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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Supplementary Figure 1. Baseline TER of healthy and CRS HNEC-ALI and effect of mucus from healthy or CRS on the TER and CBF on HNEC-ALI from controls

Supplementary Table 1. Patient Demographics

Supplementary Methods

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Antimicrobial peptide derived from insulin-like growth factor-binding protein 5 activates mast cells via Mas-related G protein-coupled receptor X2

To the Editor,

In addition to their antimicrobial activity, antimicrobial peptides (AMPs) regulate inflammatory responses, cytokine/chemokine secretion, cell migration, and wound healing.¹ (E1). AMP-IBP5 is a product of the cleavage of insulin-like growth factor-binding protein 5 (IGFBP-5) by serine proteases.² IGFBP-5 is detected in connective tissues and skin and influences cell migration, tissue remodeling and wound healing.^{2,3} Although AMP-IBP5 displays antimicrobial properties similar those of human β -defensins (hBDs) and LL-37,² its

immunomodulatory activity remains unclear. AMP-mediated immunomodulation is regulated through various receptors, including Mas-related G protein-coupled receptor X2 (MrgprX2).⁴ MrgprXs include MrgprX1-MrgprX4, which are found in sensory neurons, mast cells (MCs) and keratinocytes, and control pain, itchiness, and immune responses.^{4,5}

Although MCs participate in inflammatory and allergic reactions, they also contribute to wound healing.⁶ Given that hBDs and LL-37, which promote wound healing, activate MCs¹ and because

AMP-IBP5 contributes to wound healing,⁵ we hypothesized that AMP-IBP5 would stimulate MCs. To address this issue, we exposed human MC cell line LAD2 to AMP-IBP5 to study the induced immune

responses. We propose that in addition to its antimicrobial activity, AMP-IBP5 may modulate immune responses through the recruitment and activation of MCs at inflammation sites and wounds.

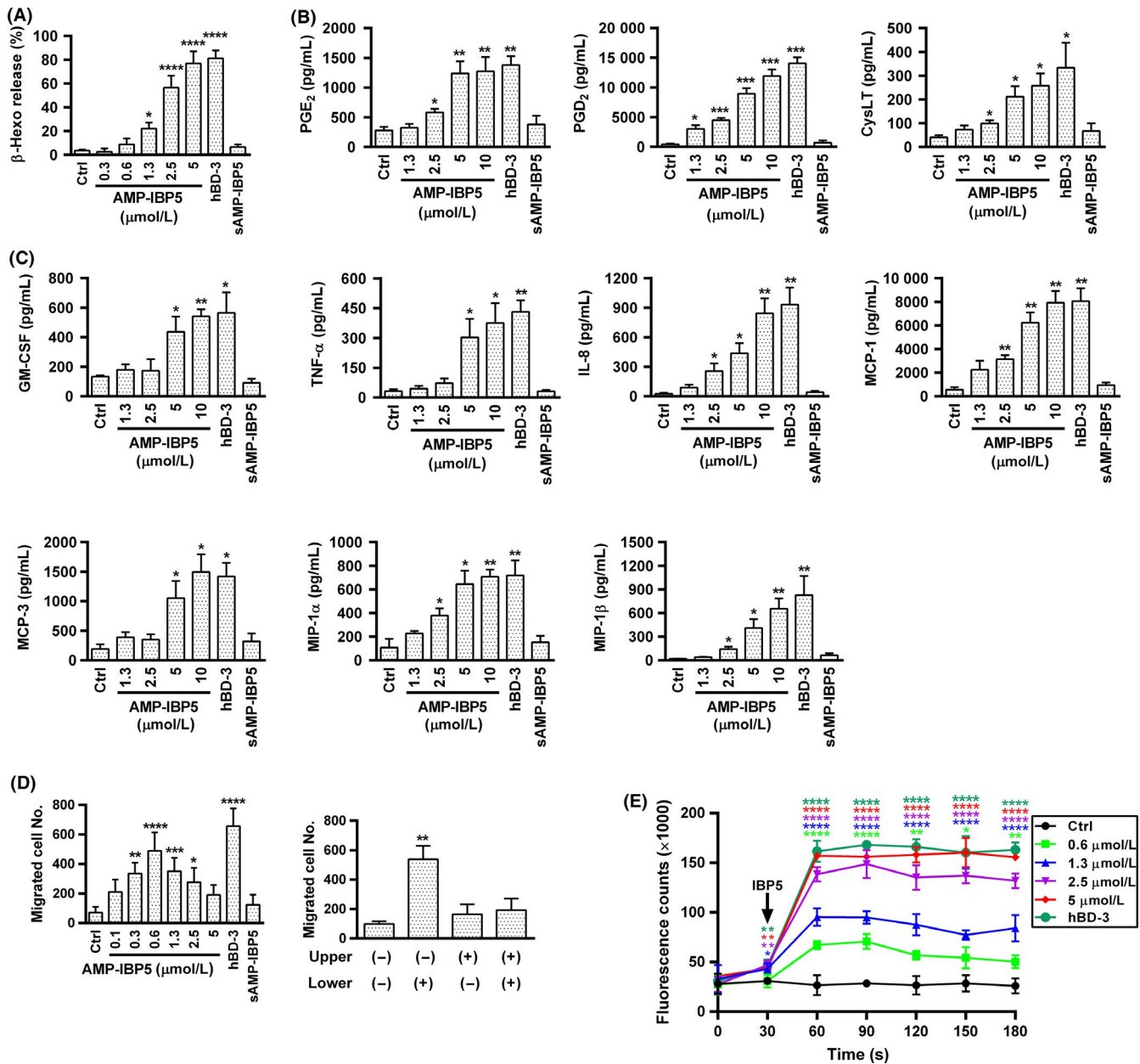


FIGURE 1 AMP-IBP5 induces mast cell (MC) degranulation, secretion of lipid mediators, production of cytokines/chemokines, chemotaxis, and intracellular Ca²⁺ mobilization. A, MCs were incubated with 0.3–5 μ mol/L AMP-IBP5, 2.5 μ mol/L hBD-3, 5 μ mol/L sAMP-IBP5, or diluent 0.01% acetic acid (Ctrl, control). The levels of β -hexosaminidase in the supernatants were measured following 40 min of stimulation. B, C, MCs were incubated with 1.3–10 μ mol/L AMP-IBP5, 5 μ mol/L hBD-3, 10 μ mol/L sAMP-IBP5, or diluent for 30 min (B) or 3 h (C). The amounts of CysLT, PGD₂, and PGE₂ released into the supernatants were quantified by an enzyme immunoassay, whereas GM-CSF, TNF- α , IL-8, MCP-1, MCP-3, MIP-1 α , and MIP-1 β were analyzed by ELISA. D, Left: MCs were seeded in the upper wells of a chemotaxis microchamber and allowed to migrate toward 0.1–5 μ mol/L AMP-IBP5, 0.5 μ mol/L hBD-3, 5 μ mol/L sAMP-IBP5, or diluent for 3 h. Right: 0.6 μ mol/L AMP-IBP5 was added to the lower chamber and/or upper chamber containing MCs, and the migratory response was measured after 3 h of incubation. MCs that migrated through the membrane were counted under a light microscope in three randomly selected high-power fields (HPF). The values are presented as the means $\pm$ SD and compared between different treatments and the diluent control treatment (Ctrl, control). * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001, n = 4–6. E, MCs were incubated for 1 h in Hank's balanced salt solution containing HEPES, probenecid and a calcium 3 reagent. The cells were then loaded with 0.6–5 μ mol/L AMP-IBP5, 2.5 μ mol/L hBD-3, or diluent. The results are presented as the means $\pm$ SD and compared between different treatments and the diluent control treatment (Ctrl, control). **** P < 0.0001, n = 4

Materials and methods are provided in supplementary information (Data S1).

Mast cells are associated with epithelial and endothelial barriers, strategically making them the first cells to encounter invading pathogens. Following activation, MCs immediately release preformed mediators (eg, histamine), secrete leukotrienes (LTs) and

prostaglandins (PGs) within minutes, and cytokines/chemokines within hours.⁶ First, we investigated the effect of AMP-IBP5 on LAD2 cell degranulation. AMP-IBP5 dose dependently increased release of β -hexosaminidase, a MC degranulation marker (E2). The control peptide, scrambled sAMP-IBP5, had no effect, suggesting that AMP-IBP5-mediated MC activation was specific. hBD-3

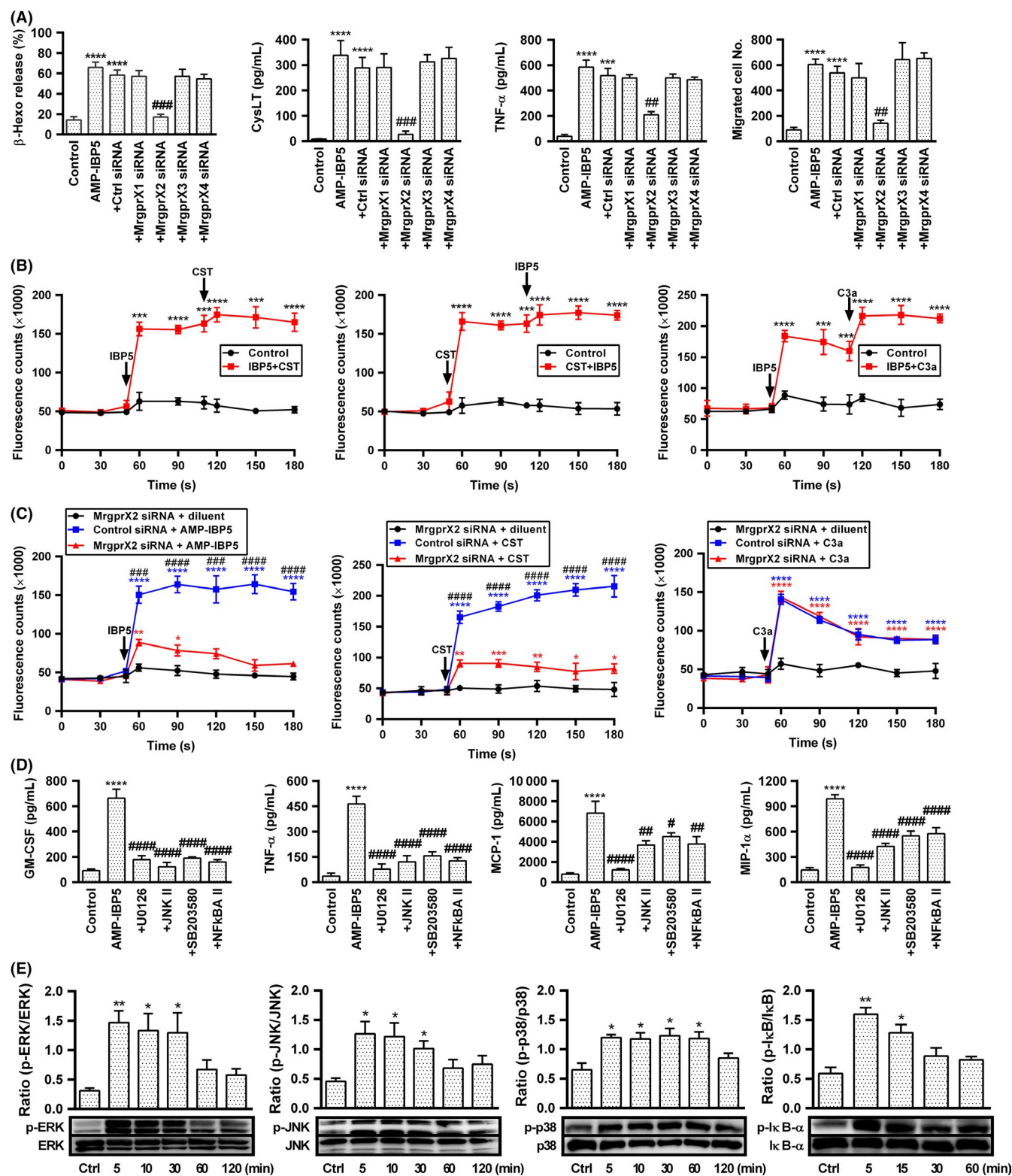


FIGURE 2 AMP-IBP5 induced mast cell (MC) activation is mediated by MrgprX receptors and MAPK and NF- κ B pathways. A, MCs were transfected with 200 nmol/L MrgprX1–MrgprX4 siRNAs or control siRNA for 48 h and stimulated with 5 μ mol/L AMP-IBP5 for β -hexosaminidase release assay or 10 μ mol/L for enzyme immunoassay and ELISA. The amounts of CysLT were quantified by an enzyme immunoassay, whereas TNF- α and MCP-1 were analyzed by ELISA. The values obtained using the nonstimulated (Control) and stimulated cells (AMP-IBP5 and +Ctrl siRNA) were compared, *** $P < 0.001$, **** $P < 0.0001$, $n = 4 \sim 6$. MrgprX siRNA-transfected and control siRNA-transfected cells (+Ctrl siRNA) were also compared, ## $P < 0.01$, ### $P < 0.001$, #### $P < 0.0001$. B, Cross-desensitization of intracellular Ca^{2+} . Calcium 3 reagent-loaded MCs were sequentially stimulated with 2.5 μ mol/L AMP-IBP5 (IBP5) and cortistatin (CST) (left panel), 2.5 μ mol/L cortistatin and AMP-IBP5 (middle panel), or 2.5 μ mol/L AMP-IBP5 and 50 nmol/L C3a (right panel). The values are presented as the means $\pm$ SD and compared between different treatments and the diluent control treatment (Ctrl, control). *** $P < 0.001$, **** $P < 0.0001$, $n = 3$. (C) Effect of MrgprX2 siRNA on intracellular Ca^{2+} mobilization. MCs transfected with MrgprX2 siRNA or control siRNA were stimulated with 2.5 μ mol/L AMP-IBP5, 2.5 μ mol/L cortistatin, 50 nmol/L C3a, or diluent 0.01% acetic acid (Control). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ between MrgprX2 siRNA + diluent (black) and control siRNA + AMP-IBP5 or cortistatin or C3a (blue), and between MrgprX2 siRNA + diluent and MrgprX2 siRNA + AMP-IBP5 or cortistatin or C3a (red). ### $P < 0.001$, #### $P < 0.0001$ between control siRNA + AMP-IBP5 or cortistatin or C3a (blue) and MrgprX2 siRNA + AMP-IBP5 or cortistatin or C3a (red). $n = 4$. D, MCs were treated with 10 μ mol/L U0126, JNK inhibitor II (JNK inh II), SB203580, NF- κ B activation inhibitor II (NF κ BAlI), or 0.1% DMSO (Control) for 2 h before stimulation with 10 μ mol/L AMP-IBP5 (AMP-IBP5, +U0126, +JNK II, +SB203580, +NF κ BAlI) or diluent 0.01% acetic acid (Control) for 3 h. The amounts of cytokines and chemokines were determined by ELISA. *** $P < 0.001$, **** $P < 0.0001$ for comparisons between the untreated cells (Control) and the stimulated groups without inhibitors (AMP-IBP5). # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, and #### $P < 0.0001$ for comparisons between the presence and absence of inhibitors. $n = 4 \sim 6$. E, AMP-IBP5 enhances phosphorylation of MAPKs and I κ B. MCs were stimulated with 10 μ mol/L AMP-IBP5 or diluent (Ctrl, control), and then, equal amounts of protein were subjected to 12.5% SDS-PAGE. The membranes were stained with antibodies against phosphorylated or unphosphorylated ERK (p-ERK and ERK), JNK (p-JNK and JNK), p38 (p-p38 and p38), and I κ B (p-I κ B and I κ B). The results show one representative among five separate experiments. The bands were quantified by densitometry to correct for protein loading discrepancies. The data show the ratio of the intensity of phosphorylated protein divided by unphosphorylated protein. The values are presented as the means $\pm$ SD and compared between AMP-IBP5-stimulated and nonstimulated cells (Ctrl, control). * $P < 0.05$, ** $P < 0.01$, $n = 5$.

(2.5 μ mol/L) showed comparable potency with 5 μ mol/L AMP-IBP5 (Figure 1A). Moreover, AMP-IBP5 elevated the release of cysteinyl leukotrienes (CysLTs: LTC₄, LTD₄, and LTE₄), PGD₂, and PGE₂ (Figure 1B). We next observed that AMP-IBP5 induced the production of cytokines/chemokines GM-CSF, TNF- α , IL-8, MCP-1, MCP-3, MIP-1 α , and MIP-1 β (Figure 1C). In addition to inflammation, GM-CSF and TNF- α promote neovascularization and tissue remodeling during the wound healing process, and IL-8, MCP-1, MCP-3, MIP-1 α , and MIP-1 β recruit inflammatory cells, including MCs, to wound sites and contribute to tissue remodeling and angiogenesis.^{6,7} Therefore, we next tested whether AMP-IBP5 induces MC migration. AMP-IBP5 stimulated MC migration in a bell-shaped dose-dependent manner (Figure 1D, left), and this migration was predominantly chemotactic rather than chemokinetic, because the addition of AMP-IBP5 to the upper wells of the chemotaxis chamber failed to increase the cell migration (Figure 1D, right). Moreover, AMP-IBP5-stimulated MCs showed increase in intracellular Ca^{2+} (Figure 1E). Intracellular Ca^{2+} mobilization plays a crucial role in MC degranulation and migration.⁴

In this study, the molecular mechanism underlying AMP-IBP5-mediated MC activation suggested involvement of the G protein and phospholipase (PL) C pathways. Pertussis toxin and U-73122, inhibitors for G protein and PLC, respectively, suppressed AMP-IBP5-induced MC activation (Figure S1). Activation of PLC leads to increase in intracellular Ca^{2+} mobilization, which is indispensable for MC degranulation, eicosanoid release, and migration.⁴ The finding that AMP-IBP5 activates G protein and PLC pathways suggests that this peptide may act via a receptor-signaling pathway. Therefore, we focused on MrgprXs, because some AMPs are known to activate MrgprX2.^{4,5} We found that in addition to the

MrgprX1 and MrgprX2 that are reported to be expressed in MCs,⁴ both MrgprX3 and MrgprX4 were also detected in MCs both at the mRNA and at the protein levels (Figure S2A,B). To determine whether MrgprXs contribute to AMP-IBP5-mediated activation, we used siRNAs to silence the MrgprX expression (Figure S3). Interestingly, transfection of LAD2 cells with MrgprX siRNAs showed that only MrgprX2 siRNA abolished MC degranulation, secretion of eicosanoids, and cytokines/chemokines (Figures 2, S4A,B). However, even at higher concentrations, AMP-IBP5 could not activate HMC-1 (Figure S5). HMC-1 is an immature human mast cell line that lacks functional MrgprX2 and does not respond to MrgprX2 activators such as LL-37 (E3). AMP-IBP5 did not affect MrgprX2 expression in MCs (Figure S6). MrgprX2 requirement was further demonstrated by the finding that AMP-IBP5 induced degranulation of mouse peritoneal MCs via MrgprB2, a mouse counterpart of MrgprX2⁴ (Figure S7A,B).

In addition, desensitization of Ca^{2+} mobilization studies using AMP-IBP5 and the MrgprX2 endogenous ligand, cortistatin,⁴ showed that AMP-IBP5 and cortistatin mutually inhibited each other's signaling (Figure 2B, left and middle), while there was no desensitization between AMP-IBP5 and C3a, which utilizes C3a receptor (right). The simultaneous administration of both AMP-IBP5 and cortistatin did not result in a greater response than that to either AMP-IBP5 or cortistatin alone (Figure S8A). MrgprX2 siRNA suppressed AMP-IBP5- and cortistatin-mediated intracellular Ca^{2+} mobilization (Figure 2C, left and middle), but had no effect on C3a (right). As expected, MrgprX1, MrgprX3, and MrgprX4 siRNAs did not influence AMP-IBP5-induced Ca^{2+} mobilization (Figure S8B). Therefore, we suggest that MrgprX2 is a functional receptor for AMP-IBP5.

To elucidate the downstream signaling, we focused on MAPK and NF- κ B pathways. U0126 (ERK inhibitor), SB203580 (p38 inhibitor), JNK inhibitor II, and NF- κ B activation inhibitor II suppressed the AMP-IBP5-induced production of cytokines/chemokines, including GM-CSF, TNF- α , MCP-1, and MIP-1 α (Figure 2D). We confirmed that AMP-IBP5-mediated MC stimulation led to phosphorylation of both MAPK and NF- κ B pathways (Figure 2E).

Mast cell-derived histamine, eicosanoids, cytokines/chemokines are involved not only in the promotion of inflammation but also in wound healing.^{8,9} Therefore, AMP-IBP5 may participate in the promotion of wound healing through its ability to recruit and activate MCs at wound sites. This was supported by the fact that AMP-IBP5 induced the production of fibroblast growth factor, platelet-derived growth factor, and vascular endothelial growth factor, which promote angiogenesis and wound healing^{6,7} (Figure S9).

Altogether, because MCs participate in inflammatory responses, regulation of immune system and promotion of wound healing, and because AMP-IBP5 accelerates wound healing, our findings provide evidence for the role of AMP-IBP5 in immune responses and wound healing by recruiting and activating MCs.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Antimicrobial peptide derived from insulin-like growth factor-binding protein 5
activates mast cells via Mas-related G protein-coupled receptor X2**

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Short title: AMP-IBP5 activates mast cells

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mast cell, wound healing

Materials and methods

1. Reagents

AMP-IBP5 (AVYLPNCDRKGIFYKRKQCKPSR-NH₂) was purchased from the Peptide Institute (Osaka, Japan), and its inactive scrambled form, sAMP-IBP5 (KFDRYLKPPNQCRVKGCSKYRA-NH₂), was synthesized using the solid-phase method with a peptide synthesizer (Model PSSM-8; Shimadzu, Kyoto, Japan). Pertussis toxin, U-73122, U0126, SB203580, JNK inhibitor II and NF- κ B activation inhibitor II were purchased from Calbiochem (La Jolla, CA). Anti-phosphorylated and anti-unphosphorylated ERK, JNK, p38 and I κ B antibodies were obtained from Cell Signaling Technology (Beverly, MA). The ELISA kits for cytokines and chemokines were from R&D Systems (Minneapolis, MN), whereas enzyme immunoassay kits for cysteinyl leukotriene (CysLT, which measures LTC₄, LTD₄, and LTE₄), prostaglandin (PG)D₂ and PGE₂ were purchased from Cayman Chemical Company (Ann Arbor, MI). The calcium assay kit was purchased from Molecular Devices (Sunnyvale, CA).

2. Mice

C57BL/6 mice purchased from Japan SLC (Hamamatsu, Japan) were maintained in specific pathogen-free conditions. All animals received humane care, and all procedures were approved by the Institutional Review Committee of Juntendo University (approval no. 300235).

3. Cells

Human MC line LAD2 was provided by Dr. A. Kirshenbaum at the National Institutes of Health (Bethesda, MD) (E4), whereas HMC-1 cell line was kindly supplied by Dr. J.H. Butterfield (E5). LAD2 cells were cultured in a Stem Pro-34 serum-free basal medium (Invitrogen, Carlsbad, CA) supplemented with Stem Pro-34 nutrient supplements and recombinant human stem cell factor (Wako, Osaka, Japan), as previously reported (E6, E7). HMC-1 were cultured at in basal Iscove's Medium containing 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 0.002% α -monothioglycerol.

Mice were intraperitoneally injected with Tyrode's buffer containing 0.1% gelatin and peritoneal MCs were isolated from total peritoneal cells by a density-gradient centrifugation with 22.5% histodenz (Sigma-Aldrich) as previously described (E8), with >90% purity and >98% viability. Peritoneal MCs were identified by toluidine blue staining and flow cytometry analysis of cell surface expression of c-Kit and Fc ϵ RI.

4. Measurement of MC degranulation

MC degranulation was assessed by measuring the release of β -hexosaminidase. Cells at the concentration of 2×10^5 cells/100 µl were suspended in Tyrode's buffer containing 0.1% BSA, 1 mM CaCl₂ and 0.6 mM MgCl₂ and then challenged with AMP-IBP5 for 40 min at 37°C. Following stimulation, the cell culture supernatants were treated with 1.3 mg/ml of 4-nitrophenyl-N-acetyl- β -D-glucosaminide (Sigma-Aldrich, St. Louis, MO) for 90 min, and

the enzymatic activity of β -hexosaminidase was measured at the absorbance of 405 nm using a plate reader. The percentage of β -hexosaminidase released into the supernatants was calculated using the following formula: percent release = (OD of the stimulated supernatant – OD of the unstimulated supernatant) $\times$ 100 / (OD of the total cell lysate from cells stimulated with 1% Triton X-100 – OD of the unstimulated supernatant).

5. Enzyme immunoassay and ELISA

MCs (5×10^5 cells) were challenged to AMP-IBP5 for 30 min and 3 h for the enzyme immunoassay and ELISA, respectively, at 37°C. Following stimulation, the cell-free supernatants were assayed for the secretion of CysLT, PGD₂ and PGE₂ by enzyme immunoassay, and for GM-CSF, TNF- α , IL-8, MCP-1, MCP-3, MIP-1 α and MIP-1 β by ELISA. In some experiments, the cells were treated with inhibitors or siRNAs, stimulated with AMP-IBP5, and enzyme immunoassay or ELISA were subsequently performed as described above.

6. MC migration

MC migration assay was performed using 48-well chemotaxis microchamber (Neuro Probe, Cabin John, MD). The upper chambers containing MCs (1.5×10^5 cells/50 μ l) were separated from the lower chambers containing AMP-IBP5 by an 8- μ m pore-size polyvinylpyrrolidone-free polycarbonate membrane (Neuro Probe). In some experiments, AMP-IBP5 was added to the upper compartments of the microchamber to determine whether

cell migration occurs by chemotaxis or chemokinesis. After 3 h of incubation, the migrated cells were stained with DiffQuick (Kokusai Shiyaku, Kobe, Japan) and counted under a light microscope.

7. Intracellular Ca^{2+} mobilization

MCs (2×10^5 cells/100 μ l) were cultured into 96-well plates coated with poly-D-lysine (Becton-Dickinson, NJ), and 100 μ l of HBSS buffer containing 20 mM HEPES, 2.5 mM probenecid, and calcium 3 reagent (Molecular Devices, Menlo Park, CA) was added into each well. After 1 h of incubation, changes in fluorescence following the addition of AMP-IBP5 were measured using FlexStation II (Molecular Devices).

8. Western blot analysis

Following incubation with AMP-IBP5, MCs (1×10^6 cells) were lysed in RIPA buffer (Cell Signaling Technology). The proteins in the cells lysates were separated by SDS-PAGE and transferred to a polyvinylidene fluoride membrane. The membranes were subsequently incubated with various antibodies overnight at 4°C and visualized with the Luminata Forte Western HRP substrate (Millipore, Billerica, MA). The blots were imaged by LAS-4000 Plus (Fujifilm, Tokyo, Japan) and densitometry analysis using the software program Image Gauge (LAS-4000 Plus) was performed to quantify the band intensities.

9. RNA extraction and real-time PCR

Total RNA from MCs was extracted using an RNeasy Plus Micro kit (Qiagen). First-strand

cDNA was synthesized from 1 µg of total RNA using a ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan). Real-time PCR was performed with the Applied Biosystems StepOne Plus Real-time PCR Systems (Thermo Fisher Scientific, Waltham, Mass) by using TaqMan Universal PCR Master Mix and Assays-on-Demand primer/probe sets for MrgprX1 (Hs00600918_s1), MrgprX2 (Hs00365019_s1), MrgprX3 (Hs00754695_s1), and MrgprX4 (Hs00607779_s1). The mRNA expression levels were quantified by using the comparative method with StepOne software and normalized to the housekeeping gene β -actin.

10. siRNA transfection

MCs (2×10^6 cells) were transfected with 200 nM of one of three different siRNA duplexes targeting each MrgprX or with nontargeting control siRNA (no. 12935-300, Invitrogen). MrgprX primers used were: MrgprX1 siRNA forward AGAAAGCUCAGGCCUGCAAAGUAGG, reverse GAGGAGACUCUUUGCUACAAGCAGA; MrgprX2 siRNA forward UCAUGGUUCUCUGUGGGUCCAGUCU, reverse AGACUGGACCCACAGAGAACCAUGA; MrgprX3 siRNA forward GACCUUUCCCUACUUUAUAGGCCUA, reverse UAGGCCUAUAAAGUAGGGAAAGGUC; MrgprX4 siRNA forward GGUGCUGAUUCUAGUUGGUGUGAAA, reverse UUUCACACCAACUAGAAUCAGCACC. The cells were transfected using a Neon

transfection system (Invitrogen) for 48 h and the gene silencing efficiency was determined by real-time PCR and confirmed a protein level by flow cytometric analysis. After transfection, MCs were challenged with AMP-IBP5 and tested for degranulation, enzyme immunoassay, ELISA and intracellular Ca^{2+} mobilization.

11. Flow cytometric analysis

Unstimulated or AMP-IBP5-treated MCs (1×10^6 cells) were suspended in BD Cytotfix/Cytoperm solution (BD Biosciences Pharmingen, San Diego, CA) for 20 minutes according to the manufacturer's instructions. Following one wash with BD Perm/Wash buffer, MrgprX antibodies (LifeSpan BioSciences, Seattle, WA): MrgprX1 (LS-C151375), MrgprX2 (LS-B11741), MrgprX3 (LS-B11748), MrgprX4 (LS-B11749) or an isotype control rabbit IgG (LS-C88566) were added for 1 h at 4°C. The expression of MrgprX receptors was evaluated by FACSCalibur (BD Biosciences) after staining with Alexa Fluor 647-conjugated goat anti-rabbit IgG (H+L, Invitrogen).

12. Statistical analysis

Data are presented as the means $\pm$ SD. The statistical significance of was determined by either one-way ANOVA followed by the appropriate post hoc test or Student's *t*-test using Prism 7 (GraphPad Software, San Diego, CA). A *P* value of <0.05 was considered statistically significant.

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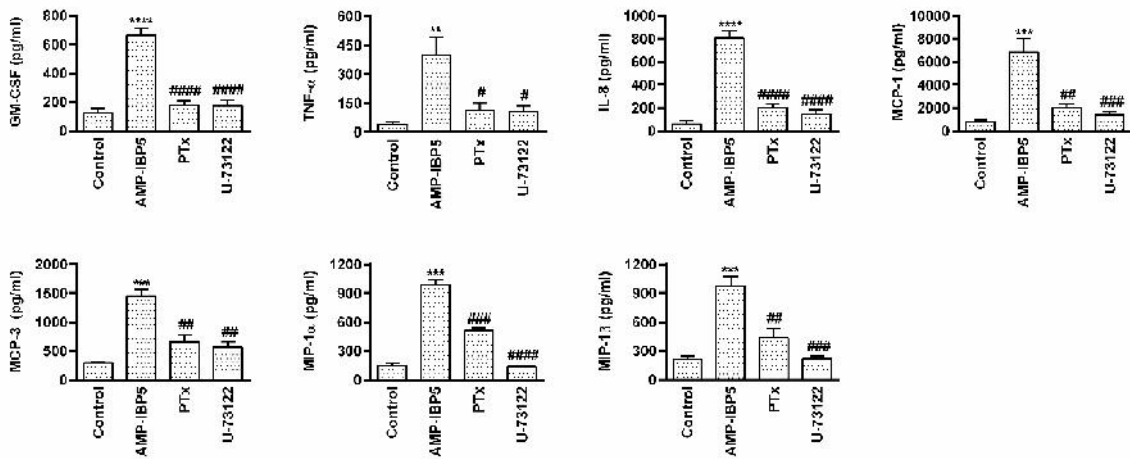


Figure S1

Figure S1. Pertussis toxin and U-73122 inhibit AMP-IBP5-induced MC activation

MCs were preincubated with 200 ng/ml pertussis toxin (PTx), 20 μ M U-73122 or 0.1% DMSO for 2 h. After, the cells were stimulated with 10 μ M AMP-IBP5 or diluent 0.01% acetic acid (Control) for 3 h, and the levels of GM-CSF, TNF- α , IL-8, MCP-1, MCP-3, MIP-1 α and MIP-1 β released into the supernatants were determined by ELISA. The values are presented as the means $\pm$ SD. ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ for comparisons between the untreated cells (Control) and the stimulated groups without the inhibitors (AMP-IBP5). ## $P < 0.01$, ### $P < 0.001$, and #### $P < 0.0001$ for comparisons between the presence and absence of the inhibitors. n= 5.

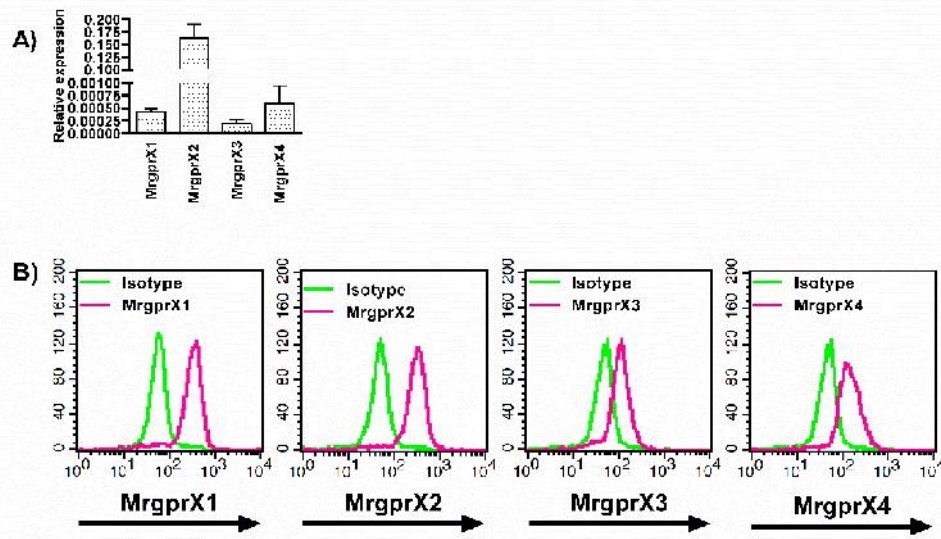


Figure S2

Figure S2. MrgprXs are expressed in mast cells

(A) MrgprX1–MrgprX4 mRNA levels in unstimulated mast cells were analyzed by real-time PCR. The values represent relative expression compared between MrgprX1–MrgprX4 and human β -actin. $n = 9$. **(B)** Protein expression of MrgprX1–MrgprX4 in LAD2 cells was analyzed by flow cytometry. Representative of 3 independent experiments.

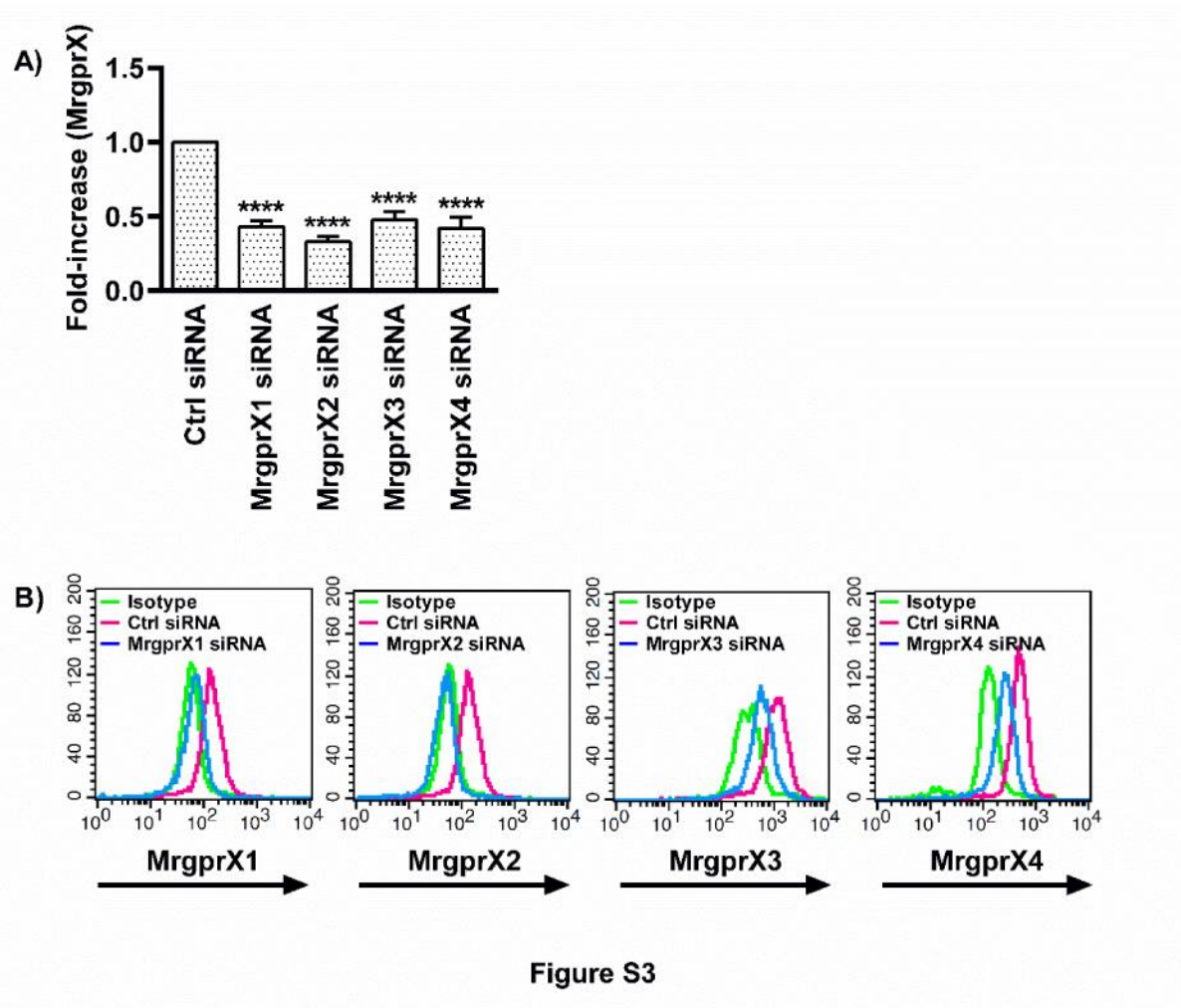


Figure S3

Figure S3. RNA interference with MrgprX siRNAs

LAD2 cells were stably transfected with control siRNA or siRNAs targeting MrgprX1–MrgprX4. **(A)** MrgprX1–MrgprX4 mRNA expression was evaluated by real-time PCR using specific primers/probes. Values are the mean $\pm$ SD and compared between MrgprX1–X4 siRNA-transfected and control siRNA-transfected cells (Control siRNA). **** $P < 0.0001$, $n = 4$. **(B)** MrgprX1–MrgprX4 expression at the protein level was analyzed by flow cytometry. Representative of 3 independent experiments.

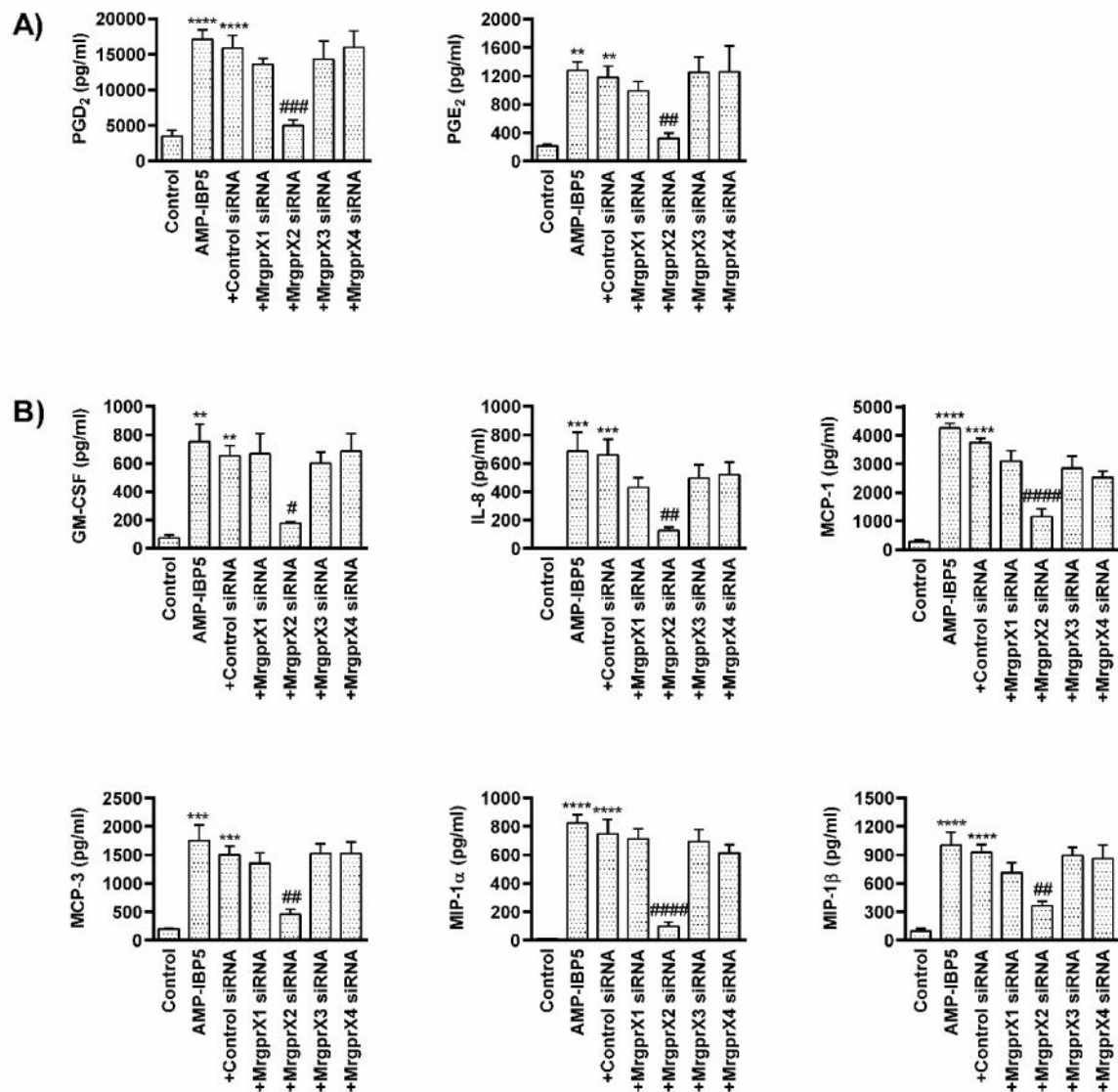


Figure S4

Figure S4. MrgprX2 is required for AMP-IBP5-mediated MC activation

MCs were transfected with 200 nM MrgprX1–MrgprX4 siRNAs or control siRNA for 48 h.

(A) Cells were then stimulated with 10 μ M AMP-IBP5 for enzyme immunoassay to quantify the amounts of PGD₂ and PGE₂ released into the culture supernatants. **(B)** MCs were also stimulated with 10 μ M AMP-IBP5 for GM-CSF, IL-8, MCP-3, MIP- α and MIP-1 β quantification by ELISA. ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ for comparisons

1 between the untreated cells (Control) and the stimulated cells (AMP-IBP5 and +Control
2 siRNA). # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, #### $P < 0.0001$ for comparisons between
3 MrgprX siRNA-transfected and control siRNA-transfected cells (+Control siRNA). n= 4 ~ 6.
4

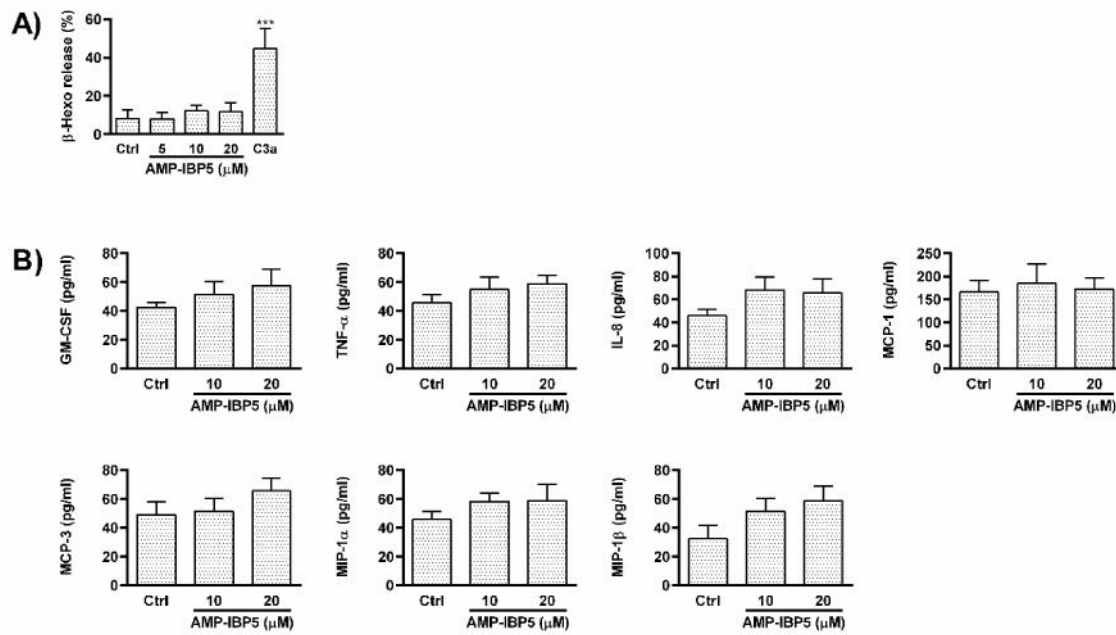


Figure S5

Figure S5. AMP-IBP5 has no effect on HMC-1 activation

(A) HMC-1 cells were incubated with 5 to 20 μ M AMP-IBP5, 50 nM C3a or diluent 0.01% acetic acid (Ctrl, control). The levels of β -hexosaminidase in the supernatants were measured following 40 min of stimulation. Values are the mean $\pm$ SD and were compared between stimulated and non-stimulated cells (Ctrl, control). *** $P < 0.001$, $n = 3$. **(B)** Cells were incubated with 10 to 20 μ M AMP-IBP5 or diluent for 3 h, and the amounts of cytokines and chemokines released into the supernatants were analyzed by ELISA.

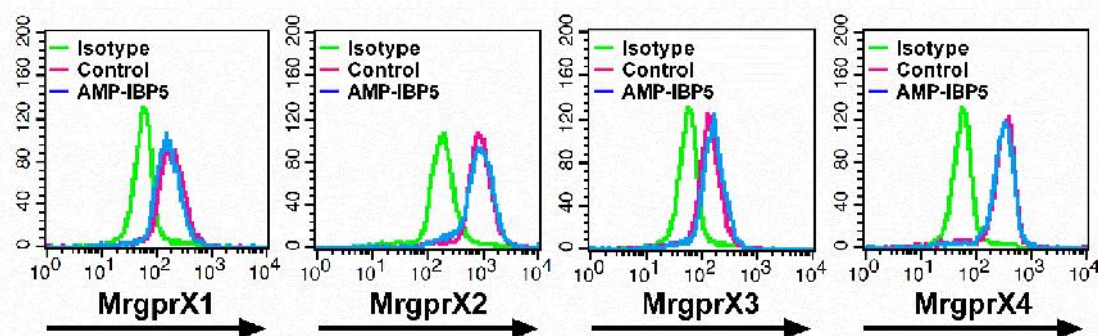


Figure S6

Figure S6. Effect of AMP-IBP5 on MrgprX expression

LAD2 cells were incubated with 10 μ M AMP-IBP5 or diluent 0.01% acetic acid (Control).

MrgprX1–MrgprX4 expression was analyzed by flow cytometry. Representative of 3 independent experiments.

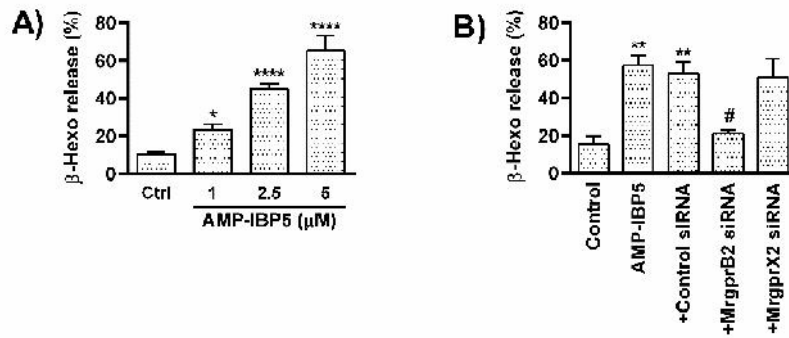


Figure S7

Figure S7. AMP-IBP5 induces degranulation of mouse peritoneal MCs via MrgprB2

(A) Mouse peritoneal MCs were incubated with 1 to 5 μ M AMP-IBP5 and the levels of β -hexosaminidase in the supernatants were measured. The values were compared between stimulated and non-stimulated cells (Ctrl, control). * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$. n= 4. **(B)** Cells were transfected with control siRNA or siRNAs targeting MrgprB2 siRNA or MrgprX2 and then stimulated with 5 μ M AMP-IBP5. ** $P < 0.01$ for comparisons between the untreated cells (Control) and the stimulated cells (AMP-IBP5 and +Control siRNA). # $P < 0.05$ for comparisons between MrgprB2 or MrgprX2 siRNA-transfected and control siRNA-transfected cells (+Control siRNA). n= 4.

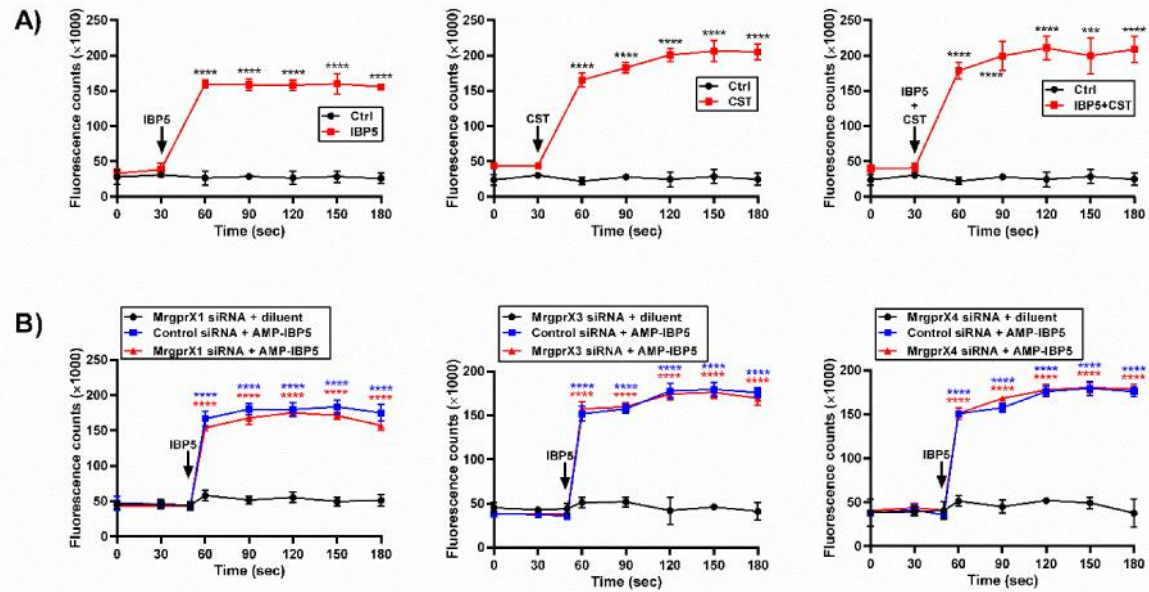


Figure S8

Figure S8. Effects of MrgprX siRNAs on AMP-IBP5-mediated MC activation

(A) LAD2 cells were loaded with 2.5 AMP-IBP5 (IBP5), 2.5 μ M cortistatin (CST) or both AMP-IBP5 and cortistatin (IBP5+CST) and intracellular Ca^{2+} was measured. The results are presented as the means $\pm$ SD and compared with stimulated and non-stimulated cells (Ctrl, control). **** $P < 0.0001$, $n = 3$. (B) MCs transfected with siRNAs targeting MrgprX1, MrgprX3 and MrgprX4 or control siRNA were stimulated with 2.5 μ M AMP-IBP5 (IBP5) or diluent 0.01% acetic acid (Diluent). **** $P < 0.0001$ between MrgprX siRNA + diluent (black) and control siRNA + AMP-IBP5 (blue), and between MrgprX siRNA + diluent and MrgprX siRNA + AMP-IBP5 (red). $n = 4$.

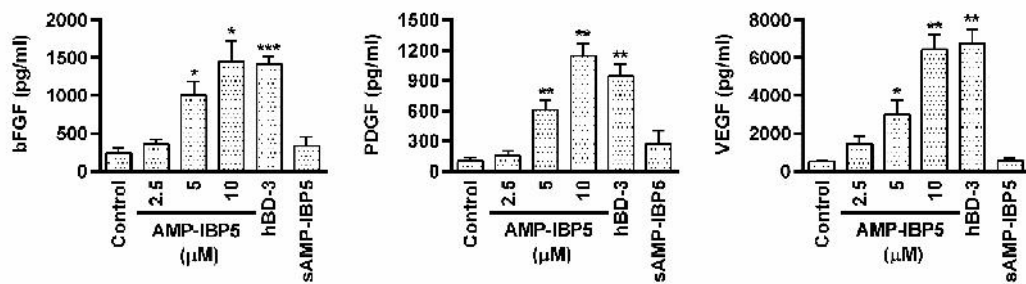


Figure S9

Figure S9. AMP-IBP5 induces the angiogenic growth factors

MCs were stimulated with 2.5 to 10 μ M AMP-IBP5, 5 μ M hBD-3, 10 μ M sAMP-IBP5, or diluent 0.01% acetic acid (Control) for 24 h. The concentrations of basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) released into the culture supernatants were analyzed by ELISA. The values are presented as the means $\pm$ SD and compared between the stimulated and non-stimulated cells (Control). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. n= 4.