

1 Article

# 2 Suppression of IgE-independent degranulation of 3 murine connective tissue-type mast cells by 4 dexamethasone

5 Keiko Yamada<sup>1</sup>, Hitomi Sato<sup>1</sup>, Kazuma Sakamaki<sup>1</sup>, Mayumi Kamada<sup>2</sup>, Yasushi Okuno<sup>2</sup>,  
6 Nobuyuki Fukuishi<sup>3</sup>, Kazuyuki Furuta<sup>1</sup>, and Satoshi Tanaka<sup>4\*</sup>

7 <sup>1</sup> Department of Immunobiology, Division of Pharmaceutical Sciences, Okayama University Graduate  
8 School of Medicine, Dentistry, and Pharmaceutical Sciences, Tsushima-naka 1-1-1, Kita-ku,  
9 Okayama 700-8530, Japan.

10 <sup>2</sup> Department of Biomedical Data Intelligence, Graduate School of Medicine, Kyoto University,  
11 Shogoin-Kawaharacho, Sakyo-ku, Kyoto 606-8507, Japan.

12 <sup>3</sup> Department of Pharmacology, College of Pharmacy, Kinjo Gakuin University, 2-1723 Omori,  
13 Moriyama-ku, Nagoya, Aichi, 463-8521, Japan.

14 <sup>4</sup> Department of Pharmacology, Division of Pathological Sciences, Kyoto Pharmaceutical University,  
15 Misasagi Nakauchi-cho 5, Yamashina-ku, Kyoto 607-8414, Japan.

16 \* Department of Pharmacology, Division of Pathological Sciences, Kyoto Pharmaceutical University,  
17 Misasagi Nakauchi-cho 5, Yamashina-ku, Kyoto 607-8414, Japan.: tanaka-s@mb.kyoto-phu.ac.jp;  
18 Tel.: +81-75-595-4667

19

20 **Abstract:** Steroidal anti-inflammatory drugs are widely used for treatment of chronic cutaneous  
21 inflammation, such as atopic dermatitis, although it remains unknown how they modulate  
22 cutaneous mast cell functions. Murine connective tissue-type mast cells, which were sensitive to  
23 mast cell secretagogues, such as compound 48/80 and substance P, were generated by co-culture of  
24 bone marrow-derived mast cells with Swiss 3T3 fibroblasts in the presence of stem cell factor. This  
25 process was accompanied by up-regulation of  $\alpha$  subunit of a trimeric G protein,  $G_{\alpha i1}$ , and several  
26 Mas-related G protein-coupled receptor (Mrgpr) subtypes. Secretagogue-induced degranulation  
27 and up-regulation of these genes were suppressed when they were cultured in the presence of a  
28 synthetic glucocorticoid, dexamethasone. The profiles of granule constituents were drastically  
29 altered by dexamethasone. Several Mrgpr subtypes were found to be expressed in the cutaneous  
30 tissues and their expression levels were decreased in response to topical application of  
31 dexamethasone. The numbers of degranulated cutaneous mast cells in response to compound 48/80  
32 were decreased in mice treated with dexamethasone. These results suggest that mast cell-mediated  
33 IgE-independent cutaneous inflammation could be suppressed by steroidal anti-inflammatory  
34 drugs through down-regulation of  $G_{\alpha i1}$  and several Mrgpr subtypes in mast cells.

35 **Keywords:** mast cell; dexamethasone; trimeric G protein; Mrgpr, skin, inflammation

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## 38 1. Introduction

39 Glucocorticoid was found to have a potential to suppress inflammation in 1940s and synthetic  
40 glucocorticoids, steroidal anti-inflammatory drugs, have widely prescribed for the treatments of  
41 various chronic inflammatory diseases, such as atopic dermatitis and autoimmune disorders [1, 2]. A  
42 large part of glucocorticoid-mediated effects arises through its binding to glucocorticoid receptor (GR,  
43 Nr3c1). Because GR is ubiquitously expressed in a variety of cells, it is quite difficult to attribute the  
44 anti-inflammatory effects of glucocorticoids to the actions on the specific cell types. Steroidal anti-  
45 inflammatory drugs have been frequently used for therapy of various cutaneous inflammatory

46 diseases, in some of which cutaneous mast cells were found to play critical roles, although it remains  
47 to be fully clarified how they act on cutaneous mast cells.

48 Mast cells originate in the hematopoietic stem cells in the bone marrow and undergo terminal  
49 differentiation in the tissue, in which they infiltrate from the circulation [3]. These findings indicated  
50 that tissue mast cells should have a greater diversity. We previously established a murine bone  
51 marrow-derived cultured mast cell model, which had similar characteristics with cutaneous mast  
52 cells, through modification of the previous models [4]. We found that this model could undergo  
53 degranulation in response to mast cell secretagogues, such as compound 48/80 and substance P.  
54 Sensitivity to mast cell secretagogues is one of the signatures of connective tissue-type mast cells.  
55 Because no suitable culture models have been developed, the signaling pathways involved in  
56 secretagogue-induced degranulation remained largely unknown [5]. Tatemoto et al. first  
57 demonstrated that Mas-related G protein-coupled receptor (Mrgpr) X2 should be involved in  
58 secretagogue-induced degranulation of mast cells. Recently, one of murine orthologues of MrgprX2,  
59 MrgprB2, was found to be responsible for pseudo allergic drug responses induced by mast cell  
60 degranulation using the gene-targeted mice, indicating that Mrgpr family should be involved in  
61 secretagogue-induced mast cell degranulation [6]. MrgprX2 was found to be up-regulated in the  
62 cutaneous mast cells of the patient with severe chronic urticaria [7]. Accumulating evidence suggests  
63 that IgE-independent activation of mast cells should play critical roles in a wide variety of cutaneous  
64 inflammatory diseases; about 20% of the patients with atopic dermatitis were reported to have no  
65 IgE-sensitization to environmental antigens and low serum IgE levels (intrinsic atopic dermatitis) [8],  
66 and about 50% of the patients with chronic urticaria were found to spontaneously develop the  
67 symptoms [9]. Steroidal anti-inflammatory drugs have been one of the primary therapeutic agents  
68 for these inflammatory diseases [10,11].

69 We here investigated the effects of a synthetic glucocorticoid, dexamethasone, on our connective  
70 tissue-type cultured mast cells and on the cutaneous vascular responses in mice in order to clarify  
71 how glucocorticoid modulate the functions of cutaneous mast cells.  
72

## 73 2. Materials and Methods

### 74 Mice

75 Specific-pathogen-free, 8-10 week-old male BALB/c mice were obtained from Japan SLC  
76 (Hamamatsu, Japan), and all mice were kept in a specific-pathogen-free animal facility at Okayama  
77 University. This study was approved by the Committee on Animal Experiments of Okayama  
78 University (Approved #OKU-2012218, 2015040, and 2015430).  
79

### 80 Materials

81 The following materials were commercially obtained from the sources indicated:  
82 dexamethasone, *p*-nitrophenyl- $\beta$ -*D*-2-acetoamide-2-deoxyglucopyranoside, compound 48/80, an anti-  
83 dinitrophenyl IgE antibody (clone SPE-7), *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA, mitomycin C, substance  
84 P, and dinitrophenyl human serum albumin (DNP-HSA) from Sigma-Aldrich (St. Louis, MO),  
85 Toluidine blue, Safranin-O, and Evans blue from Wako Pure Chemical Industries (Osaka, Japan), an  
86 anti-trinitrophenyl IgE antibody (clone IgE-3) from BD Biosciences (San Diego, CA), trinitrophenyl  
87 bovine serum albumin (TNP-BSA) from LSL (Tokyo, Japan), H-*D*-Ile-Pro-Arg-*p*NA (S-2288) from  
88 Chromogenix (Milano, Italy), *N*-(4-Methoxyphenylazoformyl)-Phe-OH potassium salt (M-2245) from  
89 Bachem AG (Bubendorf, Switzerland), an anti-*G* $\alpha$ 2 antibody from Santa Cruz Biotechnology (Dallas,  
90 TX), an anti-pan-actin antibody (clone C4), an anti-*G* $\alpha$ 1 antibody, an anti-*G* $\alpha$ 3 antibody, and  
91 thapsigargin from Merck Millipore (Billerica, MA), and recombinant mouse IL-3 from R&D Systems  
92 (Minneapolis, MN). All other chemicals were commercial products of reagent grade.  
93

### 94 Preparation of bone marrow-derived cultured mast cells

95 Preparation of IL-3-dependent bone marrow-derived cultured mast cells (BMMCs) and  
96 connective tissue type mast cell-like cultured mast cells (CTMC-like MCs) was performed as

97 described [4]. Briefly, bone marrow cells obtained from male BALB/c mice were cultured in the  
98 presence of 10 ng/ml IL-3 for ~30 days. Greater than 95% of the cells exhibited metachromasy by the  
99 acidic toluidine blue staining and were FcεRI<sup>+</sup>c-kit<sup>+</sup> on the flow cytometry. CTMC-like MCs were  
100 obtained through 16-days of co-culture of BMMCs with mitomycin C-treated Swiss 3T3 fibroblasts  
101 in the presence of 100 ng/ml recombinant murine stem cell factor (SCF). Greater than 90% of the cells  
102 were confirmed as mature mast cells by Safranin-O staining on Day-16. Dexamethasone (final  
103 concentration, 1 μM) was added to the culture medium simultaneously with SCF with 48 hr of  
104 interval thorough the co-culture period.

105

#### 106 **Measurement of degranulation**

107 Cultured mast cells were suspended in 25 mM PIPES-NaOH, pH 7.4 containing 125 mM NaCl,  
108 2.7 mM KCl, 1 mM CaCl<sub>2</sub>, 5.6 mM glucose, and 0.1% bovine serum albumin, and then stimulated for  
109 30 min at 37°C. Degranulation was evaluated by measuring enzyme activity of a granule enzyme, β-  
110 hexosaminidase using the specific substrate, *p*-nitrophenyl-β-D-2-acetoamide-2-  
111 deoxyglucopyranoside.

112

#### 113 **Measurement of histamine and IL-6**

114 The amount of histamine was determined by the fluorometrical method with *o*-phthalaldehyde  
115 [13]. Tissues were homogenized using a Polytron homogenizer (Kinematica AG, Schweiz,  
116 Switzerland) in phosphate buffered saline containing 2 M NaCl and the resultant homogenate was  
117 treated with 0.5% Triton X-100. The soluble fraction was subjected to histamine assay. The amount of  
118 IL-6 in the medium was measured using the ELISA system (BD Biosciences) according to the  
119 manufacturer's instruction.

120

#### 121 **Measurement of granule protease activities**

122 Three categories of granule protease activities were measured using their specific substrates  
123 basically as described previously [4]. In this study, *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA was used as the  
124 substrate for chymase instead of S-2586.

125

#### 126 **Quantitative PCR analysis**

127 Messenger RNA levels of various granule proteases and *Mrgpr* gene family were analyzed by  
128 quantitative reverse transcription (RT)-PCR with DNase-treated total RNAs. Total RNAs were  
129 prepared using NucleoSpin RNA kit (TaKaRa Bio, Kusatsu, Japan). PCR was performed using  
130 StepOne Plus (Thermo Fisher Scientific, Waltham, MA) with KOD SYBR qPCR Mix (TOYOBO,  
131 Osaka, Japan) or Fast SYBR Green Master Mix (Thermo Fisher Scientific) the specific primer pairs  
132 (forward, reverse); *Mcpt4*, (5'-CCT TAC ATG GCC CAT CT-3', 5'-CTT CCC CGG CTT GAT A-3'),  
133 *Mcpt5*, (5'-AGA ACT ACC TGT CGG C-3', 5'-GTC GTG GAC AAC CAA AT-3'), *Mcpt6*, (5'-CTT TGA  
134 ACC GGA TCG T-3', 5'-CTC GTC ATT ATC AAT GTC GC-3'), *Mcpt7*, (5'-AGC TAT GAC ACG AGA  
135 AGG-3', 5'-GCT TAC GGA GCT GTA CT-3'), *Cpa3*, (5'-GAT GTC TCG TGG GAC T-3', 5'-GCC GTA  
136 GAT GTA ACG GG-3'), *Mrgpra4*, (5'-CCT GTG TGC TGT GAT CTG GT-3', 5'-TCA CGG TTA ATC  
137 CAG GGC AC-3'), *Mrgprb1*, (5'-GAC ACA GAG CAA ATT ACC ATC TTC-3', 5'-CAA GGT TGA  
138 GGA TGT AGA CAG AG-3'), *Mrgprb2*, (5'-TGC TTG TCT GTA ATA TGG CCC-3', 5'-GTC ACA TAC  
139 AGC CTG GTC ATA G-3'), *Mrgprb10*, (5'-CCC AGG TTG GTG GAA CTG TT-3', 5'-GCC AGA AGC  
140 CTG ACA GTA GG-3'), *Mrgprc11*, (5'-CTA GCA TCC ACA ACC CCA G-3', 5'-TGT TTC CTG CCA  
141 GTC CAA C-3'), *Mrgpre*, (5'-AGA ACT ACC TGT CGG C-3', 5'-TTG CCT TCT GGC AGT GAT-3')  
142 and *Gapdh*, (5'-TGT GTC CGT CGT GGA TCT GA-3', 5'-TTG CTG TTG AAG TCG CAG GAG-3').

143

#### 144 **Immunoblot analyses**

145 Immunoblot analyses were performed as described previously [14]. SDS-PAGE was performed  
146 using 10% slab gels and PVDF membrane transfer was carried out by the semi-dry blotting method.  
147 Immunoreactive bands were detected by horseradish peroxidase-conjugated secondary antibodies  
148 and were visualized by ECL Western Blotting Detection Reagents (GE healthcare, Chicago, IL).

149

**150 Gene expression analysis by next generation sequencing**

151 Sequence reads from each group were individually aligned to the mm10 genome assembly  
152 (GRCm38) using TopHat v2.1.0 [15] with default parameters. Aligned read counts were calculated  
153 using HTSeq version 0.6.1 [16] with the RefSeq gene annotations obtained from the UCSC Genome  
154 Browser [17]. Read counts were then analyzed using DESeq version 1.20.0 [17] to detect differential  
155 expression genes (DEGs). Briefly, DESeq normalizes the raw read counts for each sample using size  
156 factors, which are calculated from the median of the ratio of observed count to geometric mean for  
157 each gene across all samples, and then infers DEGs based on the negative binomial distribution with  
158 estimated dispersion and mean linked by local regression. For without biological replicates situation,  
159 DESeq estimates dispersion using the samples from the different conditions as replicates. Three  
160 comparisons were made between BMMCs and CTMC-like MCs, between BMMC and CTMC-like  
161 MCs treated with dexamethasone, and between CTMC-like MCs and CTMC-like MCs treated with  
162 dexamethasone. The detected DEGs for each comparison were filtered to those having a false  
163 discovery rate (FDR) < 0.1, and divided into up-regulated genes and down-regulated genes based on  
164 the logarithmic fold change of normalized counts.

165

**166 Dexamethasone treatment**

167 Dexamethasone (20 µl/site, dissolved in acetone) was daily applied to the surface of the ear  
168 tissues of mice for 6 days. A series of experiments were performed 24 hr after the last application of  
169 dexamethasone.

170

**171 Evaluation of cutaneous extravasation induced upon IgE-dependent passive cutaneous  
172 anaphylaxis, and treatment with compound 48/80 or histamine**

173 Mice were intracutaneously sensitized with IgE (30 ng/site, clone SPE-7) in the ear tissues 24 hr  
174 before the challenge with intravenous injection of 60 µg DNP-HSA in 0.2 ml saline containing 1 mg  
175 Evans blue. The ear tissues were collected 30 min after the challenge and lysed in 3 N KOH. The  
176 amounts of Evans blue dye were determined by measuring the value of OD<sub>620</sub>. Extravasation  
177 responses induced by IgE-independent stimulus were determined by monitoring the dye leakage as  
178 described above when non-sensitized male BALB/c mice were intracutaneously injected with  
179 compound 48/80 (30 µg/site) or histamine (30 µg/site).

180

**181 Histological evaluation of cutaneous mast cells**

182 IgE-mediated antigen challenge and compound 48/80 stimulation were performed as described  
183 above without injection of Evans blue dye and the ear tissues were collected 3 min after the  
184 stimulation. Cutaneous mast cells were visualized by the acidic toluidine blue staining (pH 3.3) and  
185 the numbers of degranulated and intact mast cells were respectively counted.

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**187 Statistical analysis**

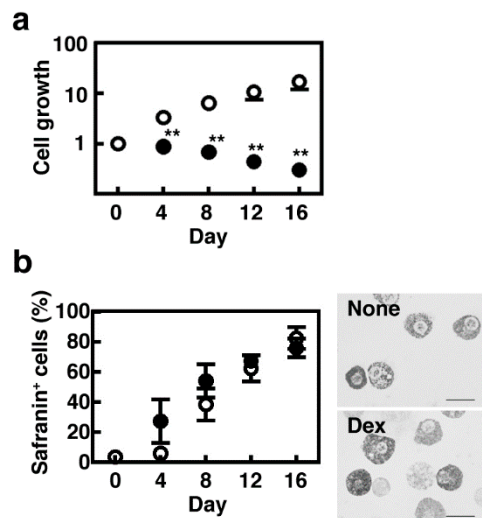
188 Data are presented as the means ± SEM. Statistical significance for comparisons was determined  
189 using one-way ANOVA. Additional comparisons were made with Dunnett multiple comparison test  
190 for comparison with the control groups or Tukey-Kramer multiple comparison test for all pairs of  
191 column comparison. Two-tailed unpaired Student's *t* test was used for comparison between two  
192 populations.

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**194 3. Results****195 3.1. Characteristic changes of the co-cultured mast cells induced by prolonged treatment with dexamethasone**

196 We first investigated the effects of dexamethasone on proliferation of BMMCs when they were  
197 co-cultured with Swiss 3T3 fibroblasts in the presence of SCF. The number of mast cells was  
198 increased during the co-culture period, whereas it remained unchanged in the presence of

199 dexamethasone (Fig. 1a). A slight decrease in the number of mast cells cultured in the presence of  
 200 dexamethasone resulted from the loss during the repeated subculture processes. The cell viability  
 201 was unchanged during the co-culture periods (> 95%). The granule maturation was monitored by  
 202 the Safranin staining, which reflects the amount of hypersulfated proteoglycans, such as heparin,  
 203 stored in the granules. Dexamethasone did not affect the percentages of Safranin-positive cells and  
 204 the granule number and morphology (Fig. 1b). We previously reported that granule protease  
 205 activities were drastically increased during the co-culture periods [4]. Prolonged treatment with  
 206 dexamethasone abolished the induction of chymotryptic activity but enhanced the  
 207 carboxypeptidase A activity in CTMC-like MCs (Fig. 2a and 2c). The tryptic activity was not  
 208 changed until Day-12, but was significantly decreased at Day-16 in the cells co-cultured in the  
 209 presence of dexamethasone (Fig. 2b). Expression of *Mcpt4*, *Mcpt6*, and *Mcpt7* were all up-regulated  
 210 in CTMC-like MCs and were suppressed in the presence of dexamethasone, indicating that  
 211 dexamethasone should affect the granule protease expression at the transcriptional levels, whereas  
 212 no significant changes were observed in the expression levels of *Mcpt5* and *Cpa3* (Fig. 2d). Cellular  
 213 histamine content was drastically increased in the presence of dexamethasone (> 8 fold at Day-16,  
 214 Fig. 3a), which is consistent with a previous study that exhibited the dexamethasone-mediated  
 215 induction of histidine decarboxylase (HDC), which is the rate-limiting enzyme for histamine  
 216 synthesis, in a mouse mastocytoma, P-815 [18]. We, indeed, confirmed that the enzymatic activity of  
 217 HDC was increased 5-fold in the cells cultured in the presence of dexamethasone (HDC activity at  
 218 Day-4 (nmol/min/mg protein), Control,  $0.42 \pm 0.138$ , +Dexamethasone,  $2.11^* \pm 0.424$ , \* $P < 0.05$ ,  $n = 3$ ).  
 219 Unexpectedly, enzymatic activity of  $\beta$ -hexosaminidase, a lysosomal enzyme, which might play  
 220 critical roles in bactericidal actions [19] and is often used for monitoring degranulation levels, was  
 221 significantly up-regulated in CTMC-like MCs obtained in the presence of dexamethasone (Fig. 3b).



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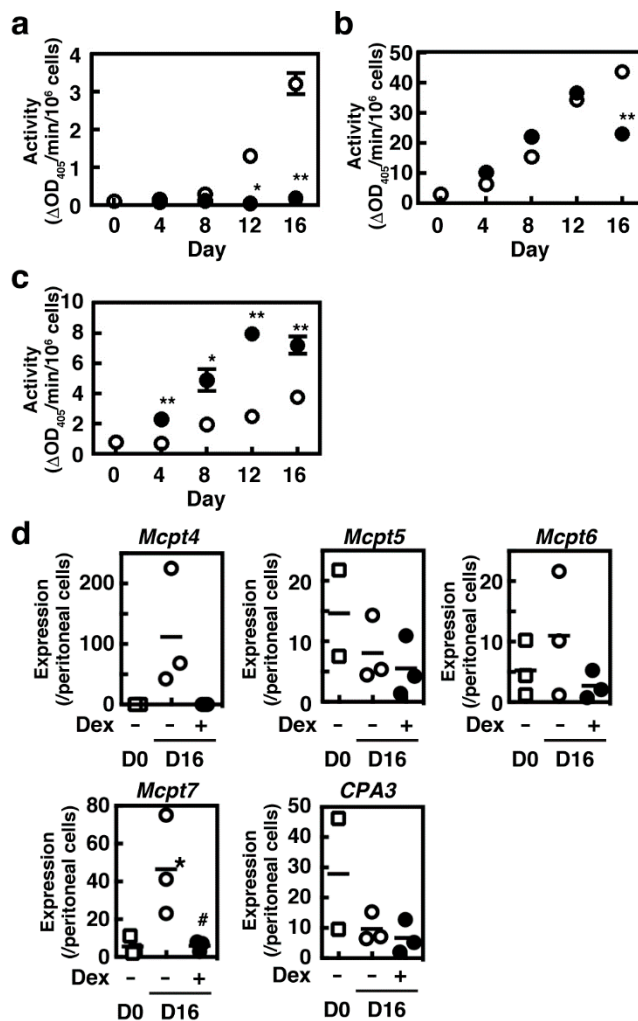
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**Figure 1.** BMMCs were co-cultured with Swiss 3T3 fibroblasts in the presence (closed circles) or absence (open circles) of  $1 \mu\text{M}$  dexamethasone for 16 days as described in Materials and Methods. (a) The numbers of the cultured mast cells were counted on Day-0, 4, 8, 12, and 16. Values were presented as the means  $\pm$  SEMs ( $n = 4$ ). (b) The ratios of the Safranin-positive cells were determined. Values were presented as the means  $\pm$  SEMs ( $n = 4$ ).



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**Figure 2.** BMMCs were co-cultured with Swiss 3T3 fibroblasts in the presence (closed circles or columns) or absence (open circles or columns) of 1  $\mu$ M dexamethasone for 16 days as described in Materials and Methods. (a-c) Enzymatic activities of three kinds of granule proteases (a; chymotrypsin activity, b; trypsin activity, and c; carboxypeptidase A activity) were measured. Values with \* $p$  < 0.05 and \*\* $p$  < 0.01 are regarded as significant. (d) Expression levels of granule protease genes (*Mcpt4*, 5, 6, 7, and *CPA3*) were determined by quantitative RT-PCR analyses. Values with \* $p$  < 0.05 (vs. D0) and # $p$  < 0.05 (vs. D16, (-)Dex) are regarded as significant.

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### 3.2. Suppression of $G_i$ -mediated degranulation in mast cells cultured in the presence of dexamethasone

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BMMCs co-cultured with Swiss 3T3 fibroblasts were found to undergo degranulation in response to basic secretagogues, such as compound 48/80 and substance P, which is one of the characteristics of CTMCs and is mediated by pertussis toxin-sensitive  $G_i$  proteins [5]. Degranulation induced by these secretagogues was abolished in the cells co-cultured in the presence of dexamethasone, whereas that upon IgE-mediated antigen stimulation remained unchanged (Fig. 3c and 3d). Suppressive effects of dexamethasone on the  $G_i$ -dependent degranulation were not observed when dexamethasone was added 24 hr before stimulation (Fig. 3f), indicating that dexamethasone-mediated suppression of  $G_i$ -dependent degranulation should require long-term characteristic changes.

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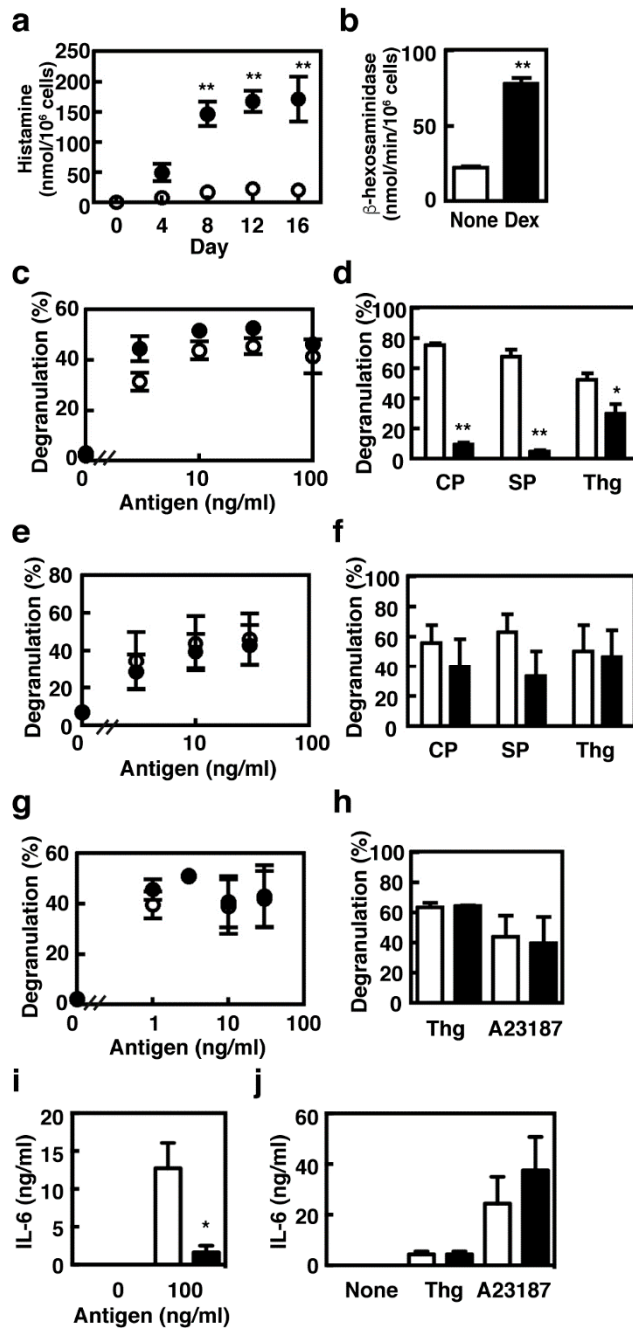
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Previous studies demonstrated that treatment with dexamethasone down-modulated the surface expression of Fc $\epsilon$ RI and thereby suppressed antigen-induced degranulation in mast cells [20,21]. However, no significant changes were observed in the levels of degranulation of activated BMMCs, which were treated with dexamethasone 24 hr before the stimulation (Fig. 3g and 3h).

250 Surface expression levels of FcεRI were comparable between the control and dexamethasone-  
 251 treated BMDCs (mean fluorescent intensity; Control,  $47.2 \pm 4.01$ , +Dexamethasone,  $41.8 \pm 0.768$ ,  
 252  $n=3$ ), whereas those of c-kit were significantly decreased in the dexamethasone-treated cells (mean  
 253 fluorescent intensity; Control,  $104 \pm 3.55$ , +Dexamethasone,  $81.5 \pm 0.379$ ,  $n=3$ ,  $*p < 0.05$ ). In contrast  
 254 to unchanged levels of degranulation, treatment with dexamethasone for 24 hr significantly  
 255 suppressed antigen-induced IL-6 production whereas  $Ca^{2+}$  influx-induced IL-6 production was  
 256 unchanged in the presence of dexamethasone (Fig. 3i and 3j).

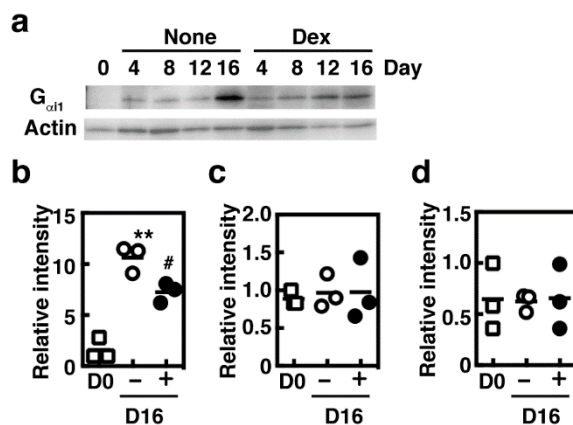


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258 **Figure 3.** (a, b) The cellular histamine contents and enzymatic activities of β-hexosaminidase in the  
 259 mast cells co-cultured for 16 days in the presence (closed circles) or absence (open circles) of 1 μM  
 260 dexamethasone were measured. (c-f) The co-cultured mast cells were sensitized with IgE (1 μg/ml,  
 261 clone IgE-3) for 3 hr and then stimulated with the indicated concentrations of the antigen, or  
 262 stimulated with compound 48/80 (CP, 10 μg/ml), substance P (SP, 100 μM), or thapsigargin (Thg, 300  
 263 nM) without sensitization. Degranulation upon IgE-mediated antigen stimulation (c) and treatment  
 264 with compound 48/80, substance P, or thapsigargin (d) was measured in the mast cells co-cultured

265 for 16 days in the presence (closed circles or columns) or absence (open circles or columns) of 1  $\mu$ M  
 266 dexamethasone. (e, f) BMMCs were co-cultured for 16 days and were treated with 1  $\mu$ M  
 267 dexamethasone during the last 24 hr (closed circles and columns). Degranulation was then measured  
 268 as described above. (g-j) BMMCs were treated without (open circles or columns) or with 1  $\mu$ M  
 269 dexamethasone (closed circles or columns) for 24 hr. The cells were then sensitized with 1  $\mu$ g/ml IgE  
 270 (clone IgE-3) for 3 hr and stimulated with the indicated concentrations of the antigen or stimulated  
 271 with thapsigargin (Thg, 300 nM) or A23187 (A23187, 1  $\mu$ M). Degranulation (g, h) and IL-6 release (i,  
 272 j) were measured. The degree of degranulation was determined by measuring  $\beta$ -hexosaminidase  
 273 activity. Values were presented as the means  $\pm$  SEMs (n=3). Values with \* $p$  < 0.05 and \*\* $p$  < 0.01 are  
 274 regarded as significant.

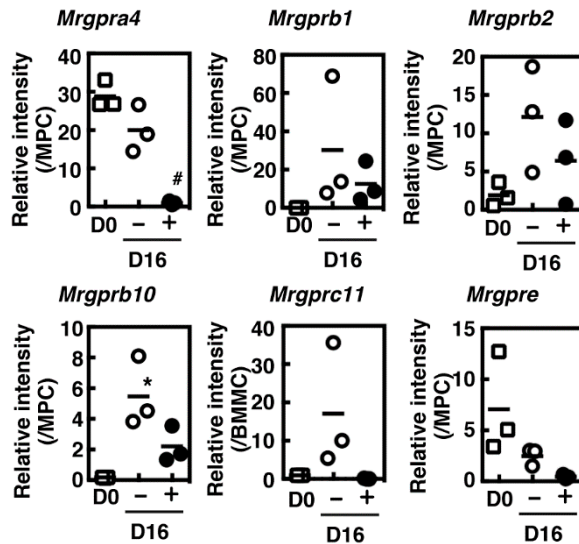
275 Because  $G_{\alpha i1}$  protein was found to be up-regulated during the co-culture period [4], we then  
 276 investigated the effects of dexamethasone on the expression of three subtypes of  $\alpha$  subunit of  
 277 trimeric  $G_i$ . Expression of  $G_{\alpha i1}$  protein was exclusively induced during the co-culture period as  
 278 previously reported and significantly suppressed by prolonged treatment with dexamethasone,  
 279 whereas no obvious changes in the other  $G_{\alpha i}$  protein expression were observed (Fig. 4a-4d).  
 280 Recently, the possible candidates for the receptors of mast cell secretagogues have been identified;  
 281 various secretagogues were found to act as the agonists of MRGPRX2 [6], and one of its murine  
 282 orthologues, MrgprB2, was identified as the primary receptor for various mast cell secretagogues  
 283 using the gene targeted mice [7]. We investigated the expression levels of 6 murine Mrgpr family  
 284 genes based on the results obtained through the next generation sequencing analysis. Messenger  
 285 RNA expression of *Mrgpra4*, *b1*, *b2*, *b10*, and *e* were detected in the cultured mast cells and murine  
 286 peritoneal cells. *Mrgprc11* was not detected in murine peritoneal cells, but expressed in the cultured  
 287 mast cells. *Mrgprb1*, *b2*, *b10*, and *c11* were found to be up-regulated during the co-culture period  
 288 whereas *Mrgpra4* and *e* were down-regulated (Fig. 5). Overall, the presence of dexamethasone  
 289 during the co-cultured period suppressed mRNA expression of these *Mrgpr* genes.



290

291 **Figure 4.** BMMCs were co-cultured with Swiss 3T3 fibroblasts in the presence (Dex) or absence (None)  
 292 of 1  $\mu$ M dexamethasone for 16 days as described in Materials and Methods. (a) Expression of  $G_{\alpha i1}$  was  
 293 visualized by immunoblot analyses using an anti- $G_{\alpha i1}$  antibody. Expression of actin was measured as  
 294 the loading control. (b-d) Expression levels of various  $G_{\alpha i}$  proteins (b;  $G_{\alpha i1}$ , c;  $G_{\alpha i2}$ , and d;  $G_{\alpha i3}$ ) in  
 295 BMMCs (D0), and the Day-16 co-cultured mast cells prepared in the presence (+) or absence (-) of 1  
 296  $\mu$ M dexamethasone were densitometrically determined. Values with \*\* $p$  < 0.01 (vs. D0) and # $p$  < 0.05  
 297 (vs. D16, (-)Dex) is regarded as significant.





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**Figure 5.** BMMCs were co-cultured with Swiss 3T3 fibroblasts in the presence (Dex) or absence (None) of 1  $\mu$ M dexamethasone for 16 days as described in Materials and Methods. Expression levels of *Mrgpr* family genes in BMMCs (D0), and the Day-16 co-cultured mast cells prepared in the presence (+) or absence (-) of 1  $\mu$ M dexamethasone were measured using quantitative RT-PCR. Relative expression levels were calculated based on the expression levels in mouse peritoneal cells (MPC) or those in BMMCs (BMMC). Values with \* $p < 0.05$  (vs. D0) and # $p < 0.05$  (vs. D16, (-)Dex) are regarded as significant.

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### 3.3. Effects of dexamethasone on gene expression profiles of cultured mast cells

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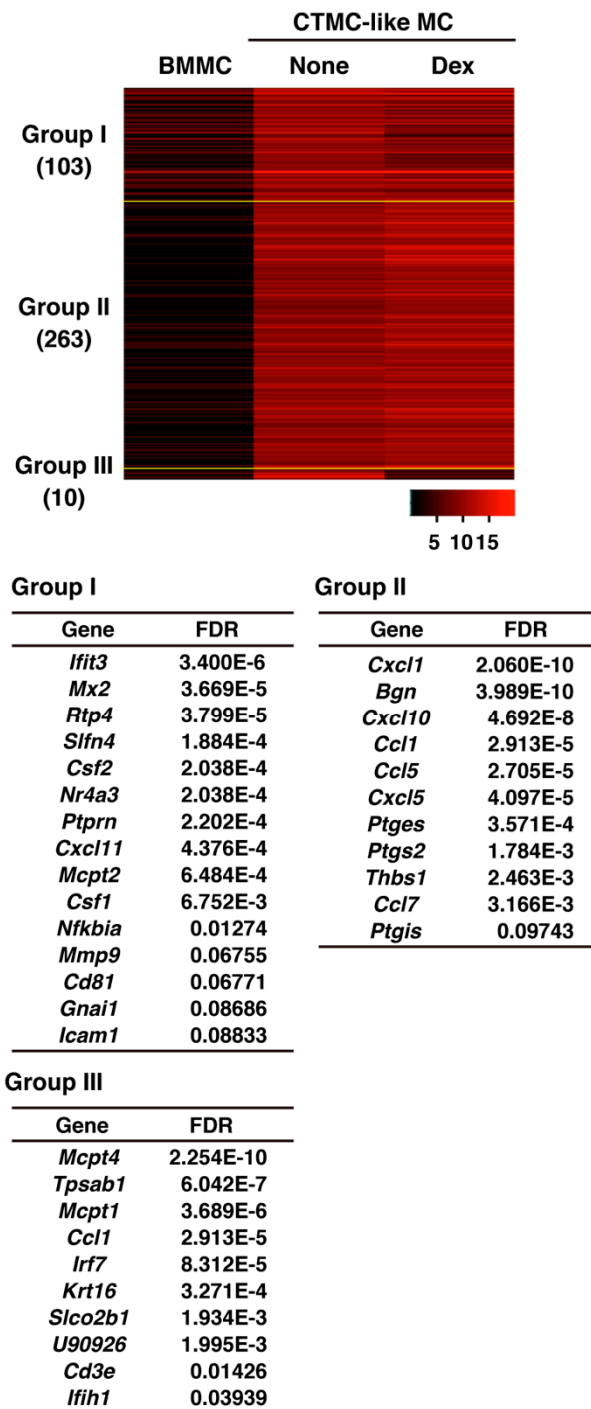
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We then investigated the gene expression profiles by the next generation sequencing analysis. 376 genes were extracted as the up-regulated genes (FDR < 0.1) in CTMC-like MCs in comparison with BMMCs. This population included the characteristic genes of CTMCs, which we previously identified by the microarray analyses [4], such as *Bgn*, *Cd81*, *Gnai1*, *Icam1*, *Mcpt4*, *Ptges*, *Ptgis*, *Ptgs2*, *Thbs1*, and *Tpsab1*. Among them, 113 genes were not induced in the cells cultured in the presence of dexamethasone (Fig. 6, Group I and III). These dexamethasone-sensitive groups included the genes, such as *Cd81*, *Gnai1*, *Icam1*, *Mcpt4*, *Mmp9*, *Nfkb1a*, and *Tpsab1*. The number of genes, which were up-regulated in CTMC-like MCs but were insensitive to dexamethasone, were 263 (Fig. 6, Group II). They included the genes involved in arachidonic acid metabolism, such as *Ptges*, *Ptgs2*, and *Ptgis*.



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**Figure 6.** Each RNA sample was collected from the cultured mast cells (BMMCs, CTMC-like MCs, and CTMC-like MCs prepared in the presence of Dex) and gene expression analyses were performed by the next generation sequencing. Differentially expressed genes between BMMCs and CTMC-like MCs were extracted with the false discovery rate (FDR) < 0.1. The heat map presents the expression profiles of the genes, of which expression were increased in CTMC-like MCs. The extracted genes are classified into three clusters based on the expression patterns.

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#### 3.4. Effects of dexamethasone on murine cutaneous vascular responses

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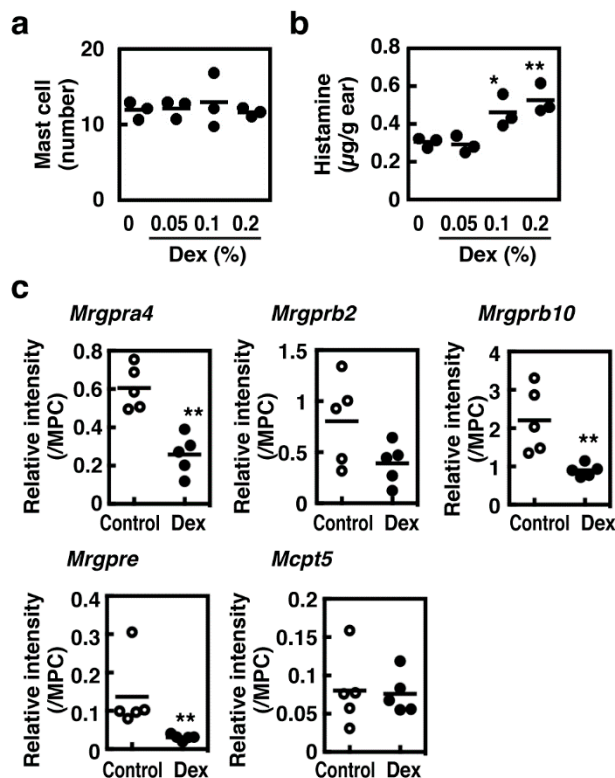
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We then investigated the effects of dexamethasone on cutaneous vascular responses. Six days of topical application of dexamethasone did not affect the number of cutaneous tissue mast cells, whereas cutaneous histamine content was significantly increased (Fig. 7a and 7b). Four *Mrgpr* genes, *Mrgpra4*, *b2*, *b10*, and *e*, were expressed in the ear tissues whereas expression of *Mrgprb1* and

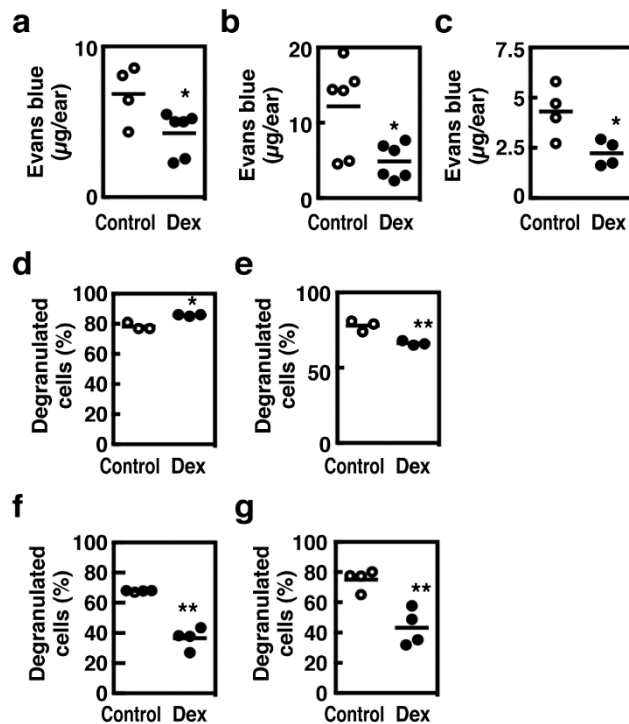
328 *c11* could not be detected by RT-PCR (Fig. 7c). Prolonged treatment with dexamethasone down-  
 329 regulated all *Mrgpr* genes expressed in the ear tissues.



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331 **Figure 7.** (a, b) The indicated concentrations of dexamethasone (20  $\mu\text{l/site}$ , dissolved in acetone) were  
 332 daily applied to the surface of the ear tissues for 6 days. The ear tissues were collected 24 hr after the  
 333 last administration and the numbers of cutaneous mast cells (a) and tissue histamine content (b) were  
 334 measured. Values with \* $p < 0.05$  and \*\* $p < 0.01$  are regarded as significant. (c) Expression levels of  
 335 *Mrgpr* family genes and *Mcpt5* in the ear tissues were measured using quantitative RT-PCR. Relative  
 336 expression levels were calculated based on the expression levels in mouse peritoneal cells (MPC).  
 337 Values with \*\* $p < 0.01$  and \* $p < 0.05$  are regarded as significant.

338 Extravasation responses evaluated by Evans blue dye leakage upon IgE-mediated antigen  
 339 stimulation or compound 48/80 were significantly attenuated in the mice daily treated with 0.05%  
 340 dexamethasone (Fig. 8a and 8b). We then investigated the sensitivity to histamine, which is the  
 341 major vasoactive mediator derived from mast cells, and found that histamine-induced  
 342 extravasation responses was also attenuated in the dexamethasone-treated mice (Fig. 8c). These  
 343 findings implied that decreased dye leakages observed in the dexamethasone-treated mice might  
 344 result from impaired vascular responses to histamine rather than impaired degranulation. Previous  
 345 studies demonstrated that glucocorticoids could augment the functions of tight junctions of  
 346 vascular endothelial cells [22,23]. We, therefore, assessed the frequencies of degranulation of tissue  
 347 mast cells by histological analyses with the acidic toluidine blue staining. In the mice daily treated  
 348 with 0.05% dexamethasone, the number of degranulated mast cells was slightly but significantly  
 349 increased upon IgE-mediated antigen stimulation, whereas that was significantly decreased upon  
 350 the compound 48/80 application (Fig. 8d and 8e). Suppression of compound 48/80-induced  
 351 degranulation was more pronounced in the mice daily treated with 0.2% dexamethasone, whereas  
 352 antigen-induced degranulation was also suppressed under this condition (Fig. 8f and 8g).



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**Figure 8.** (a-c) After the daily application of 0.05% dexamethasone to the ear tissues for 6 days, extravasation in the ear tissues was evaluated by Evans blue dye leakage 24 hr after the last application. Mice were subjected to IgE-dependent passive cutaneous anaphylaxis (PCA) reactions (a), and intracutaneous injections of compound 48/80 (b, 30 µg/site) or histamine (c, 30 µg/site). Values with \* $p < 0.05$  are regarded as significant. (d-g) Mice were daily treated without (Control) or with 0.05% (d and e) or 0.2% (f and g) of dexamethasone (Dex, 20 µl/site, dissolved in acetone) on the surface of ear tissues for 6 days. Mice were then subjected to IgE-dependent PCA reactions (d and f) or an intracutaneous injection of compound 48/80 (e and g, 30 µg/site). The ear tissues were collected 3 min after the stimulation and the degrees of degranulation of cutaneous mast cells were determined based on the acidic Toluidine blue staining. Values with \* $p < 0.05$  and \*\* $p < 0.01$  are regarded as significant.

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#### 4. Discussion

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We demonstrated here that prolonged treatment with a synthetic glucocorticoid, dexamethasone, could suppress secretagogue-induced degranulation of murine mast cells. Because glucocorticoid receptor is ubiquitously expressed, it remains unknown how glucocorticoid suppress cutaneous inflammation. Our findings strongly suggest that down-regulation of *Gnai1* and *Mrgpr* family in mast cells should be involved in impaired secretagogue-induced degranulation. A large part of secretagogue-induced degranulation was found to be sensitive to pertussis toxin [5]. Aridor et al. suggested that  $G_{i3}$  should be involved in secretagogue-induced degranulation of rat peritoneal mast cells [24]. It is likely that cooperation between several *Mrgpr* subtypes and  $G_{i1}$  should be responsible for secretagogue-induced degranulation of murine mast cells. This mechanism might account at least in part for the therapeutic effects of steroidal anti-inflammatory drugs on IgE-independent cutaneous inflammation including intrinsic atopic dermatitis and contact dermatitis.

The effects of glucocorticoids on degranulation induced by IgE-mediated antigen stimulation have been extensively investigated. A majority of studies demonstrated that dexamethasone could significantly suppress degranulation of mast cells upon antigen stimulation [20,21,25-27]. It remains to be determined how dexamethasone should act on the signaling pathways involved in degranulation. Some of these studies suggested that the surface expression of  $Fc\epsilon R1$  was decreased in the presence of dexamethasone. We observed here that prolonged treatment with dexamethasone did not affect antigen-induced degranulation of cultured mast cells but significantly suppressed that of cutaneous mast cells. We could not reproduce the previously reported findings that 24-hours of

385 treatment with dexamethasone suppressed antigen-induced degranulation of BMMCs [20,21].  
386 Antigen-induced IL-6 release was abolished by 24-hours of treatment with dexamethasone, excluding  
387 the possibility that BMMCs should be insensitive to glucocorticoids in our system. We have no good  
388 explanation for this discrepancy, although the methods for preparation of BMMCs are slightly  
389 different from our model.

390 It remains controversial how glucocorticoid affect the number of mast cells. Eklund et al.  
391 reported that dexamethasone suppressed proliferation of murine BMMCs induced by SCF or IL-3,  
392 which is consistent with our findings [28]. Methylprednisolone could deplete intestinal mast cells but  
393 did not affect the number of connective tissue-type mast cells in rats [29]. In cutaneous tissues, the  
394 effects of glucocorticoids on fibroblasts might be involved in regulation of the mast cell number.  
395 Chronic topical treatments with fluocinonide decreased the number of cutaneous mast cells through  
396 down-regulation of SCF in the fibroblasts, which induced apoptosis of the mast cells [30]. It is likely  
397 that the expression levels of SCF in Swiss 3T3 were down-regulated in the presence of dexamethasone  
398 in our system. However, the presence of a large amount of soluble exogenous SCF may prevent  
399 apoptic cell death of CTMC-like MCs. Clinical studies demonstrated that prolonged treatment with  
400 clobetasol-17-propionate and fluocinonide could down-modulate the number of cutaneous mast cells  
401 [31,32]. Because no changes in the number of cutaneous mast cells were observed in our system,  
402 dexamethasone might not affect the viability of them in the range of concentrations used here. We  
403 used Swiss 3T3 fibroblasts as the feeder cells to prepare CTMC-like MCs, raising the possibility that  
404 dexamethasone should affect the phenotype of mast cells indirectly through the effects on Swiss 3T3  
405 cells. It is quite difficult to distinguish the direct effects from those through the fibroblasts. Kusunose  
406 et al. reported that treatment of murine fibroblasts with mitomycin c should attenuate the nuclear  
407 translocation of GR [33]. Because Swiss 3T3 cells were pretreated with mitomycin c before the co-  
408 culture in our system, the effects of dexamethasone on them may be relatively small.

409 We observed here that granule enzymes, such as carboxypeptidase A and  $\beta$ -hexosaminidase,  
410 and histamine contents were significantly up-regulated in the cultured mast cells generated in the  
411 presence of dexamethasone. Increase in histamine content was also observed in the cutaneous tissues  
412 of mice treated with dexamethasone. Accumulating evidence suggests that glucocorticoids could not  
413 only suppress inflammatory responses but also enhance the innate immune responses [34,35].  
414 Carboxypeptidase A and a granule proteoglycan core, serglycin, were found to be up-regulated in  
415 BMMCs treated with dexamethasone [28]. Mast cells were found to contribute to wound healing  
416 through release of their mediators including histamine [36]. Dexamethasone-induced up-regulation  
417 of the granule mediators may be associated with its therapeutic effects.

418 We found that a variety of murine Mrgpr family was expressed in cultured mast cells in addition  
419 to *Mrgprb2* and that their expression levels were dynamically changed. Although McNeil et al.  
420 highlighted the critical roles of MrgprB2 using the gene targeted mice [7], it is plausible that the other  
421 Mrgpr family should be involved in IgE-independent degranulation of mature mast cells in  
422 cooperation with MrgprB2. Mrgpr family may respond to various secretagogues through its  
423 heterodimerization. We also detected mRNA expression of Mrgpr family in murine cutaneous tissues,  
424 the levels of which were decreased in the presence of dexamethasone. Because previous studies  
425 indicated that Mrgpr family was expressed exclusively in the sensory nerve, of which cell body is  
426 localized in the dorsal root ganglion, except that mast cells expressed a part of it, mRNA expression  
427 of Mrgpr subtypes in the cutaneous tissues might indicate that cutaneous mast cells should express  
428 them. Characterization of these Mrgpr subtypes in addition to MrgprB2 is necessary for  
429 understanding the mechanism of IgE-independent inflammatory responses.

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432 N.F.; Writing-Original Draft Preparation, H.S., K.S. and S.T.; Writing-Review & Editing, K.F. and S.T.;  
433 Supervision, S.T.; Project Administration, S.T.; Funding Acquisition, S.T.

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