

INTERLEUKIN-3 PROMOTES THE EXPRESSION OF E-NPP3/CD203C ON HUMAN BLOOD BASOPHILS IN HEALTHY SUBJECTS AND IN PATIENTS WITH BIRCH POLLEN ALLERGY

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We recently identified the ectoenzyme CD203c as a novel basophil activation antigen that is up-regulated in response to FcεRI cross-linkage. We investigated the effects of various interleukins (ILs) on expression of CD203c on blood basophils using an antibody against CD203c and flow cytometry. Of all cytokines tested, only IL-3 was found to upregulate expression of CD203c on basophils above baseline levels. The effects of IL-3 were dose- and time-dependent (EC₅₀: 0.1-1 ng/ml) without differences observed between healthy and allergic donors. Whereas anti-IgE induced maximum upregulation of CD203c within 15 minutes, the IL-3-induced upregulation showed a maximum after 180 minutes. IgE-receptor cross-linking resulted in enhanced expression of both CD63 and CD203c, whereas IL-3 enhanced the levels of CD203c without promoting expression of CD63. The IL-3-induced upregulation of CD203c was also observed in highly enriched basophils and was counteracted by a blocking antibody against the alpha chain of the IL-3 receptor (CD123). The IL-3-induced upregulation of CD203c was also found to depend on the presence of calcium. To analyze signaling pathways involved in IL-3-induced upregulation of CD203c, pharmacologic inhibitors were applied. The PI3-kinase inhibitors, wortmannin and LY294002 counteracted the IL-3-induced expression of CD203c, whereas MEK- and PKC inhibitors showed no effects. In conclusion, IL-3 upregulates expression of CD203c on basophils through a specific receptor and via a PI3-kinase-dependent signaling-pathway. Compared to FcεRI-mediated cell activation, IL-3-induced upregulation of CD203c is a late(r) event and is not accompanied by upregulation of CD63.

Basophils are important effector cells of allergic reactions (1-4). These cells contain a number of proinflammatory mediators in their granules and express high affinity IgE-binding sites (FcεRI) (1-

4). During an allergic reaction, FcεRI is cross-linked on basophils resulting in mediator-release (1-5). Apart from FcεRI, basophils express a number of other important surface antigens including adhesion

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molecules and receptors for various cytokines (2-4, 6-10). Among these are the interleukin (IL)-3 receptor, IL-5 receptor, and GM-CSF receptor (6-9). The respective cytokines promote survival in basophils and their ability to respond to IgE-dependent stimuli (6, 10-15). In addition, some of these cytokines directly induce mediator-secretion from basophils in allergic individuals (15-16). Furthermore, such cytokines can promote differentiation, adhesion and migration in basophils (17-20).

The ectonucleotide pyrophosphatase/phosphodiesterase 3 (ENPP-3 = CD203c), has recently been identified as activation-antigen that is expressed on blood basophils and their progenitors, and less abundantly on mast cells, whereas other leukocytes do not express CD203c (21-24). In response to IgE-receptor cross-linking by allergen or anti-IgE, the levels of CD203c on basophils increase (21, 25-26). Based on this notion and the specificity of CD203c for basophils, this new marker has recently been considered for the development of a flow cytometry-based allergy-test (25-26). However, so far it remains unknown whether CD203c is exclusively upregulated by IgE-dependent stimuli.

The aims of the present study are to analyze the effects of various recombinant human (rh) cytokines on expression of CD203c on human basophils, and to compare these effects with the effects of an anti-IgE antibody. The results of our study show that IL-3 upregulates the expression of CD203c on human basophils in healthy subjects as well as in allergic patients.

MATERIALS AND METHODS

Reagents and monoclonal antibodies (mAb)

The PE-labeled mAb 97A6 (CD203c), FITC-conjugated mAb CLB-gran12 (CD63) as well as the anti-IgE mAb E124.2.8 (De2) and the CD2 mAb 6F10.3 were purchased from Coulter-Immunotech (Marseille, France), and a polyclonal goat-anti-PI3-kinase antibody (human p85 α -subunit) from Santa Cruz Biotechnology (Santa Cruz, CA), the anti-IL-3 receptor mAb 7G3 (CD123) and the FITC-labeled CDw17 mAb G035 were from Becton Dickinson (San Diego, CA), recombinant human (rh) interleukin(IL)-3 from Strathmann Biotech (Hamburg, Germany), rhIL-5 and rhIL-8 from PeproTech (Rocky Hill, NJ), rhGM-CSF from Sandoz (Basel, Switzerland), and EDTA, EGTA, actinomycin D, and cycloheximide from Sigma (St. Louis, MO). Actinomycin D was

dissolved in methanol, and cycloheximide in DMSO. The blocking CD203c mAb 3A2C2 was produced at the University of Tübingen. Wortmannin, piceatannol, LY294002, staurosporine, PD98059, TPCK, RO-318220, and rapamycin were from Calbiochem (San Diego, CA), the farnesyl transferase inhibitor (FTI) tipifarnib (R115777) from Johnson&Johnson Pharmaceutical Research and Development, LLC (Titusville, NJ, USA), and RPMI 1640 medium and fetal calf serum (FCS) from PAA laboratories (Linz, Austria).

Preparation and enrichment of basophils

Peripheral blood was obtained from 28 healthy individuals and 11 birch-pollen-allergic donors (in the winter season), after informed consent was given. Allergy was confirmed by RAST and skin prick tests. Blood was collected in heparin-containing tubes and layered over Ficoll (density 1.077) to isolate mononuclear cells (MNC). In select experiments, blood (healthy donors, n=3) was prepared in either Ca²⁺-free medium (supplemented with EDTA or EGTA, each 10 μ M) or Ca²⁺-containing medium. The percentage of basophils in the MNC-preparations ranged between 0.1% and 1.3%. Cell viability was always >90% by trypan blue-exclusion. In 18 healthy donors, blood basophils were enriched by flow cytometry according to published techniques (21, 27) using antibodies to CDw17 or CD203c, and a FACS-ARIA sorter (Becton Dickinson). Basophil purity ranged between 20 and 75% using CDw17 mAb for sorting, and between 95% and 99% using the CD203c mAb 97A6 for cell-sorting.

Incubation of basophils with anti-IgE, cytokines, and inhibitors

Cells (impure cells or sorted basophils) were incubated with various concentrations of anti-IgE (0.001–10 μ g/ml), rhIL-3 (0.0001–500 ng/ml), rhIL-5 (0.01–100 ng/ml), rhIL-8 (0.01–100 ng/ml), or rhGM-CSF (0.01–100 ng/ml) in RPMI 1640 medium at 5% CO₂ and 37°C. In typical experiments, cells were incubated with anti-IgE for 15 minutes, and with cytokines for 90 minutes. In time course experiments, cytokines and anti-IgE were applied for up to 72 hours. In blocking experiments, basophils from healthy donors (n=3) were preincubated with anti-IL-3 receptor mAb 7G3 (2 or 20 μ g/ml), an isotype-matched control-antibody (CD2, 20 μ g/ml) or control medium for 30 minutes, washed, and then incubated with rhIL-3 (100 ng/ml) at 37°C for 90 minutes.

To study signaling-pathways underlying anti-IgE-mediated or IL-3-induced up-regulation of CD203c, cells were preincubated with pharmacologic inhibitors (wortmannin, 10 nM; LY294002, 10 μ M; staurosporine, 100 nM; PD98059, 10 μ M; piceatannol, 100 μ M; TPCK,

50 μ M; RO-318220, 3 μ M; rapamycin, 20 nM; tipifarnib, 10 μ M) for 15 to 30 minutes prior to incubation with rhIL-3 or anti-IgE.

In selected experiments, basophils were incubated with IL-3 (90 minutes) or anti-IgE (15 minutes) in the presence or absence of actinomycin-D (10 μ g/ml), methanol (0.1%) (solvent-control for actinomycin-D), cycloheximide (10 μ g/ml), or DMSO (0.1%) (solvent-control for cycloheximide). After stimulation with cytokines or anti-IgE (with or without inhibitors), cells were washed and subjected to flow cytometry.

Antibody staining experiments

Cell surface staining was performed by two-color flow cytometry as described (26). In brief, cells were incubated with 10 μ l of PE-labeled CD203c mAb 97A6 and 20 μ l of FITC-labeled CD63 mAb (CLB-gran 12) for 15 minutes (RT). Cells were then washed, re-suspended, and analyzed on a FACScan (Becton Dickinson) (26). Expression of surface antigens was expressed as mean fluorescence intensity (MFI).

Immunocytochemistry was performed on normal enriched (sorted CD203c+) basophils and the basophil cell line KU812 kindly provided by Dr. Kenji Kishi (Niigata University, Niigata, Japan). KU812 cells were kept in RPMI 1640 medium (PAA laboratories) with 10% FCS. Prior to staining, KU812 cells were pre-incubated with LY294002 (10 μ M) or control medium at 37°C for 24 hours. FACS-enriched basophils were pre-incubated with LY294002 (10 μ M) at 37°C for 12 hours, and were then exposed to IL-3 (100 ng/ml) or control medium for 90 minutes. Thereafter, cells were spun on cytospin slides. Immunocytochemistry was performed as described (28). In brief, slides were incubated with an anti PI3-kinase antibody (work dilution: 1:100) for 60 minutes. Slides were then washed and incubated with a biotinylated second-step rabbit anti-goat IgG antibody (Biocarta, San Diego, CA). As chromogen, alkaline phosphatase complex (Biocarta) was used. Antibody-reactivity was made visible using Neofuchsin (Nichirei, Tokyo, Japan).

Histamine release experiments

Basophils were enriched from 3 healthy controls and 3 allergic donors by Dextran sedimentation, washed, and re-suspended in histamine release buffer. All donors gave informed consent before blood donation. Basophils of allergic donors were exposed to rhIL-3 (100 ng/ml) in the presence or absence of inhibitors (wortmannin, 100 nM; LY294002, 20 μ M; PD98059, 50 μ M; rapamycin, 20 nM; R115777, 1 μ M; TPCK, 50 μ M; piceatannol, 100 μ M; staurosporin, 100 nM; RO-318220, 3 μ M) at 37°C for 90 minutes. Normal basophils were exposed to anti-IgE mAb E124.2.8 (1 μ g/ml) in the presence or

absence of inhibitors (same concentrations as used for IL-3 stimulation, see above) for 30 minutes at 37°C. In a separate set of experiments, basophils from 3 non-allergic donors were pre-incubated with control-buffer or CD203c mAb 3A2C2 (1 μ g/ml) for 10 minutes at 4°C, washed, then incubated in IL-3 (100 ng/ml) or control-buffer for 15 minutes, and thereafter were exposed to various concentrations of anti-IgE at 37°C for 30 minutes. In 2 allergic donors, basophils were pre-incubated with control-buffer or CD203c mAb 3A2C2 (1 μ g/ml) for 10 minutes at 4°C, washed, and then incubated with IL-3 (100 ng/ml) or control-buffer for 90 minutes.

After incubation, the cells were centrifuged at 4°C for 10 minutes. Histamine was measured in cell-free supernatants and cell-lysates by radioimmunoassay (RIA) (Immunotech). Histamine release was expressed as percentage of total histamine.

Statistical analysis

To determine significance levels in differences in expression of CD203c or CD63 on basophils after incubation with compounds or inhibitors, appropriate statistical tests including the Wilcoxon's matched pairs signed rank test and ANOVA, were applied.

RESULTS

IL-3 promotes expression of CD203c on blood basophils

Unstimulated basophils expressed measurable baseline-levels of CD203c in all non-allergic donors (n=28) and all allergic donors examined (n=11) without a significant difference between the two groups (MFI in non-allergic donors: 68 $\pm$ 55 [range: 33-267] versus allergic patients: 69 $\pm$ 45 [range: 25-181]). Of all cytokines tested, only IL-3 was found to upregulate expression of CD203c on basophils (p<0.05) (Fig. 1A). The effect of IL-3 was observed in all healthy donors (p<0.05, Fig. 1B) and all allergic patients (p<0.05, Fig. 1C). In addition, we were able to show that IL-3 upregulates expression of CD203c in pure basophils (p<0.05) (Fig. 1D and 1E).

The effects of IL-3 on CD203c-expression in unfractionated basophils (Fig. 2A) and enriched basophils (Fig. 2B) were dose-dependent. The effect of IL-3 was also time-dependent with maximum up-regulation seen after 180 minutes (Fig. 2C). Thereafter, the levels of CD203c decreased back to baseline. Anti-IgE also induced upregulation of CD203c on basophils in all donors (not shown).

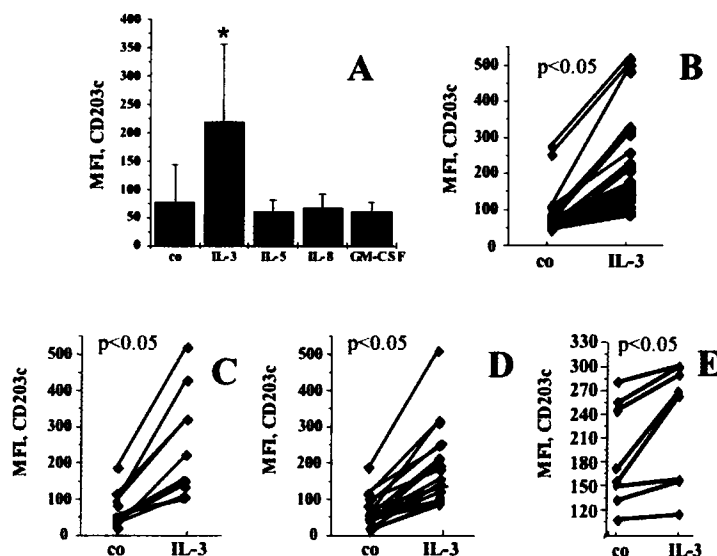


Fig. 1. IL-3 induced upregulation of CD203c on basophils. (A) Effects of cytokines on expression of CD203c on unfractionated normal blood basophils determined by flow cytometry and the CD203c antibody 97A6. Prior to staining, basophils were incubated with recombinant cytokines (each 100 ng/ml) for 90 minutes at 37°C. Data are shown as mean fluorescence intensities (MFI) and represent the mean values $\pm$ S.D. from 5 donors (asterisk = $p < 0.05$ compared to control). (B) Basophils (unfractionated) from 28 healthy donors were incubated with IL-3 (100 ng/ml) or control medium (co) for 90 minutes at 37°C, and then were examined for expression of CD203c (MFI) by flow cytometry. (C) Effects of IL-3 on expression of CD203c on impure basophils in 11 birch pollen allergic donors. (D) Effect of IL-3 (100 ng/ml, 90 minutes, 37°C) on expression of CD203c in FACS-enriched (CDw17+) basophils in 18 healthy donors. (E) Effect of IL-3 (100 ng/ml, 90 minutes, 37°C) on expression of CD203c in highly enriched (purity: 91%–99%) CD203c+ normal basophils ($n=8$). MFI, mean fluorescence intensity; co, medium control.

As observed with IL-3, the effect of anti-IgE on expression of CD203c on basophils was time-dependent (Fig. 2D). However, in contrast to IL-3-exposed basophils, the maximum-upregulation of CD203c induced by anti-IgE was measured already at 15 minutes (Fig. 2D). Thus, the time kinetics of FcεRI-mediated and IL-3-induced enhancement of CD203c on basophils differ from each other. As shown in Fig. 3, neither actinomycin D nor cycloheximide were found to inhibit the IL-3-induced or anti-IgE-induced increase of CD203c, suggesting that upregulation was not a result of increased protein synthesis. The solvent controls (methanol, DMSO) also failed to reduce or promote IL-3-induced or anti-IgE-induced upregulation of CD203c on basophils.

IL-3 upregulates expression of CD203c on basophils through a specific receptor

Confirming previous data (9), basophils were found to react with a mAb against the IL-3

receptor alpha-chain CD123 in all donors tested. To demonstrate that the effects of IL-3 on CD203c-expression were mediated through this receptor, a blocking CD123 antibody (7G3) was applied. This antibody was found to inhibit the IL-3-induced upregulation of CD203c on enriched basophils ($p < 0.05$), whereas no effects were seen with a control antibody (Fig. 4).

The IL-3-induced expression of CD203c on basophils is Ca^{2+} -dependent and inhibited by PI3-kinase-targeting compounds

IL-3 was found to promote expression of CD203c on basophils in the presence but not in the absence of Ca^{2+} , i.e. after depletion of Ca^{2+} by EDTA or EGTA (Fig. 5). Confirming previous data (26), the same result was obtained for anti-IgE-dependent expression of CD203c (not shown). To study signaling pathways underlying IL-3-induced upregulation of CD203c, pharmacologic inhibitors were applied. Baseline expression of CD203c on

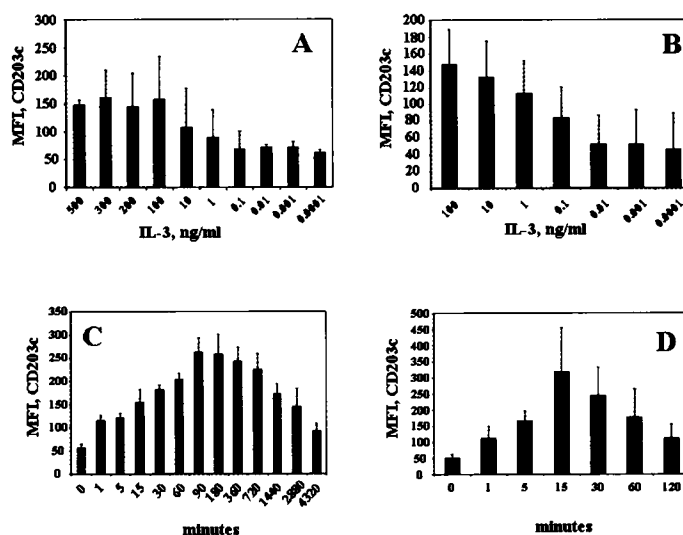


Fig. 2. Dose- and time-dependent effects of IL-3 on expression of CD203c. (A) Dose-dependent effect of IL-3 on expression of CD203c on basophils. Basophils were incubated with various concentrations of IL-3 (as indicated) for 90 minutes at 37°C. Maximum upregulation of CD203c was observed at IL-3 concentrations of ≥ 100 ng/ml, with an EC_{50} of approximately 0.1-1 ng/ml. (B) Dose-dependent effect of IL-3 (0.0001-100 ng/ml) on expression of CD203c in FACS-enriched (CDw17+) basophils. (C) Expression of CD203c on unfraktionated blood basophils incubated with rhIL-3 (100 ng/ml) at 37°C for various time periods as indicated. (D) Time-dependent effect of anti-IgE (1 µg/ml, 37°C) on expression of CD203c on (unfraktionated) basophils. Expression of CD203c on basophils was analyzed by flow cytometry using the antibody 97A6. MFI, mean fluorescence intensity. In each image (A, B, C, D), results represent the mean $\pm$ S.D. values (MFI) from 3 donors.

basophils was not influenced by any inhibitor. Pre-incubation of basophils with the PI3-kinase inhibitors wortmannin (10 nM) and LY294002 (10 µM) resulted in inhibition of IL-3-induced upregulation of CD203c ($p < 0.05$) on unfraktionated and enriched basophils (Fig. 6A, 6B). By contrast, little if any effects were seen with the other inhibitors (Table I). These data suggest that activation of the PI3-kinase is required for IL-3-induced upregulation of CD203c on basophils. The anti-IgE-induced upregulation of CD203c was also inhibited by LY294002 and wortmannin (Table I). To confirm that exposure of basophils to IL-3 is associated with PI3-kinase-activation, we performed immunocytochemical staining experiments using an antibody against phosphorylated (p) PI3-kinase. In these experiments, basophils were found to express activated pPI3-kinase after incubation with IL-3 (100 ng/ml) for 90 minutes (Fig. 6C, 6D). As expected, the KU812 cell line also expressed pPI3-kinase (Fig. 6E). Pre-incubation of KU812 cells with LY294002 (10 µM)

resulted in a decrease in expression of pPI3-kinase (Fig. 6E, 6F), and the same result was obtained with primary basophils (not shown).

Comparison between CD203c upregulation and histamine release

We next compared IL-3-induced upregulation of CD203c with histamine release. In these experiments we found, that IL-3-induced release of histamine in basophils obtained from allergic donors is inhibitable by the LY294002 and wortmannin, whereas other inhibitors showed no effects (Fig. 7A, Table I). A corresponding result was obtained for anti-IgE-dependent cell-activation. Again, only the PI3-kinase-targeting compounds showed significant inhibitory effects on anti-IgE-induced expression of CD203c and anti-IgE-induced histamine release (Fig. 7B). To examine the possible role of CD203c in IL-3-induced and IgE-dependent histamine release, we applied the blocking CD203c mAb 3A2C2. However, 3A2C2 neither inhibited IL-3-induced

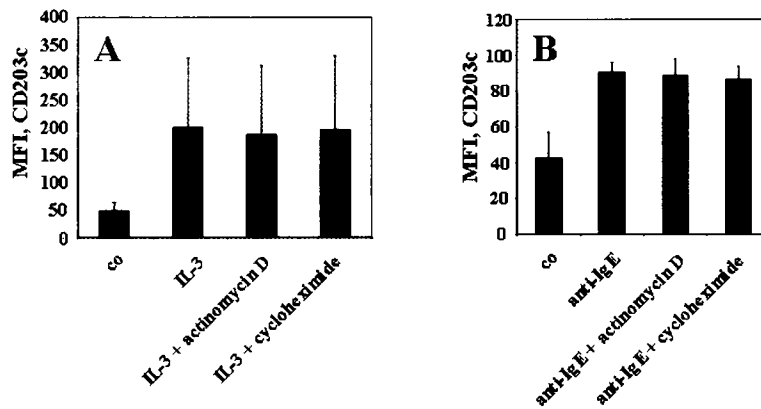


Fig. 3. Effects of actinomycin D and cycloheximide on upregulation of CD203c. (A) Blood basophils obtained from 3 healthy donors were incubated with control medium (co) or recombinant IL-3 (100 ng/ml) at 37°C in the absence or presence of actinomycin-D (10 µg/ml) or cycloheximide (10 µg/ml) as indicated, for 90 minutes. (B) Basophils were incubated with control medium (co) or with an anti-IgE antibody (1 µg/ml) at 37°C in the absence or presence of actinomycin-D (10 µg/ml) or cycloheximide (10 µg/ml), for 15 minutes. Then, basophils were stained with a CD203c antibody and analyzed by flow cytometry. Results represent the mean±S.D. values (mean fluorescence intensities = MFI) from 3 donors. No significant effects of actinomycin-D and cycloheximide on IL-3- or anti-IgE-induced upregulation of CD203c were observed ($p>0.05$).

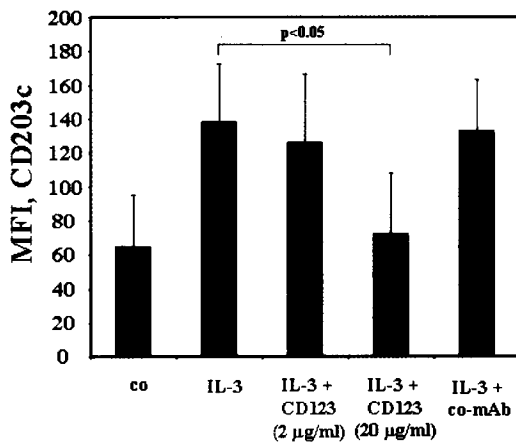


Fig. 4. Blocking of IL-3-induced upregulation of CD203c by the CD123 antibody 7G3. Highly enriched (FACS-sorted) blood basophils obtained from 3 healthy donors were pre-incubated with control medium, with mAb 7G3 (at 2 or 20 µg/ml) directed against the IL-3 receptor alpha chain (CD123), or with an isotype-matched control antibody, 20 µg/ml (co-mAb), for 30 minutes at room temperature. Then, cells were washed and incubated with or without IL-3 (100 ng/ml) for 90 minutes at 37°C, prior to labeling with PE-conjugated CD203c antibody 97A6. co = medium control. Expression of CD203c was determined by flow cytometry. As visible, the blocking CD123 antibody 7G3 inhibited the IL-3-induced upregulation of CD203c ($p<0.05$). Results represent the mean±S.D. values (mean fluorescence intensities = MFI) from 3 donors.

or anti-IgE-induced release of histamine from basophils nor the effect of IL-3 on anti-IgE-induced release (releasability) in basophils (Fig. 7C, 7D).

Differential effects of anti-IgE and IL-3 on expression of CD63

We next compared IL-3-induced expression of CD203c with that of CD63. Incubation of normal basophils with anti-IgE resulted in upregulation of both CD63 and CD203c, whereas exposure to IL-3 was followed by an increase in expression of CD203c, but was not accompanied by upregulation of CD63 (Fig. 8). The other cytokines tested (rhIL-5, rhIL-8, rhGM-CSF) also failed to upregulate expression of CD63 on basophils. These data suggest that, in contrast to CD203c, CD63-upregulation is selectively induced by IgE-dependent stimuli.

DISCUSSION

The ectoenzyme CD203c has recently been identified as a basophil-specific antigen that is rapidly upregulated in response to FcERI cross-linking (21, 25-26). So far, upregulation of CD203c was considered to occur specifically after IgE-dependent cell activation. Our results show that, in addition, activation of basophils through the IL-3

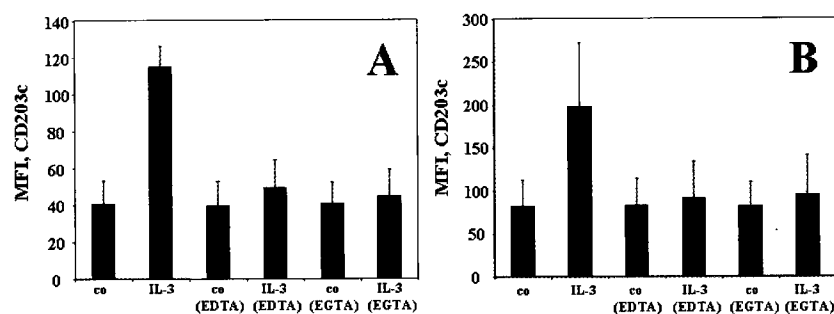


Fig. 5. IL-3-induced upregulation of CD203c on basophils requires the presence of Ca²⁺. Unfractionated basophils (5A) and FACS-enriched basophils (5B) obtained from 3 healthy individuals were incubated with IL-3 (100 ng/ml) or control buffer (co) in the presence of Ca²⁺ or in the absence of Ca²⁺ (i.e. in EDTA or EGTA) as indicated. After 90 minutes, cells were examined for expression of CD203c by flow cytometry using the PE- conjugated antibody 97A6. Results represent the mean $\pm$ S.D. values (mean fluorescence intensities = MFI) from 3 donors. As visible, a significant effect of IL-3 ($p < 0.05$) was observed in the presence of calcium, whereas no significant effect of IL-3 (compared to control) was observed in the presence of EDTA or EGTA ($p > 0.05$).

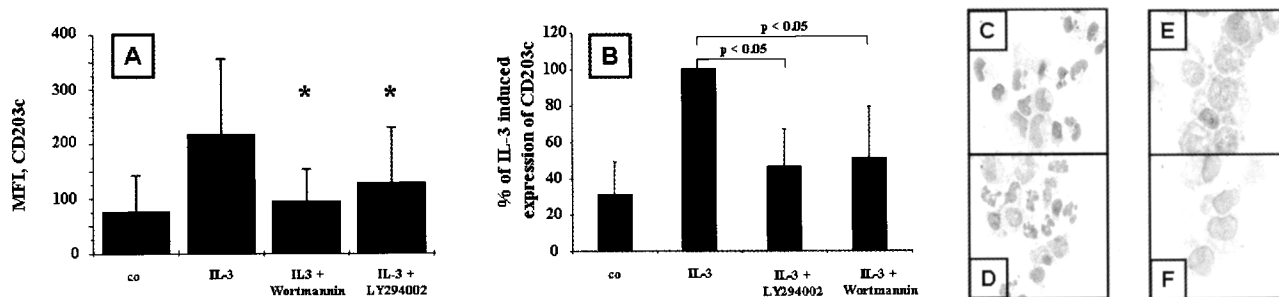


Fig. 6. Effects of PI3-kinase inhibitors on IL-3-induced expression of CD203c. (A) Unfractionated basophils from non-allergic donors ($n=10$) were pre-incubated with control medium, wortmannin (10 nM), or LY294002 (10 μ M) for 30 minutes. Then, cells were washed and incubated with IL-3, 100 ng/ml (or control medium) for 90 minutes. co = medium control. Expression of CD203c was analyzed by flow cytometry using the PE-labeled mAb 97A6. Results represent the mean $\pm$ S.D. from 10 experiments (10 donors). Asterisk: $p < 0.05$ compared to IL-3 alone. (B) Effects of wortmannin (10 nM) and LY294002 (10 μ M) on IL-3-induced upregulation of CD203c in FACS-enriched basophils in 5 donors (conditions were the same as in Figure 6A) Results are expressed as percentage of CD203c-expression (mean fluorescence intensity = MFI) measured on basophils after incubation in IL-3. (6C+6D) Enriched basophils were incubated with control medium (6C) or recombinant IL-3 (100 ng/ml) (6D) at 37°C for 90 minutes, and were then stained with an antibody against the phosphorylated PI3-kinase. (6E+6F) KU812 cells (basophil cell line) were incubated with control medium (6E) or LY294002 (10 μ M) (6F) for 24 hours, and then stained with the antibody against phosphorylated PI3-kinase.

receptor is associated with upregulation of CD203c. However, the IL-3-induced upregulation of CD203c requires a longer incubation-time than FcERI-dependent upregulation, and is not accompanied by upregulation of CD63.

A number of different cytokines including IL-3, IL-5, GM-CSF, and IL-8 have been reported to induce basophil-activation (6, 10-16). In most instances, IL-3 is a most potent agonist. In the present study, we have investigated the effects of

various cytokines on expression of CD203c on basophils. Interestingly, of all cytokines tested, only IL-3 was found to be capable of inducing significant upregulation of CD203c. This observation points to an IL-3-specific effect. Thus, even IL-5 and GM-CSF, both of which show overlapping effects with IL-3 on basophil growth and function (13-16) failed to promote expression of CD203c, and the same result was obtained for IL-8, a chemokine that binds to a specific receptor on basophils (10). However, not

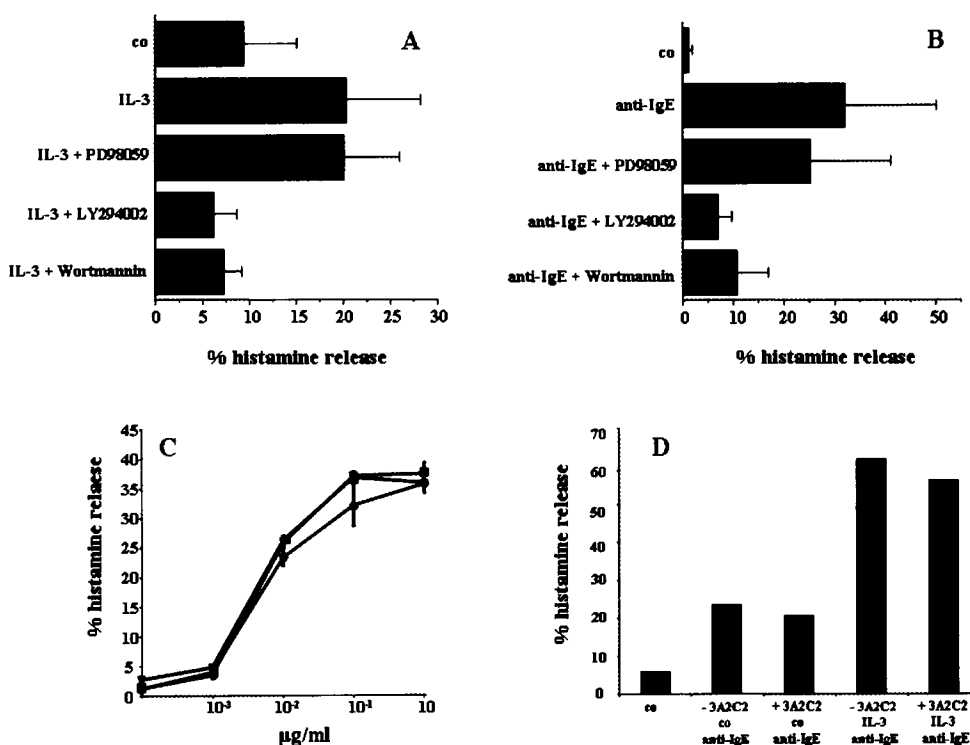


Fig. 7. Effects of inhibitors on histamine release in blood basophils. (A) Effects of inhibitors on IL-3-induced histamine release from basophils obtained from allergic donors ($n=3$). Basophils were pre-incubated with PD98059 (50 μ M), LY294002 (20 μ M), or wortmannin (100 nM) for 60 minutes at 37°C. Thereafter, cells were incubated with IL-3 (100 ng/ml) at 37°C for 90 minutes, centrifuged, and cell-free supernatants examined for histamine. co = baseline histamine release in the absence of IL-3 and inhibitors. Histamine release is expressed as percentage (%) of total histamine. Results represent the mean $\pm$ S.D. from 3 independent experiments. (B) Normal basophils were pre-incubated with PD98059 (50 μ M), LY294002 (20 μ M), or wortmannin (100 nM) for 60 minutes at 37°C. Thereafter, cells were incubated with anti-IgE (1 μ g/ml) at 37°C for 30 minutes, centrifuged, and cell-free supernatants examined for (released) histamine. Histamine release is expressed as percentage (%) of total histamine. Results represent the mean $\pm$ S.D. of 3 independent experiments (3 donors). co = non-specific release from basophils (in buffer without any compound). As visible, the PI3-kinase inhibitors LY294002 and wortmannin counteracted IL-3-induced- and anti-IgE-induced release of histamine ($p<0.05$) whereas no effect was seen with the MEK inhibitor PD98059. (C) Anti-IgE-induced release of histamine (percent of total histamine) from normal basophils that had been pre-incubated with control buffer (■—■), an isotype-matched control antibody (▼—▼) or the blocking CD203c mAb 3A2C2 (●—●). The anti-IgE antibody was applied at various concentrations (as indicated) at 37°C for 30 minutes. Results represent the mean $\pm$ S.D. of triplicates in one donor. (D) Effect of the blocking CD203c antibody 3A2C3 on anti-IgE-induced release of histamine in basophils primed with IL-3 in one donor. Basophils were first incubated in the absence (– 3A2C2) or presence (+ 3A2C2) of the blocking CD203c antibody 3A2C2 for 10 minutes at 4°C, then were washed and incubated with control buffer (co) or IL-3 (100 ng/ml) for 15 minutes at 37°C (priming), and thereafter were exposed to an anti-IgE antibody (1 μ g/ml) for 30 minutes. The non-specific release of histamine (control buffer = co in the absence of any compound) is also shown. Results represent the percentage (%) of released histamine relative to the total histamine content of basophils. As visible, no significant effect of the antibody on anti-IgE-induced (C) or IL-3-induced (D) histamine release was found ($p>0.05$).

all currently available basophil-activating cytokines were tested. Therefore, we cannot exclude effects of other cytokines or chemokines on expression of CD203c in basophils. Moreover, it remains to be determined whether combinations of cytokines and

other compounds can (also) upregulate the levels of CD203c on basophils.

A number of different studies have shown that human basophils express a specific receptor for IL-3 (6-9). This receptor is composed of a ligand-

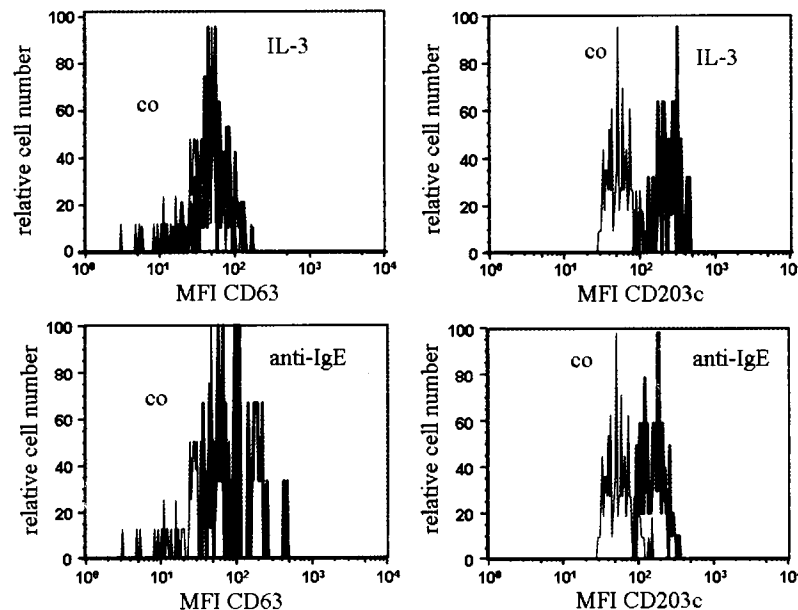


Fig. 8. Differential effects of IL-3 and anti-IgE on expression of CD203c and CD63. Normal blood basophils were incubated with IL-3 (100 ng/ml) (bold line) or control buffer (thin line) for 90 minutes, or with anti-IgE (1 μ g/ml) (bold line) versus control buffer (thin line) for 15 minutes at 37°C. After incubation, cells were washed and examined for expression of CD63 and CD203c by flow cytometry (as indicated) using specific mAbs. Whereas anti-IgE was found to promote the expression of both CD63 and CD203c on basophils, IL-3 was found to upregulate expression of CD203c without promoting expression of CD63 on basophils. MFI, mean fluorescence intensity.

specific alpha-chain (=CD123) and a beta-chain shared by IL-3-, IL-5-, and GM-CSF-receptors (7, 29). In this study, pre-incubation of basophils with a blocking antibody directed against the IL-3 receptor alpha-chain (CD123) inhibited the IL-3-induced upregulation of CD203c. This observation suggests that IL-3 promotes expression of CD203c through this receptor. In addition, we found that IL-3 promotes expression of CD203c on enriched basophils, which suggests a direct effect on basophils. Moreover, we were able to demonstrate surface expression of the alpha chain (CD123) on basophils by flow cytometry, confirming our previous studies (9, 23).

A number of different signal-transduction pathways are involved in Fc ϵ RI-dependent and IL-3 receptor-dependent activation of basophils (2-3, 30-31). These pathways are in part overlapping and show significant cross-interactions. We found that the IL-3-induced upregulation of CD203c on basophils is inhibited by pre-incubation with PI3-kinase-targeting compounds, whereas little or no effects were exerted by other inhibitors. This observation suggests that activation of the PI3-kinase is an

important step in signaling-events underlying IL-3-induced upregulation of CD203c on basophils. This observation is also in line with the notion that the PI3-kinase plays a role in IL-3 dependent signaling in various other models and cell types.

A number of previous and more recent studies have shown that IgE-dependent activation of basophils is associated with increased expression of CD63 (32-35). We therefore compared cytokine effects on expression of CD63 and CD203c on basophils. In respective experiments, we found that IL-3 upregulates expression of CD203c without promoting expression of CD63 in normal subjects as well as in allergic patients. In addition, none of the other cytokines tested appeared to be capable of upregulating CD63 on basophils. This observation is of particular interest and leads to the conclusion that CD63 is a rather specific marker for IgE-dependent basophil-activation, whereas expression of CD203c also depends on other (IgE-independent) factors. Thus, when considering CD63 and CD203c as tools for an allergen-based activation-test, CD63 may be judged as the more specific compound

Table 1. Effects of various pharmacologic inhibitors on anti-IgE-induced and rhIL-3-mediated histamine release and upregulation of CD203c in human basophils.

inhibitor	target signaling molecule	inhibitory effect on histamine release induced by		inhibitory effect on upregulation of CD203c induced by	
		anti-IgE	rhIL-3	anti-IgE	rhIL-3
PD98059	MEK	+/-	-	-	-
LY294002	PI3K	+	+	+	+
Wortmannin	PI3K	+	+	+	+
Staurosporine	PKC	-	-	-	-
Piceatannol	Syk	+/-	+/-	-	-
TPCK	serine proteases	-	-	+/-	+/-
RO-318220	PKC	+/-	-	-	-
Rapamycin	mTOR	-	+/-	+/-	-
Tipifarnib	RAS	-	-	-	-

Basophils were pre-incubated with standard concentrations of inhibitors (for details see text) before being exposed to anti-IgE (1 µg/ml) or rhIL-3 (100 ng/ml). Anti-IgE was applied to normal basophils at 37°C for 15 minutes (CD203c upregulation) or for 30 minutes (histamine release). RhIL-3 was applied to basophils of allergic donors at 37°C for 90 minutes (for both CD203c induction and histamine release). rhIL-3, recombinant interleukin-3; score: +, >50% inhibition; +/- 10-50% inhibition; -, <10% inhibition. MEK, MAP kinase / ERK kinase; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; mTOR, mammalian target of rapamycin.

regarding dependence on IgE, whereas CD203c (but not CD63) is a basophil-specific compound and probably a more sensitive marker compared to CD63 (21, 25-26). Based on all these results it seems a most straightforward approach to recommend the use of both markers in one test to determine IgE-dependent activation of basophils in sensitized individuals.

CD203c has recently been characterized as the ecto-enzyme E-NPP3 (22). This enzyme is expressed on basophil progenitors as well as mature basophils, whereas other blood leukocytes do not express CD203c (21-22). The biologic significance of E-NPP3-expression on basophils remains at present unknown. Likewise, it is unknown whether IL-3-induced upregulation of CD203c on basophils is associated with specific changes in cell functions. We found that IL-3-induced upregulation of CD203c and the IL-3-induced upregulation of histamine-release have similar time-kinetics. Moreover, the IL-3-induced upregulation of CD203c and the IL-3-induced release of histamine were found to utilize similar signaling pathways. However, a blocking CD203c antibody was unable to counteract IL-3-dependent or IgE-mediated histamine release, which

would argue against a role of CD203c in secretory responses in basophils. On the other hand, the lack of an effect of the anti-CD203c antibody does not exclude with certainty a possible role of CD203c in IL-3-dependent histamine release, as the antibody may not have access to critical intracellular domains of CD203c involved in the secretory response.

In summary, our data show that IL-3 upregulates expression of CD203c on human basophils through a specific receptor and through a PI3-kinase-dependent signaling pathway. In contrast to IgE-mediated activation, IL-3-induced upregulation of CD203c is a late(r) event, and is not accompanied by upregulation of CD63.

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