



SUBJECT AREAS:  
INNATE IMMUNITY  
PATHOLOGY  
BACTERIA  
ANTIMICROBIALS

# Formylpeptide receptors are critical for rapid neutrophil mobilization in host defense against *Listeria monocytogenes*

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Received  
15 August 2012

Accepted  
27 September 2012

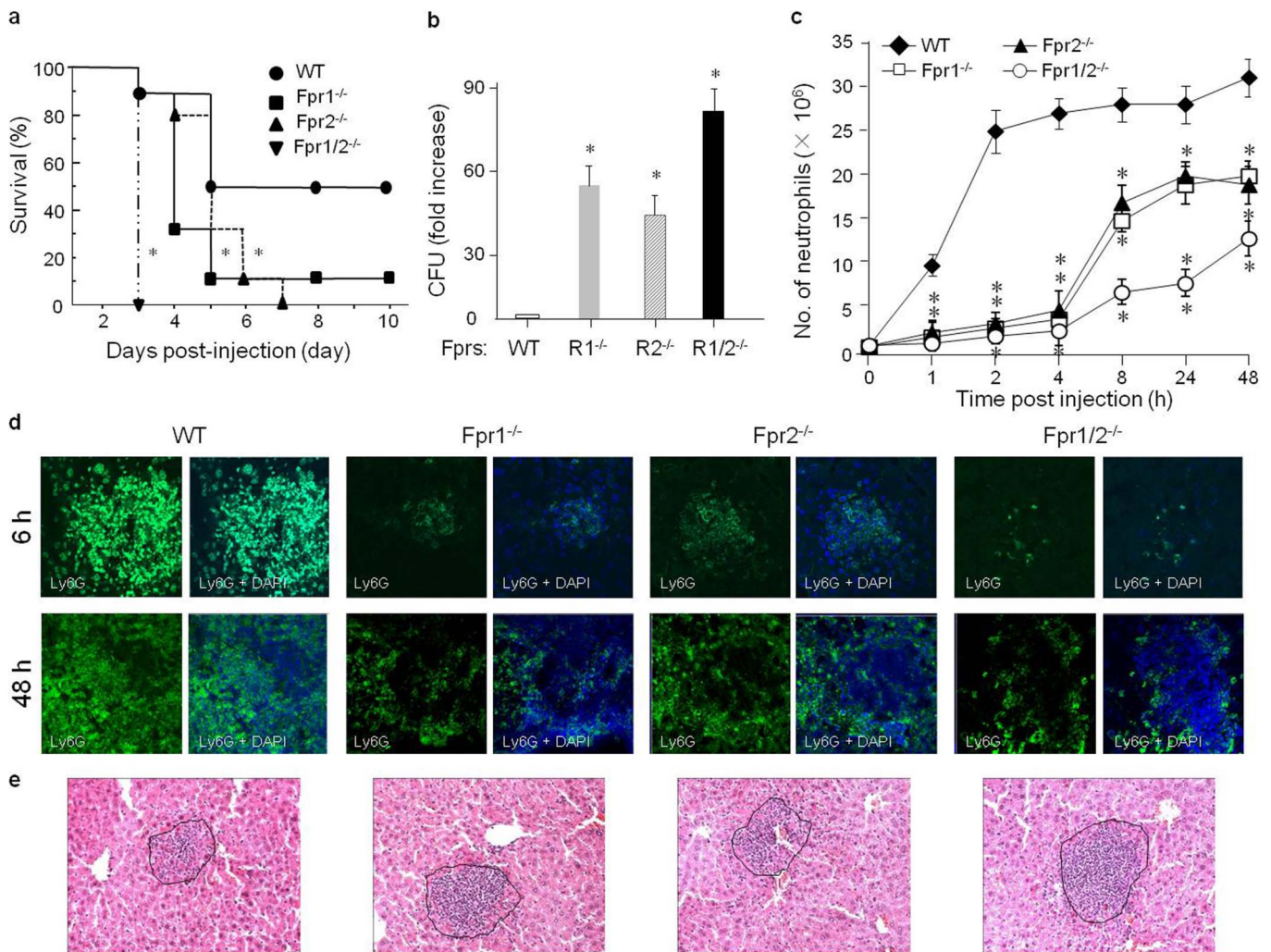
Published  
8 November 2012

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***Listeria monocytogenes* (*Listeria*) causes opportunistic infection in immunocompromised hosts with high mortality. Resistance to *Listeria* depends on immune responses and recruitment of neutrophils of the immune system into infected sites is an early and critical step. Mouse neutrophils express two G protein-coupled formylpeptide receptor subtypes Fpr1 and Fpr2 that recognize bacterial and host-derived chemotactic molecules including *Listeria* peptides for cell migration and activation. Here we report deficiency in Fprs exacerbated the severity of the infection and increased the mortality of infected mice. The mechanism involved impaired early neutrophil recruitment to the liver with Fpr1 and Fpr2 being sole receptors for neutrophils to sense *Listeria* chemoattractant signals and for production of bactericidal superoxide. Thus, Fprs are essential sentinels to guide the first wave of neutrophil infiltration in the liver of *Listeria*-infected mice for effective elimination of the invading pathogen.**

**L**isteria monocytogenes (*Listeria*) is an opportunistic pathogen that causes severe infections in immunocompromised individuals<sup>1</sup>. The incidence of listeriosis in human is 3–5 per million<sup>2,3</sup>, but the lethality rate is as high as 30% in infected patients<sup>4,5</sup>. *Listeria* enter a variety of mammalian cells where the bacteria replicate and spread from one cell to the next to escape host immune surveillance<sup>6–13</sup>. The resistance to *Listeria* infection is dependent on mobilization of immune responses. Recruitment of phagocytes, in particular neutrophils, into the infected site is the first and key step of host defense<sup>9,14,15</sup>.

During bacterial infection, pattern recognition receptors (PRRs) such as TLRs on innate immune cells recognize pathogen-derived danger signals and initiate anti-bacterial host responses, characterized by the accumulation of neutrophils and their release of reactive oxygen species (ROS) and proteolytic enzymes for pathogen clearance<sup>16,17</sup>. In inflammatory responses, the recruitment of neutrophils is mediated by G-protein coupled receptors (GPCR), including formylated peptide receptors (FPRs), which also exhibit PRR properties by sensing a plethora of pathogen- and host-derived chemotactic and activating molecular patterns<sup>18</sup>. FPRs are expressed at high levels on neutrophils. Human FPR1 and FPR2, as well as their mouse counterparts Fpr1 and Fpr2, share a number of chemotactic ligands, including mitochondrial peptides and peptides derived from some bacterial species, such as *Listeria* and *Staphylococcus aureus*<sup>19</sup>. Activation of FPRs by their agonist peptides elicits a signaling cascade that culminates in neutrophil migration, increased phagocytosis and release of superoxide. In *Listeria* infection, while the PRR TLR2 has been reported as a mediator of host resistance by activating inflammasome pathways in immune cells<sup>20</sup>, mice deficient in Fpr1 (Fpr1<sup>-/-</sup>) also were more susceptible<sup>21</sup>, albeit with unclear role in phagocyte recruitment at the site of bacterial infection. On the other hand, although Fpr2 has recently been implicated in sustaining innate and adaptive host immune responses<sup>22</sup>, whether it also participates in host defense against *Listeria* is unknown. In this study, we examined the mechanistic basis for Fpr1 to confer anti-*Listeria* host defense and the potential participation by Fpr2. Here we report that Fpr1 and Fpr2 are sole sensors of the neutrophil chemotactic activity of *Listeria* components and are critical for the early wave of neutrophil accumulation in infected mouse liver required for elimination of invading pathogen.



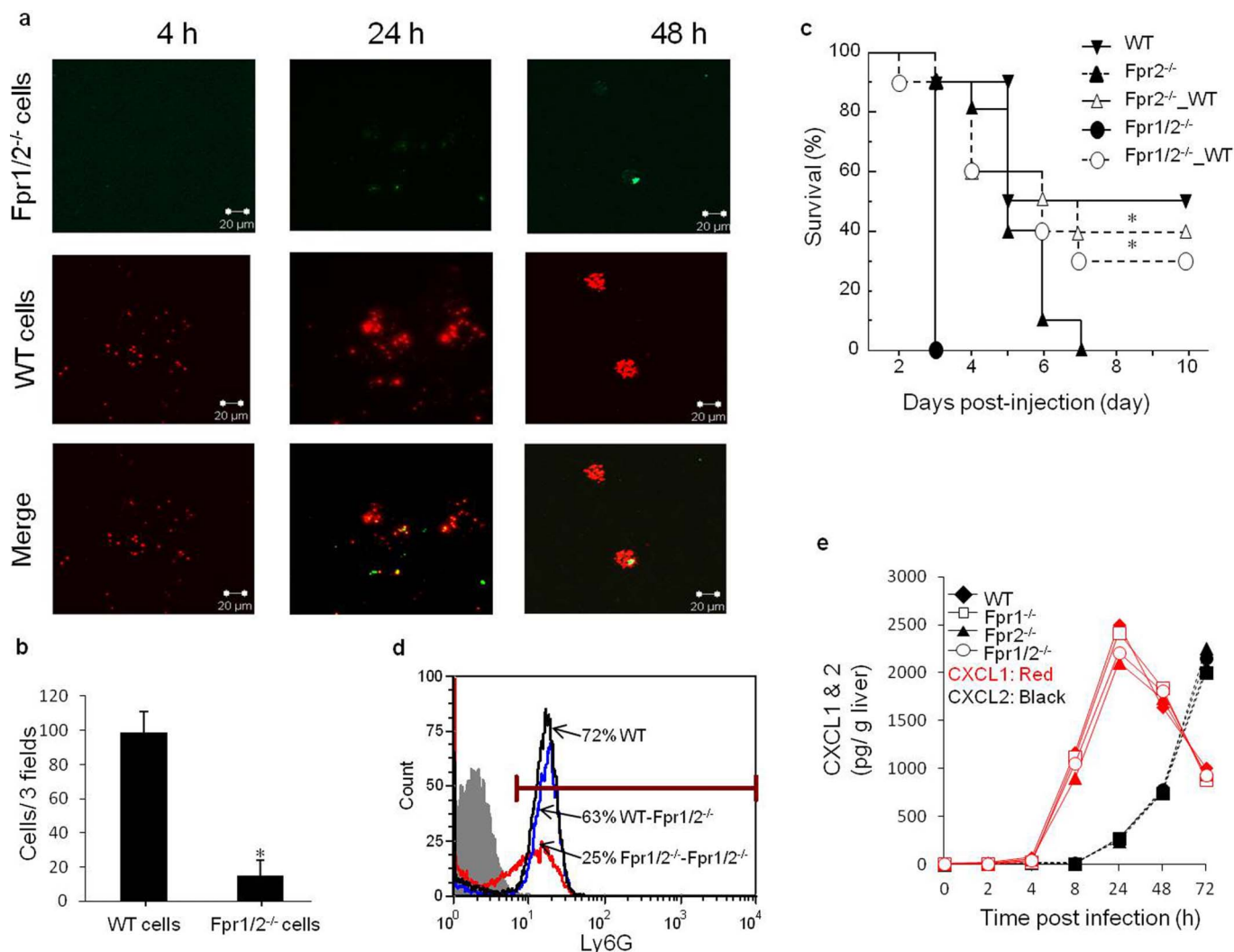
**Figure 1 | Increased susceptibility and *Listeria* load in Fpr-deficient mice.** (a) The survival of mice post *Listeria* infection. Mice were *i.v.* injected with  $2 \times 10^4$  *Listeria* in 100  $\mu$ l DPBS and observed for up to 10 days. Results shown are the means of three experiments. \* significantly reduced survival of Fpr-deficient mice compared with WT littermates,  $p = 0.031$ .  $n = 8$  mice for each group in each experiment. (b) *Listeria* load in the liver. Mouse livers were harvested 3 d after infection and homogenized in DPBS. The tissue suspension was diluted, inoculated and incubated in agar plates at 37°C for 24 h. The bacterial colony forming units (CFUs) were counted.  $n = 3-4$  mice per group in each experiment. \* significantly increased *Listeria* CFUs formed by liver lysates from Fpr-deficient mice compared with WT mice ( $p = 0.008$ ). Data are the mean  $\pm$  SD from a representative experiment out of three performed. (c) Neutrophils in the liver of *Listeria*-infected mice. Mice were *i.v.* injected with  $2 \times 10^4$  *Listeria* in 100  $\mu$ l DPBS. Neutrophils in the liver were purified and analyzed with flow cytometry at different time points.  $n = 5$  mice per group in each experiment. \* significantly decreased neutrophils in the liver of Fpr-deficient mice at all measurement time points as compared with WT mice ( $p = 0.006$ ). (d) Immunofluorescence staining of infiltrating neutrophils. The livers of mice were cryosectioned and stained with Ly6G (Green) and DAPI (Blue) 6 h and 48 h after *Listeria* infection (400 $\times$ ). (e) Abscess formation in the liver of *Listeria*-infected mice. Mice were injected with  $2 \times 10^4$  *Listeria* and the livers were harvested at 48 h. Paraffin liver sections (5  $\mu$ m) were stained with H&E. Marked areas delineate the edges of abscesses (200 $\times$ ).

## Results

**Fpr-deficiency impairs host resistance to *Listeria* infection.** Firstly, we confirmed increased susceptibility of Fpr1<sup>-/-</sup> mice to *Listeria* with a 90% death rate at day 7 after intravenous infection with a bacterial dose causing 50% death in wild type (WT) mice at day 10. Infection with the same *Listeria* dose resulted in 100% death in Fpr2<sup>-/-</sup> mice at day 7. All mice deficient in both Fprs (Fpr1/2<sup>-/-</sup>) were dead by day 3 after infection (Fig. 1a). The *Listeria* load in the liver was 50-, 40- and 80-fold higher in Fpr1<sup>-/-</sup>, Fpr2<sup>-/-</sup> and Fpr1/2<sup>-/-</sup> mice than in WT mice (Fig. 1b). Thus, Fprs cooperatively confer mice with anti-*Listeria* resistance. We also performed sub-lethal *Listeria* dose experiments. At a *Listeria* dose ( $1 \times 10^4$ ) that did not cause any death in WT mice, Fpr1/2<sup>-/-</sup> mice showed a 50% death rate at day 7.

**Fprs are responsible for the rapid neutrophil infiltration of infected liver.** In investigating the mechanisms involved in Fpr-mediated

anti-*Listeria* resistance, we detected a rapid wave of neutrophil accumulation in the WT mouse liver, initiating at 30 min and peaking at 4 h post infection (Fig. 1c). In contrast, in the liver of Fpr single- or double-deficient mice, neutrophil accumulation was markedly reduced. Despite a subsequent slow increase of neutrophils in the liver of Fpr-deficient mice up to 48 h, the cell number remained significantly lower than in WT mice (Fig. 1c and d). Histological examination revealed increased abscess formation in the liver of Fpr-deficient mice with substantially reduced neutrophils surrounding the core of injured hepatocytes (Fig. 1d and e). Competitive repopulation of neutrophils in *Listeria*-infected Fpr1/2<sup>-/-</sup> mice showed greatly increased WT cells infiltrating the infected liver (Fig. 2a and b). Merged images in the lower panels of Fig. 2a clearly demonstrate a predominant repopulation of WT over Fpr1/2<sup>-/-</sup> mouse neutrophils in the infected liver. In addition, transplantation of WT mouse bone marrow markedly increased the survival rate of Fpr2<sup>-/-</sup> and



**Figure 2 | Competitive repopulation of neutrophils and chemokine production in the liver of *Listeria*-infected mice.** (a) Competitive repopulation of WT and Fpr-deficient mouse neutrophils in *Listeria*-infected Fpr1/2<sup>-/-</sup> mice. Bone marrow cells ( $1 \times 10^7$ ) from WT (Red) and Fpr1/2<sup>-/-</sup> mice (Green) were labeled and mixed at ratio 1: 1 then were *i.v.* injected into Fpr1/2<sup>-/-</sup> mice immediately after *Listeria* infection. The livers were harvested and analyzed by immunofluorescence microscopy at 4, 24 and 48 h.  $n = 10$  (400 $\times$ ). (b) Quantification of infiltrating cells in the Fpr1/2<sup>-/-</sup> mouse liver at 4 h. Fluorescence stained cells in 3 high powered fields (400 $\times$ ) in liver sections were counted with Image J. \* significantly decreased repopulation of Fpr1/2<sup>-/-</sup> mouse cells (green) as compared with WT mouse cells (red) ( $p = 0.0004$ ). (c) Survival rate of Fpr-deficient mice receiving transfer of WT mouse bone marrow. Fpr2<sup>-/-</sup> and Fpr1/2<sup>-/-</sup> mice were irradiated and transferred with  $1 \times 10^7$  WT mouse bone marrow cells. All recipient mice were then *i.v.* injected with  $2 \times 10^4$  *Listeria*. \* significantly increased survival rate of Fpr-deficient mice receiving WT mouse bone marrow (Fpr2<sup>-/-</sup>\_WT-BM, and Fpr1/2<sup>-/-</sup>\_WT-BM) as compared with mice without WT bone marrow transfer (Fpr2<sup>-/-</sup> and Fpr1/2<sup>-/-</sup>) ( $p = 0.001$ ).  $n = 10$  mice per group in each experiment. (d) Restoration of Ly6G<sup>+</sup> cell infiltration in the liver Fpr1/2<sup>-/-</sup> mice after transfer of WT mouse bone marrow cells. Myeloid cells purified from infected mice and labeled with CD45, CD11b and Ly6G. The percentage of Ly6G<sup>+</sup> cells in CD45<sup>+</sup>CD11b<sup>+</sup> cells was analyzed. Grey areas: isotype control; Black line: cells from infected WT mouse liver; Blue: cells from the liver of infected Fpr1/2<sup>-/-</sup> mice receiving bone marrow cells from WT mice; Red line: cells from the liver of infected Fpr1/2<sup>-/-</sup> mice receiving bone marrow cells from Fpr1/2<sup>-/-</sup> mice. (e) CXCL1 and CXCL2 production in the liver of *Listeria*-infected mice. The livers of *Listeria*-infected mice were homogenized. CXCL1 and CXCL2 in the supernatant were measured with ELISA.  $n = 5$  mice per group in each experiment.

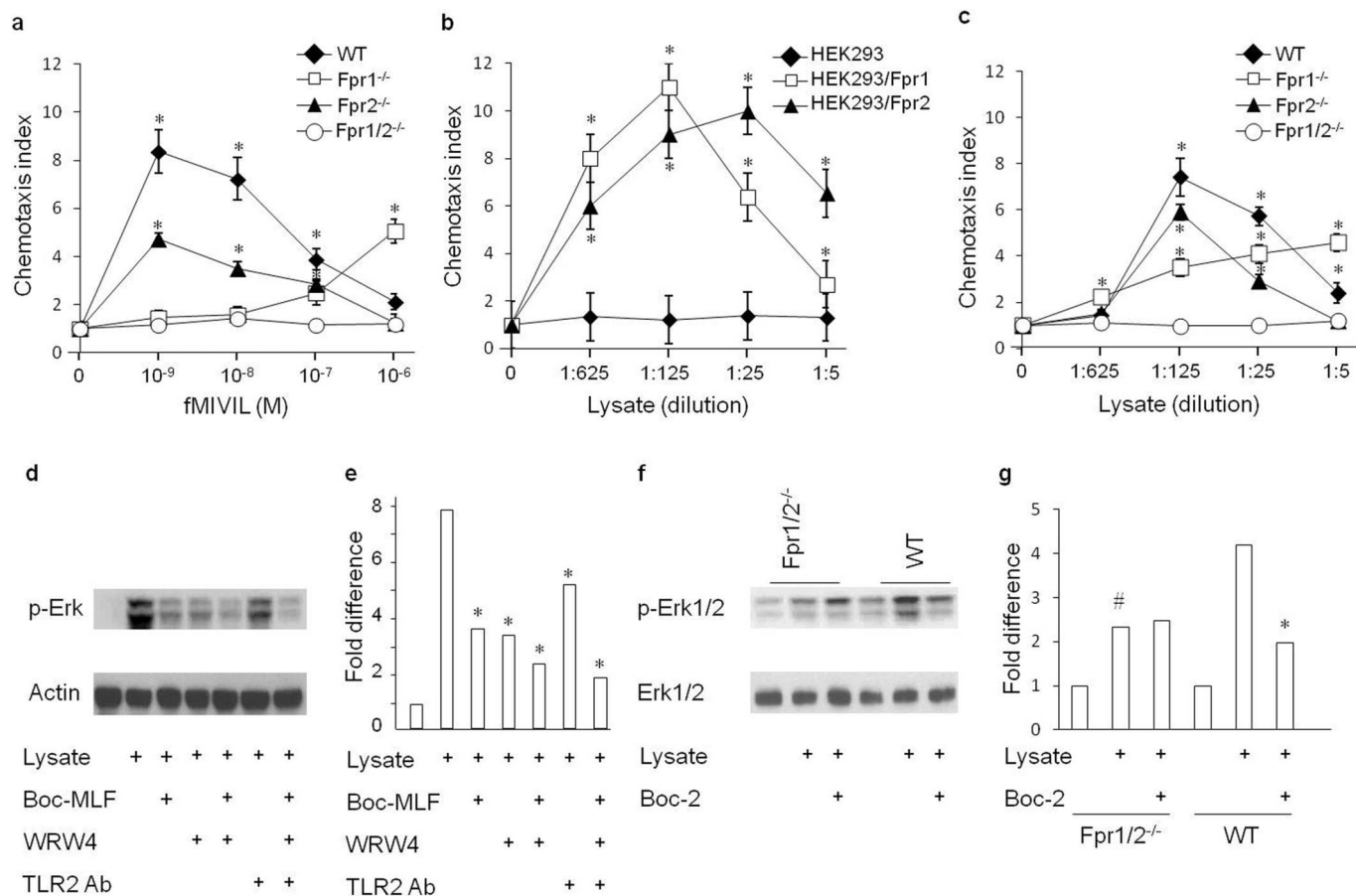
Fpr1/2<sup>-/-</sup> mice from 0 at day 10 post infection to 50% and 40%, respectively (Fig. 2c), with substantial restoration of neutrophil infiltration in the liver (Fig. 2d). These results indicate the requirement for Fprs in neutrophil accumulation in the liver and in host resistance to *Listeria* infection. We also observed similar defects of rapid neutrophil infiltration in the spleen in Fpr deficient mice after infection.

We then examined the nature of chemoattractants responsible for neutrophil infiltration in the liver. In the liver of WT mice, despite rapid infiltration of neutrophils, the production of neutrophil specific chemokines CXCL1 and CXCL2 was not detectable at 8 h and 24 h post infection, respectively (Fig. 2e). There was no difference in

CXCL1 and CXCL2 levels in the infected liver of Fpr-deficient mice and WT mice. Therefore, the “first wave” neutrophil infiltration in the liver of *Listeria*-infected WT mice is not dependent on CXCL1 and CXCL2, but rather, Fpr ligands are likely responsible.

#### Fprs are sole receptors for *Listeria*-derived chemotactic signals.

To test this possibility, we found that Fpr1<sup>-/-</sup> or Fpr2<sup>-/-</sup> mouse neutrophils exhibited decreased chemotaxis to a *Listeria* peptide (Fig. 3a) that was reported to activate both Fprs<sup>21</sup>. Fpr1/2<sup>-/-</sup> mouse neutrophils failed to respond to the peptide. However, Fpr-deficient mouse neutrophils retained normal chemotaxis induced by ligands using other GPCRs (Supplementary Fig. 1). In addition,



**Figure 3 | The neutrophil chemotactic and activating effects of *Listeria* products.** (a) Chemotactic activity of *Listeria*-derived peptide fMIVIL for mouse neutrophils. \* significantly increased cell migration in response to the peptide as compared with medium control (0) ( $p = 0.0007$ ). (b) Chemotactic activity of *Listeria* lysate for HEK293 cells transfected with Fprs. \* significantly increased cell chemotaxis in response to *Listeria* lysate as compared with medium control (0) ( $p = 0.004$ ). (c) Migration of mouse neutrophils to *Listeria* lysate. \* significantly increased chemotaxis response of neutrophils to lysates as compared with medium control (0) ( $p = 0.003$ ). (d) *Listeria* lysate-induced phosphorylation of Erk1/2 in WT mouse neutrophils in the presence or absence of Fpr antagonists and a TLR2 neutralizing antibody. (e) Semiquantitative analysis of phosphorylated Erk1/2. \* significantly decreased Erk1/2 phosphorylation compared with cells stimulated with *Listeria* lysate at 1:10 dilution in the absence of Fpr antagonists or TLR2 antibody. Results are from 1 experiment out of 3 performed. (f) Induction of Erk1/2 phosphorylation in WT and Fpr1/2<sup>-/-</sup> mouse neutrophils by *Listeria* lysate (at 1:10 dilution) in the presence or absence of an Fpr antagonist Boc-2. (g) Semiquantitative analysis of phosphorylated Erk1/2. # significantly reduced Erk1/2 phosphorylation in Fpr1/2<sup>-/-</sup> mouse neutrophils as compared with WT mouse neutrophils. \* significantly decreased Erk1/2 phosphorylation in WT mouse neutrophils stimulated with *Listeria* lysate in the presence of Fpr antagonist Boc-2 as compared with cells stimulated with *Listeria* lysate alone ( $p = 0.001$ ).

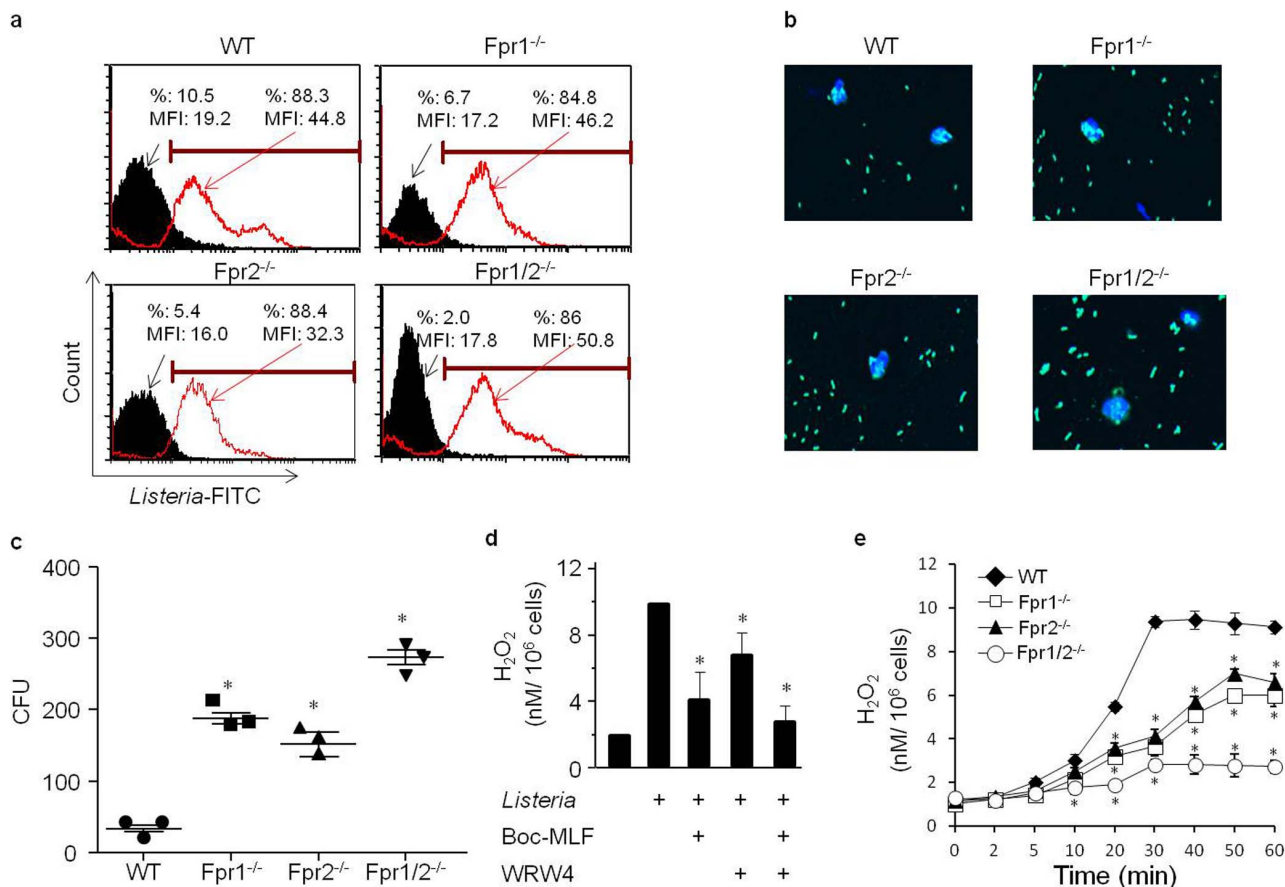
*Listeria* lysate induced migration of HEK293 cells transfected to express Fprs, but not the parental HEK293 cells (Fig. 3b). WT mouse neutrophils also migrated potently to *Listeria* lysate (Fig. 3c). In contrast, Fpr1<sup>-/-</sup> or Fpr2<sup>-/-</sup> mouse cells showed reduced chemotaxis to *Listeria* lysate, with complete absence of response of Fpr1/2<sup>-/-</sup> mouse cells. The requirement of Fprs by *Listeria* to induce neutrophil chemotaxis was supported by decreased neutrophil exudation elicited by bacteria injected into the peritoneal cavity of Fpr1<sup>-/-</sup> or Fpr2<sup>-/-</sup> mice, with no neutrophil exudation in Fpr1/2<sup>-/-</sup> mice (Supplementary Fig. 2). These observations confirm that Fpr1 and Fpr2 are sole receptors on neutrophils to recognize *Listeria*-produced chemotactic signals.

In addition to chemotaxis, Fprs also mediate neutrophil activation by *Listeria* lysate as shown by increased phosphorylation of extracellular regulatory kinase (Erk) 1/2 in WT mouse neutrophils, which was reduced by Fpr1 or Fpr2 antagonist (Fig. 3d and e). A TLR2 antibody also partially inhibited *Listeria* lysate-induced Erk1/2 phosphorylation in WT mouse neutrophils. Combination of three inhibitors completely abrogated Erk1/2 phosphorylation. In neutrophils from Fpr1/2<sup>-/-</sup> mice, *Listeria*

lysate-induced Erk1/2 phosphorylation was diminished (Fig. 3f and g). These results indicate that *Listeria* lysate activates Fprs and TLR2 in neutrophils. However, TLR2 does not mediate neutrophil chemotaxis in response to *Listeria* components therefore its effect on neutrophil recruitment is indirect.

#### Fprs mediate H<sub>2</sub>O<sub>2</sub>-dependent *Listeria* killing by neutrophils.

Since neutrophils are major effectors for *Listeria* clearance, we examined *Listeria* phagocytosis and killing capabilities of neutrophils from Fpr-deficient mice. There was no difference in phagocytosis of both live and heat-inactivated bacteria by neutrophils from WT and Fpr-deficient mice (Fig. 4a and b). However, the killing of *Listeria* by Fpr1<sup>-/-</sup> and Fpr2<sup>-/-</sup> neutrophils was considerably reduced with even greater reduction in killing by Fpr1/2<sup>-/-</sup> neutrophils (Fig. 4c). Therefore, *Listeria* phagocytosis by neutrophils did not require Fprs. Instead, Fprs were required for *Listeria* killing as shown by increased bacterial colonies released from Fpr-deficient neutrophils previously exposed to the bacteria. The *Listeria* killing capacity of neutrophils was correlated with their H<sub>2</sub>O<sub>2</sub> production in response to heat-inactivated bacteria. In WT neutrophils, *Listeria*-induced H<sub>2</sub>O<sub>2</sub> production was



**Figure 4** | *Listeria* phagocytosis, killing and *Listeria*-induced H<sub>2</sub>O<sub>2</sub> production by neutrophils. (a) *Listeria* phagocytosis by mouse neutrophils. Neutrophils were incubated with 100-fold heat-inactivated *Listeria* for 1 h at 37°C then were stained with anti-Ly6G and anti-*Listeria* (ANTIBODY-ONLINE.com, Cat#ABIN576776) antibodies. The cells were then analyzed for % positivity and mean fluorescence index (MFI) with flow cytometry. Cells with *Listeria* on ice were used as control (Black areas: neutrophils with *Listeria* on ice; Red lines: neutrophils with phagocytosed *Listeria*). (b) Immunofluorescence of *Listeria* phagocytosed by mouse neutrophils. Neutrophils were attached to chamber slides. *Listeria* were pre-treated with anti-*Listeria* antibody for 30 min and then incubated with neutrophils for 1 h followed by analysis with immunofluorescence confocal microscopy. Neutrophil nuclei were counterstained with DAPI (Green: *Listeria*; Blue: DAPI). (c) *Listeria* killing capacity of neutrophils. Mouse neutrophils were incubated with 100-fold live *Listeria* for 1 h at 37°C and non-phagocytosed bacteria were removed by washing. Permeabilized neutrophils were inoculated on agar plates and *Listeria* CFUs released by neutrophils were counted after 24 h. \* significantly increased CFUs released by Fpr-deficient mouse neutrophils as compared with WT mouse cells ( $p = 0.006$ ). (d) *Listeria*-induced H<sub>2</sub>O<sub>2</sub> production by WT mouse neutrophils. Neutrophils ( $5 \times 10^6$  cells) from WT mice were primed with 1 ng/ml GM-CSF for 60 minutes then were incubated with an Fpr1-specific antagonist Boc-MLF (1  $\mu$ M, 10 min), an Fpr2 specific antagonist WRW4 (2  $\mu$ M, 10 min) or a TLR2 neutralizing antibody (100 ng/ml, 30 min) followed by stimulation with 100-fold heat-inactivated *Listeria* for 30 min at 37°C. \* significantly decreased H<sub>2</sub>O<sub>2</sub> production by neutrophils pretreated with Fpr antagonists or anti-TLR2 antibody as compared with neutrophils treated with inactivated *Listeria* only ( $p = 0.001$ ). (e) Neutrophil production of H<sub>2</sub>O<sub>2</sub> in response to heat-inactivated *Listeria*. Neutrophils ( $5 \times 10^6$ ) primed with 1 ng/ml GM-CSF for 60 minutes were incubated with 100-fold heat-inactivated *Listeria*. H<sub>2</sub>O<sub>2</sub> production was measured at indicated time points. \* significantly decreased H<sub>2</sub>O<sub>2</sub> production by Fpr-deficient mouse neutrophils as compared with WT mouse neutrophils ( $p = 0.007$ ,  $n = 3$ ).

partially inhibited by Fpr1 or Fpr2 antagonist, with further reduction by combination of two antagonists (Fig. 4d). In Fpr-deficient mice, absence of a single Fpr substantially reduced neutrophil H<sub>2</sub>O<sub>2</sub> production induced by *Listeria*, with complete loss of production by Fpr1/2<sup>-/-</sup> cells (Fig. 4e). In support of the specificity of Fprs in *Listeria*-stimulated H<sub>2</sub>O<sub>2</sub> production, WT and Fpr-deficient neutrophils responded equally to phorbol ester (PMA), which was not inhibited by Fpr antagonists (Supplementary Fig. 3). Therefore, Fpr-mediated H<sub>2</sub>O<sub>2</sub> production by neutrophils is directly correlated with their bacterial killing capabilities. The importance of H<sub>2</sub>O<sub>2</sub> in *Listeria* resistance was supported by increased susceptibility to infection of mice lacking a superoxide metabolism enzyme NADPH oxidase<sup>23</sup>.

## Discussion

In addition to PRRs including TLRs, FPRs have emerged as a novel set of PRR-like molecules that directly interact with pathogen and host derived chemotactic molecules<sup>18,24</sup>. The importance of FPRs in

host defense against bacterial infections is clearly illustrated in our studies in which Fpr1 and Fpr2 both contribute to the accumulation of neutrophils at the site of infection, the production of superoxide and the elimination of the bacteria by rapidly responding to the chemotactic agonists released by the bacteria. Although the chemokines CXCL1 and CXCL2 have been reported to induce neutrophil infiltration at the sites of *Listeria* infection following TLR2-mediated pro-inflammatory cascade<sup>25-32</sup>, we demonstrated that in normal mice, neutrophil accumulation in the liver of *Listeria*-infected mice initiates within 30 min and reaches the highest level at 4 h. However, CXCL1 and 2 were produced in a time frame far behind the rapid phase neutrophil accumulation. In contrast, in Fpr deficient mice, although the production of CXCL1 and CXCL2 in the infected liver showed kinetics and magnitude similar to the liver of WT mice, there was a markedly reduced early phase neutrophil accumulation. Therefore, Fprs are critical for the rapid recruitment of neutrophils in *Listeria* infected liver, which is critical for elimination of the invading pathogen.



It remains unclear whether Fpr deficiency may have caused intrinsic defects in the overall responsiveness of neutrophils to *Listeria* infection. However, several lines of evidence obtained in our study showed a minimal impact of Fpr deletion on the overall viability and function of neutrophils. For instance, neutrophils from Fpr1/2<sup>-/-</sup> mice showed normal chemotaxis responses to chemoattractants that do not use Fprs (Supplementary Figure 1); neutrophils from Fpr1/2<sup>-/-</sup> mice showed normal H<sub>2</sub>O<sub>2</sub> production in response to PMA; and neutrophils from Fpr deficient mice showed phagocytosis comparable to the cells from WT mice. However, further study is undergoing to more fully evaluate the neutrophil responses in additional models of inflammatory and infectious diseases.

It is interesting to note that in a model of sterile injury in the liver, while an intravascular gradient of the chemokine CXCL2 mediates neutrophil accumulation proximal to the border of necrotic tissue, the directional cell migration into the core of the lesion is dependent on Fpr1<sup>33</sup>, presumably in response to agonists released by damaged tissue. This relay of chemotactic signals by different GPCRs guiding neutrophil infiltration into the inflammatory lesion constitutes a finely tuned innate host response during injurious insult<sup>33</sup>. On the other hand, Fpr2 also plays an important role in mediating leukocyte recruitment in vivo as shown in our previous study in which Fpr2<sup>-/-</sup> mice manifested markedly reduced severity of allergic inflammation due to impaired dendritic cell infiltration into the inflamed airway and draining lymph nodes, possibly mediated by Fpr2 agonists present in the airway tissue<sup>22</sup>. In our present study, Fprs on neutrophils clearly override chemokine GPCRs in directly sensing the *Listeria* chemotactic signals to rapidly induce neutrophil recruitment into the infected liver, which should be beneficial for subsequent interaction of the bacteria components with TLR2 to amplify anti-bacteria responses by inducing pro-inflammatory cytokines and chemokines. Thus, Fprs play differential roles in bacterial infection and tissue injury in the sequence of neutrophil recruitment but they are both essential as the first line host defense.

It is worth noting that Fprs are expressed by cell types other than neutrophils, such as monocytes/macrophages. Our results did not exclude the involvement of other cell types in host defense, because monocytes or macrophages of our Fpr deficient mice also no longer express these receptors that sense *Listeria* components. However, our observations do demonstrate that neutrophils are the inflammatory cell type that rapidly infiltrate *Listeria* infected mouse liver, which antecede monocyte/macrophage accumulation. In addition, lacking the rapid infiltration of neutrophils in Fpr deficient mice did exacerbate the mortality of the infected mice. We also detected a much slower (maximal at 48 h after infection) and comparable increase in monocyte/macrophage infiltration in the liver of both *Listeria*-infected WT mice and all strains of Fpr deficient mice. In addition, the monocyte/macrophage infiltration was correlated with an increase in the chemokine CCL2 in kinetics behind CXCL1 and CXCL2 production (data not shown). We therefore believe neutrophils are important, at least for the initial stage of host responses to *Listeria*, which requires rapid neutrophil mobilization. Despite our observation of a clear role of neutrophils in anti-*Listeria* host defense, a recent study suggested that in systemic infection, inflammatory monocytes are critical while neutrophils are dispensable<sup>34</sup>. The reasons for the discrepancy in the role of neutrophil in *Listeria* infection remain to be clarified in further studies. We however noticed that in the above mentioned study, the neutrophil depleting antibody was given upon infection, not prior to infection, and the earliest data presented were from 24 h after infection. Therefore, the possibility may exist that some neutrophils were not eliminated at a very early stage of infection. Given the fact that in our study and those of the others<sup>1,35,36</sup>, neutrophils accumulate at infection foci within hours, it is plausible that neutrophils and monocytes play a sequential role in host defense against *Listeria* infection.

## Methods

**Animals and reagents.** Mouse strains deficient in Fpr1 (mFPR1<sup>-/-</sup>) or Fpr2 (mFPR2<sup>-/-</sup>) were generated as described<sup>21,22</sup>. Fpr1/2 double deficient mice were generated by replacing the 7 kb fragment containing exon 1 of the Fpr1 gene, the promoter regions of Fpr1 and 2 genes with a neo gene cassette in order to construct a targeting vector. The neo gene was subsequently deleted by crossing with β-actin Cre transgenic mice (Yoshimura et al, unpublished observation). Mice were crossed to C57/B6 background for at least 8 generations before use. All mice were housed in the animal facility at Frederick National Laboratory for Cancer Research and were used at 8–12 week of age.

Mouse experiments were approved by Animal Care and Use committee of National Cancer Institute at Frederick and performed in accordance with the procedures outlined in the “Guide for Care and Use of Laboratory Animals” (National Research Council; 1996; National Academy Press, Washington D.C.).

Formyl-Met-Ile-Val-Ile-Leu (fMIVIL) from *Listeria* and Formyl-Met-Ile-Phe-Leu (fMIFL) from *Staphylococcus* were kind gifts from Dr. R. D. Ye (University of Illinois at Chicago). The other reagents and sources were: Rat anti-mouse Ly6G, F4/80, goat anti-rat serum antibodies, mouse CXCL1 and CXCL2 ELISA kits (eBioscience, San Diego, CA); 4,6-diamidino-2-phenylindole (DAPI, Molecular Probe, Eugene, OR); Alexa Fluora 488-labelled goat anti-rat IgG, ProLong antifade reagent, amplex red hydrogen peroxide/peroxidase assay kit, Qtracker<sup>®</sup> Cell Labeling Kits (Invitrogen, Eugene, OR); rat anti-mouse Erk1/2, rat anti-mouse phospho-Erk1/2, rat anti-mouse p38, rat anti-mouse phospho-p38, goat anti-rat HRP-IgG antibodies (Cell signaling, Beverly, MA); Boc-MLF, Boc-2, TLR2 antibody, MMK-1, CCL2, CXCL2, phorbol myristate acetate (PMA) (Tocris, Ellisville, MO); Igepal CA-630 nonionic detergent (R&D, Minneapolis, MN); Formyl-Met-Leu-Phe (fMLF), Percoll, Saponin, Casein and Thioglycollate, (Sigma-Aldrich, St. Louis, MO); Brain Heart Infusion Broth and Brain Heart Infusion Agar (BD, Franklin Lakes, New Jersey); FITC-labeled anti-*Listeria* polyclonal antibody (Antibodies-online, Atlanta, GA).

**Infection with *Listeria*.** *Listeria* strain EGD was cultured in Brain Heart Infusion broth and stored in 30% glycerol at -80°C at a concentration of 5 × 10<sup>8</sup> CFU/ml. Heat-inactivated *Listeria* were prepared by incubating the bacteria at 60°C for 1 h. Bacteria lysate was obtained from heat-inactivated *Listeria*. For infection, male mice were *i.v.* injected with 100 μl *Listeria* suspension via the tail vein. An LD50 of *Listeria* determined in wild type mice was 2 × 10<sup>4</sup>. To examine bacterial load, mice were euthanized by CO<sub>2</sub> inhalation 48 h after infection. The mouse liver was removed and homogenized in distilled water with 0.01% Triton X-100, and the number of viable *Listeria* was determined by plating serial dilutions of organ homogenates on Brain-Heart Infusion agar after overnight growth at 37°C. To examine the animal survival, mice injected *i.v.* with 2 × 10<sup>4</sup> or 1 × 10<sup>4</sup> CFU *Listeria* were monitored for up to 10 days.

**Myeloid cell infiltration in the liver and peritoneal cavity.** Mice injected *i.v.* with *Listeria* were euthanized at different time points. Mouse liver and spleen were harvested and homogenized. Following lysis of red blood cells, myeloid cells were purified with Percoll gradient centrifugation. Cells were counted and labeled with F4/80 or Ly6G and analyzed with flow cytometry. Immunofluorescence microscopy was used to determine the infiltration of myeloid cells in the liver tissue sections. Heat-inactivated *Listeria* (5 × 10<sup>5</sup> in 100 μl PBS) were also injected into the peritoneal cavity to elicit exuding neutrophils. Mice were euthanized 3 h after injection and peritoneal cells were collected and neutrophils were counted by flow cytometry.

**Isolation of mouse neutrophils and macrophages.** Donor mice were *i.p.* injected with 1 ml 9% Casein. After 12 h, each mouse received a second *i.p.* injection of 1 ml Casein solution for an additional 3 h. The peritoneal cavity of CO<sub>2</sub> euthanized mice was then flushed with 3 ml DPBS/mouse and exuding cells, which contained > 90% neutrophils were collected. For collecting peritoneal macrophages, donor mice were *i.p.* injected with 1 ml 3% thioglycollate. After 72 h, mice were euthanized with CO<sub>2</sub> and the peritoneal cavity was flushed with DPBS to collect exuding macrophages with the purity of > 90%.

**Chemotaxis.** The chemotaxis of phagocytes and HEK293 cells was analyzed using polycarbonate membranes with 5-μm (phagocytes) or 8-μm pore size (HEK293 cells) in 48-well chambers (NeuroProbe, Gaithersburg, MD). An aliquot of 29 μl chemoattractants was placed in the lower wells of the chamber, and 50 μl of cells (1.5 × 10<sup>6</sup>/ml) suspended in RPMI 1640 with 0.5% BSA were placed in the upper wells. After incubation (45 min for neutrophils, 90 min for monocytes and 240 min for HEK293 cells) at 37°C, the membranes were removed, rinsed with PBS, fixed, and stained with Diff-Quick. Migrated cells were counted in 3 random fields at 400 magnification under light microscopy. The results are expressed as the mean ± SEM of the chemotaxis index (CI), representing the fold increase in the number of migrated cells in response to chemoattractants over spontaneous cell migration (to control medium).

***Listeria* killing by neutrophils.** Mouse neutrophils (1 × 10<sup>4</sup>) were incubated with 100 fold *Listeria* for 1 h at 37°C. Non-phagocytosed bacteria were removed by washing. Permeabilized neutrophils were diluted and inoculated on Agar plates for incubation at 30°C. Colony forming units (CFUs) were counted after 24 h<sup>20</sup>.

**H<sub>2</sub>O<sub>2</sub> production by neutrophils.** Mouse neutrophils (5 × 10<sup>6</sup> cells) were incubated in 1 ml RPMI1640 medium with 10% FCS. After priming with 1 ng/ml GM-CSF for



60 minutes, 100 fold heat-inactivated *Listeria* or PMA (100 ng/ml, 15 min) were added into the cells for 30 min and H<sub>2</sub>O<sub>2</sub> was measured by spectroscopy (FluoStar Omega, BMG Labtech, Ortenberg, Germany) at 550 nm. H<sub>2</sub>O<sub>2</sub> was expressed in nanomoles of O<sub>2</sub><sup>-</sup> produced by 1 × 10<sup>6</sup> cells<sup>37</sup>. To examine the contribution of Fprs in neutrophil production of H<sub>2</sub>O<sub>2</sub> in response to *Listeria*, an Fpr1 specific antagonist Boc-MLF (1 μM, 10 min), an Fpr2 specific antagonist WRW4 (2 μM, 10 min) and a TLR2 neutralizing antibody (100 ng/ml, 30 min) were incubated with neutrophils prior to the addition of *Listeria*.

**Phagocytosis of *Listeria* by neutrophils.** Mouse neutrophils (1 × 10<sup>6</sup>) were incubated in RPMI1640 medium in the presence of 100 fold heat-inactivated *Listeria* for 1 h at 37°C. Neutrophils incubated on ice in the presence of *Listeria* were used as controls. After removal of non-phagocytosed bacteria by washing, the cells were permeabilized with 10% Saponin for 10 min at room temperature and stained with an anti-*Listeria* antibody for flow cytometry and immunofluorescence microscopy analyses.

**Chemokine production.** Mouse livers were weighed and 2 ml of ice-cold endotoxin-free PBS containing 0.1% Igepal CA-630 nonionic detergent were added to the tissues for 10 minutes before homogenization. The tissues were centrifuged at 12000 rpm for 5 min and the supernatants were collected for measurement of the chemokines CXCL1 and CXCL2 by ELISA.

**Phosphorylation of Erk1/2 induced by *Listeria* in neutrophils.** Mouse neutrophils (2 × 10<sup>6</sup>) were stimulated with *Listeria* lysate (5 × 10<sup>7</sup> bacteria/ml) for 10 min and the phosphorylated Erk1/2 was detected by Western blotting. Specific antagonists for Fprs and a TLR2 antibody were added 30 min prior to the addition of *Listeria* lysate to neutrophils to test the receptor specificity.

**Competitive repopulation of neutrophils and bone marrow transfer.** Mouse bone marrow cells (1 × 10<sup>7</sup>) were labelled with different colours and *i.v.* injected through tail vein immediately after *Listeria* infection. The sections of livers were observed with immunofluorescence microscopy at 4, 24 and 48 h. The colored spots at 4 h were counted. Fpr-deficient mice were also subjected to 1000 rads of irradiation (from a 137 Cesium Gammacell source) once and *i.v.* injected with bone marrow cells from WT mice (1 × 10<sup>7</sup> cells in 100 μL RPMI1640) 4 h later. All recipient mice were infected with 1 × 10<sup>7</sup> *Listeria* 6 weeks after irradiation and bone marrow transfer to measure the survival rate.

**Statistical analysis.** All experiments were repeated at least three times with reproducible results. Results shown were from representative experiments. Statistical differences among testing and control groups were analysed by Student's *t*-test. A *P* value < 0.05 was considered as statistically significant.

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## Acknowledgement

We thank Drs. J.J. Oppenheim and G. Trinchieri for critically reviewing the manuscript; C. Lamb for secretarial assistance. This project was funded in part by federal funds from the National Cancer Institute, National Institutes of Health, under Contract No. HHSN261200800001E and was supported in part by the Intramural Research Program of the NCI, NIH.

## Author contributions

Liu M. and Wang JM. planned experiments and wrote the main manuscript text. Chen K., Liu Y., Wang A. and Gong W. discussed the experimental findings and interpretation of results; Yoshimura T. generated Fpr1/2<sup>-/-</sup> mice. Gao J. and Murphy P. generated Fpr1<sup>-/-</sup> mice.

## Additional information

**Supplementary information** accompanies this paper at <http://www.nature.com/scientificreports>

**Competing financial interests:** The authors declare no competing financial interests.

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**How to cite this article:** Liu, M. *et al.* Formylpeptide receptors are critical for rapid neutrophil mobilization in host defense against *Listeria monocytogenes*. *Sci. Rep.* **2**, 786; DOI:10.1038/srep00786 (2012).