

Letter to the Editor

Mechanism of phosphatidylserine inhibition of IgE/FcεRI-dependent anaphylactic human basophil degranulation via CD300a

To the Editor:

The process that leads to histamine release from human basophils constitutes a continuum that starts with piecemeal degranulation and eventually converts to anaphylactic degranulation (AND).^{1,2} These degranulation events are accompanied by mobilization of distinct cellular compartments and expression of surface molecules that are not or barely expressed on resting basophils. Two different histamine-containing compartments, which show distinct early signaling requirements and kinetics, have been described, namely, the CD203c compartment and the CD63 compartment.^{2,3} The appearance of CD63 is invariably associated with AND.² Mechanisms that regulate basophil activation likely relate to activation conditions (IgE/FcεRI-dependent and -independent), mode of degranulation (piecemeal degranulation vs AND), and changes in functional properties of inhibitory receptors.⁴ The inhibitory receptor CD300a (IRp60) is constitutively expressed on human basophils and rapidly upregulated in response to IgE/FcεRI cross-linking; its engagement selectively suppresses AND without a significant effect on CD203c.⁵ Moreover, recently CD300a has been shown to be a receptor for phosphatidylserine (PS) and phosphatidylethanolamine expressed on the membrane of apoptotic cells.⁶

This study aimed at investigating the effect of PS recognition by CD300a on IgE/FcεRI-dependent basophilic activation. Three experimental models addressed (1) the effect of apoptotic K562 cells on IgE/FcεRI-mediated activation of basophils in whole blood, (2) the putative role of PS, and finally (3) the effect of the PS-binding molecule Annexin V and CD300a-blocking antibody on inhibitory signals delivered by apoptotic PBMCs on separated basophils, excluding interference of other cells or humoral factors.

For detailed information on experimental design, see the [Methods](http://www.jacionline.org) section in the Online Repository at www.jacionline.org.

Aliquots of whole blood from 5 patients and 5 controls were preincubated with serial dilutions of viable or apoptotic K562 cells (from $20 \times 10^7/\text{mL}$ to $20 \times 10^2/\text{mL}$; 37°C, 30 minutes) before stimulation with buffer (negative control) or with anti-IgE (positive control) and, in patients, rBet v 1, the recombinant major allergen from birch (*Betula verrucosa*) pollen (for detailed information, see the [Methods](http://www.jacionline.org) section). This resulted in a significant dose-dependent inhibition of CD63 upregulation (Fig 1, A) that was more pronounced in healthy subjects than in allergic patients (Fig 1, B; $P < .05$). Similar results were obtained with patients' basophils stimulated with rBet v 1 (Fig 1, C). There was no significant effect of viable or apoptotic K562 on the spontaneous expression of CD63 and CD203c on basophils (data not shown).

Fluorescein-stained liposomes, noncoated or coated with PS or phosphorylcholine, were added to magnetic bead-isolated basophils⁷ of 2 healthy donors (4°C, 20 minutes) and immediately assessed by flow cytometry (for detailed information about liposomes preparation, see the [Methods](http://www.jacionline.org) section). Next, the functional effect of noncoated and PS- and phosphorylcholine-coated liposomes was assessed by preincubating different concentrations

of nonstained liposomes with isolated basophils⁷ at 37°C for 30 minutes before anti-IgE stimulation. These experiments revealed that only PS-coated liposomes bind basophilic membranes (Fig 1, D) and inhibit IgE/FcεRI-mediated activation of the cells in a dose-dependent manner, starting with a ratio liposomes/basophil 20/1. As with apoptotic K562 cells, this effect was more pronounced on the CD63 compartment (Fig 1, E; see Fig E1 in this article's Online Repository at www.jacionline.org).

To study the effect of the PS-binding molecule Annexin V and CD300a-blocking antibody (clone TX49), apoptotic autologous PBMCs (ratio PBMCs/basophil 20/1) were used ($n = 3$). To bind PS on the membrane of apoptotic PBMCs (aPBMCs), cells were incubated with different concentrations of recombinant human Annexin V at 37°C for 30 minutes; thereafter, basophils were added for 30 minutes at 37°C. Subsequently, cells were stimulated with buffer or anti-IgE. Inversely, to block CD300a receptor on isolated basophils, cells were exposed to different concentrations of the monoclonal anti-human CD300a-blocking antibody TX-49⁶ at 37°C for 30 minutes before adding aPBMC followed by anti-IgE activation. These experiments showed that blocking CD300a resulted in dose-dependent reversal of the magnitude of inhibition up to 77% (Fig 2, A and C; $P = .05$). Preincubation with anti-CD300a alone did not result in any significant effect on basophil activation markers (data not shown). This is not in contrast with our previous study,⁵ in which a CD300a receptor-activating antibody (clone MEM-260) was used, resulting in an inhibition of CD63 upregulation. Similarly, preincubating the cells with recombinant human Annexin V reversed the magnitude of aPBMC-induced inhibition up to 82% (Fig 2, B and D; $P = .04$).

Taken together, these results led us to conclude that the interaction between PS and CD300a inhibits IgE/FcεRI-dependent anaphylactic basophil degranulation. Nevertheless, the biological or clinical relevance of basophilic response/inhibition in IgE-mediated hypersensitivity needs to be elucidated.

The externalization of PS in the absence of apoptosis as a consequence of granular membrane fusion with the cell membrane involving inversion of membrane leaflet polarity⁸ and upregulation of the inhibitory receptor CD300a during basophilic degranulation⁵ on a single cell level may contribute to self-terminating mechanisms through *cis* recognition.

Whether the finding of increased apoptosis of monocytes on day 3 of venom immunotherapy⁹ suggests the involvement of PS-CD300a interaction on the early suppression of basophils and mast cells is a hypothesis that deserves investigation.

Finally, the modulation of basophilic phagocytic activity through CD300a-PS interaction seems difficult to postulate because these cells express low phagocytic activity.

In conclusion, we have for the first time demonstrated that the apoptosis-related signal PS suppresses human basophil anaphylactic degranulation via the inhibitory receptor CD300a (IRp60). These data provide interesting input to study the role of phospholipids recognition in modulation of basophilic responses and endorse the role of CD300a as a pharmacologic target for the treatment of allergic disease.

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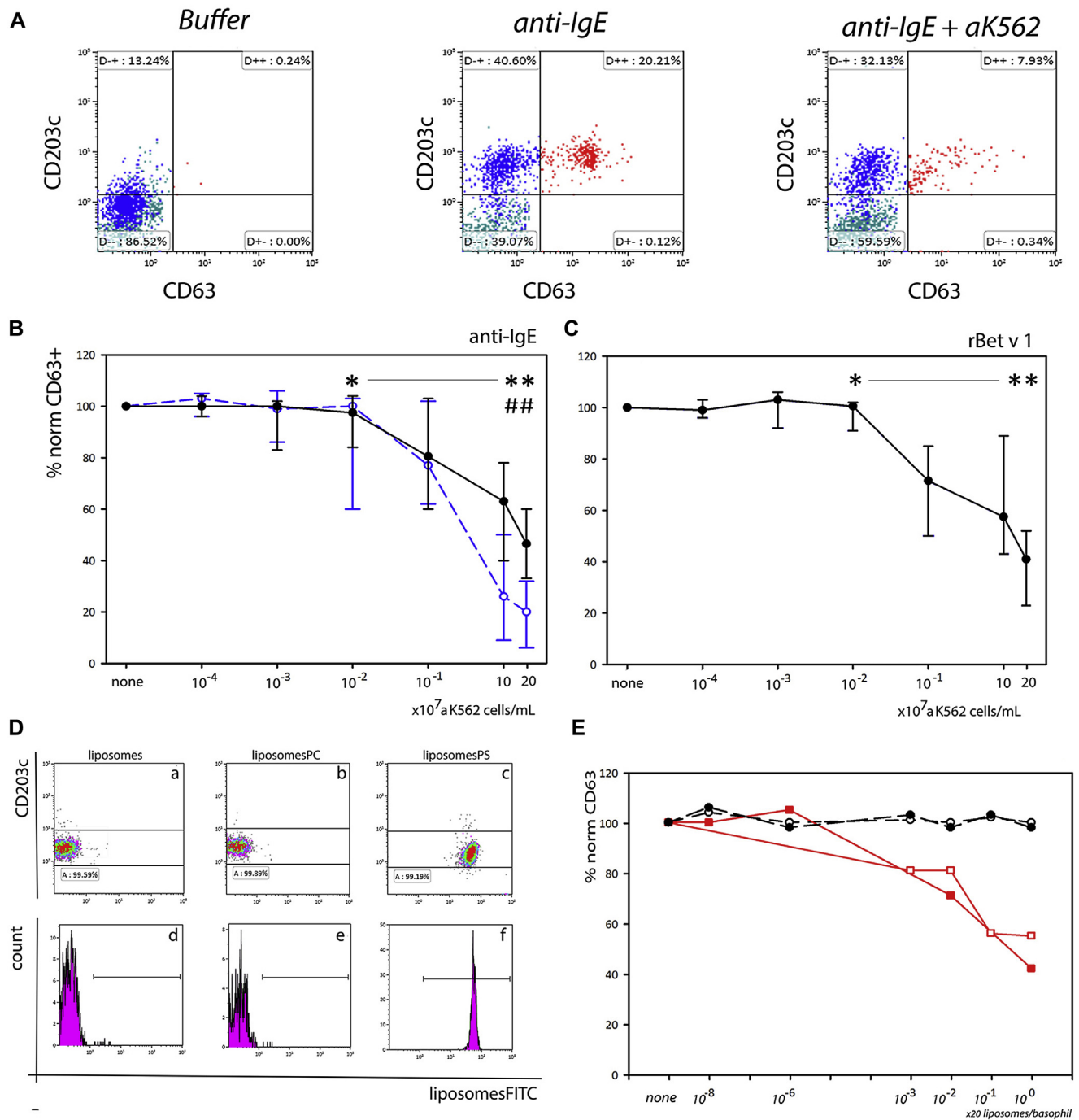


FIG 1. Effect of apoptotic cells on IgE-mediated basophil activation in patients and controls and the specific role of PS. **A**, Representative of 10 experiments. Apoptotic (a) K562 cells induce selective inhibition of CD63. **B**, Inhibition of CD63 upregulation after anti-IgE stimulation in patients allergic to birch pollen (*closed circles*) and controls (*open circles*) (** $P < .05$ for aK562 concentrations $20 \times 10^7/\text{mL}$ and $10 \times 10^7/\text{mL}$). The inhibitory effect is more pronounced in controls (** $P < .05$ controls vs patients). Results are expressed as median (range). **C**, Inhibition of Bet v 1-induced CD63 upregulation (** $P < .05$ for aK562 concentrations $20 \times 10^7/\text{mL}$ and $10 \times 10^7/\text{mL}$). Results are expressed as median (range). **D**, Only fluorescent PS- but not PC-coated liposomes or noncoated liposomes stain basophilic membranes. **E**, Results of 2 independent experiments with different concentration of PS- and PC-coated liposomes (closed and open squares and closed and open circles, respectively). Only PS-coated liposomes downregulate CD63 appearance on isolated basophils stimulated with anti-IgE. PC, Phosphorylcholine; PS, phosphatidylserine.

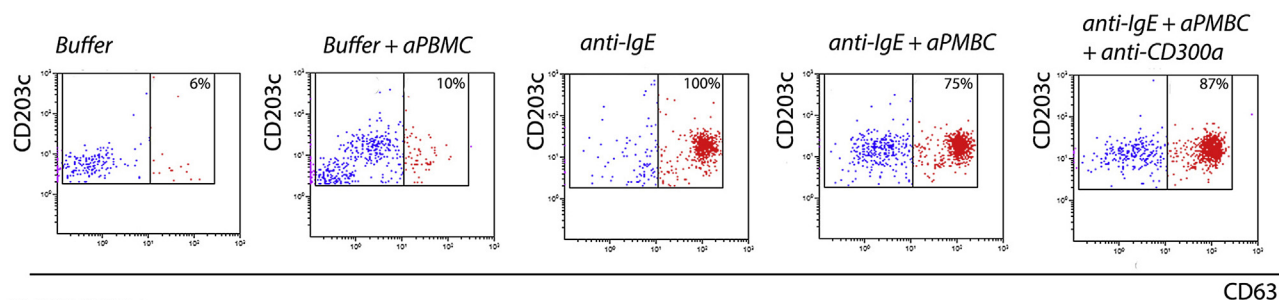
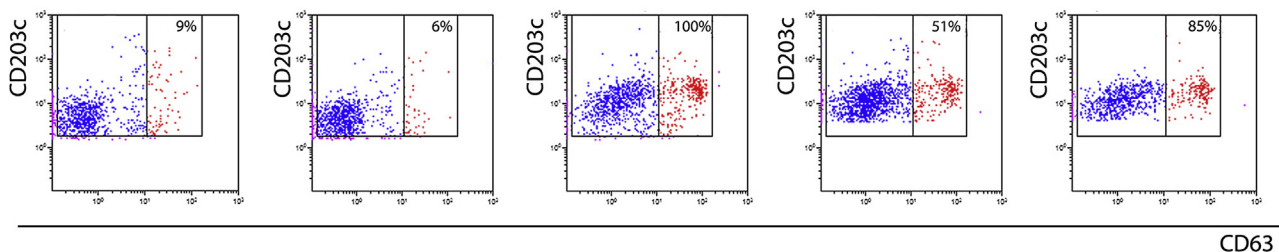
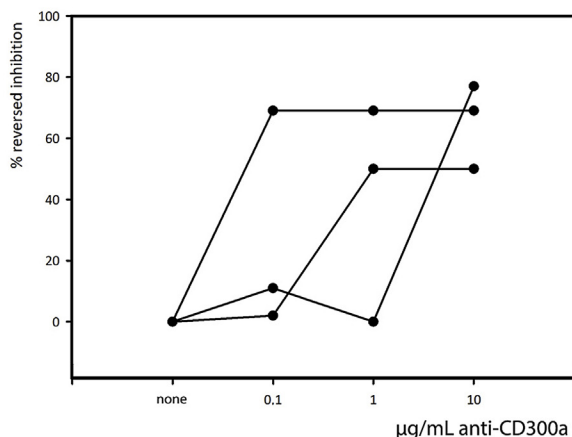
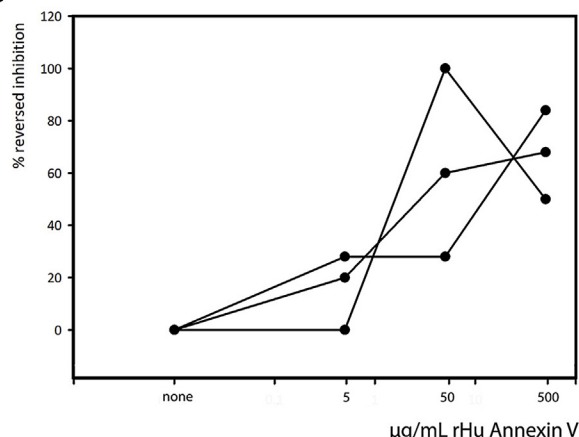
A *anti-CD300a***B** *rANNEXIN V***C****D**

FIG 2. CD300a-PS interaction: attenuation of the inhibitory signals delivered by apoptotic cells. **A** and **C**, CD300a-blocking assays. A representative experiment with anti-IgE-stimulated basophils without aPBMC and in the presence of aPBMC with or without blocking with the anti-CD300a TX-49 clone (10 $\mu\text{g}/\text{mL}$) (Fig 2, A). Individual results from 3 different experiments (Fig 2, C). CD300a blocking attenuates the magnitude of inhibitory signals delivered by aPBMC in a dose-dependent manner (Fig 2, C; $P = .05$). **B** and **D**, Results from human recombinant Annexin V-blocking assays. A representative experiment with anti-IgE-stimulated basophils in the absence of aPBMC, in the presence of Annexin V, and in the presence of Annexin V-blocked aPBMC (500 $\mu\text{g}/\text{mL}$) (Fig 2, B). Individual results from 3 different experiments are displayed (Fig 2, D). Similarly to the CD300a-blocking experiments, Annexin V blocking attenuates the magnitude of inhibitory signals delivered by aPBMC in a dose-dependent manner ($P = .04$).

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METHODS

Patients and ethics

Blood samples were drawn from 5 patients allergic to birch pollen (2 men, median age, 36 year; range, 26–48 years) and 10 healthy volunteers (3 men, median age, 40 year; range, 21–61 years).

The selection of patients was based on the history of spring-related rhinoconjunctivitis and documented by standardized skin tests and quantification of specific IgE.^{E1} Experiments were performed outside the birch pollen season.

Approval for this study was obtained from the local ethical committee (B300201318240). Patients and healthy control individuals provided an informed consent.

Basophils

In the first experimental model, peripheral basophils in endotoxin-free heparinized whole blood were studied. Subsequently, purified basophils were obtained via a previously described immunomagnetic bead separation protocol (EasySep Human Basophil Enrichment Kit; StemCell Technologies, Vancouver, British Columbia, Canada).⁷

Experimental design

In the first set of experiments, we aimed at investigating the effect of apoptotic cells on human peripheral blood basophils. Because of competition with other peripheral blood cells, and as observed in preliminary tests, a relatively high number of apoptotic cells was needed to alter basophil responses. Therefore, in the whole blood experiment, we applied the tumoral cell-line K562. On quantitative footing, when related to RBC, the ratio K562/RBC is approximately 1/25 and 1/50 for the concentrations $20 \times 10^7/\text{mL}$ and $10 \times 10^7/\text{mL}$, respectively.

To exclude whether the findings in these whole blood experiments could result from alternative humoral factors or engagement by other peripheral blood cells, we continued our investigations with isolated basophils. This enabled us to reduce the apoptotic load while applying autologous apoptotic PBMCs and to use liposomes as a source of PS.

Because of the artificial nature of liposomes, we chose apoptotic autologous PBMCs as a potential natural source of PS to explore whether inhibitory signals could be reverted by PS-binding molecules (such as Annexin V) and a CD300a-blocking antibody (TX49).

Induction and quantification of apoptosis

To induce apoptosis in K562 cells, cells were treated in FCS-free RPMI (Invitrogen, Paisely, United Kingdom) with $10 \mu\text{mol/L}$ H_2O_2 for 15 minutes at 37°C . Subsequently, cells were spun and resuspended in RPMI with 2% FCS and left in the CO_2 incubator at 37°C for 24 hours. Finally, cells were washed and suspended in Tyrode's buffer (Sigma-Aldrich, Seelze, Germany) and used immediately.

Apoptosis was induced in Histopaque (Sigma)-isolated PBMCs with $10 \mu\text{mol/L}$ H_2O_2 for 2 hours. Cells were then washed and immediately used in Tyrode's buffer. Apoptosis was quantified with fluorescein isothiocyanate-conjugated Annexin V and propidium iodide (BD Biosciences, Erembodegem, Belgium). Experiments were performed only if more than 90% of K562 cells or PBMCs were Annexin V positive.

Preparation of liposomes

Phosphatidylcholine and PS unilamellar liposomes were formed by a method adopted by de la Maza.^{E2}

Initially 1,2-dioleoyl, L- α -Phosphatidylcholine (DOPC; Sigma-Aldrich) and 1,2-diacyl-sn-glycero-3-phospho-L-serine (PS; Sigma-Aldrich) were dissolved in a 2:1 % v/v chloroform: methanol solution. DOPC and PS were mixed in a round-bottom flask to achieve a 70:30 % molar ratio. DOPC liposomes alone were formed by the addition of DOPC alone to the round-bottom flask (100 % mol). Total lipid added was 5 mg in all cases.

The organic solvent was then evaporated under a stream of nitrogen gas to achieve a lipid film. Residual solvent was removed by drying the round-bottom flask for 2 hours at 50°C .

The lipid film was then rehydrated using 3 mL HBSS with 25 mmol/L HEPES (Invitrogen) at 50°C to achieve a final lipid concentration of 10 mmol/L. Rehydration took place over 2 hours in a 45°C water bath. Following this, the liposome suspension was subject to 21 cycles of extrusion through a 100-nm filter to achieve a 100 nm unilamellar liposome suspension. Extrusion took place at 45°C . The formed liposome suspension was then sterilized using a 200-nm filter before application to the cells.

Where appropriate, 5(6)-carboxyfluorescein (CF; Sigma-Aldrich) was encapsulated into the DOPC/PS and DOPC-only liposomes. This occurred at the rehydration stage, whereby CF was dissolved in HBSS with 25 mmol/L HEPES to achieve a 1.32 mmol/L solution. Unencapsulated dye was removed by centrifugation of the liposome suspension at 13,000 rpm for 30 minutes. The pellet was then resuspended in 3 mL HBSS with 25 mmol/L HEPES. CF-loaded liposomes were also extruded through a 100-nm filter at 45°C to achieve a unilamellar liposome population and sterilized before use.

Interaction of liposomes with basophils

Fluorescein-stained liposomes, noncoated or coated with PS or phosphorylcholine, were added to isolated basophils of 2 healthy donors at 4°C for 20 minutes and immediately assessed by flow cytometry to evaluate the specific binding of PS.

Next, the functional effect of noncoated and PS- and phosphorylcholine-coated nonstained liposomes was assessed by preincubating different concentrations of liposomes (starting from a ratio basophils/liposomes of 1/20) with isolated basophils at 37°C for 30 minutes before anti-IgE stimulation (see below).

Stimulation conditions

Whole blood or isolated basophils were challenged (37°C , 20 minutes) with $100 \mu\text{L}$ of anti-IgE ($10 \mu\text{g/mL}$; clone G7-18, BD Biosciences) and activation buffer. In allergic patients, additional stimulation experiments involved recombinant *Betula verrucosa* 1 (rBet v 1; $1 \mu\text{g/mL}$; Stallergenes, Antony, France), a concentration optimized elsewhere.^{E1} Cells were stimulated for 20 minutes at 37°C .

Immunophenotyping and flow cytometric analysis

Activation was stopped by cooling the cells on ice. Thereafter, basophils were stained with $20 \mu\text{L}$ anti-human IgE (clone GE-1, Sigma Aldrich) labeled with Alexa Fluor 405 (Molecular Probes, Invitrogen), $10 \mu\text{L}$ anti-human CD63-fluorescein isothiocyanate (clone H5C6, BD Biosciences), and $10 \mu\text{L}$ anti-human CD203c-APC (clone NP4D6 Biologend, San Diego, Calif) for 20 minutes on ice. Red blood cells were lysed and white blood cells were fixed (FACS, BD) for 20 minutes at room temperature.

Flow cytometric quantification of upregulation of CD63 and CD203c was performed with a FACSCanto II flow cytometer (BD Immunocytometry Systems, San Jose, Calif) using a selective region on basophils. Basophils were selected as low SSC-A, IgE⁺/CD203c⁺ cells, excluding aggregates. In preliminary experiments, dead cells, stained by near-IR live/dead dye, were visualized in the gate of double-negative basophils (CD203c^{dim}CD63⁻). To exclude the possible interference of apoptotic cells in the basophil region during analysis, CD63 upregulation measurement was restricted to the CD203c^{bright} basophils.

At least 1000 CD203c^{bright} basophils were acquired (interassay variability, $\pm 3\%$). Results (in %) of CD63 upregulation are normalized according to the formula: normalized % CD63⁺ basophils = (%CD63⁺ experiment/% CD63⁺ with anti-IgE stimulation) $\times 100$.

Statistical analysis

All the data were expressed as median (range). Nonparametric Mann-Whitney and Wilcoxon tests were applied where appropriate. *P* values of less than .05 were considered as significant.

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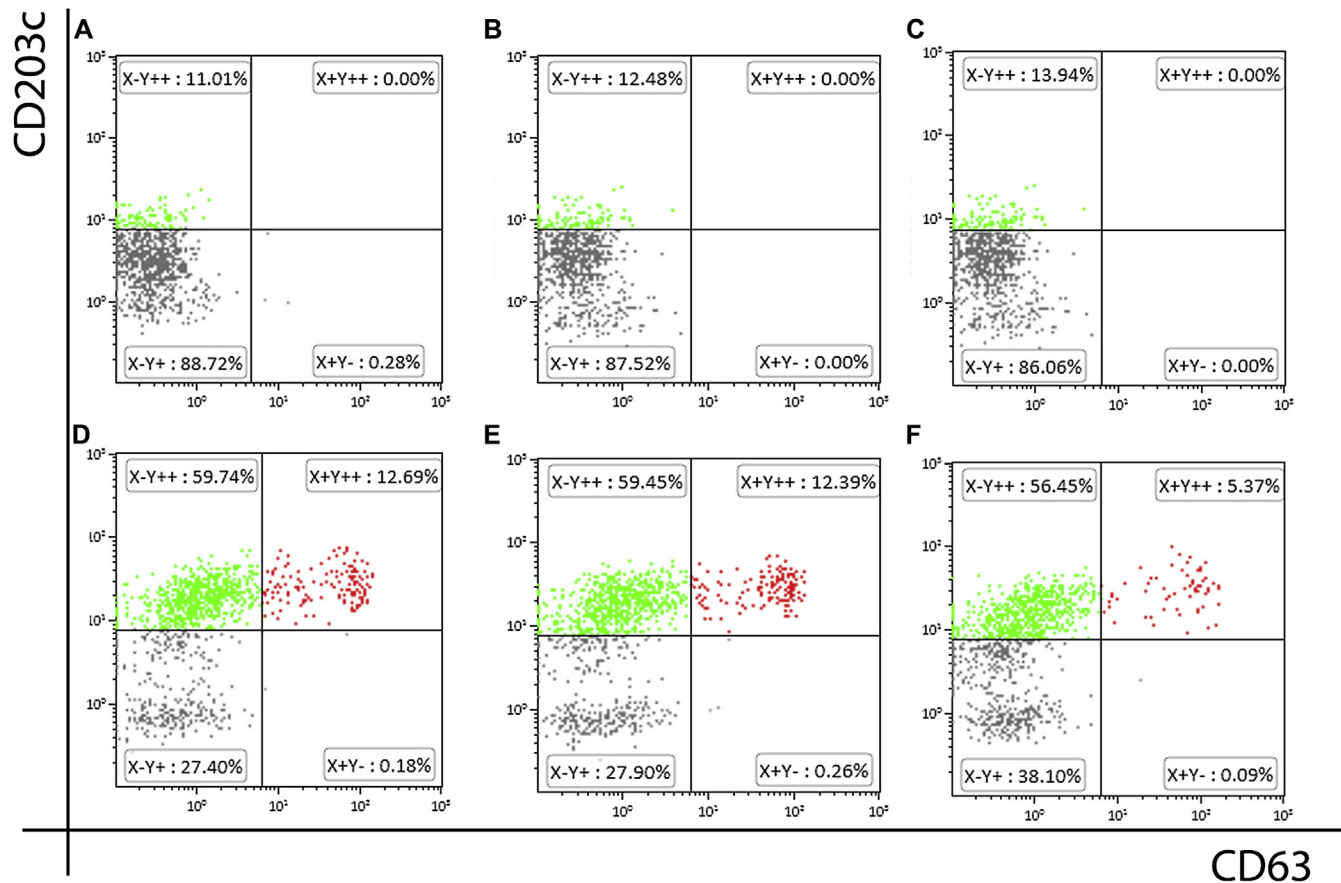


FIG E1. A representative example of liposomes and basophil interaction experiments. The plots display spontaneous expression of CD203c and CD63 on unstimulated (A-C) and anti-IgE-stimulated (D-F) isolated basophils. Noncoated (Fig E1, A), PC-coated (Fig E1, B), and PS-coated (Fig E1, C) liposomes do not alter the spontaneous expression of CD63 and CD203c. In contrast to noncoated (Fig E1, D) and PC-coated (Fig E1, E) liposomes, PS-coated liposomes exert an inhibitory effect on anti-IgE-dependent CD63 upregulation (Fig E1, F). *PC*, Phosphorylcholine; *PS*, phosphatidylserine.