The pseudoallergen receptor MRGPRX2 on peripheral blood basophils and eosinophils: Expression and function

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Abstract

Background: Mas-related G protein-coupled receptor X2 (MRGPRX2) is regarded as a mast cell-specific receptor mediating non-IgE-dependent activation. We aimed to investigate whether human basophils and eosinophils express functional MRGPRX2.

Methods: Flow cytometry, immunocytochemistry, immunofluorescence, Western blot, and RT-PCR were performed in highly purified peripheral blood basophils and eosinophils of atopic and nonatopic donors. To assess functional activity, fluorescent avidin-based degranulation assay, calcium mobilization, cytokine production in supernatants, assessment of viability/apoptosis, and tricolor granulocyte activation test were used.

Results: MRGPRX2 was significantly expressed by basophils and eosinophils but not neutrophils. Functional capacity was shown by anti-MRGPRX2 mAb-induced calcium influx and concentration-dependent induction of degranulation. Sequential stimulation in the calcium mobilization assay gave no evidence for desensitization or receptor internalization. Anti-MRGPRX2 mAb significantly promoted survival. Inhibition of apoptosis could be due to released IL-3, IL-5, and GM-CSF found in supernatants. Short-term incubation with IL-3 dose-dependently upregulated MRGPRX2 expression in both, stimulation for 24 hours with anti-IgE, C5a, fMLP, and IL-3 in basophils and by IL-3, IL-5, and IL-33 in eosinophils. Among known mast cell MRGPRX2 agonists ciprofloxacin but not PMX-53 was functional on basophils and eosinophils. In basophils of allergic subjects, tricolor granulocyte activation test using grass pollen demonstrated MRGPRX2 upregulation associated with degranulation and CD63 expression.

Conclusion: Unraveling the regulation and signaling mechanisms of MRGPRX2 on basophils and eosinophils might enable the development of new therapeutic strategies.
to prevent or inhibit allergic and nonallergic hypersensitivity. Moreover, addressing MRGPRX2 might have potential for diagnostic purposes in (drug) hypersensitivity.

**KEYWORDS**
basophil, ciprofloxacin, eosinophil, function, MRGPRX2

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**GRAPHICAL ABSTRACT**
MRGPRX2 is not mast cell specific, as it is expressed on comparable levels on peripheral blood basophils and eosinophils, but not neutrophils, from atopic and non-atopic donors. Engagement of MRGPRX2 by monoclonal antibodies results in calcium influx, enhanced survival, and degranulation with release of cytokines. Mast cell MRGPRX2 ligand ciprofloxacin, but not PMX-53, shows similar effects. Granulocyte activation test with grass pollen in allergic subjects results in basophil and eosinophil MRGPRX2 upregulation similar to CD63 upregulation. # survival not assessed with ciprofloxacin, cytokine release not assessed with PMX-53;
Abbreviations: MRGPRX2, mas-related G-protein coupled receptor X2; PMX-53, C5a receptor antagonist.

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**1 | INTRODUCTION**

Mas-related G protein-coupled receptor X2 (MRGPRX2, formerly known as MRGX2), whose adaptive evolution in humans was a relatively recent event, is expressed on sensory neurons in dorsal root ganglia and involved in nociception. In addition, MRGPRX2 has been found to be expressed on the plasma membrane and intracellular sites of human tryptase and chymase-expressing mast cells (MC\textsubscript{TC}) in the dermis of the skin or submucosa of the gut.\textsuperscript{2,3} Mast cell MRGPRX2 are regarded to be of importance in mediating non-IgE-mediated (so-called pseudoallergic or anaphylactoid) hypersensitivity reactions, neurogenic inflammation, pain, and itch and also in promoting the innate immune response against diverse skin and gut-penetrating noxious stimuli or invading pathogens.\textsuperscript{3-8} Identified ligands for MRGPRX2 in mast cells and mast cell lines are neuropeptides (eg substance P, cortistatin), antimicrobial host-defense peptides (eg LL37), eosinophil granule proteins (eg major basic protein, eosinophil peroxidase), and peptidergic drugs (eg ciprofloxacin, icatibant).\textsuperscript{3,4,9} It was anticipated that targeting MRGPRX2, for example, by small molecules, might reduce a subset of drug-induced adverse effects.\textsuperscript{6} However, the exact role of MRGPRX2 in mast cells and its validated endogenous ligands is still unclear. The MRGPRX2 is classified as an orphan receptor, and unlike most GPCRs, it recognizes a wide range of basic molecules. Thus, there still might be several unknown ligands for the receptor.

It has been stated in the literature that among human immunocytes, MRGPRX2 is exclusively expressed on mast cells.\textsuperscript{4,10} However, data for granulocytes have not been shown in detail. In addition to mast cells, basophils and eosinophils are central effector cells in allergic and nonallergic inflammation, as well as in innate and adaptive immunity.\textsuperscript{11} Therefore, in this study, we aimed to investigate whether human basophils and eosinophils express MRGPRX2 and, if present, whether it possesses a functional capacity.

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**2 | MATERIALS AND METHODS**

**2.1 | Antibodies**

If not stated otherwise, all antibodies were purchased from Biolegend. The main experiments were performed using MRGPRX2
(clone K125H4, mouse IgG2b, and PE-conjugated). CD63 (clone CLBGran/12, mouse-IgG1k, and FITC-conjugated) was from Beckman Coulter, Brea, CA, USA. Alexa488-conjugated avidin was purchased from Thermo Scientific. Stimulation experiments were performed with unconjugated anti-MRGPRX2 antibody (clone K125H4, mouse IgG2b) and mouse IgG2b control antibody.

2.2 | Isolation and stimulation of eosinophils, basophils, and LAD2 mast cell line

After informed consent (approved by the ethics committee of the Hannover Medical School, (7814_BO_S_2018), peripheral venous blood was obtained between 8 and 10 o’clock. Basophils and eosinophils of nonatopic volunteers (no personal history of atopy, negative blood was obtained between 8 and 10 o’clock. Basophils and eosinophils, and LAD2 mast cell line

2.2.1 | Isolation of eosinophils

Isolation of eosinophils was performed with EasySep™ Human Eosinophil Enrichment (Stem Cell Technologies). For basophils, for most experiments we used a EasySep™ Human Basophil Enrichment Kit (Stem Cell Technologies). All kits were used according to the manufacturer’s instructions. Purity and viability were consistently ≥98% for both basophils and eosinophils, as assessed by staining with Kimura, alcian blue, and trypan blue, respectively. 1 × 10⁵ cells were washed in PBS and resuspended in 150 μL PBS. Cell suspensions were centrifuged onto slides (500 rpm, 5 minutes) using a cytospin-3 cytocentrifuge (Shandon Southern Instruments). As control, we prepared cytospins with the human mast cell line LAD2 (1.5 × 10⁶ cells).

2.3 | Cytospin preparation

To perform cytospins, 1 × 10⁵ cells were washed in PBS and resuspended in 150 μL PBS. Cell suspensions were centrifuged onto slides (500 rpm, 5 minutes) using a cytospin-3 cytocentrifuge (Shandon Southern Instruments). As control, we prepared cytospins with the human mast cell line LAD2 (1.5 × 10⁵ cells).

2.4 | Immunocytochemistry and immunofluorescence

Immunocytochemical (ICC) and immunofluorescence (IF) staining of cytospins was performed using mouse anti-human MRGPRX2 antibody (1:20 - 25 μg/mL). An isotype control antibody or incubations without primary antibody served as experimental controls. Incubation with Abs was performed over night at 4°C. To visualize nuclei, slides were stained with DAPI (300 nmol/L, (Molecular Probes, Invitrogen, Thermo Scientific) for 5 minutes at room temperature.

ICC visualization was done according to the instructions of the manufacturer using either Dako EnVision+ System-HRP (AEC) resulting in a red-colored precipitate at the antigen or Liquid DAB+ Substrate Chromogen System (Dako) yielding a brown reaction end product at the site of the target antigen.

Images were acquired using Olympus BX43 light/fluorescence microscope equipped with an Olympus XC30 camera using cellSens imaging Software (Olympus).

Images were captured using an automatic digital slide scanner (Pannoramic MIDI II BF, Sysmex Deutschland GmbH).

2.5 | Flow cytometric analysis for surface receptor expression

Flow cytometric surface staining procedures have been described previously. The expression of surface expression was assessed using a PE-conjugated mouse anti-MRGPRX2 antibody (15 μg/mL, clone K125H4, isotype IgG2b, Biolegend) or the respective isotype as described. Staining was performed for 1 hour at 4°C.

Since all experiments were performed with highly purified cells (≥98%), no specific gating antibodies were required. Cells were analyzed using a BD FACScan™ II platform and BD Cell Quest™ Pro software (Becton Dickinson). Day-to-day instrument variability was monitored using BD FACS 7-color Setup Beads (BD Biosciences). For statistical calculation, percentage of positive cells and geo mean fluorescence intensities (geo MFI) were calculated after subtraction of the values obtained with the respective isotype antibody.

2.6 | Western blot

1 × 10⁶ eosinophils, basophils, or neutrophils (purity 99%) were stimulated with medium (control), IL-3 (0.1-10 ng/mL), fMLP (1 μmol/L), IL-3...
(10 ng/mL) + IL-3 (20 ng/mL), anti-IgE (100 ng/mL), or PMA (10 ng/mL) for 30 minutes and then lysed as well as 0.5 × 10^6 LAD2 cells in M-Per Mammalian Extraction Reagent (Pierce/Thermo Scientific). The protein concentration was determined according to the Lowry method. Equal protein extracts (15 µg per sample) were separated by a SDS-PAGE using 4%-20% gradient Mini-Protean TGX Precast (Bio-Rad Laboratories). Proteins were transferred to a nitrocellulose membrane (Pierce/Thermo Scientific), blocked with blocking buffer (1 × TBST with 5% BSA), and immunoblotted with polyclonal mouse anti-MRGPRX2 antibodies (1:500; ABNOVA) followed by HRP-conjugated goat anti-mouse secondary antibodies (1:2000, Cell Signaling Technology). Mouse anti-human histone H3 (clone mAbcam 24834 Nuclear Loading Control) monoclonal antibodies (1:1000, Abcam) were used to detect housekeeping protein. The blots were visualized using a chemiluminescence kit according to the instructions of the manufacturer (Pierce/Thermo Scientific) and documented with imaging system Aequoria, including dark box and CCD camera and the HoKaWo imaging software (Hamamatsu Photonics). Detected bands were quantified using Image J image analysis software (National Institutes of Health).  

2.9 | Monitoring of basophil and eosinophil degranulation by using fluorescent avidin

As recently described, a fluorescent avidin-based method provides better results in monitoring mast cell and basophil degranulation when compared with CD63 expression.16,17 Avidin directly stains cell-bound granules upon degranulation, and the AlexaFluor488-labeled avidin (Av.A488) fluorescence intensity provides a measure of the degranulation magnitude.17 Although this has not been described before, we used this method for eosinophils too.

After in vitro stimulation of cells at 37°C as indicated, avidin-Alexa488 (Av.A488) staining was performed as described in detail16 using a final avidin-Alexa488 concentration of 2.5 µg/mL. Anti-IgG2b isotype was used as control antibody. After first results demonstrated no difference between a concentration of 2.5 and 5 µg/mL we used 2.5 µg/mL in further experiments.

2.10 | Flow cytometric annexin V/propidium iodide staining

Basophil and eosinophil (2 × 10^5 cells) apoptotic cell death after incubation with anti-MRGPRX2 mAb, an IgG2b isotype antibody, PMX-53 (100 nm) or IL-3 (2 ng/mL), and dexamethasone (1 µmol/L) as controls was assessed by annexin V/propidium iodide staining (Annexin V kit, Pharmingen) as previously described.18

2.11 | Cytokine production

The basophil and eosinophil supernatants from the flow cytometric Annexin V/propidium iodide staining experiments were immediately frozen at ~80°C and later used to detect production of IL-3 (Peprotech, detection range 31.25-2000 pg/mL), IL-5 (R & D, detection range 23.44-1500 pg/mL), and GM-CSF (R & D, detection range 15.6-1000 pg/mL) by ELISA technique according to the manufacturer’s instructions.

2.12 | Multicolor granulocyte activation test

Granulocyte activation test was performed using heparinized whole blood of six grass pollen-allergic donors (specific IgE against g6, timothy grass CAP-class ≥2, ImmunoCAP®, Thermo Scientific). A 100 µL whole blood was incubated with stimulation buffer (control) for 10 minutes at 37°C; then, either stimulation buffer (control), 7 grass mix (Meadow Fescue, Orchard, Redtop, Perennial Rye, Sweet Vernal, Kentucky Blue, and Timoth), in equal parts, 100 000 BAU/mL ≥ 600 µg/mL) (BASOTEST™, BD Bio-Science/Glycotope Biotechnology) at indicated concentrations or anti-FcεRI (0.33 µg/mL), or fMLP (1 µmol/L) as positive controls were added for 20 minutes at 37°C. Degranulation was stopped by incubation on ice for 5 minutes. Cells were incubated for 20 minutes on ice with FcεR1-PerCP, CD63-PE, and MRGPRX2-APC, thereafter lysed, and fixed and immediately analyzed using a BD
FACSCanto II platform and BD Cell Quest™ Pro software (Becton Dickinson). Per sample, at least 1,000 cells expressing low (eosinophils) or high amounts of FcεRlα (basophils) were live gated.

3 | RESULTS

3.1 | MRGPRX2 is constitutively expressed on peripheral blood basophils and eosinophils

Using flow cytometry (Figure 1A,B), immunocytochemistry, and immunofluorescence (Figure 1C), we found significant surface expression by both, freshly isolated peripheral blood basophils and eosinophils, but not neutrophils (data not shown). MRGPRX2 expressing LAD2 mast cells were used as positive control. There was no apparent difference in MRGPRX2 expression regarding the isolation method (see methods). Moreover, there was no significant difference between atopic (patients with inhalant allergy or extrinsic atopic dermatitis) and nonatopic subjects (Figure 1A). Pooling atopic and nonatopic results (n = 19-27), there was no statistically significant difference to basophils and eosinophils regarding %MRGPRX2pos cells (mean ± SEM: 28.96 ± 5.0 and 30.93 ± 6.44, respectively) or geo MFI (mean ± SEM: 1.3 ± 0.22 and 2.38 ± 0.46, respectively), as assessed by nonparametric Wilcoxon/Kruskal-Wallis test.

Constitutive MRGPRX2 protein and gene expression were confirmed in both, freshly isolated and unstimulated basophils and eosinophils, by Western blot analysis (Figure 1D-H) and RT-PCR in all donors demonstrating melting temperature peak curves at 69°C for MRGPRX2 in relation to 77°C for the ribosomal protein RPS 20 control (not shown). Gel electrophoresis showed distinct single bands for the amplified products (not shown).

Western blot analysis demonstrated constitutive MRGPRX2 protein expression in pooled basophils and eosinophils, that was enhanced by 30-min stimulation with fMLP (1 µmol/L), a combination of IL-3 (10 ng/mL) + IL-33 (30 ng/mL), anti-IgE (100 ng/mL), and PMA (10 ng/mL) (Figure 1D and G). In contrast, pooled neutrophils (n = 7) did not express MRGPRX2 protein and MRGPRX2 protein was not induced by stimulation with fMLP (1 µmol/L). Relative MRGPRX2 Western blot density of basophils, eosinophils, and neutrophils (n = 10) compared to LAD2 (relative density set = 1, dotted reference line) are shown in Figure 1E. The mean relative density ± SD for basophils was 0.265 ± 0.146; for eosinophils 0.263 ± 0.107; and 0.038 ± 0.039 for neutrophils in relation to 1.0 for LAD2. Individual relative Western blot densities of basophils and eosinophils and means (bars) in response to stimulation with fMLP, IL-3 + IL-33, anti-IgE, and PMA of 7 subjects are shown in Figure 1F. Moreover, 30-min stimulation with IL-3 dose-dependently increased MRGPRX2 protein in both, basophils and eosinophils (Figure 1H, n = 2).

3.2 | Calcium influx after MRGPRX2 engagement

In the next series of experiments, we investigated whether basophil and eosinophil MRGPRX2 is functionally activated by ligation. Engagement of MRGPRX2 using anti-MRGPRX2 mAb but not medium control or control antibody of the same isotype immediately induced significant calcium influx in basophils and eosinophils (Figure 2A.C-E) that was less compared to C5a (0.1 µmol/L, not shown), anti-IgE (100 ng/mL), and fMLP (1 µmol/L) as shown in Figure 2C,D,E). However, in co-stimulation with anti-MRGPRX2 mAb calcium influx was potentiated (Figure 2C,D,E). Moreover, calcium influx induced by stimulation with anti-MRGPRX2 mAb (2.5 µg/mL), anti-IgE, anti-FcεRI mAb (2.5 µg/mL), or fMLP (1 µmol/L) after 3–5 seconds could be again enhanced by a second stimulation with anti-MRGPRX2 mAb (2.5 µg/mL) after 60 seconds (Figure 2B). The second subsequent stimulation with anti-MRGPRX2 mAb resulted in higher calcium influx compared to the first stimulus when the first stimulus was IgE-independent, that is fMLP or anti-MRGPRX2-mAb.

3.3 | Degranulation induced by MRGPRX2 antibodies

Basophil and eosinophil activation was determined by using a new fluorescent avidin-based method reflecting degranulation in basophils. Avidin directly stains cell-bound granules upon degranulation, and the AlexaFluor488-labeled avidin (Av.A488) fluorescence intensity provides a measure of the degranulation magnitude. Crosslinking of MRGPRX2 by anti-MRGPRX2 mAb, but not by anti-IgG2b isotype control for 30 minutes, resulted in a significant concentration-dependent increase of Av.A488 fluorescence-positive cells reflecting degranulation (Figure 3A), *P < .05, **P < .01, paired t test vs medium and isotype. Figure 3B demonstrates spaghetti plots of all eight individual donor experiments, and Figure 3C,D a representative dot plot of anti-MRGPRX2 mAb (concentration as indicated) induced Av.A488 staining of basophils (C) and eosinophils (D).

3.4 | MRGPRX2 engagement enhanced survival/delayed apoptosis

We next compared anti-MRGPRX2 mAb-induced effects regarding viability/apoptosis compared to survival-promoting IL-3 (2 ng/mL) and apoptosis-inducing dexamethasone (1 µmol/L). In contrast to IgG2b isotype, anti-MRGPRX2 mAb (2.5 µg/mL) significantly promoted survival after 24 hours in basophils and eosinophils as assessed by Annexin V/PI staining (Figure 4A) and trypan blue dye exclusion (Figure 4B). *P < .05, **P < .01 compared to medium and isotype (Wilcoxon signed-rank sum test), n = 6. A representative dot plot of basophil (top row) and eosinophil (bottom row) Annexin-/PI staining is shown in Figure 4C.

3.5 | Cytokine release after MRGPRX2 engagement

Assessing basophil and eosinophil cytokine release in the supernatants of the viability/apoptosis assay, we found that 24-hour
stimulation with anti-MRGPRX2 mAb dose-dependently resulted in a statistically significant release of IL-3, IL-5, and GM-CSF compared to medium and respective isotype (Figure 4D, black bars, n = 6, Wilcoxon signed-rank test). P-values for IL-3 in basophils at 1 µg/mL: *P < .05, at 2.5 µg/mL: **P < .01; in eosinophils at 1 µg/mL and at 2.5 µg/mL: *P < .05, for IL-5 in basophils at 1 µg/mL: n.s., at 2.5 µg/mL: ***P < .001; in eosinophils at 1 µg/mL: *P < .05 and at 2.5 µg/mL: **P < .01, and for GM-CSF in basophils at 1 µg/mL: *P < .05, at 2.5 µg/mL: ***P < .001; in eosinophils at 1 µg/mL and at 2.5 µg/mL: *P < .01. Moreover, in eosinophils even after 4 hours, significant amounts of IL-3 (at 1 µg/mL and at 2.5 µg/mL: *P < .05) and GM-CSF (at 1 µg/mL and at 2.5 µg/mL: *P < .05) were found. Figure 4D, bottom figure, horizontal striped bars, n = 6, Wilcoxon signed-rank sum test. Basophils could not be assessed after 4 hours due to fewer available cells. The effect of anti-MRGPRX2 mAb-induced cytokine release was very potent compared to stimulation with the potent eosinophil and basophil secretagogue IL-3 (2 ng/mL). In contrast, dexamethasone while inducing apoptosis significantly reduced the cytokine release (negative control) in basophils after 24h of GM-CSF (***P < .01), whereas after 24h in eosinophils, release of all, IL-3, IL-5 (*P < .05), and GM-CSF (***P < .01), was reduced, in addition to GM-CSF after 4 hours (*P < .05).
3.6 | Modulation of surface MRGPRX2 expression

A short incubation for 30 minutes with IL-3 (0.1, 0.5, 1.2, 5, and 10 ng/mL) dose-dependently and significantly (*P < .05 for each concentration of IL-3 from 0.1 to 10 ng/mL vs incubation without IL-3, paired t test) upregulated MRGPRX2 surface expression of basophils (optimal concentration 2 ng/mL) (Figure 4E, n = 3) and eosinophils (optimal concentration 2-4 ng/mL) (Figure 4F, n = 4).

In the next series of experiments, we examined the effects of several known basophil and eosinophil-activating stimuli on MRGPRX2 expression after 24 hours of incubation at 37°C. As shown in Figure 4G, MRGPRX2 surface expression was significantly upregulated in basophils (n = 9-13) by 24-hour stimulation with anti-IgE (100 ng/mL, P = .005, Mann-Whitney rank sum test), C5a (0.1 µmol/L, P < .001), fMLP (1 µmol/L, P < .001), and IL-3 (10 ng/mL, P < .001), whereas no effect was found by IL-5 (10 ng/mL), IL-33 (20 ng/mL), or anti-FcεRI mAb (330 ng/mL).

Moreover, after 24-hour stimulation of eosinophils MRGPRX2 surface expression was significantly upregulated by IL-3, IL-5, and IL-33, but not by anti-IgE, anti-FcεRI mAb, C5a, or fMLP (concentrations like in basophils, n = 6, P < .05, Mann-Whitney rank sum test, Figure 4H).
3.7 Evaluation of effects of mast cell MRGPRX2 agonists, PMX-53, and ciprofloxacin, on basophils and eosinophils

PMX-53 (10, 100, and 1000 nmol/L) did not result in a modulation of MRGPRX2 expression, calcium influx, degranulation, or survival in basophils or eosinophils \((n = 4-5, \text{data not shown})\). Functionality of the PMX-53 preparation was proven by demonstrating an immediate dose-dependent \((50, 100, 1, 10 \mu \text{mol/L}, \text{but not 10 nm})\) calcium mobilization in LAD2 cells as previously demonstrated.\(^9\) Moreover, being a C5aR antagonist, PMX-53 given 55 seconds before C5a \(10^{-8} \text{mol/L}\) demonstrated a clear dose-dependent inhibitory effect of calcium release in LAD2, basophils, and eosinophils \((n = 3-4 \text{ for each cell type, Figure S1})\). In contrast to PMX-53, ciprofloxacin dose-dependently \((20-200 \mu \text{g/mL}, \text{but not 400 } \mu \text{g/mL})\) enhanced MRGPRX2 surface expression on basophils and eosinophils (Figure 5A). Stimulation with ciprofloxacin \(200 \mu \text{g/mL}\) resulted in a 1.6-fold increase of %MRGPRX2\(^{pos}\) basophils \((\text{mean } \pm \text{ SEM } 32.69 \pm 8.19 \text{ vs } 51.83 \pm 5.17)\) and eosinophils \((23.11 \pm 4.46 \text{ vs } 36.65 \pm 2.71), P = 0.006\) for both, paired t test, \(n = 7\).

In basophils, there was a significant correlation of MRGPRX2-positive and Av.A488-positive cells (Figure 5B top, r\(^2\) = .688, \(P < .0001\)) but not in eosinophils (Figure 5B bottom, r\(^2\) = .032, \(P = .3852\)). In further experiments in basophils, dose-dependent ciprofloxacin enhanced MRGPRX2 expression was similar to enhanced CD63 expression (Figure 5C), whereas in eosinophils ciprofloxacin did not show significant effects on MRGPRX2 or CD69 \((n = 3)\). Direct stimulation with ciprofloxacin in the calcium mobilization assay demonstrated significant dose-dependent calcium influx in both, basophils and eosinophils (Figure 5D), at all tested concentrations \((basophils 20-400 \mu \text{g/mL} \text{ ciprofloxacin}: P < .05; \text{eosinophils at } 20-400 \mu \text{g/mL}: P < .001)\). We next tried to block ciprofloxacin effects in calcium mobilization assay by prior 10 minutes with anti-MRGPRX2 mAb at high concentrations \((10 \mu \text{g/mL})\). This resulted in a significant inhibition for ciprofloxacin at 100 \(\mu \text{g/mL}\) in basophils \((P < .05)\) and at 100 and 200 \(\mu \text{g/mL}\) (both \(P < .01)\) in eosinophils (Figure 5E), indicating that ciprofloxacin might mediate its effect by binding to MRGPRX2.

3.8 Grass pollen allergen-induced basophils and eosinophil CD63 and MRGPRX2 expression

Using multicolor granulocyte activation test with simultaneous labeling of FceRIα, CD63, and MRGPRX2 of whole blood of nine grass pollen-allergic donors, we were able to demonstrate a statistically significant enhancement of MRGPRX2 expression \((P < .05)\) after allergen stimulation with grass pollen extract \((0.01, 0.1, 1, \text{ and } 10 \text{ ng/mL})\) that was similar to increased CD63 expression (Figure 6A) and higher in basophils compared to eosinophils. Regression analysis (Figure 6B) demonstrated a correlation of grass pollen-induced MRGPRX2 and CD63 surface expression on basophils \((\text{RMSE: } 9.15, r^2 = .499, P < .0001)\) and eosinophils \((\text{RMSE: } 5.93, r^2 = .509, P < .0001)\).

4 DISCUSSION

Mas-related G protein-coupled receptor X2 (MRGPRX2) is regarded to be exclusively expressed by mast cells and to mediate non-IgE-dependent activation.\(^4\,10\) It’s role on host defense, chronic
inflammatory diseases such as urticaria and asthma, on drug-induced anaphylactoid reactions, neurogenic inflammation, itch, and pain is emerging. In this study, using a specific monoclonal antibody, we have demonstrated by flow cytometry, immunocytochemistry, immunofluorescence, and Western blot analysis that human peripheral blood basophils and eosinophils, but not neutrophils, consistently expressed MRGPRX2. Flow cytometric percentages of MRGPRX2 surface expression and geo MFI were not significantly different between atopic and nonatopic donors. Basophils and eosinophils of all donors investigated showed MRGPRX2 expression but the amount was heterogeneous as can be also seen from the whiskers in the outlier box plots shown in Figure 1A and the histograms of six donors in Figure 1B. Interestingly, very recently, in primary human mast cells isolated from different anatomic sites heterogeneous expression and function of MRGPRX2 receptor has been demonstrated.

Constitutive MRGPRX2 expression in basophils and eosinophils was confirmed by real-time PCR in all donors. To the best of our knowledge, this is the first demonstration of MRGPRX2 expression on basophils and eosinophils. In neutrophils, stimulation with fMLP for 30 minutes was not able to induce MRGPRX2 protein expression. However, in basophils and eosinophils stimulation with fMLP, IL-3 + IL-33, anti-IgE, and PMA for 30 minutes enhanced MRGPRX2 expression. In addition, in both cell types, IL-3, even in an optimal concentrations of 2 ng/mL, significantly enhanced MRGPRX2 protein expression.
FIGURE 5  Ciprofloxacin dose-dependently enhanced MRGPRX2 surface expression and induced degranulation and calcium influx. A, Ciprofloxacin 20-200 µg/mL but not 400 µg/mL enhanced % MRGPRX2-positive basophils and to a lesser extent eosinophils, *P < .05, paired t test. B, Double-staining of MRGPRX2 and Av.A488 demonstrated a significant correlation of MRGPRX expression (%MRGPRX<sup>pos</sup>) and degranulation (%Av.A488<sup>pos</sup>) in basophils but not eosinophils. C, In basophils (left figure), ciprofloxacin effects were similar in enhancing MRGPRX2 expression and CD63 expression, whereas in eosinophils (right figure) CD69 expression was not enhanced. C5a (0.1 µmol/L), anti-IgE (100 ng/mL), and fMLP (1 µmol/L) served as controls; Iso, isotype (IgG2b mAb), Med, medium control; Ci, ciprofloxacin n = 3. D, Stimulation with anti-MRGPRX2 mAb or ciprofloxacin significantly (as indicated) and dose-dependently induced calcium influx demonstrated by sum of fluorescence intensity counts over 0-60 s. C5a (0.1 µmol/L) served as positive control. Symbols represent individual subjects (n = 4-5). E, Ciprofloxacin-induced calcium influx could be significantly blocked by prior 10-min incubation with anti-MRGPRX2 mAb (10 µg/mL) at a concentration of 100 µg/mL (Ci 100) in basophils (n = 4) and of 100 and 200 µg/mL in eosinophils (n = 5). Bars ± SEM. *P < .05, **P < .01, without (w/o) vs with prior anti-MRGPRX2

FIGURE 6  Grass pollen extract induced significant and comparable MRGPRX2 and CD63 surface expression of basophils and eosinophils. A, Whole blood of nine grass pollen-allergic donors was stimulated with medium control (0), anti-FcεRI (FceRI, 330 ng/mL), fMLP (1 µmol/L), or grass pollen extract (0.01, 0.1, 1, 10 ng/mL) for 20 min and analyzed by tricolor granulocyte activation test (CCR3-FITC, CD63-PE, and MRGPRX2-APC). Bars show mean ± SEM of grass pollen induced % MRGPRX2-positive (black bars) and CD63-positive (bars with black diamonds) basophils (left figure) and eosinophils (right figure). Black stars for MRGPRX2, gray stars for CD63, *P < .05, **P < .01, ***P < .001 (Mann-Whitney rank sum test), all vs medium control (0). B, Regression analysis demonstrated significant correlation of MRGPRX2- and CD63-positive basophils (left figure, filled dots) and eosinophils (right figure, unfilled dots). RMSE, R<sup>2</sup>, F, and P values are indicated in the figure
Increases in calcium induce profound effects in granulocytes, including the initiation of cytoskeletal changes, degranulation, presentation of adhesion molecules, and oxidative burst. Calcium influx in human mast cells has been demonstrated using MRGPRX2 ligands²¹⁹ prompting us to investigate whether engagement of MRGPRX2 mobilizes intracellular calcium. Crosslinking of MRGPRX2 by a monoclonal antibody resulted in calcium mobilization in both, basophils and eosinophils. It should be noted that in both granulocyte subtypes MRGPRX2 crosslinking before or after was capable to further enhance calcium influx induced by potent stimuli such as anti-IgE and fMLP. These data indicate that ligation of MRGPRX2 on basophils and eosinophils could enhance the activating effects of exposure (before and after) to non-IgE- and IgE-dependent stimuli. Whether this effect is additive or synergistic has to be further investigated. We demonstrated anti-IgE and anti-FcεRI mAb-induced calcium influx in both, basophils and eosinophils. Whereas expression of the low-affinity IgE receptor, CD23, on eosinophils is known to be involved in activation and degranulation, expression of FcεRI on the surface of human eosinophil is still controversial.²²²³ The majority of studies suggest that surface expression is low. However, eosinophil expression of FcεRI is reported in diseases associated with elevated circulating IgE and eosinophilia.²⁴⁻²⁹ The putative interaction between IgE and eosinophils is a primary focus in current studies in bullous pemphigoid.²⁶ Moreover, these authors have previously demonstrated occasionally co-expression of FcεRI and IgE on enriched peripheral eosinophils from controls and within normal skin. Moreover, robust staining was observed when surface-bound IgE was evaluated by flow cytometry.

Next, we assessed the effects of sequential stimulation in the calcium mobilization assay and found no evidence for desensitization or internalization of MRGPRX2, a feature of many GPCRs.³⁰ This is in line with data demonstrating no evidence for internalization of MRGPRX2 in a human MRGPRX2 transfected mast cell line (HMC-1) using mast cell MRGPRX2 agonists.¹⁹ Using the same concentration, a second sequential stimulation with anti-MRGPRX2 55 seconds after the first stimulation resulted in a similar (eosinophils) and even increased (basophils) calcium mobilization compared to the first stimulation. This indicates that MRGPRX2 activation might have significant pro-inflammatory impact in allergic or nonallergic inflammation.

To address activation and degranulation, we used a recently described fluorescent avidin-based method that according to its discoverers provides better results in monitoring mast cell²⁵ and basophil degranulation when compared with CD63 expression.¹⁶¹⁷ In addition to basophils, we investigated eosinophils and showed for the first time that this method is also applicable for eosinophils. In contrast to medium control and anti-IgG2b control antibody, stimulation with anti-MRGPRX2 antibody dose-dependently resulted in increased fluorescence intensity of Av:A488 reflecting degranulation in purified basophils and eosinophils. The linear-linear dose response of MRGPRX2 was different from that of an allergen, where we have a linear-logarithmic response.

Moreover, MRGPRX2 engagement by its mAb resulted in enhanced basophil and eosinophil survival and delay of apoptosis as revealed by Annexin V/PI staining and trypan blue dye exclusion. Analyzing basophil and eosinophil supernatants of the viability/apoptosis experiments, we found significant amounts of IL-3, IL-5, and GM-CSF after MRGPRX2 ligation with its mAb. It is well known that these cytokines rapidly and transiently activate several canonical signaling pathways in both basophils and eosinophils in a manner that is consistent with the capacity of these cytokines to promote some early cellular functions in both cell types.³¹ We now provide evidence that engagement of MRGPRX2 is able to prime basophils and eosinophils that thereafter might be able to respond to stimuli that by itself are not able to result in mediator release.

Confirming Western blot results by flow cytometry analysis, we showed that low concentrations of IL-3 significantly and dose-dependently upregulated MRGPRX2 surface expression after 30 minutes in both granulocyte types. Our data pointing to a pivotal role of IL-3 in enhancing MRGPRX2 expression are in line with recent observations that IL-3 transcriptionally regulates surface levels of FcεRI in human primary basophils.³² Moreover, in most assays measuring basophil functions, IL-3 appears to be among the most potent (complete or incomplete) agonists³¹,³³ and is also a well-known agonist for eosinophils.³¹,³² At least, the normal circulating basophil is balanced between a state of high and low IL-3 exposure and thus is very sensitive to changes in IL-3.³⁴

Among different known potent basophilic and eosinophilic stimuli, incubation for 24 hours with anti-IgE, C5a, fMLP, and IL-3 in basophils and IL-3, IL-5, and IL-33 in eosinophils was able to significantly upregulate MRGPRX2 surface expression. It is tempting to speculate that, in vivo MRGPRX2 surface expression might be upregulated by a certain cytokine milieu in the peripheral blood. However, we did not find a significant difference of MRGPRX expression on fresh peripheral blood granulocytes among atopic and nonatopic donors with inhalant allergy or atopic dermatitis in which at least eosinophils are known to be primed.³⁵,³⁶ However, subdivision according to phenotype was not performed in all experiments in this study and it should be considered that we found a consistent but heterogeneous amount of MRGPRX2 expression.

We also investigated the effect of PMX-53 which has been demonstrated not only to act as C5a receptor antagonist but also as agonist for MRGPRX2 in human mast cells.⁹ PMX-53 at concentration ≥30 nmol/L caused degranulation and at 100 nmol/L and 1 µmol/L calcium mobilization in LAD2 mast cells, CD34 cell-derived mast cells, and RBL-2H3 cells stably expressing MRGPRX2.⁹ PMX-53 is a potent C5a receptor antagonist in human neutrophils and macrophages in vitro²⁷,²⁸ but no effects of PMX-53 on basophils and eosinophils have been described so far, although both are expressing C5a receptors⁹,⁶⁰ and although PMX-53 has reached phase I clinical trials.⁴¹ However, we did not find any effects of PMX-53 (10 and 100 nmol/L) on basophils or eosinophils by means of calcium influx, activation/degranulation, viability/apoptosis, or modulation of MRGPRX2 surface expression. Functionality of these concentrations of PMX-53 was shown by inhibition of
C5a-induced calcium influx in both cell types and LAD2 mast cells (Figure S1).

In contrast, ciprofloxacin at concentrations of 20-200 µg/mL, another known mast cell MRGPRX2 agonist, significantly enhanced MRGPRX2 surface expression and induced degranulation and calcium influx in basophils and to a lesser extent in eosinophils. Double-staining of MRGPRX2 and Av.A488 demonstrated a significant correlation of enhanced MRGPRX expression and degranulation in basophils but not eosinophils. Further experiments showed that ciprofloxacin-induced MRGPRX2 expression was associated with increased CD63 expression in basophils but not CD69 expression in eosinophils. Ciprofloxacin concentrations are in line with concentrations used in mouse mast cells and diagnostic basophil activation test (BAT) in ciprofloxacin hypersensitive subjects. Our blood donors were not sensitized to ciprofloxacin, some of them were exposed, some not. The data show that ciprofloxacin generally activates basophils. This is reflected by difficulties to identify quinolone sensitive patients by basophil activation test or skin tests. Immediate type reactions to quinolones such as ciprofloxacin are suggested to be IgE-mediated but this has never been clearly established. Skin tests often induce false-positive results, probably because of the capacity to directly induce histamine release, and commercial in vitro test are not well validated.

Most studies diagnosed immediate hypersensitivity solely by positive skin test and/or positive basophil activation test, but did not prove this by drug provocation test. Looking at recent more reliable data using the gold standard drug provocation, basophil activation test was not regarded as useful diagnostic tool. Confirming our data, Fernandez et al demonstrated basophil CD63 upregulation in basophil activation tests of nonhypersensitive controls using similar ciprofloxacin concentrations.

It has been shown in human LAD2 mast cell line that ciprofloxacin induced calcium influx. In our hands, in both granulocyte subtypes, direct stimulation with ciprofloxacin significantly and dose-dependently induced calcium influx that could be significantly blocked by prior blocking with anti-MRGPRX2 mAb indicating that ciprofloxacin might act through MRGPRX2 receptor. However, this has to be studied in more detail in future experiments.

Using grass pollen stimulation in multicolor granulocyte activation test of whole blood, we were able to demonstrate a dose-dependent comparable and correlated enhancement of MRGPRX2 expression and CD63 expression by ciprofloxacin. These preliminary data give evidence that in vivo human basophil MRGPRX2 might not only play a role in IgE-independent activation as has been previously described for human mast cells, but also in IgE-dependent mechanisms. Recently, it has been speculated that MRGPRX1 and MRGPRX2 may contribute to house dust mite allergy as stimulation of mouse mast cells with Der p 1 resulted in activation of MRGPRX1, MrgrpC11, and PAR2 as determined by ratiometric calcium imaging with Fura2. However, no effects of Der p 1 were demonstrated in MRGPRX2 transfected human HeLa cells. With regard to ciprofloxacin in subjects with hypersensitivity, the role of specific IgE is debated. Future experiments might clarify whether addressing MRGPRX2 is useful for diagnostic purposes in allergic and pseudoallergic reactions like CD63 or CD203c. In addition, it might be interesting to investigate whether anergic nonresponder basophils to anti-FceRI respond to MRGPRX2 engagement.

Taken together, our results clearly show constitutive and IgE- and non-IgE-dependent stimulated expression of MRGPRX2 by human peripheral blood basophils and eosinophils. Engagement of MRGPRX2 is associated with pro-inflammatory basophil and eosinophil effects such as calcium mobilization, enhanced survival, and degranulation with cytokine release. Among the cytokines released, at least IL-5 and, particularly, IL-3 were shown to further enhance MRGPRX2 expression which might result in a pro-inflammatory vicious circle. Moreover, calcium mobilization assay demonstrated that MRGPRX2 activation is able to further enhance effects of potent calcium inducing stimuli. It is tempting to speculate that activation of MRGPRX2 might enhance IgE- and non-IgE-mediated inflammatory reactions. However, for basophils and eosinophils endogenous ligands still have to be defined. Moreover, the localization of MRGPRX2 in resting allergic effector cells (eg in CD203c-associated granules or CD63-associated secretory lysosomes) should be examined. In view of our results, the role of MRGPRX2 upregulation and function should be further assessed not only in human pseudoallergic anaphylactoid but also in IgE-mediated allergic reactions. As has been shown in human and mouse mast cells, MRGPRX2 recognizes a wide range of basic molecules, unlike most GPCRs. There still might be several unknown ligands for this receptor.54 Activation not only of mast cell but also of basophil and eosinophil MRGPRX2 might contribute to hypersensitivity reactions. Nevertheless, at the moment, most data indicating MRGPRX-mediated anaphylactoid reactions are derived from mouse models or cell lines. Recently, human skin mast cell MRGPRX2 upregulation has been demonstrated in severe chronic spontaneous urticaria, a disease in which the role of specific IgE against allergens is negligible and against autoallergens debated. Not only mast cells, but also basophils and eosinophils are increasingly regarded as potent effector cells in chronic urticaria. Therefore, it will be very interesting to investigate the role of MRGPRX2 expression of basophils and eosinophils in the peripheral blood and skin of different urticaria subtypes. Fujisawa et al were not able to identify a responsible stimulus for upregulation of MRGPRX2 on human skin mast cells in vitro by assessing histamine, SP, epithelium-derived cytokine IL-33, and thymic stromal lymphopoietin (TSLP).

It is of outmost importance to know that MRGPRX2 is not exclusively expressed on human mast cells but also on basophils and eosinophils and that it mediates degranulation. Deciphering the downstream signaling mechanisms of MRGPRX2 in basophils and eosinophils might enable the development of new therapeutic strategies to prevent or inhibit allergic and nonallergic hypersensitivity. Moreover, targeting MRGPRX2 might have potential for diagnostic purposes in (drug) hypersensitivity.
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CONFLICTS OF INTEREST
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS
BW designed, analyzed, and supervised experiments, and wrote the manuscript. MG performed, and analyzed experiments, and contributed to writing of the method section. AK contributed to the analysis interpretation and edited the manuscript. All authors revised and approved the final version.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.