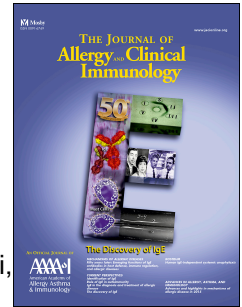


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A new fluorescent-avidin-based method for quantifying basophil activation in whole blood

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TITLE: A new fluorescent-avidin-based method for quantifying basophil activation in whole blood.

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CAPSULE SUMMARY:

We report herein a simple new fluorescent-avidin-based method to detect activated basophils in the whole blood of normal or allergic subjects, and compare this method to a basophil activation test based on detection of CD63.

LIST OF KEY WORDS:

Basophil; avidin; CD63; flow cytometry; peanut allergy.

ABBREVIATIONS USED:

Basophil activation tests, BATs; mast cells, MCs; AlexaFluor488-labeled avidin, Av.A488.

To the Editor:

Secretion of cytoplasmic granules (i.e., “degranulation”) by mast cells (MCs) and basophils activated via the FcεRI plays a key role in diverse IgE-mediated immune responses including asthma, hay fever, food allergies and anaphylaxis. In both cells, secretory granule structure is thought to reflect in part electrostatic complexes between proteases (positively-charged) and proteoglycans (negatively-charged), which associate with and thereby help to store multiple bioactive molecules (e.g., histamine and certain cytokines).^{1, 2} Even though basophils usually represent <1% of circulating peripheral blood leukocytes, analysis of basophil activation (i.e., using basophil activation tests [BATs]) has become popular,^{3, 4} both because basophils may play specific roles during type 2 immune responses and allergic disorders and because blood basophils are much more readily available for analysis than are tissue-resident MCs.

Classical BATs are based on detection of proteins, e.g., the vesicular marker CD63, on the surface of activated basophils. However, CD63 is expressed by most peripheral blood leukocytes and platelets, and activated platelets can bind to various leukocytes, raising concerns that platelet binding to basophils can falsely elevate levels of “basophil” CD63.³⁻⁶ We therefore sought to develop an alternative method that ideally could be both simple to perform and be more specific and/or more sensitive than classical CD63-based BATs. We recently reported that AlexaFluor488-labeled avidin (Av.A488, a positively-charged molecule which binds to highly negatively-charged molecules) can be used to monitor degranulation of human or mouse MCs in real time, using flow cytometry *in vitro* or intravital imaging *in vivo*.⁷ We report herein that Av.A488 also can be used to detect activated basophils in whole blood of anonymous blood donors or subjects suffering from allergies, and can quantify the extent of such basophil activation.

First, we purified basophils from the whole blood of anonymous blood donors and used single cell confocal imaging to determine whether Av.A488 could stain basophils stimulated with anti-IgE, which results in basophil degranulation. In accord with our previous reports on primary human and mouse MCs,⁷ we observed that, compared to RPMI-challenged control basophils, anti-IgE-stimulated basophils exhibited enhanced staining with Av.A488, with a pattern of staining consistent with the exteriorization and cell surface association of negatively-charged granule constituents (e.g. chondroitin sulfate- and/or heparan sulfate-containing proteoglycans) (Fig 1, A).

We recently reported a simple protocol which permits BATs to be performed, either by conventional flow cytometry or by Cytometry by Time-of-Flight mass spectrometry (CyTOF), on whole blood stored at 4°C for up to 24 h before analysis.⁸ We used those conditions of blood storage to determine whether Av.A488 staining could be used to monitor basophil activation in

whole blood from a group of 19 anonymous blood donors. We mixed 100 μ L of whole blood with 1 μ g/mL anti-IgE (in 100 μ L of RPMI) or with 100 μ L RPMI (control) alone, then gated on basophils as CD123-positive and HLA-DR-negative cells (two stable markers that, compared to Fc ϵ RI or CD203c, do not vary upon basophil activation, Fig 1, B),⁸ and compared detection of activated basophils by flow cytometry using either FITC-labeled anti-CD63 antibody or Av.A488 (both using the same detection channel; we defined “activated basophils” as those with a positive fluorescence signal for FITC-labeled anti-CD63 or for Av.488 as compared to the unstained and non-stimulated conditions) (Fig 1, C-D). As illustrated in Fig 1, C-D, we found that both Av.A488 and FITC-labeled anti-CD63 antibody readily detected anti-IgE-induced basophil activation in the whole blood of such subjects, whose allergy status is unknown. Importantly, no significant increase in Av.A488 staining was observed in other granulocyte populations, i.e., neutrophils and eosinophils (Fig E1, A-B), in blood incubated with anti-IgE.

Using co-staining with APC-labelled anti-CD63 antibody and Av.A488, we next assessed whether the two methods were identifying the same cell subsets within the population of anti-IgE-activated basophils in five anonymous blood donors. Notably, we identified three subsets of CD63⁺ or Av.A488⁺ basophils (i.e., CD63⁺Av.A488⁺, CD63⁻Av.A488⁺ and CD63⁺Av.A488⁻) and found that each of the 5 donors exhibited different percentages of each subset (Fig. 1, E). We also found, testing blood from 10 anonymous blood donors, that there were statistically significant positive correlations between the percentages of anti-IgE induced CD63⁺ or Av.A488⁺ basophils and between each of these percentages and the amount of histamine released in the same anti-IgE challenged specimens (Fig. 1, F). Finally, we tested whether exposure to IL-3 altered the reactivity of basophils with anti-CD63 antibody vs. Av.A488. While FITC-labeled anti-CD63 antibody did not label IL-3-stimulated basophils (a finding in accord with previous reports⁸), we detected a modest but significant increase (vs. RPMI stimulation) in the

percentage of Av.A488⁺ basophils after IL-3 challenge (at 20 ng/mL) (Fig. E1, C-D); however, no histamine release above background was detected after such IL-3 challenge (data not shown).

We next investigated whether Av.A488 could be used to monitor basophil activation in whole blood from 41 of the peanut allergic patients enrolled in one of two IRB-approved clinical trials of oral immunotherapy (OIT) (32 from POISED ClinicalTrials.gov Identifier: NCT02103270 and 9 from MAP-X ClinicalTrials.gov Identifier: NCT02643862) whose demographic features are shown in Table E1 in the Online Repository. Peanut allergy in both trials was defined as having both a clinical reaction to ≤ 500 mg total of peanut protein in a double-blind, placebo-controlled food challenge (DBPCFC) to peanut and a positive skin prick test to peanut ($\geq$ to 5 mm).⁸

Using 100 μ L of whole blood per patient, as described in Fig. 2, A-B (and in ref⁸ and the Online Repository), we used flow cytometry to compare the ability of FITC-labeled anti-CD63 antibody vs. Av.A488 to monitor changes in whole blood basophils after their exposure *ex vivo* to either RPMI (control), 1 μ g/mL anti-IgE (for 41 patients) or 100 ng/mL peanut extract (for 19 of the 41 patients). Compared to CD63⁺ basophils, we detected a higher percentage of Av.A488⁺ basophils in 38 of 41 patients tested, both at baseline (i.e., after RPMI incubation) and after basophil stimulation with anti-IgE, and in 17 of the 19 patients tested after basophil stimulation with peanut extract (Fig 2, B-D). We also compared the detection of Av.A488⁺ basophils in the whole blood of anonymous blood donors vs. subjects suffering from peanut allergies, both at baseline (i.e., after RPMI incubation) and 30 min after anti-IgE treatment. Anti-IgE stimulation increased the percentage of Av.A488⁺ basophils to similar levels in healthy donors and peanut allergic patients (Fig 2, E). However, the percentage of Av.A488⁺ basophils at baseline was

significantly higher in the blood of allergic subjects (global mean ~11.4%) vs. anonymous blood donors (global mean ~3.8%) (Fig 2, E, far left panel). In some of the same blood specimens from peanut allergic patients shown in Fig 2, E, we also analyzed basophil CD203c (a surface marker reported to be associated with basophil activation)^{3, 8} and found a positive correlation ($P=0.01$) between the percentage of detected Av.A488⁺ basophils and the intensity of CD203c staining at baseline (i.e., after exposure to RPMI) but this correlation did not achieve statistical significance ($P=0.07$) after activation by anti-IgE (Fig. E1, E). Finally, we did a pilot experiment to compare, in 14 peanut allergic patients tested either at week 104 or 117 in the POISED trial, the percentage of Av.A488⁺ basophils detected at baseline (i.e., after incubation with RPMI) and the ability of those subjects to tolerate peanut flour in a DBPCFC performed on the same day (after the phlebotomy). We found that subjects who passed the DBPCFC (i.e., defined by tolerance of 4000 mg of ingested peanut flour) had lower levels of Av.A488⁺ basophils at baseline than the patients who tolerated lower amounts of food challenge (i.e., developed a clinical reaction when challenged with ≤ 1900 mg of ingested peanut flour) (Fig. 2, F). Notably, 1 of the 14 patients tested failed the DBPCFC (i.e., reacted to the lowest amount of peanut flour tested) and this patient exhibited the highest level of Av.A488⁺ basophils at baseline among all of the 41 peanut allergic patients analyzed in this study.

Taken together, these results show that fluorochrome-labelled avidin can be used to monitor basophil activation in small amounts of whole blood *ex vivo* and in basophils stimulated either with anti-IgE or (in peanut allergic subjects) with the patients' offending allergen. Our data also suggest that this method might represent a more specific and sensitive alternative to CD63 detection for monitoring the activation status of blood basophils, and might even be useful in assessing the extent to which food allergic subjects are at risk for exhibiting strong clinical reactions to their offending allergen.

152

153 Some of our findings raise interesting questions that can only be answered by performing
154 additional studies involving substantially larger groups of subjects. For example, since avidin is
155 a constituent of egg white, the extent to which the avidin-based method can be used to detect
156 basophil activation by egg allergen in egg allergic patients, or can be used in tests of basophil
157 reactivity with other negatively-charged allergens, will have to be determined. It also will be
158 important to try to understand the biological significance of the distinct basophil subsets we
159 have observed following anti-IgE challenge (Fig. 1, E). This might reflect, at least in part, the fact
160 that avidin molecules specifically label exteriorized basophil granule matrix (i.e., providing a
161 direct assessment of the release of basophil granule contents), while anti-CD63 antibodies
162 detect CD63⁺ vesicles which have fused with the plasma membrane during secretion (i.e.,
163 representing a more indirect and non-specific assessment of release of basophil granule
164 contents). Therefore, it is tempting to speculate that different basophil subpopulations may co-
165 exist in variable proportions in different people (and/or perhaps in the same person under
166 different conditions), and that some of these basophils contain a pool of CD63-negative
167 secretory granules (most of them enriched in negatively-charged constituents [e.g.,
168 proteoglycans]) which can be externalized in response to anti-IgE challenge, whereas other
169 basophils may exteriorize CD63 from granules/vesicles that contain low levels of Av.A488-
170 binding content.

171 Finally, it will be important to explore further the possibility that the detection of high “baseline”
172 levels of Av.A488⁺ basophils in food allergic patients may help to identify those subjects at risk
173 to exhibit strong clinical reactions to the offending allergens, including patients who are enrolled
174 in immunotherapy protocols. Perhaps the detection of Av.A488⁺ basophils in specimens
175 incubated only with RPMI reflects low “basal” levels of activation even without *ex vivo* antigen
176 stimulation and/or persistent effects of prior, perhaps subclinical, episodes of activation (e.g.,

related to the oral immunotherapy treatments or because of inadvertent exposure to small amounts of offending food allergens), and/or is due to particular intrinsic features of the basophils of some allergic subjects. It also is possible that some of the differences observed at “baseline” in the extent of Av.A488-binding vs. CD63 expression on basophils from various groups of peanut allergic subjects may reflect a longer persistence of Av.A488-binding content on the basophil surface in such subjects compared to elevated levels of CD63. These and other possible explanations of our findings will be analyzed in future studies of larger groups of subjects. However, whatever the mechanism(s) accounting for the Av.A488⁺ basophils observed at baseline in the blood of many of the peanut allergic subjects, such evidence of potential ongoing (or past) activation was not observed in the same specimens by assessment of CD63.

AUTHORS

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MATERIAL AND METHODS

Material and Methods are described in the “Supplemental Material” section.

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FIGURES AND LEGENDS

Figure 1. Anti-IgE challenge increases the binding of fluorochrome-labelled avidin to human blood basophils. (A) Protocol for basophil isolation from whole blood and representative micrograph of purified basophils stimulated with RPMI or 1 µg/ml anti-IgE. Av.A488 fluorescence (green) is merged with Differential Interference Contrast (DIC). Bar=5 µm. (B) Protocol for assessing basophil activation by flow cytometry in whole blood of anonymous donors⁸. (C) Representative dot plot of HLA-DR⁺CD123⁺ basophils from an anonymous donor incubated with RPMI or anti-IgE and stained with anti-CD63-FITC or Av.A488. (D) Percentage of CD63⁺ (black) or Av.A488⁺ (green) basophils after RPMI or anti-IgE incubation in anonymous donors. Mean + SEM. Mann-Whitney U tests; ***P < .0005; **P < .005; *P < .05. (E) Percentage of Av.A488⁺CD63⁺, Av.A488⁺CD63⁻ or Av.A488⁻CD63⁺ basophils detected simultaneously with anti-CD63-APC and Av.A488 after anti-IgE challenge in 5 anonymous donors. (F) Correlation analysis, for results from anti-IgE challenged blood specimens from 10 anonymous donors, of the percentages (%) of CD63⁺ or Av.A488⁺ basophils and the amount of histamine released (nM)/Number of basophils. Nonparametric Spearman R correlation, we considered P < .05 as statistically significant. Pairs of open or green circles are the results from the single donors analyzed.

Figure 2. Using fluorochrome-labelled avidin to monitor the status of basophil activation in whole blood of peanut allergic patients. (A) Protocol for assessing basophil activation by flow cytometry in whole blood.⁸ (B) Representative dot plot of HLA-DR⁺CD123⁺ basophils incubated with RPMI, anti-IgE or peanut flour and stained with anti-CD63-FITC or Av.A488. (C) Percentage of CD63⁺ (black) or Av.A488⁺ (green) basophils after RPMI or anti-IgE or peanut extract challenge in 41 peanut allergic patients. Lower/higher 5% values are plotted as individual dots. Boxes extend from the 25th to 75th percentiles and whiskers represent 5th and 95th percentiles. Bars indicate medians. (D) Comparison of CD63⁺ (black) or Av.A488⁺ (green) basophils after RPMI (left) or anti-IgE (right) for each patient. Linked dots represent results from the individual patients. (E) Percentage of Av.A488⁺ basophils in 19 anonymous donors (open) versus 41 peanut allergic patients (black). (F) Y axis: Percentage of Av.A488⁺ basophils after RPMI (baseline) incubation in 14 peanut allergic patients in the POISED oral immunotherapy (OIT) trial that were challenged with increasing amounts of peanut flour; X axis: amount of tolerated peanut flour (mg) as per Bock's criteria.⁹ Mean + SEM; Mann-Whitney U tests; ***P < .0005; **P < .005; *P < .05.

Table E1

Age (y)	Range 4-53; median = 11, mean = 13
Sex	76% Male
Ethnicity*	98% Not Hispanic / Latino
Race*	73% Caucasian, 22% Asian and 5% multi-racial

*Subjects self-identified ethnicity and race.

Figure 2

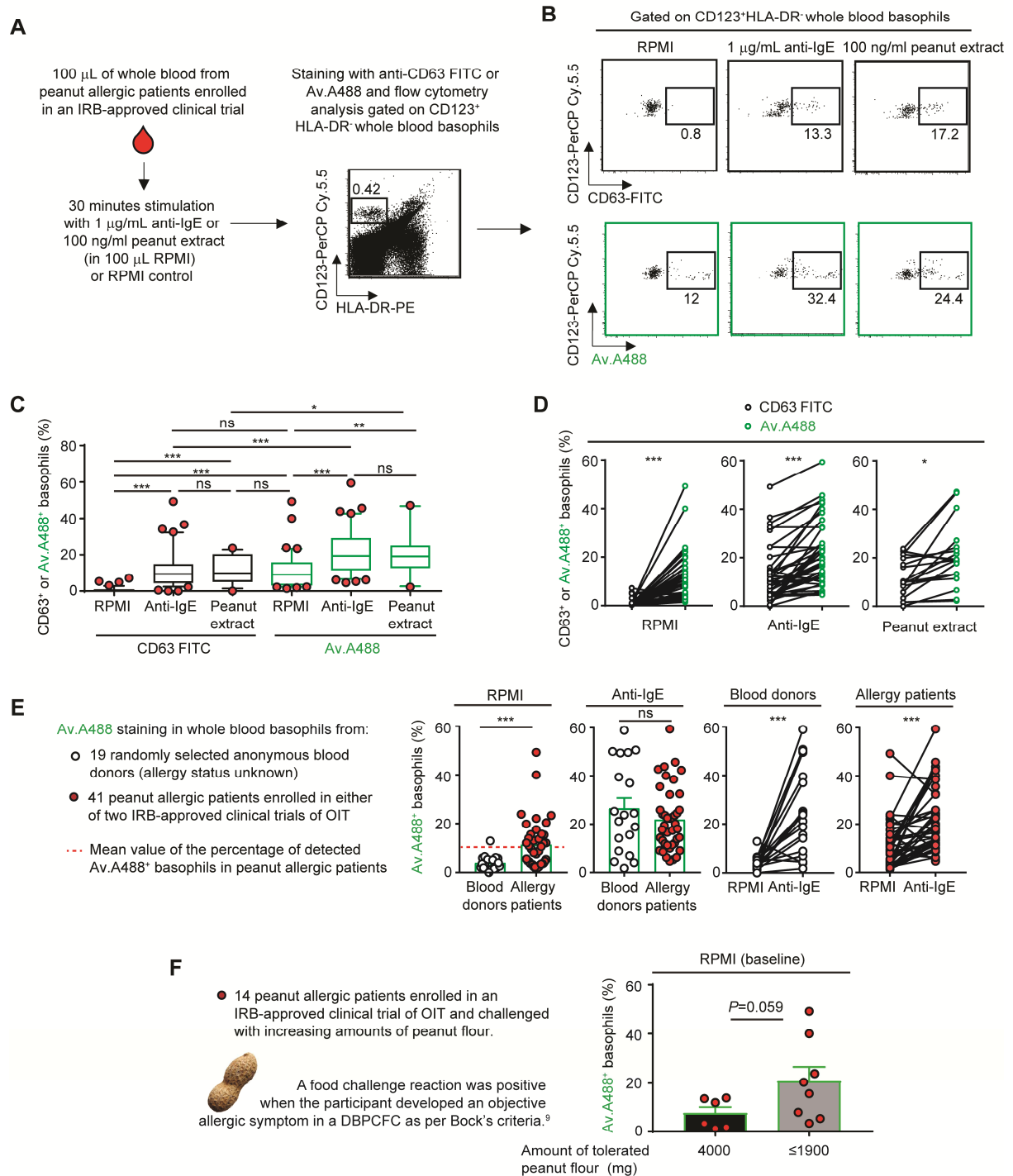
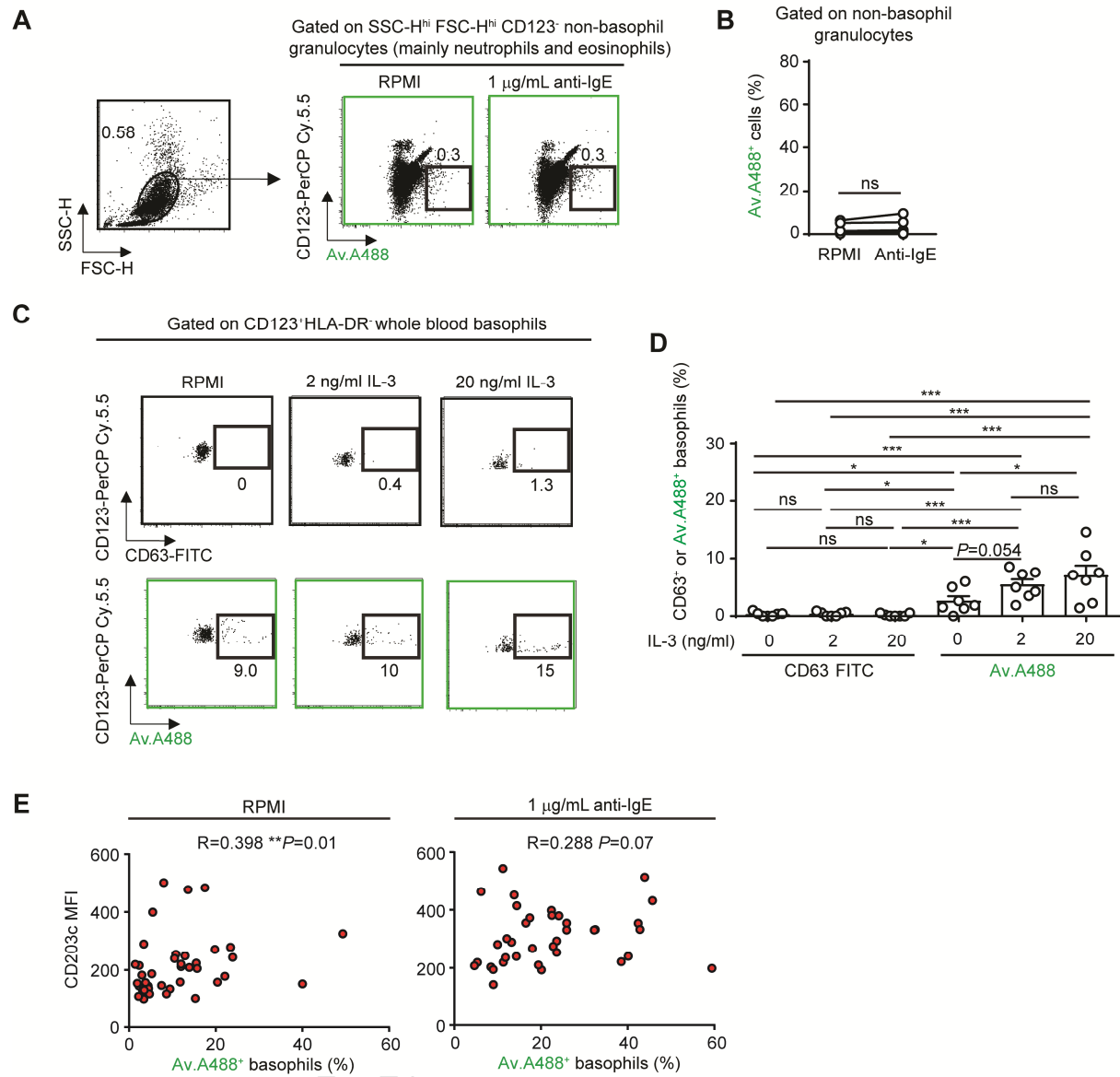


Figure E1



1 **METHODS**

2

3 **Blood specimens**

4 Blood from randomly selected anonymous healthy blood donors (allergy status
5 unknown) was obtained from the Stanford Blood Center (Palo Alto, CA, USA) and blood
6 from 41 peanut allergic patients (Table E1) was obtained as part of their participation in
7 two IRB-approved clinical trials (32 from POISED ClinicalTrials.gov Identifier:
8 NCT02103270 and 9 from MAP-X ClinicalTrials.gov Identifier: NCT02643862). The
9 POISED trial is not yet unblinded and it includes subjects randomized into oral
10 immunotherapy (OIT) or placebo groups. Following escalation to 4,000 mg peanut
11 protein/day and double blind, placebo controlled food challenges (DBPCFCs) at week
12 104, some subjects decreased their maintenance dose to 300 mg peanut protein/day,
13 whereas, others received placebo (peanut avoidance group). Accordingly, for 32 of the
14 41 subjects whose results are show in Fig. 2, C and for all of the 14 subjects whose
15 results are shown in Fig. 2, F we do not know to which treatment (or placebo) group the
16 individual subjects belonged. All blood samples were collected in heparin tubes and
17 stored at 4°C for ~24 hours with gentle agitation before analysis.⁸ Peanut allergy was
18 defined as having a clinical reaction to peanut during a double-blind, placebo-controlled
19 food challenge to peanut (the reaction occurring at ≤ 500 mg total of peanut protein) and
20 a positive skin prick test to peanut (≥ 5 mm).

21

22 **Reagents**

23 RPMI-1640 medium was purchased from Gibco, Grand Island, NY, USA. Polyclonal
24 rabbit anti-human IgE (Bethyl Laboratories, Montgomery, TX, USA) was used for BATs.
25 The antibody cocktail for surface staining for BATs consisted of FITC-conjugated anti-

CD63 mAb (clone: H5C6 from BD Bioscience, San Jose, CA, USA), anti-CD203c (clone: NP4D6), APC-conjugated CD63 antibody (clone: H5C6), PE-conjugated anti-HLA-DR mAb (clone: G46-6 from BD Bioscience), and PerCP-conjugated anti-CD123 mAb (clone: 7G3 from BD Bioscience). Alexa488-conjugated avidin was purchased from ThermoFisher Scientific (Carlsbad, CA USA). Calcium/magnesium-free PBS (CMF-PBS) was purchased from Corning Cellgro, Mediatech, Manassas, VA, USA. 0.5 M EDTA was purchased from Invitrogen Life Technologies, Carlsbad, CA, USA. Bovine serum albumin (BSA) was purchased from Sigma, St. Louis, MO, USA. Round bottomed tubes (352058) were purchased from BD Falcon, San Jose, CA, USA. Fixation/Permeabilization Concentrate and Diluent, and Permeabilization Buffer (10X), were purchased from eBioscience, San Diego, CA, USA. Staining buffer refers to 5% BSA and 2 mM EDTA in CMF-PBS. Histamine assay EIA kits were purchased from Cayman Chemical, USA. IL-3 was purchased from Perprotech, USA. All reagents were kept sterile at 4°C except for IL-3 that was preserved sterile at -20°C.

Basophil Activation Tests

Blood specimens were gently rotated at 4°C for ~24 h after blood collection. Immediately before starting BAT assays, samples were put into a water bath at 37°C for 30 seconds. 100 µL of whole blood were mixed with 100 µL of RPMI or with 100 µL of RPMI containing anti-IgE (to achieve a final concentration, after mixing with blood, of 1 µg/mL) or IL-3 (to achieve a final concentration, after mixing with blood, of 2 or 20 ng/mL) or peanut extract (to achieve a final concentration of 100 ng/mL) in round-bottomed tubes with loose lids as described in Ref⁸. In a pilot study employing blood obtained from 8 healthy blood donors, we found that anti-IgE induced elevations of basophil surface CD63 expression and Alexa488-avidin (Av.A488) binding with similar kinetics, reaching

peak levels at approximately 30 min, but with levels of Av.A488 binding, but not CD63 expression, starting to diminish by 45 min. We therefore used 30 min as our standard period of *ex vivo* stimulation of blood basophils. After a 30-min incubation at 37°C in a 5% CO₂ incubator (Panasonic, Osaka, Japan), reactions were stopped by adding 900 µL of cold 2.5 mM EDTA/CMF-PBS, followed by centrifuging for 5 min at 4°C (all centrifuge runs were done with these conditions). After removal of supernatants, the antibody cocktail for surface staining (5 µL of each antibody mentioned above, total 20 µL), or avidin-Alexa488 (final concentration: 1 µg/mL) instead of FITC-labeled CD63 antibody, or avidin-Alexa488 and APC-labeled CD63 antibody, was added and mixed with the cell pellets, then incubated on ice for 20 min. After incubation, 3 mL of staining buffer was added and the tubes were centrifuged, the supernatant was removed, and 1 mL of Fix/Perm solution was added and mixed, and incubated for 30 min on ice. 2 mL of permeabilization buffer was added after the incubation and the tubes were centrifuged, supernatants were removed, and 150 µL of staining buffer was added, and then flow cytometry was performed with a FACS Canto II (BD Bioscience). Data were analyzed with FlowJo (TreeStar, Ashland, OR, USA) by gating basophils as CD123 (IL-3 receptor alpha chain) positive and HLA-DR negative cells, and then measuring expression of and CD63 or avidin positive populations (Fig 1, A).

Histamine release

100 µL of whole blood were processed as described above in the paragraph “**Basophil Activation Tests**”. Measurements of histamine release were performed in some of the same experiments which assessed BATs, so that we could determine the extent of any correlation between detection of CD63⁺ or Av.A488⁺ basophils by flow cytometry with basophil histamine release in the same anti-IgE-challenged specimens from anonymous

blood donors. Measurements of histamine were performed according to the manufacturer's instructions and results were normalized to the number of basophils contained in 100 μ L of whole blood for each donor.

Basophil analysis by confocal microscopy

The human basophil enrichment kit (STEMCELL Technologies, Vancouver, Canada) was used for purification of basophils as per the manufacturer's instructions. 5×10^4 Purified basophils were placed into poly-D-Lysine-coated (5 μ g/ml in water, #P6407, Sigma Aldrich, USA) Nunc Lab-Tek 1.0 borosilicate cover glass system 8 chambers (#155411, ThermoFisher Scientific, USA) in RPMI medium supplemented with 1 μ g/ml of Av.A488 in a controlled atmosphere (using a Zeiss stage-top incubation system with an objective heater, at 37°C and in humidified 5% CO₂).⁷ Stimuli were added as described above and single cell images were taken 30 minutes later using a Zeiss LSM780 Meta inverted confocal laser-scanning microscope and a 63x/1.40 Oil DIC M27 objective and electronic zoom 3 (dimension x:512 y:512, scaling x= 0.264 μ m and y= 0.264 μ m).⁷

Statistical Analysis

Nonparametric Mann-Whitney *U* tests or Spearman *R* 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.

SUPPLEMENTARY FIGURE LEGENDS

Figure E1. Fluorochrome-labelled avidin staining of non-basophil granulocytes and IL-3-stimulated basophils, and comparison of staining with anti-CD203c-FITC or Av.A488 in basophils of peanut allergic patients. (A) Representative dot plot of gated SSC-H^{hi}FSC-H^{hi}CD123⁻ whole blood non-basophil granulocytes (i.e., neutrophils and eosinophils) incubated with RPMI or anti-IgE and stained with Av.A488. (B) Detection of Av.A488⁺ cells among non-basophil granulocytes after RPMI or anti-IgE incubation in whole blood of 11 anonymous donors. (C) Representative dot plot of gated HLA-DR⁻CD123⁺ whole blood basophils from an anonymous donor incubated with RPMI containing 0, 2 or 20 ng/mL IL-3 and stained with anti-CD63-FITC or Av.A488. (D) Percentage of CD63⁺ (black) or Av.A488⁺ (green) basophils in whole blood of anonymous donors after incubation with RPMI containing 0, 2 or 20 ng/mL IL-3. Mean + SEM. Mann-Whitney U tests; ***P < .0005; *P < .05. (E) Correlation analysis between the percentage of Av.A488⁺ basophils and mean fluorescence intensity (MFI) of CD203c staining after anti-IgE or RPMI incubation in whole blood specimens from 41 peanut allergic patients. Nonparametric Spearman R correlation, we considered P < .05 as statistically significant. Each dot represents one patient analyzed.

Table E1. Demographic characteristics of patients with peanut allergy reported in Fig. 2 and Fig. E1, E.