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RESEARCH ARTICLE

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Targeted inhibition of allergen-induced histamine production by neutrophils

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Abstract

Histamine is a critical inflammatory mediator in allergic diseases. We showed in a previous work that neutrophils from allergic patients produce histamine in response to allergens to which the patients were sensitized. Here, we investigate the molecular mechanisms involved in this process using peripheral blood neutrophils. We challenged these cells in vitro with allergens and analyzed histamine release in the culture supernatants. We also explored the effect of common therapeutic drugs that ameliorate allergic symptoms, as well as allergen-specific immunotherapy. Additionally, we examined the expression of histidine decarboxylase and diamine oxidase, critical enzymes in the metabolism of histamine, under allergen challenge. We show that allergen-induced histamine release is dependent on the activation of the phosphoinositide 3-kinase, mitogen-activated protein kinase p38, and extracellular signal-regulated kinase 1/2 signaling pathways. We also found a contribution of the phosphatase calcineurin to lesser extent. Anti-histamines, glucocorticoids, anti-M3-muscarinic receptor antagonists, and mainly β_2-receptor agonists abolished the allergen-dependent histamine release. Interestingly, allergen-specific immunotherapy canceled the histamine release through the downregulation of histidine decarboxylase expression. Our observations describe novel molecular mechanisms involved in the allergen-dependent histamine release by human neutrophils and provide new targets to inhibit histamine production.

Abbreviations: Ag, allergen; DAO, diamine oxidase; HDC, histidine decarboxylase; IL, interleukin; IT, immunotherapy; MAPKs, mitogen-activated protein kinases; PI3K, phosphoinositide 3-kinase.

Pedro Chacón and Antonio Vega-Rioja contributed equally to this work.

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KEYWORDS
allergen immunotherapy, asthma, diamine oxidase, histamine, histidine decarboxylase

1 | INTRODUCTION

Histamine plays a pivotal role in allergic diseases. It is a biogenic amine generated by one-step decarboxylation of histidine by the rate-limiting enzyme histidine decarboxylase (HDC) and degraded by diamine oxidase.[1] Histamine regulates multiple physiological functions including cell differentiation and proliferation, vascular permeability, gastric acid secretion, or immune cell chemotaxis.[2-4] It also has an important role in the pathophysiology of immune diseases regulating the release of proinflammatory mediators from most immune cells, fibroblasts, epithelial, or endothelial cells.[2,3,5] Histamine is also responsible of most allergic symptoms including rhinitis, bronchoconstriction, diarrhea, and cutaneous wheal and flare responses.[2-4] When released from granules of FεRI$^+$ cells, it plays a critical role in both early and late responses to allergens (Ags).[2-4] It enhances the production of T_H2 (IL-4, IL-5, IL-10, and IL-13) and inhibits T_H1 (IL-2 and IFN-γ and IL-12) cytokines.[6] Ag-specific immunotherapy (IT) and Ag avoidance are the only approaches to diminish the progression of IgE-mediated diseases such as allergic rhinitis, allergic asthma, or atopic dermatitis.[7,8] In addition, treatments with anti-histamines, glucocorticoids, and bronchodilators (muscarinic antagonists and β_2-agonists) are used to reduce the symptoms.[9-11] Previous work from our laboratory describes higher HDC expression in neutrophils from allergic patients, which were able to produce and release histamine in response to specific Ags. This production is dependent on the presence of Ag-specific IgE on the cell surface and is mediated by FcεRI and FcεRII/CD23 receptors.[12] In the present work, we analyze the molecular mechanisms involved in the Ag-dependent histamine release and we assess the effect of IT and common drugs used to treat allergic diseases.

2 | MATERIALS AND METHODS

2.1 | Materials

The Ags extracts used in this work were *Olea europaea* (T_9) and Alt a1, the major antigen of *Alternaria alternata. Alt a1* reacts with serum IgE in 82% to 100% of the cases[13] (Diater, Madrid, Spain). The Fix and Perm cell permeabilization kit, Ficoll-Hypaque, phosphate-buffered saline (PBS), RPMI 1640 medium, heat-inactivated fetal bovine serum, penicillin, and streptomycin were purchased from Thermo Fisher Invitrogen (San Diego, CA, USA). Fluorescein isothiocyanate (FITC)-conjugated mouse anti (α)-human-CD66b and phycoerythrin (PE)-conjugated goat α-rabbit IgG were from Immunotech-IZASA (Barcelona, Spain). Rabbit polyclonal antibody α-human HDC was purchased from ACris GmbH (Herford, Germany). PE-conjugated rabbit α-human diamine oxidase (DAO)/AOC-1 was from VWR (Barcelona, Spain). Inhibitors (wortmannin, SB203580, PD098059, Cyclosporin A (CsA)) and medicals drugs (loratadine, budesonide, tiotropium bromide, and salmeterol) were purchased from Sigma-Aldrich (Madrid, Spain). All culture reagents had endotoxin levels of ≤0.01 ng/mL, as tested by the Coatest Limulus lysate assay (Chromogenix, Mölndal, Sweden).

2.2 | Patients and controls

The groups studied included adult IT-treated allergic patients with Alt a1 allergic asthma, non-IT-treated allergic patients with Alt a1 allergic asthma, and healthy volunteer controls (Table 1). The asthmatic patients had positive results in skin-prick test (Diater) and serum-specific IgE (HYTEC 288, Hycor Biomedical-IZASA) to Alt a1. The sensitivity of the assay was 0.24 ng/mL to Alt a1. The non-IT group had not received specific hyposensitization and did not experienced episodes of respiratory infections for the last 4 weeks before blood extraction. The IT group received Alt a1-IT (Diater) for the previous three years and continued to receive a maintenance dose within the highest dose of the extract. Allergic patients were not allowed to take any inhaled bronchodilators within 8 hours before cell isolation and the in vitro cellular challenge, oral bronchodilators for 24 hours or anti-histamines, oral corticosteroids, disodium cromoglycate, or nedocromil sodium in the previous week. The healthy group had no history of allergy or bronchial symptoms and had both negative skin-prick tests and specific IgE to a battery of inhalant Ags (house-dust mites, pollens, animal danders, and molds, including Alt a1). The Hospital Universitario Virgen Macarena Ethics Committee approved the study, and each subject gave a written informed consent.

TABLE 1 Demographic characteristics of the study groups

Parameter	HD (n = 10)	Non-IT AP (n = 28)	IT- AP (n = 15)
Age*	42 ± 5.3	40.1 ± 4.4	38.4 ± 5
Gender (male/female)	6/4	14/14	8/7
Caucasian (%)	100	100	100
IgE (KU/L)*	9.7 ± 1.4	205.4 ± 37.2	214 ± 23.7

Abbreviations: AP, allergic patients; HD, healthy donors.
*Mean ± SEM

2.3 | Cell isolation and culture

Purified human neutrophils were isolated as previously described.[14] Neutrophil preparations were further purified by MACs using the neutrophil isolation kit (Miltenyi Biotec SL, Madrid, Spain) following the manufacturer's instructions. The purity of the neutrophil preparations was on average >99%. Human basophils were purified using the Basophil Isolation Kit II (Miltenyi Biotec SL, Madrid, Spain) following the manufacturer's instructions. Neutrophils were cultured in RPMI 1640 medium supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin and maintained at 37°C in an atmosphere of 95% O_2 and 5% CO_2. For the stimulation treatments, neutrophils were incubated with 1 μg/mL Alt a1 Ag for 30 minutes. Inhibitors or medical drugs were added to the culture 1 hour prior to the addition of the Ag. 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.

2.4 | Analysis of histamine release

Histamine was measured in the culture supernatant (5×10^6 cells/300 μL) by ELISA (Beckman Coulter-IZASA) according to the manufacturer's instructions.

2.5 | Analysis of DAO and HDC mRNA expression by real-time PCR

Total RNA was isolated from highly purified neutrophils and basophils using TRIzol reagent (Invitrogen Thermo Fisher, CA, USA) and further purified using the RNeasy mini kit (Qiagen, CA, USA) including a DNAse I treatment. cDNA was prepared from 1 μg total RNA using random hexamers (Promega, WI, USA) and Superscript III first-strand synthesis system (Invitrogen). Quantitative PCR was performed on the StepOne system (Applied Biosystems, Invitrogen) using TaqMan probes and primers for human L-HDC (Hs01113381_g1), DAO (Hs00175631_m1), and rRNA18S (Hs03003631_g1) genes (Applied Biosystem, Invitrogen). Relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method and the housekeeping gene rRNA18S for normalization.

2.6 | Analysis of HDC and DAO by flow cytometry

For intracellular detection of HDC and DAO by flow cytometry, human neutrophils (2×10^6 cells) were incubated with α-CD66b-FITC Abs, or the mouse isotype control Ab. Cells were then fixed for 30 minutes and permeabilized for 20 minutes by the Fix and Perm cell permeabilization kit (Thermo Fisher Invitrogen) following the manufacturer's instructions. Samples were incubated with primary antibodies against intracellular antigens (unconjugated rabbit α-human HDC or PE-conjugated rabbit α-human DAO) for 1 hour at 4°C. Cells were then washed with PBS and incubated for 30 minutes at 4°C with PE-conjugated goat α-rabbit IgG. Cells were then washed, resuspended in PBS, and analyzed by flow cytometry (Coulter- IZASA, Barcelona, Spain). Secondary antibodies were used as isotype control when primary Abs were omitted.

2.7 | Protein extraction and Western blot analysis

10^7 cells were lysed for 30 minutes in an ice-cold buffer containing 20 mM Tris-HCl, pH 7.4, 50 mM β_2-mercaptoethanol, 2 mM EDTA, 25 mM NaF, 1% NP-40, 1% Triton X-100, 2 mM diisopropylfluorophosphate (DFP), supplemented with a protease and phosphatase inhibitor cocktail mix (Sigma-Aldrich). Cells were then sonicated (1 pulse, 50% amplitude) and centrifuged to remove insoluble debris. For the analysis of PI-3K and MAPKs, cells were left untreated for 18 hours to reduce basal phosphorylation levels usually found in our preparations. 80 μg of total proteins were separated on 4%-12% NuPAGE gradient gels containing SDS (Thermo Fisher Invitrogen) and transferred to nitrocellulose membranes using a semi-dry Trans-Blot unit (Bio-Rad). Blots were then blocked for 2 hours at RT with 3% blocking reagent (Thermo Fisher Invitrogen) and 3% BSA in TBS-T and then probed overnight at 4°C with rabbit α-phosphor-p38MAPK (Thr180/Tyr182) (Cell signaling Tech-IZASA), mouse α-phospho-ERK1/2 (Thr202/Tyr204) (Cell signaling), rabbit α-phopsho-PI3K p85/p55 (Tyr458, Tyr199) (Invitrogen) or chicken α-β-actin (Abcam, UK) at 1:1,000 dilution in blocking reagent. Primary antibodies were visualized using donkey α-mouse (1:5,000), donkey α-rabbit (1:5,000) (Promega, Madison, WI), or goat α-chicken horseradish (HRP)-conjugated secondary Abs (1:2,000) (Abcam). Chemiluminescence was detected using the ECL™ Prime Western Blotting System (Sigma-Aldrich).

2.8 | CaN phosphatase assay

CaN phosphatase activity was measured as previously described.[15] Reaction mixtures containing 2 μM ^{32}P-labeled phosphopeptide corresponding to a segment of the RII subunit of cAMP-dependent kinase[16] and 80 μg of whole-cell extract

were incubated in a total volume of 60 μL of assay buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 6 mM $MgCl_2$, 0.5 mM DTT, 0.1 mM $CaCl_2$, 0.5 μM okadaic acid, and 0.1 mg/mL bovine serum albumin) for 15 minutes at 30°C. The reactions were stopped by the addition of 500 μL of 100 mM potassium phosphate buffer, pH 7.0, containing 5% trichloroacetic acid. The reaction mixtures were passed through columns (500 μL) of activated Dowex cation-exchange resin, and free radiolabeled inorganic phosphate (^{32}Pi) was quantified in the eluates by scintillation counting. Linearity of the assays after 15 minutes of reaction was adequately verified. Assays were performed in triplicate, and the c.p.m., measured in blank assays lacking neutrophil lysate, was subtracted. Data were expressed as the number of pmol of inorganic phosphate released per minute per mg of protein.

2.9 | Statistical analysis

All statistical analyses were performed using GraphPad Prism 4.00 for Windows (GraphPad Software, San Diego, CA, USA). Pair-wise comparisons were made using a Student's *t* test (two tailed). For non-parametric comparisons, the Mann-Whitney *U* test was used. Data are expressed as means ± SEM. A level of $P < .05$ was considered significant. $*P < .05$; $**P < .01$; $***P < .001$.

3 | RESULTS

3.1 | Cell signaling pathways involved in Ag-dependent histamine release by human neutrophils

One of the first steps in the phagocyte activation is an increase in PI3K-dependent phosphoinositides.[17] The mitogen-activated protein kinases (MAPKs) p38 and extracellular signal-regulated kinase 1/2 and calcineurin/phosphatase 2B are also major signaling pathways in different neutrophil functions.[18] We explored the possible contribution of these signaling pathways to elucidate the molecular mechanisms underlying the Ags-dependent histamine production. To this purpose, neutrophils from allergic patients sensitized to the major Ag(s) present in an extract of the fungus *Alternaria alternata* (Alt a1) were treated for 30 minutes with this Ag. We subsequently analyzed the phosphorylation levels of p85 subunit of PI3K, p38, and ERK1/2 MAPKs by Western blotting using phospho-specific Ab. Our experiments indicated that the p85 subunit of the PI3K complex was phosphorylated and thereby activated in response to Alt a1, but not in response to an extract of *Olea europaea* (T_9), to which the patients were not sensitized (Figure 1A). The same effect was observed for p38 and ERK1/2 MAPKs (Figure 1B,C, respectively).[19] To

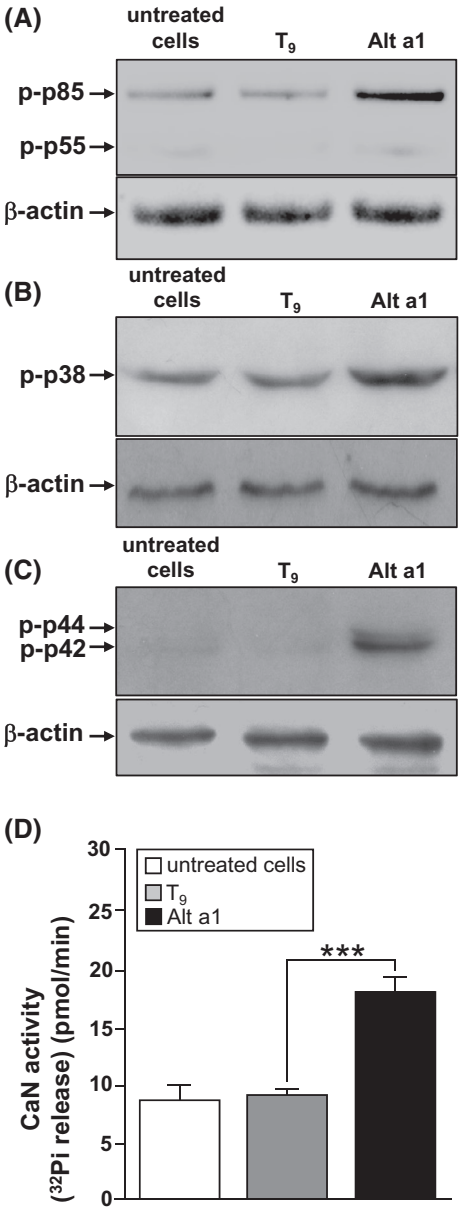


FIGURE 1 Cell signaling pathways activated in response to Alt a1 Ag. A-C, Neutrophils from allergic patients sensitized to Alt a1 were left untreated or treated with 10 μg/mL T_9 (to which the patients were not sensitized), or with 1 μg/mL Alt a1 for 30 minutes. Phosphorylated PI3K (p-p55 and p-p85), p38 MAPK (p-p38), and ERK1/2 (p-p42 and p-p44) were subsequently detected. β-actin was used as loading control. The results shown in each panel are representative of three separate experiments with similar results. D, Neutrophils from allergic patients sensitized to Alt a1 were left untreated or treated with 10 μg/mL T_9 (to which the patients were not sensitized) or with 1 μg/mL Alt a1 for 30 minutes. After cell lysis, CaN activity was analyzed. Values are the means ± SEM from four separate experiments performed in triplicate. $***P < .001$

study the activation of the phosphatase calcineurin, we assayed its enzymatic activity after 30-min treatment with Alt a1. We found that Alt a1, but not T_9, induced a strong activation of calcineurin in neutrophils from patients sensitized

to this Ag but not to T_9 (Figure 1D). These results are consistent with our previous findings describing the activation of both pathways in human neutrophils by an IgE-dependent mechanism.[14,19] To further evaluate the involvement of these signaling pathways in the Ag-dependent histamine production, we examined the effect of different kinases and phosphatase inhibitors: wortmannin (PI3K inhibitor[17]), SB20358 (p38 MAPK[20]), PD098059 (MEK inhibitor, the upstream kinase of ERK[21]), and cyclosporin A (CsA, a specific inhibitor of calcineurin[22]). The action of these compounds was tested by pretreatment of neutrophils from Alt a1 allergic patients, prior to the stimulation with Alt a1 Ag. We analyzed the resulting histamine levels in the culture supernatant by ELISA. Our experiments revealed that wortmannin (at doses from 1 to 100 nM), and SB203580 and PD098059 (both at doses from 0.1 to 10 μM) significantly prevented the histamine release in a dose-dependent manner (Figure 2A-C). Conversely, CsA, even at a high dose of 1 μg/mL, only partially decreased the levels of histamine release in response to Alt a1 (Figure 2D).

3.2 | Effect of common therapeutic drugs on the Ag-dependent histamine release

Anti-histamines,[23] corticosteroids,[6,10,11,24] and bronchodilators (long-acting M3-muscarinic receptor antagonists[25] and long-acting β_2-adrenoceptor agonists)[26,27] are effective drugs against allergic symptoms. Their common use led us to investigate whether they can modulate the Ag-induced histamine release. We first analyzed the effect of loratadine, a classic second-generation H_1-anti-histamine. To test this drug, we preincubated neutrophils from allergic patients sensitized to Alt a 1 with different doses of loratadine prior to de addition of the Ag. We then quantified the histamine production in the culture supernatant. 1 and 10 μM concentrations of this drug decreased histamine release in response to Alt a1 in a dose-dependent manner (Figure 3A). However, concentrations lower than 1 μM did not have any significant effect on histamine production. We next tested the action of budesonide (a glucocorticoid) and tiotropium (a long-acting M3-muscarinic

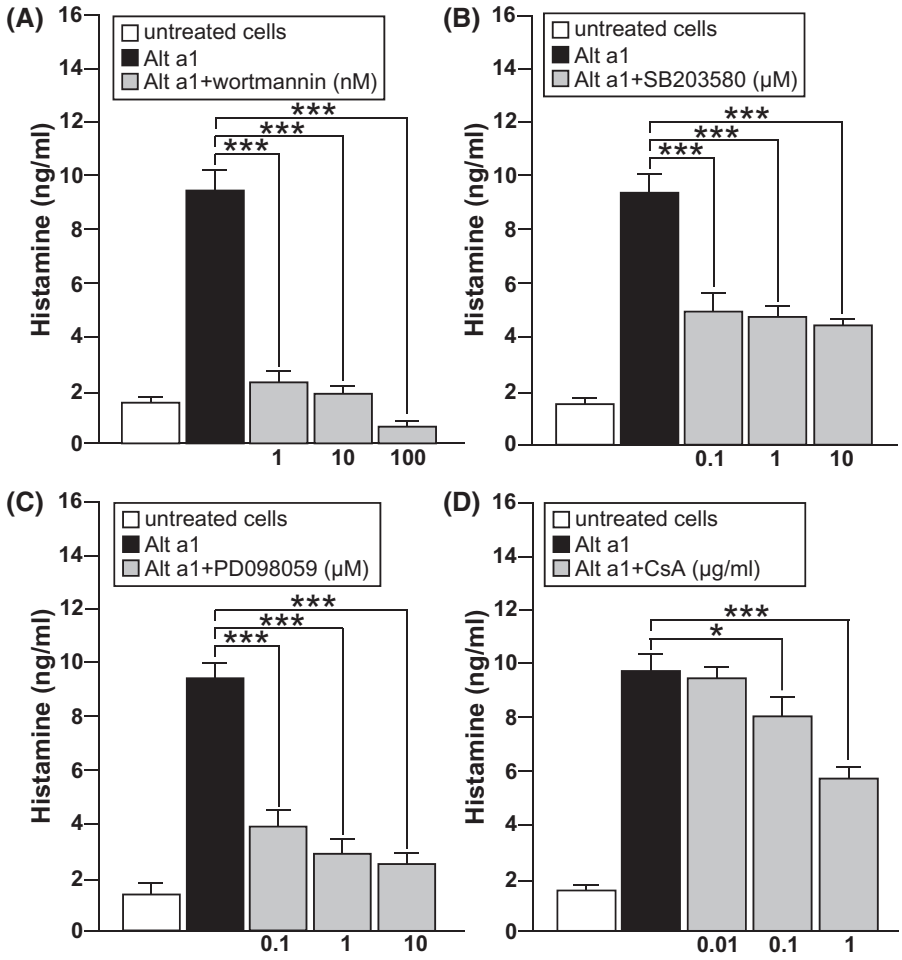


FIGURE 2 Cell-signaling pathways involved in the Ag-dependent histamine production. A-D, Neutrophils from allergic patients sensitized to Alt a1 (n = 10) were pre-incubated for 1 hour with vehicle (untreated cells) or with wortmannin (A), SB203580 (B), PD098059 (C) or cyclosporin A (CsA) (D) at the indicated doses. Cells were left untreated or treated with 1 μg/mL Alt a1 for 30 minutes. We measured histamine release in the culture supernatant by ELISA (for calculation see Material and methods). Values are the means ± SEM of four independent experiments in which each measurement was performed in triplicate. *$P < .05$ and ***$P < .001$

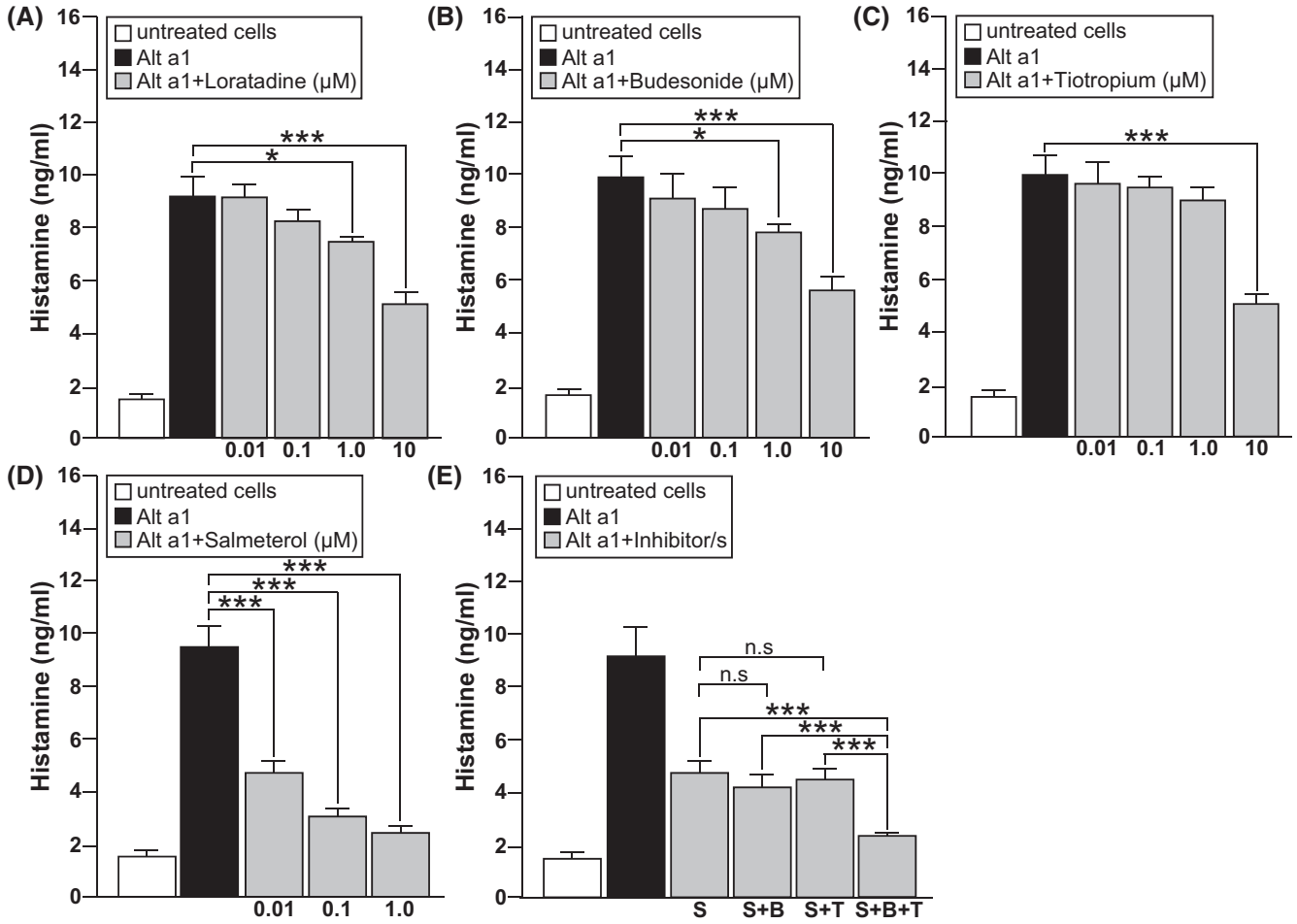


FIGURE 3 Effect of medical treatments in the Ag-dependent histamine production. A-D, Neutrophils from allergic patients (n = 10) sensitized to Alt a1 were pre-incubated for 1 hour with vehicle (untreated cells) or with loratadine, budesonide, tiotropium, or salmeterol at the indicated doses (μM). E, Cells were pre-incubated for 1 hour with vehicle or 0.01 μM salmeterol (S), 0.01 μM salmeterol plus 1 μM budesonide (S + B), 0.01 μM salmeterol plus 1 μM tiotropium (S + T), or 0.01 μM salmeterol plus 1 μM budesonide plus 1 μM tiotropium (S + B + T). Then, neutrophils were left untreated or incubated with 1 μg/mL Alt a1 for 30 minutes. Histamine release was measured in the culture supernatant by ELISA. The results shown are the means ± SEM four independent experiments in which each measurement was performed in triplicate. *$P < .05$ and ***$P < .001$. n.s: non-significant

antagonist). Whereas budesonide displayed a similar pattern than loratadine, with a significant inhibitory effect at doses of 1 μM and 10 μM (Figure 3B), tiotropium was only effective at the highest dose (10 μM) (Figure 3C). We also examined the effect of salmeterol, a long-acting β_2-agonist. This compound also caused a dose-dependent inhibition on Ag-dependent histamine release (Figure 3D). Our experiments show that the effect of salmeterol was particularly strong, by ~10^4 fold stronger than the other drugs, hampering histamine production to concentrations as low as 0.01 μM. Current medical practice guidelines for patients suffering non-controlled asthma (eg, Global Initiative for Asthma) recommend a triple therapy consisting on the administration of long-acting β-agonists together with glucocorticoids and long-acting muscarinic antagonists.[28] In this context, we performed the same experiments using combinations of the different drugs. The inhibitory action of a suboptimal dose of salmeterol (0.01 μM) was tested in the presence of maximal doses (10 μM) of either budesonide, or tiotropium or a combination of both budesonide and tiotropium. The ability of salmeterol (0.01 μM) to reduce the Ag-induced histamine production by ~50% remained unchanged when used together with budesonide or tiotropium (Figure 3E). However, the cocktail combining all three compounds resulted on a decrease of ~75% in histamine levels.

3.3 | Effect of Ag-specific IT on the Ag-dependent histamine release. Role of HDC and DAO

It is well known that IT inhibits histamine release by human basophils,[29] but no similar studies have been hitherto reported in human neutrophils. To investigate common

regulatory links between both cell types, we analyzed the histamine release in response to Alt a1 in neutrophils from three groups of subjects: non-IT Alt a1 allergic patients, IT-treated Alt a1 allergic patients, and healthy donors. Our analysis showed that the group of IT-treated allergic patients experienced a ~70% reduction of histamine release induced by Ag, compared with the non-IT-treated patient group. However, histamine released by neutrophils from IT-treated patients did not reach similar basal levels of healthy donors, where the Ags were ineffective (Figure 4A). We reported previously higher HDC mRNA and protein expression in neutrophils from allergic patients than those from healthy subjects.[12] Thus, we analyzed by real-time PCR whether the modulating effect of the IT on the production of histamine was coupled with changes in HDC expression. The levels of HDC mRNA were lower in neutrophils from IT-treated patients than in neutrophils from non-IT-treated patients. However, the levels of HDC mRNA in neutrophils from non-IT-treated patients did not reach the basal levels found in healthy subjects, in line with our previous findings[12] (Figure 4B).

We also analyzed the expression of HDC protein by flow cytometry. Whereas 98% of the $CD66b^+$ (neutrophils) were HDC^+ in the non-IT-treated group (Figure 4C), only 18% and 12% were HDC^+ in the IT-treated group (Figure 4D) and in the healthy donors (Figure 4E), respectively. Statistical analysis showed that IT significantly reduced the HDC expression levels in neutrophils from the IT-treated group as compared to the non-IT-treated group ($P < .001$). Although we observed a trend for a higher expression in IT-treated patients *vs* healthy subjects, the differential values did not reach statistical significance ($P = .056$) (Figure 4F).

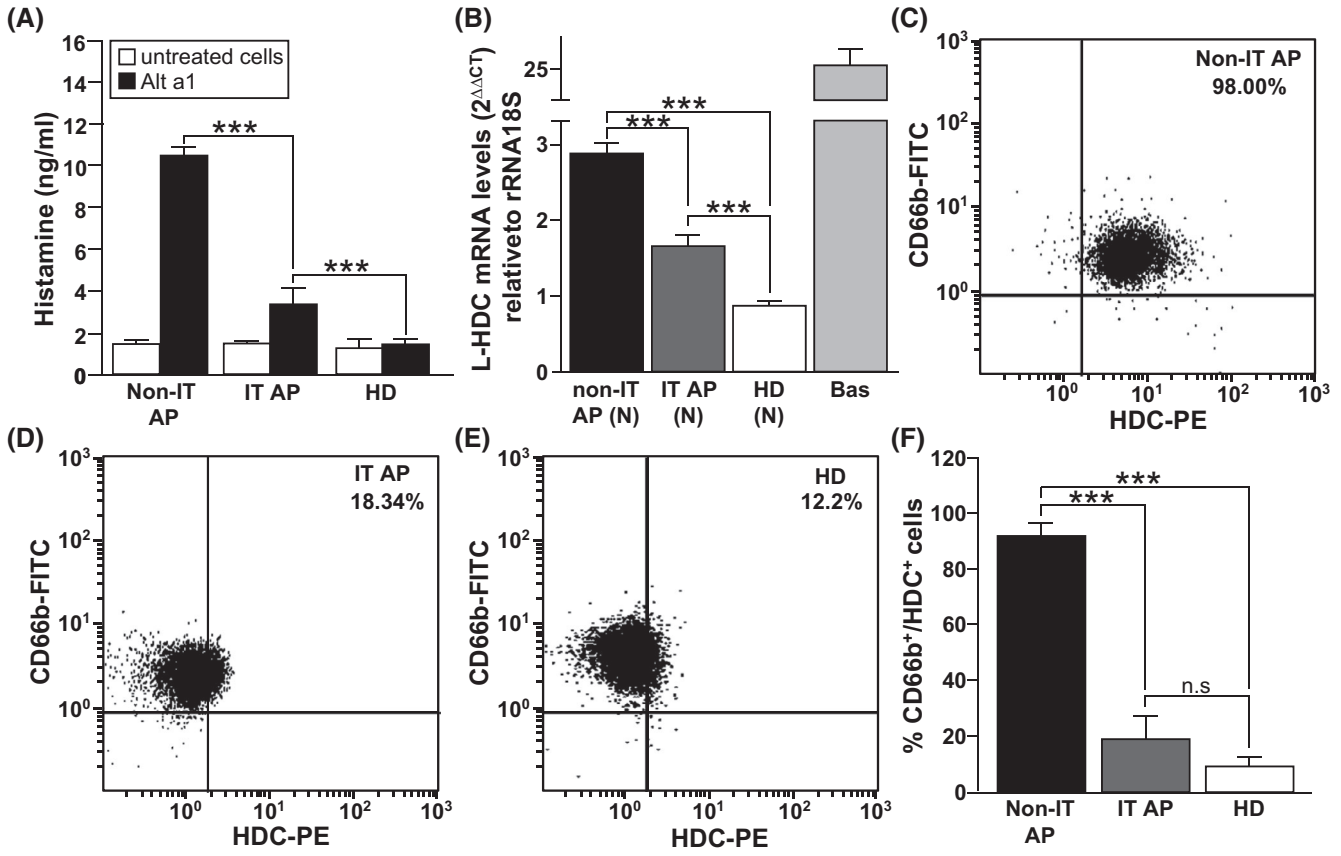


FIGURE 4 IT decreases histamine release and downregulates the Ags-induced HDC expression. A, Neutrophils from non-IT-treated allergic patients sensitized to Alt a1 (Non-IT AP; n = 10), IT-treated allergic patients sensitized to Alt a1 (IT AP; n = 15) or healthy donors (HD; n = 10) were left untreated or incubated with 1 μg/mL Alt a1 for 30 minutes. Histamine release was measured in the culture supernatant by ELISA. Values are the means ± SEM four independent experiments in which each measurement was performed in triplicate. ***$P < .001$. B, HDC mRNA expression analyzed by real-time PCR in neutrophils from non-IT-treated allergic patients sensitized to Alt a1 (non-IT AP (N)), IT-treated allergic patients sensitized to Alt a1 (IT AP (N)) or healthy donors (HD (N)). We standardized the expression against the housekeeping gene rRNA18S. mRNA from basophils (Bas) was used as positive control. Values are the mean values ± SEM quantified in triplicated (n = 5/group). C, Neutrophils from non-IT-treated allergic patients (Non-IT AP) sensitized to Alt a1, (D) IT-treated allergic patients (IT AP) sensitized to Alt a1 or (E) healthy donors (HD) were analyzed by flow cytometry for CD66b-FITC and HDC-PE expression. Representative dot plots indicating the percentage of double-positive cells are shown. F, Statistical analysis of the HDC expression within the three groups (n = 10/group). Values are the mean of percentage of double-positive cells ± SEM. ***$P < .001$. n.s: non-significant

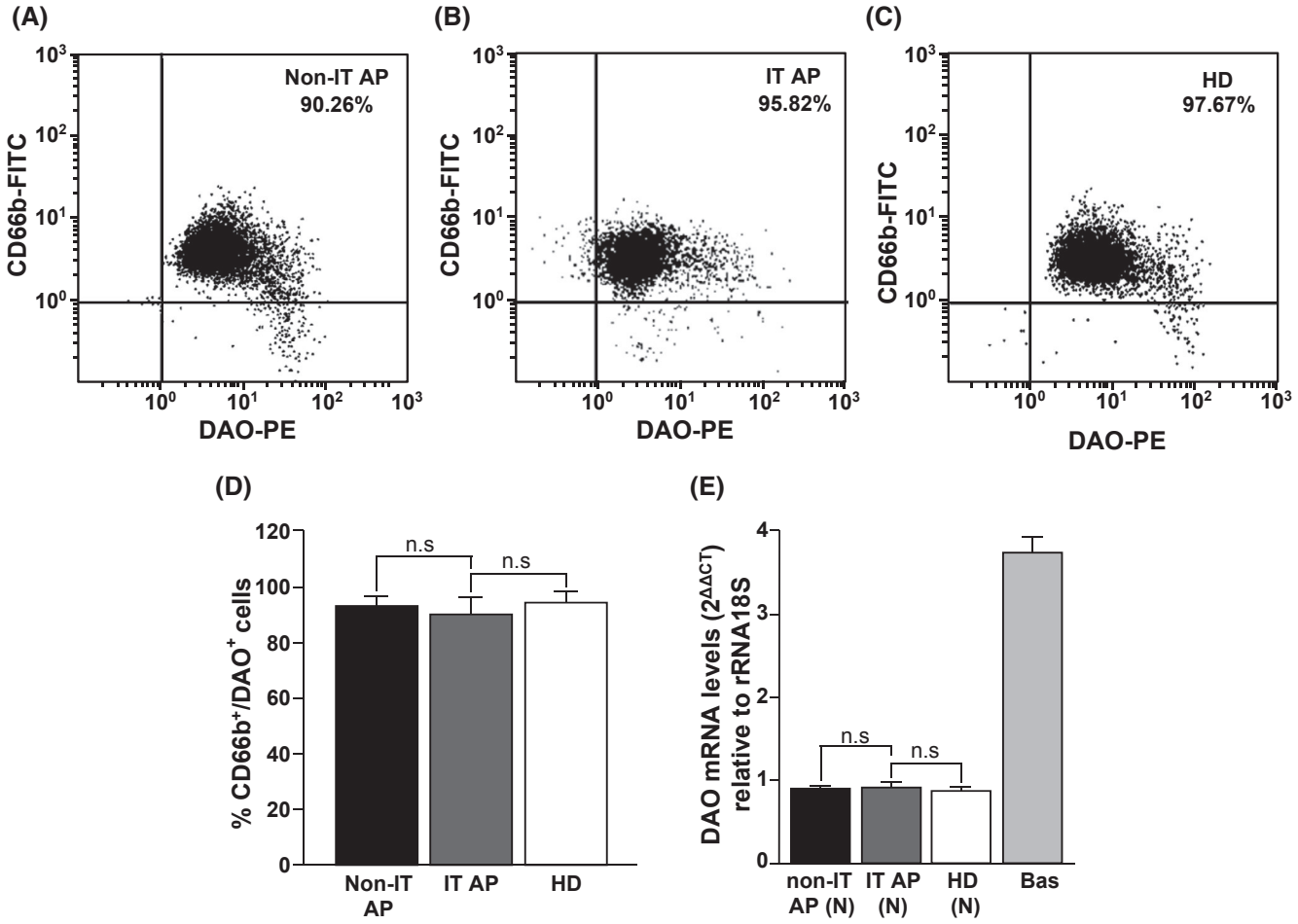


FIGURE 5 IT treatment has no effect on DAO expression by human neutrophils. A, Neutrophils from non-IT-treated allergic patients sensitized to Alt a1 (Non-IT AP), (B) IT-treated allergic patients sensitized to Alt a1 (IT AP) or (C) healthy donors (HD) were analyzed by flow cytometry using α-CD66b-FITC and α-DAO-PE. Representative dot plots indicating the percentage of double-positive cells are shown. D, Statistical analysis of DAO expression by the three groups (n = 8/group). Values are the mean of percentage of double-positive cells ± SEM. E, DAO mRNA expression was analyzed by real-time PCR in neutrophils from non-IT-treated allergic patients sensitized to Alt a1 (non-IT AP (N)), IT-treated allergic patients sensitized to Alt a1 (IT AP (N)), and healthy donors (HD (N)). We standardized expression against rRNA18S. mRNA from basophils (Bas) was used as positive control. Values are the mean ± SEM quantified in triplicates (n = 5/group). n.s: non-significant

The expression of DAO, the enzyme responsible of histamine degradation, is increased by IT in basophils and it is associated with a reduction of responsiveness in allergic patients.[30] To explore whether the same effect occurs in human neutrophils, we analyzed the expression of DAO by flow cytometry in the 3 subject groups. Although we observed high levels of DAO protein expression in $CD66^+$ cells from the three groups (Figure 5A-C), we found no significant differences between them ($P = .08$) (Figure 5D). Additional analysis of DAO mRNA expression by real-time PCR showed similar results (Figure 5E).

4 | DISCUSSION

It is largely accepted that histamine plays a central role in the pathogenesis of allergic diseases, where tissue mast cells and circulating basophils are traditionally considered the major producers of histamine.[5] We have previously reported that neutrophils from allergic patients also produce and store histamine, which is released in response to Ags to which they are sensitized.[12] Neutrophils are the most abundant circulating leukocytes and the first recruited cells to the site of an allergic reaction, where they perform an inflammatory function.[31,32] Although histamine content in neutrophils is three times less than in basophils and mast cells,[12] it may also contribute to the development and maintenance of allergic inflammation. Therefore, we sustain that it is important to understand the intracellular signaling pathways involved in the histamine released by Ags in human neutrophils. Our results should be taken into consideration in order to determine whether current therapeutic drugs and approaches are capable of modulating histamine production. Our work explores possible molecules involved in this

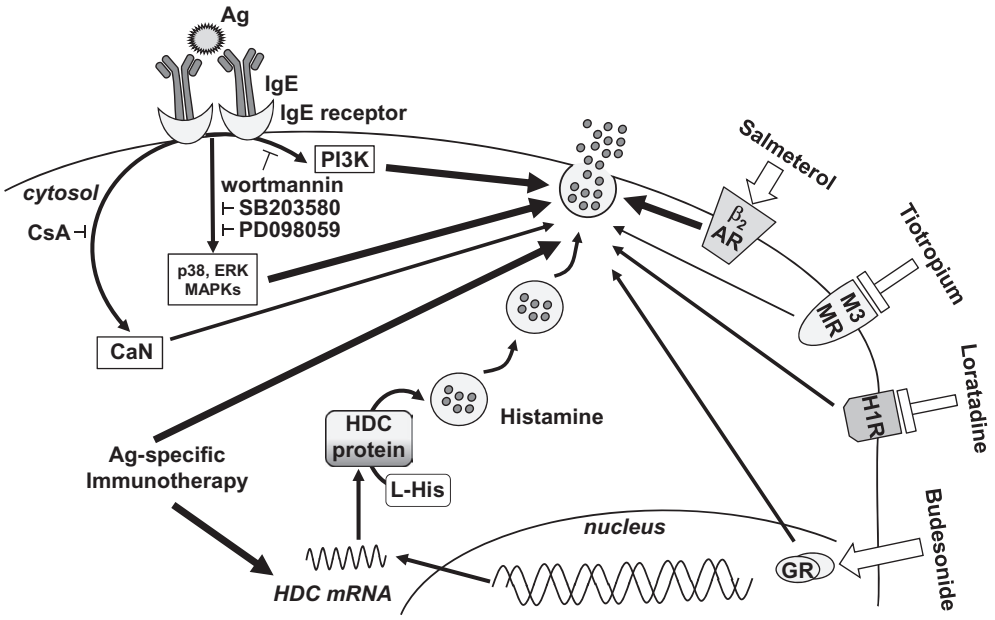


FIGURE 6 Schematic summary of the signaling pathways involved in the Ag-induced histamine release by human neutrophils. Modulation by therapeutic medical drugs and Ag-specific IT. The binding of an Ag to the specific IgE/IgE receptors complex on the neutrophil surface initiate a cascade of events activating the PI3K, MAPKs (p38 and ERK), and phosphatase CaN that lead to the histamine release. The effect of anti-histamines, glucocorticoids, bronchodilators (M3 antagonists and β2 agonists), and Ag-specific is also shown. Note that the thickness of the arrows represents the relative contribution of each pathway/drug to histamine production

process, suggesting a significant role for PI3K, p38, and ERK 1/2 MAPKs (see scheme in Figure 6). These proteins are activated in response to Ags, and their targeted inhibition canceled the Ag-dependent histamine release. We published previous reports that support these data, where PI3K, p38, and ERK 1/2 MAPKs are involved in the Ags and α-IgE-dependent downmodulation of L-selectin,[33] NF-κB activation, COX-2 expression,[19] and MMP-9 release[34] in human neutrophils. Other studies also showed the involvement of the PI3K in the production of histamine by Toll like receptor-activated mouse neutrophils[35] or by α-IgE-, IL-3-, and GM-CSF-activated human basophils.[36] Regarding MAPKs, the α-IgE-mediated histamine production by human basophils depends exclusively on p38 MAPK.[37,38] However, in line with our findings, in mouse macrophage line RAW 264.7 activated by TPA or thapsigargin, the histamine production depends on both p38 and ERK1/2.[39] In addition to these pathways, we also demonstrate that Ag challenge enhanced the activity of CaN phosphatase in neutrophils from allergic patients. Although we have previously described the involvement of this enzyme in the α-IgE-mediated NFAT activation and IL-8 release,[14,40] we now show evidence suggesting that histamine production is only under its partial control. By contrast, CaN is fully required for the production of histamine in other cells, such as human mast cells[41] or human basophils.[42]

The symptoms of allergic diseases are generally treated with three different pharmacological approaches: first, blocking the action of preformed mediators (anti-histamines); second, inhibiting or enhancing the generation of proinflammatory or anti-inflammatory mediators (glucocorticoids); and third, preventing the effect on target organs as airways with bronchodilators (anti-M3 muscarinic receptor antagonists and β-2 adrenoceptor agonists). Human neutrophils express histamine and glucocorticoid receptors.[43,44] Through them, the anti-histamines and glucocorticoids control multiple phenomena of the neutrophil physiology such as cell migration, cellular activation, respiratory burst, and apoptosis.[45,46] In addition, both therapeutic agents are able to regulate the Ag-dependent O_2^- and IL-8 production by neutrophils from allergic patients (PC, AV, and JM Unpublished data). In this work, we extend the modulating properties of anti-histamines and glucocorticoids, illustrating that the anti-histamine loratadine and the glucocorticoid budesonide also prevent, at least partially, Alt a1-dependent histamine release (Figure 6). Other studies have indicated a similar effect for loratadine and budesonide in decreasing the production of histamine in Ag/α-IgE-activated human basophils[10] and in Ag-activated rat and human mast cells.[11,47] Similarly, M3-muscarinic and β_2-adrenergic receptors are detectable on circulating neutrophils[48,49]; their respective inactivation (M3 receptors) or activation (β_2 receptors) leads to anti-inflammatory actions, including a downregulation of both cytokine production and chemotactic activity.[50-53] In this context, we show that Alt a1-induced histamine production is only partially decreased by high concentrations of the

muscarinic antagonist tiotropium. This result contrasts with previous reports describing the lack of response to such drug in Ag-activated human mast cells.[54] Conversely, β_2-agonist salmeterol was, among all tested drugs, the most effective inhibitor in hampering histamine release in response to Ags. Interestingly, its effect, previously reported in α-IgE-stimulated human and pig mast cells,[9,55] was enhanced in combination with budesonide and tiotropium. Taken together, these results suggest that β_2 agonists may play a master regulator role on signaling events operating in histamine production by human neutrophils. Conversely, muscarinic antagonists and glucocorticoids may only partially control these events or might act upon different putative ones in a synergistic fashion.

Another therapeutic approach that modifies allergic diseases progression is IT, which provides long-term clinical benefits.[56] The molecular mechanisms regulated by IT are not fully elucidated. To this end, our laboratory has performed several studies about the effect of IT in non-IgE and IgE-mediated neutrophils functions. For instance, we found that IT inhibits responses elicited by lipopolysaccharide (LPS) or fMLP, including NF-κB activation, IL-8/TXA_2 production,[57] and myeloperoxidase (MPO) release.[58] IT also reduces the MPO release,[59] respiratory burst,[60] L-selectin shedding,[33] and upregulation of HLA-DR[61] promoted by Ags. We now present evidence that IT is capable of reducing Ag-induced histamine release in neutrophils. Our data matches previous studies in Ag-activated human basophils.[29] In these cells, the IT effect is mediated by an upregulation of DAO expression.[30] To date, DAO expression has been described in colon, small bowel, kidney, placenta,[1] mast cells, and basophils.[62] Here, we provide first proof of DAO mRNA and protein expression in human neutrophils and show that, unlike reported in basophils, their levels remain unchanged after IT treatment. By contrast, we do show that IT downregulates HDC mRNA, the key enzyme for histamine synthesis. While HDC mRNA is upregulated in neutrophils from rodents exposed to *Mycoplasma pulmonis*,[63] here we report for the first time that IT is capable of downregulating its expression. Our results pinpoint histamine and HDC expression as key targets for IT and propose the potential use of the histamine produced by human neutrophils as a predictive marker for the IT success.

A detailed identification of the molecular mechanisms involved in allergic processes is required for a full understanding of the processes leading to inflammation. Here, we reveal novel signaling pathways controlling histamine release in human neutrophils from allergic patients, and we establish the most effective therapeutic strategies to modulate histamine production in these cells. We believe that future studies targeting these pathways will improve the effectiveness of current therapies.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

P. Chacón and A. Vega-Rioja designed and performed most of the research, analyzed the data, and helped with the preparation of the manuscript. B. Doukkali and L. Alcañiz performed some research. V. Bellido and Y. Puente were in charge of the diagnosis, selection, and recruitment of the patients. D. Rodríguez, R. Palacios, and J.A. Cornejo-García analyzed the data and wrote the manuscript. J. Monteseirín designed the research, analyzed the data, and wrote the manuscript. A. del Valle Rodriguez and D. Rivas-Pérez analyzed the data and wrote the manuscript.

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