FROUTN Is a Common Regulator of CCR2 and CCR5 Signaling to Control Directional Migration

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**FROUNT Is a Common Regulator of CCR2 and CCR5 Signaling to Control Directional Migration**

Etsuko Toda, Yuya Terashima, Tsuyoshi Sato, Kenzo Hirose, Shiro Kanegasaki, and Kouji Matsushima

FROUNT is a known CCR2-binding protein that facilitates monocyte/macrophage infiltration. Here we report that FROUNT also binds to the C-terminal region of CCR5 and enhances CCR5-mediated cellular chemotaxis. We show that FROUNT overexpression increases the directionality of chemotaxis, while FROUNT suppression results in impaired responsiveness. Furthermore, we found an increase in consolidated pseudopodium formation in FROUNT-overexpressing cells (FNT cells) on uniform stimulation with CCL4 (MIP1-β), a specific ligand of CCR5. In most FNT cells, one to two pseudopodia directed toward higher chemokine concentration were found, whereas most FNT-suppressed cells had multiple pseudopodia. The data indicate that FROUNT is involved in sensing and amplifying a shallow extracellular chemokine gradient that leads to a limited number of accurate pseudopodia directed toward the chemokine concentration. In addition to its separate roles in CCR2- and CCR5-mediated chemotaxis, FROUNT, as a common regulator of these receptors, possibly plays a crucial role in the recruitment of immune cells expressing these receptors. *The Journal of Immunology, 2009, 183: 6387–6394.*

**D**irectional migration is essential for immune cells to infiltrate to sites of inflammation (1). This migration is achieved by chemokine signaling through a specific receptor (called a chemokine receptor) that belongs to the seven-transmembrane G protein-coupled receptor family (2, 3). In contrast to the current level of knowledge about chemokine and chemokine receptor networks, further elucidation is needed for the intracellular signaling cascade that follows ligand-receptor interaction, especially the initial events, including binding of intracellular molecules to the cytoplasmic C-terminal domain of the receptor, that initiate the cascade.

Directional migration is achieved by maintaining a persistent leading edge of the cell accurately in the appropriate direction of a chemoattractant gradient. Defects in cell polarization have been reported to prevent the consolidation of one leading edge, resulting in pseudopodia protruding in multiple directions (4). In the classical view, PI3K was considered as the most upstream molecule that is relocalized to the leading edge (5–7), although there is a gap between gradients of activated receptors and the steep gradient of PI3K (8). For the CCR2-mediated chemotaxis system, we previously reported that FROUNT promotes CCR2-mediated chemotaxis by promoting PI3K activation. In other words, FROUNT is the factor that lies immediately downstream of the chemokine-receptor interaction in chemotaxis signaling and links activated CCR2 and the PI3K/Rac/lamellipodium cascade (9). However, it has not been assessed whether FROUNT is involved in the generation of directionality of cellular chemotaxis.

FROUNT was originally cloned from a library of the human monocyte cell line THP-1 in an attempt to identify CCR2-binding molecules that positively regulate chemotaxis signaling. We demonstrated further that infiltration of macrophages depends on FROUNT function in an animal model of in vivo peritonitis. Recently, another group reported that mRNA levels of both FROUNIT and CCR2 were up-regulated in biopsy tissue samples from patients with heart failure where monocytes/macrophages play important roles (10). In addition to CCR2, monocytes and macrophages are known to express various chemokine receptors such as CCR1, CCR3, CCR5, and CXCR4. Whether FROUNT regulates only CCR2 signaling or has an overlapping function on other chemokine receptors is not known. Besides its role in monocytes/macrophages, FROUNT has also been shown to be required for the migration and recruitment of CCR2-expressing bone marrow-derived mesenchymal stem cells to injured heart (11). FROUNT has also been reported to mediate transendothelial migration of prostate carcinoma through activation of the small G protein Rac (12). Information about the range of chemokine receptors to which FROUNT binds has significance for understanding the role of FROUNT in vivo in the migration of various cells as stated above.

The C-terminal domain, especially the membrane-proximal C-terminal (Pro-C) region of chemokine receptors, has been reported to play an important role in the directional migration of cells (13–15). In experiments using various truncated mutants of CCR5, Oppermann and colleagues showed that truncation of the C terminus after residue 320 did not affect cellular chemotaxis, whereas truncation at the Pro-C region, residues 313–320, abrogated the ability of CCR5 to mediate cell migration (14). This region corresponds to the CCR2 region that is essential for

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*Abbreviations used in this paper: Pro-C, membrane-proximal C-terminal; FNT, FROUNT; HOS, human osteosarcoma; siRNA, small interfering RNA.

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ROLE OF FROUNT IN DIRECTIONAL MIGRATION

FROUNT binding, raising the possibility that FROUNT also regulates CCR5 signaling. Among the 20 known chemokine receptor genes, those encoding CCR2 and CCR5 are located in close proximity on human chromosome 3p21 and share 71% sequence identity (16, 17). Despite this high homology, the function of these receptors seems to be different. For example, the chemokines that bind to CCR2 are CCL2 (MCP-1), CCL8 (MCP-2), and CCL7 (MCP-3), whereas those binding to CCR5 are CCL3 (MIP-1α), CCL4 (MIP-1β), and CCL5 (RANTES) (2). Different levels of CCR2 and CCR5 expression in a subset of macrophages and different chemokine usage of macrophages have been reported using pathological models (18, 19). Although CCR2 seems to play a more important role in macrophage migration in these models, it has been reported that heterodimeric complexes of these receptors exhibit more efficient signaling (20). The evidence also suggests that there is crosstalk between the CCR2 and CCR5 signaling pathways. We are therefore interested in whether FROUNT might also play a role in CCR5-mediated chemotaxis as a common regulatory linker in signal transduction.

In the present study, we show that FROUNT binds to the C-terminal domain of CCR5 in addition to that of CCR2. Our results from chemotaxis imaging reveal that FROUNT enhances chemotaxis by promoting the directionality of the cells. Additionally, we have further analyzed the precise morphological changes in the protrusion of pseudopodia caused by overexpression of FROUNT or blockade of its function, the results of which indicate that FROUNT promotes directional and consolidated pseudopodial protrusion. The results provide significant insight into the molecular mechanisms involved in directional sensing and suggest that FROUNT, as a common regulator of CCR2 and CCR5 signaling, is a powerful target for drug search to treat wide range of diseases in which monocytes/macrophages and other cells expressing these receptors are involved.

Materials and Methods

Cells and reagents

The human osteosarcoma cell line (HOS) expressing CD4 and CCR5 was obtained from National Institutes of Health AIDS research. HOS cells were retransfected with the human CCR5 gene using PMX retrovirus vector to enhance CCR5 expression. A mouse B cell line, L1.2 cells stably expressing human CCR5, was provided by Dr. Yoshiie (Kinki University School of Medicine, Osaka, Japan). Human CCL4 was purchased from PeproTech. Rabbit anti-FROUNT polyclonal Ab was purchased from Novus Biologicals. Goat anti-human CCR5 Ab was purchased from Santa Cruz Biotechnology. Biotinylated anti-human CCR5 mAb (2D7) was purchased from BD Biosciences. Alexa Fluor-conjugated secondary Abs and phalloidin-XX-biotin were purchased from Molecular Probes.

Phylogenetic tree analysis

The C-terminal amino acid sequences of human chemokine receptors were extracted from National Center for Biotechnology Information, and the phylogenetic tree was constructed using DNASIS Higgins algorithms (Hitachi Software Engineering).

 Yeast two-hybrid system
cDNAs encoding the C-terminal domain of CCR1, CCR2, CCR3, CCR5, or CXCR4 were subcloned into pAS2-1 (Clontech). Each vector and the pACT vector (Clontech) carrying cDNA encoding amino acids 500–656 of human FROUNT were cotransfected into yeast Y190 cells using a lithium acetate protocol. Positive clones were selected using growth in selection medium free of tryptophan and leucine. Interactions of each chemokine receptor and FROUNT in yeast cells were tested for histidine auxotrophy and β-galactosidase activity using the filter lift assay. A semiquantitative liquid β-galactosidase assay using ONPG (o-nitrophenyl-β-D-galactopyranoside) as a substrate was performed according to the protocol described for the Matchmaker System 2 (Clontech).

 Immunoprecipitation assay

CCR5-expressing HOS cells or THP-1 cells were stimulated with CCL4 for 1, 5, or 20 min and then lysed in a detergent buffer containing 20 mM triethanolamine (pH 8.0), 300 mM NaCl, 2 mM EDTA, 20% glycerol, and 1% digitonin, supplemented with a cocktail of protease inhibitors (0.5 mM aprotinin, 1 mM leupeptin, 1 μM pepstatin, and 1 mM PMSF). As a control, HOS cells without stimulation were used. Samples were then centrifuged at 13,000 g for 10 min at 4°C. The soluble fraction was incubated at 4°C with mouse anti-human CCR5 (D-6) Ab or anti-human FROUNT Ab for 30 min, and then treated with Sepharose-protein G (GE Healthcare) for 2 h with continuous rocking. Immunoprecipitates were centrifuged (13,000 × g for 5 s at 4°C), subjected to SDS-PAGE, transferred onto polyvinylidene difluoride membranes, and stained with anti-human FROUNT Ab or anti-CCR5 Ab (C-20).

 Transfection

Retrovirus vectors carrying cDNA for human FROUNT, antisense mutant FROUNT (AS-FNT), truncated mutant FROUNT (DN-FNT), or silencing small interfering RNA (FNT-siRNA) were constructed as described previously (9) (supplemental Fig. 2A). A siRNA library of human CCR5 sequences was generated using EPRIL technology (21). The best siRNA construct with efficient silencing activity of FROUNT expression was screened as described (21). FNT-siRNA was cloned into retrovirus vector pNAMA containing the gene encoding DsRed (supplemental Fig. 1A). Murine pre-B cell line L1.2 cells stably expressing human CCR5 were transfected with a control vector, or vectors carrying cDNA for human FROUNT or DN-FNT, or FNT-siRNA. CCR5-expressing HOS cells were transfected using control vector or a vector encoding human FROUNT, DN-FNT, or AS-FNT. Transfected cells were purified using a cell sorter (Epics Altra; Beckman Coulter). Samples consisting of >95% purified cells were used for all experiments. FROUNT protein in transfected cells was quantified by Western blotting (supplemental Fig. 1B).

Chemotaxis assay using TAXIScan technology

Control, FNT cells, and two cell lines in which FROUNT was suppressed by transduction of cDNA encoding DN-FNT or FNT-siRNA originated from L1.2 cells expressing CCR5 were tested for chemotaxis using the TAXIScan system (22, 23). The TAXIScan device (ECI) is an optically accessible horizontal chemotaxis apparatus consisting of an etched silicon substrate and a flat glass plate, which forms two compartments with a 5-μm deep microchannel in between. Cells were applied through a hole connected to a compartment and aligned along the start line on the edge of the channel. CCL4 (10 ng/ml) applied through a hole connected to the other compartment formed a concentration gradient in the channel. Time-lapse images were recorded every 30 s for 30 min and cell movement was analyzed using Metamorph (Universal Imaging) and Velocity software (Improvision).

Localization of FROUNT, CCR5, and F-actin

CCR5-expressing HOS cells in culture medium were seeded into chamber slides (Nunc) and incubated at 37°C for 12 h to allow adherence to the slide. They were stimulated by incubation with 50 ng/ml CCL4 at 37°C for 1, 5, or 20 min, fixed, and permeabilized. FROUNT and CCR5 were stained with rabbit anti-human FROUNT Ab and biotinylated anti-human CCR5 Ab, followed by Alexa Fluor 546-labeled anti-rabbit Ig and streptavidin-Alexa Fluor 488, respectively. For staining of F-actin, slides were incubated with phallolidin-XX-biotin (Molecular Probes) and then streptavidin-Alexa Fluor 488. Fluorescence images were acquired with a confocal microscope (FV300+IX-70 system; Olympus). Colocalization was analyzed using Fluoview software (Olympus).

Morphological analysis of pseudopodia formation

CCR5-expressing HOS cells overexpressing human FROUNT or DN and AS-FNT were seeded and stimulated as described above. Cells were stained with phallolidin to visualize cytoskeletal organization, and the numbers of pseudopodia were counted. For stimulation with chemokine gradients, cells were applied to the TAXIScan device and stimulated with 100 ng/ml CCL4. Time-lapse images were obtained every 30 s for 75 min.

Visualization of receptor clustering

CCR5-expressing HOS cells overexpressing human FROUNT or DN and AS-FNT were seeded and incubated with biotinylated anti-human CCR5

5 The online version of this article contains supplemental material.
membrane-proximal C-terminal domain. indicates the seven-transmembrane domain, and Pro-C indicates the Bold characters show amino acids identical to those of CCR2. 7TM Amino acid sequences of CCR1, CCR3, CCR2b, CCR5, and CXCR4. the DNASIS Higgins algorithm (Hitachi Software Engineering). mation database, and phylogenetic tree analysis was performed using data were extracted from the National Center for Biotechnology Informa- tion database. B, Amino acid sequences of CCR1, CCR3, CCR2b, CCR5, and CXCR4. Bold characters show amino acids identical to those of CCR2. 7TM indicates the seven-transmembrane domain, and Pro-C indicates the membrane-proximal C-terminal domain.

We confirmed that FROUNT binds to CCR5 by coimmunoprecipitation assays using human HOS cell line that expressed CCR5 and contained endogenous FROUNT (Fig. 3). Even in unstimulated cells, some interaction was observed and FROUNT was coimmunoprecipitated with CCR5. However, the amount of precipitate increased when the system was activated by CCL4 (MIP-1β), a ligand specific for CCR5 (Fig. 3A). The results of reciprocal immunoprecipitations also supported this interaction (Fig. 3B). We further confirmed the interaction of endogenous FROUNT and endogenous CCR5 in a monocyte cell line (supplemental Fig. 3). The interaction slightly but significantly increased upon stimulation with a CCR5-selective ligand.

Promotion of CCR5-mediated directional migration mediated by the FROUNT/CCR5 complex

CCR5 is expressed on the cell surface of monocytes, dendritic cells, and T cells and is considered to function in the recruitment of these cells to sites of inflammation. To determine whether FROUNT is functionally associated with CCR5-mediated leukocyte chemotaxis, we used the murine pre-B cell line L1.2 stably expressing human CCR5 to establish FROUNT-overexpressing cells (FNT cells) and cells with defective FROUNT function (FNT-suppressed cells) by transfection with a gene encoding truncated mutant FROUNT (DN-FNT) or with silencing siRNA (FNT-siRNA). Using a TAXIScan device (22, 23), in which movement of each cell in a horizontal channel can be tracked, chemotaxis of these cells was assessed (Fig. 4). By applying 10 ng/ml CCL4 to the compartment opposite to that containing the cells, a concentration gradient of the chemokine formed in the channel in between the compartments and the cells began to migrate along this gradient.

Despite homogeneous expression of CCR5 in these cells (supplemental Fig. 4A), not all cells responded to CCL4; ~40% of control cells and FNT cells in the channel migrated under the experimental conditions used (Table I and supplemental Fig. 5).
FROUNT suppression resulted in decreased responsiveness: only 11% of DN-FNT cells and 26% of FNT-siRNA responded, and no cell (among >300 cells examined) was observed to migrate as far as the FNT cells and control cells. Fig. 4, A and B, shows the tracks of the three most active cells (Fig. 4A) and the translated migration pattern of the 10 most motile cells from a starting point (Fig. 4B). Some FNT cells migrated faster and more directly along the chemokine gradient, indicating facilitation of directional migration. These results indicate that FROUNT enhances CCR5-mediated directional cell migration.

**Accumulation of FROUNT in the ruffling membrane and its colocalization with CCR5 and F-actin**

To determine the mechanism underlying the directional cell movement mediated by FROUNT, we evaluated the dynamics and colocalization of FROUNT with the receptor CCR5 on stimulation with a chemokine in HOS cells expressing exogenous CCR5 and endogenous FROUNT (Fig. 5). Before stimulation, FROUNT was diffusely distributed within the cytosol and showed little colocalization with membrane CCR5. Upon exposure to CCL4, ruffling membranes appeared within 1 min and strong FROUNT signals appeared in the vicinity and partly colocalized with CCR5 (Fig. 5A, magnified image, and Fig. 5B). After 5 min, FROUNT accumulated in the ruffling membrane and colocalized CCR5/FROUNT was widely observed in the pseudopodial area (between two asterisks). Twenty minutes after stimulation, FROUNT signals at the ruffling membrane became weak and FROUNT/CCR5 colocalization appeared more in the inner cellular space near the nucleus, suggesting that CCR5/FROUNT complexes are maintained during receptor internalization. These analyses revealed dynamic changes of FROUNT localization upon chemokine stimulation and a stimulation-dependent increase in FROUNT/CCR5 interactions, consistent with the data obtained from the in vitro coimmunoprecipitation assays.

We then investigated colocalization of FROUNT with F-actin by phalloidin staining using the same cells. Pseudopodial protrusion with accumulated F-actin and other molecules (namely, lamellipodial formation) in the correct direction is important in directional cell migration. FROUNT was observed in the pseudopodial area between 1 and 5 min after CCR5-ligand stimulation conducted as in Fig. 5, and FROUNT was colocalized with F-actin in this area (Fig. 6). Although the pseudopodia persisted over 20 min, as above the FROUNT/F-actin signals therein weakened and colocalization with F-actin was no longer apparent. This observation suggests that FROUNT interacts with F-actin at an early stage and is involved in cytoskeletal reorganization and lamellipodial formation.

**Promotion by FROUNT of unidirectional and polarized pseudopodial protrusion toward chemokine concentration**

Next, we studied the formation of pseudopodia to elucidate the mechanism by which FROUNT promotes directional migration. We established FNT cells and FNT-suppressed cells (DN-FNT and AS-FNT cells) from HOS cells expressing CCR5 by transfection and studied the formation of pseudopodia in these cells. Expression of CCR5 on all these cell lines was comparable (supplemental Fig. 4B). The number of pseudopodia produced before and after uniform stimulation with CCL4 (no chemokine gradient) was
counted. Most of the cells had no pseudopodium before stimulation. Examples of each cell type are shown in the left column (untreated control) in Fig. 7A, and quantitation of number of pseudopodia is shown in Fig. 7B and C. Upon exposure to the chemokine, several control and FNT cells exhibited a single, unidirectional protrusion of pseudopodium/lamellipodium and the number of such cells was greater in the FNT cell population than in control cell population (Fig. 7C). Interestingly, both DN-FNT and AS-FNT cells exhibited multiple protrusions of pseudopodia (Fig. 7A, middle). Magnified images (Fig. 7A, right) showed, however, that the pseudopodium in FNT cells comprised thick actin-rich lamellipodium, whereas the multiple pseudopodia in DN-FNT and AS-FNT cells showed filopodia-like structures. These results suggest that FROUNT facilitates unidirectional protrusion of lamellipodium even in the absence of a chemokine gradient.

We next assessed whether FROUNT regulates the pseudopodial protrusion in an appropriate direction along a chemokine gradient by using the TAXIScan device. More persistent pseudopodia were formed in FNT cells, but now they were directed toward the higher concentration of chemokine (Fig. 8A). The percentage of FNT cells with a single pseudopodium was similar to that in controls, whereas the proportion of the cells forming two pseudopodia increased and that with more than three multiple pseudopodia substantially decreased (Fig. 8B). In contrast, FROUNT-suppressed cells produced multiple pseudopodia extruding in all directions (Fig. 8, A and B). FROUNT overexpression increased the number of cells with a pseudopodial protrusion directed toward the higher...
chemokine concentration (Fig. 8C). These results indicate that FROUNT promotes consolidated pseudopodial protrusion toward higher concentrations of CCL4.

**Discussion**

We report in this paper that the CCR2-binding protein FROUNT also binds to CCR5 at the C-terminal domain, as in the case of CCR2. By chemotaxis imaging using CCR5-expressing cells, we have shown here for the first time that FROUNT promotes the directionality of chemotaxis. Furthermore, we reveal that FROUNT amplifies signals to form mainly one leading edge protrusion of pseudopodia toward higher chemokine concentrations. It seems that FROUNT promotes the formation of consolidated pseudopodia by facilitating receptor clustering, producing high-density areas of the receptor where the consolidated pseudopodia will protrude. Thus, FROUNT is a common regulator of both CCR2 and CCR5 and plays a key role in directional migration.
The result of three independent experiments was calculated (were obtained when the experiment was repeated. It has been pos-
ing from the chemoattractant gradient (29 –31). It has been pos-
s the signal originating from the chemoattractant gradient (29–31). It has been pos-

These observations suggest that FROUNT participates in the initial polarization of lamellipodial protrusions mediates directional migration (26 –28). Our findings now add FROUNT to the list of molecular components for this proposed mechanism of directional migration.

In a chemokine gradient, FROUNT amplifies signals to form accurate control of the directional migration in each receptor-mediated chemotaxis.

We previously reported that, when macrophages or CCR2-expressing cells are exposed to a chemokine gradient, FROUNT interacts with CCR2 in migrating cells and induces PI3K activation. This process is then coupled to activation of Rac, a small G protein of the Rho GTPase family. In this paper, we have further shown, by using CCR5-expressing cells, that FROUNT facilitates the directionality of cell migration. This directionality appears to be mediated by the persistent and accurate formation of a limited number of pseudopodia (one or two) in an appropriate direction, a process accompanied by actin reorganization. Even when stimulated uniformly with chemokine rather than with a chemokine gradient, FROUNT consolidates the direction of lamellipodial formation. Analogously, under uniform stimulation, FROUNT facilitates receptor clustering and produces a cell-surface area of high receptor density. Taking the data together, we hypothesize that, upon stimulation, FROUNT concentrates the distribution of receptor/FROUNT complex, which leads to local activation of the receptors, resulting in the formation of consolidated pseudopodia. Recent studies have shown that control of the number and orientation of lamellipodial protrusions mediates directional migration (26–28). Our findings now add FROUNT to the list of molecular components for this proposed mechanism of directional migration.

In a chemokine gradient, FROUNT amplifies signals to form mainly one leading edge protrusion of pseudopodia toward higher chemokine concentrations. Because FROUNT directly binds to chemokine receptors proximal to the cell membrane and upstream of PI3K activation, it seems that FROUNT functions at an early stage of receptor signaling. We previously demonstrated that the FROUNT-chemokine receptor complex selectively locates to the more concentrated side of a chemoattractant gradient in a cell (9). These observations suggest that FROUNT participates in the initial amplification signal, which senses and magnifies the signal originating from the chemoattractant gradient (29–31). It has been postulated that a "molecular compass" determining the direction of polarity exists (5). The Pro-C region where FROUNT binds seems to serve as a scaffold for such a molecular compass. Local activation of the small G protein Ras is reported to regulate PI3K and cell polarity in *Dictyostelium* (32). The GTP exchange factor Vav interacts with CXCR4 and controls lymphocyte shape and chemotaxis (33). The relationship of FROUNT to molecules such as these remains to be elucidated.

The cytoplasmic C-terminal domain of chemokine receptors is known to be involved in efficient chemotaxis. Truncation of the cytoplasmic carboxyl tail of CCR2 to 20 aa had little or no effect on chemotaxis or signal transduction, but further truncation resulted in marked functional defects (13). A CCR5 mutant with truncation after the membrane-Pro-C region 313–352, but not after the 320–352 region, resulted in impaired chemotaxis despite comparable expression to the wild-type receptor at the cell surface (14). Impaired chemotaxis in these mutants of the Pro-C region of CCR2 and CCR5 may be explained by the loss of interaction with FROUNT and its function.

In other chemokine receptors, the C-terminal domain LLKIL (325–329) motif of CXCR2 has been reported to be necessary for early signals during chemotaxis (14, 34). Another CXCR2 mutant exhibiting the loss of C-terminal residues (342–355) no longer undergoes ligand-enhanced receptor phosphorylation or desensitization, as monitored by Ca2+ mobilization in ligand stimulation (35). A CCR3 mutant lacking residues 310–355, which include the membrane-proximal region, exhibits impaired chemotaxis, although a mutant lacking residues 325–355 displays normal chemotaxis (36); additionally, CCR7 lacking the whole C terminus is not able to transmit signals leading to cell migration, while a mutant lacking part of the C terminus exhibits normal chemotaxis (37). These defects in truncation mutants of the C terminus, especially the Pro-C region, have been thought to be due to the inability of the protein to activate G proteins. The specific binding of FROUNT to homologous receptors implies the existence of "FROUNT-like" molecules that bind to the Pro-C region of other chemokine receptors and regulate the early signaling of chemotactic responses dependent or independent of G protein activation.

CCR2 and CCR5 have been shown to form heterodimeric complexes, resulting in more efficient signaling (20). The C-terminal sequences of CCR2 and CCR5 are structurally related and are partially overlapping, but their functions differ due to different chemokine usage (19, 38). CCR2 is expressed mainly in monocytes and macrophages, whereas CCR5 is expressed in NK cells, CD4+,-, CD8+,-, and Th1-type lymphocytes, and immature dendritic cells, in addition to monocytes/macrophages. Monocytes/macrophages and some types of T lymphocytes express both CCR2 and CCR5 together. It has been shown that CCR2/CCR5-ligand double knockout mice exhibit an increased survival in a model of pulmonary inflammation than do CCR2 single knockout mice (18). We have reported previously that FROUNT is expressed mainly in the red pulp of mouse spleens together with CCR2 and a macrophage marker, and that, in mice whose FROUNT functions in hematopoietic cells are defective, infiltration of macrophages into the inflammation sites is reduced (9). It is possible that, in those experiments, a combination of CCR5 and CCR2 should have worked efficiently for macrophage recruitment. Macrophages are the cells that are involved in various chronic inflammatory diseases such as atherosclerosis, obesity, multiple sclerosis, and rheumatoid arthritis, and they generate a large amount of inflammatory cytokines that may affect prognosis (19, 39–41). Because the sequence of the internal Pro-C region in CCR2 and CCR5 is conserved among animal species including humans and mice, an inhibitor that blocks the FROUNT-CCR2/ FROUNT-CCR5 interaction could overcome species specificity.
The physiological role of FROUNT in vivo as a common regulator of CCR2 and CCR5 has not yet been assessed. Future analysis of FROUNT-transgenic or FROUNT-knockout mice will provide crucial information on this issue. Our findings indicate the significance of FROUNT as a therapeutic target for a broad range of diseases associated with chemokine signaling.

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Disclosures
The authors have no financial conflicts of interest.

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