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Formyl Peptide Receptor Type 2 Deficiency in Myeloid Cells Amplifies Sepsis-Induced Cardiac Dysfunction

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Highlights

- hFPR2 deficiency in myeloid cells amplified cardiac dysfunction, worsened clinical outcome, and impaired bacterial clearance in mice subjected to CLP-induced polymicrobial sepsis.
- Myeloid cell-specific hFPR2 KO led to an imbalance between pro-inflammatory and pro-resolving immune cell recruitment both within the hearts and in peritoneal cavity in septic mice.
- The cardioprotective effects of AnxA1 are blunted in myeloid cell-specific hFPR2 KO mice, where it failed to polarize macrophages toward an MHC II^{-} phenotype.

Keywords
Cardiomyopathy · Cardiac function · Polymicrobial sepsis · Septic shock · FPR2 · Annexin A1 · Resolution pharmacology · Macrophages · Myeloid cells · Immune response

Abstract
Using a global formyl peptide receptor (Fpr) 2 knockout mouse colony, we have reported the modulatory properties of this pro-resolving receptor in polymicrobial sepsis. Herein, we have used a humanized FPR2 (hFPR2) mouse colony, bearing an intact or a selective receptor deficiency in myeloid cells to dwell on the cellular mechanisms. hFPR2 mice and myeloid cell-specific hFPR2 KO (KO) mice were subjected to cecal ligation and puncture (CLP)-induced polymicrobial sepsis. Compared with hFPR2 mice, CLP caused exacerbated cardiac dysfunction (assessed by echocardiography), worsened clinical outcome, and impaired bacterial clearance in KO mice. This pathological scenario was paralleled by increased recruitment of pro-inflammatory monocytes and reduced M2-like macrophages within the KO hearts. In peritoneal exudates of KO mice, we quantified increased neutrophil and MHC II^{+} macrophage numbers but decreased monocyte/macrophage and MHC II^{-} macrophage recruitment. hFPR2 upregulation was absent in myeloid cells, and local production of lipoxin A4 was reduced in septic KO mice. Administration of the FPR2 agonist annexin A1 (AnxA1) improved cardiac function in hFPR2

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septic mice but had limited beneficial effects in KO mice, in which the FPR2 ligand failed to polarize macrophages toward an MHC II$^-$ phenotype. In conclusion, FPR2 deficiency in myeloid cells exacerbates cardiac dysfunction and worsens clinical outcome in polymicrobial sepsis. The improvement of cardiac function and the host immune response by AnxA1 is more effective in hFPR2-competent septic mice.

Introduction

Sepsis is characterized by a systemic inflammatory response to microbial infection which, when excessive, may lead to multiple organ failure and death [1]. Approximately 50% of patients with sepsis exhibit signs of cardiac dysfunction [1, 2], and presence of cardiac dysfunction in septic patients raises the mortality rate from 40% to 70% [3]. Increased levels of pro-inflammatory cytokines contribute to the development of cardiac dysfunction in septic patients, yet therapeutic strategies targeting pro-inflammatory cytokines failed to improve the cardiac outcome [4]. Therefore, current therapy for cardiac dysfunction in sepsis remains mainly supportive, and this includes fluid resuscitation. A greater understanding of the systemic and tissue-specific cellular mechanisms operative during sepsis may help develop novel pharmacological strategies and reduce sepsis-induced cardiac (organ) dysfunction.

Acute inflammation is a protective reaction of our body to invading pathogens or tissue damage, which often leads to healing and recovery of biological function. To be successful, completed on time, and limited in space, the physiological inflammatory process is characterized by a pro-inflammatory phase followed by a resolution phase. The latter is governed by endogenous pro-resolving mediators, some of which signal through G protein-coupled receptors (GPCRs). Particularly, the formyl peptide receptor (FPR) family of GPCRs plays crucial roles in host defence, regulation of inflammation, and its resolution [5]. Of relevance, FPR2 agonists such as annexin A1 (AnxA1) [6], lipoxin A4 (LXA4) [7], and resolving D1 [8] exert protective actions in murine models of sepsis. In patients, the rs11666254 polymorphism of the FPR2 gene is linked to decreased FPR2 mRNA and protein expression and is functionally associated with higher susceptibility to post-traumatic sepsis [9]. Using a global mouse Fpr2/3 null (−/−) mouse colony, we have described an impaired host response and exacerbated organ dysfunctions, particularly cardiac dysfunction, in cecal ligation and puncture (CLP) sepsis. In this model, a pro-resolving peptide derived from the N-terminal region of AnxA1 afforded cardioprotection in wild-type (WT) but not in Fpr2/3−/− mice [10].

The pro-resolving receptor FPR2 is expressed at high levels in myeloid cells including neutrophils, macrophages, and monocytes and promotes key processes of resolution of biology; thus, FPR2 activation limits neutrophil extravasation, promotes non-phlogistic monocyte recruitment, enhances phagocytosis, augments neutrophil apoptosis and efferocytosis [11]. Because of the important role of FPR2 in resolution of inflammation and the fact that FPR2 is highly expressed in myeloid cells, the present study aimed to investigate whether FPR2 deficiency specifically in myeloid cells would impair resolution and worsen cardiac outcome in experimental polymicrobial sepsis.

Materials and Methods

Animals

The humanized Fpr2 (hFPR2) heterozygous mice with C57BL/6 background were generous gifts from Idorsia Pharmaceuticals. In these mice, the mouse endogenous Fpr2/3 locus has been replaced by human FPR2 which is flanked by loxP sequences (online suppl. Fig. S1; for all online suppl. material, see https://doi.org/10.1159/000530284) [12]. hFPR2 homozygous mice (hFPR2 loxP/loxP) were crossed with mice expressing Cre recombinase under control of the lysozyme M promoter (LysM-Cre) to generate LysM-Cre/+ hFPR2 loxP/+ mice. These mice were then backcrossed with hFPR2 homozygous mice to generate myeloid cell-specific hFPR2 KO mice (LysM-Cre/+; hFPR2 loxP/loxP; also referred to as KO) (online suppl. Fig. S2) [12]. All mice were bred in house, maintained on a standard chow pellet diet, and had access to water ad libitum, with a 12-hour light-dark cycle. Cages had clean bedding and cardboard rolls to allow dark areas for mice to shelter.
Model of Polymicrobial Sepsis Caused by CLP and Human-Recombinant AnxA1 Treatment

CLP surgery was conducted in 10-week-old male and female mice as described previously [10, 13]. Based on previous evidence and preliminary data, an 18-G needle was used with the double-puncture technique in order to generate reproducible cardiac dysfunction during the early phase of sepsis (24 h). Briefly, mice were anesthetized i.p. with 1.5 mL/kg of a ketamine (100 mg/mL)/xylazine (20 mg/mL) solution in a 2:1 ratio. Buprenorphine (0.05 mg/kg i.p.) was injected additionally to provide adequate analgesia. The rectal temperature of the animals was maintained at 37°C with a homoeothermic blanket. The abdomen was opened via a 1.5 cm midline incision and the caecum exposed. The caecum was ligated just below the ileocaecal valve and punctured at both opposite ends. After a small amount of fecal matter was extruded from both ends, the caecum was placed back in its anatomical position, and the abdomen was sutured. Ringer's solution was given s.c. for resuscitation directly after surgery

(30 mL/kg) and 6 h and 18 h after surgery (0.5 mL/mouse). Treatment with an antibiotic (imipenem/cilastatin; 20 mg/kg s.c.) and an analgesic (buprenorphine; 0.05 mg/kg i.p.) was done at 6 h and 18 h after surgery, respectively. Sham-operated mice were not subjected to ligation or perforation of the caecum but were otherwise treated the same way. In all cases, 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 presence of the following signs: lethargy, piloerection, tremors, periorbital exudates, respiratory distress, and diarrhoea. At 24 h after surgical procedures, cardiac function was assessed by echocardiography in vivo (see below). To study the role of myeloid hFPR2 in sepsis-induced cardiac dysfunction, mice were randomly allocated into different groups: (i) hFPR2 + sham operation (n = 5) and (ii) hFPR2 + CLP (n = 7). Age-matched myeloid cell-specific hFPR2 KO were randomly allocated into two different groups: (i) hFPR2 KO + sham operation (n = 5) and (ii) hFPR2 KO + CLP (n = 7).

In the human-recombinant AnxA1 (hrAnxA1) therapeutic study, 1-hour and 6-hour after CLP or sham operation, mice were treated either with hrAnxA1 (2 μg/mouse i.v.) [14, 15] or vehicle (100 μL saline). Thus, hFPR2 mice were randomly allocated into two different groups for echocardiography and flow cytometry: (i) hFPR2 + CLP + vehicle (n = 4) and (ii) hFPR2 + CLP + hrAnxA1 (n = 5). Age-matched myeloid cell-specific hFPR2 KO were randomly allocated into two different groups: (i) hFPR2 KO + CLP + vehicle (n = 4) and (ii) hFPR2 KO + CLP + hrAnxA1 (n = 4).

Assessment of Cardiac Function in vivo

Cardiac function was assessed in mice by in vivo echocardiography as reported previously [10, 16]. At 24 h after CLP, anaesthesia was induced with 3% isoflurane and maintained at 0.5–0.7% for the duration of the procedure. Before assessment of cardiac function, mice were allowed to stabilize for at least 10 min. During echocardiography, the heart rate was obtained from electrocardiogram tracing, and the temperature was monitored with a rectal thermometer. M-mode echocardiography images were recorded using a Vevo 3100 imaging system (VisualSonics, Toronto, ON, Canada). Percentage ejection fraction (EF) and percentage FS were calculated from the M-mode measurements in the parasternal short axis view at the level of the papillary muscles. Calculation of percentage EF and FS requires the measurements of left ventricle internal dimension (LVID) in diastolic and systolic phases from M-mode.

Quantification of Circulating Leukocytes

Mice were anaesthetized with 3% isoflurane; approximately 0.7 mL of blood was collected by cardiac puncture and immediately decanted into EDTA blood tubes (Sarstedt AG & Co., Germany). Approximately 0.1 mL of blood was used for analyses of white blood cell counting by ProCyte Dx Haematology Analyser (IDEXX Laboratories, UK). Mice were then euthanized with CO_2.

Flow Cytometry

Mice were euthanized with CO_2. Hearts were collected, residual blood was cleared by retrograde perfusion with 5 mL ice-cold normal saline, and cut into 1-mm^2 pieces, digested in 1 mL Hanks medium, containing 608 U/mL collagenase I, 187.5 U/mL collagenase XI, 90 U/mL hyaluronidase, and 90 U/mL DNase, for 1 h at 37°C with agitation as described [15, 17]. Digestion was quenched and filtered through a 70-μm nylon filter (BD Biosciences, San Jose, USA) using 3 mL FACS buffer ($PBS^{-/-}$ buffer containing 0.02% bovine serum albumin). Cells were then centrifuged at 1,200 rpm for 10 min at 4°C and were resuspended in 500 μL FACS buffer for staining. Cardiac immune cells were differentiated using anti-CD45 (clone 30-F11; BioLegend, London, UK), anti-CD64 (clone X54-5/7.1; BioLegend), anti-Ly6G (clone 1A8; BioLegend), anti-I-A/I-E (clone M5/114.15.2; BioLegend), anti-CD206 (clone C068C2; BioLegend), anti-Ly6C (clone HK1.4; eBioscience, Dartford, UK), anti-CCR2 (clone 475301; R&D Systems), anti-CD3 (clone 17A2; BioLegend), and anti-CD19 (clone 6D5; BioLegend). Zombie NIR™ Fixable Viability Kit (BioLegend) was used to identify live cells. 10,000 live CD45 events were acquired with a FACSCalibur (BD Biosciences).

Peritoneal exudate was collected from mice that underwent CLP surgery. Peritoneal cells were differentiated using anti-CD45 (clone 30-F11; BioLegend), anti-CD64 (clone X54-5/7.1; BioLegend), anti-Ly6G (clone 1A8; BioLegend), anti-I-A/I-E (clone M5/114.15.2; BioLegend), anti-CD3 (clone 17A2; BioLegend), and anti-CD19 (clone 6D5; BioLegend). Zombie NIR™ Fixable Viability Kit (BioLegend) was used to identify live cells. 10,000 live CD45 events were acquired for analysis.

In a separate set of experiments, cell surface expression of hFPR2 was determined on myeloid cells and lymphocytes collected from the heart and peritoneal cavity. Myeloid cells and lymphocytes were differentiated using anti-CD45 (clone 30-F11; BioLegend), anti-CD11b (clone M1/70; eBioscience), and anti-CD3 (clone 17A2; BioLegend). Human hFPR2 monoclonal antibody (clone GM1D6; Aldevron, Bath, UK) was conjugated with AF647 fluorophore using an Alexa Fluor™ 647 Antibody Labeling Kit (Thermo Fisher Scientific).

Positive populations were gated based on fluorescence minus one controls, in which samples were stained with all the fluorophores in the antibody panel but minus one of them. The fluorescence minus one control scatter dot plots are shown in online supplementary Figure 3.

All FCS files were analysed using FlowJo analysis software (version 9.2, TreeStar Inc., Ashland, USA). Median fluorescence intensity (MFI) of hFPR2 was calculated by FlowJo on the gated myeloid cells and lymphocytes, respectively.

Bacteria Counting

The mice were euthanized with CO_2 at 24 h after the CLP. The skin of the abdomen was cut open after disinfection, without causing damage to the muscle layer, and the peritoneal cavity was washed with 4 mL of sterile PBS with 2 mM EDTA. Aliquots of serial log dilutions of the obtained peritoneal lavage were plated on LB broth with agar plates, colony-forming units were counted after incubation in a humidified incubator at 37°C for 24 h; the results were expressed as the number of colony-forming units per mL [18].

AnxA1 and LXA_4 ELISA

Quantification of mouse AnxA1 in peritoneal lavages was obtained using ELISA kit purchased from Cloud-Clone Corp., Houston, USA. LXA_4 was assayed using ELISA kit purchased from Cambridge Bioscience, Cambridge, UK. In both cases, procedures were carried out according to the manufacturer's instructions.

Polymerase Chain Reaction

Fpr2 WT and humanized alleles and Cre were detected by polymerase chain reaction (PCR) (online suppl. Fig. S4a, b). DNA from the ear clips was extracted using an Extract-N-Amp Tissue PCR Kit (Merck Life Science UK Limited). Primers were purchased from Merck Life Science UK Limited (online suppl. Table S1). PCR amplification was conducted using REDExtract-N-Amp™ PCR ReadyMix™ (Merck Life Science UK Limited). The amplified samples were run on 3.5% agarose gel with GeneRuler DNA Ladder Mix (Thermo Fisher Scientific) at 100 voltage.

Real-Time PCR RNA Quantification

The mRNA expression of *hFpr2* and *mFpr2* was determined in mouse liver tissue. mRNA was extracted with an RNeasy Mini Kit (Qiagen, Manchester, UK), and total RNA concentration and quality were determined with an ND-1000 spectrophotometer (Nano Drop Technologies, Wilmington, DE, USA). cDNA was synthesized from 1,000 ng RNA using the SuperScript™ VILO™ cDNA Synthesis Kit (Invitrogen, Dartford, UK) according to the manufacturer's instructions. Real-time quantitative PCR was performed using SYBR Green ROX Mix (Thermo Fisher Scientific) in the StepOnePlus™ thermal cycler (Applied Biosystems, Dartford, UK). Primers were purchased from Merck Life Science UK Limited (Dorset, UK) (online suppl. Table S2). Each sample was measured in triplicate. Cycle threshold values were determined by StepOne software. Ct values were normalized using *GAPDH* as housekeeping gene. Fold changes of *mFpr2* and *hFpr2* in hFpr2 mice were calculated relative to age-matched C57BL/6 WT mice.

Statistics

All values described in the text and figures are presented as mean ± standard error of the mean of n observations, where n represents the number of animals studied. Statistical analysis was performed using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA). One-way ANOVA followed by Bonferroni's post hoc test or unpaired Student's t-test was used to compare intergroup differences. Comparing results were considered statistically significant when $p < 0.05$.

Results

hFPR2 Deficiency in Myeloid Cells Exacerbates Cardiac Dysfunction and Clinical Outcome in Mice with Polymicrobial Sepsis

To study whether hFPR2 in myeloid cells plays essential roles in the host response to sepsis, hFPR2 mice and myeloid cell-specific hFPR2 KO mice were generated and subjected to sham or CLP surgery. In hFPR2 mice, mRNA expression of hFpr2 in the liver was ~52-fold higher relative to age-matched C57BL/6 WT mice, whereas mFpr2 was ~0.2-fold lower (online suppl. Fig. S5). Fpr2 WT and humanized alleles and Cre were detected by PCR (online suppl. Fig. S4a, b). No difference was detected in the number of circulating leukocytes between hFPR2 mice and myeloid cell-specific hFPR2 KO mice, and these include neutrophils, lymphocytes, monocytes, and eosinophils (Table 1).

Left ventricular functionality was analysed in vivo using echocardiography. Mice subjected to sham surgery did not show significant differences in percentage EF or FS (Fig. 1a–c) between the two genotypes. hFPR2 CLP mice showed significant reduction in both parameters, indicating the development of systolic dysfunction (Fig. 1a–c). When compared with hFPR2 CLP mice, KO deficiency specifically in myeloid cells exacerbated cardiac dysfunction as indicated by a significant reduction in percentage for both EF and FS (Fig. 1a–c). Additionally, when compared with hFPR2 mice, myeloid cell-specific hFPR2 KO mice presented worse clinical scores (Fig. 1d). These data suggest that hFPR2 deficiency in myeloid cells aggravates cardiac dysfunction and clinical outcome in response to microbial sepsis.

hFPR2 Deficiency in Myeloid Cells Modulates Cardiac Immune Cell Infiltration in Mice with Polymicrobial Sepsis

To gain a better mechanistic insight into the aggravated cardiac dysfunction, we investigated whether cardiac immune cell infiltration was altered in myeloid cell-specific hFPR2 KO CLP mice by flow cytometry (Fig. 2a). No significant difference was observed in cardiac $Ly6G^+CD64^-$ neutrophils (Fig. 2b) or $CD64^+Ly6G^-$ monocytes/macrophages infiltration (Fig. 2c). However, when compared with hFPR2 CLP mice, KO CLP mice had significantly increased $CD64^+MHCII^-CCR2^+Ly6C^+$ pro-inflammatory monocytes within the heart (Fig. 2d, e). Moreover, there was a trend for a reduction of cardiac $CD64^+Ly6G^-CD206^+$ M2-like macrophages in hFPR2 KO CLP mice ($p = 0.05$; Fig. 2f, g). No significant changes were observed in cardiac $CD3^+$ T cells or $CD19^+$ B cells (Fig. 2h, i).

hFPR2 Deficiency in Myeloid Cells Aggravates the Host Immune Response to Polymicrobial Sepsis in Peritoneum and Increases Bacterial Load

Analysis of peritoneal exudates by flow cytometry showed a trend for increased peritoneal $Ly6G^+CD64^{-}$ neutrophils at 24 h post-CLP in myeloid cell-specific hFPR2 KO CLP mice compared to hFPR2 CLP mice ($p = 0.09$; Fig. 3a, b), while $CD64^+Ly6G^-$ monocytes/macrophages were significantly decreased (Fig. 3a, c). Additionally, when compared with hFPR2 CLP mice, MHC II^+ macrophages were increased in hFpr2 KO CLP mice, while MHC II^- macrophages were reduced (Fig. 3d–f). No significant change was observed in peritoneal $CD3^+$ T cells or $CD19^+$ B cells (Fig. 3g, h).

Table 1. Characterization of circulating leukocytes in hFPR2 mice (hFPR2 flox/flox) and myeloid cell-specific hFPR2 KO mice (LysM-cre; hFPR2 flox/flox)

Parameter	hFpr2 mice	Myeloid cell-specific hFpr2 KO mice
White blood cells, K/μL	7.41±0.47	7.79±1.37
Neutrophils, K/μL	1.08±0.28	1.20±0.50
Lymphocytes, K/μL	5.84±0.61	6.08±1.21
Monocytes, K/μL	0.28±0.06	0.27±0.07
Eosinophils, K/μL	0.19±0.04	0.24±0.03
Neutrophils, %	15.48±4.73	14.82±5.03
Lymphocytes, %	78.15±4.8	78.38±5.74
Monocytes, %	3.83±0.72	3.76±0.61
Eosinophils, %	2.50±0.48	2.74±0.54

All data are expressed as means ± SEM for *n* number of observations. *N* = 6 for hFPR2 mice group, *n* = 5 for myeloid cell-specific hFPR2 KO mice group. Data were analysed by unpaired Student's *t*-test.

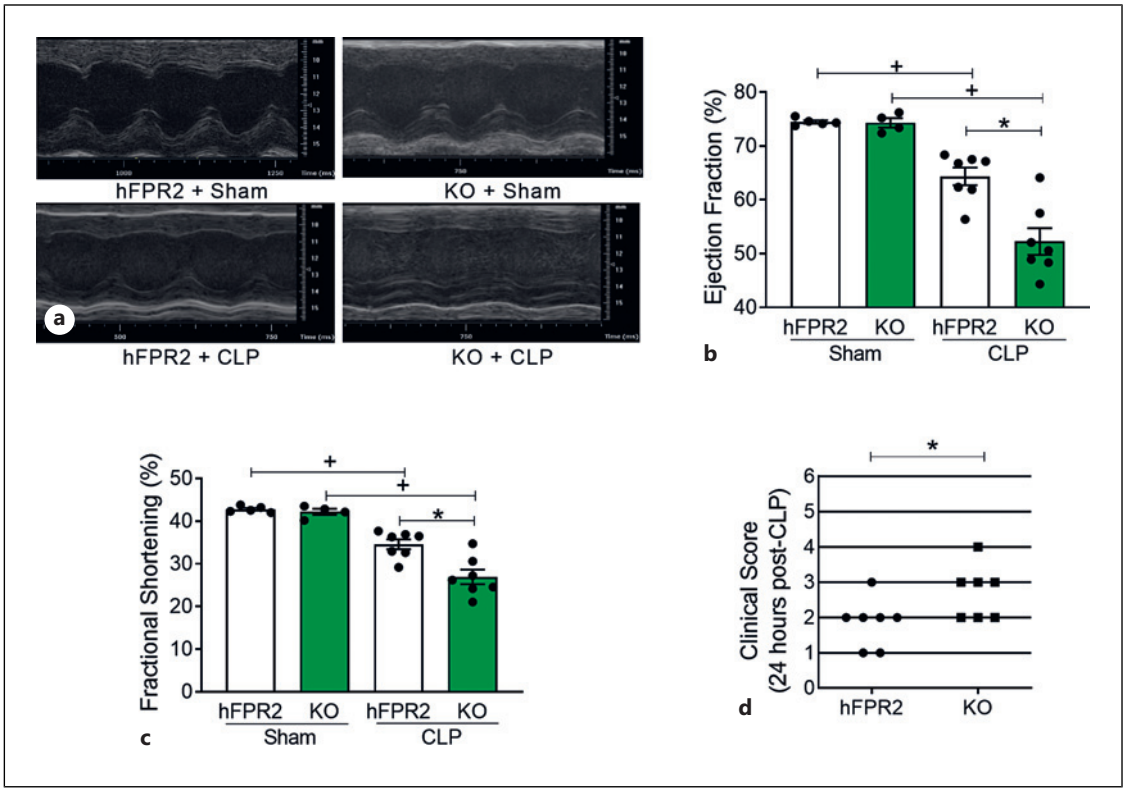

Fig. 1. hFPR2 deficiency in myeloid cells exacerbates polymicrobial sepsis-induced cardiac dysfunction and sepsis-associated clinical scores in mice. hFPR2 mice (hFPR2 loxP/loxP) and myeloid cell-specific hFPR2 KO mice (LysM-Cre/+; hFPR2 loxP/loxP) underwent sham or CLP surgery. Cardiac function was assessed at 24 h. **a** Representative M-mode echocardiograms and percentages of (**b**) ejection fraction and (**c**) fractional shortening. The following groups were studied: hFPR2 + sham (n = 5); hFPR2 KO + sham (n = 4); hFPR2 + CLP (n = 7), and hFPR2 KO + CLP (n = 7). All data are represented as means ± SEM. Data were analysed by one-way ANOVA followed by Bonferroni's post hoc test. $^{+}p < 0.05$ represents comparison versus respective sham groups; $^{*}p < 0.05$ represents comparison versus hFpr2 + CLP group. **d** At 24 h post-CLP, mice were scored for the presence or absence of six different macroscopic signs of sepsis, namely, lethargy, piloerection, tremors, periorbital exudates, respiratory distress, and diarrhoea. A clinical score >3 is considered as severe sepsis. Data are from 14 mice and were analysed by unpaired Student's *t*-test. $^{*}p < 0.05$ versus hFPR2 + CLP group. SEM, standard error of the mean.

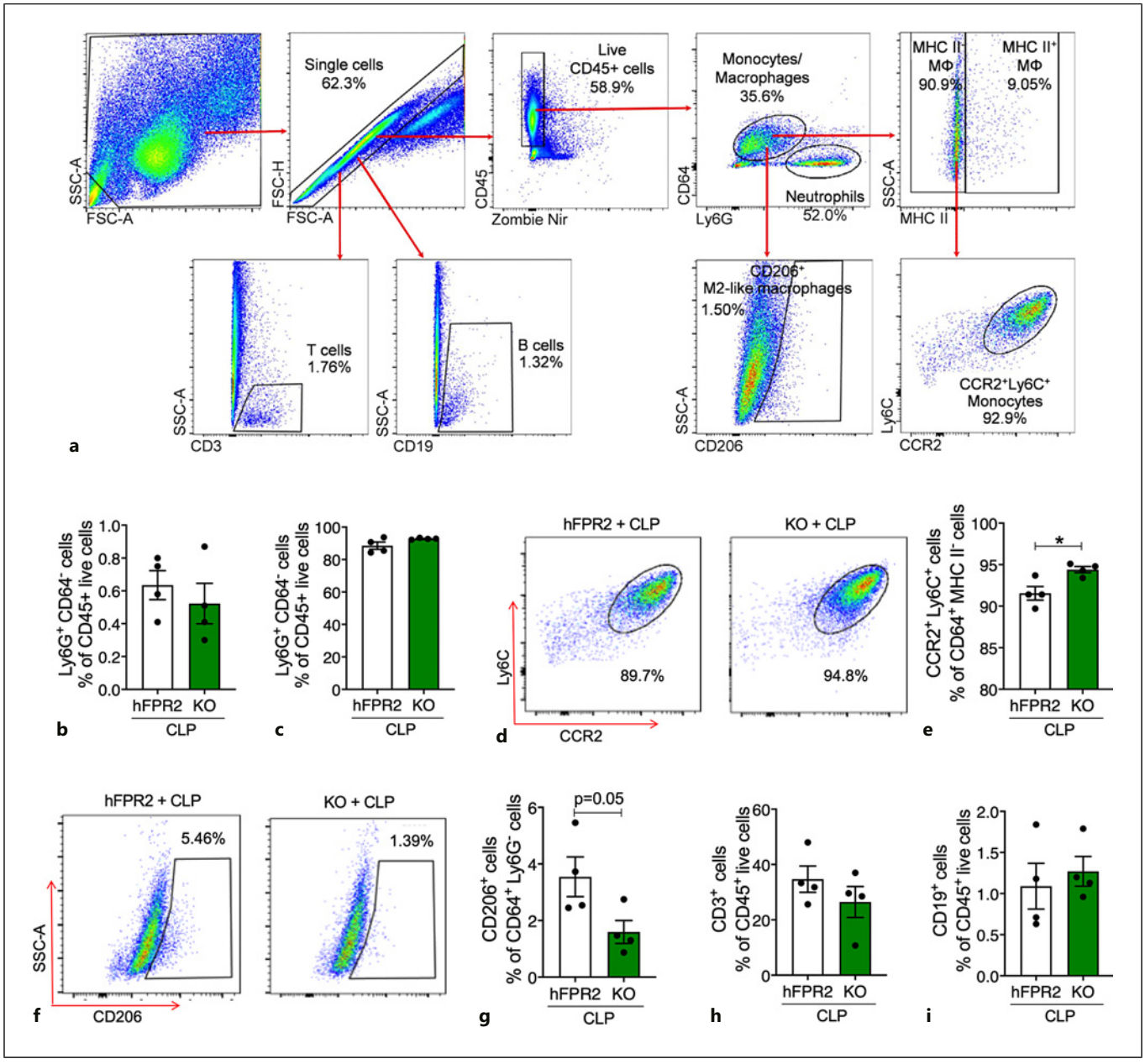

Fig. 2. Increased CCR2$^+$Ly6C$^+$ pro-inflammatory monocytes and reduced CD206$^+$ macrophages in myeloid cell-specific hFPR2 KO mouse heart underwent CLP. hFPR2 mice (hFPR2 loxP/loxP) and myeloid cell-specific hFPR2 KO mice (LysM-Cre/+; hFPR2 loxP/loxP) underwent sham or CLP surgery. Heart samples were collected at 24 h. **a** Flow cytometry gating strategy. Peritoneal leukocyte samples were used to demonstrate the gating strategy, apart from that for cardiac CD64$^+$MHCII$^-$CCR2$^+$Ly6C$^+$ pro-inflammatory monocytes, which was demonstrated using a heart sample. The rest of the gating strategy was also applied to the heart. Values shown in the scattergrams are the mean values of the data collected from both hFPR2 and hFPR2 KO mice subjected to CLP. **b** Cumulative data for cardiac Ly6G$^+$CD64$^-$ neutrophils and (**c**) CD64$^+$Ly6G$^-$ monocytes/macrophages in hFPR2 and hFPR2 KO mice subjected to CLP. **d** Scattergrams illustrating pro-inflammatory monocyte (identified as CD64$^+$MHCII$^-$CCR2$^+$ Ly6C$^+$) positive events in the heart from hFpr2 and KO mice subjected to CLP. **e** Cumulative data for cardiac CD64$^+$MHCII$^-$ CCR2$^+$Ly6C$^+$ pro-inflammatory monocytes. **f** Scattergrams illustrating M2-like macrophage (identified as CD64$^+$Ly6G$^-$CD206$^+$) positive events in the heart from hFPR2 and hFPR2 KO mice subjected to CLP. **g** Cumulative data for cardiac CD64$^+$Ly6G$^-$CD206$^+$ M2-like macrophages. Cumulative data for (**h**) cardiac CD3$^+$ T cells and (**i**) CD19$^+$ B cells. $N = 4$ per group. All data are represented as means ± SEM. Data were analysed by unpaired Student's t-test. *$p <$ 0.05 versus hFPR2 + CLP group.

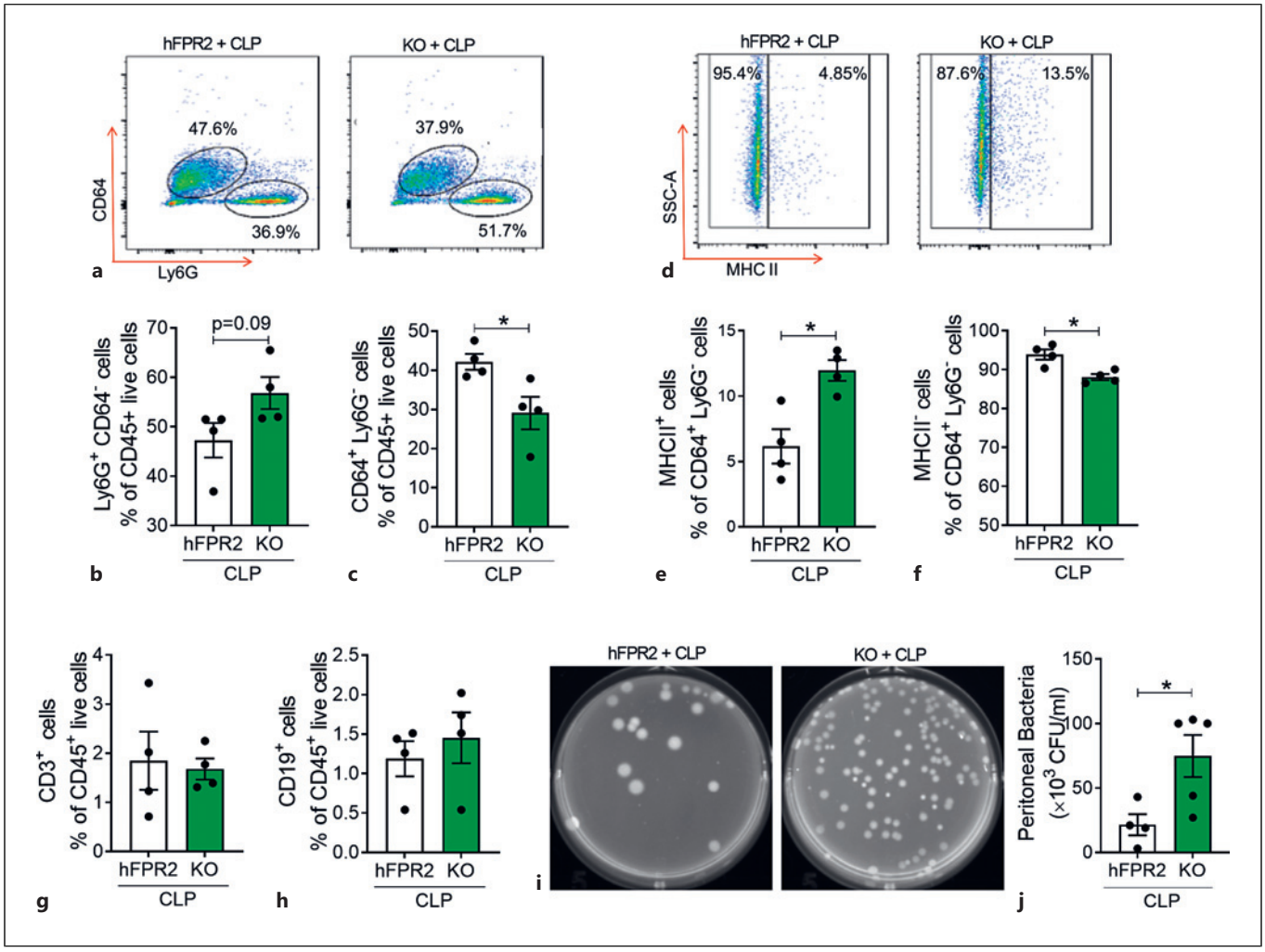

Fig. 3. hFPR2 deficiency in myeloid cells reduced peritoneal monocytes/macrophages recruitment, reduced MHC II$^-$ macrophage subset, and impaired peritoneal bacterial clearance in mice with CLP. hFPR2 mice (hFpr2 loxP/loxP) and myeloid cell-specific hFPR2 KO mice (LysM-Cre/+; hFpr2 loxP/loxP) underwent sham or CLP surgery. Peritoneal exudates were collected at 24 h. **a** Scattergrams illustrating peritoneal Ly6G$^+$CD64$^-$ neutrophils and CD64$^+$Ly6G$^-$ monocytes/macrophages in hFPR2 and myeloid cell-specific hFPR2 KO mice subjected to CLP. **b**, **c** Cumulative data for peritoneal Ly6G$^+$CD64$^-$ neutrophils and CD64$^+$Ly6G$^-$ monocytes/macrophages. **d** Scattergrams illustrating MHC II$^-$ macrophage and MHC II$^+$ macrophage positive events in peritoneal lavages from hFpr2 and KO mice subjected to CLP. **e**, **f** Cumulative data for peritoneal MHC II$^-$ macrophages and MHC II$^+$ macrophages. **g**, **h** Cumulative data for peritoneal CD3$^+$ T cells and CD19$^+$ B cells. **i** Representative images of colony-forming units (CFU). **j** Measurement of bacteria levels in peritoneal lavages 24 h post-CLP from hFPR2 and hFPR2 KO mice subjected to CLP. N = 4–5 per group. All data are represented as means ± SEM. Data were analysed by unpaired Student's t-test. *$p < 0.05$ versus hFPR2 + CLP group.

As uncontrolled bacterial load contributes to a worse outcome in sepsis [10], we investigated whether hFPR2 deficiency in myeloid cells could affect bacterial load in mice subjected to polymicrobial sepsis. Interestingly, when compared to hFPR2 CLP mice, myeloid cell-specific hFPR2 KO CLP mice exhibited higher peritoneal bacterial load as quantified by colony counts (Fig. 3i, j), indicative of impaired bacterial clearance during experimental sepsis.

Following CLP, the levels of MFI of hFPR2 on the surface of both cardiac and peritoneal myeloid cells from hFPR2 KO mice were comparable to the hFPR2 MFI in unstained cells (shown in the histograms of Fig. 4a, b), indicating the full knockout of hFPR2 from myeloid cells. Notably, a ~twofold increase in hFPR2 cell surface expression was measured in both cardiac and peritoneal myeloid cells, relative to myeloid cell-specific hFPR2 KO CLP mice

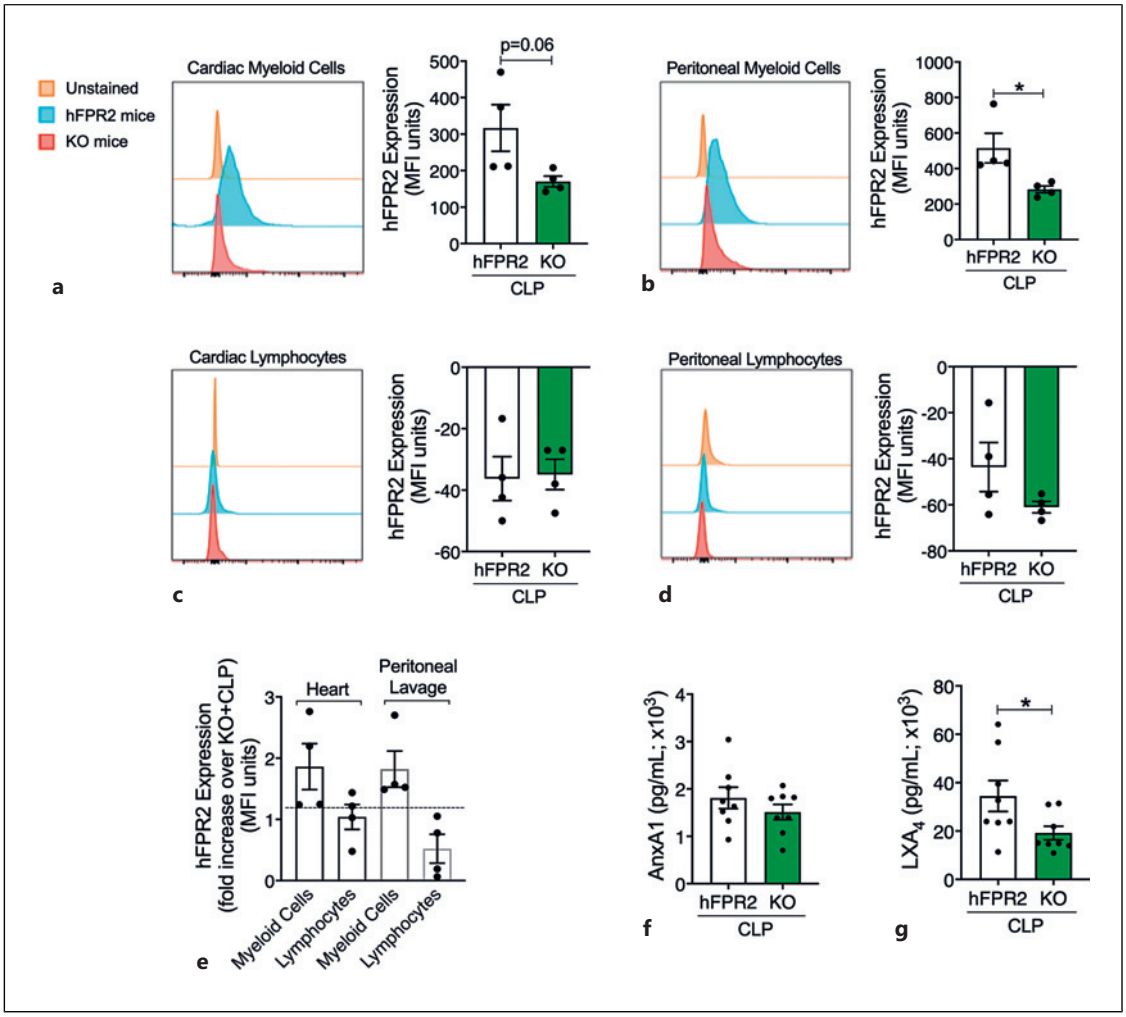

Fig. 4. Lack of hFPR2 upregulation and lipoxin A_4 production in myeloid cell-specific hFPR2 KO mice subjected to CLP. hFPR2 mice (hFPR2 loxP/loxP) and myeloid cell-specific hFPR2 KO mice (LysM-Cre/+; hFPR2 loxP/loxP) underwent CLP surgery. The heart and peritoneal exudates were collected at 24 h. hFpr2 cell surface expression was assessed in both $CD11b^+$ myeloid cells and $CD3^+$ lymphocytes in the heart (**a**, **b**, **e**) and peritoneal lavage (**c**, **d**, **e**) in hFPR2 and hFPR2 KO mice subjected to CLP. **e** Fold changes of hFPR2 expression in different cell types in hFPR2 mice + CLP were calculated relative to hFPR2 KO mice + CLP. Annexin A1 (AnxA1) (**f**) and lipoxin A_4 (**g**) levels in peritoneal exudates during polymicrobial sepsis. **a**–**e** $N = 4$ per group. **f**, **g** $N = 8$ per group. All data are represented as means ± SEM. Data were analysed by unpaired Student's *t*-test. $^*p < 0.05$ versus hFPR2 + CLP group.

(Fig. 4a, b, e). This indicates presence and upregulation of hFPR2 in myeloid cells that play non-redundant roles in preserving cardiac function and improving host immune response in hFpr2-competent CLP mice. However, there was no difference in hFPR2 expression in lymphocytes between genotypes (Fig. 4c, d).

We then analysed the levels of the FPR2 agonists AnxA1 and LXA_4 in mice subjected to CLP. At 24 h post-CLP, peritoneal levels of AnxA1 were comparable between genotypes. However, LXA_4 levels were significantly reduced in myeloid cell-specific hFPR2 KO mice (Fig. 4f, g). Together, these data demonstrate that myeloid FPR2 plays a crucial role in dampening cardiac dysfunction and improving host immune response.

hrAnxA1 Improves Cardiac Function in hFPR2 Mice Subjected to Polymicrobial Sepsis but Has Limited Beneficial Effects in Myeloid Cell-Specific hFPR2 KO Mice

In the second part of the study, we investigated the therapeutic potential of hrAnxA1 in hFPR2 mice, complementing our previous study where a peptide was used [10]

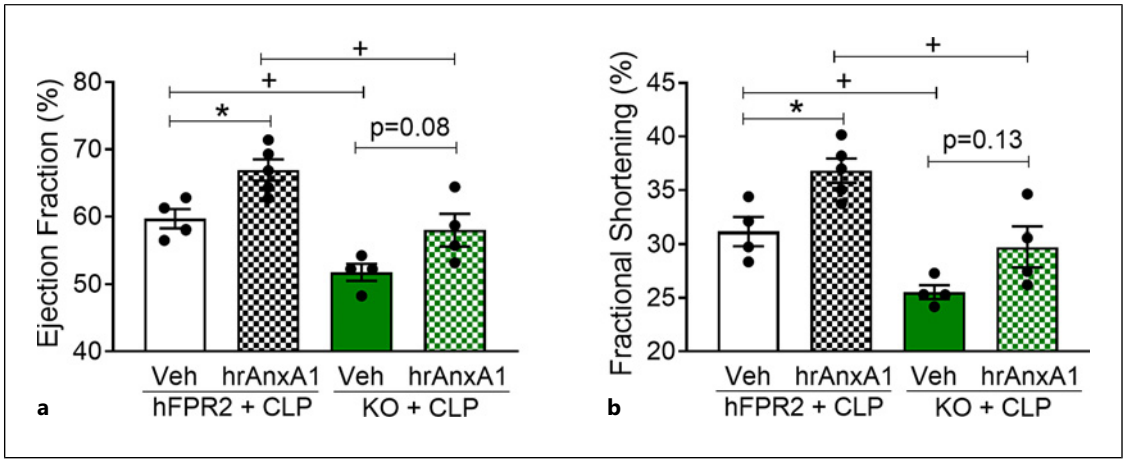

Fig. 5. Human-recombinant annexin A1 (hrAnxA1) improves cardiac function in hFPR2 mice subjected to polymicrobial sepsis but has limited beneficial effects in myeloid cell-specific hFPR2 KO mice. hFPR2 mice and myeloid cell-specific hFpr2 KO mice were subjected to CLP surgery at time 0 and treated with hrAnxA1 (2 μg/mouse, i.v.) or vehicle (100 μL saline) at 1-hour and 6-hour post-CLP surgery. Cardiac function was assessed at 24 h. Percentages of (**a**) ejection fraction and (**b**) fractional shortening. The following groups were studied: hFPR2 + CLP + vehicle ($n = 4$); hFPR2 + CLP + hrAnxA1 ($n = 5$); hFPR2 KO + CLP + vehicle ($n = 4$); and hFPR2 KO + CLP + hrAnxA1 ($n = 4$). All data are represented as means ± SEM. Data were analysed by one-way ANOVA followed by Bonferroni's post hoc test. $^{*}p < 0.05$ represents comparison versus vehicle groups; $^{+}p < 0.05$ represents comparison between genotypes.

and a more recent study on cardiomyopathy in arthritis, where this dose of AnxA1 was used [15]. Administration of hrAnxA1 (1 μg per mouse, i.v.) improved cardiac function (Fig. 5a, b), whereas no significant improvement was quantified in myeloid cell-specific hFPR2 KO mice. When compared with vehicle groups, administration of hrAnxA1 reduced cardiac $Ly6G^{+}CD64^{-}$ neutrophils in both mouse genotypes (Fig. 6a), whereas cardiac $CD64^{+}Ly6G^{-}$ monocytes/macrophages were only upregulated in hFPR2 CLP mice (Fig. 6b). The protein did not reduce cardiac $CD64^{+}MHCII^{-}CCR2^{+}Ly6C^{+}$ pro-inflammatory monocytes in either mouse genotype (Fig. 6c), which means the increased number of this cell population within the heart (Fig. 2d, e) persisted in KO CLP mice. hrAnxA1 increased cardiac $CD64^{+}Ly6G^{-}CD206^{+}$ M2-like macrophages in both phenotypes (Fig. 6d).

In peritoneal exudates, administration of hrAnxA1 reduced $Ly6G^{+}CD64^{-}$ neutrophils in myeloid cell-specific hFPR2 KO CLP mice (Fig. 6e) and increased $CD64^{+}Ly6G^{-}$ monocytes/macrophages in both mouse genotypes (Fig. 6f). However, hrAnxA1 reduced MHC II^{+} and increased MHC II^{-} macrophages only in hFPR2 CLP but not in KO CLP mice (Fig. 6g, h), indicating activation of myeloid hFPR2 is important for macrophage maturation and/or phenotype switch. No significant difference was observed in peritoneal bacterial load (Fig. 6i).

Discussion

Impaired resolution of inflammation has been associated with the development of cardiac dysfunction in sepsis [10, 18, 19], which predicts high mortality in septic patients [3]. Moreover, the differential expression of endogenous pro-resolving mediators correlates with survival in septic patients [20]. We have reported that a global knockout for Fpr2 impaired host response and aggravated sepsis-induced cardiac dysfunction in mice [10]. This prototypic pro-resolving receptor is highly expressed in myeloid cells and plays crucial roles in promoting resolution of inflammation [11]. In this study, we took advantage of an hFPR2 mouse colony to generate hFPR2 deficiency, specifically in myeloid cells which displays an impaired resolution and exacerbated polymicrobial sepsis-induced cardiac dysfunction.

Fpr2 plays a non-redundant protective role against cardiac function in the setting of sepsis; moreover, Fpr2 global knockout also showed exacerbated kidney injury indicated by increased plasma urea and creatinine, as well as augmented liver dysfunction indicated by increased levels of plasma alanine aminotransferase and aspartate aminotransferase markers following CLP [10]. In the brain, Fpr2 knockout demonstrated exaggerated cerebrovascular responses to LPS indicated by increased leukocyte rolling and adhesion in mice [21]. Of great relevance, in patients

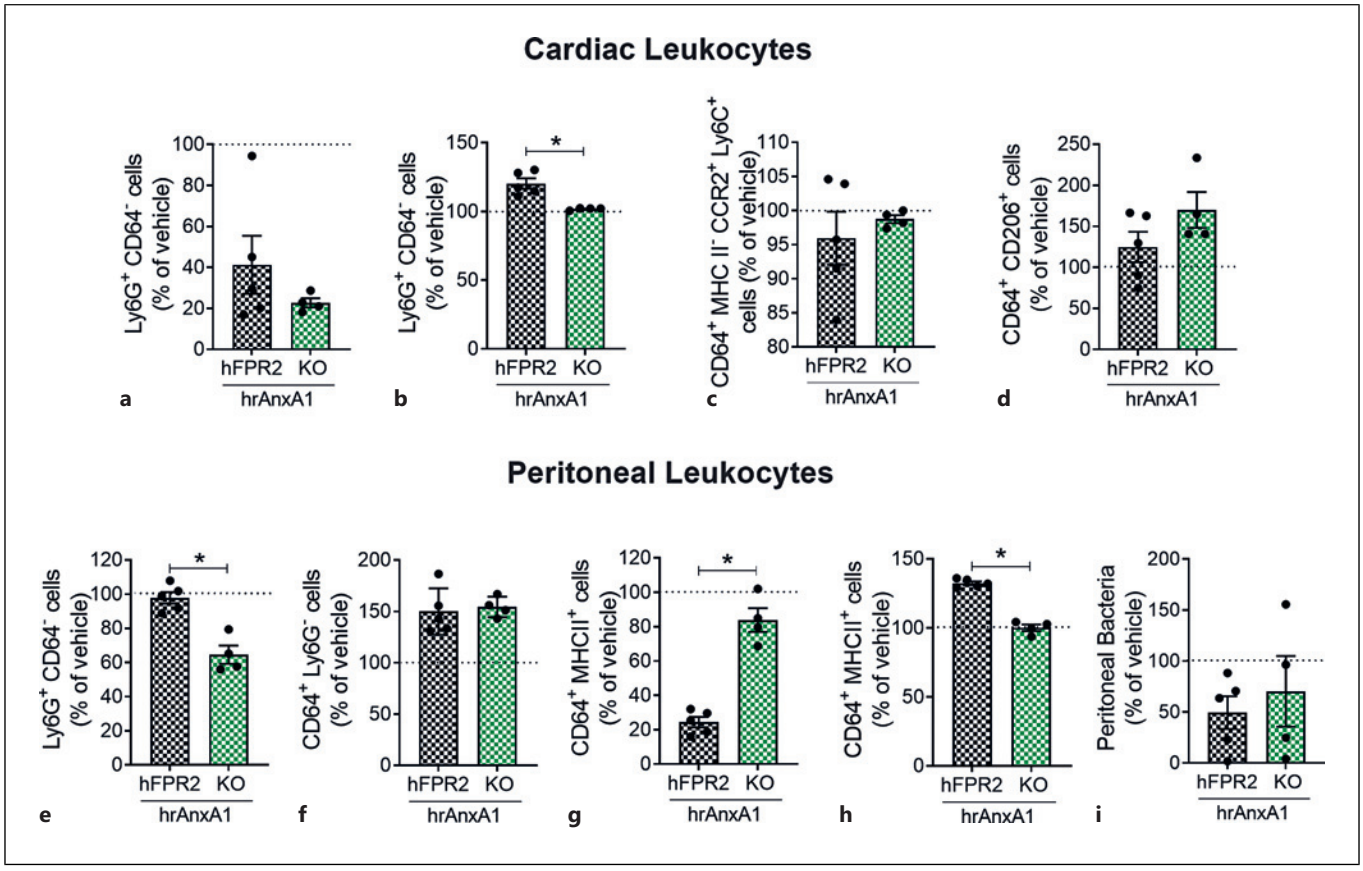

Fig. 6. hrAnxA1 modulates the host response in both hFPR2 mice and myeloid cell-specific hFPR2 KO mice subjected to polymicrobial sepsis. hFPR2 mice and myeloid cell-specific hFPR2 KO mice were subjected to CLP surgery at time 0 and treated with hrAnxA1 (2 μg/mouse, i.v.) or vehicle (100 μL saline) at 1-hour and 6-hour post-CLP. The heart and peritoneal exudates were collected at 24 h. **a**–**d** Cardiac immune cells from hFPR2 and hFPR2 KO mice subjected to CLP and treated with hrAnxA1 or vehicle. Cumulative data for cardiac $Ly6G^+CD64^-$ neutrophils (**a**), $CD64^+Ly6G^-$ monocytes/macrophages (**b**), $CD64^+MHCII^-CCR2^+Ly6C^+$ pro-inflammatory monocytes (**c**), and $CD64^+Ly6G^-CD206^+$ M2-like macrophages (**d**). **e**–**h** Peritoneal leukocytes. Cumulative data for peritoneal (**e**) $Ly6G^+CD64^-$ neutrophils, $CD64^+Ly6G^-$ monocytes/macrophages (**f**), MHC II^+ macrophages (**g**), and MHC II^- macrophages (**h**). **i** Measurement of bacteria levels in peritoneal lavages 24 h post-CLP from hFPR2 and hFPR2 KO mice treated with hrAnxA1 or vehicle. $N = 4–5$ per group. All data are represented as means ± SEM. Data were analysed by unpaired Student's *t*-test. $^*p < 0.05$ versus hFPR2 flox/flox mice + CLP + hrAnxA1 group.

with severe trauma, genetic variants of the FPR2 gene are associated with significantly higher risk of sepsis; in particular, patients who carry the A allele of rs11666254 are more susceptible to sepsis than individuals who carry the G allele. Interestingly, this single-nucleotide polymorphism could not only reduce the promoter activity of the FPR2 gene, but it is also associated with lower FPR2 expression and higher TNF-a production from circulating leukocyte in response to bacterial lipoprotein stimulation [9].

In the current study, absence of hFPR2 in myeloid cells was associated with a significant increase in $CD64^+MHCII^-CCR2^+Ly6C^+$ pro-inflammatory monocytes within the heart, as well as increased peritoneal $Ly6G^+CD64^-$neutrophils: these cellular changes could exacerbate systemic and local cardiac inflammation, which leads to aggravated cardiac dysfunction [22]. Cardiac $CD64^+Ly6G^-CD206^+$ M2-like macrophages were reduced in myeloid cell-specific hFPR2 KO CLP mice. This is in line with our finding in the peritoneal exudates, where MHC II^+ macrophages were increased in myeloid cell-specific hFPR2 KO CLP mice, while MHC II^- macrophages were reduced. MHC II^- macrophages express more M2-associated genes including CD36, CD163, MerTK, and Arg1, while MHC II^+ macrophages express more M1-associated genes including CD80, CD86, and Il1b [23]. Classically activated M1-like macrophages express pro-inflammatory cytokines

(e.g., TNF-α, IL-1β, IL-6) which promote inflammation, whereas the alternatively activated M2-like macrophages express anti-inflammatory cytokines (e.g., IL-10, TGF-β) and are involved in the resolution of inflammation and tissue repair [24]. Additionally, M2 macrophages produce more pro-resolving lipid mediators like RvE2, RvD5, MaR1, and LXA_4 compared with M1 but less pro-inflammatory prostanoids and leukotrienes [25]. FPR2 is highly expressed in macrophages [26]; therefore, in our study, the reduced MHC II^- macrophages or M2-like macrophages were likely a direct consequence of hFPR2 deficiency in this cell type, indicating that FPR2 can control the phenotypic switch of this cell type. Consistent with our observation, in myeloid cell-specific hFPR2 KO mice underwent hindlimb ischaemia, and macrophages isolated from ischaemic muscles show downregulated vascularization-related genes, indicating FPR2 expression in macrophages is also required for the adoption of a pro-angiogenic phenotype [12].

In the pharmacological study, hrAnxA1 reduced MHC II^+ and increased MHC II^- macrophages only in hFPR2 septic mice, but this macrophage phenotype switch was absent in myeloid cell-specific hFpr2 KO septic mice; again, it suggests that presence of FPR2 is crucial for phenotype switch in macrophages. Recent studies have reported the ability of endogenous AnxA1 to induce a macrophage phenotype switch. For instance, in settings of muscle injury and repair, hrAnxA1 promotes tissue repair by inducing a reparative phenotype of macrophage, an effect mediated through FPR2 activation and AMPK signalling [26]. In the same study, human monocyte-derived macrophages expressed high levels of FPR2 when polarized toward an inflammatory phenotype, but they express much lower FPR2 when polarized to a reparative phenotype, suggesting the biological function of the receptor was no longer needed in the latter case. Congruent with these observations, in settings of myocardial infarction over a 7-day protocol, delivery of hrAnxA1 exerts cardioprotection by inducing an angiogenic phenotype of heart macrophages [27]; this effect resonates with the present study, conducted with myeloid cell-specific hFPR2 KO mice which underwent hindlimb ischaemia. A recent study has shown that a unique subpopulation of cardiac resident macrophages (CD163+RETNLA+) with high expression of TREM2 is protective against sepsis-induced cardiomyopathy by scavenging cardiomyocyte-ejected dysfunctional mitochondria [28]. Therefore, it would be interesting to investigate whether hFPR2 deficiency in myeloid cells and hrAnxA1 treatment would impact also on this macrophage subpopulation in future studies.

The reduced $CD64^+Ly6G^-$ monocytes/macrophages recruitment to the peritoneal cavity in myeloid cell-specific hFPR2 KO septic mice is functionally linked to a higher neutrophil to monocytes/macrophages ratio and insufficient bacterial removal. Altogether, this is indicative of an inadequate host response, so that, as a consequence, bacterial clearance from the peritoneal cavity remains significantly impaired in these mice. Such an inadequate dealing with the infection leads to a vicious cycle contributing to amplification of inflammation as well as impaired resolution. It is tempting to propose that, in absence of FPR2, monocyte recruitment is defective, and this delays resolution through an ineffectual bacterial killing and removal. In addition, Fpr2/3 deficiency in neutrophils directly impaired their phagocytic functions [10] which may, at least in part, contribute to the increased bacterial count in our setting. Other studies have shown that AnxA1, LXA_4, and resolving D1 enhance particle phagocytosis and efferocytosis in immune cells in a FPR2-dependent manner [29, 30].

Relevantly, two FPR2 ligands like LXA_4 and AnxA1 have been described to promote non-phlogistic recruitment of monocytes [31, 32]. We have observed that LXA_4 levels in peritoneal lavage were reduced in myeloid cell-specific hFPR2 KO septic mice, which may have contributed to the reduced monocytes/macrophages recruitment. This dataset indicates the existence of a feed-forward circuit centred on FPR2 which then, once activated, can lead to generation of a pro-resolving agonist like LXA_4. We could speculate that in our experimental settings, peritoneal AnxA1 (which is not modified between hFPR2 mice and KO mice) could activate endogenous FPR2 to incite the signal. Such a control function of FPR2 has been reported in the context of ischaemia reperfusion, where aggregation of platelets with neutrophils would provide the biosynthetic pathway for LXA_4 synthesis [33]. In this study, it was noted that FPR2 expression in neutrophils, instead of platelets, was central to the generation of LXA_4. We can speculate the transcellular elements in the CLP peritoneum which could underpin the biosynthesis of this lipid mediator, but large presence of myeloid cells together with stromal cells and endothelial cells would bring the two enzymes required for the synthesis. Finally, it is worth remarking here that this AnxA1-FPR2-LXA_4 protective circuit can also operate in the other direction, with both in vitro and in vivo data showing how the lipid mediator agonist can mobilize AnxA1 from target cells and tissues [34, 35].

Having characterized that hFPR2 deficiency specifically in myeloid cells impaired host response and amplified polymicrobial sepsis-induced cardiac dysfunction, in the second part of the study, we investigated the therapeutic

effects of hrAnxA1 in both hFPR2 and myeloid cell-specific hFPR2 KO mice. We have a long-dated interest in FPR2 and its agonist AnxA1, which stems from the observation, made in 2002, that the pro-resolving protein AnxA1 activates this specific GPCR to downregulate neutrophil recruitment both in vitro and in vivo [36]. Further studies then identified this receptor as a major target for AnxA1 on a variety of pro-resolving effects (see recent reviews [11, 37]). AnxA1 binds FPR2 selectively but not FPR1 [38] and by and large through this receptor favours the resolution process by inhibiting neutrophil trafficking, enhancing macrophage microorganism phagocytosis, promoting neutrophil apoptosis and subsequent macrophage efferocytosis [39]. The significant improvement of cardiac function afforded by hrAnxA1 in the present study could be an indirect effect through reduced systemic inflammation and/or a direct action on the myocardium. For instance, reduction of neutrophil recruitment [40], promotion of monocyte recruitment [32], subsequent favouring of macrophage polarization toward an M2-like phonotype [26] could all contribute to an adequate host response enabling proper dealing with the infection. At the same time, AnxA1 and AnxA1 peptide Ac2-26 also exert direct effects on the heart, as seen in experimental myocardial ischaemia reperfusion injury [41, 42]. These acute (~2 h) cardioprotective effects are downstream of FPR2, but not FPR1, engagement [42]. hrAnxA1 is also protective against type 1 diabetes-induced cardiomyopathy, mediated through inhibiting cardiac MAPK signalling and promoting the pro-survival Akt pathway [14]. More recently, we have shown that hrAnxA1 halts the development of cardiac diastolic dysfunction in inflammatory arthritis through modulation of both fibroblast and immune cell phenotype within the heart [15].

Apart from cardioprotective effects, the protection afforded by FPR2 activation is also evident in other organ injuries. In settings of sepsis, AnxA1 peptide Ac2-26 improved kidney function by reducing the levels of NF-κB, TNF-03B1;, IL-103B2;, IL-6, and cleaved caspase −3 and −8 in kidney tissue, while the protective role of Ac2-26 is abolished by the administration of FPR2 antagonist WRW4 or Fpr2-siRNA [43]. In the brain, Ac2-26 mitigated LPS-induced leukocyte adhesion in cerebral venules in WT mouse; however, Ac2-26 loses its effect in Fpr2 global KO mice [21]. FPR2 activation by its agonist WKYMVm not only reverses LPS-induced vascular hyperactivity in mouse aorta rings via reducing NO production but also improves the survival rate in mice bearing pneumosepsis [44]. In settings of hindlimb ischaemia, exogenous RvD1 promotes perfusion recovery in hFPR2-competent mice by inducing pro-angiogenic phenotype of macrophages (discussed in detail as above); however, this protection is abrogated in myeloid cell-specific hFPR2 KO mice secondary to impaired vascularization of ischaemic skeletal muscle and cutaneous wounds [12].

It is noteworthy that human-recombinant AnxA1 we used here is a selective agonist at FPR2, while it does not activate FPR1 [38]. The present study is a continuation of previous work conducted with the global Fpr2 KO mouse [10], where a non-redundant function was revealed for this receptor, in settings of sepsis. In that study, we proved efficacy of an AnxA1-derived peptide (e.g., cleavage-resistant Ac2-50) which also retains the selectivity of the whole protein, thus activating FPR2 and not FPR1 [45]. On these bases, we did not consider the potential engagement of FPR1 in our experiments since it was irrelevant for the therapeutic tools employed. Some confusion has been created due to the fact that the short N-terminal peptide Ac2-26 binds to both FPR2 and FPR1 [38]; however, AnxA1 effects on FPR1 should not be assigned based on data produced with Ac2-26.

Limitation of the Study

Firstly, some of the tissue-protective properties of AnxA1 were lost in myeloid cell-specific hFPR2 KO mice, like the lack of improvement in cardiac function, but others were retained. In fact, hrAnxA1 was able to modulate immune cell responses in myeloid cell-specific hFPR2 KO mice including reducing cardiac and peritoneal neutrophil infiltration and increasing peritoneal macrophages. These effects might be mediated through AnxA1 engagement of FPR2 on other cell types, such as endothelial cells, epithelial cells, smooth muscle cells, and fibroblasts [37]. Therefore, the roles of FPR2 in those cell types in the context of sepsis warrant future investigations. Secondly, while AnxA1 is a selective FPR2 agonist, one cannot exclude the possibility that AnxA1-derived peptides from the N-terminal domain may be generated in vivo and act through mouse Fpr1, which may evoke relevant biological effects on monocytes and neutrophils. Thirdly, this study did not show the mRNA downregulation in myeloid cells due to the technical challenges in sorting highly purified myeloid cells. Notwithstanding this, the functional data provide unequivocable engagement of myeloid FPR2 in the cardioprotection, together with a non-redundant role in the control of the host response.

Conclusions

Selective FPR2 deficiency in myeloid cells exacerbates cardiac dysfunction and clinical outcome in a model of polymicrobial sepsis. These detrimental effects are

attributed to impaired host immune response both at the site of infection and within the heart. Administration of hrAnxA1 improves cardiac function in hFPR2 mice with sepsis but has limited beneficial effects in myeloid cell-specific hFPR2 KO mice, a lack of effect associated with reduced or absent macrophage phenotype switch, a fundamental phenomenon in resolution and tissue repair which seems to be chiefly regulated by this pro-resolving receptor.

Acknowledgments

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Statement of Ethics

This study protocol was reviewed and approved by Animal Welfare Ethical Review Body, Queen Mary University of London, in accordance with the derivatives of both the "Home Office Guidance on the Operation of Animals (Scientific Procedures) Act 1986", and the "Guide for the Care and Use of Laboratory Animals" of the National Research Council. The approval number is PA672E0EE.

Conflict of Interest Statement

M.P. is on the Scientific Advisory Board of ResoTher Pharma AS, who is interested in the development of AnxA1-derived peptides for cardiovascular settings. M.P. consults for Bristol Myers Squibb.

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Author Contributions

J. Chen and M. Perretti conceived and designed research; J. Chen, S. Austin-Williams, C. E. O'Riordan, M. Sugimoto, and P. Claria-Ribas performed the research and acquired the data; J. Chen, C. E. O'Riordan, M. Sugimoto, and P. Claria-Ribas analysed data; L. V. Norling validated hFPR2 transgenic mice; C. Thiemermann provided advice on the echocardiography; all authors contributed to the writing of the paper.

Data Availability Statement

The data that support the findings of this study are available in the methods and supplementary material of this article. Further enquiries can be directed to the corresponding authors.

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