

Letter to the Editor

Expression of activation markers in circulating basophils and the relationship to allergen-induced bronchoconstriction in subjects with mild allergic asthma

To the Editor:

Asthma is a chronic respiratory disease characterized by reversible airway obstruction, airway hyperresponsiveness, and eosinophilic airway inflammation. In sensitized individuals, basophils play an important role in airway responses to allergens through IgE-mediated release of histamine and cysteinyl leukotrienes, which are both potent mediators of bronchoconstriction. The number of basophils in the airways has been reported to be significantly higher in patients with asthma than in healthy subjects,¹ and this number increases during asthmatic exacerbation.² Furthermore, basophil infiltration of the airways increases during the late-phase asthmatic response (LAR) following allergen inhalation,^{3,4} where basophils provide a significant source of IL-4.⁵ In recent years, the basophil activation test has been developed to assess human basophil activation in cases of immediate-type hypersensitivity allergic responses. These tests monitor surface marker expression of CD63, CD203c, and CD69 after immediate-type sensitivity reactions. Given that the use of a basophil activation test in the context of allergic asthma has not been established, we examined the change in circulating basophil activation markers after allergen inhalation in subjects with allergic asthma.

Nineteen subjects with mild allergic asthma were enrolled in a diluent-controlled allergen bronchoprovocation study (see Table E1 in this article's Online Repository at www.jacionline.org). Subjects were required to have a skin prick test positive to common aeroallergens, methacholine PC₂₀ of 16 mg/mL or less, and FEV₁ of at least 70% of predicted. All subjects developed a fall in FEV₁ of 20% or more within 2 hours of allergen inhalation. Those with a subsequent fall of less than 15% in FEV₁ between 3 and 7 hours after allergen inhalation were considered to be isolated early responders, whereas those who developed a subsequent fall in FEV₁ of 15% or more between 3 and 7 hours after allergen inhalation were defined as dual responders.

Baseline lung function and peripheral blood was measured on day 1. Subjects were then randomized to inhale diluent or allergen on day 2, followed by blood sample collection at 3, 7, and 24 hours postchallenge. This was repeated with allergen or diluent after a 2-week recovery period. Airway challenges are described in this article's Methods section in the Online Repository at www.jacionline.org. Basophils were defined as CD45⁺/HLA-DR⁺/CD123⁺ (see Fig E1 in this article's Online Repository at www.jacionline.org); whole blood was immunostained with mAbs to CD45-Alexa Fluor 700 (BD Biosciences, Franklin Lakes, NJ), HLA-DR-APCH7 (BD Biosciences), and CD123-PE Cy7 (Ebioscience, San Diego, Calif), and markers of activation (CD203c-APC [MACS Miltenyi, San Diego, Calif], CD63-V450 [BD Biosciences], and CD69-PE [BD Biosciences]). Cells were acquired with an LSR II flow cytometer (BD Biosciences), and analyses were performed using Flow-Jo software (TreeStar, Ashland, Ore), as described in the Methods section. Statistical analysis is described in the Methods section.

Six subjects developed isolated early responses with a 32.7% ± 4.6% maximum fall in FEV₁ 0 to 2 hours after allergen

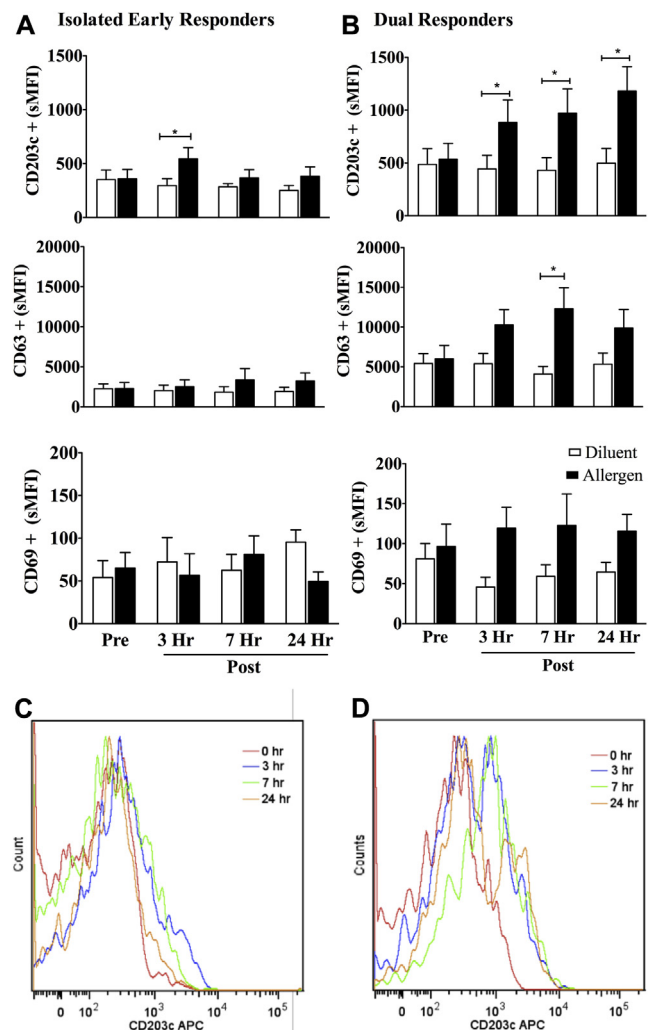


FIG 1. Effects of allergen inhalation on the expression of basophil activation markers. Basophils' sMFI expression of CD203c, CD63, and CD69 before allergen challenge and 3, 7, and 24 hours after allergen challenge (black bars), compared with diluent (open bars) in isolated early responders (A, N = 6) and dual responders (B, N = 13), as well as CD203c APC concatenate histograms for isolated early responders (C) and dual responders (D). Data are presented as mean ± SEM. *Significantly different from diluent control ($P < .05$). APC, Allophycocyanin.

challenge ($P < .05$ vs diluent) and had no difference from diluent challenge during the LAR at 3 to 7 hours after allergen challenge (see Fig E2, A, in this article's Online Repository at www.jacionline.org). Thirteen subjects developed dual responses, with a 32.3% ± 3.2% maximum fall in FEV₁ during the early response and a 23.5% ± 3.4% maximum fall in FEV₁ during the late response, and no change following diluent inhalation (see Fig E2, B).

Dual responders had significantly higher preallergen CD63 specific mean fluorescent intensity (sMFI) expression than did isolated early responders (see Fig E4 in this article's Online Repository at www.jacionline.org). Compared with diluent challenge, there was a significant increase in CD203c sMFI expression in basophils from isolated early responders at 3 hours

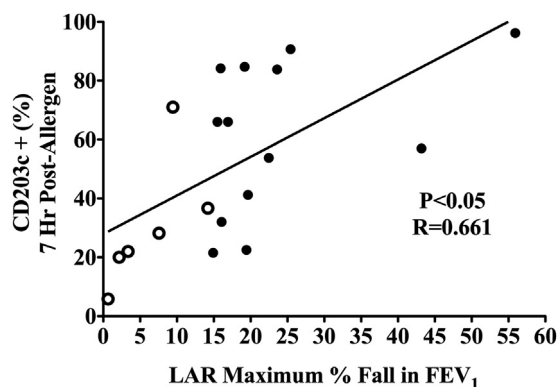


FIG 2. Relationship between CD203c expression on circulating basophils and maximum percent fall in FEV₁ during the LAR. Expression of CD203c on circulating basophils from isolated early responders (*open circle*) (N = 6) and dual responders (*closed circle*) (N = 13) at 7 hours after allergen challenge compared with maximum percent fall in FEV₁ during the LAR.

after allergen challenge, whereas CD69 and CD63 showed no change (Fig 1, A and C). A similar response was observed in basophils of dual responders at 3 hours; however, CD203c levels remained elevated at 7 and 24 hours after allergen challenge (Fig 1, B and D). In addition, dual responders had a peak in circulating CD63 sMFI basophil expression at 7 hours after allergen challenge, compared with diluent (Fig 1, B), whereas no change was seen in CD69 expression after allergen challenge. The same results were found when activation markers were expressed as percent positive basophils (see Fig E3 in this article's Online Repository at www.jacionline.org). When measures of basophil activation were compared with lung function, there was a positive correlation between the maximum percent fall in FEV₁ during the LAR (7 hours after allergen challenge) and CD203c expression ($P = .0018$; $R = 0.661$) (Fig 2). Last, there was a significant correlation between the predicted FEV₁ (%) and pre-allergen challenge CD203c percent cell expression ($P = .0248$; $R = 0.265$) (see Fig E5 in this article's Online Repository at www.jacionline.org).

Ono et al⁶ used flow cytometry to compare spontaneous and stimulated expression of CD63, CD69, and CD203c on basophils from healthy subjects and from patients with stable or exacerbated asthma. Spontaneous CD203c basophil expression was significantly higher in patients with asthma exacerbation than in patients with stable asthma or healthy subjects, whereas no differences were found in CD63 or CD69 expression. *In vitro* anti-IgE-induced basophils had a significantly higher expression of CD63 and CD203c in patients with asthma exacerbation than in healthy controls. Stimulation with *Der p 1* or IL-3 upregulated CD203c expression in a dose-dependent manner, with patients with clinically improved asthma experiencing less of an increase versus patients with asthma exacerbation. Last, patients with clinical improvement demonstrated a significant correlation between CD203c expression on basophil and predicted FEV₁ values. Ono et al⁶ established that there are differences in CD203c expression on basophils at baseline across asthma severity groups and healthy controls, and this continued to be notable after *in vitro* stimulation.

The present study aimed to expand on the above findings by examination of the change in the expression of activation markers on circulating basophils after allergen inhalation, and by comparing subjects with isolated early versus dual responses to

allergen. Subjects with isolated early responses showed a significant increase in basophil CD203c expression at 3 hours after allergen challenge compared with diluent; a return of CD203c expression to baseline by 7 and 24 hours after allergen challenge is consistent with the lack of LAR in these subjects. Following allergen inhalation, subjects with dual responses experienced a significant upregulation of CD203c from 3 to 24 hours after allergen challenge, which is consistent with the allergen-induced bronchoconstriction during the early asthmatic response and LAR. These findings are also consistent with our previous reports demonstrating that circulating basophil expression of CD203c remains elevated for up to 24 hours after allergen inhalation in dual responders.⁴

To conclude, this study has demonstrated that CD203c expression on circulating basophils after allergen challenge can be used to differentiate between subjects with asthma with isolated early responses and subjects with asthma with dual responses to an inhaled allergen. Given that dual responders had significantly higher basophil activation before allergen challenge compared with isolated early responders, and that pre-allergen challenge basophil activation correlated with percent predicted FEV₁, this suggests that basophil activation tests can be used as a marker to reflect pre-allergen challenge airflow limitation and the occurrence of LAR. Furthermore, changes in basophil CD203c levels together with a positive correlation between allergen-induced bronchoconstriction and CD203c expression suggests that activated basophils contribute to the decline in lung function during LAR.

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METHODS

Inclusion criteria

Subjects were excluded if they were current or ex-smokers with more than 10 pack-years, developed lower respiratory tract infections, or used inhaled or oral steroids 4 weeks before the study. Antihistamines, caffeine, and nonsteroidal anti-inflammatory agents were prohibited 48 hours before study visits.

Methacholine challenge

The methacholine inhalation challenge was carried out via tidal breathing from a Wright nebulizer, as previously described.^{E1} Doubling concentrations of methacholine chloride (Methapharm) were inhaled orally from a Hans Rudolph valve every 2 minutes. The FEV₁ was measured after inhalation at 30 and 90 seconds or until it stopped falling. The percent fall was calculated from the postdiluent FEV₁ value. The test was terminated when a fall of at least 20% of the lowest postsaline value in FEV₁ occurred. The methacholine PC₂₀ was then calculated using linear interpolation.

Allergen and diluent challenge

Allergen challenges were conducted by administering doubling inhaled concentrations of allergen extract, as previously described,^{E2} whereas diluent challenges were conducted using 3 inhalations of 0.9% saline for 2 minutes each. The early asthmatic response was the largest percent fall in FEV₁ between 0 and 2 hours, and the LAR was the largest percent fall in FEV₁ between 3 and 7 hours postchallenge.

Flow cytometry staining and analysis

Whole-blood samples were immunostained with isotype or specific mAbs to CD45-Alexa Fluor 700 (BD Biosciences), HLA-DR-APCH7 (BD Biosciences), and IL-3R α -PE Cy7 (Ebioscience), CD203c-APC (MACS Miltenyi), CD63-V450 (BD Biosciences), and CD69-PE (BD Biosciences) for 30 minutes at 4°C. After incubation the samples were washed with fluorescence-activated cell sorting buffer at 300g for 10 minutes at 4°C, resuspended in 2 mL of fluorescence-activated cell sorting buffer, and stored at 4°C

until flow cytometry could be conducted. Cells were acquired with an LSR II flow cytometer (BD Biosciences), and analyses were performed using FlowJo software (TreeStar). Basophils were identified through a commonly used basophil gating strategy as the CD45⁺/HLA-DR⁻/IL-3R α ⁺ population (Fig E1), as previously described.^{E3} A first gate was made in the side scatter (SSC) versus full scatter dot plot around the monocyte/lymphocyte populations, which are known to contain basophils (Fig E1, A). A second gate was placed around the CD45⁺ population in the SSC versus CD45 AF700 dot plot (Fig E1, B), followed by gating around the HLA-DR⁻ population in the SSC versus HLA-DR APCH7 (Fig E1, C), and a final gate around the IL-3R α ⁺ population in the SSC versus IL-3R α PE Cy7 plot (Fig E1, D). The isotype control for the markers of interest was set to 2%, which was compared with the specific markers to detect the percentage of cells expressing the marker (Fig E1, E and F). To maintain consistency across samples, at least 300,000 CD45⁺ events were acquired, with collection of the same cell number for each time point pre-/postdiluent/allergen challenge per subject.

Statistical analysis

Statistical analysis was conducted using Graph Pad Prism. All data are presented as mean $\pm$ SEM. The flow cytometry results are expressed as mean percent positive cells or sMFI. Statistical analysis for the inhalation challenges was performed using a 2-way ANOVA and post hoc Bonferroni test. Correlation analyses were conducted using a Pearson correlation test. Statistical significance was set to $P < .05$.

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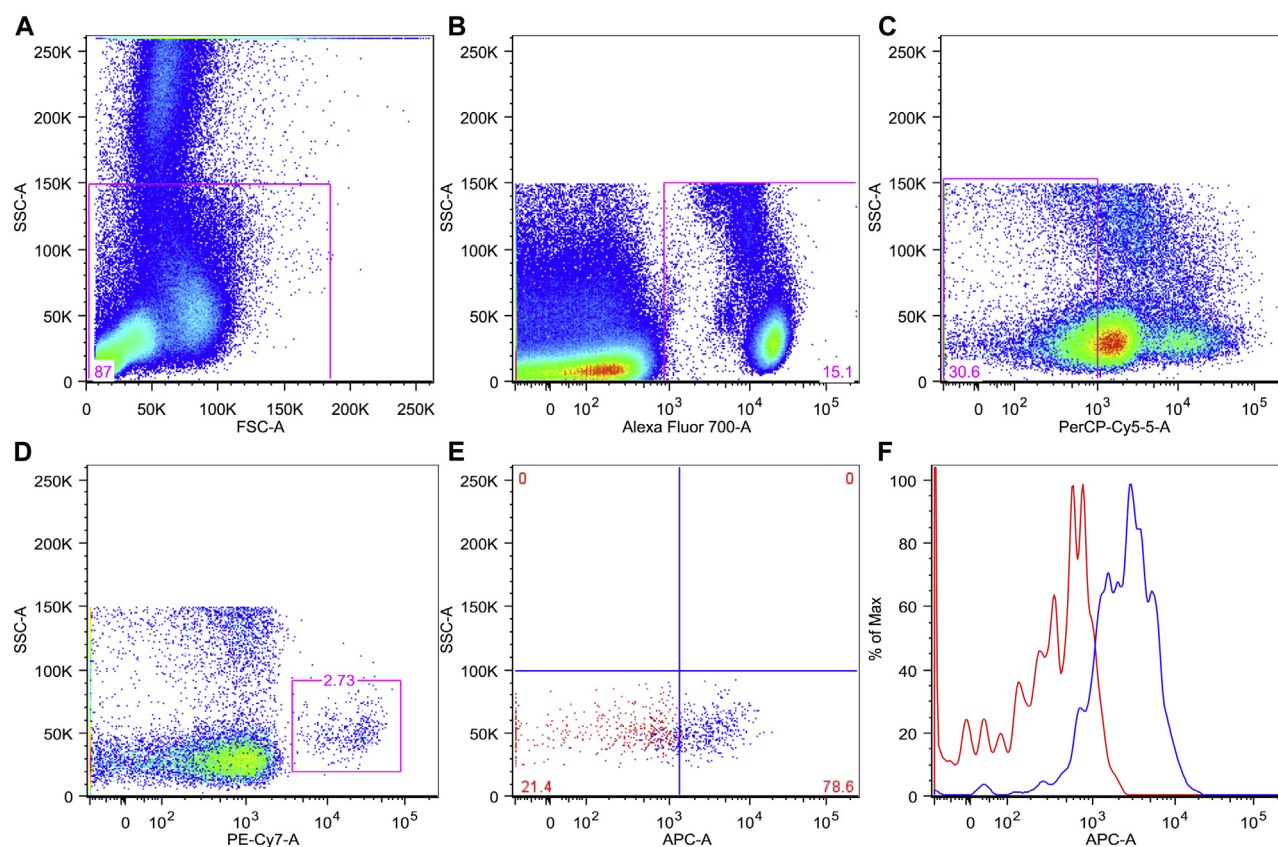


FIG E1. Basophil gating strategy. Representative whole-blood dot plots showing basophil gating strategy used for flow cytometry analysis. **A**, SSC vs FSC. **B**, SSC vs CD45 Alexa Fluor. **C**, SSC vs HLA-DR PerCP Cy5.5. **D**, SSC vs IL-3R α PE Cy7. **E**, Dot plot demonstrating isotype vs specific CD203c APC expression. **F**, Histogram demonstrating isotype vs specific CD203c APC expression on basophils. *APC*, Allophycocyanin; *FSC*, forward scatter.

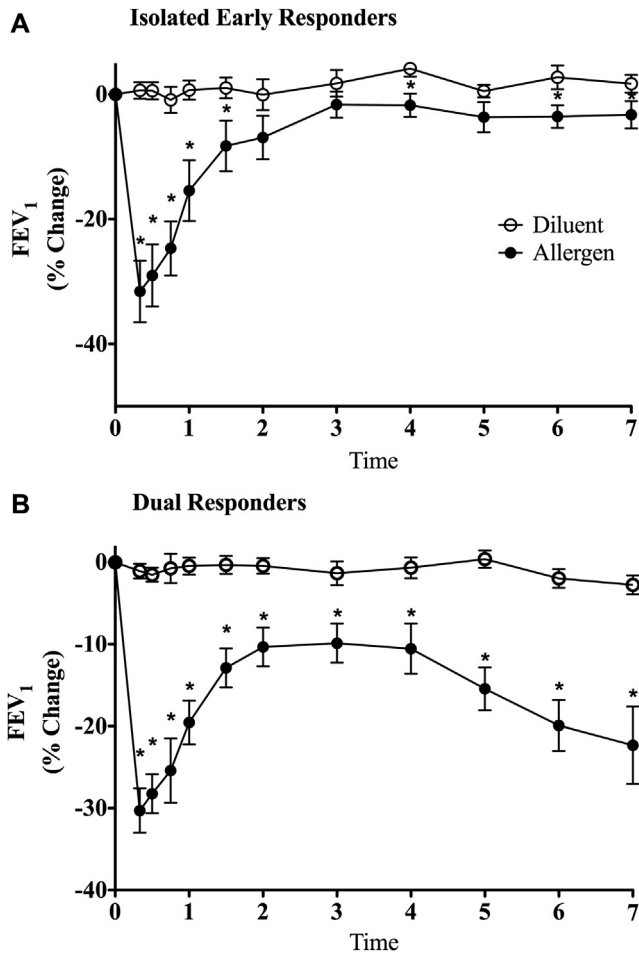


FIG E2. Allergen inhalation induces airway changes in patients with mild allergic asthma. Percent change in FEV₁ in isolated early responders (**A**, N = 6) and dual responders (**B**, N = 13). Data presented as mean $\pm$ SEM. *Significantly different from diluent control ($P < .05$).

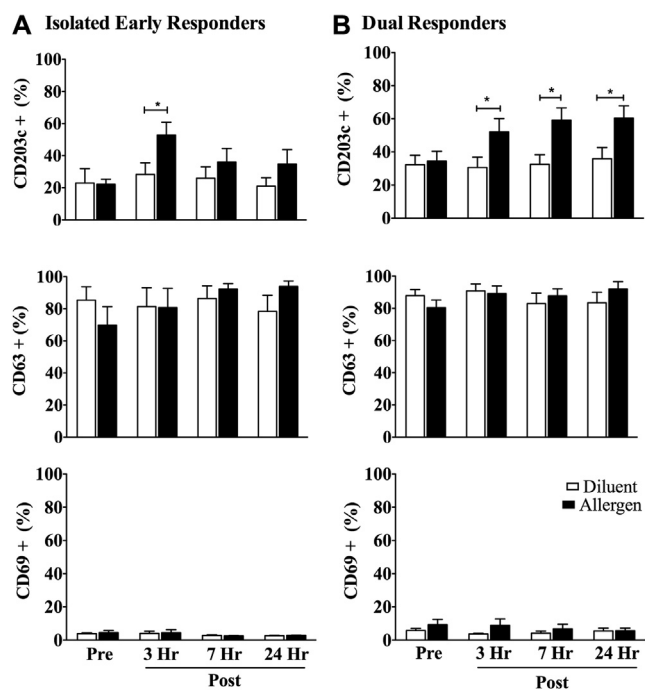


FIG E3. Effects of allergen inhalation on percentage of basophils expressing activation markers. Percentage of basophils expressing CD203c, CD63, and CD69 before allergen challenge and 3, 7, and 24 hours after allergen challenge (*black bars*), compared with diluent (*open bars*) in isolated early responders (**A**, $N = 6$) and dual responders (**B**, $N = 13$). Data are presented as mean $\pm$ SEM. *Significantly different from diluent control ($P < .05$).

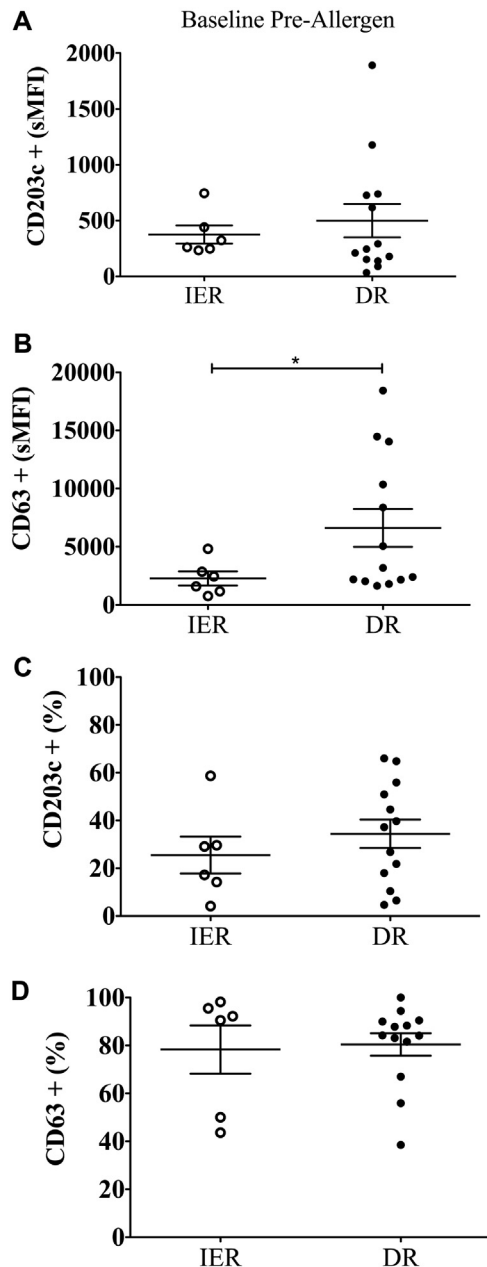


FIG E4. Preallergen basophil expression of activation markers of isolated early versus dual responders. Basophil CD203c sMFI (**A**), CD63 sMFI (**B**), CD203c % cell expression (**C**), and CD63 % cell expression (**D**) before allergen challenge of IER (N = 6) (*open circle*) compared with DR (N = 13) (*closed circle*). Data are presented as mean $\pm$ SEM. DR, Dual responder; IER, isolated early responder. *Baseline of IER and DR is statistically significant if $P < .05$.

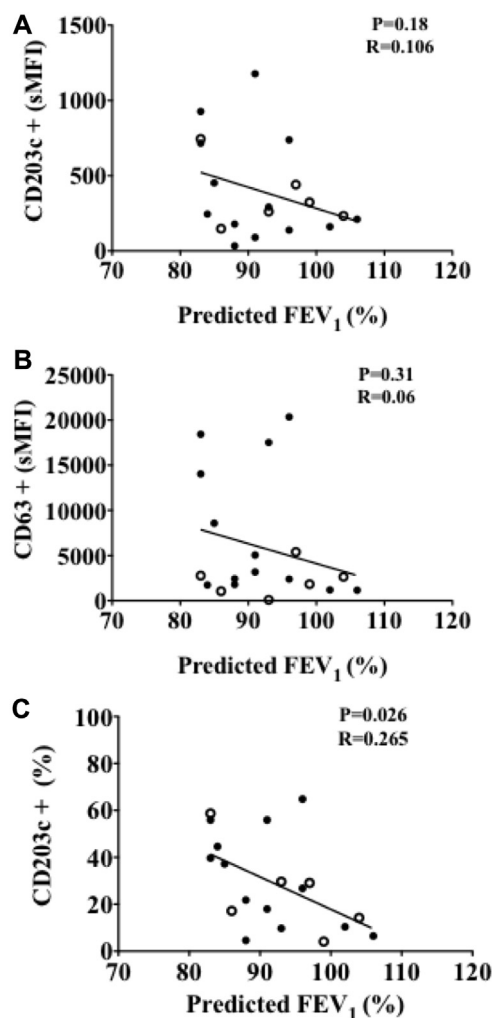


FIG E5. Relationship between CD203c and CD63 expression on circulating basophils before allergen challenge and predicted FEV₁ (%). Circulating basophil CD203c sMFI (**A**), CD63 sMFI (**B**), and CD63 % cell expression (**C**) from isolated early responders (*open circle*) (N = 6) and dual responders (*closed circle*) (N = 13) before allergen challenge compared with predicted FEV₁ (%). Data are presented as mean $\pm$ SEM.

TABLE E1. Subjects' characteristics

Sex	Age (y)	Predicted FEV ₁ (%)	Baseline methacholine PC ₂₀ (mg/mL)*	Postdiluent methacholine PC ₂₀ (mg/mL)*	Post-allergen challenge methacholine PC ₂₀ (mg/mL)*	Allergen extract inhaled	Dilution of inhaled extract	Early asthmatic response (%)	LAR (%)	Subject classification
M	24	99	2.3	2.6	6.7	Grass	1:128	22	3	IER
F	21	83	13	10	3.0	Ragweed	1:16	32	9	IER
M	36	88	5.7	9.8	12	Grass	1:512	64	19	DR
F	22	93	1.4	1.2	0.80	Trees	1:64	43	8	IER
M	25	86	0.43	0.74	0.68	Horse	1:1024	29	0	IER
F	19	97	1.2	0.51	0.34	HDM	1:8	21	2	IER
F	21	91	5.3	3.3	7.8	HDM	1:64	31	25	DR
F	47	84	0.41	0.46	0.16	Cat	1:256	22	43	DR
M	29	104	0.39	0.36	0.38	Ragweed	1:8	49	14	IER
M	63	88	5.9	6.2	11	Cat	1:128	34	16	DR
M	28	85	3.6	2.8	1.8	HDM	1:64	22	22	DR
M	23	83	0.48	1.6	0.29	HDM	1:128	35	19	DR
F	43	91	4.2	3.2	1.9	Ragweed	1:32	22	15	DR
F	18	102	2.4	8.6	9.4	HDM	1:128	26	15	DR
F	21	106	8.6	17	0.50	Ragweed	1:16	36	23	DR
M	50	96	19	6.9	5.4	Cat	1:64	37	19	DR
M	53	96	1.1	0.61	0.6	HDM	1:32	27	16	DR
F	27	93	6.2	9.7	14	HDM	1:4	41	17	DR
F	21	83	5.6	3.02	1.38	HDM	1:1024	23	56	DR
Mean ± SEM	31 ± 3	92 ± 2	2.6	2.6	1.6			32.4 ± 3.1	18 ± 3.7	

DR, Dual responder; F, female; HDM, house dust mite; IER, isolated early responder; M, male.

*Geometric mean.