

REVIEW

New strategies for treatment of infectious sepsis

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Abstract

In this mini review, we describe the molecular mechanisms in polymicrobial sepsis that lead to a series of adverse events including activation of inflammatory and prothrombotic pathways, a faulty innate immune system, and multiorgan dysfunction. Complement activation is a well-established feature of sepsis, especially involving generation of C5a and C5b-9, along with engagement of relevant receptors for C5a. Activation of neutrophils by C5a leads to extrusion of DNA, forming neutrophil extracellular traps that contain myeloperoxidase and oxidases, along with extracellular histones. Generation of the distal complement activation product, C5b-9 (known as the membrane attack complex, MAC), also occurs in sepsis. C5b-9 activates the NLRP3 inflammasome, which damages mitochondria, together with appearance in plasma of IL-1 β and IL-18. Histones are strongly proinflammatory as well as being prothrombotic, leading to activation of platelets and development of venous thrombosis. Multiorgan dysfunction is also a feature of sepsis. It is well known that septic cardiomyopathy, which if severe, can lead to death. This complication in sepsis is linked to reduced levels in cardiomyocytes of three critical proteins (SERCA2, NCX, Na⁺/K⁺-ATPase). The reductions in these three key proteins are complement- and histone-dependent. Dysfunction of these ATPases is linked to the cardiomyopathy of sepsis. These data suggest novel targets in the setting of sepsis in humans.

KEYWORDS

complement receptors, complement, histones, NLRP3 inflammasome

1 | INTRODUCTION

Infectious sepsis in North America involves more than 500,000 humans annually,¹ with a death rate of ~40% depending on the severity of sepsis.² These infections chiefly are triggered by bacteria, but viruses, fungi, and protozoa may also cause sepsis.^{3,4} In spite of extensive research in septic animals and humans, there has yet to be a Federal Drug Administration (FDA)-approved drug specifically for treatment of sepsis. Supportive care such as fluid resuscitation, broad spectrum antibiotics delivered early, and safer techniques for mechanical ventilation have improved clinical outcomes in septic patients.⁵ This mini review describes information gained from the study of polymicrobial sepsis in mice, which is providing new insights

into interventions that may pave the way for new strategies for treatment of humans with sepsis.

It is important to mention the extent to which genomic studies in humans and in mice have influenced our understanding of sepsis. A few years ago, a consortium of investigators compared genomic analysis using blood leukocytes from humans and mice that had experimental sepsis, burn injury, and other “inflammatory stresses.” The report by Seok et al.⁶ concluded that “genomic responses in mouse models poorly mimic human inflammatory diseases.” This led to many reports in newspapers, suggesting that use of mice was essentially irrelevant for trying to understand human sepsis. In 2015, a group from Japan using the same database came to an opposite conclusion that “genomic response in mouse models greatly mimic human inflammatory diseases.”⁷ This controversy has, as of 2018, not been resolved and represents a troubling problem, especially since both articles were published in a respected scientific journal (*Proceedings of the National Academy of Sciences of the United States of America*). As of now, there is no consensus that genomic analysis of mice with sepsis can reliably be extrapolated to humans with sepsis, and this has caused large pharma

Abbreviations: C5a, complement C5a anaphylatoxin; C5aRs, complement C5a receptors 1 and 2; C5b-9, complement product of distal complement pathways; CLP, cecal ligation and puncture; FDA, federal drug administration; KO, knockout; MAC, membrane attack complex, C5b-9; Na⁺/K⁺-ATPase, sodium (Na⁺) and potassium (K⁺)-ATPase; NCX, sodium-calcium exchanger; NET, neutrophil extracellular traps; PMN, polymorphonuclear leukocytes (neutrophils); ROS, reactive oxygen species; SERCA2, sarco/endoplasmic reticulum Ca²⁺-ATPase type 2

companies to avoid research investment in the field of sepsis, whether the activity involves mice with polymicrobial sepsis or septic humans. On the other hand, it should be emphasized that the use of septic mice has allowed studies that could not be done in humans with sepsis (such as measurements of regulatory proteins in cardiomyocytes, CMs). Septic mice have provided important information on signaling pathways and how these pathways can be manipulated to reduce organ dysfunction in sepsis, at least in mice. Finally, studies show a link between complement product of C5a and adverse outcomes in septic mice and in septic humans. Use of knockout (KO) mice (lacking C5a receptors; C5aRs) has been shown to protect against adverse events in the heart developing in sepsis.^{8,9} This emphasizes that certain manipulations can be done in septic mice that add important information on how sepsis causes organ dysfunction and might have application in septic humans.

2 | ROLE OF COMPLEMENT IN POLYMICROBIAL SEPSIS

Polymicrobial sepsis has been frequently used for the study of sepsis in rats and mice, especially sepsis induced by cecal ligation and puncture (CLP), which often closely mimics the clinical picture of humans with sepsis.¹⁰⁻¹³ The procedure, originally described nearly 40 years ago,¹⁴ has been widely used, in part due to the availability of KO mice. The technique involves a through-and-through puncture of the cecum, with extrusion of small amounts of feces into the peritoneal cavity. The intensity of sepsis can be controlled by the numbers of punctures and the size (gauge) of the needle.¹⁵ "Polymicrobial" refers to the presence of both aerobic and anaerobic bacteria that appear in the peritoneal cavity and in blood of septic mice.¹⁶ The polymicrobial sepsis model has rarely been used in larger animals or subhuman primates, in part because in such cases an ICU setting would have to be available, requiring animal care around the clock, all of which would result in exceptionally high operating costs. In addition, obtaining institutional approval for use of CLP in subhuman primates would be highly problematic.

Complement activation products and relevant receptors involved in polymicrobial sepsis are described in Fig. 1. The onset of sepsis triggers activation of several of the complement pathways, with the "classical" and the "alternative" pathways being activated in polymicrobial sepsis.¹⁷ It remains to be determined what precisely triggers activation of these pathways. The C5 convertase in the classical pathway involves C4b and C2a, while in the alternative pathway C3b and Bb represent the C5 convertase. In either case, C5a and C5b are generated. It is not clear what triggers complement activation in infectious sepsis involving mice with polymicrobial sepsis or septic humans. It is well established that Gram negative bacteria often activate complement by interaction via the lectin pathway. This features the mannose-binding lectin interacting with carbohydrates on the surfaces of bacteria. Related to the innate immune system, there are many pattern-associated molecular patterns and damage-associated molecular patterns that can activate complement. For example, bacterial LPS from Gram negative bacteria is a well-known bacterial product that activates the alternative complement pathway. LPS interacts with

Role of Complement in Polymicrobial Sepsis

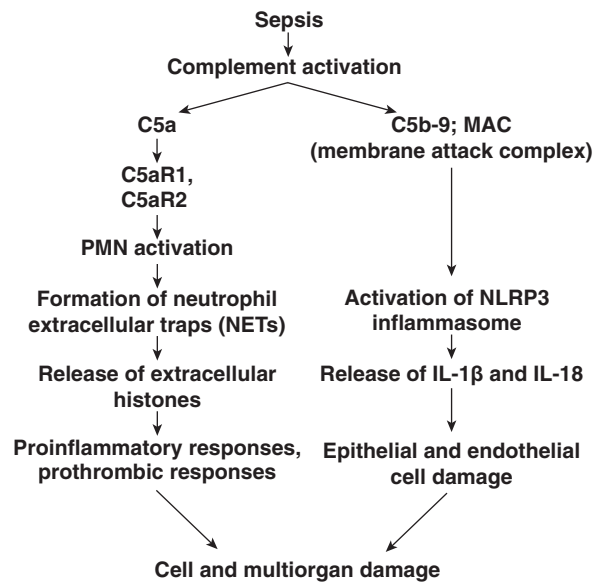


FIGURE 1 Sequence of events developing during infectious sepsis and the important role of complement activation, which results in generation of two major products, C5a and C5b-9 (membrane attack complex). C5a activates polymorphonuclear leukocytes (neutrophils) that form NETs, which release extracellular histones and proinflammatory peptides. C5b-9 activates the NLRP3 inflammasome, leading to release of two potent cytokines, IL-1 β and IL-18

Mechanisms of Histone-Induced Cell Damage in Sepsis

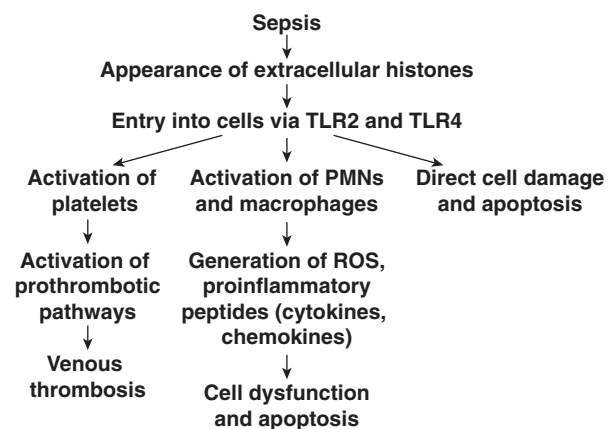


FIGURE 2 Infectious sepsis leads to the appearance of extracellular histones which interact with TLR2 and TLR4. This results in activation of the clotting system with thrombus formation, activation of neutrophils and macrophages that trigger a series of proinflammatory outcomes, together with ensuing cell damage and apoptosis

TLR2 and TLR4 causing activation of signaling pathways in a variety of cells (Fig. 2). Finally, certain Gram positive bacteria (e.g., *Staphylococcus aureus*) secrete proteins that block complement activation molecules. The third pathway ("lectin" pathway) is sometimes the chief pathway of complement activation in bacterial-induced sepsis.¹⁷ Whatever pathway is being triggered, there is appearance of C5a, the powerful anaphylatoxin, which reacts with its receptors (C5aR1 and C5aR2) that are especially prevalent on neutrophils (PMNs).^{18,19} A consequence

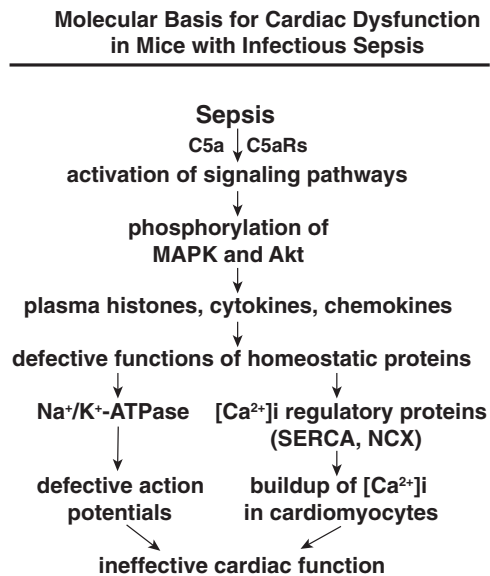


FIGURE 3 Events in infectious sepsis that cause the cardiomyopathy of sepsis, which is defined as contractile and relaxation defects in cardiomyocytes (CMs). The outcomes can be traced in MAPK (p38) and Akt activation in CMs, leading to cardiac dysfunction. These defects are associated with defective action potentials (reduced Na^+/K^+ -ATPase) in CMs, together with reduced activity of $[\text{Ca}^{2+}]_i$ regulatory proteins, SERCA2 and $\text{Na}^+/\text{Ca}^{2+}$ exchanger

of these events is strong activation of PMNs and, to a lesser extent, macrophages.^{18,19} Activated PMNs release long strands of DNA forming neutrophil extracellular traps (NETs) along with release of histones as well as products of PMN-specific granules (myeloperoxidase, proteases, etc.).^{20–22} Histones (H1, H2A, H2B, H3, H4) are molecules with potent proinflammatory and prothrombotic activities inducing biological responses.²³ As emphasized in Fig. 2, extracellular histones play key effector roles in human and mouse sepsis. Histones can cause diffuse vascular and epithelial cell damage and play important roles in multiorgan dysfunction that develops in sepsis. Downstream complement activation also results in generation of C5b-9 (membrane attack complex; MAC) (Fig. 3).

It is important to emphasize that complement activation via either the classical or the alternative pathway results in generation of C5 convertase, which generates both C5a and C5b. C5a reacts with C5aRs on PMNs, causing production of NETs, which release histones that have powerful proinflammatory functions. Generation of C5b interacts with C6, C7, C8, and C9 to form C5b-9. This complex is known to activate the NLRP3 inflammasome that results in inflammasome activation, mitochondrial damage, and release of $\text{IL-1}\beta$ and IL-18 , which are powerful proinflammatory cytokines. The NLRP3 inflammasome in its precursor mode exists in a variety of cells (PMNs, macrophages, cardiomyocytes, astrocytes, etc.). Briefly, the inflammasome protocol consists of the “priming” phase in which cells are exposed to LPS (100 ng/mL) for 4 h at 37°C. This results in very little release of $\text{IL-1}\beta$. Thereafter, the cells are exposed to 1 mM ATP for 45 min, which serves as an “activator,” resulting in very substantial release of $\text{IL-1}\beta$.^{22,23} Suresh et al. also demonstrated that complement-mediated “bystander” damage, during the phagocytosis of

complement-opsonized particles in macrophages, initiates NLRP3 inflammasome activation, resulting in caspase-1 activation and $\text{IL-1}\beta$ and IL-18 secretion.²⁴ To an extent, complement-dependent activation or inflammasome activation can cause acute cellular and organ dysfunction via C5a and C5b-9 by the mechanisms described above. Such adverse events can occur whenever complement or inflammasome activation occurs. In this sense, cell damage does not occur in a targeted manner, but in the vicinity of where activation of complement or inflammasome is occurring, such that a variety of cells may be damaged.

Regarding bacteria and the complement system, it is well established that Gram positive bacteria, such as *S. aureus*, are resistant to effects of complement activation while Gram negative bacteria are sensitive to effects of complement activation. For instance, some *S. aureus* bacteria are resistant to methicillin and may pose a grave danger to individuals. It should also be emphasized that many Gram negative bacteria release LPS that react with TLRs (2, 3, 4, and 9) that in the setting of sepsis may collectively contribute to the adverse events in infectious sepsis.²⁵ Complement activation products such as C3b and iC3b promote phagocytosis of bacteria and their intracellular killing by PMNs and macrophages, often via NADPH oxidase and its products, oxygen-derived free radicals.^{26,27} In addition, as mentioned above, MAC also has cytolytic effects on bacteria via MAC or via the lectin and NLRP3 inflammasome pathways. Bacteria can also activate the lectin pathway of complement, resulting in antibacterial effects described above. It should also be pointed out that certain Gram positive bacteria (such as *S. aureus*) release peptides that block complement activation or complement activation products.²⁸

The issue of whether complement complexes such as C5b-7 or C5b-8 assert cell damaging activity has by-in-large not been convincingly shown in experimental studies. A recent study by Morgan's group showed that in the absence of C9, sublytic MAC cannot be generated so cannot drive inflammation and apoptosis in human lung epithelial cells as found by studying cytokine production, NLRP3 inflammasome activation, cytosolic Ca^{2+} increase, mitochondrial dysfunction, and cytochrome c release.²⁹ It should be mentioned that most of the published information related to C5b-7 and C5b-8 fails to show damaging effects on mammalian cells.

3 | MECHANISMS OF HISTONE-INDUCED CELL DAMAGE IN SEPSIS

Figure 2 describes our recent work that has defined signaling pathways occurring in the heart after onset of CLP-induced sepsis. A major event is activation of PMNs by C5a, resulting in appearance of NETs and extracellular histones. It has been established that histones bind to TLRs in various cell types,^{30–33} including CMs.³⁴ This results in activation of platelets and development of thrombosis. Another major event occurring in the presence of extracellular histones is activation of PMNs and macrophages, causing generation of intracellular oxidants (reactive oxygen species, ROS) in a variety of cells, as well as appearance of proinflammatory peptides (cytokines, chemokines, and other factors), all of which cause cell dysfunction and, often,

apoptosis, especially in the setting of infectious sepsis.³⁵ Other damaging features of histones is that they can directly cause cell damage and apoptosis and are very prothrombotic and proinflammatory.³⁶ Several studies showed that histones bind to different TLRs (mainly TLR2 and TLR4) and interact with these receptors on various cell types.^{30–33,37} It should be mentioned that depending on the experimental setting and cell lines used, there are additional data about TLR2 or TLR4 being activated by histones. The study by Xu et al showed TLR4, but not TLR2, was the major receptor for a histone-induced cytokine inflammatory response after histone infusion into TLR4 KO mice.³⁰ The study by Ekanev et al.³⁸ also showed that blocking TLR4 resulted in decreased cellular cytotoxicity on human endothelial cells (as measured by lactate dehydrogenase measurement and propidium iodide staining). It has been shown that combination of TLR2 and TLR4 blocking Abs suppressed the cytotoxic and antiangiogenic effects on endothelial cells.³⁹ We have also found the macrophages from double TLR2 and TLR4 KO mice had dramatically reduced IL-1 β release induced by inflammasome protocol after the exposure to individual histones compared to single KO of TLR2 or TLR4.²³

4 | MOLECULAR BASIS OF CARDIAC DYSFUNCTION DEVELOPING DURING POLYMICROBIAL SEPSIS

Figure 3 describes how infectious sepsis activates a series of signaling pathways that have detrimental effects on cardiac function in mice. Initially, the MAPK, especially p38, and Akt signaling pathways are activated, followed by increased plasma levels of various cytokines, chemokines, and extracellular histones. The results of these events caused significant dysfunction of CMs. The basis for defects in CM function was linked to p38 activation, since a water soluble inhibitor of p38 prevented development of sepsis-induced heart dysfunction, as defined by echo-Doppler parameters.⁴⁰ Reasons for septic cardiomyopathy could be traced to defects in Na⁺/K⁺-ATPase, which is critical for effective action potentials in CMs and in other cell types. Defects in sarco/endoplasmic reticulum Ca²⁺ ATPase type 2 (SERCA2) and Na⁺/Ca²⁺ (NCX) exchanger led to diastolic buildup of [Ca²⁺]_i in CMs in a manner that was associated with the inability to clear cytosolic [Ca²⁺]_i in CMs during diastole.^{8,9,34} Such events caused substantial dysfunction and apoptosis of CMs. We believe these events likely occurred in other organs (brain, liver, lungs, etc.), resulting in multiorgan dysfunction in sepsis. Based on this information, blocking p38 activation might be a strategy to prevent cardiac dysfunction developing in sepsis in humans.

5 | CONCLUSIONS

The data in Figs. 1–3 emphasize the appearance of complement activation products and histones during sepsis, leading to cardiac dysfunction. Since C5a is such a key factor in many induced defects developing in septic cardiomyopathy, *in vivo* blockade of either C5a or C5aRs in septic mice would be an obvious potential therapeutic

strategy. Such inhibitors are not currently available but are under development. In severe sepsis in humans, most events leading to lethality occur during the first 3–5 days after onset of sepsis, especially in elderly septic patients. Therapeutic application of C5a or signaling pathway inhibitors would only be for a limited period of time (3–5 days). Currently, several pharmaceutical companies are developing small molecular weight compounds that block C5a or C5, although clinical trials have not yet been done in order to assess safety and efficacy of these compounds. In terms of blockade of C5b-9, there do not yet appear to be any FDA-approved drugs, except Ab to C5, that result in blockade of generation of C5b-9. However, the use of mAb to human C5 (eculizumab), which should block generation of C5a and C5b-9, is also associated with predilection to development of meningococcal meningitis. In some diseases affecting the complement system, such as paroxysmal nocturnal hemoglobinuria and atypical hemolytic uremic syndrome, clinical trials showed that eculizumab is effective and safe.⁴¹ However, in the setting of infectious sepsis associated with bacteremia, use of anti-C5 would likely face a very high barrier for FDA approval that would require very expensive clinical trials on the safety of such an intervention. In the short term, it seems unlikely that FDA approval would be granted. Development of small molecule inhibitors than block the activation epitope of C9 would be an attractive strategy to prevent the biological activity of C5b-9. There are currently mAbs that block the epitope of activated C9, but this mAb has not been used in humans. Targeting IL-1 produced by the activated NLRP3 inflammasome has already been tried unsuccessfully in septic humans using a compound IL-1 receptor agonist (IL-1ra).^{42,43} One study in septic mice applying combination therapy targeting IL-1 and IL-18 showed promising data.⁴⁴ Vanden Berghe et al., using CLP or LPS-induced endotoxemia in mice, showed that only the combined neutralization of IL-1r antagonist and anti-IL-18 conferred complete protection against endotoxin-induced lethality in the mice. They also found similar results after applying the mice genetically deficient for both IL-1 β and IL-18.⁴⁴ Although clinical trials using these combination therapies would be needed to confirm the mice data, the results of the failed clinical trials using blockade of IL-1 have discouraged additional clinical trials featuring blockade of IL-1ra. It is important to suggest that, beyond sepsis, blocking C5a or its receptors or C5b-9 might have relevance in many other situations in humans, such as acute cardiac ischemia, acute lung injury, autoimmune diseases (e.g., rheumatoid arthritis, systemic lupus erythematosus), and early allograft rejection.

AUTHORSHIP

Both authors contributed equally to this mini review.

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DISCLOSURES

The authors declare no conflicts of interest and no relevant financial disclosures.

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