Reduction of NETosis by targeting CXCR1/2 reduces thrombosis, lung injury and mortality in experimental sepsis.

Mohmad Alsabani[1, 2 #]; Simon T Abrams[1,3 #]; Zhenxing Cheng[4,5 #]; Ben Morton[6, 7]; Steven Lane[8]; Samar Alosaimi[1]; Weiping Yu[4]; Guozheng Wang[1,3*]; and Cheng-Hock Toh[1, 9*]

[1]Department of Clinical Infection, Microbiology and Immunology, University of Liverpool, Liverpool, L69 7BE, UK.

[2]College of Applied Medical Sciences, King Saud bin Abdulaziz University for Health Sciences, Riyadh, Saudi Arabia.

[3]Coagulation, Liverpool University Hospitals NHS Foundation Trust, Liverpool, L7 8XP, UK.

[4]The Medical School, Southeast University, Nanjing 210009, China.

[5]Department of Gastroenterology, the First Affiliated Hospital, Anhui Medical University, Hefei 230032, P.R. China

[6]Department of Clinical Science, Liverpool School of Tropical Medicine, Liverpool L3 5QA, UK.

[7]Malawi-Liverpool Wellcome Trust Clinical Research Programme, Blantyre, Malawi.

[8]Medical Statistics, Institute of Translational Medicine, University of Liverpool, Liverpool L7 8XP, UK.

[9]Roald Dahl Haemostasis & Thrombosis Centre, Liverpool University Hospitals NHS Foundation Trust, Liverpool, L7 8XP, UK.

[#]These authors contributed equally to this work.

*Correspondence to Cheng-Hock Toh, Department of Clinical Infection, Microbiology and Immunology, University of Liverpool, Liverpool L69 7BE, UK. Email: toh@liverpool.ac.uk or Guozheng Wang, Department of Clinical Infection, Microbiology and Immunology, Institute of Infection and Global Health, University of Liverpool, Liverpool L69 7BE, UK. Email: wangg@liverpool.ac.uk

Keywords: CXCR1/2, IL-8, NETs, neutrophil depletion, organ injury, reparixin, sepsis.

Short running title: Ex vivo NETs assay for anti-NETosis therapy

Word count: (3280)

EDITORS KEYPOINTS

- Reducing the formation of neutrophils extracellular traps (termed NETosis) may reduce organ injury in sepsis.
- The authors examined whether CXCR1/2 signalling contributes to NETosis in human and murine models of sepsis.
- Circulating CXCR1/2 ligands correlated with ex vivo assays for NETosis in both patients and mice.
- Pharmacological inhibition of CXCR1/2 induced netosis reduced organ injury without impairing bacterial clearance and prevented 100% mortality in mice.
- Assays targeting NETosis may guide personalised therapy in sepsis.

Abstract

Background. Neutrophils extracellular traps (NETs) facilitate bacterial clearance but also promote thrombosis and organ injury in sepsis. We quantified *ex vivo* NET induction in septic humans and murine models of sepsis to identify signalling pathways that may be modulated to improve outcome in human sepsis.

Methods. NET formation in human donor neutrophils was quantified after incubation with plasma obtained from patients with sepsis or systemic inflammation (double-blinded assessment of extracellular DNA by immunofluorescence microscopy). NET formation was correlated with plasma cytokine levels (MultiPlex assay). Experimental sepsis was induced by caecal ligation and puncture or intraperitoneal injection of Escherichia coli in C57/BL6 male mice. The effect of pharmacological inhibition of CXCR1/2 signalling (reparixin) on NET formation, organ injury (hepatic, renal and cardiac biomarkers) and survival in septic mice was examined.

Results. NETs formation was higher following incubation with septic plasma from patients (median:25.0 [10.5-46.5]), compared to plasma obtained from patients with systemic inflammation (median:14.0 [4.0-23.3]; P=0.02). Similar results were observed following incubation of plasma from septic mice with neutrophils isolated from normal mice. Circulating CXCR1/2 ligands correlated with NETosis in patients (interleukin-8; r=0.643) and mice (macrophage inflammatory protein-2, r=0.902). In experimental sepsis, NETs were primarily observed in the lungs, correlating with fibrin deposition (r=0.702) and lung injury (r=0.692). Inhibition of CXCR1/2 using reparixin in mouse sepsis models reduced NET formation, multi-organ injury and mortality, without impairing bacterial clearance.

Conclusion. CXCR1/2 signalling-induced NET formation is a potential therapeutic target in sepsis, which may be guided by *ex vivo* NET assays.

Introduction

Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection [1] that causes an estimated 11 million deaths worldwide each year.[2] A key pathological process is the interaction between inflammation, coagulation and innate immune activation, termed immunothrombosis.[3] Neutrophils kill pathogens through phagocytosis or expulsion of neutrophil extracellular traps (NETs), which contain DNA together with antimicrobial proteins and enzymes.[4, 5] Excessive NETs can potentiate microvascular occlusion and the development of multiple organ dysfunction syndrome (MODS) and disseminated intravascular coagulation (DIC).[6-8] Moreover, NETs and the associated proteins and enzymes can directly induce cytotoxic effects on tissues which, in turn, propagate inflammation and cause organ injury.[9]

Although many factors induce NETosis, [4, 5, 10] which is an established feature of sepsis, [11, 12] the major driving factors for this process are unclear. We recently identified that IL-8 is a major NETosis-driving factor in sepsis, using a novel *ex vivo* NETs assay.[13] IL-8 is a chemokine that binds to CXCR1/2 to recruit and activate neutrophils, mainly to the lungs during infection.[14, 15] CXCR1/2 also contributes to NETosis in chronic obstructive pulmonary disease.[16]

Here, we explored the role of CXCR1/2 signalling in driving NETs in sepsis using the *ex vivo* NETs assay. We first confirmed the role of IL-8, the main ligand of CXCR1/2, in driving *ex vivo* NETs formation in ICU patients with sepsis. Complementary *in vivo* experiments were performed to assess the *ex vivo* and *in vivo* NETs levels in septic mouse models after neutrophil depletion, digestion of NETs and inhibition of the CXCR1/2 signalling pathway.

Methods

Study design-patients

A retrospective analysis was performed of prospectively-collected samples of 94 patients admitted with sepsis or SIRS between 2013 and 2014 admitted to the general intensive care unit (ICU) at the Royal Liverpool University Hospital, UK. Written informed consent was obtained for all participants. Demographics, clinical data and blood samples were collected on admission according to the protocol permitted by the NRES Committee North West - Greater Manchester West and Liverpool Central (Ref:13/NW/0089). Human citrated plasma was collected from the clinical laboratory and neutrophils were isolated from healthy donors according to the protocol (RETH000685) approved by the Research Ethics Committee of University of Liverpool. All plasma samples were aliquoted and stored at -80^{o}C to minimize the effects of storage on parameters analysed.

Laboratory models of sepsis

We adhered to ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. C57/BL6 male, 8-10 week-old mice for experimental sepsis were purchased from Beijing Vital River Laboratory Animal Technology and housed with free access to water and food in sterile conditions at the Research Centre of Genetically Modified Mice, Southeast University, Nanjing, China. All procedures were performed according to State laws under License (Jiangsu province, 2151981, to ZXC) and the use of animals was monitored by local inspectors. Sepsis was induced by either caecal ligation and puncture (CLP)[17] or intraperitoneal injection (i.p) of *Escherichia coli* (*E. coli*) K12 strain, as described previously.[18]

Neutrophil depletion

To evaluate the overall contribution of neutrophils to NETs, we injected (i.p.) the Ly6G-specific monoclonal antibody 1A8 (BioXcell, New Hampshire, United States) (1.0 mg per mouse) to selectively deplete neutrophils[19] 24h prior to sepsis induction. Antibody efficacy for neutrophil depletion was 90% evaluated using a routine automated haematology analyser. When mouse IgG was used as control for neutrophil depletion, no significant changes in circulating neutrophils was detected.

Inhibition of CXCR1/2 signalling.

Reparixin (Cambridge Bioscience, Cambridge, UK) (20 mg kg^{-1} per dose) was given subcutaneously 1h before, and 2h, 4h after sepsis was generated.

Digestion of NETs

Fifty mg kg^{-1} DNase I (Merck, Hertfordshire, UK) was injected (i.p.) at 1h and 5h after CLP induction and the mice were euthanized by cervical dislocation at 10h.

Ex vivo NETs formation assay in human and murine samples.

Ex vivo NETs formation and cytokine analysis was performed on ICU admission samples using citrated plasma. In mice, neutrophils and plasma was isolated after cervical dislocation in naïve mice (Animal Unit, University of Liverpool). Neutrophils were isolated using immunomagnetic negative selection kit (Stemcell Technologies, Cambridge, UK). Cells ($2x10^5$) were then incubated with plasma to induce NETs formation, quantified as described previously.[13] To investigate the role of CXCR1/2 signaling *ex vivo*, IL-8 (100 pg ml^{-1}; Sigma-Aldrich, Dorset, UK), CXCL2 (8ng mL^{-1}; R&D systems, Abingdon, UK) or reparixin (250 μg mL^{-1}) were added to plasma. 100 nM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) was used as a positive control.

Laboratory protocol

Mice were euthanized at 4h, 6h and 10h (3 mice per group per time point) after sepsis induction for pathological and organ injury markers; for neutrophil depletion, only the 10h time point after CLP was used (6 mice/group). For the survival study, mice were observed and scored every 2h[20] for a total of 72h after CLP induction without and with reparixin (Cambridge Bioscience, Cambridge, UK). Euthanasia of severely septic mice was undertaken according to local welfare standards.

Primary clinical outcome

The primary clinical study outcome was NETs formation induced by plasma from patients with sepsis versus plasma obtained from patients with systemic inflammation.

Laboratory outcomes

Online supplementary material provides detailed additional methods on the measurement of pathological and organ injury markers. *In vivo* NETs formation was analysed by immunohistochemical (IHC) staining of mouse organ sections from both CLP and i.p injection of *E. coli* sepsis models for citrullinated histone H3 (Cit-H3), a specific marker of NETs.[21] To clarify the roles of NETs formation in promoting coagulation[22] during sepsis, we quantified fibrin deposition.

Sample size estimation

The same age and sex of mice were used to minimise variability. The minimal sample sizes in each experimental group were calculated for a power of 80% and significance of 5% using values that have been determined in our previous experiments. Thus, for analysis of difference between groups, 6-8 mice per group were used.

Statistical analysis

Data were assessed for normality using the Shapiro-Wilk test. Normally distributed data was reported as mean ± standard deviation (SD). Non-normally distributed data were reported as median and interquartile range (IQR). Categorical variables were reported as number (%). Comparison between two groups was assessed by Mann-Whitney U test, Wilcoxon test or Chi-square test, where appropriate. Kruskal-Wallis analysis of variance (ANOVA) test was used for comparison of more than two groups. Kaplan-Meier plots was used for survival analysis and were compared using log-rank tests. Spearman's rank correlation was used for correlation analysis. Statistical analysis was performed using SPSS (version 25). P value <0.05 was considered statistically significant.

Results

NETs formation in vivo positively correlates with the ex vivo NETs assay.

NETs formation induced by plasma from patients with sepsis (median=25.0 [10.5-46.5]) was higher than plasma obtained from patients with systemic inflammation (median=14.0 [4.0-23.3]; P=0.02). We also observed typical NETs structures following incubation of plasma from septic mice with neutrophils isolated from normal mice, but not with plasma from mock control mice (Figure 1A-D, Supplemental Figure S1). *In vivo*, NETs formation occurred mainly in the lungs (Figure 1E-H) within 4h of inducing sepsis in both mouse models, which persisted throughout the 10h study. No NETs staining was found in liver, kidneys and heart (Supplemental Figure S2). The levels of *ex vivo* NETs correlated with the extent of *in vivo* NETs formation over the time course following sepsis induction (4 to 10h) in both mouse models ($r=0.869$, $p<0.001$ in CLP; $r=0.781$, $p<0.001$ in *E. coli* injection model).

NETs formation is associated with fibrin deposition and lung injury in murine sepsis.

IHC staining with anti-Cit-H3 and anti-fibrin showed NETs and fibrin deposition in the CLP model, but no NETs or fibrin deposition were found in CLP mice with either neutrophil depletion or DNase I treatment (Figure 2A and B). Histopathological examination (Figure 2C) demonstrated that CLP-induced sepsis caused significant lung injury, which was reduced by neutrophil depletion and DNase I treatment. Neutrophil depletion and DNase I treatment reduced NETs formation (Figure 2D), fibrin deposition in lungs (Figure 2E) and severity of lung injury (Figure 2F), using a modified lung injury score which excluded neutrophil counts (Supplemental Figure S3).

CXCR1/2 ligands are elevated in both patients with sepsis and mouse sepsis models and associated with ex vivo NETs formation

Levels of *ex vivo* NETs formation and 27 cytokines were correlated in a cohort of patients with sepsis and SIRS (n=94) recruited in ICU (Supplemental Table 1). IL-8 was the only NETs-inducing factor that was associated with *ex vivo* NETs formation (r=0.643; Table 1). Since MIP-2 is the major murine homologue of human IL-8 [24] to activate CXCR1/2 signalling, we measured circulating MIP-2 levels, which were persistently elevated from 4h after CLP (Figure 3A) and *E. coli*-induced sepsis (Figure 3B). MIP-2 levels correlated positively with the levels of *ex vivo* NETs formation in both CLP-induced sepsis (n=18, r= 0.902; P<0.001) and *E. coli*-induced sepsis (n=18, r= 0.904; P<0.001). NETosis in human and mouse samples was also induced by pathologically-relevant concentrations of IL-8 (100 pg ml^{-1}) and MIP-2 (8000 pg ml^{-1}) pathological levels of CXCR1/2 ligands (Figure 3C).

Blocking CXCR1/2 signalling using reparixin.

Reparixin inhibited both *ex vivo and in vivo* NETs formation (Figure 4A-D). The total number of NETs and the percentage of NETs forming neutrophils were both reduced within the lungs by reparixin treatment (Supplemental Figure S4). Similarly, directly adding reparixin to the plasma from septic mice inhibited its capacity to induce *ex vivo* NETs (Supplemental Figure S5). Similar numbers of colony-forming units (CFU) were observed in all organs between the CLP-induced sepsis and CLP following reparixin treatment (Figure 4E).

Blocking CXCR1/2 signalling reduces fibrin deposition, organ injury and improves survival in sepsis mice

In CLP-induced sepsis, lung appearance was improved following reparixin treatment (Figure 5A). Haematoxylin and eosin (H&E) sections confirmed less severe lung injury (Figure 5B). Fibrin deposition within the lungs was reduced by reparixin (Figure 5C). Lung injury scores (Figure 5D) and the severity of hepatic, renal and cardiac injury (as assessed by plasma

biomarkers) were lower in CLP following reparixin treatment (Figure 5E-G). Similar results were found in *E. coli*-induced sepsis (Supplemental Figure S6). Reparixin reduced mortality after CLP, compared to 100% mortality in mice without treatment (log rank test, p=0.036; Figure 5H).

Discussion

In this study, we have demonstrated that NETs formation is found predominantly in the lungs of sepsis mice in conjunction with strong fibrin deposition. By depleting neutrophils and inhibiting CXCR1/2 signalling to reduce NETosis, as well as use of DNase I to digest NETs, we have confirmed that neutrophil infiltration and NETs formation are critical contributors to fibrin deposition, microvascular impairment and lung injury.

NETs help to prevent bacterial spread and facilitate bacterial clearance. However, excessive NETs formation promotes immunothrombosis which impairs the local microcirculation and causes organ injury.[5, 13, 25] NETs have been shown to play important pathological roles in sepsis.[26, 27] This is consistent with NETs release being widely present in lung tissues to provide a scaffold for formation of intrapulmonary thrombi[23] and clinical reports that patients with adult respiratory distress syndrome have the highest NETs formation.[28, 29]

Our data also demonstrated that NETosis in sepsis is mainly driven by factors within the circulation. CXCR2 ligand, i.e. IL-8 in human and MIP-2 in mice, is one of the major factors. Therefore, NETs formation within the lungs in mouse models can be semi-quantified by this *ex vivo* NETs assay. This observation has validated our previous finding that *ex vivo* NETs assay is of more translational relevance by independently predicting the development of DIC and mortality in patients with acute critical illness,[13] compared to current indirect markers of NETs, e.g. cell-free DNA and MPO-DNA complexes.[30-32] MODS is a deleterious consequence of sepsis, and organ crosstalk can significantly influence the mortality and morbidity in these patients. [33] In sepsis, the first affected organ is often the lung and deleterious organ crosstalk between lungs and distal organs may play important roles in MODS.[34]

Targeting NETs using PAD4 inhibitors,[35, 36] activated protein C (APC),[37] DNase [38] and anti-histone antibodies [6], have been explored by many laboratories but not much progress has been made, particularly in sepsis. In our previous publication, blocking CXCR1/2

using reparixin significantly reduced *ex vivo* NETs formation induced by septic patient plasma.[13, 39] In mice, the CXC chemokines including CXCL1/keratinocyte-derived chemokine (KC), CXCL2/macrophage inflammatory protein-2 (MIP-2) and CXCL5-6/LPS-induced chemokine (LIX) are regarded as functional homologues of IL-8, which can activate neutrophils by binding only to CXCR2.[24] These chemokines are similarly elevated during infection,[40] particularly circulating MIP-2 levels in experimental sepsis.[41] Our previous publication showed that high levels of *ex vivo* NETs formation were strongly associated with circulating IL-8 as well as with SOFA scores, which suggests that IL-8-CXCR1/2 signalling-induced NETs formation plays a major pathological role in patients with sepsis. In mouse models of sepsis, MIP-2 increased 300-1000 fold and the pathological levels of MIP-2 can induce significant NETs formation both *in vitro* and *ex vivo*.

Reparixin is a specific non-competitive allosteric inhibitor of CXCR1/2 and blocks the intracellular signal transduction events activated by MIP-2 in mice and by IL-8 in humans.[39] Reparixin reduced murine lung injury induced by LPS, which was attributed to the reduction of neutrophils in lungs by 50% [42] or downregulating substance P.[43] In this study, we have demonstrated that reparixin not only reduced the recruitment of neutrophils but also reduced the NETs formation in lungs in mouse sepsis models. The reduction of NETs and associated fibrin deposition could be the major mechanism of reparixin-reduced lung injury in patients with sepsis. This may explain the negative results of reparixin in human LPS studies, because the low dose of LPS used in humans may not be sufficient to induce NETosis in the lungs (even though certain proinflammatory cytokines were increased).[44] Reparixin potentially benefits other patients with IL-8 elevation, such as trauma or complicated surgical procedures, which cause release of histones and elevated IL-6, IL-8 and IL-10 levels.[45] Reparixin inhibition of IL-8-induced NETosis may reduce NETs-mediated cancer cell metastasis and recurrence after lumpectomy.[46, 47]

CXCR1 and CXCR2 are expressed by several cell types, especially neutrophils, fibroblasts and vascular endothelial cells [48]. In humans, CXCR1/2 is expressed on the surface of neutrophils [49]. Compared with normal donors, CXCR2 surface expression was down-regulated by 50% on polymorphonuclear cells from septic patients, while CXCR1 expression persisted [50]. In both our septic mouse models, CXCR2 expression also increased within the lungs compared to mock controls, and reparixin did not reduce CXCR2 expression (Supplemental Figure S7). The increase in the numbers of CXCR2 positive cells may be partially due to neutrophil infiltration in lungs.

Although a major function of NETs is to prevent bacterial spread, we found no sign of impaired bacterial clearance after administration of reparixin. However, CFUs only indicate the number of viable bacteria and dead bacteria in circulation might also contribute to disease progression. No clear side effects of reparixin in mice were identified in our study. In contrast, the widely recognised NETs-digesting agent, DNase I, promotes inflammation if it is used in the early phase of sepsis and impairs bacterial clearance.[11] Reparixin may therefore be a better treatment to target NETosis in patients with sepsis.

In summary, our study shows that *ex vivo* NETs formation reflects the degree of *in vivo* NETs formation in murine and human models. Reparixin inhibits CXCR1/2 signalling, reduces NETs formation, organ injury and mortality in severe murine sepsis. These data suggest that CXCR1/2 signalling-induced NETosis is a potential therapeutic target in sepsis, which may be guided by *ex vivo* NETs assays.

Authors' contributions

Study conception/design: STA, GW, CHT.

Conduct of experiments: MA, ZXC, STA.

Assistance in performing some IHC experiments: SA

Guidance of animal experiments: WY, GW.

Data analysis: STA, MA, SL.

Drafting of paper: MA, STA, GW.

Editing/revision of paper: BM, CHT.

Supervision of work: CHT, GW, STA.

Acknowledgements

Thanks to all clinical staff and patients involved in this study. Thanks to Professor Haibo Qiu and Professor Yi Yang for their support in animal experiments. Thanks to Professor Philip Rudland for his critical reading and Angela Platt-Higgins for her assistance in mouse tissue processing.

Declaration of interests: The authors have no potential conflicts of interest.

Funding: This study was supported the British Heart Foundation (BHF) (PG/14/19/30751, PG/16/65/32313) and received scholarship from the Royal Embassy of Saudi Arabia Cultural Bureau in London, UK, to MA.

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Table 1. Correlation between ex vivo neutrophil extracellular traps formation and cytokines in systemic inflammatory response syndrome and sepsis patients.

	Correlation (R value)	SIRS	Sepsis	P value*
NETs-inducing factors				
Total number (n)		35	59	
IL-1β (pg/ml)	0.423	5.0 [3.2-6.7]	5.1 [3.7-9.8]	0.449
IL-6 (pg/ml)	0.276	113.7 [54.5-245.1]	375.1 [120.3-1380.2]	**0.006**
IL-8 (pg/ml)	**0.643**	58.4 [42.3-107.8]	163.8 [69.7-345.3] [a]	**0.003**
TNFα (pg/ml)	0.362	103.6 [50.6-125.5]	81.3 [55.0-155.0]	0.815
General activation				
IL-1ra (pg/ml)	0.243	607.5 [229.0-2026.8]	1179.6 [439.3-6256.0]	**0.045**
IL-2 (pg/ml)	-0.224	0.0 [0.0-2.1]	0.0 [0.0-3.0]	0.518
IL-15 (pg/ml)	0.100	12.1 [4.9-17.0]	8.3 [2.5-13.9]	0.305
Chemokines				
IP-10 (pg/ml)	0.473	788.4 [347.4-1283.5]	1187.3 680.7-3621.1]	**0.030**
MCP-1 (pg/ml)	**0.554**	48.9 [19.2-118.6]	98.2 [15.4-218.8]	0.370
MIP-1α (pg/ml)	0.528	5.0 [3.2-6.3]	4.9 [4.1-7.5]	0.587
MIP-1β (pg/ml)	**0.580**	93.3 [57.2-145.1	119.6 [80.8-211.9]	0.096
RANTES (pg/ml)	-0.358	5352.7 [2859.0-6830.9]	4777.3 [2714.5-6630.7]	0.654
T cell-related				
IL-4 (pg/ml)	0.392	6.4 [5.0-8.1]	6.8 [4.9-8.4]	0.662
IL-5 (pg/ml)	0.100	12.1 [4.9-17.0]	8.3 [2.5-13.9]	0.305
IL-9 (pg/ml)	0.522	32.7 [17.4-56.5]	50.4 [23.6-96.3]	0.201
IL-10 (pg/ml)	-0.255	24.5 [18.4-61.5]	30.5 [15.7-51.3]	0.839
IL-12 (p70) (pg/ml)	0.148	9.7 [6.9-23.0]	14.9 [9.8-19.6]	0.337
IL-13 (pg/ml)	-0.386	7.3 [3.5-18.9]	4.7 [2.3-8.4]	0.135
IL-17 (pg/ml)	0.318	36.5 [20.7-67.0]	43.4 [19.1-68.2]	0.601
Eotaxin (pg/ml)	0.272	103.8 [72.4-130.3]	100.2 [71.7-132.4]	0.662
IFNγ (pg/ml)	0.239	155.3 [127.0-198.4]	146.2 [110.1-246.8]	0.915
Bone marrow-derived				
IL-7 (pg/ml)	0.259	12.2 [8.4-33.2]	15.3 [7.7-22.6]	0.890
GM-CSF (pg/ml)	0.316	15.8 [0.0-114.1]	35.0 [0.0-150.2]	0.488
G-CSF (pg/ml)	0.420	129.4 [84.6-272.2]	161.3 [83.6-3531.8]	0.216
Angiogenic factors				
FGF-2 (pg/ml)	0.295	48.9 [21.2-86.1]	65.6 [36.9-87.8]	0.316
PDGF-bb (pg/ml)	-0.446	564.0 [374.8-799.7]	693.7 [375.2-1122.2]	0.495
VEGF (pg/ml)	0.390	28.5 [14.8-76.2]	83.0 [42.5-107.3]	**0.009**

Data are presented as median [Q1-Q3]. SIRS: systemic inflammatory response syndrome; NETs: neutrophil extracellular traps; IL: interleukin; TNF: tumour necrosis factor; IP: interferon gamma-induced protein; MCP: monocyte chemoattractant protein; MIP: macrophage inflammatory protein; IFNγ: interferon gamma; GM-CSF: granulocyte-macrophage colony-stimulating factor; G-CSF: granulocyte colony-stimulating factor; FGF-2: basic fibroblast growth factor; PDGF: platelet-derived growth factor; VEGF: vascular endothelial growth factor. Mann-Whitney U test, * P value for comparisons of SIRS vs sepsis patients.

Figure Legends

Figure 1. *Ex vivo* neutrophil extracellular traps formation reflects *in vivo* NETosis. C57BL/6 male mice were anesthetized and sepsis was induced either by caecal ligation and puncture (CLP) or intraperitoneal (i.p) injection of *Escherichia coli* (*E. coli*). Blood and tissues were collected at 4, 6 and 10h following sepsis induction with mock CLP and saline (i.p) as controls (3 mice per time point per group). **(A-D):** *ex vivo* NETs formation assay with neutrophils isolated from normal mice and plasma from the mouse models and controls. Typical images of NETs formation are presented in **A** and **B**, white arrows indicate normal neutrophils, yellow indicates NETs. Scale bar= 50µm. NETs formation was quantified as the percentage of neutrophils forming NETs per microscopic field and means (SD) are presented in C and **D**. Mann-Whitney U test, *P<0.05 increase NETs formation compared to mock controls. (**E-F)** *In vivo* neutrophil extracellular traps (NETs) quantification. Representative images of immunohistochemically (IHC)-stained lung sections with an anti-citrullinated histone H3 (Cit-H3) antibody are presented **(E, F).** Arrows (yellow) indicate NETs. Scale bar= 20µm. Cit-H3 staining intensity was quantified and means ± SD are presented **(G, H)**. Mann-Whitney U test, *P<0.05 increase NETs formation compared to mock controls.

Figure 2. Neutrophil depletion reduces fibrin deposition and organ injury in sepsis. Typical images of lungs sections stained with anti-citrullinated histone H3 (Cit-H3) for neutrophil extracellular traps (NETs) **(A)** and anti-fibrin for fibrin deposition **(B)** (Scale bar= 20µm). Yellow arrows indicate NETs and red arrows indicate fibrin deposition. **(C)** Lungs sections were haematoxylin and eosin (H&E) stained**.** Typical images are presented. Red arrows indicate interstitial neutrophil, black arrows indicate membrane thickness, green arrows indicate proteinaceous debris, yellow arrows indicate hyaline membrane. Scale bar= 50µm. NETs

formation **(D)**, fibrin score **(E)** and lung injury score **(F)** are presented. Six mice in each group. Mann-Whitney U test, *P<0.05 compared to mock group; #P<0.05 compared to CLP group.

Figure 3. CXCL2-CXCR2 is important for neutrophil extracellular traps formation in sepsis. Circulating macrophage inflammatory protein-2 (MIP-2; a CXCL2 in mice) was measured in both caecal ligation and puncture (CLP) and *Escherichia coli* (*E. coli*)-induced sepsis models and controls **(A, B)** (n=3 per time point). Means ± SD are presented over the time course of the experiment. *P<0.05 increase in circulating MIP-2 compared to mock controls. **(C)** Representative fluorescence microscopy images showing the capability of phorbol 12-myristate 13-acetate (PMA) (positive control), Interleukin (IL)-8 (100 pg ml^{-1}) or MIP-2 (8000pg ml^{-1})-spiked normal mouse plasma in inducing neutrophil extracellular traps (NETs) formation of isolated neutrophils from normal mice. Scale bar= 50 µm.

Figure 4. Reparixin, a CXCR2 inhibitor, reduces neutrophil extracellular traps formation in sepsis mice. Sepsis were induced in C57BL/6 male mice and treated with reparixin or saline as control (6 mice per group). Blood and tissues were collected 10h after sepsis induction. **(A)** Typical images of *ex vivo* neutrophil extracellular traps (NETs) formation are presented. **(B)** Typical images of anti-citrullinated histone H3 (Cit-H3) staining of lung sections for quantification of *in vivo* NETs formation are presented. White arrows indicate normal neutrophils; yellow arrows indicate NETs. Scale bar= 50µm. Quantification of *ex vivo* **(C)** and *in vivo* **(D)** NETs formation are presented. *Mann-Whitney U test, P<0.05 compared sepsis model without (-inhibitor) to with reparixin treatment (+inhibitor). **(E)** Bacterial cultures of organ tissues, including heart, liver, spleen, lungs and kidneys at 10h after cecal ligation and puncture (CLP)-sepsis induction. The means of colony-forming unit (CFU) from the organ tissues of septic mice treated with saline (-inhibitor) were set up as 100% and the relative

percentage of CFU from the organ tissues of sepsis mice treated with reparixin (+inhibitor). No statistical difference was found.

Figure 5. Blocking CXCR2 reduces fibrin deposition, organ injury and improves survival times of sepsis mice. Typical photos of lungs 10h after CLP (CLP) and CLP treated with reparixin (CLP +Inhibitor) **(A)** and typical images of haematoxylin and eosin (H&E) stained lung sections **(B)** are presented. Semi-quantified fibrin deposition in lung sections immunohistochemically (IHC)-stained with anti-fibrin antibody **(C),** lung injury scores **(D),** alanine aminotransferase **(**ALT) **(E)**, blood urea nitrogen (BUN) **(F)** and cardiac troponin I (cTnI) **(G)** in mice 10h after mock or CLP induction are presented. Mann-Whitney U test, *$P<0.05$ compared to mock group, $^{\#}P<0.05$ compared to CLP group without treatment. **(H)** Kaplan-Meier survival curve is presented to compare CLP (n=8) to CLP treated with reparixin (n=6), log-rank test.