

Assessing basophil activation by using flow cytometry and mass cytometry in blood stored 24 hours before analysis

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Background: Basophil activation tests (BATs) have promise for research and for clinical monitoring of patients with allergies. However, BAT protocols vary in blood anticoagulant used and temperature and time of storage before testing, complicating comparisons of results from various studies.

Objective: We attempted to establish a BAT protocol that would permit analysis of blood within 24 hours of obtaining the sample.

Methods: Blood from 46 healthy donors and 120 patients with peanut allergy was collected into EDTA or heparin tubes, and samples were stored at 4°C or room temperature for 4 or 24 hours before performing BATs.

Results: Stimulation with anti-IgE or IL-3 resulted in strong upregulation of basophil CD203c in samples collected in EDTA or heparin, stored at 4°C, and analyzed 24 hours after sample collection. However, a CD63^{hi} population of basophils was not observed in any conditions in EDTA-treated samples unless exogenous calcium/magnesium was added at the time of anti-IgE stimulation. By contrast, blood samples collected in heparin tubes were adequate for quantification of upregulation of basophil CD203c and identification of a population of CD63^{hi} basophils, irrespective of whether the specimens were analyzed by means of conventional flow cytometry or cytometry by time-of-flight mass spectrometry, and such tests could be performed after blood was stored for 24 hours at 4°C.

Conclusion: BATs to measure upregulation of basophil CD203c and induction of a CD63^{hi} basophil population can be conducted with blood obtained in heparin tubes and stored at 4°C for 24 hours. (J Allergy Clin Immunol 2016;■■■:■■■-■■■.)

Key words: Basophils, CD63, CD203c, anti-coagulants, heparin, EDTA, peanut allergy, cytometry by time-of-flight mass spectrometry, platelets

Basophils and mast cells are major effector cells of IgE-dependent immune and allergic responses.¹⁻³ These cells express large numbers of the high-affinity IgE receptor FcεRI on their surfaces, and crosslinking of their FcεRI-bound IgE by bivalent or multivalent allergens induces the secretion of multiple stored and newly synthesized mediators, cytokines, and chemokines.⁴⁻⁷ Although basophils typically represent less than 1% of peripheral blood leukocytes, analysis of basophil function has become increasingly popular, both because basophils can have certain unique roles in immunity and allergic diseases⁸⁻¹⁰ and because blood basophils are much more readily available for analysis than tissue-resident mast cells.

Studies of basophil activation *ex vivo* (ie, basophil activation tests [BATs]) are flow cytometry-based assays to assess basophil activation by various stimuli. The BAT was first developed as a diagnostic test in 1991, and the use of such tests has subsequently increased.¹¹⁻¹³ However, several different BATs are now used, including commercial kits and tests developed and used by research groups. Various BATs differ in the choice of anticoagulant, temperature and duration of blood storage, activation markers measured, consideration of the effects of possible platelet attachment to basophils, reproducibility, whether basophils are studied in whole blood or after various purification steps, and stimulants used to activate the cells.^{12,14-21}

However, to compare the most meaningful results of BATs obtained with different patient populations analyzed at various sites, it would be optimal to use standardized methods at all sites. Moreover, such protocols could be used ideally at reference laboratory sites after overnight shipment of samples from widespread clinical sites. We developed a simple protocol that permitted us to perform BATs on whole blood stored for up to 24 hours before analysis and showed that this protocol could be used to analyze basophils both by means of conventional flow cytometry and by means of the newly introduced method of cytometry by time-of-flight mass spectrometry (CyTOF).²²

METHODS

Outline of experiments

We used blood specimens from both healthy donors and patients with peanut allergy to compare BAT results obtained in blood anticoagulated with EDTA versus heparin and stored before BAT analysis at room (or, for shipped specimens, ambient) temperature or at 4°C for 4 or 24 hours. See Table E1 in this article's Online Repository at www.jacionline.org for a summary of the

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Abbreviations used

BAT:	Basophil activation test
CMF-PBS:	Calcium/magnesium-free PBS
CyTOF:	Cytometry by time-of-flight mass spectrometry
DAPI:	4',6-Diamidino-2-phenylindole
FITC:	Fluorescein isothiocyanate
MFI:	Mean fluorescence intensity
OIT:	Oral immunotherapy
PE:	Phycoerythrin
PerCP:	Peridinin chlorophyll protein
RT:	room temperature

design of the experiments depicted in the various regular and supplemental figures.

Blood specimens

Blood from randomly selected anonymous donors (allergy status unknown) was obtained from the Stanford Blood Center (Palo Alto, Calif), and blood from patients with peanut allergy (see [Tables E2](#) and [E3](#) in this article's Online Repository at www.jacionline.org) was obtained as part of enrollment into an institutional review board–approved clinical trial (ClinicalTrials.gov Identifier: NCT02103270). Peanut allergy was defined as having a reaction to a double-blind, placebo-controlled food challenge to peanut (up to 500 mg of peanut protein) and a positive skin prick test response to peanut (≥ 5 mm).

BATs

Blood specimens were gently rotated at room temperature (RT) or 4°C for 4 or 24 hours after blood collection. Immediately before starting BAT assays, samples were put into a water bath at 37°C for 30 seconds. One hundred microliters of whole blood was mixed with 100 μ L of medium only or each stimulant. More details about the BAT protocols and the reagents used can be found in the [Methods](#) section in this article's Online Repository at www.jacionline.org.

CyTOF

Metal-labeled antibodies used for CyTOF analysis are shown in [Table E4](#) in this article's Online Repository at www.jacionline.org. Other details can be found in the [Methods](#) section in this article's Online Repository. In addition, details on basophil quantification with fluorescence beads, basophil and platelet analysis by means of confocal microscopy, and preparation of peanut extract can be found in the [Methods](#) section in this article's Online Repository.²³

Statistical analysis

Mann-Whitney *U* tests were performed (the groups analyzed are described in the figure legends), and the results are reported in figure legends. We considered a *P* value of less than .05 to be statistically significant.

RESULTS**BATs can be performed 24 hours after collection of heparin-anticoagulated blood stored at 4°C**

We sought to identify conditions of blood collection and storage that would permit conducting BATs with specimens stored as long as 24 hours before analysis. This interval would permit shipping specimens obtained at one location to another for analysis. We performed anti-IgE or IL-3 stimulation of basophils in whole blood and used changes in CD203c²⁴⁻²⁶ and CD63^{11,27,28} values as basophil activation markers. Basophils were gated as

CD123⁺ and HLA-DR⁺ cells,²⁹ and expression of CD203c and CD63 in gated basophils was shown in histograms ([Fig 1](#)).

Basophils exhibited upregulation of both CD203c and CD63 on anti-IgE or IL-3 stimulation in samples from normal blood donors that were collected in either EDTA or heparin, although the CD63 upregulation in EDTA was minimal ([Fig 1](#)). CD203c was uniformly upregulated in both EDTA and heparin specimens. In heparin, but not EDTA, specimens anti-IgE stimulation induced a strongly bimodal upregulation of CD63, yielding a basophil population with high levels of fluorescence intensity (in [Fig 1](#), the “CD63^{hi}” basophil population represented 0.02% in EDTA and 22% in heparin specimens, respectively). In subsequent experiments we compared the intensity of responses under different protocols of testing by using mean fluorescence intensity (MFI) to quantify CD203c and the percentage of CD63^{hi} basophils to quantify CD63.

We first compared results obtained 4 or 24 hours after blood storage at 4°C or RT. When blood samples in EDTA were stimulated with anti-IgE or IL-3, the most significant and largest differences in basophil CD203c expression (Δ CD203c) were in specimens stored for 24 hours at 4°C (see [Fig E1, A](#), in this article's Online Repository at www.jacionline.org). In heparin specimens, Δ CD203c was similar under all 4 conditions after anti-IgE stimulation, but as with EDTA specimens, the most significant and substantial Δ CD203c after IL-3 stimulation was in specimens stored for 24 hours at 4°C (see [Fig E1, A](#)). CD63^{hi} basophils were observed only in specimens collected with heparin and stimulated with anti-IgE, but the results obtained in the 4 conditions of storage were very similar ([Fig 1](#) and see [Fig E1, B](#)). Absolute MFI values for CD203c without stimulation (RPMI media) were low under all conditions, with values in EDTA specimens being higher in samples stored for 24 hours at either temperature, whereas the opposite was the case for specimens collected in heparin (see [Fig E1, C](#)). No obvious CD63^{hi} populations were observed without anti-IgE or IL-3 stimulation in any of the conditions (see [Fig E1, D](#)). However, in tests of heparin specimens, 3 of 11 donors barely responded to anti-IgE stimulation (both Δ CD203c and percentage of CD63^{hi} basophils were nearly zero), but the cells responded to IL-3 stimulation. Therefore we considered these 3 subjects to be nonreleasers.³⁰⁻³⁴

Importance of exogenous or added calcium/magnesium for BAT measurements

In tests of blood collected in either EDTA or heparin tubes from the same healthy donors, no significant difference was observed in Δ CD203c between EDTA and heparin specimens (*P* = .0627) on anti-IgE stimulation, but anti-IgE significantly induced CD63^{hi} basophils only in the heparin specimens (*P* < .0001; [Fig 2, A](#)). Absolute levels of CD203c MFI were higher in basophils in heparin than in EDTA specimens ([Fig 2, B](#)) both without stimulation and after anti-IgE stimulation (data not shown), which resulted in similar values for Δ CD203c in the EDTA and heparin specimens.

The addition of calcium/magnesium to blood collected in EDTA tubes just before anti-IgE stimulation resulted in induction of a CD63^{hi} population in response to anti-IgE that was similar to that induced in heparin specimens ([Fig 3, A and B](#); EDTA vs heparin; *P* = .0079 without calcium/magnesium, *P* > .05 with calcium/magnesium). By contrast, addition of calcium/magnesium

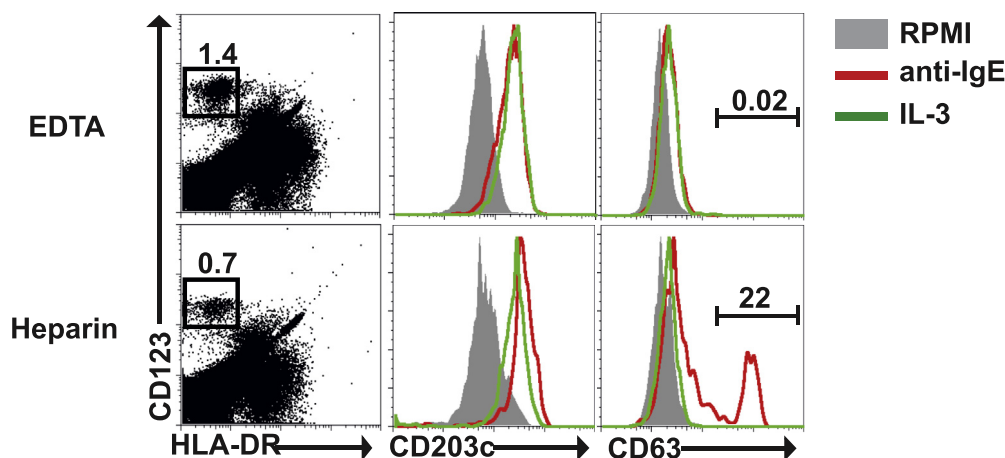


FIG 1. Overview of BATs. Blood from each subject was collected separately into EDTA and heparin tubes, stored at 4°C for 24 hours, and then incubated with RPMI, anti-IgE, or IL-3. CD123⁺HLA-DR⁺ cells are gated as basophils (left panels), and histograms show their expression of CD203c (middle panels) and CD63 (right panels). Gray histograms show RPMI (unstimulated) cells, red lines show anti-IgE stimulation, and green lines show IL-3 stimulation.

did not significantly influence the percentage of CD63^{hi} basophils in heparin specimens ($P > .05$, percentage of CD63^{hi} basophils in anti-IgE vs anti-IgE plus calcium/magnesium conditions in heparin). We next examined whether calcium/magnesium also affected CD203c expression in EDTA, heparin, or both specimens because it has been reported that calcium ionophore can induce CD203c upregulation.²⁹ Without stimulation, CD203c levels were higher in heparin versus EDTA specimens (Fig 2, B, and Fig 3, C and D). Adding only calcium/magnesium significantly upregulated CD203c in basophils in EDTA ($P = .008$, RPMI vs calcium/magnesium only) but not in heparin ($P = .222$, RPMI vs calcium/magnesium only) specimens. Also, although anti-IgE stimulation elicited higher absolute CD203c expression in heparin compared with standard EDTA specimens, adding calcium/magnesium to anti-IgE resulted in induction of similar absolute levels of basophil CD203c MFI in the 2 anticoagulants. These results indicate that calcium/magnesium contributes to both baseline levels and upregulation of basophil CD203c surface expression.

Because RPMI contains calcium nitrate and magnesium sulfate, we compared RPMI and calcium/magnesium-free PBS (CMF-PBS) as vehicles. We found no differences between RPMI and CMF-PBS for either CD203c upregulation or induction of CD63^{hi} basophils (data not shown). This provided additional evidence that chelation of extracellular calcium/magnesium by EDTA was the reason for the failure of anti-IgE stimulation to induce CD63^{hi} basophils in EDTA specimens. Thus our data indicate that addition of calcium/magnesium is essential to obtain CD63^{hi} basophils in EDTA specimens.

Finally, we found no significant differences in basophil percentages among the 4 conditions of storage in EDTA or heparin specimens (see Fig E2, A, in this article's Online Repository at www.jacionline.org). Nor were there significant differences in the percentage of basophils in EDTA and heparin specimens after storage of blood at 4°C for 24 hours (see Fig E2, C). However, there were slightly lower numbers of basophils in heparin specimens after storage at 4°C for 24 hours (see Fig E2, B).

CD63 expression is predominantly caused by basophils rather than platelets

CD63 can be expressed on platelets in addition to basophils and mast cells,^{27,28,35} and activated platelets can bind to various leukocytes, raising concerns that platelet binding to basophils can falsely increase levels of “basophil” CD63.^{15,17,36-38} We used the platelet/megakaryocyte-specific marker CD41 (gpIIB) to search for CD41 staining associated with basophils. Immunocytochemical analysis revealed that platelets were attached to some basophils in both EDTA and heparin specimens (Fig 4, A and B). Adding calcium/magnesium to EDTA specimens caused extensive aggregation of platelets, and some of these large aggregates attached to basophils (Fig 4, B). By using flow cytometry, basophils in heparin samples exhibited more platelet attachment than did those in standard EDTA specimens (Fig 4, C and D). This is consistent with a report that the majority of CD63⁺ basophils were CD41⁺ when EDTA, which is known to inhibit platelet activation,^{39,40} was used as an anticoagulant. The mean percentage of basophils that were CD41⁺ after anti-IgE stimulation was somewhat higher in the CD63^{hi} than in the CD63^{lo} population (Fig 4, D). However, basophils exhibited similar levels of high or low CD63 expression regardless of the expression level of CD41 (Fig 4, E). Furthermore, we confirmed morphologically that CD63 was expressed on basophils after anti-IgE but not IL-3 stimulation in heparin but not at all in EDTA unless exogenous calcium/magnesium was added (Fig 4, F). Our observations are consistent with those of others, who concluded that CD63 is expressed on the basophil itself when the cells are activated through FcεRI.^{11,41} Moreover, in heparin specimens CD41 was positive on some basophils after IL-3 stimulation, even though IL-3 did not increase basophil CD63 expression. Finally, we found that leukocytes other than basophils were also CD41⁺ (between 21% and 73% of cells; Fig 4, E), but CD63 was expressed only on basophils by using flow cytometry or direct microscopy. Together, these results indicate that the appearance of many CD63^{hi} basophils is predominantly or entirely due to basophil-derived rather than platelet-derived CD63.

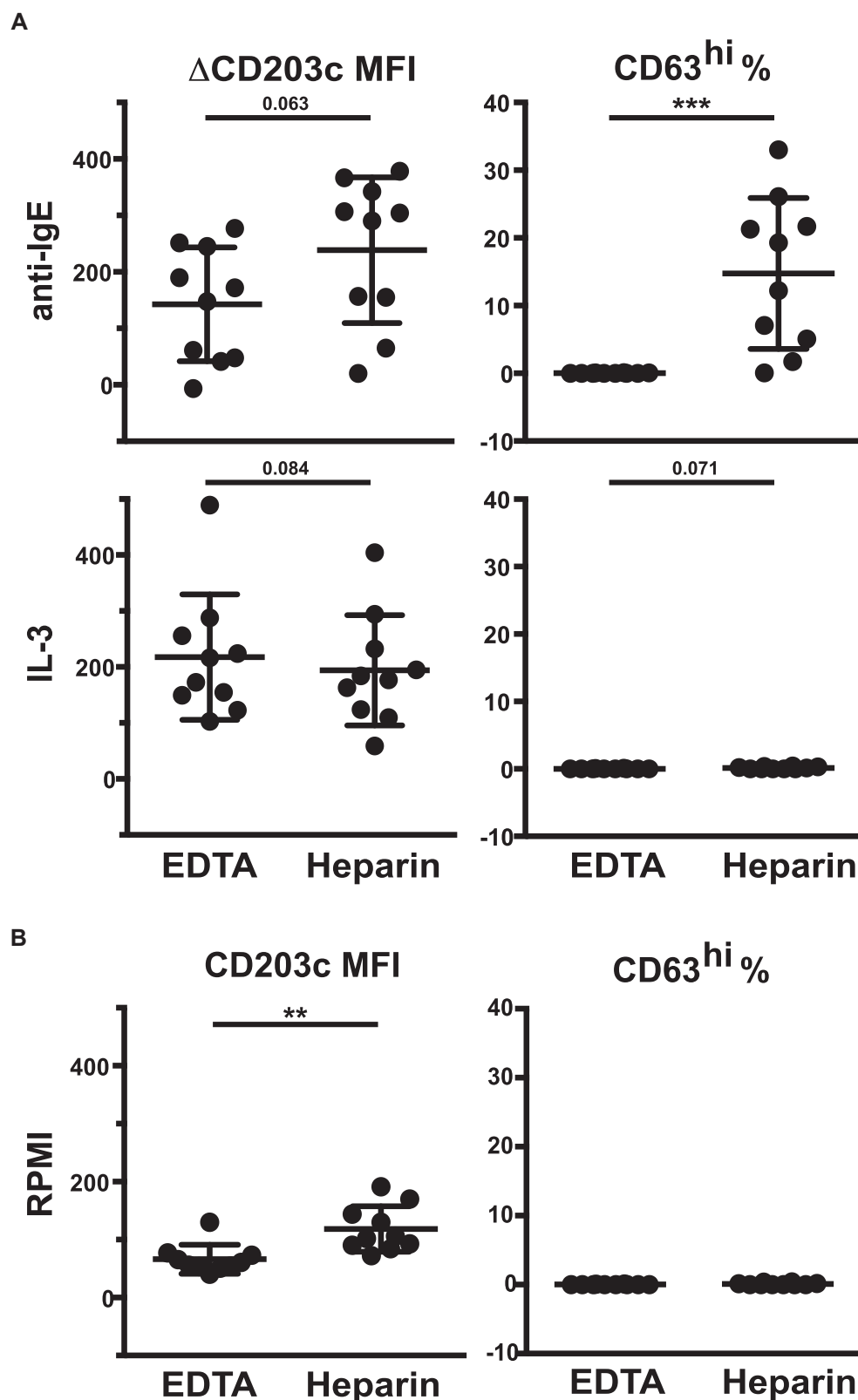


FIG 2. Comparison of anticoagulants. Blood of the same 10 healthy donors anticoagulated with EDTA or heparin was stored at 4°C for 24 hours before cells were stimulated with IL-3 or anti-IgE. **A, Left,** Δ CD203c MFI; **right,** percentage of CD63^{hi} basophils. **B, Left,** CD203c MFI; **right,** percentage of CD63^{hi} basophils after incubation with RPMI alone. Data shown (individual values [dots] and means $\pm$ SDs) are the combined results of all the measurements performed in 3 independent experiments. * $P < .05$, ** $P < .005$, and *** $P < .0005$. No asterisks, $P > .05$. P values are stated when they are between .05 and .1. MFI, Mean fluorescence intensity.

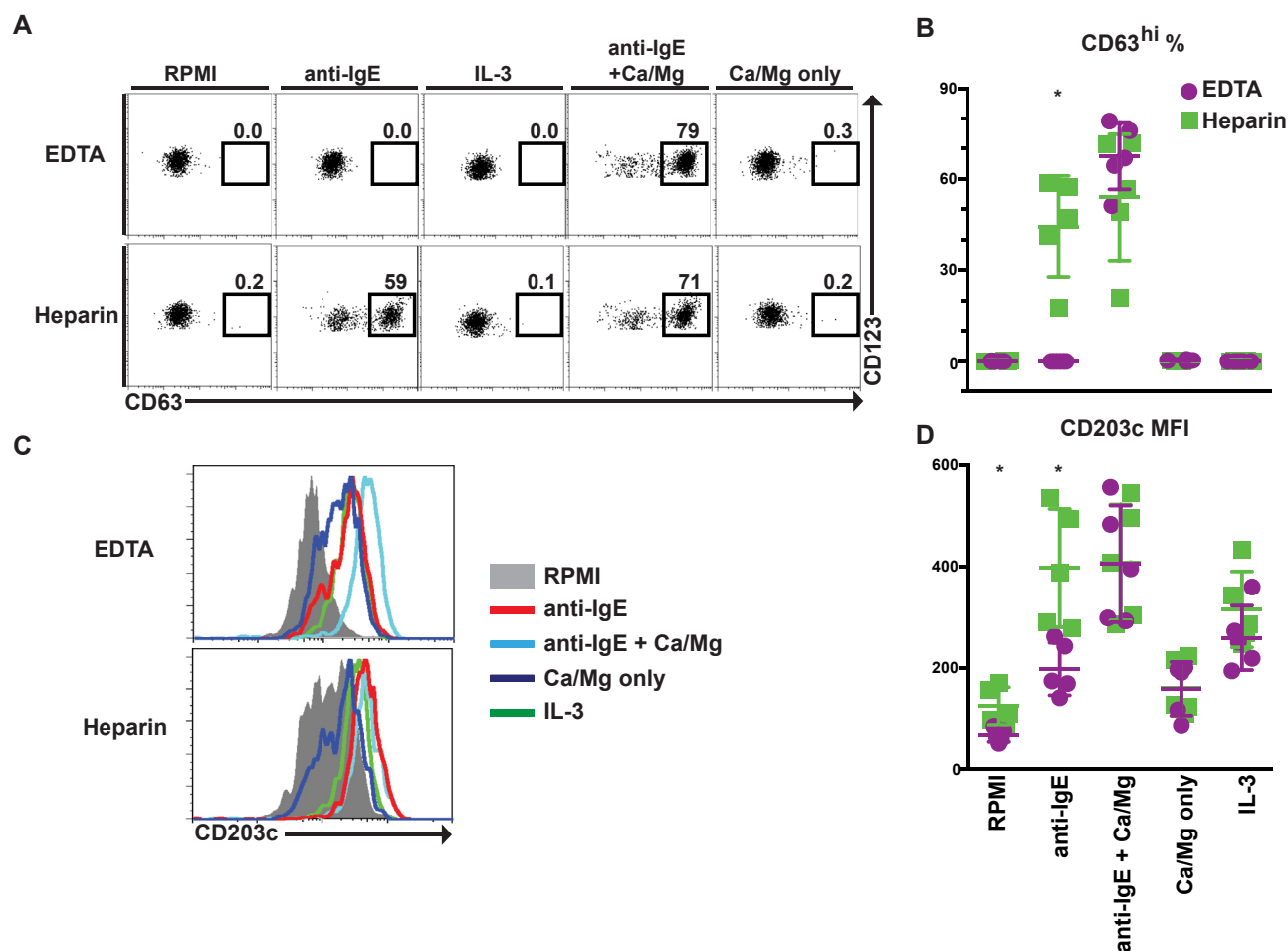


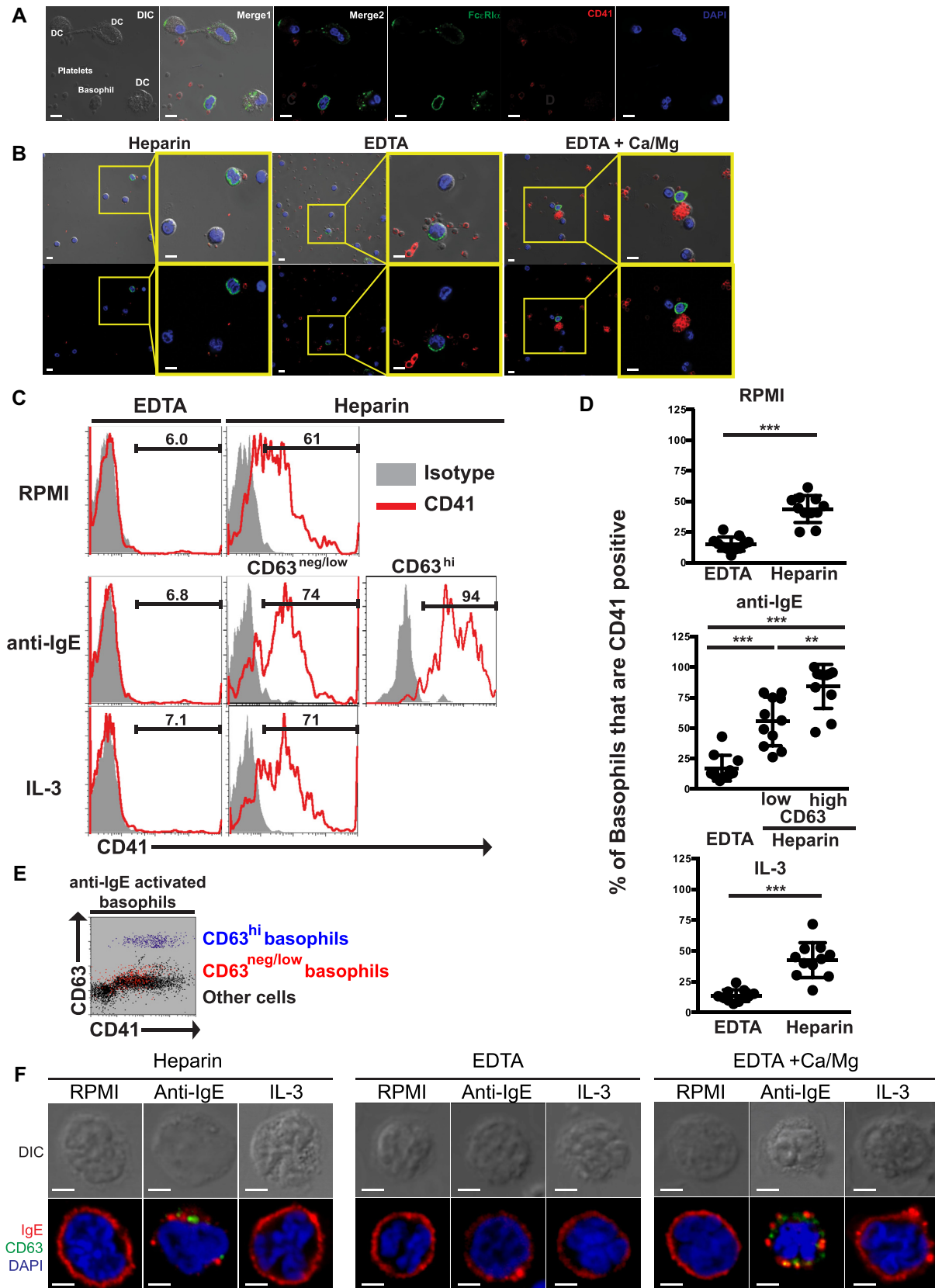
FIG 3. Effect of calcium/magnesium on CD203c and CD63. Blood of healthy donors was treated with IL-3, anti-IgE with or without calcium/magnesium, or calcium/magnesium only. **A**, Representative basophil plots of CD63 for 1 donor. **B**, CD63 results from 5 donors. **C**, Representative basophil histograms of CD203c for 1 donor. **D**, CD203c MFI of 5 donors. Fig 3, **B** and **D**, share the x-axis labels in Fig 3, **D**. Data shown (individual values [dots] and means $\pm$ SDs) are from 1 of 3 independent experiments, each of which produced similar results. * $P < .05$, ** $P < .005$, and *** $P < .0005$. No asterisks, $P > .05$ between EDTA and heparin for each condition of stimulation. None of the P values for any other comparisons are between .05 and .1. MFI, Mean fluorescence intensity.

BATs using flow cytometry in patients with peanut allergy

We next tested blood collected in heparin tubes from 21 participants with peanut allergy enrolled in a trial of oral immunotherapy (OIT) for peanut allergy (see Table E2) after storage of the blood for 24 hours at 4°C or RT. Values for anti-IgE-induced Δ CD203c were not significantly different among the 4 conditions of blood storage (see Fig E3, A, in this article's Online Repository at www.jacionline.org), as with blood specimens from healthy donors (see Fig E1, A). In cells stimulated with the 3 highest concentrations of peanut extract, those stored at RT for 24 hours had lower Δ CD203c values versus those at 4°C, but there were no significant differences between the corresponding 4- and 24-hour values at 4°C. Values for CD63^{hi} basophils after treatment with anti-IgE or the 3 highest concentrations of peanut extract were not significantly different between 4 and 24 hours at either 4°C or RT (see Fig E1, B). Slightly higher values for CD63^{hi} basophils were observed after incubation with RPMI, IL-3, or a low (1 ng/mL) concentration of peanut extract at 24 versus 4 hours at 4°C, but these changes were more marked in

RT specimens, even in nonreleasers (see Fig E3, B), a finding we did not observe in blood from healthy donors (see Fig E1, D). Finally, higher mean basal levels of CD63^{hi} basophils (in specimens treated only with RPMI) and higher variation in the individual values were especially notable in RT specimens in comparisons between 4°C and RT at 24 hours (see Fig E3, C).

Given those results and in light of other possible pitfalls of performing BATs after blood stored at RT for 24 hours^{18,42} and considering that there were few or no differences in results obtained with blood stored at 4°C for 4 or 24 hours, we decided to store blood routinely at 4°C for 24 hours before performing BATs. We tested heparin- versus EDTA-anticoagulated blood obtained in the same venipuncture from each of 98 patients with peanut allergy (see Table E3 for their demographic characteristics) at their screening for entry into the OIT trial. On stimulation with anti-IgE, IL-3, or 5 concentrations of peanut extract, CD203c upregulation occurred in basophils in blood collected in either anticoagulant (Fig 5, A). Δ CD203 values on IL-3 stimulation were slightly lower in heparin versus EDTA specimens, but all doses of peanut extract resulted in significantly



higher values in heparin versus EDTA specimens. As in specimens from healthy blood donors (Fig 2, B), levels of CD203c surface expression without stimulation were slightly higher in heparin versus EDTA specimens ($P < .0001$; Fig 5, B), and induction of many CD63^{hi} basophils after stimulation with anti-IgE or peanut extract was observed only in heparin (Fig 5, C). However, we observed a small amount of CD63 upregulation at lower fluorescence intensity both in EDTA and heparin, as with specimens from healthy blood donors (Fig 1, data not shown). As with healthy blood donors, 9 of the 98 patients were nonreleasers, with basophils responding neither to anti-IgE nor peanut antigen (Fig 5, D).

We found that very similar results were obtained in BATs done in duplicate at the same time with blood from the same patients (see Fig E4, A, in this article's Online Repository at www.jacionline.org) or when we tested blood from the same subjects at screening or weeks later at the beginning (week 0) of the OIT trial (see Fig E4, B). To examine the feasibility of performing BATs in specimens after shipment at 4°C, we conducted BATs 24 hours after blood collection in aliquots of blood from the same subjects with or without overnight shipment (over a distance of 130 km) at 4°C or at room or ambient temperature. Δ CD203c on anti-IgE stimulation did not differ significantly between specimens kept at different temperatures with or without shipment, but IL-3-induced Δ CD203c values were lower in specimens that had been kept at room/ambient temperature (regardless of shipment), as in Fig E1 (see Fig E5, A, in this article's Online Repository at www.jacionline.org). CD63^{hi} basophil values were more variable at RT with or without shipment, also as in Fig E1 (see Fig E5, B).

BATs can be performed by using CyTOF in blood stored 24 hours before analysis

Finally, we investigated whether our protocol for preparing whole blood for BATs could also be used for the recently established CyTOF approach, which uses metal-labeled antibody probes instead of antibodies labeled with fluorescent compounds.²² The appearance of a CD63^{hi} basophil population on stimulation with anti-IgE was observed in blood from healthy donors that was obtained with heparin but not with EDTA (Fig 6, A and B), which is consistent with the results we obtained using conventional flow cytometry (Figs 1 and 2 and see Fig E1). Notably, it appeared that few platelets were attached to basophils analyzed by using CyTOF (only 0.6% to 6% of

basophils were positive for the platelet marker CD61; Fig 6, C, left). Moreover, the expression pattern of CD63 was equivalent between CD61⁺ and CD61⁻ basophils (Fig 6, C, right).

These findings support the conclusion that CD63 expression is predominantly due to basophils rather than platelets. In blood from donors with peanut allergy obtained in heparin tubes, CyTOF can be used to assess upregulation of CD63 and the appearance of a CD63^{hi} population of basophils in specimens stimulated with peanut extract (Fig 6, D), and the results obtained with CyTOF correlate very well with those obtained by mean of flow cytometry (Fig 6, E).

DISCUSSION

We wished to define a practical protocol for performing BATs in blood stored for up to 24 hours before analysis. We used whole blood because of its convenience compared with first having to purify basophils (which also might result in some changes in basophil function and activation parameters) and also because tests conducted with whole blood might be more physiologically relevant. We decided against using IgE or Fc ϵ RI α as gating markers both because their expression could markedly change depending on the concentration of serum IgE and because other cells can express these markers, particularly in allergic patients.⁴³⁻⁴⁷ Also, Fc ϵ RI crosslinking by allergen causes their internalization, and this could interfere with basophil gating.⁴⁸

Our findings indicate that, for conducting BATs 24 hours after sample collection, using heparin as the anticoagulant and storage at 4°C are the optimal conditions among those tested. Using heparin (vs EDTA) permitted a much more robust induction of CD63^{hi} basophils after anti-IgE. Moreover, performing BATs after storage of heparinized blood for 24 hours at 4°C also permitted strong anti-IgE- or IL-3-induced upregulation of CD203c surface expression. Notably, there appears to be no consensus about the optimal time at which to conduct BATs. Sturm et al^{19,20} reported a time-dependent decrease in basophil reactivity in EDTA specimens stored at 4°C. However, Sousa et al⁴⁹ observed that the basophil response was intact at 24 hours after blood collection using the same conditions as Sturm et al.^{19,20}

We confirmed that upregulation of CD63 requires physiologic extracellular concentrations of calcium/magnesium (Fig 3), which is consistent with the current understanding that degranulation of basophils and mast cells is dependent on extracellular calcium/magnesium.⁵⁰⁻⁵³ The lack of induction of

FIG 4. Assessment of platelet attachment to basophils. Blood of healthy donors anticoagulated with EDTA or heparin was incubated with RPMI or CaCl₂/MgCl₂. **A**, Representative photographs of the individual blood cell types identified. *Left*, Differential interference contrast (DIC) with labels indicating 1 basophil, several platelets, and 3 dendritic cells. The 3 panels on far right indicate Fc ϵ RI α , CD41, and DAPI staining alone. The *second and third panels from the left* indicate merged staining indicated as Merge1 (DIC plus antibody staining) and Merge2 (antibody staining alone). **B**, Representative pictures of basophils and platelets after incubating blood with RPMI (heparin, EDTA, *left and middle panel*) or RPMI with calcium/magnesium (EDTA plus calcium/magnesium, *right panel*). Pictures highlighted by yellow borders are higher magnifications of the corresponding picture. Scale bars in Fig 4, A and B = 10 μ m. Data shown in Fig 4, A and B, are from 1 of 3 independent experiments, each of which produced similar results. **C** and **D**, Blood was treated as in Fig 1, and CD41⁺ basophils were assessed by means of flow cytometry of the cells of 1 subject (Fig 4, C) or by assessing the cells of 11 subjects (Fig 4, D). * $P < .05$, ** $P < .005$, and *** $P < .0005$. No asterisks, $P > .05$. **E**, Blood anticoagulated with heparin was incubated with anti-IgE and stained for CD63 and CD41. Representative *dots plots* are displayed. *Blue dots*, CD63^{hi} basophils; *red dots*, CD63^{-/low} basophils; and *black dots*, all other cells. **F**, Blood anticoagulated with EDTA or heparin was incubated with RPMI or CaCl₂/MgCl₂ and stimulated with RPMI, anti-IgE, or IL-3 for 30 minutes. Representative images of differential interference contrast (*upper panel*) and merged IgE, CD63, and DAPI staining (*lower panel*) are shown. Scale bars = 10 μ m.

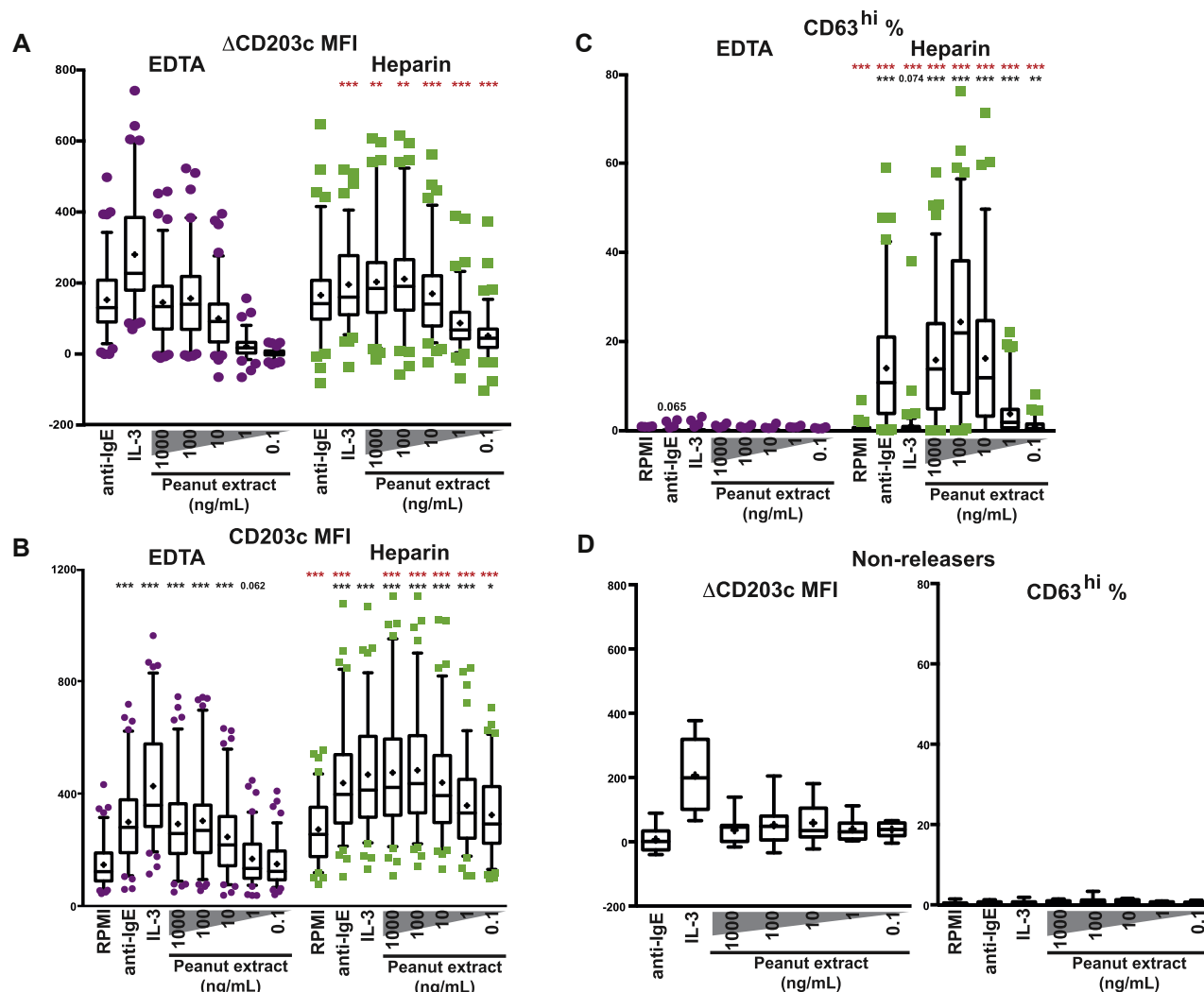


FIG 5. Comparison of anticoagulants for performing BATs in patients with peanut allergy. Blood from patients with peanut allergy was treated with IL-3 or anti-IgE or peanut extract. **A**, Δ CD203c MFI. **B**, Absolute CD203c MFI values. **C**, Percentage of CD63^{hi} basophils. **D**, Δ CD203c MFI (left) and percentage of CD63^{hi} basophils (right) of nonreleasers identified among patients with peanut allergy. Lower/higher 5% of all values are plotted as individual values (dots). Boxes extend from the 25th to 75th percentiles, and whiskers represent 5th and 95th percentiles. Bars in boxes indicate medians, and crosses indicate means. Fig 5, A-C, n = 98. Fig 5, D, n = 9. * $P < .05$, ** $P < .005$, and *** $P < .0005$. No asterisks, $P > .05$. P values are stated when they are between .05 and .1. Red asterisks are comparisons between EDTA and heparin at each condition of stimulation. Black asterisks are comparisons of CD203c MFI (Fig 5, B) or percentage of CD63 high basophils (Fig 5, C) between RPMI and each condition of stimulation for cells analyzed in the same anticoagulant.

CD63^{hi} basophils in EDTA samples can be compensated by addition of exogenous calcium/magnesium. Indeed, some groups and commercial kits use EDTA as an anticoagulant and add calcium/magnesium to enable the stimulus-dependent induction of a CD63^{hi} basophil population.^{54,55} However, adding exogenous calcium/magnesium lengthens the procedure and could cause variable results. Moreover, we found that calcium/magnesium affected not only CD63 but also CD203c expression levels, including baseline levels (Fig 3, C and D). Finally, addition of calcium/magnesium caused extensive platelet aggregation, resulting in large aggregates of platelets that might affect BAT results (Fig 4, B).

Some BATs are conducted with IL-3 priming.⁵⁶⁻⁵⁸ Because stimulation with IL-3 alone induced upregulation of surface

CD203 (Figs 1 and 2 and see Fig E1, A), this has the potential to partially mask CD203c upregulation in response to allergens.⁵⁹ Therefore we recommend not adding IL-3 routinely during BATs, particularly when assessing CD203 responses.

Importantly, we examined whether our protocol (4°C/24 hours) could be used to perform BATs in allergic patients, including assessing stimulation with peanut antigen. When we assessed BATs in blood samples from 98 patients with peanut allergy collected separately into EDTA or heparin, we obtained results with anti-IgE or IL-3 stimulation that were essentially the same as those from blood of healthy donors. Because some subjects are nonreleasers,³⁰⁻³⁴ it is essential to include a non-FcεRI-mediated stimulant, such as IL-3, as a positive control. Consistent with prior reports, we found that approximately 10% to 20% of healthy

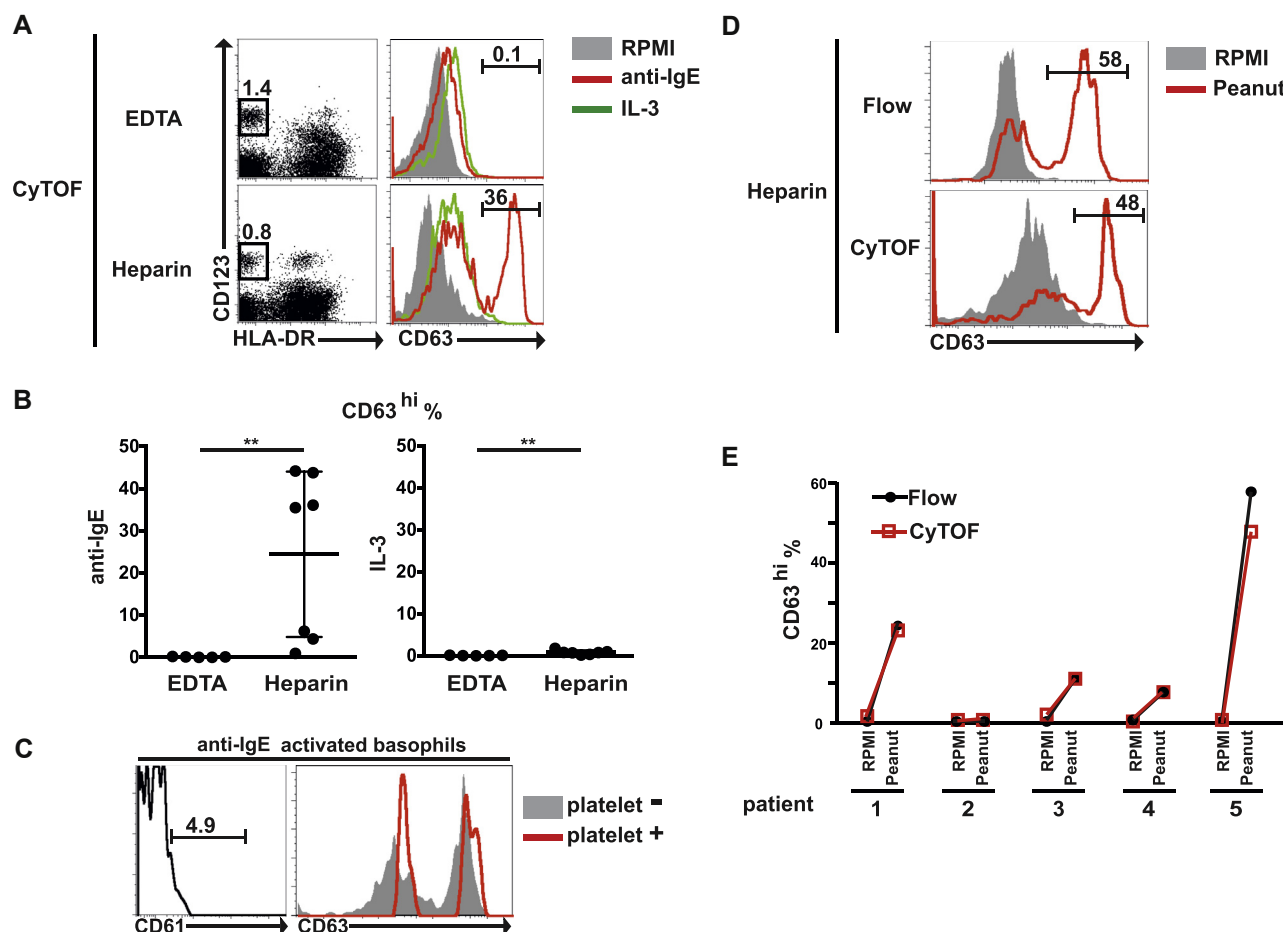


FIG 6. BAT with the CyTOF platform. **A**, Blood from healthy donors was collected separately into EDTA and heparin tubes, stored at 4°C for 24 hours, and then incubated with RPMI, anti-IgE, or IL-3. DNA⁺CD235⁻CD61⁻CD45⁺CD123⁺HLA-DR⁻ cells were gated as basophils (left panels), and histograms show their expression of CD63 (right panels) after mock stimulation with RPMI (gray shaded histograms) or after stimulation with anti-IgE (red lines) or IL-3 (green lines). **B**, Percentage of CD63^{hi} basophils on stimulation with anti-IgE (left panel) or IL-3 (right panel; n = 5 for EDTA, n = 7 for heparin). Individual values are plotted (dots) with means ± SDs. **P < .005. **C**, Left, Blood anticoagulated with heparin was incubated with anti-IgE. Representative CD61 staining after basophils were gated as (DNA⁺CD123⁺HLA-DR⁻). Right, Comparison of CD63 expression between platelet-negative (CD61⁻) and platelet-positive (CD61⁺) basophils. **D**, Blood from patients with peanut allergy was collected into heparin tubes, stored at 4°C for 24 hours, and then stimulated with peanut extract (100 ng/mL) for 30 minutes (Flow, fluorescence-based flow cytometry; upper panel) or 20 minutes (CyTOF; lower panel). Histograms show basophil CD63 from 1 representative patient. **E**, Data from 5 representative patients showing percentage of CD63^{hi} basophils with conventional flow cytometry versus CyTOF.

donors and allergic patients are nonreleasers who do not respond to FcεRI-mediated stimulation (data from such nonreleasers are included in the data shown in our figures).

In conclusion, we report that BATs can be performed by using flow cytometry in blood obtained with heparin as an anticoagulant and after storage for 24 hours at 4°C. Such specimens can be analyzed by means of both conventional flow cytometry and CyTOF. Although the 2 methods produced very similar results for stimulus-induced changes in basophil CD63 surface expression, CyTOF can permit multiple additional basophil surface structures or intracellular proteins to be analyzed simultaneously. Given the cost of the equipment and the expertise needed for performing CyTOF analyses, it is useful that these tests, as well as conventional flow cytometric BATs, can be performed on blood stored for 24 hours before analysis.

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Key messages

- Heparin is superior to EDTA as an anticoagulant for BATs because heparin permits assessment of both CD203c upregulation and CD63^{hi} basophils.
- BATs can be performed with heparinized blood stored for 24 hours at 4°C.
- By using heparinized blood, BATs can be performed either by means of conventional flow cytometry or CyTOF.

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METHODS

Reagents

RPMI 1640 medium was purchased from Gibco (Grand Island, NY). Polyclonal rabbit anti-human IgE (Bethyl Laboratories, Montgomery, Tex) was used for BATs. The antibody cocktail for surface staining for BATs consisted of fluorescein isothiocyanate (FITC)-conjugated anti-CD63 mAb (clone H5C6; BD Biosciences, San Jose, Calif), phycoerythrin (PE)-conjugated anti-HLA-DR mAb (clone G46-6, BD Biosciences), peridinin chlorophyll protein (PerCP)-conjugated anti-CD123 mAb (clone 7G3, BD Biosciences), and allophycocyanin-conjugated CD203c mAb (clone NP4D6; BioLegend, San Diego, Calif). For platelet staining, the antibody cocktail consisted of FITC-conjugated anti-CD63 mAb (clone H5C6, BD Biosciences), PE-conjugated anti-CD41 mAb (clone HIP8, BioLegend), PerCP-conjugated anti-CD123 mAb (clone 7G3, BD Biosciences), and allophycocyanin-conjugated anti-HLA-DR mAb (clone G46-6, BD Biosciences). CMF-PBS was purchased from Corning Cellgro, Mediatech (Manassas, Va). EDTA (0.5 mol/L) was purchased from Invitrogen Life Technologies (Carlsbad, Calif). IL-3 was purchased from PeproTech (Rocky Hill, NJ). BSA was purchased from Sigma (St Louis, Mo). Round-bottom tubes (352058) were purchased from BD Falcon (San Jose, Calif). Fixation/Permeabilization Concentrate and Diluent and Permeabilization Buffer (10×) were purchased from eBioscience (San Diego, Calif). Staining buffer was 5% BSA and 2 mmol/L EDTA in CMF-PBS. All reagents were kept sterile at 4°C.

BATs

One hundred microliters of whole blood was mixed with 100 μ L of RPMI or anti-IgE (final concentration, 2 μ g/mL), IL-3 (final concentration, 2 ng/mL), or peanut extract (final concentrations, 1000, 100, 10, 1, and 0.1 ng/mL) dissolved in 100 μ L of RPMI in round-bottom tubes with loose lids. After a 30-minute incubation at 37°C in a 5% CO₂ incubator (Panasonic, Osaka, Japan), reactions were stopped by adding 900 μ L of cold 2.5 mmol/L EDTA/CMF-PBS, followed by centrifugation for 5 minutes at 4°C (all centrifuge runs were done with these conditions). After removal of supernatants, the antibody cocktail for surface staining (5 μ L of each antibody mentioned above; total, 20 μ L) was added and mixed with pellets and then incubated on ice for 20 minutes. After incubation, 3 mL of staining buffer was added; the tubes were centrifuged, the supernatant was removed, and 1 mL of Fix/Perm solution was added and mixed and incubated for 30 minutes on ice. Two milliliters of permeabilization buffer was added after the incubation. The tubes were centrifuged, supernatants were removed, and 150 μ L of staining buffer was added, and then flow cytometry was performed with a FACSCalibur (BD Biosciences). Data were analyzed with FlowJo software (TreeStar, Ashland, Ore) by gating basophils as CD123⁺ (IL-3 receptor α chain) and HLA-DR⁺ cells and then measuring expression of CD203c and CD63^{hi} populations (Fig 1). Δ CD203c is defined as CD203c MFI after anti-IgE or IL-3 stimulation minus CD203c MFI incubated with RPMI. For some experiments, platelets were stained with an antibody cocktail (5 μ L of each antibody; total, 20 μ L) to assess platelet attachment to basophils. For some experiments, to examine the influence of calcium/magnesium on BATs, CaCl₂ and MgCl₂ (final concentrations were 1 mmol/L each, both from Sigma-Aldrich) were added into the samples just before starting the BAT assay.

CytoF

Blood from healthy donors (allergy status unknown) from the Stanford Blood Center was obtained by means of venipuncture in heparin or EDTA tubes and stored at 4°C for 24 hours. Blood from patients with peanut allergy was collected by means of venipuncture in heparin tubes and stored at 4°C for 24 hours. Stimulation of specimens for BATs was performed, as described above in the main text. After stimulation for BATs in whole blood, red blood cells were removed by means of hypotonic lysis by using a 10× RBC lysis buffer (BioLegend). Cells were then stained, washed, intercalated, and analyzed by using mass cytometry, as previously described.^{E1} Briefly, the remaining cells were then stained with metal-conjugated antibodies, fixed

with 1.6% paraformaldehyde, intercalated in the presence of 0.02% saponin, and acquired on a CyTOF2 mass cytometer (Fluidigm Sciences, South San Francisco, Calif) at an event rate of less than 500 cells per second.

Basophil quantification with fluorescence beads

Basophils were counted by using fluorescent beads with flow cytometry (123count eBeads, eBioscience). Basophil (CD123⁺HLA-DR⁺) numbers were calculated according to the manufacturer's manual after staining with PE-conjugated anti-HLA-DR mAb (clone G46-6, BD Biosciences) and PerCP-conjugated anti-CD123 mAb (clone 7G3, BD Biosciences).

Basophil and platelet analysis by using confocal microscopy

For staining of platelets and basophils (Fig 4, A and B), 20 μ L of whole blood from healthy donors was placed into each well of a 96-well U-bottom plate (BD Falcon) with or without CaCl₂ and MgCl₂ (1 mmol/L each) in RPMI medium and incubated for 30 minutes. Then samples were plated on poly-D-lysine-coated slides and fixed with 4% paraformaldehyde, as previously described.^{E2} Cells were first permeabilized and blocked in PBS containing 0.5% BSA (Sigma-Aldrich) and 0.1% saponin (Sigma-Aldrich). Cells were stained with the following antibodies for 45 minutes at RT in PBS with 0.5% BSA and 0.1% saponin: purified anti-CD41 (clone HIP8, BioLegend) and anti-Fc ϵ RI-FITC (clone AER37, eBioscience). Cells were extensively washed and then incubated with the following reagents for 30 minutes at RT in PBS with 0.5% BSA and 0.1% saponin: goat anti-mouse IgG₁-Alexa Fluor 546 and 4',6-diamidino-2-phenylindole (DAPI; both from Life technologies). Samples then were mounted and examined with a Zeiss LSM780 Meta inverted confocal laser-scanning microscope, 63×/1.40 Oil differential interference contrast M27 objective, and electronic zoom 1 (dimension: x, 512; y, 512; scaling: x, 0.264 μ m; y, 0.264 μ m). Images were processed with Zen and ImageJ software.

For CD63 and basophil staining (Fig 4, E), for each point, 20 μ L of whole blood from healthy donors was lysed with Pharm Lyse lysing buffer (BD Biosciences) and washed with RPMI. Cell pellets were suspended in 50 μ L of RPMI and placed on warmed poly-D-lysine-coated slides at 37°C. Thirty minutes later, 5 μ L of RPMI or RPMI with stimulants, in both cases supplemented with soluble FITC-conjugated anti-CD63 mAb (which will bind to extracellular CD63 molecules exteriorized on the cell surface during secretion), were added at 37°C. Stimulants were anti-IgE (final concentration, 5 μ g/mL) or IL-3 (final concentration, 2 ng/mL) with or without CaCl₂ and MgCl₂ (final concentration, 1 mmol/L each), which was the same as used for BATs.

Thirty minutes later, cells were fixed with 4% paraformaldehyde and then permeabilized and blocked with PBS with 0.5% BSA and 0.1% saponin. Cells were then incubated with a purified rabbit anti-human IgE (Bethyl Laboratories, Montgomery, Tex) for 45 minutes at RT in PBS with 0.5% BSA and 0.1% saponin. Cells were extensively washed and then incubated with the following reagents for 30 minutes at RT in PBS with 0.5% BSA and 0.1% saponin: goat anti-rabbit IgG-Alexa Fluor 594 and DAPI (both from Life Technologies). Samples were then mounted and examined by using a Zeiss LSM780 Meta inverted confocal laser-scanning microscope, 63×/1.40 Oil differential interference contrast (DIC) M27 objective and electronic zoom 1, 2, or 3 (dimension: x, 512; y, 512; scaling: x, 0.264 μ m; y, 0.264 μ m). Image were processed with Zen and ImageJ software.

Peanut extract preparation

Two grams of defatted peanut flour from Byrd Mill (Ashland, Va) was dissolved in CMF-PBS and vortexed vigorously and rotated on a shaker at RT (23°C) for 1 hour, vortexed again, centrifuged at 250g for 5 minutes at 23°C, and filtered through a 0.22- μ m filter (Millipore, Billerica, Mass). The protein concentration was determined by using the bicinchoninic acid assay (Thermo Fisher Scientific, Waltham, Mass). The peanut antigen solution was placed in aliquots and stored at -80°C. The antigen solution was thawed as needed and stored at 4°C for up to 1 week before use in experiments.

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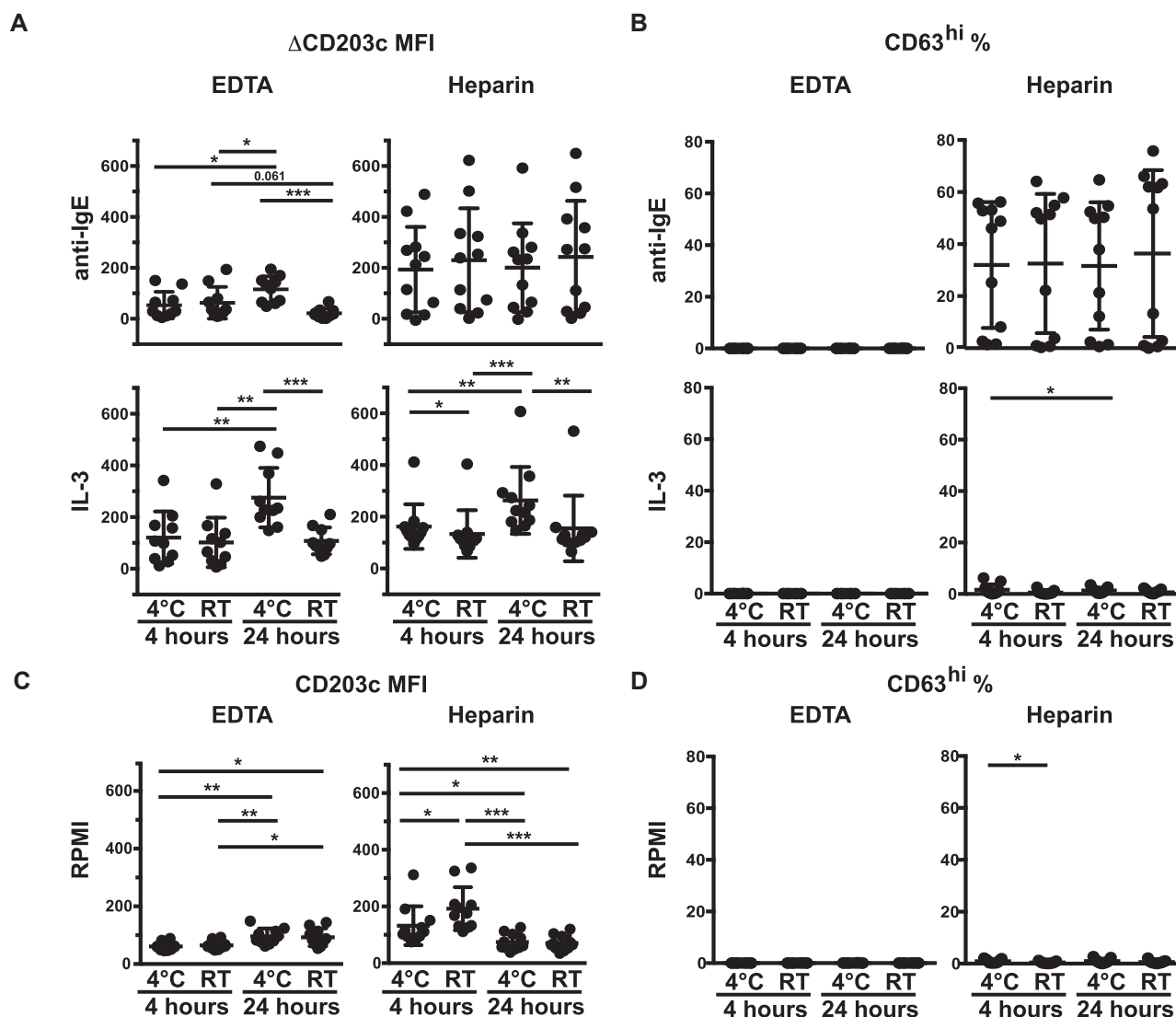


FIG E1. Comparison of time and temperature of blood storage. Blood from the same healthy donors was stored at 4°C or RT and then analyzed at 4 and 24 hours. Blood (from separate donors) that was anticoagulated with EDTA ($n = 10$ donors) or heparin ($n = 11$ donors) was incubated with RPMI (medium only control), anti-IgE, or IL-3. **A**, Δ CD203c MFI. **B**, Percentage of CD63^{hi} basophils. **C**, CD203c MFI. **D**, Percentage of CD63^{hi} basophils after incubation with RPMI medium only. Data shown (individual values [dots] and means $\pm$ SDs) are the combined results from 3 independent experiments. * $P < .05$, ** $P < .005$, and *** $P < .0005$. No asterisks, $P > .05$. P values are stated when they are between .05 and .1.

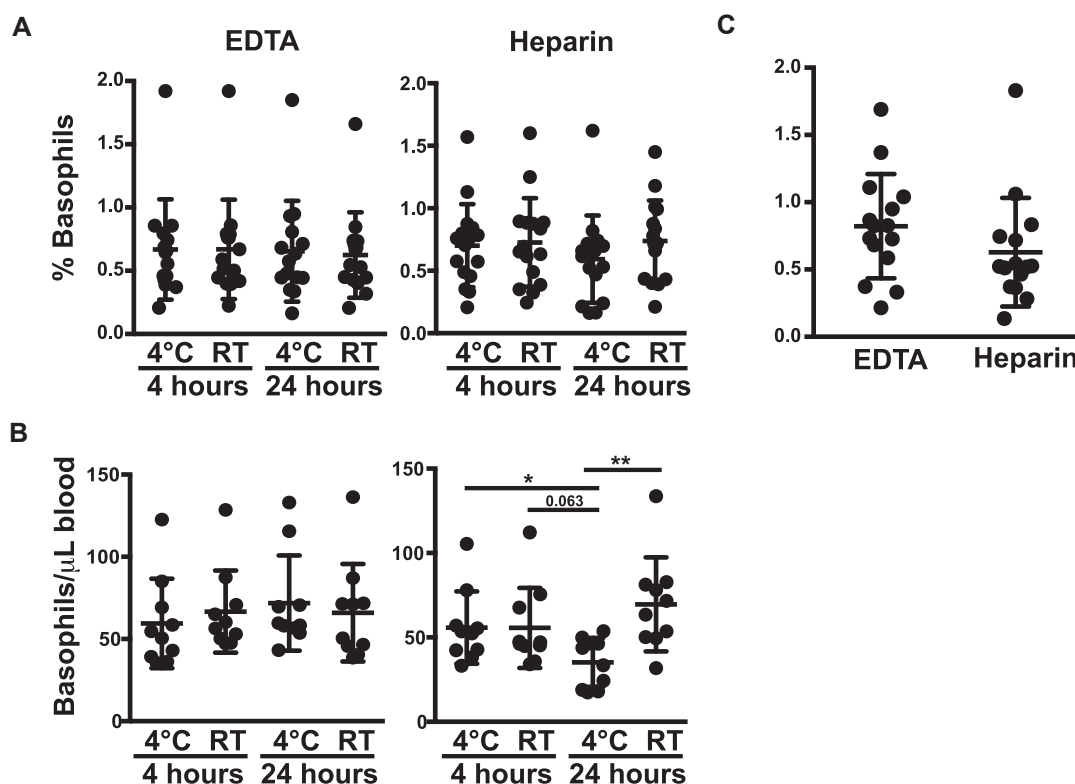


FIG E2. Comparison of basophil percentages and numbers in different conditions of specimen preparation. Percentage of basophils (**A**) and basophil numbers (**B**) in the 4 conditions of temperature and time of blood storage tested and comparison of percentage of basophils in EDTA versus heparin specimens stored at 4°C for 24 hours (**C**) are shown. Blood from the same healthy donors was stored at 4°C or RT and then analyzed at 4 and 24 hours (Fig E2, A and B). Fig E2, A, EDTA, $n = 15$; heparin, $n = 16$. Fig E2, B, $n = 10$. Fig E2, C, $n = 15$. Data shown (individual values [dots] and means $\pm$ SDs) are combined results from 3 independent experiments. * $P < .05$, ** $P < .005$, and *** $P < .0005$. No asterisks, $P > .05$. P values are stated when they are between .05 and .1.

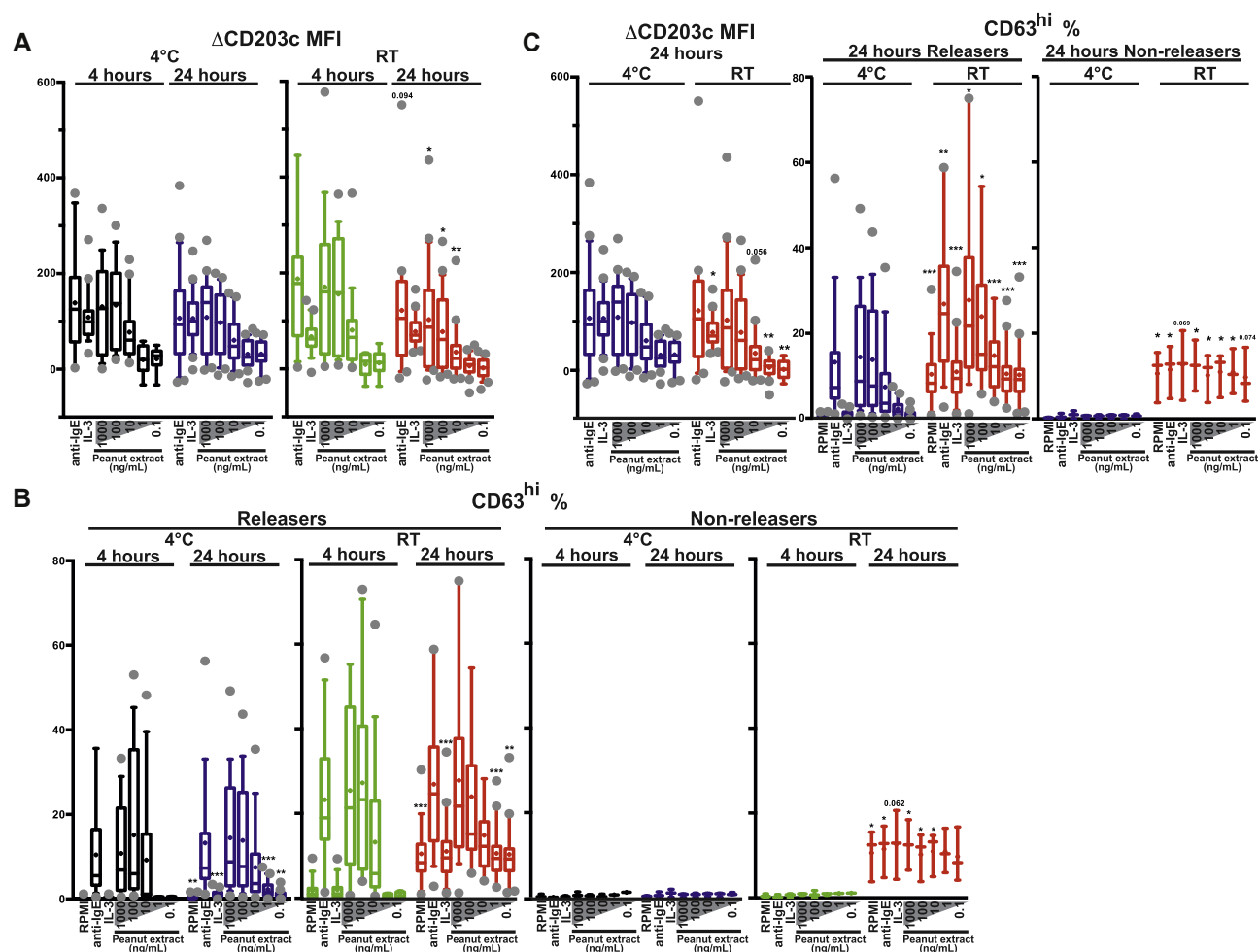


FIG E3. Comparison of time and temperature of specimen storage in patients with peanut allergy. Heparin-anti-coagulated blood from patients with peanut allergy was stored at 4°C or RT and then analyzed at 4 and 24 hours after treatment with IL-3, anti-IgE, or peanut extract. **A** and **B**, Δ CD203c MFI (Fig E3, A) and percentage of CD63^{hi} basophils (Fig E3, B). Lower/higher 5% of all values are plotted as individual values (*dots*). Boxes extend from the 25th to 75th percentiles, and *whiskers* represent 5th and 95th percentiles. Bars in boxes indicate medians, and crosses indicate means. Fig E3, A, $n = 19$ for 4 hours (4°C/RT) and $n = 21$ (including the 19 subjects studied at 4 hours) for 24 hours (4°C/RT). Fig E3, B, $n = 16$ for 4 hours (4°C/RT), $n = 18$ (including the 16 subjects studied at 4 hours) for 24 hours (4°C/RT) for releasers, and $n = 3$ for non-releasers. **C**, Comparison of Δ CD203c MFI and CD63^{hi} percentage basophils between 4°C and RT at 24 hours from Fig E3, A and B. * $P < .05$, ** $P < .005$, and *** $P < .0005$. No asterisks, $P > .05$. P values are stated when they are between .05 and .1.

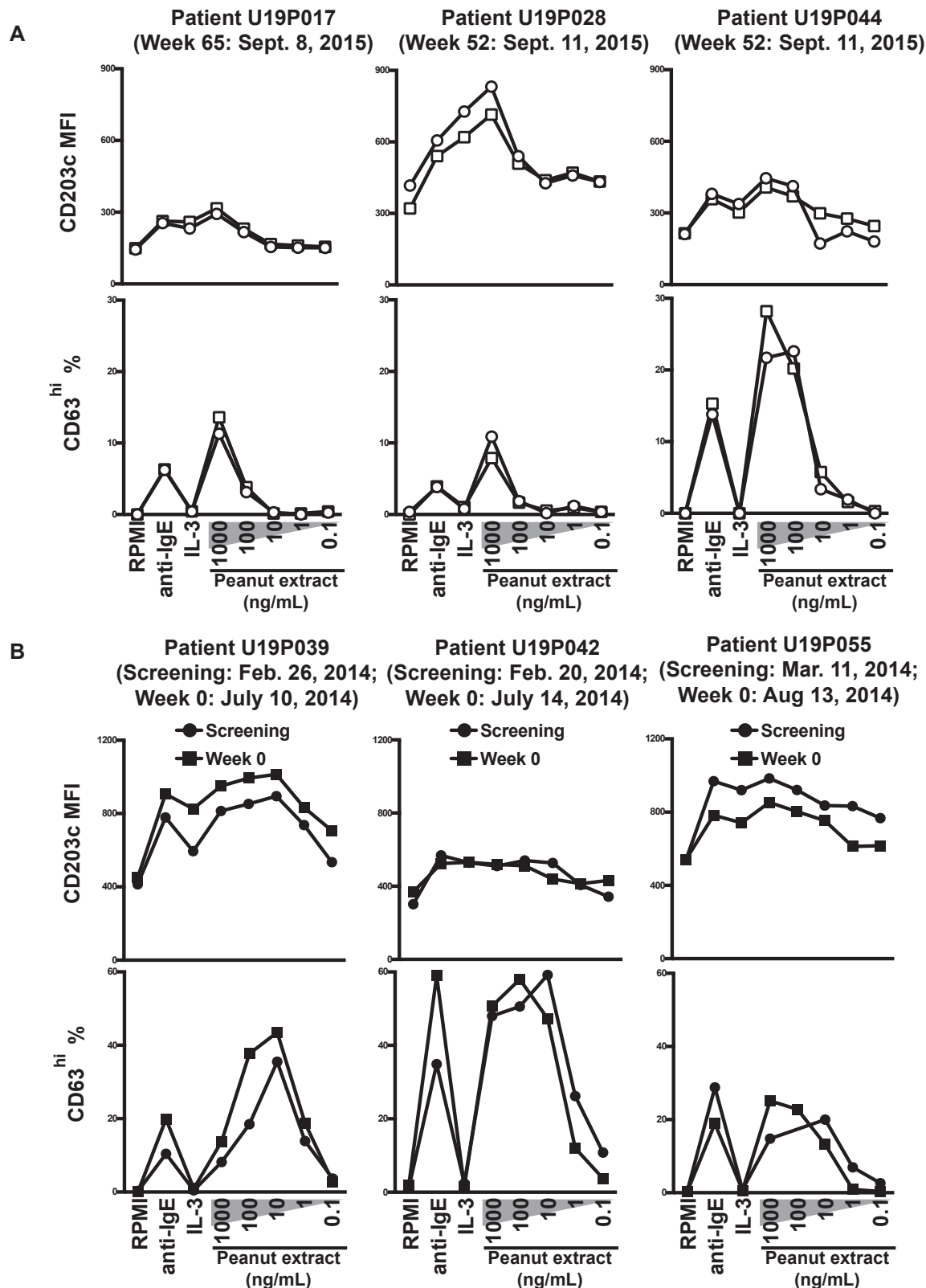


FIG E4. Assessment of reproducibility of BATs in the same subjects. Blood from patients with peanut allergy anticoagulated with heparin was stored at 4°C for 24 hours before treating the cells as in Fig 5. **A**, Duplicate samples from the same specimens were analyzed at the same time. Values shown with open circles or open squares are from the 2 duplicate tubes. **B**, Specimens from the same patients tested at 2 different times (as indicated in the figure). Solid black circles are values obtained at screening, and solid black squares are values obtained at week 0 of the OIT trial. Both panels show absolute CD203c MFI values (upper panel) and percentages of CD63^{hi} basophils (lower panel).

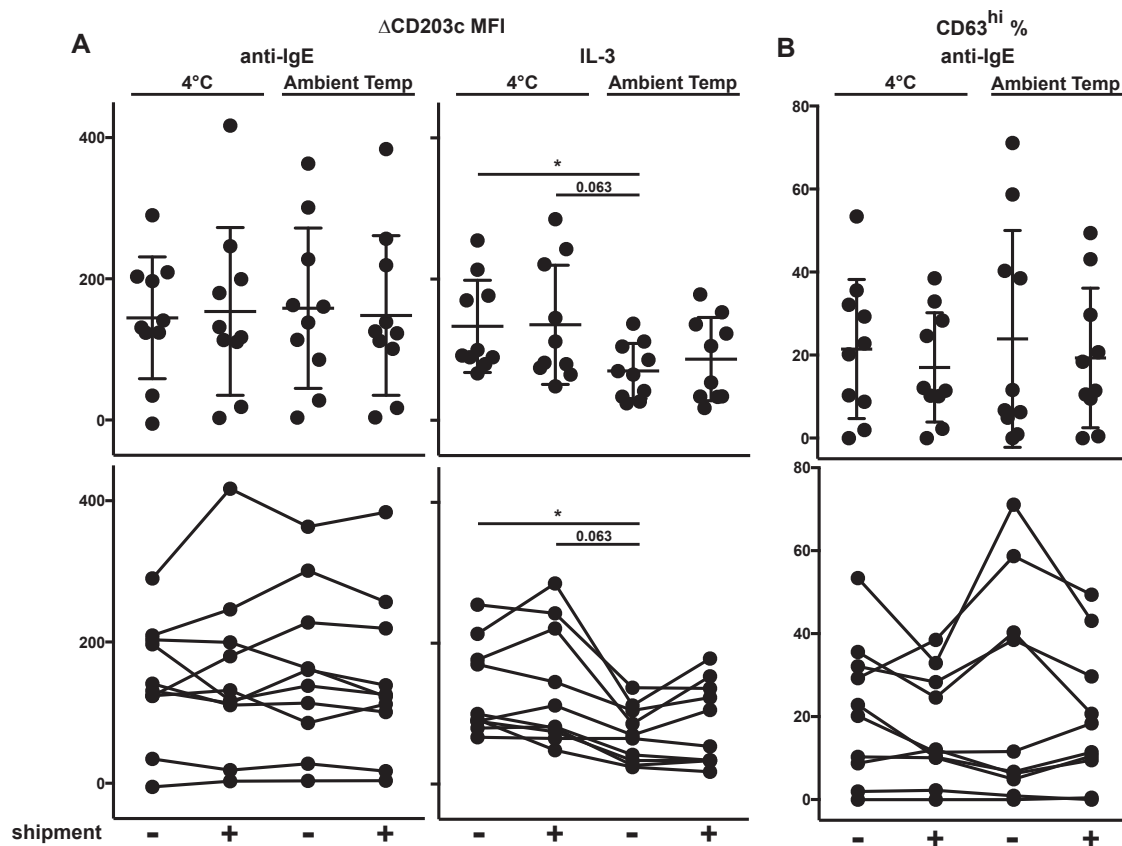


FIG E5. Assessment of BATs in blood shipped at 4°C or ambient temperature. Blood from the same healthy donors was stored at 4°C or RT without shipment in the laboratory or during overnight shipment for 130 km at 4°C or ambient temperature (*Ambient Temp*) and then analyzed at 24 hours. Blood that was anticoagulated with heparin ($n = 10$) was incubated with RPMI (medium only control), anti-IgE, or IL-3. **A**, Δ CD203c MFI after incubation with anti-IgE (*left*) or IL-3 (*right*). **B**, Percentage of CD63^{hi} basophils after incubation with anti-IgE. Data shown (*upper panel*, individual values [*dots*] and means $\pm$ SDs; *lower panel*, individual values [*dots*] from aliquots of blood from the same subjects are connected by *lines*) are the combined results from 2 independent experiments (ie, in 2 experiments in each of which blood from 5 different subjects was aliquoted and then shipped or maintained in our laboratory before performing BATs). * $P < .05$, No asterisks, $P > .05$. P values are stated when they are between .05 and .1.

TABLE E1. Summary of experimental design

	Samples	Purpose	Anticoagulants	No. of subjects (n)	Temperature/time
Fig 1	Healthy donors	Overview of BATs analyzed	EDTA vs heparin		4°C/24 h
Fig 2	Healthy donors	Comparison of anticoagulants	EDTA vs heparin	n = 10	4°C/24 h
Fig 3	Healthy donors	Effects of calcium/ magnesium on BATs	EDTA vs heparin	n = 5	4°C/24 h
Fig 4	Healthy donors	Analysis of platelet attachment	EDTA vs heparin	n = 11	4°C/24 h
Fig 5	Allergic patients	Comparison of anticoagulants	EDTA vs heparin	n = 98	4°C/24 h
Fig 6	Healthy donors (A-C) Allergic patients (D and E)	Comparison of BAT results with flow cytometry vs CyTOF	EDTA vs heparin	n = 7 healthy donors (EDTA: n = 5, heparin: n = 7) n = 5 allergic patients	4°C/24 h
Fig E1	Healthy donors	Comparison of 4 combinations of temperature and time of blood storage	EDTA vs heparin	n = 10 (EDTA) n = 11 (heparin)	4°C vs RT/4 h vs 24 h
Fig E2	Healthy donors	Percentages and numbers of basophils in EDTA vs heparin in 4 combinations of temperature and time of blood storage (A and B) or in EDTA specimens stored at 4°C for 24 h (C)	EDTA vs heparin	(A) n = 15 (EDTA) n = 16 (heparin) (B) n = 10 (C) n = 15	(A and B) 4°C vs RT/4 h vs 24 h (C) 4°C/24 h
Fig E3	Allergic patients	Comparison of 4 combinations of temperature and time of blood storage; serial analysis	Heparin	n = 21	4°C vs RT/4 h vs 24 h
Fig E4	Allergic patients	Assessment of BAT reproducibility over time	Heparin	n = 6	4°C/24 h
Fig E5	Healthy donors	Assessment of BATs in blood shipped before testing	Heparin	n = 10	4°C vs ambient temperature/ 24 h
Table E1		Summary of experimental design			
Table E2	Allergic patients	Demographic data for Fig E3			
Table E3	Allergic patients	Demographic data for Fig 5			
Table E4		Antibodies used in CyTOF			

TABLE E2. Demographic characteristics of patients with peanut allergy reported in [Fig E3](#)

Age (y)	Range, 7-53; median, 12; mean, 19
Sex	67% Male
Ethnicity*	95% Non-Hispanic/Latino
Race*	57% White, 38% Asian, and 5% African American

*Subjects self-identified ethnicity and race.

TABLE E3. Demographic characteristics of patients with peanut allergy reported in [Fig 5](#)

Age (y)	Range, 7-17; median, 9; mean, 10
Sex	65% Male
Ethnicity*	97% Non-Hispanic/Latino
Race*	70% White, 36% Asian, 2% African American, and 1% Hawaiian/Pacific Islander

*Subjects self-identified ethnicity and race, and several identified with more than 1 race; thus race percentages do not add up to 100%.

TABLE E4. Metal-labeled antibodies used for CyTOF analysis

Target	Clone	Manufacturer	Metal isotope
CD45	HI30	Fluidigm Science, South San Francisco, Calif	Gd156
CD61	VI-PL2	BD Biosciences, San Jose, Calif	La 139
CD63	H5C6	BD Biosciences	Er 170
CD123	6H6	Fluidigm Science	Eu 151
CD235ab	HIR2	BioLegend, San Diego, Calif	In 113
HLA-DR	L243	Fluidigm Science	Yb 174
DNA			Ir 191
Viability			Pt195 or In115*

*Maleimide-2,2',2''-(10-(2-((2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)amino)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl) triacetic acid (DOTA) loaded.