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# Kallistatin in Sepsis: Protective Actions and Potential Therapeutic Applications

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<http://dx.doi.org/10.5772/67988>

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## Abstract

Sepsis is a systemic inflammatory response to infection, leading to multiorgan injury and mortality. Kallistatin is an endogenous protein expressed in the liver and tissues relevant to cardiovascular function. Kallistatin levels are markedly reduced in patients with sepsis and liver disease and in lipopolysaccharide (LPS)-induced septic mice. Kallistatin administration attenuates inflammation, multiorgan damage, and lethality in septic mice with LPS treatment, group A streptococcal, or polymicrobial infection. Importantly, kallistatin treatment not only prevents but also reverses organ injury and lethality in septic mice. Kallistatin decreases sepsis-induced inflammatory responses and tissue damage by modulating differential signaling pathways, including: (1) stimulating endothelial nitric oxide (eNOS) and sirtuin 1 (SIRT) synthesis, and NO formation; (2) increasing suppressor of cytokine signaling-3 (SOCS3) expression; (3) antagonizing tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and high mobility group box 1 (HMGB1)-mediated oxidative stress and inflammatory gene expression; and (4) displaying bactericidal effects by stimulating superoxide formation. Therefore, kallistatin's multifactorial activities provide effective protection during septic shock in animal models. As kallistatin displays no apparent cytotoxicity, kallistatin therapy may provide a promising approach for the treatment of sepsis in humans.

**Keywords:** kallistatin, sepsis, oxidative stress, inflammation, organ injury

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## 1. Introduction

Sepsis is a major contributor to the morbidity and mortality of intensive care patients and a leading cause of death worldwide [1, 2]. Sepsis is a systemic inflammatory response to infection that can lead to multiorgan dysfunction and is mediated by both early (tumor necrosis factor- $\alpha$ , TNF- $\alpha$ ) and late (high mobility group box 1, HMGB1) inflammatory cytokines [2, 3]. Although the underlying pathophysiology of sepsis has not been completely elucidated,

TNF- $\alpha$  and HMGB1 upregulation is known to play a critical role in the inflammatory response [4–6]. Moreover, suppressor of cytokine signaling-3 (SOCS3), a feedback inhibitor of lipopolysaccharide (LPS)-induced inflammation, has been shown to be a key player in inhibiting nuclear factor (NF)- $\kappa$ B-mediated pro-inflammatory cytokine production [7–9]. Anti-inflammatory strategies have been investigated with favorable effects in animal models of sepsis but with marginal results in humans [10]. Consequently, clinical trials for sepsis have been referred to as the “graveyard for pharmaceutical companies” [10]. Because numerous signaling cascades are triggered during septic shock, selective blocking of inflammatory mediators is not sufficient to arrest this process. Therefore, the development of novel strategies is vital to the effective treatment of sepsis patients.

Kallistatin was first identified in human plasma as a tissue kallikrein-binding protein (KBP) and a new serine proteinase inhibitor (serpin) [11–13]. Kallistatin modulates a wide spectrum of biological activities independent of tissue kallikrein [14–20]. Kallistatin is mainly expressed in the liver but is also present in the heart, kidney, and blood vessel [21–23]. Kallistatin protein contains two structural elements: an active site and a heparin-binding domain [24–26]. The active site of kallistatin is necessary for complex formation with tissue kallikrein and thus inhibition of tissue kallikrein activity and bioavailability [13, 27]. Kallistatin's heparin-binding domain, however, is essential for antagonizing signaling pathways mediated by vascular endothelial growth factor (VEGF), TNF- $\alpha$ , HMGB1, and transforming growth factor (TGF)- $\beta$  [16, 20, 28, 29]. Kallistatin levels are markedly reduced in hypertensive or normotensive rodents with cardiac and renal injury or streptozotocin-induced diabetes [11, 18, 30–32]. Circulatory kallistatin levels are also diminished in patients with liver disease, septic syndrome, diabetic retinopathy, severe pneumonia, inflammatory bowel disease, obesity, prostate, and colon cancer [33–40]. Kallistatin administration by gene or protein delivery protects against the pathogenesis of hypertension, heart and kidney damage, arthritis, influenza virus infection, tumor growth, and metastasis in animal models [15–19, 41–46]. Conversely, depletion of endogenous kallistatin by neutralizing antibody injection exacerbates cardiovascular and renal injury in hypertensive rats [47]. These findings indicate that kallistatin modulates a wide spectrum of biological activities, such as blood pressure, inflammation, multiorgan injury, and cancer.

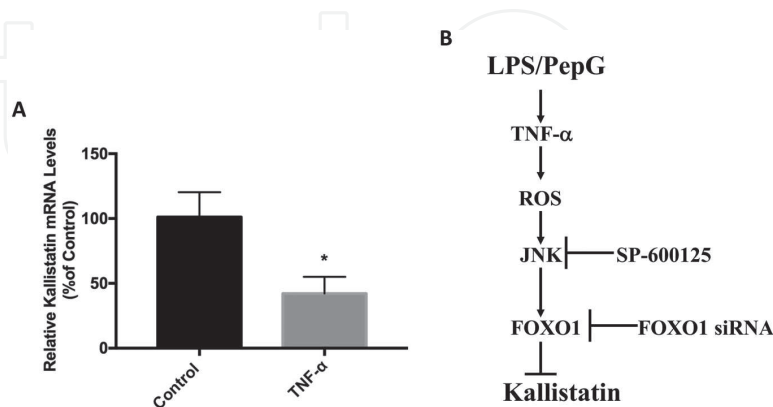
## **2. Kallistatin via its structural elements regulates differential signaling pathways**

Kallistatin through its two functional domains modulates numerous signaling pathways and biological activities. Kallistatin's active site is crucial for: (1) complex formation with tissue kallikrein and inhibiting tissue kallikrein activity and bioavailability [13, 27]; (2) increasing eNOS and SIRT1 expression and activation, leading to elevated NO formation [28]; (3) stimulating SOCS3 expression [48]; and (4) interacting with a tyrosine kinase [28, 48]. Kallistatin via its heparin-binding domain interacts with cell surface heparan sulfate proteoglycans, thereby antagonizing the following biological effects: (1) VEGF-mediated angiogenesis and

vascular permeability [16, 20]; (2) TNF- $\alpha$ -induced NF- $\kappa$ B activation, inflammation, oxidative stress, and apoptosis [20]; (3) HMGB1-induced inflammatory gene expression and oxidative stress [29]; (4) TGF- $\beta$ -induced endothelial-mesenchymal transition (EndMT), and epithelial-mesenchymal transition (EMT) [28]; (5) Wnt-mediated cancer cell proliferation, migration, invasion, and autophagy [42, 44]; and (6) EGF-induced cancer cell migration and invasion (unpublished results). Thus, kallistatin, with its multifactorial activities, regulates a wide spectrum of biological processes, such as angiogenesis, inflammation, oxidative stress, apoptosis, fibrosis, and cancer development.

### 3. Depleted kallistatin expression and levels in organ damage and sepsis

Kallistatin is a member of the serpin family, which also includes  $\alpha$ 1-antitrypsin and  $\alpha$ 1-antichymotrypsin [21]. In contrast to  $\alpha$ 1-antitrypsin, kallistatin is a negative acute-phase protein, as kallistatin expression in rat liver is rapidly downregulated within 24 h after lipopolysaccharide (LPS)-induced endotoxemia [31]. Kallistatin levels are depleted in animal models with hypertension, diabetes, cardiovascular, and renal damage [11, 18, 29–31]. Circulatory kallistatin levels are markedly reduced in patients with septic syndrome, liver disease, diabetic retinopathy, inflammatory bowel disease, and severe pneumonia [33, 34, 36–38]. LPS (endotoxin) from Gram-negative bacteria, or peptidoglycan (PepG) and lipoteichoic acid (LTA) from Gram-positive bacteria, are capable of inducing TNF- $\alpha$  synthesis and reactive oxygen species (ROS) formation [49–54]. TNF- $\alpha$  stimulates ROS information in endothelial cells and endothelial progenitor cells [17, 43, 55, 56]. Kallistatin expression and levels are negatively regulated by H<sub>2</sub>O<sub>2</sub> through JNK-dependent FOXO1 activation in endothelial cells [57]. Like ROS, TNF- $\alpha$  dramatically suppresses kallistatin expression in endothelial cells (**Figure 1A**). Therefore, kallistatin levels are depleted by the activation of the TNF- $\alpha$ -ROS signaling pathway during septic shock (**Figure 1B**).



**Figure 1.** (A) TNF- $\alpha$  inhibits kallistatin expression in endothelial cells. TNF- $\alpha$  (10 ng/ml) significantly decreases kallistatin expression in endothelial cells.  $n = 3$ ,  $*P < 0.05$  vs. control groups. (B) LPS/PepG exposure leads to TNF- $\alpha$  synthesis and inhibition of kallistatin expression through a ROS-JNK signaling pathway.

#### 4. Kallistatin is a potent anti-inflammatory agent

Sepsis is a systemic inflammation in response to bacterial infection. Exogenous kallistatin attenuates inflammation and multiorgan damage in animals with cardiovascular and renal disorders [14, 17–20, 41]. For example, kallistatin gene delivery significantly reduces inflammatory responses and joint swelling in arthritic rats [19]. Kallistatin administration also suppresses inflammatory cell infiltration and prevents complement factor C5a-induced paw edema and vascular leakage in mice [20]. Local delivery of the kallistatin gene into rat heart enhances cardiac performance and attenuates inflammatory cell infiltration after acute myocardial ischemia/reperfusion (I/R) [41]. In salt-induced hypertensive rats, kallistatin therapy attenuates vascular and renal damage and reduces oxidative stress and inflammation [17, 18], while depletion of endogenous kallistatin augments organ injury and accentuates oxidative stress, inflammation, and fibrosis [47].

Kallistatin suppresses inflammatory responses by modulating differential signaling pathways. Kallistatin's active site is responsible for upregulating eNOS and SIRT1 expression, and thus increasing NO production [28]. NO, in turn, blocks TNF- $\alpha$ -induced ROS formation by inhibiting NADPH oxidase activity and NF- $\kappa$ B activation [58, 59]. Kallistatin via its active site induces the expression of SOCS3, a negative regulator of inflammation [48], thereby inhibiting LPS-induced TNF- $\alpha$  production in cultured macrophages [48, 60]. Moreover, kallistatin via its heparin-binding site antagonizes TNF- $\alpha$ -induced oxidative stress, NF- $\kappa$ B activation and inflammatory gene expression *in vitro* [20]. Likewise, kallistatin blocks HMGB1-mediated synthesis of inflammatory genes in endothelial cells [29]. Moreover, kallistatin ameliorates inflammation by blocking VEGF-induced endothelial cell permeability [16, 20]. Thus, kallistatin via its two structural domains exerts anti-inflammatory actions by: (1) stimulating eNOS and SIRT1 synthesis and NO formation; (2) increasing SOCS3 expression; (3) antagonizing TNF- $\alpha$ - and HMGB1-mediated inflammatory gene expression and oxidative stress; and (4) blocking VEGF-induced vascular permeability. Together, these findings indicate that kallistatin protects against organ damage by inhibiting inflammatory responses through multiple mechanisms.

#### 5. Kallistatin attenuates organ damage and mortality in septic animal models

In Gram-negative infections, the cell wall component LPS (endotoxin) is the main initiator of the cascade of cellular reactions that lead to circulatory failure and organ injury [61]. *Staphylococcus aureus* is one of the most common Gram-positive bacteria isolated from patients with sepsis [61–63]. Gram-positive bacteria contain two major cell wall components, PepG and LTA, which cause sepsis and multiple organ injury in the absence of LPS [49–53]. A human kallistatin gene polymorphism is correlated with a decreased risk of developing acute kidney injury in patients with septic shock [64]. Kallistatin treatment exerts beneficial effects in Gram-negative and Gram-positive bacteremia, as well as “mixed” polymicrobial



infection [29, 35, 48, 65]. Transgenic mice expressing kallistatin are highly resistant to mortality induced by LPS [66]. Moreover, kallistatin gene transfer reduces mortality, bacterial counts, and inflammatory cell numbers, as well as skin and liver damage, in a mouse model of streptococcal infection [65]. Kallistatin treatment in septic mice with polymicrobial infection attenuates lethality, peritoneal bacterial counts, renal injury and inflammation, and splenic apoptosis [29]. The protective effects of kallistatin in the kidney occurred in conjunction with reduced expression of TNF- $\alpha$  and HMGB1 and increased eNOS synthesis and NO levels [29]. NO inhibits oxidative organ damage by inactivating NAD(P)H oxidase activity [59]. Furthermore, delayed kallistatin administration after the onset of sepsis attenuates mortality, kidney and liver injury in mouse models of polymicrobial sepsis and endotoxemia [48]. Kallistatin treatment inhibits systemic inflammation by reducing circulatory levels of TNF- $\alpha$  and HMGB1, and dramatically upregulating SOCS3 expression in the kidney and lung [48]. Kallistatin delivery improves mortality, attenuates acute lung damage in LPS-induced septic mice, and inhibits ROS-mediated inflammation and apoptosis in cultured lung epithelial cells [35]. These findings indicate that kallistatin administration significantly enhances survival and protects against multiorgan damage during sepsis.

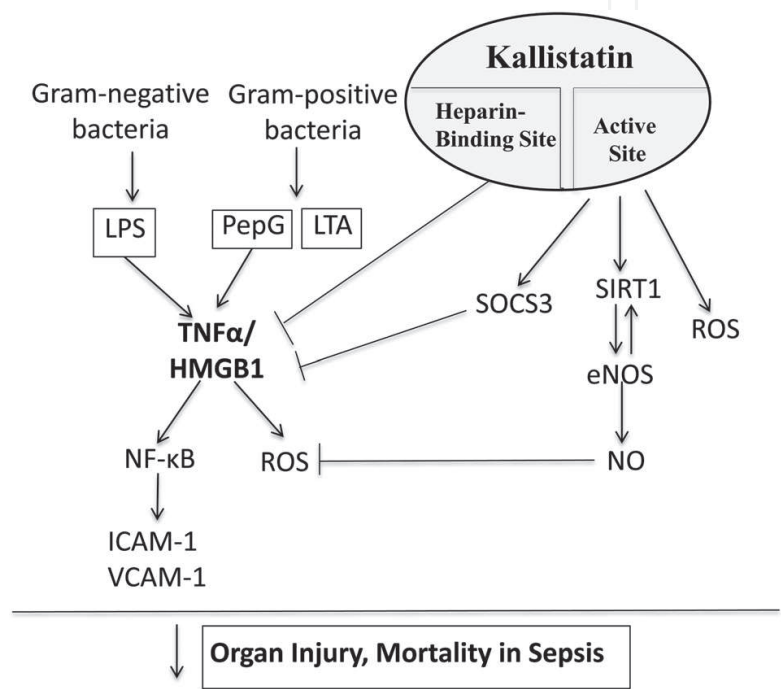
## 6. Kallistatin enhances bacterial killing by elevated oxidative stress

Oxidative stress has been shown to have beneficial effects with bacterial killing activity in inflammatory disorders [67, 68]. Human kallistatin treatment significantly enhances bacterial clearance and antimicrobial activity in group A streptococcus-infected mice by increasing superoxide production in immune cells [65]. Likewise, kallistatin administration leads to more than 10-fold decrease of bacterial counts in the peritoneal fluid of mice with polymicrobial sepsis [29]. The bactericidal activity of kallistatin is most likely attributed to elevated ROS formation in peritoneal neutrophils [61]. Kallistatin also enhances immune cell viability and reduces their apoptosis [65]. Kallistatin through its active site increases SOCS3 expression in macrophages and SIRT1 synthesis in endothelial cells, but the effects are blocked by genistein [28]. Genistein also abolishes kallistatin's stimulation on endogenous H<sub>2</sub>O<sub>2</sub> formation in vascular smooth muscle cells (unpublished results). These combined findings implicate a role of kallistatin's active site in enhancing ROS formation by interaction with a cell surface tyrosine kinase. Thus, kallistatin has a double-edged role in oxidative stress, depending on the pathological conditions. In addition to inhibiting oxidative stress and multiorgan damage, kallistatin is capable of exerting potent bactericidal activity by stimulating ROS formation in immune cells of animals with bacterial infection.

## 7. Signaling mechanisms mediated by kallistatin in sepsis

LPS (endotoxin) derived from Gram-negative bacteria, or PepG and LTA from Gram-positive bacteria, can lead to multiorgan injury and lethality by inducing the synthesis of TNF- $\alpha$  and HMGB1, thus elevating ROS formation [49–54]. TNF- $\alpha$  activates pro-inflammatory

transcription factor NF- $\kappa$ B and the expression of inflammatory genes, such as ICAM-1 and VCAM-1. Kallistatin via its active site stimulates eNOS and SIRT1 expression and activation and increases NO formation in endothelial cells; NO in turn inhibits ROS formation [28, 59]. Kallistatin's active site is also crucial for increasing SOCS3 expression, leading to inhibition of LPS-induced TNF- $\alpha$  synthesis [48, 60]. Kallistatin via its heparin-binding domain blocks TNF- $\alpha$ - and HMGB1-induced NF- $\kappa$ B activation, inflammatory gene expression, and ROS formation [20, 29]. However, kallistatin's active site is involved in superoxide formation in immune cells, leading to a bacterial killing effect [65]. The signaling pathways mediated by kallistatin in the protection against sepsis are shown in **Figure 2**.



**Figure 2.** Signaling pathways mediated by kallistatin in sepsis. Kallistatin via its heparin-binding domain blocks TNF- $\alpha$ - and HMGB1-induced NF- $\kappa$ B activation, inflammatory gene expression, and ROS formation. Kallistatin via its active site stimulates eNOS, SIRT1 and SOCS3 expression, and NO formation, leading to inhibition of oxidative stress. Kallistatin increases ROS formation in immune cells. Kallistatin protects against organ injury and lethality during sepsis by modulating these differential signaling cascades.

## 8. Therapeutic implications of kallistatin

Sepsis is a systemic inflammatory response to infection. Kallistatin is an effective anti-inflammatory agent by triggering multiple signaling cascades in protection against septic shock. Reduction of plasma kallistatin levels is observed in patients with sepsis syndrome and is associated with severity of community-acquired pneumonia [33, 36]. Therefore, circulating kallistatin levels may serve as a biomarker for patients with severe sepsis and septic shock. Although the beneficial properties of kallistatin treatment in sepsis have been shown in animal studies, they still have yet to be confirmed in humans. Therapeutic application of kallistatin may provide a new approach for the treatment of sepsis and septic shock in humans.

## 9. Concluding remarks

Kallistatin levels are markedly reduced in septic shock and inflammatory disease. Kallistatin administration exerts beneficial effects during septic shock induced by Gram-negative bacteremia, Gram-positive bacteremia, or polymicrobial infection. Kallistatin possesses potent anti-inflammatory activities. Kallistatin via its two structural elements ameliorates inflammatory responses by regulating differential signaling cascades. Kallistatin's active site is crucial for stimulating eNOS, SIRT1, and SOCS3 expression and/or activation. Kallistatin via the heparin-binding site antagonizes both early (TNF- $\alpha$ ) and late (HMGB1) cytokine-induced oxidative stress and inflammatory gene synthesis. Interestingly, kallistatin has a double-edged role in oxidative stress. In addition to suppressing oxidative stress, kallistatin exerts a marked bactericidal effect by stimulating ROS production in immune cells of mice with microbial infection. Kallistatin with its pleiotropic activities is an effective therapeutic agent in tissue injury and consequences of septic shock.

## Acknowledgements

This work was supported by the National Institutes of Health grants HL118516 and HL 44083.

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