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Biological Role of the N-Formyl Peptide Receptors

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Ligation of N-formyl-methionyl-leucyl-phenylalanine (fMLP) to its specific cell surface receptors triggers different cascades of biochemical events, eventually leading to cellular activation. The formyl peptide receptors (FPRs) are members of the seven-transmembrane, G-protein coupled receptors superfamily, expressed at high levels on polymorphonuclear and mononuclear phagocytes. The main responses elicited upon ligation of formylated peptides, referred to as cellular activation, are those of morphological polarization, locomotion, production of reactive-oxygen species and release of proteolytic enzymes. FPRs have in recent years been shown to be expressed also in several non myelocytic populations, suggesting other unidentified functions for this receptor family, independent of the inflammatory response. Finally, a number of ligands acting as exogenous or host-derived agonists for FPRs, as well as ligands acting as FPRs antagonists, have been described, indicating that these receptors may be differentially modulated by distinct molecules.

Keywords Cell Membrane Receptors, Seven-transmembrane Receptors, Chemokine Receptors, Viruses, Conserved Sequences, Evolution, Formyl Peptide Receptors, 18S rRNA.

Phagocytes, such as neutrophils and monocytes, play a pivotal role in the host defenses against invading microbial pathogens through the release of proteolytic enzymes and other aggressive molecules. Leukocyte recruitment into an infected area is the hallmark of the innate immune responses and is dependent on the presence of chemotactic factor gradients. A number of chemotactic agents, including N-formylated peptides, the complement C5a fragment, leukotriene B4, the platelet-activating factor (PAF) and a superfamily of chemokines, are able to recruit phagocytes to a site of infection or inflammation, stimulate respiratory burst activity and induce release of lysosomal enzymes.[1,2] Several natural formylated peptides, identified as low molecular weight chemoattractants, have been purified from bacterial supernatants, providing evidence that they are able to activate human phagocytes.[3] In particular,

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the tripeptide N-formyl-methionyl-leucyl-phenylalanine (fMLP) isolated from *Escherichia coli* cultures is the most extensively studied member of the formyl peptide family, due to its capacity to activate a complex program in mammalian phagocytic leukocytes, including directed cell movement, phagocytosis, release of proteolytic enzymes and other aggressive proteins and generation of reactive oxygen intermediates.[4] Mitochondrial proteins are also N-formylated and are chemotactic for neutrophils, representing a possible source of endogenous chemoattractants.[5] Genes for chemoattractant receptors have been cloned and sequenced, and all are members of the superfamily of G protein-coupled receptors (GPCRs), containing seven transmembrane (7TM) domains with an extracellular domain and an intracellular C-terminus tail separated by three intracellular loops and three extracellular loops.[6,7] In human phagocytes formyl peptides bind at least two GPCRs, the high-affinity formyl peptide receptor (FPR) and its low-affinity variant FPR-like 1. Although the chemotactic FPRs were identified and cloned a number of years ago, their significance and functional role is poorly understood especially as regards their expression in non leukocyte cells. The purpose of this review is to briefly summarize the state of the art regarding the understanding of the biological actions of receptors for bacteria-derived peptides.

N-FORMYL PEPTIDE RECEPTORS

In humans two functional receptors for the formyl peptide, referred to as formyl peptide receptor 1, FPR1 (UniProtKB/Swiss-Prot entry FPR1_HUMAN [P21462]) and its variant formyl peptide receptor-like 1 (lipoxin A4 receptor), FPRL1 (FPRL1_HUMAN [P25090]), and in addition a putative formylpeptide receptor-like 2, FPRL2 (FPRL2_HUMAN [P25089]) have been identified. All three genes, FPR1, FPRL1 and FPRL2 are clustered on chromosome 19q13.3.[8] FPR1 was cloned in 1990 by Boulay et al. from a differentiated HL-60 myeloid leukemia-cell cDNA library. FPR binds fMLP with high affinity and is activated by picomolar to low nanomolar concentrations of fMLP to mediate chemotaxis and calcium ion mobilizing responses in human phagocytic leukocytes.[9] FPRL1 and FPRL2 genes were isolated by low-stringency hybridization using FPR cDNA as a probe. FPRL1 is defined as a low-affinity fMLP receptor because only high concentrations (mM range) of fMLP are able to induce Ca^{2+} mobilization, and it is poorly chemotactic even in the micromolar concentration. FPR and FPRL1 share 69% identity at the amino acid level. The FPRL2 gene encodes a putative protein with 56% amino acid sequence identity to human FPR and 83% to FPRL1. FPRL2 transfected in Xenopus oocytes does not elicit a response to fMLP, and, although it is expressed in monocytes but not in neutrophils, its functional agonists remain unclear. Functional FPRL2 is also expressed in mature dendritic cells (DCs), which express reduced levels of FPR but do not appear to express FPRL1.[10,11]

Recently, a novel chemoattractant peptide, acting specifically through FPRL2, has been identified. Migeotte et al. isolated F2L, an acetylated amino-terminal peptide derived from the cleavage of the human heme-binding protein, an intracellular tetrapyrrole-binding protein.[12] The peptide binds and activates FPRL2 in the low nanomolar range, triggering intracellular calcium release, inhibition of cAMP accumulation, and phosphorylation of extracellular signal-regulated kinase 1/2 mitogen-activated protein kinases through the G_i class of heterotrimeric G proteins. When tested on monocytes and monocyte-derived DCs, F2L promotes calcium mobilization and chemotaxis. Therefore, F2L appears to be a natural chemoattractant peptide for DCs and monocytes and the first potent and specific agonist of FPRL2.[12]

FPR1s genes have been also identified in the neutrophils of some non-human primates (Pan troglodytes [chimpanzee], FPR1_PANTR [P79241]; Gorilla gorilla gorilla [lowland gorilla], FPR1_GORGO [P79176]; Macaca mulatta [rhesus monkey], FPR1_MACMU [P79189]; Pongo pygmaeus [orangutan], FPR1_PONPY [P79235]), in the rabbit (Oryctolagus cuniculus, FPR1_RABIT [Q05394]) and mouse (Mus musculus, FPR1_MOUSE [P33766]), showing that FPR1 has been highly conserved throughout mammal species. In rabbit and mouse, FPR1s share 78% and 76% identity with human FPR1, respectively.[13] Snyderman and Pike reported that equine neutrophils respond to fMLP with potent degranulation, but the same ligand fails to induce chemotaxis.[14]

FPR1 and FPR2, the murine counterparts of human FPR1 and FPRL1, respectively, have been shown to interact with fMLP with a similar pattern to that of the human receptors.[15,16] Although the biological role of these receptors has not been fully defined, FPR1-depleted mice are more susceptible to bacterial infections.[17] Primary murine microglial cells expressing FPR1 and FPR2 genes show a FPR2-mediated activation only after treatment with lipopolysaccharide (LPS), demonstrating that bacterial endotoxin selectively modulates the function of chemotactic peptide receptors in murine microglial cells. The low responsiveness of nonstimulated microglial cells to FPR2 agonists may have a considerable biological significance for the homeostasis of the central nervous system. In normal conditions this compartment is protected by the blood-brain barrier, and it is not readily exposed to pathogens whereas in the course of endotoxemia, when microvessels form an incomplete blood-brain barrier, circulating LPS is able to enter into the nervous parenchyma and stimulate microglial cells. These, in turn, become activated to assume the characteristics of tissue macrophages, thus playing a critical role in the inflammation process.[18]

Expression of formylated peptide receptors in nonphagocytic cells was demonstrated in the 1990s. In fact, receptors with structural homologies with the neutrophil and monocyte FPRs have been shown to be expressed in different human tissues, suggesting that FPRs may be involved in cellular mechanisms other than inflammatory responses. For instance, FPR was identified in

astrocytes and hepatocytes, but since chemotaxis is not a perogative of these nonhematopoietic cells, the precise role of FPR in these cells remains to be defined.[19] Moreover, in the isolated heart, fMLP was reported to slightly decrease the rate of contraction and the coronary flow[20] while transient contractions have been described in isolated human coronary arteries following fMLP challenging.[21] The toxicity of fMLP during embryonal development has been studied by our group. In particular, in the chick embryo, fMLP is highly toxic to the heart musculature during a well defined developmental period. In fact, treatment with fMLP causes a transient increase in the rate of contraction, followed by arrhythmic contractions and cessation of the contractile activity. All these actions are mediated through a G-protein since the pertussis toxin completely abrogates the cardiac effects of fMLP, but the putative receptor is likely to be dissimilar to the FPR of mammalian granulocytes (since it is not impaired by specific antagonists). This fact raises the question of a possible role of putative endogenous agonists for an FPR-equivalent during well-defined stages of organogenesis.[22]

Using a rabbit polyclonal antiserum directed against the C-terminus of the human FPR, Becker et al. have observed that FPR is widely localized in different organs, including the spleen, thymus, appendix, lymph nodes, bone marrow, liver, lung, placenta, heart and the tunica media of coronary arteries, although the identity of the cell type expressing the receptor is not well defined.[23] In astrocytes and microglia cells FPR expression increases at sites of multiple sclerosis lesions, suggesting that chemotactic receptors may play a role in inflammatory responses in this disease and likely in other central nervous system diseases.[24]

Expression of FPR was also demonstrated in DCs, whose FPR-stimulated migration may be important for the modulation of T-cell activation.[25]

Functional FPR expression in the human liver cell line HepG2 was shown to regulate hepatic acute phase genes.[26]

Recently, a report demonstrated that functional FPRs are also expressed by normal human lung and skin fibroblasts. In particular, fMLP triggers dose-dependent migration in these cells, suggesting a possible role for nonleukocyte cell types, expressing functional FPRs, in innate immune responses.[27]

FORMYL PEPTIDE RECEPTOR SIGNAL TRANSDUCTION

Studies in leukocytes and in transfected cell lines indicate that FPR-mediated cell responses can be inhibited by agents capable to ADP-ribosylate G-proteins, such as pertussis toxin. In fact, FPR is functionally coupled to G proteins, $G_{i\alpha1}$, $G_{i\alpha2}$ and $G_{i\alpha3}$.[28–31] fMLP-receptor interaction results in the activation of phospholipase C (PLC) and phosphatidylinositol 3-kinase (PI3K). PI3K converts the membrane phosphatidylinositol 4,5–biphosphate (PIP_2) into phosphatidylinositol 3,4,5-triphosphate (PIP_3). PLC catalyzes PIP_3 into the secondary

messengers inositol triphosphate (IP_3) and diacylglycerol (DAG). DAG activates a Ca-dependent protein kinase C (PKC), whereas IP_3 regulates calcium mobilization from intracellular stores. A Ca^{2+} increase seems to be one of the earliest events of neutrophil response to formyl peptides. The activation of human neutrophils by chemotactic peptides evokes a rapid change in membrane potential and an increase in cytoplasmic Ca^{2+} levels. These events are followed up to a minute later by the release of detectable levels of microbicidal agents formed by the oxidative burst. The depolarization is maximal at 40 s after stimulation with fMLP. In contrast, the cytosolic Ca^{2+} concentration, albeit fMLP-dose dependent, is maximal at 10 s and is already rapidly decreasing by the time the cell reaches its lowest potential. Thus, Ca^{2+} release into the cytoplasm is the earliest evidence of neutrophil stimulation by fMLP and occurs in close association with an apparent membrane hyperpolarization.[32] However, studying the calcium requirements for chemotaxis Laffafian and Hallet demonstrated that neutrophils, moving toward a source of formylated peptide, change shape and display chemotaxis without any significant or persistent global or localized increase of cytosolic free calcium.[33] An abrupt rise in intracellular calcium concentration was observed when cells change shape as a consequence of membrane deformation probably related to the stretch-activated channels observed in some other cells.[34]

Several studies demonstrated that stimulation of neutrophils with fMLP results in an increase of the levels of PIP_3, which may contribute to activation of the oxidative burst, suggesting a critical role for PI3K in the fMLP-FPR interaction.[35] Browing et al. demonstrated that fMLP activates the transcription factor NF-kB in leukocytes and that this response is cell type- and developmental stage-specific.[36] In addition, fMLP is able to stimulate PI3K activity in monocytes: in fact inhibition of PI3K with wortmannin blocks the transcription factor NF-kB activation and interleukin (IL) 1β gene expression, indicating that PI3K is a necessary signal transducer for fMLP-induced NF-kB activation and proinflammatory cytokine gene expression in activated human monocytes.[37] Synthesis of PIP_3 by PI3K contributes to asymmetric F-actin synthesis and cell polarization during neutrophil chemotaxis. In fact, the selective PI3K delta inhibitor, IC87114, is able to inhibit polarized morphology of neutrophils, fMLP-stimulated PIP_3 production and chemotaxis although PI3K inhibition does not block F-actin synthesis or neutrophil adhesion. Therefore, PI3K seems to play a selective role in the amplification of PIP_3 levels that lead to neutrophil polarization and directional migration in response to fMLP stimulation.[38]

Several additional enzymes that regulate the production of lipid signalling molecules are activated in chemoattractant-stimulated neutrophils. These include phospholipase A_2 (PLA_2) and phospholipase D (PLD). Neutrophil cytosolic PLA_2 hydrolyses phospholipids containing the arachidonyl moiety at the sn-2 position to liberate arachidonic acid and a lysophospholipid, both products serving as precursors for additional inflammatory mediators.[39]

Inhibition of PLA_2 has been shown to inhibit oxidant production and degranulation; however, its role in the migratory response remains to be clarified.[40–42]

Also, PLD plays a pivotal role in the signal transduction pathway of the chemoattractant-receptor complex involved in the neutrophil activation in the same way as PI3K does. PLD catalyzes the hydrolysis of phospholipids to produce phosphatidic acid and the corresponding polar head group. In neutrophils, phosphatidic acid is a signalling molecule, and it appears to directly activate a kinase that phosphorylates a component of the NADPH oxidase complex; therefore, PLD seems to be essential for O_2- production from fMLP-stimulated human neutrophils.[43]

Studies on the kinetics of these enzymes revealed a differential timing in the action of PI3K and PLD. These enzymes produce second messengers which are required for subsequent superoxide production in human neutrophils stimulated by chemotactic peptide. In fact, NADPH oxidase is activated in a PI3K-dependent manner in the early phase of cellular response whereas PLD activity follows PI3K, and in human neutrophils, it is related to superoxide production in the late phase after stimulation with fMLP.[44,45]

Two groups of mitogen-activated protein kinase (MAPK) cascades, the extracellular signal-regulated kinases (ERKs) and p38 kinases are stimulated in polymorphonuclear cells (PMN) by chemoattractants.[46–49] ERKs participate in PMN adherence and oxidative metabolism whereas p38 kinases are involved in PMN adherence, chemotaxis and respiratory burst activation.[50,46] The pertussis toxin is reported to inhibit ERK activation but not p38 kinase activation by fMLP in human PMN.

In addition, chemoattractants stimulate distinct patterns of intracellular signalling involving a selective activation of MAPK activity, such as IL-8 and PAF, which stimulate a weaker ERK response than C5a and fMLP. This suggests that specific domains of chemoattractant receptors regulate MAPK activity through different G protein-coupled pathways.[47,51] In particular, the C-terminus tail of FPR is necessary for ligand-mediated activation of G_i proteins and MAPK cascades.[52] These results demonstrate distinct patterns of intracellular signalling for chemoattractants and suggest that selective activation of intracellular signalling cascades may underlie different patterns of functional responses.

Recently, the role of PKC in the fMLP-stimulated signalling events leading to the activation of NF-kB has been investigated. Huang et al. reported that fMLP-induced activation of NF-kB in human peripheral blood monocytes requires the activity of the small GTPase, RhoA. In particular, exposure of monocytes to fMLP causes increased activity of PKCe, PKC isoform.[53] The inhibition of PKCe activity blocks fMLP-stimulated activation of NF-kB. Moreover, RhoA associates with PKCe in fMLP-stimulated monocytes and the fMLP-induced PKCe activity is blocked by an inhibitor of RhoA. These findings demonstrate that fMLP-induced activation of NF-kB utilizes a signalling

pathway which requires activity of PKCe, which acts as a signalling component in cytokine gene transcription stimulated by chemoattractants, suggesting a novel mechanism through which fMLP not only attracts leukocytes but may also directly contribute to inflammation.[54]

AGONISTS FOR FORMYL PEPTIDE RECEPTORS

A number of agonists for FPR have been identified and isolated from exogenous sources or by artificial synthesis and used as probes for the study of leukocyte receptor expression and activation. For example, Bae et al. reported that a novel chemoattractant, the synthetic peptide His-Phe-Tyr-Leu-Pro-Met-NH2 (HFYLPM) is able to stimulate monocytes and neutrophils to induce chemotaxis and produce reactive oxygen intermediates through binding to the FPRL1 on human phagocytes.[55] Recently, Trp-Lys-Tyr-Val-D-Met (WKYMVm), a hexapeptide isolated and modified from a peptide library has been reported to be a very potent stimulant of several human leukocytic cell lines, including neutrophils. WKYMVm is a synthetic leukocyte-activating peptide postulated to use 7TM GPCR(s) able to induce marked chemotaxis and calcium flux in human phagocytes. Both FPR- and FPRL1-expressing cells mobilize calcium in response to picomolar concentrations of WKYMVm. While FPRL1-expressing cells migrated to picomolar concentrations of WKYMVm, nanomolar concentrations of the peptide were required to induce migration of FPR-expressing cells. Thus, WKYMVm uses both FPR and FPRL1 to stimulate phagocytes with a markedly higher efficacy for FPRL1, suggesting that FPR and FPRL1 in phagocytes react to a broad spectrum of agonists and that WKYMVm, as a remarkably potent agonist, provides a valuable tool for studying leukocyte signalling via these receptors.[56]

Another peptide library-derived sequence is MMK, a potent specific agonist for FPRL1; MMK acts as a chemotactic and calcium-mobilizing agonist for human monocytes, neutrophils, and FPRL1-transfected human embryonic kidney (HEK) 293 cells but is inactive in cells transfected with FPR.[57]

The synthetic methyl ester fMLP derivative, fMLP-OMe, is a potent chemoattractant for phagocytes with activities which are identical to those of fMLP, inducing a full response by neutrophils and may therefore be adopted as a reference model for evaluating the activity of newly synthesized analogues.[58] Several fMLP-OMe analogues have been synthesized in order to characterize the formylpeptide-receptor interaction in phagocytes and consequent cellular activation. Recently, the new disulfur-bridged peptide for-Met-Leu-Cys(OMe)-Cys(OMe)-Leu-Met-for has been synthesized, and its biological properties resulting from binding to the FPR of human neutrophils have been characterized, in terms of cell migration (i.e., chemotaxis), superoxide anion production and lysozyme enzyme release. Chemotaxis is triggered at low concentrations while both superoxide anion production and lysosomal enzyme release are elicited only at high concentrations and never reach the response peak observed for the prototype peptide at physiologically relevant

concentrations. The derivative appears to bind with a good affinity to FPRs, providing new information regarding the structure-activity relationship of the FPR.[59,60]

Despite 20 years of study, a number of findings have increased the uncertainty about the biological role of FPR, in part regarding the identity of its natural ligands. In fact, the demonstration that N-formylation of peptides is not necessary to activate FPR suggests a broad spectrum of potential natural ligands for fMLP receptors, not restricted to bacterial or mitochondrial sources. Recent observations provide evidences that HIV-envelope proteins contain domains able to interact with classical nonchemokine chemoattractant receptors on host phagocytes and have been identified as novel exogenous agonists for FPR. Among these, three peptide domains of gp41, namely T20/DP178, T21/DP107 and N36, are potent chemoattractants and activators of human peripheral blood phagocytes. T20/DP178 specifically activates FPR; T21/DP107 activates both receptors but has a much higher affinity for FPRL1 while N36 uses only FPRL1 as a functional receptor, suggesting that these peptide domains of the HIV-1 gp41 may have the potential to activate the host innate immune response by interacting with FPR and FPRL1 on phagocytes.[61–63]

Recently, Bylund et al. demonstrated that the cecropin-like *Helicobacter(H) pylori* peptide, Hp(2–20), induces activation of NADPH oxidase in human neutrophils via FPRL1.[64] Moreover, this nonformylated peptide fragment produced by *H. pylori* was reported to be a monocyte chemoattractant and activated the monocyte NADPH-oxidase to produce oxygen radicals.[65]

In addition to a number of exogenous molecules able to interact with fMLP receptors, important progress has been made in identifying host-derived agonists. Among these, the glucocorticoid-regulated protein annexin I (lipocortin I) has been shown to mediate the anti-inflammatory activities of glucocorticoids, acting through the FPR on human neutrophils. Peptides derived from the unique N-terminal domain of annexin I serve as FPR ligands and trigger different signalling pathways in a dose-dependent manner. These findings identify annexin I peptides as novel, endogenous FPR ligands and establish a mechanistic basis of annexin I-mediated anti-inflammatory effects.[66] Ernst et al. observed that the annexin 1 peptide initiates chemotactic responses in human monocytes that express all three FPR family members and also desensitizes the cells toward subsequent stimulation with bacterial peptide agonists. Experiments using HEK 293 cells stably expressing a single FPR family member reveal in addition to FPR, FPRL1 and FPRL2 can be activated and desensitized by the N-terminal annexin 1 peptide. These observations identify the annexin 1 peptide as the first endogenous ligand for chemotactic peptide receptors and indicate that annexin 1 is probably involved in regulating leukocyte migration into inflamed tissue by activating or desensitizing different receptors of the FPR family.[67]

Other endogenous ligands for FPRs have been identified and resulted of particular interest because they are associated with various pathological conditions. These molecules include three amyloidogenic proteins (SAA, $A\beta_{42}$ and a prion protein fragment PrP106–126), which act as chemoattractants and stimulate human phagocytes through FPRL1 so that a role has been attributed to this receptor in amyloidogenic diseases.[61,63,68–70]

Recently, certain nonformylated peptides have been observed to bind and activate neutrophils via FPR. In fact, it has been shown that acetylated fMLP analogues as well as non-acetylated, nonformylated fMLP analogues may be recognized by FPRs.[61,71,72,16]

ANTAGONISTS FOR FORMYL PEPTIDE RECEPTORS

Several molecules acting as antagonists for FPR have been described. For example, the t-butyloxycarbonyl (t-Boc) peptide derivative t-Boc-Phe-D-Leu-Phe-D-Leu-Phe (BocPLPLP) is able to block the fMLP activation of phagocytes, both in man and rabbit, through a competitive binding of the antagonist to FPR. Replacement of the formyl group of fMLP with a t-Boc group yields peptides that can block the interaction of fMLP with its receptor.[73,74]

The cyclic undecapeptide, cyclosporin (Cs) H, is a potent inhibitor of fMLP-induced superoxide anion (O_2-) formation in human neutrophils, and much more effective than other well known FPR antagonists, such as Boc-PLPLP or other Cs. In addition to impairing the O_2-release, CsH is able to inhibit the stimulatory effects of fMLP on the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), β-glucuronidase and lysozyme release.[29,75]

A series of amino-terminal carbamate analogues of the peptide Met-Leu-Phe (MLF) have been synthesized in order to determine the structural requirements for imparting agonist or antagonist activity to the human neutrophil FPR by evaluating receptor binding, superoxide anion release, and cell adhesion. Unbranched carbamates (methoxycarbonyl, ethoxycarbonyl, and n-butyloxycarbonyl) act as agonists, whereas branched carbamates (iso-butyloxycarbonyl, tert-butyloxycarbonyl, and benzyloxycarbonyl) act as antagonists. These results indicate that the switch from agonist to antagonist activity can be achieved by modifying the overall size and shape of the amino-terminal group and that modifications at both the amino and carboxy termini can alter the functional selectivity of the peptide. This suggests the possibility of development of antagonist molecules for diagnostic applications.[76]

Finally, deoxycholic acid (DCA) and chenodeoxycholic acid (CDCA), both hydrophobic bile acids, commonly used in Chinese traditional medicine for their immunoregulatory and antiinflammatory effects, selectively inhibit fMLP functions and regulate chemotaxis of human leukocytes. DCA inhibits fMLP-induced human monocyte and neutrophil migration and calcium mobilization, apparently through a blockage of fMLP binding to its receptors on

leukocytes. CDCA competitively inhibits [^{3}H]-fMLP binding to human monocytes and reduces both the chemotactic and calcium flux responses induced by fMLP. CDCA not only inhibits the effects of fMLP but also the effects of the W peptide (a potent agonist of fMLP receptors that lacks the N-formyl structure), probably by steric hindrance, suggesting a mechanism inducing inhibition of inflammation and suppression of the innate immune response.[77,78]

Formyl peptide receptor desensitisation

FPR has been shown to be rapidly (a few minutes) desensitized upon exposure to fMLP (homologous desensitization), with a consequent reduction of phagocytic cell responses, such as chemotaxis, calcium transient, and respiratory burst, when cell are restimulated with fMLP. Homologous desensitization is associated with receptor phosphorylation and internalization.[79]

To assess the role of phosphorylation in receptor function, U937 promonocytic cells were stably transfected to express the recombinant human FPR. Activation and desensitization of three mutant forms of FPR lacking specific serine and threonine residues in the C-terminus were studied, and the results indicated that phosphorylation of FPR is a necessary and sufficient step in desensitization that multiple phosphorylation sites are involved and that redundant desensitization does not occur downstream of G protein activation in the signalling cascade.[80,81] In addition, the role of receptor phosphorylation in FPR internalization and leukocyte chemotaxis has been examined. Whereas the wild type receptor is rapidly internalized upon stimulation, the phosphorylation-deficient mutant remains entirely on the cell surface. In addition, contrary to the hypothesis that receptor processing and recycling are required for chemotaxis, no defect in the ability of the mutant FPR to migrate up to a fMLP concentration gradient was found. Therefore, although FPR phosphorylation is a necessary step in receptor internalization, receptor phosphorylation, desensitization, and internalization are not required for chemotaxis.[82]

In addition to the homologous desensitization of chemoattractant receptors, where a ligand desensitizes only its own receptor, heterologous desensitization, where an activated receptor desensitizes one or more other inactive receptors, has also been demonstrated to occur between chemoattractant receptors.[83,84] This latter form of desensitization is at least in part mediated by second messenger-activated kinases, such as PKC.[85] Studies on the existence of a novel form of desensitization were carried out utilizing stably co-expressed receptors for the chemoattractants fMLP, C5a and IL-8 in a rat basophilic leukemia (RBL-2H3) cell line; a desensitization of FPR-mediated IP_3 generation and calcium mobilization by C5a and IL-8 in the absence of FPR phosphorylation was observed.[86] C5a and IL-8 treatment, however, does not result in desensitization of GTPS binding, a measure of receptor-G protein coupling. This suggests that the C5a- or IL-8-mediated desensitization of

FPR-mediated signalling occurs at the level of either G protein effector coupling or PLC activation. In addition, since fMLP, C5a, and IL-8 all appear to utilize similar signal transduction pathways, stimulation by any one of these three ligands should engage the described downstream desensitization mechanism(s).[86] Thus, it was predicted that fMLP stimulation would result in FPR desensitization regardless of receptor phosphorylation.

Role of N formyl peptide receptor in host immune response

In neutrophils and monocytes fMLP binding to its specific cell surface receptors triggers and modulates various cellular responses associated with inflammation, including transendothelial migration, degranulation, cytokine production, secretion of lysosomal enzymes, generation of lipid mediators and reactive oxygen species production. All these activities can serve to orchestrate immune cell development and are essential to host defence against invading micro-organisms.[22] Neutrophils, the cell type in which the role of FPR has been most extensively studied, constitute the first defense line against foreign organisms. The ability of these phagocytes to concentrate at inflammation sites is the result of cell movement from the blood across capillary walls (diapedesis) followed by directional movement (chemotaxis) towards the source of a concentration gradient. The initial step of chemotaxis features shape changes of a migrating cell, which can also be observed in the absence of a chemotactic gradient. In fact, upon exposure to fMLP, neutrophils develop a characteristic surface ruffling and acquire an elongated shape, indicated as "polarization," in which modifications in the actin cytoskeleton are implicated.[87,88] We analyzed mathematically the cell contour of fMLP-stimulated human granulocytes stimulated with fMLP under non-gradient conditions and demonstrated that the geometry of surface ruffling, as examined by power spectral analysis of the cell outline, exhibits well defined periodicities.[89] Such modifications are considered to be at least partly responsible for receptor trafficking and the modulation of some activation responses of granulocytes.[80]

Arbour et al. demonstrated that fMLP induces the secretion of IL-1 α, IL-1 β and IL-6 in human peripheral blood mononuclear cells (PBMC): northern analysis confirms that fMLP induces IL-1 α, IL-1 β and IL-6 gene expression in human PBMC. The fMLP-induced IL-1 α and IL-1 β gene expression and IL-6 secretion are abolished by pertussis toxin pre-treatment, suggesting that fMLP induction of cytokine is also mediated via a G_i protein, through a mechanism similar to that of other chemotactic factors (C5a, MCP-1, PAF, IL-8) which are able to modulate cytokines and whose receptors belong to the same superfamily as the fMLP receptors.[90]

The inflammatory response produced by infecting bacteria or microbial products involves leukocyte gene expression tightly regulated by the activities

of transcription factors, such as nuclear factor-kappa B (NF-kB), 1 NF-IL-6, and AP-1. NF-kB is of paramount importance to immune cell function owing to its ability to activate the transcription of many proinflammatory immediate-early genes.[91,92] Indeed, fMLP-induced activation of NF-kB appears to be essential for proinflammatory cytokine synthesis in the phagocytic cell. The activation of NF-kB appears to be cell-specific and different from the activation of NF-kB by other stimulant agents, such as TNFα. Neutrophil preparations that respond to fMLP, TNFα, and LPS with IL-8 secretion do not show NF-kB activation, whereas FPR-transfected HL-60 cells were responsive to TNFα but not to fMLP for NF-kB activation. Differentiation of FPR-transfected HL-60 cells with dimethyl sulfoxide for 3–5 days conferred the capability of the cells to activate NF-kB in response to fMLP without a significant increase in the amount of FPRs. These results identify NF-kB as a transcription factor that can be activated by the prototypic chemotactic peptide and demonstrate that this function is both highly regulated and dependent on signalling components specifically expressed during myeloid differentiation.[36]

Accordingly, the professional phagocytes (granulocytes, monocytes, macrophages) that form the first line of defense against invading microbes express such pattern recognition receptors, for which the N-formylated methionyl group is a critical determinant. Since the signals generated by the occupied FPRs induce chemotaxis, it has been widely held that these receptors evolved to mediate trafficking of phagocytes to sites of bacterial infection. This notion is supported by the fact that N-formyl peptides, in addition to being chemotactic, also possess other proinflammatory properties, such as the ability to activate phagocytes and trigger the release of antimicrobial peptides and oxidants. The importance of proper recognition of formylated peptides is clearly illustrated by the fact that FPR deficiencies are associated with increased susceptibility to bacterial infections.[93,94]

Neutrophils from patients with localized juvenile periodontitis (LJP) exhibit decreased binding and responsiveness to various chemotactic agents, including fMLP. In fact, a molecular alteration in the second intracellular loop of the fMLP receptor molecules in LJP patients seems to play a role in the decreased chemotactic activity reported for some LJP patients. This altered reaction of neutrophils is thought to account, at least in part, for the increased susceptibility of LJP patients to infections by periodontal organisms.[95] The role of FPR in host defense was confirmed by comparing the susceptibility of FPR–/– and FPR+/+ mice to infection with *Listeria monocytogenes*. When challenged with this microorganism, FPR-deficient mice experienced an increased bacterial burden in the liver and spleen soon after infection and earlier death, which suggests a role for FPR in host defense, specifically through regulation of innate immunity. This is consistent with the expression of FPR on phagocytes.[17]

ROLE OF FORMYL PEPTIDE RECEPTORS IN DISEASE STATES

Neurodegenerative diseases

FPRL1 acts as a functional receptor for at least three amyloidogenic peptides, serum amyloid A (SAA), $A\beta_{42}$ and PrP106–126. All three peptides are able to chemoattract and activate human phagocytes.[62,69,70]

SAA, an acute phase protein secreted mainly by hepatocytes, is normally present in serum at 0.1-μM levels but increases by 1,000-fold in systemic inflammatory conditions.[96] The optimal concentrations for SAA to induce leukocyte migration, adhesion, and tissue infiltration ranged from 0.8 to 4 μM,[97] which are higher than the SAA levels present in normal serum but well below the concentrations observed during a systemic acute phase response.[96–98] Increased serum levels of SAA have been observed in a number of inflammatory and infectious diseases as well as after organ transplantation.[99] A rapid increase in the concentration of locally produced SAA could establish a gradient of free active SAA with consequent recruitment of leukocytes into inflammatory sites. Chronic inflammatory conditions with elevated serum SAA may culminate in amyloidosis, characterized by enzymatic cleavage of SAA into fragments, with consequent deposition of "amyloid" fibrils in tissues, associated with progressive destruction of organ function.[100,101] Su et al. demonstrated that SAA is the first chemotactic ligand identified for FPRL1, thus suggesting that this receptor could mediate phagocyte migration in response to SAA.[62] Because phagocytes are the source of SAA cleaving enzymes and these cells are present at the sites of amyloid deposits, it is possible that at local inflammatory sites elevated SAA concentrations can attract and activate leukocytes through FPRL1 for the clearance of amyloid deposits. This process may also cause tissue injury, as observed in the course of amyloidosis.

A recent study reported that bacterial fMLP and antagonists against the high-affinity fMLP receptor FPR attenuate the production of proinflammatory cytokines induced by amyloid β (Aβ) peptides in microglial and THP-1 monocytes, suggesting that Aβ peptides may activate an FPR-like cellular receptor.[102] In fact, Aβ peptides have previously been shown to elicit a number of proinflammatory responses in mononuclear phagocytes, including microglial cells, monocytes, and monocytic cell lines. These include induction of cell adhesion, migration,[103–106] accumulation at sites of injection in the brain,[107] Ca^{2+} mobilization,[108] phagocytosis,[109] release of reactive oxygen intermediates, and increased production of neurotoxic or proinflammatory cytokines.[110–113] Signal transduction in monocytes involves activation of G-proteins, PKC,[106,112] and tyrosine kinases,[108,114–116,108] which are known to be activated by 7TM receptors including FPR and FPRL1.[80] $A\beta_{42}$ is an enzymatic cleavage fragment of the amyloid precursor protein (APP), and its aggregated form is a major component of the senile plaques observed in the brain tissue of patients with Alzheimer's disease (AD). AD is a progressive, neurodegenerative

disease characterized by the presence of multiple senile plaques in the brain, which are also associated with considerable inflammatory infiltrates. Although the precise mechanisms of the pathogenesis of AD remain to be determined, the overproduction and precipitation of a 42 amino acid form of $A\beta_{42}$ in plaques have implicated $A\beta_{42}$ in the neurodegeneration and proinflammatory responses seen in the AD brain. Recent studies revealed that the activation of FPRL1 by $A\beta_{42}$ may be responsible for accumulation and activation of mononuclear phagocytes (monocytes and microglia). In fact, upon binding to FPRL1, $A\beta_{42}$ is rapidly internalized into the cytoplasmic compartment in the form of $A\beta_{42}$/FPRL1 complexes. Persistent exposure of FPRL1-expressing cells to $A\beta_{42}$ resulted in intracellular retention of $A\beta_{42}$/FPRL1 complexes and the formation of Congo red-positive fibrils in mononuclear phagocytes, suggesting that FPRL1 may not only mediate the proinflammatory activity of $A\beta_{42}$ but also actively participate in $A\beta_{42}$ uptake and the resultant fibrillar formation.[117] Although Aβ has been reported to be directly neurotoxic, it also causes indirect neuronal damage by activating mononuclear phagocytes (microglia) that accumulate in and around senile plaques. The identification of FPRL1 as a functional receptor for $A\beta_{42}$ and detection of FPRL1 mRNA in mononuclear phagocytes infiltrating senile plaques provide a molecular basis for inflammation in AD. In fact, FPRL1 is expressed at high levels by inflammatory cells infiltrating senile plaques in brain tissues from AD patients.[69] The hypothesis that the pathogenesis of AD involves a proinflammatory response is linked to the observations that the 42 amino acid form of amyloid β is a chemotactic agonist for FPRL1, which is expressed on human mononuclear phagocytes. Therefore, FPRL1 may constitute an additional molecular target for the development of therapeutic agents for AD.[63,68] Whether an increased microglial response to amyloidogenic peptides results in a beneficial clearance of noxious agents or exacerbates the disease by promoting inflammation is not yet known.[68]

Prion diseases are transmissible and fatal neurodegenerative disorders which, similarly to AD, involve infiltration and activation of mononuclear phagocytes at the brain lesions. A 20 amino acid fragment of the human cellular prion protein, PrP(106–126), was reported to mimic the biological activity of the pathologic prion isoform and activate mononuclear phagocytes.[118] The cell surface receptor(s) mediating the activity of PrP(106–126) is unknown. In a recent study, it was demonstrated that PrP(106–126) is chemotactic for human monocytes through ligation of FPRL1. Upon stimulation by PrP(106–126), FPRL1 is rapidly internalized and an enhanced monocyte production of proinflammatory cytokines, inhibited by pertussis toxin, has been described. These observations suggest that FPRL1, acting as a "pattern recognition" receptor, is able to interact with multiple pathologic agents and may probably be involved in the inflammatory processes observed in prion diseases.[70]

HIV-1 infection

HIV-1 envelope proteins contain multiple domains that act as chemotactic agonists for FPR and FPRL1, thus suggesting a possible implication of FPRs in host responses during HIV-1 infection.[61,62,119] In fact, several synthetic peptides corresponding to amino acid sequences of HIV-1 envelope proteins gp41 and gp120 have been reported to down regulate the expression and function of the receptors for fMLP and a variety of chemokines on monocytes. The inhibitory effect of HIV-1 envelope proteins on monocytes was apparently due to a mechanism resembling PKC-mediated receptor desensitization and required the presence of CD4, a primary receptor for HIV-1, since the effect of gp41 was only observed in $CD4^+$ monocytes and in HEK293 cells cotransfected with chemokine receptors and an intact CD4, but not in the presence of a CD4 lacking its cytoplasmic domain.[120,121] The mechanism of action is not yet clear: gp41 could directly interact with FPRs or, alternatively, the interaction of gp41 with cellular CD4 may lead to the exposure of epitopes to FPRs.[61] Although there is no experimental evidence of direct interaction between intact HIV-1 envelope proteins and the FPRs, recent reports suggest the possible *in vivo* generation of agonist fragments through envelope proteins proteolysis. For example, both synthetic T20/DP178 and T21/DP107 epitopes could be recognized by sera of HIV-infected patients, suggesting that the epitopes of gp41 can likely become accessible to host immune cells.[122] In particular, T20 functions as a phagocyte chemoattractant and a chemotactic agonist at the phagocyte FPR. Furthermore, antibodies recognizing HIV-1 envelope epitopes were detected in early phases of HIV-1 infection.[123,124] T20 has been tested in clinical trials because it significantly reduces viral load in AIDS patients.[125] Moreover T20 and T21 have been demonstrated to inhibit viral fusion in vitro.[121,125,126] Although HIV-1 envelope proteins gp41 and gp120 have been shown to be chemotactic agonists for FPR and/or FPRL1, it is not yet clear whether these interactions between FPRs and viral proteins also occur *in vivo*, then interfering with the immune responses.[16,127]

Interestingly, interactions between FPRs and their agonists play a critical role in the complex host responses to infections. Chemokine receptor CCR5 and CXCR4 act as key fusion cofactors used by the human immunodeficiency virus type 1 (HIV-1). Chemokine ligands specific for CCR5 and antibodies recognizing this receptor have been shown to inhibit HIV-1 entry and replication.[119,128]

Alternatively, HIV-1 resistance exhibited by some exposed but uninfected individuals is due, in part, to a 32 base-pair deletion in the CCR5 gene which results in a truncated protein that is not expressed on the cell surface.[129]

The fMLP binding to its receptor, FPR, results in a significant attenuation of cell responses to CCR5 ligands and in inhibition of HIV-1-envelope-glycoprotein-mediated fusion and infection of cells expressing CD4, CCR5, and FPR. In

particular, it was observed that fMLP rapidly induces a PKC-mediated serine phosphorylation and down-regulation of CCR5. Therefore, increased levels of CCR5 phosphorylation are accompanied by down regulation of the surface expression and function of CCR5 in monocytes. However, treatment of monocytes with CCR5 ligands does not substantially compromise the cell response to fMLP. These results support the idea of a "hierarchy phenomenon" observed among chemoattractant receptors, suggesting an important role for FPR in the orchestration of the host responses in the presence of multiple leukocyte chemoattractants at sites of local inflammation.[128,130]

Conclusions

Among all the chemoattractant receptors discovered so far, the FPRs are unique in many aspects. Firstly, they represent the only receptors for the exogenous chemoattractant, fMLP. Secondly, a host of experimental observations suggests the involvement of FPRs in antimicrobial defense. Thirdly, these receptors play a role in pathological conditions, as observed in some neurodegenerative diseases, in HIV infection or in LJP. However, many questions remain to be answered; for example, the exact role of FPRs in different tissues and cell types, including non leukocytic cells, requires further investigation, which is likely to reveal novel biological functions mediated by these receptors. On the other hand, the identification of novel ligands for FPRs, such as nonformylated peptides, host-derived molecules and lipid agonists, seems to indicate that these receptors constitute a family of molecules implicated in complex biological responses. In this context, the discovery that FPRs are modulated by distinct peptide ligands, acting as agonists or antagonists, and that this modulation leads to differential cellular signalling and different functional cellular responses may be important for the development of therapeutic strategies, opening up new possibilities for the development of novel antiinflammatory and microbicidal approaches.

ABBREVIATIONS

7TM	seven-transmembrane
AD	Alzheimer's disease
BocPLPLP	t-Boc-Phe-D-Leu-Phe-D-Leu-Phe
CDCA	chenodeoxycholic acid
DAG	diacylglycerol
DCA	deoxycholic acid
DCs	dendritic cells
ERKs	extracellular signal-regulated kinases
fMLP	N-formyl-methionyl-leucyl-phenylalanine
FPR	formyl peptide receptor
FPR1	formyl peptide receptor 1

FPRL1	formyl peptide receptor-like 1 (lipoxin A4 receptor)
FPRL2	formyl peptide receptor-like 2
GPCRs	G protein-coupled receptors
HFYLPM	His-Phe-Tyr-Leu-Pro-Met-NH2
IL	interleukin
IP_3	inositol triphosphate
LJP	localised juvenile periodontitis
LPS	lypopolysaccharide
MLF	Met-Leu-Phe
NF-kB	nuclear factor-kappa B
PBMC	human peripheral blood mononuclear cells
PI3K	phosphatidylinositol 3-kinase
PIP_2	phosphatidylinositol 4,5-biphosphate
PIP_3	phosphatidylinositol 3,4,5-triphosphate
PKC	protein kinase C
PLA_2	phospholipase A_2PLCphospholipase C
PLD	phospholipase D
PMN	polymorphonuclear cells
SAA	serum amyloid A
WKYMVm	Trp-Lys-Tyr-Val-D-Met

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