

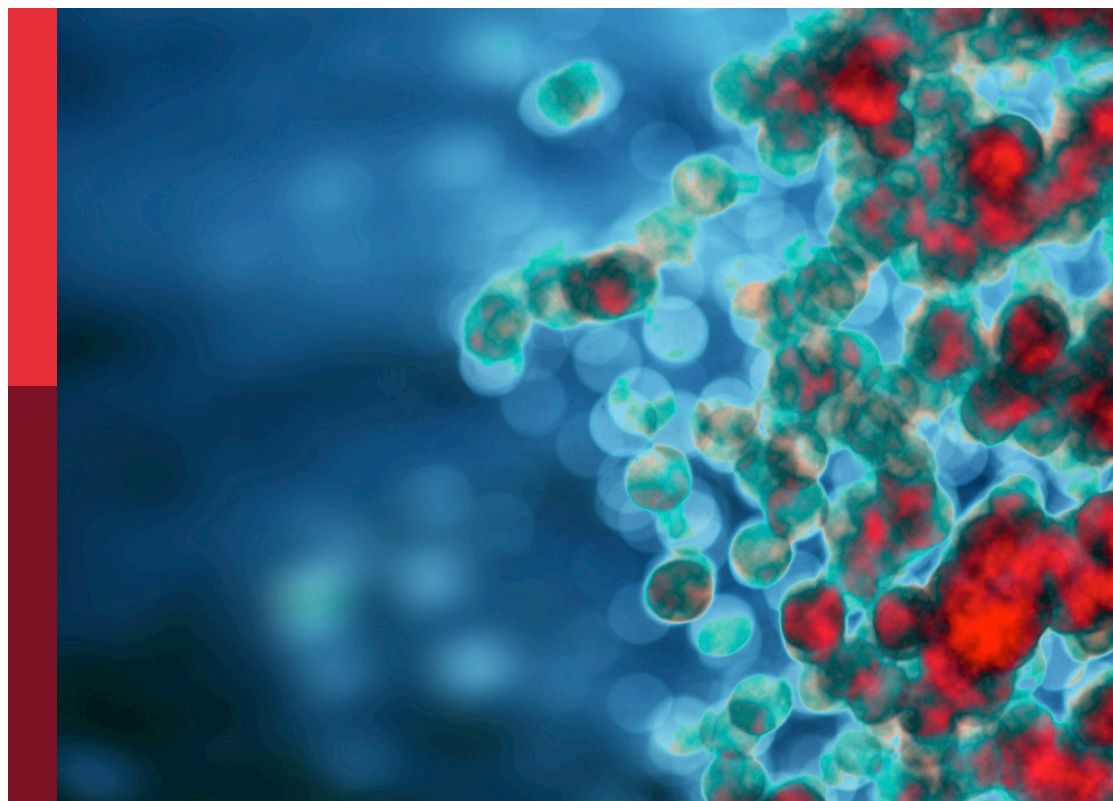
Role of endogenous regulators of innate immunity in sepsis

Edited by

Haichao Wang, Xianzhong Meng, Daolin Tang and
Yong Ming Yao

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Role of endogenous regulators of innate immunity in sepsis

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Editorial: Role of endogenous regulators of innate immunity in sepsis

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hemichannel, extracellular vesicle, serum amyloid A, TREM2, IL-8, glycogenin-1,
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Editorial on the Research Topic

Role of endogenous regulators of innate immunity in sepsis

Sepsis, a life-threatening syndrome arising from a dysregulated host response to infection, remains a leading cause of mortality and long-term morbidity worldwide. The profound immune dysfunction characterizing sepsis—oscillating between hyperinflammation and immunosuppression—underscores the critical need to unravel the intricate network of endogenous regulators that govern innate immunity during this complex condition. This Research Topic in *Frontiers in Immunology* presents a collection of seven research and five review articles that significantly advance our understanding of these endogenous mechanisms, offering new insights into sepsis pathophysiology, novel diagnostic biomarkers, and promising therapeutic avenues.

Orchestrating cellular responses: channels, vesicles, and alarmins

Several contributions highlight the critical role of endogenous molecular entities in orchestrating cellular communication and shaping the innate immune response. [Li et al.](#) reviewed Connexin 43 and Pannexin 1 hemichannels in macrophages, revealing how bacterial lipopolysaccharide (LPS) and serum amyloid A (SAA) upregulate these channels, leading to ATP efflux. This efflux intensifies inflammasome activation, pyroptosis, and the release of pathogenic damage-associated molecular patterns (DAMPs) like high mobility group box 1 (HMGB1), thereby fueling the inflammatory cascade. Crucially, the authors demonstrate that mimetic peptides targeting these hemichannels can modulate their activity and impact sepsis-induced lethality, positioning them as significant therapeutic targets.

Expanding on intercellular communication, [You et al.](#) reviewed the pathogenic and therapeutic roles of extracellular vesicles (EVs) in sepsis. EVs, released by both host cells and bacteria, act as vital paracrine components, delivering bioactive materials that can

either promote inflammatory responses or serve as tools for therapeutic cargo delivery. This dual role underscores their intricate involvement in both disease progression and tissue repair, suggesting engineered EVs as a novel strategy for diagnosis and targeted intervention. This concept is further elaborated by [Jiao et al.](#), who focused on tubular epithelial cell (TEC)-derived EVs carrying SAA1. This study uncovers a novel mechanism where these EVs exacerbate sepsis-associated acute kidney injury (SA-AKI) by promoting neutrophil extracellular trap (NET) formation via the TLR4/p38 MAPK pathway. Importantly, targeting EV secretion or SAA1 within TECs alleviates both renal and remote lung injury, indicating kidney-lung crosstalk and highlighting TEC-EVs/SAA1 as a potential prognostic index for SA-AKI.

The versatile protein SAA emerges as a recurrent endogenous regulator across the topic. [Mohanty et al.](#) delved into the specific roles of SAA proteins in sterile and infectious diseases, demonstrating their dual functionality. SAA amplifies cytokine and chemokine responses during sterile inflammation (where SAA-deficient mice showed better survival) but is essential for bacterial clearance in infectious conditions (where SAA-deficient mice were more susceptible). This intricate balance is mediated via NF- κ B signaling, highlighting SAA as a key modulator whose context-dependent actions are critical. The explicit link between SAA and hemichannels, and SAA1 carried by TEC-EVs, illustrates the complex interplay and functional convergence of these endogenous mediators.

Decoding immune cell dynamics and dysfunction

The heterogeneity of immune cell responses and their impact on patient outcomes is a central theme. [Chen et al.](#) identified a specific subset of CD10-CD121b⁺ neutrophils in septic shock patients that correlated with severity and predicted immunosuppression. In these immunosuppressive neutrophils, CD121b blockade led to increased proinflammatory cytokine production. This identifies CD121b as both a predictive indicator and a therapeutic target, particularly as its expression is amenable to down-regulation by all-trans retinoic acid (ATRA).

In the context of specific organ injury, [Shen et al.](#) reviewed the TREM2 signaling pathway in sepsis-induced acute lung injury (ALI). TREM2, expressed predominantly on myeloid cells, plays a pivotal role in modulating inflammation. Understanding its functions and mechanisms in both physiological and septic lung injury scenarios, along with evaluating TREM2-targeted therapies, is crucial for addressing this severe septic complication. Complementing this, [Wang et al.](#) focused on lymphocyte subsets as predictors for SA-AKI. This study meticulously identified significant alterations in peripheral blood lymphocyte subsets – including various T cell activation/exhaustion markers and myeloid-derived suppressor cells (MDSCs) – that predicted the incidence and severity of SA-AKI. The developed predictive model, incorporating these immunological parameters with clinical data, offers a robust tool for early warning and clinical decision-making.

The search for precise diagnostic tools is also addressed. [Herminghaus et al.](#) investigated the diagnostic utility of IL-18 plasma levels in distinguishing abdominal from non-abdominal sepsis. While several inflammatory cytokines were elevated in both sepsis types, IL-18 showed moderate predictive accuracy, particularly for identifying non-abdominal sepsis when its level was below a certain threshold. It suggests IL-18 as a useful additional biomarker, highlighting the importance of understanding specific inflammatory signatures based on infection source.

Metabolic and neuro-immune axes in sepsis

Beyond direct immune cell interactions, systemic physiological changes significantly impact innate immunity. The comprehensive analysis of metabolism-related genes (MRGs) in sepsis by [Zheng et al.](#) revealed critical metabolic-immune heterogeneity. By stratifying patients based on MRGs, this multi-omics study identified a high metabolic risk group characterized by a neutrophil-dominant and lymphocyte-suppressed immune landscape. Glycogenin-1 (GYG1) emerged as a key hub gene, highly expressed in monocytes and neutrophils, and its knockdown significantly improved survival in a murine sepsis model. This positions GYG1 as a metabolic driver of innate immune hyperactivation and a promising therapeutic target.

The often-overlooked neuro-immune axis receives attention from the review paper by [Wang et al.](#), which highlights dopamine as an endogenous regulator of innate immunity in sepsis. Traditionally known for its role in the central nervous system, dopamine is shown to significantly influence the immune response, specifically regulating aconitate decarboxylase 1 (ACOD1). This mini-review underscores the critical, yet under-explored, interplay between the nervous and immune systems in sepsis.

Furthermore, [Guo et al.](#) explored the role of scavenger receptor class B type I (SR-BI) in sepsis, linking glucocorticoid (GC) biology to precision steroid therapy. This perspective review sheds light on how SR-BI mediates the adrenal stress response, which is crucial for controlling inflammation. By understanding SR-BI's mechanisms, the authors advocate for a precision medicine approach to GC therapy, reserving it for septic patients with adrenal insufficiency rather than broad application.

Harnessing host defense for therapeutic gains

Finally, the topic introduces potential therapeutic strategies derived from endogenous host defense mechanisms. [Chen et al.](#) presented pro-dermcidin and its derivatives as potential therapeutics for lethal experimental sepsis. While not directly bactericidal, pro-dermcidin and derivatives protected against sepsis by reducing inflammation, decreasing bacterial counts, and activating autophagy and phagosome maturation. This highlights a

novel approach using host-derived peptides to modulate immune responses and improve outcomes.

Interconnections, synthesis, and future directions

Collectively, the papers within this special topic paint a picture of sepsis as a highly dynamic and heterogeneous syndrome, driven by a complex interplay of endogenous regulators. A key recurring theme is the dual nature of many of these regulators – molecules like SAA, EVs, and hemichannels can be both beneficial and detrimental depending on the context, timing, and concentration. The metabolic-immune axis, exemplified by GYG1, and the neuro-immune axis, highlighted by dopamine, represent crucial systemic influences that modulate innate immune responses. The emphasis on specific immune cell subsets (CD121b⁺ neutrophils, lymphocyte populations in SA-AKI) and organ-specific complications (ALI, AKI) underscores the importance of a nuanced understanding of immune dysfunction beyond a generic “inflammatory response”.

The clear connections between studies are particularly exciting: SAA’s dual role is further contextualized by its ability to upregulate hemichannels and its packaging into TEC-derived EVs to drive organ injury. EVs, generally explored in the review paper by [You et al.](#), gain specific pathogenic function through SAA1 in the context of AKI (3). The metabolic underpinnings of immune cell behavior likely intersect with the activation states and phenotypes described for neutrophils and lymphocytes.

Looking ahead, these investigations pave the way for several critical future directions. First, continued integration of multi-omics data with single-cell analyses will be essential to map the precise regulatory networks governing sepsis heterogeneity and to develop personalized diagnostic as well as prognostic tools. Second, translating these findings into precision therapies requires careful consideration of the context-dependent roles of endogenous regulators. Targeting hemichannels, engineered EVs, or metabolic drivers like GYG1 offers exciting possibilities, but their therapeutic applications will likely benefit from patient stratification based on identified biomarkers (e.g., CD121b, IL-18, lymphocyte subsets, metabolic risk scores). Third, further exploration of less-understood axes, such as the neuro-immune system (dopamine), will undoubtedly uncover additional endogenous regulators with

therapeutic potential. Finally, moving these promising preclinical findings into robust clinical trials will be the ultimate test of their impact on improving patient outcomes in sepsis. This Research Topic represents a significant step forward in our quest to tame the dysregulated host response in sepsis by understanding and strategically modulating its endogenous regulators.

Author contributions

HW: Writing – original draft. XM: Writing – review & editing. DT: Writing – review & editing. YY: Writing – review & editing.

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Connexin 43 and Pannexin 1 hemichannels as endogenous regulators of innate immunity in sepsis

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Sepsis is a life-threatening organ dysfunction resulting from a dysregulated host response to infections that is initiated by the body's innate immune system. Nearly a decade ago, we discovered that bacterial lipopolysaccharide (LPS) and serum amyloid A (SAA) upregulated Connexin 43 (Cx43) and Pannexin 1 (Panx1) hemichannels in macrophages. When overexpressed, these hemichannels contribute to sepsis pathogenesis by promoting ATP efflux, which intensifies the double-stranded RNA-activated protein kinase R (PKR)-dependent inflammasome activation, pyroptosis, and the release of pathogenic damage-associated molecular pattern (DAMP) molecules, such as HMGB1. Mimetic peptides targeting specific regions of Cx43 and Panx1 can distinctly modulate hemichannel activity *in vitro*, and diversely impact sepsis-induced lethality *in vivo*. Along with extensive supporting evidence from others, we now propose that hemichannel molecules play critical roles as endogenous regulators of innate immunity in sepsis.

KEYWORDS

Connexin 43, Pannexin 1, hemichannel, ATP, HMGB1, inflammasome, mimetic peptide, innate immune cells

Introduction

Microbial infections and resultant sepsis account for nearly 20% of deaths worldwide (1), and annually cost over \$60 billion in healthcare in the U.S. alone. Despite the urgent need, effective therapies remain elusive, as setbacks in sepsis clinical trials have dampened enthusiasm for both preclinical investigation of complex sepsis pathophysiology and clinical development of innovative sepsis therapies. However, prior pre-clinical studies of pathogenic cytokines (e.g., TNF) in inflammatory diseases (2) have led to the Nobel Prize-Winning anti-TNF therapies for patients with rheumatoid arthritis (3, 4), inspiring an improved understanding of the complex pathogenic mechanism of sepsis. As a multifactorial

disorder, sepsis is initiated by the innate immune system, the body's first line of defense against microbial infections (5, 6).

Monocytes, originating from hematopoietic stem cells in the bone marrow, circulate continuously in the bloodstream to search for invading pathogens or damaged tissues. Upon detecting pathogen- or damage-associated molecular pattern molecules (PAMPs or DAMPs), they infiltrate affected tissues and differentiate into resident macrophages (7, 8). Macrophages and monocytes are equipped with various pattern recognition receptors (PRR) such as the Toll-like receptor 4 (TLR4) and TLR9 (9–11) to detect PAMPs, including bacterial lipopolysaccharides (LPS) and CpG-DNA (12, 13). For instance, upon encountering small amounts of LPS, an LPS-binding protein (LBP) (14) aids in its delivery to a co-receptor, cluster of differentiation 14 (CD14) (15), which then presents it to TLR4 (10). This interaction rapidly initiates the release of “early” proinflammatory cytokines such as tumor necrosis factor (TNF) (2), interleukin-1 β (IL-1 β) (16) and interferon- γ (IFN- γ) (17). While early cytokine responses can be protective against microbial infections (18–21), excessive cytokine production (e.g., TNF) can contribute to the pathogenesis of sepsis (22) and septic shock (2). Despite attempts to neutralize TNF in clinical settings, therapeutic windows for early cytokines are narrow, motivating the search for “late” mediators that may provide broader therapeutic opportunities in treating lethal infections. In 1999, we discovered that high mobility group box 1 (HMGB1) was released by endotoxin-stimulated macrophages and monocytes, and functioned as a late mediator of lethal endotoxemia (23). In animal models of lethal sepsis induced by cecal ligation and puncture (CLP), circulating HMGB1 levels peaked between 24–36

hours (24). Pharmacological inhibition of HMGB1 with neutralizing antibodies conferred protection against lethal sepsis, even when administered 24 hours post-CLP (24, 25), establishing HMGB1 as a “late” mediator of sepsis with a broader therapeutic window (26–30).

Role of inflammasome activation in the regulation of HMGB1 release

Lacking a signal sequence, HMGB1 cannot be secreted through the classical Endoplasmic Reticulum (ER) - Golgi exocytotic pathways (23). Instead, inflammatory stimuli promoted HMGB1 acetylation (31), lactylation (31) or phosphorylation (32, 33), facilitating its translocation to cytoplasmic vesicles and secretion through non-classical exosomal secretory pathways (31, 34–37). In addition, HMGB1 can be passively released via pyroptosis (Figure 1) (38, 39), a programmed necrotic cell death pathway mediated by Casp-1-dependent canonical and Casp-11/4/5-dependent non-canonical inflammasome activation pathways (40).

Accumulative evidence has supported the critical role of inflammasome activation in regulating LPS/ATP-induced HMGB1 release, as genetic disruption of key inflammasome components like Casp-1 or Nalp3 significantly impairs this process (41). Additionally, the double-stranded RNA-activated protein kinase R (PKR) has been identified as a key regulator of inflammasome activation and HMGB1 release (Figure 1) (42). Genetic deletion of PKR expression or pharmacological inhibition of its phosphorylation similarly disrupts inflammasome activation,

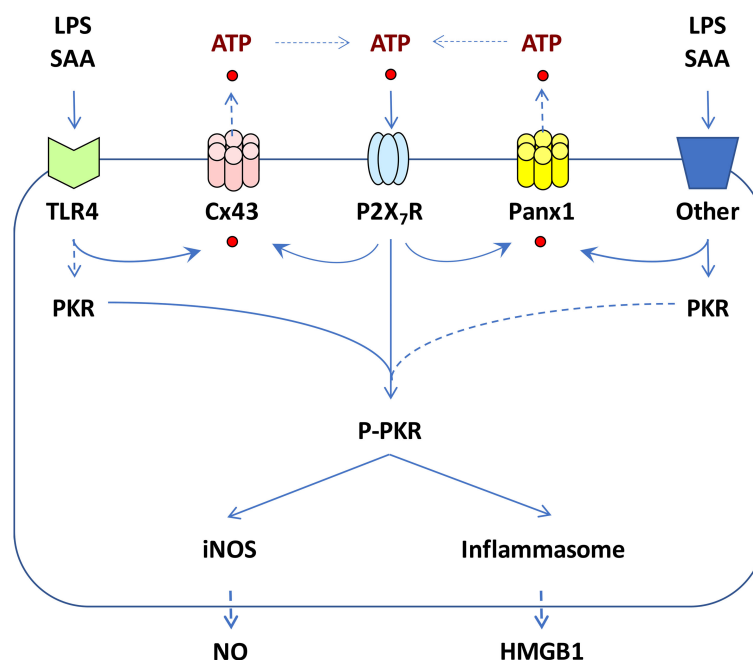


FIGURE 1

Proposed roles of Cx43 and Panx1 hemichannels in regulating ATP release and inflammasome activation. Prolonged exposure to crude LPS or SAA induced upregulation of Cx43 and Panx1 hemichannels, potentially contributing to extracellular ATP efflux, P2X7R-mediated PKR phosphorylation, pyroptosis, and the release of DAMPs such as HMGB1. Concurrently, PKR activation led to increased expression of inducible nitric oxide synthase (iNOS) and parallel production of nitric oxide (NO) in macrophage cultures.

pyroptosis, and HMGB1 release (42, 43). Notably, bacterial toxins are believed to activate inflammasome signaling pathways partly by triggering passive ATP leakage (44). Indeed, ultrapure LPS, free of contaminating bacterial proteins and nucleic acids, can induce early cytokine production (e.g., TNF), but cannot trigger nitric oxide (NO) production and HMGB1 secretion unless the LPS priming is accompanied by a second stimulus, ATP (41, 42, 45, 46). Similarly, while ATP itself can induce PKR phosphorylation (42), it cannot independently trigger the release of HMGB1 or other inflammasome-dependent cytokines (e.g., IL-1 β and IL-18) without prior LPS priming (41, 47–49).

Role of extracellular ATP in the regulation of inflammasome activation

Extracellular ATP is believed to activate the inflammasome by binding to the purinergic P2X7 receptor (P2X7R) (50), which rapidly opens the ATP-gated P2X7R channel, permitting the influx of small cationic ions. This activation subsequently recruits and activates connexin 43 (Cx43) and pannexin 1 (Panx1) hemichannels (Figure 1), allowing the passage of larger anionic molecules up to 900 Da, including ATP (51–53). This ATP-mediated, feed-forward release of ATP amplifies LPS-stimulated inflammasome activation (54) and subsequent release of inflammasome-dependent cytokines (e.g., IL-1 β and IL-18) (47–49, 55).

Our previous work demonstrated that both crude LPS and purified SAA upregulated the expression of Cx43 and Panx1 hemichannels in macrophage cultures (56, 57), and effectively induced PKR-dependent inflammasome activation and HMGB1 release (Figure 1) (56). Connexins and pannexins are transmembrane proteins that form hemichannels on the surface of monocytes and macrophages. When hemichannels from neighboring cells align, they can create a gap junction (GJ), enabling direct exchange of ions, small molecules, and signaling molecules between the cytoplasm of adjacent cells. However, hemichannels also exist in an unpaired state on the cell surface, typically remaining closed in quiescent cells to prevent uncontrolled release of intracellular contents. In response to inflammatory signals, however, these hemichannels can open, allowing ions and small molecules—such as ATP—to pass between the intracellular and extracellular spaces. Among hemichannel proteins, Cx43 and Panx1 are the most extensively studied, especially in macrophages and monocytes, where they play critical roles in regulating immune activation and cytokine production.

In light of our observation that a hemichannel blocker, carbenoxolone (CBX), dose-dependently inhibited both endotoxin- and SAA-induced PKR activation and HMGB1 release (56, 58, 59), we proposed that hemichannel molecules like Cx43 and Panx1 may play important roles in regulating the release of ATP, which intensifies inflammatory responses through PKR-dependent inflammasome activation, pyroptosis, and HMGB1 release (Figure 1). Below we discuss the distinct and shared roles of Cx43 and Panx1 in modulating innate immunity during sepsis, emphasizing their tissue-specific contributions to inflammation and sepsis progression.

Role of Cx43 as an endogenous regulator of innate immunity in sepsis

Cx43, a member of the connexin family, is widely expressed in innate immune cells such as monocytes (60) and macrophages (61). In sepsis, Cx43 forms unpaired hemichannels that enable the release of signaling molecules, such as ATP, into the extracellular space, thereby amplifying innate immune responses.

Up-regulation of Cx43

To investigate the potential role of Cx43 hemichannel in regulating innate immunity, we measured Cx43 expression in wild-type and TLR4-deficient macrophages after prolonged stimulation with crude endotoxins or highly purified human serum amyloid A (SAA) (56). While quiescent macrophages constitutively expressed Cx43 at relative low levels, exposure to crude LPS and SAA resulted in a marked increase in Cx43 expression (by 45–60 folds) in wild-type, but not in the TLR4-deficient macrophages, suggesting that LPS and SAA induced Cx43 expression in a TLR4-dependent fashion (Figure 1) (56). Our findings were consistent with previous studies showing that bacterial endotoxins and proinflammatory cytokines (e.g., TNF and IFN- γ) up-regulated Cx43 expression in macrophages/monocytes (60, 62) and Kupffer cells (63). Given the expression of Cx43 in Kupffer cells (63)—the liver's resident macrophages that play a crucial role in mediating hepatic immune responses—it is plausible that Cx43 may also play a significant role in regulating inflammasome activation in these cells. Indeed, inflammatory stimuli such as LPS or heat stroke have been shown to induce NLRP3-dependent inflammasome activation in Kupffer cells, thereby contributing to inflammatory hepatic injury (63–67). Recently, these findings are further supported by studies showing that LPS increased Cx43 expression (68, 69), stimulated ATP release (68), increased NLRP3 inflammasome activation (70), and contributed to septic lethality (68). In light of the notion that diabetes and hyperglycemia are frequently linked to poorer outcomes in experimental (71, 72) and clinical (73–75) sepsis, high glucose levels have also been shown to amplify cytokine-induced Cx43 expression, ATP release, and NLRP3 activation (76).

In vivo, endotoxin increases Cx43 expression in the lungs and kidneys (77), but transiently decrease it in the heart (78) (Table 1). The mechanisms underlying the tissue-specific regulation of Cx43 expression by endotoxins are not yet understood and remain an intriguing avenue for future investigation. Additionally, Cx43 was found to be upregulated in macrophage cultures by endotoxins and cytokines (56, 60, 62) or septic insults in animals or patients with experimental or clinical peritonitis (68). Conversely, conditional knockout of Cx43 specifically in macrophages reduce LPS-induced ATP release and inflammation in animal models of lethal endotoxemia and CLP sepsis, resulting in improved survival rates (68). Furthermore, genetic deletion or pharmacological inhibition of Cx43 conferred protection against CLP-induced intestinal (79) and lung injury (80), as well as against high-fat diet (HFD)-induced

TABLE 1 Divergent regulation of Cx43 and Panx1 expression in sepsis.

Cells or Tissues	Cx43	Panx1
Macrophages/Monocytes	↑ by LPS, SAA, TNF, and IFN- γ	↑ by LPS, SAA, TNF, and IFN- γ
Lung	↑ by LPS and sepsis	↓ by sepsis
Kidney	↑ by LPS and sepsis	↓ by sepsis
Heart	↓ by LPS and sepsis	↑ by sepsis

downward arrow: decrease; upward arrow: increase.

inflammasome activation and inflammation (81), chronic colitis (82), and tubular inflammatory injury (83). Collectively, these findings underscore the compartmentalized regulation of Cx43 during sepsis and suggest that selectively inhibiting Cx43 hemichannels may serve as a potential therapeutic strategy to reduce inflammation in specific tissues.

Role of Cx43 in the regulation of ATP release

Almost two decades ago, it was proposed that Cx43 hemichannels play a crucial role in facilitating the release of ATP (84), which acts as a pro-inflammatory signal by activating purinergic receptors on adjacent immune cells. In innate immune cells such as macrophages, Cx43 exists predominantly in the form of hemichannels that connect cell interior to extracellular milieu (44, 85, 86). Conditional knockout of Cx43 in lung macrophages led to a significant reduction in ATP release and a diminished calcium response to bleomycin-induced injury (61). Therefore, macrophage Cx43 hemichannel-mediated ATP release is essential for inflammasome activation, and the release of DAMPs (e.g., HMGB1, Figure 1) or other inflammasome-dependent cytokines (such as IL-1 β and IL-18).

Divergent effects of Cx43 mimetic peptides on hemichannel activation and sepsis outcomes

Cx43 carries four transmembrane domains that encompass two extracellular loops (ELs), one intracellular cytoplasmic loop, and the intracellular N- and C-termini (Figure 2A) (87). The cytoplasmic membrane-bound Cx43 can oligomerize to form hexameric hemichannels (Figure 2B) (88), which can dock with hemichannels on adjacent cells to create gap junction (GJ) channels, particularly in non-immune cells of the heart, brain, and vasculature, facilitating intercellular communication (89, 90). Since innate immune cells do not form GJ with one another (86), the upregulation of Cx43 expression may lead to increased hemichannel activity. To explore this, we assessed changes in hemichannel activity using Cx43 mimetic peptides, including GAP26, which corresponds to sequence of the first extracellular loop (EL1) of Cx43 (Figure 2A) (91), and GAP19 (Figure 2A), derived from a nine-residue segment of the cytoplasmic loop of

Cx43 (92). Under calcium-depleted conditions, LPS significantly increased Cx43 hemichannel activity, which was distinctly influenced by pre-incubation with GAP26 and TAT-GAP19 (56). Consequently, these two different Cx43 mimetic peptides, GAP26 and TAT-GAP19, had different effects on LPS-induced hemichannel activation *in vitro*, and varied impacts on the outcomes of lethal sepsis *in vivo* (56).

To further investigate the potential role of Cx43 hemichannels in sepsis, we synthesized a series of hexamer peptides overlapping with the first extracellular loop (EL1) of Cx43 and screened them for their ability to modulate macrophage hemichannel activities. Among these, the cysteine-containing peptide P5 (ENVCYD, Figure 2A) significantly inhibited LPS-induced increase in hemichannel activity, as evidenced by a marked reduction in cellular lucifer yellow dye uptake (56). Additionally, the P5 peptide significantly diminished LPS-induced ATP release and trypan blue uptake, indicating its effectiveness in impairing the LPS-mediated elevation of hemichannel activity, cell death and HMGB1 release (56). It is thus possible that crude LPS- and SAA-induced Cx43 upregulation and hemichannel activation may contribute to the efflux of ATP, which activates the purinergic P2X7R to mediate PKR phosphorylation and HMGB1 secretion (Figure 1). Furthermore, mimetic peptides such as GAP26 and P5 (ENVCYD) may hold potential as therapeutic agents for the clinical treatment of human sepsis (56).

Role of Cx43 in innate immune cell communication with non-immune cells

Cx43 hexameric hemichannels can dock with the hemichannels on adjacent cells to form GJ channels to facilitate intercellular communications in various tissues such as the heart, brain, and vasculature (85, 89, 90). Under inflammatory conditions, macrophages form Cx43-containing GJ channel with cardiomyocytes (93), epithelial cells (94), and endothelial cells (62), suggesting an exciting possibility that innate immune cells may communicate with non-immune cells through Cx43-containing GJ channels to orchestrate inflammatory responses. For instance, a subset of alveolar macrophages form Cx43-containing gap junctions with the lung epithelial cells, allowing them to communicate during LPS-induced inflammation (94). This intercommunication resulted in a reduction of neutrophil recruitment and cytokine secretion, thereby serving as a novel immunomodulatory mechanism. Similarly, macrophage and lymphocytes can both express Cx43 and can potentially form GJ channels between innate and adaptive immune cells (95), suggesting a possible role of GJ-mediated communication in immune regulation. Additionally, Cx43 has been implicated in the formation of tunneling nanotubes (96, 97), long membranous channels that can transfer organelles and signaling molecules between immune cells to facilitate intercellular communication over long distances. The Cx43-mediated transfer of cellular contents is especially important during viral infections, as rapid cellular communication is essential for mounting effective inflammatory responses.

A Amino acid sequence and structural domains of human Cx43 and Panx1.

MGDWSALGKLLDKVQAYSTAGGKVLVSVLFIIFRILLGTAVE**SAWGEQSAFRCNTQQPGC**^{"P5"}**ENVCYDKSFPISHVR**^{"GAP26"}FWVLQIIFVSVPTL
 "Extracellular Loop 1" (EL1)
 LYLAHVYFVMRKEEKLNKKEEELKVAQTDGVNVDML**KQIEIKKFKY**^{"GAP19"}GIEEHGKVKMRGGLRITYIISILFKSIFEVAFLLIQWYIYGFS
 "Cytoplasmic Loop"
 LSAV**YTCRDPCHQVDCFLSRPTEKTIF**IIFMLVSVSLALNI IELFYVFFKGVKDRVKGKSDPYHATSGALS PAKDCGSQKYAYFNG
 "Extracellular Loop 2" (EL2)
 CSSPTAPLSFMSPPGYKLTGDRNNSCRNYNKQASEQNWANYSAEQNRMGQAGSTISNSHAQPFDFPDNDQNSKKLAAGHELQPLAIVD
 QRPSSRASSRASSRPPDDEI (Human Cx43)

MAIAHLATEYVFSDFLLKEPTEPKFGLRLELAVDKMVTICAVGLPLLISLAFQAEISIGT**QISCFSPSSFSWRQA**^{"10Panx"}**AFVDSY**^{"GAP26"}CWAAVQQ
 "Extracellular Loop 1" (EL1)
 KNSLQSESGNLPLWLHKFFPYILLFALLYLPLFW**RFAAAPHICSDLKFIME**ELDKVYNRAIKAASARDLMDRGACSVPGVTENLG
 "Cytoplasmic Loop"
 QSLWEVSESHFKYPIVEQYLTKKNSNLIKYISCRLLTLIIILLACIYLGYYFSLSSLSDEFVCSIKSGILRNDSTVPDQFQCKLIAV
 "Extracellular Loop 2" (EL2)
 GIFQLLSVINLVVYVLLAPVVVYTLFVFPFRQKTDVLKVEILEFTFDVLHFKSEGYNDLSYLNLFLEENISEVKSCKLVLENIKSSGGQ
 IDPMLLLTNLGMKMDVVDGKTPMSAEMREEQGNQTAELQGMNIDSETKANNGEKNARQRLDSSC (human Panx1)

B Structure of human Cx43 and Panx1 hemichannels.

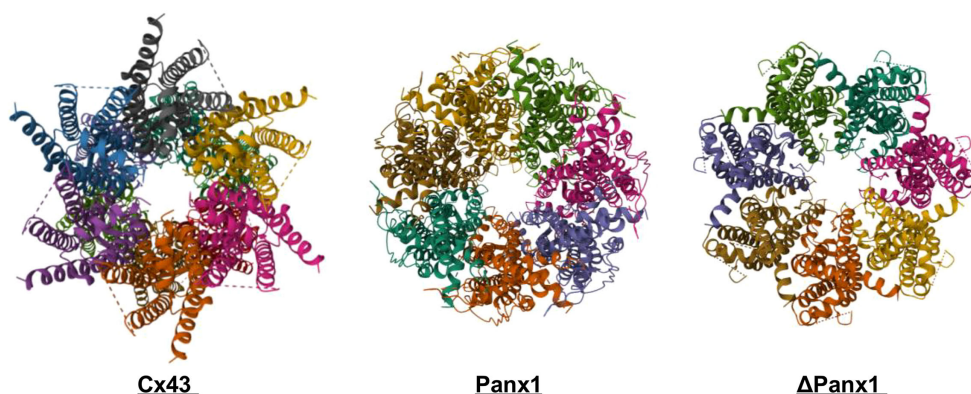


FIGURE 2

Amino acid sequence and structural domains of human Cx43 and Panx1. (A) highlights various mimetic peptides that correspond to specific regions within the extracellular or cytoplasmic loops of Cx43 or Panx1 protein. (B) presents the three-dimensional structures of Cx43 and Panx1 hemichannels, including the Panx1 variant lacking the C-terminal tail (Δ Panx1).

Role of Pannexin 1 as an endogenous regulator of innate immunity in sepsis

Pannexins, originally identified in flatworms as innexin-like membrane channels, also form hexameric hemichannels in vertebrates that similarly enable ATP release (98–100). The Pannexin (Panx) family comprises three members: Panx1, which is widely expressed across diverse tissues; Panx2, primarily restricted to the central nervous system (CNS); and Panx3, predominantly found in skin and skeletal tissues (101).

Regulation of Panx1 expression and processing

Panx1 is constitutively expressed in quiescent monocytes and macrophages typically at low levels. Exposure to crude LPS or purified SAA markedly elevated Panx1 protein levels in macrophages and monocytes, evidenced by increased Panx1-specific punctate staining in the cytoplasm (57). This finding aligns with prior reports that Panx1 hemichannel expression in microglia was upregulated by proinflammatory cytokines such as

TNF, IL-1, and IFN- γ (101). Under non-inflammatory conditions, Panx1 hemichannels remain closed, with the C-terminal tail physically plugging the hemichannel pore from the cytoplasmic side. Upon exposure to inflammatory stimuli, caspase activation can result in the cleavage of the C-terminal tail (102, 103), leading to gradual opening of Panx1 hemichannels and the release of larger molecules like ATP (104, 105). This extracellular ATP acts as an essential danger and “find me” signal (104), attracting innate immune cells to sites of tissue injury or infection, and binding P2X7 receptor to trigger inflammasome activation (51, 103), pyroptosis (106), and the release of HMGB1 (107) and other inflammasome-dependent cytokines such as IL-1 β and IL-18 (108–110). Thus, the Panx1-mediated ATP release also plays an important role in promoting innate immune cell chemotaxis (111), inflammasome activation (112), pyroptosis (103, 113), and the inflammatory response (Figure 1) (114–116).

Additionally, we observed that both crude LPS and human SAA induced Panx1 release in macrophage cultures (57). This extracellular release of Panx1 was accompanied by the appearance of a lower molecular weight band (12 kDa), detected by a monoclonal antibody specific to the C-terminus of Panx1, suggesting it as a possible degradation product of Panx1. This finding aligns with subsequent report that LPS triggered Casp-11-mediated cleavage of the Panx1 hemichannel in macrophage cultures (103).

While Cx43 and Panx1 share roles in mediating ATP release and promoting inflammation, they display distinct regulatory mechanisms and tissue-specific expression patterns during sepsis. As aforementioned, endotoxin has been shown to reduce Cx43 expression in the heart (78), while increasing it in the kidney and lung (77). In contrast, Panx1 mRNA expression was markedly upregulated in the heart but downregulated in the kidney, lung, and spleen of septic animals (57) (Table 1). These findings not only confirm the widespread tissue distribution of Panx1, but also suggest that distinct hemichannel proteins, such as Cx43 and Panx1, are differentially regulated during experimental sepsis. The parallel decrease in circulating Panx1 protein levels and its mRNA expression in macrophage-rich organs, such as the lungs and spleen, suggested that these tissues may contribute to systemic Panx1 accumulation under normal conditions (57).

Role of Panx1 in the regulation of ATP release

Almost two decades ago, Panx1 was proposed as a signaling molecule essential for Casp-1 processing (117), inflammasome activation, and consequent release of ATP (118) and inflammasome-dependent cytokines (e.g. IL-1 β) (51, 109). Subsequently, Panx1 was also identified as a channel for intracellular delivery of muramyl dipeptide, a small molecular component of bacterial peptidoglycan, to trigger Casp-1- and NLRP3-dependent inflammasome activation (119). Beyond inflammasome activation, Panx1 channels also contribute to innate immune cell apoptosis and the release of apoptotic metabolites as signaling molecules (120). For example, Panx1

hemichannels facilitate ATP release from apoptotic cells, guiding macrophages to clear apoptotic bodies (120). This release is triggered by caspase-mediated cleavage of the C-terminal inhibitory domain (102, 120), propelled via an autocrine loop of P2X7 receptor activation (51, 103), and further amplified by Panx1 hemichannel opening (121).

Role of Panx1 in non-immune cells

Panx1 hemichannels have also been implicated in inflammasome activation in non-immune cells including airway epithelial cells (122), neurons (112), astrocytes (112), and endothelial cells (107). In endothelial cells, Panx1 similarly serves as a channel for ATP release, which activates macrophages through P2X7 receptors, promoting the release of IL-1 β and HMGB1 (107). Recently, an Panx1-mediated communication circuit between epithelial cells and macrophages has also been proposed as crucial for promoting epithelial regeneration after injury (123). Suppressing Panx1 expression in epithelial cells with lentivirus-mediated shRNA (122) significantly reduced hypotonic stress-induced ATP release, supporting an important role of Panx1 hemichannels in mediating ATP release in non-immune cells.

Roles of Panx1 in sepsis and other inflammatory diseases

In animal models of sepsis, Panx1 expression exhibits tissue-specific regulation, with upregulation observed in certain tissues like the heart and downregulation in others, such as the lung and spleen (57) (Table 1). This differential regulation of Panx1 expression suggests its tissue-dependent roles in sepsis and other inflammatory diseases. For example, Panx1 has been shown to exacerbate the inflammatory response in animal models of sepsis by promoting the excessive release of ATP, thereby enhancing innate immune cell recruitment and activation. Inhibition of Panx1 conferred protection against sepsis-associated encephalopathy and mortality (113, 124). However, genetic disruption of Panx1 expression rendered animals resistant to lethal endotoxemia (103), but impaired inflammation-mediated microbial clearance (109) and ultimately worsened the outcome of lethal sepsis (125). Similarly, liver-specific knockout of Panx1 also worsened the outcome of lethal endotoxemia (126), suggesting that Panx1 may still be needed for mounting potentially protective innate immune responses against lethal bacterial infections.

The 10Panx, a synthetic peptide that mimics a sequence (WRQAAFVDSY) in the first extracellular loop of Panx1 (Figure 2A), is known to inhibit Panx1 hemichannel-mediated ATP release and inflammasome activation in microglia (127) and neurons (51, 128). The administration of 10Panx has been shown to reduce inflammasome activation and attenuate the severity of inflammation in animal models of brain ischemic injury (129), liver toxemia (130, 131), neuropathic pain (132), and epilepsy (133). However, we observed that 10Panx consistently worsened CLP-induced animal lethality, especially when administered at relatively

lower doses (10–50 mg/kg) (57). This detrimental effect of 10Panx was associated with an enhancement of the LPS- or SAA-induced hemichannel activation, ATP release and NO production (57). Our finding aligns with the critical role of ATP in PKR-dependent inflammasome activation (42, 59, 103) and NO synthesis (46, 134–136), enforcing the notion that excessive macrophage Panx1 hemichannel activation may contribute to the pathogenesis of lethal systemic inflammation (116). Our finding was also consistent with a previous report that 10Panx did not inhibit macrophage hemichannel activities at a relatively lower concentration (e.g., 100 μ M) (137).

Clinical applicability of Cx43- and Panx1-targeting mimetic peptides

Macrophage hemichannel molecules, such as Cx43 and Panx1, have emerged as pivotal regulators of ATP release, which amplifies innate immune responses in conditions such as sepsis, rheumatoid arthritis, and other inflammatory diseases. Mimetic peptides targeting these hemichannels represent a promising class of therapeutics, offering the potential to selectively modulate macrophage-mediated inflammation. By mimicking endogenous regulatory motifs, these peptides can inhibit pathological hemichannel opening without broadly suppressing innate immune function. This precision allows for effective treatment of diseases characterized by excessive inflammation, such as sepsis and autoimmune disorders, while preserving essential innate immune response required for host defense. Beyond systemic inflammation, hemichannels are also implicated in a range of other conditions, including ischemia-reperfusion injury and neuroinflammation. Mimetic peptides targeting these channels could thus serve as versatile agents for multiple indications, particularly when combining with existing anti-inflammatory agents (e.g., cytokine inhibitors) that could provide synergistic effects. A significant advantage of mimetic peptides lies in their derivation from endogenous proteins, which reduces the risk of immunogenicity and adverse reactions compared to synthetic drugs. This feature enhances their tolerability and facilitates regulatory approval, making them an attractive option for clinical development.

Challenges in developing mimetic peptides

Despite their promise, the clinical development of mimetic peptides faces several major hurdles. For instance, mimetic peptides often suffer from poor stability *in vivo* due to enzymatic degradation and rapid clearance. Thus, developing stable formulations or introducing structural modifications, such as peptide cyclization or pegylation, is essential to improve their half-life. Additionally, achieving effective delivery to target macrophages in specific tissues remains challenging, particularly for systemic inflammatory diseases where broad biodistribution may dilute their efficacy. While hemichannels are a promising target, Cx43 and Panx1 are ubiquitously expressed in various tissues, including the heart and nervous system. The potentially

non-specific inhibition of these channels could result in unintended side effects, such as impaired cardiac conduction or neuronal signaling. Thus, designing peptides with macrophage-specific activity or achieving tissue-specific delivery is essential to avoid these complications. Furthermore, mimetic peptides often exhibit variable pharmacokinetics, complicating dosing strategies. Identifying the appropriate dosing regimen to achieve sustained therapeutic levels without toxicity is a key challenge, especially for conditions like sepsis, where timing is critical. Additionally, obtaining regulatory approval for peptide-based therapies is inherently complex, as these agents often straddle the boundary between small molecules and biologics, requiring comprehensive preclinical testing to demonstrate their safety, efficacy, and manufacturability. Finally, although mimetic peptides are derived from endogenous proteins, modifications to enhance stability or efficacy could inadvertently increase their immunogenic potential, thereby interfering with the efficacy of mimetic peptide therapy.

Strategies to overcome challenges

To address these challenges, a multidisciplinary approach is needed, such as peptide engineering to incorporate chemical modifications, such as non-natural amino acids or conjugation to delivery vehicles to enhance stability and specificity. Additionally, targeted delivery systems such as nanoparticles, liposomes, or antibody-drug conjugates can be employed to localize peptides to macrophages, thereby minimizing off-target effects and enhancing therapeutic concentration at the disease site. Similarly, combinational therapies of several mimetic peptides as adjuncts to existing treatments (e.g., cytokine inhibitors) can enhance efficacy while mitigating risks. Thus, mimetic peptides targeting macrophage hemichannel molecules hold significant potential as novel therapeutics for inflammatory diseases. Their specificity, versatility, and potential for synergy with existing therapies highlight their clinical applicability. However, challenges related to stability, specificity, and immunogenicity must be addressed to ensure successful translation into clinical practice. Through advancements in peptide engineering, targeted delivery systems, and preclinical validation, these obstacles can be surmounted, paving the way for innovative and effective treatments for inflammatory and immune-mediated diseases.

Conclusions

Cx43 and Panx1 hemichannels play critical roles in regulating innate immunity during sepsis partly by mediating the release of ATP and other inflammatory signals. Although both hemichannels amplify innate immune responses, their contributions are tissue-specific and disease context-dependent. In septic conditions, Cx43 expression increases in the lung and kidney but decreases in the heart, whereas Panx1 expression is elevated in the heart but reduced in the lung and spleen (Table 1). This complex regulation of Cx43 and Panx1 suggests that selective targeting of these hemichannels may offer therapeutic potential for controlling systemic inflammation in

tissue-specific fashion. Various inhibitors of Cx43 and Panx1 have shown promise in modulating inflammation across sepsis and other disease models. However, caution is warranted when using mimetic peptides to influence macrophage hemichannel activities, as effects can vary with dose regimens and experimental conditions. Therefore, additional research is necessary to fully elucidate these hemichannels' multifaceted roles during different phases of sepsis and to develop effective hemichannel-targeted therapies for this devastating condition.

Author contributions

JL: Writing – original draft. LL: Writing – original draft. WC: Writing – review & editing. XQ: Writing – review & editing. CZ: Writing – review & editing. HW: Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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Pathogenic and therapeutic roles of extracellular vesicles in sepsis

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Sepsis is a systemic injury resulting in vascular dysfunction, which can lead to multiple organ dysfunction, even shock and death. Extracellular vesicles (EVs) released by mammalian cells and bacteria have been shown to play important roles in intercellular communication and progression of various diseases. In past decades, the functional role of EVs in sepsis and its complications has been well explored. EVs are one of the paracrine components of cells. By delivering bioactive materials, EVs can promote immune responses, particularly the development of inflammation. In addition, EVs can serve as beneficial tools for delivering therapeutic cargos. In this review, we discuss the dual role of EVs in the progression and treatment of sepsis, exploring their intricate involvement in both inflammation and tissue repair processes. Specifically, the remarkable role of engineered strategies based on EVs in the treatment of sepsis is highlighted. The engineering EVs-mediated drug delivery and release strategies offer broad prospects for the effective treatment of sepsis. EVs-based approaches provide a novel avenue for diagnosing sepsis and offer opportunities for more precise intervention.

KEYWORDS

sepsis, extracellular vesicles, intercellular communication, treatment, engineering strategies

1 Introduction

Sepsis is a medical emergency without a clearly defined treatment method. It is one of the most common causes of death, closely associated with uncontrolled systemic inflammation (1, 2). The first modern definition of sepsis, proposed in 1992, described it as an excessive inflammatory response to infection, identified by the presence of the systemic inflammatory response syndrome (SIRS) (3). SIRS is characterized by two or more abnormalities in temperature, heart rate, respiratory rate, or white blood cell count. The pathobiology of sepsis is characterized by simultaneous inflammation and impaired immune function, along with significant microvascular damage (4). A pathogen is identified in about 60 to 70% of cases (5). The most frequent cause is bacterial infection, either gram-positive or gram-negative, followed by fungal or viral infections. In 2017, there were reported 11 million sepsis-related deaths globally, accounting for 19.7% of all global deaths (6). Over the years, sepsis has remained a highly fatal condition with suboptimal

diagnostic accuracy and limited therapeutic decision-making capabilities. The outcome of the pathobiological process of sepsis is organ damage, frequently leading to multiorgan failure. Sepsis is often accompanied by widespread acute lung injury (ALI), acute kidney injury (AKI), liver injury and myocardial injury, among other life-threatening complications, for which timely and effective pharmacological interventions are still lacking (7–9). Therefore, studying the pathogenesis and intervention methods of sepsis is of great significance for improving the prognosis of sepsis.

Extracellular vesicles (EVs) are small vesicles secreted by various cells, consisting of a double-layered membrane structure (10). According to the recommendation of the International Society for Extracellular Vesicles (ISEV), EVs could be defined into subtypes based on physical characteristics (e.g., size and density), biochemical composition, descriptions of conditions, or cell of origin (11). In previous studies, EVs have been commonly referred to as exosomes, microvesicles, and apoptotic bodies (12). In recent years, new types of EVs, such as retractosomes and migrasomes, have been identified (13, 14). However, their biogenesis and physiological functions remain largely unknown. In this manuscript, we use the names of EVs as they appear in their original articles. The formation and release of different types of EVs are illustrated in the Figure 1. They contain a variety of bioactive components such as proteins, RNA, lipids, etc., and play significant roles in intracellular and intercellular communication (15–17). The research indicates that EVs primarily deliver their cargo to target cells via ligand-receptor interactions, endocytosis, and direct fusion with the plasma membrane (18). Recent studies have shown that EVs are deeply involved in the occurrence and development of sepsis (19). On one hand, EVs secreted between cells under disease conditions promote sepsis-induced inflammatory storms and organ damage by interacting with recipient cells. On the other hand, exogenous EVs can deliver therapeutic molecules to inhibit systemic inflammation and promote tissue function recovery, thus alleviating disease progression.

Abbreviations: EVs, Extracellular vesicles; SIRS, Systemic inflammatory response syndrome; AKI, Acute kidney injury; ALI, Acute lung injury; ISEV, International Society for Extracellular Vesicles; MVBs, Multivesicular bodies; CLP, Cecal ligation and puncture; TNF- α , tumor necrosis factor- α ; IL-6, Interleukin-6; IL-1 β , Interleukin-1 β ; EC-EVs, Endothelial cell-derived EVs; TLR4, Toll-like receptor 4; ROS, Reactive oxygen species; PMNs, Polymorphonuclear neutrophils; NF- κ B, Nuclear factor κ B; RTECs, Renal tubular epithelial cells; HMGB1, High mobility group box protein 1; DAMP, Damage-associated molecular pattern; DGKK, Diacylglycerol kinase kappa; NET, Neutrophil extracellular trap; MSC, Mesenchymal stem cells; HMVECs, Human microvascular endothelial cells; HMGB1, High mobility group box 1; ADSC-EVs, Adipose MSC-derived EVs; BMSC-EVs, Bone marrow MSC-derived EVs; PGE2, Prostaglandin E2; apoVs, Apoptotic vesicles; DIC, Disseminated intravascular coagulation; VCAM1, Vascular cell adhesion molecule 1; ITGA4, Integrin subunit alpha 4; Gran-MVs, Granulocyte-derived microvesicles; PGC, Platelet granule content; LPS, Lipopolysaccharide; ER, Endoplasmic reticulum; HIF-1 α , Hypoxia-inducible factor 1 α ; L-Exo, LPS-pretreated BMSC-derived exosomes; srIkB, Super-repressor IkB; ATG2B, Autophagy-related protein 2 homolog B; RIPC, Remote ischemic preconditioning.

In this review, we will focus on the role of EVs as messengers and therapeutic tools in the context of sepsis, with particular attention to the advancements in engineering modifications to enhance the efficacy of EVs. A deeper understanding of the potential of EVs in the pathogenesis and therapeutic interventions of sepsis can contribute to the timely resolution of this medical challenge.

2 EVs-mediated intercellular communication in sepsis

EVs are rich in various bioactive components and play a significant role in intercellular communication. Current research indicates that EVs participate in the occurrence and progression of sepsis through multiple pathways (20). Increased plasma EVs levels correlate with the severity of organ failure and can serve as predictors of mortality in patients with sepsis (21). EVs could serve as an innovative mode of intercellular communication in the process of sepsis. In this section, we focus on the changes in cargo of EVs in sepsis and how EVs from different sources participate in various pathological processes and organ dysfunction in sepsis.

Circulating EVs in the body, which carry biomarkers and mediators of sepsis, have garnered interest. EVs containing an abundance of cytokines and chemokines play crucial roles in T cell differentiation, proliferation, and chemotaxis throughout the course of sepsis (22). EVs derived from individuals experiencing septic shock carry miRNAs and mRNAs associated with pathogenic pathways, such as inflammatory response, oxidative stress, and cell cycle regulation (23). Circulating EVs notably exacerbate the inflammatory response to sepsis in both serum and lung tissue by enhancing the production of proinflammatory factors such as tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), and interleukin-1 β (IL-1 β) (24). Compared with healthy individuals, 354 proteins, 195 mRNAs, 82 lncRNAs, and 55 miRNAs were found to be differentially expressed in serum exosomes from septic patients (25). Furthermore, pretreating septic mice with serum exosomes obtained from mice undergoing cecal ligation and puncture (CLP) notably suppressed the expression of proinflammatory cytokines and mitigated tissue damage. Caspase-1, a member of the cysteine protease family, is essential for regulating inflammatory responses (26). miR-126 could enhance the outcomes of acute lung injury induced by lipopolysaccharide and sepsis induced by cecal ligation and puncture (27). Increased caspase-1 activity and decreased levels of miR-126 in circulating extracellular vesicles are correlated with organ failure and mortality in sepsis (28). The combination of serum exosomal miR-483-3p and let-7d-3p showed diagnostic value for sepsis (29). The multi-omics analyses above indicate that EVs may play a significant role in the progression of sepsis. During sepsis, endothelial-derived microvesicles exacerbate endothelial inflammation by enhancing the adhesion between neutrophils and the endothelium and facilitating the release of neutrophil extracellular traps that contain citrullinated histones and myeloperoxidase (30). Endothelial EVs induce a pro-inflammatory phenotype in monocytes through aberrant expression of miR-99a and miR-99b, thus

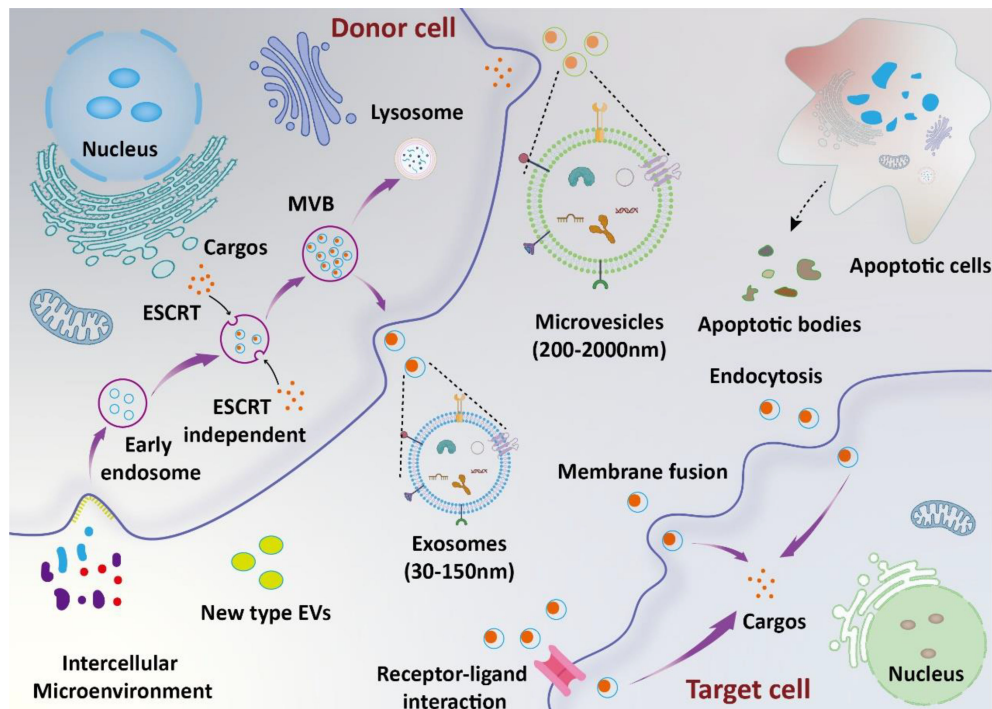


FIGURE 1

Biosynthesis and function of EVs. The biosynthesis of exosomes involves several stages, including lipid raft-mediated endocytosis, the formation of multivesicular bodies (MVBs), and exosome release. Microvesicles are formed through the direct budding of the cell membrane. Apoptotic bodies are produced by apoptotic cells. Extracellular vesicles (EVs) deliver their cargos into target cells through mechanisms such as ligand-receptor interaction, endocytosis, and direct fusion with the plasma membrane.

inhibiting mTOR expression (31). Lipinski et al. demonstrated that inflammasome-activated macrophages-derived EVs were able to transmit a robust IL-1 β -dependent inflammatory response in sepsis mice (32). The shuttle of EVs is beneficial for the transmission of inflammatory signals in sepsis, thereby accelerating the progression of sepsis. The development of EVs-based detection technologies may provide valuable diagnostic and therapeutic insights for the ongoing battle against sepsis.

Sepsis-induced ALI stands as a critical complication and the primary contributor to mortality (33). Recent findings suggest that overactivation of macrophages could lead to detrimental lung inflammation implicated in sepsis-induced ALI (34). During sepsis, endothelial cell-derived EVs (EC-EVs) with heightened levels of vascular cell adhesion molecule 1 (VCAM1) stimulate the NF- κ B pathway upon binding to integrin subunit alpha 4 (ITGA4) receptors on monocytes (35). This interaction modulates monocyte differentiation, shifting them toward a proinflammatory M1 macrophage phenotype, thereby promoting sepsis-related ALI. EVs originating from CD4⁺ T cells delivered diacylglycerol kinase kappa (DGKK) to induce apoptotic cell death, oxidative damage, and inflammation in alveolar epithelial cells, exerting toxic effects (36). The messenger role of EVs in mediating the crosstalk between alveolar epithelial cells and alveolar macrophages is crucial for ALI progression. Liu et al. showed alveolar epithelial cells-derived exosomes could deliver miR-92a-3p to activate macrophages, resulting in activation of the NF- κ B pathway and downregulation of PTEN expression along with pulmonary inflammation (37). Gong

et al. demonstrated that alveolar epithelial cells derived exosomes containing tenascin-C (TNC) bind to Toll-like receptor 4 (TLR4) on macrophages, leading to increased production of reactive oxygen species (ROS) and subsequent activation of the NF- κ B pathway (38). The processes ultimately result in macrophage pyroptosis, thus exacerbating the release of inflammatory cytokines. On the other hand, exosomal APN/CD13 derived from macrophages regulates necroptosis in lung epithelial cells by interacting with the cell surface receptor TLR4, thereby triggering ROS production, mitochondrial dysfunction, and NF- κ B activation (39). Polymorphonuclear neutrophils (PMNs) are pivotal in sepsis-related ALI (40). Jiao et al. revealed that exosomal miR-30d-5p from PMNs contributed to sepsis-related ALI by inducing M1 macrophage polarization and triggering macrophage pyroptosis through the activation of NF- κ B signaling (41). In another study, M2 macrophage-derived exosomes (M2-Exos) were able to inhibit PMN migration and neutrophil extracellular trap (NET) formation, thus alleviate lung injury and reduce mortality in a sepsis mouse model (42). Mechanistically, the secreted prostaglandin E2 (PGE2) contained within M2-Exos could bind to the EP4 receptor expressed on PMNs, thereby upregulating the expression of 15-LO in PMNs. In addition to EVs in the pulmonary microenvironment, circulating EVs are also involved in the progression of sepsis-induced ALI. Plasma extracellular vesicles deliver miR-210-3p, targeting ATG7 to enhance sepsis-induced ALI by modulating autophagy and triggering inflammation in macrophages (43). In addition, serum exosomes could transfer miR-155 to macrophages, triggering the activation of nuclear factor

κ B (NF- κ B) and prompting the secretion of TNF- α and IL-6 in ALI (44). Sepsis plasma-derived exosomal miR-1-3p was able to induce endothelial cell dysfunction by targeting SERP1 (45). This inhibition results in decreased cell proliferation, increased apoptosis, cytoskeleton contraction, heightened monolayer endothelial cell permeability, and membrane injury. Together, these investigations highlight the significance of circulating EVs released into the bloodstream as crucial mediators of septic lung injury through the transport of cargo via EVs (Figure 2).

Sepsis-induced AKI and cardiomyopathy are common complications of sepsis. Sepsis-associated AKI is a critical complication with high morbidity and mortality rates among critically ill patients (46). Sepsis-induced cardiomyopathy is prevalent among septic patients, and is distinguished by a decreased ejection fraction (47). Studies have shown that EVs are involved in kidney and myocardial damage during the progression of sepsis. Renal tubular epithelial cells (RTECs) are the predominant cell type in the kidney and have a vital role in pathological renal injuries (48). Plasma EVs in circulation primarily derive from platelets and can contribute to organ dysfunction during sepsis. EVs loaded with ARF6 activate ERK/Smad3/p53 signaling in RTECs, exacerbating sepsis-induced AKI (49). M1 and M2 macrophages exosomal miR-93-5p could regulate the TXNIP expression directly to influence the pyroptosis in RTECs (50). During sepsis-induced cardiomyopathy, endothelial HSPA12B participates in regulating macrophage pro-inflammatory responses via EVs (51). miRNAs originating from extracellular vesicles derived from neutrophils play a significant role in the progression of septic disease severity towards

cardiomyopathy. Ye et al. found that 38 miRNAs showed differentially expression between the septic cardiomyopathy and without cardiomyopathy patients, especially the independent predictor potential of miR-150-5p (52). In septic myocardial depression, circulating EVs promoted the pyroptosis of cardiomyocyte through miR-885-5p via HMBOX1 (53). Septic exosomes were found to be highly enriched with ROS, which can be transferred to endothelial cells. This transfer leads to the formation of podosome clusters in target endothelial cells, resulting in the relocation of ZO-1, vascular leakage, and cardiac dysfunction (54). Overall, these studies emphasize the crucial roles of EVs in septic development towards cardiomyopathy.

In addition to the aforementioned complications, liver injury is also a common organ dysfunction in sepsis, manifesting in around 50% of septic patients (55). EVs have been shown to be involved in sepsis induced liver injury. High mobility group box protein 1 (HMGB1) serves as a prototypical damage-associated molecular pattern (DAMP) molecule, exerting cytotoxic effects that result in cell death and tissue injury (56). Utilizing transferrin-mediated endocytosis, macrophage-derived EVs transport HMGB1 to target cells, inducing hepatocyte pyroptosis through the activation of NLRP3 inflammasomes (57). Platelet-derived exosomes deliver HMGB1 and/or miR-15b-5p and miR-378a-3p to induce NET formation and subsequent organ injury by the activation of Akt/mTOR autophagy pathway (58). Yang and colleagues discovered that macrophages possess the capability to absorb extracellular lactate using monocarboxylate transporters (MCTs), thus facilitating HMGB1 lactylation through a p300/CBP-dependent pathway (59). The lactylated/acetylated HMGB1 is subsequently

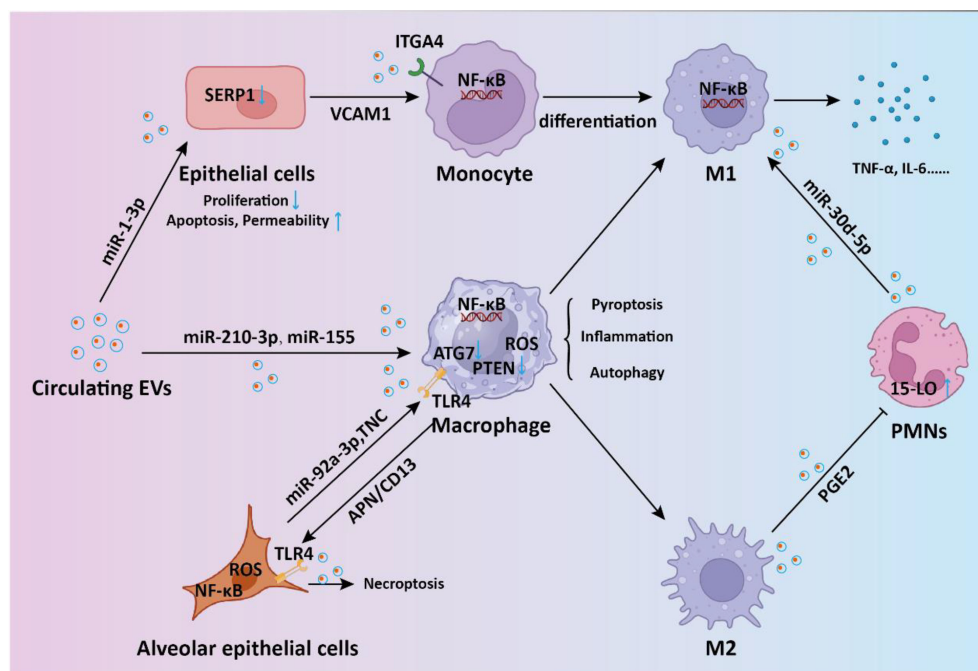


FIGURE 2

The intercellular communication mediated by EVs in sepsis-induced acute lung injury. During the process of sepsis-induced acute lung injury, EVs-mediated shuttling of miRNA and proteins regulates the signaling between endothelial cells, macrophages, monocytes, and neutrophils, promoting inflammatory responses and thereby accelerating lung injury.

discharged from macrophages through exosome secretion, consequently enhancing endothelium permeability.

3 The beneficial effects of EVs in sepsis treatment

Patients with severe sepsis often exhibit a heightened inflammatory response, which is a key factor leading to multiple organ dysfunction (60). Therefore, anti-inflammatory intervention has been considered an effective approach in the treatment of sepsis. Mesenchymal stem cells (MSC) are the most common source of EVs used in the treatment of sepsis. MSC-derived exosomes could mitigate the destructive effects of sepsis-induced inflammation by reducing inflammatory factors and tissue damage, leading to decreased levels of cytokines such as IL-6, IL-1 β , and TNF- α (61). miRNAs packaged within EVs have been implicated in the pathophysiology of sepsis. Sun et al. revealed that MSC-derived exosomes containing miR-27b suppressed sepsis progression by reducing the production of pro-inflammatory cytokines (62). This effect was achieved through the downregulation of JMJD3 and inhibition of the NF- κ B signaling pathway in bone marrow-derived macrophages. Similarly, adipose MSC-derived EVs (ADSC-EVs) were able to attenuate sepsis-induced inflammation by modulating the Notch/miR-148a-3p signaling pathway, which also decreased macrophage polarization to M1 (63). In addition to inflammation response, oxidative stress also deeply participates in the progression of sepsis. ADSC exosomes exerted protective effect in sepsis by relieving inflammatory cytokines storm and oxidative stress (64). Mechanistically, ADSC exosomes could regulate Nrf2/HO-1 axis in macrophages, thus promoting the polarization of macrophages from the M1 to M2 phenotype. Bone marrow MSC-derived EVs (BMSC-EVs) therapy enhanced survival, decreased sepsis-induced inflammation, reduced pulmonary capillary permeability, and improved liver and kidney function via delivery of miR-21a-5p (65). Therefore, the intervention effect of EVs on inflammatory response is a key factor in the treatment of sepsis. MSC-derived apoptotic vesicles (apoVs) could improve survival and alleviate multiple organ dysfunction in septic mice (66). Mechanistically, it is discovered that apoVs infused through the tail vein predominantly gather in the bone marrow of septic mice through electrostatic interactions with positively charged NET. Additionally, apoVs induce a shift in neutrophils from NETosis to apoptosis via the apoV-Fas ligand (FasL)-activated Fas pathway. Pyroptosis is an inflammatory programmed cell death process (67). EVs derived from pyroptotic MSC show promising benefits in the treatment of sepsis. Huang et al. showed that pyroptotic EVs specifically express pyroptotic maker ASC and bind to B cells to repress cell death by repressing Toll-like receptor 4 (68). The pyroptotic EVs were found to alleviate inflammatory responses and improve the survival rate of mice with sepsis.

In addition to MSC, EVs derived from other cell sources have also been shown to have anti-sepsis effects. Zhou and colleagues demonstrated that endothelial progenitor cells derived exosomes may prevent microvascular dysfunction and improve sepsis outcomes potentially through the delivery of miR-126 (69).

Mechanistically, miR-126-5p and 3p inhibited the levels of lipopolysaccharide (LPS)-induced HMGB1 and VCAM1, respectively, in human microvascular endothelial cells (HMVECs). Endothelial progenitor cell-derived EVs transferred lncRNA TUG1, facilitating M2 macrophage polarization by disrupting miR-9-5p-mediated inhibition of SIRT1 (70). Septic shock is marked by profound systemic inflammation, heightened coagulation activation, and impaired fibrinolysis, culminating in disseminated intravascular coagulation (DIC) (4). Cointe et al. demonstrated that elevated levels of platelet granule content (PGC) in granulocyte-derived microvesicles (Gran-MVs) reduce thrombus formation and enhance survival, indicating a protective function of Gran-MVs in sepsis (71). DIC is a common severe complication of sepsis, currently lacking effective treatment methods. In another study, Bao and colleagues demonstrated that neutrophils mitigate sepsis-associated coagulopathy via EVs containing superoxide dismutase 2 (72). Mechanistic investigations revealed that superoxide dismutase 2 is essential for inducing neutrophil anti-thrombotic function by preventing endothelial reactive oxygen species accumulation and relieving endothelial dysfunction (Figure 3).

EVs derived from multiple sources have been used for the intervention of sepsis-induced ALI. A study compared the protective effects of exosomes derived from adipose tissue, bone marrow, and umbilical cord on sepsis-induced ALI (73). The results showed that all three types of exosomes effectively downregulated sepsis-induced macrophage glycolysis and inflammation, improved lung pathological damage, and enhanced the survival rate of septic mice. Research has found that ADSC-derived exosomes deliver miR-125b-5p to inhibit Keap1, thereby upregulating Nrf2/GPX4, reducing ferroptosis in microvascular endothelial cells, ultimately alleviating lung tissue damage and lowering mortality rates (74). Additionally, ADSC-derived exosomes could suppress macrophage aggregation and IL-27 secretion in lung tissues, leading to reduced pulmonary edema and pulmonary vascular leakage (75). The excessive expression of SAA1 in BMSC-derived exosomes suppressed lung injury by reducing the levels of endotoxin, TNF- α , and IL-6 induced by CLP or LPS (76). Exosomes from endothelial progenitor cells were able to enhance the prognosis of lipopolysaccharide-induced ALI by delivering miR-126 to the injured alveoli (77). Endoplasmic reticulum (ER) stress exacerbates sepsis-induced ALI. Chiang et al. showed that human placental MSC-derived exosomes could alleviate lipopolysaccharide-induced ER stress, inflammation, and lung injury in mice (78).

Some studies have explored the beneficial effects of EVs in sepsis-related AKI. The most common contributing factor for AKI among ICU patients is septic shock, accounting for approximately 47.5%, and is associated with a high in-hospital mortality rate (79). Traditional methods to address septic shock, such as fluid administration, antibiotic use, and vasopressors, have not led to improved outcomes in cases of sepsis-related AKI (80). Therefore, EVs-based biologic therapy has significant clinical value in improving sepsis-associated AKI. BMSC-derived exosomes could alleviate inflammation and apoptosis in sepsis-associated AKI through activating autophagy (81). ADSC-derived exosomes were

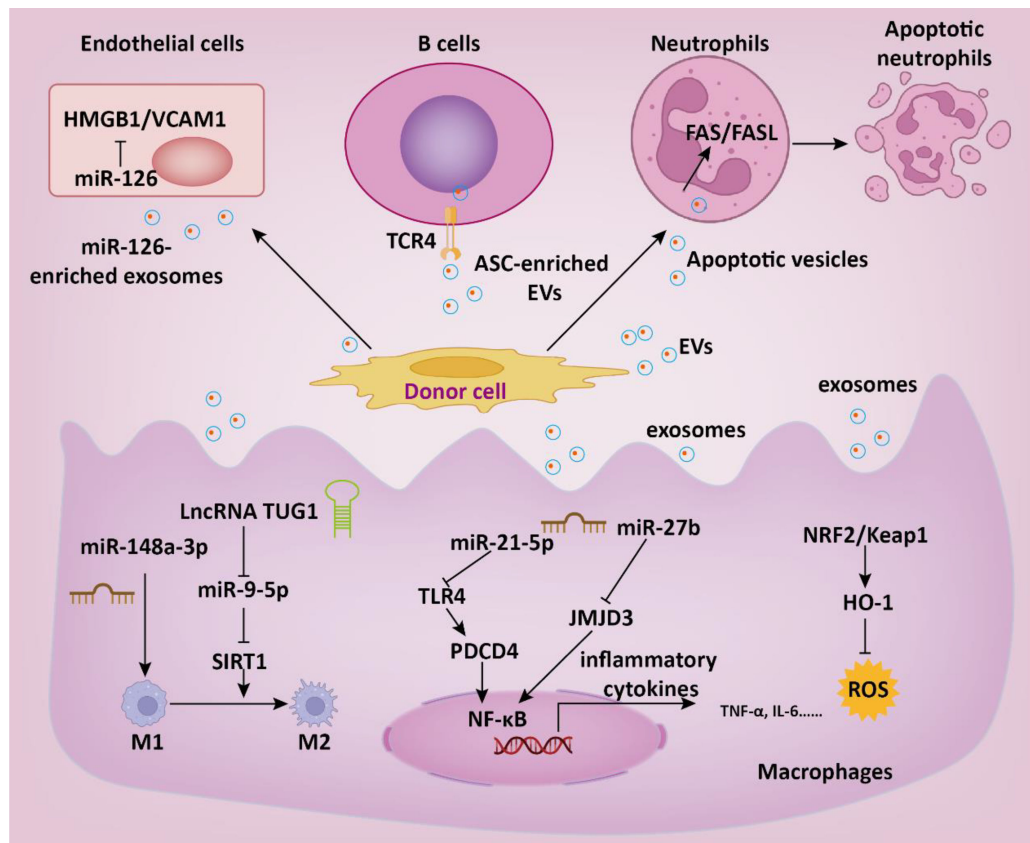


FIGURE 3

The role of EVs in the therapeutic intervention of sepsis. EVs regulate multiple intracellular signaling pathways in target cells by delivering functional cargoes such as miRNA, lncRNA, proteins, etc., thereby suppressing inflammatory responses, reducing thrombosis formation, and consequently slowing down the progression of sepsis.

able to protect against sepsis induced inflammation and apoptosis in AKI via activation of SIRT1 signaling pathway (82). Zhang et al. found that endothelial progenitor cells-derived exosomes could release miR-21-5p and miR-93-5p to mitigate sepsis-induced AKI by suppressing RUNX1 expression and KDM6B/H3K27me3/TNF- α axis, thereby enhancing renal function and ameliorating renal tissue pathology (83, 84). This intervention attenuated serum inflammatory response and diminishes apoptosis and oxidative stress in renal tissues.

EVs have also shown great potential for application in sepsis-induced myocardial and liver injuries. In a study, Li et al. found that MSC-derived exosomes prevented sepsis-induced myocardial injury by delivering circRTN4 to inhibit miR-497-5p, thereby upregulating MG53 in cardiomyocytes (85). In another research, Zhou and colleagues demonstrated that umbilical cord MSC-derived exosomes transported Pink1 mRNA to recipient cardiomyocytes, enhancing PINK1 expression (86). This process helped mitigate mitochondrial calcium overload in sepsis by restoring mitochondrial calcium efflux through the PINK1-PKA-NCLX axis. For acute liver injury, treatment with MSC-EVs led to a notable decrease in the expression of multiple glycolysis-related enzymes and suppressed glycolytic flux by inhibiting the expression of hypoxia-inducible factor 1 α (HIF-1 α), thereby effectively dampening macrophage inflammatory responses (87). MSC-

derived exosomal miR-26a-5p could significantly protect against hepatocyte death and liver injury caused by sepsis through targeting lncRNA MALAT1 (88). LPS-pretreated BMSC-derived exosomes (L-Exo) notably alleviated septic liver injury induced by cecal ligation and puncture and suppressed macrophage STING signaling (89). Mechanistically, L-Exo inhibited macrophage STING signaling by delivering ATG2B to promote mitophagy and prevent the release of mtDNA into the cytosol. In addition, the combination of MSC and hepatocyte-derived exosomes with imipenem was able to improve the inflammatory response and liver damage in sepsis, thus increasing survival rates (90). Compared to treating with a single drug, combination therapy offers an alternative strategy (Figure 4).

4 Engineering methods for improving treatment effect of EVs in sepsis

EVs hold promise in the treatment of sepsis, yet they suffer from limitations such as low therapeutic cargoes and weak targeting. To overcome these shortcomings, various engineering strategies for modifying EVs have been developed. In this section, we introduce genetic and non-genetic methods for remodeling EVs and focus on the enhanced role of engineered EVs in sepsis therapy.

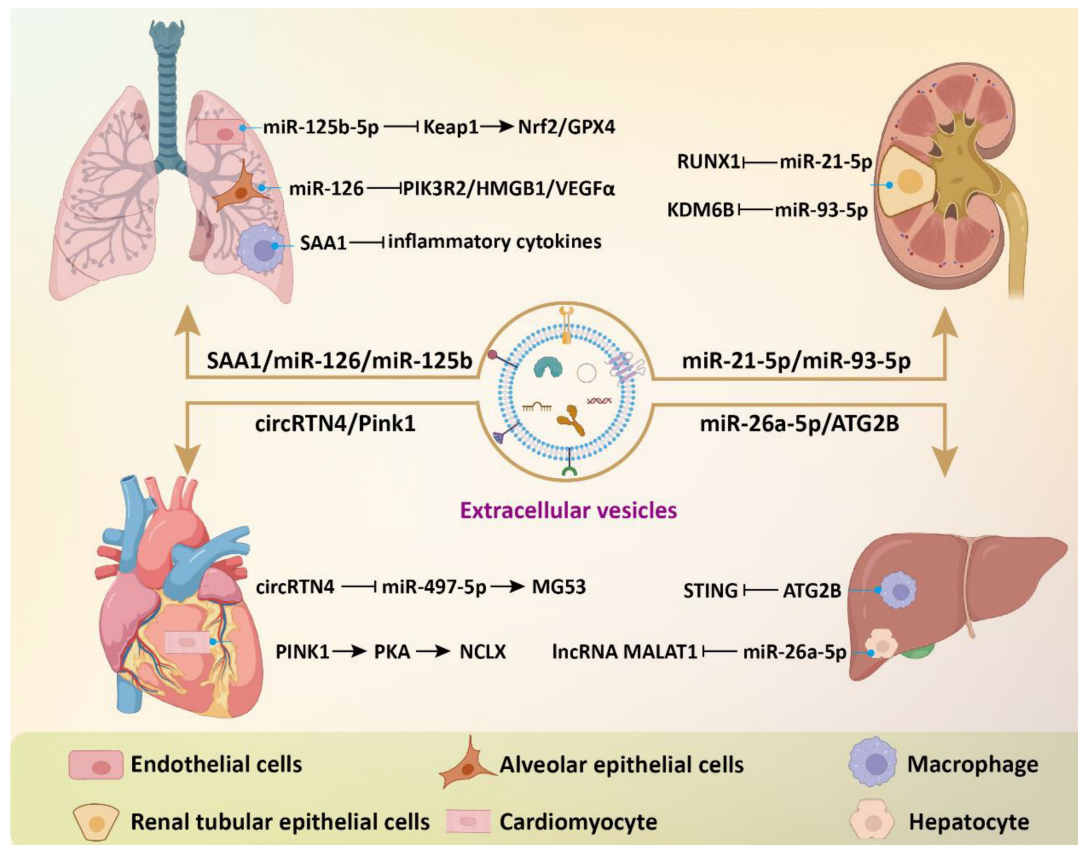


FIGURE 4

The beneficial effects of EVs in sepsis-related complications. EVs modulate intracellular signaling in effector cells of target organs by delivering bioactive components, thereby inhibiting inflammatory responses, reducing cell death and oxidative stress, and consequently alleviating tissue damage.

To increase the functional cargos within EVs, exogenous manipulation of donor cells or isolated EVs is employed. Li et al. discovered that tumor cell-secreted exosomes produced after LPS treatment are more effective in improving sepsis compared to normal secretory exosomes due to the presence of protective miRNAs (91). To mimic these exosomes, they developed exosome mimics by incorporating the mentioned microRNAs into hyaluronic acid-polyethylenimine nanoparticles. These exosome mimics, with specific miRNA compositions, mitigate sepsis in mice and cynomolgus monkeys, suggesting that biomimetic simulation of tumor-suppressive exosomes may offer a promising therapeutic approach for treating sepsis and cytokine-storm-related conditions. MicroRNA-146a is a widely reported negative immunoregulatory small noncoding RNA (92). In another study, researchers developed an engineered macrophages-derived apoEVs based multifunctional agents for sepsis treatment (93). ApoEVs, engineered with mesoporous silica nanoparticles carrying miR-146a, captured iron and neutralized α -toxin using their natural membrane, and modulated inflammation by releasing miR-146a in phagocytes. These engineered apoEVs exhibited a high capacity for capturing toxins and iron, ultimately providing protection against sepsis associated with high iron loads. Dysregulation of M1 macrophage polarization in sepsis leads to serious inflammation. In a study, exosomes were modified through introduction of super-

repressor I κ B (srI κ B) by an optogenetically engineered exosome system (94). In septic mouse models, intraperitoneal administration of purified srI κ B-loaded exosomes alleviates mortality and systemic inflammation, potentially by blocking the translocation of NF- κ B into the nucleus in neutrophils and monocytes. In another study, EVs derived from immortalized bone marrow-derived macrophages are loaded with siRNA targeting the chemokine receptor CCR2 for targeted drug delivery (95). These EVs containing siCCR2 not only inhibited the chemotaxis of inflammatory monocytes/macrophages but also alleviated septic symptoms in mice by reducing the mobilization of splenic inflammatory monocytes and attenuating the subsequent serum cytokine storm.

In addition to direct manipulation of isolated EVs, modifying the cell culture environment or implementing exogenous pretreatment is an effective strategy to enhance the therapeutic efficacy of EVs. EVs derived from pretreated MSC have demonstrated significant promise in addressing diverse inflammatory conditions. Pre-treated with LPS, BMSC-EVs significantly alleviated septic liver injury induced by cecal ligation and puncture through the inhibition of macrophage STING signaling (89). These BMSC-EVs delivered autophagy-related protein 2 homolog B (ATG2B) for promoting mitophagy and suppressing the release of mtDNA into the cytosol. Additionally,

IL-1 β -primed MSC-derived exosomes exhibited beneficial effects on macrophage polarization and sepsis (96). These exosomes delivered miR-21 to macrophages, targeting PDCD4 and inducing M2 polarization. These studies underscore a novel basis for inducing immunomodulation for the therapeutic application of MSC-EVs in sepsis. Hypoxia triggers the upregulation of genes associated with proliferation and angiogenesis in MSC, enhancing their differentiation capacity and altering the content of EVs (97–99). Cao et al. showed that hypoxia-preconditioned exosomes derived from ADSC exhibited greater suppression of renal vascular leakage and reduced renal dysfunction compared to control exosomes, thereby improving the survival rate of septic mice (100). These protective effects could be partially attributed to the renal microvascular protective role of exosome-derived circ_0001295.

Aside from the direct administration of exogenous EVs, increased serum EVs induced by remote ischemic preconditioning (RIPC) have been shown to be beneficial in treating sepsis. Pan et al. demonstrated that RIPC, induced by short periods of ischemia and reperfusion in femoral arteries, confers protective benefits against sepsis-induced AKI through the transportation of miR-21 via serum exosomes (101). Mechanistically, exosomal miR-21 was assimilated into renal tubular epithelial cells and subsequently aimed at the downstream PDCD4/NF- κ B and PTEN/AKT pathways, subsequently exerting anti-inflammatory and anti-apoptotic effects. For sepsis induced ALI, RIPC could attenuate pulmonary edema, and inflammatory cell infiltration into lung tissues (102). The elevated expression miR-142-5p in serum EVs could target PTEN to activate the PI3K/Akt signaling pathway, thus alleviating ALI. These studies propose novel approaches for intervening in sepsis and highlight the crucial role of EVs in mediating therapeutic benefits.

The weak targeting ability of EVs is one of the main obstacles hindering their application *in vivo*. Li and colleagues demonstrated that exosomes derived from CD5L lentivirus infection and kidney tubular cell targeting peptide LTH modified fibroblastic reticular cells showed enhanced binding specificity to kidney tubular cell (103). The CD5L-enriched exosomes selectively adhered to primary kidney tubular cells, fostering kinase PINK-ubiquitin ligase Parkin-mediated mitophagy. This process suppressed pyroptosis and enhanced kidney function by impeding NLRP3 inflammasome activation, ultimately ameliorating the sepsis survival rate. Membrane fusion technology is also widely applied to improve the therapeutic targeting of EVs. Neutrophils are the initial cells recruited to the site of injury during an inflammatory response. Neutrophil membrane was performed on panax ginseng root-derived exosomes following by miR-182-5p electroporation to obtain engineered exosomes (104). Compared with control exosomes, engineered exosomes significantly improved sepsis-induced ALI via target regulation of NOX4/Drp-1/NLRP3 signal pathway.

To target the activated macrophages in sepsis, Fan et al. developed a method involving exosomes encapsulation with circRNA mSCAR and electroporation with TPP-PDL, promoting targeted delivery into mitochondria for macrophage polarization towards the M2 subtype (105). This targeted delivery system attenuates systemic inflammation and reduces mortality in septic

mouse and cell models. In order to inhibit overactivated macrophages, Zheng et al. developed folate-functionalized exosomes to target inflammatory macrophages during sepsis in the lungs (106). They found that folate-functionalized exosomes co-loaded with resveratrol and celastrol demonstrated potent anti-inflammatory and immunosuppressive effects against sepsis-stimulated M1 macrophages, thus reducing acute lung injury. These effects were mediated through the regulation of NF- κ B and ERK1/2 signaling pathways. Collectively, the strategy of engineered EVs for targeting inflammatory macrophages during sepsis development offers a new avenue for sepsis treatment (Figure 5).

5 Discussion

Sepsis results from a dysregulated host response to infection, leading to life-threatening organ dysfunction. Despite progress in treatment, the high mortality rate persists due to rising antibiotic resistance and an aging population. Sepsis patients and normal controls exhibit significant differential expression of molecules in serum EVs, including proteins, mRNA, miRNAs, and lncRNAs. An integrative multiomics analysis revealed that components of EVs were linked to cytokine storm, complement and coagulation cascades and endothelial barrier function (25). These studies suggest that EVs could potentially serve as new markers for the diagnosis and monitoring of sepsis. EVs, particularly those derived from MSC, have been shown to promote the restoration of homeostasis in sepsis and alleviate organ damage, facilitating functional recovery. However, the mechanisms by which EVs promote tissue recovery during sepsis have not been fully elucidated. Numerous studies have demonstrated the effectiveness of administration of EVs *in vivo*, there is a lack of detailed elucidation of specific signaling pathways, which introduces many unknown and ambiguous areas for potential clinical translation research.

To further enhance the therapeutic efficacy of EVs and overcome the limitations of natural EVs, various engineering modifications are employed to boost the therapeutic molecular activity, targeting specificity, and biocompatibility of EVs. Collectively, strategies to modify EVs could be a new avenue in developing therapeutics against sepsis and its complications. Currently, the main methods employed involve genetic or non-genetic modifications of parent cells or isolated EVs. Manipulating donor cells to obtain engineered EVs can preserve most of the biophysical characteristics of EVs. However, modification of parent cells may have unforeseen consequences on the biology of EVs, ultimately interfering with EV biogenesis and altering other biological properties. Therefore, an increasing number of studies are focusing on the direct manipulation of EVs. Direct modification of isolated EVs does not alter the biological origin of EVs and allows for the direct impartation of desired biological properties using various methods as needed.

Multiple studies have reported the beneficial effects of EVs, especially those derived from mesenchymal stem cells, in the treatment of sepsis. However, the therapeutic effects of EVs have not yet been widely recognized. A meta-analysis suggests that the treatment of MSC-EVs may be associated with lower mortality rates in septic animal models (107). Nevertheless, there is a lack of

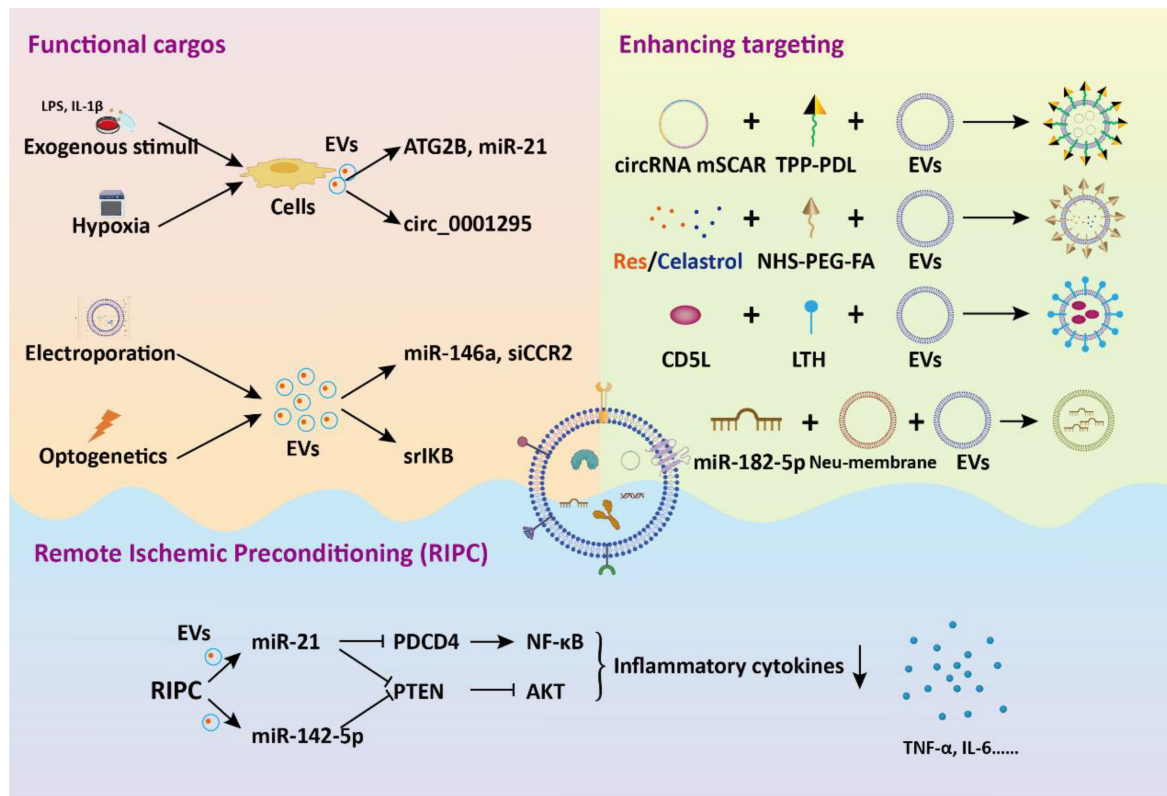


FIGURE 5

The application of engineered EVs in the treatment of sepsis. Genetic or non-genetic modification strategies enhance the bioactivity or targeting of EVs. These engineered EVs deliver functional cargos to target cells, eliciting biological effects, thereby alleviating sepsis and associated tissue dysfunction.

standardized data on the dosage, source, and timing of EVs administration for comparison. The therapeutic effects of EVs derived from MSC may vary depending on their source, thus more research is needed to further investigate the complex cargo functionality within EVs (108). Due to the heterogeneous nature of EVs, a more precise definition and classification of EVs subtypes may help enhance the feasibility and therapeutic advantages of EVs in clinical applications. Additionally, there is controversy surrounding the outcomes of interrupting the cascade of sepsis-induced inflammatory responses. More studies are needed to understand the origin and characteristics of EVs, laying the groundwork for comprehending their diversity and complexity. Therefore, it is essential to comprehend the mechanisms of inflammation and develop new treatment approaches to improve the prognosis of sepsis.

In addition to EVs released by mammalian cells during sepsis, numerous studies have highlighted the multifaceted roles of bacterial EVs in this condition. As key mediators of intercellular communication, bacterial EVs impact bacterial pathogenicity, disease mechanisms, and the modulation of the host immune response (109). During sepsis progression, bacterial EVs contribute to pro-inflammatory responses by activating pattern recognition receptors and regulating cytokine release pathways (110). Furthermore, EVs derived from probiotic or commensal bacteria may promote anti-inflammatory responses by modulating anti-inflammatory TLR

pathways and cytokine production. For instance, EVs released from probiotic bacteria can enhance macrophage phagocytosis in polymicrobial sepsis through activation of the FPR1/2 pathway (111). Additionally, outer membrane vesicles derived from *Escherichia coli* Nissle 1917 have been shown to improve immunomodulation and antimicrobial activity in RAW264.7 macrophages (112). These findings underscore the potential of bacterial EVs in modulating host immune cell functions, thus enriching our understanding of EVs as a critical mechanism in both the development and therapeutic management of sepsis.

Overall, EVs-based therapies hold great promise as a potential treatment strategy for sepsis and related complications. EVs also have tremendous potential for early diagnosis and dynamic monitoring of sepsis. However, there is still a long way to go before clinical translation. Extra efforts are needed in standardizing EVs research, understanding their pathogenic mechanisms, and implementing long-term monitoring.

Author contributions

BY: Funding acquisition, Writing – original draft, Writing – review & editing. YY: Writing – original draft. JW: Writing – review & editing. CZ: Writing – review & editing. SD: Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Balancing inflammation: the specific roles of serum amyloid A proteins in sterile and infectious diseases

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Serum Amyloid A (SAA) proteins are acute-phase reactants with critical roles in sterile and bacterial inflammation. Through *in vitro* and *in vivo* experiments, we demonstrate that SAA proteins amplify cytokine and chemokine responses during sterile inflammation and enhance bacterial clearance in infectious conditions. Mechanistically, SAA proteins augment NF- κ B signaling, driving pro and anti-inflammatory mediator production. SAA^{-/-} mice carrying a deletion of the *Saa1*, *Saa2*, *Saa3*, and *Saa4* serum amyloid A genes have better survival rates in sterile sepsis but are more prone to bacterial sepsis than their SAA^{+/+} counterparts, emphasizing their dual functionality in immune regulation. Overexpression of *Saa1*, *Saa2*, *Saa3*, and *Saa4* in macrophages enhances NF- κ B-mediated pro-inflammatory cytokine production and bacterial clearance during infection. Together, our results show that SAA proteins are key modulators of inflammation, with distinct mechanisms tailored to sterile and infectious contexts.

KEYWORDS

innate immunity, PAMPs (pathogen associated molecular patterns), inflammation, serum amyloid proteins, cytokines

Introduction

The family of Serum Amyloid A (SAA) proteins consists of small, evolutionarily conserved acute-phase proteins with important roles in inflammation, immunity, and lipid metabolism (1). SAA proteins respond rapidly to pro-inflammatory signals, such as cytokines like IL-1, IL-6, and TNF α . During inflammatory episodes, their plasma levels can increase over 1000-fold (2). In humans, there are four primary isoforms (SAA1-4): SAA1 and SAA2 are acute-phase proteins and most responsive to inflammation. These two SAAs are primarily expressed in the liver (3). SAA3, while functional in some animal

species, is considered a pseudogene in humans. SAA4 is constitutively expressed at low levels in the liver, but maintains basal functions without major upregulation in response to inflammation (4). The four SAA isoforms participate in diverse processes, from High-Density Lipoprotein (HDL)-mediated lipid transport to innate immune signaling (5). The differential expression patterns and functional diversity among the isoforms highlight their adaptive roles in various physiological and pathological states, such as infections, chronic inflammatory diseases, and amyloidosis (6).

Notably, SAA proteins are emerging as significant biomarkers in precision medicine, particularly for predicting disease outcomes and personalizing treatment. In cancer, hepatocyte-derived SAA proteins are linked to immune evasion by reducing T-cell infiltration into tumors, influencing tumor progression and patient prognosis (7). Genetic predispositions also play an important role, as specific SAA gene variants increase susceptibility to chronic inflammatory diseases. In addition, high SAA protein levels are associated with relapse risk and treatment responses, aiding personalized care decisions in conditions like inflammatory bowel disease (8, 9). SAA protein levels are similarly valuable in acute infections and hyper-inflammatory states, where they indicate disease severity and guide tailored interventions, with COVID-19 being one such example (10). It has therefore been suggested that SAA proteins could serve as vital markers in infectious diseases including sepsis (11).

Under inflammatory conditions, SAA proteins play a pivotal role in influencing immune cell behavior and amplifying inflammatory signaling pathways. For instance, SAA1 has been shown to exacerbate airway inflammation by promoting neutrophil and macrophage activation, contributing to the pathogenesis of airway diseases (12, 13). In asthma, elevated SAA1 levels were found to drive airway hyperresponsiveness and increase the expression of inflammatory cytokines such as IL-6 and TNF α , which further amplifies the inflammatory response (14). Similarly, in kidney diseases, SAA2 has been reported to play a role in promoting podocyte injury through the activation of the NF- κ B signaling pathway, highlighting its contribution to chronic inflammation in renal tissues (15).

Using a murine endotoxin model, SAA3 has been implicated in LPS-induced ocular inflammation, where it activates retinal microglia and promotes a pro-inflammatory state via Toll-like receptor 4 (TLR4) signaling. These results highlight the diverse cellular pathways that SAA proteins engage across different tissues (16). SAA proteins also affect lipid metabolism, as seen in patients with chronic inflammatory conditions, where SAA-modified high-density lipoprotein (HDL) particles lose their anti-inflammatory properties and become pro-inflammatory, further contributing to disease severity (17). Together the multifaceted role of SAA proteins in inflammation positions them as central mediators of both acute and chronic inflammatory diseases.

In the context of sepsis, SAA proteins have become increasingly recognized for their critical role in modulating the immune response through their rapid upregulation. Studies have demonstrated that patients with sepsis exhibit significantly higher levels of SAA proteins, which correlate with disease severity and the likelihood of developing septic shock (11). Therefore, elevated SAA

protein levels are strongly associated with the severity of sepsis and are used as diagnostic and prognostic biomarkers. In particular, SAA protein levels have been shown to predict multiple organ dysfunction and mortality, making them valuable for assessing patient prognosis in the intensive care unit (ICU) (18).

The combined use of SAA proteins with other biomarkers, such as C-reactive protein (CRP) and procalcitonin (PCT), enhances diagnostic accuracy for early detection of sepsis, particularly in cases involving severe trauma or infections like urinary tract infections (19). In neonatal sepsis, point-of-care measurements of SAA levels have proven to be effective in early diagnosis, providing a faster and more accurate approach than traditional methods (20).

Moreover, role of SAA proteins extends beyond diagnosis to prognostication; studies have indicated that dynamic monitoring of SAA protein levels can help predict outcomes more reliably than other markers like nitric oxide or CRP alone (11). By closely tracking SAA protein levels over time, clinicians can better assess the severity of systemic inflammation, enabling more targeted therapeutic interventions to reduce mortality in septic patients.

This comprehensive role of SAA proteins in sepsis, from diagnosis to prognosis, underscores its potential as a crucial tool in the management of systemic inflammatory responses, particularly in critical conditions like septic shock.

The present study was undertaken to investigate the role of SAA proteins in modulating inflammatory responses under sterile and bacterial conditions. To this end, we stimulated bone marrow-derived macrophages (BMDMs) with a diverse panel of PAMPs and performed *in vivo* experiments using LPS-induced endotoxemia and bacterial infections.

Materials and method

Ethical statement

All experiments on mice were performed according to Swedish Animal Welfare Act SFS 1988:534 and were approved by the Animal Ethics Committee of Malmö/Lund, Sweden (permit numbers Dnr 5.8.18-18-01753/2022). Mice were housed in Innovive IVC Rodent Caging System on a 12/12 h light/dark cycle. The ambient temperature ranged between 19 and 23 °C with humidity 55 $\pm$ 10%. All animals had free access to water and chow. Technicians and staff did not enter the room during the dark cycle unless strictly required to collect cages for health monitoring. All animals received care according to the USA Principles of Laboratory Animal Care of the National Society for Medical Research, Guide for the Care and Use of Laboratory Animals, National Academies Press (1996) (46).

Animals

SAA^{+/+} (C57BL/6NJ) and SAA^{-/-} (C57BL/6-Del(7Saa3-Saa2)738Lvh/J) mice (8 to 12 weeks of age, weighing 18 to 22 g (The Jackson laboratory, USA) were maintained under specific-pathogen-free conditions and had free access to commercial chow

and water. *SAA*^{-/-} mice genotype has been confirmed using a specific SAA ELISA.

Isolation of bone marrow-derived macrophages

SAA^{+/+} and *SAA*^{-/-} knockout mice were sacrificed. The hind legs were removed and stored in HBSS + 1% BSA on ice. The femurs were extracted, and the upper and lower joints were cut using sterile scissors. The bone marrow was then flushed out with HBSS including 1% BSA solution using a syringe. The resulting cell suspension was passed through a 100 µm cell strainer into a new Falcon tube and centrifuged at 1600 rpm for 10 minutes. The supernatant was discarded, and 5 ml of sterile water was added for 5 seconds to osmotically lyse erythrocytes. The solution was then brought up to 50 ml with HBSS including 1% BSA and centrifuged again at 1600 rpm for 10 minutes. After discarding the supernatant, the cell pellet was resuspended in HBSS including 1% BSA, and the cells were counted. The cells were then transferred into T75 flasks containing 15 ml of DMEM medium (10% FBS, 1% Antibiotic-Antimycotic, and 50 mg/ml M-CSF).

Plasmid restriction digestion and purification

The expression plasmids with Myc-DDK tags for SAA1 (MR200611, Saa1 (NM_009117) Mouse Tagged ORF Clone), SAA2 (MR219150, Saa2 (NM_011314) Mouse Tagged ORF Clone), SAA3 (MR200605, Saa3 (NM_011315) Mouse Tagged ORF Clone), and SAA4 (MR200725, Saa4 (NM_011316) Mouse Tagged ORF Clone) were purchased from Origene Technologies. BsrGI-HF® (#R3575S) from New England Biolabs.

Generation of SAA DDK stable macrophage cell lines

100,000 RAW Blue cells per well were seeded in a 24-well plate with 1 ml of DMEM (10% FBS, 1% Antibiotic-Antimycotic) per well. After 24 hours of incubation, NATE Transfection Enhancer (Cat. no: lycen-ate; Invivogen™) were added to each well and incubated for 30 minutes. Transfection was performed according to the instructions of the Lipofectamine LTX with Plus Reagent Kit (Cat. no: 15338100; Invitrogen™) to transfect the digested DDK-tagged SAA plasmids. For the selection of transfected cells after 48 hours after transfection, the plasmids containing G418 resistance genes were cultured in DMEM (10% FBS, 1% Antibiotic-Antimycotic, 2 mg/mL G418 (Cat. no: ant-gn-1; Invivogen™)) for 21 days. The medium with the added puromycin was changed every 3 days.

Stimulation of macrophages

100,000 cells of each genotype were seeded in triplicates in a 96-well plate. The cells were stimulated with the reagents indicated in

Supplementary Table 1 or RAW cells stimulated with SAA proteins with or without LPS. After overnight incubation, the supernatants were collected and analyzed for cytokine levels.

Stimulation of *SAA*^{+/+} and *SAA*^{-/-} KO BMDMs

100,000 cells of each genotype were seeded in triplicates in a 96-well plate. The cells were stimulated with the reagents indicated in Supplementary Table 1 or RAW cells stimulated with SAA proteins (Nordic Biosite) with or without LPS. After overnight incubation, the supernatants were collected and analyzed for cytokine levels. The activators used in this study were chosen to model diverse inflammatory conditions. LPS, a TLR4 ligand, mimics sterile endotoxemia, while FSL-1 and Pam3CSK4, TLR2 agonists, represent bacterial and fungal infections. Zymosan, a β-glucan, was included to simulate sterile inflammation via TLR2 and dectin-1 pathways. These activators reflect key inflammatory contexts, enabling the evaluation of SAA-mediated immune modulation under both sterile and infectious conditions.

Nuclear factor kappa B activity

After stimulation, RAW cells were spun down at 350 x g for 5 min and 20 µl of the supernatant was used for the NF-κB activation assay. After adding 180 µl of Quanti-Blue (Invivogen), samples were incubated at 37°C for 30 min and the absorbance was measured at 600 nm. Untreated cells served as a negative control. Cytokine heatmap was generated using the R package ComplexHeatmap (v2.22.0). Briefly, the mean of absorbance values (600nm) from three replicates (n=3) from different stimulations were obtained. The blank control value (n=3) was then subtracted from the means and ratios were taken between the stimulants versus untreated control samples (n=3). The ratios were then log2-transformed and presented in the heatmap.

Murine endotoxemia model

SAA^{+/+} and *SAA*^{-/-} mice were injected intraperitoneally with LPS (8 mg/kg) in 200 µl PBS. Survival rates were monitored for 7 days. To determine cytokine levels, mice were sacrificed after 18 hours. Blood was collected via heart puncture into EDTA tubes, and mouse lungs were fixed in Histofix (Histolab Products AB, Gothenburg, Sweden) for immunohistochemistry. Blood samples were centrifuged at 1000 x g for 10 minutes at 4°C and stored at -80°C for further analysis.

Pseudomonas aeruginosa infection mouse model

P. aeruginosa (strain Xen 41, derived from the parental pleural isolate *P. aeruginosa* PAO1; PerkinElmer, Waltham, MA) was

grown aerobically in Todd-Hewitt (TH) broth at 37°C to the logarithmic growth phase (optical density at 620 nm [OD₆₂₀] of ~0.5), harvested, washed in phosphate-buffered saline (PBS), and diluted to 2 × 10⁹ cfu/ml in the same buffer. Each animal was infected with 50 µl of the bacterial solution by intranasal instillation, whereas uninfected control animals received PBS alone. In order to maintain a consistent inoculum throughout the experiments, bacteria at a fixed OD₆₂₀ nm value were used to infect the animals, and the cfu were confirmed by plating in every experiment. The mice were euthanized on the following day and the blood, BALF, lung, kidney, spleen and liver samples were collected for further processing. To quantify bactericidal activity, serial dilutions of the incubation mixtures were plated on TH agar, followed by incubation at 37°C overnight and determination of the number of cfu.

Enzyme-linked immunosorbent assay

The ELISA was performed separately for SAA1, SAA2, SAA3, and SAA4 proteins using specific ELISA kits from Nordic Biosite. The following kits were used: Mouse SAA/SAA1 ELISA Kit (#KBB-9Y3TR5-96), Mouse SAA2 (Serum Amyloid A-2 Protein) ELISA Kit (#EKX-7XRF2Z-96), Mouse SAA3 (Serum Amyloid A-3 Protein) ELISA Kit (#EKX-4YOM8J-96), and Mouse SAA4 (Serum Amyloid A-4 Protein) ELISA Kit (#EKX-IZ7HDW-96). All four ELISAs followed a similar protocol. The protein concentrations were measured by using human antigen specific ELISA Kits according to manufacturer's protocol. The absorbance was measured at 450 nm using a Victor Microplate Reader.

Cytokine analysis using Bioplex

Cytokine levels in plasma and cell supernatants were analyzed using multiplex immunoassay kits (36-Plex ProcartaPlex Panel or 23-plex Bioplex ProTM, Invitrogen). The assays were performed according to the manufacturer's instructions using a Bio-PlexTM 200 system (Bio-Rad).

Hematoxylin and eosin staining

The left lungs were harvested in histofix, rehydrated, and embedded in paraffin. Sections of 4 µm thickness were placed on polylysine-coated glass slides, deparaffinized in histolab-clear (Histolab Products AB), rehydrated in graded alcohols and stained with hematoxylin and eosin by routine procedures.

Statistical analysis

Experiments were done at least in two independent trials. Data were analyzed with GraphPad Prism 10.0 (GraphPad Software, San Diego, CA). All results are presented as mean values ± SEM with the

number of independent experiments and mice per group indicated in the figure legends. Comparison of data was performed by one-way or two-way ANOVA and Tukey's multiple comparison test.

Results

SAA proteins modulate cytokine and chemokine responses to diverse inflammatory stimuli

To explore the role of SAA proteins in modulating inflammatory responses, bone marrow-derived macrophages (BMDMs) from SAA^{+/+} and SAA^{-/-} mice were stimulated with a diverse set of PAMPs, including LPS, FSL-1, Pam3CSK4, and zymosan, to mimic bacterial, viral, fungal, and sterile inflammatory conditions. Cytokine and chemokine levels in the supernatant were quantified using multiplex immunoassays to assess the differential impact of SAA proteins on inflammatory signaling. Details about the specific ligands, their associated receptors, and the inflammatory contexts are summarized in [Supplementary Table 1](#), providing a comprehensive overview of the experimental setup.

As shown in [Figure 1](#), SAA^{+/+} cells exhibited significantly elevated levels of pro-inflammatory cytokines, including TNFα, IL-6, MCP-1, and RANTES, compared to SAA^{-/-} cells across most conditions. This effect was particularly pronounced with sterile inflammatory mimetics such as LPS, which elicited the highest cytokine responses. Other pro-inflammatory mediators, such as G-CSF and IL-31, also demonstrated significant genotype-dependent differences, though with greater variability across stimulators, suggesting that SAA proteins broadly enhance inflammatory pathways. In contrast, anti-inflammatory cytokines, including IL-10 and IL-27, displayed less consistent elevation between genotypes. This selective amplification of pro-inflammatory mediators highlights the role of SAA proteins in promoting robust immune activation in sterile inflammation while potentially maintaining balanced responses during bacterial challenges. Furthermore, immune mediators like LIF and M-CSF, which are associated with tissue repair and macrophage survival, were also elevated in SAA^{+/+} cells under specific conditions, indicating potential additional roles for SAA proteins in cellular homeostasis during inflammation.

NF-κB activation by SAA isoforms during sterile inflammation

To better understand the mechanisms underlying these observations, we investigated NF-κB activation in RAW 264.7 cells under various conditions. NF-κB is a pivotal transcription factor involved in the regulation of cytokine production and innate immune responses, particularly in response to microbial stimuli such as LPS (21). LPS was chosen because it is a well-characterized inducer of TLR4-mediated inflammatory responses, mimicking

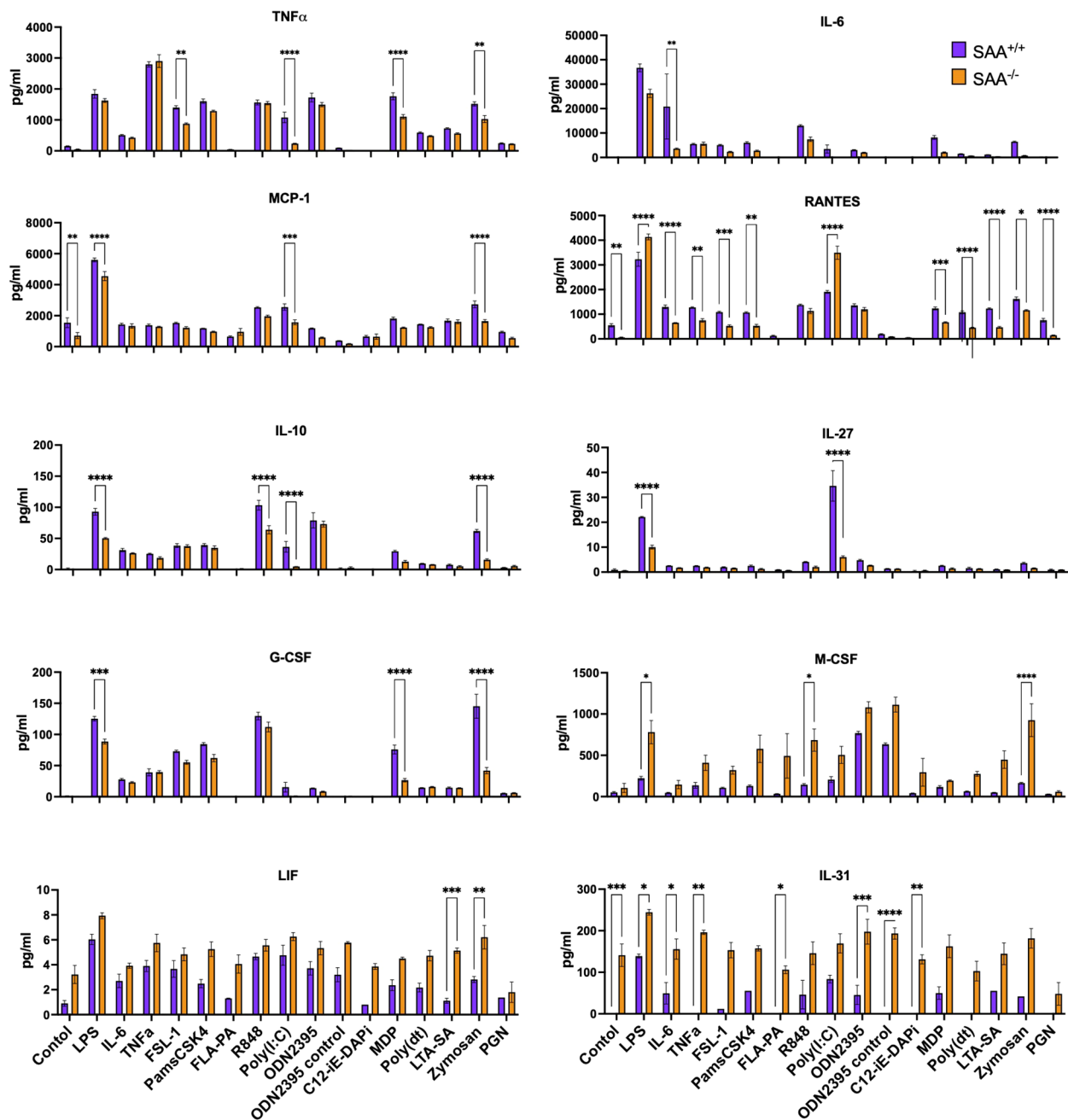


FIGURE 1

Measurement of cytokine and chemokine levels in response to stimulation with various immune ligands. SAA^{+/+} and SAA^{-/-} mice BMDMs were stimulated with a single dose of LPS, IL-6, TNF α , FSL-1, Pam3CSK4, FLA-PA, R-848, Poly(I), ODN2395, C12-iE-DAP, MDP, Poly dT, LTA-SA, Zymosan, and Peptidoglycan overnight. Untreated BMDM cells served as controls. After overnight incubation of cells, the concentrations of TNF α , IL-6, MCP-1, RANTES, IL-10, IL-27, G-CSF, M-CSF, LIF, and IL-31 were determined. Significant differences between conditions are indicated by asterisks: * (p < 0.05), ** (p < 0.01), *** (p < 0.001), and **** (p < 0.0001), one of the two representative experiment was presented (n=10 mice). A full list of the ligands, their corresponding receptors, and the type of infection they mimic (bacterial, viral, fungal, or sterile) is provided as [Supplementary Table 1](#).

sterile endotoxemia and triggering the release of inflammatory mediators (22). RAW cells were either genetically modified to overexpress SAA genes (SAA1, SAA2, SAA3, and SAA4) or treated with purified SAA proteins, with and without LPS stimulation. These experiments aimed to assess whether SAA

proteins directly or indirectly enhance NF- κ B activity during sterile inflammation.

Figure 2 illustrates the results of these experiments. Figure 2A, a heatmap shows NF- κ B activity in RAW cells overexpressing different SAA isoforms. In the absence of agonists, NF- κ B activity

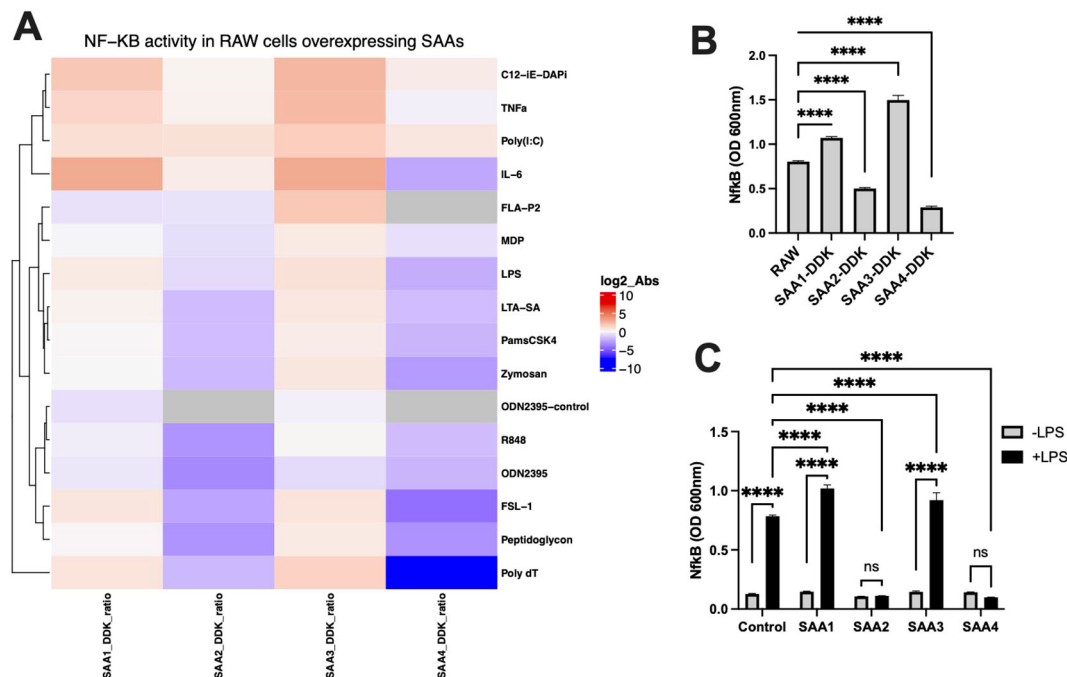


FIGURE 2

NF-κB activation in RAW cells overexpressing SAA proteins was assessed in response to stimulation with various immune ligands, or treatment with purified SAA proteins, compared to the response to LPS in control RAW cells. RAW-Blue™ cells (RAW 264.7) overexpressing SAA proteins were treated with a single dose of LPS, IL-6, TNFα, FSL-1, Pam3CSK4, FLAP2, R-848, Poly(I), ODN2395, C12-iE-DAP, MDP, Poly dT, LTA-SA, Zymosan, and Peptidoglycan overnight to activate NF-κB. Non-stimulated cells served as negative controls. **(A)** Heatmap showing NF-κB activity in RAW cells overexpressing SAA proteins (SAA1, SAA2, SAA3, SAA4) normalized against control RAW cells, with log2-transformed absorbance values (@600nm) displayed. **(B)** Representative bar graph of NF-κB activity in response to LPS stimulation in RAW cells overexpressing DDK-tagged SAA proteins (SAA1-DDK to SAA4-DDK) compared to unmodified RAW cells (n = 3). **(C)** NF-κB activity in RAW cells treated with purified SAA proteins (SAA1-100ng/ml, SAA2-10ng/ml, SAA3-10ng/ml, SAA4-100ng/ml) in the absence or presence of LPS (n = 3). Data represent mean ± SEM. Statistical significance is denoted as * (p < 0.05), ** (p < 0.01), *** (p < 0.001), and **** (p < 0.0001). (ns = non-significant).

remained low across all groups. Upon different agonists stimulation, however, cells overexpressing SAA1 and SAA3 proteins exhibited significantly elevated NF-κB activity compared to SAA2 and SAA4 overexpressed cells. **Figure 2B**, a bar graph depicts NF-κB activity in RAW cells over-expressing DDK-tagged SAA isoforms. This finding aligns with previous reports that LPS activates TLR4-mediated pathways, with downstream activation of NF-κB as a key regulator of pro-inflammatory cytokine production (21, 23). Consistent with *Panel A*, all DDK-tagged SAA groups showed significant changes in NF-κB activity upon LPS stimulation compared to unmodified RAW cells. These results further validate the role of SAA proteins in modulating inflammatory signaling during sterile inflammation.

Figure 2C examines the effects of treating RAW cells with purified SAA proteins. Without LPS, purified SAA proteins did not significantly activate NF-κB, regardless of the isoform or concentration. However, under LPS stimulation, RAW cells treated with purified SAA1 and SAA3 proteins exhibited altered NF-κB activity compared to untreated RAW cells, whereas cells treated with SAA2 and SAA4 showed no significant changes in NF-κB activity due to stimulation. These findings underscore the dependency of SAA-driven NF-κB activation on microbial stimulation, reinforcing their role in amplifying sterile inflammatory responses (24, 25). Together

our result show that SAA1 and SAA3 proteins amplify NF-κB activation and pro-inflammatory cytokine responses, whereas SAA2 and SAA4 proteins inhibit pro-inflammatory cytokine responses particularly under LPS-stimulated conditions.

Survival and cytokine profiles following LPS challenge

To investigate the systemic implications of SAA proteins during sterile inflammation, we assessed survival and cytokine profiles in SAA^{+/+} and SAA^{-/-} mice following an intraperitoneal LPS injection (8 mg/kg). These experiments were designed to evaluate whether SAA proteins modulate survival and systemic inflammation under these conditions.

As shown in **Figure 3A**, SAA^{-/-} mice exhibited significantly improved survival rates compared to SAA^{+/+} mice over the 7-day observation period. This survival benefit highlights a potential inflammatory role of SAA proteins in mitigating lethal systemic inflammation.

To explore the underlying mechanisms, we quantified plasma levels of key cytokines and chemokines 16 hours post-LPS injection. **Figures 3C, D** reveal significantly elevated levels of pro-

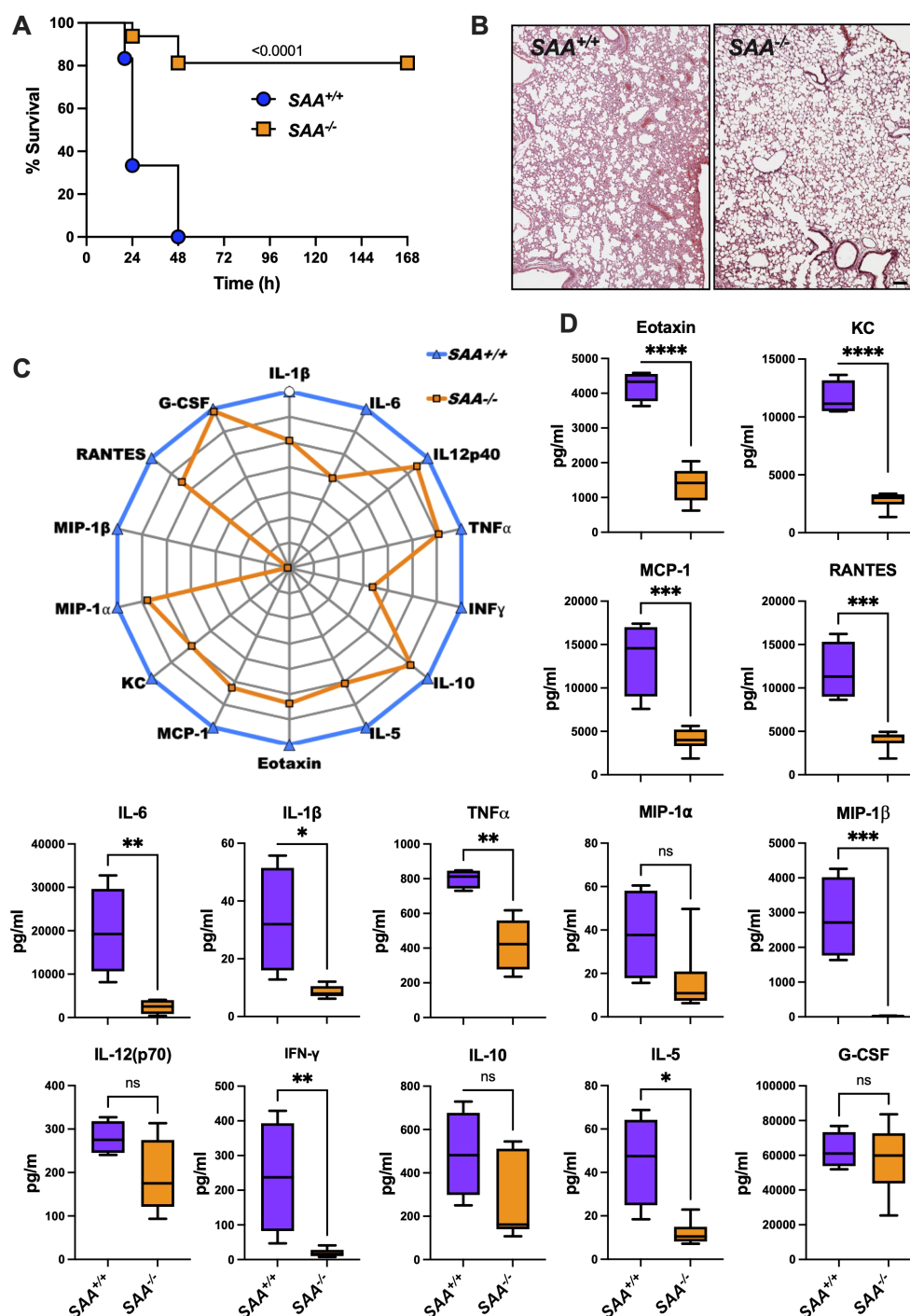


FIGURE 3

Survival, lung histology, and cytokine profiles in $SAA^{+/+}$ and $SAA^{-/-}$ mice following LPS challenge. (A) Kaplan-Meier survival curves of $SAA^{+/+}$ (n=6; blue) and $SAA^{-/-}$ (n=16; orange) mice i.p. injected with LPS (8 mg/kg). Mice were monitored over a 7-day period post-injection to assess survival rates. (B) Representative histological images of lung tissue from $SAA^{+/+}$ and $SAA^{-/-}$ mice 16 hours after LPS injection. Hematoxylin and eosin (H&E) staining shows tissue architecture, with each panel displaying one representative section from each genotype. (C) $SAA^{+/+}$ and $SAA^{-/-}$ mice were injected for 16 hours with LPS before blood was drawn. Normalized radar plot depicting the plasma levels of cytokines and chemokines, including IL-1 β , IL-6, IL-12p40, TNF α , INF- γ , IL-10, IL-5, Eotaxin, G-CSF, MCP-1, KC, MIP-1 α , MIP-1 β , RANTES, and G-CSF in $SAA^{+/+}$ (blue line) and $SAA^{-/-}$ (orange line) mice. Cytokine levels were measured 16 hours after LPS injection ($SAA^{+/+}$ (n=6) and $SAA^{-/-}$ (n=4)). (D) Box plots showing quantitative comparisons of cytokine concentrations (pg/ml) between $SAA^{+/+}$ (purple) and $SAA^{-/-}$ (orange) mice for Eotaxin, KC, MCP-1, RANTES, IL-6, IL-1 β , TNF α , MIP-1 α , MIP-1 β , IL-12(p70), INF- γ , IL-10, IL-5, G-CSF. Data represent individual measurements with median and interquartile ranges indicated. Data are presented as mean $\pm$ SEM. Statistical significance is indicated by asterisks: * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), and **** ($p < 0.0001$).

inflammatory mediators, including TNF α , IL-6, and IL-1 β , in SAA^{+/+} mice compared to SAA^{-/-} mice. These cytokines are critical drivers of systemic inflammation, and their elevation suggests that SAA proteins amplify inflammatory signaling in response to LPS (11, 19). Interestingly, anti-inflammatory cytokines such as IL-10 showed no significant differences between genotypes, indicating a selective enhancement of pro-inflammatory pathways by SAA proteins.

Chemokine analysis demonstrated higher levels of MCP-1, KC, and RANTES in SAA^{+/+} mice, supporting enhanced monocyte and neutrophil recruitment. Notably, eotaxin levels were also significantly elevated in SAA^{+/+} mice, suggesting a broader regulatory role for SAA proteins in immune cell recruitment (14).

Histological analysis of lung tissue collected 16 hours after LPS injection (Figure 3B) showed less severe inflammatory damage in SAA^{-/-} mice compared to SAA^{+/+} mice. These observations suggest that SAA proteins not only amplify inflammatory signaling but may also play a role in protecting against end-organ damage during systemic inflammation. To conclude, the results demonstrate that SAA proteins enhance systemic inflammatory signaling while leading to a survival disadvantage in LPS-induced endotoxemia. This dual role highlights their importance in modulating inflammatory responses during sterile inflammation.

Role of SAA proteins in bacterial clearance and cytokine modulation

To investigate the role of SAA proteins during bacterial infections, we evaluated bacterial clearance, cytokine responses, and organ-specific inflammatory changes in SAA^{+/+} and SAA^{-/-} mice following intranasal infection with *P. aeruginosa*. These experiments aimed to determine whether SAA proteins modulate immune responses to bacterial pathogens distinctively compared to sterile inflammation. As shown in Figure 4A, bacterial burden in the lungs of SAA^{+/+} mice was significantly lower than in SAA^{-/-} mice 24 hours post-infection, indicating enhanced bacterial clearance in the presence of SAA proteins. Similarly, bacterial counts were reduced in extrapulmonary organs, including the liver, spleen, and kidney, suggesting that SAA proteins play a systemic role in controlling bacterial dissemination.

Cytokine analysis of plasma revealed significantly higher levels of IL-6, TNF α , and MCP-1 in SAA^{-/-} mice compared to SAA^{+/+} mice (Figure 4B). These cytokines are key mediators of the innate immune response, facilitating pathogen clearance through immune cell activation and recruitment (26, 27). Interestingly, anti-inflammatory cytokines such as IL-10 exhibited no significant differences between genotypes, highlighting a pro-inflammatory bias associated with SAA proteins during bacterial infections. Histological examination of lung tissue (Figure 4C) revealed reduced inflammatory damage in SAA^{+/+} mice compared to SAA^{-/-} mice, characterized by less alveolar infiltration of neutrophils and fewer areas of necrosis. This suggests that SAA proteins may mitigate excessive tissue damage while supporting effective pathogen elimination. To summarize, these results highlight that SAA proteins enhance bacterial clearance and

regulate inflammatory cytokine responses, facilitating pathogen elimination while minimizing severe tissue damage.

Differential expression of SAA isoforms in response to inflammation and infection

To examine the expression patterns of SAA isoforms under different inflammatory conditions, we measured SAA1, SAA2, SAA3, and SAA4 levels in SAA^{+/+} mice across three experimental groups: healthy controls, LPS-treated (sterile inflammation), and *P. aeruginosa*-infected (bacterial infection). These experiments aimed to determine whether distinct inflammatory stimuli elicit isoform-specific responses and how these differences contribute to systemic immune modulation. As shown in Figure 5, baseline SAA1-4 protein levels were undetectable in healthy control mice. Following LPS treatment, SAA1 and SAA2 levels were significantly elevated compared to baseline, reflecting their acute-phase response to sterile inflammation (28, 29). In contrast, SAA3 showed minimal induction, and SAA4 levels increased only modestly. In response to *P. aeruginosa* infection, SAA1 and SAA2 levels were also significantly upregulated but to a lesser extent than in the LPS-treated group. These findings suggest that SAA proteins respond more robustly to sterile inflammation than to bacterial infections, consistent with the notion that SAA1 and SAA2 are highly inducible under acute-phase conditions (30, 31).

These findings demonstrate that SAA1, SAA2 and SAA4 are key acute-phase responders, with their expression patterns varying significantly between sterile and bacterial inflammatory stimuli, reflecting their distinct roles in systemic immune regulation. Together, these findings reveal that SAA proteins play distinct roles in sterile and non-sterile inflammation, amplifying cytokine production and NF- κ B activation in sterile conditions while promoting bacterial clearance and minimizing tissue damage during infections. This highlights their ability to tailor immune responses to specific inflammatory stimuli.

Discussion

Key findings

In this study, we investigated how SAA proteins modulate inflammatory responses during sterile endotoxemia and bacterial infections. Comprehensive *in vitro* and *in vivo* experiments were conducted to evaluate the effects of SAA proteins on cytokine and chemokine production, immune signaling pathways, and survival outcomes. Our findings demonstrate that SAA proteins exhibit context-dependent roles, amplifying inflammation in sterile conditions while promoting bacterial clearance during infection (32).

Understanding the distinct roles of SAA proteins in sterile and non-sterile inflammation is pivotal for bridging gaps in our knowledge of immune regulation and disease pathogenesis. Sterile inflammation, driven by damage-associated molecular patterns (DAMPs), underpins conditions such as sepsis, atherosclerosis,

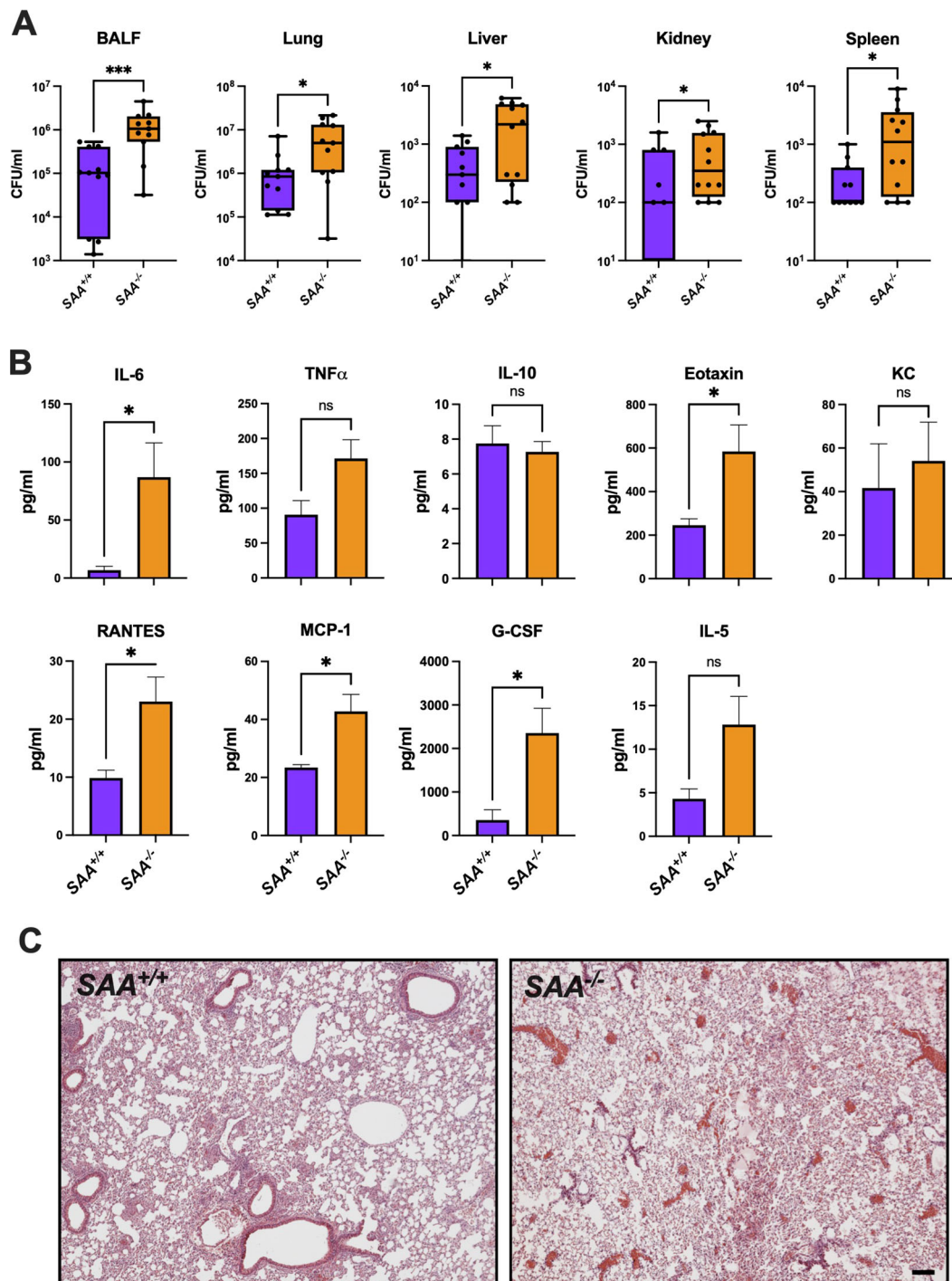


FIGURE 4

Bacterial burden, lung histology, and cytokine concentrations in SAA^{+/+} and SAA^{-/-} mice. SAA^{+/+} and SAA^{-/-} mice were infected i.n. with *P. aeruginosa* for (16 h). **(A)** Bacterial cfu counts in BALF, lung, liver, kidney, and spleen from *P. aeruginosa* infected SAA^{+/+} and SAA^{-/-} mice (n = 11) are shown. **(B)** The levels of inflammatory (IL-6, TNF α , RANTES, Eotaxin, KC, MCP-1, G-CSF, IL-5, IL-10) in SAA^{+/+} (n = 4) and SAA^{-/-} (n = 5) mice infected with *P. aeruginosa* were determined. **(C)** Representative lung histology sections (n = 3) from SAA^{+/+} and SAA^{-/-} mice treated with LPS are depicted. Data are presented as mean $\pm$ SEM. Statistical significance is indicated by * (p < 0.05), ** (p < 0.01), *** (p < 0.001), and **** (p < 0.0001). (ns = non-significant).

and autoimmune diseases, where unchecked immune responses lead to tissue damage and adverse clinical outcomes (32). In contrast, non-sterile inflammation, triggered by pathogen-associated molecular patterns (PAMPs) during microbial infections, requires a delicate balance between inflammatory

responses and pathogen clearance to prevent systemic immune disruption (33). Positioned at the intersection of these inflammatory cascades, SAA proteins act as acute-phase reactants, orchestrating cytokine and chemokine networks to shape immune dynamics (34, 35).

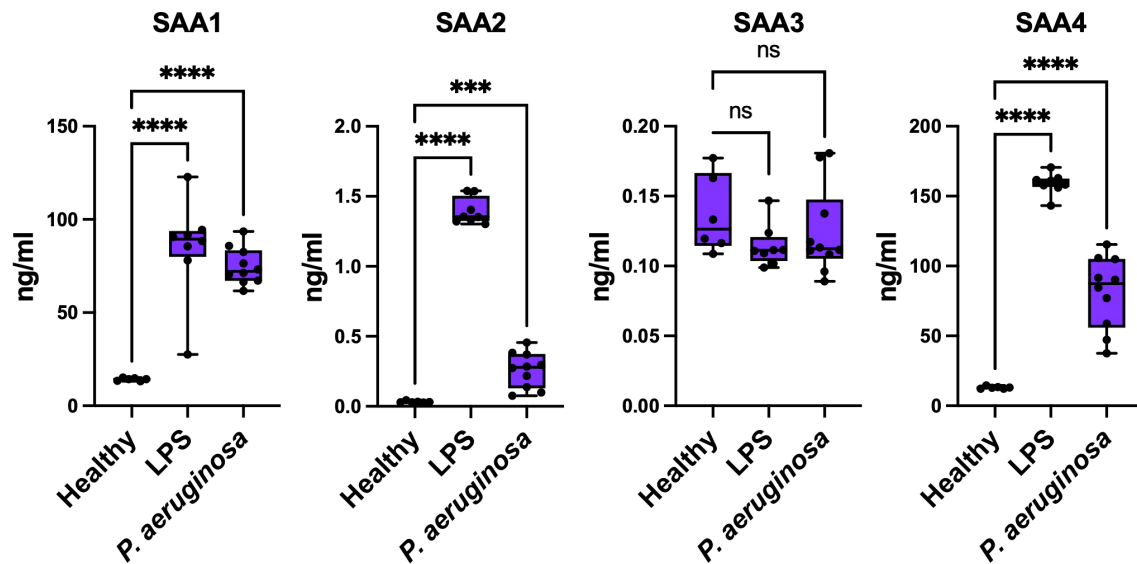


FIGURE 5

Concentrations of SAA proteins in response to LPS and *P. aeruginosa* infection. Concentrations of SAA1, SAA2, SAA3, and SAA4 were measured in samples from Healthy, LPS-treated, and *P. aeruginosa*-infected conditions (Healthy $n = 6$; LPS $n=8$; and *P. aeruginosa* $n=10$). Data represent mean $\pm$ SEM. Statistical significance is indicated by * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), and **** ($p < 0.0001$). (ns = non-significant).

Mechanisms of action

To investigate how SAA proteins regulate inflammatory signaling, immune cell recruitment, and survival across these contrasting contexts, we conducted comprehensive *in vitro* and *in vivo* experiments. Our findings reveal that SAA proteins amplify cytokine and chemokine responses, a function particularly critical in sterile inflammation driven by DAMPs such as LPS (1). Cytokine and chemokine profiling as outlined in Figure 1 demonstrates significantly higher levels of pro-inflammatory mediators, such as IL-6 and MCP-1, in SAA^{+/+} macrophages compared to SAA^{-/-} cells. These results reinforce the established role of SAA proteins in enhancing immune signaling networks (36) and align with previous studies linking proteins activity to immune cell recruitment during inflammatory responses (37).

Mechanistically, the activation of NF- κ B by SAA proteins, as shown in Figure 2, offers valuable insights into how these acute-phase reactants amplify inflammatory signaling. NF- κ B serves as a central regulator of pro-inflammatory cytokine production and plays a critical role in mediating sterile inflammation. The results demonstrate that RAW cells overexpressing SAA1 and SAA3 protein isoforms exhibited significantly enhanced NF- κ B activity following LPS stimulation. Our finding aligns with previous reports suggesting that SAA proteins may not directly engage TLR4 but instead enhance its activation indirectly through interactions with LPS. Studies by others demonstrated that the binding of SAA proteins bind to LPS, can stabilize the LPS-TLR4 complex that amplifies downstream signaling pathways (38). This indirect mechanism provides a plausible explanation for the observed NF- κ B activation in our study, particularly in the context of sterile

inflammation where LPS is a primary agonist. To conclude, isoform-specific differences in NF- κ B activation observed in our study point to potential specialization among SAA proteins, a hypothesis that warrants further investigation (39).

Functional implications

Functionally, the survival analysis in LPS-induced endotoxemia, presented in Figure 3, provides compelling evidence for the protective role of SAA proteins in sterile inflammation.

SAA^{-/-} mice exhibited significantly improved survival compared to their SAA^{+/+} counterparts, likely reflecting a balance between enhanced immune activation and the mitigation of excessive tissue damage. This dual role of SAA proteins in amplifying inflammation while promoting its resolution, has also been observed in clinical studies linking SAA protein activity to better outcomes in sepsis and other inflammatory disorders (1). Collectively, these findings underscore SAA proteins as key modulators of inflammation, with distinct mechanisms tailored to sterile and infectious contexts. By integrating cytokine profiling, mechanistic insights, and survival outcomes, our results provide a comprehensive framework for understanding the dual functionality of SAA proteins and their therapeutic potential.

Building on these findings, our study further highlights the versatility of SAA proteins in adapting to distinct inflammatory demands. During sterile endotoxemia, SAA proteins amplify cytokine production, while in infectious contexts, they facilitate bacterial clearance. This evolutionarily conserved ability to modulate immune responses underscores their critical role as acute-phase reactants in maintaining immune homeostasis (40).

Clinical implications

In sterile inflammation, the engagement of TLR4 by SAA proteins triggers NF- κ B activation, driving the production of pro-inflammatory cytokines such as IL-6 and MCP-1 (41). This mechanism underscores the pivotal role of SAA proteins in sterile inflammatory pathways and its significance in systemic conditions such as sepsis and autoimmune diseases. Conversely, in infectious inflammation, SAA proteins interact with bacterial PAMPs to enhance immune cell recruitment and pathogen clearance. This dual role is exemplified by the opsonizing properties of SAA proteins, which promote phagocytosis and pathogen neutralization (42). Our findings on bacterial burden reduction in SAA^{+/+} mice further support the role of SAA proteins in strengthening innate immune defenses (43).

The context-specific versatility of SAA proteins highlights their importance in balancing inflammatory amplification and resolution, reflecting a broader principle of immune homeostasis. These findings also emphasize the therapeutic potential of SAA proteins or its analogs, particularly in disorders like sepsis and bacterial infections. However, this adaptability warrants caution in therapeutic strategies; targeting SAA-TLR4 interactions or NF- κ B signaling must preserve the protective functions of SAA proteins while mitigating excessive inflammation.

Future research should focus on isoform-specific targeting of SAA proteins to optimize therapeutic efficacy while minimizing potential risks. For instance, SAA1 and SAA2, which are robustly induced during inflammation, likely differ functionally from SAA4, whose constitutive expression suggests a distinct role in maintaining immune homeostasis (39). A deeper understanding of these isoform-specific functions could facilitate the development of precision therapies for inflammatory and infectious diseases.

Limitations

Despite the valuable insights provided by our study into the roles of SAA proteins in sterile and bacterial inflammation, several limitations should be acknowledged. While murine models are indispensable for dissecting immune mechanisms, their relevance to human inflammatory responses requires further validation. Moreover, the focus on NF- κ B signaling in this study leaves other pathways, such as JAK/STAT and MAPK, relatively underexplored. Additionally, the analysis was limited to specific cytokines and chemokines, and broader profiling or time-course studies could capture more comprehensive inflammatory dynamics. Direct evaluation of SAA protein-targeted interventions remains an important area for future research.

Future directions

Future studies should focus on isoform-specific functions of SAA proteins, particularly SAA1 and SAA3, to clarify their distinct roles in immune regulation (24, 36). Investigating alternative pathways, such as JAK/STAT and RAGE, and validating findings in human systems will be critical for translating preclinical insights into clinical applications (44, 45). From a therapeutic perspective,

targeting SAA protein-mediated pathways offers promising opportunities for managing inflammation in both sterile and infectious conditions. For instance, modulating TLR4-SAA protein interactions or developing isoform-specific inhibitors could mitigate excessive inflammation during sterile endotoxemia while preserving the protective functions of SAA proteins in bacterial clearance. Additionally, SAA proteins hold potential as biomarkers for assessing inflammation severity and therapeutic response, particularly in diseases such as sepsis and COVID-19, where precise immune modulation is essential (10). By addressing these gaps, future research could position SAA proteins as key targets for diagnostic and therapeutic innovations, providing novel strategies for managing inflammatory diseases.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was approved by Animal Ethics Committee of Malmö/Lund, Sweden. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

TM: Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing. KM: Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. HG: Data curation, Investigation, Methodology, Writing – original draft, Writing – review & editing. AT: Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. MP: Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. PP: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. HH: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2025.1544085/full#supplementary-material>

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CD121b-positive neutrophils predict immunosuppression in septic shock

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Background: Septic shock is linked with high mortality and significant long-term morbidity in survivors. However, the specific role of neutrophils in septic shock pathophysiology remains scarce in recent research.

Methods: Peripheral blood immune cells from healthy donors and patients with septic shock were analyzed using single-cell RNA sequencing and batch RNA sequencing. We measured serum CD121b in both patients and healthy donors. Peripheral immune cells were isolated and exposed to either a CD121b recombinant protein or a CD121b blocking antibody to evaluate the expression of inflammatory factors. Additionally, in a humanized mouse sepsis model, the expression of CD121b in neutrophils across different tissues was assessed following treatment with all-trans retinoic acid (ATRA).

Results: This study identified a subset of CD10⁺CD121b⁺ neutrophils in the peripheral blood of patients with septic shock. These patients exhibited elevated concentrations of soluble CD121b in serum and urine. Furthermore, outcomes revealed that the presence of CD121b⁺ neutrophils positively correlated with the severity of septic shock. These cells displayed immunosuppressive characteristics; after blocking CD121b, proinflammatory cytokines increased in peripheral immune cells. Additionally, we found that treatment with ATRA down-regulated the expression of CD121b.

Conclusions: CD121b is closely associated with the progression of septic shock and may serve as a potential predictor indicator of immunosuppression for the condition.

KEYWORDS

septic shock, neutrophils, immunosuppression, CD121b, cytokine

Introduction

Sepsis, a life-threatening condition, is marked by a dysregulated immune response that involves the simultaneous occurrence of hyperinflammation and immune suppression (1, 2). Septic shock, the utmost severe form of sepsis, remains a major global healthcare challenge, leading to significant morbidity and mortality (3–5). While initially underrecognized, severe immunosuppression in sepsis patients is now well-researched and linked to harmful outcomes, primarily due to an increased susceptibility to secondary hospital-acquired infections (6, 7).

Outcomes from immunomodulatory treatments have been underwhelming due to the difficulty in identifying when patients enter the immunosuppressive phase of sepsis. *Research gap*: there is an urgent need for new and effective biomarkers that can definitively diagnose sepsis-induced immunosuppression in clinical settings.

CD121b designates the recombinant protein for IL-1 receptor II (IL1R2), encoded by the IL1R2 (8). It is a vital component of the IL-1 receptor family. In contrast to other family members, IL-1R2 possesses a shorter cytoplasmic domain. It lacks the Toll/IL-1 receptor (TIR) domain, rendering it incapable of initiating signal transduction (9, 10). The IL-1R2 protein has two forms: a membrane-bound receptor and a soluble variant (11). Its primary mechanism of action as a decoy receptor involves competitively capturing IL-1 at the cell surface and binding IL-1 in the microenvironment, thereby effectively inhibiting IL-1 activity (12). Recent research has identified immature CD121b⁺ neutrophils in patients with sepsis (13). CD121b is a decoy receptor for IL-1, suggesting that the subpopulation of CD121b⁺ neutrophils may have immunosuppressive effects (12, 14). However, researchers have not yet thoroughly investigated the impact and underlying mechanisms of CD121b⁺ neutrophils on sepsis progression. The present *research aimed* to assess the extent of CD121b expression in patients with septic shock and investigate its relationship with the severity of sepsis. Additionally, we examined whether targeting CD121b could effectively treat sepsis-induced immunosuppression. The *core hypotheses* of this study are as follows: 1) CD121b⁺ neutrophils are significantly elevated in patients with septic shock compared to those with sepsis alone, 2) The impact of CD121b⁺ neutrophils on the progression of sepsis is associated with immune suppression, 3) All-trans retinoic acid (ATRA) could downregulate CD121b expression on neutrophils and can be utilized for alleviating sepsis-induced immunosuppression.

Abbreviations: IL1R2, IL-1 receptor II; TIR, Toll/IL-1 receptor; ATRA, All-trans retinoic acid; HDs, Healthy donors; RBC, red blood cell; PBS, phosphate-buffered saline; FACS, Flow Cytometry and Fluorescence-Activated Cell Sorting; ELISAs, Enzyme-linked immunosorbent assays; LPS, Lipopolysaccharide; FBS, fetal bovine serum; G-CSF, granulocyte-colony stimulating factor; CRP, C-reactive protein; PCT, procalcitonin; G-MDSCs, Granulocytic myeloid-derived suppressor cells; WBCs, white blood cells; sCD121b, soluble CD121b.

Materials and methods

Patient recruitment

From September 2021 to December 2023, every consecutive patient admitted to the Intensive Care Unit of the First Affiliated Hospital of the University of Science and Technology of China, Hefei, China, was enrolled. All patients (aged 18–90 years, sex not specified) met the clinical criteria defined by Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). The segregation criteria were defined as follows: (1) pregnancy or lactation, (2) advanced-stage cancer with cachexia, and (3) granulocytopenia. We collected blood samples from ICU-admitted patients (ICU-Ad) within the first 24 hours of ICU admission. We obtained samples from ICU survivors (ICU-Survivor) within 24 hours of ICU discharge, meanwhile collecting samples from patients in recovery (Recovery) within 24 hours of hospital discharge.

Moreover, blood samples from stabilized ICU patients (ICU-Hosp) were collected within 24 hours of circulatory stabilization. The control group comprised healthy donors (HDs) aged 18 to 90 years, with no sex specification. Entire patients and their legal representatives gave written informed consent. **Table 1** outlines the clinical data for patients suffering from sepsis and for HDs. The *SI Appendix, Supplementary Table S1*, shows detailed information on patients.

Mice and treatment

Female NOD/ShiLtJGpt-Prkdc^{em26Cd52}Il2rg^{em26Cd22}/Gpt (NCG) mice (6–8 weeks; catalog number: T001475) were purchased from GemPharmatech (Nanjing, China) for the generation of humanized mouse models. We housed all mice under specific pathogen-free conditions. White blood cells (WBCs) (1×10^7) from patients with septic shock were intravenously injected into NCG mice through the tail vein to create a humanized mouse model. We gave mice ATRA (ATRA treatment group; 20mg/kg) or the corresponding solvent (Vehicle group); 24 hours later, we collected the liver, lungs, and spleen of mice for flow cytometry.

Ethics

The Medical Research Ethics Committee of the First Affiliated Hospital of the University of Science and Technology of China approved this study (2022KY Ethics number 158). The Ethics Committee of the University of Science and Technology of China (Beijing, China) approved all animal experimental procedures, and these were undertaken following the National Guidelines for Animal Usage in Research in China (USTCACUC25120122038).

TABLE 1 Baseline characteristics of patients, stratified according to study group.

	Healthy donors (n=113)	Non-sepsis (n=37)	Septic non-shock (n=46)	Septic shock (n=127)
Age, years, mean($\pm$ SEM)	51.0 ($\pm$ 1.3)	62.1 ($\pm$ 2.5)	64.5 ($\pm$ 2.0)	66.3 ($\pm$ 1.1)
Sex				
Male	75 (66.4%)	20 (54.1%)	31 (67.4%)	89 (70.1%)
Female	38 (33.6%)	17 (45.9%)	15 (32.6%)	38 (29.9%)
Source of infection				
Pulmonary	/	/	39	51
Abdominal	/	/	5	41
Urinary	/	/	1	9
Blood	/	/	0	6
Other	/	/	1	20
APACHE II, median (IQR)	/	12.0 (8.0 to 16.5)	16.0 (12.0 to 22.0)	21.0 (17.0 to 26.0)
SOFA, median (IQR)	/	0.0 (0.0 to 3.0)	6.0 (4.0 to 8.0)	6.0 (4.0 to 9.0)
Lactate, mmol/L, median (IQR)	/	/	2.0 (1.3 to 3.1)	10.0 (5.0 to 16.0)
ICU LOS, days, median (IQR)	/	2.0 (1.0 to 4.0)	11.0 (7.0 to 20.3)	3.4 (2.5 to 5.7)

Data are presented as n (%); mean ($\pm$ SEM) or median with IQR (25th to 75th percentiles). APACHE II, acute physiology and chronic health evaluation score; SOFA, Sequential Organ Failure Assessment; LOS, length of stay.

Blood sample collection, preparation

The authors collected peripheral blood samples from donors following ethical guidelines and under sterile conditions. Samples were centrifuged at $600 \times g$ for 10 minutes at room temperature to separate serum. For red blood cell (RBC) lysis, 2 mL of RBC Lysis Buffer was added to each tube containing up to 100 μ L of whole blood, followed by gentle vortexing. The samples were then incubated at room temperature, protected from light, for 10 minutes. After incubation, we centrifuged the cell suspension at $350 \times g$ for 10 minutes and carefully aspirated the supernatant without disturbing the cell pellet. We resuspended the resulting cell pellet in phosphate-buffered saline (PBS) for subsequent experiments.

Flow cytometry and fluorescence-activated cell sorting

Human WBCs or mouse mononuclear cells isolated from the liver, lungs, and spleen resuspended in 100 μ L of PBS containing a pre-mixed antibody cocktail. Cells were incubated at 4°C for 30 minutes, followed by two washes with cold PBS. Flow cytometric analysis occurred on a FACSCelesta™ system (BD Biosciences, Franklin Lakes, NJ, USA). For FACS purification, cells were sorted on a FACS Aria™ III Cell Sorter (BD Biosciences) using 1 $\times$ PBS as the sheath fluid. Live cells were first gated by excluding zombie dye-positive cells, then removing debris using forward scatter area (FSC-A) and side scatter area (SSC-A) parameters. We excluded cell doublets and aggregates using FSC-width (FSC-W) and SSC-width

(SSC-W) parameters. Neutrophils were identified and isolated based on CD45⁺CD14⁺CD16⁺ expression.

Enzyme-linked immunosorbent assays

Serum levels of IL-1 β , IL-4, IL-8, IL-10, IL-1R2, and IL-1RA were quantified using ELISA following the manufacturer's protocols. Briefly, serum samples were applied to pre-coated 96-well plates and incubated with detection antibodies for 60 minutes at room temperature. After three washes, we incubated the plates with streptavidin-horseradish peroxidase for 20 minutes at room temperature. We added 3,3',5,5'-Tetramethylbenzidine substrate solution and stopped the enzymatic reaction after 2–5 minutes. Absorbance was measured at 450 nm using a plate reader.

RT-qPCR

We extracted total RNA using TsingZol Reagent (TSP401; Tsingke Biotech, Beijing, China) and reverse transcribed into complementary DNA (cDNA) using MonScript™ RTIII All-in-One Mix with dsDNase (MR05101M; Monad Laboratories, New York, NY, USA). We used gene-specific primers to amplify target genes and quantified relative gene expression using real-time quantitative PCR (RT-qPCR) with the SYBR Green Pre-mix Pro Taq HS qPCR Kit (AG11746; Accurate Biology, Salem, NC, USA). We used β -actin as the internal control for normalization. Table 2 shows the primer sequences used for qRT-PCR.

TABLE 2 qRT-PCR primers used in this study, related to STAR Methods.

Targeted gene	NCBI Gene ID.	Primer sequence
Human		
<i>ACTB</i>	60	Forward: TTGCCGACAGGATGCAGAA
		Reverse: GCCGATCCACACGAGTACTT
<i>IL1R2</i>	7850	Forward: ATGTTGCGCTTGTACGTGTG
		Reverse: CCCGCTTGTAATGCCTCCC
<i>IL1B</i>	3553	Forward: ATGATGGCTTATTACAGTGCCAA
		Reverse: GTCGGAGATTCGTAGCTGGA
<i>IL6</i>	3569	Forward: ACTCACCTCTTCAGAACGAATTG
		Reverse: CCATCTTTGGAAGGTTCAAGTTG
<i>TNF</i>	7124	Forward: CCTCTCTCTAATCAGCCCTCTG
		Reverse: GAGGACCTGGGAGTAGATGAG
<i>IL8</i>	3576	Forward: TTTTGCCAAGGAGTGCTAAAG
		Reverse: AACCTCTGCACCCAGTTTTC
<i>CD10</i>	4311	Forward: AGAAGAAACAGCGATGGACTCC
		Reverse: CATAGAGTGCGATCATTGTCACA

Peripheral blood smears and Wright–Giemsa staining

A 50-μL aliquot of human peripheral blood was aspirated and placed at one end of a glass slide using a micropipette. A second glass slide (the spreader) was positioned at an approximate 30° angle and gently brought into contact with the blood droplet. We retracted the spreader slide to allow even distribution of the blood between the two slides, followed by a forward motion to create a uniform smear. Wright–Giemsa stain (0.2–0.6 mL) was then applied, covering the entire smear, and left for 1 minute. We added an equal volume of PBS to the stain, mixed it gently, and incubated it for 3 minutes. We rinsed the slides with water, air-dried them, and subsequently examined them under a microscope for morphological analysis.

Bulk RNA sequence

We extracted total RNAs from human neutrophils using TRIzol™ Reagent (15596026; Invitrogen, Carlsbad, CA, USA). We carried out DNA digestion after RNA extraction using DNaseI. We determined RNA quality by measuring absorbance at an excitation wavelength of 260 nm and an emission wavelength of 280 nm with a spectrophotometer (Nanodrop™; Thermo Fisher Scientific, Waltham, MA, USA). We confirmed RNA integrity by electrophoresis using 1.5% agarose gels. We quantified qualified RNAs by Qubit3.0 with the Qubit™ RNA Broad Range Assay kit (Q10210; Life Technologies, Carlsbad, CA, USA). We used 2 μg of total RNAs to prepare RNA sequencing libraries using the KC-Digital™ Stranded mRNA Library Prep Kit (DR08502; Wuhan Seqhealth,

Wuhan, China) for an Illumina (San Diego, CA, USA) platform following the manufacturer's instructions. This kit eliminated duplication bias in PCR and sequencing steps using a unique molecular identifier (UMI) of eight random bases to label pre-amplified cDNA molecules. Library products corresponding to 200–500 bps were enriched, quantified, and sequenced on a DNBSEQ-T7 sequencer (MGI Tech, Beijing, China) with the PE150 model. We started by filtering the raw sequencing data, eliminating low-quality reads and reducing adaptor-sequence-contaminated reads. Clean reads were treated with in-house scripts to eliminate the duplication bias introduced in library preparation and sequencing. In brief, clean reads were first clustered according to UMI sequences (i.e., reads with the same UMI sequence were grouped into the same cluster). Reads in the same cluster were compared by pairwise alignment, and then reads with sequence identity >95% were extracted to a new sub-cluster. After generating all sub-clusters, we carried multiple sequence alignments to obtain one consensus sequence for each sub-cluster. After these steps, we eliminated any errors and biases introduced by PCR amplification or sequencing.

Single-cell collection, construction and sequencing of the library

Single-cell suspensions (2×10^5 cells/mL) with PBS were loaded onto a microwell chip using the Matrix® Single Cell Processing System (Singleron, Nanjing, China). Barcoding beads were collected from the microwell chip, followed by reverse transcription of the mRNA captured by the barcoding beads to obtain cDNA and PCR amplification. Then, we fragmented the amplified cDNA, ligated it with sequencing adapters, and constructed scRNA-sequencing libraries according to the protocol of the GEXSCOPE® Single Cell RNA Library Kit (Singleron). Donor libraries were diluted to 4 nM, pooled, and sequenced on the NovaSeq 6000 system (Illumina) with 150-bp paired-end reads. Raw reads from scRNA-sequencing were processed to generate gene expression matrices using the CeleScope v1.9.0 pipeline on the reference genome GRCh38 to generate expression matrix files for subsequent analyses.

scRNA sequencing analyses and workflows

We imported expression matrix files into the R package (R Institute of Statistical Computing, Vienna, Austria) Seurat (version 4.3.0 and version 4) and the python-based toolkit scanpy (version 3.11.4 and version 1.9.3) for normalization, scaling, integration, Louvain clustering, dimensionality reduction, analysis of differential expression, and visualization. We considered cells with abnormal transcriptional complexity (<500 UMIs, >30,000 UMIs, or >25% of mitochondrial reads) as artefacts and removed them from subsequent analyses. Cell clusters were merged for protein marker-based annotation of major known immune-cell types at a broad level and kept at the clustering resolution of choice for acceptable annotation, in which neutrophil clusters were annotated manually by querying known markers (i.e., CSF3R,

CD177, S100A8 and S100A9). We confirmed annotation results with SingleR (v.1.6.1) assignments.

For RNA velocity, a BAM file containing the reference genome GRCh38 (hg38) was used in the analysis with velocity v0.2.3 and scVelo v0.17.17 in Python with default parameters. We projected the result to the t-distributed stochastic neighbor embedding (tSNE) plot from Seurat clustering analysis for visualization consistency. The Python package decoupler did pseudo-bulk functional analysis with default processing pipeline and parameters, in which we analyzed pathway-activity inferences based on PROGENy, a comprehensive resource containing a curated collection of pathways and their target genes with weights for each interaction.

Serum stimulation

WBCs from HDs were resuspended in a mixture of 70% RPMI 1640 medium and 30% serum obtained from either HDs or patients with septic shock. Lipopolysaccharide (LPS) was added to achieve a final 10 ng/mL concentration. A total of 1 mL of the cell suspension was plated in each well of a 24-well culture plate. After a 24-hour stimulation period, we collected cells and centrifuged to form a pellet. Subsequently, the pellet was resuspended in an appropriate volume of TsingZol Reagent, ensuring thorough mixing to promote cell lysis and RNA stabilization. IL-1B and IL-6 gene expression levels were quantified using real-time RT-qPCR.

Stimulation with recombinant human CD121b protein

WBCs from HDs were resuspended in complete RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). LPS was added to a final concentration of 10 ng/mL and recombinant human CD121b protein at a final concentration of 500 ng/mL. Following a 24-hour stimulation period, cells were collected as previously described. Real-time RT-qPCR assessed the expression levels of IL-1B and IL-6.

Antibody blockade of CD121b

WBCs from HDs were resuspended in a mixture of 70% RPMI 1640 medium and 30% serum derived from patients with septic shock. LPS was added to achieve a final concentration of 10 ng/mL and an anti-human CD121b blockade antibody at 10 µg/mL. After a 24-hour stimulation period, cells were collected following previously described protocols. IL1B, IL6, IL8, and TNF expression levels were analyzed using real-time RT-qPCR.

Drug treatments of cells

WBCs isolated from patients with septic shock were resuspended in complete RPMI 1640 medium supplemented with

10% FBS. Several pharmacological agents were selected for cell stimulation, including L-arginine (final concentration: 1 mM), dexamethasone (final concentration: 100 µM), and ATRA (final concentration: 50 µM). Stimulation experiments were conducted with and without adding human granulocyte-colony stimulating factor (G-CSF) at a final concentration of 50 ng/mL. Following a 24-hour stimulation period, cells were collected according to the aforementioned procedures for subsequent single-cell RNA sequencing. The gene expression levels of MME and IL-1R2 were assessed by real-time RT-qPCR.

Statistical analyses

Statistical analyses were performed using GraphPad Prism 8.0 (GraphPad, La Jolla, CA, USA). Data normality was assessed with the Shapiro–Wilk test, while homogeneity of variance was evaluated using the Brown–Forsythe test. For normally distributed and homoscedastic data, statistical comparisons were made using a 2-tailed Student's t-test, paired t-test, or one-way ANOVA, followed by Bonferroni *post hoc* correction. Data are expressed as mean ± standard error of the mean (SEM). In cases where the data did not meet normality assumptions, results are presented as median with interquartile range (IQR) and analyzed using the Mann–Whitney U test or the Kruskal–Wallis test, followed by Dunn's *post hoc* test. A P value of less than 0.05 was considered statistically significant.

Role of funders

The funding sources did not influence the study's design, data collection, data analysis, interpretation of results, or manuscript writing.

Result

CD66b⁺CD10[−] neutrophils are associated with the progression of septic shock

To investigate the impact of the immune response on the progression of sepsis, we retrospectively assessed the proportions and absolute numbers of various immune cell subsets in the peripheral blood of HDs and patients in the ICU. A significant increase in the proportion of neutrophils was observed in septic shock patients, accompanied by a corresponding decrease in lymphocytes and monocytes proportions (Supplementary Figure 1A). Consistent with previous studies (15), the neutrophil count in patients with septic shock was significantly elevated (Supplementary Figure 1B). These results implied that neutrophils may play a key role in the pathogenesis of septic shock.

To further investigate the characteristics of peripheral blood immune cells in septic shock patients, we collected blood samples from various groups for comparative analysis. The study cohort consisted of three HDs, one patient shortly after ICU admission

(ICU-Ad) and the same patient upon clinical improvement at ICU discharge (ICU-Survivor), one septic shock patient discharged from the hospital (Recovery), and one septic shock patient who deteriorated and subsequently passed away shortly after ICU admission (ICU-Non-survivor). Single-cell RNA-seq analysis revealed a significant increase in neutrophil counts and a notable reduction in other immune cell populations in ICU patients compared to the HDs. This trend was even more pronounced in the non-survivor group (Figures 1A–C, E). We clustered single-cell sequencing data. We focused on neutrophil subsets from different samples for dimensionality reduction analysis. We observed increased expression of *MME*, which encodes CD10, in neutrophils from HDs.

In contrast, neutrophils from ICU-Ad and ICU-Non-survivor exhibited reduced *MME* expression. At the same time, a significant increase was observed in ICU-Survivor and the Recovery group (Figure 1D). Based on these observations; we conducted bulk sequencing of neutrophils isolated from three HDs and four septic shock patients at various stages of illness: upon ICU admission (ICU-Ad), during ICU hospitalization (ICU-Hosp), and before ICU discharge following clinical improvement (ICU-Survivor). Principal component analysis (PCA) revealed significant transcriptomic differences between the HDs, ICU-Ad, ICU-Hosp, and ICU-Survivor (Supplementary Figure 2A).

Flow cytometry analysis of CD10 expression on neutrophils revealed elevated CD10 levels in HDs. At the same time, septic shock patients exhibited markedly reduced CD10 expression (Figures 1F, G; Supplementary Figure 3A). To further investigate the association between CD10 expression and disease severity, we performed longitudinal monitoring of neutrophil phenotypes in two septic shock patients. Patient 119 showed clinical improvement, with a corresponding decrease in the proportion of CD66b⁺CD10[−] neutrophils, ultimately leading to discharge from the ICU (Figure 1H). In surviving patients, a decrease in CD10[−] neutrophils was associated with recovery and a reduction in inflammatory markers, including C-reactive protein (CRP) and procalcitonin (PCT) (Figure 1I). In contrast, Pat 120 exhibited a sustained increase in the proportion of CD66b⁺CD10[−] neutrophils, which remained elevated as the patient's condition progressively deteriorated (Figure 1J). Analysis of clinical data from deceased septic shock patients revealed persistently elevated levels of CD10[−] neutrophils, which were associated with increased inflammatory markers, worsening disease progression, and eventual death (Figure 1K). These results suggested a strong association between CD66b⁺CD10[−] neutrophils and the severity of septic shock.

Arrest of neutrophilic development in patients with septic shock

We performed Wright-Giemsa staining on peripheral blood smears to investigate the morphological characteristics of neutrophils in patients with septic shock. The results show a significant increase in immature neutrophils, including myelocytes and metamyelocytes, in patients with septic shock (Figures 2A, B). To further investigate neutrophil subpopulations

in the peripheral blood of septic shock patients, neutrophils were isolated from all samples for comprehensive follow-up analyses. Cell clustering analysis identified six distinct neutrophil clusters (Figure 2C). The distribution of these clusters is illustrated in a percentage bar chart (Figure 2D). The neutrotime signature (16) reveals a progressive maturation of neutrophils from clusters C1 to C6 (Supplementary Figure 4A). Notably, significant enrichment of immature neutrophils was observed in ICU-Ad and the ICU-Non-survivor groups compared to HDs and Recovery group. Conversely, as patient conditions improved (from ICU-Survivor to Recovery), the proportion of C6 increased (Figure 2E). Neutrophil development was blocked in ICU-Ad and ICU-Non-survivor patients. RNA velocity analysis further elucidated a distinct developmental trajectory from C1 to C6 (Figure 2F). We identified well-established marker genes for neutrophil maturation (*MME*, *CXCR2*, *FCGR3B*) within sequencing data, revealing that the C6 clusters exhibited the highest degree of maturation. C1 and C2 clusters exhibited elevated expression of *LCN2* (17) and *IL1R2* (13), while C3, C4, and C5 clusters, which represent an intermediate evolutionary state, demonstrated characteristic expression of *CD163* (18), *S100A4* (19), and *AIF1* (20) (Figure 2G). These results indicate that the blockade of the maturation and differentiation pathways in circulating neutrophils was an essential feature of the immune response in patients with septic shock.

Identification of CD121b⁺ neutrophils in septic shock

We aimed to investigate the genes implicated in the progression of sepsis within neutrophils. By integrating scRNA-sequencing data across various disease states, we assessed the correlations between different genes and disease progression. *IL1R2*, which encodes CD121b, was identified as one of the genes most closely correlated with sepsis progression (Figures 3A–C). Furthermore, we demonstrated a significant upregulation of *IL1R2* expression in patients with septic shock at mRNA and protein levels (Figures 3D–F). Consistent with our previous findings, bulk RNA sequencing data revealed distinct transcriptional profiles among the HDs, ICU-Ad, ICU-Hosp, and ICU-Survivor (Supplementary Figure 5A). Additionally, we documented alterations in the expression of genes from the *S100A*, *MMP* and *IL1* families across these groups (Supplementary Figures 5B–D). Notably, the changes in *IL1R2* expression paralleled those observed at the single-cell level, further highlighting the characteristic upregulation of *IL1R2* in patients with septic shock (Supplementary Figure 5D). These findings indicated that genes from these families were expressed at elevated levels in ICU-Ad and ICU-Hosp, returning to baseline levels in ICU survivors.

Furthermore, we utilized flow cytometry to dynamically analyze neutrophil phenotypes in patients who improved to those who deteriorated and subsequently died. The proportion of CD10[−]CD121b⁺ neutrophils decreased progressively in septic shock patients who exhibited clinical improvement from ICU admission to discharge (Figures 3G, H). In contrast, patients who deteriorated

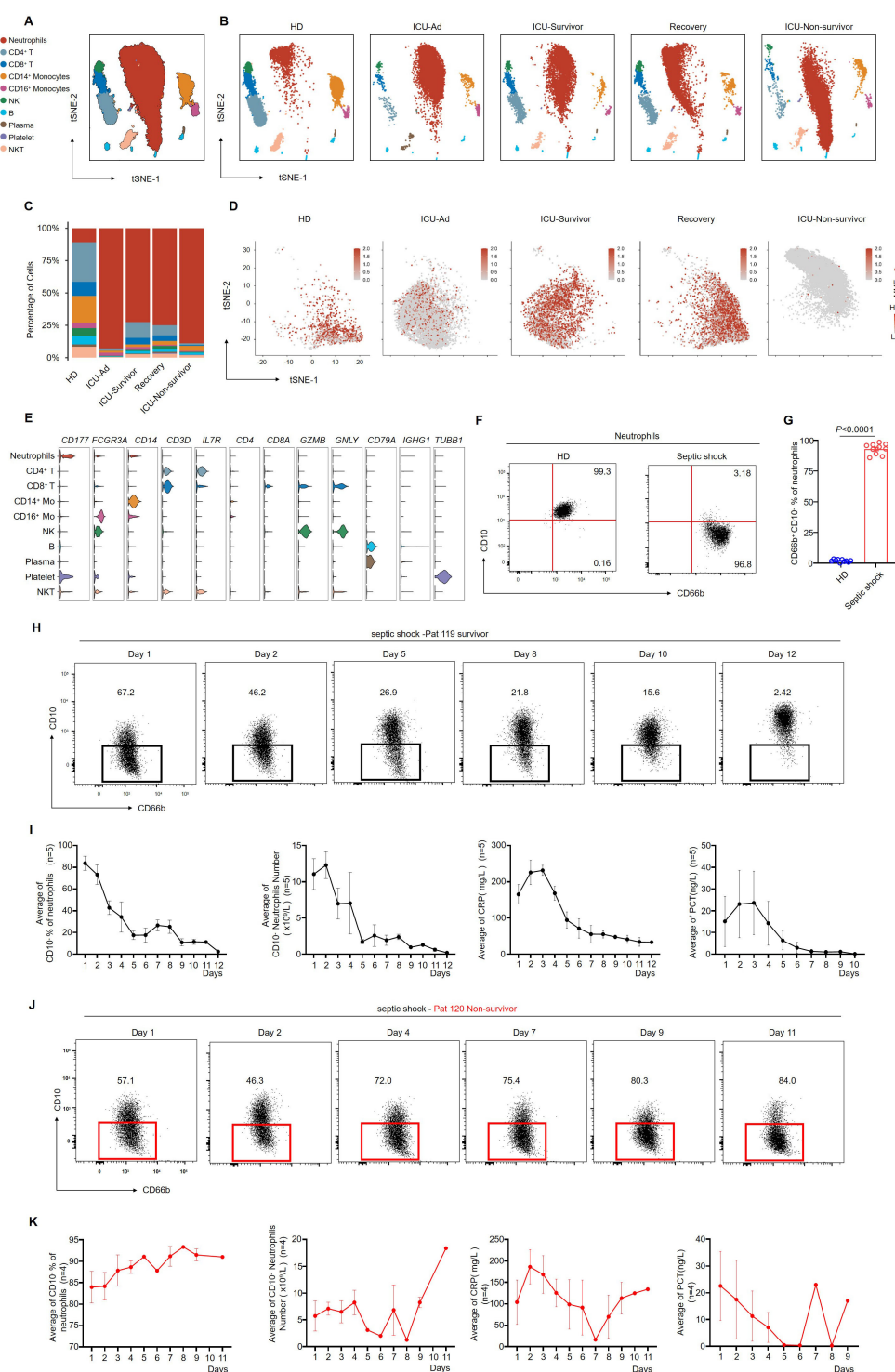


FIGURE 1

CD66b⁺ CD10⁻ neutrophils are related to the progression of septic shock. **(A)** the t-SNE plot of single-cell sequencing data (33,472 cells) from peripheral blood of patients with septic shock and HDs, annotated by distinct cell populations. **(B)** t-SNE plots for individual sample groups. HDs, n = 3; ICU Admission (ICU-Ad), n = 1; ICU-Survivor, n = 1; Recovery, n = 1; ICU Non-survivor, n = 1. **(C)** Changes in the proportions of immune cell subpopulations across conditions. **(D)** MME expression pattern projected on the t-SNE plot, showing neutrophils only. **(E)** Violin plots displaying a scaled expression of selected signature genes across clusters. **(F)** Representative flow cytometry plots showing the frequencies of CD66b⁺CD10⁻ neutrophils in healthy donors (left) and a patient with septic shock (right). **(G)** Quantification of CD66b⁺CD10⁻ neutrophils in healthy donors (n = 10) and septic shock patients (n = 10). **(H)** Dot plots showing dynamic changes in the proportion of CD66b⁺CD10⁻ neutrophils in surviving septic shock patients. **(I)** Dynamic changes in the average proportion of CD10⁻ neutrophils, absolute counts of CD10⁻ neutrophils, serum CRP, and PCT levels in septic shock survivors (n = 5). **(J)** Dot plots showing dynamic changes in the proportion of CD66b⁺CD10⁻ neutrophils in septic shock patients who died. **(K)** Dynamic changes in the average proportion of CD10⁻ neutrophils among total neutrophils, absolute CD10⁻ neutrophil counts, serum CRP, and PCT levels in patients who succumbed to septic shock (n = 4). Three or more independent experiments were performed. Statistical analyses were performed using two-tailed unpaired Student's t-test **(G)**.

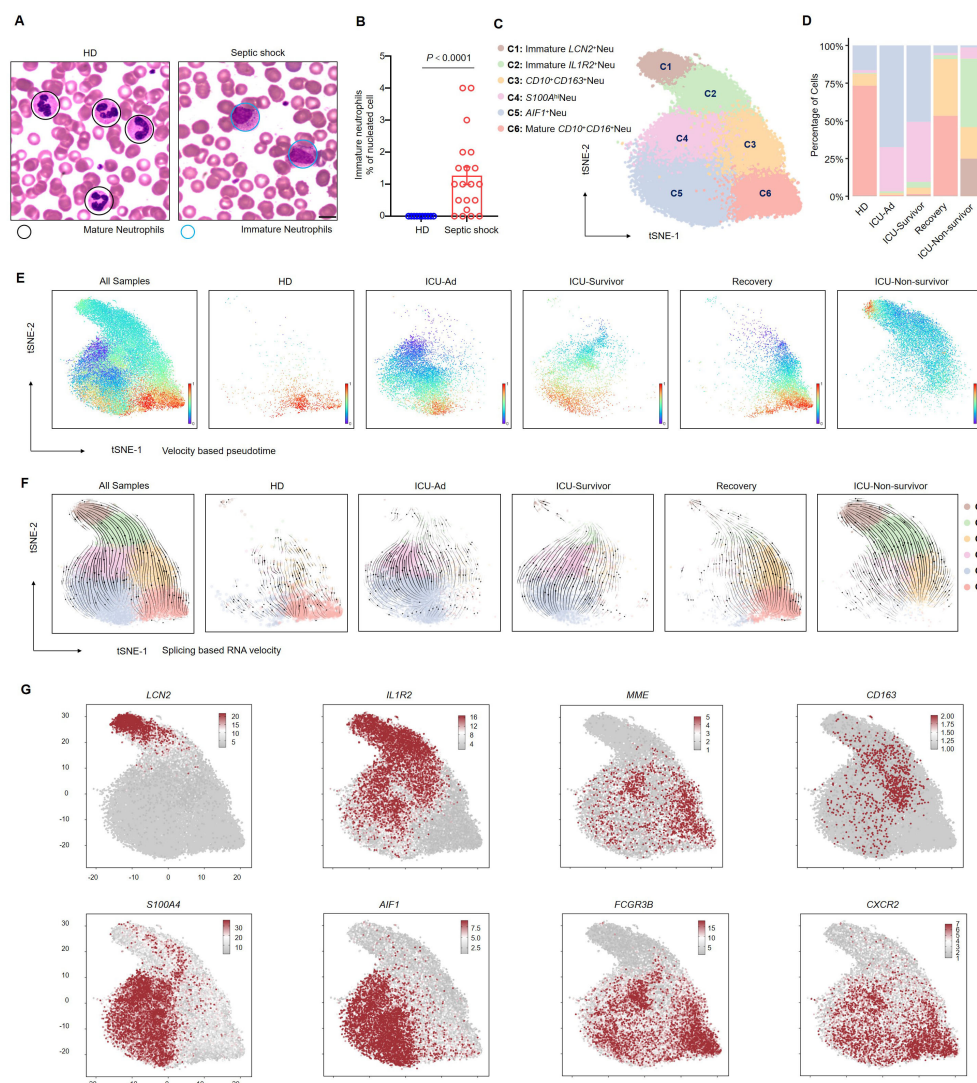


FIGURE 2

Blockade of the development of $CD66b^{+}CD10^{+}$ neutrophils in septic shock. (A) Representative Wright–Giemsa–stained peripheral blood smears showing mature neutrophils in a healthy donor (left) and immature neutrophils in a patient with septic shock (right); scale bar, 10 μ m. (B) The ratio of the proportion of immature neutrophils to nucleated cells in the peripheral blood of healthy donors ($n = 10$) and septic shock patients ($n = 20$). (C) t-SNE plot identifying six neutrophil clusters. (D) Proportions of neutrophil clusters across five patient groups. (E, F) Latent time analysis (E) and neutrophil RNA velocity (F) are displayed for each sample group on the t-SNE plot. (G) t-SNE plots illustrating the expression of selected marker genes across six neutrophil clusters. Statistical analyses were performed using the Mann–Whitney test (B).

and ultimately died exhibited a sustained elevation of $CD10^{-}CD121b^{+}$ neutrophils throughout their hospitalization, with levels remaining high until death (Figures 3I, J). These data suggested a correlation between the reduction of $CD10^{-}CD121b^{+}$ neutrophils and clinical improvement in septic shock patients. To evaluate the chemotactic, activation, and degranulation functions of neutrophils in patients with septic shock, we utilized flow cytometry to analyze the expression of CD10, CD121b, CD16, CD62L, and CD63 on neutrophils from HDs and septic shock patients. Our findings demonstrated that septic shock patients exhibited a significant reduction in CD10 and CD16 expression compared to HDs, while CD121b expression was notably elevated. These findings suggest impaired neutrophil maturation in septic shock patients. Additionally, the observed downregulation of CD62L and CD63

indicates compromised migratory and degranulation functions of neutrophils (Figures 3K, L). In contrast, serum levels of IL-10, IL-1RA, and inflammatory cytokines (including IL-8) were significantly elevated in septic shock patients compared to HDs (Figure 3M).

CD121b⁺ neutrophils exhibit immunosuppressive features

We defined cells with counts exceeding the upper quartile as “ $IL1R2^{hi}$ ” neutrophils” in our single-cell analysis. Among the five groups, neutrophils from HDs, ICU-Survivor, and Recovery patients predominantly exhibited the $IL1R2^{lo}$ phenotype. In contrast,

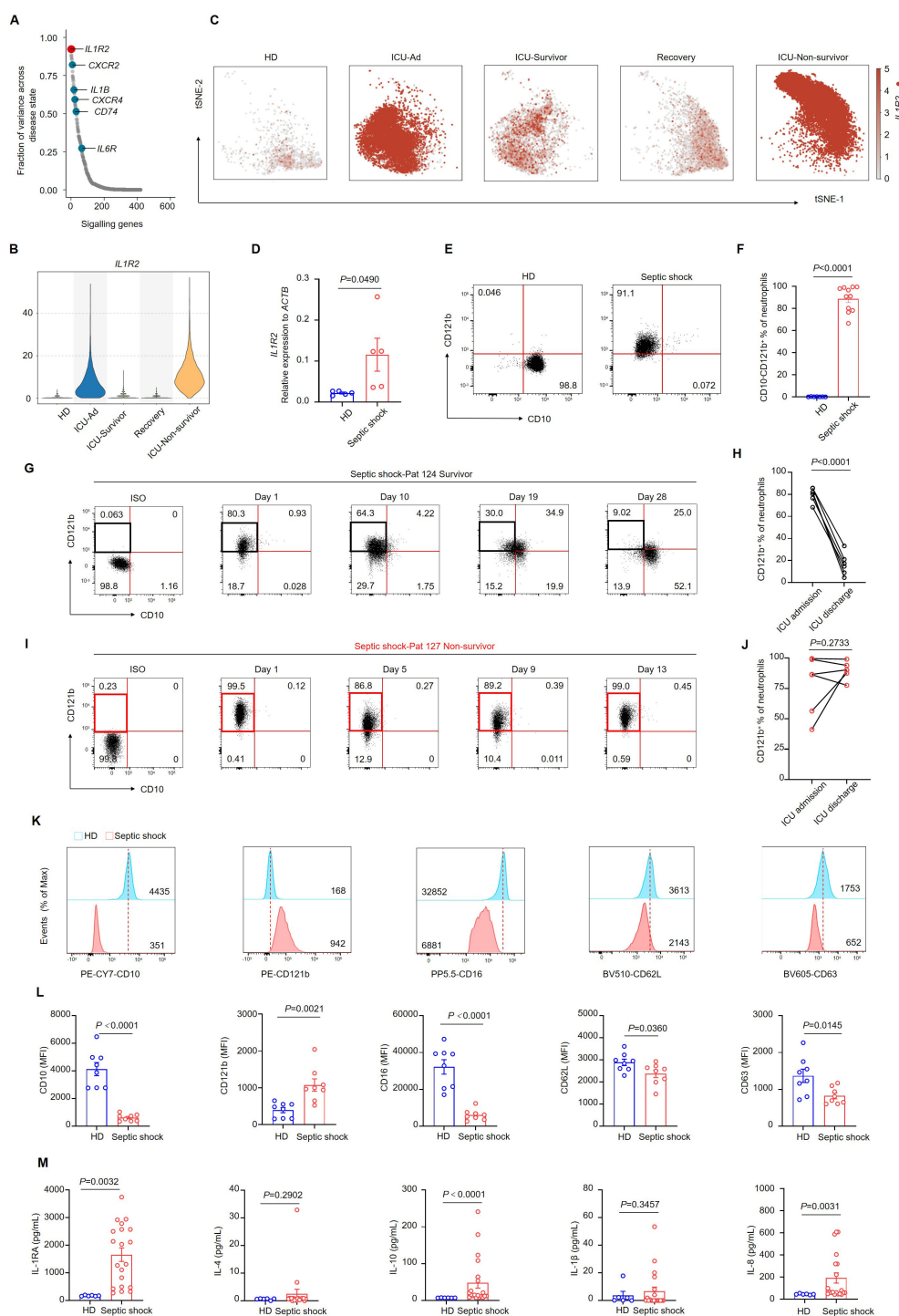


FIGURE 3

CD10⁻CD121b⁺ neutrophils are positively correlated with mortality risk in septic shock. **(A)** Differential gene expression in neutrophils across distinct phases of sepsis, organized by the magnitude of change, with *IL1R2* showing the most pronounced alteration. **(B)** Violin plot depicting *IL1R2* expression in neutrophils across different phases of sepsis and healthy donors. **(C)** *IL1R2* expression projected on the t-SNE plot, highlighting neutrophils. **(D)** *IL1R2* expression in peripheral blood neutrophils from HDs (n = 5) and patients with septic shock (n = 5). **(E)** Representative flow cytometry plots showing CD10⁻CD121b⁺ neutrophil frequencies in a healthy donor (left) and a patient with septic shock (right). **(F)** Quantification of CD10⁻CD121b⁺ neutrophils in healthy donors (n = 7) and septic shock patients (n = 11). **(G, H)** Representative flow cytometry plots **(G)** and statistical analysis **(H)** showing the dynamics of CD10⁻CD121b⁺ neutrophil frequencies in septic shock survivors from ICU admission to discharge (n = 6). **(I, J)** Representative flow cytometry plots **(I)** and statistical analysis **(J)** of CD10⁻CD121b⁺ neutrophils in patients with septic shock leading to death (n = 6). **(K)** Histograms of CD10, CD121b, CD16, CD62L, and CD63 expression in peripheral blood neutrophils from healthy donors (upper) and septic shock patients (lower). **(L)** Mean fluorescence intensity (MFI) of CD10, CD121b, CD16, CD62L, and CD63 in neutrophils from healthy donors (n = 8) and septic shock patients (n = 8). **(M)** Serum concentrations of IL-1RA, IL-4, IL-10, IL-1 β , and IL-8 in healthy donors (n = 6) and septic shock patients (n = 20). Three or more independent experiments were conducted. Statistical analyses were performed using two-tailed unpaired Student's t-test **(D, F, K)**, paired t-test **(H, J)**, and Mann-Whitney test **(L)**.

neutrophils from ICU-Ad and ICU-Non-survivor primarily exhibited the *IL1R2*^{hi} phenotype (Figure 4A). Utilizing the characteristic genes of *IL1R2*^{hi} neutrophils as a gene set, we performed gene set variation analysis to assess the enrichment of this gene set in bulk sequencing data from neutrophils of HDs, ICU-Ad, and ICU-Survivor. We observed a significantly higher enrichment score for this gene set in the ICU-Ad (Figure 4B). These findings indicate a strong association between *IL1R2*^{hi} neutrophils and the disease state, suggesting that this subpopulation may serve as a predictive biomarker for disease progression. Further analysis of the *IL1R2*^{hi} and *IL1R2*^{lo} neutrophils subsets revealed differentially expressed genes, and pathway-activity inferences were derived from the PROGENy resource, identifying distinct signaling pathways (Figures 4C, D). In the *IL1R2*^{hi} subgroup, we observed upregulated of signaling pathways such as “TGF- β ”, “Androgen”, “PI3K”, and “Hypoxia”, while pathways including “JAK-STAT”, “NF- κ B”, “TNF- α ”, and “P53” was downregulated. The expression of downstream genes associated with the JAK-STAT pathway, including *IFIT2*, *GBP1*, *ISG15*, and *IFIT3*, was significantly decreased. These findings suggest a disruption of the interferon signaling in the *IL1R2*^{hi} subgroup.

Granulocytic myeloid-derived suppressor cells (G-MDSCs) are recognized as a subset of neutrophils characterized by their significant immunosuppressive properties, albeit with distinct features (21, 22). To evaluate the immunophenotype of neutrophils in sepsis, we utilized a gene set representative of G-MDSCs (23). Upon scoring the two previously mentioned subsets, we found that *IL1R2*^{hi} neutrophils exhibited more pronounced GMDSC-like characteristics, evidenced by significantly upregulated expression of the signature genes *TSPO* and *CD177* (Figure 4E). To investigate the functional characteristics of *IL1R2*^{hi} neutrophils, we assessed the expression of classical immunosuppressive molecules in both *IL1R2*^{hi} neutrophils and *IL1R2*^{lo} neutrophils using single-cell sequencing data. Notably, we observed significant upregulation of genes associated with immunosuppressive functions, including *ARG1*, *PADI4*, *CD47*, and *LGALS1*, in the *IL1R2*^{hi} subgroup (Figure 4F), indicating enhanced expression of immunosuppressive genes in these neutrophils. Furthermore, we analyzed the expression of key immune checkpoint molecules on T cells and markers associated with T-cell activation. We found that major immune checkpoint molecules, such as *PDCD1*, *TIGIT*, and *LAG3*, were upregulated in T cells from deceased patients, while expression of activation markers, including *CD3E*, *ZAP70*, and *CD27*, was downregulated. The expression of functional markers such as IFNG, GZMA, and GZMK, along with the capacity to produce cytokines including IL1B, IL6, and TNF, was significantly downregulated (Figure 4G). These molecules are critical in mediating T-cell activation pathways in response to antigenic stimulation (24). The observed reduction in intercellular communication may reflect compromised coordination among immune cells and a diminished efficacy of the immune response (25). We isolated peripheral blood white blood cells (WBCs) from HDs and stimulated them with LPS *in vitro*. In the experimental group, we supplemented the serum from septic shock patients and co-cultured for 24 hours. Subsequent RT-PCR analysis of the WBCs revealed a marked suppression of proinflammatory gene expression,

including IL1B and IL6, in cells exposed to serum from critically ill patients (Figure 4H). The results indicate the presence of immunosuppressive factors in the serum of septic shock patients. To further explore whether CD121b could inhibit the transduction of inflammatory signals and exert immunosuppressive effects, we isolated peripheral blood immune cells from HDs and stimulated them with LPS *in vitro*. In this experimental setup, soluble CD121b (sCD121b) protein was added, and the cells were co-cultured for 24 hours. RT-qPCR analysis demonstrated significant downregulation of *IL1B* and *IL6* expression in the experimental group (Figure 4I). Following the introduction of a blocking antibody against CD121b, we observed a reversal of the serum immunosuppressive effects, with restored expression of *ILB*, *IL6*, *IL8*, and *TNF*. These findings strongly suggested that CD121b was a major mediator of immunosuppressive responses in septic shock (Figure 4J). Our data confirmed that the CD121b protein was a key source of immunosuppressive signals in patients with septic shock, indicating that *IL1R2*^{hi} neutrophils may have contributed to this immunosuppression in severe sepsis.

ATRA rescues the immunosuppressive effect of neutrophils

ATRA, a naturally occurring vitamin A derivative, was crucial in regulating cell differentiation, proliferation, and apoptosis (26–28). To investigate whether ATRA could ameliorate the immunosuppressive state of patients by promoting neutrophil differentiation, we isolated WBCs from the peripheral blood of patients with septic shock and cultured them for 24 hours. In this culture system, we introduced ATRA, L-Arginine, and Dexamethasone. Additionally, we established a parallel system incorporating the same drug combinations, supplemented with G-CSF. We observed a reduction in the expression of *MME* in immune cells treated with G-CSF, indicating an enhanced generation of immature neutrophils (Figure 5A). Among the three drugs, both dexamethasone and L-Arginine upregulated the expression of *IL1R2* in neutrophils. Intriguingly, ATRA downregulated *IL1R2* expression regardless of adding G-CSF, suggesting its potential as a therapeutic agent for septic shock. (Figure 5B). we collected WBCs from four groups (Control, Dexamethasone, L-arginine, and ATRA) and performed single-cell sequencing to investigate this phenomenon further. We extracted neutrophil subpopulations from the sequencing data and mapped them onto single-cell sequencing data derived from neutrophils in human peripheral blood (Figures 2C, 5C). Contour plots revealed an increase in the proportion of C6 neutrophils following ATRA stimulation, indicating that ATRA facilitated the development and maturation of neutrophils of septic shock patients. In contrast, the proportion of C6 neutrophils decreased after dexamethasone stimulation, suggesting that dexamethasone inhibited neutrophil maturation (Figure 5C). Based on this subgrouping result, we generated a bar graph depicting the percentages of C1–C6 subsets across the four sample groups (Figure 5D).

We extracted neutrophil datasets from the single-cell sequencing data of the four samples and labeled the expression of four key molecules: *MME*, *IL1R2*, *ARG1*, and *PADI4*. We observed

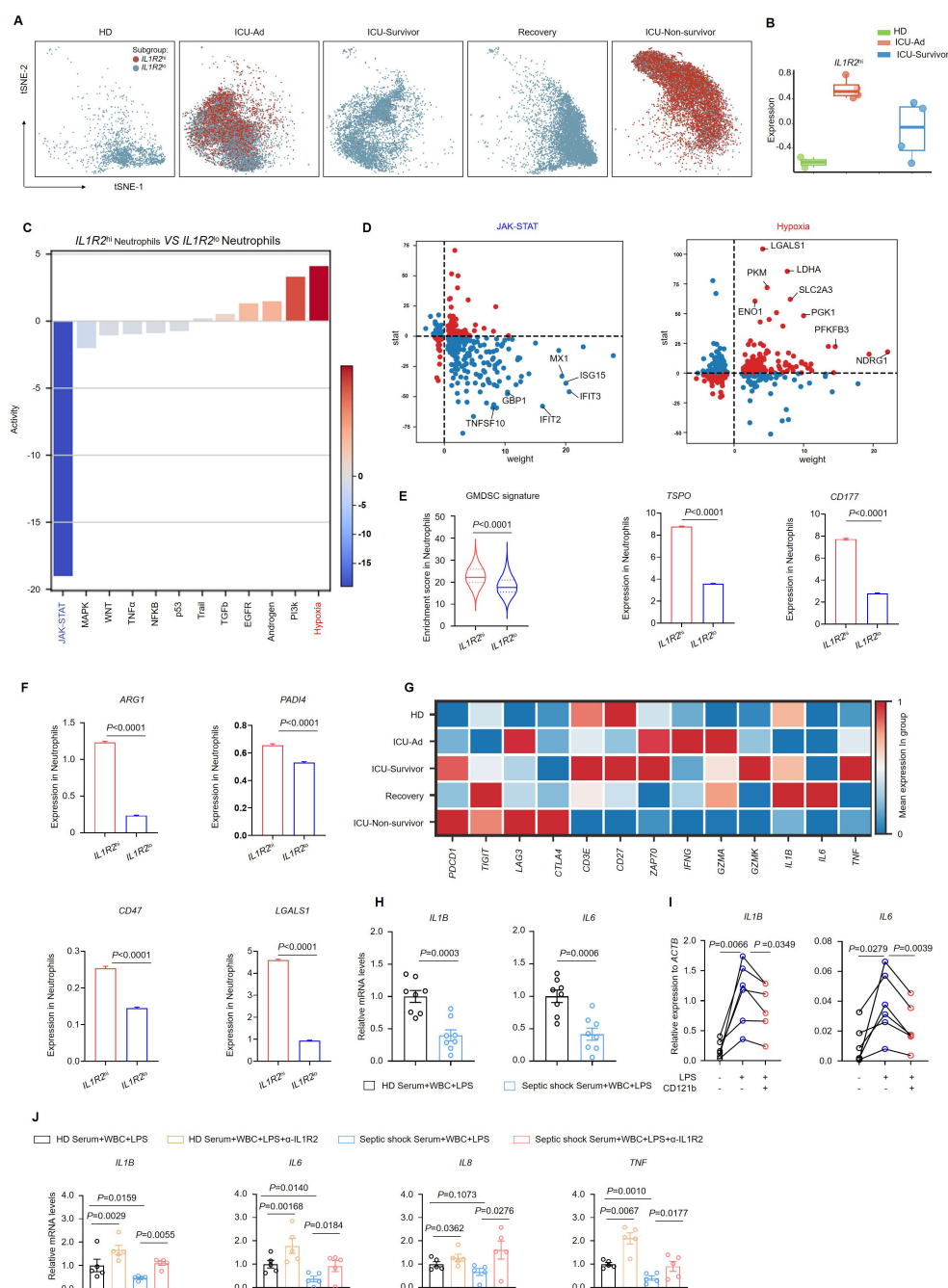


FIGURE 4

CD121b^{hi} neutrophils have an immunosuppression role in septic shock. (A) t-SNE plot displaying two distinct neutrophil populations separated by *IL1R2* expression across five sample groups. (B) Based on RNA-sequencing data, a bar graph illustrating gene-signature enrichment scores of highly expressed *IL1R2* in neutrophils. (C) Inferred pathway activity among neutrophil subclusters using the PROGENy platform. (D) Differentially expressed genes specific to the JAK-STAT and hypoxia signaling pathways. (E) Enrichment scores of the GMDSC signature in neutrophil subclusters, with bar graphs showing variation in the expression of *TSPO* and *CD177*. (F) Bar graphs depicting variation in the expression of immunosuppressive genes in neutrophils. (G) Heatmap displaying a differential expression of immune-related genes in T cells across five sample groups. (H) Gene expression of *IL1B* and *IL6* in white blood cells from HDs following 24 h of LPS stimulation in serum from HDs (n = 8) or septic shock patients (n = 8). (I) Gene expression of *IL1B* and *IL6* in white blood cells from HDs (n = 6) following 24 h of LPS stimulation in FBS with or without recombinant human CD121b protein. (J) Gene expression of *IL1B*, *IL6*, *IL8*, and *TNF* in white blood cells from HDs following 24 h of LPS stimulation in serum from HDs (n = 5) or septic shock patients (n = 5) with or without the addition of CD121b (*IL1R2*)-blocking antibodies. Three or more independent experiments were performed. Statistical analyses were conducted using two-tailed unpaired Student's t-test (E, F, H), paired t-test (I, J), and Mann-Whitney test (J).

upregulation of *MME* expression in the ATRA group, indicating that ATRA induced neutrophil maturation. Additionally, *IL1R2*, *ARG1*, and *PADI4* expression were downregulated following ATRA stimulation, suggesting that ATRA reversed the immunosuppressive characteristics of neutrophils in patients with septic shock. *IL1R2* expression was upregulated after dexamethasone stimulation, which aligned with our understanding of the immunosuppressive function of dexamethasone (Figure 5E). These results demonstrated the potent *in vitro* effects of ATRA in restoring neutrophil maturation and reversing immunosuppression. We utilized a characteristic gene set of GMDSC and the feature gene *TSPO* to assess the immunosuppressive level of neutrophils (Figure 5F). The immunosuppressive state of septic shock patients was reversed following ATRA induction. Moreover, we analyzed signaling-pathway enrichment based on differentially expressed genes in neutrophils. After ATRA stimulation, the expression of the inflammatory signaling pathways, including MAPK and NF- κ B, was enhanced, indicating neutrophil activation. Concurrently, the expression of the TGF- β -signaling and hypoxia-signaling pathways was downregulated, suggesting a weakened immunosuppressive effect and the restoration of neutrophil metabolic capacity alongside impaired function (Figure 5G). Detailed analysis of the differentially expressed genes within these signaling pathways consistently revealed similar phenomena (Figure 5H). Next, we examined the gene expression changes following dexamethasone stimulation. In contrast to ATRA, dexamethasone stimulation markedly increased immunosuppressive signals, notably the TGF- β and androgen signaling pathways, both exhibited significant upregulation (Figures 5I, J).

We established a sepsis-humanized mouse model to investigate whether ATRA could reduce CD121b expression on neutrophils. Immune cells from septic shock patients were transferred to severely immunodeficient mice (NCG), which were subsequently treated with ATRA. Twenty-four hours post-ATRA administration, we collected the mice's spleen, lung, and liver for flow cytometry analysis. Compared to the vehicle group, CD121b expression in human neutrophils was downregulated in the ATRA-treated group (Figures 5K–P). These results indicated that ATRA enhanced the function and development of neutrophils *in vitro* and *in vivo*.

CD121b can be a potential indicator of immunosuppression in septic shock

We evaluated CD121b expression on various immune cell types in peripheral blood. CD121b was minimally expressed in healthy donors' and septic shock patients' T cells. To determine whether neutrophils could produce sCD121b, we cultured neutrophils isolated from healthy donors and septic shock patients *in vitro*. Neutrophils derived from septic shock patients demonstrated the capacity to produce sCD121b (Supplementary Figures 6A–C). This observation supports that neutrophils are a source of immunosuppressive signaling in septic shock. To evaluate whether CD121b could function as a serum marker for immunosuppression, we collected samples from three groups: HDs, septic non-shock, and septic shock. We quantified the concentration of sCD121b in each group using

ELISAs. Notably, the serum levels of sCD121b were significantly elevated in patients with septic shock compared to both HDs and septic non-shock patients (Figure 6A). Additionally, receiver operating characteristic (ROC) curves were constructed to assess the diagnostic performance of sCD121b for distinguishing between septic non-shock and septic shock. The area under the ROC curve (AUC) was calculated to be 0.971, indicating a high diagnostic accuracy (Figure 6B). Furthermore, we obtained consistent results in urine samples (Figure 6C). We observed an elevation in the concentration of sCD121b in the urine of patients with septic shock, with an area under the ROC curve (AUC) of 0.910 (Figure 6D). Soluble CD121b detection in plasma and urine, and its close association with clinical outcomes in septic patients, suggest its potential as a non-invasive marker for sepsis-related complications. We also measured the concentrations of IL-6 and LCN2, establishing serum markers of sepsis (29, 30). Our results revealed a significant increase in LCN2 levels in the serum of septic shock patients; however, no significant differences were found between those with and without septic shock. (Figures 6E, F). Conversely, IL-6 levels were markedly low in the serum of HDs but showed a substantial increase in both septic non-shock and septic shock patients (Figure 6G). ROC analysis demonstrated the diagnostic efficacy of IL-6 in differentiating HDs from patients with sepsis, yielding an AUC of 0.972 (Figure 6H). While IL-6 is a reliable marker of inflammation, it does not adequately differentiate between immunosuppressed states in septic shock patients.

Discussion

Sepsis is characterized by disrupted homeostasis in two opposing directions: excessive inflammation and immune suppression (31). This complex immune response evolves over time, with many septic patients quickly developing severe immunosuppression, which is linked to poor outcomes (32). (i) This study identified CD121b⁺ neutrophils as key mediators of immunosuppression in septic shock, strongly correlating with worse outcomes. Blockading of downstream CD121b signaling, either with a CD121b antibody or ATRA, CD121b expression in neutrophils was successfully downregulated both *in vitro* and in a humanized mouse model.

(ii) We performed single-cell sequencing of whole blood and transcriptomic analysis to dynamically track changes in immune cell composition throughout different stages of disease progression. In contrast to studies utilizing randomly selected samples (33, 34), our investigation focused specifically on patients with septic shock, emphasizing continuous monitoring throughout the disease. We observed a significant increase in immature neutrophils in these patients and a reduction in lymphocyte counts—a hallmark of immunosuppression. Furthermore, the subset of CD10⁺ CD121b⁺ neutrophils was strongly associated with disease severity, and elevated CD121b expression on neutrophils emerged as a potential predictor of poor outcomes. In a study by Julian et al., two distinct sepsis response signature groups (SRS1 and SRS2) were identified (34). Consistent with our results, their single-cell sequencing data on sepsis revealed enrichment of CD121b⁺ neutrophils in patients with higher

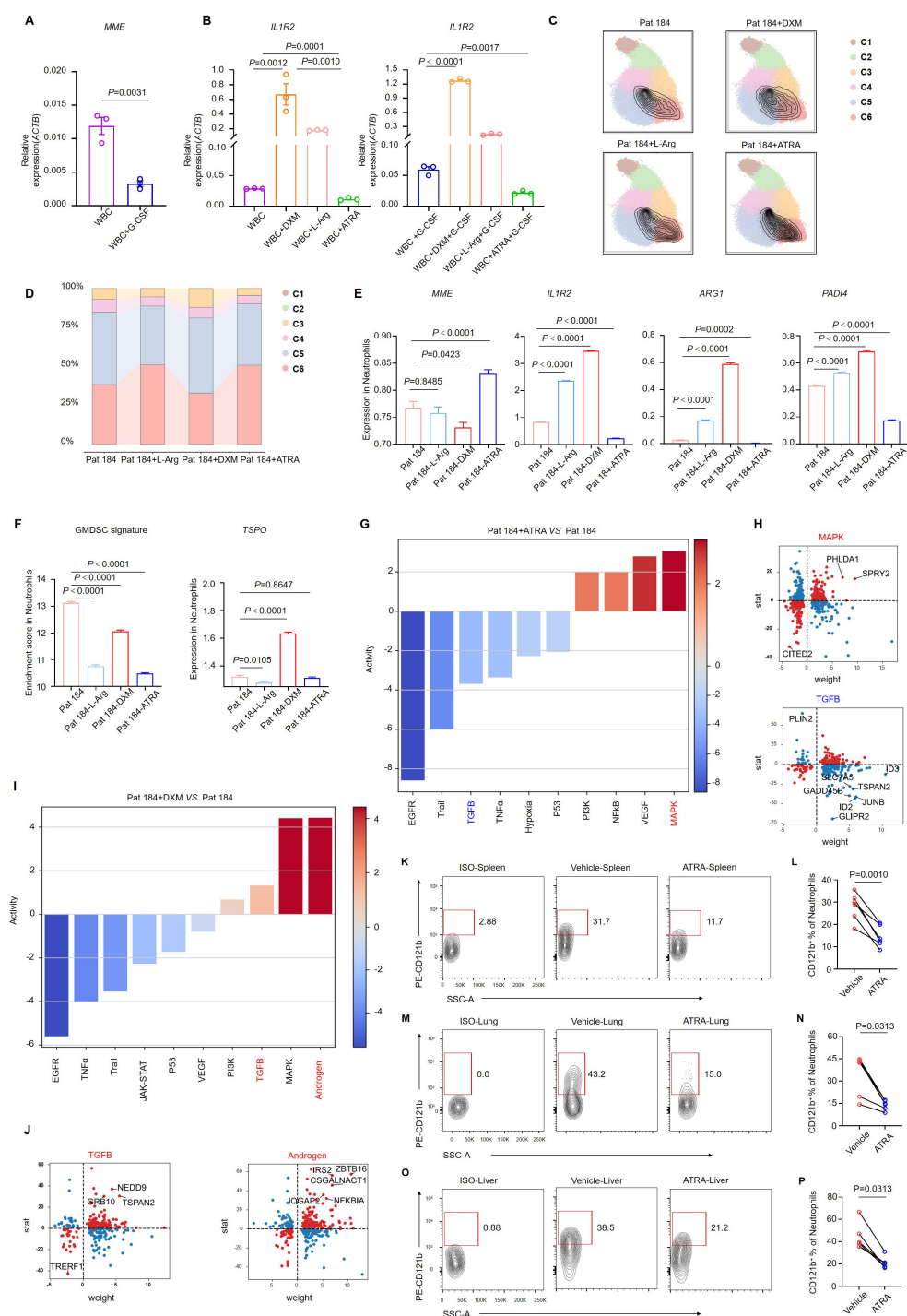


FIGURE 5

ATRA alleviates the immunosuppressive effect of neutrophils. (A) MME expression in white blood cells from septic shock patients (n = 3) after 24 h of G-CSF stimulation. (B) *IL1R2* expression in white blood cells from septic shock patients (n = 3) following 24 h of stimulation with dexamethasone, L-arginine, or ATRA, with or without G-CSF. (C) Single-cell sequencing of neutrophils from septic shock patients after *in vitro* stimulation with DXM, L-Arg, or ATRA, with neutrophil subpopulations displaying maturation features circled in black. (D) Changes in the proportions of neutrophil subclusters. (E) Bar graphs showing variation in the expression of key genes in neutrophils of single-cell sequencing data. (F) GMDSC signature enrichment in neutrophils, with a bar graph showing variation in *TSPO* expression of single-cell sequencing data. (G, H) Pathway activity changes (G) and differentially expressed genes (H) in the MAPK and TGF-β signaling pathways in neutrophils treated with ATRA. (I, J) Pathway activity changes (I) and differentially expressed genes (J) in the TGF-β and androgen signaling pathways in neutrophils treated with DXM. (K–O) Representative flow cytometry plots showing the proportion of CD121b⁺ human neutrophils in the spleen (K), lung (M), and liver (O) of NCG mice 24 h after ATRA administration. (L, N, P) Statistical analysis of CD121b⁺ human neutrophil proportions in the spleen (L), lung (N), and liver (P) of vehicle-treated (n = 6) and ATRA-treated (n = 6) NCG mice. Three or more independent experiments were performed. Statistical analyses were conducted using two-tailed unpaired Student's t-test (A, E, F), one-way ANOVA (B), paired t-test (L), and Wilcoxon test (N, P).

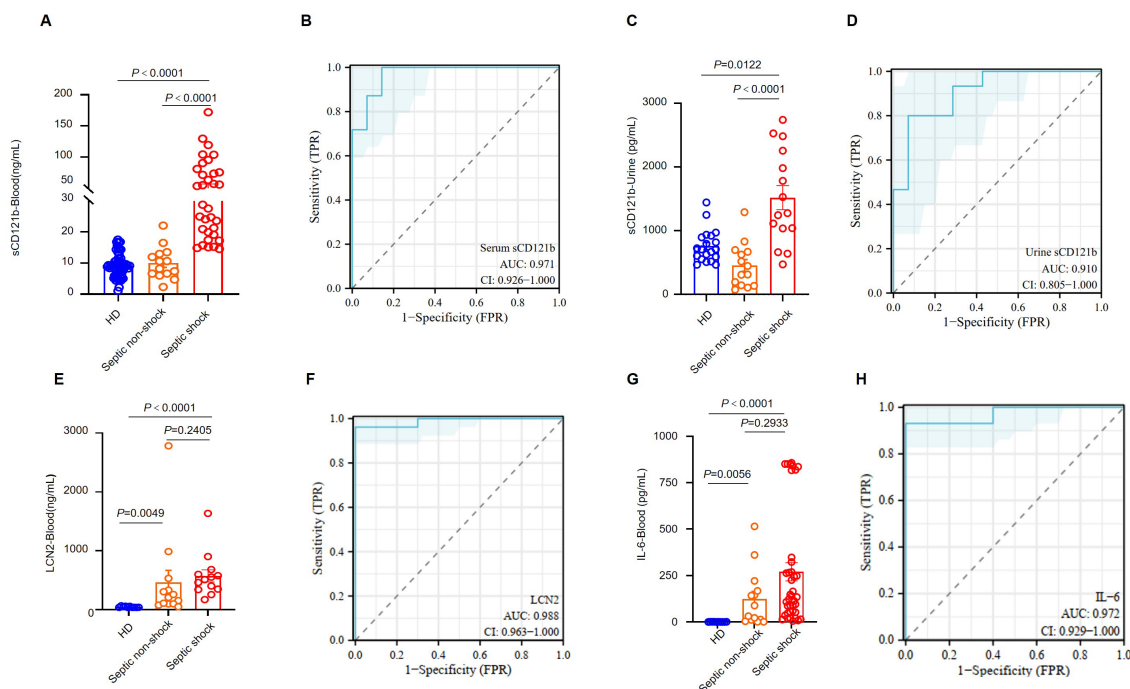


FIGURE 6

CD121b as a biomarker for the immunosuppressive state in patients with septic shock. (A) Serum levels of sCD121b in HDs ($n = 57$), non-septic patients ($n = 14$), and septic shock patients ($n = 39$). (B) The ROC curve for serum CD121b levels comparing non-septic patients and septic shock patients. (C) Urine levels of sCD121b in HDs ($n = 20$), non-septic patients ($n = 14$), and septic shock patients ($n = 15$). (D) ROC curve for urine CD121b levels comparing non-septic and septic shock patients. (E) Serum levels of LCN2 in HDs ($n = 10$), non-septic patients ($n = 13$), and septic shock patients ($n = 13$). (F) ROC curve for serum LCN2 levels comparing HDs and sepsis patients (including non-septic and septic shock patients). (G) Serum levels of IL-6 in HDs ($n = 10$), non-septic patients ($n = 14$), and septic shock patients ($n = 39$). (H) ROC curve for serum IL-6 levels comparing HDs and sepsis patients (including non-septic and septic shock patients). Three or more independent experiments were performed. Statistical analyses were performed using the Kruskal-Wallis test (A, C, E, G) and AUC analysis (B, D, F, H).

immunosuppression scores (SRS1 type) (13). Our study highlights the significant enrichment of $CD10^+ CD121b^+$ neutrophils in patients with poor prognoses characterized by an exaggerated septic response. We further validated these findings at the protein level through comprehensive analysis.

(iii) Research indicates that sCD121b exerts a more potent immunosuppressive effect than its membrane-bound counterpart (35, 36). sCD121b binds directly to interleukin-1 (IL-1) molecules, effectively inhibiting the intracellular transmission of IL-1 signaling (37). Our study demonstrates that sCD121b significantly reduces the expression of proinflammatory cytokines, including IL-6 and IL-1 β , in immune cells. Conversely, the administration of CD121b-blocking antibodies disrupted the immunosuppressive effects observed in the serum of patients, compromising the anti-inflammatory response. The biological role of sCD121b signifies its potential as a marker for the transition from proinflammatory activation to anti-inflammatory suppression throughout the disease course. This observation underscores the promise of CD121b as a therapeutic target for enhancing immunosuppression in patients with septic shock.

(iv) Research also indicates that sepsis is characterized by immune system dysregulation. However, the distinction between excessive immune activation and immunosuppression remains ambiguous, directly impacting clinical diagnostic and therapeutic approaches. Our findings demonstrate that serum levels of

sCD121b can effectively differentiate healthy donors from patients without septic shock and those with septic shock. Developing a molecular detection system for CD121b in serum, along with a potential non-invasive urine-based assay, could facilitate the assessment of immune status in patients with sepsis.

Recent investigations are exploring the potential of immunostimulatory agents for managing sepsis (7, 38), with a primary focus on cytokines (39–41), immune checkpoint inhibitors (42) (43), and immunoglobulins (44). However, these treatment modalities, which predominantly target adaptive immunity, often overlook the pivotal role of neutrophils, which dominate the immune landscape in the peripheral blood during the late stages of sepsis. Therefore, a promising strategy may involve ‘retraining’ immature neutrophils. ATRA, known for its ability to induce granulocyte maturation in acute promyelocytic leukemia treatment, presents a potential therapeutic candidate (45–48). Our findings demonstrate that ATRA enhances neutrophil maturation in the peripheral blood of patients, as evidenced by a significant reduction in CD121b expression at both the gene and protein levels. The use of glucocorticoids in the treatment of septic shock remains controversial, particularly concerning dosing, timing of initiation, and treatment duration (49–51). Notably, our data indicate that dexamethasone tends to drive neutrophils towards a more immature and immunosuppressive phenotype, which may explain why a substantial

proportion of patients do not experience clinical benefit from this therapeutic approach.

A *primary limitation of our investigation* is the absence of single-cell data regarding bone marrow hematopoiesis, which is essential for elucidating the developmental origin of CD121b⁺ neutrophils. Further experimental evidence is needed to delineate the immunosuppressive functions of CD121b⁺ neutrophils, particularly concerning their direct interactions with lymphocytes. Furthermore, animal models and clinical trials will be necessary to determine the potential clinical benefits of ATRA.

Conclusions

This study demonstrated that CD121b⁺ neutrophils positively correlated with septic shock severity. These cells displayed immunosuppressive characteristics; after blocking CD121b, proinflammatory cytokines increased in peripheral immune cells. Treatment with ATRA was found to down-regulate the expression of CD121b. CD121b may serve as a viable therapeutic target for intervention. These findings provide novel insights and directions for developing pharmacological strategies to mitigate immunosuppression in sepsis.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: HRA007054, HRA007050, HRA007060 (GSA; <https://ngdc.cncb.ac.cn/gsa-human>) (52, 53).

Ethics statement

The studies involving humans were approved by The Medical Research Ethics Committee of the First Affiliated Hospital of the University of Science and Technology of China. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. The animal study was approved by The Ethics Committee of the University of Science and Technology of China. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

HW: Funding acquisition, Writing – original draft, Writing – review & editing. JC: Data curation, Formal Analysis, Methodology, Writing – original draft, Writing – review & editing, Conceptualization, Investigation. JZ: Data curation, Writing – original draft. SC: Data curation, Writing – original draft. JQ: Formal Analysis, Writing – original draft. XH: Methodology, Writing – original draft. SX: Conceptualization, Writing – original

draft. LZ: Software, Writing – original draft. MZ: Software, Writing – original draft. YZ: Methodology, Writing – original draft. BF: Data curation, Writing – original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2025.1565797/full#supplementary-material>

SUPPLEMENTARY FIGURE 1

(related to Figure 1). Increased proportion and absolute number of neutrophils in patients with septic shock. (A) Proportion neutrophils, lymphocytes, and monocytes in peripheral blood of HDs, patients surviving from septic shock (ICU-Survivor-Septic shock) and patients with septic shock leading to fatal outcome (ICU-Non-Survivor-Septic shock). n = 10, 8 and 10

for HD, ICU-Survivor-sepsis and ICU-Non-survivor-sepsis, respectively. **(B)** Cell count of neutrophils, lymphocytes, and monocytes in peripheral blood of HDs, non-sepsis ICU patients (ICU-non-Sepsis), patients without septic shock (ICU-Septic non-shock) and patients with septic shock (and ICU-Septic shock). $n = 7, 37, 32$ and 18 for HD, ICU-non-sepsis, ICU-Septic non-shock and ICU-Septic shock, respectively. Statistical analyses were performed using the Kruskal–Wallis test **(A, B)**.

SUPPLEMENTARY FIGURE 2

(related to **Figure 1**). PCA analysis of transcriptome sequencing features in septic shock patients. **(A)** PCA of peripheral blood neutrophils from healthy donors and septic patients across disease states.

SUPPLEMENTARY FIGURE 3

(related to **Figure 1**). Flow cytometry gate strategy for neutrophils in human peripheral blood. **(A)**Gating strategy for flow cytometry analysis.

SUPPLEMENTARY FIGURE 4

(related to **Figure 2**). Differences in various subsets of neutrophils. **(A)** Violin plots showing pseudotime of neutrophils in human PBs across subsets, tested with student t-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

SUPPLEMENTARY FIGURE 5

(related to **Figure 3**). Differences in gene expression of neutrophils between healthy donors and septic patients Differences in various subsets of neutrophils. **(A)** Neutrophil gene expression in septic patients at varying stages displays noteworthy differences. Only genes with counts > 1 are presented above. **(B–D)** Heatmaps displaying the expression patterns of selected genes related to the IL-1 receptor **(B)**, MMP **(C)**, and TLR **(D)**.

SUPPLEMENTARY FIGURE 6

(related to **Figure 6**). Neutrophils generate soluble CD121 in patients with septic shock. **(A)** Representative flow cytometry analysis of the frequencies of CD121b⁺ neutrophils in healthy donor (middle panel) and septic shock patient (lower panel). **(B)** Representative flow cytometry analysis of the frequencies of CD121b⁺ T cells in healthy donor (middle panel) and septic shock patient (lower panel). **(C)** Soluble IL1R2 (sCD121b) concentrations secreted by peripheral blood neutrophils from healthy donors ($n=5$) and patients with septic shock ($n=5$) after 6 h or 18 h of *in vitro* culture. Statistical analyses were performed using two-tailed unpaired Student's t test (C) and Mann–Whitney test (C).

SUPPLEMENTARY TABLE 2

Key resources table.

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Diagnostic utility of IL-18 plasma levels in distinguishing abdominal from non- abdominal sepsis

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Background: Abdominal sepsis is a critical and high-risk condition in intensive care, characterized by diagnostic challenges, complex treatment, and high mortality. Non-specific symptoms and the difficulty of discriminating harmful bacteria from the normal flora complicate a timely diagnosis and treatment. Although timely interventions are crucial, the best timing of surgery remains uncertain, especially in unstable patients. Diagnostic markers like C-reactive protein, procalcitonin, and interleukins help guide diagnosis but often lack specificity of an abdominal focus. This study aims to identify possible additional markers for earlier detection of abdominal sepsis.

Methods: Plasma samples were collected from 47 sepsis patients at the day of sepsis diagnosis, and from 10 healthy controls. Patients were retrospectively categorized into those with abdominal (n=23) and those with non-abdominal (n=24) sepsis. Patient's characteristics, clinical outcomes, physiological and laboratory parameters, and cytokine levels were assessed. Receiver operating characteristic curves and Spearman correlation analyses were conducted.

Results: Age and sex proportions were comparable across the sepsis groups, as were the chronic disease prevalence, the severity of illness and mortality rates. Patients with abdominal sepsis were more likely to undergo emergency surgeries. Pro-inflammatory cytokines like IL-6, MCP-1, and IL-18 were elevated, as was the anti-inflammatory IL-10 in both sepsis cohorts compared to healthy controls. IL-18 was particularly associated with a more severe inflammatory response in non-abdominal sepsis. IL-18 levels below 1892.00 pg/mL showed 82.6% sensitivity and 56.5% specificity for identifying patients with abdominal sepsis, with a significant diagnostic accuracy (AUC 0.68, p = 0.034). This suggests IL-18 as a useful additional moderate predictor for critical cases.

Conclusion: The results demonstrate that IL-18, IL-6, MCP-1, and IL-10 are increased in sepsis, while IL-18 may serve as an additional biomarker for distinguishing abdominal from non-abdominal sepsis.

KEYWORDS

prediction, inflammation, cytokines, outcome, IL-18

1 Introduction

Despite continuous progress in intensive care medicine, sepsis remains a major cause of morbidity and mortality in the intensive care unit (ICU), with outcomes strongly influenced by the timelines and appropriateness of early management (1). One of the most critical early steps in managing sepsis is identifying the source of infection, which directly informs decisions regarding antimicrobial therapy and the need for surgical or procedural source control. In particular, distinguishing between abdominal and non-abdominal sources is essential, as intra-abdominal infections frequently necessitate urgent interventions, whereas non-abdominal infections may follow a different clinical trajectory.

Abdominal sepsis is a specific and particularly severe form of sepsis, often caused by intra-abdominal abnormalities such as gastrointestinal perforation, ischemic bowel, or cholecystitis; associated with a high mortality rate of nearly 30% (2). These infections are clinically challenging due to varied manifestations and frequently require complex therapeutic regimes including antibiotic treatment and surgical source control. They can involve a broad spectrum of pathogens, including Gram-positive and Gram-negative bacteria, fungi, and parasites, necessitating microbiological diagnostics that distinguish commensal flora from potential pathogens (3, 4). In contrast, non-abdominal infections like pneumonia, urinary tract infections, or catheter-related bloodstream infections typically demand a different therapeutic approach. In a large cohort, Reitz et al. showed that initiating source control within six hours of recognition reduced the adjusted odds of 90-day mortality by 29% compared to delayed intervention (5). These findings align with earlier studies showing that inappropriate antimicrobial therapy and delayed source control significantly increase mortality in sepsis, particularly in abdominal cases (6–8). Surgical source control, although critical, is complicated by the patient's hemodynamic stability and the risk associated with urgent surgery (7). Failure to identify and manage intra-abdominal sources in a timely manner is associated with increased morbidity and mortality (9). For instance, the multicenter observational study (AbSeS) found that emergency surgery within 2 hours of peritonitis diagnosis was associated with higher mortality compared to urgent intervention within 2–6 hours after diagnosis (7), whereas another study revealed a 100% mortality when surgical source control was delayed beyond 6 hours after gastrointestinal perforation (5, 6, 10).

Conversely, Martinez et al. found no clear survival benefit from early (<12 hours) versus late intervention (11).

Effective source control is also needed to avoid the development of antimicrobial resistance. Lack of source control often implies prolonged antimicrobial therapy, which is a key driver of antimicrobial resistance (12). Notably, the empiric antibiotic regimen in abdominal sepsis often differs from that used in non-abdominal sepsis. Intra-abdominal infections tend to be polymicrobial and may include anaerobes and resistant Gram-negative organisms, requiring broader initial antimicrobial coverage (3, 7, 9). In contrast, non-abdominal infections like pneumonia or urinary tract infections may require a different, narrow spectrum antibiotic empiric strategy. Misguided therapy can lead to prolonged courses of antibiotics, increasing the risk for antimicrobial resistance and complications like *Clostridioides difficile* infection (13). The increasing prevalence of extended-spectrum β -lactamase (ESBL)-producing *Enterobacteriaceae* in intra-abdominal infections underscores the importance of timely, targeted therapy based on early source identification (14).

Prognostically, abdominal sepsis carries a significant burden. A large-scale cohort analysis reported an in-hospital mortality rate of 18.93% for abdominal sepsis—comparable to pulmonary sources (19.27%) but higher than renal (12.81%) or catheter-related infections (12), underscoring the prognostic importance of early source differentiation. This stratification can help clinicians prioritize risk assessment, prognostication, and resource allocation—such as ICU bed triage or surgical prioritization. Moreover, up to 31% of patients undergoing emergency surgery for peritonitis had undiagnosed sepsis prior to focused screening using SOFA scores, highlighting persistent diagnostic delays in abdominal sepsis (15). These delays can compromise resuscitation and preoperative optimization, especially in resource-limited settings.

Understanding the peritoneal immune response and the pathophysiology of abdominal sepsis is key to improving clinical outcomes. Early identification of the infection source is critical, yet intra-abdominal infections often present with a broad spectrum of symptoms, ranging from mild discomfort in localized abscesses to rapid progression to septic shock in cases of gastrointestinal perforation (16). Tools like the Mannheim Peritonitis Index may help identify high-risk patients, while early recognition, fluid resuscitation, appropriate antibiotic therapy, and prompt surgical intervention remain foundational elements of management (5–7,

17). Laboratory markers such as white blood count, C-reactive protein (CRP), and procalcitonin (PCT) are commonly used, but each has limitations. CRP and PCT may rise during bacterial infections, yet lack specificity and can be affected by trauma or surgery (16). Serum lactate indicates tissue hypoperfusion but is not specific for abdominal causes (18). Interleukin (IL)-6, a standard marker in many ICUs, correlates with an poor outcomes in sepsis but does not differentiate infection sources (19). Recently, IL-18, a proinflammatory cytokine of the IL-1 family, has gained attention as a potential biomarker in septic states (20, 21). It plays a role in innate immune responses and mucosal inflammation (22–24). However, its relevance in abdominal sepsis remains unclear. Some data suggest that IL-18 may help differentiate sepsis origins, offering a path toward source-specific diagnosis (20, 21).

Given these challenges, improved diagnostic tools are needed. This study aimed to evaluate whether IL-18 levels differ between abdominal and non-abdominal sepsis, and assess its utility as an early diagnostic indicator in critically ill patients.

2 Material and methods

2.1 Ethics

All patients included in the study were treated in the ICU at the University Hospital of the University Dueseldorf. The institutional ethics committee approved the study under the number: 2018094832. The healthy subjects were included at the University Hospital of the University Ulm upon the approval of the study by the institutional ethics committee (number: 420/23). The study was performed in accordance with the Declaration of Helsinki and following STROBE-guidelines. In alignment with the ethical standards, written informed consent was obtained from all enrolled subjects, and all enrolled subjects signed the informed consent forms themselves or informed consent was obtained from the nominated legally authorized representative, who consented on the behalf of participants as approved by the ethical committees. This study was conducted as a mixed retrospective, non-interventional observational study.

2.2 Study design and patient classification

This was a mixed retrospective observational study involving critically ill patients with sepsis admitted to the ICU. For blood sampling, patients were prospectively enrolled; however, the cytokine assessments were conducted retrospectively. The study population consisted of patients who were retrospectively classified into two subgroups based on the presumed or microbiologically confirmed infection source: Abdominal sepsis was defined as sepsis with an intra-abdominal focus, including but not limited to gastrointestinal or gastric perforation, anastomotic leakage, appendicitis, intestinal ischemia, or cholecystitis. Non-abdominal sepsis included all other sepsis etiologies such as pneumonia,

urosepsis, and empyema, in the absence of confirmed abdominal pathology.

The diagnosis of sepsis in all patients was based on the Sepsis-3 criteria (25), which includes suspected or confirmed infection plus an increase in SOFA score ≥ 2 from baseline.

2.3 Inclusion and exclusion criteria

A total of forty-seven patients between 18 and 80 years of age were included. The study exclusion criteria were: age under 18 years, lack of or withdrawal of informed consent, refusal to participate in the study, expected ICU discharge within 72 hours, palliative care patients or those with established therapeutic limitations, foreseeable and unavoidable death at the time of screening, readmission after prior study inclusion (no repeat enrollment). Ten sex-matched healthy subjects (3 female and 7 male) with a mean age of 37.5 from the volunteering hospital staff were included as controls for cytokine analysis.

2.4 Initial patient assessment, treatment and clinical data acquisition

Patients were managed according to international sepsis guidelines (13). Clinical data including the Sequential Organ Failure Assessment Score (SOFA) score, Acute Physiology And Chronic Health Evaluation (APACHE) II, blood pressure, heart and respiratory rate, body temperature, routine blood gas analysis (including pH and lactate levels), glucose, as well as coagulation parameters (thromboplastin time, TPT; partial TPT, PTT; international normalized ratio, INR; fibrinogen, and platelets, PLT) were recorded at ICU admission and on the day of sepsis diagnosis (d0), and tracked up to 7 days post-diagnosis. Functional organ parameters including creatinine, bilirubin, glutamate dehydrogenase (GLDH), gamma-glutamyl transferase (GGT), creatine kinase (CK), and the numbers of transfused packed red blood cell (PRBC) units and fresh frozen plasma (FFP) were recorded. Furthermore, C-reactive protein (CRP) and procalcitonin (PCT) were determined.

Additional diagnostic imaging (X-rays, CT scans, MRI, bronchoscopy, endoscopy, etc.) was performed as clinically indicated. Information on emergency surgical interventions and infection source was recorded for classification and comparative analysis. The length of hospital stay before ICU admission, ICU length of stay, and in-hospital mortality were also documented.

2.5 Blood processing and analyses

Blood samples were obtained for clinical routine diagnostic parameters at ICU admission (ICU adm.) and at the day of sepsis diagnosis (d0) in citrate and ethylenediaminetetraacetic acid (EDTA) tubes (BD vacutainer, Becton Dickinson Diagnostics, Aalst, Belgium). Plasma for cytokine analyses was obtained from

EDTA tubes at d0 processed via centrifugation at 2000 × g for 15 minutes at 4°C. Samples were stored at -80°C until analysis. The same procedure was applied for healthy controls.

2.6 Analysis of circulating cytokines and chemokines

Thirteen inflammatory mediators including IL-1β, IFN-α2, IFN-γ, TNF-α, MCP-1, IL-6, IL-8, IL-10, IL-12p (70), IL-17A, IL-18, IL-23 and IL-33 were measured using the commercially available LEGENDplex Human Inflammation Panel 1 kit (BioLegend, San Diego, California), according to manufacturer’s instructions. Samples were analyzed using the Attune CytPix flow cytometer (Thermo Fisher Scientific, Germany). Concentrations of each parameter were calculated based on standard curves and logarithmic transformation.

2.7 Statistical analysis

All data were tested for normality using the Kolmogorov-Smirnov test. To assess statistical differences between the groups, the unpaired non-parametric Mann Whitney U test was applied. Chi-square test was applied for the analyses of proportions. Correlation analysis was performed using Spearman’s correlation coefficient and Spearman’s r-test. Receiver–operator curves (ROC) were generated to analyze the optimal cutoff levels. Data are presented as the median ± interquartile range (IQR), mean ± standard error of the mean (sem) unless otherwise stated. A p-value < 0.05 was considered statistically significant. GraphPad Prism 10.0 software (GraphPad Software Inc. San Diego, CA) was used for statistical analyses.

3 Results

3.1 Patient characteristics and outcomes

The comparison between patients with abdominal sepsis and those with non-abdominal sepsis reveals that both groups had a similar number of patients (23 vs. 24), and the median age for abdominal sepsis patients was slightly higher (75 years vs. 67 years), though this difference was not statistically significant. Both groups had a similar proportion of female patients, around 30%, and the prevalence of chronic diseases was also comparable (26% in abdominal sepsis and 29% in non-abdominal), with no significant difference (Table 1).

The infection sites differed markedly between the two groups. In the abdominal sepsis group, in 100% of patients, abdominal infections were confirmed, while none of the patients in the non-abdominal sepsis group had infections in this site. Conversely, 50% of the non-abdominal sepsis group had lung infections, compared to 4% in the abdominal sepsis group (p = 0.0007, Table 1). Additionally, 50% of the non-abdominal sepsis patients had infections in other sites, while no patients in the abdominal sepsis

TABLE 1 Overview of the characteristics of patients with abdominal and non-abdominal (non-abd.) sepsis.

Characteristics	Abdominal sepsis	Non-abd. sepsis	p <0.05
Number of patients	23	24	n.s.
Age (years) (IQR)	75.00 (61.00-81.00)	67.00 (55.25-74.75)	n.s.
Sex (female) (n, %)	7, 30.43%	7, 29.17%	n.s.
Chronic disease (n, %)	6, 26.09%	7, 29.17%	n.s.
Infection site			
Blood (n, %)	8, 34.78%	10, 41.67%	n.s.
Lung (n, %)	1, 4.35%	12, 50.00%	0.0007
Abdomen (n, %)	23, 100.00%	0, 0%	<0.0001
Other (n, %)	0, 0%	12, 50.00%	<0.0001
Surgical operation (n, %)	14, 60.87%	18, 75.00%	n.s.
Emergency surgical operation (n, %)	21, 91.30%	8, 33.33%	<0.0001
SOFA score ICU adm.	10.00 (7.50-12.00)	7.00 (5.00-11.00)	n.s.
SOFA score d0	9.00 (7.00-12.00)	9.00 (5.00-11.00)	n.s.
APACHE II ICU adm.	25.00 (16.75-29.75)	27.00 (19.00-31.00)	n.s.
APACHE II d0	27.00 (17.75-31.25)	25.00 (17.00-30.00)	n.s.
Length of hospital stay prior ICU (days)	0.00 (0.00-1.00)	8.50 (0.00-30.00)	0.0029
ICU days ≥7 (n, %)	16, 69.57%	15, 62.50%	n.s.
Length of ICU stay (days)	15.00 (4.00-25.00)	9.50 (4.00-22.75)	n.s.
Length of hospital stay (days)	28.00 (15.00-58.00)	39.50 (18.75-67.75)	n.s.
Mortality rate (n, %)	8, 34.78%	9, 37.5%	n.s.

A comparative analysis of demographic, clinical, and outcome parameters between patients diagnosed with abdominal and non-abdominal sepsis at the admission to the intensive care unit (ICU adm.) or at the day of sepsis diagnosis (d0) is demonstrated. Variables include the number of patients, age (median and interquartile range, IQR), sex distribution, presence of chronic disease, infection site (blood, lung, abdomen, or other), incidence of surgical intervention and emergency surgery, severity scores (Sequential Organ Failure Assessment Score, SOFA and Acute Physiology And Chronic Health Evaluation, APACHE II), length of hospital stay before ICU admission, duration of ICU stay, total hospital stay, and mortality rate. Data are given as median and interquartile range (IQR) with 25% and 75% percentile. Statistical comparisons between groups are indicated, with p-values less than 0.05 denoting significant differences. Non-significant results are labeled as “n.s.”.

group had infections outside the abdomen (p < 0.0001, Table 1). Blood infections were observed at similar rates in both groups (35% in abdominal sepsis and 42% in non-abdominal sepsis), with no significant difference (Table 1).

The overall rate of performed surgery was not significantly different between the two groups. However, emergency surgeries were more common in abdominal sepsis patients, with 91% undergoing emergency operations compared to 33% in the non-abdominal sepsis group (p < 0.0001, Table 1).

Regarding disease severity, there were no significant differences in the SOFA and APACHE II scores at ICU admission or on d0, indicating that both groups had similar severity of illness at these points (Table 1).

Length of hospital stay before ICU admission was significantly shorter for abdominal sepsis patients, with a median of 0 days compared to 8.5 days in the non-abdominal sepsis group ($p = 0.0029$, Table 1). However, there were no significant differences between the groups in terms of total ICU stay, total hospital stay, or the proportion of patients staying in the ICU for 7 days or more (Table 1).

Finally, the hospital mortality rates were similar between the two groups, with 34.78% mortality in the abdominal sepsis group and 37.5% in the non-abdominal sepsis group, showing no significant difference in outcomes (Table 1).

In summary, patients with abdominal sepsis were more likely to have emergency surgeries, had distinct infection sites, and shorter hospital stays prior to ICU admission compared to those with non-abdominal sepsis. However, the overall severity of illness and mortality were similar between the two groups.

Figure 1 displays a comparison between abdominal and non-abdominal sepsis patients over time in terms of several clinical severity scores: SOFA and APACHE II. Figure 1A shows a significant increase in SOFA scores in non-abdominal sepsis group over time, with notable statistical differences at d3 and d5 ($p < 0.05$). The APACHE II score reflecting the overall severity of illness, shows high scores at ICU admission in both groups. However, there are no significant differences between the two groups in this parameter (Figure 1B).

3.2 Physiological and laboratory parameters

The comparison of physiological and laboratory parameters between patients with abdominal sepsis and those with non-abdominal sepsis reveals several notable findings. In terms of blood pressure and heart rate, there were no significant differences between the two groups. Systolic blood pressure (SBP), shock index, and heart rate at ICU admission, on d0 (day of sepsis

diagnosis) were similar in both groups (Table 2). Mean arterial blood pressure values followed a similar trend, showing no significant differences between abdominal and non-abdominal sepsis patients at any of the measured time points (Table 2).

Regarding oxygenation and ventilation, no significant differences were observed in the fraction of inspired oxygen (FiO_2) or partial pressure of oxygen (PO_2) between the two groups at ICU admission, or on d0. However, partial pressure of carbon dioxide (PCO_2) was marginally higher in abdominal sepsis patients at ICU admission ($p = 0.0812$, Table 2). A significant difference was found in positive end-expiratory pressure (PEEP), which was higher in abdominal sepsis patients at ICU admission ($p = 0.0494$), indicating more aggressive ventilatory support (Table 2).

For hemoglobin and hematocrit levels, abdominal sepsis patients had significantly higher hemoglobin on d0 ($p = 0.0134$, Table 2). Hematocrit levels were also significantly higher in this group on d0 ($p = 0.0255$, Table 2). Lactate levels were borderline higher in abdominal sepsis patients at ICU admission ($p = 0.0540$) and d0 ($p = 0.0542$), which could suggest higher levels of tissue hypoxia or metabolic stress, though these differences did not reach statistical significance (Table 2). Glucose levels remained similar between the two groups at all time points. In terms of acid-base balance and electrolytes, bicarbonate (HCO_3) levels were not significantly different (Table 2). Additionally, pH values were marginally lower in abdominal sepsis patients at ICU admission ($p = 0.0568$), suggesting slight acidosis in this group (Table 2).

Clotting parameters showed no significant differences between the groups, indicating that coagulation function was comparable between the two groups at all time points (Table 2).

Regarding the inflammatory markers, both procalcitonin and CRP levels were similar between abdominal sepsis and non-abdominal sepsis patients, showing no significant differences in the inflammatory response (Table 2).

For liver function, there was one notable difference: GGT was significantly lower in abdominal sepsis patients on d0 ($p = 0.0179$, Table 2). However, other liver function markers, including bilirubin and GLDH levels were similar between the groups (Table 2).

Finally, there were no significant differences between the two groups in terms of red blood cell transfusions, fresh frozen plasma

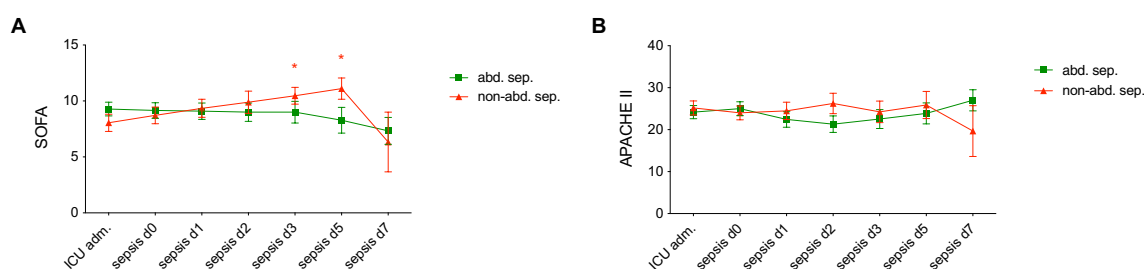


FIGURE 1
Comparison of (A) Sequential Organ Failure Assessment (SOFA) scores and (B) Acute Physiology and Chronic Health Evaluation II (APACHE II) scores in patients with abdominal sepsis (abd. sep.) and non-abdominal (non-abd. sep.) sepsis at various time points: admission to the intensive care unit (ICU adm.), on day of the sepsis diagnosis (d0), and on days 1, 2, 3, 5, and 7 post-sepsis onset. Each data point represents the mean score, with error bars indicating standard error of the mean. Statistically significant differences are marked as * $p < 0.05$ vs. corresponding matched-pair at the ICU adm.

TABLE 2 An overview of the physiological characteristics and laboratory parameters of the study population with abdominal and non-abdominal (non-abd.) sepsis at the admission to the intensive care unit (ICU adm.) and at the day of sepsis diagnosis (d0) is shown.

Physiological and laboratory parameters	Abdominal sepsis	Non-abd. sepsis	p <0.05
SBP (mm Hg) ICU adm.	107.40 ± 21.58	99.17 ± 22.05	n.s.
SBP (mm Hg) d0	99.77 ± 15.92	96.67 ± 15.01	n.s.
Shock index (HR / SBP) ICU adm.	1.002 ± 0.381	1.163 ± 0.572	n.s.
Shock index (HR / SBP) d0	0.975 ± 0.246	0.988 ± 0.365	n.s.
Heart rate ICU adm.	100.40 ± 25.49	107.50 ± 29.27	n.s.
Heart rate d0	97.50 ± 20.16	93.75 ± 24.51	n.s.
MABP (mm Hg) ICU adm.	71.48 ± 13.61	65.58 ± 13.44	n.s.
MABP (mm Hg) d0	67.77 ± 9.22	64.42 ± 9.17	n.s.
FiO ₂ (%) ICU adm.	55.74 ± 19.03	47.39 ± 27.64	n.s.
FiO ₂ (%) d0	42.59 ± 16.17	42.39 ± 24.68	n.s.
PO ₂ (%) ICU adm.	114.30 ± 57.10	94.88 ± 45.04	n.s.
PO ₂ (%) d0	89.95 ± 23.58	86.65 ± 27.58	n.s.
PCO ₂ (%) ICU adm.	42.83 ± 11.28	39.83 ± 12.28	n.s.
PCO ₂ (%) d0	44.77 ± 9.72	43.13 ± 11.79	n.s.
Horowitz index ICU adm.	245.60 ± 116.60	303.60 ± 226.20	n.s.
Horowitz index d0	254.50 ± 81.04	262.30 ± 140.30	n.s.
PEEP (cm H ₂ O) ICU adm.	6.95 ± 3.68	4.52 ± 4.86	0.0494
PEEP (cm H ₂ O) d0	7.30 ± 2.90	6.10 ± 6.10	n.s.
Hemoglobin (g / dL) ICU adm.	11.17 ± 2.59	9.88 ± 1.25	n.s.
Hemoglobin (g / dL) d0	10.42 ± 2.00	9.07 ± 1.26	0.0134
Hematocrit (%) ICU adm.	35.73 ± 8.74	30.92 ± 3.18	n.s.
Hematocrit (%) d0	33.65 ± 7.20	29.66 ± 4.08	0.0255
Glucose (mg / dL) ICU adm.	136.90 ± 55.16	147.50 ± 55.81	n.s.
Glucose (mg / dL) d0	131.00 ± 33.16	128.20 ± 38.01	n.s.
Lactate (g / dL) ICU adm.	4.88 ± 4.12	3.23 ± 2.98	n.s.
Lactate (g / dL) d0	4.28 ± 3.59	3.37 ± 4.27	n.s.
HCO ₃ ICU adm.	22.49 ± 4.73	22.58 ± 3.16	n.s.
HCO ₃ d0	25.83 ± 4.75	24.93 ± 3.68	n.s.
Base excess ICU adm.	-2.109 ± 5.436	-2.200 ± 3.868	n.s.
Base excess d0	1.536 ± 5.656	0.104 ± 4.26	n.s.
PH value ICU adm.	7.347 ± 0.081	7.390 ± 0.101	n.s.
PH value d0	7.375 ± 0.091	7.402 ± 0.069	n.s.
TPT (%) ICU adm.	24.00 ± 9.16	40.79 ± 44.36	n.s.

(Continued)

TABLE 2 Continued

Physiological and laboratory parameters	Abdominal sepsis	Non-abd. sepsis	p <0.05
TPT (%) d0	30.95 ± 31.83	37.91 ± 44.91	n.s.
INR (sec.) ICU adm.	1.277 ± 0.271	1.642 ± 0.949	n.s.
INR (sec.) d0	1.365 ± 0.313	1.567 ± 0.603	n.s.
PTT (sec.) ICU adm.	30.22 ± 9.11	38.58 ± 16.18	n.s.
PTT (sec.) d0	34.59 ± 17.96	38.04 ± 11.17	n.s.
TT (sec.) ICU adm.	28.26 ± 27.49	39.65 ± 42.88	n.s.
TT (sec.) d0	30.39 ± 30.44	38.64 ± 44.84	n.s.
Fibrinogen (mg / dL) ICU adm.	433.10 ± 112.30	430.90 ± 221.50	n.s.
Fibrinogen (mg / dL) d0	433.60 ± 134.80	496.10 ± 228.60	n.s.
Leukocytes (U / nL) ICU adm.	13.95 ± 8.46	18.67 ± 10.44	n.s.
Leukocytes (U / nL) d0	13.74 ± 8.83	16.68 ± 9.45	n.s.
PLT count (U / nL) ICU adm.	258.40 ± 156.60	262.50 ± 145.20	n.s.
PLT count (U / nL) d0	251.40 ± 146.10	233.00 ± 136.60	n.s.
Erythrocytes (U / nL) ICU adm.	4.22 ± 2.41	3.32 ± 0.38	n.s.
Erythrocytes (U / nL) d0	3.97 ± 2.17	3.12 ± 0.36	0.0086
Creatinine (mg / dL) ICU adm.	1.499 ± 1.01	2.179 ± 2.531	n.s.
Creatinine (mg / dL) d0	1.414 ± 0.86	1.862 ± 1.841	n.s.
Bilirubin (mg / dL) ICU adm.	1.35 ± 1.37	1.33 ± 1.84	n.s.
Bilirubin (mg / dL) d0	1.37 ± 1.33	1.93 ± 2.56	n.s.
GLDH (U / L) ICU adm.	344.10 ± 247.40	381.20 ± 269.4	n.s.
GLDH (U / L) d0	352.50 ± 262.00	390.30 ± 239.40	n.s.
GGT (U / L) ICU adm.	82.85 ± 92.85	109.20 ± 82.24	n.s.
GGT (U / L) d0	66.26 ± 59.44	146.50 ± 147.00	0.0179
CK (U / L) ICU adm.	393.50 ± 755.00	216.70 ± 257.90	n.s.
CK (U / L) d0	507.20 ± 1083.00	217.50 ± 236.80	n.s.
Procalcitonin ICU adm.	21.88 ± 31.07	15.93 ± 27.50	n.s.
Procalcitonin d0	17.05 ± 24.41	32.99 ± 42.07	n.s.
CRP (mg / dL) ICU adm.	12.36 ± 12.83	15.34 ± 11.59	n.s.
CRP (mg / dL) d0	11.98 ± 10.42	17.23 ± 12.63	n.s.
Noradrenalin (mL / h) ICU adm.	14.09 ± 10.80	10.06 ± 10.48	n.s.

(Continued)

TABLE 2 Continued

Physiological and laboratory parameters	Abdominal sepsis	Non-abd. sepsis	p < 0.05
Noradrenalin (mL / h) d0	12.55 ± 10.27	11.38 ± 11.38	n.s.
PRBC transfusion (U) ICU adm.	0.17 ± 0.49	0.0 ± 0.0	n.s.
PRBC transfusion (U) d0	0.50 ± 0.80	0.42 ± 1.06	n.s.
FFP transfusion (U) ICU adm.	0.74 ± 1.10	0.50 ± 1.22	n.s.
FFP transfusion (U) d0	1.23 ± 1.60	0.83 ± 1.74	n.s.
Electrolytes (mL) ICU adm.	1519.00 ± 651.60	1693.00 ± 1167.00	n.s.
Electrolytes (mL) d0	2009.00 ± 1208.00	1946.00 ± 1179.00	n.s.

APACHE II, Acute Physiology and Chronic Health Evaluation II; CK, Creatine Kinase; CRP, C-Reactive Protein; FiO₂, Fraction of Inspired Oxygen; FFP, Fresh Frozen Plasma; GGT, Gamma-Glutamyl Transferase; GLDH, Glutamate Dehydrogenase; HCO₃, Bicarbonate; HR, Heart Rate; INR, International Normalized Ratio; MABP, Mean Arterial Blood Pressure; PCO₂, Partial Pressure of Carbon Dioxide; PEEP, Positive End-Expiratory Pressure; PLT, Platelet; PO₂, Partial Pressure of Oxygen; PRBC, Packed Red Blood Cells; SBP, Systolic Blood Pressure; SOFA, Sequential Organ Failure Assessment; TPT, Thrombin Time Percentage; TT, Thrombin Time; PTT, Partial Thromboplastin Time. Data are given as mean ± standard error of the mean, and significant differences between groups are indicated by p-values less than 0.05; non-significant results are marked as “n.s.”.

transfusions, or electrolyte volumes administered at any time point (Table 2).

3.3 Analyses of cytokines

The results in Figure 2 show the levels of various cytokines in peripheral blood between patients with abdominal sepsis (abd. sep.), non-abdominal sepsis (non-abd. sep.), and healthy controls at the day of sepsis diagnosis (d0). The cytokines measured include pro-inflammatory markers such as IL-1β, IL-6, IL-8, and TNF-α, among others.

IL-1β, IFN-α2, IFN-γ, TNF-α, IL-8, IL-33, IL12p70, IL-17A, and IL-23 do not show significant differences between the sepsis groups or compared to healthy controls at d0, suggesting these markers may not play a central role in distinguishing between sepsis types (Figure 2).

MCP-1, IL-6, and IL-10 levels are significantly elevated at d0 in both sepsis groups compared to healthy controls (p < 0.05, Figure 2). MCP-1, which is responsible for recruiting monocytes to sites of infection, plays a vital role in the immune response to sepsis. Enhanced levels of IL-6, a key mediator of inflammation which is commonly associated with the acute phase of sepsis, suggest its critical role in driving the systemic inflammatory response.

IL-18 is significantly elevated in non-abdominal sepsis group compared to both abdominal sepsis group and healthy controls, highlighting a marked inflammatory response in this group (p < 0.05, Figure 2). IL-18 as a potent pro-inflammatory cytokine promotes the production of IFN-γ, which enhances the immune response. The elevated IL-18 levels in the non-abdominal sepsis

group suggest its involvement in the more severe inflammatory response associated with another type of infection than abdominal causes.

3.4 Correlation analyses for patients with abdominal sepsis and clinical and experimental parameters

The correlation analysis for patients with sepsis reveals important relationships between certain clinical parameters and the stratification of sepsis (abdominal vs. non-abdominal sites). In this analysis, negative correlations indicate that an increase in a measured value is more strongly associated with abdominal sepsis. Conversely, positive correlations suggest that an increase in a parameter is more strongly linked to other forms of sepsis.

Additionally, when a decrease in a measured value is associated with abdominal sepsis, it is considered a positive correlation, meaning that lower values of the parameter are more common in abdominal sepsis patients. On the other hand, if lower values are linked to other types of sepsis, this is defined as a negative correlation. This approach helps to clarify which clinical parameters are more characteristic of either abdominal or other forms of sepsis based on whether values increase or decrease in association with each type.

Older age correlated negatively with non-abdominal sepsis (r = -0.369, p = 0.008, Table 3). In contrast, a higher BMI was positively correlated with non-abdominal sepsis (r = 0.443, p = 0.010, Table 3).

When examining blood pressure, higher diastolic readings at ICU admission (r = -0.361, p = 0.009), and on d0 (r = -0.377, p = 0.007) showed an association with abdominal sepsis, meaning lower diastolic blood pressure was linked to non-abdominal sepsis types (Table 3).

There was also a positive correlation between heart rate at ICU admission and outcomes (r = 0.287, p = 0.043), suggesting that higher heart rates were associated with non-abdominal sepsis types (Table 3).

Regarding ventilation and oxygenation, there were no correlations between FiO₂ and PCO₂ on d0 (Table 3). PEEP at ICU admission (r = -0.493, p = 0.001) and d0 (r = -0.357, p = 0.016) was negatively correlated, indicating that higher PEEP was associated with abdominal sepsis (Table 3).

The analysis showed significant correlations for hemoglobin levels at ICU admission (r = -0.395, p = 0.005), and d0 (r = -0.451, p = 0.001) with non-abdominal sepsis. Lower hemoglobin levels were linked to non-abdominal sepsis. Similarly, higher lactate levels at ICU admission (r = -0.420, p = 0.002) and d0 (r = -0.421, p = 0.003) were associated with abdominal sepsis (Table 3).

In terms of acid-base balance, lower base excess on d0 (r = -0.300, p = 0.034), as well as lower pH at ICU as well as on d0 correlated negatively with non-abdominal sepsis (Table 3).

Several clotting factors were positively correlated with the type of sepsis, including TPT (r = 0.304, p = 0.040) and fibrinogen (r = 0.328, p = 0.026) on d0, suggesting a link between clotting ability

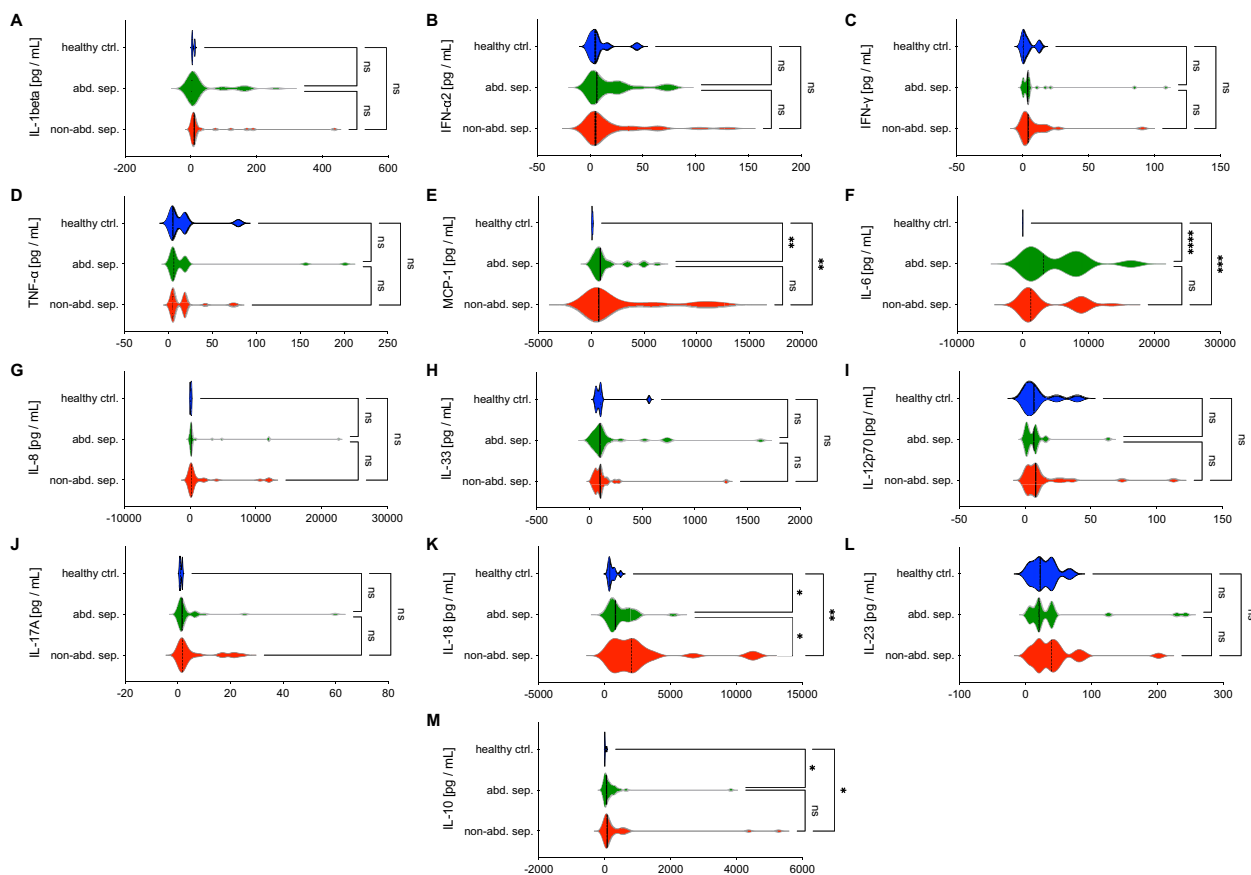


FIGURE 2

Comparative analysis of cytokine and chemokine levels in abdominal sepsis (abd. sep.) and non-abdominal sepsis (non-abd. sep.) patients versus healthy controls (ctrl.). Panels (A–M) show the levels of various parameters, including (A) IL-1 β , (B) IFN- α 2, (C) IFN- γ , (D) TNF- α , (E) MCP-1, (F) IL-6, (G) IL-8, (H) IL-33, (I) IL-12p70, (J) IL-17A, (K) IL-18, (L) IL-23, and (M) IL-10 across the three groups. Cytokine concentrations are measured in picograms per milliliter (pg/mL) at the day of the sepsis diagnosis. Statistically significant differences are marked as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$, while "ns" denotes non-significant comparisons.

and as cause rather non-abdominal than abdominal sepsis site (Table 3).

Markers of organ injury as well as liver function showed significant positive correlations, with GLDH ($r = 0.320$, $p = 0.028$) at d0, and GGT at ICU admission ($r = 0.347$, $p = 0.006$) and d0 ($r = 0.561$, $p = 0.031$, Table 3).

C-reactive protein positively correlated with non-abdominal sepsis at ICU admission ($r = 0.395$, $p = 0.004$) and d0 ($r = 0.440$, $p = 0.002$).

Finally, a significant and positive correlation between IL-18 and non-abdominal sepsis is observed ($r = 0.316$, $p = 0.032$, Table 3).

3.5 Analyses and diagnostic performance of IL-18 for non-abdominal sepsis

Table 4 shows a threshold of < 1892.00 pg/mL, with a sensitivity at 82.61% (CI 62.86–93.02) and a specificity of 56.52% (CI 36.81–74.37), with an AUC of 0.6825 ($p = 0.0340$), to predict abdominal sepsis (Table 4). This cut-off value was chosen based on the highest

likelihood ratio for corresponding possible highest sensitivity and specificity values.

Figure 3 presents the diagnostic performance of IL-18 levels as a biomarker for distinguishing abdominal from non-abdominal sepsis cases. Figure 3A shows the real distribution of IL-18 concentrations across patient samples, highlighting a threshold at 1892.00 pg/mL, where IL-18 levels are categorized into two groups: values below and above the threshold. In the abdominal sepsis group 19 out of 23, and in the non-abdominal sepsis group 10 out of 23 patients had IL-18 levels below the cut-off (Figure 3A). Figure 3B illustrates the diagnostic accuracy with correct and incorrect classifications of abdominal sepsis cases (Figure 3B). Thirty-three patients were correctly and 14 out of 47 patients were incorrectly diagnosed according to the IL-18 cut-off at < 1892.00 pg/mL for abdominal sepsis.

A receiver operating characteristic (ROC) curve for IL-18, showing an area under the curve (AUC) of 0.682 (95% CI: 0.526 to 0.839), indicates its discriminative ability (Figure 3C). The statistical significance is confirmed with a p -value of 0.034. This

TABLE 3 Correlation analyses of physiological, laboratory, and clinical parameters in patients with abdominal and non-abdominal (non-abd.) sepsis at the admission to the intensive care unit (ICU adm.) or at the day of sepsis diagnosis (d0) showing results for abdominal sepsis.

Correlation analyses (abd. sep.)	Spearman r	p value
Age	-0.369	0.008
Sex	-0.297	0.034
BMI	0.443	0.010
RR diast. (mm Hg) ICU adm.	-0.361	0.009
RR diast. (mm Hg) d0	-0.377	0.007
Heart rate ICU adm.	0.287	0.043
Hypothermia (<36°C) ICU adm.	-0.333	0.017
Hypothermia (<36°C) d0	-0.420	0.002
PEEP (cm H ₂ O) ICU adm.	-0.493	0.001
PEEP cm H ₂ O) d0	-0.357	0.016
Hemoglobin ICU adm.	-0.395	0.005
Hemoglobin (g / dL) d0	-0.451	0.001
Glucose (mg / dL) ICU adm.	0.284	0.044
Lactate (g / dL) ICU adm.	-0.420	0.002
Lactate (g / dL) d0	-0.421	0.003
Base excess d0	-0.300	0.034
Acidosis (pH <7.35) ICU adm.	-0.327	0.019
Acidosis (pH <7.35) d0	-0.378	0.007
TPT (%) d0	0.304	0.040
Fibrinogen (mg / dL) d0	0.328	0.026
GLDH (U / L) ICU adm.	0.320	0.028
GGT (U / L) ICU adm.	0.347	0.006
GGT (U / L) d0	0.561	0.031
CRP (mg / dL) ICU adm.	0.395	0.004
CRP (mg / dL) d0	0.440	0.002
Hospital days before ICU	0.432	0.002
IL-18 (pg / m L) d0	0.316	0.032

The Spearman correlation coefficient (r) and p-values for various factors including demographic characteristics (age, sex, BMI), vital signs (diastolic blood pressure, heart rate, hypothermia status), respiratory support parameters, laboratory markers, and clinical are given. Abbreviations: BMI, Body Mass Index; CRP, C-Reactive Protein; GGT, Gamma-Glutamyl Transferase; GLDH, Glutamate Dehydrogenase; IL-18, Interleukin-18; PEEP, Positive End-Expiratory Pressure; RR diast., Diastolic Blood Pressure; TPT, Thrombin Time Percentage.

TABLE 4 Cut-off value of Interleukin-18 (IL-18) with corresponding sensitivity and specificity for distinguishing abdominal sepsis from non-abdominal sepsis at the day of sepsis diagnosis (d0).

Parameter	Value	Sensitivity %	95% CI	Specificity %	95% CI	Likelihood ratio	AUC	p value
IL-18 d0	<1892.00	82.61	62.86 to 93.02	56.52	36.81 to 74.37	1.900	0.6824	0.0340

AUC, Area Under the Curve; CI, Confidence Interval. A p-values less than 0 is considered significant.

analysis underscores the potential of IL-18 as a biomarker for identifying abdominal sepsis (Figure 3).

3.6 Correlation analyses of IL-18 with clinical and inflammatory markers

The correlation analysis between IL-18 levels and a range of clinical and laboratory parameters reveals several significant associations, suggesting IL-18's potential role as a biomarker for outcomes in ICU settings. Specifically, hematocrit levels at ICU admission were found to have a modest yet significant inverse correlation with IL-18 levels ($r = -0.302$, $p = 0.041$, Table 5). Similarly, calcium levels, measured both at ICU admission and on d0, demonstrated moderate negative correlations with IL-18 ($r = -0.348$, $p = 0.018$, and $r = -0.311$, $p = 0.038$, respectively), suggesting a potential relationship between IL-18 levels and altered calcium homeostasis in severe cases.

Coagulation parameters, including INR and PTT, exhibited positive correlations with IL-18. Specifically, INR at ICU admission was positively correlated ($r = 0.379$, $p = 0.010$), as were PTT measurements at ICU admission ($r = 0.351$, $p = 0.017$) and on d0 ($r = 0.339$, $p = 0.023$), potentially indicating a link between elevated IL-18 and coagulopathy or prolonged clotting times in critically ill patients.

Transaminase levels showed particularly strong positive correlations with IL-18, with GLDH levels at ICU admission ($r = 0.461$, $p = 0.002$) and d0 ($r = 0.445$, $p = 0.002$, Table 5) being significant. Additionally, GGT levels on d0 also positively correlated with IL-18 ($r = 0.337$, $p = 0.041$), highlighting a possible association between IL-18 and liver function or injury markers.

Troponin T, a key cardiac biomarker, presented one of the most substantial correlations with IL-18 at ICU admission ($r = 0.700$, $p = 0.016$, Table 5), suggesting a potential link between IL-18 and myocardial stress or damage in the critically ill cohort.

Furthermore, SOFA scores, both at ICU admission ($r = 0.389$, $p = 0.015$ and on d0 ($r = 0.376$, $p = 0.017$, Table 5), were positively correlated with IL-18 levels, indicating that higher IL-18 may be associated with greater illness severity as measured by this standard scoring system.

Hospitalization metrics also correlated significantly with IL-18 levels; both the length of total hospital stay to ICU admission ($r = 0.391$, $p = 0.007$) and total length of hospital stay ($r = 0.412$, $p = 0.004$, Table 5) were positively associated with IL-18. This trend may reflect a link between elevated IL-18 and prolonged recovery or severity of illness. Additionally, mortality was positively correlated

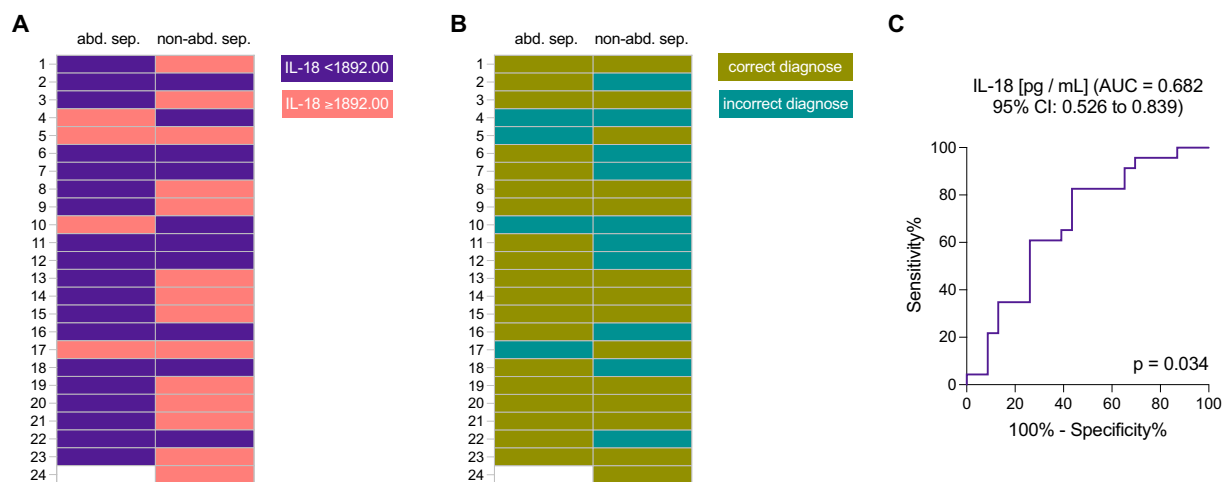


FIGURE 3

The diagnostic efficacy of interleukin-18 (IL-18) levels in distinguishing abdominal sepsis (abd. sep.) from non-abdominal sepsis (non-abd. sep.) patients. (A) Distribution of IL-18 levels (threshold of 1892.00 pg/mL) in patients with abdominal sepsis versus non-abdominal sepsis; and (B) diagnostic accuracy illustrated by correct and incorrect classifications based on IL-18 levels, with a threshold set at 1892.00 pg/mL. (C) Receiver Operating Characteristic (ROC) curve assessing the sensitivity and specificity of IL-18 as a biomarker for abdominal sepsis, showing the area under the curve (AUC).

with IL-18 ($r = 0.375$, $p = 0.010$, Table 5), further suggesting IL-18's potential utility as a prognostic marker for adverse outcomes.

Lastly, IL-18 levels correlated with a spectrum of cytokines and chemokines involved in inflammation and immune response, including MCP-1, IL-6, and IL-10 (Table 5). In Figure 4, correlations between IL-18 levels and inflammatory markers that have shown clearly detectable levels above the minimal expression of the detection array are shown, highlighting relationships with MCP-1, IL-6, and IL-10 ($p < 0.05$, Figure 4 and Table 5). Figure 4A shows a positive correlation between IL-18 and MCP-1 levels ($r = 0.525$, $p < 0.001$), indicating a notable association. Figure 4B presents a positive correlation between IL-18 and IL-6 with a moderate but significant correlation ($r = 0.339$, $p = 0.021$). Also, a positive correlation between IL-18 and IL-10 with a strong correlation ($r = 0.460$, $p = 0.0001$) is highlighted in Figure 4C. These findings suggest that IL-18 may play a central role in the inflammatory process, correlating with multiple cytokines that are critical in both pro-inflammatory and regulatory pathways.

4 Discussion

Abdominal infection is a significant clinical problem, representing the second most common infection site in ICU patients (26). The diverse clinical presentations and lack of specific biomarkers make diagnosis particularly challenging and complicate the timely decision-making required for potentially early life-saving surgical infection source control.

In this mixed retrospective clinical study, we analyzed two patient groups: one with abdominal sepsis and another with sepsis from other origins. Our aim was to assess whether IL-18 could serve as a biomarker to differentiate these subtypes. Interestingly, we observed significantly higher IL-18 levels in

patients with non-abdominal sepsis. While both groups were comparable in terms of age, comorbidities, and initial clinical status, the abdominal sepsis group had a length significantly shorter hospital stay prior to sepsis diagnosis, suggesting a more aggressive disease course leading to earlier ICU admission. Despite a higher rate of emergency surgical interventions in abdominal sepsis group—reflecting the need for surgical source control—SOFA and APACHE II scores at diagnosis, ICU length of stay, total hospitalization, and in-hospital mortality rates were similar between the groups. Similarly, both groups exhibited similar hemodynamic parameters, while hemoglobin and hematocrit levels in abdominal sepsis were significantly higher, likely due to pre-existing dehydration from intra-abdominal pathology. Standard inflammatory markers such as leukocyte count, PCT, and CRP, were elevated in both groups without significant distinction—indicating that these are rather not suitable to differ the sepsis subtypes. The data from respiratory parameters is limited by the higher proportion of intubated patients in the abdominal sepsis group, both at ICU admission and at the day of sepsis diagnosis.

Our primary objective was to evaluate IL-18 as a diagnostic biomarker for differentiating abdominal from non-abdominal sepsis. Surprisingly, IL-18 levels were significantly higher in patients with non-abdominal sepsis, a result that contrasts the study by Mierzchala-Pasierb et al., who reported elevated IL-18 levels in abdominal sepsis compared to pulmonary sepsis (20). This discrepancy may stem from methodological differences, including their limited sample size ($n=9$ for abdominal sepsis, $n=8$ for pulmonary sepsis) and the notably higher SOFA scores in the abdominal sepsis group. Furthermore, while their comparison focused solely on abdominal versus pulmonary sources, our non-abdominal cohort was more diverse, encompassing pneumonia, urosepsis, and empyema. IL-18 levels in our cohort correlated with

TABLE 5 Correlation analyses of Interleukin-18 (IL-18) with physiological, laboratory, and clinical parameters in patients with abdominal and non-abdominal (non-abd.) sepsis at the admission to the intensive care unit (ICU adm.) or at the day of sepsis diagnosis (d0).

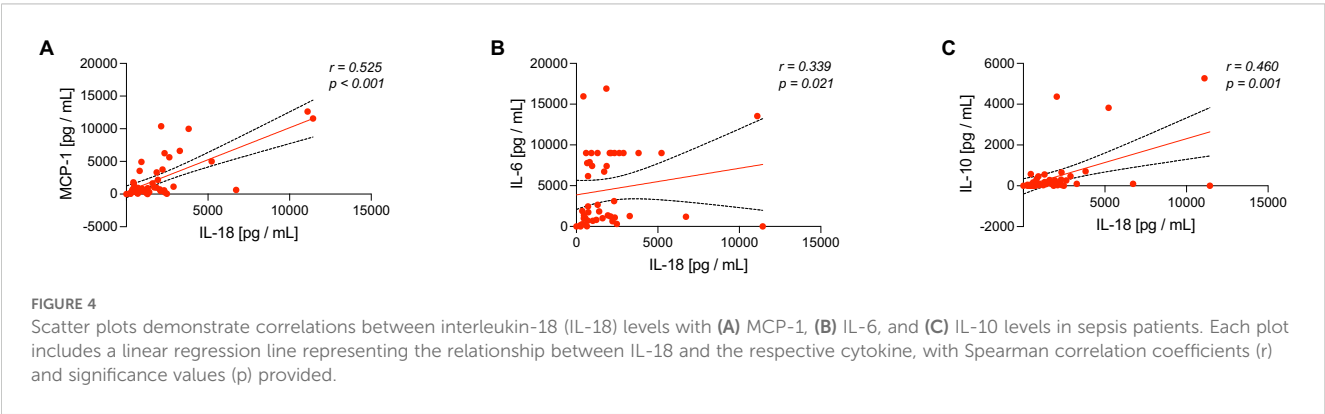
Correlation analyses (IL-18)	Spearman r	p value
Hematocrite (%) ICU adm.	-0.302	0.041
Ca ICU adm.	-0.348	0.018
Ca d0	-0.311	0.038
INR ICU adm.	0.379	0.010
PTT (sec.) ICU adm.	0.351	0.017
PTT (sec.) d0	0.339	0.023
GLDH (U / L) ICU adm.	0.461	0.002
GLDH (U / L) d0	0.445	0.002
GGT (U / L) d0	0.337	0.041
Troponin T ICU adm.	0.700	0.016
SOFA score ICU adm.	0.389	0.015
SOFA d0	0.376	0.017
Length of hospital stay prior ICU (days)	0.391	0.007
Length of hospital stay (days)	0.412	0.004
Mortality	0.375	0.010
MCP-1	0.525	<0.001
IL-6	0.339	0.021
IL-10	0.460	0.001

Ca, Calcium; GGT, Gamma-Glutamyl Transferase; GLDH, Glutamate Dehydrogenase; IL, Interleukin; INR, International Normalized Ratio; MCP-1, Monocyte Chemoattractant Protein-1; PTT, Partial Thromboplastin Time; SOFA, Sequential Organ Failure Assessment; Troponin T, Cardiac Troponin T. Significant correlations are highlighted with p-values, where $p < 0.05$ indicates a statistically significant association. The Spearman correlation coefficient (r) and p-values for various hematologic and biochemical markers, clinical scores, length of hospital stay, and inflammatory cytokines are shown.

several key inflammatory mediators, including MCP-1, IL-6, and IL-10, suggesting its broader immunologic role in the septic response. Several pro-inflammatory cytokines, such as MCP-1 and IL-6, were significantly elevated in both sepsis groups compared to healthy controls. MCP-1, expressed by various cells,

including fibroblasts, endothelial cells, and smooth muscle cells, plays a pivotal role in the immune response to sepsis (27). It correlates with SOFA scores (28) and 28-days mortality (29). IL-6, a multifunctional cytokine, initiates acute-phase reactions, enhances leukocyte recruitment, and increased vascular permeability, all of which can contribute to tissue damage and organ dysfunction in septic shock (30). IL-6 also promotes the production of IL-10, an anti-inflammatory cytokine that helps control excessive immune activation (30). While IL-6 is a sensitive diagnostic biomarker for sepsis (85.0% sensitivity; and 62.0% specificity) (31) and correlates with sepsis-related mortality (32), it lacks specificity for abdominal sepsis. Therefore, while both MCP-1 and IL-6 hold prognostic value, they are not suitable for distinguishing abdominal sepsis.

IL-18's behavior in different sepsis remains complex and may depend on microbial etiology. Previous studies have reported conflicting data: Mierzczyńska-Pasierb et al. found no significant IL-18 differences between Gram-positive and Gram-negative bacterial infections, while Oberholzer et al. noted elevated IL-18 in Gram-positive infections (20, 33). These inconsistencies underscore the heterogeneity of IL-18 responses in sepsis and suggest a need for larger, more stratified studies. From a biological standpoint, IL-18 is a highly plausible candidate as a sepsis biomarker. As a proinflammatory cytokine in the IL-1 family, it plays a critical role in innate immunity and stimulates interferon-gamma release (20–22). It also activates the NF- κ B pathway, promoting the expression of adhesion molecules, chemokines, and Fas ligand, all central to the inflammatory cascade in sepsis (22). In experimental models, IL-18 has been linked to gut barrier disruption, increased bacteremia, and sepsis-related mortality (34). In fact, simultaneous blockade of IL-1 and IL-18 has been shown to confer full protection in murine models of sepsis induced by LPS or cecal ligation and puncture (35) (36). Clinically, IL-18 correlates with critical severity indicators such as age, temperature, respiratory rate, and CRP (37). Elevated IL-18 levels have been observed in both adult and neonatal sepsis, especially in non-survivors (34). Compared to traditional markers like CRP, PCT, and white blood cell count (WBC), IL-18 has demonstrated better diagnostic discrimination for sepsis and septic shock (20). Given the limited sensitivity and specificity of leukocytosis and limited specificity of existing markers like CRP and PCT—both of which can be elevated in trauma and postoperative



states (16)—IL-18 may offer a more precise means of early diagnosis and should be further elaborated in larger studies. Similarly, lactate though assessing perfusion deficits, remains non-specific for infection source (18), and although IL-6 is widely used and linked to sepsis severity, it also lacks specificity for distinguishing abdominal from non-abdominal sepsis (19).

IL-18's established role in mucosal immunity, particularly in inflammatory bowel disease (22–24), further supports its potential relevance in abdominal infections. However, its prognostic utility remains controversial. While Mierzchala-Pasierb et al. and Esquerdo et al., found no significant difference between survivors and non-survivors (20, 38), others—such as Eidt et al.—reported a positive association between IL-18 and mortality (39). Our data support the latter, reinforcing the potential of IL-18 not only as a diagnostic marker but also as a prognostic tool in critically ill patients. Finally, the broader clinical relevance of IL-18 extends beyond sepsis. It has been implicated in various inflammatory and autoimmune diseases, including rheumatoid arthritis, psoriasis, systemic lupus erythematosus, adult-onset Still's disease, and dermatomyositis (40), which further highlights its broader immunological role.

In conclusion, our findings add to the growing body of evidence supporting IL-18 as a promising biomarker in sepsis. Despite conflicting reports in the field, its strong biological plausibility, clinical correlations, and potential for infection source differentiation justify further investigation, particularly in distinguishing abdominal from non-abdominal sepsis.

5 Limitations

The study has several limitations that should be acknowledged. First, the retrospective design may introduce selection bias and limit the generalizability of the results to broader ICU populations or different healthcare systems. Second, the overall sample size was relatively small ($n=47$), which reduces statistical power and may increase the likelihood of some errors like type II, particularly in subgroup comparisons. Third, the non-abdominal group was heterogeneous, including infections such as pneumonia, urosepsis, and emphysema, which may have contributed to variability in inflammatory responses and limited the ability to detect source-specific cytokine patterns. Of limiting importance is the younger age in the group of healthy controls which should be matched in future studies. Furthermore, the study relies on single-time-point measurements of cytokines (on the day of sepsis diagnosis), which may not capture the dynamic changes in respiratory and inflammatory parameters over the course of the disease. This limitation is especially relevant for cytokines such as IL-18, where fluctuations over time could provide additional diagnostic and prognostic value. While IL-18 showed moderate diagnostic accuracy in our study (AUC 0.68), its sensitivity and specificity were rather insufficient to be used as a sole biomarker for abdominal sepsis. Additional confounding factors were not fully addressed. For instance, differences in ventilatory support strategies—evidenced by

the unequal distribution of ventilated patients and higher PEEP in abdominal sepsis patients—as well as the unaccounted effects of sedation and catecholamine administration, could influence the observed hemodynamic parameters. Moreover, other factors such as prior antibiotic therapy, variability in the timing of surgical interventions, and other clinical management practices were not standardized across patients and may have affected cytokine levels and clinical outcomes. Also, the study was not adjusted for those potential confounders (e.g., timing of antibiotic administration, immunosuppression, comorbidities), which limits the validity of the cytokine comparisons. Although we evaluated several commonly used biomarkers, pathogen-specific analyses (e.g., Gram-positive versus Gram-negative infections) were not performed. Given that microbial etiology can influence immune responses, this represents a missed opportunity for further stratification. Finally, the findings were not validated in an external cohort, and larger, prospective, and multicenter studies with longitudinal biomarker profiling and standardized treatment protocols are needed to confirm the diagnostic and prognostic utility of IL-18 and other cytokines in sepsis.

6 Conclusions

This study evaluated the clinical profiles and immunological responses of patients with abdominal versus non-abdominal sepsis, with a particular focus on the diagnostic utility of IL-18. While standard clinical parameters and inflammatory markers such as CRP, PCT, and IL-6 were elevated in both groups, they lacked the specificity to distinguish sepsis origin. IL-18 levels were significantly higher in non-abdominal sepsis and correlated with other key cytokines, including IL-6, IL-10, and MCP-1, suggesting a broader role in systemic inflammation. Despite showing moderate diagnostic accuracy (AUC 0.68), IL-18 alone may not be sufficient as a standalone biomarker for identifying abdominal sepsis. However, its biological plausibility, correlation with disease severity, and ability to reflect distinct immunologic profiles support its continued investigation. These findings underscore the need for a multimodal diagnostic approach combining clinical evaluation with a panel of biomarkers. Given the limitations of this retrospective study—including small sample size, heterogeneous infection sources, and single time-point cytokine measurements—larger prospective studies with longitudinal assessments are warranted to validate IL-18's diagnostic and prognostic value in sepsis, particularly in distinguishing abdominal from non-abdominal infection sources.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

All patients were included at the University Hospital of the University Düsseldorf with the institutional ethics committee approval with the number: 2018094832. The healthy subjects were included at the University Hospital of the University Ulm as approved by the institutional ethics committee with the number: 420/23. In alignment with the ethical standards, written informed consent was obtained from all enrolled subjects, and all enrolled subjects signed the informed consent forms themselves or informed consent was obtained from the nominated legally authorized representative consented on the behalf of participants as approved by the ethical committees. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

AH: Conceptualization, Formal Analysis, Investigation, Project administration, Supervision, Visualization, Writing – original draft. MT: Data curation, Investigation, Methodology, Visualization, Writing – review & editing. CV: Data curation, Investigation, Methodology, Resources, Writing – review & editing. TD: Data curation, Investigation, Methodology, Resources, Writing – review & editing. TB: Data curation, Investigation, Methodology, Resources, Writing – review & editing. AK: Methodology, Writing – review & editing. HR: Methodology, Writing – review & editing. CB: Methodology, Writing – review & editing. IV: Formal Analysis, Methodology, Writing – review & editing. JB: Methodology, Writing – review & editing. NB: Methodology, Writing – review & editing. BR: Conceptualization, Data curation,

Formal Analysis, Investigation, Project administration, Resources, Supervision, Visualization, Writing – original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

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Pro-dermcidin and derivatives as potential therapeutics for lethal experimental sepsis

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A 110-amino acid precursor of dermcidin (pre-dermcidin, pre-DCD) with a 19-residue N-terminal leader signal sequence can be secreted by human eccrine sweat glands as a leader-less pro-domain-containing peptide (pro-DCD), which is enzymatically cleaved to generate C-terminal anti-microbial peptides (dermcidin-1, DCD-1) capable of killing various bacteria. Previously, it was unknown whether pro-DCD could be pharmacologically developed as potential therapeutics for lethal sepsis. Here, we demonstrated that pharmacological suppression of pro-DCD with polyclonal antibodies worsened sepsis-induced inflammation and liver injury, whereas supplementation of pro-DCD or its PEGylation derivatives significantly protected against sepsis, even when given 2–24 h after disease onset. These protective effects were associated with a significant reduction in circulating levels of surrogate biomarkers [e.g., Granulocyte Colony Stimulating Factor (G-CSF), Interleukin-6 (IL-6), keratinocytes-derived chemokine (KC), Monocyte Chemoattractant Protein 1 (MCP-1), Macrophage Inflammatory Protein-2 (MIP-2), and Soluble Tumor Necrosis Factor Receptor I (sTNFRI)], tissue injury, and blood bacterial counts. Although pro-DCD or its PEGylation derivatives failed to directly kill bacteria across a wide range of concentrations, they were able to activate microtubule-associated protein 1A/1B-light chain 3 (LC3), a marker of autophagy and phagosome maturation in LC3-associated bacterial phagocytosis. Our findings suggest that pro-DCD-derived agents hold promise as potential therapies for clinical sepsis.

KEYWORDS

innate immune cells, pro-dermcidin, PEGylation, anti-bacterial, anti-inflammation, LC3 activation

Introduction

Throughout evolution, primates and humans have developed eccrine sweat glands that produce sweat as an efficient mechanism for evaporative heat loss and thermoregulation. In humans, these eccrine sweat glands also express various antibiotic peptides, including pre-dermcidin (pre-DCD) (1), a 110-amino acid precursor protein with a 19-residue N-terminal leader signal sequence. Once this leader signal sequence is removed, the leaderless pro-dermcidin (pro-DCD) is secreted by the eccrine sweat glands and enzymatically cleaved to remove the pro-domain to produce the mature form dermcidin (DCD), which can kill several bacteria such as *Escherichia coli*, *Enterococcus faecalis*, and *Staphylococcus aureus* (2).

In addition to eccrine sweat glands (1, 2), there is evidence (2) that pro-DCD may also be up-regulated and/or secreted by other cell types, such as monocytes (3), bone marrow-derived mesenchymal stromal cells (BM-MSCs) (4), and skeletal muscles (5) in response to various challenges, including HIV infection (3), *E. coli* exposure (4), or facioscapulohumeral muscular dystrophy (FSHD) (5). Consistently, pro-DCD has been detected in the blood of patients with diverse conditions such as ischemic stroke (4), FSHD (5), melanoma (6), and obstructive sleep apnea (7). Even in rodents, there is some evidence for the possible expression of pro-DCD in the sweat gland of the footpad (8), peripheral blood mononuclear cells (PBMCs) (8), and skeletal muscles in both healthy rats (8) and murine models of myocardial ischemia/reperfusion (9). Previously, it was entirely unknown whether pro-DCD could be developed into potential therapeutics for lethal sepsis. Here, we presented compelling evidence to support a therapeutic potential of pro-DCD and PEGylation derivatives in pre-clinical model of sepsis by attenuating dysregulated inflammation and possibly facilitating LC3-associated phagocytosis and/or autophagic degradation of engulfed pathogens.

Materials and methods

Materials

Murine macrophage RAW 264.7 cell line was purchased from the American Type Culture Collection (ATCC). Macrophage cultures were routinely cultured in DMEM media containing 1% streptomycin/penicillin and 10% fetal bovine serum. When cell densities reached 80–90% confluence, adherent macrophages were cultured in serum-free OPTI-MEM I medium and stimulated with recombinant human pro-DCD-C34S. The intracellular levels of LC3-I and lipidated LC3-II were respectively measured by Western blotting using specific antibodies.

Western blotting

The concentrations of cellular LC3-I and lipidated LC3-II in pro-DCD-stimulated murine macrophage-like RAW 264.7 cells

were measured by Western blotting using rabbit anti-mouse LC3A/B monoclonal antibodies (Cat. # 12741, Cell Signaling) as previously described (10). Briefly, equal volume of cell-conditioned culture medium or murine/human serum were resolved on sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes. After blocking with 5% nonfat milk, the membranes were incubated with the appropriate antibodies (anti-pro-DCD, 1:1000; anti-LC3A/B, 1:1000) overnight. Subsequently, the membranes were incubated with secondary antibodies (mouse-anti-rabbit IgG-HRP, Cat.# sc-2357, Santa Cruz; or donkey-anti-rabbit IgG-HRP, Cat. # NA934, GH Healthcare), and the immune-reactive bands were visualized by chemiluminescence. The relative levels of specific proteins were determined using the UN-SCAN-IT Gel Analysis Software Version 7.1 (Silk Scientific Inc., Orem, UT, USA) and expressed in arbitrary units (AU).

Preparation of recombinant human pro-DCD and pro-DCD-C34S proteins

The cDNA encoding human pro-dermcidin (residue 20–110) or a mutant with a Cys (C)→Ser (S) substitution at residue 34 (pro-DCD-C34S) was cloned into a pReceiver expression vector downstream of a T7 promoter with an N-histidine tag, and expressed in *E. coli* BL21 (DE3) pLysS cells as previously described (11, 12). The inclusion body-associated recombinant pro-DCD or pro-DCD-C34S protein was isolated by differential centrifugation and urea solubilization, followed by refolding in Tris buffer (pH 8.0) containing N-lauroylsarcosine. The recombinant pro-DCD and pro-DCD-C34S were further purified using histidine-affinity chromatography, followed by extensive Triton X-114 extractions to remove contaminating endotoxins. The recombinant pro-DCD or pro-DCD-C34S protein were tested for LPS content by the chromogenic *Limulus* amebocyte lysate assay (Endochrome; Charles River), and the endotoxin content was less than 0.01 U per microgram of recombinant protein.

Generation of anti-pro-DCD polyclonal antibodies

Polyclonal antibodies were generated in Female New Zealand White Rabbits by the Covance Inc. (Princeton, NJ, USA) using recombinant human pro-DCD combined with Freund's complete adjuvant following standard procedures. Blood samples were collected in 3-week cycles of immunization and bleeding, and the antibody titers were determined by direct pro-DCD ELISA. Total IgGs and pro-DCD antigen-binding IgGs were purified from the anti-pro-DCD rabbit serum using Protein A and pro-DCD-affinity column chromatography, respectively. Briefly, rabbit serum was pre-buffered with PBS and slowly loaded onto the Protein A/G Sepharose (Cat. # ab193262) column to allow sufficient binding of IgGs. After washing with 1×PBS to remove unbound serum components, the IgGs were eluted with acidic buffer (0.1 M

glycine-HCl, pH 2.8), and then immediately dialyzed into 1×PBS buffer at 4°C overnight. For pro-DCD antigen-affinity purification, recombinant human pro-DCD was conjugated to cyanogen bromide (CNBr)-activated Sepharose4 agarose beads (Cat. # 17098101, GE Healthcare), which were then loaded into columns. After repeated washings with acid buffer (0.1 M Acetic/Sodium Acetate, 0.5 M NaCl, pH 4.0) and alkali buffer (0.1 M Tris-HCl, 0.5 M NaCl, pH 8.0), anti-pro-DCD total IgGs were slowly loaded onto the column, and the flow-through fractions were collected. After repetitive washing with 1×PBS buffer, the pro-DCD-binding antibodies were eluted with acidic elution buffer, and immediately neutralized in 1×PBS solution.

Peptide dot blotting

A library of 13 synthetic peptides corresponding to various regions of human pro-DCD sequence was synthesized at the Genscript, and spotted (0.1 µg in 2.5 µl) onto a nitrocellulose membrane (Thermo Scientific, Cat No. 88013). Subsequently, the membrane was probed with anti-pro-DCD rabbit serum or pro-DCD antigen affinity-purified IgGs, following a standard protocol as previously described (13, 14).

Cytokine Antibody Arrays

Murine Cytokine Antibody Arrays (Cat. No. M0308003, RayBiotech Inc., Norcross, GA, USA), which simultaneously detect 62 cytokines on one membrane, were used to measure relative cytokine concentrations in murine serum as described previously (13–15).

PEGylation of pro-DCD-C34S

Recombinant pro-DCD-C34S was pegylated using methyl-PEG₂₄-NHS ester (MW of 1214.39 Daltons, Cat.# 22687, Thermo Scientific, Rockford, IL, USA) according to manufacturer's instructions. Briefly, methyl-PEG₂₄-NHS ester was dissolved in DMSO (5.0 mg/ml) and mixed with a solution of pro-DCD-C34S at a 20-fold molar excess to the protein, ensuring the final DMSO concentration was below 10%. After incubating the mixture on ice for two hours, the solution was dialyzed overnight at 4°C in 1×PBS buffer to remove any unreacted PEG NHS ester.

Animal model of experimental sepsis

Every effort was made to minimize the number of animals used in this study in accordance with the ARRIVE guidelines developed by the British National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs). Additionally, all experiments were conducted following the International Expert Consensus Initiative for Improvement of Animal Modeling in

Sepsis - Minimum Quality Threshold in Pre-Clinical Sepsis Studies (MQTiPSS) (16). This includes practices such as randomizing animals within each experimental group, implementing delayed therapeutic interventions (with agents like pegylated pro-DCD-C34S) (17), establishing specific criteria for euthanizing moribund septic animals (e.g., labored breathing, minimized response to human touch, and immobility), and administering fluid resuscitation and antibiotics (18). This study received administrative approval from the IACUC of the Feinstein Institutes for Medical Research (FIMR, Protocol # 2017–038 Term II; Date of Approval, February 22, 2021).

Adult male and female Balb/C mice (7–8 weeks old, 20–25 g body weight) were purchased from Charles River Laboratories (Wilmington, MA), and acclimated for at least 5–7 days before usage. To induce experimental sepsis, Balb/C mice underwent a surgical procedure known as “cecal ligation and puncture” (CLP) as previously described (13, 14, 19). Prior to CLP surgery, all animals received a buprenorphine injection (0.05 mg/kg, subcutaneously) to manage immediate surgical pain, because repetitive use of buprenorphine in the CLP model could paradoxically elevate sepsis surrogate markers and increase animal lethality (20, 21). Approximately 30 min post CLP surgery, all experimental animals were subcutaneously injected with a dose of imipenem/cilastatin (0.5 mg/mouse) (Primaxin, Merck & Co., Inc.) to avoid their potential adverse effects on the therapeutic efficacy of pro-DCD-C34S or derivatives. Animals were randomly assigned to control vehicle and experimental groups, and pro-DCD, pro-DCD-C34S, or pegylated pro-DCD-C34S was intraperitoneally injected to septic mice at various time points post-CLP. Animal survival was monitored for two weeks to ensure no late death occurred. To elucidate the potential protective mechanisms of peg-pro-DCD-C34S, a separate group of Balb/C mice underwent CLP and received peg-pro-DCD-C34S (0.2 mg/kg) at 2 h and 20 h post-CLP. At 24 h post CLP, animals were euthanized to collect blood and measure: i) serum levels of various cytokines and chemokines using murine Cytokine Antibody Arrays; ii) markers of liver injury using specific colorimetric enzymatic assays; and iii) blood bacterial colony forming units as previously described (13).

Male and female mice were not mixed in the same experiments to avoid potential influences of female hormones on the host immune response to infections. Instead, all experiments were initially conducted using male mice and subsequently replicated in age-matched female mice in separate studies to assess potential gender-specific differences. When no gender-specific differences were observed, the results from both male and female mice were pooled to reduce the number of animals used while ensuring robust and reliable conclusions.

Measurement of liver injury markers

Blood samples were collected at 24 h post-CLP following intraperitoneal administrations of pegylated pro-DCD-C34S (0.2 mg/kg) at 2 h and 20 h post-CLP. The samples were centrifuged at 3000 × g for 10 min to collect serum, and serum levels of liver injury

markers such as aspartate aminotransferase (AST, Cat. No. 7561) and alanine aminotransferase (ALT, Cat. No. 7526) were measured using specific colorimetric enzymatic assays (Pointe Scientific, Canton, MI) according to manufacturer's instructions as previously described (13, 22).

Liver histological analysis

The liver samples were collected at 6 h post CLP, fixed in 10% buffered formalin, and embedded in paraffin. The paraffin-embedded tissues were sectioned into 5- μ m slices, stained with hematoxylin-eosin, and examined under light microscopy. Liver parenchymal injury was assessed in a blinded fashion by summing three different Suzuki scores (ranging from 0–4) for sinusoidal congestion, hepatocyte cytoplasmic vacuolization, and parenchymal necrosis as previously described (12, 14). The parameter scores were calculated using a weighted equation with a maximum score of 100 per field and then averaged to determine the final liver injury score for each experimental group.

Evaluation of bactericidal activity

Escherichia coli BL21 (DE) strain was inoculated into Luria Bertani Broth (LB broth), incubated at 37°C until the culture reached the mid-log phase. Pro-DCD or peg-pro-DCD-C34S was dissolved in 1 $\times$ PBS buffer at various dilutions and mixed with the *E. coli* suspension in 1 $\times$ PBS. The mixture was incubated at 37°C for 4 h in sterile test tubes as previously described (1, 23). After incubation, the bacterial suspension was serially diluted in sterile 1 $\times$ PBS solution and spread onto LB agar plates. Following incubation 37°C for 16–24 h, the number of colony-forming units (CFUs) on each plate was counted to determine whether pro-DCD or its derivatives reduced the CFU count of the *E. coli* suspension.

Visualization of LC3-positive punctate structures

Murine macrophage-like RAW 264.7 cells stably transfected with GFP-LC3 were stimulated with pro-DCD (1.0 μ g/ml) for 16 h, and the formation of GFP-LC3 punctate structures was examined under a fluorescence microscope as previously described (10). The ratio between the 18-kD cytosolic LC3-I and 16-kD lipidated LC3-II was determined by Western blotting analysis following previously described methods (10).

Statistical analysis

All data were first assessed for normality using the Shapiro-Wilk test before applying the appropriate statistical analyses. The Student's *t* test was employed to compare two independent experimental groups. For comparison among multiple groups with non-normal (skewed)

distribution, the Kruskal-Wallis ANOVA test followed by Dunn's *post hoc* test was used to evaluate statistical differences. The Kaplan-Meier method, along with the nonparametric log-rank test, was used to compare mortality rates between different groups. Statistical significance was defined as a *P* value less than 0.05.

Results

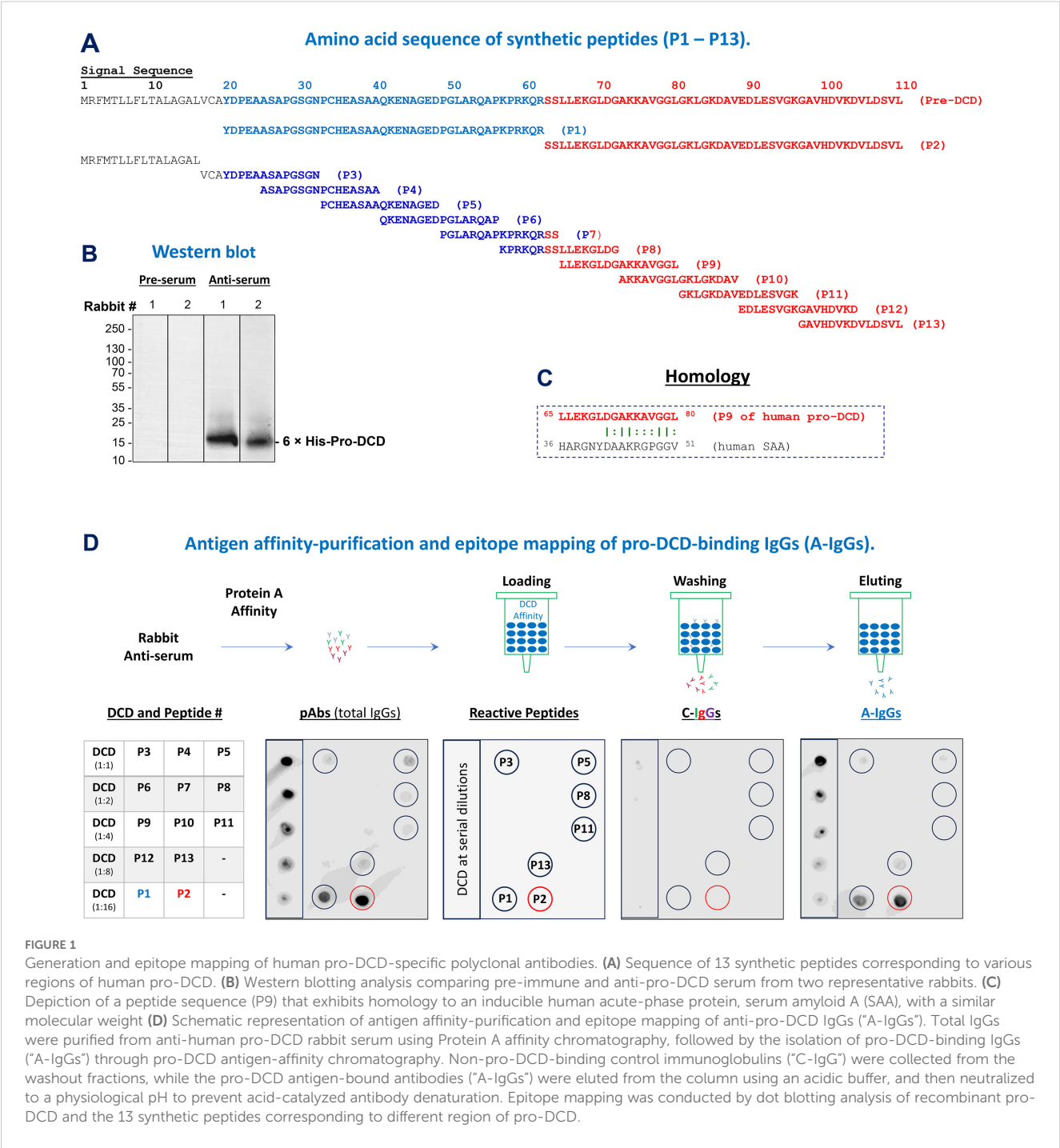
Generation of human pro-DCD-specific polyclonal antibodies

To understand the extracellular role of pro-DCD in sepsis, we synthesized thirteen peptides corresponding to different regions of human pro-DCD (Figure 1A) and used them to profile the epitopes of rabbit polyclonal antibodies that we raised against human pro-DCD (Figure 1B). As predicted, immunoblotting of recombinant pro-DCD with pre-immune rabbit serum did not reveal any immune-reactive band (Figure 1B), indicating that these rabbits did not produce pro-DCD-reactive autoantibodies. However, Western blotting with anti-pro-DCD serum revealed a strong band corresponding to the molecular weight of recombinant pro-DCD with an N-terminal 6 $\times$ His tag (11, 12) (Figure 1B). Consistently, our home-made pro-DCD protein was recognized by some commercial polyclonal antibodies (ab175519) raised against a unique peptide sequence (residue 96–110) of human pro-DCD (Supplementary Figure S1).

To obtain pro-DCD antigen-affinity purified total IgGs ("A-IgGs"), we first subjected anti-pro-DCD rabbit serum to Protein-A-affinity chromatography to obtain total IgGs ("pAbs", Figure 1D, Top Panel), followed by pro-DCD-antigen-affinity chromatography to isolate the antigen-binding IgGs ("A-IgGs") (Figure 1D, Top Panel). Dot blotting analyses demonstrated that these anti-pro-DCD pAbs were reactive with recombinant pro-DCD at various dilutions, as well as several synthetic peptides (e.g., P1, P2, P3, P5, P8, P11 and P13) corresponding to different regions of pro-DCD (Figure 1D), confirming their specificity for human pro-DCD. Additionally, the epitope profiles of pro-DCD antigen-affinity purified IgGs ("A-IgGs") were consistent with those of the crude anti-pro-DCD serum (Figure 1D), validating the specificity of our home-made anti-pro-DCD pAbs to multiple pro-DCD peptides, including epitopes corresponding to residue 97–110 of pro-DCD (i.e., "P13", Figure 1D). Despite some homology between two short peptides of pro-DCD (residue 65–80 or "P9") and the acute phase protein serum amyloid A (SAA, residue 36–51, Figure 1C), our anti-pro-DCD pAbs did not react with the P9 peptide, suggesting that our home-made anti-pro-DCD pAbs were unlikely to cross-react with human SAAs.

Pro-DCD-reactive polyclonal antibodies worsened the outcome of experimental sepsis

To explore the role of pro-DCD in experimental sepsis, we assessed the impact of antigen affinity-purified anti-pro-DCD IgGs



on sepsis-induced systemic inflammation and tissue injury. Notably, pro-DCD-specific IgGs (“A-IgGs”) significantly elevated sepsis-induced systemic accumulation of G-CSF, IL-6 and MCP-1 (Figure 2A), three surrogate markers of experimental (24, 25) and clinical sepsis (26). Consistently, these anti-pro-DCD antibodies exacerbated sepsis-induced liver injury (Figures 2B, C) and appeared to dose-dependently increase sepsis-induced animal lethality (Figure 2D). Collectively, these findings have suggested a potentially beneficial role of pro-DCD in an animal model of experimental sepsis.

Pro-DCD and PEGylation derivatives conferred a significant protection against lethal sepsis

To assess the therapeutic potential of pro-DCD, we generated recombinant pro-DCD corresponding to residue 20-110 (excluding the signal leader peptide, Figure 3A) with an N-terminal 6×His tag as previously described (11, 12). Because recombinant pro-DCD could form dimers via disulfide bond at Cys (C) 34, we also created a pro-DCD variant with a Cys (C)→Ser (S) substitution at residue

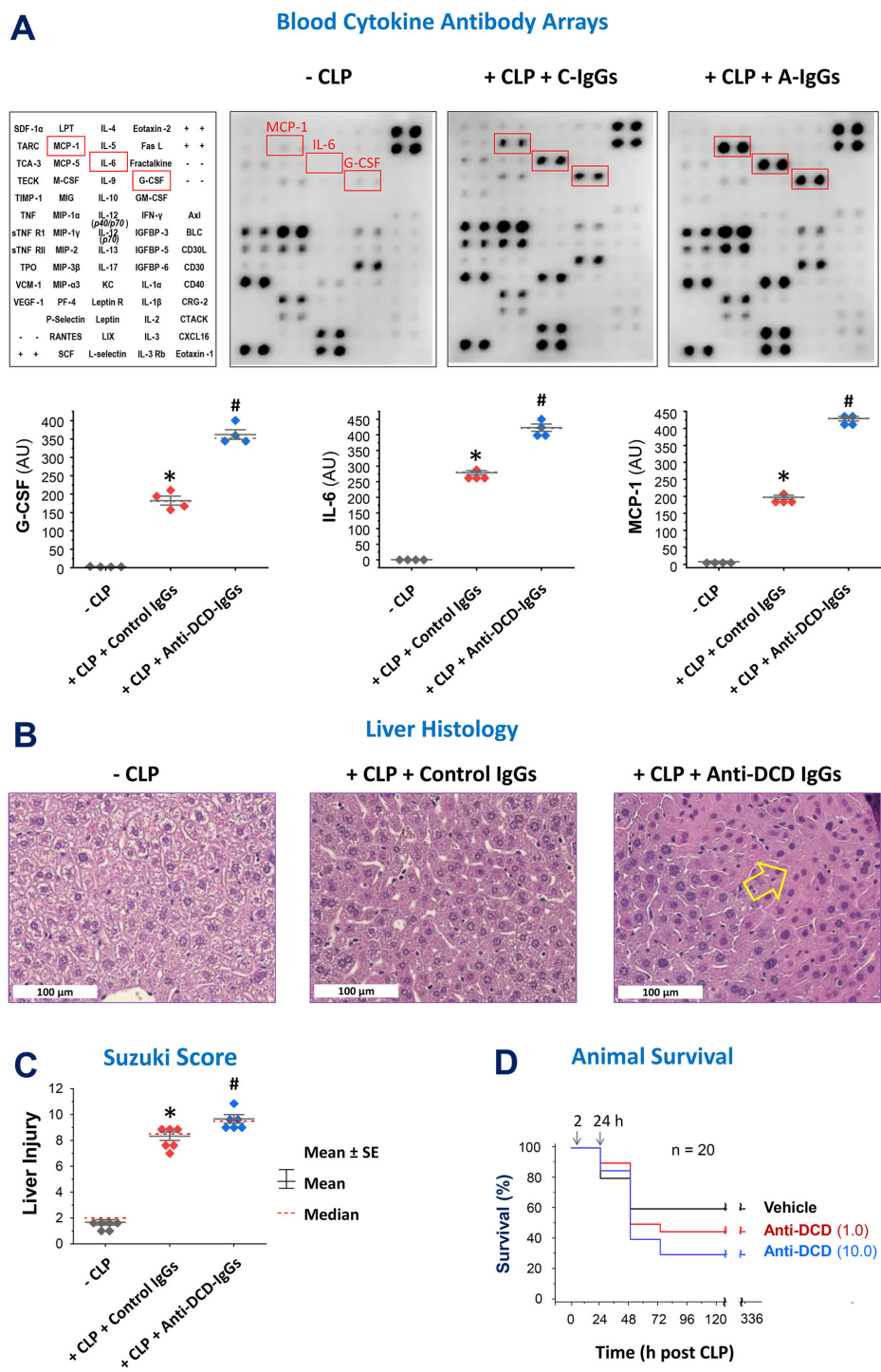


FIGURE 2 Pro-DCD-reactive IgGs worsened sepsis-induced inflammation and tissue injury in mice. **(A–C)** Pro-DCD-specific IgGs aggravated sepsis-induced inflammation and tissue injury. Balb/C mice were subjected to CLP-induced experimental sepsis, followed by intraperitoneal administration of either pro-DCD-non-reactive control antibodies (“C-IgGs”) or antigen-affinity purified anti-pro-DCD IgGs (“A-IgGs”, 10 mg/kg) at 2 h post-CLP. At 6 h post-CLP, the animals were sacrificed to harvest blood and liver tissues to measure blood levels of various cytokines and chemokines using Cytokine Antibody Arrays **(A)** and assess liver injury through histological analysis **(B)** and assessment of Suzuki Injury Scores **(C)**. *, $P < 0.05$ vs. negative control (“-CLP”); #, $P < 0.05$ vs. positive control (“Control IgGs”). non-parametric Kruskal-Wallis ANOVA test. **(D)** Antigen affinity-purified anti-DCD antibodies (“A-IgGs”) appeared to increase sepsis-induced mortality in a dose-dependent manner.

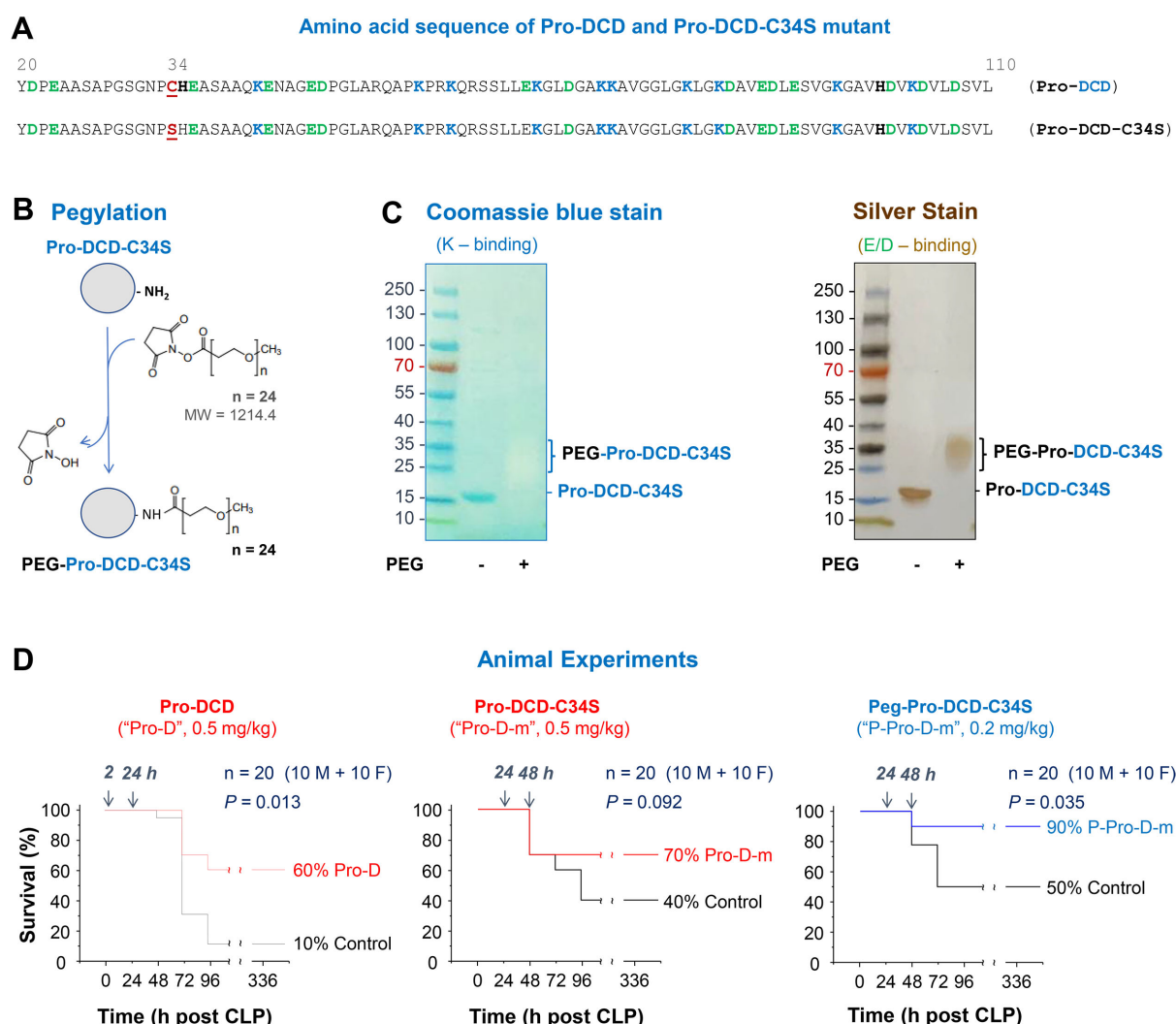


FIGURE 3

Generation of pro-DCD-C34S mutant and PEGylation derivatives for treating experimental sepsis. **(A)** Amino sequence of recombinant pro-DCD mutant containing a Cys (C)→Ser (S) substitution at residue 34 (pro-DCD-C34S). **(B)** PEGylation of pro-DCD-C34S using methyl-PEG₂₄-NHS ester potentially targeting ten Lys (K) residues. **(C)** Detection of pegylated pro-DCD-C34S (Peg-pro-DCD-C34S) through SDS-PAGE gel electrophoresis and silver staining. The PEG-DCD-C34S was not visible with Coomassie blue staining due to the PEGylation of amine groups of Lys (K) residues, which prevented its electrostatic interaction with the Coomassie Brilliant Blue G-250 dye. However, pegylated pro-DCD-C34S was detectable by silver staining as silver nitrate could still interact with other residues, such as the carboxylic acid groups of Asp (D) and Glu (E) or imidazole of His (H) of pro-DCD-C34S. **(D)** Pro-DCD and PEG-pro-DCD-C34S rescued mice from lethal sepsis. Balb/C mice (male or female, 8–10 weeks, 20–25 g) mice were subjected to CLP-induced sepsis, and highly purified recombinant pro-DCD, pro-DCD-C34S, or pegylated pro-DCD-C34S were administered intraperitoneally at specified doses and time points after CLP. Animals were monitored for two weeks to ensure long-lasting effects. *, $P < 0.05$ versus saline control group.

34 (pro-DCD-C34S, Figure 3A), which migrated as a 16-kDa monomer on SDS-PAGE even in the absence of a reducing agent, DTT (Figure 3C). To extend the half-life of pro-DCD-C34S, we chemically conjugated highly hydrophilic polyethylene glycol (PEG) chains to the Lys (K) residue of this peptide (Figure 3B). This modification is expected to shield pro-DCD-C34S from enzymatic degradation and prevent it from renal clearance. As anticipated, pegylated pro-DCD-C34S was not detected by Coomassie blue staining (Figure 3C, Left Panel), because PEGylation of amine groups of Lys (K) residues obstructed its electrostatic interaction with the Coomassie Brilliant Blue G-250 dye. However, the pegylated pro-DCD-C34S was still detected by

silver staining as larger molecules ranging 25 to 37 kDa (Figure 3C, Right Panel), since silver nitrate could still interact with other residues such as the carboxylic acid groups of Asp (D) and Glu (E) or imidazole of His (H) of pro-DCD-C34S.

After extensive extraction with Triton X-114 to remove endotoxin contaminants, we evaluated the therapeutic efficacy of highly purified pro-DCD, pro-DCD-C34S, and pegylated pro-DCD-C34S in a murine model of experimental sepsis. Administration of recombinant pro-DCD at 2 h and 24 h post-CLP significantly improved animal survival rate, increasing it from 10% in the saline control to 60% in the pro-DCD-treatment group (Figure 3D, Left Panel). When the 1st dose was delayed to 24 h post-

CLP, the pro-DCD-C34S analog showed a similar trend towards increasing animal survival at the same dose (0.5 mg/kg BW, **Figure 3D**, Middle Panel). Notably, pegylated pro-DCD-C34S provided a significant protection against lethal sepsis even when given in a delayed fashion (24 h post-CLP) at a dose (0.2 mg/kg) that was approximately 5-fold lower in molar concentration compared to pro-DCD-C34S (**Figure 3D**, Right Panel), considering the almost two-fold difference in molecular weight between pro-DCD-C34S (~15 kDa) and PEG-pro-DCD-C34S (25–37 kDa, **Figure 3C**). Collectively, these results indicate that both pro-DCD and pro-DCD-C34S derivatives offer significant protection against lethal sepsis.

Peg-pro-DCD-C34S reduced blood levels of surrogate inflammatory and liver markers and bacterial counts

To investigate the mechanisms underlying pro-DCD-mediated protection, we assessed the effects of pegylated pro-DCD-C34S on sepsis-induced systemic inflammation, tissue injury, and bacterial dissemination. Intraperitoneal administration of peg-pro-DCD-C34S significantly reduced sepsis-induced systemic accumulation of G-CSF, IL-6, KC, MCP-1, MIP-2, and sTNFRI (**Figure 4A**), indicating that pro-DCD derivatives confer protection against sepsis by mitigating systemic inflammation of key sepsis surrogate biomarkers of experimental (24, 25) and clinical sepsis (26). Additionally, peg-pro-DCD-C34S significantly attenuated sepsis-induced elevation of liver enzymes such as AST and ALT (**Figure 4B**), suggesting that pro-DCD derivatives provide protection against lethal sepsis partly by alleviating both inflammation and tissue injury.

To further elucidate the protective mechanism of pro-DCD, we also examined the impact of peg-pro-DCD-C34S on blood bacterial counts. As predicted, CLP led to a substantial increase in blood bacterial levels (**Figures 4C, D**), reflecting cecal bacterial spillage into the blood stream. However, treatment with peg-pro-DCD-C34S almost completely diminished blood bacterial counts (**Figures 4C, D**), suggesting that pro-DCD conferred protection against lethal sepsis partly by facilitating bacterial clearance.

3 Pro-DCD failed to directly kill bacteria but induced LC activation in macrophage cultures

To understand how pro-DCD facilitates bacterial elimination in septic animals, we incubated pro-DCD or peg-pro-DCD-C34S with *E. coli* for 3–4 hours and plated the mixture onto LB broth agar at serial dilutions to count the colony forming unit (CFU). At concentrations up to 200 µg/ml, neither pro-DCD nor peg-pro-DCD-C34S reduced CFU (**Figure 5A**), suggesting that pro-DCD or peg-pro-DCD-C34S could not directly kill bacteria in septic animals. To explore alternative mechanisms by which pro-DCD might enhance bacterial clearance, we examined its effect on the

activation of microtubule-associated protein 1A/1B-light chain 3 (MAP1LC3, LC3) in macrophage cultures. Pro-DCD stimulation led to the formation of LC3-positive cytoplasmic puncta (**Figure 5B**), and the conversion of LC3-I to the lipidated LC3-II form (**Figure 5C**). It raised an interesting possibility that pro-DCD may promote bacterial elimination by promoting LC3-associated phagocytosis and/or autophagy, two highly conserved processes that clear extracellular and intracellular bacteria by many phagocytes.

Discussion

Currently, there are still no effective therapies for clinical sepsis beyond adjunctive care, such as antibiotic administration and fluid resuscitation (27–29). Therefore, it is crucial to use animal models of sepsis (30) to explore novel protective proteins that can eliminate invading pathogens and mitigate sepsis-induced dysregulated inflammatory injury. In this study, we demonstrated a beneficial role pro-DCD in an experimental model of lethal bacterial infections. On one hand, pro-DCD antigen-affinity purified pAbs exacerbated sepsis-induced systemic inflammation and tissue injury. On the other hand, recombinant pro-DCD conferred a significant protection against lethal sepsis when administered repeatedly at 2 h or 24 h post-onset. Because pro-DCD contains a Cys (C) residue at position 34 that promotes the formation of pro-DCD dimers through disulfide cross-linking, we generated a pro-DCD mutant with a Cys (C)→Ser (S) substitution at this position. The resultant pro-DCD-C34S mutant remained monomeric in solution even in the absence of any reducing agents. Given the relatively short half-life (< 24 h) of a 105-amino acid pro-DCD isomer 2 with 75% sequence homology to pro-DCD (31), we chemically conjugated water-soluble PEG side-chains to the Lys (K) residue of pro-DCD-C34S. The pegylated pro-DCD-C34S effectively rescued both male and female mice from microbial infections, even when given 24 hours post-CLP at a molar concentration five-fold lower than pro-DCD or pro-DCD-C34S. These findings mirrored previous observations that systemic administration of pro-DCD-C34S attenuated hepatic ischemia-reperfusion injury (12). Therefore, it will be important to determine whether systemic administration of pro-DCD-C34S derivatives can confer protection against lethal infections or ischemia-reperfusion injuries in clinical settings.

Currently, the exact mechanism for pro-DCD-C34S-mediated protection remains unclear but may attribute to the attenuation of sepsis-induced dysregulated inflammation and tissue injury. Indeed, pegylated pro-DCD-C34S significantly attenuated sepsis-induced accumulation of G-CSF, IL-6, KC/GRO-α, MCP-1, MIP-2/GRO-β, and sTNFRI, six surrogate markers of experimental sepsis (20, 26, 32). These findings aligned with our previous reports that pro-DCD or pro-DCD-C34S attenuated LPS- or ischemia-reperfusion-induced production of chemokines such as KC/GRO-α (11) and MIP-2/GRO-β (12). Additionally, pegylated pro-DCD-C34S significantly attenuated sepsis-induced liver injury, and markedly facilitated bacterial elimination in septic animals.

The mechanisms underlying the facilitation of bacterial elimination by pro-DCD-C34S remains an intriguing subject for

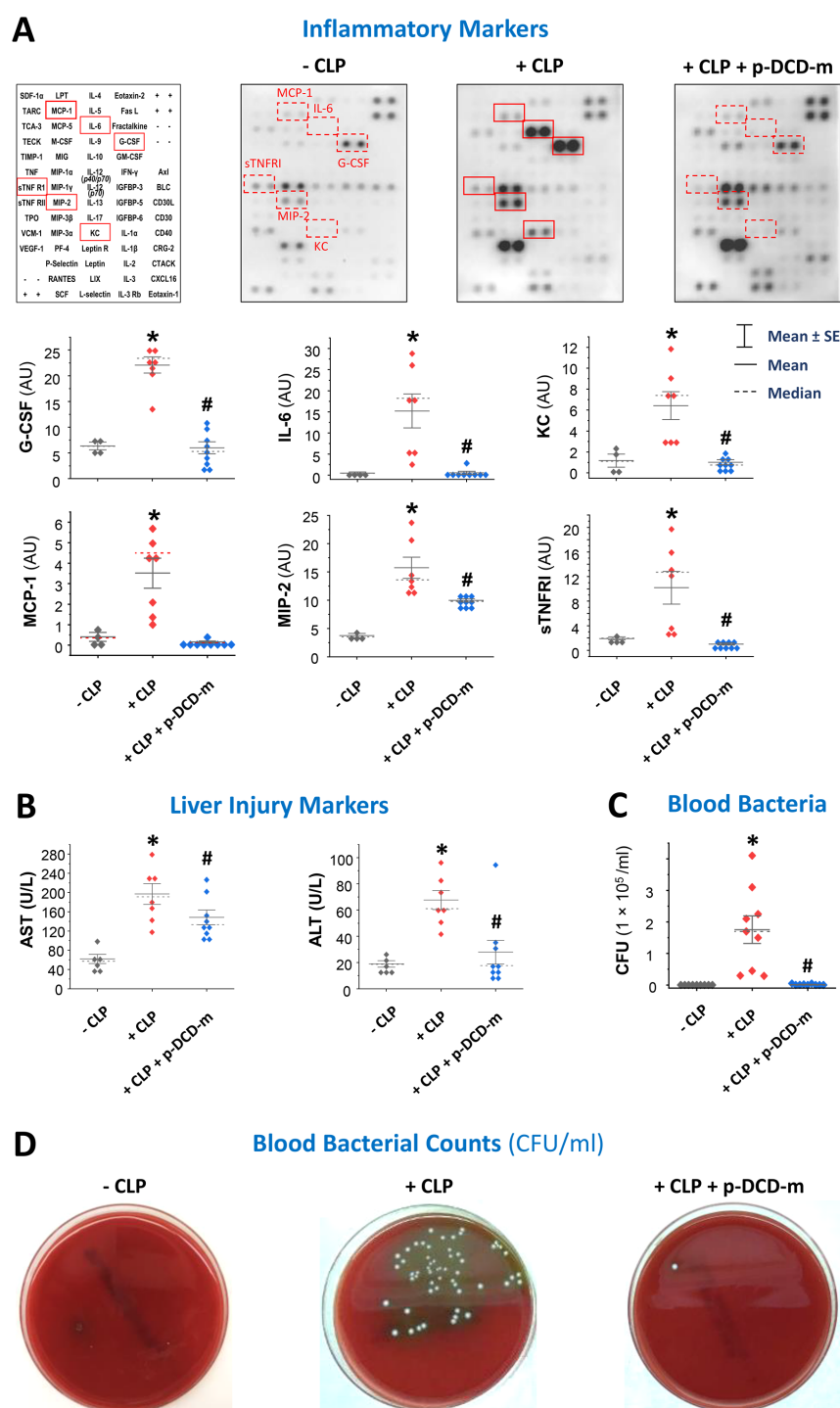


FIGURE 4

Pegylated pro-DGD-C34S significantly reduced sepsis-induced systemic inflammation, liver injury, and blood bacterial loads. (A, B) Pegylated pro-DGD-C34S significantly attenuated sepsis-induced systemic inflammation and liver injury. Balb/C mice were subjected to CLP-induced sepsis, and pegylated pro-DGD-C34S (0.2 mg/kg) was given twice at 2 and 20 h post-CLP. At 24 h post-CLP, animals were sacrificed to harvest blood to measure various cytokines and chemokines (A) and liver injury markers such as AST and ALT (B). * $P < 0.05$ vs. negative control ("– CLP"); # $P < 0.05$ vs. positive control ("+ CLP") group, non-parametric Kruskal-Wallis ANOVA test. (C, D) Pegylated pro-DGD-C34S markedly reduced blood bacterial load in septic animals. Balb/C mice were subjected to CLP-induced sepsis, and pegylated pro-DGD-C34S (0.2 mg/kg) was given twice at 2 and 20 h post-CLP. At 24 h post-CLP, animals were sacrificed to harvest blood to count bacterial colony forming unit (CFU) after serial dilution and plating on LB agar plates. $P < 0.05$ vs. negative control ("– CLP"); # $P < 0.05$ vs. positive control ("+ CLP"), non-parametric Kruskal-Wallis ANOVA test.

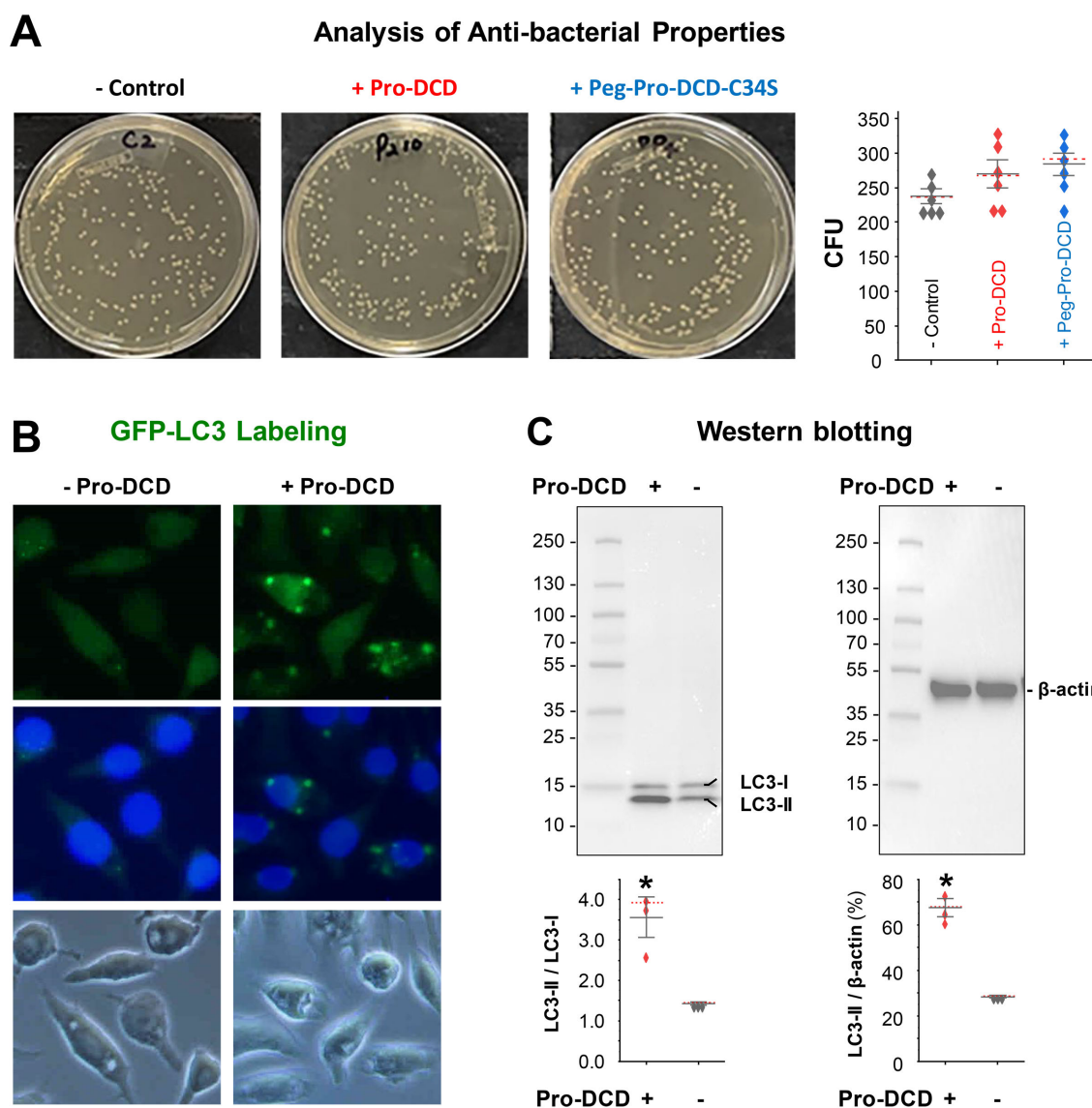


FIGURE 5

Pro-DCD failed to directly kill bacteria but significantly activated LC3 in macrophage cultures. **(A)** Pro-DCD failed to directly kill *E. coli*. Pro-DCD-C34S or its pegylated derivatives were incubated with *E. coli* strain at various concentrations (up to 200 $\mu\text{g/ml}$) for 4 hours, and then plated onto LB agar plates. After overnight incubation at 37°C, the number of colony forming units (CFU) was counted. **(B)** Pro-DCD-C34S induced the formation of LC3 puncta in macrophage cultures. GFP-LC3-transfected murine macrophage-like RAW 264.7 cells were stimulated with pro-DCD-C34S (0.2 $\mu\text{g/ml}$) for 16 h, and the formation of LC3 puncta was observed under fluorescent microscopy. **(C)** Pro-DCD-C34S induced LC3-II production in macrophage cultures. Macrophage-like RAW264.7 cells were stimulated with pro-DCD-C34S for 16 h, and cellular levels of LC3-I and LC3-II were determined by Western blotting analysis with reference to β -actin. * $P < 0.05$ versus negative control ("– pro-DCD"), non-parametric Kruskal-Wallis ANOVA test.

future investigations. It has been proposed that the C-terminal anti-microbial domains (residue 63–110) of pro-DCD can form hexameric structures that may interact with bacterial phospholipids, leading to membrane depolarization and bacterial cell death (33–35). However, in contrast to the reported antibacterial activities of C-terminal anti-microbial domains of pro-DCD (1, 2, 23), our findings showed that pro-DCD and its PEGylation derivatives failed to directly kill *E. coli* even at concentrations up to 200 $\mu\text{g/ml}$. It remains elusive whether the presence of the pro-domain may physically hinder the formation of

hexameric structures in the antimicrobial domains of pro-DCD, which might be necessary for disrupting bacterial membranes and inducing bacterial cell death (33–35).

Alternatively, pro-DCD may enhance bacterial elimination by promoting LC3-associated phagocytosis and autophagic degradation of engulfed pathogens. Indeed, macrophages and monocytes utilize two highly conserved processes, phagocytosis and a special form of autophagy termed as "xenophagy", to eliminate extracellular pathogens and destroy intracellular organisms (36–38). Specifically, the engulfment of extracellular

bacteria leads to the recruitment of lipidated LC3-II to the single-membrane phagosome, which can fuse with lysosomes, resulting in rapid acidification and destruction of the ingested organism (38). Xenophagy, on the other hand, involves the formation of a double-membrane structure called the autophagosome, which also facilitates the degradation of invasive bacteria following fusion with lysosomes (39–41), a process that share similarities with the maturation of phagosome during LC3-associated phagocytosis to destroy engulfed pathogens. Therefore, LC3 activation facilitates its recruitment to both single-membrane phagosomes and double-membranes autophagosomes during LC3-associated phagocytosis and autophagy. Consistent with a previous report that DCD-containing extracellular vesicles promoted LC3-associated phagocytosis of *E. coli* by macrophages (4), we observed that pro-DCD significantly induced LC3-II production and the formation of LC3-containing puncta in macrophage cultures. It suggests that pro-DCD may enhance bacterial elimination by promoting LC3-associated phagocytosis and autophagic degradation of engulfed pathogens. This possibility is further exemplified by previous findings that various agents capable of inducing autophagy (e.g., antibiotics, endotoxins, bedaquiline, seriniquinone, or anti-bacterial peptides) can enhance antimicrobial responses (42–47). However, future independent studies are needed to investigate whether pro-DCD loses its protective effects against lethal sepsis when LC3 is pharmacologically inhibited or genetically disrupted in experimental animals.

There are a few limitations in the current study: i) It remains elusive whether pro-DCD facilitates bacterial elimination by promoting LC3-associated phagocytosis and/or autophagic degradation of engulfed pathogens. ii) We did not assess systemic inflammatory cytokine profiles at later stages of sepsis, because many septic animals in the control group likely succumbed to the disease between 24 and 48 h post-CLP (as shown in Figure 3D). This made blood sampling at later time points, especially after the death of some animals in the control group, practically infeasible (22). Despite these limitations, our findings of pro-DCD as an inducible and beneficial protein have presented a promising opportunity to develop pro-DCD-C34S-based therapeutics for treating lethal microbial infections. Therefore, it is crucial to advance the development of pro-DCD-C34S derivatives and further translate our pre-clinical research into clinical treatments for bacterial infections.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was approved by Feinstein Institutes for Medical Research. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

WC: Investigation, Writing – review & editing. XQ: Investigation, Writing – review & editing. CZ: Investigation, Writing – review & editing. JL: Resources, Writing – review & editing. LL: Investigation, Writing – review & editing. PW: Conceptualization, Methodology, Writing – review & editing. KT: Conceptualization, Resources, Writing – review & editing. HW: Conceptualization, Formal Analysis, Funding acquisition, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2025.1621633/full#supplementary-material>

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TREM2 signaling pathway in sepsis-induced acute lung injury: physiology, pathology, and therapeutic applications

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Sepsis emerges as a formidable and life-threatening condition, born from an unregulated immune response to infection, presenting a significant challenge to global health. A notable complication of sepsis is acute lung injury (ALI), marked by profound hypoxia, rampant inflammation, and the accumulation of fluid within the pulmonary system. ALI harbors the potential to escalate into acute respiratory distress syndrome (ARDS), thereby exacerbating the severity of sepsis. The triggering receptor expressed on myeloid cells 2 (TREM2), predominantly situated within various myeloid cell types, plays a pivotal role in the modulation of neurodegeneration, inflammation, neoplasms, and other pathologies. Recent investigations have illuminated TREM2's considerable involvement in septic lung injury; however, the precise mechanisms and therapeutic implications within this context demand further scrutiny. This article endeavors to elucidate the intricate interplay between sepsis, lung injury, and TREM2's role in immune modulation. It will furnish an overview of the TREM2 signaling pathway's functions and mechanisms in both physiological and septic lung injury scenarios, while also evaluating the current status and advancements in TREM2-targeted therapies.

KEYWORDS

TREM2, sepsis, ALI, immunomodulation, therapeutic application

Introduction

Sepsis is characterized as a life-threatening condition triggered by infection that culminates in organ dysfunction (1). The clinical tableau presents a diverse array of symptoms, encompassing fever, tachycardia, tachypnea, and alterations in mental status. Absent prompt intervention, sepsis may rapidly escalate into septic shock, with the ominous potential for multiple organ failure (2). Epidemiological insights reveal a concerning rise in sepsis incidence each year, particularly within the confines of intensive care units, where it is estimated that a staggering 30% to 50% of patients may grapple with this affliction. Furthermore, as organ dysfunction deteriorates, the risk of mortality escalates significantly (3, 4). Thus, the imperative for early identification and management of sepsis becomes paramount, serving as a crucial determinant for enhancing patient prognosis and mitigating mortality rates.

Acute lung injury (ALI) frequently manifests in patients beset by sepsis, characterized by symptoms such as dyspnea, hypoxemia, and pulmonary infiltrates (5). The emergence of ALI within the sepsis context is intricately linked to the systemic inflammatory response. The release of inflammatory mediators precipitates damage to alveolar epithelial cells and engenders an increase in pulmonary capillary permeability, ultimately culminating in

pulmonary edema and compromised gas exchange (6). The advent of ALI exacerbates the plight of sepsis patients, prolonging their hospital stays and inflating healthcare costs (7). Hence, a profound understanding of the mechanisms and etiologies underlying acute lung injury in sepsis is essential for the formulation of effective treatment strategies.

Triggering Receptor Expressed on Myeloid Cells 2 (TREM2) predominantly resides in microglia and peripheral macrophages. Since its identification in 2000, TREM2 has garnered recognition as a pivotal regulator of immune cell function (8). For instance, during the tumultuous course of sepsis and its associated acute lung injury, the activation of TREM2 amplifies phagocytic activity and cultivates an anti-inflammatory milieu, potentially mitigating the severity of injury (9). In the realm of neurodegenerative diseases, TREM2 activation not only bolsters microglial survival and functionality but also enhances their phagocytic prowess (10). Moreover, an expanding body of evidence implicates TREM2 in the dynamics of tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs), which conspire to forge an immunosuppressive tumor microenvironment, thereby influencing tumor progression and outcomes (11). Despite the considerable focus on the TREM2 signaling pathway's regulation of immune cells in neurodegenerative diseases and tumors, its role in peripheral inflammatory diseases remains largely uncharted.

This review endeavors to illuminate the role of TREM2 in the context of acute lung injury associated with sepsis. It aspires to elucidate the potential mechanisms at play. We systematically assess the expression levels of TREM2 in sepsis patients and scrutinize its correlation with clinical outcomes, drawing upon current research findings. Furthermore, we delve into strategies to harness TREM2 for the early diagnosis and intervention in septic lung injury, thereby offering novel insights for clinical treatment.

TREM2 signaling pathway

Molecular structure of TREM2

TREM2, or triggering receptor expressed on myeloid cells 2, emerges as a transmembrane receptor belonging to the illustrious immunoglobulin superfamily, residing on human chromosome 6p21. The entirety of the gene spans 4,676 base pairs, intricately composed of five exons. These exons give rise to a glycoprotein predominantly localized within the microglia of the brain and peripheral macrophages. The architectural design of the TREM2 receptor encompasses a signal peptide, an extracellular domain replete with an immunoglobulin domain and a succinct handle sequence, alongside a transmembrane helix and a cytoplasmic tail. The extracellular domain plays a pivotal role in facilitating ligand binding, showcasing a highly glycosylated region that enhances its stability and functionality (12) (Figure 1). TREM2 engages with a diverse spectrum of ligands, encompassing various anionic molecules, whether free or affixed to the plasma membrane, including bacterial products, DNA, lipoproteins—such as low-density lipoprotein (LDL), apolipoproteins (APOE)—and phospholipids present in the body under normal physiological conditions (11). Investigations reveal that TREM2 undergoes proteolytic cleavage, yielding a soluble form (sTREM2) detectable

in biological fluids, serving as an indicator of TREM2 activation and the cellular response to inflammation (13). The intricate structure of TREM2, coupled with the vast array of ligands, renders the prediction of its binding effects a complex endeavor. A profound understanding of the molecular architecture of TREM2 is imperative for the formulation of therapeutic strategies aimed at augmenting its function or compensating for its loss in pathological states.

Activation of the TREM2 signaling pathway

Recent investigations have illuminated the intricate workings of TREM2, revealing its dual nature as both an independent entity and a participant in a multifaceted signaling network. This receptor is intricately woven into a tapestry of biological processes, encompassing phagocytosis, metabolism, cell survival, and anti-inflammatory responses, all of which culminate in transformative shifts in cellular phenotypes and functions. TREM2 emerges as a sentinel against the perils of unchecked inflammation, wielding the capacity to temper pro-inflammatory signaling cascades, particularly those orchestrated by toll-like receptors (TLRs). Through the downregulation of TLR4 expression, TREM2 plays a pivotal role in mitigating inflammatory responses, thereby safeguarding tissues from the ravages of acute inflammatory injuries (14). Emerging research suggests that TREM2 engages with adapter proteins DNAX-activating protein 10 (DAP10) and DAP12, utilizing oppositely charged residues within its transmembrane domain to forge heterodimers with either DAP10 or DAP12. The full-length TREM2 undergoes cleavage by ADAM17/10, yielding soluble TREM2 (sTREM2), which subsequently permeates the intercellular milieu to activate immune cells. Concurrently, γ -secretase cleaves the remaining carboxyl terminus of TREM2, facilitating the release of DAP12. This DAP12 then orchestrates the activation of tyrosine protein kinase Syk, setting in motion a cascade of tyrosine phosphorylation events that ultimately activate downstream mediators such as phospholipase $\text{C}\gamma 2$ (PLC $\gamma 2$), phosphatidylinositol-3-kinase (PI3K), mammalian target of rapamycin (mTOR), and mitogen-activated protein kinase (MAPK), culminating in cellular activation. DAP10 complements this process by recruiting PI3K, thereby activating Akt and ERK to enhance signaling (15, 16). Recent findings have elucidated that TREM2-dependent phagocytosis necessitates the activation of the SYK/PI3K/AKT/PLC γ pathway, while the inhibition of NF κ B activation by TREM2 operates independently of SYK, PI3K, and PLC γ activities. This study underscores the notion that TREM2 governs phagocytic activity through a pathway distinct from its anti-inflammatory function, thereby accentuating the receptor's multifaceted role within the realm of immune responses (17).

TREM2 orchestrates the migration of microglia toward sites of injury through the activation of specific signaling pathways, notably FAK (Focal Adhesion Kinase), Rac1, and Cdc42-GTPase. FAK, a pivotal molecule in cell adhesion and signal transduction, undergoes phosphorylation upon the activation of TREM2 receptors. This phosphorylation cascade subsequently activates Rac1 and Cdc42, leading to a profound remodeling of the cytoskeleton. Consequently, microglia are empowered to migrate

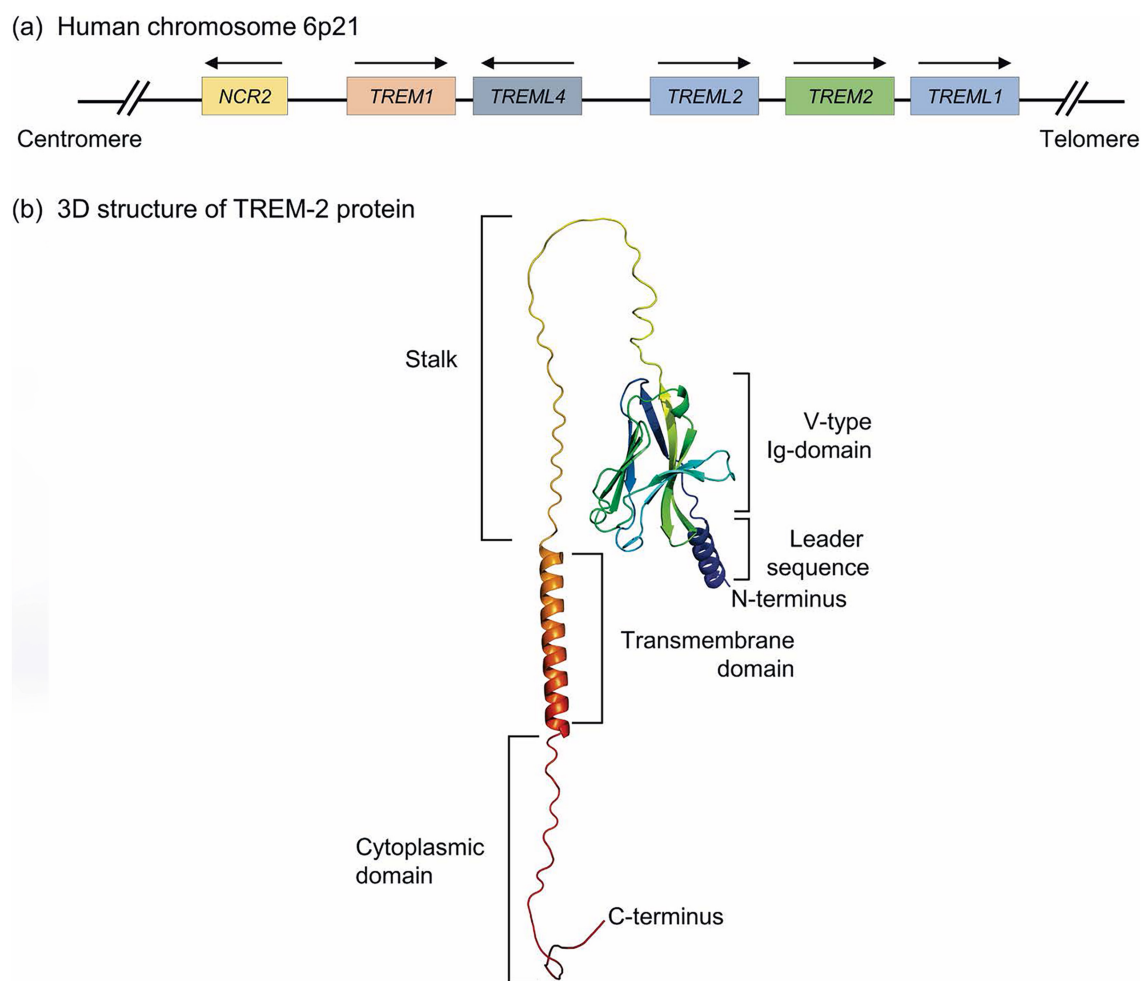


FIGURE 1

Genomic location and molecular structure of TREM-2 receptor. **(a)** Human TREM gene clusters located on human chromosome 6p21 locus, including TREM1, TREM2, TREML1, TREML2, and TREML4 genes. Also, this locus contains the NCR2 gene encoding NKp44, a typical cell-surface receptor of Natural killer (NK) cells and group 3 innate lymphoid cells. **(b)** A three-dimensional structure of the full-length TREM-2 protein, which is predicted by AlphaFold. Herein, the extracellular structure of TREM-2 contains a V-like Ig domain linked with a long stalk followed by a TM domain. Also, the N-terminus leader sequence and C-terminus along with cytoplasmic domains are depicted in the predicted TREM-2 protein model (12).

with enhanced efficacy, responding swiftly to the exigencies of tissue damage and inflammation (18).

The signaling pathways engaged by TREM2, particularly those entwined with NF- κ B, are of paramount importance in the orchestration of inflammatory responses, playing a critical role in both the initiation and resolution of sepsis (19). Following activation, TREM2 exerts an inhibitory influence on NF- κ B activation by modulating intracellular signal transduction, thereby curtailing the expression of pro-inflammatory factors. This inhibitory mechanism serves to regulate the intracellular immune response, averting the perils of excessive inflammation and subsequent tissue damage. While TREM2 predominantly inhibits NF- κ B activation, it is noteworthy that it can, on occasion, contribute to the emergence of inflammatory responses (17).

Moreover, TREM2 engages with a multitude of other signaling pathways, thereby influencing their functional dynamics. For instance, TREM2 is implicated in the deposition of Alzheimer's disease-associated A β plaques and tau proteins, thereby modulating

the neurodegenerative process through the clearance of these pathological entities (20). Additionally, TREM2 plays a significant role in regulating the release of inflammatory mediators within macrophages, thereby shaping the inflammatory milieu of the local microenvironment (21). Furthermore, the activation of TREM2 is intricately linked to the NLRP3 signaling pathway, which modulates the inflammatory response of microglia in the context of elevated glucose levels. This connection suggests that TREM2 may serve as a crucial nexus between metabolic disorders and neuroinflammation (9) (Figure 2).

TREM2 and immune cells

TREM2 finds its primary expression within a diverse array of myeloid cells, encompassing granulocytes, bone marrow-derived macrophages (BMDMs), monocyte-derived macrophages (MDMs), alveolar macrophages, and osteoclasts (OCs). Notably,

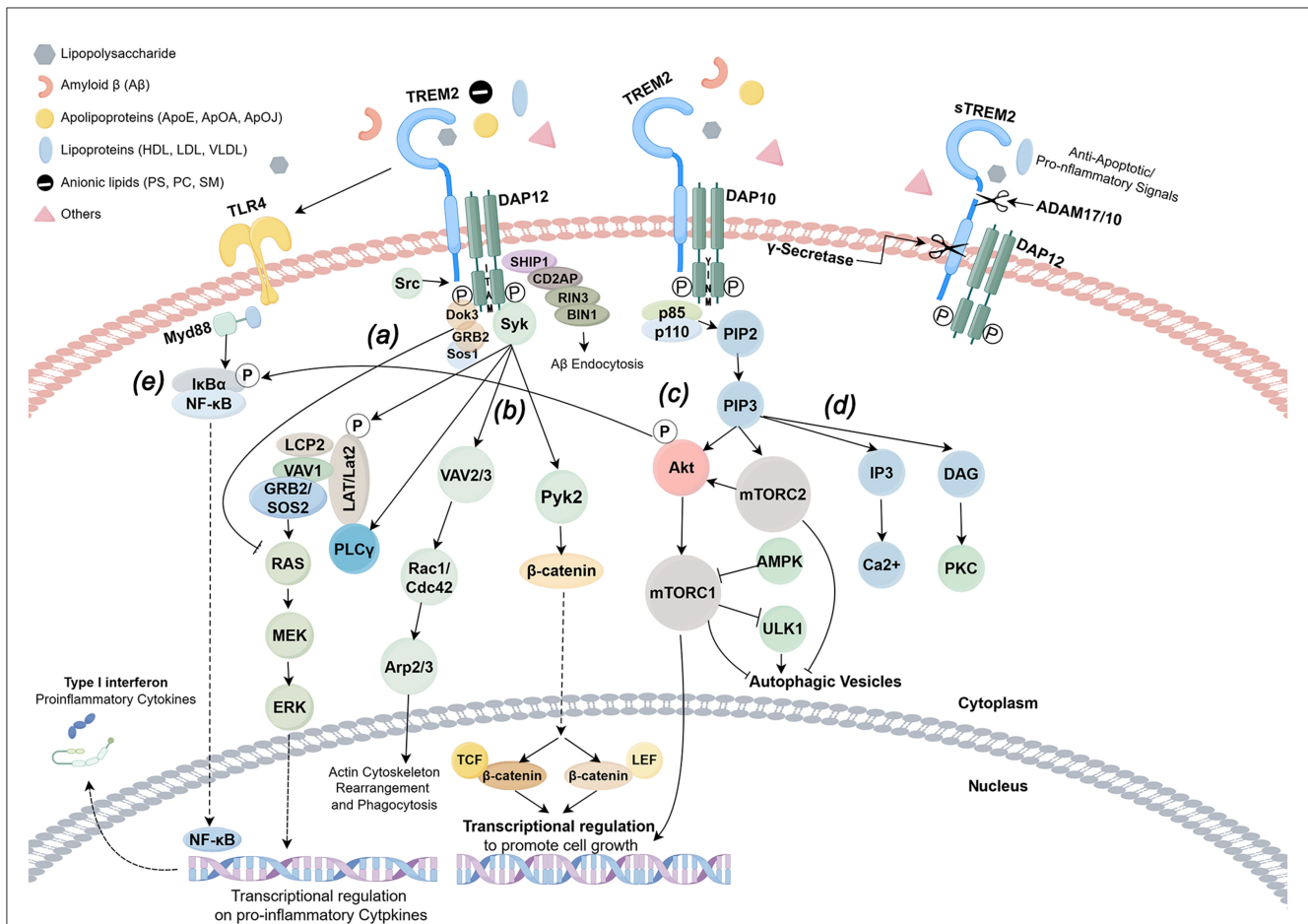


FIGURE 2

TREM2 signaling pathway (by Figdraw). TREM-2 orchestrates the activation of signaling cascades within myeloid cells through the engagement of DAP12, DAP10, and an array of other receptors upon the detection of ligands. Notably, DAP12 is endowed with an immunoreceptor tyrosine-based activation motif (ITAM), facilitating downstream signaling, whereas DAP10 is devoid of such a motif. The formation of heterodimers between TREM-2 and either DAP12 or DAP10 catalyzes the phosphorylation of adaptor proteins, thereby igniting a cascade of downstream pathways subsequent to ligand binding. **(a)** In the intricate signaling network of TREM-2/DAP12, the Src kinase activates DAP12, which in turn recruits the Sos1/2 and GRB2 junction proteins, leading to the inhibition of the RAS/MEK/ERK (or MAPK) pathway, a process that curtails the release of proinflammatory cytokines. **(b)** TREM-2 further establishes a complex with DAP12 or DAP10, engaging in crosslinking with receptors such as CSF1R, which modulates TREM-2/DAP12 signaling through the phosphorylation of DAP12/ITAM by Src kinase. This interaction recruits spleen tyrosine kinase (SYK), setting in motion downstream signaling pathways that involve guanine nucleotide exchange factors Vav2/3, Pyk2, and PI3K, with the assistance of DAP10. **(c)** Conversely, the interplay between DAP12 and DOK3 facilitates the recruitment of SHIP1, an inositol phosphatase characterized by a Src homology 2 (SH2) domain, which catalyzes the conversion of PIP3 to PIP2. This pivotal process governs the involvement and activation of intracellular signaling proteins associated with the membrane through PIP3. **(d)** The regulatory mechanisms of transcription are mediated by β-catenin and mTORC1. The activation of AKT and mTORC2 by PIP2/3 underpins metabolic homeostasis, while the RAS/MEK/ERK pathway modulates proinflammatory gene expression, with Ca²⁺ modulation facilitating actin remodeling. **(e)** TREM2 plays a crucial role in the dual responses of immune cells—both pro-inflammatory and anti-inflammatory—triggered by TLR4. In the nascent stages of bacterial infection, various antigenic substances can directly activate the TLR4 receptor, instigating the NF-κB signaling pathway that augments the expression of inflammatory factors. Concurrently, the TREM2-DAP12-SYK pathway activates the IKK complex, promoting the degradation of IκBα and enabling NF-κB to translocate into the nucleus, thereby driving the inflammatory response. Upon activation, TREM2 recruits SHP-1, a protein that dephosphorylates critical molecules within the NF-κB pathway, such as IKKβ and p65, thereby obstructing their nuclear translocation. This mechanism effectively inhibits the excessive activation of NF-κB instigated by TLR4, leading to a reduction in the release of pro-inflammatory factors such as TNF-α and IL-6. Furthermore, it curtails glycolysis in macrophages via the AKT/mTOR pathway, diminishing the production of pro-inflammatory factors reliant on NF-κB.

TREM2 is also richly present in microglia, while its expression remains absent in lymphocytes (12). This differential expression of TREM2 across immune cell types underscores its pivotal role in orchestrating the immune response to a multitude of conditions, including various infectious diseases, Alzheimer's disease, and tumors (22–25). As contemporary research predominantly centers on peripheral macrophages and microglia, this section shall delve into the intricate role of TREM2 within these two cell types.

TREM2 and macrophages

TREM2 emerges as a pivotal immune regulatory factor, intricately influencing the activation, polarization, phagocytosis, and metabolic processes of macrophages. Upon binding to its ligand, TREM2 initiates a cascade of intracellular signaling pathways, notably the PI3K/AKT pathway, which serves to bolster macrophage survival and proliferation (26). Empirical investigations illuminate TREM2's role in attenuating the

activation of the NF- κ B pathway, a fundamental mechanism governing the synthesis of pro-inflammatory cytokines during inflammatory responses. This attenuation culminates in a reduction of inflammatory mediators such as TNF- α and IL-6, thereby mitigating the excessive inflammation that can precipitate tissue damage and chronic diseases (21). Furthermore, TREM2 curtails the inflammatory response by inhibiting the activation of the NLRP3 inflammasome, thereby diminishing the release of inflammatory factors (27). A further study elucidated that TREM2/ β -catenin orchestrates macrophage pyroptosis by suppressing NLRP3 inflammasome expression, thereby enhancing the macrophage-mediated clearance of pyogenic bacteria (28).

Conversely, TREM2 possesses the capacity to steer the polarization of macrophages from a pro-inflammatory M1 phenotype toward an anti-inflammatory M2 phenotype, activating a myriad of downstream signaling pathways, including the NF- κ B/CXCL3 axis, thus amplifying their anti-inflammatory effects and reparative capabilities (21, 29). Moreover, in models of kidney disease, the absence of TREM2 precipitates macrophage apoptosis and polarization via the JAK-STAT pathway, thereby exacerbating kidney injury (30).

In the realm of bacterial infection, TREM2 emerges as a pivotal enhancer of macrophage efficacy, orchestrating the clearance of pathogens and cellular debris through the regulation of their metabolic state. This modulation fortifies the antibacterial defenses of macrophages (19, 26, 31). The absence of TREM2 is intricately linked to a diminished capacity for bacterial clearance and an escalated vulnerability to infection, thereby underscoring its protective essence within macrophage functionality (26). Moreover, the deficiency of TREM2 incites a reprogramming of macrophages, which exacerbates inflammatory responses and hinders tissue repair, further highlighting its critical role in sustaining immune equilibrium (32). Compelling research has illuminated the role of the transcription factor P53 in the upregulation of TREM2 expression, thereby enhancing macrophage lipid metabolism, activity, and phagocytic prowess (33). In a murine model of sepsis intertwined with non-alcoholic fatty liver disease (NAFLD), TREM2 is instrumental in preserving the metabolic harmony between macrophages and hepatocytes, ultimately improving liver energy supply and outcomes in sepsis (34). Furthermore, TREM2 is intricately associated with steroidogenesis in adrenocortical cells during the tumultuous phase of lipopolysaccharide-induced septic shock (35).

In addition, TREM2 plays a vital role in tissue repair and regeneration by deftly modulating the metabolic status of macrophages. For instance, TREM2 facilitates the healing process following cardiac injury by influencing lipid metabolism within macrophages (36). The TREM2hi Mac1 cells actively engage in the clearance of dysfunctional mitochondria expelled from cardiomyocytes, thereby preserving the delicate homeostasis of cardiomyocytes and safeguarding the heart amidst septic conditions (37). Conversely, the absence of TREM2 can severely compromise macrophage functionality, culminating in exacerbated tissue damage and inflammatory responses, as demonstrated across various disease models (30).

In summation, TREM2 stands as a crucial regulatory molecule, intricately interacting with macrophages and shaping the immune response. Its influence on macrophage functionality is profound,

playing a significant role in diverse pathological conditions and thereby affecting the overarching immune response.

TREM2 and other immune cells

Regarding TREM2 and other immune cells, investigations indicate that the maturation of dendritic cells (DCs) mediated by TREM2/DAP12 can enhance partial T cell activation even in the absence of external antigens, a process that is critical for sustaining T cell homeostasis and survival (38). Additionally, the expression of TREM2 within nephritis DCs catalyzes the production of nitric oxide (NO), which subsequently inhibits the differentiation of Th17 cells and mitigates the progression of chronic kidney disease (CKD) (39). Moreover, it has been documented that TREM2 is abundantly expressed in CD4+ T cells amidst infection and inflammation. The TREM2 receptor complex incites a pro-inflammatory Th1 response through the activation of the STAT1/STAT4 pathway, thereby orchestrating the regulatory mechanisms inherent to adaptive immunity and host inflammatory responses (40). A multitude of studies have elucidated the extensive regulatory influence of mesenchymal stem cells on immune responses, revealing that the overexpression of TREM-2 significantly curtails the production of inflammatory cytokines, such as TNF- α and IL-1 β , while simultaneously fostering the elevated secretion of the anti-inflammatory cytokine IL-10 (41). Nevertheless, the intricate mechanisms underlying these processes remain to be thoroughly explored. In a separate investigation concerning tumor immunity, it was discovered that ApoE, released by prostate tumor cells, engages with the immunosuppressive TREM2+ neutrophil subpopulation—also recognized as polymorphic nuclear myeloid-derived suppressor cells (PMN-MDSCs)—thereby promoting its senescence (42). This finding prompts contemplation regarding the potential existence of analogous regulatory mechanisms in inflammation-related diseases, which may significantly influence immune responses.

The aforementioned summary fully underscores the vital biological functions of TREM2 across a spectrum of immune cells, particularly within macrophages, microglia, and dendritic cells. Its regulatory scope encompasses phagocytosis, polarization transitions, cell survival, and proliferation, as well as antigen presentation. In pathological states such as sepsis, the role of TREM2 is indispensable for immunomodulation and tissue repair. Thus, delving into the mechanisms of TREM2 across diverse immune cells is paramount for unraveling the complexities of sepsis-induced lung injury and for identifying novel therapeutic targets in clinical practice.

TREM2 and sepsis-induced acute lung injury

About sepsis-induced acute lung injury

Sepsis is a systemic inflammatory response syndrome caused by infection, and its pathophysiological changes involve complex responses of multiple systems. The genesis of sepsis unfolds with the invasion of pathogens, accompanied by the aberrant

activation of the host immune response, culminating in the tempest of a cytokine storm. Upon the onset of infection, the host immune system, through the vigilant eyes of pattern recognition receptors (PRRs), including the toll-like receptors (TLRs), discerns the presence of pathogens. This recognition of pathogen-associated molecular patterns (PAMPs) ignites the activation of macrophages and other immune sentinels, setting in motion an inflammatory cascade that releases a plethora of inflammatory mediators, such as tumor necrosis factor (TNF) and interleukins (IL-6, IL-1 β), among others. These inflammatory agents exert a profound influence on local tissues, instigating endothelial dysfunction and amplifying vascular permeability. Moreover, they can incite systemic inflammatory reactions coursing through the bloodstream, leading to organ dysfunction, with acute lung injury standing as a particularly grievous consequence (19, 43, 44). Furthermore, recent investigations illuminate the pivotal roles of mitochondrial dysfunction and oxidative stress, suggesting that compromised mitochondrial function may precipitate energy deficits within the cells of those afflicted by sepsis (34, 45).

Sepsis-induced Acute lung injury (SI-ALI) is a prevalent and harrowing complication of sepsis, manifesting as a severe respiratory affliction characterized by profound hypoxemia, an unchecked inflammatory response, and the accumulation of both alveolar and interstitial edema, potentially culminating in the development of acute respiratory distress syndrome (ARDS). The pathophysiological underpinnings of SI-ALI are multifaceted, with the release of inflammatory mediators serving as a critical catalyst in the onset of acute lung injury (46). This cascade of events can inflict damage upon the alveolar-capillary membrane, heightening permeability and leading to pulmonary edema, thereby impairing gas exchange (47). In addition, the activation of inflammatory mediators incites a response from other immune cells residing within the alveoli and endothelial cells, compelling them to secrete an array of chemokines and cytokines, thus perpetuating a vicious cycle that exacerbates lung injury (48). For example, the infiltration of neutrophils into the pulmonary landscape serves as a hallmark of sepsis-induced lung injury, wherein activated neutrophils unleash reactive oxygen species (ROS) and proteolytic enzymes, further intensifying tissue damage (49, 50).

Moreover, pyroptosis—a distinct form of programmed cell death—plays a crucial role in the context of septic lung injury. Recent studies have elucidated that the activation of the NLRP3 inflammasome within lung epithelial cells triggers the release of inflammatory cytokines, culminating in further detriment to lung tissue (51, 52). Thus, the role of inflammatory mediators is indispensable in the progression of septic lung injury. A comprehensive understanding of the regulatory mechanisms governing these mediators may pave the way for mitigating the inflammatory response and curtailing organ damage, ultimately enhancing the prognosis for patients grappling with septic lung injury.

Mechanism of TREM2 in SI-ALI

TREM2 assumes a dual role within the intricate tapestry of the inflammatory response, nurturing anti-inflammatory processes

while simultaneously providing a protective shield amidst the tumult of inflammation. As a central figure in immune regulation, TREM2 has captured the attention of researchers delving into the complexities of septic lung injury. Compelling evidence indicates that TREM2 tempers excessive inflammatory responses through the nuanced modulation of macrophage activity and functionality, thereby alleviating the burdens of sepsis-induced acute lung injury. For instance, the activation of TREM2 can catalyze a transformation in macrophages toward an anti-inflammatory phenotype, culminating in a reduction of pro-inflammatory factor release and a diminishment of the pulmonary inflammatory response (26). Moreover, TREM2 fortifies the immune response against sepsis by engaging its ligand and activating signaling pathways that bolster macrophage survival and functionality (53).

Recent studies illuminate that Rhein fosters M2 polarization in macrophages by targeting the NFATc1/TREM2 pathway, a mechanism pivotal in regulating the inflammatory response and prognosis following acute lung injury (54). Galectin-3 emerges as a significant upstream factor implicated in lung ischemia-reperfusion injury (LIRI), and within this framework, the enhancement of TREM2 expression reveals potential in mitigating lung injury (55). In addition, grape seed proanthocyanidins orchestrate M2a macrophage polarization through the TREM2/PI3K/Akt pathway, thereby ameliorating LPS-induced acute lung injury (56). Related investigations underscore that plasma sTREM2 may serve as a prognostic marker in the assessment of acute lung injury, intricately regulating inflammation alongside the downstream TREM2 response (57). Furthermore, the vasoactive intestinal peptide has been demonstrated to recalibrate the TREM-1/TREM-2 ratio within lung cells, influencing the onset of acute lung injury (58).

TREM2, conversely, orchestrates the antimicrobial defense mechanisms of macrophages, fortifying their survival and enhancing their functionality amidst the presence of pathogens. For instance, the absence of TREM2 is linked to compromised bacterial clearance and an escalated vulnerability to infection, thereby underscoring its pivotal role in the protective effects against sepsis (26). Moreover, TREM2 influences the metabolic interplay between macrophages and hepatocytes, a critical factor in sustaining homeostasis during the tumultuous state of sepsis (34). Intriguingly, a study concerning acne has posited that squalene elevates the expression of TREM2 within macrophages residing in lesions, thereby augmenting their phagocytic capacity to engulf lipids and bacteria. Nevertheless, given that squalene possesses the ability to scavenge reactive oxygen species (ROS) and downregulate the enzymes implicated in ROS generation and its subsequent reactions, it inadvertently curtails the antibacterial efficacy of TREM2-expressing macrophages, resulting in a notable deficiency in antibacterial functionality (59).

Further investigations have illuminated that in the context of sepsis-induced acute lung injury in murine models, TRIM21 (tripartite motif-containing protein 21) attenuates the expression of IRF1 (interferon regulatory factor 1) through the mechanism of ubiquitination, thereby reinstating TREM2 expression and ameliorating sepsis-induced acute lung injury (SI-ALI) (60). In stark contrast, it has been documented that pro-inflammatory conditions, while suppressing TREM2 expression, concurrently diminish the expression levels of the STAT6 transcription factor

downstream of the IL-4 pathway, thereby obstructing its anti-inflammatory capabilities (61). In a study focused on LPS-induced septic lung injury in mice, it was noted that the silencing of TREM2 could precipitate the downregulation of SHP1 and an increase in the phosphorylation of STAT3; conversely, the overexpression of TREM2 could mitigate oxidative stress and ferroptosis via the SHP1/STAT3 pathway, thus significantly alleviating sepsis-induced acute lung injury (62). Furthermore, the absence of TREM2 precipitates a dysregulation of macrophage function, exacerbating lung injury associated with sepsis, thereby indicating that the protective role of TREM2 in SI-ALI warrants considerable attention (63). Additionally, TREM2 deficiency has been shown to exacerbate kidney injury and LPS-induced acute lung injury in murine models (30). While TREM2 is widely acknowledged for its protective role in immune regulation, excessive activation of TREM2 may yield immunosuppressive effects, potentially impairing immune responses and heightening the risk of secondary infections following sepsis (64).

In summary, while TREM2 assumes a protective role in the modulation of immune responses during sepsis, its dysregulation may also culminate in adverse outcomes. This duality accentuates the significance of TREM2 in maintaining a delicate balance of pro-inflammatory signals, a crucial endeavor to avert excessive tissue damage in the context of sepsis. Thus, the intricate interplay of TREM2 in sepsis may herald a promising avenue for targeted therapeutic interventions aimed at the immune response in future endeavors.

Strategies for TREM2 therapeutic agents in SI-ALI

As a pivotal element in immune regulation, the burgeoning interest in the development of pharmacological agents targeting TREM2 has become increasingly pronounced. Yet, the complexity inherent in the TREM2 signaling cascade, along with its unique mechanisms across various pathologies, renders the advancement and applicability of these therapies a formidable challenge. Currently, the majority of pharmacological agents directed at the TREM2 receptor are primarily examined within the context of Alzheimer's Disease (AD), with scant exploration in the realm of septic lung injury. Nevertheless, the insights derived from the investigation of TREM2-targeted therapies in AD may illuminate pathways for addressing septic lung injuries (Table 1).

In Alzheimer's disease, the imperative to foster phagocytosis while mitigating inflammation emerges; however, the anti-inflammatory signaling of TREM2 may inadvertently disrupt the body's intrinsic anti-tumor defenses or exacerbate pro-fibrotic responses in the aftermath of liver injury (15). Thus, it becomes essential to strike a delicate balance in the activation of the TREM2 signaling pathway to attain the most favorable therapeutic outcomes. A selection of monoclonal agonistic anti-TREM2 antibodies has been crafted to amplify the protective transduction of TREM2 signals within microglia.

In the milieu of immune cells, the enzymes ADAM10 and ADAM17 engage in the cleavage of TREM2, resulting in the shedding of its extracellular domain and the release of soluble

TREM2 (sTREM2), thereby culminating in the cessation of cell-autonomous signaling. The agonistic anti-TREM2 antibody, conceived from this understanding, is designed to bind to the upstream regions of the ADAM10 and ADAM17 cleavage sites, thwarting the release of sTREM2 and thereby promoting TREM2 signaling. In accordance with this mechanism, a poorly cleavable mutant of TREM2 has been engineered to enhance its presence on the cell surface, exhibiting efficacy akin to that of an agonistic antibody (65).

AL002, a humanized monoclonal antibody, has demonstrated the capacity to elevate cell survival and proliferation through the activation of TREM2 signaling, thereby counteracting dementia potentially arising from compromised TREM2 functionality, while simultaneously invigorating microglial activity. However, the prolonged administration of AL002 has been associated with an escalation in the dissemination of phosphorylated tau forms, which may, in turn, precipitate cognitive decline (66). Despite AL002 exhibiting a favorable pharmacokinetic/pharmacodynamic profile in healthy individuals, the drug has recently faltered in its ability to alleviate Alzheimer's symptoms during Alector's clinical trial.

Despite the promising therapeutic effects exhibited by currently available targeted drugs, it is noteworthy that only approximately 0.1% of antibodies for neurological disorders successfully traverse the blood-brain barrier and penetrate the brain parenchyma, necessitating the administration of significantly high antibody doses (67). Yet, recent investigations have unveiled an ingeniously engineered bispecific antibody, adeptly targeting both TREM2 and the transferrin receptor—designated as Ab18 TVD-Ig/aTfR—which remarkably amplifies the efficiency of central nervous system entry by over 10-fold, achieving a pervasive distribution throughout the brain parenchyma (68). These revelations serve as a poignant reminder that the tissue-specific delivery of pharmacological agents profoundly influences both therapeutic efficacy and safety. For instance, it is essential to deliberate whether the agents within SI-ALI can be judiciously directed toward the lungs to elicit pulmonary therapeutic effects, rather than being indiscriminately absorbed, thereby engendering unpredictable outcomes. Given that the majority of contemporary research remains ensconced within the confines of *in vitro* experimentation, uncertainty looms regarding the *in vivo* efficacy of these drugs and the ramifications of the body's complex physiological milieu on drug delivery.

Certain studies suggest that excessive stimulation of anti-TREM2 antibodies may perturb TREM2 expression within peripheral tissues, potentially undermining overall immune functionality. In response, researchers have devised an engineered antibody (ATV: TREM2) incorporating the transferrin receptor binding site to bolster endocytosis. Current investigations reveal that modest doses of this antibody yield improvements in amyloidosis. The distribution of ATV: TREM2 is contingent upon transferrin receptor expression, enabling its ingress into the spleen, bone marrow, plasma, brain, and other tissues to exert its influence. Moreover, this antibody is well-tolerated, exhibiting no deleterious effects on tissue integrity (69).

PY314, another humanized monoclonal antibody, functions as a TREM2 antagonist, effectively depleting tumor-associated macrophages. Clinical trial outcomes have indicated that PY314 is well-tolerated, suggesting that patients exhibit a favorable

TABLE 1 Research progress on TREM2-targeted drugs.

Medicine	Mechanism	Disease	Development phase	References
AL002	Anti-TREM2 McAb	Alzheimer	Clinical phase II (Terminated)	(66)
Iluzanebart (VGL101)	Anti-TREM2 McAb	Hereditary diffuse leukoencephalopathy combined with axonal spheroid degeneration; leukoencephalopathy	Clinical phase II	(73, 74)
VG-3927	TREM2 agonist	Alzheimer	Clinical phase I	(72)
VHB937	Anti-TREM2 McAb	Neurodegenerative disease; Amyotrophic lateral sclerosis	Preclinical	-
PY314	Anti-TREM2 McAb	Ovarian cancer; Gastric cancer; Colorectal cancer; Triple-negative breast cancer; Lung adenocarcinoma; Renal cell carcinoma	Clinical phase I	(70, 71)
DNL919	Anti-TREM2 McAb; Tfr ligand	Alzheimer	Clinical phase I	(76)
Ab18 TVD-Ig/atfr	Anti-TREM2/Tfr bispecific antibody	Alzheimer	Preclinical	(68)
ATV: TREM2	Transferrin receptor binding site antibody	Neurodegenerative disease	Preclinical	(69)
OPA	Small molecule TREM2 inhibitor	Colorectal cancer	Preclinical	(24)
68Ga-NOTA-COG1410	PET drugs labeled 68Ga; anti-TREM2 Polypeptide conjugated nuclides	PET imaging; Cancer	Preclinical	-

adaptation to treatment with this agent, accompanied by a reduction in adverse effects. This characteristic positions PY314 advantageously for broader applications within tumor therapy (70, 71). OPA, a platinum (IV)-based compound derived from Oxaliplatin (OP) and Artesunate (ART), has been shown to diminish TREM2 expression in macrophages, facilitating their polarization from an immunosuppressive M2 phenotype to an anti-tumor M1 phenotype. Furthermore, this compound fosters the maturation of dendritic cells, augments the functionality of natural killer (NK) cells, and amplifies the presence of CD8+ T cells within tumor tissues, thereby manifesting potent chemotherapeutic and immunostimulatory effects against cancer (24).

Furthermore, small molecule TREM2 activators such as VG-3927 (72) and humanized monoclonal antibody VGL101 (73, 74) are presently navigating the intricate landscape of clinical trials aimed at neurodegenerative disorders. Preliminary findings suggest that the synergistic combination of VGL101 with TREM2 engenders a selective activation of the receptor, thereby initiating a cascade of downstream signaling pathways that not only promote neuroprotection but also enhance the homeostatic functions of microglia. Notably, VG-3927 has demonstrated a significant reduction in soluble TREM2 levels within the cerebrospinal fluid of patients, illuminating its potential efficacy in clinical contexts targeting TREM2. Moreover, TREM2 antagonists may serve to alleviate neurotoxicity by tempering the excessive activation of microglia (75).

It is imperative to acknowledge that, despite the promising therapeutic effects exhibited by these agonists, no applicable drugs have yet attained approval. This underscores the necessity for further exploration, given the intricate mechanisms of action

associated with the TREM2 target and its dual-sided effects, to thoroughly assess the safety and efficacy of its targeted pharmacological agents.

Conclusion and prospect

TREM2 unveils a realm of promising potential in the treatment of sepsis-induced acute lung injury, its central role in immunomodulation and tissue repair establishing it as a formidable therapeutic target. Recent explorations have illuminated TREM2's dual contribution to the regulation of the immune system and its protective influence amidst the inflammatory tempest of sepsis. Thus, therapeutic strategies aimed at TREM2 may pave new pathways for the management of patients grappling with sepsis. Presently, the lion's share of mechanistic inquiries and drug development endeavors surrounding TREM2 predominantly fixates on neurological disorders. In stark contrast, our comprehension of its implications in sepsis-related maladies remains markedly less thorough. While this may appear as a daunting challenge, we can still draw upon the therapeutic effects and mechanisms of TREM2-targeted agents in other conditions to forge effective treatments for the intricate responses elicited by sepsis.

Although existing research has provided initial validation for TREM2's application in sepsis, it is imperative to acknowledge the potential variances in interpretations and outcomes across diverse studies. For instance, certain investigations may accentuate TREM2's capacity to mitigate the progression of lung injury, while others may underscore its multifaceted role in the nascent

immune response. Therefore, future inquiries should endeavor to unravel the mechanisms governing TREM2's function, particularly its functional transformations across a spectrum of pathological states. Researchers ought to refine therapeutic approaches, honing in on the effective activation or inhibition of TREM2 signaling pathways to attain the most advantageous therapeutic outcomes.

While the prospect of targeted therapy involving TREM2 shines with promise, a profound investigation into the intricate signaling pathways is paramount to elucidate its mechanisms of action across diverse pathological states, as well as to evaluate the safety and efficacy of its clinical applications. Presently, a cadre of TREM2-specific pharmaceuticals is advancing through the stages of development and research. Notable among these, VG3927, VGL101, and VHB937 have exhibited encouraging therapeutic effects in clinical trials. Yet, the landscape remains devoid of any drug that has received approval and formal licensing for patient treatment. Consequently, ethical quandaries and barriers to clinical application loom as formidable challenges in TREM2-focused research. This prevailing scenario can be attributed to several pivotal factors. Firstly, investigations involving human genome editing and immunotherapy often incite ethical dilemmas, particularly in light of the uncertainties surrounding the long-term health ramifications for patients. Secondly, the design and execution of clinical trials must adhere to stringent ethical standards to ensure the comprehensive safeguarding of patients' informed consent and rights. Nevertheless, due to TREM2's intricate involvement in a multitude of diseases, researchers frequently find themselves navigating the delicate balance between scientific inquiry and ethical obligations. Furthermore, impediments to clinical application include the absence of effective biomarkers for monitoring TREM2 activity and its fluctuations during disease progression, resulting in a dearth of guidance for clinicians regarding the implementation of pertinent therapies.

It is well acknowledged that sepsis-related diseases encompass complex pathological processes, and the ensuing multi-organ damage presents significant challenges to treatment. TREM2, with its extensive array of ligands, exhibits functionality that is contingent upon the distribution of immune cells. Coupled with its diverse mechanisms of action, it undoubtedly injects uncertainty into the treatment of sepsis-related maladies. Thus, the pressing challenge remains: how to efficiently and specifically direct drugs to the intended organs while monitoring the site of drug action and effective concentration. The trajectory of TREM2-related research may be swayed by various patient-related factors. Therefore, it is imperative that forthcoming investigations not only address the scientific dimensions but also the ethical considerations to facilitate the clinical translation of TREM2.

Author contributions

H-LS: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Visualization, Writing – original draft, Writing – review & editing. JH: Data curation,

Investigation, Methodology, Resources, Visualization, Writing – original draft, Writing – review & editing. FL: Conceptualization, Funding acquisition, Project administration, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Dopamine as an endogenous regulator of innate immunity in sepsis

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Sepsis, a life-threatening organ dysfunction caused by a dysregulated host response to infection, presents a major clinical challenge. While the complex interplay of inflammatory mediators and immune cells during sepsis is increasingly understood, the role of neurotransmitters, particularly dopamine, in modulating the innate immune response is emerging as a crucial area of investigation. Dopamine, traditionally recognized for its role in the central nervous system, acts as an endogenous regulator of innate immunity, significantly influencing the course and outcome of sepsis. In this mini-review, we highlight our recent finding of dopamine's critical role in regulating aconitate decarboxylase 1 (ACOD1) in sepsis.

KEYWORDS

dopamine, sepsis, aconitate decarboxylase 1, CD274, innate immunity

Introduction

Sepsis is a life-threatening condition that arises when the body's response to infections triggers a dysregulated production of various inflammatory cytokines (1). It accounts for almost 20% of total deaths worldwide (2), and annually cost >\$60 billion in the U.S. alone. Animal models remain indispensable in sepsis research, providing a controlled environment to unravel complex pathophysiological mechanisms, identify therapeutic targets, and evaluate novel interventions (3). The intricate interplay of various innate immune cells, inflammatory mediators, and signaling pathways contributes to the pathogenesis and progression of sepsis (4, 5). While the inflammatory cascade initiated by the innate immune system is crucial for pathogen clearance, its dysregulation can lead to detrimental systemic inflammation and subsequent immunosuppression (6, 7). Understanding the intricate mechanisms governing this immune response is critical for developing effective therapeutic strategies.

Dopamine (DA), traditionally recognized for its role in the central nervous system (8), acts as an endogenous regulator of innate immunity through a complex interplay with various immune cells expressing dopamine receptors, primarily D1-like (DRD1 and DRD5) and D2-like (DRD2, DRD3, and DRD4) receptors (9), thereby significantly

influencing the course and outcome of sepsis. Depending on the receptor subtype and the specific context, dopamine can trigger intracellular signaling cascades that can either enhance or suppress immune cell activities.

Dopamine as a bridge between the nervous and innate immune systems

Dopamine (DA) is a central nervous system (CNS) neurotransmitter involved in the control of several key functions, such as cognition and movement. In the periphery, DA is produced by neuroendocrine cells, the adrenal glands, and neuronal fibers, and influence functions like blood pressure, sodium balance and adrenal and renal functions (10, 11), as well as glucose homeostasis and body weight (12). In addition to its primary role in the CNS, DA is now recognized as a key modulator of the innate immune response in sepsis. For instance, dopamine is produced and released by various types of immune cells including lymphocytes, macrophages, peripheral blood mononuclear cells (PBMCs), and dendritic cells (8, 13–17) during inflammation (18). This localized production suggests dopamine's involvement in modulating immune responses through paracrine or autocrine signaling, regulating both neurological and immunological responses (16, 19, 20).

It is plausible that dopamine release is tightly regulated and triggered by specific immunological stimuli, such as pathogen-associated molecular pattern molecules (PAMPs) or damage-associated molecular pattern molecules (DAMPs). Understanding the temporal dynamics of dopamine production—when and for how long it is released in response to specific immune challenges—is crucial. Furthermore, different tissues might exhibit varying abundances of these cells, leading to tissue-specific effects of dopamine signaling. Therefore, a deeper understanding of this interplay between temporal and spatial dynamics of dopamine production is essential for developing targeted therapies that effectively harness the immunomodulatory potential of dopamine signaling in immune-related diseases. This includes further research into the precise regulation of dopamine release, variations in its production across different immune cell types and tissues, and the intricate relationship between neuronal and immune-derived dopamine.

DA interacts with D1-like (DRD1, DRD5) and D2-like (DRD2, DRD3, DRD4) receptors on immune cells, triggering specific intracellular signaling cascades and influencing immune cell activity depending on receptor subtype and the specific immune cell involved. For instance, DA modulates innate immunity, in part, by influencing neutrophil functions. As the first line of defense against invading pathogens, neutrophils are crucial for bacterial clearance. Expressing DRD3, DRD5, and to a lesser extent, DRD2 and DRD4 dopamine receptors (21), neutrophils are responsive to dopamine signaling. For instance, acting via D1-like receptors, DA inhibits neutrophil chemotaxis and phagocytosis (22), potentially mitigating excessive inflammation and tissue damage (23). While this inhibition may be beneficial in early sepsis by attenuating an overwhelming inflammatory response, prolonged suppression of

neutrophil activity can compromise bacterial clearance and increase the risk of secondary infections. Furthermore, DA reduces neutrophil activity by decreasing endothelial adherence, reactive oxygen species and cytokine production (18), and impairing cell migration and phagocytosis (22, 24–27).

Human monocytes predominantly express DRD2 and DRD3, with lower expression of DRD4 and DRD5 (21). Consequently, DA suppresses LPS-mediated NF- κ B activation and cytokine production in these cells (28). Additionally, DA modulates macrophage polarization towards the M2 phenotype, which contribute to tissue repair and resolution of inflammation (9), suggesting a potential role for DA in the later stages of sepsis by fostering an anti-inflammatory action. *In vivo*, DA and its agonists suppress inflammatory responses in mice, reducing LPS-induced production of IL-12p40 (29), TNF (30), IFN- γ , and nitric oxide in macrophages (31) primarily via D2-like receptors (DRD2/DRD3/DRD4) (32). Conversely, DA stimulates the production of anti-inflammatory cytokines, such as IL-10, in macrophages (32, 33), mounting an anti-inflammatory response.

Therapeutic potential of dopamine-based agents

DA exerts cardiovascular effects by acting on α - and β -adrenergic receptors, increasing cardiac output, systemic vascular resistance, and blood pressure (34), thereby counteracting the hypotension and hypoperfusion characteristic of organ dysfunction. Consequently, DA is often a first-line vasopressor in sepsis and septic shock during overwhelming immune responses to bacterial infections (35). While both DA and norepinephrine (NE) are commonly used as first-line vasopressors in the treatment of septic shock (36–39), NE may demonstrate superior efficacy in clinical settings (37, 40).

In vivo, pharmacological DA administration modulates the secretion of hormones such as prolactin (41, 42), restores hepatic blood flow (43), and improves hemodynamics by increasing blood pressure/flow and causing vasodilatation (34). DA suppresses systemic inflammation by blocking the TRAF6/NF- κ B pathway via a DRD5 receptor-mediated signaling axis involving ARRB2 and PP2A (44). Consistently, a dopamine D1-like receptor-specific agonist improves survival of septic mice, partly by inhibiting TNF, IL-1 β (45), IL-6, and IFN- γ (46). Similarly, electroacupuncture of the sciatic nerve increases adrenal DA production in mice, which subsequently acts on DRD1 receptors to reduce systemic inflammation and protect against lethal sepsis (47). Clinically, low-dose DA may benefit splanchnic blood flow and oxygen consumption in patients with septic shock (48), aligning with the potential therapeutic benefits of dopaminergic agonists in septic diabetic patients by controlling both hyperglycemia and systemic inflammation (49). However, high-dose DA, compared to norepinephrine, is associated with increased arrhythmic events and mortality (50–52). Independent of norepinephrine (NE) use, DA administration is associated with higher mortality (51, 53) and a greater incidence of arrhythmic events compared to NE administration (40).

While the role of dopamine in modulating immune responses is increasingly recognized, it is presently unclear whether systemic and locally produced dopamine exert distinct effects on immune function during sepsis. Addressing this important question is crucial for refining our understanding of sepsis pathophysiology and developing targeted therapeutic interventions. As aforementioned, systemic dopamine, primarily derived from the nervous system, circulates throughout the body and can interact with dopamine receptors expressed on various immune cells. These interactions can modulate immune cell activity, including cytokine production, phagocytosis, and lymphocyte proliferation. In the context of sepsis, extensive evidence indicates that DA might play a protective role by modulating inflammatory responses (43, 46, 54). In contrast to systemic dopamine, locally produced dopamine is synthesized and released by immune cells themselves, acting within the immediate microenvironment. This localized production allows for precise and targeted modulation of immune responses within specific tissues or at the site of infection, influencing the activity of neighboring immune cells.

The distinct effects of systemic versus local dopamine in sepsis may stem from several factors. First, the concentration of dopamine at the site of action may differ significantly. Locally produced dopamine can achieve high concentrations within the immune microenvironment, potentially exceeding those achieved by circulating dopamine. Second, the specific dopamine receptor subtypes expressed on different immune cell populations and within different tissues may vary, leading to diverse downstream effects. Finally, the interplay between dopamine and other signaling molecules present in the local microenvironment, such as cytokines and chemokines, could further influence the net effect of dopamine on immune function. Therefore, disentangling the roles of systemic and locally produced dopamine in sepsis requires sophisticated experimental approaches. For instance, studies using conditional knockout mice, where dopamine production is selectively ablated in specific cell types or tissues, could help elucidate the distinct contributions of systemic and local dopamine. Furthermore, *in vitro* studies using co-culture systems of immune cells and other relevant cell types, such as endothelial cells, can provide valuable insights into the interplay between dopamine and other signaling pathways within the immune microenvironment.

Therefore, understanding the differential effects of systemic versus locally produced dopamine in sepsis has significant implications for developing targeted therapeutic strategies. Manipulating dopamine signaling pathways could offer novel approaches to modulating immune function and improving outcomes in sepsis patients. For instance, selectively enhancing local dopamine production by immune cells at the site of infection could promote bacterial clearance and dampen excessive inflammation. Conversely, modulating systemic dopamine levels or targeting specific dopamine receptor subtypes might be beneficial in mitigating the systemic inflammatory response and preventing organ damage. Therefore, deciphering the distinct roles of systemic and locally produced dopamine in sepsis is a critical area of future research. This knowledge will not only enhance our understanding of the complex immunopathology of sepsis but also pave the way for developing innovative therapeutic strategies that harness the immunomodulatory potential of dopamine signaling.

Novel role of DA in the regulation of ACOD1 expression

Aconitate decarboxylase 1 (ACOD1, also known as immune-responsive gene 1, IRG1) is a critical regulator of immunometabolism and inflammation, particularly in the context of infection and injury. While initially recognized for its role in generating the anti-inflammatory metabolite itaconate, ACOD1's functions have proven to be multifaceted, encompassing both itaconate-dependent and -independent mechanisms (Figure 1). Initially, the well-characterized function of ACOD1 relates to its catalysis of cis-aconitate to itaconate within the mitochondria. This activity is markedly upregulated in macrophages and other immune cells upon stimulation with inflammatory stimuli like lipopolysaccharide (LPS) (55). Itaconate, in turn, exerts a range of anti-inflammatory effects through multiple mechanisms such as: 1) competitive inhibition of succinate dehydrogenase (SDH), leading to succinate accumulation and stabilization of hypoxia-inducible factor-1 α (HIF-1 α) which promotes anti-inflammatory gene expression (56); 2) direct alkylation of proteins like Kelch-like ECH-associated protein 1 (KEAP1), resulting in activation of nuclear factor erythroid 2-related factor 2 (Nrf2) and subsequent antioxidant and anti-inflammatory responses (Figure 1) (57); and 3) inhibition of glycolysis, contributing to the metabolic reprogramming of activated immune cells (58). Collectively, through these mechanisms enable itaconate to dampen inflammation and promotes tissue repair.

Our recent research has uncovered itaconate-independent functions of ACOD1 (59), adding complexity to its immunoregulatory role in sepsis. Specifically, an LPS-stimulated, JUN-regulated, pro-inflammatory function of ACOD1 has been identified, involving an interplay between CDK2, RACK1, and MAPK8 (59). Mechanistically, LPS triggers the formation of a CDK2-RACK1-MAPK8 complex, leading to MAPK8 activation and JUN phosphorylation (Figure 1). Phosphorylated JUN then translocates to the nucleus and promotes ACOD1 expression (Figure 1) (59, 60). In a mouse model of sepsis induced by cecal ligation and puncture (CLP), global or myeloid-specific genetic knockout of either CDK2 or ACOD1 significantly improved survival (59), attenuating systemic inflammation, organ dysfunction, and coagulopathy. These protective effects have been observed even in the absence of itaconate production, supporting the existence of itaconate-independent mechanisms. Pharmacological inhibition of CDK2, a kinase upstream of ACOD1, with dinaciclib has replicated these benefits in CLP and other clinically relevant sepsis models (e.g., *E. coli* and *S. pneumoniae*) (59), suggesting that targeting the CDK2-ACOD1 axis may be a promising therapeutic strategy.

Recently, we have identified a key role for DA in regulating ACOD1 expression through a comprehensive screening of neurotransmitters for their ability to modulate LPS-induced expression of ACOD1 (54). Dopamine's inhibitory effect was observed at both the mRNA and protein levels, suggesting a mechanism of transcriptional regulation. Furthermore, we

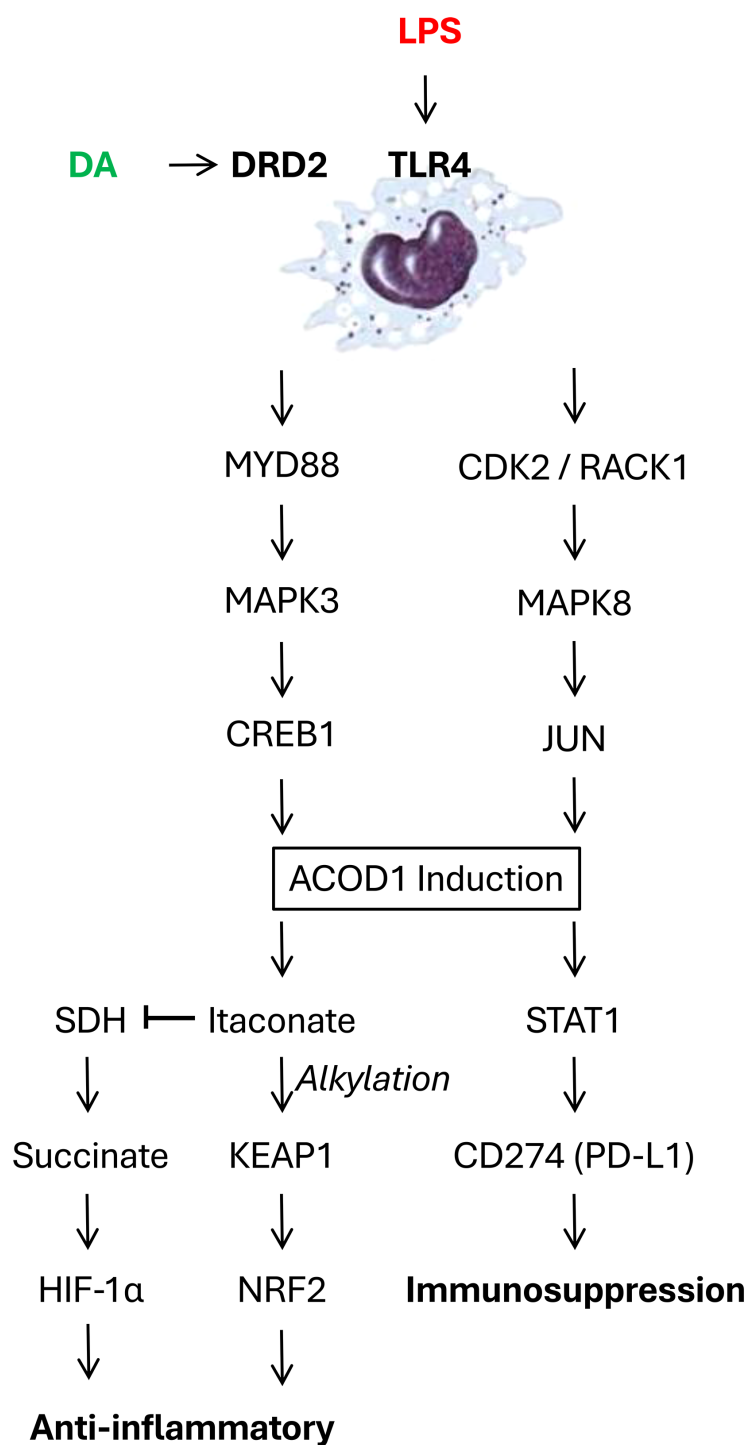


FIGURE 1

Dopamine (DA) counter-regulates bacterial endotoxins (LPS)-induced aconitate decarboxylase 1 (ACOD 1) expression in innate immune cells. LPS engages Toll-like receptor 4 (TLR4), activating either the MYD88-MAPK3-CREB1 or CDK2-RACK1-MAPK8-JUN signaling pathway, ultimately upregulating ACOD1 expression in innate immune cells. This ACOD1 upregulation increases itaconate production, which exerts anti-inflammatory effects by promoting direct alkylating Kelch-like ECH-associated protein 1 (KEAP1), activating nuclear factor erythroid 2-related factor 2 (Nrf2) and driving antioxidant and anti-inflammatory responses. Concurrently, ACOD1 upregulation also competitively inhibits succinate dehydrogenase (SDH), resulting in succinate accumulation which stabilizes hypoxia-inducible factor-1 α (HIF-1 α) and promotes anti-inflammatory gene expression. Furthermore, ACOD1 can promote the expression of the immune checkpoint inhibitor CD274 (PD-L1) via STAT1 activation. The engagement of DRD2 by dopamine or agonists can disrupt TLR4-MYD88 interaction, inhibit ACOD1 expression, thereby conferring protection against lethal sepsis.

pinpointed DRD2 as the specific receptor mediating dopamine's inhibitory effect on ACOD1 expression (Figure 1) (54). Consistently, DRD2 knockdown and knockout reversed dopamine's suppression on ACOD1 expression, while the DRD2 agonist ropinirole mimicked dopamine's effect (54). These findings establish DRD2 as a crucial receptor in mediating DA's immunomodulatory function in sepsis, opening avenues for targeted therapeutic interventions.

To elucidate the molecular mechanism underlying dopamine-mediated suppression of ACOD1 expression, we assessed the potential involvement of the TLR4-MYD88-MAPK3-CREB1 signaling pathway. A transcription factor, CREB1, was identified as a critical regulator of ACOD1 expression (54). Dopamine, acting through DRD2, inhibits CREB1 phosphorylation at Ser133, thereby suppressing ACOD1 transcription (Figure 1). Upstream of CREB1, the canonical TLR4-MYD88-MAPK3 pathway also plays a central role, with dopamine disrupting the interaction between TLR4-MYD88 interaction, leading to decreased MAPK3 activation and subsequent reduction in CREB1 phosphorylation (Figure 1) (54).

While ACOD1 is known for its role in producing the anti-inflammatory metabolite itaconate (61), our study also revealed an itaconate-independent function of ACOD1 in regulating the expression of CD274 (PD-L1), a crucial immune checkpoint inhibitor (Figure 1). ACOD1 promotes CD274 expression via STAT1 activation (54). Consequently, dopamine, by inhibiting ACOD1 upregulation, indirectly suppresses CD274 expression. This finding has significant implications for understanding the immunosuppressive phase of sepsis, as CD274 contributes to T-cell exhaustion and dysfunction. This itaconate-independent role of ACOD1 highlights its multifaceted involvement in immune regulation. At present, it is entirely unknown whether DA inhibits CD274 expression partly by inhibiting itaconate-independent activity of ACOD1.

We also explored the therapeutic potential of modulating DA signaling in sepsis. Pramipexole, a DRD2 agonist, conferred a significant protection in mouse models of endotoxemia and polymicrobial sepsis (54). Even when administered after the onset of sepsis, pramipexole significantly improved survival rates, reduced pro-inflammatory cytokine levels, attenuated organ damage, and downregulated ACOD1 and CD274 expression (54). These results suggest that enhancing dopamine signaling through DRD2 agonism, specifically using pramipexole, could represent a promising therapeutic strategy for sepsis.

These preclinical findings were corroborated by clinical data from sepsis patients. Non-survivors exhibited lower circulating dopamine levels and higher ACOD1 expression in peripheral blood mononuclear cells compared to survivors (54). This inverse correlation between dopamine and ACOD1 expression in human sepsis underscores the clinical relevance of our animal studies. The observed association of ACOD1 and CD274 with inflammatory markers in patients further reinforces the potential role of this axis in sepsis pathogenesis (54). However, larger and well-controlled clinical trials are needed to evaluate the efficacy and safety of DRD2 agonists, like pramipexole, in diverse sepsis patient populations, considering factors like sepsis stage, infection source, and comorbidities.

Conclusions

Our recent studies have confirmed a crucial immunoregulatory role for dopamine in sepsis via the DRD2-TLR4-ACOD1-CD274 axis. Dopamine, acting through DRD2, inhibits the TLR4-MYD88-MAPK3 pathway, suppressing CREB1 phosphorylation and downregulating ACOD1 (Figure 1). This, in turn, impacts both the inflammatory and immunosuppressive phases of sepsis, influencing cytokine production, and ultimately animal survival. While these new findings offer promising new avenues for sepsis treatment, further investigation is crucial to translate these findings into clinical practice. It will be important to fully elucidate the complex interplay of dopamine and innate immunity in sepsis, including the roles of specific dopamine receptor subtypes, dopamine production by immune cells, and its impact on distinct immune cell subsets. A more comprehensive understanding of dopamine's multifaceted effects on the innate immune response during sepsis is essential for developing effective therapeutic strategies.

Author contributions

HW: Writing – original draft, Writing – review & editing. RK: Writing – review & editing. DT: Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The Role of SR-BI in sepsis: leveraging mechanistic insights to advance precision steroid therapy

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According to the Surviving Sepsis Campaign, 50.3% of septic shock patients received steroid/glucocorticoid (GC) therapy. However, whether GC therapy is beneficial and who might benefit from it are hotly debated. Initial guidelines recommended GC therapy for septic patients with adrenal insufficiency, but this has since been retracted. Recent studies using animal models of adrenal insufficiency have shed light on the mechanisms, demonstrating that the adrenal stress response is a part of the host response that is essential for control inflammatory response in sepsis and the adrenal insufficiency is a risk factor for sepsis. This perspective review explores the limitations of GC therapy through the lens of GC biology, with a particular focus on the role of scavenger receptor class B type I (SR-BI) in mediating the adrenal stress response. We highlight the mechanisms of how SR-BI-mediated adrenal stress response contributes to the regulation of hyperinflammation and innate immune responses. By integrating mechanistic insights with the limitations of GC therapy, we advocate for a precision medicine approach to GC therapy in sepsis—selectively applying GC therapy for patients with adrenal insufficiency, not without.

KEYWORDS

precision medicine, sepsis, scavenger receptor BI, adrenal stress response, glucocorticoid

Background

Sepsis is a major cause of mortality and morbidity, affecting 49 million people annually (1). It is caused by a dysregulated host response to infection (2). When an infection happens, immune cells recognize the invading microorganism using pattern recognition receptors. This triggers the innate immune system, releasing cytokines, chemokines and nitric oxide, to fight infection. However, hyper activation of the host response causes organ injury, leading to organ dysfunction and death (3, 4).

Despite the significant role of inflammation in organ injury, many trials targeting inflammatory signaling have had little impact on patient survival (5). One potential limitation is that these therapies were applied nonselectively to all septic patients. In reality, septic patients have heterogeneous subtypes; some exhibit a hyperinflammatory response while others show a hypoinflammatory response. Anti-inflammation therapy may benefit those with a hyperinflammatory response but could harm those with a hypoinflammatory response. Additionally, the inflammatory response can shift from hyperinflammation to hypoinflammation depending on the stage of sepsis, requiring timely targeting of the inflammatory response (6). Given the complexity of sepsis, there is a growing call for an endotype-based precision medicine approach for sepsis therapy (7–11). In this translational review, we discuss the potential limitations of steroid/glucocorticoid (GC) therapy for sepsis regarding the target and timing of GC therapy, review the role of scavenger receptor BI (SR-BI)-mediated adrenal stress response in sepsis. By integrating mechanistic insights with the limitations of GC therapy, we advocate for a precision medicine approach to GC therapy in sepsis—selectively applying GC therapy for patients with adrenal insufficiency, not without.

Glucocorticoid, inducible glucocorticoid and adrenal insufficiency in sepsis

GC is produced in adrenal gland and presents in circulation at 20–200 ng/ml at physiological conditions (12). GC has potent activity in regulation of inflammatory response (please refer to review article for mechanism of GC regulation of inflammatory signaling (13)). A lack of physiological levels of GC is lethal without treatment, as shown in patients with Addison's disease and in mice undergoing adrenalectomy (14). GC acts through its receptor, glucocorticoid receptor (GR) (15). Mice lacking GR in macrophage, endothelial, dendritic, or T cells are susceptible to sepsis (16–19). These early studies clearly demonstrated critical protective roles of GC-GR signaling in sepsis.

A striking feature of GC in sepsis is its inducible nature. GC production is rapidly upregulated by 5–10-fold in response to septic stress (20). We call this inducible or induced GC (iGC) (8, 9, 21). Importantly, iGC is closely related to a common condition/phenotype in septic patients, called relative adrenal insufficiency (RAI). Patients with RAI have insufficient iGC production in response to stress, which is diagnosed by a delta total cortisol of < 9 µg/dL post-ACTH stimulation (22). 25–60% of septic patients

develop RAI (15, 22–24). Numerous clinical studies showed that RAI is associated with a poor prognosis of sepsis (22, 25), but some studies failed to find such correlation (26). Nevertheless, the contribution of RAI, the major type of adrenal insufficiency, to sepsis and the pathogenesis caused by RAI are largely unknown.

In 2008, the term “RAI” was replaced by “critical illness-related corticosteroid insufficiency” (CIRCI) (27). CIRCI is defined by inadequate cellular corticosteroid activity for the severity of the patient's critical illness. It includes all types of adrenal insufficiency: absolute adrenal insufficiency, RAI and GC resistance. It is diagnosed when a seriously ill patient has very low cortisol levels (less than 10 µg/dl) or a delta cortisol < 9 g/dl upon ACTH stimulation test. Despite some disagreement, the ACTH test is commonly used to diagnose CIRCI (28).

The absolute adrenal insufficiency refers to low plasma cortisol level and is uncommon. GC resistance refers to impaired cellular GC-GR signaling. An early study by Liberty's group reported genome-wide GC resistance in sepsis (29). In their study, mice were subjected to cecal ligation and puncture (CLP) or sham surgery, followed by dexamethasone (DEX) treatment six hours later. RNA sequencing was performed two hours post-treatment to assess gene expression changes. The authors observed no significant gene induction following DEX administration in septic mice and concluded that sepsis induces genome-wide GC resistance. However, several issues in the experimental design and interpretation of the data may undermine this conclusion. At 8 hours post-CLP, mice were under significant septic stress, and endogenous GC levels had already peaked. According to Figure S2 of RNA-seq data, 60% of genes upregulated by DEX in sham mice were also upregulated by CLP alone (227/376 genes), and 68% of genes downregulated by DEX in sham mice were similarly downregulated by CLP alone (113/165 genes). These findings show that GC/GR signaling remains active at this time point, challenging the assertion of genome-wide GC resistance. GC resistance is typically defined as impaired GR signaling despite the presence of GCs. In this context, the relevance of additional GR activation by exogenous DEX, when endogenous GC levels are already maximal, is unclear. To test the hypothesis that early GR activation leads to exhaustion, the authors performed adrenalectomy (ADX) and found that septic ADX mice failed to respond to DEX (Figure S2C). They interpreted this as evidence to support the hypothesis that early HPA axis activation causes GC resistance. However, this experiment is problematic: 90% of ADX mice died within 10 hours post-CLP as indicated in Figure S1C of the article, raising concerns about the validity of RNA-seq data collected just 2 hours before death. Moreover, DEX supplementation rescued septic ADX mice (Figure S1K), which is inconsistent with the presence of functional GR resistance. Another earlier study has also shown that GC supplementation as late as 18 hours post-CLP rescues mice with adrenal insufficiency (30). Given these inconsistencies, the data presented in the article does not convincingly support the conclusion of genome-wide GR resistance in sepsis. While GC resistance has been extensively studied in chronic diseases, it remains an underexplored area in the context of sepsis/critical illness.

Abbreviations: ACTH, Adrenocorticotropic Hormone; AP-1, Activator Protein 1; CIRCI, Critical Illness-Related Corticosteroid Insufficiency; CLP, Cecal Ligation and Puncture; GC, Glucocorticoid; HDL, High-Density Lipoprotein; iGC, Induced Glucocorticoid; LDL, Low-Density Lipoprotein; NF-κB, Nuclear Factor kappa-light-chain-enhancer of activated B cells; RAI, Relative Adrenal Insufficiency; SR-BI, Scavenger Receptor BI; TLRs, Toll-Like Receptors.

As the absolute adrenal insufficiency is less common and no method to diagnose GC resistance in septic patients, we focus on RAI/CIRCI in this review.

GC therapy for sepsis is highly controversial

GC therapy has been extensively investigated in septic patients. Early studies using high-dose GC failed to demonstrate a survival benefit (31). Given the high prevalence of relative adrenal insufficiency (RAI) in sepsis, subsequent approaches have focused on administering low-dose GC in septic shock to meet the presumed increased physiological demand (13, 15, 23, 24). The French trial reported a significant reduction in mortality among septic shock patients with RAI who received GC therapy (32). However, the CORTICUS trial, which included a less severely ill cohort, did not replicate these findings (33). Meta-analyses have attempted to reconcile these conflicting results, but conclusions remain inconclusive (34–36). The HYPRESS trial evaluated hydrocortisone in patients with sepsis without shock and found no reduction in the progression to septic shock or improvement in survival within 14 days (37). In contrast, the APROCCHSS trial, which tested a combination of hydrocortisone and fludrocortisone in patients with severe vasopressor-dependent septic shock, demonstrated a significant reduction in both 90- and 180-day mortality (38). The large-scale ADRENAL trial, involving over 3,800 patients with septic shock, did not show a survival benefit from GC therapy (39). Notably, this trial did not stratify patients based on RAI or CIRCI status. Overall, the efficacy of GC therapy in sepsis remains controversial, particularly regarding whether treatment should be tailored based on adrenal function status (40, 41). For a comprehensive overview, refer to Table 1, which summarizes key clinical trials of GC therapy in sepsis.

The Problematic diagnosis of RAI/ CIRCI and its implications for GC therapy in sepsis

RAI or CIRCI is defined as “insufficient glucocorticoid (GC) relative to increased physiological demand” or “inadequate cellular corticosteroid activity for the severity of critical illness.” The ACTH stimulation test is widely used to diagnose RAI/CIRCI, but its application in septic patients remains controversial. Several experts have questioned the validity of using the ACTH test in the context of sepsis (72, 73), and the most recent clinical guidelines have not reached a consensus on its utility for diagnosing CIRCI (28). The test is designed to assess the adrenal stress response by measuring inducible GC (iGC) production stimulated by ACTH. While appropriate for non-septic patients, its interpretation in septic patients is problematic. These patients are already under extreme physiological stress and typically exhibit elevated levels of endogenous iGC. In this context, the ACTH test evaluates the

incremental adrenal response—referred to as “delta iGC”—on top of an already heightened iGC. This raises critical questions: What is the physiological significance of a low delta iGC (e.g., < 9 µg/dL)? Does it truly reflect “insufficient GC relative to demand”?

To investigate the reliability of the ACTH stimulation test in sepsis, we conducted the test in a murine model of sepsis (74). Strikingly, the ACTH test identified the majority of mice as having adrenal insufficiency during the early and intermediate stages of sepsis—even those with a demonstrably intact adrenal stress response. More concerning, ACTH administration significantly elevated inflammatory cytokine levels to lethal thresholds, resulting in a moderate but measurable increase in mortality. These findings highlight critical flaws in the use of the ACTH test for diagnosing RAI/CIRCI in sepsis. Not only does the test risk misclassifying patients and misleading GC therapy, but it may also provoke a harmful inflammatory response under septic conditions. This raises the possibility that the inconclusive outcomes of clinical trials on GC therapy may stem not from the ineffectiveness of targeting adrenal insufficiency, but from the flawed diagnostic criteria used to identify it. This underscores the urgent need for a deeper understanding of GC biology in the context of sepsis—particularly the role of the adrenal stress response (iGC production). Mechanistic studies in animal models deficient in iGC production offer a promising avenue to elucidate the precise contribution of adrenal insufficiency to sepsis pathophysiology and to refine therapeutic strategies accordingly.

Scavenger receptor BI protects against sepsis

Scavenger receptor BI (SR-BI) is a membrane protein (75, 76). It is abundantly expressed in liver, endothelial cells and steroidogenic tissues. SR-BI functions as a high-density lipoprotein (HDL) receptor, mediating the uptake of cholesteryl ester from HDL, which is essential for reverse cholesterol transport in the liver. In SR-BI null mice, the deficiency in this receptor leads to elevated HDL levels, female infertility, autoimmune disorders when aging (77), and susceptible to atherosclerosis (78–81). Similarly, humans with loss-of-function mutations in SR-BI also exhibit impaired uptake of cholesteryl esters from HDL, elevated HDL levels, and an increased risk of coronary heart disease (82, 83). This indicates that SR-BI has similar functions in both humans and rodents [please refer to SR-BI review articles (76, 84)].

Dr. Li's laboratory first reported SR-BI as a protective factor in sepsis (85, 86). They reported that LPS induces 90% fatality in SR-BI null mice versus 0% in wild type controls (85), and cecal ligation and puncture (CLP) induces 100% fatality in SR-BI null mice versus 20% in wild type controls (86). Using an LPS model, Dr van der Westhuyzen's group confirmed the protective role of SR-BI in endotoxemia and showed that SR-BI null mice are susceptible to LPS-induced endotoxic death due to uncontrolled inflammation (66). They further found that SR-BI null mice lack GC production upon ACTH stimulation or LPS challenge and pretreatment of SR-BI null mice with dexamethasone 8 hours prior LPS challenge prevented the mice from LPS induced endotoxic death. Dr. Huby's group generated adrenal specific SR-BI null (SF1CreHypoSR-BI^{fl/fl})

TABLE 1 Summary of literature on clinical trials of glucocorticoid (GC) therapy.

Study/source	Model/population	GC used	Key findings
Schumer et al., Ann Surg, 1976 (42)	Septic shock	dexamethasone or methylprednisolone	Corticosteroids significantly reduced mortality in septic shock.
Sprung et al., N Engl J Med, 1984 (43)	Septic shock	Methylprednisolone, dexamethasone	Corticosteroids do not improve overall survival in patients with severe septic shock, but they may be beneficial when used in the early stages or in specific patient subgroups.
Bone et al., N Engl J Med, 1987 (31)	Severe sepsis and septic shock	Methylprednisolone	High-dose corticosteroids offer no benefit in treating severe sepsis and shock.
Slotman et al., Crit Care Med, 1993 (44)	Severe sepsis and septic shock	Methylprednisolone	High-dose methylprednisolone significantly increases blood urea nitrogen and bilirubin levels in severe sepsis. Its potential adverse effects should be considered.
Bollaert et al., Crit Care Med, 1998 (45)	Septic shock	Hydrocortisone	Modest-dose hydrocortisone improves hemodynamics and survival in pressor-dependent septic shock, independent of adrenal insufficiency.
Briegel et al., Crit Care Med, 1999 (46)	Septic shock	Hydrocortisone	Stress-dose hydrocortisone shortened vasopressor duration and hastened organ recovery in septic shock.
Anname et al., Jama, 2002 (32)	Septic shock and relative adrenal insufficiency	Hydrocortisone	Seven-day low-dose hydrocortisone and fludrocortisone therapy reduced mortality in septic shock patients with relative adrenal insufficiency, without increasing adverse events.
Keh et al., Am J Respir Crit Care Med, 2003 (47)	Septic shock	Hydrocortisone	Hydrocortisone restored hemodynamic stability and promoted an anti-inflammatory, rather than immunosuppressive, response to stress.
Oppert et al., Crit Care Med, 2005 (48)	Septic shock	Hydrocortisone	Low-dose hydrocortisone accelerates shock reversal and reduces proinflammatory cytokines in early hyperdynamic septic shock, with hemodynamic effects linked to cortisol levels and immune effects independent of adrenal reserve.
Fernández et al., Hepatology, 2006 (49)	Cirrhosis and septic shock	Hydrocortisone	Relative adrenal insufficiency is common in advanced cirrhosis with septic shock, and hydrocortisone treatment is associated with frequent shock resolution and improved survival.
Anname et al., Crit Care Med, 2006 (50)	Septic shock	Hydrocortisone	7-day low-dose corticosteroids improved outcomes in septic shock patients with early ARDS who were nonresponders, but not in responders or those without ARDS.
Loisa et al., Crit Care, 2007 (51)	Septic shock	Hydrocortisone	Continuous hydrocortisone infusion in septic shock facilitates strict normoglycemia and reduces nursing workload for glucose control.
Weber-Carstens et al., Intensive Care Med, 2007 (52)	Septic shock	Hydrocortisone	Hydrocortisone bolus injections can cause variable, significant blood glucose spikes in septic shock patients, potentially leading to fluctuations. Continuous infusion is therefore preferred for better glycemic control.
Sprung et al., N Engl J Med, 2008 (33)	Septic shock and relative adrenal insufficiency	Hydrocortisone	Hydrocortisone did not improve survival or shock reversal overall in septic shock patients, including corticotropin nonresponders, although it hastened shock reversal in those who eventually recovered.
Arabi et al., Cmaj, 2010 (53)	Cirrhosis and septic shock	Hydrocortisone	In cirrhotic patients with septic shock, hydrocortisone initially improved hemodynamics, it did not reduce mortality and was linked to increased adverse effects.
Huh et al., Respiriology, 2011 (54)	Septic shock	Hydrocortisone	No difference in 28-day mortality was observed between septic shock patients with relative adrenal insufficiency treated with low-dose hydrocortisone for 3 or 7 days.
Moreno et al., Intensive Care Med 2011 (55)	Septic shock	Hydrocortisone	Hydrocortisone-treated patients showed faster reduction in overall organ dysfunction, mainly due to quicker cardiovascular recovery, but this did not translate into lower mortality.
Keh et al., Jama, 2016 (37)	Severe sepsis	Hydrocortisone	In adults with severe sepsis without septic shock, hydrocortisone did not reduce the 14-day risk of septic shock, providing no support for its use in this group.
Anname et al., N Engl J Med, 2018 (38)	Septic shock	Hydrocortisone plus fludrocortisone	In septic shock patients, 90-day all-cause mortality was lower with hydrocortisone plus fludrocortisone treatment compared to placebo.
Antcliffe et al., Am J Respir Crit Care Med, 2019 (56)	Transcriptomic sepsis response signatures (SRSs)	hydrocortisone	Septic shock transcriptomic profiles predicted corticosteroid response; patients with the immunocompetent SRS2 endotype had higher mortality when treated with corticosteroids versus placebo.

(Continued)

TABLE 1 Continued

Study/source	Model/population	GC used	Key findings
Venkatesh et al., Anesthesiology, 2019 (57)	Septic shock (Sepsis-3) diagnostic criteria or APROCCHSS inclusion criteria	Hydrocortisone	In Sepsis-3 or APROCCHSS subjects, continuous hydrocortisone infusion did not reduce 90-day mortality compared to placebo in septic shock.
Moskowitz et al., Intensive Care Med, 2020 (58)	Septic shock	Combination, hydrocortisone	In septic shock patients, ascorbic acid-corticosteroid-thiamine combo did not significantly reduce SOFA scores compared to placebo, providing no support for routine use.
Meduri et al., Intensive Care Med, 2020 (59)	ARDS patients	Methylprednisolone	Early, prolonged GC therapy improves survival and reduces inflammation.
Sevransky et al., Jama, 2021 (60)	Sepsis-induced respiratory and/or cardiovascular dysfunction	Combination, hydrocortisone	In critically ill sepsis patients, vitamin C, thiamine, and hydrocortisone treatment did not significantly increase ventilator- or vasopressor-free days within 30 days; however, early trial termination may have limited detection of meaningful effects.
Wong et al., Crit Care Med, 2021 (61)	Gene expression-based endotypes of pediatric septic shock	Hydrocortisone	corticosteroid exposure may increase mortality in septic shock patients with endotype A.
Cohen et al., Intensive Care Med, 2021 (62)	Septic shock	Hydrocortisone	Adrenocortical candidate gene expression was not linked to mortality. Higher GLCCI1 predicted faster shock resolution and higher BHSD1 predicted slower resolution with hydrocortisone.
Walsham et al., Intensive Care Med, 2024 (63)	Septic shock	Hydrocortisone, enteral fludrocortisone	Enteral fludrocortisone achieved variable plasma levels in septic shock patients, reflecting inconsistent absorption; its addition to hydrocortisone did not shorten time to shock resolution.
Heming et al., Lancet Respir Med, 2024 (64)	Community acquired pneumonia (CAP) and septic shock	Hydrocortisone plus fludrocortisone	hydrocortisone plus fludrocortisone reduced mortality in septic shock patients with CAP. The analysis was underpowered to separate ARDS and CAP effects, and no mortality benefit was seen in the non-CAP subgroup.
Donaldson et al., JAMA Netw Open, 2025 (65)	Septic shock	Hydrocortisone	In septic shock patients, IV hydrocortisone was linked to a reduced risk of requiring new kidney replacement therapy.

mice and showed that the mice are more susceptible to CLP-induced septic death than control (HypoSR-BI^{fl/fl}) mice (67). Using a bacterial pneumonia sepsis model (87), Gowdy et al. reported that SR-BI null mice suffer increased mortality associated with higher bacterial burden in the lung and blood, deficient in corticosterone production, higher serum cytokines, and organ injury. SR-BI null mice had significantly increased PMN recruitment and cytokine production in the infected airspace. Early efforts have revealed that SR-BI exerts its protection through multiple mechanisms including preventing nitric oxide-induced cytotoxicity (85), promoting neutrophil recruitment and LPS clearance (66, 86, 87), regulating cholesterol metabolism in liver (88) and suppressing TLR4 signaling in macrophages (86, 89, 90). These early studies establish SR-BI as a multiple protective molecule in sepsis [for detail, please refer to review article (91)].

Scavenger receptor BI-HDL pathway is a key regulator of the adrenal stress response in sepsis

In adrenal gland, GC production is markedly induced in response to septic stress (92). In our previous study, we specifically defined iGC

production as an adrenal stress response in sepsis (8). GC is derived from intracellular cholesterol. The intracellular cholesterol comes from three resources: 1) endocytosis from LDL through LDL receptor; 2) up taken from HDL through SR-BI; and 3) *de novo* synthesis. SR-BI-HDL pathway appears playing an essential role in iGC production. SR-BI null mice maintain normal basal GC levels at physiological conditions, but lack iGC under stress conditions induced by factors like LPS (66), ACTH stimulation (66), cecal ligation and puncture (CLP) (30, 67, 86), or long-term fasting (93, 94). The SR-BI null mice had normal expression in other key genes related to cholesterol *de novo* synthesis (66). Considering that rodents mainly have HDL with very low LDL in circulation, Dr. Li's laboratory generated humanized SR-BI^{-/-}ApoBtg mice (SR-BI null mice expressing ApoB) with high LDL in circulation. The mice did not produce iGC in response to ACTH stimulation or under sepsis conditions (95). Regarding the role of SR-BI in human, an early report showed that human carriers SR-BI P297S mutant, which has a 50% reduction in the uptake of cholesterol from HDL, displays a 50% reduction in iGC production to ACTH stimulation (83). In contrast, a 50% reduction in LDL receptor in familial hypercholesterolemia patients does not hinder cholesterol delivery to the adrenal cortex (96). These studies establish SR-BI-HDL pathway as a key regulator of iGC production in sepsis.

Scavenger receptor BI-mediated adrenal stress response is an essential host response against sepsis

As discussed above, SR-BI null mice have normal basal GC levels at physiological conditions, but lack iGC under stress conditions induced by ACTH stimulation (66) or CLP (30, 67, 86). Thus, SR-BI null mice are RAI/CIRCI. Dr. Li's laboratory generated adrenal specific SR-BI null mice by adrenal transplantation and used the mice as the first RAI animal model to determine if GC therapy benefits mice with RAI (30). They demonstrated that mice deficient in adrenal SR-BI lack iGC production in response to CLP challenge and are more susceptible to CLP-induced septic death and kidney injury. Importantly, GC treatment 2- and 18-hours post CLP effectively rescued adrenal specific SR-BI null mice. Interestingly, GC treatment caused more death in wild type, which was associated with lower plasma IL-6 levels and higher bacterial load in the blood and in the peritoneal fluid, suggesting immunosuppression in GC-treated wildtype mice.

Considering that the adrenal transplantation may disrupt catecholamine production by adrenal gland, Dr. Li's laboratory generated adrenal specific SR-BI null (SF1CreSR-BI^{fl/fl}) mice using new floxed SR-BI mice (8, 97). The SF1CreSR-BI^{fl/fl} mice were deficient in adrenal SR-BI expression but had normal SR-BI expression in other tissues. Using this new SF1CreSR-BI^{fl/fl} mice, they showed that adrenal SR-BI-specific knockout mice have impaired iGC production in response to ACTH stimulation and to CLP-induced sepsis. They demonstrated that while both wild-type and RAI mice exhibit a hyperinflammatory phenotype in the early stage of sepsis, iGC keeps the inflammatory response under control in wild-type mice. However,

RAI mice experience uncontrolled hyperinflammation due to a lack of iGC. Supplementing with GC restores control of the inflammatory response in RAI mice. SF1CreSR-BI^{fl/fl} mice were susceptible to CLP-induced sepsis (6.7% survival in SF1CreSR-BI^{fl/fl} mice versus 86.4% in SRBI^{fl/fl} mice). Supplementation of a low stress dose of GC to SF1CreSR-BI^{fl/fl} mice kept the inflammatory response under control and rescued the mice. However, SR-BI^{fl/fl} mice receiving GC treatment exhibited significantly less survival compared to SR-BI^{fl/fl} mice without GC treatment.

The importance of SR-BI-mediated iGC production in pediatric sepsis was assessed in 21-day-old mice (9). Mice deficient in adrenal SR-BI were susceptible to both CLP and cecal slurry induced septic death, with survival of 88.9% in SRBI^{fl/fl} mice versus 15.4% in SF1CreSRBI^{fl/fl} mice in CLP model; 33% SRBI^{fl/fl} mice versus 0% in SF1CreSRBI^{fl/fl} mice in cecal slurry model. SF1CreSRBI^{fl/fl} mice featured persistent inflammatory responses, and were effectively rescued by administering GC 2 hours post CLP. GC treatment did not improve survival in CLP-challenged wild type mice. While GC has been shown to suppress many inflammatory signaling pathways *in vitro*, using an unbiased RNA-seq analysis, the study found that a lack of iGC production in SF1CreSRBI^{fl/fl} mice causes persistent inflammatory responses mainly due to transcriptional dysregulation of AP-1 and NF- κ B (9). In addition, the study found that iGC functions to control cytokine-induced secondary inflammatory response (9).

In sum, SR-BI mediates iGC production in sepsis. Using SR-BI null mice as a model for adrenal insufficiency, early studies demonstrated that the adrenal stress response is an essential host response, which functions to keep the inflammatory response under control. GC therapy benefits mice with adrenal insufficiency but harms mice without it (Figure 1).

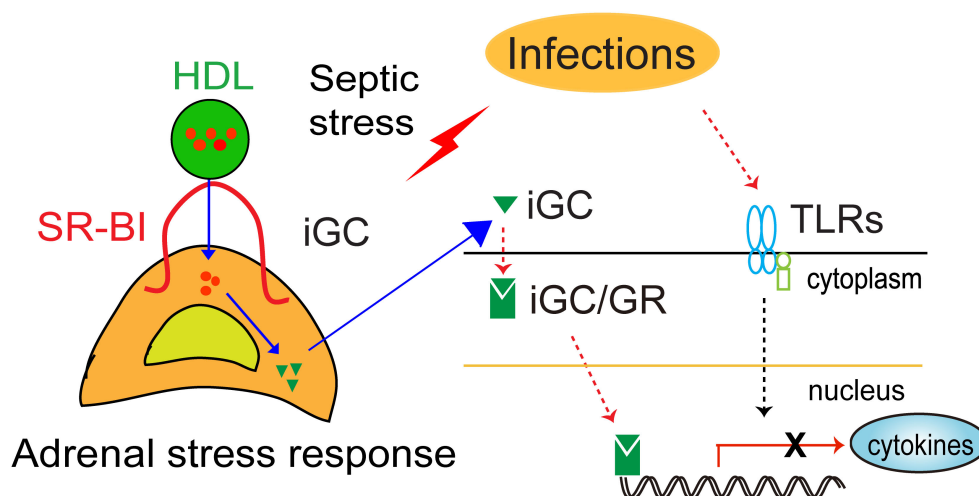


FIGURE 1

Schematic model of SR-BI-mediated adrenal stress response (iGC production) protection against sepsis. Upon infection, immune cells recognize the invading microorganism using pattern recognition receptors (TLRs). This triggers the innate immune system, releasing cytokines to fight infection. However, dysregulation of the host response causes organ injury, leading to organ dysfunction and death. In response to septic stress, adrenal SR-BI mediates the uptake of cholesterol from HDL into adrenal gland for induced glucocorticoid (iGC) production, which functions to keep the inflammatory response under control.

Translation of the mechanistic studies into a precision medicine approach to guide GC therapy for sepsis

The mechanistic findings using adrenal SR-BI null mice as a RAI model provide proof-of-concept that targeting RAI/CIRCI with GC can be an effective therapy for sepsis. However, clinical trials did not show a survival benefit of GC therapy in septic patients. There are a number of disconnections between GC therapy and the mechanisms of GC function, which may render GC therapy less effective. Let's examine it through the lens of GC biology in sepsis.

1) Adrenal insufficiency versus diagnosis of RAI/CIRCI: RAI or CIRCI is commonly defined as “insufficient GC activity relative to increased physiological demand” or “inadequate cellular corticosteroid activity for the severity of critical illness.” However, these definitions are conceptually vague and lack mechanistic specificity, limiting their utility in guiding GC therapy. Mechanistically, sepsis triggers a robust host response, including activation of the adrenal stress axis and increased inducible glucocorticoid (iGC) production. As previously discussed, failure to mount this adrenal stress response is a recognized risk factor in sepsis, and targeting patients with impaired iGC production may

improve outcomes. In clinical practice, the ACTH stimulation test is used to assess adrenal function. While appropriate for non-septic patients, its application in septic patients—who already exhibit elevated endogenous iGC due to extreme stress—raises concerns. In this context, the test measures the incremental adrenal response (delta iGC) on top of endogenous iGC. Our recent findings indicate that the ACTH test fails to elicit additional iGC production in mice with normal adrenal stress response during the early and intermediate phases of sepsis. Moreover, under septic conditions, ACTH administration can exacerbate cytokine production, potentially worsening patient outcomes (74). Consequently, the ACTH test may misclassify septic patients with an intact adrenal stress response as adrenal insufficient, leading to inappropriate GC therapy.

2) Functions of GC versus targets of GC therapy: Mechanistic studies have established that inducible glucocorticoids (iGC) play a critical role in modulating inflammation. Current clinical guidelines recommend GC therapy for septic patients experiencing shock (41), with hypotension serving as the primary criterion for intervention. Table 2 summarizes literature on phenotype-based GC therapy in animal models. While GCs are known to support blood pressure regulation, this recommendation is largely grounded in clinical observations rather than mechanistic understanding. Hypotension in sepsis is typically a downstream consequence of organ dysfunction,

TABLE 2 Summary of literature on phenotype-based glucocorticoid (GC) therapy in animal models.

Study/source	Model/population	GC used	Key findings	Precision strategy
Cai et al, J Clin Invest, 2008 (66)	SR-BI-null mice	Corticosterone	SR-BI-null mice lack inducible glucocorticoid synthesis. SR-BI is essential for the anti-inflammatory response to endotoxemic shock via its roles in glucocorticoid production and LPS clearance.	Corticosterone supplementation decreased the sensitivity of SR-BI-null mice to LPS.
Gilibert et al, J Immunol, 2014 (67)	Hypo-adrenal SR-BI null mice		adrenal SR-BI is essential for HPA axis function, enabling effective glucocorticoid-mediated host defense following endotoxemic shock or bacterial infection.	
KM Gowdy et al, Mucosal Immunology, 2015 (68)	SR-BI null mice (adrenal insufficiency model)	Corticosterone	SR-BI null mice showed impaired stress-induced GC production, increased mortality, and exaggerated inflammation during bacterial pneumonia. Corticosterone replacement corrected neutrophil trafficking but not mortality.	Use of genetic model to dissect adrenal contribution; GC replacement to isolate adrenal effects.
Ai et al, Crit Care Med, 2015 (69)	adrenal-specific SR-BI null mice (adrenal transplantation)	Corticosterone	Corticosteroid treatment benefits mice with adrenal insufficiency but harms mice without adrenal insufficiency.	Corticosteroids may benefit septic patients with adrenal insufficiency but harm those without, supporting the need for clinical studies to test this hypothesis.
Quatrini & Ugolini, Cellular and Molecular Immunology, 2021 (70)	Preclinical (rodent & cellular models)	Corticosterone, Dexamethasone	GC effects are highly cell- and tissue-specific; GR signaling varies by context.	Targeting GR isoforms and cell-specific delivery.
Wu et al, Front Immunol, 2022 (8)	adrenal-specific SR-BI null mice (SF1CreSR-BI ^{fl/fl} mice)	Hydrocortisone	Glucocorticoid treatment benefits mice with relative adrenal insufficiency (RAI) but is harmful in mice with normal adrenal function.	Selectively applying GC therapy for a subgroup of patients with RAI.
Hobson et al., Research in Autism Spectrum Disorders, 2023 (71)	Preclinical (molecular modeling)	SEGRMs	Selective GR modulators reduce side effects while preserving efficacy.	Ligand design for selective GR activation.
Hao et al, J Infect Dis 2023 (9)	21-day-old SF1CreSR-BI ^{fl/fl} mice	Hydrocortisone	Relative adrenal insufficiency mice exhibited significantly higher mortality and were effectively rescued by glucocorticoid therapy.	Use of glucocorticoids (GCs) in sepsis based on the status of relative adrenal insufficiency.

which is often driven by hyperinflammation. Given that iGCs function primarily to control inflammatory responses, a mechanistically informed approach would suggest that targeting patients with iGC insufficiency and a hyperinflammatory phenotype may be more effective than relying solely on the presence of hypotension as an indicator for GC therapy. This perspective raises an important question: could a precision medicine strategy that identifies and treats patients with impaired iGC production and hyperinflammation yield better outcomes than the current one-size-fits-all approach?

3) Timing of GC action versus timing of GC therapy: Following infection, immune effector cells rapidly initiate a robust inflammatory response characterized by the release of high levels of cytokines and chemokines. While essential for pathogen clearance, this response can become detrimental if not properly regulated, leading to tissue damage and organ dysfunction. Mechanistic studies have shown that inducible glucocorticoids (iGC) are produced early in the course of infection and play a critical role in modulating inflammation during the early and intermediate stages of sepsis. In contrast, current clinical guidelines recommend initiating GC therapy in septic patients who develop shock (41), —a condition that typically manifests in the later stages of sepsis. This temporal disconnect raises an important concern: is GC therapy being administered too late in the disease course to exert its full therapeutic benefit? If iGC's anti-inflammatory effects are most critical during the early phases of sepsis, delayed intervention may limit the efficacy of exogenous GC therapy. This discrepancy underscores the need to re-evaluate the timing of GC administration and consider earlier, targeted intervention based on mechanistic insights.

Conclusions - reevaluating GC therapy in sepsis through a precision medicine lens

Despite extensive clinical trials, glucocorticoid (GC) therapy has demonstrated limited impact on patient survival (5). The efficacy of GC treatment in sepsis and whether its use should be stratified based on adrenal insufficiency—remains a subject of ongoing debate (40, 41). This controversy is further complicated by the limitations of the ACTH stimulation test, which may not reliably diagnose adrenal insufficiency in septic patients.

Given the complexity and heterogeneity of sepsis, there is increasing support—including from our own studies (7–9, 21, 30)—for adopting a precision medicine approach to sepsis management (98, 99). A cornerstone of this strategy is the identification and targeted treatment of patient subgroups defined by specific endotypes (100, 101). Mechanistic studies using adrenal SR-BI knockout mice, a validated model of adrenal insufficiency, have identified adrenal insufficiency as both a risk factor and a

distinct endotype in sepsis. Building on these insights, we advocate for two key shifts in clinical practice: 1) Redefining and developing improved diagnostic criteria for adrenal insufficiency in septic patients, moving beyond the limitations of current testing methods; 2) Implementing a precision medicine framework to guide GC therapy—administering treatment in a timely and selective manner to patients with confirmed adrenal insufficiency, while avoiding unnecessary use in those with an intact adrenal stress response.

Author contributions

LG: Formal Analysis, Validation, Methodology, Writing – review & editing, Conceptualization, Project administration, Data curation, Writing – original draft, Investigation, Resources. QW: Writing – review & editing. X-AL: Investigation, Project administration, Funding acquisition, Data curation, Conceptualization, Writing – review & editing, Formal Analysis, Writing – original draft.

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Tubular epithelial cell-derived extracellular vesicles carrying serum amyloid A1 exacerbate sepsis-associated acute kidney injury by promoting NETs formation

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Introduction: Sepsis-associated acute kidney injury (SA-AKI) is a highly lethal condition with a rapid onset, and effective treatments are lacking because the molecular pathogenesis remains unclear. Tubular epithelial cells (TECs) have increasingly been recognized as driving forces in the progression of kidney diseases, partly through the release of extracellular vesicles (EVs) carrying proinflammatory cargos. However, the role of TEC-derived EVs on neutrophil extracellular traps (NETs) formation, which is an established feature of sepsis, and SA-AKI remains unclear.

Methods: EVs isolated from phosphate buffer saline (PBS)/lipopolysaccharide (LPS)-treated TECs were injected intravenously into C57BL/6J wild type mice to determine whether TECs-derived EVs can directly induce NETs formation and kidney injury. Proteomics and single-cell RNA sequencing analysis were used to screen the key molecules that mediate the effects of TECs-derived EVs. EVs secretion from TECs and serum amyloid A1 (SAA1) expression in TECs were specifically inhibited via adeno-associated virus (AAVs). Finally, the association between SAA1 level in plasma EVs and clinical features of septic patients was determined.

Results: This study demonstrated that EVs secreted from LPS-stimulated TECs exacerbated AKI by promoting NETs formation. Specifically blocking EVs secretion from TECs via AAVs reduced NETs formation and alleviated LPS-induced AKI. Bioinformatics analysis suggested that LPS increased SAA1 expression in TECs, and then released extracellularly through EVs. Further mechanistic studies revealed that SAA1 packaged in TECs-derived EVs was responsible for NETs formation and AKI via activation of the TLR4/p38 MAPK signaling pathway in neutrophils. Specifically inhibiting SAA1 upregulation in TECs via AAVs also reduced NETs formation and alleviated LPS-induced AKI. Interestingly, modulating EVs release from TECs or SAA1 expression in TECs also alleviated remote lung injury induced by LPS, indicated that TECs-derived EVs may participate in kidney–lung crosstalk during sepsis. Furthermore, plasma

TECs-derived EVs proportion and SAA1 expression in plasma EVs may be promising prognostic indexes for SA-AKI patients.

Discussion: Here, we explored a new mode of TECs-neutrophils crosstalk mediated by EVs during SA-AKI, and strategies to modify TECs-derived EVs and the cargo SAA1 could be a new avenue for developing therapeutics against SA-AKI.

KEYWORDS

sepsis-associated acute kidney injury, tubular epithelial cells, extracellular vesicles, neutrophil extracellular traps, serum amyloid A1

Introduction

Sepsis—a life-threatening global health crisis—is a leading cause of hospital mortality. It arises from infection-induced immune hyperactivation and cytokine storms, resulting in tissue damage and multi-organ dysfunction, including acute kidney injury (AKI). Sepsis-associated acute kidney injury (SA-AKI) affects nearly 60% of septic patients, significantly compounding morbidity and mortality risks. However, the pathophysiology of SA-AKI remains incompletely understood (1).

Neutrophils are important innate immune cells playing crucial roles in the pathogenesis of sepsis (2). Neutrophils activated locally execute pathogen defense through oxidative burst, phagocytosis, and NETs formation (3). Composed of DNA, histones and granule proteins, NETs in sepsis function dually: constraining pathogens but causing tissue injury when overproduced (4). One preclinical study propose that interrupting NETs formation in combination with monoclonal antibody might be a feasible therapeutic strategy for septic AKI (5). Therefore, reducing NETs formation is crucial for alleviating kidney injury.

Extracellular vesicles (EVs)-mediated intercellular communication critically regulates kidney injury progression (6, 7). Our previous studies have shown that EVs from innate immune cells could modulate NETs formation and thus participate in the pathogenesis of sepsis. EVs-carrying microRNAs and CCL-2

mRNA have the tropism to macrophages and provoke tubulointerstitial inflammation and kidney injury (8–10). However, the interplay among NETs formation, SA-AKI and EVs needs to be further addressed.

SA-AKI is hallmarked by renal inflammation, apoptosis, and oxidative injury (11). Tubular epithelial cells (TECs) are the most populous cell type in the kidney and now recognized not only as injury victims but also as pathogenic mediators accelerating kidney disease progression. Chemokines/cytokines from damaged TECs recruit mainly neutrophils and macrophages to injured kidneys. These infiltrating cells release cytokines that drive further tubular injury, creating a vicious cycle (10). Therefore, we hypothesize that TECs-derived EVs modulate NETs formation and promote SA-AKI progression.

Serum amyloid A (SAA) is a family of acute-phase proteins, the plasma levels of which may increase >1000-fold in acute inflammatory states. SAA1 plus several conventional biomarkers including C-reactive protein, procalcitonin are extensively investigated to diagnose sepsis (12, 13). In addition to be regarded as biomarkers, recent study showed that SAA1 could induce neutrophilic airway inflammation by activating neutrophils along with NETs formation (14). Previous study also suggested that SAA1 was enriched in plasma EVs obtained from septic patients (15). In this study, we aimed to investigate whether TECs-derived EVs could deliver SAA1 to induce NETs formation and SA-AKI progression.

Materials and methods

Patient samples and ethics statement

We recruited 26 patients with early (less than 24 h) diagnosis of sepsis who were admitted to the intensive care unit (ICU) of Nanjing Drum Tower Hospital (Nanjing, China) between February and May 2024 (Supplementary Table S1). Sepsis was diagnosed according to the Sepsis-3 criteria (16). Acute kidney injury (AKI) was defined using the KDIGO criteria (an increase in serum creatinine (Scr) of ≥ 0.3 mg/dl within 48 hours or $\geq 50\%$ from

Abbreviations: SA-AKI: Sepsis-associated acute kidney injury; TECs: Tubular epithelial cells; EVs: Extracellular vesicles; PBS: Phosphate buffer saline; LPS: Lipopolysaccharide; NETs: Neutrophil extracellular traps; scRNA-seq: Single-cell RNA sequencing; SAA1: Serum amyloid A1; MODS: Multiple organ dysfunction syndrome; ICU: Intensive care unit; KDIGO: Kidney Disease: Improving Global Outcomes; Scr: Serum creatinine; *i.p.*: Intraperitoneal; *i.v.*: Intravenously; Cr: Creatinine; BUN: Blood urea nitrogen; H&E: Hematoxylin and eosin; CitH3: Citrullinated histone H3; AAV9: Adeno-associated viruses; NC: Negative control; HK-2: Human proximal tubule cell line; FBS: Fetal bovine serum; CLP: Cecal ligation and puncture; MPO: Myeloperoxidase; FDR: False discovery rate; UMAP: Uniform Manifold Approximation and Projection; FPR2: Formyl peptide receptor 2; RT-PCR: Real-time polymerase chain reaction; SEM: Standard error of mean; WT: Wild-type; ELISA: Enzyme-linked immunosorbent assay.

baseline) (17). Pregnant women and patients under 18 years of age and those with existing AKI before admission to the ICU, severe anemia, active bleeding, or chemotherapy were excluded. Healthy age-/sex-matched donors served as controls for septic patients. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the institutional ethics and review board of Nanjing Drum Tower Hospital (Approval No. 2024-102-02). Informed consent was obtained from the participating volunteers, patients or their representatives.

Animals

Male C57BL/6J mice (6–8 weeks old, weighting 20–25 g) were purchased from (Vital River Laboratories, Zhejiang, China) were fed under a specific pathogen-free environment in the Laboratory Animal Center of Nanjing First Hospital. All animal experiments were conducted under the rules approved by the Ethics Committee of Nanjing First Hospital (Approval No. DWSY-23011315). The septic AKI mice models were induced by intraperitoneal (*i.p.*) injection of LPS (10 mg/kg of body weight, Sigma-Aldrich, USA) as previously described (18). The control mice were injected with *i.p.* an equal volume of phosphate buffer saline (PBS). To degrade NETs or inhibit EVs release, DNase I (5 mg/kg) or GW4869 (2.5 µg/g) was given with an intraperitoneal injection. To explore EVs function *in vivo*, mice were intravenously (*i.v.*) injected with TECs-derived EVs (300 µg/mouse) using 31-gauge insulin syringes. 24 h later, blood samples were collected for plasma creatinine (Cr), blood urea nitrogen (BUN) and NETs components (MPO-DNA complexes and dsDNA) measurement. Kidney samples were collected for histological analysis and NETs evaluation.

Knockdown of Rab27a and SAA1 in TECs *in vivo*

To specifically knockdown Rab27a and SAA1 in renal TECs, as previously described (19), we conducted adeno-associated viruses (AAV9) carrying short hairpin RNA against Rab27a/SAA1 and Ksp-cadherin (a unique, tissue-specific member of the cadherin family that is exclusively expressed in renal tubular epithelial cells) which was used as the upstream promoter of Rab27a/SAA1 (GeneChem, Shanghai, China). GFP linked to each shRNA cassette was cloned downstream of the Ksp-cadherin promoter in the AAV9 vector plasmid. Eight-week-old male C57BL/6J mice were administrated 1×10^{12} gv/mouse AAVs via tail vein injection one week before LPS injection. The target sequence for Rab27a was 5'-GCTTAACCACTGCATTCTTCA-3'. The target sequences for SAA1 were 5'-GAAGAGAGGCCTTTCAGGAAT-3', 5'-GGACATGAGGACACCATTGCT-3', 5'-CTCCTATTAGCTCAGTAGGTT-3'. The insert sequence for negative control (NC) was 5'-TTCTCCGAACGTGTACAGT-3'.

Cell culture and treatment

The human proximal tubule cell line (HK-2) was cultured in DMEM/F12 medium (Gibco, USA) supplemented with 1% (v/v) penicillin streptomycin (P/S, Gibco, USA) and 10% fetal bovine serum (FBS, Viva cell, China) at 37°C. An immortalized mouse renal tubular epithelial cells (TCMK-1 cells) were maintained in DMEM (Gibco, USA) supplemented with 1% (v/v) P/S (Gibco, USA) and 10% FBS (Viva cell, China) at 37°C. Both HK-2 and TCMK-1 cells were purchased from the American Type Culture Collection. To obtain TECs-derived EVs, HK-2 or TCMK-1 cells were treated with PBS or LPS (1 µg/ml) at 37°C for 24 hours (h) in medium containing 10% exosome-free FBS (#EXO-FBS-50A-1; System Biosciences, Palo Alto, CA, USA), and the supernatant were collected for EVs isolation.

siRNA and transfection

To delete SAA1 expression in TECs-EVs, HK-2 or TCMK-1 cells were transfected with 50 nM of siRNA against human or mouse SAA1 respectively (RiboBio, Guangzhou, China) using Lipomaster 3000 Transfection Reagent (Vazyme, Nanjing, China) per manufacturer's recommendations. A scramble sense-control was used as negative control. The target sequences of siRNAs for human SAA1 were as follows: (1) 5'-GATCAGGCTGCCAATG AAT-3'; (2) 5'-GAGAGAATATCCAGAGATT-3'; (3) 5'-GCGATGCCAGAGAGAATAT-3'. The target sequences of siRNAs for mouse SAA1 were as follows: (1) 5'-CUCCUGGACUGCCUGA CAA-3'; (2) 5'-GAACUAUGAUGCUGCUCAA-3'; (3) 5'-UGUUCACGAGGCUUCCAA-3'. After 24 h of siRNA treatment, the culture medium was changed to medium containing 10% exosome-free FBS plus LPS (1 µg/ml) for another 24 h. At the end of the experiments, cells and EVs in the supernatant were collected and the level of protein expression was evaluated by western blotting.

EVs isolation and characterization

EVs were isolated from the supernatant of HK-2 or TCMK-1 cells treated with PBS (Ctrl-EVs) or LPS (LPS-EVs) using ExoQuick-TC (#EXOTC10A-1; System Biosciences, USA) according to the manufacturer's instructions. For kidney EVs extraction, we conducted a mouse model of cecal ligation and puncture (CLP) to mimic clinical sepsis as described previously (20). At 24 h after CLP, 100 mg of kidney cortex was collected and submitted to tissue digestion with collagenase and trypsin for 120 minutes at 37°C (10). Then the sample was submitted for EVs extraction using ExoQuick-TC according to the manufacturer's instructions. Plasma samples from septic patients were collected for EVs purification using differential ultracentrifugation as described previously (21). Plasma was sequentially centrifuged

(300 × g/10 min, 2000 × g/20 min, 10,000 × g/30 min; 4°C), filtered (0.22 µm; Merck Millipore SLGP033RB), and ultracentrifuged (100,000 × g/3 h; SW28 Ti rotor). The pellet was washed via PBS resuspension/re-ultracentrifugation (100,000 × g/3 h) and finally resuspended in 200 µl PBS. EVs characterization methods are in [Supplementary Materials](#).

Neutrophil isolation and *in vitro* co-culture experiments

Neutrophils were isolated from healthy donor venous blood using Polymorphprep™ (#1114742, Axis-Shield, Norway) per manufacturer's protocol. Isolated cells (90% purity, 95% viability by flow cytometry/Trypan blue) were resuspended in complete RPMI 1640 (1% FBS, 50 µg/ml penicillin/streptomycin) at 10⁶ cells/ml.

Freshly isolated neutrophils were co-cultured with Ctrl-EVs or LPS-EVs (100 µg/ml) for 3 h at 37°C. PBS was used as negative control. To inhibit MAPK pathway activation, SB203580 was added to the culture medium at a final concentration of 30 µM about 30 min before co-cultured with EVs. To block formyl peptide receptor 2 (FPR2), Toll-like receptor 2 (TLR2) or TLR4 activation, WRW4 (10 µM), C29 (50 µM) or TAK-242 (50 µM) were added to the culture medium about 30 min before co-cultured with EVs respectively.

Statistical analysis

Data normality was assessed by Shapiro-Wilk test. Normally distributed data are expressed as mean ± SEM and analyzed by two-tailed Student's t-test (two groups) or one-way ANOVA with Tukey's *post hoc* test (multiple groups). Non-normal data are presented as median ± IQR and analyzed by Mann-Whitney U test (two groups) or Kruskal-Wallis with Dunn's test (multiple groups). Correlations used Spearman's method. Statistical significance was defined as $P < 0.05$ (GraphPad Prism 8).

More detailed methods are described in [Supplementary Methods](#).

Results

NETs contributed to kidney injury in an LPS-induced AKI model

First, a mouse model of sepsis induced by LPS challenge was used to detect the formation of NETs. As shown in [Figure 1A](#), immunofluorescence labeling via anti-citrullinated histone H3 (citH3) and anti-Ly6G antibodies revealed increased NETs formation in the kidneys induced by LPS, and the levels of NETs components in the plasma were also significantly increased following LPS challenge ([Figures 1B-C](#)). For determination of the exact role of NETs in LPS-induced AKI, NETs were degraded by DNase I (a well-established inhibitor of NETs formation) 30 min after LPS administration, and as demonstrated by immunofluorescence,

Picogreen and ELISA, NETs formation was significantly decreased ([Figures 1A-C](#)). Furthermore, H&E staining revealed that DNase I significantly ameliorated the kidney damage induced by LPS ([Figure 1D](#)) and reduced the plasma Cr and BUN levels ([Figures 1E-F](#)). qPCR analyses indicated that DNase I treatment attenuated the changes in kidney injury markers (NGAL and KIM-1) and inflammatory factors (IL-1β, IL-6 and TNF-α) caused by LPS challenge ([Figures 1G-I](#)). Collectively, these findings indicate that LPS stimulates NETs formation to induce kidney injury.

Inhibition of total EVs release by GW4869 reduced NETs formation and alleviated LPS-induced AKI

Next, we investigated the relationship between EVs release and NETs formation during SA-AKI. GW4869, a noncompetitive phospholipase inhibitor, was used to block total EVs synthesis/secretion, and NETs formation was substantially inhibited in the kidneys and plasma of septic mice ([Figures 2A-C](#)). More importantly, the tubular injury scores, plasma Cr and BUN levels, kidney injury markers and inflammatory factors in the kidneys were all reduced by GW4869 ([Figures 2D-I](#)), indicating that EVs are involved in NETs formation and kidney injury during LPS-induced AKI.

EVs secreted from LPS-stimulated TECs promoted NETs formation and exacerbated AKI *in vivo*

Previous studies have shown that TECs-derived EVs function as active vectors that play essential roles in the pathogenesis of kidney disease (22). However, whether EVs contribute to the communication between tubules and neutrophils during AKI remains unknown. To address this question, we treated mouse renal TECs (TCMK-1 cells) with LPS (1 µg/ml) to simulate sepsis *in vitro*. After 24 h, EVs in the supernatant were isolated and characterized via transmission electron microscopy, NTA, and western blotting ([Figures 3A-D](#)). For determination of whether TECs-derived EVs can directly induce NETs formation and kidney injury, TECs-derived EVs were injected *i.v.* into C57BL/6J WT mice, and fluorescence imaging revealed that Dil-labeled EVs accumulated in kidney tissues ([Figure 3E](#)). Immunofluorescence revealed that LPS-EVs (EVs from LPS-stimulated TCMK-1 cells) promoted NETs formation in kidney tissues ([Figure 3F](#)) and increased the NETs concentration in the plasma ([Figures 3G-H](#)). Moreover, more histological lesions were observed in the mouse kidney after the administration of LPS-EVs than after administration of Ctrl-EVs ([Figure 3I](#)). The plasma Cr and BUN levels, kidney injury markers and inflammatory factors in kidney tissues were significantly increased in the LPS-EVs group ([Figures 3J-N](#)), suggesting that the kidney injury induced by LPS-EVs occurred in parallel with NETs formation.

To further establish a causal relationship between LPS-EVs-induced NETs formation and kidney injury, we administered

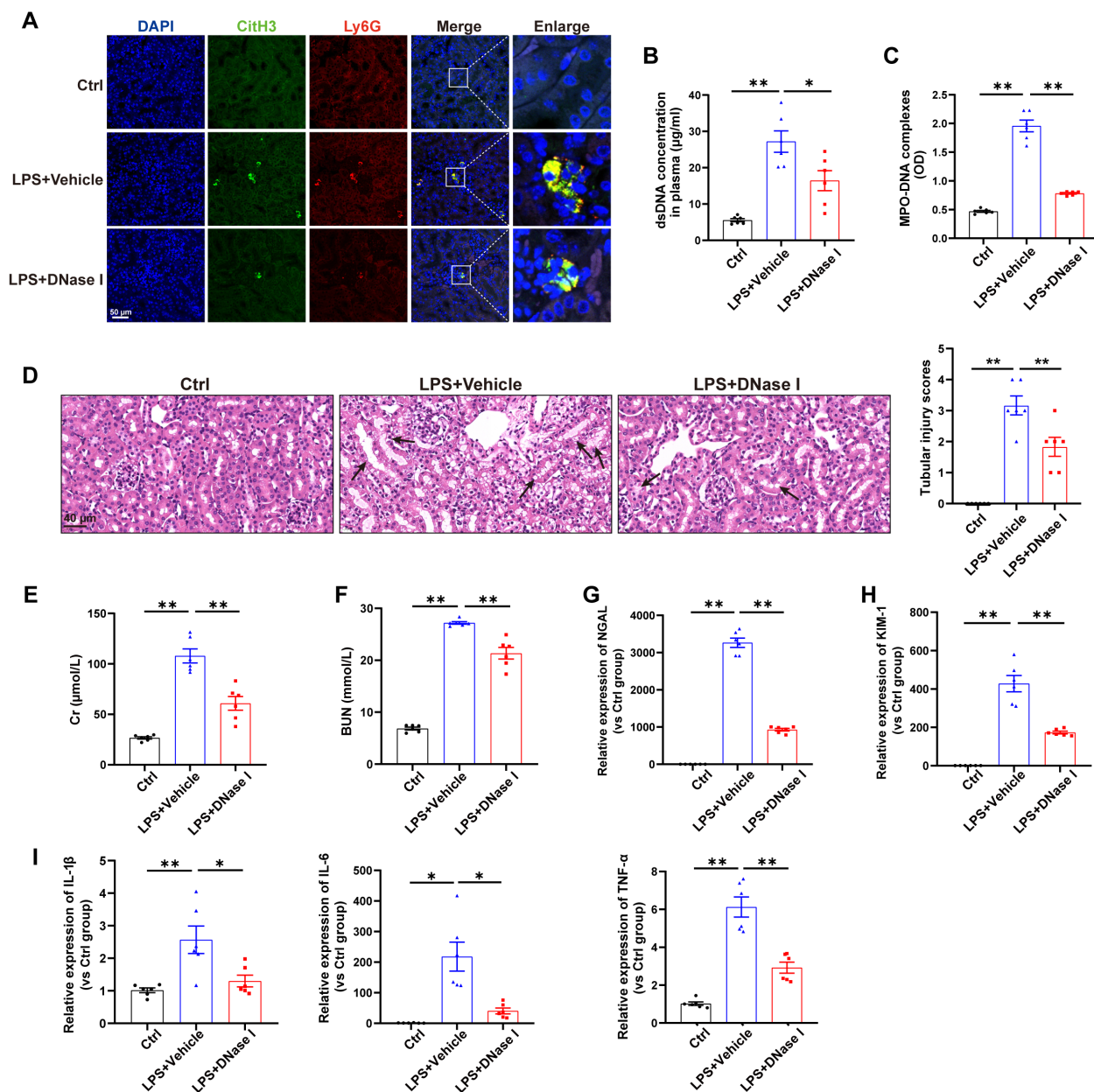


FIGURE 1

NETs contribute to kidney injury in an LPS-induced AKI model. WT C57BL/6J mice were intraperitoneally injected with LPS (10 mg/kg body weight) for 24 h. The control mice were injected with an equal volume of PBS. DNase I (5 mg/kg) was given via an *i.p.* injection 30 min after LPS, and an equal volume of normal saline was used as the vehicle. (A) Representative images showing the presence of NETs (Ly6G, red; citrullinated H3, green) in kidney tissues. Nuclei were counterstained with DAPI (blue). Scale bar, 50 μ m. (B, C) Quantification of dsDNA and circulating NETs structures (MPO-DNA complexes) in the plasma of mice via PicoGreen fluorescence quantification and ELISA, respectively. (D) Representative images of H&E-stained kidneys (original magnification, 400 $\times$). Scale bars: 40 μ m. The black arrows indicate tubule damage. Quantification of tubular injury via H&E staining. (E, F) Quantitation of Cr and BUN in blood samples from mice in each group. (G-I) RT-qPCR analysis of NGAL, KIM-1, IL-1 β , IL-6 and TNF- α mRNA levels in the kidney. One-way analysis of variance with Tukey's multiple comparisons test was used for the analysis. The graphs present the means $\pm$ SEMs, $n = 6$; * $P < 0.05$, ** $P < 0.01$ compared between two groups.

DNase I 30 min after LPS-EVs injection to degrade NETs. The results showed that DNase I successfully reduced NETs formation (Supplementary Figures S1A-C) and alleviated kidney injury induced by LPS-EVs (Supplementary Figures S1D-I). These data indicate that EVs secreted from LPS-stimulated TECs exacerbated AKI by promoting NETs formation.

EVs secreted from LPS-stimulated TECs promoted NETs formation *in vitro*

To further demonstrate the ability of LPS-EVs to stimulate NETs formation, we established an *in vitro* coculture system by coculturing neutrophils freshly isolated from healthy volunteers with TECs-

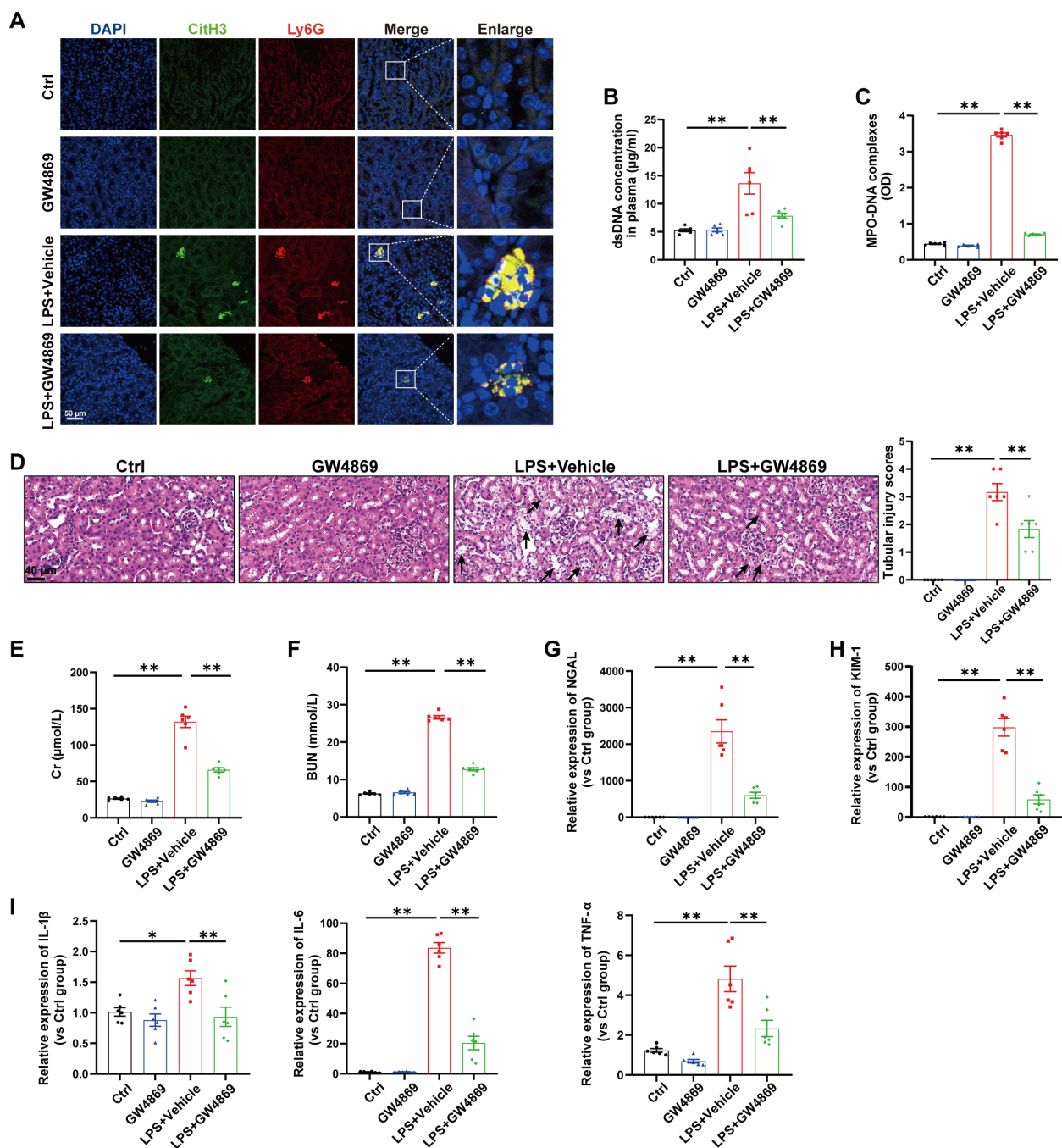


FIGURE 2

Inhibition of total EVs release by GW4869 reduced NETs formation and alleviated LPS-induced AKI. GW4869 (2.5 μ g/g) was given via an *i.p.* injection 1 h before LPS administration, and an equal volume of DMSO was used as the vehicle. **(A)** Representative images showing the presence of NETs (Ly6G, red; citrullinated H3, green) in kidney tissues. Nuclei were counterstained with DAPI (blue). Scale bar, 50 μ m. **(B, C)** Quantification of dsDNA and circulating NETs structures (MPO–DNA complexes) in the plasma of mice via PicoGreen fluorescence quantification and ELISA, respectively. **(D)** Representative images of H&E-stained kidneys (original magnification, 400 $\times$). Scale bars: 40 μ m. The black arrows indicate tubule damage. Quantification of tubular injury via H&E staining. **(E, F)** Quantitation of Cr and BUN in blood samples from mice in each group. **(G–I)** RT–qPCR analysis of NGAL, KIM-1, IL-1 β , IL-6 and TNF- α mRNA levels in the kidney. One-way analysis of variance with Tukey's multiple comparisons test was used for the analysis. The graphs present the means $\pm$ SEMs, $n = 6$; * $P < 0.05$, ** $P < 0.01$ compared between two groups.

derived EVs. The same species of cell line, the human proximal tubule cell line (HK-2), was used in this part. First, we isolated EVs from HK-2 cells treated with PBS/LPS (1 μ g/ml) for 24 h. The isolated EVs were validated via transmission electron microscopy, NTA, and western blotting (Figures 4A–D). After incubating neutrophils *in*

vitro with Dil-labeled EVs for 3 h, we observed neutrophil internalization of TECs-derived EVs (Figure 4E), and the uptake efficacy of LPS-EVs by neutrophils was greater than that of Ctrl-EVs (Figure 4F). Confocal microscopy revealed obvious NETs formation following LPS-EVs treatment *in vitro* (Figure 4G), and the NETs

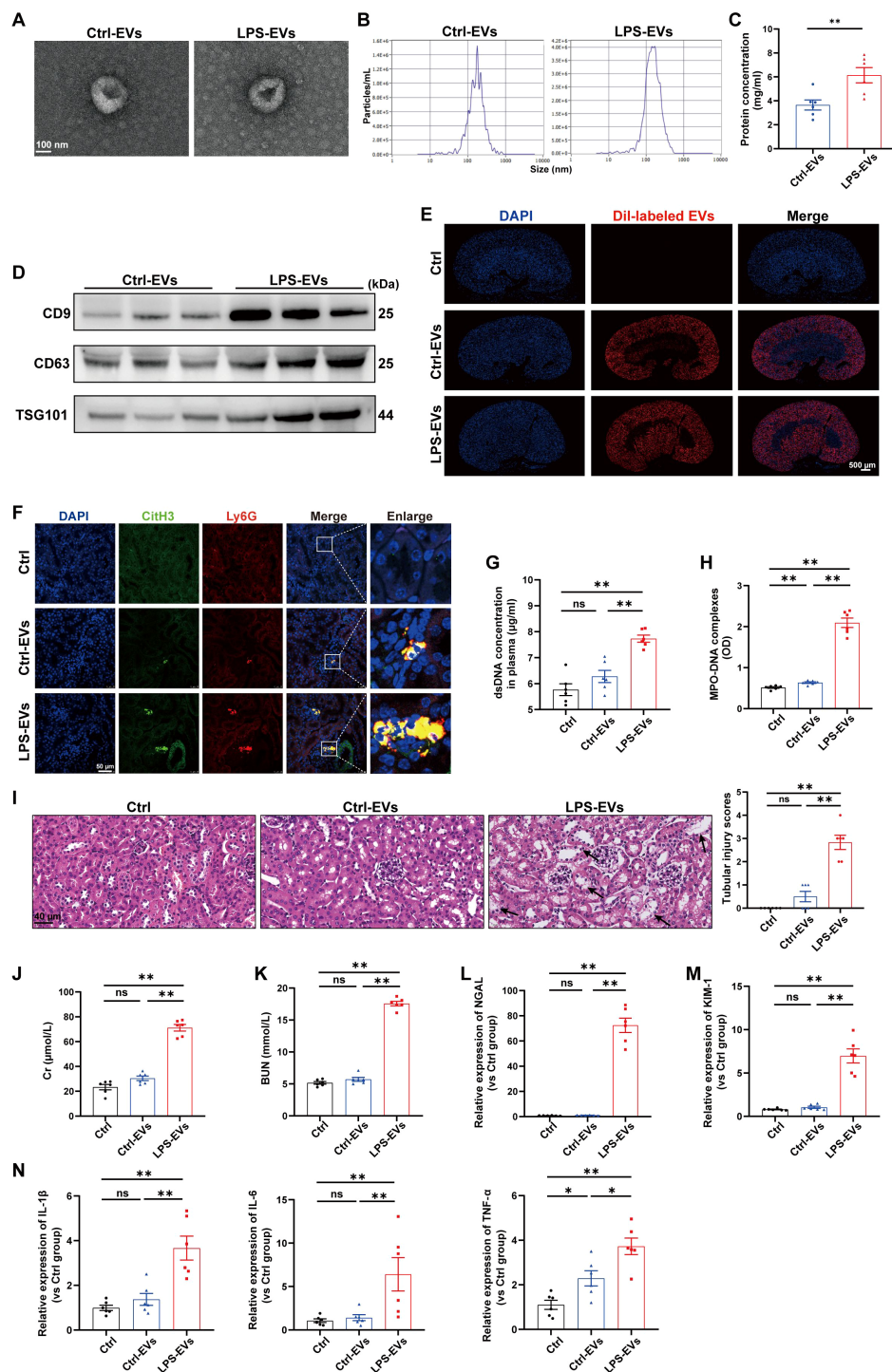


FIGURE 3

EVs secreted from LPS-stimulated TECs promoted NETs formation and aggravated AKI *in vivo*. (A) Electron micrograph of EVs isolated from the supernatant of PBS/LPS (1 $\mu\text{g}/\text{ml}$)-treated TCMK-1 cells (Ctrl-EVs/LPS-EVs). Scale bar, 100 nm. (B) EVs size distribution was measured via NanoSight tracking analysis. (C) The protein concentration of EVs was determined via a BCA protein assay kit. (D) CD9, CD63 and TSG101 protein expression in EVs was quantified by Western blotting with equal amounts of EVs protein (20 μg). (E–N) WT C57BL/6J mice were *i.v.* administered PBS (Ctrl group), Ctrl-EVs or LPS-EVs (300 $\mu\text{g}/\text{mouse}$) for 24 h (E) Representative images of fluorescent signals in the kidneys of mice *i.v.* injected with DiI-labeled EVs (red). Nuclei were counterstained with DAPI (blue). Scale bar, 500 μm . (F) Representative images showing the presence of NETs (Ly6G, red; citrullinated H3, green) in kidney tissues. Nuclei were counterstained with DAPI (blue). Scale bar, 50 μm . (G, H) Quantification of dsDNA and MPO-DNA complexes in the plasma of the mice. (I) Representative images of H&E-stained kidneys (original magnification, 400X). Scale bars: 40 μm . The black arrows indicate tubule damage. Quantification of tubular injury via H&E staining. (J, K) Quantitation of Cr and BUN in blood samples from mice in each group. (L–N) RT-qPCR analysis of NGAL, KIM-1, IL-1 β , IL-6 and TNF- α mRNA levels in the kidney. Student's t test (C) or one-way analysis of variance with Tukey's multiple comparisons test (G–N) was used for the analysis. The graphs present the means $\pm$ SEMs, $n = 6$; * $P < 0.05$, ** $P < 0.01$ compared between two groups. ns, not significant.

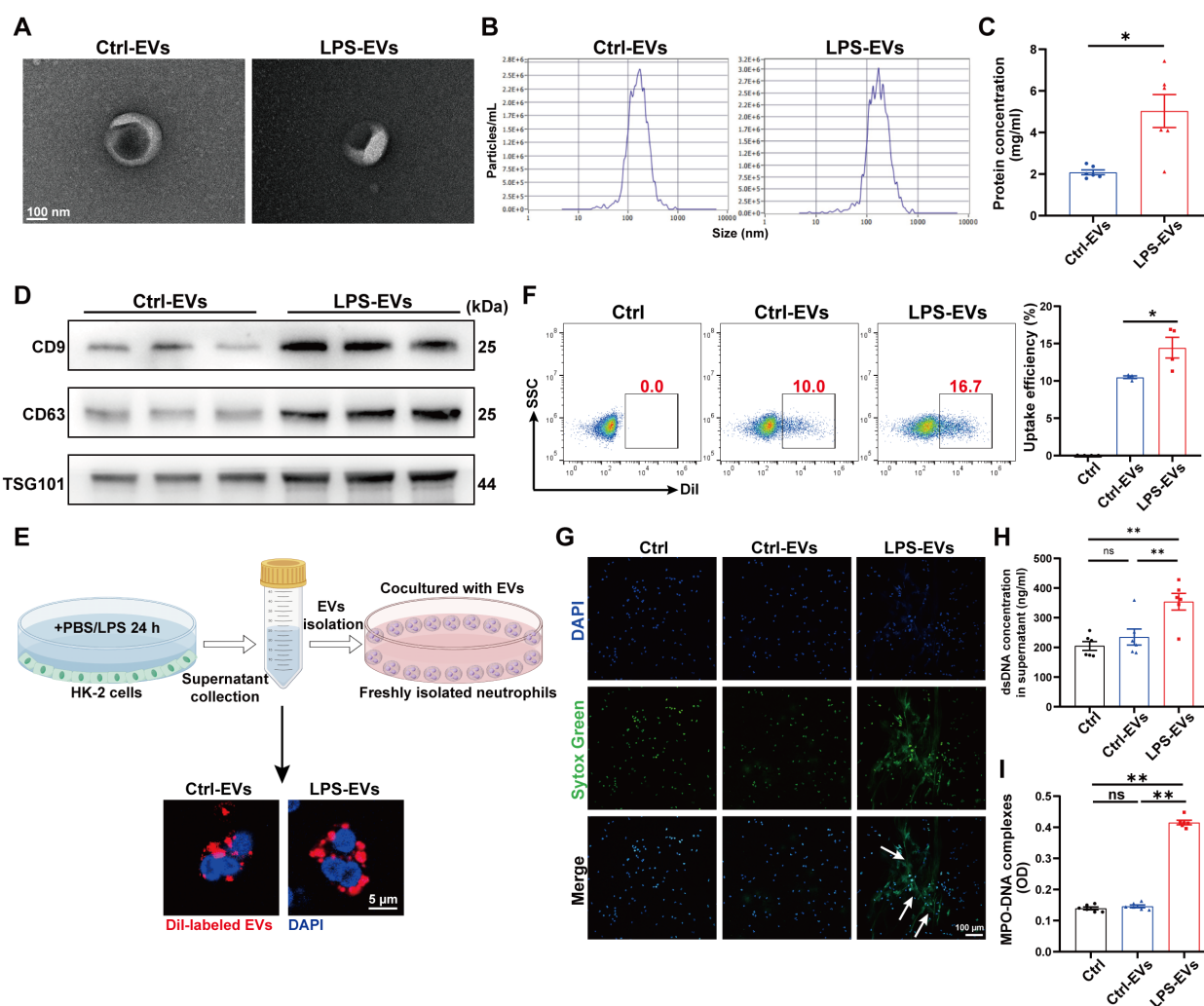


FIGURE 4

EVs secreted from LPS-stimulated TECs promoted NETs formation *in vitro*. **(A)** Electron micrograph of EVs isolated from the supernatant of PBS/LPS (1 µg/ml)-treated HK-2 cells (Ctrl-EVs/LPS-EVs). Scale bar, 100 nm. **(B)** EVs size distribution was measured via NanoSight tracking analysis. **(C)** The protein concentration of EVs was determined via a BCA protein assay kit. **(D)** CD9, CD63 and TSG101 protein expression in EVs was quantified by Western blotting with equal amounts of EVs protein (20 µg). **(E)** Immunofluorescence images showing neutrophils freshly isolated from healthy volunteers incubated with DiI-labeled EVs (red) for 3 h. Nuclei were counterstained with DAPI (blue). Scale bar, 5 µm. **(F)** Uptake efficiency of DiI-labeled EVs by neutrophils was measured by flow cytometry. SSC, side scatter. **(G)** Typical images of NETs formation following 3 h of treatment with Ctrl-EVs/LPS-EVs (100 µg/ml) were obtained via confocal microscopy via Sytox Green (green), where the white arrows indicate NETs. Scale bar, 100 µm. **(H, I)** Quantification of dsDNA and NETs components (MPO–DNA complexes) in the supernatants of cultured neutrophils via the PicoGreen assay and ELISA, respectively. Student's t test **(C)** or one-way analysis of variance with Tukey's multiple comparisons test **(F–I)** was used for the analysis. The graphs present the means ± SEMs, n = 4–6; *P < 0.05, **P < 0.01 compared between two groups. ns, not significant.

concentration was significantly increased in the supernatant of the LPS-EVs-treated group (Figures 4H, I).

Blocking EVs secretion from TECs reduced NETs formation and alleviated LPS-induced AKI

We subsequently investigated whether impaired EVs secretion from TECs could inhibit NETs formation and then alleviate LPS-induced AKI. Since the small GTPase Rab27a plays a well-established role in EVs release (23), we generated AAV9 carrying shRNAs against Rab27a and Ksp-cadherin (a unique, tissue-specific

member of the cadherin family that is exclusively expressed in renal TECs), which was used as the upstream promoter of Rab27a to specifically inhibit EVs secretion from TECs, as previously described (19). As shown in Figure 5A, C57BL/6J mice were injected via the tail vein with AAV9-Ksp-GFP-shRab27a (AAV9-shRab27a) or the control vector AAV9-Ksp-GFP-shScramble (AAV9-shNC). One week after injection, these mice were used to establish the LPS-AKI model. GFP fluorescence was clearly observed in nearly 100% of the tubular cells but was not detected in the lung tissue (Figure 5B). AAV9-shRab27a significantly decreased the Rab27a mRNA and protein levels in the kidney (Figures 5C–D), whereas AAV9-shRab27a did not affect Rab27a mRNA expression in the lung (Supplementary Figure S2A),

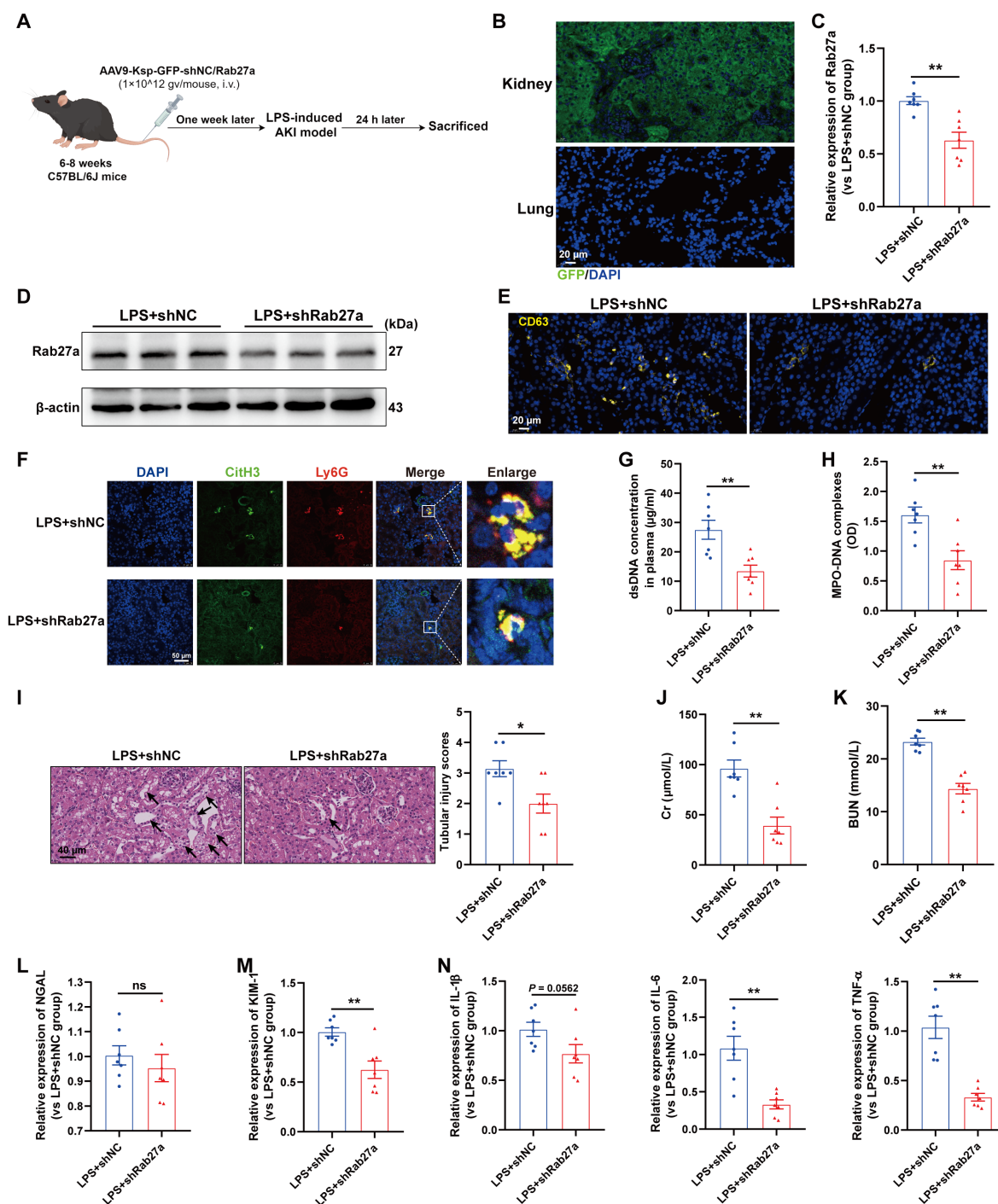


FIGURE 5

Blocking EVs secretion from TECs reduced NETs formation and alleviated LPS-induced AKI. Briefly, the mice were subjected to LPS stimulation at 7 days after AAV9-Ksp-GFP-shNC/Rab27a injection and were sacrificed at 24 h after LPS. Schematic was created with Figdraw.com. (B) Representative images of GFP fluorescence in the kidney and lung tissues of the AAV9-GFP-injected mice. Scale bar, 20 μ m. (C, D) Rab27a mRNA and protein expression in kidney tissues was determined after the mice were treated with AAV9-shNC/Rab27a. (E) Representative images of staining for the classic EVs marker CD63 in kidneys. Scale bar, 20 μ m. (F) Representative images showing the presence of NETs (Ly6G, red; citrullinated H3, green) in kidney tissues. Nuclei were counterstained with DAPI (blue). Scale bar, 50 μ m. (G, H) Quantification of dsDNA and MPO-DNA complexes in the plasma of the mice. (I) Representative images of H&E-stained kidneys (original magnification, 400 $\times$). Scale bars: 40 μ m. The black arrows indicate tubule damage. Quantification of tubular injury via H&E staining. (J, K) Quantitation of Cr and BUN in blood samples from mice in each group. (L–N) RT-qPCR analysis of NGAL, KIM-1, IL-1 β , IL-6 and TNF- α mRNA levels in the kidney. Student's t test was used for the analysis. The graphs present the means $\pm$ SEMs, $n = 7$; * $P < 0.05$, ** $P < 0.01$ compared between two groups. ns, not significant.

indicating that AAV9-shRab27a-induced Rab27 knockdown was specific to TECs. Further immunofluorescence staining revealed that the expression of the EVs marker CD63 was significantly decreased in the kidneys of the AAV9-shRab27a LPS-AKI mice (Figure 5E). In addition, a notable decrease in renal NETs formation and the plasma NETs concentration was detected in the AAV9-shRab27a LPS-AKI group compared with the control group (Figures 5F-H). Moreover, histological lesions, plasma Cr and BUN levels, kidney injury markers and inflammatory factors in kidney tissues were decreased in the AAV9-shRab27a LPS-AKI mice (Figures 5I-N). Thus, the inhibition of EVs secretion from TECs reduced NETs formation and alleviated LPS-induced AKI.

Interestingly, we also evaluated lung tissues and found that specifically blocking EVs secretion from TECs decreased NETs formation in the lung and alleviated lung injury induced by LPS (Supplementary Figures S2B-D), indicating that during sepsis, TECs-derived EVs could mediate remote organ injury.

SAA1 was enriched in EVs secreted from LPS-stimulated TECs

Next, we investigated the key molecules that mediate the effects of TECs-derived EVs on NETs formation. Given that proteins packaged in EVs are involved in regulating specific cellular functions under numerous physiological and pathological conditions, we performed 4D label-free proteomic analysis of EVs isolated from the kidney cortex of the sham and cecal ligation and puncture (CLP)-treated mice (as shown in Figure 6A). The isolated EVs were validated via transmission electron microscopy, NTA, and western blotting (Supplementary Figure S3). The top 20 changed proteins (fold change > 2 or < 0.5) are shown in Figure 6B. Among them, SAA1 was reported to induce neutrophilic airway inflammation by activating neutrophils along with NETs formation (14). SAA1 is shown in the volcano map (Figure 6C), and we further validated the enrichment of SAA1 in EVs isolated from the kidney cortex of LPS-treated mice (Supplementary Figure S4). EVs from LPS-stimulated HK-2 and TCMK-1 cells also contained more SAA1. (Figures 6D, E).

We also reanalyzed previously published scRNA-seq data of the kidney following LPS stimulation for 16 h. On the basis of the cluster-defining markers shown in previous work (24), the UMAP-based computational layout of epithelial clusters recapitulated the normal tubular segmental order of the nephron (Figure 6F). We next examined the differential gene expression between the control and LPS groups and found that all three segments (S1, S2, and S3) of the proximal tubules presented increased expression of SAA1 (Figure 6G). Violin plots and feature plots revealed that SAA1 was expressed mainly in the proximal tubule clusters of the LPS-stimulated mice (Figures 6H, I). As validated in our LPS-induced AKI model, the mRNA and protein levels of SAA1 in kidney tissue were significantly increased (Supplementary Figure S5). Double staining for SAA1 and AQP-1 (a marker for proximal renal tubular cells) further confirmed the significant upregulation of SAA1 in proximal tubules following LPS stimulation (Figure 6J).

Western blot analysis also revealed markedly increased levels of SAA1 in LPS-stimulated HK-2 and TCMK-1 cells (Figures 6K, L). These results collectively suggest that LPS increases SAA1 expression in TECs, after which SAA1 is released extracellularly through EVs.

EVs secreted from LPS-stimulated TECs promoted NETs formation and exacerbated AKI through SAA1

To validate whether SAA1 packaged in EVs is responsible for NETs induction, we transfected SAA1-siRNAs into HK-2 cells before LPS stimulation, and SAA1 expression was effectively downregulated by si-SAA1-2 (Figures 7A, B). Afterward, EVs in the supernatant were isolated, and as shown by western blot, the expression of SAA1 was significantly reduced in EVs from the si-SAA1-2-transfected HK-2 cells (Figure 7C). After coculturing these EVs with neutrophils freshly isolated from healthy volunteers, we found that NETs formation was significantly inhibited in EVs from the SAA1 knockdown cell-treated group (Figures 7D-F).

Furthermore, we examined the effects of SAA1 packaged in TECs-derived EVs *in vivo*. TCMK-1 cells were also transfected with SAA1-siRNAs before LPS stimulation, and the mRNA and protein levels of SAA1 were efficiently decreased by si-SAA1-2 (Figures 7G, H). The expression of SAA1 in EVs from the si-SAA1-2-transfected cells was also significantly reduced (Figure 7I). Following the injection of these EVs, we observed significant inhibition of NETs formation both in kidney tissue and in plasma (Figures 7J-L) and alleviation of kidney injury and inflammation in EVs from the SAA1 knockdown cell-treated group (Figures 7M-R).

SAA1 knockdown in TECs reduced NETs formation and alleviated LPS-induced AKI

Next, we investigated whether the knockdown of SAA1 in TECs could reduce NETs formation and alleviate LPS-induced AKI *in vivo*. We generated an AAV9 vector carrying shRNAs against SAA1 and Ksp-cadherin, which was also used as the upstream promoter of SAA1 to specifically reduce SAA1 levels in TECs (Figure 8A). One week after injection, GFP fluorescence was clearly observed in nearly 100% of the tubular cells but was not detected in the lung tissue (Figure 8B). AAV9-shSAA1 significantly decreased the mRNA and protein levels of SAA1 in the kidney (Figures 8C, D), whereas did not affect SAA1 mRNA expression in the lung (Supplementary Figure S6A). Double-labeling of AQP-1 and SAA1 also revealed that SAA1 expression in TECs was lower in the AAV9-shSAA1 group than in the NC group (Figure 8E). As shown in Figures 8F-H, SAA1 knockdown in TECs significantly suppressed NETs formation at 24 h after LPS administration. Kidney histological lesions, plasma Cr and BUN levels, kidney injury markers and inflammatory factors in kidney tissues were all decreased in the AAV9-shSAA1 LPS-AKI mice (Figures 8I-N). These data indicated that the inhibition of SAA1

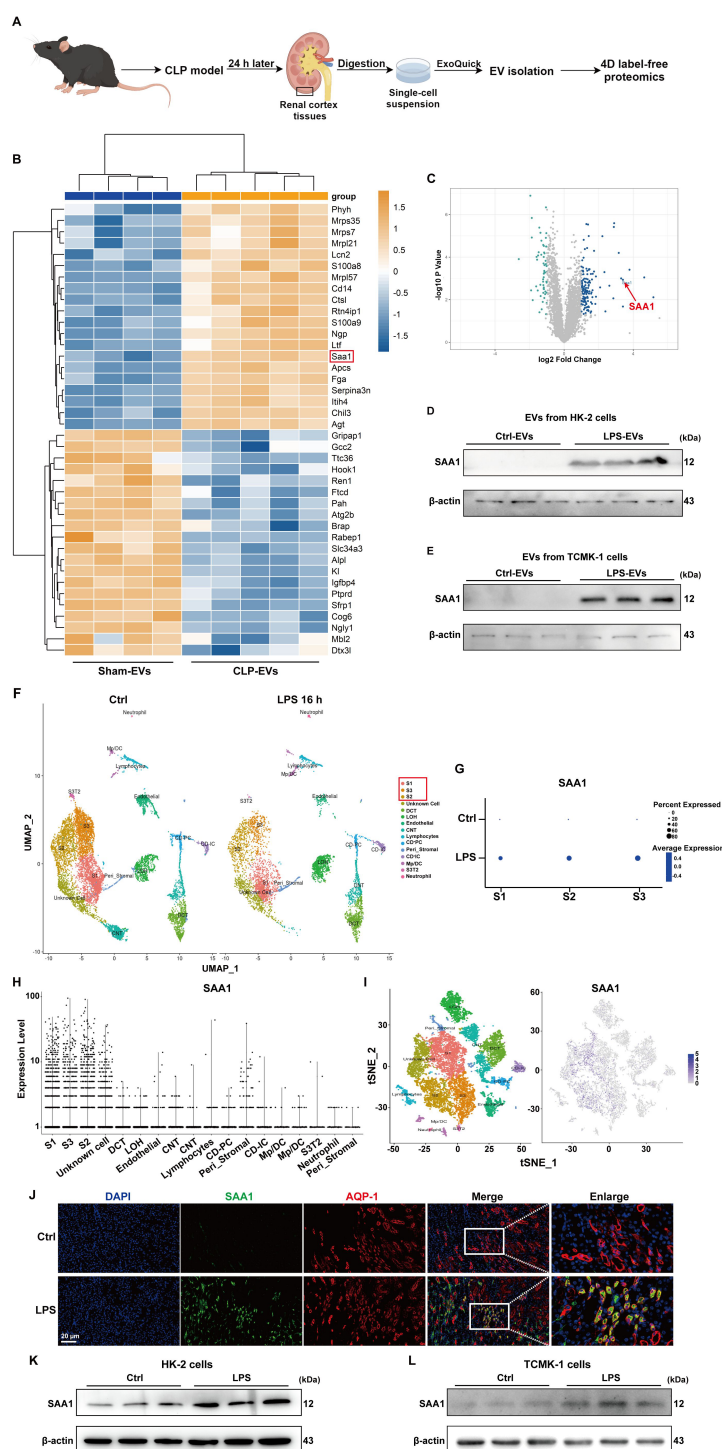


FIGURE 6

SAA1 was enriched in EVs secreted from LPS-stimulated TECs. **(A)** Schematic illustration of the experimental design. In brief, renal EVs were purified from the same weight of digested renal cortex tissues of the sham and CLP-treated mice via ExoQuick-TC reagent. Schematic was created with Figdraw.com. **(B)** Heatmap showing the top 20 proteins whose expression changed between the Sham-EVs and CLP-EVs (fold change > 2 or < 0.5). **(C)** Volcano plot displaying differentially expressed proteins between the Sham-EVs and CLP-EVs. **(D, E)** SAA1 expression in EVs isolated from the PBS/LPS-treated HK-2 and TCMK-1 cells was determined via western blotting. **(F)** UMAP plot showing clusters from the kidneys of the Ctrl and LPS groups. **(G)** Dot plot showing SAA1 expression across the three proximal tubule clusters (S1, S2, and S3) between the Ctrl and LPS groups. SAA1 gene expression in all clusters of LPS-treated kidneys was visualized via violin plots **(H)** and feature plots **(I, J)** Representative images of costaining for the proximal renal tubular cell marker AQP-1 (red) and SAA1 (green) in the kidneys of the Ctrl and LPS groups. Scale bar, 20 μ m. **(K, L)** SAA1 expression in the PBS/LPS-treated HK-2 and TCMK-1 cells was determined via western blotting. S1, first segment of proximal tubule. S2, second segment of proximal tubule. S3, third segment of proximal tubule. DCT, distal convoluted tubule. LOH, Loop of Henle. CNT, connecting tubule. CD-PC, collecting duct-principal cells. Peri_Stromal, mixed pericyte and stromal cells. CD-IC, collecting duct-intercalated cells. Mp/DC, macrophage-dendritic cells. S3T2, S3 type 2 cells.

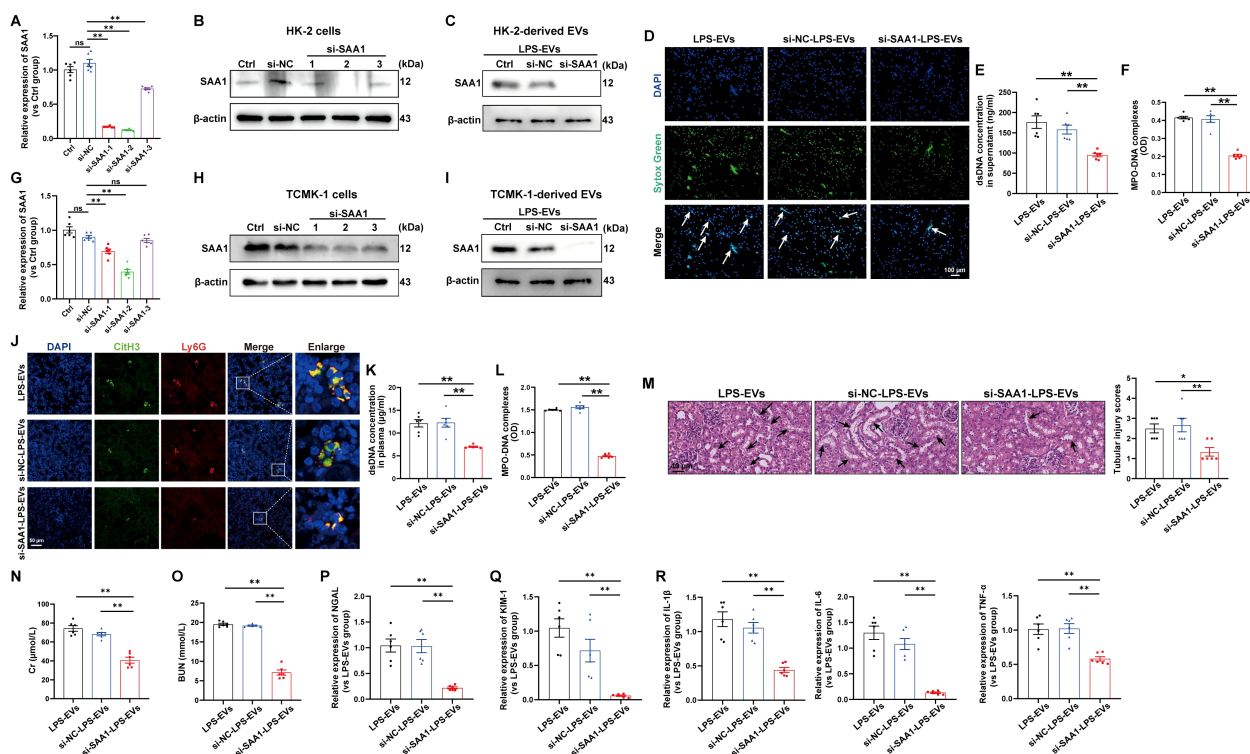


FIGURE 7

EVs secreted from LPS-stimulated TECs promoted NETs formation and exacerbated AKI through SAA1. SAA1-siRNAs were transfected into HK-2 cells before LPS stimulation. (A, B) At 24 h after LPS stimulation, SAA1 mRNA and protein expression in HK-2 cells was determined via RT-qPCR and western blotting. (C) SAA1 expression in EVs isolated from the siRNA-transfected HK-2 cells was assessed by western blotting. (D) Typical images of NETs formation were obtained via confocal microscopy via Sytox Green (green), where the white arrows indicate NETs. Scale bar, 100 μ m. (E, F) Quantification of dsDNA and NETs components (MPO-DNA complexes) in the supernatants of cultured neutrophils via the PicoGreen assay and ELISA, respectively. (G-R) SAA1 siRNAs were transfected into TCMK-1 cells before LPS stimulation. (G, H) SAA1 mRNA and protein expression in TCMK-1 cells was determined 24 h after LPS stimulation. (I) SAA1 expression in EVs isolated from the siRNA-transfected TCMK-1 cells was assessed by western blotting. (J) Representative images showing the presence of NETs (Ly6G, red; citrullinated H3, green) in kidney tissues. Scale bar, 100 μ m. (K, L) Quantification of dsDNA and MPO-DNA complexes in the plasma of the mice. (M) Representative images of H&E-stained kidneys (original magnification, 400x). Scale bar: 40 μ m. The black arrows indicate tubule damage. Quantification of tubular injury via H&E staining. (N, O) Quantitation of Cr and BUN in blood samples from the mice in each group. (P-R) RT-qPCR analysis of NGAL, KIM-1, IL-1 β , IL-6 and TNF- α mRNA levels in the kidney. One-way analysis of variance with Tukey's multiple comparisons test was used for the analysis. The graphs present the means $\pm$ SEMs, $n = 6$; * $P < 0.05$, ** $P < 0.01$ compared between two groups. ns, not significant.

upregulation in TECs reduced NETs formation and alleviated LPS-induced AKI.

In addition, SAA1 knockdown in TECs decreased NETs formation in the lung and alleviated lung injury induced by LPS (Supplementary Figures S6B-D), indicating that, during sepsis, SAA1 released by TECs can mediate remote organ injury.

SAA1 enriched in TECs-derived EVs promoted NETs formation via the TLR4/MAPK signaling pathway

To examine the signaling pathways that mediate NETs formation via SAA1 packaged in TECs-derived EVs, we reanalyzed the scRNA-seq data of the kidney as mentioned above (Figure 6F). Enrichment analysis of differentially expressed genes in neutrophils revealed significant enrichment of the MAPK signaling pathway (Figure 9A), indicating that LPS activates MAPK signaling pathways in neutrophils, which is consistent with a previously

published study showing that activation of the MAPK pathway is required for NETs formation (25). Using western blot analysis, we also demonstrated that the phosphorylation levels of p38, JNK and ERK were increased following LPS-EVs treatment compared with that in the control group (Figure 9B), whereas EVs from SAA1 knockdown cells failed to increase p38, JNK and ERK phosphorylation compared with that in the NC group (Figure 9C). SB203580, a specific small molecule inhibitor of p38 MAPK phosphorylation (26), was sufficient to suppress p38 MAPK phosphorylation (Figure 9D) and NETs formation induced by LPS-EVs (Figures 9E-G).

Formyl peptide receptor 2 (FPR2) and Toll-like receptors (TLR2, 4) have been reported to be the receptors for SAA1 (27). The effects of LPS-EVs on NETs formation were significantly diminished after the cells were treated with the TLR4 antagonist TAK-242, while not the TLR2 antagonist C29 or the FPR2 inhibitor WRW4 (Figures 9H, I). Further, TLR4 antagonist also reversed the activation of MAPK pathway induced by LPS-EVs (Figure 9J). Taken together, our findings confirmed that SAA1 enriched in

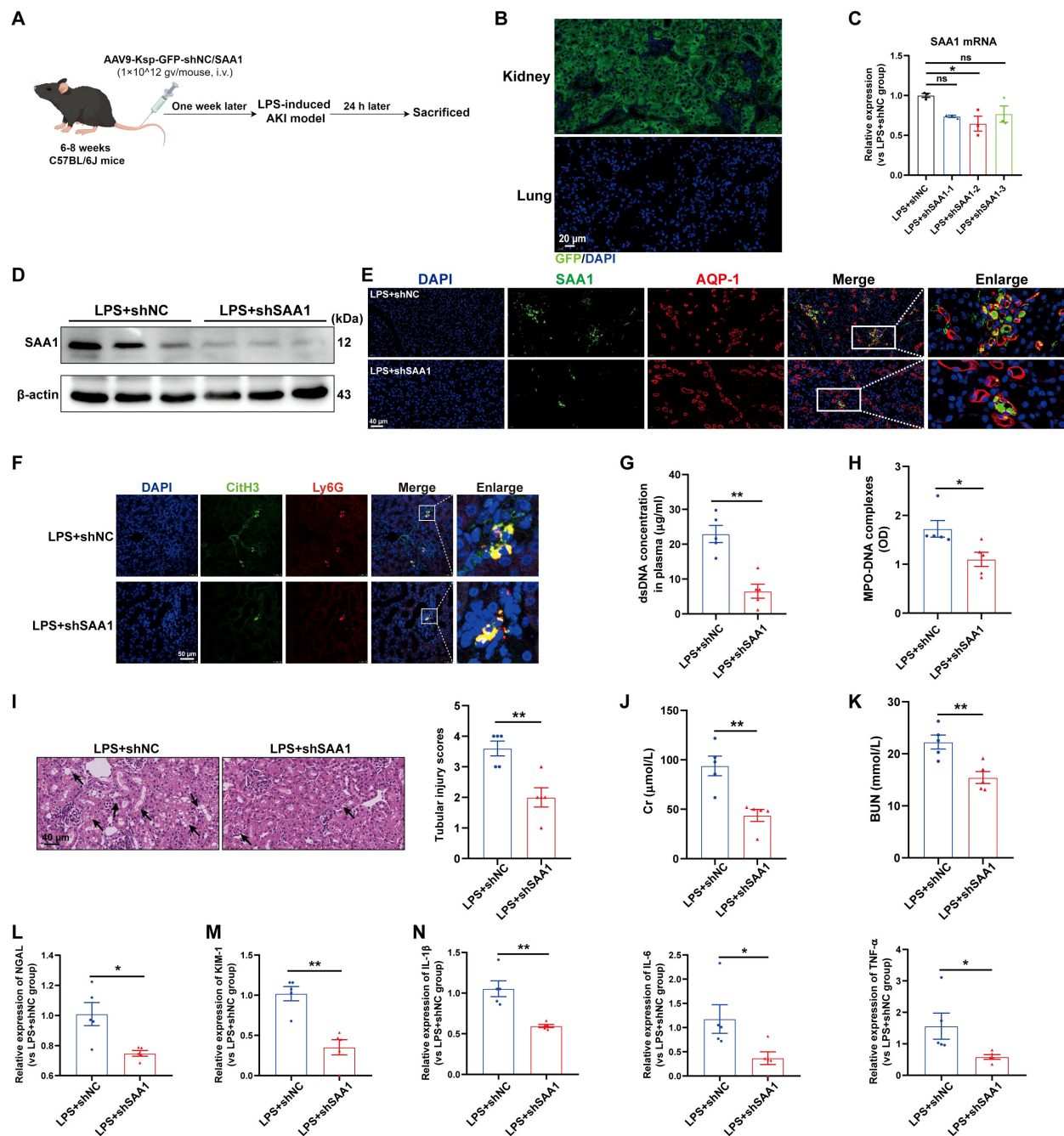


FIGURE 8

Knockdown of SAA1 in TECs reduced NETs formation and alleviated LPS-induced AKI. **(A)** Schematic diagram of the experimental design. Briefly, the mice were subjected to LPS stimulation at 7 days after AAV9-Ksp-GFP-shNC/SAA1 injection and were sacrificed at 24 h after LPS. Schematic was created with Figdraw.com. **(B)** Representative images of GFP fluorescence in the kidney and lung tissues of the AAV9-GFP-injected mice. Scale bar, 20 μ m. **(C, D)** SAA1 mRNA and protein expression in kidney tissues was determined after the mice were treated with AAV9-shNC/SAA1. **(E)** Representative images of costaining for AQP-1 (red) and SAA1 (green) in the kidneys of the AAV9-shNC/SAA1-injected mice. Scale bar, 40 μ m. **(F)** Representative images showing the presence of NETs (Ly6G, red; citrullinated H3, green) in kidney tissues. Nuclei were counterstained with DAPI (blue). Scale bar, 50 μ m. **(G, H)** Quantification of dsDNA and MPO-DNA complexes in the plasma of the mice. **(I)** Representative images of H&E-stained kidneys (original magnification, 400 $\times$). Scale bars: 40 μ m. The black arrows indicate tubule damage. Quantification of tubular injury via H&E staining. **(J, K)** Quantification of Cr and BUN in blood samples from mice in each group. **(L–N)** RT-qPCR analysis of NGAL, KIM-1, IL-1 β , IL-6 and TNF- α mRNA levels in the kidney. One-way analysis of variance with Tukey's multiple comparisons test **(C)** or Student's *t* test **(G–N)** was used for the analysis. The graphs represent the means $\pm$ SEMs, *n* = 5; * P < 0.05, ** P < 0.01 compared between two groups. ns, not significant.

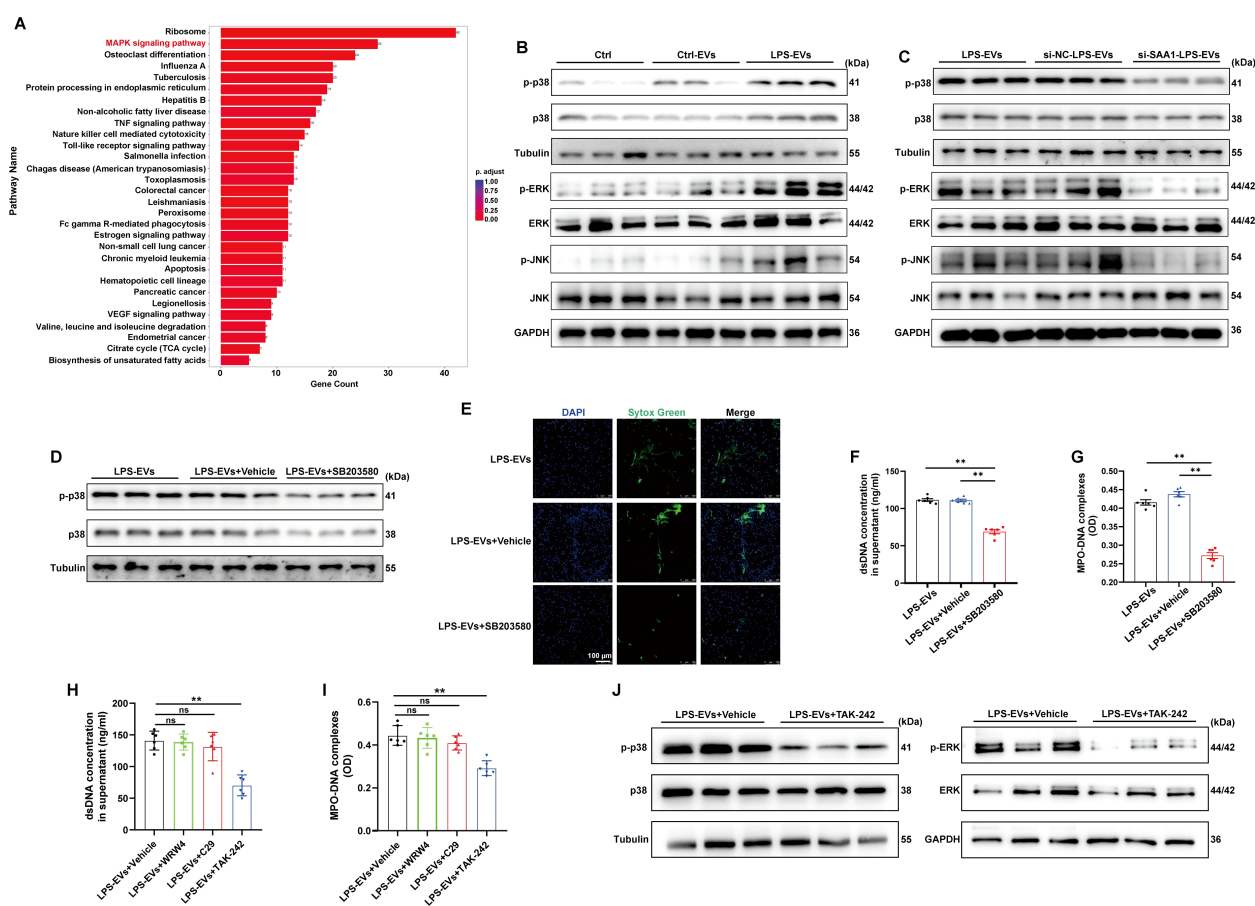


FIGURE 9

SAA1 enriched in TECs-derived EVs promoted NETs formation via the TLR4/MAPK signaling pathway. **(A)** Enrichment analysis of differentially expressed genes in neutrophils in the scRNA-seq data from the kidneys of the PBS/LPS-treated mice. **(B–D)** Representative western blot of MAPK pathway in neutrophils treated with PBS, Ctrl-EVs, LPS-EVs, and LPS-EVs from SAA1 knockdown HK-2 cells and LPS-EVs + SB203580 (a specific small molecule inhibitor of p38 MAPK phosphorylation, 30 μ M). **(E)** Typical images of NETs formation were obtained via confocal microscopy via Sytox Green (green). Scale bar, 100 μ m. **(F, G)** Quantification of dsDNA and NETs components (MPO–DNA complexes) in the supernatants of cultured neutrophils via the PicoGreen assay and ELISA, respectively. **(H–J)** WRW4 (10 μ M), C29 (50 μ M) or TAK-242 (50 μ M) were added to the culture medium about 30 min before co-cultured with LPS-EVs. **(H, I)** Quantification of dsDNA and NETs components (MPO–DNA complexes) in the supernatants of cultured neutrophils via the PicoGreen assay and ELISA, respectively. **(J)** Representative western blot of MAPK pathway in neutrophils. One-way analysis of variance with Tukey's multiple comparisons test was used for the analysis. The graphs present the means $\pm$ SEMs, $n \geq 3$; ** $P < 0.01$ compared between two groups. ns means "ns, not significant"

TECs-derived EVs promoted NETs formation via the TLR4/MAPK signaling pathway.

Plasma TECs-derived EVs proportion and SAA1 level in plasma EVs were positively correlated with plasma NETs levels and poor outcomes in septic patients

We isolated and purified plasma EVs from 21 healthy volunteers and 26 patients with sepsis. The septic patients were divided into two groups according to the occurrence of AKI (13 vs. 13). The total protein concentration of EVs among the three groups had no significant difference (Figure 10A). The proportion of EVs derived from TECs, identified by KIM-1, and the level of SAA1 in plasma EVs were higher in septic patients with AKI than those in

healthy volunteers and septic patients without AKI as assessed by flow cytometry and ELISA respectively (Figures 10B, C). The proportion of TECs-derived EVs was positively related to the level of SAA1 in plasma EVs (Figure 10D). Correlation analysis revealed that plasma TECs-derived EVs proportion and SAA1 expression were positively correlated with the Scr level and the SOFA score in septic patients (Figures 10E–H). Plasma TECs-derived EVs proportion and SAA1 expression were also higher in the non-survivors among septic patients (Figures 10I, J). Furthermore, the plasma NETs concentration tended to increase in septic patients with AKI (Figure 10K) and was positively correlated with increased plasma TECs-derived EVs proportion and SAA1 expression in plasma EVs (Figures 10L, M). Additionally, we found that plasma TECs-derived EVs and SAA1 expression in plasma EVs were negative related to oxygenation index of septic patients (Supplementary Figure S7).

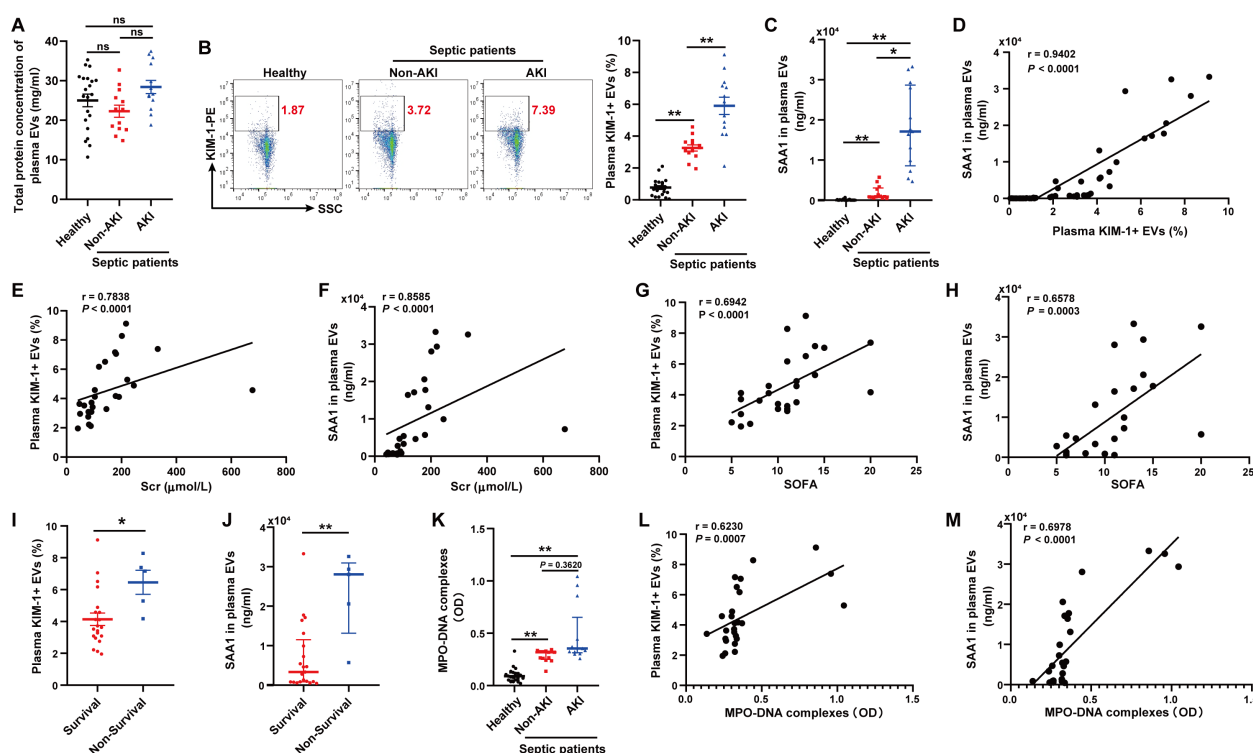


FIGURE 10

Plasma TECs-derived EVs proportion and SAA1 level in plasma EVs were positively correlated with plasma NETs levels and poor outcomes in septic patients. (A) Total protein concentration of EVs from equal plasma volumes of healthy controls ($n = 21$) and septic patients with or without AKI ($n = 26$). (B) Proportion of TECs-derived EVs identified in the plasma of healthy controls and septic patients with or without AKI as assessed by flow cytometry after gating by CD63-APC. (C) SAA1 concentration in EVs from equal plasma volumes as assessed via ELISAs. (D) Correlation of the SAA1 concentration in plasma EVs with the proportion of plasma TECs-derived EVs. (E–H) Association of the plasma TECs-derived EVs proportion or SAA1 concentration in plasma EVs with the Scr level and the SOFA score of septic patients. (I, J) The comparison of the plasma TECs-derived EVs proportion and SAA1 concentration in plasma EVs between the survival and non-survival septic patients. (K) Quantification of MPO-DNA complexes in the plasma of healthy controls and septic patients with or without AKI. (L, M) Correlation of the plasma TECs-derived EVs proportion and SAA1 concentration in plasma EVs with MPO-DNA complexes in plasma. Statistics: One-way analysis of variance with Tukey's multiple comparisons test in (A, B); the Kruskal–Wallis test followed by Dunn's *post hoc* test in (C, K); the Student's *t* test in (I); the Mann–Whitney *U* test in (J); and the Spearman order correlation analysis in (D–H, L, M). The data are presented as the means $\pm$ SEMs (A, B, I) or medians (25th–75th percentiles) (C, J, K). * $P < 0.05$, ** $P < 0.01$ compared between two groups. ns, not significant.

Discussion

In this study, we firstly demonstrated that EVs secreted from LPS-stimulated TECs aggravated AKI through promoting NETs formation. Combined proteomics and scRNA-seq analysis, we found that LPS increased SAA1 expression in TECs and then released extracellularly through EVs. Further mechanistic study showed that SAA1 packaged in TECs-derived EVs was responsible for NETs formation and AKI via activation of TLR4/MAPK signaling pathway in neutrophils (Figure 11). Specifically blocking EVs secretion from TECs or inhibiting SAA1 upregulation in TECs by AAV9s reduced NETs formation and alleviated LPS-induced AKI and remote lung injury. More importantly, we propose that the proportion of plasma TECs-derived EVs and SAA1 expression levels in EVs may serve as promising prognostic biomarkers for SA-AKI patients.

Neutrophils can expulse NETs to trap and kill pathogens, while excessive NETs formation can directly induce cytotoxic effects on tissues which, in turn, propagate inflammation and cause organ injury (28). Previous study has demonstrated that NETs induced

tubular epithelial cell death in ischemia-reperfusion injury in the mouse kidney, and pretreatment with inhibitors of NETs formation reduced kidney injury (29). For SA-AKI, Ni et al., demonstrated that NETs are enriched in murine AKI of three distinct sepsis procedures, including CLP, LPS-induced endotoxemia and multidrug-resistant sepsis, and either pharmacological or genetic NETs interruption in combination with Fn14 blockade prolong mice survival and provide renal protection against sepsis (5). Consistent with their study, our study also showed that NETs formation was significantly enhanced in LPS-induced AKI, and NETs degrader DNase I could efficiently ameliorate kidney inflammation and tissue injury. Our study highlighted the significance of overzealous NETs formation in exacerbating sepsis and NETs can be an immune checkpoint for SA-AKI.

Increasing evidence has demonstrated that TECs-derived EVs play fundamental roles in the pathogenesis of kidney disease. Previous studies in both AKI and chronic kidney disease experimental models have shown that EVs-mediated TECs-neighbor cell crosstalk can cause the progression of tubulointerstitial inflammation and fibrosis (8, 10, 30). Here, we

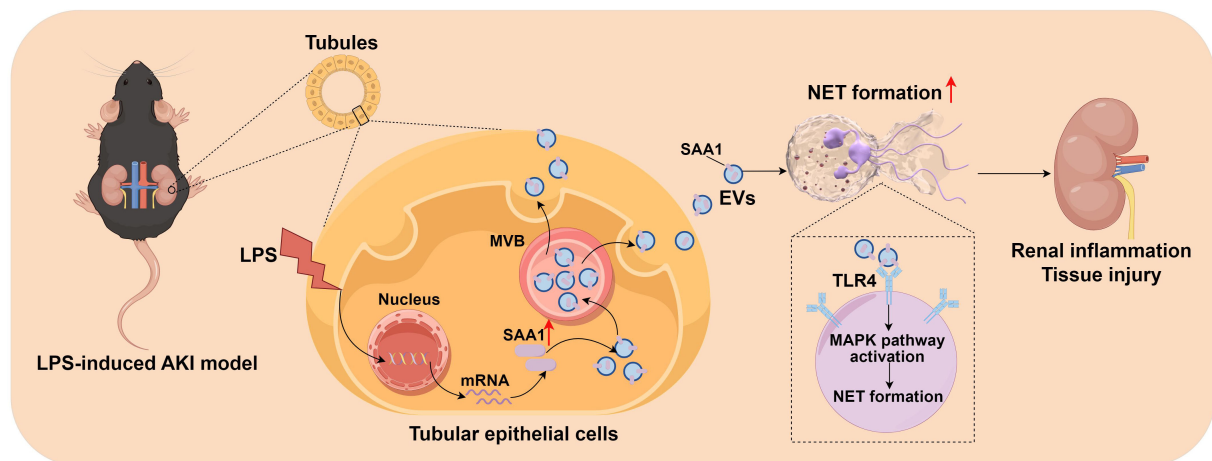


FIGURE 11

Schematic depiction of SAA1 delivered by tubular epithelial cell-derived extracellular vesicles exacerbates sepsis-associated acute kidney injury by promoting NETs formation. The graph was drawn on the Figdraw online website (Export ID: TIOORe3519).

demonstrated that TECs exposed to LPS during SA-AKI communicated with neutrophils via EVs and led to NETs formation and kidney injury. Rab27a is known to be involved in the regulation of EVs secretion (31). As expected, the inhibition of EVs release from TECs via silence of Rab27a in TECs mitigated the LPS-induced NETs formation and AKI. These findings suggest a new role for TECs-derived EVs in aggravating SA-AKI by activating neutrophils.

Proteins can be sorted into EVs and selectively induce specific signals in recipient cells to modulate numerous processes (32). Our *in vitro* and *in vivo* experiments using siRNAs and AAVs revealed that SAA1-packaged in EVs released by LPS-stimulated TECs augmented the proinflammatory response during SA-AKI by inducing NETs formation. Aside by been regarded as a prognostic indicator for inflammatory diseases, SAA1 obtained multiple biological activities, including functioning as a pro-inflammatory molecular by attracting immune cells to the inflammation sites (33), and playing a promoting tumor progression role in liver and bladder cancers (34, 35). Those studies demonstrated that SAA1 mainly functions through unbound form to direct contact between cells, while our findings introduce a novel mode of action for SAA1 to be transported through EVs. A recent study also revealed that SAA stimulates renal dysfunction through promoting the IFN- γ -iNOS-p38 MAPK axis (36), which was consistent with our study that SAA1 enriched in TECs-derived EVs promoted NETs formation via MAPK signaling pathway.

Interestingly, we demonstrated that AAV9-mediated blockade of TECs-EVs secretion or SAA1 upregulation attenuates LPS-induced lung injury. This suggests sepsis-injured TECs release SAA1-carrying EVs that disseminate via circulation, delivering pathogenic SAA1 to distant recipient cells. Our findings align with emerging consensus that EVs-facilitated interorgan communication contributes to septic kidney-lung axis derangement, offering insights into unified pathways of organ failure (37).

Recent studies have demonstrated a significant correlation between plasma EVs concentrations and the severity of organ

dysfunction, as well as the prognostic mortality in critically ill septic patients (38). Consistently, we confirmed a significant increase of plasma TECs-derived EVs proportion and SAA1 concentration in plasma EVs, and positively correlated with poor prognosis and severity in septic patients. We further speculated that plasma TECs-derived EVs proportion and SAA1 concentration in plasma EVs may be attractive biomarkers for SA-AKI.

Our study had several limitations. First, TECs-derived EVs were isolated from the supernatant of *in vitro* culturing cells to evaluate their effects on NETs formation and AKI both *in vitro* and *in vivo*, not from the kidney cortex of septic mice using immunomagnetic beads or flow cytometry, due to their extreme fragility during the sorting process. Second, we only evaluated the effects of blocking EVs secretion from TECs or inhibiting SAA1 upregulation in TECs on AKI and remote lung injury following LPS challenge, while the other organs could also be influenced by TECs-derived EVs and thus need to be further addressed. Lastly, our clinical sample sizes are relatively small, larger clinical samples are needed to further validate the prognostic value of plasma TECs-derived EVs and SAA1 in plasma EVs for SA-AKI.

Conclusion

This study revealed that TECs-derived EVs containing SAA1 exacerbated SA-AKI by promoting NETs formation through activation of the TLR4/MAPK pathway. TECs-derived EVs are also likely to play a role in kidney-lung crosstalk during sepsis. Additionally, plasma TECs-derived EVs proportion and SAA1 concentration in plasma EVs may be promising biomarkers for SA-AKI patients. These findings may add a new element to TECs-neutrophil crosstalk, and strategies to modify TECs-derived EVs and the cargo SAA1 could be a new avenue for developing therapeutics against SA-AKI.

Data availability statement

The proteomic data have been deposited to the ProteomeXchange Consortium via the iProX repository with the dataset identifier PXD055037. Any remaining information can be obtained from the corresponding author upon reasonable request.

Ethics statement

The studies involving humans were approved by Institutional ethics and review board of Nanjing Drum Tower Hospital. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. The animal study was approved by Ethics Committee of Nanjing First Hospital. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

YJ: Investigation, Funding acquisition, Data curation, Formal analysis, Methodology, Writing – original draft. ML: Validation, Methodology, Investigation, Writing – original draft, Formal analysis, Data curation. XX: Validation, Methodology, Data curation, Formal analysis, Writing – original draft, Investigation. MP: Writing – original draft, Validation, Investigation. LZ: Writing – original draft. WZ: Methodology, Writing – original draft, Resources. JS: Funding acquisition, Resources, Writing – original draft, Supervision. TZ: Funding acquisition, Writing – review & editing, Conceptualization, Supervision. ZM: Conceptualization, Writing – review & editing, Funding acquisition, Methodology. XG: Conceptualization, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Establishment and validation of the prediction model based on lymphocyte subsets for acute kidney injury in sepsis patients

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Purpose: This study aimed to construct a risk predictive model for acute kidney injury in sepsis based on peripheral blood lymphocyte subsets.

Methods: This prospective study included patients with sepsis admitted to the ICU from March to August 2024 (483 for training and 146 for validation), and 125 patients from September to December 2024 as the external test cohort. Clinical data and peripheral blood samples on days 1 and 3 were collected after ICU admission. Lymphocyte subsets were analyzed using flow cytometry, covering T cell, B cell, NK cell populations. Differences in clinical variables and lymphocyte subsets between AKI and non-AKI groups were analyzed. A predictive model was developed using LASSO and multivariate logistic regression and validated internally (5-fold cross-validation) and externally. Model performance was assessed using ROC curves, calibration plots, and decision curve analysis (DCA). A nomogram was constructed for clinical applications.

Results: Among the 483 patients, the incidence of AKI was 54.66%. Compared to non-AKI patients, the AKI group had significantly higher SOFA and APACHE II scores and lower GCS scores. Laboratory findings showed higher neutrophil and monocyte counts, and elevated serum creatinine in the AKI group. On day 1, several lymphocyte subsets were significantly altered in the AKI group, including increased CD4⁺CD38⁺T%, CD8⁺CD38⁺T%, CD155⁺T%, CD4⁺TeM⁺T%, CD8⁺TIGIT⁺T%, and M-MDSC, and decreased CD4⁺LAG3⁺T%, CD4⁺TN⁺T%, and Th17 cells. On day 3, AKI patients exhibited further distinct changes in NK cells and T cell activation/exhaustion markers. A predictive model incorporating key clinical (APACHE II and creatinine) and lymphocyte subsets (CD15⁺T%_{1st}, CD4⁺LAG3⁺T%_{1st}, Th17_{1st}, CD8⁺PD1⁺T%_{3rd}, CD8⁺TIGIT⁺T%_{3rd}, E_MDSC_{3rd}, CD8⁺CCR7⁺CD45RA⁺T%_{3rd}, CD4⁺CTLA4⁺T%_{3rd}, CD4⁺TIM3⁺T%_{3rd}, PMN_MDSC_{3rd}, and M_MDSC_{3rd}) achieved high accuracy, with an AUC of 0.989 in the training set, 0.895 in the validation set, and 0.906 in the test set. Calibration curves and DCA confirmed the model's reliability and clinical utility.

Conclusion: Peripheral blood lymphocyte subsets are significantly altered in patients who develop SA-AKI and can serve as potential early biomarkers. The developed predictive model based on clinical and immunological parameters demonstrated robust performance in identifying patients at high risk of SA-AKI, offering a practical tool for early warning and clinical decision-making.

KEYWORDS

acute kidney injury, sepsis, lymphocyte subsets, prediction model, nomogram

Introduction

Sepsis is a life-threatening condition characterized by organ dysfunction resulting from a dysregulated host response to infection, and it remains a leading cause of mortality in intensive care units (ICUs) (1). According to the Global Burden of Disease Study, approximately 49 million cases of sepsis occur globally each year, with an estimated 11 million sepsis-related deaths. Among critically ill patients, the mortality rate associated with severe sepsis can reach as high as 30–50% (2). Due to its high incidence and mortality, sepsis imposes a substantial economic burden on patients, families, and healthcare systems.

Sepsis is not merely a systemic inflammatory or immune dysregulation process; it is frequently accompanied by multi-organ dysfunction. Among its complications, acute kidney injury (AKI) is one of the most common and severe. When AKI occurs as a consequence of sepsis, it is referred to as sepsis-associated acute kidney injury (SA-AKI). SA-AKI is defined by a rapid decline in renal function, leading to the accumulation of nitrogenous waste products and disturbances in electrolyte and acid-base homeostasis (3). Compared to AKI resulting from other causes, SA-AKI is associated with significantly prolonged hospital stays and increased in-hospital mortality (4). Notably, the mortality rate in septic patients with concurrent AKI may be as high as 70% (5). Despite advances in supportive care and diagnostic technologies, the prognosis of SA-AKI remains poor, and its diagnosis still relies on conventional indicators such as changes in serum creatinine and urine output. These markers lack sensitivity and specificity for detecting early pathophysiological changes (6). The underlying mechanisms of SA-AKI are complex and heterogeneous, involving inflammation, complement activation, dysregulation of the renin–angiotensin–aldosterone system (RAAS), mitochondrial dysfunction, microcirculatory disturbances, and metabolic reprogramming (7). Over recent decades, various biomarkers have been explored for the early prediction of AKI, including neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule-1 (KIM-1), tissue inhibitor of metalloproteinase-2 (TIMP-2), and insulin-like growth factor-binding protein 7 (IGFBP7), and C-reactive protein-albumin-lymphocyte index (8–11). However, many of these biomarkers are limited in clinical utility due to high cost, technical complexity, and lack of accessibility. Therefore, there is

an urgent need for simple, reliable, and widely available biomarkers for the early identification and risk stratification of SA-AKI.

Lymphocyte subsets, a group of white blood cells, are essential components of the immune system and play pivotal roles in immune regulation. During immune responses, lymphocytes differentiate into functional subtypes, including T lymphocytes, B lymphocytes, and natural killer (NK) cells (12). Flow cytometry is routinely used in clinical practice to assess immune status by quantifying these subsets. T cells are primarily responsible for eliminating infected and malignant cells; B cells generate antibodies for antigen-specific responses; and NK cells target virus-infected cells by recognizing stress ligands (12). Alterations in lymphocyte subset counts have been associated with the severity and prognosis of a variety of diseases, including sepsis (13–15). Accumulating evidence suggests that the onset and progression of sepsis are closely tied to immune dysfunction, particularly immunosuppression mediated by decreased T cell reactivity (16, 17). Sepsis-induced immune dysregulation, often underpinned by systemic inflammatory response syndrome (SIRS), is characterized by widespread lymphocyte apoptosis and functional exhaustion. T cell apoptosis contributes significantly to immune paralysis and T cell clonal anergy (18, 19). Among T cell subsets, CD3⁺ T cells reflect overall cellular immunity, CD4⁺ T cells (helper T cells) assist in coordinating the immune response, and CD8⁺ T cells (cytotoxic/suppressor T cells) are involved in immune inhibition (20). Thus, alterations in the absolute counts and ratios of CD3⁺, CD4⁺, CD8⁺ T cells, and the CD4⁺/CD8⁺ ratio can reflect changes in cellular immune status (21). Recent clinical studies have reported that septic patients with AKI exhibit distinct alterations in CD4⁺ T cell subsets (22), and that sepsis-induced AKI is often accompanied by T lymphopenia (23). Animal studies have further shown that peripheral T cell apoptosis is mechanistically linked to the development of SA-AKI, providing a theoretical foundation for further investigation (24). Given the clinical availability and simplicity of lymphocyte subset testing, these parameters represent a promising avenue for non-invasive, early detection of SA-AKI. However, their predictive value remains underexplored in this context.

In this study, we performed a comprehensive analysis of peripheral blood lymphocyte subsets including T cells, B cells, and NK cells in patients with sepsis, using flow cytometry. By

integrating lymphocyte profiles with clinical features, we constructed a predictive model for SA-AKI occurrence. The model underwent both internal and external validation, culminating in an individualized risk assessment tool for SA-AKI. This work aims to provide a practical framework for early warning, improved clinical decision-making, and optimized management of patients with sepsis-associated acute kidney injury.

Material and methods

Study population

This study was designed as a prospective investigation. Baseline data and blood samples were collected from 767 sepsis patients admitted to the intensive care unit (ICU) of our hospital between March 7, 2024, and August 30, 2024. To reduce the potential impact of confounding factors, predefined inclusion and exclusion criteria were applied to identify eligible participants. Sepsis was diagnosed according to the Sepsis-3 criteria (25). The inclusion criteria were as follows: (1) Diagnosis of sepsis based on Sepsis-3, defined as an increase of ≥ 2 points in the Sequential Organ Failure Assessment (SOFA) score from baseline in patients with confirmed or suspected infection; (2) Age >18 years.

(3) ICU stays longer than 24 hours. The exclusion criteria were: (1) Age <18 years; (2) ICU stay <24 hours or development of AKI within 36 hours of admission; (3) History of kidney disease, dialysis, cancer, hematologic or immune system disorders, or severe liver dysfunction; (4) Presence of AKI before ICU admission; (5) Missing baseline data or loss to follow-up, or missing $>10\%$ of laboratory data. Based on these inclusion and exclusion criteria, 629 patients with sepsis were assigned to the training set ($n=483$) and validation set ($n=146$). For binary logistic regression, the empirical rule of thumb requires the sample size (N) to be 10–20 times the number of independent variables (k) $N \geq 10-20 \times k$. In this study, 23 independent variables were included in the initial screening ($k = 23$). Therefore, the minimum required sample size was estimated as: $N_{\min} = 10 \times 23 \times (1 + 10\%) = 253$, where 10% was added to account for potential loss to follow-up. Finally, 629 participants were included in the analysis, which substantially exceeds the minimum requirement, ensuring adequate statistical power. An external test cohort consisting of 125 additional sepsis patients from the same institution was identified between September 1, 2024, and December 31, 2024. The follow-up period for all patients was one month. Figure 1 presented the study process.

Data collection and definitions

The data collection included clinical information and blood samples. The clinical information consisted of age (year), gender (male vs female), body mass index ($BMI = \text{weight}(\text{kg})/\text{height}(\text{m})^2$), SOFA score, acute physiology and chronic health evaluation II (APACHEII), and Glasgow Coma scale (GCS). The history of diseases: chronic obstructive pulmonary disease (COPD),

hypertension (Systolic blood pressure ≥ 140 mm Hg and/or diastolic blood pressure ≥ 90 mm Hg), diabetes (Fasting plasma glucose ≥ 7.0 mmol/L or Oral glucose tolerance test ≥ 11.1 mmol/L), coronary heart disease (CHD), primary infection, septic shock, (Yes vs No), continuous renal replacement therapy (CRRT) and vasoactive drugs usage. The blood samples were collected on the first day and the third day after admission. The biochemical parameters were analyzed, including white blood cells (WBC, 10^9), hemoglobin (g/L), platelet (10^9), neutrophil (10^9), lymphocyte (10^9), monocyte (10^9), C-reactive protein (mg/L), procalcitonin (ng/L), total bilirubin ($\mu\text{mol/L}$), albumin (g/L), globulin (g/L), blood urea nitrogen (BUN, mmol/L), creatinine ($\mu\text{mol/L}$), and lactate (mmol/L).

The peripheral blood lymphocyte subsets of patients with sepsis on the first and third days were detected by flow cytometry. To comprehensively evaluate the role of lymphocyte subgroups in the development of AKI among patients with sepsis, we employed established detection methods to systematically measure the relevant indicators within each lymphocyte subset. The lymphocyte subsets included T cell subsets (CD3+T%, CD4+T%, CD8+T%, CD4+CD8+T%, CD4-CD8-T%, CD3+T count, CD4+T count, CD8+T count, CD4/CD8, CD4+CD8+T count, CD4-CD8-T count, CD4+CD28+T%, CD4+CD38+T%, CD4+CD69+T%, CD8+CD28+T%, CD8+CD38+T%, CD8+CD69+T%, CD155+T%, CD4+BTLA+T%, CD4+CTLA4+T%, CD4+HLADR+T%, CD4+LAG3+T%, CD4+PD1+T%, CD4+TIGIT+T%, CD4+TIM3+T%, CD4+TcM+T%, CD4+TeM+T%, CD4+TeMRA+T%, CD4+TN+T%, CD8+BTLA+T%, CD8+CTLA4+T%, CD8+HLADR+T%, CD8+LAG3+T%, CD8+PD1+T%, CD8+TIGIT+T%, CD8+TIM3+T%, CD8+TcM+T%, CD8+TeM+T%, CD8+TeMRA+T%, CD8+TN+T%, MDSC, PMN_MDSC, M_MDSC, e_MDSC, Th1, Th2, Th17, Treg, CD4+CD45RA+T%, CD4+CD45RO+T%, CD8+CD45RA+T%, CD8+CD45RO+T%, CD4+CCR7+CD45+T%, CD4+CCR7+CD45-T%, CD4+CCR7-CD45+T%, CD4+CCR7-CD45-T%, CD8+CCR7+CD45+T%, CD8+CCR7+CD45-T%, CD8+CCR7-CD45+T%, and CD8+CCR7-CD45-T%), B cells subsets (CD19+B count), lymphocyte count, NK cells subsets (NKT count, CD16+CD56+NK%, CD16+CD56+NK count, NKT%), and B cells subsets (CD19+B%), and Neutrophil CD64 index (nCD64).

According to the diagnostic criteria of Kidney Disease Improving Global Outcomes (KDIGO) (26), the acute kidney injury (AKI) is defined as follows: Serum creatinine increase of ≥ 0.3 mg/dL (within 48 hours) and/or $\geq 50\%$ increase from baseline creatinine (within 7 days).

Statistical analysis

All data analyses were performed using R version 4.4.3. The online tool Sangerbox 2 was also used to plot the forest plot (27). For continuous variables with a normal distribution (age, BMI, SOFA, APACHEII, GCS, WBC, hemoglobin, platelet, neutrophil, lymphocyte, monocyte, C-reactive protein, procalcitonin, total bilirubin, albumin, globulin, BUN, creatinine, and lactate), data are presented as mean $\pm$ standard deviation, and comparisons between two groups were conducted using the independent

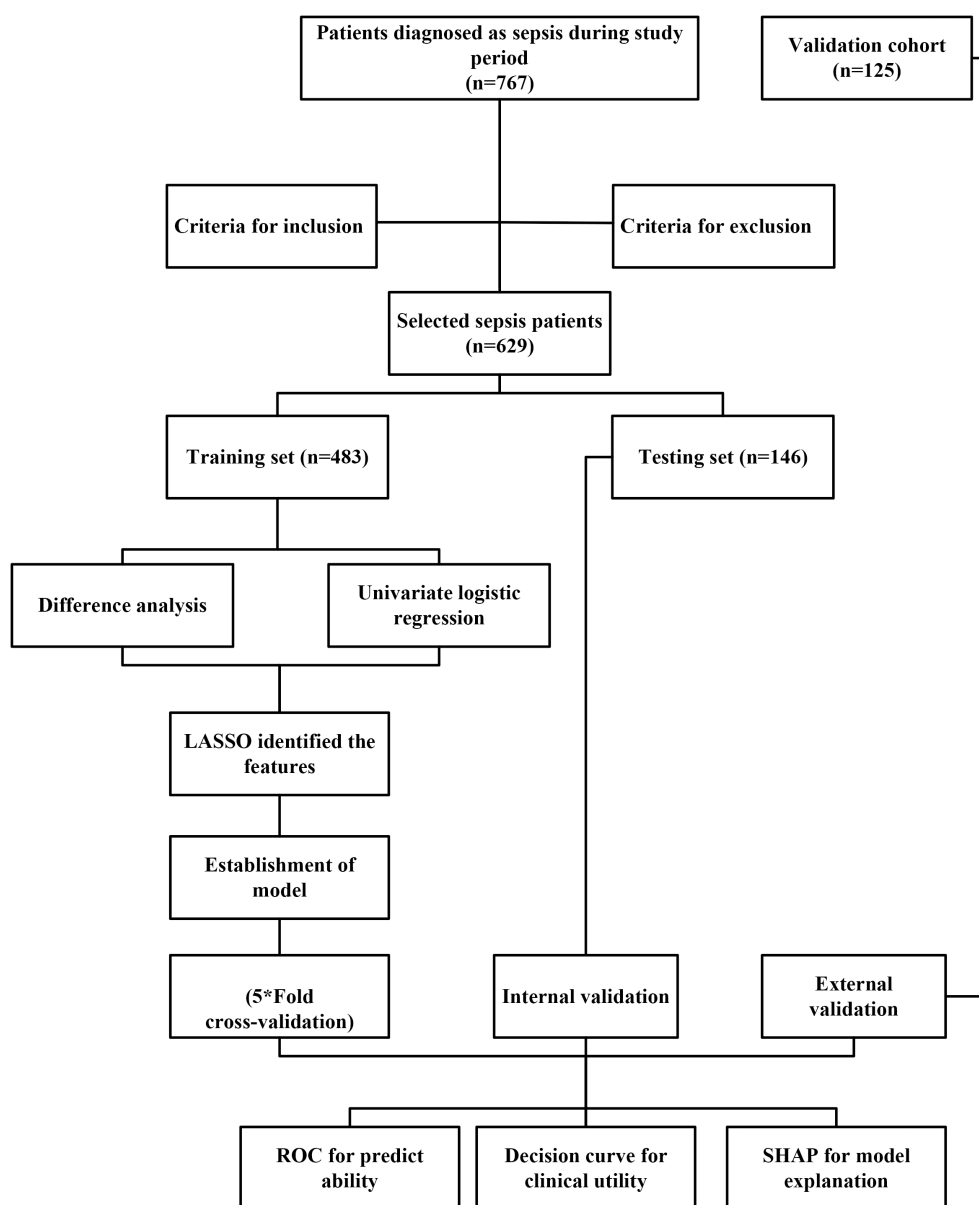


FIGURE 1
Flow chart of the whole study.

samples t-test. For non-normally distributed continuous variables (lymphocyte subsets), data are presented as median and interquartile range, and comparisons between groups were performed using the Wilcoxon test. The false discovery rate was applied for multiple comparisons of lymphocyte subsets. Categorical variables are presented as counts and percentages (gender, COPD, hypertension, diabetes, CHD, primary infection, septic shock, CRRT, and vasoactive drugs usage), and comparisons between groups were conducted using the Chi-square test. $P < 0.05$ was considered a significant level.

To develop a predictive model for AKI in patients with sepsis, we used the training set to identify overlapping variables between those showing significant differences between the AKI and non-AKI

groups and those identified as significant in univariate logistic regression. These overlapping variables were then further refined using the least absolute shrinkage and selection operator (LASSO) regression. Subsequently, multivariate logistic regression with forward stepwise selection was performed. Variables with a p -value < 0.05 were retained in the final predictive model.

The model was validated using two approaches. For internal validation, five random subsets were extracted from the training set and subjected to 5-fold cross-validation. For external validation, an independent cohort dataset was used. Model performance was assessed using the receiver operating characteristic (ROC) curve to evaluate discriminative ability. Calibration plots for both the training and validation sets were generated to assess the agreement

between predicted and observed probabilities, demonstrating the model’s stability.

A nomogram for predicting the risk of AKI was constructed using the R “rms” package based on the multivariate logistic regression model. Finally, decision curve analysis was conducted to evaluate the clinical utility of the model by weighing its potential benefits and risks, thereby determining its applicability in clinical decision-making. The SHapley Additive explanation (SHAP) method was used for global and local explanations for the model explanation.

Results

Clinical characteristics between non-AKI and AKI groups

Based on the predefined inclusion and exclusion criteria, a total of 483 patients with sepsis were included in the training set. The incidence of acute kidney injury (AKI) was 54.66% (264/483). **Table 1** summarizes the comparisons of clinical characteristics between the AKI and non-AKI groups. No significant differences were observed in age ($P = 0.971$), gender distribution ($P = 0.286$), or body mass index (BMI) ($P = 0.509$) between the two groups. However, the SOFA score (8.94 ± 3.67 vs. 6.89 ± 3.48 , $P < 0.001$) and the APACHE II score (22.86 ± 6.93 vs. 17.00 ± 7.87 , $P < 0.001$) were significantly higher in the AKI group compared to the non-AKI group. In contrast, the GCS score was significantly lower in the AKI group (10.16 ± 3.60 vs. 11.52 ± 3.76 , $P = 0.020$). There were no significant differences in the prevalence of hypertension ($P = 0.214$), diabetes mellitus ($P = 0.573$), or coronary heart disease (CHD) ($P = 0.702$) between the groups. However, the prevalence of chronic obstructive pulmonary disease (COPD) (11.36% vs. 0.00% , $P = 0.008$), use of continuous renal replacement therapy (CRRT) (39.77% vs. 20.55% , $P = 0.009$), administration of vasoactive drugs (77.27% vs. 56.16% , $P = 0.004$), occurrence of septic shock (80.68% vs. 54.79% , $P < 0.001$), and rate of primary infection (94.32% vs. 83.56% , $P = 0.027$) were all significantly higher in the AKI group.

In terms of laboratory findings, both neutrophil count (20.77 ± 30.56 vs. 12.41 ± 11.38 , $P = 0.019$) and monocyte count (0.84 ± 0.91 vs. 0.53 ± 0.40 , $P = 0.005$) were significantly elevated in the AKI group. Serum creatinine levels were also markedly higher in the AKI group (261.19 ± 259.45 vs. 128.09 ± 132.35 , $P < 0.001$). No significant differences were found between the groups in white blood cell count ($P = 0.098$), hemoglobin level ($P = 0.258$), platelet count ($P = 0.665$), lymphocyte count ($P = 0.056$), C-reactive protein ($P = 0.803$), procalcitonin ($P = 0.142$), total bilirubin ($P = 0.542$), albumin ($P = 0.348$), globulin ($P = 0.711$), blood urea nitrogen (BUN) ($P = 0.052$), or lactate level ($P = 0.301$).

Lymphocyte subsets between non-AKI and AKI groups

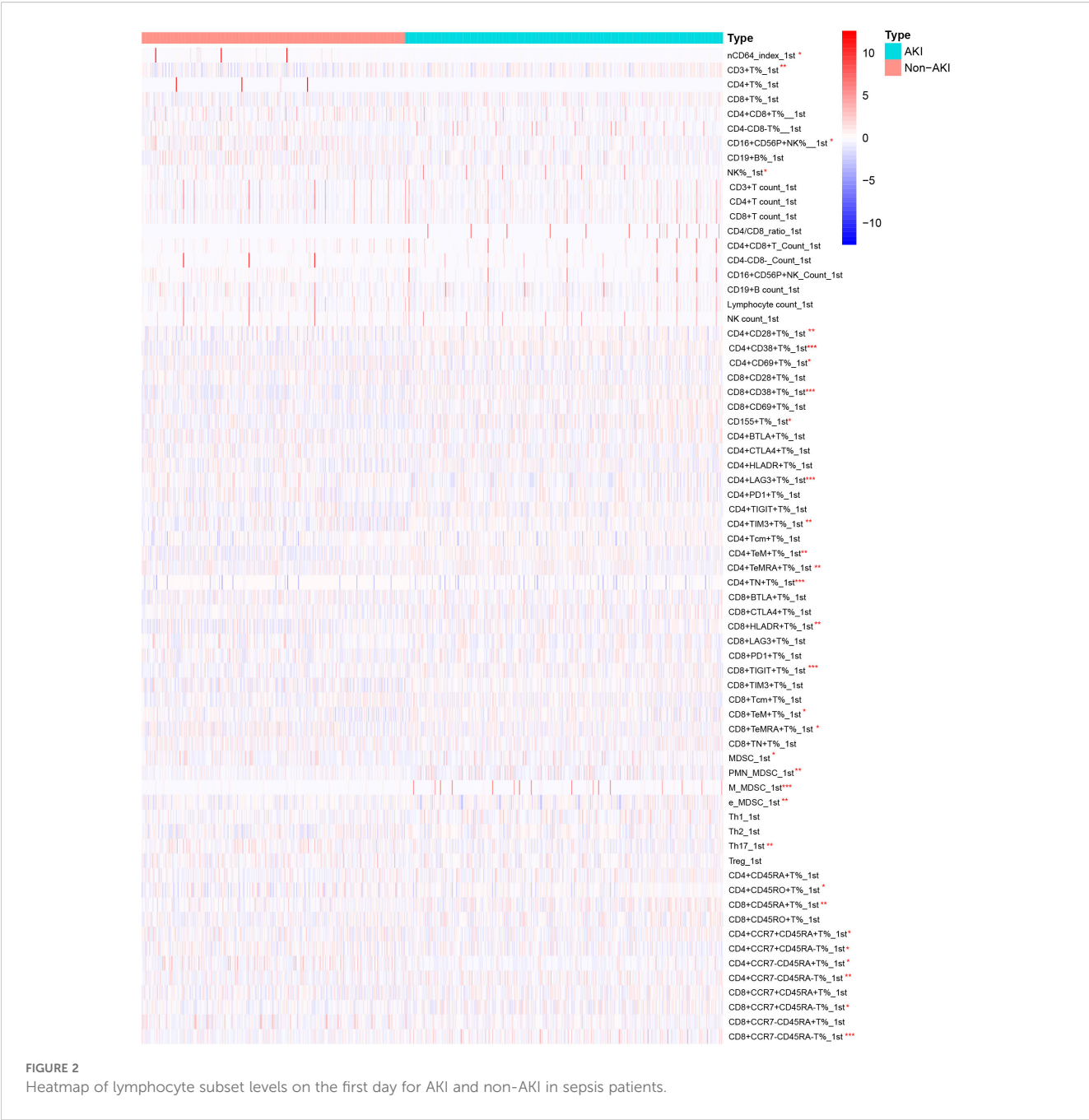
The first day and third day lymphocyte subsets were compared between non-AKI and AKI groups. **Figure 2** shows the heatmap of the

TABLE 1 Comparisons of clinical characteristics between non-AKI and AKI groups.

Variables	Non-AKI	AKI	<i>P</i>
Age, year	66.07 ± 17.21	65.98 ± 13.94	0.950
Gender, Male (%)	90 (41.10)	87 (32.95)	0.065
BMI, kg/m ²	24.82 ± 3.52	25.24 ± 4.57	0.251
SOFA	6.89 ± 3.47	8.94 ± 3.66	<0.001
APACHEII	17.00 ± 7.84	22.86 ± 6.90	<0.001
GCS	11.52 ± 3.74	10.16 ± 3.59	<0.001
COPD, n (%)	0 (0.00)	30 (11.36)	<0.001
Hypertension, n (%)	93 (42.47)	87 (32.95)	0.031
Diabetes, n (%)	66 (30.14)	69 (26.14)	0.329
CHD, n (%)	66 (30.14)	87 (32.95)	0.508
CRRT, n (%)	45 (20.55)	105 (39.77)	<0.001
Vasoactive drugs, n (%)	123 (56.16)	204 (77.27)	<0.001
Septic shock, n (%)	40 (54.79)	71 (80.68)	<0.001
Primary Infection, n (%)	183 (83.56)	249 (94.32)	<0.001
White blood cell, 10 ⁹ /L	12.90 ± 7.09	15.03 ± 8.76	0.003
Hemoglobin, g/L	113.37 ± 31.48	107.86 ± 29.67	0.049
Platelet, 10 ⁹ /L	171.53 ± 92.37	164.33 ± 113.35	0.442
Neutrophil, 10 ⁹ /L	12.41 ± 11.33	20.77 ± 30.45	<0.001
Lymphocyte, 10 ⁹ /L	0.90 ± 0.84	1.68 ± 3.32	<0.001
Monocyte, 10 ⁹ /L	0.53 ± 0.40	0.84 ± 0.91	<0.001
C-reactive protein, mg/L	134.95 ± 84.79	138.76 ± 104.18	0.658
Procalcitonin, ng/L	20.51 ± 30.80	28.11 ± 33.66	0.010
Total bilirubin, μmol/L	42.87 ± 48.67	38.48 ± 41.95	0.288
Albumin, g/L	30.57 ± 6.62	29.65 ± 5.75	0.102
Globulin, g/L	26.79 ± 4.68	27.09 ± 5.22	0.515
Blood urea nitrogen, mmol/L	14.51 ± 9.10	17.94 ± 12.36	<0.001
Creatinine, μmol/L	128.09 ± 131.74	261.19 ± 258.47	<0.001
Lactate, mmol/L	3.06 ± 2.75	5.33 ± 18.43	0.071

lymphocyte subsets on the first day between two groups. Compared with the non-AKI group, the AKI group exhibited elevated levels of CD4+CD38+T% ($P < 0.011$), CD8+CD38+T% ($P < 0.001$), and CD155+T% ($P = 0.016$). Additionally, CD4+TeM+T% ($P = 0.001$), CD8+TIGIT+T% ($P = 0.012$), M-MDSC ($P < 0.001$), and CD8+CCR7-CD45RA-T% ($P = 0.026$) levels were increased in the AKI group. In contrast, CD4+LAG3+T% ($P = 0.017$), CD4+TN+T% ($P < 0.001$), and Th17 ($P = 0.014$) levels were decreased in the AKI group. No significant differences were observed in other lymphocyte subsets between the two groups ($P > 0.005$). More detailed information is provided in **Supplementary Table S1**.

Figure 3 showed the heatmap of the lymphocyte subsets on the third day between the two groups. Compared with the non-AKI



group, the AKI group had lower levels of CD16+CD56+NK% ($P = 0.036$), CD16+CD56+NK count ($P = 0.005$), CD4+CTLA4+T% ($P < 0.001$), CD4+TIM3+T% ($P = 0.002$), CD8+CTLA4+T% ($P = 0.009$), CD8+TIM3+T% ($P < 0.001$), CD8+TeMRA+T% ($P = 0.038$), MDSC ($P = 0.043$), e_MDSC ($P = 0.017$), and CD8+CCR7-CD45-T% ($P = 0.001$). However, the levels of CD4+TeMRA+T% ($P = 0.043$), CD8+PD1+T% ($P = 0.001$), CD8+TIGIT+T% ($P = 0.005$), PMN_MDSC ($P = 0.002$), M_MDSC ($P = 0.004$), CD4+CD45RA+T% ($P = 0.002$), CD4+CCR7-CD45+T% ($P = 0.008$), and CD8+CCR7+CD45+T% ($P = 0.006$) were significantly higher in the AKI group than in the

non-AKI group. No significant differences were observed for other lymphocyte subsets, and more detailed information can be found in [Supplementary Table S2](#).

Establishment of the prediction model for AKI in sepsis patients

We identified 32 factors by overlapping the variables that showed significant differences ([Supplementary Table S1, S2](#)) between the AKI and non-AKI groups with those found to be significant in univariate

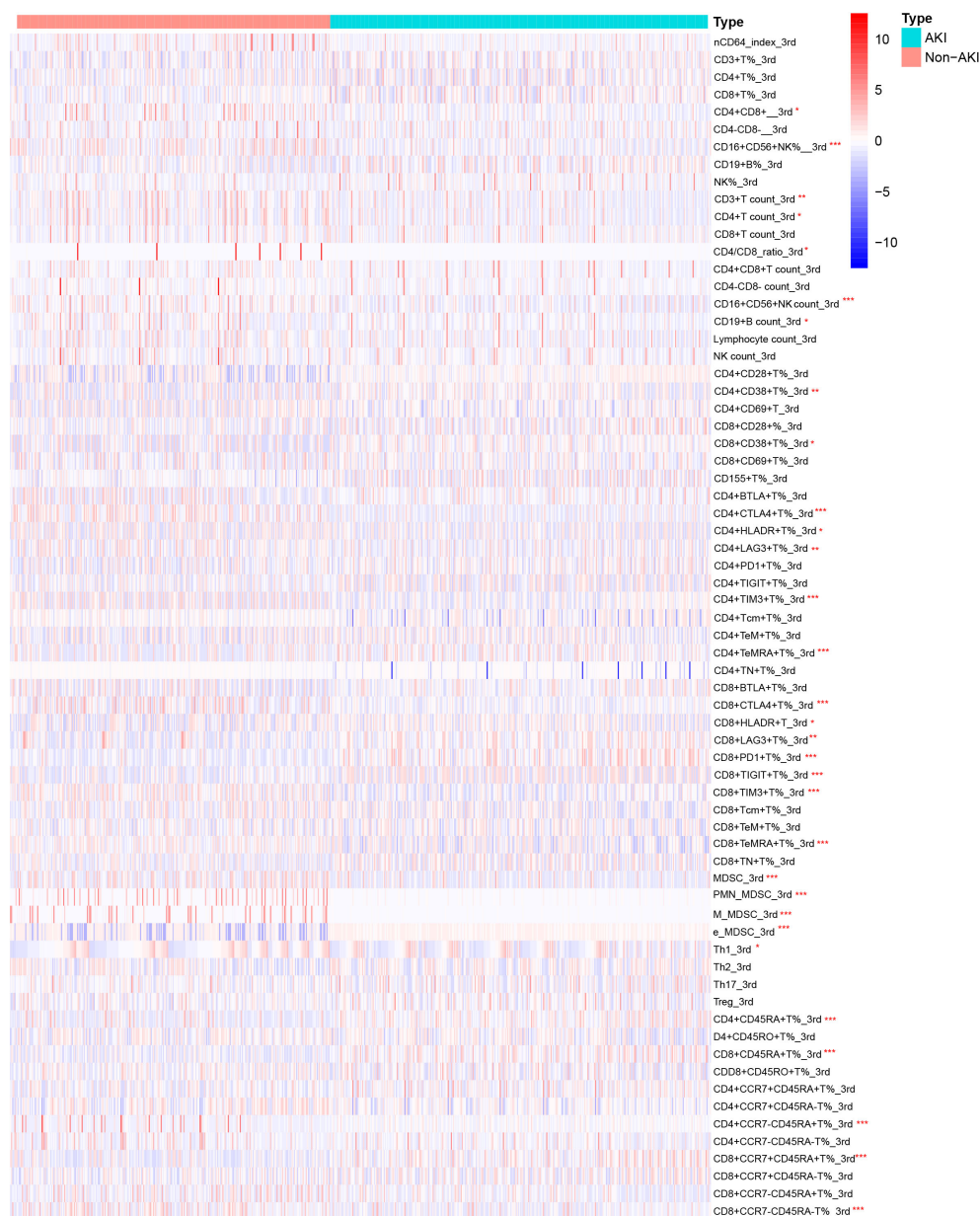


FIGURE 3
Heatmap of lymphocyte subset levels on the third day for AKI and non-AKI in sepsis patients.

logistic regression (Supplementary Table S3). These 32 factors were then subjected to LASSO regression, resulting in the selection of 23 variables for further analysis (Figures 4A, B). A multivariate logistic regression using a forward stepwise approach identified 13 factors associated with the risk of AKI in sepsis patients. Specifically, higher values of APACHE II (OR: 1.379, 95% CI: 1.152-1.649), creatinine (OR: 1.011, 95% CI: 1.004-1.018), CD15⁺T%_{1st} (OR: 1.066, 95% CI: 1.004-1.132), CD8⁺PD1⁺T%_{3rd} (OR: 1.279, 95% CI: 1.115-1.468), CD8⁺TIGIT⁺T%_{3rd} (OR: 1.033, 95% CI: 1.001-1.066), E_MDSC_{3rd}, and CD8⁺CCR7⁺CD45RA⁺T%_{3rd} were associated with an increased risk of AKI. In contrast, higher levels of CD4⁺LAG3⁺T%_{1st} (OR: 0.924, 95% CI: 0.872-0.978), Th17_{1st} (OR: 0.744, 95% CI: 0.616-0.898), CD4⁺CTLA4⁺T%_{3rd} (OR:

0.830, 95% CI: 0.747-0.923), CD4⁺TIM3⁺T%_{3rd} (OR: 0.928, 95% CI: 0.874-0.986), PMN_MDSC_{3rd} (OR: 0.757, 95% CI: 0.595-0.962), and M_MDSC_{3rd} (OR: 0.748, 95% CI: 0.627-0.891) were associated with a decreased risk of AKI (Figure 4C).

Validation and assessment of the prediction model for AKI in sepsis patients

We first compared the clinical characteristics and lymphocyte subsets in the training, validation and test sets and found no significant differences among the three groups. There is good comparability between among the training, the validation and test

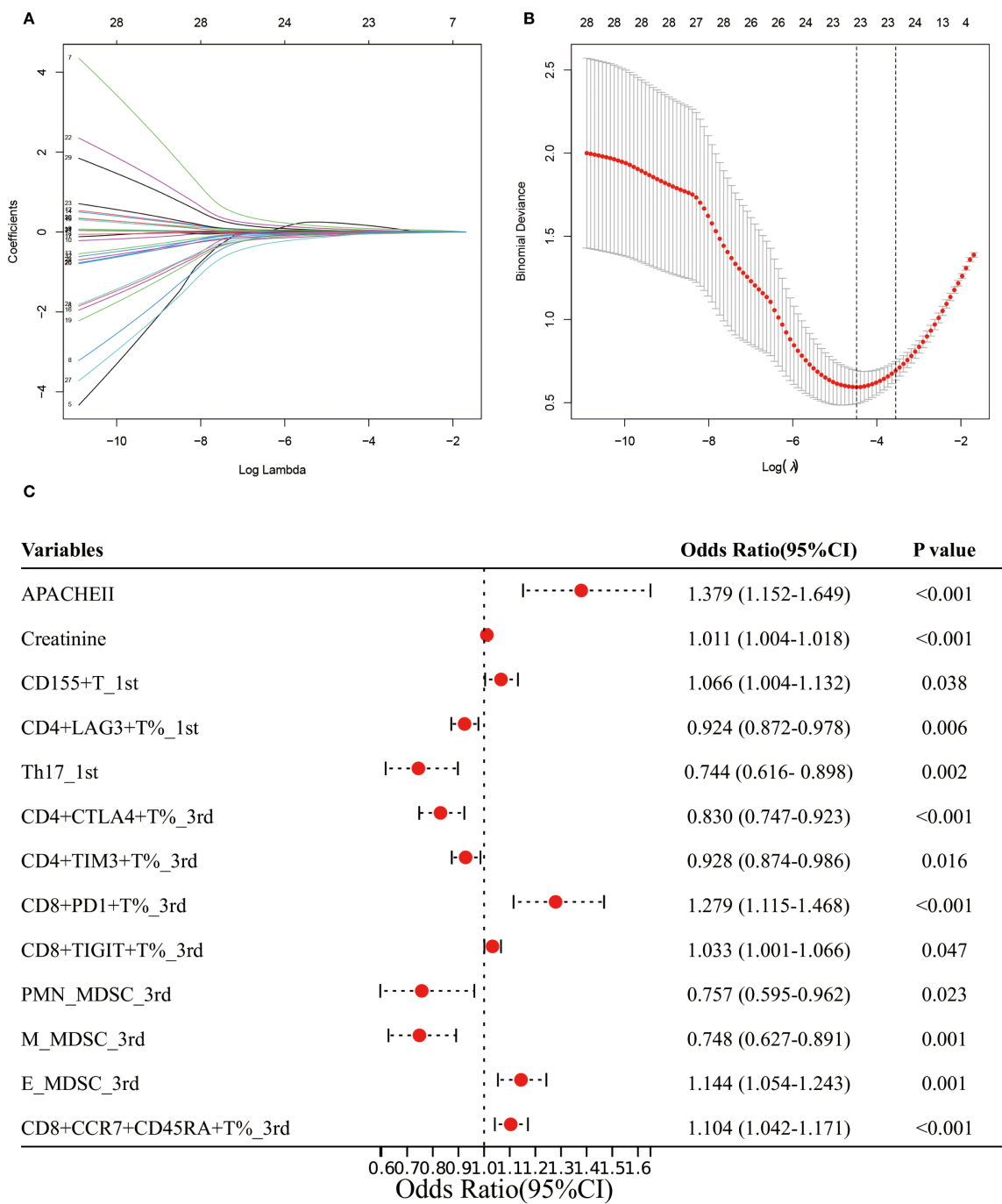


FIGURE 4 Establishment of the prediction model for AKI in sepsis patients. (A, B) LASSO regression identified the potential model factors. (C) Forest plot of multivariate logistic regression for AKI in sepsis patients.

sets (Supplementary Tables S4–S6). ROC analysis showed that the AUC was 0.989 (95% CI: 0.977–1.000) in the training set, 0.895 (95% CI: 0.789–1.000) in the validation set (Figure 5A), and 0.906(95%CI: 0.849–0.963) in the test set (Figure 5B). To further evaluate the model’s robustness, we performed five-fold cross-validation. The AUCs for Fold 1 to Fold 5 were 0.963 (95% CI: 0.945–0.981), 0.925 (95% CI: 0.907–0.943), 0.950 (95% CI: 0.932–0.968), 0.973 (95% CI: 0.955–0.991), and 0.977 (95% CI: 0.959–0.995), respectively (Figure 5C).

Unadjusted calibration plots for both the training, validation and test sets demonstrated good agreement between predicted and observed probabilities (Figures 5D–F). After adjustment, the calibration plots showed reduced bias and improved consistency (Figures 5G–I). Based on the 13 predictors included in the final model, we developed an individualized risk scoring system (Figure 6A). Decision curve analysis indicated that patients in the training set could benefit from clinical decisions guided by the

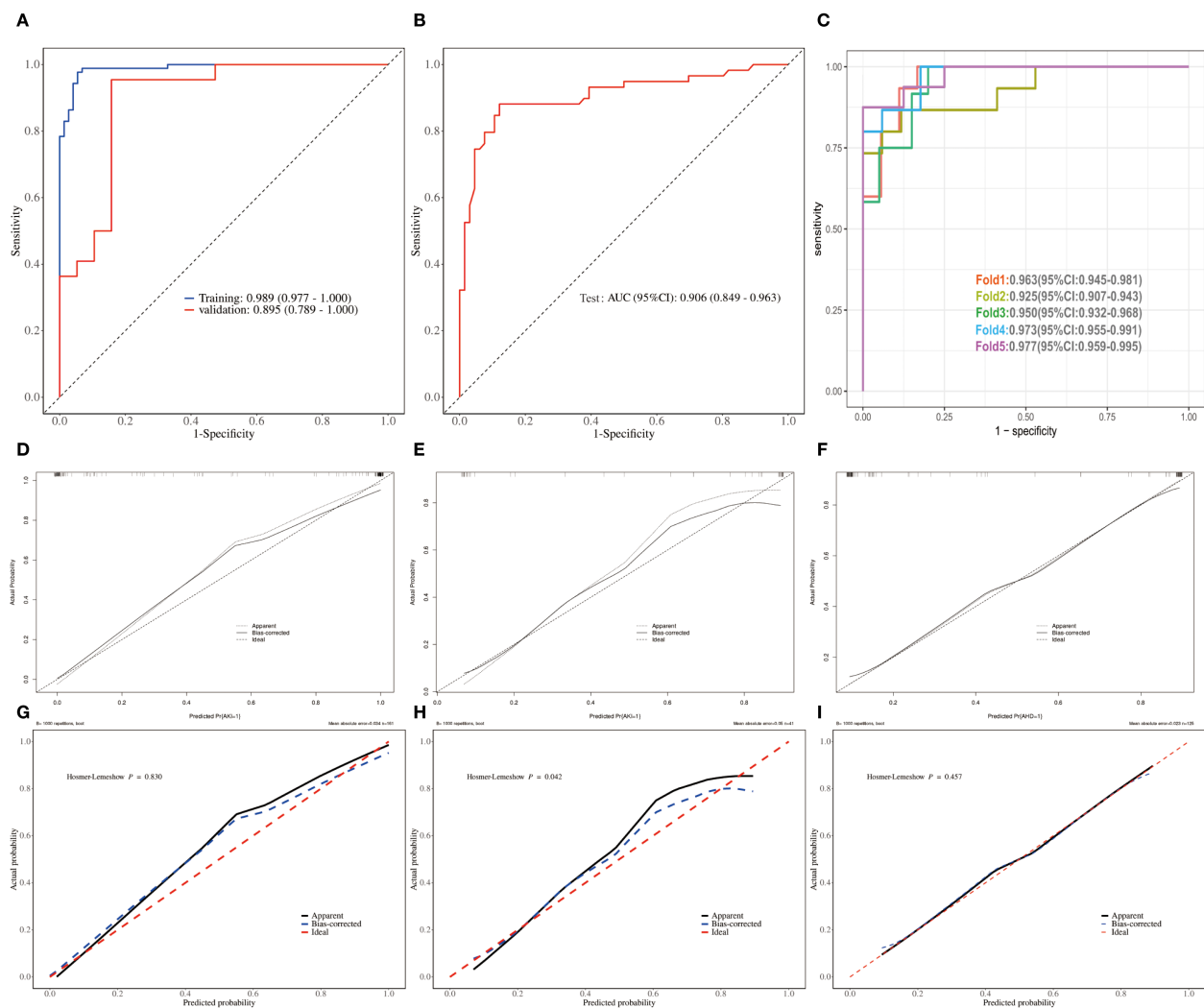


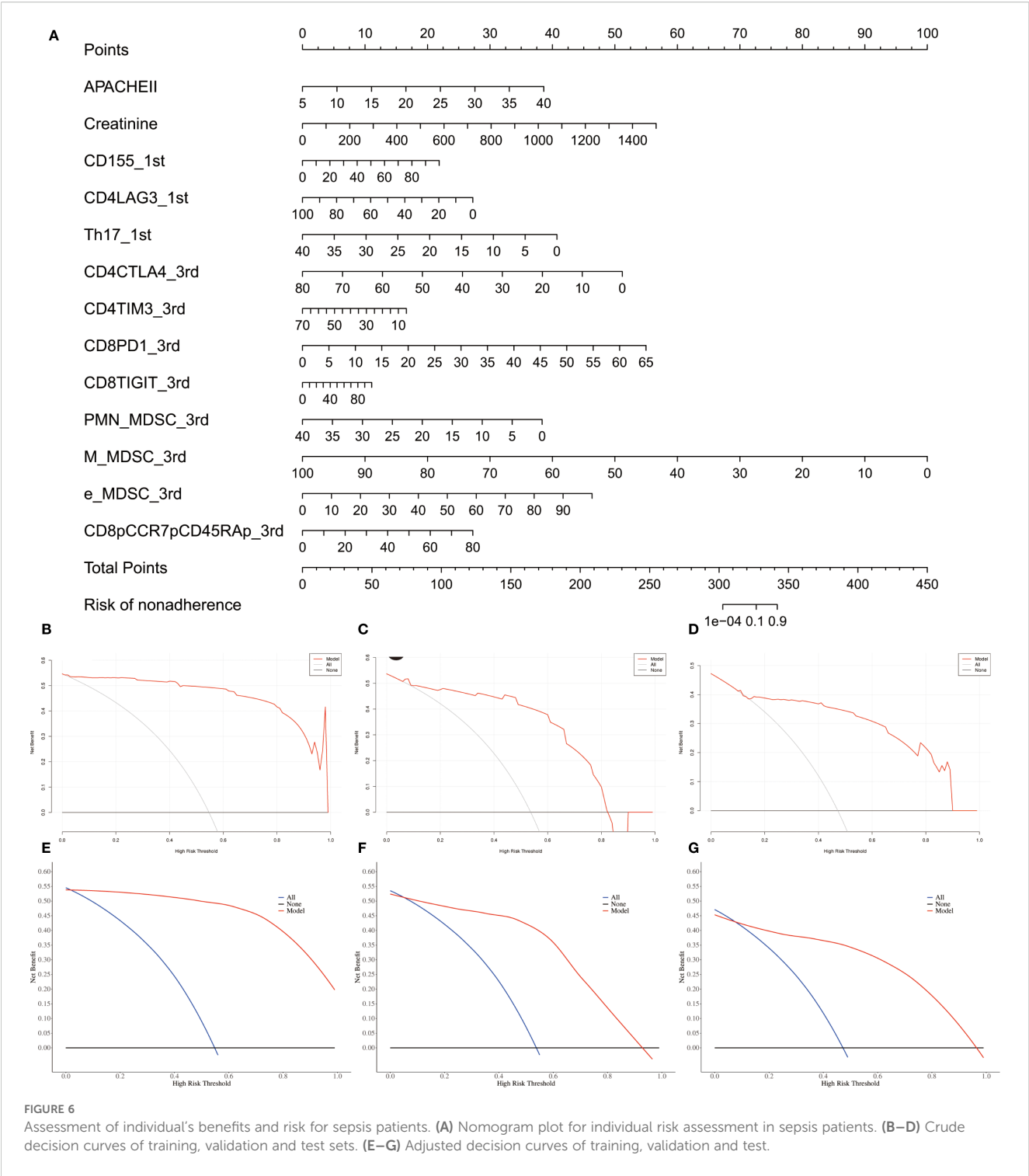
FIGURE 5 Validation and assessment of the predict model for AKI in sepsis patients. (A, B) ROCs of training, validation and test sets. (C) ROCs of 5-fold cross-validation. (D–F) Crude calibration plots of crude for training, validation and test set. (G–I) Corrected calibration plots of crude for training and validation set.

model (Figure 6B). In the validation and test set, the model provided clinical net benefit when the decision threshold ranged from 0.10 to 0.84 (Figures 6C, D). After adjustment, the model continued to show clinical utility, with net benefit observed across a decision threshold range of 10% to 90% (Figures 6E–G). As shown in SHAP summary plots (Figures 7A, B), the importance of variables was evaluated using the contribution of variables to the model. The larger the absolute SHAP value, the greater the contribution to the model and the more important the feature is. The SHAP dot plot can judge the effect of variables on the AKI. The high feature value can increase the risk of AKI, such as patients with high APACHE II, creatinine, CD155+T₁st, CD8+PD1+T%₃rd, CD8+TIGIT+T%₃rd, E_MDSC₃rd, and CD8+CCR7+CD45RA+T% (SHAP value>0), which pushed the decision towards the AKI group. In contrast, patients with the low levels of CD4+LAG3+T%₁st, Th17₁st, CD4+CTLA4+T%₃rd, CD4+TIM3+T

%₃rd, PMN_MDSC₃rd, and M_MDSC₃rd had SHAP values lower than zero, which pushed the decision towards the AKI group.

Discussion

Sepsis-associated acute kidney injury (SA-AKI) is a severe and life-threatening condition characterized by high mortality and a lack of specific clinical features. The pathophysiology of sepsis is complex and often involves multiple organ systems, complicating both diagnosis and treatment. Due to the unique pathophysiological characteristics of SA-AKI, its clinical management is particularly challenging, with high mortality rates and poor prognosis (28, 29). Sepsis is one of the most common causes of AKI, and conversely, AKI can serve as an early indicator of sepsis. However, elucidating the molecular mechanisms underlying SA-AKI remains extremely



difficult (30). Currently, there are no specific or standardized therapeutic strategies for the treatment of SA-AKI, posing significant challenges to early diagnosis and effective intervention (31). These challenges ultimately contribute to poor outcomes in affected patients. Therefore, early assessment and timely treatment of SA-AKI are critical for improving patient prognosis. This study investigated the clinical characteristics and lymphocyte subset changes in sepsis patients with Acute Kidney Injury (AKI), aiming to develop and validate a predictive model for AKI based

on these factors. Our study will offer a promising tool for early AKI risk assessment in sepsis patients.

Previous studies reported that the incidence of AKI was approximately 50% in sepsis patients. In a large prospective cohort study spanning 24 European countries and 198 ICUs, 1177 sepsis patients were observed to have a 51% incidence of AKI and a 41% ICU mortality rate (32). A retrospective study in China, encompassing 146,148 sepsis patients, reported an AKI incidence of 47.1% (33). An ancillary analysis of a multicenter

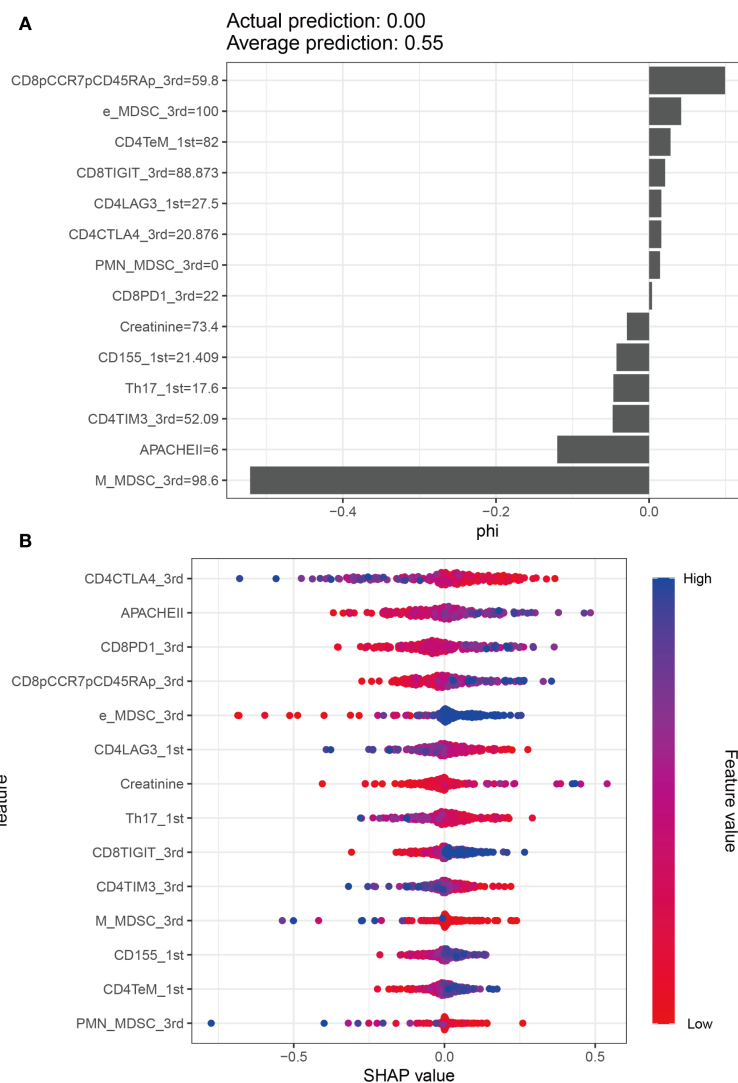


FIGURE 7
Model explanation by the SHAP method. (A) SHAP summary bar plot. (B) SHAP summary dot plot.

RCT on septic shock, encompassing 1,243 patients, found that 50.4% had AKI upon emergency department admission, with a further 18.7% developing AKI within the subsequent seven days (34, 35). The incidence of AKI among septic patients in our cohort was 54.66%, which is consistent with previously reported rates in critically ill populations. We found Some clinical characteristics were significantly different between AKI and non-AKI groups. Compared to the non-AKI group, AKI patients exhibited significantly higher SOFA and APACHE II scores, but lower GCS scores. The SOFA and APACHE II scores were associated with diseases severity and prognosis, and the lower GCS was related to the degree of coma (36, 37). Regarding comorbidities and interventions, the AKI group showed significantly higher prevalence of COPD, CRRT use, vasoactive drug administration, septic shock occurrence, and primary infection rates. Laboratory analyses revealed elevated neutrophil count, monocyte count, and serum creatinine levels in the AKI group. These changes in the clinical characteristics of AKI patients could be associated with

reduced bilateral glomerular perfusion, inflammatory responses, metabolic adaptations, and impaired microcirculatory function, which are key mechanisms of organ damage in sepsis patients (38). However, only APACHE II and creatinine were included in the final prediction model for AKI in sepsis. That makes sense, as serum creatinine is a primary indicator for diagnosing sepsis-associated AKI, while the APACHE II score reflects the overall severity of illness in sepsis patients (5, 39).

Although the precise role of lymphocyte subsets in sepsis-induced acute kidney injury (AKI) remains unclear, several studies provide important insights. It has been suggested that damage-associated molecular patterns (DAMPs), such as high mobility group box 1 (HMGB1), cell-free DNA (cfDNA), and histones, can be released as a result of widespread immune cell death. These molecules can trigger endothelial injury and microcirculatory dysfunction, thereby contributing to the progression of multiple organ failure in patients with sepsis (40). In our study, we observed significant alterations in lymphocyte subsets between the AKI and non-AKI groups on both

the first- and third days following sepsis onset. On day 1, the AKI group exhibited significantly elevated levels of CD4⁺CD38⁺ T%, CD8⁺CD38⁺ T%, CD15⁺ T%, CD4⁺TeM⁺ T%, CD8⁺TIGIT⁺ T%, monocytic myeloid-derived suppressor cells (M-MDSCs), and CD8⁺CCR7⁻CD45RA⁻ T%. In contrast, levels of CD4⁺LAG3⁺ T%, CD4⁺TN⁺ T%, and Th17 cells were significantly reduced. By day 3, the AKI group showed notably decreased levels of CD16⁺CD56⁺ NK% and NK cell count, CD4⁺CTLA4⁺ T%, CD4⁺TIM3⁺ T%, CD8⁺CTLA4⁺ T%, CD8⁺TIM3⁺ T%, CD8⁺TeMRA⁺ T%, total MDSCs, and early-stage MDSCs (e-MDSCs). Conversely, the same group had significantly increased levels of CD4⁺TeMRA⁺ T%, CD8⁺PD1⁺ T%, CD8⁺TIGIT⁺ T%, polymorphonuclear MDSCs (PMN-MDSCs), M-MDSCs, CD4⁺CD45RA⁺ T%, CD4⁺CCR7⁻CD45⁺ T%, and CD8⁺CCR7⁺CD45⁺ T%. These findings suggest that lymphocyte subset dysfunction is not limited to peripheral blood but may also affect target organs through systemic circulation. Such dysfunction has been associated with the dysregulated release of immunoregulatory molecules, including excessive pro-inflammatory cytokines, which can intensify tissue inflammation and injury (41). For instance, Akcay et al. reported that CD4⁺ T cells contribute to neutrophil recruitment and apoptosis in a murine model of cisplatin-induced AKI. These cells also upregulated IL-33 expression and promoted inflammatory factor release, ultimately causing immune-mediated injury to the peritubular and glomerular capillary networks (42). Mesenchymal stem cells attenuate sepsis-AKI by changing the balance of Th17 cells/Tregs via Gal-9/Tim-3 (43). Conversely, certain interventions have shown promise in mitigating this damage. Curcumin, a well-known anti-inflammatory compound, has been shown to alleviate kidney and lung inflammation by enhancing the suppressive activity of regulatory T cells (Tregs) (44). Similarly, glutamine supplementation can attenuate renal injury by promoting balanced T cell polarization and reducing T cell apoptosis (44). Immune regulation is further modulated by immune checkpoint molecules expressed on lymphocytes and antigen-presenting cells, such as PD-1 and PD-L1, CD40 and CD40L, CD28, cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), B and T lymphocyte attenuator (BTLA), and members of the T cell immunoglobulin and mucin domain (Tim) family. These molecules provide essential co-stimulatory signals for T cell activation and immune homeostasis. Recent studies *in vitro* particularly highlight that excessive interaction between PD-1 and PD-L1 can induce lymphocyte apoptosis, leading to immunosuppression. This immune dysfunction may contribute to tubular epithelial cell damage and the development of septic AKI (45). Consequently, therapeutic strategies aimed at restoring lymphocyte functions such as anti-PD-L1 therapy or lactate receptor blockade, may offer promising avenues for the treatment of sepsis-associated AKI. Drawing on this evidence, it is reasonable to propose that reestablishing the function of immune cells, especially T and B lymphocytes, may play a crucial role in mitigating sepsis-related organ damage and enhancing patient prognosis. The multivariate logistic regression analysis further revealed two distinct cellular patterns associated with AKI risk in sepsis patients: an AKI-promoting pattern and an AKI-protective pattern. Patients with elevated proportions of CD8⁺ T cells expressing inhibitory/exhaustion markers (PD1⁺, TIGIT⁺, TIM3⁺, CCR7⁺CD45RA⁺) and

E-MDSCs were more likely to develop AKI, suggesting that immune exhaustion and expansion of immunosuppressive myeloid subsets contribute to renal vulnerability. Conversely, patients with higher levels of checkpoint-positive CD4⁺ T cells (LAG3⁺, CTLA4⁺, TIM3⁺) and specific MDSC subsets (PMN-MDSCs, M-MDSCs) exhibited a lower risk of AKI, indicating that these regulatory mechanisms may buffer excessive immune activation and provide renal protection. While renal impairment and disease severity are well-recognized contributors to AKI, our findings underscore the critical role of immune dysregulation. Taken together, these results suggest that AKI development is tightly linked to an imbalance between pro-injury immune exhaustion and compensatory immunoregulation. This highlights the potential value of targeting immune modulation as a therapeutic strategy to prevent or attenuate AKI in sepsis.

In this study, we identified 13 key predictive factors for AKI risk in sepsis patients using the least absolute shrinkage and selection operator (LASSO) and multivariate logistic regression. The resulting model demonstrated excellent predictive performance, with areas under the curve (AUCs) of 0.989 in the training set and 0.895 in the validation set. Its robustness and accuracy were further validated through five-fold cross-validation, calibration plots, and decision curve analysis, highlighting its potential clinical utility. Compared to previous research, our study presents several distinct advantages. A prior study investigated the association between T-lymphocyte subsets and sepsis-induced AKI, focusing on a limited set of immune parameters (CD3⁺, CD3⁺CD4⁻, CD3⁺CD8⁺, CD4⁺/CD8⁺ ratio, and CD3⁺ percentage) (23). In contrast, our study evaluated 68 types of lymphocyte subsets, including T, B, and natural killer (NK) cells. Furthermore, while the previous study primarily explored the relationship between selected T-lymphocyte subsets and in-hospital mortality, it also reported that CD3⁺ and CD3⁺CD8⁺ T-lymphocyte counts had good predictive value for AKI, with AUCs of 0.849 and 0.856, respectively. In the present study, we developed a comprehensive predictive model for AKI based on lymphocyte subsets measured on both day 1 and day 3 after sepsis onset. Unlike the previous study, we conducted both internal and external validation of our model, further ensuring its reliability. Additionally, our sample size was more than twice that of the earlier study, enhancing the generalizability of our findings. Several other studies have also developed predictive models for sepsis-associated AKI using various approaches. Lin et al. constructed a logistic regression model based on clinical parameters, achieving an AUC of 0.835 in a retrospective analysis (46). Zhang et al. employed a support vector machine model incorporating 43 genes selected via a genetic algorithm, yielding an AUC of 0.948 (47). However, this model lacked external validation. Yue et al. applied the XGBoost algorithm using data from the MIMIC-III database and reported an AUC of 0.821, but similarly did not include a validation cohort (48). Other reported models include logistic regression (49), lightGBM model (50), and pooled analyses without adjustment for confounders (51), with AUCs ranging from 0.712 to 0.873. Compared to these models, our lymphocyte subset-based prediction model demonstrated superior performance and validation, making it a promising tool for the early identification of AKI in sepsis patients.

The present study still has several limitations. First, this is a single-center, observational study with limited sample size. While we performed rigorous cross-validation and internal validation, external validation with a larger, more diverse cohort is essential to confirm the model's widespread applicability. Second, although we identified several lymphocyte subsets significantly associated with AKI, their precise role remains to be fully elucidated. It's unclear whether these cells directly contribute to kidney injury or merely reflect the systemic immune status. Future functional research, incorporating *in vitro* and *in vivo* assays, alongside animal experiments, will be crucial to clarify the causal role of these cell subsets in sepsis-induced AKI. Finally, our analysis did not include certain potentially relevant variables, such as genetic predisposition, detailed hemodynamic parameters, or specific urine biomarkers. Incorporating these factors in future studies could further refine risk prediction and reveal complementary pathophysiological mechanisms.

In conclusion, this study has revealed the unique characteristics of lymphocyte subsets in patients with sepsis-related AKI and successfully constructed a predictive model integrating lymphocyte subsets and clinical indicators, which has good performance and clinical practical value. Future studies should further expand the sample size, strengthen external validation and explore potential molecular mechanisms, with the expectation of promoting the early identification and precise intervention of AKI.

Data availability statement

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

Ethics statement

This study was approved by the Ethics Committee of People's Hospital of Xinjiang Uygur Autonomous Region, and informed consent was obtained from all participants. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

LW: Data curation, Investigation, Writing – original draft. QL: Formal Analysis, Methodology, Software, Validation, Writing –

review & editing. CW: Data curation, Visualization, Writing – review & editing. MH: Project administration, Resources, Visualization, Writing – review & editing. ZL: Conceptualization, Funding acquisition, Project administration, Writing – review & editing.

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The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2025.1674673/full#supplementary-material>

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Comprehensive analysis of metabolism-related genes in sepsis reveals metabolic-immune heterogeneity and highlights GYG1 as a potential therapeutic target

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Background: Sepsis is a life-threatening syndrome characterized by dysregulated host immune responses, yet the metabolic drivers of immune dysfunction remain poorly understood.

Methods: Here we systematically profiled metabolism-related genes (MRGs) in sepsis using bulk transcriptomic data and stratified patients into two subgroups with distinct immune infiltration profiles by MRGs, as assessed by CIBERSORT and single-cell RNA-seq integration. Machine learning identified five hub metabolic genes for constructing a metabolic risk score, whose prognostic relevance was robustly validated in an external cohort. Single cell analyses, cell-cell communication, and cell-type-specific differential expression analyses were performed to dissect the immunological context. Finally, *in vivo* validation was conducted using an LPS-induced sepsis mouse model.

Results: Patients in the high metabolic risk group exhibited a neutrophil-dominant and lymphocyte-suppressed immune landscape, consistent across bulk and single-cell analyses. Among the five hub genes (ALPL, CYP1B1, GYG1, OLAH, VNN1), GYG1 demonstrated the strongest predictive performance and was highly expressed in monocytes, neutrophils, and proliferating myeloid cells. High-risk patients displayed intensified monocyte-dendritic cell interactions and transcriptional programs enriched in neutrophil degranulation pathways. *In vivo*, Gyg1 was markedly upregulated in septic mice, and LNP-mediated siRNA knockdown of Gyg1 significantly improved survival in the LPS model. Mechanistically, Gyg1 knockdown significantly reduced glycogen content in myeloid cells, attenuated IL-6 and TNF- α production, alleviated LPS-induced neutrophil, and modestly decreased CD40 expression in monocytes and dendritic cells. These results collectively suggest that Gyg1 regulates metabolic fueling of inflammatory activation and intercellular communication during sepsis.

Conclusions: This integrative multi-omics study established a robust immune-metabolic risk score system to predict sepsis patient outcomes and identified

GYG1 as a metabolic driver of innate immune hyperactivation. Targeting GYG1 via LNP–siRNA delivery reduces glycogen availability and inflammatory output in myeloid cells, mitigating immune overactivation and improving disease outcomes *in vivo*, thereby highlighting its potential as a novel therapeutic target for sepsis.

KEYWORDS

sepsis, metabolism, immune infiltration, risk score, lipid nanoparticles, GYG1

Introduction

Sepsis is a life-threatening syndrome characterized by dysregulated host responses to infection, leading to life-threatening organ dysfunction and high mortality worldwide (1–3). Despite advances in supportive care, effective targeted therapies remain elusive, largely due to the substantial biological heterogeneity of the disease. This heterogeneity is driven by complex interactions between immune and metabolic pathways, which together determine the trajectory from infection to systemic inflammation, immune suppression, and multi-organ failure (4).

Metabolic reprogramming is now recognized as a hallmark of immune cell activation and function during sepsis (5–7). Innate immune cells, such as neutrophils and monocytes, rapidly shift their metabolic profiles to meet the energy demands of pathogen clearance, whereas adaptive immune cells undergo distinct metabolic adaptations that influence survival and effector function (8, 9). Disruption of these tightly regulated metabolic–immune interactions can exacerbate inflammation or promote immune paralysis, both of which contribute to poor clinical outcomes. However, the precise metabolic drivers of immune dysregulation in sepsis remain incompletely understood.

High-throughput transcriptomic profiling has enabled comprehensive interrogation of gene expression programs underlying sepsis pathophysiology (10–12). While previous studies have examined immune-related genes or signaling pathways, few have systematically explored metabolism-related genes (MRGs) in the context of sepsis, particularly in relation to immune cell composition, intercellular communication, and patient prognosis (13–15). Furthermore, integration of bulk transcriptomics with single-cell RNA sequencing (scRNA-seq) offers an unprecedented opportunity to link transcriptional alterations to specific immune cell populations and to identify cell-type-specific therapeutic targets.

In this study, we performed an integrative multi-omics analysis of MRGs in sepsis, combining bulk RNA-seq, external cohort validation, and single-cell transcriptomic datasets. We established a robust immune–metabolic risk score system that stratifies patients into distinct metabolic–immune subtypes with divergent immune infiltration patterns and predicted outcomes. Through machine

learning-based feature selection, we identified five hub genes driving this stratification and validated their prognostic performance in an independent dataset. Among them, GYG1 emerged as a key gene associated with innate immune hyperactivation, predominantly expressed in neutrophils and monocytes. Functional validation in an LPS-induced sepsis mouse model demonstrated that lipid nanoparticle (LNP)–mediated siRNA silencing of *Gyg1* significantly ameliorated disease severity. Our findings provide new insights into the metabolic–immune heterogeneity of sepsis and highlight GYG1 as a promising therapeutic target.

Materials and methods

Bulk RNA-seq data acquisition and processing

Gene expression data of sepsis patients were retrieved from the GEO database (GSE57065) (16) and normalized using the Robust Multi-array Average (RMA) algorithm in R. Differentially expressed genes (DEGs) at 24 h and 48 h post-disease onset were identified relative to baseline (0 h) using the “limma” package (17), with $|\log_2 \text{fold change}| > 1$ and adjusted $p < 0.05$ as cutoffs. A list of metabolism-related genes (MRGs) was compiled from MSigDB metabolic gene sets and relevant literature (18), and the intersection between DEGs and MRGs was calculated. Heatmaps of intersected MRGs were generated using the “pheatmap” package with hierarchical clustering (19).

Consensus clustering and immune infiltration analysis

Patients were classified into metabolic subgroups based on the expression of intersected MRGs using consensus clustering (ConsensusClusterPlus) (20). Immune cell composition was estimated via the CIBERSORT algorithm (21), and differences between subgroups were assessed by Wilcoxon rank-sum test. Gene Set Enrichment Analysis (GSEA) and Gene Ontology (GO)

enrichment were performed to explore functional differences between clusters (22, 23). Volcano plots and functional enrichment bubble charts were created in R.

Machine learning and construction of the metabolic risk score

Least Absolute Shrinkage and Selection Operator (LASSO) regression was applied to DEGs between metabolic clusters to identify hub genes using the “glmnet” package (24). The metabolic risk score was calculated as a weighted sum of hub gene expression, with coefficients derived from LASSO regression. The predictive performance of each gene was evaluated by receiver operating characteristic (ROC) curve analysis in the external dataset GSE95233 (25).

Single-cell RNA-seq data processing and annotation

Single-cell transcriptomic data of peripheral blood mononuclear cells from sepsis patients and healthy controls were obtained from published datasets GSE167363 (26). Data were processed using Seurat in R (27), including quality filtering, normalization, scaling, and principal component analysis. Cells were clustered via graph-based methods and visualized using Uniform Manifold Approximation and Projection (UMAP) (28). Cell types were annotated with canonical marker genes, and platelet/erythroid lineages were excluded from downstream analyses. The distribution of immune subsets was compared between high- and low-risk groups. Cell clusters were annotated based on the expression of canonical marker genes. Clusters exhibiting dominant expression of platelet markers (PPBP, PF4) or erythroid markers (HBB) were excluded from subsequent analysis to focus on nucleated immune cell populations. Hub gene expression patterns across immune cell subsets were assessed using violin plots.

Cell–cell communication analysis

Cell–cell interaction networks were inferred using the CellChat package (29). Ligand–receptor interaction frequencies and strengths were compared between high- and low-risk groups. Differential communication patterns were visualized via heatmaps and network diagrams, with a focus on monocyte–dendritic cell interactions.

In vivo validation and LNP–siRNA delivery

Eight-week-old female C57BL/6 mice were used for an LPS-induced sepsis model. Mice were randomly assigned to control, LNP-control siRNA, and LNP–siGyg1 groups (n = 10 per group).

Lipid nanoparticles (LNPs) containing siRNA targeting Gyg1 were formulated using DLin-MC3-DMA as the cationic lipid component (30). LNP–siGyg1 was administered intravenously 24 hours prior to LPS injection (10 mg/kg, intraperitoneal) and repeated every 24 h. SiRNA targeting Gyg1 was acquired from Thermo Fisher. qPCR was performed on peripheral blood samples to measure hub gene expression. All qPCR analyses in this study were normalized to β -actin, which served as the internal reference gene. The relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. Total leukocytes (RBC-lysed peripheral blood cells) were collected from mice treated with either control LNPs or LNP–siGyg1. Flow cytometric sorting was subsequently performed to purify monocytes (CD11b⁺Ly6G[−]) and neutrophils (Ly6G⁺) from the same samples. Survival was monitored for every 40 hours post-LPS injection, and differences were analyzed by Kaplan–Meier curves. All mice were obtained from The Jackson Laboratory and housed under specific pathogen-free conditions at the Zunyi Medical University animal facility. All animal procedures were approved by the Zunyi Medical University ethics committee with the approved number (2024) 1-068.

Flow cytometry, glycogen quantification, serum cytokine ELISA and cell migration assay

Peripheral blood cells were collected into heparinized tubes and subjected to red blood cell lysis (ACK buffer, Thermo Fisher). Total leukocytes were stained with fluorescent antibodies against CD45, CD11b, Ly6G, CD11c, MHC-II, CD4, CD8, NK1.1, and B220 (BioLegend). The markers are as follows: Neutrophils (CD11b⁺Ly6G⁺), Monocytes (CD11b⁺Ly6G[−]), Dendritic cells (CD11c⁺), T cells (CD4⁺ or CD8⁺), B/NK cells (B220⁺ or NK1.1⁺). Flow cytometry and sorting were performed on BD FACSARIA III. Data were analyzed with FlowJo v10.

As for glycogen quantification, Sorted neutrophils and monocytes were lysed, and glycogen content was determined using a Glycogen Assay Kit (Sigma-Aldrich, MAK016) following the manufacturer’s instructions. Absorbance was measured at 570 nm, and results were normalized to cell number and expressed as $\mu\text{g}/\mu\text{L}$ glycogen. Serum levels of IL-6, TNF- α , and IL-1 β were quantified 6h after LPS treatment using mouse ELISA kits (R&D Systems) according to the manufacturer’s protocol. Cytokine concentrations were determined by standard curves and expressed in pg/mL.

To assess the migratory capacity of immune cells after Gyg1 knockdown, neutrophils and monocytes were isolated from peripheral blood of LNP–siGyg1- or LNP–ctrl-treated mice using FACS sorting. Migration assays were performed using 24-well Transwell chambers with 5- μm pore polycarbonate membranes (Corning, 3421). Cells (1 million per insert) were suspended in serum-free RPMI-1640 medium and placed in the upper chamber, while the lower chamber contained medium supplemented with CCL2 (100 ng/mL) for monocytes or CXCL1 (100 ng/mL) for neutrophils (PeproTech). After incubation for 30 min or 3 hours at

37 °C, cells that migrated to the lower chamber were collected and counted using a hemocytometer or flow cytometry. Migration efficiency was expressed as the percentage of input cells migrated. All experiments were performed in triplicate.

Statistical analysis

Statistical analyses were performed using R (v4.1.2) and Python (v3.8). Data are expressed as mean $\pm$ standard deviation (SD). Two-group comparisons were conducted with Wilcoxon rank-sum tests, while multi-group comparisons used one-way ANOVA followed by Tukey's *post hoc* test. Survival differences were assessed by log-rank test. A *p*-value < 0.05 was considered statistically significant.

Results

Identification and characterization of metabolism-related genes in sepsis

To systematically explore the role of metabolism-related genes (MRGs) in sepsis, we first analyzed transcriptional changes at three time points—0 h, 24 h, and 48 h—using the whole blood transcriptomic dataset GSE57065. Differential expression analysis was conducted by comparing patient samples to healthy samples (Figure 1A–C). A total of 70 differentially expressed genes (DEGs) were identified across all time points, indicating a dynamic transcriptomic response during the early progression of sepsis. To specifically evaluate the contribution of metabolic pathways, we intersected these DEGs with a curated list of metabolism-related genes, yielding a panel of metabolic DEGs with potential relevance to sepsis pathophysiology (Figure 1D). Notably, many of these genes are involved in key processes such as mitochondrial function, glycolysis, and amino acid metabolism. We next visualized the expression of these metabolic DEGs across all patient samples (Figure 1E). Heatmap clustering analysis revealed that several genes exhibited consistent temporal trends, suggesting potential regulatory programs associated with metabolic reprogramming in sepsis (Figure 1F). These genes may serve as candidate biomarkers or therapeutic targets, warranting further validation.

Metabolism-related gene expression stratifies sepsis patients into distinct immunological subgroups

To determine whether metabolism-related genes (MRGs) could distinguish biologically distinct subgroups of sepsis patients, we performed unsupervised hierarchical clustering based on the expression of previously identified MRGs. This approach stratified patients into two major clusters, designated Cluster 1 and Cluster 2 (Figure 2A). The clustering revealed substantial transcriptional heterogeneity, suggesting differential metabolic states among patients. We then identified differentially expressed

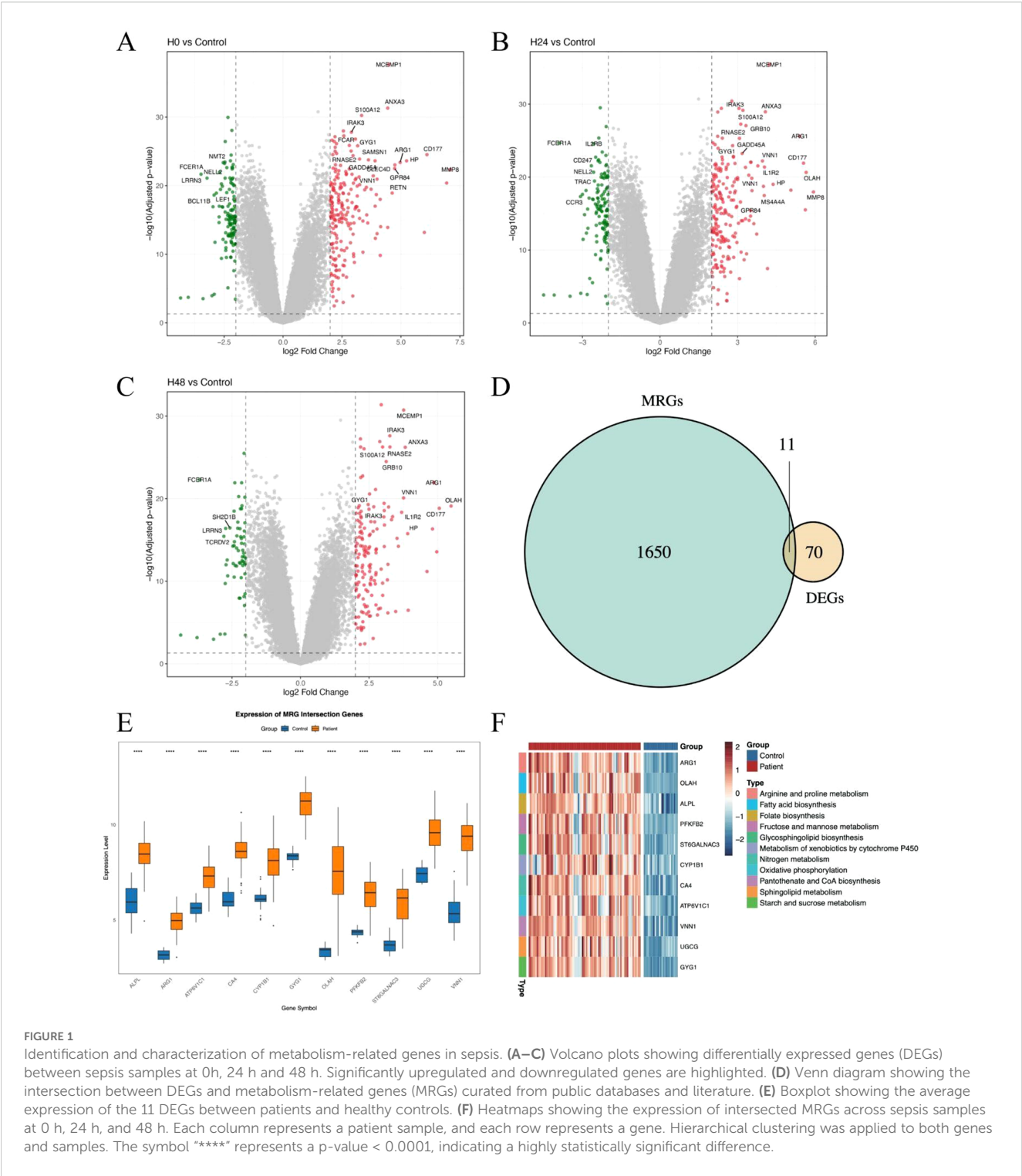
genes (DEGs) between the two clusters to investigate their molecular differences. A large number of DEGs were observed, with both significantly upregulated and downregulated genes distinguishing the two groups (Figure 2B). Subsequent Gene Ontology (GO) enrichment analysis of the DEGs revealed that Cluster 1 was characterized by enrichment in a neutrophil-dominant immune response state (Figure 2C). Furthermore, Gene Set Enrichment Analysis (GSEA) reinforced these findings: Cluster 1 showed significant enrichment in neutrophil responses and defense against pathogens (Figure 2D), whereas Cluster 2 was more enriched in T cell responses (Figure 2E). Together, these findings suggest that metabolism-based clustering captures biologically meaningful immunological differences and may help explain the variable clinical outcomes observed in sepsis.

Identification of hub genes and construction of a metabolic risk score model in sepsis

To further refine clinically relevant metabolic signatures in sepsis, we applied a machine learning approach to the 11 differentially expressed genes (DEGs) identified between Cluster 1 and Cluster 2. Least absolute shrinkage and selection operator (LASSO) regression was performed to reduce feature redundancy and select the most informative genes (Figures 3A, B). Five hub genes—ALPL, CYP1B1, GYG1, OLAH, and VNN1—were identified as optimal predictors based on the minimum binomial deviance criteria. We then constructed a metabolic risk score model based on the expression levels of these five genes. The risk score formula was defined as: Risk score = $0.231 \times \text{ALPL} + 0.218 \times \text{CYP1B1} + 1.930 \times \text{GYG1} + 0.0829 \times \text{OLAH} + 0.277 \times \text{VNN1}$. This score effectively stratified patients from Cluster 1 and Cluster 2, with significantly higher scores observed in patients from the neutrophil-dominant Cluster 1 (Figure 3C), indicating that the hub gene signature captures cluster-specific immunometabolic phenotypes. To further examine the expression patterns of the hub genes, we visualized their expression in all samples (Figure 3D). Notably, all five hub genes were significantly upregulated in Cluster 1 compared to Cluster 2 (Figures 3E–I), suggesting that elevated expression of these genes is associated with the hyperinflammatory metabolic state seen in neutrophil-dominant sepsis subtypes.

Hub genes demonstrate robust and accurate diagnostic performance in an independent validation cohort

To validate the diagnostic potential of the five identified hub genes, we applied the model to an independent external dataset (GSE95233, Supplementary Table 1), which includes peripheral blood samples from septic patients and healthy controls. All five hub genes—ALPL, CYP1B1, GYG1, OLAH, and VNN1—were significantly upregulated in septic patients compared to healthy individuals (Figure 4A), consistent with findings from the discovery cohort. We further evaluated the predictive performance of each gene using receiver



operating characteristic (ROC) curve analysis. Strikingly, all five genes demonstrated excellent discriminative power, with area under the curve (AUC) values of 0.981 for ALPL, 0.951 for CYP11B1, 1.000 for GY1, 0.978 for OLAH, and 0.994 for VNN1, respectively (Figure 4B). The consistently high AUC values suggest that this metabolic gene signature may serve as a robust biomarker panel for sepsis detection. It is worth noting that the perfect classification performance of GY1 (AUC = 1.0) in the validation cohort may be partly attributed to the limited sample size and needs to be interpreted with caution. Nonetheless, these findings support the strong diagnostic relevance of the identified hub genes. Our model also achieved an AUC of 0.673 (Day 1) and 0.722 (Day 3) in predicting the survival of the sepsis patients in the external validation cohort (new Figure 4C). This performance is comparable to the reported efficacy of gold-standard clinical scores like SOFA and APACHE II from large-scale studies, which typically show AUCs in the range of 0.65-0.75 for survival prediction (31, 32).

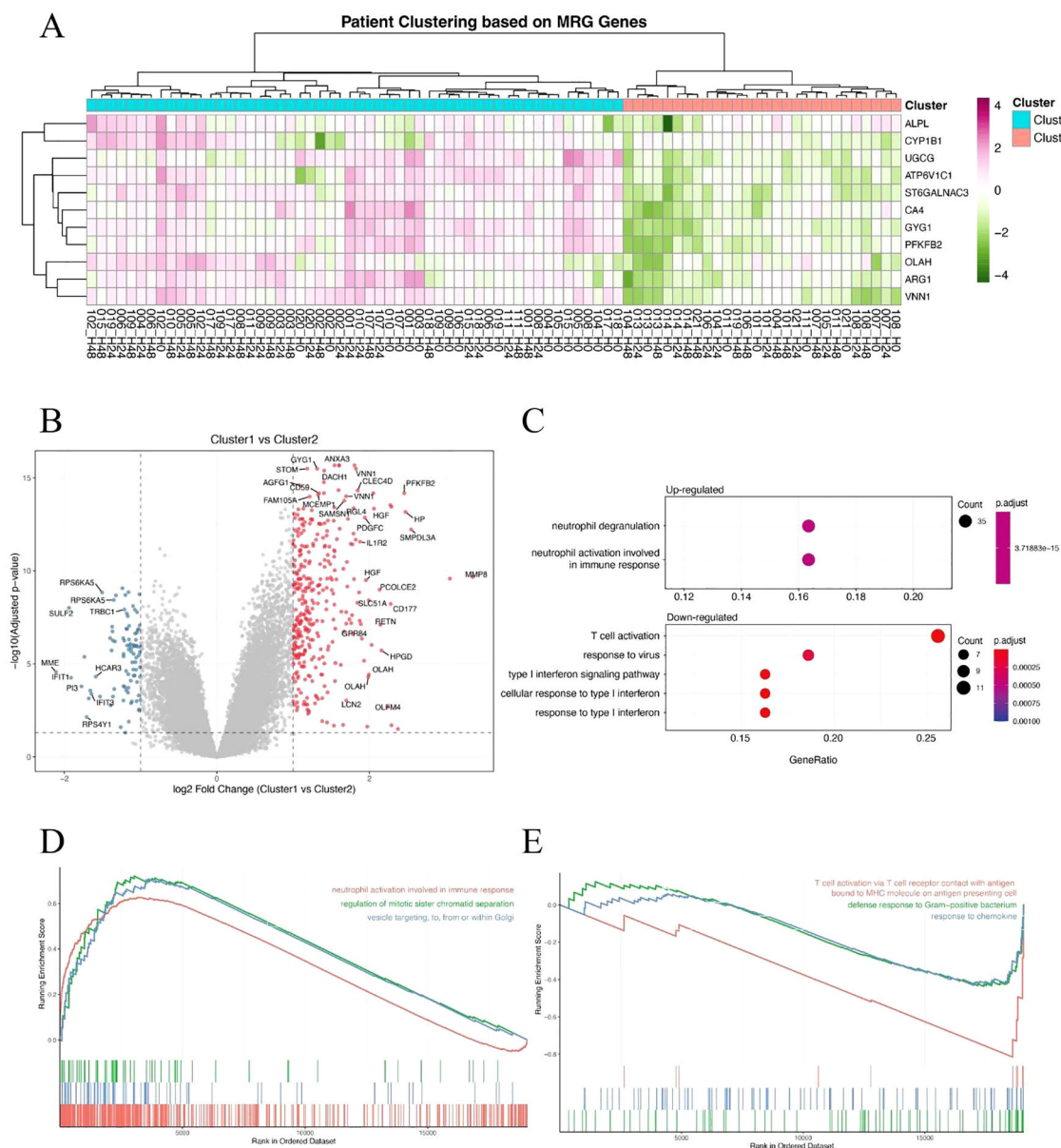


FIGURE 2

Metabolism-related gene expression stratifies sepsis patients into distinct immunological subgroups. (A) Hierarchical clustering of sepsis patients based on metabolism-related gene (MRG) expression, revealing two clusters (Cluster 1 and Cluster 2). (B) Volcano plot showing differentially expressed genes (DEGs) between Cluster 1 and Cluster 2. Red and blue dots represent significantly upregulated and downregulated genes, respectively. (C) GO biological process enrichment analysis (GSEA) of hallmarks or GO terms between the two clusters. Cluster 1 shows enrichment in neutrophil activation and antibacterial responses (D), while Cluster 2 is associated with T cell-mediated immune pathways (E).

High metabolic risk score in sepsis is associated with neutrophil and monocyte-dominant and lymphocyte-suppressed immune infiltration

To explore the immunological differences between metabolic subgroups of sepsis, we investigated the immune cell infiltration landscape of high- and low-risk patients defined by our five-gene risk score model. Using the CIBERSORT deconvolution algorithm, we estimated the relative proportions of 22 immune cell types across

all patients. Significant differences in immune infiltration were observed between the two groups (Figure 5A). High-risk patients exhibited markedly elevated levels of neutrophils and monocytes, alongside reduced proportions of natural killer (NK) cells and multiple T cell subpopulations, including CD4⁺ and CD8⁺ T cells. These findings suggest that high metabolic risk in sepsis is associated with a neutrophil-dominant, lymphocyte-suppressed immune landscape. To further elucidate the immunological relevance of each hub gene, we performed Pearson correlation analysis between gene expression levels and estimated immune

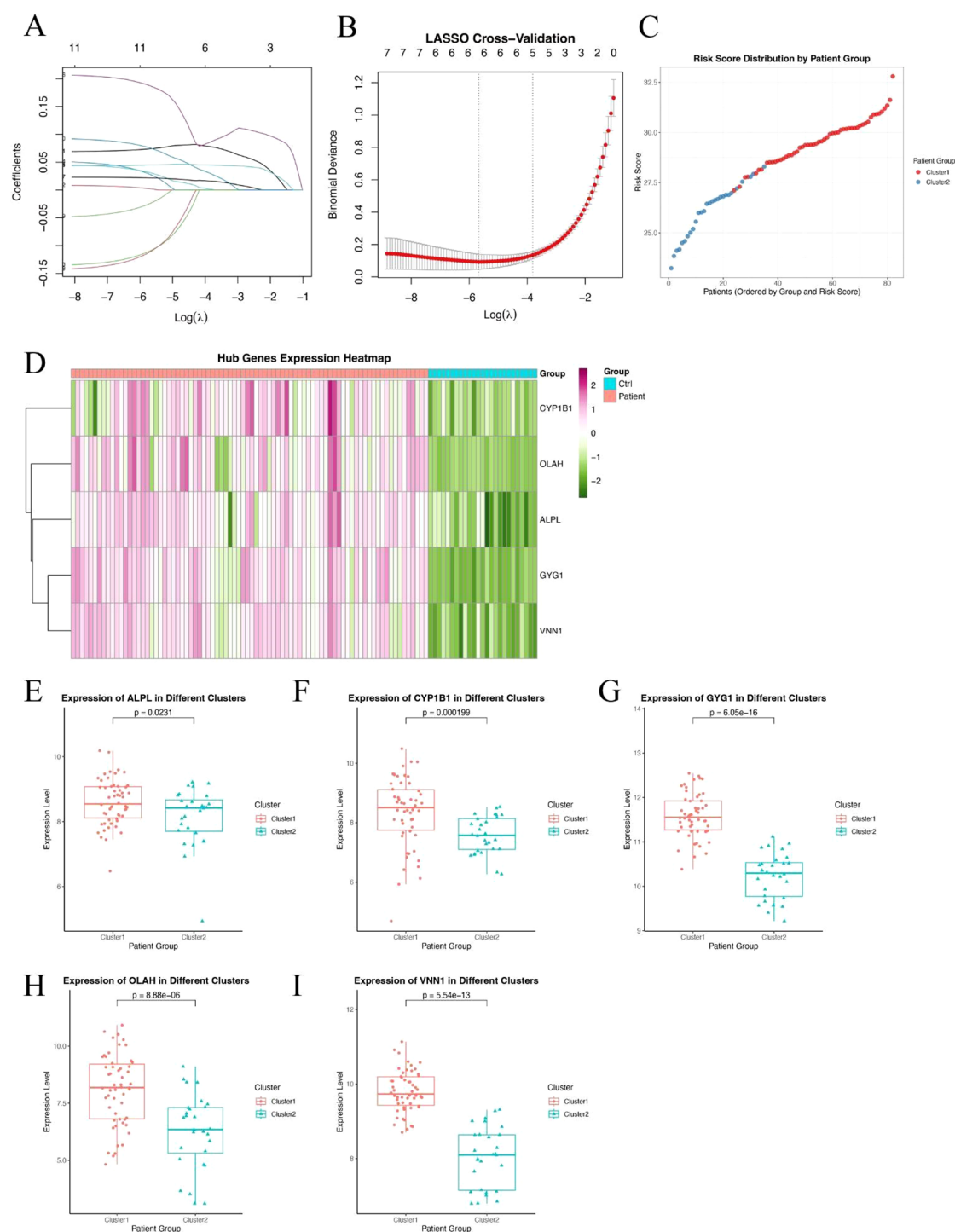


FIGURE 3

Identification of hub genes and construction of a metabolic risk score model. (A, B) LASSO regression and cross-validation plots based on 11 DEGs between Cluster 1 and Cluster 2. Five hub genes were selected based on the optimal lambda value. (C) Distribution of the calculated risk score across patients from Cluster 1 and Cluster 2, showing higher scores in neutrophil-dominant Cluster 1. (D) Heatmap showing expression patterns of the five hub genes (ALPL, CYP1B1, GYG1, OLAH, VNN1) across all sepsis samples. (E–I) Box plots showing significantly higher expression levels of ALPL (E), CYP1B1 (F), GYG1 (G), OLAH (H), and VNN1 (I) in Cluster 1 compared to Cluster 2. P-values are indicated above each comparison.

cell fractions (Figure 5B). All five risk genes—ALPL, CYP1B1, GYG1, OLAH, and VNN1—showed strong positive correlations with neutrophils, monocytes, and macrophages, and consistent negative correlations with T cells and NK cells. These results

suggest that the severity of sepsis may be linked to an imbalance between innate and adaptive immune responses, with high-risk patients exhibiting a shift toward an innate immunity-dominated profile.

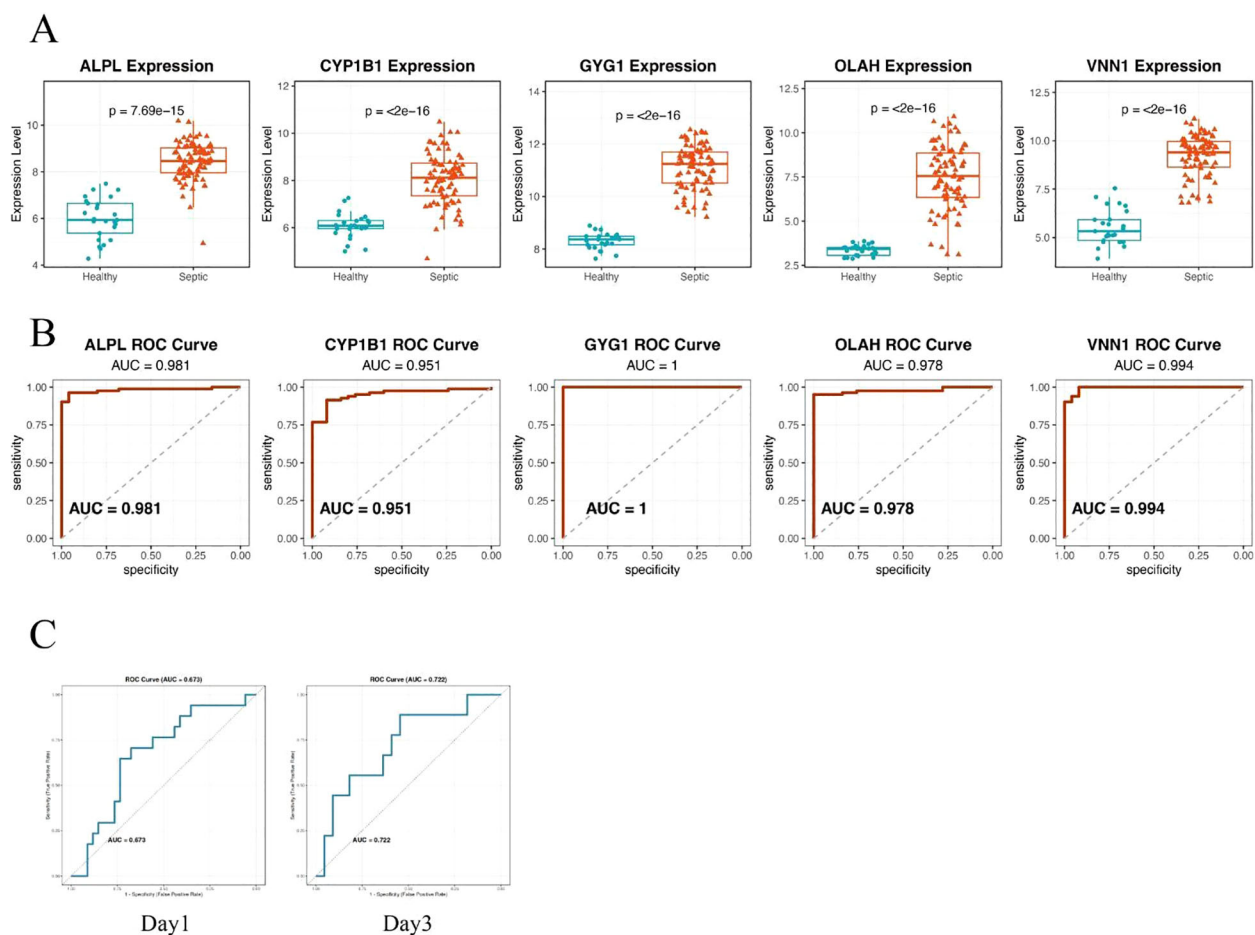


FIGURE 4

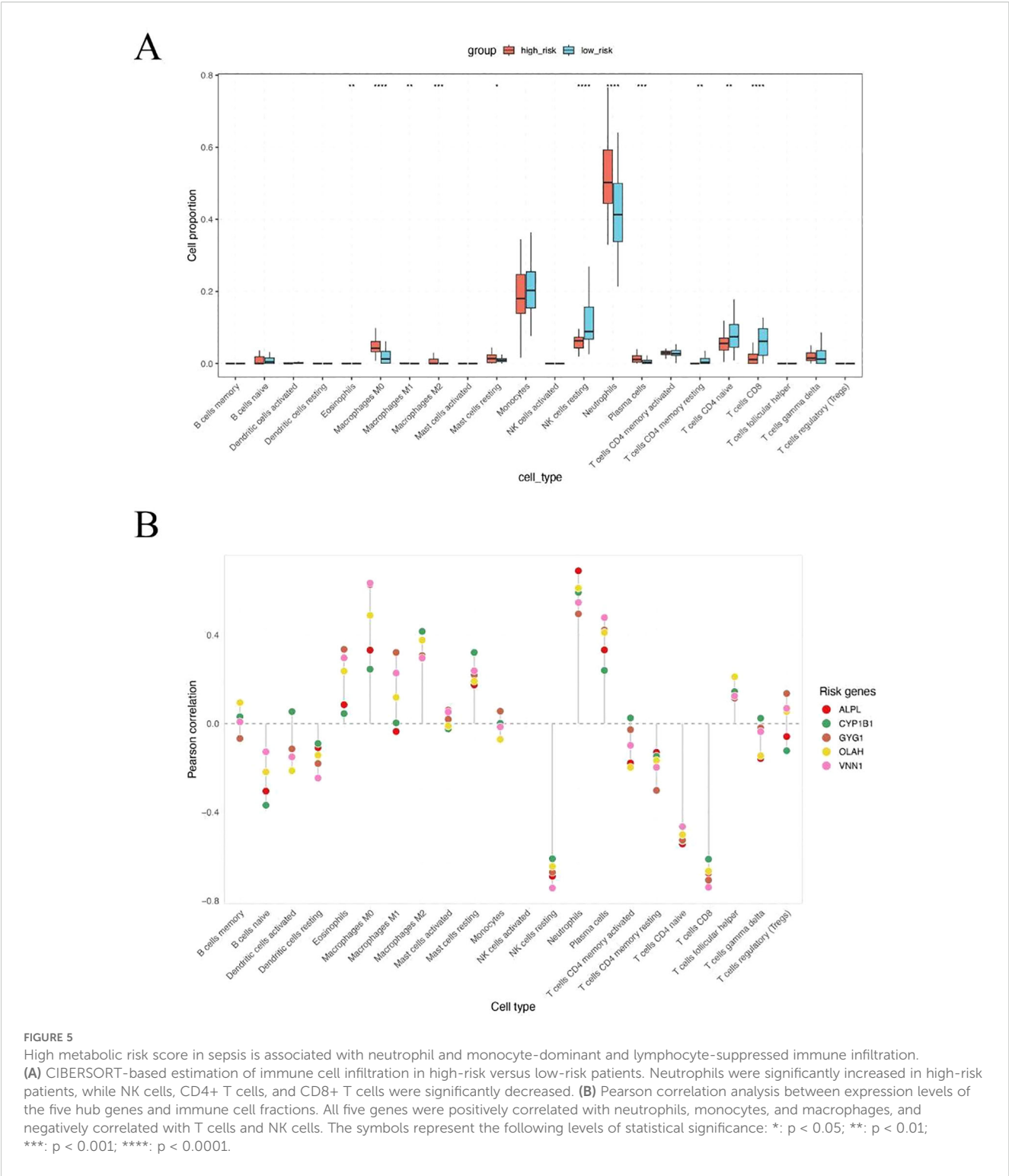
Hub genes demonstrate robust and accurate diagnostic performance in an independent validation cohort. **(A)** Box plots showing significantly higher expression levels of the five hub genes—ALPL, CYP1B1, GYG1, OLAH, and VNN1—in septic patients compared to healthy controls. **(B)** ROC curve analysis demonstrating the diagnostic performance of each gene in distinguishing sepsis from healthy controls. All five genes exhibited high predictive accuracy, with AUC values exceeding 0.95. **(C)** ROC curve of the metabolic risk score model in predicting the survival of sepsis patients based on the blood samples of day 1 (left) or day 3 (right).

Single-cell analysis of immune cell composition in septic patients

To further validate the association between metabolic risk and immune cell remodeling in sepsis, we performed integrative single-cell transcriptomic analysis using published peripheral blood mononuclear cell (PBMC) datasets. After standard quality control, normalization, and dimensionality reduction, immune cells were clustered and visualized using UMAP (Figures 6A, B). Cell identities were annotated based on canonical marker genes, and platelet/erythroid lineage cells were excluded to focus on immunologically relevant populations (Figure 6C). Comparative analysis of cell composition across clinical groups revealed that non-surviving septic patients exhibited a markedly higher proportion of neutrophils compared to both healthy controls and survivors (Figure 6D). This observation is consistent with our earlier findings based on CIBERSORT deconvolution of bulk transcriptomic data, reinforcing the association between

neutrophil and innate immune dominance and poor clinical outcome in sepsis. To further investigate the cellular origin of the risk score components, we analyzed the expression patterns of the five hub genes across different immune subsets (Figure 6E). Three of the five genes—ALPL, CYP1B1, and GYG1—were predominantly expressed in monocytes, neutrophils, and proliferating myeloid cells, consistent with their potential roles in innate immune activation. In contrast, OLAH and VNN1 showed low expression across all major immune cell types, suggesting that these genes may be expressed in non-immune blood components (e.g., endothelial cells or platelets) and contribute to sepsis progression through indirect mechanisms.

To better characterize the immune context of metabolic risk in sepsis, we compared the single-cell distribution patterns between high- and low-risk patients. Immune cells were first annotated based on canonical marker expression (Figure 6F). Density estimation maps revealed that high-risk patients showed marked expansion of monocytes, along with a notable reduction in T cells



compared to the low-risk group (Figures 6G, H). These findings reinforce the hypothesis that severe sepsis is associated with a shift toward innate immunity dominance, coupled with adaptive immune suppression. These results suggest that innate immune hyperactivation, particularly neutrophil and monocyte expansion, may play a critical role in driving sepsis severity and mortality.

Single-cell communication analysis reveals intensified monocyte–DC interactions in high-risk sepsis patients

To investigate potential alterations in intercellular communication associated with metabolic risk, we performed ligand–receptor

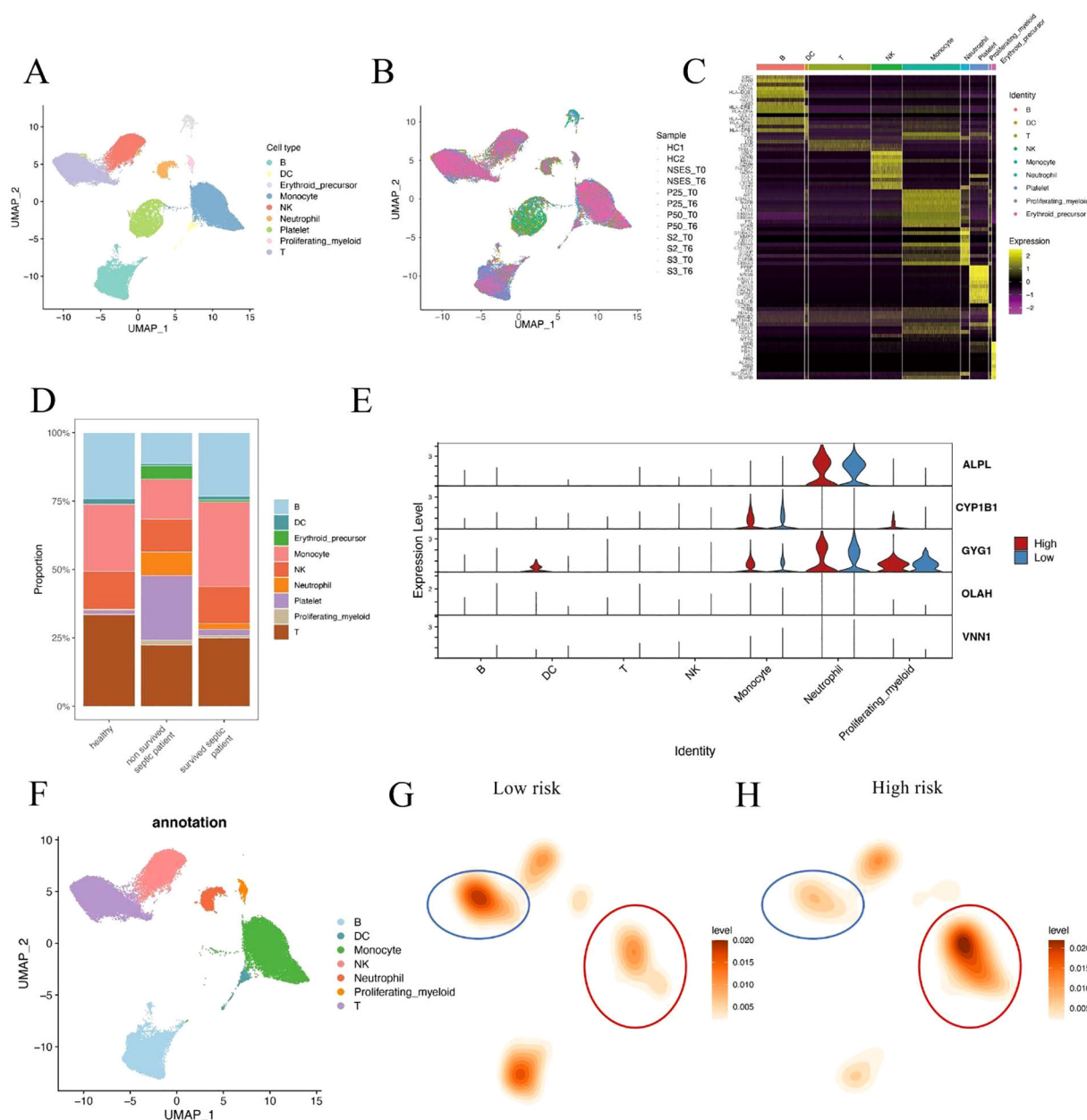


FIGURE 6

Single-cell analysis of immune cell composition in septic patients. (A, B) UMAP visualization of integrated single-cell RNA-seq data from septic patients and healthy controls. Cells were clustered and annotated based on canonical immune markers. Platelet and erythroid cells were excluded from downstream analysis. (C) Heatmap of representative marker gene expression used for immune cell type annotation. (D) Cell composition comparison across clinical groups shows a significantly increased neutrophil fraction in non-surviving sepsis patients relative to survivors and healthy controls. (E) Violin plots showing expression of hub genes (ALPL, CYP1B1, GYG1, OLAH, VNN1) across immune cell subsets in high- and low-risk patients. Three genes were enriched in innate immune cells, while two showed minimal expression in immune lineages. (F) UMAP plot showing immune cell type annotations used in downstream comparison. (G, H) Density maps illustrating differences in immune cell distributions between low-risk (G) and high-risk (H) patients. Monocytes were expanded in high-risk patients, while T cell density was reduced.

interaction inference based on single-cell expression profiles in high- and low-risk sepsis patients. A total of 608 inferred interactions were detected in the high-risk group, compared to 526 in the low-risk group (Figure 7A), indicating a globally enhanced signaling environment in high-risk patients. Relative information flow analysis revealed that high-risk patients exhibited greater activity across a range of signaling pathways (Figures 7B, C), particularly those associated with

inflammation and antigen presentation (e.g., CD40, MIF, SELPLG). In contrast, several homeostatic or regulatory pathways (e.g., CCL, CD46) were more active in the low-risk group. Heatmap visualization of outgoing signaling patterns further demonstrated that high-risk patients had enhanced pathway activity originating from monocytes and dendritic cells (DCs) (Figures 7D, E). Network topology analysis revealed a dense interaction hub centered around these two cell types

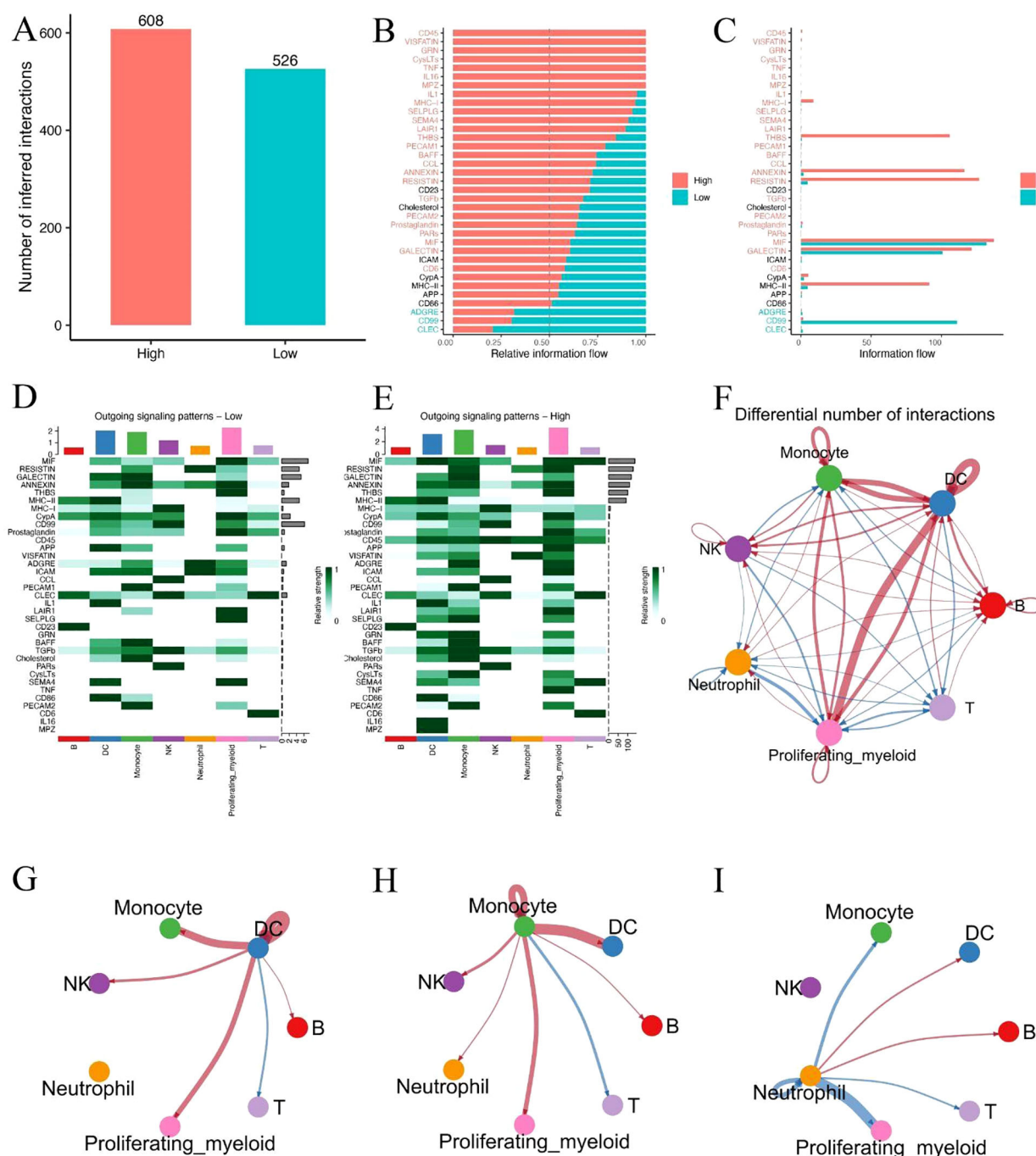


FIGURE 7

Single-cell communication analysis reveals intensified monocyte–DC interactions in high-risk sepsis patients. (A) Total number of predicted ligand–receptor interactions in high- and low-risk groups. (B, C) Relative and absolute information flow across signaling pathways in both groups. (D, E) Heatmaps showing outgoing signaling strength from each cell type in low-risk (D) and high-risk (E) patients. (F) Overall cell–cell communication network based on differential interaction strength. (G–I) Focused visualization of directed intercellular signaling networks, highlighting intensified monocyte–DC interactions in the high-risk group.

in high-risk patients, with significantly elevated communication frequency and intensity compared to low-risk individuals (Figure 7F). Notably, focused cell–cell interaction mapping confirmed that monocyte–DC signaling was markedly stronger in the high-risk

group (Figures 7G–I). These findings suggest that increased monocyte–DC crosstalk may contribute to the hyperinflammatory immune environment and disease progression in metabolically high-risk sepsis patients.

GYG1 is highly expressed in innate immune cells and associated with pro-inflammatory transcriptional programs in high-risk patients

Given the substantial differences in dendritic cells (DCs), monocytes, and neutrophils between high- and low-risk patients, we examined the expression of the five hub genes across these three cell types. Among them, GYG1 was consistently expressed in all three cell populations and showed significantly higher expression in the high-risk group (Figure 8A), suggesting that it may play a key role in orchestrating innate immune activation in metabolically high-risk patients. We next performed differential gene expression (DEG) analysis between high- and low-risk patients within each of the three cell types. The volcano plots revealed a large number of upregulated genes in high-risk DCs (Figure 8B), monocytes (Figure 8C), and neutrophils (Figure 8D), many of which are associated with innate immune functions. Gene Ontology enrichment analyses of the upregulated DEGs in each cell type showed consistent results: neutrophil degranulation and activation-related pathways were significantly enriched in all three cell populations (Figures 8E–G). These findings suggest that GYG1 may be involved in driving pro-inflammatory transcriptional programs in key innate immune subsets, potentially contributing to disease severity in high-risk sepsis patients.

LNP-mediated Gyg1 silencing improves survival in LPS-induced sepsis mouse model

To validate the *in vivo* relevance of the hub genes identified in our computational analyses, we first measured their expression in peripheral blood from an LPS-induced sepsis mouse model using qPCR. Among the five hub genes (*Alpl*, *Cyp1b1*, *Gyg1*, *Olah*, *Vnn1*), all except *Olah* and *Vnn1* were significantly upregulated in septic mice compared to controls, with *Gyg1* showing the most pronounced increase (Figures 9A–E). Given the strong predictive performance of *Gyg1* and its association with pro-inflammatory innate immune programs, we next investigated whether targeting *Gyg1* could ameliorate sepsis outcomes. We formulated lipid nanoparticles (LNPs) encapsulating siRNA specific to *Gyg1* (LNP-SiGyg1) and administered them intravenously 24 hours prior to LPS injection (10 mg/kg) and at 24-hour intervals thereafter (Figure 9F). To confirm the cellular specificity and efficiency of LNP-SiGyg1 delivery, we evaluated GYG1 protein expression in distinct peripheral immune subsets. Western blot analysis demonstrated a marked reduction of GYG1 protein in both monocytes and neutrophils following LNP-SiGyg1 treatment, whereas total leukocytes also exhibited an overall decrease, confirming effective knockdown of *Gyg1* in circulating myeloid cells (Figure 9G). Kaplan–Meier survival analysis revealed that *Gyg1* silencing markedly improved survival compared to both untreated and LNP-control siRNA-treated groups (Figure 9H), suggesting that metabolic targeting of *Gyg1* may offer therapeutic benefit in sepsis.

GYG1 knockdown reduces glycogen content and inflammatory activation in myeloid cells *in vivo*

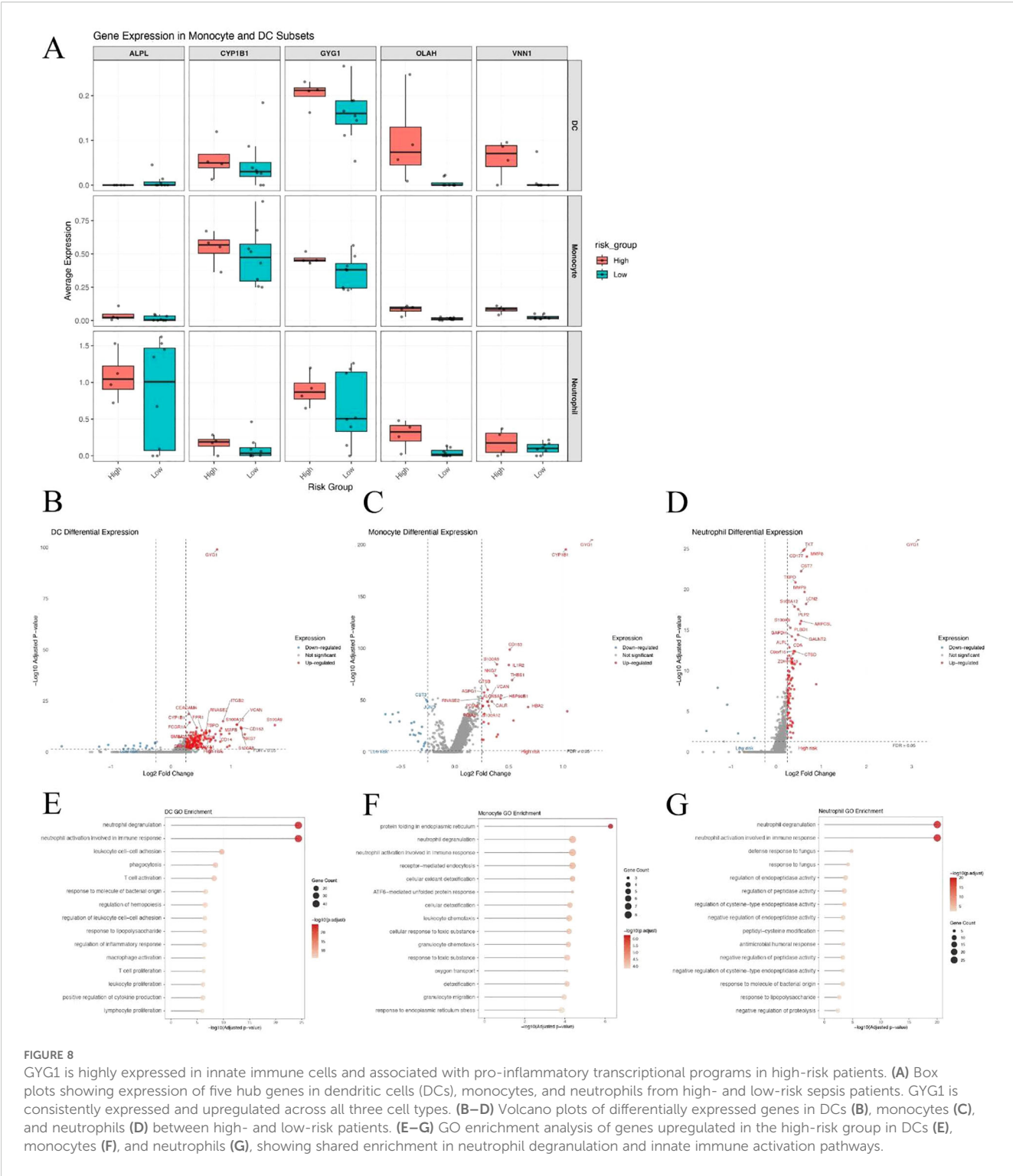
To further explore the mechanisms of *Gyg1* knockdown's impact on immune metabolism and inflammation, we examined glycogen levels and inflammatory responses in myeloid cells from LPS-induced septic mice treated with LNP-SiGyg1. Quantification of glycogen content revealed that *Gyg1* silencing significantly reduced intracellular glycogen levels in both neutrophils and monocytes compared with control LNPs (Figure 10A), confirming the metabolic efficacy of our knockdown strategy. Next, we evaluated the transcriptional and systemic inflammatory response following *Gyg1* inhibition. qPCR analysis showed that *Gyg1* knockdown markedly decreased the mRNA expression of IL-6, TNF- α , and IL-1 β in peripheral monocytes and neutrophils from LPS-challenged mice (Figure 10B). Consistently, ELISA of serum samples demonstrated reduced protein levels of IL-6 and TNF- α , whereas IL-1 β levels were not significantly affected (Figure 10C).

To further investigate immune cellular changes, we performed flow cytometry profiling of major leukocyte subsets. *Gyg1* knockdown strongly attenuated the LPS-induced expansion of neutrophils, while the decreased proportions of T and B/NK cells were partially rescued (Figure 10D). Moreover, *Gyg1* silencing slightly reduced CD40 expression in both monocytes and dendritic cells (Figure 10E), suggesting that diminished glycogen metabolism may suppress pro-inflammatory activation and antigen-presenting potential in myeloid populations. Together, these data demonstrate that *Gyg1* depletion alleviates hyperinflammation in sepsis by reducing metabolic fuel availability and dampening myeloid activation.

Discussion

In this study, we performed a comprehensive analysis of metabolism-related genes (MRGs) in sepsis and developed an immune-metabolic risk score capable of stratifying patients into distinct subgroups with divergent immune landscapes and predicted outcomes. Through machine learning-based feature selection, we identified five hub genes—ALPL, CYP1B1, GYG1, OLAH, and VNN1—that exhibited strong predictive performance, both in the discovery and independent validation cohorts. Single-cell transcriptomic analyses further demonstrated the immune cell-specific distribution of these genes and their correlation with innate and adaptive immune components, providing mechanistic insights into the metabolic-immune heterogeneity observed in sepsis.

Among these genes, GYG1 emerged as a particularly notable candidate. GYG1 encodes glycogenin 1, a core enzyme in glycogen biosynthesis, catalyzing the attachment of glucose residues to a protein primer to initiate glycogen polymerization (33). Beyond its canonical metabolic role, glycogen metabolism has been increasingly recognized as a regulator of immune cell activation, particularly in myeloid cells (34). Our single-cell analysis revealed high expression of GYG1 in monocytes, neutrophils, and



proliferating myeloid cells—cell populations that dominate the high-risk, innate immunity-driven sepsis subtype. Functional enrichment analysis of high-risk innate immune cells consistently identified neutrophil degranulation and activation pathways, suggesting that GYG1 may facilitate rapid energy supply to fuel hyperinflammatory responses. The observation that GYG1

knockdown via LNP-siRNA delivery ameliorated disease severity in an LPS-induced sepsis model supports its potential as a metabolic driver of immune dysregulation. Previous studies have established that glycogen metabolism modulates inflammatory signaling during sepsis largely through GSK3 β activity, which integrates upstream signals to regulate transcriptional responses via NF- κ B and CREB

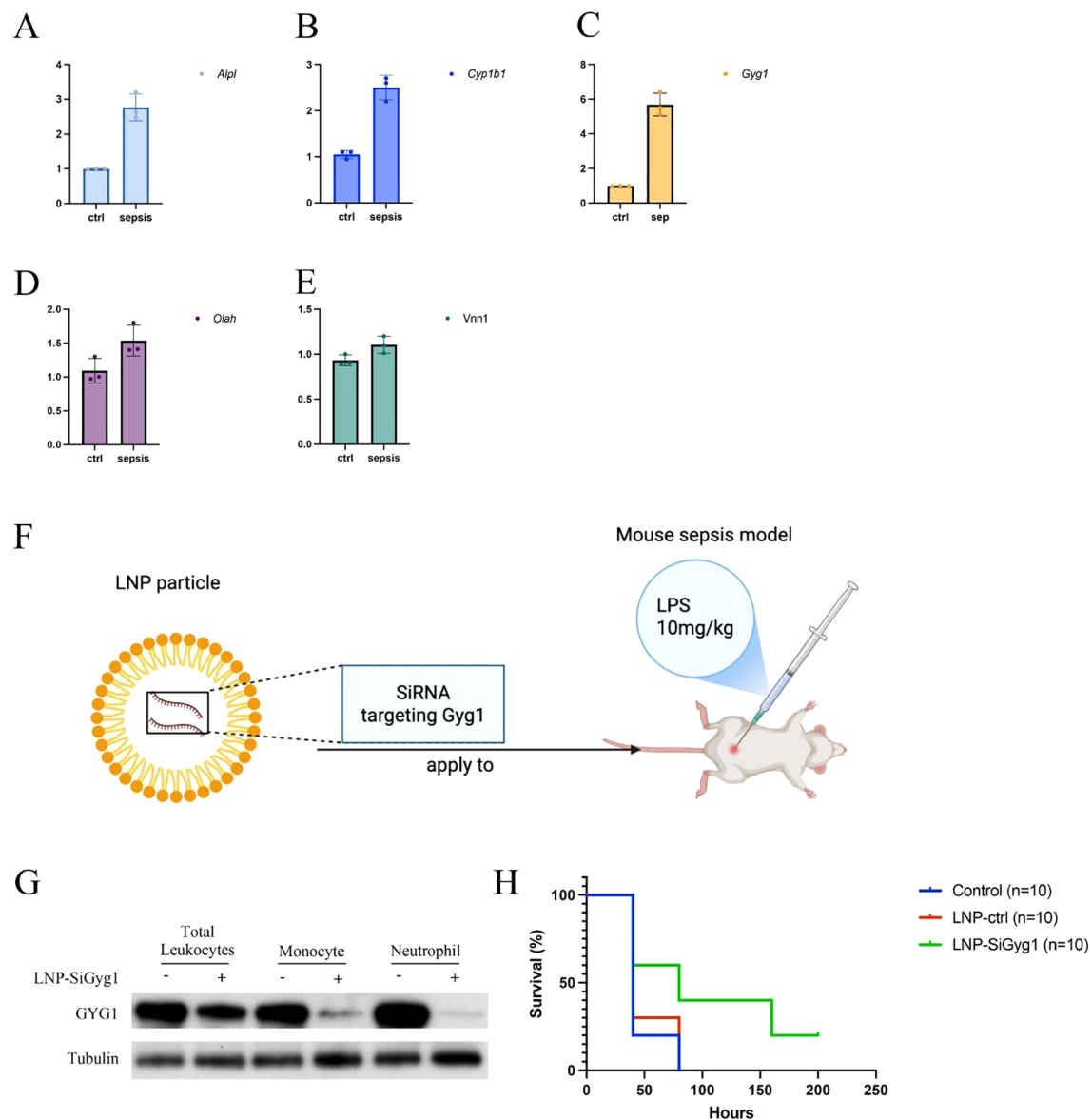


FIGURE 9

LNP-mediated *Gyg1* silencing improves survival in an LPS-induced sepsis mouse model. (A–E) qPCR analysis of hub gene expression in peripheral blood from control and LPS-induced sepsis mice. *Gyg1* exhibited the most pronounced upregulation among the five hub genes. (F) Schematic of LNP formulation and dosing strategy for *Gyg1* siRNA delivery in the LPS sepsis model. LNPs were administered intravenously 24 h before and every 24 h after LPS injection (10 mg/kg). (G, H) Kaplan–Meier survival curves showing improved survival in the LNP-SiGyg1 group compared to control and LNP-control siRNA groups.

pathways (35, 36). In contrast, *GYG1* functions at a distinct metabolic level by catalyzing the priming step of glycogen synthesis, thereby determining the cellular glycogen reserve available for rapid glycolytic activation. Our results indicate that *Gyg1* depletion reduces intracellular glycogen content and dampens cytokine production without altering canonical signaling molecules such as GSK3 β . Thus, *GYG1* complements GSK3 β -mediated immune regulation by controlling the metabolic substrate pool that sustains pro-inflammatory effector functions. This distinction highlights *GYG1* as a unique upstream regulator of immunometabolic homeostasis and a potentially novel therapeutic target in sepsis.

The other four hub genes also have plausible roles in shaping immune responses. ALPL (alkaline phosphatase, tissue-nonspecific isozyme) has been implicated in detoxifying lipopolysaccharide and modulating inflammatory signaling (37). CYP1B1, a cytochrome P450 enzyme, can influence oxidative stress and lipid mediator metabolism, thereby affecting immune cell activation (38). OLAH (oleoyl-ACP hydrolase) is involved in fatty acid metabolism, and altered lipid handling has been linked to immune suppression in late-stage sepsis (39). VNN1 (vanin-1) participates in pantothenic acid metabolism and oxidative stress regulation, and its activity may influence leukocyte recruitment (40). Collectively, these genes

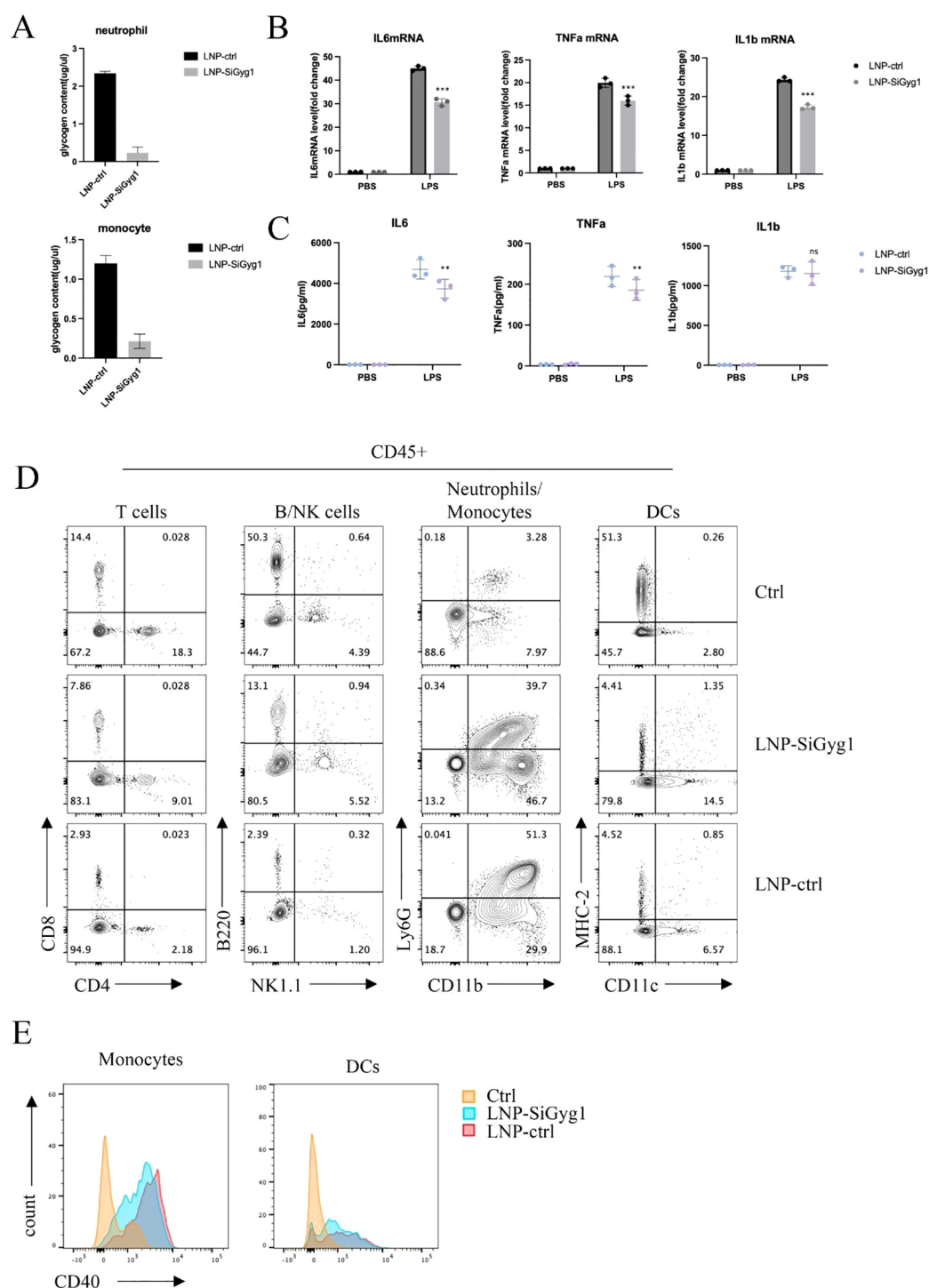


FIGURE 10

GYG1 knockdown reduces glycogen metabolism and inflammatory activation in myeloid cells *in vivo*. **(A)** Quantification of glycogen content in sorted neutrophils and monocytes from mice treated with LNP-siGyg1 or control LNPs. GYG1 knockdown significantly decreased intracellular glycogen levels in both cell types. **(B)** Relative mRNA expression of IL6, TNFα, and IL1β in peripheral monocytes and neutrophils from LPS-challenged mice (6h post LPS inject) with or without GYG1 silencing. Data were normalized to β-actin. **(C)** Serum concentrations of IL-6, TNF-α, and IL-1β measured by ELISA (6h post LPS inject). GYG1 knockdown markedly reduced IL-6 and TNF-α levels, with minor effects on IL-1β. **(D)** Flow cytometry profiling of major immune subsets (T cells, B/NK cells, neutrophils/monocytes, and dendritic cells) in septic mice treated with control or GYG1-targeting LNPs. GYG1 silencing attenuated LPS-induced neutrophil expansion. **(E)** Flow cytometric analysis of CD40 expression in monocytes and dendritic cells. GYG1 knockdown decreased CD40 expression, suggesting reduced pro-inflammatory activation. Data represent mean ± SD from three independent mice per group. $p < 0.05$, $p < 0.01$, $p < 0.001$ by one-way ANOVA with Tukey's *post hoc* test.

represent interconnected metabolic nodes that can modulate both innate and adaptive immune functions during sepsis.

Our findings align with and expand current understanding of sepsis pathophysiology. Traditionally, sepsis has been conceptualized as a biphasic process, beginning with systemic hyperinflammation driven by innate immune activation, followed by a phase of immune suppression dominated by lymphocyte exhaustion. Our work highlights metabolic heterogeneity as a determinant of these immune states, with high-risk patients showing pronounced neutrophil dominance and T/NK cell suppression. Notably, our single-cell data also revealed increased platelet abundance in high-risk patients, consistent with earlier hypotheses that coagulopathy and platelet activation contribute to sepsis-related mortality. While this observation was not a primary focus of our study, it reinforces the multifaceted nature of sepsis pathobiology, in which coagulation, metabolism, and immunity are intricately interconnected.

Therapeutically, sepsis management remains largely supportive, relying on timely antibiotics, hemodynamic stabilization, and organ support. Targeted immunomodulatory therapies have had limited success, in part due to patient heterogeneity. Our immune-metabolic risk score provides a framework for patient stratification, which could inform more personalized therapeutic approaches. Recent studies have also emphasized the translational potential of biomarkers and transcriptomic risk models for patient stratification, further supporting our findings (41–44). The proof-of-concept intervention targeting GYG1 via LNP-siRNA delivery represents a novel strategy to modulate immune metabolism in sepsis. Although further preclinical optimization is required, such approaches may complement existing therapies by selectively dampening hyperactive innate immune responses without broadly suppressing immunity.

This study has several limitations. First, although we integrated bulk and single-cell transcriptomic datasets from multiple cohorts, the patient sample size for some analyses was modest, and the external validation was limited to available public datasets. Second, our *in vivo* functional validation was performed in an LPS-induced model, which recapitulates aspects of hyperinflammation but does not fully capture the complexity of clinical sepsis. Additionally, dissecting the upstream regulatory networks and detailed downstream effectors of GYG1 in immune cells may reveal broader therapeutic opportunities.

Conclusion

In conclusion, our integrative multi-omics approach uncovered metabolic-immune heterogeneity in sepsis, established a robust immune-metabolic risk score system, and identified GYG1 as a potential metabolic driver of innate immune hyperactivation. These findings advance our understanding of the metabolic underpinnings of sepsis pathophysiology and open avenues for metabolism-targeted interventions in this complex and deadly syndrome.

Data availability statement

All the datasets analyzed in this study can be found in the Gene Expression Omnibus (GEO) at <https://www.ncbi.nlm.nih.gov/geo/> with accession numbers GSE57065, GSE95233, and GSE167363.

Ethics statement

The animal study was approved by Zunyi Medical University ethics committee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

JZ: Formal Analysis, Resources, Investigation, Software, Validation, Writing – review & editing, Data curation, Writing – original draft, Methodology, Conceptualization, Visualization, Project administration. KQ: Writing – review & editing, Writing – original draft, Conceptualization, Investigation, Formal Analysis, Data curation. XW: Resources, Writing – original draft, Writing – review & editing, Formal Analysis, Conceptualization, Data curation. BF: Writing – original draft, Writing – review & editing, Software, Methodology. YZ: Formal Analysis, Writing – review & editing, Writing – original draft, Validation. YW: Methodology, Data curation, Writing – original draft, Writing – review & editing. HQ: Writing – review & editing, Writing – original draft, Data curation. QD: Methodology, Writing – original draft, Writing – review & editing. XL: Writing – review & editing, Writing – original draft, Conceptualization. KY: Software, Writing – original draft, Conceptualization, Writing – review & editing, Validation. SQ: Funding acquisition, Resources, Data curation, Visualization, Validation, Formal Analysis, Project administration, Writing – original draft, Conceptualization, Investigation, Supervision, Writing – review & editing, Methodology, Software.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

The author(s) declare that Generative AI was used in the creation of this manuscript. The authors verify and take full responsibility for the use of generative AI in preparing this manuscript. We used GPT-5 to assist with language polishing (grammar, clarity, and style only). The tool was not used to generate data, perform analyses, create figures, or draw scientific conclusions, and it is not credited as an author. All content was critically reviewed and verified by the authors for accuracy and integrity, in compliance with journal policies.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2025.1682846/full#supplementary-material>

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