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**Title:**

**The pseudoallergen receptor MRGPRX2 on peripheral blood basophils and eosinophils: expression and function.**

**Short running title:**

MRGPRX2 expression by basophils and eosinophils

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## **Abstract** (248 words)

**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 non-atopic donors. To assess functional activity, fluorescent-avidin-based degranulation assay, calcium mobilization, cytokine production in supernatants, assessment of viability/apoptosis and tricolour 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 tricolour granulocyte activation test using grass pollen demonstrated MRGPRX2 upregulation associated with degranulation and CD63 expression.

**Conclusion:** Unravelling 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 has potential for diagnostic purposes in (drug) hypersensitivity.

## **Key words (5):**

MRGPRX2, basophil, eosinophil, function, ciprofloxacin

## Abbreviations

## Abbreviations

|                           |                                                  |
|---------------------------|--------------------------------------------------|
| $\alpha$                  | anti                                             |
| APC                       | allophycocyanin                                  |
| AT                        | atopic                                           |
| Av.A488                   | alexaFluor488-labeled avidin                     |
| C5a                       | complement 5a                                    |
| CD                        | cluster differentiation                          |
| Ci                        | ciprofloxacin                                    |
| DAB                       | 3,3'-Diaminobenzidine                            |
| DAPI                      | 4',6-diamidino-2-phenylindole                    |
| F                         | F-test                                           |
| FACS                      | fluorescence activated cell sorter               |
| Fc $\epsilon$ RI $\alpha$ | high affinity IgE receptor alpha chain           |
| FEIA                      | fluorescence enzyme immunoassay                  |
| FITC                      | fluorescein isothiocyanate                       |
| FLUO-4 AM                 | fluo-4 acetoxymethyl ester                       |
| fMLP                      | n-formyl-Met-Leu-Phe chemotactic peptide         |
| geo                       | geometric                                        |
| GM-CSF                    | granulocyte macrophage colony stimulating factor |
| HRP                       | horse-radish peroxidase                          |
| ICC                       | immunocytochemical                               |
| IF                        | immunofluorescence                               |
| IL-                       | interleukin-                                     |
| iso                       | isotype                                          |
| mAb                       | monoclonal antibody                              |
| med                       | medium                                           |
| MFI                       | mean fluorescence intensity                      |
| min                       | minutes                                          |
| MRGPRX2                   | mas-related G protein coupled receptor X2        |
| NA                        | non-atopic                                       |
| <sup>neg</sup>            | negative                                         |
| p                         | calculated probability                           |

|                |                                                 |
|----------------|-------------------------------------------------|
| PE             | phycoerythrin                                   |
| PerCP          | peridinin chlorophyll protein complex           |
| PI             | propidium iodide                                |
| PMA            | phorbol 12-myristate 13-acetate                 |
| PMX-53         | Ac-Phe-cyclo(Orn-Pro-D-Cha-Trp-Arg              |
| pos            | positive                                        |
| R <sup>2</sup> | R Squared                                       |
| RMSE           | root mean square error                          |
| RNA            | ribonucleic acid                                |
| RPMI           | Roswell Park Memorial Institute medium          |
| RT-PCR         | reverse transcription polymerase chain reaction |
| SCF            | stem cell factor                                |
| sec            | seconds                                         |
| TBST           | Tris-buffered saline with Tween20               |

## Main text

Mas-related G protein coupled receptor X2 (MRGPRX2, formerly known as MRGX2), whose adaptive evolution in humans was a relatively recent event <sup>1</sup>, 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<sub>TC</sub>) in the dermis of the skin or submucosa of the gut <sup>2,3</sup>. 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 <sup>3-8</sup>. Identified ligands for MRGPRX2 in mast cells and mast cell lines are neuropeptides (e. g. Substance P, cortistatin), antimicrobial host defence peptides (e.g. LL37), eosinophil granule proteins (e. g. major basic protein, eosinophil peroxidase), and peptidergic drugs (e. g. ciprofloxacin, icatibant) <sup>3,4,9</sup>. It was anticipated that targeting MRGPRX2 for example by small molecules might reduce a subset of drug-induced adverse effects <sup>6</sup>. However, the exact role of MRGPRX2 in mast cells and its validated endogenous ligands are 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 <sup>4,10</sup>. 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 non-allergic inflammation, as well as in innate and adaptive immunity <sup>11</sup>. Therefore, in this study, we aimed to investigate whether human basophils and eosinophils express MRGPRX2 and, if present, whether it possesses a functional capacity.

## Materials and Methods

### Antibodies

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

### **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 non-atopic volunteers (no personal history of atopy, negative atopy screen sxl Phadiatop®, CAP-FEIA, Thermo Fisher Scientific), patients with inhalant allergy, (personal history of rhinoconjunctivitis and/or allergic asthma and specific IgE to aeroallergens) and extrinsic atopic dermatitis (personal history of atopy, high levels of serum IgE, and specific IgEs to food or aeroallergens) were purified using density gradient centrifugation, erythrocyte hypotonic lysis, and immunomagnetic negative selection, as described <sup>12-14</sup>. For eosinophils, immunomagnetic negative selection via CD16 MicroBeads (Milteny Biotec, Bergisch Gladbach, Germany) was used for most experiments, for some we used EasySep™ Human Eosinophil Enrichment (Stem Cell Technologies, Grenoble, France). For basophils, for most experiments we used a two-step isolation (immunomagnetic negative selection and thereafter positive selection via CD123 MicroBeads, Diamond Basophil Isolation Kit, Miltenyi Biotec, Bergisch Gladbach, Germany) and for some experiments we used EasySep™ Human Basophil Enrichment Kit (Stem Cell Technologies, Grenoble, France). 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 with trypan blue, respectively. 1 x 10<sup>5</sup> cells were cultured for indicated time periods at 37 °C in 5% CO<sub>2</sub> incubator in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 0,5% Gentamicin, 1% L-Glutamine, non-essential amino acids (all from Biochrom GmbH, Berlin, Germany) in the absence or presence of goat anti-human-IgE (100 ng/ml, ε-chain specific, I6284), n-formyl-Met-Leu-Phe chemotactic peptide (1 μM), complement C5a (0,1-0,5 μM), dexamethasone (1 μM), ciprofloxacin (20-400 μg/ml) (all from Merck Sigma-Aldrich St. Louis, USA), anti-FcεRIα (0,33 μg/ml; Bühlmann Laboratories, Schönenbuch, Switzerland), interleukin (IL)-33 (20 ng/ml), (from PeproTech, New York, USA), IL-3 (1-20 ng/ml, ImmunoTools, Friesoythe, Germany), IL-5 (10 ng/ml), and PMX-53 (10, 100, 1000 nM, R&D Systems/Tocris). In some experiments a combination of stimuli were used as indicated. The concentration of the stimuli was used according to published results demonstrating functional activity in basophils/eosinophils and/or after own optimal concentration testing.

The human mast cell line LAD2 (kindly provided by Dr. D. Metcalfe, National Institutes of Health, Bethesda, MD, USA) was grown in Stem Pro-34 growth medium (Invitrogen) containing Stem Pro-34 nutrient supplement, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine and 100 ng/ml of stem cell factor (SCF) (Sigma-Aldrich) at 37 °C in 5% CO<sub>2</sub>.

### **Cytospin Preparation**

To perform cytopins,  $1 \times 10^5$  cells were washed in PBS and resuspended in 150  $\mu$ l PBS. Cell suspensions were centrifuged onto slides (500 rpm, 5 min) using a Cytospin-3 cytocentrifuge (Shandon Southern Instruments, Sewickley, PA, USA). As control we prepared cytopins with the human mast cell line LAD2 ( $1,5 \times 10^5$  cells).

### **Immunocytochemistry and immunofluorescence**

Immunocytochemical (ICC) and immunofluorescence (IF) staining of cytopins was performed using mouse anti-human MRGPRX2 antibody (1: 20  $\equiv$  25  $\mu$ g/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 nM, (Molecular Probes, Invitrogen, ThermoScientific) for 5 min 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, Waldbronn, Germany) 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, Hamburg, Germany).

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

### **Flow cytometric analysis for surface receptor expression**

Flow cytometric surface staining procedures have been described previously<sup>12-14</sup>. The expression of surface expression was assessed using a PE-conjugated mouse anti-MRGPRX2 antibody (15  $\mu$ g/ml, clone K125H4, isotype IgG2b, Biolegend) or the respective isotype as described. Staining was performed for 1h at 4 °C.

Since all experiments were performed with highly purified cells ( $\geq 98\%$ ) no specific gating antibodies were required. Cells were analysed using a BD FACSCanto II platform and BD Cell Quest™ Pro software (Becton Dickinson, San Jose, CA, USA). 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.

### **Western blot**

1 x 10<sup>6</sup> eosinophils, basophils or neutrophils (purity 99%) were stimulated with medium (control), IL-3 (0,1 ng/ml to 10 ng/ml) , fMLP (1 µM), IL-3 (10 ng/ml) + IL-33 (20 ng/ml), anti-IgE (100 ng/ml) or PMA (10 ng/ml) for 30 min and then lysed as well as 0,5 x 10<sup>6</sup> LAD2 cells in M-Per Mammalian Extraction Reagent (Pierce/ ThermoScientific). 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, CA, USA). Proteins were transferred to a nitrocellulose membrane (Pierce/ThermoScientific), blocked with blocking buffer (1 x TBST with 5 % BSA) and immunoblotted with polyclonal mouse anti-MRGPRX2 antibodies (1:500; Abnova, Taipei, Japan) followed by HRP-conjugated goat anti-mouse secondary antibodies (1:2000, Cell Signaling Technology, Danvers, MA, USA). Mouse anti-human histone H3 (clone mAbcam 24834 Nuclear Loading Control) monoclonal antibodies (1:1000, Abcam, Cambridge, UK) were used to detect housekeeping protein. The blots were visualised 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, Japan). Detected bands were quantified using Image J image analysis software (National Institutes of Health).

### **RNA isolation and RT-PCR**

mRNA was extracted from highly purified (≥ 98%) non-stimulated or stimulated (as indicated) human basophils (1-2 x 10<sup>6</sup>) or eosinophils (2 x 10<sup>6</sup>) using an RNeasy Plus Micro Kit (Quiagen, Germantown, MD) according to the manufacturer's protocol. cDNA synthesis was performed using a QuantiTect Reverse Transcription Kit (Quiagen, Germantown, MD). Yield and purity of extracted RNAs and synthesized cDNAs were assessed using a Nanodrop 2000 spectrophotometer at 260 and 280 nm (Pqlab/ThermoScientific). RT-PCR conditions followed standard protocols, a Rotor-Gene Q MDx cyclor (software 2.1.0.9) being used (Quiagen). KiCqStart® SYBR® Green Primer sequences for human MRGPRX2 were purchased from SIGMA-Aldrich (H\_MRGPRX2\_2) and for Ribosomal Protein S20 (RPS20) from Quiagen. After amplification reaction, products were controlled and separated on 2% agarose gels, stained with ethidium bromide, and photographed under UV illumination (not shown).

### **Calcium influx**

Fluorescence (excitation, 485 nm; emission, 520 nm) was measured as intensity counts per second for 120 s at 24.8-25.0 °C in the FluoStar plate reader (BMG Lab Technologies GmbH, Jena, Germany). 5 x 10<sup>5</sup> purified (≥99%) basophils or eosinophils were labelled with the green fluorescent calcium indicator FLUO-4AM (Invitrogen GmbH, Karlsruhe, Germany) and after 3-5

sec of measurement stimulated with either medium control, C5a (0,1  $\mu$ M), C5a (0,1  $\mu$ M) + IL-3 (10 ng/ml), anti-IgE (100 ng/ml), or fMLP (1  $\mu$ M) with or without 30 min pre-incubation with mouse anti-MRGPRX2 monoclonal antibodies (Biolegend 2,5  $\mu$ g/ml). A control antibody of the same isotype (mouse anti-IgG2b) did not result in calcium mobilization (data not shown).

### **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<sup>15</sup> and basophil degranulation when compared with CD63 expression<sup>16, 17</sup>. Avidin directly stains cell-bound granules upon degranulation and the AlexaFluor488-labeled avidin (Av.A488) fluorescence intensity provides a measure of the degranulation magnitude<sup>17</sup>. 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 detail<sup>16</sup> using a final avidin-Alexa488 concentration of 2,5  $\mu$ g/ml. Anti-IgG2b isotype was used as control antibody. After first experiments demonstrated no difference between a concentration of 2,5  $\mu$ g/ml and 5  $\mu$ g/ml we used 2,5  $\mu$ g/ml in further experiments.

### **Flow cytometric Annexin V / propidium iodide staining**

Basophil and eosinophil (2 x 10<sup>5</sup> 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  $\mu$ M) as controls was assessed by annexin V/ propidium iodide staining (Annexin V kit, Pharmingen, San Diego, USA) as previously described<sup>18</sup>.

### **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 pg/ml to 2000 pg/ml), IL-5 (R & D, detection range 23.44 pg/ml to 1500 pg/ml), and GM-CSF (R & D, detection range 15.6 pg/ml to 1000 pg/ml), by ELISA technique according to the manufacturers instructions.

### **Multicolour 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  $\geq$ 2, ImmunoCAP®, ThermoScientific). 100  $\mu$ l whole blood was incubated with stimulation buffer (control) for 10 min at 37 °C, then either stimulation buffer (control), 7 grass mix (Meadow Fescue, Orchard, Redtop, Perennial Rye, Sweet Vernal, Kentucky Blue, and Timothy, in equal parts, 100,000 BAU/ml  $\cong$  600

µg/ml) (BASOTEST™, BD Bio-Science/Glycotope Biotechnology) at indicated concentrations or anti-FcεRI (0.33 µg/ml) or fMLP (1 µM) as positive controls were added for 20 min at 37 °C. Degranulation was stopped by incubation on ice for 5 min. Cells were incubated for 20 min on ice with FcεRIα-PerCP, CD63-PE and MRGPRX2-APC, thereafter lysed and fixed and immediately analysed using a BD FACSCanto II platform and BD Cell Quest™ Pro software (Becton Dickinson, San Jose, CA, USA). Per sample at least 1.000 cells expressing low (eosinophils) or high amounts of FcεRIα (basophils) were live gated.

## Results

### **MRGPRX2 is constitutively expressed on peripheral blood basophils and eosinophils**

Using flow cytometry (Fig. 1 A, B), immunocytochemistry and immunofluorescence (Fig. 1 C) 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 <sup>19</sup>. There was no apparent difference of 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 non-atopic subjects (Fig. 1 A). Pooling atopic and non-atopic results (n = 19-27) there was no statistically significant difference between basophils and eosinophils regarding %MRGPRX2<sup>pos</sup> 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 non-parametric Wilcoxon/Kruskal-Wallis test.

Constitutive MRGPRX2 protein and gene expression was confirmed in both, freshly isolated and unstimulated basophils and eosinophils, by western blot analysis (Fig. 1 D to 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 µM), a combination of IL-3 (10 ng/ml) + IL-33 (30 ng/ml), anti-IgE (100 ng/ml), and PMA (10 ng/ml) (Fig. 1 D and G). In contrast, pooled neutrophils (n = 7) did not express MRGPRX protein and MRGPRX2 protein was not induced by stimulation with fMLP (1 µM). 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 Fig. 1 E. 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 1 F. Moreover, 30 min stimulation with IL-3 dose-dependently increased MRGPRX2 protein in both, basophils and eosinophils (Fig 1 H, n = 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 (Fig. 2 A, C-E) that was less compared to C5a (0,1  $\mu$ M, not shown), anti-IgE (100 ng/ml) and fMLP (1  $\mu$ M) as shown in Fig. 2 C, D, and E). However, in co-stimulation with anti-MRGPRX2 mAb calcium influx was potentiated (Fig. 2 C, D, and E). Moreover, calcium influx induced by stimulation with anti-MRGPRX2 mAb (2,5  $\mu$ g/ml), anti-IgE, anti-Fc $\epsilon$ RI mAb (2,5  $\mu$ g/ml) or fMLP (1  $\mu$ M) after 3-5 sec could be again enhanced by a second stimulation with anti-MRGPRX2 mAb (2,5  $\mu$ g/ml) after 60 sec (Fig. 2 B). 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, i. e. fMLP or anti-MRGPRX2-mAb.

### **Degranulation induced by MRGPRX2 antibodies**

Basophil and eosinophil activation was determined by using a new fluorescent-avidin-based method reflecting degranulation in basophils<sup>16, 17</sup>. Avidin directly stains cell-bound granules upon degranulation and the AlexaFluor488-labeled avidin (Av.A488) fluorescence intensity provides a measure of the degranulation magnitude. Cross-linking of MRGPRX2 by anti-MRGPRX2 mAb, but not by anti-IgG2b isotype control for 30 min resulted in a significant concentration-dependent increase of Av.A488 fluorescence positive cells reflecting degranulation (Fig. 3 A), \* $p < 0,05$ , \*\* $p < 0,01$ , paired t-test versus medium and isotype. Figure 3 B demonstrates spaghetti plots of all eight individual donor experiments and Figure 3 C and D a representative dot plot of anti-MRGPRX2 mAb (concentration as indicated) induced Av.A488 staining of basophils (C) and eosinophils (D).

### **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  $\mu$ M). In contrast to IgG2b isotype anti-MRGPRX2 mAb (2,5  $\mu$ g/ml) significantly promoted survival after 24 h in basophils and eosinophils as assessed by Annexin V/PI staining (Fig. 4 A) and trypan blue dye

exclusion (Fig. 4 B), \* $p < 0,05$ , \*\* $p < 0,01$  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 Fig. 4 C.

### **Cytokine release after MRGPRX2 engagement**

Assessing basophil and eosinophil cytokine release in the supernatants of the viability/apoptosis assay, we found that 24 h 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 (Fig. 4 D, black bars,  $n = 6$ , Wilcoxon signed rank test). P-values for IL-3 in basophils at 1  $\mu\text{g/ml}$ : \* $p < 0,05$ , at 2,5  $\mu\text{g/ml}$ : \*\* $p < 0,01$ ; in eosinophils at 1  $\mu\text{g/ml}$  and at 2,5  $\mu\text{g/ml}$ : \* $p < 0,05$ , for IL-5 in basophils at 1  $\mu\text{g/ml}$ : n.s., at 2,5  $\mu\text{g/ml}$ : \*\*\* $p < 0,001$ ; in eosinophils at 1  $\mu\text{g/ml}$ : \* $p < 0,05$  and at 2,5  $\mu\text{g/ml}$ : \*\*\* $p < 0,001$ , and for GM-CSF in basophils at 1  $\mu\text{g/ml}$ : \* $p < 0,05$ , at 2,5  $\mu\text{g/ml}$ : \*\*\* $p < 0,001$ ; in eosinophils at 1  $\mu\text{g/ml}$  and at 2,5  $\mu\text{g/ml}$ : \*\* $p < 0,01$ . Moreover, in eosinophils even after 4 h significant amounts of IL-3 (at 1  $\mu\text{g/ml}$  and at 2,5  $\mu\text{g/ml}$ : \* $p < 0,05$ ) and GM-CSF (at 1  $\mu\text{g/ml}$  and at 2,5  $\mu\text{g/ml}$ : \* $p < 0,05$ ) were found (Fig. 4 D, bottom figure, horizontal striped bars,  $n = 6$ , Wilcoxon signed rank sum test). Basophils could not be assessed after 4 h 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 (\*\* $p < 0,01$ ), whereas after 24h in eosinophils release of all, IL-3, IL-5 (\* $p < 0,05$ ), and GM-CSF (\*\* $p < 0,01$ ) was reduced, in addition to GM-CSF after 4 h (\* $p < 0,05$ ).

### **Modulation of surface MRGPRX2 expression**

A short incubation for 30 min with IL-3 (0,1, 0,5 , 1, 2, 5 and 10 ng/ml) dose-dependently and significantly (\* $p < 0,05$  for each concentration of IL-3 from 0,1 ng/ml to 10 ng/ml versus incubation without IL-3, paired t-test) upregulated MRGPRX2 surface expression of basophils (optimal concentration 2 ng/ml) (Fig. 4 E,  $n = 3$ ) and eosinophils (optimal concentration 2-4 ng/ml) (Fig. 4 F,  $n = 4$ ).

In the next series of experiments, we examined the effects of several known basophil and eosinophil-activating stimuli on MRGPRX2 expression after 24 h of incubation at 37 °C. As shown in Fig. 4 G, MRGPRX2 surface expression was significantly upregulated in basophils ( $n = 9-13$ )

by 24 h stimulation with anti-IgE (100 ng/ml,  $p=0,005$ , Mann-Whitney Rank Sum test), C5a (0,1  $\mu$ M,  $p<0,001$ ), fMLP (1  $\mu$ M,  $p<0,001$ ), and IL-3 (10 ng/ml,  $p<0,001$ ), whereas no effect was found by IL-5 (10 ng/ml), IL-33 (20 ng/ml), or anti-Fc $\epsilon$ RI mAb (330 ng/ml).

Moreover, after 24 h stimulation of eosinophils MRGPRX2 surface expression was significantly upregulated by IL-3, IL-5, and IL-33, but not by anti-IgE, anti-Fc $\epsilon$ RI mAb, C5a or fMLP (concentrations like in basophils,  $n = 6$ ,  $p<0,05$ , Mann Whitney Rank Sum test, Fig. 4 H).

### **Evaluation of effects of mast-cell MRGPRX2 agonists, PMX-53 and ciprofloxacin, on basophils and eosinophils**

PMX-53 (10 nM, 100 nM, and 1000 nM) did not result in a modulation of MRGPRX2 expression, calcium influx, degranulation or survival in basophils or eosinophils ( $n = 4-5$ , data not shown). Functionality of the PMX-53 preparation was proven by demonstrating an immediate dose-dependent (50 nM, 100 nM, 1  $\mu$ M, 10  $\mu$ M, but not 10 nM) calcium mobilization in LAD2 cells as previously demonstrated<sup>9</sup>. Moreover, being a C5aR antagonist, PMX-53 given 55 sec before C5a  $10^{-8}$  M demonstrated a clear dose-dependent inhibitory effect of calcium release in LAD2, basophils and eosinophils ( $n = 3-4$  for each cell type, supplemental Fig. S1). In contrast to PMX-53, ciprofloxacin dose-dependently (20-200  $\mu$ g/ml, but not 400  $\mu$ g/ml) enhanced MRGPRX2 surface expression on basophils and eosinophils (Fig. 5 A). Stimulation with ciprofloxacin 200  $\mu$ g/ml resulted in a 1,6-fold-increase of %MRGPRX2<sup>pos</sup> basophils (mean  $\pm$  SEM  $32,69 \pm 8,19$  vs.  $51,83 \pm 5,17$ ) and eosinophils ( $23,11 \pm 4,46$  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 (Fig. 5 B top,  $R^2 = 0,688$ ,  $p<0,0001$ ) but not in eosinophils (Fig. 5 B bottom,  $R^2 = 0,032$ ,  $p=0,3852$ ). In further experiments in basophils, dose-dependent ciprofloxacin enhanced MRGPRX2 expression was similar to enhanced CD63 expression (Fig. 5 C) 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 (Fig. 5 D), at all tested concentrations (basophils at 20-400  $\mu$ g/ml ciprofloxacin:  $p<0,05$ ; eosinophils at 20-400  $\mu$ g/ml:  $p<0,001$ ). We next tried to block ciprofloxacin effects in calcium mobilization assay by prior 10 min with anti-MRGPRX2 mAb at high concentrations (10  $\mu$ g/ml). This resulted in a significant inhibition for ciprofloxacin at 100  $\mu$ g/ml in basophils ( $p<0,05$ ) and at 100  $\mu$ g/ml and 200  $\mu$ g/ml (both  $p<0,01$ ) in eosinophils (Fig. 5 E), indicating that ciprofloxacin might mediate its effect by binding to MRGPRX2.

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

Using multicolor granulocyte activation test with simultaneous labelling of FcεRIα, CD63 and MRGPRX2 of whole blood of nine grass pollen allergic donors we were able to demonstrate a statistically significant enhancement of MRGPRX2 expression ( $p < 0,05$ ) after allergen stimulation with grass pollen extract (0,01, 0,1, 1, and 10 ng/ml) that was similar to increased CD63 expression (Fig. 6 A) and higher in basophils compared to eosinophils. Regression analysis (Fig. 6 B) demonstrated a correlation of grass pollen induced MRGPRX2- and CD63 surface expression on basophils (RMSE: 9,15,  $R^2 = 0,499$ ,  $p < 0,0001$ ) and eosinophils (RMSE: 5,93,  $R^2 = 0,509$ ,  $p < 0,0001$ ).

### 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<sup>4, 10</sup>. 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<sup>4, 20</sup>.

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 non-atopic 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 Fig. 1 A and the histograms of six donors in Fig. 1 B. Interestingly, very recently, in primary human mast cells isolated from different anatomic sites heterogeneous expression and function of MRGPRX2 receptor has been demonstrated<sup>21</sup>. 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 min 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 min 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.

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 ligands<sup>2, 19</sup>

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 <sup>22, 23</sup>. 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 <sup>24-29</sup>. The putative interaction between IgE and eosinophils is a primary focus in current studies in bullous pemphigoid <sup>26</sup>. 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 <sup>30</sup>. 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 <sup>19</sup>. Using the same concentration, a second sequential stimulation with anti-MRGPRX2 55 sec 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 non-allergic 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 cell <sup>15</sup> and basophil degranulation when compared with CD63 expression <sup>16, 17</sup>. 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. Analysing 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 signalling 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<sup>31</sup>. 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 min 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<sup>32</sup>. Moreover, in most assays measuring basophil functions, IL-3 appears to be among the most potent (complete or incomplete) agonists<sup>31, 33</sup> and is also a well-known agonist for eosinophils<sup>31, 32</sup>. 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<sup>34</sup>.

Among different known potent basophilic and eosinophilic stimuli, incubation for 24 h with anti-IgE, C5a, fMLP, and IL-3 in basophils, and IL-3, IL-5, and IL-33 in eosinophils, were 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 non-atopic donors with inhalant allergy or atopic dermatitis in which at least eosinophils are known to be primed<sup>35, 36</sup>. 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<sup>9</sup>. PMX-53 at concentration  $\geq 30$  nM caused degranulation and at 100 nM and 1  $\mu$ M calcium mobilization in LAD2 mast cells, CD34 cell-derived mast cells, and RBL-2H3 cells stably expressing MRGPRX2<sup>9</sup>. PMX-53 is a potent C5a receptor antagonist in human neutrophils and macrophages *in vitro*<sup>37, 38</sup> but no effects of PMX-53 on basophils and eosinophils have been described so far, although both are expressing C5a receptors<sup>39, 40</sup> and although PMX-53 has reached phase I clinical trials

<sup>41</sup>. However, we did not find any effects of PMX-53 (10 nM and 100 nM) 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 (Supplemental Fig. S1).

In contrast, ciprofloxacin at concentrations of 20 to 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 <sup>6, 42</sup> and diagnostic basophil activation test (BAT) in ciprofloxacin hypersensitive subjects <sup>43-45</sup>. 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 <sup>46</sup>. 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 <sup>47, 48</sup>. 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 <sup>49, 50</sup>. Confirming our data, Fernandez et al. <sup>51</sup> demonstrated basophil CD63 upregulation in basophil activation tests of non-hypersensitive controls using similar ciprofloxacin concentrations.

It has been shown in human LAD2 mast cell line that ciprofloxacin induced calcium influx <sup>52</sup>. 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 multicolour 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<sup>53</sup> as stimulation of mouse mast cells with Der p 1 resulted in activation of MRGPRX1, MrgprC11, 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<sup>53</sup>. With regard to ciprofloxacin in subjects with hypersensitivity the role of specific IgE is debated<sup>43</sup>. Future experiments might clarify whether addressing MRGPRX2 is useful for diagnostic purposes in allergic and pseudoallergic reactions like CD63 or CD203c<sup>54</sup>. In addition, it might be interesting to investigate whether anergic non-responder basophils to anti-FcεRI<sup>55</sup> 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 (for example 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<sup>56</sup>. 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<sup>6, 20, 42, 57, 58</sup>. Recently, human skin mast cell MRGPRX2 upregulation has been demonstrated in severe chronic spontaneous urticaria<sup>10</sup>, a disease in which the role of specific IgE against allergens are negligible and against autoallergens debated<sup>59-61</sup>. Not only mast cells, but also basophils<sup>62-65</sup> and eosinophils<sup>66-68</sup> 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.<sup>10</sup>, 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 has potential for diagnostic purposes in (drug) hypersensitivity.

### **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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## Figure legends

### Figure 1.

#### **MRGPRX2 expression on basophils and eosinophils measured at baseline and after**

**stimulation.** (A) Flow cytometric % MRGPRX2 surface expression (left figure) and geometric MFI (right figure) after subtraction of IgG2b isotype values on basophils (grey bars) and eosinophils (striped bars) of atopic (AT) and non-atopic (NA) donors. Outlier box plots of 11 to 16 different donors (end of the boxes demonstrate 25<sup>th</sup> and 75<sup>th</sup> quantiles, horizontal line within the box represents median),  $p < 0,001$  compared to isotype (not shown), Mann-Whitney Rank Sum test. Differences between AT and NA were statistically not significant. (B) Histograms demonstrating MRGPRX2 and IgG2b isotype expression of freshly isolated basophils (top figure) and eosinophils (bottom figure) of six different donors. (C) Representative photographs of cytopins of  $1 \times 10^5$  fresh unstimulated basophils (purity 99%) (i, ii) or eosinophils (purity 99%) (iii, iv) using an unconjugated (i, iii) or PE-conjugated (ii, iv) monoclonal mouse anti-human MRGPRX2 antibody and visualization with immunocytochemistry using DAB resulting in brown-colored precipitates, 20x (i, iii) or with immunofluorescence resulting in red fluorescence (ii, iv), blue DAPI staining of nuclei, 20x. (D) Western blot demonstrating MRGPRX2 protein (37 kD) in unstimulated (med) and fMLP (1  $\mu$ M), IL-3 (10 ng/ml) + IL-33 (20 ng/ml), anti-IgE (100 ng/ml) or PMA (10 ng/ml) stimulated (30 min) basophils and eosinophils (pool of 3 different donors, each). Unstimulated LAD2 mast cells ( $n = 7$  pooled) and unstimulated and fMLP-stimulated neutrophils (7 donors pooled) served as control cells. Housekeeping protein Histone H3 (18 kD). (E) Relative western blot density of basophils, eosinophils and neutrophils ( $n = 10$ ) compared to LAD2 (relative density = 1, dotted reference line). (F) relative western blot densities of basophils and eosinophils stimulated with fMLP, IL-3 + IL-33, anti-IgE, and PMA (for conc. see D) compared to medium (relative density = 1). Bars represent means of the seven donors (individual dots). (G) Representative western blot of two demonstrating MRGPRX2 protein (37 kD) in unstimulated and IL-3 incubated (concentration as indicated, 30 min) basophils and eosinophils. LAD2 mast cells served as control. Housekeeping protein Histone H3 (18 kD). (H) Relative western blot densities of two different donors (dots) after 30 min stimulation with IL-3 in indicated concentrations. Lines represent mean.

### Figure 2.

#### **Calcium mobilization after engagement of MRGPRX2.**

(A) Ligation of MRGPRX2 using anti-MRGPRX2 mAb (1,0  $\mu$ g/ml and 2,5  $\mu$ g/ml) but not medium control or isotype (IgG2b, 2,5  $\mu$ g/ml) immediately induced calcium influx in basophils and eosinophils. Number 1 indicates stimulation after 3-5 seconds. (B) Calcium influx induced by stimulation with anti-MRGPRX2 mAb (2,5

μg/ml), anti-IgE (100 ng/ml), anti-FcεRIα mAb (2,5 μg/ml) or fMLP (1 μM) after 3-5 sec (number 1) was further enhanced by a second stimulation with anti-MRGPRX2 mAb (2,5 μg/ml) after 60 sec (number 2). One representative example of three. IgG2b isotype control for anti-MRGPRX2 mAb and IgG1 isotype control for anti-FcεRIα were similar to medium control (not shown). When basophils (C) and eosinophils (D) were pre-stimulated for 10 min with anti-IgE (100 ng/ml) or fMLP (1 μM) before calcium mobilization assay and subsequently stimulated with anti-MRGPRX2 mAb (2,5 μg/ml) (number 1) calcium influx was enhanced compared to stimulation with anti-IgE or fMLP alone. Highest calcium influx was shown when the stimulation was vice versa (10 min preincubation with anti-MRGPRX2 and stimulation (number 1) with anti-IgE or fMLP. The data shown are representative of four (basophils) and four to six (eosinophils) independent analyses from different donors. (E) Sum of intensity counts representing calcium influx over the whole period of 0-120 sec of all donors. Two-tailed p-test, p<0,05: MRGPRX2 and fMLP versus medium, anti-IgE versus medium, MRGPRX2+fMLP versus MRGPRX2 and fMLP alone, MRGPRX2+anti-IgE versus MRGPRX2 and anti-IgE alone.

### Figure 3.

**Engagement of MRGPRX2 for 30 min results in degranulation.** (A) In contrast to medium control (= 0) and anti-IgG2b control Ab (isotype, 2,5 μg/ml, only shown in C and D, no difference to medium), stimulation with anti-MRGPRX2 mAb for 30 min dose-dependently resulted in increased fluorescence intensity of Av.A488 reflecting degranulation in fresh highly purified basophils and eosinophils (n = 8, each). p<0,05, \*\*p<0,01, paired t-test versus medium and isotype. (B) Spaghetti plots of all individual donors. (C) Representative dot plot of anti-MRGPRX2 mAb (concentration as indicated) induced Av.A488 staining of basophils and eosinophils (D).

### Figure 4.

**MRGPRX2 engagement for 24 h enhanced survival accompanied by cytokine release.** (A) In contrast to medium control (med) and anti-IgG2b control antibody (iso, 2,5 μg/ml), 24 h stimulation with anti-MRGPRX2 mAb (MRGPRX2) 2,5 μg/ml resulted in significantly enhanced survival of purified basophils (grey bars) and eosinophils (striped bars) ± SEM as assessed by Annexin V-negative and PI-negative (Annexin V<sup>neg</sup>/ PI<sup>neg</sup>) cells indicating non-apoptotic/non-necrotic cells. Survival-enhancing IL-3 (2 ng/ml) and apoptosis-inducing dexamethasone (Dexa, 1 μM) served as controls. N = 6, \*p<0,05, \*\*p<0,01 compared to medium and isotype (Wilcoxon signed rank sum test). (B) Accordingly, non-viable basophils (grey bars) and eosinophils (striped bars) significantly decreased after 24h incubation with anti-MRGPRX2 mAb (concentrations like in A), as determined by trypan blue exclusion test. N = 6, \*p<0,05, \*\*p<0,01 compared to medium and isotype (Wilcoxon signed rank sum test). (C) Representative dot plot of six for basophils (top

figure) and eosinophils (bottom figure). For experimental details see A. (D) IL-3, IL-5, and GM-CSF release in 4 h (horizontal striped bars) and 24 h supernatants (black bars) of the stimulated basophils and eosinophils demonstrated in A as assessed by ELISA.  $n = 6$ ,  $*p < 0,05$ ,  $**p < 0,01$ ,  $***p < 0,001$  compared to medium and isotype (Wilcoxon signed rank sum test). **Enhancement of MRGPRX surface expression after 30 min and 24 h stimulation.** IL-3 stimulation for 30 min dose-dependently upregulated MRGPRX2 surface expression in basophils (E,  $n = 3$ ) and eosinophils (F,  $n = 4$ ).  $*p < 0,05$  for each concentration of IL-3 from 0,1 ng/ml to 10 ng/ml versus incubation without IL-3, paired t-test. Dotted line represents expression of IgG2b isotype. Percentage of MRGPRX2<sup>pos</sup> basophils (G, grey bars) and eosinophils (H, striped bars) after 24 h incubation with medium, anti-IgE (100 ng/ml), anti-Fc $\epsilon$ RI (anti-Fc $\epsilon$ RI, 330 ng/ml), C5a (0,1  $\mu$ M), fMLP (1  $\mu$ M), IL-3 (10 ng/ml), IL-5 (10 ng/ml), IL-33 (20 ng/ml), Bars show mean  $\pm$  SEM of 9-13 (basophils) or 6 (eosinophils) independent experiments.  $*p = 0,036$  (two tailed t-test),  $**p = 0,005$ ,  $***p < 0,001$  (Mann-Whitney Rank Sum test), all versus medium control.

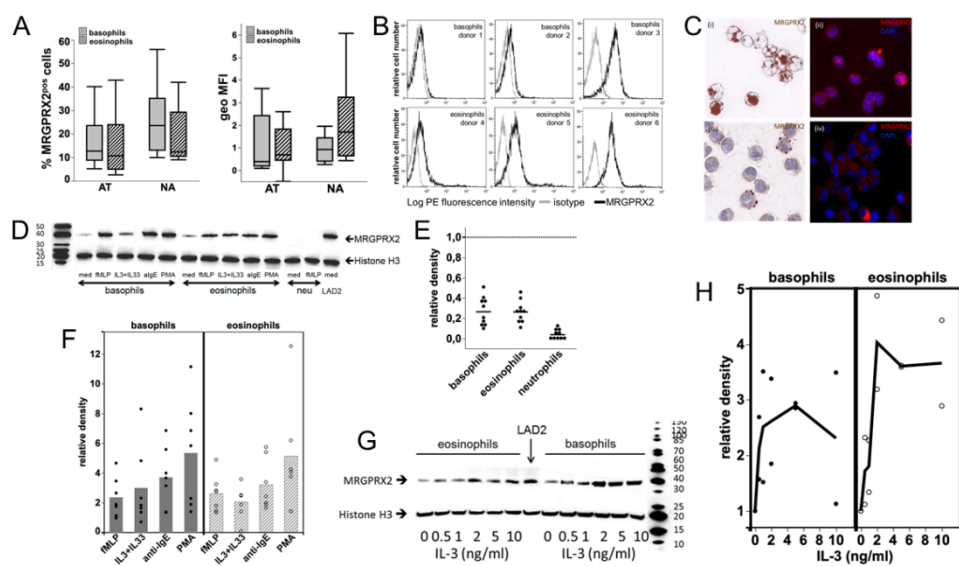
### Figure 5.

**Ciprofloxacin dose-dependently enhanced MRGPRX2 surface expression and induced degranulation and calcium influx.** (A) Ciprofloxacin 20 to 200  $\mu$ g/ml but not 400  $\mu$ g/ml enhanced % MRGPRX2-positive basophils and to a lesser extent eosinophils,  $p < 0,05$ , 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  $\mu$ M), anti-IgE (100 ng/ml) and fMLP (1  $\mu$ M) 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 sec. C5a (0,1  $\mu$ M) 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  $\mu$ g/ml) at a concentration of 100  $\mu$ g/ml (Ci 100) in basophils ( $n = 4$ ) and of 100  $\mu$ g/ml and 200  $\mu$ g/ml in eosinophils ( $n = 5$ ). Bars  $\pm$  SEM.  $*p < 0,05$ ,  $**p < 0,01$ , without (w/o) versus with prior anti-MRGPRX2.

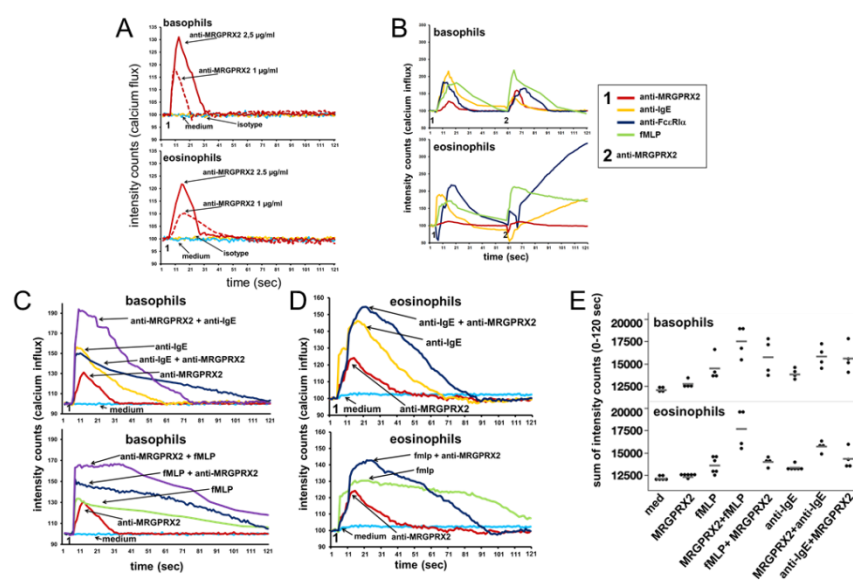
### Figure 6.

**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

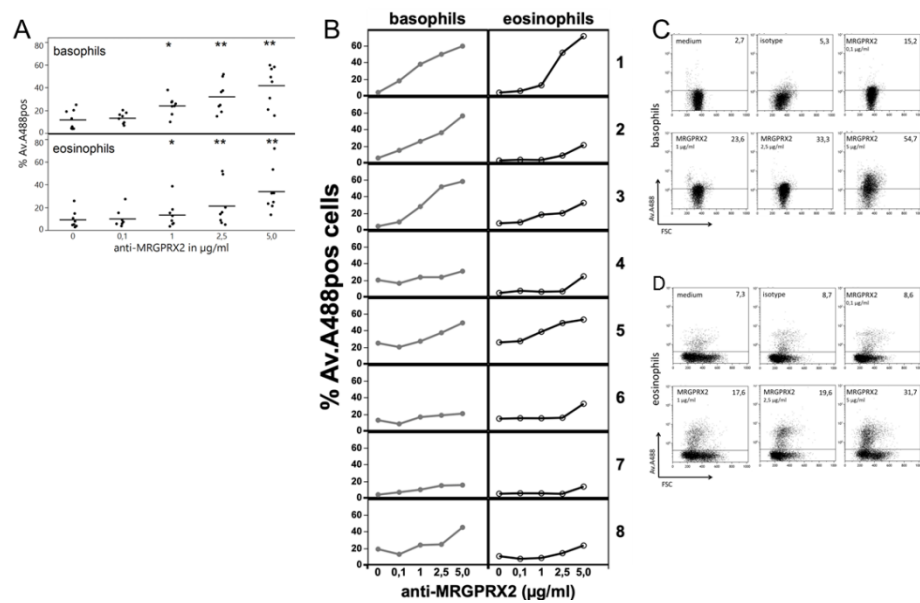
was stimulated with medium control (0), anti-FcεRI (FceRI, 330 ng/ml), fMLP (1 μM) or grass pollen extract (0,01, 0,1 ng/ml, 1 ng/ml, 10 ng/ml) for 20 min and analyzed by tricolor granulocyte activation test (CCR3-FITC, CD63-PE, MRGPRX2-APC). Bars show mean ± SEM of grass-pollen induced % MRGPRX2-positive (black bars) and CD63-positive (bars with black diamonds) basophils (left figure) and eosinophils (right figure). Black stars for MRGPRX2, grey stars for CD63, \*p<0,05, \*\*p<0,01, \*\*\*p<0,001 (Mann Whitney Rank Sum test), all versus medium control (0). (C) 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.



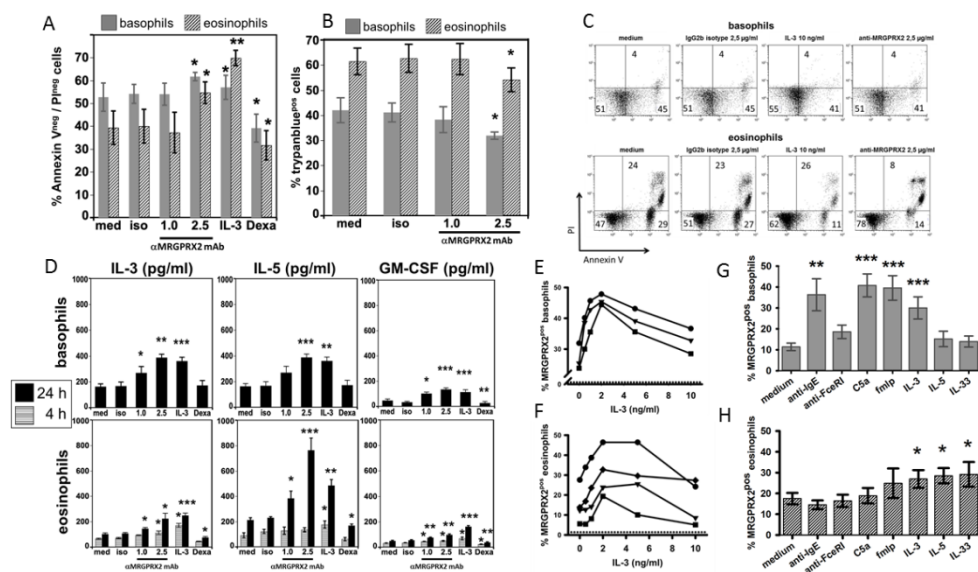
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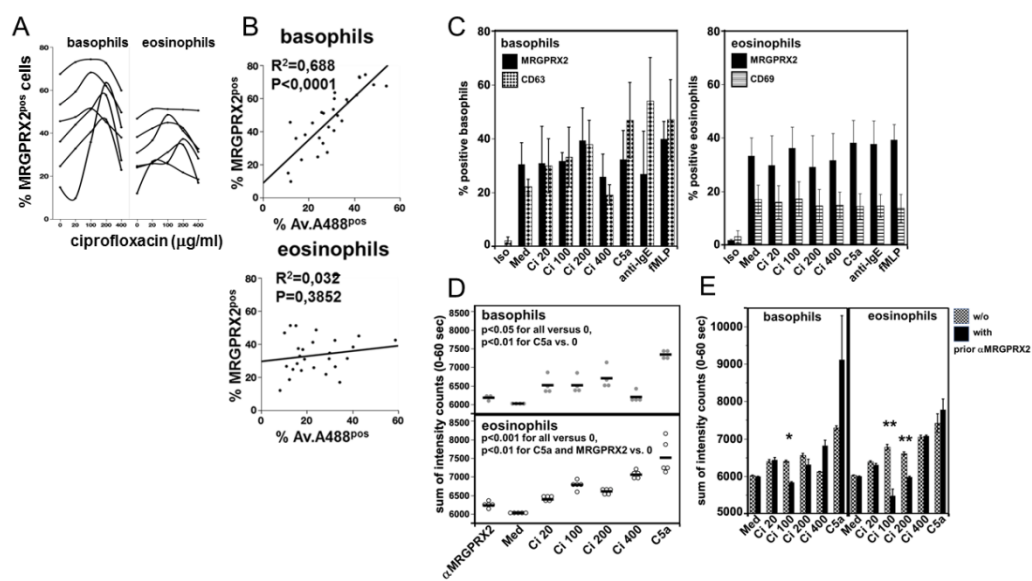
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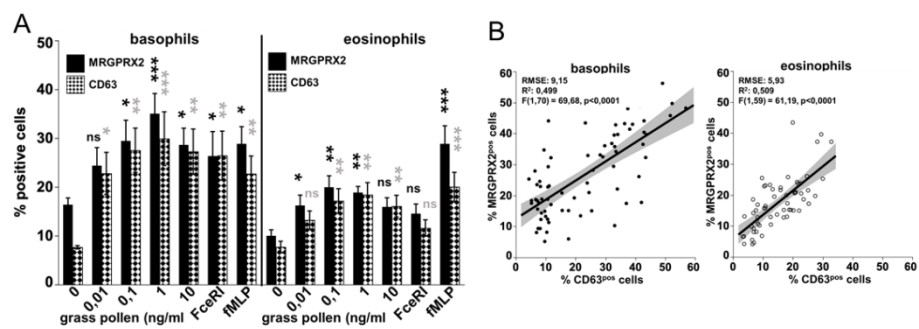
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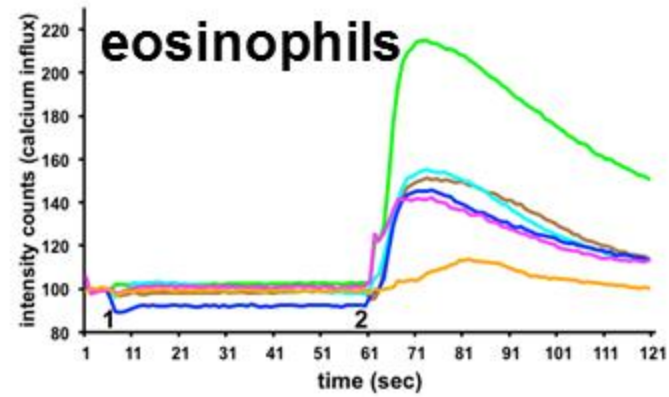
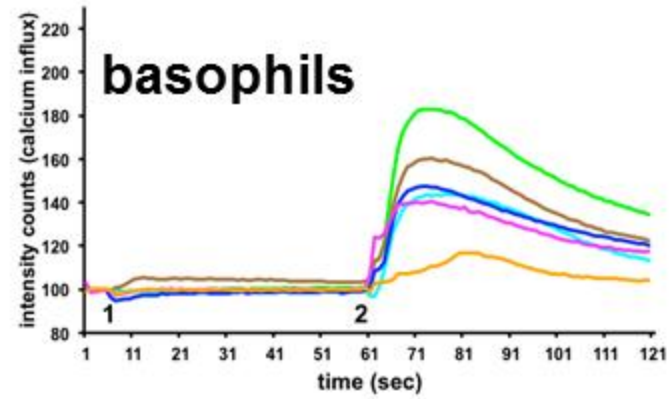
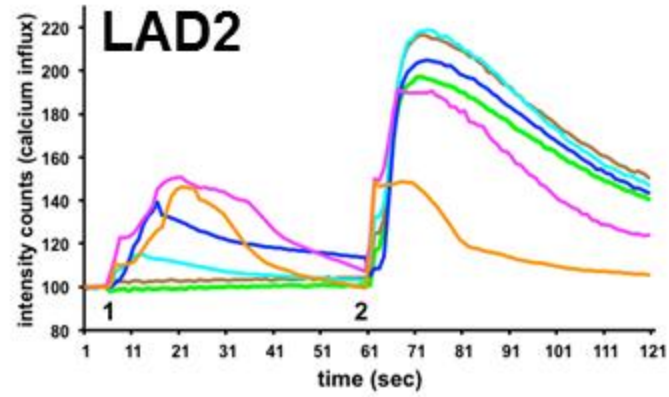
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1: medium  
1: PMX 10 nM  
1: PMX 50 nM  
1: PMX 100 nM  
1: PMX 1  $\mu$ M  
1: PMX 10  $\mu$ M  
2 for all: C5a  $10^{-8}$  M

### **Supplemental Figure legend for Figure S1**

**Fig. S1. Calcium mobilization induced by PMX-53.** Stimulation with PMX-53 after 3-5 sec (indicated as number 1) at indicated concentrations induced calcium influx only in LAD2 mast cells (top figure), but not in basophils (middle) or eosinophils (bottom figure). PMX-53 was functional as it dose-dependently inhibited C5a ( $10^{-8}$  M) induced calcium influx (second stimulus 55 sec later, indicated as number 2) in all three cell types. One representative experiment of four.