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Functional Expression of Formyl Peptide Receptor Family in Human NK Cells

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We determined the expression of the formyl peptide receptor (FPR) family and the functional roles of the FPR family in NK cells. All tested human NK cells express two members of the FPR family (FPR1 and FPR2). The expression of FPR3 was noted to occur that is primarily expressed in phagocytic cells, such as neutrophils, monocytes, and dendritic cells (DCs). Members of the FPR family also play an important role in host defense against pathogens (8, 9) and have been identified in association with three cell surface G proteins as follows: FPR1 (FPR), FPR2 (FPRL1), and FPR3 (FPRL2) (8, 9). Additionally, several ligands in the FPR family have been identified, including a bacterial peptide N-formyl-Met-Leu-Phe (fMLF), HIV-1 envelope peptides (T20 and T21), host-derived agonist annexin I, and a synthetic peptide (Trp-Lys-Tyr-Met-Val-O-Met (WKYMVm)) (10–13). The FPR2 ligand includes host-derived agonists (LL-37, lipoxin A₄, and serum amyloid A) and synthetic peptides (WKYMVm and MMK-1) (14–18). Moreover, the FPR3 ligand includes Helicobacter pylori-derived Hp2–20 (19), the heme-binding protein cleavage product F2L (Ac-ML GMIKNSLFGSVETPWQVL) (20), and a synthetic peptide (WKYMVm) (21). Members of the FPR family play an important immunologic function, including host defense against pathogens via the modulation of chemotactic migration and superoxide generation in human monocytes, as well as in neutrophils (8, 9). Furthermore, DCs, which play a key role in the regulation of adaptive immune responses, have also been found to express receptors from members of the FPR family (22). Also, activation of the FPR family induces the chemotactic migration of DCs (21), and activation of FPR by the HIV-derived peptide T-20 suppresses IL-12 production via human monocytes (23). Although members of the FPR family play an important role in immune responses via the modulation of cellular activities in monocytes, neutrophils, and DCs, the expression of FPR family members and the effects of their ligands on NK cell activity have not been fully investigated.

In the present study, we investigated the expression of members of the FPR family in NK cells for the functional role of FPR family

N atural killer cells play an important role in innate and adaptive immune responses. Initially, NK cells were discovered as a small population of blood lymphocytes (1). Previous studies have demonstrated that NK cells kill tumor cells, as well as virally infected cells (2, 3). As a result of this activity, NK cells have been regarded as potential agents in the treatment of cancer and infectious diseases. NK cells are primarily found in circulating blood and in the spleen; however, NK cell-specific activity requires the migration of NK cells toward the infected sites or tumor cell-localizing sites. It has been reported that several chemokines and lysophospholipids stimulate the chemotactic migration of NK cells (4–7). However, the extracellular signals that regulate NK cell migration and lysis activity have not been fully identified.

A formyl peptide receptor (FPR) is a chemoattractant receptor that is primarily expressed in phagocytic cells, such as neutrophils, monocytes, and dendritic cells (DCs). Members of the FPR family also play an important role in host defense against pathogens (8, 9) and have been identified in association with three cell surface G proteins as follows: FPR1 (FPR), FPR2 (FPRL1), and FPR3 (FPRL2) (8, 9). Additionally, several ligands in the FPR family have been identified, including a bacterial peptide N-formyl-Met-Leu-Phe (fMLF), HIV-1 envelope peptides (T20 and T21), host-derived agonist annexin I, and a synthetic peptide (Trp-Lys-Tyr-Met-Val-O-Met (WKYMVm)) (10–13). The FPR2 ligand includes host-derived agonists (LL-37, lipoxin A₄, and serum amyloid A) and synthetic peptides (WKYMVm and MMK-1) (14–18). Moreover, the FPR3 ligand includes Helicobacter pylori-derived Hp2–20 (19), the heme-binding protein cleavage product F2L (Ac-ML GMIKNSLFGSVETPWQVL) (20), and a synthetic peptide (WKYMVm) (21). Members of the FPR family play an important immunologic function, including host defense against pathogens via the modulation of chemotactic migration and superoxide generation in human monocytes, as well as in neutrophils (8, 9). Furthermore, DCs, which play a key role in the regulation of adaptive immune responses, have also been found to express receptors from members of the FPR family (22). Also, activation of the FPR family induces the chemotactic migration of DCs (21), and activation of FPR by the HIV-derived peptide T-20 suppresses IL-12 production via human monocytes (23). Although members of the FPR family play an important role in immune responses via the modulation of cellular activities in monocytes, neutrophils, and DCs, the expression of FPR family members and the effects of their ligands on NK cell activity have not been fully investigated.

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agonists in NK cells, as well as the signaling pathway involved in this process.

Materials and Methods

Materials

RPMI 1640 medium and FBS were obtained from Invitrogen. The synthetic peptides WKYMVm (24), MMK-1 (18), and acetylated F2L (20) were synthesized by Anygen. Pertussis toxin (PTX), 2-αminoo-3-me-thyloxyflavone (PD98059), 4-(4-fluorophenyl)-2-(4-methylsulfonylphen-yl)-5-(4-pyridyl)1H-imidazole (SB203580), and antibr[1,9-cd]pyrra-zol-6(2H)-one (SP600125) were purchased from Calbiochem. All of the Abs for phospho-MAPKs were purchased from Cell Signaling Technology. Mouse anti-FPR1, anti-FPR2, anti-FPR3, and FITC-conjugated anti-mouse IgG were purchased from R&D Systems. PE-Cy5-conjugated anti-CD107a was obtained from BD Biosciences. The HRP-conjugated Abs to rabbit IgG were purchased from Kirkegaard & Perry Laboratories.

The isolation of NK cells from peripheral blood

Peripheral blood was collected from healthy donors, and the human peripheral mononuclear cells were isolated by a lymphocyte separation medium, as described previously (25). Peripheral blood NK cells were isolated using a blood NK cell isolation kit (Miltenyi Biotec) from peripheral mononuclear cells. Briefly, non-NK cells (i.e., T cells, B cells, stem cells, DCs, monocytes, granulocytes, and erythroid cells) were magnetically labeled and depleted using a cocktail of biotin-conjugated Abs and the NK Cell MicroBead Cocktail, according to the manufacturer’s instructions (Miltenyi Biotec). The purity of isolated NK cells was analyzed with a FACS analysis using Abs against CD3 and CD56. The purity of isolated CD56+CD3− cells was >97%.

Isolation of human monocytes and neutrophils

Peripheral blood was collected from healthy donors, and human peripheral mononuclear cells were separated on a Histopaque-1077 gradient. After two washes with HBSS, 50% without Ca2+ and Mg2+, the peripheral mononuclear cells were suspended in 10% FBS containing RPMI 1640 and incubated for 60 min at 37°C to let the monocytes attach to the culture dish. The cells were washed five times with warmed RPMI 1640 medium to washout lymphocytes, and then the attached monocytes were collected as described previously (26, 27). Human neutrophils were isolated according to the standard procedures of dextran sedimentation, hypotonic lysis of erythrocytes, and a lymphocyte separation medium gradient as described previously (27, 28). The isolated human leukocytes were then used promptly.

FACS analysis

Isolated NK cells were incubated with mAbs against FPR1, FPR2, or FPR3 for 30 min, followed by washing with cold PBS, and incubated with FITC-conjugated anti-mouse IgG for 20 min. The samples were analyzed using a FACSCalibur (Beckman Coulter). The expression of CD107a was analyzed according to a previous report (29). Purified resting NK cells were resuspended at 2 × 106 cells/ml in RPMI 1640 containing 10% FBS, 2 mM t-glutamine, and 50 IU/ml penicillin. The cells were incubated with K562 cells at an E:T ratio of 10:1 and then stimulated with medium alone or with several concentrations of WKYMVm (0, 0.01, 0.1, or 1 µM). CD107a-PE-Cy5 Ab was added directly to the tubes. The cells were incubated for 1 h at 37°C in 5% CO2, after which monensin was added at a final concentration of 6 µg/ml and incubated for an additional 5 h at 37°C in 5% CO2. The NK cells were then analyzed by way of a FACS analysis (Beckman Coulter).

NK cytolytic assay

The cytolytic activity of NK cells was determined in a colorimetric assay based on the measurement of lactate dehydrogenase (LDH) activity released from the cytols of lysed K562 target cells (a human erythroleukemia cell line) into the supernatant with a cytotoxicity detection kit (Roche Diagnostics), as described previously (24). Briefly, 100 µl of NK cells (2 × 104 cells/ml) and 100 µl of K562 cells (2 × 105 cells/ml) were incubated in round-bottom microtiter plates in triplicate for 4 h (E:T ratio of 10:1) in a humidified atmosphere (5% CO2 at 37°C). The plates were then centrifuged for 10 min at 250 × g, and 100 µl of the supernatant was transferred to a 96-well flat-bottom microtiter plate. The activity, as a result of released LDH, was determined using the cytotoxicity detection kit. The maximum release was determined by the incubation of 100 µl of target cells in addition to 100 µl of medium at a final concentration of 1% Triton X-100.

Stimulation of NK cells with WKYMVm for Western blot analysis

NK cells (2 × 106) were stimulated with WKYMVm at several concentrations at predetermined times. After stimulation, the cells were washed with serum-free RPMI 1640 medium and lysed in lysis buffer (20 mM HEPES (pH 7.2), 10% glycerol, 150 mM NaCl, 1% Triton X-100, 50 mM NaF, 1 mM Na3VO4, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 1 mM PMSF). Detergent-insoluble materials were pelleted by centrifugation (12,000 rpm for 15 min at 4°C), followed by the removal of the soluble supernatant fraction, and then used immediately or stored at −80°C until subsequent use. The protein concentrations in the lysates were determined using the Bradford protein assay reagent.

Electrophoresis and immunoblot analysis

Protein samples were prepared for electrophoresis and subsequently separated using a 10% SDS-polyacrylamide gel and buffer system, as described previously (30). Following electrophoresis, the protein samples were blotted onto a nitrocellulose membrane, which were then blocked by incubation in Tris-buffered saline and 0.05% Tween 20 (TBST) containing 5% nonfat dried milk. Next, the membranes were incubated with anti-phospho-ERK Ab, anti-phospho-Akt kinase Ab, or anti-ERK Ab and then washed with TBST. The Ag-Ab complexes were visualized using an ECL detection system by incubating membranes with 1:5000 diluted goat anti-rabbit IgG or goat anti-mouse IgG Ab coupled with HRP.

Cytokine assays

NK cells were pretreated with a MEK inhibitor (50 µM PD98059), a p38 MAPK inhibitor (20 µM SB203580), or a JNK inhibitor (20 µM SP600125) before WKYMVm stimulation for 24 h. Next, NK cells (5 × 106 cells/ml) were treated with vehicle or WKYMVm for 24 h. Lastly, culture supernatants were analyzed by ELISA for IFN-γ, according to the manufacturer’s instructions (BD Biosciences).

Chemotaxis assay

Chemotaxis assays were performed in multiwell chambers (Neuroprobe), as described previously (30). Briefly, isolated human NK cells were incubated with 100 IU/ml IL-2 containing RPMI 1640 medium for 4 h before the chemotaxis assay. Then, the cells were harvested, suspended in RPMI 1640 at 1 × 106 cells/ml, and placed into the upper well of a chamber. Next, the aliquot was separated by a 5-µm polyhydrocarbon filter from the peptide-containing lower well. The migrated cells were comprised of stained cells with hematoxylin, which were subsequently counted in five randomly chosen high-power fields (×400) (30). The results are presented as chemotaxis indexes, which represent the number of stimuli-induced migrated cells per number of RPMI 1640 medium-induced cells.

Statistics

The differences between treatment groups were analyzed using Student’s t test.

Results

Expression of FPR family in NK cells

In this study, we determined which of the three members of the FPR receptor family are expressed on NK cells by performing FACS analysis using three Abs against the FPR family. As shown in Fig. 1, isolated human monocytes were stained with anti-FPR1, anti-FPR2, or anti-FPR3 Ab. Human neutrophils were stained with anti-FPR1 and anti-FPR2, but not with anti-FPR3 Ab. Taken together, these results confirm that human monocytes express FPR1, FPR2, and FPR3; human neutrophils express FPR1 and FPR2, but not FPR3. When we isolated NK cells from each FPR family Ab, NK cells stained with anti-FPR1 Ab or anti-FPR2 Ab (Fig. 1). Only some donor-derived NK cells were stained with anti-FPR3 Ab; the other donor-derived NK cells were not stained with anti-FPR3 Ab (Fig. 1).

Activation of FPR family induces cytolytic activity of NK cells

NK cells perform a crucial role in cancer immune responses by way of cytolytic activity (2, 3). In this study, we determined the effect of the FPR receptor family on NK cell cytolytic activity.
FIGURE 1. Expression of FPR family in human NK cells. Cell surface expression of FPR1, FPR2, and FPR3 was determined by flow cytometric analysis. The results are representative of at least three independent experiments.

Using several FPR family agonists. The stimulation of resting NK cells with fMLF, MMK-1, F2L, and WKYMVm increased the cytolytic activity in a concentration-dependent manner (Fig. 2A). The EC_{50} of each agonist was 9.2, 11.3, 30.3, and 131.9 nM for WKYMVm, fMLF, MMK-1, and F2L, respectively. Stimulation of IL-2-activated NK cells with WKYMVm did not increase cytolytic activity (data not shown). Up-regulation of CD107a was associated with degranulation and cytolytic activity from NK cells. The effect of WKYMVm on the up-regulation of CD107a was measured. Stimulation of NK cells with WKYMVm stimulated the up-regulation of CD107a in a concentration-dependent manner in resting NK cells (Fig. 2B). Since WKYMVm has been reported to bind the FPR family (13, 17, 21), we determined whether WKYMVm stimulates cytolytic activity in resting NK cells via the FPR family using the two FPR family-selective antagonists (cyclosporin H (CsH) and WRW4 (WRW^4)) (31, 32). WKYMVm-induced NK cell cytolytic activity was partly inhibited by CsH or WRW^4 (Fig. 2C). Moreover, the preincubation of resting NK cells with both of the antagonists completely inhibited WKYMVm-induced NK cell cytolytic activity (Fig. 2C). CsH also completely inhibited the cytolytic activity of fMLF, a FPR1 agonist. WRW^4 inhibited the cytolytic activity of MMK-1, an FPR2 ligand (data not shown). Taken together, the results suggest that WKYMVm stimulates cytolytic activity in NK cells via the FPR family.

We next investigated the regulatory mechanism of WKYMVm-elicted cytolytic activity in NK cells. Previous studies have reported that PTX-sensitive G proteins are involved in FPR family-mediated cellular responses, including chemotactic migration (13, 17, 20). We found that the preincubation of resting NK cells with 100 ng/ml PTX before the cytolytic assay did not affect WKYMVm-stimulated cytolytic activity in NK cells (Fig. 2D), thereby indicating PTX-sensitive G protein independence.

**JNK is involved in WKYMVm-induced cytolytic activity of NK cells**

We examined whether WKYMVm stimulates MAPKs by Western blotting using the anti-phospho-specific Abs against each enzyme. When the resting NK cells were stimulated with 1 \mu M WKYMVm at different times (0, 2, 5, 10, or 30 min), the levels of phosphorylation of ERK, p38 MAPK, and JNK were transiently increased, showing dramatic activity after 2–10 min of stimulation (Fig. 3A). Furthermore, preincubation of resting NK cells with 100 ng/ml PTX before WKYMVm stimulation did not have a significant effect on WKYMVm-stimulated MAPK phosphorylation in NK cells (Fig. 3B), thereby indicating that WKYMVm stimulates MAPK activity independent of PTX-sensitive G proteins. We also examined the roles of ERK, p38 MAPK, and JNK on WKYMVm-induced cytolytic activity of NK cells. We measured the effect of several concentrations of PD98059, SB203580, and SP600125 on WKYMVm-induced NK cell cytolytic activity. Only SP600125 strongly inhibited NK cell-mediated cytolsysis in a concentration-dependent manner (Fig. 3C). These results indicate that JNK-mediated signaling is involved in WKYMVm-induced cytolytic activity of NK cells.

**Stimulation of NK cells with WKYMVm induces IFN-\(\gamma\) production**

IFN-\(\gamma\) is a key cytokine in the polarization of T cells toward the Th1 phenotype and in NK cell activation (33). Therefore, we determined whether FPR family agonists alter IFN-\(\gamma\) production in NK cells. When resting NK cells were stimulated with fMLF, MMK-1, F2L, and WKYMVm, IFN-\(\gamma\) secretion was dramatically up-regulated (Fig. 4A). Moreover, we determined the role of PTX-sensitive G proteins on WKYMVm-induced IFN-\(\gamma\) production. The results indicated that preincubation of resting NK cells with 100 ng/ml PTX did not affect WKYMVm-stimulated IFN-\(\gamma\) production (Fig. 4B); thus, WKYMVm stimulates IFN-\(\gamma\) production independent of PTX-sensitive G proteins.
Stimulation of the FPR family induces IL-2-activated NK cell chemotaxis via PTX-sensitive G proteins

Since the FPR family members are chemoattractant receptors, they are involved in the chemotactic migration of phagocytes (8, 9). We investigated the effect of FPR family agonists on NK cell chemotaxis. All of the tested FPR family agonists (fMLF, MMK-1, F2L, and WKYMVm) failed to stimulate the chemotactic migration of resting NK cells (data not shown). Several studies have demonstrated that IL-2 is necessary for proper functioning of NK cells (4, 34). We also investigated the effect of FPR family agonists on the chemotactic migration of NK cells, which were cultured in the presence of IL-2. All of the tested FPR family agonists induced the chemotactic migration of IL-2-activated NK cells as a function of concentration (Fig. 5).

We found that WKYMVm dramatically stimulated chemotactic migration in IL-2-activated NK cells (Fig. 5). Since WKYMVm has been reported to bind the three members of the FPR receptor family (13, 17, 21), we tested whether WKYMVm stimulates chemotactic migration in NK cells via the FPR family using the two FPR family-selective antagonists CsH and WRW4. WKYMVm-induced NK cell chemotaxis was partly inhibited by CsH or WRW4 (Fig. 6A). Moreover, the preincubation of IL-2-activated NK cells with both antagonists completely inhibited WKYMVm-induced NK cell chemotaxis (Fig. 6A), suggesting that WKYMVm stimulates the chemotactic migration in NK cells via the FPR family.

Stimulation of the FPR family induces IL-2-activated NK cell chemotaxis via PTX-sensitive G proteins

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FIGURE 3. Regulation of lysis activity by WKYMVm in human NK cells. Resting NK cells were incubated in the absence (A) or presence (B) of 100 ng/ml PTX, and cells were subsequently stimulated with 1 μM WKYMVm for various times (0, 2, 5, 10, or 30 min). A Western blot analysis was performed using anti-phospho-ERK, anti-phospho-p38 kinase, or anti-phospho-JNK Abs. The results shown are representative of at least six independent experiments (A and B). Resting NK cells were preincubated in the presence or presence of several concentrations of PD98059, SB203580, or SP600125 for 15 min (60 min for PD98059) (C). One hundred microliters of NK cells (2 × 10⁶ cells/assay) was used for the cytolytic assay in the presence of 1 μM WKYMVm (C). The activity of the released LDH was determined using the cytotoxicity detection kit. Data are expressed as the means ± SE of three independent experiments performed in duplicate (C). *, p < 0.05 from the control (vehicle treated).

FIGURE 4. IFN-γ production by WKYMVm in human NK cells. Resting NK cells were incubated with vehicle (DMSO), fMLF (1 μM), MMK-1 (1 μM), F2L (10 μM), or WKYMVm (1 μM) for 48 h (A). Isolated human NK cells were preincubated in the presence or absence of PTX (100 ng/ml) for 20 h. The cells were incubated with vehicle (DMSO) or WKYMVm (1 μM) for 48 h (B). The supernatants were harvested from cultures and evaluated for IFN-γ production by ELISA. The data are expressed as the means ± SE of three independent experiments performed in duplicate (A and B). *, p < 0.05 from the control (vehicle treated).

FIGURE 5. Stimulation of NK cell chemotaxis by FPR family agonists. IL-2-activated NK cells (1 × 10⁶ cells/ml in serum-free RPMI 1640) were used for the chemotaxis assay in the presence of several concentrations (0, 0.001, 0.1, or 1 μM for fMLF, MMK-1, and WKYMVm; 0, 0.1, 1, 10, or 20 μM for F2L) of peptides for 4 h at 37°C (A–D). The number of cells that migrated was determined by counting five high-power fields (×400). The data are expressed as the means ± SE of three independent experiments performed in duplicate (A–D). *, p < 0.05 from the control (vehicle treated).
receptors elicits NK cell activation. Cells express the FPR receptor family, and the activation of these receptors results in the up-regulation of CD107a from NK cells. Collectively, human NK cells expressed two members of the FPR receptor family (FPR1 and FPR2); however, some donor-derived NK cells expressed FPR3. From a functional point of view, we observed that the stimulation of NK cells with FPR family agonists stimulated cytolytic activity in resting NK cells, but not in IL-2-activated NK cells, and chemotactic migration of IL-2-activated NK cells, but not of resting NK cells. The agonists also induced IFN-γ production and the up-regulation of CD107a from NK cells. Collectively, human NK cells express the FPR receptor family, and the activation of these receptors elicits NK cell activation.

Additionally, the preincubation of IL-2-activated NK cells with PTX (100 ng/ml) before the chemotaxis assay resulted in a dramatic reduction in the number of cells migrating toward each agonist (Fig. 6A), which strongly suggests the involvement of PTX-sensitive G proteins in this process.

**WKYMVm-induced NK cell chemotaxis is mediated by ERK**

We determined whether WKYMVm stimulates MAPKs in IL-2-activated NK cells by Western blotting with the anti-phospho-specific Abs of each enzyme. When the NK cells were stimulated with 1 μM WKYMVm at different times (0, 2, 5, 10, or 30 min), the levels of phosphorylation of ERK, p38 MAPK, and JNK were transiently increased (Fig. 7A). The preincubation of IL-2-activated NK cells with PTX (100 ng/ml) before WKYMVm stimulation resulted in an almost complete inhibition of WKYMVm-stimulated ERK and JNK phosphorylation (Fig. 7B), thereby indicating the dependence of PTX-sensitive G proteins.

Several chemoattractants stimulate MAPK activities, which in turn are involved in the chemoatactic migration of cells (35, 36). We also examined the roles of ERK, p38 MAPK, and JNK on WKYMVm-induced NK cell chemotaxis. The preincubation of IL-2-activated NK cells with several concentrations (0, 1, 5, 10, 20, or 50 μM) of PD98059 before the chemotaxis assay resulted in the dramatic blocking of WKYMVm-induced NK cell chemotaxis (Fig. 7C). However, WKYMVm-induced NK cell chemotaxis was not inhibited by SB203580 or SP600125 (Fig. 7C). The results indicate that ERK-mediated signaling is involved in WKYMVm-induced NK cell chemotaxis.

**Discussion**

In this study, we determined the functional expression of the FPR family in human NK cells. All tested samples of human NK cells expressed two members of the FPR receptor family (FPR1 and FPR2); however, some donor-derived NK cells expressed FPR3. From a functional point of view, we observed that the stimulation of NK cells with FPR family agonists stimulated cytolytic activity in resting NK cells, but not in IL-2-activated NK cells, and chemotactic migration of IL-2-activated NK cells, but not of resting NK cells. The agonists also induced IFN-γ production and the up-regulation of CD107a from NK cells. Collectively, human NK cells express the FPR receptor family, and the activation of these receptors elicits NK cell activation.

The FPR family is considered to contain an important set of chemoattractant receptors for several human phagocytic cells, for example, monocytes, neutrophils, and DCs (8, 9, 22). However, the expression of FPR family in NK cells and functional roles of the receptors in NK cells has not been fully determined. In this study, we confirmed that NK cells express the FPR family (Fig. 1). All tested NK cells from several donors expressed FPR1 and FPR2 (Fig. 1). However, in the case of FPR3, only some donor-derived NK cells expressed FPR3, showing donor variation (Fig. 1). At this point, it is not clear what caused this differential expression pattern of FPR3 in NK cells from several different donors. Because some previous reports have demonstrated that certain extracellular stimuli can cause the expression of some chemoattractant receptors...
(37, 38), we assumed that FPR3 is selectively expressed in NK cells from donors who had been exposed to certain inflammatory or infectious agents. To test this possibility, we investigated the effect of IL-2 or LPS on the expression of FPR3 in NK cells. Stimulation of resting NK cells with IL-2 or LPS did not affect the expression of FPR3 in NK cells (data not shown). It will be interesting to determine the molecular mechanism involved in the differential expression of FPR3 in different donors.

We also found that the activation of these FPR family members, via their selective agonists, stimulated the cytolytic activity of NK cells (Fig. 2). We tested the effect of FPR agonists on target cells directly by measuring LDH release. No FPR agonists affected LDH release from target cells (data not shown), indicating that FPR agonists stimulate NK cells, resulting in cytolytic activity. An early publication involving large granular lymphocytes has described the chemotaxis toward formylated peptides and binding to a rat NK cell line, supporting our findings with respect to the functional expression of FPR family members (39). Even though we detected that WKYMVM stimulates cytolytic activity in resting NK cells, we failed to see any positive effect in IL-2-activated NK cells (data not shown). Since stimulation of NK cells with IL-2 induces cytolytic activity, addition of WKYMVM in such conditions might not induce a further increase in cytolytic activity.

IFN-γ functions as a linkage between the innate and adaptive immune systems (40). Additionally, IFN-γ polarizes the immune system toward a primary Th1 response (41, 42). Thus, IFN-γ production by NK cells plays a key role in the determination of subsequent immune responses. In this study, we measured IFN-γ production by NK cells and found that the IFN-γ levels dramatically increased within 48 h of exposure to WKYMVM (Fig. 4). Recently, Sung and colleagues (43) demonstrated that WKYMVM enhances the surface expression of CD80 on mouse bone marrow-derived dendritic cells. Moreover, the coinjection of WKYMVM with HIV, HBV, and influenza DNA vaccines enhanced vaccine-induced CD8+ T cell responses in terms of IFN-γ secretion and cytolytic activity, thereby suggesting that WKYMVM can function as a novel adjuvant for a DNA vaccine (43). In this study, we showed that stimulation of human NK cells via a FPR family agonist (WKYMVM) dramatically stimulated IFN-γ production (Fig. 4A). Recently, a study has shown that NK cells and DCs reciprocally activate one another during immune responses (44). Mocikat et al. (45) demonstrated that NK cells were required for IL-12 production by DCs. Moreover, the interaction between NK cells and DCs is required for the generation of CD8+ T cell responses. These results suggest that the activation of the FPR family members by WKYMVM positively modulates the immune responses against tumor Ags or pathogens via activation of NK cells. NK cell-derived IFN-γ production is also important for anti-infective activity (44). IFN-γ mediates host defense against microbes by stimulating macrophage bactericidal activity (46, 47). In this study, we demonstrated that the stimulation of FPR family members via their specific ligands increased IFN-γ production from NK cells (Fig. 4). These results also suggest that the activation of FPR family members enhances anti-infective activity by activating NK cells. It is feasible, therefore, that FPR family ligands can be ultimately developed as anti-infective drugs.

Previous studies have demonstrated that WKYMVM stimulates a variety of intracellular signaling molecules; perhaps the best characterized function is its role in the MAPK pathway (48, 49). In this study, we showed that FPR family activation by WKYMVM induces ERK, p38 MAPK, and JNK activation in human NK cells. To identify the pathway leading to the cytolytic activity and chemotactic migration of NK cells, we treated NK cells with MAPK inhibitors. In the case of the intracellular signaling mechanism for WKYMVM-induced NK cell activation, we found that the ERK and JNK pathways are essential for cytolytic activity or chemotactic migration, respectively, via the activation of the FPR family by WKYMVM in human NK cells (Figs. 3 and 7).

In this study, we found that WKYMVM stimulated chemotactic migration in IL-2-activated NK cells, but not in resting NK cells (Fig. 5 and data not shown). Thus, it is unclear what causes these differential effects by WKYMVM. Since resting NK cells also express the FPR family and are stimulated by WKYMVM, resulting in cytolytic activity, it is reasonable to assume that addition of IL-2 to resting NK cells may cause some changes in cellular content, which are required for the proper migration of NK cells to WKYMVM. Some previous reports have also demonstrated that certain chemokines induce chemotactic migration in IL-2-activated NK cells, but not in resting NK cells (50, 51).

It has been demonstrated that FPR1 and FPR2 mediate the chemotactic migration of phagocytes in a PTX-sensitive manner, indicating that these receptors couple with the G1i subfamily of G proteins (8, 9). More recently, FPR3 has also been reported to induce dendritic cell chemotaxis in a PTX-sensitive manner (20). In this study, we also observed that the preincubation of IL-2-activated NK cells with PTX completely inhibited the WKYMVM-induced chemotactic migration of NK cells (Fig. 6B). From our results and previous reports, there is very strong evidence suggesting that the chemotactic migration of human leukocytic cells via the FPR family is mediated by PTX-sensitive Gi proteins. In our study, however, we found that WKYMVM-stimulated cytolytic activity was inhibited by FPR family antagonists and not by PTX, indicating that WKYMVM stimulates cytolytic activity via the FPR family, but not PTX-sensitive G proteins (Fig. 2, C and D). Collectively, our results strongly indicate that two different signaling pathways are essential for the regulation of chemotactic migration or cytolytic activity in resting or IL-2-activated NK cells downstream of the FPR family. As shown in Fig. 2, WKYMVM stimulated cytolytic activity in resting NK cells, which was not inhibited by PTX (Fig. 2); however, WKYMVM stimulated chemotactic migration in IL-2-activated NK cells, which was inhibited by PTX (Fig. 6B). These results suggest that the FPR family couples with PTX-insensitive G proteins in resting NK cells, yet couple with PTX-sensitive Gi proteins in IL-2-activated NK cells.

In conclusion, our findings provide a new perspective on the roles of the FPR family in the regulation of immune responses via their stimulatory effects on NK cell activity. Furthermore, these findings indicate that the FPR family should be regarded as important therapeutic targets with respect to the modulation of various diseases in which NK cells play a role.

Disclosures
The authors have no financial conflicts of interest.

References


