

Original article

Expression of activation markers on basophils in a controlled model of anaphylaxis

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Background: Anaphylaxis has variable clinical presentations and lacks reliable biomarkers. Expression of activation markers on basophils has been useful in assessing sensitization in IgE-mediated diseases but has not been examined *in vivo* in anaphylaxis.

Objective: The study's goals were to assess the baseline expression of activation markers on basophils in individuals with a sting reaction history, the degree of change in expression of these markers after intentional sting challenge, and the relationship between *in vitro* and *in vivo* activation marker expression.

Methods: Patients allergic to insect venom were enrolled and grouped by clinical category defined by a history of a systemic or large local reaction and use of venom immunotherapy. Blood was collected before and after sting challenge. Enriched basophils were analyzed for activation marker expression. In select subjects, basophils were examined after *in vitro* stimulation with insect venom for activation marker expression and histamine release.

Results: Of 35 sting-challenge participants, 21 provided adequate samples for analysis. Pre-sting basophil CD63 expression was significantly higher in systemic reactors on immunotherapy. Following sting challenge, the rise in basophil CD69 expression and CD203c was significantly higher in systemic reactors on immunotherapy. Levels of activation markers on basophils were greater after *in vitro* venom stimulation than after *in vivo* challenge.

Conclusion: Broader shifts in expression of basophil activation markers after *in vivo* challenge occurred among subjects with a history of *in vivo* systemic anaphylaxis despite venom immunotherapy.

Clinical implications: Basophil activation markers may be potential biomarkers for anaphylaxis. (J Allergy Clin Immunol 2007;119:1016-1022.)

Key words: Anaphylaxis, basophil, activation marker

A recent consensus report on anaphylaxis highlighted the need for more sensitive biomarkers to confirm as well as predict anaphylaxis and less severe allergic reactions.¹ Anaphylaxis is an unexpected, life-threatening, IgE-mediated reaction occurring after exposure to allergen. Anaphylactic reactions can vary in the target organ affected, allergen threshold, occurrence of prolonged or biphasic anaphylaxis, and response to therapy.¹ Such clinical variability supports a need for confirmatory tests for anaphylaxis. Currently, elevation in serum tryptase is a useful biomarker in severe anaphylaxis but may be unreliable in milder forms of anaphylaxis or in food allergy.² Blood histamine levels are also elevated early in anaphylaxis but quickly return to baseline, typically within 1 hour of the start of symptoms.^{3,4}

Recently, flow cytometry-based basophil assays have gained in popularity as a means to predict sensitization in IgE-mediated diseases such as food, drug, and insect venom allergy.⁵⁻¹⁰ In particular, increased expression of certain surface molecules on basophils has been used to assess cell activation following allergen binding to specific IgE.⁸ CD63, a member of the transmembrane-4 superfamily, is rapidly mobilized on the basophil surface by IL-3, polyclonal anti-IgE, and allergen, as well as other degranulation stimuli.^{11,12} In contrast, CD69 is slowly induced by IL-3 and is thought to require *de novo* synthesis. *In vivo*, CD69 is also increased on basophils from the bronchoalveolar lavage of patients with asthma, and both CD69 and CD63 are elevated on blood basophils from chronic idiopathic urticaria as well as allergic subjects.^{12,13} Most recently, cell-surface expression of CD203c (ectonucleotide pyrophosphatase 3) has been shown to be rapidly enhanced by cross-linking anti-IgE antibody and allergen and slowly induced by IL-3.^{8,14-17} Further, its expression is unique to basophils, mast cells, and their progenitors.¹⁸ To date, shifts in the expression of these activation markers on basophils have not been examined in subjects experiencing anaphylaxis.

Intentional insect-sting challenges have been extensively used in the past both to study sting anaphylaxis and to document the efficacy of venom immunotherapy.¹⁹ In the present study, we adopted this established model of intentional sting challenge to examine the degree of activation marker expression on basophils as a consequence

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Abbreviations used

FITC: Fluorescein isothiocyanate
 LL: Large local
 LTC₄: Leukotriene C₄
 MFI: Mean fluorescence intensity
 S: Systemic

of experimental *in vivo* insect-sting challenges. Additionally, we were interested in the baseline expression of activation markers in basophils in individuals with varied histories of sting reactions and the relationship between *in vitro* and *in vivo* basophil activation.

METHODS

Subjects

Thirty-five adult volunteers (age ≥ 18 years) with a history of systemic (S) or large local (LL) reactions to insect venom stings were recruited from an ongoing study of sting challenge in treated and untreated patients. For this protocol, subjects with LL reactions were defined by: (1) a history of severe local swelling contiguous to the insect sting site; (2) positive venom skin tests; and (3) an LL reaction of at least 16 cm in diameter within 48 hours after initial sting challenge. Subjects with S reactions were defined by a history of systemic symptoms such as urticaria, respiratory distress, and hypotension. Subjects with a history of loss of consciousness or a near-fatal systemic reaction—defined as prolonged unconsciousness, extreme hypotension, severe respiratory distress, or intubation—were excluded. Of the 35 subjects, 26 were on specific venom immunotherapy at the time of specific insect sting challenge, either at standard (monthly) or extended-dosing intervals (12–20 weeks). All subjects were required to have a positive skin test to insect venom for inclusion in the current study. Select subjects were invited to participate in the *in vitro* arm of the study. All subjects gave informed consent, as approved by the Johns Hopkins Institutional Review Board, before undergoing a sting challenge. Subjects separately consented to participate in the present study approved by the Western Institutional Review Board.

In vivo sting challenge

The sting challenges were performed at the Johns Hopkins Bayview General Clinical Research Center between the months of July and October in 2004, 2005, and 2006, as previously described.²⁰ Participants underwent a specific insect-sting challenge based on earlier positive venom skin testing and clinical sting history. Vital signs and intravenous access were obtained in all subjects before sting challenge. Live insects were captured and provided by an entomologist who performed the sting challenges using a specialized sting chamber.²⁰ Patients were monitored for sting reactions, including a change in vital signs, following the sting challenge. Blood was sampled immediately before and 45 minutes after the sting challenge. The time of sampling for marker expression was based on the reported kinetics of the activation markers studied as well as on pilot studies.^{12,17,21} After sting challenge, all patients were monitored for at least 1 hour and discharged as long as vital signs were stable; subjects experiencing a systemic reaction after sting challenge were kept a minimum of 1 hour from the start of the reaction and monitored until vital signs were stable.

Basophil isolation

For both the *in vivo* and the *in vitro* studies, venous blood samples were drawn into syringes containing 10 mmol/L EDTA to prevent coagulation. Enriched basophils were obtained by density-gradient

sedimentation, using Accuspin separation tubes (Sigma-Aldrich, St Louis, Mo) and a discontinuous double Percoll gradient (GE Healthcare Biosciences, Piscataway, NJ, as previously described.¹³ Basophils (basophil purity $8.35 \pm 0.95\%$) were enumerated by Alcian blue staining.²²

Dual-color flow cytometry

Enriched basophil suspensions were labeled for direct and indirect immunofluorescence for flow cytometry. Antibodies used include an fluorescein isothiocyanate (FITC)-conjugated polyclonal goat anti-human IgE and its control, FITC-conjugated normal goat antihuman IgG, from Kirkegaard and Perry (Gaithersburg, Md), an irrelevant mouse IgG1 control (clone 679.1Mc7), mouse anti-CD63 (IgG1, clone CLB-gran12), mouse anti-CD69 (IgG2b, clone TP1.55.3), phycoerythrin (PE)-conjugated mouse anti-CD203c (IgG1, clone 97A6), and PE-conjugated IgG1 control (clone 679.1Mc7), all purchased from Immunotech (Marseille, France). On select subjects, platelet contamination of enriched basophil preparations was monitored by using mouse anti-CD42b (IgG1, clone SZ2) from Immunotech. Cells were incubated with monoclonal antibodies specific to each surface marker for 30 minutes at 4°C in the presence of FITC-conjugated antihuman IgE. Cells were washed and, if necessary, labeled with PE-conjugated antimouse (Biosource, Camarillo, Calif) for an additional 30 minutes. Samples were analyzed on a Becton Dickinson (Franklin Lakes, NJ) FACS Caliber flow cytometer employing Cell Quest software (BD Biosciences, Franklin Lakes, NJ). Cells were initially gated based on FITC-IgE-positive cells to select the basophil population and then analyzed for specific activation markers (Fig 1).¹³ Data are expressed as net mean fluorescence intensity (MFI) (actual MFI – MFI of irrelevant IgG control).

Histamine, leukotriene C₄, and IL-13 measurement

Basophil-enriched cell suspensions were isolated from pre-sting blood samples and cultured at 37°C in a 96-well flat-bottom microtiter plate. After 4 hours of incubation, cell-free supernatants were harvested, acid-precipitated overnight at 4°C, and analyzed for histamine release via automated fluorometry. Leukotriene C₄ (LTC₄) was assayed by an in-house radioimmune assay.²³ Interleukin-13 in the culture supernatants of cells incubated overnight (18–20 hours) was measured by enzyme-linked immunosorbent assay (Immunotech).²⁴ Both spontaneous and insect venom-induced (0.01–10 $\mu\text{g/mL}$) mediator release were measured.

In vitro venom studies

After basophil isolation, cells were either immediately stained for expression of activation markers or incubated with yellow jacket or honeybee venom (0.01–1 $\mu\text{g/mL}$) (ALK-Abelló, Hørsholm, Denmark) (depending on known donor sensitivity) in a 37°C water bath for 30 minutes. After *in vitro* venom challenge, basophils were assessed for activation marker expression. In parallel, basophils were stimulated for histamine release to purified yellow jacket or honeybee venom (0.01–1 $\mu\text{g/mL}$) or polyclonal goat antihuman IgE (0.1 $\mu\text{g/mL}$) in a 37°C water bath for 45 minutes.¹³ Histamine was quantified from supernatants using an automated fluorometric assay. Results are presented as the percentage of total histamine content minus spontaneous release.¹³

Statistics

Statistics were performed using the Student *t* test, and a *P* value of $\leq .05$ was considered to be significant. The Spearman rank test was used to assess correlation of histamine, LTC₄, and IL-13 to basophil activation markers. Results are presented as mean $\pm$ standard error of the mean given.

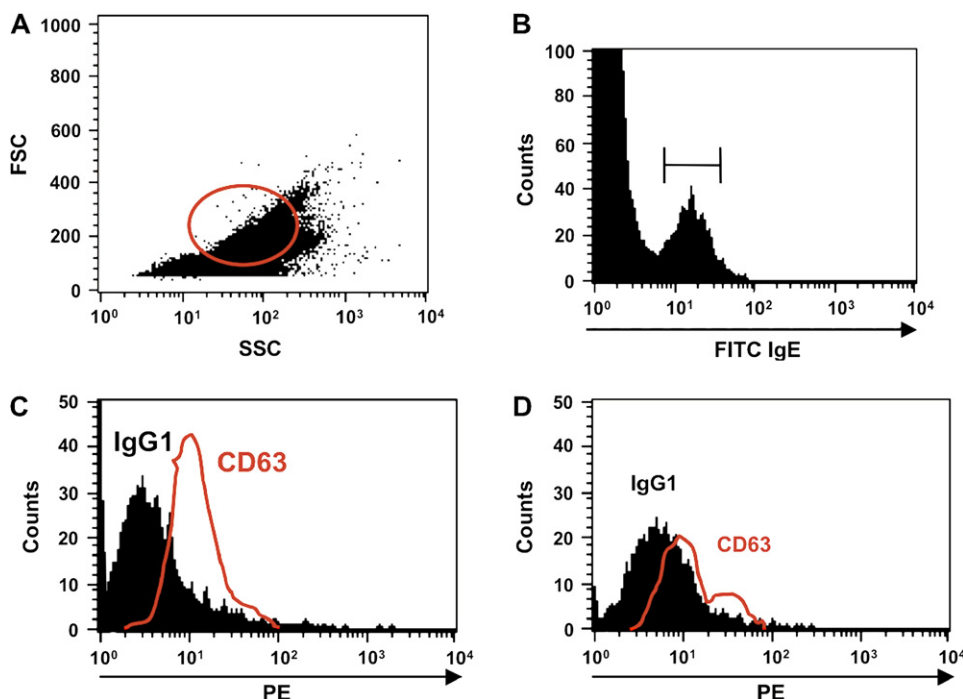


FIG 1. Flow cytometry analysis and bimodal CD63 expression. **A** and **B**, Basophils were selected on the basis of scatter and presence of surface IgE. **C**, The majority of subjects displayed unimodal CD63 expression on their basophils. Subject 11 is shown. **D**, A few subjects demonstrated bimodal CD63 expression. Subject 10 is shown.

RESULTS

In vivo sting challenge

Clinical reaction. Of 35 enrolled participants, 21 provided adequate pre- and post-sting samples for basophil analysis (LL/immunotherapy, $n = 12$; S/immunotherapy, $n = 5$; no immunotherapy, $n = 4$). The most common reason for exclusion of a sample was insufficient basophil recovery. For a given subject, basophil number and percentage purity were similar in pre- and post-sting samples. The clinical characteristics and reactions following experimental sting challenge for the included subjects are shown in Table I and are similar to the excluded subjects (Table II). Of note, only 1 of the 35 subjects had a systemic reaction following sting challenge, consisting of hypotension, with no sign of a major clinical reaction in the other 34 subjects.

Basophil activation markers. Expression of activation markers was examined on basophils isolated at baseline and after sting challenge. Subjects were grouped by clinical category and use of immunotherapy. Baseline basophil CD63 expression was highest in the S/immunotherapy group (mean 33.1 ± 11.8 ; $n = 5$) compared with the LL/immunotherapy (mean 8.1 ± 3.1 ; $n = 12$; $P < .02$) and no immunotherapy (mean 20.2 ± 17.5 ; $n = 3$) groups (Fig 2, A). Baseline basophil CD69 expression was also greatest in the S/immunotherapy group (mean 22.7 ± 13 , $n = 5$) followed by no immunotherapy (mean 17.3 ± 10.1 ; $n = 4$) and LL/immunotherapy (mean 9.2 ± 7.4 ;

$n = 12$; Fig 2, B). Baseline basophil CD203c expression was not significantly different among groups but higher in the no immunotherapy group (mean 2.2 ± 0.9 ; $n = 4$) than in the LL/immunotherapy (mean 1.6 ± 0.4 ; $n = 12$) and S/immunotherapy (mean 0.7 ± 0.6 ; $n = 5$) groups (Fig 2, C).

We next examined the net shift in basophil surface activation marker expression following sting challenge in each clinical category (Fig 3). Despite a significant difference at baseline, the rise in CD63 was similar across groups and independent of platelet contamination. The rise in CD69 following sting challenge was highest in the S/immunotherapy group (mean 23.2 ± 9.5 ; $n = 5$) followed by no immunotherapy (mean 13.5 ± 8.2 ; $n = 4$) and LL/immunotherapy (mean 2.8 ± 3.6 ; $n = 12$; $P < .03$). Despite the lowest basal expression, the rise in CD203c was significantly higher in the S/immunotherapy group (mean 2.9 ± 1.6 ; $n = 5$) versus LL/immunotherapy (mean -0.5 ± 0.4 ; $n = 12$; $P < .01$) and higher than no immunotherapy (mean -0.3 ± 0.3 ; $n = 4$). Basophil mediator release, including IL-13, LTC₄, and histamine, was examined in conjunction with surface marker expression data in baseline samples, and we found no significant correlations (data not shown).

Of the 14 subjects who participated in sting challenges in 2004, 4 subjects had repeat basophil activation marker measurements following a second sting challenge in 2005. A comparison of the results from both years of *in vivo* sting challenges suggests that the pattern of basophil

TABLE I. Subject characteristics and reaction to experimental sting challenge

Subject no.	Clinical category	Time on venom immunotherapy	Insect	Atopic history	Type of reaction
1	LL	2 y	YJ	Yes	LL
2	LL	2 y	HB	No	LL
3	LL	1 y	YJ	Yes	LL
4	LL	2 y	HB	No	LL
5	LL	2 y	HB	Yes	LL
6	LL	2 y	YJ	Yes	LL
7	LL	2 y	YJ	No	LL
8	LL	2 y	YJ	No	LL
9	LL	1 y	HB	No	LL
10	LL	3 y	YJ	No	LL
11	LL	1 y	YJ	No	LL
12	LL	1 y	YJ	Yes	LL
13	S	16 y	YJ	No	None
14	S	11 y	YJ	Yes	None
15	S	10 y	YJ	Yes	None
16	S	12 y	YJ	No	None
17	S	15 y	YJ	No	None
18	S	No immunotherapy	Pol	Yes	None
19	S	No immunotherapy	YJ	Yes	None
20	LL	No immunotherapy	YJ	Yes	LL
21	LL	No immunotherapy	YJ	Yes	LL

YJ, Yellow jacket; HB, honeybee; Pol, *Polistes fuscatus*.

activation marker expression in subjects is reproducible (data not shown).

Basophil activation markers: *In vitro* versus *in vivo* challenge

Given the common use of activation marker expression on basophils as a potential diagnostic assay, we compared the relationship between *in vivo* and *in vitro* allergen-induced expression of these markers on basophils. We studied basophils from a total of 7 subjects *in vitro* who also participated in the *in vivo* sting challenge portion of the study. The time between the *in vivo* sting challenge and *in vitro* studies was at least 3 months. Table III summarizes the results of the *in vitro* challenge in relation to the allergen dose, histamine release, and activation marker expression. Basophil histamine release to specific venom occurred in a concentration-dependent manner, most often reaching maximal release at a venom concentration of 1 μ g/mL, which was as previously reported.^{25,26} In addition, we noted increased expression of activation markers at allergen doses suboptimal for histamine release in all subjects. When comparing *in vivo* activation marker expression in basophils to *in vitro* expression, in general, we observed: (1) a similar range of CD63 expression; (2) higher CD69 expression with *in vivo* allergen challenge; (3) higher CD203c expression *in vitro*; and (4) a wider range of activation marker expression overall *in vitro* (Table III). Also, we observed bimodal expression of CD63 in select subjects (Fig 1, D). This did not correlate with clinical category and was present in a minority of samples challenged *in vivo* as well as *in vitro*. Overall,

TABLE II. Excluded subject characteristics and sting reactions

Subject no.	Clinical category	Time on venom immunotherapy	Insect	Atopic history	Type of reaction
1	LL	2 y	YJ	No	LL
2	LL	2 y	YJ	Yes	LL
3	LL	3 y	HB	No	LL
4	LL	1 y	HB	Yes	LL
5	LL	No immunotherapy	HB	Yes	LL
6	LL	No immunotherapy	YJ	Yes	LL
7	S	15 y	YJ	Yes	None
8	S	17 y	YJ	Yes	None
9	S	12 y	YJ	Yes	None
10	S	29 y	YJ	No	None
11	S	12 y (off last 8 y)	YJ	Yes	None
12	S	No immunotherapy	WH	Yes	Hypotension
13	S	No immunotherapy	YJ	No	None
14	S	No immunotherapy	YJ	No	None

YJ, Yellow jacket; HB, honeybee; WH, white hornet.

we observed that *in vivo* basophil surface marker shift was modest compared with *in vitro* expression (Table III).

DISCUSSION

In this study, we examined the expression of activation markers on circulating basophils in the setting of an established human model of anaphylaxis. Specifically, individuals with a history of insect venom allergy had basophils examined before and after intentional sting challenge. At baseline, we found that the basophils of subjects with a history of a systemic reaction had the highest CD63 expression. Surprisingly, the net rise in basophil CD63 expression after sting challenge was remarkably comparable in all clinical categories, independent of reaction history or immunotherapy use. However, the shifts in CD69 and CD203c expression were greatest in subjects with a history of a systemic reaction. The pattern of activation marker expression after sting challenge was reproducible in a small number of subjects stung in 2 consecutive years. The advantages of the insect model of anaphylaxis are the controlled environment for sting challenge and the similarity of the allergen exposure, both in dose and in route of administration, to an unintentional insect sting.

Basophils of allergic subjects have been shown to exhibit a primed phenotype, as defined by elevations in spontaneous mediator release, and perhaps priming explains why the systemic group exhibited the highest CD63 expression at baseline.^{24,27,28} For example, we previously demonstrated that repeated intranasal allergen challenge in subjects with allergic rhinitis led to increased spontaneous IL-13 secretion for a period of days after the last challenge.²⁴ In the present study, expression of CD63 was most elevated at baseline in the systemic group, perhaps indicating basophil priming. In contrast, we did not find

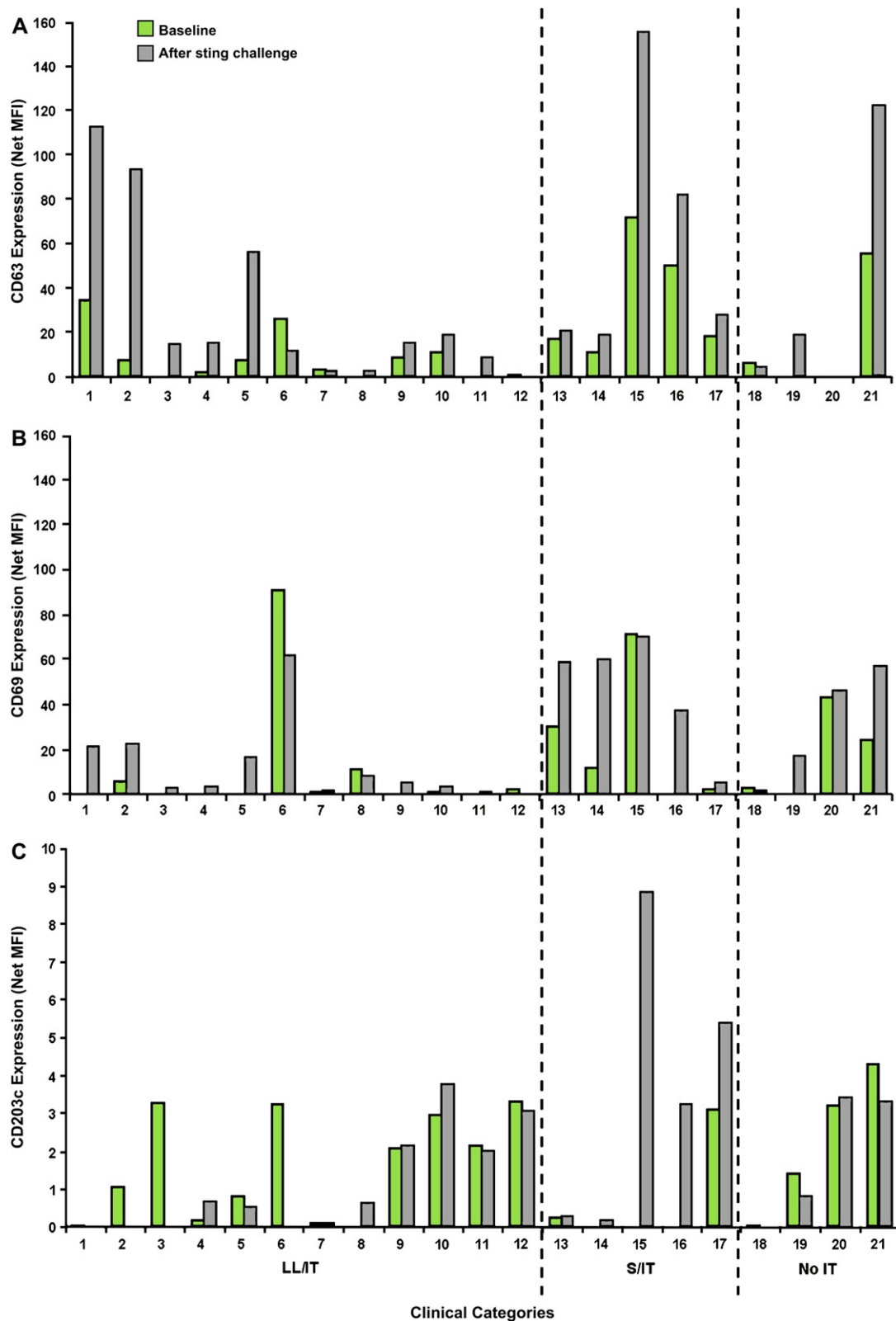


FIG 2. Baseline basophil activation marker expression. Basophil activation markers were measured at baseline and after sting challenge. **A**, Baseline CD63 expression was significantly higher in S/immunotherapy (mean 33.1 ± 11.8 ; $n = 5$; $P < .02$). Subject 20 was omitted owing to an inadequate baseline CD63 measurement. **B** and **C**, Baseline CD69 and CD203c expression were not significantly different among groups. Of note, CD203c expression was 10-fold lower compared with other markers.

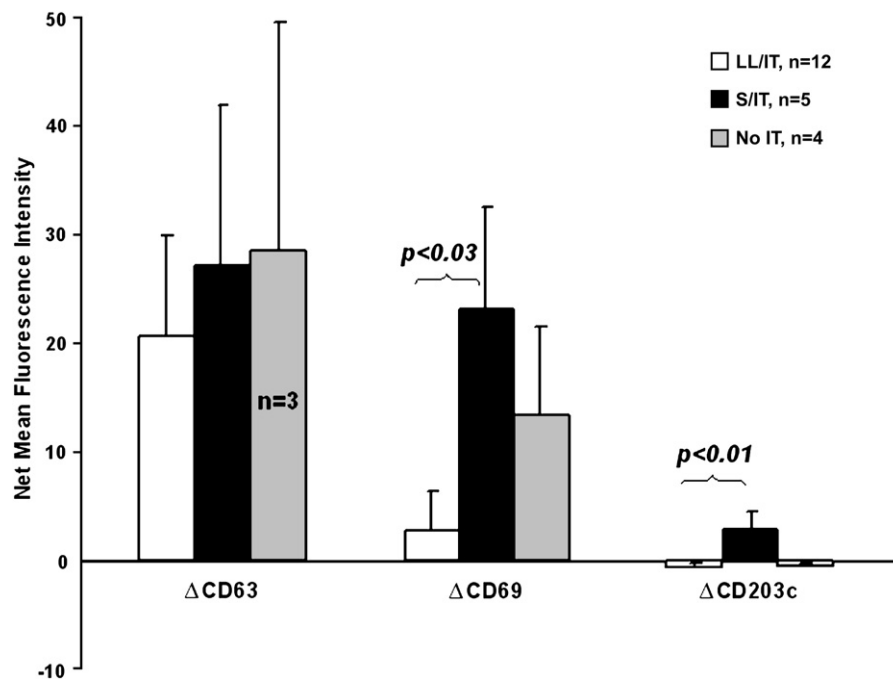


FIG 3. Shift in expression of activation markers on basophils after sting challenge. The shift in basophil activation marker expression was measured following *in vivo* sting challenge. CD63 expression was similar across groups. CD69 was higher in the S/immunotherapy group ($P < .03$). CD203c was significantly higher in the S/immunotherapy group ($P < .01$).

TABLE III. *In vitro* versus *in vivo* venom challenge

Subject no.	Specific venom (μg/mL)	Net histamine release (%)	<i>In vitro</i> challenge			<i>In vivo</i> challenge		
			ΔCD63	ΔCD69	ΔCD203c	ΔCD63	ΔCD69	ΔCD203c
1	1	18	—	—	—	78.79	21.17	0
	0.1	0	—	—	—			
	0.01	0	95.84	0	4.91			
	0.001	—	227.44	0	11.84			
3	1	0	−5.85	0	5.69	14.04	2.56	−3.24
	0.1	5	20.07	2.01	2.82			
	0.01	0	18.54	0	5.27			
5	1	100	—	—	—	48.3	16.2	0
	0.1	80	850.31	−0.07	37.5			
	0.01	63	834.15	2.87	39.21			
9	1	47	—	—	—	6.28	4.69	0.06
	0.1	16	74.39	1.75	22.01			
	0.01	11	58.19	1.04	22.6			
13	1	31	—	—	—	3.38	28.54	0.05
	0.1	2	6.35	−1.49	9.88			
	0.01	1	—	—	—			
15	1	6	—	—	—	83.4	−1	8.8
	0.1	7	31.15	273.16	11.04			
	0.01	9	85.69	−11.34	9.33			
16	1	49	—	—	—	31.6	36.9	3.2
	0.1	9	4.43	0.97	10.78			
	0.01	5	—	—	—			

a correlation between basophil secretion of IL-13, LTC₄, or histamine and activation marker expression on basophils. An earlier study of subjects with allergic rhinitis undergoing immunotherapy demonstrated decreased

histamine release as a consequence of immunotherapy.²⁷ Because the majority of subjects in the present study had a history of prolonged immunotherapy, a reduction in venom-induced mediator production is not surprising.

Among the disadvantages of the venom model used in the present study are the high frequency of subjects on active immunotherapy and the exclusion of high-risk subjects owing to safety concerns.

Given the pattern and range of activation markers observed *in vivo*, we explored the relationship between *in vivo* versus *in vitro* challenge. As expected, we observed that basophil histamine release occurred in a concentration-dependent manner after *in vitro* challenge with insect venom. Hypothetically, a honeybee delivers an estimated 50 μg of venom protein *in vivo* and a yellow jacket delivers between 2–3 μg in an average adult, achieving an estimated *in vivo* blood venom concentration of approximately 0.01 $\mu\text{g}/\text{mL}$ per honeybee and 0.005 $\mu\text{g}/\text{mL}$ per yellow jacket sting.²⁹ These *in vivo* estimates are much lower than concentrations (0.01–1 $\mu\text{g}/\text{mL}$) often used for basophil *in vitro* studies. To our knowledge, no direct measures of *in vivo* concentrations of insect venom have been reported.

Although the pattern of basophil activation marker expression was comparable across individuals *in vivo* and *in vitro*, the magnitude in activation marker elevation was greater *in vitro* (Table III). This holds implications for the use of the basophil activation test and the range of concentrations of allergen selected.³⁰ Typically, the allergen dose has been selected based on the concentration needed to induce histamine and LT release assays. Both of these have been shown to have a dose-dependent relationship to CD63 and CD203c expression to allergen.^{5,30,31} However, we have clearly seen that activation marker expression may occur at allergen concentrations suboptimal for inducing histamine release and at concentrations that are possibly more relevant to those achieved after *in vivo* stings. The variability in concentration response when studying basophil activation markers may miss an individual at serious risk for anaphylaxis if a basophil activation test is performed with only 1 concentration of allergen. Perhaps it would be better to perform the test with multiple allergen concentrations or develop standard dose-response curves for each allergen used.^{32,33} The *in vitro* results in the present study also reiterate the broad variability in basophil mediator response between different donors, as has been observed for IgE receptor activation.³⁴

Owing to the enrollment criteria for intentional sting challenge which excluded subjects with severe systemic reactions but included those on extended venom immunotherapy and those with large local reactions, we did not expect to see a high frequency of significant clinical reactions in this study. In fact, only 1 out of 35 subjects who underwent sting challenge experienced a systemic reaction. Nonetheless, basophil activation marker shifts were seen and were greatest in subjects with a history of systemic reactions despite numerous years of venom immunotherapy. Similarly, in a study of 25 patients with a history of a systemic reaction following a sting and 6 months on venom immunotherapy, subjects underwent a sting challenge, and none of the subjects had a systemic reaction although 23 out of 25 had a significant rise in basophil CD63 expression following *in vitro* pre-sting

stimulation.³¹ That suggests that allergen sensing by basophils, as demonstrated by CD63 expression, persists despite the use of immunotherapy.

We have also observed that subjects with a history of a systemic reaction to venom demonstrate broader activation marker expression on basophils after *in vivo* sting challenge. The magnitude of activation marker expression in basophils indicates that this may be a useful biomarker to assist in the diagnosis of certain forms of anaphylaxis. It is also possible that the magnitude of shift in expression would be higher in subjects not on active immunotherapy. Additional studies are clearly needed to establish the risk of anaphylaxis with intentional allergen challenge as predicted by patterns of *in vitro* basophil marker expression. In the future, patterns of *in vitro* basophil activation markers may also be useful in following the natural history of food allergy where no immunotherapy is available to patients and food challenges can result in severe anaphylaxis. In a study of subjects presenting to an emergency department with clinically confirmed anaphylaxis, only 42% had elevations in blood histamine and 21% elevated serum tryptase levels.³⁵ In the urgent care or emergency room setting, assessment of basophil activation markers could also serve as an important adjunct confirmatory test of anaphylaxis for physicians.

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