Nonredundant protective properties of FPR2/ALX in polymicrobial murine sepsis

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Sepsis is characterized by overlapping phases of excessive inflammation temporally aligned with an immunosuppressed state, defining a complex clinical scenario that explains the lack of successful therapeutic options. Here we tested whether the formyl-peptide receptor 2/3 (Fpr2/3)—ortholog to human FPR2/ALX (receptor for lipoxin A4)—exerted regulatory and organ-protective functions in experimental sepsis. Coecal ligation and puncture performed to obtain nonlethal polymicrobial sepsis, with animals receiving antibiotics and analgesics. Clinical symptoms, temperature, and heart function were monitored up to 24 h. Peritoneal lavage and plasma samples were analyzed for proinflammatory and proresolving markers of inflammation and organ dysfunction. Compared with wild-type mice, Fpr2/3\textsuperscript{−/−} animals exhibited exacerbaration of disease severity, including hypothermia and cardiac dysfunction. This scenario was paralleled by higher levels of cytokines [CXCL1 (CXC receptor ligand 1), CCL2 (CC receptor ligand 2), and TNFα] as quantified in cell-free biological fluids. Reduced monocyte recruitment in peritoneal lavages of Fpr2/3\textsuperscript{−/−} animals was reflected by a higher granulocyte/monocyte ratio. Monitoring Fpr2/3\textsuperscript{−/−} gene promoter activity with a GFP proxy marker revealed an over threefold increase in granulocyte and monocyte signals at 24 h post coecal ligation and puncture, a response mediated by TNFα. Treatment with a receptor peptido-agonist conferred protection against myocardial dysfunction in wild-type, but not Fpr2/3\textsuperscript{−/−}, animals. Therefore, coordinated physio-pharmacological analyses indicate nonredundant modulatory functions for Fpr2/3 in experimental sepsis, opening new opportunities to manipulate the host response for therapeutic development.

Significance

Sepsis defines a syndrome with poor clinical management characterized by overlapping phases of excessive inflammation temporally aligned with an immunosuppressed state. We define an endogenous pathway centered on formyl-peptide receptor 2/3 (Fpr2/3)—ortholog to human FPR2/ALX (receptor for lipoxin A4)—that protects the host against polymicrobial sepsis. Using null mice and proof-of-concept experiments with a peptido-agonist, we demonstrate how engagement of Fpr2/3 is crucial to enact nonredundant functions that span from control of cell recruitment and phagocytosis, modulation of soluble mediator generation, to containment of bacteremia, thus preventing spreading to vital organs and opening new opportunities to manipulate the host response in sepsis.

Author contributions: T.G., R.J.F., C.T., and M.P. designed research; T.G., S.M.C., J.C., S.M., P.J.F., and N.C. performed research; T.G., S.M.C., J.C., S.M., P.J.F., and N.C. analyzed data; and T.G. and M.P. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. F.Q.C. is a guest editor invited by the Editorial Board.

Freely available online through the PNAS open access option.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1410938111/-/DCSupplemental.

www.pnas.org/cgi/doi/10.1073/pnas.1410938111

PNAS | December 30, 2014 | vol. 111 | no. 52 | 18685–18690
Results

Fpr2/3 Deficiency Aggravates the Host Response to Microbial Sepsis.

Induction of polymicrobial sepsis yielded worse clinical scores for Fpr2/3−/− mice compared with WT animals. At 24 h post-coecal ligature and puncture (CLP), WT mice developed moderate sepsis (82%; score, ≤3), whereas 73% of Fpr2/3−/− mice recorded a score >3, indicating severe sepsis (Fig. L4). Rectal temperature 12 h post-CLP was decreased in both genotypes, however Fpr2/3−/− mice displayed prolonged hypothermia (Fig. 1B).

Analyses of peritoneal exudates by flow cytometry (Fig. 1C) demonstrated increased peritoneal leukocyte recruitment 12 h post-CLP in Fpr2/3−/− mice (Fig. SL4), largely due to increased Ly6G+ neutrophil recruitment (Fig. 1D). At 24 h post-CLP, neutrophil numbers did not differ between genotypes, but the total number of F4/80low monocytes was lower in Fpr2/3−/− mice (Fig. IE), which was confirmed by immunohistochemistry (Fig. S1B) and yielded a marked increase in neutrophil/monocyte ratio in Fpr2/3−/− mice (Fig. IE, Inset). Analysis of T-lymphocyte and B-lymphocyte numbers revealed no difference between genotypes (Fig. S1 C and D).

Thus, Fpr2/3 deficiency aggravates the host response to microbial sepsis and impairs the timely resolution of peritoneal inflammation. The augmented Ly6G+ neutrophil recruitment observed following CLP in Fpr2/3−/− mice was not indicative of indiscriminate higher extents of cell trafficking, as leukotriene B4, KC [keratinocyte chemokine, also termed CXCL1 (CXC receptor ligand 1)], and TNFα evoked similar recruitment in the two genotypes (Fig. S2).

Soluble Mediator Generation in Fpr2/3−/− Mice. Assessment of exudate cytokine levels revealed selected alterations in Fpr2/3−/− mice with higher levels of KC, MCP-1 [monocyte chemotactic protein 1, also termed CCL2 (CC receptor ligand 2)], and IL-6. TNFα, IFNγ, IL-10, and IL-17a levels increased to a similar degree at 12 h, yet they remained elevated at 24 h post-CLP in Fpr2/3−/− animals (Fig. S3). In the plasma, specific cytokines were detected, with increased levels of KC, IL-6, TNFα, and IFNγ 24 h post-CLP in Fpr2/3−/− mice (Fig. S4). Liquid chromatography–MS/MS (LC–MS/MS) spectroscopy allowed determination of multiple bioactive lipids with significantly augmented levels of PGE2 and 6-keto-PGF1α in peritoneal exudates of Fpr2/3−/− mice (Table S1).

We then analyzed the presence of the Fpr2/3 agonists AnxA1 and LXA4. At the time points under observation, peritoneal levels of AnxA1 increased more than fourfold, over sham, with no difference between genotypes. LXA4 levels were markedly increased in Fpr2/3−/− mice 24 h post-CLP, whereas levels were much lower in WT animals (Fig. S5).

Fpr2/3 Absence Impairs Bacterial Clearance. As a worse outcome of sepsis is associated with bacteremia (16) and Fpr2/3 agonists promote bacterial phagocytosis in vitro (17), we investigated whether Fpr2/3−/− mice displayed altered bacterial clearance in vivo. Fig. 2 shows that Fpr2/3−/− mice exhibited higher peritoneal bacteria content following CLP as quantified by flow cytometry (Fig. 2 A and B and Fig. S6A), a result confirmed also through peritoneal bacterial colony counts (Fig. S6B). Blood bacteria counts were also elevated in Fpr2/3−/− mice (Fig. S6C).

As neutrophils were the principal cell type present in the inflammatory exudates of Fpr2/3−/− mice post-CLP, we investigated whether the phagocytic ability of this cell type was compromised. Exudate neutrophils from Fpr2/3−/− mice phagocytosed much less Escherichia coli than WT cells (Fig. 2 C and D). Addition of AnxA1 to WT neutrophils incremented both the proportion of cells with internalized bacteria (Fig. 2E) and their median fluorescence intensity (a sign for higher engulfment per cell; Fig. 2F), effects absent in neutrophils lacking Fpr2/3. Efficiency of bacteria killing was investigated next. Following 2 h of incubation, WT exudate neutrophils (SI Methods) killed ~13% of phagocytized bacteria, whereas Fpr2/3−/− cells displayed minimal bactericidal capability (Fig. 2G). Collectively, these data demonstrate fundamental properties for host Fpr2/3 to manage peritoneal bacterial infection.

Fpr2/3 Gene Modulation in Immune Cells During Sepsis. Fpr2/3−/− mice bear an in-frame GFP reporter construct (12). Following CLP, a ~threefold increase in GFP signal was measured in F4/80+ monocytes, with >twofold in Ly6G+ neutrophils (Fig. 3A). To investigate the activating role of soluble mediators, Fpr2/3−/− peritoneal macrophages were incubated with peritoneal lavages from CLP WT or Fpr2/3−/− animals. At 24 h, increased GFP expression was measured, whereas lavages from sham-operated animals were inactive (Fig. S7A). This outcome was irrespective of genotype.

Next, macrophages were incubated with single proinflammatory cytokines, yet Fpr2/3 gene activation was solely induced by TNFα, as shown by (i) increased GFP fluorescence intensity (using Fpr2/3−/− cells; Fig. 3B) and (ii) elevated Fpr2/3 mRNA message in WT but not TNFα type I receptor null macrophages (Fig. S7B). Similar results could be replicated in mouse

Fig. 1. Fpr2/3 deficiency aggravates response to microbial sepsis. WT and Fpr2/3−/− mice were scored for the presence or absence of six different macroscopic signs of sepsis (SI Methods). A clinical score >3 is considered as severe sepsis. Data are from 11 mice; *P < 0.05 (Fisher’s exact test). (B) Temporal changes in rectal temperature post-CLP in WT and Fpr2/3−/− mice. (C) Scattergrams illustrating neutrophil (identified as Ly6G-F4/80-) and monocyte-macrophage (identified as Ly6G-F4/80+) positive events in peritoneal lavages from WT and Fpr2/3−/− mice at 24 h post-CLP. (D) Cumulative data for peritoneal Ly6G+ and F4/80+ cells. (Inset) Ratios of neutrophils/monocytes. Data are mean ± SEM of six mice per genotype. ***P < 0.001 versus respective WT value (two-way ANOVA, post hoc Tukey test).
bone marrow-derived neutrophils (Fig. S7C). TNFα treatment of human primary blood monocytes enhanced cell surface FPR2/ALX receptor expression (Fig. S7D).

**Cardiac Dysfunction Following Polymicrobial Sepsis Is Exacerbated by Fpr2/3 Deficiency.** The principal cause of mortality in sepsis is organ failure, with heart, liver, and kidney being among the most commonly affected organs (16). In our settings, CLP had no effect on plasma creatinine and urea levels (indicative of renal dysfunction) in WT animals, except for a transient increase in heart rate (Fig. 4A). Despite the compromised cardiac function, WT animals did not show increased plasma levels of Troponin-I at 24 h post-CLP, however these were elevated in Fpr2/3−/− mice, indicative of unaltered cardiac dysfunction (Fig. 4F). Finally, both Fpr2 and Fpr1 mRNA expression was increased in WT heart tissue samples 24 h post-CLP (Fig. 4G).

Together, these data demonstrate that endogenous activation of Fpr2/3 plays a major role in dampening dysfunction in distinct organs during polymicrobial sepsis, with a particular efficacy in preserving cardiac function.

Finally, we investigated the therapeutic potential of a stable AnxA1-based FPR2/ALX agonist (18). Administration of CR-Ac2−50 (3 μg or 550 pmol per mouse) improved cardiac function: These protective actions were not observed in mice lacking Fpr2/3 (Fig. 5A–C). Treatment with the peptide did not alter markers of liver dysfunction (Fig. S9 A and B), whereas an effect on kidney markers was evident at 24 h post-CLP. Peptide CR-Ac2−50 improved the clinical status of WT animals, again with no efficacy in Fpr2/3−/− animals (Fig. S9C). Administration of peptide CR-Ac2−50 decreased the total number of both neutrophils and monocytes recruited 24 h post-CLP in WT mice (Fig. S8 D–F). A tendency in the reduction of peritoneal bacteremia was seen post-CR-Ac2−50 administration (Fig. S9G). Assessment of exudate cytokines demonstrated that although IL-6, IL-1α, IFNγ, IL-17α, KC, and MCP1 content was not altered (values comparable to vehicle group), CR-Ac2−50 decreased (−35%) TNFα levels in WT, but not Fpr2/3−/− mice.
mice (Fig. S9H). Plasma levels of TNFα and IL-6 levels were also reduced (Fig. S9 J and J).

Together, these data demonstrate that exogenous activation of Fpr2/3 affords marked improvement of cardiac dysfunction following sepsis, possibly consequent to modulation of the inflammatory response both locally and in the circulation.

Discussion

We characterize here a nonredundant endogenous pathway that protects the host against disseminated polymicrobial sepsis. Engagement of Fpr2/3 (mouse ortholog of human FPR2/ALX) is crucial to enact nonredundant functions that span from cell recruitment to phagocytosis, from control of soluble mediator generation to containment of inflammation within the site, thus preventing spreading to vital organs. The augmented impaired function detected in the kidney and heart is indicative of fundamental protective properties evoked by FPR2/3, which could be harnessed by the exogenous administration of peptide CR-AnxA2,50.

The consensus definition of sepsis as a SIRS that occurs during infection (19) propelled the trialing of different anti-inflammatory approaches ranging from corticosteroids (20) to neutralizing anti-endotoxin strategies (21) and from TNFα and IL-1 receptor antagonist (23). Unfortunately, none of these treatments resulted in effectively reducing mortality; rather, in some cases, they were detrimental (22). Therefore, sepsis remains a major clinical challenge, and the need for new treatments is urgent.

CLP, the gold standard model of sepsis, produces a polymicrobial infection with ensuing immune, hemodynamic, and biochemical responses that replicate those observed in patients with sepsis (24). Older patients are more frequently affected by sepsis, and the treatment at intensive care units is, except for early administration of antibiotics, mostly of a supportive nature, including vasopressor therapy and fluid resuscitation (25). In this study, we simulated these conditions by performing CLP in 8-mo-old mice treated with a large spectrum of antibiotic and fluids (26, 27). Fpr2/3−/− mice developed longer lasting macroscopic signs of severe sepsis, as evident from 24 h of hypothermia, than WT animals.

The homeostatic functions of proreresolving and tissue-protective pathways are now emerging in several immune contexts, including arthritis, colitis, periodontal pathologies (5), and ischemia/reperfusion damage (13). The complex biological functions affected by Fpr2/3 afforded efficacy against polymicrobial sepsis downstream of positive modulation of multiple life-saving processes through a single receptor determinant. Indeed, the appreciation that sepsis is not just SIRS but rather a complex clinical setting with an overlapping CARS indicates that innovative approaches must be proposed.

There is experimental evidence that endogenous agonists of Fpr2/3 are protective in experimental sepsis. The omega-6 derivative LXA4 administered to CLP rats increases survival rates and attenuates tissue injury (9). The omega-3 derivative resolvin D1 improves the outcome of sepsis (28). Work from our group has identified marked AnxA1 gene activation in experimental endotoxaemia, together with a higher toxic response in AnxA1−/− mice, a phenotype rescued by exogenous application of the protein (8). From a therapeutic perspective, a recent study conducted with peptide WKYMVM, a panagonist at FPRs, described the control of severe sepsis after microbial infection (29). Albeit interesting, this study does not provide target validation to the properties of FPR2/ALX agonists and focuses solely on the host immune response rather than complementing it with organ functionality. These pharmacological studies indicate a role for Fpr2/3, but the mechanism(s) it might control in the contest of sepsis remained unexplored.

The Fpr2/3 agonists AnxA1 and LXA4 could be detected in peritoneal infected exudates with an interesting “overshooting” of the latter in Fpr2/3−/− mice, likely indicative of a “frustrated” compensatory loop to dampen the exacerbated inflammatory response. This would also suggest that LXA4 is the pivotal agonist in these settings or, rather, that a temporal distinction for agonist exposure exists, when one compares profiles of AnxA1 and LXA4 levels in WT mice. Following detection of endogenous agonists, we noted that a variety of host responses were altered when the Fpr2/3 axis was not engaged, pointing again to nonredundant multiple
regulated functions. Thus, selected changes in cytokine and chemokine levels were associated with augmented bioactive lipid generations. Of interest, major differences were measured for KC (CXCL1), MCP-1 (CCL2), and IL-6. High MCP-1 levels were not manifested by an efficient monocyte influx (quite the opposite), indicating the requirement for other pathways to bring in monocytes, a crucial process for the in situ differentiation into phagocytizing macrophages that regulate resolution (30). It is plausible that CCR2 independent pathways might be altered. Relevantly, LXA₄ can promote nonphlogistic monocyte migration (31), and the same occurs for another Fpr2/3 ligand, the cathelicidin LL-37 (32).

The poor recruitment of monocytes observed in Fpr2/3−/− animals is functionally linked to a lower number of macrophages, higher neutrophil/monocyte–macrophage ratios, and inadequate bacterial removal. Congruently, clearance of bacteria from the peritoneal cavity was markedly reduced in Fpr2/3−/− mice, possibly enacting a vicious circle leading to potentiation of inflammation and delayed resolution, as evident from the higher cfu formation in Fpr2/3−/− peritoneal fluids. Bacteria counts were also elevated in the circulation. Analyses of cell behavior indicated, besides defective recruitment, a direct impairment of phagocyte functions in Fpr2/3−/− neutrophils. Although not in these settings, studies have demonstrated the importance of AnxA1, LXA₄, and more recently resolvin D₁ in promoting particle phagocytosis and efferocytosis by immune cells (17, 33, 34) together with ineffectiveness in cells lacking Fpr2/3 (35).

The ultimate cause of death in patients with sepsis is multiple organ failure. Absence of Fpr2/3 was associated with major changes in distant organ injury, with a particular effect upon heart and kidney. An exacerbated myocardial dysfunction occurred in the transgenic, as reflected by a marked profound change in systolic contractility. This effect can reflect an indirect consequence of a direct protective action on the myocardium, preserving its contractile function (45, 46). None of these biological properties were retained in Fpr2/3−/− mice that could underlie organ dysfunction: There are indications that unabated circulating cytokines are predictive of early mortality in sepsis (38). Some of them, like TNFα and IL-1β, can exert a direct effect through increased expression of inducible NO synthase (39, 40). In any case, the augmented myocardial injury determined by Fpr2/3 absence was functional, as evident with poor systolic pressure and pulse.

Whereas liver and lungs were mildly affected in the CLP model, with little or no difference between the two genotypes, different responses were quantified for the kidney parameters. The kidney is relatively resistant, and indeed markers of injury were not elevated in WT mice subjected to our protocol of sepsis. In contrast, elevated urea and creatinine plasma levels were measured in Fpr2/3−/− mice. Kidney MPO activity, an index of granulocyte tissue recruitment, was increased in the null animal, prompting speculation that—at least in part—in check inflammation could be the cause of acute kidney injury experienced by Fpr2/3−/− mice. Cellular infiltrates, particularly neutrophils, damage tissue directly by realizing lysosomal enzymes and superoxide-derived radicals (41). It is also plausible that the hypotension/vasodilatation derived from impaired cardiac function could at least contribute to kidney damage.

Two sets of data merit further discussion. The first one entails the pivotal role played by TNFα, but not other cytokines tested, in activating the Fpr2/3 gene promoter, as demonstrated using a combination of reporter assays ex vivo and in vitro, with analyses in mouse and human cells. The appreciation that proinflammatory mediators set in motion the resolution and protective phase of inflammation is emerging (42), yet our data and others suggest this link and identify TNFα as the culprit, acting through the type I receptor. This set of data extends previous in vitro observations in synovial fibroblasts (43) and mouse microglial cells (44). It could be speculated that failure of anti-TNFα strategies in sepsis may, at least in part, be due to inadequate induction of a protective pathway in the host, including AnxA1, the agonist (8), and its receptor Fpr2/3 (this study).

The second comment relates to the pharmacological experiments with CR-Ac₂₋₅₀. Therapeutic delivery of this peptide, highly selectively for human FPR2/ALX (IC₅₀ ~10 nM) over FR1 (18), afforded marked protection. It is relevant here that CR-Ac₂₋₅₀ inhibited neutrophil trafficking while enhancing macrophage efferocytosis in sterile inflammation (18), possessing therefore a few of the necessary properties, as discussed above, to elicit host protection. Control of the immune response by CR-Ac₂₋₅₀ was complemented by a significant attenuation of the impairment in systolic contractility. This effect can reflect an indirect protection through reduced inflammation but also could be the consequence of a direct protective action on the myocardium, preserving its contractile function (45, 46). None of these biological properties were retained in Fpr2/3−/− mice. The documented increase in myocardial mRNA for Fpr1 and Fpr2 can be explored in future investigations. These data complement recent studies conducted with panagonists of formyl peptide receptors (28, 29).

In conclusion, we describe nonredundant properties of endogenous Fpr2/3, the receptor determinant for agonists of the resolution of inflammation process, in a model of polymicrobial sepsis. This nonredundant role stems from multiple biological functions controlled by this master receptor, which is able to set in motion an integrated battery of host-protective effects operating both at the site of infection and in distant vital organs (Fig. 6). Therapeutic innovation in the management of sepsis can derive from the development of selective Fpr2/3 (and FPR2/ ALX in man) agonists, and this could include small molecules, peptidomimetics, as well as bioactive lipid derivatives (47).

**Methods**

**SI Methods** provides an extended version of the experimental procedures.

**Polymicrobial Sepsis.** CLP was performed in 8-mo-old male C57BL/6 or Fpr2/3−/− mice. Kidney MPO activity, an index of granulocyte tissue recruitment, was increased in the null animal, prompting speculation that—at least in part—in check inflammation could be the cause of acute kidney injury experienced by Fpr2/3−/− mice. Cellular infiltrates, particularly neutrophils, damage tissue directly by realizing lysosomal enzymes and superoxide-derived radicals (41). It is also plausible that the hypotension/vasodilatation derived from impaired cardiac function could at least contribute to kidney damage.

**Fig. 6.** Loss of host control on polymicrobial sepsis in the absence of Fpr2/3. The control exerted by endogenous engagement of FPR2/ALX (mimicked here by its ortholog Fpr2/3) is dual: regulation for an optimal local reaction with proper host immune cells with the bacteria load, and modulation of circulating mediators and distant organ functionality (heart and kidney).
after surgery (27). In some experiments, mice were treated with peptide CR-AnxA1-26. A clinical score for monitoring the health of experimental mice was used. Animals were housed 12 or 24 h later. All animal experiments were approved by the local Animal Use and Care Committee in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986. Human cells were prepared according to an approved protocol (East London & the City Local Research Ethics Committee; no. 06/Q0605/0/00029).

Assessment of Cardiac Function in Vivo. Cardiac function was assessed in mice by echocardiography (48).

Bacteria Counting. Accurate enumeration of bacteria in peritoneal lavages was performed by flow cytometry using the SYTO BC bacteria counting kit. Blood and peritoneal bacteria loads were also determined by growth on a tryptic soy agar plate, as reported (10).

Supporting Information

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SI Methods

Animals. Male C57BL/6 or Fpr2/3−/− mice (thereafter referred to as Fpr2/3−/−) were maintained on a standard chow diet with free access to water and a 12-h light–dark cycle. All animal experiments were approved by the local Animal Use and Care Committee in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986. Human cells were prepared according to an approved protocol (East London & the City Local Research Ethics Committee; no. 06/Q0605/40, F/00/029).

In Vivo Models.

Polymicrobial sepsis. Coecal ligature and puncture (CLP) was performed in 8-mo-old male C57BL/6 or Fpr2/3−/− mice using our published protocol that mimics clinical management in intensive care units (2), including fluid resuscitation (Acetatet Ringer’s solution; 0.5 mL per mouse), antibiotic therapy (Imipenem/Cilastin; 20 mg/kg body weight s.c.), and analgesia (buprenorphine; 0.05 mg/kg body weight i.p.) at 6 h and 18 h after surgery (2). In some experiments, mice were treated 1 and 9 h after CLP with a stable AnxA1-derived peptide, termed CR-AnxA12−50 (3 μg per mouse i.p.), or vehicle (saline/0.1% DMSO). Sham-operated mice underwent the same procedure but without CLP.

A clinical score for monitoring the health of experimental mice was used to evaluate the symptoms consistent with murine sepsis. The maximum score of 6 comprised the presence of the following signs: lethargy, piloerection, tremors, periorbital eduates, respiratory distress, and diarrhea. Mice with a clinical score >3 were defined as exhibiting severe sepsis, against a moderate sepsis for a score ≤3. Mice were killed 12 or 24 h later after assessment of cardiac function by echocardiography. Blood was collected by cardiac puncture and peritoneal lavage fluid, and kidney and lung tissue were also collected and stored at −80 °C for further analysis. Plasma and peritoneal cytokine levels were determined by cytokine beads assay (bBioscience). Peritoneal cells were counted using Turks solution and differentiated by flow cytometric analysis, as described below. Plasma was analyzed for levels of serum creatinine, urea, aspartate aminotransferase, and alanine transaminase (IDEXX Laboratories) for analysis. Plasma and peritoneal cytokine levels were determined by cytokine beads assay (eBioscience). Peritoneal cells were labeled with anti-Ly6G, and the number of phagocytosing cells was measured by flow cytometry.

Neutrophil phagocytosis and killing assay. Exudate neutrophils, obtained 6 h after i.p. injection of zymosan (3), were plated in 96-well plates at a density of 0.5 × 10⁶ cells per well in complete medium. pH-sensitive Phrodu E. coli bioparticles (Invitrogen) were added to the cells. Neutrophils were labeled with anti-Ly6G, and the number of phagocytosing cells was measured by flow cytometry.

Neutrophil bactericidal activity was measured by incubating 2 × 10⁶ neutrophils with 2 × 10⁷ opsonized E. coli for 1 h. After infection, gentamicin (5 μg/mL) was added to the plates for 1 h, allowing the antibiotic to kill all bacteria that were not able to penetrate the cells and remained outside. The wells were then washed to remove the dead bacteria. Next the neutrophils were lysed using 0.1% Triton X-100, releasing the bacteria that penetrated the cells and remained alive. Lysates were then plated on solid medium plates, and the number or viable bacteria was determined by colony counting of live bacteria.

Bacteria counting. Accurate enumeration of bacteria in peritoneal lavages was performed by flow cytometry using the SYTO BC bacteria counting kit (Invitrogen) according to the manufacturer’s instructions. Blood and peritoneal bacteria loads were also determined by growth on tryptic soy agar plate as reported previously (4).

Assessment of cardiac function in vivo (echocardiography). Cardiac function was assessed in mice by echocardiography in vivo as reported previously (5). At 24 h after CLP, anesthesia was induced with 3% (vol/vol) isoflurane and maintained at 0.5–1% for the duration of the procedure. Before assessment of cardiac function, mice were allowed to stabilize for at least 10 min. 2D and M-mode echocardiography images were recorded using a Vevo-770 imaging system (VisualSonic). Percentage fractional area change was assessed with a 2D trace of left ventricle (LV) and was derived by 100 × (LV end-diastolic area − LV end-systolic area) / LV end-diastolic area. The method involves tracing the endocardial surface of the LV in the parasternal short axis view at the level of papillary muscles. Percentage ejection fraction (EF) and percentage fractional shortening (FS) were calculated from the M-mode measurements of the LV internal dimension (LVID) in the diastolic (d) and systolic (d) phase, in the parasternal short axis view at the level of the papillary muscles. Percentage EF was calculated using the formula: 100 × [(LVID (d) − LVID (s))/ LVID (d)]%; percentage FS was derived using the formula: 100 × [(LVID (d) − LVID (s))/ LVID (d)] (2, 5, 6).

Primary Macrophage and Human Monocyte Cultures. Mice were injected with 1 mL of 2% Bio-Gel (Bio-Rad) i.p., and 4 d later, peritoneal lavages were harvested with 4 mL of EDTA (3 mM) in PBS. Cells (0.5 × 10⁶) were plated in 24-well plates in RPMI medium 1640 containing 10% (vol/vol) FCS and 50 mg/mL of gentamicin. After 2 h at 37 °C, nonadherent cells were washed and adherent cells (>90% macrophages) were incubated in RPMI 1640 ± 1% FCS supplemented with specific cytokines (all at 50 ng/mL)—as indicated later—or vehicle. Cytokines were purchased from PeproTech. In a set of experiments, TNFα type I receptor null cells (from the colony described in ref. 7) were prepared and compared with macrophages prepared from C57BL/6 mice.

Primary human monocytes were extracted from the whole blood of healthy donors using the RosetteSep negative selection assay (Stem Cell Technologies Inc.) according to the manufacturer’s protocols. Cells were resuspended in RPMI medium 1640, supplemented with 0.1% BSA, 100 U/mL penicillin, and 100 μg/mL streptomycin for all assays. Extractions were performed immediately before use in experiments.

Immunofluorescence Analyses. Peritoneal cells were fixed by incubation with 2% formaldehyde for 15 min at room temperature, washed, and immunostained for the monocye/macrophage marker protein Iba1. Briefly, after 30 min at room temperature in 10% normal goat serum containing 0.025% saponin, cells were incubated for 1 h with a rabbit anti-mouse Iba1 polyclonal antibody (1/1,000; Wako Chemicals). After washing, cells were incubated for 30 min with AF594-conjugated goat anti-rabbit IgG secondary antibody (1/300), before further washing and mounting under glass coverslips using Moviol mounting agent (Sigma-Aldrich). Cells were then examined at 20× magnification using EVOS microscope (Life Technologies).

Biochemical Analyses.

Flow cytometry. Peritoneal cells were differentiated using anti-F4/80 (clone BM8; BioLegend), anti-Ly6G (clone 1A8; BioLegend), anti-CD3 (clone 145–2C11; bBioscience), and anti-B220 (clone...
Quantification of 6kPGF
Sepsis clinical score analysis was performed method. 2 tables). Statistical differences
<β6(4):–×461(7268):1287 in septic shock. 364(6440):798
Total RNA from biogel-elicited macro-
in peritoneal lavages was
2o f8 Nature
Autoregulation mode with optimized conditions (ion optics and collision
dient elution. Data were acquired in Multiple Reaction Moni-
tography) System (Agilent LC1290 Infinity) coupled to Agilent
MS/MS analysis was per-
formed on the UHPLC (ultra high performance liquid chroma-
tography) System (Agilent LC1290 Infinity) coupled to Agilent
6460 triple quadruple MS (Agilent Technologies) equipped with
electrospray ionization operating in negative mode. Reverse-
phase UHPLC was performed using ZorBAX SB-C18 column
(2.1 mm, 50 mm, 1.8 μm) (Agilent Technologies) with a gra-
dient elution. Data were acquired in Multiple Reaction Moni-
toring mode with optimized conditions (ion optics and collision
energy). Peak detection, integration, and quantitative analysis
were done using Mass Hunter Quantitative analysis software (Agilent Technologies). For each standard, calibration curves were
built using 10 solutions at concentrations ranging from 0.95 ng/mL
to 500 ng/mL.
Determination of MPO activity in heart, kidney, and lung tissue. Myelo-
peroxidase (MPO) activity was measured as an index of blood-
borne PMN infiltration as described (11).
Real-time PCR analysis. Total RNA from biogel-elicited macrophages was isolated with TRIzol reagent (Invitrogen) according to
the manufacturer’s protocol. In all cases, total RNA (1 μg) was reverse-transcribed with random hexamer oligonucleotides and SuperScript III (Invitrogen). Amplification was performed with a LightCycler 480 using a SYBR Green I Master Kit (Qiagen Ltd.) and the mouse Fpr2 primer set (QT00171514, Qiagen).
Relative expression of the target gene was normalized to expres-
sion of the Hypoxanthine Phosphoribosyltransferase (Hpri)
gene, using the ΔΔCt method.
Statistical Analyses. Sepsis clinical score analysis was performed using Fisher’s exact test (2 × 2 tables). Statistical differences
were determined using one- or two-way analysis of variance, as
appropriate, followed by Tukey’s postest. A P value < 0.05 was
taken as significant for rejection of the null hypothesis.

2. Coldewey SM, et al. (2013) Erythropoietin a
3. Perrett M, Solito E, Parente L (1992) Evidence that endogenous interleukin-1 is in-
7. Rothe J, et al. (1993) Mice lacking the tumour necrosis factor receptor 1 are resistant to TNF-mediated toxicity but highly susceptible to infection by Listeria mono-
9. Gobbetti T, et al. (2013) Polyunsaturated fatty acid metabolism signature in ischemia-
10. Le Faouder P, et al. (2013) LC-MS/MS method for rapid and concomitant quantifica-
**Fig. S1.** Cell phenotype during ongoing CLP. WT and Fpr2/3−/− mice were subjected to CLP at time 0 (Methods). (A) Temporal changes in total number of cells recruited in the peritoneal cavity post-CLP in WT and Fpr2/3−/− animals. Data are mean ± SEM of six mice per genotype. ***P < 0.001 versus respective WT value (two-way ANOVA, post hoc Tukey test). (B) Immunofluorescent microscopic analysis of Iba1-positive cells (arrowheads) in peritoneal exudates 24 h post-CLP from WT and Fpr2/3−/− mice. (C and D) Cumulative data for CD3+ (T cells) and B220+ (B cells) in the peritoneal cavity post-CLP from WT and Fpr2/3−/− mice. Data are mean ± SEM of six mice per genotype.

**Fig. S2.** Fpr2/3 deficiency does not affect neutrophil recruitment for classical chemoattractants. Mice were injected i.p. with three classical neutrophil chemoattractants (LTB4, 100 ng; TNFa, 1 μg; KC, 1 μg) or vehicle (V; 100 μL). Four hours after the injection, peritoneal lavages were collected. Cells were stained for Ly6G or F4/80. Data are mean ± SEM of 4–5 mice per group.
Fig. S3. Peritoneal soluble inflammatory mediator generation in Fpr2/3−/− mice during polymicrobial sepsis. WT and Fpr2/3−/− mice were subjected to CLP at time 0 (Methods). (A–H) At 24 h post-CLP, blood was collected and plasma samples prepared and tested for cytokine levels using the cytokine bead assay by flow cytometry. Data are mean ± SEM of six mice per group. **P < 0.01, ***P < 0.001 versus correspondent WT value (two-way ANOVA, post hoc Tukey test).
Fig. S4. Plasma soluble inflammatory mediator generation in Fpr2/3−/− mice during polymicrobial sepsis. WT and Fpr2/3−/− mice were subjected to CLP at time 0 (Methods). (A–F) At 24 h post-CLP, blood was collected and plasma samples prepared and tested for cytokine levels using the cytokine bead assay by flow cytometry. Data are mean ± SEM of six mice per group. **P < 0.01, ***P < 0.001 versus correspondent WT value (two-way ANOVA, post hoc Tukey test).

Fig. S5. Annexin A1 and Lipoxin A4 levels in peritoneal exudates during polymicrobial sepsis. WT and Fpr2/3−/− mice were subjected to CLP at time 0 (Methods). (A) Annexin A1 was quantified by sandwich ELISA. Quantitative Annexin A1 levels over the time course of the CLP response. (B) Lipoxin A4 (LXA4) was identified and quantified by LC-MS/MS-based lipidomic analysis. Quantitative LXA4 levels over the time course of the CLP response. (C) Spectrum of relative peaks for LXA4. (D) LXA4 signature fragment ions reported as m/z, highlighting the two diagnostic fragments at 115 and 351 (351 is the ion mass). Data are mean ± SEM of six mice. ***P < 0.001 versus correspondent WT value (two-way ANOVA, post hoc Tukey test).
Fig. S6. Sham settings and bacterial levels in CLP. WT and Fpr2/3−/− mice were subjected to CLP at time 0 (Methods). (A) Representative flow cytometry scattergrams illustrating bacteria (SYTO BC bacteria dye) positive events in sham peritoneal exudates from WT (Left) and Fpr2/3−/− (Right) mice. The density of bacteria in the experimental samples was determined from the ratio of bacterial to microsphere signals. (B) Measurement of aerobic bacteria levels in peritoneal lavages 24 h post-CLP from WT and Fpr2/3−/− mice. Data are mean ± SEM of six mice. *P < 0.05 versus correspondent WT value (Student t test). (C) Measurement of bacterial levels 24 h after CLP in plasma from WT and Fpr2/3−/− mice. Data are mean ± SEM of six mice. *P < 0.05 versus correspondent WT value (Student t test).

Fig. S7. Fpr2 modulation in mouse and human cells. (A) Biogel-elicited macrophages from Fpr2/3−/− mice were incubated with sham or 24-h CLP lavages (collected from WT or Fpr2/3−/− animals) for 24 h at 37 °C, before assessment of GFP fluorescence by flow cytometry. Data are mean ± SEM of six mice per group. **P < 0.01, ***P < 0.001 versus correspondent sham value (two-way ANOVA, post hoc Tukey test). (B) Biogel-elicited macrophages from WT and TNFαR1−/− mice were incubated for 24 h with 50 ng/mL TNFα before Fpr2/3 mRNA quantification by real-time PCR. Data are mean ± SEM of 4–6 distinct cell preparations. ***P < 0.001 versus WT value (two-way ANOVA, post hoc Tukey test). (C) Zymosan-elicited PMN from Fpr2/3−/− mice were incubated for 24 h with 50 ng/mL TNFα before assessment of GFP fluorescence by flow cytometry, used as a reporter for Fpr2/3 gene promoter activity. (D) Human monocytes were incubated for 24 h with 50 ng/mL TNFα before assessment of FPR2/ALX expression by flow cytometry. Data are mean ± SEM of 3–4 distinct cell preparations. *P < 0.05, **P < 0.01 versus control (Student t test).
Fig. 58. Organ injury modulation between the two genotypes. WT and Fpr2/3−/− mice were subjected to CLP at time 0 (Methods). (A and B) Temporal changes in plasma urea and creatinine post-CLP in WT and Fpr2/3−/− animals. (C and D) Temporal changes in plasma alanine aminotransferase and aspartate aminotransferase post-CLP in WT and Fpr2/3−/− animals. (E and F) Kidney and lung MPO activity at different times post-CLP. Data are mean ± SEM of six mice. **P < 0.01, ***P < 0.001 between genotypes (two-way ANOVA, post hoc Tukey test).
Fpr2/3 agonism modulates the host response to polymicrobial sepsis. WT and Fpr2/3−/− mice were subjected to CLP at time 0 (Methods) and treated with an AnxA1-based peptide CR-Ac2–50 1 h and 9 h postsurgery, using a dose of 90 μg/kg i.p., or with vehicle (100 μL i.p.), and killed at 24 h post-CLP. (A and B) Plasma alanine aminotransferase and aspartate aminotransferase values in WT and Fpr2/3−/− animals. (C) Presence or absence of six different macroscopic signs of sepsis—namely, lethargy, piloerection, tremors, periorbital exudates, respiratory distress, and diarrhea. (D–F) Cumulative data for total number of cells, Ly6G+ cells (neutrophils), and monocyte-macrophage (F4/80+ cells; Mo) counts in peritoneal exudates from WT and Fpr2/3−/− mice treated i.p. with vehicle or CR-Ac2–50. (G) Peritoneal bacterial load. (H) CR-Ac2–50 treatment reduces exudate TNFα, compared with vehicle, in WT but not Fpr2/3−/− mice. (I and J) CR-Ac2–50 treatment reduces plasma TNFα and IL-6 levels, compared with vehicle, in WT but not Fpr2/3−/− mice. Data are mean ± SEM of six mice per group. *P < 0.05 versus corresponding WT value (Student t test).

### Table S1. Bioactive lipid mediator quantification in peritoneal exudates

<table>
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<tr>
<th>Genotype</th>
<th>Treatment</th>
<th>PGE2, pg/mL</th>
<th>TXB2, pg/mL</th>
<th>6-keto-PGF1α, pg/mL</th>
<th>LTB4, pg/mL</th>
<th>PGD2, pg/mL</th>
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<tbody>
<tr>
<td>WT</td>
<td>Sham</td>
<td>106 ± 8</td>
<td>62 ± 38</td>
<td>362 ± 83</td>
<td>30 ± 20</td>
<td>105 ± 7</td>
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<td></td>
<td>CLP 12 h</td>
<td>426 ± 148</td>
<td>439 ± 70</td>
<td>5,229 ± 1,365</td>
<td>210 ± 46</td>
<td>13 ± 13</td>
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<td></td>
<td>CLP 24 h</td>
<td>813 ± 258</td>
<td>628 ± 187</td>
<td>4,066 ± 11</td>
<td>348 ± 103</td>
<td>839 ± 401</td>
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<tr>
<td>Fpr2/3−/−</td>
<td>Sham</td>
<td>126 ± 24</td>
<td>30 ± 25</td>
<td>346 ± 103</td>
<td>30 ± 18</td>
<td>120 ± 32</td>
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<tr>
<td></td>
<td>CLP 12 h</td>
<td>1,026 ± 382</td>
<td>710 ± 161</td>
<td>10,616 ± 2,370*</td>
<td>630 ± 186</td>
<td>48 ± 39</td>
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<tr>
<td></td>
<td>CLP 24 h</td>
<td>5,297 ± 2,257***</td>
<td>1,405 ± 493</td>
<td>20,628 ± 5,360**</td>
<td>746 ± 292</td>
<td>2,458 ± 1,015</td>
</tr>
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WT and Fpr2/3−/− mice were subjected to CLP at time 0 (Methods), with sham animals being operated without cecum damage. Cell-free lavage fluids were analyzed by LC-MS/MS-based lipidomic analysis for multiple lipid mediators. Data (mean ± SEM of six mice per group) are expressed as concentrations. Results are mean ± SEM of six mice. *P < 0.05, **P < 0.01, ***P < 0.001 versus respective WT value (two-way ANOVA, post hoc Tukey test).