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Proteomic Profiling of Serological Responses to *Aspergillus fumigatus* Antigens in Patients with Invasive Aspergillosis

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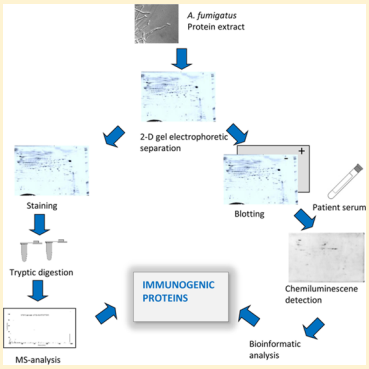
Supporting Information

ABSTRACT: *Aspergillus fumigatus* is the species that most commonly causes the opportunistic infection invasive aspergillosis (IA) in patients being treated for hematological malignancies. Little is known about the *A. fumigatus* proteins that trigger the production of *Aspergillus*-specific IgG antibodies during the course of IA. To characterize the serological response to *A. fumigatus* protein antigens, mycelial proteins were separated by 2-D gel electrophoresis. The gels were immunoblotted with sera from patients with probable and proven IA and control patients without IA. We identified 49 different fungal proteins, which gave a positive IgG antibody signal. Most of these antigens play a role in primary metabolism and stress responses. Overall, our analysis identified 18 novel protein antigens from *A. fumigatus*. To determine whether these antigens can be used as diagnostic or prognostic markers or exhibit a protective activity, we employed supervised machine learning with decision trees. We identified two candidates for further analysis, the protein antigens CpcB and Shm2. Heterologously produced Shm2 induced a strongly proinflammatory response in human peripheral blood mononuclear cells after *in vitro* stimulation. In contrast, CpcB did not activate the immune response of PBMCs. These findings could serve as the basis for the development of an immunotherapy of IA.



KEYWORDS: *Aspergillus fumigatus, protein antigens, immunoproteomics, invasive aspergillosis, decision trees*

INTRODUCTION

The filamentous fungus *Aspergillus fumigatus* is a ubiquitously distributed saprophyte, which degrades organic matter in terrestrial habitats such as soil and compost heaps. It usually propagates by the formation of asexually produced spores, which are called conidia. These are relatively small in size and are easily inhaled by humans, where they can reach the alveoli of the lung. In general, this does not pose a danger to healthy individuals, and spores can be easily cleared by the innate immune system.[1,2] However, *A. fumigatus* is able to cause allergic sensitization in immunocompetent individuals and life-threatening invasive pulmonary aspergillosis (IA) in patients with immunodeficiency or immunosuppression. Patients at risk of developing IA include those with hematological malignancies, hematopoietic stem cell or solid organ transplants, and chronic obstructive pulmonary disease.[3] It is estimated that worldwide around 200 000 cases of IA occur each year.[4] Despite the introduction of new antifungal compounds, IA still has a high mortality rate of around 50%.[4] One major reason for this unacceptably low survival is the late detection of IA. Clinical symptoms are often nonspecific, and high-resolution computed tomography (CT) scans of the chest have limited specificity.[5] The introduction of PCR-based assays and the testing of serum to detect the *Aspergillus* cell wall component galactomannan have improved diagnosis of IA in high risk patients, but sensitivity and specificity are still only moderate.[6] An alternative, culture-independent approach to diagnose

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fungal infections is to characterize the protein antigens reactive to serum from patients. Over 20 allergenic proteins from *A. fumigatus* with IgE binding activity have been identified in patients with asthma and allergic bronchopulmonary aspergillosis (ABPA). These allergens include heat shock and ribosomal proteins and metabolic as well as antioxidative enzymes.[7,8] Recent immunoproteomic studies, which combined 2-D gel electrophoresis with Western blotting to detect fungal target antigens, expanded the list of IgE reactive antigens including cell wall and secreted proteins as well as many metabolic enzymes by using sera from patients with ABPA.[9−12] Surprisingly little is known about proteins from *A. fumigatus* produced during the course of IA that are recognized by IgG antibodies. Although antigen presentation is impaired in many immunocompromised individuals at risk for IA;[13] an exploration of anti-*Aspergillus* antibodies in this patient group may lead to the discovery of prognostic antibody markers and protective antigens which may be used for vaccine development.[14−16] Indeed, patients with IA do produce anti-*Aspergillus* IgG antibodies.[17] A previous study of three recombinant *A. fumigatus* antigens by Sarfati et al. showed that a high level of anti-*A. fumigatus* protein antibodies in serum of patients at their admission to hospital was positively correlated with the occurrence of proven/probable IA.[18] A subsequent study confirmed an association between IgG responses against *A. fumigatus* protein antigens and the subsequent development of IA.[19]

The first attempt at a large-scale characterization of the serological response to *A. fumigatus* proteins in patients with IA using immunoblot analysis was performed in the 1980s.[20] However, the detected protein antigen was not characterized further. Later, de Repentigny et al. established a rabbit model of IA and detected antibodies against an immunodominant protein antigen in serum of infected animals.[21] Recently, with the advancement of protein identification technology, and the release of the *A. fumigatus* genome, 59 different *A. fumigatus* proteins were found, which provoked a protective immune response in rabbits challenged three times with *A. fumigatus* spores at intervals of several weeks.[22,23] In two recent studies, 17 and 22 different extracellular protein antigens from *A. fumigatus* were described, respectively. All were recognized by IgG antibodies from pooled sera of patients with probable/proven IA.[24,25] Among these proteins, the gliotoxin biosynthesis enzyme GliT turned out to have diagnostic value for the discrimination of IA patients from uninfected individuals.[26]

Here, we performed the first evaluation of the potential of immunoproteomic profiling of *Aspergillus* antibodies for diagnosis and outcome prediction in individual patients. Proteomics using 2-D gel electrophoretic separations of mycelial *A. fumigatus* proteins combined with immunoblotting revealed 49 different protein antigens in serum from 43 different patients (2 with proven and 22 with probable IA) at risk for the development of IA. Eighteen *A. fumigatus* proteins have not been described before as protein antigens. On the basis of decision tree classification, some of these proteins may exhibit an immunoprotective function and thus could be considered as potential immunotherapeutic agents. From these protein antigens, the metabolic enzyme serine hydroxymethyltransferase Shm2 exhibited high immunostimulatory activity in human PBMCs, illustrated by the expression of pro-inflammatory cytokine genes.

EXPERIMENTAL PROCEDURES

Patient Samples

Prior approval for the study was obtained from the St James's Hospital (SJH) Joint Research Ethics committee, Ireland, and the Ethical Committee of the University Hospital of Wuerzburg (UKW), Germany. Serum samples of patients undergoing remission-induction chemotherapy for acute leukemia, lymphoma, or myeloma, allogeneic bone marrow, or stem cell transplant (ASCT) were used for the detection of *A. fumigatus* specific IgG antibodies. Serum samples from 14 patients (63 serum samples from different time points) were collected from SJH and from 29 patients (92 serum samples from different time points) from UKW. The EORTC/MSG definitions were used for categorization of patients with invasive fungal diseases including IA.[27] For the categorization of patients, all CT scans were reported by local radiologists as part of clinical management and by an independent expert. Furthermore, PCR assays for the detection of *A. fumigatus* DNA and the Platelia sandwich enzyme-linked immunoabsorbent assay for the testing of galactomannan were performed as described by Rogers et al.[6]

Cultivation of *Aspergillus fumigatus*

A. fumigatus ATTC 46645[28] was grown in YPD broth (Carl Roth, Germany) at 37 °C as described by Brakhage and Van den Brulle.[29] Mycelium was harvested after 18−20 h by filtering the culture through Miracloth (Calbiochem). Mycelium was immediately frozen in liquid nitrogen and stored at −80 °C.

Sample Preparation for 2-D Gel Electrophoresis

Frozen mycelium was ground in a mortar in the presence of liquid nitrogen. Trichloroacetic acid (TCA)/acetone were added to 100 mg of this cell homogenate to precipitate proteins as described previously by Kniemeyer et al.[30] Protein pellets were air-dried and resuspended in 300 μL of sample buffer (7 M urea, 2 M thiourea, 2% [wt/vol] CHAPS (3-[3-choloamidopropyl)-dimethylammonio]-1-propanesulfonate), 1% [wt/vol] Zwittergent 3−10 and 20 mM Tris), sonicated for 10 min in an ultrasonic bath, and incubated for 1 h at room temperature. After centrifugation at 20 000 × *g* for 30 min at 16 °C, the supernatant was collected. The protein concentration was determined according to the method of Bradford[31] using the BIO-RAD protein assay (BIO-RAD Lab., Hartfordshire, USA).

2-D Gel Electrophoresis Analysis

A total of 100 μg of protein was applied via anodic cup loading to 11 cm IPG strips covering a nonlinear pH gradient from 3−11 (GE Healthcare Bio-Sciences). The IPG strips had been rehydrated overnight in the following buffer: 7 M urea, 2 M thiourea, 2% [wt/vol] CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate), 1% [wt/vol] Zwittergent 3−10, 0.002% [wt/vol] bromophenol blue, 0.5% [vol/vol] IPG buffer 3−11 NL, 1.2% [vol/vol] De-Streak reagent [GE Healthcare Bio-Sciences]). The following protocol was used for isoelectric focusing with an IPGphor II device (GE Healthcare Bio-Sciences): 4 h at 300 V, 4 h at 600 V, 4 h at 1000 V, 4 h at 8000 V, and 24 000 V hours at 8000 V. After separation by the first dimension, the strips were equilibrated for 15 min in 10 mL of equilibration buffer (6 M urea, 30% [vol/vol] glycerol, 2% [wt/vol] SDS (sodium dodecyl sulfate), 75 mM Tris, 0.002% [wt/vol] bromophenol blue) containing 1% (wt/vol) dithiothreitol, and subsequently for 15 min in 10 mL of equilibration buffer containing 2.5% (wt/vol) iodoacetamide.

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For the second dimension, the Criterion Dodeca cell system (Bio-Rad) was used for separating proteins on precast Criterion 12.5% Tris-HCl gels (stain-free formulation with a fluorescent trihalo compound). Separation was performed at 200 V. Proteins in the polyacrylamide gels and on the blotting membrane (after electrotransfer) were visualized by detecting the haloalkylated tryptophan fluorescence signals with the aid of the Criterion Stain Free Imager (Bio-Rad). In parallel, protein samples separated by gel electrophoresis were visualized by poststaining with colloidal Coomassie using a protocol from Candiano et al.[32] with slight modifications as described by Teutschbein et al.[33] Coomassie-stained gels were used for spot picking and protein identification. Image analysis was performed with Delta2D Version 3.6 (DECODON, Greifswald, Germany). Western blot images were matched first with the corresponding complete 2-D gel fluorescence image captured from the blotting membrane followed by matching with the fluorescence image captured from the gel. Finally gel images were matched with the Coomassie-stained master gel produced in parallel. Only spots found reproducibly in three replicate gels and Western blot signals on at least two replicate membranes were considered for further analyses. Western blot signals were quantified based on gray scale volumes excluding background using Delta 2D.

Western Blot Analysis

After two-dimensional separation, proteins were transferred onto a polyvinylidene fluoride (PVDF) blotting membrane for 2 h at 0.6 A using the Criterion Blotter system (Bio-Rad). Afterward, the membrane was equilibrated in 1 × TBS buffer (0.3 M NaCl, 20 mM Tris pH8, 1% [vol/vol] Tween 80), blocked for 1 h with 5% [wt/vol] BSA (Fraction V, Sigma) in 1 × TBS buffer, and finally incubated with patient serum (1:2000) overnight at 4 °C. Human IgG antibodies were detected with a goat antihuman IgG antibody (HRP conjugated, γ-chain specific, Millipore) that was diluted 1:10 000 in blocking buffer. After 1 h, the blotting membrane was washed three times with 1 × TBS-Tween, and remaining antibody conjugates were detected by using the Immobilon Western HRP Substrate (Merck Millipore) as detection reagent. Chemiluminescence signals were detected by the VersaDoc 4000 imaging system (BioRad) for 270 s. Three technical replicates were performed per serum sample.

Protein Identification by MALDI-TOF/TOF Analysis

Protein spots reacting with patient antibodies were excised from Coomassie-stained gels, which had been run in parallel to the blotted gels. Protein spots were digested with trypsin according to the protocol of Shevchenko et al.[34] Extracted peptides were identified on an Ultraflex I MALDI-TOF/TOF device using flexControl 3.0 for data collection and flexAnalysis 3.0 spectra analysis/peak list generation (Bruker Daltonics, Germany). Peptide mass fingerprint (PMF) and peptide fragmentation fingerprint (PFF) spectra were submitted to the MASCOT server (MASCOT 2.1.03, Matrix Science, UK), searching the NCBI database limited to the taxon Fungi. With respect to the sample preparation, fixed modifications of cysteine thiols to S-carbamidomethyl derivatives and variable methionine oxidation were defined for the database search. Further, up to one missed cleavage and a peptide mass tolerance of 50 ppm was allowed. Results were regarded as significant with an allowed likelihood for a random hit of $p \leq 0.05$, according to the MASCOT score. Identified proteins were classified with the FungiFun annotation tool as described by Priebe et al. based on the Functional Catalogue (FunCat) annotation scheme.[35] All proteome data were imported into our in-house data warehouse Omnifung (http://www.omnifung.hki-jena.de).

Recombinant Protein Production of CpcB and Shm2

The recombinant proteins CpcB and Shm2 were produced as described by Bacher et al.[36] In summary, the open reading frames of the genes *shm2* (AFUA_3G09320) and *cpcB* (AFUA_4G13170) were amplified from cDNA derived from *A. fumigatus* strain ATCC46645 and cloned into the expression vector pET43.1H6 for recombinant expression as HIS-tagged proteins. Both proteins were purified by affinity chromatography using an Äkta explorer purification system (GE Healthcare). Endotoxin content of the purified proteins was determined (Pierce LAL Chromogenic Endotoxin Quantitation Kit, Thermo Fisher Scientific) and minimized to <0.15 ng/mL (equivalent to 1.5 EU/ml; Pierce High Capacity Endotoxin Removal Resin, Thermo Fisher Scientific) for subsequent stimulation experiments with human peripheral blood mononuclear cells (PBMC).

Isolation of PBMCs, Stimulation with Purified *A. fumigatus* Proteins, and Characterization of Immune Cell Activation

PBMCs were freshly isolated from leukocyte concentrates obtained from healthy volunteers using Ficoll-Hypaque (Biochrome AG) density gradient centrifugation. PBMCs (2.5×10^6 in 1 mL) were incubated in serum-free Cellgro DC medium (CellGenix) at 37 °C for 18 h in the presence of different concentrations of Shm2 (0.5−5 μg/mL) and CpcB (1−10 μg/mL) or without any stimulus. PBMCs were analyzed for cell surface activation marker levels, cytokine release, and cytokine gene expression profiles. For the analysis of surface activation markers, PBMCs (5×10^5) were stained with fluorescent-labeled antibodies (CD80-APC, CD69-APC, CD25-FITC) and analyzed by flow cytometry (FACS Calibur, BD). In parallel, PBMC culture supernatants were analyzed by ELISA assays for Interferon gamma (IFN-γ) quantification (Human IFN-γ ELISA, Biolegend). The remaining PBMCs (2×10^6) were taken for cytokine gene expression profiling of *TNFA* (tumor necrosis factor alpha), *IL1B* (interleukin 1 beta), *IFNG* (interferon gamma), *IL4* (interleukin 4), and *IL10* (interleukin 10), respectively. RNA was isolated (RNeasy Mini Kit, Qiagen), *in vitro* transcribed into cDNA (First Strand cDNA Synthesis Kit, Thermo Fisher Scientific), and analyzed by qPCR assays (iTaq Universal SYBR Green Supermix, Bio Rad; StepOnePlus, Applied Biosystems) using the primers (TNFA 5′-TGCTTGTTCCTCAGCCTCTT-3′ and 5′-TGGGCTACAGGCTTGTCACT-3′; IL1B 5′-GGACAAGCTGAGGAAGATGC-3′ and 5′-TAGTTATCCCATGTGTCGAA-3′; IFNG 5′-GCATCCAAAAGAGTGTGGAG-3′ and 5′-GCAGGCAGGACAACCATTAC-3′; IL4 5′-TGTGCTCCGGCAGTTCTACA-3′ and 5′-GACAGGAATTCAAGCCCGCC-3′; IL10 5′-TTACCTGGAGGAGGTGATGC-3′ and 5′-GGCCTTGCTCTTGTTTTCAC-3′). Results were normalized against the house keeping gene ALAS (aminolevulinic acid synthase1; primers 5′-GGCAGCACAGATGAATCAGA-3′ and 5′-CCTCCATCGGTTTTCACACT-3′).

Bioinformatic Analyses

The immunoblot data were analyzed with supervised machine learning based on decision tree induction. For the induction of decision trees, the WEKA[37] java implementation J48 of the C4.5 algorithm of Quinlan in revision 8 was used through an

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interface provided by the R package RWeka.[38,39] We calculated different decision trees based on different classifications: (i) Revised EORTC definitions of IA,[27] (ii) *Aspergillus* galactomannan (GM) ELISA test results, (iii) GM test result and/or the ITS *Aspergillus*-PCR assay, and (iv) outcome of the disease. Decision trees were constructed for the entire set of patient data and different subgroups of patients. Positive signals for protein antigen detection were only included in the analysis if they were found in at least two different patients. For the induction of decision trees for the prediction of disease outcome, only patients with probable or proven IA were included. For each classification (i–iv and different groups), different parameter settings were used. We selected the best tree per classification and validated final decision trees with leave-one-out cross-validation (LOOCV).

RESULTS

Characterization of the Patients

Sera from a total number of 43 patients undergoing chemotherapy for acute leukemia, lymphoma, myeloma, or allogeneic stem cell transplant were selected for immunoproteomic analysis. The numbers of cases of proven, probable IA were 2 and 22, respectively, according to EORTC/MSG criteria.[27] Nineteen patients were diagnosed as unclassified and were regarded as negative controls. The majority of probable cases in both centers were ASCT recipients (for details see also Table 1 and Rogers et al.[6]). Between three and five serum samples per patient collected at different time points were used for Western blot analysis.

Table 1. Characteristics of the Patients Included in This Study

parameter	value(s)
no. of patients	43
no. of males	22
no. of females	21
median age (yr) of males (range)	50 (20–69)
median age (yr) of females (range)	48 (19–69)
no. of AML patients	22
no. of ALL patients	7
no. of patients with other underlying diseases[a]	14
no. of allogenic stem cell recipients	26

[a]Including chronic lymphoblastic leukemia (CLL) (n = 3), multiple myeloma (n = 3), lymphoma (n = 5), immunocytoma (n = 1), and myelofibrosis (n = 2).

Identified Immunoreactive Proteins

The analysis of Western-blot signals (Figure 1) and the comparison with the corresponding Coomassie-stained gels revealed a total of 57 distinct immunoreactive spots separated from mycelial protein extracts of *A. fumigatus* (Figure 2; Supplementary Table S1). These spots corresponded to 49 individual proteins, many of which were involved in central metabolic pathways (Table 2). Several proteins were identified in more than one spot, which may be explained by posttranslational modifications. In other cases, more than one protein was detected from one Coomassie-stained spot (spot numbers followed by a character in Table 2). This could be the result of incompletely separated proteins. In a few cases, Western blot signals could not be unambiguously matched to a corresponding protein spot, or could not be reproduced in technical replicates or protein spots could not be identified by mass spectrometry.

Peptide mass fingerprinting (PMF) data were evaluated based on Mascot scores and sequence coverage, which ranged from 43.7–210 and 16.6%–75%, respectively (see Supplementary Table S2). For 26 proteins, PMF identification data were confirmed by MS/MS analysis. Of the 49 identified proteins, 32 have already been described as allergens or antigens in patients suffering from ABPA or IA[12,24,25] or in a rabbit model of IA.[22] For example, enzymes of the glycolytic pathway have often been described as immunogenic.[12,22] Among them is enolase, which is known to provoke an immune response in patients with allergic *Aspergillus* infections and is designated as allergen Asp F22.[40] Also enzymes of the TCA-cycle, amino acid metabolism, the oxidative stress response, and ribosomal proteins are known antigens (Figure S1). However, we identified 18 novel protein antigens that reacted with IgGs of human serum and that substantially broaden the list of *A. fumigatus* antigens (Table 2). On the basis of functional catalogue (FunCat) categorization with the FungiFun-tool, several of these new protein antigens fall into the category of mitochondrial proteins, enzymes involved in carbohydrate interconversion, translation, and amino acid metabolism (Figure S1). Interestingly, proteins occurring only in filamentous fungi were found among the detected *A. fumigatus* antigens: the major Woronin body protein HexA[41] and the putative UDP-galactopyranose mutase AFUA_3G12690/GlfA.[42] Woronin bodies are involved in occluding pores of septa between hyphal compartments, whereas GlfA catalyzes the second step in the biosynthesis of the cell wall compound galactofuranose.

Recently, a 2-D proteome reference map was published for the mycelial growth form of *A. fumigatus*.[43] Interestingly, among the 10% of proteins with the highest abundance (39 different proteins) 21 were recognized in the immunoblots shown here by anti-*Aspergillus* IgG antibodies from patient sera (Table 2). In another study, the hyphal proteome of *A. fumigatus* was investigated via LC–MS/MS.[15] The 20 most abundant proteins from the *A. fumigatus* protein extract were quantified. Again, 13 of the top 20 proteins were found to provoke an immune response in our experiments (Table 2). Fourteen immunogenic proteins have also been shown to be prevalent in the proteome of dormant conidia (Table 2).[33] This suggests that highly abundant fungal proteins have a higher probability of inducing a humoral immune response in humans.

Serum from both patients with proven/probable IA and from negative controls contained IgGs recognizing *A. fumigatus* protein spots. The number of different antigen signals varied from patient to patient and ranged between 0 and 19 different signals. Small differences were observed in the type and number of anti-*A. fumigatus* IgGs between serum samples from each patient. Eight different proteins were detected by 25% or more of the patient sera tested (Table 2 and Figure 3). For example, the mitochondrial processing peptidase AFUA_1G14200 (spot 20) provoked an immune response in more than half of the investigated patients (n = 28), followed by spot 12, the translation elongation factor eEF-1 alpha subunit (19 patients), and spot 10, the serine hydroxymethyltransferase Shm2 (17 patients). All three protein spots showed high levels of immunoreactivity when considering immunoblot signal intensities (Figure 3). Moreover, 12 out of 19 patients with anti-eEF-1 alpha antibodies, 16 out of 26 patients with antipeptidase AFUA_1G14200 antibodies and 6 out of 17 patients with anti-

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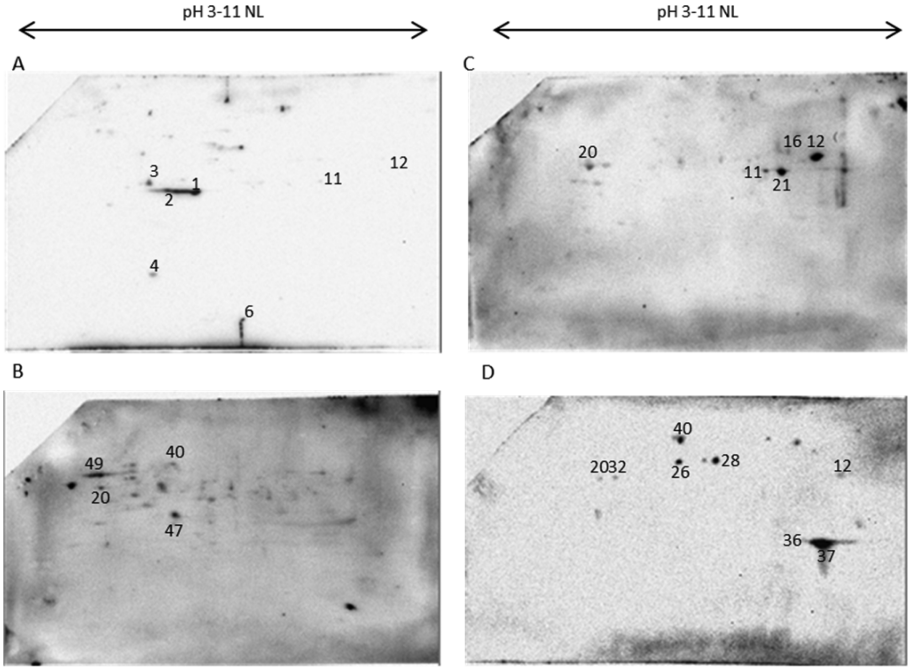

Figure 1. 2-D immunoblots of sera (W09−08_1.1, W06−09_6.1) from patients with indeterminate diagnosis (A, B) or patients (W03−09_2.2, W03−09_4.3) with probable IA (C, D). The numbers next to the chemiluminescence signals refer to the protein spots listed in Table 2. NL = nonlinear.

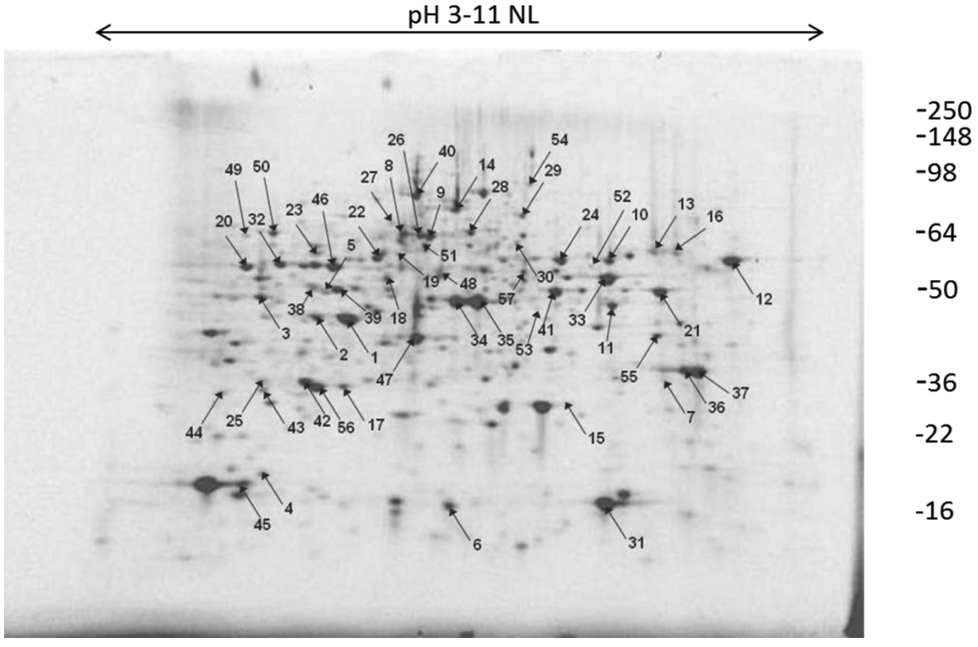

Figure 2. Coomassie-stained 2-D map of the *A. fumigatus* mycelial proteome. Labeled spots represent the *A. fumigatus* antigenic proteins that were detected with serum from the patient population. Spot numbers refer to those listed in Table 2.

Shm2 antibodies, respectively, were diagnosed with probable IA. Nevertheless, on first examination, no clear difference in the antigen pattern could be observed between patients with proven/probable aspergillosis and the control group. Even when prevalence and average volumes of immunogenic protein spots were plotted (Figure 3), no clear picture emerged. However, it is evident from the plot that many protein antigen-specific *A. fumigatus* antibodies occur only in a very limited number of patients. Because of these difficulties, we employed different bioinformatic approaches to evaluate the data in more detail and to elucidate whether the information on the number and type of anti-*A. fumigatus* IgGs present in patients could be used to diagnose IA or to predict the outcome of infection. Therefore, in further analyses, we applied supervised machine learning techniques.

Bioinformatic Analysis of the Serological Response Pattern

The immunoblot data were analyzed with the supervised machine learning method decision trees. Supervised learning methods use data sets for which an outcome is known (training data) to learn decision rules for the classification of new, unseen

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Table 2. Antigenic Proteins Identified through Immunoblotting of *A. fumigatus* Mycelial Extract with Sera from Patients with Haematological Malignancies[a]

spot-ID	Mr	pI	locus tag	Incidence	description
Glycolysis/Fermentation					
2	39.8	5.5	AFUA_3G11690	4	Fructose-bisphosphate aldolase, class II[c,f,g,h]
8b, 26	63.0	6.1	**AFUA_3G11070**	**11**	**Pyruvate decarboxylase PdcA**[b,e]
9	60.5	6.3	AFUA_3G11830	2	Phosphoglucomutase PgmA[e]
11	37.2	8.7	AFUA_5G06240	7	Alcohol dehydrogenase AlcC[c,e,g]
25, 42, 43, 56b	28.1	5.8	AFUA_5G13450	2	Triosephosphate isomerase[c,e]
32	47.3	5.3	AFUA_6G06770	10	Enolase/allergen Asp F22[b,c,d,e,g,h]
48	44.7	6.3	AFUA_1G10350	7	Phosphoglycerate kinase PgkA[b,c,d,e,g,h]
49	52.0	6.3	AFUA_7G05720	3	Pyruvate dehydrogenase complex, dihydrolipoamide acetyltransferase[g,h]
50	57.4	5.4	AFUA_3G09290	1	2,3-Bisphosphoglycerate-independent phosphoglycerate mutase[d,e,g,h]
53	36.9	6.7	AFUA_4G08240	2	Zinc-containing alcohol dehydrogenase[g]
Glycerol Metabolism					
1	36.8	5.9	AFUA_4G11730	4	Glycerol-dehydrogenase GldB[I,d]
TCA Cycle					
27	71.1	6.5	AFUA_3G07810	3	Succinate dehydrogenase subunit Sdh1[e,h]
33	52.1	9.2	AFUA_5G04230	5	Citrate synthase Cit1[c,d,e,g,h]
40	85.5	6.3	AFUA_6G12930	7	Mitochondrial aconitate hydratase AcoA[c,d,e,g]
Electron Transport and Oxidative Phosphorylation					
4	21.5	6.2	AFUA_2G03010	2	Cytochrome C subunit Vb[I]
7, 36, 37	36.9	10.0	**AFUA_4G06910**	**13**	**Outer mitochondrial membrane protein porin**[I,c]
13, 16	59.9	9.7	**AFUA_8G05320**	**12**	**Mitochondrial F1 ATPase subunit alpha**[I,c,d]
20	53.2	5.9	**AFUA_1G14200**	**26**	**Mitochondrial processing peptidase**[d,h]
55	36.2	9.6	AFUA_1G04540	1	NADH-cytochrome b5 reductase[I]
Pentose Phosphate Pathway					
14	74.8	6.1	AFUA_1G13500	2	Transketolase TktA[b,c,d,e]
Carbohydrate Interconversion					
30	56.9	6.4	AFUA_7G01830	1	UTP-glucose-1-phosphate uridylyltransferase Ugp1[I]
51a	60.4	5.7	AFUA_3G12690	2	UDP-galactopyranose mutase GlfA (galactofuranose biosynthesis)[I]
Vitamin and Cofactor Biosynthesis					
38a, 39b	50.1	7.9	AFUA_1G07480	1	Coproporphyrinogen III oxidase[e]
C1-Metabolism					
34, 35	45.7	9.2	AFUA_6G04920	2	NAD-dependent formate dehydrogenase AciA/Fdh[b,d,e,g]
Amino Acid Metabolism					
3	39.9	5.4	AFUA_4G13120	3	Glutamine synthetase[b,c,g,h]
10	51.8	8.6	**AFUA_3G09320**	**17**	**Serin hydroxymethyltransferase Shm2**[c,e,g]
18	48.5	5.8	AFUA_1G10130	7	Adenosylhomocysteinase[b,c,g]
19	50.2	5.8	AFUA_2G04220	1	Homogentisate 1,2 dioxygenase (HmgA)[I]
21, 41	47.9	9.5	AFUA_4G10410	10	Aspartate aminotransferase[c,e]
22, 23	55.1	5.6	AFUA_6G07770	2	Alanine aminotransferase[c,g]
24, 52	55.2	9.4	**AFUA_5G06680**	**15**	**4-Aminobutyrate transaminase**[I]
38b	42.2	5.6	AFUA_1G10630	1	S-Adenosylmethionine synthetase[c,g]
46	49.3	5.7	AFUA_4G06620	1	Glutamate/Leucine/Phenylalanine/Valine dehydrogenase[b,c,e,g,h]
Purine, Pyrimidine Biosynthesis					
31	16.9	7.4	AFUA_5G03490	1	Nucleoside diphosphate kinase[I,b,c]
Translation and Protein Biosynthesis					
12	54.0	9.2	**AFUA_1G06390**	**19**	**Translation elongation factor eEF-1 alpha subunit**[I,b,c,d]
29	71.0	6.2	AFUA_2G16010	2	Prolyl-tRNA synthetase[I]
47	35.0	6.1	**AFUA_4G13170**	**12**	**G-protein complex beta subunit CpcB**[d,f,h]
54	93.1	6.5	AFUA_2G13530	4	Translation elongation factor EF-2 subunit[b,c,d,g,h]
57	54.1	7.8	AFUA_6G04570	4	Translation elongation factor eEF-1 subunit gamma[b,h]
Protein Fate					
5, 38c, 39a	69.6	4.9	AFUA_1G07440	1	Molecular chaperon HSP 70[b,c,g,h]
6	16.5	6.4	AFUA_3G06030	3	Ubiquitin-conjugating enzyme[I]
Cell Rescue, Defense, Virulence					
43b, 44, 56	23.4	5.3	AFUA_4G08580	1	Mitochondrial peroxiredoxin Prx1[I]
45	18.5	5.4	AFUA_6G02280		Allergen AspF3[j,b,c,e]

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Table 2. continued

spot-ID	Mr	pI	locus tag	Incidence	description
Secondary Metabolism					
17	20.9	5.9	AFUA_6G12220	1	Isochorismatase family hydrolase[I]
Cytoskeleton/Septum-Associated Structure					
15	61.4	9.3	AFUA_5G08830	3	Woronin body protein HexA[I]
Miscellaneous					
8	60.2	6.4	AFUA_8G04090	2	Choline oxidase (CodA)[I]
22b	52.9	5.9	AFUA_6G10660	2	ATP citrate lyase subunit[g]
28	58.1	6.3	AFUA_8G05580	4	Putative coenzyme A transferase CoaT[I]
51b	54.7	6.1	AFUA_2G11940	2	Adenylosuccinate lyase Ade13[e]

[a]Proteins written in bold provoked a response in at least 25% of all patients (≥11). [b]High abundant protein in the study of Champer et al.[15] [c]High abundant mycelial protein in the study of Vödisch et al.[43] [d]High abundant conidial protein in the study of Teutschbein et al.[33] [e]Described as immunogenic in rabbit model of IA by Asif et al.[22] [f]Described as immunogenic in IA patients by Shi et al.[24] [g]Described as allergic by Singh et al.[11,12] [h]Described as immunogenic in IA patients by Virginio et al.[25] [I]First described here as *A. fumigatus* protein antigen. [j]Antibodies had been found in patient, which were later on excluded from the analysis.

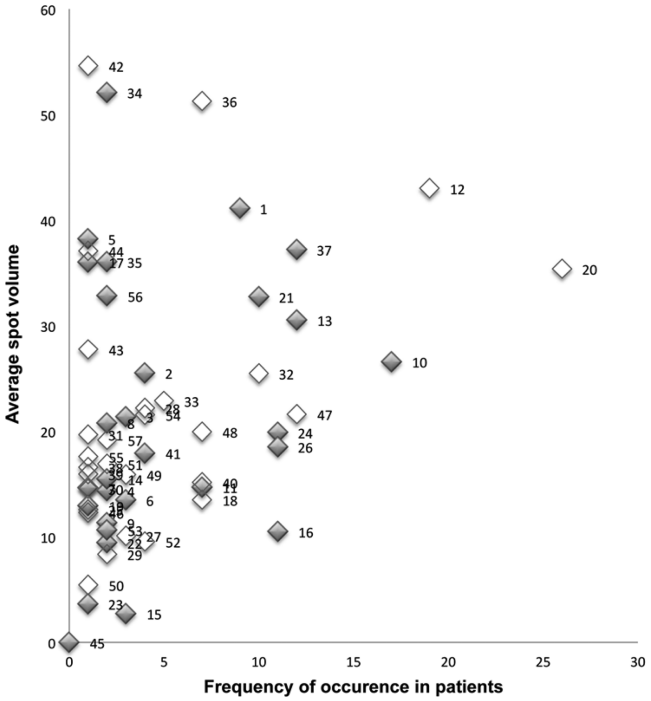

Figure 3. Prevalence and average volume of immunogenic protein spots of *Aspergillus fumigatus* in 2-D immunoblots with sera from patients with proven or probable IA and controls (at-risk patients without evidence of IA). The relation between prevalence of protein antigen-specific antibodies in patients and average volume of Western blot signals is presented. Open diamonds represent protein spots, which were predominantly recognized by sera from patients with probable or proven IA (more than 50%).

data sets and thereby describing the structural patterns of data. These patterns may reflect potential relationships and markers. For the induction of decision trees, the WEKA java implementation J48 of the C4.5 algorithm of Quinlan in revision 8 was used through an interface provided by the R package RWeka.[38,39] We calculated different decision trees based on different classifications: (i) revised EORTC definitions of IA (indeterminate versus proven/probable), (ii) *Aspergillus* galactomannan (GM) ELISA test results, (iii) GM test result or the ITS *Aspergillus*-PCR assay, and (iv) survival of patients with probable/proven IA. Decision trees were constructed for (a) the entire set of patient data and for the following subgroups of patients: (b) patients from UKW, (c) patients from SJH, (d) patients with ASCT, (e) patients without ASCT, (f) patients with an age above 60 years, and (g) patients with younger age (between 19 and 60). With these different groups (a−g), we checked whether specific subgroups of patients allow to build an improved predictive model. For each classification (i−iv) and group (a−g), a set decision tree was created with different parameter settings. We selected the best tree per classification and group in the following ways. (1) We selected all small trees with at maximum eight leafs to obtain interpretable trees. (2) We selected the tree out of set 1 with the best G-mean.[44] The G-mean is the geometric mean of the TP rates, thus taking into account all classifications [e.g., indeterminate vs proven/probable (i) or alive vs deceased (iv)]. Compared to the arithmetic mean, the geometric mean has the advantage that it gives smaller values more weight. The final decision trees were validated with LOOCV.

Decision trees for the classification of EORTC criteria and patient's disease outcome showed the best true positive rates (weighted average) and were considered further. The true positive rates (TP rate) for the calculated decision trees for the EORTC criteria (indeterminate vs proven/probable) varied between 0.780 and 0.967 (weighted average, LOOCV: 0.605−0.900). Interestingly, decision trees for patients from only one location, either UKW or SJH, had TP rates of 0.967 and 0.867, respectively (0.900 and 0.667 LOOCV), thus higher values than the trees for the complete patient data set (Figure S2). This finding suggests that some differences in the patient characteristics exist between the two hospitals and illustrates that heterogeneity in the underlying disease of patients and immune response impedes the construction of robust decision trees for diagnosis. In summary, more patient data are required for the construction of decision trees with an overall good performance for diagnostic classification. Therefore, we investigated the prognostic potential of our approach instead of the diagnostic potential in more detail and evaluated the ability to find protein antigens that exhibit immunoprotective activity.

Decision trees calculated for the prediction of disease outcome were more robust, and TP rates between 0.833 and 1 were calculated (Figure 4 and data not shown). The decision tree based on all patient data revealed that a serological response to spot 47 (WD repeat protein CpcB), spot 36 (outer mitochondrial membrane protein porin), and spot 21 (aspartate aminotransferase) was associated with survival of patients with probable IA. In contrast, the occurrence of IgGs directed against spot 15 (Woronin body protein HexA), spot

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Class	TP Rate	FP Rate	Precision	F-Measure
Alive	0.868 (0.830)	0.226 (0.419)	0.868 (0.772)	0.868 (0.800)
Deceased	0.774 (0.581)	0.132 (0.170)	0.774 (0.581)	0.774 (0.621)
Weighted Avg.	0.833 (0.783)	0.191 (0.327)	0.833 (0.733)	0.833 (0.734)

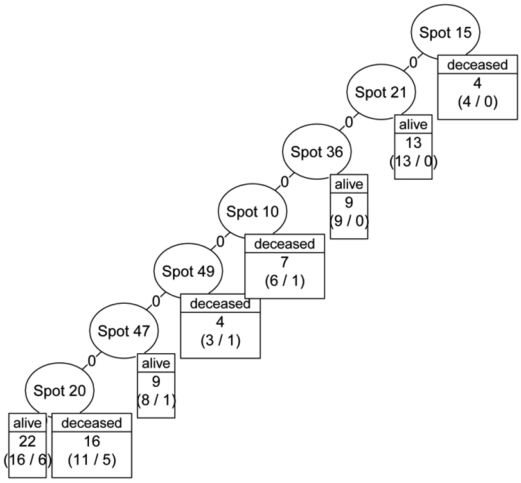

Figure 4. Best decision tree for the classification of the serum of patients for outcome prediction (death/survival) of patients suffering from IA based on immunoblot signal patterns. The true positive (TP) rate and the complementary false positive (FP) rate, precision (TP/[TP/FP]), and the F-Measure (a measure of the test's accuracy) were used for the evaluation of the decision tree (values of the leave-one-out cross validation are given in brackets). The numbers under the leaf nodes state the number of observations classified within this leaf node. In brackets, the number of true positive and false positive classified samples is given (TP/FP).

10 (serine hydroxyl methyltransferase Shm2), spot 49 (pyruvate dehydrogenase complex), and spot 20 (mitochondrial processing peptidase beta subunit) predicted a fatal outcome in patients with IA. Some of these proteins may have potential as prognostic markers and may also be suitable for immunotherapy against IA. To further characterize the features of these *A. fumigatus* protein antigens we produced recombinant CpcB (spot 47) and serine hydroxyl methyltransferase (Shm2, spot 10) in *E. coli*. The immunological properties of the purified proteins were tested in coincubation experiments with human PBMCs.

Shm2 but Not CpcB Activates the Immune Response of PBMCs

Stimulation of human PBMCs with Shm2 induced a pro-inflammatory response. In contrast, CpcB did not provoke any PBMC activation (Figure 5). Shm2 induced the expression of myeloid (CD80) as well as lymphoid (CD69, CD25) activation markers (Figure 5A–C) on PBMCs. Lymphoid activation markers were induced dependent on the antigen concentration (0.5, 1, 5 μg/mL); CpcB did not induce any activation markers. Expression of pro-inflammatory cytokine genes (*TNFA*, *IL1B*, and *IFNG*) was induced after stimulation with 0.5 μg/mL of Shm2 (Figure 5E–H). Interestingly, Shm2 did not alter gene expression of the anti-inflammatory cytokine *IL10* and reduced the expression level of *IL4*. CpcB did not alter the expression of the pro- and anti-inflammatory cytokines analyzed in this study. In addition, the cytokine concentrations of culture supernatants of PBMCs, stimulated with Shm2 or CpcB were analyzed (Figure 5I). Again, Shm2 induced the secretion of IFN-γ, whereas PBMCs treated with CpcB did not alter cytokine levels, confirming our gene expression profiling data.

DISCUSSION

The early diagnosis of IA is essential for the successful treatment of patients but remains difficult.[45] Various attempts have been made to use circulating anti-*Aspergillus* antibodies in the serum of patients for the diagnosis of IA. However, results from these studies have been contradictory, and doubts were raised about whether the poor antibody response seen in severely immunocompromised patients could be used for diagnosis.[13,46] Nevertheless, recent immunoproteomic studies with pooled sera from patients with IA[24,25] revealed that a relatively high diversity of IgG anti-*A. fumigatus* antibodies is present in patients with IA and that IgG antibodies specific to the secreted *A. fumigatus* gliotoxin oxidase GliT have diagnostic value in non-neutropenic IA patients. Previous studies on the serological response to *A. fumigatus* protein antigens either investigated only pooled sera[24,25] or selected protein antigens.[18,47,48] The aim of our study was (1) to broaden the list of *A. fumigatus* protein antigens, (2) to assess the frequency of occurrence of specific anti-*A. fumigatus* IgG antibodies among risk patients, and (3) to find candidate proteins suitable for diagnosis, prognosis, or for an immunotherapy of IA. The mycelium we used for our experiments was harvested at the trophophase after 18–20 h of cultivation, when a network of hyphae (mycelium) was formed. Hyphal growth is a hallmark of an invasive infection of *A. fumigatus* and serum antibodies directed against hyphal-specific *A. fumigatus* proteins may represent potential biomarkers for the diagnosis of IA.

Our immunoproteomics approach found in total 49 different protein antigens *from A. fumigatus*, of which 31 had already been described before by other groups.[11,12,22,24,25] Among these, antibodies were most frequently found against the well-characterized allergen enolase Asp F22[7] and the pyruvate decarboxylase PdcA. The latter as well as the antigenic alcohol dehydrogenase AlcC represent the primary enzymes involved in *A. fumigatus* ethanol fermentation.[49] The presence of anti-PdcA and anti-AlcC antibodies in human serum suggests that these enzymes are also formed and consequently ethanol is produced under *in vivo* conditions.

However, among the newly identified antigens, many mitochondrial proteins provoked an immune response in patients. The mitochondrial processing peptidase AFUA_1G14200 was the most frequently found protein antigen in patient sera (26 out of 43). It had already been described before as antigenic,[25] and it is part of the matrix processing peptidase complex of mitochondria[50] and belongs to the 40 most abundant proteins in the proteome of dormant conidia.[33] This may explain the frequent appearance of antibodies in sera of patients since conidia of *A. fumigatus* are widely distributed in the environment and are inhaled daily.[51] Also 13 other proteins with antigenic activity including the new antigens F1 ATPase subunit alpha and the translation elongation factor eEF-1 alpha subunit belong to this group of proteins that occur in large quantities in resting conidia (Table 2). In general, it is striking that many immunological response provoking proteins in humans belong to the most abundant proteins in the proteome of *A. fumigatus* conidia or mycelium (see Table 2). This supports the hypothesis that the efficiency of antigen presentation via the major histocompatibility complex (MHC) correlates with the abundance of the mature protein.[52] However, this statement must be qualified by saying that the 2-D gel electrophoresis technique is less efficient at

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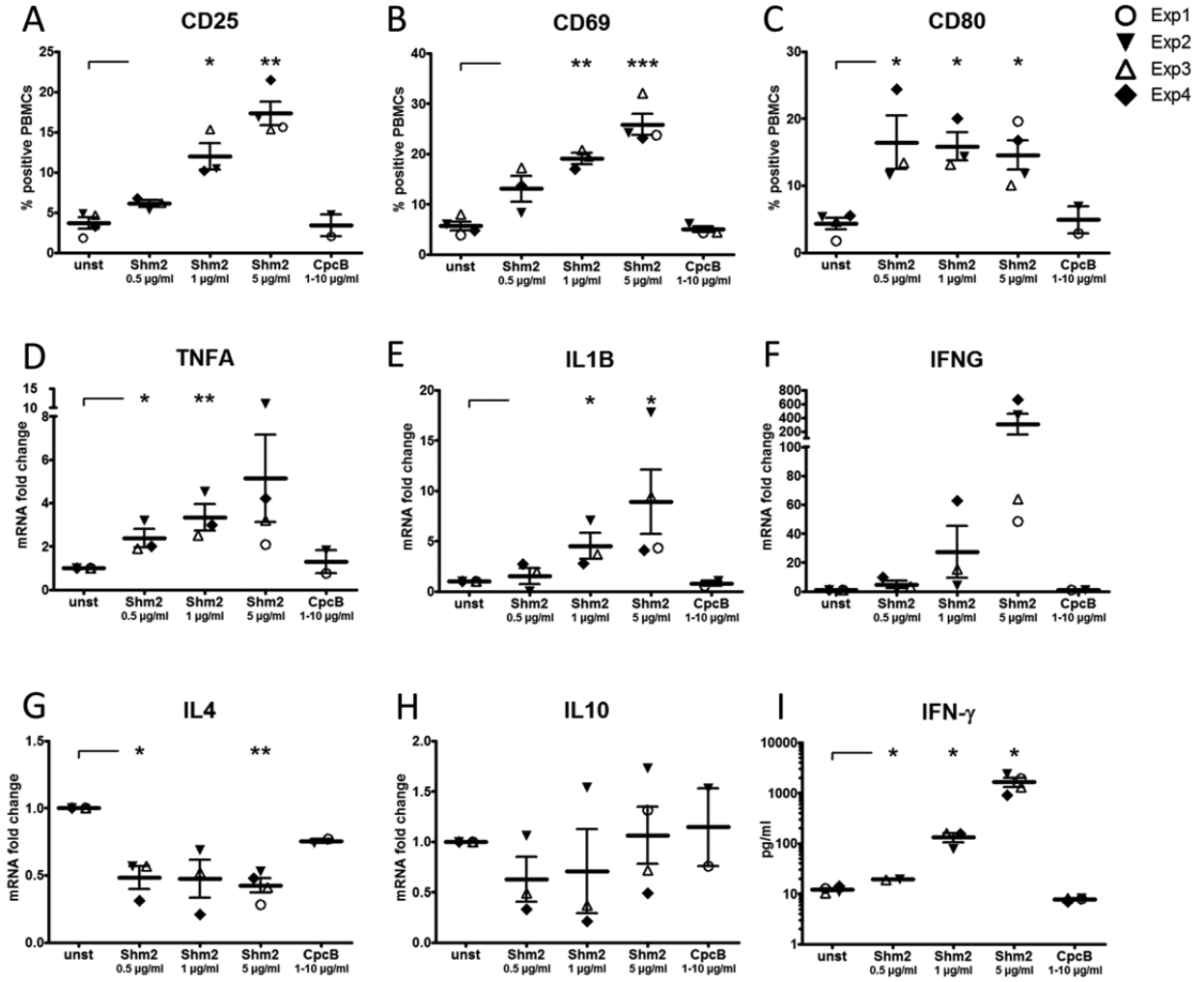

Figure 5. Shm2 induced immune response of human PBMCs. (A–C) Shm2 up-regulated surface activation markers CD25, CD69, and CD80. (D–H) Shm2 induces mRNA expression of pro-inflammatory cytokines TNFA, IL1B, and IFNG. Gene expression of anti-inflammatory cytokines IL4 and IL10 was reduced or not altered, respectively. (I) IFN-γ secretion to culture supernatant was induced by Shm2 but not by CpcB. Data collected in four independent experiments are plotted, and mean-values ± SEM are given. Significant differences are indicated by asterisks (∗, $p < 0.05$; ∗∗, $p < 0.01$; ∗∗∗, $p < 0.001$; one way repeated measures ANOVA followed by Bonferroni's multiple comparison test). Three different concentrations were tested for CpcB (1, 5, and 10 μg), and average values are depicted.

separating low abundance proteins and 2-D gels are dominated by proteins with high abundance.

Many of the antigenic proteins identified in *A. fumigatus* are highly conserved among fungi and other eukaryotic microorganisms. For this reason, cross reactivity with antigens from different fungi cannot be excluded and has already been reported.[53,54] Therefore, sera from control patients are probably able to detect *A. fumigatus* proteins either due to induction of antibodies by commensal fungi (e.g., *Candida albicans*) or due to the fact that humans inhale numerous conidia from *A. fumigatus* or other molds daily.[55] Nevertheless, some of the newly described protein antigens detected in this study are found exclusively in filamentous fungi, namely, UDP-galactopyranose mutase GlfA and the Woronin body protein HexA. However, anti-HexA antibodies were identified in both noninfected and putatively infected patients, while anti-GlfA antibodies were only found in two patients, who were classified as probable cases of IA.

Until now, the immunoproteomic profiling of individual patient sera did not lead to the discovery of an immunodominant *A. fumigatus* protein antigen that can induce an antibody response in the majority of patients. Overall, the anti-*A. fumigatus* IgG antibody profile varies considerably between individual IA risk patients. Even the aforementioned mitochondrial processing peptidase only provoked a humoral response in 60% of all patients. It can, therefore, be concluded that a single protein antigen from *A. fumigatus* is not appropriate for the diagnosis or prognosis of IA. This may explain at least in part why no clear picture emerged from clinical studies that compared IgG responses against selected recombinant *A. fumigatus* proteins in sera of hematopoietic stem cell transplant recipients.[13,18,47] Moreover, our data demonstrate that, with one exception, all the investigated patient sera contained anti-*A. fumigatus* antibodies, which suggests that the patient's immune system was consequently able to mount an antibody response. Similar results have been reported from a previous study, which followed up the anti-*Aspergillus* antibody response in bone marrow transplant patients.[17] However, neither the number of different anti-*A. fumigatus* antibodies nor the dynamic changes of anti-*Aspergillus* IgGs in patient serum correlated well with the infection state (negative versus proven/probable cases) or the disease outcome.

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For a deeper exploration of the immunoproteomic data, we applied decision tree induction as a supervised learning method. Altogether, patient antibody responses were found to be of only limited diagnostic value, when all samples were included in the analysis. Rather, the IgG profiling revealed the problem that IA diagnosis remains challenging due to the high heterogeneity of the patient group. This is illustrated by the fact that the decision trees calculated for each single hospital resulted in trees with much higher diagnostic precision; here, in particular, the decision tree for patients of the UKW gave good TP and FP rates. Thus, serological profiling of the anti-*Aspergillus* response is likely more suitable for a homogeneous patient cohort. A critical point of immunoproteomic studies, which are based on 2D-gel electrophoresis, is the allocation of Western blot signals to the stained protein spots from 2D gels. We took advantage of the stain-free technology to optimize this procedure. Nevertheless, further studies with additional patient sera are necessary for the validation of the results and for further narrowing down the list of suitable biomarker candidates.

Besides diagnosis, the antibody response against *A. fumigatus* could also be used as a prognostic tool and as a method to discover immunoprotective protein antigens. Although cell-mediated immunity is regarded as the predominant host defense mechanism against fungi, there is growing evidence that certain antibodies can modify the course of fungal infection by either being protective or disease-enhancing.[56] Our decision tree, which set prediction rules for disease outcome in patients with IA, revealed that some specific anti-*Aspergillus* antibodies are more frequently present in patients who survived a probable IA infection (e.g., anti-CpcB antibodies), whereas the occurrence of other antibodies (e.g., anti-Shm2 antibodies) were correlated with a fatal disease outcome. Surprisingly, Shm2 induced a strongly proinflammatory response in human PBMCs after *in vitro* stimulation, but it was associated with particularly strong response in sera from patients who died of IA. In contrast, CpcB did not exhibit any immune-stimulating activity, although anti-CpcB antibodies were primarily found in patients who survived IA. These results are consistent with a previous classification of these two proteins based on the characterization of protein-specific T cells in healthy donors.[36] In that study, Shm2 elicited strong memory T cell responses and CpcB T cell exhaustion. One possible explanation for the observed correlation of antibody responses and disease outcome is that some anti-*A. fumigatus* antibodies, such as Shm2, trigger the host defense toward a detrimental response. In line with this assumption, clearance of *A. fumigatus* from the lungs was shown to be more efficient in mice deficient of B-cells and antibodies.[57] Without doubt, additional studies are needed to uncover the definitive role of specific *A. fumigatus* antigens and their contribution to condition the host immune response.

In conclusion, our analyses indicated that immunoproteomic studies of individual patients help to expand the knowledge about the serological response against *A. fumigatus* in patients with IA. Furthermore, this study provides valuable data for the development of prognostic indicators and immunotherapeutic tools to combat *A. fumigatus* infections as shown by the examples of Shm2.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.6b00042.

FunCat annotation (2nd level) for all 49 detected protein antigens; decision tree for the classification (indeterminate vs proven/probable IA) of serum from patients at risk for IA based on immunoblot signal patterns (PDF)

Patient data and immunoblot spot pattern for tested sera from Wuerzburg or Dublin (XLSX)

Protein identification of immunogenic proteins by MALDI-TOF/TOF analysis (XLS)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

IA, invasive aspergillosis; CT, computer tomography; ABPA, allergic bronchopulmonary aspergillosis; PBMCs, peripheral blood mononuclear cells; ASCT, allogeneic stem cell transplantation; EORTC/MSG, European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group; AMM, Aspergillus minimal medium; TCA, trichloroacetic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; IPG, immobilized pH gradient; SDS, sodium dodecyl sulfate; PVDF, polyvinylidene fluoride; TBS, Tris-buffered saline; PMF, peptide mass fingerprinting; PFF, peptide fraction fingerprinting; FunCat, functional catalogue; ELISA, enzyme-linked immunosorbent assay

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