (Rolle et al., 2016; Thornton, 2008).

In a more extensive study, 17 mAbs generated against *A. fumigatus* mycelial, conidial and secreted antigens were selected based on their ability to label the fungal cell wall (Hao et al., 2008). Competitive binding analysis demonstrated that some of these mAbs shared the same epitope. Although, none of the antibodies bound to galactomannans, some of them recognized 25 and 75 kDa proteins from a ConA-purified fraction which were also specifically detected by the galactomannan-specific antibody EB-A2 (Stynen et al., 1992). These data suggest that the cross-reacting proteins contain distinct and unrelated epitopes recognized separately by EB-A2 and the novel antibodies.

Fratamico and coworkers identified an immunodominant 58 kDa protein that is present in 90% of serum samples from IA patients (Fratamico and Buckley, 1991; Fratamico et al., 1991). This protein was

purified by ConA affinity chromatography and used to generate mAb 2-2-9. Further characterization of the antigen revealed a protein-to-carbohydrate ratio of 1.16:1 and showed that the carbohydrate moiety consisted of mannose, galactose and glucose in a 2:1:2 molar ratio. The authors observed strong cross-reactivity to a *C. albicans* mannoprotein but were able to abolish mAb 2-2-9 binding by treating the antigen with periodate, indicating that the shared epitope was a carbohydrate containing mannose residues. Similarly, the *A. fumigatus*-specific IgMs (mAb 1 and mAb 40) were characterized by a combination of proteolytic cleavage and exposure to periodate, revealing that both antibodies recognize carbohydrate epitopes (Ste-Marie et al., 1990). Cross-reactivity to other fungal pathogens and the results of lectin binding assays suggested that mAb 1 and mAb 40 detect the oligogalactoside and mannosylpyranosyl side chains of galactomannans, respectively. A carbohydrate epitope was also identified for IgM AK-14 (Kumar and Shukla, 2015). In other cases where immunoblots revealed multiple or fuzzy bands, the chemical nature of the epitope remains unknown (Candlish et al., 1997; Hetherington et al., 1994; Jensen et al., 1996; Kumar and Kurup, 1993; Kumar et al., 1993).

5.3. Carbohydrate antigens

Carbohydrate epitopes associated with the cell wall or secreted as exoantigens can be linked to glycolipids or polysaccharides instead of glycoproteins. Such non-protein antigens are rarely detected by SDS-PAGE and more sophisticated methods are required for their analysis (Fig. 4). Carbohydrate structures can be stained using certain chemical dyes or carbohydrate-specific antibodies that detect chitin/chitosan (Schubert et al., 2010), mannan (Cassone et al., 1988), melanin (Youngchim et al., 2004) or β -glucan (Rachini et al., 2007; Torosantucci et al., 2009). Although many carbohydrate molecules are found in a wide range of fungi, some are characteristic or unique to certain species. In *Aspergillus* spp., the cell wall contains carbohydrates such as GAG and galactomannans with terminal β -1,5-Galf residues that are not found in other fungal genera, and these can be used to raise antibodies that solely detect *Aspergillus* cells.

EB-A2 (IgM) was the first and best-characterized galactomannan-specific antibody (Stynen et al., 1992) used in the Pastorex *Aspergillus* latex agglutination assay (Van Cutsem et al., 1990) and in the Bio-Rad Platelia *Aspergillus* ELISA (Stynen et al., 1992) for the laboratory diagnosis of IA. This antibody is FDA-approved and validated for the detection of galactomannan in clinical samples of urine, bronchoalveolar fluid and sera from IA patients, and is still the “gold standard” for the serological detection of IA. The epitope recognized by EB-A2 was determined based on the known components of the *A. fumigatus* cell wall (Stynen et al., 1992). Galactomannan preparations subjected to mild hydrolysis with hydrochloric acid to remove the galactose side chains were no longer recognized by EB-A2. Subsequent ELISA analysis using synthetic β -1,5-Galf oligomers with different degrees of polymerization confirmed that the EB-A2 epitope consists at least of a Galf tetramer.

However, a drawback of IgM antibodies such as EB-A2 is an isotype-dependent lower specificity and stability compared to IgG antibodies. Recently, mAb AP3 was described as a Galf-specific IgG for the detection of *Aspergillus* contamination in food (Rettcher et al., unpublished). A combination of proteolysis and periodate treatment confirmed that the antigen of AP3 is a carbohydrate (Schubert et al., unpublished). Most importantly, experimental proof was provided by binding analysis using a mutant strain of *A. fumigatus* lacking UDP:Galf mutase activity (*AglfA*) (Schmalhorst et al., 2008). The binding analysis of the *AglfA* mutant confirmed that AP3 binds to an epitope of the Galf-branch of *Aspergillus* galactomannan and Galf-containing *Aspergillus* glycoproteins (Schubert et al., unpublished).

Recently, Matveev and coworkers (Matveev et al., 2018), have generated Galf-specific antibodies using a synthetic pentasaccharide β -d-Galf-(1,5)-[β -d-Galf-(1,5)]3- α -d-Manp for immunization. The IgG antibody mAb 7B8 specifically recognize constituting parts of the Galf-

trisaccharide, whereas mAb 8G4 detects the parental pentasaccharide. Both antibodies do not react with *Bifidobacterium longum* and demonstrate less cross reactivity compared to EB-A2.

The Galf-specific IgMs described by McCormick (McCormick et al., 2010) and Heesemann (Heesemann et al., 2011) show that carbohydrate epitopes with different structures can be distinguished using specific mAbs. Detailed immunofluorescence analysis of the *ΔglfA* mutant and wild-type strains showed that mAb L-10 stained the wild-type cell wall homogeneously, whereas mAb L99-13 produced a punctate staining pattern, indicating that distinct Galf epitopes occupy different sites on the fungal cell wall. Galf-containing molecules with β -1,6-linkages have been isolated from *Fusarium* spp. (Chen et al., 2015) and the Galf-specific IgM AB135-8 generated against killed *A. fumigatus* germ tubes bound weakly to Galf released into the *A. fumigatus* culture supernatant (Wiedemann et al., 2016) but strongly to *Fusarium* hyphae and Galf secreted by *Fusarium* spp. In contrast to EB-A2, mAb AB135-8 can distinguish between *Fusarium* and *Aspergillus* Galf epitopes.

GAG is a recently characterized *Aspergillus* cell wall polysaccharide that is present mainly in the extracellular matrix but also secreted into the medium (Fontaine et al., 2011; Reichhardt et al., 2015). GAG plays an important role in cell adhesion and is a virulence factor that suppresses the host immune response by shielding the fungus (Gravelat et al., 2013; Lee et al., 2015). The structure of GAG was determined by sequential hydrolysis of the polysaccharide antigen followed by gas chromatography/mass spectrometry (GC–MS) and nuclear magnetic resonance (NMR) spectroscopy (Fontaine et al., 2011). A GAG-specific antibody was then generated, and competitive binding studies demonstrated that the antibody does not bind to galactomannan, β -1,3-glucan or α -1,3-glucan, whereas oligo-*N*-acetylgalactosamine (GalNAc) completely inhibited its ability to bind GAG.

5.4. Further methods to identify antigens and characterize antibody–antigen interactions

As soon as the antigen recognized by an *Aspergillus*-specific antibody has been identified, further methods can be used to (i) characterize the association of the antigen with the fungal cell wall, (ii) determine the structure of the antibody–antigen complex, and (iii) analyze the function of the antigen in the fungal lifecycle (Fig. 4). The linkage of an antigen to the fungal cell wall can be determined by sequential isolation (Fig. 2) and the analysis of each fraction to detect the antigen (Fig. 4).

As an example, PhoAp was initially identified as a major *A. fumigatus* GPI-anchored protein (Bruneau et al., 2001). Further characterization (Bernard et al., 2002) indicated that the 80 kDa protein carries *N*-linked glycans and is strongly bound to β -1,3-glucan (probably in a non-covalent manner), and can be secreted into the culture medium. However, despite its enzymatic activity, PhoAp does not appear to be involved in cell wall morphogenesis. The function of the galactomannan-containing proteins phospholipase C and phytase was experimentally determined by substrate inhibition assays (Morelle et al., 2005). Interestingly, the analysis of these proteins by matrix-assisted laser desorption mass spectrometry revealed the absence of β -1,5- Galf chains, but both proteins were recognized by the IgM EB-A2, which is specific for this structure (Stynen et al., 1992). This suggests that EB-A2 can also detect oligomannans substituted with Galf at the non-reducing end (Morelle et al., 2005).

Proteinaceous epitopes can also be identified using peptide arrays, and if the native structures are preserved (as is the case in phage display) this approach can even reveal conformational epitopes. The structure of an antibody–antigen complex can be resolved by the crystallization of solutions containing the antigen and a corresponding Fab fragment or scFv. The analysis of epitopes by X-ray crystallography or NMR spectroscopy can determine the three-dimensional structure of globular proteins and oligosaccharides (Abbott et al., 2014). The 3D

structures of antibody–glycan complexes can also be resolved using these methods. However, to the authors' knowledge X-ray crystallography or NMR spectroscopy of a specific antibody and a fungal antigen has not been done so far. Empirical structures and in silico models can be consulted in reference datasets, such as those housed by the Protein Data Bank (<http://www.rcsb.org/pdb>).

Antigens containing glycans can be released, profiled and fractionated to reveal the glycan structures and their linkage (Mulloy and Stanley, 2009), whereas lipid analysis can reveal the structure of sphingolipids and glycosylinositol phosphorylceramides (Fontaine et al., 2009; Simenel et al., 2008). Acidic glycosphingolipid components have been isolated from *A. fumigatus* whole cell extracts using butanol, and separated by high performance thin layer chromatography (HPTLC) (Toledo et al., 2007). Eight *A. fumigatus* glycosylinositol phosphorylceramides were detected by this method, and two bands corresponding to proteins Af-3b and Af-4 were recognized by MEST-1, a β -1,6-Galf-specific IgG3 generated against *Paracoccidioides brasiliensis* glycolipids (Suzuki et al., 1997).

Bioassays using *Aspergillus*-specific antibodies and/or their targets can reveal information about the antigen's function (Fig. 4). The function of a specific protein can be investigated by gene overexpression using classical transformation (Stringer et al., 1991) or much more rapidly using gene knockdown approaches such as RNAi (Kalleda et al., 2013) and genome editing (Fuller et al., 2015). Neutralization (Luther et al., 2006) and adhesion assays (Peñalver et al., 1996) as well as animal models of aspergillosis (Paulussen et al., 2014) can be used to investigate the therapeutic applications of *Aspergillus*-specific antibodies.

6. Conclusion

The fungal kingdom has the broadest host range in our biosphere. Global warming, globalization, broad resistance to antibiotics and the ability to exploit new hosts will promote rare and emerging fungal infections and these will have a strong impact on our food security and ecosystems (Fisher et al., 2016; Fisher et al., 2012). New and efficient tools for the diagnosis and treatment of infections caused by these fungal pathogens will be indispensable, and pathogen-specific antibodies will certainly play an important role. Strategies to fractionate and identify antigens and characterize new targets for *Aspergillus*-specific antibodies generated from complex antigen mixtures can be used as a guideline for the identification of other fungal pathogen-specific antibodies with unknown antigens to track and diagnose fungal infections in plants, humans, insects and other animals. In basic research, antibodies specific to certain targets can be used to investigate the biosynthesis and development of the fungal cell, improving our understanding of the function of fungal proteins, carbohydrates and secondary metabolites. Additionally fungal pathogen-specific antibodies can help to identify unique targets that facilitate rapid and reliable diagnosis, e.g. using lateral flow devices (Sajid et al., 2015) and biosensors (Byrne et al., 2009). New antifungal strategies based on specific antibodies can be developed or the target antigens can be used to design more efficacious drugs (Magliani et al., 2012; Medici and Del Poeta, 2015). Moreover, these potential targets can be incorporated into plant protection strategies using non-transgenic approaches such as mutagenesis, or transgenic approaches such as RNAi (Koch et al., 2016), host-induced gene silencing (Chen et al., 2016) and genome editing to avoid crop losses by fungal pathogens.

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