

The effect of cationic charge on release of eosinophil mediators

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Background: In patients with atopic diseases, cationic-charged eosinophil proteins are deposited in inflamed tissues. Although the role of cytokines in cell activation is well established, the presence of cationic-charged tissue can also be an important factor in inflammatory cell function.

Objectives: We sought to determine whether increased cationic charge seen in an atopic microenvironment plays a role in the activation of eosinophils.

Methods: Human eosinophils were incubated with Sepharose beads coated with cationic or anionic compounds in the presence and absence of a cytokine cocktail (IL-3, IL-5, and GM-CSF) to simulate the milieu of inflammation. Eosinophil peroxidase and eosinophil-derived neurotoxin (EDN) release were compared with eosinophil morphology and expression of CD18, as determined by means of confocal microscopy.

Results: Cytokines with positively charged beads caused greater eosinophil peroxidase release (lysine coated, 44.2 nmol/L; compound 48/80, 40.0 nmol/L; or EDN coated, 49.1 nmol/L) than cytokines alone (14.9 nmol/L). Beads coated with heparin, dextran sulfate, and aspartic acid did not show this effect. EDN release was also induced by lysine-coated beads with cytokines (67.1 ng/100 μ L) and blocked by heparin. Eosinophil incubation with wortmannin, genistein, and the src kinase inhibitor PP1 blocked cationic signaling. Eosinophils adherent to cationic-charged beads but not anionic-charged beads show polarization of CD18 expression toward the bead's surface.

Conclusion: Cationic-charged surfaces induce increased eosinophil mediator release by increasing the density of CD18 expression available at the target surface. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Eosinophil, cationic charge, heparin, eosinophil peroxidase, eosinophil-derived neurotoxin, CD18

Eosinophils are associated with a range of human pathologies in many organ systems. The eosinophil contains 5 cationic-charged proteins: major basic proteins (MBPs) 1 and 2, eosinophil peroxidase (EPO), eosinophil-derived neurotoxin (EDN), and eosinophil cationic protein.^{1,2} In human subjects and animals inhalation of cationic substances induces airway hyperreactivity.^{3,4} It has been surmised that the presence of extracellular eosinophil MBP seen in the tissue of patients with atopic disease⁵⁻⁷ and around parasitic worms⁸ plays a role in inflammatory cell activation. MBP induces mediator release from neutrophils by using phosphatidylinositol-3 (PI3) kinase- and tyrosine kinase-dependent pathways.⁹ MBP can also induce eosinophil mediator release, although the mechanism remains unclear.¹⁰

As eosinophils migrate through tissue, they interact with other cells and structural proteins, which induce a state of activation.¹¹ Furthermore, the milieu of cytokines present in inflamed tissue plays an important role in eosinophil activation and survival.¹² Expression of cell-surface CD18 is upregulated in tissue-dwelling eosinophils,¹³ and signaling through CD18 appears essential for eosinophil degranulation *in vitro*.^{14,15}

In this study we attempted to reproduce the atopic tissue microenvironment and investigate the role of cationic charge within the context of eosinophil-activating cytokines (IL-3, IL-5, and GM-CSF) likely present in inflamed tissues. Our results indicate that exposure of eosinophils to cytokines and to a positively charged surface strikingly increases eosinophil degranulation. The mechanism of this sensitivity is through polarization of CD18 toward cationic surfaces, increasing the density of CD18 at the target.

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METHODS

Please see the **Methods** section of the Online Repository at www.jacionline.org for more complete methodology.

Eosinophil isolation

Human peripheral blood eosinophils were isolated as previously described (>99% purity).^{16,17}

Measurement of EPO release

Eppendorf tubes were precoated with 2.5% human serum albumin (HSA) to block nonspecific cell activation.¹⁸ The release of EPO was measured by using a standardized assay with purified human EPO¹⁹ and peroxidase substrate solution containing *o*-phenylenediamine (OPD), as previously described.¹⁷

Abbreviations used

CNBr:	Cyanogen bromide
DB-LysBD:	Dextran sulfate–blocked lysine-coated CNBr bead
DIC:	Differential interference contrast
EDN:	Eosinophil-derived neurotoxin
EPO:	Eosinophil peroxidase
HB-LysBD:	Heparin-blocked lysine-coated CNBr beads
HSA:	Human serum albumin
LysBD:	Lysine-coated CNBr bead
MBP:	Major basic protein
OPD:	<i>o</i> -Phenylenediamine
PI3:	Phosphatidylinositol-3
ROI:	Region of interest
RT:	Room temperature
sIgA:	Secretory IgA
TMB:	3,3',5,5'-Tetramethyl-benzidine

Measurement of EDN release

EDN release was measured by using an immunoluminometric assay with 2 mAbs. Briefly, microtiter plates were coated with anti-EDN capture antibody. After blocking and washing, standards, samples, and controls were added. Acridinium-labeled anti-EDN secondary antibody was added. After incubations, the plate was read in an LmaxII luminometer (Molecular Designs, Sunnyvale, Calif).

Cationic-charged bead preparation and eosinophil stimulation

Cyanogen bromide (CNBr)–coupled Sepharose 4 Fast Flow beads (CNBr beads; Amersham Biosciences, Uppsala, Sweden) are typically used in secretory IgA (sIgA) stimulation protocols with eosinophils. To create a cationic surface, we adapted the protocol of Abu-Ghazaleh et al,²⁰ using various cationic suspensions in place of sIgA (0.1 mol/L lysine monohydrochloride, compound 48/80 [5 µg/mL], or EDN [purified from human eosinophils, 2.5 mmol/L]). These cationic compounds were also studied directly in solution without the beads (lysine, 0.003 mol/L; compound 48/80, 1 µg/mL; EDN, 0.06 mmol/L). EPO release was also measured from eosinophils and CNBr-coupled beads without cationic coating. Some cationic-coated beads were blocked with heparin (2500 IU/mL) or dextran sulfate (5000 µg/mL). In place of lysine, HSA (0.5%) or aspartic acid (0.037 mol/L) was used for some bead preparations. EPO and EDN release was measured from eosinophils incubated with or without the various types of beads in the presence or absence of the cytokine cocktail.

Cytokine cocktail stimulation

Some eosinophils were incubated with a cocktail of IL-3 (5 ng/mL), IL-5 (2.5 ng/mL), and GM-CSF (5 ng/mL) with or without CNBr-coupled beads. This was compared with calcium ionophore A23187.

Inhibition of intracellular signaling

Eosinophils were preincubated with various concentrations of the PI3 kinase inhibitor wortmannin (0–25 nmol/L), the broad-spectrum tyrosine kinase inhibitor genistein (0–25 µmol/L), the src kinase family inhibitor PP1 (0–100 nmol/L) and the syk kinase family inhibitor piceatannol (0–1000 nmol/L), each for 30 minutes at 20°C before the protocols above. Controls were incubated similarly.

CD18 expression by means of flow cytometry

Eosinophils were incubated with mouse anti-human CD18 IgG2a or isotype control to measure CD18 expression before and after treatment with cytokine cocktail. Goat anti-mouse phycoerythrin-labeled IgG (1:250) was used to quantify CD18 expression by means of FACScan analysis (Becton-Dickinson, Franklin Lakes, NJ).

Blockade of CD18

Eosinophils were preincubated with mouse anti-human CD18 or the isotype control (mouse anti-human IgG2a, sodium azide free) before use in some stimulation protocols.

Live-cell confocal microscopy

By using live-cell real-time photography, eosinophils were observed for morphology and movement during the experimental protocols. Petri dishes with glass coverslip bottoms (MatTek Corp, Ashland, Mass) were precoated with 2.5% HSA. A laser-scanning microscope created differential interference contrast (DIC) images (×160, Olympus FluoView FV1000; Olympus, Markham, Ontario, Canada).

Eosinophil adhesion assay

Eosinophils (5×10^5 /well) stimulated with the cytokine cocktail were added to wells precoated with 2.5% HSA and then coated with either lysine or heparin. After washing away nonadherent cells, 3,3',5,5'-tetramethylbenzidine (TMB) was added. TMB permeates the cell membrane; thus the color change represents the reaction of TMB with EPO from adherent cells (Softmax, 450-nm wavelength).

CD18 expression by means of confocal microscopy

Eosinophils alone without beads or cytokine-treated eosinophils with beads were incubated with rat anti-human CD18 (1 µg/100 µL) or rat purified IgG before Alexa Fluor 488–labeled chicken anti-rat IgG (H+L, 2.5 µg/100 µL; Molecular Probes, Eugene, Ore). Samples were fixed in 1% paraformaldehyde. CD18 localization and quantification of average fluorescence intensity were determined by using confocal laser scanning microscopy with Fluoview software (×480 images, scanned to a thickness of 0.95 µm with the FV10-ASW, Olympus). Images were selected. Freehand region of interest (ROI) selection was set to contain cell membrane contacting or not contacting the bead surface (see Fig E1 in the Online Repository at www.jacionline.org). Average fluorescence intensity negates the possible effects of small differences in ROI area.

Statistical analysis

Data are expressed as means $\pm$ SEMs, with *n* values representing the number of individual donors and their measured EPO or EDN values within the specified experimental condition. Microplate Manager software (Bio-Rad, Hercules, Calif) was used for linear correlation between optical density and EPO molarity values. Comparisons of mean fluorescent intensity and EPO and EDN activity between the groups were analyzed by using ANOVA (Statview 5.0; SAS Institute, Cary, NC). A *P* value of less than .05 was considered statistically significant.

RESULTS**Cationic charge induces EPO release from cytokine cocktail-stimulated eosinophils**

Individually, IL-5, GM-CSF, and IL-3 are known to increase eosinophil survival and activation.^{11,21} Compared with unstimulated control eosinophils (5.1 nmol/L), the cytokine cocktail induced a small but consistent release of EPO (15.3 nmol/L, *P* < .02, Fig 1), which was greater than the release from IL-3, IL-5, and GM-CSF used individually (5.5, 7.5, 9.1 nmol/L, respectively; *n* = 6 each). In the absence of cytokines, CNBr beads with or without lysine coating did not induce EPO release (7.3 ± 0.86 nmol/L vs 5.5 ± 0.9 nmol/L, respectively). In the absence of lysine coating, CNBr beads did not induce EPO release from cytokine-stimulated eosinophils greater than that seen with cocktail alone (16.7 nmol/L). In contrast, the cytokine cocktail together with lysine-coated CNBr beads caused significant EPO release

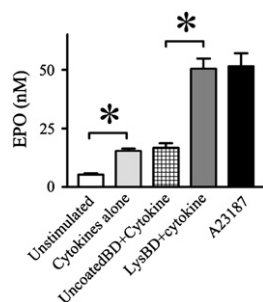


FIG 1. Eosinophils incubated with or without cytokines and CNBr beads (BD). Cytokine cocktail induced a small release of EPO ($n = 20$; $P < .02$) compared with unstimulated control ($n = 20$). Without lysine coating, CNBr beads did not induce EPO release from cytokine-treated eosinophils (UncoatedBD, $n = 19$). Lysine-coated CNBr beads caused significant EPO release from LysBDs ($n = 23$; $P < .0001$) similar to the calcium ionophore A23187 ($n = 14$). SEMs are shown by vertical bars.

(51.4 nmol/L) when compared with that seen with the cocktail alone ($P < .0001$). This was similar to the release induced by the calcium ionophore A23187 (51.4 nmol/L). Based on data from lysed eosinophils,¹⁷ this amount of release is equivalent to 14% total EPO release. IL-3, IL-5, or GM-CSF used individually with the lysine-coated beads increased EPO release, although the potency of each cytokine was not equivalent and was less than that seen with the combined cocktail (19.2, 37.5, and 43.4 nmol/L, respectively; $n = 6$ each).

Other cationic-charged proteins induce EPO release from cytokine-treated eosinophils

To confirm that cationic charge induces eosinophil mediator release, we used compound 48/80, a well-established cationic stimulus for mast cell exocytosis,^{22,23} and one of the cationic eosinophil granule proteins, EDN, to coat beads (Fig 2). Compound 48/80- or EDN-coated beads induced release of EPO from cytokine cocktail treated eosinophils (40.2 and 49.1 nmol/L, respectively) compared with that seen with compound 48/80- or EDN-coated beads incubated without cytokine-treated eosinophils (7.7 and 8.1 nmol/L, respectively; $P < .0001$ each). Eosinophils incubated with compound 48/80 in solution at an effective concentration for mast cells²³ did not release EPO (4.2 nmol/L, $n = 11$). Furthermore, lysine and EDN in solution showed no effect ($n = 3$ each, data not shown).

Inhibition of EPO release by polyanions

The effects of polyanions were studied to further test the hypothesis that induction of EPO release was due to the presence of cationic charge. Lysine-coated beads blocked with heparin or dextran sulfate before incubation with cytokine cocktail-treated eosinophils no longer induced EPO release greater than that seen with cytokine cocktail treatment alone (17.4 nmol/L and 11.1 nmol/L, respectively; $P < .0001$; Fig 3, A). Beads prepared with 0.5% HSA, a noncationic protein, or aspartic acid, an anionic-charged amino acid, also failed to induce EPO release from cytokine-treated eosinophils greater than that seen with cocktail alone (18.3 nmol/L and 9.53 nmol/L, respectively; $P < .0001$). Beads coated with compound 48/80 and blocked with heparin (17.4 nmol/L) or dextran sulfate (11.1 nmol/L) no longer induced EPO release from cocktail-treated eosinophils greater than that observed with the cocktail alone ($P < .0001$ and $P = .0004$,

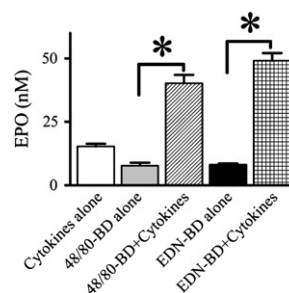


FIG 2. Compound 48/80 and EDN were coated on CNBr beads (BD) in place of the lysine. Compound 48/80- and EDN-coated beads induced a greater release of EPO from cytokine cocktail-treated eosinophils ($n = 15$ and $n = 3$, respectively) compared with that seen in compound 48/80- and EDN-coated beads in the absence of cytokines ($n = 12$ and $n = 3$, respectively; $P < .0001$ each). SEMs are shown by vertical bars.

respectively; Fig 3, B). Purified human EPO was mixed with heparin-coated beads to determine whether heparin had a direct effect on EPO enzymatic activity. EPO added to heparin-coated beads expressed equal amounts of peroxidase activity as EPO mixed with nonheparinized beads (data not shown).

Sensitivity of cytokine cocktail-treated eosinophils to cationic charge is mediated through eosinophil CD18 signaling

The role of eosinophil integrin function in the release of eosinophil granule-stored proteins is well established.¹⁴ Based on the above results and the results of others using IL-5, IL-3, and GM-CSF individually,²⁴ we hypothesized that the cytokine cocktail increases CD18 expression on eosinophils. Eosinophils incubated with the cytokine cocktail of IL-5, IL-3, and GM-CSF showed increased expression of CD18 by means of flow cytometry, which was greater than that described previously²⁴; the mean fluorescent intensity of untreated cells increased from 91.5 to 221.2 ($P = .03$) after cytokine treatment. In keeping with these data, eosinophils preincubated with an antibody to CD18 showed reduced EPO release in response to cytokines and cationic-charged lysine-coated beads (23.5 nmol/L) compared with those preincubated with the appropriate IgG isotype control (48.4 nmol/L, $P < .0001$, Fig 4).

Increased release of EPO from eosinophils incubated with cytokines and lysine-coated beads is not solely dependent on eosinophil adhesion

Under live-cell confocal microscopy, we observed cytokine-treated eosinophils adhering to the lysine-coated beads. This adhesion only occurred in the presence of cytokine cocktail (Fig 5, A). Similar to the blockade of EPO release (Fig 4), anti-CD18 antibody entirely blocks the ability of cytokine-treated eosinophils to adhere to the lysine-coated beads (Fig 5, B). The effect is striking. In contrast, despite inhibition of EPO release (Fig 3, A), the adhesion of eosinophils was not inhibited by heparin blockade of lysine-coated beads (Fig 5, C). Cytokine-stimulated eosinophils were incubated on lysine- or heparin-coated plates to confirm that heparin was acting through the inhibition of cationic charge and not inhibition of eosinophil adhesion. Inhibition of cationic charge did not inhibit adhesion because the numbers of cytokine-treated eosinophils adherent to HSA-, lysine-, and

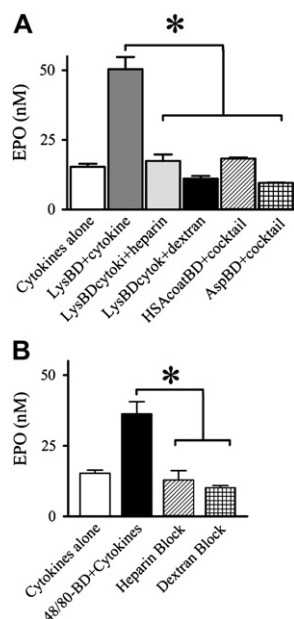


FIG 3. A, LysBDs blocked with heparin ($n = 11$) or dextran sulfate ($n = 4$) did not induce EPO release from cytokine-treated eosinophils compared with unblocked LysBDs ($n = 11$ and $P < .0001$ each). In place of lysine, beads with 0.5% HSA ($n = 3$) or aspartic acid ($n = 9$) failed to induce EPO release from cytokine-treated eosinophils ($P < .0001$ each). B, Compound 48/80-coated beads blocked with heparin ($n = 9$) or dextran sulfate ($n = 3$) did not induce EPO release from cocktail-treated eosinophils ($P < .0001$ and $P < .0004$, respectively). SEMs are shown by vertical bars.

heparin-coated wells were similar (optical densities of 0.70, 0.93, and 0.85, respectively; Fig 5, D). Thus it appeared that adhesion was necessary but not sufficient for EPO release.

Cationic charge polarizes the eosinophil, increasing the density of CD18 toward the bead surface

Increased adhesion did not appear to be the sole mechanism for the effect of cationic charge. Thus we considered that cationic charge could have other effects on CD18 function. We hypothesized that cationic charge might polarize the cell, increasing CD18 density toward the bead surface, and thus potentiate increased CD18 function. In the absence of beads (control), eosinophils demonstrate uniform cell-surface staining for CD18 (Fig 6 and see Fig E2 in the Online Repository at www.jacionline.org). After incubation with lysine-coated beads (LysBDs), adherent cytokine-treated eosinophils showed increased density of CD18 expression toward the bead surface. This was quantified as a 3-fold increase in the average signal intensity of CD18 expression in the cell membrane at the bead surface relative to the intensity of cell membrane not in contact with the bead surface (ratio of 3.15 ± 0.48 ; see Fig E2 in the Online Repository at www.jacionline.org). In contrast, the expression of CD18 on eosinophils adherent to lysine beads blocked with heparin (HB-LysBDs) was not densely directed toward the bead surface but remained uniform over the entire eosinophil surface. The ratio of CD18 signal intensity at the bead surface relative to that at membrane not in contact with the bead surface was 0.89 ± 0.05 , which was significantly different from the lysine-coated bead ratio ($P < .0001$). Similar to heparin treatment, dextran sulfate-blocked beads (DB-LysBDs) also showed an unpolarized surface staining pattern for CD18, with a ratio of 1.16 ± 0.06 ($P < .0001$).

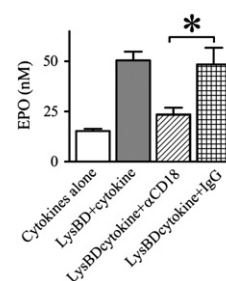


FIG 4. Eosinophils preincubated with an antibody to CD18 had significantly reduced response to cytokines and cationic-charged lysine-coated beads ($n = 16$) compared with the appropriate IgG isotype control ($n = 16$; $P < .0001$). SEMs are shown by vertical bars.

PI3 kinase and tyrosine kinases, including the src family of kinases, signal the effect of cationic charge on cytokine-treated eosinophils

To confirm that the activation of cytokine-treated eosinophils by cationic charge is a physiologically regulated event, we examined the potential intracellular signaling mechanisms. Both wortmannin (Fig 7, A) and genistein (Fig 7, B) inhibited EPO release in a dose-dependent manner from eosinophils subsequently incubated with the cytokine cocktail and lysine-coated beads ($P < .0001$, except for the lowest dose of genistein [$P = .004$]). Although we found no effect of inhibition of the syk family with piceatannol (data not shown), inhibition of the src family kinases with PP1 did show dose-dependent inhibition (Fig 7, C; $P < .0001$, except for lowest dose of PP1 [$P = .01$]).

Cationic charge mediates the release of EDN from eosinophils incubated with cytokines and CNBr beads

To determine whether cationic charge is involved in the release of other mediators from cytokine-stimulated eosinophils, we measured the release of another granule-stored protein, EDN (Fig 8). Similar to EPO release, cytokine cocktail, together with CNBr beads coated with lysine or compound 48/80, induced significant EDN release (67.1 and 54.4 ng/100 μ L, respectively) compared with that seen in unstimulated eosinophils (6.1 ng/100 μ L, $P < .0001$) or cytokine cocktail-stimulated eosinophils alone (26.5 ng/100 μ L, $P = .0002$). Again similar to the EPO data, cationic charge was involved in the release of EDN from cytokine-treated eosinophils because lysine-coated beads blocked with heparin (22.7 ng/100 μ L) no longer induced EDN release from cytokine-treated eosinophils greater than that seen with the cytokine cocktail alone. Wortmannin treatment prevented the release of EDN from eosinophils incubated with the cytokine cocktail and lysine-coated beads (12.4 ng/100 μ L, $P < .0001$).

DISCUSSION

Blood eosinophils become activated as they migrate into tissue and airway luminal spaces, and their functions appear to be seen especially at mucosal surfaces.^{12,25} We hypothesized that cationic-charged tissue surfaces play a role in eosinophil activation, likely in combination with the cytokines present in inflamed tissues. In this report we demonstrate that proinflammatory cytokines induce eosinophils to respond to cationic charge

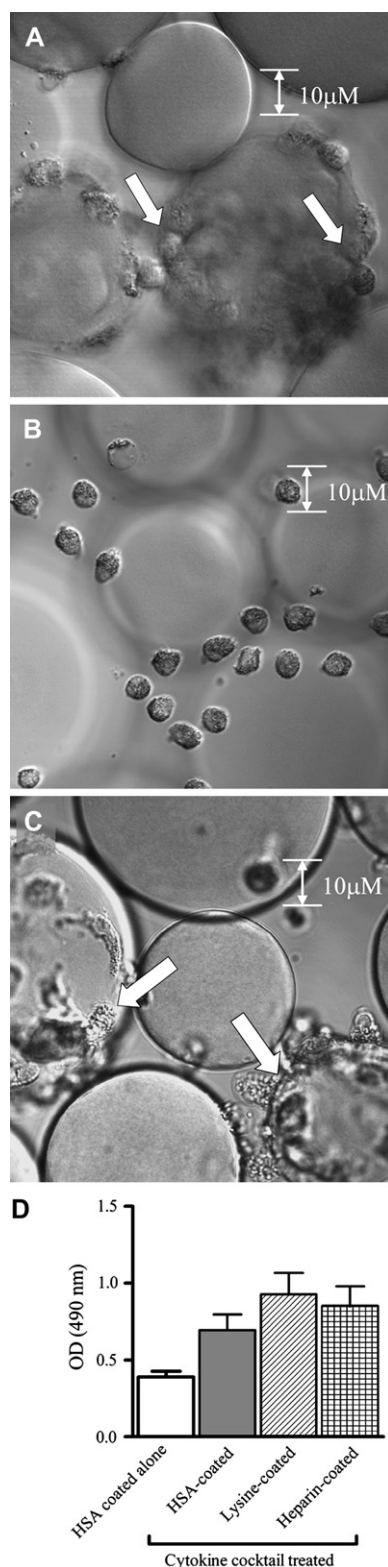


FIG 5. Live-cell confocal microscopy shows cytokine-treated eosinophils adherent around lysine-coated beads (**A**; representative picture of $n = 4$, $\times 160$). Anti-CD18 blocked adhesion (**B**; $n = 3$). Heparin blocking does not inhibit adhesion (**C**; $n = 3$). Eosinophils incubated in plates coated with HSA, lysine, or heparin with or without cytokines (**D**, $n = 4$ each) shows that anionic coating does not inhibit adhesion of cytokine-treated eosinophils. SEMs are shown by vertical bars.

with release of granule-stored proteins. This is the first report investigating the mechanisms of the effect of cationic charge on eosinophil mediator release.

Cationic surface coating of beads with lysine, compound 48/80, or EDN induced EPO release (as a marker of exocytosis) from eosinophils incubated with a cytokine cocktail. Compound 48/80 is a well-established cationic stimulus for mast cell degranulation.^{22,23,26} In the absence of cationic surface coating, the uncoated CNBr beads did not induce EPO release, even in the presence of the cytokine cocktail. We further demonstrated that beads coated with anions, including heparin sulfate, dextran sulfate, HSA, and aspartic acid, were unable to stimulate the cytokine-treated eosinophils.

To determine the mechanism of cationic charge-induced release in cytokine-stimulated eosinophils, we first examined the role of established pathways in eosinophil granule protein signaling. Integrins play an essential role in the release of granule-stored proteins, and release of these proteins is often correlated with eosinophil adhesion.²⁷ *In vitro* IL-3, IL-5, and GM-CSF individually induce increased expression of CD11 and CD18.²⁴ We were intrigued by the finding that combining these cytokines induced greater EPO release and greater surface CD18 expression. We suspect that the combination of cytokines either enhances common β -chain stimulation (CD131) or that this is associated with a small but significant level of signaling through the α -chain. IL-5 stimulation is reported to induce adhesion in the form of clustering eosinophils around sIgA-coated beads.²⁸ Our live-cell confocal images provide further supportive evidence for adhesion of eosinophils to bead surfaces when cytokines are present. However, for the first time, our study presents evidence that eosinophil granule protein release is associated with this visual pattern of eosinophil adhesion. Incubation with anti-CD18 blocked adhesion to the beads and the release of EPO, suggesting that part of the effect of cytokines on increased cationic charge-induced EPO release is secondary to increased eosinophil integrin function.

We expected that inhibition of cationic charge coating would be associated with inhibition of adhesion. To our surprise, adhesion of eosinophils around the beads still occurred, even after heparin treatment. The eosinophil adhesion assay confirmed that cytokine-treated eosinophils bind to heparin-coated surfaces comparably with lysine-coated surfaces. Thus although integrin function appeared important in the release of EPO, adhesion alone did not explain the effect of cationic charge. Heparin sulfate has effects on inflammatory cell migration,²⁹ but rather than inhibiting adhesion, heparin is known as a ligand for Mac-1 (CD11b/CD18).³⁰ Because cationic charge did not increase integrin signaling through adhesion alone, we hypothesized that a cationic surface might induce polarization of integrin receptors, increasing the density and degree of stimulation. Such polarization of integrins has been well described in neutrophils.^{31,32} Similar to reports on neutrophils, confocal microscopy demonstrated increased CD18 density in the eosinophil cell membrane adherent to the bead in this study. In contrast, lysine-coated beads blocked with heparin or dextran sulfate did not induce this polarization.

We believe that the amount of integrin expression at the cell surface could explain the apparent disconnect between adhesion and degranulation. When the surface is negatively charged, the expression of CD18 is still enough for adhesion but insufficient for maximal release of granule proteins as that generated by a

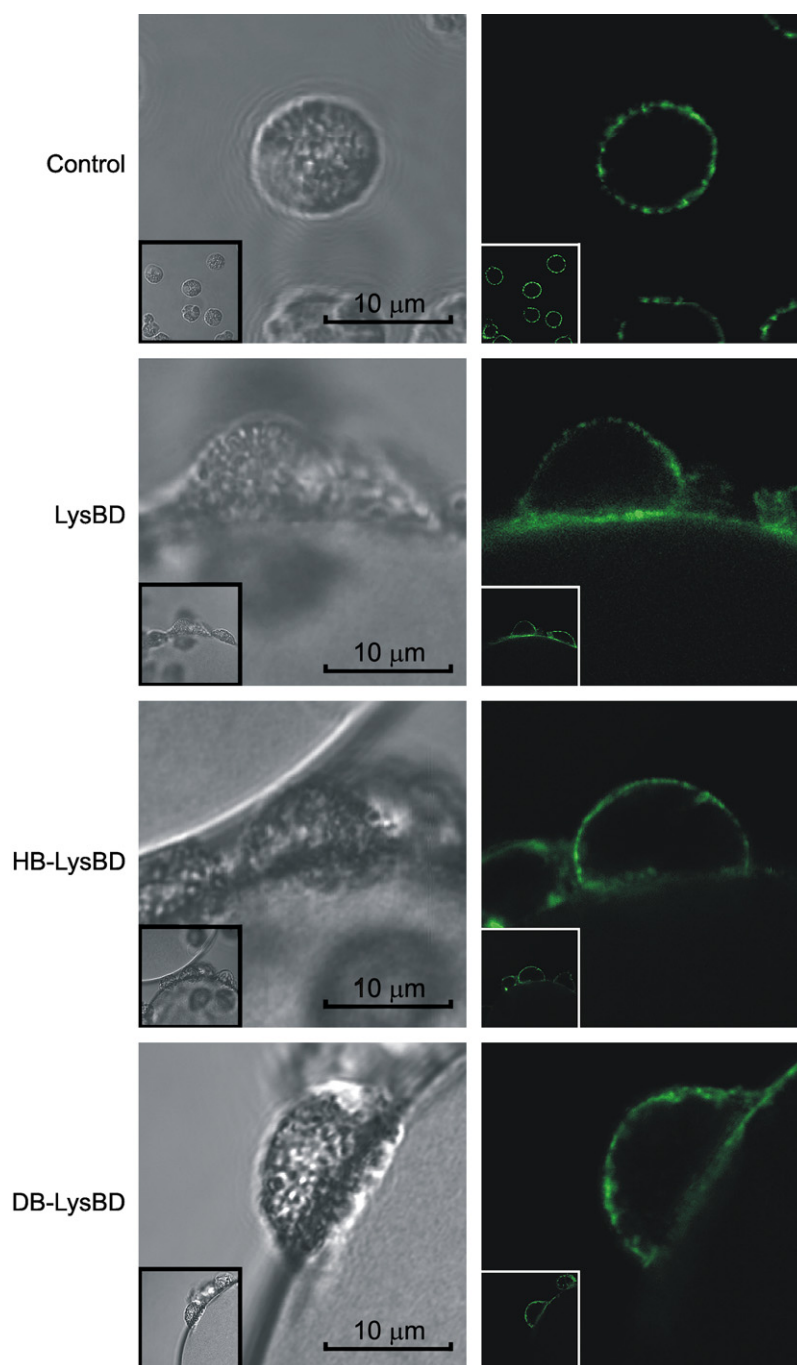


FIG 6. CD18 expression measured by means of confocal microscopy (DIC images on *left*; fluorescence on *right*; $\times 480$). In the absence of CNBr beads (*Control*, $n = 15$), eosinophils demonstrate uniform cell membrane staining for CD18. Cytokine-treated eosinophils adherent to LysBDs ($n = 15$) show increased CD18 density in membrane on the bead surface. In contrast, to eosinophils adherent to HB-LysBDs ($n = 15$) or DB-LysBDs ($n = 3$), the density of CD18 remains uniform over the entire eosinophil surface. Isotype controls are shown in the [Online Repository](http://www.jacionline.org) at www.jacionline.org. Each n value represents 1 eosinophil donor, with 5 to 10 images per donor for each condition.

cationic surface. Density of integrin expression correlates with the amount of granule protein release. Such increased cell membrane density of integrins associated with altered cell function has also been described in lymphocytes, which is referred to as *capping*.³³ Although the role of surface integrins is well described in eosinophil granule protein release, we have

noted an additional important effect of extracellular cationic charge, namely the polarization of CD18 toward a cationic-charged surface. When one considers that eosinophils are present and adhering to tissues as part of their pattern of margination and entry into tissue, it becomes obvious that adhesion alone is not enough for mediator release. If it were, there would be severe

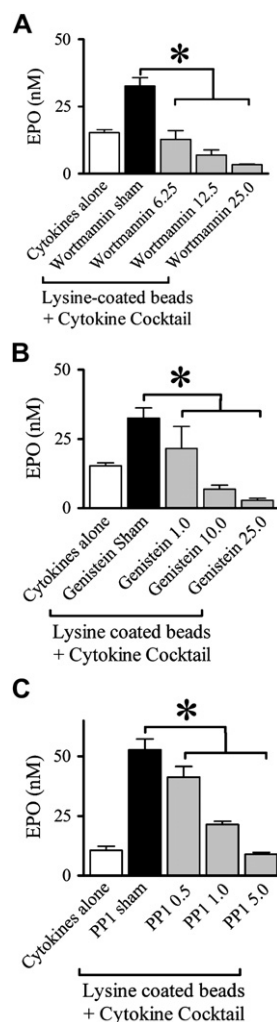


FIG 7. PI3 kinase and tyrosine kinases, including the src family of kinases, are involved in the signaling of cationic charge. Wortmannin ($n = 8$; 0-25 nmol/L; **A**), genistein ($n = 4$; 0-25 μ mol/L; **B**), and PP1 ($n = 4$; 0-100 nmol/L; **C**) caused dose-dependent inhibition of EPO release from eosinophils subsequently incubated with cytokine cocktail- and lysine-coated beads. SEMs are shown by vertical bars.

inflammatory reactions from the mere presence of eosinophilia, which clearly is not the case.

We also investigated the intracellular signaling pathways involved in cationic charge stimulation of eosinophils. IL-5 stimulation of eosinophils leads to a cascade of tyrosine kinase activity with subsequent PI3 kinase stimulation and ultimately increased p38 mitogen-activated protein kinase activity.^{28,34} Individually, IL-3, IL-5, and GM-CSF upregulate eosinophil CD18 expression through p38 mitogen-activated protein kinase phosphorylation.²⁴ Our data are consistent with these observations. In neutrophils β_2 -integrin surface signaling involves the syk and src kinases.³⁵ Because the role for these kinases has not been previously investigated for eosinophil granule protein release, our findings are novel. Cell-surface heparin sulfate is required for neutrophil chemotaxis, and selective inhibition by using PP1 inhibits this chemotaxis pathway.³⁶ Given our data, we suggest that changes in surface charge might also have a role in signaling events through src kinase activity. Overall, the increased sensitivity of cytokine-treated eosinophils to cationic

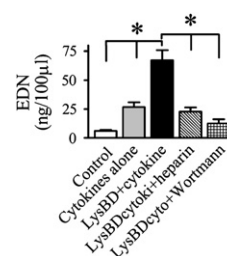


FIG 8. Eosinophils incubated with cytokines and lysine-coated beads release EDN ($n = 5$) compared with unstimulated eosinophils ($n = 6$; $P < .0001$) or the cytokines alone ($n = 3$; $P = .0002$). Heparin-blocked, lysine-coated beads ($n = 3$) did not induce EDN release from cytokine-treated eosinophils. Wortmannin treatment also prevented release of EDN from cytokine/lysine bead-stimulated eosinophils ($n = 5$; $P < .0001$). SEMs are shown by vertical bars.

charge is regulated in a physiologic manner based on established intracellular signaling pathways.

Similarly to the effects on EPO release, cytokine cocktail-treated eosinophils responded to the cationic-charged beads with significant EDN release. Here, too, both heparin coating of the lysine beads or wortmannin pretreatment of the eosinophils blocked EDN release. Thus the pathways described in this article for release of EPO appear to apply equally to other granule-stored proteins, including EDN. Whether other eosinophil mediators are released through similar mechanisms remains unknown.

Biopsy specimens of damaged tissue in eosinophil-associated disease demonstrate extracellular cationic material, including “sheets” of MBP.^{7,37-40} Opsonized helminth parasites, targeted by eosinophils, acquire eosinophil-derived cationic proteins on their surface.^{41,42} Eosinophil adhesion to and killing of parasitic worm targets is greatly enhanced if the worm surface is coated with MBP.⁸ Heparin blocks helminthic parasite killing *in vitro*.⁴³ In addition, heparin might have a role in treating asthma.^{44,45} Our study proposes an important role for a cationic environment in eosinophil mediator release. Although the association of eosinophils and extracellular cationic charge was suggested almost 4 decades ago,^{46,47} this is the first study to determine the important nature of eosinophil-cationic charge interactions at a functional level. Our data suggest that eosinophil cationic products might induce a positive feedback loop to activate eosinophils further by increasing cell-surface receptor polarization to a target. Because eosinophils are a rich source of cationic proteins, their sensitivity to positive charge might be an important element in maintaining autocrine activation and mediator release in atopic tissues. Because other inflammatory cells, including neutrophils, mast cells, and basophils, also respond to charged proteins,^{9,22,48} eosinophil proteins might play a wider role in inflammation, and substances that neutralize charge should be clinically relevant.

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Clinical implications: Sensitivity to cationic charge might be physiologically relevant, enhancing *in situ* autocrine activation and degranulation.

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METHODS

Eosinophil isolation

Human eosinophils were isolated from peripheral blood, as previously described.^{E1,E2} Briefly, blood was obtained from patients with atopy who displayed blood eosinophilia and were not receiving oral corticosteroids. All subjects provided written informed consent, as approved by the University of Alberta Health Research Ethics Board and according to the Helsinki protocol. After erythrocyte sedimentation in 6% dextran (100,000–200,000 MW; Sigma-Aldrich, Oakville, Ontario, Canada), the remaining leukocytes were subjected to density centrifugation on Ficoll (Pharmacia Biotech, Uppsala, Sweden). Eosinophils were purified from the granulocyte pellet by using a brief erythrocyte lysis step (10 seconds in sterile distilled water before resuspension in EDTA containing RPMI). Erythrocyte lysis, although necessary, can impair the quality of eosinophils recovered,^{E3} and therefore we minimized the injury with a very brief exposure. The resuspended pellet was subjected to immunomagnetic selection by using the MACS system (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), resulting in highly purified CD16⁺ eosinophils (>99%). Contamination by mononuclear cells and lymphocytes was avoided by means of cocubation with anti-CD14- and anti-CD3-coated micromagnetic beads (Miltenyi Biotec). Cells were washed and resuspended in color-free RPMI with 0.1% HSA and 25 mmol/L HEPES at 1×10^6 eosinophils/mL.

Measurement of EPO release

The release of EPO was measured by using a standardized assay with purified human EPO^{E4} and freshly prepared peroxidase substrate solution containing OPD. Briefly, peroxidase substrate solution for the measurement of EPO was prepared by adding 800 μ L of 5 mmol/L OPD HCl (Sigma, St Louis, Mo) to 4 mL of 1 mol/L Tris buffer (pH 8.0) and 1.25 μ L of 30% hydrogen peroxide (Sigma). Distilled water was added to a total volume of 10 mL. The OPD solution was protected from light and was prepared fresh before each experiment. The OPD assay was standardized by using purified human EPO.^{E4} Stock EPO (125 nmol/L) was diluted in serial half dilutions to a final concentration of 2 nM in a total tube volume of 500 μ L. OPD solution (500 μ L) was added for 2 minutes, and the reaction was stopped with the addition of 1 mL of 4 mol/L H₂SO₄. The optical density of the EPO-OPD reaction was measured in duplicate in 96-well plates (Softmax, 490-nm wavelength). The reaction was invariably linear ($r = 0.99$) within the EPO concentration range, which we find in nonlysed eosinophils responding to our stimulation protocols.^{E5} Eppendorf tubes were precoated with 1 mL of 2.5% HSA for 2 hours at room temperature (RT) to block nonspecific activation of eosinophils.^{E6} Tubes were then washed with 1 mL of PBS once before aliquots of eosinophil suspensions (500 μ L of 1×10^6 eosinophils/mL in color-free RPMI with 0.1% HSA and 25 mM HEPES) were added to HSA-coated tubes. After the various cell incubation protocols were complete (see below), OPD solution was added at 1:1 dilution (500:500 μ L) directly to cell suspensions. Unstimulated control cells were sham treated with RPMI vehicle and subsequently incubated similarly to stimulated cells. The remainder of the EPO measurement was performed as per the above EPO standard. The EPO assay has been described in detail previously.^{E5}

Measurement of EDN release

Microtiter plates (White Enhanced Binding, ThermoLabsystems, West Palm Beach, Fla) were coated overnight at 4°C with the anti-EDN capture antibody J167-6C5 (10 μ g/mL in PBS). Wells were washed with buffer (0.1 mol/L phosphate buffer and 10 mL/L Tween 20, pH 7.4) and blocked with PPF-E (0.1 mol/L phosphate buffer, 0.5% FCS, 0.1% sodium azide, 0.01 M EDTA, and 0.1% protamine sulfate, pH 7.5) for 1 hour at RT. After this, wells were washed again before standards, samples, and controls were added at 100 μ L/well in duplicate for an overnight incubation at 4°C. After washing, the acridinium-labeled anti-EDN secondary antibody, J167-2G4 was added (100 μ L/well and 50 ng/mL in PPF-E), and the plate was incubated at RT for 3 hours. The plate was washed and read in an LmaxII luminometer with 100 μ L of the following solutions: trigger 1, 0.1 mol/L HNO₃ and 0.5% hydrogen peroxide; trigger 2, 0.25 mol/L NaOH and 1% cetyl trimethylammonium chloride.

Cytokine cocktail stimulation

To approximate this tissue environment in which EPO release would occur, we incubated eosinophils with a cocktail of IL-3 (5 ng/mL), IL-5 (2.5 ng/mL), and GM-CSF (5 ng/mL) at RT for 2 hours with or without CNBr beads. This cocktail was compared with the nonphysiologic but potent stimulus calcium ionophore A23187 (5 μ mol/L, 30 minutes at 37°C). The cytokine doses were selected after performing dose-response curves over 4 orders of magnitude (data not shown). EPO release was most consistent at the reported doses. Based on the doses above, eosinophils were also incubated with the cytokines individually.

Cationic-charged bead preparation and eosinophil stimulation

CNBr-coupled Sepharose 4 Fast Flow beads (CNBr beads) are typically used in sIgA stimulation protocols with eosinophils. To create a cationic surface, we adapted the protocol of Abu-Ghazaleh et al^{E7} by using various cationic suspensions in place of sIgA. Briefly, CNBr-Sepharose 4 Fast Flow beads (CNBr beads) were preswollen in 1 mmol/L HCl. Lysine monohydrochloride (Sigma, 0.1 mol/L), compound 48/80 (Sigma, 5 μ g/mL), or EDN (purified from human eosinophils, 2.5 mmol/L) was dissolved in a coupling buffer (pH 8.6) containing 0.2 mol/L borate was incubated with preswollen washed beads at RT for 2 hours while mixing end over end. All beads were washed in 3 cycles of borate-coupling buffer (pH 8.6) and then 0.1 mol/L sodium acetate buffer (pH 4). Beads were stored in borate-coupling buffer (pH 8.6) to stabilize the bound protein. For stimulation, eosinophil suspensions were incubated with the various coated beads (RT, 2 hours, 1:40 beads to eosinophil suspension). These cationic compounds were also studied directly in solution without the beads (lysine, 0.003 mol/L; compound 48/80, 1 μ g/mL; EDN, 0.06 mmol/L) in solution at RT. As a control, EPO release was measured from eosinophils and CNBr beads, with or without the cationic-charge coating. EPO and EDN release was measured from eosinophils incubated with or without the various types of beads in the presence or absence of the cytokine cocktail.

Flow cytometric analysis of CD18 expression

Eosinophils (1×10^6 /mL as isolated above) were incubated with either mouse anti-human CD18 IgG2a or the isotype control to confirm the presence of CD18 on eosinophils (5 μ g/mL; Calbiochem, La Jolla, Calif). Some eosinophils were treated with the cytokine cocktail (60 minutes at RT) before incubation with anti-CD18. After 30 minutes at 4°C, cells were washed and secondary goat anti-mouse phycoerythrin-labeled IgG (1:250) was added (30 minutes at 21°C). After washing, cells were fixed with 1% formalin overnight. Data were acquired by means of FACScan and analyzed with Flojo software (Tree Star, San Carlos, Calif).

Inhibition of EPO release through blockade of CD18

Some eosinophils were preincubated with mouse anti-human CD18 for 5 minutes at RT before addition of stimuli. Mouse anti-human IgG2a served as the isotype control. Both antibodies were sodium azide free to avoid nonspecific blockade of EPO activity.

Confirmation of the role of cationic charge in eosinophil degranulation

Some lysine- and compound 48/80-coated beads were blocked with heparin (2500 IU/mL in borate coupling buffer for 30 minutes at RT) or dextran sulfate (5 mg/mL in borate coupling buffer for 30 minutes at RT then washed). We also substituted 0.5% HSA and aspartic acid (0.05 mol/L) in place of lysine for a number of experiments. All beads were washed with borate coupling buffer (pH 8.6) twice and stored in this buffer before use. EPO and EDN release was measured from eosinophils incubated with or without the various types of beads in the presence or absence of the cytokine cocktail (2 hours at RT).

Live-cell confocal microscopy

By using live-cell real-time photography, eosinophils were observed for morphology and movement according to the same experimental protocols as

above. Petri dishes (35 mm) with glass coverslip bottoms were precoated with 2.5% HSA (4°C overnight). Plates were washed with PBS before adding 200 μ L of the eosinophil suspension. Cells were observed under a $\times 40$ oil objective (Zeiss, Toronto, Ontario, Canada; NA 1.3) by using a Zeiss laser-scanning microscope (LSM 510, HeNe laser 543 nmol/L) creating DIC images ($\times 160$ from $\times 40$ optical and $\times 4$ digital). Live images (captured at 1.5-second intervals) and still images were saved for analysis.

Eosinophil adhesion assay

To further determine whether heparin inhibition of eosinophil mediator release was through inhibition of positive charge or inhibition of eosinophil adhesion, we measured EPO release from eosinophils stimulated in 96-well tissue-culture plates. All wells were precoated with 2.5% HSA. Similar to the bead protocol, wells in duplicate were coated with either lysine (0.1 mol/L) or heparin (1000 IU/mL). Plates were washed before adding cytokine cocktail-stimulated eosinophils (5×10^5 /well). After washing away nonadherent cells, TMB was added for 5 minutes. The reaction was stopped with 4M H_2SO_4 , and the amount of adherent cells was measured (Softmax, 490-nm wavelength). Because TMB and its diluent permeates the cell membrane, colorimetric analysis represents the number of remaining adherent cells.^{E8}

Intracellular signaling pathways in cation-induced release of EPO

Eosinophils were preincubated with various concentrations of the PI3 kinase inhibitor wortmannin (0–25 nmol/L), the broad-spectrum tyrosine kinase inhibitor genistein (0–25 μ mol/L), the src kinase family inhibitor PP1 (0–100 nmol/L, Calbiochem), and the syk kinase family inhibitor piceatannol (Sigma, 0–1000 nmol/L), each for 30 minutes at 20°C before the stimulation protocol used above. Appropriate controls were incubated similarly.

Localization of CD18 by means of confocal microscopy

Eosinophils (1×10^6 /mL) alone without CNBr beads or cytokine-treated eosinophils with CNBr beads were incubated with rat anti-human CD18 (1 μ g/100 μ L; AbD Serotec, Raleigh, NC). After incubation (60 minutes on ice), cells and beads were washed once (PBS with 0.5% HSA) and stained with Alexa Fluor 488-labeled chicken anti-rat IgG (H+L, 2.5 μ g/100 μ L, 45 minutes on ice). Rat purified IgG (Sigma) served as a control. All samples were incubated with 0.5% gelatin (30 minutes on ice) before staining to block nonspecific binding. After staining, samples were fixed in 1% paraformaldehyde. CD18 localization and quantification of average fluorescence intensity

were determined by means of confocal laser scanning microscopy with Fluoview software ($\times 480$ images [$\times 60$ optical, $\times 8$ digital] were scanned to a thickness of 0.95 μ m by using FV10-ASW Version 01.06.01.00). Images of single cells without beads (control) or attached to the surface of lysine-coated, lysine with heparin-blocked, or noncoated CNBr beads were selected. Free-hand ROI selection was set to contain the membrane areas contacting the bead surface in contrast to cell membrane not in contact (Fig E2). Use of average fluorescence intensity negates any effects of differences in ROI area.

Statistical analysis

All data are expressed as means $\pm$ SEMs. Each donor supplied eosinophils, which were used for the various experiments. Control samples of eosinophils were performed at the same time as these treated groups. Thus *n* values represent the number of individual donors and their measured value of EPO or EDN under the specified experimental condition. Comparison of mean fluorescent intensity and EPO and EDN activity between the groups was analyzed by using ANOVA (Statview 5.0). A *P* value of less than .05 was considered statistically significant. Calculation for linear correlation between optical density and EPO molarity values was performed with the Microplate Manager software (Bio-Rad).

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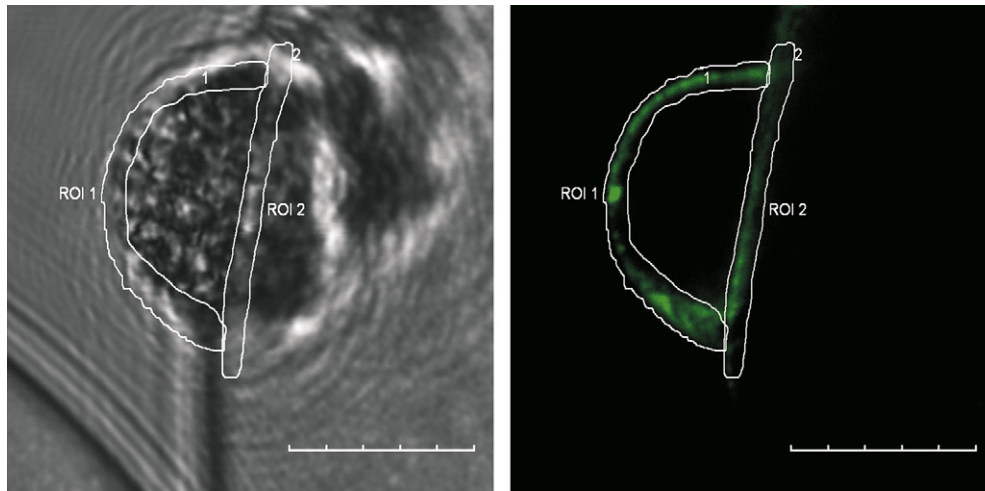


FIG E1. Measurement of fluorescence intensity in confocal acquired images ($\times 480$ magnification). Freehand ROIs were set to contain the membrane areas that were not contacting the bead surface (*ROI 1*) in contrast to cell membrane in contact with it (*ROI 2*). Average intensities of CD18 per ROI were analyzed.

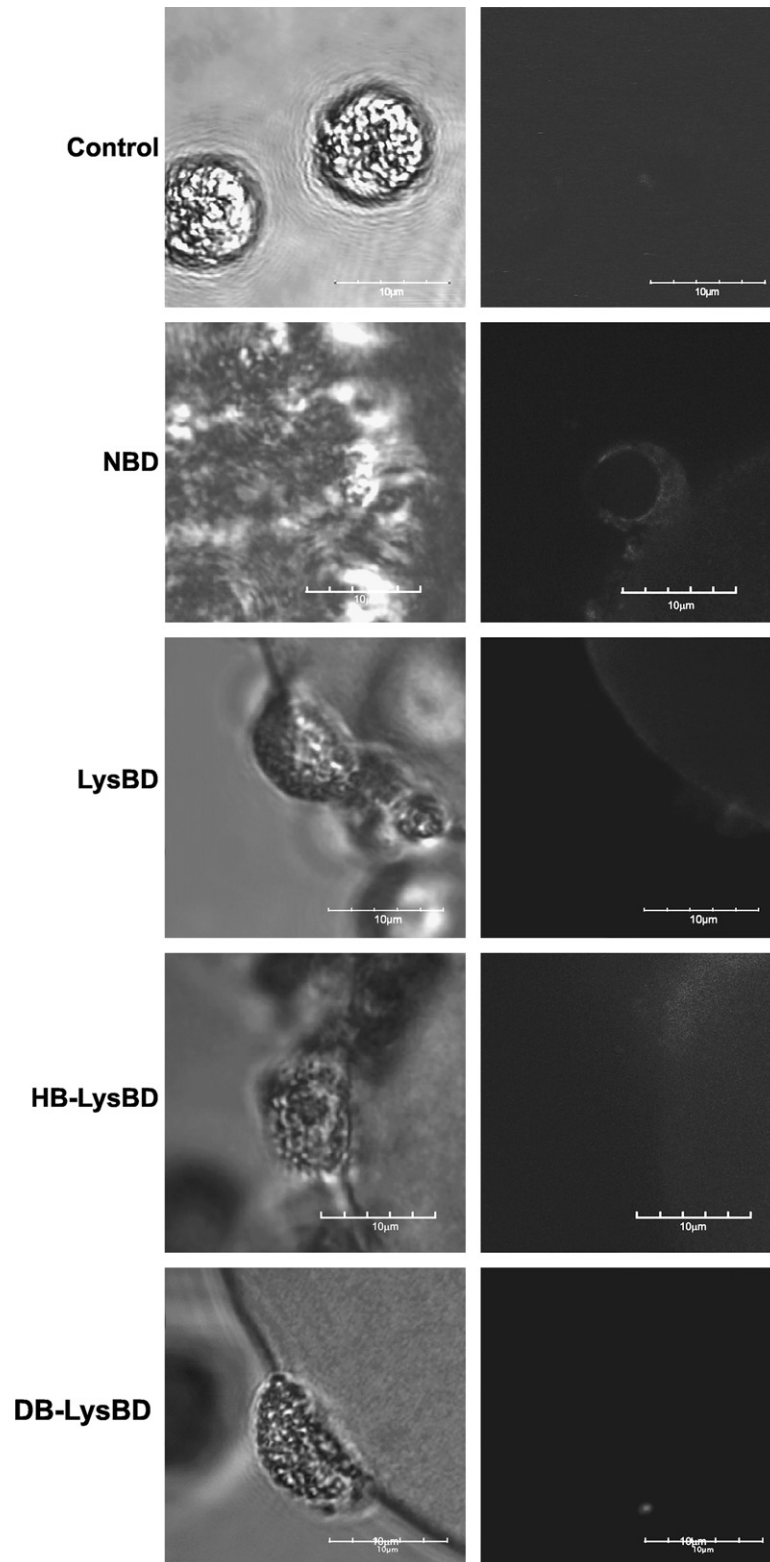


FIG E2. Isotype control images of eosinophils alone (*Control*) and those cytokine-treated eosinophils incubated with noncoated CNBr beads (*NBD*), LysBDs, HB-LysBDs, or DB-LysBDs ($\times 480$ magnification). DIC images (*left*) and pooled rat IgG-Alexa 488 (*right*) are shown.