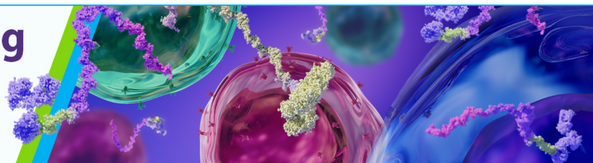

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Hariharan Subramanian, Kshitij Gupta, Donguk Lee, Arzu K. Bayir, Harry Ahn and Hydar Ali

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β-Defensins Activate Human Mast Cells via Mas-Related Gene X2

Hariharan Subramanian, Kshitij Gupta, Donguk Lee, Arzu K. Bayir, Harry Ahn, and Hydar Ali

Human β-defensins (hBDs) stimulate degranulation in rat peritoneal mast cells in vitro and cause increased vascular permeability in rats in vivo. In this study, we sought to determine whether hBDs activate murine and human mast cells and to delineate the mechanisms of their regulation. hBD2 and hBD3 did not induce degranulation in murine peritoneal or bone marrow–derived mast cells (BMMC) in vitro and had no effect on vascular permeability in vivo. By contrast, these peptides induced sustained Ca^{2+} mobilization and substantial degranulation in human mast cells, with hBD3 being more potent. Pertussis toxin (PTx) had no effect on hBD-induced Ca^{2+} mobilization, but La^{3+} and 2-aminoethoxydiphenyl borate (a dual inhibitor of inositol 1,4,5-triphosphate receptor and transient receptor potential channels) caused substantial inhibition of this response. Interestingly, degranulation induced by hBDs was substantially inhibited by PTx, La^{3+}, or 2-aminoethoxydiphenyl borate. Whereas human mast cells endogenously express G protein–coupled receptor, Mas-related gene X2 (MrgX2), rat basophilic leukemia, RBL-2H3 cells, and murine BMMCs do not. Silencing the expression of MrgX2 in human mast cells inhibited hBD-induced degranulation, but had no effect on anaphylatoxin C3a-induced response. Furthermore, ectopic expression of MrgX2 in RBL-2H3 and murine BMMCs rendered these cells responsive to hBDs for degranulation. This study demonstrates that hBDs activate human mast cells via MrgX2, which couples to both PTx-sensitive and insensitive signaling pathways most likely involving Gαq and Gαi to induce degranulation. Furthermore, murine mast cells are resistant to hBDs for degranulation, and this reflects the absence of MrgX2 in these cells. ***The Journal of Immunology*, 2013, 191: 345–352.**

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Human β-defensins (hBDs) are small cationic antimicrobial peptides (AMPs) that are produced by epithelial cells and platelets and play an important role in host defense (1, 2). Of the four members of this family (hBD1–4), hBD-1 is constitutively expressed, whereas the others are induced by bacteria, viruses, and cytokines. In addition to their host defense functions, hBD2 and hBD3 display immunomodulatory properties and induce the expression of costimulatory molecules on dendritic cells in a TLR-dependent manner (3, 4). Furthermore, they promote chemotaxis of $CD4^+$ T lymphocytes and macrophages via the activation of chemokine receptor, CCR2 (5, 6). hBD3 causes the recruitment of $CD11c^+$ dendritic cell precursors via CCR6 into tumorigenic locations where VEGF-A transforms them into endothelial cells, resulting in neovascularization, tumor development, and progression (7).

Mast cells are multifunctional immune cells, and in humans two subtypes are recognized based on the composition of their secretory granules (8, 9). Thus, mast cell granules that contain both tryptase and chymase are designated MC_{TC}, whereas those containing only tryptase are known as MC_T. Rat peritoneal mast cells (PMCs) display phenotypic and functional properties similar to human MC_{TC}, and both hBD2 and hBD3 induce degranulation in these cells (10, 11). Moreover, hBD3 causes increased cutaneous vascular permeability in wild-type, but not mast cell–deficient W^s/W^s rats (11). Studies with pertussis toxin (PTx) indicated the involvement of G proteins, but the G protein–coupled receptors (GPCRs) via which hBDs activate rat mast cells remain unknown (11). We have recently shown that the antimicrobial peptide LL-37 activates human mast cells via a novel GPCR, known as MrgX2 (12). This raises the interesting possibility that hBDs can also activate human mast cells via MrgX2 or a related GPCR (13). However, the possibility that hBDs activate primary murine mast cells has not been reported.

The purpose of the current study was to determine whether hBD2 and hBD3 activate murine and human mast cells and to determine the mechanisms of their regulation. Surprisingly, we found that murine mast cells are resistant to activation by hBDs in vitro and in vivo. By contrast, hBDs caused degranulation in human mast cells via MrgX2, which couples to both PTx–sensitive and insensitive G proteins, most likely Gαq and Gαi.

Materials and Methods

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 PTx were purchased from Invitrogen (Gaithersburg, MD). Amaxa transfection kit (Kit V) was purchased from Lonza (Gaithersburg, MD). All recombinant human cytokines were purchased from PeproTech (Rocky Hill, NJ). Cortistatin (CST)-14 was obtained from American Peptide (Vista, CA). Native complement C3a was from Complement Technology (Tyler, TX). LL-37 and mouse cathelin-related AMP (mCRAMP) was from Anaspec (Freemont, CA). hBD2 and hBD3 were from Peptide International (Louisville, KY). MrgX2 Ab was purchased from Novus Biologicals (Littleton, CO). Bisindolylmaleimide (GFX; GF109203X)

Department of Pathology, School of Dental Medicine, University of Pennsylvania, Philadelphia, PA 19104

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Address correspondence and reprint requests to Dr. Hydar Ali, Department of Pathology, University of Pennsylvania School of Dental Medicine, 240 South 40th Street, Philadelphia, PA 19104-6030. E-mail address: alih@upenn.edu

Abbreviations used in this article: AMP, antimicrobial peptide; 2-APB, 2-aminoethoxydiphenyl borate; BMMC, bone marrow–derived mast cell; CST, cortistatin; GFX, bisindolylmaleimide; GPCR, G protein–coupled receptor; HA, hemagglutinin; hBD, human β-defensin; mCRAMP, mouse cathelin-related AMP; PKC, protein kinase C; PMC, peritoneal mast cell; PTx, pertussis toxin; rhIL, human rIL; rhSCF, recombinant human stem cell factor; shRNA, short hairpin RNA.

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and 2-aminoethoxydiphenyl borate (2-APB) were obtained from Santa Cruz Biotechnology (Dallas, TX).

Generation of murine bone marrow–derived mast cells and PMCs

Bone marrow–derived mast cells (BMMCs) were obtained by flushing bone marrow cells from the femurs of C57BL/6 mice (The Jackson Laboratory) and culturing the cells for 4–6 wk in IMDM supplemented with 10% FCS, L-glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100 μg/ml), and murine IL-3 (10 ng/ml) (PeproTech). Peritoneal cells were collected from mice injected (i.p.) with 2 ml IMDM complete medium. Cells were seeded at 1×10^6/ml in complete IMDM supplemented with murine stem cell factor (10 ng/ml) and murine IL-3 (10 ng/ml). BMMCs and PMCs were used within 4–8 wk.

Passive cutaneous anaphylaxis

Four- to 6-wk-old C57BL/6 mice and C57BL/6J-*Kit*$^{W\text{-}sh}$ (W^{sh}/W^{sh}) mice weighing 20–22 g were used throughout the study. All animal experiments were performed according to a protocol approved by the Institutional Animal Care and Use Committee that conforms to the ethical standards formulated in the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health. Induction of passive cutaneous anaphylaxis was performed as described previously with minor modifications (14). Briefly, anti–DNP-BSA–specific IgE (Sigma-Aldrich; SPE-7, 20 ng) was intradermally injected into the mouse left ear, or PBS as a control in the right ear. After 24 h, mice were challenged with an i.v. injection of 100 μg Ag (DNP-BSA) in 200 μl PBS containing 1% Evans blue (Sigma-Aldrich) through the tail vein. Thirty minutes following the Ag challenge, the mice were euthanized; the ears were removed, weighed, dissolved in 500 μl formamide, and incubated at 55˚C overnight. After shaking, the supernatant was collected by centrifugation at $4000 \times g$ for 10 min, and absorbance was measured at 650 nm. For some experiments, mice were i.v. injected with 200 μl 1% Evans blue 5 min before intradermal injection of hBD3 (150 ng) in left ear and vehicle PBS in the right ear. After 30 min, mice were euthanized, and absorbance of Evans blue extracted from mouse ear was determined.

Differentiation of human mast cells from CD34$^+$ progenitors and culture of human mast cell lines

To generate primary mast cells, human CD34$^+$ progenitors were cultured in StemPro-34 medium (Life Technologies, Rockville, MD) supplemented with L-glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100 μg/ml), recombinant human stem cell factor (rhSCF) (100 ng/ml), human rIL (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) (15). Cells were used for experiments after 7–10 wk in culture. LAD2 cells were maintained in complete StemPro-34 medium supplemented with 100 ng/ml rhSCF (16). RBL-2H3 and HEK293 cells were maintained as monolayer cultures in DMEM supplemented with 10% FBS, L-glutamine (2 mM), penicillin (100 IU/ml), and streptomycin (100 μg/ml) (17).

Lentivirus-mediated knockdown of MrgX2 in LAD2 mast cells

MrgX2-targeted Mission short hairpin RNA (shRNA) lentiviral plasmids were purchased from Sigma-Aldrich. The clone that gave the highest knockdown efficiency (TRCN0000009174) was used (12). A nontarget vector (SHC002) was used as a control. Lentivirus generation was performed, according to the manufacturer's manual. Cell transduction was

A

Sequence	Peptide
GIGDPVTCLKSGAICHPVFCPRRYKQIGTCGLPGTKCCKKP	(hBD2)
GIINTLQKYYCRVRGGRCAVLSCLPKEEQIGKCSTRGRKCCRRKK	(hBD3)
PCKNFFWKTFSSCK	(CST)
LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	(LL-37)
GLVRKGGEKFGEKLRKIGQKIKEFFQKLALEIEQ	(mCRAMP)

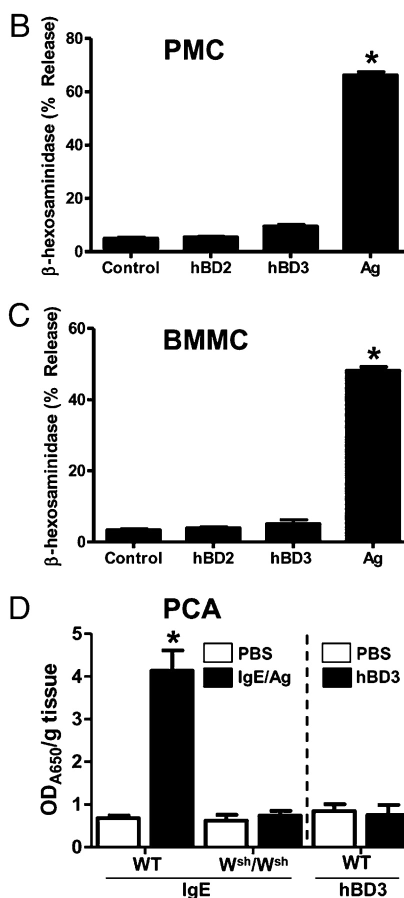

FIGURE 1. hBDs do not induce degranulation in murine mast cells in vitro or in vivo. (**A**) Amino acid sequences of hBD2, hBD3, CST, LL-37, and mCRAMP. Positively charged amino acids are underlined. (**B**) Murine PMCs or (**C**) BMMCs were incubated with mouse IgE (1 μg/ml, 16 h) and then stimulated with hBD2 (5 μM), hBD3 (3 μM), or Ag (DNP-BSA, 100 ng/ml), and degranulation was determined. (**D**) For IgE-mediated passive cutaneous anaphylaxis, C57BL/6 mice or mast cell–deficient W^{sh}/W^{sh} mice were passively sensitized in the ear with PBS (open white bars) or IgE (closed black bars) (20 ng for 16 h) and challenged with an i.v. injection of a 100 μg Ag (DNP-BSA) in 200 μl PBS containing 1% Evans blue. For defensin, C57BL/6 mice were i.v. injected with 200 μl 0.5% Evans blue before intradermal injection of hBD3 (closed black bars) (150 ng) into one side of the ear and vehicle PBS (open white bars) into the other ear. The mice were killed 30 min after injection, and Evans blue dye contents in the ear tissues were measured. The results are expressed as OD_{A650}/g tissue. Data shown are representative of three similar experiments. Statistical significance was determined by two-way ANOVA with Bonferroni's posttest. *$p < 0.01$.

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conducted by mixing 1.5 ml viral supernatant with 3.5 ml LAD2 (5×10^6) cells. Eight hours postinfection, medium was changed to virus-free complete medium, and antibiotic (puromycin, 4 μg/ml; Sigma-Aldrich) selection was initiated 16 h later. Cells were analyzed for MrgX2 knockdown by Western blotting.

Transfection of RBL-2H3, HEK293 cells, and BMMCs

RBL-2H3 cells were transfected with plasmids encoding hemagglutinin (HA)-tagged MrgX2 using the Amaxa nucleofector device and Amaxa kit V, according to the manufacturer's protocol. HEK293 cells were transfected with the same plasmid using Lipofectamine reagent (Invitrogen). Following transfection, cells were cultured in the presence of G-418 (1 mg/ml), and cells expressing equivalent receptors were sorted using an FITC-conjugated anti-HA–specific Ab (12CA5) and used for studies on Ca^{2+} mobilization and degranulation (18, 19).

Mature BMMCs (2.0×10^6) were transfected with plasmids encoding HA-tagged MrgX2 (3 μg) using the Amaxa nucleofector device and Amaxa kit V (program T020). Twenty-four hours following transfection, cells were used for degranulation studies.

Calcium mobilization

Ca^{2+} mobilization was determined, as described previously (17). Briefly, cells (human mast cells; 0.2×10^6, RBL-2H3 and HEK293 cells; 1.0×10^6) were loaded with 1 μM indo-1 AM for 30 min at room temperature. Cells were washed and resuspended in 1.5 ml 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 (20).

Degranulation

BMMCs and PMCs were sensitized overnight with anti-DNP mouse IgE (SPE-7, 1 μg/ml) in cytokine-free medium. The cells were rinsed three times with buffer containing BSA (Sigma-Aldrich) to remove excess IgE. Human mast cells (5×10^3) and RBL-2H3 cells (5×10^4) 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 PTx (EMD Millipore, Billerica, MA; 100 ng/ml for 16 h) or La^{3+} (lanthanum chloride, 1 μM for 5 min). For total β-hexosaminidase release, unstimulated cells were lysed in 50 μl 0.1% Triton X-100. Aliquots (20 μl) of supernatant or cell lysates were incubated with 20 μl 1 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosamine for 1.5 h at 37°C. Reaction was stopped by adding 250 μl 0.1 M Na_2CO_3/0.1 M $NaHCO_3$ buffer, and absorbance was measured at 405 nm (17).

Results

Effects of hBDs on murine mast cells in vitro and in vivo

hBD2 and hBD3 are small cationic peptides that play an important role in innate immunity by directly killing microbes (21). Compared with hBD2, hBD3 contains more positive charges and possesses a broader spectrum of antimicrobial activity (Fig. 1A) (22). In rat PMCs, both hBD2 and hBD3 induce degranulation in a dose-dependent manner, with hBD2 being more potent (11, 23). We therefore sought to determine whether these peptides also induce degranulation in murine mast cells. For these studies, we tested the effects of hBDs on degranulation in murine PMCs and BMMCs. As shown in Fig. 1B and 1C, whereas Ag caused substantial degranulation in both types of mast cells, hBD2 (5 μM) and hBD3 (3 μM) were without effect. Local cutaneous administration of hBD3 (150 ng) increases vascular permeability in wild-type rats, but this response was absent in mast cell–deficient W^s/W^s rats (11). We

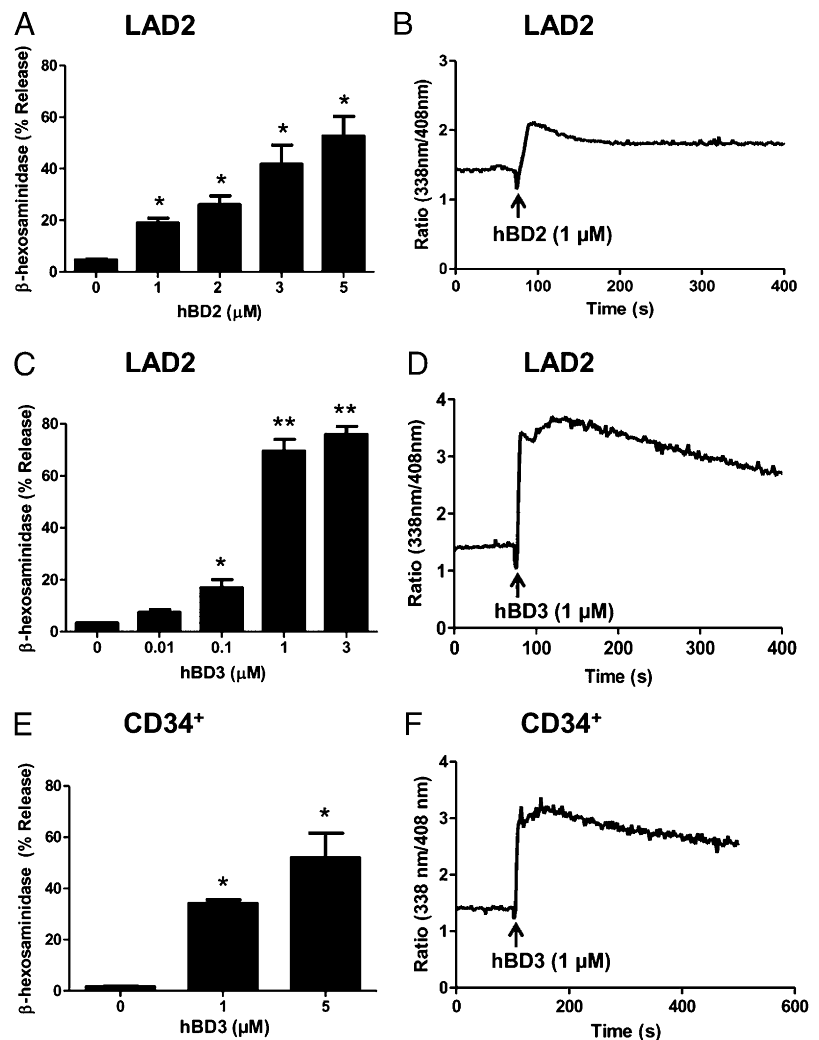

FIGURE 2. hBDs induce degranulation and Ca^{2+} mobilization in LAD2- and $CD34^+$-derived human mast cells. LAD2 mast cells were stimulated with different concentrations of (**A**) hBD2 or (**C**) hBD3, and percentage of degranulation (β-hexosaminidase release) was determined. Data are mean ± SEM of three experiments. (**B** and **D**) LAD2 cells were loaded with indo-1 AM, and Ca^{2+} mobilization in response to hBD2 or hBD3 was determined. (**E**) $CD34^+$ cell-derived primary mast cells were exposed to different concentrations of hBD3, and degranulation was determined. (**F**) $CD34^+$ cell-derived mast cells were loaded with indo-1, and Ca^{2+} mobilization in response to hBD3 was determined. Data shown are representative of three similar experiments. Statistical significance was determined by two-way ANOVA with Bonferroni's posttest. *$p < 0.01$, **$p < 0.001$.

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found that mouse IgE and Ag caused a passive cutaneous anaphylactic reaction in wild-type C57BL/6 mice and that this response was absent in mast cell–deficient W^{sh}/W^{sh} mice (Fig. 1D). However, hBD3 did not induce increased vascular permeability in C57BL/6 mice. These findings clearly demonstrated that, unlike the situations in rats (10, 11), hBDs do not induce degranulation in murine mast cells in vitro or in vivo.

hBD2 and hBD3 induce degranulation in human mast cells

In rat PMCs, hBD2 is more potent in inducing degranulation than hBD3 (10, 11). We therefore sought to determine the dose-response effects of these AMPs on degranulation in human mast cells using LAD2 cells, which are widely used as a model to study human mast cell function in vitro. We found that, unlike the situation with rat mast cells, but consistent with greater net positive charge on hBD3 (Fig. 1A), it was more potent than hBD2 in inducing degranulation in LAD2 cells. Thus, hBD2, at a concentration of 1 μM, caused ~20% degranulation, but hBD3 at the same concentration caused ~75% response (Fig. 2A, 2C). This difference in the extent of degranulation correlated with a greater Ca^{2+} mobilization by hBD3 than hBD2 (Fig. 2B, 2D). To confirm the biological relevance of the studies with LAD2 cells, we repeated selected experiments in $CD34^+$-derived primary human mast cells. We found that, as for LAD2 cells, hBD3 induced degranulation in $CD34^+$-derived mast cells, but the magnitude of the response was ~50% lower (Fig. 2C, 2E). Interestingly, hBD3 induced a Ca^{2+} response in $CD34^+$-derived human mast cells that was similar in profile to that observed in LAD2 cells (Fig. 2D, 2F).

Role of PTx-sensitive G protein–dependent and independent pathways on hBD2/3-induced degranulation

In rat PMCs, hBD2- and hBD3-induced Ca^{2+} mobilization and degranulation are inhibited by PTx (10, 11). The signaling pathway via which hBD2 and hBD3 induce degranulation in human mast cells is unknown. We first tested the effects of PTx on hBD2- and hBD3-induced Ca^{2+} mobilization in LAD2 mast cells. We used the anaphylatoxin C3a as a control, which is known to induce a Ca^{2+} response via a PTx-sensitive G protein. We found that whereas C3a-induced Ca^{2+} response was substantially blocked by PTx, it had little or no effect on the response to hBD2 or hBD3 (Fig. 3A, 3B, 3E, 3F). La^{3+}, an inhibitor of Ca^{2+} release-activated Ca^{2+} channels, has been shown to inhibit both Ca^{2+} influx and mast cell degranulation (24, 25). We found that La^{3+} had no effect on the early Ca^{2+} spike in response to C3a, but it completely blocked the Ca^{2+} responses to both hBD2 and hBD3 (Fig. 3C, 3G). Interestingly, treatment of cells with either PTx or La^{3+} almost completely blocked degranulation induced by C3a, hBD2, and hBD3 (Fig. 3D, 3H). These findings demonstrate that, unlike the situation in rat mast cells, hBD2 and hBD3 cause degranulation in human mast cells via the interaction of a Gαi-independent Ca^{2+} influx and an unknown Gαi-mediated pathway, most likely via protein kinase C (PKC) (17). We used a pharmacologic approach to test this possibility. The 2-APB inhibits mast cell degranulation via its action as a dual inhibitor of inositol 1,4,5-triphosphate receptor and transient receptor potential channels (26–28). We found that 2-APB blocked both the initial Ca^{2+} spike and the sustained Ca^{2+} influx in response to hBD3, and this was associated with a substantial inhibition of degranulation (Fig. 4A, 4B, 4D). By contrast, a PKC inhibitor, GFX, had little or no effect on hBD3-induced Ca^{2+} mobilization, but caused significant inhibition of degranulation (Fig. 4A, 4C, 4D).

hBD2 and hBD3 activate human mast cells via MrgX2

hBDs activate dendritic cells, T cells, and monocytes via CCR2 and CCR6 and TLRs (3, 5, 29). However, in rat PMCs, hBD-induced responses are not mediated via CCR6 (30). Furthermore, RBL-2H3 cells stably expressing CCR6 are unresponsive to hBD2 and hBD3 (31). In addition, we found that CCL2, a ligand for CCR2, failed to induce Ca^{2+} mobilization in LAD2 mast cells (data not shown). These findings clearly demonstrate that effects of hBDs in human mast cells are mediated independently of CCR2 or CCR6. It is noteworthy that mast cells are the only known cells outside

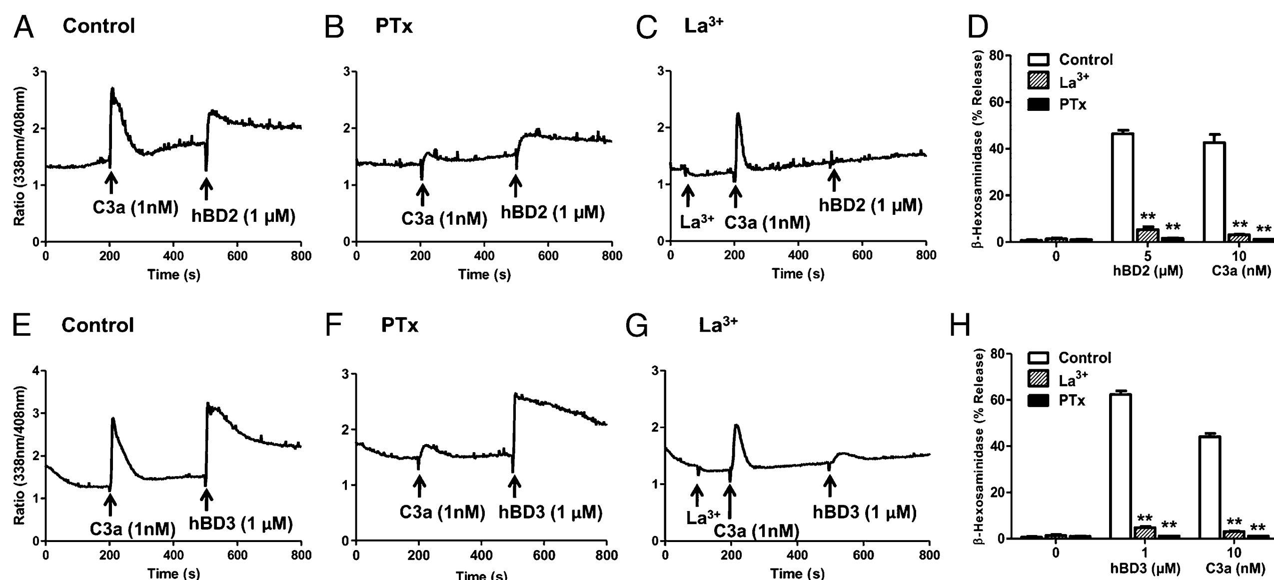

FIGURE 3. Effects of PTx and La^{3+} on C3a-, hBD2-, and hBD3-induced Ca^{2+} mobilization and degranulation in human mast cells. (**A** and **E**) Indo-1–loaded LAD2 cells were exposed to C3a, followed by hBD2 or hBD3, and intracellular Ca^{2+} mobilization was determined. (**B** and **F**) Cells were treated with PTx (100 ng/ml, 16 h), and effects of C3a, hBD2, or hBD3 on Ca^{2+} mobilization were determined. (**C** and **G**) Indo-1–loaded LAD2 cells were exposed to La^{3+} (lanthanum chloride, 1 μM), and C3a-, hBD2-, or hBD3-induced Ca^{2+} mobilization was determined. Traces are representative of three independent experiments. (**D** and **H**) LAD2 mast cells were exposed to buffer (control) or PTx (100 ng/ml, 16 h) or (La^{3+} (lanthanum chloride, 1 μM, 30 min), and C3a-, hBD2-, and hBD3-induced degranulation was determined. Data are mean ± SEM of three experiments. Statistical significance was determined by two-way ANOVA with Bonferroni's posttest. $**p < 0.001$.

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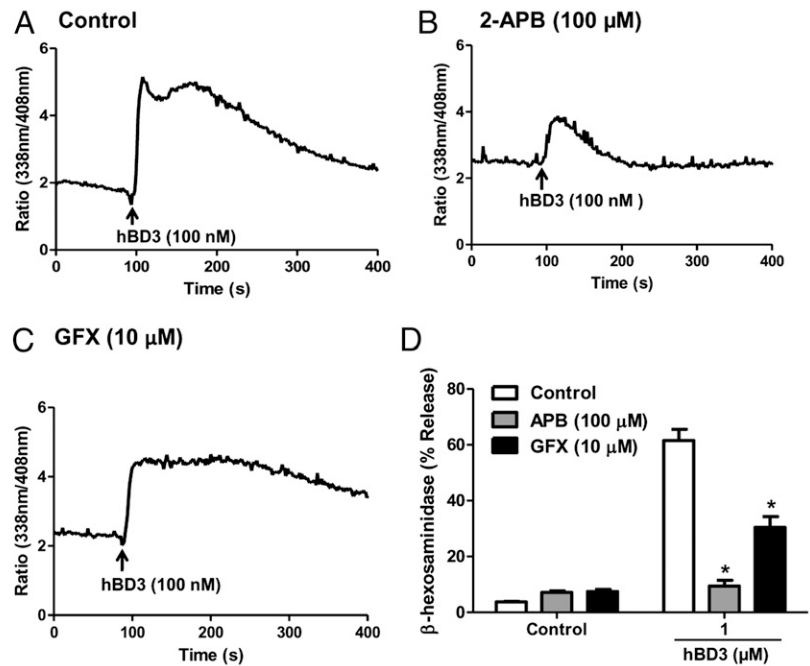

FIGURE 4. Effects of 2-APB and GFX on hBD3-induced Ca^{2+} mobilization and degranulation in human mast cells. (**A**–**C**) Indo-1–loaded LAD2 cells were left untreated (control) or pretreated with 2-APB or GFX, and hBD3-induced Ca^{2+} mobilization was determined. Traces are representative of three independent experiments. (**D**) LAD2 mast cells were pretreated with buffer control or 2-APB or GFX, and degranulation response to hBD3 was determined. Data are mean ± SEM of three experiments. Statistical significance was determined by one-way ANOVA with Bonferroni's posttest. *$p < 0.01$.

the dorsal ganglia that express MrgX2 (32). Furthermore, this receptor is activated by basic peptides, including the amphipathic antimicrobial peptide, LL-37 (12, 32). To determine whether hBDs activate human mast cells via MrgX2, we used shRNA to silence the expression of MrgX2 in LAD2 mast cells (12). We found that MrgX2-shRNA caused a substantial reduction in the expression of MrgX2 when compared with control shRNA-transduced cells (Fig. 5A). Furthermore, hBD2-, hBD3-, and CST (a known ligand for MrgX2)-induced degranulation was significantly inhibited in MrgX2-silenced cells when compared with shRNA control. By contrast, degranulation to C3a, which activates mast cells via C3aR, was not affected (Fig. 5B). We have previously shown that native RBL-2H3 cells, which are highly responsive to Ag/IgE for degranulation, do not endogenously express MrgX2 and are unresponsive to MrgX2 ligands such as CST unless the cells are transfected with cDNA encoding MrgX2 (18, 19). To further confirm the role of MrgX2 on hBD-induced degranulation, we used RBL-2H3 cells stably expressing human MrgX2 (18). In this system, hBD2, hBD3, and CST induced substantial mast cell degranulation (Fig. 6A), and this was associated with increased intracellular Ca^{2+} mobilization (Fig. 6B–D). hBD3 and CST also induced Ca^{2+} mobilization in HEK293 cells stably expressing MrgX2 (Fig. 6E, 6F), but this response was absent in untransfected cells (data not shown).

Previous studies showed that human β-defensins and the human cathelicidin LL-37 induce signaling and degranulation in rat PMCs (11, 33). However, the resistance of murine PMCs or BMMCs to human antimicrobial peptides (Fig. 1B, 1C) could reflect the inability of human peptides to activate a murine Mrg receptor. Murine analogs of human antimicrobial peptides are not well characterized, with the exception of mCRAMP, which is the murine analog of human LL-37 (34–36). We have recently shown that LL-37 induces human mast cell degranulation via MrgX2 (12). To test the possibility that mCRAMP could activate mast cells via MrgX2, we transiently expressed HA-MrgX2 in murine BMMCs and confirmed cell surface expression via flow cytometry (Fig. 7A). Cells expressing MrgX2 responded to mCRAMP, hBD3, and CST with an increased degranulation response as compared with control vector-transfected BMMCs (Fig. 7B). In addition, mCRAMP caused substantial Ca^{2+} mobilization and dose-dependent degranulation in LAD2 mast cells (Fig. 7C, 7D). Collectively, these data suggest that hBDs, LL-37, and mCRAMP activate mast cells via MrgX2 and that resistance of native murine PMCs and BMMCs reflects the absence of this receptor in mouse mast cells.

Discussion

hBDs are multifunctional AMPs produced by epithelial cells and platelets and promote innate immunity, adaptive immunity, angiogenesis, and tumor metastasis, and modulate sepsis (1, 37). Most of the effects of hBDs in immune cells appear to be mediated via the activation of TLRs, CCR2, and CCR6 (4–6, 38). hBDs have been shown to induce chemotaxis and degranulation in rat PMCs (10, 11, 31). Given the functional heterogeneity that exists between mast cells of different species (39–41), data obtained

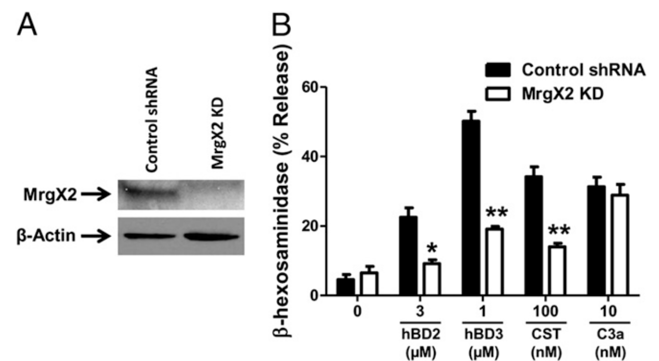

FIGURE 5. Knockdown of MrgX2 inhibits hBD2, hBD3, and cortistatin, but not C3a-induced mast cell degranulation. LAD2 mast cells were stably transduced with scrambled shRNA control lentivirus or shRNA lentivirus targeted against MrgX2. (**A**) Western blotting was performed to determine MrgX2 expression in control and MrgX2 knockdown (KD) cells. (**B**) shRNA control and MrgX2 KD cells were stimulated with hBD2, hBD3, CST, or C3a, and percentage of degranulation (β-hexosaminidase release) was determined. Data are mean ± SEM of three experiments. Statistical significance was determined by one-way ANOVA with Bonferroni's posttest. *$p < 0.01$, **$p < 0.001$.

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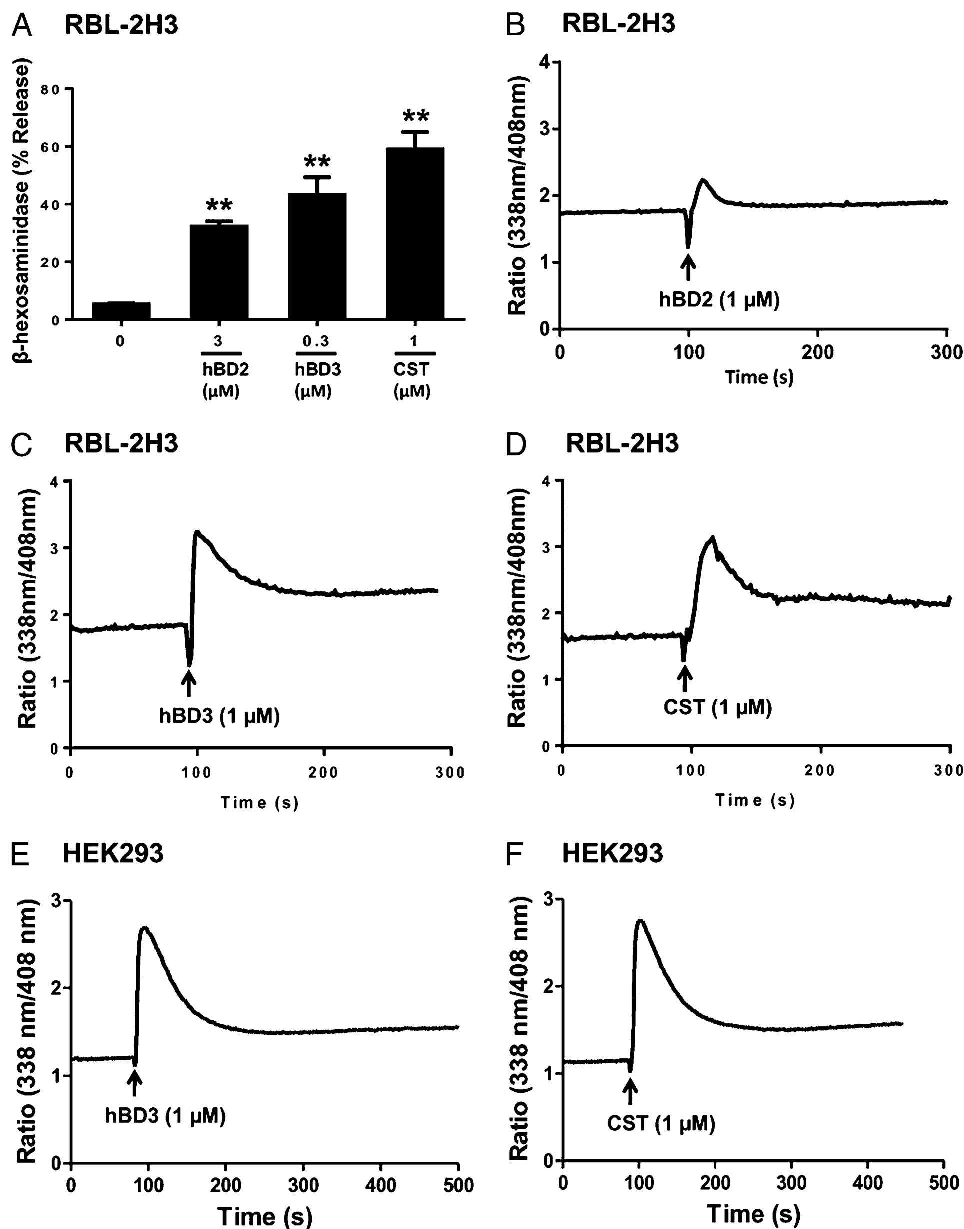

FIGURE 6. hBDs activate RBL-2H3 and HEK293 cells expressing MrgX2. (**A**) RBL-2H3 cells stably expressing MrgX2 were stimulated with buffer, hBD2, hBD3, or CST for 30 min, and β-hexosaminidase release was measured. Data shown are representative of three similar experiments. Statistical significance was determined by one-way ANOVA with Bonferroni's posttest. $^{**}p < 0.001$. RBL-2H3 cells stably expressing MrgX2 were loaded with indo-1 AM, and Ca^{2+} mobilization in response to (**B**) hBD2, (**C**) hBD3, or (**D**) CST was determined. HEK293 cells stably expressing MrgX2 were loaded with indo-1 AM, and Ca^{2+} mobilization in response to (**E**) hBD3 or (**F**) CST was determined. Traces shown are representative of three individual experiments.

with rat mast cells may not replicate in mouse and human mast cells. In the current study, we demonstrate that hBD2 and hBD3 do not activate murine mast cells, but induce substantial degranulation in human mast cells. Our studies also demonstrate that G protein and signaling pathways via which hBDs activate human mast cells are different from those reported for rat mast cells and may reflect differences in the activation of cell surface receptors. In this study, we identify MrgX2 as a novel GPCR via which hBD2 and hBD3 activate human mast cells.

Mrg receptors belong to the GPCR family, and, in humans, four Mrg genes, MrgX1–X4, are known (13, 42). Although originally thought to be specifically expressed in dorsal root ganglia, it now appears that human skin mast cells, cord blood–derived mast cells, $CD34^+$ cell-derived mast cells, and a human mast cell line, LAD2, express MrgX2 (18, 32, 43). Most interestingly, this receptor is not present in human lymph node, spleen, or peripheral blood leukocytes (32). In fact, of the 42 human cell types tested, only mast cells express MrgX2 (32). hBD2 and hBD3 are amphipathic peptides, and, given the recent demonstration that MrgX2 serves as a receptor for a variety of cationic peptides (18, 32), we hypothesized that it could serve as a receptor for hBDs 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 hBDs for Ca^{2+} mobilization and degranulation. Second, shRNA-mediated silencing of MrgX2 caused a significant decrease of hBD2- and hBD3-induced responses. Third, ectopic expression of MrgX2 in RBL-2H3 cells, murine BMMCs, and HEK293 cells renders these cells responsive to hBDs.

Consistent with previous reports in rat PMCs (10), we found that hBD2- and hBD3-induced degranulation in human mast cells is inhibited by PTx. However, an important difference was that whereas hBD2-induced Ca^{2+} influx in rat PMCs was blocked by PTx (10), it had no effect on the Ca^{2+} response in human mast cells. It is noteworthy that MrgX2 couples to Gαq family of G proteins for Ca^{2+} mobilization in transfected HEK293 cells (44). We found that La^{3+} or 2-APB blocked hBD3-induced Ca^{2+} mobilization and degranulation. By contrast, a PKC inhibitor, which had little or no effect on hBD3-induced Ca^{2+} mobilization, caused significant inhibition of degranulation. This raises the interesting possibility

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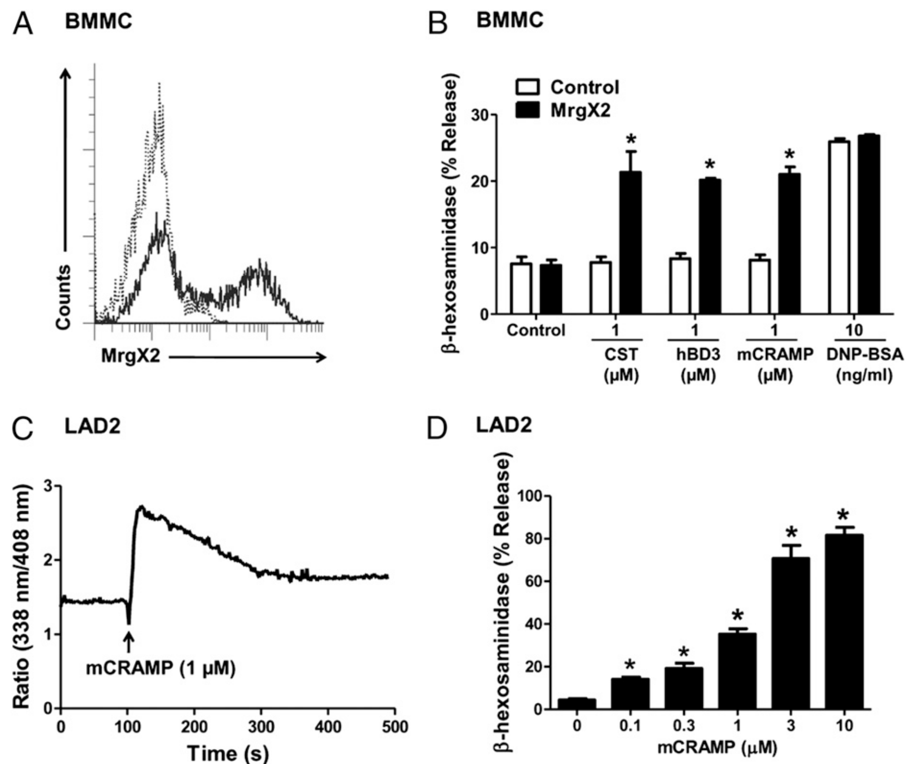

FIGURE 7. hBD3 and mCRAMP activate murine BMMCs expressing MrgX2. (**A**) BMMCs were transiently transfected with HA-tagged MrgX2 (solid line) or control plasmid vector (broken line), and MrgX2 receptor expression level was analyzed using flow cytometry. A representative histogram is shown. (**B**) Control and MrgX2 expressing BMMCs were incubated with DNP-specific mouse IgE (1 μg/ml, 16 h). Cells were exposed to buffer (control), CST, hBD3, mCRAMP, or DNP-BSA (10 ng/ml) for 30 min, and β-hexosaminidase release was measured. LAD2 cells were stimulated with mCRAMP, and (**C**) intracellular Ca^{2+} mobilization or (**D**) degranulation was determined. Traces are representative of three independent experiments. Bar graphs represent mean ± SEM of three experiments. Statistical significance was determined by one-way ANOVA with Bonferroni's posttest. $*p < 0.01$.

that, unlike the situation in rat mast cells, hBDs activate Gαq to promote Ca^{2+} mobilization (inhibited by La^{3+} and 2-APB) and that this response functions in concert with PTx-sensitive signals, at least in part via PKC, to promote degranulation in human mast cells. The reason for the difference in specificity of hBDs for G protein coupling between rat and human mast cells is unknown, but could reflect the utilization of different GPCRs. Interestingly, unlike most GPCRs, Mrg receptors display substantial species-specific differences. Thus, human Mrg receptors share only 45–65% amino acid sequence identity with rat receptors. In addition, whereas there are only four Mrg genes known in humans, the rat genome possesses one each of the MrgA, MrgC, and MrgD genes and 10 MrgB genes (45). Rat PMCs express a number of Mrg receptors, including MrgB1, MrgB2, MrgB3, MrgB6, MrgB8, and MrgB9. Furthermore, basic peptides induce Ca^{2+} mobilization only in HEK293 cells expressing MrgB3 (32). Thus, whereas hBD2 and hBD3 activate human mast cells via MrgX2, it is likely that they activate rat PMCs via MrgB3, and this difference is reflected in the differences in G protein–coupling specificities.

A surprising observation of the current study was that, unlike the situation in human mast cells, hBDs did not induce degranulation in murine PMCs in vitro and had no effect on skin mast cell in vivo as measured by changes in vascular permeability. Because human defensins were used throughout this study, it is possible that the resistance of murine mast cells reflects differences between humans and murine peptides. This possibility is, however, unlikely. In addition to hBDs, the human cathelicidin LL-37 also activates degranulation in rat and human mast cells (33, 46). We have recently shown that LL-37 induces degranulation in human mast cells via MrgX2 (12). The murine analog of human LL-37 (mCRAMP) displays biological activities very similar to those of LL-37 (47). In the current study, we have shown that mCRAMP causes Ca^{2+} mobilization and degranulation in human mast cells. Furthermore, transfection of murine BMMCs with cDNA encoding MrgX2 renders them responsive to CST, LL-37, hBD3, and mCRAMP for degranulation. These findings suggest that hBDs and LL-37 activate human mast cells via MrgX2 and that the resistance of murine mast cells reflects absence of this receptor in murine mast cells (13, 42).

This study has important implications for mast cell–mediated host defense. Rapid mast cell degranulation following bacterial infection provides an important mechanism for host defense in vivo (48). It has been proposed that, following bacterial infection, the anaphylatoxins C3a and C5a are generated, which cause mast cell degranulation and contribute to the recruitment of neutrophils at the site of infection. Given that the anaphylatoxins induce degranulation from both human and murine mast cells (14, 49), the initial mechanism for the mast cell activation in the context of innate immunity is likely to be similar for both humans and mice. However, mast cell degranulation induces hBD2 and hBD3 in human epithelial cells (50), which most likely contributes to further human mast cell degranulation via MrgX2. This suggests that innate function of mast cells most likely involves two phases, one mediated by anaphylatoxins and the other defensins. The differences between the abilities of AMPs to activate human and murine mast cells suggest important species-specific differences in the mechanism of regulation of innate immunity.

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Disclosures

The authors have no financial conflicts of interest.

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