Pleurocidin, a novel antimicrobial peptide, induces human mast cell activation through the FPRL1 receptor

P Pundir[1,2], A Catalli[1], C Leggiadro[3], SE Douglas[3] and M Kulka[1,4]

Pleurocidins are a novel family of α-helical cationic antimicrobial peptides (CAPs) that are structurally and functionally similar to cathelicidins, one of the major CAP families. As cathelicidins stimulate mast cell chemotaxis and mediator release, we postulated that pleurocidins similarly activate mast cells. A screen of 20 pleurocidin peptides revealed that some were capable of degranulating the human mast cell line LAD2 (Laboratory of Allergic Diseases 2). Pleurocidin NRC-04 caused LAD2 to adhere, migrate, degranulate, and release cysteinyl leukotrienes and prostaglandin D_2. Moreover, pleurocidin increased intracellular Ca^{2+} mobilization in mast cells and induced the production of proinflammatory chemokines such as monocyte chemotactic protein-1/C-C motif chemokine ligand 2 (CCL2) and macrophage inflammatory protein-1β/CCL4. Our evaluation of possible cellular mechanisms suggested that G proteins, phosphoinositol-3 kinase (PI3K), phospholipase C (PLC), and phosphokinase C (PKC) were involved in pleurocidin-induced mast cell activation as evidenced by the inhibitory effects of pertussis toxin (G protein inhibitor), wortmanin (PI3K inhibitor), U-73122 (PLC inhibitor), and Ro-31-8220 (PKC inhibitor), respectively. We also found that human mast cells expressed the N-formyl-peptide receptor 1 (FPRL1) receptor and FPRL1-specific inhibitor affected pleurocidin-mediated activation of mast cell. Our finding that the novel CAP pleurocidin activated human mast cell through G protein–coupled receptor signaling suggests that this peptide might have immunomodulatory functions.

INTRODUCTION

The innate immune system is the first line of defense against invading pathogens and is dependent upon invariant receptors that recognizes pathogen-associated molecular patterns. Mast cells are immune cells resident in tissues throughout the body but most common at sites exposed to the external environment, i.e., skin, mucosa of airways, and intestine.[1,2] Considered key players in asthma and allergy pathology, recent studies have highlighted their importance in the protection against infection.[3] They promote innate immune responses by rapidly and selectively producing proinflammatory mediators, which are divided into three major categories: (i) preformed mediators: i.e., histamine, heparin, proteoglycans, and antimicrobial peptides; (ii) *de novo* generated lipid mediators: i.e., prostaglandins, leukotrienes, and platelet-activating factor; and (iii) cytokines, chemokines, and growth factors.[1] Stimulation with G protein–coupled receptor (GPCR) ligands can dramatically potentiate mast cell mediator release against pathogenic organisms, thus helping to limit the pathology associated with infection.[4]

Given the need for alternate sources of multifunctional antibiotics and vaccine adjuvants, a number of novel GPCR ligands have recently been investigated for their potential to activate mast cells. Cationic antimicrobial peptides (CAPs), including defensins and cathelicidins, are compounds mainly expressed in epithelial cells and cause functional changes in mast cells. Defensins and cathelicidins induce mast cell chemotaxis, degranulation, and chemokine production[5–7] in a pertussis toxin–sensitive manner which indicates that they may use GPCRs to exert their effects.[6,8] Mast cells are not only a target but are also a source of cathelicidins;[9] therefore, the expression and release of CAPs and their subsequent action is likely an important autocrine mechanism in the innate immune functions of mast cells.

[1]National Research Council, Charlottetown, Prince Edward Island, Canada. [2]Department of Biomedical Sciences, Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, Prince Edward Island, Canada. [3]National Research Council, Halifax, Nova Scotia, Canada and [4]National Research Council, Edmonton, Alberta, Canada. Correspondence: M Kulka (Marianna.Kulka@nrc-cnrc.gc.ca)

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In 2003, 20 pleurocidin-like CAPs (NRC-01 to -20) were identified in various Atlantic flounder species and screened for antimicrobial activity.[10] Structurally similar to cathelicidins, pleurocidins are mucus-derived CAPs constituting a large family of linear, positively charged, α-helical cationic peptides found in flatfish.[11] Recent findings provide evidence that these proteins are important as a first line of defense in vertebrates. As with cathelicidins, pleurocidins are generated in epithelial surfaces in response to bacterial infection and exert a potent, broad-spectrum antibacterial and antifungal activity.[12] Furthermore, pleurocidin has been localized to circulating cells resembling mast cells in winter flounder, where it resides in cytoplasmic granules.[13] Related CAPs in the piscidin family also have been detected in fish mast cells.[14] Fish mast cells (also known as eosinophilic granule cells) are similar to their mammalian counterparts in that they are filled with metachromatic granules containing a diverse array of proinflammatory mediators that are released during degranulation.[15] As pleurocidin occur in mast cells of winter flounder, we were interested to see whether it can initiate innate defenses in human mast cells.

Given the structural and functional similarities between pleurocidins and cathelicidin LL-37, we postulated that the pleurocidins family of CAPs also activates diverse functions of human mast cells in a G protein–dependent manner. The aims of our study were to determine: (i) the ability of different pleurocidin variants in stimulating mast cell degranulation, (ii) whether pleurocidins stimulate mast cell eicosanoids, cytokine, and chemokine production, (iii) whether pleurocidins influence mast cell adhesion and migration, and (iv) whether pleurocidin-mediated mast cell activation is GPCR dependent.

Table 1 Amino-acid sequences and lengths of the 20 pleurocidin peptides tested

Code	Amino-acid sequence	No. of residues
NRC-01	GKGRWLERIGKAGGIIIGGALDHL-NH_2	24
NRC-02	WLRRIGKGVKIIGGAALDHL-NH_2	20
NRC-03	GRRKRKWLRRIGKGVKIIGGAALDHL-NH_2	26
NRC-04	GWGSFFKKAAHVGKHVGKAALTHYL-NH_2	25
NRC-05	FLGALIKGAIHGGRFIHGMIQNHH-NH_2	24
NRC-06	GWGSIFKHGRHAAKHIGHAAVNHYL-NH_2	25
NRC-07	RWGKWFKKATHVGKHVGKAALTAYL-NH_2	25
NRC-08	RSTEDIIKSISGGGFLNAMNA-NH_2	21
NRC-09	FFRLLFHGVHHGGGYLNAA-NH_2	19
NRC-10	FFRLLFHGVHHVGKIKPRA-NH_2	19
NRC-11	GWKSVFRKAKKVGKTVGGLALDHYL-NH_2	25
NRC-12	GWKKWFNRAKKVGKTVGGLAVDHYL-NH_2	25
NRC-13	GWRTLLKKAEVKTVGKLALKHYL-NH_2	23
NRC-14	AGWGSIFKHIFKAGKFIHGAIQAHND-NH_2	26
NRC-15	GFWGKLFKLGLHGIGLLHLHL-NH_2	21
NRC-16	GWKKWLRKGAKHLGQAAIK-NH_2	19
NRC-17	GWKKWLRKGAKHLGQAAIKGLAS-NH_2	23
NRC-18	GWKKWFTKGERLSQRHFA-NH_2	18
NRC-19	FLGLLFHGVHHVGKWIHGLIHGHH-NH_2	24
NRC-20	GFLGILFHGVHHGRKKALHMNSERRS-NH_2	26

RESULTS

Effect of pleurocidin peptides on mast cell degranulation

Twenty different pleurocidin peptides (NRC-01 to NRC-20) were screened for their ability to induce human mast cell degranulation using a degranulation assay. The peptides differ in their total length and amino-acid sequence (**Table 1**). Following stimulation of LAD2 (Laboratory of Allergic Diseases 2) cells with various concentrations of each of the pleurocidin peptides, we observed that not all were capable of degranulating LAD2 cells, as assessed by the release of β-hexosaminidase enzyme, a marker of histamine release. Only 11 of the tested peptides NRC-02, -03, -04, -07, -10, -12, -13, -16, -17, -18, and -20 induced significant degranulation at a concentration of 1 μM (**Figure 1a**).

To determine whether these peptides had any cytotoxic effects on mast cells, we incubated cells with 1 μM of each peptide and measured the metabolic activity of cells using the resazurin assay. There was no change in cell metabolic activity following 30 min, 3, and 24 h stimulation (data not shown).

Mast cell degranulation in response to pleurocidin NRC-04 is concentration and time dependent

Out of the 11 pleurocidin peptides that activated mast cell degranulation, NRC-04 stimulated the highest amount of degranulation and it was chosen for further studies. NRC-04 degranulated LAD2 cells in a concentration-dependent manner, with maximum degranulation (58.36 ± 3.23%) at 1 μM (**Figure 1b**). NRC-04 also notably caused degranulation of human peripheral blood $CD34^+$ cell–derived mast cells (HuMC; **Figure 1b**); however, these cells had a weaker response (21.89 ± 1.1%) to pleurocidin when compared with LAD2 cells. We further characterized the kinetics of NRC-04–mediated LAD2 degranulation and found that degranulation reached a maximum (71.07 ± 3.03%) within 5 min of stimulation (**Figure 1c**). Since degranulation reached its maximum by 5 min, we evaluated the effect of NRC-04 at shorter time periods. NRC-04 induced gradual degranulation in LAD2 cells from 1 to 5 min (**Figure 1d**).

To study the morphological changes associated with pleurocidin activation, we used electron microscopy to analyze intracellular changes in granularity and tomography. As shown in **Figure 1e**, stimulation with NRC-04 caused marked changes in the surface configuration of LAD2 cells, with loss in membrane integrity and the appearance of pores leading to the interior of the cell.

Pleurocidin NRC-04 induces intracellular calcium mobilization

As increase in intracellular Ca^{2+} is indispensable for the induction of mast cell degranulation, we next tested whether NRC-04 had the capacity to mobilize intracellular Ca^{2+}. LAD2

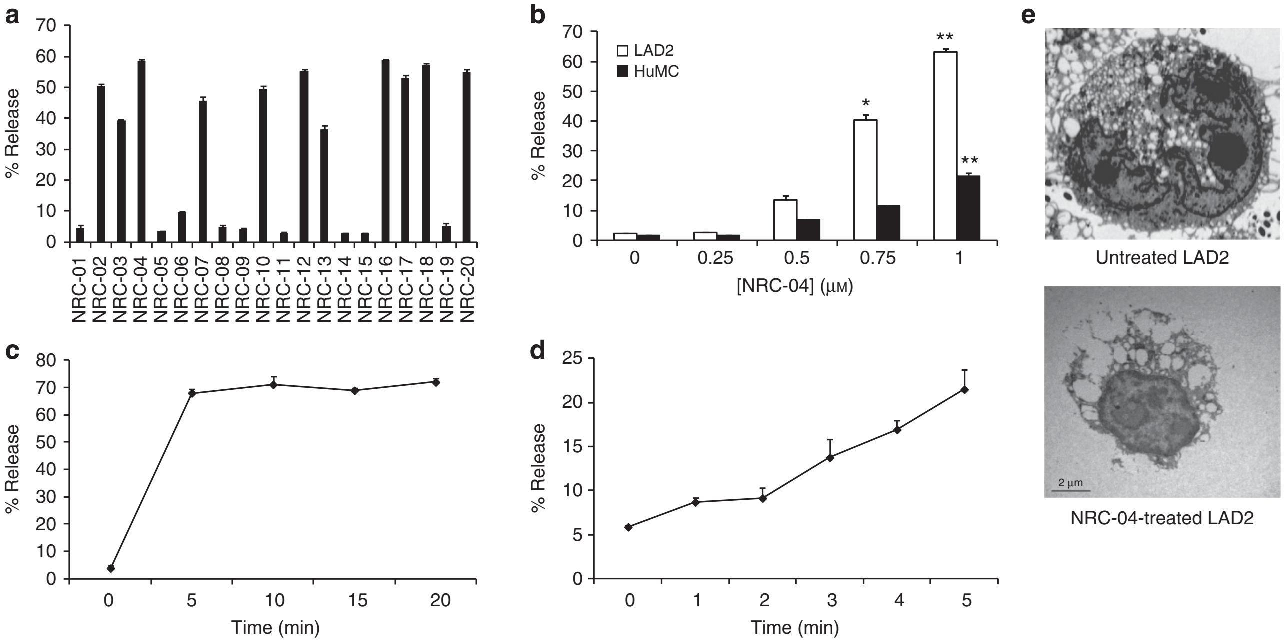

Figure 1 Pleurocidin peptides degranulate human mast cells. (**a**) Laboratory of Allergic Diseases 2 (LAD2) cells were stimulated for 30 min with 1 μM pleurocidin peptides NRC-01 to NRC-20, and the percentage of β-hexosaminidase released was measured as described in Methods section ($n=4$). (**b**) Human peripheral blood CD34$^+$ cell–derived mast cells (HuMC) and LAD2 cells were stimulated for 30 min with NRC-04 at indicated concentrations or (**c**, **d**) LAD2 cells were stimulated with 1 μM NRC-04 for indicated times and β-hexosaminidase release was measured ($n=3$). (**e**) Electron micrograph showing morphological changes in LAD2 cells either unstimulated (left) or stimulated (right) for 30 min with 1 μM NRC-04. $^*P<0.05$, $^{**}P<0.01$ compared with untreated.

cells loaded with the Ca^{2+}-sensing fluorescent dye fura-2 acetoxymethyl (AM) showed a rapid increase in intracellular Ca^{2+}, reaching a peak within 60 s after NRC-04 exposure. As expected, c48/80 (positive control) caused additional Ca^{2+} influx (**Figure 2a,b**).

Pleurocidin NRC-04 induces the release of lipid mediators cysteinyl leukotrienes (CysLTs) and prostaglandin D_2 (PGD_2)

Following degranulation, activated mast cells release arachidonic acid–derived lipid mediators. Given that NRC-04 induced mast cell degranulation, we examined its ability to initiate the release of leukotrienes and prostaglandins. NRC-04 induced significant release of CysLTs (147.11 ± 4.55 pg ml^{-1}) and PGD_2 (935.48 ± 75.6 pg ml^{-1}) from LAD2 cells. Immunoglobulin E (IgE)/anti-IgE stimulated similar amounts of CysLTs and PGD_2 release while the G protein agonist c48/80 had no significant effect (**Figure 2c,d**). Although LAD2 cells showed high spontaneous release of PGD_2, this was not attributable to any cell medium component as shown by low PGD_2 concentrations in the cell-free medium control (**Figure 2d**) and NRC-04 in medium alone control (data not shown).

Pleurocidin NRC-04 stimulates gene expression and protein production of chemokines

We further measured the expression of cytokines and chemokines by LAD2 cells following 3 h stimulation with NRC-04 by quantitative PCR (qPCR). Although no effect was observed on mRNA expression of cytokines (**Figure 3a**), mRNA expression levels of chemokines monocyte chemotactic protein (MCP)-1 and macrophage inflammatory protein (MIP)-1β increased significantly (**Figure 3b**). In addition to the cytokines and chemokines examined in **Figure 3a,b**, we also tested whether NRC-04 induced the expression of interleukin (IL)-4, IL-17, GM-CSF (granulocyte macrophage colony-stimulating factor), IP-10 (inducible protein-10), IL-1β, MIG (monokine induced by interferon-γ), RANTES (regulated on activation, normal, T-cell-expressed, and secreted), or IL-8. There was no change in the mRNA expression levels of these mediators following NRC-04 stimulation (data not shown). After observing the enhanced expression of MCP-1 and MIP-1β, the stimulatory effects on protein production of the respective chemokines was confirmed using an enzyme-linked immunosorbent assay (ELISA), showing that NRC-04 enhanced the production of both MCP-1 (**Figure 3c**) and MIP-1β (**Figure 3d**). Interestingly, the effects of NRC-04 on chemokine production were comparable with that of IgE/anti-IgE but considerably lower than c48/80.

To further support our hypothesis that NRC-04 preferentially activates chemokine production, we performed cytometric bead arrays for IL-12p70, tumor necrosis factor (TNF), IL-10, IL-6, and IL-1β and IP-10, MCP-1, MIG, RANTES, and IL-8. NRC-04 induced the production of MCP-1 only (data not shown). We next evaluated the action of NRC-04 on HuMC and observed no production of TNF, MCP-1, or MIP-1β (**Figure 3e**). In comparison, NRC-05 did not result in mast cell degranulation and also failed to stimulate the production of TNF, MCP-1, and MIP-1β by LAD2 cells. These cells were

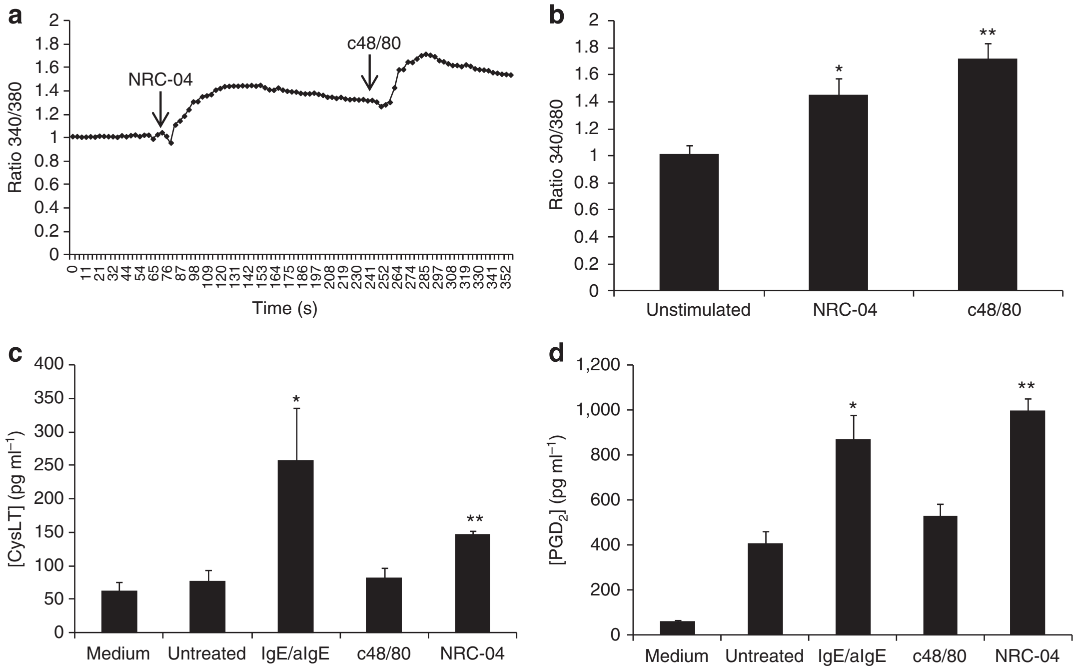

Figure 2 Pleurocidin NRC-04 mediates intracellular Ca^{2+} mobilization and release of lipid mediators. (**a**) Fura-2-loaded Laboratory of Allergic Diseases 2 (LAD2) cells were stimulated with 1 μM NRC-04 at time point 70 s followed with 0.5 μg ml^{-1} compound 48/80 (c48/80) at time point 256 s and mobilization of intracellular Ca^{2+} was measured ($n=4$). (**b**) Calcium response in 20 randomly selected individual LAD2 cells reported as mean ± s.e.m. of ratio 340/380 ($n=4$). LAD2 cells were stimulated with 0.5 μg ml^{-1} immunoglobulin E (IgE)/100 μg ml^{-1} anti-IgE (aIgE), 1 μg ml^{-1} c48/80, or 1 μM NRC-04. After 3 h treatment, production of (**c**) cysteinyl leukotriene (CysLT) and (**d**) prostaglandin D_2 (PGD_2) in cell-free supernatants was measured by enzyme immunoassay ($n=5$). *$P<0.05$, **$P<0.01$ compared with untreated.

responsive to c48/80, which was used as a positive control (**Figure 3f**).

Pleurocidin NRC-04 induces mast cell adhesion and chemotaxis

Activation of mast cell degranulation in response to infection requires mast cell migration and accumulation at the sites of infection. As CAPs evoke mast cell adhesion, chemotaxis, and subsequent degranulation, we evaluated whether NRC-04 could also promote these functions. Comparable with stem cell factor (SCF), NRC-04 activated LAD2 cells to adhere to fibronectin, an extracellular matrix protein (**Figure 4a**). As adhesion is closely related to cytoskeletal reorganization and migration, we measured LAD2 chemotaxis to either SCF or NRC-04. Results showed that similar to SCF, NRC-04 activated LAD2 chemotaxis (**Figure 4b**).

Human mast cell activation by pleurocidin NRC-04 is sensitive to inhibitors of G protein signaling

Although the signaling pathways responsible for cathelicidin activation of mast cells are poorly understood, a number of specific secondary messengers such as G proteins and lipid kinases have been implicated. We examined whether G protein, phosphoinositol-3 kinase (PI3K), phospholipase C (PLC) and/or phosphokinase C (PKC) pathways were involved by pretreating the cells with pathway specific inhibitors, pertussis toxin, wortmannin, U-73122, and Ro-31-8220, respectively before activation with NRC-04. All four inhibitors significantly inhibited NRC-04-activated mast cell degranulation (**Figure 5a–d**), and pretreatment with pertussis toxin significantly suppressed NRC-04-induced intracellular Ca^{2+} mobilization in LAD2 cells (**Figure 5e,f**).

Human mast cells express N-formyl-peptide receptor 1 (FPRL1) and $P2X_7$ receptors

Pertussis sensitivity suggested that NRC-04 was activating G protein signaling mediated by the activation of functional GPCR. Previous studies have shown that cathelicidin LL-37 activates mast cell chemotaxis via FPRL1 and mast cell cytokine release via purinoreceptor $P2X_7$. We hypothesized that FPRL1 and/or $P2X_7$ receptors might therefore be involved in NRC-04-mediated mast cell activation. qPCR analysis showed that LAD2 cells expressed mRNA for both FPRL1 and $P2X_7$ receptors (**Figure 6a**), and flow cytometry analysis confirmed cell surface expression of FPRL1 (**Figure 6b**).

To determine whether NRC-04 activation was FPRL1 and $P2X_7$ dependent, we pretreated LAD2 cells with WRW4 (FPRL1 antagonist) or KN-62 ($P2X_7$ antagonist) before activation with NRC-04. WRW4 inhibited NRC-04-induced degranulation (**Figure 6c**), whereas KN-62 had no effect (**Figure 6d**).

Fluorescence-resonance energy transfer (FRET)-based detection of FPRL1 activation by pleurocidin NRC-04

To determine whether NRC-04 could directly activate the FPRL1 receptor, we used a reporter assay in which FPRL1 is stably integrated into the Gα15-NFAT-*bla* CHO-K1 cell line. This cell line contains a beta-lactamase (*bla*) reporter gene under the control of a NFAT (nuclear factor of activated T-cell) response element and a promiscuous G protein, Gα15, stably

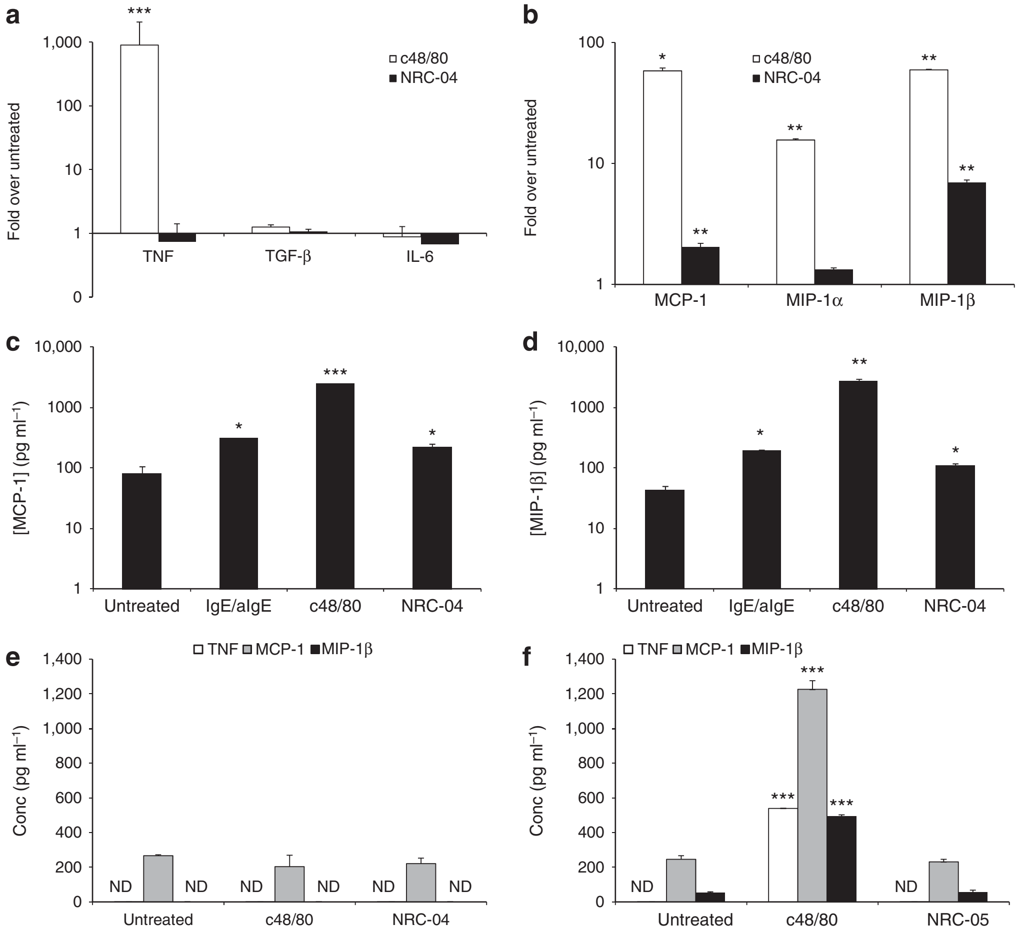

Figure 3 Pleurocidin NRC-04 induces the production and release of proinflammatory mediators. After 3 h treatment, mRNA expression of (**a**) tumor necrosis factor (TNF), transforming growth factor (TGF)-β, interleukin (IL)-6, (**b**) monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, and MIP-1β was measured by real-time PCR ($n=3$). Data were normalized to glyceraldehyde 3-phosphate dehydrogenase mRNA levels and are expressed as fold increase over unstimulated controls. After 24 h treatment, production of (**c**) MCP-1 and (**d**) MIP-1β in cell-free supernatants was measured by enzyme-linked immunosorbent assay (ELISA) ($n=3$). Production of TNF, MCP-1, and MIP-1β in cell-free supernatant from (**e**) c48/80 or NRC-04-treated HuMC (human peripheral blood $CD34^+$ cell–derived mast cells) and (**f**) c48/80 or NRC-05-treated LAD2 (Laboratory of Allergic Diseases 2) cells was measured by ELISA ($n=3$). *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared with untreated. IgE, immunoglobulin E; aIgE, anti-IgE.

integrated into CHO-K1 cells. In the absence of *bla* expression (unstimulated) cells appear green fluorescent (530 nm emission); however, in the presence of *bla* expression (stimulated), the FRET-enabled substrate is cleaved and cells appear blue fluorescent (460 nm emission). Incubation of cells with NRC-04 resulted in a significant increase in 460/530 (Blue/Green) emission ratio in a concentration-dependent manner. WKYMVM (W(m)) peptide was used a positive control. WRW4 and pertussis toxin were able to inhibit NRC-04 activity (**Figure 6e**), indicating a specific interaction between NRC-04 and FPRL1.

DISCUSSION

In the present study, we investigated the pleurocidin family of CAPs in innate immune responses based on its ability to activate human mast cells. We demonstrated that pleurocidin NRC-04 induced mast cell migration, adhesion, degranulation, release of lipid mediators, and the production of chemokines. By contrast, NRC-05 did not activate LAD2 cell degranulation and chemokine production, suggesting that these two functions are controlled by similar signaling pathways. Our data further showed that pleurocidins activate G proteins and PI3K, PLC, and PKC signaling pathways in human mast cells. A specific GPCR, FPRL1, is expressed in human mast cells and likely to be functional in pleurocidin-induced mast cell activation.

Among the mucus-derived fish antimicrobial peptides, the pleurocidin family is probably the best characterized and has been under investigation by several research groups. A study by Chiou *et al.*[16] reported the potential immunoregulatory effects of pleurocidin on the expression of immune-relevant genes, IL-1 and cyclooxygenase-2, in a fish macrophage cell line, suggesting a proinflammatory role of pleurocidin in the vertebrate immune system. In other studies, pleurocidin CAPs exhibited cytolytic activity against breast cancer cells and multiple myeloma cells and impaired the growth of tumor xenografts in immune-deficient mice,[17] indicating that these peptides may also stimulate an antitumor immune response.

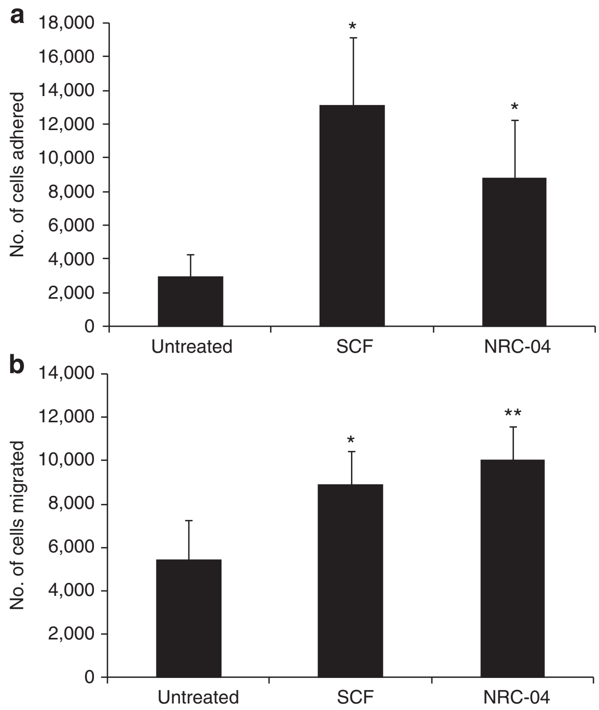

Figure 4 Pleurocidin NRC-04 mediates human mast cell adhesion and migration. (**a**) Laboratory of Allergic Diseases 2 (LAD2) cells were allowed to adhere for 2 h to fibronectin-coated wells in the presence of medium alone, 100 ng ml $^{-1}$ stem cell factor (SCF), or 1 μM NRC-04, and the number of adherent cells was assessed as described in Methods section ($n=3$). (**b**) LAD2 cells were allowed to migrate for 6 h across inserts towards medium alone, 100 ng ml $^{-1}$ SCF, or 1 μM NRC-04, and migration was assessed as described in Methods section ($n=3$). *$P<0.05$, **$P<0.01$ compared with untreated.

Similar to cathelicidins, a family of mammalian CAPs, pleurocidin genes share highly conserved N-terminal signal peptide (*pre*) and C-terminal acid peptide (*pro*) sequences, in addition to the mature pleurocidin sequences;[18–20] this results in an initial *pre*-pleurocidin-*pro* peptide, which is the precursor of mature and fully bioactive pleurocidin. Both mature cathelicidins[18] and mature pleurocidins form α-helixes and kill Gram-positive and Gram-negative bacteria by forming pores in their membranes,[12,21] causing cell lysis.[11,22] Peptides belonging to the pleurocidin family are only found in pleuronectid flatfish (Winter flounder, Yellowtail flounder, American plaice, and Halibut) and share a common signal and proregion amino-acid sequences but differ in core antimicrobial sequences.[10] Pleurocidin family peptides exhibit a wide range of antimicrobial activities, ranging from highly potent (minimum inhibitory concentration $<1\ \mu g\ ml^{-1}$) to inactive. Peptides NRC-03, -04, -06, -07, -11, -12, -13, -15, -16, and -17 exhibit significant inhibitory activities against pathogens, and the peptide sequences of NRC-04, -07, -11, -12, and -13 CAPs consist of an XVGK motif, where X is H, K, or T, all of which are relatively bulky residues. In the case of NRC-04, the motif is even doubled as HVGKHVGK, which is probably compatible with a highly bactericidal amphipathic helix.[10] Interestingly, the widely divergent antimicrobial activities of pleurocidins extend also to their ability to activate mast cells. Our initial screen of the 20 pleurocidin peptides revealed that some peptides were capable of degranulating human mast cells. Eleven of the 20 pleurocidin peptides tested (NRC-02, -03, -04, -07, -10, -12, -13, -16, -17, -18, and -20) induced significant LAD2 degranulation. Pleurocidin NRC-04 showed the highest degranulation activity, and as it is also a potent CAP, it was used for subsequent studies.

A hallmark of mast cell activation is the rise in intracellular Ca^{2+} upon agonist stimulation, and polybasic compounds such as c48/80 are potent activators of mast cell degranulation and calcium influx.[23] Intracellular Ca^{2+} is thought to have a key role in mast cell migration, release of histamine and lipid mediators.[24] Influx of Ca^{2+} is required for histamine release by human β-defensin-2 and LL-37.[7] In this study, pleurocidin increased intracellular Ca^{2+} concentration in mast cells, causing them to degranulate and release proinflammatory mediators.

De novo synthesized lipid mediators are mainly secreted by activated mast cells and are mediators of proinflammatory reactions.[25] We showed that pleurocidin activated the release of CysLTs and PGD_2 in human mast cells, in contrast to LL-37 which does not induce PGD_2 production.[7] However, the study involving LL-37 used rat peritoneal mast cells; thus, differences in mast cell phenotypes should be considered. Our results with c48/80 were consistent with a previous report that in mast cells, c48/80 induced intracellular Ca^{2+} mobilization, high secretion of histamine, and no release of PGD_2.[26] The failure of c48/80 to induce secretion of lipid mediators suggests that, unlike c48/80 that activates human mast cells in a receptor-independent manner by directly acting on G proteins, pleurocidin utilizes a receptor for their action.

Our findings demonstrated that pleurocidin activated the production of chemokines MCP-1 and MIP-1β, but not cytokines, implying that stimulation may be selective for a limited number of proinflammatory chemokines. MCP-1 and MIP-1β are involved in recruitment and activation of various immune cells such as neutrophils, basophils, eosinophils, monocytes, and T cells to sites of tissue injury, infection, and inflammation.[27] Taken together, our results suggest that in addition to histamine and lipid mediators release, pleurocidin may also participate in the regulation of inflammatory immune response by the production of chemokines and subsequent immune cell recruitment.

During inflammation, localized increase in the number of mast cells occurs, and the accumulation requires directed migration of these cells.[1,2] The present study showed that pleurocidin served as a potent chemoattractant for human mast cells. Moreover, it promoted mast cell adhesion to fibronectin, indicating its importance in tissue localization of mast cells and a possible role in process, such as wound healing and fibrosis.

To understand the molecular mechanisms underlying the activities of pleurocidin, we investigated the requirement for G proteins, PI3K, PLC, and PKC as their roles in CAPs–stimulated mast cell activation has been previously reported.[4] The G protein inhibitor pertussis toxin, the PI3K inhibitor

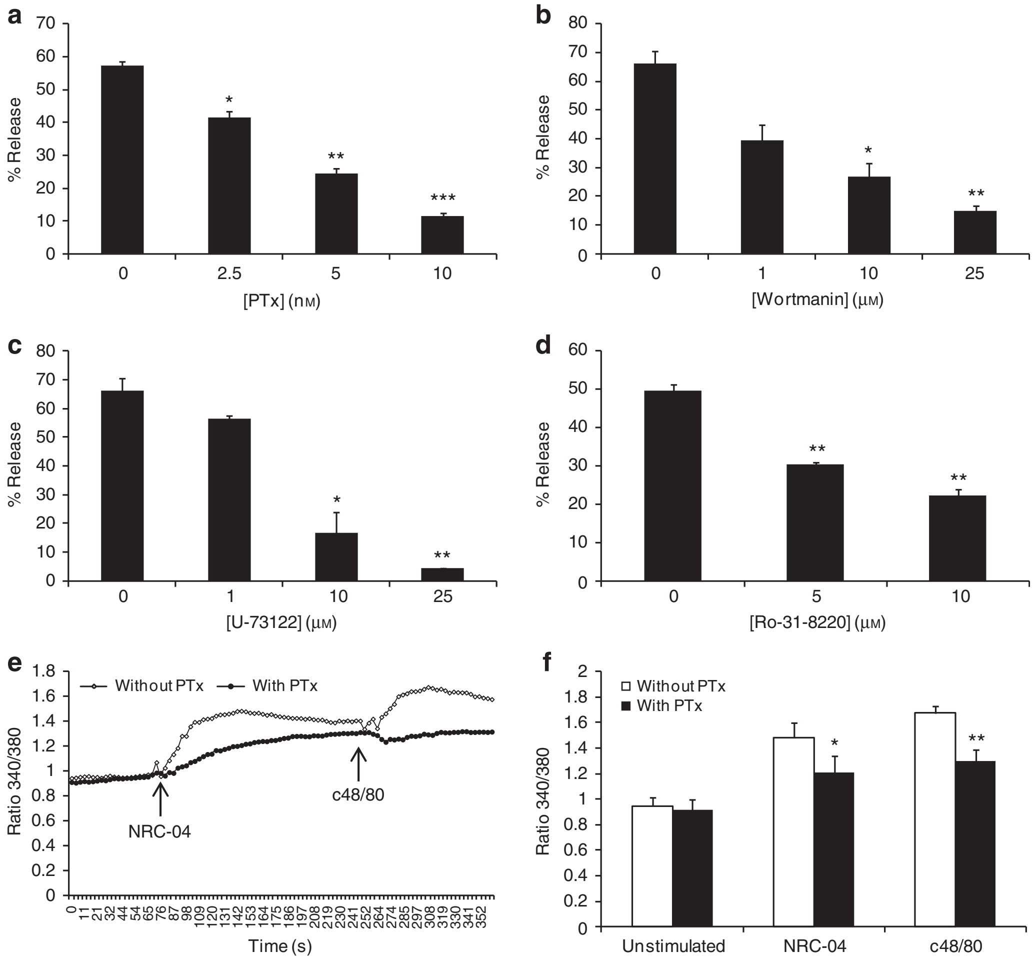

Figure 5 Pleurocidin NRC-04-induced human mast cell degranulation is G protein, PI3K (phosphoinositol-3 kinase), PLC (phospholipase C) and PKC (phosphokinase C) dependent. Laboratory of Allergic Diseases 2 (LAD2) cells were treated with the indicated concentrations of (**a**) pertussis toxin (PTx), (**b**) wortmannin, (**c**) U-73122, or (**d**) Ro-31-8220 before stimulation with 1 μM NRC-04, and percentage of β-hexosaminidase released was measured ($n=3$). (**e**) LAD2 cells were pretreated for 2 h with 5 nM PTx, loaded with fura-2, stimulated with 1 μM NRC-04 at time point 70 s followed with 0.5 μg ml^{-1} c48/80 at time point 256 s, and mobilization of intracellular Ca^{2+} was measured. (**f**) Ca^{2+} response in 20 randomly selected individual LAD2 cells reported as mean ± s.e.m. of ratio 340/380 ($n=5$). *$P<0.05$, **$P<0.01$, ***$P<0.001$, compared with untreated.

wortmannin, the PLC inhibitor U-73122 and the PKC inhibitor Ro-31-8220 showed inhibitory effects on pleurocidin-mediated mast cell degranulation. In addition, pertussis toxin also inhibited the increase in intracellular Ca^{2+} mobilization. Cationic peptides stimulating G proteins lead to the activation of PLC and PI3K; activated PLC catalyzes the generation of inositol triphosphate and diacylglycerol leading to Ca^{2+} mobilization and PKC activation, respectively.[23] Among isoenzymes of PLC identified, only β subtype PLC is activated by G proteins, typically $G_{q\alpha}$. In addition, βγ subunits of G proteins dissociated from $G_{i/o\alpha}$ also regulate PLC β activity,[28,29] and as pertussis toxin is known to inhibit $G_{i/o\alpha}$ but not $G_{q\alpha}$,[30] our findings suggest that G_i and/or $G_{io\alpha}$ type G proteins are involved in pleurocidin-induced activation of PLC in mast cells. Thus, the activation of G protein–coupled PLC β pathway likely has a role in the pleurocidin-elicited intracellular Ca^{2+} mobilization leading to mast cell activation. We also tested the effect of fluticasone propionate, salmeterol xinafoate, and tranilast on NRC-04-activated mast cell degranulation but found that none of these inhibitors had an effect on mast cell activation (data not shown).

Next we identified a functional receptor for pleurocidin in mast cells. LL-37 chemoattracts immune and inflammatory cells, including neutrophils, monocytes, and T cells via ligation of a low-affinity formyl peptide receptor, FPRL1.[31] LL-37 also interacts with the purinoreceptor $P2X_7$, which is predominantly expressed on monocytes, macrophages, and dendritic cells, and induces the processing and release of the potent cytokine IL-1β[32] and IL-8 in human gingival fibroblasts.[33] Thus, we addressed the question as to whether the pleurocidin-induced mast cell degranulation was mediated by FPRL1 and/or $P2X_7$. We found that both FPRL1 and $P2X_7$ were expressed by human mast cells. The FPRL1 antagonist WRW4 inhibited pleurocidin-induced mast cell degranulation but the

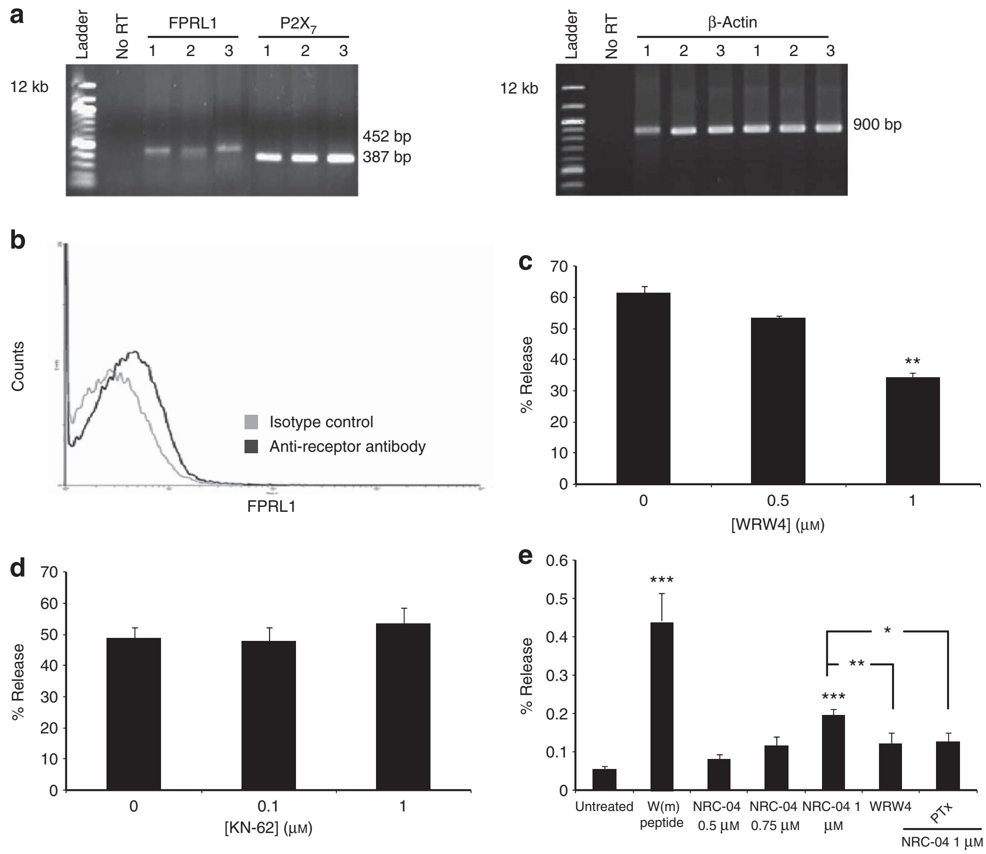

Figure 6 N-formyl-peptide receptor 1 (FPRL1) and $P2X_7$ receptors in NRC-04-mediated human mast cell activation. (**a**) Total RNA was isolated from Laboratory of Allergic Diseases 2 (LAD2) cells, and FPRL1 and $P2X_7$ receptors mRNA expression was examined by reverse transcriptase (RT) PCR. β-actin was used as a positive control. (**b**) The expression of FPRL1 on LAD2 cell surface was evaluated by flow cytometry. LAD2 cells were pretreated for 2 h with (**c**) WRW4, (**d**) KN-62, or vehicle, stimulated with 1 μM NRC-04, and percentage of β-hexosaminidase released was measured ($n=3$). (**e**) FPRL-1-Gα15-NFAT-*bla* CHO-K1 cells were incubated for 2 h with WRW4 or pertussis toxin (PTx), stimulated with WKYMVM (W(m)) peptide or NRC-04 and fluorescence-resonance energy transfer assay was performed as described in Methods section ($n=3$). *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared with untreated.

$P2X_7$ antagonist KN-62 had no effect. Thus, pleurocidin utilizes FPRL1 as a receptor to activate mast cells. In a fluorescent reporter assay utilizing FPRL1 stably integrated into the Gα15-NFAT-*bla* CHO-K1 cell line under the control of beta-lactamase reporter gene, we showed that pleurocidin activated a conformational change in FPRL1, which was sensitive to a pharmacological inhibitor of FPRL1 and pertussis toxin. FPRL1 belongs to the GPCR superfamily that activates host defense mechanism in immune cells, including migration, adhesion, and degranulation. It mediates these cellular functions in a pertussis toxin–sensitive manner, indicating coupling to one or more members of the G_i subfamily of G proteins. The novel ability of FPRL1 to convey both pro- and anti-inflammatory signaling makes it an intriguing, yet unusual GPCR.[34] Not surprisingly, numerous peptide ligands of FPRL1 have been identified as potential therapeutic interventions for human disease. Our study describes a novel, naturally occurring proinflammatory agonist for FPRL1.

In conclusion, our studies show that pleurocidin could effectively recruit and activate human mast cells by binding and signaling through FPRL1 receptor. Pleurocidin activation of mast cells not only activated degranulation and release of preformed granule-contained mediators, but it also induced the production of chemokines. Pleurocidin-induced release of mast cell mediators can possibly recruit and activate T lymphocytes, eosinophils, and other inflammatory cells possibly contributing to innate and adaptive immune responses. With the global emergence of many new infectious diseases, as well as concerns about the antibiotic resistance of an increasing number of microbial pathogens, the peptide antibiotics group of anti-microbials warrants attention and further studies. Our data may prove useful in suggesting novel ways of activating mast cells to boost immune responses.

METHODS

Pleurocidin peptides. The amino-acid sequences were predicted from nucleic acid sequences and those predicted to contain a C-terminal glycine were amidated (**Table 1**). Peptides NRC-01 to NRC-20 were synthesized by N-9-fluorenylmethoxycarbonyl (Fmoc) chemistry at Dalton Chemical Laboratories (Toronto, ON, Canada). Peptide purity (95%) was confirmed by high-performance liquid chromatography and mass spectrometry. All peptide stocks were prepared in phosphatebuffered saline (PBS) and stored at −80 °C.

Table 2 Sequences of oligonucleotides used for PCR

Gene	Forward primer	Reverse primer	Probe: FAM/TAMRA (GAPDH: MAX/BHQ)
GAPDH	5′-TCGTGGAAGGACTCATGAC-3′	5′-CCATCACGCCACAGTTT-3′	5′-/5MAXN/AGTCCATGCCATCACTGCCAC/3IABlk_FQ/-3′
GM-CSF	5′-CAGCCCTGGGAGCATGTG-3′	5′-ATTCATCTCAGCAG-CAGTGTCTCTA-3′	5′-/56-FAM/AGGCCCGGCGTCTCCTGAACC/36-TAMSp/-3′
IL-1β	5′-TGAGCTCGCCAGTGAAATGA-3′	5′-TGAGCTCGCCAGTGAAATGA-3′	5′-/56-FAM/GAAGCTGATGGCCCTAAACAGAT-GAAGTGCT/36-TAMSp/-3′
IL-4	5′-CTGCAAATCGACACCTAT-TAATGG-3′	5′-GCACATGCTAGCAGGAA-GAACA-3′	5′-/56-FAM/TCTCACCTCCCAACTGCTTCCCCC/36-TAMSp/-3′
IL-6	5′-AGCCACTCACCTCTTCAGAAC-GAA-3′	5′-AGTGCCTCTTTGCTGCTTTCA-CAC-3′	5′-/56-FAM/ACGGCATCTCAGCCCTGAGAAAGGA GA/36-TAMSp/-3′
IL-8	5′-CTGGCCGTGGCTCTCTTG-3′	5′-TTGGCAAAACTGTTTAG-CACTCC-3′	5′-/56-FAM/CAGCCTTCCTGATTTCTGCAGCTCTG TGT/36-TAMSp/-3′
IP-10	5′-CGATTCTGATTTGCTGCCTT ATC-3′	5′-TGATTACTAATGCTGATGCAGG-TACA-3′	5′-/56-FAM/TGGCATTCAAGGAGTACCTCTCTCT AGAACCGT/36-TAMSp/-3′
MCP-1	5′-TCTCTGCCGCCCTTCTGT-3′	5′-GCCTCTGCACTGAGATCTT CCT-3′	5′-/56-FAM/CTGCTCATAGCAGCCACCTTCATTC CC/36TAMSp/-3′
MIG	5′-GGAGTGCAAGGAACCCCAG TA-3′	5′-TCTTTCAAGGATTGTAGGTG GATAGTC-3′	5′-/56-FAM/TCGCTGTTCCTGCATCAGCACCA/36-TAMSp/-3′
MIP-1α	5′-CTACACCTCCCGGCAGATTC-3′	5′-CCGGCTTCGCTTGGTTAG-3′	5′-/56-FAM/CAGTGCTCCAAGCCCGGTGTCATC/36TAMSp/-3′
MIP-1β	5′-CAGCGCTCTCAGCACCAA-3′	5′-TTCCTCGCGGTGTAAGAAA AG-3′	5′-/56-FAM/CTCAGACCCTCCCACCGCCTGC/36-TAMSp/-3′
RANTES	5′-TCGCTGTCATCCTCATTGCTA-3′	5′-GCACTTGCCACTGGTGTA-GAAA-3′	5′-/56-FAM/CTGGGACACCACACCCTGCTGC/36-TAMSp/-3′
TGF-β	5′-CTCTCCGACCTGCAACAGA-3′	5′-AACCTAGATGGGCGCGATCT-3′	5′-CCCTATTCAAGACCACCCACCTTCTGGT-3′
TNF	5′-TCTGGCCCAGGCAGTCA-3′	5′-GCTTGAGGGTTTGCTACAA-CATC-3′	5′-/56-FAM/CTTCTCGAACCCCGAGTGACAAG CC/36T-AMSp/-3′
FPRL1	5′-TGTGAGTCTGGCCATGAAGG TGAT-3′	5′-AGCTCGTTGGGTTAACCA GGATGT-3′	–
$P2X_7$	5′-TACAGCTGCTTAGAAA GGAGGCGA-3′	5′-ACTGCCCTTCACTCTTCGG AA-3′	–
β-Actin	5′-ATCTGGACCACACCTTCT ACAATGAGCTGCG-3′	5′-CGTCATACTCCTGCTTG CTGATCCACATCTGC-3′	–

Abbreviations: FPRL1, N-formyl-peptide receptor 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase,; GM-CSF, granulocyte macrophage colony-stimulating factor; IL, interleukin; IP-10, inducible protein-10; MCP-1, monocyte chemotactic protein-1; MIG, monokine induced by interferon-γ; MIP, monocyte inflammatory protein; RANTES, regulated on activation, normal, T-cell-expressed, and secreted; TGF-β, transforming growth factor-beta; TNF, tumor necrosis factor.

Cell culture. LAD2 human mast cells[35] were cultured in serum-free medium (StemPro-34 SFM, Life Technologies, Rockville, MD) supplemented with 2 mM L-glutamine, 100 U ml^{-1} penicillin, 50 μg ml^{-1} streptomycin, and 100 ng ml^{-1} recombinant human SCF (Peprotech, Rocky Hill, NJ). Cells were maintained at 1×10^5 cells ml^{-1} at 37 °C/5% CO_2. Cells were periodically tested for expression of c-Kit and FcεRI by flow cytometry. Human peripheral blood CD34$^+$ cells–derived mast cells were kindly provided by Dr Dean Befus. These cells were cultured in StemSpan SFEM (StemCell Technologies, Vancouver, BC, Canada) supplemented with 2 mM L-glutamine, 100 U ml^{-1} penicillin, 50 μg ml^{-1} streptomycin, 100 ng ml^{-1} SCF, and 100 ng ml^{-1} recombinant human IL-6 (Peprotech). Recombinant human IL-3 (30 ng ml^{-1}) was added for the first week. At 8–10 weeks, cultures consisted of >99% mast cells.

Degranulation. Cells were washed, resuspended in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (10 mM HEPES, 137 mM NaCl, 2.7 mM KCl, 0.38 mM $Na_2HPO_4.7H_2O$, 5.6 mM glucose, 1.8 mM $CaCl_2.H_2O$, 1.3 mM $MgSO_4.7H_2O$, 0.4% bovine serum albumin (BSA), pH 7.4) at 2.5×10^4 cells per well and stimulated for 30 min with serial dilutions (0.0001–1 μM) of each pleurocidin at 37 °C/5% CO_2. β-Hexosaminidase released into the supernatants and in total cell lysates solubilized with 0.01% Triton X-100 was quantified by hydrolysis of p-nitrophenyl N-acetyl-β-D-glucosamide (Sigma-Aldrich, Oakville, ON, Canada) in 0.1 M sodium citrate buffer (pH 4.5) for 90 min at 37 °C/5% CO_2. The percentage of β-hexosaminidase released was calculated as a percentage of the total content.

In some experiments, LAD2 cells were pretreated for 30 min with wortmannin (0.1–25 μM; Sigma-Aldrich), U-73122 (0.1–25 μM; Calbiochem, Gibbstown, NJ), Ro-31-8220 (0.01–10 μM; Sigma-Aldrich), or vehicle (0.1–1% dimethyl sulfoxide); or for 2 h with pertussis toxin (0.1–10 nM, Sigma-Aldrich), WRW4 (0.5–1 μM; Biotrend Chemicals, Zurich, Switzerland), KN-62 (10–100 nM, Sigma-Aldrich), or vehicle. Cells were then stimulated for 30 min with NRC-04 and β-hexosaminidase released was measured.

Electron microscopy. LAD2 cells were treated for 30 min with 1 μM NRC-04 at 37 °C/5% CO_2 and fixed for 20 min in glutaraldehyde fixative (2.5% glutaraldehyde in 0.05 M cacodylate buffer with 0.1 M NaCl, pH 7.5) at room temperature. Sections were cut using a microtome and observed by using a S300N transmission electron microscope (Hitachi, Tokyo, Japan).

Intracellular Ca^{2+} mobilization. LAD2 cells were loaded for 30 min with 1 μM fura-2 AM (Invitrogen, Burlington, ON, Canada) in HEPES buffer, washed, and incubated for 15 min in BSA-free HEPES at 37 °C/5% CO_2. A total of 4×10^6 cells were placed in glass-bottommed culture dish under an inverted microscope (Axiovert 200, Carl Zeiss Canada Ltd., Toronto, ON, Canada). Fura-2 was excited at 340 and 380 nm alternatively. The Ca^{2+} response was recorded at 100-ms intervals using SlideBook for Stallion, version 4.26.04 software (Intelligent Imaging Innovations, Denver, CO). In all, 1 μM NRC-04 was added at the time point of 70 s and 0.5 μg ml^{-1} c48/80 at the time point of 256 s. Ca^{2+} response of 20 randomly selected cells was analyzed for each experiment and plotted as 340/380 ratio vs. time.

ELISA. In all, 1×10^6 cells were stimulated for either 3 or 24 h with 1 μM pleurocidins or 1 μg ml^{-1} c48/80 at 37 °C/5% CO_2. Cell-free supernatants were isolated and analyzed for CysLTs, PGD_2, or chemokine production using the following commercial competitive enzyme immunoassay (EIA) kits: Correlate-EIA cysteinyl leukotriene kit (Assay Designs, Ann Arbor, MI), prostaglandin D_2 EIA kit (Cayman Chemicals, Ann Arbor, MI), human CCL2 (C-C motif chemokine ligand 2; MCP-1) ELISA kit (eBioscience, San Diego, CA), and quantikine human MIP-1beta ELISA kit (R&D Systems, Minneapolis, MN). The minimum detection limits are 78.1 pg ml^{-1} for CysLT, 200 pg ml^{-1} for PGD_2, 7 pg ml^{-1} for MCP-1, and 4 pg ml^{-1} for MIP-1β.

Isolation of RNA and generation of cDNA by reverse transcription. LAD2 cells were stimulated for 3 h with 1 μM NRC-04 or 1 μg ml^{-1} c48/80 at 37 °C/5% CO_2. Total RNA was isolated using the Tri Reagent method (Sigma-Aldrich). In all, 1 μg of total cellular RNA was reverse-transcribed to cDNA using M-MLV Reverse Transcriptase (Invitrogen) in a 20-μl reaction mix according to the manufacturer's recommendation.

Reverse transcription PCR (RT-PCR). RT-PCR was performed on a Peltier Thermal Cycler System (Bio-Rad, Hercules, CA) using Invitrogen reagents. FRPL1, $P2X_7$, and β-actin primers (**Table 2**) were designed using the Primer Express software (Applied Biosystems, Foster City, CA). Amplification was carried out for 30 cycles: denaturation for 45 s at 94 °C, annealing for 30 s at 56 °C for FPRL1, $P2X_7$, and 60 °C for β-actin and extension for 60 s at 72 °C. PCR amplicons were analyzed by 1% agarose gel electrophoresis and visualized by ethidium bromide staining.

Real-time qPCR. Gene expression was analyzed using real-time qPCR on a StepOnePlus system (Applied Biosystems). For each qPCR assay, a total of 50 ng of cDNA was used. Primer sets (**Table 2**) for PCR amplifications were designed using Primer Express software. All reactions were performed in triplicate for 40 cycles as per the manufacturer's recommendation. All data were normalized to glyceraldehyde 3-phosphate dehydrogenase internal controls[36] and are reported as fold change in expression over untreated cells.

Cytometric bead array assay. In all, 1×10^6 cells were stimulated for 24 h with 1 μM NRC-04 or 1 μg ml^{-1} c48/80 at 37 °C/5% CO_2. Cell-free supernatants were isolated and analyzed for human cytokine and chemokine expression using the following commercial cytometric bead array kits; Human Inflammatory Cytokine Kit and Human Chemokine Kit (BD Biosciences, Mississauga, ON, Canada). The minimum detection levels for the cytokines and chemokines are IL-1β, 7.2 pg ml^{-1}; IL-6, 2.5 pg ml^{-1}; IL-10, 3.3 pg ml^{-1}; TNF, 3.7 pg ml^{-1}; IL-12p70, 1.9 pg ml^{-1}; IL-8, 0.2 pg ml^{-1}; RANTES, 1.0 pg ml^{-1}; MIG, 2.5 pg ml^{-1}; MCP-1, 2.7 pg ml^{-1}; and IP-10, 2.9 pg ml^{-1}.

Adhesion assay. Maxisorp 96-well plates (NUNC, Naperville, IL) were coated for 16 h with 10 μg ml^{-1} human fibronectin (Sigma-Aldrich) in PBS at 4 °C, washed three times with PBS, blocked for 1 h with 3% BSA in HEPES buffer at 37 °C/5% CO_2, and then washed three times with HEPES buffer. LAD2 cells were washed with HEPES buffer, suspended at 1×10^6 cells ml^{-1}, and labeled for 20 min with 5 μM calcein-AM (Invitrogen) at 37 °C/5% CO_2. After labeling, cells were washed and resuspended at 1×10^6 cells ml^{-1} in HEPES buffer. Cell suspension (5×10^4 cells) ± HEPES buffer containing 1 μM NRC-04 or 100 ng ml^{-1} SCF was added and incubated for 2 h at 37 °C/5% CO_2. After incubation, non-adherent cells were washed away with warm HEPES buffer. Fluorescence emission at 530 nm (485 nm excitation) was measured using a fluorescence plate reader (Varioskan Flash Multimode Reader, Thermo Fisher Scientific, Nepean, ON, Canada). The number of adhered cells was calculated from a standard curve consisting of increasing LAD2 cell numbers.

Chemotaxis assay. Performed using the 96-Well Monocyte Cell Migration kit (Calbiochem) with slight modifications. LAD2 cells were incubated overnight in SCF-free media,washed with HEPES buffer, and suspended at 0.25×10^6 cells ml^{-1}. Agonists (1 μM NRC-04 or 100 ng ml^{-1} SCF) were added to the lower chamber of 96-well tray and cell suspension (2.5×10^4 cells) was added to the insert (upper chamber). Cells were allowed to migrate towards the agonists for 6 h at 37 °C/5% CO_2. After incubation, the insert was washed twice with warm HEPES buffer. Cells were labeled for 30 min with 5 μM calcein-AM at 37 °C/5% CO_2. After labeling, cells migrated across the insert membrane were detached using 0.01% Triton X-100. Fluorescence emission was measured at 530 nm (485 nm excitation). The number of migrated cells was calculated from a standard curve consisting of increasing LAD2 cell numbers.

Cell viability assay. Cells were pretreated with 1 μM NRC-04 for 30 min, 3 and 24 h, followed by incubation for 2 h with Resazurin dye (In vitro Toxicological Assay kit, Sigma-Aldrich) at 37 °C/5% CO_2. Fluorescence emission was measured at 590 nm (560 nm excitation).

Flow cytometric analysis. LAD2 cells were washed, suspended at 2×10^5 cells ml^{-1} in 0.1% BSA/PBS and incubated for 1 h with either anti-FPRL1 antibody or isotype control antibody (R&D Systems) at 4 °C. Cells were washed twice, resuspended in 0.1% BSA/PBS, and analyzed on a FACSArray (BD Biosciences).

FRET analysis. FPRL1-Gα15-NFAT-*bla* CHO-K1 cells (Invitrogen) were cultured in growth medium (Dulbecco's modified Eagle's medium (DMEM; high-glucose), with GlutaMAX (Invitrogen) supplemented with 10% dialyzed fetal bovine serum, 0.1 mM non-essential amino acid, 25 mM HEPES, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 100 μg ml^{-1} zeocin, 5 μg ml^{-1} blasticidin, and 600 μg ml^{-1} geneticin). Cells were washed, resuspended in plating medium (DMEM (high-glucose), with GlutaMAX + 10% charcoal-stripped fetal bovine serum) and plated at 5,000 cells per well in a 384-well plate and incubated at 37 °C/5% CO_2 for 16 h. After incubation, the plating media was removed and replenished with an assay medium (DMEM (high-glucose), with GlutaMAX). Cells were pretreated for 2 h with the antagonists (WRW4 and pertussis toxin) followed by stimulation for 4 h with either 1 μM NRC-04 or 5 nM WKYMVM (W(m)) peptide (Phoenix Pharmaceuticals, Burlingame, CA) at 37 °C/5% CO_2. Following stimulation, cells were loaded in the dark for 2 h at room temperature with LiveBLAzer-FRET B/G substrate (Invitrogen). Fluorescence emissions were measured at 460 nm (409 nm excitation, Blue channel) and 530 nm (409 nm excitation, Green channel).

Statistical analysis. Each experiment was performed at least three separate times. Statistical significance was determined by two-tailed Student's paired *t*-test and $P < 0.05$ was considered significant. The results are shown as mean ± s.e.m.

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DISCLOSURE

The authors declared no conflict of interest.

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