MrgX2-mediated internalization of LL-37 and degranulation of human LAD2 mast cells

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Abstract. LL-37 is the sole antimicrobial peptide of human cathelicidin comprising 37 amino acids, which is expressed mainly in epithelial cells and neutrophils, and activates mast cells. In the present study, in order to elucidate the mechanism of mast cell activation by LL-37, the associations between the internalization of LL-37 and Mas-related gene X2 (MrgX2)-mediated mast cell activation (degranulation) was investigated using the human mast cell line, LAD2. LL-37 was rapidly internalized into the cells, and induced degranulation, as assessed by the extracellular release of β-hexosaminidase. Pertussis toxin, a G-protein inhibitor, significantly suppressed the internalization of LL-37 and the degranulation of LAD2 cells. Furthermore, small interfering (si)-RNA-mediated knockdown of MrgX2, a putative G protein-coupled receptor for LL-37, inhibited the internalization of LL-37 and degranulation of LAD2 cells. Notably, LL-37 internalization was enhanced by the stable expression of MrgX2 in HMC-1 and 293 cells. In addition, the internalized LL-37 mainly colocalized with MrgX2 in the perinuclear region of LAD2 cells. Furthermore, neuraminidase treatment, which removes negatively charged sialic acid from the cell surface, markedly reduced the internalization of LL-37 and degranulation of LAD2 cells, and clathrin-mediated endocytosis inhibitors (dynasore and chlorpromazine) inhibited the internalization and degranulation of LAD2 cells. Taken together, these observations indicated that LL-37 may bind the negatively charged cell surface molecules, rapidly internalize into the cells via clathrin-mediated endocytosis and interact with MrgX2 to activate mast cells (LAD2 cells).

Introduction

Mammalian cells express a number of peptide antibiotics that function as effector components in innate host defense systems (1-3). Cathelicidin is a family of antimicrobial peptides, characterized by the highly conserved cathelin-like prosequences and variable C-terminal sequences that correspond to the mature antibacterial peptides (4). LL-37 is the sole antibacterial peptide of human cathelicidin comprising of 37 amino acids, which is expressed mainly in epithelial cells and neutrophils, and cleaved from the 18-kDa human cationic antibacterial polypeptide (5). LL-37 has an α-helical amphiphilic structure, and can disrupt the outer and inner membranes of bacteria. In addition its broad killing activity against bacteria, fungi, and certain viruses (6), LL-37 has diverse immunomodulatory effects, including the regulation of pro- and anti-inflammatory mediator production (7,8), wound healing (9), angiogenesis (10,11), and expression of nerve elongation factors (12). Additionally, it was reported that LL-37 induces chemotaxis and histamine release by mast cells (13).

Mast cells are usually present in submucosal tissues and connective tissues, and play a pivotal role in innate immunity by releasing several mediators such as histamine, leukotrienes, and tryptase (14,15). We previously found that LL-37 activates mast cells to induce chemotaxis, degranulation, and the production of cytokines and inflammatory mediators (13,16,17). As mast cells and LL-37-expressing epidermal cells are located close to each other, we hypothesized that LL-37 activates mast cells locally at the sites of infection/inflammation, and controls the immune response. Recently, a G protein-coupled receptor, Mas-related gene X2 (MrgX2), was identified as a putative receptor for LL-37 for mast cell degranulation (18). This suggests that LL-37 interacts with MrgX2 and activates the G protein signaling cascade. However, little is known about how LL-37 activates MrgX2, thereby leading to mast cell degranulation. In contrast, some pruritogenic basic peptides, such as

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substance P, have been reported to induce mast cell degranulation by translocating (internalizing) into the cells (19). LL-37 has affinity for the cell membrane based on its α-helical and amphipathic structure (20). Thus, we speculate that LL-37 also internalizes into the cells and activates MrgX2, thereby inducing the degranulation of mast cells. Therefore, in this study, we investigated the relationship between the internalization of LL-37 and MrgX2-mediated mast cell degranulation using the LAD2 human mast cell line.

Materials and methods

Reagents and antibodies. Chlorpromazine hydrochloride and genistein were purchased from Nacalai Tesque (Kyoto, Japan). Dynasore and neuraminidase were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Pertussis toxin was purchased from Fujifilm Wako Pure Chemical (Osaka, Japan). A 37-mer peptide of hCAP18 (LL-37; L[l]LGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES[37]) was synthesized by the solid-phase method on a peptide synthesizer (model PSSM-8; Shimadzu Scientific Instruments, Kyoto, Japan) by fluorenylmethoxycarbonyl chemistry, as described previously (21). The concentration of the LL-37 stock solution was measured using the bicinchoninic acid method with bovine serum albumin (BSA) as a standard (Pierce BCA Protein Assay kit; Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Anti-LL-37 serum was raised in rabbits using LL-37 covalently coupled to keyhole limpet hemocyanin, as described previously (5). Rabbit anti-human MrgX2 polyclonal antibodies (pAbs) were purchased from Abcam (ab129548, Cambridge, MA, USA) and MyBioSource (MBS7006480; San Diego, CA, USA). Mouse anti-LL-37 monoclonal antibody (mAb) was purchased from Santa Cruz Biotechnology, Inc. (sc-166770; Santa Cruz, CA, USA). Phycoerythrin-conjugated mouse anti-human MrgX2 mAb and its isotype control were purchased from BioLegend (359004 and 400314; San Diego, CA, USA).

Cell culture. The human mast cell line LAD2 was a kind gift from Dr Dean D. Metcalfe (National Institutes of Health, Bethesda, MD, USA), and was maintained in StemPro-34 serum-free media (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with penicillin (50 IU/ml), streptomycin (50 μg/ml), L-glutamine (2 mM), and recombinant human stem cell factor (100 ng/ml). Another human mast cell line, HMC-1, was obtained from Merck Millipore (Darmstadt, Germany), and maintained in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 IU/ml), streptomycin (100 μg/ml), and α-thioglycerol (1.2 mM). The human embryonic kidney cell line 293 was supplied by American Type Culture Collection (Manassas, VA, USA), and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, penicillin (100 IU/ml), and streptomycin (100 μg/ml).

β-Hexosaminidase release. LAD2 mast cells ($1x10^5$ cells) were suspended in 200 μl of Tyrode's buffer (130 mM NaCl, 5 mM KCl, 3.2 mM KH_2PO_4, 1.4 mM $CaCl_2.2H_2O$, 1 mM $MgCl_2.6H_2O$, 10 mM HEPES, 5.6 mM D-glucose, and 0.1% BSA), and then stimulated with differing concentrations of LL-37 (1-10 μM) for 40 min at 37°C. The activity of β-hexosaminidase in the supernatants and total cell lysates, which were solubilized with 1% Triton X-100, was quantified by hydrolysis of 4 mM p-nitrophenyl-N-acetyl-β-D-glucopy ranoside in 0.1 M sodium citrate buffer (pH 4.5) for 30 min at 37°C, and the reaction was stopped by the addition 0.2 M glycine buffer (pH 11), as previously reported (22). The absorbance was measured with a microplate reader at a wavelength of 405 nm. The percentage of β-hexosaminidase release was calculated using the formula: % release=(OD of stimulated supernatant-OD of unstimulated supernatant) x100/(OD of total cell lysate-OD of unstimulated supernatant). In some experiments, mast cells were pre-treated with pertussis toxin (250 ng/ml) for 60 min, endocytosis inhibitors for 30 min, or neuraminidase for 40 min; thereafter, the cells were washed and stimulated with LL-37.

Determination of LL-37 internalization. LAD2 mast cells ($1x10^5$ cells) were suspended in 200 μl of Tyrode's buffer and then incubated with LL-37 (5 μM) for 40 min at 37°C, unless otherwise stated. Cells were washed twice with ice-cold phosphate-buffered saline (PBS) containing 1% BSA and resuspended in PBS. Cells were cytocentrifuged with Cytospin 4 (Shandon, Runcorn, UK) at 300 x g for 2 min, fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.2% saponin, and blocked by Blocking One (Nacalai Tesque, Kyoto, Japan). Cells were then incubated with rabbit anti-LL-37 serum or mouse anti-LL-37 mAb overnight at 1:1,000, followed by incubation with the respective secondary antibodies conjugated with Alexa Fluor 488 or 594 overnight at 1:1,000 at 4°C. Alternatively, for double staining of LL-37 and MrgX2, cells were further incubated with anti-MrgX2 rabbit polyclonal antibody (MyBioSource) overnight at 1:200, followed by incubation with the secondary antibodies conjugated with Alexa Fluor 488. After washing, the cells were mounted in Vectorshield mounting media containing 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA). Images were obtained by using the fluorescence microscope BZ-X700 (Keyence Japan, Osaka, Japan). The percentage of LL-37-internilized cells was calculated using the following formula: The LL-37-containing cell number x100/the total cell number. Alternatively, the internalization of LL-37 was determined by measuring the fluorescence intensities using ImageJ software (NIH Image, National Institutes of Health, Bethesda, MD, USA). The fluorescence intensity values were calculated as the average of two distinct regions on the same slide glass. Data represent the ratio of controls cells incubated with LL-37, but not treated with pertussis toxin, MrgX2 knockdown, MrgX2 stable expression, neuraminidase, or endocytosis inhibitors.

siRNA-mediated knockdown of MrgX2. Short-interfering RNA products (MISSION esiRNA) for MrgX2 (EHU145411) and universal negative control siRNA (SIC-001) were purchased from Sigma-Aldrich (Merck KGaA). The complex of siRNAs and Lipofectamine 3000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) were formed in Opti-MEM (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. After incubating for 5 min at room temperature, the complexes were added to

LAD2 cells suspended in StemPro-34 media containing antibiotics. The knockdown efficiency of MrgX2 was confirmed after 72 h by Western blotting. For western blotting, LAD2 cells ($2x10^5$) were washed twice with Tyrode's buffer, and lysed in 50 μl RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 150 mM NaCl, and 10 mM Tris-HCl, pH 7.2) containing protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan). Lysed samples were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes (Immobilon-P; Merck Millipore). The membranes were blocked with Blocking One (Nacalai Tesque), and sequentially probed with a 1:250 dilution of rabbit anti-MrgX2 polyclonal antibody (ab129548; Abcam) and a 1:5,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (Merck Millipore). The expression of GAPDH was evaluated as an internal control. The membranes were reprobed with a 1:5,000 dilution of anti-GAPDH mAb (MAB374, Merck Millipore) and a 1:5,000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG (115-055-044; Jackson ImmunoResearch, West Grove, PA, USA). Signals were detected with Super Signal West Dura Chemiluminescent Substrate (Pierce; Thermo Fisher Scientific, Inc.), and the detected bands were analyzed with Image Studio software Ver 4.0 and C-DiGit blot scanner (LI-COR, Lincoln, NB, USA). Using Mrgx2-knockdown cells, LL-37-induced β-hexosaminidase release and LL-37 internalization were evaluated as described above.

Preparation of MrgX2-expressing stable transfectants. Total RNA was extracted from LAD2 mast cells with RNeasy (Qiagen, Hilden, Germany) and reverse transcribed into cDNA using Oligo(dT) primer (KOD-Plus-; Toyobo, Osaka, Japan). MrgX2 cDNA was amplified with the following primers: Forward primer 5'-TATAAGCTTACCATGGAT CCAACCACCCCGGC-3' and reverse primer 5'-GCCGAA TTCCTACACCAGACTGCTTCTCGACATC-3'. The forward and reverse primers contained the sequences for *Hin*dIII and *Eco*RI digestion sites, respectively. The amplified product was digested and ligated into the pcDNA3 mammalian expression vector (Sigma-Aldrich; Merck KGaA) with Ligation high ver.2 (Toyobo) for subcloning of pcDNA3-MrgX2. 293 and HMC-1 cells were transfected with pcDNA3-MrgX2 using Lipofectamine 3000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) based on the manufacturer's protocol, and Mrgx2-expressing 293 and HMC-1 cells were selected in the presence of 0.4 and 1 mg/ml G418, respectively. MrgX2 expression was confirmed by flow cytometry as follows: The selected cells ($5x10^5$) were stained with 1:200 diluted phycoerythrin-labelled anti-MrgX2 mAb or isotype control IgG, and were measured by using BD FACS Calibur (BD Biosciences, Franklin Lakes, NJ, USA). Internalization of LL-37 was evaluated as described above.

Statistical analysis. Data are shown as the mean ± standard error of the mean. Significance was determined by one-way analysis of variance with Tukey's post hoc test using GraphPad Prism 7.0 software (GraphPad Software, Inc., La Jolla, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

LL-37-induced degranulation and LL-37 internalization by the human mast cell line LAD2. We first examined whether LL-37-stimulation induces the degranulation of LAD2. As shown in Fig. 1A, LL-37 induced degranulation, as determined by β-hexosaminidase release, in time- and concentration-dependent manners. β-Hexosaminidase release was observed within 5-10 min after LL-37 stimulation and reached a plateau at 20 min; 13.4% after 40-min stimulation with 5 μM of LL-37. Next, to investigate the relationship between the LL-37 internalization and mast cell degranulation, LAD2 cells were incubated with or without LL-37 (5 μM) for 40 min, and internalized LL-37 was detected by anti-LL-37 rabbit pAbs and Alexa Fluor 488-conjugated secondary antibody. As shown in Fig. 1B, the intracellular fluorescence signals of LL-37 were visualized as dot patterns (middle panel and right panel, an enlarged photo of the boxed area); however, the control cells without LL-37 incubation had no signal (left panel). Of note, the percentage of LL-37-internilized cells reached a plateau within 2-5 min, and prolonged incubation up to 30 min did not increase the internalization of LL-37 (Fig. 1C), indicating that LL-37 is quickly internalized into mast cells prior to degranulation.

It has been reported that the mast cell degranulation response by LL-37 is susceptible to pertussis toxin (PTx) and involves G protein-coupled receptor activation (13). However, it is unclear whether the internalization of LL-37 is also susceptible to pertussis toxin. Thus, LAD2 cells were stimulated with LL-37 (5 μM) in the presence or absence of pertussis toxin, and degranulation and LL-37-internalization were evaluated. As previously reported (13), degranulation was significantly suppressed by pertussis toxin (Fig. 1D). Moreover, LL-37-internalization was also significantly suppressed by pertussis toxin (Fig. 1E).

These observations suggest that not only the LL-37-induced degranulation but also the internalization of LL-37 are elicited via the G protein-coupled pathway.

Effects of MrgX2 knockdown on the degranulation and LL-37-internalization of LAD2. It was previously reported that a G protein-coupled receptor, MrgX2, functions in the LL-37-induced mast cell degranulation (18). Thus, we next investigated whether MrgX2 is also involved in the internalization of LL-37 into LAD2 cells using siRNA-mediated MrgX2-knockdown cells. The expression of MrgX2 in MrgX2-knockdown cells was reduced to approximately 30% of that in control siRNA-transfected cells (Fig. 2A). Based on the reduced MrgX2 expression, the β-hexosaminidase release was slightly reduced in MrgX2-knockdown cells by 10 and 20% at 1 and 2 μM LL-37, respectively, compared with that in control siRNA-transfected cells (Fig. 2B). Similarly, the LL-37 internalization was markedly suppressed by MrgX2-knockdown (Fig. 2C), and reduced in MrgX2-knockdown cells by 30 and 20% at 1 and 2 μM LL-37, respectively, compared with that in control siRNA-transfected cells (Fig. 2D). These observations suggest that MrgX2 is likely involved in both the degranulation and internalization of LL-37 by LAD2 cells.

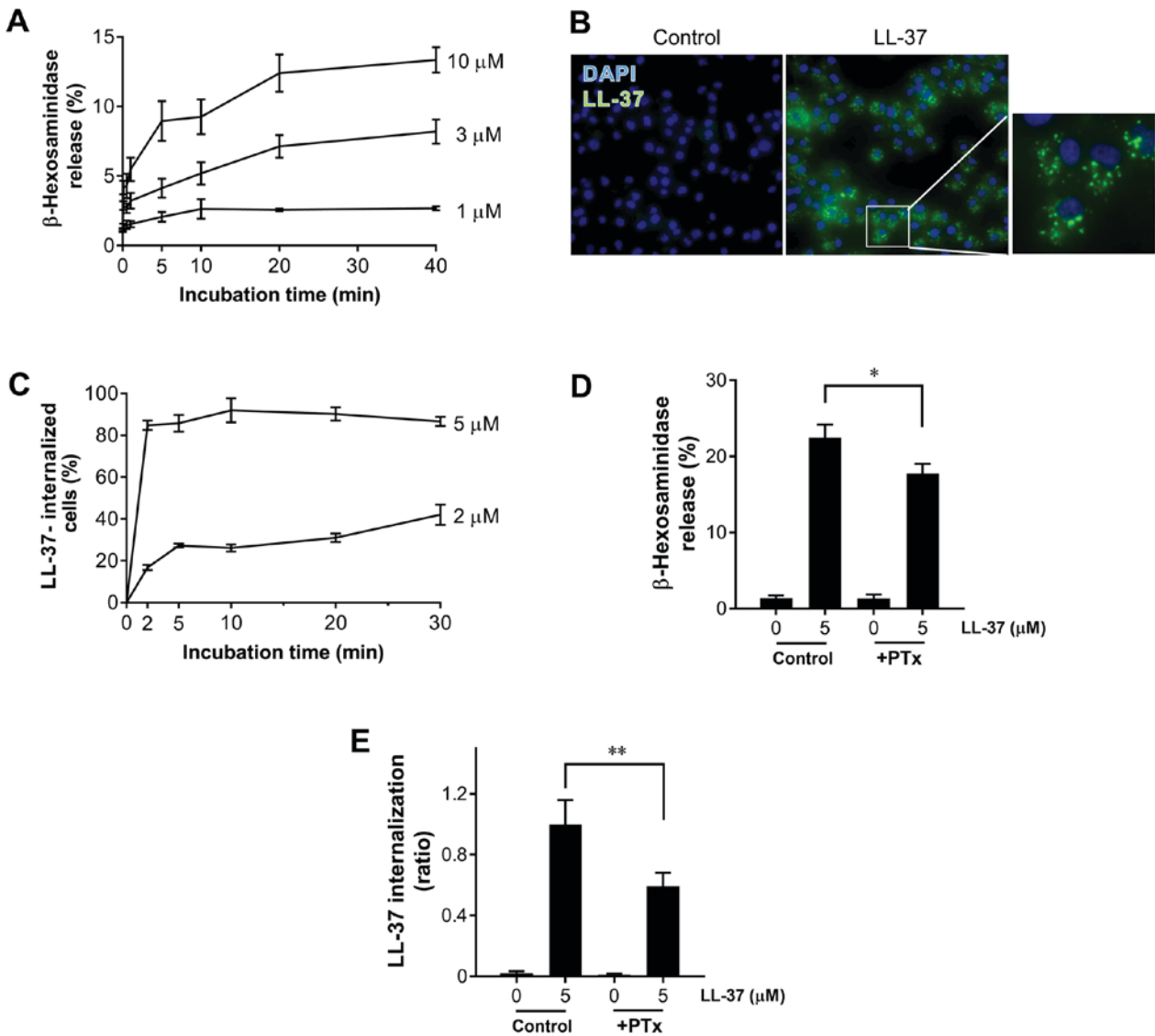*Effects of stable MrgX2 expression on the LL-37-internalization by HMC-1 and 293 cells.* To further

Figure 1. LL-37 induces degranulation and internalization into LAD2 cells. LAD2 cells were incubated with LL-37 (1-10 μM) at 37˚C for the indicated time periods, and (A) the release of β-hexosaminidase was measured. (B) LAD2 cells were cytocentrifuged following incubation without (Control, left panel) or with 5 μM LL-37 (LL-37, middle panel) at 37˚C for 40 min, and LL-37 was stained with anti-LL-37 rabbit polyclonal antibodies followed by anti-rabbit Immunoglobulin-G conjugated with Alexa Fluor 488 (green staining). Magnification, x400. The right-hand panel presents the enlarged photo of the boxed area in the middle panel. LAD2 cells were incubated with LL-37 (2-5 μM) at 37˚C for the indicated time periods, and (C) the percentage of LL-37-internilized cells was calculated using the following formula: (LL-37-containing cell number x100)/the total cell number. LAD2 cells were untreated (Control) or treated with PTx (250 ng/ml, +PTx) at 37˚C for 1 h, and further incubated with or without LL-37 (5 μM) for 40 min. The release of (D) β-hexosaminidase and (E) LL-37 internalization was evaluated. The internalization of LL-37 was determined by measuring the fluorescence intensities using ImageJ software, and represented as a ratio of controls cells incubated with LL-37 (5 μM) but not treated with pertussis toxin. Data are presented as the mean ± standard error of the mean of 4 to 8 independent experiments. *$P<0.05$ and **$P<0.01$, as indicated. PTx, pertussis toxin.

clarify the involvement of MrgX2 expression in LL-37 internalization, we established MrgX2-stable transfectants of the human mast cell line HMC-1 and embryonic kidney 293 cells, both of which do not endogenously express MrgX2. As shown in Figs. 3A and 4A, the cells transfected with the MrgX2-expression plasmid exhibited the augmented fluorescence intensity of MrgX2 as compared with the cells transfected with the control plasmid on flow cytometry using phycoerythrin-labeled anti-MrgX2 antibody. Furthermore, when stimulated with 5 μM LL-37, both MrgX2-expressing HMC-1 and 293 cells demonstrated enhanced LL-37 internalization compared with control plasmid-transfected HMC-1 and 293 cells (Figs. 3B and C, and 4B and C). It

should be noted that LL-37 internalization was moderately observed in control plasmid-transfected HMC-1 and 293 cells, suggesting that LL-37 is internalized into the cells independent of MrgX2 (Figs. 3B and C, and 4B and C). These observations clarified that MrgX2 plays a role in the LL-37 internalization, although LL-37 is also internalized into the cells in an MrgX2-independent manner. In addition, in separate experiments, we confirmed that LL-37 cannot induce β-hexosaminidase release by MrgX2-expressing HMC-1 and 293 cells nor by control plasmid-transfected HMC-1 and 293 cells, although these cells possess comparable β-hexosaminidase activity with LAD2 cells (data not shown). These observations suggest that signaling molecules
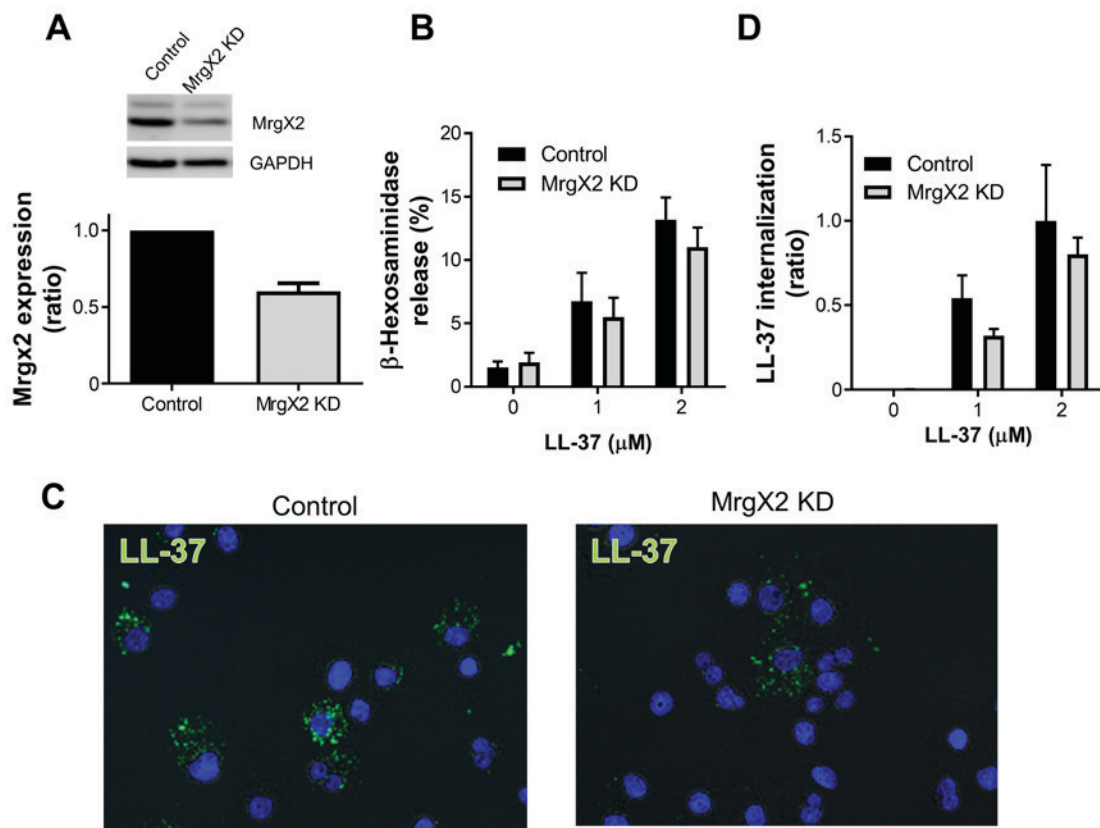
Figure 2. MrgX2 knockdown reduces degranulation and LL-37 internalization. Mrgx2 was knocked-down by MrgX2-targeted siRNA transfection, and confirmed by western blotting. The expression of GAPDH was evaluated as the internal control. Images are representative of 3 independent experiments. (A) MrgX2 expression was quantified by densitometry, and the expression in MrgX2-knockdown LAD2 cells (MrgX2 KD group) was expressed as a ratio of that in the control siRNA-transfected cells (Control group). (B) The control siRNA-transfected LAD2 cells (Control group) and MrgX2-knockdown cells (MrgX2 KD group) were incubated with or without LL-37 (1-2 μM) at 37˚C for 40 min, and the release of β-hexosaminidase was measured. (C) The control siRNA-transfected LAD2 cells (Control group, left panel) and MrgX2-knockdown cells (MrgX2 KD group, right panel) were cytocentrifuged following incubation with LL-37 (5 μM) at 37˚C for 40 min, and LL-37 was stained with anti-LL-37 rabbit polyclonal antibodies followed by anti-rabbit Immunoglobulin-G-Alexa Fluor 488 (green staining). Nuclei were stained with DAPI (blue staining). Images are representative of 3 independent experiments. Magnification, x400. (D) The control siRNA-transfected LAD2 cells (Control group) and MrgX2-knockdown cells (MrgX2 KD group) were incubated with or without LL-37 (1-2 μM) at 37˚C for 40 min. The internalization of LL-37 was determined by measuring the fluorescence intensities using ImageJ software, and represented as a ratio of the control siRNA-transfected LAD2 cells (Control group) incubated with LL-37 (2 μM). Data are presented as the mean ± standard error of the mean of 3 independent experiments. MrgX2, Mas-related gene X2; siRNA, small interfering RNA; KD, knockdown.

or machineries essential for LL-37-induced degranulation are missing in these cells regardless of the expression of MrgX2.

Colocalization of internalized LL-37 and MrgX2. A pruritogenic basic peptide, substance P, was previously found to be rapidly internalized after interaction and form a ligand-receptor complex with its receptor, NK$_1$R (23). Thus, to clarify the interaction of LL-37 with MrgX2, we next examined the localization of LL-37 and MrgX2 in the cells by immunofluorescence double staining of LL-37 and MrgX2. As shown in Fig. 5A, MrgX2 was diffusely detected in the cytoplasmic region or localized in the perinuclear region of the control cells without LL-37 stimulation. When cells were stimulated with LL-37, internalized LL-37 was detected as dot patterns in the perinuclear region (Fig. 5B). Notably, internalized LL-37 mainly colocalized with MrgX2, although not all MrgX2 colocalized with the internalized LL-37 (Fig. 5C).

Effects of neuraminidase-treatment and endocytosis inhibitors on the degranulation and LL-37 internalization of LAD2. Lastly, we evaluated the effects of neuraminidase-treatment and endocytosis inhibitors on the degranulation and LL-37 internalization of LAD2. Notably, neuraminidase treatment (0.2 U/ml) markedly reduced the degranulation and LL-37 internalization (Fig. 6A and B). Moreover, the clathrin-mediated endocytosis inhibitors dynasore (10 μM) and chlorpromazine (2.5 μM) inhibited degranulation, and chlorpromazine similarly suppressed LL-37 internalization. In contrast, a caveolar-mediated endocytosis inhibitor, genistein (10 μM), did not affect degranulation or LL-37 internalization (Fig. 6C and D).

Discussion

Mast cells are usually present in submucosal tissues and connective tissues, and play a central role in innate immunity and allergic reactions by releasing several mediators such as histamine, leukotrienes, tryptase, and several pro- and anti-inflammatory cytokines (24). For mast cell activation, two main pathways, immunoglobulin E-dependent and -independent, have been identified. Immunoglobulin E/antigen-dependent activation occurs in response to antigens, and is mediated by the
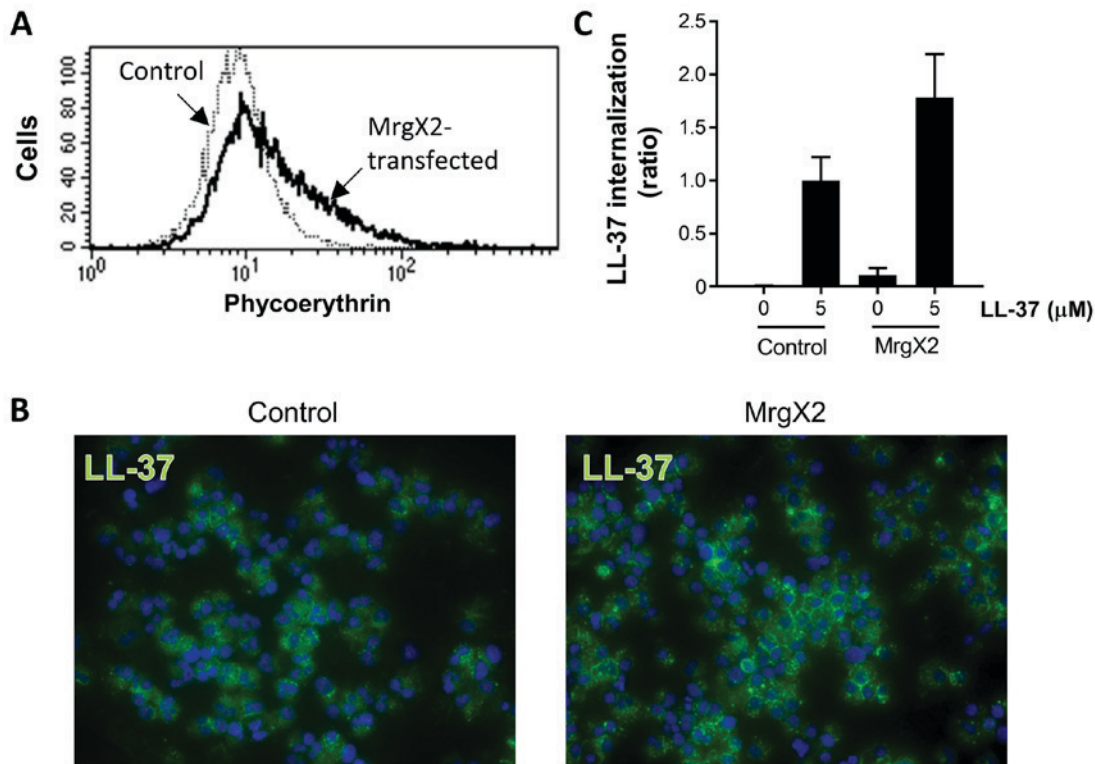
Figure 3. Stable expression of MrgX2 in HMC-1 cells enhances LL-37-internalization. The control plasmid-transfected HMC-1 cells (Control group) and MrgX2-expressing HMC-1 cells (MrgX2-transfected group) were incubated with phycoerythrin-conjugated anti-MrgX2 mouse mAbs, and (A) analyzed by flow cytometry. (B) Control plasmid-transfected HMC-1 cells (Control, left panel) and MrgX2-expressing HMC-1 cells (MrgX2, right panel) were cytocentri-fuged following incubation with LL-37 (5 μM) at 37˚C for 40 min, and LL-37 was stained with anti-LL-37 rabbit pAbs followed by anti-rabbit IgG-Alexa Fluor 488 (green staining). Nuclei were stained with DAPI (blue staining). Images are representative of 6 independent experiments. Magnification, x400. (C) Control plasmid-transfected HMC-1 cells (Control group) and MrgX2-expressing HMC-1 cells (MrgX2 group) were incubated with or without LL-37 (5 μM) at 37˚C for 40 min. The internalization of LL-37 was determined by measuring the fluorescence intensities using ImageJ software, and represented as a ratio of control plasmid-transfected HMC-1 cells (Control) incubated with LL-37 (5 μM). Data are presented as the mean ± standard deviation of 6 independent experiments. m/pAbs, mono/polyclonal antibodies; MrgX2, Mas-related gene X2.

cross-linking of specific immunoglobulin E-bound FcεRI on the cell surface (25). Although this classical activation pathway is well investigated, less is known about the immunoglobulin E-independent pathway. The activators of the FcεRI-independent pathway mainly comprise basic secretagogues, such as substance P, venom peptide mastoparan, neuropeptide Y, and compound 48/80, and some antibacterial peptides, including LL-37. They commonly activate a pertussis toxin-sensitive G-protein in mast cells and lead to degranulation; however, the receptor and signaling mechanism are unclear (24).

LL-37 is the sole anti-microbial peptide of the cathelicidin family in humans, and is cleaved from an 18-kDa precursor human cationic antimicrobial polypeptide. In addition to its broad spectrum of bactericidal activities, LL-37 can alter numerous immune responses. It has been reported that LL-37 utilizes formyl peptide receptor-like 1 (FPRL1) to chemoat-tract human neutrophils, T cells, and monocytes (26). FPRL1 also functions in the LL-37-mediated angiogenic activity of endothelial cells (11). Furthermore, LL-37 suppresses neutro-phil apoptosis via action on both FPRL1 and P2X7 (27). Based on this, we investigated whether WRW4, an FPRL1 antagonist, and KN-62, a P2X7 antagonist, can inhibit the LL-37-induced degranulation; however, both WRW4 and KN-62 did not inhibit the degranulation of a LAD2 human mast cell line (data not shown). The failure of these antagonists to inhibit the LL-37-mediated mast cell activation suggests that FPRL1 and P2X7 are not functional, at least regarding LL-37-mediated mast cell activation.

Recently, MrgX2, a member of the mas-related genes primarily expressed in mast cells and dorsal root ganglia, was identified as a putative receptor for LL-37 involved in mast cell activation; however, the mechanism by which LL-37 activates MrgX2 remains unclear. In contrast, some pruritogenic basic peptides, such as substance P, induce mast cell degranulation by internalizing into the cells (23). We recently demonstrated that LL-37 enhances the uptake of bacterial lipopolysac-charide by liver sinusoidal cells by forming a complex with the lipopolysaccharide and internalizing into the cells via an endocytosis-mediated mechanism (28). Thus, we speculated that LL-37 also internalizes into mast cells and activates MrgX2, thereby inducing degranulation. In the current study, we investigated the relationship between the internalization of LL-37 and MrgX2-mediated mast cell degranulation using a LAD2 mast cell line, and found that LL-37 internalizes into LAD2 mast cells in dose- and time-dependent manners, and induces degranulation, possibly via the pertussis toxin-sensi-tive G protein-coupled pathway (Fig. 1). Furthermore, based on the results using siRNA-mediated knockdown cells, MrgX2 may function in both the degranulation and internalization of LL-37 (Fig. 2), and this possibility was further supported by the finding that LL-37 internalization is enhanced in stably MrgX2-expressing HMC-1 cells and 293 cells (Figs. 3 and 4).
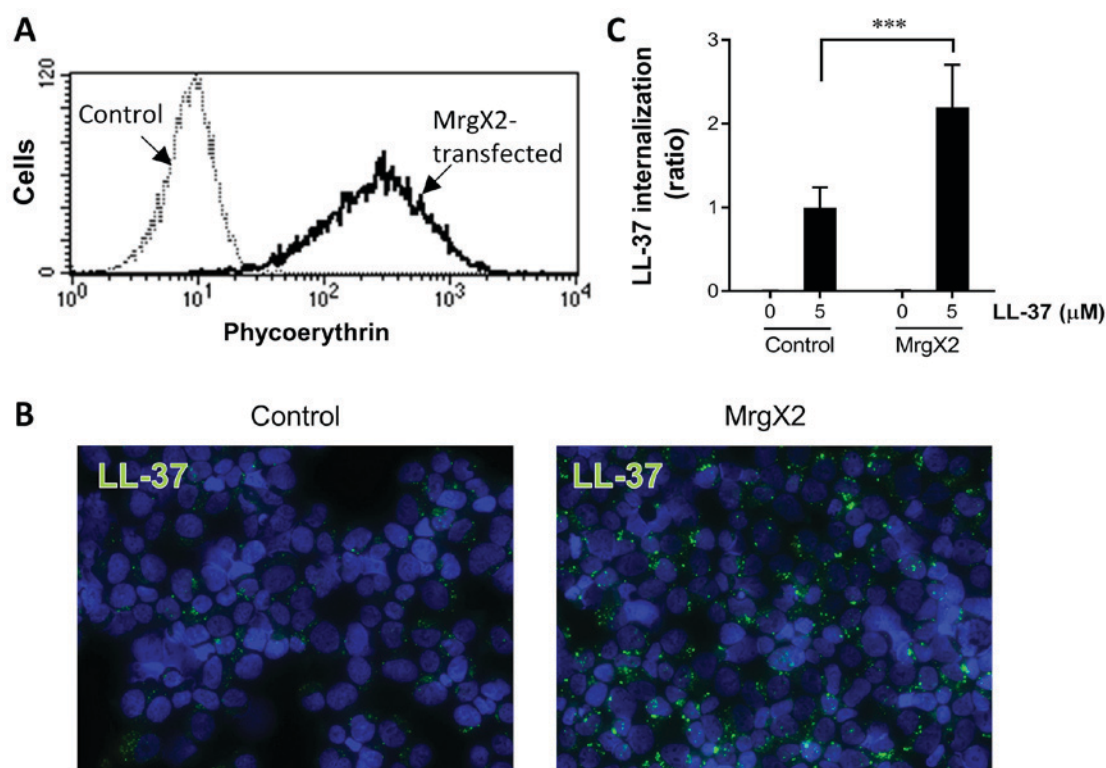
Figure 4. Stable expression of MrgX2 in 293 cells enhances LL-37-internalization. (A) The control plasmid-transfected 293 cells (Control group) and MrgX2-expressing 293 cells (MrgX2-transfected group) were incubated with phycoerythrin-conjugated anti-MrgX2 mouse mAbs, and analyzed by flow cytometry. (B) Control plasmid-transfected 293 cells (Control, left panel) and MrgX2-expressing 293 cells (MrgX2, right panel) were cytocentrifuged following incubation with LL-37 (5 μM) at 37°C for 40 min, and LL-37 was stained with anti-LL-37 rabbit pAbs followed by anti-rabbit Immunoglobulin G-Alexa Fluor 488 (green staining). Nuclei were stained with DAPI (blue staining). Images are representative of 6 independent experiments. Magnification, x400. (C) Control plasmid-transfected 293 cells (Control group) and MrgX2-expressing 293 cells (MrgX2 group) were incubated with or without LL-37 (5 μM) at 37°C for 40 min. The internalization of LL-37 was determined by measuring the fluorescence intensities using ImageJ software, and represented as a ratio of control plasmid-transfected 293 cells (Control) incubated with LL-37 (5 μM). Data are presented as the mean ± standard error of the mean of 6 independent experiments. ***$P<0.001$, as indicated. m/pAbs, mono/polyclonal antibodies; MrgX2, Mas-related gene X2.
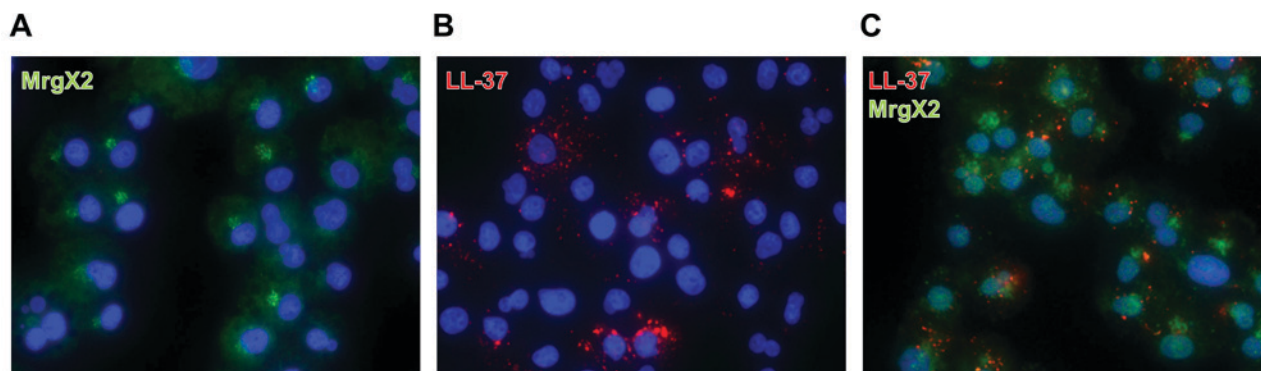

Figure 5. Colocalization of LL-37 and MrgX2 in LAD2 cells. LAD2 cells were cytocentrifuged following incubation without LL-37 at 37°C for 40 min. (A) Cells were incubated with anti-MrgX2 pAbs, followed by anti-rabbit IgG-Alexa Fluor 488 (green staining). (B) LAD2 cells were cytocentrifuged following incubation with LL-37 (5 μM) at 37°C for 40 min. Cells were incubated with anti-LL-37 mAbs followed by anti-mouse IgG-Alexa Fluor 594 (red staining). (C) LAD2 cells incubated with LL-37 (5 μM) at 37°C for 40 min were double stained with anti-MrgX2 rabbit pAbs and anti-LL-37 mouse mAbs, followed by secondary Abs conjugated with Alexa Fluor 488 and 594, respectively Nuclei were stained with DAPI (blue staining). Images are representative of 4 independent experiments. Magnification, x400. IgG, immunoglobulin G; m/pAbs, mono/polyclonal antibodies; MrgX2, Mas-related gene X2.

To further clarify the interaction of LL-37 with MrgX2, the localization of LL-37 and MrgX2 in LAD2 cells was examined by the immunofluorescence double staining of LL-37 and MrgX2. MrgX2 was diffusely detected in the cytoplasmic region or localized in the perinuclear region of control cells without LL-37 stimulation (Fig. 5A). Notably, when cells were stimulated with LL-37, internalized LL-37 was detected as dot patterns and colocalized with MrgX2 in the perinuclear region, although not all MrgX2 colocalized with the internalized LL-37 (Fig. 5B and C). These observations suggest that LL-37 interacts with MrgX2 after internalization into the cells for mast cell activation.
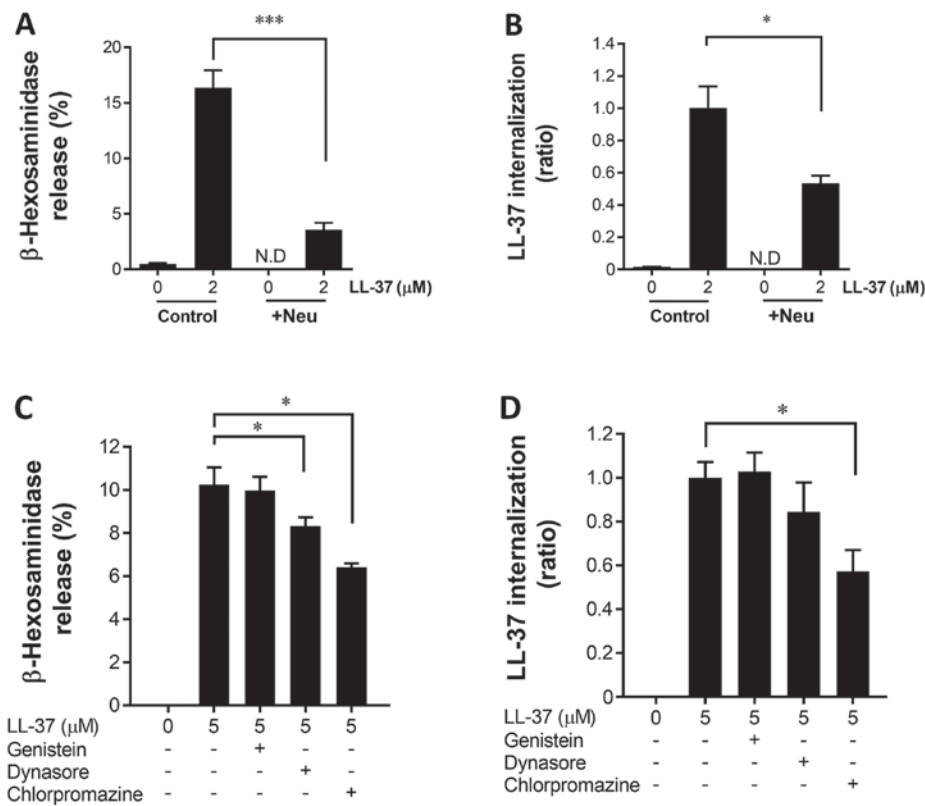
Figure 6. Effects of neuraminidase treatment and endocytosis inhibitors on the degranulation and LL-37 internalization of LAD2 cells. LAD2 cells were treated with neuraminidase (0.2 U/ml, +Neu) or without (Control) at 37˚C for 40 min, and further incubated with or without LL-37 (2 μM) for 40 min. (A) Thereafter, the release of β-hexosaminidase was measured. Alternatively, the cells were cytocentrifuged, and LL-37 was stained with anti-LL-37 rabbit pAbs followed by anti-rabbit IgG-Alexa Fluor 488. (B) The internalization of LL-37 was determined by measuring the fluorescence intensities using ImageJ software, and represented as a ratio of the control cells (Control) incubated with LL-37 (2 μM) but without neuraminidase treatment. (C) In addition, LAD2 cells were treated with or without genistein (10 μM), and dynasore (10 μM) or chlorpromazine (2.5 μM), and further incubated with or without LL-37 (5 μM) at 37˚C for 40 min, and the β-hexosaminidase release was measured. Alternatively, the cells were cytocentrifuged, and LL-37 was stained with anti-LL-37 rabbit pAbs followed by anti-rabbit IgG-Alexa Fluor 488. (D) The internalization of LL-37 was determined by measuring the fluorescence intensities using ImageJ software, and represented as a ratio of the cells incubated with LL-37 (5 μM) but without endocytosis inhibitors. Data are presented as the mean ± standard error of the mean of 4 to 8 independent experiments. *$P<0.05$ and ***$P<0.001$, as indicated. IgG, immunoglobulin G; pAbs, polyclonal antibodies; N.D, not detected; MrgX2, Mas-related gene X2.

It has been reported that a pruritogenic peptide, substance P, is internalized into cells by endocytosis for mast cell activation (23). Moreover, positively charged amino acid residues (Arg and Lys) in the N-terminal region and hydrophobic amino acid residues (Phe, Leu, and Met) in the C-terminal region of substance P are essential for the binding of substance P to the cell surface and subsequent internalization into the cells. This is because neuraminidase treatment, which can remove negatively charged sialic acid from the cell surface, inhibits the binding of substance P to the cell surface and mast cell activation (29). As LL-37 is an amphipathic molecule with cationic and hydrophobic features, neuraminidase treatment was expected to inhibit LL-37 internalization and mast cell activation. Thus, we evaluated the effects of neuraminidase treatment and endocytosis inhibitors on LL-37 internalization and LAD2 cell activation. Indeed, neuraminidase and clathrin-mediated endocytosis inhibitors suppressed the LL-37-internalization and degranulation of LAD2 (Fig. 6). These observations suggest that LL-37, a positively charged amphipathic molecule, interacts with the negatively charged cell surface molecules, such as sialic acid, and internalizes into the cells via clathrin-mediated endocytosis for mast cell activation.

In conclusion, to elucidate the mechanism of mast cell activation by LL-37, the relationship between the internalization of LL-37 and MrgX2-mediated mast cell activation was evaluated in the present study. We found that LL-37 likely binds with the negatively charged cell surface molecules, rapidly internalizes into the cells via clathrin-mediated endocytosis, and interacts with MrgX2 for mast cell activation. Further studies are needed to clarify the mechanism by which the internalized LL-37 induces the signal leading to mast cell activation after interacting with MrgX2.

Acknowledgements

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

TM, KS and IN designed the research. TM and KS performed the experiments. TM and IN analyzed the data. HyT, FN, JR and HsT interpreted and discussed the data. TM and IN prepared the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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