

CD300c costimulates IgE-mediated basophil activation, and its expression is increased in patients with cow's milk allergy



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Background: Basophils express high-affinity IgE receptors (FcεRI), which play an essential role in allergic diseases. It is important to characterize new cell-surface receptors that modulate IgE-mediated basophil activation threshold to design promising immunomodulatory therapies.

Objectives: We sought to analyze the expression of CD300 receptors on human basophils and their implication in IgE-mediated basophil activation processes.

Methods: Blood samples from healthy subjects and patients with cow's milk allergy were collected through the Basque Biobank under an institutional review board–approved protocol. PBMCs were obtained by means of density centrifugation, basophils were purified with a specific isolation kit, and phenotypic and functional studies were performed by using flow cytometry.

Results: We demonstrate that basophils express the activating receptor CD300c, which is specifically upregulated in response to IL-3. CD300c works as a costimulatory molecule during IgE-mediated basophil activation, as shown by a significant increase in degranulation and cytokine production when

basophils are activated in the presence of CD300c cross-linking compared with activation through the IgE/FcεRI axis alone. Coligation of FcεRI and CD300c increased intracellular calcium mobilization and phosphorylation of signaling intermediates evoked only by FcεRI ligation. We show that the natural ligands of CD300c, phosphatidylserine and phosphatidylethanolamine, modulate IgE-mediated basophil activation. Furthermore, we have observed that CD300c expression in children with cow's milk allergy is increased compared with that in healthy control subjects and that the intensity of expression correlates with the severity of the hypersensitivity symptoms.

Conclusion: CD300c could be considered a biomarker and therapeutic target in patients with IgE-mediated allergic diseases because it seems to be involved in the modulation of IgE-mediated basophil activation. (*J Allergy Clin Immunol* 2019;143:700-11.)

Key words: Basophils, CD300, CD300a, CD300c, allergy, IgE-mediated activation, IL-3, activation threshold

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Basophils are major effector cells of IgE-dependent immune and allergic responses.¹⁻³ They express high levels of the high-affinity IgE receptor (FcεRI) on their surface, and cross-linking of FcεRI-bound IgE by bivalent or multivalent allergens induces the secretion of multiple stored and newly synthesized inflammatory mediators, including vasoactive amines (eg, histamine), lipid metabolites (eg, leukotriene C₄), and type 2 cytokines (eg, IL-4 and IL-13).⁴⁻⁶ These substances contribute to the development of allergy and other forms of inflammation.

In allergic reactions basophil degranulation and consequent release of various mediators occur after loss of the balance between activating and inhibitory signals. However, the means that govern this equilibrium are not completely elucidated. Among other mechanisms, the balance could be accomplished by signals originating from cell-surface receptors with activating and inhibitory capabilities. Some inhibitory receptors are characterized for having a long cytoplasmic tail with immunoreceptor tyrosine-based inhibitory motifs (ITIMs),⁷⁻⁹ whereas many activating receptors are characterized for having a short cytoplasmic tail and a charged amino acid residue that allows their association with immunoreceptor tyrosine-based activating motifs (ITAM)–bearing adaptors, which transduce activation signals and other adaptors that bind to, for example, phosphoinositide 3-kinase.⁷⁻¹¹

The human CD300 family consists of 8 receptors encoded in chromosome 17, and they are expressed in both myeloid

Abbreviations used

APC:	Allophycocyanin
cIMDM:	Complete Iscove modified Dulbecco medium
Erk:	Extracellular signal-regulated kinase
fMLP:	Formyl-methionyl-leucyl-phenylalanine
FMO:	Fluorescence minus one
ITAM:	Immunoreceptor tyrosine-based activating motif
ITIM:	Immunoreceptor tyrosine-based inhibitory motif
JAK:	Janus kinase
PC:	Phosphatidylcholine
PE:	Phosphatidylethanolamine
PerCP:	Peridinin-chlorophyll-protein
PS:	Phosphatidylserine
STAT:	Signal transducer and activator of transcription
TSLP:	Thymic stromal lymphopoietin

and lymphoid lineages, except CD300g, which is expressed on endothelial cells.¹²⁻¹⁶ The CD300 molecules are type I transmembrane proteins with a single IgV-like extracellular domain. CD300a and CD300f contain ITIMs in their cytoplasmic tail and are considered inhibitory receptors, although CD300f is also able to deliver activating signals through motifs reported to bind the p85 α regulatory subunit of phosphoinositide 3-kinase.¹⁷ On the other hand, CD300b, CD300c, CD300d, CD300e, and CD300h are activating receptors. The mouse CD300 molecules are encoded by 9 genes located on mouse chromosome 11, the syntenic region of human chromosome 17.¹²

CD300 receptors, including CD300a, CD300b, CD300c, and CD300f, are capable of recognizing phospholipids, such as phosphatidylethanolamine (PE) and phosphatidylserine (PS), which are exposed on the outer leaflet of the plasma membranes of dead and activated cells.¹⁸⁻²³ In addition, other lipids, such as ceramide and sphingomyelin, have been proved to bind CD300f and CD300e.²⁴⁻²⁶ Through its lipid binding, this receptor family is poised to have an important role in host responses in patients with severe pathologic conditions and complex biological processes.^{12,15}

Over the last years, the biological and clinical significance of CD300 molecules and their participation in the pathogenesis of several diseases (allergy among others) have been documented both in human and mouse models.^{12,15} In fact, the expression and function of several members of the CD300 receptor family have been described on cell types, such as mast cells, eosinophils, and basophils, that have important roles in the initiation, regulation, and effector phases of allergic responses. Thus far, several authors have reported that mast cells constitutively express the inhibitory receptors CD300a^{10,27} and CD300f^{25,26,28} and the activating members CD300b,²⁹ CD300c,²³ and CD300d²⁸ and that at least the inhibitory receptors CD300a³⁰ and CD300f^{31,32} are expressed on eosinophils.

Regarding human basophils, it has been shown that they express the inhibitory receptor CD300a.³³⁻³⁵ Furthermore, it has been described that basal expression of CD300a is lower in basophils from patients with birch pollen allergy than in basophils from healthy control subjects.³³ Interestingly, apoptotic cells expressing the CD300a ligands PS and PE inhibited anti-IgE-mediated basophil degranulation in healthy donors more efficiently than in allergic patients.³⁵ Altogether, these data suggest that CD300a could have an important role in the modulation of the activation threshold of basophils during allergic reactions.³³⁻³⁵

Despite the relevant role of CD300a in human basophil activation processes, the expression and function of other members of the CD300 family have not been determined. Here we have analyzed the expression of different members of the CD300 family and found that, in addition to the CD300a receptor, human basophils also express the CD300f inhibitory receptor and the CD300c activating receptor, with the latter being the only member of the family that increases its expression levels in response to IL-3. We have demonstrated that CD300c acts as a costimulatory molecule during basophil activation through the IgE/Fc ϵ RI axis. Importantly, we show that expression levels of CD300c on basophils from children with cow's milk allergy are greater than those in healthy control children and that there is an association between the severity of the hypersensitivity symptoms and levels of CD300c expression on basophils.

METHODS

Subjects and samples

Blood samples from healthy donors and donors with cow's milk allergy (both adults and children) were collected through the Basque Biobank (<http://www.biobancovasco.org>). All the patients included in the study presented IgE-mediated allergy symptoms, positive skin prick test responses, and *in vitro*-specific IgE to cow's milk, α -lactalbumin, β -lactoglobulin, and casein in the 6 months before blood extraction (Table I). Hypersensitivity to cow's milk was classified as grade I (local reaction with perioral and/or contact urticaria), grade II (systemic reaction without cardiovascular and/or respiratory involvement with gastrointestinal symptoms), or grade III (severe systemic reaction with cardiovascular and/or respiratory involvement) at the time of diagnosis, according to the criteria described by Niggemann and Beyer.³⁶ Samples from control children (Table II) were collected from healthy siblings of patients with a diagnosis of celiac disease who participated in a common type of study.

The Basque Biobank complies with the quality management, traceability, and biosecurity set out in the Spanish Law 14/2007 of Biomedical Research and the Royal Decree 1716/2011. All subjects provided written and signed informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Basque Ethics Committee for Clinical Research (PI2015182; version 2; November 18, 2015).

Functional experiments

For functional experiments, nonprimed and IL-3-primed PBMCs, purified basophils, or both were used. For priming, cells were cultured in complete Iscove modified Dulbecco medium (cIMDM) and stimulated with IL-3 for 2 hours. Then, they were washed thoroughly and starved from IL-3 for 22 additional hours. Functional experiments were performed the next day.

The detailed list of antibodies and reagents used for the experiments, as well as experimental protocols for flow cytometric analyses and functional experiments, such as calcium mobilization assay, phosphorylation assay, cross-linking of CD300c, and measurement of activation markers and cytokine production; lipid stimulation experiments; stimulation of basophils in the presence or absence of apoptotic K562 cells; and data analysis are detailed in the Methods section in this article's Online Repository at www.jacionline.org.

RESULTS

Human basophils express CD300a, CD300c, and CD300f receptors

Despite a few studies showing that human basophils express the inhibitory receptor CD300a,^{33,34} expression of other members of the CD300 receptor family has not been determined. We analyzed

TABLE I. Clinical data of children with cow's milk allergy

Patient no.	Sex	Age	Hypersensitivity	IgE, milk	IgE, α -lactalbumin	IgE, β -lactoglobulin	IgE, casein
1	Male	18 mo	Grade III	3.37	0.09	0.26	1.06
2	Female	4 y	Grade III	4.43	3.73	1.53	0.99
3	Male	16 mo	Grade I	4.44	5.78	0.06	0.27
4	Female	6 y	Grade II	46.3	10.9	4.9	46
5	Male	5 y	Grade II	0.69	0.79	0.26	0.11
6	Female	17 mo	Grade I	5.13	1.42	6.55	1.06
7	Female	3 y	Grade II	8.91	5.01	1.74	7.18
8	Male	4 mo	Grade I	5.79	0.32	0.47	0.01
9	Female	22 mo	Grade III	0.92	0.92	0.11	0.64
10	Male	7 y	Grade III	62.5	4.48	20.7	64.5
11	Male	3 y	Grade III	22.1	6.93	1.18	7.99
12	Male	6 mo	Grade I	6.72	2.95	2	4.58
13	Male	13 mo	Grade I	0.48	0.3	0.14	0.31
14	Male	15 y	Grade III	15.3	11	3.69	14
15	Female	12 mo	Grade I	2.42	1.18	1.64	3.5
16	Male	12 y	Grade I	3.26	0.3	0.05	0.79
17	Male	2 y	Grade I	3.25	3.25	0.47	1.39

TABLE II. Clinical data of healthy children

Control no.	Sex	Age (y)
1	Male	6
2	Male	3
3	Female	4
4	Female	9
5	Male	12

the expression pattern of CD300a, CD300c, CD300e, and CD300f on circulating basophils from healthy donors' peripheral blood.

To identify basophils among the PBMCs, we used a staining strategy based on expression of the surface receptors Fc ϵ RI and CD203c within lineage-negative (CD3⁻CD14⁻CD19⁻CD20⁻) cells (Fig 1, A). We also tested a staining strategy based on surface expression of CD123 and the absence of HLA-DR and concluded that both strategies were equally valid for basophil identification (data not shown). Regarding CD300 receptor expression, we observed that basophils express not only the CD300a inhibitory receptor but also CD300f and the CD300c activating receptor at lower levels, although they do not express CD300e (Fig 1, A).

Expression of CD300c on basophils increases in response to IL-3

Next, we studied regulation of the expression of CD300c, CD300e, and CD300f in response to several IgE-dependent and IgE-independent basophil activators, such as anti-IgE mAb, IL-3, thymic stromal lymphopoietin (TSLP), IL-33, and formyl-methionyl-leucyl-phenylalanine (fMLP). We did not study regulation of CD300a expression because it has been described previously by others.³³ Interestingly, except for a very significant upregulation on CD300c expression in response to IL-3 (Fig 1, B), stimulation of basophils for 18 hours did not induce any other significant change in expression of CD300c, CD300e, or CD300f. When we tested the effect of IL-3 on CD300c expression over a short time course, we did not see any change in the first 3 hours of stimulation (Fig 1, C). This suggests

that IL-3-mediated upregulation of CD300c expression involves transcriptional and/or translational mechanisms.

We also tested the effect of IL-3 on purified basophils and found that the expression of CD300c was induced after 18 hours, demonstrating a direct effect of IL-3 on modulation of CD300c expression on basophils (Fig 1, D). Nonetheless, IL-3-induced CD300c levels were lower in purified basophils, suggesting that, in addition to a direct influence, IL-3 also has effects on other cell types among PBMCs, which could indirectly affect regulation of CD300c expression on basophils.

IL-3 is a cytokine that signals through the Janus kinase 2 (JAK2)/signal transducer and activator of transcription 5 (STAT5) pathway.^{37,38} Hence we wanted to investigate whether upregulation of CD300c in response to IL-3 was also dependent on the JAK/STAT pathway. To do this, we used the JAK1/2-specific inhibitor ruxolitinib³⁹ over 18 hours of stimulation with IL-3. We used a concentration (1 μ mol/L) that corresponds to intermediate serum levels achieved in treated patients.⁴⁰ We observed that not only was the IL-3-dependent activation of basophils inhibited (data not shown) but also upregulation of CD300c was abrogated in the presence of ruxolitinib (Fig 1, E), demonstrating that upregulation of CD300c in response to IL-3 is dependent on the JAK/STAT pathway.

CD300c costimulates basophil IgE-dependent degranulation and cytokine production

Once we described the regulation of CD300c expression on basophils, we wanted to study the role of this activating receptor in basophil function. It has been described previously that CD300c acts as an activating receptor in several human immune cells, such as monocytes, mast cells, and natural killer cells.^{16,19,23,41} Therefore we hypothesized that CD300c could also have an activating and costimulatory role in basophils.

To determine the activation status of basophils, we analyzed the surface expression of CD63 and CD203c markers associated with anaphylactic degranulation and activation of basophils,

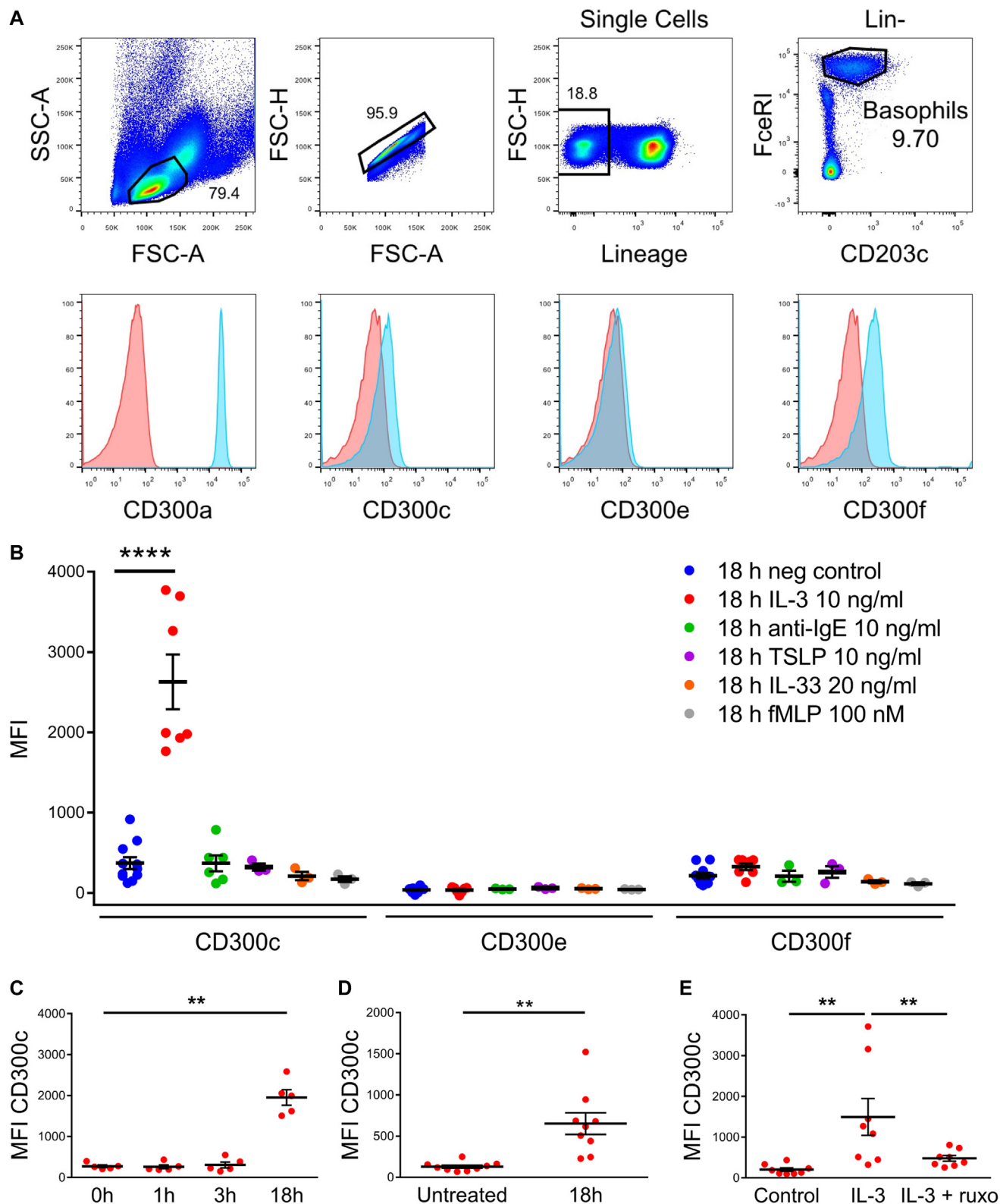


FIG 1. CD300 molecule expression on human basophils. **A**, Expression of CD300 receptors on basophils. Gating strategy is shown. *Blue histograms* represent binding of anti-CD300 mAbs, and *red histograms* represent FMO control. A representative example from data obtained from 12 to 15 healthy donors is shown. *FSC*, Forward scatter; *SSC*, side scatter. **B**, Median fluorescence intensity (*MFI*) of CD300c, CD300e, and CD300f expression on basophils in response to IL-3, anti-IgE mAb, TSLP, IL-33, and fMLP during 18 hours. Each *dot* represents a different donor, and means $\pm$ SEMs are represented. **C**, MFI of CD300c expression on basophils before and after stimulation with IL-3 (10 ng/mL) during 1, 3, and 18 hours. Each *dot* represents a different donor, and means $\pm$ SEMs are represented. **D**, MFI of CD300c expression on untreated and IL-3-treated purified basophils. Basophils were treated with 10 ng/mL IL-3 for 18 hours. Means $\pm$ SEMs are represented. Data obtained from 9 independent experiments are represented. **E**, MFI of CD300c expression on basophils before and after stimulation with IL-3 (10 ng/mL) during 18 hours in the absence and presence of ruxolitinib (*ruxo*; 1 μ mol/L). Each *dot* represents a different donor. Means $\pm$ SEMs are represented. ** $P < .01$ and **** $P < .0001$.

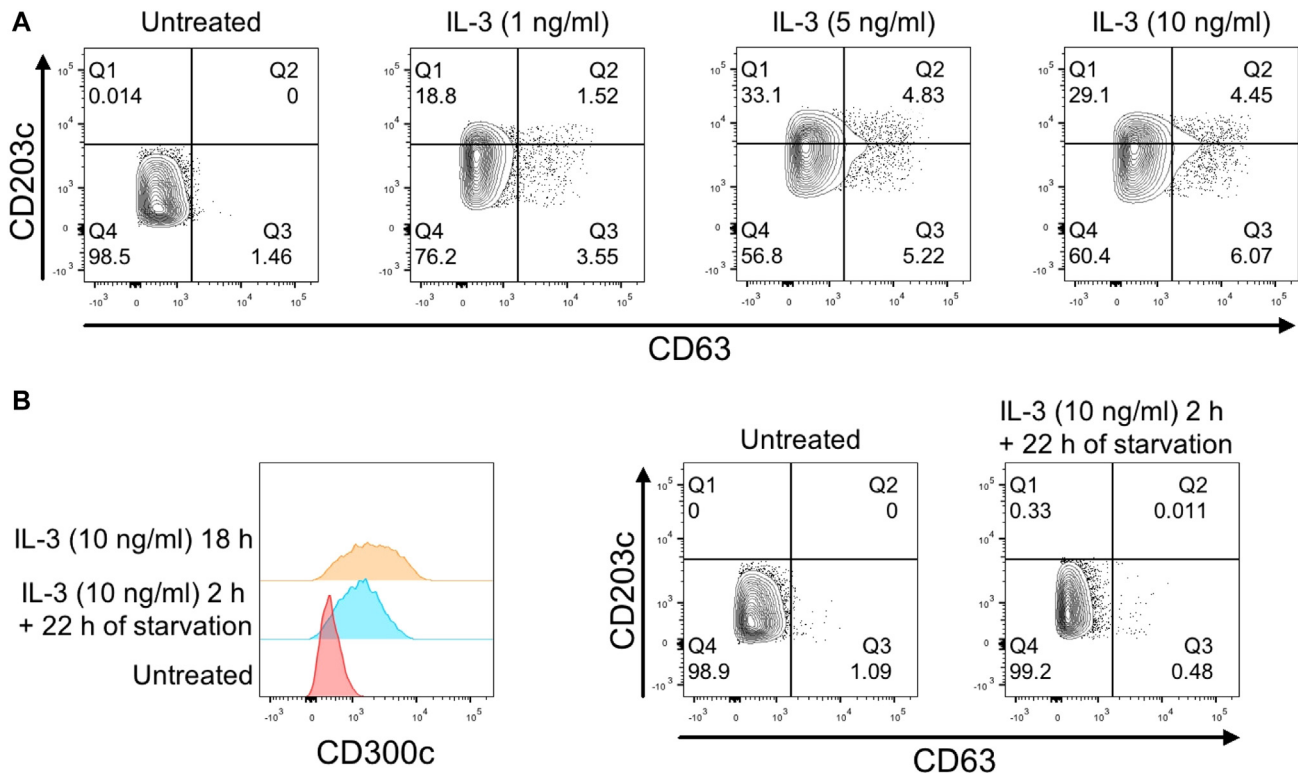


FIG 2. Priming basophils with IL-3 for 2 hours induces late CD300c expression upregulation. **A**, Contour plots representing expression of the basophil activation markers CD63 and CD203c in response to different concentrations of IL-3 during 18 hours. Contour plots are representative of data obtained from 6 independent experiments. **B**, Histograms (left) showing expression of CD300c on untreated (red histogram) and IL-3-treated basophils. Blue histogram shows expression of CD300c on basophils treated with 10 ng/mL IL-3 for only 2 hours, washed, and cultured for 22 hours in the absence of IL-3 (IL-3-primed basophils), and orange histogram shows expression of CD300c on basophils treated with 10 ng/mL IL-3 for 18 hours. Contour plots (right) representing expression of CD63 and CD203c of untreated and IL-3-primed basophils. Contour plots and histograms are representative of data obtained from 6 independent experiments.

respectively.⁴² First, we wanted to design an experimental protocol to study the role of CD300c in basophils. As shown in Fig 1, A and B, basal expression of CD300c on basophils is relatively low and increases in response to 18 hours of stimulation with 10 ng/mL IL-3. However, that stimulus also activates basophils, as indicated by upregulation of the expression of surface molecules that are not or are barely expressed on resting basophils (ie, CD63 and CD203c; Fig 2, A). Our objective was to obtain high levels of CD300c while avoiding basophil activation. Thus, we first checked the effect of different concentrations of IL-3 during 18 hours of culture. Despite lower doses of IL-3 reducing the percentage of activated basophils, they did not return to a basal resting status (Fig 2, A).

Next, we stimulated PBMCs with 10 ng/mL IL-3 only for 2 hours, washed them thoroughly, and cultured them for 22 additional hours in the absence of IL-3. We could see that when using the latter protocol, expression levels of CD300c on basophils were very similar to those obtained when cells were stimulated with IL-3 for 18 hours, and additionally, expression of the activation markers CD203c and CD63 did not change (Fig 2, B). From here on, we used this protocol in which cells were first primed for 2 hours with IL-3 and then washed thoroughly and starved from IL-3 for 22 hours to study CD300c function in IL-3-primed basophils. Notwithstanding the above,

on some occasions, experiments with non-IL-3-primed basophils were also performed as controls.

To analyze whether CD300c receptor contributes to the IgE-dependent basophil activation, we measured upregulation in the expression of CD63 and CD203c, as well as production of the cytokines IL-4 and IL-13, 2 interleukins produced by basophils in response to IgE-dependent activation, after stimulation of basophils with anti-IgE or anti-IgE plus IL-3 in the presence and absence of CD300c cross-linking with specific mAbs. We observed that the frequency of CD63⁺ basophils in response to IgE-mediated stimulation was significantly greater when CD300c was cross-linked than when an isotype control mAb was used (Fig 3, A, upper line, and see Fig E1, A, in this article's Online Repository at www.jacionline.org), indicating a costimulatory role of CD300c in response to IgE-dependent basophil activation. We also observed an increase in CD203c expression on basophils, although it was not statistically significant, when they were stimulated through FcεRI in the presence of CD300c cross-linking (Fig 3, A, lower line, and see Fig E1, B). When we activated IL-3-primed purified basophils in an IgE-dependent manner in the presence of anti-CD300c antibodies we could still see the CD300c costimulatory role (see Fig E1, C). Yet, the results were not as significant as those obtained with PBMCs (Fig 3, A), probably because of the lower

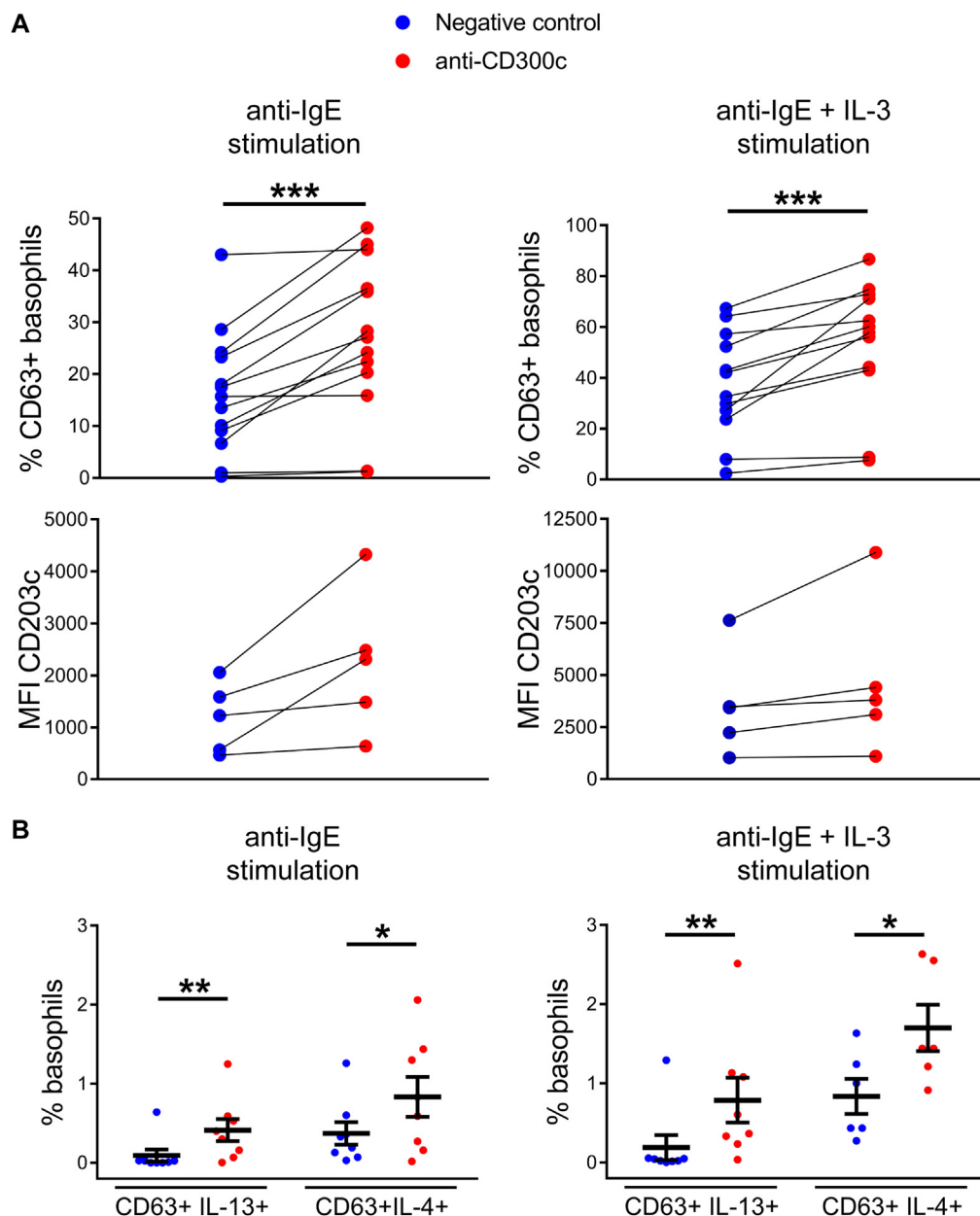


FIG 3. CD300c cross-linking enhances IgE-dependent basophil activation marker expression and cytokine production. **A**, Dot graphs show percentages of CD63⁺ basophils (upper line) and median fluorescence intensity (MFI) of CD203c (lower line) in the presence (red dots) and absence (blue dots) of cross-linking of CD300c after stimulation with anti-IgE (10 ng/mL) or anti-IgE (10 ng/mL) plus IL-3 (2 ng/mL). Each pair of dots represents a different experiment. Data obtained from 12 independent experiments are represented. **B**, Dot graphs show percentages of CD63⁺IL-13⁺ and CD63⁺IL-4⁺ basophils in the presence (red dots) and absence (blue dots) of CD300c cross-linking after stimulation with anti-IgE (10 ng/mL; left graph) or anti-IgE (10 ng/mL) plus IL-3 (2 ng/mL; right graph). Each pair of dots represents a different experiment. Data obtained from 8 independent experiments and means $\pm$ SEMs are represented. * $P < .05$, ** $P < .01$, and *** $P < .001$.

levels of CD300c expression on IL-3–primed purified basophils (Fig 1, D, and see Fig E1, D). In addition, a higher percentage of CD63⁺IL-13⁺ and CD63⁺IL-4⁺ basophils was observed in response to IgE-mediated stimulation in the presence of cross-linking of CD300c (Fig 3, B). Taken together, these results indicate that the CD300c receptor acts as a costimulator in IgE-dependent basophil activation processes, inducing not only the degranulation of basophils but also the production of at least 2 key cytokines for development of allergic inflammation.

CD300c costimulation strengthens FcεRI-mediated activation of signaling intermediates in basophils

Once we demonstrated that CD300c has a costimulatory role in the FcεRI-mediated activation of basophils, we studied the molecular basis of this influence. We analyzed whether CD300c cross-linking was able to modulate the IgE/FcεRI-mediated signaling pathway in human basophils from peripheral blood.

During the first steps of the FcεRI signaling cascade, there is recruitment of the protein tyrosine kinase Syk to the

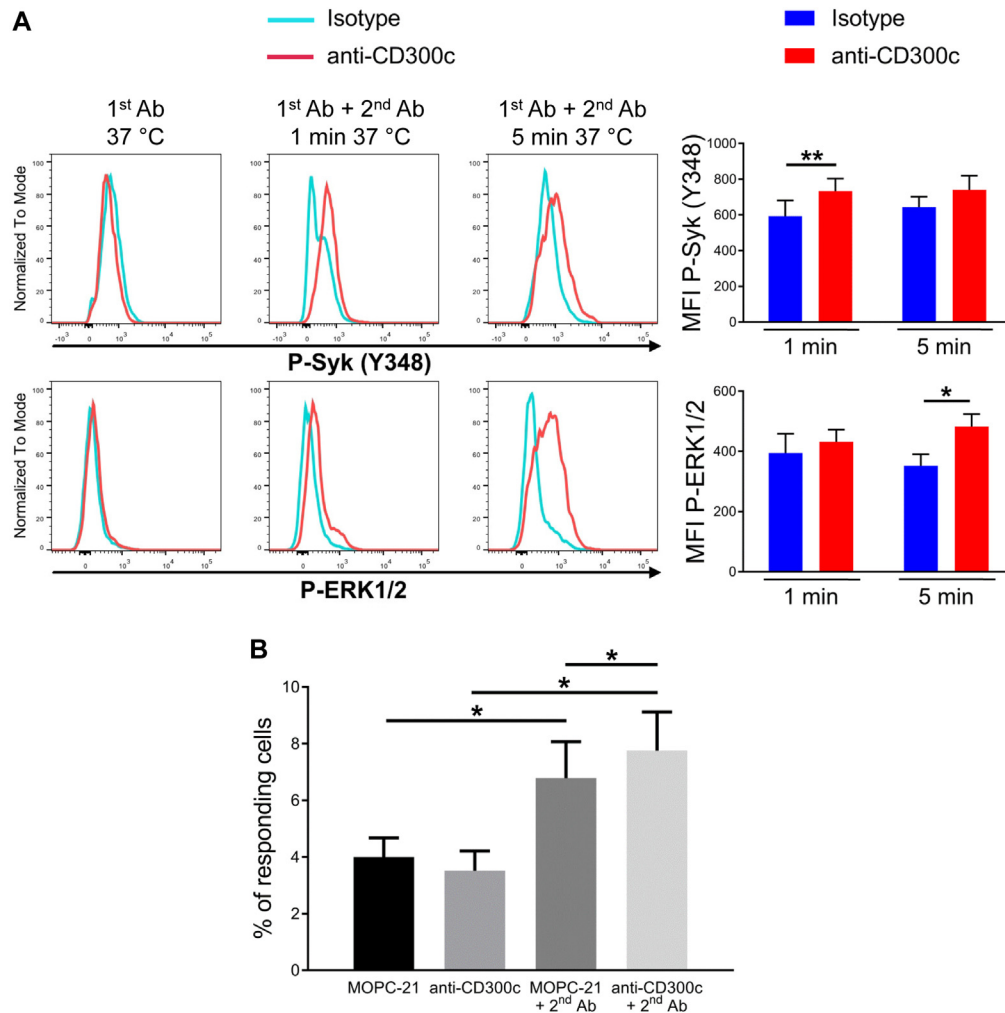


FIG 4. CD300c stimulation strengthens FcεRI-mediated activation of signaling intermediates. **A**, Representative histograms showing phosphorylation levels of Syk (P-Syk; upper line) and phosphorylation levels of Erk (P-ERK1/2; lower line) in basophils in which the primary mAbs anti-CD300c (in red) or isotype-matched control MOPC-21 (in blue) were added and then not cross-linked (left column) or cross-linked with secondary antibody (Ab) for 1 (middle column) or 5 (right column) minutes. Bar graphs show MFIs of P-Syk (upper graph) and P-ERK1/2 (lower graph) in basophils in which the primary mAbs anti-CD300c (in red) or isotype-matched control (in blue) were added and then cross-linked with secondary antibody for 1 or 5 minutes. Data are from 9 independent experiments, and means $\pm$ SEM are represented. MFI, Median fluorescence intensity. * $P < .05$ and ** $P < .01$. **B**, Basophil Ca^{2+} mobilization. FcεRI-positive cells were first acquired for 30 seconds, at which point the primary mAb anti-CD300c or isotype-matched control were added. Then, the primary mAbs were cross-linked with secondary antibody (Ab), and fluorescence was measured. Percentages of responding cells from 60 to 210 seconds were measured. Data are from 6 independent experiments, and means $\pm$ SEMs are represented. * $P < .05$.

phosphorylated tyrosine residues in the ITAMs of the β and γ subunits of FcεRI. Then Syk becomes activated and initiates the signaling cascade that involves phosphorylation and activation of several downstream proteins, such as mitogen-activated protein kinases, and release of Ca^{2+} from internal stores.⁴³⁻⁴⁵

To have an idea of the kinetics of the FcεRI signaling pathway in the presence and absence of CD300c cross-linking, we measured phosphorylation levels of Syk, which is an early event in the FcεRI signaling cascade, and phosphorylation of extracellular signal-regulated kinase (Erk) 1/2, which appears more downstream in the pathway. We saw that phosphorylation levels of the intermediates Syk and Erk1/2 were significantly

greater in the presence of CD300c cross-linking after 1 and 5 minutes of FcεRI-mediated stimulation, respectively (Fig 4, A).

We also investigated the ability of CD300c to modulate intracellular calcium mobilization. In basophils that are activated through FcεRI, engagement of CD300c with soluble anti-CD300c mAbs, followed by cross-linking with anti-mouse IgG F(ab')₂, induced a transient and rapid increase in intracellular calcium levels that was significantly greater than that observed when cells were stimulated with an isotype-matched control (Fig 4, B).

Altogether, these results demonstrate that CD300c has a costimulatory role in the activation of the FcεRI downstream signaling pathway, which is mediated in part by an increase in

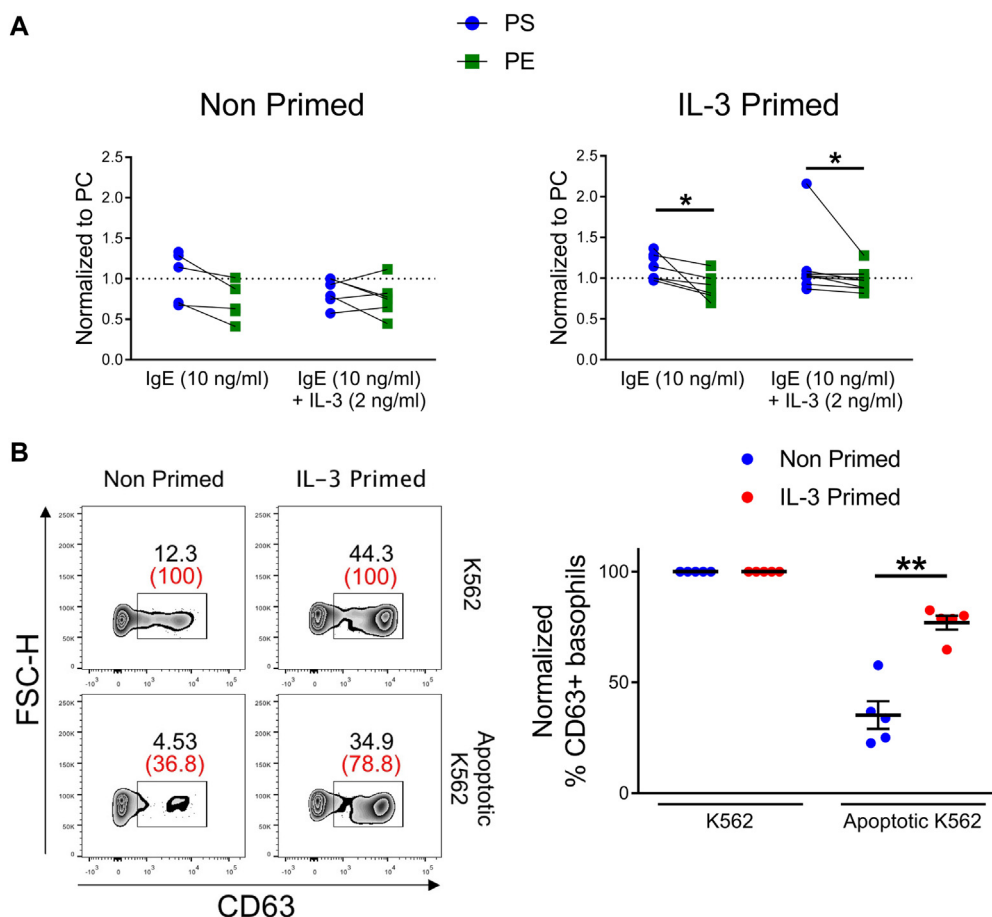


FIG 5. PS and PE modulate IgE-dependent basophil activation. **A**, Relative values of basophil activation in response to anti-IgE and anti-IgE plus IL-3 in the presence of PS (blue dots) and PE (green dots). Values of relative activation were measured by dividing the percentage of CD63⁺ cells in the presence of PS and PE by the percentage of CD63⁺ cells in the presence of the control PC. Results of non-IL-3-primed (left panel) and IL-3-primed (right panel) basophils are shown. Values above and below the dotted line indicate that the percentage of CD63⁺ basophils was higher and lower, respectively, than the percentage obtained in the presence of the PC phospholipid control. **B**, Left, Zebra plot showing a representative experiment of percentage of CD63⁺ basophils (numbers in black) in response to anti-IgE plus IL-3 in the presence of nonapoptotic and apoptotic K562 cells. Normalized values are shown in red and parentheses. Right, Dot graph showing normalized percentage of CD63⁺ basophils. Results of non-IL-3-primed (blue) and IL-3-primed (red) basophils are shown. Results were normalized to the percentage of CD63⁺ basophils obtained in the presence of nonapoptotic K562 cells. Means $\pm$ SEMs are represented. * $P < .05$ and ** $P < .01$.

FcεRI-mediated intracellular calcium mobilization and Syk and Erk1/2 activation.

PS and PE binding to CD300c modulate IgE-dependent basophil activation

As it has been demonstrated by us and others,^{19,23} the binding affinity of CD300a and CD300c to PS is very similar, whereas CD300a has a higher affinity for PE than CD300c. We wanted to explore the IgE-dependent costimulatory role of CD300c on basophils by stimulating them in the presence of CD300c natural ligands (ie, PS and PE). The phospholipid phosphatidylcholine (PC) served as a negative control. On the one hand, IL-3-primed and nonprimed basophils were incubated in lipid-coated plates and were also stimulated with anti-IgE or anti-IgE plus IL-3. After 2 to 3 hours, cells were stained with the appropriate mAbs to measure activation levels of basophils. We saw that in

non-IL-3-primed cells IgE/FcεRI-mediated activation of basophils was lower, especially when activated in the presence of PE, compared with activation levels observed in the presence of control PC (Fig 5, A, and see Fig E2, A, in this article's Online Repository at www.jacionline.org). These results suggest that in the absence of high levels of CD300c, the ligands PS and PE generally induced an inhibitory effect on IgE/FcεRI-dependent basophil degranulation, probably because of their binding mostly to the CD300a inhibitory receptor. Interestingly, in IL-3-primed basophils, in which CD300c expression was increased significantly (Fig 2, B), the decrease in the percentage of CD63⁺ basophils after FcεRI-mediated activation in the presence of PS and PE was not so marked compared with that in the presence of PC (Fig 5, A, and see Fig E2, B). This suggests that greater levels of CD300c counteract the inhibitory role of CD300a. Moreover, it was very interesting to see that in IL-3-primed basophils costimulation with PS resulted in greater

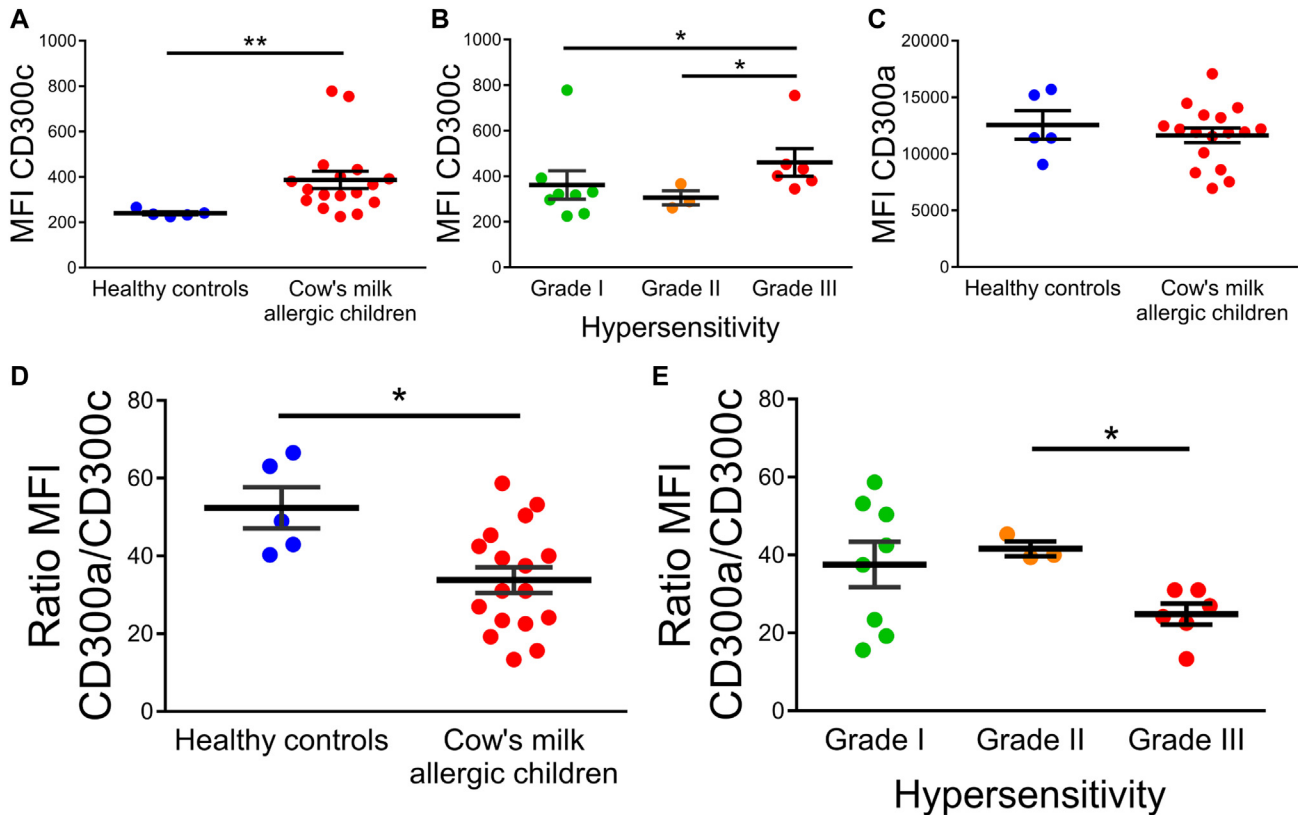


FIG 6. Basophils from children with cow's milk allergy exhibit greater expression of CD300c. **A**, Dot graph showing basal median fluorescence intensity (MFI) of CD300c on basophils from healthy children (blue) and children with cow's milk allergy (red). **B**, Dot graph showing basal MFI of CD300c on basophils in children with cow's milk allergy classified by the severity of the symptoms of hypersensitivity as grade I (green), grade II (orange), and grade III (red). **C**, Dot graph showing basal MFI of CD300a on basophils from healthy children (blue) and children with cow's milk allergy (red). **D**, Dot graphs representing MFI CD300a/CD300c ratio on basophils from healthy children and children with cow's milk allergy. **E**, Dot graphs representing the MFI CD300a/CD300c ratio on basophils from children with cow's milk allergy classified by the severity of the symptoms of hypersensitivity as grade I (green), grade II (orange), and grade III (red). Each dot represents a donor, and means $\pm$ SEMs are shown. * $P < .05$ and ** $P < .01$.

basophil activation compared with activation levels obtained with the control lipid PC, whereas in the presence of PE, which has a greater affinity for the CD300a inhibitory receptor, there was still an inhibitory effect on basophil activation, although at lower levels than in nonprimed cells (Fig 5, A).

We also stimulated basophils in the presence of nonapoptotic or apoptotic K562 cells, which expose PS and PE in the outer leaflet of the plasma membrane. We saw, as has been described previously by Sabato et al,³⁵ a significant decrease in the frequency of CD63⁺ cells in nonprimed IgE/FcεRI-activated basophils activated in the presence of apoptotic cells. However, in IL-3-primed basophils expressing greater levels of CD300c, the decrease in the frequency of CD63⁺ basophils in the presence of apoptotic cells was significantly lower (Fig 5, B).

Taken together, these results led us to conclude that the interaction of PE with CD300a is predominant over the interaction with CD300c, which leads to an inhibitory signal. However, importantly, cross-linking of CD300c by PS on activated basophils counteracts and even overrides cross-linking of CD300a, resulting several times in a net positive signal and

therefore decreasing the threshold of basophils FcεRI-mediated activation.

Allergic subjects displayed a significantly greater expression of CD300c

It has been described before that basal expression of the CD300a inhibitory receptor is lower in basophils of patients with birch pollen allergy than in healthy donors.³³ Here we compared the expression of CD300c on basophils in healthy children with those from children with cow's milk allergy. Clinical data of allergic patients, which consist mainly of the degree of hypersensitivity symptoms and levels of specific IgE, are shown in Table I. In Table II data of healthy children are shown. We saw that basal expression of CD300c is significantly greater in allergic children than in healthy control subjects (Fig 6, A). Importantly, we observed that CD300c expression increases with the severity of the symptoms of hypersensitivity in allergic children, especially in children with more severe symptomatology (Fig 6, B). We verified the costimulatory role of CD300c in fresh and nonprimed basophils from allergic patients (see Fig E3 in this

article's Online Repository at www.jacionline.org). We did not see significant differences between healthy and allergic children in the CD300a inhibitory receptor expression on basophils (Fig 6, C). However, if we calculate the CD300a/CD300c ratio, we can observe a significant decrease in this ratio in patients with more severe symptoms (Fig 6, D and E). A lower CD300a/CD300c ratio could be interpreted as a lower basophil activation threshold. Altogether, these results suggest that the CD300c molecule might have a role in modulating the activation threshold of basophils in allergic patients and possibly in the clinical manifestations of hypersensitivity.

DISCUSSION

Basophils are one of the target cells that have been shown to participate in allergic diseases through their ability to bind IgE. Because human basophils express high levels of FcεRI, blocking their interaction with IgE has been a main goal in antiallergic therapy. In this context, clinical responses after administration of the anti-IgE mAb omalizumab to patients with food allergy that correlated with basophil suppression have been described, as measured by using the allergen-induced basophil activation test. Clinical trials with omalizumab in patients with food allergy, including milk allergy, resulted in achieving tolerance to higher amounts of the allergen in some patients.⁴⁶⁻⁴⁸ In addition, cell-surface activating and inhibitory receptors, such as CD48 and CD300a, respectively, hold promising therapeutic possibilities based on preclinical studies.^{11,49} In this line inhibition of activating receptors, such as CD300c, can prevent development of allergic reactions by increasing the threshold of IgE-mediated basophil activation.

Several *in vivo* mouse models have demonstrated the role of certain CD300 molecules (ie, CD300a and CD300f) in the regulation of activation of key effector cells, such as mast cells, in allergic responses. Furthermore, genetic approaches and use of bispecific antibody fragments against CD300a have shown the potential of these molecules as therapeutic targets.^{26,49-51} However, less is known about the role of human CD300 molecules in controlling activation status of basophils, eosinophils, and mast cells during allergic responses. Specifically for basophils, only the ability of the CD300a inhibitory receptor to downregulate IgE-mediated activation of these cells has been described.³³⁻³⁵ Here, for the first time, we show that basophils express not only the CD300a inhibitory receptor but also CD300f and the CD300c activating receptor at lower levels. Our results differ from those reported by Takahashi et al.²³ We believe that modifications in the experimental setup, including staining strategies and using different mAbs, could explain the differences between our results and those published by Takahashi et al.

Apart from the phenotypic analysis, the present study has addressed the question of whether CD300c activating receptor expressed in human basophils works as a costimulatory receptor for FcεRI-mediated signals. Our results have provided evidence that CD300c increases degranulation, cytokine production, intracellular calcium mobilization, and phosphorylation of signaling intermediates induced by FcεRI ligation alone. The costimulatory role of CD300c was demonstrated by ligation with specific mAbs and by the ability of its 2 natural ligands, PS and PE, to modulate IgE-mediated basophil activation. Supporting the costimulatory role of CD300c, we have also found that its

expression in children with cow's milk allergy is significantly increased compared with that in healthy control subjects and that it correlates with symptom severity.

IL-3 is a very important cytokine for the development, maturation, and survival of basophils.⁵²⁻⁵⁵ Furthermore, IL-3 markedly increases the activation and release of mediators from basophils in response to cross-linking of FcεRIα through IgE⁵⁴⁻⁵⁶ and also activates them in an IgE-independent manner.^{57,58} We have shown that IL-3 is the only tested stimulus able to upregulate CD300c expression on human basophils. IL-3-induced expression of CD300c was apparent after 18 to 24 hours of stimulation, somehow suggesting that it involves transcriptional and translational mechanisms. Other tested stimuli, including anti-IgE mAb, did not have any effect on CD300c cell-surface levels. This is in contrast to the fast upregulation (3 minutes) of CD300a inhibitory receptor expression in IgE/FcεRI-activated basophils,³³ which probably indicates that there is an intracellular pool of CD300a ready to go to the cell surface after stimulation, in a similar manner as observed in human neutrophils.⁵⁹ Given our results, we propose that one of the mechanisms through which IL-3 increases basophil activation is by inducing CD300c activating receptor expression, which has a costimulatory role during IgE-mediated activation.

Signaling cascades initiated from cell-surface receptors modulate the activation threshold of basophils. Previously, it was described that CD300a inhibits IgE-mediated basophil activation,³³⁻³⁵ and here we have shown that CD300c has a costimulatory role. Human CD300a and CD300c are paired receptors that possess highly homologous sequences in the extracellular domain and have opposing functions in immune regulation, which is explained by the presence of ITIM motifs in the intracellular tail of CD300a and by association of CD300c with adaptor proteins containing ITAM motifs.^{12,15}

We and others have demonstrated previously the recognition of the same ligands (ie, PS and PE) by CD300a and CD300c receptors, although with different affinities.^{19,22,23} CD300a exhibits a stronger binding to PE than CD300c. On the other hand, both CD300a and CD300c exhibit similar binding affinity to PS. In human basophils our results indicate that in the presence of the natural ligands PS and PE, the CD300a-mediated inhibitory signal is predominant, although the degree of this preponderance depends on the expression levels of CD300c and priming of cells with IL-3. This is particularly evident when IL-3-primed basophils were IgE stimulated in the presence of PS, resulting in a greater activation when compared with the control lipid PC. Even in the presence of PE, we observed that the inhibition was lower when basophils were primed with IL-3 and expressed higher levels of CD300c compared with non-IL-3-primed cells. Similar results were observed when basophils were stimulated in the presence of apoptotic cells. This led us to propose that the interplay between the paired receptors CD300a and CD300c has an important role in determining the threshold of IgE-mediated activation of basophils. This notion might have relevance in disease situations, where levels of the CD300a inhibitory receptor are diminished and levels of the CD300c activating receptor are increased. Good examples of such clinical conditions are the description of low levels of CD300a inhibitory receptor expression on basophils from patients allergic to birch pollen³³ and high levels of CD300c activating receptor on

basophils from children with cow's milk allergy (Fig 6). The relationship between the inhibitory and activating capabilities of CD300a and CD300c, respectively, might also be important in the success of allergen-specific immunotherapy because previous studies have shown that there is an increase in the number of apoptotic monocytes expressing the ligands of CD300a and CD300c (ie, PS and PE) early in the induction of protective and tolerogenic allergen-specific immunotherapy.⁶⁰

Food allergy is characterized by altered responses and the development of immediate hypersensitivity reactions to ingested foods. IgE, basophils, and mast cells have an important role in driving immediate hypersensitivity reactions, with the consequent release of vasoactive mediators in response to food allergens.⁶¹ Here we demonstrate that CD300c increases IgE-mediated basophil degranulation. Importantly, our data with a cohort of 17 children allergic to cow's milk have shown that there is a correlation between expression levels of CD300c on basophils and the severity of hypersensitivity symptoms,³⁶ especially in children with more severe symptomatology. These symptoms are the clinical indicator of granule release, suggesting that CD300c might have a role in the clinical manifestations of allergic reactions by decreasing the activation threshold of basophils. However, it would be very important to study a larger cohort of patients allergic to cow's milk and other allergens to obtain a definitive conclusion on the role of CD300c receptor in allergy. Moreover, it would be interesting to analyze IL-3 levels in sera of allergic subjects. They can be increased in allergic patients, which could be the reason for which the expression of CD300c appears increased in basophils from allergic children.

In addition, basophil activation through IgE/FcεRI also leads to a secondary late-phase reaction characterized by cytokine production. Basophils are a main source of the T_H2 cytokines IL-4 and IL-13, which are crucial for isotype class-switching to IgE and amplification of T_H2 responses that in turn aggravate chronic allergic inflammation.^{6,62} Also, IL-13 produced by basophils in response to IgE/FcεRI-mediated activation might have a role in the development of intestinal inflammation.⁶³ Our results indicate that CD300c-mediated signals are able to diminish the threshold of basophils activation through IgE/FcεRI, increasing the production of IL-4 and IL-13 and indicating a possible role of CD300c in the maintenance of allergic inflammation.

Taken together, our results led us to conclude that the interaction between PS and PE with CD300c costimulates IgE/FcεRI-dependent basophil degranulation. Nonetheless, more studies, especially in the context of allergic and inflammatory diseases, are required to completely understand the biological relevance of these findings. The clinical relevance is shown by the high expression of CD300c on basophils from children with cow's milk allergy, although a larger cohort of patients is required to confirm these results. Our data provide interesting contributions to study the role of phospholipid recognition in the modulation of basophil responses and endorse the possible role of CD300c as a biomarker of disease activity and as a pharmacologic target for the treatment of allergic diseases and possibly for other pathologic situations, such as autoimmunity.

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Key message

- CD300c decreases the IgE-mediated activation threshold, and its expression is increased in children with cow's milk allergy.

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METHODS

Cell isolation, purification, and culture

Adult PBMCs were obtained by using Ficoll density centrifugation from buffy coats. Isolated PBMCs and purified basophils (>95% of basophils) were cultured in Iscove modified Dulbecco medium (Thermo Fisher Scientific, Waltham, Mass) supplemented with 10% human AB serum (Invitrogen, Carlsbad, Calif) and 1% GlutaMAX (Thermo Fisher Scientific), hereafter referred to as cIMDM. Basophil enrichment was carried out by using a negative selection method with the Human Basophil Isolation Kit II from Miltenyi Biotec (Bergisch Gladbach, Germany). Fresh whole blood samples from children were collected in sodium citrate-containing tubes and used only for determination of surface expression of CD300c on basophils.

Antibodies and reagents

The following anti-human mAbs were used for flow cytometric analysis: fluorescein isothiocyanate anti-CD63 (clone H5C6), fluorescein isothiocyanate Lineage Cocktail 3 (lin 3; CD3, CD14, CD19, and CD20), Brilliant Violet 412 anti-CD123 (clone 9F5), and peridinin-chlorophyll-protein (PerCP)-Cy5.5 anti-HLA-DR (clone G46-6) from BD Biosciences (San Jose, Calif); phycoerythrin anti-CD203c (clone 97A6) and phycoerythrin anti-CD300a (clone E59.126) from Beckman Coulter (Fullerton, Calif); allophycocyanin (APC) anti-CD300c (clone UP-H2) and phycoerythrin and APC anti-CD300f (clone UP-D2) from Miltenyi Biotec; eFluor660 anti-CD300c (clone TX45), phycoerythrin-Cy7 anti-FcεRI (clone AER-37), PerCP-eFluor710 anti-phosphorylated ERK1/2 (clone MILAN8R), and APC anti-P-Syk (Y348; clone moch1ct) from eBioscience (San Diego, Calif); and Brilliant Violet 421 anti-human IL-4 (clone MP4-25D2), PerCP-Cy5.5 anti-human IL-13 (clone JES10-5A2), purified anti-CD300c (clone TX45), and purified isotype control (clone MOPC-21) from BioLegend (San Diego, Calif).

The following reagents were also used: human recombinant IL-3, human TSLP, and human recombinant IL-33 from Miltenyi Biotec; human anti-IgE from Abbiotec (San Diego, Calif); fMLP from Sigma (St Louis, Mo); 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS or PS), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE or PE), and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC or PC) from Avanti Polar Lipids (Alabaster, Ala); BD GolgiStop Protein Transport Inhibitor (monensin), BD GolgiPlug Protein Transport Inhibitor (brefeldin A), BD Cytofix/Cytoperm Plus Kit, BD Cytofix Fixation Buffer, BD Phosflow Perm Buffer III, and BD Lysing buffer from BD Biosciences; Fluo-4 cell permeant from Thermo Fisher Scientific; and ruxolitinib and camptothecin from Selleckchem (Houston, Tex).

Flow cytometric analyses

Adult PBMCs and purified basophils were washed with staining buffer containing PBS and 1% BSA to block Fc receptors and stained with the respective fluorochrome-conjugated mAbs for 30 minutes on ice. For intracellular staining, cells were permeabilized after extracellular staining with the BD Cytofix/Cytoperm Plus Kit, according to the manufacturer's protocol, and incubated with different labeled mAbs for the detection of intracellular cytokines. Then, cells were washed to remove unbound mAbs and further acquired in a FACSCanto II Flow cytometer (BD Biosciences). Whole blood from healthy children and children with cow's milk allergy was used to determine CD300c expression. Briefly, 100 μ L of whole blood was stained with the respective fluorochrome-conjugated mAbs for 20 minutes at room temperature. Next, red blood cells were lysed with BD FACS Lysing buffer for 15 minutes at room temperature. Then, cells were washed to remove unbound mAbs and further acquired in a FACSCanto II Flow cytometer (BD Biosciences). We used fluorescence minus one (FMO) control, which contains all the fluorochrome-conjugated mAbs in a panel, except for the one that is being measured. FMO control is used to identify and gate cells in the context of data spread caused by the multiple fluorochromes in a given panel. Flow cytometric data were analyzed with FlowJo software, version 10.0.7 (TreeStar, Ashland, Ore).

Functional experiments

For functional experiments, nonprimed and IL-3-primed PBMCs and purified basophils were used. For priming cells with IL-3, freshly isolated PBMCs were cultured in cIMDM and stimulated with IL-3 for 2 hours. Then, they were washed thoroughly and starved from IL-3 for 22 hours. Then, functional experiments were performed.

Calcium mobilization assay. IL-3-primed cells were washed and resuspended in RPMI 1640 containing 10% FBS (5×10^6 cells/mL). Next, cells were labeled with Fluo-4 (2 μ g/mL) from Life Technologies (Grand Island, NY) for 30 minutes at 30°C while protected from light, adding the PE-Cy7-conjugated anti-FcεRI mAb during the last 15 minutes for basophil identification. Then, cells were washed twice and resuspended at 2×10^6 cell/mL. Aliquots of 1 mL were warmed at 37°C for 5 minutes, followed by acquisition in a FACSCanto II flow cytometer.

To establish a baseline, cells were first acquired for 30 seconds without stimuli, and then 5 μ g of anti-CD300c mAb or isotype control was added and acquired for 30 seconds, followed by cross-linking with the addition of 8.5 μ g of goat anti-mouse IgG F(ab')₂. Cells were further acquired for 6 more minutes. Basophils were electronically gated based on their forward- and side-scatters properties and FcεRI expression. The percentage of responding cells was determined by electronically gating basophils that had higher Fluo-4 fluorescence intensity than the baseline between 60 seconds (after cross-linking of receptors) and 210 seconds once cytoplasmic Ca⁺⁺ levels reached basal values. Data were analyzed with FlowJo software, version 7.6.5 and 10.0.7 (TreeStar).

Phosphorylation assay. IL-3-primed PBMCs were washed with PBS and stained for the surface markers FcεRI and CD203c for 15 minutes in cIMDM. Then, cells were resuspended at 10^6 cells/mL and cross-linked with either 5 μ g of anti-CD300c or isotype control, followed by addition of 8.5 μ g of goat anti-mouse IgG F(ab')₂ for 1 and 5 minutes at 37°C. After the incubation time, cells were harvested and washed with PBS before fixing them with the BD Cytofix buffer at 37°C for 10 minutes. Cells were washed again 2 times and permeabilized with the BD Phosflow Perm buffer III for 30 minutes on ice, and then they were incubated for 30 minutes on ice with specific labelled phospho-mAbs for detection of phosphorylated intermediates. Finally, cells were washed to remove unbound mAbs and further acquired in a FACSCanto II Flow cytometer. FMO control was determined. Data were analyzed with FlowJo software.

Cross-linking of CD300c and measurement of activation markers and cytokine production. Culture plates (24 wells) were coated with 5 μ g of either anti-human CD300c or isotype control at 10 μ g/mL concentrations for 2 to 3 hours at 37°C. Adult nonprimed and IL-3-primed PBMCs and purified basophils were then added to the mAb-coated plates (2×10^6 cells/mL) in cIMDM. PBMCs and purified basophils were also stimulated with anti-IgE (10 ng/mL) or anti-IgE (10 ng/mL) plus IL-3 (2 ng/mL). For measurement of activation markers, after 2 to 3 hours of stimulation, cells were stained with appropriate mAbs against CD203, CD63, and FcεRI surface receptors. To measure cytokine production, PBMCs were incubated at 37°C for 6 hours in the presence of BD GolgiStop (monensin) and BD GolgiPlug (Brefeldin A), and cells were harvested and stained with different labeled mAbs for the detection of activation markers and cytokine production, as described above. Sample acquisition and data analysis were carried out, as described previously.

Lipid stimulation experiments. For lipid stimulation experiments, the lipids PC, PS, and PE were diluted in 100% methanol, plated in 24-well plates at 5 μ g/mL, and air-dried for 2 to 3 hours. Later, the IL-3-primed and nonprimed PBMCs (2×10^6 cells/mL) were added to the lipid-coated plates and also stimulated with anti-IgE (10 ng/mL) or anti-IgE (10 ng/mL) plus IL-3 (2 ng/mL). After 2 to 3 hours, cells were stained with appropriate mAbs against CD203, CD63, and FcεRI surface receptors to measure basophil activation.

Stimulation of basophils in the presence or absence of apoptotic K562 cells. K562 human erythroleukemic cells (5×10^5 cells/mL) were cultured overnight at 37°C in the presence of

3 $\mu\text{mol/L}$ camptothecin to induce apoptosis in K562 cells. Induction of apoptosis was determined by using Annexin V staining with a flow cytometer. The next day, IL-3–primed and nonprimed PBMCs (5×10^5 cells/well) were cocultured with normal or apoptotic K562 cells (10^7 cells/well; ratio 1:20) in 48-well plates and stimulated with anti-IgE (10 ng/mL) or anti-IgE (10 ng/mL) plus IL-3 (2 ng/mL) in a final volume of 1 mL. After 2 to 3 hours, cells were stained with appropriate mAbs against surface receptors, CD63, and Fc ϵ RI to measure basophil activation.

Statistical analysis and graphic representation

GraphPad Prism software (version 6.01; GraphPad Software, La Jolla, Calif) was used for graphic representation and statistical analysis. Data were represented in dot plot graphs and bar graphs showing means $\pm$ SEMs. Values obtained from different experiments were compared with the nonparametric Wilcoxon matched-pairs signed-rank test. For comparison between healthy and allergic children, we removed from the analysis the outliers identified with the robust regression and outlier removal (ROUT) method, and then the nonparametric unpaired Mann-Whitney rank test was used.

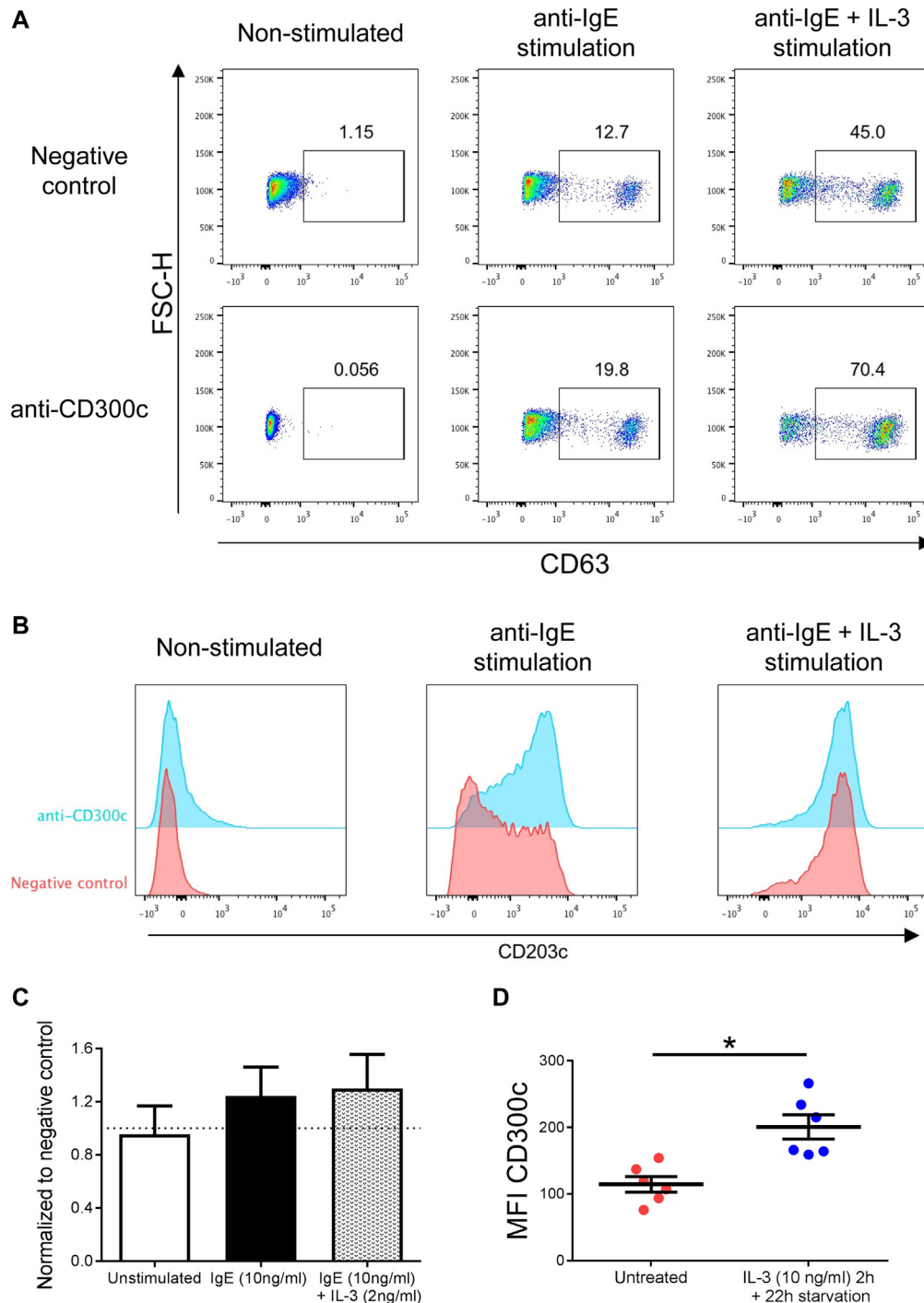


FIG E1. CD300c cross-linking enhances IgE-dependent basophil activation marker expression. **A**, Percentages of CD63⁺ basophils in the presence and absence of cross-linking of CD300c in nonstimulated conditions and after stimulation with anti-IgE or anti-IgE plus IL-3. *FSC*, Forward scatter. **B**, Representative histograms showing expression of CD203c in the presence (blue) and absence (red) of cross-linking of CD300c in nonstimulated conditions and after stimulation with anti-IgE or anti-IgE plus IL-3. **C**, Bars graph showing relative values of basophils activation in response to anti-IgE and anti-IgE plus IL-3 in the presence of anti-CD300c mAb. Values of relative activation were measured by dividing the percentage of CD63⁺ cells in the presence of anti-CD300c mAb by the percentage of CD63⁺ cells in the presence of the isotype control. Results using IL-3-primed purified basophils are shown. Values above and below the dotted line indicate that the percentage of CD63⁺ basophils was higher and lower, respectively, than the percentage obtained in the presence of the isotype control. Means $\pm$ SEMs are represented. **D**, Dot graph showing the median fluorescence intensity (MFI) of CD300c on untreated (red) and IL-3-primed (blue) purified basophils. Means $\pm$ SEMs are shown. * $P < .05$.

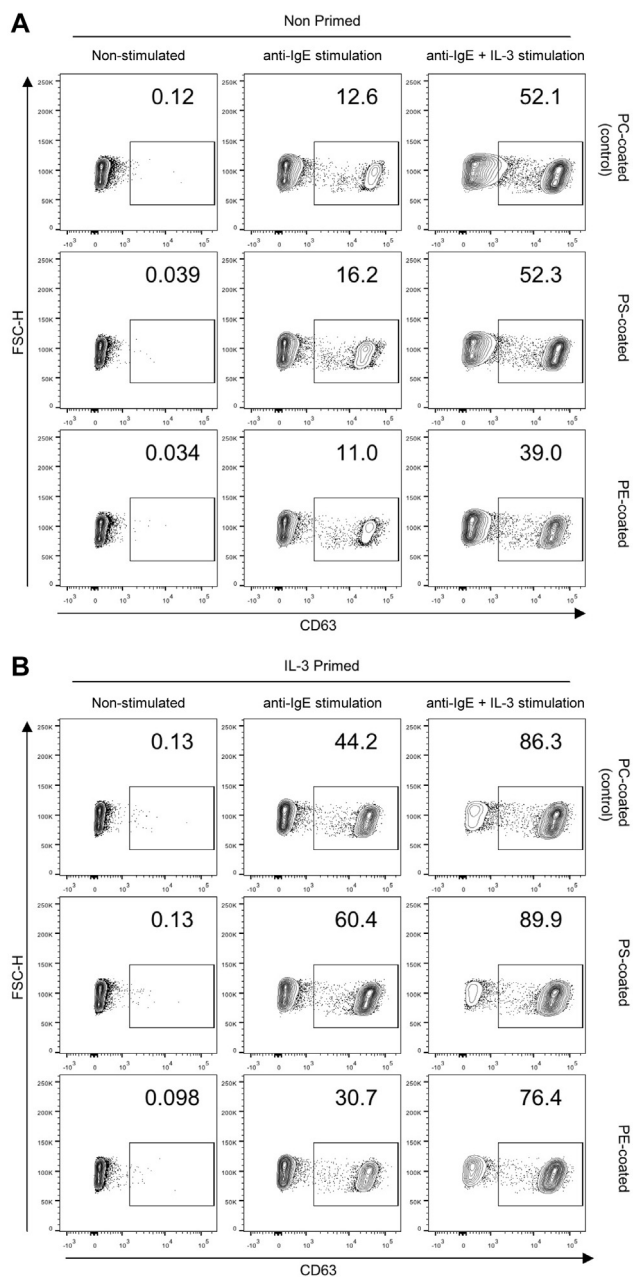


FIG E2. PS and PE modulate IgE-dependent basophil activation. Representative contour plots showing percentages of CD63⁺ basophils in non-IL-3-primed (**A**) and IL-3-primed (**B**) unstimulated (*left column*) or stimulated with anti-IgE (10 ng/mL; *middle column*) and anti-IgE (10 ng/mL) plus IL-3 (2 ng/mL; *right column*) in the presence of the phospholipids PC (control), PS, and PE. FSC, Forward scatter.

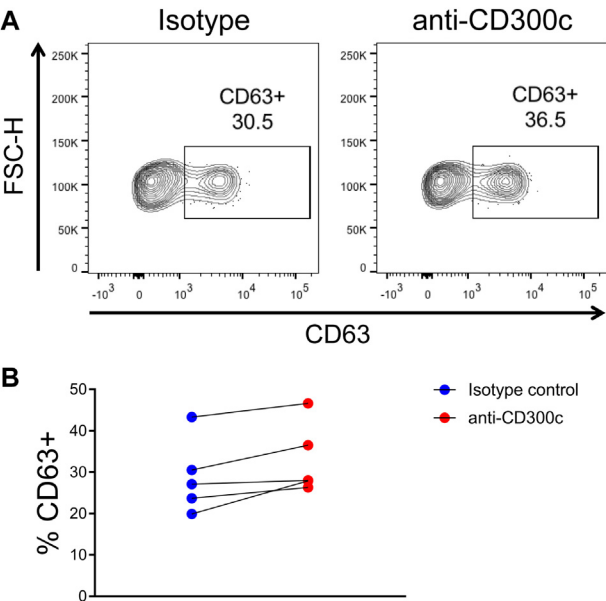


FIG E3. CD300c cross-linking enhances IgE-dependent basophil activation marker expression in basophils from allergic patients. **A**, Contour plots showing percentages of CD63⁺ basophils in the presence and absence of cross-linking of CD300c after stimulation with anti-IgE (10 ng/mL) plus IL-3 (2 ng/mL). Contour plots are representative of data obtained from 5 independent experiments. **B**, Dot graphs showing percentages of CD63⁺ basophils in the presence (*red dots*) and absence (*blue dots*) of cross-linking of CD300c after stimulation with anti-IgE (10 ng/mL) plus IL-3 (2 ng/mL). Each *pair of dots* represents a different experiment.