

Histamine production by human neutrophils

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ABSTRACT Histamine is an important mediator in the development of allergic reactions. Only a small subset of human cell types is able to produce histamine. No previous studies have shown that human neutrophils are among them. The present work was undertaken to analyze whether human neutrophils produce histamine, and to determine what agonists are involved in histamine production by human neutrophils. The expression of histidine decarboxylase in human neutrophils was established by quantitative PCR, Western blotting, and flow cytometry analysis. The activity of the enzyme was determined by ELISA, which measured histamine in the culture supernatant of neutrophils stimulated with a set of classical agonists. Human neutrophils are *bona fide* histamine-producing cells. Neutrophils store ~0.29 pg/cell and release ~50% of the histamine content in an antigen-dependent manner and on stimulation with other neutrophil agonists. Basal expression of histidine decarboxylase, the rate-limiting enzyme in histamine production, is higher in neutrophils from patients with allergies than from healthy donors. Our results cannot be ascribed to cell contamination for several reasons. LPS failed to induce histamine release by basophils, whereas it induced histamine release by neutrophils; and we did not detect basophils, monocytes, or lymphocytes in our neutrophil preparations. Eosinophils, albeit detected, were only 0.001–0.004% of the final cell population, and they did not store or release histamine on antigen or LPS stimulation. Antigens to which patients with allergies were sensitized stimulated release of histamine from neutro-

phils. These observations represent a novel view of neutrophils as possible source of histamine in the allergic diseases.—Alcañiz, L., Vega, A., Chacón, P., El Bekay, R., Ventura, I., Aroca, R., Blanca, M., Bergstralh, D. T., Monteseirín, J. Histamine production by human neutrophils. *FASEB J.* 27, 000–000 (2013). www.fasebj.org

Key Words: antigens • IgE • histidine decarboxylase

HISTAMINE IS A BIOGENIC AMINE that regulates a wide range of physiological functions, including cell proliferation and differentiation, embryonic development, and regeneration (1, 2). It can influence most cells of the immune system and selectively recruit them into tissue sites, affecting their maturation, activation, polarization, and effector functions, and leading to chronic inflammation (1, 2).

In human pathology, histamine triggers acute symptoms, including acute rhinitis, bronchoconstriction, diarrhea, and cutaneous wheal and flare responses, due to its very rapid activity on vascular endothelium and bronchial and smooth muscle cells. Histamine also significantly modulates chronic-phase inflammatory events (1, 2). The important role of histamine in the early-phase response to antigens (Ags) is well defined (3). Stimulation of the mucous glands and increased vascular permeability with plasma exudation cause an increase in nasal secretions. Vasodilation results in a sensation of congestion, while histamine-induced stimulation of sensory nerves located in epithelial tight junctions leads to sneezing and itching. Histamine is also important at late phase of the allergic response, when it is involved in recruitment, adherence, and activation of inflammatory cells, generation of leukotri-

Abbreviations: Ag, antigen; BAL, bronchoalveolar lavage; CD, cluster of differentiation; ECP, eosinophil cationic protein; FITC, fluorescein isothiocyanate; GAM, goat anti-mouse; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GAR, goat anti-rabbit; HDC, histidine decarboxylase; Ig, immunoglobulin; IL, interleukin; LPS, lipopolysaccharide; MACS, magnetic cell-sorting system; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SPT, skin-prick test; TLR, toll-like receptor

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enes, induction of interleukin (IL) secretion by endothelial cells, and up-regulation of the expression and mobilization of cell adhesion molecules (4). Airway hyperresponsiveness to histamine is a hallmark of asthma (5). Plasma histamine concentrations are elevated in the early and late bronchoconstriction responses to inhaled Ags.

We have previously shown that neutrophils isolated from patients with allergies produce a functional response to those Ags that produce clinical symptoms. For example, in neutrophils, Ags induce the release of myeloperoxidase, IL-8, and elastase, the down-modulation of L-selectin, and the respiratory burst and intracellular increase of Ca^{2+} levels (6, 7). In addition, they induce the activation of important inflammatory transcription factors, like nuclear factor κB (NF- κB) and nuclear factor of activated T cells (NFAT) (7, 8), and the expression of allergic inflammatory mediators, such as cyclooxygenase-2 (9, 10) and eosinophil cationic protein (ECP; refs. 9, 11).

To date, only a small subset of human cell types has been shown to produce histamine: neurons, enterochromaffin-like cells, gastrin-containing cells, mast cells, basophils, monocytes/macrophages, dendritic cells, and platelets (12–14). However, studies in mast cell-deficient mice have shown that neutrophils are an important source of histamine in mice under *Mycoplasma* infection, which causes chronic inflammation (15). *Mycoplasma* infection induces the expression of histidine decarboxylase (HDC), the rate-limiting enzyme in histamine production (16), resulting in histamine production. Neutrophils become a major source of airway histamine by ramping up histamine production in each cell in response to bacteria, and by increasing their numbers to achieve overwhelming numerical superiority over other histamine-producing cells. This conclusion is consistent with studies in rats suggesting that leukocytes infiltrating foci of allergic inflammation express HDC transcripts by *in situ* mRNA hybridization and also contribute to increased HDC activity (17). However, the expression of HDC and release of histamine by human neutrophils has not been observed. Discovery of a functional HDC in human neutrophils, the majority leukocyte in blood, would provide substantial insight into the allergic condition. Determination of the cell-signaling mechanisms involved in HDC modulation could illuminate important therapeutic targets. The present study was undertaken to analyze these questions.

MATERIALS AND METHODS

Materials

The Ags were commercially available Ag extracts, including D_1 (*Dermatophagoides pteronyssinus*), the European house dust mite; G_3 (*Dactylis glomerata*), orchard grass; T_9 (*Olea europaea*), olive tree pollen; and M_6 (*Alternaria alternata*), a widespread fungal mold. They were purchased from Bial-Arístegui (Bilbao, Spain). Fix and Perm cell permeabilization kit was from

Caltag Laboratories (Burlingame, CA, USA). Lipopolysaccharide (LPS) from *Escherichia coli* (L2630), mouse monoclonal Ab for β -actin, and mouse immunoglobulin G (IgG) were from Sigma-Aldrich (Madrid, Spain). Ficoll-Hypaque, phosphate-buffered saline (PBS), RPMI 1640, heat-inactivated fetal bovine serum, penicillin, and streptomycin were purchased from Lonza (Verviers, Belgium). PCR primers, random primers, and *Taq* polymerase were obtained from Roche (Madrid, Spain). Rabbit polyclonal Ab for HDC was purchased from Acris GmbH (Herford, Germany). Goat anti-mouse (GAM) IgG-coated micromagnetic beads, goat anti-rabbit (GAR) IgG-fluorescein isothiocyanate (FITC), mouse monoclonal Abs specific for Fc ϵ R2 [cluster of differentiation 23 (CD23)] clone 9P.25, CD9, CD14, CD203c, CD66b, and CD16 were purchased from Immunotech-IZASA (Barcelona, Spain). Mouse monoclonal Ab specific for Fc ϵ R1 (α chain) clone AER-37 (also known as clone CRA-1) was obtained from eBioscience (San Diego, CA, USA). Anti-galectin-3 (also known as Mac-2) monoclonal clone A3A12 was purchased from AbCam (Cambridge, UK). Horseradish peroxidase-conjugated horse anti-mouse IgG and GAR IgG secondary Abs were purchased from Promega (Madison, WI, USA). All culture reagents (including Ags) used in this work had endotoxin levels of ≤ 0.01 ng/ml, as verified by the Coatest *Limulus* lysate assay (Chromogenix, Mölndal, Sweden).

Patients and controls

Two groups were examined and compared: adult atopic patients with intermittent bronchial asthma, and healthy nonatopic volunteer controls (11). The group of patients with asthma had positive skin-prick test (SPT) results (Bial-Arístegui) and specific-IgE (Hytec 288; Hycor Biomedical-IZASA, Barcelona, Spain) to at least one common Ags (house dust mites and pollens). The patients had not received Ag immunotherapy and were not allowed to take any bronchodilators within the 8 h before challenge of cells *in vitro*. Oral bronchodilators were withheld for 24 h, and none of the donors had taken corticosteroids, disodium cromoglycate, nedocromil sodium, or antihistamines in the previous week. These donors had not experienced episodes of asthma for ≥ 3 mo, or respiratory tract infections in the 4 wk before blood sampling. The healthy subjects had no history of allergy or bronchial symptoms, and were negative for the skin-prick and specific-IgE tests toward a battery of inhalant Ags (house-dust mites, pollens, molds and animal danders). The Hospital Universitario Virgen Macarena ethics committee approved the study, and each donor gave informed consent.

Cell isolation and culture

Highly purified human neutrophils were isolated exactly as described previously (8), which is a modification of an earlier method (18). Briefly, after isolation, neutrophil preparations were further purified, depleting CD9^+ cells (eosinophils), CD203c^+ cells (basophils), and CD14^+ cells (monocytes) using a magnetic cell-sorting system (MACS) by 4 rounds of incubation with mouse anti-human CD9, anti-human CD203c, and anti-human CD14 Abs, and then with anti-mouse IgG micromagnetic beads. The purity of the neutrophil preparations was determined as described previously (18). This purification method reduced contaminating eosinophils to 0.001–0.004% of the final cell population. The purity of neutrophils was on average $>99\%$. Monocytes, basophils, and lymphocytes were not

detected in the neutrophil preparations, as previously shown (8). Human basophils were purified using the Basophil Isolation Kit II (Miltenyi Biotec S.L., Madrid, Spain) following the manufacturer's instructions. Human lymphocytes were isolated as described previously (19). Highly purified human eosinophils were isolated using the Eosinophil Isolation Kit (Miltenyi Biotec) following the manufacturer's instructions, which is based on a previously described method (20). Neutrophils, basophils, and eosinophils were cultured in a histidine-free buffer containing 25 mM PIPES, 110 mM NaCl, 5 mM KCl, 0.1% glucose, 0.003% HSA, and 5 mM CaCl₂ (21), and maintained at 37°C in an atmosphere of 95% O₂ and 5% CO₂ for 30 min, or for up to 18 h. None of the reagents affected the viability of the cells at the concentrations used in this work, as confirmed by the trypan blue dye-exclusion test.

Dissociation of neutrophil-bound Igs

Ig molecules were dissociated from the surface of neutrophils as described previously (7,11). Specific IgE and IgG were determined by enzyme-linked immunosorbent assay (ELISA; Hycor).

Nucleic acid extraction and conventional RT-PCR analysis

Total RNA was isolated from highly purified neutrophils and basophils using the Trizol RNA isolation method (Invitrogen, Carlsbad, CA, USA) and further purified using the RNeasy Lipid kit (Qiagen, Valencia, CA, USA). Total RNA (1 µg) was reverse-transcribed to first-strand cDNA using random primers. The first-strand cDNA was amplified with primer sets for human HDC (accession number: NM_002112.3): 5'-CTCCACATCGATGCTGCTTA-3' and 5'-AGAGTGGCAGCATCTCGAAT-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH; accession number: J04038): 5'-CCACCCATGGCAAATTCATGGCA-3' and 5'-TCTAGACGGCAGGTCAGGTCCACC-3'. Aliquots (20 µl) taken after 35 cycles were electrophoresed on agarose gels, visualized on ethidium bromide-stained agarose gels, and photographed under UV light.

Nucleic acid extraction and real-time PCR

Total RNA was isolated and reverse-transcribed (4 µg) from human neutrophils or basophils as above. Subsequently, cDNA amplifications were carried out using a MicroAmp optical 96-well reaction plate (PE Applied Biosystems, Foster City, CA, USA) on an ABI 7500 Real-Time PCR System (PE Applied Biosystems). qRT-PCR reactions were carried out for all genes using specific TaqMan Gene Expression Assays (PE Applied Biosystems). A negative PCR control without template was done in each assay. During PCR, the C_t values (the cycle number at which the detected fluorescence exceeds the threshold) for each amplified product were determined using a threshold value of 0.1. The specific signals of HDC (NM_002112.3) were normalized by constitutively expressed β -actin (NM_001101.2) signals using the formula $2^{-\Delta C_t}$, where $\Delta C_t = C_t$ (HDC) - C_t (β -actin).

Preparation of whole-cell extracts

For the analysis of HDC and β -actin expression, neutrophils (5×10^6 cells) and lymphocytes or basophils (5×10^6 cells) were resuspended in 50 µl of ice-cold lysis buffer (50 mM Tris, pH 7.4; 10 mM EGTA; 50 mM NaCl; 1% Triton X-100; and the following protease-inhibitor mixture: 10 µg/ml aprotinin, 10

µg/ml leupeptin, 10 µg/ml soybean trypsin inhibitor, 10 µg/ml N-tosyl-L-phenylalanyl-chloromethyl ketone, 10 µg/ml captopril, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamide, and 10 mM iodoacetamide), and vortexed for 10 s following sonication for 10 s. After centrifugation at 12,000 *g* for 2 min at 4°C, the supernatant obtained constituted the whole-cell extract. Protein concentration was determined by the Bradford procedure.

Western blotting analyses

For the analysis of HDC and β -actin, whole-cell extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 50 µg/lane) and electrophoretically transferred to polyvinylidene difluoride membranes. The membranes were probed for 2 h at room temperature with specific Abs against human HDC or β -actin, at a 1:2000 dilution in PBS containing 1% bovine serum albumin and 0.02% Tween 20. Then, after washing with PBS, they were incubated for 30 min with horseradish peroxidase-conjugated horse anti-mouse IgG or GAR IgG secondary Abs at a 1:5000 dilution. Protein signals were detected by electrochemiluminescence detection Quantity One software (Bio-Rad Laboratories).

Analysis of HDC by flow cytometry

For intracellular detection of HDC by flow cytometry, human neutrophils (2×10^6 cells), basophils (2×10^6 cells), lymphocytes (2×10^6 cells), or eosinophils (2×10^6 cells) were incubated, where indicated, with Abs against a cell marker, CD66b (neutrophils), CD203 (basophils), CD3 (lymphocytes), or the mouse isotype control Ab. Cells were then fixed and permeabilized using the Fix and Perm cell permeabilization kit (Caltag Laboratories), following the manufacturer's instructions. Samples were incubated with rabbit anti-HDC for 30 min. Then, samples were washed with PBS, and incubated for 30 min with GAR-FITC. After washing with PBS, cells were resuspended in PBS and analyzed by flow cytometry (Coulter-IZASA, Barcelona, Spain). GAR-FITC was used as isotype control in cells when HDC Ab was omitted.

Analysis of total and released histamine

Histamine was determined in the culture supernatant or the cellular pellets using an ELISA histamine kit from Coulter-IZASA following the manufacturer's instructions.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 4.00 for Windows (GraphPad Software, San Diego, CA, USA). Normality distribution was first examined using Shapiro-Wilk normality test before analysis of statistical significance. Data are expressed as means $\pm$ SE. A Student's *t* test or 1-way ANOVA was used to make comparisons between groups. A level of $P < 0.05$ was considered significant.

RESULTS

Histidine decarboxylase is expressed by human neutrophils

The expression of HDC mRNA was examined in total RNA extracted from neutrophils from patients with

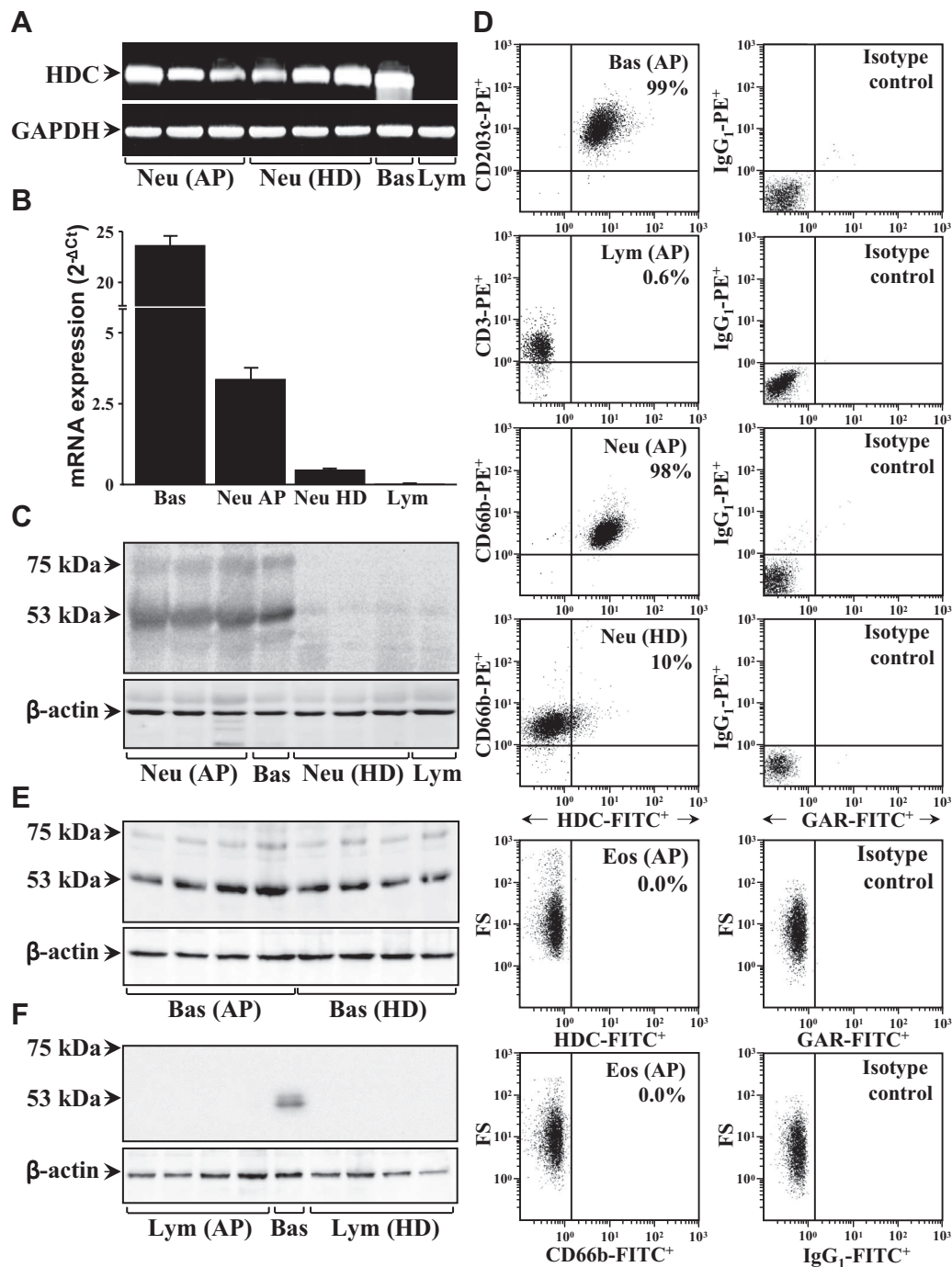


Figure 1. Human neutrophils express HDC. *A*) RT-PCR reveals that HDC mRNA is present in highly purified unstimulated neutrophils (Neu) isolated from 3 human patients with allergies (AP) and three healthy donors (HD). As a positive control, HDC was amplified from highly purified basophils (Bas). RNA from lymphocytes (Lym) was used as a negative control. Results from amplification of the GAPDH housekeeping gene transcript are shown. *B*) Expression of HDC mRNA was also analyzed by real-time PCR analysis ($n=10$ /group). Expression was standardized against β -actin mRNA. Values are means $\pm$ SE. *C*) HDC protein expression was examined by Western blot. β -Actin was used as loading control. Results shown are representative of 3 independent experiments with similar results. *D*) Neutrophils, basophils, lymphocytes, and eosinophils (Eos) from patients with allergies healthy donors ($n=10$ /group) were analyzed by flow cytometry for HDC-FITC, CD66b-PE, CD203c-PE, and CD3-PE, where indicated; representative dot-plot graphs are shown. Percentage of double-positive or single-positive cells is indicated. *E*, *F*) HDC protein expression was examined by Western blot in basophils (*E*) and lymphocytes (*F*) from patients with allergies and healthy donors. Results are representative of two experiments with similar results ($n=8$ /group).

allergies and from healthy donors. As shown in **Fig. 1A**, HDC mRNA was detected in human neutrophils. Expression of HDC mRNA in basophils was measured as a

positive control, while lymphocytes were used as a negative control. Quantitative PCR was performed to examine differences in HDC expression between neu-

trophils from patients with allergies and from healthy donors. Neutrophils from allergic donors showed ~4-fold higher HDC expression than those from healthy donors (Fig. 1B). Basophils showed ~20-fold higher expression than neutrophils from patients with allergies, and lymphocytes showed almost nondetectable levels.

Western blotting was performed to confirm the presence of HDC protein in human neutrophils. As shown in Fig. 1C, neutrophils from patients with allergies and basophils showed similar HDC protein levels. We detected a major band of 74 kDa, corresponding to the precursor form of the protein, and another band of 53 kDa, corresponding to the mature protein, as described previously (22). Neutrophils from healthy donors showed lower HDC protein levels than from patients with allergies.

Additional analysis by flow cytometry showed similar results. Figure 1D shows a representative dot-plot comparison between neutrophils from patients with allergies, neutrophils from healthy donors, basophils, lymphocytes, and eosinophils. Some 98% of the CD66b⁺ cells from highly purified neutrophils from patients with allergies and asthma were positive for HDC compared to 10% from healthy donors. Similar results were obtained for CD203c⁺ cells (basophils). Almost no expression was observed in CD3⁺ cells (lymphocytes). No HDC expression was observed in highly purified eosinophils, in which we could not detect CD66b expression. Additional analysis by Western blotting showed no difference in HDC expression in either basophils from allergic asthmatic patients or healthy donors (Fig. 1E). The same analysis performed in lymphocytes from patients with allergies and healthy donors showed no detectable HDC expression in lymphocytes (Fig. 1F). We did not find differences in HDC expression in neutrophils from patients with allergies and asthma (depending on the severity of symptoms) or patients with allergic rhinitis (data not shown).

Histamine release by human neutrophils

We next analyzed the activity of HDC from neutrophils by measuring production and release of histamine into the culture supernatant by ELISA. Two specific agonists induce functional responses in these cells: the specific Ags to which the patients were sensitized and LPS (6–11). The effect of both agonists was examined. As shown in Fig. 2A, LPS induced release of histamine by neutrophils, but not by basophils. The Ag extracts to which the patients were sensitized strongly stimulated histamine release by neutrophils (Fig. 2A). Other Ags to which the patients with allergies were not sensitized (irrelevant Ags) did not affect histamine release (Fig. 2A). The same analysis was performed in neutrophils from healthy donors with negative results; however, they did respond to LPS (Fig. 2A). A similar analysis was performed in eosinophils with negative results. We could not detect stored histamine in the cell pellets or released to the supernatant after Ag/LPS stimulation.

Further experiments were performed to characterize the importance of the Ag-dependent response to histamine release. As shown in Fig. 2B, C, neutrophils released histamine in a time- and dose-dependent manner after exposure to the same Ags that induce clinical allergic symptoms in the patient. Histamine release was detected from 5 min, with a maximum histamine level at 30 min, decreasing at 40 min, and returning to basal levels at 18 h. Histamine release was detected at an Ag dose of 5 µg/ml, reaching the maximum at a dose of 10 µg/ml.

The presence of specific IgE and IgG on the neutrophil cell surface was measured as previously (7, 11). Only patients with positive SPT results and serum specific IgE to the studied Ags showed specific IgE on the neutrophil surface (data not shown). We could not detect specific IgG to the studied Ags on the neutrophil surface. On *in vitro* Ag challenge, histamine was released from only those neutrophils having cell surface IgE specific to the Ag. Histamine release was not

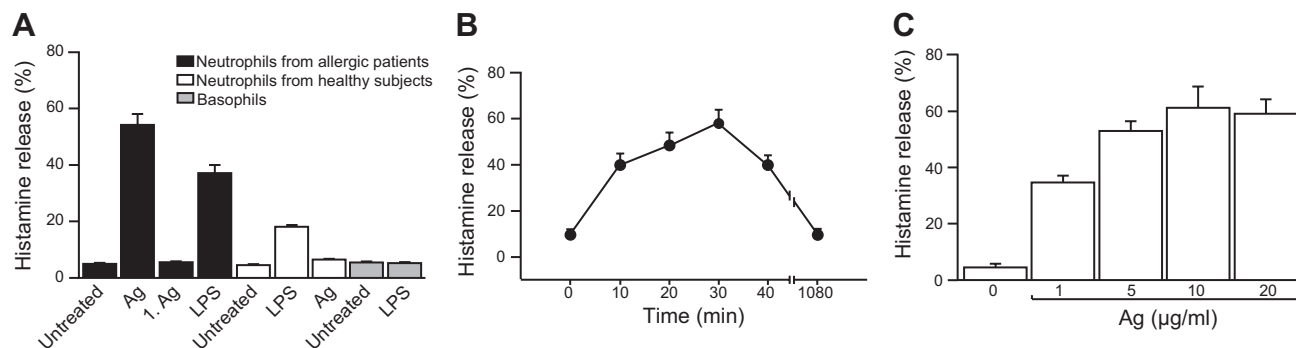


Figure 2. Human neutrophils release histamine. A) Human neutrophils from patients with allergies ($n=11$) were incubated with LPS (1 µg/ml), an Ag to which the patient was sensitized (10 µg/ml), or an irrelevant Ag (I. Ag) to which the patient was not sensitized for 30 min. Human neutrophils from healthy donors ($n=10$) were cultured with LPS (1 µg/ml) or an Ag (10 µg/ml) for 30 min. Human basophils from patients with allergies ($n=11$) were left untreated or cultured with LPS (1 µg/ml) for 30 min. Histamine release was measured in the culture supernatant by ELISA. B, C) Neutrophils from patients with allergies ($n=5$) were incubated with 10 µg/ml of an Ag to which the patient was sensitized for the indicated times or with the indicated doses for 30 min, and histamine release was measured as in A. Results are means $\pm$ SE. Mean stored histamine per neutrophil was 0.29 ± 0.036 pg ($n=43$), and per basophil was 0.66 ± 0.0779 pg ($n=11$).

detected when IgE molecules were stripped from the neutrophil surface before Ag challenge but was detected when neutrophils were incubated with LPS (Fig. 3A).

There are 3 defined types of IgE receptors, all previously described in neutrophils (FcεRI, FcεRII/CD23, and galectin-3; refs 23–41). Experiments were thus performed in order to identify the main IgE receptors involved in Ag-dependent histamine release by human neutrophils. The effect of agonist antibodies against each of the identified IgE receptors was examined (6–8, 11). The antibodies used were CRA1 (anti-FcεRI-chain), 9P.25 (anti-CD23/FcεRII), and A3A12 (anti-galectin-3/Mac-2). Antibodies against FcεRI or FcεRII promoted release of histamine into the medium, while anti-galectin-3 did not have significant effect (Fig. 3B). A nonspecific mouse IgG antibody was used as a negative control (Fig. 3B).

DISCUSSION

On Ag challenge of the airway, histamine levels are rapidly increased both in the airway and in plasma (42). There is increasing evidence of the participation of neutrophils in allergic processes in general and in asthma in particular. Because neutrophils are the first cells recruited to the site of an allergic reaction, they may influence clinical presentation and play a role in development of severe chronic asthma and the onset of sudden severe attacks (9).

The results described here show, for the first time, that human neutrophils are able to produce and release histamine. Several lines of evidence verify this claim. HDC expression is detected at both the transcript and protein levels, and histamine release is measured by ELISA. Previous studies have shown that histamine is produced by other types of human cells, namely basophils and mast cells (13, 16, 17). Thus, a

critical concern in performing this study and evaluating its results was the potential for contamination of our preparations by other cell types. Our results are exclusively due to neutrophils, and cannot be ascribed to a possible cell contamination for several reasons. In agreement with previous reports (43), LPS failed to induce histamine release by basophils, whereas it induced histamine release by neutrophils; we did not detect basophils, monocytes, or lymphocytes in our neutrophil preparations. Eosinophils, albeit detected, were only 0.001–0.004% of the final cell population. Furthermore, we could not detect HDC expression in highly purified eosinophils, which neither stored nor released histamine on Ag or LPS stimulation. While some early studies suggested possible production of histamine by eosinophils (44, 45), our data using updated cell purification techniques do not support that claim. We found that CD66b⁺ cells express HDC, and we could not detect CD66b⁺ cells in our highly purified eosinophil preparations. However, there is controversy about whether CD66 is a specific marker of eosinophils as opposed to neutrophils (46–48).

To date, histamine release from neutrophils has only been observed in rodents (15, 17), in which HDC protein expression is inducible in response to *Mycoplasma pulmonis* (15), *Pseudomonas aeruginosa* (49), and Toll-like receptor (TLR) challenge (50), and histamine production is completely dependent on HDC activity and protein levels. In addition, although we found higher levels of HDC mRNA and HDC activity in human basophils, expression of HDC protein seems to be similar to that found in neutrophils. Given the importance of HDC activity, these results suggest the possibility of tight post-translational control. High HDC expression in neutrophils is a potential marker of the allergic condition. We detected higher HDC mRNA and protein levels in human neutrophils from patients with allergies than healthy donors, in whom neutrophil

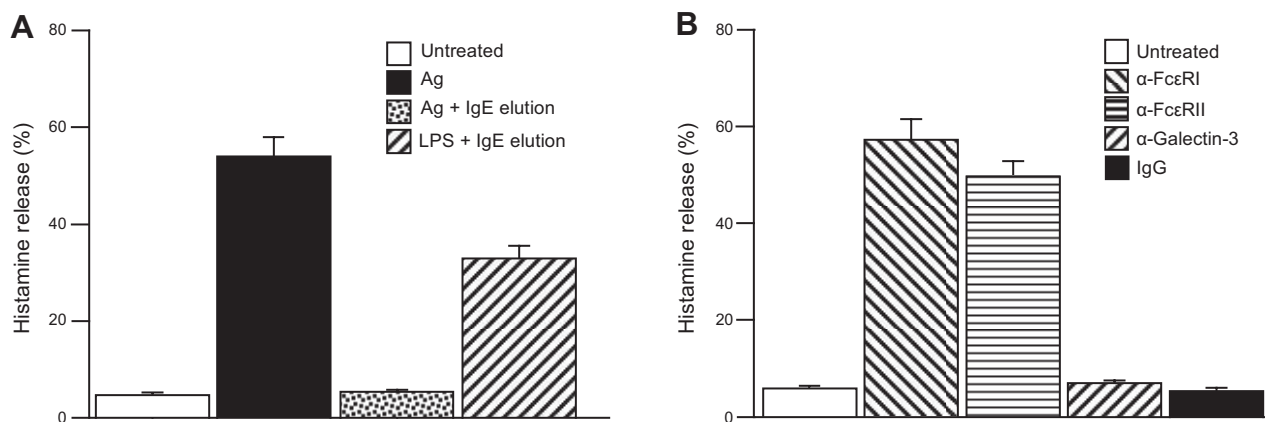


Figure 3. Histamine release in response to Ags is IgE-dependent. IgE receptors involved in the process. A) Neutrophils from patients with allergies ($n=10$) were treated, where indicated, to elute the Igs from the cell surface, and then incubated with an Ag (10 $\mu\text{g}/\text{ml}$) to which the patient was sensitized or LPS (1 $\mu\text{g}/\text{ml}$) for 30 min, and histamine release was measured in the culture supernatant by ELISA. Specific IgE and IgG for the above Ags were determined in the elution solution by ELISA. B) Neutrophils from patients with allergies ($n=7$) were incubated with anti-FcεRI (CRA 1), anti-CD23 (9P.25), or anti-galectin-3 (A3A12) antibodies at 5 $\mu\text{g}/\text{ml}$ for 30 min, and histamine release was measured as in A. Values are means $\pm$ SE.

HDC was almost nondetectable. We cannot exclude the possibility of inducible HDC expression under the nonsymptomatic inflammatory conditions of the patients tested. Increased HDC expression likely represents a priming of neutrophils to respond to allergic stimuli. In basophils, higher HDC expression in patients with allergies has not been described.

Human basophils and mast cells store, on average, ~ 1 and $\sim 1\text{--}3$ pg histamine/cell, respectively. However, the amount of stored histamine may vary widely. For this reason, the level of histamine release is commonly expressed as a percentage of the total amount (51, 52). We could detect stored histamine of 0.66 ± 0.27 pg/cell ($n=11$) in basophils and 0.29 ± 0.125 ($n=43$) in neutrophils. The clinical relevance of the histamine release by neutrophils may be important in the context of allergic inflamed tissues, where mast cells modulate certain features of the allergic asthma disease, by recruiting and activating neutrophils, which actively contribute to the inflammatory response. For instance, mast cell chymases and tryptases (53) are potent chemoattractants for neutrophils (54, 55). In addition, histamine promotes neutrophil recruitment by increasing CD62P expression on the endothelium (56, 57). Thus, mast cell activation provides an amplification loop for the neutrophil recruitment and activation in IgE-mediated reactions (58, 59). Neutrophils have a very short half-life of 6–18 h during circulation. This short half-life arises from the fact that bloodstream neutrophils constitutively undergo apoptosis. In contrast to the short half-life of circulating cells, neutrophils recruited into tissues have a much longer survival time (several days) because they become rescued from the constitutive apoptotic pathway (60, 61). Multiple studies support the view that neutrophil apoptosis and/or clearance may be defective in patients with asthma and show survival factors in the airway lining fluid which are specifically enriched in the context of severe asthma (61, 62). Mast cells can enhance the survival of neutrophils due to produced factors, such as tumor necrosis factor α (TNF- α) and granulocyte macrophage-colony stimulating factor (GM-CSF), which delay their apoptosis (62). Perturbation of the normal apoptosis pathway of tissue neutrophils can enhance their survival times and thereby extend their capacity to secrete their products, such as histamine, which may contribute to potentiate the role of mast cells in tissues.

Neutrophils from mice demonstrated the capacity to generate histamine in response to *M. pulmonis* (15), *P. aeruginosa* (49), and in response to specific TLR stimuli (50). Furthermore, the level of histamine itself was shown to correlate with neutrophil infiltration into the lung during the course of a *Mycoplasma* pneumonia infection, even in mast cell-deficient mice (15); these researchers demonstrated *in vitro* histamine production from neutrophils stimulated by either live or dead *Mycoplasma*. This study does not support the hypothesis that *M. pulmonis* degranulates mast cells, because these live organisms did not affect cultured mast cells and did not increase histamine levels in bronchoalveolar lavage

(BAL) at early time points after infection *in vivo*. BAL and lung histamine levels rose progressively in mast cell-deficient mice at later points, especially 1–2 wk after infection, when levels came to equal or exceed those in non-mast cell-deficient mice. The histopathological analysis established that these increases were not caused by the appearance of mast cells in mast cell deficient mice and coincided with progressive worsening of neutrophilic tracheobronchitis and pneumonia. Similarly they cannot attribute the large increases in mast cell deficient mice to recruitment of basophils, which decreased in number as inflammation progressed, as shown by FACS. Indeed, the principal source of histamine appeared to be neutrophils, which populated the airway and alveolar lumen in large numbers, and which tracked with the rise in histamine. Up-regulated production of histamine has also been observed in neutrophils recruited to sites of Ag-induced inflammation in rats (17) and mice (63). An increase in histamine content in the pouch fluid during the anaphylaxis phase (0 ± 30 min after Ag challenge) was caused by the degranulation of subcutaneous mast cells by an IgE-dependent mechanism. In contrast, at the late phase (4 ± 24 h after the Ag challenge), histamine production, is not caused by mast cell degranulation. It was suggested that the neutrophils infiltrating the inflammatory locus are the major histamine-producing cells at the late phase of allergic reactions (17).

In summary, our study shows that neutrophils possess the capacity to generate histamine in response to specific Ag stimuli. While the level of histamine per cell is relatively low when compared to mast cells or basophils, neutrophils may possess the capacity to exert histamine-dependent immunomodulatory influences by the cumulative effects of their massive influx and accumulation during inflammation. Consequently, neutrophil-derived histamine may have important roles to play in directing allergy-immune responses. **[F]**

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REFERENCES

- Jutel, M., Watanabe, T., Akdis, M., Blaser, K., and Akdis, C. A. (2002) Immune regulation by histamine. *Curr. Opin. Immunol.* **14**, 735–740
- Akdis, C. A., and Blaser, K. (2003) Histamine in the immune regulation of allergic inflammation. *J. Allergy Clin. Immunol.* **112**, 15–22
- Andersson, M., Greiff, L., and Svensson, C. (1994) Allergic rhinoconjunctivitis: the role of histamine. *Mediators Inflamm.* **3**, 171–175
- Naclerio, R. M., Hubbard, W., Lichtenstein, L. M., Kagey-Sobotka, A., and Proud, D. (1996) Origin of late phase histamine release. *J. Allergy Clin. Immunol.* **98**, 721–723
- Holgate, S. T. (1994) Antihistamines in the treatment of asthma. *Clin. Rev. Allergy* **12**, 65–78
- Monteseirin, J., Chacon, P., Vega, A., Sanchez-Monteseirin, H., Asturias, J. A., Martinez, A., Guardia, P., Perez-Cano, R., and Conde, J. (2005) L-selectin expression on neutrophils from allergic patients. *Clin. Exp. Allergy* **35**, 1204–1213
- Monteseirin, J., Chacon, P., Vega, A., El Bekay, R., Alvarez, M., Alba, G., Conde, M., Jimenez, J., Asturias, J. A., Martinez, A., Conde, J., Pintado, E., Bedoya, F. J., and Sobrino, F. (2004) Human neutrophils synthesize IL-8 in an IgE-mediated activation. *J. Leukoc. Biol.* **76**, 692–700
- Vega, A., Chacon, P., Monteseirin, J., El Bekay, R., Alba, G., Martin-Nieto, J., and Sobrino, F. (2007) Expression of the transcription factor NFAT2 in human neutrophils: IgE-dependent, Ca²⁺- and calcineurin-mediated NFAT2 activation. *J. Cell Sci.* **120**, 2328–2337
- Monteseirin, J. (2009) Neutrophils and asthma. *J. Investig. Allergol. Clin. Immunol.* **19**, 340–354
- Vega, A., Chacon, P., Alba, G., El Bekay, R., Martin-Nieto, J., and Sobrino, F. (2006) Modulation of IgE-dependent COX-2 gene expression by reactive oxygen species in human neutrophils. *J. Leukoc. Biol.* **80**, 152–163
- Monteseirin, J., Vega, A., Chacon, P., Camacho, M. J., El Bekay, R., Asturias, J. A., Martinez, A., Guardia, P., Perez-Cano, R., and Conde, J. (2007) Neutrophils as a novel source of eosinophil cationic protein in IgE-mediated processes. *J. Immunol.* **179**, 2634–2641
- Laszlo, V., Rothe, G., Hegyesi, H., Szeberenyi, J. B., Orso, E., Schmitz, G., and Falus, A. (2001) Increased histidine decarboxylase expression during in vitro monocyte maturation; a possible role of endogenously synthesised histamine in monocyte/macrophage differentiation. *Inflamm. Res.* **50**, 428–434
- Saxena, S. P., Brandes, L. J., Becker, A. B., Simons, K. J., LaBella, F. S., and Gerrard, J. M. (1989) Histamine is an intracellular messenger mediating platelet aggregation. *Science* **243**, 1596–1599
- Szeberenyi, J. B., Laszlo, V., Pallinger, E., Orso, E., Rothe, G., Schmitz, G., and Falus, A. (2001) Intracellular histamine content increases during in vitro dendritic cell differentiation. *Inflamm. Res.* **50**(Suppl. 2), S112–S113
- Xu, X., Zhang, D., Zhang, H., Wolters, P. J., Killeen, N. P., Sullivan, B. M., Locksley, R. M., Lowell, C. A., and Caughey, G. H. (2006) Neutrophil histamine contributes to inflammation in mycoplasma pneumonia. *J. Exp. Med.* **203**, 2907–2917
- Oh, C., Suzuki, S., Nakashima, I., Yamashita, K., and Nakano, K. (1988) Histamine synthesis by non-mast cells through mitogen-dependent induction of histidine decarboxylase. *Immunology* **65**, 143–148
- Shiraishi, M., Hirasawa, N., Oikawa, S., Kobayashi, Y., and Ohuchi, K. (2000) Analysis of histamine-producing cells at the late phase of allergic inflammation in rats. *Immunology* **99**, 600–606
- Sur, S., Glitz, D. G., Kita, H., Kujawa, S. M., Peterson, E. A., Weiler, D. A., Kephart, G. M., Wagner, J. M., George, T. J., Gleich, G. J., and Leiferman, K. M. (1998) Localization of eosinophil-derived neurotoxin and eosinophil cationic protein in neutrophilic leukocytes. *J. Leukoc. Biol.* **63**, 715–722
- Chacon, P., Vega, A., Monteseirin, J., El Bekay, R., Alba, G., Perez-Formoso, J. L., Msartinez, A., Asturias, J. A., Perez-Cano, R., Sobrino, F., and Conde, J. (2005) Induction of cyclooxygenase-2 expression by allergens in lymphocytes from allergic patients. *Eur. J. Immunol.* **35**, 2313–2324
- Hansel, T. T., De Vries, I. J., Iff, T., Rihs, S., Wandzilak, M., Betz, S., Blaser, K., and Walker, C. (1991) An improved immunomagnetic procedure for the isolation of highly purified human blood eosinophils. *J. Immunol. Methods* **145**, 105–110
- Vonakis, B. M., Macglashan, D. W., Jr., Vilarino, N., Langdon, J. M., Scott, R. S., and MacDonald, S. M. (2008) Distinct characteristics of signal transduction events by histamine-releasing factor/translationally controlled tumor protein (HRF/TCTP)-induced priming and activation of human basophils. *Blood* **111**, 1789–1796
- Tanaka, S., Nemoto, K., Yamamura, E., and Ichikawa, A. (1998) Intracellular localization of the 74- and 53-kDa forms of L-histidine decarboxylase in a rat basophilic/mast cell line, RBL-2H3. *J. Biol. Chem.* **273**, 8177–8182
- Alphonse, M. P. (2005) *Regulation of the high affinity receptor for IgE (FcεRI) in human neutrophils*. Masters of Science thesis, Department of Immunology, University of Manitoba
- Alphonse, M. P., Saffar, A. S., Shan, L., HayGlass, K. T., Simons, F. E., and Gounni, A. S. (2008) Regulation of the high affinity IgE receptor (Fc epsilonRI) in human neutrophils: role of seasonal allergen exposure and Th-2 cytokines. *PLoS One* **3**, e1921
- Benoni, G., Adami, A., Vella, A., Ariosto, E., Ortolani, R., and Cuzzolin, L. (2001) CD23 and CD69 expression on human neutrophils of healthy subjects and patients with peripheral arterial occlusive disease. *Int. J. Immunopathol. Pharmacol.* **14**, 161–167
- Dehlink, E., Baker, A. H., Yen, E., Nurko, S., and Fiebiger, E. (2010) Relationships between levels of serum IgE, cell-bound IgE, and IgE-receptors on peripheral blood cells in a pediatric population. *PLoS One* **5**, e12204
- Gounni, A. S., Lamkhioued, B., Koussih, L., Ra, C., Renzi, P. M., and Hamid, Q. (2001) Human neutrophils express the high-affinity receptor for immunoglobulin E (Fc epsilon RI): role in asthma. *FASEB J.* **15**, 940–949
- Kita, H., Kaneko, M., Bartemes, K. R., Weiler, D. A., Schimming, A. W., Reed, C. E., and Gleich, G. J. (1999) Does IgE bind to and activate eosinophils from patients with allergy? *J. Immunol.* **162**, 6901–6911
- Liu, F. T., Hsu, D. K., Zuberi, R. I., Hill, P. N., Shenhav, A., Kuwabara, I., and Chen, S. S. (1996) Modulation of functional properties of galectin-3 by monoclonal antibodies binding to the non-lectin domains. *Biochemistry* **35**, 6073–6079
- Nakajima, T., Iikura, M., Okayama, Y., Matsumoto, K., Uchiyama, C., Shirakawa, T., Yang, X., Adra, C. N., Hirai, K., and Saito, H. (2004) Identification of granulocyte subtype-selective receptors and ion channels by using a high-density oligonucleotide probe array. *J. Allergy Clin. Immunol.* **113**, 528–535
- Nishimura, M., Mitsunaga, S., Ishikawa, Y., and Satake, M. (2003) Possible mechanisms underlying development of trans-fusion-related acute lung injury: roles of anti-major histocompatibility complex class II DR antibody. *Transfus. Med.* **13**, 141–147
- Novak, N., Kraft, S., and Bieber, T. (2001) IgE receptors. *Curr. Opin. Immunol.* **13**, 721–726
- Porcherie, A., Mathieu, C., Peronet, R., Schneider, E., Claver, J., Commere, P. H., Kiefer-Biasizzo, H., Karasuyama, H., Milon, G., Dy, M., Kinet, J. P., Louis, J., Blank, U., and Mecheri, S. (2011) Critical role of the neutrophil-associated high-affinity receptor for IgE in the pathogenesis of experimental cerebral malaria. *J. Exp. Med.* **208**, 2225–2236
- Saffar, A. S., Alphonse, M. P., Shan, L., Hayglass, K. T., Simons, F. E., and Gounni, A. S. (2007) IgE modulates neutrophil survival in asthma: role of mitochondrial pathway. *J. Immunol.* **178**, 2535–2541
- Scholl, P. R., and Geha, R. S. (1993) Physical association between the high-affinity IgG receptor (Fc gamma RI) and the gamma subunit of the high-affinity IgE receptor (Fc epsilon RI gamma). *Proc. Natl. Acad. Sci. U. S. A.* **90**, 8847–8850
- Sugita, N., Suzuki, T., Yoshie, H., Yoshida, N., Adachi, M., and Hara, K. (1993) Differential expression of CR3, Fc epsilon RII and Fc gamma RIII on polymorphonuclear leukocytes in gingival crevicular fluid. *J. Periodontal Res.* **28**, 363–372
- Truong, M. J., Gruart, V., Kusnier, J. P., Papin, J. P., Loiseau, S., Capron, A., and Capron, M. (1993) Human neutrophils express immunoglobulin E (IgE)-binding proteins (Mac-2/epsilon BP)

- of the S-type lectin family: role in IgE-dependent activation. *J. Exp. Med.* **177**, 243–248
38. Truong, M. J., Liu, F. T., and Capron, M. (1994) Human granulocytes express functional IgE-binding molecules, Mac-2/epsilon BP. *Ann. N. Y. Acad. Sci.* **725**, 234–246
 39. Vella, A., Bellavite, P., Adami, A., Ortolani, R., Benoni, G., Carletto, A., Biasi, D., Caramaschi, P., and Tridente, G. (1999) Expression of FCepsilonII/CD23 on human neutrophils isolated from rheumatoid arthritis patients. *Inflammation* **23**, 471–479
 40. Yamaoka, A., Kuwabara, I., Frigeri, L. G., and Liu, F. T. (1995) A human lectin, galectin-3 (epsilon bp/Mac-2), stimulates superoxide production by neutrophils. *J. Immunol.* **154**, 3479–3487
 41. Yamaoka, K. A., Arock, M., Issaly, F., Dugas, N., Le Goff, L., and Kolb, J. P. (1996) Granulocyte macrophage colony stimulating factor induces Fc epsilon RII/CD23 expression on normal human polymorphonuclear neutrophils. *Int. Immunol.* **8**, 479–490
 42. Busse, W. W., and Swenson, C. A. (1989) The relationship between plasma histamine concentrations and bronchial obstruction to antigen challenge in allergic rhinitis. *J. Allergy Clin. Immunol.* **84**, 658–666
 43. Saini, S., Bloom, D. C., Bieneman, A., Vasagar, K., Togias, A., and Schroeder, J. (2004) Systemic effects of allergen exposure on blood basophil IL-13 secretion and FcepsilonRIIbeta. *J. Allergy Clin. Immunol.* **114**, 768–774
 44. Graham, H. T., Lowry, O. H., Wheelwright, F., Lenz, M. A., and Parish, H. H., Jr. (1955) Distribution of histamine among leukocytes and platelets. *Blood* **10**, 467–481
 45. Krauss, S., Gilbert, H. S., and Wasserman, L. R. (1968) Leukocyte histidine decarboxylase: properties and activity in myeloproliferative disorders. *Blood* **31**, 699–709
 46. Schmidt, T., Zundorf, J., Gruger, T., Brandenburg, K., Reinert, A. L., Zinserling, J., and Schnitzler, N. (2012) CD66b overexpression and homotypic aggregation of human peripheral blood neutrophils after activation by a gram-positive stimulus. *J. Leukoc. Biol.* **91**, 791–802
 47. Yoon, J., Terada, A., and Kita, H. (2007) CD66b regulates adhesion and activation of human eosinophils. *J. Immunol.* **179**, 8454–8462
 48. Torsteinsdottir, I., Arvidson, N. G., Hallgren, R., and Hakansson, L. (1999) Enhanced expression of integrins and CD66b on peripheral blood neutrophils and eosinophils in patients with rheumatoid arthritis, and the effect of glucocorticoids. *Scand. J. Immunol.* **50**, 433–439
 49. Xu, X., Zhang, H., Song, Y., Lynch, S. V., Lowell, C. A., Wiener-Kronish, J. P., and Caughey, G. H. (2012) Strain-dependent induction of neutrophil histamine production and cell death by *Pseudomonas aeruginosa*. *J. Leukoc. Biol.* **91**, 275–284
 50. Smuda, C., Wechsler, J. B., and Bryce, P. J. (2011) TLR-induced activation of neutrophils promotes histamine production via a PI3 kinase dependent mechanism. *Immunol. Lett.* **141**, 102–108
 51. Barret, K. E., Pearce, F. L. (1993) Mast cell heterogeneity. In *Immunopharmacology of Mast Cells and Basophils* (Foreman, J. C., ed) pp 29–42, Academic Press, London
 52. Schroeder, J. T., Lichtenstein, L. (2003) Biology of basophils. In *Middleton's Allergy Principles and Practice*, 6th Ed. (Adkinson Jr., N. F., Yunginger, J. W., Busse, W. W., Bochner, B. S., Holgate, S. T., and Simons, F. E. R., eds) pp 347–359, Mosby, Philadelphia
 53. Caughey, G. H. (2007) Mast cell tryptases and chymases in inflammation and host defense. *Immunol. Rev.* **217**, 141–154
 54. Huang, C., De Sanctis, G. T., O'Brien, P. J., Mizgerd, J. P., Friend, D. S., Drazen, J. M., Brass, L. F., and Stevens, R. L. (2001) Evaluation of the substrate specificity of human mast cell tryptase beta I and demonstration of its importance in bacterial infections of the lung. *J. Biol. Chem.* **276**, 26276–26284
 55. Tani, K., Ogushi, F., Kido, H., Kawano, T., Kunori, Y., Kamimura, T., Cui, P., and Sone, S. (2000) Chymase is a potent chemoattractant for human monocytes and neutrophils. *J. Leukoc. Biol.* **67**, 585–589
 56. Gaboury, J. P., Johnston, B., Niu, X. F., and Kubes, P. (1995) Mechanisms underlying acute mast cell-induced leukocyte rolling and adhesion in vivo. *J. Immunol.* **154**, 804–813
 57. Kubes, P., and Granger, D. N. (1996) Leukocyte-endothelial cell interactions evoked by mast cells. *Cardiovasc. Res.* **32**, 699–708
 58. Moreno, A. N., Jamur, M. C., Oliver, C., and Roque-Barreira, M. C. (2003) Mast cell degranulation induced by lectins: effect on neutrophil recruitment. *Int. Arch. Allergy Immunol.* **132**, 221–230
 59. Schramm, R., and Thorlacius, H. (2004) Neutrophil recruitment in mast cell-dependent inflammation: inhibitory mechanisms of glucocorticoids. *Inflamm. Res.* **53**, 644–652
 60. Edwards, S. W., Moulding, D. A., Derouet, M., and Moots, R. J. (2003) Regulation of neutrophil apoptosis. *Chem. Immunol. Allergy* **83**, 204–224
 61. Yang, E. J., Choi, E., Ko, J., Kim, D. H., Lee, J. S., and Kim, I. S. (2012) Differential effect of CCL2 on constitutive neutrophil apoptosis between normal and asthmatic subjects. *J. Cell. Physiol.* **227**, 2567–2577
 62. Uddin, M., Nong, G., Ward, J., Seumois, G., Prince, L. R., Wilson, S. J., Cornelius, V., Dent, G., and Djukanovic, R. (2010) Prosurvival activity for airway neutrophils in severe asthma. *Thorax* **65**, 684–689
 63. Tanaka, S., Deai, K., Konomi, A., Takahashi, K., Yamane, H., Sugimoto, Y., and Ichikawa, A. (2004) Expression of L-histidine decarboxylase in granules of elicited mouse polymorphonuclear leukocytes. *Eur. J. Immunol.* **34**, 1472–1482

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