Identification of FAM3D as a novel endogenous chemotaxis agonist for the FPRs (formyl peptide receptors)

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Abstract

The family with sequence similarity 3 (FAM3) gene family is a cytokine-like gene family with four members FAM3A, FAM3B, FAM3C, and FAM3D. In this study, we found that FAM3D strongly chemoattracted human peripheral blood neutrophils and monocytes. To identify FAM3D receptor, we used chemotaxis, receptor internalization, calcium flux and radioligand-binding assays in FAM3D-stimulated HEK293 cells that transiently expressed FPR1 or FPR2 to show that FAM3D was a high affinity ligand of formyl peptide receptors (FPR1 and FPR2), both of which were highly expressed on the surface of neutrophils and monocytes/macrophages. After being injected into the mouse peritoneal cavity, FAM3D chemoattracted CD11b+Ly6G+ neutrophils in a short time. In response to FAM3D stimulation, p-ERK and p-p38 were up-regulated in the mouse neutrophils, which could be inhibited by an inhibitor of FPR1 or FPR2. FAM3D was reported to be constitutively expressed in the gastrointestinal tract. We found that FAM3D expression increased significantly in dextran sulfate sodium-induced colitis. Taken together, we propose that FAM3D plays a role in gastrointestinal homeostasis and inflammation through its receptors FPR1 and FPR2.

Keywords: FAM3D, FPR1, FPR2, neutrophils, colitis;
Introduction

Chemokines comprise a large family of structurally homologous small cytokines. The classification of chemokines is based on the distance between the first two of the four to six conserved cysteine residues. Four subfamilies of chemokines have been discovered to date: CXC (α), CC (β), C (γ), and CX3C (Zlotnik and Yoshie, 2012). Chemokines can mediate their activities through G protein-coupled receptors, which have a characteristic seven-transmembrane structure and transduce their signals to the inside of the cell through heterotrimeric G proteins (Rollins, 1997) that are grouped into two families depending on their sensitivity to pertussis toxin (PTX). The $G_{i/o}$ family is sensitive to PTX, whereas the $G_q$ family are not (Wu et al., 1993; Venkatakrishnan et al., 2000).

The formyl peptide receptors (FPRs) mediate chemotaxis activity. The three known FPRs are seven-transmembrane-domain G protein-coupled receptors that are important in host defense and inflammation (Ye et al., 2009). FPR1 and FPR2 expression was first described in neutrophils and monocytes (Durstin et al., 1994). The expression profiles of FPR1 and FPR2 are similar. They were later observed in immature dendritic cells (DCs), microglial cells, spleen and bone marrow and some other tissues at lower levels (Migeotte et al., 2006). FPR3 transcripts are not found in neutrophils but can be detected in monocytes (Durstin et al., 1994). The tripeptide N-formyl-Met-Leu-Phe (fMLF) is a prototype of formylated chemoattractant peptides for neutrophils owing to its ability to bind and activate FPR1. At subnanomolar to
nanomolar concentrations, this binding event translates into directional movement of neutrophils (Nanamori et al., 2004). Recently, a number of FPR ligands have been identified, such as the synthetic hexapeptides WKYMVM and WKYMVm, which activate FPR1 and FPR2. Annexin A1, a glucocorticoid-regulated protein, and its cleavage peptides Ac2-26 bind FPRs with affinities in the low micromolar range (Walther et al., 2000). These receptors also have other endogenous ligands, such as cathepsin G for FPR1 and lipoxin A4, SAA, and LL37 for FPR2 (Migeotte et al., 2006).

We have employed a peripheral blood mononuclear cell (PBMC) chemoattractant platform to identify candidate chemoattractant cytokines and found that PBMCs and neutrophils exhibited chemotaxis toward family with sequence similarity 3 member D (FAM3D), a protein whose function is currently unknown. FAM3D belongs to a cytokine-like family that shares a four-helix-bundle structure (Zhu et al., 2002). The family consists of four members: FAM3A, FAM3B, FAM3C and FAM3D. FAM3D shows 53%, 28% and 50% amino acid sequence identity to FAM3A, FAM3B and FAM3C, respectively. FAM3B, also called pancreatic-derived factor, is an important regulator of glucose and lipid metabolism (Yang and Guan, 2013). FAM3D is constitutively expressed in the gastrointestinal tract and its function is linked to nutritional regulation (de Wit et al., 2012), but its chemotaxis function remains unknown. Here, we found that human peripheral blood neutrophils and monocytes have strong chemotaxis activity to FAM3D. The chemoattractant receptors for
FAM3D were determined to be FPR1 and FPR2 through functional screens for the classical and other chemoattractant receptors. Furthermore, we found that FAM3D expression increased significantly in dextran sulfate sodium (DSS)-induced colitis. The present study has demonstrated that chemokines and chemokine receptors play a key role in gastrointestinal homeostasis and inflammation (Griffith et al., 2014; Hill and Artis, 2010); an endogenous FPR ligand, Annexin A1, has been shown to regulate intestinal mucosal injury, inflammation, and repair (Babbin et al., 2008). Therefore, we propose that FAM3D may function in gastrointestinal homeostasis and inflammation.

Results

FAM3D is a classical secretory protein and strongly chemoattracts PMNs and PBMNs

FAM3D belongs to the family with sequence similarity 3 (FAM3), which is a cytokine-like family. However, whether it is a classical secretory protein is unknown. We produced a secreted recombinant FAM3D protein through the PEI transfection system. The purified FAM3D protein exhibited a purity of at least 90% as assessed by the optical density assay (Fig. 1A). Furthermore, as shown in Figure 1B, the secretion of FAM3D was inhibited by the Golgi blocker BFA, suggesting FAM3D is secreted through the classical ER-Golgi pathway. The N-terminal testing result showed that the mature protein sequence started from the 39th amino acid. The first 38 amino acid residues were a signal peptide sequence (Fig. 1C, underlined). However, this was not consistent with the prediction made by SignalP-HMM software, which was a 25
Because the supernatant of HEK293T cells transfected with pcDB-FAM3D induced the migration of PBMCs (Fig. S1), we wanted to know the leukocyte cell chemotaxis type. Thus, we separated PMNs, PBMs and PBLs from human peripheral blood. As shown in Figure 1D, when we used the purified recombinant FAM3D protein to determine the chemoattractant effect, both PMNs and PBMs were strongly chemoattracted by FAM3D, but the chemoattraction to PBLs was not strong.

**FAM3D is an agonist in FPR1- and FPR2-mediated chemotaxis**

The major function of chemokines is to induce directional migration of cells expressing that chemokine’s receptors. Therefore, a chemotaxis assay with HEK293 cells transiently transfected with different receptors was used to investigate the agonistic properties of FAM3D. FAM3D chemoattracted FPR1- and FPR2-expressing HEK293 cells (Fig. 2A, 2B), but HEK293 cells transiently expressing CXCR1 or CXCR2, both of which are highly expressed on the cell surface of neutrophils, were not chemoattracted by FAM3D (Fig. 2C, 2D). FPR3-overexpressing cells were not chemoattracted by FAM3D (Fig. S1). When we used cyclosporine H, a specific inhibitor of FPR1, the chemoattractant ability of both FAM3D and fMLF disappeared (Fig. 2A). As Figure 2B shown, the chemoattractant ability of FAM3D and WKYMVm to FPR2 was inhibited by WRW4, an inhibitor of FPR2. To further evaluate the relationship between FAM3D and FPR, we prepared mouse monoclonal antibodies targeting FAM3D and found that the antibodies 5E12 and 6D7 effectively
neutralized FAM3D’s chemoattractant ability toward FPR1-expressing HEK293 cells (Fig. 2E). Interesting, only 6D7 effectively neutralized FAM3D’s chemoattractant ability to FPR2 (Fig. 2F), thus suggesting the binding sites of the two monoclonal antibodies to FAM3D are not exactly the same. Furthermore, we have detected both Fpr1 and Fpr2 are expressed by mouse peritoneal neutrophils, and 6D7 could block the chemotaxis action of FAM3D completely, while 5E12 could not (Fig. S3).

Because fMLF- or WKYMVm-induced responses require the physical association of FPR1 or FPR2 with a PTX-sensitive $G_{i/o}$ protein in leukocytes (Bommakanti et al., 1992; Schreiber et al., 1993), we also investigated the chemoattractant activities of FAM3D to HEK293-FPR1 and HEK293-FPR2. Both were inhibited by PTX (Fig. 2G, 2H), which suggests that the pathway induced by FAM3D relies on the $G_{i/o}$ protein.

**FAM3D stimulates internalization of FPR1 and FPR2**

G protein-coupled receptors rapidly internalize upon ligand binding (von Zastrow, 2003). Based on this property, we examined FAM3D for its ability to bind to FPR1 or FPR2 and stimulate their internalization. We used the recombinant 100 nM and 1000 nM FAM3D to stimulate HEK293 cells transiently expressing FPR1-EGFP for 1 h. Confocal microscopy showed that FAM3D and fMLF effectively stimulated the internalization of FPR1 (Fig. 3A).

We next examined the surface expression of FPR1 on the transfected HEK293 cells by flow cytometry. It reduced after 1 h of 100 nM or 1000 nM FAM3D stimulation (Fig. 3B). In comparison, fMLF, which is an agonist of FPR1, also induced the
When we stimulated HEK293 cells overexpressing FPR2-EGFP with 10 nM or 100 nM FAM3D or WKYMVm for 1 h, the phenomenon of receptor internalization was captured clearly by confocal microscopy (Fig. 4A). Next, the surface expression of FPR2 on the transfected HEK293 cells was examined by flow cytometry after stimulation with FAM3D or WKYMVm (Fig. 4B), as we can see, the expression of FPR2 was decreased by FAM3D or WKYMVm. After three repeated experiments, the rate of internalization of FPR1 or FPR2 was calculated, which showed that FAM3D strongly stimulated internalization of FPR1 and FPR2 (Fig. 3C, 4C).

**FAM3D can induce FPR1- or FPR2-mediated calcium flux in HEK293 cells transiently transfected with FPR1 or FPR2**

As FPR1 mediated fMLF-induced calcium mobilization in a pertussis toxin-sensitive manner (Prossnitz et al., 1991), we examined whether FAM3D also affected intracellular calcium levels of FPR1- or FPR2-transfected HEK293 cells. The cells were loaded with the Ca^{2+}-sensitive fluorescent probe fluo-3 AM and stimulated either with FAM3D and fMLF or with FAM3D and WKYMVm. At 200 nM, FAM3D induced Ca^{2+} mobilization with a magnitude similar to that induced by 10 nM fMLF (Fig. 5A, 5B). Furthermore, 200 nM FAM3D and 10 nM fMLF cross-desensitized calcium flux induced by each other (Fig. 5A, 5B). As shown in Fig. 5C and 5D, at 200 nM, FAM3D induced FPR2-mediated calcium flux in HEK293-FPR2 cells. The addition of both 200 nM FAM3D and 10 nM WKYMVm cross-desensitized calcium
flux induced by each other.

**Radioactive binding assay confirms the receptors of FAM3D are FPR1 and FPR2**

To further confirm that FPR1 and FPR2 are specific receptors for FAM3D, we performed a radioactive binding assay (RBA). After the recombinant mature FAM3D was labeled with $^{125}$I, we calculated a FAM3D-FPR1 saturation binding curve (Fig. 6A) by reacting $^{125}$I-FAM3D with varying quantities of FPR1-EGFP-HEK293 membrane extract. We calculated a FAM3D-FPR2 saturation binding curve in the same way (Fig. 6B). A competitive binding assay was conducted with FAM3D, fMLF, cyclosporine H or CCL2 competing with $^{125}$I-labeled FAM3D to bind to FPR1. As shown in Fig. 6A, FAM3D, fMLF or cyclosporine H inhibited binding of $^{125}$I-FAM3D to FPR1. As a more potent agonist for FPR1, fMLF competed with the binding at a lower concentration. However, the uncorrelated control CCL2 did not compete with $^{125}$I-FAM3D. Similarly, in the FPR2 competitive binding assay, FAM3D, WKYMVm and WRW4 competed with $^{125}$I-FAM3D for binding to FPR2 to similar degrees, while the uncorrelated control RS102895, a specific CCR2B inhibitor, did not (Fig. 6B). As the ligand for FPR1, fMLF had a $K_d$ of 0.04 nM (Fay et al., 1991), according to the ratio of the $K_i$ for fMLF ($K_i=0.1934$ nM) to the $K_i$ for FAM3D ($K_i=34.46$ nM) (calculated from the competition binding analysis), the calculated dissociation constant $K_d$ of FAM3D to FPR1 was 7.13 nM. As the ligand for FPR2, WKYMVm had a $K_d$ of 1 pM (Migeotte et al., 2006), according to the ratio of the $K_i$ for WKYMVm ($K_i=21.53$ nM) to the $K_i$ for FAM3D ($K_i=136.7$ nM), the calculated dissociation constant $K_d$ of FAM3D to FPR2 was 6.35 pM.
**FAM3D can chemoattract neutrophils in peritoneal cellular recruitment**

As FAM3D could chemoattract PMNs and PBMs *in vitro*, we wanted to know if they could be recruited when FAM3D was injected into the peritoneal cavity of mice. Six hours after intraperitoneal injection, compared to 14.0 percent CD11b+Ly6G+ neutrophils in control (Fig. 7A), 46.6 percent CD11b+Ly6G+ neutrophils were collected in the FAM3D group (Fig. 7B). Furthermore, 86.8 percent of the neutrophils chemoattracted by FAM3D were Fpr2+, and 19.7 percent were Fpr1+ (Fig. 7C). 82.6 percent of CD11b-Ly6G- cells were Fpr1-Fpr2- (Fig. 7D). Finally, we calculated the relative numbers of various types of leukocytes, including neutrophils, T lymphocytes (CD3), B cells (B220), dendritic cells (CD11c), and macrophages (F4/80) (Fig. 7E). Except for neutrophils, the number of other leukocytes had no obvious change.

**ERK1/2 and p38 signaling is crucial for FAM3D induced neutrophil activation**

Activation of neutrophils through formyl peptide receptors depends on MAPK signaling (Hauser et al., 2010). However, whether the activation of the MAPKs is required for FAM3D-induced neutrophil activation and function has not been determined. After peritoneal neutrophils were isolated from the peritoneal cavity after thioglycollate injection, we pretreated freshly isolated neutrophils with cyclosporine H and/or WRW4 before FAM3D stimulation. FAM3D activated p-ERK1/2 and p-p38 in 2 or 5 min (Fig. 7F). When neutrophils were pretreated with cyclosporine H, a selective antagonist of formyl peptide receptor 1, the up-regulation of p-ERK1/2 did not change, but p-p38 was inhibited. When the cells were pretreated with WRW4, a selective antagonist of FPR2, FAM3D could no longer up-regulate either p-ERK1/2 or
p-p38 (Fig. 7F). This suggests the activation of neutrophils by FAM3D occurs via p38 and ERK1/2 MAPK, the activation of ERK is dependent on FPR2, but the activation of p38 is both FPR1 and FPR2 dependent.

**FAM3D is up-regulated in the colon of mice in a DSS model**

FAM3D expressions were evaluated in Multiple Tissue cDNA Panels from Clontech Laboratories by real-time PCR. In general, FAM3D had a high level of expression in tonsil, a moderate level of expression in lung and pancreas, and a low level of expression in other human tissues we detected (Fig. 8A) Other lab has reported that FAM3D is expressed in gastrointestinal tract tissues and as a gut-secreted protein displaying nutritional status-dependent regulation (de Wit et al., 2012). To explore the role of FAM3D under physiological and pathological status, we detected FAM3D in mouse DSS-induced colitis by IHC and found that its expression increased significantly (Fig. 8B). Real-time PCR confirmed this result (Fig. 8C). As the DSS-induced colitis model is widely used because of its simplicity and many similarities with human ulcerative colitis (Chassaing et al., 2014), FAM3D may be related to gastrointestinal inflammation processes.

**Discussion**

Since the first chemokine, CXCL8, was described in 1987, 48 chemokines have been identified in humans, which is the largest subfamily of cytokines (Zlotnik and Yoshie, 2012). They are directly involved in the migration and activation of leukocytes, especially phagocytes and lymphocytes, playing an important role in the inflammatory response. Several chemokine-like function (CLF) chemokines cannot
be classified into known chemokine subfamilies. For example, MIF (Bernhagen et al., 2007), β-defensins (Yang et al., 1999), and a tyrosyl-tRNA-synthetase fragment (Wakasugi and Schimmel, 1999), among others, are also involved in leukocyte chemotaxis and the migration of normal cells and tumor cells.

In our lab, using a PBMC chemoattractant platform to screen for unknown function cytokines with chemokine function, we found that FAM3D exhibits chemoattractant ability. PC3-Secreted Microprotein (PSMP) exerts its chemoattractant activity via CCR2, which was also found by our platform (Pei et al., 2014). Here, we found that FAM3D was classically secreted to yield an amino acid mature form. Purified recombinant FAM3D protein strongly induced the chemotactic migration of PMNs and PBMs, which may be the target cells of FAM3D. Furthermore, FAM3D chemoattracted numerous CD11b+Ly6G+ neutrophils within 6 h in vivo, which is consistent with FAM3D protein’s strong induction of the chemotactic migration of PMNs in vitro.

Because the amino acid structure of mature FAM3D does not have the typical structural domain of classical chemokines, FAM3D should be a new CLF. Chemokines always exert their function by binding the GPCR receptors on the surface of immune cells. We decided to search for the potential receptors of FAM3D through a Boyden chamber chemotaxis assay and found that FAM3D induced the chemotaxis of HEK293 cells expressing FPR1 or FPR2, but CXCR1- or CXCR2-expressing
HEK293 cells were not chemoattracted by FAM3D. In addition, FAM3D could not
chemoattract many other classical chemokine receptors. The results of chemotaxis,
receptor internalization, calcium flux, and RBA assays led us to conclude that
FAM3D is a novel agonist of FPR1 and FPR2. Additionally, the chemotaxis
phenotype was inhibited by an inhibitor of the relevant receptor. Furthermore, when
we used monoclonal antibodies against FAM3D (5E12 and 6D7), the chemoattractant
activity of FPR1 was neutralized by 5E12 and 6D7. On the other hand, the
chemoattractant activity of FPR2 was neutralized by 6D7 completely, but not 5E12.
These data show that FAM3D has a ligand-receptor relationship with FPR1 and FPR2.
Finally, the chemoattractant activity of FAM3D to HEK293-FPR1 or FPR2 was
inhibited by PTX, thus suggesting the pathway induced by FAM3D is $G_{i/o}$ protein
dependent. As FPR1 and FPR2 are similarly highly expressed on the cell surface of
neutrophils and monocytes but are expressed to a lesser degree in the lymphocytes
(Migeotte et al., 2006), they might not be the only two receptors of FAM3D.

FPR1 and FPR2 are mainly expressed in neutrophils and monocytes, and they have
been known to transduce proinflammatory (Karlsson et al., 2009; Partida-Sanchez et
al., 2004) as well as anti-inflammatory signals (Herrera et al., 2015; Wang et al.,
2015). During a response to infection, accumulation and activation of neutrophil
granulocytes at inflammatory sites are of profound importance (Bidula et al., 2015).
FPR-related signaling pathways, including MAPK (Hazeldine et al., 2015),
PI3K/AKT (Wang et al., 2015), and JAK/STAT/SOCS (Pupjalís et al., 2011) in
neutrophils or macrophages, participate in the proliferation, metastasis, or survival of these leukocytes. Our results indicate that FAM3D activates ERK1/2 and p38 MAPK in neutrophils through FPR1 or FPR2.

Formylated peptides produced by bacteria are the exogenous ligands of FPR1 and FPR2 with high-affinity. They play critical roles in infectious diseases (Molloy et al., 2013). Other high-affinity ligands of FPR2 include WKYMVm and WKYMVM from random peptide libraries. Like FAM3D, Annexin A1 or its N-terminal peptide Ac 2-26 is also an endogenous ligand of FPR1 and FPR2 (Ernst et al., 2004). The endogenous peptide Ac2-26 is cleaved from Annexin A1, but its Kd for FPR1 or FPR2 is in the low micromolar range (Migeotte et al., 2006). In acute and chronic inflammation, Annexin A1 is activated during the process of neutrophil extravasation (Oliani et al., 2001). It also mediates the rapid anti-inflammatory effects of neutrophil-derived microparticles (Dalli et al., 2008). Annexin A1 is expressed in many organs of the body, and highly in the intestinal tract. In DSS colitis, Annexin A1−/− mice are more susceptible to develop enteritis and recover slowly than wild-type mice when the DSS is withdrawn (Babbin et al., 2008), which suggests that Annexin A1 regulates intestinal mucosal injury, inflammation, and repair. WKYMVm, a FPR2 synthetic agonist from a random peptide library, reverses decreases in body weight, bleeding score and stool score in DSS-treated mice; these functions are inhibited by WRW4 (Kim et al., 2013). Chemokines and chemokine receptors play a key role in gastrointestinal homeostasis and inflammation (Griffith et al., 2014; Hill and Artis,
An endogenous ligand of FPR1 and FPR2, Annexin A1 regulates intestinal mucosal injury, inflammation, and repair (Babbin et al., 2008; Fay et al., 1991), also, Fpr2−/− mice showed diminished PMN infiltration into the colonic mucosa in acute DSS induced colitis, delayed mucosal restoration after injury, and increased azoxymethane-induced tumorigenesis, suggesting Fpr2 contributes to colonic epithelial homeostasis, inflammation, and tumorigenesis (Chen et al., 2013). FAM3D is highly expressed in the gastrointestinal tract (de Wit et al., 2012) and our data demonstrated FAM3D up-regulated in colon tissue following DSS treatment. Therefore, we proposed that FAM3D may play a role in gastrointestinal-related inflammation processes.

In conclusion, our work identifies FAM3D as a novel chemotaxis agonist for the G protein-coupled formyl peptide receptors FPR1 and FPR2. Moreover, FAM3D can activate ERK1/2 and p38 MAPK signaling in neutrophils via FPR1 or FPR2. As FAM3D is constitutively expressed in the gastrointestinal tract and up-regulated in DSS-induced colitis, FAM3D may play an important role in intestinal homeostasis and gastrointestinal inflammation.

**Materials and Methods**

**Animals**

All mice used in this study were between 6–8 weeks old and were feeding at the Peking University Health Science Center, which were of a C57BL/6 background, under specific pathogen-free conditions. The animal experimental procedures were
approved by the ethics committee of the Peking University Health Science Center.

**Reagents and chemicals**

RPMI 1640 medium and fetal bovine serum (FBS) were purchased from Life Technologies, Inc. Dulbecco's Modified Eagle's medium (DMEM), Brefeldin A (BFA), Bovine Serum Albumin (BSA), thioglycollate, polyethyleneimine (PEI) and mouse IgG were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mouse mAb of His (clone OGHis) for the Western blot were obtained from MEDICAL & BIOLOGICAL LABORATORIES CO. (Japan). Pertussis toxin (PTX) was purchased from Alexis Biochemical Corporation. $^{125}$I Na was obtained from DuPont. CXCL8, CXCL12, CCL2 and CCL23 were purchased from PeproTech Inc. Fluo-3 AM was obtained from Invitrogen. Goat anti-FAM3D (No. AF2869), mouse anti-FPR1 (No. MAB3744), anti-FPR2 (No. MAB3479), PE anti-FPR1 (No. FAB3744P), APC anti-FPR2 (No. FAB3479A) and fMLF, WKYMVM, WRW4, RS102895 were obtained from R&D System, Inc. Cyclosporine H was produced at Fujian Institute of Microbiology (Fuzhou, China) as described (Yan et al., 2006). FITC anti-mouse CD45 (clone 30-F11), FITC anti-mouse CD11b (clone M1/70), PE anti-mouse B220 (clone RA3-6B2), APC anti-mouse F4/80 (clone BM8), APC anti-mouse CD11c (clone N418) were purchased from Biolegend, PE anti-mouse IgG (clone M1-14D12), Percp/cy5.5 anti-mouse CD3 (clone 145-2C11), Percp/cy5.5 anti-mouse Ly6G (clone RB6-8C5) were purchased from eBioscience. The protease inhibitor cocktail and the PhosSTOP cocktail were obtained from Roche (Rotkreuz, Switzerland). Rabbit monoclonal antibodies (mAbs) of p38 (No. 8690) and Phospho-p38 (No. 4511) and
Rabbit mAb of ERK (clone 4695) and Mouse mAb of Phospho-ERK (clone 9106) for the Western blot were obtained from Cell signaling Technology (Danvers, MA, USA).

**Plasmids**

The pcDNA3.1-FPR2 and pCEP4-CXCR1 expression plasmids were kindly provided by Professor Philip M. Murphy from the Laboratory of Molecular Immunology, National Institutes of Health. The pcDNA3.1-FPR1, pcDNA3.1-CXCR2, pEGFP-N1-FPR1, pEGFP-N1-FPR2 and pcDNA3.1-FAM3D-myc-his plasmids were constructed in our laboratory.

**Cells**

HEK293 and HEK293T cells were purchased from ATCC (Manassas, VA, USA) and were cultured in complete Dulbecco’s Modification of Eagle’s Medium (DMEM) with 10% FBS (inactivated FBS for the latter).

**Production of recombinant human FAM3D protein**

The FAM3D DNA sequence was inserted into the pcDNA3.1-myc-his plasmid. The pcDNA3.1-FAM3D-myc-his plasmid was transfected with polyethyleneimine (PEI) into the HEK293T cell line according to the manufacturer’s recommended protocol. Sixteen hours after transfection, HEK293T culture medium was replaced with Hektor S medium (Cell Culture Technologies, Switzerland) with 2% glutamate (Sigma-Aldrich, St. Louis, MO, USA). The culture medium of the transfected HEK293T cells was collected at 120 h after the replacement of medium. Recombinant human FAM3D was purified using the Ni Sepharose High Performance system according to manufacturer’s instructions (GE Healthcare). Finally, FAM3D protein
was eluted by 500 mM imidazole in 10 ml buffer (pH 7.4), and high-concentration imidazole was replaced with phosphate-buffered saline (PBS) (PH 7.2) by ultrafiltration device (3,000 m.w Millipore, Billerica, MA, USA). All glassware used to purify FAM3D was pretreated at 180°C for 4 h to eliminate endotoxin. The recombinant FAM3D was dissolved in PBS (pH 7.2) at 400 μg/ml and stored at -80°C.

**N-terminal sequencing**

The purified recombinant FAM3D protein was separated by SDS-PAGE and transferred onto a hydrophobic polyvinylidene fluoride (PVDF) membrane (Whatman). Then, the membrane was stained by Coomassie blue, and the N-terminal sequencing was analyzed by Shanghai GeneCore BioTechnologies Co., Ltd.

**The separation of PBMC and PMN from human peripheral blood cells**

Human peripheral blood was obtained from the Beijing Red Cross Blood Center. Peripheral leukocytes were separated by Polymorphprep (Axis-Shield, Norway) at a 1.077 density following the manufacturer’s instructions. PBMCs were located at the interface between RPMI 1640 and Polymorphprep, and polymorphonuclear neutrophils (PMNs) were located at the interface between Polymorphprep and red blood cells. After washing PBMCs and PMNs twice with RPMI 1640, the PBMCs were cultured in RPMI 1640 with 10% inactivated FBS. Then, the PMNs were used in the chemotaxis assay. After culture overnight, the PBMCs were separated into peripheral blood monocytes (PBMs) and peripheral blood lymphocytes (PBLs). PBMs grew attached to the dish, PBLs grew in suspension. The PBMs and PBLs were
washed separately, at which point they were tested for their chemoattraction to FAM3D in Boyden chambers.

**The preparation of mouse anti-FAM3D monoclonal antibodies**

The FAM3D eukaryotic protein and prokaryotic protein were used to immunize mice. After screening hundreds of monoclonal antibodies, we choose several of the best specific antibodies for the experiments and purified them by protein-G.

**Receptor-transfected HEK293 cells**

HEK293 cells (ATCC) were grown in RPMI 1640 supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml). 4×10^6 HEK293 cells in 400 μl were transiently transfected by electroporation with 20 μg of the expression plasmid at 120 volts for 20 milliseconds using an electric pulse generator (Electro Square Porator ECM 830, BTX, SanDiego, CA) and chemotaxis assays, receptor internalization, calcium mobilization and radioligand binding assay were performed 48 h later.

**Chemotaxis assay**

The chemotaxis assay was performed using a 48-well microchemotaxis chamber (Neutroprobe; Cabin John, MD, U.S.A.) as described previously (Sarafi et al., 1997). In brief, chemoattractants were diluted in RPMI 1640 medium supplemented with 0.5% BSA and placed in the lower wells (27 μl/well). PMN, PBM, PBL or HEK293 cells transfected with plasmid were resuspended in the same medium at 1×10^6 cells/ml and added to the upper wells (55 μl/well), which were separated from the lower wells by a polyvinylpyrrolidone-free polycarbonate filter with pores. After
incubation at 37°C in 5% CO₂ for several hours, cells that had migrated into the lower chamber were counted by a Veritas Microplate Luminometer (Turner Systems) or the membranes were stained by the three-step staining buffer. The chemotaxis index (CI) was calculated from the number of cells that migrated to the control. Significant chemotaxis was defined as CI >2.

**Receptor internalization assay**

We used confocal microscopy to screen the receptor internalization directly. An FPR1-EGFP or FPR2-EGFP construct was prepared by ligation of FPR1 or FPR2 cDNA to the N-terminus of EGFP (BD Biosciences Clontech, Palo Alto, CA). A HEK293 cell line expressing FPR1-EGFP or FPR2-EGFP was generated by transient transfection as described above. For the internalization assay, the HEK293-FPR1-EGFP or HEK293-FPR2-EGFP cells were grown on glass coverslips for 16 h in RPMI 1640 supplemented with 10% FBS. The transfected cells were stimulated with FAM3D, fMLF, WKYMVm, or BSA for 1 h in supernatant. Internalization was terminated by adding fixation buffer (3% paraformaldehyde in PBS) followed by incubation at room temperature for 15 min. The cells were then washed twice with PBS, and the Diaminobenzidine Detection System (DAKO code k5007) was used to visualize expression. The subcellular localization of EGFP was observed under a confocal microscope (Leica TCS SP8).

We also used flow cytometry to detect the receptor internalization. After electro-transfecting pcDB-FPR1 or pcDB-FPR2 into HEK293 cells as described
above, HEK293-FPR1 or HEK293-FPR2 cells were stimulated with fMLF or WKYMVm, FAM3D, or BSA in suspension for 1 h. The cells were then washed with PBS twice and incubated in PBS with 20% FBS and 1 μg/ml human IgG for blocking Fc on the cell surface. Then, cells were washed with cold PBS and incubated with anti-FPR1, anti-FPR2 or mouse IgG primary antibody at 4°C for 1 h. After being washed twice in PBS, the cells were incubated with goat anti-mouse-IgG-PE as the detection antibody for 30 min, then washed twice in PBS and resuspended in cold PBS. Finally, 10,000 cells per experiment were analyzed by flow cytometry using a FACSCalibur, the CellQuest software (BD Biosciences), and the FlowJo 7.6.1 software.

**Calcium flux assay**

After electro-transfection with pcDB-FPR1 or pcDB-FPR2, HEK293 cells were cultured in 35 mm confocal dishes for 48 h. Then, the cells were incubated with 10 μM fluo-3AM in RPMI 1640 at 37°C for 1 h in the dark and examined for calcium mobilization in response to the compounds using a Leica SP8 confocal laser-scanning microscope. HEK293 cells were loaded with fluo-3AM, washed twice with PBS, and stimulated by 200 nM FAM3D, 10 nM fMLF, or 10 nM WKYMVm. Calcium flux changes were recorded using a Leica SP8 confocal laser-scanning microscope; images were acquired every 5 seconds, and the first 40 s were used as the basal calcium flux line before any stimulation was added. Calcium flux was recorded for 3 min before the second stimulation. Calcium flux intensity was assessed by Leica System Analysis software.
Radioligand binding assay

First we established an HEK293 cell line stably expressing FPR1-EGFP or FPR2-EGFP. FAM3D was labeled with $^{125}$I. In the saturation experiment, $^{125}$I-FAM3D was incubated with varying quantities of the FPR1 or FPR2 cell membrane extract in binding buffer. In the competition experiment, $^{125}$I-FAM3D was incubated with varying quantities of unlabeled FAM3D, fMLF, WKYMVM, CCL2, or RS102895. The radioactivity assay was evaluated as described previously (Pei et al., 2014).

Peritoneal cellular recruitment

Cellular recruitment was induced by i.p. injection of female C57BL/6 mice (6–8 weeks old) with 10 μg of FAM3D protein diluted in 0.1 ml of sterile, LPS-free PBS. Basal peritoneal cells were counted by the administration of 0.1 ml of sterile, LPS-free PBS. Six hours later, mice were sacrificed and peritoneal lavage was performed with three washes of 5 ml of sterile, ice-cold PBS. Samples were centrifuged at 600× g for 10 min. The cells were resuspended in a final volume of 0.5 ml, and the total leukocytes were incubated with specific antibodies to identify the cell types as follows: FITC anti-mouse CD11b and Percp/cy5.5 anti-mouse Ly6G for neutrophils, FITC anti-mouse CD45 for leukocytes, APC anti-mouse F4/80 for macrophages, APC anti-mouse CD11c for dendritic cells, PE anti-mouse B220 for B lymphocytes, Percp/cy5.5 anti-mouse CD3 for T lymphocytes. All antibodies of the CD45, CD11b, Ly6G, FPR1, FPR2, F4/80, CD11c, CD3, and B220 were used in accordance with the instructions of Biolegend or R&D or eBioscience. Finally, 10,000 leukocytes per experiment were analyzed by flow cytometry using a FACSCalibur,
CellQuest software (BD Biosciences) and FlowJo 7.6.1 software.

**Preparation of peritoneal neutrophils and stimulated it by FAM3D**

For the preparation of peritoneal neutrophils, cells were isolated from the peritoneal cavity 4 h after 1 ml 4% thioglycollate injection and cultured for 18 h. Then, nonadherent cells were harvested and stimulated as described (Takeda et al., 1999). The neutrophils were stimulated by 200 ng/ml FAM3D, or before stimulating, cells were treated with the FPR1 inhibitor cyclosporine H or the FPR2 inhibitor WRW4 for 1 h. After being stimulated 2 min or 5 min, the cell lysates were obtained for western blot.

**Western blot**

Cell lysates were extracted with ice-cold RIPA lysis buffer for 30 min and cleared by centrifugation at 6,000×g for 5 min at 4°C. Total protein concentration was measured using a bicinchoninic acid protein assay kit (Pierce). Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes (HybondTMECLTM, Amersham Biosciences). After blocking the nitrocellulose membranes in ODYSSEY block buffer for 1 h, membranes were incubated with primary antibodies at 4°C overnight and then with ODYSSEY 700/800-labeled IgG secondary antibody at room temperature for 1 h in the dark. Finally, the fluorescence intensity on the membranes was detected with the LI-COR infrared imaging system and was analyzed using ODYSSEY software.
**Real-time PCR**

The expression of FAM3D mRNA was examined using real-time PCR with the UPL probes and GAPDH as the internal reference. The following primers were used:

- FAM3D-F: 5’-gtaaagccctttgagcagt-3’
- FAM3D-R: 5’-ggccatcctcgtatttgt-3’
- Fpr1-F: 5’-tgccagctgtgtaaatgt-3’
- Fpr1-R: 5’-ttcatgagttcactgcagact-3’
- Fpr2-F: 5’-ccacaggaaccgagagtt-3’
- Fpr2-R: 5’-ccacaccttcatccttca-3’
- GAPDH-F: 5’-tccacttgcctctcact-3’
- GAPDH-R: 5’-ggcagagatgatgaccctttt-3’

Real-time PCR was performed in the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) using the human Universal Probe Library system (Roche) and Taqman Gene Expression Master Mix (Applied Biosystems). The samples were run in triplicate with the following cycling conditions: 10 min denaturing at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C.

**Immunohistochemistry (IHC)**

Tissue paraffin sections were pretreated in 70°C for at least 3 h. After soaking in dimethylbenzene for 15 min, the sections were hydrated in 100%, 95%, 90%, 80%, 75%, and 70% ethanol. Antigen retrieval was performed using a high-pressure method in citrate buffer (pH 6.0). Peroxidase was removed by 3% H$_2$O$_2$ for 10 min. Samples
were blocked with rabbit serum in PBS for 40 min at room temperature, and 1 μg/ml goat anti-FAM3D antibody or IgG was applied to the section for 1 h at 37°C. The tissue section was washed three times in PBS (pH 7.2), and then anti-goat HRP-conjugated second antibody was applied for 30 min. All antibodies were diluted in PBS. After washing three times in PBS, the Diaminobenzidine Detection System (DAKO code k5007) was used to visualize expression.

**DSS-induced experimental colitis in mice**

Ulcerative colitis was induced by feeding C57BL/6 mice (6 weeks old) 3% (w/v) DSS (molecular weight, 36,000–50,000 Da; MP Biomedicals, Solon, OH, USA) in drinking water. The mice were given free access to water containing DSS for 7 days. Control mice received water without DSS. The mice were weighed and monitored for the appearance of diarrhea and blood in the stool throughout the experimental period.

**Statistical analysis**

Statistical analysis was performed using the two-tailed Student’s *t* test (unpaired) for determining differences between two samples. Data were expressed as mean ± standard error of the mean (SEM). Significant differences between groups are represented by *0.01 < P < 0.05, **0.001 < P < 0.01, and ***P < 0.001. Comparative analysis was analyzed in Prism 5.0 (GraphPad Software).
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Author contributions

XJP and EQX conceived or designed the experiments, performed experiments, and wrote the manuscript. WWL performed the experiment and analyzed the data. XLP, DXC and DFZ analyzed the data. YZ, CZ, SPS, and JM interpreted the data. PZW screened candidate genes of chemokine in biological information analysis. YZ establish the screening platform of the chemokine. XNM finished the work of real-time PCR and analyzed this data. YMZ gave many advice for culturing cells. DLM provided many advice of the work. YW conceived or designed the experiments and wrote the manuscript.

Conflict of interest

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

**Figure 1 - FAM3D is a classical secretory protein and can chemoattract both PBMCs and PMNs.**

**A** The purified recombinant FAM3D protein secreted by pcDB-FAM3D-transfected HEK293T cells was analyzed by SDS-PAGE. The eukaryotic FAM3D protein was >90% pure.

**B** The HEK293T cells were transfected with pcDB-FAM3D, FAM3D protein was detected by anti-His Ab in both supernatant and cell lysates by Western blot. BFA (10 μg/ml) was added to the cell culture medium 24 h prior to harvesting.

**C** The signal peptide (underlined) and the sequence of FAM3D protein. Purified eukaryotic FAM3D protein was used for N-terminal sequencing.

**D** The chemoattractant activity of FAM3D on human PMN and PBMC. The chemoattractant effects of various concentrations of recombinant FAM3D (2, 20, 200,
2000 ng/ml) on PMNs, PBMs, and PBLs were evaluated, *0.01 < P < 0.05, **0.001 < P < 0.01, and ***P < 0.001.
Figure 2 - FAM3D is an agonist in FPR1-orFPR2-mediated chemotaxis.

A, B  The chemoattractant activity of HEK293-FPR1 or HEK293-FPR2 cells toward FAM3D. In a Boyden chamber system, the chemoattractant effects of various concentrations of recombinant FAM3D (1, 10, 100 nM) or fMLF, WKYMVnm were tested for their ability to chemoattract HEK293-FPR1 or HEK293-FPR2 cells, and the chemotaxis index and the significant difference compared with the medium were calculated. The cells were pretreated with or without 5 μM cyclosporine H or 10μM WRW4 for 1 h.

C, D  FAM3D (2, 20, 200, or 2000 ng/ml) or CXCL8 (200 ng/ml) was tested for its ability to chemoattract HEK293-CXCR1 or HEK293-CXCR2 cells, and the chemotaxis index and the significant difference compared with the medium were calculated.

E, F  Mouse monoclonal antibodies targeting FAM3D (5E12 and 6D7) were applied in the chemotaxis assay. FAM3D (20, 200, or 2000 ng/ml) was pretreated with 5E12 or 6D7 (10 or 50 μg/ml) for 30 min, and the chemotaxis index was calculated for HEK293-FPR1 or HEK293-FPR2 cells.
FAM3D (10nM) was tested for its ability to chemoattract HEK293-FPR1 or HEK293-FPR2 cells with or without PTX pretreatment (1, 10, or 100 ng/ml) at 37°C for 30 min, and the chemotaxis index was calculated.

In A-H, the significant difference was displayed by *0.01 < P < 0.05, **0.001 < P < 0.01, and ***P < 0.001.
Figure 3 - FAM3D stimulates internalization of FPR1.

A  BSA, FAM3D, or fMLF (100 nM or 1000 nM) was used to stimulate HEK293 cells expressing FPR1-EGFP for 1 h. Cells were then fixed and analyzed by confocal microscopy. Scalar bars, 25 μm.

B  Flow-cytometric analysis using anti-FPR1 as the primary antibody to detect the quantity of FPR1 on the cell surface after the stimulation with BSA, FAM3D, or fMLF (100 nM or 1000 nM) for 1 h.

C  After three independently repeated experiments of HEK293-FPR1 stimulated by 100 nM or 1000 nM fMLF, FAM3D or BSA, the quantity of FPR1 on the cell surface was evaluated by flow cytometry and the rate of FPR1 internalization was calculated, *0.01 < P < 0.05, **0.001 < P < 0.01, and ***P < 0.001.
Figure 4 - FAM3D stimulates internalization of FPR2.

A  BSA, FAM3D, or WKYMVm (10 nM or 100 nM) was used to stimulate HEK293 cells expressing FPR2-EGFP for 1 h. Cells were then fixed and analyzed by confocal microscopy. Scale bars, 25 μm.

B  Flow-cytometric analysis using anti-FPR2 as the primary antibody to detect the quantity of FPR2 on the cell surface after the stimulation with BSA, FAM3D, or WKYMVm (10 nM or 100 nM) for 1 h.

C  After three independently repeated experiments in which HEK293-FPR2 cells were stimulated by 10 nM or 100 nM WKYMVm, FAM3D or BSA, the quantity of FPR2 on the cell surface was evaluated by flow cytometry and the rate of FPR2 internalization was calculated, *0.01 < P < 0.05, **0.001 < P < 0.01, and ***P < 0.001.
Figure 5 - FAM3D can induce FPR1- or FPR2-mediated calcium flux in HEK293 cells transiently transfected with FPR1 or FPR2.

A, B  HEK293-FPR1 cells were loaded with 10 μM fluo-3 AM for 0.5 h. After cells were washed with PBS three times and images of the unstimulated state were obtained by confocal microscopy, cells were stimulated by FAM3D (200 nM) or fMLF (10 nM) and constantly observed for 1.5 min. The second stimulation was then added, and the cells were constantly observed for another 1.5 min. The fluorescence intensity of the cells before and after the first and second stimulation was evaluated and analyzed by the Leica System Analysis Software. Scale bars, 200 μm, n means
the cell number we analyze.

C, D HEK293-FPR2 cells were loaded with 10 μM fluo-3 AM for 0.5 h. After cells were washed with PBS three times and images of the unstimulated state were obtained by confocal microscopy, cells were stimulated by FAM3D (200 nM) or WKYMVm (10 nM) and constantly observed for 1.5 min. The second stimulation was then added, and the cells were constantly observed for another 1.5 min. The fluorescence intensity of the cells before and after the first and second stimulation was evaluated and analyzed by the Leica System Analysis Software. Scale bars, 200 μm, n means the cell number we analyze.
Figure 6 - Radioactive binding assay (RBA) confirms the interaction of FPR1 or FPR2 with FAM3D.

A  RBA for HEK293-FPR1 cells. In the saturation experiments, equivalent quantities of $^{125}$I-FAM3D were incubated with varying quantities of FPR1 cell membrane extract in binding buffer. The y-axis represents the radioactivity of the binding, and the x-axis represents the logarithmic form of the concentration of FAM3D. In the competitive-binding assays, equivalent quantities of cell membrane extract and $^{125}$I-FAM3D were incubated with varying quantities of unlabeled FAM3D, fMLF, cyclosporine H or CCL2. The y-axis represents the radioactivity of the specific binding complexes, and the x-axis represents the logarithmic form of the concentration of the competitors.
B RBA for HEK293-FPR2 cells. In the saturation experiments, equivalent quantities of \( ^{125}\text{I}-\text{FAM3D} \) were incubated with varying quantities of FPR2 cell membrane extract in binding buffer. The y-axis represents the radioactivity of the binding, and the x-axis represents the logarithmic form of the concentration of FAM3D. In the competitive-binding assays, equivalent quantities of cell membrane extract and \( ^{125}\text{I}-\text{FAM3D} \) were incubated with varying quantities of unlabeled FAM3D, WKYMVM, WRW4 or RS102895. The y-axis represents the radioactivity of the specific binding complexes, and the x-axis represents the logarithmic form of the concentration of the competitors.
Figure 7 - Flow-cytometric analysis of the cellular composition of peritoneal cellular recruitment and FAM3D activates ERK1/2 and p38 signaling in mouse neutrophils.

A, B  The flow-cytometric analysis of the peritoneal cellular recruitment 6 h after intraperitoneal injection of PBS or FAM3D. The x-axis represents CD11b, and the y-axis represents Ly6G.

C, D  The flow-cytometric analysis of G1 or G2 from Fig. 7B. The x-axis represents Fpr1, and the y-axis represents Fpr2.
E  Cells were stained for flow cytometry with fluorochrome-conjugated mAbs specific for macrophages (F4/80), dendritic cells (CD11c), T lymphocytes (CD3) and B cells (B220). Data are mean ± SEM (n = 3). The number of cells above were calculated, *0.01 < P < 0.05, **0.001 < P < 0.01, and ***P < 0.001.

F  Activation of ERK1/2 and p38 signaling as assessed by western blotting in freshly isolated neutrophils treated with 200 ng/ml FAM3D over a 2 or 5 min time course and pretreated with or without 5 μM cyclosporine H or WRW4 for 1 h.
Figure 8 - FAM3D is up-regulated significantly in the colon of mice of the DSS model.

A  FAM3D mRNA expression levels in normal tissues from the tissue library was measured by real-time PCR using specific primers with the UPL probes and GAPDH as the internal reference.

B  IHC for FAM3D in colon of control or 3%DSS water-fed mice by IHC.

C  Real-time PCR for FAM3D in colon of control or 3%DSS water-fed mice, using specific primers with the UPL probes and GAPDH as the internal reference. Data are mean ± SEM (n = 4), *0.01 < P < 0.05, **0.001 < P < 0.01, and ***P < 0.001.
References


