

Elevated Levels of the Neutrophil Chemoattractant Pro-Platelet Basic Protein in Macrophages From Individuals With Chronic and Allergic Aspergillosis

Nicola L. D. Smith,^{1,3,4,5} Michael J. Bromley,^{1,3,4,5} David W. Denning,^{1,2,3,4,5} Angela Simpson,^{2,3,4,5} and Paul Bowyer^{1,3,4,5}

¹Manchester Fungal Infection Group and ²Respiratory and Allergy Centre, Faculty of Medical and Human Science, University of Manchester, ³Manchester Academic Health Science Centre, ⁴National Aspergillosis Centre, University Hospital South Manchester NHS Foundation Trust, and ⁵NIHR South Manchester Respiratory and Allergy Clinical Research Facility, United Kingdom

Background. *Aspergillus fumigatus* causes chronic cavitary pulmonary aspergillosis (CCPA) and allergic bronchopulmonary aspergillosis (ABPA) in overtly immunocompetent and atopic individuals, respectively. Disease mechanisms are poorly understood but may be related to increased neutrophil presence and activation. Pro-platelet basic protein (PPBP) is a potent neutrophil chemoattractant and activator whose expression is repressed by interleukin 10 (IL-10).

Methods. PPBP expression by monocyte-derived macrophages from patients with ABPA or CCPA and asthmatic and healthy controls (10 individuals per group) was analyzed using reverse-transcription polymerase chain reaction. PPBP and IL-10 protein levels in cell culture supernatants were measured by enzyme-linked immunosorbent assay. Two PPBP single-nucleotide polymorphisms (SNPs) were genotyped in 638 individuals. The gene was resequenced in 20 individuals.

Results. PPBP expression and protein levels were significantly increased in the ABPA (19.7-fold) and CCPA (27.7-fold) groups, compared with the control groups. PPBP SNPs were not associated with disease. IL-10 protein levels were significantly lower in the ABPA and CCPA groups, compared with the healthy group, suggesting that differences in PPBP levels may result from regulatory mechanisms.

Conclusions. The results suggest a role for increased PPBP expression in ABPA and CCPA. Repression of PPBP expression may benefit some patients. Increased PPBP expression in ABPA and CCPA may be useful as a future diagnostic tool or possible target for novel therapeutics.

Keywords. PPBP expression; aspergillosis; ABPA; CCPA; *Aspergillus fumigatus*.

The fungus *Aspergillus fumigatus* is a common cause of life-threatening infections in immunocompromised individuals and also causes devastating diseases in overtly immunocompetent and/or atopic individuals. These diseases are typified by chronic cavitary pulmonary aspergillosis (CCPA) and allergic bronchopulmonary

aspergillosis (ABPA) [1–3]. *A. fumigatus* is ubiquitous in the environment, and humans are estimated to inhale several hundreds of spores daily [1, 4–6]. These spores are small (diameter, 2–4 µm), allowing their deposition deep within the lung [1, 7]. In addition, the presence of surface galactosaminogalactan appears to confer particular adhesive and immune evasion capabilities of *A. fumigatus*, compared with other inhaled fungi [8]. In the majority of individuals these spores are cleared without causing disease, but in certain individuals disease such as CCPA or ABPA may ensue.

CCPA occurs in overtly immunocompetent individuals, usually those with some form of prior lung disease (eg, tuberculosis, sarcoidosis, or pneumothorax), and involves the formation of pulmonary cavities [3, 9].

Received 17 April 2014; accepted 19 August 2014; electronically published 5 September 2014.

Correspondence: Paul Bowyer, PhD, University of Manchester, 2nd Fl, Education and Research Centre, University Hospital South Manchester, Manchester M23 9LT, UK (paul.bowyer@manchester.ac.uk).

The Journal of Infectious Diseases® 2015;211:651–60

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DOI: 10.1093/infdis/jiu490

These cavities increase in size and number slowly, over a period of months to years [3]. Aspergillomas, or fungal balls, may be observed within the cavities but are not required for diagnosis of CCPA. The cavity walls show chronic inflammation with a mixture of inflammatory cells, including neutrophils, but few eosinophils or granulomas [3].

ABPA occurs in overtly immunocompetent but atopic individuals [2]. It often occurs in patients with cystic fibrosis (approximately 15% of adults with cystic fibrosis) or asthma (1%–3.5% of individuals with asthma seen in hospital referral clinics) but occasionally occurs independently of these [1, 2, 10–13]. ABPA usually presents as wheezing, poor asthma control, and expectoration of mucus plugs, occasionally with fatigue or increasing breathlessness [14]. Central bronchiectasis and/or mucoid impaction of bronchi with highly attenuated mucus may occur. The presence of *A. fumigatus* may be detected in the sputum of patients, either by fungal culture or by polymerase chain reaction (PCR), and skin reactivity to *A. fumigatus* or raised *Aspergillus*-specific immunoglobulin E (IgE) can also be demonstrated [2, 13, 15, 16]. In addition, sputa from patients with ABPA usually contains raised numbers of eosinophils and neutrophils, compared with controls [17, 18].

The human innate immune response to *A. fumigatus* involves both macrophages and neutrophils, as well as other immune cells. Macrophages prevent germination and rapidly ingest and kill resting conidia [19]. They are less effective at killing swollen conidia and hyphae, but they do ingest these forms of *A. fumigatus* and produce inflammatory cytokines in response to them, as well as acting as primary antigen-presenting cells for adaptive immune responses [20, 21]. Neutrophils are recruited to the lungs following infection with swollen conidia and hyphae, probably in response to the inflammatory cytokine or chemokine production of macrophages and other cells (eg, dendritic cells), and can kill *A. fumigatus* hyphae [19–22]. They may also be involved in the response to resting conidia [23]. However, although neutrophils are important for clearance of *A. fumigatus*, there may also be negative effects to their activation, as the oxidants and proteases they release are harmful to the host tissues, as well as to the pathogen they are designed to combat [24].

Human pro–platelet basic protein (PPBP; also known as CXCL7) and its many derivatives are potent neutrophil chemoattractants and activators [25]. Increased levels of PPBP have been found in bronchial submucosa and biopsy samples from patients with chronic obstructive pulmonary disorder (COPD) and are associated with increased neutrophil activation [25]. As neutrophils are important in the response to *A. fumigatus*, we hypothesized that levels of PPBP are important in the pathogenesis of aspergillosis.

In the current study, we investigate the expression of PPBP by human macrophages from patients with ABPA or CCPA and controls both before and after stimulation with *A. fumigatus*.

We also analyze PPBP and interleukin 10 (IL-10) protein levels in the supernatant from these cultures, as well as the PPBP protein level in the plasma from these subjects. In addition, we investigate single-nucleotide polymorphisms (SNPs) in the gene encoding PPBP in relation to susceptibility to ABPA and CCPA.

MATERIALS AND METHODS

Subjects

Individuals with ABPA, CCPA, atopic asthma, or nonatopic asthma and healthy controls were defined as described in Table 1. Patients with both ABPA and CCPA were excluded from the study. Subjects with ABPA, CCPA, or asthma were recruited from the North West Lung Centre and the National Aspergillosis Centre (University Hospital of South Manchester [UHSM], United Kingdom) between March 2006 and August 2010. In addition to these subjects, previously recruited asthmatic and healthy control subjects were used [26–29]. Additional healthy controls were recruited from the UHSM staff. Informed consent was obtained from all subjects. Subject demographic characteristics are shown in Supplementary Table 1.

DNA Extraction

Whole-blood specimens were collected in EDTA-treated blood collection tubes (BD, Oxford, United Kingdom) and centrifuged to separate plasma and cellular sections. DNA was extracted from the latter, using a phenol chloroform extraction method. Both plasma and DNA specimens were stored at -80°C . For the previously recruited asthmatic and healthy control subjects, DNA had been collected previously [29].

Peripheral Blood Mononuclear Cell (PBMC) Extraction

Whole-blood specimens were collected in sodium heparin-treated blood collection tubes (BD), and PBMCs were extracted using a Ficoll-Paque Plus density gradient (GE Lifesciences, Buckinghamshire, United Kingdom). PBMCs were stored in liquid nitrogen.

Collection of Bronchoalveolar Lavage (BAL) Supernatants

Subjects invited for bronchoscopy needed to have stable disease with no recent exacerbations (within 4 weeks) and needed to meet the safety criteria for bronchoscopy. Informed consent was obtained for all subjects. Flexible bronchoscopy was completed by physicians at the UHSM, using local guidelines and British Thoracic Society standard procedures [30]. Local anesthesia and conscious sedation was used with intravenous midazolam (average dose, 2 mg) and topical lignocaine spray. Two percent lignocaine gel was applied to the nostril of choice when the nasal approach was used (the method preferred by most patients). Where possible, high-volume lavage was collected from lobes with significant radiological abnormality. Where no

Table 1. Diagnostic Criteria for Recruited Subjects

| Study Group | Diagnostic Criteria |
|---|---|
| Patients with ABPA | <p>All of the following are required:</p> <ul style="list-style-type: none"> • Total serum IgE level >500 IU/mL, usually >1000 IU/mL • Either positive SPT for <i>Aspergillus</i> or <i>Aspergillus</i>-specific IgE • Either recurrent obstruction (mucoïd impaction) or episodes coughing up plugs of thick mucus (containing hyphae and eosinophils) • Current or historical evidence of eosinophilia <p>Further indicators:</p> <ul style="list-style-type: none"> • Most patients have asthma (or cystic fibrosis [not enrolled in this study]) and a large proportion have central bronchiectasis detected by CT, but these were not required for inclusion |
| Patients with CCPA | <p>All of the following are required:</p> <ul style="list-style-type: none"> • Chronic pulmonary or systemic symptoms (3 mo) compatible with chronic pulmonary aspergillosis, including at least one of the follow: weight loss, productive cough, or hemoptysis • Cavitory pulmonary lesion with evidence of paracavitory infiltrates or expansion of cavity size over time • Either positive serum <i>Aspergillus</i> IgG findings and/or precipitating antibody test OR isolation OR visualization of <i>Aspergillus</i> in pulmonary or pleural cavity specimens • Elevated inflammatory markers (C-reactive protein, plasma viscosity, or erythrocyte sedimentation rate) • No overt immunocompromising conditions (eg, HIV infection, leukemia, and chronic granulomatous disease) <p>Further indicators:</p> <ul style="list-style-type: none"> • Often multiple cavities, with more forming over time, if untreated • A fungal ball, or aspergilloma, might be present |
| Nonatopic asthmatics | <p>All of the following are required:</p> <ul style="list-style-type: none"> • Physician-diagnosed asthma • No diagnosis of aspergillosis • Negative SPT result (at 3-mm cutoff) and/or IgE (<0.4) to all allergens tested, including mite, cat, dog, and grasses • Negative SPT result (at 3-mm cutoff) and/or IgE (<0.4) to all fungi tested, including <i>A. alternata</i>, <i>C. albicans</i>, <i>C. herbarum</i>, <i>P. chrysogenum</i> (<i>notatum</i>), <i>T. rubrum</i>, and <i>A. fumigatus</i> |
| Atopic asthmatics (not sensitized to fungi) | <p>All of the following are required:</p> <ul style="list-style-type: none"> • Physician-diagnosed asthma • No diagnosis of aspergillosis • Negative SPT result (at 3-mm cutoff) and/or IgE (<0.4) to all fungi tested, including <i>A. alternata</i>, <i>C. albicans</i>, <i>C. herbarum</i>, <i>P. chrysogenum</i>, <i>T. rubrum</i>, and <i>A. fumigatus</i> • Either positive SPT result (at 3-mm cutoff) and/or IgE (<0.4) to any allergen nonfungal allergen tested (eg, mite, cat, dog and grasses) OR nonfungal allergens not tested |
| Healthy controls | <p>All of the following are required:</p> <ul style="list-style-type: none"> • No diagnosis of asthma • No diagnosis of aspergillosis • Negative SPT result (at 3-mm cutoff) and/or IgE (<0.4) to all allergens tested, including mite, cat, dog, and grasses • Negative SPT result (at 3-mm cutoff) and/or IgE (<0.4) to all fungi tested, including <i>A. alternata</i>, <i>C. albicans</i>, <i>C. herbarum</i>, <i>P. chrysogenum</i>, <i>T. rubrum</i>, and <i>A. fumigatus</i> |

Abbreviations: *A. alternata*, *Alternaria alternata*; ABPA, allergic bronchopulmonary aspergillosis; *A. fumigatus*, *Aspergillus fumigatus*; *C. albicans*, *Candida albicans*; CCPA, chronic cavitory pulmonary aspergillosis; *C. herbarum*, *Cladosporium herbarum*; CT, computed tomography; HIV, human immunodeficiency virus; IgE, immunoglobulin E; IgG, immunoglobulin G; *P. chrysogenum*, *Penicillium chrysogenum* (*notatum*); SPT, skin prick test; *T. rubrum*, *Trichophyton rubrum*.

radiological abnormalities were present (eg, in most asthmatics), samples were collected from the right lower lobes. A maximum of 240 mL (in 4 × 60-mL aliquots) of prewarmed sterile 0.9% sodium chloride (saline) solution was instilled into each chosen lobe. Aspirated BAL fluid was stored on ice before filtration (using a 100-µm-pore filter, BD Biosciences; Oxford, England). The filtrate was centrifuged (400 ×g for 10 minutes at 4°C), and the resulting supernatants were stored at –80°C.

Generation of *A. fumigatus* Conidia

A. fumigatus (AF293 strain) was cultured at 37°C for 48–72 hours on Sabouraud dextrose agar (Oxoid, Basingstoke, United

Kingdom) until green in color, at which time conidia were harvested using growth medium (Roswell Park Memorial Institute 1640 medium, supplemented with Pen Strep and 5% fetal bovine serum).

Macrophage–*A. fumigatus* Coculture

For these experiments, we selected patients with ABPA who had a total IgE level of >1000 IU/mL and patients with CCPA who had a total IgE level of <150 IU/mL, to avoid any phenotypic overlap between groups. PBMCs were plated into 24-well plates (2 × 10⁶ cells/well), and monocyte-derived macrophages (MDMs) were generated as described previously [31]. Live *A. fumigatus* conidia were added (4 × 10⁵ conidia/well) to mature

MDMs. RNA was extracted at baseline and at 0.5 hours, 1 hour, 3 hours, 6 hours, and 9 hours after infection, using the RNeasy kit (Qiagen, Crawley, United Kingdom). Time points of 0.5–3 hours, 6 hours, and 9 hours represent exposure to conidia, germ tubes, and hyphae, respectively [31].

Measuring PPBP Expression by Macrophages

Initially, PPBP expression was measured in pooled ($n = 10$) RNA samples (1 μg each) from each disease group, using the human innate and adaptive immune responses RT2 profiler PCR array (SABiosciences [now Qiagen]), according to the manufacturer's instructions. HRPT1, RPL13A, and GAPDH were used for normalization. HPRT1 was variable in the healthy 9 hours samples, and only RPL13A and GAPDH were used for the normalization of this array. Reverse-transcription PCR (RT-PCR) was completed in triplicate, and the data were analyzed according to the manufacturer's instructions, using their online RT-PCR data analysis tool (<http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php>). This uses the $\Delta\Delta\text{Ct}$ method. Fold-changes were calculated relative to the mean value for healthy controls at baseline.

Subsequently, PPBP expression was measured in individual RNA samples, using RT-PCR and previously reported primers [32]. ACTB and GAPDH were used for normalization. A total of 50 ng of RNA was used in a 25- μL reaction with 2 \times Brilliant II SYBR QRT-PCR master mix (Agilent, Wokingham, United Kingdom), RT/RNase block enzyme mixture (Agilent), and the appropriate primers (Supplementary Table 2) at 20-nM final concentration. RT-PCR was performed in triplicate with the Stratagene Mx3005p real-time quantitative PCR instrument, using the cycling conditions specified in Supplementary Table 3. The data were analyzed in the same way as for the pooled samples.

Measuring PPBP Protein Level in Cell Culture Supernatants, Plasma Specimens, and BAL Supernatants

PPBP protein levels were measured using the human CXCL7/NAP-2 DuoSet enzyme-linked immunosorbent assay (ELISA) Development System (R&D Systems, Minneapolis, MN). This

was used in accordance with the manufacturer's instructions, with the exception that the concentrations of both the capture and detection antibody were doubled. Samples were diluted as required.

Genotyping of the Gene Encoding PPBP

Two haplotype-tagging SNPs in PPBP were genotyped (rs9291191 and rs183028). Each tagged the remainder of the SNPs in PPBP (assessed with the Genome Variation Server; <http://gvs.gs.washington.edu/GVS/>). Genotyping was completed successfully in 638 individuals (112 with CCPA, 95 with ABPA, 279 healthy controls, and 152 with atopic asthma), using the Sequenom MassArray iPLEX Gold system, and results were analyzed in SNP and Variation Suite (SVS; version 7.4.3, Golden Helix), using Fisher exact and χ^2 tests. Both SNPs were in Hardy-Weinberg equilibrium ($P > .0001$).

Resequencing of the Gene Encoding PPBP

The PPBP gene and 1000 bp immediately flanking it was amplified by PCR, using Taq mastermix (Promega, Madison, WI) and 0.1- μM primers (Table 2), with cycling conditions of 95°C for 5 minutes, then 95°C for 1 minute, 52°C–57°C for 1 minute (annealing temperatures are specified in Table 2), and 72°C for 1 minute (35 cycles), and then 72°C for 2 minutes. PCR products were cleaned using the Wizard DNA Clean-up kit (Promega) and were sequenced using the same primers as for PCR amplification (forward and reverse) by Beckman Coulter Genomics (High Wycombe, United Kingdom). Sequences were compared to the Ensembl sequence (ENSG00000163736.3), using ClustalW2 online sequence comparison software (EMBL-EBI) [33]. Resequencing was completed for 5 individuals each from the ABPA, CCPA, asthmatic, and healthy control groups.

Measuring IL-10 Protein Levels in Cell Culture Supernatants

IL-10 protein levels were measured using the Human TH1/TH2 10-plex assay from Meso Scale Discovery (MSD, Rockville, MD) according to the manufacturer's instructions. Samples were diluted as required.

Table 2. Primers for Amplification of the Gene Encoding Pro-Platelet Basic Protein for Resequencing

| Amplicon Number | Primer | Amplicon Size, bp | Annealing Temperature, °C |
|-----------------|--|-------------------|---------------------------|
| 1 | Forward: TGACCTGAAAAGATAAGGAACACA; reverse: CTGAAGGCACAAACATGTGG | 629 | 55 |
| 2 | Forward: GGAGGATGAGTTTCATTTGTAG; reverse: ACCAGAAGACCTCTGAGTGAG | 563 | 55 |
| 3 | Forward: CCACTTATCTGCAGACTTGAG; reverse: TCCAGAGGTTGTCTTTATACACAT | 605 | 52 |
| 4 | Forward: AGGTACAATTGCCCTCAATCTGC; reverse: GCCATCTTTAAACCAACCTTCTCAG | 642 | 57 |
| 5 | Forward: CCAGGAAGGGTAGAATTTTGAACCC; reverse: GTCTAGTAAGGTTGAAGTCC | 681 | 55 |
| 6 | Forward: TGTGGTTAGCTGGTTTAAAGTGAA; reverse: GGCAATATTATCCCCATTTTAGG | 650 | 55 |

Statistical Analysis

Statistical analysis was completed using SPSS (version 16; SPSS) and GraphPad Prism (Version 5.02; GraphPad Software). Expression data was analyzed using the *t* test, repeated-measures 1-way analysis of variance (ANOVA), and 2-way ANOVA. PPBP and IL-10 protein levels were not normally distributed and were analyzed using the Mann–Whitney and Friedman tests. Correlation between protein levels and expression was analyzed by 2-tailed Spearman rank correlation.

RESULTS

Expression of PPBP in Pooled RNA Samples Is Higher in Macrophages From Patients With ABPA or CCPA Than in Controls

Baseline expression of PPBP was lowest in the healthy group and was 2.65-fold higher in the asthma groups ($P = .0004$), 19.7-fold higher in the ABPA group ($P = .0002$), and 27.9-fold higher in the CCPA group ($P = .00006$; Figure 1A). In addition,

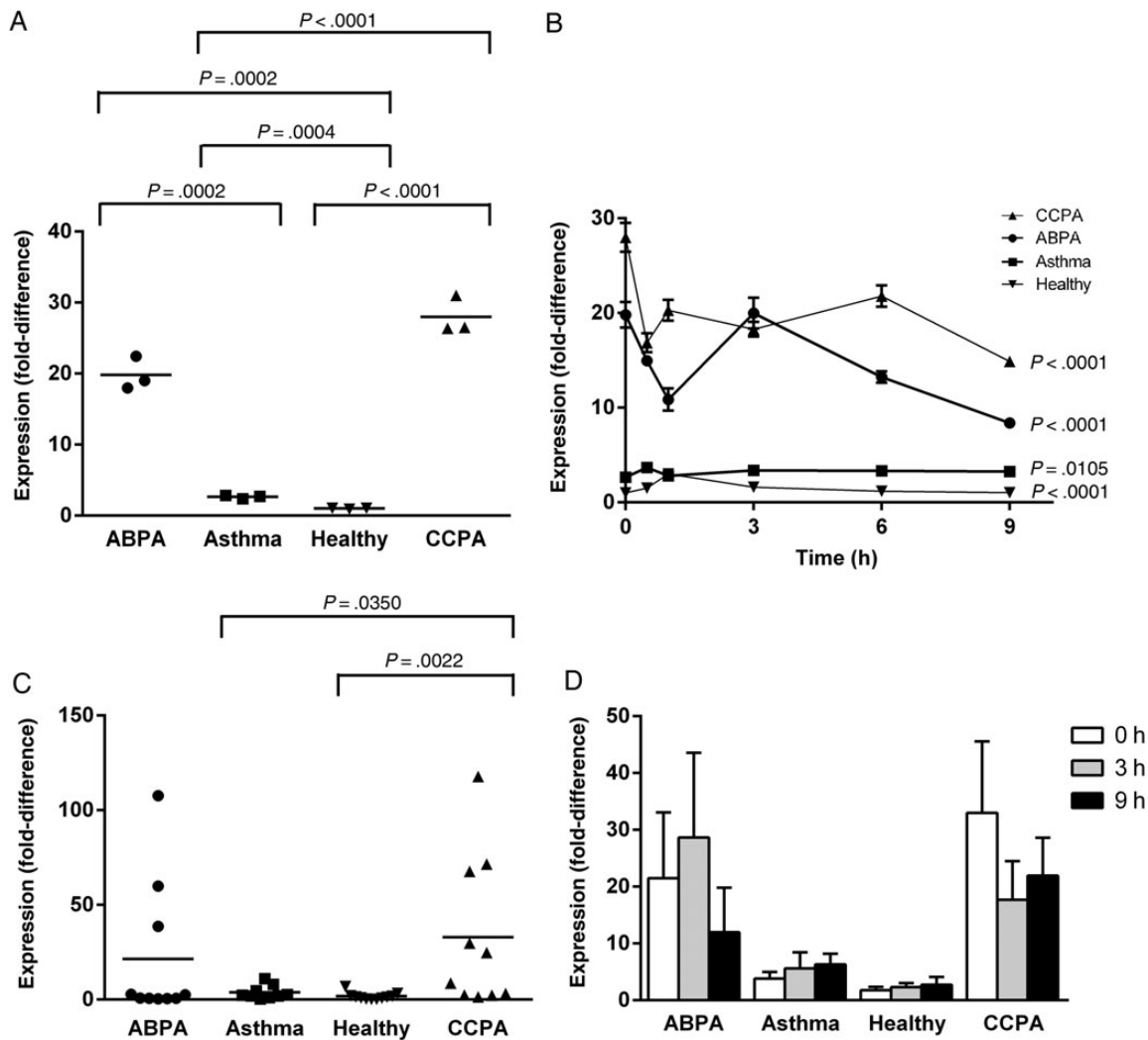


Figure 1. Expression of pro-platelet basic protein (PPBP) by macrophages from patients with allergic bronchopulmonary aspergillosis (ABPA) or chronic cavitary pulmonary aspergillosis (CCPA), compared with healthy and asthmatic controls. Fold-difference is calculated, compared with the average at baseline for healthy controls. **A**, Baseline expression (pooled). Each point is 1 array (10 subjects pooled together), and the bar represents the mean. Means were compared using the *t* test, and *P* values are given. There were significant differences between all groups. **B**, Expression after stimulation with *Aspergillus fumigatus* (pooled). Each point represents the mean, and the error bars represent the standard error of the mean (SEM) for the 3 replicate arrays. Change in expression over time was analyzed using repeated-measures 1-way analysis of variance, and *P* values are given for each group. Expression changed over time in all groups but remained higher in the CCPA and ABPA groups, compared with the asthmatic and healthy control groups. **C**, Baseline expression levels (individual subjects). Each point represents a single subject. Bars represent the mean. Means were compared using the Mann–Whitney test. Expression in the CCPA group was significantly increased, compared with the asthmatic and healthy control groups. **D**, Expression after stimulation with *A. fumigatus* (individual subjects). Shown are the mean and SEM results for each the ABPA ($n = 10$), CCPA ($n = 10$), healthy control ($n = 11$), and asthmatic control ($n = 9$) groups. The change in expression over time for each group was analyzed using a Friedman test. Changes were not significant for any group.

baseline expression of PPBP in the ABPA group was 7.45-fold higher than that in the asthma groups ($P = .0002$).

After stimulation with *A. fumigatus*, expression varied significantly over time in all study groups but remained higher in the CCPA and ABPA groups, compared with the control groups, at all time points (Figure 1B).

The Expression Pattern Observed in the Pooled RNA Can Be Replicated Using Individual RNA

To determine whether the data from the pooled experiment were representative of the entire patient population, we performed RT-PCR on the individual RNA samples (Figure 1C and 1D). Expression of PPBP was variable between subjects but was shown to reflect the pooled expression data, with a trend toward higher PPBP expression in patients with ABPA

or CCPA, compared with controls. However, this was only significant for the subjects with CCPA (Figure 1C and 1D).

PPBP Protein Levels Are Increased in Cell Culture Supernatants From Macrophages From Subjects With CCPA or ABPA, Compared With Controls

To determine whether the increased expression observed in the macrophages from patients with aspergillosis resulted in increased protein production, we performed ELISA on supernatants from the cell cultures. Baseline PPBP protein levels were found to be significantly higher in supernatant from cells from subjects with ABPA or CCPA than in supernatant from cells from asthmatic or healthy subjects (Figure 2A). Protein levels did not change significantly over time after stimulation with *A. fumigatus* in any group except the ABPA group, but

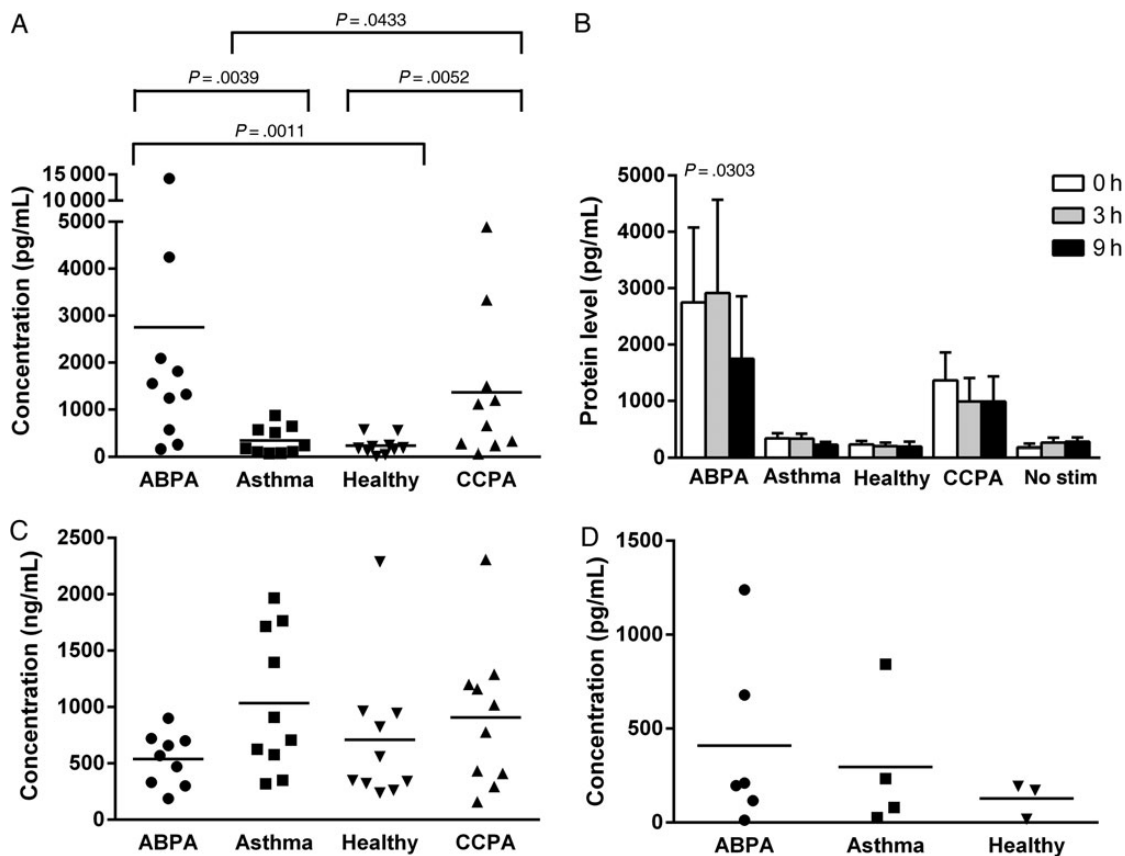


Figure 2. Pro-platelet basic protein (PPBP) levels in supernatant from cell cultures (A and B), plasma specimens (C), and bronchoalveolar lavage (BAL) fluid (D). A, Baseline protein levels. Each point represents a single subject ($n = 10$). Bars represent the mean and were compared using a Mann–Whitney test (significant P values are shown). PPBP protein levels in the allergic bronchopulmonary aspergillosis (ABPA) and chronic cavitary pulmonary aspergillosis (CCPA) groups are significantly higher than those in the asthmatic and healthy control groups. Levels in the asthmatic control group were not significantly different from those in the healthy control group. B, Protein levels over time. Shown are the mean and standard error of the mean results for each disease group ($n = 10$). The change in protein level over time for each group was analyzed using a Friedman test. This was only significant for the ABPA group. Unstim, unstimulated samples ($n = 20$). C, PPBP protein levels in plasma. Means were compared using Mann–Whitney tests, and no significant differences were found ($n = 10$ for the asthma control group, healthy control group, and CCPA group; $n = 9$ for the ABPA group). D, PPBP protein levels in BAL supernatants. Means were compared using Mann–Whitney tests, and no significant differences were found ($n = 4$ for the asthma control group, $n = 3$ for the healthy control group, and $n = 6$ for the ABPA group).

the levels remained higher in the CCPA and ABPA groups than in the asthma and healthy groups (Figure 2B). As would be expected, there was strong correlation between PPBP expression by the MDMs and PPBP protein level in the supernatant (both overall and at each time point; overall $P < .0001$).

PPBP Protein Levels in Plasma and BAL Are Not Significantly Different Between Patients and Controls

Next we investigated whether the observed increased production of PPBP protein by macrophages from subjects with CCPA or ABPA resulted in differences in blood plasma or BAL supernatants collected directly from these patients, again by ELISA. PPBP protein levels were much higher in the plasma than in the BAL supernatants, possibly because of the presence of platelets in the blood, but were not significantly different between groups in either plasma or supernatant (Figure 2C and 2D). There was no correlation between MDM PPBP expression or protein level at baseline and protein levels in plasma or BAL.

SNPs in PPBP Are Not Associated With CCPA or ABPA, and No Novel Polymorphisms Are Identified in the Gene Encoding PPBP

Neither SNP genotyped (rs9291191 and rs183028) showed an association with either ABPA or CCPA (genotype frequencies are given in Table 3). Resequencing of PPBP in a subset of 5 subjects with ABPA, 5 with CCPA, 5 with asthma, and 5 healthy controls did not identify any novel polymorphisms or any differences between subjects.

IL-10 Protein Levels Are Lower in Cell Culture Supernatants From Macrophages From Subjects With CCPA or ABPA, Compared With Healthy Controls

As IL-10 negatively regulates expression of PPBP, we investigated the IL-10 protein levels in the supernatants from our cell-culture experiments. Baseline IL-10 levels were higher in the healthy group, compared with the disease groups (Figure 3). IL-10 expression was altered significantly over time after stimulation with *A. fumigatus* in the ABPA, CCPA, and asthmatic groups but not in the healthy group.

DISCUSSION

This study demonstrates that PPBP expression by human MDMs from patients with ABPA or CCPA is higher than that expressed by MDMs from healthy or asthmatic controls, with MDMs from subjects with CCPA expressing on average >25-fold more PPBP than MDMs from healthy control subjects. For the subjects with CCPA, this was statistically significant at both the messenger RNA (mRNA) and protein levels, while for the ABPA group the difference was significant only at the protein level. We believe this is the first study to investigate gene expression by macrophages derived from patients with ABPA or CCPA and to demonstrate that differences can be found

Table 3. Genotype Frequencies in Patients With Chronic Cavitary Pulmonary Aspergillosis (CCPA), Patients With allergic Bronchopulmonary Aspergillosis (ABPA), and Asthmatic and Healthy Controls

| Variable | SNP | |
|-------------------------------------|---|--|
| | rs9291191 | rs183028 |
| Alleles, major/minor | A/G | G/A ^a |
| CCPA group vs healthy control group | | |
| Genotype frequency | | |
| CCPA group | AA: 102 (94) AG: 7 (6) GG: 0 (0) | GG: 87 (78) GA: 23 (20) AA: 2 (2) |
| Healthy control group | AA: 259 (95) AG: 13 (5) GG: 0 (0) | GG: 225 (82) GA: 45 (16) AA: 6 (2) |
| Dominant model | | |
| Model | AA vs GG+AG | GG vs AA+GA |
| <i>P</i> | .5771 | .3880 |
| OR (95% CI) | 0.764 (.297–1.968) | 0.789 (.460–1.352) |
| Recessive model | | |
| Model | NA ^b | AA vs GG+GA |
| <i>P</i> | NA ^b | .8076 |
| OR (95% CI) | NA ^b | 0.818 (.163–4.116) |
| ABPA group vs atopic asthma group | | |
| Genotype frequency | | |
| ABPA group | AA: 90 (97) AG: 3 (3) GG: 0 (0) | GG: 76 (80) GA: 18 (19) AA: 1 (1) |
| Atopic asthma group | AA: 139 (93) AG: 11 (7) GG: 0 (0) | GG: 123 (81) GA: 26 (17) AA: 3 (2) |
| Dominant model | | |
| Model | AA vs GG+AG | GG vs AA+GA |
| <i>P</i> | .1521 | .8590 |
| OR (95% CI) | 2.5 (.688–9.90) | 0.943 (.495–1.798) |
| Recessive model | | |
| Model | NA ^b | AA vs GG+GA |
| <i>P</i> | NA ^b | .5777 |
| OR (95% CI) | NA ^b | 0.528 (.054–5.155) |

Data are no. (%) of study participants, unless otherwise indicated.

Abbreviations: CI, confidence interval; NA, not available; OR, odds ratio; SNP, single-nucleotide polymorphism.

^a The SNP is actually A/G/T, but only G and A alleles were identified in our population.

^b The recessive model could not be completed for this SNP because no individuals carried the GG (minor homozygous) genotype. Neither of SNP is significantly associated with either CCPA or ABPA in either a dominant or recessive model.

between these and control cells. We have also shown that pooled expression data can be useful as a cost- and resource-efficient method of directing research (ie, more genes can be analyzed from a smaller number of cells) and that it may reflect the individual data well, as shown for our CCPA group.

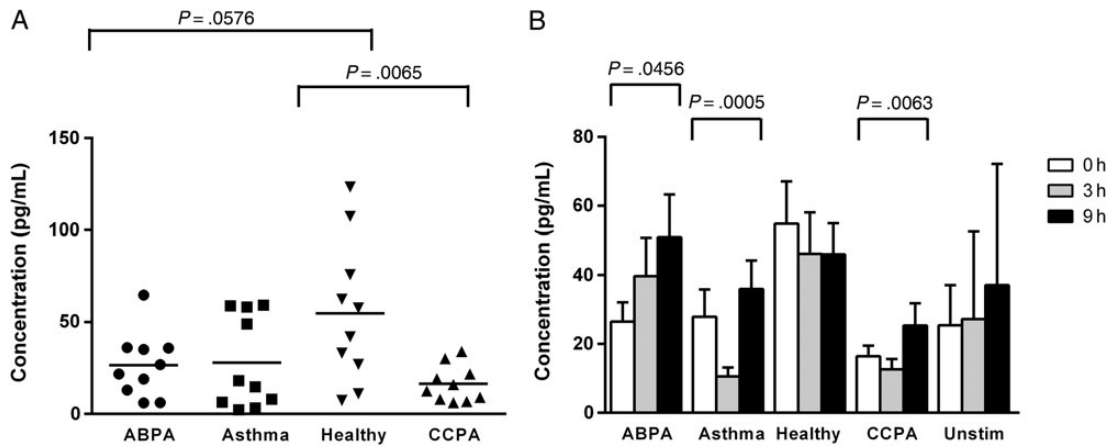


Figure 3. Interleukin 10 (IL-10) protein levels in cell culture supernatants. *A*, Baseline IL-10 levels. Each point represents a single subject ($n = 10$). Bars represent the mean and were compared using a Mann–Whitney test (significant P values are shown). IL-10 protein levels in the chronic cavitary pulmonary aspergillosis (CCPA) group were significantly lower than those in the healthy control group. A similar trend was seen for the allergic bronchopulmonary aspergillosis (ABPA) group. *B*, Protein levels over time after stimulation with *A. fumigatus*. Shown are the mean and standard error of the mean for each disease group ($n = 10$). The change in protein level over time for each group was analyzed using a Friedman test. This was significant for the ABPA, CCPA, and asthmatic control groups. Unstim, unstimulated samples ($n = 5$).

The increased expression of PPBP observed in aspergillosis has not been reported previously. PPBP is a small protein produced by platelets, monocytes, and macrophages. Expression by macrophages and monocytes has been shown to be increased in response to bacteria or microbial components and to involve protease-activated receptor stimulation [34]. PPBP is a precursor to a wide variety of derivatives, including platelet basic protein (PBP), neutrophil-activating peptide-2 (NAP-2), connective tissue-activating peptide-III, β -thromboglobulin, and thrombospondin-1 (TC-1) and TC-2 [35]. These are produced by posttranslational cleavage and have various antifungal and antibacterial properties [34–39]. In particular, NAP-2 is a neutrophil activator, and the TCs are cationic antimicrobial peptides, which kill many bacteria and fungi [34, 36, 37].

PPBP and its derivatives are also potent neutrophil chemoattractants and activators [25, 40–43], and neutrophils are important in the host defense against *A. fumigatus*. This may suggest that increased levels of PPBP should be beneficial, but it may also be that uncontrolled, persistent recruitment of these cells is detrimental and could be central to understanding the pathogenesis of all forms of pulmonary aspergillosis in nonimmunocompromised patients. For example, studies have noted increased sputum neutrophil numbers in subjects with ABPA, compared with controls (60% vs 29%), and in *A. fumigatus*-sensitized asthmatics, compared with nonsensitized asthmatics (81% vs 50%), and the proportion of neutrophils in sputum of patients with ABPA correlates with disease markers such as bronchiectasis and interleukin 8 expression [17, 18, 44]. In both ABPA and CCPA, uncontrolled, ongoing neutrophil recruitment and presence could result in an ongoing inflammatory

response, resulting in localized host damage, prolonging disease and preventing resolution of symptoms.

We have shown that levels of PPBP mRNA are high in macrophages from patients with ABPA or CCPA before stimulation with *A. fumigatus*, suggesting that PPBP is not being produced in response to the fungus but is instead constitutively expressed at higher levels in these patients. A recent study suggested the possibility of memory in innate immune cells, which in turn suggests the possibility that the high level of PPBP observed may be a result of the priming of these cells to fungus before sampling [45]; however, we do not believe this was likely in our study, as the cells were frozen, thawed, and then cultured for 15 days without fungus before measurement of the cytokine level and it is unlikely that any priming effect would remain by this stage. In addition, although levels decreased over time during the experiments, which may have been related to differing responses to different morphologies (hyphae predominate by the 9-hour time point), levels remained very high in the disease groups until the end of the experiment. We were unable to gather data beyond 9 hours because the live fungus overgrew the culture plate after this time. Within the host, conidia are continually inhaled and levels may remain higher.

PPBP levels have previously been found to be increased in the lungs of patients with COPD, a finding that was also associated with increased neutrophil activation [25]. This study used bronchial submucosa and biopsy samples. We found increased mRNA and protein levels in macrophage cultures but did not find differences in PPBP protein level in peripheral blood plasma specimens from our patients. In addition, there was no significant difference in PPBP protein level or size in plasma tested

by Western blot (data not shown). Because PPBP is produced in large amounts by platelets, it may be that the presence of these in peripheral blood reduces the ability to detect differences in the plasma PPBP levels of patients and controls [34, 35]. It is likely that investigating levels directly in the lung rather than in peripheral blood will be more relevant and may identify increased levels. We investigated levels of PPBP in BAL samples from patients with ABPA, asthmatics, and healthy subjects but did not find a difference in the level of PPBP. We were only able to obtain BAL samples from 4 asthmatics, 3 healthy subjects, and 6 subjects with ABPA, and it is possible that we did not have sufficient power with these small numbers to show a statistically significant difference.

PPBP is negatively regulated by interleukin 4 and IL-10 [34, 35], and mRNA levels are reduced following stimulation with these cytokines [35]. mRNA levels are also reduced following stimulation with interferon γ (IFN- γ), although the decrease is not significant [35]. Many patients with chronic pulmonary aspergillosis in our center have reduced IFN- γ production, and treatment with interferon gamma can be beneficial to patients [3, 46]. We have demonstrated here that the IL-10 levels in the cell culture supernatants were lower in groups with higher PPBP levels. Because we did not find any genetic association of SNPs in PPBP with either ABPA or CCPA and also did not identify any novel polymorphisms in the PPBP gene, it appears that the increased levels of PPBP are a result of constitutive differences in upstream regulatory systems rather than PPBP gene polymorphisms. IL-10 may be involved in one of these mechanisms, but it is likely that others are also involved.

Both PPBP mRNA and expression of PBP and other derivatives have been demonstrated to be strongly repressed by corticosteroids (dexamethasone) in monocytes and platelets in vitro [35]. After removal of the glucocorticoids, levels of PPBP mRNA are shown to increase back to baseline levels, suggesting that the repression is due to transcriptional regulation. This repression has also been demonstrated in vivo. Monocytes extracted from healthy volunteers who had received clinically relevant doses of dexamethasone (25 mg per day from baseline to day 3, 50 mg from days 3 to 6, and 100 mg from days 6 to 9) showed reduced PPBP mRNA levels, compared with those extracted from the volunteers before dexamethasone treatment. Monocytes extracted 4 days after dexamethasone treatment showed increasing levels of PPBP mRNA, correlating with the in vitro data suggesting the response is temporary.

It may be therefore that treatment with corticosteroids or interferon gamma is beneficial in subjects with ABPA or CCPA who have increased PPBP mRNA or protein levels. Use of prednisolone and inhaled steroids (eg, beclomethasone and fluticasone) is currently routine for treatment of asthma and ABPA [2, 15], and the effect on PPBP expression may be one mechanism for the benefit observed in these patients. Similarly, repression of PPBP may be part of the reason that many patients with CCPA

respond to treatment with interferon gamma [3]. Because a proportion of patients with ABPA in our clinic are prescribed corticosteroid therapy, it may be that some of those tested are currently taking this medication, which may explain the lower PPBP level observed for some of the macrophages in the ABPA group; however, because the effect is temporary, we would expect it to be lost after 15 days of culture. Corticosteroids are not currently used routinely for treatment of CCPA but may be beneficial for some patients, based on the data presented here.

In conclusion, the current study demonstrated increased levels of PPBP in macrophage cultures from patients with ABPA or CCPA, compared with asthmatic and healthy subjects, and suggested that this may be a result of reduced transcriptional regulation by IL-10. Further work is required to ascertain whether these increased PPBP levels are detrimental and involved in disease production or progression or whether they are beneficial and due to priming within the subject, demonstrating a response to fungal exposure occurring within the host. If the former is found to be correct, then treatment with corticosteroids or blockade of the PPBP receptor CXCR2 may be beneficial in patients with ABPA or CCPA who have raised PPBP levels.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (<http://jid.oxfordjournals.org>). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

Acknowledgments. We thank the patients and control subjects, for their participation; Dr Livingstone Chishimba, for providing the BAL samples; and the staff at the Centre for Integrated Genomics Research (Manchester, United Kingdom), who completed the genotyping.

Disclaimer. The views expressed in this publication are those of the author(s) and not necessarily those of the NHS, the National Institute for Health Research, or the Department of Health.

Financial support. This work was supported by the European Union's Seventh Framework Programme (FP7/2007-2013, under grant agreement HEALTH-2010-260338 [ALLFUN]), the Medical Research Council, the Fungal Research Trust, the National Aspergillosis Centre, the National Institute for Health Research, and the JP Moulton Charitable Foundation. This report is independent research supported by the National Institute for Health Research Clinical Research Facility at University Hospital of South Manchester NHS Foundation Trust.

Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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