Temporin A and Related Frog Antimicrobial Peptides Use Formyl Peptide Receptor-Like 1 as a Receptor to Chemoattract Phagocytes

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Many mammalian antimicrobial peptides (AMPs) have multiple effects on antimicrobial immunity. We found that temporin A (TA), a representative frog-derived AMP, induced the migration of human monocytes, neutrophils, and macrophages with a bell-shaped response curve in a pertussis toxin-sensitive manner, activated p44/42 MAPK, and stimulated Ca\(^{2+}\) flux in monocytes, suggesting that TA is capable of chemotactically phagocytic leukocytes by the use of a G\(_i\) protein-coupled receptor. TA-induced Ca\(^{2+}\) flux in monocytes was cross-desensitized by an agonistic ligand MMK-1 specific for formyl peptide receptor-like 1 (FPRL1) and vice versa, suggesting that TA uses FPRL1 as a receptor. This conclusion was confirmed by data showing that TA selectively stimulated chemotaxis of HEK 293 cells transfected with human FPRL1 or its mouse ortholog, murine formyl peptide receptor 2. In addition, TA elicited the infiltration of neutrophils and monocytes into the injection site of mice, indicating that TA is also functionally chemotactic in vivo. Examination of two additional temporin revealed that Rana-6 was also able to attract human phagocytes using FPRL1, but temporin 1P selectively induced the migration of neutrophils using a distinct receptor. Comparison of the chemotactic and antimicrobial activities of several synthetic analogues suggested that these activities are likely to rely on different structural characteristics. Overall, the results demonstrate that certain frog-derived temporin have the capacity to chemotactically phagocytes by the use of human FPRL1 (or its orthologs in other species), providing the first evidence suggesting the potential participation of certain amphibian antimicrobial peptides in host antimicrobial immunity. The Journal of Immunology, 2004, 173: 2652–2659.

For the past 30 years, over 700 host gene-encoded peptides and proteins with direct microbicidal activities against bacteria, fungi, and some parasites have been isolated and characterized from multicellular organisms (1–4). These antimicrobial peptides (AMPs),\(^3\) although structurally diverse, can roughly be grouped into two categories: linear AMPs (e.g., cathelicidins) and AMPs with 1–4 intramolecular disulfide bonds (e.g., defensins) (3, 4). In recent years, a number of mammalian AMPs have been found to have, in addition to microbicidal activities, effects on host cells such as promoting chemotaxis and activating leukocytes, enhancing angiogenesis, accelerating wound healing, and neutralizing toxic microbial products (3, 5, 6). These effects of mammalian AMPs on host cells are mediated by receptors (7–11). The receptors that mediate the chemotactic effects of certain β-defensins and human cathelicidin/LL-37 have been identified as the chemokine receptor CCR6 and formyl peptide receptor-like (FPRL1) 1, respectively (7–9). Furthermore, the use of transgenic or knockout models has shown that a number of defensins and cathelicidin confer resistance of the host to microbial challenges (12–17) and cathelicidin also contributes to angiogenesis (11), supporting the notion that AMPs function as essential multifunctional endogenous effectors of host innate defense against the invasion of pathogenic microorganisms.

AMPs are generated not only by mammals, but also by lower vertebrates, such as amphibians, and by insects (1–4). Temporins are a group of naturally occurring AMPs originally isolated from the skin secretions of the European frog, Rana temporaria (18). Temporin and temporin-like AMPs are short (10–13 amino acid residues), linear, basic, and highly hydrophobic (18–21). Temporins are most active against Gram-positive bacteria, but some are active against Gram-negatives and fungi, and they exhibit varying degrees of hemolytic activity (18–22). Studies with synthetic analogues of the temporins are beginning to reveal structural features of these peptides that are important for their antimicrobial and hemolytic properties (21–26).

By far, it is not clear whether AMPs of lower vertebrate also act on leukocytes to promote chemotaxis and/or activation. In this study, we investigated whether temporins could attract leukocytes, and if so, the potential underlining mechanism. Our data show that temporin A (TA), temporin Rana-6, and several temporin-like synthetic AMPs act as chemoattractants for phagocytic leukocytes using the FPRL1 or murine formyl peptide receptor 2 (mFPRL2), the

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\(^3\)Abbreviations used in this paper: AMP, antimicrobial peptide; FPRL, formyl peptide receptor-like; mFPR2, murine formyl peptide receptor 2; rh, recombinant human; DC, dendritic cell; HPF, high power field; PTX, pertussis toxin; Aβ, amyloid β.

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mouse ortholog of FPRL1, as the receptor, suggesting that certain amphibian AMPs can also act on host leukocytes. Furthermore, the chemotaxing activity appears to be able to be dissociated from their antimicrobial activity by modification of their structures.

**Materials and Methods**

**Reagents**

RPMI 1640 and DMEM were purchased from BioWhittaker (Walkersville, MD). FBS was purchased from HyClone Laboratories (Logan, UT). Recombinant human (rh) GM-CSF, rhIL-4, and rhM-CSF were obtained from PeproTech (Rocky Hill, NJ). Pertussis toxin (PTX) was from Sigma-Aldrich (St. Louis, MO). Amyloid β_{1-42} (Aβ) peptide was purchased from American Peptide Company (Vista, CA). [125I]-Aβ was from Amersham Biosciences (Piscataway, NJ).

MMK-1, an FPRL1-specific ligand of 13 amino acids (LESIFRSLL-PFVM) (27), and W peptide, a chemotactic hexapeptide (WKYMVm, mFPR1) (27), were characterized by amino acid analysis and electrospray ionization mass spectrometry. The peptides were purified and characterized as previously described (22, 24, 25). The peptides were purified by reverse-phase HPLC and characterized by amino acid analysis and electrospray ionization mass spectrometry.

**Cell preparation and maintenance**

Human PBMC were isolated from leukopacks of normal blood donors (courtesy of the Transfusion Medicine Department, Clinic Center, National Institutes of Health, Bethesda, MD) by routine Ficoll-Hypaque density gradient centrifugation. Monocytes were purified from PBMC using of a MACS CD14 monocyte isolation kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer’s recommendation. Neutrophils (purity ≥97%) were isolated from the same leukopacks by 3% dextran sedimentation as previously described (8). The in vitro generation of monocyte-derived macrophages and immature dendritic cells (DCs) was conducted as previously described (32, 33). Briefly, monocytes were cultured at 1 × 10^6/ml in complete RPMI 1640 medium (RPMI 1640 plus 10% FBS, 2 mM glutamine, 25 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin) in the presence of either rhM-CSF or a combination of rhGM-CSF and rhIL-4 at 37°C in a humidified atmosphere of 5% CO_2 and 95% air for 7 days for the generation of macrophages or immature DCs.

Human embryonic kidney (HEK) 293 cells stably transfected with human FPRL1 receptor (hereafter designated FPRL1/HEK293 cells) or mFPRL2 (hereafter designated mFPRL2/HEK293 cells) were obtained from Dr. J. M. Wang (National Cancer Institute, Frederick, MD), and were originally generous gifts of Drs. P. M. Murphy and J.-L. Gao (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD). The parental HEK 293 cells were maintained in complete DMEM (DMEM plus 10% FBS, 2 mM glutamine, 25 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin). Both FPRL1/HEK293 and mFPRL2/HEK293 cells were maintained in complete DMEM in the presence of 1%/ml G418 (Invitrogen Life Technologies, Rockville, MD).

**Chemotaxis assay**

Migration of monocytes, neutrophils, DCs, macrophages, and HEK 293, FPRL1/HEK293, and mFPRL2/HEK293 cells in response to chemotactic factor was evaluated using a 48-well microchemotaxis chamber (Neuro Probe, Gaithersburg, MD) as previously described (8, 32, 33). Briefly, different concentrations of chemotactic factors diluted in chemotaxis medium (RPMI 1640 containing 1% BSA) were placed in the lower wells of the chamber, and the cells suspended in chemotaxis medium were put in the upper wells. In some experiments, monocytes were pretreated for 30 min at 37°C in the absence or presence of PTX (final concentration = 200 ng/ml) before usage. For checkerboard analysis, TA was simultaneously added into the upper compartments. The lower compartment was separated from the upper compartment by a polycarbonate membrane (NeuroProbe) of different pore size based on the target cells. For neutrophils, monocytes, DCs, and macrophages, 5-μm uncoated membranes were used. For evaluating the migration of HEK 293, FPRL1/HEK293, and mFPRL2/HEK293 cells, polycarbonate membranes were coated at 37°C with type 1 rat tail collagen (BD Biosciences, Bedford, MA) at the final concentration of 50 μg/ml, air-dried, and used. After incubation at 37°C in humidified air with 5% CO_2 for a period of time (60 min for neutrophils, 90 min for monocytes, DCs, and macrophages, 300 min for HEK 293, FPRL1/HEK293 and mFPRL2/HEK293 cells), the membranes were removed, scraped in 0.1% Triton X-100, and washed. The membranes were determined using a BioQuant semiautomatic counting system (Nashville, TN). The results are presented as the number of cells per high power field (HPF).

**Measurement of Ca^{2+} flux**

Monocytes were loaded by incubating with 5 μM fura 2 (Molecular Probes, Eugene, OR) at 24°C for 30 min in the dark. Subsequently, the cells were washed with and resuspended in 10% saline buffer (138 mM NaCl, 6 mM KCl, 1 mM CaCl_2, 10 mM HEPES, 5 mM glucose, and 1% BSA, pH 7.4). Each 2 ml of loaded monocytes were transferred into a quartz cuvette, which was placed in a luminescence spectrometer LS50 B (PerkinElmer, Wellesley, MA). Ca^{2+} flux of the cells in response to chemotactic factors was measured by recording the ratio of fluorescence emitted at 510 nm after sequential excitation at 340 and 380 nm.

**Detection of MAPK activation**

Human monocytes were washed three times with PBS and suspended in serum-free RPMI 1640. Monocytes (2 × 10^6/sample) were incubated at 37°C in the absence or presence of indicated concentrations of TA, d-TA, or MMK-1 (as a positive control) for a period of time as specified. At the end of incubation, a large amount of ice-cold PBS (10-fold) was added to stop the stimulation, and the monocytes were spun down at 1500 × g for 5 min at 4°C. After complete removal of the supernatant, the monocytes were lysed by adding 50 μl of SDS sample buffer (62.5 mM Tris-HCl, pH 6.8 at 25°C, 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% bromphenol blue). The lysate was sonicated for 10 s on ice to shear DNA and boiled for 10 min afterward. The lysates were loaded (20 μl)ane and separated on a 4–12% NuPAGE Bis-Tris Gel (Invitrogen, Carlsbad, CA) using NuPAGE MES SDS Running buffer (Invitrogen) as the electrode buffer. SeeBlue Plus2 (Invitrogen) was used as the molecular size marker. After the electrophoresis, proteins in the gel were electrotransferred (30 V constant for 1 h) onto a piece of Immobilon membrane (Millipore, Bedford, MA) using NuPAGE transfer buffer (12 mM Tris, 96 mM glycine, pH 8.3, 0.1% v/v methanol, 0.5% w/v methanol). Phosphorylated MAPK was detected by Western blotting as previously described (34). Briefly, the membrane was sequentially washed, blocked for 1 h at room temperature, washed, and incubated at 4°C overnight in the presence of 1/1000 dilution of rabbit-anti-phospho-p44/42 (catalogue no. 9101; Cell Signaling Technology, Beverly, MA). On the next day, the membrane was washed and incubated with 1/2000 dilution of HRP-conjugated anti-rabbit IgG (catalogue no. 7074; Cell Signaling Technology) for 1 h. The membrane was washed and incubated with 10 ml working solution of ECL Plus Western Blotting Detection System (Amersham Biosciences) for 5 min at room temperature, and exposed to a piece of BioMax x-ray film (Kodak, Rochester, NY) for 5 s. The x-ray film was developed using an automatic processor (Kodak X-OMAT 200A). The same membrane was stripped and probed for p44/42 protein essentially in the same manner except using rabbit anti-p44/42 (catalogue no. 9102; Cell Signaling Technology) as the primary Ab.

**Binding assay**

Competitive binding was performed in triplicate by adding constant amount of iodinated Aβ, [125I]-Aβ, and increasing amounts of Aβ or TA to individual 1.5-ml microtubes, each containing 2 × 10^6 FPRL1/HEK293 transfected cells suspended in chemotaxis medium. After incubation at room temperature with constant mixing, the membranes were centrifuged through a 10% sucrose/PBS cushion and the cell-associated radioactivity was measured with a 1217 Wallac gamma counter. The percentage of binding was calculated by the formula: Percentage of binding = [(cpm in the presence of unlabeled ligand)/cpm in the absence of unlabeled ligand] × 100.

**Air pouch experiments**

Six to eight-wk-old BALB/c mice were obtained from the Animal Production Facility, National Cancer Institute (Frederick, MD) and maintained...
under specific pathogen-free conditions in the Experimental Animal Facility at the National Cancer Institute for several weeks to allow acclimation before the experiments. The animal studies were conducted in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Animals. Air pouches were raised on the dorsum as previously described (34). Mice with a well-formed air pouch were randomized into two groups. Each air pouch was injected with 1 ml of endotoxin-free PBS alone or PBS containing 1 μM TA. Four hours later, the mice were killed by CO₂ asphyxiation, and cells in the air pouches were washed out and enumerated following trypan blue staining. Differential counting of leukocyte subpopulations migrating into the pouch space was performed after eosin-methylene blue staining of cytospins.

Results

TA is chemotactic for human monocytes and neutrophils

Of the temporins and temporin-like amphibian linear antimicrobial peptides identified so far, TA is one of the very first three temporins identified (18) and has been extensively investigated in terms of antimicrobial activity and the relationship between its structure and antimicrobial activity (21–24, 26, 35). To investigate whether TA has the capacity to induce chemotaxis, the migration of human peripheral blood monocytes in response to TA was examined using the 48-well microchemotaxis chamber. As shown in Fig. 1A, TA induced monocyte migration with a bell-shaped dose-response curve. The peak response was observed at 250 nM. In the same experiment, two synthetic analogues of TA, α-TA, TA composed of all d-amino acid residues, and Rev-TA, TA with reversed sequence, did not induce the migration of monocytes over the range of concentrations tested, suggesting that TA-induced monocyte migration was based on a chiral interaction. To determine whether TA-induced migration of monocytes was due to chemotaxis or chemokinesis, checkerboard analysis was performed (Table I). Increasing concentrations of TA added simultaneously with taxis or chemokinesis, checkerboard analysis was performed (Tacyte migration was based on a chiral interaction. To determine sequence, did not induce the migration of monocytes over the absorption or presence of the specified concentration of TA were added to the upper wells of the chemotaxis chamber. The result of one experiment representative of three is shown as the average (mean ± SD) of migrated monocytes of six high-power microscopic fields of triplicate wells.

To investigate whether monocytes other than monocytes could also respond to TA, we tested whether neutrophils could be chemotaxically attracted by TA. Similar to monocytes, neutrophils migrated in response to TA with a bell-shaped dose-response curve (Fig. 1B), indicating that TA is also an agonistic chemotaxant for human neutrophils.

TA uses a G protein (Gαi)-coupled receptor

To find out whether TA-induced phagocyte chemotaxis was mediated by a Gαi-coupled receptor, we next examined whether TA-induced monocyte chemotaxis could be inhibited by PTX, a toxin that specifically inhibits Gαi-coupled receptor signaling by ADP-ribosylating Gαi protein (36). Incubation of monocytes at 37°C for 60 min in the presence of PTX (200 ng/ml) before chemotaxis assay completely inhibited the migration of monocytes in response to TA (Table II). Pretreatment with PTX did not affect the absolute motility of monocytes because there was no difference in spontaneous migration (in response to chemotaxis medium) between PTX-pretreated or control monocytes. Furthermore, the inhibition was not due to the preincubation time per se because control incubation of monocytes in the absence of PTX did not inhibit migration in response to TA. As expected, fMLP-induced monocyte migration was also greatly inhibited by PTX. Therefore, TA-induced monocyte chemotaxis seems to be mediated by a Gαi-coupled receptor.

Besides chemotaxis, activation of a Gαi-coupled receptor by its chemotactic agonist ligands often results in activation of MAPK activation in target cells (37, 38). Therefore, we investigated whether TA could also induce the activation of p44/42 MAPK in monocytes (Fig. 2). PBS-treated monocytes displayed basal level of phospho-p42 with very little phospho-p44 (Fig. 2, lane I, top).

Table I. Checkerboard analysis of TA-induced migration of human monocytes

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<th>TA in the Lower Wells (nM)</th>
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* TA at specified concentration was added to the lower wells of the chemotaxis chamber, and monocytes at 10⁶/ml in the absence or presence of the specified concentration of TA were added to the upper wells of the chemotaxis chamber. The result of one experiment representative of three is shown as the average (mean ± SD) of migrated monocytes of six high-power microscopic fields of triplicate wells.

* TA Usage of FPR1 to Attract Phagocytes

To investigate whether phagocytes other than monocytes could also respond to TA, we tested whether neutrophils could be chemotaxically attracted by TA. Similar to monocytes, neutrophils migrated in response to TA with a bell-shaped dose-response curve (Fig. 1B), indicating that TA is also an agonistic chemotaxant for human neutrophils.
Treatment of monocytes with MMK-1 at $10^{-7}$ M for 5 min (as a positive control) resulted in a marked increase of both phospho-p42 and phospho-p44 as expected (Fig. 2, lanes 2–4, top). Phosphorylation of p44/42 MAPK in monocytes in response to 1 μM of TA begun to increase upon TA treatment for as short as 1 min (Fig. 2, lanes 5–7, upper panel). Similar to the inability of α-TA to induce monocyte chemotaxis (Fig. 1A), α-TA did not activate p44/42 MAPK (Fig. 2, lane 8, top). Probing the same membrane with anti-p44/42 Ab after stripping revealed identical amounts of p44/42 MAPK on each lane (Fig. 2, top). Phosphorylation of p44/42 MAPK in monocytes with TA for 5 min enhanced the levels of both phospho-p42 and phospho-p44 MAPK as expected (Fig. 2, lane 9). Probing the same membrane with anti-phospho-p44/42 Ab after stripping revealed identical amounts of p44/42 MAPK on each lane (Fig. 2, bottom). The exposure time for the autoradiographs was 30 s. Shown are the results of one experiment representative of three.

**FPRL1 functions as a receptor for TA**

As the first step to identify the receptor for TA, we examined whether differentiation of monocytes to either DCs or macrophages (Mφ) would affect their responsiveness to TA. Monocytes purified from the peripheral blood of the same healthy donor were cultured in the presence of M-CSF or a combination of GM-CSF and IL-4 for 7 days for the generation of monocyte-derived Mφ or monocyte-derived immature DCs as previously described (33). Monocyte-derived Mφ migrated chemotactically in response to TA (Fig. 3, upper panel), whereas DCs differentiated from the same starting population of monocytes failed to do so (Fig. 3, lower panel). The failure of monocyte-derived immature DCs to migrate in response to TA was not due to the lack of motility because those immature DCs migrated in response to another chemottractant, W peptide (Fig. 3, lower panel). Therefore, the Gαs-coupled receptor used by TA must be a receptor that is expressed by monocytes, neutrophils, and monocyte-derived Mφ, but its expression must be down-regulated when monocytes are differentiated to immature DCs in vitro.

Analysis of the expression patterns of all known Gαs-coupled receptors based on published reports revealed one possible candidate, FPRL1, which is expressed by monocytes (29, 33, 39, 40), neutrophils (29, 39, 40), and monocyte-derived Mφ (33), but down-regulated on immature DCs differentiated from monocytes (33). To test whether TA indeed acts on FPRL1 in monocytes, we investigated whether TA could induce Ca$^{2+}$ flux in monocytes, and if so, whether its capacity to induce Ca$^{2+}$ flux in monocytes could be homologously desensitized by an FPRL1-specific agonistic ligand, MMK-1 (27). As expected, both MMK-1 and TA induced a dose-dependent Ca$^{2+}$ flux in monocytes (Fig. 4, A and B). In complete agreement with the results showing that α-TA and Rev-TA could not induce chemotaxis of monocytes, neither α-TA nor γ-TA could induce Ca$^{2+}$ flux in monocytes (Fig. 4, C and D). Interestingly, the Ca$^{2+}$ flux induced by TA at 1 μM was completely desensitized by pretreatment of monocytes with 100 nM MMK-1 (Fig. 4E). Conversely, TA at 3 μM also completely desensitized the Ca$^{2+}$ flux in response to 5 nM MMK-1 (Fig. 4F). Because homologous cross-desensitization of Ca$^{2+}$ flux is often due to two agonistic ligands acting on the same receptor, these data indicate that TA uses FPRL1 as a functional receptor.

To directly test whether TA uses FPRL1 as a receptor, we examined whether TA could chemoattract HEK 293 cells stably transfected to express FPRL1. Indeed, TA dose-dependently induced the migration of FPRL1/HEK293 cells, but not the parental monocytes.

**FIGURE 2.** TA activation of p44/42 MAPK in monocytes. Freshly isolated human peripheral monocytes received no treatment or were treated with indicated concentrations of TA, α-TA, and MMK-1 for a period of time as specified before lysis. The cell lysates were separated on a 4–12% Bis-Tris Gel, transferred to polyvinylidene difluoride membrane, and the phosphorylated p44/42 MAPKs were detected by Western blotting with anti-phospho-p44/42 (top). The same membrane was stripped and blotted with anti-p44/42 (bottom). The exposure time for the autoradiographs was 30 s. Shown are the results of one experiment representative of three.

**FIGURE 3.** TA induction of chemotaxis of monocyte-derived macrophages. The chemotaxis of macrophages (Mφ) and immature dendritic cells (iDCs) differentiated from monocytes of the same blood donor in response to TA was evaluated by chemotaxis assay. The results are shown as the average cell migration (mean ± SD) of triplicate wells. Similar results were obtained from two separate experiments.

**FIGURE 4.** TA stimulation of Ca$^{2+}$ flux in monocytes and cross-desensitization. Ca$^{2+}$ flux of fura 2-loaded human monocytes in response to MMK-1 (A), TA (B), α-TA (C), and Rev-TA (D) was measured by recording the ratio of emission at $λ_{380}$ after simultaneous excitation at $λ_{340}$ and $λ_{380}$. Cross-desensitization of Ca$^{2+}$ flux was determined by sequential adding MMK-1 and TA at indicated concentrations (E) or vice versa (F).
HEK 293 cells lacking FPRL1 expression (Fig. 5A) or HEK 293 stably transfected to express several other \(G_{i}\)-coupled receptors (data not shown). To confirm whether TA binds to FPRL1, we investigated whether TA could inhibit the binding of iodinated A\(_{\beta}\), a selective agonistic ligand for FPRL1 (41), to FPRL1/HEK293 cells (Fig. 5B). As expected, unlabeled A\(_{\beta}\) dose-dependently inhibited the binding of iodinated A\(_{\beta}\) to FPRL1/HEK293 cells. TA, although less potent than A\(_{\beta}\), also displaced the binding of iodinated A\(_{\beta}\) to FPRL1/HEK293 cells in a dose-dependent manner with an EC\(_{50}\) of \(\sim 2 \mu M\). These data collectively confirm that TA uses FPRL1 as a receptor.

**TA acts as a phagocyte chemoattractant in vivo**

To investigate whether TA could chemoattract phagocytes in vivo in mice, we first examined whether mFPR2, the mouse ortholog for human FPRL1 (39, 41–44), could host TA as an agonistic ligand. As shown by Fig. 6A, TA dose-dependently induced the migration of mFPR2/HEK293 cells, indicating that TA can function as an agonistic ligand for mFPR2. Accordingly, we reasoned that TA should also be active in mice because mouse leukocytes express mFPR2 mRNA (42), and mouse phagocytes can be activated by agonistic ligands for FPRL1 (43–45). Therefore, the potential in vivo chemoattracting activity of TA was studied by the use of mouse air pouch model. One milliliter of PBS or TA diluted in PBS at 1 \(\mu M\) was injected into each air pouch on the back of mice and the cells recruited into the pouches were lavaged out and evaluated 4 h after the injection. As shown by Fig. 6B, TA markedly increased the number of leukocytes in the air pouches when compared with PBS control group, indicating that TA functions as a leukocyte chemoattractant in vivo. Based on the microscopic morphologies and differential counting, \(\sim 95\%\) of the leukocytes recruited by TA into the air pouches were phagocytes, including neutrophils (73\%) and monocytes (22\%), which was in agreement with its in vitro chemotactic activities for both neutrophils and monocytes and macrophages (Fig. 7C). Thus, TA is chemotactic for phagocytes both in vitro and in vivo and uses FPRL1 or its ortholog (mFPR2 in mice) as its receptor.

**Chemotraction of human phagocytes by other temporins or synthetic analogues and receptor use**

To address whether the phagocyte-attracting activity was unique for TA, we investigated whether two additional temporins, Rana-6 and T1P, and two consensus synthetic analogues deduced from all temporins identified so far (21) could also induce the in vitro migration of human phagocytes. Of the four AMPs tested, Rana-6, I4S10-C, and I4G10-C induced the migration of human monocytes and neutrophils with an optimal dose from 0.5–5 \(\mu M\) (Fig. 7A). T1P chemoattracted neutrophils, but not monocytes (Fig. 7A). To investigate whether these AMPs also use FPRL1, we examined whether they could induce the migration of FPRL1/HEK293 cells using MMK-1, TA, d-TA, and Rev-TA for positive and negative controls (Fig. 7B). Similar to TA, I4S10-C, I4G10-C, and Rana-6 induced the migration of FPRL1/HEK293 cells, suggesting that these AMPs also use FPRL1 as the chemotactic receptor. d-TA and Rev-TA, as negative controls did not induce the migration of FPRL1/HEK293 cells. T1P, although chemotactic for neutrophils (Fig. 7A), did not induce the migration of FPRL1/HEK293 cells, suggesting that it uses a receptor other than FPRL1 on neutrophils.

**Discussion**

In this study, we have shown that TA, a representative frog-derived temporin, is a chemoattractant for phagocytic leukocytes including neutrophils, monocytes, and macrophages both in vitro and in vivo, and identified the receptor that TA uses to mediate its chemotactic effect as human FPRL1/mFPR2. Similar to TA, Rana-6, another temporin, and I4S10-C and I4G10-C, two position 10 isomers of the consensus sequences of temporin-like peptides (21), are also capable of chemoattracting human monocytes and neutrophils by activating FPRL1 (Fig. 7). T1P, another frog-derived temporin, although unable to chemoattract monocytes, is a chemoattractant for human neutrophils, presumably using a receptor other than FPRL1 (Fig. 7). Numerous studies have revealed...
that defensins and cathelicidins, two major categories of mammalian AMPs, function both as direct endogenous antibiotics and as multifunctional mediators of the immune system by recruiting and/or activating various types of leukocytes (3, 5, 6, 8–10, 34, 46–48). Although only human and mouse leukocytes were used in this study and it remains to be determined whether TA and related frog AMPs also act as chemoattractants for frog leukocytes, our results provide the first evidence indicating that at least some amphibian AMPs such as TA and Rana-6 act across species barriers and can function as chemotactic recruiters for mammalian phagocytic leukocytes expressing FPRL1-type receptor(s).

FPRL1/mFPR2 is a member of the formyl peptide receptor subfamily (39, 41). Recently a variety of agonistic ligands have been identified for this receptor, among which naturally occurring agonistic ligands consist of two categories: host-derived mediators including serum amyloid A, lipoxin A4, LL-37, amyloid A, and annexin 1-derived peptide (8, 41, 43, 49–52). Many vertebrate species, including human cathelicidin/LL-37 (55) and can be recognized by anti-human cathelicidin/LL-37 Abs (56). Therefore, it appears that many vertebrate α-helical AMPs may function as agonistic ligands for FPRL1/mFPR2 type of receptor. The AMPs identified so far to act as ligands for FPRL1/mFPR2, such as cathelicidins and temporins, are capable of inducing chemotaxis and activation of phagocytes, suggesting a role of the interaction between FPRL1 (or its orthologs) and agonistic AMPs in the promotion of innate host defenses by recruiting phagocytes to sites of microbial invasion in which AMPs are present as a result of constitutive or induced production.

Of the natural ligands for FPRL1/mFPR2 identified so far, most are peptides and proteins capable of inducing chemotaxis and activation of phagocytes and have thus been considered to promote inflammatory responses in the context of host defense or pathogenesis of diseases (8, 41, 43, 49, 52–54). However, many eicosanoid metabolites such as lipoxin A4, aspirin-triggered 15-epi-lipoxin A4, and their stable synthetic analogues also act as ligands for FPRL1-type receptor (hence FPRL1 is also called LXA4R) (50, 57). However, these lipid ligands and certain glucocorticoid-induced annexin 1-derived peptides (51), by interacting with FPRL1/LXA4R-type receptor, predominantly cause inhibition, rather than promotion, of the recruitment and activation of inflammatory phagocytes (50, 51, 57–61). Therefore, FPRL1/LXA4R appears to be capable of mediating both pro- and anti-inflammatory signals, which is dependent on the type as well as temporal and spatial availability of ligands under various pathophysiological conditions (40, 62).

Due to the emergence of antibiotic-resistant pathogenic strains of bacteria, and because many AMPs can efficiently kill, at least in vitro, clinical isolates of such antibiotic-resistant pathogens, AMPs have been investigated as a source of new antibiotics (63, 64). Topical application of the P113 gel whose effective ingredient is histatin, a linear antimicrobial peptide physiologically produced by salivary glands, has been shown to be safe and able to reduce experimental gingivitis in humans (65). Topical oral administration of iseganan (formerly known IB367), a modified synthetic analogue of protegrin, has been shown to reduce the occurrence of oral mucositis and to reduce ventilator-associated pneumonia in patients receiving stomatotoxic chemotherapy in phase III clinical trial (66). In this regard, temporins are good candidates for the development of clinically applicable peptide antibiotics for local and/or systemic use because 1) they are small (13 amino acid residues) linear AMPs so that they can be easily synthesized in considerable amount using well-established solid-phase chemistry and have a potential good bioavailability if orally administered, and 2) they are active on Gram-positive and Gram-negative bacteria, and fungi (18–20, 25, 35) including methicillin-resistant Staphylococcus aureus and vancomycin-resistant Enterococcus faecium (21, 23, 24). The discovery of temporin chemotactic activities and the identification of their receptor in this study suggest that their effects on phagocytic leukocytes should also be taken into consideration in the process of developing potential applicable antibiotics because the effect on leukocytes can lead to undesirable proinflammatory consequence. Interestingly, the available data indicate that the antimicrobial and receptor-mediated effects on phagocytic leukocytes seem to be based on different structural requirements. For instance, TA has both antimicrobial (21, 23, 25, 35) and phagocyte-activating effects leading to chemotaxis (Figs. 1 and 3 and Table I), Ca2+ influx (Fig. 4), and MAPK activation (Fig. 2). 1,10-TA, although showing similar antibacterial activity as TA (21, 24), does not have any phagocyte-activating effects (Figs. 1, 2, and 4) and cannot trigger FPRL1-expressing HEK 293 cells (Fig. 7B). Conversely, 14S10-Con and 14G10-Con maintain the FPRL1-mediated phagocyte-activating effect of TA (Fig. 7), whereas showing more potent antibacterial activities than TA (21, 24, 25). Thus, two types of potential clinically applicable antibiotics can be
References


