MAS-Related gene X2 (MrgX2) is a novel G protein coupled Receptor for the antimicrobial Peptide LL-37 in Human Mast Cells: Resistance to Receptor Phosphorylation, Desensitization and Internalization

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Running head: MrgX2 as a novel G protein coupled receptor for LL-37

Background: Antimicrobial peptide LL-37 activates leukocytes, mast cells and endothelial cells.

Results: LL-37 induces chemotaxis, degranulation and chemokine production in human mast cells expressing MrgX2 but it does not induce receptor desensitization.

Conclusion: MrgX2 is a novel receptor for LL-37 in human mast cells and this receptor is resistant to regulation.

Significance: This study has important implications for innate immunity and inflammation.

SUMMARY

Human LL-37 is a multifunctional antimicrobial peptide that promotes inflammation, angiogenesis, wound healing and tumor metastasis. Most effects of LL-37 are mediated via the activation of cell surface G protein coupled receptor, FPR2 on leukocytes and endothelial cells. Although LL-37 induces chemotaxis, degranulation and chemokine production in mast cells, the receptor involved and the mechanism of its regulation remains unknown. MrgX2 is a member of Mas-related genes that is primarily expressed in human dorsal root ganglia and mast cells. We found that a human mast cell line LAD2 and CD34+ cell-derived primary mast cells caused a substantial reduction in LL-37-induced degranulation. Furthermore, mast cell lines stably expressing MrgX2 responded to LL-37 for chemotaxis, degranulation and CCL4 production. Surprisingly, MrgX2 was resistant to LL-37-induced phosphorylation, desensitization and internalization. In addition, shRNA-mediated knockdown of the G protein coupled receptor kinases (GRK2 and GRK3) had no effect on LL-37-induced mast cell degranulation. This study identified MrgX2 as a novel GPCR for the antibacterial peptide LL-37 and demonstrates that unlike most GPCRs it is resistant to agonist-induced receptor phosphorylation, desensitization and internalization.

Antimicrobial peptides such as defensins and cathelicidins are secreted by activated epithelial cells as well as by invading leukocytes and play an important role in host defense (1). Cathelicidins consist of a putative N-terminal signal peptide, a highly conserved cathelin-like domain and a C-terminal antimicrobial domain corresponding to the mature antibacterial peptide. About 30 cathelicidin members have been identified in mammals. However, only one cathelicidin, hCAP18 (human cationic antibacterial protein of 18 kDa), has been found in humans thus far, and its C-terminal mature antibacterial peptide (LL-37), comprising 37 amino acid residues, has direct
antibacterial effect against Gram positive and Gram negative bacteria (2). In addition, LL-37 displays immunomodulatory properties via the recruitment of monocytes and T cells (3, 4). LL-37 stimulates angiogenesis to promote wound healing and tumor invasiveness (5-8). Most effects of LL-37 appear to be mediated via the activation of G-protein coupled formyl peptide receptor 2 (FPR2; earlier known as FPRL1) on monocytes, T cells and endothelial cells (3, 8-11). LL-37 also activates chemokine receptor CXCR2 in human neutrophils (12), purinergic receptor P2X7 in fibroblasts (13) and insulin growth factor receptors (IGFR) in epithelial cells (14).

Mast cells are known to play a critical role in innate immunity and this function requires mast cell degranulation and subsequent neutrophil recruitment (15, 16). LL-37 induces Ca\(^{2+}\) mobilization, chemotaxis and degranulation in rat peritoneal mast cells (17, 18). It also causes increased vascular permeability in wild-type but not in mast cell-deficient rats (19). Thus, LL-37-induced mast cell activation could contribute to the innate immune function of mast cells. LL-37 is also thought to be involved in chronic inflammatory diseases. The level of LL-37 in human skin increases dramatically from 1 µM in normal individuals to ~304 µM in psoriatic patients (20, 21). LL-37 induces degranulation and pruritogenic cytokine generation in human mast cells (22, 23). Although LL-37 activates mast cells via pertussis toxin sensitive G protein and phospholipase C-mediated signaling pathway (18), the GPCR it utilizes have not been determined.

A large family of GPCRs called Mas-related genes (Mrgs, also known as sensory neuron-specific receptors, SNSR) has recently been identified in rodents (24, 25). These receptors are selectively expressed in small-diameter sensory neurons of dorsal root ganglia and are thought to be involved in the sensation and modulation of pain. Interestingly, a subgroup of these receptors (MrgX1 - MrgX4), are expressed in human but not in murine neurons (24, 26). Recent studies have shown that MrgX2 is also expressed in human mast cells and activated by basics peptides (27, 28). Given that LL-37 also displays basic properties, we hypothesized that it activates human mast cells via MrgX2. Using, shRNA-mediated knockdown of MrgX2 in human mast cells we provide the novel finding that LL-37 causes sustained Ca\(^{2+}\) mobilization and degranulation via MrgX2. Moreover, using transfected mast cell lines that do not natively express MrgX2 (27), we demonstrate that LL-37 promotes chemotaxis and induces chemokine, CCL4 via MrgX2.

Agonist occupied GPCRs are phosphorylated by a family of protein kinases, collectively known as G protein coupled receptor kinases (GRKs) (29). Of the seven known GRKs, four (GRK2, GRK3, GRK5 and GRK6) are expressed ubiquitously. It is well established that GPCR phosphorylation by GRKs leads to the recruitment of β-arrestin, which results in receptor desensitization and internalization (29). A goal of the present study was to determine the role of agonist-induced receptor phosphorylation on the regulation of MrgX2. Our studies clearly demonstrate that unlike most other known GPCRs, MrgX2 is resistant to LL-37-induced receptor phosphorylation, desensitization and internalization. It is noteworthy that LL-37-induced mast cell degranulation leads to the release of tryptase, which degrades LL-37 rendering it inactive (23). Thus, in the absence of MrgX2 receptor desensitization, enhanced mast cells degranulation likely provides a novel feedback mechanism to regulate receptor function by limiting ligand availability for the receptor.

**Experimental procedures**

**Materials:** Frozen human G-CSF-mobilized peripheral blood CD34\(^+\) progenitors were obtained from The Fred Hutchinson Cancer Center (Seattle, WA). All cell culture reagents and pertussis toxin were purchased from Invitrogen (Gaithersburg, MD). Amaxa cell transfection kits and reagents were purchased from Lonza (Gaithersburg, MD). Anti HA antibody (12CA5) and anti HA (HA-7) agarose beads were purchased from Roche (Mannheim, Germany) and Sigma-Aldrich (St. Louis, MO) respectively. All recombinant human cytokines were purchased from Peprotech (Rocky Hill, NJ). Phorbol-12-myristate-13-acetate (PMA) was purchased from Calbiochem (EMD,
Gibbstown, NJ). Anti-human C3a receptor antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Phycoerythrin (PE)-labeled donkey anti-mouse IgG was purchased from eBioscience (San Diego, CA). Bovine Adrenal Medulla Docosapeptide (BAM-22P) and Cortistatin-14 (CST) were obtained from American Peptide (Vista, CA). Native complement C3a was from Complement Technology (Tyler, TX). LL-37 (Leu-Leu-Gly-Asp-Phe-Phe-Arg-Lys-Ser-Lys-Glu-Lys-Ile-Gly-Lys-Glu-Phe-Lys-Arg-Ile-Val-Gln-Arg-Ile-Lys-Asp-Phe-Leu-Arg-Leu-Val-Pro-Arg-Thr-Glu-Ser) was from Anaspec (Freemont, CA).

**Differentiation of human mast cells from CD34⁺ progenitors and culture of human mast cell lines:** To obtain primary mast cells, human CD34⁺ progenitors were cultured in StemPro-34 medium supplemented with L-glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100 µg/ml), rhSCF (100 ng/ml), rhIL-6 (100 ng/ml) and rhIL-3 (30 ng/ml) (first week only). Hemidepletions were performed weekly with media containing rhSCF (100 ng/ml) and rhIL-6 (100 ng/ml) (30). Cells were used for experiments after 7-10 weeks in culture. HMC-1 cells were cultured in Iscove’s Modified Dulbecco’s Medium (IMDM) supplemented with 10% FCS, glutamine (2 mM), penicillin (100 IU/ml) and streptomycin (100 µg/ml) (31). LAD2 cells were maintained in complete StemPro-34 medium supplemented with 100 ng/ml rhSCF (32). RBL-2H3 and HEK-293T cells were maintained as monolayer cultures in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS, L-glutamine (2 mM), penicillin (100 IU/ml) and streptomycin (100 µg/ml) (33).

**Lentiviral-mediated knockdown of MrgX2, GRK2 and GKR3 in human mast cells:** MrgX2 targeted Mission shRNA lentiviral plasmids were purchased from Sigma. The clone that gave the highest knockdown efficiency (TRCN0000009174) was used. A scrambled control non-target vector (SHC002) which does not bind to any known human mRNAs was also purchased from Sigma. Lentivirus generation was performed according to the manufacturer’s manual. Cell transduction was conducted by mixing 1.5 ml of viral supernatant with 3.5 ml of LAD2 (5 x 10⁶ cells) or CD34⁺-mast cells (3 x 10⁶ cells). Eight hours post-infection, medium was changed to virus-free complete medium, and antibiotic (puromycin, 2 µg/ml, Sigma) selection was initiated 16 hr later. Knockdown of GRK2 and GKR3 in LAD2 cells were performed as described previously (34). Cells were analyzed for MrgX2, GRK2 or GKR3 knockdown and used for subsequent assays 4 days following initiation of puromycin selection.

**Quantitative PCR:** Total RNA from mast cells was extracted using TRIzol (Invitrogen), treated with DNase I, and reverse transcribed to cDNA using first strand cDNA synthesis kit (GE Healthcare Life Sciences, Piscataway, NJ). Gene expression was analyzed using real time PCR with Taqman® Fast Universal PCR Master Mix on a Taqman 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Taqman probe for hGAPDH, hMrgX2 and hGRK3 were used for real time PCR to analyze the knockdown efficiency. The amplification conditions were as follows: initial denaturation at 95°C for 20 sec, followed by 40 cycles of amplification: 95°C for 3 sec, 60°C for 30 sec. Analysis was performed according to ∆∆-Ct method. The results are expressed as a ratio of MrgX2 or GRK3 to GAPDH (34, 35).

**Western Blotting:** Protein extracts of control and GRK2 knock down LAD2 cells following lysis with RIPA buffer were separated by SDS-PAGE and transferred to Hybond ECL nitrocellulose membranes (GE Healthcare Life Sciences). Blots were incubated with anti human GRK2 antibody (Santa Cruz Biotechnology) in blocking buffer (PBS, 0.5% Tween 20, 5% skim milk) followed by HRP-labeled goat anti-rabbit IgG (Thermo Scientific, 1:5000 in blocking buffer) secondary antibody. Bound antibody was detected using the SuperSignal® West Femto Maximum Sensitivity Substrate kit (Thermo Scientific) according to the manufacturer’s protocol.

**Stable transfection of RBL-2H3 and HMC-1 cells:** RBL-2H3 cells stably expressing MrgX1
and MrgX2 were generated as described previously (27, 36). For HMC-1 cells, 2 x 10^6 cells were transfected with plasmids encoding HA-tagged MrgX2 using the Amaza nucleofector device and Amaza kit V according to the manufacturer’s protocol. Following nucleofection, cells were cultured in the presence of G418 (1 mg/ml) and cells expressing equivalent receptors were sorted using an anti-HA specific antibody 12CA5/FITC-conjugated anti-mouse-IgG and used for studies on Ca^{2+} mobilization, chemotaxis, receptor internalization and CCL4 chemokine generation.

**Calcium mobilization:** Ca^{2+} mobilization was determined as described previously (37, 38). Briefly, cells (human mast cells; 0.2 x 10^6) and (RBL-2H3 or HMC-1 cells; 1.0 x 10^6) were loaded with 1 µM indo-1 AM for 30 min at room temperature. Cells were washed and resuspended in 1.5 ml of HEPES-buffered saline. Ca^{2+} mobilization was measured in a Hitachi F-2500 spectrophotometer with an excitation wavelength of 355 nm and an emission wavelength of 410 nm (38).

**Degranulation:** Human mast cells (5 x 10^5) and RBL-2H3 cells (5 x 10^5) were seeded into 96-well plates in a total volume of 50 µl HEPES buffer containing 0.1% BSA and exposed to different concentrations of peptides. In some assays cells were pretreated with pertussis toxin (100 ng/ml; 16 hr) or La^{3+} (lanthanum chloride, 1 µM; 5 min). For total β-hexosaminidase release, unstimulated cells were lysed in 50 µl of 0.1% Triton X-100. Aliquots (20 µl) of supernatants or cell lysates were incubated with 20 µl of 1 mM p-nitrophenyl-N-acetyl-β-D-glucosamine for 1.5 hr at 37°C. Reaction was stopped by adding 250 µl of a 0.1 M Na_2CO_3/0.1 M NaHCO_3 buffer and absorbance was measured at 405 nm (33).

**Chemotaxis assay:** LL-37 or buffer (30 µl) was added to the lower wells of a 96-well chemotaxis chamber (8 µm pore size; NeuroProbe, Gaithersburg, MD). Mock transfected HMC-1 cells or cells expressing MrgX2 (0.5 x 10^6 in 50 µl buffer) were placed on the upper chamber. After 3 hr incubation at 37°C migrated cells were collected from the lower chambers. Triplicate wells were pooled and the cells were resuspended in thirty microliters of complete IMDM. The cells were counted with a hemocytometer and the results are expressed as absolute number of cells that had migrated.

**Receptor internalization:** Cells (0.25 x 10^6) were exposed to buffer or different agonists for indicated time intervals at 37°C. Cells were washed twice with ice-cold FACS buffer (PBS containing 2% FBS) and labeled with anti-HA-antibody (12CA5) or Isotype control and incubated on ice for 30 min. After washing twice with cold FACS buffer, cells were stained with PE-labeled anti-mouse IgG antibody on ice for 30 min. Cells were washed twice and fixed in 300 µl of 2% formaldehyde solution. The samples were acquired and analyzed using BD LSR II flow cytometer (BD Biosciences).

**Chemokine CCL4 generation:** Chemokine release assay was performed as described previously (38). Briefly, mock or MrgX2 transfected cells (0.2 x 10^6) were stimulated with indicated concentrations of LL-37 for 6 hrs. CCL4 chemokine levels in the supernatants were quantified by DuoSet ELISA kit from R&D systems (Minneapolis, MN) according to the manufacturer’s protocol.

**Receptor expression and phosphorylation:** Transient transfections were performed on 80% confluent HEK-293T monolayers in 60-mm dishes in 4 ml of Optimem® medium (Gibco, Invitrogen) containing 1 µg of plasmid DNA (encoding hemagglutinin (HA)-tagged human MrgX2 and C3aR) and 7 µl of Lipofectamine reagent (Invitrogen). To detect receptor expression, cells (0.5 x 10^6) were incubated with anti-HA (12CA5) antibody followed by phycoerythrin (PE)-conjugated anti mouse IgG as the secondary antibody at 4°C for 30 min and analyzed by flow cytometry.
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Results

**LL-37 induces degranulation in human mast cells via Pertussis toxin-sensitive G Protein-dependent and independent pathways.** Previous studies demonstrated that LL-37 causes substantial degranulation in primary human lung mast cells in a dose-dependent manner within a concentration range of 1 - 10 µM (23, 40). As shown in Fig. 1A, we also found that LL-37 stimulates degranulation in LAD2 mast cells within the same concentration range. In rat peritoneal mast cells, LL-37 induces a sustained Ca²⁺ mobilization and degranulation and both of these responses are inhibited by pertussis toxin (PTx), indicating the involvement of a Gi-family of G proteins (18). However, the signaling pathway via which LL-37 induces degranulation in human mast cells is unknown. We therefore tested the effects of PTx on LL-37-induced Ca²⁺ mobilization and degranulation in LAD2 mast cells. We found that LL-37 caused sustained Ca²⁺ mobilization in LAD2 cells but PTx had no effect on this response (Fig. 1B). By contrast, PTx caused substantial inhibition of LL-37-induced degranulation (Fig. 1C). La³⁺ has been shown to inhibit both Ca²⁺ influx and mast degranulation (41, 42). As shown in Fig. 1B, unlike PTx, La³⁺ (1 µM) almost completely inhibited LL-37-induced Ca²⁺ response and this was associated with a substantial inhibition of LL-37-induced mast cell degranulation (Fig. 1D). These findings suggest that in contrast to the situation in rat peritoneal mast cells, LL-37 causes degranulation in human mast cells via the synergistic interaction of a Gai-independent Ca²⁺ influx and another Gi-mediated pathway (33).

**LL-37 activates human mast cells via MrgX2.** LL-37 activates neutrophils, monocytes, eosinophils and T cells via FPR2 (3, 9). Although mast cells express transcript for FPR2, effects of LL-37 do not appear to be mediated via this receptor (17, 40). It is noteworthy that mast cells are the only known cells outside the dorsal ganglia that express MrgX2 (28). Furthermore, this receptor is activated by basic peptides (27, 28). Given that LL-37 displays basic properties, we hypothesized that it could activate mast cells via MrgX2. While LAD2 cells endogenously express MrgX2, an immature human mast cell line HMC-1 cells do not express functional receptor (27). By contrast both cell lines express GPCR for C3a (C3aR) (43). We therefore used these cell lines to determine if there was a correlation between MrgX2 expression and responsiveness to LL-37. As shown in Fig. 2 (A and D) C3a caused transient Ca²⁺ mobilization in both LAD2 and HMC-1 cells. However, the known MrgX2 neuropeptide ligand cortistatin (CST) and LL-37 caused sustained Ca²⁺ responses in LAD2 cells (Fig. 2 B and C) but not in HMC-1 cells (Fig. 2 E and F). These findings suggest that MrgX2 could serve as a receptor for LL-37 in LAD2 mast cells.

To confirm the role of MrgX2 on LL-37-induced mast cell responses in human mast cells, we generated stable transfectants expressing this receptor in HMC-1 cells. FACS analysis demonstrated cell surface expression of HA-tagged MrgX2 in receptor transfected but not in mock-transfected cells (Fig. 3A). As shown in Fig. 3, B-D, LL-37 induced sustained Ca²⁺ mobilization, chemotaxis and chemokine CCL4 in cells expressing MrgX2 but not in mock transfected cells. Our next goal was to test the role of MrgX2 on LL-37-induced mast cell degranulation. Since HMC-1 cells do not have the capacity to undergo degranulation, we used rat basophilic leukemia, RBL-2H3 cells stably expressing human MrgX2 (27). In this system, both CST and LL-37 induced substantial mast cell degranulation (Fig. 4A). In addition to MrgX2, human mast cells express MrgX1 (27, 36). To determine the specificity of LL-37 for MrgX2, we
also utilized RBL-2H3 cells stably expressing MrgX1 (36). As shown in Fig. 4B, cells expressing MrgX1 responded to its known ligand BAM-22P for degranulation but they were resistant to LL-37. Moreover, cells expressing MrgX2 did not respond to BAM-22P. These findings support the notion that LL-37 utilizes human MrgX2 to activate mast cells.

To further confirm the role of MrgX2 on LL-37-induced mast cell degranulation, we used lentiviral Mission shRNA to knockdown the expression of MrgX2 in LAD2 cells. Cells were transduced with 5 different shRNA constructs targeting different regions of MrgX2. For control, we used a scrambled shRNA construct. After transduction and selection with puromycin, quantitative PCR was performed to determine the extent of MrgX2 knockdown. We found that, clone #4 (TRCN0000009174) was the most effective in knocking down MrgX2 expression. We therefore used this clone for subsequent studies. As shown in Fig. 5A, we were able to knockdown the mRNA expression for MrgX2 by ~60%. We could not determine MrgX2 protein expression due to the absence of a suitable antibody for Western blotting studies. When compared to shRNA control cells, knockdown of MrgX2 resulted in almost complete inhibition of LL-37-induced Ca\textsuperscript{2+} mobilization. This effect was specific for MrgX2 as silencing the expression of this receptor had little or no effect on C3a-induced Ca\textsuperscript{2+} response (Fig. 5 B and C). At a functional level, silencing MrgX2 expression caused substantial inhibition of LL-37 and CST but not C3a-induced mast cell degranulation (Fig. 5D).

**MrgX2 is resistant to agonist-induced receptor phosphorylation desensitization and internalization.** C3aR is highly susceptible to agonist-induced GRK-mediated phosphorylation (39, 44). Although GRKs have no clear consensus sequence in their receptor substrates for phosphorylation, some prefer acidic residues whereas others require basic residues in close proximity to serine/threonine residues (29). As shown in Fig. 6A, the carboxyl terminus of C3aR possesses ten serine/threonine residues whereas MrgX2 possesses only five. Furthermore, MrgX2 carboxyl terminus contains fewer acidic and basic residues than C3aR. To compare agonist-induced phosphorylation of MrgX2 with C3aR, we generated transfectants expressing HA-tagged receptors in HEK-293T cells. Flow cytometry analysis using an antibody specific to HA tag indicated that MrgX2 is expressed at a higher level than C3aR (Fig. 6B and C).\textsuperscript{32}P labeled cells were stimulated with the appropriate ligand; C3a, CST or LL-37 and receptor phosphorylation was determined by immunoprecipitation followed by autoradiography. As expected, C3a caused robust phosphorylation of its receptor (Fig. 6D). Despite the fact that MrgX2 was expressed at higher level than C3aR, MrgX2 was relatively resistant to CST and LL-37 for phosphorylation (Fig. 6D).

Using intracellular Ca\textsuperscript{2+} mobilization as an assay, we have previously shown that C3aR and platelet-activating factor receptor (PAFR) are highly susceptible to desensitization (33, 45, 46). Thus, while the wild-type receptors expressed in RBL-2H3 cells respond to ligand for transient Ca\textsuperscript{2+} mobilization, cells expressing phosphorylation-deficient receptors respond to ligand for sustained Ca\textsuperscript{2+} response. We have recently shown that RBL-2H3 cells expressing wild-type MrgX2 respond to its ligands for sustained Ca\textsuperscript{2+} mobilization (27). Furthermore, unlike the situation for C3a, both LL-37 and CST induce sustained Ca\textsuperscript{2+} responses in LAD2 mast cells (Fig. 2 A-C). These findings suggest that, consistent with the relative resistance of MrgX2 to undergo phosphorylation (Fig. 6D), it does not undergo desensitization.

We performed additional sets of experiments to assess desensitization of MrgX2. In each case we used C3aR for control. In the absence of added extracellular Ca\textsuperscript{2+}, C3a and LL-37 induced transient Ca\textsuperscript{2+} responses that returned to basal levels within ~1 min (Fig. 7, A and B). Adding back Ca\textsuperscript{2+} (1 mM) at 5 min after the initial stimulation with C3a resulted only in a marginal increase in intracellular Ca\textsuperscript{2+} (Fig. 7A). However, for LL-37, subsequent addition of Ca\textsuperscript{2+} resulted in substantial increase in Ca\textsuperscript{2+} mobilization (Fig. 7, B).

Agonist-induced phosphorylation of most GPCRs is associated with their internalization.
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(29). To determine if MrgX2 is internalized following LL-37 exposure, we utilized HMC-1 cells stably expressing HA-tagged MrgX2. Because these cells endogenously express C3aR, which is very sensitive to internalization (34), we used C3a as control. Consistent with phosphorylation, C3aR was internalized by ~75% upon stimulation with C3a for 1-5 min (Fig. 7C). By contrast, LL-37 did not induce internalization of MrgX2 even after cells were exposed to the ligand for up to 30 min (Fig. 7D). To assess if MrgX2 receptor was internalized by heterologous mechanisms, we stimulated the cells with the protein kinase C activator PMA. As for LL-37, PMA did not promote MrgX2 internalization (Fig 7E).

For most GPCRs, agonist-induced receptor phosphorylation and desensitization are mediated by G protein coupled receptor kinases (GRKs) (29). We have recently shown that LAD2 mast cells express GRK2 and GRK3 and that these protein kinases are involved in C3aR desensitization (34). We therefore, generated LAD2 mast cells with stable knockdown of GRK2 and GRK3. As shown in Fig. 8A, lentiviral shRNA almost completely silenced GRK2 protein expression. Because we were unable to utilize any of the commercially available antibodies to assess GRK3 knockdown, we performed qPCR. As shown in Fig. 8B, we were able to achieve ~75% knockdown of GRK3 mRNA expression. Consistent with our previous findings (34), GRK2 and GRK3 knockdown enhanced C3a-induced mast cell degranulation (Fig. 8C). However, silencing the expression of these GRKs had no effect on CST or LL-37-induced degranulation (Fig. 8D). These findings clearly demonstrate that MrgX2 is resistant to agonist-induced receptor phosphorylation and desensitization.

**LL-37 induces signaling and degranulation in human CD34^+ cell-derived mast cells via MrgX2.**

All of the signaling and functional studies described above were performed with a human mast cell line that endogenously expresses MrgX2 as well as transfected HMC-1 cells, RBL-2H3 cells and HEK-293T cells. To confirm the biological relevance of these studies, we repeated selected experiments in CD34^+ derived primary human mast cells. We found that, as for LAD2 cells (Fig.1A), LL-37 induced degranulation in CD34^+ derived mast cells in the concentration range of 1 – 10 µM. However, the maximum response observed in CD34^+ derived mast cells was approximately 50% of that found in LAD2 cells (compare Fig. 1A with Fig. 9A). Similar to the situation in LAD2 cells, LL-37 induced a transient Ca^{2+} response in primary mast cells in the absence of extracellular Ca^{2+} but restoration of extracellular Ca^{2+}, resulted in a sustained intracellular Ca^{2+} response (compare Fig. 7B to Fig. 9B). Most importantly, we were able to knockdown the expression of MrgX2 in primary mast cells (Fig. 9C). Furthermore, silencing MrgX2 expression resulted in a substantial inhibition of LL-37-induced degranulation (Fig. 9D).

**Discussion**

Human LL-37 is a multifunctional antimicrobial peptide that promotes innate immunity, inflammation, angiogenesis, wound healing and tumor metastasis. Although most effects of LL-37 in immune cells and endothelial cells appear to be mediated via the activation of the GPCR, FPR2 it also activates CXCR2, P2X7 IGFR (8, 11-14, 47). LL-37 induces chemotaxis, degranulation and chemokine production in mast cells (17-19, 22, 23). Although much effort has been directed towards delineating the signaling pathways via which LL-37 activates mast cells, little progress has been made due to the lack of information regarding the receptor type involved (17-19, 22, 48). In the present study, we have identified MrgX2 as a novel GPCR for LL-37 in human mast cells. We also demonstrated that unlike most GPCR, MrgX2 is resistant to agonist-induced phosphorylation, desensitization and internalization. We propose a novel mechanism for the regulation of mast cell activation by LL-37.

Mrg receptors belong to the GPCR family and they are also known as the sensory neuron-specific GPCRs. In humans, four MrgX genes, MrgX1 - X4 are known (24, 26). Although originally thought to be specifically expressed in dorsal root ganglia, it now appears that human skin mast cells, cord blood-derived mast cells,
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CD34+ cell-derived mast cells and a human mast cell line, LAD2 express MrgX2 (27, 28, 49). Most interestingly, this receptor is not present in human lymph node, spleen or peripheral blood leukocytes (28). In fact, of the 42 human cell types tested, only mast cells express MrgX2 (28). LL-37 is an amphipathic peptide and given the recent demonstrations that MrgX2 serves as a receptor for a variety of cationic peptides (27, 28), we hypothesized that it could serve as a receptor for LL-37 in human mast cells. Indeed, three lines of evidence clearly support this contention. First, LAD2 and CD34+ cell-derived human mast cells that endogenously express MrgX2 responded to LL-37 for Ca2+ mobilization and degranulation. Second, mast cell lines stably expressing MrgX2 responded to LL-37 for chemotaxis, degranulation and chemokine production. Third, shRNA-mediated knockdown of MrgX2 in LAD2 cells and CD34+ cell-derived primary mast cells resulted in substantial decreases in LL-37-induced Ca2+ mobilization and degranulation.

Consistent with previous reports in rat peritoneal mast cells (18), we found that LL-37-induced degranulation in human mast cells is inhibited by PTx. However, an important difference was that while LL-37-induced Ca2+ influx in rat peritoneal mast cells was blocked by PTx (18), it had no effect on the Ca2+ response in human mast cells (Fig. 1). It is noteworthy that MrgX2 couples to Gαq family of G proteins for Ca2+ mobilization in transfected HEK293 cells (50). This raises the interesting possibility that unlike the situation in rat mast cells, MrgX2 couples to Gαq to promote Ca2+ influx (inhibited by La3+) and that this response synergizes with PTx-sensitive signals likely protein kinase C to promote degranulation in human mast cells. The reason for the difference in specificity of LL-37 for G protein coupling between rat and human mast cells is unknown but could reflect the utilization of different GPCRs. It is noteworthy that unlike most GPCRs, Mrg receptors display substantial species specific differences. Interestingly, human Mrg receptors share only 45 - 65% amino acid sequence identity with rat receptors. In addition, while there are only four Mrg genes known in humans, the rat genome possesses one each of the MrgA, MrgC, and MrgD genes and ten MrgB genes (51). Rat peritoneal mast cells express a number of Mrg receptors including MrgB1, MrgB2, MrgB3, MrgB6, MrgB8 and MrgB9 (28). Thus, while LL-37 couples to MrgX2 in human mast cells it likely activates one or more of the Mrg receptors expressed in rat peritoneal mast cells to induce degranulation.

An important property of most GPCRs is that upon ligand stimulation they undergo GRK-mediated receptor phosphorylation, desensitization and receptor internalization (52). Accordingly, the uridine nucleotide-activated P2Y6 receptor, which does not possess a consensus phosphorylation site for GRKs is resistant to agonist-induced desensitization and internalization (53). Furthermore, PKC activation by PMA also has no effect on the regulation of P2Y6. In the present study, we have demonstrated that MrgX2 is also resistant to agonist-induced receptor phosphorylation, desensitization and internalization. We also showed that silencing of GRK2 and GRK3 had no effect on LL-37-induced mast cell degranulation. Furthermore, similar to the situation for P2Y6 receptor (53), MrgX2 was resistant to internalization in response to PKC activation by PMA. These findings suggest that MrgX2 and P2Y6 are among only few GPCRs, which are resistant to regulation by receptor phosphorylation. It is noteworthy that both MrgX2 and P2Y6 are expressed in human mast cells (27, 54). Furthermore, P2Y6 contributes to leukotriene-mediated mast cell activation and survival (54). Thus, lack of desensitization of these receptors likely has important consequences for their biological functions in mast cells.

It is important to note that outside the dorsal root ganglia mast cells are the only cell type that expresses MrgX2 (28). Human peripheral blood leukocytes, thyroid, bone marrow cells, monocytes, T cells, fibroblasts and epithelial cells have all been shown not to express MrgX2 (28). Many of these cells respond to LL-37 for chemotaxis and cytokine generation via the activation of GPCRs and growth factor receptors. Mast cells are unique among LL-37 responsive cells for two important reasons. First, they respond to LL-37 via MrgX2 and second the
resulting release of mast cell protease degrades and inactivates LL-37 (23). This raises the interesting possibility that protease released by LL-37-activated mast cells not only provides a negative feedback loop to control further mast cell activation but cross-regulates other effects of LL-37 such as angiogenesis, wound healing, tumor metastasis and inflammation.

REFERENCES


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FOOTNOTES
Abbreviations used in this paper: GPCR, G protein coupled receptor; CST, cortistatin-14; Mrg, Mas-related gene and PTx, pertussis toxin.

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Figure Legends

**Fig. 1: LL-37 stimulates degranulation and Ca\(^{2+}\) mobilization in LAD2 mast cells.** (A) LAD2 mast cells were stimulated with different concentrations of LL-37 (0.1 - 10 µM) and percent degranulation (β-hexosaminidase release) was determined. (B) Cells were treated with or without pertussis toxin (PTx; 100 ng/ml, 16h), loaded with Indo-1-AM and Ca\(^{2+}\) mobilization in response to LL-37 (1 µM) was determined. Indo-1 loaded cells were also exposed to La\(^{3+}\) (lanthanum chloride, 1 µM) and LL-37-induced Ca\(^{2+}\) mobilization was determined. (C and D) LAD2 cells were pretreated with vehicle or (C) pertussis toxin (PTx, 100 ng/mL) or (D) La\(^{3+}\) (1 µM; 5 min), stimulated with different concentrations of LL-37 (1 - 10 µM) and percent degranulation was determined. Data are mean ± SEM of three experiments. Statistical significance was determined by one-way ANOVA (A) or two-way ANOVA with Bonferroni’s post test (C and D). ** indicates p<0.001.

**Fig. 2: Cortistatin and LL-37 induce Ca\(^{2+}\) mobilization in LAD2 mast cells but not in HMC-1 cells.** (A - C) LAD2 and (D - F) HMC-1 cells were loaded with Indo-1-AM and stimulated with C3a (10 nM; A and D), cortistatin (CST; 1 µM) (B and E) or 10 µM LL-37 (C and F) and intracellular Ca\(^{2+}\) mobilization was determined. For panels E and F, cells were also stimulated at 500 s with 10 nM C3a following initial stimulation. Data shown are representative of 3 similar experiments.

**Fig. 3: LL-37 induces Ca\(^{2+}\) mobilization, chemotaxis and chemokine CCL4 generation in HMC-1 cells stably expressing MrgX2.** (A) Representative histogram of expression level of HA-MrgX2 (MrgX2) in receptor transfected (thick bold line) or mock transfected control cells (dotted line) as analyzed by flow cytometry is shown. (B) Mock transfected HMC-1 cells or cells expressing MrgX2 were loaded with indo-1 and intracellular Ca\(^{2+}\) mobilization in response to LL-37 (3 µM) was determined. (C and D) Cells (as in B) were exposed to different concentrations of LL-37 and chemotaxis as well as chemokine CCL4 generation were determined. Data are mean ± SEM of three experiments. Statistical significance was determined by two-way ANOVA with Bonferroni’s post test. * indicates p<0.01 and ** indicates p<0.001.

**Fig. 4: LL-37 induces degranulation in RBL-2H3 cells stably expressing MrgX2 but not MrgX1.** RBL-2H3 cells stably expressing (A) MrgX2 or (B) MrgX1 were stimulated with buffer (C), BAM-22P, cortistatin (CST) (both at 1 µM) or LL-37 (3 µM) for 30 min and β-hexosaminidase release was measured. Data are mean ± SEM of three experiments. Statistical significance was determined by one-way ANOVA with Bonferroni’s post test. ** indicates p<0.001.

**Fig. 5: Knockdown of MrgX2 inhibits LL-37-induced Ca\(^{2+}\) mobilization and degranulation in LAD2 mast cells.** LAD2 mast cells were stably transduced with scrambled shRNA control lentivirus or shRNA lentivirus targeted against MrgX2. (A) Quantitative PCR was performed to determine mRNA expression in control and MrgX2 knockdown (KD) cells. Results are expressed as a ratio of MrgX2 to GAPDH mRNA levels. (B) shRNA control and (C) MrgX2 KD cells were loaded with Indo-1 and stimulated with LL-37 (100 nM) at 100 s followed by a second
stimulation of C3a (10 nM) at 300 s and intracellular Ca\(^{2+}\) mobilization was determined. (D) shRNA control and MrgX2 KD cells were stimulated with different concentrations of LL-37 (1 - 10 µM), CST (10 and 100 nM) or C3a (1 nM) and percent degranulation (β-hexosaminidase release) was determined. Data are mean ± SEM of three experiments. Statistical significance was determined by t-test (A) or two-way ANOVA with Bonferroni’s post test (D). * indicates p<0.01 and ** indicates p<0.001.

**Fig 6: MrgX2 is resistant to agonist-induced phosphorylation.** (A) Sequence comparison of the carboxyl termini of C3aR and MrgX2 are shown. (B and C) HEK-293T cells transiently expressing HA-C3aR or HA-MrgX2 were incubated with anti-HA antibody followed by phycoerythrin (PE) conjugated anti-mouse IgG. Representative histograms of expression levels of (B) C3aR and (C) MrgX2 (thick bold lines) in receptor transfected and mock transfected cells (dotted lines) are shown. (D) HEK-293T cells expressing C3aR were labeled with \(^{32}\)P and stimulated with C3a (100 nM) and C3aR phosphorylation was determined. Similarly, cells expressing MrgX2 were stimulated with CST (100 nM) or LL-37 (10 µM) for 5 min and receptor phosphorylation was determined. An autoradiograph from a 3 h exposure is shown. Data shown are representative of 3 independent experiments.

**Fig 7: MrgX2 is resistant to desensitization and internalization.** (A-B) LAD2 cells were incubated with Indo-1AM, washed in a Ca\(^{2+}\)-free buffer and stimulated with (A) C3a (10 nM) and (B) LL-37 (3 µM) and Ca\(^{2+}\) mobilization in the absence of extracellular Ca\(^{2+}\) was determined. After 5 min, cells were exposed to Ca\(^{2+}\) at a final concentration of 1mM and intracellular Ca\(^{2+}\) mobilization was again determined. Traces represent results from 3 similar experiments. (C) MrgX2 expressing HMC-1 cells were exposed to C3a (100 nM) for different time periods and cell surface C3aR expression was determined by flow cytometry. (D) MrgX2-expressing HMC-1 cells were exposed to LL-37 (1 µM), or (E) PMA (50 ng/mL) for different time periods and MrgX2 expression levels were determined by flow cytometry using 12CA5 antibody. Data is represented as percent receptor surface expression levels of control. Data are mean ± SEM of three experiments. Statistical significance was determined by one-way ANOVA with Bonferroni’s post test. ** indicates p<0.001.

**Fig 8: Knockdown of GRK2 or GRK3 has no effect on Cortistatin and LL-37-induced degranulation in LAD2 mast cells.** LAD2 mast cells were stably transduced with scrambled shRNA control lentivirus or shRNA lentivirus targeted against GRK2 or GRK3. (A) Representative immunoblot of LAD2 cells with GRK2 knockdown is shown. (B) Quantitative PCR was performed to assess GRK3 mRNA levels in shRNA control and GRK3 knockdown (KD) cells. Results are expressed as a ratio of GRK3 to GAPDH mRNA levels. (C-D) Control shRNA, GRK2 and GRK3 KD cells were stimulated with (C) different concentrations of C3a (0.1 and 1 nM) or (D) LL-37 (1 and 3 µM) and CST (100 nM) and percent degranulation was determined. Data are mean ± SEM of three experiments. Statistical significance was determined by t-test (B) and two-way ANOVA with Bonferroni’s post test. * indicates p<0.01 and ** indicates p<0.001.
Fig 9: LL-37 activates CD34+ cell-derived primary mast cells via MrgX2. (A) CD34+ primary mast cells were stimulated with different concentrations of LL-37 (1 - 10 µM) and percent degranulation was determined. (B) Cells were incubated with Indo-1AM in Ca²⁺ free buffer and stimulated with 3 µM LL-37 at 100-200 s and intracellular Ca²⁺ mobilization was determined. After 5 min, cells were exposed to 1 mM Ca²⁺ and intracellular Ca²⁺ mobilization was determined. (C) Mast cells were stably transduced with scrambled shRNA control lentivirus or shRNA lentivirus targeted against MrgX2. Quantitative PCR was performed to assess MrgX2 mRNA levels in shRNA control and MrgX2 knockdown (KD) cells. Results are expressed as a ratio of MrgX2 to GAPDH mRNA levels. (D) Control shRNA or MrgX2 KD cells were stimulated with different concentrations of LL-37 (3 and 10 µM) and percent degranulation was determined. Data are mean ± SEM of three experiments. Statistical significance was determined by one-way ANOVA (A), t-test (C) or two-way ANOVA with Bonferroni’s post test (D). ** indicates p<0.001.
**Fig. 1**

A. 

Graph showing the relationship between LL-37 concentration (µM) and β-hexosaminidase release (%). Two data points are significantly different from the control (**). The concentration of LL-37 is plotted on the x-axis, ranging from 0 to 10 µM. The β-hexosaminidase release is shown on the y-axis, ranging from 0 to 100 %.

B. 

Graph showing the time course of changes in the ratio of 338 nm to 408 nm with and without PTx and La³⁺. The time (sec) is shown on the x-axis, ranging from 0 to 300 sec. The ratio of 338 nm to 408 nm is shown on the y-axis, ranging from 0 to 4.

C. 

Bar graph comparing β-hexosaminidase release (% Release) in different conditions: Vehicle and PTx. The concentration of LL-37 is plotted on the x-axis, ranging from 0 to 10 µM. The release of β-hexosaminidase is shown on the y-axis, ranging from 0 to 100 %.

D. 

Bar graph showing β-hexosaminidase release (% Release) with and without La³⁺ at different concentrations of LL-37. The concentration of LL-37 is plotted on the x-axis, ranging from 0 to 10 µM. The release of β-hexosaminidase is shown on the y-axis, ranging from 0 to 100 %.
Fig. 2
Fig. 3

A. 

B. 

C. 

D. 

**

D.

CCL4 (pg/mL)
Fig. 4
Fig. 5
A

C3aR: LGKDFRKARQSIQGILEAAFSEELTRSTHCPSSNVISERSNSTTV
MrgX2: LQQPIKLALQRALQDIAEVDHSEGFRQGTPEMS---------RSSLV-

B. C3aR

C3aR transfected

C. MrgX2

MrgX2 transfected

D. C3aR

MrgX2

C3aR →

MrgX2 →

Fig. 6
Fig. 7
**Fig. 8**

A. Western blot images showing control and GRK2 KD conditions for GRK2 and β-Actin.

B. Bar graph showing relative expression levels of GRK3 to GAPDH (ΔΔCT).

C. Bar graph showing β-hexosaminidase release in response to C3a at different concentrations.

D. Bar graph showing β-hexosaminidase release in response to LL-37 and CST at different concentrations.
**Fig. 9**

A. 

Bar graph showing 

\[ \beta\text{-hexosaminidase (% Release)} \]

vs.

\[ \text{LL-37 (\text{\( \mu \text{M} \)})} \]

with significant increases at 3 and 10 \( \mu \text{M} \).

B. 

Graph showing the ratio (338nm/408 nm) vs. time (s).

C. 

Bar graph showing relative expression levels (\( \Delta \Delta \text{CT} \)) vs.

\[ \text{shRNA control, MrgX2 KD} \]

with significant increases at MrgX2 KD.

D. 

Bar graph showing \( \beta\text{-hexosaminidase (% Release)} \) vs.

\[ \text{LL-37 (\text{\( \mu \text{M} \)})} \]

with significant increases at 3 and 10 \( \mu \text{M} \).
MAS-related gene X2 (MrgX2) is a novel G protein coupled receptor for the antimicrobial peptide LL-37 in human mast cells: resistance to receptor phosphorylation, desensitization and internalization
Hariharan Subramanian, Kshitij Gupta, Qiang Guo, Ryan Price and Hydar Ali

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