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

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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.

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*Listeria monocytogenes* (*Listeria*) is an opportunistic pathogen that causes severe infections in immunocompromised individuals¹. The incidence of listeriosis in human is 3–5 per million²,³, but the lethality rate is as high as 30% in infected patients⁴,⁵. *Listeria* enter a variety of mammalian cells where the bacteria replicate and spread from one cell to the next to escape host immune surveillance⁶–¹³. 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 defense⁶,⁷,¹⁴,¹⁵.

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 clearance¹⁶–¹⁷. 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 patterns¹⁸. 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*¹⁹. 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 cells²⁰, mice deficient in Fpr1 (Fpr1−/−) also were more susceptible²¹, 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 responses²², 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.
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 × 10<sup>7</sup>) 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 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 × 10<sup>5</sup> Listeria in 100 μl 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 ± 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 × 10<sup>5</sup> Listeria in 100 μl 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×). (e) Abscess formation in the liver of Listeria-infected mice. Mice were injected with 2 × 10<sup>5</sup> Listeria and the livers were harvested at 48 h. Paraffin liver sections (5 μm) were stained with H&E. Marked areas delineate the edges of abscesses (200 ×).
Fpr1/2−/− 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−/− or Fpr2−/− mouse neutrophils exhibited decreased chemotaxis to a Listeria peptide (Fig. 3a) that was reported to activate both Fprs.21 Fpr1/2−/− 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).
**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 of Fpr antagonist 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^−/− 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^−/− 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^−/− or Fpr2^−/− mouse cells showed reduced chemotaxis to *Listeria* lysate, with complete absence of response of Fpr1^−/− 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^−/− or Fpr2^−/− mice, with no neutrophil exudation in Fpr1^−/− 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^−/− 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 H₂O₂-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^−/− and Fpr2^−/− neutrophils was considerably reduced with even greater reduction in killing by Fpr1^−/−/Fpr2^−/− 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 H₂O₂ production in response to heat-inactivated bacteria. In WT neutrophils, *Listeria*-induced H₂O₂ production was...
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 H2O2 production induced by Listeria, with complete loss of production by Fpr1/2 cells (Fig. 4e). In support of the specificity of Fprs in Listeria-stimulated H2O2 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 H2O2 production by neutrophils is directly correlated with their bacterial killing capabilities. The importance of H2O2 in Listeria resistance was supported by increased susceptibility to infection of mice lacking a superoxide metabolism enzyme NADPH oxidase.

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. 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 proinflammatory cascade, 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−/− mice showed normal chemotaxis responses to chemottractants that do not use Frps (Supplementary Figure 1); neutrophils from Fpr1/2−/− mice showed normal H2O2 production in response to PMA; and neutrophils from Fpr deficient mice showed phagocytosis comparable to the cells from WT mice. However, further study is needed 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, 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 injuries that require fast neutrocytic recruitment in vivo as shown in our previous study in which Fpr2−/− 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. In our present study, Frps 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, Frps 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 Frps 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. 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, 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−/−) or Fpr2 (mFpr2−/−) were generated as described.21,22 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. The neonates and 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. Mice were backcrossed with C57BL/6 for at least 8 generations before use. All animals 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 DC). Rodent isolates of Listeria monocytogenes and Listeria pneumoniae were grown on Brain–Heart Infusion agar at 37°C. 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 (Molecular Probes, Eugene, OR; Eugen, OR); 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); L-Arg CA-630 nonionic detergent (R&D, Minneapolis, MN); Formyl-Met-Leu-Phe (FMLP), Percoll, Saponin, Casein and Trygloycollate, 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 × 109 CFU/ml. Heat-inactivated Listeria was prepared by incubating of the bacteria at 65°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 L50 of Listeria determined in wild type mice was 2 × 10^3. To examine bacterial load, mice were euthanized by CO2 inhalation 48 h after infection. The mouse liver was removed and homogenized in distilled water to 0.01% Triton X-100, and bacterial number was counted. 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 × 10^3 or 1 × 10^4 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. Mice 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 examine the infiltration of myeloid cells in the liver tissue sections. Heat-inactivated Listeria (5 × 10^6 in 100 μl PBS) were also injected into the peritoneal cavity to elicit exuding neutrophils. Mice were euthanized 3 h after infection 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 CO2 euthanized mice was then flushed with 5 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% thioglycolate. After 72 h, mice were euthanized with CO2 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 filtrate from tumor cell suspensions treated with 0.5% BSA was 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 experiments are expressed as the mean ± SEM of the chemotaxis index (CI), representing the fold increase in the number of migrated cells in response to chemotaxants over spontaneous cell migration (to control medium).

#### Listeria killing by neutrophils

Mouse neutrophils (1 × 10^6) 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.

H2O2 production by neutrophils

Mouse neutrophils (5 × 10^5 cells) were incubated in 1 ml RPMI1640 medium with 10% FCS. After priming with 1 μg/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 H2O2 was measured by spectroscopy (FluoStar Omega, BMG Labtech, Ortenberg, Germany) at 550 nm. H2O2 was expressed in nanomoles of O2·− produced by 1×106 cells. To examine the contribution of Fpr1 in neutrophil production of H2O2 in response to Listeria, an Fpr1 specific antagonist Boc-MLF (1 μM, 10 min), an Fpr2 specific antagonist WRF4 (2 μM, 10 min) and a TLR2 specific antagonist anti-TLR2 (100 ng/ml, 30 min) were incubated with neutrophils prior to the addition of Listeria.

Phagocytosis of Listeria by neutrophils. Mouse neutrophils (1×106) 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 (2×106) were stimulated with Listeria lysate (5×108 bacteria/ml) for 10 min and the phosphorylated Erk1/2 was detected by Western blotting. Specific antagonists for Fpr 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 (1×106) were labelled with different colours and i.v. injected through tail veins 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−/− mice immediately 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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Additional information

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