

Evaluation of CCR3 as a Basophil Activation Marker

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ABSTRACT

Objectives: Recent reports have provided conflicting evidence on the stability of CCR3 expression on the surface of basophils. Hence we wanted to independently evaluate the diagnostic usefulness of CCR3 as a surrogate marker of basophil activation and function.

Methods: We examined the correlative relationship between CCR3 expression on the surface of donor basophils and histamine release after donor basophils were treated with agonistic antibodies directed against the high-affinity IgE-Fc receptor and serum samples from 80 individuals displaying symptoms of chronic urticaria (CU).

Results: We observed that CCR3 was significantly downregulated on donor basophils treated with the agonistic antibody and CU-patient serum that demonstrated positive "histamine-releasing activity" (HRA scores >10). However, CCR3 downregulation was also observed on donor basophils incubated with more than 40% of CU-patient serum samples with HRA scores less than or equal to 10.

Conclusions: Overall our data show that CCR3 demonstrates adequate sensitivity (83%) but weak specificity (59%) in its ability to reliably identify histamine-releasing activated basophils.

Upon completion of this activity you will be able to:

- define the phenotypic and functional correlates of basophil activation status.
- outline the rationale for assays.
- set up the assays used to assess basophil activation and function.

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Basophils are a small cellular subset in the peripheral blood that actively participate in the host response to allergens.¹ They express Fc receptors for immunoglobulin (Ig) E as well as IgG on their surface, and the interaction of these molecules with their appropriate ligands tethered to specific antigens regulate basophil responsiveness.² Engagement and cross-linking of the high-affinity IgE-Fc receptor, FcεR1, results in an intracellular signaling cascade that induces basophil activation, which culminates in the release of preformed vasoactive mediators, such as histamine and leukotriene C4 residing in cytosolic granules.^{3,4} Basophil stimulation through this pathway also initiates a transcriptional program to synthesize interleukin (IL) 4 and IL-13, which are released later.⁵

Given the critical role the basophils play in allergic diseases, many diagnostic laboratories have made efforts to use these cells in assays for diagnosing specific allergic conditions.⁶⁻¹¹ Flow cytometry is a useful technique that has gained

popularity in diagnostic laboratories to track the physical presence and evaluate the functional fitness of the cellular subsets of interest.¹² However, this technique requires a priori knowledge of specific cell surface and/or intracellular markers to pinpoint the cellular population to be analyzed.¹² In the case of basophils, monoclonal antibodies targeting CD203c (a molecule specific for cells of the basophil lineage) and basophil surface-bound IgE are commonly used in conjunction with CD45 (a marker for cells of hematopoietic origin) to accurately identify basophils using flow cytometry.^{6,13} The advantage of including CD203c in such analyses is that, in addition to being a basophil-specific marker, it also provides useful information on the basophil activation status. This occurs because its expression is enhanced on basophils that release histamine after stimulation.^{6,13} Other molecules that have been evaluated to identify activated basophils include markers of degranulation CD63 and CD107a as well as the adhesion molecule CD164.⁷⁻⁹ Similar to CD203c, their expression is enhanced after basophil activation. However, none of these molecules are specific to basophils and generally require other markers to be used in conjunction to positively identify basophils. In addition, even the correlation of CD203c upregulation with histamine release, albeit currently acceptable (Spearman $\rho \sim 0.6-0.7$), is not particularly strong. This indicates the need for further studies to find better markers.⁶ CCR3 is a chemokine receptor that is expressed at high levels on resting human basophils and eosinophils.^{10,14} Reports on the stability of its expression on the surface of basophils are discrepant; at least 1 report has shown surface expression of CCR3 to be downregulated after basophil activation.^{10,11} In this report we analyzed the potential efficacy of CCR3 as a reliable marker of basophil activation status using both commercially available basophil activation reagents as well as clinical samples obtained from patients suffering from chronic urticaria (CU).

Materials and Methods

Patient Samples

Aliquots of serum samples from more than 80 individuals exhibiting CU symptoms were analyzed for their ability to stimulate donor basophils. Donor basophil stimulation was determined using enzyme-linked immunosorbent assay to measure histamine release, and phenotypic changes in CCR3 and CD203c expression on the surface of basophils were determined by flow cytometry. Serum aliquots were stored at -70°C prior to analysis. The procurement and analysis of all samples were approved by the institutional review board at the University of Utah. All procedures involving human subjects were in accord with the Declaration of Helsinki of 1975.

Basophil Donors

Heparinized whole blood samples from 2 individuals were used as the source of donor basophils. This selection was made on the basis of reproducibility and accuracy studies performed on 24 donors who reported a history of allergies. All donors provided informed consent, and a 5-mL sample of heparinized blood from the selected donor was drawn once a week for the duration of this study.

Antibodies and Reagents

Fluorochrome-conjugated monoclonal antibodies used for the flow cytometric analysis of basophils included fluorescein isothiocyanate-conjugated goat antihuman IgE (Invitrogen, Camarillo, CA), peridinin chlorophyll protein-conjugated antihuman CD45, phycoerythrin (PE)-conjugated antihuman CD203c, PE-mouse IgG1, κ , allophycocyanin (APC)-conjugated antihuman CCR3, APC mouse IgG2b, κ , APC antihuman CD3, and APC anti-human CD14 (all from Biolegend, San Diego, CA). PE-Cy7 antihuman CD19 was obtained from BD-Pharmingen (San Jose, CA). The positive control rabbit polyclonal antibody to human Fc ϵ R1 α was obtained from Millipore (Billerica, MA). Antibody staining buffer (FACS buffer) was prepared using 1X-Dulbecco phosphate-buffered saline (Invitrogen) and 1% human-AB serum (Cellgro, Mediatech, Manassas, VA) + 0.1% sodium-azide. Basophil stimulation buffer (negative control reagent) was prepared as described previously.⁶

Basophil Histamine Release Assay

Histamine released from donor basophils present in whole blood samples treated with stimulants (polyclonal antibody to Fc ϵ R1 α , CU-patient serum samples) was measured using the histamine-enzyme immunoassay (EIA) kit from Immunotech (Beckman-Coulter, Marseille, France) as per manufacturer's instructions and previous description.⁶ EIA plates were read using an EIA plate reader (Molecular Devices, Sunnyvale, CA). Histamine present in the patient serum samples used for stimulating donor basophils was measured separately, and this value was subtracted from the histamine measured in the serum and donor basophil mixtures to obtain the accurate value of histamine produced by the activated donor basophils. Basal histamine release was measured using donor basophils treated with buffer alone. The stimulants (polyclonal antibody to Fc ϵ R1 α , CU-patient serum) used to induce basophil histamine release were assigned a "histamine releasing activity score" (HRA score). HRA scores greater than 10 were considered positive. An HRA score of 10 is equivalent to 15% of maximal histamine release. A similar cutoff for positivity was used in a previous report.⁶ Maximal histamine release was determined by diluting an aliquot of donor whole blood 1:20 in distilled water and freeze-thawing the sample twice before using the supernatant from the lysed cell suspension in the

assay.⁶ Basal histamine release was always between 5% and 8% of total histamine released.

Basophil-Associated CCR3 Expression

The expression of CCR3 on the surface of basophils was analyzed using flow cytometry. CD203c expression was also measured for comparison and used as an internal control.⁶ Briefly, 100 μ L of undiluted heparinized whole blood from selected donors was mixed with 20 μ L of 1:50 dilution of polyclonal antibody to Fc ϵ R1 or 20 μ L of either undiluted patient serum or basophil stimulation buffer (negative control). This mixture was incubated for 60 minutes at 37°C, following which it was stained with an antibody cocktail targeting CD45, IgE, CD203c, and CCR3. Before it was used, the antibody cocktail was absorbed with a 2% vol/vol mixture of normal mouse, rabbit, goat, and human serum samples. Following a 30-minute incubation at 4°C in the dark, red blood cells were lysed using FACS lysing solution (BD Biosciences, San Jose, CA), and the cell-suspension was washed using FACS buffer and resuspended in 1% paraformaldehyde. Samples were acquired using a FACS Canto-II flow cytometer (BD Biosciences) and analyzed using Flow-Jo software (Tree Star, Ashland, OR). A minimum of 720,000 total events were acquired for each sample. Representative zebra plots are used to depict the basophil gating strategy in whole blood samples. Geometric mean fluorescence intensity (gMFI) of CCR3 and CD203c expression was analyzed on gated SSClo, CD45+, IgE+, CD203c+ cells using histogram plots. Select data are presented as a gMFI ratio (gMFI of marker on basophils incubated with polyclonal antibody to Fc ϵ R1 or patient serum divided by gMFI of marker on basophils incubated with negative control buffer). Data in bar graphs depicting gMFI ratios are displayed as means $\pm$ standard error of the mean. Dashed lines denote the gMFI ratio cutoffs for positivity for the markers (≤ 0.91 for CCR3 and ≥ 1.33 for CD203c).

Statistical Analysis

Differences in the gMFI ratios for CCR3 and CD203c expression on donor basophils after treatment with patient serum samples with HRA index values greater than 10 or HRA index values less than or equal to 10 were analyzed. The unpaired Student 2-tailed *t* test (GraphPad Software, La Jolla, CA) was used for this analysis. The correlative relationship between CCR3 downregulation or CD203c upregulation and histamine release was assessed using scatter plots and determining the Spearman ρ correlation coefficient. In addition, a receiver operating characteristics (ROC) curve was generated for CD203c and CCR3. Statistical difference in the ROC area under the curve (AUC) between the 2 markers was determined using the χ^2 test. Optimal cutoffs for determining basophil activation (gMFI ratio of ≤ 0.91 for CCR3 and ≥ 1.33 for CD203c) were derived by using the maximum Youden

score after generating ROC curves. This maximum Youden score is the value with the maximum combined sensitivity and specificity (Youden score = sensitivity + specificity – 1). A *P* value less than .05 was considered statistically significant.

Results

Engagement of Basophil-Associated Fc ϵ R1 α Induces Marked CCR3 Downregulation

CCR3 is a chemokine receptor that is expressed at high levels on the surface of peripheral blood basophils.¹⁴ A recent report indicated that CCR3 on its own, without the inclusion of additional markers, can be used to reliably identify peripheral blood basophils using flow cytometry.¹⁰ This report further demonstrated that activation of basophils with f-Met-Leu-Phe (fMLP), anti-IgE, or birch pollen allergen in whole blood samples obtained from birch pollen-allergic individuals did not significantly alter basophil-associated CCR3 expression.¹⁰ A subsequent report demonstrated that CCR3 is downregulated after treatment of basophils with fMLP and anti-IgE.¹¹ To resolve these discrepant findings, we activated basophils in whole blood samples obtained from 24 donors who reported a history of allergy, using an agonistic polyclonal antibody targeting the high-affinity IgE-Fc receptor (Fc ϵ R1 α). As a negative control, whole blood samples of these same individuals were treated with buffer alone. Activation of basophils with this method resulted in a 40% reduction in CCR3 expression assessed by gMFI measurement in comparison with buffer-treated basophils (Figure 1A, Figure 1B (top), and Figure 1C). The activation status of basophils was also monitored by evaluating CD203c surface expression and by measuring histamine release (Figure 1). Similar to previous reports, treatment of whole blood samples with this polyclonal antibody resulted in significant upregulation of CD203c on basophils and robust histamine release (Figures 1B [bottom] and 1C).⁶ These findings suggest that basophil-associated CCR3 expression may not be as stable as previously reported.¹⁰

Treatment of Basophils With Serum Samples From Patients With CU Induces CCR3 Downregulation

CU is a common and complex dermatologic condition in which a subset of patients harbor IgG autoantibodies that target Fc ϵ R1 α and/or IgE.^{3,4} The engagement of these autoantibodies with their targets induces basophil activation marked by CD203c upregulation and histamine release.⁶ We wanted to determine if treatment of donor basophils with CU-patient serum samples induces CCR3 downregulation as we observed in basophils treated with the polyclonal antibody to Fc ϵ R1 α . Donor basophils were incubated with

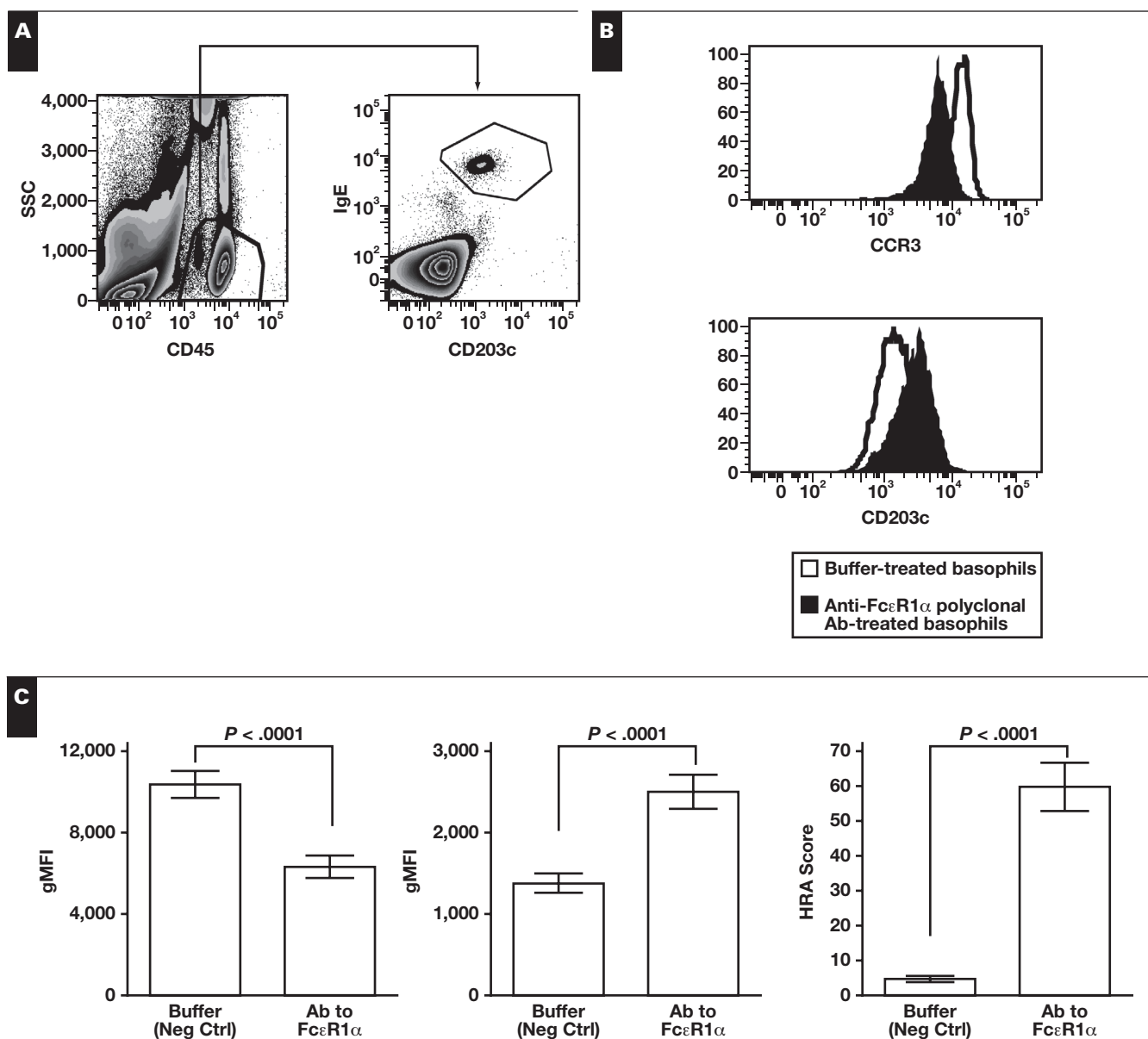


Figure 1 Significant downregulation of CCR3 on donor basophils was seen after treatment with purified polyclonal anti-FcεR1α. **A**, Donor whole blood was stained with relevant antibodies, and cells were initially gated based on CD45 and SSC properties (left). Basophils were identified by further subgating cells based on IgE and CD203c expression (right). **B**, Representative histograms depict (top) CCR3 and (bottom) CD203c expression patterns on the surface of basophils, with each plot depicting histogram overlays of donor basophils incubated with buffer alone and donor basophils incubated with anti-FcεR1α polyclonal Ab. **C**, Bar graphs depict the geometric mean fluorescence intensity of CCR3 (left) and CD203c (center) expression on donor basophils as well as histamine released (right) by donor basophils after treatment with buffer alone and anti-FcεR1α polyclonal antibody. Data are shown as means ± SEM (n = 24).

CU-patient serum samples as indicated in the “Materials and Methods” section. Their activation status was evaluated by measuring changes in the levels of CCR3 and CD203c expression and histamine release. We observed a significant reduction in CCR3 expression and CD203c upregulation on donor basophils treated with CU-patient serum

samples that also induced robust histamine release (HRA scores >10): CCR3 gMFI ratio = 0.79 ± 0.12 (cutoff for positivity ≤ 0.91); CD203c gMFI ratio = 1.64 ± 0.40 (cutoff for positivity ≥ 1.33). **Figure 2**. CU-patient samples that did not provoke strong histamine release from donor basophils (HRA scores ≤ 10) did not significantly change

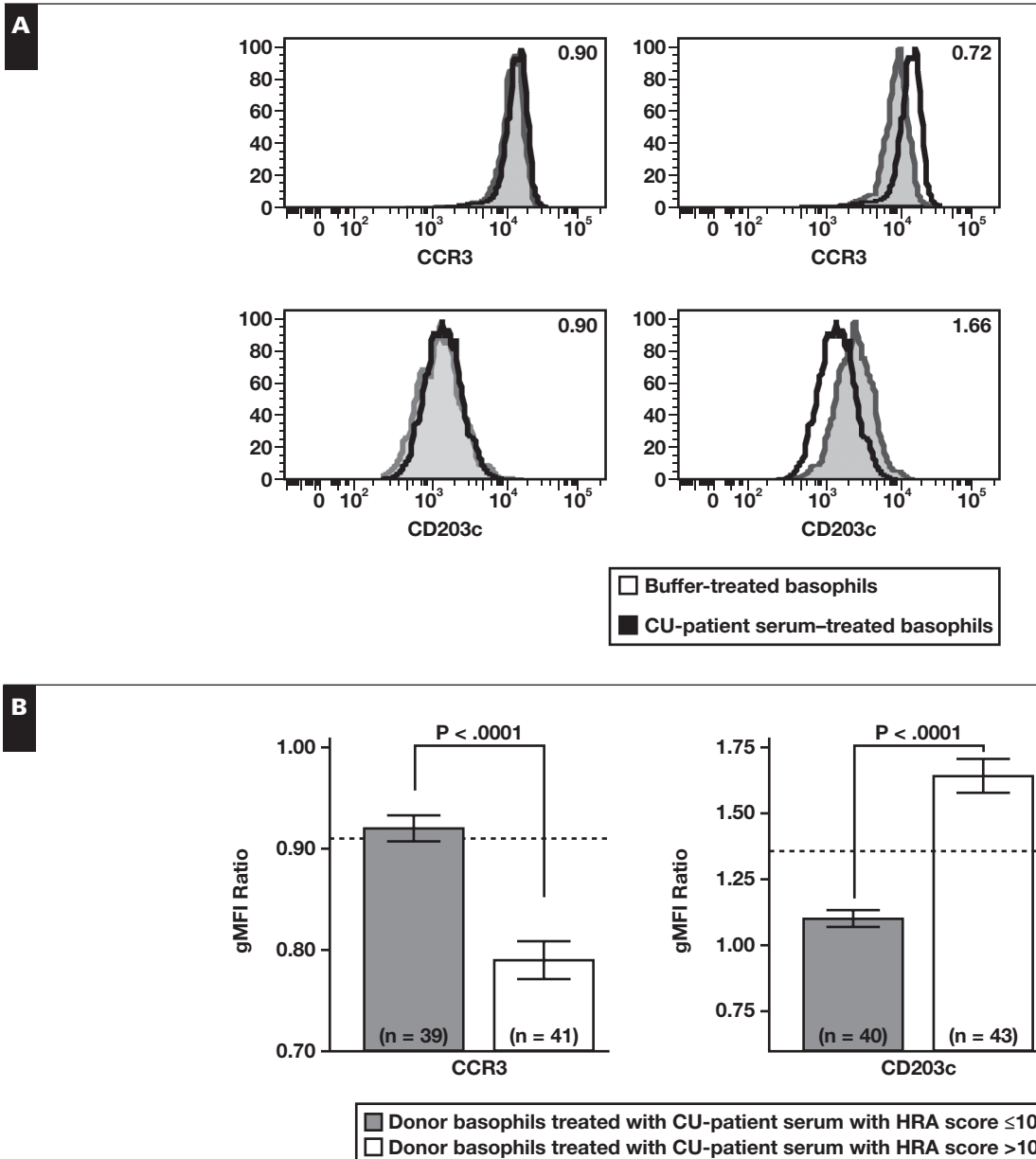


Figure 2 CCR3 downregulation on donor basophils treated with serum samples from patients with chronic urticaria (CU).

A, Representative histograms depict CCR3 and CD203c expression patterns on the surface of donor basophil, with each plot depicting histogram overlays of donor basophils incubated with buffer alone and serum samples from patients with CU (left, histamine-releasing activity [HRA] score ≤ 10 ; right, HRA score = 41). Numbers in each plot depict geometric mean fluorescence intensity (gMFI) ratios. **B**, Bar graphs depict the fold change in MFI (gMFI ratio) for CCR3 and CD203c on donor basophils incubated with patient serum that induced histamine release (HRA scores > 10) or did not induce histamine release (HRA scores ≤ 10). Data are shown as means $\pm$ SEM. Dashed lines denote the cutoffs for gMFI ratios identifying activated basophils (≥ 1.33 for CD203c and ≤ 0.91 for CCR3).

basophil-associated CCR3 (gMFI ratio = 0.92 ± 0.08) and CD203c (gMFI ratio = 1.10 ± 0.19) expression compared with buffer-treated controls (Figure 2). These results raised the possibility that analysis of changes in CCR3 expression could be potentially incorporated in clinical assays evaluating basophil activation status.

Reduction in CCR3 Expression Lacks Adequate Specificity to Reliably Measure Basophil Activation Status

Based on the data described earlier, we wished to further analyze the correlative relationship between basophil activation status, specifically histamine production, and CCR3 downregulation after treating donor basophils with

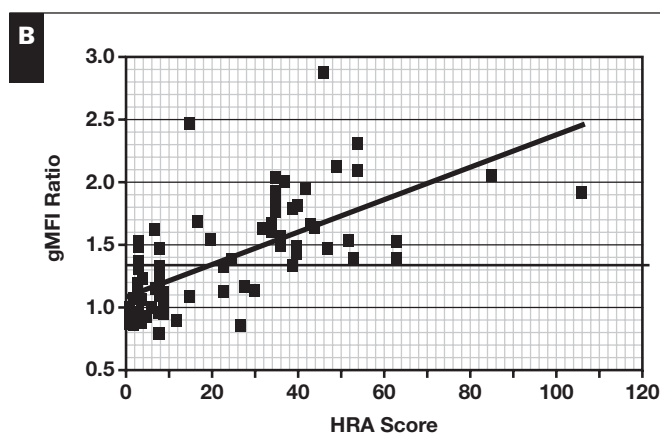
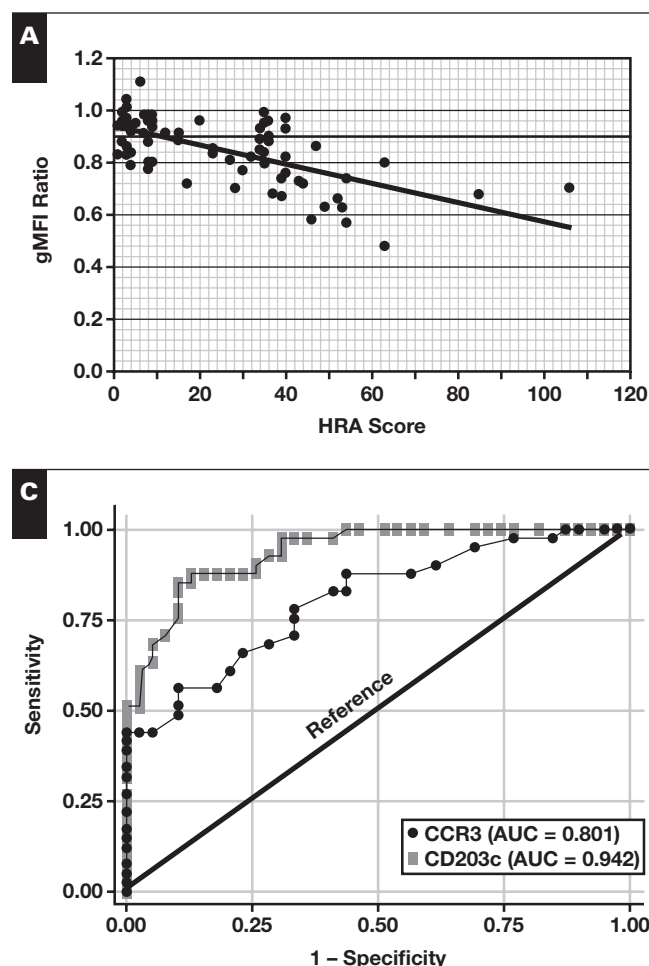


Figure 3 Correlative relationship between basophil-associated CCR3 expression and histamine release. Scatter plots illustrating the relationship between histamine release by basophils and CCR3 (**A**; Spearman $\rho = -0.5756$; $P < .0001$) and CD203c (**B**; Spearman $\rho = 0.7144$; $P < .0001$) expression are shown. **C**, A receiver operating characteristics curve was generated that demonstrated the association of CCR3 and CD203c with histamine release. Differences in the CCR3 and CD203c areas under the curve (AUC) are depicted ($P = .0127$ for AUC difference). gMFI, geometric mean fluorescence intensity; HRA, histamine-releasing activity.

Table 1
Relationship Between CCR3 Downregulation and Histamine Release by Donor Basophils^a

HRA Score ^b	CCR3 gMFI Ratio	
	≤ 0.91	> 0.91
> 10	34	7
≤ 10	16	23

gMFI, geometric mean fluorescence intensity; HRA, histamine-releasing activity.

^a Sensitivity [(+) % agreement (34/41)] = 82.9; specificity [(-) % agreement (23/39)] = 58.97; overall % agreement (57/80) = 71.25.

^b HRA score > 10 identifies serum samples that scored as positive for basophil activation-associated histamine release.

Table 2
Relationship Between CD203c Upregulation and Histamine Release by Donor Basophils^a

HRA Score ^b	CD203c gMFI Ratio	
	≥ 1.33	< 1.33
> 10	36	7
≤ 10	5	35

gMFI, geometric mean fluorescence intensity; HRA, histamine-releasing activity.

^a Sensitivity [(+) % agreement (36/43)] = 83.7; specificity [(-) % agreement (35/40)] = 87.5; overall % agreement (71/83) = 85.5.

^b HRA score > 10 identifies serum samples that scored as positive for basophil activation-associated histamine release.

CU-patient serum samples. This was achieved by plotting out data points in the form of XY scatter plots, an ROC curve, and 2×2 tables (Figure 3, Table 1, and Table 2). As expected, we observed a negative correlative relationship between CCR3 downregulation and basophil activation-associated histamine production. In contrast, we observed that CD203c had a positive correlative relationship (Figures 3A and 3B). However, the statistical correlation between CCR3 downregulation and histamine production (Spearman $\rho = -0.5756$) was not as robust as that observed between CD203c upregulation and histamine production (Spearman $\rho = 0.7144$) (Figures 3A and 3B). The superiority of CD203c as a more reliable marker of basophil activation status was further borne out when the data were expressed in the form of an ROC curve, in which the AUC associated with CD203c expression was closer to 1 than the AUC associated with CCR3 expression (Figure 3C). The weaker fidelity between CCR3 expression and basophil activation-induced histamine release was due to the presence of a greater proportion of CU-patient serum-treated donor basophils that downregulated CCR3 without concomitantly elaborating histamine (Tables 1 and 2). These data suggest that basophil-associated CCR3

measurement has limited use in assays that aim to accurately measure basophil activation status.

Discussion

We describe herein the relationship between basophil-associated CCR3 expression and basophil activation status. Our investigation reveals that although CCR3 is expressed at high levels on nonactivated basophils, it is significantly downregulated after activation (Figures 1 and 2) **Figure 4**. This is in direct contrast to findings from a recently published report.¹⁰ Although the measurement of CCR3 expression is adequately sensitive in identifying activated basophils, it lacks the specificity of the internal control, CD203c, in accurately identifying functionally responsive basophils.

One of the intriguing observations of our study is the downregulation of CCR3 on basophils after treatment with purified agonistic antibody to FcεR1α. One explanation for this finding could be the induction of CCR3-binding chemokines by other leukocytes present in the whole blood samples after histamine stimulation. Histamine is a bioamine that affects multiple physiologic processes in the body, including immune function.¹⁵ Consequently, histamine receptors are widely distributed throughout the body, and most types of histamine receptors (H1, H2, and H4, but not H3) are prominently expressed on neutrophils, eosinophils, monocytes, and T and B cells.¹⁵ It has been demonstrated that histamine enhances the secretion of a number of proinflammatory cytokines and CCR3-binding chemokines from various cell types.¹⁶ Elaboration of any of the CC chemokines that bind CCR3, such as eotaxin, eotaxin-3, MCP-3 and 4, and RANTES, can cause internalization of surface-bound CCR3 by the basophils.¹⁷

About 40% of patients with CU harbor IgG autoantibodies directed against the FcεR1 and 5% have antibodies that target IgE.^{3,4} Having observed significant downregulation of CCR3 using a purified polyclonal antibody directed against FcεR1, we also examined the effect of CU-patient serum treatment of donor basophils and subsequent changes in donor basophil-associated CCR3 expression. A similar approach was used previously to identify patients with CU who harbor IgG autoantibodies that trigger basophil activation.⁶ CCR3 surface expression was significantly reduced on donor basophils treated with CU-patient serum that also induced robust histamine release (Figure 2B). However, serum samples from 39 of the 80 patients displaying symptoms of CU did not induce robust histamine release after incubation with donor basophils. These individuals likely represent the cohort of patients with CU in whom no causal factor was identified (idiopathic chronic urticaria).^{3,4} Nonetheless, of these 39 serum samples, 16 induced CCR3 downregulation on donor

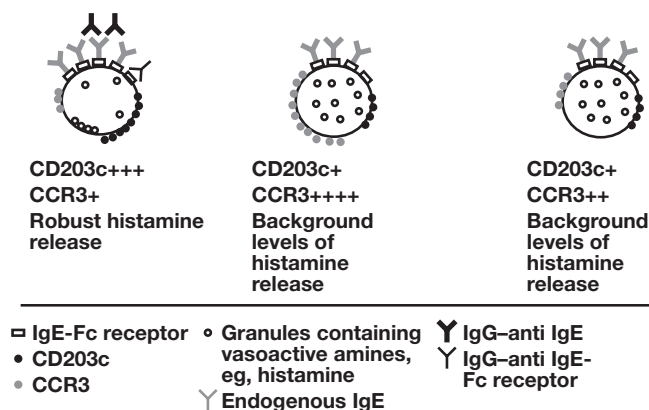


Figure 4 Monitoring basophil responsiveness with flow cytometry. This summary figure demonstrates that donor basophils incubated with patient serum with histamine-releasing activity (HRA) scores greater than 10 (left) display an “activated” phenotype as indicated. Treatment of donor basophils with patient serum with HRA scores less than or equal to 10 (center) maintains a basal state of responsiveness. However the presence of an inflammatory milieu (right) that may or may not be directly related to chronic urticaria can selectively induce CCR3 downregulation without provoking histamine release or CD203c upregulation.

basophils beyond the threshold established for determining basophil activation (gMFI ratio ≤ 0.91) (Table 1). A possible explanation for this might be that the existence of CCR3-binding chemokines in the patient serum sample itself is the result of an independent or related coexisting inflammatory condition (Figure 4).

Collectively, our data demonstrate that CCR3 expression on basophils lacks the stability reported in a previous study that examined basophils in patients with birch pollen-associated allergies.¹⁰ Although CCR3 is significantly downregulated after basophil activation, we observed a less-than-optimal correlative relationship between basophil activation-associated CCR3 downregulation and histamine release. Therefore, CCR3 has limited usefulness as a dependable marker of basophil identity, activation, and function in allergic responses.

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