Recognition of Bacterial Signal Peptides by Mammalian Formyl Peptide Receptors: A New Mechanism for Sensing Pathogens

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Background: The function of formyl peptide receptors (FPRs) is incompletely understood.

Results: We report the identification of bacterial signal peptides as potent activators of mammalian FPRs and innate immune responses and define critical features underlying FPR peptide recognition.

Conclusion: These findings identify a molecular signature for FPR activation.

Significance: Our results define a novel mechanism for sensing bacteria.

SUMMARY

Formyl peptide receptors (FPRs) are G-protein-coupled receptors (GPCRs) that function as chemotactic receptors in innate immune responses. Here we perform systematic structure-function analyses of FPRs from six mammalian species using structurally diverse FPR peptide agonists and identify a common set of conserved agonist properties with typical features of pathogen-associated molecular patterns. Guided by these results, we discover bacterial signal peptides, normally used to translocate proteins across cytoplasmic membranes, as a vast family of natural FPR agonists. N-terminally formylated signal peptides fragments with variable sequence and length activate human and mouse FPR1 and FPR2 at low nanomolar concentrations, thus establishing FPR1 and FPR2 as sensitive and broad signal peptide receptors. The vomeronasal receptor mFpr-rs1 and its sequence orthologue hFPR3 also react to signal peptides but are much more narrowly tuned in signal peptide recognition. Furthermore, all signal peptides examined here function as potent activators of the innate immune system. They elicit robust, FPR-dependent calcium mobilization in human and mouse leukocytes and trigger a range of classical innate defense mechanisms such as the production of reactive oxygen species, metalloprotease release, and chemotaxis. Thus, bacterial signal peptides constitute a novel class of immune activators that are likely to contribute to mammalian immune defense against bacteria. This evolutionarily conserved detection mechanism combines structural promiscuity with high specificity, and enables discrimination between bacterial and eukaryotic signal sequences. With at least 175,542 predicted sequences, bacterial signal peptides represent the largest and structurally most heterogeneous class of GPCR agonists currently known for the innate immune system.
The initial sensing of infection depends on innate pattern recognition receptors (PRRs) that recognize evolutionary conserved structures of microorganisms known as pathogen-associated molecular patterns (PAMPs). Toll-like receptors represent a prime example for such PRRs (1,2). Formyl peptide receptors (FPRs) belong to a class of G-protein-coupled receptors (GPCRs) involved in host defense against pathogens in the innate immune system (3-5). FPR function is best known in phagocytic leukocytes (e.g. neutrophils and monocytes) that, in response to microbial chemoattractants, migrate and accumulate at sites of infection where they release reactive oxygen species (ROS) and other factors to combat invading microorganisms (6-8). Activation of FPRs triggers classical GPCR signaling cascades involving G-protein-dependent phospholipase C stimulation leading to intracellular Ca²⁺ mobilization (9-11). Humans are known to express three FPR genes – FPR1, FPR2, and FPR3 – but this number varies across mammalian species (12,13).

FPRs have been proposed to function as PRRs (5,7,14,15) but their pathogen-associated molecular pattern remains unclear. In fact, one of the most puzzling features of the FPR family is their unusually high degree of molecular promiscuity (3,7,16). Although FPRs have been named according to their capability to detect formylated peptides (5,17), these receptors can recognize structurally diverse agonists with no obvious common pattern in amino acid sequence or natural origin (3,16). Such ligands include N-formylated, C-amidated, and unmodified peptides from bacterial and viral pathogens as well as host-endogenous mitochondrial peptides and several non-peptide agonists such as resolvin D1 and lipoxin A4 (3,5,18). FPRs detect a wide range of structurally diverse pro- and anti-inflammatory ligands associated with important human diseases such as amyloidosis, Alzheimer’s disease, HIV, and inflammatory pain (19,20).

Although FPR expression has been initially described in immune cells, it is becoming increasingly clear that FPRs are also expressed in other cell types and tissues, from the nervous system (13,21-23) to internal organs including lung and gut (24,25), suggesting that FPRs could be generally involved in the sensing and management of the microbiome of the body. An important development has been the finding that a set of FPR-like proteins represents a distinct receptor family in chemoitative neurons of the mouse vomeronasal organ (VNO) (13,23) and that some of these neurons recognize formylated peptides (23,26), suggesting an evolutionary relationship between recognition mechanisms in chemotactic immune cells and subsets of sensory neurons of the VNO (27-29).

To gain new insight into the function and recognition capabilities of FPRs and to better understand the molecular promiscuity of these receptors, we investigated conserved features in the structure of disparate peptide agonists required for activation of human and mouse FPRs. Through this approach, we generated critical knowledge leading to the discovery of an unsuspected, extremely large family of natural FPR agonists with common structural properties: bacterial signal peptides and their short breakdown products. Signal peptides are N-terminal protein signatures that are required for directing the transfer of bacterial proteins through the plasma membrane during which they are cleaved off to give rise to the native form of membrane-associated or secreted proteins (30,31). We observed a remarkably high degree of sensitivity, selectivity, and functional conservation of signal peptide recognition across species and receptor subtypes for FPR1 and FPR2, strongly arguing for an important role of this novel agonist family during evolution of mammalian FPR function. Dynamic measurements in human and mouse innate immune cells demonstrate that all tested signal peptides are recognized by these cells and trigger classical innate immune responses such as intracellular Ca²⁺ mobilization, generation of reactive oxygen species, release of metallopeptidase, and chemotactic cell migration. These observations argue that mammalian FPRs may have evolved originally as germline-encoded pattern recognition receptors that recognize structurally conserved export motifs of bacterial signal sequences as their cognate, pathogen-associated molecular pattern.

**EXPERIMENTAL PROCEDURES**

**Cloning of FPR genes**–All FPR genes were cloned as described previously (32). Sequences of the coding regions are annotated in supplemental Table S1.

**Cell culture and transient transfection**–HEK293T PEAKrapid cells (ATCC) cells were cultivated as previously described (32). For immunostaining and intracellular Ca²⁺ measurements, cells were seeded at 20 - 30% confluency on poly-D-lysine coated 96-well μ-
clear plates (Greiner). Cells were grown to 50 - 70% confluency and transfected with equal amounts of receptor and G-protein subunit Ga16 using jetPEI (PeqLab) according to manufacturer’s protocol.

Isolation and cultivation of human monocytes—Peripheral blood leukocytes (PBLS) enriched in leukocyte-reducing-system chambers were kindly provided by the Institute of Clinical Haemostaseology and Transfusion Medicine, University of Saarland School of Medicine. Monocytes were isolated by Ficoll (PAA) density gradient centrifugation in Leucosep filter columns (Gibco) according to the manufacturer’s protocol. Following centrifugation, leukocytes were washed with HBSS (Sigma), erythrocytes and thrombocytes were lysed by resuspension in lysis buffer (in mM: 155 NH₄Cl, 10 KHCO₃ and 0.13 EDTA in H₂O, pH 7.3) for 2 min. Cells were harvested by centrifugation, the pellet was rinsed with HBSS and resuspended in PBS with 0.5% BSA. Subsequently, monocytes were further enriched through an adhesion protocol. Cells were first kept in standard culture flasks containing RPMI-1640 with 10% FCS and 1% Penicillin/Streptomycin. After 2 h the medium was exchanged to remove non-adhesive cells. The remaining cell fraction was further incubated overnight in culture medium. On the following day, cells were rinsed with PBS plus 0.5% BSA, scratched from the flask surface, and seeded in Ultra-Low cluster 24-well plates (Corning) at a density of 1 - 4 x 10⁶ cells/ml in culture medium. After an additional overnight incubation, monocytes were harvested by gentle trituration and seeded at 30,000 cells/well on uncoated µ-clear plates (Greiner). The cell-pellet was resuspended by gentle swirling. For erythrocyte lysis, the pellet was gently resuspended in 5 ml of sterile water and immediately mixed with 45 ml DPBS. After centrifugation (10 min for 339 g at 4°C) the supernatant was removed and the lysis-procedure was repeated. The pellet was gently resuspended in RPMI-1640 (Gibco) supplemented with 2 mM L-Glutamine, 1% Penicillin/Streptomycin, 1% Na-Pyruvate, and 1% autologous human serum.

Isolation of mouse leukocytes—Three different mouse strains were used in these experiments: (1) wild type mice (C57BL/6J); (2) mice harboring a global deficiency in Fpr1 (C57BL/6NTac-Fpr1<sup>Tm1GaoN6</sup>, denoted as Fpr1<sup>−/−</sup> mice) (Taconic) (12); (3) and their heterozygous littermate controls (Fpr1<sup><i>+/−</i></sup>). To obtain sufficient quantities of cells from these strains, leukocytes were extracted from bone marrow (33). Mice were killed and femur and tibia from both hind legs were isolated. The distal tips of each bone were cut off and the bone marrow was obtained by forced rinsing with ice cold Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free HBSS buffer. The cell suspension was filtrated with a 100 µm cell strainer (BD) and then centrifuged (300 g for 8 min at 4°C). After removal of the supernatant, cells were resuspended in HBSS containing 1 mM Ca<sup>2+</sup>, 1 mM Mg<sup>2+</sup>, and 2 µM Fluo-4 AM. Approximately 150,000 cells per well were seeded on µ-clear plates (Greiner) and incubated for 45 min at room temperature. Cells were rinsed two times with HBSS before Ca<sup>2+</sup> recordings were performed. Staining with nuclear dyes and Ly6G antibody (a neutrophil marker) showed that this leukocyte preparation contains 50% mature neutrophils in wild type, Fpr1<sup>+/−</sup>, or Fpr1<sup>−/−</sup> mice. When we isolated mouse neutrophils directly from peripheral blood using Ly6G beads (pluriSelect), the cell number was reduced by 10-fold but these cells showed comparable Ca<sup>2+</sup> responses.

Immunocytochemistry—Cells were seeded in 96 well pates as described above. For cell surface expression studies, cells were fixed for 4 min in methanol-free paraformaldehyde (Polyscience Inc), 4% in PBS. For immunostaining, cells were blocked with 5% heat inactivated FCS in PBS for 30 min and incubated overnight with the following monoclonal primary antibodies: anti-human FPR1 mouse IgG<sub>2A</sub> (R&D Systems, MAB3744, 1 µg/ml); mouse IgG<sub>2A</sub> isotype control (R&D Systems, MAB0031, 1 µg/ml); anti-human CD14 mouse IgG1,κ (Biolegend, 325601, 0.5 µg/ml); mouse IgG1,κ isotype control (Biolegend, 400101, 0.5 µg/ml). Staining was obtained by 45-min incubation with a polyclonal donkey anti-mouse Alexa Fluor 555 antibody (Invitrogen, 2 µg/ml).
throughput Ca²⁺ measurements of cell Ca²⁺-dependent fluorescence changes of cells Fura2-AM (Molecular Probes) for 1 h at RT. Bioscience) (32). Cells were loaded with 2 µM Pathway Bioimager 855 system (BD with single-cell resolution, we used the BD imaging of human monocytes and HEK cells immediately following the experiment to 5 min by addition of 2 % paraformaldehyde was used. To correlate Ca²⁺ responses with receptor expression we used posthoc antibody staining. Cells were fixed for 5 min by addition of 2 % paraformaldehyde immediately following the experiment to prevent receptor internalization.

**Calcium imaging**—For automated high throughput Ca²⁺ measurements of cell populations using a FLIPR (fluorescence imaging plate reader) system (Molecular Devices), mean Ca²⁺ changes of cell populations (cells/well: HEK293T, ~50,000; human monocytes, ~30,000; human granulocytes, 100,000; mouse leukocytes, 150,000) were recorded essentially as described previously (32). Briefly, HEK cells were loaded 48-h post transfection with 2 µM Fluo4-AM (Molecular Probes) in C1 solution (130 mM NaCl, 10 mM HEPES, 5 mM KCl, 2 mM CaCl₂, 5 mM glucose, pH 7.2) at RT for 2 h. Monocytes were loaded in Ringer for 1 h whereas human and mouse granulocytes were loaded for 45 min in RPMI or HBSS, respectively. For automated high content Ca²⁺ imaging of human monocytes and HEK cells with single-cell resolution, we used the BD Pathway Bioimager 855 system (BD Bioscience) (32). Cells were loaded with 2 µM Fura2-AM (Molecular Probes) for 1 h at RT. Ca²⁺-dependent fluorescence changes of cells were recorded at 0.5 Hz and quantified either with BD-image Explorer software (BD Bioscience) or ImageJ 1.46r (32). Monocytes were treated as described above for HEK cell experiments except that 3% methanol-free paraformaldehyde was used. To correlate Ca²⁺ responses with receptor expression we used posthoc antibody staining. Cells were fixed for 5 min by addition of 2 % paraformaldehyde immediately following the experiment to prevent receptor internalization.

**Chemotactic cell migration**—Granulocyte migration assays were performed in duplicates using 96-well HTS transwell chambers (Corning) with 3 µm pore size according to the manufacturer’s protocol. Directly after isolation 100,000 cells per well were seeded in the upper chamber in 75 µl RPMI medium and subsequently maintained for 2h in a cell culture incubator. Thereafter, the number of transmigrated cells was determined by automated counting using the BD Pathway Bioimager 855 system.

**Ligands**—Custom made peptides were routinely synthesized by VCPBIO LAB, United Peptides, and Genscript Corporation. The sources of commercially available agonists and antagonists are listed in Table SIII. Purity of ligands was typically >95%. Agonists were routinely dissolved in C1 buffer or in ringer as a 0.2 - 1 mM stock solution. Strongly hydrophobic substances were dissolved as 10 mM stocks in DMSO (99.7%, Sigma). Stock solutions were routinely stored in small aliquots at -20°C until use. More details on sequence, purity, source, solvents and storage conditions are given in Table 1. To increase solubility, the highest concentration of the working aliquot was routinely heated for 3 min to 80°C prior to further dilutions.

**Data analysis**—Experiments were routinely performed as duplicates and averaged over at least three independent transfections/donors. Response amplitudes (ΔF/F₀) were calculated by dividing maximal fluorescence change after ligand application by baseline fluorescence. Maximal amplitudes were set to 100% and curves were calculated with Graph Pad Prism 5.0 using the equation for sigmoidal dose response with variable slope. Standard deviation was calculated as averages from independently obtained EC₅₀ values using the STDEV.P function of Microsoft Excel 2010. Single-cell imaging videos of monocytes and analysis of posthoc immunochemistry was done coated overnight with a mouse anti-human MMP-9 (1 µg/ml) antibody. On the following day, the plates were first incubated with 50 µl/well of the test samples for 2 h followed by a 2 h-incubation with a biotinylated goat anti-human antibody (100 ng/ml) and a 20-min incubation with Streptavidin-HRP (1:200). For detection, a tetramethylbenzidine containing substrate solution was added. The reaction was stopped after 20 min and the optical density was determined with a microplate reader (2030 Multilable Reader Victor X4, Perkin Elmer) at 450 nm excitation and a wavelength correction set to 540 nm. Measurements were always carried out as duplicates. MMP-9 concentrations were determined via concentration standards.

**Matrix metallopeptidase release**—The release of MMP-9 from human granulocytes was determined using the Human Total MMP-9 DuoSet (R&D Systems) according to the manufacturers protocol. 96-well plates were...
with ImageJ 1.46r. All cells responding to Ringer bath application (<5%) were excluded from evaluations. Posthoc correlations of Ca²⁺ signals and receptor staining were plotted with Origin8.6.

Estimating the size of the ligand family–To provide an estimate of the size of the signal peptide ligand family, we tested a set of twelve randomly selected signal sequences. All peptides were chosen independently of their amino acid sequence among all 1,168 experimentally confirmed signal peptides present in the database (www.signalpeptide.com). We found that all twelve hexapeptides were activators of FPRs. We then calculated the probability to obtain this result by chance using the basic urn model. In this approach we randomly draw 12 peptides and assumed different percentages of FPR activators among the total number of peptides in the library. We calculated the probability for a range from 100 to 70% of FPR activators using the formula: $x^{12} = p \times (x$ denotes the percentages of FPR agonists and $p$ denotes the probability that this ratio is obtained by chance). If we assume that 90%, 80% or 70% of the sequences are agonists, the respective p-values for an x of 90%, 80% or 70% are $0.90^{12} = 0.28$, $0.80^{12} = 0.07$, $0.70^{12} = 0.01$. Thus, the respective probability to obtain our result by chance is 28%, 7% or 1%, respectively. Calculation of the 95% confidence interval ($0.78^{12} = 0.05$) leads to the prediction that 78% of the peptides in the database should be FPR activators with a confidence level of 95%.

Fluorescence-based hydrogen peroxide measurement–Monocyte-dependent hydrogen peroxide ($H_2O_2$) production was measured using $H_2O_2$ reactive Amplex®UltraRed (Molecular Probes) that forms the fluorescent dye Resorufin during the reaction. Signals were recorded at 535 nm excitation and 590 nm emission in a Tecan GENios Pro plate reader with bottom reading settings using black 96-well plates (Greiner). All measurements were performed as duplicates with ~30,000 monocytes per well in Ringer supplied with 50 µM Amplex®UltraRed, 0.1 U/ml horseradish peroxidase and 10 U/ml superoxide dismutase. $H_2O_2$ concentrations were calculated from relative fluorescent units (RFU) 10 min after application using calibration curves.

Peptide modeling–The secondary structure of the peptides was modeled with the molecular graphics software package Accelrys Discovery Studio v1.6 and Visualizer 3.5 installed on a PC. For comparison, selected structures were also modeled with QUANTA (Release 4.1, MSI) running on a Silicon Graphics Indy work station. For the modified groups (e.g., formylation, amidation) the atom types were adequately adjusted. Each structure was minimized in energy by the implemented Dreiding or charmm22 force field using the “steepest descent” algorithm until a threshold was achieved. Selected structures were further processed by molecular dynamics calculations (QUANTA) to monitor structural flexibility (34). The minimal energy structures of the various peptides were superimposed by fit of C-a-pairs (formylated or amidated peptides) or by tethers on backbone atoms (formylated and amidated cases) for minimal rms (root mean square) superposition. For comparison of formylated and amidated petides rotamers of the side chains were selected to achieve close similarity of side chain orientation. These structures were again minimized in energy. From the NMR structures of f-MLF (1Q7O.pdb) one conformational state was selected and minimized in energy after editing atom types. This structure was overlaid on the first a turn of a reference peptide. The side chain orientations were adjusted by rotation around freely rotatable bonds. The methionine (FME1) was rotated by 184 degrees and for MTY3, the phenyl group was rotated around N-CA by 64 and around CA-CB by 58 degrees. After minimization both structures showed nearly identical energies (51.19 vs 51.52). The adjusted and minimized structure was used for final overlay to the reference peptide. To visualize the space-filling of the relevant side groups a transparent soft solvent surface was rendered for each peptide. Chemical structures of ligands were drawn with Accelrys Draw, 4.1 (accelrys).

Electron paramagnetic resonance spectrometry –These experiments were performed with a Bruker spectrometer (ESP300e) equipped with a standard 4102ST cavity which holds the capillary support quartz glass finger. The glass finger temperature was controlled by a BioIII-TGC device (Noxygen) and set to 37 °C for work with cells. The modulation amplitude was set to 0.1 mT and the microwave power to 20 mW as standard condition for all experiments. Spectra were recorded with scan times of 60 s and stored consecutively to monitor the kinetic behavior of the signal. All experiments were performed in Ringer’s buffer at 37 °C with an oxygen concentration of about 200 µM. The activity of monocytes was initially tested with phorbol ester (PMA, 1µM). The redox activated cyclic hydroxylamine spin trap 1-
hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH) was added last in 300 μM concentration for all experiments to monitor superoxide production. 1 x 10^5 to 2.5 x 10^5 monocytes per experiment were stimulated with the designated peptides and immediately transferred into a 50 μl glass capillary in the capillary holder. The reduction of CMH by superoxide leads to formation of a three line EPR signal typical for the nitroxide centered radical. The temporal evolution of the CM-radical signals was evaluated with the in-house program Medeia which automatically determines the double integral of one or several lines of the radical visible in the time series with a resolution of 1 s. In the absence of line width changes and saturation effects, the information can be translated to radical concentration by comparing to the stable nitroxide radical TEMPOL at 100 µM. The output data were further processed with Origin 8.5 for evaluation and presentation. In control experiments each component of the assays and CMH were tested for unwanted radical production. The superoxide radical was identified as the reacting species by suppression of the CMH signal by superoxide dismutase SOD (100 U/ml) as scavenger.

**RESULTS**

**Structurally dissimilar peptide agonists imply a conserved FPR recognition mechanism**—To investigate molecular mechanisms underlying FPR ligand recognition and activation, we first used heterologous expression of 13 FPRs from 6 mammalian species in combination with high-throughput measurements of intracellular Ca^{2+} mobilization and examined ligand-induced activation (32). This strategy enabled us to systematically investigate the effects of a large number of combinations between potential ligands and receptors.

Initial experiments revealed that two C-terminally amidated peptides exhibiting highly divergent chemical structures, MMHWAm (M-peptide) (35) and WKYMVm (W-peptide) (36) (Fig. 1A), and their stereoisomers caused surprisingly similar activation of mFpr-rs1, one of the FPRs expressed in mouse VNO, with respect to concentration, stereoselective preference, and the relative potency of D- versus L-isoforms (Fig. 1B-D). Recognition capabilities towards M- and W-peptide seemed to be highly conserved between different receptor subtypes and different species. Both peptides activated FPR1 from mouse, human, rat, dog, rabbit and hamster (Fig. 1E). Conserved activation patterns extended also to human and mouse FPR2 as well as rFpr-rs2a, rFpr-rs2b, and rFpr-rs2c from rat (Fig. 1E). Thus, recognition capabilities of distinct FPRs from different species to these structurally divergent synthetic peptides were closely similar, resembling that of the best-known natural ligand f-MLF, whereas the response profile to another synthetic peptide agonist, MMK-1, showed very little correlation (Fig. 1E). Further support for a strong degree of functional conservation came from concentration-response measurements of W- and M-peptide using human and mouse FPR1 and FPR2 (Fig. 1F and G).

Thus, despite major structural differences of M- and W-peptide, they activate some FPRs in a strikingly similar manner suggesting shared structural properties that could not be deduced from their amino acid sequences. This high level of conservation across different species is unexpected because there should be no evolutionary pressure in mammals towards maintaining sensitive detection of these synthetic molecules. Hence, we reasoned that these findings could reflect the existence of a conserved recognition mechanism for a family of natural FPR agonists exhibiting mutual structural features.

**A conserved secondary structure of FPR peptide agonists**—To define the conserved structural features essential for agonist activity, we next used a combination of experimental and molecular modeling approaches. We previously identified a core agonist motif for mFpr-rs1 activation (32). To explore if a similar agonist motif is recognized by immune FPRs, we performed a detailed comparison of all five human and mouse immune FPRs with mFpr-rs1 by analyzing EC_{50} values derived from 343 distinct concentration-response experiments to stimulation with W-peptide and 22 W-peptide derivatives (Fig. 2A).

For hFPR1, hFPR2, mFpr1 and mFpr2, the results revealed remarkably potent activation by almost all tested peptides (Fig. 2A). By contrast, hFPR3 and mFpr-rs1 reacted to fewer peptides and, if they reacted, responses were less sensitive (Fig. 2A). Despite these differences, through systematic variation of W-peptide, we arrived at the conclusion that all six receptors detect similar agonist key positions (Fig. 2B). Both human and mouse FPR1 and FPR2 tolerate N-terminal elongations by three amino acids and deletions by two residues without a strong loss of agonist potency (Fig. 2A). Deletion of the third and fourth...
N-terminal residue drastically reduced the agonist potency for both FPRs. This suggests that these amino acids are especially important for receptor-ligand interactions. Furthermore, D-methionine (met, m) at C6, methionine (Met, M) at C4, tyrosine (Tyr, Y) at C3, and the C-terminal amidation (CONH$_2$) all critically influence agonist potency. Nonetheless, a considerable amount of structural variation is tolerated at the C3 and C4 positions (Fig. 2A). However, larger residues are preferred suggesting that these amino acids preferentially contribute to van der Waals interactions. By contrast, little variation at C6 is permitted. This implies that methionine at C6 is a key element in the agonist structure. The C-terminal amide group (CONH$_2$) represents another key structure because its removal results in a strong loss of agonist potency (Fig. 3A). Surprisingly, replacement of this CONH$_2$-group by methyl ester (COOCH$_3$) or aldehyde (CHO) did not drastically change the agonist potency (Fig. 3B). This argues against a direct interaction of the amine (NH$_2$) with the receptor and favors a model in which NH$_2$ influences the charge properties of the adjacent carbonyl group (C=O). A C=O group with similar donor/acceptor properties is also contained in the methyl ester and aldehyde derivative (Fig. 3C and F). In the drastically less potent COO' peptide, the C=O donor/acceptor properties are clearly altered through the negative charge of this group (Fig. 3D).

We next compared M- and W-peptide at the key residues C6, C4 and C3. Both peptides have an amidated methionine (m-CONH$_2$) at C6, further supporting our hypothesis that this residue is the central element. M-peptide exhibits tryptophane (Trp, W) instead of Met at C4 and histidine (His, H) instead of tyrosine at C3. Despite considerable structural variations, all these residues have a large molecular surface that permits van der Waals interactions. This indicates that the residues at C3 and C4, together with met at C6, may form a spatially conserved 3D structure instead of a linear motif. We therefore employed 3D modeling to investigate the spatial orientation of key residues between W- and M-peptide. Assuming that these peptides form an α-turn, the most frequent conformation occurring in similar peptides (37), alignment of both peptides revealed that the residues at C4 and C3 are indeed closely overlapping (Fig. 3E). Tyr at C3 of W-peptide has a similar spatial orientation and molecular surface as His at C3 of M-peptide. At C4 the non-polar residues Trp and Met are in close proximity. Together, the three key residues at C6, C4 and C3 form a predominantly hydrophobic tripartite structure that is oriented symmetrically around a carbonyl group in a claw-like fashion. Interestingly, an N-terminally formylated Met resembles a C-terminal met with an aldehyde substitution (Fig. 3F).

The symmetrical organization of this motif (Fig. 3E), together with our finding that C-terminal CHO and CONH$_2$ likely act through the C=O group, suggest a mechanism in which an amidated peptide first interacts with the receptor binding pocket via its C-terminal met whereas a formylated peptide binds first through its N-terminus. Comparison of the model structure of W-peptide with a de novo peptide in which the amino acid sequence is reversed (f-MVMYKW or rev-W-peptide) demonstrates that the three N-terminal residues at C1, C3 and C4 of this peptide form a similar hydrophobic tripartite structure as that formed by C6, C4 and C3 at the C-terminus of W-peptide (Fig. 3G), predicting that rev-W-peptide should function as a potent FPR activator. When we tested this, we found indeed nearly identical EC$_{50}$ values for this peptide on hFPR1 and mFpr1 (Fig. 3H). With an EC$_{50}$ of 0.19 ± 0.13 (n = 3) for hFPR1 and 0.09 ± 0.12 (n = 6) for mFpr1, the reversed form of M-peptide (rev-M-peptide or f-MAWHMM) was even ~100-fold more potent than M-peptide that had EC$_{50}$ values of 205 ± 25 (n = 3) for hFPR1 and 11 ± 6.9 (n = 5) for mFpr1. Together, these results demonstrate that the position of certain functional groups and their spatial arrangement are more important for FPR-ligand interactions than their linear amino acid sequences. These results offer a plausible mechanistic explanation for our finding that FPRs can be activated with equal sensitivity by sequence-divergent peptides that are either N-terminally formylated or C-terminally amidated.

Importantly, the structural constraints deduced from our analysis of synthetic peptides apply also to natural FPR ligands. We compared the NMR structure of f-MLF (38) (accession: PDB ID 1Q70) with the model of rev-W-peptide and found clear similarities in shape and spatial orientation (Fig. 3I). 3D modeling using two known mitochondrial FPR agonists, humanin (39) and ND1 (40), revealed a tripartite claw-like motif at the N-terminus of ND1 and humanin that showed clear structural similarities to our synthetic peptides (Fig. 3I). Subsequent functional measurements indeed confirmed that peptide fragments containing the N-terminus of humanin and ND1
activate human and mouse FPRs (Fig. 3J, K) and, as expected, addition of an N-terminal formyl group substantially improved the potency of humanin (Fig. 3K). Hence, we hypothesized that these results define a common structural basis – or molecular signature – for FPR activation that could be used by FPRs to detect natural molecules associated with the presence of pathogens.

**Bacterial signal peptides constitute a novel family of FPR agonists**–Guided by the results illustrated in Figs. 1 - 3, we proposed the existence of an as yet undiscovered family of natural FPR ligands that are most likely released by bacteria and had a significant impact on shaping FPR structure and function during mammalian evolution. Our strategy to identify such ligands was based on the following assumptions: (1) They are likely to be short peptides with variable amino acid sequence and a minimal length of three amino acids. (2) Their N-terminus should consist of a formylated methionine or their C-terminus should terminate with an amidated methionine. (3) The second and/or third residue next to this methionine should comprise amino acids that preferentially allow for van der Waals interactions. (4) These ligands should possess a well-defined secondary structure, likely containing an α-turn, which resembles the trilobular motif identified above. (5) The agonist structure should be highly conserved across many different pathogens and such pathogens should affect a wide range of mammalian species including humans and mice.

These predictions enabled us to identify classical bacterial signal peptides, normally used to target newly synthesized proteins to the membrane transport machinery, as novel natural agonists of human and mouse FPRs (Figs. 4, 5 and Tables 2, 3). Such signal peptides are variable in their amino acid sequence but have a conserved secondary structure that is largely α-helical (41). They contain three typical domains: a 3 - 6 amino acid long N-terminal region (n) starting with a methionine; an α-helical hydrophobic h-region (h); and a c-region (c) containing a conserved signal peptidease (SPase) recognition motif (Fig. 4A) (30,41).

Through database analyses (signalpeptide.com), we first identified N-terminal signal peptide fragments on the basis of their similarity with our core agonist motif (Fig. 4B) and initially tested nine hexapeptides (SP1 - SP9) derived from distinct bacterial strains (Table 2). All nine peptides turned out to function as potent activators of both hFPR1 and hFPR2 (Fig. 4C-E and Table 2). We examined sensitivity and selectivity of hFPR1 and hFPR2 for these peptides by analyzing full-concentration-response curves (Fig. 4D, E and Table 2). *Streptococcus*-SP1 potently activated hFPR1 with an EC$_{50}$ value of 1.8 ± 1.6 nM whereas the EC$_{50}$ value of hFPR2 was 1343 ± 614 nM. A similar preference for hFPR1 was observed by *Staphylococcus*-SP3, with EC$_{50}$ values of 61 ± 31 nM for hFRP1 and 293 ± 143 nM for hFRP2. By contrast *Bacillus*-SP2, *Salmonella*-SP4 and *Haemophilus*-SP5 all preferentially activated hFPR2, with EC$_{50}$ values of 568 nM, 31 nM, and 8 nM, respectively, whereas they activated hFPR1 at ~100-fold higher concentrations. Thus, at low nanomolar concentrations, these signal peptides are selectively recognized by either of the two receptors whereas, at higher concentrations, they activate both receptors.

We found that the sequence of the classical FPR agonist f-MLF is contained in signal peptides of at least 305 distinct bacterial strains, including the highly pathogenic *Yersinia pestis*, *Clostridium botulinum* and *Bacillus cereus*. These signal peptides could constitute a potential natural source for f-MLF and its derivatives. We therefore tested three signal peptides (SP6 - SP8) that contain an f-MLF at their N-terminus: f-MLFYS from *Psychromonas ingrahamii*, f-MLFKYS from *Shewanella baltica*, and f-MLFYLA from *Desulfotomaculum reducens* (Fig. 4E and Table 2). For hFPR1 and hFPR2, EC$_{50}$ values were 0.15 ± 0.06 nM and 38 ± 34 nM for *Psychromonas*-SP6, 7.7 ± 4.5 nM and 6.7 ± 4.0 nM for *Shewanella*-SP7, and 0.25 ± 0.17 nM and 34 ± 25 nM for *Desulfotomaculum*-SP8, respectively. Thus, two of these tested peptides are among the most potent natural FPR agonists identified thus far.

To determine in more detail signal peptide key features crucial for FPR activation, we next analyzed signal peptides of different lengths, fragments from different signal peptide domains, and the effects of formylation (Fig. 4F-H). We compared concentration-response curves of hFPR1 to the full-length, 36 and 23 amino acid-containing signal peptides of *Streptococcus*-SP1 and *Desulfotomaculum*-SP8 with their corresponding N-terminal hexapeptide fragments (Fig. 4F and Table 2). In case of *Streptococcus*-SP1, the hexapeptide and the full-length signal peptide activated hFPR1 at comparable concentrations. For *Desulfotomaculum*-SP8, the small N-terminal fragment was considerably more potent than the...
full-length signal peptides. However, both full-length signal peptides could activate hFPR1 at nanomolar concentrations (Table 2), demonstrating that cleavage products of variable lengths can be recognized. To test whether peptides derived from different domains of a given signal peptide are detected by FPRs, we compared activation of several FPRs to Streptococcus-SP1 vs. fragments from the n-, h-, and c-region of the corresponding full-length signal peptide. Whereas N-terminal Streptococcus full-length signal peptide. Whereas N-terminal the n-, h-, and c-region of the corresponding more potently on hFPR2 preferentially by mFpr1. Similarly, peptides that reacted activated hFPR1 it was usually also preferred…

## The size of the ligand family—Given that all nine signal peptides that we initially tested are activators of human and mouse FPR1 and FPR2 (Table 2), we asked how many of the currently annotated 175,542 bacterial signal peptides are potentially recognized by these two receptors. Database searches using the first four amino acids of SP1 - SP9 (containing the key motif) revealed 55 identical hits for the hexapeptides and 717 hits for the core sequence comprising the first four amino acids (Table 3). These results imply that the number of bacterial signal peptides functioning as FPR activators could be very large. All peptides that we tested thus far were selected on the basis of structural considerations. To provide a more unbiased estimate of the size of the ligand family, we next used an additional set of twelve signal sequences (SP10 - SP21) that were randomly chosen from the 1,168 experimentally confirmed signal peptides present in the database (Table 3 and Fig. 5). For this peptide selection, database searches revealed another 123 identical hits for the hexapeptide and 3,576 hits for their core motifs (Table 3). Remarkably, all twelve hexapeptides turned out to function as activators of human and mouse FPR1 and FPR2, although with differential activation thresholds and selectivity for each receptor (Fig. 5B, and Table 3). These results allowed us to estimate the size of the ligand family for FPR1 and FPR2. Probability calculations predict with a confidence level of 95% that these two receptors should be capable of detecting at least 78% of the 175,542 sequences currently annotated in the signal peptide database (see Experimental Procedures).

### Strongly restricted signal peptide recognition by hFPR3 and mFpr-rs1—Having established that human and mouse FPR1 and FPR2 are both capable of recognizing a wide range of bacterial signal peptides, we asked if this applies also to hFPR3 and mFpr-rs1. These receptors did not recognize all tested signal peptides but we identified seven signal peptides that activated hFPR3 (SP6, SP8, SP10, SP15, SP16, SP18, and SP19). Three of these peptides also activated mFpr2 (Table 2). Furthermore, responses to Streptococcus-SP1, Psychromonas-SP6, and Desulfotomaculum-SP8 were all nearly identical for both receptors between both species (Fig. 4C - 4E, 5A and Table II). These observations strongly argue for an evolutionary conserved recognition mechanism of this agonist family by human and mouse FPR1 and FPR2.

Signal peptide recognition is evolutionary conserved between human and mouse FPRs—Our findings obtained with human FPRs extend also to mouse FPRs. Comparison of activation profiles of hFPR1 and hFPR2 with mFpr1 and mFpr2 revealed several striking similarities (Fig. 4C-H, 5A and Table 2). Streptococcus-SP1 hexapeptide robustly activated hFPR1 and hFPR2 at nanomolar concentrations, none of the tested peptides from other domains were potent agonists (Fig. 4G). Moreover, in order to function as efficient agonists, signal peptides require a formylated N-terminal methionine. Complete removal of this residue abolished the response of hFPR1 and hFPR2 (Fig. 4G) whereas removal of the N-terminal formylation alone was sufficient to reduce the activation of hFPR1 by 770-fold (Fig. 4H). Interestingly, this latter result enables FPR discrimination between bacterial and host-endogenous signal peptides: whereas bacteria use an N-terminally formylated methionine to initiate protein biosynthesis, this methionine in eukaryotic cells remains normally unformylated (42).

### Strongly restricted signal peptide recognition by hFPR3 and mFpr-rs1—Having established that human and mouse FPR1 and FPR2 are both capable of recognizing a wide range of bacterial signal peptides, we asked if this applies also to hFPR3 and mFpr-rs1. These receptors did not recognize all tested signal peptides but we identified seven signal peptides that activated hFPR3 (SP6, SP8, SP10, SP15, SP16, SP18, and SP19). Three of these peptides also activated mFpr2 (Table 2). Furthermore, responses to Streptococcus-SP1, Psychromonas-SP6, and Desulfotomaculum-SP8 were all nearly identical for both receptors between both species (Fig. 4C - 4E, 5A and Table II). These observations strongly argue for an evolutionary conserved recognition mechanism of this agonist family by human and mouse FPR1 and FPR2.

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activated by Psychromonas-SP6 and Desulfotomaculum-SP8 with EC_{50} values of 284 ± 79 nM and 269 ± 245 nM, respectively (Fig. 5D and Table 3). These results indicate that hFPR3 and mFpr-rs1 could also function as signal peptide receptors but, compared to FPR1 and FPR2, their recognition capabilities seem to be far more restricted as they detect only a limited number of the peptides tested thus far.

Signal peptides trigger innate defense mechanisms in phagocytic leukocytes—To further validate the biological significance of our findings, we investigated recognition capabilities of bacterial signal peptides by native human and mouse immune cells. We first examined primary human monocytes which naturally express FPR1 and FPR2 (3) and performed single-cell Ca^{2+} imaging experiments. Exposure to Streptococcus-SP1 produced robust intracellular Ca^{2+} mobilization in ~89% of monocytes (Fig. 6A). Consistent with our results from the in vitro assay, monocytes recognize a wide range of signal peptides (Fig. 6B) and short hexapeptides are more potent than the corresponding full-length signal peptides (Fig. 6C and Table 2).

Monocyte Ca^{2+} responses to Streptococcus-SP1 and f-MLF were inhibited by the established FPR antagonists t-Boc2 and cyclosporine H (Fig. 6D). Streptococcus-SP1 and f-MLF strongly cross-desensitized each other whereas RANTES, a CCR1 receptor agonist (43), did not affect subsequent Ca^{2+} responses to Streptococcus-SP1 and f-MLF in monocytes (not shown). We next combined Ca^{2+} imaging with posthoc immunocytochemistry using antibodies specific for hFPR1 (Fig. 6E, 6F, and Suppl. Video 1) and found that the vast majority of all cells expressing hFPR1 (92 ± 10%) responded to Streptococcus-SP1 (Fig. 6F). There was a clear correlation between receptor staining intensity and strength of the Ca^{2+} signal (Fig. 6F). We compared concentration-response curves for six signal peptides in human monocytes with curves obtained from the in vitro experiments and found them to be remarkably similar (Fig. 6G, 6L and Table 2). Equivalent Ca^{2+} responses to bacterial signal peptides were also observed in human granulocytes (Fig. 6H), another immune cell type known to naturally express FPR1 and FPR2 (5).

To directly demonstrate that FPR1 is required for signal peptide recognition by immune cells, we also performed some mouse experiments comparing Ca^{2+} responses in leukocytes of wild type, Fpr1^{−/−}, and Fpr1^{+/−} mice (Fig. 6I). These results demonstrate that Streptococcus-SP1 can be recognized by mouse leukocytes and that this response, along with the response to f-MLF, is selectively abolished in Fpr1^{−/−} cells whereas the response to MIP1-α, a chemokine receptor agonist (44), remains normal (Fig. 6I). Together, our results indicate that bacterial signal peptides are recognized by native immune cells and that this recognition process depends on FPR function.

As the physiological function of monocytes is host defense, we tested whether bacterial signal peptides are capable of activating known classical innate defense mechanisms. To accomplish this, we first examined whether signal peptides trigger the production of superoxide radicals (O_{2}^{·−}) and other reactive oxygen species which are produced by leukocytes during immune responses (5). In human monocytes, we monitored the kinetics of short-lived free O_{2}^{·−} production in response to Streptococcus-SP1 using electron paramagnetic resonance (EPR) spectroscopy (45). Streptococcus-SP1 induced dose-dependent production of O_{2}^{·−} with a kinetic response very similar to that of f-MLF (Fig. 6J). For a larger selection of stimuli, we also used a 96-well Amplex-based ROS assay that monitors fluorescence changes induced by H_{2}O_{2} (45). All four tested signal peptides caused robust H_{2}O_{2} production (Fig. 6K). Direct comparison of concentration-response curves for Ca^{2+} mobilization and H_{2}O_{2} production obtained with Streptococcus-SP1 and f-MLF stimulation revealed a ~10-fold shift between H_{2}O_{2} and Ca^{2+} responses in monocytes and granulocytes (Fig. 6L), consistent with previous studies of f-MLF-mediated ROS formation (46). Further tests with human granulocytes revealed that signal peptides also elicit other classical innate defense reactions such as the release of matrix metallopeptidase 9 (MMP-9) as well as chemotactic cell migration (Fig. 6M and 6N). Hence, these results demonstrate that bacterial signal peptides are potent molecular triggers of classical innate defense mechanisms against pathogen invasion.

DISCUSSION

FPRs are a unique family of GPCRs with an exceptionally broad and promiscuous agonist spectrum showing no obvious common pattern in primary structure or natural origin (3,7,8). Because of this molecular promiscuity, there is little understanding of what the biological targets of these receptors are and why FPRs detect a range of
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Our experiments establish that FPR1 and FPR2 function as broad signal peptide receptors. A goal of future experiments will be to determine whether this finding applies to other FPR subtypes as well. The vomeronasal receptor mFpr-rs1 and its sequence orthologue hFPR3 seem to be much more narrowly tuned in the detection of signal peptides. Compared to FPR1 and FPR2, these receptors reacted to a relatively small subset of signal peptides and required higher concentrations. Possibly, hFPR3 and mFpr-rs1 focus on the detection of a specific set of bacterial pathogens whereas FPR1 and FPR2 function as more general sensors of bacteria.

FPRs were originally discovered as receptors for formylated peptides such as f-MLF present in the supernatant of bacterial cultures (6,47). However, the precise source and release pathway of such peptides has never been identified (5). Our finding that the sequences of these ligands are contained within bacterial signal peptides and that these signal peptides and their N-terminal breakdown products function as potent activators of FPRs identifies a likely natural source for these molecules. Signal peptides are cleaved off the maturing protein by extracellular proteases during membrane targeting and subsequently digested into short fragments (30). Although the fate of bacterial signal peptides after their cleavage is currently not well understood (30), recent mass spectrometry studies of bacterial secretomes found complete signal peptides as well as N-terminal fragments in the extracellular medium of bacteria cultures, demonstrating that these molecules indeed can be secreted by bacteria (48-50). One possibility of how this happens is through lysis, either through autolysis during bacterial growth (51) or through immune cell-mediated lysis during an infection (52). Both mechanisms are well established and, therefore, represent likely pathways by which signal peptides can be made available for the recognition by FPRs. Whether other more specific release mechanisms exist for bacterial signal peptides remains a goal of future research. Compared to eukaryotes, bacteria employ highly complex secretion mechanisms and their secretory pathways are less well examined (53,54).

The ability of FPRs to recognize thousands of peptides with distinct amino acid sequences yet maintaining selectivity requires a compromise between broad specificity and high affinity. Our results suggest a remarkable solution to this problem. We provide evidence for an FPR detection mechanism that seems to focus on the recognition of a conserved 3D motif rather than the linear peptide sequences. Several critical features

structurally dissimilar molecules. Here, we report the ability of FPRs to recognize bacterial signal peptides, a vast family of novel natural FPR agonists that differ extensively in their primary structure but exhibit a well-conserved secondary structure. Detailed structural variation analysis of peptide agonists defined the key features that enable both promiscuity and high specificity of FPR molecular detection. Finally, we argue that these results identify a common structural basis or molecular signature for FPR activation that is used to detect natural pathogens. These results provide new insight into the function of these receptors and their molecular promiscuity and make specific inferences about their biological roles and evolution.

Our results indicate that bacterial signal peptides provide an exceptionally large pool of sequence-divergent FPR agonists that all contain a conserved secondary structure. The f-MLF motif alone exists at the N-terminus of several hundred signal peptides whereas the core agonist motif of all 21 signal peptides used in this study occurs at the N-terminus of a total of 4,293 peptides (Table 3). Moreover, our results suggest that FPR1 and FPR2 together may recognize far more than 100,000 distinct signal peptides. In fact, there is currently no reason for ruling out that FPR1 and FPR2 could detect the entire set of bacterial signal peptides. As such, bacterial signal peptides represent the largest family of natural FPR agonists and one of the largest family of GPCR ligands known to date. They are also one of the most complex classes of natural activators of the innate immune system discovered thus far.

Our findings not only demonstrate that bacterial signal peptides are recognized by human and mouse FPR1 and FPR2 in vitro, but they also provide extensive evidence that this recognition process occurs in primary leukocytes of the human and mouse innate immune systems. These cells are equipped with native FPRs and we provide clear evidence that bacterial signal peptides are detected in a sensitive manner by monocytes and granulocytes and that they trigger a range of classical innate immune defense mechanisms including chemotactic migration, ROS production, and matrix metalloproteinase release. Pharmacologic and genetic manipulation indicates that this recognition process is indeed FPR-dependent.
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...of this mechanism can now be defined. For example, our data predict that FPR peptide agonists possess a well-defined secondary structure, likely containing an α-turn. Such agonists can vary considerably in their length but a minimal size of three amino acids is required. The first (or last) amino acid residue of a given peptide represents a key element for agonist potency because this residue has the most stringent spatial and chemical limitations. The N-terminal residue should consist of a formylated methionine or, alternatively, the C-terminal residue should be an amidated methionine. Rather than site-specific hydrogen donor/acceptor or ionic bonds, flexible shape-oriented van der Waals bonds, flexible shape-oriented van der Waals interactions of most other amino acids side chains determine agonist potency. Especially the second and/or third residue next to this methionine should comprise amino acids that preferentially permit forming a hydrophobic, tripartite claw-like structure with an α-turn oriented around a carbonyl group. The symmetrical organization of this agonist motif, together with the observation that a similar carbonyl group exists in both the N-terminal formyl and the C-terminal amidate group (Fig. 3F), provides an explanation for the result that FPRs recognize in very similar ways both N-terminally formylated and C-terminally amidated peptides. We predict that a C-terminally amidated peptide interacts first with the receptor binding pocket via its C-terminal methionine whereas an N-terminally formylated peptide binds first through its N-terminus. Experimental evidence for the validity of this mechanism is provided by our demonstration that C-terminally amidated peptides are equally potent FPR agonists as corresponding peptides in which the amino acid sequence has been reversed and now comprises an N-terminal formylation.

The fact that this mechanistic concept enabled us to identify bacterial signal peptides as a novel class of FPR agonists provides direct support for its usefulness. Bacterial signal peptides have structural features that fit particularly well to our model predictions, including a highly variable primary structure that contains a largely hydrophobic alphahelical domain close to a conserved N-terminus starting with a formylated methionine (41). Importantly, key features derived from our analyses can also be found in other previously identified FPR agonists such as mitochondrial peptides, suggesting that these results could be of general significance. N-terminally formylated mitochondrial peptides from membrane proteins such as ND1, ND4, ND6 and CO1 are well known to function as FPR agonists (9). Mitochondria are of ancient bacterial origin (55) and we predict that these mitochondrial peptides served originally as signal peptides enabling protein translocation through the mitochondrial membrane. Consistent with this idea, the ND1 N-terminus reveals clear structural similarities to our agonist model (Fig. 3I). Further structural comparisons demonstrate that other known FPR ligands such as humanin and f-MLF also display striking similarities with our agonist model (Fig. 3I). Independent support for this concept comes from a recent study using none-agonist peptides to identify a potential binding motif in FPR2 (56). Moreover, other FPR agonists such as phenol-soluble modulin (PSM) peptide toxins (15), amyloid-β 1-42 (8), or µPAR (57) may also fit into this scheme. For example, a recent study demonstrated that the signal peptide of the S. aureus quorum-sensing signal, AgrD, shares clear structural and functional similarities to the PSM family (48-50). The fact that critical agonist features as described here extend to a number of distinct FPR agonists suggests that our results should prove useful in future developments such as the identification of the FPR ligand binding site and the discovery of subtype-selective FPR antagonists.

The mammalian innate immune system as the first line of host defense has evolved multiple strategies to detect pathogen-associated molecules to subsequently eliminate infective pathogens in the body. A major challenge for the innate immune system is the recognition of a multitude of rapidly adapting microorganisms via a limited number of germline-encoded PRRs. Therefore PRRs focus on the recognition of highly conserved microbial components, so-called PAMPs, which are difficult to alter because they are essential for the survival of an invading pathogen. Bacterial signal peptides show a number of specific molecular properties that are typical hallmarks of PAMPs. First, export processes initiated by signal peptides represent an evolutionary conserved mechanism that is essential for bacterial survival (30). Second, bacteria use an N-terminally formylated methionine to initiate translation whereas this methionine in eukaryotic cells is unformylated (42). As the formylation is critical for FPR recognition, this difference provides an elegant solution to enable discrimination between host-endogenous and
microbial signal peptides. Third, our studies using human and mouse leukocytes provide clear evidence that signal peptides are indeed capable of triggering classical innate immune responses mediated by FPRs. Fourth, we observed a remarkably high degree of conservation in signal peptide recognition between human and mouse FPR1 and FPR2 thus arguing for an important role of bacterial signal peptides during evolution of FPR function. Consistent with this concept, human and mouse FPR1 and FPR2 both show relatively broad tuning and respond to a large number of signal peptides and these receptors display striking similarities in their sensitivity and selectivity towards structurally divergent peptides. In summary, the recognition capabilities that mammalian FPRs have evolved are perfect for sensing the molecular properties of bacterial signal peptides which combine an exceptionally high sequence-variability with a conserved secondary structure. Hence, we propose that mammalian FPRs may have evolved originally as germline-encoded PRRs that recognize structurally conserved export motifs of bacterial signal sequences as their cognate, pathogen-associated molecular pattern.

Both FPRs and the Toll-like receptors focus on the recognition of invading bacteria. However, these receptors trigger distinct signal transduction cascades. It is tempting to speculate that there could be an interaction between both systems. A critical feature of FPRs is their ability to detect not only molecular patterns associated with pathogens such as bacterial signal peptides, but also host-endogenous mitochondrial peptides. This specific property enables the FPRs to function not only as PAMP receptors but also as sensors of danger-associated molecular patterns (known as DAMPs), for example in case of tissue damage. This aspect could be useful in the general management of the microbiome of the body, consistent with the large distribution of FPRs in multiple tissues and organs. Taken together, our findings provide an essential foundation for understanding the function of FPRs, with far-reaching consequences for their biological roles.
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The abbreviations used are: FPR, formyl peptide receptor; VNO, vomeronasal organ; GPCR, G-protein-coupled receptor; f-MLF, N-formylmethionine-leucine-phenylalanine; PRR, pattern recognition receptor; PAMP, pathogen-associated molecular pattern; ROS, reactive oxygen species; EPR, electron paramagnetic resonance spectroscopy; ND, NADH-reductase subunit; RANTES, regulated on activation, normal T-cell expressed and secreted cytokine; CCR, C-C chemokine receptor; PMS, phenol-soluble modulin; μPAR, urokinase receptor; DAMP, danger-associated molecular pattern.

TABLES

TABLE 1. Source, sequence and purity of the FPR agonist and antagonists used in this study. Peptide sequences of all essential amino acids are given in one-letter code. Capital letters denote an L-isomer; lowercase letters denote a D-isomer. Abbreviations for non-essential amino acids are hcy for homocysteine and orn for ornithine. N- and C-terminal modifications are abbreviated as follows: (f), formylated N-terminus; (CONH$_2$), C-terminus with amidation; (CHO), aldehyde group; (COOCH$_3$), methyl ester group; (Ac), acetylation.

TABLE 2. Bacterial signal peptides as FPR agonists. Listed is a set of nine bacterial signal peptides that we initially identified, their sources, sequences, accession numbers (UniProt database), and mean EC$_{50}$ values (in nM) with respect to the activation of heterologously expressed hFPR1, mFpr1, hFPR2, mFpr2, hFPR3, and mFpr-rs1. Sequences of all essential amino acids are given in one-letter code. EC$_{50}$ values were obtained by measuring full concentration-response curves. Number of experiments (n) is given in parentheses. n.d., no data; n.r., no response with the highest tested concentration (30 μM).

TABLE 3. Summary of FPR response profiles for 21 bacterial signal peptides identified in this study. Listed are sequences, sources, pathogenicity, frequency of database hits for residues 1-6 or 1-4, and the response profiles of hFPR1, hFPR2, hFPR3, mFpr1, mFpr2, and mFpr-rs1 for 21 N-terminal peptide fragments. Database hits refer to the number of identical sequence entries in the signal peptide database for the first four or six amino acids of a given signal sequence. Accession numbers refer to the UniProt database. Quantification of receptor sensitivity: Full large circles, activation threshold ≤100 nM; full small circles, activation threshold ≤1000 nM; open small circles, activation threshold ≤30000 nM; minus sign, no response at 30 μM.

FIGURE LEGENDS

FIGURE 1. Activation by disparate peptides suggests a conserved FPR recognition mechanism. A. Chemical structures of W- and M-peptide. Amino acid sequences are given in one-letter code. H, L-
histidine; Y, L-tyrosine; M, L-methionine; W, L-tryptophan; K, L-lysine; A, L-alanine; V, L-valine. Both peptides contain a C-terminal D-methionine with an amidation (m-NH2). In the L-isooform of both peptides the D-methionine is replaced by an amidated L-methionine. B. Representative single cell imaging traces demonstrating relative cytosolic Ca2+ changes of HEK293T cells transiently transfected with mFpr-rs1. Red traces: cells responding to M-peptide and W-peptide; grey traces: cells not responding to both stimuli; black trace: mean response. X-scale, 1 min; Y-scale, 10% increase in A340/380 ratio. Cells were extensively rinsed for 3 min with 20-fold bath exchange prior to the next stimulus application. Similar results were obtained in 3 independent experiments. C. Percentage of M-peptide responding cells that also responded to a second stimulus. Data are representative of three experiments. Numbers above each bar show the total amount of cells responding to a given stimulus. Note that 97% of the M-peptide-responding cells also reacted to W-peptide whereas responses to typical agonists for immune FPRs were below 3%. Error bars indicate SD. D. Mean concentration-response curves of mFprs-rs1 activation by W-peptide (green) or M-peptide (red). Error bars represent SD, experimental numbers are given in parentheses. E. Ca2+ peak responses representing activation of FPRs from six different species upon stimulation with M-peptide, W-peptide, f-MLF, and MMK-1 (each at 10 μM). Similar results were obtained in three independent experiments. F. Concentration-response curves of human (green) and mouse (black ) FPR1 and FPR2 activation by W- and M-peptide. Curves denote mean responses from 3 - 9 independent experiments. Error bars indicate SD. G. Concentration-response curves of hFPR2 activation by W- (green) and M-peptide (red).

FIGURE 2. Key residues in the sequence of W-peptide required for FPR activation. A. EC50 values (in nM, with standard deviation) obtained from concentration-response curves of hFPR1, hFPR2, hFPR3, mFpr1, mFpr2, and mFpr-rs1 for different W-peptide derivatives. Number of experiments (n) is given in parentheses. Color code indicates the loss in receptor sensitivity relative to the W-peptide response (grey): light blue, 6 to 15-fold reduced activity; green, 25 to 600-fold reduced activity; dark blue, ≥1000-fold reduced activity. B. Key residues in W-peptide that are important for activation of human and mouse FPRs. The analysis of dose-response experiments using 22 W-peptide derivatives identified residues at C3, C4, and C6 as crucial for agonist-receptor interaction. Importance of residues for receptor activation is indicated by color code and letter size. C3, C4, and C6 denote the distance of the positions to the N-terminal residue of W-peptide. Peptide sequences of all essential amino acids are given in one-letter code. Capital letters denote an L-isomer; lowercase letters denote a D-isomer. Abbreviations for non-essential amino acids are hcy for homocysteine and orn for ornithine. N- and C-terminal modifications are abbreviated as follows: (f), formylated N-terminus; (CONH2), C-terminus with amidation; (CHO), aldehyde group; (COOCH3), methyl ester group.

FIGURE 3. A conserved secondary structure of FPR peptide agonists. A. Mean concentration-response curves of hFPR1 (red) and mFpr1 (black) activation by W-peptide and a derivative lacking C-terminal amidation. Experimental numbers are given in parentheses. Error bars indicate SD. B. Mean concentration-response curves to derivatives in which the amidation at the C-terminal methionine was replaced by a methyl ester or an aldehyde. C. Chemical structure of methionine carrying a C-terminal amide or methylester. D. Structure of methionine with a C-terminal COOH group without modifications. E. Comparison between the predicted 3D structure of M- (red) and W-peptide (green). Both peptides have similarities in their spatial orientation, shape and chemical properties that cannot be easily predicted from their primary sequences. The residues at C3, C4 and C6 form a predominantly hydrophobic trilobular structure indicated by a grey dotted line. F. Structural similarities between methionine carrying a C-terminal aldehyde group (blue circle) and N-terminally formylated methionine (red circle). G. Comparison between the predicted 3D structure of W-peptide (green) and a formylated derivative with reversed amino acid sequence (rev-W-peptide, blue). The C-terminal methionine of W-peptide is aligned with the N-terminal methionine of rev-W-peptide. Despite large variations in linear amino acid chain key residues, both peptides show a similar spatial orientation forming a characteristic trilobular structure indicated by the dotted line. H. Comparison of the effects of W-peptide (green) and rev-W-peptide (blue) on the activation of mFpr1 (upper curves) and hFPR1 (lower curves). I. Comparison between the 3D structure of rev-W-peptide based on our modeling (blue) and an experimentally determined NMR structure for f-MLF (left, cyan), the N-terminus of the mitochondrial protein NADH reductase subunit 1 (ND1, middle, pink), a potent FPR agonist, and the N-terminus of the neuroprotective mitochondrial protein humanin (right, yellow), a
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less potent FPR agonist. Note that the C3 lobe in humanin appears considerably smaller. J. Representative Ca\(^{2+}\) responses of mouse and human immune FPRs to the formylated N-terminus of the mitochondrial protein NADH reductase subunit 1 (ND1). Y-scale, 100,000 RFU/div; X-scale, 3 min/div.). Similar results were obtained in 4 independent experiments. K. Representative Ca\(^{2+}\) responses of human and immune FPRs to the formylated (left) or unformylated (right) N-terminus of humanin. Y-scale, 50,000 RFU/div; X-scale, 3 min/div. Similar results were obtained in 3 independent experiments.

**FIGURE 4.** Bacterial signal peptides activate hFPR1 and hFPR2. A. Signal peptides dependent on the Sec protein-translocation pathway have a typical motif permitting a high degree of sequence flexibility in combination with a conserved three dimensional topology. Bacterial signal peptides consist of an n-region (blue) with an N-terminal formylated methionine, an \(\alpha\)-helical hydrophobic h-region (green), and a c-region (red) containing a conserved signal peptidase (SPase) recognition motif. B. Comparison of the calculated van der Waals surface between the first six amino acids of a signal peptide (purple) and the de novo agonist rev-W-peptide (blue). C. The N-terminal hexapeptide fragment from the signal peptide of *Streptococcus suis* hyaluronidase elicits robust dose-dependent Ca\(^{2+}\) transients in HEK-cells transfected with human and mouse FPR1 or FPR2. D. Specificity and sensitivity of hFPR1 (circles, solid lines) and hFPR2 (squares, dotted lines) to structurally diverse N-terminal signal peptide fragments as observed through mean concentration-response curves. Numbers of experiments are given in parentheses. Error bars represent SD. E. Mean concentration-response curves of hFPR1 (full circles) and hFPR2 (full squares, dotted lines) activation by three signal peptides starting with an f-MLF motif. F. Mean concentration-response curves of hFPR1 activation by N-terminal hexapeptide fragment (blue squares) vs. the full-length signal peptide of *Streptococcus*-SP1 comprising 36 residues (grey circles). G. Mean Ca\(^{2+}\) peak responses (n = 3) of different human and mouse FPRs to stimulation with the indicated bacterial signal peptide fragments from the n- (blue), h- (green), or c- (red) regions of *Streptococcus*-SP1 (full-length sequence indicated in the figure). Error bars indicate SD. H. Mean concentration-response curves of hFPR1 and mFPR1 to the formylated (blue circles) and non-formylated peptide (grey squares) comprising the first six N-terminal residues of *Streptococcus*-SP1.

**FIGURE 5.** Conserved signal peptide recognition by mFpr1 and mFpr2, the effects of randomly chosen signal peptide fragments, and signal peptide recognition by hFPR3 and mFpr-rs1. A. Mean concentration-response curves of mFpr1 (grey circles) and mFpr2 (grey squares) activation by several signal peptides. Corresponding curves for hFPR1 and hFPR2 are plotted in Fig. 4D and 3E. Numbers of experiments are given in parentheses. B. Comparison of mean Ca\(^{2+}\) peak responses (n = 4) of hFPR1 and hFPR2 (red) with mFpr1 and mFpr2 (grey) to the formylated N-termini of twelve randomly chosen bacterial signal peptides (each at 30 µM except for *Corynebacterium*-SP14 which was used 100 µM). Mock control (black) indicates empty vector transfection. Error bars indicate SD. C. Mean Ca\(^{2+}\) peak responses (n = 3) of hFPR3 and mFpr-rs1 to 21 bacterial signal peptides (each at 30 µM). Error bars indicate SD. D. Mean concentration-response curves of mFpr-rs1 activation by two distinct signal peptides as indicated.

**FIGURE 6.** Human immune cells recognize bacterial signal peptides. A. Overlay of single-cell Ca\(^{2+}\) responses obtained from 186 human leukocytes present in the visual field of a representative experiment (n = 3). Of these, 167 cells responded to *Streptococcus*-SP1 (70 nM). B. Average Ca\(^{2+}\) peak responses of human monocytes stimulated with 10 µM of the indicated peptides. Number of experiments (each from a different human donor) is shown in parentheses. Error bars indicate SD. C. Concentration-response curves of human monocytes to the N-terminal hexapeptide and the full-length signal peptide of *Streptococcus*-SP1 comprising 36 residues. D. Inhibition of signal peptide-induced Ca\(^{2+}\) responses by FPR antagonists. 100 nM *Streptococcus*-SP1 was applied either alone or in combination with the hFPR1 inhibitors CsH (1 µM) or tBoc2 (10 µM). E. Left: Representative images showing immunostainings of hFPR1 (green) and the corresponding mock staining using an isotype KLH antibody as negative control. Similar results were obtained in 3 independent experiments. Cell nuclei (red); scale bar, 50 µm. Right: Percentage of cells expressing hFPR1 or the monocyte marker hCD14 (n = 3). As isotype controls, we used a KLH antibody for hFPR1 and a MOPC-21 antibody for hCD14. F. Left: Representative correlation between intensity of hFPR1 staining in individual cells and peak amplitude of Ca\(^{2+}\) responses to *Streptococcus*-SP1. Similar results were obtained in 3
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independent experiments. The black line indicates a best curve fit of the data points. Right: Quantitative comparison of monocyte $Ca^{2+}$ responses to Streptococcus-SP1 and post hoc hFPR1 immunostaining. G. Concentration-response curves of human monocytes (red) and heterologously expressed hFPR1 (black) to the indicated signal peptides. Curves denote average responses ± SD. H. Concentration-response curves of human granulocytes (green) and heterologously expressed hFPR1 (black) to the indicated signal peptide. Error bars indicate SD. I. Mean $Ca^{2+}$ peak responses of leukocytes from wild type, $Fpr1^{+/–}$, and $Fpr1^{–/–}$ mice to stimulation with Streptococcus-SP1 (1 µM) or control buffer (mock). Stimulation with the FPR agonist f-MLF (1 µM) and the macrophage inflammatory protein 1α (MIP-1α, 10 µM) that activates a chemokine receptor served as positive controls. Bars denote mean responses ± SD from two independent experiments. J. Representative electron paramagnetic resonance spectrometry measurements of $•O_2^–$ production by human monocytes upon stimulation with 10 µM Streptococcus-SP1, 10 µM f-MLF, or control buffer (mock). Similar results were obtained in 3 independent experiments. K. Amplex-measurements of H2O2 production by human monocytes stimulated with 4 different signal peptides (each at 10 µM except Borrelia-SP9 which was used at 100 µM) and rev-W-peptide. W-peptide and f-MLF (each at 10 µM) were used as positive controls. Bath application served as mock control. L. Comparison of concentration dependence between $Ca^{2+}$ signals of human monocytes (red, left panel) or granulocytes (green, right panel) and their respective $O_2^–$ production (blue) ± SD upon stimulation with Streptococcus-SP1. M. Release of MMP-9 by human neutrophils. Left, MMP-9 release ± SD upon stimulation with Streptococcus-SP1 (1 µM), Hydrogenobacter-SP16 (1 µM). Stimulation with lipopolysaccharide (LPS 100 ng/ml) served as positive and medium as negative control. Right: Stimulus-response curves for MMP-9 release (dark blue) and $Ca^{2+}$ mobilization (green) in human granulocytes following stimulation with Streptococcus-SP1. N. Chemotactic response of human granulocytes to stimulation with signal peptides. Left: Representative images showing accumulation of human neutrophils following exposure to Streptococcus-SP1 (100 nM). Culture medium served as negative control; scale bar, 100 µm. Similar results were obtained in 3 independent experiments. Right: Number of migrated granulocytes ± SD after stimulation with Streptococcus-SP1 (10 nM), Salmonella-SP4 (100 nM), or Hydrogenobacter-SP16 (10 nM). Numbers denote the total amount of cells counted.
| Organic compounds | Storage | Source and purity | Solvent |
|-------------------|---------|------------------|---------|
| AG09/1 C2H2N4O5S | 4°C | Sigma, ≥ 98% | DMF |
| A14  | 4°C | Tocris, 98% | DMF |

### W-peptide library

| W-peptide | Storage | Source and purity | Solvent |
|-----------|---------|------------------|---------|
| WKYMVm-NH2 | -20°C | VCPBIO, >98.5% | C1 |
| L-W-Peptide | -20°C | Tocris, 92.9% | C1 |
| M-peptide | -20°C | GenScript, >98% | C1 |
| L-M-peptide | -20°C | GenScript, ≥ 98% | C1 |
| rev-W-peptide | -20°C | VCPBIO, >98.47% | C1 |
| Library peptide 1 | -20°C | GenScript, >98.3% | C1 |
| Library peptide 2 | -20°C | GenScript, >96.3% | C1 |
| Library peptide 3 | -20°C | GenScript, >96% | C1 |
| Library peptide 4 | -20°C | GenScript, >96% | C1 |
| Library peptide 5 | -20°C | GenScript, >95% | C1 |
| Library peptide 6 | -20°C | GenScript, >95% | C1 |
| Library peptide 7 | -20°C | GenScript, >95% | C1 |
| Library peptide 8 | -20°C | GenScript, >94% | C1 |
| Library peptide 9 | -20°C | GenScript, >93% | C1 |
| Library peptide 10 | -20°C | GenScript, >92% | C1 |
| Library peptide 11 | -20°C | GenScript, >92% | C1 |
| Library peptide 12 | -20°C | GenScript, >91% | C1 |
| Library peptide 13 | -20°C | GenScript, >90% | C1 |
| Library peptide 14 | -20°C | GenScript, >90% | C1 |
| Library peptide 15 | -20°C | GenScript, >89% | C1 |
| Library peptide 16 | -20°C | GenScript, >88% | C1 |
| Library peptide 17 | -20°C | GenScript, >88% | C1 |
| Library peptide 18 | -20°C | GenScript, >87% | C1 |
| Library peptide 19 | -20°C | GenScript, >87% | C1 |

### Bacterial signal peptides

| Bacterial signal peptides | Storage | Source and purity | Solvent |
|---------------------------|---------|------------------|---------|
| Streptococcus-SP1 f-MGFFIS | -20°C | VCPBIO, >95.43% | C1 |
| Streptococcus-SP1 (7-12) GSQHDF | -20°C | United Biosystems, >95.23% | C1 |
| Streptococcus-SP1 (20-25) GSCVAL | -20°C | United Biosystems, >95.23% | C1 |
| Streptococcus-SP1 (32-37) GTRVAA-NH2 | -20°C | United Biosystems, >99.9% | C1 |
| Streptococcus-SP1 full f-MGFFISQSKQHYGIRKYKVGVCSALIALSILGTRVAA | -20°C | VCPBIO, >95.18% | Ringer |
| Bacillus-SP2 f-MKNFKG | -20°C | VCPBIO, >96.42% | C1 |
| Staphylococcus-SP3 f-MFIYYCK | -20°C | VCPBIO, >97.24% | C1 |
| Salmonella-SP4 f-MMKNKL | -20°C | VCPBIO, >96.14% | C1 |
| Haemophilus-SP5 f-MVFKFX | -20°C | VCPBIO, >96.74% | C1 |
| Psychrosomonas-SP6 f-MLFYFS | -20°C | VCPBIO, >96.6% | DMF |
| Shewanella-SP7 f-MLFXV | -20°C | VCPBIO, >96.18% | C1 |
| Desulfitomaculum-SP8 f-MLFYLA | -20°C | VCPBIO, >97.04% | C1 |
| Desulfitomaculum-SP8 full f-MLFYALPCTVLFHAFASKALYAY | -20°C | VCPBIO, >96.59% | Ringer |
| Borella-SP9 f-MLKXYX | -20°C | VCPBIO, >96.24% | C1 |
| Vibrio-SP10 f-MPKXR | -20°C | United Biosystems, >95.32% | Ringer |
| Vibrio-SP11 f-MLVF | -20°C | United Biosystems, >96.3% | Ringer |
| Staphylococcus-SP12 f-MNKILL | -20°C | United Biosystems, >95.21% | C1 |
| Clostridium-SP13 f-MMNKLN | -20°C | United Biosystems, >95.67% | Ringer |
| Corynebacterium-SP14 f-MEQQNK | -20°C | United Biosystems, >95.72% | Ringer |
| Shewanella-SP15 f-MVPSI | -20°C | United Biosystems, >95.85% | Ringer |
| Hydrogenobacter-SP16 f-MKWW1 | -20°C | United Biosystems, >95.78% | Ringer |
| Bacillus-SP17 f-MMMKEG | -20°C | United Biosystems, >96.38% | C1 |
| Listeria-SP18 f-MKWL | -20°C | United Biosystems, >95.27% | Ringer |
| Desulfitobactera-SP19 f-MKFC3A | -20°C | United Biosystems, >95.44% | C1 |
| Zymomonas-SP20 f-MTNKSV | -20°C | United Biosystems, >95.91% | Ringer |
| Gensera-SP21 f-MKTBR | -20°C | United Biosystems, >95.04% | Ringer |

### Other peptides and proteins

| Other peptides and proteins | Storage | Source and purity | Solvent |
|-----------------------------|---------|------------------|---------|
| f-MLF f-MLF | -20°C | Sigma, ≥ 95% | C1 |
| OC1  | -20°C | GenScript, >98.6% | DMF |
| ND1  | -20°C | GenScript, >98.2% | DMF |
| T20  | -20°C | Anaspec, ≥ 95% | C1 |
| Ac26 | -20°C | Tocris, ≥ 95% | C1 |
| MMK-1 | -20°C | Tocris, ≥ 95% | C1 |
| uPAR | -20°C | Anaspec, ≥ 95% | C1 |
| Humanin | -20°C | VCPBIO, ≥ 94% | C1 |
| iHumanin | -20°C | VCPBIO, ≥ 90.02% | C1 |
| Humanin (1-6) | -20°C | VCPBIO, ≥ 90% | C1 |
| Mouse MIP1-α | -20°C | Peprotech, ≥ 90% | Ringer |
| RANTES | -20°C | Peprotech, ≥ 90% | Ringer |

### Antagonists

| Antagonists | Storage | Source and purity | Solvent |
|-------------|---------|------------------|---------|
| t-Boc2 N-tertbutoxy-FlFlF | -20°C | Bachem, ≥ 98% | DMF |
| Cyclosporin H | -20°C | Santa Cruz Biotech, ≥ 95% | DMF |

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**Table 1**
| Signal peptide  | Source                  | Species                  | Symptoms                        | Monocytes | hFPR1  | mFpr1  | hFPR2  | mFpr2  | hFPR3  | mFpr-rs1 |
|----------------|-------------------------|--------------------------|---------------------------------|-----------|--------|--------|--------|--------|--------|----------|
| Streptococcus-SP1 | Hyaluronidase (Q8VL08)  | Streptococcus spp.   | Meningitis, sepsis, skin lesions | 24±17 (4) | 30±29 (2) | n.d.  | 54±35 (2) | n.d.  | n.d.  | n.d.  |
| Streptococcus-SP1-full | Hyaluronidase (Q8VL08)  | Streptococcus spp.   | Meningitis, sepsis, skin lesions | 12850 (1) | 1981±732 (3) | 568 (1) | 71±34 (3) | n.d.  | n.d.  | n.d.  |
| Bacillus-SP2    | Hemolysin (O34100)     | Bacillus cereus         | Food poisoning                   | n.d.      | 6.4±7.7 (13) | 1.8±1.6 (4) | n.d.  | 1013±84 (3) | n.d.  | n.d.  | n.d.  |
| Staphylococcus-SP3 | Cell wall hydrolase LytN (Q6GHT8) | Staphylococcus aureus | Abscess, cellulitis, pneumonia | 164±134 (3) | 61±31 (3) | 26±15 (3) | 299±143 (3) | 437±105 (3) | n.d.  | n.d.  |
| Salmonella-SP4  | Membrane protein YaeF (O26N3) | Salmonella typhimurium | Enteric fever                    | n.d.      | 11520±188 (2) | 6862±3985 (3) | 31±16 (2) | 417±318 (4) | n.d.  | n.d.  |
| Haemophilus-SP5  | Tail-specific protease (P45369) | Haemophilus influenzae | Fever, meningitis, bronchitis   | 232±97 (5) | 70±238 (2) | 100±40 (3) | 8±2.0 (2) | 62±60 (3) | n.d.  | n.d.  |
| Psychromonas-SP6 | Phosphatase transferase (A1ST24) | Psychromonas agariphilus | n.d.                            | 0.19±0.14 (3) | 0.15±0.06 (4) | 0.07±0.03 (4) | 38±34 (2) | 9±3±56 (4) | >10000 (4) | 28±7 (4) |
| Shewanella-SP7  | Siderophore receptor (A9WJ37) | Shewanella baltica      | n.d.                            | 64±32 (3) | 7.7±4.5 (3) | 2.4±3.1 (3) | 6.7±4.0 (3) | 68±9 (3) | n.d.  | n.d.  |
| Haemophilus-SP8  | Membrane protein (A4J7E6) | Desulfotomaculum reducens | n.d.                            | 0.09±0.04 (7) | 0.25±0.17 (4) | 0.17±0.26 (4) | 34±25 (4) | 144±226 (4) | >10000 (4) | 28±7 (4) |
| Haemophilus-SP8-full | Membrane protein (A4J7E6) | Desulfotomaculum reducens | n.d.                            | 735±210 (4) | 100±6 (2) | n.d.  | 15±249 (2) | n.d.  | n.d.  | n.d.  |
| Borrelia-SP9    | Basic membrane protein D (Q44743) | Borrelia burgdorferi | Lyme borreliosis                  | n.d.      | 1137±9 (3) | 580±3150 (4) | 957±640 (3) | 1087±584 (3) | n.d.  | n.d.  |

Table 2
| Signal peptide | Example Organism | Protein and accession | Virulence factor | Sequence Database hits for residues | Receptor response |
|----------------|------------------|-----------------------|------------------|-------------------------------------|-------------------|
| Streptococcus-SP1 | Streptococcus | Hyalurondase/Hyaluronate Lyase (Q8VLQ8) | yes | f-MKIFFS 5 24 | hFPR1 |
| Bacillus-SP2 | Bacillus | Hemolysin (Q45105) | yes | f-MKFNKG 1 117 | hFPR2 |
| Staphylococcus-SP3 | Staphylococcus | Probable cell wall hydrolase LytN (Q6QH98) | n.d. | f-MFYYCK 3 9 | hFPR3 |
| Salmonella-SP4 | Salmonella typhi | Assembly factor yaeT (Q82993) | n.d. | f-MAWHFL 40 55 | hFPR4 |
| Haemophilus-SP5 | Haemophilus influenzae | Tail-specific protease (P43306) | n.d. | f-MVANFK 1 14 | hFPR5 |
| Psychrobacillus-SP6 | Psychrobacillus ingrahami | Phosphatase (A1ST24) | n.d. | f-MFYFS 1 5 | hFPR6 |
| Shewanella-SP7 | Shewanella | TorB-dependent siderophore receptor (A09F97) | n.d. | f-MLRYYS 1 38 | hFPR7 |
| Desulfitomaculum-SP8 | Desulfitomaculum reducens | Putative uncharacterized protein (A4Q769) | n.d. | f-MFLYLA 2 5 | hFPR8 |
| Borrelia-SP9 | Borrelia burgdorferi | Basic membrane protein D (Q47473) | n.d. | f-MLKKVY 1 450 | hFPR9 |
| Vibrio-SP10 | Vibrio cholerae | Cholera enterotoxin subunit A (P01565) | yes | f-MVXIF 2 30 | hFPR10 |
| Staphylococcus-SP12 | Staphylococcus aureus | Toxic shock syndrome toxin-1 (P08660) | yes | f-MNOLL 32 426 | hFPR11 |
| Clostridium-SP13 | Clostridium perfringens | Epsilon-toxin type B (Q20207) | yes | f-MVNLV 5 423 | hFPR12 |
| Corynebacterium-SP14 | Corynebacterium glutamicum | Cytochrome c oxidase subunit 2 (Q87566) | n.d. | f-MEGONK 2 5 | hFPR13 |
| Streptomyces-SP15 | Streptomyces carinostaticus | Nicotinamide (P0A939) | n.d. | f-MVIPSI 1 6 | hFPR14 |
| Hydrogenobacter-SP16 | Hydrogenobacter thermophilus | Cytochrome c-552 (P15452) | n.d. | f-MKFLG 41 664 | hFPR15 |
| Bacillus-SP17 | Bacillus amyloquefaciens | Ribonuclease (P03458) | n.d. | f-MAIKSTFEG 1 41 | hFPR16 |
| Listeria-SP18 | Listeria monocyotogenes | Listersycsin O (P13128) | yes | f-MKML 22 1570 | hFPR17 |
| Desulfovibrio-SP19 | Desulfovibrio gigas | NifH hydrogenase small subunit (P13263) | n.d. | f-MKFTCA 1 10 | hFPR18 |
| Zymomonas-SP20 | Zymomonas mobilis | Glucose-fructose oxidoreductase (Q27982) | n.d. | f-MTNOS 6 68 | hFPR19 |
| Neisseria-SP21 | Neisseria gonorrhoeae | Major ferric iron-binding protein (P17259) | yes | f-MKTSSR 8 255 | hFPR20 |

Table 3
Figure 1
### Table A

| Amino acid sequence | C-terminus | mFpr1 | mFpr2 | mFpr2 - res1 | hFPR1 | hFPR2 | hFPR3 | Maximum |
|---------------------|------------|-------|-------|-------------|-------|-------|-------|---------|
| A A A W K Y M V m | CO(NH)     | 5.0±3.9 | 3.4±4.3 | 1.5±0.9 | 4.5±3.2 | 0±1.8 | 3.0±0.2 | 3000    |
| A A W K Y M V m  | CO(NH)     | 2.2±1.3 | 1.7±1.3 | 1.2±1.1 | 2.2±1.1 | 1.2±1.1 | 1.0±0.8 | 3000    |
| A W K Y M V m   | CO(NH)     | 1.2±1.3 | 1.1±1.9 | 1.3±1.0 | 1.0±1.3 | 1.4±0.4 | 1.0±0.4 | 3000    |
| W K Y M V m     | CO(NH)     | 1.4±1.3 | 1.1±1.9 | 0±0.4   | 1.2±1.1 | 1.2±1.1 | 1.0±0.8 | 3000    |
| - K Y M V m     | CO(NH)     | 0±0.4   | 2.6±3.0 | 2.6±3.0 | 2.6±3.0 | 2.6±3.0 | 2.6±3.0 | 3000    |
| - Y M V m      | CO(NH)     | 0.8±0.2 | 1.6±0.2 | 1.6±0.2 | 1.6±0.2 | 1.6±0.2 | 1.6±0.2 | 3000    |
| - - - M V m  | CO(NH)     | 2.9±0.2 | 1.0±0.4 | 0.9±0.5 | 2.9±0.4 | 3.0±0.5 | 2.9±0.4 | 3000    |
| - - - V m       | CO(NH)     | -     | -     | -     | -     | -     | -     | 2    |
| W K F M V m     | CO(NH)     | 2.4±1.0 | 1.1±1.0 | 1.1±1.0 | 1.1±1.0 | 1.1±1.0 | 1.1±1.0 | 1000    |
| W K K K V m     | CO(NH)     | 0±0.4   | 2.6±3.0 | 2.6±3.0 | 2.6±3.0 | 2.6±3.0 | 2.6±3.0 | 3000    |
| W K E M V m     | CO(NH)     | 7.1±3.0 | 1.1±3.0 | 1.0±3.0 | 1.0±3.0 | 1.0±3.0 | 1.0±3.0 | 3000    |
| W K Q M V m     | CO(CO)     | 9.3±0.4 | 9.3±0.4 | 9.3±0.4 | 9.3±0.4 | 9.3±0.4 | 9.3±0.4 | 3000    |
| W K K M V m     | CO(CO)     | 11.4±1.3 | 7.4±3.0 | 7.4±3.0 | 7.4±3.0 | 7.4±3.0 | 7.4±3.0 | 3000    |
| W K Y M V m     | hcy (CO)   | 14.5±3.0 | 4.6±3.0 | 4.6±3.0 | 4.6±3.0 | 4.6±3.0 | 4.6±3.0 | 3000    |
| W K Y M V c     | CO(H)      | 4.8±3.0 | 1.7±3.0 | 1.7±3.0 | 1.7±3.0 | 1.7±3.0 | 1.7±3.0 | 3000    |
| W K Y M V c     | CO(H)      | 6.8±3.0 | 2.6±3.0 | 2.6±3.0 | 2.6±3.0 | 2.6±3.0 | 2.6±3.0 | 3000    |
| W K Y M V c     | CO(H)      | 3.8±3.0 | 1.6±3.0 | 1.6±3.0 | 1.6±3.0 | 1.6±3.0 | 1.6±3.0 | 3000    |
| W K Y M V c     | CO(H)      | 3.8±3.0 | 1.6±3.0 | 1.6±3.0 | 1.6±3.0 | 1.6±3.0 | 1.6±3.0 | 3000    |
| W K Y M V c     | CO(H)      | 3.8±3.0 | 1.6±3.0 | 1.6±3.0 | 1.6±3.0 | 1.6±3.0 | 1.6±3.0 | 3000    |

### Table B

| Key residues | C3 | C4 | C6 | C3 | C4 | C6 |
|--------------|----|----|----|----|----|----|
| mFpr1        | N-N-A-W-K-Y-M-N-<sub>(NH2)</sub> | N-H-P-R1 | N-N-A-W-K-Y-M-N-<sub>(NH2)</sub> | hFPR1 | N-N-A-W-K-Y-M-N-<sub>(NH2)</sub> | hFPR2 |
| mFpr2        | N-N-A-W-K-Y-M-N-<sub>(NH2)</sub> | hFPR3 | N-N-A-W-K-Y-M-N-<sub>(NH2)</sub> | hFPR2 | N-N-A-W-K-Y-M-N-<sub>(NH2)</sub> | hFPR3 |

- > 1000 fold
- 25 - 600 fold
- 6 - 15 fold
- no effect
- not analyzed

### Figure 2
Figure 3
Figure 4
Randomly chosen N-terminal signal peptide fragments from different bacterial strains

A

B

C

D

Figure 5
Figure 6
Recognition of bacterial signal peptides by mammalian formyl peptide receptors: a new mechanism for sensing pathogens
Bernd Bufe, Timo Schumann, Reinhard Kappl, Ivan Bogeski, Carsten Kummerow, Marta Podgórska, Sigrun Smola, Markus Hoth and Frank Zufall

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