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Research article

Differential neutrophil responses in murine following intraperitoneal injections of *Escherichia coli* and *Staphylococcus aureus*

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ABSTRACT

Objective: This study aimed to investigate the proportion of neutrophils among leukocytes, in various tissues following intraperitoneal injection of *Escherichia coli (E. coli)* and *Staphylococcus aureus (S. aureus)* in mice.

Methods: Twelve specific-pathogen free (SPF) male mice, aged eight weeks, were segregated into three groups, each containing four mice. Two of these groups were subjected to intraperitoneal injections of *E. coli* and *S. aureus*, both in high concentrations, to establish mouse models of inflammation. The remaining group, which received an intraperitoneal injection of phosphate buffered saline (PBS), served as the control group. Observe the mice every half hour. Then mice were anesthetized, and samples from peripheral blood, liver, and brain tissues were carefully collected nearing death. These samples underwent a digestion process to produce single-cell suspensions. Subsequently, these suspensions were stained with fluorescent antibodies targeting CD45, Ly6G, and CD11b. A flow cytometric analyzer was then employed to enumerate and compare the neutrophil alterations across each group (Fig. 1).

Results: The results indicated a significant variation in the ratio of $CD11b^+$ $Ly6G^+$ neutrophils to $CD45^+$ leukocytes among the groups. In peripheral blood, the control group showed a neutrophil proportion of approximately 1.44 %, while the *E. coli* and *S. aureus* groups exhibited increased proportions of 6.53 % and 3.82 %, respectively. In liver tissue, a marked elevation was observed

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in the experimental groups, with ratios of 19.20 % and 20.40 % for *E. coli* and *S. aureus*, respectively, compared to 1.64 % in the control. In brain tissue, the increments were more modest but noticeable, with the experimental groups showing 2.40 % and 1.11 % in contrast to 0.13 % in the control group.

Conclusions: These findings suggest neutrophils are involved in the response after intraperitoneal injection of *E. coli* and *S. aureus*, with marked differences in neutrophil responses in different tissues. This study enhances our understanding of the acute inflammatory response to bacterial infection.

1. Introduction

Escherichia coli (E. coli) and *Staphylococcus aureus (S. aureus)* are widely recognized as significant causes of bacterial infections, triggering various immune responses. *E. coli*, a gram-negative bacterium, is known for causing infections in the urinary tract and other parts of the body, while *S. aureus*, a gram-positive bacterium, is often associated with skin infections and can lead to more severe conditions [1–3]. Both *E. coli* and *S. aureus* have strains that are resistant to multiple antibiotics, which poses a significant challenge in clinical settings [4,5]. These antibiotic-resistant strains, like Methicillin-resistant *Staphylococcus aureus* (MRSA) and Extended-spectrum beta-lactamases (ESBL)-producing *E. coli*, complicate treatment and increase the risk of severe outcomes, especially in hospital environments where infections can be more easily spread [6–9]. These pathogens elicit distinct immune responses, after they succeed to adhere and pass the mucosal barrier of the host. Neutrophils, as the first line of defense against pathogens, play a pivotal role in the innate immune response [10–12] Upon infection, neutrophils are rapidly recruited to the site of inflammation, where they perform critical functions such as phagocytosis, degranulation, and the release of neutrophil extracellular traps (NETs) [13,14]. Understanding the distribution and activation of neutrophils during bacterial infections is crucial for comprehending the dynamics of the immune response. However, there is a gap in comprehensive, comparative studies examining neutrophil populations in various tissues following exposure to these bacteria. Flow cytometry stands out as a powerful tool in immunological studies due to its ability to rapidly quantify and characterize cells in various tissues [15–21]. This technique allows for the detailed analysis of cell populations based on size, granularity, and fluorescence intensity, thus providing an in-depth understanding of immune cell dynamics in different physiological and pathological conditions [22,23].

In this study, we aimed to quantitatively assess neutrophil counts in peripheral blood, liver, and brain tissues in a mouse model of sepsis. This model was developed through the intraperitoneal injection of two common bacterial pathogens: *E. coli* and *S. aureus*. Our method involved the use of flow cytometry, a technique that enabled precise measurement of neutrophil levels in these different tissues. By comparing these levels across various tissues, we were able to observe distinct patterns of neutrophil mobilization in response to sepsis induced by different bacteria. This comparative analysis is key to enhancing our understanding of the immune response in bacterial infections. Such insights are crucial as they contribute to a better understanding of the body's defense mechanisms against bacterial sepsis and could inform future research in this area.

2. Materials and methods

2.1. Bacterial preparation

E. coli standard strain (ATCC 25922) and *S. aureus* standard strain (ATCC 25923) were cultured on Luria-Bertani (LB) solid medium and blood agar plates separately. Pick a single colony and inoculate it into a 50 mL centrifuge tube containing 15 mL of LB liquid medium respectively. These cultures were incubated overnight at 37 °C with a shaking speed of 160 rpm for 12 h. Then transfer 10 mL of bacterial suspension from the centrifuge tube to a 15 mL centrifuge tube and centrifuge at 5000 g for 5 min at 4 °C. Carefully discard the supernatant and resuspend the bacterial pellet in PBS by gentle pipetting to achieve an optical density (OD600) of approximately 1.0.

2.2. Animal model

Male C57BL-6 mice, aged 8 weeks, were utilized. The mice were housed at a controlled temperature of 23–26 °C with a 12-h light/dark cycle. Control mice received an intraperitoneal injection of 400 μL PBS. For experimental groups, mice were administered 400 μL of *E. coli* (concentration: 1×10^{10} CFU) and *S. aureus* (concentration: 1×10^{10} CFU) respectively. Observations were conducted every half hour, noting behavioral changes, physical appearance, and time of death. This study has been approved by the Research Ethics Committee of Yuebei People's Hospital, Shantou University Medical College, Shaoguan, China.

2.3. Sample collection and handling

Mice in the experimental groups were processed upon morbidity, while those in the control were processed in the sixth hour. Anesthesia was administered using isoflurane, followed by blood collection via eyeball puncture using a 2 mL heparinized syringe. Post blood collection, mice were disinfected with alcohol, and thoracic and abdominal cavities were dissected to extract the liver, lung,

brain, and spleen. Excised tissues were perfused with PBS containing 2 % fetal bovine serum (FBS) and minced using a surgical blade. Tissue fragments were further dissociated in tissue dissociation solution at 37 °C for 30 min, with intermittent agitation. Cell resuspended in PBS with 2 % FBS. Mouse blood was centrifuged at 400 g for 5 min, plasma discarded, and cells resuspended in diluent. A gradient centrifugation using mouse mononuclear cell separation solution was performed to isolate peripheral blood mononuclear cells (PBMCs). Cells were subjected to red blood cell lysis, followed by washing and resuspension in PBS with 2 % FBS. Trypan blue staining was employed to assess cell viability and count cell suspensions from each organ.

2.4. Flow cytometry

Cell suspensions were washed twice with PBS and resuspended in flow cytometry staining buffer. Cells were incubated for 30 min at 4 °C in the dark with the following Fixable Viability Stain 510 for cell viability assessment, and a panel of fluorochrome-conjugated antibodies including Fixable Viability Stain 510, APC-Cy™7 Rat Anti-Mouse CD45, BV421 anti-mouse Ly6G, and CD11b FITC M1/70 for specific cell marker identification. Leukocyte were first gated based on forward scatter (FSC) and side scatter (SSC) properties. $CD11b^+$ $Ly6G^+$ Neutrophils populations were identified within the $CD45^+$ gate. Data analysis was performed using FCS 3.0 software (Tree Star). Statistical analyses were performed using GraphPad Prism.

3. Results

3.1. Rapid and lethal outcomes in mice following exposure to E. coli and S. aureus

The results revealed significant differences in the health outcomes of the mice across the control and experimental groups. In the control group, the mice exhibited normal behavior and remained asymptomatic throughout the duration of the experiment. This group served as a baseline for comparison with the experimental groups. In contrast, the mice in the experimental groups, which were exposed to *E. coli* and *S. aureus*, exhibited severe health outcomes. In the *E. coli* group, the mice met a tragic end within a remarkably short duration of 5–5.5 h post-infection, underscoring the lethal potency of this bacterial pathogen. In a similar vein, the mice in the *S. aureus* group succumbed within a slightly extended timeframe of 5.5–6 h post-infection, further highlighting the deadly nature of these bacteria. As the experiment progressed, the mice in the experimental group began to exhibit severe symptoms that ominously signaled their impending demise. A noticeable change in their behavior was evident as they displayed signs of depression, a stark

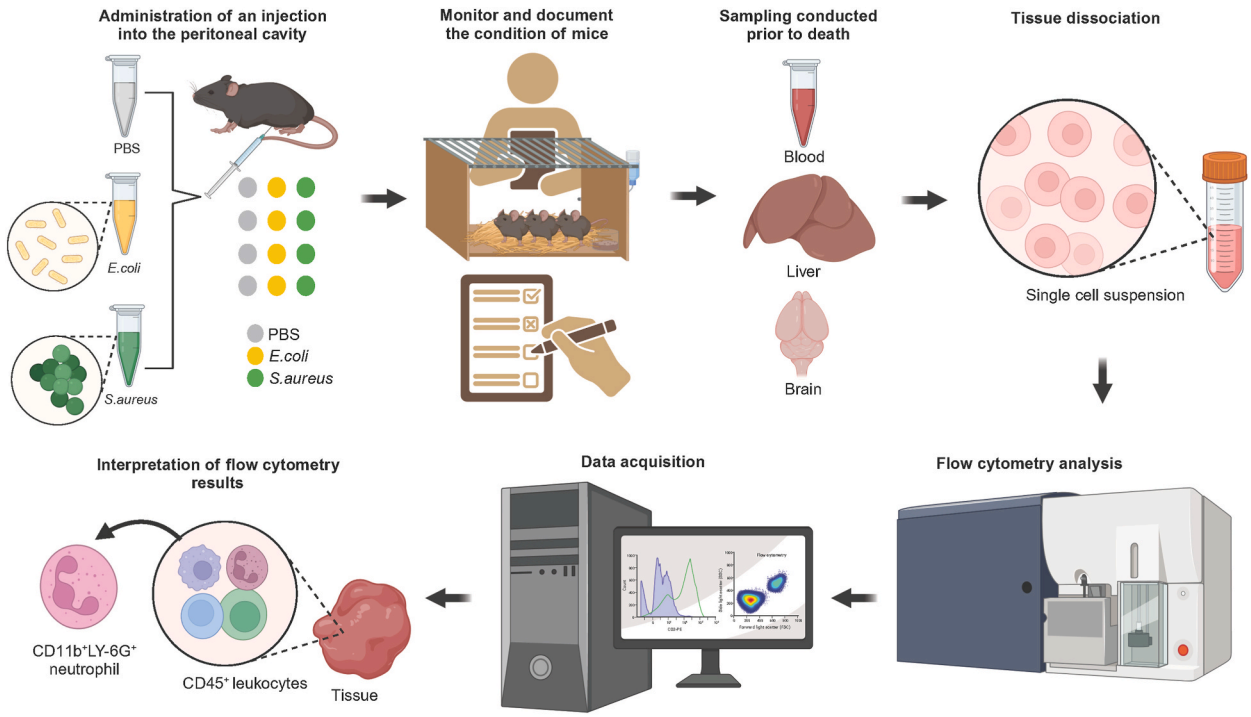

Fig. 1. Schematic diagram of experimental process. Twelve mice were divided into three groups (n = 4 per group). Each group received an intraperitoneal injection of PBS, *E. coli*, or *S. aureus* solution, separately. Following the injections, peripheral blood, liver, and brain tissues were collected from each mouse. These collected samples were then processed to prepare single cell suspensions. The single-cell suspensions were then subjected to a flow cytometry assay. Finally, the data from the flow cytometry assay were obtained and interpreted to understand the effects of the different injections on the mice.

contrast to their usual active nature. Their appetite dwindled, further exacerbating their weakened state. A small amount of purulent discharge was observed from their eyes, a clear indication of the infection's toll on their bodies. Most alarmingly, upon examination, there were signs of congestion and bruising of the internal organs, painting a grim picture of the internal damage inflicted by the infection (see Fig. 1).

3.2. Neutrophil response in peripheral blood to E. coli and S. aureus infections

The study unveiled a striking diversity in the distribution of $CD11b^+$ $Ly6G^+$ neutrophils to $CD45^+$ leukocytes in the peripheral blood among different test subjects. Within the control ensemble, the neutrophil to leukocyte ratio was a modest 1.44 %. In stark contrast, the *E. coli* -infected cohort witnessed a remarkable surge in this ratio, reaching 6.53 %, while the *S. aureus*-infected group saw a moderate elevation to 3.82 % (Fig. 2). These observations are in harmony with prior scholarly discourse, underscoring an intensified neutrophil response in the wake of bacterial infections [24–26]. Interestingly, the elevated counts of $CD11b^+$ $Ly6G^+$ neutrophils in the peripheral blood serve as a hallmark of the body's reaction to pulmonary cryptococcus infections and exposure to lipopolysaccharide (LPS) [27,28].

3.3. Elevated neutrophil proportions in liver tissue post E. coli and S. aureus inoculation

Delving into liver tissue analysis, the control group showed a neutrophil to leukocyte ratio of 1.64 %. However, a striking escalation was noted in both *E. coli* (19.20 %) and *S. aureus* (20.40 %) infected groups, suggesting a pronounced hepatic neutrophilic response to bacterial incursion (Fig. 3). This trend of heightened neutrophil proportions in liver tissues post *E. coli* and *S. aureus* inoculation aligns well with the liver's pivotal role as an immunological sentinel, filtering bloodborne pathogens [29,30].

3.4. Enhanced neutrophil infiltration in brain tissue in response to E. coli and S. aureus

Turning to brain tissues, the control group manifested a negligible neutrophil presence at 0.13 %. However, the presence of *E. coli* and *S. aureus* elevated these ratios to 2.40 % and 1.11 %, respectively (Fig. 4). This finding resonates with the existing mosaic of scientific inquiry, which consistently observes a marked increase in neutrophil infiltration following bacterial invasions in the brain [31], painting a complex picture of the body's multifaceted response to bacterial threats.

4. Discussion

Both *E. coli* and *S. aureus* are known to cause infections. Intraperitoneal injections of bacteria like *E. coli* and *S. aureus* can pose several dangers, primarily due to the potential for causing severe infections and triggering a systemic immune response [32]. When injected intraperitoneally, they can lead to peritonitis (inflammation of the peritoneum, the lining of the abdominal cavity) [33,34]. This condition can be severe and may progress to sepsis, a life-threatening response to infection that can lead to tissue damage, organ failure, and death [35–37]. Neutrophils, a pivotal type of white blood cell, stand at the forefront of the body's intricate defense mechanism against bacterial invasions. These cells, remarkable for their rapid response and potent antimicrobial actions, are key players in the immune system's arsenal [38]. Neutrophils may respond differently depending on the tissue or organ in which the

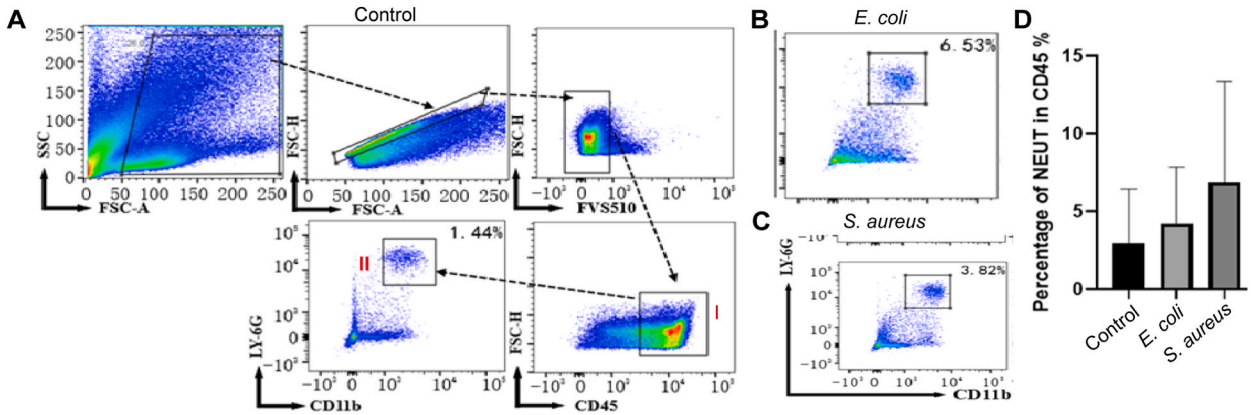

Fig. 2. Neutrophil proportions in peripheral blood. (A) Representative example of the supervised gating and analysis strategy of flow cytometry data. Arrows describe the hierarchical sequences of analysis (*i.e.* gating strategy). Identified cell subsets: $CD45^+$ leukocytes (I), $CD11b^+$ $Ly6G^+$ neutrophils (II) of peripheral blood in control group. Two cell subsets are identified and highlighted with a black frame. (B) Pseudo-color plots of $Ly6G^+$ vs $CD11b^+$ expression of peripheral blood in *E. coli* group. (C) Pseudo-color plots of $Ly6G^+$ vs $CD11b^+$ expression of peripheral blood in *S. aureus* group. (D) Percentages of the $CD11b^+$ $Ly6G^+$ neutrophils. Data represent the means ± SD from three independent experiments. Student's t-test was performed using GraphPad Prism 8.0 software to calculated *P*-value. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. Control.

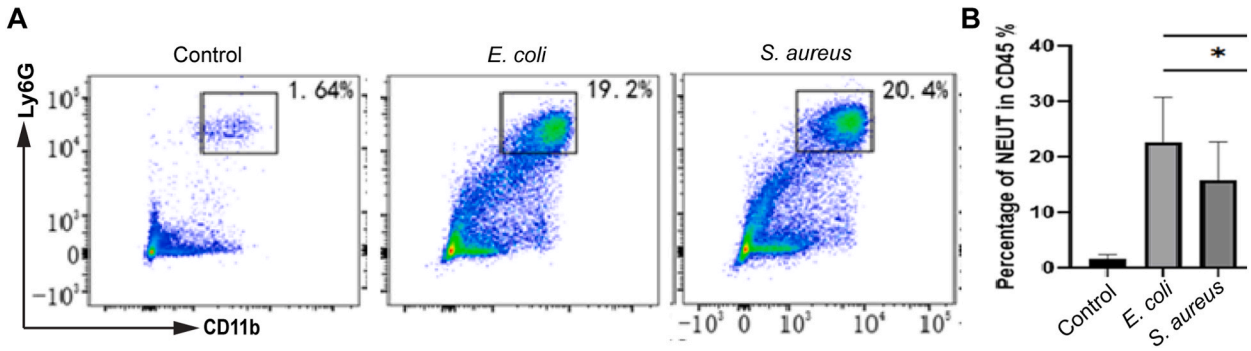

Fig. 3. Liver tissue neutrophil proportions. (A) Representative flow cytometry results for $CD11b^+$ $Ly6G^+$ neutrophils after infection with PBS, *E. coli* or *S. aureus*. (B) Percentages of the $CD11b^+$ $Ly6G^+$ neutrophils. Data represent the means ± SD from three independent experiments. Student's t-test was performed using GraphPad Prism 8.0 software to calculated *P*-value. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. Control.

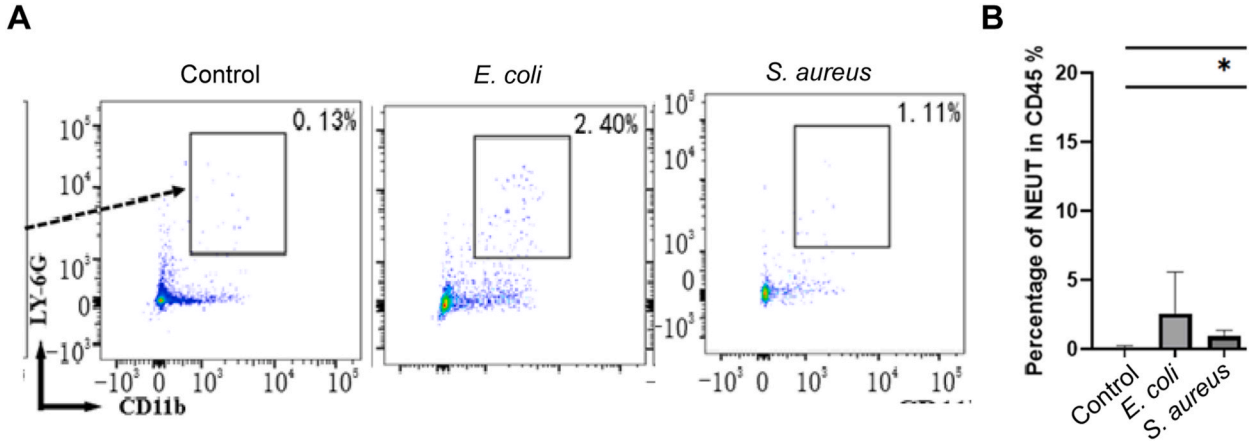

Fig. 4. Brain tissue neutrophil proportions. (A) Representative flow cytometry results for $CD11b^+$ $Ly6G^+$ neutrophils after infection with PBS, *E. coli* or *S. aureus*. (B) Percentages of the $CD11b^+$ $Ly6G^+$ neutrophils. Data represent the means ± SD from four independent experiments. Student's t-test was performed using GraphPad Prism 8.0 software to calculated *P*-value. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. Control.

infection occurs. Different tissues and organs have unique microenvironments that may affect neutrophil behavior, such as recruitment, activation, and effectiveness in combating pathogens [39,40]. In conditions like sepsis, where an infection in one organ can spiral into a full-blown systemic inflammatory response [41]. It's a puzzle where each piece – each organ's response – influences the outcome of the disease. Thus, understanding the tissue-specific roles of neutrophils is vital. In the present study, the number of hepatic neutrophils was significantly increased before the death of the mice suggesting that neutrophils may exert an excessive pro-inflammatory effect, leading to hepatic injury, which is consistent with previous reports [42,43]. Neutrophils present various dysfunctions at the late stage of sepsis, including of apoptosis, seriously damaged chemotaxis, and extensive infiltration into the tissues. Neutrophils are a key part of the body's defense against infection, but they can also contribute to tissue damage and organ dysfunction during sepsis [44]. Excessive neutrophil recruitment contributes to tissue damage due to their arsenal of molecular weapons that do not distinguish between host and pathogen [45]. The antibacterial arsenal of neutrophils can contribute to tissue damage and the development of organ dysfunction during sepsis. This is due to the release of effector molecules such as reactive oxygen species, myeloperoxidase, and neutrophil extracellular traps. These traps are produced to contain and kill invading pathogens, but can paradoxically promote further tissue damage [46,47]. This is a key aspect of the pathogenesis of sepsis. Several studies have elucidated the specific mechanisms by which neutrophils contribute to organ dysfunction in sepsis. Işeri demonstrated that oxytocin protects against sepsis-induced multiple organ damage by modulating neutrophil activity [48]. Building on this foundation, Martin et al. identified phosphoinositide-3 kinase gamma (PI3Kγ) as a pivotal molecule in sepsis pathogenesis and organ damage, thereby suggesting it as a therapeutic target [49]. Further supporting this line of investigation, research has highlighted the essential role of the chemokine receptor CCR2 in driving neutrophil infiltration and tissue damage in remote organs during sepsis [50]. Continuing this exploration, studies have revealed that antioxidant treatment can reverse organ failure in a rat sepsis model by restoring the balance of antioxidant enzymes, reducing neutrophil infiltration, and alleviating oxidative stress [51]. Additionally, contributions have shown that neutrophil extracellular traps (NETs) exacerbate organ dysfunction through tissue damage [52]. Expanding on this, research has demonstrated that NETs promote disseminated intravascular coagulation in sepsis, leading to microvascular hypoperfusion and

end-organ damage [53]. Moreover, investigations have explored the role of thrombopoietin (TPO) in organ damage during endotoxemia and sepsis, finding that blocking TPO reduces organ damage in experimental models [54]. A novel development involves the creation of a NET-inhibiting therapeutic antibody targeting citrullinated histones, designed for the treatment of autoimmune diseases characterized by aberrant NET formation [55]. Further, studies have examined the impact of Nbeal2 deficiency on organ damage and host defense during gram-negative pneumonia-derived sepsis, underscoring the complex interplay between neutrophils and platelets [56]. Additionally, research has delved into the role of hepatocyte AMPKα1 in sepsis, revealing that hepatocyte-specific deletion of AMPKα1 exacerbates outcomes in septic mice, with sex-specific effects on organ injury and neutrophil infiltration [57]. Overall, these studies collectively underscore the critical role of neutrophils in sepsis-induced organ damage and identify several potential therapeutic targets for mitigating the detrimental effects of neutrophil activation in multiorgan dysfunction associated with sepsis. On the other hand, during sepsis, neutrophils can become impaired, leading to a condition known as "neutrophil paralysis". This impairment can prevent neutrophils from effectively migrating to the site of infection, resulting in the host's inability to contain and eliminate the infection [58]. Furthermore, as sepsis, neutrophil gene expression is altered, leading to suppression of proinflammatory and immunomodulatory genes, as well as decreased production of reactive oxygen species [59]. There was also a single-cell RNA sequencing that found a previously unknown immunosuppressive subset of neutrophils as inhibitory neutrophil in sepsis [60]. In brief, in the context of sepsis, the role of neutrophils is complex and paradoxical.

E. coli is commonly used to replicate gram-negative sepsis, whereas *S. aureus* represents another common, gram-positive human pathogen [61,62]. In this experiment, there was no significant difference in the increase in neutrophils between the *E. coli* and *S. aureus* groups. But some differences of the pathogenic mechanisms of sepsis caused by gram-negative and gram-positive bacteria and the response of neutrophils can be observed. One study found that the serum concentrations of C-reactive protein (CRP), procalcitonin, and tumor necrosis factor-alpha (TNF-α) in the group infected with gram-negative bacteria were significantly higher than those in the group infected with gram-positive bacteria. However, there was no significant difference in interleukin-6 (IL-6) and white blood cell (WBC) count between the two groups [63]. Furthermore, gene expression profiles in neutrophils can also differ in sepsis caused by gram-negative and gram-positive bacteria. These differences in gene expression can further contribute to the dysregulated immune response observed in sepsis [64]. The interplay between neutrophils and bacteria, particularly *E. coli* and *S. aureus*, has garnered significant attention in immunological research. Studies have revealed distinct mechanisms in how neutrophils respond to these two pathogens. For instance, Ramamoorthy et al. demonstrated that blocking CD18 adhesion impaired bacterial clearance and neutrophil recruitment following intrapulmonary *E. coli* infection but had no such effect on *S. aureus* infection [65]. This finding underscores variations in the immune response to *E. coli* and *S. aureus*. Similarly, Karzai et al. observed contrasting effects of G-CSF during *E. coli* and *S. aureus* pneumonia in rats, further illustrating the differential host responses to these bacteria [66,67]. Research on lactating bovine udders infected with *E. coli* or *S. aureus* has also shown that E. coli-infected animals exhibit higher levels of complement fragment C5a and cytokines in milk compared to those infected with *S. aureus*, indicating a more pronounced inflammatory reaction in the former [68]. Moreover, studies examining organism-specific neutrophil-endothelial cell interactions have found variations in polymorphonuclear neutrophil (PMN) migration across an endothelial monolayer in response to *E. coli*, Streptococcus pneumoniae, and *S. aureus* [69]. Additionally, research on *Staphylococcus aureus* cardiolipin synthases has highlighted differences in lipid metabolism between *E. coli* and *S. aureus* [70]. The role of olfactomedin 4 in modulating neutrophil killing of *S. aureus* and *E. coli* has also been investigated, revealing the involvement of regulatory molecules in the bactericidal capabilities of neutrophils [71]. Collectively, these studies highlight the necessity of understanding the nuanced interactions between neutrophils and *E. coli* versus *S. aureus* to better comprehend the differences in immune responses and pathogenesis.

In conclusion, the role of neutrophils in sepsis is multifaceted and contradictory. On one hand, the activation of neutrophils, can be excessive. And this overzealous response can lead to tissue damage. On the other hand, neutrophils can also become impaired during sepsis, leading to "neutrophil paralysis", which hinders their ability to effectively combat infection. Besides, this finding indicates a similar neutrophilic response despite the type of bacteria involved. In essence, the study reveals that while neutrophils play a critical role in the progression and severity of sepsis, their functions and impacts are highly complex and can vary depending on the type of underlying bacterial infection.

CRediT authorship contribution statement

Yanyan Zhu: Writing – original draft, Validation, Software, Resources, Methodology, Investigation, Formal analysis, Data curation. **Jingya Luo:** Writing – original draft, Visualization, Software, Resources, Methodology. **Xianzhu Xia:** Writing – review & editing, Supervision, Resources, Project administration, Investigation. **Hao Feng:** Writing – review & editing, Supervision, Software, Resources, Project administration, Methodology, Investigation, Formal analysis. **Pingsen Zhao:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Availability of data and materials

The data sets used and analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

This study has been approved by the Clinical Research Ethics Committee of Yuebei People's Hospital Affiliated to Shantou

University Medical College (registration number: SUMC2021-332).

Consent for publication

Not applicable.

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Declaration of competing interest

All authors declare that there are no potential conflicts of interest. All authors have reviewed and approved the manuscript and are willing to attest to their qualifications as authors, disclose potential conflicts of interest, and release copyright should the manuscript be accepted for publication.

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None.

Abbreviations

CD11b	Cluster of differentiation molecule 11b
CD45	Cluster of differentiation molecule 45
E. coli	*Escherichia coli*
ESBL	Extended-spectrum beta-lactamases
FBS	Fetal bovine serum
FSC	Forward scatter
IL-6	Interleukin-6
LB medium	Luria-Bertani medium
LPS	Lipopolysaccharide
Ly6G	Lymphocyte antigen 6 complex, locus G
MRSA	Methicillin-resistant *Staphylococcus aureus*
NETs	Neutrophil extracellular traps
OD	Optical density
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
S. aureus	*Staphylococcus aureus*
SPF	Specific-pathogen free
SSC	Side scatter
TNF-α	Tumor necrosis factor-alpha
WBC	White blood cell

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