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Régis Joulia, PhD, Claire Mailhol, MD, Salvatore Valitutti, MD, Alain Didier, MD, Eric Espinosa, PhD



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Title:

Direct monitoring of basophil degranulation by using avidin-based probes

Régis Joulia, PhD¹, Claire Mailhol, MD², Salvatore Valitutti, MD¹, Alain Didier, MD² and Eric Espinosa, PhD¹

1 Inserm, U1043, Toulouse, F-31300, France; Université de Toulouse, UPS, Centre de Physiopathologie de Toulouse Purpan (CPTP), Toulouse, F-31300, France.

2 Pôle des Voies Respiratoires, CHU de Toulouse, France.

Corresponding author

Eric Espinosa, PhD
INSERM U1043
CPTP CHU Purpan
BP3028
31024 Toulouse, FRANCE
eric.espinosa@inserm.fr
☎ +33 (0)562 748 304
Fax: +33 (0)562 744 558

Capsule Summary:

We show that fluorescent avidin binds to basophil cell surface upon degranulation and can be used to set up a new basophil activation test (BAT). This new assay provides results comparable to those provided by the CD63 exposure-based BAT, but has the advantage to directly monitor granule exteriorization.

Key words:

Basophil degranulation; avidin; BAT (basophil activation test); allergy

This work was supported by INSERM.

Abbreviations used

Ab, antibody; BAT, basophil activation test; IFI, Integrated fluorescence intensity; gMFI, geometric mean fluorescence intensity; pDC, plasmacytoid dendritic cell; WBC, white blood cell.

To the Editor:

Basophils are multifunctional effector cells involved in allergic and inflammatory reactions¹. Basophil degranulation is classically monitored by measuring the amount of mediators (such as histamine) that are released in the extracellular milieu. Alternative methods based on measurement of basophil degranulation using flow cytometry are currently used in clinics²⁻⁴. Most of those available commercial basophil activation tests (BATs) rely on the exposure of CD63 or on the upregulation of CD203c on the basophil surface upon allergen challenge⁵. Yet, these assays do not provide a direct measure of the granule exteriorization process and their results do not always correlate with histamine release². To make a step further, we set up a method that allows to measure the final step of the degranulation process (granule exteriorization) in individual basophils. We have previously shown that fluorescent avidin (that binds the negatively charged proteoglycans composing granule matrix) can be employed to stain exteriorized granules on the degranulated mast cell surface and to quantify degranulation by flow cytometry^{6, 7}. Similarly to mast cells, the basophil granule matrix is composed of negatively charged proteoglycans. We thus investigated whether avidin could be used to monitor basophil degranulation.

IgE-sensitized basophils were stimulated with anti-IgE Abs. Avidin-sulforhodamine (Av.SRho) was added to the incubation medium to monitor degranulation dynamics by time-lapse confocal laser scanning microscopy. Five minutes after stimulation, basophils underwent morphologic changes (e.g. increase of cell perimeter and cell spreading) while exteriorized granules were detected on the cell surface (Fig. 1A, Video E1 and Fig. E1A). The Av.SRho integrated fluorescence intensity (IFI) of the degranulating basophil augmented progressively to reach a plateau 15 min after stimulation (Fig. E1B). Taken together, these results show that the addition of Av.SRho to the culture medium allows to monitor basophil degranulation.

To directly measure basophil specific activation among white blood cells (WBCs), we analyzed fluorescent avidin binding by flow cytometry. Freshly isolated peripheral WBCs from healthy donors were stimulated with anti-IgE Abs. 20 minutes after stimulation, cells were stained with avidin-A488 (Av.A488), anti-CD203c, anti-FcεRI, anti-CD123 and anti-CD63 mAbs and analyzed by flow cytometry. Basophils were identified as CD203c⁺ FcεRI⁺ CD123⁺ cells. Degranulated basophils stained positive for Av.A488 (Fig. 1B). Analysis of the degranulation as assessed by measurement of CD63 exposure or Av.A488 binding provided similar results, by showing that a substantial fraction of the basophil population degranulated for 10 donors in 11 (Fig 1C-D). In addition, we analyzed in parallel avidin staining, CD63 staining and histamine released from purified basophils. Scatter plot representations showed a good correlation between the amount of histamine released and the percentage of avidin⁺ or CD63⁺ cells. This analysis shows that fluorescent avidin binding matches the amount of histamine

released (Fig. 1E). We next compared Av.A488 staining to CD203c upregulation on basophil surface following stimulation. Basophils from two donors in 18 did not respond to anti-IgE stimulation as measured by both methods (Fig. E2). Noticeably, we observed a discrepancy among the two methods in two donors (donor #1 and #6) with an increase of avidin staining and no difference in CD203c staining before and after stimulation (Fig. E2). To analyze the relative increase of MFI for the two staining procedures, we took advantage of the two non-responder donors (donors #2 and #17) to set the threshold of these tests to rMFI=0.1. This analysis showed that the rMFI calculated using the avidin-based assay did not correlate with rMFI calculated using the CD203c-based assay. In other words, the two methods do not identify the same donors as high responder donors. The discrepancies observed when comparing CD203c- and avidin-based assays could be explained by the fact that avidin staining is strictly associated to degranulation whereas CD203c is a more generic marker of activation. Because priming factors such as IL-3 could impact CD203c upregulation, we investigated whether IL-3 pretreatment affects avidin staining. As shown in Fig E3, IL-3 used alone or in combination with anti-IgE stimulation did not affect avidin staining (Fig. E3).

Since other immune cells might be activated following stimulus application to the whole WBC population, we initially investigated whether other cells might bind avidin. In the same set of experiments, we analyzed FcεRI⁺ monocytes and plasmacytoid dendritic cells (pDC) that are present in the low SSC gate. We observed no avidin-A488 staining on FcεRI^{low} monocytes and on CD203c⁺ CD123⁺ FcεRI⁺ pDC (Figure E4). In a second approach, we analyzed avidin binding on the two other granulocyte populations, i.e. neutrophils (FSC^{high} SSC^{high} CD16^{high}) and eosinophils (FSC^{high} SSC^{high} CD16⁺ FcεRI⁺) (Fig. E4). We did not detect Av.A488⁺ neutrophils following PMA/ionomycin stimulation for 9 donors in 14 and a very low percentage of slightly Av.A488⁺ neutrophils for 5 donors in 14 (Fig. E5B-C). We observed that a small fraction of the eosinophils stained dimly for avidin-A488 for all the donors upon PMA/ionomycin stimulation only (Fig. E5D-E).

We next tested the avidin-based method on blood samples from patients with confirmed grass pollen allergy. Patients' WBC were challenged with an array of allergens and analyzed using the three methods (avidin, CD63 or CD203c staining). The avidin-based method allowed to efficiently detect patients' response to allergens and provided results comparable to those obtained using CD63 and CD203c staining (Fig 2B-D). Interestingly, when compared to CD203c staining, avidin binding appeared to be a more sensitive method to evaluate the extent of degranulation in response to different allergens (Fig. 2D). The analysis of avidin rMFI upon different allergen challenges (either single allergens or allergen mixtures) allowed us to establish the response pattern of each patient (Fig 2E). Avidin rMFI and Prick-test values obtained following 5-grass mix stimulation showed significant correlation (Fig. 2F). Moreover, avidin and CD63 rMFIs showed an excellent correlation

(Fig. 2G) confirming that these two assays exhibit similar sensitivity for the diagnosis of allergic patients.

In conclusion, we described a new test easy to perform that provides similar results when compared to routinely used CD63-based test. It has been reported that CD63 expression does not always correlate with histamine release^{2, 8, 9}. Accordingly we observed, in mast cells, that degranulation as detected by avidin binding (but not by CD63 staining) correlates with β -hexosaminidase release⁷. Additional caveats might apply to CD63-based assays. First, CD63 exposure upon stimulation is not restricted to basophils, underlying the necessity of accurate gating strategies. Second, platelets express CD63 and can bind to basophils upon activation, thus providing a source of potential artifact².

Finally, the avidin-based method is a suitable alternative to current methods. Its advantage resides in the fact that avidin directly stains cell-bound granules upon degranulation and that the Av.A488 FI provide a measure of the degranulation magnitude.

Authors:

Régis Joulia, PhD¹, Claire Mailhol, MD², Salvatore Valitutti, MD¹, Alain Didier, MD² and Eric Espinosa, PhD¹

1 Inserm, U1043, Toulouse, F-31300, France; Université de Toulouse, UPS, Centre de Physiopathologie de Toulouse Purpan (CPTP), Toulouse, F-31300, France.

2 Pôle des Voies Respiratoires CHU de Toulouse

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Figure Legends

Fig 1. Fluorescent avidin allows to monitor basophil degranulation. (A), Sequence of snapshots from confocal videomicroscopy of IgE-sensitized basophil stimulated with anti-IgE Abs. B-D, flow cytometry analysis of basophil stimulated with anti-IgE Abs. Gating strategy and representative FACS profiles (B). Percentages of av.A488⁺ or CD63⁺ basophils (C) and gMFI of gated basophils following Av.A488 or CD63 stainings (D). Percentages in the FACS profiles indicate the frequency of gated cells. Each point represents a donor, bars represent median. Two-tailed paired *t*-test, *** <0.001 , **** <0.0001 . (E) Correlation between avidin or CD63-based assays and histamine release following stimulation with 1 $\mu\text{g/mL}$ (circles), 2.5 $\mu\text{g/mL}$ (square) and 5 $\mu\text{g/mL}$ (diamonds) anti-IgE, pooled data from 7 healthy donors, Spearman rank correlation.

Fig 2. Analysis of basophil degranulation in allergic patients. (A-D), 1×10^6 WBC were stimulated or not with anti-IgE Abs (2.5 $\mu\text{g/mL}$) or with indicated allergen preparations. Basophils were gated as in Fig 1, representative FACS profiles (A), Avidin⁺ basophil frequency (B), CD63⁺ basophil frequency (C) and CD203c or avidin fluorescence rMFI following stimulation (D). (E) avidin rMFI and Prick test results from tested patients. (F-G) Correlation analysis between Prick test results and avidin rMFI (F) or CD63 rMFI (G) for 5 grasses mix stimulation, Spearman rank correlation.

Figure 1

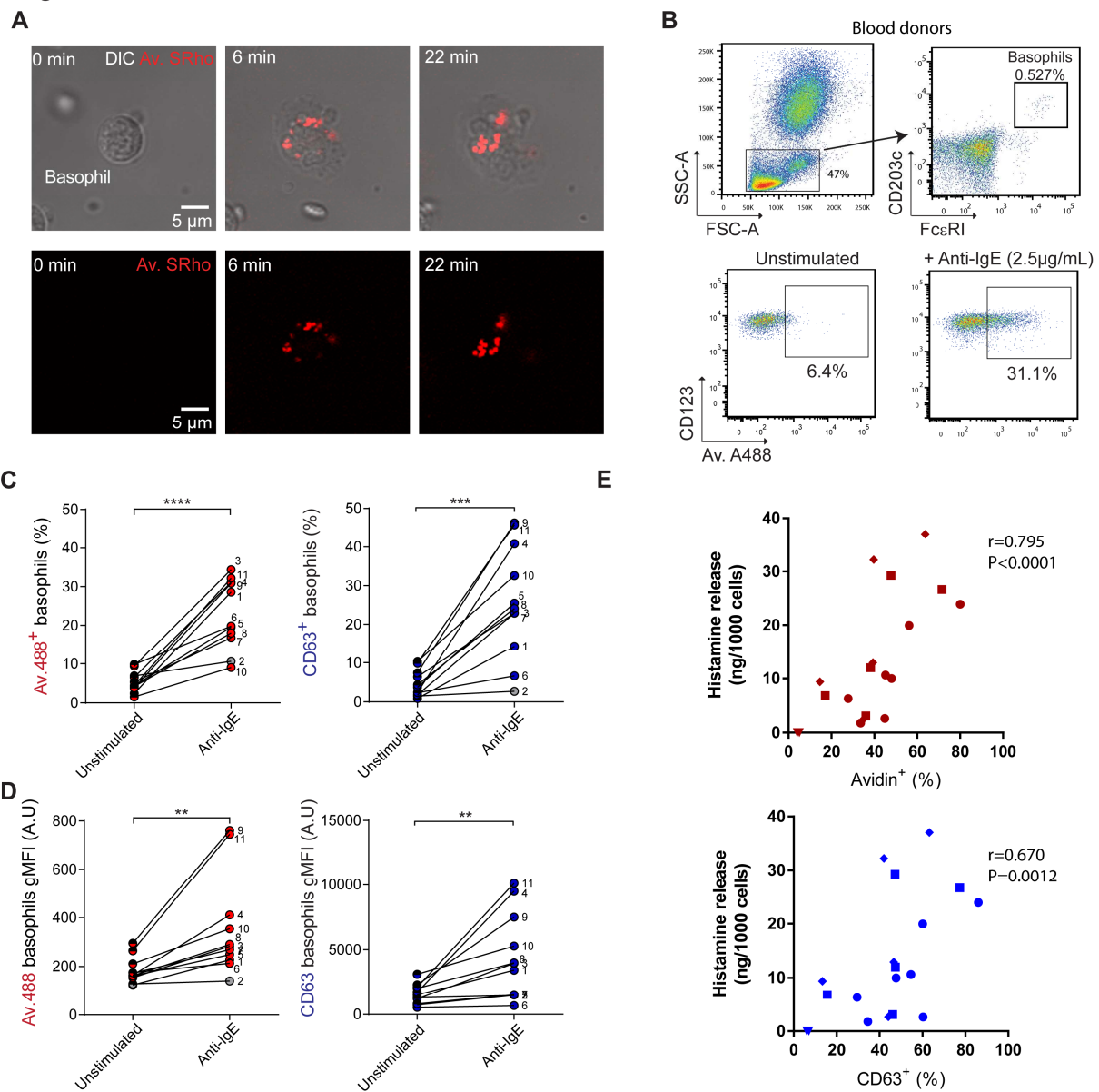


Figure 2

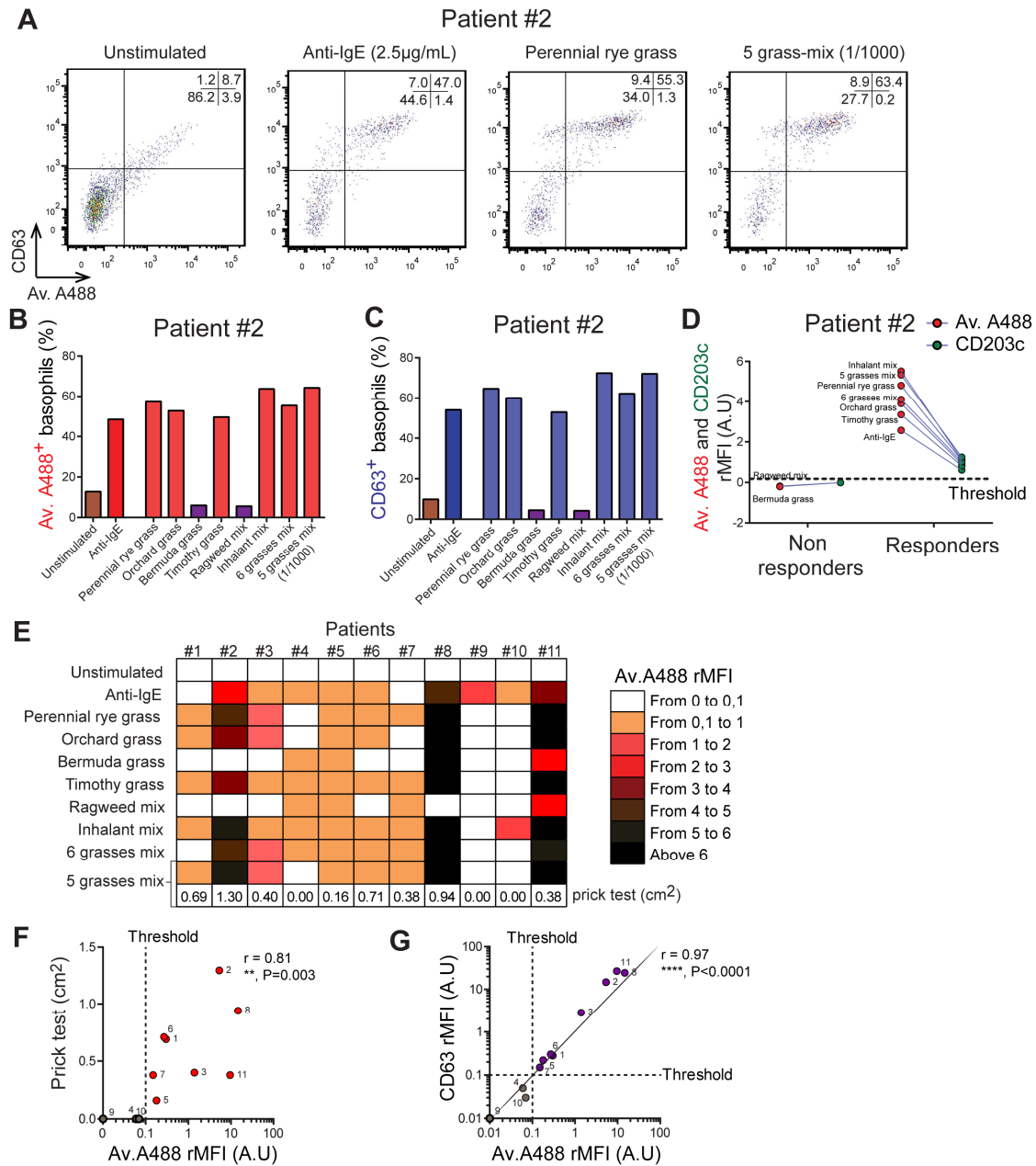


Figure E1

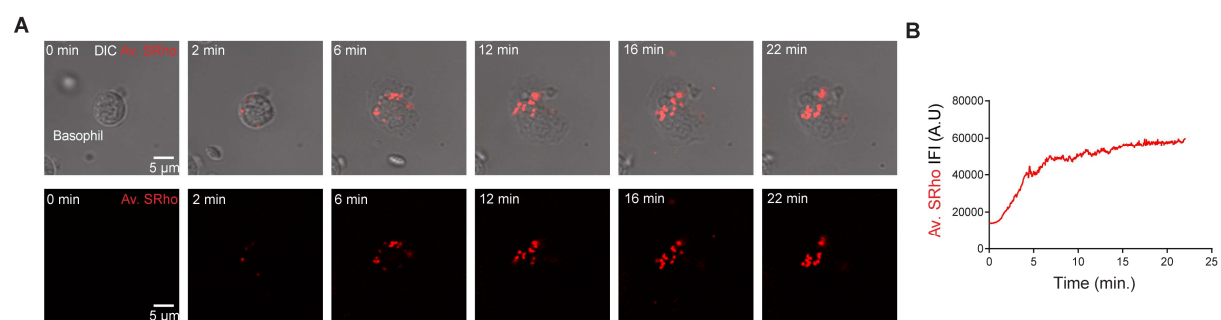


Figure E2

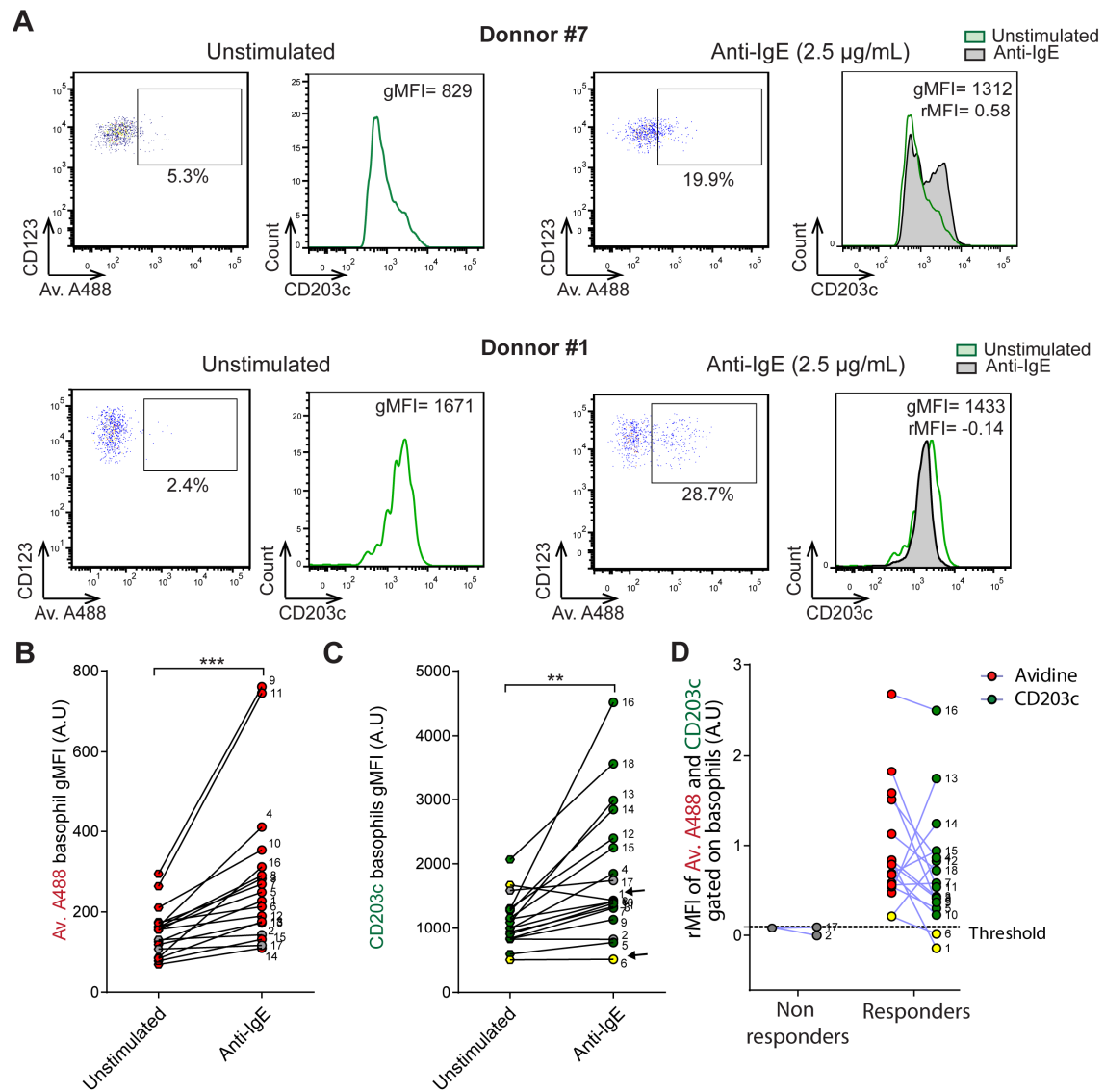


Figure E3

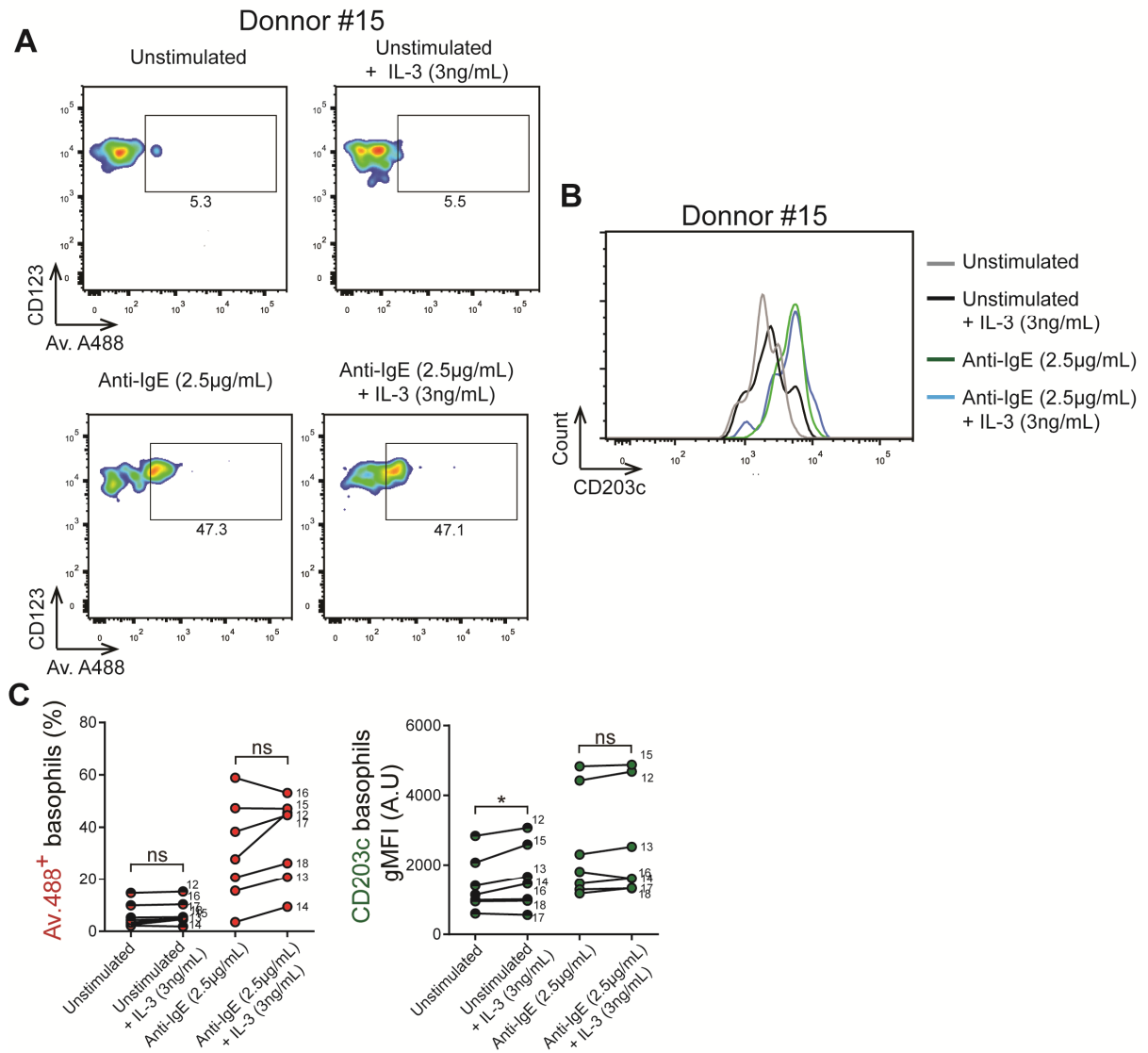


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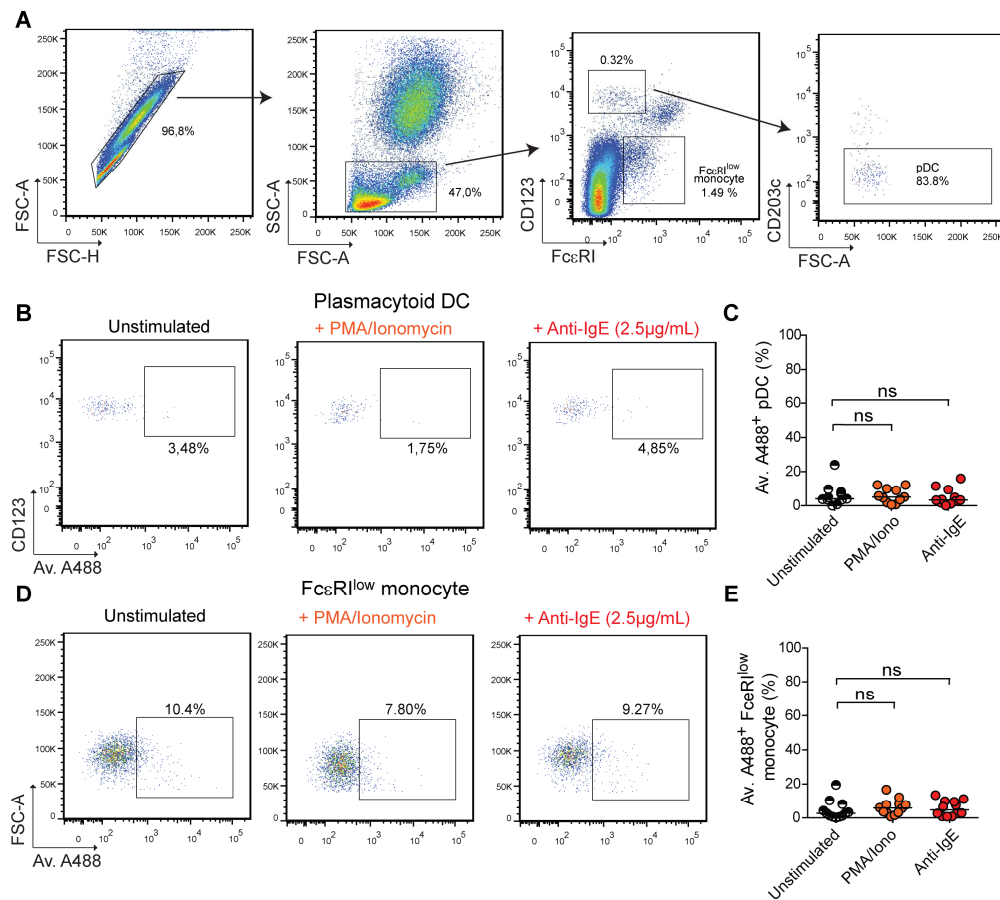
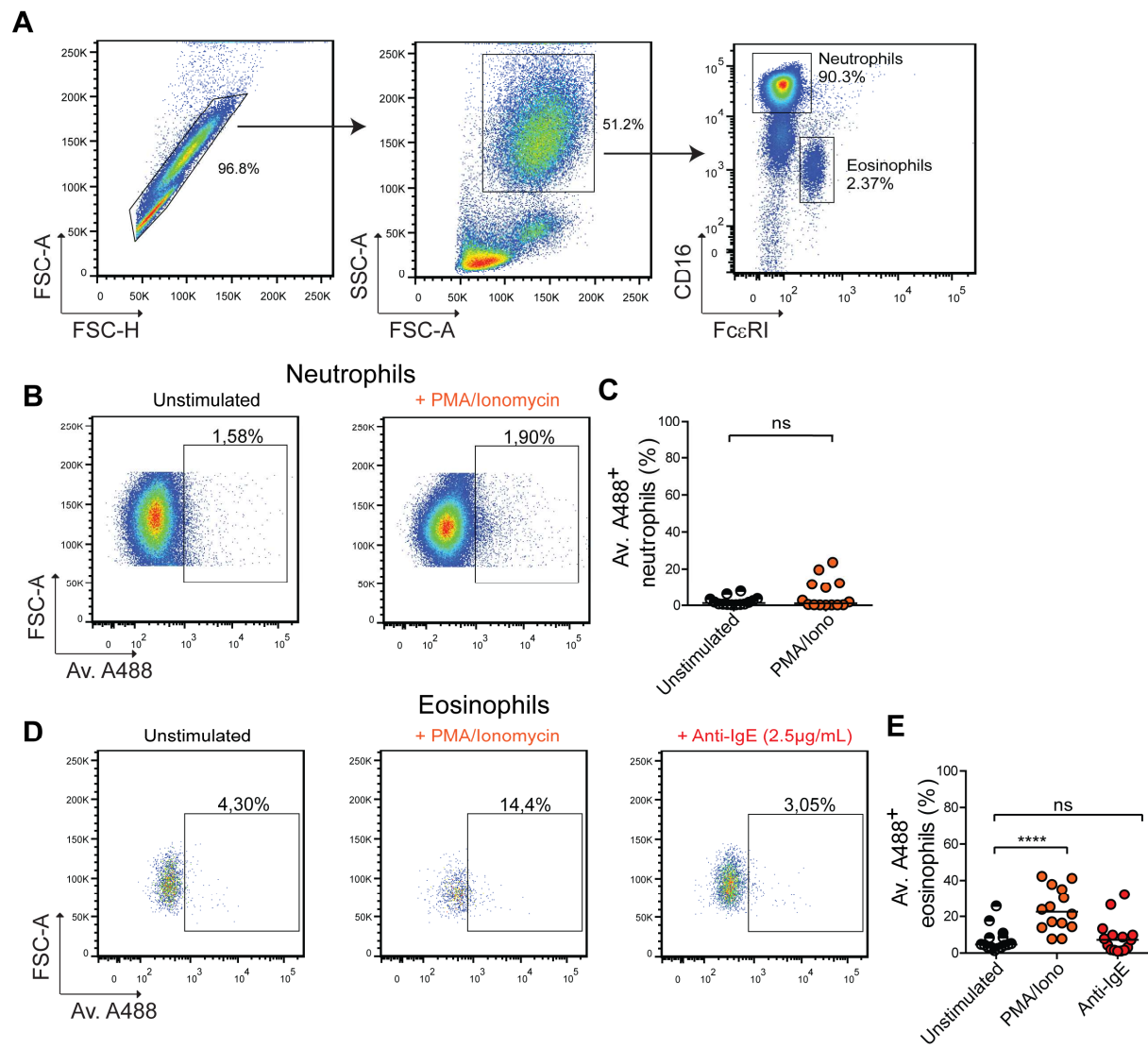


Figure E5



Appendix

Supplementary figure Legends

Fig E1. Avidin binds to human basophil cell surface following activation. **A**, Sequence of snapshots from time lapse analysis of IgE-sensitized basophil stimulated with anti-IgE Abs. **B**, Single cell analysis of Av.SRho Integrated FI. Data are from one representative experiment out of three.

Fig E2. Avidin staining is not always correlated with the CD203c upregulation-based assay. 1×10^6 Human WBC were stimulated or not with anti-IgE Abs ($2.5\mu\text{g/mL}$). Basophils were gated as in Fig 1. **A**, Representative FACS profiles of donor #7 and #1. **B-C**, gMFI of Av. A488 (B) and CD203c (C) staining on basophils (pooled data, $n=18$), arrows indicate discordant results for donors #1 and #6 (yellow circles). **D**, rMFI of Av. A488⁺ (red) and CD203c (green) (pooled data, $n=18$). Threshold (rMFI=0.1, dashed line) was determined using non responder donors (grey circles). Percentages in the FACS profiles indicate the frequency of gated cells. Each point represents a donor, Arrows indicate donor #13. Two-tailed paired *t*-test, ** <0.01 *** <0.001 .

Fig E3. IL-3 priming did not affect avidin staining. 1×10^6 Human WBC were stimulated or not with anti-IgE Abs ($2.5\mu\text{g/mL}$) in absence or in presence of IL-3 (3ng/mL). Basophils were gated as in Fig 1. (A-B), Representative FACS profiles of donor #15. (C-D) Percentages of Av. A488⁺ basophil (C) and CD203c gMFI (D) (pooled data, $n=7$). Percentages in the FACS profiles indicate the frequency of gated cells. Each point represents a donor. Two-tailed paired *t*-test, ns >0.05 * <0.05 .

Fig E4. pDC and FcεRI^{low} monocyte are not stained with avidin upon stimulation. 1×10^6 Human PWBC were stimulated or not with either PMA/Ionomycin or anti-IgE mAb ($2.5\mu\text{g/mL}$). (A) Gating strategy used to isolate pDC and FcεRI^{low} monocyte. Doublets were excluded using FSC-A and FSC-H parameters, then low SSC-A and FSC-A cells were gated; FcεRI^{low} monocyte and CD123⁺ cells were selected. To further separate pDC from basophil, pDC were gated as CD203c⁻ cells. Representative FACS profiles of gated CD123⁺ pDCs (B) or FcεRI^{low} monocytes (D) for Av.A488 staining following stimulation with PMA/Ionomycin or anti-IgE. Percentages of Av.488⁺ pDCs (C) or FcεRI^{low} monocytes (E) from pooled data $n=11$. Percentages in the FACS profiles indicate the frequency of gated cells. Each point represents a donor, bars represent median. Two-tailed paired *t*-test, ns $P>0.05$.

Fig E5. A small fraction of eosinophils or neutrophils stained dimly positive for avidin following stimulation. 1×10^6 Human PWBC were stimulated or not with either PMA/Ionomycin or anti-IgE Abs (2.5 µg/mL). (A) Gating strategy used to isolate neutrophils and eosinophils. Doublets were excluded using FSC-A and FSC-H parameters, CD16^{high}/FcεRI⁺ neutrophils and CD16⁺/FcεRI⁺ eosinophils were gated from high SSC-A and FSC-A cells. Representative FACS profiles of gated neutrophils (B) or eosinophil (D) for Avidin-A488 staining following stimulation with PMA/Ionomycin or anti-IgE. Percentages of Av.488⁺ neutrophils (C) or eosinophils (E) from pooled data n=14. Percentages in the FACS profiles indicate the frequency of gated cells. Each point represents a donor, bars represent median. Two-tailed paired *t*-test, ns $P > 0.05$, **** $P < 0.0001$.

Video E1. Basophil degranulation following stimulation with anti-IgE Abs. (Video E1 corresponds to FIG 1 and E1). IgE-sensitized basophils stimulated with 2.5 µg/mL anti-IgE Abs (added at $t=0$) in the presence of 8 µg/mL Av.SRho were inspected using time-lapse confocal microscopy. Image sequences of the time-lapse recording were processed using Zen software. This video is from one representative experiment out of five. Bars, 5 µm

Methods:

Reagents. Primary antibodies used for immunostaining: anti-FcεRI efluor[®] 450 (clone AER-37, eBioscience), anti-CD203c BV510 (2.5 µL per test, clone NP4D6, BD Biosciences), anti-CD123 PE-Cy5 (clone 9F5, BD Biosciences), anti-CD16 Alexa 700 (clone 3G8, Beckman Coulter). Reagents used to stimulate peripheral blood cells were as follows: Goat anti-human IgE (Life Technologie), PMA, phorbol 12-myristate 13-acetate and ionomycin (Sigma-Aldrich), IL-3 (PeproTech, Rocky Hill, NJ). Allergens extracts: bermuda grass (BAG-G2), orchard grass (BAG-G3), perennial rye grass (BAG-G5), timothy grass (BAG-G6), 6-grass mix (BAG-GX1: Orchard grass, perennial rye grass, Timothy grass, meadow fescue, meadow grass, velvet grass), ragweed mix (BAG-WX1: common ragweed, giant ragweed), all from Bühlmann laboratories and 5-grass mix (Alyostal: *Phleum pratense*, *Dactylis glomerata*, *Anthoxanthum odoratum*, *Lolium perenne*, *Poa pratensis*, Stallergenes). Avidin-Alexa 488 (Invitrogen) or anti-CD63 PE (clone H5C6, BD Biosciences) were used to monitor basophils degranulation. Basophils were purified using EasySep[™] Human Basophil Enrichment Kit (#19069, Stemcell[™]) according to the manufacturer's recommendations. Histamine secretion was analyzed using Histamine EIA kit (Cayman Chemical, Ann Harbor).

Blood donors. Blood from randomly selected anonymous donors (allergy status unknown) was obtained from buffy coats or fresh heparinated blood (Etablissement Français du Sang). Eleven patients (4 men, 7 women, at the age of 19 to 57 years, mean age: 33 years, median age: 29 years) with a confirmed grass pollen allergy (positive history of rhinoconjunctivitis and positive skin prick test to grass pollen, 5-grass mix, Stallergenes) or irrelevant allergic patients (positive history of rhinoconjunctivitis and negative skin prick test to grass pollen, 5-grass mix, Stallergenes) were included in this study. Venous blood was collected in 10 mL EDTA tubes. The study was approved by the INSERM national ethics committee (# 16-280).

White blood cell preparation. Blood was centrifuged, the plasma discarded and red blood cells were lysed using red blood cells lysis buffer (15 mmol/L NH_4Cl , 1 mmol/L KHCO_3 , 10 mmol/L EDTA). After centrifugation, white blood cells were washed in PBS and distributed in 96-well V-bottom plate in Tyrode's buffer (1×10^6 cells in 100 μL). The cells were adapted to 37°C for 15 minutes before stimulation.

Cell stimulation for confocal analysis. 1×10^4 purified basophils were sensitized with human IgE for two hours. Then cells were plated on Poly-D-Lysine (Sigma)-coated Lab-Tek™ chambered coverglass (Nunc) in Tyrode's buffer supplemented with 8 $\mu\text{g mL}^{-1}$ avidin-sulforhodamine 101 (Av.SRho) and warmed at 37°C for 20 min. Cells were stimulated with 2.5 $\mu\text{g/mL}$ goat anti-human IgE at time=0. Fluorescence was acquired every 2.3 seconds using Zeiss LSM 710 confocal microscope and ZEN software, environmental chamber (37°C and 5% CO_2), 63x Plan-Apochromat objective (1.4 oil).

Cell stimulation for FACS analysis. Cells were stimulated with 100 μL of Tyrode's buffer containing either anti-IgE Abs (final concentration 2.5 $\mu\text{g/mL}$) or a combination of phorbol 12-myristate 13-acetate and ionomycin (final concentration, 50 ng/mL and 1 $\mu\text{g/mL}$ respectively) or allergen extract (recommended concentration or 1/1000 for 5-grass mix) or IL-3 (final concentration 3 ng/mL) or a combination of IL-3 and anti-IgE Abs (final concentration 3 ng/mL and 2.5 $\mu\text{g/mL}$ respectively) for 20 minutes at 37°C. Cells were washed and stained with a combination of anti-Fc ϵ RI efluor® 450, anti-CD203c BV510, anti-CD123 PE-Cy5, anti-CD16 Alexa 700, avidin-Alexa 488 and anti-CD63 PE diluted in 50 μL FACS buffer (PBS plus 0.1% bovine serum albumin) for 30 minutes at 4°C. Data were acquired by flow cytometry on a BD LSR-II cytometer and were analyzed by using FlowJo software (Tree Star, Inc, Ashland, Ore).

Cell stimulation for histamine release: 1×10^4 purified basophils were sensitized with human IgE for one hour. Cells were stimulated with 100 μL of Tyrode's buffer containing anti-IgE Abs (final concentration 1, 2.5 and 5 $\mu\text{g/mL}$) for 20 minutes at 37°C. Supernatants were harvested for histamine secretion analysis by ELISA (Cayman Chemical, Ann Arbor) and cells were washed and

97 stained with a combination of anti-CD203c BV510, avidin-Alexa 488 and anti-CD63 PE diluted in 50 μ L
98 FACS buffer (PBS plus 0.1% bovine serum albumin) for 30 minutes at 4°C. Data were acquired by flow
99 cytometry on a BD LSR-II cytometer and were analyzed by using FlowJo software (Tree Star, Inc,
100 Ashland, Ore).

101 **Statistical analysis.** Relative MFI was calculated as follow: $rMFI = (gMFI \text{ of stimulated condition} -$
102 $gMFI \text{ of unstimulated condition}) / gMFI \text{ of unstimulated condition}$. Positive rMFI threshold was
103 determined using non responder donors (Av. A488⁻, no CD203c or CD63 upregulation). Two-tailed
104 paired Student's *t*-tests were used for comparing two groups (Prism 5, GraphPad Software). *P*-value
105 range is indicated: ns>0.05 **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001. Shown flow cytometry
106 profiles and microscopy images are representative of repeated experiments.