De-escalation Antibiotic Therapy Alleviates Organ Injury through Modulation of NET Formation during Sepsis

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Abstract

Empiric broad-spectrum antimicrobials therapy is suggested to be started immediately for sepsis patients. Empiric antimicrobial therapy should be narrowed once pathogen identification and sensitivities are established. However, the detail mechanisms of de-escalation strategy are still unclear. Here we hypothesized neutrophil extracellular trap (NETs) played an essential role and de-escalation strategy might alleviate organs injury through regulation of NETs formation in sepsis. We evaluated the effect of imipenem and ceftriaxone on NETs formation in vitro and examined the role of reactive oxygen species (ROS). Next, we designed de-escalation and escalation strategy based on their effects on NETs formation in CLP model. Organ injury, inflammatory cytokines, NETs levels were compared and evaluated. The in vitro study showed that imipenem and ceftriaxone had opposite effects on NETs formation in activated neutrophils. De-escalation therapy resulted in an evaluated MPO-DNA during early stage and decreased MPO-DNA during late stage, which exerted the reverse effects in escalation therapy sepsis animal model. Inflammatory response and organ injury exacerbated when eliminated NETs with DNAseI during early stage of sepsis ($p<0.01$). Histopathological analysis showed decreased injury in lung, liver and intestine in de-escalation therapy compared with escalation therapy ($p<0.01$). De-escalation therapy results in the highest 6-day survival rate compared with the control group ($p<0.01$), however, no significant difference was found between de-escalation and escalation group ($p=0.051$). We demonstrate that de-escalation, not escalation, therapy reduces organ injury, decreases inflammatory response by promoting NETs formation in the early stage and inhibiting NETs formation in the late stage of sepsis.

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Short title: Antibiotic de-escalation to treat sepsis.

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Summary

Empiric broad-spectrum antimicrobials therapy is suggested to be started immediately for sepsis patients. Empiric antimicrobial therapy should be narrowed once pathogen identification and sensitivities are established. However, the detail mechanisms of de-escalation strategy are still unclear. Here we hypothesized neutrophil extracellular trap (NETs) played an essential role and de-escalation strategy might alleviate organs injury through regulation of NETs formation in sepsis.

We evaluated the effect of imipenem and ceftriaxone on NETs formation*in vitro* and examined the role of reactive oxygen species (ROS). Next, we designed de-escalation and escalation strategy based on their effects on NETs formation in CLP model. Organ injury, inflammatory cytokines, NETs levels were compared and evaluated.

The *in vitro* study showed that imipenem and ceftriaxone had opposite effects on NETs formation in activated neutrophils. De-escalation therapy resulted in an evaluated MPO-DNA during early stage and decreased MPO-DNA during late stage, which exerted the reverse effects in escalation therapy sepsis animal model. Inflammatory response and organ injury exacerbated when eliminated NETs with DNAseI during early stage of sepsis ($p<0.01$). Histopathological analysis showed decreased injury in lung, liver and intestine in de-escalation therapy compared with escalation therapy ($p<0.01$). De-escalation therapy results in the highest 6-day survival rate compared with the control group ($p<0.01$), however, no significant difference was found between de-escalation and escalation group ($p=0.051$).

We demonstrate that de-escalation, not escalation, therapy reduces organ injury, decreases inflammatory response by promoting NETs formation in the early stage and inhibiting NETs formation in the late stage of sepsis.

Key words : Inflammation, Neutrophils, Reactive Oxygen Species, Cytokines, Lung.

INTRODUCTION

Sepsis is a life-threatening disease that is characterized by organ dysfunction and caused by a dysregulated host response to infection; sepsis remains a distressing public health care problem and ranks among the top 10 causes of death worldwide [1]. Currently, it is well known that immunoparalysis is more than the overwhelming pro-inflammatory response that endangers critically ill patients [2]. The mechanisms of sepsis-induced immunoparalysis remain unclear, but functional defects of leukocytes, excessive expression of inhibitory receptors, and dysregulated production of cytokines may play an important role in the immune dysfunction in sepsis.

Neutrophil extracellular traps (NETs) are a new antimicrobial function of neutrophils; NETs are web-like structures accompanied by many proteins, histones, and DNA [3]. The formation of NETs is a double-edged sword in sepsis; NETs trap pathogens in the early stage and cause NET-associated injuries, such as coagulation, thrombotic disorders, and organ injury, in the later stage [4-6]. The balance of NET formation and clearance plays a crucial role in sepsis, and treatments that target the clearance of NETs in the late stage, such as DNase I and Cl-amidine, have been confirmed to ameliorate the severity of sepsis [7, 8]. In addition, NETs have been shown to link innate and adaptive immune responses by regulating the activation of apoptosis in CD4+ and CD8+ T cells [9]. In lipopolysaccharide-induced activation of monocytes, NETs can downregulate the maturation of monocyte-derived dendritic cells, thus reducing the production of cytokines (TNF-α, IL-6, IL-12, IL-23) [10]. These may be potential mechanisms of the role of NETs in sepsis.

Empiric broad-spectrum therapy with one or more intravenous antimicrobials to should be started immediately for patients presenting with sepsis [11]. Broad-spectrum antimicrobial therapy should be narrowed when pathogen identification and sensitivities have been established or discontinued if a decision is made that the patient does not have infection, which was termed as de-escalation therapy. The link between early administration of antibiotics for suspected infection and antibiotic stewardship remains an essential aspect of high-quality sepsis management [12]. Although both de-escalation and escalation antibiotic therapy kill bacteria during sepsis [13], it is unclear why de-escalation, not escalation, therapy reduces mortality, and the detailed mechanism by which these changes in the sequence of antibiotic drug administration or the changes in the drugs themselves affect clinical outcomes are also unknown.

Previous studies have shown that some antibiotics themselves may exert immunomodulatory effects on phagocytes, cytokines, immunoglobulins, and cellular immunity [14, 15]. Although specific immunomodulatory therapy targeting inflammatory cytokines has been confirmed in sepsis models, clinical trials on the blockade of TNF, IL-1 and other cytokines failed [16]. Recent studies focused on the optimization of immunomodulatory effects of NET formation during sepsis, enhancement of NET formation to trap and eradicate all bacteria in the early stage and the attenuation of excessive NET formation to prevent NET-associated injury in the later stage [17]. In this study, we hypothesized that antibiotics might manifest both antimicrobial and immunomodulatory functions in the treatment of sepsis and that de-escalation antibiotic therapy might alleviate organ damage and inflammatory responses through the modulation of NET formation in the different stages of sepsis.

METHODS

Definitions

De-escalation therapy: De-escalation therapy was defined as either a switch to a narrower spectrum agent or the reduction in the number of antibiotics or the early arrest of antibiotic treatment [13]. The guidelines recommend that using broad-spectrum antibiotics when the pathogen is not identified [12], otherwise using narrow-spectrum antibiotics. In our study, antibiotic de-escalation therapy is defined as the use of broad-spectrum antibiotic (imipenem) for the first 3 days and then switch to narrow-spectrum antibiotic (ceftriaxone) according to previous research [4, 13], because the bacterial culture results usually take 48-72h to obtain.

Escalation therapy: Escalation therapy is the opposite of de-escalation therapy. In our study, we defined escalation therapy as the use of narrow-spectrum antibiotic (ceftriaxone) in the first three days of sepsis, followed by broad-spectrum antibiotic (imipenem).

Neutrophil isolation

Peripheral blood collected from healthy volunteers was layered on Polymorphprep (Axis-Shield, Norway) and centrifuged for 30 min at $500g$. The lower interphase having granulocytes was collected and washed with PBS. For purification, twice lysis of red blood cells was done using Red blood cell lysis Buffer (Beyotime, Shanghai, China) followed by two washes with cold PBS. Isolated PMNs were suspended in RPMI 1640 (Gibco, NY, USA) medium containing 1% HEPES (Gibco, NY, USA). The protocol was approved by the institutional ethics committee at Jinling Hospital (No. 2019-NJGKJ-047).

Animals

Adult male C57BL/6J mice weighing between 25 and 30 g were purchased from the Experimental Animal Center, Jinling Hospital, Nanjing, China. All the experimental procedures in our study were reviewed and approved by the Institutional Animal Care and Use Committee of Jinling Hospital. All the mice received food and water ad libitum.

Sepsis models

CLP sepsis models were established as described [4, 7]. Briefly, the mice were anesthetized using 3.5% isoflurane in 100% oxygen. A midline laparotomy was used, and the cecum was ligated with a 4-0 silk suture

located at either 50% (low-grade) or 75% (high-grade) between the ileocecal junction and the distal end of the cecum. A through-and-through puncture was made using a 21-G needle. A small amount of the cecal contents were extruded to ensure the patency of the hole before returning the cecum to the abdominal cavity. The peritoneum was then closed. After surgery, the mice were resuscitated with 0.9% sterile saline (500 ml SC). The mice received buprenorphine (0.05 mg/kg SC) as an analgesic. The sham animals underwent the same procedure except for the ligation and puncture of the cecum. The control animals did not undergo surgery and were euthanized alongside the CLP animals.

Study design

Mice were randomly divided into 2 independent experiment studies, early sepsis stage experiment and late sepsis stage experiment. Early sepsis stage was defined as 3 days after CLP procedure and late sepsis stage was defined as 6 days or more in terms of antibiotics recommendation in sepsis and study design [4, 12, 13].

In early sepsis experiment, mice were randomly divided into 5 groups, as followed: DE for de-escalation group, ES for escalation group, DNase+DE for DNaseI+ de-escalation group, Sham group and Control group. In DE, ES and DNase+DE group, imipenem (Merck & Co., New Jersey, USA) (ip.,25mg/kg, twice a day), ceftriaxone (Pfizer, New York, USA) (ip.,75mg/kg, twice a day) and imipenem (ip.,25mg/kg, twice a day) + DNaseI (Thermo Fisher, Waltham, MA, USA)(ip.,5mg/kg, once a day) were used during all 3 days after CLP procedure and no antibiotics or DNaseI were used in control group. There was no CLP procedure for sham group. Animal were euthanized at day 4 morning. Blood and organs were collected for further analysis.

In late sepsis experiment, mice were randomly divided into 4 groups as DE for de-escalation group, ES for escalation group, Sham group and Control group. In the first 3 days, the antibiotics usage was the same as the early sepsis experiment. At day 4 after CLP procedure, ceftriaxone (ip.,75mg/kg, twice a day) was used in DE group while imipenem (ip.,25mg/kg, twice a day) was used in ES group. Namely, imipenem-initial and ceftriaxone-initial strategy were evaluated. Animals were euthanized at day 7 morning. Blood and organs were collected for further analysis.

Cytokines and MPO-DNA quantification

Blood was collected into EDTAK2 anticoagulant (10% v/v). After centrifugation of whole blood at 1000G for 5 minutes at 4, plasma was collected, and the cell pellet was removed. NET-associated MPO was captured on coated 96-well plates from the mouse MPO ELISA kit (Abcam, Cambridge, UK, Roche, Basel, Switzerland). Cytokines: Interleukin (IL)-6, IL-10, Interferon-γ (IFN-γ), Tumor Necrosis Factor-alpha (TNF-α), Macrophage Inflammatory Protein 2 (MIP-2) and Monocyte Chemoattractant Protein-1(MCP-1) were measured using Luminex ® xMAP according to the manufacturer's instructions. A standard curve was used to calculate the concentrations of MPO-DNA and cytokines.

Determination of biochemical indexes

On day 3 and day 6 after the last administration, blood samples were taken from the peripheral blood of the mice and centrifuged to obtain the serum. The alanine transferase (ALT), aspartic acid transferase (AST), creatinine (Cr), and lactate dehydrogenase (LDH) activities in the serum were detected according to the kit (Amyjet Scientific, Wuhan, China) instructions.

HE staining

The lung, liver, and intestine tissues of the mice were fixed with 10% neutral formalin, embedded with paraffin, sliced, stained with hematoxylin-eosin (HE) and observed under a light microscope. A semi-quantitative analysis was carried out in a blinded fashion by an experienced pathologist who was unaware of the groups to quantify the results, defined as: 0 = normal, + = mild, ++ = moderate, +++ = severe histological changes. The tissues and parameters assessed included the lungs (thickening of the septum, edema, congestion and intestinal leukocyte infiltration); the liver (enlarged sinusoids, increased volume of endothelial cells, luminal leukocyte infiltration, hydropic degeneration, Kupffer cell hypertrophy and hyperplasia) and the intestine

(edema of mucosal villi, infiltration of necrotic epithelial and inflammatory cells, injury of intestinal glands, blood and lymph vessels expanded).

Immunohistochemistry

CitH3 has been identified as an important biomarker of NETs. Thus, immunohistochemistry analysis was performed to determine the expression levels of citH3. Immunohistochemistry was performed using standard protocols. Briefly, paraffin sections were deparaffinized and rehydrated, washed three times with PBS (PH7.4). Sections were placed in 3% hydrogen peroxide solution was incubated for ten minutes. After washing with PBS, 4% goat serum was added dropwise to block, at room temperature for 30 minutes. Add a sufficient amount of primary antibody (CitH3, Abcam, Cambridge, MA, USA) to the section and place it in a humidified box, and incubate at room temperature for 2 hours. After washing three times with PBS, add secondary antibody (goat anti-rabbit IgG, Abcam, Cambridge, MA, USA) and incubate for 30 minutes. Finally, stain with DAB solution and observe under the microscope, the positive signal is brown.

Detection of cell apoptosis

Apoptosis of liver and intestine cells was detected by terminal deoxynucleotidyl. Transferase dUTP nick-end labeling assay (TUNEL) with the In-Situ Cell Death Detection kit (Roche, Basel, Switzerland) according to the kit instructions. Briefly, the cells on coverslips were washed with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature. After washing, the cells were incubated in permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate, freshly prepared) for 2 min on ice. The cells were then incubated with the TUNEL reaction mixture in a humidified chamber at 37°C for 1 h. Subsequently, the cells were briefly rinsed with PBS and counterstained with DAPI for 5 min in order to visualize the nuclei.

NET Quantification and ROS detection

NET quantification was performed as previously described (18). In brief, the cells were seeded at 5.2 × 10ˆ4 cells per well in a 96-well plate in the culture media and were preincubated with antibiotics for 2 h at 37 °C in 5% CO2. Then, PMA (100 nM) was added in the presence of 5 μM SYTOX Green cell-impermeable nucleic acid stain (Life Technologies). The fluorescence intensity (RFU) was measured using a SpectraMax i3x fluorescence microplate reader (Molecular Devices LLC) at specific time intervals for up to 200 min after the activation of the cells. For the inhibitory assays, an extra 1-h preincubation with DPI or Pyro was needed. The NETotic index was used to calculate the index of NET formation and is expressed as the RFU ratio of each group. Some cells were lysed with 0.5% Triton X-100 at each time point, and this sample represented 100% DNA release.

In order to quantify reactive oxygen species (ROS) generation, isolated PMNs were suspended in RPMI 1640 medium containing 1% HEPES and pretreated with Dihydrorhodamine 123 (DHR123, 1 ummol/L, Beyotime, Shanghai, China) for 30 minutes. Then washing PMNs three times with PBS after centrifugation. The same number of PMNs were then resuspended with PBS and transferred into a black 96-well microplate. Fluorescence intensity was quantified every 10 minutes after treated with PMA, which using SpectraMax i3x fluorescence microplate reader (Molecular Devices LLC) at an excitation wavelength of 488nm and an emission wavelength of 530nm.

NET Visualization

NET visualization was performed according to previous protocols. The neutrophils used for the plate reader assay were fixed with 4% PFA in PBS buffer for 30 min and permeabilized with 0.1% Triton X-100 for 30 min. Then, blocking was performed with 10% normal goat serum for 30 min at RT. The cells were stained using an anti-myeloperoxidase antibody (Abcam ab25989) and an anti-histone H3 (citrulline2+8+17) antibody (Abcam ab5103). A goat anti-mouse IgG H&L-Alexa Fluor® 647 antibody (Abcam, ab150115) and a goat anti-rabbit IgG H&L-Alexa Fluor® 488 antibody (Abcam, ab150077) were utilized as secondary antibodies. The DNA was already stained with SYTOX Green after permeabilization. The images were captured using a Leica DMi8 inverted microscope.

Statistics

The data are expressed as the means ± SEMs and analyzed using Student's t-test or one-way ANOVA followed by the Student–Newman–Keuls test. P values < 0.05 were considered significant. All the statistical analyses were performed using GraphPad Prism (version 8.01; GraphPad Software, Inc., La Jolla, CA).

RESULTS

β-λαcταμ αντιβιοτιcς μοδυλατε τηε φορματιον οφ NETc ιν ΠΜΑ-αcτιατεδ ΠΜΝc

To determine the potential effect of β-lactam antibiotics on NET formation, we preincubated purified peripheral blood neutrophils with imipenem and ceftriaxone for 2 h. Then, the neutrophils were activated with PMA and monitored for 3 h. The NETotic index curves are shown in Fig 1A. Unlike the activated neutrophils, the neutrophils (without PMA) treated with antibiotics alone did not form NETs (Fig 1B). To confirm the existence of NETs, both fluorescence and immunofluorescence images were obtained to determine the morphology and composition of the NETs (Fig 1C). All the data suggested that imipenem and ceftriaxone have opposite effects on the formation of NETs in activated neutrophils.

NADPH oxidase (NOX) is a crucial enzyme in the process of NET formation; during the classic process of NET formation, which proceeds in a ROS-dependent manner, NOX may modulate the generation of ROS. β-Lactams were proven to influence the activity of NOX. Thereafter, we detected the activity of NOX to explore the mechanism by which β-lactam antibiotics participate in NET formation. DHR123, an indicator of ROS, was used to measure the generation of ROS. In both the resting and activated (activated by PMA) neutrophils, the levels of ROS were increased in the imipenem group and decreased in the ceftriaxone group compared with the control group (Fig 1D). Then, we used the NOX inhibitor DPI to explore the relationship between ROS and NET formation in response to β-lactam antibiotics (Fig 1E). Consistent with the results described above, DPI inhibited the formation of NETs and the generation of ROS in all the groups. Once the exogenous ROS H_2O_2 was added, the formation of NETs increased in all the groups (Fig 1F), which indicated that ROS are involved in β-lactam antibiotic-induced NET formation.

All these data showed that β-lactam antibiotics modulate the formation of NETs in activated neutrophils through a ROS-dependent manner.

Effects of de-escalation and escalation antibiotic therapy on the formation of NETs during sepsis

To evaluate the function of NETs during different sequential antibiotic therapy, we determined the serum level of MPO-DNA, which is a biomarker of NETs, in CLP septic model. Mice were divided into two groups: the early stage of sepsis (the timepoint was set to 72 h after the initial CLP) and the late stage of sepsis (the timepoint was set to 6 days after the initial CLP). In the early stage, the mice were divided into 4 groups: the de-escalation group (imipenem for 3 days), the escalation group (ceftriaxone for 3 days), the de-escalation+ DNaseI group (used to disrupt the NETs) and the control group. The serum MPO-DNA levels in the control mice were much higher than those in the antibiotic-treated mice (P¡0.01), and the MPO-DNA levels in the de-escalation group were higher than those in the escalation group (Fig 2A). When DNaseI administration was added, MPO-DNA in the de-escalation group decreased to levels lower than those in the escalation group.

Next, we determined the MPO-DNA levels in the late stage of sepsis. Three groups were established: the de-escalation group (imipenem for the first 3 days and ceftriaxone for the last 3 days), the escalation group (ceftriaxone for the first 3 days and imipenem for the last 3 days) and the sham group. In the late stage, the serum levels of MPO-DNA in the de-escalation group were lower than those in the escalation group with the adaption of de-escalation therapy (Fig 2B). All the data suggested that de-escalation antibiotic therapy increased NET formation during the early stage of sepsis and decreased NET formation during the late stage of sepsis, which was consistent with the *in vitro* results described above.

De-escalation antibiotic therapy alleviates NET-associated organ injury and inflammation in

CLP model.

Considering the role of NETs in the progression of sepsis, NET-associated organ injury was assessed. In the early stage of sepsis, both the levels of AST and serum creatinine were significantly lower in the de-escalation group (imipenem for 3 days) than in the escalation group (ceftriaxone for 3 days) and the control group (Fig 3A-B). However, when DNase I was administered to the escalation group for 3 days, there was no significant difference between the levels of AST and serum creatinine in this group, although these levels were lower than those in the control groups. Next, we measured the inflammatory response. Consistent with the levels of AST and serum creatinine, the levels of serum IL-6, IL-10, and IFN-γ were lower in the de-escalation group than in the other groups (Fig 3C-E). Interestingly, the level of MCP-1 was lower in the escalation group. Then, we used DNase I in the de-escalation group. The levels of all the cytokines were elevated, and the level of MCP-1 was decreased (Fig 3F).

Next, we measured these factors in the late stage of sepsis. First, we measured the AST, ALT, LDH, and serum creatinine levels to evaluate organ injury. There were varying degrees of damage in the sepsis group compared with the sham group, and all the serum biochemical values were lower in the de-escalation group than in the escalation group (Fig 4A-D). The levels of the cytokines IL-6, IL-10, IFN-γ, MIP-2 and MCP-1 were consistent with the biochemical values in the two groups (Fig 5A-F).

These findings suggested that NETs exert a protective role in the early stage of sepsis, which can alleviate organ injury and the inflammatory response, and reduced NET production is beneficial to septic mice in the late stage.

NET formation is involved in de-escalation therapy in sepsis.

To elucidate the relationship between NET formation and organ injury in the late stage of sepsis, we performed histological and immunohistochemical staining of samples from the lung, intestine, and liver. The damage in the sepsis groups was significantly worse than that in the sham group, and compared with escalation therapy, de-escalation therapy reduced intra-alveolar hemorrhage, interstitial edema, and acute inflammatory cell infiltration. The lung injury score in the de-escalation group was lower than that in the escalation group. In the liver and intestine samples, we compared the results of the histological and immunohistochemical analyses of citH3 and apoptosis (Fig 6A-C). The injury, injury scores, and apoptosis levels were closely associated with citH3 in the liver and intestine samples of those groups. The de-escalation group had milder liver and intestine injuries and lower NETs than the escalation group. Finally, the survival rates were calculated in the four groups. Compared with the control, antibiotic administration was more effective, but de-escalation therapy in the CLP mice did not lead to significantly higher survival than escalation therapy (p=0.051, Fig 6D).

All the data suggested that de-escalation antibiotic therapy positively regulates immune function by increasing NET formation to maximize the capacity to kill bacteria during the early stage of sepsis and negatively regulates immune function by decreasing NET formation to minimize the associated overwhelming and excessive tissue injury during the late stage of sepsis.

DISCUSSION

Past clinical experience has shown that patients with sepsis benefit from de-escalation antibiotic therapy, but the mechanism has not been explored in depth. This study is the first time to elaborate the mechanism of DE therapy from animal experiments. Our results show that different β-Lactams have different effects on NETs formation both*ex vivo* and *in vivo* , and we demonstrated that de-escalation antibiotic therapy, including the early use of NET-promoting antibiotics and the late use of NET-inhibiting antibiotics, could fully exploit the immunomodulatory effects of NET formation during the different stages of sepsis. More importantly, these results underline the important role of NETs directed initial empirical antibiotic therapy in the infection of polymicrobial sepsis *in vivo* .

Similar with previous studies, our data emphasize the critical antibacterial role of NETs in the early stage and the deleterious role of NETs in the late stage of sepsis [17, 19]. Neutrophils serve as the first line

of defense in the innate immune system against bacterial pathogens. The release of intracellular contents, which is called neutrophil extracellular traps, is critical for killing bacteria in infection and sepsis [3]. Current studies have shown that NETs and their elements can cause organ injury, coagulation disorders, autoimmune diseases, and thromboembolism [20]. Although NETs are a double-edged sword in sepsis, few studies have focused on their antibacterial function in the early stage of sepsis, during which NETs can trap pathogens and prevent bacterial dissemination [17, 18, 21]. Our data show clearing NETs with DNaseI will lead to the spread of infection and increased organ damage, these results prove that NETs formation playing an important role in organ protection during the early stage of sepsis.

We demonstrated, for the first time, that in addition to macrolide and quinolone drugs, β-lactam antibiotics can also modulate the formation of NETs *in vivo* and *in vitro* . Previous studies have proven that some antibiotics may exert immunomodulatory functions, and with the discovery of NETs, more antibiotics have been found to affect the immune system [22-24]. However, no studies have shown immunomodulatory functions of β-lactam antibiotics, and some studies have shown a lack of immunomodulatory function of β-lactam antibiotics. This study has proven that different subgroups of β-lactam antibiotics have distinct effects on NET formation and that imipenem and ceftriaxone regulate NETs in a ROS-dependent manner. ROS generation is a well-recognized step in NET formation, and many studies have shown that NET-associated diseases may be alleviated by the usage of ROS scavengers or inhibitors [25]. The β-lactam antibiotic-induced NET regulation observed in this study could be inhibited by NOX inhibitors or promoted by exogenous ROS, which confirmed the involvement of ROS.

De-escalation therapy has been shown to be associated with reduced mortality in patients with severe sepsis and septic shock and has been shown to be effective and safe; however, there is no adequate, direct evidence or high-quality RCT studies to prove these claims, and the necessary studies to elucidate the mechanism of de-escalation therapy have not been conducted [26]. Although there is no consensus when the ridge line of early and late stage of sepsis is, it is acknowledged that the pathogen confirmed in 24-72h after sepsis diagnose. Thus, in our study we defined that 3 days were the early stage in according to the unknown pathogen period and discover whether the initial empiric antibiotic act differently. We confirmed that although it cannot improve the 6-day mortality of CLP mice in our study, de-escalation therapy can reduce organ injury and inflammatory responses, which is consistent with observations in human patients. According to the guidelines of sepsis treatment, antibiotics were administered 1 h after the CLP procedure and de-escalated. Escalation therapy was used under the guidance of empirical antibiotic usage in our study. We showed better reductions in the AST, ALT, serum creatine, and cytokine levels (IL-6, IL-10, TNF-α, IFN-γ, MIP-2 and MCP-1) in both the early and late stages of sepsis.

Based on the recovery of the function of β-lactam antibiotics in the regulation of NETs, we then explored the mechanism of de-escalation therapy. In our study, we found that the combination of different antibiotics affects the serum levels of MPO-DNA, which is a biomarker of NETs. With the sequential treatment with NET-promoting and NET-inhibiting antibiotics, NET formation could be regulated, ultimately maximizing the function of NETs and reducing their damage. In the experiment with imipenem-ceftriaxone de-escalation therapy, NET formation was higher in the early stage and lower in the late stage, which was accompanied by milder organ injury and inflammatory responses. In addition, the administration of DNase I with de-escalation therapy reduced the formation of NETs, but organ injury and inflammatory responses were exacerbated. To confirm the role of NETs, we conducted histological and immunohistochemical staining, and with greater infiltration of citH3, which is a credible NET biomarker, increased injury to organs and enhanced apoptosis of tissue cells were observed. It seems, therefore, that in addition to the useful antibacterial function of antibiotics, the function of de-escalation therapy in the regulation of NETs is more important in sepsis; this regulatory role enhances the antibacterial function of innate immunity and prevents further NET-associated damage in sepsis. Although there was no significant difference in survival between the DE group and the ES group due to sample size. Meanwhile, several therapeutic strategies such as anti-infection, nutrition and organ function support are employed in clinical treatment of sepsis, and antibiotic treatment alone does not reflect the overall picture of sepsis treatment. However, organ damage and the levels of inflammatory cytokines in the DE group were significantly decreased compared to the ES group.

These results show the benefits of de-escalation antibiotic therapy strategies in sepsis.

However, there are some limitations. Polymicrobial sepsis model was used and we did not examine the bacterial loads and differentiate bacterial infiltration. It is important to detect the antibacterial spectrum, which takes about 24-72h to confirm in clinical practice. The initial administration of different antibiotics may shift bacterial colonization with a certain resistance to NET-dependent killing and influence the real outcome. Besides, bacterial colonization changes may also affect homeostasis in intestine which contribute to the injury of intestine. Those speculative assumption need to be addressed in future studies. Lastly, we assessed the 6-day mortality in the DE group which was shorter compared with the real clinical setting. Extended observation time may be expected.

CONCLUSION

In this study, we firstly demonstrated that de-escalation therapy played a novel immunomodulatory effect during sepsis, which was depended on the NET formation. The early use of NET-promoting antibiotics (maximizing the capacity of capturing bacteria) in the early stage, followed by NET-inhibiting antibiotics in the late stage (preventing NET-associated organ injury), may be a reasonable treatment algorithm for the treatment of sepsis. Our study may shed lights on immunotherapy treatment in sepsis that involves the detection of NETs and usher the treatment of sepsis into the era of precision medicine and individualized therapy.

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AUTHOR CONTRIBUTION

Tian Xie : Conceptualization, Methodology, Software, Formal analysis, Writing - original draft. **Chengnan Chu** : Conceptualization, Methodology, Software, Formal analysis.**Shilong Sun** : Methodology, Software. **Xinyu Wang** : Methodology. **Zehua Duan** : Methodology. **Weiwei Ding** : Project administration, Conceptualization, Writing - review & editing.**Jieshou Li:** Project administration, Conceptualization.

CONFLICTS OF INTEREST

The authors declare that they have no competing interests related to this manuscript.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request

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FIGURE LEGENDS

Figure 1. The effects of antibiotics on ROS generation and NETs formation. Human peripheral neutrophils were pre-incubated with antibiotics or PBS for 2 hours and stimulated to form NETs with or without PMA (100nM) for 3 hours. **A.** Kinetics of NETs releasing in PMA-stimulated neutrophils was recorded (n=3).**B** . NETs release in activated (with PMA) or resting (antibiotics alone) neutrophils was measured using Sytox Green fluorescence plate reader assay at 3 hour and expression as ratio of percentage of total DNA (n=3). Both antibiotics group showed effects on the NETs formation only in activated neutrophils. **C.** To further confirm the existence of NETs, neutrophils were pre-incubated with antibiotics or PBS for 2 hours and stimulated to form NETs with PMA (100nM) for 3 hours. DNA (blue) stained with DAPI, MPO (green) stained with anti-MPO antibody and cit-H3 (red) stained with anti-cit-H3 were counterstained in neutrophils for immunofluorescence. DNA stained with Sytox Green alone in neutrophils for immunofluorescence to show the morphology of NETs. **D** . Neutrophils were pre-incubated with β-Lactams (2 mM) for 2 hour and then treated with or without PMA. Intracellular ROS were measured with DHR123 at 45 min (n=3). **E** . Neutrophils pre-cultivated with DPI, a NADPH oxidase inhibitor, were incubated with β-Lactams for 2 hour and then activated with PMA. The ROS production was measured using DHR123 fluorescence plate reader assay at time of 45min (n=3). **F** . To confirm the role of ROS, exogenous H_2O_2(30μM) was added after 2-hour incubating with β-Lactams. NETosis was measured using Sytox Green fluorescence plate reader assay at time of 3 hour and expression as relative fluorescence unit (n=3). Images are representative of three independent experiments. Bars were shown in the figure; data were analyzed by Student's t test. *P<0.05, ** P<0.01, ***P<0.001, ns, no significance.

Figure 2. De-escalation strategy exerted a diverse effect on the serum MPO-DNA at different stages of sepsis. A . At the early stage of sepsis (Day 3), blood was drawn from different groups (n=25), and serum MPO-DNA levels were determined with mouse MPO ELISA kit. DE group showed higher serum MPO-DNA levels than ES group, and both were lower that control group (p<0.001). Higher MPO-DNA levels were diminished with the administration of DNaseI (p<0.001). **B** . At the late stage of sepsis (Day 6), serum MPO-DNA levels were determined the same way (n=20). ES group showed higher levels of serum MPO-DNA than DE group (p<0.01), and both were higher than sham group (p<0.001).

Figure 3. De-escalation strategy caused less organ injuries and inflammatory response attenuated by DNaseI in early sepsis stage. A-B . The serum AST and Creatine were determined by ELISA (n=25). Reduced levels of AST and Creatine were shown in DE compared with ES group (p<0.01) , however, this effect was reversed by NETs scavenger, DNaseI (p<0.001). **C-F** . The serum levels of IL-6,

IFN-γ, IL-10 and MCP-1 were determined using Luminex ® xMAP (n=25). The inflammatory cytokines levels were lower in DE group than that in ES group ($p<0.01$), except for the level of MCP-1 ($p<0.001$). When DNaseI was administrated, all were elevated compared with DE group ($p<0.05$).

Figure 4. De-escalation strategy alleviates organ injuriesin the late sepsis stage. A-D. The serum levels of AST, creatine, ALT and LDH were measured with ELISA kit in all groups (n=17). The levels blood chemistries were lower in DE group than those in ES group ($p<0.01$). However, those were extremely higher in control group though there was 2 of 10 left at day 6.

Figure 5. De-escalation strategy allays inflammatory response in the late sepsis stage. A-F. The serum levels of MIP-2, MCP-1, TNF-α, IL-10, IL-6 and IFN-γ were measured using Luminex ® xMAP (n=17). The inflammatory cytokines levels were lower in DE group than that in ES group ($p<0.01$), which was coincident with that in early sepsis stage. And in control group (n=5), those biomarkers were extremely higher than those in antibiotic groups.

Figure 6. Morphologic changes in liver, intestine and lung tissues in late sepsis stage and survival rates in different groups (day #6). The image A-C are representative of liver, intestine and lungs from sham, DE and ES group. Mice in control group did not shown due to severe necrosis. **A.** The same liver tissues in the same mice were exanimated by HE and ICH in each group. Mice in sepsis groups showed more leukocyte infiltration and hydropic degeneration in liver tissues. And DE treatment presented alleviated pathological changes than ES treatment. And poor pathological morphologic changes were consistent with more positive citH3 and apoptotic cells in liver tissues. Stars indicate leukocyte cells infiltration. Solid triangles indicate hydropic degeneration. Arrows indicate positive cells in ICH. **B.**Intestine tissues collected in each group were treated with HE or ICH examination. Mice suffered from sepsis showed severe inflammatory cells infiltration and microvilli damage. And severer intestine injuries were correlative to more positive citH3 and apoptotic cells in intestine tissues. Stars indicate leukocyte cells infiltration. Triangles indicate microvilli damage. Arrows indicate positive cells in ICH. **C.** CLP procedure induced inflammatory cells infiltration comparing normal tissue, and DE showed less pathological changes than ES group. Stars indicate inflammatory cells infiltration. **D** . Survival rates of CLP sepsis mice treated with DE or ES comparing with control group at day 6 after CLP operation. Although there was no significant difference between DE and ES group ($p=0.051$), DE has the greatest survival benefits compared with the control group. DE, de-escalation group. ES, escalation group. N=10 per experimental group and N=5 in sham group.