

# An antimicrobial peptide with angiogenic properties, AG-30/5C, activates human mast cells through the MAPK and NF- $\kappa$ B pathways

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Published online: 10 December 2015

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**Abstract** Apart from their direct antimicrobial activities against invading pathogens, antimicrobial peptides exhibit additional protective functions that have led to their being named host defense peptides (HDPs). These functions include the stimulation of the production of cytokines/chemokines, the promotion of chemotaxis and cell proliferation and the induction of angiogenesis and wound healing. AG-30/5C is a novel angiogenic HDP that in addition to its antimicrobial activity also activates fibroblasts and endothelial cells and promotes angiogenesis and wound healing. Given that mast cells are found primarily in the vicinity of vessels, where they are intimately involved in wound healing, we hypothesized that AG-30/5C may activate mast cells. We demonstrated that AG-30/5C activated LAD2 human mast cells to degranulate and produce lipid mediators including leukotriene C<sub>4</sub>, prostaglandin D<sub>2</sub> and E<sub>2</sub>. Moreover, AG-30/5C increased mast cell chemotaxis and induced the production of the cytokines GM-CSF and TNF- $\alpha$  and various chemokines, such as IL-8, MCP-1, MCP-3, MIP-1 $\alpha$  and MIP-1 $\beta$ . The chemotaxis and cytokine/chemokine production induced by AG-30/5C were suppressed by both pertussis toxin and U-73122, suggesting the involvement of the G protein and phospholipase C pathways in AG-30/5C-induced mast cell activation. Furthermore, these pathways were activated downstream of the MAPK and NF- $\kappa$ B signaling molecules, as demonstrated by the inhibitory effects

of ERK-, JNK-, p38- and NF- $\kappa$ B-specific inhibitors on cytokine/chemokine production. Interestingly, AG-30/5C caused the phosphorylation of MAPKs and I $\kappa$ B. We suggest that the angiogenic and antimicrobial peptide AG-30/5C plays a key role in the recruitment and activation of human mast cells at inflammation and wound sites.

**Keywords** Antimicrobial peptide · Inflammation · Mast cell · MAPK/NF- $\kappa$ B · Wound healing

## Abbreviations

AG-30/5C	Angiogenic peptide-30/5C
EIA	Enzyme immunoassay
ERK	Extracellular signal-regulated kinase
GM-CSF	Granulocyte-macrophage colony-stimulating factor
hBD	Human $\beta$ -defensin
HDP	Host defense peptide
IL	Interleukin
JNK	c-Jun N-terminal kinase
LT	Leukotriene
MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemoattractant protein
MIP	Macrophage inflammatory protein
NF- $\kappa$ B	Nuclear factor- $\kappa$ B
PG	Prostaglandin
PLC	Phospholipase C
TNF	Tumor necrosis factor

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## Introduction

A large body of evidence now shows that antimicrobial peptides (AMPs) are important as a first line of defense in vertebrates against a broad spectrum of bacteria, viruses

and fungi [1]. Defensins and cathelicidins are the largest groups of AMPs that have been most extensively studied. In addition to exhibiting direct and/or indirect antimicrobial activity, AMPs, such as human  $\beta$ -defensins (hBDs) and cathelicidin LL-37, display a wide variety of protective and immunomodulatory functions that have led to AMPs being called host defense peptides (HDPs). Among their immunomodulatory functions, HDPs modulate inflammatory responses; promote chemotaxis, cell proliferation and differentiation; stimulate the production of many chemokines and certain cytokines; suppress harmful pro-inflammatory responses; and accelerate angiogenesis and wound healing [2–6].

Recently, the Kaneda laboratory developed a small angiogenic peptide (AG)-30 that exhibits both antimicrobial and wound healing activities [7]. AG-30 was screened from a human library of angiogenic factors, and it contains an amphipathic  $\alpha$ -helix structure with hydrophobic and cationic residues [7]. In addition to its antimicrobial activity against various bacteria, AG-30 induces cell growth in endothelial cells, stimulates these cells to generate angiogenesis-related cytokines and augments neovascularization [7]. However, taking into account the fact that the AG-30 molecule is unstable and easily degraded by proteases, the authors developed a modified version of the AG-30 peptide by replacing several of its amino acids with cationic amino acids, resulting in a new molecule named AG-30/5C [8]. Compared to the original AG-30 peptide, AG-30/5C shows enhanced antimicrobial activity and an enhanced ability to induce endothelial cell migration, angiogenesis and wound healing [8].

Although mast cells are known primarily for initiating allergic reactions, they also play a crucial role in host defenses against invading pathogens. Mast cells are professional immune cells that are strategically located at sites exposed to the external milieu, such as the skin, gut and airways [9, 10]. They are therefore among the first cells to encounter pathogens [9, 11]. Mast cells promote innate immune responses by releasing a panel of preformed inflammatory mediators, such as histamine, de novo-produced eicosanoids, cytokines, chemokines and growth factors [9]. Furthermore, mast cells are closely associated with vascular and lymphatic vessels, and they stimulate re-epithelialization, angiogenesis and tissue remodeling to accelerate wound healing [12].

Given that both mast cells and AG-30/5C are intimately involved in angiogenesis and wound healing [8], the aim of this study was to examine whether AG-30/5C can activate mast cells. We observed that AG-30/5C caused human mast cell degranulation and the production of eicosanoids and various cytokines and chemokines. In addition, AG-30/5C induced mast cell chemotaxis. AG-30/5C-induced mast cell activation involved the G protein, phospholipase C

(PLC), mitogen-activated protein kinase (MAPK) and nuclear factor- $\kappa$ B (NF- $\kappa$ B) pathways. These findings suggest novel role for AG-30/5C in the regulation of innate immunity whereby it recruits and activates human mast cells at inflammation and wound sites.

## Materials and methods

### Reagents

AG-30/5C was purchased from the Peptide Institute (Osaka, Japan). Antibodies against phosphorylated ERK, p38, JNK and I $\kappa$ B as well as unphosphorylated ERK, p38, JNK and I $\kappa$ B were obtained from Cell Signaling Technology (Beverly, MA). The G protein inhibitor pertussis toxin, the phospholipase C (PLC) inhibitor U-73122, the ERK inhibitor U0126, the p38 inhibitor SB203580, the JNK inhibitor II and the NF- $\kappa$ B activation inhibitor II were purchased from Calbiochem (La Jolla, CA). Enzyme immunoassay (EIA) kits for detecting leukotriene (LT) C<sub>4</sub> and prostaglandin (PG) D<sub>2</sub> and PGE<sub>2</sub> were obtained from Cayman Chemical Company (Ann Arbor, MI), whereas the cytokine and chemokine ELISA kits were obtained from R&D Systems (Minneapolis, MN).

### Mast cell culture

The human mast cell line LAD2 was a generous gift from Dr. A. Kirshenbaum at the National Institutes of Health (Bethesda, MD). Cells were maintained in serum-free Stem Pro-34 media (Invitrogen, Carlsbad, CA) containing nutrient supplements, 2 mM L-glutamine, 100 IU/ml penicillin, 100  $\mu$ g/ml streptomycin and 100 ng/ml recombinant human stem cell factor (Wako, Osaka, Japan), as previously reported [13]. The culture medium was hemi-depleted every week with fresh medium, and cells were maintained at  $1 \times 10^5$  cells/ml. The cells were periodically tested for expression of c-Kit and Fc $\epsilon$ RI (data not shown).

### $\beta$ -Hexosaminidase release assay

Mast cells were washed in Tyrode's buffer (10 mM HEPES [pH 7.4], 130 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.1 % BSA, 1 mM CaCl<sub>2</sub> and 0.6 mM MgCl<sub>2</sub>), re-suspended in Tyrode's buffer at  $2 \times 10^5$  cells/100  $\mu$ l and then stimulated with various doses of AG-30/5C for 40 min at 37 °C. To measure  $\beta$ -hexosaminidase activity, the culture supernatants were incubated with 1.3 mg/ml 4-nitrophenyl-*N*-acetyl- $\beta$ -D-glucopyranoside (Sigma-Aldrich, St. Louis, MO) for 90 min at 37 °C. The reaction was developed with 0.2 M glycine, and absorbance was measured at 405 nm.  $\beta$ -Hexosaminidase release was calculated as a

percentage of the total  $\beta$ -hexosaminidase content, which was determined after lysing the cells with 1 % Triton X-100. In some experiments, mast cells were pre-treated with various inhibitors for 2 h before stimulation with AG-30/5C.

### EIA and ELISA

Mast cells ( $1 \times 10^6$  cells) were incubated with AG-30/5C for 30 min (for EIA) or 3 h (for ELISA) at 37 °C. After stimulation, the cell cultures were centrifuged, and the cell-free supernatants were used for LTC<sub>4</sub>, PGD<sub>2</sub> and PGE<sub>2</sub> quantification using an EIA, while the concentrations of granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-8, monocyte chemoattractant protein (MCP)-1, MCP-3, macrophage-inflammatory protein (MIP)-1 $\alpha$ , MIP-1 $\beta$  and tumor necrosis factor (TNF)- $\alpha$  were determined using ELISA kits according to the manufacturer's instructions. In some experiments, cells were treated with various inhibitors for 2 h before stimulation with AG-30/5C, and the assays were then performed as above.

### Chemotaxis assay

Mast cells at  $1.5 \times 10^5$  cells/50  $\mu$ l were added to the upper wells of a 48-well chemotaxis microchamber (modified Boyden chamber, Neuroprobe, Cabin John, MD). The upper wells were separated from the lower wells, which contained various doses of AG-30/5C, by an 8- $\mu$ m pore-size polyvinylpyrrolidone-free polycarbonate membrane (Neuro Probe). Following 3-h incubation, the mast cells that had migrated and adhered to the underside of the membrane were fixed and stained using DiffQuick (Kokusai Shiyaku, Kobe, Japan). After the membranes were mounted onto slides, the migrated cells were counted under a light microscope in three randomly chosen high-power fields per membrane. In some experiments, various inhibitors were added 2 h prior to the assay, and chemotaxis was then evaluated as described above.

### Western blot analysis

Mast cells ( $1 \times 10^6$  cells) were stimulated with AG-30/5C for the indicated periods. After stimulation, the cells were lysed in RIPA buffer (Cell Signaling Technology), and equal amounts of lysate proteins were subjected to 12.5 % SDS-PAGE. After non-specific binding sites were blocked, the immunoblots were incubated with polyclonal antibodies against phosphorylated or unphosphorylated ERK, JNK, p38 and I $\kappa$ B overnight according to the manufacturer's instructions. The membranes were developed using Luminata Forte Western HRP substrate (Millipore, Billerica, MA) and imaged using Fujifilm LAS-4000 Plus.

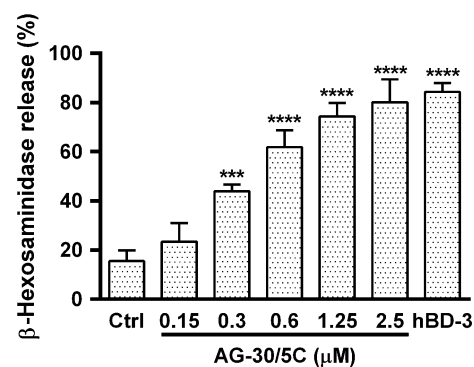
### Statistical analysis

The statistical analysis consisted of either ANOVA followed by the appropriate post hoc test or a Student's *t* test using Prism GraphPad for Windows (Prism 5, GraphPad Software, San Diego, CA).  $P < 0.05$  was considered significant. The results are presented as the mean values  $\pm$  SD.

## Results

### AG-30/5C induces human mast cell degranulation

Given that both mast cells and AG-30/5C play important roles in the wound healing process, and because HDPs such as defensins and cathelicidins, which accelerate wound healing, have been reported to activate mast cells [14–16], we hypothesized that AG-30/5C may also stimulate mast cells. We first investigated whether AG-30/5C induces mast cell degranulation. As pictured in Fig. 1, AG-30/5C potently induced mast cell degranulation, as assessed by analysis of  $\beta$ -hexosaminidase release from LAD2 human mast cells. This release was dose-dependent, and a statistically significant release of 44 % was observed at AG-30/5C concentrations as low as 0.3  $\mu$ M. hBD-3 (at a concentration of 2.5  $\mu$ M), which is known to cause mast cell degranulation [16], was used as a positive control, and it induced nearly identical degranulating potency to that of AG-30/5C at 2.5  $\mu$ M. We confirmed that the AG-30/5C doses used in this study were not cytotoxic by evaluating trypan blue dye exclusion and lactate dehydrogenase activity (data not shown).



**Fig. 1** AG-30/5C induces human mast cell degranulation. Mast cells ( $2 \times 10^5$  cells) were incubated with 0.15–2.5  $\mu$ M AG-30/5C, 2.5  $\mu$ M hBD-3 or diluent (0.01 % acetic acid; Ctrl, control). After 40 min of incubation at 37 °C,  $\beta$ -hexosaminidase release was measured in the supernatants, as described in the “Materials and methods” section. Values are shown as the mean  $\pm$  SD of four separate experiments and were compared between stimulated and non-stimulated cells (Ctrl, control). \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$

### Activation of mast cells by AG-30/5C results in the production of lipid mediators

Following activation, mast cells release preformed granules. They also de novo produce various lipid mediators, such as PGs and LTs [9, 10]. Because AG-30/5C caused mast cell degranulation, we tested whether it also induced the production of lipid mediators. AG-30/5C noticeably enhanced the production of LTC<sub>4</sub>, PGD<sub>2</sub> and PGE<sub>2</sub> in a dose-dependent manner (Fig. 2). AG-30/5C (10 μM) and hBD-3 (5 μM), which was used as a positive control, induced similar amounts of PGD<sub>2</sub> and PGE<sub>2</sub>, whereas the potency of AG-30/5C to stimulate LTC<sub>4</sub> production was 1.5-fold lower than that of hBD-3.

### AG-30/5C stimulation induces chemotaxis in human mast cells

A number of mast cell activators, including HDPs, have been shown to not only induce degranulation and the release of lipid mediators but also to cause mast cell migration [17]. Therefore, we examined whether AG-30/5C activation would induce a mast cell chemotactic response. Chemotaxis assays performed using a modified Boyden chamber demonstrated that AG-30/5C caused the directional migration of mast cells, resulting in a typical bell-shaped dose-dependent curve. The optimal concentration of AG-30/5C for maximal migration was observed to be 1.25 μM, which yielded to fivefold increase in migration compared to migration observed in the control cells (Fig. 3).

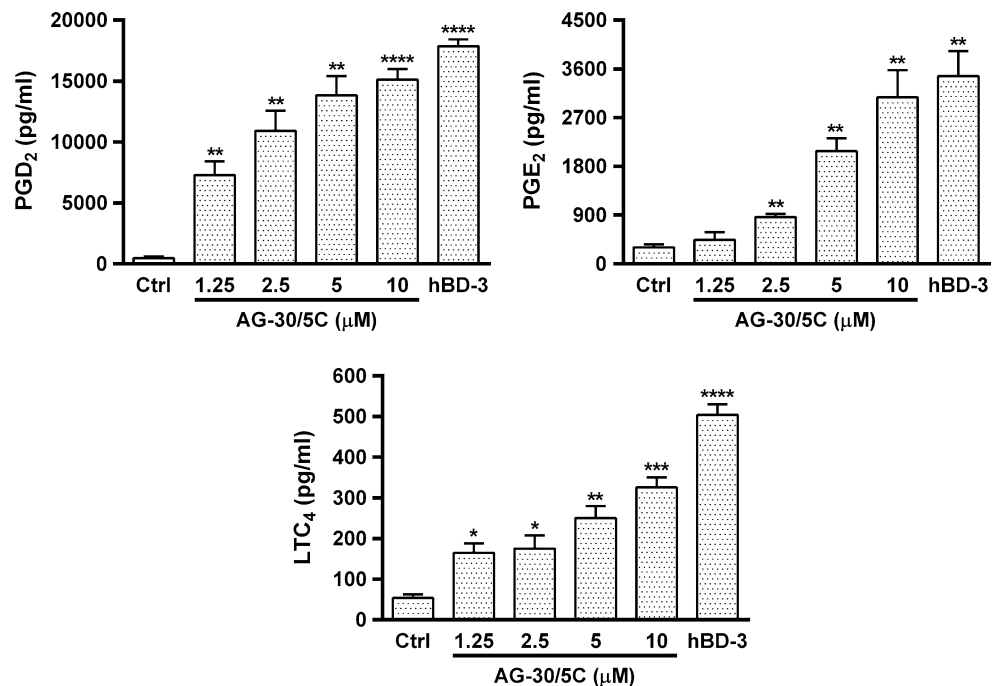
### AG-30/5C stimulates the production of cytokines and chemokines by mast cells

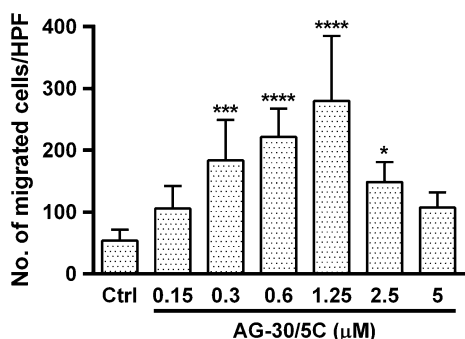
During inflammatory responses, mast cell activation also results in the coordinated production of various cytokines and chemokines. Therefore, we performed ELISA to evaluate whether AG-30/5C can stimulate mast cells to generate cytokines and chemokines. Among the cytokines and chemokines tested, we observed that AG-30/5C selectively elicited the production of the cytokines GM-CSF and TNF-α and chemokines including IL-8, MCP-1, MCP-3, MIP-1α and MIP-1β (Fig. 4). This effect was dose-dependent. In the preliminary time-course experiments, the production of cytokines and chemokines was highest after 3 h of stimulation with AG-30/5C.

### AG-30/5C-induced mast cell activation is pertussis toxin- and U-73122-sensitive

To characterize the signal transduction pathways involved in AG-30/5C-mediated mast cell activation, cells were pre-treated with a G protein inhibitor, pertussis toxin, or a PLC inhibitor, U-73122. We found that treating the mast cells with pertussis toxin or U-73122 before stimulation with AG-30/5C strongly suppressed AG-30/5C-induced mast cell degranulation (Fig. 5a), chemotaxis (Fig. 5b) and the production of GM-CSF, TNF-α, IL-8, MCP-1, MCP-3, MIP-1α and MIP-1β (Fig. 5c). Furthermore, we also confirmed that both pertussis toxin and U-73122 also abolished the AG-30/5C-induced production of LTC<sub>4</sub>, PGD<sub>2</sub> and

**Fig. 2** AG-30/5C increases the secretion of PGD<sub>2</sub>, PGE<sub>2</sub> and LTC<sub>4</sub> by human mast cells. Mast cells ( $1 \times 10^6$  cells) were stimulated for 30 min at 37 °C with 1.25–10 μM AG-30/5C, 5 μM hBD-3 or diluent (0.01 % acetic acid; Ctrl, control). The amount of PGD<sub>2</sub>, PGE<sub>2</sub> and LTC<sub>4</sub> released into the supernatants was quantified using an enzyme immunoassay. Values are shown as the mean  $\pm$  SD of three to five separate experiments and were compared between stimulated and non-stimulated cells (Ctrl, control). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$



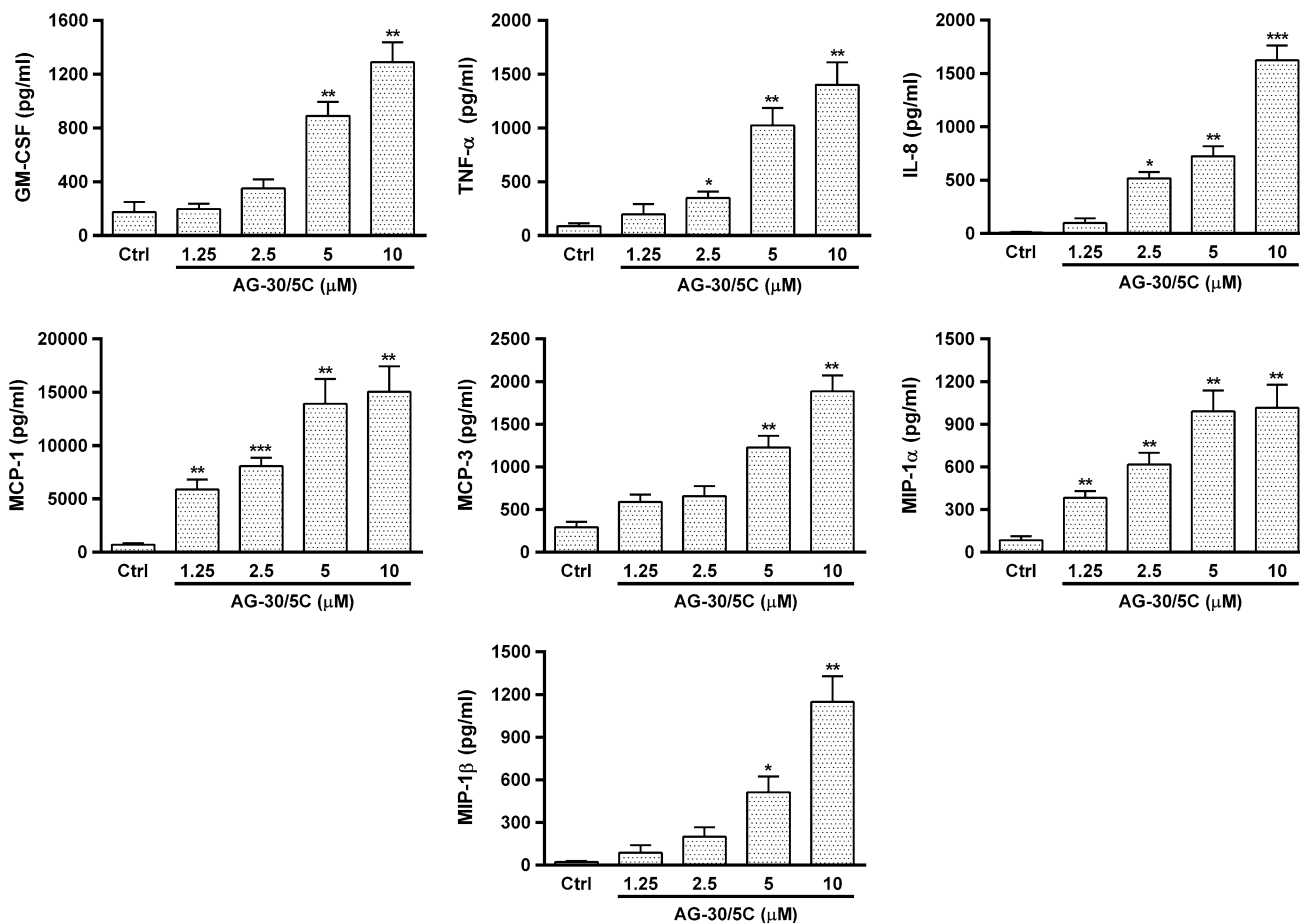


**Fig. 3** AG-30/5C mediates mast cell chemotaxis. Mast cells ( $1.5 \times 10^5$  cells) were placed in the upper wells of a chemotaxis microchamber and allowed to migrate toward 0.15–5  $\mu$ M AG-30/5C or diluent (0.01 % acetic acid; Ctrl, control) for 3 h at 37 °C. Chemotaxis was assessed by counting the number of cells that migrated through the polycarbonate membrane under a light microscope in three randomly chosen high-power fields (HPF). Values are shown as the mean  $\pm$  SD of five separate experiments and were compared between stimulated and non-stimulated cells (Ctrl, control). \* $P < 0.05$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$

PGE<sub>2</sub> (data not shown). Together, these observations suggest that AG-30/5C-mediated mast cell activation involves the G protein and PLC pathways.

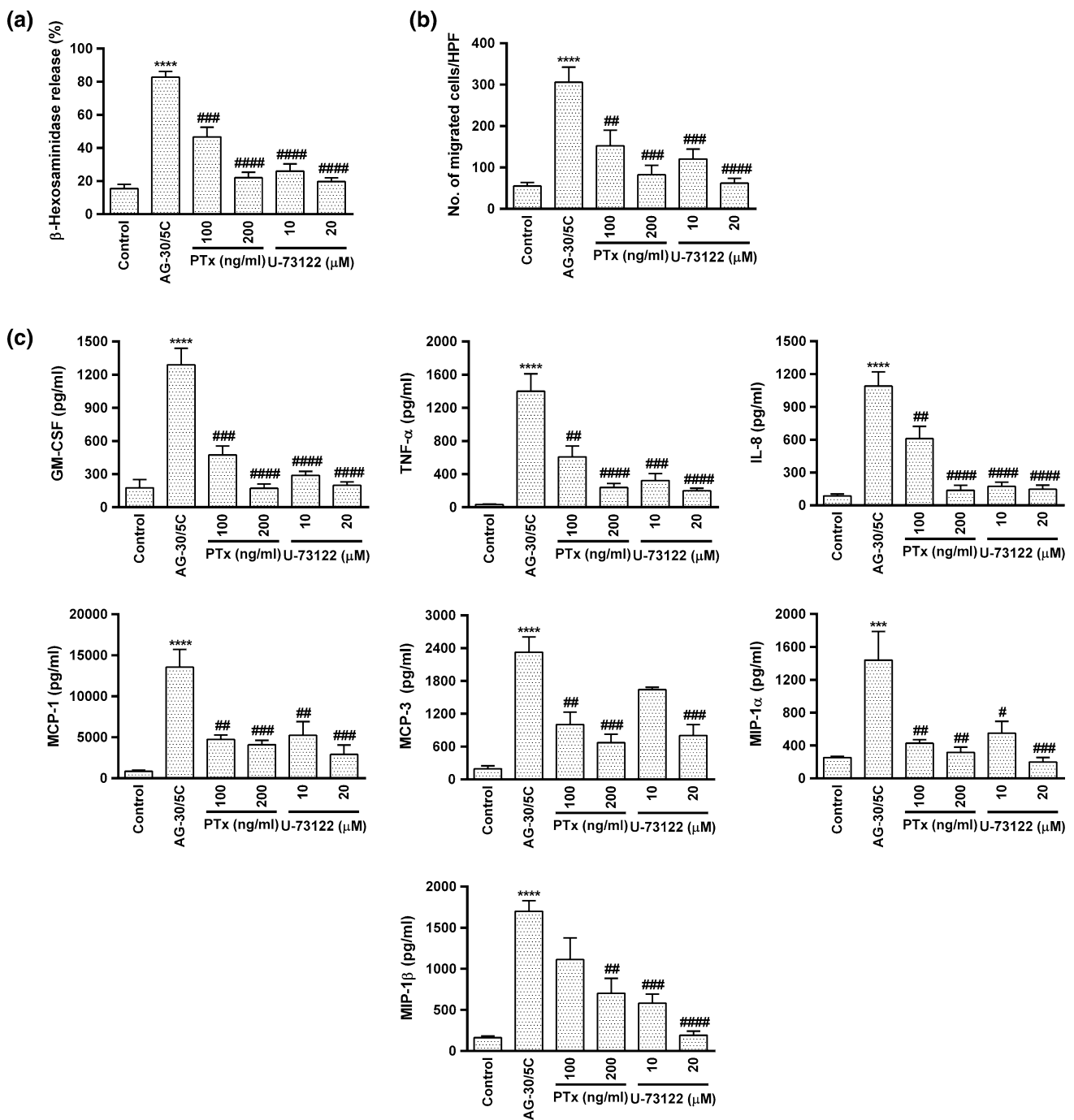
**Activation of the MAPK and NF- $\kappa$ B pathways is necessary for AG-30/5C-mediated mast cell stimulation**

The activation of mast cells involves several signaling pathways, including the activation of the MAPK and NF- $\kappa$ B pathways, which lead to the production of cytokines and chemokines [18]. To further explore AG-30/5C signaling, we examined whether this peptide could activate MAPKs and NF- $\kappa$ B because these molecules have been shown to be involved in the HDP-mediated activation of mast cells [16, 17]. As shown in Fig. 6a, AG-30/5C markedly induced the phosphorylation of the MAPKs ERK, JNK, p38 and I $\kappa$ B. Activation of ERK, JNK and I $\kappa$ B



**Fig. 4** AG-30/5C induces the production of cytokines and chemokines in mast cells. Mast cells ( $1 \times 10^6$  cells) were incubated with 1.25–10  $\mu$ M AG-30/5C or diluent (Ctrl, control) for 3 h. Following incubation, the concentrations of GM-CSF, TNF- $\alpha$ , IL-8, MCP-1, MCP-3, MIP-1 $\alpha$  and MIP-1 $\beta$  released into the culture supernatants

were determined using ELISA. The values are shown as the mean  $\pm$  SD of four to six separate experiments and were compared between the stimulated and non-stimulated cells (Ctrl, control). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$



**Fig. 5** Effects of pertussis toxin and U-73122 on AG-30/5C-induced mast cell activation. Cells were pre-treated with 100–200 ng/ml pertussis toxin (PTx), 10–20  $\mu$ M U-73122 or 0.1 % DMSO for 2 h. **a** Pre-treated cells ( $2 \times 10^5$ ) were stimulated with 2.5  $\mu$ M AG-30/5C or diluent (0.01 % acetic acid; Ctrl, control) for 40 min, and  $\beta$ -hexosaminidase release was then measured. **b** Pre-treated cells were incubated with 1.25  $\mu$ M AG-30/5C or diluent (0.01 % acetic acid; Ctrl, control) for 3 h, and a chemotaxis assay was then performed. **c** Pre-treated cells were also evaluated for cytokine and chemokine

production following 3 h of stimulation with 10  $\mu$ M AG-30/5C or diluent (0.01 % acetic acid; Ctrl, control), and the levels of GM-CSF, TNF- $\alpha$ , IL-8, MCP-1, MCP-3, MIP-1 $\alpha$  and MIP-1 $\beta$  released into the supernatants were determined using ELISA. Values are shown as the mean  $\pm$  SD of four to six separate experiments. \*\*\*\* $P$  < 0.001 and \*\*\*\* $P$  < 0.0001 for comparisons between untreated cells (Ctrl, control) and stimulated groups without inhibitor (AG-30/5C). # $P$  < 0.05, ## $P$  < 0.01, ### $P$  < 0.001 and #### $P$  < 0.0001 for comparisons between the presence and absence of inhibitors

was observed as early as 5 min after stimulation, while activation of p38 was detected at 15 min post-stimulation. Furthermore, we confirmed that activation of the MAPK

and NF- $\kappa$ B pathways was necessary for AG-30/5C-mediated cytokine and chemokine production by pre-treating mast cells with specific inhibitors of MAPKs and NF- $\kappa$ B.



U0126 (an ERK inhibitor), a JNK inhibitor, SB203580 (a p38 inhibitor) and an inhibitor of NF- $\kappa$ B activation all markedly suppressed the AG-30/5C-induced production of IL-8, MCP-1, MCP-3, MIP-1 $\alpha$  and MIP-1 $\beta$ . We observed similar inhibitory effects when we used MAPK and NF- $\kappa$ B inhibitors on the AG-30/5C-induced production of GM-CSF and TNF- $\alpha$  (data not shown).

## Discussion

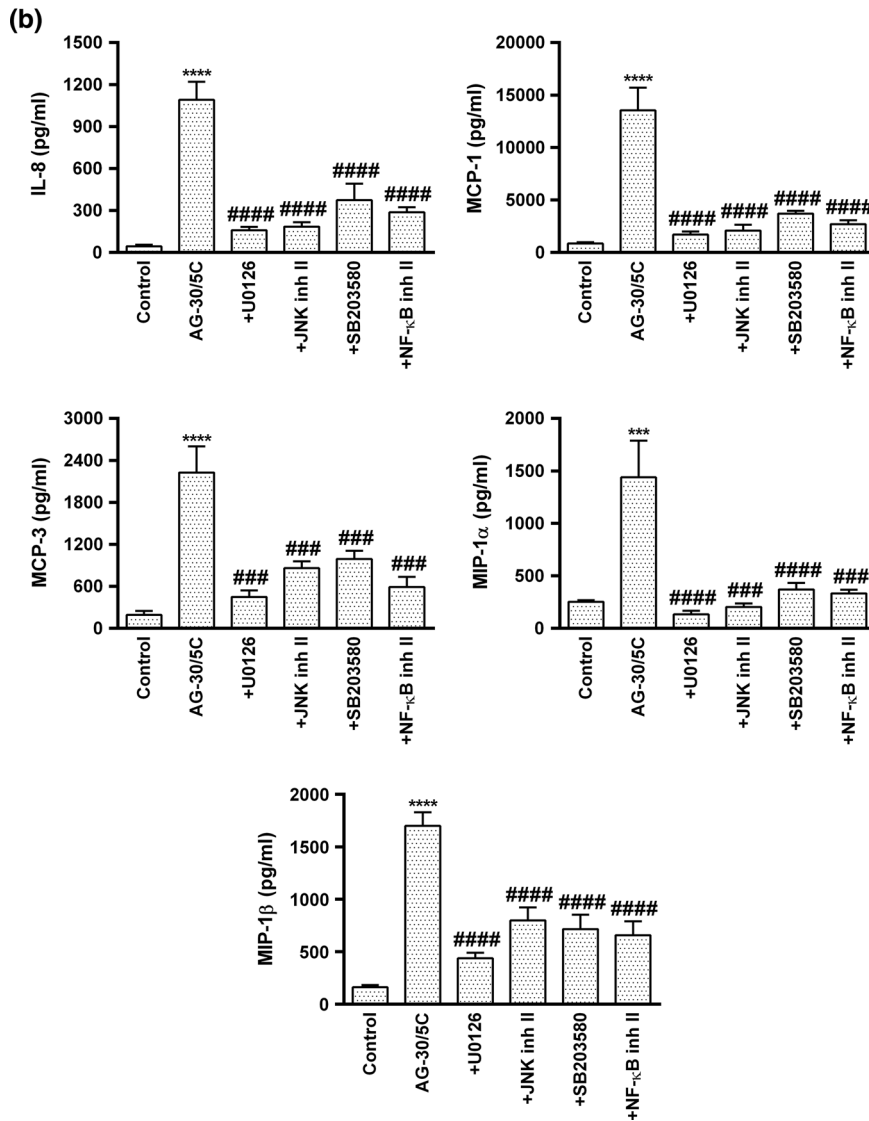
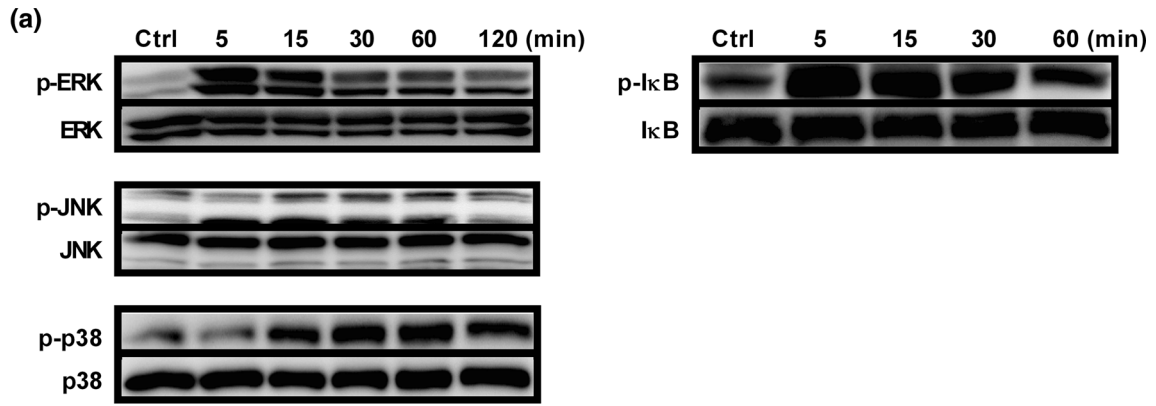
Given that mast cells are intimately involved in angiogenesis and wound healing and that an angiogenic antimicrobial peptide, AG-30/5C, increases angiogenesis and accelerates wound healing [8], we hypothesized that this peptide could activate mast cells. Here, we show that AG-30/5C stimulates mast cells to migrate, degranulate and release de novo-synthesized lipid mediators, such as LTs and PGs, and various cytokines and chemokines. Studies of the molecular mechanisms involved in these processes suggest that AG-30/5C-mediated mast cell activation occurs through the G protein, PLC, MAPK and NF- $\kappa$ B pathways. These observations imply that AG-30/5C may recruit and activate human mast cells at inflammation and wound sites.

When tissue is injured, mast cell numbers significantly increase during the inflammation phase of wounding healing, and these cells can become activated [12]. Upon activation, mast cells release a wide variety of preformed and newly generated inflammatory mediators, including LTs and PGs, which not only promote inflammation and vasodilation but also accelerate tissue repair [19, 20]. The release of these inflammatory mediators can also contribute to the formation of scars [21, 22]. Furthermore, mast cells produce several growth factors, such as vascular endothelial growth factor, fibroblast growth factor and transforming growth factor- $\beta$ , which have the potential to contribute to wound healing through effects on blood vessels [23]. We found that AG-30/5C induced mast cell migration and caused degranulation and the secretion of eicosanoids. Furthermore, in unpublished data, we also confirmed that AG-30/5C increased the production of vascular endothelial growth factor and fibroblast growth factor. Because AG-30/5C is known to promote wound healing [8], our data suggest that this HDP may accelerate the wound healing process by activating mast cells. Studies on the direct role that AG-30/5C-mediated mast cell activation plays in wound healing are therefore necessary.

Following activation, mast cells also produce numerous inflammatory cytokines and chemokines. Among the various cytokines and chemokines we tested, AG-30/5C selectively induced the production of the cytokines GM-CSF and TNF- $\alpha$  and chemokines, such as IL-8, MCP-1,

MCP-3, MIP-1 $\alpha$  and MIP-1 $\beta$ , which are implicated in many inflammatory processes, including those associated with wound healing. Indeed, in addition to their roles in the regulation of inflammation, both GM-CSF and TNF- $\alpha$  have been proposed to contribute to neovascularization, tissue remodeling or re-epithelialization, which are involved in wound healing [24, 25]. As for the chemokines IL-8, MCP-1, MCP-3, MIP-1 $\alpha$  and MIP-1 $\beta$ , they are known to participate in the regulation of epithelialization, tissue remodeling and angiogenesis, and to contribute to wound healing through their tightly regulated ability to recruit neutrophils, macrophages, lymphocytes and mast cells to inflammatory and wound sites (reviewed in [26]). For instance, IL-8 plays a crucial role in re-epithelialization and tissue remodeling by increasing cell migration and proliferation and inducing metalloproteinase expression in leukocytes [27]. MCP-1 specifically accelerates re-epithelialization and angiogenesis, and a lack of MCP-1 in vivo has been shown to delay wound healing [23]. As for MCP-3, it induces the migration of circulating angiogenic cells and stimulates angiogenesis [28]. Furthermore, similar to IL-8 and MCP-1, MIP-1 $\alpha$  and MIP-1 $\beta$  induce the expression of metalloproteinases to regulate tissue remodeling [29]. Taken together, the current study suggests that the angiogenic and antimicrobial peptide AG-30/5C may contribute to wound healing by stimulating mast cells to release histamine, eicosanoids, cytokines and chemokines.

To increase our understanding of the mechanisms underlying AG-30/5C-mediated mast cell activation, the involvement of the G protein and PLC pathways was evaluated because numerous mast cell stimulators, including HDPs, activate mast cells through the G protein and PLC pathways [14, 16]. Both pertussis toxin and U-73122, specific inhibitors of the G protein and PLC pathways, respectively, suppressed AG-30/5C-mediated mast cell activation, indicating that AG-30/5C exerts its stimulatory effects on human mast cells via the G protein and PLC pathways. It is currently unclear whether a receptor for AG-30/5C exists; therefore, studies on AG-30/5C-mediated receptor pathway are necessary. However, similar to other mast cell secretagogues, it is also possible that AG-30/5C may act through non-selective membrane receptors, or it may directly bind to and activate G proteins coupled to PLC [30]. We showed that AG-30/5C-mediated downstream signaling involved the MAPK and NF- $\kappa$ B pathways by demonstrating that AG-30/5C induced the activation of ERK, JNK, p38 and NF- $\kappa$ B and that the specific inhibition of these molecules abolished AG-30/5C-mediated mast cell stimulation. Activation of the MAPK and NF- $\kappa$ B pathways in human mast cells has been reported to result in the production of various cytokines and chemokines, including those found to be induced by AG-30/5C in the current study [18, 31]. Importantly, hBDs





**Fig. 6** AG-30/5C stimulates mast cell chemokine production via the MAPK and NF- $\kappa$ B pathways. **a** AG-30/5C induces the phosphorylation of MAPKs and I $\kappa$ B. Mast cells were stimulated with 5  $\mu$ M of AG-30/5C or diluent (Ctrl, control) for 5–120 min and then lysed. Equal amounts of protein were subjected to 12.5 % SDS-PAGE using antibodies directed against phosphorylated or unphosphorylated ERK (p-ERK and ERK), JNK (p-JNK and JNK), p38 (p-p38 and p38) and I $\kappa$ B (p-I $\kappa$ B and I $\kappa$ B). The results show one representative experiment from four independent experiments that yielded similar results. **b** Mast cells were pre-treated with 10  $\mu$ M U0126, JNK inhibitor II (JNK inh II), SB203580, NF- $\kappa$ B activation inhibitor II (NF- $\kappa$ B inh II) or 0.1 % DMSO for 2 h. The cells were then exposed to 10  $\mu$ M AG-30/5C or diluent (Ctrl, control) for 3 h, and the levels of chemokines released into the supernatants were determined using ELISA. Values are shown as the mean  $\pm$  SD of five to seven separate experiments. \*\*\* $P$  < 0.001 and \*\*\*\* $P$  < 0.0001 for comparisons between untreated cells (Ctrl, control) and stimulated groups without inhibitor (AG-30/5C). #### $P$  < 0.001 and ##### $P$  < 0.0001 for comparisons between the presence and absence of inhibitors

and LL-37 have also been reported to induce the production of cytokines and chemokines by activating the MAPK and NF- $\kappa$ B pathways [13, 32–34], suggesting similarities between HDPs in their immunomodulatory functions.

In summary, the current study demonstrates that AG-30/5C causes human mast cell migration, degranulation and the release of eicosanoids and cytokines/chemokines. Therefore, in addition to its antimicrobial activity, we propose that the novel angiogenic antimicrobial peptide AG-30/5C may contribute to the acceleration of the wound healing process through at least during the recruitment and activation of mast cells.

**Acknowledgments** We would like to express our deepest gratitude to all members of the Atopy (Allergy) Research Center, Juntendo University Graduate School of Medicine for their comments and Michio Matsumoto for secretarial assistance. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (Grant Number: 26461703 to F.N.) and by the Atopy (Allergy) Research Center, Juntendo University, Tokyo, Japan.

#### Compliance with ethical standards

**Conflicts of interest** The authors declare that they have no conflict of interest.

## References

- Hancock RE, Chapple DS. Peptide antibiotics. *Antimicrob Agents Chemother*. 1999;43(6):1317–23.
- Niyonsaba F, Nagaoka I, Ogawa H. Human defensins and cathelicidins in the skin: beyond direct antimicrobial properties. *Crit Rev Immunol*. 2006;26(6):545–76.
- Niyonsaba F, Nagaoka I, Ogawa H, Okumura K. Multifunctional antimicrobial proteins and peptides: natural activators of immune systems. *Curr Pharm Des*. 2009;15(21):2393–413.
- Oppenheim JJ, Yang D. Alarmins: chemotactic activators of immune responses. *Curr Opin Immunol*. 2005;17(4):359–65.
- Scott MG, Dullaghan E, Mookherjee N, Glavas N, Waldbrook M, Thompson A, et al. An anti-infective peptide that selectively modulates the innate immune response. *Nat Biotechnol*. 2007;25(4):465–72.
- Diamond G, Beckloff N, Weinberg A, Kisich KO. The roles of antimicrobial peptides in innate host defense. *Curr Pharm Des*. 2009;15(21):2377–92.
- Nishikawa T, Nakagami H, Maeda A, Morishita R, Miyazaki N, Ogawa T, et al. Development of a novel antimicrobial peptide, AG-30, with angiogenic properties. *J Cell Mol Med*. 2009;13(3):535–46.
- Nakagami H, Nishikawa T, Tamura N, Maeda A, Hibino H, Mochizuki M, et al. Modification of a novel angiogenic peptide, AG30, for the development of novel therapeutic agents. *J Cell Mol Med*. 2012;16(7):1629–39.
- Abraham SN, St John AL. Mast cell-orchestrated immunity to pathogens. *Nat Rev Immunol*. 2010;10(6):440–52.
- Galli SJ, Kalesnikoff J, Grimbaldston MA, Piliponsky AM, Williams CM, Tsai M. Mast cells as “tunable” effector and immunoregulatory cells: recent advances. *Annu Rev Immunol*. 2005;23:749–86.
- Kunder CA, St John AL, Abraham SN. Mast cell modulation of the vascular and lymphatic endothelium. *Blood*. 2011;118(20):5383–93.
- Wulff BC, Wilgus TA. Mast cell activity in the healing wound: more than meets the eye? *Exp Dermatol*. 2013;22(8):507–10.
- Niyonsaba F, Ushio H, Hara M, Yokoi H, Tominaga M, Takamori K, et al. Antimicrobial peptides human beta-defensins and cathelicidin LL-37 induce the secretion of a pruritogenic cytokine IL-31 by human mast cells. *J Immunol*. 2010;184(7):3526–34.
- Niyonsaba F, Someya A, Hirata M, Ogawa H, Nagaoka I. Evaluation of the effects of peptide antibiotics human beta-defensin-1/2 and LL-37 on histamine release and prostaglandin D(2) production from mast cells. *Eur J Immunol*. 2001;31(4):1066–75.
- Niyonsaba F, Iwabuchi K, Someya A, Hirata M, Matsuda H, Ogawa H, et al. A cathelicidin family of human antibacterial peptide LL-37 induces mast cell chemotaxis. *Immunology*. 2002;106(1):20–6.
- Chen X, Niyonsaba F, Ushio H, Hara M, Yokoi H, Matsumoto K, et al. Antimicrobial peptides human beta-defensin (hBD)-3 and hBD-4 activate mast cells and increase skin vascular permeability. *Eur J Immunol*. 2007;37(2):434–44.
- Pundir P, Kulka M. The role of G protein-coupled receptors in mast cell activation by antimicrobial peptides: is there a connection? *Immunol Cell Biol*. 2010;88(6):632–40.
- Wong CK, Tsang CM, Ip WK, Lam CW. Molecular mechanisms for the release of chemokines from human leukemic mast cell line (HMC)-1 cells activated by SCF and TNF-alpha: roles of ERK, p38 MAPK, and NF- $\kappa$ B. *Allergy*. 2006;61(3):289–97.
- Noli C, Miolo A. The mast cell in wound healing. *Vet Dermatol*. 2001;12(6):303–13.
- Coneely J, Kennelly R, Bouchier-Hayes D, Winter DC. Mast cell degranulation is essential for anastomotic healing in well perfused and poorly perfused rat colon. *J Surg Res*. 2010;164(1):e73–6.
- van der Veer WM, Bloemen MC, Ulrich MM, Molema G, van Zuijlen PP, Middelkoop E, et al. Potential cellular and molecular causes of hypertrophic scar formation. *Burns*. 2009;35(1):15–29.
- Wulff BC, Parent AE, Meleski MA, DiPietro LA, Schrementi ME, Wilgus TA. Mast cells contribute to scar formation during fetal wound healing. *J Invest Dermatol*. 2012;132(2):458–65.
- Barrientos S, Stojadinovic O, Golinko MS, Brem H, Tomic-Canic M. Growth factors and cytokines in wound healing. *Wound Repair Regen*. 2008;16(5):585–601.
- Williams CM, Galli SJ. Mast cells can amplify airway reactivity and features of chronic inflammation in an asthma model in mice. *J Exp Med*. 2000;192(3):455–62.
- Mann A, Niekisch K, Schirmacher P, Blessing M. Granulocyte-macrophage colony-stimulating factor is essential for normal

- wound healing. *J Investig Dermatol Symp Proc.* 2006;11(1): 87–92.
26. Gillitzer R, Goebeler M. Chemokines in cutaneous wound healing. *J Leukoc Biol.* 2001;69(4):513–21.
  27. Engelhardt E, Toksoy A, Goebeler M, Debus S, Brocker EB, Gillitzer R. Chemokines IL-8, GROalpha, MCP-1, IP-10, and Mig are sequentially and differentially expressed during phase-specific infiltration of leukocyte subsets in human wound healing. *Am J Pathol.* 1998;153(6):1849–60.
  28. Bousquenaud M, Schwartz C, Leonard F, Rolland-Turner M, Wagner D, Devaux Y. Monocyte chemotactic protein 3 is a homing factor for circulating angiogenic cells. *Cardiovasc Res.* 2012;94(3):519–25.
  29. Johnatty RN, Taub DD, Reeder SP, Turcovski-Corrales SM, Cottam DW, Stephenson TJ, et al. Cytokine and chemokine regulation of proMMP-9 and TIMP-1 production by human peripheral blood lymphocytes. *J Immunol.* 1997;158(5):2327–33.
  30. Ferry X, Brehin S, Kamel R, Landry Y. G protein-dependent activation of mast cell by peptides and basic secretagogues. *Peptides.* 2002;23(8):1507–15.
  31. Shakoory B, Fitzgerald SM, Lee SA, Chi DS, Krishnaswamy G. The role of human mast cell-derived cytokines in eosinophil biology. *J Interferon Cytokine Res.* 2004;24(5):271–81.
  32. Niyonsaba F, Ushio H, Nagaoka I, Okumura K, Ogawa H. The human beta-defensins (-1, -2, -3, -4) and cathelicidin LL-37 induce IL-18 secretion through p38 and ERK MAPK activation in primary human keratinocytes. *J Immunol.* 2005;175(3):1776–84.
  33. Smithrithee R, Niyonsaba F, Kiatsurayanon C, Ushio H, Ikeda S, Okumura K, et al. Human beta-defensin-3 increases the expression of interleukin-37 through CCR6 in human keratinocytes. *J Dermatol Sci.* 2015;77(1):46–53.
  34. Babolewska E, Brzezinska-Blaszczyk E. Human-derived cathelicidin LL-37 directly activates mast cells to proinflammatory mediator synthesis and migratory response. *Cell Immunol.* 2015; 293(2):67–73.