

Letter to the Editor

Mass cytometry profiling the response of basophils and the complete peripheral blood compartment to peanut

To the Editor:

Anaphylaxis is a serious, life-threatening immediate hypersensitivity reaction that is caused by cross-linking of IgE bound to FcεRI on mast cells and basophils. However, it has been described that anaphylaxis can also be induced by alternative pathways, mediated by IgG-antigen immune complexes, FcγRIII, and basophils, neutrophils, and/or macrophages.¹⁻³ The availability of human data regarding the acute response to allergens by different leukocyte populations in allergic patients is scarce. Mass cytometry uses heavy metal-labeled antibodies and time of flight mass spectrometry for detection, allowing for simultaneous detection of more than 40 markers. This multiplexing capacity allows for simultaneous profiling of all hematopoietic cells, and provides a unique platform with which to comprehensively study the human immune response to allergens.

Whole blood from peanut-allergic patients ($n = 6$) or healthy controls ($n = 3$) was stimulated with peanut extract, anti-IgE, or media control for 15 or 30 minutes before fixation and red blood cell lysis. For details, see this article's **Methods** section in the Online Repository at www.jacionline.org. Samples were barcoded (Fluidigm, San Francisco, Calif), pooled, blocked with heparin, stained with cell surface and intracellular phosphoprotein antibodies listed in the Online Repository (see **Table E1** in this article's Online Repository at www.jacionline.org), and acquired on a CyTOF2 mass cytometer (Fluidigm). Following data normalization and debarcoding, CD45⁺ cell events were manually gated and analyzed using spanning-tree progression analysis of density-normalized events, an algorithm that organizes multidimensional single-cell data to clusters displayed as a 2-dimensional tree plot.^{4,5} Differences between groups were analyzed by Mann-Whitney *U* test. Data analysis was done by using Prism software (GraphPad, San Diego, Calif). Results are expressed as mean $\pm$ SEM, and a value of $P < .05$ was considered significant.

Identification of cell subsets on the spanning-tree progression analysis of density-normalized events tree was performed manually by iteratively evaluating the expression of specific cell markers (see **Fig E1** and **Table E2** in this article's Online Repository at www.jacionline.org). This approach unambiguously identified basophils, eosinophils, neutrophils, monocyte subsets, plasmacytoid DCs (pDCs), conventional DCs (cDCs), natural killer (NK) cell subsets, NK T cells, CD4 and CD8 T cells, and B cells. We compared the frequencies of populations with conventional flow cytometry in a separate group of subjects, and could identify the same subsets by both techniques (data not shown). Eosinophils and B cells were increased in frequency in peanut-allergic subjects versus healthy controls, whereas neutrophils were reduced (see **Fig E2** in this article's Online Repository at www.jacionline.org).

Basophils were clearly the most responsive cells in blood to peanut stimulation in peanut-allergic patients (**Fig 1, A**; see **Fig E3** in this article's Online Repository at www.jacionline.org). In addition to upregulation of CD63 (**Fig 1, A**), a conventional marker of basophil degranulation, basophils from peanut-

allergic subjects responded to peanut with regulation of IgE (FcεRI, CD23) and IgG (CD16, CD32) receptors, which could modulate the ability of basophils to respond to a second challenge. CD25 and CD38 were downregulated on peanut exposure in basophils (**Fig 1, A**). Basophils from healthy controls had minimal response to peanut stimulation. However, they responded similarly to anti-IgE stimulation (see **Fig E4** in this article's Online Repository at www.jacionline.org), indicating that they are able to respond to the same extent as those from allergic subjects. The response of peanut-allergic subjects to peanut stimulation was identical in pattern to the anti-IgE response.

Basophils from peanut-allergic patients but not healthy controls became strongly positive for CD61, a platelet-specific marker, after peanut or anti-IgE stimulation (**Fig 1, A and B**). In addition, there was an upregulation of CD141 (**Fig 1, A**), correspondent to thrombomodulin, a cofactor of thrombin expressed in platelets among other cell types. These results suggest that there is a physical interaction between basophils and platelets after activation. The same result was observed by conventional flow cytometry using another platelet-specific marker, CD42b (see **Fig E5** in this article's Online Repository at www.jacionline.org). Furthermore, we confirmed the physical interaction between basophils and platelets using imaging flow cytometry (**Fig 1, F**). Platelet-basophil complexes had substantially greater CD63 expression and phospho-p38 than free basophils (**Fig 1, C-E**). This suggests that either basophils must be activated to form complexes with platelets, or that the formation of complexes contributes to basophil activation. Although activated platelet adhesion to basophils has been described as a potential pitfall or artifact of basophil activation assays,⁶ we observed that platelet adhesion to basophils was observed only on basophils activated by allergen or anti-IgE. Furthermore, there was no evidence of CD63 expression by platelets not adherent to basophils. By imaging flow cytometry, we observed that CD63 signal was increased in the area of platelet adherence, suggesting that platelets contribute to CD63 expression (**Fig 1, F**). On the contrary, phospho-p38 signal was observed only within the basophils (**Fig 1, F**; see **Fig E6** in this article's Online Repository at www.jacionline.org). Platelet-neutrophil complexes have been shown to contribute to aspirin-exacerbated respiratory disease through cooperative production of leukotriene C4.⁷ We speculate that similar cooperative function between platelets and basophils *in vivo* may contribute to anaphylaxis, and that this platelet-basophil interaction may be biologically meaningful. It has been described that levels of the platelet-activating factor (PAF) correlate with severity of anaphylaxis and that deficiency of the enzyme that hydrolyzes PAF, the PAF acetylhydrolase, has been linked to death from anaphylaxis.⁸ The fact that platelets are the major target of PAF suggests the importance of platelets in anaphylactic responses.

After stimulation with peanut, basophils from peanut-allergic patients showed uniquely increased phosphorylation of pERK and p38 (**Fig 1, G**), which was not observed in any other cell population (**Fig E3**). There was also increased phosphorylation of S6 (**Fig 1, G**), which was also observed in monocytes, cDCs, and pDCs (**Fig 2, A and B**) of peanut-allergic subjects but not healthy controls in response to peanut, and in all subjects in response to anti-IgE. Although cDCs and pDCs express FcεRI (**Fig E1**), phospho-S6 in monocytes did not correlate with FcεRI or FcεRII

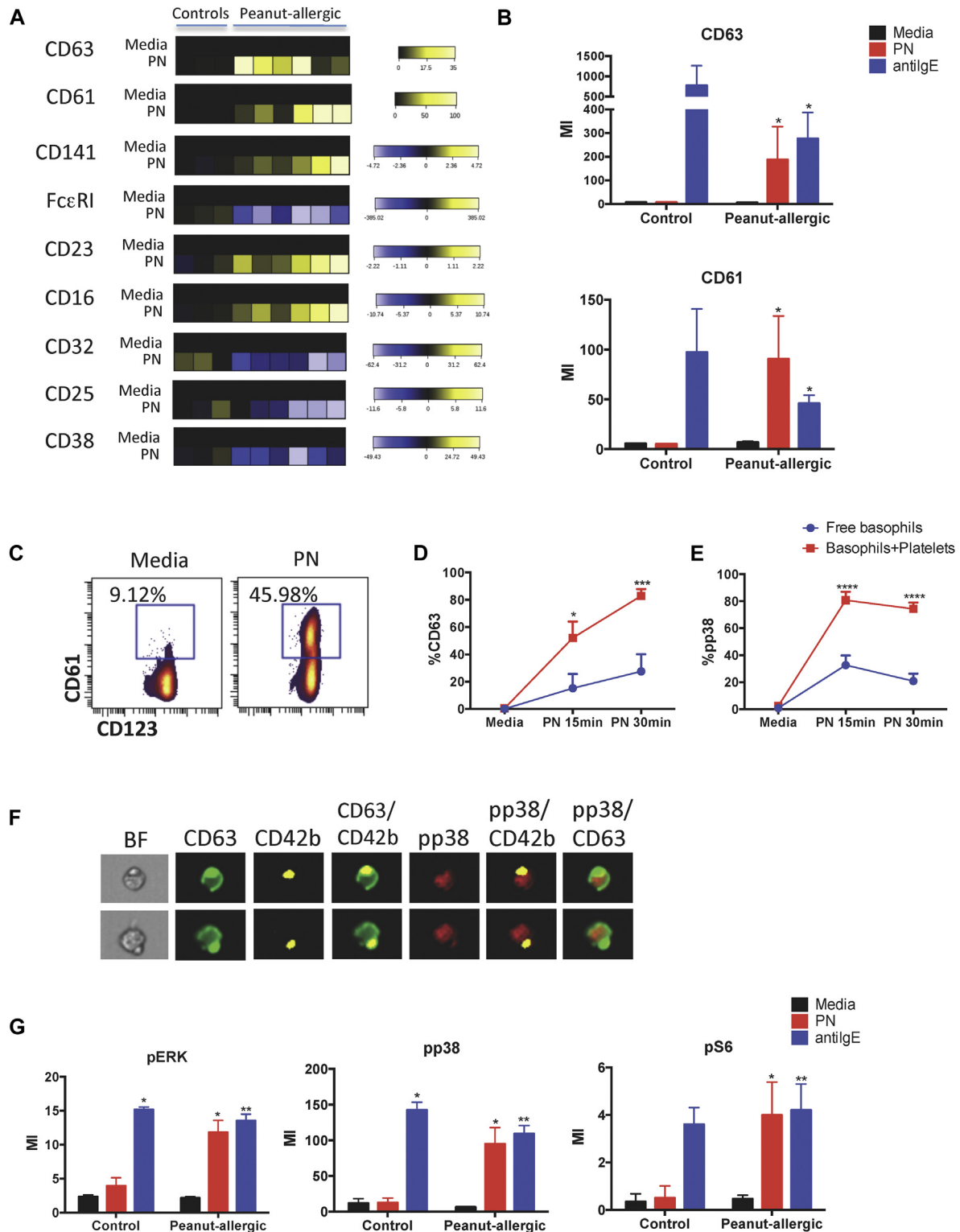


FIG 1. Basophil activation and association with platelets after peanut stimulation. **A**, Expression of each marker by basophils stimulated with peanut (Δ median intensity [MI] vs media control). Expression at 15 minutes is shown for all markers except FcεRI, CD32, and CD38, for which expression at 30 minutes is shown. **B**, Expression of CD63 and CD61 (MI) by basophils after 15-minute stimulation. * $P < .05$ vs media. **C**, Representative plot of CD61 expression (%) by basophils from peanut-allergic subjects. **D** and **E**, Expression of CD63 and pp38 (%) by free basophils (CD61[−]) or basophils + platelets (CD61⁺) from peanut-allergic patients. * $P < .05$, *** $P < .001$, **** $P < .0001$ vs free basophils. **F**, Imaging flow cytometry of basophil-platelet complexes from a healthy control stimulated with anti-IgE for 30 minutes. Two representative images of activated basophils are shown. **G**, Expression of p-ERK (15 minutes), pp38 (15 minutes), and pS6 (30 minutes) by basophils. BF, Bright field; PN, peanut extract. * $P < .05$, and ** $P < .01$.

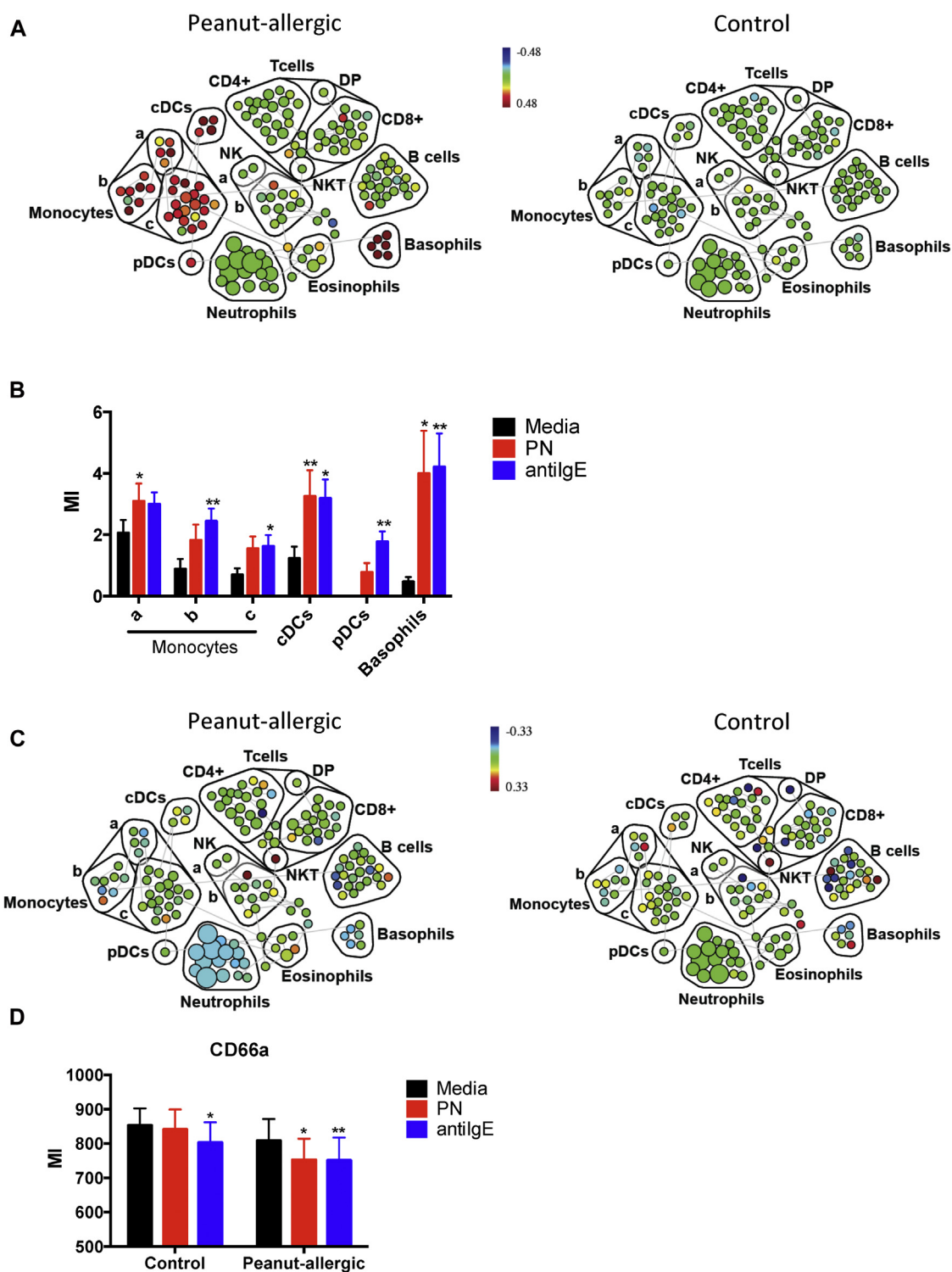


FIG 2. Myeloid cells are activated by peanut in peanut-allergic patients. **A**, Representative SPADE trees of pS6 expression by hematopoietic cells in a peanut-allergic subject and a healthy control after peanut stimulation. Colors represent fold change vs media control. **B**, Expression of pS6 (median intensity [MI]) by monocytes, pDCs, cDCs, and basophils from peanut-allergic patients after 30-minute stimulation. **C**, Representative SPADE trees of CD66a expression by the different cell populations in a peanut-allergic and a healthy control after peanut stimulation. Colors represent fold change vs media control. **D**, Expression of CD66a (MI) by neutrophils after 15-minute stimulation. Monocytes: a, CD11c⁺CD16⁺CD14⁺; b, CD11c⁺CD16⁺CD14⁻; c, CD11c⁺CD16⁻CD14⁺. NK cells: a, CD56bright; b, CD56dim. DP, Double positive; PN, peanut extract; SPADE, spanning-tree progression analysis of density-normalized events. **P* < .05, ***P* < .01 with respect to the media.

expression, suggesting that this may be a secondary response perhaps downstream of basophil activation. Human neutrophils have been shown to be activated by allergen-IgG complexes.⁹ We observed a very modest but significant downregulation of CD66a in neutrophils in response to not only peanut in peanut-allergic subjects but also anti-IgE (Fig 2, C and D). Because neutrophils do not express FcεRI, this may therefore be a secondary effect of basophil activation.

The major novel finding of this study is that basophils and platelets physically interact after peanut allergen exposure. The functional consequence of this interaction in anaphylaxis will be addressed in future studies. It is possible that by modulating the formation of these complexes anaphylaxis responses could be reduced in severity. Second, we observed that myeloid cells are activated on allergen exposure in allergic individuals. Additional studies are needed to determine whether these cells directly respond to antigen, and the consequence of this activation in anaphylaxis and antigen presentation.

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METHODS

Subjects and samples

Peanut-allergic subjects were recruited through the Jaffe Food Allergy Institute, and as controls we recruited healthy adult volunteers. The study and consent forms were approved by the Institutional Review Board at the Icahn School of Medicine at Mount Sinai. Blood was drawn in heparinized vacutainer tubes and used for experiments within 3 hours of blood draw.

Whole blood stimulation and antibody staining

All the antibodies used in this study were either purchased preconjugated from Fluidigm or were conjugated using X8 MaxPar conjugation kits (Fluidigm) according to the manufacturer's protocol. All antibodies were added after fixation except CD63 and CRTH2, which were added during stimulation.

Blood (1 mL/condition) was added to 1 mL of RPMI and stimulated with 1 μ g/mL of peanut extract, 1 μ g/mL of anti-IgE (Bethyl Laboratories, Montgomery, Tex), or media control for 15 or 30 minutes at 37°C in the presence of 2 ng/mL of IL-3 (R&D Systems, Minneapolis, Minn). Then, the samples were fixed and lysed using BD Phosflow Lyse/Fix Buffer (BD Biosciences, San Diego, Calif) and barcoded with the Cell-ID 20-Plex Pd Barcoding Kit (Fluidigm) following manufacturer's instructions.

Samples were pooled and blocked with 100 U/mL of heparin to inhibit nonspecific binding to eosinophils,^{E1} and stained with a cocktail of metal-conjugated antibodies for surface staining for 20 minutes at room temperature. Then, samples were first washed and permeabilized with ice-cold methanol for 30 minutes and then washed and stained with intracellular phosphoprotein antibodies for 30 minutes on ice, with prior heparin blocking.

After washing, the samples were incubated with 0.125 nM Ir nucleic acid intercalator (Fluidigm) to enable cell identification based on DNA content, and stored in PBS with freshly diluted 2% formaldehyde (Electron Microscopy Sciences, Hatfield, Pa) until acquisition.

Data acquisition and analysis

Immediately before acquisition, the samples were washed once with PBS, once with deionized water, and resuspended at a concentration of 600,000 cells/mL in water containing a 1/20 dilution of EQ 4 element beads (Fluidigm). Following routine autotuning according to the manufacturer's recommendations, the samples were acquired on a CyTOF2 mass cytometer (Fluidigm) using a SuperSampler (VictorianAirships, Alamo, Calif) fluidics system at a flow rate of 0.045 mL/min. For quality control, the acquisition event rate was maintained under 400 events/s, and the EQ beads were confirmed to have a median Eu151 intensity of more than 1000 to ensure appropriate mass sensitivity. The resulting FCS files were concatenated, normalized, and debarcoded using the bead-based normalization and debarcoding tools in the CyTOF2 software and uploaded to Cytobank for analysis. Cells events were identified as Ir191/193 DNA + Ce140 – events, and doublets were excluded on the basis of higher DNA content and longer event length.

Imaging flow cytometry

Whole blood from healthy controls was stimulated with anti-IgE for 30 minutes and processed as described above. All antibodies were from BioLegend (San Diego, Calif) unless otherwise stated. After lysis/fixation, cells were stained with anti-CD123 (6H6), anti-Bdca2 (201A), anti-CD42b (HIP1), and anti-CD63 (H5C6). Cells were permeabilized with methanol and stained with phospho-p38 (4NIT4KK; eBioscience, San Diego, Calif). Before acquisition, nuclei were stained with Hoechst (BD Pharmingen, San Jose, Calif). Imaging flow cytometry was performed by acquiring cells in the Image Stream 100 (Amnis, Seattle, Wash) imaging flow cytometry. Complexes between basophils and platelets and the expression of the different markers were visualized using the IDEAS software (Amnis).

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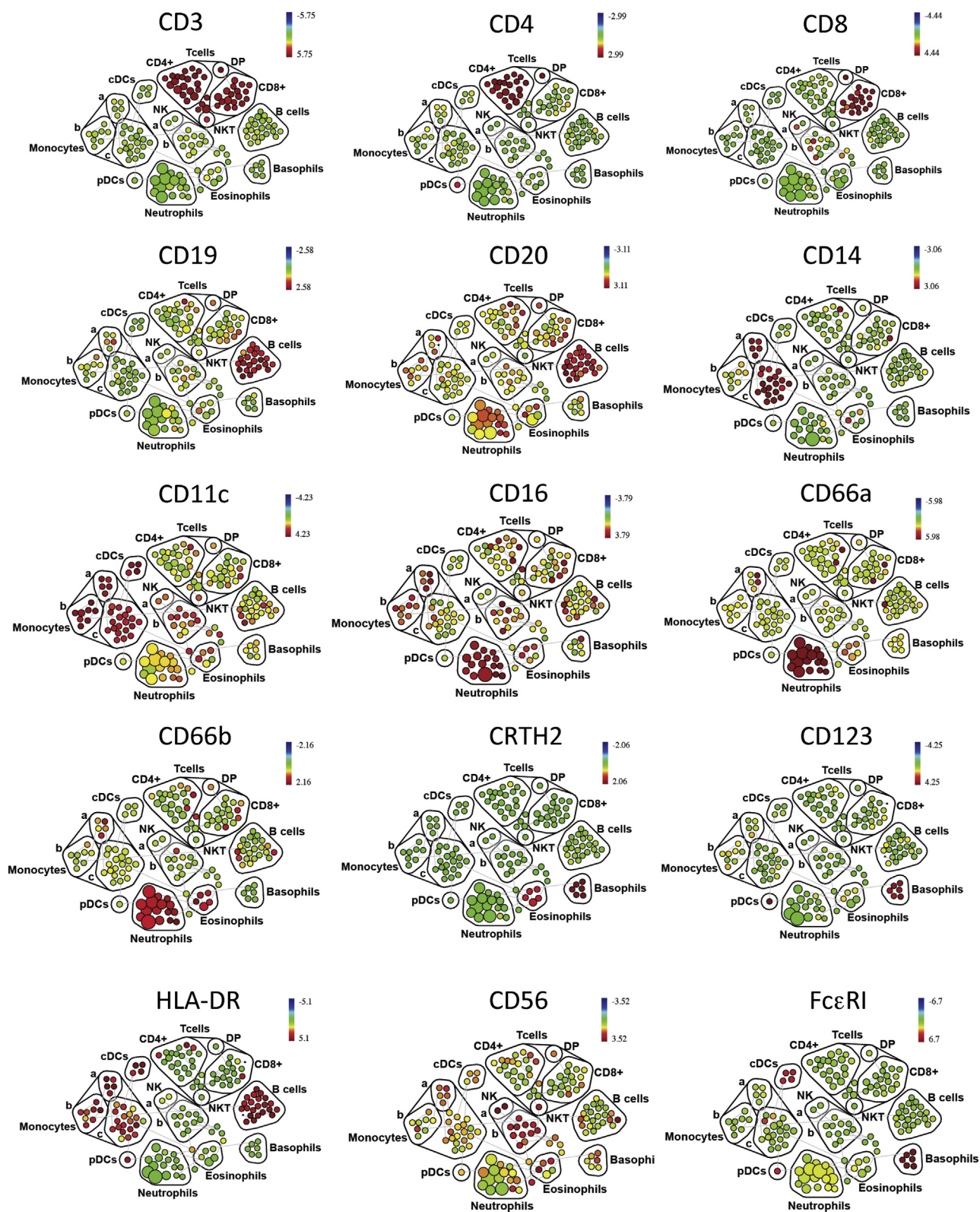


FIG E1. Markers used for gating strategy in SPADE representation. Colors represent MI. Monocytes: a, CD11c⁺CD16⁺CD14⁺; b, CD11c⁺CD16⁺CD14⁻; c, CD11c⁺CD16⁻CD14⁺. NK cells: a, CD56bright; b, CD56dim. DP, Double positive; MI, median intensity; SPADE, spanning-tree progression analysis of density-normalized events.

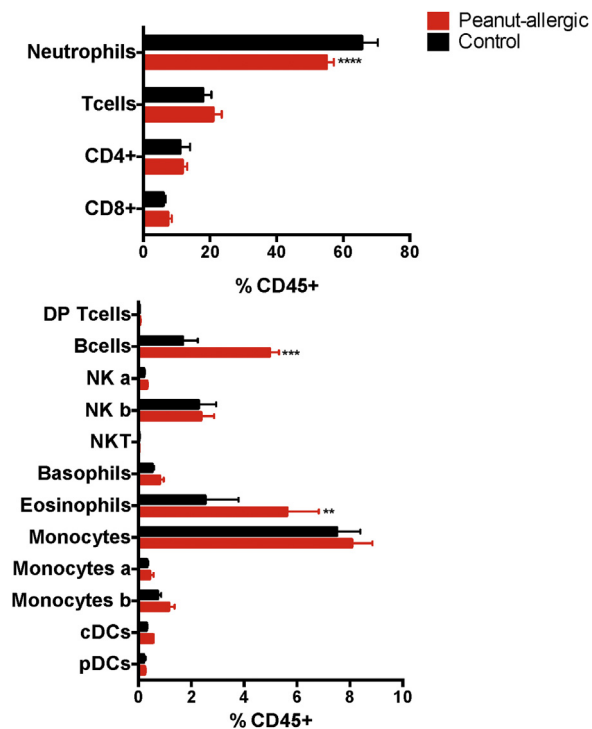


FIG E2. Percentage of each cell population in peripheral blood from peanut-allergic patients or healthy controls. *DP*, Double positive. ** $P < .01$, *** $P < .001$, **** $P < .0001$ vs healthy controls.

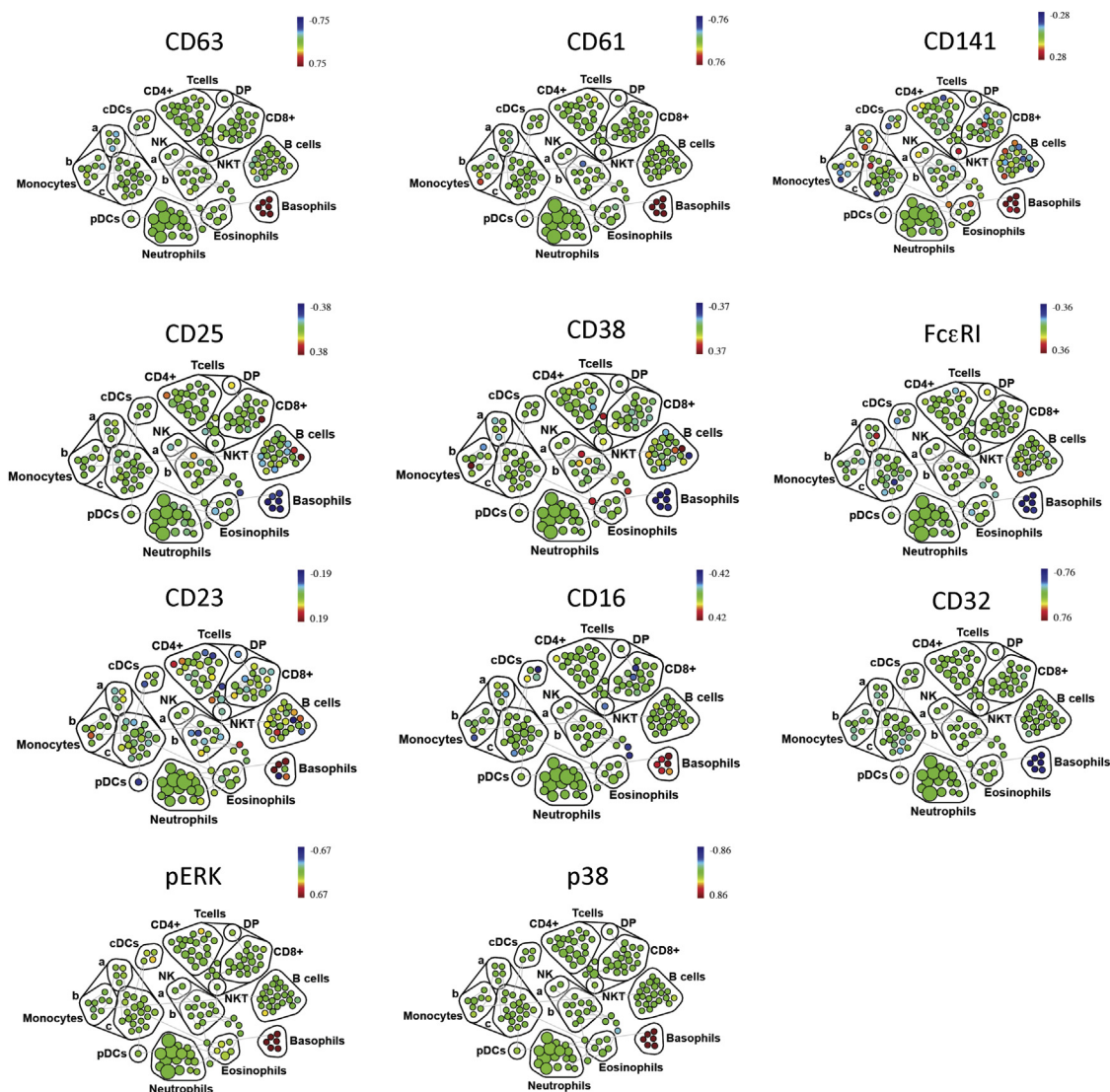


FIG E3. Expression of markers by the different cell populations from a peanut-allergic patient. Representative SPADE trees of a peanut-allergic patient. Colors represent fold change in MI vs media. Monocytes: a, CD11c⁺CD16⁺CD14⁺; b, CD11c⁺CD16⁺CD14⁻; c, CD11c⁺CD16⁻CD14⁺. NK cells: a, CD56bright; b, CD56dim. DP, Double positive; MI, median intensity; SPADE, spanning-tree progression analysis of density-normalized events.

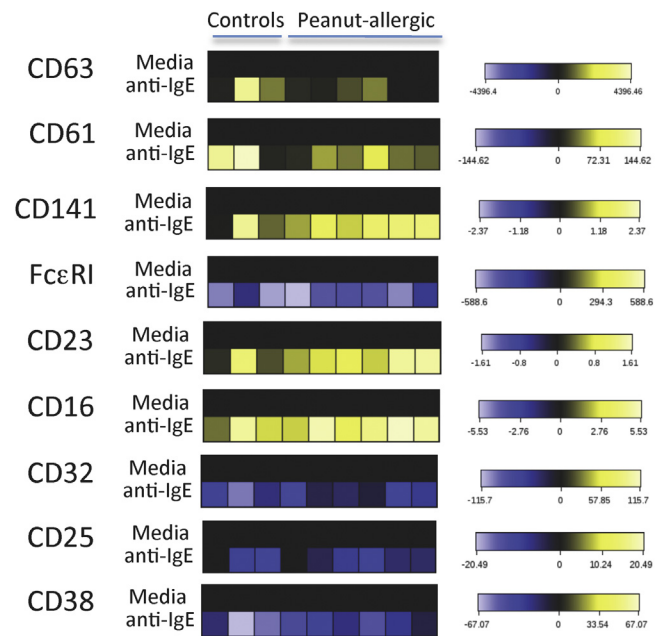


FIG E4. Basophil activation after anti-IgE stimulation. Expression of each marker by basophils stimulated with anti-IgE (Δ median intensity [MI] vs media control). Expression at 15 minutes is shown for all markers except FcεRI, CD32 and CD38, for which expression at 30 minutes is shown.

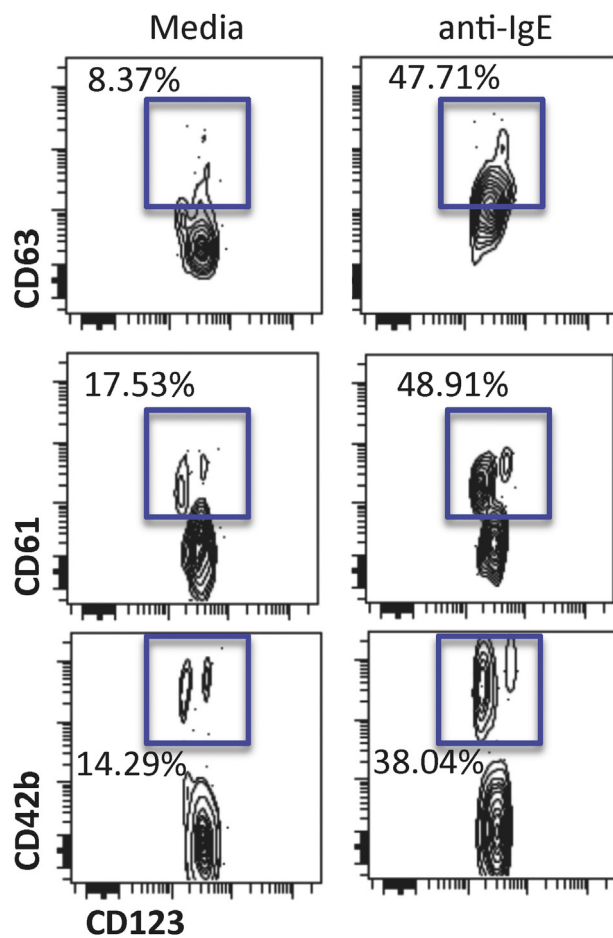


FIG E5. Basophils upregulate CD63 and the platelets markers CD61 and CD42b after anti-IgE stimulation. Representative plots of the expression of CD63, CD61, and CD42b by basophils from a healthy volunteer after stimulation with anti-IgE for 30 minutes.

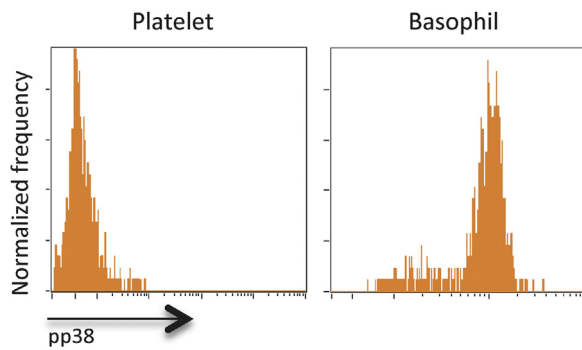


FIG E6. Expression of phospho-p38 by platelets or activated basophils. Representative plots of peripheral blood from a healthy donor stimulated with anti-IgE for 30 minutes and measured by imaging flow cytometry. Expression of p38 was assayed by applying cell-specific masks on the platelet-basophil complexes.

TABLE E1. Antibody information

Antibody target	Metal tag
CD45	154Sm
CD19	142Nd
CD4	145Nd
CD8	146Nd
CD16	148Nd
CD123	151Eu
CD66b	113In
CD14	160Gd
CD56	173Yb
CD25	169Tm
CD3	170Er
CD38	172Yb
HLA-DR	174Yb
CD61	150Nd
CD141	144Nd
CD20	147Sm
CD66a	149Sm
CD23	153Eu
p-p38	156Gd
CD11c	159Tb
CD32	162Dy
CRTH2	163Dy
FcεRI	165Ho
CD63	166Er
p-ERK	171Yb
p-S6	175Lu
HLA-ABC	115In
CD45RA	143Nd
CD27	155Gd
IκBa	164Dy

TABLE E2. SPADE gating strategy (SPADE done on CD45⁺ cells)

Cell type	Markers
T cells	CD3 ⁺
CD4 ⁺ T cells	CD3 ⁺ CD4 ⁺ CD8 ⁻
CD8 ⁺ T cells	CD3 ⁺ CD4 ⁻ CD8 ⁺
DP T cells (double positive)	CD3 ⁺ CD4 ⁺ CD8 ⁺
NK T cells	CD3 ⁺ CD4 ⁻ CD8 ⁻ CD56 ⁺
B cells	CD3 ⁻ CD19 ⁺ CD20 ⁺ HLA-DR ⁺
Basophils	CD3 ⁻ CD19 ⁻ CD123 ⁺ HLA-DR ⁻ CRTH2 ⁺ FcεRI ⁺
pDCs	CD3 ⁻ CD19 ⁻ CD123 ⁺ HLA-DR ⁺ CRTH2 ⁻
Neutrophils	CD3 ⁻ CD19 ⁻ CD123 ⁻ CD16 ⁺ CD66a ⁺ CD66b ⁺ CRTH2 ⁻
Eosinophils	CD3 ⁻ CD19 ⁻ CD123 ⁻ CD16 ^{low} CD66b ⁺ CRTH2 ⁺
cDCs	CD3 ⁻ CD19 ⁻ CD123 ⁻ CRTH2 ⁻ CD11c ⁺ CD14 ⁻ FcεRI ⁺ HLA-DR ⁺
Monocytes	CD3 ⁻ CD19 ⁻ CD123 ⁻ CRTH2 ⁻ CD11c ⁺ FcεRI ⁻ HLA-DR ⁺
Monocytes a	CD3 ⁻ CD19 ⁻ CD123 ⁻ CRTH2 ⁻ CD11c ⁺ CD16 ⁺ CD14 ⁺ HLA-DR ⁺
Monocytes b	CD3 ⁻ CD19 ⁻ CD123 ⁻ CRTH2 ⁻ CD11c ⁺ CD16 ⁺ CD14 ⁻ HLA-DR ⁺
Monocytes c	CD3 ⁻ CD19 ⁻ CD123 ⁻ CRTH2 ⁻ CD11c ⁺ CD16 ⁻ CD14 ⁺ HLA-DR ⁺
NK cells	CD3 ⁻ CD19 ⁻ CD123 ⁻ CRTH2 ⁻ CD14 ⁻ CD66b ⁻ CD56 ⁺
NK cells a	CD3 ⁻ CD19 ⁻ CD123 ⁻ CRTH2 ⁻ CD14 ⁻ CD66b ⁻ CD56 ^{bright}
NK cells b	CD3 ⁻ CD19 ⁻ CD123 ⁻ CRTH2 ⁻ CD14 ⁻ CD66b ⁻ CD56 ^{dim}

SPADE, Spanning-tree progression analysis of density-normalized events.