Activation of formyl peptide receptor 2 by WKYMV\text{m} enhances emergency granulopoiesis through phospholipase C activity

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Emergency granulopoiesis is a very important strategy to supply efficient neutrophil number in response to infection. However, molecular mechanism involved in this process remains unclear. Here, we found that administration of WKYMV\text{m}, an immune modulating peptide, to septic mice strongly increased neutrophil number through augmented emergency granulopoiesis, WKYMV\text{m}-induced emergency granulopoiesis was blocked not only by a formyl peptide receptor 2 (FPR2) antagonist (WRW4), but also by FPR2 deficiency. As progenitors of neutrophils, Lin$^-c$-kit$^+$Sca-1$^-$ cells expressed FPR2. WKYMV\text{m}-induced emergency granulopoiesis was also blocked by a phospholipase C inhibitor (U-73122). These results suggest that WKYMV\text{m} can stimulate emergency granulopoiesis via FPR2 and phospholipase C enzymatic activity. [BMB Reports 2018; 51(8): 418-423]

INTRODUCTION

Neutrophils play an essential role in host defense against bacterial infection (1, 2). Neutrophils can engulf invading pathogens and remove them via several different mechanisms (1, 2). Neutrophils generate reactive oxygen species (ROS) through activation of NADPH oxidase and these ROS can kill invading pathogens (3). Since neutrophils play important innate immune responses against pathogen infection (1, 2), regulation of de novo neutrophil generation is a hot issue.

Neutrophils are generated from hematopoietic stem cells and their subsequent intermediate progenitors such as common myeloid progenitors (CMP) and granulocyte-macrophage progenitors (GMP) (4, 5). Such de novo neutrophil generation has been observed in steady-state granulopoiesis in local bacterial infection and in emergency granulopoiesis for systemic bacterial infection (6). Unlike steady-state granulopoiesis, emergency granulopoiesis rapidly generates an increased number of neutrophils in response to an emergency alert signal following systemic bacterial infection (6). Therefore, it is of great interest to reveal molecular mechanisms involved in the regulation of emergency granulopoiesis.

WKYMV\text{m} is a synthetic peptide that stimulates innate immune cells such as neutrophils, monocytes, and macrophages (7-10). WKYMV\text{m} stimulates chemotactic migration and ROS generation in neutrophils and monocytes (7-9). On cell surface receptor for this peptide, it binds to formyl peptide receptor (FPR) family members including FPR1 and FPR2 (11, 12). Previously, we have demonstrated that WKYMV\text{m} administration has a therapeutic effect against sepsis mainly through FPR2 (13). Regarding the mechanism involved in the antiseptic activity of WKYMV\text{m}, the peptide can stimulate IFN-$\gamma$ production and suppress proinflammatory cytokine production such as the production of TNF-$\alpha$ and IL-1$\beta$ (13). However, the functional role of WKYMV\text{m} and its cognitive receptor(s) in emergency granulopoiesis remain unclear.

Phospholipase C (PLC) is an important cell signaling enzyme that can be activated by stimulation of cell surface receptors such as G-protein coupled receptors or receptor tyrosine kinase (14). PLC hydrolyzes membrane phospholipid phosphoinositide 4,5-bisphosphate to inositol 1,4,5-trisphosphate and diacylglycerol. The generated inositol 1,4,5-trisphosphate can subsequently induce cytosolic calcium increase while diacylglycerol can activate protein kinase C (14). Several different isoforms of PLC have been reported, including PLC$\beta$ (B1-B4), PLC$\gamma$ (\gamma1,\gamma2), PLC$\delta$ (D1-D4), PLC$\epsilon$, and PLC$\zeta$. These PLC isoforms can mediate diverse cellular responses including cell proliferation and cell migration (15).
Some PLC isoforms are also associated with tumorigenesis and cancer metastasis (16, 17). However, the functional role of PLC in the regulation of emergency granulopoiesis has not been reported yet.

The objective of this study was to determine whether WKYMVM administration could stimulate emergency granulopoiesis and characterize the functional role of FPR2 and PLC in WKYMVM peptide-stimulated emergency granulopoiesis.

RESULTS

WKYMVM increases neutrophil number in peritoneal fluid and peripheral blood in sepsis

Neutrophils (CD11b+ Ly6G+) play essential roles in defense against polymicrobial sepsis by killing invading bacteria (18). Since WKYMVM administration elicited strong bacterial killing activity in CLP sepsis mice (13), we investigated whether this peptide could affect neutrophil recruitment into the event area. CLP surgery caused an increase of neutrophils in peritoneal fluid. Administration of WKYMVM in CLP mice further increased the number of neutrophils in peritoneal fluid (Fig. 1A). Maximal activity of neutrophil increase in the peritoneal fluid was observed after administration of WKYMVM at 8 mg/kg (Fig. 1A). We also found that WKYMVM increased neutrophil number in peripheral blood in CLP mice in a dose-dependent manner, showing maximal activity at 8 mg/kg (Fig. 1B).

WKYMVM stimulates emergency granulopoiesis

Since we found that WKYMVM administration elicited an increase of neutrophil count in peripheral blood in the CLP sepsis model (Fig. 1B), we investigated whether WKYMVM could affect neutrophil number in CLP mice. Neutrophil numbers in bone marrow (BM) were compared between vehicle- and WKYMVM-administrated CLP mice. WKYMVM administration increased neutrophil number in the BM in the CLP sepsis model (Fig. 2A). We then examined effects of WKYMVM administration on the generation of GMP, a neutrophil progenitor. After gating Lin+ populations, LK (Lin−c-Kit−Sca-1−) cell population was further analyzed by staining with anti-CD34 and anti-FcγRI/III (CD16/32). GMP was identified as CD34+CD16/32+ as shown in Fig. 2B. WKYMVM administration significantly increased the number of GMP in CLP sepsis mice (Fig. 2B, 2C). We also examined whether WKYMVM administration increased GMP population in non-CLP mice. Administration of WKYMVM at different doses in non-CLP mice did not significantly affect GMP population (Fig. 2D). These results suggest that WKYMVM can specifically stimulate emergency granulopoiesis by increasing GMP population.

WKYMVM-stimulated emergency granulopoiesis is mediated by FPR2

WKYMVM administration increased neutrophil number in the CLP sepsis model (Fig. 1). The stimulatory effect of WKYMVM on the generation of neutrophils was mediated by enhancing
the generation of neutrophil precursors, GMP (Fig. 2B, 2C). It has been previously reported that WKYMVm can bind to FPR1 and FPR2 in mice (11, 12). In this study, we examined whether LK cells, progenitors of neutrophils, could express FPR1 and FPR2 using RT-PCR analysis. As shown in Fig. 3A, LK cells expressed both FPR1 and FPR2. Since WKYMVm enhanced emergency granulopoiesis in CLP mice, we investigated whether FPR expression in LK cells was changed in septic mice. FPR2 expression was not affected by CLP operation. However, FPR1 expression in LK cells was slightly decreased (Fig. 3B). We then investigated which receptor was involved in this peptide-stimulated granulopoiesis using an FPR1 antagonist (cyclosporine H, CsH) or an FPR2 antagonist (WRW4) (19). As shown in Fig. 3C, WKYMVm-stimulated emergency granulopoiesis was significantly inhibited by WRW4, but not by CsH. These results suggest that FPR2 can mediate WKYMVm-stimulated emergency granulopoiesis in CLP sepsis mice. WKYMVm-elicted emergency granulopoiesis was only observed from littermate wild-type (WT) mice, but not from FPR2 knockout (KO) mice (Fig. 3D). These results strongly support the notion that FPR2 plays an essential role in WKYMVm-elicted emergency granulopoiesis.

PLC plays a role in WKYMVm-stimulated emergency granulopoiesis

WKYMVm is known to stimulate PLC activity, resulting in strong increase of calcium signaling (7, 9). Thus, we investigated the role of PLC in WKYMVm-induced neutrophil recruitment and neutrophil number increase in CLP mice. We found that WKYMVm-injected further increase of neutrophil recruitment into peritoneal fluid was blocked by U-73122 (a PLC inhibitor), but not by U-73343 (inactive control of U-73122) (Fig. 4A). WKYMVm-induced neutrophil number increase in peripheral blood in CLP mice was also blocked by U-73122 (Fig. 4B).

We also examined the effect of PLC activity on WKYMVm-induced emergency granulopoiesis using a PLC inhibitor. Administration of a PLC inhibitor U-73122 prior to WKYMVm administration inhibited WKYMVm-induced increase in the percentage of c-kit− sca-1− GMP (Fig. 4C). WKYMVm-induced increase in the absolute number of GMP was also significantly blocked by U-73122, but not by U-73343 (Fig. 4D). These results suggest that WKYMVm-stimulated emergency granulopoiesis is mediated by PLC activity.
DISCUSSION

Although emergency granulopoiesis is important for rapid generation of neutrophils in the presence of a systemic bacterial infection (6, 20), the regulatory mechanism has not been characterized yet. Which cell surface receptor is involved in the positive regulation of emergency granulopoiesis remains unclear. In this study, we demonstrate that WKYMVm known to bind to FPR family members can stimulate emergency granulopoiesis. As neutrophil progenitors, LK cells expressed FPR1 and FPR2 (Fig. 3A). WKYMVm-stimulated emergency granulopoiesis was specifically blocked by WRW4, but not by CsH (Fig. 3C). Furthermore, WKYMVm-induced emergency granulopoiesis was significantly blocked by FPR2 deficiency (Fig. 3D). Since WKYMVm is a surrogate agonist for FPR1 and FPR2 in mouse phagocytic cells such as neutrophils and monocytes, our results suggest that activation of FPR family members (especially FPR2) can induce emergency granulopoiesis. To the best of our knowledge, this is the first report to demonstrate functional regulation of emergency granulopoiesis by a chemoattractant G-protein coupled receptor.

FPR recognizes bacterial formyl peptides (21, 22). Systemic bacterial infection may induce the activation of FPR in BM neutrophil precursors such as CMP and GMP. Since FPR can detect bacteria-derived N-formylated peptide (21), recognition of this bacteria-derived entity may initiate an alarm signal to induce emergency granulopoiesis. In this study, we found that the addition of FPR2 antagonist (WRW4) to CLP model could decrease GMP fraction below the level without WKYMVm addition (Fig. 3C). Similarly, FPR2-deficient mice showed decreased GMP fraction without peptide administration (Fig. 3D). These results suggest that FPR2 plays a critical role in granulopoiesis in the absence of exogenous stimuli. However, activation of FPR is insufficient to induce emergency granulopoiesis without CLP operation because WKYMVm administration alone does not induce emergency granulopoiesis (Fig. 2D). WKYMVm-induced emergency granulopoiesis requires CLP. These results suggest that CLP operation-induced septic conditions may contribute to the initiation of emergency granulopoiesis which is mediated by FPR2, at least partly, and that WKYMVm can augment this CLP-initiated emergency granulopoiesis.

The role of PLC activity in emergency granulopoiesis has not been reported yet. In this study, we demonstrated that activation of PLC by WKYMVm stimulation could elicit an increase in the number of neutrophils by upregulating emergency granulopoiesis. To the best of our knowledge, this is the first report to demonstrate the functional role of PLC in GMP generation and emergency granulopoiesis. Since previous studies have demonstrated that stimulation of G-protein coupled receptors can induce the activation of PLCβ isoforms (23, 24), which specific isoform of PLC is involved in WKYMVm-induced emergency granulopoiesis needs to be investigated in the future.

In conclusion, we demonstrate that activation of FPR2 by its surrogate agonist, WKYMVm, can augment emergency granulopoiesis in a CLP model. We also revealed the functional role of FPR2 and PLC in WKYMVm peptide-stimulated emergency granulopoiesis. Our results provide a novel insight into the important function of FPR2 and PLC in neutrophil generation.

MATERIALS AND METHODS

Materials

WKYMVm and WRW4 were synthesized by Anygen (Gwangju, Korea). PLC inhibitor U-73122 and its inactive analogue U-73343 were obtained from Calbiochem (San Diego, CA, USA). CsH was purchased from Enzo life sciences (Farmingdale, NY, USA).

Mice and CLP experimental sepsis model

Male WT 8-week-old C57BL/6 mice were purchased from Orient Bio (Seongnam, Korea). FPR2 KO mice were generated as described previously (25). All experiments involving animals adhered to relevant guidelines and received approval from the Institutional Review Committee for Animal Care and Use at Sungkyunkwan University (Suwon, Korea). The experimental CLP sepsis model was induced as described previously (26, 27). After midline laparotomy, we exposed the cecum, ligated below the ileocecal valve without causing intestinal obstruction, and then punctured the cecum twice with a 22-gauge needle. Sham mice were subjected to the same procedure without puncture of the cecum.

Emergency granulopoiesis and neutrophil population analysis

Emergency granulopoiesis was analyzed according to a previous report (20). Briefly, WT, littermate WT, and FPR2 KO mice used for analysis were males from 7 to 9 weeks old. Cells were surface-stained in PEB buffer (PBS supplemented with 2 mM EDTA and 0.5% BSA) for 30-45 min on ice. Antibodies used for flow cytometry included the following: Percp-Cy5.5-conjugated lineage markers specific for CD3e (Clone: 145-2C110), CD4 (Clone: RM4-5), CD8a (Clone: 53-6.7), CD11b (Clone: M1/70), B220 (Clone: RA3-68B), GR-1 (Clone: RB6-8C5), and Ter119 (Clone: TER119) from Thermo Fisher scientific (Waltham, MA, USA) or BioLegend (San Diego, CA, USA). Other antibodies included those against APC- or PE-conjugated Sca-1 (Clone: D7), c-kit (Clone: 2B8), FITC- or PE-Cy7-conjugated CD16/32 (Clone: 93), and CD34 (Clone: RAM34). Neutrophils were gated by CD11b+ and Ly6G+ (Clone: 1A8). Unstained cells were used as negative controls to establish flow cytometer voltage settings and single-color positive controls were used to adjust the compensation. Flow cytometric data were acquired using the flow cytometer equipped with a laser at Sungkyunkwan University (Suwon, Korea).

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BM neutrophil and LK cell sorting
BM cells were isolated from femurs using HBSS-EDTA solution. The cell suspension was centrifuged at 460 g for 5 min. Resuspended cells were carefully loaded onto 40%, 47%, and 60% Percoll gradient and centrifuged at 2,900 g for 30 min without braking. Cells were isolated on the 60%/47% interface layer and red blood cells were removed by hypotonic lysis. Isolated cells were over 90% Ly6G-positive by flow cytometry. For LK cell sorting, BM cells were stained with Percp-Cy5.5-conjugated lineage-specific markers, PE-conjugated c-kit (Clone: 2B8), and APC conjugated Sca-1 (Clone: D7). Cells were then sorted through Lin c-kit Sca-1 for LK cells using FACS AriaIII equipped with FACS Diva software (BD Bioscience).

Reverse transcription-PCR (RT-PCR) analysis
Primer used for RT-PCR included the following: FPR1 forward, 5'-ACAGCCTGACTTTCGAC-3', FPR1 reverse, 5'-CTGGAAAGTTAGAGCCCGTC-3'; FPR2 forward, 5'-GTCAGATCACAAGAAGAAACC-3'; FPR2 reverse, 5'-GGGCTCTCTCAGACATAAAGG-3'; GAPDH forward, 5'-TCCACCCGCTGTGCTGTA-3'; GAPDH reverse, 5'-AATGTGTCCGTCGTGGATCT-3'. RT-PCR was performed with 34 PCR cycles of 94°C (denaturation, 30 s), 62°C (annealing, 45 s), and 72°C (extension, 1 min). PCR products were electrophoresed on 1% or 2% agarose gel and visualized after ethidium bromide staining.

Statistical analysis
All results were evaluated using GraphPad prism software. Statistical analysis was done using one-way analysis of variance (ANOVA) followed by Student's t-test. All results are expressed as mean ± SEM. A P value < 0.05 was considered statistically significant.

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CONFLICTS OF INTEREST
The authors have no conflicting interests.

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