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Mechanisms of Innate Immunity in Polymicrobial Sepsis

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MECHANISMS OF INNATE IMMUNITY IN POLYMICROBIAL SEPSIS

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Dedicated to my parents, brother, wife and baby Ryan

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Commonly Used Abbreviations

AST	aspartate aminotransferase
ALT	alanine aminotransferase
ROS	Reactive oxygen species
NLRP3	NLR family, pyrin domain containing 3
i.p.	Intraperitoneal
MCP-1	Monocyte chemoattractant protein-1
rMCP-1	Recombinant MCP-1
CCR2	Chemokine (C-C motif) receptor 2
CXCR-2	Chemokine (CXC motif) receptor 2
CXCL1	Chemokine (C-X-C motif) ligand 1
KC	Keratinocyte cell-derived chemokines
MIP-2	Macrophage inflammatory protein-2
G-CSF	Granulocyte colony stimulating factor
LTB4	Leukotriene B4
LIX	Lipopolysaccharide-induced CXC chemokines
ICAM-1	Intracellular cell-adhesion molecule-1
VCAM-1	Vascular cell-adhesion molecule-1
BALF	Bronchoalveolar lavage fluid
MPO	Myeloperoxidase
NF-ĸB	Nuclear Factor kappa B
MAPKs	Mitogen associated protein kinases
JNK	C-Jun N-terminal kinase
PRRs	Pattern recognition receptors
PAMPs	Pathogen associated molecular patterns
PYD	Pyrin domain
TLRs	Toll-like receptors
TIR	Toll-interleukin (IL)-1 receptor homology domain
MyD88	Myeloid differentiation primary response gene (88)
MD-2	Myeloid differentiation protein-2
TRIF	TIR-domain-containing adapter-inducing interferon-β

TIRAP	Toll-interleukin 1 receptor (TIR) domain-containing adapter protein
TRAM	TRIF related adaptor molecule
LRRs	Leucine rich repeats
NLRs	Nod like receptors
RIP2	Receptor interacting protein 2
NALPS	NACHT, LRR and PYD domain-containing proteins
NAIP5	NLR family apoptosis inhibitory protein 5
NACHT	Neuronal apoptosis inhibitor protein CIITA, HET-E and TP-1
TNF-	Tumor necrotic factor alpha
IL-6	Interlukin 6
IL-17	Interlukin 17
LPS	Lipopolysaccharide
PMN	Polymorphonuclear leukocytes
AP-1	Activating protein-1
STAT	Signal transduction and transcription proteins
STAT3	Signal transduction and transcription protein 3
IRF	Interferon regulatory factor
IFN-	Interferon gamma
IRAKs	Interleukin-1 receptor-associated kinases
AM	Alveolar macrophages
WT	Wild type
CFU	Colony forming unit
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
RBC	Red blood cells
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
BSA	Bovine serum albumin
FBS	Fetal bovine serum
DMEM	Dulbecco’s modified Eagle’s medium
FACS	Flourescence activated cell sorting
PBS	Phosphate buffered saline
OD	Optical density

DAMPs	Danger associated molecular patterns
CLP	Cecal ligation and puncture
LC3	Microtubule-associated protein 1A/1B-light chain 3
LAMP1	Lysosomal-associated membrane protein 1
MARCO	Macrophage receptor with collagenous structure
MBL	Mannose-binding lectin
NADPH	Nicotinamide adenine dinucleotide phosphate
P62/SQSTM1	Sequestosome 1
ATG7	Autophagy-related protein 7
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
PMS	Polymicrobial Sepsis

Abstract

Severe bacterial sepsis leads to a pro-inflammatory condition that can manifest as septic shock, multiple organ failure, and death. Neutrophils are critical for the rapid elimination of bacteria, however, the role of neutrophil chemoattractant CXCL1, pattern recognition receptors (PRRs)-NLR protein 3 (NLRP3) and alcohol in bacterial clearance during sepsis remains elusive. We demonstrate that CXCL1 plays a pivotal role in mediating host defense to polymicrobial sepsis following cecal ligation and puncture (CLP) in gene-deficient mice. CXCL1 appears to be essential for restricting bacterial outgrowth and preventing multiple organ failure and death in mice. Moreover, CXCL1 is essential for neutrophil migration, expression of pro-inflammatory mediators, Recombinant interleukin 17 (IL-17) rescued impaired host defenses in *cxcl1*$^{-/-}$ mice. CXCL1 is important for IL-17A production via Th17 differentiation. CXCL1 is essential for reactive oxygen species production and neutrophil extracellular trap (NET) formation. This study reveals a novel role for CXCL1 in neutrophil recruitment via modulating T cell function and neutrophil-related bactericidal functions. These studies suggest that modulation of CXCL1 levels could reduce bacterial burden and excessive inflammatory injury in sepsis. NLRP3$^{-/-}$ mice or mice treated with NLRP3 inhibitor were protected in response to polymicrobial sepsis. NLRP3$^{-/-}$ mice showed reduced bacterial burden and production of proinflammatory cytokines. Intriguingly, neutrophils obtained from NLRP3$^{-/-}$ or NLRP3-inhibited mice display impaired critical functions of neutrophils, including phagocytosis, bacterial killing, NET formation, autophagy, chemotaxis, and cell death. These unique and novel findings position NLRP3 as a critical linker between neutrophil function and bacterial clearance, highlighting NLRP3 as a therapeutic target to control infection in polymicrobial sepsis. Alcoholics are more susceptible to bacterial sepsis and thus have higher mortality rate as compared to non-alcoholics. In this study, acute alcohol intoxication prior to the induction of polymicrobial sepsis show reduced NETosis. Diminished NETosis was consistent with attenuated ROS production and bacterial clearance in alcohol-challenged CLP-induced mice. Our findings demonstrate that alcohol-suppressed NETosis and NET-mediated extracellular killing of bacteria contribute to the pathogenesis of polymicrobial sepsis, and thus, furthers our understanding on alcohol-induced immune defect during bacterial infection.

Chapter 1: Introduction

Sepsis is the leading cause of death in patients in the intensive care unit, and accompany with multiple organ dysfunction syndrome (MODS). The annual incidence of sepsis is 750,000 cases and result in 210,000 deaths in the United States. The average cost of sepsis case was as high as $22,000 with total costs of $16.7 billion at the national level (1, 2). Furthermore, recent study of hospital records shows that the total number of sepsis patients who are dying is increasing. Furthermore, Steven M. Opal demonstrated that the incidence of sepsis is increasing overall and projected to increase with the age (6). Although there have been substantial extensive basic research and clinical studies, the pathophysiology of sepsis is still not well understood. The host response in sepsis involves a complex interplay between pro and anti-inflammatory cytokine production. The failure of pro-inflammatory cytokine blockade to improve survival, and the growing recognition that sepsis is associated with impaired neutrophil (PMN) function highlights the importance of pathogen control by innate immune effector cells in sepsis (4, 5). Therefore, it is critical to understand the innate immune response during sepsis to augment the immune system to combat bacterial pathogens.

Recruitment of leukocytes including neutrophils involves a complex signaling mechanism. Cytokines and chemokines drive the recruitment of these immune cells to the site of infection or inflammation. According to the arrangement of cysteine motif positioned near the N terminus, chemokines can be divided into four groups: C, CC, CXC, and CX3C. These groups are further characterized based on the position of the ELR motif (glutamic acid-leucine-arginine) before the CXC sequence. ELR positive CXC chemokines are specific neutrophil chemoattractants, and seven of this group have been identified in human: interleukin (IL)-8; neutrophil-activating peptide 2 (NAP-2); epithelium-derived neutrophil-activating peptide 78 (ENA-78); growth-related oncogenes (GRO)-α, ß, and γ; and granulocyte chemotactic protein 2 (GCP-2). In general, IL-8 is the most significant neutrophil chemoattractant in humans. Though a homolog of human IL-8 has not been discovered,

CXCL1/KC, macrophage inflammatory protein (MIP)-2 and LPS-Induced CXC chemokine (LIX) have been identified to be important neutrophil chemoattractants in mice [9]. The murine chemokine CXCL1 [aka: keratinocyte cell-derived chemokine (KC); homolog of human IL-8] has been recognized as one of the important neutrophil chemoattractants (7,8). Nevertheless, the role of KC has not been examined in the context of sepsis. In this investigation, we determined the role of KC in polymicrobial sepsis.

Toll-like receptors (TLRs) and cytosolic NOD-like receptor (NLRs) are amongst the four main families of pattern recognition receptors which are critical for the initiate pro-inflammatory signaling cascades (9). NLRs are the components of large intracellular multiprotein complexes known as inflmmasomes. Inflammasomes are made up by the NLR protein they contain, for example NLRP1 inflmmasome comprises NLRP1, ASC, caspase-1 and includes NLRP3, CARDINAL, ASC and Caspase-1. However NLRC4/IPAF is only composed of NLRP4 and caspase-1. Members of cytosolic NLRs activate caspase-1 within the inflammasome complex in a unique manner and direct a successful host defense. Furthermore, animal and human studies have proved the importance of the inflammasome pathways in the inflammatory response to sepsis. The mechanisms related to the activation of inflammasomes by pathogen associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) during sepsis are still poorly understood (10-14). However, the role of NLRP3 has not been determined in the context of sepsis. In this study, we examined the role of NLRP3 in polymicrobial sepsis.

Alcohol ranks the third leading cause of preventable death in the United States. While alcohol use disorders（AUD）are associated with medical conditions that doctors can diagnose when a patient’s drinking causes substantial morbidity and mortality (15, 16). AUD are common problems in most of the western countries. In the United States, 7% of the population has AUD, and 21 to 42% of the subjects admitted to hospitals have alcohol related problems(17-19). The ethanol-induced immune dysfunction was proved to result in the increase of both frequency and severity of infections in AUD individuals（20，21）.

Ethanol exposure compromises multiple aspects of immune system. Bautista (27) showed that cells of the monocyte lineage was extremely sensitive to the immunomodulatory effects of ethanol. In alcohol liver disease, the function of Kupffer cell is affected, leading to impaired phagocytosis ability, the increased production of proinflammatory cytokines, free radical production, and the release of suppressive factors. Furthermore, patients with AUD are predisposed to developing sepsis, have higher frequency to require mechanical ventilation, and have a higher risk of death (22, 23).The most common alcohol-related record in the emergency care departments is acute alcohol intoxication (AAI). AAI is a clinically harmful condition that is usually caused by consuming a great amount of ethanol in a short time. Previous studies suggest that consumption of ethanol is associated with an increased incidence and severity of infections in human and experimental animals. Recently, a study from the Ohio State University reported that patients with alcoholism or alcohol withdrawal were at a higher risk of developing sepsis and septic shock and were more likely to die during hospital stay. Furthermore, Waldschmidt *et al* that ethanol played an important role on keys cell of the immune system and B cell response (28). Recent studies indicated that ethanol consumption suppresses innate immunity and inflammation, all together increase the risk of mortality in patients suffered sepsis (29-30).

Neutrophils are considered as a most important part of innate immunity, the first line of defense against invading microbes. Neutrophils combat pathogens by phagocytosis, degranulation, and release of neutrophil extracellular traps (NETs). NETs are fibrous network composed by DNA and antimicrobial factors that are forming a specific neutrophil death process named NETosis. This network structure can trap and kill pathogens, such as, Gram - positive, Gram-negative as well as fungi. NETs are involved in immune defense, sepsis, and autoimmunity (24-26).

Most of the studies available on the interaction between ethanol and sepsis are associated with chronic alcohol dependence with sepsis. However, the consequence of acute alcohol intoxication in innate immune response using CLP sepsis model have not been elucidated.

Furthermore, the interaction of neutrophil function, especially the NETs with alcohol during sepsis has never been studied. Therefore, we investigated the role of alcohol in neutrophil recruitment and function during polymicrobial sepsis.

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Chapter 2: CXCL1 Contributes to Host Defense in Polymicrobial Sepsis via Modulating T cell and Neutrophil Functions

Introduction

Despite improvements in antibiotic therapies and critical care management, sepsis remains a leading cause of infectious death in intensive care units (1). The pathogenesis of sepsis is characterized by an early, overwhelming systemic pro-inflammatory response that leads to multiple organ damage; the later phases are characterized by anti-inflammatory responses and negative regulation of immune signaling pathways (2). However, the molecular and cellular mechanisms that regulate immune responses to polymicrobial sepsis are not completely understood. Prior studies have shown that deletion of nucleotide-binding oligomerization domain-2 (NOD2) receptors or the scavenger receptor class A leads to host protection (3, 4). In contrast, reduced survival and diminished production of cytokines and chemokines was observed in *Stat-2*$^{-/-}$ mice during lipopolysaccharide (LPS)-mediated endotoxic shock, indicating a crucial role for STAT-2 in host defense (5).

Neutrophils are a pivotal arm of the innate immune response during polymicrobial sepsis, and play a critical role in bacterial elimination (6, 7). Impaired neutrophil transmigration is associated with increased mortality and higher bacterial burden in peritoneal exudates and blood, as demonstrated during sepsis induced by cecal ligation and puncture (CLP) (7, 8) whereas excessive influx of neutrophils can cause unwanted tissue damage and organ dysfunction (9). Our group has demonstrated an important role for CXCL1 [also known as keratinocyte cell-derived chemokine (KC)] in pulmonary defense during pneumonia caused by *Klebsiella pneumoniae* (10). CXCL1 was found to be critical for neutrophil-dependent bacterial elimination via induction of reactive oxygen species (11) and reactive nitrogen species in the lung (12). Neutrophil migration to multiple organs is impaired during severe sepsis, due to down-regulation of the CXCL1 receptor, CXCR2, resulting in failed pathogen clearance (13). These findings suggest a potential role for

CXCR2, or its ligands, including CXCL1 (KC), CXCL2 (MIP-2), and CXCL5 (LIX), in controlling sepsis. To better understand the role of CXCL1 in neutrophil influx and function during fatal polymicrobial sepsis (PMS), we employed a murine model of PMS, induced by CLP in $CXCL^{-/-}$ mice. In addition, we addressed the role of bone marrow cell versus resident cell-derived CXCL1 in bacterial clearance following PMS through the use of bone marrow chimeras.

Materials and Methods

Animal studies: All animal experiments were approved by the Louisiana State University Animal Welfare Committee. CXCL1 gene-deficient (*cxcl1*$^{-/-}$) and C57Bl/6 wild type (WT) male mice (8-12 weeks) were generated as previously described (10, 14). PMS was induced by the CLP method as previously described (15). In brief, male mice were anesthetized, the cecum was punctured with a 21-gauge needle, a small amount of fecal material was extruded through the puncture, and the cecum was repositioned into the peritoneal cavity. Animals with sham surgery underwent the same protocol without CLP. Survival of both *cxcl1*$^{-/-}$ and WT mice that underwent CLP or sham surgery was monitored every 12 h up to 10 days. In additional experiments, recombinant CXCL1 (rCXCL1), rIL-17A, or bovine serum albumen (BSA) control was injected intraperitoneally immediately following CLP, and survival was monitored for 10 days. Bacterial burdens were determined by enumerating bacterial numbers in colony forming units (CFUs) on tryptic soy agar plates as previously described (10). Cytokine and chemokine levels in peritoneal lavage fluid and serum were measured by a double-ligand enzyme-linked immunosorbent assay (ELISA) (10, 12).

Bone marrow chimeras: Generation of CXCL1 chimeras has been described in our prior reports (10, 12). We found that greater than 80% of blood leukocytes were derived from donor mice at the time of experiments.

Western blotting: At the designated times, the lungs, livers, spleens, and kidneys were harvested and homogenized in 1 mL of phosphate buffered saline (PBS) containing 0.1%

Triton X-100 supplemented with complete protease and phosphatase inhibitor cocktail as described (12).

Immunofluorescence and electron microscopy: Immunofluorescent of the cells from *cxcl1*$^{-/-}$ and WT mice was performed to detect Neutrophil Extracellular Trap (NET) formation as described previously (16). Scanning electron microscopy (16) (FEI Quanta 200, USA) was performed to examine NET formation in peritoneal and bone marrow neutrophils following the protocols described in a previous publication (16). *E. coli* (ATCC 25922) was used to stimulate neutrophils as described previously (17).

Flow cytometry: The detection of reactive oxygen species (11), OH^-, and O_2^- producing neutrophils in the peritoneal fluid from CLP WT and KO mice was performed as described earlier (18). For isolation and detection of IL-17 producing T-cells, lungs and kidneys were first minced, digested with collagenase for 90 min, and made into a single-cell suspension, followed by staining. Cells were surface stained for markers of IL-17A–producing T cells (γδ, NK1.1, CD4, and CD8α). FlowJo software was used to analyze the data.

Th1, Th2 and Th17 differentiation: The method for IL-17 differentiation has been previously reported (19). Recombinant CXCL1 (rCXCL1) (1 mg/mL) was added on days 1 and 3. Cells were washed and resuspended in PBS followed by blocking with Fc receptor blocking reagent at 3, 6 and 12 days post-stimulation. IFN-γ, IL-4 or IL-17-producing cells were surface stained with anti-CD4 fluorescein isothiocyanate (FITC). Flowjo was used for data analysis. In another set of experiments, supernatant was collected for IL-17A or IL-17F assay by ELISA at 3 day post-stimulation with rCXCL1.

Analysis of NET-derived DNA: To analyze NET-derived DNA, peritoneal fluid was taken from the post-CLP or sham mice at 24 h and subjected to agarose gel electrophoresis. To confirm whether extracellular DNA was present in the cell-free supernatant, peritoneal fluid was treated with DNase (50 mg/ mL) for 1 h and subjected

to gel electrophoresis. The observation of DNA in the samples, but not in DNase-treated samples, was judged to be NET-derived DNA.

Determination of Reactive Oxygen Species (11), H_2O_2 and O_2 production by neutrophils: ROS and H_2O_2 levels in peritoneal neutrophils of *cxcl1*$^{-/-}$ and WT mice after CLP were measured using the Fluorescent H_2O_2/Peroxidase Detection Kit.

Bacterial killing assay: A neutrophil-dependent killing assay was performed as reported earlier (12). Briefly, 1 X 10^6 neutrophils were suspended in RPMI 1640 with 10% v/v fetal bovine serum (FBS), and 1 X 10^6 opsonized bacteria were added (1 multiplicity of infection (MOI)). Samples were incubated at 37℃ with continuous agitation. Samples were harvested at 60 min post-infection, treated with gentamycin (100 mg/mL) for 15 min to kill extracellular bacteria and plated to determine colony forming units (CFU) (12).

Data analysis: Data are expressed as mean +/- standard error (SE). Data were analyzed with the Student's t-test (between two groups) or with the two-way analysis of variance (ANOVA) (>2 groups). Survival curves were compared by the Wilcoxon rank sign test. Differences in values were defined as significant at a *p* value of less than 0.05. Experiments were repeated 2-3 times in each group in each time point.

Results

CXCL1 contributes to protection during polymicrobial sepsis. The role of CXCL1 in the pathogenesis of polymicrobial sepsis is unclear. To explore this, *cxcl1*$^{-/-}$ and WT mice were subjected to CLP, and the survival of animals was monitored up to 10 days post-CLP. As shown in Figure. 2.1A, *cxcl1*$^{-/-}$ mice displayed a reduction in survival rate compared to WT mice after CLP. To examine whether reduced survival of *cxcl1*$^{-/-}$ mice was due to an impaired ability to recruit immune cells to the peritoneum, total and differential cell counts as well as myeloperoxidase activity (MPO) activity were determined in the peritoneal fluid. In *cxcl1*$^{-/-}$ mice, total white blood cells (WBCs), neutrophil number, and MPO activity were attenuated at 24 h post-CLP as compared to

WT mice (Figure. 2.1C). In the control (sham) group, no cellular influx was observed in the peritoneal cavity of either *cxcl1*$^{-/-}$ or WT mice (Figure. 2.1C). To determine whether the reduced recruitment of WBCs and neutrophils contributed to bacterial growth and subsequent dissemination to extraperitoneal organs, bacterial numbers in the peritoneal fluid, blood, spleen, lung, kidney, and liver were enumerated 24 h post-CLP. As compared to WT mice, *cxcl1*$^{-/-}$ mice had a higher bacterial burden in all tissues (Figure. 2.1B). Liver enzymes AST and ALT were elevated in the serum of *cxcl1*$^{-/-}$ CLP-treated mice, suggesting that a higher bacterial burden contributed to higher liver damage (Figure.2. 1D). Furthermore, mice transplanted with either WT or KO marrow showed attenuated bacterial clearance in peritoneal fluid (PF), suggesting that both hematopoietic (radiosensitive) and non-hematopietic (radioresistant) cells are important for bacterial clearance (Figure. 2.1E).

CXCL1 contributes to cytokine and chemokine production. Next, we determined the role of CXCL1 in the production of cytokines and chemokines in peritoneal fluid (PF) and serum, by assessing levels in WT and *cxcl1*$^{-/-}$ mice at 6 h and 24 h post-CLP. As shown in Figure. 2.2, cytokine and chemokine levels were reduced in PF and in serum samples of *cxcl1*$^{-/-}$ mice 24 h post-CLP, except the LIX level, which was unchanged in serum. Intriguingly, the production of IL-17A and IL-17F in PF and serum of *cxcl1*$^{-/-}$ was reduced at 24 h post-CLP (Figure. 2.2).

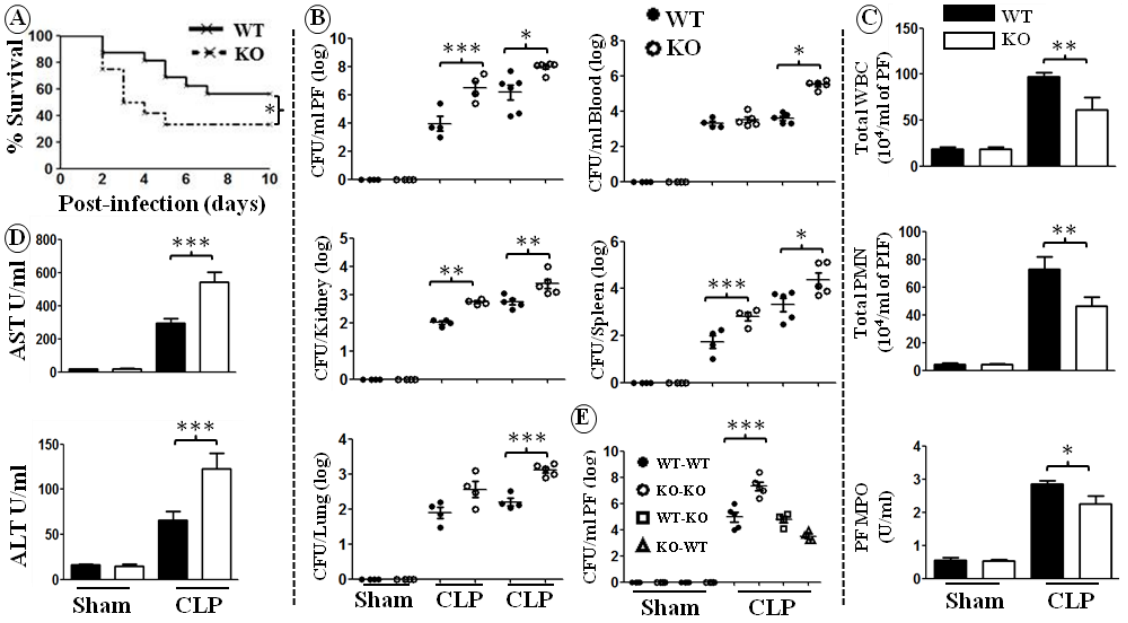

Figure 2.1. CXCL1 deficient mice are susceptible to polymicrobial sepsis (PMS) than WT mice and are defective for cellular infiltration and bacterial clearance. A, Enhanced mortality of *cxcl1*$^{-/-}$ mice after CLP-induced PMS. *cxcl1*$^{-/-}$ and WT control mice were subjected to sham or PMS. The survival rates of mice were determined every 12 h until 10 days after PMS. The results are expressed as percent for 20 animals per group. Significance between groups was examined by Wilcoxon rank test. B, Impaired bacterial clearance in *cxcl1*$^{-/-}$ mice. The CFUs were examined in peritoneal lavage fluid and blood or the homogenates obtained from kidneys, livers, lungs, and spleens of *cxcl1*$^{-/-}$ as well as WT mice at 6 and 24 h post-PMS. The results are expressed as mean log of CFU/mL (n=5/group). C, *cxcl1*$^{-/-}$ mice have reduced total WBC and neutrophil accumulation at the peritoneum after PMS, as measured by direct cell counts (for total WBC and neutrophils) or MPO activity (for neutrophil influx). The results are expressed as mean/mL (n=5-8/group). D, *cxcl1*$^{-/-}$ mice have higher organ damage than WT. Serum levels of AST and ALT of both *cxcl1*$^{-/-}$ as well as WT mice were measured at 6 h and 24 h post-PMS. The results are expressed as mean ± SEM (n=5-8/group). E, Both hematopoietic and non-hematopoietic cells are essential for bacterial clearance. Bone marrow chimeras were generated as described in *Methods* and show bacterial CFUs at 24 h post-PMS or sham (n=5group). *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

CXCL1 activates NF-κB, MAPK, and NADPH oxidase, and induces ICAM-1 expression. Activation of both Nuclear Factor κ B (NF-κB) and Mitogen-activated Protein Kinase (MAPK) is critical for controlling bacterial infections (20, 21). NF-κB activation was reduced in *cxcl1*$^{-/-}$ mice after CLP at both 6 and 24 h, as assessed by reduced phosphorylation of NF-κB/p65 (Ser 536), , and the degradation of IκBα in the liver, lung, spleen, and kidney (Figure. 2.3). Similarly, *cxcl1*$^{-/-}$ mice showed reduced activation of the p38, Extracellular Signal-regulated Kinase (ERK), and c-Jun N-terminal Kinase (JNK) MAPKs in the liver, lung, spleen, and kidney at 24 h post-CLP (Figure. 2.3). At 6

h post-CLP, *cxcl1*$^{-/-}$ mice exhibited reduced activation of MAPK. However, activation of p38 in the lung, spleen, and kidney; JNK in the liver, lung, and spleen; and ERK in the kidney, were the same as WT at 6 h post-CLP (Figure. 2.3).

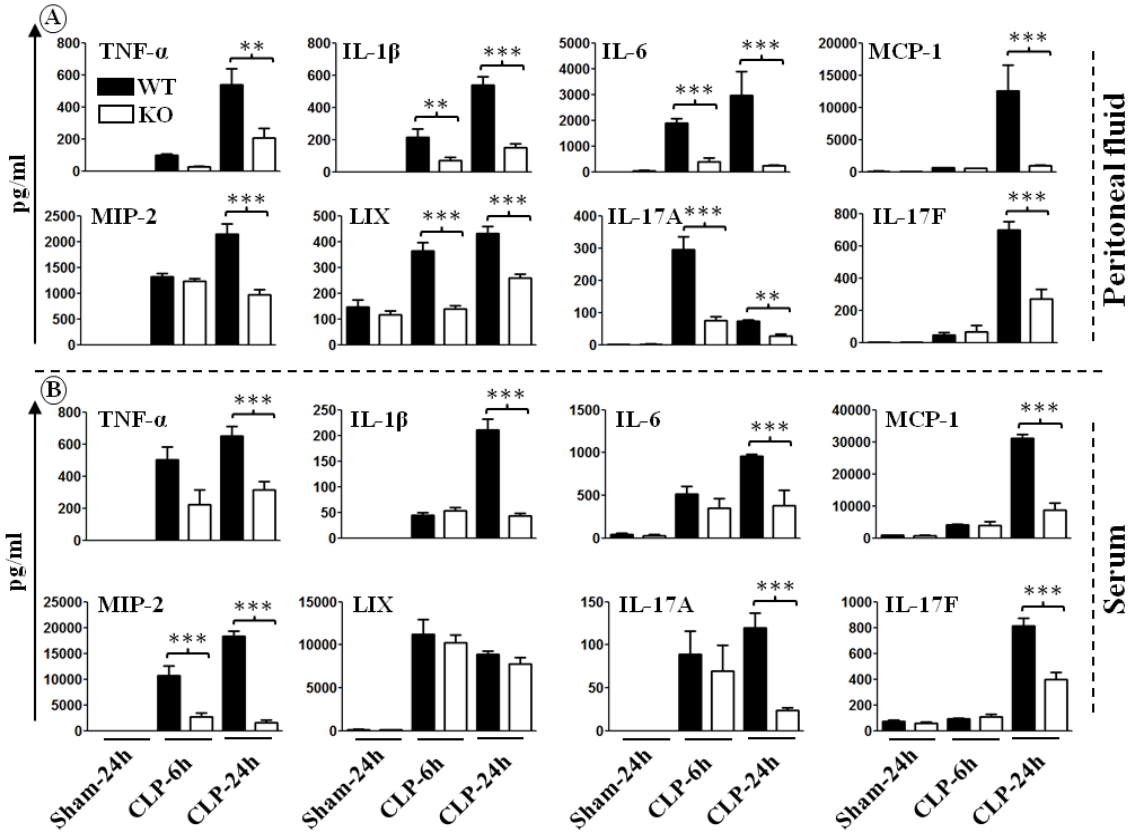

Figure 2.2. Diminished production of cytokines and chemokines in *cxcl1*$^{-/-}$ mice after induction of PMS. *cxcl1*$^{-/-}$ and their WT control mice were subjected to sham or PMS. The concentration (in pg/mL) of TNF-α, IL-1β, IL-6, IL-17A, IL-1F, MCP-1, LIX, and MIP-2 were quantified in the peritoneal fluid (A) and serum (B) for the time points of 6 and 24 h post-PMS. The results are expressed as mean ± SEM (n=5-8/group; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

We next examined whether defective bacterial clearance in the organs of *cxcl1*$^{-/-}$ mice was due to reduced activation of NADPH (nicotinamide adenine dinucleotide phosphate) oxidase, an enzyme complex consisting of membrane subunits (gp91^{phox} and p22^{phox}) and cytoplasmic subunits (p47^{phox}, p67^{phox}, and p40^{phox}, and rac) that are assembled upon cellular activation to produce microbicidal ROS (22). We observed that CXCL1 deficiency impairs the expression of p22^{phox}, p67^{phox}, and p47^{phox}, as well as Nox2, in different organs post-CLP (Figure. 2.3).

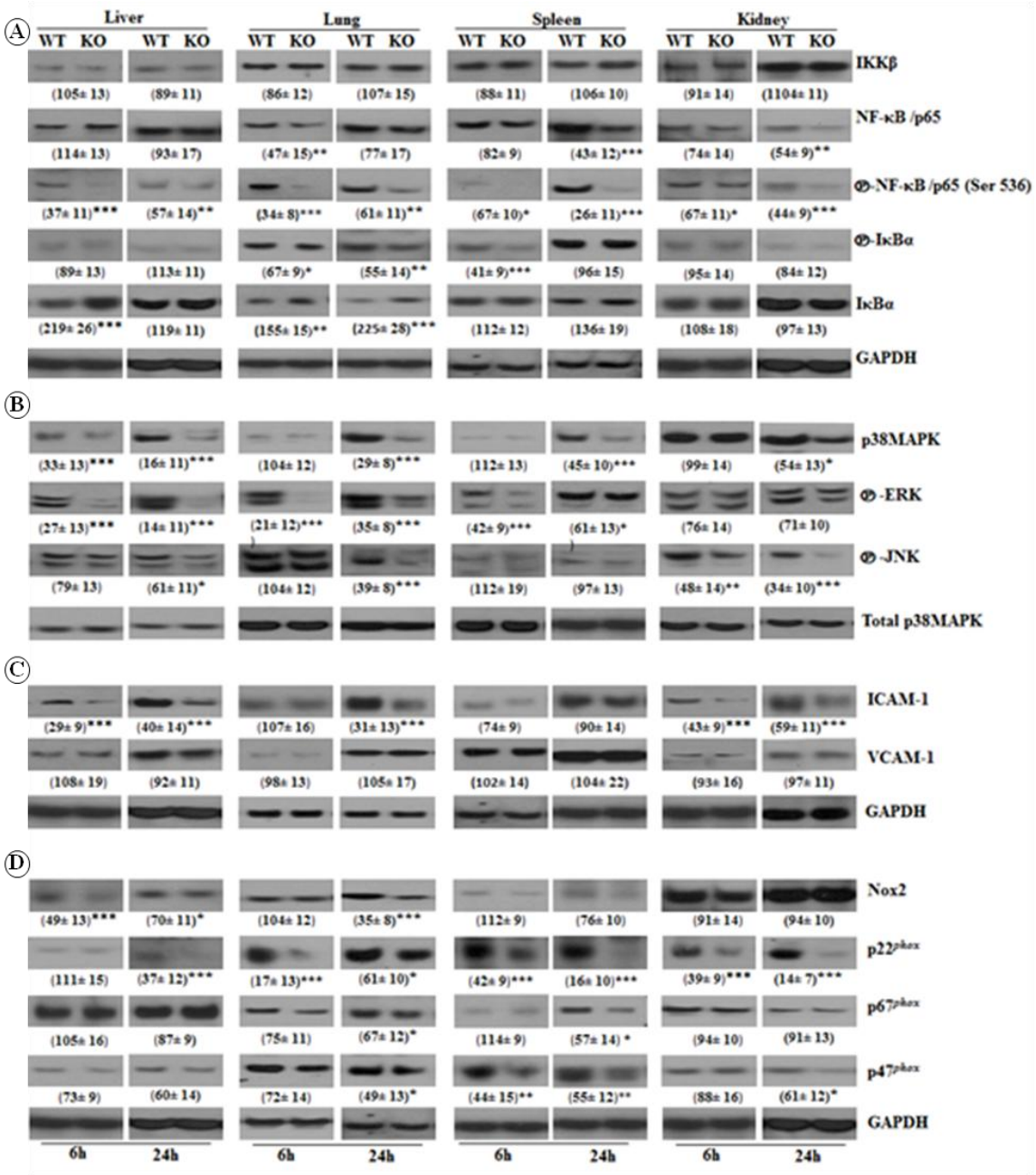

Figure 2.3. Activation of NF-κB, MAPK, and NADPH oxidase, and expression of adhesion molecule ICAM-1 were impaired in *cxcl1*$^{-/-}$ mice following PMS. A, CXCL1 is essential for the activation of NF-κB during PMS induction. phosphorylation ofNF-κB/p65 (ser 536), and IκBα in the homogenates from liver, lung, spleen, and kidney from *cxcl1*$^{-/-}$ as compared to WT mice. B, Attenuated activation of MAPKs in the organs of *cxcl1*$^{-/-}$ mice. Phosphorylated p38 MAPK, ERK, and JNK in the homogenates from different organs from *cxcl1*$^{-/-}$ and WT mice were probed with their respective antibodies. **C,** Expression of adhesion molecule ICAM-1 but not VCAM-1 is reduced in *cxcl1*$^{-/-}$ mice. The expression level of ICAM-1 and VCAM-1 were determined in the organs of *cxcl1*$^{-/-}$ and WT control mice following PMS for time points 6 and 24 h. **D,** The activation of NADPH oxidase is impaired in *cxcl1*$^{-/-}$ mice. The expression levels of Nox4, p22^{phox}, p67^{phox}, and p47^{phox} were determined in the homogenates of organs of *cxcl1*$^{-/-}$ and WT control mice by immunobloting at 6 and 24 h post-PMS. GAPDH or total p38 expression levels were assessed in all samples as internal loading control and the blots are representative of three independent experiments with similar results (A, B, C, and D). Densitometric analysis of 3 separate blots from 3 independent experiments is shown in parenthesis as mean ± SE. (n=4-6/group; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

Cell adhesion molecules are important for neutrophil migration, thus we investigated the expression levels of Intercellular Adhesion Molecule-1 (ICAM-1) and Vascular Cell Adhesion Protein-1 (VCAM-1) after CLP. We found that the expression of ICAM-1, but not VCAM-1, was diminished in the liver, lung, spleen, and kidney tissues from *cxcl1*$^{-/-}$ mice, in comparison to their WT counterparts at 6 h, as well as 24 h post-CLP (Figure. 2.3).

CXCL1 mediates IL-17A and IL-17F production via activation of CD4, CD8, NK, and γδ cells. Since both IL-17A and IL-17F were reduced in PF and serum of *cxcl1*$^{-/-}$ mice following CLP, the specific T cell subsets that produce IL-17A or IL-17F in the peritoneum following CLP were determined. Our results show that CD4, CD8, natural killer (NK) or NK T cells, and γδ cells all produce IL-17A and IL-17F in the lungs, kidney, and spleen of WT mice 24 h after sepsis (Figure.2.4A-B). There were fewer IL-17A and IL-17F-producing cells in *cxcl1*$^{-/-}$ mice following sepsis compared to WT mice (Figures. 2.4A-B). In order to confirm the positive regulation of IL-17A and IL-17F by CXCL1, we treated *cxcl1*$^{-/-}$ mice with recombinant CXCL1 (rCXCL1). Administration of rCXCL1/KC immediately following CLP rescued IL-17A but not IL-17F producing, T cell subsets in the lungs, kidney and spleen of *cxcl1*$^{-/-}$ mice (Figure.2.4). Since the enhanced number of T cell subsets in *cxcl1*$^{-/-}$ mice following CXCL1 administration may be due to T cell recruitment to the lungs and/or IL-17 differentiation by rCXCL1.

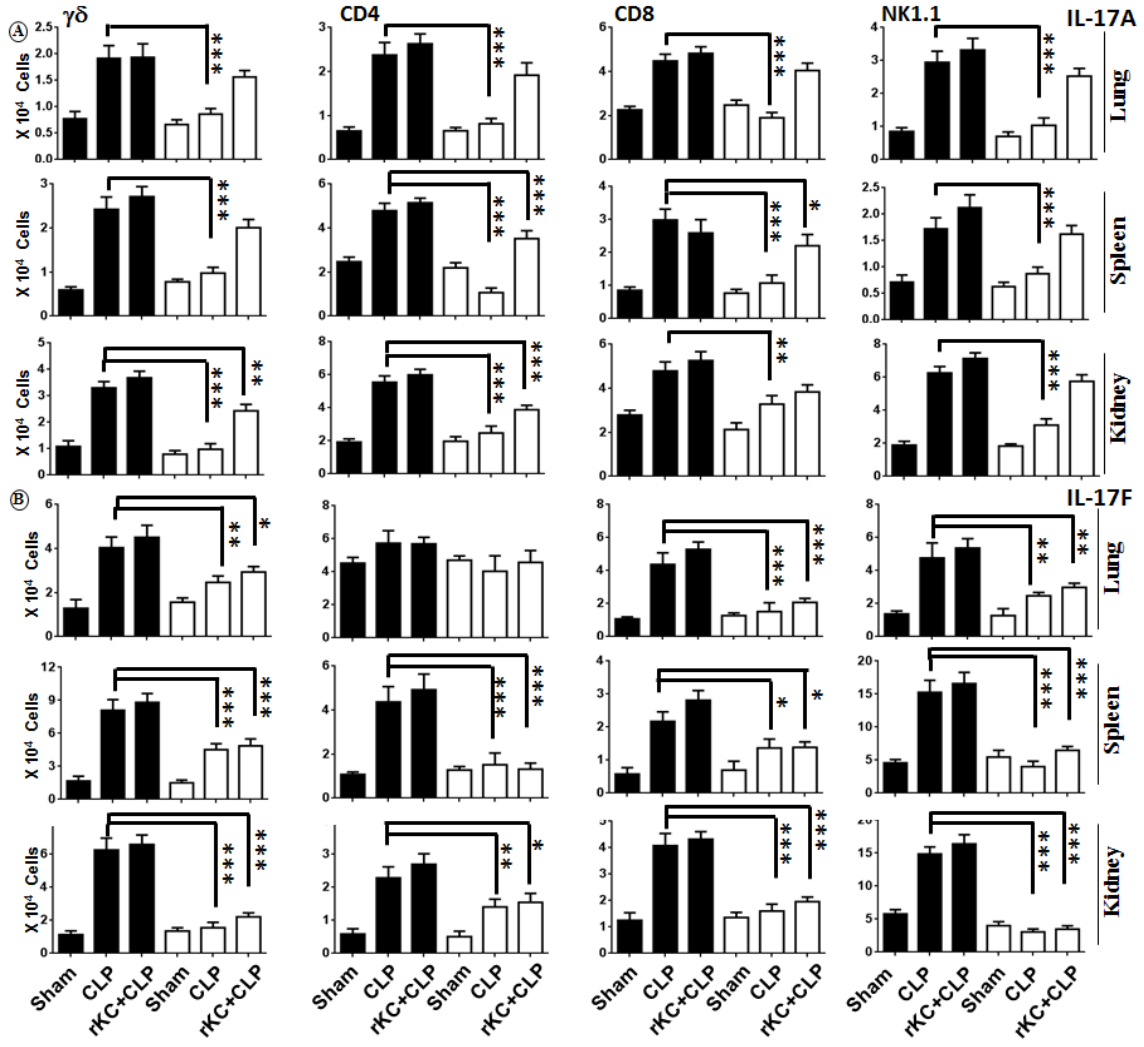

Figure 2.4. CD4, CD8, NK, and γδ cells are the major source of CXCL1-mediated IL-17A and IL-17F production and rCXCL1 rescues T cell subsets in *cxcl1*$^{-/-}$ mice in response to PMS. Intracellular IL-17A (A) and IL-17F (B) in gated CD4^{+}, CD8a, γδ, and NK1.1 cells from lung, spleen and kidney of WT, *cxcl1*$^{-/-}$ mice, and *cxcl1*$^{-/-}$ mice after rCXCL1/KC administration at time 6 and 24 h post-PMS was analyzed by flow cytometry. The results are expressed as mean ± SE from three independent experiments (n=5-8/group; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

To explore these possibilities, we performed Th17 cells differentiation assays using naïve CD4+ T cells obtained from WT and KO mice. Intriguingly, we specifically found that rCXCL1 enhances Th17 (IL-17A-producing) but not Th1 or Th2 differentiation (Figure. 2.5). Moreover, we collected supernatants at 3 days post-stimulation with rCXCL1 and found that differentiated cells produce substantial IL-17A and IL-17F (Figure. 2.5D), validating the data related to Th17 differentiation using flow cytometry.

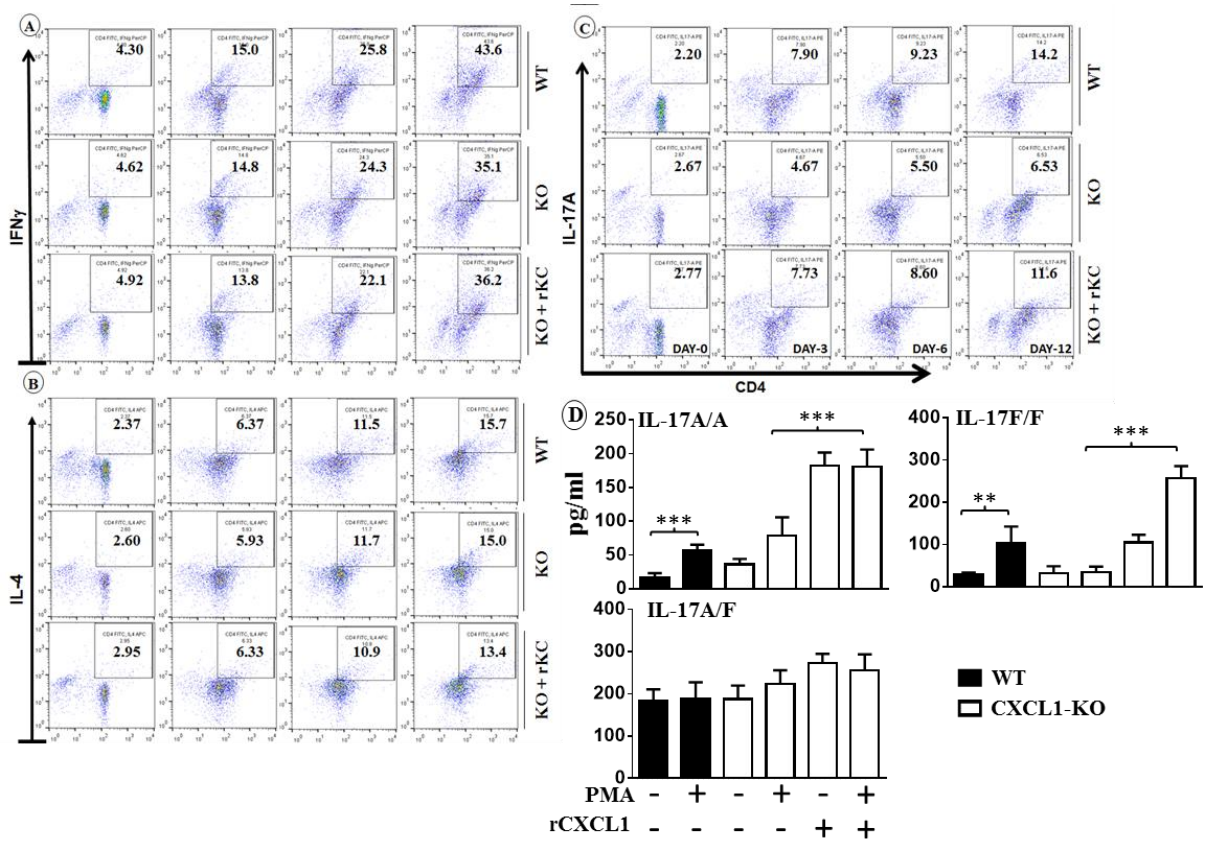

Figure 2.5. Recombinant CXCL1 increases T cell differentiation and the production of IL-17A. A-C, Naive CD4+ T cells from WT and KO mice were isolated and stimulated with PMA in the presence or absence of recombinant CXCL1 as described in Materials and Methods, and a representative dot blot is shown. Quantitation of IFN-γ (A), IL-4 (B) or IL-17A producing CD4+ T cells (C) (n=5/group). D, Production of IL-17A, IL-17A/F (heterodimer) or IL-17F at the protein level by differentiated CD4+ T cells from WT and KO mice after PMA stimulation in the absence or presence of recombinant CXCL1 at 3 days post-stimulation. (n=5-6/group; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

Recombinant IL-17A administration rescues host defense in *cxcl1*$^{-/-}$ mice. Previous studies have shown that early intraperitoneal injection of chemokines after CLP results in rapid neutrophil recruitment and subsequently lower peritoneal bacterial burdens (7).

To determine whether exogenous CXCL1 or IL-17A administration rescues neutrophil-mediated host defense in *cxcl1*$^{-/-}$ mice, *cxcl1*$^{-/-}$ mice were subjected to CLP, and immediately followed by intraperitoneal administration with either saline, rCXCL1, or rIL-17A, and assessed for survival through day 10. Intriguingly, both CXCL1-treated and IL-17A-treated *cxcl1*$^{-/-}$ mice showed improved survival rates compared to saline-treated controls (Figure. 2.6A and B). Furthermore, mice treated with rIL-17A showed lower CFUs in the peritoneal fluid and blood in *cxcl1*$^{-/-}$ mice (Figures. 2.6C-D).

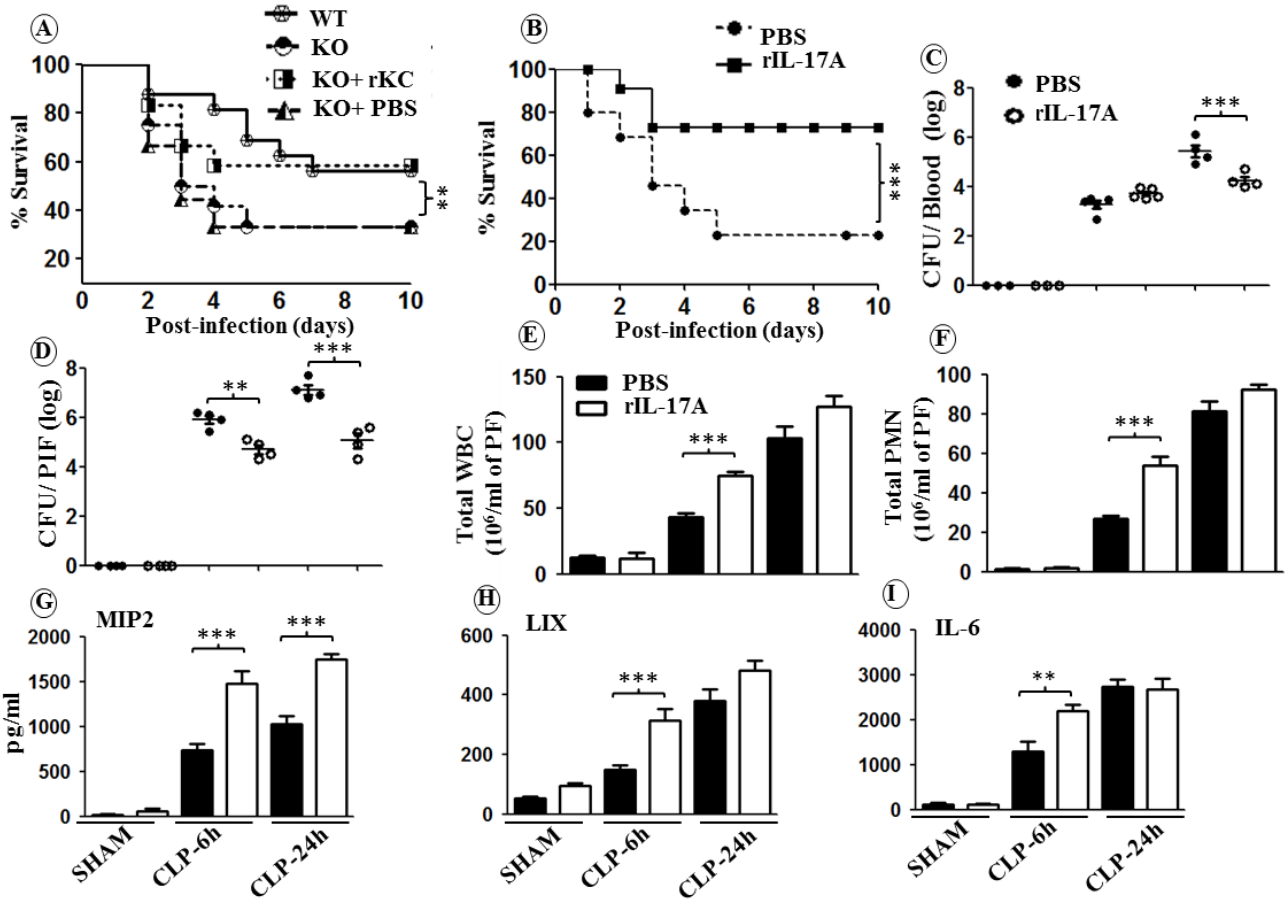

Figure 2.6. Enhanced mortality, higher bacterial burden, reduced leukocyte recruitment, and attenuated cytokine/chemokine production in peritoneal fluid (PF) are rescued in *cxcl1*$^{-/-}$ mice following PMS after rCXCL1 or rIL-17A administration. A-B, *cxcl1*$^{-/-}$ and WT control mice were subjected to sham surgery or PMS. PBS, rCXCL1, or rIL-17A was immediately injected intraperitoneally. B. Survival was assessed every 12 h up to 10 days after PMS. The results are expressed as percentage for 20 animals per group. Significance between groups was examined by Wilcoxon rank test. C-D, Bacterial clearance in *cxcl1*$^{-/-}$ mice after CLP is rescued by exogenous rCXCL1 or rIL-17A administration. CFUs were determined in the peritoneal fluid and blood of *cxcl1*$^{-/-}$ mice as well as WT mice at 6 and 24 h post-PMS. E-F, Total WBC and neutrophil accumulation at peritoneum in *cxcl1*$^{-/-}$ mice following PMS are rescued after administration of rIL-17A. G-I. Production of cytokine and chemokines production in PF of are rescued after administration of rIL-17A. The results are expressed as mean ± SE (n=5-8/group; *, p<0.05; **, p<0.01; ***, p<0.001).

Furthermore, mice treated with CXCL1 showed increased neutrophil counts (Figures. 2. 6E-F) and cytokine/chemokines expression in peritoneal fluid of *cxcl1*$^{-/-}$ mice (Figures. 2.6G-I). These results suggest that IL-17 controls neutrophil-dependent host defense during sepsis via the production of neutrophil chemoattractants such as CXCL2/MIP-2 and CXCL5/LIX.

ROS, H_2O_2, and O_2^- production by neutrophils is attenuated in *cxcl1*$^{-/-}$ mice. Proper function of neutrophils is critical to bacterial elimination from tissues and NADPH activity in

neutrophils is shown to be crucial in this process. To examine NADPH oxidase activity in *cxcl1*$^{-/-}$ mice after CLP, we measured ROS production in peritoneal fluid and in neutrophils, because these cells are a primary source for ROS production (21). Immunocytochemistry results showed a reduced presence of ROS-positive neutrophils (green) in *cxcl1*$^{-/-}$ mice compared to WT mice (Figure. 2.7A). These results are consistent with data indicating a low level of ROS and H_2O_2 production by peritoneal neutrophils and in the peritoneal fluid of *cxcl1*$^{-/-}$ mice (Figure. 2.7B). We also found reduced production of ROS, H_2O_2, and O_2^- by peritoneal neutrophils from *cxcl1*$^{-/-}$ mice via FACS analysis (Figure. 2.7C).

cxcl1$^{-/-}$ neutrophils exhibit decreased NET formation and NET-mediated bacterial killing. The antibacterial function of neutrophils is not only mediated by ROS production, but also by the formation of NETs, termed NETosis (23). NETs are composed of DNA

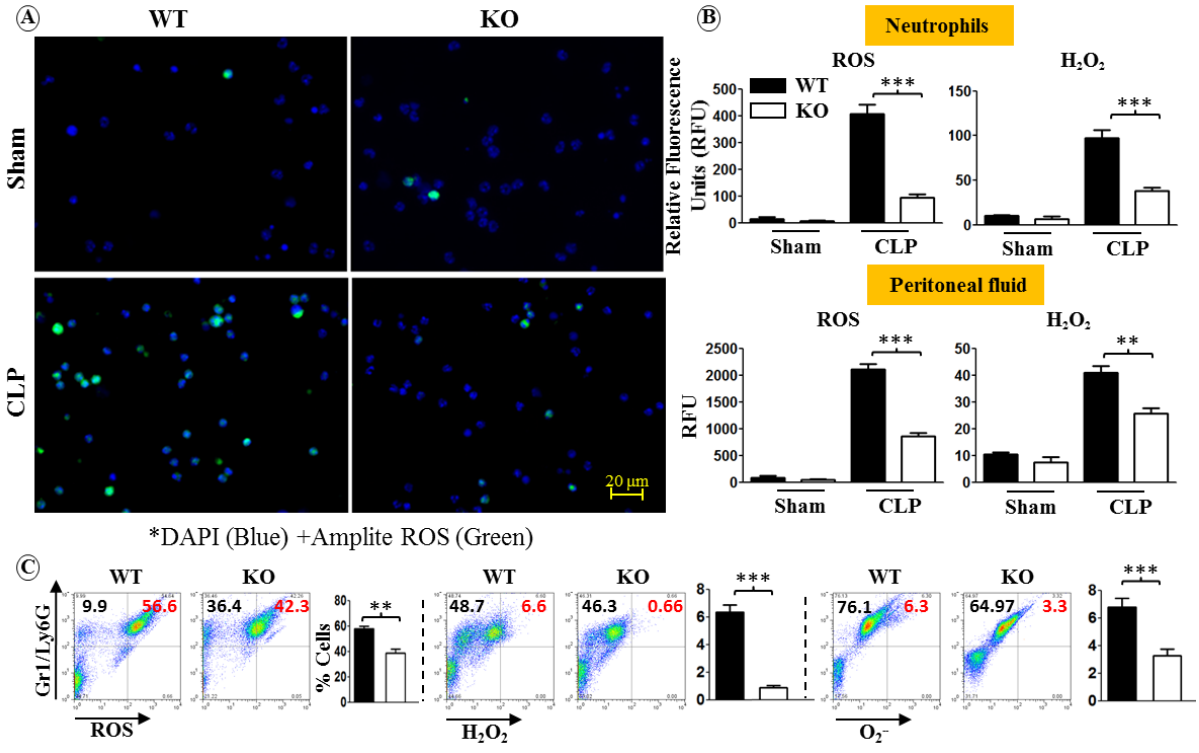

Figure 2.7. ROS production by neutrophils of *cxcl1*$^{-/-}$ mice is attenuated during PMS. A, ROS$^+$ neutrophils were identified by fluorescence microscopy after intracellular staining for ROS in peritoneal neutrophils from *cxcl1*$^{-/-}$ and WT control mice. The results are representative of 20 microscopic views of 3 independent experiments (ROS+ cells are indicated as green, ROS$^-$ cells indicated as blue). B, the production of ROS and H_2O_2 in peritoneal fluid and by peritoneal neutrophils of *cxcl1*$^{-/-}$ and WT control mice following PMS induction were quantified based upon relative fluorescence intensity using commercial kits. The results are expressed as mean ± SE (n=5-8/group). C, ROS$^+$, $H_2O_2^+$ and OH^-/O_2^{-+} neutrophils were quantitated in the peritoneal fluid from *cxcl1*$^{-/-}$ and WT

control mice after PMS by FACS using anti-Gr1/Ly6G Ab for staining neutrophils. The results are expressed as mean ± SE (n=5-8/group; *, p<0.05; **, p<0.01; ***, p<0.001).

studded with many granular proteins that have antimicrobial activity, and their formation is mediated by the induction of ROS in neutrophils (23). Decondensed chromatin with *NET* morphology was shown to have high levels o*f* histone citrullination (Cit-H3 or H3-Cit), indicating that this event is a marker of NETosis (18). In this regard, we isolated peritoneal neutrophils from *cxcl1*$^{-/-}$ and WT mice following CLP and examined them for NET formation and NET-mediated bacterial killing. For evidence of NET formation, we performed agarose gel electrophoresis of peritoneal fluid in the absence and presence of DNase. The results showed increased DNA content in the fluid from WT mice compared to *cxcl1*$^{-/-}$ mice (Figure. 2.8A). The presence of extracellular DNA in the fluid of both groups of mice was further confirmed by the disappearance of DNA bands after DNase treatment in the gel (Figure. 2.8A), suggesting different levels of NET formation in both groups during sepsis. Kinetic analysis of NET formation showed a reduction in NET formation rate by *cxcl1*$^{-/-}$ neutrophils through the initial 8 h (Figure. 2.8B). To further demonstrate unequal NET formation in the neutrophils from *cxcl1*$^{-/-}$ and WT mice, we visualized the DNA in neutrophils from both groups after staining with Sytox green nucleic acid stain and anti-citrullinated histone H3 Ab up to 8 hours post-CLP. Utilizing fluorescence microscopy, long string-like extracellular DNA with citrullinated H3 was most evident in WT neutrophils (indicated by arrowheads) (Figure. 2.8C). The percentage of neutrophils positive for extracellular DNA and citrullinated H3 was reduced among *cxcl1*$^{-/-}$ mice compared to WT mice (Figure. 2.8D). Scanning Electron Micrograph analyses showed typical NET structures (cables, threads, and globular domains), as defined by Brinkmann *et al*. (18), associated with peritoneal neutrophils derived from WT mice after CLP; those structures were largely undetected with peritoneal neutrophils derived from *cxcl1*$^{-/-}$ mice (Figure. 2.8E). Furthermore, the expression of citrullinated H3 and peptidylarginine deiminase-4 (PAD-4), associated with NET formation, was reduced in neutrophils from *cxcl1*$^{-/-}$ mice compared to WT mice after CLP (Figure. 2.8F).

To determine whether CXCL1-mediated NET formation is essential for bacterial killing, *E. coli* was incubated with *cxcl1*$^{-/-}$ or WT neutrophils, and bacterial killing was analyzed. We found a decrease in NET-mediated bacterial killing and relative phagocytosis in *cxcl1*$^{-/-}$ neutrophils compared with WT neutrophils (Figures. 2.8G-H). Because DNase can be used to inhibit NET formation, neutrophils were pretreated with DNase in one set of experiments. WT neutrophils without DNase treatment showed effective NET-mediated bacterial killing compared to the DNase-treated control, whereas no difference in bacterial killing was observed in the DNase-treated *cxcl1*$^{-/-}$ neutrophils (Figure. 2.8G). In addition, the percentage of neutrophils positive for extracellular DNA and citrullinated H3 was lower among *cxcl1*$^{-/-}$ mice following LPS stimulation (10 ng or 100 ng) or *E. coli* (MOI 1) infection (Figure. 2.8I).

Discussion

Brisk neutrophil migration to the site of infection and local ROS production are multistep processes and pivotal events in host defense during bacterial infection (24). The severity of sepsis induced by CLP correlates with decreased neutrophil recruitment and consequent diminished bactericidal activity in the peritoneum (7, 8, 25-27). Recruitment and activation of neutrophils has been demonstrated to be critical for controlling CLP-induced polymicrobial sepsis (PMS) by suppressing bacterial growth in the peritoneum and extraperitoneal organs (7).

In this report, we investigated the role of CXCL1 in neutrophil recruitment and function during sepsis induced by CLP. Mice deficient in CXCL1 exhibited a reduction in total leukocytes and neutrophils in the peritoneum after CLP, and a reduction in bacterial clearance from the peritoneum and extra-peritoneal organs, leading to reduced survival.

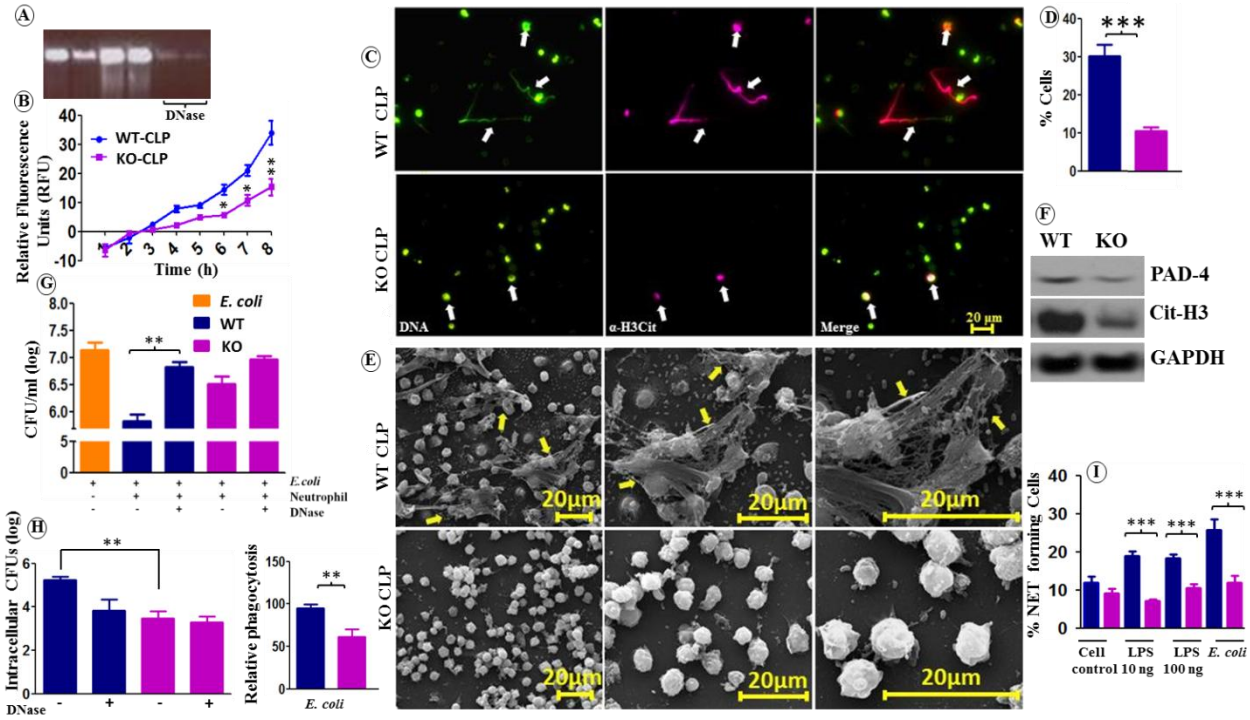

Figure 2.8. *cxcl1*$^{-/-}$ neutrophils exhibit decreased NET formation and NET-mediated bacterial killing in PMS. A, Agarose gel electrophoresis of peritoneal fluid of *cxcl1*$^{-/-}$ mice exhibited reduced amounts of extracellular DNA compared to their WT counterpart after PMS. B, Kinetic analysis of NET formation by peritoneal neutrophils harvested from *cxcl1*$^{-/-}$ and WT mice. Relative fluorescent intensity was determined to evaluate NET formation each hour until 8 h of *in vitro* culture of neutrophils (n=6-9/group). C, Peritoneal neutrophils harvested from *cxcl1*$^{-/-}$ or WT mice after PMS, allowed to undergo NET formation and fixed at 8 h. Neutrophils were stained with Sytox green DNA stain and citrullinated histone H3 Ab to visualize citrullinated DNA after the cells were fixed with 4% (v/v) paraformaldehyde. Scale bars, 20 μm. Long strands of DNA (arrowhead) are evidence of NET formation. Images presented are representative images of three independent experiments (n=5-7/group). D, A total of 20 images were selected from one experiment and quantified for the presence of NET-positive neutrophils from *cxcl1*$^{-/-}$ and WT mice. The results are expressed as mean ± SE (n=5-8/group). E, Evaluation of NET formation by SEM. Peritoneal neutrophils harvested from *cxcl1*$^{-/-}$ and WT mice after induction of PMS were analyzed by SEM. Presence of long thread-like structures (arrowhead) is evidence of NET formation. Scale bars, 20 μm. Images presented are representative of two independent experiments (n=5-8/group). F, Western blot of PAD-4 and cittrullinated-H3 in the peritoneal neutrophils from *cxcl1*$^{-/-}$ and WT mice at 6 h ad 24 h post-PMS. This blot is representative of 3 independent experiments with similar results. G-H, Bone marrow neutrophils from *cxcl1*$^{-/-}$ mice exhibited diminished extracellular bacterial killing activity. Bacterial killing capacity of *E. coli*-infected bone marrow neutrophils from WT and CXCL1-deficient mice was determined by assessing extracellular (G) and intracellular CFUs (H) at 60 min after infection with *E. coli* (MOI 1). Relative phagocytosis of *E. coli*-infected WT and CXCL1-deficient neutrophils at 60 min post-treatment (MOI 1). A total of 4–5 mice/group were used.. I, the percentage of NET-forming bone marrow neutrophils following LPS and *E. coli* stimulations were quantified after staining with Sytox green DNA stain and anti-α H3-Cit antibody as described above (B). The results are expressed as mean ± SE from two independent experiments (n=5-8/group: *, p<0.05; **, p<0.01; ***, p<0.001).

These findings are consistent with previous studies, which showed a critical protective role for CXCL1 in pulmonary host defense against *Klebsiella pneumoniae* infection (10). Inhibition of CXCL1 attenuates lung neutrophil accumulation following intratracheal LPS administration (28). Moreover, CXCL1-transgenic mice that constitutively express lung CXCL1 show enhanced survival after *K. pneumoniae* challenge, as well as enhanced neutrophil recruitment and bacterial clearance in the lungs (28). In addition, CXCL1/CXCL1 mRNA was found to be strongly and rapidly induced in the liver and lungs, which was associated with heightened neutrophil infiltration into these organs (29). The relative contribution of myeloid cells versus stromal cells in neutrophil accumulation following PMS is unclear. Myeloid cells in tissues produce a battery of neutrophil chemotactic substances such as CXCL1 (10, 11) and MIP-2 (12, 13) and resident cells, including epithelial and endothelial cells, produce other neutrophil chemoattractants, such as LIX (14). The findings in this investigation are consistent with our previous studies, demonstrating that hematopoietic and nonhematopoietic cell-derived CXCL1 is essential for neutrophil-dependent bacterial clearance (10, 12)

We found that the local and systemic inflammatory response to PMS was dependent on CXCL1, and correlated with higher cellular influx, bacterial clearance, and reduced mortality. In addition, the expression of key inflammatory cytokines and chemokines was dependent upon CXCL1 during PMS. Interestingly, we observed that the expression of IL-1β, IL-6, LIX, and IL-17A was mediated by CXCL1 even during the early phase of sepsis (6 h). These findings are in agreement with previous studies that showed the expression of CXCL2 and CXCL5, but not TNF-α, to be dependent on CXCL1 expression in *K. pneumoniae*-infected lungs (10).

Activation of transcription factors is a central feature of inflammation and host response (20), and NF-κB is a well-studied transcription factor (21). We found reduced activity of NF-κB in the organs from *cxcl1*$^{-/-}$ mice post-CLP. Although NF-κB activity is important for survival after PMS (4, 30), it is questionable whether this phenomenon contributes to

efficient neutrophil recruitment and bacterial clearance. NF-κB activation has been demonstrated to be essential for neutrophil recruitment due to its contribution to the expression of adhesion molecules, such as ICAM-1 (31). Our results reveal a strong correlation between CXCL1 expression after PMS, NF-κB activation, and ICAM-1 expression. This suggests that CXCL1-mediated NF-κB activation plays a crucial role in neutrophil recruitment at early and late phases of polymicrobial sepsis.

MAPKs are important enzymes that enhance the expression of pro-inflammatory cytokines, chemokines, adhesion molecules, and antibacterial effectors by activating transcription factors, such as AP-1, c-Jun, and STAT-1 (32). Our results show reduced activation of p38 (at 24 h post-CLP), JNK, and ERK MAP kinases in the organs from *cxcl1*$^{-/-}$ mice. These results are consistent with previous findings that showed a crucial role for CXCL1 in MAPK activation during *K. pneumonaie* pulmonary infection (10).

IL-17-producing CD4+ T cells control bacterial infection by secretion of neutrophil chamoattractants and granulopoietic factors such as CXC chemokine CXCL2/MIP-2 and G-CSF, which cause neutrophil recruitment (33). We found not only γδ T cells, but also CD4, CD8, and NK1.1 cells as major IL-17-producers in WT mice 24 h post-PMS. This suggests that CXCL1 regulates both IL-17A and IL-17F expression by major T cell subsets during sepsis. This possibility was confirmed when we performed intracellular staining for IL-17A in T cell subsets. Because we observed increased numbers of IL-17A-producing CD4 cells and increased IL-17A and IL-17F secretion after treatment with recombinant CXCL1, our results suggest that CXCL1 is an essential chemokine for differentiation of naïve CD4 T-cells to Th17. To validate this hypothesis, we differentiated Th0 cells to Th1, Th2 and Th17 *in vitro* in the absence and presence of CXCL1. Intriguingly, our findings demonstrated that CXCL1 selectively augments Th17 differentiation. The limitation of this study however noted. It is not possible to use either Gram-positive or Gram-negative bacterium in these cultures because these bacteria induced substantial cell death (data not shown).

To examine whether rCXCL1 or rIL-17 can restore the protective effect on *cxcl1*$^{-/-}$ mice, we administered each of these chemokines immediately after PMS, resulting in enhanced survival of *cxcl1*$^{-/-}$ mice. Additionally, enhanced neutrophil influx and bacterial clearance at the peritoneum were observed following rCXCL1 or rIL-17 administration. Our observation of host protection by treatment with IL-17 in *cxcl1*$^{-/-}$ mice agrees in part with a previous investigation that demonstrated reduced survival rates, lower neutrophil influx, and bactericidal capacity in IL-17R gene deficient mice following PMS (though cytokine and chemokine expression was enhanced in these mice) (8).

We observed reduced expression and activation of NADPH oxidase components Nox2, p22^{phox}, p67^{phox}, and p47^{phox} in *cxcl1*$^{-/-}$ mice following PMS, which may due to the reduced accumulation of neutrophils at the infectious focus in *cxcl1*$^{-/-}$ mice. These observations are in agreement with a previous investigation that demonstrated reduced expression of NADPH oxidase components following neutrophil depletion in WT mice after *K. pneumoniae* challenge (12). CXCL1-mediated activation and expression of NADPH oxidase is consistent with ROS production by neutrophils, as we observed decreased ROS production in peritoneal fluid and by peritoneal neutrophils in *cxcl1*$^{-/-}$ mice compared to WT counterparts. Additionally, we observed a substantial reduction of ROS^+, $H_2O_2^+$, and O_2^+ producing neutrophils from *cxcl1*$^{-/-}$ mice following PMS. These findings are consistent with a previous study that demonstrated an essential role of CXCL1 in *Klebsiella*-induced expression of ROS by neutrophils (12).

NETosis is a unique host defense mechanism employed by neutrophils to trap and kill extracellular pathogens (34). In the present study, we found a strong correlation between reduced NADPH oxidase-mediated ROS production and reduced NET formation by neutrophils from *cxcl1*$^{-/-}$ mice following PMS. Additionally, NET formation and NET-positive peritoneal neutrophils were reduced in *cxcl1*$^{-/-}$ mice compared to WT. To confirm this result, we observed reduced formation of typical NET structures associated with *cxcl1*$^{-/-}$ neutrophils by SEM. In addition, we observed reduced *E. coli* killing by

bone marrow neutrophils treated with DNase, confirming the link between NET formation and bacterial killing. The results linking neutrophil ROS production with NETosis and subsequent bacterial killing are consistent with several prior reports (18, 35, 36). Our finding that neutrophils harvested from *cxcl1*$^{-/-}$ mice are deficient for bacterial killing confirms the crucial role of CXCL1 in the containment of infection by migrating neutrophils.

The data in this study illustrate a number of advancements in our understanding of the biological role of CXCL1 in host defense during PMS. CXCL1 appears to be essential for host survival, activation of NF-κB, MAPK, and NADPH oxidase, expression of cytokines and chemokines essential for host defense, ROS production, as well as NET formation by peritoneal neutrophils and NET-mediated bacterial killing (Figure. 2.9). More importantly, CXCL1 regulates IL-17A production by enhancing Th17 differentiation in order to control neutrophil influx via the production of CXCL2/MIP and CXCL5/LIX (Figure. 2.9).

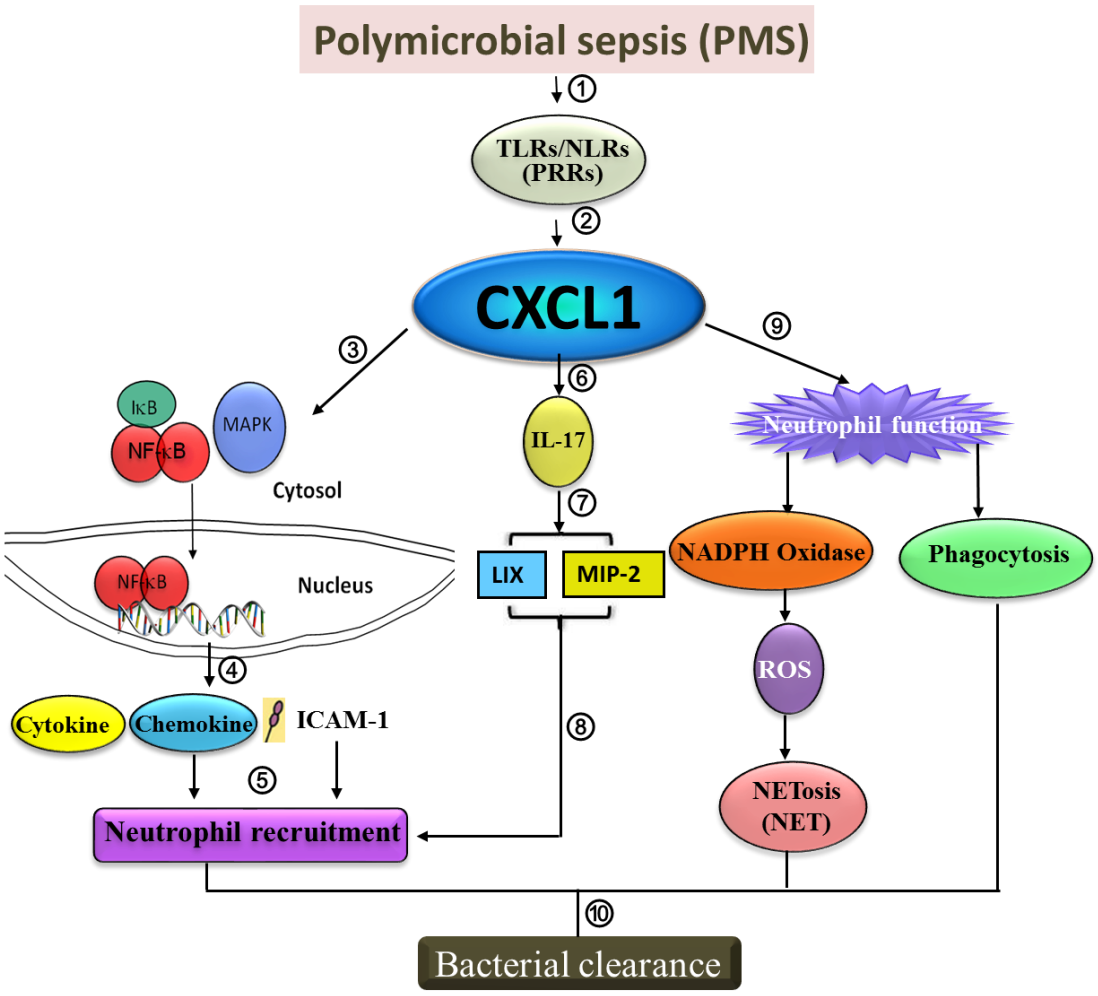

Figure 2.9. Proposed scheme for KC-mediated signaling cascades leading to bacterial clearance in the organs in response to CLP. Bacteria in the peritoneum during polymicrobial sepsis interact with pattern recognition receptors (1) resulting in CXCL1 production by hematopoietic and resident cells (2). In one arm, CXCL1 activates NF-κB, MAPK (3) and upregulation of cell adhesion molecules (ICAM-1) along with the production of cytokines/chemokines (4), including IL-17 in order to induce neutrophil recruitment to the tissues from the bloodstream (5). In the other arm, CXCL1 modulates neutrophil function (6) via activating NADPH oxidase (7) leading to the production of ROS and eventual NETosis (8) and augmenting phagocytosis (9). Both of these arms are important for bacterial clearance in organs.

Acknowledgments

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Chapter 3: NLRP3 Does not Regulate Neutrophil Recruitment but Modulates Neutrophil Function in Peritoneum during Polymicrobial Sepsis

Introduction

Despite extensive scientific and clinical care management, sepsis continues to be one of the leading causes of infectious deaths in United States. It is estimated more than 210,000 people die because of sepsis per year with annual healthcare costs for treatment of sepsis exceeding $17 billion in United States (1). Common causes for sepsis include traumatic injury, severe burns and bacterial infections. Furthermore, sepsis can also affect elderly, ill, pediatric and postsurgical patients in ICU. Clinical studies have revealed that immune responses play an important role in sepsis. Hence sepsis is defined as an overwhelming systemic inflammatory response syndrome (SIRS) after serious microbial infection. The innate immune system contributes to the migration of leukocytes in inflamed tissue, involving release of various cytokines/chemokines and adhesion molecules. However, there is a shift towards immune-suppressive condition during the course of sepsis due to apoptosis of immune cells and over-production of anti-inflammatory cytokines (2-3). The environment in sepsis can cause disseminated intravascular coagulation (DIC), however, thrombin secreted by infiltering leukocytes triggers the further release of chemokines and adhesion molecules through endothelial cells, which represents a positive feedback mechanism for innate immune responses. Events which follow sepsis represents the stages in progressive condition, therefore relationship between the cytokine/chemokine cascades and sepsis have long been under scrutiny. New therapeutic strategies for sepsis therefore, try to establish a well-balanced immune response. Intervention is accomplished through inhibition of inflammatory cytokines, their receptors or through activation of immunostimulatory responses. Neutrophils are important cell type in sepsis resulting in the release of cytokines, chemokines and antimicrobial proteins, and phagocytose microbial pathogens. Neutrophil recruitment to the site of infection has been shown to be reduced during severe sepsis, which is associated with reduction in adherence, chemotaxis, phagocytosis, and production of reactive oxygen

intermediates (ROIs) (4-8). Thus, investigating effector function of neutrophils during sepsis is essential to understanding the mechanisms associated with the pathophysiology of sepsis. Recently, besides phagocytosis, a novel antimicrobial mechanism of neutrophils also termed as neutrophil extracellular traps (NETs) was identified by Brinkmann (9). NETs are networks of extracellular fibers, primarily composed of DNA from neutrophils which bind pathogens. NETs are studded with antimicrobial proteins like elastase, myeloperoxidase eand NETs kill exracellular pathogens independent of their phagocytic ability (10-11). Furthermore, NETs may also provide a physical barrier to limit the spread of rapidly disseminating pathogens and antimicrobial proteins to regulate tissue damage (12-13). Innate immunity, the first line of host defense that employs specific pattern recognition receptors (PRRs) to recognize pathogen-associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs) emanating from host cells affected by pathogenic onslaught. The recognition of PAMPs as well as DAMPs by PRRs activates immune response pathways resulting in inflammation. There are three major families of PRRs have been recognized; the trans-membrane toll-like receptors (TLRs), the RIG-I-like receptors (RLRs), and the intracellular NOD-like receptors (NLRs). The role of various TLRs in the pathophysiology of sepsis is well established, and their potential as therapeutic targets has been explored (14). In contrast we are only beginning to appreciate the importance of NLRs in clinical course of sepsis. NLRs are important in the context of sepsis because these PRRs recognize PAMPs, as well as a variety of DAMPs. Of these, NLRP3 is of particular interest as it forms a caspase-1 activating molecular complex termed “inflammasome” (14, 15). Caspase-1 activation by the inflammasome promotes the maturation of IL-1β. Animal and human studies have highlighted the importance of the inflammasome pathways in the inflammatory response to sepsis (16, 17). For example, caspase-1-/- mice are protected from endotoxic shock and *E. coli* induced sepsis (18). Similarly, pyrin the protein mutated in familial Mediterranean fever has been shown to regulate production of mature IL-1β by complexing with pro-caspase-1 and ASC-1. Since caspase-1 can be activated by multiple NLRs, in this investigation, we

speculated that the NLRP3 inflammasome might be positioned to modulate numerous critical checkpoints in the host defense cascades to Gram-negative and Gram-positive pathogens in vivo during polymicrobial sepsis using the cecal ligation and puncture (CLP) surgery.

Here, we demonstrate that mice with deletion or inhibition of NLRP3 protected from polymicrobial sepsis and display lower bacterial load, attenuated production of proinflammatory cytokines but no change in neutrophil accumulation. However, NLRP3-/- PMNs show altered neutrophil functions including phagocytosis, bacterial killing, neutrophil extracellular trap (NET) formation, chemotaxis, and cell death. Taken together, these findings suggest that NLRP3 modulates host defense against sepsis through the regulation of neutrophil function in organs, therefore positioning NLRP3 as a critical linker between host defense and neutrophil function.

Materials and Methods

Animal care and use: Eight- to ten-week-old NLRP3 gene-deficient ($NLRP3^{-/-}$) male mice were back-crossed 10 times with C57BL/6 mice for this study and C57BL/6 (WT) male mice were used as age- and gender-matched controls. Animals were handled in accordance with Louisiana State University Animal Welfare Committee's approved protocol.

Cecal ligation and puncture (CLP), *E. coli* peritonitis and endotoxic shock:. In this experiment, cecal ligation and puncture (CLP) was used as animal sepsis model. In short, mice were anesthetized with ketamine (100 mg/kg) /xylazine (10 mg/kg) mixture. A 1-cm midline incision was made in the lower part of abdomen. The peritoneum was opened and cecum was delivered to a sterile operative field on abdominal surface. Using 3-0 silk suture, cecum was ligated at its proximal aspect without occlusion of the intestinal lumen. The cecum was punctured through by through with 21 gauge needle and a small amount of decal material was extruded through the puncture sites. Then, the cecum was repositioned into the peritoneal cavity and the abdomen was stitched up with suture clips. Finally, mice were resuscitated by injection of 1ml of warm isotonic sodium chloride. Animal with sham operation underwent the same protocol without cecal ligation and puncture. For LPS-induced

endotoxin shock, mice were injected i.p. with *E. coli* LPS at a dose of 5 or 10 mg/kg body weight. The survival of mice was checked at every 6 h after injection. For *E. coli*-induced peritonitis, mice were injected i.p. with *E. coli* at a dose of 5x 10^8CFU/kg body weight. The survival of mice was checked at every 6 h after injection.

Survival studies: Both NLRP3$^{-/-}$ and WT mice were underwent CLP or sham, in another group WT were given glybenclamide or DMSO immediately after CLP, survival was monitored every 12 h up to 10 days.

Determination of bacterial CFU: Bacterial count was assessed as previously described. The lungs and spleens of control and infected mice were weighed and homogenized in 1 ml of 0.9% saline using a tissue homogenizer. The solid tissue was allowed to sediment for 10 min at room temperature and the supernatants were serially diluted. Twenty milliliter aliquots of each sample were plated on TSA plates. The number of colonies was enumerated after incubation at 37℃ overnight.

Immunohistochemistry and immunostaining for NLRP3: Immunohistochemistry was performed on formalin-fixed paraffin-embedded tissue sections. 5μm thick sections were subject to heated antigen unmasking solution for 30 minutes, all sections for immunohistochemisty were removed paraffin and hydrated by different graded concentration of ethanol to deinoized water. All sections were washed gently with deionized water, after that slides were transferred in to 0.05 M Tris-based solution in 0.15M NaCl with 0.1% v/v Triton-X-100, pH 7.6 (TBST).

BM transplantation: Six to eight week old donor and recipient animals were used to generate chimeras as described in previous reports. Recipient mice were gamma irradiated from a cesium source in two 525-rad doses 3 h apart. BM cells (8×10^6/mouse) were injected into the irradiated recipients via the tail vein. Chimeric mice were maintained on 0.2% neomycin sulfate for the first 2 weeks. The transplanted mice were used for experiments at 8 wk after reconstitution. We found that >88% of blood leukocytes were derived from donor animals at

the time for experiments. Irradiated mice that were not reconstituted with donor cells died between days 22 and 24 post-transplantation.

Peritoneal lavage: Peritoneal exudate cells were recovered by peritoneal lavage with 4 ml of sterile, warm, heparinized RPMI 1640 medium (GIBCO/BRL, Bethesda, Md.) and PMN percentage were counted manually by a hemocytometer.

Cytokine and chemokine measurements: The concentrations cytokines and chemokines in peritoneal lavage and serum were determined by using a commercially obtained ELISA kit according to the instructions of the manufacturer (R & D Systems). The minimum detection limit is 8-pg/ml of cytokine protein.

Flow cytometry: Immediately after mice were euthanized, 6 ml of physiological saline was injected into peritoneal cavity and was lavaged repeatedly. Peritoneal cells were collected, and subsequently stained for flow cytometry using anti-Gr1-PerCP, anti-CD11b-PE, and anti-F4/80-APC purchased from ebiosiences or BD unless otherwise stated. All flow cytometry data was analyzed using FlowJo VX software.

ROS production: In an attempt to see the changes in ROS production following CLP by $CXCL1^{-/-}$ and $CXCL1^{+/+}$ neutrophils, PMNs were isolated from peritoneal fluid and resuspended in HHBS buffer. Cells were loaded with Amplite™ ROS green, Amplite™ green peroxide sensor or ROS Brite™ 570 as per manufacturer's instructions and incubated at 37℃ for 1 h. Data was acquired on a BD FACSCalibur flow cytometer (BD Biosciences) and was analyzed with FlowJo Version 7.6 software (TreeStar).

Phagocytosis and bacterial killing activity of neutrophils: The phagocytic activity of peritoneal neutrophils and macrophages was measured by the uptake of red fluorescent pHrodo *Escherichia coli* bioparticles (Invitrogen) as described by Wan et al. Briefly, 1x 10^6 macrophages or neutrophils were suspended in 100 uL of HBSS containing 20mM HEPES, pH 7.4, and mixed with 5uL of pHrodo *E coli* bioparticles. The mixture was incubated for 30 minutes at either 37℃ for uptake activity. After incubation, macrophages or neutrophils were washed twice with component C (Invitrogen), then resuspended in 100 uL of component C.

Absorbance change at different time points was recorded with a spectrophotometer (U-2001;Hitachi,Tokyo, Japan). The neutrophil mediated killing was performed as previously described with slight modification. Neutrophils isolated from bone marrow ($1x10^6$) were suspended in RPMI 1640 with 10%v/v FBS and were incubated with $1x10^6$ (1 MOI) opsonized bacteria in a shaking water bath at 37℃ for 120 min with continuous agitation. Samples were harvested at 0, 30, 60 or 120 min, and a portion of the sample was spun at 100x g for 10 min to collect the bacteria in media. The neutrophil pallet was resuspended in 1ml cold PBS, and the debris was broken up by using a homogenizer to rate engulfted bacteria. Colony counting of viable bacteria was conducted by plating 20-ul aliquots of each sample on trypticase soy agar plates. The number of colonies was countered after incubation at 37℃ overnight.

Purification of peripheral polymorphonuclear leukocytes (neutrophils): Following cervical dislocation both hind limbs of a C57Bl/6 mouse were prepared and the whole BM was flushed out of both, tibia and femur using 21-gauge needle attached to a 10 ml syringe filled with PBS. After centrifugation (300× g/5 min/room temperature (RT)) erythrocytes removed by incubation in 5 ml RBC lysis buffer. Subsequently, cells were washed with 25 ml PBS and resuspended into ROBOSEP buffer. Cells were then resuspended in an appropriate volume of antibody cocktail and incubated at 4℃ for 15 min. Samples were centrifuged and (250× g/5 min/RT) the cell pellet was resuspended in 2.5 ml ROBOSEP buffer for washing. Cells were then resuspended in ROBOSEP buffer containing biotin selection cocktail for another 15 min. A desired volume of anti-Biotin-microbeads (Stem Cell Technologies, Canada) per 107 total cells were added. Subsequently the cell suspension was applied to EasySep magnet for 3 mins and neutrophil enriched fraction was eluted. The obtained cells were washed with PBS and neutrophil cell number was assessed.

NETs formation: Cells were isolated from peritoneal fluid obtained from $NLRP3^{-/-}$ or $NLRP3^{+/+}$ male mice post CLP or sham by centrifugation at 400 x g. PMNs were isolated by magnetic separation using the EasySep negative selection system (StemCell Technologies).

Purified neutrophils were stained with 5 μM Sytox Green (Invitrogen) and cultured in DMEM with 1 mM HEPES buffer in a 96-well black/clear bottom special optics plate from BD Falcon. NETs were allowed to form for 8 h and the kinetics was monitored using fluorescence reader.

Immunostaining and electron microscopy: Samples were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA) after determining NETs kinetics. To immunostain for citrullinated histone H3, samples were blocked with BSA (5% in PBS for 1h), washed with PBS and incubated with anti-α-H3Cit (Abcam; 1:200 in 1% BSA) for 2 h. Alexa Flour 594 $(ab')_2$ fragment of goat anti-rabbit IgG (H+L) was used as secondary Ab for citrullinated histone H3 Ab. After staining, cells were washed with PBS four to five times. Images were taken using Zeiss Axio Observer Z1 Live Imaging research microscope (Carl Zeiss USA) with differential interference contrast, and operated by Axiovision software (Carl Zeiss USA).

Scanning electron microscopy: PMNs isolated from peritoneal cavity of CLP induced or Sham mice were cultured for 8h in DMEM containing 1 mM HEPES. Cells were fixed with 2.5% glutaraldehyde and 1% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) overnight at 4 degrees. In some experiments the fixed cells were washed twice with sodium cacodylate and postfixed with 1% osmium tetroxide and 1% tannic acid, dehydrated with a graded series of ethanol, and then dried in hexamethyldisalizane. The samples were subsequently mounted on colloidal graphite, sputter-coated, and visualized using a scanning electron microscope (S2460 N; Hitachi, San Jose, CA).

Annexin-V staining for cell death: Cell death was determined by staining cells with Annexin-V-fluorescein isothiocyanate (FITC, Ex/Em=488 nm/519 nm; Invitrogen Molecular Probes, Eugene, OR). In brief, $1x10^6$ peritoneal cells were washed twice with cold PBS and then resuspended in 500 μl of binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, 2.5 mM CaCl2) at a concentration of $1x10^6$ cells/ml. Annexin-V-FITC (5 μl) was then added to

these cells, which were analyzed with a FACStar flow cytometer (Becton-Dickinson, Franklin Lakes, NJ).

Glybenclamide (NLRP3 Inflammasome Inhibitor ） administration: NLRP3 inhibitor Glybenclamide (Invivogen, San Diego, CA) was prepared in DMSO to a final concentration of 25 mg/mL. A total of 40 ml/mouse (1mg/mouse) was administered intraperitoneally (i.p.) immediately after CLP. In control group, DMSO was used instead of glybenclamide.

Western Blotting: An assessment of Histone Citrullination and PAD-4 activation in the isolated neutrophils from peritoneal lavage fluid and human neutrophils post CLP was done by extracting cellular proteins using Urea/CHAPS/Tris (lysis) buffer. Briefly, the harvested cells were lysed using lysis buffer containing and complete protease and phosphatase inhibitor cocktail (Roche Co, Indianapolis, IN) for 15 mins in cold conditions. Samples were spun at maximum speed in eppendorf centrifuge to remove cellular debris. To ensure equal loading of protein onto the gel, a Bradford protein assay was performed (Bio-Rad, Herculus, CA). Proteins were fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred electrophoretically to Immobilon-P transfer membrane (Millipore UK, Watford, United Kingdom) by conventional wet blotting. Membranes were incubated in blocking buffer (1×TBS, 5% [wt/vol] non-fat dry milk, 0.1% Tween-20) for 1 hour followed by overnight incubation with primary antibodies viz. citrullinated histone and PAD-4 (at a 1 in 1000 dilution) in blocking buffer at 4℃. Antibody to GAPDH was added to the concentration of 1:5000. The membranes were washed extensively for 3 times 10 minutes each in wash buffer. Incubation with species-specific horseradish peroxidase (HRP)–conjugated secondary antibodies at a 1 in 2000 dilution was performed in blocking buffer for 1 h. Labeling was detected using the enhanced chemiluminescence (ECL) reagent (Amersham Biosciences, Amersham, United Kingdom).

Statistics: Data are expressed as means $\pm$ SE. Statistical analyses were performed using GraphPad Prism® (v.5.03). Tests for significance (p value) were calculated using Student's t-test (between two groups) or with the two-way ANOVA (more than 2 groups). Survival

curves were analyzed by the Wilcoxon rank sign test. Differences in values were defined as significant at a *p* value of less than 0.05 using Kaleidagraph (Synergy software, PA).

Results

NLRP3 protein is upregulated post sepsis.To demonstrated the important role of NLRP3 during sepsis, by immunohistochemistry , specificity of the antibody NLRP3 proved all the tissue(lung, liver, spleen and kidney) from sepsis mice strongly express NLRP3 protein but very low levels of NLRP3 was found in the sham group(Figure. 3.1). during sepsis, by immunohistochemistry , specificity of the antibody NLRP3 proved all the tissue(lung, liver, spleen and kidney) from sepsis mice strongly express NLRP3 protein but very low levels of NLRP3 was found in the sham group(Figure. 3.1).

$NLRP3^{-/-}$ mice were resistant to CLP-induced lethality. To determine the requirement of NLRP3 in host defense against sepsis, C57Bl/6 and $NLRP3^{-/-}$ mice underwent CLP and the up to 10 days. We observed that all $NLRP3^{-/-}$ mice survived to 10 days whereas only 56% of C57Bl/6 survived (Figure. 3.2A). All sham controls from both $NLRP3^{-/-}$ and WT were survived (data not shown). We next determined whether $NLRP3^{-/-}$mice show resistance to other models of sepsis. In this regard, we injected $5x10^8$ CFU/Kg *E. coli* intraperitoneally (i.p) or two doses of LPS i.p. (5mg/Kg or 10mg/Kg). In both sepsis models, $NLRP3^{-/-}$ mice were observed to be less susceptible as compared with WT controls (Figure. 3.2B-C). Furthermore, we investigated whether resistance to CLP-induced sepsis in $NLRP3^{-/-}$ mice resulted from enhanced bacterial clearance. For this CLP was performed on the groups of *C57Bl/6* and $NLRP3^{-/-}$ mice. Mice were anesthetized and exsanguinated *at* 6 h and 24 h post CLP. As shown in Figure. 3.2D, bacterial clearance in peritoneal lavage fluid (PLF) and extra-peritoneal organs such as the blood, lung, liver, spleen and kidney of $NLRP3^{-/-}$ mice was enhanced at 24 h. Bacterial clearance in blood and kidney was augmented even at 6 h (Figure. 3.2D).

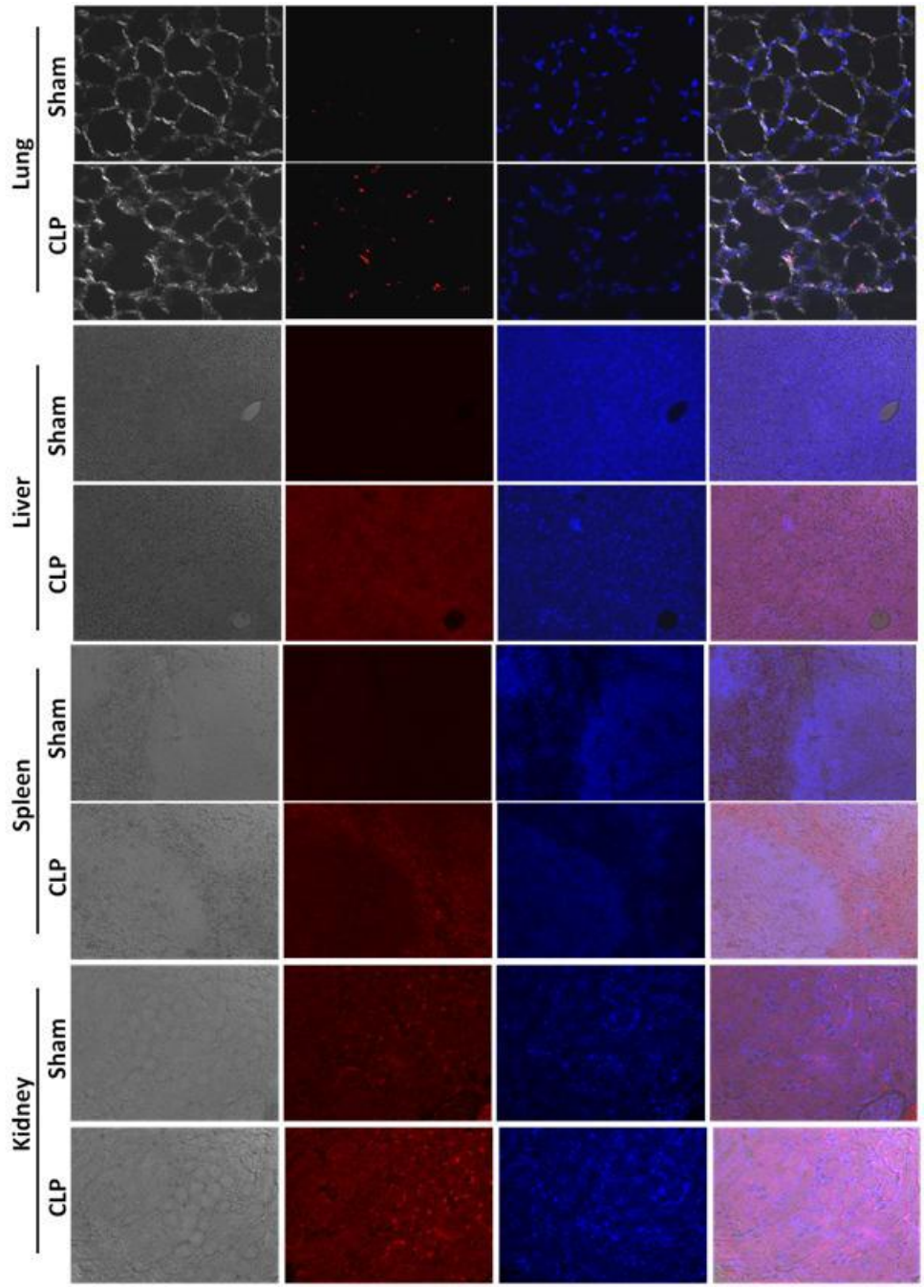

Figure 3.1. NLRP3 protein expression in organs after sepsis. First panel shows histology of normal organs, second panel shows the specific antibody staining NLRP3-red, the third shows DAPI staining, right panel shows merging of NLRP3 antibody and DAPI stains (A-D).

NLRP3$^{-/-}$ is dispensable for neutrophil recruitment to the peritoneal cavity. To assess the myeloid cells recruitment to the peritoneal cavity of NLRP3$^{-/-}$ and WT mice after polymicrobial sepsis, peritoneal lavage was collected at 24h post CLP and then enumerated the recruited cells. In NLRP3$^{-/-}$ mice, total white blood cell and neutrophil recruitment to the peritoneal cavity was observed to be at the similar level at 24h following sepsis challenge. In

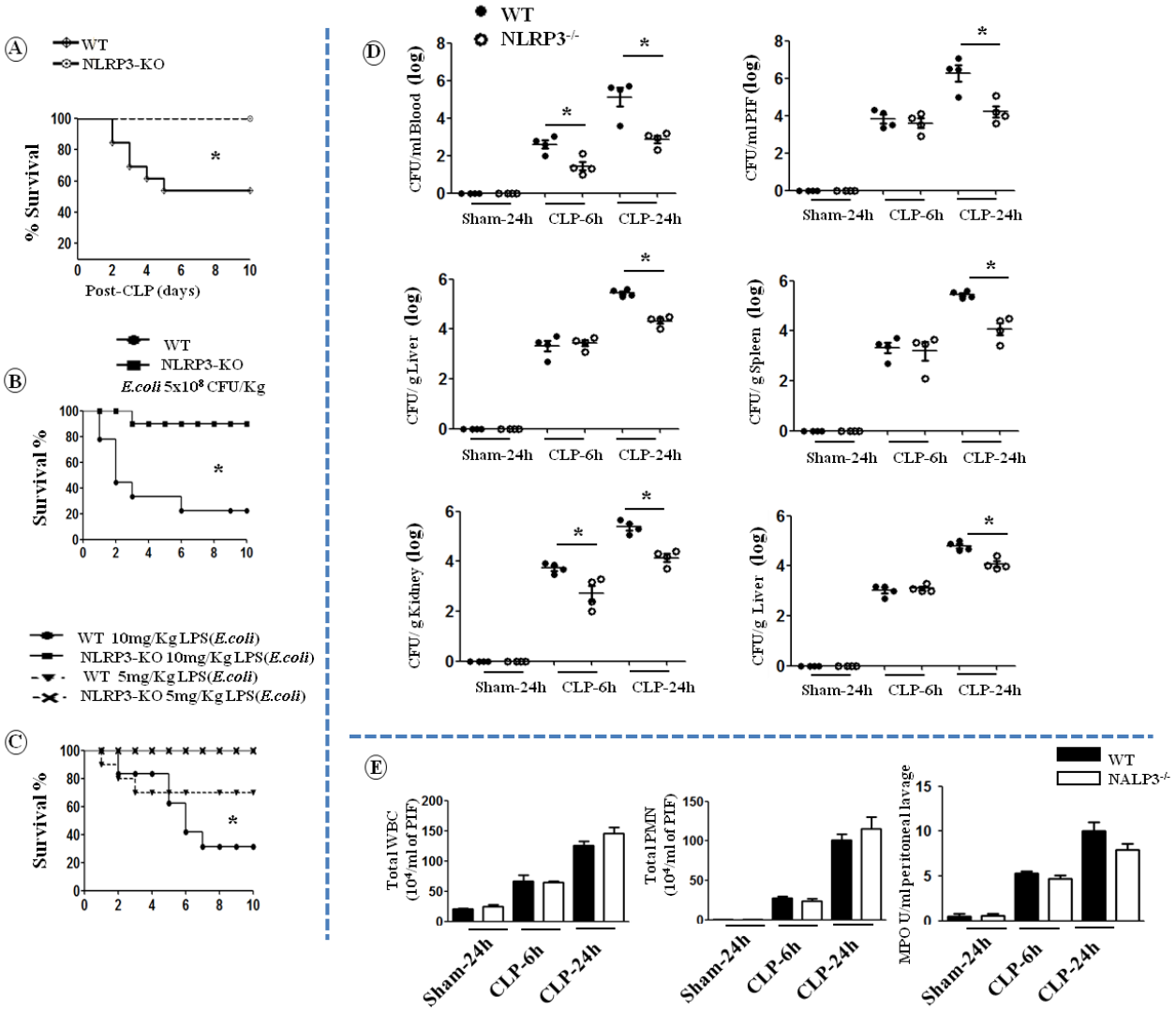

Figure 3.2. Importance of NLRP3 in sepsis. A, Increased survival rate in NLRP3$^{-/-}$ mice following sepsis. The survival rates in NLRP3$^{+/+}$ (WT) and NLRP3$^{-/-}$ (KO) mice were evaluated for 10 days after CLP. B, WT and NLRP3$^{-/-}$ mice were subjected to *E. coli* (5x10^{8} CFU/Kg body weight) injection as described in material and methods on Day 0. Mice were monitored for a course of 10 days for survival. C. WT and NLRP3$^{-/-}$ mice were received i.p. injections of LPS either 5mg/kg or 10mg/kg. Mortality was monitored. *p<0.05,log-rank test.(D) NLRP3$^{-/-}$ mice show less bacterial burden in the peritoneum and dissemination during CLP induced sepsis. The horizontal bar indicates the mean for each group. NLRP3$^{-/-}$ and WT mice were subjected to CLP using 21-gauge needles. After 6h and 24 h, they were killed to obtain blood, peritoneal wash fluid, and organs homogenates. Bacterial colony

counts were obtained as described in Materials and Methods. The results are expressed in CFU/ml of blood, peritoneal lavage fluid, CFU/g of liver, lung, spleen and kidney. (E). Cellular infiltration in peritoneum at 6h and 24h after CLP or sham. The data are data from three pooled experiments, and each group contained six to eight mice.*$p<0.05$ for NLRP3$^{-/-}$ vs WTcontrol (sham) groups, however no significant cellular influx in the peritoneal cavity was observed in both NLRP3$^{-/-}$ and WT mice (Figure3.2E). We also measured myeloperoxidase (MPO) activity in the peritoneal lavage after CLP in NLRP3$^{-/-}$ and WT mice and observed no significant change in activity in the PF of NLRP3$^{-/-}$ mice as compared with their WT counterparts (Figure3.2E), demonstrating dispensable role of NLRP3$^{-/-}$ in neutrophil recruitment.

Abrogated inflammatory response in NLRP3$^{-/-}$ mice during sepsis. Innate immune response which leads to the generation of cytokines/chemokines is the first line of self defense system against bacterial infections. However, defective production of cytokines/chemokines is the major cause of septic death. In order to understand why NLRP3$^{-/-}$ mice are more resistant to septic death, we assessed the cytokine/chemokines production in NLRP3$^{-/-}$ mice and C57Bl/6 littermates. As shown in Figure3.2, NLRP3$^{-/-}$ and C57Bl/6 mice exhibited distinct inflammatory responses during sepsis. As compared to C57Bl/6 mice, NLRP3$^{-/-}$ mice showed subdued production of cytokines and chemokines in the peritoneal fluid and blood (Figure3.3A-B). Although IL-12 and IL-10 are known to play protective roles during sepsis we did observed elevated levels of these chemokines, suggesting that these cytokines are dispensable for survival benefit in NLRP3$^{-/-}$ mice. These data indicate that the deficiency of NLRP3 leads to the lower systemic inflammatory response syndrome and which may result in severe organ injury observed in sepsis.

NLRP3-expressing neutrophils show augmented phagocytosis and bacterial killing. To investigate whether NLRP3 regulates neutrophil function that contributes to bacterial burden in response to CLP-induced sepsis, we determined the uptake of pHrodo™ Red *E. coli* and *S. aureus* BioParticles® by purified peritoneal neutrophils from NLRP3$^{-/-}$ and WT mice 24h post-CLP. The pHrodo™ dye increases in fluorescence in a pH-dependent way as the BioParticles® reach the acidic lysosome during phagocytosis, and acidification of phagosomes can be measured by the change of Relative Fluorescence Unit (RFU). Firstly, we

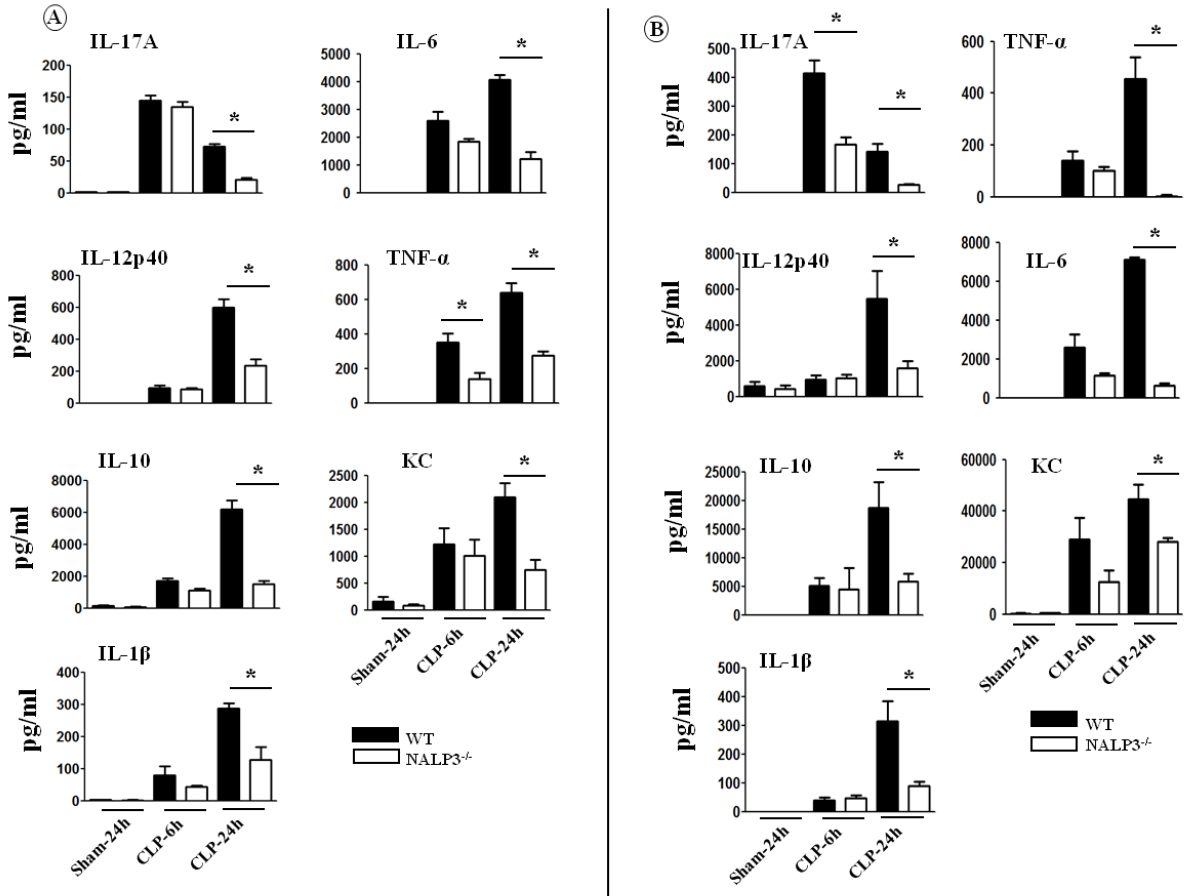

Figure 3. 3. Role of NLRP3 in inflammatory cytokine and chemokine levels in sepsis. (A and B) NLRP3$^{-/-}$and WT mice were killed and bled at 6h or 24 h post-CLP, and their peritonea were washed with5 ml of PBS. After centrifugation at 500 x *g* for 5 min, cytokine concentrations in the peritoneum (top panels) and serum were determined by ELISA as mentioned in material and methods. The data are expressed as pg cytokine/ml of peritoneal wash fluid or serum. The data are means and standard errors of the means. The results of three independent experiments were similar, and therefore the data for six to eight mice/group were pooled. $^{*}p<0.05$(compared with NLRP3$^{-/-}$ samples).

compared phagocytosis of opsonized *E. coli* (Gram positive) and *S. aureus* (Gram negative) in WT and NLRP3$^{-/-}$ neutrophils from peritoneum after CLP, we found that at 1 h, phagocytosis of *E. coli* and *S. aureus* was no different both sets of neutrophils whereas at 2 h NLRP3$^{-/-}$ neutrophils showed much higher phagosome acidification compared to WT groups (Figure.3A). Although NLRP3 contributed to the enhanced phagocytosis of *E. coli* and *S. aureus* BioParticles®, the killing ability and phagocytosis to live bacteria has not been determined. Hence, we assessed the bacterial killing and phagocytosis by bone marrow neutrophils by using live E.coi and S. aureus. Our results showed augmented phagocytosis

and bacterial killing to Gram+ and Gram- bacteria in NLRP3 deficient neutrophils as compared to WT.

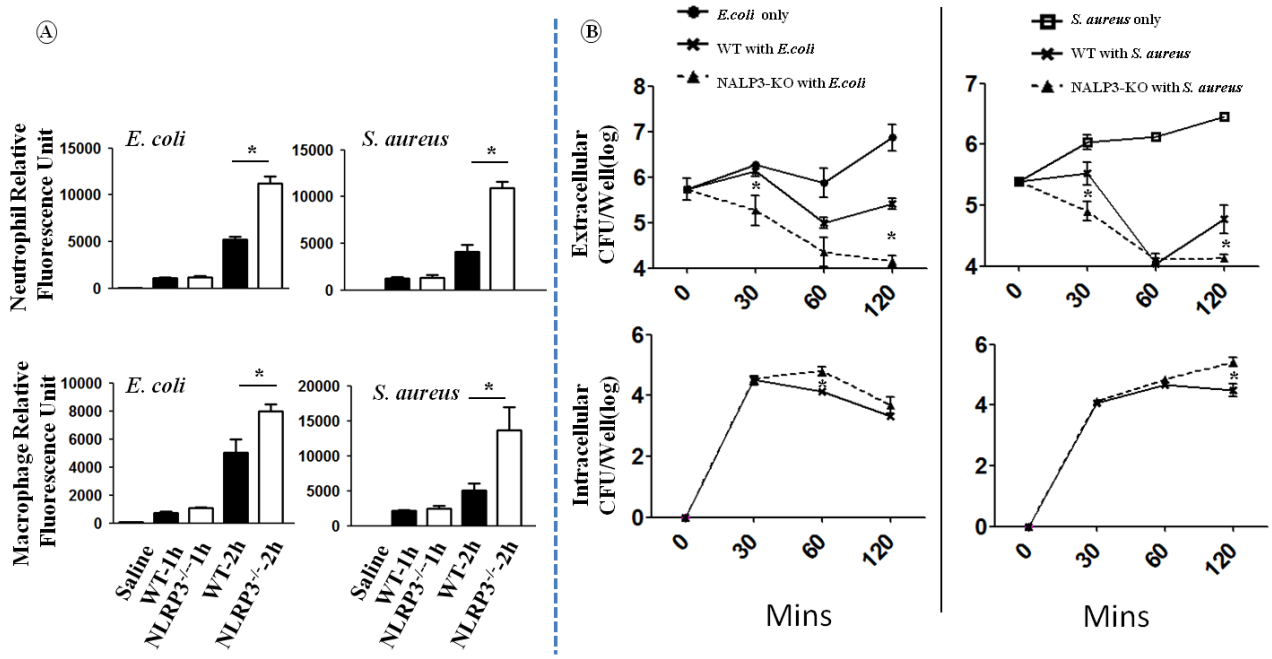

Figure 3.4. Bacterial killing capacity and phagocytosis were determined in neutrophils obtained from NLRP3$^{-/-}$ and WT mice. A.NLRP3$^{-/-}$ and WT peritoneal nuetrophils and macrophages were incubated with pHrodo™ Red *E. coli* BioParticles® and *S. aureus* BioParticles® for up to 2hs. Phagocytosis was measured as RFUs. B. Bacterial killing capacity of E.coi and *S. aureus*-infected bone marrow neutrophils from WT and NLRP3$^{-/-}$ mice was determined by assessing extracellular and intracellular CFUs at 30, 60 and 120 min after infection. $^{*}p<0.05$ (compared with NLRP3$^{-/-}$ neutrophils or macrophages).

NLRP3 is important for ROS production, NET formation in sepsis. Next, we determined the neutrophil effector functions such as ROS, $O2^{-}$ and H2O2 production in peritoneal cells during sepsis. The percentage of total ROS and H202 positive cells were decreased in NLRP3$^{-/-}$ neutrophils and macrophages (Figure. 3.5). Flow cytometry data showed intracellular $O2^{-}$ was also decreased in both neutrophils and macrophages (Figure. 3.5). It is well known that reactive oxygen species (ROS) are important mediators of NETs formation. Furthermore, it has been reported that NETs formation is induced after polymicrobial sepsis in mice peritoneal neutrophils. To determine whether NLRP3 is required for chromatin decondensation and NET formation after sepsis, we performed immunocytochemistry. In this regard, we isolated neutrophils from peritoneal fluid 24 h after CLP to determine the

formation of NETs. A previous report showed that binding of MPO to chromatin is an important trigger for NET release. To further confirm our finding, we used 3 stains: DAPI (blue) for DNA, Cit H3 (green) and MPO (red) to label NETs forming in neutrophils. After 8h culture, 39.5% of neutrophils were found positively stained for hisitone H3 citrullination, myeloperoxidasse and DNA Sytox Green, and long stringlike extracellular DNA structures colocolized with CitH3 and MPO were visible under fluorescence microscopy. On the other hand, NLRP3$^{-/-}$ neutrophils and NLRP3 inhibitor treated neutrophils showed only 14.8% and 15.2% NETs forming capability with no long network of extracellular traps detected in these neutrophils (Figures. 3.6A-B). For the quantitative NETs kinetic analysis (Figure. 3.6C), NETs formation was observed by determining the increase in the relative fluorescence unit in

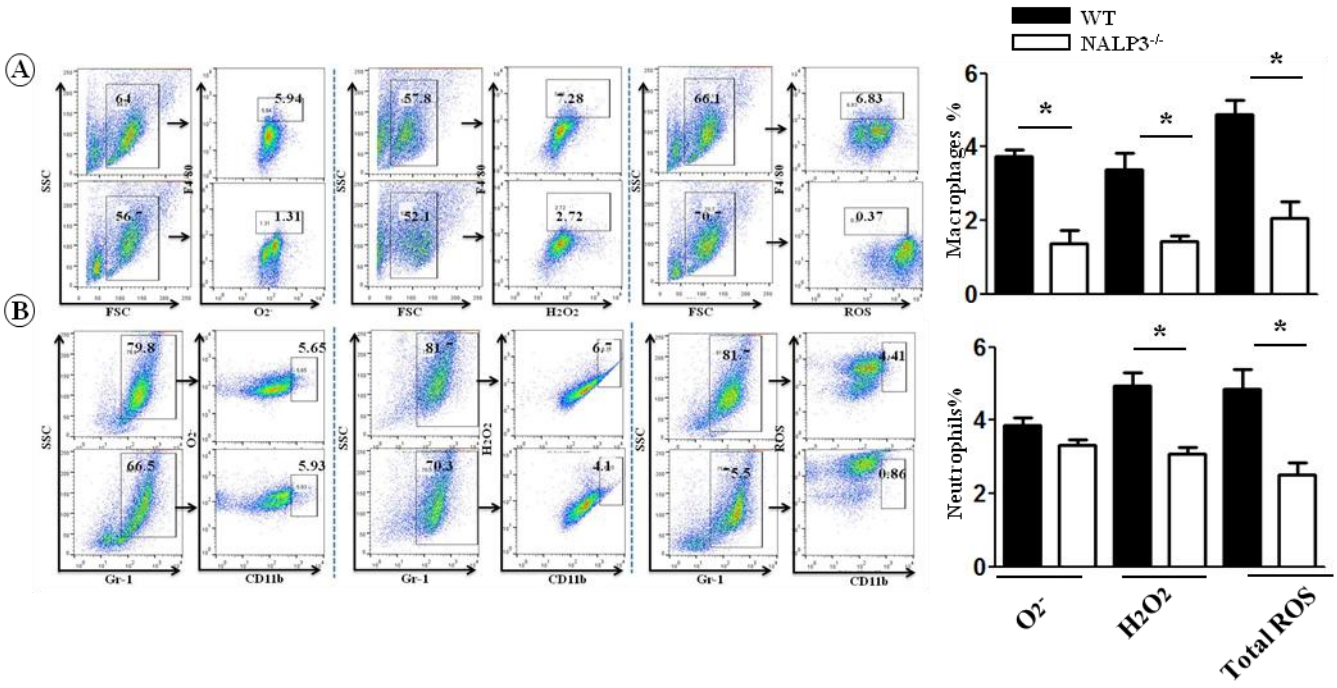

Figure 3.5. ROS production by neutrophils and macrophages of NLRP3$^{-/-}$ mice is attenuated during PMS. A-B, ROS^{+}, $H_2O_2^{+}$ and $OH^{\cdot}/O_2^{-+}$ neutrophils were quantitated in the peritoneal fluid from *NLRP3*$^{-/-}$ and WT control mice after PMS by FACS using anti-Gr1/Ly6G Ab for staining neutrophils and anti-F4/80. Ab for staining neutrophils. The results are expressed as mean ± SE (n=5-8/group; *, p<0.05; **, p<0.01; ***, p<0.001).

the presence of Sytox Green extracellular DNA dye (5 mM; Invitrogen) every hour till 8 hrs. Compared to NLRP3$^{-/-}$ and NLRP3 inhibitor treated WT groups, both WT and WT plus DMSO treated neutrophils showed steady and rapid increase in fluorescence. Overall, our

data illustrate that NALP3 is not only important for NET formation but also crucial for its initiation. Scanning Electron Micrograph analyses showed typical NET Structures (cables, threads, and globular domains), as defined by Brinkmann *et al*. (2), associated with peritoneal neutrophils derived from WT mice after CLP; those structures were largely undetected with

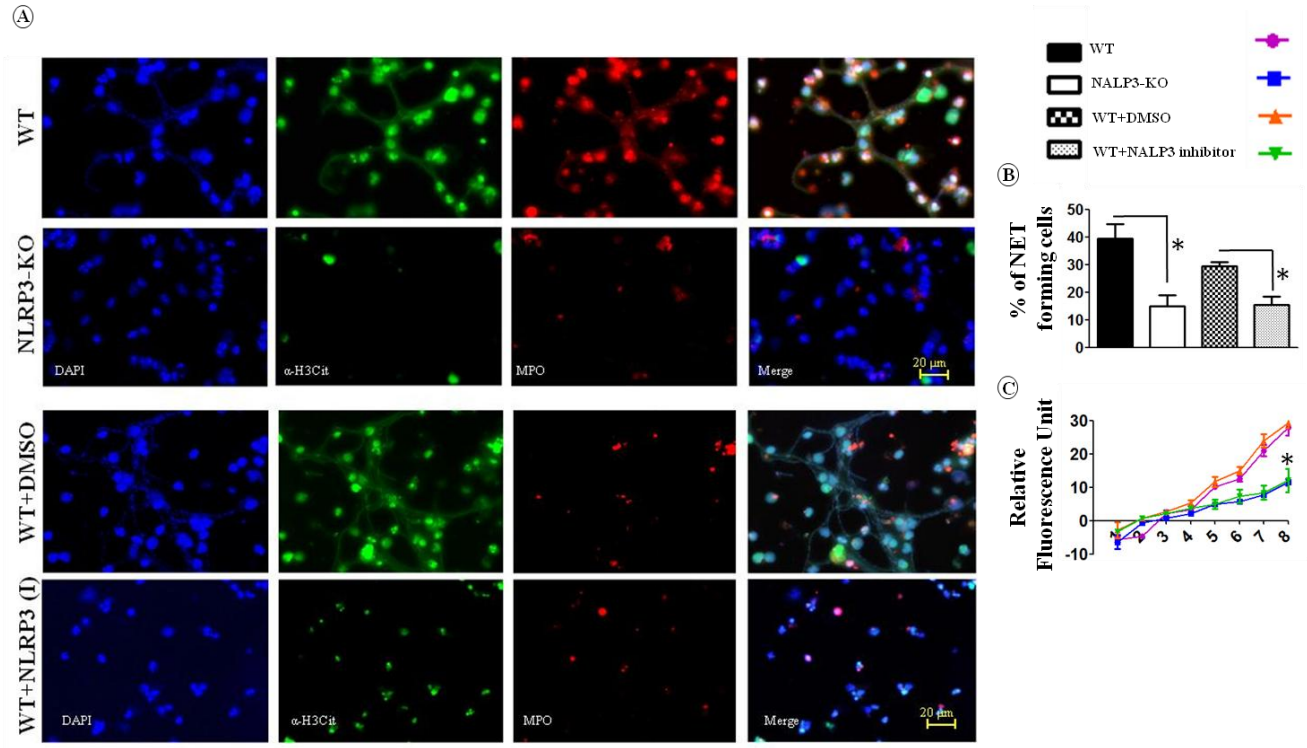

Figure 3.6. *NLRP3*$^{-/-}$ neutrophils or neutrophils treated with NLPR3 inhibitor exhibit decreased NET formation. A, Peritoneal neutrophils harvested from *NLRP3*$^{-/-}$, WT mice with NLRP3 inhibitor or WT mice after PMS, allowed to undergo NET formation and fixed at 8 h. Neutrophils were stained with Sytox green DNA stain, citrullinated histone H3 Ab and MPO Ab to visualize citrullinated DNA after the cells were fixed with 4% (v/v) paraformaldehyde. Scale bars, 20 μm. Long strands of DNA (arrowhead) are evidence of NET formation. Images presented are representative images of three independent experiments (n=5-7/group). B, A total of 20 images were selected from one experiment and quantified for the presence of NET-positive neutrophils from *NLRP3*$^{-/-}$, WT mice treated with NLRP3 inhibitor and WT mice control. The results are expressed as mean ± SE (n=5-8/group). C, Kinetic analysis of NET formation by peritoneal neutrophils harvested from *NLRP3*$^{-/-}$, WT mice treated with NLRP3 inhibitor and WT mice control. Relative fluorescent intensity was determined to evaluate NET formation each hour until 8 h of *in vitro* culture of neutrophils (n=6-9/group).The results are expressed as mean ± SE from two independent experiments (n=5-8/group: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

peritoneal neutrophils derived from *NLPR3*$^{-/-}$ mice or mice treated with NLPR3 inhibitor(Figure. 3.7A).To determine whether NLRP3-mediated NET formation is essential for bacterial killing, *E. coli* or *Staphylococcus aureus* was incubated with *NLRP3*$^{-/-}$ or WT neutrophils, and bacterial killing was analyzed. We found a decrease in NET-mediated bacterial killing and relative phagocytosis in *NLPR3*$^{-/-}$ neutrophils compared with WT neutrophils (Figures. 3.7 B-C). Because DNase can be used to inhibit NET formation, neutrophils were pretreated with DNase in one set of experiments. WT neutrophils without DNase treatment showed effective NET-mediated bacterial killing compared to the DNase-treated control, whereas no difference in bacterial killing was observed in the DNase-treated NLPR3$^{-/-}$ neutrophils (Figures. 3.7 B-C).

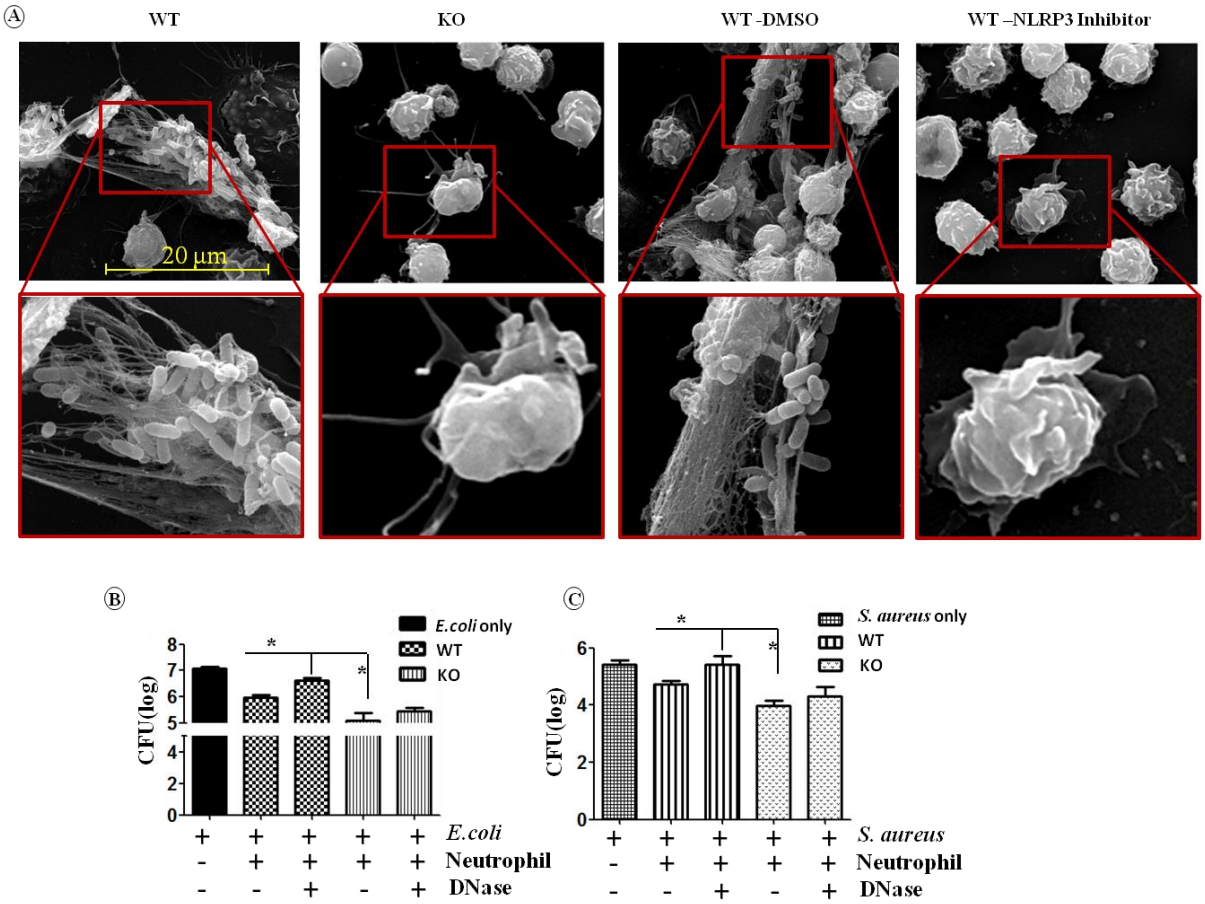

Figure 3.7. Role of NLRP3 in NET formation and NETs-mediated bacterial killing. A, NETs structure (arrows) trapping bacteria are detected in scanning electron microscopy of WT mouse. The neutrophils from NLRP3 knockout mice and WT neutrophils with NLRP3 inhibitor are devoid of NETs (B-C). NETs-mediated bacterial killing in neutrophil infected with *E.coi* or *S. aureus*. DNase was added as NETs inhibitor.

NLRP3 gene deficiency reduces autophagy but enhance phagocytosis. Having determined

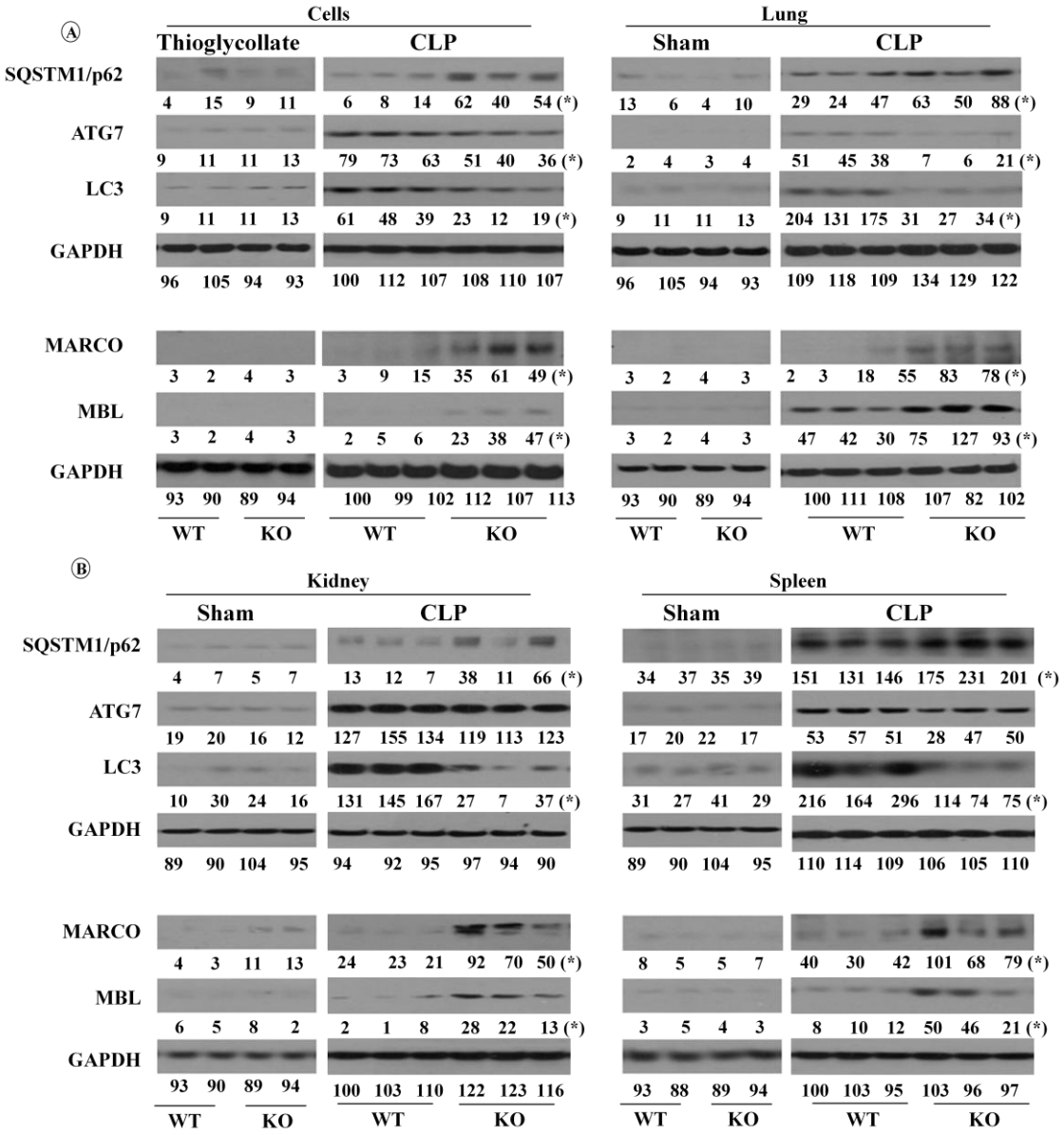

Figure 3.8. Expression of P62, ATG7, LC3, MARCO and MBL were determined in *NLRP3*$^{-/-}$ mice following PMS from peritoneal cells, lung, kidney and spleen's homogenates. Densitometric analysis of 3 separate blots from 3 independent experiments is shown in parenthesis as mean ± SE. (n=4-6/group; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

that NLRP3 was involved in the initiation of NETosis, we sought to determine whether NLRP3 was important for the morphologically change of nuetrophils. We use western blot to measure the expression of P62/SQSTM1 (Sequestosome 1), ATG7 (Autophagy-related protein 7), LC3 (Microtubule-associated protein 1A/1B-light chain 3), MARCO (Macrophage receptor with collagenous structure) and MBL (Mannose-binding lectin) in *NLRP3*$^{-/-}$ and WT mice following PMS. Peritoneal cells, lung, kidney and spleen's homogenates were used for analysis. We find that the gene LC3 as significantly reduced in peritoneal cell, lung, spleen and kidney in NLPR3 gene deficiency mice, which was important for autophagy.

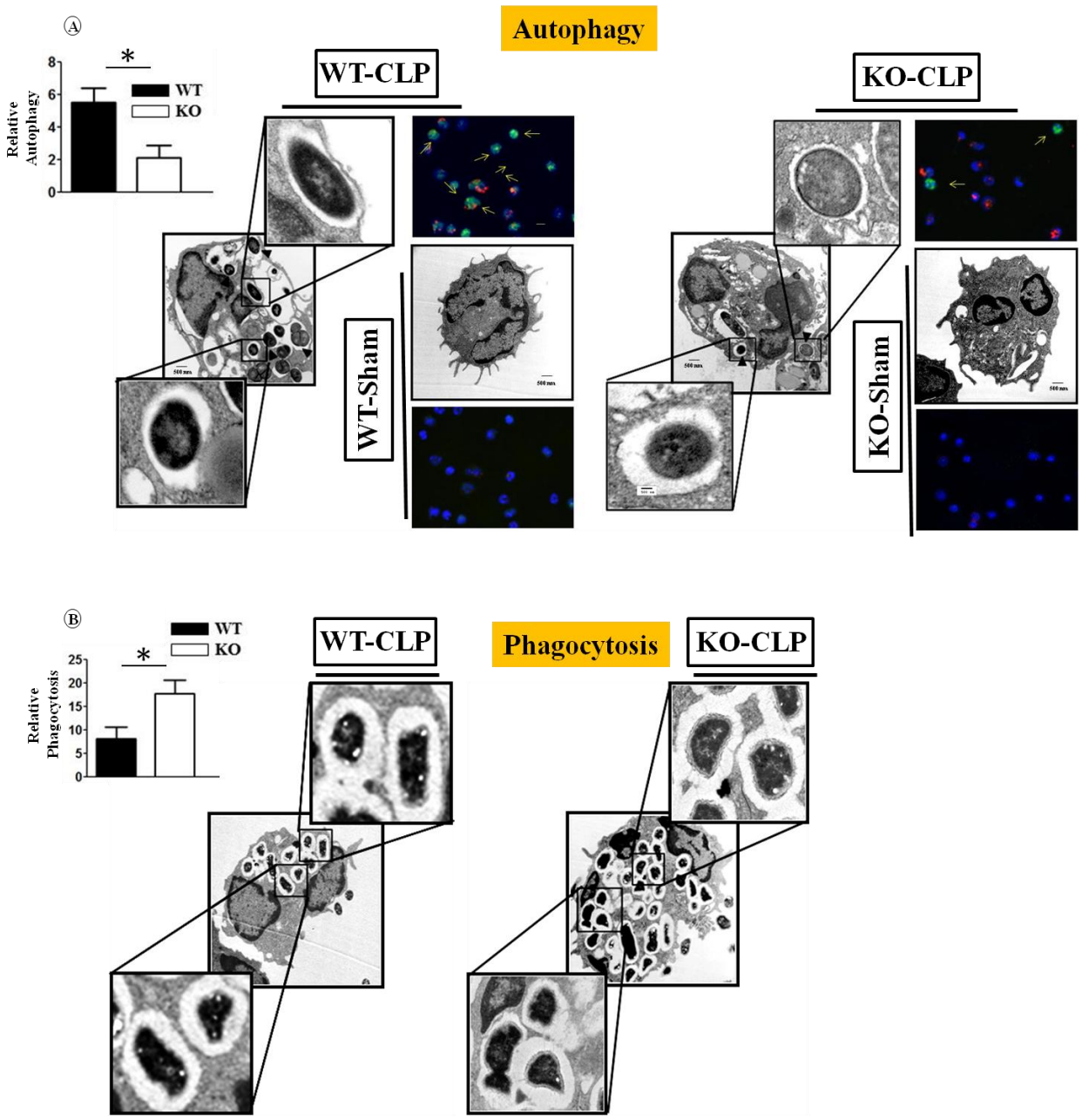

Figure 3.9. Fusion of autophagosomes with endosomes or lysosomes of neutrophils from NLRP3-KO and WT control mice after sepsis. A. Peritoneal neutrophils were stained with an antibody specific for the endosomal/lysosomal marker LAMP-1(Red), and the colocalization of GFP-LC3 and LAMP-1 vesicles was examined by fluorescence microscope.TEM pictures showing WT neutrophils undergoing autophagy containing many electron-dense with their characteristic double membranes. B. TEM analysis of neutrophils phagocytosing after sepsis from NLRP3-KO and WT countrol mice. A total of 20 images were selected from one experiment and quantified for the phagocytosis ability of nuetrophils. Data analysis of 3 separate samples from 3 independent experiments is shown in parenthesis as mean $\pm$ SE. (n=4-6/group; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

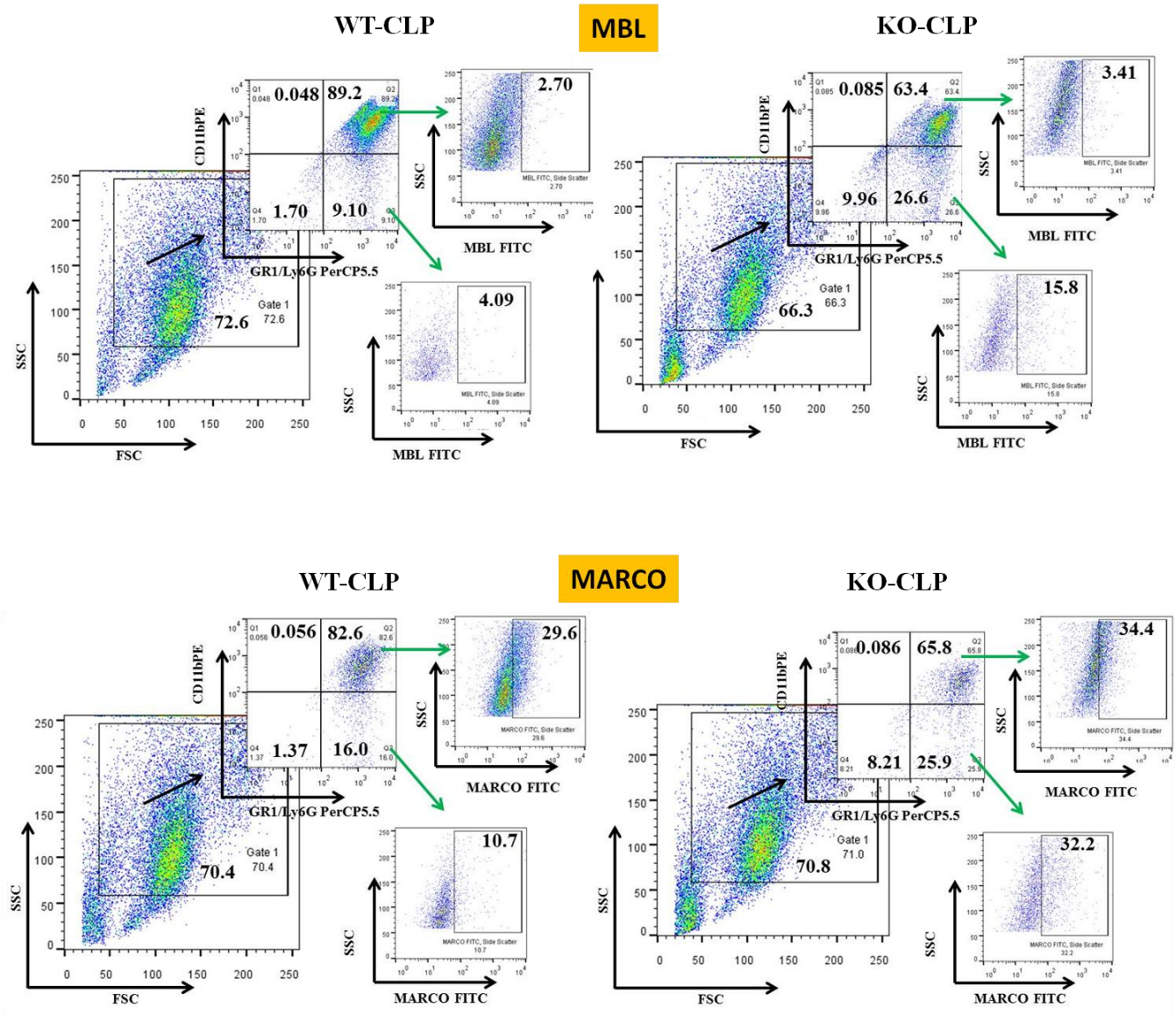

Figure 3.10. NLRP3 is important for the expression of MBL and MARCO in peritoneal neutrophils after sepsis. Peritoneal cells were isolated as described in Materials and Methods, and a representative dot blot is shown. Expression of MBL and MARCO in CD11b positive or Gr1 positive on peritoneal cell were measured by flow cytometry in NLRP3$^{-/-}$ and WT mice.

However, compared to WT, we found the scavenger receptor genes MARCO and MBL were upregulated in NLPR3 gene deficiency mice after sepsis, while these two genes were important for the process of phagocytosis (Fiure. 3.8). Similar result was confirmed by flow cytometry data (Figure3.10). To further confirm our finding, neutrophils collected from peritoneal lavage obtained from polymicrobial sepsis infection were subjected to immunofluorescence microscopy and electron microscopy analysis. Cells harvest from WT animals demonstrate several morphological features consistent with autohagy (Figure. 3.10), including more express of LC3 and lamp1 (Lysosomal-associated membrane protein 1) gene

and higher number of autophagic vesicles. In the contrast, NLPR3 gene deficiency neutrophils exhibited more phagocytosis ability, we found larger number of phagosome in NLRP3$^{-/-}$ neutrophils (Figure. 3.9). And similar result was found when we used NLRP3 inhibitor in human nuetrophils, by immunofluorescence microscopy we find with NLPR3 inhibitor treated neutrophils showed less autophagy but more phagocytosis after *E. coli* or *S. aureus* infection (Figure. 3.11).

NLRP3 deficiency inhibits caspase-1 activity and neutrophil apoptosis during infection. NLRP3 is an important component of inflammasome, while inflammasome is a critical regulator of innate immune response during bacterial infection. Here we test the activity of

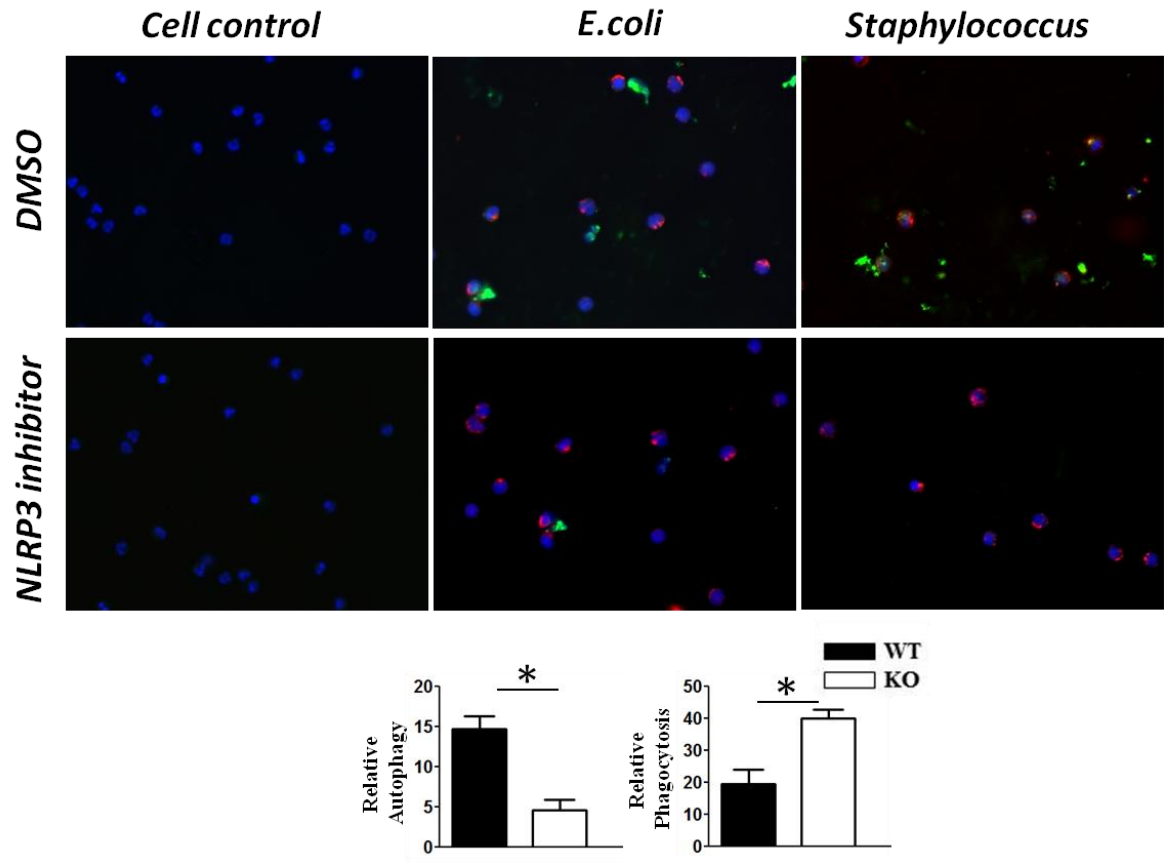

Figure 3.11. Human neutrophils exhibit reduced autophagy and enhanced phagocytosis. Human neutrophils harvested from peripheral blood were treated with NLRP3 inhibitor or DMSO control, then cells were stimulated by *E. coli* or *Staphylococcus aureus*. Neutrophils were stained with LC3 AB (Green) and LAMP-1 AB (Red) after the cells were fixed with 4% (v/v) paraformaldehyde. Scale bars, 20 μm. Images presented are representative images of three independent experiments (n=5-7/group).

caspase-1 in peritoneal cells during sepsis. As hypothesized, we observed that 24h after CLP-induced sepsis WT peritoneal macrophages and neutrophils both showed much higher percentage of capase-1 positive cells as compared to NLRP3 knock cells (Figure3.12).

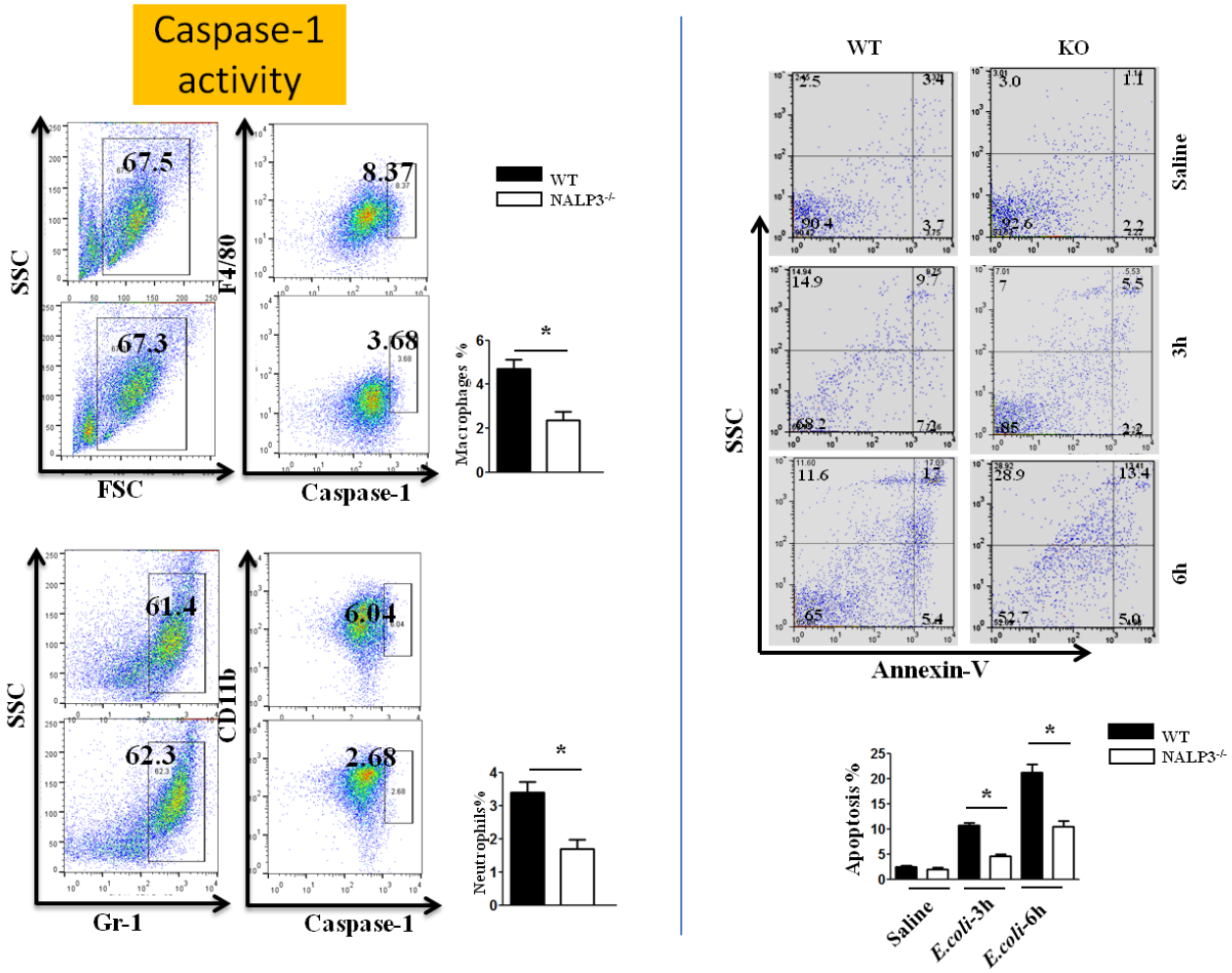

Figure 3.12. Effect of NLRP3 in caspase-1 activation and apoptosis during sepsis. A. Representative flow cytometric dot plot images of peritoneal macrophages or neutrophils. Cells were washed from peritoneal 24h after CLP then staining with F4/80, Gr-1 and caspase-1. B. Representative flow cytometric dot plot images of bone marrow neutrophils anti-coagulated with citrate and then incubated in vitro for 3 h or 6 h prior to annexin V/PI staining, fixation, red cell lysis and flow cytometric analysis. Analysis of the gated neutrophil population is shown only and demonstrates the clear separation of apoptotic and non-apoptotic cells, with minimal cell necrosis (PI positivity) and the time-dependent nature of constitutive apoptosis.

Previous studies have shown that apoptotic stimuli can activate the NLRP3 inflammasome in macrophage to induce apoptosis. To date, the effect of NLRP3 on neutrophil survival in sepsis is not determined. To test whether NLRP3 deficiency has the effect to delay neutrophil apoptosis, purified bone marrow neutrophils were infected with 1MOI *E. coli*. The neutrophil

apoptosis was examined at 3h and 6h by Annexin V/PI staining assay by using flow cytometry; a representative analysis of 5 subjects is shown in Figure 3.12. In summary, both 3h and 6h after infection, NLRP3$^{-/-}$ neutrophils showed much less apoptosis.

Administration of NLRP3 inhibitor rescues mice from sepsis. Because protective effect from sepsis was observed in NLRP3 mice, we wished to determine whether given NLRP3 inhibitor could protect WT mice following polymicrobial sepsis. To this end, WT mice were administrated a single i.p. dose of 1mg NLRP3 inhibitor immediately after CLP. Administration of NLRP3 inhibitor after sepsis resulted in increased survival (78% with inhibitor) while compared to WT (53%) or WT with DMSO (50%) (Figure3.13).This finding demonstrated that NLRP3 inhibitor could be used therapeutically to protect from sepsis-induced mortality.

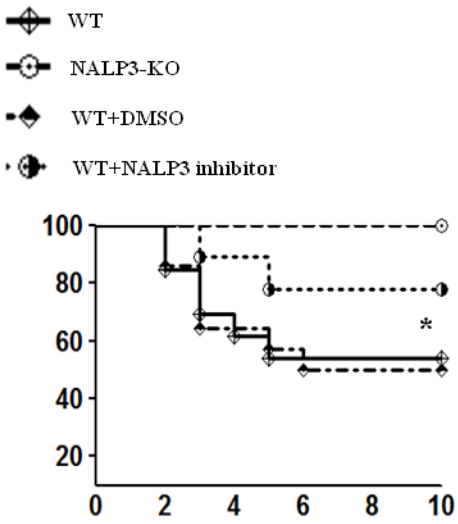

Figure 3.13. Effect of NLRP3 inhibitor in survival during sepsis. A total of 1mg NLRP3 inhibitor/mouse or the same volume of DMSO was administered intraperitoneal (i.p.) immediately after cecal ligation and puncture. Survival was monitored up to 10 d post-sepsis.

Discussion

The nucleotidebinding domain and leucine-rich repeat PYD-containing protein 3 (NLRP3) inflammasome is a multiprotein complex that contains NLRP3, the caspase recruitment domain containing protein Cardinal, apoptosis-associated speck-like protein (ASC), and caspase-1 (19). NLRP3 inflammasome is implicated in sensing a variety of stimuli ranging from silica to microbes (20). However, whether inhibition or deletion of NLRP3 is important

for sepsis-induced host responses has not been explored. However, it is well established that TLR signalling in response to overwhelming bacterial burden leads to the excessive secretion of proinflammatory mediators eventually leading to shock.

In this report, we investigated the role of NLRP3 in neutrophil function during sepsis induced by CLP. Although mice deficient in NLRP3 exhibited a no different number in total leukocytes and neutrophils in the peritoneum after CLP, but there was better ability in bacterial clearance from the peritoneum and extra-peritoneal organs, leading to increased survival. These findings are consistent with other sepsis model, like LPS shock model and bacterial sepsis model. Moreover, NLRP3 inflammasome knockout mice are found to be protected against ischemic but not cisplatin-induced acute kidney injury.

We observed reduced production ROS, $O2^-$ and H2O2 in *NLRP3*$^{-/-}$ mice following PMS. While NETosis is a unique host defense mechanism employed by neutrophils to trap and kill extracellular pathogens (21,22). In the present study, we found a strong correlation between reduced ROS production and reduced NET formation by neutrophils from *NLRP3*$^{-/-}$ mice following PMS. Additionally, NET formation and NET-positive peritoneal neutrophils were reduced in *NLRP3*$^{-/-}$ mice or mice pretreated with NLRP3 inhibitor compared to WT. To confirm this result, we observed reduced formation of typical NET structures associated with *NLRP3*$^{-/-}$ neutrophils by SEM. In addition, we observed reduced bacterial killing by bone marrow neutrophils treated with DNase, confirming the link between NET formation and bacterial killing. The results linking neutrophil ROS production with NETosis and subsequent bacterial killing are consistent with several prior reports (21, 22). Our finding that neutrophils harvested from NLRP3$^{-/-}$ mice are deficient for bacterial killing confirms the crucial role of NLRP3 in the containment of infection.

Furthermore, our data suggest that NLRP3 gene deficiency reduces autophagy but enhance phagocytosis. From cells to organs our results showed reduced autophagy gene expression LC3, and increased ability of phagocytosis. This could be an important reason to explain in NLRP3$^{-/-}$ mice had less bacterial burden.

The data in this study illustrate a number of advancements in our understanding of the biological role of NLRP3 in host defense during sepsis. NLRP3 appears to be essential for host survival, production of ROS, NET formation, NET-mediated bacterial killing, autophagy, phagocytosis and apoptosis.

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Chapter 4: Impaired Neutrophil Extracellular Trap (NET) Formation: A Novel Immunosuppressive function of alcohol to Reduce Bacterial Clearance in Polymicrobial Sepsis

Introduction

Sepsis is a complex clinical manifestation of dysregulated host inflammatory response to infection, causing the damage to vital organs that often results in death (1). Although the reported data indicate the decrease in sepsis-mediated mortality over the past decade, sepsis is still one of major cause of death in United States. It still poses greater economic burden and require prolonged intensive care treatment for patients. Much of the pathophysiology of sepsis is the result of the host response to the bacterial pathogens (1). An improved understanding of how the host response to microbial invasion is a prerequisite to successfully design therapeutic strategies to control bacterial burden in sepsis.

Clinical studies have been shown that alcoholics are more susceptible to sepsis and thus have higher mortality rate as compared to non-alcoholics with sepsis (2). Alcohol abuse is associated with a leading cause of mortality, around 100, 000 deaths per year (2, 3). In addition, previous studies have suggested that consumption of ethanol is associated with an increased incidence and severity of a broad spectrum of natural infections in human and experimental animals (1, 3). While our understanding of the many facets of the pathophysiology of sepsis continues to expand, there is still paucity in information on the fundamental mechanisms on what and how alcohol does impact to host in response to sepsis.

Formation of NETs (Neutrophil Extracellular Traps), also known as NETosis, by human PMNs has been described as a novel function of neutrophils (4). NETs are extracellular lattices of decondensed chromatin that contain antimicrobial proteases. Emerging data show that NETs trap microbes, including bacteria, fungi, and some parasites (4, 5) and subsequent investigations demonstrated that NETs kill extracellular microbes limiting the spread of pathogens (4-6). While the intracellular signaling pathways that regulate NET formation by PMNs remain largely unknown, generation of reactive oxygen species (ROS) is considered a critical event (5). NET formation is also thought to require PMN elastase (NE) activity to

initiate degradation of core histones leading to chromatin decondensation prior to plasma membrane rupture (4, 5).

Alcohol intake has a number of effects on the function of innate immune cells. For example, it has been shown that alcohol hampers the phagocytosis activity, antigen presentation capacity and the production of antimicrobial molecules by the monocytes, macrophages and dendritic cells (7). One of prominent feature of alcohol abusers with sepsis or septicemia is that they present with reduced granulocyte recruitment at infectious focus and show systemic granulocytopenia (8). The potential mechanisms for the induction of granulocytopenia by alcohol may involve cell death in sepsis. However, the other effects of alcohol on neutrophil antibacterial function including NETosis during infection remain elusive.

In this study, we used a mouse model of polymicrobial sepsis via cecal-ligation and puncture (CLP), primary mouse neutrophils and primary human neutrophils to investigate the role of alcohol in NET formation and NET-mediated bacterial killing. Our findings indicate that alcohol-suppressed NETosis and NET-mediated extracellular killing of bacteria contribute to impaired bacterial clearance in polymicrobial sepsis.

Materials and Methods

Animals: All animal experiments were approved by the Louisiana State University Animal Welfare Committee. C57Bl/6 male mice (8-12 weeks) weighing 22g to 28 g were used in all experiments.

CLP: PMS was induced by the CLP method as previously described (9). In brief, male mice were anesthetized and the cecum was punctured with a 21-gauge needle. A small amount of fecal material was extruded through the puncture, and the cecum was repositioned into the peritoneal cavity. Animals with sham surgery underwent the same protocol without CLP. Acute alcohol administration was conducted according to protocol previously reported (10). In brief, 20% ethanol at a concentration of 5g/kg was given 30 min before CLP to alcohol recipient group of mice by intraperitoneal (i. p.) injection. Control mice received an equal amount of saline.

Isolation of human PMNs: PMNs were isolated from anti-coagulated peripheral blood from healthy donors under protocol approved by Louisiana State University Institutional Review Board. Written informed consents were obtained in accordance with the Declaration of Helsinki. Purification of PMNs (>95% pure) was performed using anti-CD15 microbeads via negative selection using MACS (Miltenyl Biotech). After purification, PMNs were resuspended in RPMI 1640 medium supplemented with 10% FBS and instantly used for the experiments.

In vitro alcohol treatment: For the *in vitro* exposure of alcohol to the cells, high dose (250 mM) as well as low dose (25 mM) of ethanol was used.

Bacterial load examination: Bacterial burdens were determined by enumerating bacterial numbers in colony forming units (CFUs) as previously described (11).

Western blotting: At the designated times, the neutrophils were harvested and lyzed with cell lysis buffer containing 0.1% Triton X-100 and complete protease and phosphatase inhibitor cocktail as described (12). Equal amount of proteins were separated by SDS-PAGE, and transferred to a polyvinyl difluoride membrane. The nonspecific binding sites on the membrane were blocked with 5% skim milk () for 1 h before proteins were allowed to react with specific primary antibodies against peptidylarginine deiminase-4 (PAD-4), citrullinated histone (H) 3, and actin (Cell signaling) at 4℃ overnight. The membrane was washed three times with TBS containing 0.1% Tween 20 (0.1% TBST) and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The immmunoreactive bands in the membrane were detected by chemiluminescence method (Amersham).

Immunofluorescence and electron microscopy: Immunofluorescence of the cells with CLP or without CLP was performed to detect NET formation as described previously (13). Mouse bone marrow-derived neutrophils and human neutrophils were also similarly analyzed for NET formation after infection with *E. coli* or *S. aureus*. Scanning electron microscopy (13) (FEI Quanta 200, USA) was performed to examine NET formation in mouse peritoneal and

bone marrow neutrophils as well as in human neutrophils following the protocols described in a previous publication (13). *E. coli* (ATCC 25922) and *S. aureus* were used to stimulate neutrophils as described previously (14).

ROS measurement: The level of total ROS in neutrophils after stimulation and in peritoneal fluid after induction of CLP was measured using the Fluorescent ROS Detection Kit.

Bacterial killing assay: A neutrophil-dependent killing assay was performed as reported earlier (12). Briefly, 1 x 10^6 neutrophils were suspended in RPMI 1640 with 10% v/v fetal bovine serum (FBS), and 1 X 10^6 opsonized bacteria were added (1 multiplicity of infection (MOI)). Samples were incubated at 37 ℃ for indicated time points with continuous agitation, then at desired time points samples were harvested for plating of bacteria (12).

Data analysis: Data are expressed as mean +/- standard error (SE). Data were analyzed with the Student's t-test (between two groups). Differences in values were defined as significant at a *p* value of less than 0.05.

Results

Acute alcohol intoxication in mice leads to an increased bacterial burden, less neutrophil recruitment to the peritoneal and susceptible to sepsis. To determine the role of alcohol in sepsis, we first compared the survival rate from alcohol group and their PBS littermates to sepsis induced by cecal ligation and puncture (CLP). Acute alcohol intoxication treated mice were find to be much more susceptible to sepsis than PBS group (Figure.4.1A). Consistent with the survival assay, the number of viable bacteria in the peritoneal cavity of alcohol treated mice was significantly more than that PBS treated group. Furthermore, the number of bacteria disseminated to the blood, liver and spleen in alcohol intoxication mice was significantly higher than that in PBS treated mice postinfection for 6h and 24h (Figure.4.1B). Previously, it has been shown that neutrophil recruitment is critical for the

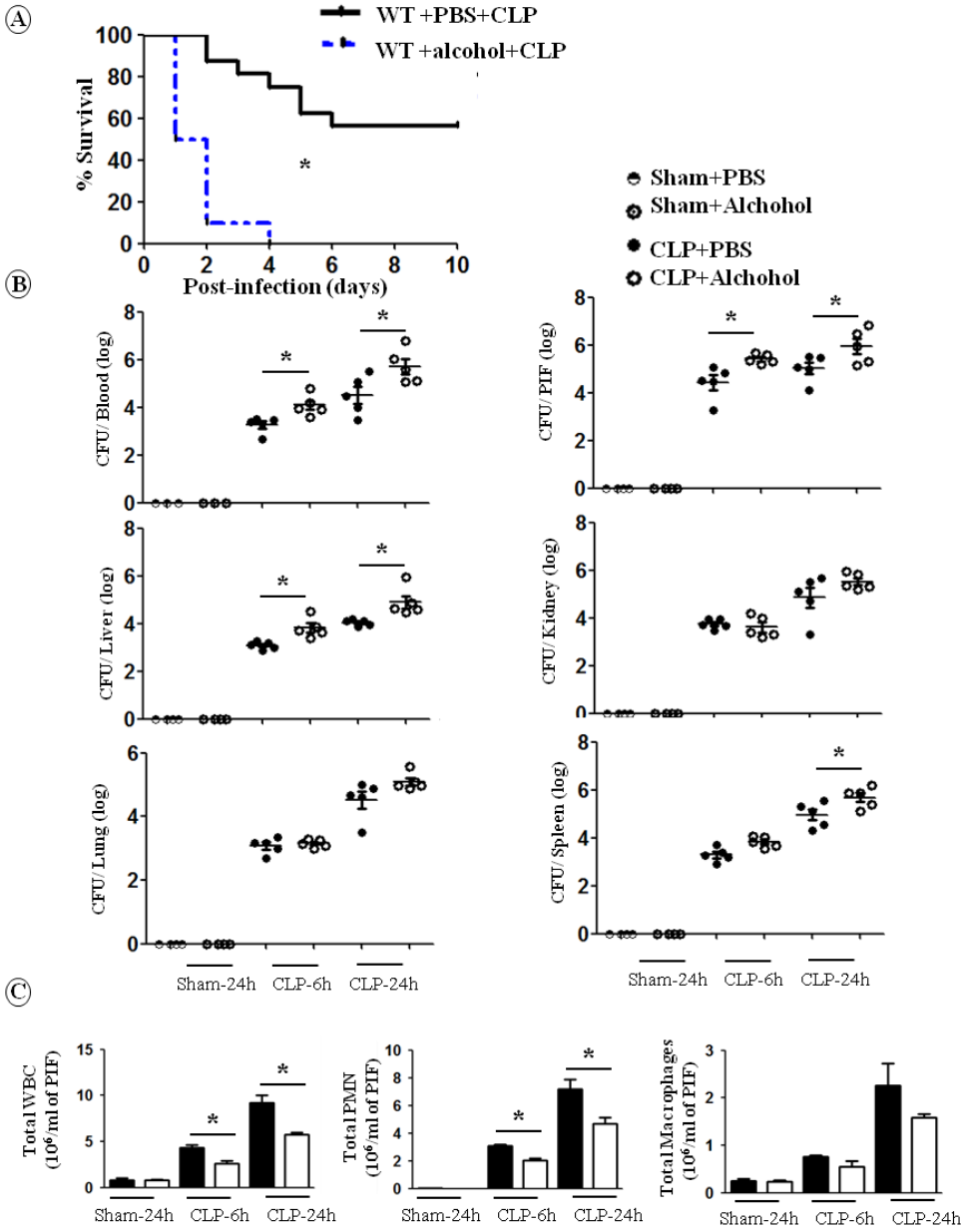

Figure 4. 1. Acute alcohol intoxication impairs host defense during sepsis. Survival after CLP in male WT mice with PBS, and WT mice on acute alcohol intoxication 30mins before CLP induced sepsis monitored for 10 The survival curve combines data from one to two independent experiments, each of which gave similar results. *p<0.05 compared with the WT with PBS group. *B*, Bacterial counts were elevated in the blood, peritoneal fluid, liver, kidney, lung and spleen of the WT acute alcohol intoxication mice 6h and 24 h after CLP compared with WT with PBS mice (n=4-5 per group; data were pooled from 2 independent experiments that gave similar results). CFU, Colony-forming unit. *p<0.05 compared with the WT control group. All values are means+SEM. C. Attenuated leukocyte and neutrophil accumulation in the peritoneum of alcohol treated mice following sepsis.

bacterial killing during sepsis. Here in, we checked the neutrophil number post infection in peritoneal cavity. From Fig4.1C, we can see that in alcohol treated group, the mice recruit much less neutrophils and total white blood cell to the peritoneal while compare with PBS treated mice at 6h and 24h post sepsis. These data suggested that acute alcohol intoxication is

a critical negative regulator of neutrophil recruitment, bacterial killing, dissemination and thus dampens mice host immune response to sepsis.

Enhanced cytokine storm in the acute ethanol intoxication groups in response to polymicrobial sepsis. Mice with acute ethanol intoxication express an enhanced cytokine profile after CLP-induced sepsis. The polymicrobial peritonitis induced by CLP is associated

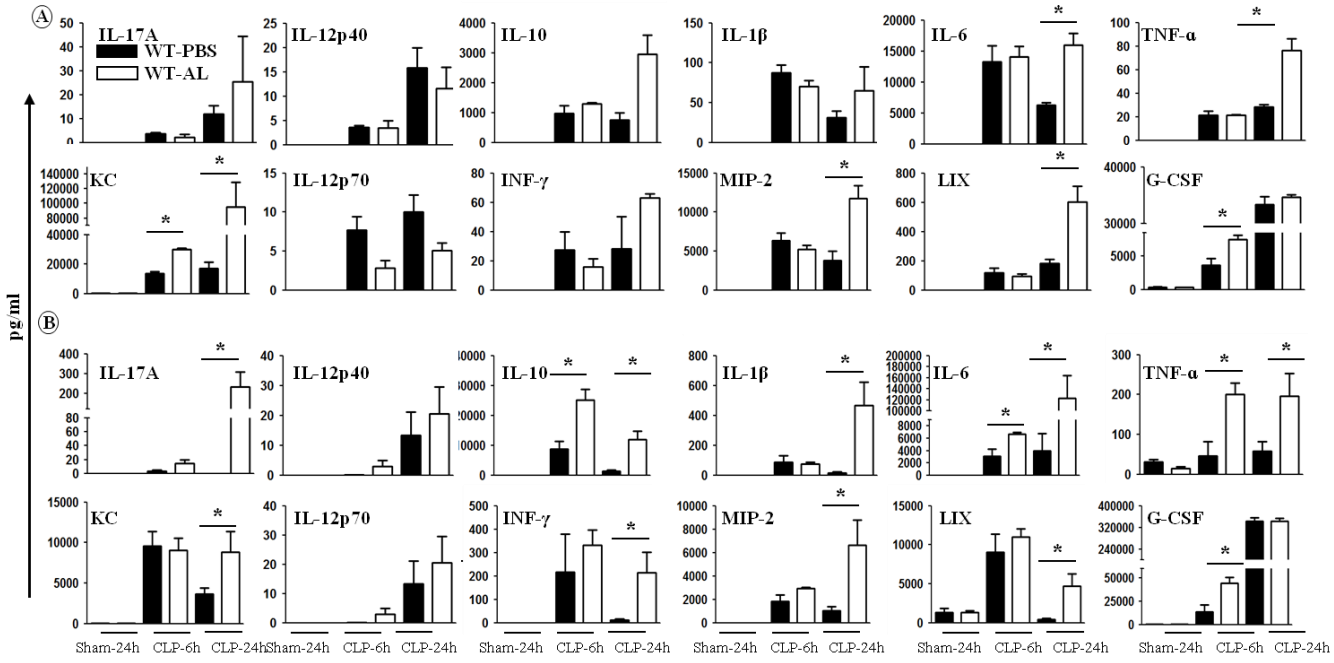

Figure 4.2. Enhanced cytokine storm in the acute ethanol intoxication groups in response to polymicrobial sepsis. A. Cytokines profile in mice with PBS and acute alcohol intoxication treated group following CLP operation. To compare the production of inflammatory cytokines in peritoneal cavities (A) and blood (B) between PBS and alcohol before CLP, ELISA was performed to measured protein levels of IL-17A, IL-12p40, IL-12p70, IL-10, IL-1β, IL-6, TNF-α, KC, FN-γ, MIP2, LIX and G-CSF in peritoneal lavage fluid and serum. * p <0.05 compared with cytokine protein levels measured in mice 6 h and 24 h after CLP, n = 5. The results shown are representative of three individual experiments.

with an amplified inflammatory response, in which the local and systemic expression of many cytokines and chemokines are augmented. We compared the cytokine profiles in peritoneal lavage fluid and blood between acute ethanol intoxication and PBS mice 6h and 24hafter CLP-induced peritonitis. Compared to PBS treated mice, acute ethanol intoxication mice produced a significantly higher amount of inflammatory cytokines, including IL-6, IL-10, TNF-α, KC, MIP-2, and G-CSF in peritoneal cavity and blood at 24h after surgery (Figures. 4.2A-B).

Alcohol interrupts ROS production. We determined whether defective NETosis in the CLP mice and in primary neutrophils infected with bacteria were due to reduced production of

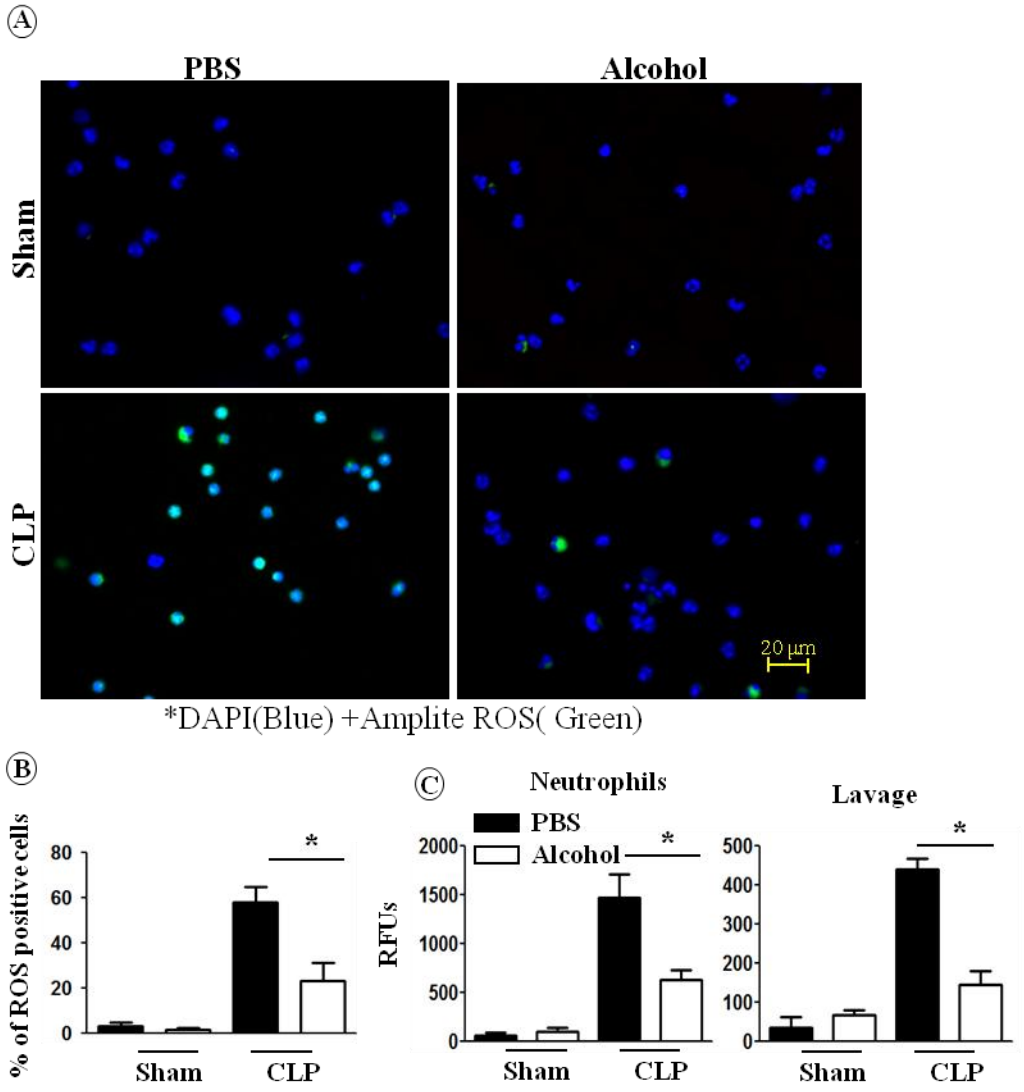

Figure 4. 3. ROS production by neutrophils of alcohol mice is attenuated during sepsis. A, ROS^+ neutrophils were identified by fluorescence microscopy after intracellular staining for ROS in peritoneal neutrophils from alcohol treated and PBS control mice. The results are representative of 20 microscopic views of 3 independent experiments (ROS+ cells are indicated as green, ROS^- cells indicated as blue). B, the percentage of ROS positive cells were calculated by counting as least 20 different views. C, the production of ROS in peritoneal fluid and by peritoneal neutrophils of alcohol treated mice or PBS control mice following PMS induction were quantified based upon relative fluorescence intensity using commercial kits. The results are expressed as mean ± SE (n=5-8/group).The results are expressed as mean ± SE (n=5-8/group; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

ROS. To explore this, 20% ethanol was administered to mice before CLP and peritoneal lavage was harvested and analyzed for ROS levels at 24h. In addition, peritoneal neutrophils isolated from C57BL/6 mice were treated with alcohol and stimulated with *E. coli* for the

determination of ROS expression in the neutrophils. The results show that alcohol administration in mice impairs the production of ROS by peritoneal cells in response to PMS (Figure 4.3A-B). The reduced percentage of ROS^+ cells among total peritoneal cells is consistent with data of diminished ROS level in peritoneal fluid at 24 h post-CLP (Figure 4.3C) as well as in peritoneal neutrophils treated with 20 mM ethanol with *E. coli* stimulation (Figure 4.3C). These observations indicate that acute alcohol treatment impairs the production of ROS by peritoneal neutrophils *in vivo* during sepsis as well as *in vitro* in response to bacterial infection.

PMNs isolated from alcohol-challenged CLP mice fail to form NETs. Although NET formation by neutrophils is a well-established concept for microbial killing mechanism during infection, it is still unknown whether alcohol can alter the NET formation in infection (15). In this regard, it is interesting to explore whether the PMNs isolated from alcohol-challenged CLP mice has an effect on NET formation. For this, we analyzed the immunofluorescence and SEM images of neutrophils from alcohol-challenged CLP mice for the evidence of NET formation and also evaluated NET formation kinetics at multiple time points. In isolated peritoneal neutrophils from alcohol-administered mice, we found a significant reduction in citrullinated histone H3 positive DNA, as measured by fluorescence microscopy, as compared to the neutrophils isolated from PBS given mice with CLP (Figure 4.4A-B). This data is consistent with the marked reduction rate of NET formation by peritoneal neutrophils isolated from alcohol-administered CLP mice than those isolated from PBS-administered CLP mice when analyzed every 1h interval till 8 h (Figure. 4.4C). Furthermore, reduced expression of citrullinated H3 and PAD-4 proteins by peritoneal neutrophils from alcohol recipient CLP mice than that PBS-recipient CLP mice also confirm the impairment of NET formation by alcohol in sepsis (Figure. 4.4E). Utilizing scanning electron microscopy, long string-like extracellular threads, a typical morphology of NETs as defined by Brinkmann *et al*. (16), entangling bacteria were most evident in peritoneal neutrophils isolated from PBS-administered CLP mice, but not by neutrophils from alcohol-

administered CLP mice (Figure. 4.4D), corroborating the above results that alcohol significantly reduce the NET formation in response to PMS in mice.

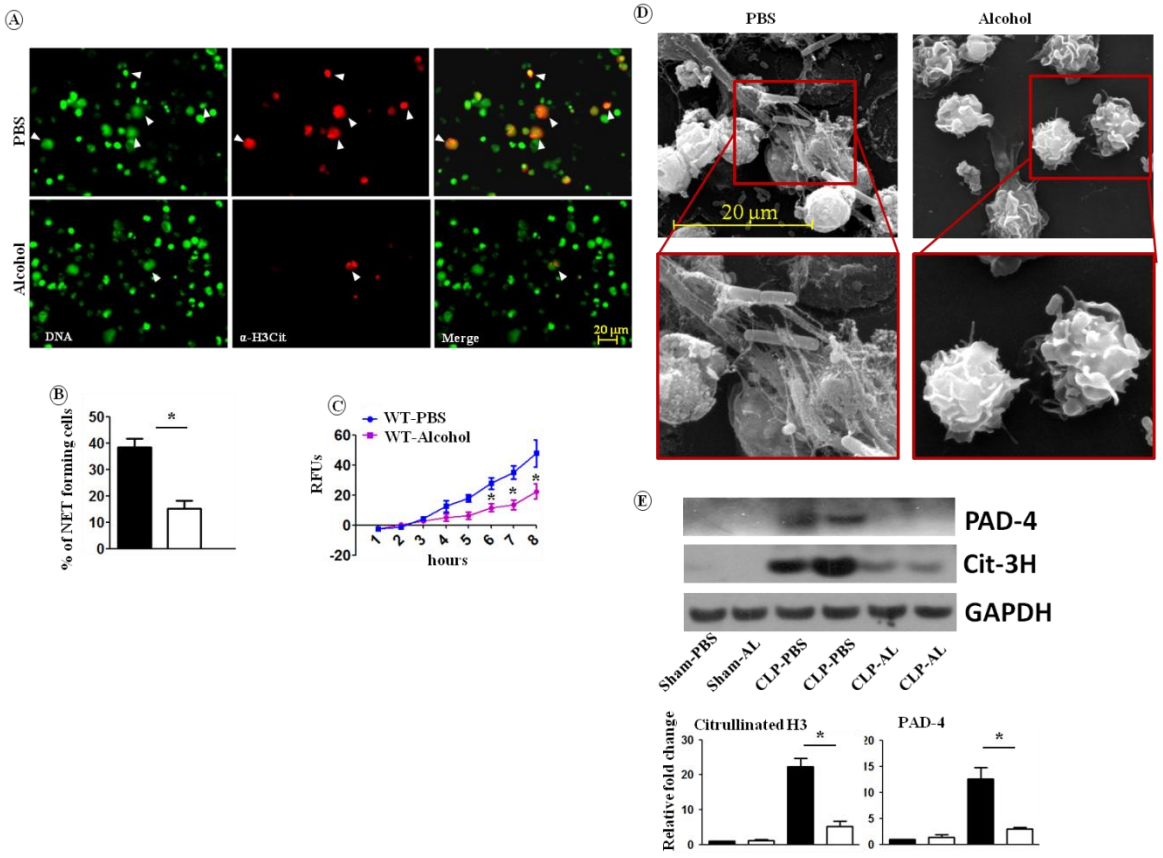

Figure 4.4. Alcohol treated neutrophils exhibit decreased NET formation after sepsis. A, Peritoneal neutrophils harvested from alcohol treated or PBS control mice after PMS, allowed to undergo NET formation and fixed at 8 h. Neutrophils were stained with Sytox green DNA stain and citrullinated histone H3 Ab to visualize citrullinated DNA after the cells were fixed with 4% (v/v) paraformaldehyde. Scale bars, 20 µm. Long strands of DNA (arrowhead) are evidence of NET formation. Images presented are representative images of three independent experiments (n=5-7/group). B, A total of 20 images were selected from one experiment and quantified for the presence of NET-positive neutrophils from alcohol treated or PBS control mice. The results are expressed as mean ± SE (n=5-8/group).C, Kinetic analysis of NET formation by peritoneal neutrophils harvested from alcohol treated or PBS control mice. Relative fluorescent intensity was determined to evaluate NET formation each hour until 8 h of *in vitro* culture of neutrophils (n=6-9/group). D, Evaluation of NET formation by SEM. Peritoneal neutrophils harvested from *cxcl1*$^{-/-}$ and WT mice after induction of PMS were analyzed by SEM. Presence of long thread-like structures (arrowhead) is evidence of NET formation. Scale bars, 20 µm. Images presented are representative of two independent experiments (n=5-8/group). E, Western blot of PAD-4 and cittrullinated-H3 in the peritoneal neutrophils from alcohol treated or PBS control mice at 24 h post-PMS. This blot is representative of 3 independent experiments with similar results. The results are expressed as mean ± SE from two independent experiments (n=5-8/group: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

The impairment of NET formation by alcohol in primary mouse and human neutrophils. Next we determined the *in vitro* effect of alcohol on NET formation utilizing mouse neutrophils and human neutrophils with bacterial infection. For these, we analyzed the NET formation in bone marrow-derived neutrophils from C57BL/6mice that were infected with *E. coli* at an MOI of 0.5 in the presence or absence of alcohol (25 mM and 250 mM).

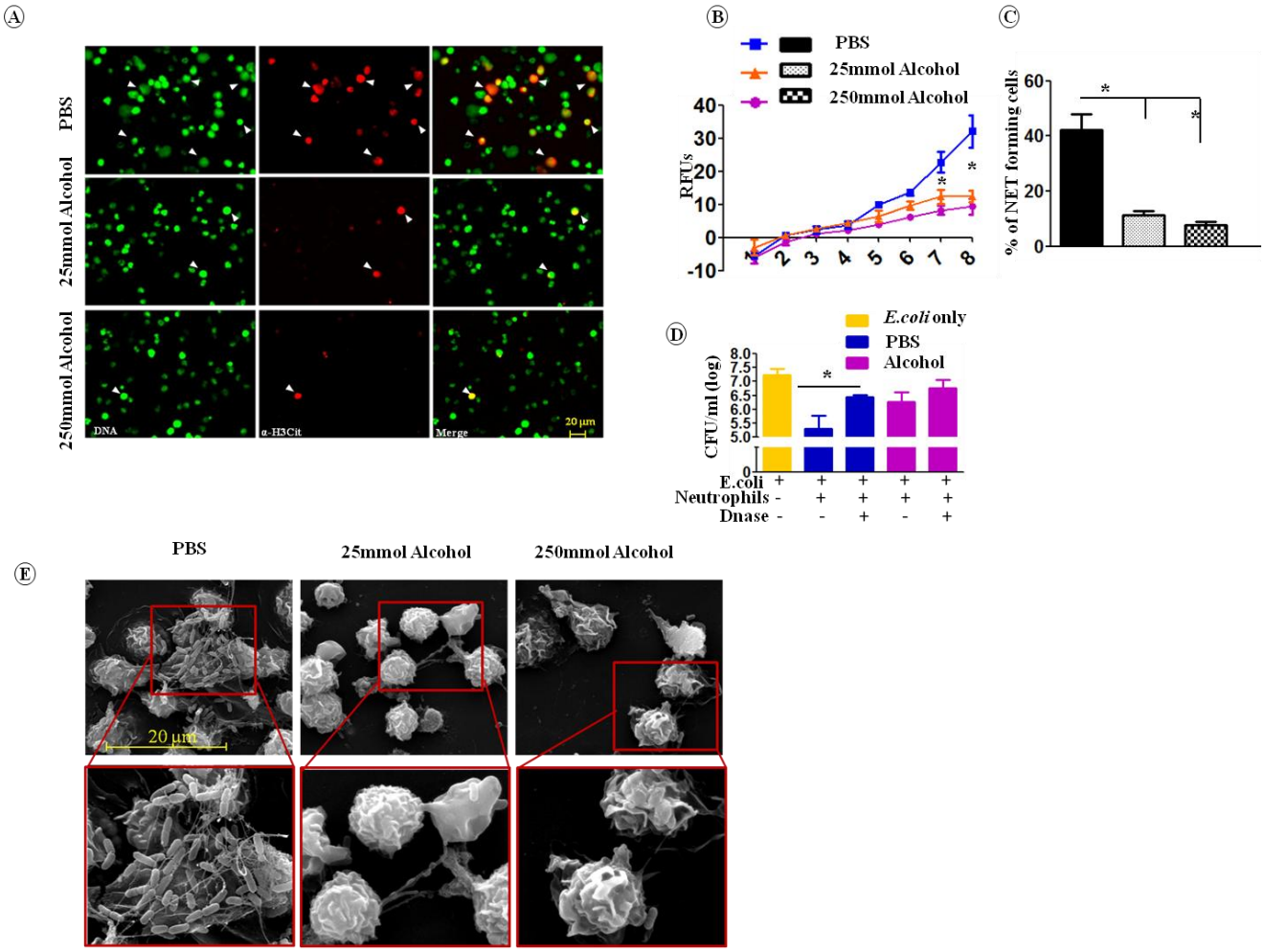

Figure 4. 5. Alcohol impairs NET formation in mouse bone marrow-derived neutrophils in response to *E. coli* infection. A, Mouse bone marrow-derived neutrophils treated with 25 mM and 250 mM alcohol exhibited diminished NET formation in response to Gram-positive and Gram-negative bacterial infection. Mouse neutrophils harvested from the C57BL/6 mice were pretreated with either 25 mM or 250 mM alcohol before infection with *E. coli* and allowed to undergo NET formation and fixed at 8 h. Neutrophils were stained with Sytox green DNA stain and citrullinated H3 Ab for immunofluorescence analyses, as described above. Images presented are representative images of three independent experiments (each in duplicate). A total of 20 images were selected from one experiment and quantified for the presence of NET-positive neutrophils. The results are expressed as mean ± SE (*, $p<0.05$). B, Kinetic analysis of NET formation by *E.coli*-infected mouse neutrophils treated with alcohol. Mouse neutrophils that were pretreated with either 25 mM or 250 mM alcohol before infection with *E. coli* and stained with Sytox green DNA stain. Relative fluorescent intensity was determined to evaluate NET formation each hour until 8 h of *in vitro* culture of neutrophils (*,

p<0.05). C, A total of 20 images were selected from one experiment and quantified for the presence of NET-positive neutrophils pretreated with either 25 mM or 250 mM alcohol before infection with *E. coli* .The results are expressed as mean ± SE (n=5-8/group) D.Bone marrow neutrophils pretreated with either 25 mM or 250 mM alcohol before infection with *E.coli* exhibited diminished extracellular bacterial killing activity. E, Evaluation of NET formation by SEM. Mouse neutrophils were pretreated with either 25 mM or 250 mM alcohol before infection with *E. coli* and allowed for NET formation till 8 h. NET formation by neutrophils were analyzed by SEM, as described above.

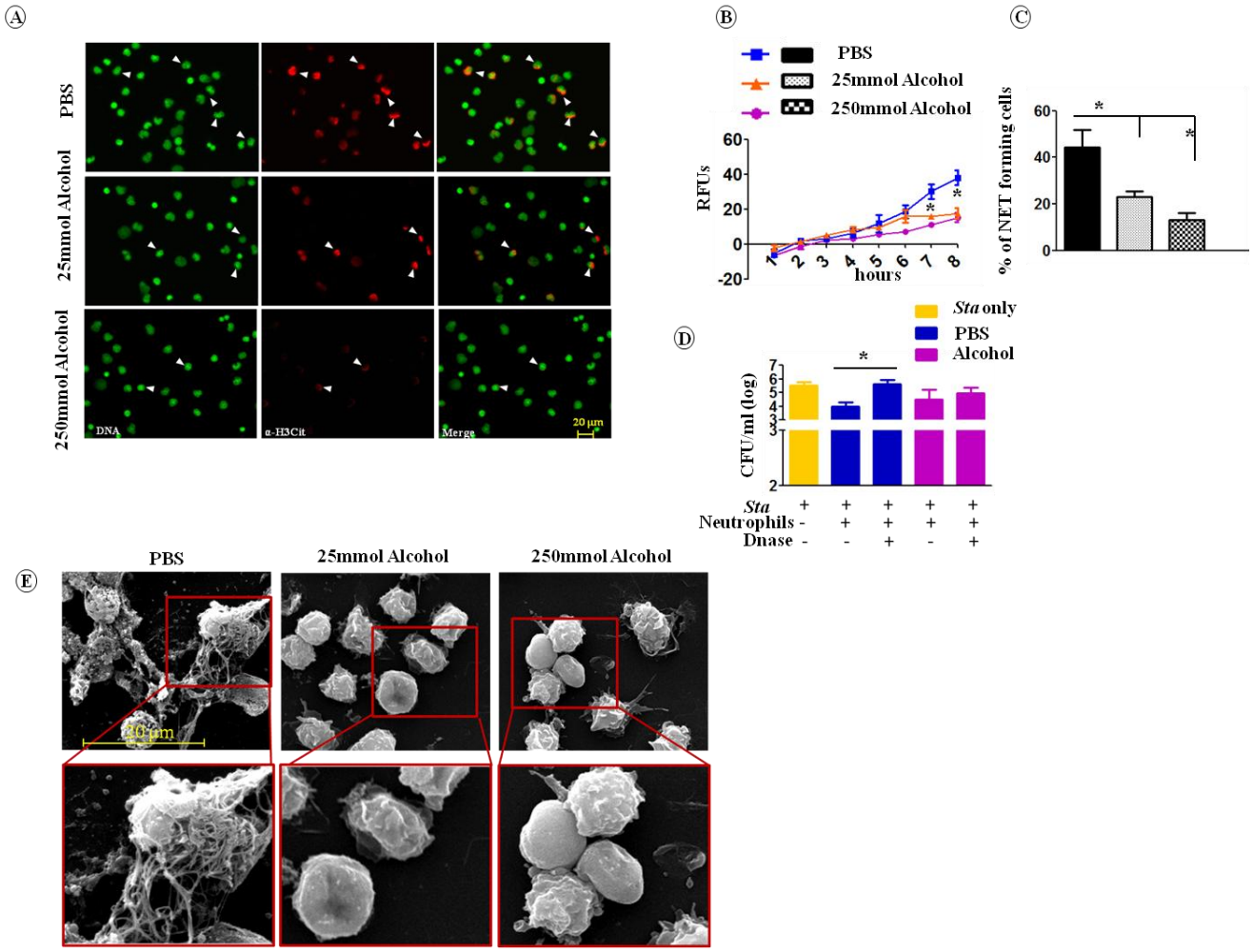

Figure 4. 6. Alcohol impairs NET formation in mouse bone marrow-derived neutrophils in response to *S. aureus* infection. A, Mouse bone marrow-derived neutrophils treated with 25 mM and 250 mM alcohol exhibited diminished NET formation in response to Gram-positive and Gram-negative bacterial infection. Mouse neutrophils harvested from the C57BL/6 mice were pretreated with either 25 mM or 250 mM alcohol before infection with *S. aureus* and allowed to undergo NET formation and fixed at 8 h. Neutrophils were stained with Sytox green DNA stain and citrullinated H3 Ab for immunofluorescence analyses, as described above. Images presented are representative images of three independent experiments (each in duplicate). A total of 20 images were selected from one experiment and quantified for the presence of NET-positive neutrophils. The results are expressed as mean ± SE (*, p<0.05). B, Kinetic analysis of NET formation by *S. aureus* -infected mouse neutrophils treated with alcohol. Mouse neutrophils that were pretreated with either 25 mM or 250 mM alcohol before infection with *S. aureus* and stained with Sytox green DNA stain. Relative fluorescent intensity was determined to evaluate NET formation each hour until 8 h of *in vitro* culture of neutrophils (*, p<0.05). C, A total of 20 images were selected from one experiment and quantified for the presence of NET-positive neutrophils pretreated with either 25 mM or 250 mM alcohol before infection with *S. aureus* .The results are expressed as mean ± SE (n=5-

8/group) D. Bone marrow neutrophils pretreated with either 25 mM or 250 mM alcohol before infection with *S. aureus* exhibited diminished extracellular bacterial killing activity. E, Evaluation of NET formation by SEM. Mouse neutrophils were pretreated with either 25 mM or 250 mM alcohol before infection with *E. coli* and allowed for NET formation till 8 h. NET formation by neutrophils were analyzed by SEM, as described above.

The results showed that the alcohol treatment dose dependently reduced the percentage of neutrophils positive for extracellular DNA and citrullinated H3 in bone marrow neutrophils following *E. coli* infection (Figure. 4.5A). In addition, SEM images also corroborate the reduced formation of NETs by alcohol-treated bone marrow-derived neutrophils (Figure. 4.5E). The reduction of NETs by alcohol was observed to be consistent over the different time periods of 8h incubation (Figure. 4.5B). And similar results were found in the mouse nuetrophil infected with *S. aureus* (Figure. 4.6).

To examine further whether the impairment of bacteria-induced NET formation by alcohol is observed in humans, we used the human neutrophils derived from peripheral blood of healthy volunteers to analyze the NET formation in response to bacterial infection. Similar to the results seen in CLP-mice and bone marrow-derived neutrophils, the percentage of neutrophils positive for extracellular DNA and citrullinated H3 was observed lower in alcohol-treated human neutrophils following *E. coli* (Figure. 4.7) or *S. aureus* (Figure. 4.8) infection. Moreover, SEM images also demonstrate much reduced or the absence of NET formation by alcohol-treated human neutrophils infected with *E. coli* (Figure 4.7E) or *S. aureus* (Figure 4.8E) at both concentration of alcohol (25 mM and 250 mM). Similarly, the kinetics studies over 6h time period also show reduced level of NET formation by alcohol-treated human neutrophils as compared to PBS-treated human neutrophils infected either *E. coli* (Figure. 4.7B) or *S. aureus* (Figure. 4.8B). Thus, our results suggest that acute alcohol exposure can inhibit the NET formation in mice as well as in primary mouse and human neutrophils.

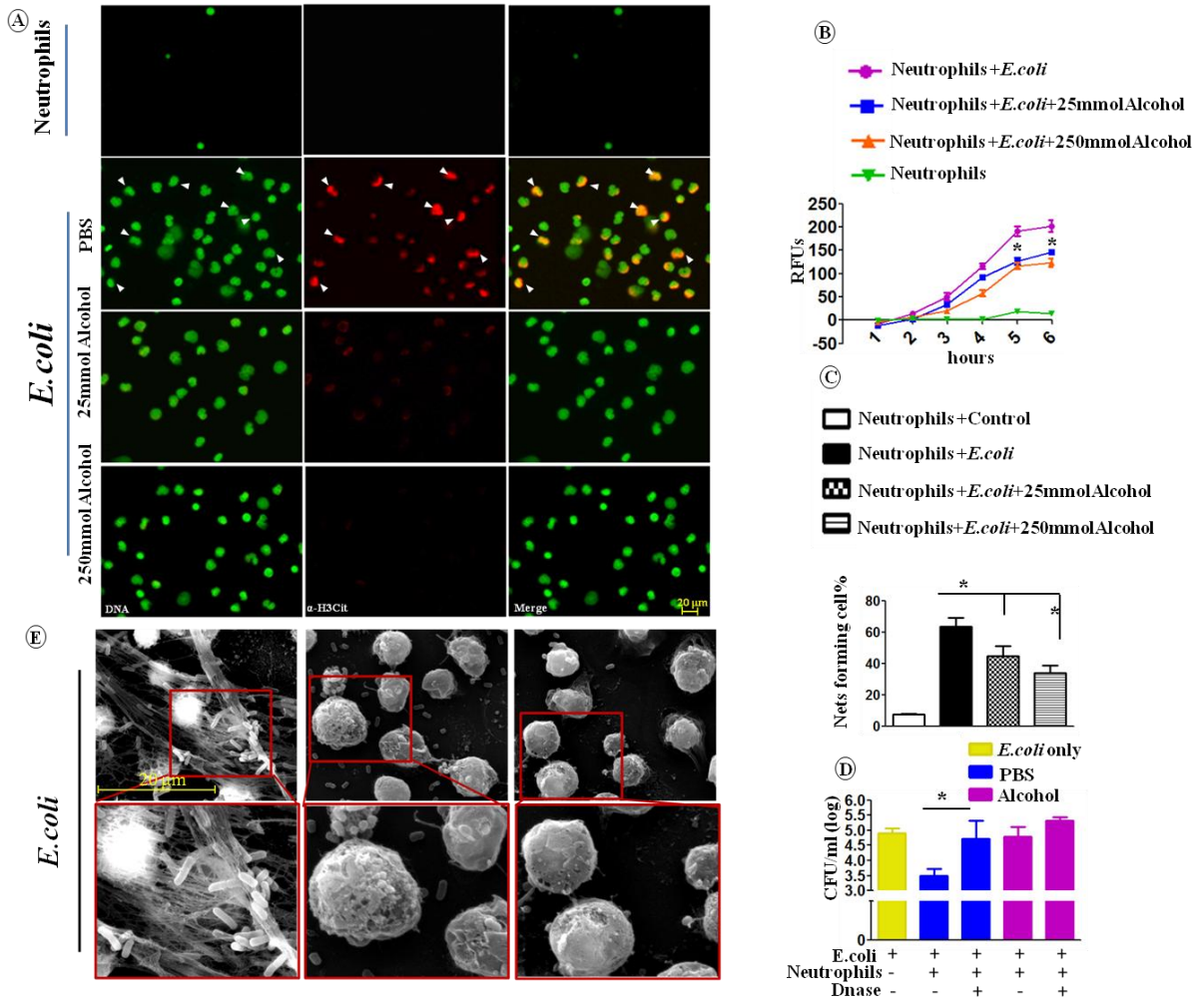

Figure 4.7. Alcohol reduces NET formation in human PMNs in response to *E. coli* infection. A, Human PMNs treated with 25 mM and 250 mM alcohol exhibited diminished NET formation in response to *E. coli* infection. Human neutrophils harvested from the blood of healthy donors were pretreated with either 25 mM or 250 mM alcohol before infection with *E. coli* and allowed to undergo NET formation and fixed at 8 h. Neutrophils were stained with Sytox green DNA stain and citrullinated histone H3 Ab for immunofluorescence analyses, as described above. Images presented are representative images of three independent experiments (each in duplicate). B, Kinetic analysis of NET formation by *E. coli*-infected human neutrophils treated with alcohol. Human neutrophils that were pretreated with either 25 mM or 250 mM alcohol before infection with *E. coli* and stained with Sytox green DNA stain. Relative fluorescent intensity was determined to evaluate NET formation each hour until 8 h of *in vitro* culture of neutrophils (*, $p<0.05$). C. A total of 20 images were selected from one experiment and quantified for the presence of NET-positive neutrophils. The results are expressed as mean ± SE (*, $p<0.05$). D. Human neutrophils pretreated with either 25 mM alcohol before infection with *E.coli* exhibited diminished extracellular bacterial killing activity. E, Evaluation of NET formation by human neutrophils by SEM. Neutrophils were pretreated with either 25 mM or 250 mM alcohol before infection with *E. coli* and allowed for NET formation till 8 h. NET formation by neutrophils were analyzed by SEM, as described above.

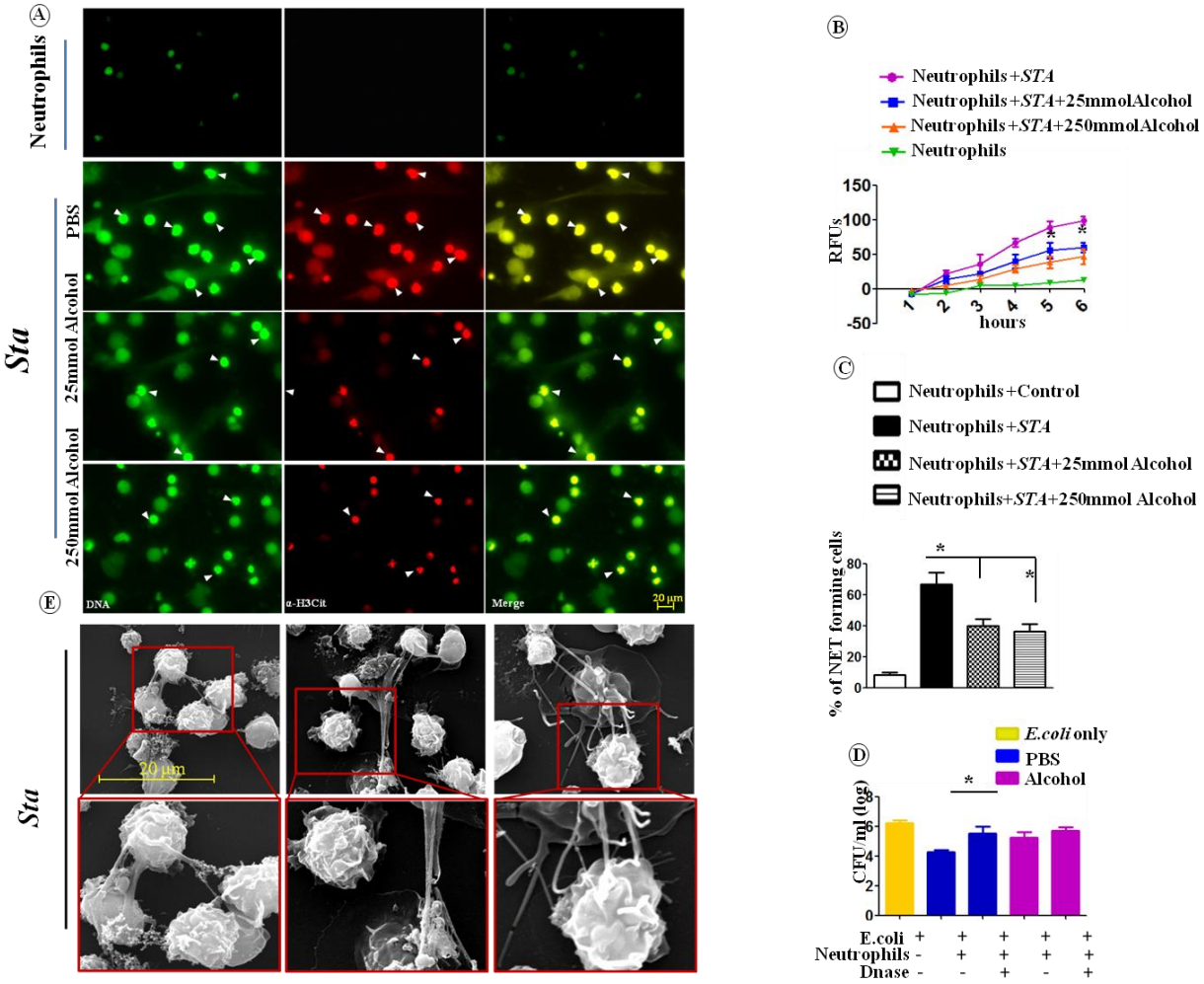

Figure 4.8. Alcohol decreases NET formation in human PMNs in response to *S. aureus* infection. A, Human PMNs treated with 25 mM and 250 mM alcohol exhibited diminished NET formation in response to *S. aureus* infection. Human neutrophils harvested from the blood of healthy donors were pretreated with either 25 mM or 250 mM alcohol before infection with *S. aureus* and allowed to undergo NET formation and fixed at 8 h. Neutrophils were stained with Sytox green DNA stain and citrullinated histone H3 Ab for immunofluorescence analyses, as described above. Images presented are representative images of three independent experiments (each in duplicate). B, Kinetic analysis of NET formation by *S. aureus*-infected human neutrophils treated with alcohol. Human neutrophils that were pretreated with either 25 mM or 250 mM alcohol before infection with *S. aureus* and stained with Sytox green DNA stain. Relative fluorescent intensity was determined to evaluate NET formation each hour until 8 h of *in vitro* culture of neutrophils (*, $p<0.05$). C. A total of 20 images were selected from one experiment and quantified for the presence of NET-positive neutrophils. The results are expressed as mean ± SE (*, $p<0.05$). D. Human neutrophils pretreated with either 25 mM alcohol before infection with *S. aureus* exhibited diminished extracellular bacterial killing activity. E, Evaluation of NET formation by human neutrophils by SEM. Neutrophils were pretreated with either 25 mM or 250 mM alcohol before infection with *S. aureus* and allowed for NET formation till 8 h. NET formation by neutrophils were analyzed by SEM, as described above.

Alcohol increases apoptosis of peritoneal cells and impairs the bacterial killing ability of neutrophils. To determine whether ethanol is involved in the cell survival during sepsis

pathway, we compare the peritoneal cell apoptosis as measured by Annexin-V binding by

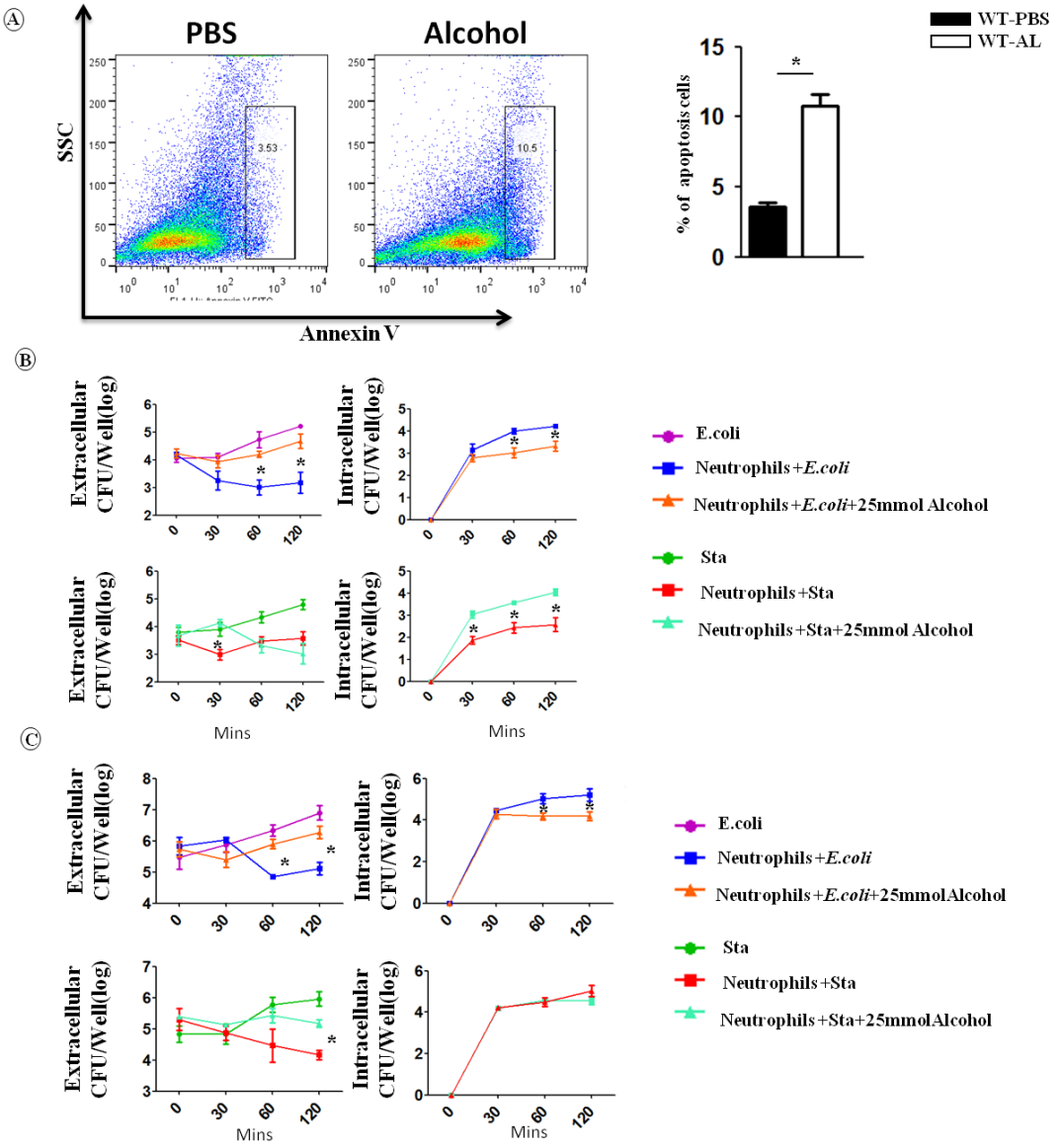

Figure 4.9. Alcohol attenuates the bacterial clearance in CLP mice and the extracellular bacterial killing ability of neutrophils. A. Impaired bacterial clearance in alcohol-challenged mice. The CFUs were examined in peritoneal lavage fluid and blood or the homogenates obtained from kidneys, livers, lungs, and spleens of alcohol-administered and unadministered mice at 6 and 24 h post-PMS. The results are expressed as mean log of CFU/mL (n=5/group; *, p<0.05). B-C, Alcohol impairs the extracellular bacterial killing activity of mouse bone marrow-derived and human neutrophils. Bacterial killing capacity of mouse neutrophils (A) and human PMNs (B) pretreated with alcohol (25 mM and 250 mM) was determined by assessing extracellular CFUs at 30, 60 and 120 min after infection with *E. coli* (MOI 1) or *S. aureus* (MOI 1). The results are expressed as mean ± SE from three independent experiments (*p <0.05).

flow cytometry 24h post sepsis. As expected, we can see the apoptosis rate was significantly increased in alcohol treated group (Figure. 4.9A). To explore the effect of alcohol in extracellular and intracellular killing ability of neutrophils, mouse bone marrow-derived

neutrophils and human neutrophils were first infected with either *E. coli* or *S. aureus* at an MOI of 0.5 in the presence or absence of 25 mM alcohol. The viable extracellular bacteria at multiple time points of infection were enumerated for extracellular killing ability of neutrophils. The results show that the alcohol significantly inhibits the extracellular killing ability of both mouse and human neutrophils, as we observed the significant higher number of viable extracellular bacteria in the wells of alcohol-treated neutrophils at 60 and 120 min of infection (Figures. 4.9 B and C). Alcohol treatment to both mouse and human neutrophils has no effect in the uptake of bacteria (as determined at 30 min of infection) (Figures. 4.9B and C), suggesting its minor role in the internalization or phagocytosis of bacteria.

Discussion

The host response to PMS involves numerous integrated processes that involve both exaggerated inflammation and immune suppression at cellular and molecular levels. It is well known fact that alcohol consumption alters the host immune response and predisposes to a variety of Gram-negative and Gram-positive bacterial infections (17-19). Although the underlying mechanisms of immune dysfunctions caused by alcohol is still not well understood, acute ethanol intoxication is known to suppress PMN recruitment to lung, the PMN phagocytosis and the oxidative burst (20). Our studies demonstrating an impaired NET formation and bacterial elimination in alcohol-challenged CLP mice, and in primary mouse and human neutrophils, are novel antimicrobial defects caused by alcohol in response to bacterial infection.

NETosis is a unique host defense mechanism employed by neutrophils to trap and kill extracellular pathogens (5). In the present study, we found a significant reduction in NETosis by peritoneal neutrophils from acutely intoxicated mice following PMS, as judged by reduced NET-positive peritoneal neutrophils (from immunofluorescence), the expression levels of PAD-4 and citrullinated H3 proteins and the NET-like structures (from SEM images) in alcohol-administered mice compared to control mice. In addition, our *in vitro* results utilizing mouse bone marrow-derived neutrophils and human neutrophils show reduced NET

formation in alcohol-challenged neutrophils exposed to bacteria, compared to PBS-treated bacteria-exposed cells. Furthermore, we observed a strong correlation between diminished NET formation ability of PMNs from alcohol-challenged CLP-induced mice to the significant increased bacterial burden in CLP mice that received alcohol.

Reactive oxygen species, which are generated by the phagocytic NADPH oxidase, is a key component of innate immunity and antibacterial defense, but can also be a harmful mediator of acute inflammation when produced in excessive amounts (21). Although studies have suggested the host deleterious role of ROS in experimental model of sepsis, it is not known whether alcohol can alter ROS production causing an effect in bacterial multiplication in mice with CLP-induced sepsis. In this context, our results of impaired ROS production and enhanced bacterial burden after acute ethanol exposure suggest that the impairment of ROS production by ethanol is critical mechanism to induce the sepsis-induced mortality. Consistent to our results, recent studies show that ethanol significantly decreases ROS production by *Acinetobacter baumannii*-treated neutrophils (22). Several previous studies have also demonstrated the attenuated ROS generation along with increased susceptibility of bacterial infection in mice with targeted disruption of NADPH oxidase components (gp91phox, p47phox), highlighting the important role of ROS in restricting bacterial multiplication *in vivo* (23, 24).

Furthermore, we demonstrated reduced NET-mediated extracellular killing ability of neutrophils for *E. coli* and *S. aureus* in alcohol-treated cells compared to PBS treated cells, suggesting a unique role of ethanol in impairing NET-mediated extracellular bacterial killing. The results linking diminished neutrophil ROS production with attenuated NETosis and subsequent reduction in bacterial killing are consistent with prior reports (16, 25, 26), but no alcohol intoxication was used in these studies. Our results of dampening role of acutely exposed alcohol in neutrophil killing ability for Gram-positive or Gram-negative bacteria is in agreement with previous report that showed increased growth of intracellular *A. baumannii* bacteria in alcohol-treated neutrophils compared to untreated *A. baumannii*-infected

neutrophils (22). However, this is the first investigation to demonstrate that the alcohol reduced NETosis and NET-mediated extracellular bacterial killing of both Gram-negative and Gram-positive pathogens as mechanism to induce immunosuppression of innate immunity.

In conclusion, the present study furthers a number of advancements in our understanding of the biological role of alcohol in host innate immune response to PMS. The present study clearly demonstrates that the acute alcohol intoxication impairs the NET formation, ROS production and thus bacterial killing in response to PMS in mice. Furthermore, alcohol-induced defect in NET formation and NET-mediated killing in mice correlates with the results observed in human PMNs.

Acknowledgments

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Chapter 5: Conclusions

Sepsis is a systemic severe infection disease involving complex immune response to microbes. It is estimated more than 210,000 people die because of sepsis per year with annual healthcare costs for treatment of sepsis exceeding $17 billion in United States alone (1,2). However, the molecular and cellular mechanisms that regulate immune responses to polymicrobial sepsis are not completely understood.

The innate immune system is the first line of host defense during infection, contributes to the migration of leukocytes in inflammed tissues/organs, involving release of various cytokines/chemokines and adhesion molecules. Neutrophils are an important arm of the innate immune response during sepsis, and play a crucial role in bacterial clearance. Impaired neutrophil migration is associated with increased mortality and higher bacterial burden in peritoneal exudates and blood, as demonstrated during sepsis induced by cecal ligation and puncture (CLP) whereas excessive influx of neutrophils can cause unwanted tissue damage and organ dysfunction (3-6). Thus, investigating effector function of neutrophils during sepsis is critical to understanding the mechanisms associated with the pathophysiology of sepsis.

Previous studies from the Lung Biology laboratory has demonstrated an important role for CXCL1 [also known as keratinocyte cell-derived chemokine (KC)] in pulmonary defense during pneumonia caused by *Klebsiella pneumonia* (7). CXCL1 was found to be critical for neutrophil-dependent bacterial elimination via induction of reactive oxygen species and reactive nitrogen species in the lung (8). Neutrophil migration to multiple organs is impaired during severe sepsis, due to down-regulation of the CXCL1 receptor, CXCR2, resulting in failed pathogen clearance. These findings suggest a potential role for CXCR2, or its ligands, including CXCL1 (KC), CXCL2 (MIP-2), and CXCL5 (LIX), in controlling sepsis.

Innate immunity also employs specific pattern recognition receptors (PRRs) to recognize pathogen-associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs) emanating from host cells affected by pathogenic onslaught. The recognition of

PAMPs as well as DAMPs by (PRRs) activates immune response pathways resulting in inflammation. There are three major families of PRRs have been recognized; the trans-membrane toll-like receptors (TLRs), the RIG-I-like receptors (RLRs), and the intracellular NOD-like receptors (NLRs) (9-10). However, we are only beginning to appreciate the importance of NLRs in clinical course of sepsis. NLRs are important in the context of sepsis because these PRRs recognize PAMPs, as well as a variety of DAMPs. Of these, NLRP3 is of particular interest as it forms a caspase-1 activating molecular complex termed "inflammasome"(11). Caspase-1 activation by the inflammasome promotes the maturation of IL-1β. Human and experimental (animal) studies have highlighted the importance of the inflammasome pathways in the inflammatory response to sepsis (12, 13). Clinical studies have been shown that alcoholics are more susceptible to sepsis and thus have higher mortality rate as compared to non-alcoholics with sepsis (14). Alcohol abuse is associated with a leading cause of mortality, around 100, 000 deaths per year. In addition, previous studies have suggested that consumption of ethanol is associated with an increased incidence and severity of a broad spectrum of natural infections in human and experimental animals (15, 16). While our understanding of the many facets of the pathophysiology of sepsis continues to expand, there is still paucity in information on the fundamental mechanisms on what and how alcohol does impact to host in response to sepsis.

In chapter 2 we have studied the role of CXCL1 in neutrophil influx and function during fatal polymicrobial sepsis (PMS). We found that CXCL1 appears to be essential for host survival, activation of NF-κB, MAPK, and NADPH oxidase, expression of cytokines and chemokines essential for host defense, ROS production, as well as NET formation by peritoneal neutrophils and NET-mediated bacterial killing. More importantly, CXCL1 regulates IL-17A production by enhancing Th17 differentiation in order to control neutrophil influx via the production of CXCL2/MIP-2 and CXCL5/LIX. Modulating KC chemokine levels (functional homologue of human IL-8) could be an effective therapeutic strategy to augment host defense in order to control sepsis.

In chapter 3 we interestingly found that NLRP3$^{-/-}$ mice or mice treated with NLRP3 inhibitor were protected in response to polymicrobial sepsis. NLRP3$^{-/-}$ mice showed reduced bacterial burden and production of proinflammatory cytokines. On the contrary, we did not observe any difference in neutrophil recruitment to the peritoneum between NLRP3$^{-/-}$ and control (wild-type) mice. Intriguingly, neutrophils (PMNs) obtained from NLRP3$^{-/-}$ or NLRP3-inhibited mice display impaired critical functions of neutrophils, including phagocytosis, bacterial killing, neutrophil extracellular trap (NET) formation, autophagy, chemotaxis, and cell death. NLRP3 inflammasome modulates antibacterial defense during sepsis through the regulation of neutrophil function, but not via neutrophil recruitment *per se*. These unique and novel findings position NLRP3 as a critical linker between neutrophil function and bacterial clearance, highlighting NLRP3 as a therapeutic target to control infection in polymicrobial sepsis.

In chapter 4 we investigated the role of acute alcohol intoxication (AAI) in polymicrobial sepsis induced by cecal ligation and puncture (CLP). We observed that AAI mice are more susceptible to CLP-induced sepsis, as evidenced by 1) reduced neutrophil recruitment into peritoneal cavity; 2) more bacterial dissemination to distal organs; 3) much higher systemic inflammatory response; and 4) increased neutrophil and macrophage apoptosis as compared with their PBS-treated counterparts. Furthermore, we are the first to report AAI impaired 1) ROS/RNS production; 2) formation of NETs and NETs-mediated bacterial killing; and 3) phagocytosis by immune cells during polymicrobial sepsis. Combined, the results of the experiments fully define a negative role for AAI in the innate immune response during polymicrobial sepsis.

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Vita

Liliang Jin was born in Dongyang, Zhejiang, China, to Yongzheng Jin and Xiujiao Jin. He has a brother, loving and supportive wife and a beautiful baby Ryan. Liliang has graduated with the Master degree of preventive veterinary medicine from the College of Veterinary Medicine at Huazhong Agriculture University at 2009. After graduation, he joined the Lung Biology Laboratory at the Department of Pathobiological Sciences, Louisiana State University to pursue his doctoral degree under the guidance of Dr. Samithamby Jeyaseelan. In lung biology laboratory, he has learned innate immune mechanisms involved with bacterial infections during sepsis. Liliang expects to graduate in December 2014 with his Doctor of Philosophy degree. After graduation, Liliang is interested in pursuing his research interests in the field of immunology of infectious diseases.