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# F2L, a peptide derived from heme-binding protein, inhibits LL-37-induced cell proliferation and tube formation in human umbilical vein endothelial cells

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**Abstract F2L, a peptide derived from heme-binding protein, was originally identified as an endogenous ligand for formyl peptide receptor-like (FPRL)2. Previously, we reported that F2L inhibits FPR and FPRL1-mediated signaling in neutrophils. Since endothelial cells express functional FPRL1, we examined the effect of F2L on LL-37 (an FPRL1 agonist)-induced signaling in human umbilical vein endothelial cells (HUVECs). F2L stimulated the chemotactic migration in HUVECs. However, F2L inhibited FPRL1 activity, resulting in the inhibition of cell proliferation and tube formation induced by LL-37 in HUVECs. We suggest that F2L will potentially be useful in the study of FPRL1 signaling and the development of drugs to treat diseases involving the FPRL1 in the vascular system.**


*Keywords:* F2L; Formyl peptide receptor-like 1; Chemotaxis; Angiogenesis; Cell proliferation; Endothelial cell

## 1. Introduction

Angiogenesis is a crucial step in the physiological and pathological processes, which include normal embryonic development, wound healing, inflammation, and the metastasis of malignant tumors [1]. Previous studies have shown that various cytokines, including vascular endothelial growth factor, tumor necrosis factor-α, and several chemokines increase chemotactic migration as well as the stimulation of angiogenic activity in endothelial cells [2–4]. Bioactive lipid molecule, sphingosine 1-phosphate (S1P), has also been reported to modulate some physiological activities in endothelial cells [5–7]. S1P induces chemotactic migration and angiogenesis in human umbilical vein endothelial cells (HUVECs) [5–7]. Since the activity of endothelial cells is vital for the regulation of several vascular biological events which result in angiogenesis and vascular inflammation, the identification of molecules inhibiting endothelial cell-mediated signaling will consequently be very important for the treatment of vascular diseases.

The formyl peptide receptor (FPR) family is a class of characteristic chemoattractant receptors, which are highly expressed in phagocytic cells such as neutrophils, monocytes, and dendritic cells [8–10]. In addition, the FPR, FPR-like (FPRL)1, and FPRL2 are three members of the FPR family which have been reported in humans [8–10]. The activation of a member of the FPR family mediates the chemotactic migration of phagocytes. On the signaling mechanism involved in FPR family-mediated chemotactic migration, pertussis toxin (PTX)-sensitive $G_i$-protein(s)-mediated signaling has been known to play a key role. Chemotactic migration of human monocytes or neutrophils by FPR family agonists including serum amyloid A and His-Arg-Tyr-Leu-Phe-Met was completely inhibited by PTX [11,12]. Furthermore, this indicates that these receptors are coupled with the $G_i$ subfamily of G proteins [8–10], and consequently play an important functional role in the regulation of defense activities against pathogen infection by modulating the activities of phagocytes. These receptors were found to be involved in chemotaxis, superoxide generation, and exocytosis in human neutrophils [8–10]. In addition, FPRL1 (a member of the FPR family) is also expressed in human endothelial cells, and its specific agonists [LL-37 and Trp-Lys-Tyr-Met-Val-D-Met (WKYMVm)] have been reported to activate FPRL1 and inducing cell proliferation and angiogenesis [13].

Previous studies have reported various types of ligand belonging to the FPR [8–10]. In particular, F2L (Ac-MLGMIKNSLFGSVETWPWQVL) has been identified as an endogenous FPRL2 ligand originating from the heme-binding

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*Abbreviations:* FPR, formyl peptide receptor; FPRL, FPR-like; PTX, pertussis toxin; WKYMVm, Trp-Lys-Tyr-Met-Val-D-Met; F2L, Ac-MLGMIKNSLFGSVETWPWQVL; HUVECs, human umbilical vein endothelial cells; EBM, endothelial basal medium; $WRW^4$, WRWWWW; FBS, fetal bovine serum; S1P, Sphingosine 1-phosphate; HPF, high power fields

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protein cleavage product [14]. F2L is also known as an agonist for the low affinity fMLF receptor, FPR2 [15]. We very recently demonstrated that F2L stimulates the chemotactic migration of human neutrophils via FPR and FPRL1; however, we also found that it blocks the calcium signaling and superoxide generation induced by fMLF, MMK-1, or WKYMVm in human neutrophils [16]. In this study, we investigated the effect of F2L on HUVECs with respect to chemotactic migration, proliferation, and tube formation.

## 2. Materials and methods

### 2.1. Reagents

The HUVECs and endothelial basal medium (EBM) were purchased from Cambrex Corp. (East Rutherford, NJ, USA). The acetylated F2L [14], LL-37 [17], and WRWWWW ($WRW^4$) [18] were synthesized from Anygen Co. Ltd. (Gwang-ju, Korea) and exceeded 95% purity. In addition, the RPMI 1640 medium and fetal bovine serum (FBS) were obtained from the Invitrogen Corp. (Carlsbad, CA, USA). Moreover, S1P was purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Finally, the PTX was purchased from Calbiochem (San Diego, CA, USA).

### 2.2. Cell cultures

The HUVECs were cultured on a 0.02% gelatin-coated (Sigma, St. Louis, MO) 10-cm dish in EBM supplemented with 10% FBS, 0.2 ml of hydrocortisone, 2 ml of recombinant human fibroblast growth factor-B, 0.5 ml of recombinant human vascular endothelial growth factor, 0.5 ml of recombinant insulin-like growth factor-1, 0.5 ml of ascorbic acid, 0.5 ml of recombinant human epidermal growth factor, 0.5 ml of gentamycin sulfate amphotericin-B, and 0.5 ml of heparin. The HUVECs were used for experiments between passages 3 and 10.

### 2.3. Chemotaxis assay

Chemotaxis assays were performed using multiwell chambers (Neuroprobe Inc., Gaithersburg, MD) as described previously [19]. In brief, we pre-coated the polycarbonate filters (8 μm pore size) with 20 μg/ml of fibronectin in a 0.25% acetic acid solution. In addition, a dry coated filter was likely placed on a 96-well chamber containing different concentrations of stimuli. The HUVECs were suspended in RPMI at a concentration of $1 \times 10^6$ cells/ml. Furthermore, 25 μl of the cell suspension was placed onto the upper well of the chamber. After a 4 h incubation period at 37 °C, non-migrating cells were removed by scarping, and cells that migrated across the filter were dehydrated, fixed, and stained with hematoxylin (Sigma). The stained cells were assigned to three randomly selected high power fields (HPF, 400×), which were then counted for each well.

### 2.4. Cell proliferation assay

Endothelial cell proliferation was determined by DNA synthesis. In brief, HUVECs were seeded at a density of $2 \times 10^4$ cells/well in gelatin-coated 24-well plates, followed by allowing for cell growth and then permitted to attach overnight. The cells were washed twice with EBM and incubated for 4 h in M199 containing 1% FBS. The cells were stimulated with each peptide for 48 h and [$^3$H]-thymidine (1 μCi) was added to each well just prior to the final 12 h of incubation [20].

### 2.5. Tube formation assay

Tube formation activity of the HUVECs was measured as previously described [21]. Briefly, 48-well culture plates were coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. HUVECs were seeded on a layer of previously polymerized Matrigel with each stimulus. After 18 h of incubation, the cell morphology was visualized via phase-contrast microscopy and photographed. The degree of tube formation was quantified by measuring the length of tubes in three randomly chosen low-power fields (50×) from each well, using the image-Pro Plus v4.5 (Media Cybernetics, San Diego, CA).

### 2.6. Statistical analysis

The results are expressed as the means ± S.E. of the number of determinations indicated. The statistical tests used to assess the differences were determined by a Student's *t*-test. Statistical significance was set at a *P*-value $<0.05$.

## 3. Results

### 3.1. F2L stimulates chemotactic migration in HUVECs

Since chemotactic migration is a representative function of endothelial cells, we examined the effect of F2L on the HUVEC chemotaxis. When we stimulated HUVECs with several concentrations of F2L, we observed that F2L induced the chemotactic migration of HUVECs in a concentration-dependent manner (Fig. 1A). The F2L-induced HUVEC chemotaxis was apparent at 1–20 μM (Fig. 1A). Another FPRL1 agonist (LL-37) also stimulated HUVEC chemotaxis in a concentration-dependent manner and showing maximal activity around 10 μM (Fig. 1B). Furthermore, chemotactic migration induced by F2L or LL-37 was completely inhibited by PTX (Fig. 1C), which highlights the importance of PTX-sensitive G-proteins in chemotactic migration.

### 3.2. F2L-induced HUVEC chemotactic migration is mediated by FPRL1

Since F2L has been reported to induce the chemotactic migration of mouse neutrophils via FPR2 (mouse counterpart of human FPRL1) [15], we tested whether F2L stimulates the chemotactic migration of HUVECs via FPRL1, with the aid of an FPRL1-selective antagonist ($WRW^4$) [18]. The F2L-induced HUVEC chemotaxis was completely inhibited by $WRW^4$ (Fig. 2). The LL-37-induced HUVEC chemotaxis was also almost completely inhibited by $WRW^4$ (Fig. 2). However, we found the S1P-induced HUVEC chemotaxis not to be inhibited by $WRW^4$, which suggests that F2L stimulates HUVEC chemotaxis via FPRL1.

### 3.3. F2L inhibits LL-37-induced endothelial cell proliferation

HUVECs are endothelial cells, which express functional FPRL1 [14,22], whilst LL-37 (an agonist for FPRL1) has previously been reported to induce endothelial cell proliferation [13,23]. We found that the stimulation of HUVECs with 5 μM of LL-37 elicited cell proliferation (Fig. 3). However, F2L alone (20 μM) did not affect HUVEC proliferation (Fig. 3). We subsequently investigated the influence of F2L on LL-37-induced endothelial cell proliferation. The stimulation of HUVECs with F2L (20 μM) prior to the incubation with LL-37 (5 μM) for 48 h, caused an almost complete inhibition of LL-37-induced cell proliferation, compared to cells treated with LL-37 alone (Fig. 3). These results indicate that F2L inhibits LL-37-induced endothelial cell proliferation. Previous reports have also indicated that S1P induces endothelial cell proliferation [24–26]. However, S1P-induced HUVEC proliferation was not inhibited by F2L, suggesting that F2L selectively inhibits HUVEC proliferation through the inhibition of FPRL1 (Fig. 3).

### 3.4. The stimulation of HUVECs with F2L inhibits angiogenesis by LL-37

LL-37 has been reported to induce physiologic and pathologic angiogenesis in vivo [13,27]. In addition, FPRL1 has been

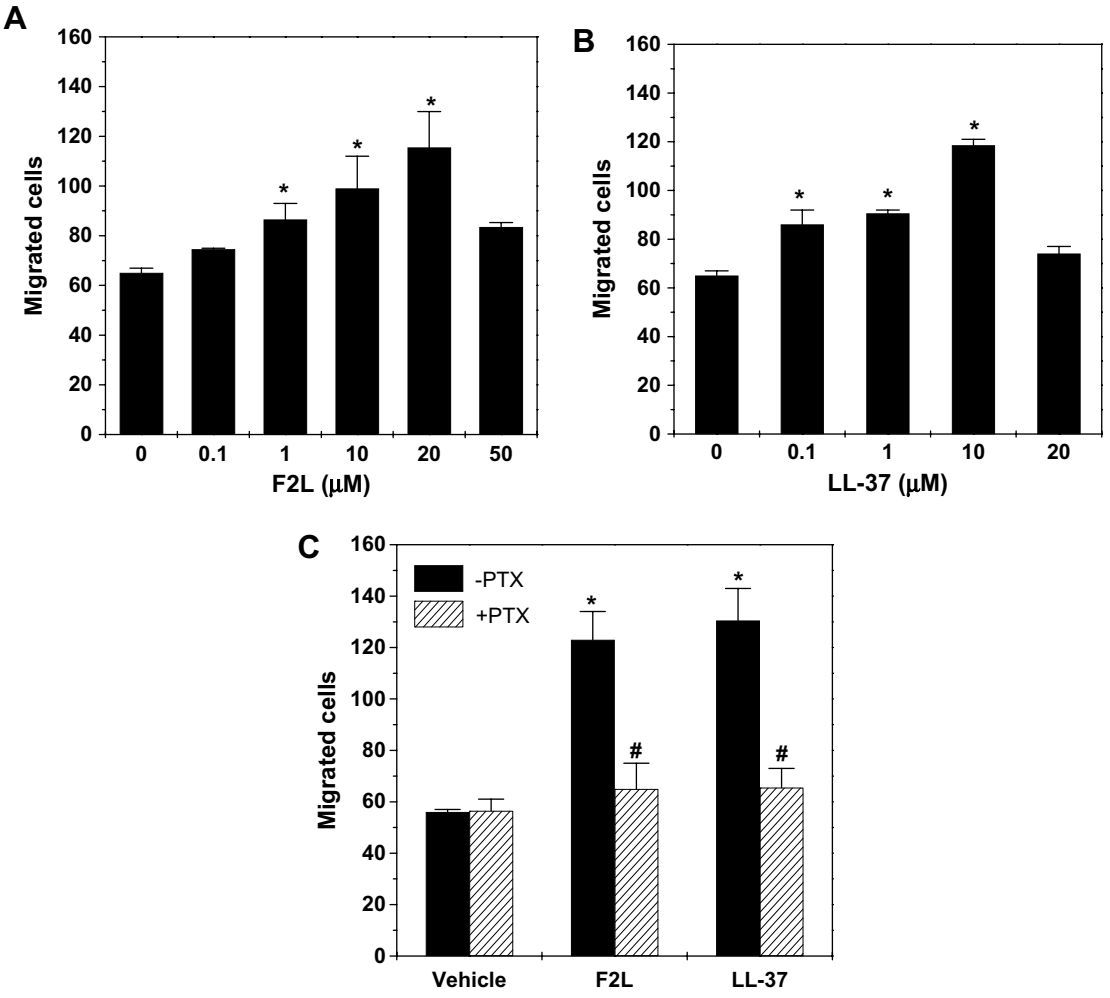


Fig. 1. The effect of F2L on the chemotactic migration in HUVECs. The HUVECs ($1 \times 10^6$ cells/ml of serum free RPMI 1640 medium) were added to the upper wells of a 96-well chemotaxis chamber and assessed for the migration across a membrane with an 8 μm pore size after incubation at 37 °C for 4 h. Various concentrations of F2L (A) or LL-37 (B) were used for the chemotaxis assays. Next, the HUVECs were incubated in the presence or absence of 100 ng/ml of PTX for 20 h. Following this, we added cultured HUVECs ($1 \times 10^6$ cells/ml of serum free RPMI 1640 medium) to the upper wells of a 96-well chemotaxis chamber, and calculated the migration across a membrane with an 8 μm pore size after incubation at 37 °C for 4 h. The number of cells which migrated across the membrane was determined by counting the cells in three HPF (400×). The data are expressed as the means ± S.E. of three independent experiments which were performed in duplicate (A–C). *Statistical significance was set at $P < 0.05$ against the control (vehicle). #Number of statistically significant values ($P < 0.05$) against the control (-PTX).

implicated in the tube formation of HUVECs stimulated with LL-37 [13,23]. Hence, we attempted to determine whether F2L alters tube formation in the LL-37-treated HUVECs. We observed significant tube formation when 5 μM LL-37 was applied during tube formation assays (Fig. 4). Moreover, F2L alone had no effect on tube formation (Fig. 4). However, the addition of 20 μM of F2L, coupled with LL-37, dramatically decreased LL-37-induced tube formation (Fig. 4). In addition, S1P induced the tube formation in HUVECs; however, it was not affected by F2L (Fig. 4). These results suggest that F2L acts upon FPRL1 to block LL-37-induced tube formation.

## 4. Discussion

We found that F2L alone stimulated the chemotactic migration of HUVECs (Fig. 1). In addition, the F2L-induced HUVEC chemotaxis was completely inhibited by an FPRL1 antagonist ($WRW^4$) (Fig. 2). The results strongly suggest that F2L stimulates HUVEC chemotaxis via FPRL1. The result that the F2L-induced cell migration was completely inhibited by PTX (Fig. 1C), supports our notion that F2L stimulates HUVEC chemotaxis via FPRL1. Despite these facts, F2L failed to stimulate cell proliferation and tube formation. Moreover, F2L inhibited signaling induced by the FPRL1 agonist LL-37 (Figs. 3 and 4). In summary, these data strongly suggest that F2L has a dual role as a partial agonist for FPRL1, as well as an inhibitor for the FPRL1 agonist LL-37-induced signalings in HUVECs.

According to previous reports, LL-37 which is made up of an antimicrobial peptide, has multiple roles in innate immunity, linking host defense and inflammation with angiogenesis and arteriogenesis [13,23,28]. In this study, we demonstrated that F2L has an inhibitory effect on the LL-37-induced proliferation of HUVECs (Fig. 3). We also showed that LL-37-induced tube formation was completely inhibited by the

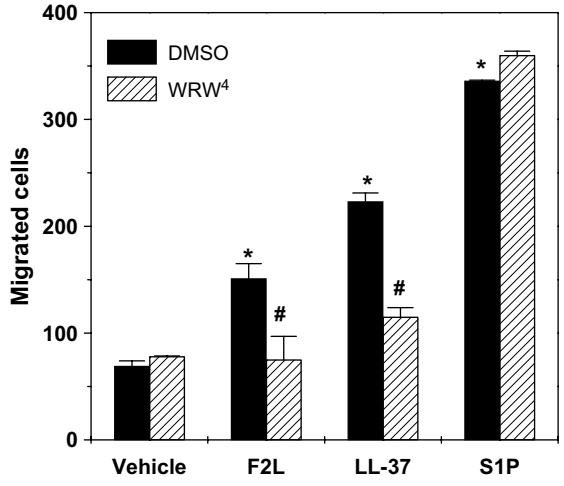


Fig. 2. The effect of the FPRL1 antagonist on the F2L-induced HUVEC chemotaxis. HUVECs were incubated in the presence or absence of 10 μM $WRW^4$ for 15 min prior to the chemotaxis assay using 20 μM of F2L, 10 μM of LL-37, or 2 μM of S1P. The number of cells that migrated across the membrane was determined by counting three HPF (400×). Moreover, the data are expressed as the means ± S.E. of three independent experiments which were performed in duplicate. *Statistical significance was set at ($P < 0.05$) against the control (vehicle). #The number of statistically significant values ($P < 0.05$) against the control (DMSO treated).

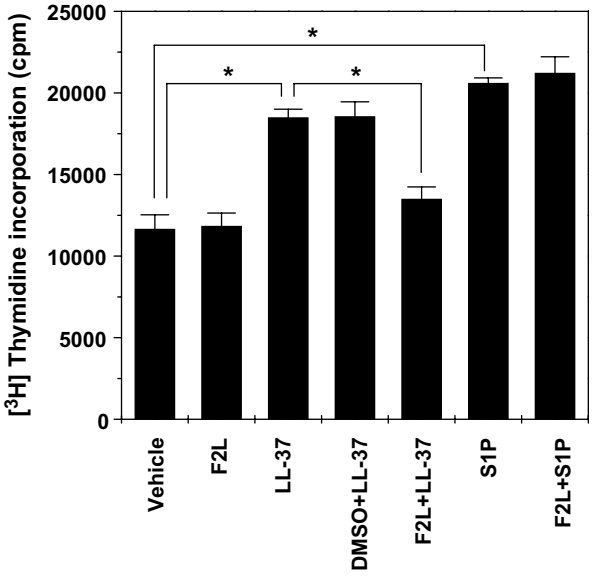


Fig. 3. The effect of F2L on HUVEC proliferation as a function of LL-37. The HUVECs were plated onto 24-well plates in triplicate, followed by stimulation with LL-37 (5 μM), or S1P (100 nM) for 48 h in the presence or absence of F2L (20 μM) pretreatment for 1 h. The cell proliferation was determined by [$^3$H]-thymidine incorporation assays. The data are expressed as the means ± S.E. of three independent experiments which were performed in duplicate. *The results indicate a significant difference at a $P$-value <0.05.

addition of F2L (Fig. 4). These results suggest a new aspect for the role of F2L in HUVECs as a negative modulator of cell proliferation and angiogenesis. The molecular mechanism involved in the LL-37-induced angiogenesis, FPRL1 was found to mediate cellular responses to LL-37 [13]. Furthermore, in a previous report, we clearly demonstrated that F2L directly

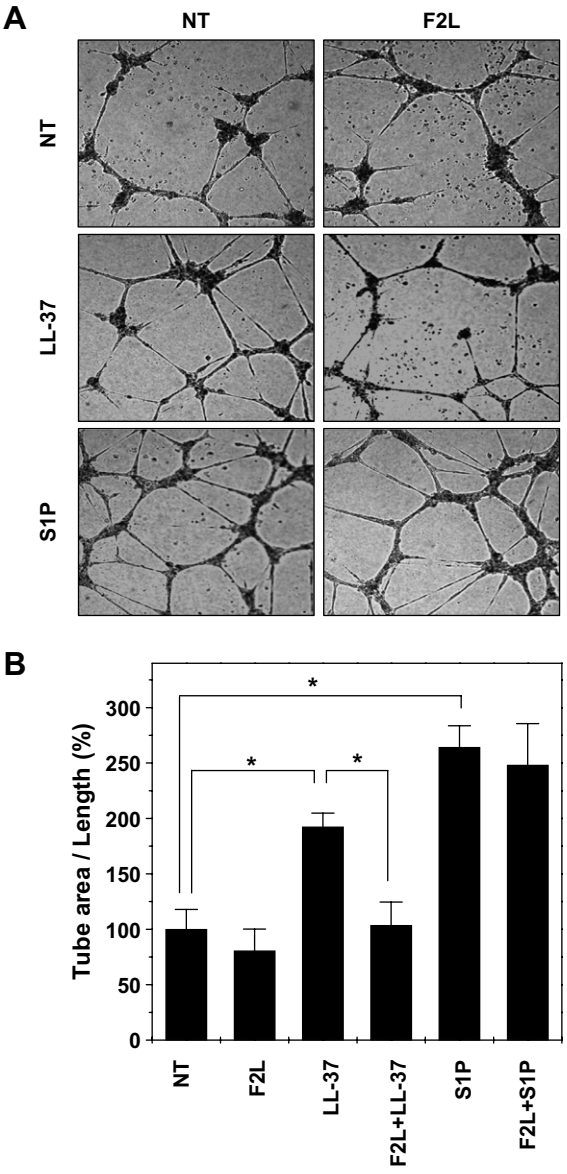


Fig. 4. The effect of F2L on the LL-37-induced tube formation in HUVECs. The HUVECs were seeded onto 48-well plates and precoated with Matrigel. Next, HUVECs was incubated with LL-37 (5 μM) or S1P (100 nM) in the presence or absence of F2L (20 μM). After an 18 h incubation period, we assessed the tube formation by obtaining photographs from an inverted phase contrast microscope image (50×) (A). The bar graph indicates the total length of the tubes formed by the HUVECs (B). The data are expressed as the means ± S.E. of three independent experiments. *The results indicate a significant difference at a $P$-value <0.05.

binds to FPRL1 and competitively inhibits the binding of WKYMVm (an agonist for FPRL1) [16]. To sum up, F2L may inhibit LL-37-induced cell proliferation and tube formation by acting on FPRL1 in HUVECs.

Originally, F2L was identified as a proteolytic fragment of the heme-binding protein [14]. Furthermore, it is known that apoptotic cells release chemotactic factors to attract phagocytes. F2L act as a chemoattractant for monocytes, and dendritic cells that would induce cellular responses against apoptotic cells [14]. Interestingly, we also found that F2L induced the chemotactic migration of endothelial cells, indicat-

ing a role of F2L in endothelial cell functioning. However, the F2L inhibited cell proliferation and tube formation induced by the activation of FPRL1 with LL-37 (an FPRL1 agonist) (Figs. 3 and 4). Since HUVECs express FPRL1, the inhibition of cell proliferation and tube formation by F2L, which is released from the heme-binding protein, must be mediated by antagonizing FPRL1. Taken together, these findings suggest a putative anti-inflammatory role for F2L, through its inhibition of FPRL1 activation in human endothelial cells for a number of vascular inflammatory diseases associated with cell death.

Several previous reports on chemokines have also demonstrated that certain chemokines have dual roles as an agonist for one chemokine receptor and as an antagonist for another receptor [29,30]. For example, CCL26 (eotaxin) which is a natural agonist of CCR3, acts as an antagonist for CCR2 and CCR5 [29,30]. CCL7 (MCP-3) is an agonist of CCR1, CCR2, and CCR3, and has been reported to be a natural antagonist at CCR5, thereby inhibiting the functional responses of CCR5 to other ligands [31]. In this study, we demonstrated that F2L (an agonist for FPRL2) can act as a negative regulator for FPRL1-mediated signaling. Based on our studies, we suggest that multiple FPR family members can also be differentially modulated by the F2L ligand. Although the functional significance of natural antagonism in the FPR family is not fully understood, it may be important for the efficient regulation of FPR family-related responses.

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