Reduced expression of TLR3, TLR10 and TREM1 by human macrophages in Chronic cavitary pulmonary aspergillosis, and novel associations of VEGFA, DENND1B and PLAT

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Abstract

Chronic cavitary pulmonary aspergillosis (CCPA) is an uncommon but serious pulmonary disease of humans, with an annual mortality rate of 10–30%. It is caused by the fungus *Aspergillus fumigatus*. Patients are overtly immunocompetent; however, some immunogenetic defect is likely. To investigate this, we performed a genetic association study analysing biologically plausible candidate genes in 112 CCPA patients and 279 healthy controls, and investigated gene expression in monocyte-derived macrophages from patients and controls at baseline and during stimulation with *A. fumigatus*. Single-nucleotide polymorphisms (SNPs) associated with CCPA were found in *TLR1*, *CLEC7A* (dectin-1), *PLAT* (n = 2), *VEGFA*, and *DENND1B*. Macrophages from CCPA patients showed low *TLR3* and *TLR10* expression and high *TREM1* expression at baseline, as compared with macrophages from healthy subjects, with major expression differences being seen in most Toll-like receptors (TLRs) during 9 h of co-culture with *A. fumigatus*. The differences in baseline expression between the healthy and CCPA groups suggest roles for TLR3 and TLR10 in susceptibility to CCPA, and the association of SNPs in *PLAT* (n = 2), *VEGFA* and *DENND1B* supports novel roles for plasminogen activation and angiogenesis and of these genes specifically in susceptibility to CCPA.

Keywords: Aspergilloma, aspergillosis, *Aspergillus fumigatus*, CCPA, gene expression, genetic susceptibility, immune response, monocyte-derived macrophages, TLR
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Introduction

Aspergillus fumigatus is the primary cause of aspergillosis, of which chronic cavitary pulmonary aspergillosis (CCPA) is one of the most devastating manifestations [1]. Although uncommon, CCPA is a serious disease affecting immunocompetent people, most of whom have underlying lung disease (e.g. previous tuberculosis (TB)) [2]. The pathogenesis of CCPA remains enigmatic [1]. The formation, expansion and coalescence of pulmonary cavities is characteristic, and occurs over months or years [1]. Cavities may contain a fungal ball (aspergilloma). Without treatment, progressive lung and pleural fibrosis occurs [1]. Haemoptysis is common. Chronic inflammation with localized fibrosis without granulomas, necrosis or eosinophilic infiltration is seen microscopically in the cavity wall, and localized networks of additional small blood vessels are generated in proximity to the cavity; however, the mechanisms behind these pathologies are unclear.

A. fumigatus interacts with the human host via pathogen recognition receptors on the surfaces of immune cells such as macrophages and neutrophils [3]. These receptors include the Toll-like receptors (TLRs), particularly TLR2, TLR4, and TLR9, but also perhaps TLR1, TLR3, and TLR6 [4,5], and the β-glucan receptor, dectin-1 [3,6]. Triggering receptor expressed on myeloid cells 1 (TREM1) is found on neutrophils and monocytes, and amplifies the inflammation induced by stimulation of

TLRs, including TLR2 and TLR4 [7,8]. In addition, *A. fumigatus* can be bound by other host proteins, including plasminogen [9]. Th1 responses (e.g. tumour necrosis factor-α (TNF-α)) appear to be beneficial, wheras uncontrolled Th2 responses are detrimental [3]. DENN/MADD domain containing 1B (DENND1B) is a negative regulator of the TNF-α receptor (TNFR1) [10]. Furthermore, angiogenesis has been shown to be inhibited by *A. fumigatus*, and inhibition of vascular endothelial growth factor A (VEGFA) may increase susceptibility to invasive aspergillosis (IA) [11,12].

The role of these receptors and molecular mediators in susceptibility and disease development in CCPA remains unclear. Small genetic association studies have found CCPA to be associated with *TNF*, *MBL2*, *TGFB1*, *IL15*, *TLR4*, and *IL10*, but further work is required [13–16]. In addition, single-nucleotide polymorphisms (SNPs) in the plasminogen gene (*PLG*) have been associated with IA, but have not been investigated in CCPA [9,17]. We performed a much larger candidate gene association study of CCPA, extensively genotyping SNPs in genes involved in the recognition of *A. fumigatus*, as well as SNPs in *PLG*, the plasminogen activator gene (*PLAT*), *VEGFA*, and *DENND1B*. In addition, we compared the expression of TLR genes by monocyte-derived macrophages (MDMs) from CCPA patients and controls, at baseline and during co-culture with *A. fumigatus*.

Materials and Methods

See Appendix S1 for further details.

Subjects

CCPA patients and healthy subjects are defined in Appendix S1. As described previously [18], CCPA patients were recruited between March 2006 and August 2010 from the National Aspergillosis Centre (NAC) in Manchester (NAC, University Hospital of South Manchester, UK). Those cases complicated by other forms of aspergillosis were excluded. Previously recruited healthy subjects were used [19]. The Local Research Ethics Committee approved the study, and written informed consent was obtained for all subjects.

DNA and peripheral blood mononuclear cell extraction

DNA and peripheral blood mononuclear cells were extracted from blood as described previously [18].

Macrophage–*A. fumigatus* co-culture

MDMs were generated, live *A. fumigatus* conidia were added (4×10^5/well) and RNA was extracted as described previously [18].

Measuring expression by MDMs

The human innate and adaptive immune responses RT2 profiler PCR array (SABiosciences, Qiagen Ltd., Crawley, UK) was used to measure expression of *TLR1*, *TLR2*, *TLR3*, *TLR4*, *TLR9*, *TLR10* and *TREM1* in pooled RNA samples (1 μg) from each group (n = 10 subjects). *HRPT1*, *RPL13A* and *GAPDH* were used as housekeeping normalizer genes. Fold changes were calculated relative to the healthy average at 0 h with the manufacturer's online analysis tool (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php).

Gene and SNP selection for genotyping

Haplotype tagging SNPs (n = 141) in 13 biologically plausible and/or previously associated candidate genes were identified by use of the Genome Variation Server (http://gvs.gs.washington.edu/GVS/) (Table S1). Sequenom genotyping was completed for 134 SNPs. After quality control and exclusion of redundant or monomorphic SNPs, 109 SNPs were analysed with Stata (Statacorp LP, College Station, TX, USA). Logistic regression was used to determine association using dominant and recessive models. The Benjamini–Hochberg correction for the false discovery rate was used to correct for multiple testing.

Statistical analysis

Statistical analysis was performed in GraphPad Prism (Version 5.02; GraphPad Software Inc., La Jolla, CA, USA), Stata, and SPSS (Version 16; SPSS Inc., Chicago, IL, USA). Ages and percentages of males were not normally distributed, and were compared by the use of Mann–Whitney tests. Statistical analysis with t-tests, repeated measures one-way ANOVA and two-way ANOVA was performed for analysis of expression data.

Results

Characteristics of the study population

Of the 396 Caucasian subjects selected for genotyping (116 CCPA; 280 healthy), 391 were genotyped successfully (112 CCPA; 279 healthy). The majority of CCPA patients had one or more underlying diseases (Table 1). The CCPA group was older than the healthy group (median age: 64.9 years vs. 47.0 years, $p < 0.0001$) and contained a higher proportion of males (60.7% vs. 40.1%, p 0.0005; Table 1). These characteristics were similar in the subset of individuals used for gene expression studies, and these are considered to be representative of the total study population (Table 2).

TABLE 1. Characteristics of patients and controls for genetic association experiments

Characteristic	CCPA	Healthy
n	112	279
Age (years), median (IQR)	64.9 (59.4–70.8)	47.0 (44.2–50.5)
Males (%)	60.7	40.1
Positive fungal culture, % (no.)	25.9 (29/112)	NA
Aspergillus fumigatus	79.3 (24/29)	NA
Aspergillus niger	3.4 (1/29)	NA
Other *Aspergillus* species	3.4 (1/29)	NA
Penicillium species[a]	13.8 (4/29)	NA
Aspergilloma presence, % (no.)		
CCPA without aspergilloma	60.7 (68/112)	NA
CCPA with aspergilloma	39.3 (44/112)	NA
Bronchiectasis[b], % (no.)	25.9 (29/112)	NA
Underlying disease, % (no.)		
COPD and/or emphysema (± bullae)	44.6 (50/112)	NA
Pneumonia[c]	20.5 (23/112)	NA
Pneumothorax (± bullae)	20.5 (23/112)	NA
Thoracic surgery	19.6 (22/112)	NA
Classical tuberculosis	17.9 (20/112)	NA
Non-tuberculous mycobacterial infection (atypical TB)	12.5 (14/112)	NA
Lung cancer survivor	11.6 (13/112)	NA
Asbestos related pleural plaques	10.7 (12/112)	NA
Asthma	8.9 (10/112)	NA
Sarcoidosis	8.0 (9/112)	NA
Other[d]	5.4 (6/112)[d]	NA
Rheumatoid arthritis	4.5 (5/112)	NA
Ankylosing spondylitis/kyphoscoliosis[e]	3.6 (4/112)[e]	NA
No underlying disease identified	1.8 (2/112)	NA

CCPA, chronic cavitary pulmonary aspergillosis; COPD, chronic obstructive pulmonary disease; IQR, interquartile range; NA, Not Applicable; TB, tuberculosis.
[a]Presumed airway colonization, rather than the causative agent of CCPA.
[b]Bronchiectasis is considered to be a co-existing, rather than underlying, disease in CCPA.
[c]Community-acquired pneumonia requiring hospitalization.
[d]Includes alcohol excess (3), chest radiotherapy without lung cancer (1), dextrocardia (1), and smoke inhalation (1).
[e]Ankylosis spondylitis (1) and kyphoscoliosis (3).

TABLE 2. Characteristics of patients and controls used for gene expression experiments

Characteristic	CCPA	Healthy
n	10	10
Age (years), median (IQR)	59.0 (55.8–68.9)	38.0 (31.2–51.1)
Males, % (no.)	70 (7/10)	40 (4/10)
Aspergilloma, % (no.)	60 (6/10)	NA
Positive fungal culture, % (no.)	50 (5/10)	NA
Aspergillus fumigatus, % (no.)	100 (5/5)	NA
Bronchiectasis, % (no.)	30 (3/10)	NA
Underlying disease, % (no.)		
COPD and/or emphysema (± bullae)	30 (3/10)	NA
Previous pneumonia[a]	30 (3/10)	NA
Previous pneumothorax (± bullae)	20 (2/10)	NA
Previous thoracic surgery	20 (2/10)	NA
Previous classic TB	20 (2/10)	NA
Previous non-tuberculous mycobacterial infection (atypical TB)	10 (1/10)	NA
Lung cancer survivor	10 (1/10)	NA
Previous asbestos exposure/asbestosis	10 (1/10)	NA
Sarcoidosis	20 (2/10)	NA
Other (alcohol excess)	10 (1/10)	NA

CCPA, chronic cavitary pulmonary aspergillosis; COPD, chronic obstructive pulmonary disease; NA, Not Applicable; IQR, interquartile range; TB, tuberculosis.
[a]Community-acquired pneumonia requiring hospitalization.

Expression of TLR genes differs between MDMs from CCPA patients and those from healthy subjects

Baseline expression of *TLR3* (−2.35-fold, p 0.003) and *TLR10* (−2.63-fold, p 0.0001) was significantly lower in MDMs from CCPA patients than in those from healthy subjects. After stimulation with *A. fumigatus*, expression of *TLR10* changed little, and remained lower in the CCPA group at all time-points (the change over time was not significantly different between the two groups), whereas expression of *TLR3* remained lower in the CCPA group up to 6 h (Fig. 1), after which expression in the healthy group decreased to the level in the CCPA group by 9 h.

Baseline expression of *TLR2*, *TLR4* and *TLR9* was only marginally different (less than two-fold) between the CCPA and healthy groups, and expression remained similar, although slightly higher in the CCPA group, during the first 6 h of stimulation with *A. fumigatus*. By the 9 h time-point, dramatic differences were observed; in the CCPA group, expression continued to be relatively stable, whereas in the healthy group expression decreased (*TLR2* and *TLR4*) or increased (*TLR9*) dramatically (Fig. 1).

TREM1 was significantly overexpressed in macrophages from CCPA patients as compared with those from healthy subjects at baseline (2.31-fold, p 0.0006). After stimulation with *A. fumigatus*, expression remained higher in the CCPA group at all time-points (Fig. 1).

Genetic association in CCPA

Six SNPs in five genes were associated with CCPA ($p < 0.05$), including a missense non-synonymous coding SNP in *TLR1* (rs4833095) and a *CLEC7A* SNP (rs7309123) previously associated with IA [20]. Three associations (*DENND1B* rs2477077, *PLAT* rs8178890, and *VEGFA* rs10434) remained significant after correction for multiple testing (Table 3; Fig. 2). The *TLR1* rs4833095 and *CLEC7A* rs7309123 associations did not survive correction for multiple testing (false discovery rate p 0.065). For further detail of SNPs implicated in CCPA, see Table S2.

No SNPs in *TLR3*, *TLR10* or *TREM1* were associated with CCPA (see Table S1 for the SNPs tested). Two SNPs previously associated with IA (*PLG* rs4252125 and *CLEC7A* rs16910526) were not associated with CCPA (Table S3).

Discussion

TLR2, TLR4 and TLR9 have long been known to be involved in the recognition of *A. fumigatus* [3,6]. More recently, studies have suggested that TLR1, TLR3 and TLR6 may also be involved [4,5]. Studies have investigated expression of the genes encoding these proteins in cells from healthy donors [4,6,17]; however, few have investigated expression in cells from CCPA patients. We used MDMs from CCPA patients and healthy donors, and investigated gene expression both before and after stimulation with live *A. fumigatus*, at various time-points when different fungal morphologies predominate

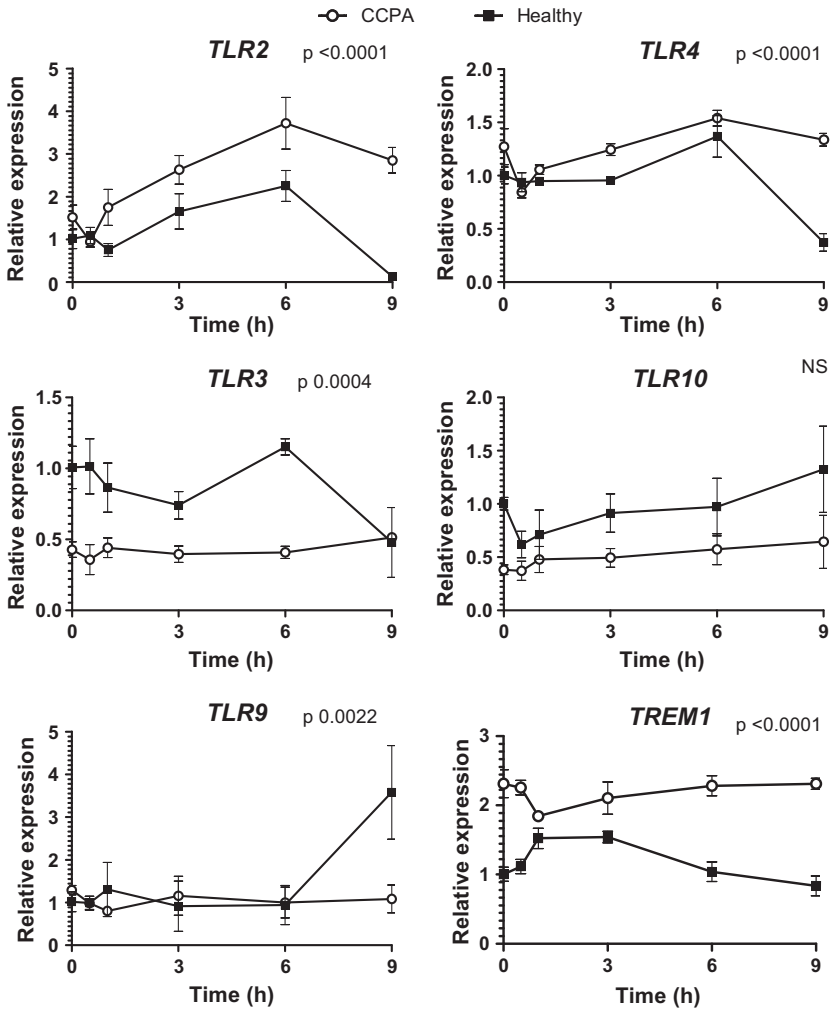

FIG. 1. Expression of the Toll-like receptor (TLR) and the triggering receptor expressed on myeloid cells 1 (TREM1) genes differs between monocyte-derived macrophages from chronic cavitary pulmonary aspergillosis patients (open circles) and healthy subjects (closed squares). Fold difference is calculated relative to the healthy 0-h value. p-Values indicate the differences in the change over time between the two groups, calculated by repeated measures two-way ANOVA. Mean and standard deviations are shown. Each group comprises ten pooled RNA samples. Mean and standard deviations are shown for the three replicates performed. NS, not significant.

TABLE 3. Single-nucleotide polymorphisms (SNPs) associated with chronic cavitary pulmonary aspergillosis (CCPA)

Gene	SNP	Alleles (M/m)	Model for association	Genotype frequency			OR (95% CI)	p-Value	FDR p-value	Location
				Genotype	CCPA, no. (%)	Healthy, no. (%)				
TLR1	rs4833095	**A**/G	AG + GG vs. AA	AA	83 (74.1)	173 (62.5)	0.58 (0.36–0.95)	0.029	0.065	Exonic (Asn/Ser)
				AG + GG	29 (25.9)	104 (37.5)				
CLEC7A (dectin-1)	rs7309123	C/**G**	CC + GC vs. GG	GG	30 (27.0)	49 (17.9)	0.59 (0.35–0.99)	0.046	0.099	Intronic
				CC + GC	81 (73.0)	225 (82.1)				
PLAT	**rs8178890**	**G**/A	AA + GA vs. GG	GG	105 (93.8)	237 (84.9)	0.38 (0.16–0.86)	0.021	**0.049**	Intronic
				AA + GA	7 (6.3)	42 (15.1)				
	rs879293	G/**A**	AA + GA vs. GG	GG	29 (25.9)	102 (36.6)	1.65 (1.01–2.69)	0.044	0.097	Intronic
				AA + GA	83 (74.1)	177 (63.4)				
DENND1B	**rs2477077**	C/**T**	CC + CT vs. TT	TT	11 (9.8)	10 (3.6)	0.34 (0.14–0.83)	0.017	**0.041**	Intronic
				CC + CT	101 (90.2)	269 (96.4)				
VEGFA	**rs10434**	**G**/A	GG + AG vs. AA	AA	15 (13.4)	69 (24.7)	2.12 (1.16–3.90)	0.015	**0.036**	3′-UTR
				GG + AG	97 (86.6)	210 (75.3)				

FDR, false discovery rate; M/m, major allele/minor allele; UTR, untranslated region.
p-Values were calculated for the model indicated by the use of logistic regression in Stata. Risk alleles are shown in bold. SNPs in bold remained significant after Benjamini–Hochberg adjustment for FDR (FDR-adjusted p-values are shown).

(0.5–3 h, conidia; 6 h, germ-tubes; 9 h, hyphae [18]). We have shown that expression of TLRs in MDMs from CCPA patients is dramatically different from expression in MDMs from healthy donors.

Our results for *TLR2* and *TLR4* expression in the healthy group are broadly similar to previous findings obtained with various cell types from healthy individuals [4,6,17]; however, we found that expression of these TLRs (*TLR2* in particular)

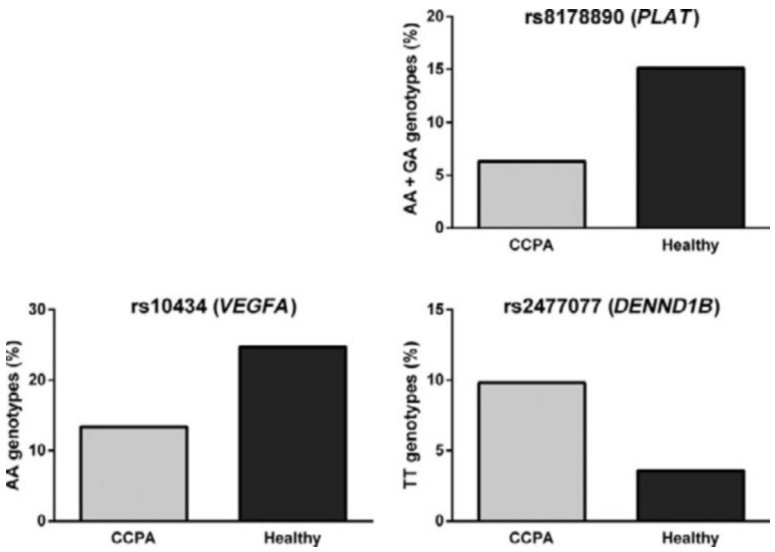

FIG. 2. Genotype frequencies of single-nucleotide polymorphisms associated with chronic cavitary pulmonary aspergillosis (CCPA): 279 healthy subjects and 112 CCPA patients were genotyped successfully. rs3917354 is an insertion–deletion mutation. DENND1B, DENN/MADD domain containing 1B; VEGFA, vascular endothelial growth factor A.

was higher in the CCPA group than in the healthy group after stimulation with *A. fumigatus*, and remained higher when expression decreased in the healthy group at 9 h. *TLR3* expression in the healthy group showed a similar decrease at 9 h, whereas *TLR9* expression in this group increased at this time-point. The changes in expression at the 9-h time-point were much less dramatic, or did not occur at all, in the CCPA group, and may represent a healthy response to the hyphal form (which is dominant after 9 h of co-culture [18]), or a response to long-term fungal exposure, which did not occur in the CCPA group. *TLR9* expression, in particular, appeared not to respond to the presence of *A. fumigatus* until 9 h, which may suggest that, in MDMs, this TLR does not respond to conidia or germ-tubes (the dominant fungal morphologies for the first 6 h of co-culture [18]), but only to hyphae. This differs from the results of studies with neutrophils from healthy donors [6]. As well as using different cell types, many previous studies have used killed fungi, whereas we used live *A. fumigatus*. The use of live fungus is important, as individuals are exposed to live fungal spores in the environment, and live *A. fumigatus* is cultured from patients, but limits the duration of the experiment to 9 h, because, after this time, the fungus overgrows the plate and the MDMs. This can be avoided by increasing the MDM/conidia ratio; however, we chose to use the 1 : 2 ratio in an attempt to ensure that all MDMs were experiencing fungal stimulation. In addition, at lower ratios, the MDMs destroy the conidia before germination, whereas the use of our methodology allows germination to occur such that the different time-points are representative of exposure to different live fungal morphologies [18]. These differences in methodology may help to explain the differences between our results and those of previous studies.

Expression of *TLR3* and *TLR10* increased only slightly after initial exposure to *A. fumigatus*, which is similar to the results of previous studies [4,6]; however, we demonstrated that baseline expression of these genes was significantly lower in the CCPA group, consistent with a role for these TLRs in protection against aspergillosis and of reduced constitutive expression with susceptibility. In addition to this, *TREM1* expression was found to be increased in MDMs from CCPA patients at baseline, and continued to be increased at all time-points after stimulation with *A. fumigatus*.

Recent studies have suggested a role for TLR3 in epithelial cell-mediated protection against *A. fumigatus* [4,21], and TLR10 is known to form heterodimers with TLR1 and TLR2 [22,23]. TREM1 is an activating receptor found on neutrophils and monocytes that amplifies the inflammation induced by stimulation of TLRs, including TLR2 and TLR4 [7,8]. TLR2 and TLR4 are important in the response to *A. fumigatus*, and TLR10 and TREM1 may play a role in the response to *A. fumigatus* via interactions with these. Deficiency in baseline expression of TLRs may affect susceptibility to CCPA by reducing the early-stage recognition of *A. fumigatus* and reducing an otherwise appropriate immune response. The increased expression of *TREM1* may be an attempt to combat this.

We acknowledge that, within the host, multiple cells are involved in the response to infection, including non-traditional immune cells such as epithelial cells [24], and that, among traditional immune cells, the alveolar macrophage would be the ideal cell type in which to study these interactions between host and pathogen; however, as discussed previously, the use of MDMs prevents the population being skewed in favour of mild CCPA, which would have occurred had we recruited only bronchoscopy-fit subjects [18]. The use of pooled samples for

gene expression experiments has also been discussed previously [18]. Although the use of these prevents analysis of interpatient variability, it allows for the analysis of a greater number of genes (owing to the limited availability of patient cells). It is likely that mean values for cases and controls would be broadly similar to those obtained with pooled samples; however, we appreciate that it is possible that our pooled value could be skewed by a minority of individuals. In addition, we acknowledge that we have used a single fungal isolate and inoculum for these experiments, in cells from multiple individuals. This is because we were interested in the differences between patients and controls rather than in differences between fungal isolates; however, we appreciate that different fungal isolates/strains may produce different gene expression responses, and that this would be interesting to investigate in future.

We have not yet performed functional assays to support the gene expression studies presented here. In particular, a functional study that correlates phenotypic expression with function would be beneficial in the future, as would validation of the gene expression results by measurement of protein levels to confirm that differences in expression are translated into differences in protein levels. However, this is predominantly a hypothesis-generating study, and, despite these limitations, we feel that the results presented here suggest that expression of TLRs differs between MDMs from CCPA patients and those from healthy subjects, both before and after stimulation with *A. fumigatus*, and that this initial finding merits further, more detailed investigation. In particular, we believe that our results support studies suggesting roles for TLRs other than TLR2, TLR4 and TLR9 in the recognition and response to *A. fumigatus*.

Given the importance of TLRs in *A. fumigatus* recognition, and the expression results presented here, it seems likely that SNPs in TLR genes affect aspergillosis susceptibility. Indeed, SNPs in *TLR1* (rs5743611 and rs4833095), *TLR4* (rs4986790 and rs4986791) and *TLR6* (rs5743611) were previously associated with IA, and one SNP in *TLR4* (rs4986790) was previously associated with CCPA [5,15,16]. Despite this, only one TLR SNP, in *TLR1* (rs4833095), was identified as being associated with CCPA in the current study, and this association did not remain significant after correction for multiple testing. This SNP was previously associated with IA [5]. Unfortunately, the previously associated *TLR4* SNP failed primer design and was not genotyped.

TLR1 forms heterodimers with TLR2 [5,25] that recognize and respond to *A. fumigatus* [6]. SNPs in *TLR1* that affect TLR1 expression, structure, or function, especially those that affect heterodimer formation with TLR2, may affect the response to *A. fumigatus*, and could affect susceptibility. rs4833095 is a non-synonymous coding SNP, and may have effects such as these. rs4833095 has been associated with a variety of diseases, and is in high linkage disequilibrium (LD; $r^2 > 0.8$) with many other SNPs (Table S2).

Despite a disappointing lack of association between TLR SNPs and CCPA, other associations were identified. SNPs in *VEGFA*, *DENND1B* and *PLAT* remained significantly associated with CCPA after correction for multiple testing. These are novel and important findings; these genes are not currently considered to be major candidates for aspergillosis susceptibility, and our results suggests novel hypotheses and novel areas for future research into this disease. Two of these SNPs are in intronic regions, which are traditionally described as non-functional DNA; however, recent research (e.g. the ENCODE project) suggests that these regions could indeed be functional [26]. Therefore, these SNPs, or those in LD with them, could indeed be important for susceptibility to CCPA.

VEGFA is a key mediator of angiogenesis, a process by which new blood vessels are formed [27]. Excessive small-vessel proliferation is a feature of CCPA, and may lead to haemoptysis. Angiogenesis can be inhibited by *A. fumigatus*, and inhibition of VEGFA may increase susceptibility to IA [11,12]. In contrast, expression of *VEGFA* is increased within aspergillomas, possibly because of hypoxia within these structures [27,28]. Recently, VEGFA has also been shown to have synergistic effects with antifungals in a murine model of acute aspergillosis [29]. Multiple isoforms of VEGFA, with varying degrees of angiogenic potential, are produced by alternative splicing [27,30]. SNPs in *VEGFA* may affect this alternative splicing and alter the isoform levels within the tissues. Alternatively, SNPs may affect the angiogenic ability of VEGFA. The 3′-untranslated region SNP identified was previously associated with measures of lung function [31]. Further work is required to determine how this SNP or those in high LD with it alter the protein expression or function, but the finding of an association with CCPA supports a role for angiogenesis and *VEGFA* in susceptibility to this disease.

DENND1B is a negative regulator of TNFR1, which is expressed by many immune cells, including monocytes and bronchial epithelial cells [10]. It binds to TNFR1, suppressing downstream signalling to modulate the Th1/Th2 response and inflammatory cytokine production of cells [10,32]. TNF-α is produced by macrophages and other immune cells in response to *A. fumigatus*, and is important in the clearance of *A. fumigatus* conidia and in neutrophil recruitment and cytokine production in response to this fungus. In one small study, the high TNF-α-producing genotype −308A/A was found to be reduced in allergic aspergillosis and CCPA patients as compared with controls, suggesting that reduced TNF-α responses are linked to aspergillosis susceptibility [14]. Sup-

pression of TNFR1 by DENND1B may affect susceptibility to aspergillosis by suppressing TNF-α-driven Th1 responses.

PLAT is a plasminogen activator that promotes the conversion of plasminogen to plasmin [17]. Plasminogen has recently been suggested to play a role in the response to *A. fumigatus*, and in susceptibility to aspergillosis [9,17]. It binds to *A. fumigatus* in a dose-dependent manner, and can be converted to plasmin while bound [9]. Variations within the *PLG* gene may influence the pathogenesis of IA in both mice and humans [9]. In this context, plasmin appears to act as a chemoattractant for monocytes and induce expression of inflammatory cytokines and chemokines by these cells [33,34]. SNPs in *PLAT* may influence susceptibility to aspergillosis by affecting the conversion of plasminogen to plasmin in response to *A. fumigatus*.

Our CCPA study population is the largest used for genetic association studies to date, despite containing only 112 patients. Many previous studies have involved only a handful of patients. This is because of the relatively rare nature of CCPA. Our patients were recruited from the NAC, which receives referrals from across the UK. The majority of CCPA patients have (often multiple) past or present underlying conditions [2], and selection of a control population presents a challenge. This has been discussed previously [18]. Ideally, a matched control subject with the same underlying diseases for each case should be recruited; however, this is practically impossible, and, consequently, healthy control populations have been used in all previous CCPA genetic association studies [13,15,18,35]. We also used healthy unmatched controls, acknowledging that, rather than increasing our power to find genetic associations with CCPA, this reduces it, and therefore does not invalidate our findings. In addition, the control population is younger than the CCPA population, and some controls could, in time, develop CCPA; however, this would, again, reduce rather than increase our power. As each underlying disease occurs in only a minority of cases, it is unlikely that any genetic associtations identified were related to an underlying disease rather than to CCPA. However, to investigate this, we compared our results with those of studies on genetic association in TB (the most common primary underlying disease in CCPA [2]; Appendix S2). Our study included SNPs that have been associated with TB previously [36]. None of these remained significantly associated with CCPA after correction for multiple testing, suggesting that the observed associations were not affected by a lack of matching for previous TB. We restricted the current study to Caucasians, in order to allow accurate comparison between cases and controls, and acknowledge that this limits the generalizability of our findings to this particular ethnic group. Further work in other ethnic groups would be required to determine the factors involved in susceptibility within these groups.

The novel associations presented here, including those with PLAT, DENND1B, and VEGFA, require replication in other populations and validation in functional studies in order to confirm them as plausible candidates for susceptibility to CCPA; however, their identification in this hypothesis-generating study suggests novel areas for future research, and increases our understanding of the factors influencing CCPA susceptibility and pathogenesis. In addition, the deficient constitutive expression of *TLR3* and *TLR10* demonstrated may increase susceptibility to CCPA by reducing the recognition of and response to *A. fumigatus*. Future work will increase this understanding further, and allow the identification of susceptible individuals or the development of treatments to combat deficient responses in CCPA patients.

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Authorship/Contribution

A. Simpson, P. Bowyer and D. W. Denning: contributed to study conception and obtained funding. A. Simpson, P. Bowyer,

D. W. Denning, and N. L. D. Smith: contributed to study design. N. L. D. Smith and J. Hankinson: contributed to acquisition of the data. N. L. D. Smith, P. Bowyer, D. W. Denning, and A. Simpson: analysed and interpreted data, and drafted the report. All authors approved the version submitted.

Transparency Declaration

There are no conflicts of interest for any author.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. List of all SNPs selected for genotyping.

Table S2. Further detail of SNPs implicated in CCPA.

Table S3. SNPs previously associated with aspergillosis that were not associated with CCPA in the current study.

Appendix S1. Additional details on methods.

Appendix S2. Comparison of genetic association with CCPA and genetic association with TB.

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