



CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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REFERENCES

- Brigham EP, Kolahdooz F, Hansel N, et al. Association between Western diet pattern and adult asthma: a focused review. *Ann Allergy Asthma Immunol* 2015;114:273-280.
- Lv N, Xiao L, Ma J. Dietary pattern and asthma: a systematic review and meta-analysis. *J Asthma Allergy*. 2014;7:105-121.
- Garcia-Larsen V, Del Giacco SR, Moreira A, et al. Asthma and dietary intake: an overview of systematic reviews. *Allergy* 2016;71:433-442.
- Kim SH, Kim MS, Lee MS, et al. Korean diet: characteristics and historical background. *J Ethn Food*. 2016;3:26-31.
- Nagel G, Weinmayr G, Kleiner A, Garcia-Marcos L, Strachan DP, Group IPTS. Effect of diet on asthma and allergic sensitisation in the International Study on Allergies and Asthma in Childhood (ISAAC) Phase Two. *Thorax* 2010;65:516-522.
- Huh IS, Kim H, Jo HK, et al. Instant noodle consumption is associated with cardiometabolic risk factors among college students in Seoul. *Nutr Res Pract*. 2017;11:232-239.
- Indicators O. *Health at a Glance 2011. OECD Indicators*. Paris, France: OECD Publishing; 2015;15:2016.

SUPPORTING INFORMATION

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Neuromedin U directly induces degranulation of skin mast cells, presumably via MRGPRX2

To the Editor,

Mast cells (MCs) play critical roles in immune responses through either the high affinity IgE receptor (FcεRI) or FcεRI independent

pathways, which involve cytokines, pathogens, and/or neuropeptides. Neuromedin U (NMU), a neuropeptide, originally isolated from porcine spinal cord and has various functions, such as regulation of stress responses and modification of ion transport in the gastrointestinal tract.¹ Recently, several groups have reported that NMU stimulates group 2 innate lymphoid cells (ILC2s) and induces type 2 inflammation in mouse gastrointestinal tract and lung.² Moriyama et al³ reported that in mouse skin, NMU released from keratinocytes induces MC degranulation with an early phase of inflammation.

Abbreviations: BMMCs, bone marrow-derived mouse mast cells; c48/80, compound 48/80; CTMCs, connective tissue-type mouse mast cells differentiated from BMMCs; FcεRI, the high affinity IgE receptor; GPCRs, G protein-coupled receptors; hsMCs, human skin-derived mast cells; ILC2s, group 2 innate lymphoid cells; MCs, mast cells; MRGPR, Mas-related G protein-coupled receptor; NMU, neuromedin U; PTx, pertussis toxin; SP, substance P; TRPV3, the transient receptor potential vanilloid 3.

However, the mechanism underlying MC activation by NMU has remained largely unknown. Previous reports have identified two G protein-coupled receptors (GPCRs) for NMU, NMUR1 and NMUR2. NMUR1 is predominately expressed in peripheral tissues, whereas the expression of NMUR2 is largely restricted to the central nervous system.¹ Nevertheless, the expression of NMUR1 in cutaneous MC has not been reported and gene knockdown of these receptors does not entirely abolish inflammatory reactions induced by NMU,⁴ suggesting the presence of other molecular target(s) for NMU. We here investigated the mechanism of human and mouse skin MC activation by NMU, the analysis of receptor proteins for NMU, and the release of NMU from human keratinocytes, *in vitro*.

We first prepared two types of mouse MCs from bone marrow: immature MCs (BMMCs) and connective tissue-type MCs (CTMCs) (Appendix S1: Figure S1a,b). Although mouse NMU, consisting of 23 peptides (NMU-23), and substance P (SP), induced significant levels of degranulation of CTMCs in a concentration-dependent manner, a high concentration of SP (500 μ M) only induced slight degranulation of BMMCs (Figure 1A). The levels of NMUR1 mRNA expression were lower in CTMCs than those in BMMCs (Figure 1B). The expression of NMUR2 mRNA was detected neither in BMMCs nor in CTMCs (data not shown). We then investigated the effect of pertussis toxin (PTx), which inactivates $G_{\alpha_{i/o}}$ -type G proteins, on degranulation of CTMCs in response to ionomycin (Io), compound 48/80 (c48/80), SP, and NMU-23. PTx potently suppressed degranulation of CTMCs in response to c48/80, SP, or NMU-23 (Figure 1C). The mRNA of Mas-related G protein-coupled receptor b2 (Mrgprb2) was found to be highly expressed in CTMCs but not in BMMCs and spleen cells (Figure 1D). We then investigated the effect of human NMU, consisting of 25 peptides (NMU-25), SP and c48/80 on degranulation of human skin-derived MCs (hsMCs) (Appendix S1: Figure S1c,d). NMU-25, SP, and c48/80 induced degranulation in a concentration-dependent manner (Figure 1E). Moreover, the degranulation of hsMCs was abolished by the treatment with PTx, suggesting that NMU-induced degranulation is mediated by G_{α_i} (Figure 1F). Similarly, hsMCs were found to express MRGPRX2, but not NMUR1 (Figure 1G). Human keratinocytes and fibroblasts expressed neither MRGPRX2 nor NMUR1.

To determine whether NMU can act as an agonist of MRGPRX2, we performed a TGF- α shedding assay⁵ using G_{α_q} , $G_{\alpha_{11}}$, $G_{\alpha_{12}}$, and $G_{\alpha_{13}}$ gene-deleted HEK293 cells ($\Delta G_{\alpha_{q/11/12/13}}$ HEK293 cells) transfected with or without the expression vectors encoding MRGPRX2 and/or chimeric $G_{\alpha_{q/11}}$ protein, consisting of the G_{α_q} backbone required for TGF- α shedding activity and carboxyl-terminal sequence (DCGLF) of $G_{\alpha_{11}}$ to be coupled with receptors. NMU-25 induced significant TGF- α shedding only in the cells expressing both MRGPRX2 and chimeric $G_{\alpha_{q/11}}$ protein, suggesting that NMU acts on MRGPRX2, with the activation of G_{α_i} . Similar responses were observed in response to SP and c48/80 (Figure 1H,I,J). To address questions where and when NMU is released in the skin, we stained human skin sections with anti-NMU antibody and measured NMU in cells cultured by ELISA. The immunohistochemistry showed an abundant expression of NMU in the epidermis, but not in the dermis

(Figure 2A). Moreover, cultured human keratinocytes, but not fibroblasts and hsMCs, expressed NMU at both mRNA (Figure 2B) and protein levels (2.32 ± 0.40 fg/cell, $n = 4$). Furthermore, NMU was released from keratinocytes within 10 min after the stimulation with Pam3CSK4 or carvacrol (Figure 2C). The NMU content in the medium did not increase during the subsequent 24 h (data not shown).

We thus revealed that NMU directly activates both CTMCs and hsMCs, in a concentration-dependent manner. Moreover, no or only slight expression of mRNA for NMUR1 in CTMCs and hsMCs was detected. On the other hand, MRGPRX2 and Mrgprb2, mouse analog of MRGPRX2, were highly expressed in hsMCs and in CTMCs, respectively.

In humans, MCs are subdivided into an MC_T subset, which expresses tryptase only and an MC_{TC} subset, which expresses tryptase and chymase. In mice, MCs are classified as connective tissue MC, comparable to human MC_{TC}, and mucosal MC. MRGPRX2 is expressed in the small neurons of sensory ganglia and human MC_{TC}.⁶ In our study, hsMCs and CTMCs expressed high levels of MRGPRX2 and Mrgprb2, respectively. Recent studies have shown that antimicrobial host defense peptides, neuropeptides, such as SP, and several drugs activate human MC_{TC} via MRGPRX2.⁶ It is noteworthy that MRGPRX2 couples to both PTx-sensitive and PTx-insensitive signaling pathways for degranulation,⁶ whereas NMUR1 is known to be coupled with $G_{\alpha_{q/11}}$, PTx-insensitive G protein. In this study, we revealed that PTx inhibits NMU-induced activation of CTMCs and hsMCs. Moreover, we demonstrated that NMU activates cells via MRGPRX2 and G_{α_i} by means of TGF- α shedding assay.

The immunohistochemistry of human skin sections showed the expression of NMU in the epidermis and keratinocytes cultured from human skin specifically expressed NMU both at mRNA and at protein levels (Figure 2). Mempel et al⁷ reported that *Staphylococcus aureus* induces IL-8 release from keratinocytes via TLR2. Szollosi et al⁸ demonstrated that human keratinocytes express the transient receptor potential vanilloid 3 (TRPV3), activated by warm temperatures, and release IL-6, IL-8, IL-1 α , and TNF- α in response to agonists of TRPV3, such as carvacrol, a plant-derived compound. In this study, we elucidated that keratinocytes store 2.32 fg NMU/cell and rapidly release it in response to Pam3CSK4 and carvacrol, *in vitro*. Considering the volume of keratinocyte, ca 1 pL and the layered structure in the epidermis, the concentration of NMU released from keratinocytes near the interface between the dermis and the epidermis where MCs reside may be readily over a few micromolar and induce degranulation of cutaneous MCs. Moreover, upregulation of MRGPRX2 in MCs, observed in patients with chronic spontaneous urticaria,⁹ may make MCs more sensitive to NMU. Thus, various stimuli which induce NMU release from keratinocytes cause the activation of MCs, presumably via MRGPRX2. The regulation of NMU synthesis and release in the skin may be involved in the pathogenesis of a wide range of non-IgE- and MC-mediated diseases. Further studies with specific antagonists for MRGPRX2 and gene knockdown experiments would help clarify a more detailed mechanism for MC activation by NMU released from keratinocytes.

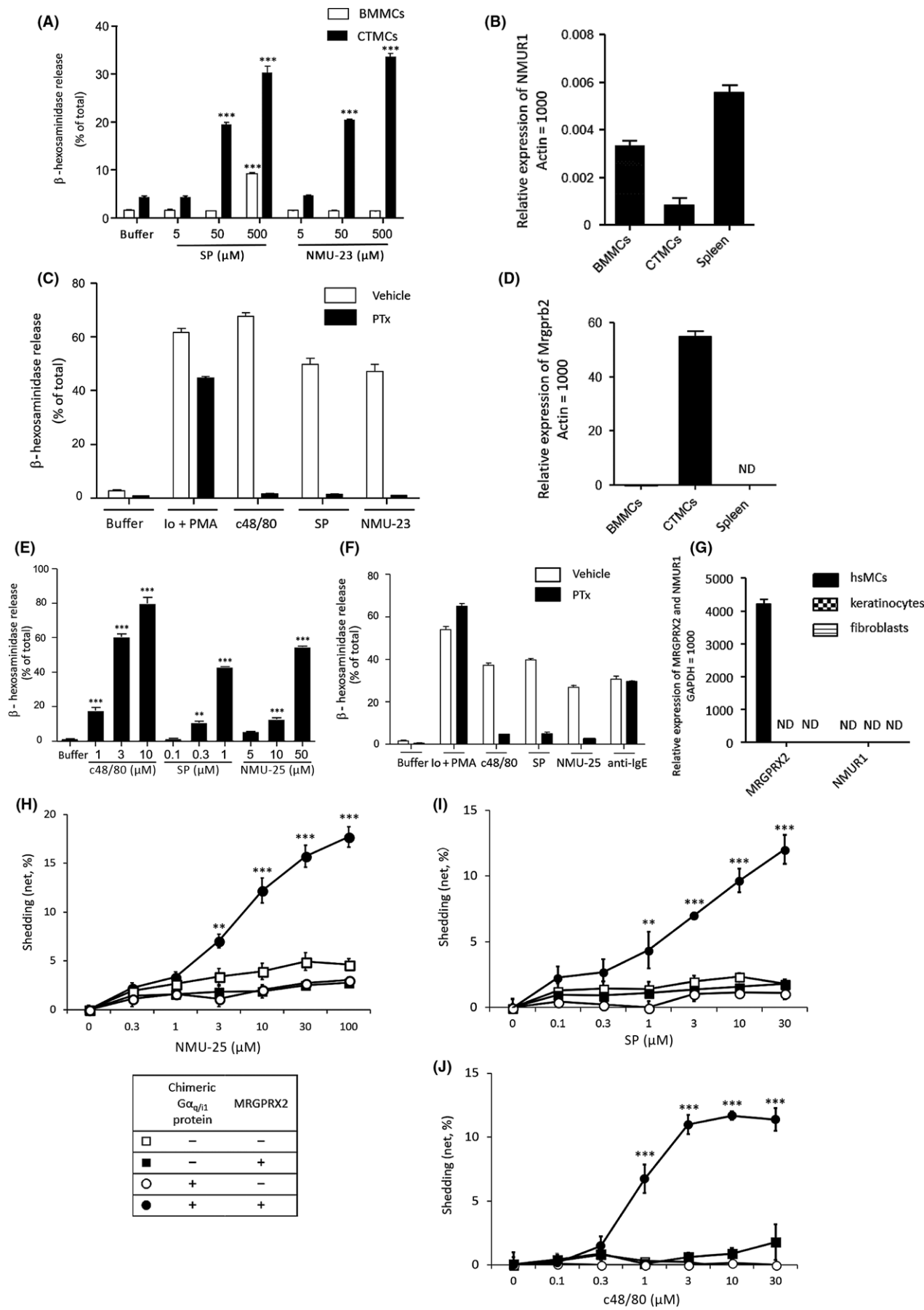


FIGURE 1 NMU induces degranulation of mouse and human skin type MCs via PTx-sensitive GPCR. NMU can binds to MRGPRX2 followed by G_{α_i} activation: (A) Mouse NMU (NMU-23) and substance P (SP) induced significant levels of degranulation of CTMCs in a concentration-dependent manner, and a high concentration of SP (500 μ M) induced only slight degranulation of BMMCs. Significant differences between buffer control and indicated concentration of stimulation. (B) Relative expression level of NMUR1-mRNA to actin-mRNA in BMMCs, CTMCs, and spleen cells. (C) Treatment with PTx (200 ng/mL) for 2 h blocked c48/80 (10 μ g/mL), SP (50 μ M), or NMU-23 (50 μ M) induced release of β -hexosaminidase from CTMCs, but not those by Io (1 μ M) and PMA (50 nM). (D) Relative expression level of Mrgprb2-mRNA to actin-mRNA in BMMCs, CTMCs, and spleen cells. (E) HsMCs cause degranulation in response to c48/80, SP, or human NMU (NMU-25) in a concentration-dependent manner. Significant differences between buffer control and indicated concentrations for stimulation. (F) Treatment with PTx (200 ng/mL) for 2 h blocked c48/80 (10 μ g/mL), SP (50 μ M), or NMU-25 (50 μ M) induced β -hexosaminidase release from hsMCs, but not those by Io (1 μ M) and PMA (50 nM). (G) Relative expression level of MRGPRX2-mRNA or NMUR1-mRNA to GAPDH-mRNA in hsMCs, keratinocytes, and fibroblasts. (H) NMU-25, (I) SP, or (J) c48/80 induces concentration-dependent TGF- α shedding from HEK293 cells only which transfected with both MRGPRX2 and chimeric $G_{\alpha_{q/11}}$ protein subunit. Significant differences between the cells expressing both MRGPRX2 and chimeric $G_{\alpha_{q/11}}$ protein (closed circles) and the cells without molecules (open squares). Values are shown as the mean $\pm$ SEM (n = 3). ND, not detected. ** P < 0.01; *** P < 0.001, one-way analysis of variance with Tukey post hoc test for multiple comparisons

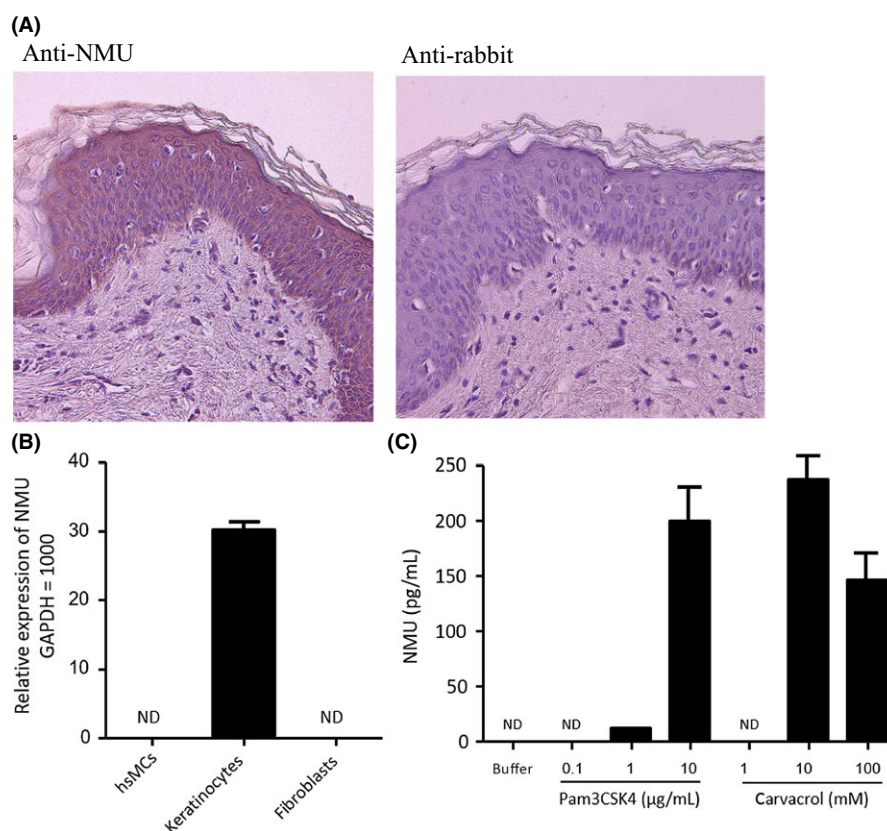


FIGURE 2 Keratinocytes in the epidermis express and rapidly release NMU in response to various stimuli. (A) Immunohistochemistry of human femur skin stained with anti-NMU antibody. (B) Relative expression level of NMU-mRNA to GAPDH-mRNA in hsMCs, keratinocytes, and fibroblasts. (C) Keratinocytes release NMU in response to indicated concentrations of Pam3CSK4 and carvacrol. Values are shown as the mean $\pm$ SEM (n = 3). ND, not detected [Colour figure can be viewed at wileyonlinelibrary.com]

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REFERENCES

1. Martinez VG, O'Driscoll L. Neuromedin U: a multifunctional neuropeptide with pleiotropic roles. *Clin Chem*. 2015;61:471-482.

2. Klose CSN, Mahlaköiv T, Moeller JB, et al. The neuropeptide neuromedin U stimulates innate lymphoid cells and type 2 inflammation. *Nature*. 2017;549:282-286.
3. Moriyama M, Sato T, Inoue H, et al. The neuropeptide neuromedin U promotes inflammation by direct activation of mast cells. *J Exp Med*. 2005;202:217-224.
4. Rao SM, Auger JL, Gaillard P, et al. The neuropeptide neuromedin U promotes autoantibody-mediated arthritis. *Arthritis Res Ther*. 2012;14:R29.
5. Inoue A, Ishiguro J, Kitamura H, et al. TGF- α shedding assay: an accurate and versatile method for detecting GPCR activation. *Nat Methods*. 2012;9:1021-1029.
6. Tatemoto K, Nozaki Y, Tsuda R, et al. Immunoglobulin E-independent activation of mast cell is mediated by Mrg receptors. *Biochem Biophys Res Commun*. 2006;349:1322-1328.
7. Mempel M, Voelcker V, Köllisch G, et al. Toll-like receptor expression in human keratinocytes: nuclear factor κ B controlled gene activation by staphylococcus aureus is toll-like receptor 2 but not toll-like receptor 4 or platelet activating factor receptor dependent. *J Invest Dermatol*. 2003;121:1389-1396.
8. Szöllösi AG, Vasas N, Angyal Á, et al. Activation of transient receptor potential vanilloid 3 regulates inflammatory actions of human epidermal keratinocytes. *J Invest Dermatol*. 2018;138:365-374.
9. Fujisawa D, Kashiwakura J, Kita H, et al. Expression of Mas-related gene X2 on mast cells is upregulated in the skin of patients with severe chronic urticaria. *J Allergy Clin Immunol*. 2014;134:622-633.

SUPPORTING INFORMATION

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Safety of ultra-rush protocols for hymenoptera venom immunotherapy in systemic mastocytosis

To the Editor,

Mastocytosis comprises a heterogeneous group of disorders characterized by the expansion and accumulation of clonal mast cells in one or more organ systems. It is a rare disease with an estimated prevalence of about 10 cases per 100 000 inhabitants.¹ Anaphylactic reactions are very common, affecting between 22% and 49% of patients with systemic mastocytosis (SM).² Hymenoptera venom allergy (HVA) seems to be particularly involved in anaphylactic reactions of patients with SM. Mastocytosis has been identified as a risk factor for severe anaphylaxis to hymenoptera venom and the prevalence of clonal mast cell disease in patients with HVA ranges from 1% to 7.9%, which is much higher than that of the general population.³ Elevation of baseline serum tryptase has been identified as a risk factor for severe anaphylaxis in HVA. Although recent EAACI (European Academy of Allergy and Clinical Immunology) guidelines on AIT recognize hymenoptera venom immunotherapy (HVI) as a safe and effective form of treatment for patients with mastocytosis

associated with anaphylactic reaction to hymenoptera venom,⁴ only a few studies are available on allergen immunotherapy (AIT) tolerance in those patients, and no protocols of HVI have been clearly recommended in patients with SM. We present hereinafter data recorded in patients with SM and anaphylactic reaction due to hymenoptera venom allergy and HVI based on an ultra-rush protocol.

Data were collected from patients with SM requiring HVI in the Mastocytosis Reference Centre at Toulouse University Hospital Centre. The inclusion criteria were as follows: (a) patients over 18 years old; (b) SM diagnosis based on World Health Organization diagnostic criteria,⁵ (c) allergy to hymenoptera venom with positive skin tests and/or positive specific IgEs to hymenoptera venom associated with a history of anaphylactic reaction following a hymenoptera sting; and (d) HVI initiation with an ultra-rush protocol as previously described.⁶

Demographic data, characteristics of the initial reaction and details of allergy skin tests were collected. Intradermal tests against

Online Supplementary Material

Supplementary Methods

Reagents

An anti-DNP IgE, compound 48/80 (c48/80), DNP-human serum albumin (DNP-HSA), bovine serum albumin (BSA), *p*-nitrophenyl-*N*-acetyl- β -glucosamine, hyaluronidase, type I DNase, anti NMU antibody (HPA025926) and carvacrol were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Ionomycin (Io) and phorbol 12-myristate 13-acetate (PMA) were from Calbiochem (La Jolla, CA). Recombinant mouse IL-3, recombinant mouse SCF and recombinant human SCF were from R&D Systems Inc (Minneapolis, MN). Substance P (SP) was from Peptide Institute (Osaka, Japan). Human NMU (NMU-25) and mouse NMU (NMU-23) were from SCRUM Inc (Tokyo, Japan). X-vivo-15 and KGM-gold were from Lonza (Walkersville, MD). The RNeasy Mini Kit and the QuantiTect Reverse transcription Kit were from Qiagen (Venlo, Netherlands). Fetal calf serum (FCS) was from Biowest (Nuaillé, France). RPMI, DMEM, Keratinocyte-SFM, penicillin, streptomycin and trypsin were from Life Technologies (Carlsbad, CA). Pertussis toxin (PTx) and Dispace II were from Wako (Osaka, Japan). Type 2 collagenase was from Worthington (Lakewood, NJ). Histofine SAB PO kit and DAB substrate kits were from Nichirei (Tokyo, Japan). Pam3CSK4 was from InvivoGen (San Diego, CA).

Establishment of BMMCs and CTMCs

BMMCs from Balb/c female mice (6-8 weeks old, Charles River Laboratories, Yokohama, Japan) were obtained as previously described.^{10, 11} Briefly, bone marrow cells were suspended at a density of 1×10^6 cells/mL in RPMI medium supplemented with 10% FCS, 50 μ M 2-mercaptoethanol, 2 mM glutamine, 100 IU/mL penicillin, 100 μ g/mL streptomycin and 5 ng/mL IL-3 for 4 weeks. CTMCs were obtained from BMMCs co-cultured with Swiss 3T3 in the presence of recombinant mouse SCF (100 ng/mL) for 2 weeks.¹²

Isolation and culture of human skin derived mast cells

HsMCs were isolated from human skin. All specimens of human skin in this study were obtained from patients with informed consent. The use of skin material from the patients was approved by the Ethics committee of Hiroshima University. Skin was cut into fragments and incubated in a solution of HBSS containing type 2 collagenase (1.5 mg/mL), hyaluronidase (0.7 mg/mL), type I DNase (0.3 mg/mL), 1% FCS and 1mM CaCl_2 for 2 hr at 37°C. The dispersed cells were separated from residual tissues by filtration through a 70 mesh sieve and suspended in X-vivo-15 media. Isolated mast cells were cultured in X-vivo-15 media with 100 ng/mL recombinant human SCF for 1-2 months.⁹

Degranulation assay

Degranulation was determined by measuring the release of β -hexosaminidase, a granule marker that hydrolyzes *p*-nitrophenyl-*N*-acetyl- β -glucosamine to the chromophore, *p*-nitrophenol, as described previously.¹³ Briefly, BMMCs, CTMCs or hsMCs were resuspended in piperazine-*N*-*N'*-bis (2-ethanesulfonic acid) (PIPES) buffer containing 119 mM NaCl, 5 mM KCl, 1.0 mM CaCl₂, 0.4 mM MgCl₂, 5.6 mM glucose, 25 mM PIPES and 1 mg/mL BSA (pH 7.2). The cells were allowed to settle onto a 96-well plate. HsMCs were sensitized with 50 ng/mL anti-DNP IgE overnight. CTMCs were sensitized with 1 μ g/mL anti-DNP IgE for 6 hr.¹⁴ After incubation with or without pertussis toxin (PTx, 200 ng/mL) for 2 hr at 37°C, CTMCs and hsMCs were stimulated with indicated concentrations of stimuli for 15 min at 37°C.

Real-time PCR

Total RNA was extracted from cells (BMMCs, CTMCs, spleen cells, hsMCs, human keratinocytes, and human fibroblasts) using the RNeasy Mini kit, and cDNA was generated using the QuantiTect Reverse transcription kit. Levels of mRNA expression of mouse NMUR1, Mrgprb2 and mouse actin were evaluated using ABI 7300 Real-time PCR system (Applied Biosystems, Carlsbad, CA) with Power SYBR Green PCR Master Mix (Applied Biosystems). The specific primer pairs were as follows, forward primer for mouse NMUR1, 5'-TCTCTTCTCTCTGCCCAACACCAGCCT-3'; reverse primer for mouse NMUR1, 5'-CGAATACCACAACCAGTGCAAACAGCA-3'; forward primer for Mrgprb2, 5'-GTCACAGACCAGTTTAACACTTCC-3'; reverse primer for Mrgprb2, 5'-CAGCCATAGCCAGGTTGAGAA-3'; forward primer for mouse actin, 5'-GCTACAGCTTCACCACCACAG-3', and reverse primer for mouse actin, 5'-GGTCTTTACGGATGTCAACGTC-3'. Expression of actin was measured as an internal control to calibrate gene expression. Levels of mRNA expression of Mcp4, human NMU, human NMUR1, MRGPRX2, human chymase and human tryptase were evaluated using ABI 7300 Real-time PCR system by taqman gene expression assay (Mm00607939_s1 for mouse actin, Mm00487636_g1 for Mcp4, Hs02758991_g1 for GAPDH, Hs00365019_s1 for MRGPRX2, Hs00183624_m1 for human NMU, Hs00173804_m1 for human NMUR1, Hs01095979_g1 for human tryptase, Hs02576518_gH, human chymase, Hs01095979_g1. All primers were purchased from Applied Biosystems).

TGF- α shedding assay

The $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{12}$ and $G\alpha_{13}$ gene-deleted HEK293 cell lines ($\Delta G\alpha_{q/11/12/13}$ HEK293 cells) were established using a CRISPR/Cas9 system as described previously.¹⁵ $\Delta G\alpha_{q/11/12/13}$ HEK293 cells were maintained in DMEM containing heat-inactivated 10% FCS, 100 IU/mL penicillin and 100

$\mu\text{g/mL}$ streptomycin at 37°C in a fully-humidified atmosphere of 5% CO_2 in air. The TGF- α shedding assay was performed as described previously⁵ with minor modifications. Transfection was performed using PEI: Polyethyleneimine “Max” (Polysciences, Inc., Warrington, PA) instead of Lipofectamine 2000. MRGPRX2 cDNA was cloned into mammalian expression vector pCAGGS. The chimeric $\text{G}\alpha$ protein consisting of the $\text{G}\alpha_q$ backbone required for TGF- α shedding activity and carboxyl-terminal sequence (DCGLF) of $\text{G}\alpha_{i1}$ to be coupled with receptors (chimeric $\text{G}\alpha_{q/i1}$ protein) was used in this study.⁵ The $\text{G}\alpha_q$ backbone may activate TGF- α converting enzyme (TACE), which induces the TGF- α shedding assay, while the DCGLF sequence of $\text{G}\alpha_{i1}$ may be associated with GPCRs.

Isolation and culture of human keratinocytes

Keratinocytes were isolated after overnight dermal–epidermal separation in 2.5 mg/mL Dispase II followed by trypsin digestion for 10 min at 37°C . Isolated keratinocytes were cultured in KGM-gold supplemented with 1% FCS.¹⁶ Keratinocytes were cultured in Keratinocyte-SFM medium containing 50 $\mu\text{g/mL}$ bovine pituitary extract (BPE), 5 ng/mL epidermal growth factor (EGF), 100 IU/mL penicillin and 100 $\mu\text{g/mL}$ streptomycin. After two passages, the cells were seeded at 2.2×10^4 cells/well in 24 wells culture clusters. Upon reaching a confluent state, the cell culture medium was replaced with fresh medium, followed by 15 min adaptation. The medium was then replaced with another batch of fresh medium containing Pam3CSK4 or carvacrol. After 10 min incubation, the medium was collected and replaced with fresh medium containing Pam3CSK4 or carvacrol, and then incubation was continued at 37°C for 24 hr.

Isolation and culture of human fibroblasts

At the start of the experiment, a piece of human skin was chopped and cultured in DMEM medium with 10% FCS, 50 μM 2-mercaptoethanol, 2 mM glutamine, 100 IU/mL penicillin and 100 $\mu\text{g/mL}$ streptomycin. Fibroblasts grown out of the chopped skin and cultured for 4–8 passages were used for these experiments.

Immunohistochemistry

Deparaffinized sections were treated in 0.3% H_2O_2 /methanol for 15 min. The sections were then rinsed with PBS for 5 min. After blocking nonspecific reactivity with rabbit serum for 15 min at room temperature (Histofine SAB PO kit), sections were incubated overnight (4°C) with primary NMU antibody (dilution 1:200). The distribution of the primary antibody was marked by subsequent application of a biotinylated antiprimary antibody (Histofine SAB PO kit) and streptavidin-peroxidase (Histofine SAB PO kit). Immunostaining was developed using DAB/ H_2O_2 solution (Histofine DAB substrate kit), and sections were counterstained with

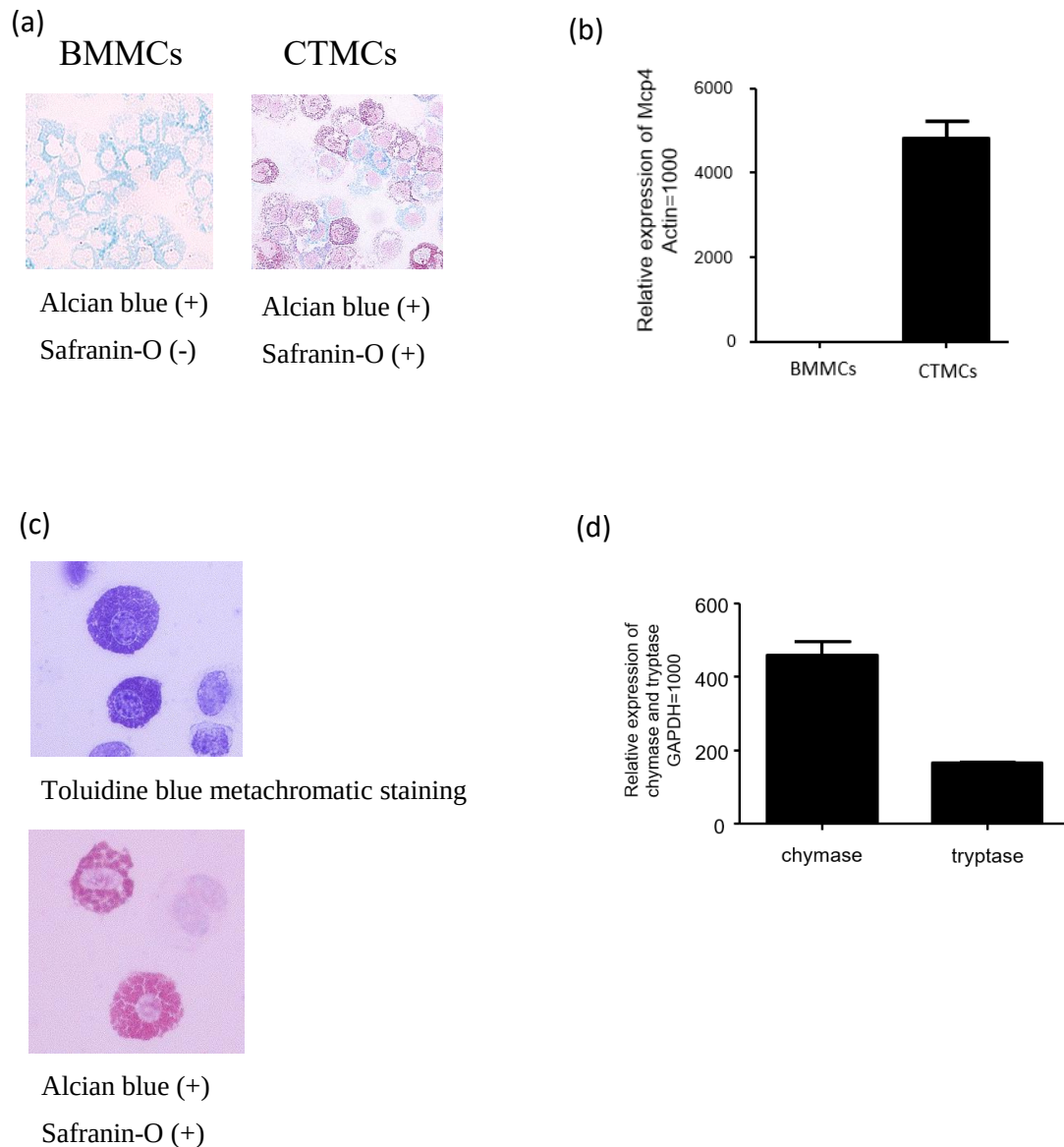
Mayer's hematoxylin. As a negative control, some sections were subjected to normal serum blocking and omission of the primary antibody.

Detection of NMU by ELISA

The concentration of NMU was measured using a human NMU (ELISA) kit (LSBio, Seattle WA) according to the manufacturer's instructions.

Reference:

10. Okabe T, Hide M, Hiragun T, Morita E, Koro O, Yamamoto S. Bone marrow derived mast cell acquire responsiveness to substance P with Ca²⁺ signals and release of leukotriene B₄ via mitogen-activated protein kinase. *J Neuroimmunol* 2006;**181**:1-12.
11. Morita E, Hiragun T, Tanaka T, Kameyoshi Y, Okabe T, Hide M, et al. Mouse bone marrow-derived mast cells acquire responsiveness to substance P after co-culture with 3T3 fibroblasts in the presence of stem cell factor. *Allergol Int* 1998; **47**: 205-212.
12. Takano H, Nakazawa S, Okuno Y, Shirata N, Tsuchiya S, Kainoh T, et al. Establishment of the culture model system that reflects the process of terminal differentiation of connective tissue-type mast cells. *FEBS Letters* 2008;**582**: 1444-1450.
13. Yanase Y, Hide I, Mihara S, Shirai Y, Saito N, Nakata Y, et al. A critical role of conventional protein kinase C in morphological changes of rodent mast cells. *Immunol Cell Biol* 2010; **89**:149-159.
14. Tanaka S, Takasu Y, Mikura S, Satoh N, Ichikawa A. Antigen-independent Induction of Histamine Synthesis by Immunoglobulin E in Mouse Bone Marrow - derived Mast Cells. *J Exp Med* 2002;**196**:229-235.
15. Devost D, Sleno R, Pé Trin D, Zhang A, Shinjo Y, Okde R, et al. Conformational profiling of the AT1 angiotensin II receptor reflects biased agonism, G protein coupling, and cellular context. *J Biol Chem* 2017 **292**:5443-5456.
16. Aasen T, Belmonte JCI. Isolation and cultivation of human keratinocytes from skin or plucked hair for the generation of induced pluripotent stem cells. *Nat Protoc* 2010; **5**:371-382.



Supplementary Fig. 1.

(a) BMMCs were safranin-O staining-negative, whereas CTMCs were safranin-O staining-positive. (b) The expression of mouse mast cell protease 4 (Mcp4) was confirmed at the transcriptional levels in CTMCs but not in BMMCs. (c) A number of dense granules were found in hsMCs stained with toluidine blue, alcian blue or safranin-O. (d) The expression of tryptase and chymase was confirmed at the transcriptional levels in hsMCs. Values are shown as the mean $\pm$ SEM (n = 3).