ORIGINAL RESEARCH

Chloride Channel 3 Channels in the Activation and Migration of Human Blood Eosinophils in Allergic Asthma

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Abstract

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is responsible for respiratory burst in immune cells. Chloride channel 3 (CLC3) has been linked to the respiratory burst in eosinophils and neutrophils. The effect of cytokines and the involvement of CLC3 in the regulation of NADPH-dependent oxidative stress and on cytokine-mediated migration of eosinophils are not known. Human peripheral blood eosinophils were isolated from healthy individuals and from individuals with asthma by negative selection. Real-time PCR was used to detect the expression of NADPH oxidases in eosinophils. Intracellular reactive oxygen species (ROS) measurement was done with flow cytometry. Superoxide generation was measured with transforming growth factor (TGF)-β, eotaxin, and CLC3 blockers. CLC3 dependence of eosinophils in TGF-β– and eotaxin-induced migration was also examined. The messenger RNA (mRNA) transcripts of NADPH oxidase (NOX) 2, dual oxidase (DUOX) 1, and DUOX2 were detected in blood eosinophils, with very low expression of NOX1, NOX3, and NOX5 and no NOX4 mRNA. The level of NOX2 mRNA transcripts increased with disease severity in the eosinophils of subjects with asthma compared with healthy nonatopic volunteers. Change in granularity and size in eosinophils, but no change in intracellular ROS, was observed with phorbol myristate acetate (PMA). PMA, TGF-β, and eotaxin used the CLC3-dependent pathway to increase superoxide radicals. TGF-β and eotaxin induced CLC3-dependent chemotaxis of eosinophils. These findings support the requirement of CLC3 in the activation and migration of human blood eosinophils and may provide a potential novel therapeutic target to regulate eosinophil hyperactivity in allergic airway inflammation in asthma.

Keywords: airway inflammation; allergic asthma; CLC3 channels; eosinophils; NADPH oxidase

Clinical Relevance

We, for the first time, report a critical role of chloride channel 3 (CLC3) channels in the migration and activation of human blood eosinophils. We also report, in addition to the known presence of nicotinamide adenine dinucleotide phosphate oxidase (NOX) 2, the existence of subtypes of NOX, including NOX1, NOX3, NOX5, dual oxidase (DUOX) 1, and DUOX2, albeit at low levels, in human blood eosinophils. However, very high levels of NOX2 messenger RNA transcripts were found in the eosinophils of subjects with moderate to severe asthma compared with subjects with mild to moderate asthma and healthy subjects, suggesting a positive correlation between the expression of NOX2 messenger RNA transcripts and disease severity. These findings provide a novel target in regulating eosinophil infiltration and activation in allergic airway inflammation in asthma.

Allergic asthma is a complex and heterogeneous disease. Eosinophils play a major role in the exacerbation of allergic asthma by migrating to the site of inflammation and releasing manifold granules with preformed and newly formed mediators that propagate excessive damage and repair cycle in the airways (1, 2). The preformed mediators consist of major basic protein, eosinophil-derived neurotoxin, eosinophil peroxidase, and eosinophil cationic protein. The newly formed

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Table 1. Information on the Recruited Volunteers for Nicotinamide Adenine Dinucleotide Phosphate Oxidase Expression Analysis

Volunteers/ Parameters	Healthy Subjects	Subjects with Mild to Moderate Asthma	Subjects with Moderate to Severe Asthma
Age, yr	21–62	19–54	30–63
Sex, M/F	3/3	0/6	3/4
Atopy	No	Yes	Yes
Symptoms	None	Up to twice per week	Daily
Lung function	N/A	FEV_1 ⩾70% FEV_1/FVC normal	FEV_1 <70% FEV_1/FVC reduced ⩾5%
Medications (at the time of blood draw)	None	None	Advair 250/50 or 500/50

Definition of abbreviation: N/A, not applicable.

mediators include cytokines and chemokines (IL-2, IL-4, IL-6, IL-10, IL-12, IL-13, IL-16, IL-18, IFN-γ, and TNF-α), prostaglandins, leukotrienes (mostly LTC_4), reactive oxygen species (ROS), and growth factors, including transforming growth factor (TGF)-β (2, 3).

Although major basic protein is highly toxic, the production and release of ROS from activated eosinophils elicit severe damage to the airway epithelium. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is a membrane-bound oxidase that triggers respiratory burst. A typical NADPH oxidase in phagocytes consists of membrane-bound $gp91^{phox}$ (NOX2) and $p22^{phox}$ in addition to cytosolic $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$ and a small GTPase Rac. Nonphagocytic NADPH oxidase (NOX) consists of different homologs of NOX2, namely NOX1, NOX3, NOX4, and NOX5, and dual oxidase (DUOX) (4–6). NADPH oxidase generates superoxide radicals ($O_2^{\bullet -}$), which are converted to H_2O_2 by superoxide dismutase. Unlike superoxide radicals, H_2O_2 can freely cross plasma membrane but has less oxidative power. Through a chemical reaction called Fenton's reaction, H_2O_2 can readily react with iron to convert it from ferrous (Fe^{2+}) to ferric (Fe^{3+}) and generate hydroxyl radicals (·OH). Alternatively, H_2O_2 can react with H^+ and Cl^- to make hypochlorous acid (HOCl) in the presence of myeloperoxidase (7) or, in the case of eosinophils, eosinophil peroxidase (8, 9), which is a powerful oxidizing agent capable of oxidizing proteins and cell membranes, leading to damage and inflammation in the airways. Chloride channel 3 (CLC3) has been shown to regulate the production of $O_2^{\bullet -}$ in human eosinophils (9). However, the precise mechanism involved in the activation of eosinophils is unclear.

Eosinophil migration and activation are carefully controlled by cytokines and chemokines that are released from damaged epithelium or other immune cells, including Th2 cells. TGF-β (10, 11), eotaxin (12, 13), and IL-5 (14, 15) are major cytokines and chemokines controlling eosinophils from their differentiation through maturity and activation in a time-, space-, and concentration-dependent manner. Ion channels could also be involved in such processes. Indeed, chloride channels are critical players in a variety of physiological and cellular processes, including pH regulation, volume homeostasis, organic solute transport, cell migration, and cell proliferation and differentiation (16–18). CLC3, a chloride channel and H^+/Cl^- antiporter, has been shown to modulate respiratory burst in eosinophils (9) and regulates the migration and activation of neutrophils (19, 20). This double-barreled antiporter (21–23) has been shown to be expressed ubiquitously in a variety of cells. Its expression has been detected on plasma membrane and intracellularly on lysosomes and nucleus (21, 24). In this study we examined the role of CLC3 on eosinophil activation involving NADPH oxidase system and on eosinophil migration.

Table 2. Primer Pairs Used in Quantitative PCR Analysis for Nicotinamide Adenine Dinucleotide Phosphate Oxidases

Primer	Accession (NCBI)	Sequence (5′–3′)	Product Size (*bp*)	Temperature (°C)
NOX1-F	NM_007052.4	TGCGCTGGACAAATGTTCCATTCC	192	60.3
NOX1-R	NM_007052.4	TCAGGAAGGCATCCACAAACAGGA	192	60.5
NOX2-F	NM_000397.3	AGACTTTGTATGGACGGCCCAACT	103	60.4
NOX2-R	NM_000397.3	AAGGCTTCAGGTCCACAGAGGAAA	103	60.2
NOX3-F	NM_015718.2	ACCGTGGAGGAGGCAATTAGACAA	109	60.2
NOX3-R	NM_015718.2	TTCCAGGTTGAAGAAATGCGCCAC	109	60.3
NOX4-F	NM_001143836.1	AGCAGAGCCTCAGCATCTGTTCTT	115	60.4
NOX4-R	NM_001143836.1	TGGTTCTCCTGCTTGGAACCTTCT	115	60.2
NOX5-F	NM_001184779.1	TGCTGCTCCTCCTCATGTTCATCT	135	60.0
NOX5-R	NM_001184779.1	TCCAGAAGTTGGGCCCATGAAAGA	135	60.5
DUOX1-F	NM_017434.3	AACAATTTGTGCGGCTACGGGATG	109	60.3
DUOX1-R	NM_017434.3	TCCTGCAGGGTGGTATTTCGGATT	109	60.3
DUOX2-F	NM_014080.4	AGTACAAGCGCTTCGTGGAGAACT	85	60.3
DUOX2-R	NM_014080.4	TCTGCAAACACGCCAACACAGATG	85	60.3
GAPDH-F	NM_002046.3	TCGACAGTCAGCCGCATCTTCTTT	94	60.6
GAPDH-R	NM_002046.3	ACCAAATCCGTTGACTCCGACCTT	94	60.5
18S-F	NR_003286.2	TCAACTTTCGATGGTAGTCGCCGT	108	60.3
18S-R	NR_003286.2	TCCTTGGATGTGGTAGCCGTTTCT	108	60.2

Definition of abbreviations: bp, base pair; NCBI, National Center for Biotechnology Information.

Materials and Methods

Human Subjects

The Institutional Review Board of Creighton University approved the protocol. Healthy volunteers and volunteers with asthma (19–65 yr of age) provided informed consent. Inclusion criteria were avoidance of short-acting β-agonists for 8 hours, long-acting β-agonists and caffeine for 2 days, antihistamines for 3 days, corticosteroids, cromolyn/nedocromil for 2 weeks, and astemizole for 3 months. Healthy subjects had no history of allergies or recent infections. Subjects with mild to moderate asthma had greater than or equal to 70% FEV_1 with no medications. Subjects with moderate to severe asthma had FEV_1 of <70% and were taking a daily dose of their prescribed Advair (GlaxoSmithKline, Philadelphia, PA) (250/50 or 500/50 μg fluticasone propionate/salmeterol). Due to the health concerns of the patients, subjects with mild to severe asthma were still taking their asthma control medicine at the time of recruitment (Table 1).

Isolation and Culture of Human Blood Eosinophils

Eosinophils were isolated by density gradient centrifugation and dextran sedimentation, followed by hypotonic lysis of venous blood with negative selection using an eosinophil isolation kit (Miltenyi Biotec, Auburn, CA) in autoMACS (Miltenyi Biotec). Purity (>99%) and viability (>98%) of eosinophils were examined by staining with Hema-Diff (StatLab Medical Products Inc., Lewisville, TX) and trypan blue (Sigma-Aldrich, St. Louis, MO), respectively.

The purified eosinophils were cultured in RPMI complete medium (RPMI + 10% FBS) at 37°C with 5% CO_2.

RNA Isolation and Reverse Transcription

Total RNA was isolated using Ambion mirVana miRNA Isolation Kit (Life Technologies, Grand Island, NY). Total RNA (500 ng) was reverse transcribed with the ImProm-II Reverse Transcription System (Promega, Madison, WI).

Real-Time PCR

Quantitative PCR was performed using a CFX96 thermocycler (Bio-Rad, Hercules, CA) with specific primers for NADPH oxidases (Table 2) and iQ SYBR Green Supermix (Bio-Rad). Fold-change in the gene expression was calculated as $2^{-\Delta\Delta cq}$ from healthy compared with asthmatic eosinophils or relative to the respective healthy untreated eosinophils in treatment groups, where $^{\Delta\Delta}Cq$ is $(Cq_{asthmatic} - Cq_{healthy})Cq_{NOX2} - (Cq_{asthmatic} - Cq_{healthy})Cq_{18S}$, or $(Cq_{treatment} - Cq_{control})Cq_{NOX2} - (Cq_{treatment} - Cq_{control})Cq_{GAPDH/18S}$.

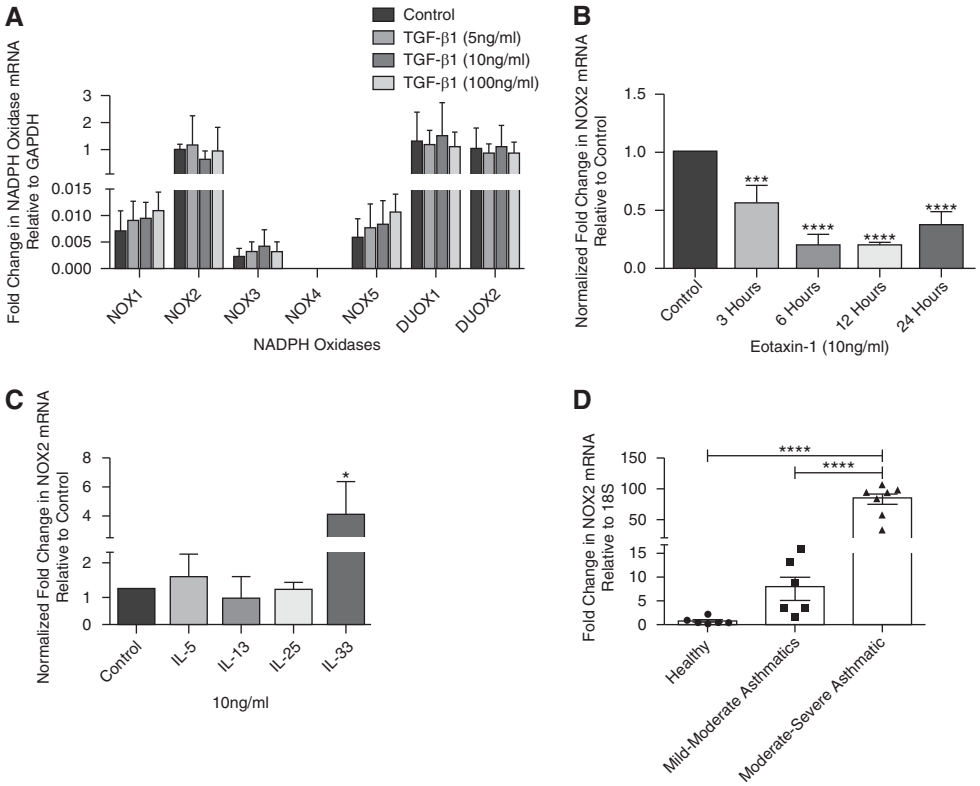

Figure 1. Expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) in blood eosinophils. (*A*) Real-time PCR exhibiting expression of NOX2, dual oxidase (DUOX) 1, and DUOX2 in blood eosinophils with low levels of NOX1, NOX3, and NOX5. No expression of NOX4 was detected. Transforming growth factor (TGF)-β1 did not have significant effect on the expression levels of the NADPH oxidase isoforms ($n = 4$). (*B*) Decreased NOX2 expression with eotaxin-1 in a time-dependent manner ($n = 4$). (*C*) The effect of IL-5, IL-13, IL-25, and IL-3 on the expression of NOX2 ($n = 5$). (*D*) A high level of NOX2 messenger RNA (mRNA) transcripts was seen in the eosinophils of subjects with moderate to severe asthma ($n = 7$) compared with subjects with mild to moderate asthma ($n = 6$) or healthy subjects ($n = 6$). There was no significant difference between eosinophils from healthy subjects and from subjects with mild to moderate asthma. *$P < 0.05$, ***$P < 0.001$, and ****$P < 0.0001$. GAPDH, glyceraldehyde phosphate dehydrogenase.

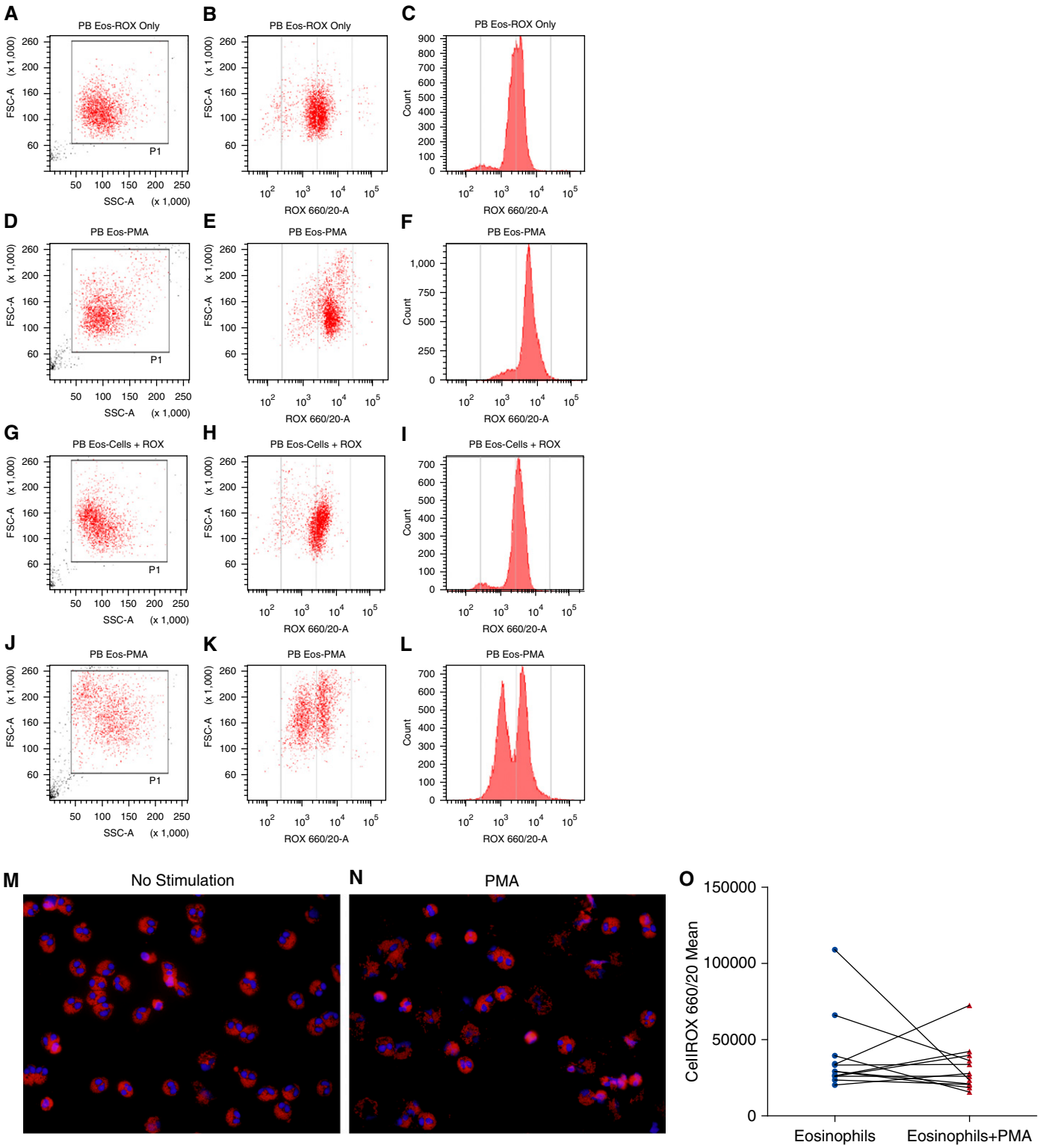

Figure 2. Intracellular reactive oxygen species (ROS) in blood eosinophils. Flow cytometry analysis of eosinophils stained with CellROX showed change in cell size and granularity with PMA. (*A–C*) Data obtained from the eosinophils from one subject. (*G–H*) Data obtained from another subject. In both cases, eosinophils were stained with CellROX without stimulation. (*D–F* and *J–L*) The corresponding data in PMA-treated and CellROX-stained eosinophils of these two individuals ($n = 12$). (*E*, *F*, *K*, and *L*) Data from two subjects with different degrees of segregation of two populations of eosinophils with respect to their ROS content after PMA stimulation. (*M* and *N*) Fluorescent micrographs of unstimulated and PMA-stimulated eosinophils, respectively, stained with CellROX and 4′,6-diamidino-2-phenylindole. (*O*) There was no change in the intracellular ROS in PMA-induced eosinophils ($n = 12$). FSC-A, forward scatter; PB Eos, peripheral blood eosinophils; PMA, phorbol myristate acetate; SSC, side scatter.

Measurement of Intracellular ROS

Cells were treated with 1 μM phorbol myristate acetate (PMA) (Sigma-Aldrich) for 30 minutes followed by 30 minutes with CellROX Deep Red Reagent (Life Technologies, Grand Island, NY). Intracellular ROS was measured with FACS analysis in the Flow Cytometry Core Facility of Creighton University.

Measurement of Extracellular ROS

After adding the cytokines and blockers, $O_2^{\bullet-}$ was measured every 5 minutes for 4 hours as the intensity of luminescence with Enspire plate reader (PerkinElmer, Waltham, MA) using a superoxide anion assay kit (Sigma-Aldrich).

Chemotaxis

A Transwell system (Costar, Corning, NY) with a 5.0-μm polycarbonate membrane was used with respective chemoattractants and blockers. Equal numbers of eosinophils were put in the upper chamber and kept in the incubator for 90 minutes at 37°C with 5% CO_2. Ten random high-power fields were chosen to count the migrating eosinophils on membranes stained with Hema-Diff (StatLab Medical Products Inc.) using a bright-field microscope (Olympus, Center Valley, PA) with 20× objective.

Data Analysis

Statistical differences of data were evaluated by Student's *t* test or by one-way or two-way ANOVA with Tukey's *post hoc* test. Data were considered significant at $P < 0.05$. Data represent means ± SEM.

Results

NADPH Oxidase Expression in Human Peripheral Blood Eosinophils

Very low levels of NOX1, NOX3, and NOX5 were detected, with no expression of NOX4 (Figure 1A). Relatively higher levels of DUOX1, DUOX2, and NOX2 messenger RNA (mRNA) transcripts were found in eosinophils, but TGF-β did not have any effect on the transcript levels of any of the NADPH isoforms (Figure 1A). Eotaxin-1 (10 ng/ml) decreased NOX2 mRNA transcripts in a time-dependent manner (Figure 1B).

Treatment of eosinophils with IL-33 (10 ng/ml) significantly increased NOX2 mRNA transcripts (~4-fold) compared with respective controls. However, there was no significant change in the NOX2 mRNA levels when eosinophils were treated with 10 ng/ml IL-5, IL-13, or IL-25 (Figure 1C). NOX2 mRNA transcripts in the eosinophils were 7-fold higher but were insignificantly different in subjects with mild to moderate asthma and 75-fold higher and significantly different in subjects with mild to severe asthma compared with healthy subjects (Figure 1D). These findings suggest a critical role of NOX2 in the activation of eosinophils in subjects with moderate to severe asthma.

Intracellular ROS in Human Peripheral Blood Eosinophils

PMA increased the granularity and cell size of eosinophils (Figures 2D, 2E, 2J, and 2K) compared with untreated eosinophils (Figures 2A, 2B, 2G, and 2H). Also, there was a remarkable increase in the cell debris (Figures 2D and 2J) with PMA outside the analysis gate compared with the unstimulated cells (Figures 2A 2G), suggesting degranulation of eosinophils. Fluorescent micrographs of CellROX- and 4′,6-diamidino-2-phenylindole–stained eosinophils further strengthened the observation of degranulating eosinophils in response to PMA (Figure 2N). Compared with the unstimulated eosinophils (Figures 2B and 2C), PMA induced and increase in ROS generation (Figures 2E and 2F) in a subset of healthy subjects but exhibited no change or decrease in intracellular ROS in the eosinophils of other volunteers (Figures 2K and 2L). Although no significant change in the intracellular ROS was observed with PMA stimulation among subjects (Figure 2O), there was a heterogeneity to the PMA-stimulated change in intracellular ROS in eosinophils, as shown by two distinct populations of eosinophils upon PMA stimulation (Figures 2K and 2L). This suggests a possibility of

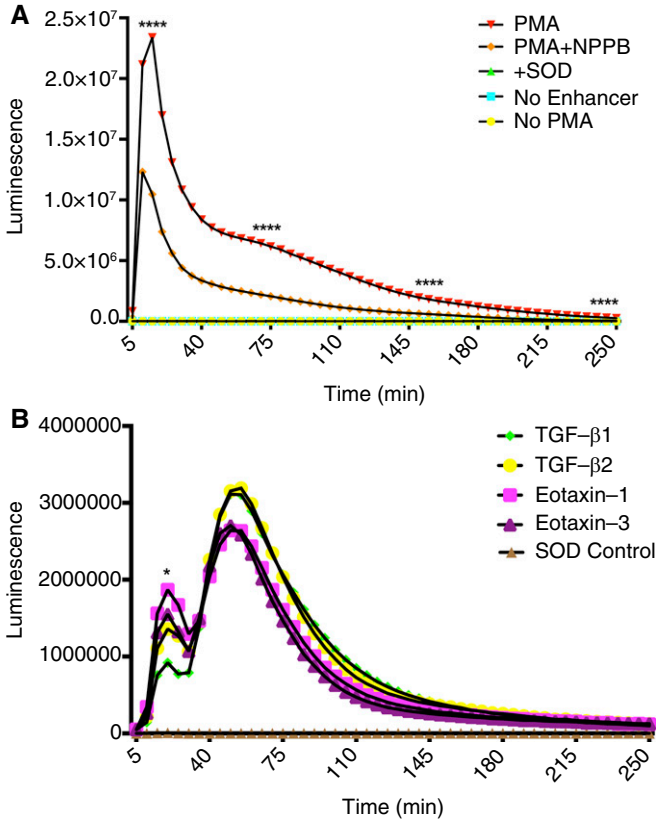

Figure 3. Extracellular ROS (superoxide [$O_2^{\bullet-}$]) in blood eosinophils. (*A*) Luminescence assay indicating a sharp increase in the $O_2^{\bullet-}$ generation with PMA that was chloride channel 3 (CLC3) dependent. Control groups contained superoxide dismutase (SOD), no enhancer, or no PMA ($n = 3$). $P < 0.05$. (*B*) Eotaxin-1, eotaxin-3, TGF-β1, and TGF-β2 increased $O_2^{\bullet-}$ release in a two-step process, which was lower in amplitude than PMA-induced $O_2^{\bullet-}$ generation ($n = 3$). *$P < 0.05$ and ****$P < 0.0001$. NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid.

segregation of the eosinophil population to hypo- and normo-dense eosinophils.

Extracellular ROS

NADPH oxidase requires an ion channel to compensate the difference in the membrane potential caused by superoxide release. CLC3 has been found to assist NADPH oxidase in the superoxide generation and charge compensation (9). Measurement of superoxide generation in human blood eosinophils revealed the dependency of NADPH oxidase activity on CLC3 in the plasma membrane of eosinophils. PMA exhibited a quick burst of $O_2^{\bullet-}$, which was partially blocked by 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), a selective CLC3 blocker (Figure 3A). The cytokines TGF-β1, TGF-β2, eotaxin-1, and eotaxin-3 induced two different bursts of $O_2^{\bullet-}$ in human peripheral blood eosinophils (Figure 3B). The first burst was generated at 20 minutes and was relatively lower in amplitude than the second burst, which was generated at 55 minutes after the addition of the respective cytokines to the eosinophils (Figure 3B). The TGF-β1–induced peak of extracellular $O_2^{\bullet-}$ generation was lower than that induced by eotaxin-1 at 20 minutes. However, there was no statistically significant difference between $O_2^{\bullet-}$ peaks induced by TGF-β1 and eotaxin-3 or $O_2^{\bullet-}$ peaks induced by TGF-β2 and eotaxin-1/eotaxin-3 at 20 or 55 minutes (Figure 3B). The amplitude of $O_2^{\bullet-}$ peak induced by all cytokines in the first burst was smaller than in the second burst.

NPPB inhibited the extracellular ROS ($O_2^{\bullet-}$) generation in response to the cytokines, suggesting the dependency of ROS generation on CLC3 (Figures 4A–4D). The NADPH oxidase inhibitor diphenyleneiodonium (DPI) also inhibited the extracellular ROS ($O_2^{\bullet-}$) generation due to TGF-β1, TGF-β2, and eotaxin-1 (Figures 4A–4C). NADPH oxidase inhibitor did not reduce the eotaxin-3–induced $O_2^{\bullet-}$ to the baseline levels as it did in other groups. DPI significantly reduced the eotaxin-3–induced $O_2^{\bullet-}$ generation in the first oxidative burst at 20 minutes. However, there was a slight delay in the peak time of eotaxin-3–induced second respiratory burst without any significant change the amplitude compared with the first respiratory burst (Figure 4D).

To determine if the $O_2^{\bullet-}$ production was mediated through CCR3, a selective blocker of CCR3 (SB328437) was used. SB328437 was able to induce $O_2^{\bullet-}$ release in eosinophils by itself. It did not change the time or amplitude of the first burst, but the second burst was even bigger than the

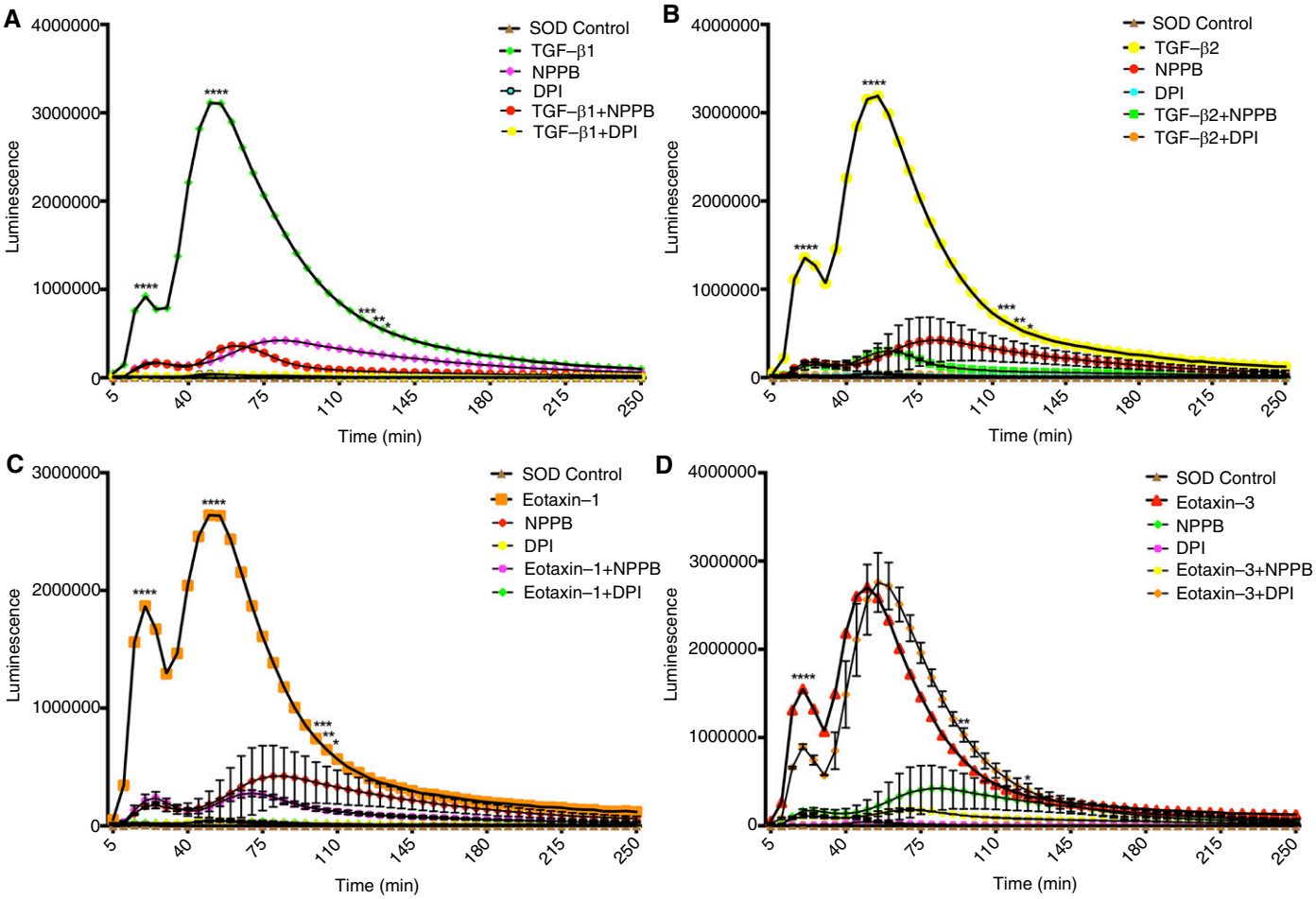

Figure 4. CLC3 dependence of cytokine-induced $O_2^{\bullet-}$ release in blood eosinophils. (*A–D*) Effect of TGF-β1, TGF-β2, eotaxin-1, and eotaxin-3 on $O_2^{\bullet-}$ release, respectively. The CLC3 blocker NPPB inhibited the increase in $O_2^{\bullet-}$ release. However, diphenyleneiodonium (DPI) blocked the $O_2^{\bullet-}$ release in all except eotaxin-3–induced eosinophils, where it partially blocked the 20-minute burst and delayed the second burst ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

eotaxin-1– and eotaxin-3–induced $O_2^{\bullet-}$. Additionally, the second burst was delayed, and there was significant difference in the $O_2^{\bullet-}$ production induced by eotaxin compared with that in the presence of CCR3 blocker (Figure 5).

Eosinophil Chemotaxis

TGF-β and eotaxin increased the migration of eosinophils. Nonetheless, the effect of eotaxin-1 and eotaxin-3 on eosinophil migration was greater than that of TGF-β1 and TGF-β2 (Figures 6A and 6B). The CCR3 blocker SB328437 had no effect on the eosinophil migration by itself but inhibited the eotaxin-1– and eotaxin-3–induced migration of eosinophils, suggesting a direct involvement of the CCR3 in eosinophil migration. The CLC3 selective blocker NPPB by itself had no significant effect on eosinophil migration. However, NPPB significantly inhibited cytokine-induced migration of eosinophils, suggesting the involvement of CLC3 (Figures 6A and 6B). Phloretin, a known inhibitor of the PKC enzyme that inhibits CLC3, also inhibited cytokine-induced migration of eosinophils (Figure 6C). This further supports the role of CLC3 in chemokine-induced migration of eosinophils.

Discussion

Eosinophil respiratory burst generates 10-fold more extracellular $O_2^{\bullet-}$ than neutrophils (25, 26). The current reports in the literature indicate the presence of only NOX2 among many NADPH oxidases in eosinophil (25). However, in addition to strong expression of NOX2 mRNA transcripts in our investigation, we found very low levels of NOX1, NOX3, and NOX5 and found no NOX4 in eosinophils. However, the low mRNA transcripts of NADPH oxidases may suggest no or a very minor role in the eosinophil respiratory burst. Unlike other NADPH oxidase homologs, DUOX1 and DUOX2 uniquely produce H_2O_2 without depending on superoxide dismutase to catalyze the reaction from $O_2^{\bullet-}$ to H_2O_2 (27). Therefore, NOX2 is the major player in eosinophil respiratory burst. Eotaxin and TGF-β are known to activate eosinophils, leading to their degranulation (2, 3). TGF-β did not elicit any effect on the mRNA levels of NOX2, suggesting that it has no role in regulating gene expression. However, TGF-β may be an activator of the NADPH oxidase protein possibly through a PKC-dependent pathway. Eotaxin, on the other hand, decreased NOX2 mRNA transcripts in a time-dependent manner, suggesting the role of eotaxin in eosinophils in early stages of allergic airway inflammation, and may also control eosinophil activation. Under *in vitro* conditions, eosinophils survive for a limited time. Therefore, extrapolation of the findings from *in vitro* experiments to *in vivo* where eosinophils remain in contact with eotaxin and perhaps other cytokines for a longer period of time might be difficult. Additionally, the presence of multiple cytokines and their complex interactions with the signaling molecules might affect NOX2 expression in patients with asthma. The increase in the mRNA levels of NOX2 with IL-33 supports the theory that NOX2 expression is increased in patients with asthma and assists in the activation of eosinophils. IL-33 is a potent cytokine involved in the development of eosinophils from $CD34^+CD117^+$ progenitors and supports production of IL-13, TGF-β, and CCL17 in an ST2-dependent pathway in eosinophils (28). Because IL-33 potentiates the expression of CCR3 on differentiating eosinophils (28) through IL-33/ST2 signaling, it is possible that IL-33 is also involved in the regulation of NOX2 expression in eosinophils. However, the precise molecular mechanism of IL-33–induced NOX2 expression warrants further attention. Massive levels of NOX2 mRNA in moderate to severe asthmatic eosinophils in comparison to the eosinophils from healthy subjects and in subjects with mild to moderate asthma indicate an increased activity level of eosinophils based on the severity of asthma. The presence of more NOX2 in the eosinophils could indicate their ability to produce more $O_2^{\bullet-}$ and hence more oxidative damage mediated by eosinophils in the airways of subjects with allergic asthma.

Sustained activation of NADPH oxidase depends on costimulation of ion channels that can compensate the charge imbalance generated by $O_2^{\bullet-}$ production from NADPH oxidase. CLC3, a Cl^-/H^+ antiporter, works to balance the depolarization generated in the process (27, 29). Phorbol esters, like PMA, produce respiratory burst in cells, including eosinophils (9, 30, 31). PMA binds to the C1 domain of conventional protein kinase C (PKC) to change its conformation (32, 33). This signaling event leads to the activation of eosinophils and assists in degranulation and $O_2^{\bullet-}$ generation. There is some evidence that PKC modulates the phosphorylation at the N-terminus of CLC3 at Ser 51 (34). Thus, the activation of PKC may be a critical step in eosinophil activation through NADPH oxidase and CLC3 activation.

Lysosomes contain CLC3 channels that are involved in their acidification via a Cl^-/H^+ antiporter mechanism (35, 36). The presence of NADPH oxidase on the phagosomes (37) and the interplay between CLC3 and NADPH oxidase in respiratory

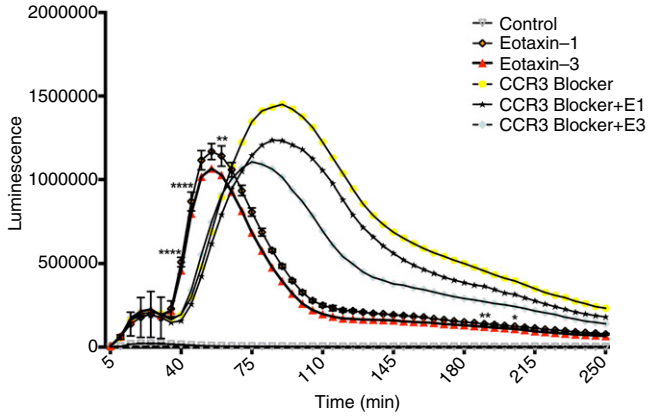

Figure 5. Effect of chemokine (C-C motif) receptor 3 (CCR3) blocker on eotaxin-induced $O_2^{\bullet-}$ release in blood eosinophils. CCR3 blocker (SB328437, 100 μM) had no effect on the first burst, but it increased the $O_2^{\bullet-}$ release alone and in the presence of eotaxin-1 and eotaxin-3. However, there was a delay in the second oxidative burst, suggesting activation of an alternate pathway. At the peak of the eotaxin-induced second oxidative burst, there was significant difference in released $O_2^{\bullet-}$ with the CCR3 blocker groups ($n = 3$). $^*P < 0.05$, $^{**}P < 0.01$, and $^{****}P < 0.0001$.

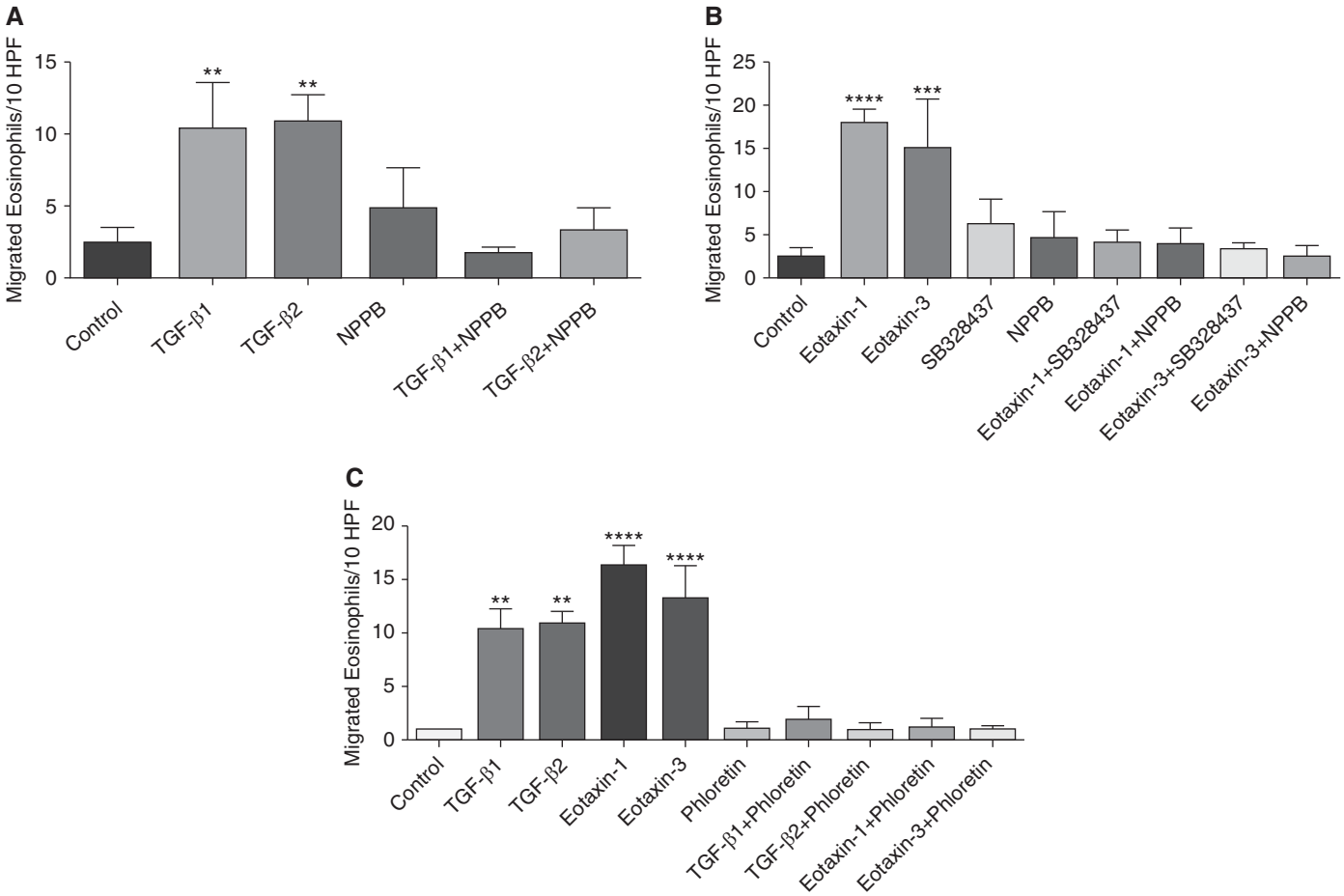

Figure 6. Chemotaxis of blood eosinophils. (*A*) TGF-β1 and TGF-β2 increased the migration of eosinophils that was blocked by the CLC3 inhibitor NPPB ($n = 3$). $^{**}P < 0.01$. (*B*) Eotaxin-1 and eotaxin-3 increased the migration of blood eosinophils that was blocked by CCR3 and CLC3 inhibitors SB328437 and NPPB, respectively ($n = 3$). $^{***}P < 0.001$ and $^{****}P < 0.0001$. (*C*) The effect of phloretin, an inhibitor of CLC3/PKC, on TGF-β1–, TGF-β2–, eotaxin-1–, and eotaxin-3–induced chemotaxis of eosinophils ($n = 4$). $^{**}P < 0.01$ and $^{****}P < 0.0001$. HPF, high-power field.

burst of neutrophils (38) and eosinophils (9) led us to examine the intracellular ROS generation in eosinophils. Although there was no significant effect of PMA in the generation of intracellular ROS, PMA-treated eosinophils exhibited heterogeneity within and between subjects (Figures 2F and 2L). This could relate to the presence of hypo- and normo-dense eosinophils in the blood (39). The heterogeneity in the eosinophil population may indicate their activated state and could be associated with increased susceptibility to the pathogenesis in allergic asthma. Additionally, an increase in the size and granularity of a subpopulation of PMA-stimulated eosinophils supports their PKC-dependent activity and further highlights the presence of multiple populations of eosinophils in a subject.

Extracellular $O_2^{\bullet-}$ generation from NADPH oxidase showed a direct dependence on PKC activation. Treatment with PMA resulted in massive $O_2^{\bullet-}$ release from eosinophils in 20 minutes, which was decreased to half in the presence of CLC3 inhibitor. However, $O_2^{\bullet-}$ generation in response to TGF-β and eotaxin showed two peaks, suggesting a two-step release of $O_2^{\bullet-}$. This two-step release of $O_2^{\bullet-}$ may relate to a sequential activation of NADPH oxidase and CLC3 in the presence of TGF-β and eotaxin. In all cases of $O_2^{\bullet-}$ release, CLC3 dependence was reiterated by the CLC3 blocker NPPB (40). Generation of $O_2^{\bullet-}$ in eosinophils is directly dependent on NADPH oxidase. DPI is an uncompetitive inhibitor of flavoenzymes (41) and therefore can only inhibit the enzyme when the substrate is bound to the active site of the enzyme. DPI significantly reduced the early peak of $O_2^{\bullet-}$ generation, but there was no significant change in the amplitude in the second burst. However, eotaxin-3–induced $O_2^{\bullet-}$ did not decrease to the baseline with the NADPH oxidase inhibitor in the first peak and delayed the second peak. This may suggest that eotaxin-3–induced NADPH oxidase activity supersedes the DPI blockade by restricting the DPI binding to the enzyme through conformational change or steric hindrance. The cause of the shift in the respiratory burst with DPI is not known but may be related to the partial masking of the DPI binding site. CLC3 blocker, on the other hand, is not affected by any of the cytokines and consistently blocks the $O_2^{\bullet-}$ generation, confirming the dependence of $O_2^{\bullet-}$ generation on CLC3. The CCR3 blocker SB328437, in the concentration that inhibited eosinophil migration, did not inhibit $O_2^{\bullet-}$ production. However, it delayed the kinetics of the burst, indicating the involvement of additional pathways in the process. Because SB328437

induced $O_2^{\bullet-}$ production by itself and in the presence of eotaxin-1 and eotaxin-3, it is possible that additional signaling pathways to induce $O_2^{\bullet-}$ production are activated upon blocking of CCR3. Involvement of an alternative pathway in eosinophil activation could be further supported by the finding of delayed $O_2^{\bullet-}$ release in the presence of SB328437. In addition, the possibility of eotaxin exerting its effect on $O_2^{\bullet-}$ release in eosinophils by binding to a site other than CCR3 cannot be ruled out. In this regard, a direct interaction of eotaxin to CLC3 is a possibility and warrants further attention.

Eosinophils migrate to the site of inflammation and release cytokines, chemokines, growth factors, chemical mediators, and ROS to support the excessive damage and repair cycle in the airways in allergic asthma. Activation of CLC3 is required for the NADPH oxidase activity. At the same time, charge imbalance potentiated by the activation of NADPH oxidase requires activation of CLC3, which transports Cl^- in and H^+ out of the cells to maintain homeostasis. However, the underlying molecular signaling involved in the coactivation of closely associated NADPH oxidase and CLC3 is unknown and warrants further attention. In addition to activation, CLC3 has been implicated in the migration of neutrophils (19). To our knowledge, this is the first report to link CLC3 to the migration of eosinophils. Eotaxin is a potent chemoattractant for eosinophils. However, the potency and selectivity varies between eotaxin-1, eotaxin-2, and eotaxin-3. Eotaxin-1 and eotaxin-3 have been implicated in the pathogenesis of asthma, especially eosinophilic asthma (13). Although eotaxin-1 and eotaxin-3 showed similar overall increases in the migration of eosinophils, the effect of eotaxin-1 was consistent in all individuals, whereas the effect of eotaxin-3 varied between the individuals, suggesting heterogeneity in eotaxin-3–induced cellular response among the subjects. Because both these chemokines use the same receptor, CCR3 blocker (SB328437) markedly reduced the number of migrated cells in the eotaxin-1 and eotaxin-3 groups. TGF-β has also been related to the migration of eosinophils. Our findings on the chemotaxis suggest that the cytokines TGF-β and eotaxin use CLC3 to induce eosinophil migration. The role of PKC in eosinophil migration could be supported by the studies using phloretin, which selectively inhibits PKCs and CLC3 (20, 40).

Uptake of Cl^- through CLC3 may result in the swelling of eosinophils, causing shape change that may lead to the cytoskeletal rearrangement and migration of eosinophils (Figure 7). Alternatively, CLC3 activation may cause migration of eosinophils through direct changes in the actin filaments. Studies have shown that hypotonic activation of CLC3b (a transcript variant) facilitates cell swelling–mediated remodeling of the actin cytoskeleton (42, 43). The cytosolic c-terminus of CLC3b directly binds to filamentous actin between amino acids 690 and 760 at proline 688 and leucine 734 (43). In addition to the direct modulation of actin, conformational changes initiated by phosphorylation/activation of CLC3 may lead to downstream signaling through

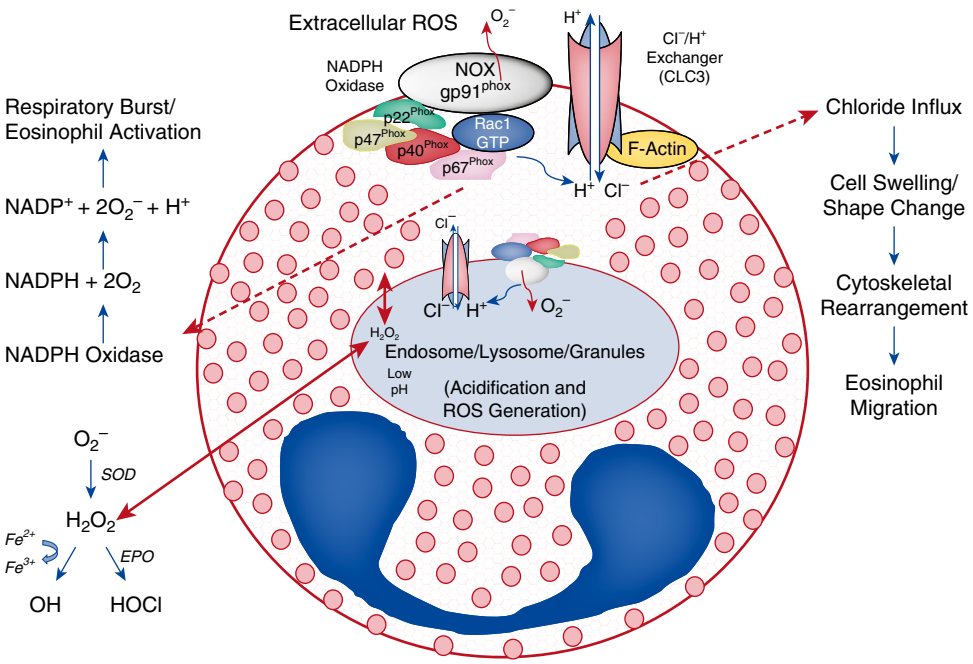

Figure 7. Mechanism of activation and migration of blood eosinophils. NOX2 generates $O_2^{\bullet-}$, causing a depolarization and charge imbalance in eosinophils, which can be compensated by the antiporter activity of the CLC3. Superoxide may dismutate to hydrogen peroxide (H_2O_2) at low pH or in the presence of SOD. Being permeable, H_2O_2 can travel through the membrane and become H_2O in presence of catalase or transform to a more oxidative hypochlorous acid (HOCl) through eosinophil peroxidase (EPO) in eosinophils. The activation of CLC3 leads to Cl^- influx, resulting in shape change and cytoskeletal rearrangement in eosinophils and supporting the migration of eosinophils in response to cytokines/chemokines. Physical interaction of CLC3 to filamentous actin (F-actin) may also result in the cytoskeletal rearrangement and migration of eosinophils upon channel activation/phosphorylation.

cytoskeletal proteins like Rac and ROCK to cause shape change and migration of eosinophils.

Novel findings related to the eotaxin and TGF-β–dependent activation and migration of eosinophils involving CLC3 in eosinophils provide a promising target for therapy in allergic asthma. More studies are required to identify the precise underlying molecular events involving the cytoskeletal proteins and their rearrangement for eosinophil transendothelial migration and in the activation of eosinophil in allergic asthma. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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