

House dust mite allergen activates human eosinophils via formyl peptide receptor and formyl peptide receptor-like 1

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The objective was to evaluate which receptors house dust mite (HDM) and birch pollen extracts engage to activate human eosinophils. Chemotaxis and degranulation were studied in eosinophils pretreated with pertussis toxin and other antagonists of G protein-coupled receptors, *e.g.* the formyl peptide receptor (FPR), CC chemokine receptor 3 (CCR3) and leukotriene receptor B4 (LTB₄R). Inhibition of the FPR as well as desensitization of the receptor rendered eosinophils anergic to activation by the allergens. Blockade of CCR3 or LTB₄R did not affect eosinophilic reactivity. It was determined by PCR that human eosinophils express the FPR family members FPR and FPR-like 1 (FPRL1). HDM, unlike birch pollen, evoked calcium fluxes in HL-60 cells transfected with FPR or FPRL1. Although both allergens gave rise to calcium transients in neutrophils, which also express FPR and FPRL1, only the HDM response was decreased by the FPR antagonist. Moreover, neutrophils migrated toward HDM but not to birch pollen. Eosinophils pretreated with inhibitors of MAPK p38, ERK1/2 or protein kinase C exhibited diminished responsiveness to the aeroallergens. This study indicates that FPR and FPRL1 mediate the activation of eosinophils by HDM, whereas birch pollen employs other pathways shared with FPR to activate human eosinophils.

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Abbreviations: **boc-MLP:** N-t-butoxycarbonyl-Met-Leu-Phe ·

CCR3: chemokine receptor 3 · **CsH:** cyclosporin H ·

EPO: eosinophil peroxidase · **FPR:** formyl peptide receptor ·

FPRL1/2: formyl peptide receptor-like 1/2 · **FMLP:** N-formyl-

Met-Leu-Phe · **GPCR:** G protein-coupled receptor · **HDM:** house dust mite · **KRG:** Kreb's Ringer glucose · **LTB₄R:** leukotriene B4 receptor · **MBP:** major basic protein · **PAF:** platelet-activating factor · **PTX:** pertussis toxin

Introduction

The eosinophils belong to the polymorphonuclear family of leukocytes called granulocytes and are in healthy humans mainly found in the gut and, to a lesser extent, in the respiratory and genitourinary tracts. Although the physiological function of the cells is incompletely elucidated, it is likely that they survey mucosal surfaces that are exposed to a hostile exterior environment. In developing countries, the most important role of eosinophils is to defend the host against helminths

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and other parasites, whereas in the developed part of the world they are more commonly associated with allergic disorders.

It is unclear why eosinophils predominate in allergic inflammatory foci, as opposed to other innate immune cells such as monocytes and neutrophils. Part of the answer may be that eosinophils appear to have a particular affinity for inhalant allergens. We and others have recently demonstrated that environmental allergens can directly activate eosinophils isolated from healthy, non-allergic individuals [1–3]. Inhalant allergens such as house dust mite (HDM), birch and grass pollens, cat dander and cockroach extracts trigger chemotactic behavior, up-regulation of cell surface-associated markers and degranulation in eosinophils [1–3] and neutrophils [4] derived from subjects devoid of allergic symptoms.

The bioactive components of the allergen extracts responsible for the activation of eosinophils appear to be distinct from the proteins to which B and T cells are sensitized. We found that whole HDM extract, but not the purified protein Der p 2, gave rise to chemotactic activity in eosinophils [1]. Similarly, Plötz *et al.* [2] could not measure eosinophilic chemotaxis toward Phl p 5b, but to grass pollen extract. In fact, the group of Behrendt have identified linoleic and α -linolenic acid to be the components of the pollen extracts that mediate the activation of eosinophils, and that the receptor employed by these lipid mediators is the leukotriene B₄ receptor (LTB₄R) [2].

In the present study, we have attempted to establish which receptors are engaged by two common inhalant allergens, HDM and birch pollen, in the activation of human eosinophils. To this end, we have used specific antagonists directed against G $\alpha_{i/o}$ protein-coupled receptors, *e.g.* pertussis toxin (PTX), two antagonists specific for the formyl peptide receptor (FPR), *i.e.* *N*-t-butoxycarbonyl-Met-Leu-Phe (boc-MLP) and cyclosporin H (CsH), and antibodies directed against two other G protein-coupled receptors (GPCR), namely the CC chemokine receptor 3 (CCR3) and the LTB₄R. We chose to examine the role of FPR family members in the activation of eosinophils by allergens because we have previously shown that this group of receptors appears to be implicated in the activation of human eosinophils by both exogenous danger signals such as a D-amino acid-containing peptide (WKYMVm) [5] and various bacterial species [6] as well as endogenous danger signals, *e.g.* necrotic epithelial cells [7]. Finally, we investigated the involvement of different kinases in the activation of eosinophils by allergens.

Results

Allergens use GPCR to activate eosinophils

Human eosinophils responded to stimulation with HDM or birch pollen extract with secretion of the granule protein eosinophil peroxidase (EPO) (Fig. 1A). In contrast, eosinophils pretreated with PTX were unable to release EPO upon stimulation with either birch or HDM allergen extracts (Fig. 1A), indicating that this process was dependent on the activation of GPCR. Platelet-activating factor (PAF), a secretagogue of eosinophils, was included as a positive control. It activates eosinophils *via* the PTX-sensitive GPCR PAF receptor. As expected, PTX blocked the PAF-induced EPO response (Fig. 1A).

Blockade of the FPR abrogates allergen-induced degranulation of eosinophils

To determine if a particular GPCR, the FPR, was involved in mediating the allergen-induced EPO release, we pretreated eosinophils with the FPR antagonist CsH. The EPO release evoked by HDM was inhibited by CsH (Fig. 1B), as was the birch allergen-induced EPO degranulation (Fig. 1B). Moreover, eosinophils pretreated with CsH were unable to release another granule protein, major basic protein (MBP), upon stimulation with HDM (Fig. 1C). The release of MBP in response to birch allergen was not tested since we have previously shown that birch pollen does not elicit MBP degranulation in human eosinophils [1].

Blockade of the FPR diminishes eosinophilic migration toward allergens

CsH completely abolished eosinophilic migration toward HDM and attenuated the migratory response to birch extract by 78% (Fig. 2). Spontaneous eosinophilic migration was not affected by pretreatment with CsH: The mean migratory index (± 1 SEM) in pretreated cells was $1.4 \pm 0.1\%$ compared to $1.5 \pm 0.3\%$ in untreated cells. To ensure that the inhibitory effect of CsH was specific, we pretreated eosinophils with another antagonist of the FPR, boc-MLP. Once again, a significant inhibition of the migration of eosinophils toward HDM (98%) and birch (88%) was seen in thus treated cells (Fig. 2). The FPR-specific ligand *N*-formyl-Met-Leu-Phe (FMLP) was included as a positive control. As expected, its capacity to attract eosinophils was abolished by pretreatment of the cells with either of the

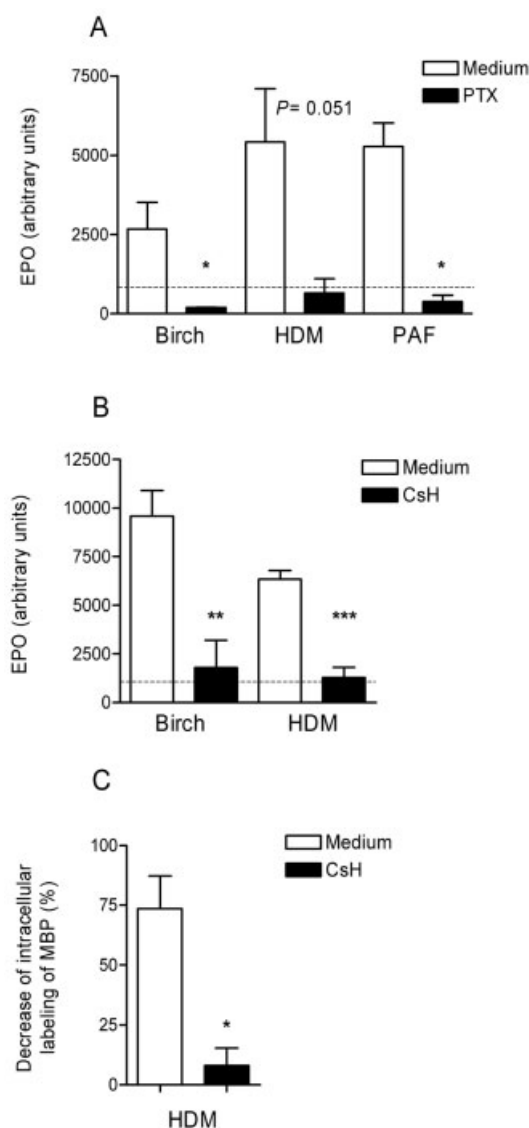


Figure 1. Capacity of eosinophils pretreated with PTX or the FPR antagonist CsH to inhibit the degranulation of eosinophils. (A) EPO release from eosinophils pretreated with medium (white bars) or PTX (200 ng/mL) (black bars) for 2 h at 37°C and then stimulated with either birch or HDM allergen extract (100 µg/mL), or PAF (10⁻⁷ M), *n* = 3 different eosinophil donors, * *p* < 0.05 versus medium. Dotted line = spontaneous release of EPO. (B) EPO release from eosinophils pretreated with medium (white bars) or CsH (10⁻⁶ M) (black bars) for 10 min at 4°C and then stimulated with allergen extracts (100 µg/mL), *n* = 5 different eosinophil donors, ** *p* < 0.01 versus medium, *** *p* < 0.001 versus medium. (C) MBP release from eosinophils pretreated with medium (white bars) or CsH (10⁻⁶ M) (black bars) and then stimulated with HDM (100 µg/mL), *n* = 3 different eosinophil donors, * *p* < 0.05 versus medium. All data are arithmetic means ± SEM.

two FPR antagonists (Fig. 2). We also included the eosinophil chemoattractants C5a and PAF as negative controls and found that neither of the FPR antagonists affected the migration of eosinophils toward these molecules (Fig. 2).

Neither CCR3 nor LTB₄R mediates chemotaxis of eosinophils toward allergens

We also examined whether two other GPCR of importance in attracting eosinophils to sites of allergic inflammation were involved in the migration of eosinophils toward allergen extracts, namely CCR3 and LTB₄R. The anti-CCR3 and anti-LTB₄R mAb chosen for this purpose were shown to be inhibitory, as reflected by their capacity to inhibit chemotaxis of eosinophils toward their natural agonists, e.g. eotaxin-1 and LTB₄, by 79% and 88%, respectively (Fig. 3A, B). However, neither of these two mAb was able to inhibit the migration of eosinophils toward the two allergen extracts (Fig. 3A, B). The migration of eosinophils toward FMLP was slightly affected by pretreatment of the cells with the anti-LTB₄R mAb.

Desensitization of the FPR decreases eosinophilic migration toward allergens

Whereas eosinophils that were pre-stimulated with FMLP were completely unable to migrate toward HDM (Fig. 4A), the migratory ability of FMLP-pretreated eosinophils in response to stimulation with birch allergen was reduced by 75% (Fig. 4A). In contrast, no such inhibitory effect was observed in eosinophils that were first desensitized with the CCR3 agonist eotaxin-1 and then exposed to either of the two allergen extracts (Fig. 4B). Further, HDM extract was unable to desensitize itself (Fig. 4C). Finally, when eosinophils were first exposed to HDM and then allowed to migrate toward FMLP, their migratory capacity was maintained (Fig. 4C).

Eosinophils express FPR and FPRL1 but not FPRL2

Since it has not been formally proven which FPR family members are expressed by human eosinophils [8], we set up a PCR method to investigate the matter. Total RNA derived from highly pure preparations of eosinophils, neutrophils and monocytes were amplified with the use of primer pairs specific for the genes encoding FPR, FPR-like 1 (FPRL1) and FPR-like 2 (FPRL2), as well as for the house-keeping gene GAPDH (Fig. 5). Single FPR cDNA bands of the expected size were amplified from total RNA of monocytes and neutrophils after 30 cycles; bands of the same size were apparent in total RNA preparations from all five tested eosinophil preparations after 40–45 cycles of amplification (Fig. 5A). Likewise, bands corresponding to FPRL1 were identified in the RNA preparations from neutrophils (not shown) and monocytes (both at 30 cycles) and four different batches of eosinophils (40–45 cycles) (Fig. 5B). Finally, FPRL2 bands were visualized in the monocyte preparation

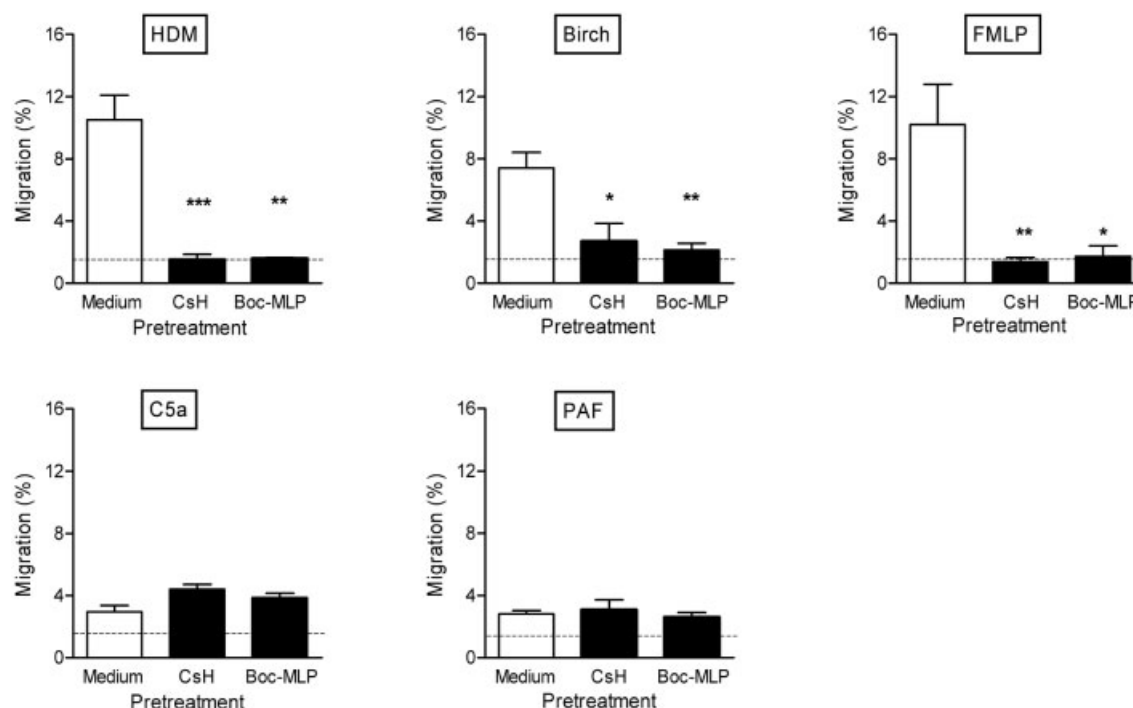


Figure 2. Chemotactic movement of eosinophils pretreated with FPR antagonists. Eosinophils were first incubated in medium, CsH (10^{-6} M) or boc-MLP (10^{-5} M) for 5 min at 37°C and then exposed for 90 min to either HDM (100 $\mu\text{g}/\text{mL}$), birch (100 $\mu\text{g}/\text{mL}$), FMLP (10^{-8} M), C5a (10^{-8} M) or PAF (10^{-8} M) in the microwell migration chambers. Spontaneous migration is indicated by the dotted line ($\approx 1.5\%$), $n = 3\text{--}6$ different eosinophil donors, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ versus medium. All data are arithmetic means $\pm$ SEM.

already after 30–35 cycles of amplification, but could not be detected in neutrophil RNA preparations (as expected), nor in any of the six different eosinophil preparations tested, not even after 60 cycles of amplification (Fig. 5C). To conclude, eosinophils expressed mRNA for FPR and FPRL1, but not for FPRL2. GAPDH transcripts determined that similar amounts of cDNA from all cell samples were loaded and amplified (Fig. 5D).

HDM, unlike birch pollen, activates FPR- or FPRL1-transfected cells

Our findings up to this point indicated that the FPR was involved in mediating the activation of eosinophils by allergens. Since FPRL1 can bind FMLP and shares a high degree of homology with the FPR, it was also a candidate receptor. Hence, we chose to study the capacity of the allergen extracts to activate promyelocytic HL-60 cells transfected with the FPR or the FPRL1. Cell activation was assessed by monitoring intracellular calcium fluxes. As expected, FMLP gave rise to calcium fluctuations in FPR-transfected cells, which were inhibited when the cells were pretreated with CsH (Fig. 6A). Likewise, calcium responses were observed in FPRL1-transfected cells that were stimulated with the peptide WKYMVm, a ligand of the FPRL1, and this response was abrogated

when cells were pretreated with the FPRL1 antagonist WRW⁴ (Fig. 6B). HDM evoked calcium fluxes in both FPR- and FPRL1-transfected HL-60 cells, and both responses were blocked by the respective inhibitors, CsH and WRW⁴ (Fig. 6C, D). In contrast, no calcium fluxes were seen in either cell type upon *in vitro* challenge with birch pollen extract (Fig. 6C, D). Although the transfected HL-60 cells are “artificial”, they do have functional properties such as the ability to migrate toward their cognate ligands (Fig. 6E, F). Nevertheless, their capacity to migrate toward the allergen extracts was poor (Fig. 6E, F).

Both allergens elicit calcium transients in neutrophils, HDM alone evokes migration

Since neutrophils are known to express both the FPR and the FPRL1, we also examined their reactivity to the allergens. Whereas both HDM (Fig. 7B) and birch pollen (Fig. 7C) extracts gave rise to intracellular calcium responses in purified neutrophils, it was only the HDM response that could be down-regulated by CsH (Fig. 7B). The FPR antagonist only caused partial inhibition of the HDM-mediated calcium transients (around 30% inhibition) and the FPRL1 antagonist exerted no effect at all (Fig. 7B). However, a combination of the two antagonists could quench the calcium response evoked by HDM

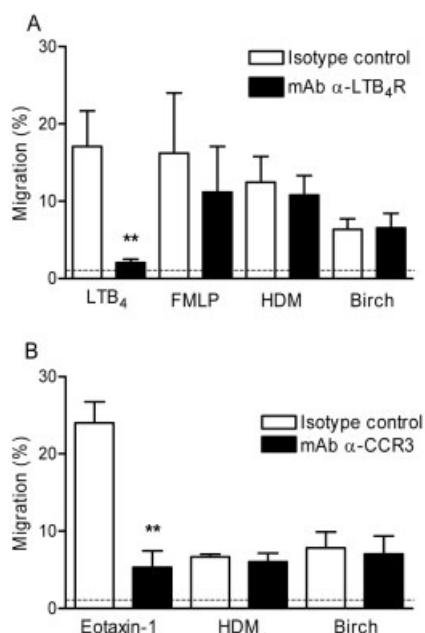


Figure 3. Chemotactic movement of eosinophils pretreated with mAb against LTB₄R or CCR3. Eosinophils were preincubated with (A) anti-LTB₄R mAb (5 µg/mL) (black bars) or (B) anti-CCR3 mAb (5 µg/mL) (black bars) for 30 min at 4°C and then exposed to HDM (100 µg/mL), birch allergen (100 µg/mL), eotaxin-1 (10⁻⁸ M), FMLP (10⁻⁸ M) or LTB₄ (5 × 10⁻⁹ M) for 90 min in the microwell migration chambers. White bars represent eosinophils pretreated with isotype control antibodies. Spontaneous migration is indicated by the dotted line (~1.0%), *n* = 3–6 different eosinophil donors, ** *p* < 0.01 versus medium. All data are arithmetic means ± SEM.

in some cases (Fig. 7B). In contrast, the calcium response caused by birch pollen was unaffected by pretreatment with CsH and WRW⁴, either singly or in combination (Fig. 7C). Finally, HDM gave rise to *in vitro* migration of neutrophils, which birch pollen did not (Fig. 7D).

Allergens and FMLP use similar signal transduction molecules to activate eosinophils

The migration of eosinophils toward HDM (Fig. 8A) and birch (Fig. 8B) extracts was significantly inhibited by the protein kinase C (PKC) inhibitor staurosporine (Fig. 8). When eosinophils were pre-incubated with the MAP ERK kinase inhibitor PD98059, their migration toward HDM was abolished, and ablated toward birch (Fig. 8A, B). Similarly, the p38 inhibitor SB203580 strongly reduced the migration of eosinophils toward the two allergen extracts (Fig. 8A, B). The intracellular signaling pathways involved in the migratory responses of eosinophils to FMLP and eotaxin-1 were studied for comparative purposes. Staurosporine, PD98059 and SB203580 abrogated the migration of eosinophils toward FMLP (Fig. 8C), and all three inhibitors halved

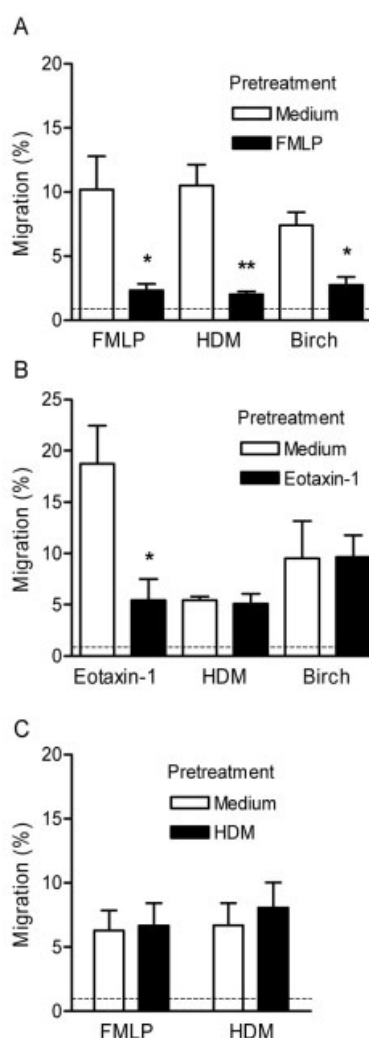


Figure 4. Desensitization of FPR and CCR3 and its effect on the migratory capacity of eosinophils toward allergens. White bars represent eosinophils pretreated with medium and black bars represent eosinophils pretreated with (A) FMLP (10⁻⁷ M), (B) eotaxin-1 (10⁻⁷ M) or (C) HDM (500 µg/mL) for 10 min at 4°C and then exposed to HDM (100 µg/mL), birch allergen (100 µg/mL), eotaxin-1 (10⁻⁸ M) or FMLP (10⁻⁸ M). The dotted line indicates spontaneously migrated eosinophils (~1%), *n* = 3–7 different eosinophil donors, * *p* < 0.05; ** *p* < 0.01 versus medium. All data are arithmetic means ± SEM.

the migration elicited by eotaxin-1 (Fig. 8D). A lower concentration of PD98059 sufficed to inhibit the eosinophilic migration toward HDM compared to what was required to abrogate the migration toward eotaxin-1 and FMLP (Fig. 8).

Discussion

The objective of this study was to unravel the identity of the receptors and pathways that mediate the activation of human eosinophils by two important inhalant allergens: birch pollen and HDM. Our initial experi-

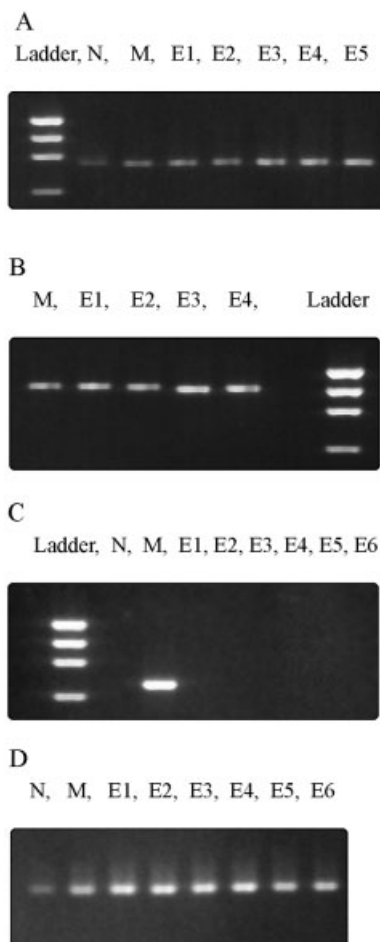


Figure 5. (A) FPR and (B) FPRL1 mRNA expression in human eosinophils purified from healthy individuals (E1–E6). Monocyte (M) and neutrophil (N) mRNA were included as positive controls. (C) FPRL2 mRNA expression is seen in human monocytes, but not in eosinophils or neutrophils. (D) Equivalent amounts of cDNA were amplified and loaded onto the agarose gels as determined by the levels of GAPDH transcripts.

ments indicated that both allergens activated eosinophils *via* a particular PTX-sensitive GPCR, the FPR. The FPR is known to be predominantly PTX-sensitive, by which is meant that PTX impedes the coupling of the FPR to certain G_i proteins *via* ADP-ribosylation, which obliterates all downstream G_i -mediated cellular responses [9]. Both boc-MLP and CsH bind reversibly to the FPR and are considered to be competitive antagonists of the cognate ligand FMLP; CsH is the more potent inhibitor of the two [10], which was confirmed by our data.

We also found that FMLP desensitized the activation of eosinophils by both HDM and birch pollen. There are at least two conceptual forms of desensitization: homologous desensitization, which results from occupation of the receptor by a ligand, and heterologous desensitization [11], also denominated receptor cross-

desensitization, in which signaling *via* one receptor triggers inhibitory signals that prevent the transmission of signals *via* an unrelated receptor [11]. One way to prove that homologous desensitization is operative is by tagging the ligand to be investigated and evaluating its ability to displace the binding of a recognized agonist of the receptor in question. Since we do not know the identity of the ligands present in our allergen extracts, this was not an approach we could undertake. An alternative approach would have been to monitor the removal of the candidate allergen receptor(s) from the surface of eosinophils, *e.g.* estimate the levels of FPR and FPRL1 expressed on eosinophils after exposure of the cells to allergen extracts. Earlier, internalization of receptor following ligand binding was claimed to be a feature of homologous desensitization, but this has been questioned recently [12]. Moreover, as HDM extract was unable to desensitize itself, this could reflect that bioactive substances in this extract may engage additional receptors besides the FPR and FPRL1. Such putative receptor complexes might be difficult to desensitize, as was recently reported for chemoattractant receptors [13].

There exists a hierarchy among chemoattractant receptors regarding the strength with which they cross-regulate each other, with the FPR being among the more potent receptors [11]. The potency of the FPR is confirmed in this study, reflected by the unidirectional desensitization of the aeroallergens by FMLP, *i.e.* FMLP could desensitize allergen responses, but not the other way around. Recently, a model has been proposed to explain the dominance of so-called end-target chemoattractants such as FMLP over intermediary chemoattractants such as LTB_4 in neutrophils: The stronger chemoattractants activate the dominant p38 MAPK pathway, which inhibits the PI3/Akt pathway favored by less potent chemoattractants [14]. However, we found that pharmacologic inhibition of either p38 or ERK1/2 abolished the chemotactic response of eosinophils to HDM extract, and halved that response to birch. Similar to our findings, Sohn *et al.* [3] reported that pretreatment of eosinophils with another p38 inhibitor attenuated the activation of human eosinophils exposed to cockroach extract. We chose to investigate these particular signal transduction molecules because previous studies had established their importance in conveying migratory responses of eosinophils to eotaxin-1 [15, 16] and FMLP [17].

To corroborate our hypothesis that both HDM and birch pollen used the FPR to activate human eosinophils, we assessed the ability of these aeroallergens to activate HL-60 cells transfected with the FPR or the closely related FPRL1. To our surprise, the transfectants were only responsive to HDM, not to birch pollen. We chose to verify this finding by examining the ability of the two

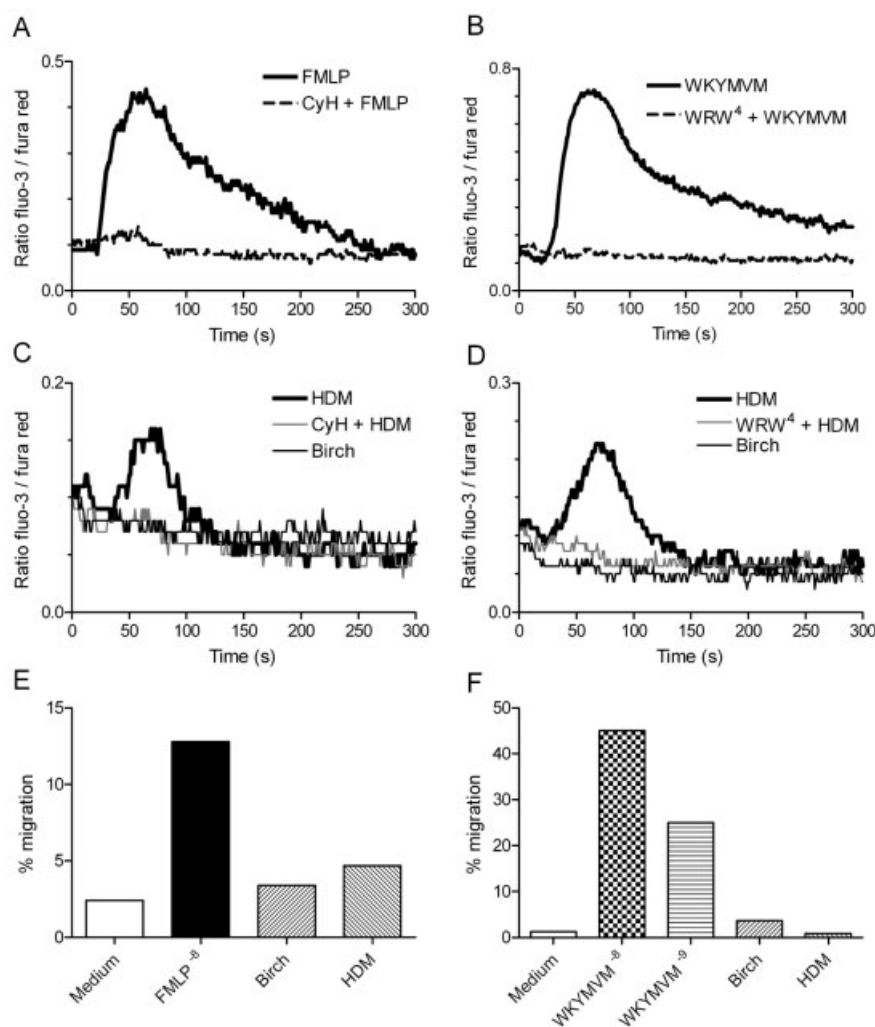


Figure 6. Intracellular calcium fluxes in HL-60 cells transfected with FPR or FPRL1 after loading with the calcium-sensitive dyes Fluo-3 and Fura Red and stimulation with allergen extracts. Calcium mobilization is expressed as the ratio between the MFI emitted by Fluo-3/Fura Red, recorded over time by flow cytometry. (A) FPR-transfected HL-60 cells stimulated with the cognate ligand of the FPR, FMLP (10^{-8} M). The specificity of the response was corroborated by pretreatment of the FPR-transfected cells with the FPR antagonist CsH (10^{-6} M). (B) FPRL1-transfected HL-60 cells stimulated with the cognate ligand of the FPRL1, WKYMVM (10^{-8} M), and pretreated with the FPRL1-specific antagonist WRW⁴ (5×10^{-6} M). (C) FPR-transfected HL-60 cells stimulated with HDM or birch pollen (100 μ g/mL), and pretreated with CsH. (D) FPRL1-transfected HL-60 cells stimulated with HDM or birch pollen (100 μ g/mL), and pretreated with WRW⁴. (E) *In vitro* migration of FPR-transfected HL-60 cells toward the cognate ligand FMLP (10^{-8} M) and allergen extracts (100 μ g/mL). (F) *In vitro* migration of FPRL1-transfected HL-60 cells toward the cognate ligand WKYMVM (10^{-8} and 10^{-9} M) and allergen extracts (100 μ g/mL). One representative experiment out of three is shown.

allergens to activate another cell type, the neutrophilic granulocyte, known to harbor both receptors. Here, HDM and birch pollen alike gave rise to cell activation measured as calcium fluxes. However, only the responses engendered by HDM could be inhibited by the FPR antagonist CsH. The FPRL1 antagonist WRW⁴ was unable to block calcium transients elicited by either of the two allergens in neutrophils. In contrast, the calcium response seen in FPRL1-transfected HL-60 cells stimulated with HDM was abolished by the FPRL1 antagonist. This apparent discrepancy between neutrophils and HL-60 cells may be explained by the finding that when both the FPR and FPRL1 pathways were blocked by dual

administration of the antagonists to neutrophils, the calcium response was completely quenched. Our interpretation is that HDM can activate cells either *via* the FPR or FPRL1, but with a preference for the FPR. Hence, when only one receptor is available, blockade of the receptor abrogates cell activation as seen in the single-transfected HL-60 cells. But when both receptors are present, as is the case in neutrophils, blockade of the FPR only reduced responses by about a third, presumably because the FPRL1 was accessible, and conversely, blockade of the FPRL1 was of little consequence since the preferred receptor FPR could be employed.

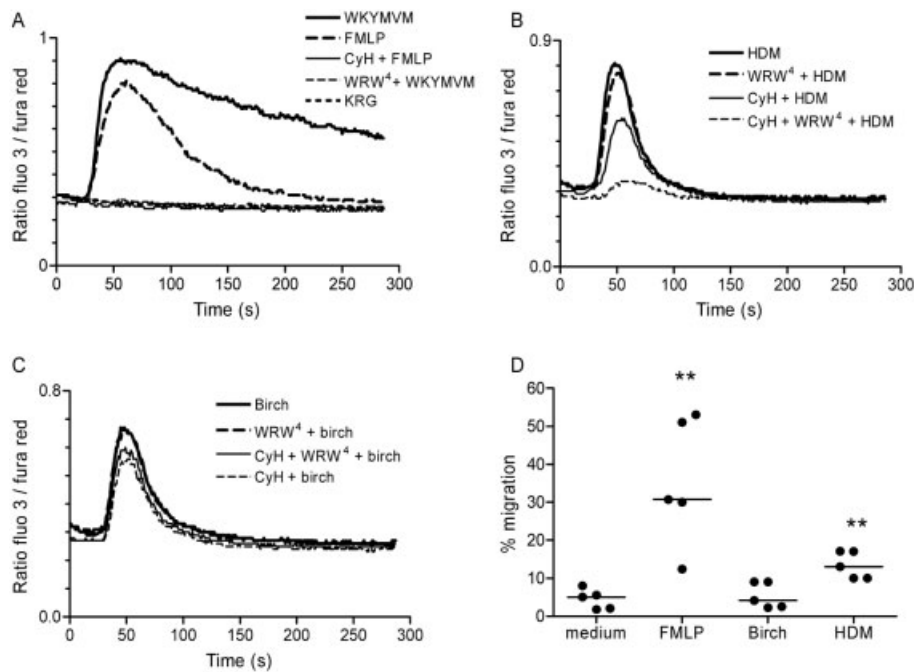


Figure 7. Calcium transients and chemotactic responses evoked by allergens in neutrophils. (A) The FPR ligand FMLP (10^{-8} M) gives rise to an intracellular calcium flux which is inhibited by the FPR antagonist CsH (10^{-6} M). The FPRL1 ligand WKYMVM (10^{-8} M) gives rise to an intracellular calcium flux which is inhibited by the FPRL1-specific antagonist WRW⁴ (5×10^{-6} M). (B) Intracellular calcium transients evoked in neutrophils exposed to HDM (400 μ g/mL) with or without pretreatment with the FPR antagonist CsH (10^{-6} M) alone, the FPRL1-specific antagonist WRW⁴ (5×10^{-6} M) alone or a combination of the two. One representative experiment is shown. (C) Intracellular calcium transients evoked in neutrophils exposed to birch pollen (400 μ g/mL) with or without pretreatment with the FPR antagonist CsH (10^{-6} M) alone, the FPRL1-specific antagonist WRW⁴ (5×10^{-6} M) or a combination of the two. One representative experiment is shown. (D) *In vitro* migration of neutrophils toward FMLP (10^{-8} M), HDM and birch pollen extract (100 μ g/mL), $n = 5$ different neutrophil donors, ** $p < 0.01$ versus medium.

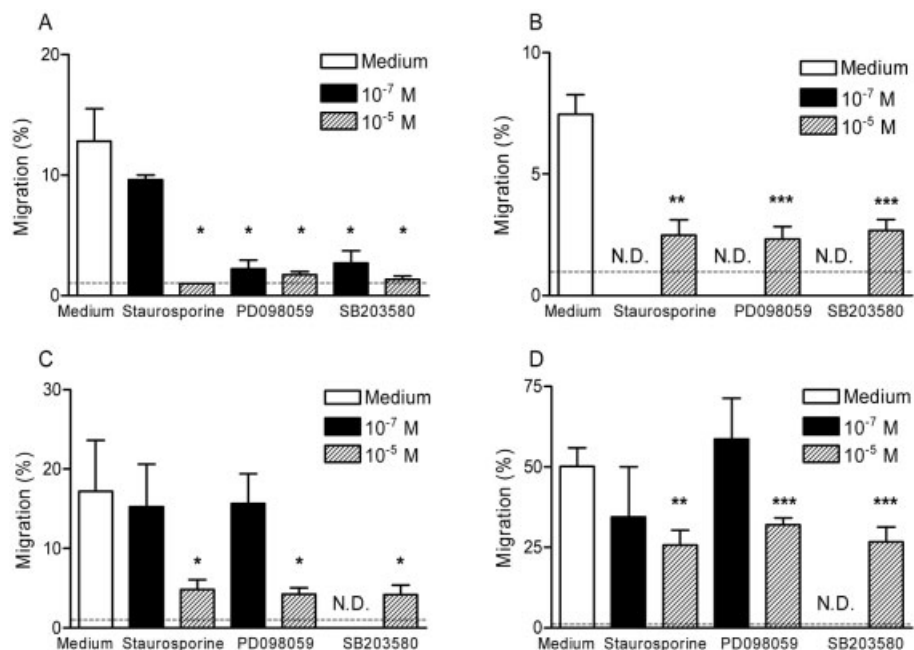


Figure 8. Inhibition of signal transduction molecules and its effect on the chemotactic activity of eosinophils. The effect of staurosporine, PD098059 and SB203580 on eosinophilic migration toward (A) HDM, (B) birch allergen, (C) FMLP, and (D) eotaxin-1. Eosinophils were pretreated with medium or pharmacologic inhibitors for 30 min at 4°C and then exposed to HDM (100 μ g/mL), birch allergen (100 μ g/mL), eotaxin-1 (10^{-8} M) or FMLP (10^{-8} M) in the microwell migration chambers for 90 min. The dotted line indicates the spontaneously migrated eosinophils ($<1\%$), $n = 3-6$ different eosinophil donors, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ versus medium. All data are arithmetic means $\pm$ SEM. N.D., not done.

It is clear from our data that birch pollen does not engage the FPR or the FPRL1 directly, as evidenced by the lack of response of transfected HL-60 cells to birch pollen and the inability of a combination of the FPR and FPRL1 antagonists to abrogate calcium fluxes evoked by pollen in neutrophils. Moreover, whereas neutrophils migrated toward HDM, they did not do so toward birch pollen. Nevertheless, antagonism of the FPR by CsH or desensitization of the same receptor by FMLP did inhibit the degranulation and migration birch pollen gave rise to in eosinophils. Similar paradoxical findings have been reported by us and others regarding eosinophils and neutrophils [5, 18, 19]. It is known that various isoforms of the human FPR exist naturally [9] – so could there be different isoforms expressed by the two types of leukocytes? Or are there important differences regarding the co-receptors and downstream signaling pathways that are coupled to the FPR and FPRL1 in eosinophils and neutrophils, respectively?

We also disclose that eosinophils express the FPR family members FPR and FPRL1 but not FPRL2. This assertion is based on the detection of mRNA specific for these receptors, and was not corroborated by detection of the proteins themselves by immunoblots of eosinophilic cell membranes, since to our knowledge all currently available antibodies exhibit significant cross-reactivity between FPRL1 and FPRL2 [12, 20]. Based on our findings, it appears as if eosinophils display the same receptor profile as neutrophils (FPR⁺, FPRL1⁺, FPRL2⁻) [8], distinct from that of basophils (FPR⁺, FPRL1⁺, FPRL2⁺) [21]. This is surprising because eosinophils are more closely related to basophils than to neutrophils [22] and seem to share similar functions as reflected by the involvement of both cell types in allergic disorders and in the defense against parasites.

Behrendt and coworkers identified derivatives of linoleic and α -linolenic acid to be the main pollen-associated lipid mediators, whose biological effects could be inhibited by the LTB₄R antagonist LY293111 [2]. Since we were unable to get access to LY293111, we instead evaluated the ability of an anti-LTB₄R mAb to block chemotactic movement of eosinophils toward allergen extracts. It is interesting in relation to our findings that LY293111 can prevent the release of reactive oxygen species from neutrophils stimulated with the FPR agonist FMLP [23]. However, since an earlier study has shown that FMLP does not bind to the LTB₄R, it is unlikely that the inhibitory effect of LY293111 is due to competitive antagonism of ligand binding to the receptor [24]. We could detect a small decrease in the chemotactic response to FMLP in eosinophils that were pretreated with the anti-LTB₄R mAb. Hence, some communication appears to exist between the LTB₄R and the FPR, although this was previously reported to be unidirectional,

e.g. FMLP could desensitize LTB₄R but not the reverse [11].

To conclude, we have established that HDM, but not birch pollen, activates eosinophils via the FPR and FPRL1. We can only speculate as to the identity of the birch pollen receptor(s) – it might be a GPCR closely related to the FPR other than the C5a receptor and the PAF receptors, alternately an FPR or FPRL1 isoform, or an altogether different receptor unrelated to the FPR but coupled to the same downstream signaling pathways as the FPR. Although p38, ERK1/2 and PKC appear to be of importance in transmitting allergen-mediated signals in eosinophils, most biological responses mediated by GPCR, one among which is the FPR, employ many-fold biochemical routes that form an intricate network of signaling pathways [25]. An increased understanding of the receptors and pathways used by aeroallergens to activate human eosinophils may be of importance in the elucidation of the complex pathophysiological mechanisms behind allergy to inhaled allergens.

Materials and methods

Allergen extracts

Aqueous extracts of HDM (*Dermatophagoides pteronyssinus*) and European white birch pollen (Greer Laboratories, Lenoir, NC) which contained 0.03 ng/mL and 1.0 ng/mL LPS, respectively, according to the *Limulus* amoebocyte lysate assay (Chromogenix AB, Mölndal, Sweden) were used. According to the manufacturer, whole bodies of HDM were crushed by ball milling, extracted in ammonium bicarbonate solution and dialyzed against distilled water. Birch pollen grains were defatted, powdered, dried and extracted as described above. Both extracts contain about 30% w/w protein.

Reagents

FMLP, boc-MLP, PTX, PAF (16 carbon atoms), PMA, PD098059, SB203580 and staurosporine were purchased from Sigma-Aldrich. Eotaxin-1/CCL11, murine anti-CCR3 mAb (clone 61828) and IgG2a isotype control mAb (clone 54447) were purchased from R&D Systems. The peptide Trp-Arg-Trp-Trp-Trp-Trp-CONH₂ (WRWWWW; WRW⁴) [26] was obtained from GenScript Corp (Piscataway, NJ) and Trp-Lys-Tyr-Met-Val-Met-CONH₂ (WKYVMV) from Alta Bioscience (Birmingham, UK). CsH and murine anti-LTB₄R mAb (clone 7B1) were kindly provided by Novartis Pharma (Basel, Switzerland) and Dr. Christer Öhman (Lund University, Sweden), respectively.

Purification of leukocytes

Eosinophils were isolated from fresh buffy coats obtained from blood donors at Sahlgrenska University Hospital, Göteborg, and Kungälv Hospital, Kungälv, or from heparinized blood derived from healthy volunteers, by negative depletion using a

mixture of magnetic beads (MACS; Miltenyi Biotec Inc.) coated with mouse anti-human CD16, CD14, CD3 and CD19 mAb (Miltenyi), as previously described [1]. Eosinophils of purity >95% determined by Diff-quick stain (Dade Behring AG) and viability >99%, assessed by Trypan blue exclusion, were used. Neutrophils were obtained by flushing out the CD16⁺ cells bound to the MACS column after passage of eosinophils. Monocytes were acquired by flushing out CD14-expressing cells from the MACS column. This study was approved by the Ethics Committee at the Medical Faculty, Göteborg University (S387-01).

Chemotaxis

Eosinophil migration was assessed in 96-well migration microplates (30 μ L volume, filter diameter 3 μ m) (ChemoTx; Neuro Probe Inc., Gaithersburg, MD) as previously described [5]. Eosinophils (3×10^4) were pretreated with either CsH (10^{-6} M), boc-MLP (10^{-5} M), FMLP (10^{-7} M) or Krebs' Ringer glucose (KRG) buffer supplemented with 0.3% BSA for 10 min at 4°C, before being allowed to migrate to the lower wells. All readings were translated to percent migrated cells and corrected for spontaneous migration. *In vitro* migration assays of the larger HL-60 cells were performed using migration microplates with filter diameter of 5 μ m, cells were allowed to migrate for 3 h instead of 1.5 h as for eosinophils, and due to poor peroxidase activity, the percentage of migrated cells was determined by counting using a microscope.

Stimulation of eosinophils

Eosinophils (1×10^5) were resuspended in X-vivo 15 buffer, put in 96-well low-binding polystyrene plates (TPP, Fisher Scientific GTF, Göteborg) and co-incubated with various allergen extracts (100 μ g/mL) or PAF (10^{-7} M) for 18 h at 37°C. The chosen concentration of allergen extract has previously been established to be optimal for activation of eosinophils *in vitro*. Eosinophil viability after stimulation was >97% by Trypan blue exclusion. Cell-free supernatants were collected and the cells were further assessed by flow cytometry.

Receptor and signal transduction blockade

Eosinophils (1×10^5) were pretreated with CsH (10^{-6} M) or boc-MLP for 10 min at 37°C, PTX (200 ng/mL) for 2 h at 37°C, and either X-vivo 15 buffer or KRG as a negative control. When mAb were used, the eosinophils were first treated with purified human Ig (1 μ g/ 10^5 cells; Pharmacia) for 30 min to block Fc receptors or with buffer alone, and then treated with mAb directed against CCR3, LTB₄R or an isotype-matched control antibody, for 45 min at 4°C. To inhibit signal transduction molecules, eosinophils were pre-incubated with staurosporine, PD098059 or SB203580 (10^{-5} or 10^{-7} M) for 30 min on ice. Subsequently, eosinophils were incubated with the allergen extracts as described above without prior washing.

Receptor desensitization

Eosinophils were first stimulated with either FMLP (10^{-7} M) or HDM (500 μ g/mL) for 10 min at 4°C; then the cells were allowed to migrate toward FMLP (10^{-8} M) or HDM (100 μ g/mL).

Release of MBP

The intracellular labeling of MBP was performed as previously described [6]. In short, eosinophils were permeabilized and stained with a mouse anti-human MBP mAb (BD Pharmingen) or anti-human CD22 antibody (Immunotech S.A., Marseille, France) as a control, followed by PE-labeled rat anti-mouse IgG₁ (BD Pharmingen) and fixation in 3.7% paraformaldehyde-PBS. MFI was analyzed using a FACScan (Becton Dickinson). A change in intracellular MBP contents was determined using the formula: (MFI of medium-treated cells – MFI of allergen-stimulated cells)/MFI of medium-treated cells, multiplied by 100 and presented as %.

Release of EPO

EPO activity in cell supernatants or lysates was measured by the addition of H₂O₂ (4 μ L of 30% H₂O₂) and *o*-phenylene-diamine (Sigma) dissolved in lysis buffer, as described [5].

Isolation of cellular mRNA and PCR analysis

Total RNA was isolated from highly purified eosinophils (>98%) (5×10^6 cells), as well as from neutrophils and monocytes, using RNeasy Mini Kit (Qiagen). Contaminating DNA was removed by the use of DNA-free (Ambion), and the RNA yield and purity were determined by spectrophotometry. The isolated RNA (0.1–5 μ g) was used for cDNA synthesis using First Strand cDNA Synthesis Kit (Fermentas, Helsingborg, Sweden), with oligo(dT)₁₈ as primer. Of each cDNA sample, 5% was subjected to PCR amplification of FPR, FPRL1, FPRL2, and GAPDH mRNA transcripts. Each 25- μ L PCR reaction contained 2.5 μ L 10 $\times$ PCR buffer II, 3 mM MgCl₂ for amplification of FPR, FPRL1 and GAPDH and 4 mM MgCl₂ for amplification of FPRL2, 0.2 mM dNTP mix (Fermentas), 0.4 μ M each of forward and reverse primer (TIB Molbiol, Berlin, Germany), and 0.625 U AmpliTaq Gold (all from Applied Biosystems, Stockholm, Sweden, except for the dNTP mix, Fermentas). FPR transcripts were amplified with the primers 5'-TACCCAGAGCAAGACCACA-3' (forward) and 5'-AAAAGGCTGCTGCGACAA-3' (reverse), FPRL1 with 5'-TGCTGGTGCTGCTGGCAA-3' (forward) and 5'-AATATCCCTGACCCCATCCTCA-3' (reverse), FPRL2 with 5'-AGTTGCTC-CACAGGAATCCA-3' (forward) and 5'-GCCAATAATGAAGTG-GAGGATCAGA-3' (reverse), and GAPDH with the primers 5'-GGCTGCTTTAACTCTGG-3' (forward) and 5'-GGAGGG-ATCTCGCTCC-3' (reverse). The amplification was performed on a Mastercycler gradient (Eppendorf Nordic). The number of cycles (30–60) was optimized for the different cell types and

primers, respectively. The PCR products (9 µL) and a molecular size standard (ΦX174 RF DNA/Hae III Fragments; Invitrogen) were resolved by electrophoresis on 1.5% agarose gels and visualized by ethidium bromide staining.

Intracellular calcium measurements in FPR- or FPRL1-transfected HL-60 cells

HL-60 cells transfected with either FPR or FPRL1 were used [27]. Intracellular calcium mobilization was measured essentially according to the method developed by Partida-Sanchez *et al.* [28]. HL-60 cells (3×10^6) were resuspended in cell-loading medium (KRG with Ca^{2+} + 1% FCS) and loaded with the fluorescent dyes Fluo-3 AM (4 µg/mL) and Fura Red AM (10 µg/mL) (Molecular Probes, Eugene, OR) for 30 min at 37°C. For 5 min prior to analysis, the cells were warmed to 37°C in a water bath. Intracellular Ca^{2+} levels were assessed by dual flow cytometry (FACScan). The fluorescence emission of Fluo-3 was monitored in the FL-1 channel and that of Fura Red in the FL-3 channel. First, baseline fluorescence was established (20 s) in a tube containing 500 000 HL-60 cells/mL. Next, the tube was rapidly removed, the stimulant added, and fluorescence emission was recorded in the tube restored to the flow cytometer. The stimulants FMLP (10^{-7} M), WKYMVm (10^{-7} M) and HDM and birch extracts (both at 100 or 400 µg/mL) were used, and in some experiments, HL-60 cells were pretreated for 5 min at 37°C with either CsH (10^{-6} M) or WRW⁴ (5×10^{-6} M). Total acquisition time was 300 s and data was collected using the software CellQuest, BD. The acquired fluorescence data was reanalyzed using the software Flow Jo 5.7.1 (Tree Star Inc., OR). The relative Ca^{2+} concentration was expressed as the ratio between Fluo-3 and Fura Red (FL-1/FL-3) MFI over time.

Statistics

Statistical analyses were performed using GraphPad Prism 4.0 software (GraphPad, San Diego, CA). The unpaired two-tailed Student's *t*-test was employed. $p < 0.05$ was considered statistically significant.

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