



Original Article

Blood basophils from cystic fibrosis patients with allergic bronchopulmonary aspergillosis are primed and hyper-responsive to stimulation by *aspergillus* allergens

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Abstract

Introduction: Fifteen to sixty percent of cystic fibrosis patients harbor *Aspergillus fumigatus* (*Af*) in their airways (CF-AC) and some will develop allergic bronchopulmonary aspergillosis (CF-ABPA). Since basophils play a key role in allergy, we hypothesized that they would display alterations in CF-ABPA patients compared to CF-AC or patients without *Af* colonization (CF).

Methods: Using flow cytometry, we measured CD203c, CD63 and CD123 levels on basophils from CF-ABPA (N=11), CF-AC (N=14), and CF (N=12) patients before and after *ex vivo* stimulation with *Af* allergens.

Results: Baseline CD203c was increased in basophils from CF-ABPA compared to CF-AC and CF patients. *Af* extract and recombinant *Asp f1* stimulated basophils from CF-ABPA patients to markedly upregulate CD203c, along with modest upregulation of CD63 and a CD123 downward trend. Plasma TARC/CCL17 at baseline and post-stimulation cell supernatant histamine levels were similar in the three groups.

Conclusions: In CF-ABPA, blood basophils are primed and hyperresponsive to *Af* allergen stimulation.

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Keywords: Degranulation; FACS; Granulocytes; Inflammation; Lung disease

Abbreviations: ABPA, allergic bronchopulmonary aspergillosis; AC, colonized with *A. fumigatus*; *Af*, *Aspergillus fumigatus*; *rAsp f1*, recombinant purified *A. fumigatus* allergen 1; CF, cystic fibrosis, without *A. fumigatus* colonization or ABPA; EDTA, ethylene diamine tetraacetic acid; HC, healthy control; IgE, immunoglobulin E; IL-3, interleukin 3; MFI, median fluorescence intensity; PBS, phosphate buffered saline; Sero, serology; TARC, Thymus and Activation-Regulated Chemokine (CCL17)

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1. Introduction

Most of the morbidity and mortality among cystic fibrosis (CF) patients is associated with airway disease [1]. CF airway disease, which starts soon after birth, results in airway obstruction caused by a pathological triad of inspissated mucus, inflammation (mostly with neutrophils) [2], and microbial colonization. Although the prokaryotic bacteria *P. aeruginosa* and *S. aureus* are the dominant microorganisms in CF [3], a large proportion of CF patients also harbor the eukaryotic filamentous fungus *Aspergillus fumigatus* (*Af*) [4–6].

The relationship between host and *Af* in CF is quite complex [7]. Some CF patients show stable *Af* colonization (CF-AC), which

Table 1
Characteristics of the patients enrolled in this study.

Group	Sex	Age	Genotype	Af	MPA	PA	SA	ITRA	OS	ZIT	TOBI	IgE (Ku/L)	FEV1 in L. (% Pr.)	FVC in L. (% Pr.)
CF	F	16	ΔF508 / ΔF508	N	N	N	Y	N	N	Y	N	33	2.42 (90)	2.97 (100)
CF	M	14	ΔF508 / ΔF508	N	N	N	Y	N	N	Y	N	5	3.49 (112)	3.87 (106)
CF	M	10	G178R / LA67P	N	N	N	Y	N	N	N	N	2	1.90 (93)	2.57 (108)
CF	F	14	ΔF508 / ΔF508	N	N	N	Y	N	N	Y	N	19	2.70 (97)	3.33 (108)
CF	M	7	ΔF508 / ΔF508	N	N	N	Y	N	N	N	N	57	1.23 (90)	1.57 (103)
CF	F	14	ΔF508 / UNK	N	N	N	Y	N	N	Y	N	247	2.20 (72)	2.80 (82)
CF	M	14	ΔF508 / 1898+7G>A	N	Y	N	N	N	N	Y	Y	34	2.32 (76)	2.79 (79)
CF	F	32	ΔF508 / ΔF508	N	N	N	Y	N	N	N	N	135	2.67 (81)	2.22 (78)
CF	F	42	ΔF508 / 749NUCT2T	N	N	N	Y	N	N	N	N	34	2.46 (76)	3.91 (76)
CF	M	15	ΔF508 / ΔF508	N	N	N	Y	N	N	Y	Y	8	3.02 (82)	4.33 (105)
CF	M	18	ΔF508 / 711 +1-G->A	N	N	Y	Y	N	N	N	Y	1008*	4.11 (92)	3.11 (94)
CF	F	14	ΔF508 / E1371X	N	N	N	Y	N	N	N	N	2	2.68 (88)	2.31(97)
CF-AC	M	13	ΔF508 / ΔF508	Y	Y	N	Y	N	N	N	N	35	2.39 (95)	2.89 (99)
CF-AC	F	18	ΔF508 / ΔF508	Y	Y	N	Y	N	N	Y	N	35	1.51 (54)	2.80 (91)
CF-AC	F	15	ΔF508 / ΔF508	Y	N	Y	N	N	N	N	N	123	2.61 (97)	3.20 (107)
CF-AC	F	19	ΔF508 / ΔF508	Y	N	Y	Y	N	N	N	N	18	2.56 (96)	3.11 (107)
CF-AC	F	22	ΔF508 / ΔF508	Y	N	N	Y	N	N	Y	N	40	3.09 (101)	3.69 (98)
CF-AC	M	17	ΔF508 / ΔF508	Y	Y	Y	Y	N	N	N	N	39	3.30 (85)	4.06 (92)
CF-AC	M	20	ΔF508 / 1717-1-G->A	Y	N	Y	N	Y	N	N	Y	204	4.11 (92)	4.96 (94)
CF-AC	F	30	ΔF508 / ΔF508	Y	N	N	Y	Y	N	Y	Y	34	2.75 (83)	3.73 (96)
CF-AC	F	46	W1282X / W1282X	Y	N	N	Y	N	N	Y	N	44	2.83 (101)	3.63 (104)
CF-AC	M	26	ΔF508 / ΔF508	Y	Y	Y	Y	N	N	Y	N	8.4	3.83 (81)	5.97 (103)
CF-AC	F	14	ΔF508 / ΔF508	Y	Y	Y	N	N	N	N	N	35	1.62 (67)	2.13 (79)
CF-AC	F	33	ΔF508 / ΔF508	Y	Y	Y	Y	N	N	Y	N	181	1.27 (37)	2.31 (56)
CF-AC	M	51	ΔF508 / ΔF508	Y	Y	Y	N	N	N	Y	N	2	2.60 (94)	4.10 (90)
CF-AC	F	51	ΔF508 / ΔF508	Y	Y	Y	N	N	N	Y	N	5	2.84 (55)	3.95 (76)
CF-ABPA	F	18	ΔF508 / W1282X	Y	N	N	Y	N	N	N	N	1417	2.84 (55)	3.95 (76)
CF-ABPA	M	28	ΔF508 / ΔF508	N	Y	Y	N	Y	N	N	N	1016	2.84 (55)	3.95 (76)
CF-ABPA	M	38	ΔF508 / 7711-G->T	Y	N	N	Y	Y	Y	Y	Y	571	0.98 (39)	1.57 (50)
CF-ABPA	M	19	ΔF508 / ΔF508	Y	N	Y	N	N	N	N	N	1204	1.94 (43)	4.30 (70)
CF-ABPA	F	17	ΔF508 / ΔF508	Y	N	N	N	N	Y	Y	Y	211**	3.03 (108)	3.41 (111)
CF-ABPA	F	16	ΔF508 / R334W	N	N	Y	N	Y	N	Y	Y	1162	1.38 (48)	2.27 (72)
CF-ABPA	F	11	G542X / C1811+1643G>T, 7T/9T poly T	Y	N	N	Y	N	N	Y	N	1932	0.73 (42)	1.37 (69)
CF-ABPA	M	8	ΔF508 / 3272-28A>G, 7T/9T poly T	N	Y	Y	Y	Y	N	N	N	1162	1.63 (37)	2.35 (44)
CF-ABPA	M	69	ΔF508 / 6194V, 7T/9T	N	Y	N	Y	N	N	Y	N	522	1.44 (54)	2.35 (52)
CF-ABPA	M	21	ΔF508 / ΔF508	N	Y	Y	Y	Y	Y	Y	N	1030	1.16 (27)	3.08 (60)
CF-ABPA	M	9	ΔF508 / ΔF508	N	N	Y	Y	N	Y	Y	Y	1106	1.19 (98)	1.65 (120)
CF omalizumab	F	11	3849+10kbC>T / UNK	Y	N	Y	N	N	Y	Y	Y	775	2.21 (56)	3.53 (76)
CF omalizumab	M	11	ΔF508 / ΔF508	Y	N	N	Y	N	Y	N	N	948	1.33 (54)	54 (78)

Af, *A. fumigatus*; F, female; FEV1, forced expiratory volume in one second; FVC, forced vital capacity; IgE, immunoglobulin E; ITRA, itraconazole; M, male; med, medication; MPA, mucoid *P. aeruginosa*; patients OS, oral steroids; PA, *P. aeruginosa* (non-mucoid); Pr., Predicted; SA, *S. aureus*; TOBI, inhaled tobramycin; ZIT, zithromax. *This patient is atopic and has many environmental allergies. He presents hives and itchy eyes anytime he is exposed to following allergens: rye grass (specific IgE: 14.5 Ku/L) and/or timothy grass (specific IgE: 38.8 Ku/L) and/or cat dander (specific IgE: 37.6 Ku/L) and/or dust mite (specific IgE: >100 Ku/L)]. **This patient had a clinically stable (annual evaluation) baseline IgE level of 24 Ku/L increasing to 211 Ku/L when she had a pulmonary exacerbation unresponsive to specific antibiotherapy with other criteria for acute ABPA.

can last for months to decades with no allergic hypersensitization and uncertain impact on the course of the disease [8–10]. In others, ABPA occurs, which severely impacts lung function [11]. The reasons underlying the progression from CF-AC to CF-ABPA are unknown but host genetic factors are likely to be involved [12,13]. Over the last two decades, cases of CF-AC and CF-ABPA have become increasingly prevalent [5,7,14]. Fifteen to 60% of all CF patients are colonized by *Af* and an additional 10% of CF patients have ABPA [4,6,7].

Despite the increasing prevalence of AC and ABPA in CF, the distinction of CF-ABPA from CF-AC is often difficult [5,15], since it is typically made after the exclusion of bacterial exacerbations as a cause of clinical deterioration, and involves the complex employment of serologic and imaging criteria [16].

Although inflammation in CF is characterized by blood and airway neutrophilia [2], ABPA is defined by a strong immunoglobulin (Ig) E-mediated allergic component. Consistent with this the allergic mediator histamine is reportedly increased in blood from ABPA patients [17]. Since histamine in blood is mostly released by basophils, we hypothesized that blood basophils may be activated in patients with CF-ABPA.

Although blood basophils represent <1% of blood leukocytes, flow cytometry can easily analyze this rare subpopulation. Blood basophils constitutively express the surface interleukin (IL)-3 receptor CD123. Upon specific allergen activation, two further markers, CD63 (a tetraspanin intracellular granule marker) and CD203c (an ecto-nucleotide pyrophosphatase/phosphodiesterase) are upregulated on the surface of basophils [18]. CD63 was

discovered in the early 1990s [19], resulting in the development and utilization of flow cytometry for monitoring allergen-specific IgE-mediated activation of basophils. More recently, CD203c was identified but its mechanism of expression has not yet been fully elucidated [20]. CD203c is expressed constitutively and increases markedly upon specific activation of blood basophils in patients with atopic diseases such as asthma [21], food allergy [22,23], drug allergy [24] and allergy to *Hymenoptera* venoms [25].

In this study, we found that CF-ABPA patients, when compared to both CF-AC and CF patients, display characteristic increases in blood basophil CD203c levels at baseline and following *ex vivo* stimulation with an *Af* allergenic extract or with recombinant *Asp fl*, a major *Af* allergen [26]. CD123 levels tended to be low in blood basophils from CF-ABPA patients at baseline and after activation, while CD63 levels were similar in the three groups. Plasma Thymus and Activation-Regulated Chemokine (TARC, a.k.a. CCL17) and plasma supernatant levels of histamine after basophil activation were similar in the three groups of patients.

2. Methods

2.1. Patients

We studied 39 patients, including 12 CF patients, 14 CF-AC patients, 11 CF-ABPA patients and 2 CF-ABPA patients treated with omalizumab (Table 1). The Stanford Administrative Panel of Human Subjects in Medical Research approved this study. All 39 CF subjects (or parents, for minors) signed informed consents before they underwent study procedures. Diagnosis of CF was by documented sweat chloride (>60 mmol/L by quantitative iontophoresis test) and/or one or more clinical features consistent with CF and/or preexisting documentation of two known CFTR mutations. CFTR genotype was confirmed on all subjects using the Elucigene CF29 assay (Tepnel Molecular Diagnostics, Abingdon, Oxon, UK). CF-AC was defined as the presence of *Af* in ≥ 2 different

sputum cultures within the past two years. One CF-AC had *Aspergillus* bronchitis (Table 1, patient 24) [10]. The diagnosis of CF-ABPA was made using published CF Foundation Consensus Conference criteria modified to include markedly increased IgE over prior stable baseline [16,27]. Total blood IgE levels were measured using the Immucap (Phadia, Portage, MI, USA) assay.

2.2. Sample collection

Blood was collected by venipuncture in EDTA Vacutainer tubes (BD Biosciences, San Jose, CA) and immediately placed on melting ice. Within 30 minutes, blood was centrifuged at 400 G for 10 minutes at 4 °C to separate the erythrocyte/leukocyte pellet (kept on melting ice) from platelet-rich plasma. Platelet-rich plasma was further spun at 3000 G for 10 minutes at 4 °C to remove platelets and yield platelet-free plasma. 400 μ l of “baseline plasma” were kept at -80 °C, for TARC measurement (see below). The erythrocyte/leukocyte pellet was reconstituted to its original volume with platelet-free plasma, yielding platelet-free, reconstituted blood. We used platelet-free reconstituted blood to assess blood basophil function, so as to avoid platelet-triggered aggregation and clotting in the course of the 10- and 30-minute, 37 °C incubation with saline or extracts.

2.3. Incubation with saline or extracts

We assessed blood basophil characteristics under 5 conditions: (i) on ice, at baseline; (ii) upon incubation with phosphate-buffered saline (PBS) as a nonimmunogenic control; (iii-iv) upon incubation with *Af* extract or *rAsp fl*; and (v) upon incubation with a non-offending (peanut) allergen extract as an immunogenic control. Clinical grade *Af* and peanut skin test allergen extracts were from Greer Laboratories (Lenoir, NC, USA). *rAsp fl* was obtained from Indoor Biotechnologies Inc. (Charlottesville, VA, USA). For each of the three types of incubation [conditions (ii), (iii-iv) and (v) listed above], 100 μ l of platelet-free, reconstituted blood, was mixed with either 3 μ l of

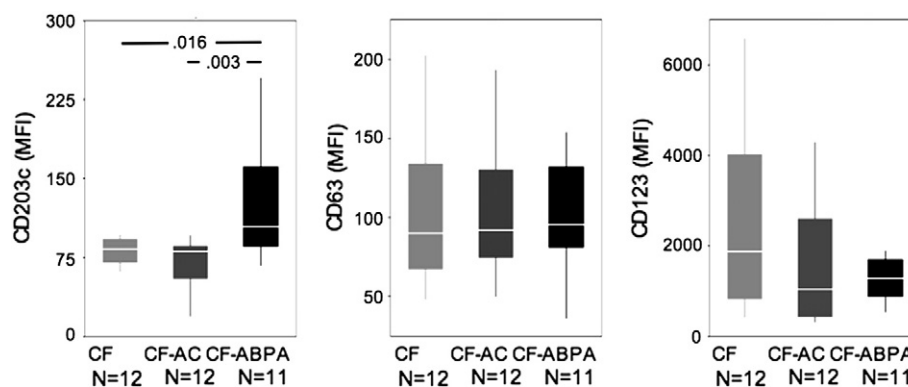


Fig. 1. Surface CD203c, CD63 and CD123 levels in blood basophils at baseline in CF-ABPA, CF-AC and CF patients. Shown are box plots (delimited by 25th and 75th percentiles, with median line and whiskers representing 10th and 90th percentiles) for CD203c, CD63 and CD123 levels in blood basophils from CF (N=12, light grey) patients, CF-AC (N=12, dark grey), CF-ABPA (N=11, black). Differences between groups were evaluated with the Wilcoxon rank-sum test. Non-significant differences are omitted.

PBS or relevant extracts for 10 and 30 minutes at 37 °C [28]. Incubations were stopped by adding ice-cold PBS with EDTA (2.5 mM final) and cells were pelleted by centrifugation (490 G, 5 minutes, 4 °C) for surface staining. Supernatants were kept at –80 °C for histamine measurement (see below).

2.4. TARC measurement by ELISA

TARC (CCL17) levels were measured in duplicate in plasma samples collected at baseline by sandwich ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions. TARC concentrations were calculated from standard curves with detection limits of 16–2,000 pg/mL.

2.5. Histamine measurement by mass spectrometry

Histamine levels were measured in supernatants from stimulated blood basophil samples using the commercially available EZ:faast amino acid analysis kit (Phenomenex, Torrance, CA, USA).

2.6. Flow cytometric analysis

Aliquots of blood from study patients were characterized using surface staining combinations for flow cytometric analysis [29] at baseline and 10 and 30 minutes after activation. Surface staining for CD203c, CD63 and CD123 was performed prior to fixation as described elsewhere (see [28]). Data was acquired

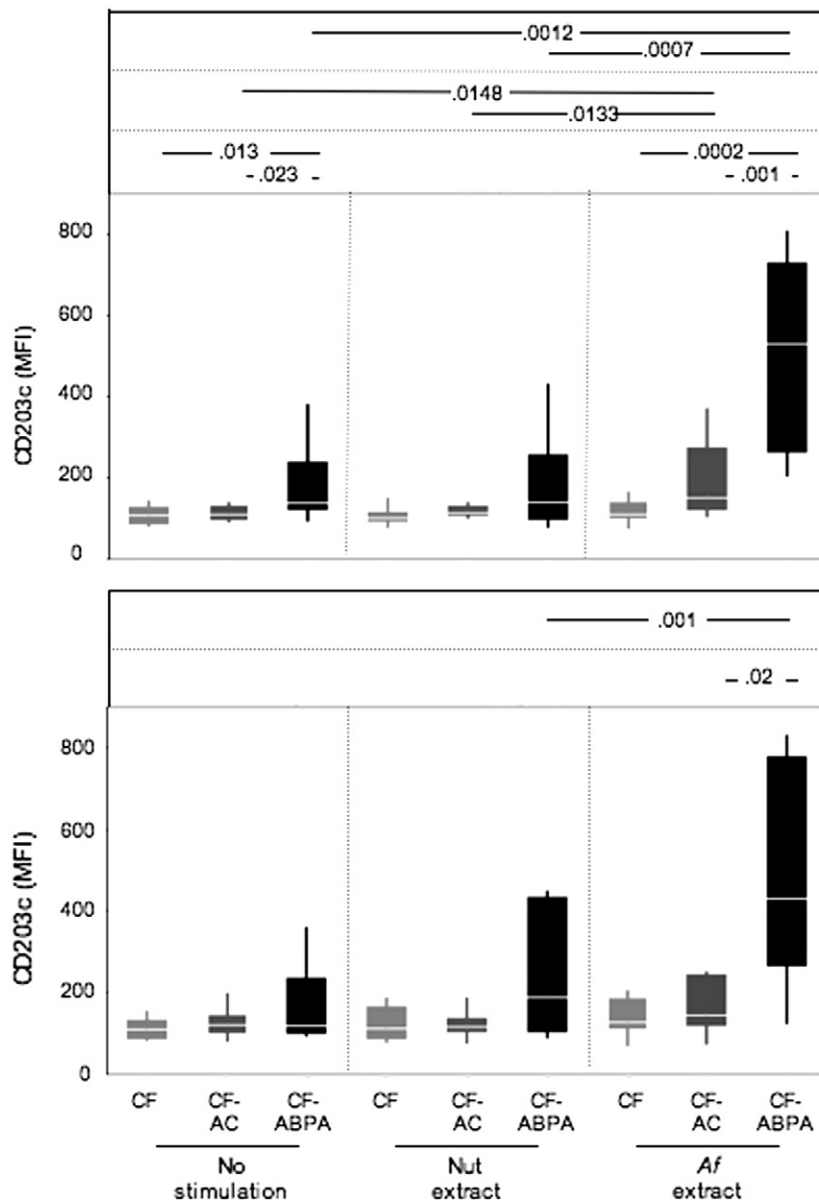


Fig. 2. Surface CD203c levels in blood basophils upon *ex vivo* stimulation with *Af* (offending) or peanut (non-offending) allergen extracts. Shown are box plots for CD203c levels in blood basophils from CF (N=12, light grey), CF-AC (N=14, dark grey) and, CF-ABPA (N=11, black) patients upon 10-minute (top) and 30 minute (bottom) allergen stimulation. Differences between groups were evaluated with the Wilcoxon rank-sum test. Differences between *Af* and peanut stimulations within each group were evaluated with the Wilcoxon signed-rank test. Non-significant differences are omitted.

using a LSRII digital flow cytometer (BD Biosciences) equipped with 4 lasers (535, 488, 633, 405 nm), 2 light scatter detectors and 18 fluorescent detectors and exported for compensation and analysis using FlowJo (Treestar, Ashland, OR, USA).

2.7. Standardization of flow cytometry data acquisition

To limit day-to-day variability, antibodies were purchased in large batches, titrated and used at saturating concentrations throughout the study. To provide fluorescence background quantification in measurement channels, one aliquot of each sample was *f*stained with the fixable viability dye but left unstained in other channels (fluorescence minus one -FMO-background control aliquots). To standardize signal output by the flow cytometer, a daily laser calibration and an additional fluorescence detector calibration procedure using a standard set of multicolor fluorescence beads was run. All MFI results were normalized to MFI from standard beads run at the beginning and end of each FACS session.

2.8. Statistics

We used JMP9 (SAS Institute, Cary, NC, USA) for all statistical analyses presented here. For between- and within-group comparisons, we used the nonparametric Wilcoxon rank-sum and signed-rank tests, respectively. Differences were considered significant with $P < .05$.

3. Results

3.1. Basophil CD203c levels are increased at baseline in patients with CF-ABPA

We used flow cytometry and a sequential gating method to discriminate live basophils from other populations of cells found in blood. We found that basophil CD203c levels at baseline (measured as median fluorescence intensity, MFI) were significantly increased in CF-ABPA compared to CF-AC and CF patients (Fig. 1; 12 CF patients, 13 CF-AC patients and 11 CF-ABPA patients, $P = .02$ between CF-ABPA and CF-AC patients, and $P = .003$ between CF-ABPA and CF patients, respectively). Baseline levels for CD63 and CD123 did not differ between the three groups. Note that baseline data from patients 19 and 23 (Table 1) were not deemed robust enough due to the very low number (< 300) of basophils in the corresponding baseline aliquots.

3.2. Ex vivo stimulation with *Af* extract markedly increases blood basophil CD203c levels in CF-ABPA compared to CF-AC and CF patients

Af extract stimulation for 10 minutes induced markedly higher increases of blood basophil CD203c levels in CF-ABPA patients than in either CF-AC (+251%, $P = .001$) or CF (+390%, $P = .0002$) patients (Fig. 2). By contrast, after stimulation with a non-offending peanut allergen extract, basophils from CF-ABPA, CF-AC, or CF patients did not exhibit significant differences in

surface levels of CD203c; this was determined by both within-group (peanut extract stimulation vs. no stimulation for each patient/subject) and between-group (peanut extract stimulation in CF-ABPA vs. CF-AC, or CF-ABPA vs. CF) comparisons. CD203c upregulation was seen in basophils from the CF-AC group following stimulation with the *Af* extract compared to stimulation with the peanut extract ($P = .0133$). After a more prolonged exposure with *Af* extract stimulation for 30 minutes, we also observed highly significant increases in surface levels of CD203c in blood basophils from CF-ABPA patients compared to CF-AC ($P = .02$) and CF patients ($P = .0001$), although the distinction between CF-ABPA and CF-AC was not as significant as that seen at 10 minutes (Fig. 2). Three CF (#1, 2 and 6, Table 1) and 3 CF-AC (#16, 18 and 22, Table 1) were sensitized to *Af* (positive *Af*-specific IgE); four did not react in the basophil assay while 2 (#6 and 18) did. The 10 minute activation CD203c assay yielded areas under the ROC curves of .91 for CF-ABPA vs CF-AC ($p = 0.0002$) and .97 for CF-ABPA vs CF ($p < 0.0001$).

3.3. Blood basophil CD63 and CD123 levels remain largely unchanged in the three groups of CF patients following an ex vivo stimulation with *Af* extract

As shown in Fig. 3 (top panels), there were no differences in CD63 levels of stimulated blood basophils in between-group comparisons. However, within-group comparisons showed that 10-minute stimulation with *Af* extract significantly increased blood basophil CD63 levels in CF-ABPA and CF-AC patients compared to the stimulation with the non-offending allergen control peanut extract ($P = .008$ and $P = .014$ within CF-ABPA and CF-AC groups, respectively). *Ex vivo* stimulation with *Af* extract tended to decrease the level of CD123 on the surface of blood basophils in patients with CF-ABPA and CF-AC compared to CF patients (between-group comparisons), but these differences were not statistically significant at either 10- or 30-minute timepoints (data not shown). There were no differences in CD123 levels on the surface of basophils after stimulation with *Af* as compared to peanut extract in within-group comparisons.

3.4. Plasma TARC (CCL17) levels at baseline and supernatant histamine levels upon stimulation do not distinguish CF-ABPA from CF-AC and CF patients

Unlike blood basophil CD203c levels, plasma TARC (CCL17) levels measured by ELISA were similar in the three patient groups (Fig. 3 panel A). We also determined the level of histamine release in the supernatant fraction of blood basophil samples following *Af* or peanut allergen stimulation. To this end, we developed a highly sensitive mass spectrometry method capable of detecting changes in histamine levels occurring upon 10- to 30-minute *ex vivo* allergen stimulation within experimental aliquots of 100 μ l of blood. *Af* allergen stimulation did not result in any significant difference in supernatant histamine release between the three groups (Fig. 3 panel B).

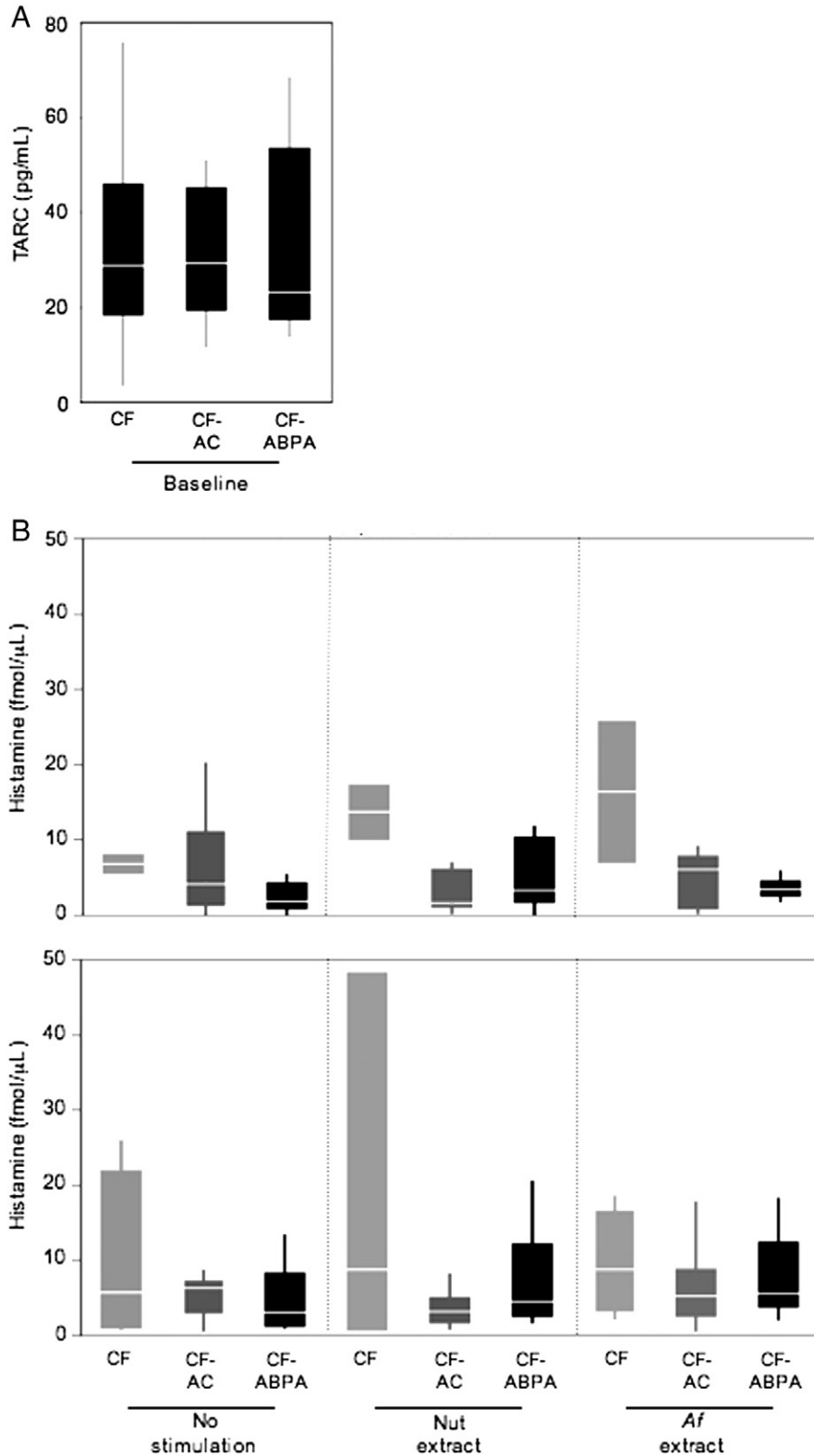


Fig. 3. Plasma TARC (CCL17) and supernatant histamine measurements in samples from patients with CF, CF-AC and CF-ABPA. (A) Shown are box plots for baseline TARC levels in plasma from CF (N=11, light grey), CF-AC (N=13, dark grey), and CF-ABPA (N=11, black) patients. There were no significant differences in within- and between-group analyses. (B) Shown are mean and standard deviation for histamine levels in blood aliquots from CF (N=8, light grey), CF-AC (N=8, dark grey), and CF-ABPA (N=9, black) patients upon 10-minute (top panels) and 30 minute (bottom panels) allergen stimulation. There were no significant differences in within- and between-group analyses.

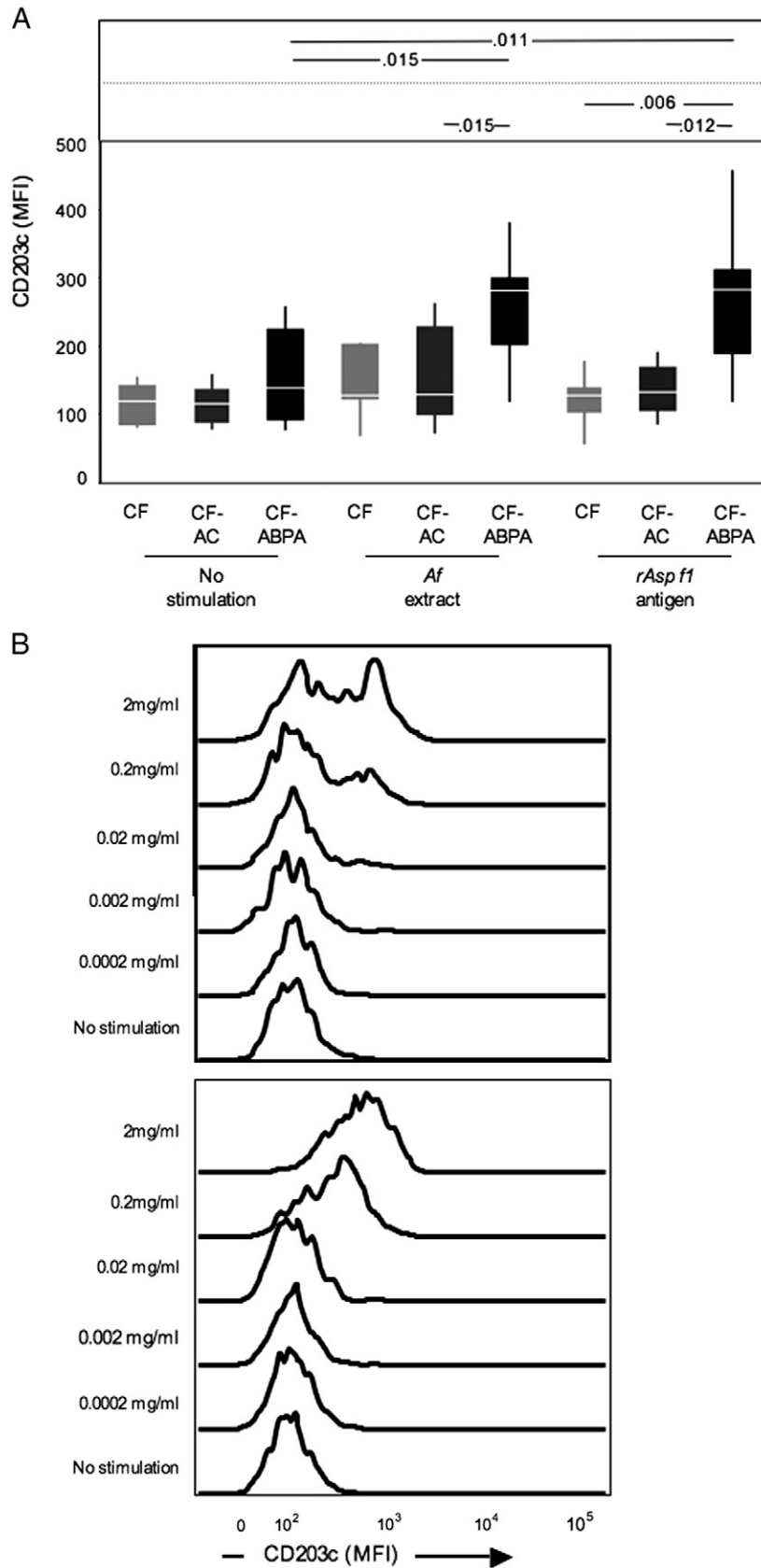


Fig. 4. Activation of blood basophils from patients with CF-ABPA following *ex vivo* stimulation with *rAsp f1*. (A) Shown are box plots for surface CD203c levels in blood basophil following *ex vivo* stimulation for 30 minutes with *Asp f1* in CF (N=9, light grey), CF-AC (N=10, dark grey) and, CF-ABPA patients (N=8, black). (B) Titration of the antigen *rAsp f1* at 10-minute (top panel) and 30-minute (bottom panel) of stimulation. Shown are histograms for CD203c surface expression in a representative subject with CF-ABPA.

3.5. *Ex vivo* stimulation with *rAsp f1* allergen also elicits a significant increase in blood basophil CD203c levels in CF-ABPA compared to CF-AC and CF patients

rAsp f1 is a major 18 kDa *Aspergillus fumigatus* allergen and a member of the mitogillin family of cytotoxins (26). Consistent with the results obtained with the *Af* extract (which contains a complex mixture of allergens), *rAsp f1* also elicited a marked upregulation of CD203c on basophils from CF-ABPA patients ($P=.02$ between CF-ABPA and CF-AC patients, Fig. 4). However, peak upregulation was seen at the 30 minute rather than the 10 minute time point (shown are results using 2 ng/mL *rAsp f1* stimulation, Fig. 4). A dose–response relationship was observed, whereby increasing amounts of *rAsp f1* elicited greater upregulation of blood basophil CD203c levels.

4. Discussion

In this study we found that CF-ABPA patients, as compared to both CF-AC and CF patients, display increases in blood basophil CD203c levels both at baseline and following 10- or 30-minute *ex vivo* stimulation with *Af* extract and/or recombinant *Asp f1*. CD123 levels tended to be low in blood basophils from CF-ABPA patients at baseline and upon activation. CD63 levels remained largely unchanged, except for an upregulation in CF-ABPA and CF-AC groups after 10-minute stimulation (within-group comparisons). Plasma TARC/CCL17 levels were not elevated in CF-ABPA. After allergen stimulation of basophils, histamine levels in the plasma supernatant remained very low and similar among the three categories of CF patients.

An increase in blood basophil CD203c levels at baseline has been reported in patients with other atopic diseases such as asthma [21], urticaria [30] and food allergy [29]. CD203c is a transmembrane ectoenzyme that is upregulated on the surface of basophils following allergen-specific activation [19,22,23]. The upregulation of this enzyme at the surface of basophils can be IgE-mediated [20]. In two CF-ABPA patients undergoing treatment with omalizumab the increase of CD203c following *ex vivo* stimulation was completely inhibited (data not shown). The upregulation of basophil CD203c thus appears to be partially, but perhaps not exclusively, IgE-mediated. Further experiments featuring more patients at baseline and after various timepoints and doses of omalizumab are needed to clarify this potential therapeutic effect.

The distinction of CF-ABPA from CF-AC remains highly challenging in the clinic, despite the consensus revision of the classic ABPA diagnostic criteria for CF patients published in 2003 [16]. The diagnosis of ABPA (and CF-ABPA) remains dependent upon a constellation of clinical, laboratory and radiological components, several of which (e.g., assay for *Af* precipitins or IgG antibodies) are not standardized [16]. Several groups have attempted to identify biomarkers that could more easily identify ABPA in CF patients, but this quest has remained inconclusive so far [15,31,32]. For example, TARC levels have been reported to be increased in blood from patients with CF-ABPA, particularly during exacerbation [15,32], but this finding has not been

reproduced [33]. We did not observe differences in plasma TARC between CF-ABPA, CF-AC and CF groups.

Other investigators have suggested that histamine may be increased in blood from ABPA patients [17]. In our assay, however, histamine levels in the plasma supernatant fraction of *ex vivo* allergen-stimulated blood basophils were not increased in the CF-ABPA group despite basophil surface marker changes. This discrepancy might be explained by the fact that microvolumes of blood are used in our assay (100 μ L of blood per condition), which may hamper the measurement of a significant change in histamine levels. Alternatively, histamine is known to not only be released by basophils but also by neutrophils, eosinophils and platelets [34], all of which were not activated in the conditions of our assay (data not shown).

Several investigators have employed immunoassays using purified or recombinant major *Af* antigens such as *Asp f1*, *Asp f2*, *Asp f4* and *Asp f6*. Whereas *Asp f4*, an endocellular protein of unknown function, and *Asp f6*, a manganese-dependent superoxide dismutase, are commonly associated with ABPA, *Asp f1* was shown to be less specific but highly sensitive for *Aspergillus*-allergic patients [31,35]. In our assay, blood basophil CD203c expression was greater following *ex vivo* stimulation with *rAsp f1* in patients with CF-ABPA than in CF-AC and CF patients, indicating that it may have utility as a standardized allergen in a potential clinical test. As two of 6 *Af*-sensitized patients without ABPA were positive in the CD203c assay long-term longitudinal data will be required to see if such patients develop ABPA over time. Analogously, one non-sensitized CF-AC patient (#25, Table 1) showed a positive response. Thus, some overlap of CD203c levels between a few CF-ABPA and CF-AC or CF patients was observed and so it is premature to assert that our assay may serve as a diagnostic test for CF-ABPA without longitudinal studies in larger and diversely sourced patient samples, and further analysis of positive predictive value, negative predictive value, sensitivity, specificity and threshold.

In conclusion, whereas CF is usually associated with pulmonary inflammation involving mostly neutrophils in airways and lymphocytes in airway walls [36], its ABPA complication involves a systemic allergic component that includes priming and hyper-responsiveness of blood basophils to *Af* allergens.

Competing interest statement

The authors declare to hold no conflict of interest with the publication of the results included in this manuscript.

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