



## REVIEW

# The Role of Formylated Peptides and Formyl Peptide Receptor 1 in Governing Neutrophil Function during Acute Inflammation



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Neutrophil migration to sites of inflammation and the subsequent execution of multiple functions are designed to contain and kill invading pathogens. These highly regulated and orchestrated processes are controlled by interactions between numerous receptors and their cognate ligands. Unraveling and identifying those that are central to inflammatory processes may represent novel therapeutic targets for the treatment of neutrophil-dominant inflammatory disorders in which dysregulated neutrophil recruitment, function, and elimination serve to potentiate rather than resolve an initial inflammatory insult. The first G protein-coupled receptor to be described on human neutrophils, formyl peptide receptor 1 (FPR1), is one such receptor that plays a significant role in the execution of these functions through multiple intracellular signaling pathways. Recent work has highlighted important observations with regard to both receptor function and the importance and functional relevance of FPR1 in the pathogenesis of a range of both sterile and infective inflammatory conditions. In this review, we explore the multiple components of neutrophil migration and function in both health and disease, with a focus on the role of FPR1 in these processes. The current understanding of FPR1 structure, function, and signaling is examined, alongside discussion of the potential importance of FPR1 in inflammatory diseases suggesting that FPR1 is a key regulator of the inflammatory environment. (*Am J Pathol* 2015, 185: 1172–1184; <http://dx.doi.org/10.1016/j.ajpath.2015.01.020>)

More than 100 years ago, Metchnikoff described microphagocytes as important responders to sterile injury in starfish larvae.<sup>1</sup> Now known to be neutrophils, they are key effector cells of the innate immune system and are pivotal in the containment and clearance of initial noxious stimuli of either infective or sterile origin.<sup>2</sup> Accounting for 50% to 70% of circulating human leukocytes, these polymorphonuclear granulocytes patrol the vasculature and rapidly migrate into tissues in response to chemotactic signals.<sup>3</sup> On arrival, neutrophils execute a variety of antimicrobial functions, including the generation of reactive oxygen species (ROS), phagocytosis of pathogens and dead and dying tissue, degranulation with the release of a variety of toxic products, expulsion of neutrophil extracellular traps, and paracrine signaling to recruit other cell types.<sup>4</sup> Disruption of these processes or overwhelming neutrophil recruitment can cause significant dysregulation of the

inflammatory cascade, resulting in failed resolution of inflammation, tissue injury, and acute or chronic disease.

The predominant mechanisms through which neutrophils sense inflammatory stimuli and surrounding environments are a plethora of cell surface receptors principally within the G protein-coupled receptor (GPCR) family. The first GPCR to be described on the human neutrophil was formyl peptide receptor 1 (FPR1) which, when activated, triggers a wide variety of functions, including chemotaxis, degranulation, ROS production, and phagocytosis.<sup>5,6</sup> Although descriptions of FPR1 functions *in vitro* are well documented, increasing attention is being paid to its *in vivo* role, influence, and

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importance in the pathogenesis of both infective and sterile acute inflammatory disease.

The principal ligands for FPR1 are bacterial and mitochondrial formylated peptides, actively secreted by invading pathogens or passively released from dead and dying host cells after tissue injury. During infection, pathogens target and destroy host tissue with the simultaneous release of both bacteria-derived (when the pathogen is of bacterial origin) and host-derived formylated peptides (from host mitochondria), thereby linking FPR1 in both infective and sterile inflammatory processes. This review therefore provides an overview of the structure, signaling, and pathological functions of FPR1 as well as a focus on the recent developments in the understanding of formylated peptides and neutrophil FPR1 in the context of infective and sterile inflammation.

## Formyl Peptide Receptors

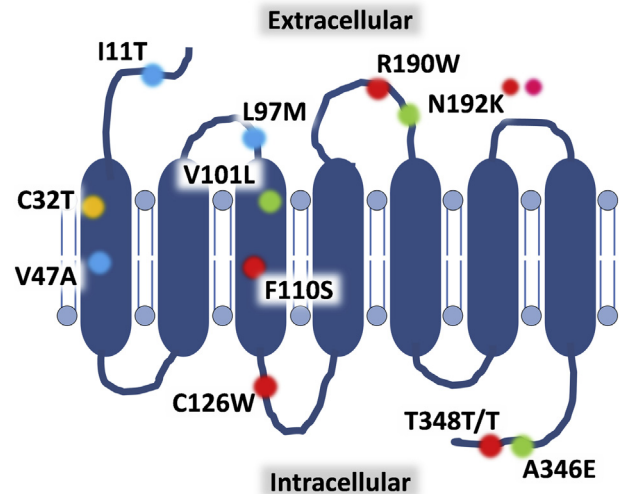
The binding of the *N*-formyl methionine motifs of bacterial- and mitochondrial-derived peptides to FPR1 was initially described over 3 decades ago and was the starting point for the subsequent dissection of the many G-protein signaling cascades within neutrophils.<sup>7,8</sup> The human FPR family constitutes FPR1, FPR2/ALX (lipoxin receptor), and FPR3, which are well-conserved GPCRs that have pluripotent and diverse roles in the initiation, propagation, and resolution of inflammation.<sup>9</sup>

FPR1 was the first neutrophil GPCR to be cloned and sequenced, identifying it as a 350-residue protein with seven hydrophobic segments (Figure 1).<sup>5</sup> A subsequent study characterized FPR1 as a seven-transmembrane receptor with the N-terminus and three loops exposed on the cell surface for ligand interactions and the C-terminus and the remaining loops found within the cytoplasm necessary for intracellular signaling.<sup>10</sup> Using low-stringency hybridization with FPR cDNA as the probe, two separate but relatively conserved low-affinity receptors, initially termed FPR-like 1 (FPRL1) and FPR-like 2 (FPR2L), were cloned from an mRNA library of neutrophil-like promyelocytic HL-60 cells. These receptors have since been renamed FPR2/ALX and FPR3, respectively, as more has become known about their distinct biochemical and physiological roles.<sup>6</sup> All three receptors are clustered together on chromosome 19q13.3 and share significant sequence homology. FPR1 has 69% amino acid identity with FPR2 and 56% with FPR3, whereas FPR2 and FPR3 share 83% identity.<sup>11</sup>

### Formyl Peptide Receptor 1

#### Regulation of FPR1 Expression

Constitutively expressed on the surface of quiescent neutrophils, FPR1 receptor expression is rapidly up-regulated in response to a wide number of inflammatory stimuli. *In vitro*, these stimuli include lipopolysaccharide (LPS), platelet-activating factor,



**Figure 1** Structure of the FPR1 receptor and key polymorphisms within the protein. Formyl peptide receptor 1 (FPR1) as a classic G-protein-coupled receptor has seven transmembrane-spanning regions with an extracellular N-terminus, whereas the C-terminus is found within the cytoplasm. Multiple amino acid substitutions have been found within the protein: recognized constitutive isoforms (green; FPR-26, FPR-98, and FPR-G6), polymorphisms associated with disease states including juvenile and aggressive periodontitis (red), hypertension in young adults (orange), gastric cancer (pink; denotes D192K), and those that have been observed but whose functional significance remains uncertain (blue).

unmethylated CpG oligonucleotides, and tumor necrosis factor  $\alpha$ <sup>12–15</sup>; FPR1 receptor up-regulation has also been described in the circulating neutrophils of patients with emphysema, Crohn disease, and sepsis.<sup>16–18</sup> Such a rapid increase in FPR1 expression indicates its preformed presence within the cell. Subcellular fractionation and the study of neutrophil transcription within the bone marrow have determined that FPR1 synthesis occurs late in neutrophil maturation, with the receptor subsequently stored in azurophilic granules (also known as primary granules) and secretory vesicles.<sup>14,19</sup> In response to agonists such as platelet-activating factor, secretory-vesicle FPR1 alone is mobilized; however, in response to powerful stimuli such as phorbol myristate acetate, azurophilic-granule FPR1 also localizes to the cell surface.<sup>14</sup>

Alongside FPR1 transport from intracellular compartments, neutrophil activation increases FPR1 protein synthesis. In nonstimulated cells, FPR1 mRNA is unstable, with a  $t_{1/2}$  of approximately 90 minutes.<sup>20</sup> After incubation with LPS, increased *FPR1* gene transcription occurs along with stabilization of the mRNA transcript ( $t_{1/2}$ , approximately 20 hours), allowing for increased synthesis of the receptor. LPS, through myeloid differentiation marker MyD88-dependent signaling pathways, is a powerful inducer of FPR1 synthesis in mature neutrophils, whereas tumor necrosis factor  $\alpha$  and other proinflammatory mediators also induce a similar response.<sup>21,22</sup> The increases in receptor cycling and protein synthesis therefore increase FPR1 at the cell surface, facilitating the perpetuation of the inflammatory response as

greater numbers of receptor are available on each cell to bind free ligand.

#### Polymorphisms within FPR1

With increasing attention being paid to the role of interindividual differences in response to infection and inflammation, variation in receptor number and function through polymorphisms within the *FPR1* gene have been studied. Three isoforms of human FPR1 exist: FPR-26 (V101, N192, E346), FPR-98 (L101, N192, A346), and FPR-G6 (V101, K192, A346). These differ in amino acids 101 (the top of the third transmembrane loop), 192 (center of the second intracellular loop), and 346 (end of the C-terminus) (Figure 1).<sup>22,23</sup> Functionally, FPR-98 and FPR-G6 have a partial defect in G<sub>i</sub>-protein coupling relative to the more constitutively active FPR-26, with the amino acid glutamic acid at position 346 essential for the G-protein signaling of the latter isoform.<sup>24</sup> Alongside these naturally occurring variants of *FPR1*, at least 30 other single-nucleotide polymorphisms (SNPs) have been described within the *FPR1* gene.<sup>6</sup>

Neutrophils isolated from individuals with localized juvenile periodontitis demonstrate impaired responsiveness to formyl-methyl-leucine-phenylalanine (fMLF; alias fMLP), perhaps explaining increased rates of periodontal infection through reduced migration or impaired bacterial killing capacity.<sup>25</sup> SNPs F110S and C126W in the second intracellular loop of the receptor may well account for this phenotype. Furthermore, a T348T/T mutation, although not altering FPR1 receptor number, was associated with impaired fMLF-induced neutrophil chemotaxis and aggressive periodontitis in an African-American population.<sup>26</sup> Meanwhile, the association of the C32T SNP with the development of hypertension in younger adults, as well as the D192K polymorphism with the onset of gastric carcinoma in an elderly Japanese population, have also been described, although their mechanistic and functional relevance is yet to be determined.<sup>27,28</sup> In contrast, the V101L SNP is associated with increased binding affinity for the FPR1 antagonist cyclosporin H.<sup>29</sup> With increasing application of genome-wide association studies, it remains to be seen whether other SNPs are associated with altered susceptibility to inflammatory diseases or infection.

#### FPR1 Ligands

Although it was initially thought that FPR1 only bound *N*-formylated peptides,<sup>7</sup> it is now widely recognized that the formyl group is not a prerequisite for receptor binding. The *N*-formylated version of any peptide containing a methionine residue at the 5' terminus is at least 100-fold more potent than the identical nonformylated peptide. However, if the peptide contains five or more amino acids, the nonformylated moieties can also bind and activate FPR1.<sup>23</sup> This finding has been further substantiated by the description of endogenous nonformylated ligands that bind FPR1, including cathepsin G, annexin A1 (AnnA1), and the recently described cytokine family with sequence similarity

**Table 1** The Principal Cell Types that Express Human Formyl Peptide Receptors and the Predominant Agonists and Antagonists of the Receptors

Receptor/cell type	Agonists	Antagonists
<b>FPR1</b>		
Neutrophils <sup>8</sup>	<i>N</i> -formylated peptides <sup>7</sup>	Cyclosporin H <sup>30</sup>
Macrophages <sup>20</sup>	Synthetic fMLF <sup>5</sup>	Cyclosporin A <sup>29</sup>
Monocytes <sup>31</sup>	Cathepsin G <sup>32</sup>	Boc-MLF (Boc-1) <sup>10</sup>
Dendritic cells <sup>33</sup>	FAM19A4 <sup>34</sup>	Boc-FLFLFL (Boc 2) <sup>10</sup>
Epithelial cells <sup>35</sup>	Annexin A1* <sup>35</sup>	CHIPS <sup>9</sup>
Hepatocytes <sup>10</sup>	Ac2-26* <sup>35</sup>	Spinorphin <sup>9</sup>
Glial cells <sup>10</sup>		
Astrocytes <sup>10</sup>		
Keratinocytes <sup>36</sup>		
<b>FPR2</b>		
Neutrophils <sup>37</sup>	<i>N</i> -formylated peptides <sup>38</sup>	Boc-MLF (Boc-1) <sup>10</sup>
Macrophages <sup>39</sup>	fMLF <sup>40</sup>	Boc-FLFLFL (Boc 2) <sup>10</sup>
Monocytes <sup>41</sup>	LL-37 <sup>41</sup>	WRW <sup>4</sup> peptide <sup>10</sup>
Dendritic cells <sup>9</sup>	Serum amyloid A <sup>9</sup>	
Microglia <sup>9</sup>	β-Amyloid <sup>9</sup>	
T lymphocytes <sup>9</sup>	HIV gp41-derived peptide <sup>9</sup>	
Epithelial cells <sup>35</sup>	Annexin A1* <sup>42</sup>	
	Ac2-26* <sup>42</sup>	
	Lipoxin A <sub>4</sub> * <sup>41</sup>	
	Humanin* <sup>43</sup>	
<b>FPR3</b>		
Macrophages <sup>10</sup>	F2L* <sup>44</sup>	WRW <sup>4</sup> peptide <sup>10</sup>
Monocytes <sup>10</sup>	Humanin* <sup>43</sup>	
Dendritic cells <sup>10</sup>		
Eosinophils <sup>9</sup>		

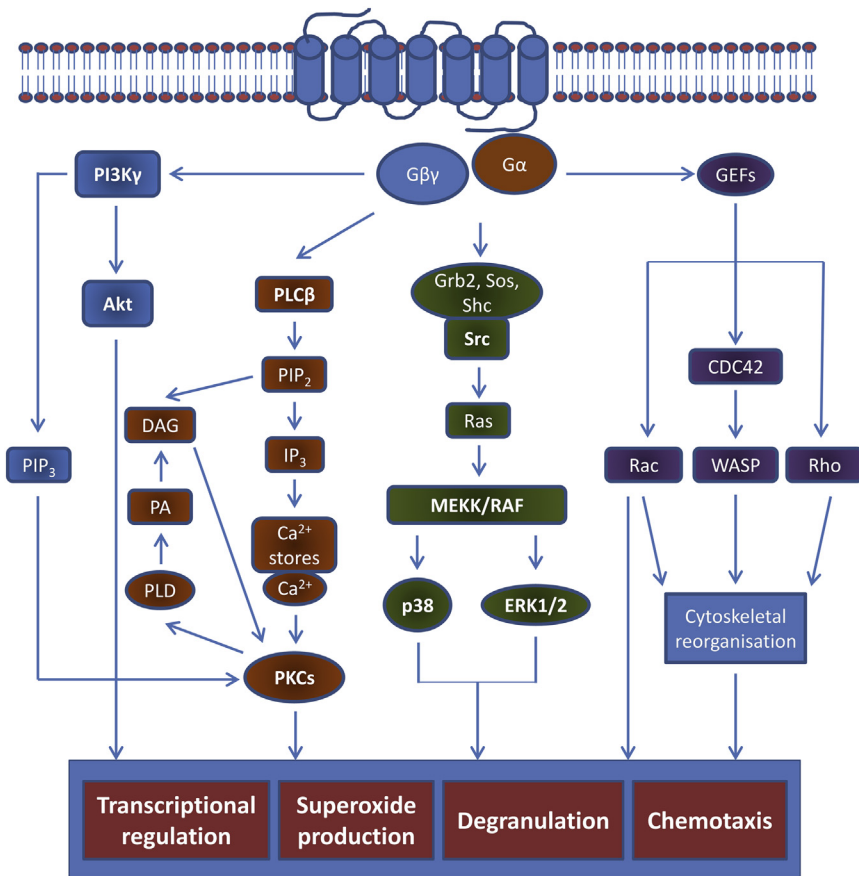
\*Anti-inflammatory agonist; the ligands described elicit functional responses in a number of cell types.

Ac2-26, N-terminal peptide of annexin 1; Boc-FLFLFL, Boc-Phe-Leu-Phe-Leu-Phe-OH; Boc-MLF, Boc-Met-Leu-Phe-OH; CHIPS, chemotaxis inhibitory protein of *Staphylococcus aureus*; FAM19A4, family with sequence similarity 19, member A4; fMLF, formyl-methyl-leucine-phenylalanine; FPR, formyl peptide receptor; gp, glycoprotein; LL-37, leucine, leucine 37; WRW<sup>4</sup>, Trp-Arg-Trp-Trp-Trp-Trp-CONH<sub>2</sub> (WRWWWW).

19, member A4, although their functional effects differ from those of fMLF/FPR1-described functions (Table 1).<sup>34,42</sup> For example, the neutrophil granule protein cathepsin G binding to FPR1 is unable to induce intracellular calcium flux but does, however, result in mitogen-activated protein kinase (MAPK) phosphorylation and neutrophil chemotaxis and dendritic cell recruitment.<sup>32</sup> It is postulated that this discrepancy in receptor function is likely attributable to low-affinity binding, thereby inducing isolated components of the inflammatory cascade.<sup>10</sup> Similarly, AnnA1 and its N-terminal peptide derivative Ac2-26 bind to FPR1 and induce anti-inflammatory and proresolution effects rather than a proinflammatory response through a variety of effects, including receptor heterodimerization with FPR2.<sup>35,42</sup>

#### FPR1 Binding Properties

Much is known about the structure and function of FPR1 at a molecular level and its interaction with the *Escherichia*



**Figure 2** Intracellular signaling events after FPR1 receptor activation. After binding of ligand to formyl peptide receptor 1 (FPR1), conversion of guanosine diphosphate to guanosine triphosphate induces dissociation of the  $\alpha$  from the  $\beta\gamma$  subunits. These trigger a range of intracellular kinase pathways, resulting in the induction of a variety of cell functions, including neutrophil chemotaxis, degranulation, superoxide anion production, and transcriptional activity. The predominant signaling pathways are those of phosphoinositide-3 kinase (PI3K), mitogen-activated protein kinase (MAPK), and phospholipase C. The latter triggers release of intracellular calcium from the endoplasmic reticulum to activate protein kinase C (PKC) and subsequent reactive oxygen species production, whereas PI3K triggers protein kinase B (alias Akt)-mediated and phosphoinositol-3,4,5-trisphosphate (PIP<sub>3</sub>)-mediated signaling, with a variety of cellular effects. With PI3K pulled toward the plasma membrane by the  $\beta$  and  $\gamma$  G-protein subunits ( $G\beta$  and  $G\gamma$ ), activation of Ras family proteins and MAPK further contributes to oxidative burst and chemotaxis. CDC, cell division control protein; DAG, diacyl glycerol;  $G\alpha$ , G-protein  $\alpha$ ; GEF, guanine nucleotide exchange factor; Grb, growth factor receptor-bound protein; IP<sub>3</sub>, inositol triphosphate; MEKK, mitogen-activated protein kinase kinase; PA, phosphatidic acid; PLC $\beta$ , phospholipase C $\beta$ ; PLD, phospholipase D; RAF, rapidly accelerated fibrosarcoma; Sos, son of sevenless; WASP, Wiskott-Aldrich syndrome protein.

*coli*-derived prototypic synthetic ligand fMLF. Firstly, the formyl group at the N-terminus allows hydrogen bond formation to a hydrogen acceptor within the binding pocket. Secondly, the methionine side chain may occupy a hydrophobic pocket within the receptor, with the sulfur atom in the ligand interacting with a positively charged portion of the receptor. Finally, further hydrophobic interactions may occur between the leucine and phenylalanine residues and FPR1.<sup>10</sup> After binding to FPR1, fMLF is internalized within approximately 30 seconds and stimulates a variety of intracellular signaling cascades, resulting in a number of cellular responses. Within neutrophils, these include chemotaxis, ROS production, degranulation, cytokine expression, phagocytosis, and changes in cell surface marker expression.<sup>23,45,46</sup>

#### FPR1 Desensitization

As discussed, regulation of GPCR function is essential *in vivo* for controlling neutrophil functions, including chemotaxis and the exact localization of cells to the inflammatory site.<sup>9,23,47</sup> After FPR1-ligand binding, the cell rapidly becomes unresponsive to subsequent stimulation by the same ligand (homologous desensitization). Multiple distinct processes account for this change in cell function, including receptor internalization and desensitization.<sup>10</sup> The latter is mediated by phosphorylation of multiple serine and threonine

residues within the carboxy-terminal, leading to G-protein dissociation and disruption of intracellular signaling pathways, a process that is governed in part by ERK1/2-GPCR kinase 2 (GPK2) and arrestin binding.<sup>48,49</sup> Meanwhile, receptor internalization predominantly occurs after C-terminal phosphorylation through  $\beta$ -arrestin-independent and non-clathrin-mediated endocytosis.<sup>6,50</sup>

Heterologous desensitization of FPR1 can also occur after activation of CD88 [a complement component 5a (C5a) receptor] or chemokine (C-X-C motif) receptor 2 CXCR2; (an IL-8 receptor) due to shared components of intracellular signaling molecules and occurs principally through protein kinase C-mediated pathways.<sup>51</sup> Due to the intracellular signaling hierarchy that exists, however, the capacity for FPR1's heterologous desensitization of other receptors far outstrips their reciprocal ability to inhibit FPR1 function,<sup>52</sup> which was recently highlighted by the observation that the activation of FPR1 in monocytes induces cross-desensitization of CCR1 but not CCR2.<sup>52</sup> This observation suggests the ability of FPR1 to act as a regulator of chemoreceptor hierarchy with regard to prioritizing not only its own signaling but also that of other potent and important agonists, such as those that bind CCR2.<sup>53</sup> Importantly, these reductions in cell surface receptor number and function can be relatively short-lived, as receptor and ligand dissociate from each other after internalization



and the receptor returns to the cell surface available for further ligand binding.

### Intracellular Signaling

Characterization of the FPR1 signaling cascades has been conducted with fMLF as the stereotypical ligand and has revealed a highly complex and integrated chain of intracellular signaling events.<sup>10,23</sup> Binding to FPR1 results in G<sub>i</sub>-type G-protein activation, with the conversion of GDP to GTP inducing the dissociation of  $\alpha$  from the  $\beta\gamma$  subunits (Figure 2). The latter liberated subunits activate both the phospholipase C $\beta$  and phosphoinositide 3-kinase  $\gamma$  (PI3K $\gamma$ ) signaling cascades. Phospholipase C $\beta$  hydrolyses membrane-bound phosphoinositol-4,5-bisphosphate into diacylglycerol and inositol trisphosphate to mediate the release of intracellular calcium stores, principally from the endoplasmic reticulum. These events subsequently activate protein kinase C and are central to NADPH oxidase ROS production.<sup>54</sup> Meanwhile, PI3K $\gamma$ -mediated conversion of phosphoinositol-4,5-bisphosphate to phosphoinositol-3,4,5-trisphosphate acts as the principal regulator of neutrophil cytoskeletal reorganization and respiratory burst after FPR1 activation as well as influences the chemotactic response.<sup>10,47</sup>

As the  $\beta\gamma$  subunits activate PI3K $\gamma$  and pull it toward the plasma membrane, the activity of Src-like tyrosine kinases increases with the phosphorylation of the Src homology 2 domain—containing (Shc) adaptor protein, which, in turn, increases the association with growth factor receptor-bound protein 2 and son of sevenless and subsequently activates MAPK signaling pathways. ERK and p38 MAPK predominantly influence chemotaxis and FPR1-mediated transcriptional activity.<sup>10</sup> Meanwhile, the activation of guanine-nucleotide exchange factors induces the activation of the Rho GTPases [Rho, Rac, and cell division control protein 42 (CDC42)]. These, in turn, regulate ROS production through control of the formation of NADPH oxidase complex<sup>6</sup> as well as influence leukocyte adhesion, transmigration, actin polymerization, and phagocytosis.<sup>55</sup>

Although much of this work is well recognized, recent work using antibodies specific to C-terminal phosphorylation sites within the FPR1 protein has demonstrated that in some cases, in particular in the context of inflammatory bowel disease, G-protein-insensitive, formylated peptide-dependent FPR1 phosphorylation may also occur. This observation was noted to be predominantly at the surface of colonic crypt abscesses, leading Leoni et al<sup>56</sup> to postulate that this possible reactivation of FPR1 at distinct sites of inflammation may contribute to perpetuation of the acute and often deleterious inflammatory response.

New facets of the conventional understanding of FPR1 signaling within neutrophils have begun to emerge in recent years, for example, the appreciation of its interconnection with autocrine release of ATP and subsequent purinergic signaling. Recent work by Bao et al<sup>57</sup> demonstrated that FPR1 signaling induces a rapid, but reversible, increase in

the mitochondrial membrane potential within neutrophils, with an associated oxidative burst and extracellular release of ATP through pannexin 1 channels. Despite the conventional understanding that neutrophil-mediated ATP production occurs through glycolysis, researchers demonstrate that the initial burst of ATP is mitochondria derived and important in initiating the FPR/purinergic response. The autocrine ATP appears to then bind purinergic P2Y<sub>2</sub> and P2X receptors to augment neutrophil chemotaxis, intracellular calcium flux, and p38 and ERK p42/44 MAPK signaling but appears to have little effect on fMLF-mediated degranulation and phagocytosis. Interestingly, pannexin 1 channels and the purinergic receptors accumulate at the leading edge of the polarized neutrophil, providing opportunity for coordinated migratory activity.<sup>57–59</sup>

Also, localizing to the leading edge of the migrating neutrophil is the receptor of urokinase-type plasminogen activator serine protease to facilitate urokinase-type plasminogen activator–mediated degradation of the extracellular matrix, thus allowing for extravascular neutrophil transit. It is understood that urokinase-type plasminogen activator receptor expression is necessary for fMLF-induced chemotaxis through a variety of means, including the colocalization of FPR1 and  $\beta_1$ -integrins at the cell surface, with urokinase-type plasminogen activator receptor acting as a bridging molecule to facilitate their respective functions.<sup>60</sup> These novel observations, therefore, provide further insight into the highly regulated and integrated crosstalk between different ligands and their receptors in fine-tuning the functional response to inflammatory agents.

### Formyl Peptide Receptor 2

In contrast to the specificity of FPR1, its relative FPR2/ALX is a highly promiscuous receptor that binds fMLF with low affinity.<sup>40</sup> It was one of the first descriptions of a receptor capable of binding lipids, peptides, and proteins with ligands including serum amyloid A, lipoxin A<sub>4</sub>, and AnnA1 and has been reviewed in detail elsewhere.<sup>9,37,61</sup> Importantly, these ligand-specific interactions are able to induce either proinflammatory or proresolution/anti-inflammatory effects. With regard to binding to formylated peptides, the binding affinity of FPR2 is determined principally by the charge of the C-terminus of peptide, contrary to FPR1 which, as discussed in *FPR1 Ligands*, is N-terminal dependent.

Binding of serum amyloid A or the cathelicidin-associated antimicrobial peptide leucine, leucine-37 (LL37) to FPR2/ALX results in proinflammatory responses with neutrophil NF- $\kappa$ B activation and cytokine release, increased neutrophil recruitment to sites of inflammation, and increased neutrophil lifespan.<sup>41,62</sup> In contrast, binding of AnnA1 inhibits neutrophil migration, promotes neutrophil apoptosis, increases the rate of macrophage phagocytosis of apoptotic cells, and skews the macrophages toward a less proinflammatory phenotype.<sup>39</sup> Lipoxin A<sub>4</sub>, again

through FPR2/ALX, inhibits neutrophil migration while concomitantly augmenting monocyte recruitment. These distinct effects elicited from ligand binding to the same receptor have recently been attributed to different dimerization states after agonist binding that alter receptor conformation and subsequent intracellular signaling.<sup>42</sup>

### Formyl Peptide Receptor 3

Distinct from the other members of the FPR family, the function of FPR3 remains relatively poorly understood. While not expressed on human neutrophils, it is found in eosinophils, monocytes, macrophages, and dendritic cells, leading to speculation that it may play a role in the pathogenesis of allergic disease.<sup>63</sup> FPR3 is relatively insensitive to formylated peptides, and few specific endogenous ligands have been identified. F2L, an endogenous 21-amino acid acetylated amino-terminal peptide, is the most specific ligand described to date.<sup>64</sup> Derived from cleavage of heme-binding protein 1 by cathepsin D, F2L activates FPR3 in low nanomolar concentrations.<sup>44</sup> In doing so, it induces monocyte intracellular calcium flux, ERK1/2 phosphorylation, and chemotaxis while also augmenting LPS-mediated IL-12 production in dendritic cells, thereby inhibiting their maturation.<sup>33,64</sup> Humanin, a neuroprotective peptide, also binds with high affinity to both FPR2 and FPR3.<sup>43</sup> Despite the high sequence homology with FPR2/ALX, the behavior of FPR3 is surprising, with significantly higher basal levels of receptor phosphorylation and internalization and relative insensitivity to common FPR2/ALX ligands.<sup>65</sup> This observation has led to the hypothesis that it may also act as a decoy receptor to bind extracellular ligands, thereby regulating the function of other formylated peptide receptors.<sup>65</sup> Although there is likely to be some functional overlap with FPR2/ALX, the true functional role of FPR3 and its relevance *in vivo* remain to be determined.

### Mouse FPR Receptors

FPR1 has been described across several species, including horse, rabbits, and rodents, with marked differences in functional responses to formylated peptides observed.<sup>6</sup> In comparison to the three FPR receptors described in humans, the mouse genome encodes multiple FPR-related receptors from chromosome 17A3.2.<sup>66</sup> Fpr1 is the murine orthologue of human FPR1, sharing 77% homology, expression on similar cell types, and induction of the same effects of neutrophil chemotaxis, degranulation, cytokine production, and phagocytosis.<sup>67</sup> Genes *Fpr2* and *Fpr3* together encode receptors that mimic human FPR2/ALX. *Fpr2* encodes the ALX receptor specific for lipoxin A<sub>4</sub>, whereas *Fpr3* encodes Fpr2, which binds formylated peptides, serum amyloid A, and other similar ligands.<sup>6</sup> Similarities between murine Fpr2 and human FPR3 with regard to responsiveness to F2L have also been observed.<sup>68</sup> At present, the function and cognate

ligands of the remaining murine Fpr receptors are poorly characterized and understood. Fpr-related sequences 3, 4, 6, and 7 may function as chemoreceptors in vomeronasal olfactory sensory neurons,<sup>69</sup> whereas Fpr-related sequence 8 appears to affect the lifespan of mice, although the reason for this remains unknown at present.<sup>66</sup>

Although there is relatively high sequence homology of FPR1 between humans and mice, and the intracellular domain structure is highly conserved, there are distinct differences in the affinity of murine Fpr1 for fMLF, which is approximately 100-fold less than that of its human counterpart.<sup>68</sup> This difference in affinity is attributed to alterations in the folding of the transmembrane and extracellular domains, as determined by the apposition of multiple noncontiguous residues.<sup>70</sup> Although differences in the affinity of a receptor to prototypic *E. coli*-derived fMLF are described, it should be noted that, with regard to other bacterial formylated peptides, murine Fpr1 remains a high-affinity receptor, in particular to *Staphylococcus aureus*, *Listeria monocytogenes*, and mitochondria-derived formylated peptides.<sup>68</sup> Although it is important to be aware of such differences, inferences from mouse models of disease remain relevant and have, to date, significantly advanced our understanding of FPR biology and neutrophil function in both the physiological and pathophysiological states.

## FPR1 in Health and Disease

### Mitochondrial Formylated Peptides

With regard to neutrophil FPR1 function, the principal proinflammatory effects are mediated through bacterial and mitochondrial formylated peptides. Given the increasing interest in the role of FPR1 in sterile inflammation, we focus on the synthesis, release, and function of the latter in greater detail. It has been proposed that mitochondria arose over 2 billion years ago after the inclusion of  $\alpha$ -proteobacteria within the ancient precursors of eukaryotic cells. Much of the initial genetic material contained within these bacteria has either been lost or transferred to the nuclear genome, which now encodes the vast majority of the approximately 1500 mitochondrial proteins.<sup>71</sup> What has been left, however, is approximately 16 Kb of CpG-rich, unmethylated mitochondrial DNA (mtDNA), which encodes for 13 mitochondrial peptides that make up the key components of the respiratory chain within the inner mitochondrial membrane. In addition, mtDNA also contains coding information of 22 tRNAs and two mitochondrial ribosome (mitoribosome)-coding RNAs, vital for the processes of translation and peptide synthesis.<sup>72</sup>

Distinct from eukaryotic cytoplasmic ribosomes, prokaryotic and mitochondrial protein synthesis require an N-terminal fMet residue to initiate translation. Mitoribosomes are located in the mitochondrial matrix, with approximately 100 per organelle, and contain 39S and 28S ribosomal

subunits.<sup>72</sup> Unlike cytoplasmic mRNA, mitochondrial mRNA has no upstream leader sequence to coordinate mitoribosome binding but instead starts at the 5' end, with the N-terminal fMet binding to the mRNA start codon. N-terminal fMet binding to the small (28S) ribosome is facilitated by mitochondrial translational initiation factor 2 in a GTP-dependent process. Importantly, it is only N-terminal fMet and not Met-tRNA that is recognized by mitochondrial translational initiation factor 2 as the initiator tRNA, hence the crucial and irreplaceable nature of fMet in mitochondrial peptide synthesis.<sup>73</sup>

Although extracellular mitochondrial formylated peptides are recognized as potent damage-associated molecular patterns, within mitochondria they are naturally subjected to post-translational modification. Some peptides, however, undergo removal of the formyl group which principally occurs through the action of peptide deformylase, a zinc- and iron-binding metalloproteinase that hydrolytically cleaves the N-terminal formyl moiety.<sup>74</sup> Inhibition of peptide deformylase causes a global reduction in the accumulation of mtDNA-encoded proteins, with impaired subsequent assembly of components of the respiratory chain and therefore mitochondrial energy production.<sup>75</sup> Despite these observations, the exact role and contribution of peptide deformylase and the balance between mature formylated and deformylated peptides within the mitochondria remain poorly understood. Importantly, it appears that peptide deformylase is not capable of deformylating all mitochondria-synthesized peptides.<sup>76</sup> Indeed, deformylation does not completely remove the presence of formylated peptides within eukaryotic cells, as both formylated and deformylated versions of the same peptides coexist.<sup>75</sup> It is also interesting to note that extracellular deformylase enzymes within rat intestinal mucosa that sequentially cleave bacterial formylated peptides to reduce the abundance of proinflammatory mediators have been described.<sup>77</sup>

At present, there is no direct evidence of active release of mitochondrial formylated peptides from cells; rather, it is considered a passive process after necrotic cell death. This method of release is obviously distinct from bacteria, which can actively secrete formylated peptides into the extracellular milieu.<sup>78</sup> Given the description of autophagy-mediated high-mobility group box protein 1 (HMGB1), IL-1 $\beta$ , mtDNA, and ATP release, it is not inconceivable that formylated peptides are also actively released by similar mechanisms.<sup>79</sup>

### FPR1 in Sterile Inflammation

The importance of mitochondrial formylated peptides in influencing neutrophil function was originally described by Carp,<sup>7</sup> who demonstrated their ability *in vitro*, distinct from nonformylated mitochondrial proteins, to induce neutrophil chemotaxis through binding to FPR1. This finding was substantiated by the subsequent demonstration of their ability to induce neutrophil-like promyelocytic HL-60 cell intracellular calcium flux and superoxide anion production

through binding to both FPR1 and FPR2/ALX.<sup>38</sup> Further appreciation of their *in vivo* significance as important damage-associated molecular patterns came with the description of elevated mtDNA levels, as a general marker of liberated mitochondrial products, in the circulation of patients with aseptic, trauma-induced systemic inflammatory response syndrome.<sup>30</sup> Alongside this observation, Zhang et al<sup>30</sup> demonstrated the FPR1-dependent peritoneal neutrophil recruitment after i.p. injection of isolated mitochondrial damage-associated molecular patterns. Furthermore, they were able to induce alveolar neutrophil accumulation and pulmonary extravascular leak after i.v. administration of mitochondrial damage-associated molecular patterns in rats. Together with further *in vitro* demonstration of mitochondrial damage-associated molecular patterns—induced FPR1-dependent chemotaxis, intracellular calcium flux, and degranulation, they provided the first robust evidence of the *in vivo* importance of mitochondrial formylated peptide-mediated neutrophil activation and migration in driving local and distant inflammation.<sup>30,80,81</sup> These findings have subsequently been supported by the discovery that, in a mouse model of paracetamol-induced liver injury in which increased levels of circulating mtDNA were detected, dual blockade of FPR1 and chemokine (C-X-C motif) receptor 2 attenuated both local hepatotoxicity and distant neutrophil migration into the lung.<sup>82</sup>

Alongside these important observations, the role of mitochondrial formylated peptides was further emphasized in eloquent work using a mouse model of focal heat-induced sterile liver injury and studied with spinning-disk intravital microscopy.<sup>83</sup> After hepatic necrosis, intravascular gradients of chemokines such as macrophage inhibitory protein 2, interacting with chemokine (C-X-C motif) receptor 2, were shown to guide neutrophils crawling along the vascular endothelium to regions of sterile inflammation. Having arrived within the vascular system, neutrophils then sensed formylated peptides, exiting the capillary bed and moving through the tissue toward the area of necrosis in an FPR1-dependent manner. This observation was confirmed by the nondirectional migration of *FPR1*<sup>-/-</sup> neutrophils within the tissue and their failure to enter the necrotic zone, distinct from wild-type neutrophils, which rapidly accumulated within the necrotic area generated by the thermal injury.<sup>83</sup> Importantly, this appears not to be an organ-specific phenomenon, as the same hierarchy of chemotactic signals was observed within areas of sterile skin inflammation even though the route of neutrophil migration was predominantly interstitial rather than intravascular.<sup>84</sup>

Understanding of the neutrophil response to infection explains the potentially significant role of mitochondrial formylated peptides in the inflammatory process. As neutrophils are essential for bacterial clearance, they need to rapidly migrate through tissues and localize invading pathogens by responding to bacteria-derived chemoattractants, including formylated peptides, and not be

distracted by endogenous factors contained within the surrounding inflammatory milieu.<sup>85</sup> The dominant role of bacterial formylated peptides, in the presence of other competing mediators, in driving neutrophil chemotaxis has been recently described.<sup>47</sup> Interstitial neutrophil migration is mediated by PI3K and p38 MAPK signaling pathways, with the latter the more dominant process. In an environment of multiple signaling mediators, neutrophil PI3K is inactivated by phosphatase and tensin homologue, allowing p38 and phospholipase A2 to drive neutrophil migration toward bacterial products.<sup>47</sup> In *Pten*<sup>-/-</sup> mice, this effect is lost and neutrophils fail to prioritize and become distracted by other chemokines, leading to impaired neutrophil migration with a subsequent reduction in bacterial clearance *in vivo*.<sup>47</sup> As mitochondrial formylated peptides appear to exert a similarly dominant hierarchical effect on neutrophil migration and function, their putative importance in the pathogenesis of sterile inflammation and tissue injury is clear.<sup>86</sup>

### FPR1 and Infection

The classic description of the role of FPR1 is in the migration of neutrophils into sites of infection for the subsequent containment and killing of the microorganism. Although this role had been suggested from *in vitro* observations,<sup>83</sup> it was the generation of the *Fpr1*<sup>-/-</sup> mouse that allowed for the demonstration of this role *in vivo*.<sup>87</sup> Replacement of a 150-bp sequence with a neomycin-resistance cassette of the *Fpr1* gene, within the part encoding for the first extracellular loop to the fourth transmembrane domain, allowed for the complete absence of Fpr1 expression at both the protein and mRNA levels. The neutrophils were shown to be completely unresponsive to formylated peptides with regard to intracellular calcium flux as well as chemotaxis both *in vitro* and *in vivo*.<sup>87</sup> Injection of i.v. *L. monocytogenes* resulted in increased bacterial load in both the liver and spleen of *Fpr1*<sup>-/-</sup> mice relative to wild-type mice, attributable to reductions in neutrophil migration and diminished ROS production.<sup>46,87</sup> Furthermore, *Streptococcus pneumoniae* meningeal infection is associated with poorer outcome in *Fpr1*<sup>-/-</sup> animals but, interestingly, a paradoxical increase in neutrophil number within the brain.<sup>88</sup> Consistent with FPR1 having a central role in pathogen containment, *S. aureus* secretes an FPR1 inhibitory protein as a potential immune-evasion strategy.<sup>89</sup>

In contrast, within the lung, susceptibility to pulmonary *S. pneumoniae* infection is not increased in *Fpr1*<sup>-/-</sup> mice or in wild-type animals treated with pharmacological inhibitors of FPR1, such as cyclosporin H.<sup>90,91</sup> Indeed, a reduction in neutrophil number after pharmacological FPR1 inhibition has instead been demonstrated to have no effect on bacterial burden.<sup>91</sup> In a model of bacterial endotoxin-mediated lung injury, *Fpr1*<sup>-/-</sup> mice had a reduction in alveolar, interstitial, and circulating neutrophil numbers, as well as extravascular leak, 4 hours after injury with nebulized LPS.<sup>92</sup> Whether these seemingly paradoxical observations are due to organ, pathogen, or model-specific differences remains to be seen.

Cigarette smoke contains a wide variety of bacterial products, including LPS and formylated peptides.<sup>93</sup> Demonstrating the importance of FPR1 in both the initiation and propagation of pulmonary inflammation, Cardini et al<sup>94</sup> found that wild-type mice chronically exposed to cigarette smoke over a 7-month period developed characteristic emphysematous changes. In contrast, *Fpr1*<sup>-/-</sup> animals were protected from emphysema, suggesting that the formylated peptides within cigarette smoke are central to disease pathogenesis. A similarly beneficial reduction in inflammatory response, with fewer migrating neutrophils and macrophages and lower proinflammatory cytokine levels, was observed after acute or subacute exposure (1 to 3 days) to cigarette smoke.<sup>94</sup> Whether the chronic changes observed are attributable to an alteration in initial inflammatory cell phenotype or are a result of a modulation of the chronic inflammatory response through both innate and adaptive immune response remains to be elucidated.

Further novel perspectives on the role of FPR1 in infection have come through the description of the essential role of neutrophil-derived IL-1 $\beta$  in abscess formation and bacterial clearance after cutaneous *S. aureus* infection.<sup>95</sup> Alongside animals deficient in Toll-like receptor (Tlr) 2 and nucleotide-binding oligomerization domain (Nod) 2, *Fpr1*<sup>-/-</sup> mice were noted to have a reduced neutrophil number at the site of infection, with an associated reduction in IL-1 $\beta$  release after direct bacterial infection *in vitro*, suggesting another mechanism through which this GPCR exerts its antibacterial functions.<sup>95</sup>

### FPR1 in Other Cell Types

The most frequently described roles for FPR1 are with regard to neutrophil chemotaxis, degranulation, and ROS production, but FPR1 activation also exerts effects in other cell types. In isolated human monocytes, FPR1 activation augments IL-8 release in a manner cooperative with other mitochondrial damage-associated molecular patterns<sup>31</sup> while also influencing dendritic cell maturation, migration, and phenotype.<sup>33,96</sup> Importantly, the number of FPR1 receptors on the macrophage surface appears linked to their phenotype, at least *in vitro*, as treatment with cytokines associated with inducing an alternatively activated phenotype (IL-4 and IL-13) reduced FPR1 through Stat6-dependent mechanisms, whereas interferon  $\gamma$  (associated with a classically activated phenotype) increased receptor expression.<sup>97,98</sup> Importantly, in the former group, fMLF-induced migration was completely abrogated, suggesting regulation of cell migration into the inflammatory environment by autocrine and paracrine manipulation of GPCR expression.

In addition to the direct regulation of immune cells, the role of bacteria-derived formylated peptides in mediating nociceptive responses to infection has recently been delineated. *S. aureus* skin infection causes hyperalgesic responses that were found to be independent of infiltrating immune cells but instead dependent on formylated peptides and the bacterial pore-forming toxin  $\alpha$ -hemolysin, with protection in *Fpr1*<sup>-/-</sup> mice noted.<sup>99</sup> Interestingly, inhibition



of nociceptor responses led to enhanced local inflammation, demonstrating that formylated peptides can have indirect immune-modulatory properties acting via neuronal cell types.<sup>99</sup>

Recent evidence has also emerged of a protective role for FPR1 in the maintenance of epithelial integrity and mucosal repair after colonic injury *in vivo*. Indeed, formylated peptides released by commensal bacteria within the colon promote enterocyte migration and proliferation through FPR1 and NADPH oxidase 1.<sup>100</sup> Furthermore, binding of AnnA1 and Ac2-26, its N-terminal derivative, to epithelial FPR1 results in Src kinase and NADPH oxidase 1 activation and increased ROS production. Subsequent oxidative inactivation of regulatory phosphatases, phosphatase and tensin homologue, and protein tyrosine phosphatase containing proline, glutamic acid, serine, and threonine residues (PTP-PEST) leads to paxillin and focal adhesion kinase-mediated epithelial migration and wound closure.<sup>35</sup> *In vivo*, delivery of exogenous AnnA1 resulted in accelerated recovery after chemical-induced colitis.<sup>35</sup>

Despite this latter observation, a similarly important role for FPR1 has been seen *in vitro* in human bronchial epithelial cell migration, with an FPR1-dependent response to mitochondrial formylated peptides observed after linear scratch injury to cells in culture.<sup>101</sup> The *in vivo* corollary and functional relevance of FPR1-mediated pulmonary epithelial homeostasis is, however, yet to be established.<sup>101</sup> Additional evidence for the importance of Fpr1 in epithelial homeostasis lies in the observation that *Fpr1*<sup>-/-</sup> mice develop spontaneous lens degeneration in the absence of any preceding inflammatory events,<sup>102</sup> whereas the presence of formylated peptides accelerates human retinal pigment epithelial cell migration after injury.<sup>103</sup> Conversely, up-regulation of expression in malignant cells may confer a more aggressive and invasive phenotype due to an increased propensity of cells to migrate.<sup>104</sup>

In keratinocytes, FPR1 has been shown, within specific clinical contexts, to play an important role in the control of necroptosis (caspase-independent programmed cell death). In patients with severe blistering skin conditions, such as Stevens-Johnson syndrome, keratinocytes, when cultured *in vitro*, were sensitive to the FPR1 agonists fMLF and AnnA1, resulting in necroptosis through ROS production and receptor-interacting protein.<sup>36</sup> The limited nature of this observation is attributed to differences in inducible expression of FPR1, which is greater in Stevens-Johnson syndrome than in other similar dermatological conditions despite no differences in promoter sequence.<sup>36</sup> This role in keratinocyte death is in contrast to the proposed beneficial effects of FPR1,<sup>35</sup> in particular on binding AnnA1, in the maintenance of gastrointestinal epithelial integrity and warrants further investigation.

## Inhibition of FPR1

Accompanying the increased understanding of the pathophysiological role of FPR1 is the appreciation of its

potential as a therapeutic target in chronic inflammatory disorders or acute sterile inflammation either through the use of agonists or antagonists, depending on the context. With regard to pharmacological antagonists, there are, at present, limited FPR1-specific compounds available. Initially, Boc-Met-Leu-Phe-OH (Boc-MLF; alias Boc-1) and subsequent Boc-Phe-Leu-Phe-Leu-Phe-OH (Boc-FLFLF; alias Boc-2) were described,<sup>105</sup> but their relative low potency and lack of specificity given associated FPR2 inhibition have led to cyclosporin H, an inverse agonist to FPR1, being the more readily used agent in preclinical studies.<sup>80,83,91,94</sup> Its specificity is limited by the off-target effects on calcium/calmodulin-dependent phosphorylation of elongation factor 2.<sup>106</sup> Studies that are based on observations made entirely using these compounds should therefore be interpreted with caution and need to be accompanied by adjunctive demonstrations of FPR1 inhibition with monoclonal antibodies or, in the murine context, the use of *Fpr1* knockout animals.

Emphasis has therefore shifted to the development of alternative synthetic peptide ligands and small molecules, agonists and antagonists that were recently reviewed elsewhere.<sup>106</sup> Several synthetic ligands inhibiting fMLF-induced neutrophil chemotaxis have been described<sup>107,108</sup>; however, their inhibitory effects may be through the inhibition of downstream signaling pathways rather than direct FPR1 antagonism. Given difficulties in the synthesis and delivery of peptides, a complementary focus has been on the development of small-molecule antagonists and the use of high-throughput screening and structure-activity relationship-directed design and synthesis.<sup>109,110</sup> Notably, this approach has recently identified particular chromones and isoflavones that are selective for FPR1 and do not alter FPR2-mediated ERK phosphorylation and intracellular calcium flux.<sup>111</sup>

## Conclusion

FPR1, as a stereotypical GPCR, has facilitated significant advances in our understanding of receptor signaling and control of multiple cell functions at a single-cell level. It has, however, been the identification of surrogate biomarkers of the release of mitochondrial products (elevated plasma mtDNA in trauma, sepsis, and systemic inflammatory response syndrome), the development and application of transgenic mouse models into a variety of disease models, and the awareness of FPR1 functions in nonmyeloid cells that has now firmly established the functional importance of FPR1 in the pathophysiology of a plethora of inflammatory diseases. Whether FPR1 antagonism, which interferes with the bactericidal capacity of neutrophils, risks precipitating or exacerbating intercurrent infection remains to be shown in appropriate animal models. However, in the context of the many neutrophil-dominant sterile inflammatory disease processes that affect humans, FPR1 antagonism may prove

beneficial. Therefore, the development of small-molecule antagonists and the appreciation of its ligand-dependent and potential proresolution effects have placed FPR1 in the arena of attractive novel therapeutic targets.

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