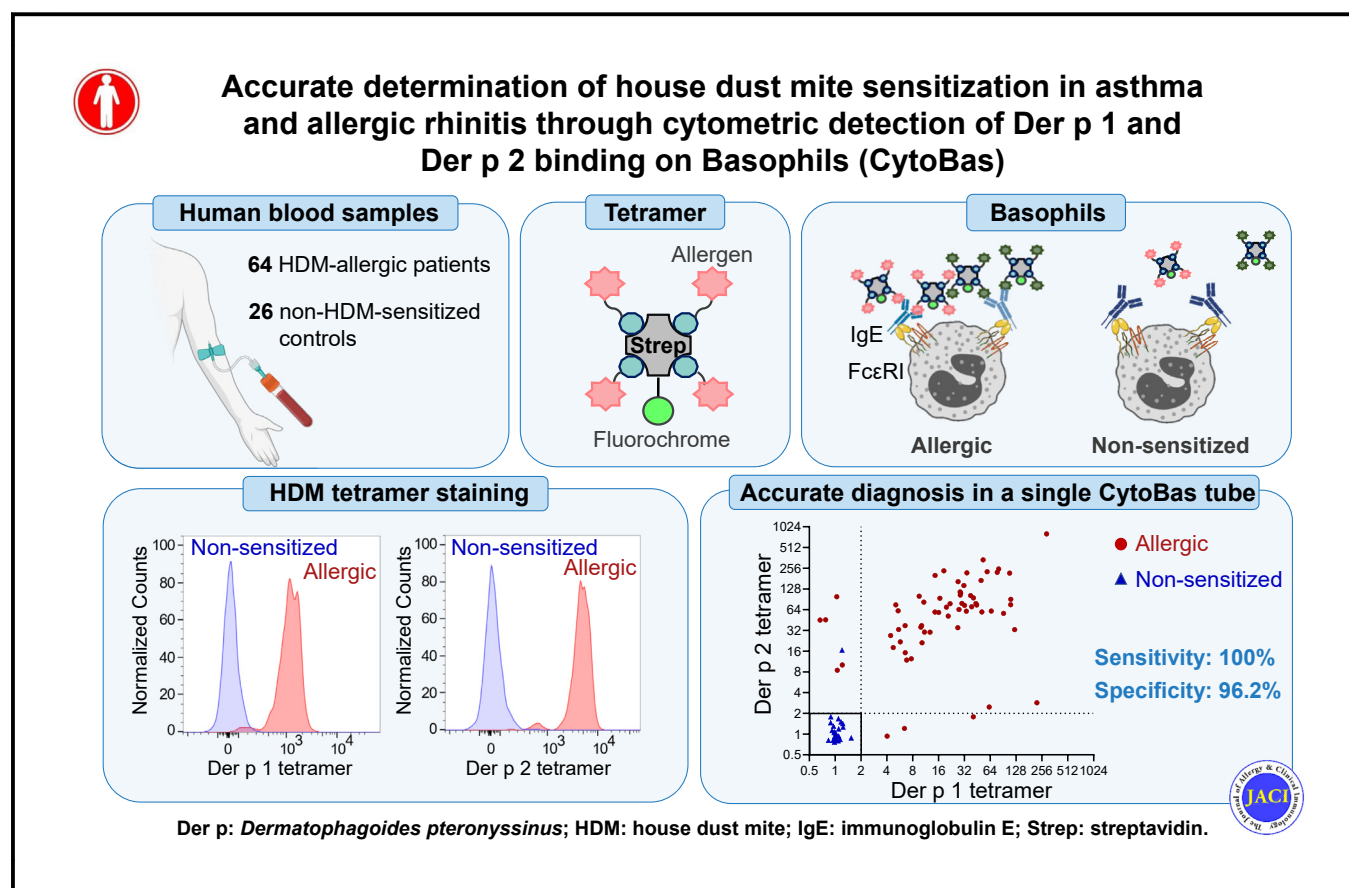


Accurate determination of house dust mite sensitization in asthma and allergic rhinitis through cytometric detection of Der p 1 and Der p 2 binding on basophils (CytoBas)

Lin Hsin, MSc, Nirupama Varese, MSc, Pei Mun Aui, BSc, Bruce D. Wines, PhD, Anouk von Borstel, PhD, Laurent Mascarell, PhD, et al

GRAPHICAL ABSTRACT



Capsule summary: A single flow cytometry stain of basophils (CytoBas) with both Der p 1 and Der p 2 provides >95% specificity and sensitivity for detection of functional HDM allergen sensitization.

Accurate determination of house dust mite sensitization in asthma and allergic rhinitis through cytometric detection of Der p 1 and Der p 2 binding on basophils (CytoBas)

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Background: House dust mite (HDM) is the most common allergen trigger globally for allergic rhinitis and atopic asthma. **Objectives:** To expedite accurate confirmation of allergen sensitization, we designed fluorescent allergen tetramers to directly stain specific IgE on basophils to detect specific allergen sensitization using the flow cytometric CytoBas assay.

Methods: Recombinant proteins of major HDM allergens (component), Der f 1, Der p 1, and Der p 2 were biotinylated and conjugated with fluorochrome streptavidins as tetramers. Blood samples from 64 patients who are HDM-allergic and 26 controls that are non-HDM-sensitized were incubated with allergen tetramers for evaluation of basophil binding (CytoBas) and activation (BAT) with flow cytometry.

Results: The tetramers effectively bound and activated basophils from patients who are allergic but not from controls who are nonsensitized. CytoBas with Der p 1 as a single allergen had comparable sensitivity and specificity (92% and 100%) to BAT (91% and 100%) in detecting allergen sensitization, as did CytoBas with Der p 2 (95% and 96%) to BAT (95% and 87%). A positive staining for Der p 1 and/or Der p 2 in CytoBas was 100% sensitive and 96% specific for HDM allergy.

Conclusions: CytoBas has diagnostic accuracy for group 1 and group 2 HDM allergens that is comparable to BAT, but with additional advantages of multiple allergen components in a single tube and no requirement for *in vitro* basophil activation. These findings endorse a single, multiplex CytoBas assay for accurate and component-resolved diagnosis of aeroallergen sensitization in patients with allergic asthma and/or rhinitis. (J Allergy Clin Immunol 2024;■■■■:■■■■-■■■■.)

Key words: Allergic rhinitis, asthma, Der p 1, Der p 2, house dust mite

Abbreviations used

AIT:	Allergen immunotherapy
APC:	Allophycocyanin
AR:	Allergic rhinitis
AUC:	Area under the curve
BAT:	Basophil activation test
BirA:	<i>Escherichia coli</i> biotin lygase
CRD:	Component-resolved diagnostics
CytoBas:	Cytometric staining of blood basophils
fMLP:	N-formylmethionine-leucyl-phenylalanine
HDM:	House dust mite
MFI:	Median fluorescence intensity
pDCs:	Plasmacytoid dendritic cells
ROC:	Receiver operating characteristic

Atopic asthma and allergic rhinitis (AR) affect about 300 million people worldwide. Impacting about 50% of these individuals, house dust mite (HDM) is the most common allergic trigger globally.^{1,2} Disease control involves minimizing allergen exposure, which is challenging, given the ubiquity of HDM.³ Pharmacotherapy is effective but requires regular administration.⁴ Allergen immunotherapy (AIT) is the only disease-modifying treatment available for HDM allergy, with the potential for a sustained response beyond the treatment period.⁵ There is thus a compelling need for early and accurate confirmation of suspected HDM allergen sensitization⁶ to facilitate optimal treatment.⁷

The 2 globally prevalent HDM species are *Dermatophagoides pteronyssinus* especially in Europe and Australia^{8,9} and *D farinae* in the United States and Asia.^{9,10} Fecal pellets are the main source of allergens,¹¹ with group 1 and group 2 allergens being the most immunodominant proteins; 95% of patients who are HDM-allergic have specific IgE against either or both.¹² The group 1 allergens, Der p 1 and Der f 1, are cysteine proteases, which have >90% protein sequence homology, resulting in high IgE cross-reactivity among patients who are allergic.¹³ Der f 2 and Der p 2 are lipid-binding proteins with 87% sequence homology.^{14,15}

Conventional diagnostics for determining atopic sensitization in allergic asthma and rhinitis include skin prick testing and allergen-specific serum IgE detection.¹⁶ However, their drawbacks include limited sensitivity due to the use of allergen extracts.¹⁷ The basophil activation test (BAT) is a functional assay used in research settings, through which the specificity of effector cell-bound IgE is evaluated.¹⁸ It involves the detection of surface

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CD63 expression,¹⁹ on *in vitro* allergen-induced cross-linking of the high-affinity FcεRI on specific IgE-loaded blood basophils. Frequencies of CD63-positive basophils correlate better ($r = 0.5$) with clinical severity than skin prick testing ($r = 0.24$) or serum-specific IgE ($r = 0.33$).^{20,21} However, BAT is labor intensive, requiring fresh blood, careful allergen titrations, and time-frame restrictions, which hinders its implementation into routine clinical practice.²²

Component-resolved diagnostics (CRD) are gaining utility in both research and clinical settings and can be highly informative for HDM allergy management.²³ Detection of specific IgE to Der p 1 and Der p 2 can identify patients who are allergic with a better positive predictive value for HDM allergy than HDM extract.^{12,17} CRD can distinguish clinically relevant allergen sensitization to HDM components from cross-reactivity with shrimp allergens,^{24,25} allowing optimal treatment prescription for patients who are HDM-allergic.²⁶

We recently developed a CRD that accurately detected functional sensitization to bee venom (Api m 1; *Apis mellifera*) and ryegrass pollen (Lol p 1 and Lol p 5), using recombinant allergen components for multiplex cytometric staining of blood basophils (CytoBas) without the need for their activation as required by BAT.^{27,28} In this study, we explored the diagnostic performance of CytoBas using Der f 1, Der p 1, and Der p 2 to detect sensitization to HDM, the most prevalent trigger for perennial atopic asthma and AR globally.

METHODS

Study participants and sample collection

Participants who are HDM-allergic ($n = 64$) were recruited from the Allergy Clinic at the Alfred Hospital in Melbourne, Victoria, Australia, on the basis of a diagnosis of perennial AR with or without asthma and serum HDM-specific IgE of ≥ 0.35 kU_A/L (ImmunoCAP, Phadia, Uppsala, Sweden). Controls who are nonsensitized ($n = 26$) were recruited based on the absence of HDM sensitization, as evidenced by the absence of clinical symptoms and a negative skin prick testing (<1 mm) or serum HDM-specific IgE levels of <0.35 kU_A/L (ImmunoCAP). Exclusion criteria for all subjects were immunodeficiency, gastrointestinal complications, AIT within the last 5 years, or prescribed beta-blockers or oral corticosteroids. The use of symptom relievers for AR, such as antihistamines and topical corticosteroids, was allowed. Lithium-heparinized blood samples were collected from all subjects and processed within 24 hours for flow cytometry (see below), serum and PBMC isolation, and cryopreservation. The study was conducted in compliance with the Declaration of Helsinki, and written informed consent was obtained from each participant prior to inclusion (Alfred Ethics, Project 297-20).

Recombinant protein production

Protein sequences of Der f 1.0101, Der p 1.0102, and Der p 2.0101 were obtained from the Allergen Nomenclature website (allergen.org).^{29,30} Full length Der f 1.0101 and Der p 1.0102 constructs were generated with the native leader sequence for secretion, an *Escherichia coli* biotin lygase (BirA) tag for biotinylation, and a 6-His tag for cobalt column binding during protein purification (Fig 1). Both constructs contained the C35S mutation to disrupt their cysteine protease activity.^{31,32} The Der p 1 construct contained an S(-63)N mutation in the prodomain

to disrupt the N-glycosylation motif (NKS from codons -65 to -63). This motif is absent from Der f 1, and its presence in Der p 1 was previously shown to impair efficient protein production.³³ The Der p 2.0101 construct was generated with an N-terminal leader sequence from Api m 1,²⁷ a 6-His tag and a BirA sequence (Fig 1). All 3 constructs were codon-optimized for *Spodopora frugiperda* and cloned into the pFastBac vector (Thermo Fisher Scientific, Waltham, Mass), before incorporating into a Bacmid for baculovirus production and purification, as described previously.²⁷ Purified proteins were run under denaturing and non-denaturing conditions on a 4% to 15% mini-PROTEAN TGX Stain-Free Protein Gel (Bio-Rad Laboratories, Hercules, Calif), and following blotting, evaluated with a 6x-His Tag mouse mAb (clone: HIS.H8; Thermo Fisher Scientific).²⁷

The purified recombinant proteins were subjected to targeted-biotinylation by overnight incubation at room temperature with a BirA enzyme (2.5 μg/mL) in 10 mmol/L TRIS containing 62.5 mmol/L Bicine-HCl, pH 8.3; 62.5 μmol/L D-Biotin; 12.5 mmol/L ATP; and 12.5 mmol/L MgOAc, followed by dialysis against 10 mmol/L TRIS (pH 8). The biotinylated proteins were tetramerized by adding fluorochrome-conjugated streptavidin (Thermo Fisher Scientific) at an allergen-streptavidin molar ratio of 4:1.^{27,34}

BAT and basophil staining for CytoBas

BAT and CytoBas were performed on fresh whole blood samples collected from all study subjects within 24 hours of collection, as described previously.²⁷ The circulating basophils were first primed with IL-3 by incubating for 10 minutes at 37°C with stimulation buffer (HEPES 20 mmol/L, NaCl 133 mmol/L, KCl 5 mmol/L, CaCl₂ 7 mmol/L, MgCl₂ 3.5 mmol/L, BSA 1 mg/mL, recombinant IL-3 2 ng/mL, and heparin 20 μL/mL; pH 7.4). Cells were then incubated with HDM extract (0.001, 0.01, 0.1, 1 μg/mL; Stallergenes Greer, London, UK), fluorescent allergen tetramers (0.01, 0.1, 1 μg/mL), or fluorochrome-conjugated streptavidin only for 20 minutes at 37°C. Stimulation with rabbit anti-human IgE (Dako, Carpinteria, Calif) and fMLP (N-formylmethionine-leucyl-phenylalanine; Sigma Aldrich, St Louis, Mo), respectively, were used as positive controls for FcεRI-dependent and FcεRI-independent basophil degranulation. Activation was halted by incubating samples on ice for 5 minutes. The cells were then washed with cold wash buffer (HEPES 20 mmol/L, NaCl 133 mmol/L, KCl 5 mmol/L, and EDTA 0.27 mmol/L; pH 7.3) before staining surface markers for flow cytometry.

Flow cytometry

Following basophil activation or direct staining, the basophils were incubated with IgE-fluorescein isothiocyanate (polyclonal; Thermo Fisher Scientific) and/or HLA-DR-allophycocyanin (APC) (L243; BioLegend, San Diego, Calif), as well as CD123-BV605 (6H6; BioLegend), Fixable live/dead dye 700 (BD Biosciences, San Jose, Calif), and CD63-PE (H5C6, BD Biosciences), on ice for 20 minutes. Subsequently, red-blood-cell lysis buffer (NH₄Cl 155 mmol/L, KHCO₃ 9.9 mmol/L, and EDTA 1 mmol/L) was added for 10 minutes at room temperature, and cells were washed (HEPES 20 mmol/L, NaCl 133 mmol/L, KCl 5 mmol/L, and EDTA 0.27 mmol/L, pH 7.3), and fixed with 1% paraformaldehyde for 20 minutes at room temperature following

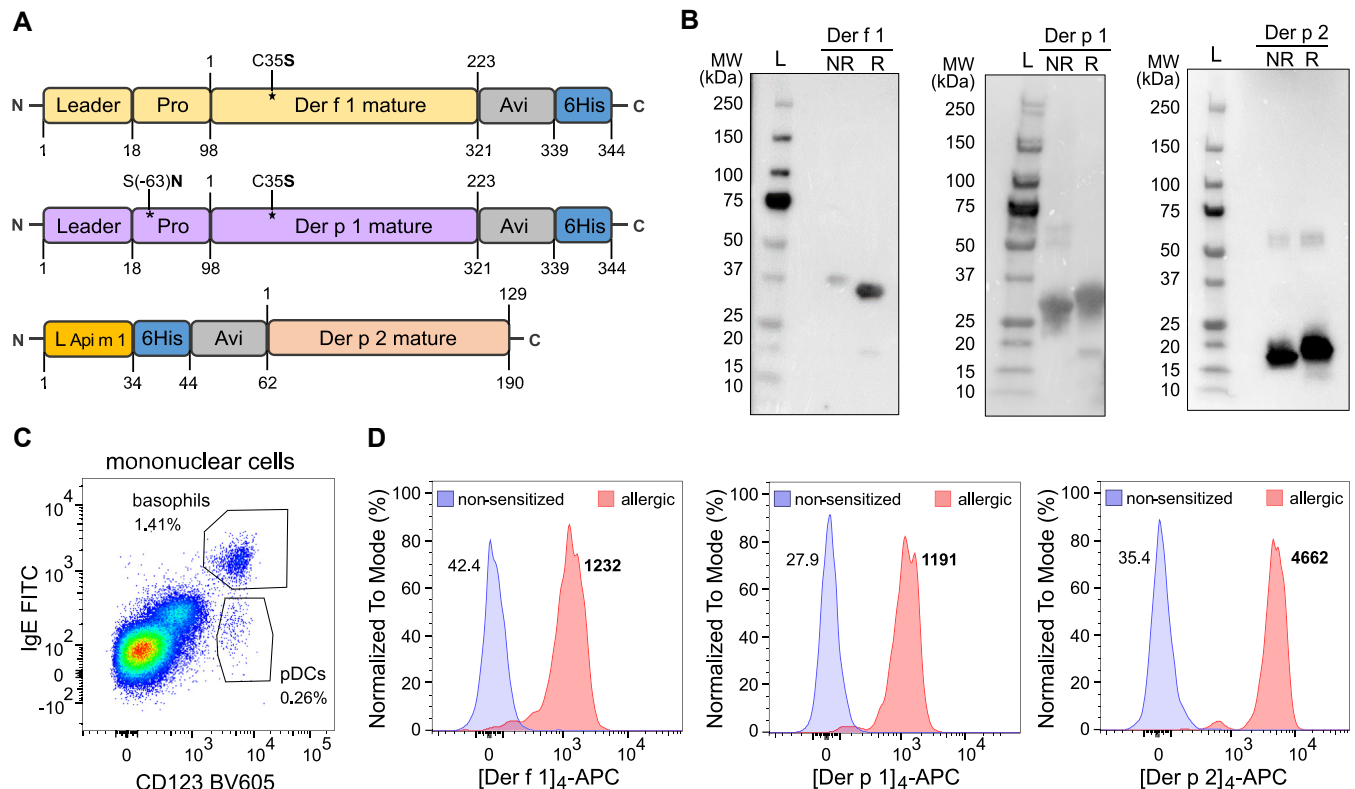


FIG 1. Detection of allergen sensitization by quantifying binding of fluorescent allergen components of HDM to basophils. **(A)** Schematic diagram of DNA constructs for Der p 1 and Der p 2 protein generation. **(B)** Anti-His Western blots of recombinant allergens in nonreduced (NR) and reduced (R) forms. Protein ladders (L) are shown to ensure proteins possess the molecular weight (MW) of the sequence-predicted sizes, Der f 1 (37 kDa); Der p 1 (37 kDa); and Der p 2 (21 kDa). **(C)** Within gating of circulating basophils from mononuclear cells, **(D)** representative flow cytometry plot of tetramer staining by [Der f 1]₄-APC, [Der p 1]₄-APC, and [Der p 2]₄-APC, respectively, between individuals who are nonsensitized and those who are allergic. FITC, Fluorescein isothiocyanate.

resuspension in 100 μ L wash buffer, they were run on the 4-laser BD LSRII (BD Biosciences). Instrument setup and calibration were performed using EuroFlow standard operation procedures.³⁵

Data analysis and statistics

Flow cytometry data were analyzed with FACS DIVA V6.3.1 software (BD Biosciences) and FlowJo v10.8.0 software packages (FlowJo LLC, Ashland, Ore). Statistical analyses were performed using GraphPad Prism (v9.0.1; GraphPad Software, Dotmatics, Boston, Mass). The nonparametric Wilcoxon signed-rank test was used for paired data, and the nonparametric Mann-Whitney *U*-test was used for nonpaired data. Receiver operating characteristic (ROC) curves were used to determine the performance capabilities (specificity and sensitivity) of allergen components in the BAT and CytoBas assays between patients who are HDM allergic and controls who are nonsensitized. *P* values of <0.05 were considered statistically significant.

RESULTS

Design and production of recombinant allergen tetramers for CytoBas

Production of full-length recombinant Der f 1 with the C35S mutation and C-terminal 6-His and BirA tags was successful (Fig

1, A). Furthermore, introduction of the S(-63)N mutation in Der p 1, to disrupt the N-glycan attachment at N-65, and model the lack of a glycosylation motif in Der f 1, resulted in successful production of this recombinant protein. The full-length Der p 2 protein was generated with the N-terminal Apm 1 leader sequence,²⁷ 6-His, and BirA tags (Fig 1, A). Purified recombinant allergens were detected at the expected molecular weights by Western blotting using an anti-6-His antibody (Thermo Fisher Scientific) (Fig 1, B). Following targeted biotinylation, these proteins were tetramerized with streptavidin-APC for BAT and CytoBas assays.

To confirm the ability of each recombinant allergen protein tetramer to bind to IgE on basophils by flow cytometry, we first gated on IgE⁺CD123⁺ basophils (Fig 1, C and Fig E1 in this article's Online Repository at www.jacionline.org). Fluorochrome-conjugated [Der f 1]₄, [Der p 1]₄, and [Der p 2]₄ positively stained basophils derived from patients who are HDM-allergic but not those from controls who are nonsensitized (Fig 1, D) or patients who are ryegrass pollen-allergic (Fig E2 in this article's Online Repository at www.jacionline.org). Binding was specifically inhibited in the presence of unconjugated proteins (Fig E3 in this article's Online Repository at www.jacionline.org) and not mediated by IgG-Fc γ R binding (Fig E4 in this article's Online Repository at www.jacionline.org). Basophils from patients who are HDM-allergic did not show staining for ryegrass pollen or dog allergens (Fig E5 in this article's Online Repository at www.jacionline.org).

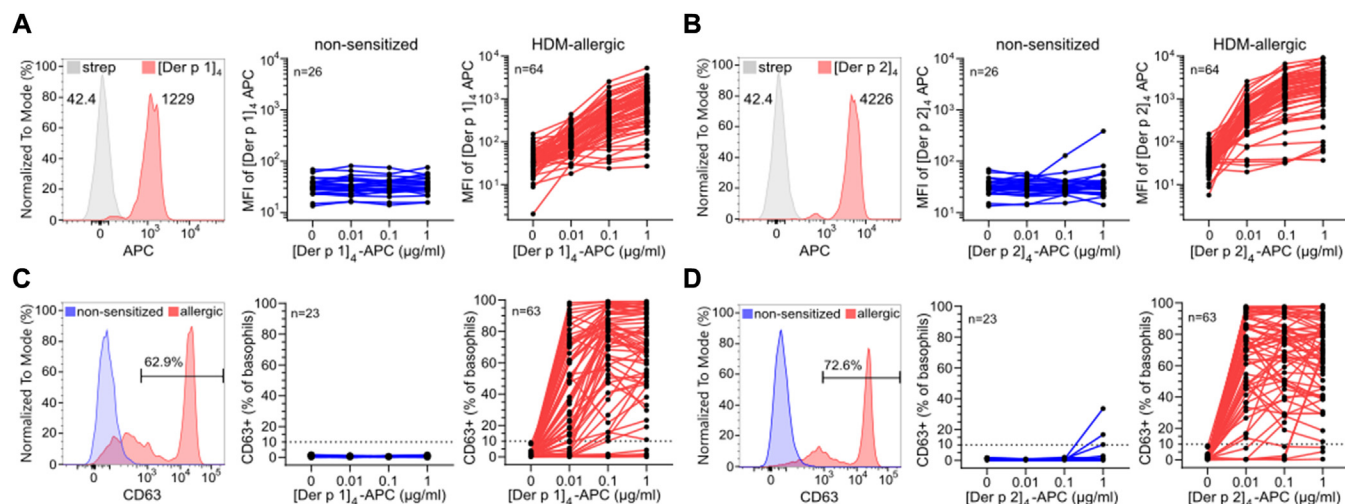


FIG 2. Recombinant Der p 1 and Der p 2 bind to basophils from patients who are HDM-allergic in a concentration-dependent manner and induce basophil activation. (A,B) Representative staining of basophils from a blood sample from a patient who is HDM-allergic, incubated with [Der p 1]₄-APC or streptavidin-APC. MFI of basophils from controls who are nonsensitized and subjects who are HDM-allergic after incubation with 1 µg/mL streptavidin-APC, presented as the baseline control "0 µg/mL" or 0.01, 0.1, and 1 µg/mL [Der p 1]₄-APC/[Der p 2]₄-APC. (C,D) Representative histograms of CD63 expression on basophils and frequencies of basophils from subjects who are nonsensitized and those who are HDM-allergic following stimulation with streptavidin-APC or ascending concentrations of [Der p 1]₄-APC/[Der p 2]₄-APC.

[jacionline.org](http://www.jacionline.org)). Thus, the recombinant HDM proteins demonstrate specific molecular binding to IgE on the surface of blood basophils.

Recombinant Der f 1, Der p 1, and Der p 2 tetramer staining can detect functional HDM-allergen sensitization

The ability of [Der f 1]₄, [Der p 1]₄, and [Der p 2]₄ to detect functional allergen sensitization was determined by measuring the allergen binding (Fig 2, A and B) and activation (CD63⁺) (Fig 2, C and D) using blood samples from 62 patients who are HDM-allergic and 26 controls who are nonsensitized (Table I). Detection of blood basophils postactivation was performed with the gating strategy shown (Fig E1), and plasmacytoid dendritic cells (pDCs) were gated as a known negative control.²⁷ Within the gated basophils, the median fluorescence intensity (MFI) of the APC-allergen tetramers was examined as the allergen-specific IgE binding intensity, and CD63 expression was used to investigate activation and degranulation. As controls for basophil activation, separate samples from each donor were incubated with serial dilutions of HDM extract, anti-IgE, or fMLP, a bacterial tripeptide that activates basophils through a G-protein coupled receptor, independent from FcεRI.²²

Following HDM extract incubation, basophils from patients who are HDM-allergic only showed an increased expression of CD63⁺ (Fig E6, A in this article's Online Repository at www.jacionline.org). Basophils from both patients who are HDM-allergic (n = 62 of 64) and individuals who are non-HDM-sensitized (n = 25 of 26) expressed CD63 after stimulation with the IgE-independent stimulus fMLP (Fig E6, B). Anti-IgE stimulation induced CD63 expression on the basophils of most patients who are HDM-allergic (n = 60 of 64) and control subjects (n =

23 of 26) (Fig E6, C). Donors whose basophils did not respond to both anti-IgE and HDM (extract or components) were classified as nonresponders and were excluded from further BAT analysis (n = 1 patient with allergy; n = 3 controls).

While there was no detectable tetramer staining in nearly all the controls, a concentration-dependent increase in MFI was evident in the HDM-allergic group following incubation with any of the following: [Der f 1]₄ (Fig E7 in this article's Online Repository at www.jacionline.org), [Der p 1]₄ (Fig 2, A), or [Der p 2]₄ (Fig 2, B). The increased staining intensity was accompanied by an increase in CD63⁺ basophil frequencies in patients who are allergic but not controls (Fig 2, C and D).²² Basophils of patients and controls expressed similar levels of IgE (Fig E8 in this article's Online Repository at www.jacionline.org). The activated (CD63⁺) and nonactivated (CD63⁻) fractions of basophils from the patients who are HDM component-specific allergic showed high binding intensities (Fig E9 in this article's Online Repository at www.jacionline.org). Thus, despite not all basophils from a single individual undergoing *in vitro* degranulation on allergen binding, these all have the same capacity to bind allergens with specific IgE. These findings confirmed that our fluorescent allergen tetramers can be applied to specifically detect HDM sensitization in patients who are allergic.

High diagnostic capacity to detect HDM allergy sensitization with basophil staining

Tetramer staining intensities were determined by calculating the ratio between MFI of allergen tetramer (1 µg/mL) and streptavidin-APC staining. The [Der p 1]₄ staining ratio was >2 for 59 of 64 patients who are allergic, compared to <2 for all 26 control subjects (Fig 3, A). Basophil activation with 1 µg/mL [Der p 1]₄ induced >10% CD63⁺ basophils in 57 of 63 patients,

TABLE I. Demographics and characteristics of study participants

	Patients who are HDM-allergic (n = 64)	Controls who are non-HDM-sensitized (n = 26)
Females	44 (68)	12 (46)
Age (y), median (range)	29 (19-57)	28 (22-54)
Allergic rhinitis	61 (97)	0 (0)
Asthma	39 (62)	0 (0)
HDM monosensitization*	10 (16)	0 (0)
Other sensitizations:	54 (84)	5 (19)
Grass pollen*	48 (75)	4 (15)
Cat*	34 (53)	2 (8)
Dog*	26 (41)	2 (8)

Values are n (%) unless otherwise indicated.

*Cutoff for sensitization: serum-specific IgE > 0.35 kUA/L.

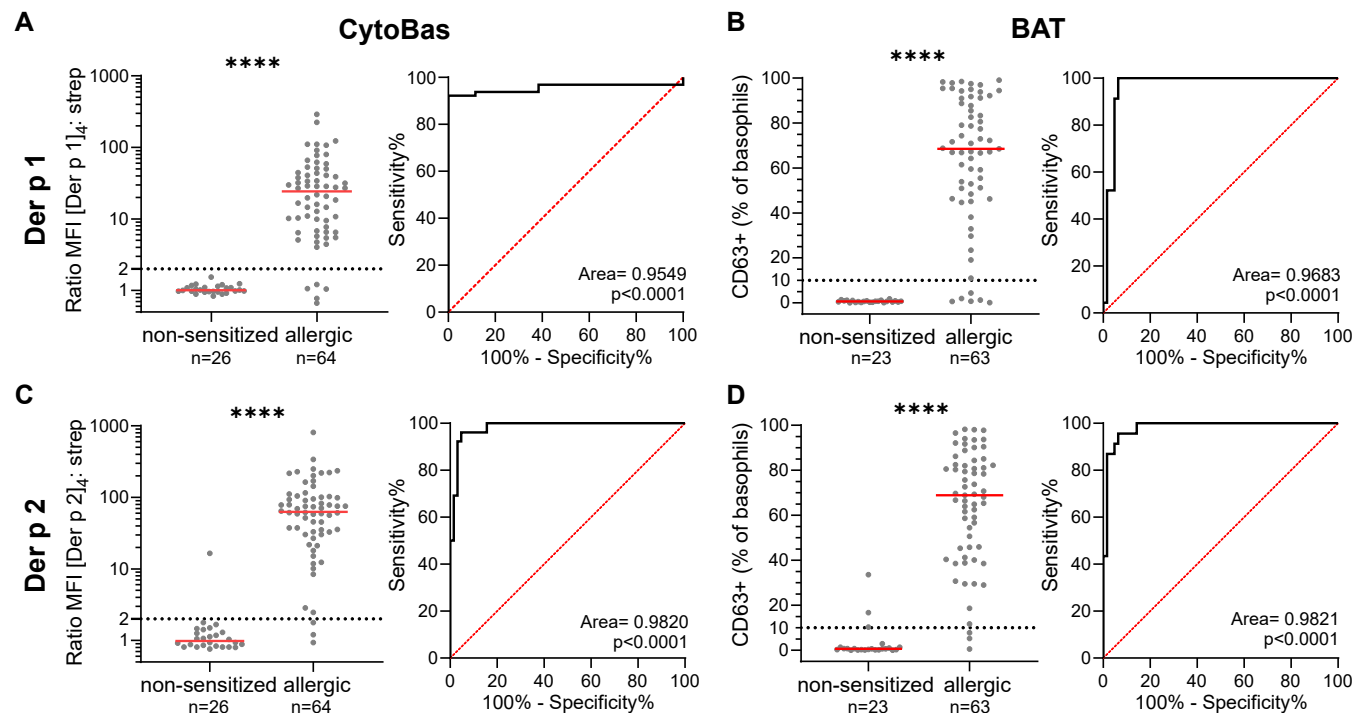


FIG 3. The sensitivity and specificity of CytoBas are comparable to BAT for HDM allergy detection. Staining intensities of HDM tetramers in CytoBas via the ratio of (A) [Der p 1]₄-APC at 1 µg/mL/streptavidin-APC, (C) [Der p 2]₄-APC at 1 µg/mL/streptavidin-APC and respective ROC on staining intensity. Frequency of CD63⁺ basophils in BAT following *in vitro* stimulation with 1 µg/mL of (B) [Der p 1]₄-APC, (D) [Der p 2]₄-APC and ROC of CD63 positivity. Statistics: Mann-Whitney *U*-test; for ROC, Wilson/Brown method for confidence level (>95%) determination. *****P* < .0001.

whereas this population was <10% in all evaluable controls (n = 23) (Fig 3, B). In a direct comparison, the [Der p 1]₄ CytoBas had comparable sensitivity (92%) to BAT (91%) for detecting HDM sensitization, while both tests demonstrated 100% specificity (Table II). In addition, ROC curve analyses showed very high area under the curve (AUC) for both [Der p 1]₄ CytoBas (0.9549) and [Der p 1]₄ BAT (0.9683).

The [Der p 2]₄ staining ratio was >2 in 61 of 64 patients who are allergic and <2 in 25 of 26 control subjects (Fig 3, C). Basophil activation with 1 µg/mL [Der p 2]₄ induced >10% CD63⁺ basophils in 60 of 64 patients, and this population was <10% in 20 of 23 controls (Fig 3, D). The sensitivity and specificity (95% and 87%) of [Der p 2]₄ CytoBas were similar to BATs (95% and 96%) (Table II). The AUC from the ROC curve analyses

demonstrated enhanced performance compared to [Der p 1]₄: 0.9821 for [Der p 2]₄ CytoBas and 0.9820 for [Der p 2]₄ BAT. The [Der p 1]₄ and [Der p 2]₄ staining ratios did not show a high correlation with basophil activation (CD63⁺), clinical presentation (visual analog score), or serum-specific IgE levels (Fig E10 in this article's Online Repository at www.jacionline.org).

Because this is not the major sensitizer in Australia, a limited set of samples were run with Der f 1 (Fig E7).¹⁰ Nevertheless, the [Der f 1]₄ staining ratio was >2 for 32 of 36 patients who are allergic, and <2 for all 17 control subjects (Fig E7, A-E). Only 27 of 35 patients showed >10% CD63⁺ basophils following stimulation with 1 µg/mL [Der f 1]₄, and all the 15 evaluable controls had <10% stimulation (Fig E7, F-J). CytoBas with [Der f 1]₄

TABLE II. Diagnostic performance of BAT and CytoBas assays with HDM components

Assay	HDM component	True positive (n)	False positive (n)	False negative (n)	True negative (n)	Sensitivity (%)	Specificity (%)	Accuracy (%)
BAT	Der f 1 (35 patients, 15 controls)	27	0	8	15	75.0	100	82.4
	Der p 1 (63 patients, 23 controls)	57	0	6	23	90.5	100	93.0
	Der p 2 (63 patients, 23 controls)	60	3	3	20	95.2	87.0	93.0
	Der p 1 and/or Der p 2 (63 patients, 23 controls)	62	3	1	20	96.9	86.9	94.2
CytoBas	Der f 1 (36 patients, 17 controls)	32	0	4	17	88.9	100	92.5
	Der p 1 (64 patients, 26 controls)	59	0	5	26	92.2	100	94.4
	Der p 2 (64 patients, 26 controls)	61	1	3	25	95.3	96.2	95.6
	Der p 1 and/or Der p 2 (64 patients, 26 controls)	64	1	0	25	100	96.2	98.9

yielded a very similar sensitivity and specificity as [Der p 1]₄ CytoBas, and large AUC in the ROC analysis (0.9379) (Fig E3, E), underlining their cross-reactivity due to the high sequence homology between the 2 proteins.³⁶

Multiplex component-resolved diagnosis with the inclusion of both Der p 1 and Der p 2

Our CytoBas approach enables the inclusion of multiple allergen components in a single assay.²⁷ To evaluate the potential of combined evaluation with 2 allergen components, we depicted the allergen: streptavidin staining ratios of [Der p 1]₄ versus [Der p 2]₄ (Fig 4, A). Detection of positive sensitization to HDM was defined as one or both MFI ratios above 2. Thus, only data points within the lower left quadrant were defined as negative. All 64 patients who are HDM-allergic were positive for one or both allergens, and 25 of 26 control subjects were below the cutoff ratio of 2 for both allergens. Despite basophil activation requiring separate tubes and multiple dilution procedures to evaluate each component, a direct comparison was made with BAT (Fig 4, B). This showed that 1 of 63 patients who are allergic was negative for both components, and 3 of 23 control individuals were positive for Der p 2 (Table II). Thus, by using 2 allergen components, the CytoBas approach achieved 100% sensitivity and 96% specificity. The BAT was equally sensitive (100%), but less specific (87%). This analysis demonstrated the capacity for CytoBas to enable CRD in a single tube for differential detection of monosensitization to either Der p 1 (3 of 64) or Der p 2 (5 of 64), or both (56 of 64) in patients who are HDM-allergic.

DISCUSSION

Here we present highly sensitive and specific detection of HDM sensitization, using 2 immunodominant HDM components as recombinant tetramers in a single CytoBas tube. The performance of CytoBas for single group 1 (Der f 1 or Der p 1) or group 2 (Der p 2) allergens was comparable to BAT. However, CytoBas performance was further enhanced by combined detection of both Der p 1 and Der p 2, which is achievable in a single CytoBas assay, with 100% sensitivity and 96% specificity.

Several key technical differences favor the use of CytoBas over BAT. First, CytoBas does not require *in vitro* stimulation of basophils. As a result, the test is rapid, less susceptible to batch or operator differences, and can be performed on thawed, live PBMCs. Second, with BAT, basophils of 5% to 10% of people fail to

respond to *in vitro* cross-linking of IgE (nonresponders), making the result uninterpretable.³⁷ CytoBas has no such limitations. Third, CytoBas is a single-tube assay and does not rely on repeat measurements for additional components or titration of the stimulus, saving time and reagents.

Here we employed the baculovirus expression system in Sf21 (*S. frugiperda*) insect cells to produce recombinant Der f 1, Der p 1, and Der p 2 proteins that were highly reactive in our BAT and CytoBas assays. Sf21 cells enable paucimannosidic glycosylation of produced proteins,³⁸ which cannot be obtained in bacterial cell systems, and could be the reason for the previously observed reduced (25-fold) immunogenicity.³⁹ While *Pichia pastoris* yeast cells are capable forming highly glycosylated Der p 1 with allergenic activities, this was also limited in inducing an immune response.⁴⁰ Similar to previously published plant and insect proteins by us and others,^{27,41} we here showed that the *S. frugiperda* cells were suited for the production of recombinant HDM recombinant protein that could be recognized by IgE of sensitized patients.

Our recombinant Der f 1 and Der p 1 proteins contained specific amino acid substitutions. Both proteins contained the C35S mutation to disrupt their protease activity and minimize the impact on protein stability and/or cell viability in our assays.^{36,41} To overcome the challenges of recombinant Der p 1 production, the additional S(-63)N mutation was introduced in the prodomain, disrupting a glycosylation site,³³ which is absent in Der f 1. In line with previous reports,^{27,33} these targeted mutations did not impair the immunoreactivity with our protein tetramers efficiently binding IgE on basophils and triggering degranulation.

CytoBas using either [Der p 1]₄ or [Der p 2]₄ exhibited comparable sensitivity and specificity for HDM-sensitization detection as BAT. While the robustness of the ROC analysis might be affected due to the higher number of patients than controls, it does demonstrate that allergen-component binding to basophils is similarly effective for detection of allergen sensitization as *in vitro* activation. In addition, the 4 nonresponders in BAT (1 patient, 3 controls) in our study were evaluable with CytoBas. Thus, basophil staining is a highly suited approach to detect functional allergen sensitization without the potential challenges of *in vitro* stimulation.

As compared to IgE serology, the CytoBas approach is more labor-intensive and costlier, which can impact clinical translation. Rather than replacing standard specific IgE tests, it can be used for specific queries requiring differential diagnosis with 8 or more components. Because these can be run in a single assay, the cost

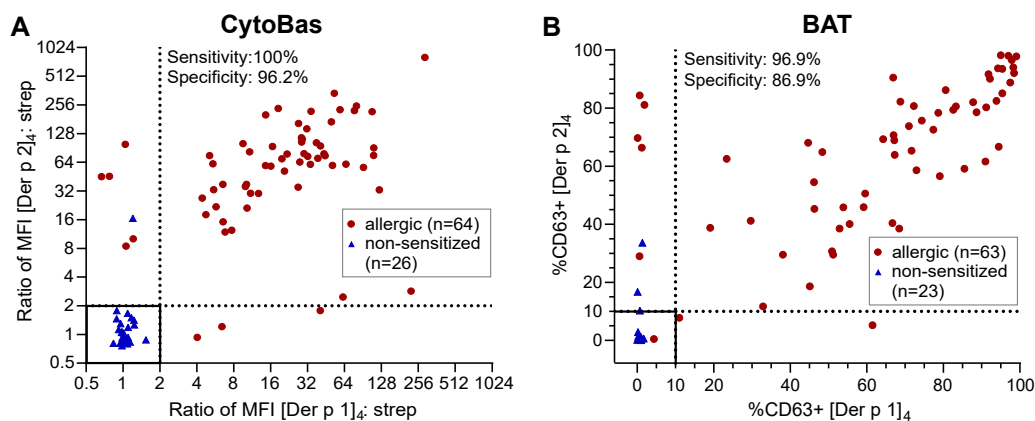


FIG 4. Combined evaluation of Der p 1 and Der p 2 tetramers for component-resolved diagnostic tool for HDM allergy. **(A)** The capacity of CytoBas to detect HDM sensitization was evaluated based on positive [Der p 1]₄ and/or [Der p 2]₄ staining. Scatterplot depicting MFI ratio of allergen: streptavidin of [Der p 1]₄-APC versus [Der p 2]₄-APC on basophils from control and HDM-allergic samples. **(B)** Scatterplot for the proportion of CD63⁺ basophils in BAT following *in vitro* stimulation with both allergens.

per component is drastically reduced. Furthermore, the assay can provide a same-day result within 2 to 3 hours in acute situations (eg, differential evaluation of β -lactam allergy), and it provides evaluation of functional, cell-bound IgE, rather than serum IgE. Because tetramerization does not always lead to full occupation of the biotin-binding sites, it is possible that allergen-streptavidin mixtures contain “free” biotinylated allergens.^{42,43} When multiple allergen-streptavidin mixtures are combined for staining immune cells, it is possible that “free” allergens bind to another fluorochrome-labeled streptavidin and result in a cross-positive signal in flow cytometry. It is therefore important to ensure that single allergen-tetramers are purified or diluted before mixing them as we have consistently done in our studies to prevent false-positive signals.

All 64 patients in our study were sensitized to Der p 1 and/or Der p 2, which is consistent with previous findings that Der p 1 and Der p 2 are the highly immunodominant components of HDM (95%).^{12,44,45} Der p 1 and Der p 2 have been described as “initiator molecules” for HDM sensitization in the context of molecular spreading⁴⁶; in other words, allergen sensitization arises first against these components and subsequently spreads to other components from the same allergen source.⁴⁷ This suggests that the detection of sensitization to Der p 1 and Der p 2 can be beneficial for early screening. Furthermore, dual-sensitization to both Der p 1 and Der p 2 is associated with more concerning morbidity and a higher risk of asthma among patients who are allergic.^{48,49} Based on our findings, combining Der p 1 and Der p 2 in the CytoBas approach, provides the best sensitivity and specificity for allergen detection of patients who are HDM-allergic, reinforcing the need to use both components for future diagnoses.

Parallel evaluation of basophil staining and activation by [Der p 1]₄ and [Der f 1]₄ enabled investigation of the immunoreactivity between the primary sensitizer (Der p 1) and a highly homologous variant (Der f 1). All patients (n = 32) who had positive staining to Der p 1 were positive for Der f 1, while 90% (n = 27 of 30) who showed basophil activation to Der p 1 were positive for Der f 1. Der p 1 and Der f 1 amino acid sequences have a 90% similarity, and the 2 structures are highly homologous,⁵⁰ so that epitopes will

be largely shared between these 2 allergens.⁵¹ Still, the Der p 1 CytoBas exhibited enhanced performance capability (AUC from ROC) than Der f 1 CytoBas in our Australian patient cohort. This underpins the importance of using relevant allergen components based on geographic region⁵² to achieve reliable diagnostic results for inhalant^{53,54} and food allergies.⁵⁵ The inclusion of geographically relevant allergens together, or in multiplexed form, such as combining Der f 1 and Der p 1, despite their largely shared epitopes, will increase the universality of CytoBas allergy detection.

While all 64 patients who are HDM-allergic were detected with CytoBas including Der p 1 and Der p 2, several studies have reported that 2% to 5% of patients who are HDM-allergic are monosensitized to Der p 23.^{53,56,57} Thus, the inclusion of other (minor) allergens, including Der p 23 and Der p 10, could be important to improve diagnosis rates. Furthermore, the inclusion of these proteins in CytoBas could inform disease prognosis. Sensitization to Der p 23 is suggested to correlate with more severe asthma symptoms and more intense airway inflammation measured via fractional exhaled nitric oxide (FENO).⁵⁸ Sensitization to Der p 10 (tropomyosin) highly relates with cross-reactivity to other tropomyosin allergens, such as shrimp and crab, which can be inducers of severe systemic anaphylaxis.⁵⁹ The early detection of sensitization against Der p 10 can advance monitoring symptoms of ingestion of cross-reactive crustacea or snails.^{60,61} Stratification of patients based on molecular sensitization profiles obtained from CRD⁶² can improve AIT success.^{63,64} Therefore, it can be considered to expand the CytoBas approach beyond group 1 and group 2 HDM allergens with additional HDM allergen components that could improve precision diagnosis and potentially inform on treatment decisions.⁶⁵

Our results expand our previous findings²⁷ that the CytoBas approach can be applied for multiplex component-resolved detection of aeroallergen sensitization.²⁸ As the major triggers of allergic asthma and AR are HDM, grass/tree pollen, and household pets,⁶⁶⁻⁶⁸ future studies should address whether these patients can be differentially diagnosed with a single CytoBas assay that includes the major components from these allergens.

DISCLOSURE STATEMENT

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Disclosure of potential conflict of interest: M. C. van Zelm, R. E. O'Hehir, and P. M. Hogarth are inventors on a patent application related to this work. The rest of the authors declare that they have no relevant conflicts of interest.

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Key messages

- Fluorescent tetramers of recombinant Der f 1, Der p 1, and Der p 2 can be used to detect functional IgE sensitization to HDM by flowcytometric detection on basophils (CytoBas).
- A single CytoBas assay with the inclusion of both Der p 1 and Der p 2 can detect HDM sensitization with >95% sensitivity and specificity.

REFERENCES

- Demir E, Akmeşe Ö, Erbay H, Taylan-Özkan A, Mumcuoğlu K. Bibliometric analysis of publications on house dust mites during 1980–2018. *Allergol Immunopathol (Madr)* 2020;48:374–83.
- Tovey ER, Chapman M, Platts-Mills T. Mite faeces are a major source of house dust allergens. *Nature* 1981;289:592–3.
- Zuiani C, Custovic A. Update on house dust mite allergen avoidance measures for asthma. *Curr Allergy Asthma Rep* 2020;20:50.
- Montuschi P. Pharmacotherapy of patients with mild persistent asthma: strategies and unresolved issues. *Front Pharmacol* 2011;2:35.
- Virchow JC, Backer V, Kuna P, Prieto L, Nolte H, Villesen HH, et al. Efficacy of a house dust mite sublingual allergen immunotherapy tablet in adults with allergic asthma: a randomized clinical trial. *JAMA* 2016;315:1715–25.
- Calderón MA, Kleine-Tebbe J, Linneberg A, De Blay F, de Rojas DHF, Virchow JC, et al. House dust mite respiratory allergy: an overview of current therapeutic strategies. *J Allergy Clin Immunol Pract* 2015;3:843–55.
- Maurer M, Zuberbier T. Undertreatment of rhinitis symptoms in Europe: findings from a cross-sectional questionnaire survey. *Allergy* 2007;62:1057–63.
- Peat J, Tovey E, Mellis C, Leeder S, Woolcock A. Importance of house dust mite and *Alternaria* allergens in childhood asthma: an epidemiological study in two climatic regions of Australia. *Clin Exp Allergy* 1993;23:812–20.
- Zock J-P, Heinrich J, Jarvis D, Verlato G, Norbäck D, Plana E, et al. Distribution and determinants of house dust mite allergens in Europe: the European Community Respiratory Health Survey II. *J Allergy Clin Immunol* 2006;118:682–90.
- Arlian LG, Morgan MS, Neal JS. Dust mite allergens: ecology and distribution. *Curr Allergy Asthma Rep* 2002;2:401–11.
- Hammad H, Plantinga M, Deswarte K, Pouliot P, Willart MA, Kool M, et al. Inflammatory dendritic cells—not basophils—are necessary and sufficient for induction of Th2 immunity to inhaled house dust mite allergen. *J Exp Med* 2010;207:2097–111.
- Pittner G, Vrtala S, Thomas W, Weghofer M, Kundi M, Horak F, et al. Component-resolved diagnosis of house-dust mite allergy with purified natural and recombinant mite allergens. *Clin Exp Allergy* 2004;34:597–603.
- Aalberse RC, Akkerdaas J, Van Ree R. Cross-reactivity of IgE antibodies to allergens. *Allergy* 2001;56:478–90.
- Reginald K, Chew FT. The major allergen Der p 2 is a cholesterol binding protein. *Sci Rep* 2019;9:1–8.
- Mueller GA, Benjamin DC, Rule GS. Tertiary structure of the major house dust mite allergen Der p 2: sequential and structural homologies. *Biochemistry* 1998;37:12707–14.
- Ansotegui IJ, Melioli G, Canonica GW, Caraballo L, Villa E, Ebisawa M, et al. IgE allergy diagnostics and other relevant tests in allergy, a World Allergy Organization position paper. *World Allergy Organ J* 2020;13:100080.
- Huang HJ, Resch-Marat Y, Rodriguez-Dominguez A, Chen KW, Kiss R, Ziegler-mayer P, et al. Underestimation of house dust mite-specific IgE with extract-based ImmunoCAPs compared with molecular ImmunoCAPs. *J Allergy Clin Immunol* 2018;142:1656–9.e9.
- Hoffmann H, Santos A, Mayorga C, Nopp A, Eberlein B, Ferrer M, et al. The clinical utility of basophil activation testing in diagnosis and monitoring of allergic disease. *Allergy* 2015;70:1393–405.
- Knol EF, Mul FP, Jansen H, Calafat J, Roos D. Monitoring human basophil activation via CD63 monoclonal antibody 435. *J Allergy Clin Immunol* 1991;88:328–38.
- Dahlén B, Nopp A, Johansson S, Eduards M, Skedinger M, Adedoyin J. Basophil allergen threshold sensitivity, CD-sens, is a measure of allergen sensitivity in asthma. *Clin Exp Allergy* 2011;41:1091–7.
- Song Y, Wang J, Leung N, Wang LX, Lisann L, Sicherer SH, et al. Correlations between basophil activation, allergen-specific IgE with outcome and severity of oral food challenges. *Ann Allergy Asthma Immunol* 2015;114:319–26.
- Santos AF, Alpan O, Hoffmann HJ. Basophil activation test: mechanisms and considerations for use in clinical trials and clinical practice. *Allergy* 2021;76:2420–32.
- Dramburg S, Hilger C, Santos AF, de las Vecillas L, Aalberse RC, Acevedo N, et al. EAACI Molecular Allergy User's Guide 2.0. *Pediatr Allergy Immunol* 2023;34(suppl 28):e13854.
- Farioli L, Losappio LM, Giuffrida MG, Pravettoni V, Micarelli G, Nichelatti M, et al. Mite-induced asthma and IgE levels to shrimp, mite, tropomyosin, arginine kinase, and Der p 10 are the most relevant risk factors for challenge-proven shrimp allergy. *Int Arch Allergy Immunol* 2017;174:133–43.
- Werfel T, Asero R, Ballmer-Weber B, Beyer K, Enrique E, Knulst A, et al. Position paper of the EAACI: food allergy due to immunological cross-reactions with common inhalant allergens. *Allergy* 2015;70:1079–90.
- Agache I, Lau S, Akdis CA, Smolinska S, Bonini M, Cavkaytar O, et al. EAACI Guidelines on allergen immunotherapy: house dust mite-driven allergic asthma. *Allergy* 2019;74:855–73.
- McKenzie CI, Varese N, Aui PM, Wines BD, Hogarth PM, Thien F, et al. CytoBas: precision component-resolved diagnostics for allergy using flow cytometric staining of basophils with recombinant allergen tetramers. *Allergy* 2021;76:3028–40.
- van Zelm MC, McKenzie CI, Varese N, Rolland JM, O'Hehir RE. Advances in allergen-specific immune cell measurements for improved detection of allergic sensitization and immunotherapy responses. *Allergy* 2021;76:3374–82.
- Marsh D, Goodfriend L, King T, Lowenstein H, Platts-Mills T. Allergen nomenclature. *Bull World Health Organ* 1986;64:767.
- Pomés A, Davies JM, Gadermaier G, Hilger C, Holzhauser T, Lidholm J, et al. WHO/IUIS allergen nomenclature: providing a common language. *Mol Immunol* 2018;100:3–13.
- Yasuhara T, Takai T, Yuuki T, Okudaira H, Okumura Y. Biologically active recombinant forms of a major house dust mite group 1 allergen Der f 1 with full activities of both cysteine protease and IgE binding. *Clin Exp Allergy* 2001;31:116–24.
- Takai T, Mineki R, Nakazawa T, Takaoka M, Yasueda H, Murayama K, et al. Maturation of the activities of recombinant mite allergens Der p 1 and Der f 1, and its implication in the blockade of proteolytic activity. *FEBS Lett* 2002;531:265–72.
- Takai T, Mizuuchi E, Kikuchi Y, Nagamune T, Okumura K, Ogawa H. Glycosylation of recombinant proforms of major house dust mite allergens Der p 1 and Der f 1 decelerates the speed of maturation. *Int Arch Allergy Immunol* 2006;139:181–7.
- Hartley GE, Edwards ESJ, Aui PM, Varese N, Stojanovic S, McMahon J, et al. Rapid generation of durable B cell memory to SARS-CoV-2 spike and nucleocapsid proteins in COVID-19 and convalescence. *Sci Immunol* 2020;5:eabf8891.
- McKenzie CI, Varese N, Aui PM, Reinwald S, Wines BD, Hogarth PM, et al. RNA sequencing of single allergen-specific memory B cells after grass pollen immunotherapy: two unique cell fates and CD29 as a biomarker for treatment effect. *Allergy* 2023;78:822–35.
- Chruszcz M, Chapman MD, Vailes LD, Stura EA, Saint-Remy J-M, Minor W, et al. Crystal structures of mite allergens Der f 1 and Der p 1 reveal differences in surface-exposed residues that may influence antibody binding. *J Mol Biol* 2009;386:520–30.
- Ebo D, Bridts C, Hagendorens M, Aerts N, De Clerck L, Stevens W. Basophil activation test by flow cytometry: present and future applications in allergology. *Cytometry B Clin Cytom* 2008;74:201–10.
- Shi X, Jarvis DL. Protein N-glycosylation in the baculovirus-insect cell system. *Curr Drug Targets* 2007;8:1116–25.
- Walgraffe D, Mattéotti C, El Bakkoury M, Garcia L, Marchand C, Bullens D, et al. A hypoallergenic variant of Der p 1 as a candidate for mite allergy vaccines. *J Allergy Clin Immunol* 2009;123:1150–6.

40. Jacquet A, Magi M, Petry H, Bollen A. High-level expression of recombinant house dust mite allergen Der p 1 in *Pichia pastoris*. *Clin Exp Allergy* 2002;32:1048-53.
41. Takahashi K, Takai T, Yasuhara T, Yokota T, Okumura Y. Effects of site-directed mutagenesis in the cysteine residues and the N-glycosylation motif in recombinant Der f 1 on secretion and protease activity. *Int Arch Allergy Immunol* 2001;124:454-60.
42. Kwok WW, Ptacek NA, Liu AW, Buckner JH. Use of class II tetramers for identification of CD4+ T cells. *J Immunol Methods* 2002;268:71-81.
43. Novak EJ, Liu AW, Gebe JA, Falk BA, Nepom GT, Koelle DM, et al. Tetramer-guided epitope mapping: rapid identification and characterization of immunodominant CD4+ T cell epitopes from complex antigens. *J Immunol* 2001;166:6665-70.
44. Weghofer M, Thomas W, Kronqvist M, Mari A, Purohit A, Pauli G, et al. Variability of IgE reactivity profiles among European mite allergic patients. *Eur J Clin Invest* 2008;38:959-65.
45. Custovic A, Sonntag H-J, Buchan IE, Belgrave D, Simpson A, Prosperi MC. Evolution pathways of IgE responses to grass and mite allergens throughout childhood. *J Allergy Clin Immunol* 2015;136:1645-52.e8.
46. Posa D, Perna S, Resch Y, Lupinek C, Panetta V, Hofmaier S, et al. Evolution and predictive value of IgE responses toward a comprehensive panel of house dust mite allergens during the first 2 decades of life. *J Allergy Clin Immunol* 2017;139:541-9.e8.
47. Hatzler L, Panetta V, Lau S, Wagner P, Bergmann RL, Illi S, et al. Molecular spreading and predictive value of preclinical IgE response to *Phleum pratense* in children with hay fever. *J Allergy Clin Immunol* 2012;130:894-901.e5.
48. Vidal C, Lojo S, Juangorena M, Gonzalez-Quintela A. Association between asthma and sensitization to allergens of *Dermatophagoides pteronyssinus*. *J Invest Allergol Clin Immunol* 2016;26:304-9.
49. Letrán A, García I, Espinazo-Romeu M, Moreno-Aguilar C, Moreno F. Cut-off value of *D. pteronyssinus* specific IgE in double negative patients Der p 1 and Der p 2 and its clinical repercussion. *Sci Rep* 2021;11:1-9.
50. De Halleux S, Stura E, VanderElst L, Carlier V, Jacquemin M, Saint-Remy J-M. Three-dimensional structure and IgE-binding properties of mature fully active Der p 1, a clinically relevant major allergen. *J Allergy Clin Immunol* 2006;117:571-6.
51. Glesner J, Vailes LD, Schlachter C, Mank N, Minor W, Osinski T, et al. Antigenic determinants of Der p 1: specificity and cross-reactivity associated with IgE antibody recognition. *J Immunol* 2017;198:1334-44.
52. Bousquet PJ, Chinn S, Janson C, Kogevinas M, Burney P, Jarvis D. Geographical variation in the prevalence of positive skin tests to environmental aeroallergens in the European Community Respiratory Health Survey I. *Allergy* 2007;62:301-9.
53. Muddaluru V, Valenta R, Vrtala S, Schleiderer T, Hindley J, Hickey P, et al. Comparison of house dust mite sensitization profiles in allergic adults from Canada, Europe, South Africa and USA. *Allergy* 2021;76:2177-88.
54. Rossi R, Melioli G, Monasterolo G, Harwanegg C, Rossi L, Canonica G, et al. Sensitization profiles in polysensitized patients from a restricted geographical area: further lessons from multiplexed component resolved diagnosis. *Eur Ann Allergy Clin Immunol* 2011;43:171.
55. Ballmer-Weber BK, Scheurer S, Fritsche P, Enrique E, Cistero-Bahima A, Haase T, et al. Component-resolved diagnosis with recombinant allergens in patients with cherry allergy. *J Allergy Clin Immunol* 2002;110:167-73.
56. González-Pérez R, Poza-Guedes P. Inferring clinical relevance of Der p 23 from the restricted effect on treatment outcomes after tablet allergy immunotherapy. *Allergy* 2021;76:1298-9.
57. Jiménez-Feijoo R, Pascal M, Moya R, Riggioni C, Domínguez O, Lózano J, et al. Molecular diagnosis in house dust mite allergic patients suggests clinical relevance of Der p 23 in asthmatic children. *J Invest Allergol Clin Immunol* 2020;30:127-32.
58. Kowal K, Pampuch A, Siergiejko G, Siergiejko Z, Swiebocka E, Schlachter CR, et al. Sensitization to major *Dermatophagoides pteronyssinus* allergens in house dust mite allergic patients from North Eastern Poland developing rhinitis or asthma. *Adv Med Sci* 2020;65:304-9.
59. Resch Y, Weghofer M, Seiberler S, Horak F, Scheibhofer S, Linhart B, et al. Molecular characterization of Der p 10: a diagnostic marker for broad sensitization in house dust mite allergy. *Clin Exp Allergy* 2011;41:1468-77.
60. Kamath SD, Scheibhofer S, Johnson CM, Machado Y, McLean T, Taki AC, et al. Effect of structural stability on endolysosomal degradation and T-cell reactivity of major shrimp allergen tropomyosin. *Allergy* 2020;75:2909-19.
61. Pajno GB, La Grutta S, Barberio G, Canonica GW, Passalacqua G. Harmful effect of immunotherapy in children with combined snail and mite allergy. *J Allergy Clin Immunol* 2002;109:627-9.
62. Stringari G, Tripodi S, Caffarelli C, Dondi A, Asero R, Businco ADR, et al. The effect of component-resolved diagnosis on specific immunotherapy prescription in children with hay fever. *J Allergy Clin Immunol* 2014;134:75-81.e2.
63. Matricardi PM, Dramburg S, Potapova E, Skevaki C, Renz H. Molecular diagnosis for allergen immunotherapy. *J Allergy Clin Immunol* 2019;143:831-43.
64. Rodríguez-Domínguez A, Berings M, Rohrbach A, Huang H-J, Curin M, Gevaert P, et al. Molecular profiling of allergen-specific antibody responses may enhance success of specific immunotherapy. *J Allergy Clin Immunol* 2020;146:1097-108.
65. Chen K-W, Zieglmayer P, Zieglmayer R, Lemell P, Horak F, Bunu CP, et al. Selection of house dust mite-allergic patients by molecular diagnosis may enhance success of specific immunotherapy. *J Allergy Clin Immunol* 2019;143:1248-52.e12.
66. Ciprandi G, Alesina R, Ariano R, Aurnia P, Borrelli P, Cadario G, et al. Characteristics of patients with allergic polysensitization: the POLISMAIL study. *Eur Ann Allergy Clin Immunol* 2008;40:77-83.
67. Dondi A, Tripodi S, Panetta V, Asero R, Businco ADR, Bianchi A, et al. Pollen-induced allergic rhinitis in 1360 Italian children: comorbidities and determinants of severity. *Pediatr Allergy Immunol* 2013;24:742-51.
68. Konradsen JR, Nordlund B, Onell A, Borres MP, Grönlund H, Hedlin G. Severe childhood asthma and allergy to furry animals: refined assessment using molecular-based allergy diagnostics. *Pediatr Allergy Immunol* 2014;25:187-92.

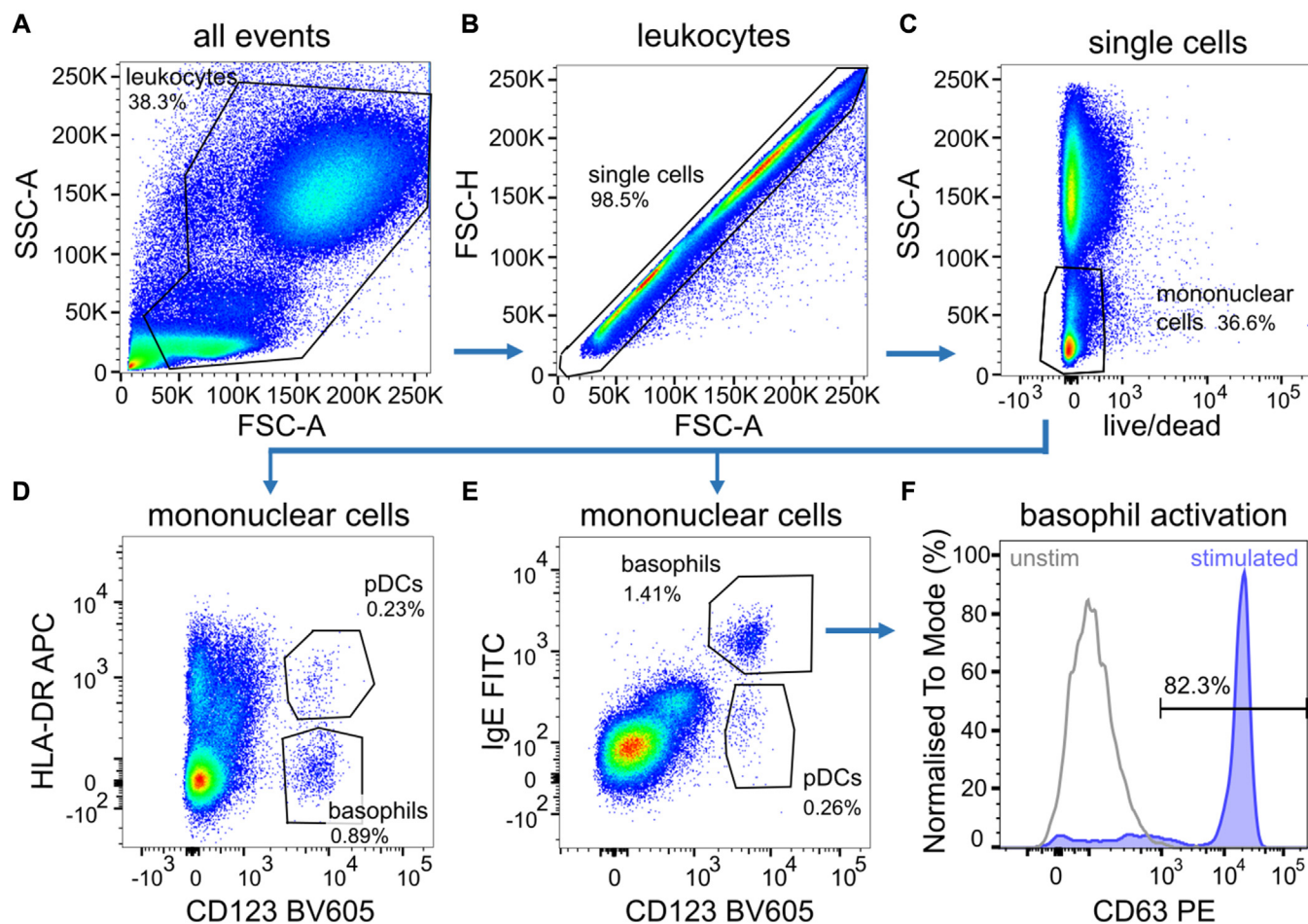


FIG E1. Gating strategy to identify activated basophils in whole blood. Stepwise gating strategy for blood immune cells to identify (A) total leukocytes, (B) single cells, and (C) live mononuclear cells. (D) Basophils are defined by the positivity for both IgE and CD123 or (E) by the expression of CD123 in the absence of HLA-DR for those samples in which basophils were activated with anti-IgE. (F) Expression of surface CD63 was used to define *in vitro* activated basophils. A, Area; H, height; FSC, forward scatter; PE, phycoerythrin; SSC, side scatter.

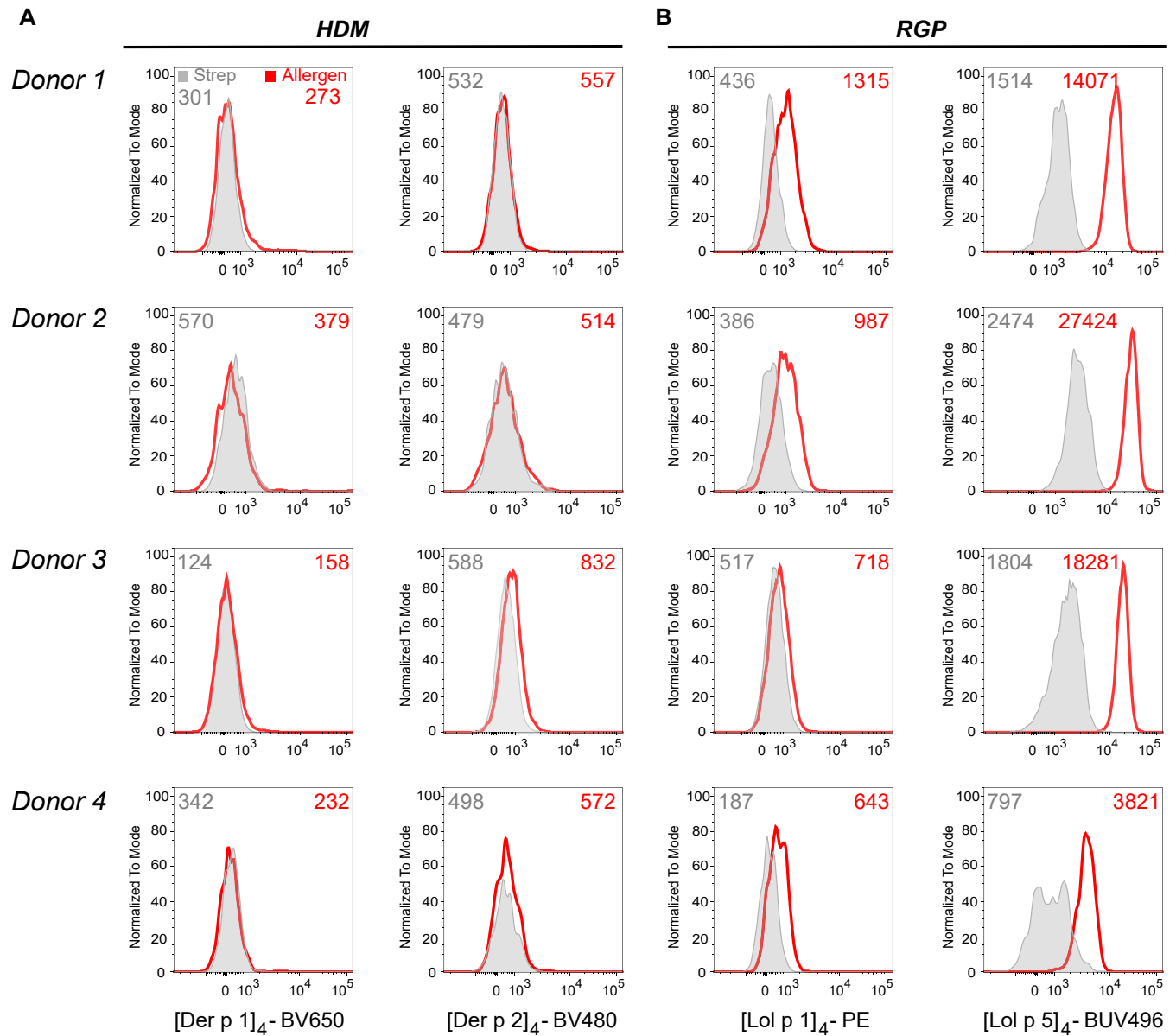


FIG E2. Multiplex CytoBas analysis of 4 patients who are ryegrass pollen (RGP)-allergic. **(A)** Staining intensities of HDM allergen components [Der p 1]₄-BV650 and [Der p 2]₄-BV480, as well as **(B)** RGP allergen components [Lol p 1]₄-PE and [Lol p 5]₄-BUV496 on basophils of patients who are RGP-allergic.²⁷ MFI for each allergen tetramer and the streptavidin-only (*strep*) control are shown within the histogram plots.

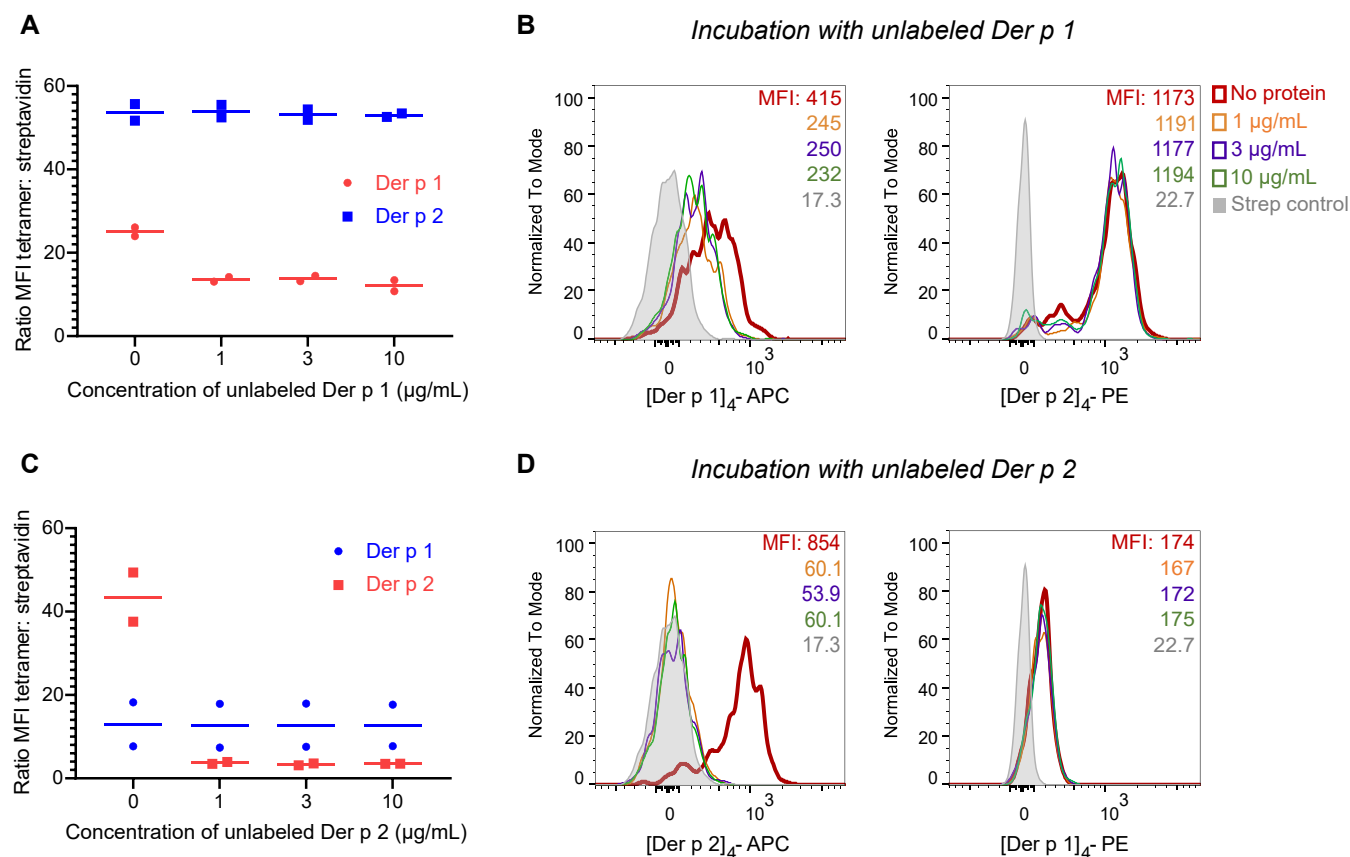


FIG E3. Molecular specificity of [Der p 1]₄ and [Der p 2]₄ binding to basophils of patients who are HDM-allergic. **(A,B)** Multiplex staining of basophils from 2 patients who are HDM-allergic with [Der p 1]₄-APC and [Der p 2]₄-PE in the presence of increasing concentrations of unlabeled Der p 1. **(C,D)** Multiplex staining of basophils from 2 patients who are HDM-allergic with [Der p 2]₄-APC and [Der p 1]₄-PE in the presence of increasing concentrations of unlabeled Der p 2. MFI for each allergen tetramer and the streptavidin-only control are shown within the histogram plots.

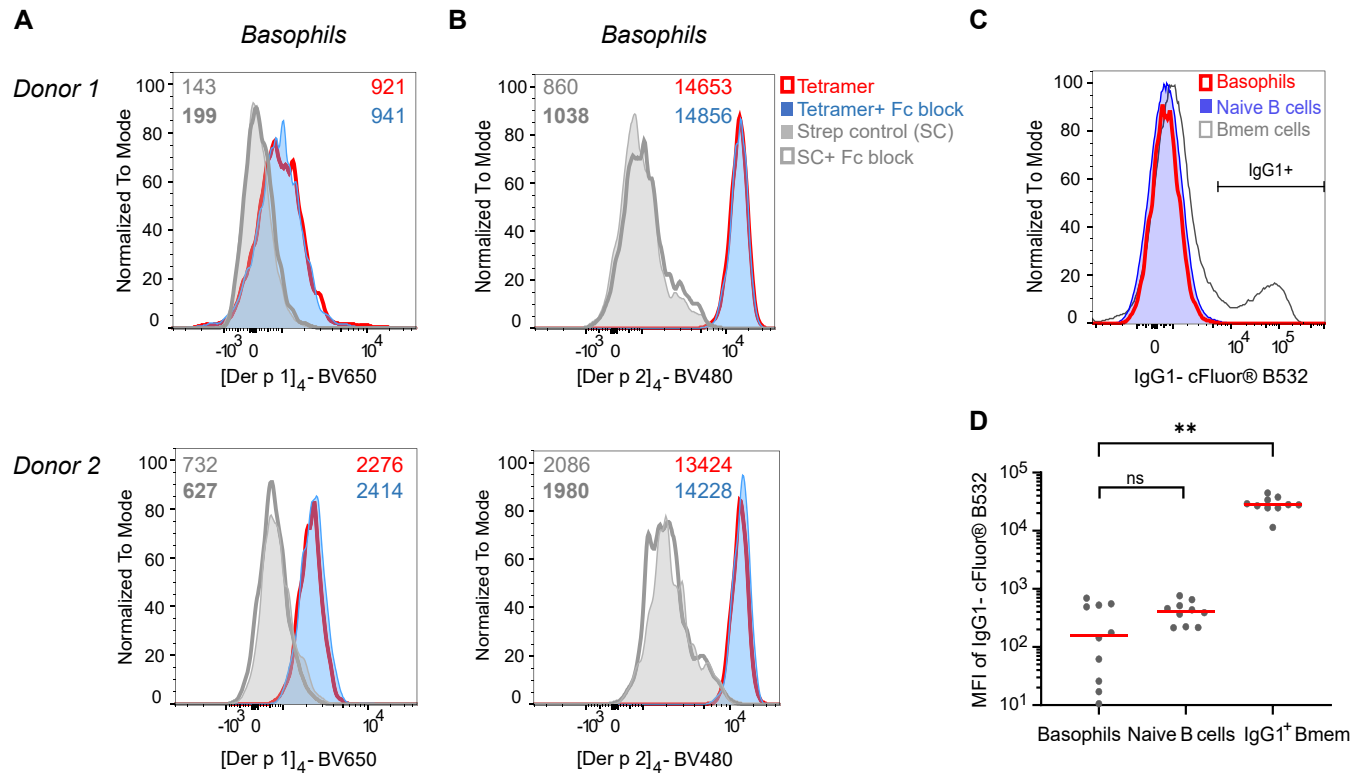


FIG E4. No role for IgG-FcγR binding in allergen-tetramer staining of basophils. Detection of (A) [Der p 1]₄ and (B) [Der p 2]₄ binding to basophils of 2 patients who are HDM-allergic in the presence or absence of Fc block, with MFI values of tetramer and strep control stainings shown in the histogram plots. (C) Representative histogram overlays of IgG₁ expression on basophils, naive B cells (as a negative control), and memory B cells (Bmem cells) from a patient who is HDM-allergic. (D) Quantitative analysis IgG₁ staining intensities on basophils, naive B cells, and IgG₁⁺ Bmem of 10 patients who are HDM-allergic. Statistics: Wilcoxon signed rank test. ***P* < .01.

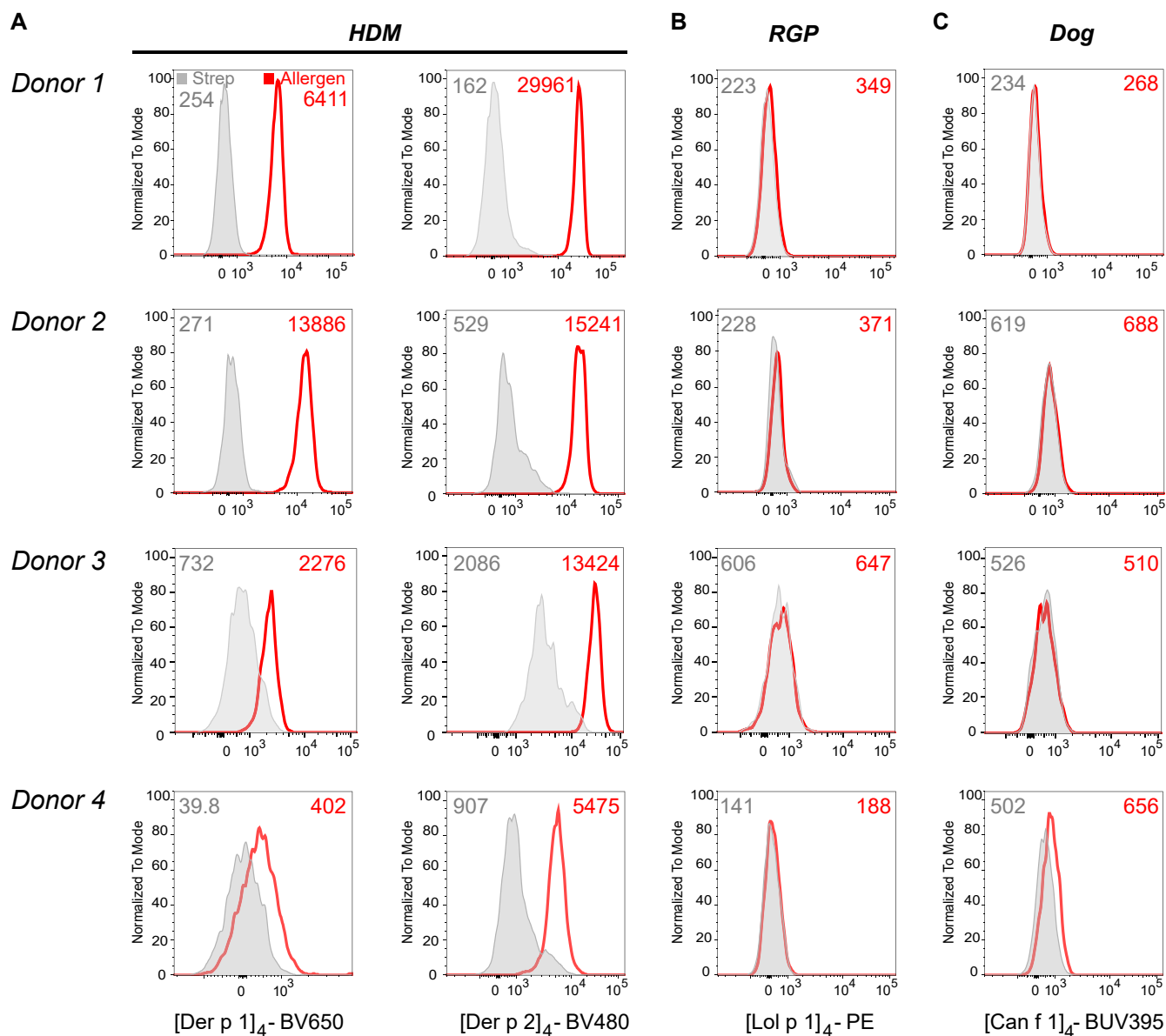


FIG E5. Specificity of allergen tetramer binding. Basophils of 4 patients who are HDM-allergic were evaluated with a multiplex CytoBas panel including (A) [Der p 1]₄-BV650 and [Der p 2]₄-BV480, (B) [Lol p 1]₄-PE, and (C) [Can f 1]₄-BUV395. MFI of each allergen tetramer staining and strep control are shown within the histogram plots.

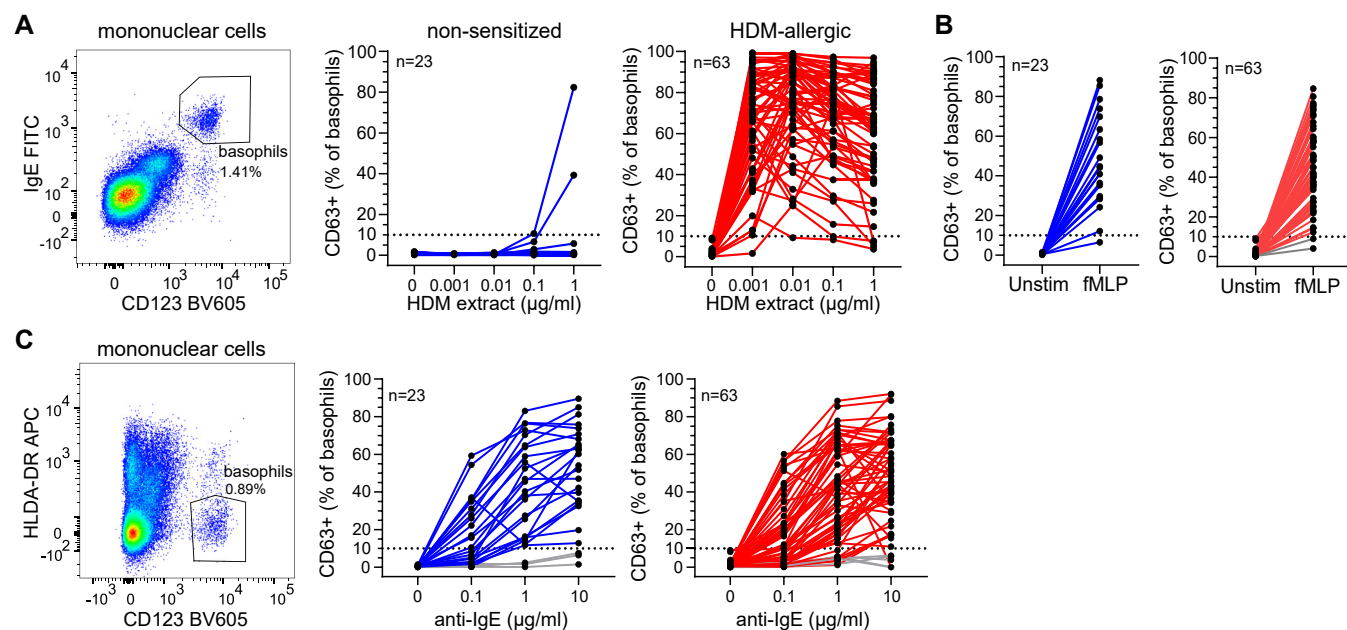


FIG E6. Basophils from both subjects who are allergic and those who are nonsensitized are functionally capable of degranulation. **(A)** Gating strategy for the detection of basophils after stimulation with HDM extract or **(B)** fMLP in individuals who are nonsensitized (*blue*) and those who are HDM-allergic (*red*). **(C)** Gating strategy using HLA-DR and CD123 for detection of basophils after anti-IgE stimulation in individuals who are nonsensitized (*blue*) and those who are HDM-allergic (*red*).

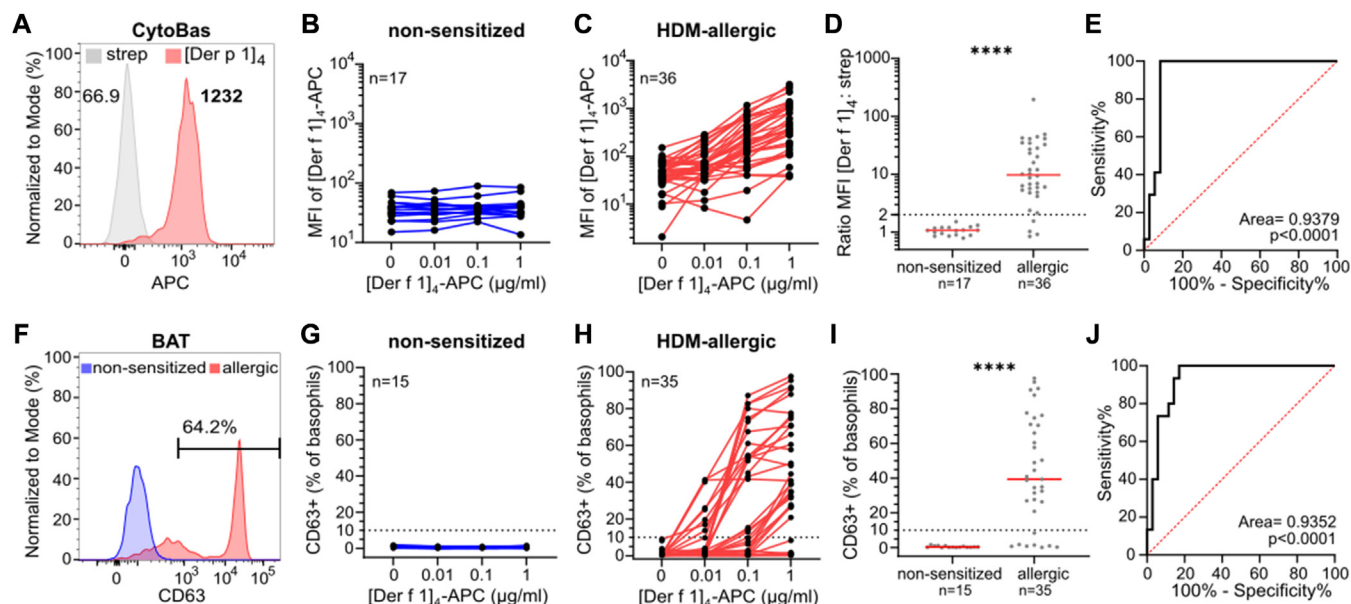


FIG E7. Performance analysis using Der f 1 tetramer in BAT and CytoBas assays. **(A)** MFI following stimulation of [Der f 1]₄-APC at 1 μg/mL. **(B,C)** Frequencies of basophils from nonsensitized (*blue*) and allergic groups (*red*) following stimulation with streptavidin-APC or increasing concentrations of [Der f 1]₄-APC. **(D)** Staining intensities of CytoBas via the MFI ratio of [Der f 1]₄-APC at 1 μg/mL over streptavidin-APC and **(E)** ROC from MFI. **(F)** Representative gating strategy of CD63⁺ on basophils and **(G,H)** frequencies of basophils following stimulation with streptavidin-APC or increasing concentrations of [Der f 1]₄-APC. **(I)** CD63 expression post stimulation with [Der f 1]₄-APC at 1 μg/mL and **(J)** the corresponding ROC of CD63 positivity. Statistics: Mann-Whitney *U*-test; for ROC, Wilson/Brown method for confidence level (>95%) determination. *****P* < .0001.

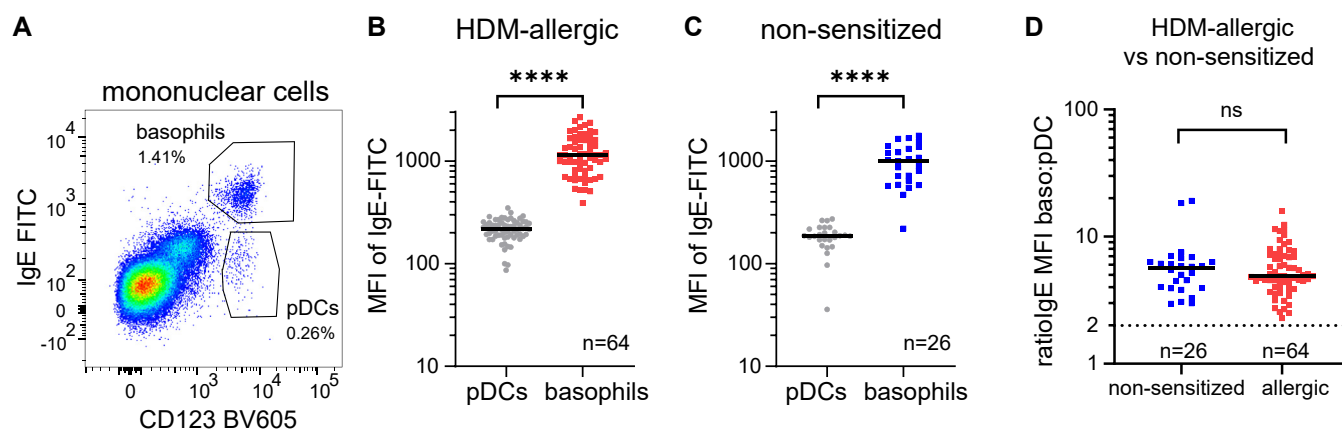


FIG E8. Surface IgE expression levels on basophils and pDCs. **(A)** Representative flow cytometry plot of basophils and pDCs with corresponding frequencies. MFI of IgE-FITC, indicating surface IgE expression from basophils and pDCs in **(B)** patients who are HDM-allergic ($n = 64$) and **(C)** controls who are nonsensitized ($n = 26$). **(D)** Ratio MFI of IgE staining of basophils over pDC from nonsensitized and allergic groups, respectively, with median shown. Cutoff ratio of 2 is indicated by the dotted line. Statistics: Wilcoxon signed rank test. **** $P < .0001$. ns, Not significant.

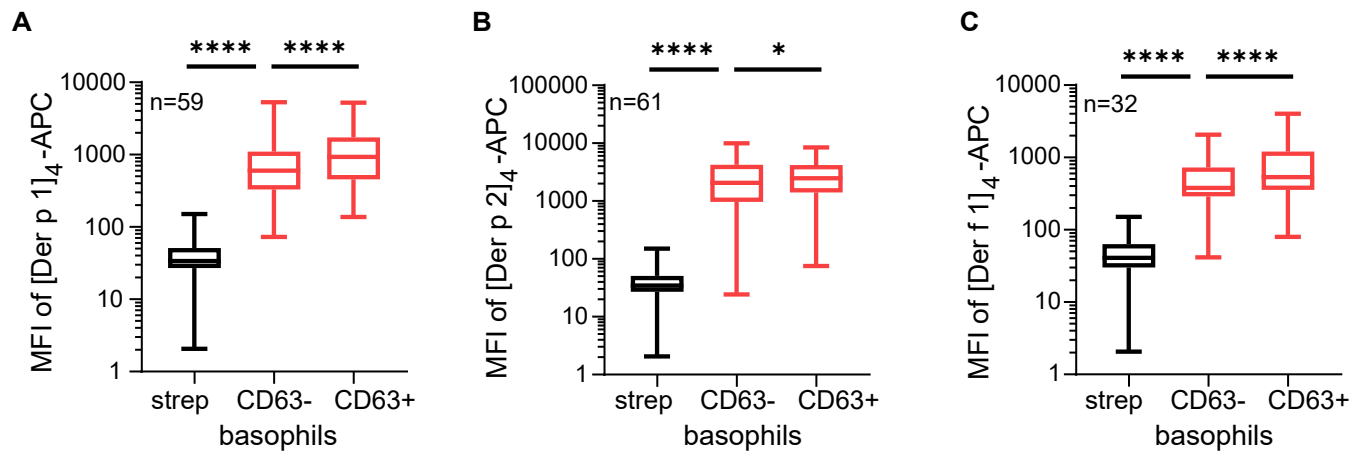


FIG E9. Allergen tetramers bind basophils in sensitized individuals irrespective of activation. MFI of allergen staining on CD63⁺ and CD63⁻ basophils following *in vitro* stimulation with streptavidin-APC and 1 µg/mL (A) [Der p 1]₄-APC, (B) [Der p 2]₄-APC, (C) [Der f 1]₄-APC. Statistics: Wilcoxon signed rank test. **P* < .05, *****P* < .0001.

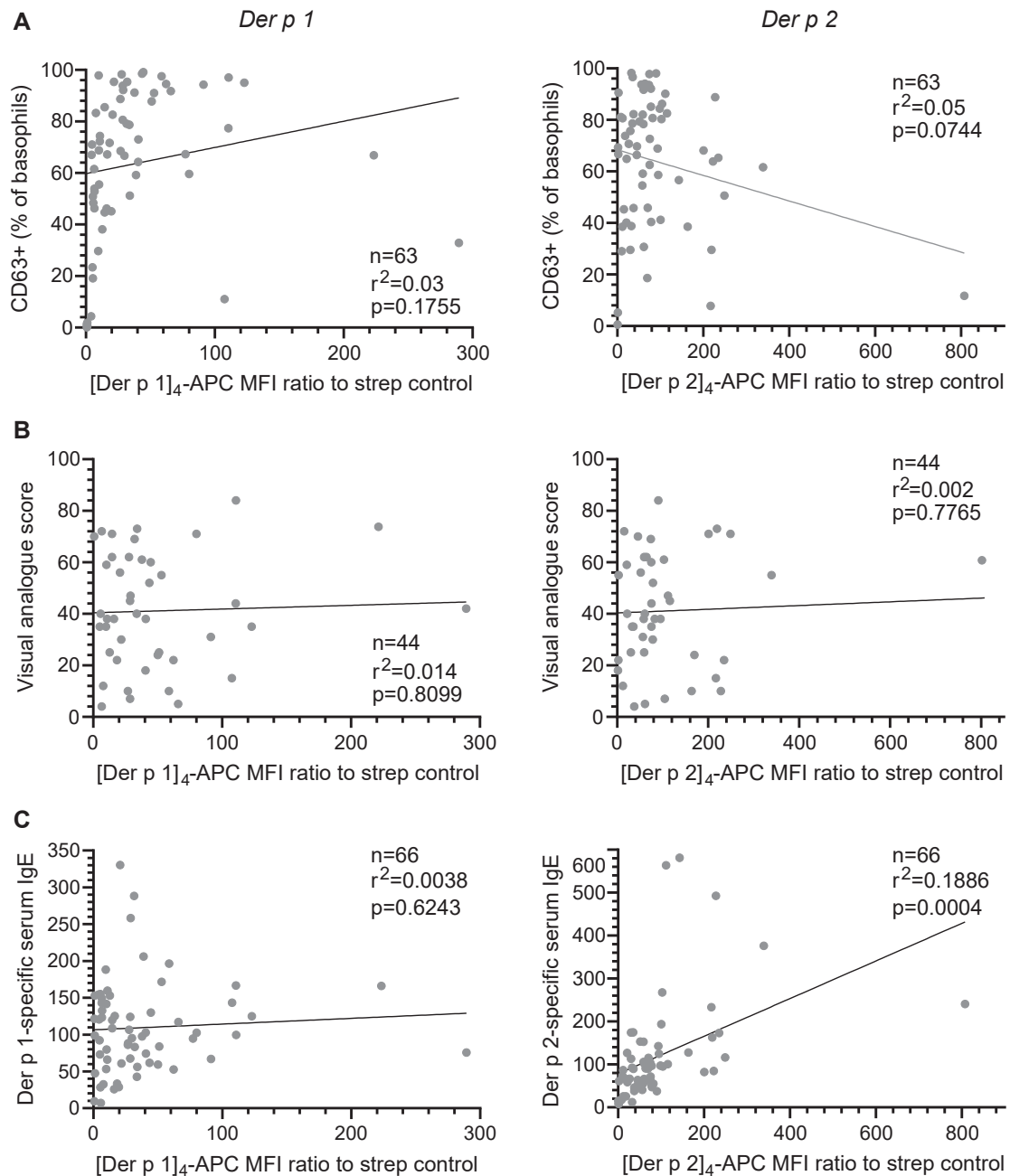


FIG E10. Correlations of [Der p 1]₄ and [Der p 2]₄ staining ratios with (A) basophil activation (CD63⁺ frequencies), (B) visual analogue scores, and (C) serum-specific IgE concentrations. Significance and *r*-square (*r*²) of correlation determined by linear regression.