A prominent role for the IL1 pathway and IL15 in susceptibility to chronic cavitary pulmonary aspergillosis

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Abstract

Chronic cavitary pulmonary aspergillosis (CCPA) is a progressive lung condition with a 10–30% annual mortality. Although overtly immunocompetent, some immunogenetic defect in patients is likely. To investigate a possible immunogenetic defect in CCPA, we analysed biologically plausible candidate genes in 112 CCPA patients and 279 healthy controls in a genetic association study of genes involved in the post-recognition immune response to *Aspergillus fumigatus*. We also compared gene expression in monocyte-derived macrophages from subjects with and without disease, both at baseline and during stimulation with *A. fumigatus*. Compared with macrophages from healthy subjects, CCPA macrophages showed unrestrained rises in *IL1A*, *IL1B*, *IL6*, *IRAK2* and *TRAF6* throughout the experiment, and a lack of expression of *TGFB1* at 9 h. Single nucleotide polymorphisms (SNPs) associated with CCPA were found in *IL1B* (n = 2), *IL1RN* and *IL15* (n = 3). Uncontrolled expression of IL1 and IL6 and continuing high levels of these cytokines may result in continuing cellular influx and pro-inflammatory responses, inhibiting disease resolution and contributing to disease progression in CCPA. The association of SNPs in *IL1B*, *IL1RN* and *IL15* with CCPA supports a role for the IL1 pathway, as well as implicating the *IL15* gene, in susceptibility to CCPA.

Keywords: Aspergillosis, *Aspergillus fumigatus*, CCPA, genetic susceptibility, IL1, IL15, immune response, monocyte-derived macrophages
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Introduction

Estimates suggest that humans inhale several hundred *Aspergillus fumigatus* conidia each day, which persist in the lung [1,2]. Most individuals do not suffer symptoms, but in a small minority this fungus causes the serious disease chronic cavitary pulmonary aspergillosis (CCPA) [3]. Affected individuals almost invariably have some prior lung disease (e.g. chronic obstructive pulmonary disorder (COPD) or pulmonary tuberculosis (TB)) but are overtly immunocompetent and do not generally have a clinical history of recurrent infection [4]. CCPA involves the formation and/or expansion of a pulmonary cavity/cavities over months or years, with progressive lung fibrosis and chronic inflammation [3]. *Aspergillus* growth on the cavity surface, without tissue invasion, may lead to fungal balls (aspergillomas). The mechanisms underlying the observed pathology are largely unknown, but development of aspergilloma(s) represents a later phase of CCPA [5].

The human immune response to *A. fumigatus* involves macrophages and neutrophils, which ingest and kill the fungus and induce pro-inflammatory cytokines and cellular influx [6–8]. As the numerically dominant cell in alveoli, macrophages are likely to be the first innate immune cell to contact invading fungal particles. Following recognition, a wide variety of cytokines and chemokines are expressed, including many in the interleukin-1 (IL1) pathway [9]. Th1 responses appear beneficial and uncontrolled Th2 responses detrimental [6,7,10].

Despite knowledge of the immune response to *A. fumigatus*, little is known about the factors affecting susceptibility, continuing inflammation or disease pathogenesis in CCPA. The most commonly cited susceptibility factor is underlying disease; however, the proportion of patients with any one disease is very small, and only a small percentage of individuals with any one underlying disease develop CCPA [4]. Previous genetic association studies involving small numbers of patients have identified associations between CCPA and *TNF*, *MBL2*, *TGFB1*, *IL15*, *TLR4* and *IL10*, [11–14] but do not explain all cases of CCPA and have proved difficult to replicate. The aim of the current study was to comprehensively genotype a broad panel of single nucleotide polymorphisms (SNPs) from genes important in the post-recognition immune response to *A. fumigatus*, in a candidate gene association study involving a large cohort of patients and healthy controls. We also investigated whether some of these genes, particularly those in the IL1 pathway, were differentially expressed by monocyte-derived macrophages (MDMs) from subjects with and without disease, at baseline and during culture with *A. fumigatus*.

Methods

See (Appendix S1) for further details.

Subjects

CCPA subjects and healthy controls are defined in Table 1. CCPA subjects complicated by allergic bronchopulmonary aspergillosis (ABPA) were excluded. CCPA subjects were recruited from the National Aspergillosis Centre (University Hospital of South Manchester, UK) between March 2006 and August 2010. Previously recruited healthy subjects were used [15]. The study was approved by the Local Research Ethics Committee and written informed consent was obtained from all subjects.

TABLE 1. Diagnostic criteria for recruited subjects

Disease	Diagnostic criteria
CCPA	All the following are required: Chronic pulmonary or systemic symptoms (3 months) compatible with CCPA, including at least one of: weight loss, productive cough or haemoptysis Cavitary pulmonary lesion with evidence of paracavitary infiltrates or expansion of cavity size over time Either positive serum *Aspergillus* precipitins test or isolation of *Aspergillus* from pulmonary or pleural cavity Elevated inflammatory markers (C-reactive protein, plasma viscosity or erythrocyte sedimentation rate) No overt immunocompromising conditions (HIV infection, leukaemia, chronic granulomatous disease, etc.) No concurrent ABPA Further indicators: Often multiple cavities, with more forming over time if untreated A fungal ball or aspergilloma may or may not be present
Healthy control	All the following are required: No diagnosis of asthma No diagnosis of aspergillosis Negative SPT (at 3 mm cut-off) and/or IgE (<0.4) to all allergens tested, including mite, cat, dog and grasses Negative SPT (at 3 mm cut-off) and/or IgE (<0.4) to all fungi tested, including *Alternaria alternata*, *Candida albicans*, *Cladosporium herbarum*, *Penicillium chrysogenum (notatum)*, *Trichophyton rubrum* and *A. fumigatus*

CCPA, chronic cavitary aspergillosis; ABPA, allergic bronchopulmonary aspergillosis.

DNA and PBMC extraction from blood

DNA was extracted using a phenol chloroform extraction method. Peripheral blood mononuclear cells (PBMCs) were extracted using a Ficoll-paque Plus (GE Lifesciences, Buckingham, UK) density gradient.

Macrophage-*Aspergillus fumigatus* co-culture

PBMCs were plated onto 24-well plates (2×10^6/well). Monocytes were selected using 1.5 h plastic adherence. Monocyte-derived macrophages (MDMs) were generated using GM-CSF and 15 days incubation. Live *A. fumigatus* conidia were added (4×10^5/well). RNA was extracted using the RNeasy kit (Qiagen Ltd, Crawley, UK).

Measuring expression by MDMs

Gene expression was measured in pooled RNA samples (1 μg) from each disease group ($n = 10$ subjects) using the human innate and adaptive immune responses RT2 profiler PCR array (SABiosciences, Qiagen Ltd, Crawley, UK). The housekeeping genes *HRPT1*, *RPL13A* and *GAPDH* were used as the normaliser genes. Data were analysed using the manufacturer's online RT-PCR data analysis tool (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php) to calculate fold changes relative to the healthy average 0 h.

Gene and SNP selection, genotyping, quality control and data analysis

Seventeen biologically plausible and/or previously associated candidate genes were selected (Table S1). Haplotype tagging SNPs ($n = 327$) were selected using the Genome Variation Server (http://gvs.gs.washington.edu/GVS/) and genotyping of 168 of these was completed on the Sequenom® (Sequenom Inc., San Diego, CA, USA). After quality control and exclusion of SNPs redundant or monomorphic within our population, 137 SNPs remained for analysis using Stata (Statacorp, College Station, TX, USA). Logistic regression was used to determine association using dominant and recessive models. Correction for multiple testing was completed using the Benjamini-Hochberg correction for False Discovery Rate (FDR).

Statistical analysis

Statistical analysis was completed in Stata, SPSS (Version 16; SPSS Inc., Chicago, IL, USA) and GraphPad Prism (Version 5.02; GraphPad Software Inc., La Jolla, CA, USA). Ages and percentage of male subjects were compared between the

groups using Mann–Whitney tests as the data were not normally distributed. For analysis of expression data, statistical analysis using *t*-test, repeated measures one-way ANOVA and two-way ANOVA was completed using GraphPad.

Results

Characteristics of the study population

In total, 116 Caucasian subjects with CCPA were recruited. A further 280 healthy Caucasian subjects were selected as controls. Of these, 112 CCPA and 279 healthy subjects were genotyped successfully. Most CCPA patients had some, and many had multiple, underlying diseases (Table 2). The CCPA group was older (median age 64.9 years vs. 47.0 years, p <0.0001) and more likely to be male (60.7% vs. 40.1%, p 0.0005) than the healthy group (Table 2). Characteristics were similar in the subset of subjects used for gene expression experiments (Table 2) and are likely to be representative of the total study population.

Expression of the IL1pathway differs between MDMs from CCPA subjects and healthy subjects

The pattern of expression of *IL1A* and *IL1B* showed a markedly different time course in CCPA patients compared with healthy controls. For healthy controls, expression peaked by 3 h and returned to baseline by 6 h. In CCPA patients there was a much slower increase in expression, which continued to 9 h (Fig. 1). Expression of the downstream signalling molecules *IRAK2* and *TRAF6* initially rose in both groups, then fell below baseline after 6 h in the healthy group but not the CCPA group (TRAF6 does reduce after 6 h in the CCPA group, but not to below baseline) (Fig. 1). Expression of the negative regulator, *IL1RN*, increased in both groups after stimulation with *A. fumigatus* (Fig. 1). Expression of *IL6* follows a similar pattern to *IL1A* and *IL1B* (Fig. 1). Additional results from the IL1 pathway are presented in Figure S1. Expression of the anti-inflammatory cytokine *TGFB1* does not change over time in CCPA group, but increases in the healthy group at 9 h.

Genetic association in CCPA

Of the 137 SNPs that passed quality control, seven SNPs in four genes were found to be associated with CCPA (p <0.05). Five remained significant after correction for multiple testing (Table 3), including three intronic mutations in *IL15* (rs6842735, rs12508866 and rs1519551), an intronic insertion-deletion mutation in *IL1B* (rs3917354) (Fig. 2) and a 3′ UTR SNP in *IL1RN* (rs4252041). Additional SNPs in *IL17A* and *IL1B* showed trends towards significance but failed to pass

TABLE 2. Characteristics of patients and controls

	Genetic association study		Gene expression study	
Characteristic	**CCPA**	**Healthy**	**CCPA**	**Healthy**
n	112	279	10	10
Age (years) (median, IQR)	64.9 (59.4–70.8)	47.0 (44.2–50.5)	59.0 (55.8–68.9)	38.0 (31.2–51.1)
% Male	60.7	40.1	70 (7/10)	40 (4/10)
Positive *Aspergillus* culture (%)	23.2 (26/112)	N/A	50 (5/10)	N/A
A. fumigatus	92.3 (24/26)	N/A	100 (5/5)	N/A
Aspergillus niger	3.8 (1/26)	N/A	0 (0/5)	N/A
Other *Aspergillus spp.*	3.8 (1/26)	N/A	0 (0/5)	N/A
CCPA type (%)				
CCPA—aspergilloma	60.7 (68/112)	N/A	40 (4/10)	N/A
CCPA + aspergilloma	39.3 (44/112)	N/A	60 (6/10)	N/A
Bronchiectasis[a] (%)	25.9 (29/112)	N/A	30 (3/10)	N/A
Underlying disease (%)				
COPD and/or emphysema (± bullae)	44.6 (50/112)	N/A	30 (3/10)	N/A
Pneumonia[b] (previous)	20.5 (23/112)	N/A	30 (3/10)	N/A
Pneumothorax (previous, ± bullae)	20.5 (23/112)	N/A	20 (2/10)	N/A
Thoracic surgery (previous)	19.6 (22/112)	N/A	20 (2/10)	N/A
Classical tuberculosis (TB, previous)	17.9 (20/112)	N/A	20 (2/10)	N/A
Non-tuberculous mycobacterial infection (atypical TB, previous)	12.5 (14/112)	N/A	10 (1/10)	N/A
Lung cancer survivor	11.6 (13/112)	N/A	10 (1/10)	N/A
Asbestos exposure/asbestosis (previous)	10.7 (12/112)	N/A	10 (1/10)	N/A
Asthma	8.9 (10/112)	N/A	0 (0/10)	N/A
Sarcoidosis	8.0 (9/112)	N/A	20 (2/10)	N/A
Other	5.4 (6/112)[c]	N/A	10 (1/10)[d]	N/A
Rheumatoid arthritis[e]	4.5 (5/112)	N/A	0 (0/10)	N/A
Ankylosing spondylitis/kyphoscoliosis	3.6 (4/112)[f]	N/A	0 (0/10)	N/A
No underlying disease identified	1.8 (2/112)	N/A	0 (0/10)	N/A

IQR, interquartile range.
[a]Bronchiectasis is considered a co-existent rather than underlying disease in CCPA.
[b]Community-acquired pneumonia requiring hospitalization.
[c]Includes alcohol excess (3), chest radiotherapy without lung cancer (1), dextrocardia (1) and smoke inhalation (1).
[d]Includes alcohol excess (1).
[e]Rheumatoid arthritis patients were receiving <7.5 mg/day prednisolone and were therefore considered immunocompetent.
[f]Ankylosing spondylitis (1), kyphoscoliosis (3). COPD, chronic obstructive pulmonary disorder.

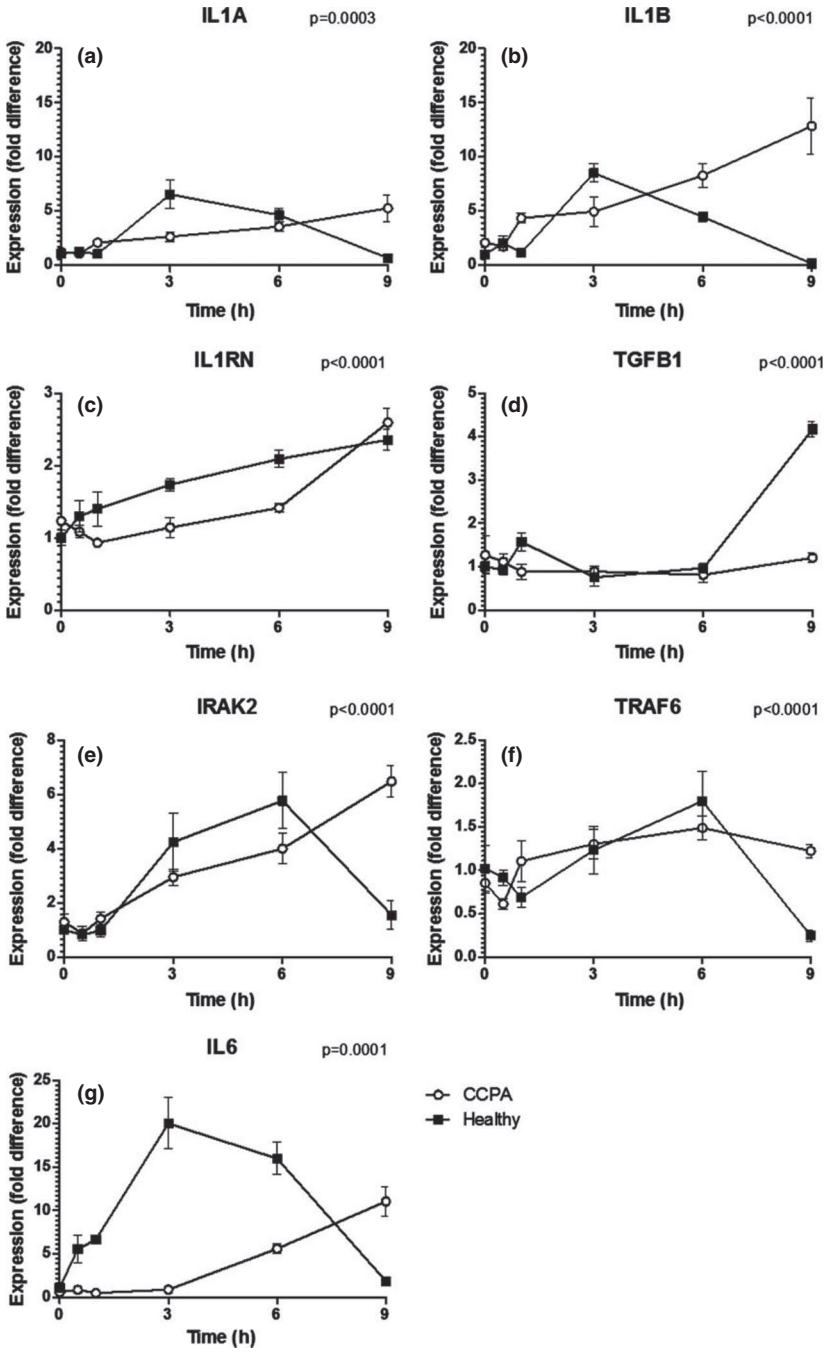

FIG. 1. Expression of the IL1 pathway differs between MDMs from CCPA (open circles) and healthy subjects (closed squares). Fold difference is calculated relative to the healthy 0 h. p-values indicate the differences in the change over time between the two groups, calculated by repeated measures two-way ANOVA. Each group comprises ten pooled RNA samples. Mean and standard deviations are shown for the three replicates completed.

correction for multiple testing (FDR-corrected p-values 0.050–0.051) (Table 3).

An IL10 SNP (rs1800896) previously associated with invasive aspergillosis (IA) was not associated with CCPA and a previous association with CCPA (*MBL2* rs5030737) could not be replicated (Table S2).

Discussion

Knowledge of the immune responses that are defective in CCPA is very limited, but is essential if we are to develop novel treatments for this progressive, often fatal, lung

TABLE 3. SNPs associated with chronic cavitary pulmonary aspergillosis (CCPA)

Gene	SNP	Alleles (M/m)	Model for association	Genotype frequency			Odds ratio (95% CI)	p-value	FDR p-value	Location
				Genotype	CCPA (%)	Healthy (%)				
IL1RN	rs4252041	**C**/T	TT + TC vs. CC	CC TT+TC	109 (97.3) 3 (2.7)	249 (89.2) 30 (10.8)	0.23 (0.07, 0.76)	0.017	**0.039**	3'UTR
IL1B	rs3136558	**A**/G	AG + GG vs. AA	AA AG + GG	82 (73.2) 30 (26.8)	170 (60.9) 109 (39.1)	0.57 (0.35, 0.92)	0.023	0.051	Intronic
	rs3917354	**T**/-	T-+-- vs. TT	TT T-+--	86 (76.8) 26 (23.2)	180 (64.5) 99 (35.5)	0.55 (0.33, 0.91)	0.020	**0.046**	Intronic
IL15	rs1519551	A/**G**	AA + AG vs. GG	GG AA + AG	34 (30.9) 76 (69.1)	48 (17.6) 225 (82.4)	0.48 (0.29, 0.79)	0.004	**0.011**	Intronic
	rs6842735	G/**T**	TT + GT vs. GG	GG TT + GT	69 (62.7) 41 (37.3)	205 (75.1) 68 (24.9)	1.79 (1.12, 2.88)	0.016	**0.038**	Intronic
	rs12508866	T/**C**	CC + TC vs. TT	TT CC + TC	55 (50) 55 (50)	172 (63.0) 101 (37.0)	1.70 (1.09, 2.66)	0.020	**0.046**	Intronic
IL17A	rs3748067	G/**A**	AA + GA vs. GG	GG AA + GA	85 (75.9) 27 (24.1)	239 (85.7) 40 (14.3)	1.90 (1.10, 3.28)	0.022	0.050	3'UTR

CI, confidence interval; M/m, Major allele/minor allele.
p-value calculated for the model indicated using logistic regression in Stata. Risk allele shown in bold. SNPs in bold remain significant after Benjamini-Hochberg adjustment for false discovery rate (FDR adjusted p-values shown).

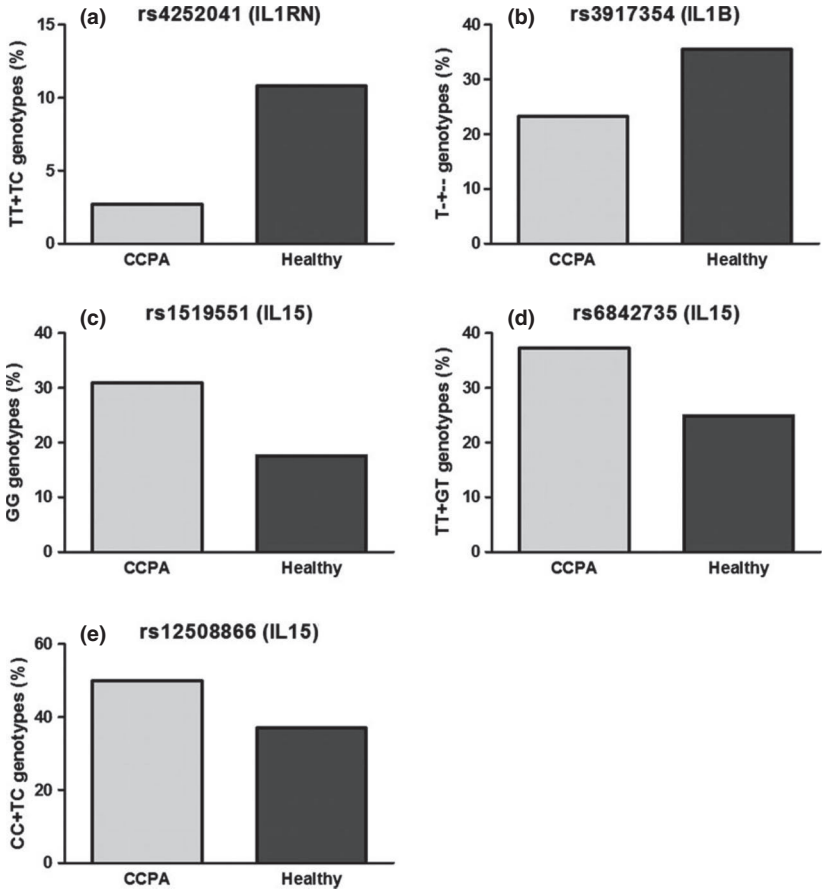

FIG. 2. Genotype frequencies of SNPs associated with CCPA; 279 healthy subjects and 112 CCPA subjects were genotyped successfully. RS3917354 is an insertion-deletion mutation.

condition. Identification of genetic susceptibility patterns may facilitate stratification of at-risk individuals, and possible disease prevention, or at least earlier diagnosis. We have shown that following stimulation of MDMs with live *A. fumigatus*, expression of cytokines from the IL1 pathway is markedly different in patients with CCPA compared with healthy controls. In addition, we identified SNPs in this pathway (as well as SNPs in IL15) that are associated with CCPA. This is the first study to investigate the possible underlying mechanisms of CCPA by comparing *in vitro* effects of *A. fumigatus* on MDMs from CCPA patients and healthy subjects; previous studies were conducted only in cells from healthy donors [6,7,9,16]. Further, this is by far the most extensive genetic association study of CCPA to date, involving three times as many patients and investigating many more SNPs.

We found that after exposing MDMs to *A. fumigatus*, expression of *IL1A* and *IL1B* peaks at 3 h for healthy subjects, then falls to baseline, whereas for CCPA subjects, expression rises more slowly but is still rising at 9 h (the experiment duration); it is unclear whether this represents the peak response. The reason that expression of these pro-inflammatory cytokines follows such different time courses in health and disease is unclear. In our co-culture model, live *A. fumigatus* conidia were introduced; until 3 h the predominant morphology was conidia, by 6 h germtubes and by 9 h almost completely hyphae (Appendix S2, Figure S2). The observed expression profiles suggest an initial pro-inflammatory IL1 response to the presence of conidia by MDMs from healthy subjects, followed by a reducing inflammatory response at 6–9 h as germtubes and hyphae predominate. It is likely in health that other immune cells (e.g. neutrophils) are recruited to clear fungus if macrophages fail [6], and we speculate that down-regulation of IL1 expression prevents a damaging on-going inflammatory response. For CCPA patients, the IL1 response during the first 3 h (when conidia predominate) was comparatively muted but continued to rise during the germtube and hyphal phases, which may reflect a lack of/delayed response to the changing fungal morphology (or the length of fungal exposure), which may result in on-going inflammation, resulting in tissue damage. The expression profiles of IRAK2, TRAF6 and IL6 also appear delayed in the CCPA group. These molecules are involved in multiple pathways, one of which is the IL1 pathway, where they are found downstream of IL1 itself. Although the expression may not be entirely related to the IL1 pathway, which could explain the differences between the TRAF6 and IRAK2 expression, the observed patterns appear to show a similar, delayed response in the CCPA group compared with the healthy group. In addition, expression of IL1 receptor accessory protein (IL1RAP) is also increased in the CCPA group compared with the healthy group at 9 h (Figure S1). This protein forms a complex with the IL1 receptor and IL1 at the cell surface, and is required for the response to IL1. Its expression correlates with IL1 responsiveness, including the IL1-induced production of inflammatory and immune responses [17,18]. We speculate that continued production of IL1 (in particular IL1β) could result in sustained cellular recruitment, proliferation and activation, and continued inflammation via both the IL1 pathway and IL17, which could promote CCPA disease progression rather than resolution.

IL1β is a key cytokine secreted and activated by the NLRP3 inflammasome following stimulation by pathogens including *A. fumigatus* [19]. IL1β is essential for the induction and expansion of the highly pro-inflammatory human Th17 cells [20], and can induce pulmonary inflammation via IL17 [21]. This is likely to be initially beneficial, and several studies (usually with a single time-point) have demonstrated that expression of the IL1 pathway (IL1α, IL1β and IL6) is increased in cells (macrophages and monocytes) from healthy donors in response to *A. fumigatus* [6,7,9]. However, uncontrolled IL1β can be detrimental and has been implicated in multiple diseases, including diabetes and gout [22]. Because of the presumed damaging effects, IL1β-targeted therapies have been attempted in several diseases [22]. For example, in arthritis, where IL1β is pivotal for sustained cellular recruitment and erosive cartilage damage [23], IL1β-targeted therapies reduce large joint inflammation and corticosteroid dosage [24]. Furthermore, in a mouse model, IL1 inhibition reduces levels of inflammatory cytokines (including IL6 and IL1β) and joint inflammation [25].

The possible delayed response and failure to control inflammation observed in the CCPA group may be influenced by *TGFB1* expression. TGF-β is an anti-inflammatory cytokine, which has previously been shown to antagonise IL1-mediated effects, such as cartilage proteoglycan synthesis in arthritic mice and lymphocyte proliferation [26]. This can occur via down-regulation of IL2-mediated proliferative signals. As IL2 stimulates production of IL1 [27], TGF-β may reduce levels of IL1 via IL2. In the current study, *TGFB1* expression increases at 9 h in the healthy group but not the CCPA group. This increased expression may be important in controlling an IL1-induced inflammatory response, and the low level in the CCPA group may represent a deficiency, which may contribute to uncontrolled inflammation in these subjects. TGF-β is also known to mediate fibrosis, and as such increased levels may be expected in CCPA patients as lung fibrosis is a feature of uncontrolled CCPA. This was not seen, and may reflect the short time course or that non-TGF-β-driven processes are involved in fibrosis in CCPA. The differential gene expression observed between the healthy and CCPA groups appears to suggest a mechanism by which disease develops and progresses as a result of continued IL1 production and continued inflammation. An alternative view would be that the observed increased expression of IL1 and other genes is due to the CCPA, rather than a cause of it. Innate immune cells such as monocytes do have an adaptive or memory component [28]; however, the MDMs used in this study are stored as frozen cells before being cultured for 15 days, and whether this memory would be retained is unclear. Despite this, it is possible that the response to *A. fumigatus* is a learned response rather than a predetermined response and as such that the differential response is a result of CCPA rather than a cause of susceptibility. However, the association of SNPs in this pathway with CCPA supports the theory that these genes are involved in susceptibility.

In addition to the gene expression observations, we identified SNPs in the IL1 pathway associated with CCPA. Previously, a haplotype including polymorphisms in *IL1RN* and *IL1B* was associated with susceptibility to IA [29]. The *IL1B* SNP associated with CCPA (rs3917354) in the current study is an intronic single base deletion that has previously been associated with ankylosing spondylitis [30]. One CCPA patient had underlying ankylosing spondylitis, but as this patient constituted only 0.9% of the patient population we do not feel this unduly influenced our results. Traditionally, intronic regions are considered non-functional; however, the ENCODE project suggests these regions could indeed be functional, and as such this SNP, or those in linkage disequilibrium (LD) with it, may affect the function of IL1β and in turn susceptibility to CCPA [31].

The *IL1RN* SNP associated with CCPA (rs4252041) is located in the 3'UTR. The CC genotype has previously been associated with higher circulating IL1Ra levels but only in patients with previous myocardial infarction (3 months to 6 years previously) and whether the higher levels had an effect (beneficial or detrimental) was not investigated [32]. A 3'UTR SNP in *IL17A* (rs3748067) was also found to be associated with CCPA, and although this SNP did not quite pass correction for multiple testing (FDR-adjusted p-value 0.050), it is interesting because of the interplay between IL1 and IL17. As mentioned, IL1β is essential for both the induction and expansion of Th17 cells [20]. These cells are highly pro-inflammatory. In addition, IL1β can induce pulmonary inflammation via IL17 [21]. As discussed, inflammation may be initially beneficial but longer term could be detrimental, and a failure to control this response could result in damaging inflammation in CCPA. Although we have not analysed expression of IL17 in the current study, the identification of associated SNPs in this gene suggests a role for IL17 in susceptibility to CCPA.

Three intronic SNPs in *IL15* are also significantly associated with CCPA. These SNPs were not in high LD with each other (all pairs $r^2 \leq 0.36$), their function remains unknown and none has been previously associated with disease, although another SNP in IL15 (not included in our study) has previously been shown to be associated with non-invasive aspergillosis [13]. We did not measure *IL15* expression; however, other studies have shown that IL15 increases the oxidative burst, IL8 release and hyphal damage of cells in response to *A. fumigatus* [33]. IL15 can act on dendritic cells (DCs), T-cells and macrophages and appears to promote IFN-γ expression and augment a Th1 response [34,35]. Th1 responses are thought to be beneficial in infections with *A. fumigatus*, and impaired IFN-γ responses are associated with aspergillosis, including chronic pulmonary aspergillosis [6,7,10,36,37]. This combined evidence supports a role for IL15 as beneficial in the response to *A. fumigatus*, and suggests that SNPs that affect functionality or expression of IL15 may increase susceptibility to CCPA.

As in a previous study by our group [38], we could not replicate an association of *MBL2* with CCPA [12]. MBL2 may be associated with CCPA complications, severity and disease progression, rather than with CCPA itself [38] (Appendix S3).

In the gene expression experiments we used pooled samples, as this allowed us to measure an increased number of cytokines from the limited number of cells available for each individual patient. Although pooling resulted in us being unable to view the distribution of individual data points for each cytokine and measure interpatient variability, it is unlikely that had we analysed samples individually and calculated a mean value for cases and for controls these would have been materially different from the pooled values. We acknowledge that it is possible that the expression level measured in the pooled samples could be skewed by a few individuals, but we nevertheless believe our data indicate that the IL1 pathway is involved in CCPA, at least in certain individuals, and merits further, more detailed, investigation. We recognize the ideal cell type for these experiments is the alveolar macrophage; however, had we recruited only subjects fit enough for bronchoscopy, the population would have been skewed towards stable patients at the mild end of the CCPA spectrum. In addition, we acknowledge that while the adherence method is a recognized method of selecting for monocytes, a number of lymphocytes may remain present in the culture.

Although our study recruited only 112 patients with CCPA, we emphasize that this is the largest genetic study of CCPA to date. The disease is relatively rare in developed countries; this population was recruited from the National Aspergillosis Centre, which receives referrals from across the UK. Selection of a control population was challenging; the range of underlying conditions in CCPA patients is broad and patients usually have multiple co-morbidities [4]. Recruiting matched controls with the same underlying diseases for every case would be ideal, but practically impossible, as demonstrated by the fact that all previous genetic association studies involving CCPA have used healthy controls [11,12,14]. We therefore selected healthy unmatched controls, recognizing that this reduces rather than increases our power to find genetic associations with CCPA and as such does not invalidate our findings. It could be argued that the associations we find are related to an underlying disease rather than to CCPA. This is unlikely, as each underling disease occurs in only a small proportion of cases; however, to investigate this we compared the results of our study with genetic studies of TB, the commonest primary underlying disease in CCPA [4] (Appendix S4). Although SNPs in genes we studied have been previously associated with TB, none of these were associated with CCPA, suggesting lack of matching

for previous TB did not affect the observed associations. We acknowledge that because our control population is younger than the patient population some could develop CCPA later in life; again, this would reduce, not increase, our power to identify associations.

In conclusion, our data indicate that expression of the IL1 pathway differs in CCPA compared with health, and that genetic variants in this pathway are associated with disease. We speculate that ongoing expression of *IL1B* (and *IL6*) may result in continuing inflammation, inhibiting disease resolution and contributing to disease progression in CCPA. As our study is relatively small, these findings need confirmation, but this pathway merits further study in this disease. CCPA is a serious and debilitating chronic condition, which requires long-term treatment with potentially toxic and often expensive antifungal agents and we hope that increased understanding of the genes and pathways involved in both susceptibility and disease progression in CCPA will lead to novel treatments and improved identification of at-risk individuals.

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Transparency Declaration

There are no conflicts of interest for any author.

Author Contributions

AS, PB and DD contributed to study conception and obtained funding. AS, PB, DD and NS contributed to study design. NS and JH contributed to acquisition of the data. NS, PB, DD and AS analysed and interpreted data and drafted the report. All authors approved the version submitted.

Supporting Information

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

Figure S1. Expression of members of the IL1 pathway differs between MDMs from CCPA (open circles) and healthy subjects (closed squares).

Figure S2. Changes in *A. fumigatus* morphology over time.

Table S1. List of all SNPs selected for genotyping.

Table S2. SNPs previously associated with aspergillosis that were not associated with CCPA in the current study

Appendix S1. Additional details on methods.

Appendix S2. Analysis of fungal morphology over time.

Appendix S3. Comparison with previous SNP association.

Appendix S4. Comparison of genetic association with CCPA to genetic association with TB.

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