

Original Article

CD300a is Expressed on Human Basophils and Seems to Inhibit IgE/FcεRI-Dependent Anaphylactic Degranulation

Vito Sabato,^{1,2} Marjoke M. Verweij,¹ Chris H. Bridts,¹ Francesca Levi-Schaffer,³ Bernhard F. Gibbs,⁴ Luc S. De Clerck,¹ Domenico Schiavino,² and Didier G. Ebo^{1*}

¹Department of Immunology, Allergology, Rheumatology, Faculty of Medicine, University of Antwerp and Antwerp University Hospital, Antwerpen, Belgium

²Department of Allergology, Faculty of Medicine, Catholic University of Sacred Heart, Rome, Italy

³Department of Pharmacology and Experimental Therapeutics, School of Pharmacy, Faculty of Medicine, The Hebrew University of Jerusalem, Jerusalem, Israel

⁴Medway School of Pharmacy, University of Kent, Chatham Maritime, United Kingdom

Background. The final response that leads to basophil degranulation results from a cross-talk between activatory and inhibitory signals. However, in the context of basophil biology, the inhibitory mechanisms that control these processes are still poorly understood.

Aim. To investigate the expression and function of the inhibitory receptor CD300a in human basophils.

Methods. Peripheral blood of 20 patients with birch pollen allergy and 10 healthy control individuals were assessed for CD300a expression before and after activation. To study the function of CD300a, basophils were pre-incubated with anti-human CD300a/c monoclonal antibodies and analyzed for the up-regulation of the activation marker CD203c and appearance of the degranulation marker CD63 following IgE-dependent and IgE-independent triggering.

Results. Basophils from allergic individuals constitutively expressed significantly less CD300a than non-allergics ($P = 0.001$). However, after cross-linking with either anti-IgE or recombinant major birch pollen allergen (rBet v 1), basophils from allergic patients demonstrated a significant and rapid (3 min) up-regulation of CD300a expression in all activated basophils that persisted for over 2 h ($P < 0.05$). Moreover, CD300a expression was significantly higher in the CD203c^{bright+}CD63^{bright+} subpopulation than in CD203c^{bright+}CD63[−] cells ($P < 0.05$). In both patients and controls, pre-incubation with anti-CD300a/c significantly inhibited IgE-mediated CD63 expression ($P < 0.05$), though it did not affect CD203c. In contrast, IgE-independent basophil activation was not inhibited by CD300a/c engagement.

Conclusion. CD300a is expressed on human peripheral blood basophils and rapidly up-regulated upon cross-linking of IgE/FcεRI and suppresses anaphylactic degranulation. © 2011 International Clinical Cytometry Society

Key terms: basophils; CD300a; inhibitory receptors; allergy; immunophenotyping; immune monitoring

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*Correspondence to: Didier G. Ebo, MD, PhD, Department of Immunology, Allergology, Rheumatology, Faculty of Medicine, University of Antwerp, Campus Drie Eiken T4.08, Universiteitsplein 1, 2610 Antwerpen, Belgium.

E-mail: immuno@ua.ac.be

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Upon stimulation, human basophils exhibit different degranulation patterns with the release of various mediators and expression of particular activation markers such as CD63 and CD203c.

The prevailing hypothesis is that basophil degranulation is a continuum, starting with piecemeal degranulation and eventually switching to anaphylactic degranulation with a more substantial release of mediators. In this model, the appearance of lysosome-associated membrane protein-3 (CD63) seems to be closely related to anaphylactic degranulation, whereas up-regulation of CD203c rather reflects basophil activation and is not *per se* associated with a significant release of mediators (1–4).

The mechanisms that govern both this switch and suppress basophil activation to avoid excessive responses remain to be elucidated. However, they are thought to be triggered by inhibitory receptors that can belong either to the immunoglobulin receptor super family or to the c-type lectin super family and are characterized by various numbers of immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in the cytoplasm (5,6).

CD300a (IRp60 or CMRF-35) belongs to a multigene family of activating/inhibitory membrane receptors that contains four ITIMs (7). This inhibitory receptor is constitutively expressed in a variety of immune cells including NK cells (8–10), T helper cells (11,12), monocytes (13), neutrophils (14), eosinophils (15), dendritic cells (10–16), and mast cells (17).

With respect to mast cells, it has been shown that cross-linking of CD300a with the high affinity receptor for IgE (FcεRI) down-regulates IgE-mediated mast cell degranulation and cytokine production (17,18), whereas linking to c-Kit abrogates c-Kit-mediated cell differentiation, survival, and activation (19). Furthermore, CD300a shares 80% structural homology with CD300c (8,9), whose function remains to be elucidated.

However, to the best of our knowledge, the function of CD300a in human basophils has not been reported. Therefore, the primary purpose of this study was to investigate the response of CD300a expression by human basophils to different IgE-dependent and IgE-independent stimuli, and finally to explore its potential to modulate basophil degranulation.

PATIENTS AND METHODS

Patients and Control Individuals

Twenty birch pollen allergic patients (11 males [M]/9 females [F]: median age 28 years; range 10–40) were selected based upon clinical history documented by standardized skin tests and quantification of sIgE as described elsewhere (20). Ten healthy control individuals (6 M/4 F: median age 25 years, range 10–40) were also included. Experiments were performed during autumn and winter, outside the birch pollen season, whilst patients were free of treatment (including allergen-specific immunotherapy). Approval for this study

was obtained from the local ethical committee. Patients and healthy control individuals were asked to approve an informed consent.

Blood Sampling

Endotoxin-free heparinized whole-blood samples were obtained from healthy donors and birch pollen allergic patients. Aliquots of 100 µL whole blood were challenged at 37°C with various IgE-dependent and IgE-independent stimuli at different time points (*vide infra*). One hundred microliters of activation buffer (Hank's balanced salt solution (Invitrogen, Paisley, UK) with 20 µM HEPES and 7.5% NaHCO₃, pH 7.4, without interleukin-3 (IL-3)) were used as negative control to assess spontaneous expression of the different markers. Reactions were terminated by chilling on ice.

IgE-Dependent Activation

Cells were challenged with 100 µL of either anti-IgE (10 ng/mL; clone G7-18 BD Bioscience, Erembodegem, Belgium), as a positive control, or recombinant major allergen from birch pollen (*Betula verrucosa*) (rBet v 1: 1 µg/mL, kindly provided by Stallergènes, Antony Cedex, France) for different time points (60 s to 120 min). The optimal stimulation concentrations of anti-IgE and rBet v 1 were established elsewhere (20).

IgE-Independent Activation with fMLP, PMA, and Ionomycin

Cells were stimulated with *N*-formyl-met-leu-phe (fMLP, Sigma-Aldrich, St-Louis, MO: 0.5 µg/mL) and phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich: 10, 100, 500 ng/mL)–ionomycin (Sigma-Aldrich: 0.25 and 2 µg/mL). In PMA + ionomycin experiments, cells were first incubated with PMA for 10 min and subsequently with ionomycin for an additional 10 min. A stimulation time of 20 min was employed in order to allow comparison with CD63 and CD230c (21,22).

Effect of Cytokines

Cells were also challenged with IL-3 (PeproTech, London, England: 5 ng/mL), IL-10 (PeproTech: 50 ng/mL) or transforming growth factor beta (TGF-β) 1 (PeproTech: 10 ng/mL) for 20–60 and 120 min with or without co-stimulation with rBet v 1 (1 µg/mL) for the last 20 min.

CD300a/c Engagement and Basophil Activation

To evaluate the potential role of CD300a in basophil activation, cells were pre-incubated with mouse monoclonal MEM-260 (AbD Serotec, Oxford, England) that recognizes CD300a and has been demonstrated to significantly inhibit IgE-dependent activation of isolated human and murine mast cells (17).

After a pre-incubation period of 30 min at 37°C, cells were subsequently stimulated with either anti-IgE (10 ng/mL), rBet v 1 (1 µg/mL), PMA 500 ng/mL + ionomycin 2 µg/mL, or fMLP (0.5 µg/mL). Concentrations of 1 and 0.1 µg/mL of anti-CD300a (AbD Serotec, Dusseldorf, Germany), dialysed against buffer with a Nanosep 3K

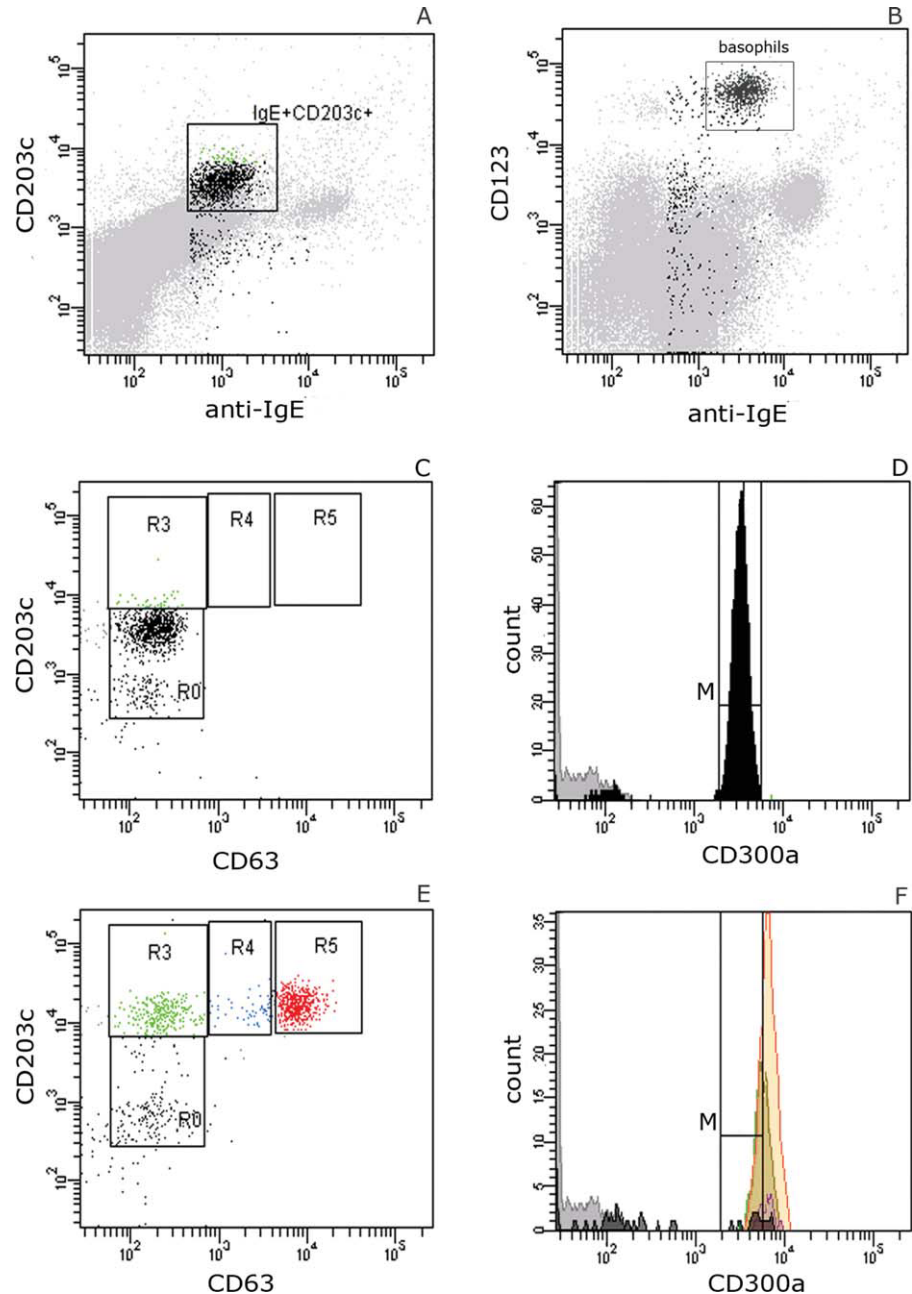


FIG. 1. Comparative analysis of CD300a, CD63, and CD203c expression on resting and activated human basophils. **A:** Basophils selection: basophils are selected as IgE⁺CD203c⁺; **(B)** = IgE⁺CD203c⁺ stained for CD123 denotes a clear basophil population. Basophils after buffer stimulation **(C, D)** and anti-IgE stimulation for 20 min **(E, F)** showing different basophil populations: R0 = CD203c^{dim+}CD63⁻; R3 = CD203c^{bright+}CD63⁻; R4 = CD203c^{bright+}CD63^{dim+}; R5 = CD203c^{bright+}CD63^{bright+}. M denotes the basal expression of CD300a on basophils before and after activation. CD300a up-regulation in cells with distinct CD63 (R5, red cells) expression is significantly higher than CD300a up-regulation in CD63⁻ cells ($P < 0.05$) (F). The gray histogram represents the background staining in the unstimulated (D) and stimulated (F) cells obtained with a FMO-sample. Histograms represent raw counts before MFI calculation. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

centrifugal device (Pall Life Sciences, Ann Arbor, MI), were selected according to preliminary dose-finding experiments.

The surface markers CD203c and CD63 were studied as readouts for basophil activation since they have been reported that up-regulation of these two molecules follows two distinct pathways (23).

Immunophenotyping and Flow Cytometric Analysis

Cells were stained with 20 μ L monoclonal anti-human IgE (clone GE-1, Sigma Aldrich GmbH, Steinheim, Germany) labeled with Alexa Fluor 405 (Molecular Probes,

Invitrogen, Paisley, UK) as described by the manufacturer. Cells were also treated with 10 μ L monoclonal anti-human CD123-PC5 (clone 9F5, BD Bioscience, San Jose, CA), 10 μ L monoclonal anti-human CD63-FITC (clone H5C6, BD Biosciences), 10 μ L monoclonal anti-human CD203c-APC (clone NP4D6 Biolegend, San Diego, CA), and 10 μ L monoclonal anti-human CD300a-PE (clone E59.126, IOTest, Beckman Coulter, US) for 20 min on ice.

Flow cytometric quantification of basophil activation was performed on a FACSCanto II flow cytometer (BD Immunocytometry Systems, San Jose, CA). Side scatter

and anti-IgE⁺/CD203c⁺ staining were applied to gate out at least 1,000 basophils. CD300a positivity was set on basophils with a fluorescence intensity above the level of the 99th percentile of a fluorescence-minus-one (FMO) sample (24).

The fluorescence intensity of CD300a-PE and CD203c-APC for each sample was standardized using Ultra Rainbow Calibration beads (URCP-38-2K, Spherotech, Inc., Lake Forest, IL) and expressed as median fluorescence intensity (MFI) as described in the protocol of the manufacturer.

Statistical Analysis

All results are expressed as median (range). Non-parametric Mann-Whitney and Wilcoxon tests were applied where appropriate. *P*-values < 0.05 were considered as significant. All statistics were performed using SPSS version 16.0 (IBM Corporation, Somers, NY).

RESULTS

Human Basophils Constitutively Express CD300a (IRp60)

As shown in Figures 1a-1d, resting basophils from both patients and healthy control individuals constitutively express the inhibitory receptor CD300a (IRp60). However, spontaneous CD300a expression in patients (MFI 29×10^3 , range $16\text{--}33 \times 10^3$) was significantly lower than in healthy control individuals (MFI 37×10^3 , range $35\text{--}39 \times 10^3$) (*P* = 0.0001) (Fig. 2).

Cross-linking of IgE/FcεRI Rapidly Up-regulates CD300a Expression on Human Basophils

After cross-linking, using either anti-IgE or allergen (rBet v 1), basophils from patients (*n* = 10) demonstrated a rapid and significant up-regulation of CD300a (Fig. 1f) that peaked within 1–3 min and persisted on a plateau for over 2 h (*P* < 0.05) (Fig. 3A).

Up-regulation of CD203c displayed similar kinetics but returned to basal levels within 2 h (Fig. 3B), whereas CD63 peaked later at 5–20 min and subsequently

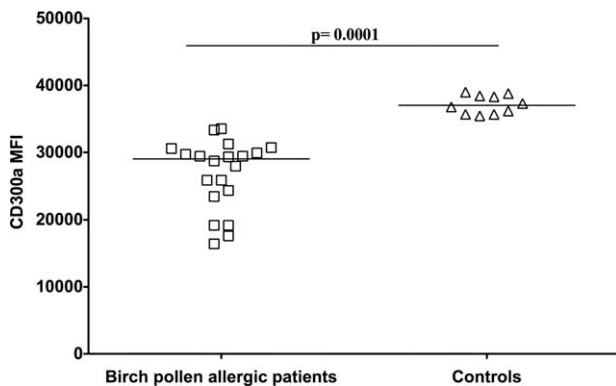


FIG. 2. Basal expression of CD300a (IRp60). Control individuals (open triangles) displayed a significantly higher basal expression of CD300a than birch pollen allergic patients (open squares).

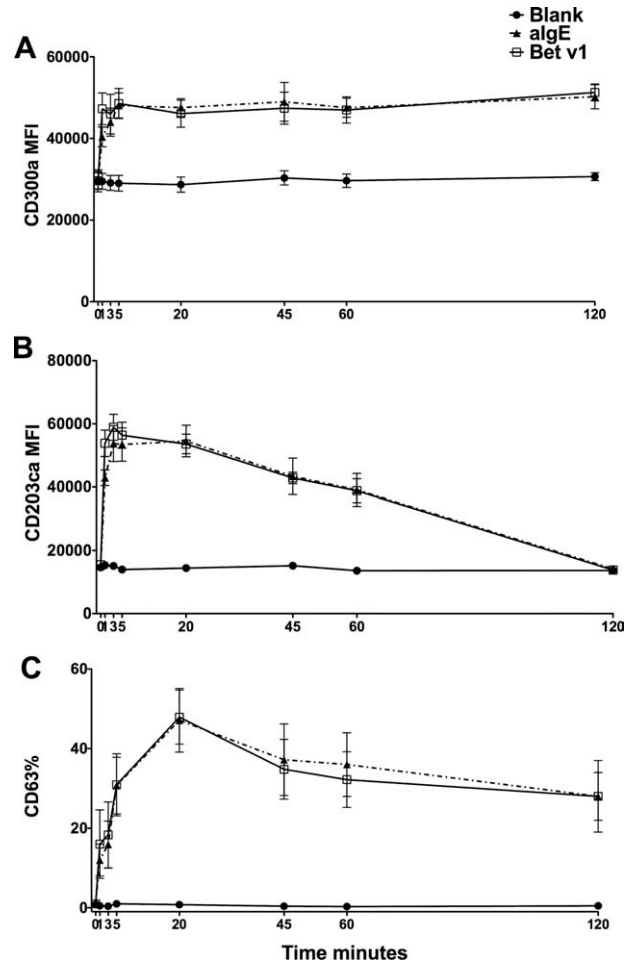


FIG. 3. Kinetics of IgE/FcεRI-dependent up-regulation of CD300a (IRp60) (A), CD203c (B), and CD63 (C) in birch pollen allergic patients (*n* = 10). Results are expressed as median (range). Up-regulation of CD300a and CD203c is fast and peaks within 1–3 min. Appearance of CD63 attains its maximum within 5–20 min. CD300a and CD63 remain on a plateau over 2 h, whereas CD203c returns to basal values within the same time frame.

declined over 2 h without returning to basal levels (Fig. 3C).

In healthy control individuals (*n* = 5), up-regulation of CD300a, CD203c, and CD63 demonstrated similar kinetics as in patients but was only demonstrable through stimulation of the cells with anti-IgE.

Effects of IgE-Independent Basophil Activation on CD300a Expressions Using fMLP and PMA + Ionomycin and Cytokines

Stimulation with fMLP, PMA 500 ng/mL, PMA 500 ng/mL + ionomycin 2 μg/mL, and PMA 100 ng/mL + ionomycin 2 μg/mL (*n* = 10) induced a significant up-regulation of CD300a compared to spontaneous expressions (*P* < 0.05) (Fig. 4). However, up-regulation of CD300a by these IgE-independent stimuli was significantly lower compared to IgE/FcεRI cross-linking (*P* < 0.05). In contrast, PMA 100 and 10 ng/mL and ionomycin 0.25 and 2

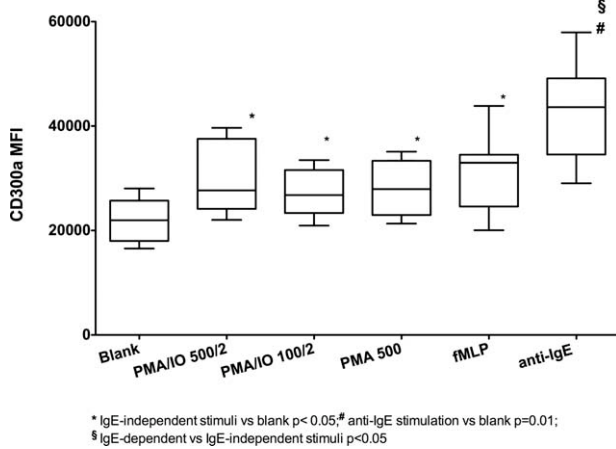


Fig. 4. Comparison of IgE-dependent and independent CD300a up-regulation. Results are expressed as median (25th–75th percentile). IgE-independent activation with PMA 500 ng/mL, PMA+IO 500/2, and 100/2 ng/g mL⁻¹ induces a significant CD300a up-regulation as compared to blank ($P < 0.05$), whereas PMA 100 and 10 ng/mL and IO alone (data not shown) does not modify the expression of this receptor on basophilic membrane. Parallel to PMA/IO experiments, fMLP stimulation shows a significant CD300a up-regulation ($P < 0.05$). Stimulation with anti-IgE induces up-regulation of CD300a that is significantly higher as compared to blank stimulation ($P = 0.01$) as well as compared to stimulation with all IgE-independent stimuli ($P < 0.05$).

μg/mL alone did not modify the expression of CD300a (Fig. 4). Incubation of basophils with IL-3, IL-10, and TGF-β1 neither affected the expression of CD300a on these cells nor modified allergen-induced CD300a up-regulation (data not shown).

Comparative Analysis of CD300a with CD63 and CD203c

Our experiments disclosed that, in addition to CD203c, all activated basophils significantly up-regulated CD300a upon stimulation with either anti-IgE or allergen. In these CD203c^{bright+}/CD300a^{bright+} cells, three subtypes of CD63 expressions were observed: (i) a population without CD63 expression (CD203c^{bright+}CD300a^{bright+}CD63⁻), (ii) a population with moderate CD63 expression (CD203c^{bright+}CD300a^{bright+}CD63^{dim+}) and, (iii) a population with substantial CD63 expression (CD203c^{bright+}CD300a^{bright+}CD63^{bright+}) (Fig. 1e). However, since the number of CD203c^{bright+}CD63^{dim+} events was below 8% in this study population, phenotyping from this subpopulation was not included in the comparative analysis.

From the comparison of CD63⁻ (gate R3) and CD63^{bright+} (gate R5) subpopulations, it emerged that MFI of CD300a up-regulation in cells with distinct CD63 expression is significantly higher than CD300a up-regulation in CD63⁻ cells ($P < 0.05$). A representative case is shown in Figure 1f.

Effect of CD300a/c Engagement on the Expression of the Basophil Activation Markers CD63 and CD203c

Figure 5 shows the effect of 30 min pre-incubation with anti-CD300a/c upon basophil activation by anti-IgE (10 ng/mL) and/or rBet v 1 (1 μg/mL) for an additional

20 min. In patients, pre-incubation with anti-CD300a/c at 1 and 0.1 μg/mL led to a significant median (range) inhibition of 43% (37–77%) and 36% (25–70%) of anti-IgE-induced CD63 expression ($P < 0.05$), respectively ($n = 5$). Furthermore, a median (range) suppression of 63% (48–69%) and 58% (35–61%) of rBet v 1-induced CD63 expression ($P < 0.05$) was observed. CD300a/c engagement in control individuals showed similar significant inhibitory effects on anti-IgE induced CD63 expression ($P < 0.05$) ($n = 5$).

In contrast, pre-incubation with anti-CD300a/c neither affected IgE-dependent CD203c up-regulation nor IgE-independent up-regulation of CD203c and/or CD63 by PMA + ionomycin or fMLP (data not shown).

DISCUSSION

The precise mechanisms that govern basophil piecemeal and anaphylactic degranulation (25) have not been fully resolved but do result from a complex cross-talk between activatory and inhibitory signals. This study demonstrates for the first time that the ITIM-containing inhibitory receptor CD300a (IRp60) is rapidly up-regulated in response to IgE-dependent and, to a lesser extent, also to IgE-independent activation of human basophils. Furthermore, it inhibits IgE/FcεRI-dependent anaphylactic basophil degranulation.

Moreover, it emerged that similar to CD203c, and unlike CD63, up-regulation of CD300a is fast and present in almost all IgE/FcεRI-activated basophils that up-regulate CD203c. However, its expression is significantly higher in the subpopulation of cells that also distinctly co-express the degranulation marker CD63. Alternatively, the expression of CD300a, like CD63, remained on a plateau for 2 h, unlike CD203c which returned to basal levels within the same time frame.

In contrast to the above, no up-regulation of CD300a was observed following incubation of basophils with IL-3, a cytokine that has been shown to prime the cells with phosphorylation of signaling molecules [e.g., p-38 mitogen-activated protein kinases (26), signal transducer and activator of transcription (STAT)-5 (27), up-regulation of CD203c (28), but without appearance of CD63 (4,26) or significant histamine release (4)].

Taken together, our data suggest that even CD300a can be considered as a “fast responder,” its up-regulation seems to be disconnected from the up-regulation of CD203c. Furthermore, as shown in Figure 4, experiments with various IgE-independent and IgE-dependent stimuli suggest that CD300a up-regulation is calcium dependent. In other words, its potential inhibitory effect could be more relevant for anaphylactic degranulation with combined expression of CD203c and CD63 than for other activation states without significant mediator release or cell priming. This presumption is further endorsed by our experiments showing that pre-incubation with the MEM-260 monoclonal antibody suppresses the appearance of CD63 but has no effect on the up-regulation of CD203c. Moreover, from our

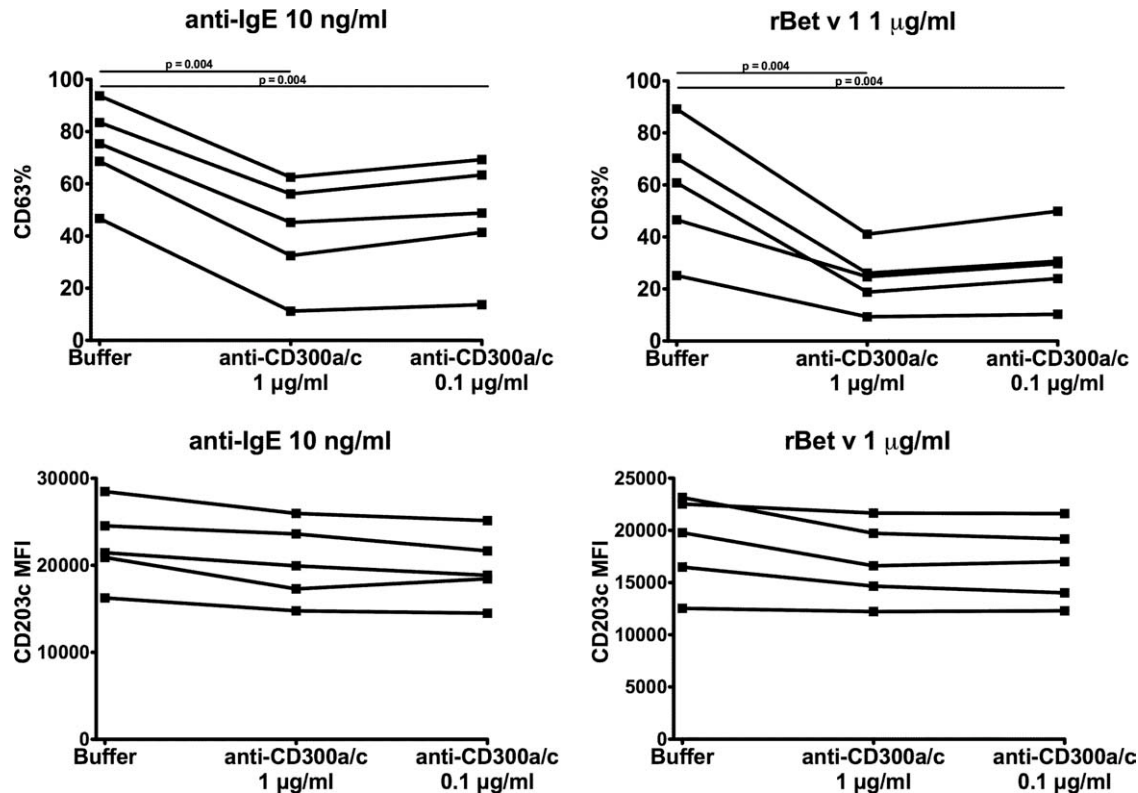


FIG. 5. Effect of a-CD300a ligation on IgE-dependent basophil activation. Individual data for five randomly selected birch pollen allergic patients are plotted. a-CD300a ligation induces a significantly lower CD63 up-regulation in all patients at optimal anti-IgE (10 ng/mL) and rBet v 1 (1 g/mL) stimulation concentration, whereas no effect is detectable on CD203c up-regulation. Similar results were obtained in control individuals (data not shown).

experiments with the various IgE-independent stimuli, it seems that the effect of the inhibitory receptor is strictly related to the anaphylactic degranulation caused by IgE/FcεRI cross-linking. This aligns with the observation that engagement of the inhibitory receptor does not suppress mast cell activation by compound 48/80 (17) and could relate to the fact that IgE-independent activation does not involve Tyr kinase phosphorylation (29–31).

A potential criticism in our study is that, in our whole blood experiments, it cannot be ruled out that engagement of CD300a/c on other cells (i.e., T cells) can have inhibited basophil activation and additional experiments on purified basophils are needed. However, in these pilot experiments we were interested to study the cells in their natural environment with a minimum of extra manipulations that could have influenced the outcome. One could argue that the monoclonal antibody MEM-260 does not discriminate between CD300a and CD300c (9). Nevertheless, the net observed effect is inhibitory and could reflect the CD300a dominance over CD300c signaling, which is in line with results found on other cells (9,14,17–19). Therefore it is clear that more specific tools are needed in characterizing the functional input from the CD300a and CD300c glycoprotein.

Another issue is that we currently only studied phenotypic alterations and need to correlate the results with

mediator release experiments. In this context, it is anticipated that the recently developed technique to analyze histamine by flow cytometry in combination with surface markers on a single cell level (4) might disclose data that are inaccessible to traditional histamine release assays in which results only represent an average from all stimulated cells. Moreover, combining the flow-assisted analyses of phenotypic alterations and histamine release with staining of intracellular signal molecules (4,26,27) might further elucidate on the principles that govern basophil activation.

In conclusion, we show that human basophils express CD300a, and it can be considered as a therapeutic target for allergic diseases as it seems to be closely involved in the inhibition of IgE-mediated anaphylactic degranulation.

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