

Factors in Homo and Heterotypic Aggregate Formation in Sepsis

Azzah Alharbi^{1,2}, Roisin Thomas³, Majid Ali⁴, Jonathan Thompson³ and Cordula Stover^{1*}

¹Department of Infection, Immunity and Inflammation, University of Leicester, UK

²King Abdulaziz University, Saudi Arabia

³Department of Cardiovascular Sciences, Division of Anaesthesia, Critical Care and Pain Management, University of Leicester, UK

⁴Thrombosis and Tissue Repair Group, University of Leeds, UK

***Corresponding author:** Cordula Stover, Department of Infection, Immunity and Inflammation, College of Medicine, Biological Sciences and Psychology, University of Leicester, UK, Email: cms13@le.ac.uk

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ABSTRACT

Sepsis, a severe systemic inflammatory response to an infection that can be bacterial, viral or fungal in origin, remains a serious condition with high mortality. The dynamics in the immune response (immune activation, over activation and exhaustion) during development and progression of sepsis pose a problem in the design of new treatment approaches. This review focuses on the understanding of molecular interactions that lead to the formation of cellular aggregates in sepsis and puts novel treatment targets in the context of these interactions.

Keywords: Sepsis; Complement; Intravital microscopy; Adhesion; Aggregate formation; Treatment

INTRODUCTION

Sepsis is a global health problem that is increasing incidence and represents a significant economic burden. People at the extremes of age, those with serious or chronic disease and immunocompromised patients are at higher risk of developing sepsis than others [1]. Early in sepsis, most cases present with symptoms of systemic inflammation includes fever, chills and shivering, tachycardia and tachypnoea. However, sepsis can progress to cause tissue hypoperfusion, organ dysfunction and septic shock, characterized by the presence of hypotension inspite of adequate fluid resuscitation.

Extrapolation of recent data from high-income-countries indicates that there may be as many as 31.5 million cases of sepsis worldwide per year, with the loss of 5.3 million lives [2]. Estimates from the UK Sepsis trust suggest that there are 150,000 cases of sepsis in the United Kingdom annually with an associated 44,000 deaths, which signifies a higher mortality rate than breast, bowel and colon cancer combined [3].The financial impact of sepsis on healthcare resources is considerable. According to the UK Department of Health, the treatment of patients with sepsis costs the NHS in England approximately £2 billion a year [4] although the true economic cost is most likely much higher, higher, when welfare costs and the loss of economic productivity for those suffering long-term health consequences are included. With an increasing incidence of cases of sepsis [5] and the development of antibiotic resistance these costs are likely to rise.

Current treatment includes antimicrobial agents (antibiotics, antifungals), control of the source of infection (surgery or abscess drainage), fluid resuscitation and more invasive interventions if the patient deteriorates and is managed in critical care units [6]. However sepsis remains a major cause of death in intensive care, as clinical trials disappoint. Improving the medical care of patients with sepsis may require even greater insight into dynamic molecular interactions in the pathogenesis of sepsis in order to propose novel therapeutic targets and agents.

SEPSIS PATHOPHYSIOLOGY

Despite the clinical importance of sepsis and extensive studies, understanding the dynamics in the pathophysiology of sepsis and its life threatening complications remains a challenge. For several years, it has been widely accepted that sepsis is the end result of exaggerated inflammatory responses to the microorganism [7]. This was concluded from animal studies in which infusion of high doses of bacteria or bacterial products leads to sudden systemic release of a wide range of inflammatory mediators. Most of these mediators have been shown to be directly related to host death, including the pro-inflammatory cytokines Tumour Necrosis Factor (**TNF**)- α and Interleukin (IL)-1. However, almost all recent clinical trials using anti-inflammatory therapies, such as anti TNF- α and IL-1 receptor antagonists, have failed to improve clinical outcomes [8]. These results reflect the complexity of the pathogenesis of sepsis [9] and the contribution of the host response to sepsis-associated mortality [10]. Importantly, comorbidities influence susceptibility to developing sepsis and also acute and long-term survival.

The pathogenesis of sepsis involves complex interactions between the endothelium, platelets, leukocytes, the coagulation system, and multiple inflammatory mediators [11]. Early in sepsis, leukocyte-endothelium-platelet complexes have been observed during inflammation in several *in vivo* and *in vitro* models of sepsis [11]. This heterotypic cell interaction is presumed to play a critical role in the pathophysiology of sepsis. Recently, it was found that interaction between LPS-activated platelets with neutrophils adherent to endothelium in a model of sepsis has a beneficial role in bacterial trapping [12]. Although it could be seen as a defence mechanism for the host to confine the infection and limit its spreading, platelet-neutrophil aggregation on activated endothelium is an important determinant of microvascular dysfunction as the inflammatory process progresses to capillary leakage and of activation of the coagulation system [13]. For example, it has been suggested that Neutrophil Extracellular Trap (**NET**) formation might be a trigger for formation of red blood cell-rich thrombi leading to Disseminated Intravascular Coagulopathy (**DIC**) and organ failure [14,15]. Furthermore, activated endothelial cells, platelets and neutrophils express Tissue Factor and release microparticles which also express Tissue Factor, leading to activation of coagulation and acceleration of microvascular injury [13]. Microparticles are vesicles of a defined size which bud off the cell membrane in an inflammatory context [16], are mainly derived from platelets during sepsis [13], and participate in the intercellular communications by transfer of mRNA, microRNA, proteins.

PATHOGEN RECOGNITION IN SEPSIS

The initial immune response to infection is triggered by recognition of conserved molecular products of pathogens termed Pathogen Associated Molecular Patterns (**PAMPs**). Pathogen Recognition Receptors (**PRRs**) are expressed by innate immune cells (monocytes, macrophages and to some extent endothelial cells), and lead to activation of intracellular signalling cascades and production of inflammatory mediators such as TNF- α , IL-1, IL-6, IL-12, and IL-8 [17]. Prolonged activation of such signaling leads to an exaggerated inflammatory response, which may result in tissue damage and their release of Damage-Associated Molecular Patterns (**DAMPs**) [18,19]. These DAMPs such as hyaluronic acid and heat shock proteins may be sensed by PRRs expressed on endothelial cells, neutrophils and platelets, thereby leading to amplification of the inflammatory response. Lipopolysaccharide (**LPS**) dependent Toll-Like Receptor 4 (**TLR4**) signaling is a key pathway in the pathogenesis of gram negative sepsis [19, 20]. In murine studies using TLR4 mutant mice, administration of purified LPS failed to induce an immune response [17]. Furthermore, it has recently been found that platelets also express TLRs that react with PAMPs and DAMPs leading to platelet activation, production of immunomodulatory agents such as TNF- α and promoting other cell activation e.g. of neutrophils and endothelial cells [12,14]. Activation of complement and release of antimicrobial peptides are significant in sepsis, pursuing roles of antimicrobial and cell instructive activities [21]. Though therapeutically trialled antimicrobial peptide (lactoferrin) was unsuccessful, there is still a demand for synthetic peptides with greater bioavailability [22].

CELLULAR AGGREGATE FORMATION IN SEPSIS

Homo- and heterotypic aggregates are formed in sepsis and can be analysed by flow cytometry. Binding of activated platelets to the inflamed microvasculature has long been known to be the initiating event in developing DIC, a condition defined by the consumption of coagulation factors in sepsis or trauma. Thanks to intensive treatment, the incidence of DIC has declined [23]. Platelets bind to inflamed endothelial cells when the Endothelial Protein C Receptor (**EPCR**) is shed, which normally participates in the activation of Protein C, when thrombin is bound to thrombomodulin. Through analytical advances we now know that microparticles can be associated with aggregates. Monocyte-platelet aggregates occur and relate to mortality from sepsis in the elderly, concomitant with elevated Il-6 and Il-8 [24]. Much data has been accrued on neutrophil-platelet aggregate formation in sepsis: Activated platelets attach to neutrophils through P-Selectin (a granule protein expressed on the platelet surface upon its activation), which binds to P-Selectin Glycoprotein Ligand-1 (**PSGL-1**) present on the neutrophil surface [14]. P-Selectin and PSGL-1 interaction results in further neutrophil activation and expression of molecules such as Lymphocyte function-associated antigen 1, LFA-1. Furthermore, activated platelet expresses CD40 Ligand (**CD40L**) and shed this into the circulation. Platelet derived-CD40L can bind to CD40 expressed on the neutrophil surface and leads to its activation and production of reactive oxygen species, ROS. It can also interact with endothelium expressed CD40 leading to stimulation of the endothelial cell to upregulate expression of various adhesion molecules, such as ICAM and VCAM, and to release chemokine, CCL2, thereby promoting recruitment of neutrophils. Additionally, activated platelets in sepsis can interact with neutrophils through Triggering Receptor Expressed on Myeloid cells (**TREM1**), which leads to further stimulation of neutrophils [14].

Early in sepsis, neutrophil-platelet aggregate formation on vascular endothelium may be mediated by a wide range of effector molecules, which include but are not limited to proinflammatory cytokines, chemokines, and secondary mediators for tissue injury such as Nitric Oxide (**NO**) and complement activation products. Local release of TNF- α and IL-1 leads to activation of vascular endothelium and increases expression of adhesion molecules such as P-Selectin which is essential for the process of tethering neutrophils [17,25]. While locally produced IL-8 plays a significant role in recruitment and activation of neutrophils, NO induces local vasodilation which results in slowing the blood flow rate and allowing neutrophil tethering to the vascular wall [17]. Neutrophils bind endothelial cells of post capillary venules through the expression of adhesion molecules [26]. Selectins promote tethering and rolling on endothelium and $\beta 2$ integrins mediate firm adhesion. When neutrophils become activated, they increase expression of adhesion molecules and exocytose properdin, amplifying LPS-induced complement activation locally, and bind to activated platelets [27]. In a static adhesion assay, neutrophils isolated from patients with sepsis adhered to unstimulated and a cytokine stimulated endothelial cell culture in a supranormal manner. In addition, plasma from patients with sepsis strengthened the attachment of neutrophils derived from healthy individuals to endothelial cells *in vitro* [26].

COMPLEMENT ACTIVATION IN SEPSIS

Cell activation, endothelial cell adherence and formation of aggregates occur in the context of complement activation during sepsis. Significant changes in levels of complement components and activities have been described in cohorts of patients with sepsis [28-31]. Complement is activated in response to invading microorganisms via three pathways, the classical, alternative, and lectin pathway, triggered by binding to LPS of some gram negative bacteria, complexes made of natural antibodies binding to pathogens, or recognising bacterial, fungal or viral sugar moieties. Subsequently, convertase complexes are generated which result in the production of anaphylatoxins and a membrane attack complex. These end products of complement activation exert various biological effects to clear the infection and instruct cellular activities, alongside the effects of cytokines. Uncontrolled complement activation might be a significant contributor to the pathogenesis of sepsis, resulting in an exaggerated inflammatory response and ensuing host tissue damage. Properdin is a serum protein produced by stimulated neutrophils [32] and binds to activated platelets [33]. Properdin amplifies LPS-induced complement activation by stabilising complement C3 convertase through its binding to C3b found in the C3 convertase complex (C3bBb) attached to the cell membrane. C3 convertase generates C3b which is bound by the C3 convertase to generate the C5 convertase, but also binds to P-Selectin [34]. Reciprocally, platelet expressed P-Selectin may activate complement *in vitro* after activation with ADP or thrombin-receptor activating peptide, TRAP [35].

Properdin stabilises the C5 convertase to generate C5a, which leads to expression of procoagulant Tissue Factor in neutrophils [36]. C5aR is expressed by microvascular endothelial cells [37]. Signalling through C5aR results in cell activation to generate ROS (O_2^- , H_2O_2 , HO^\cdot) that are toxic to other cells, pathogens and connective tissue components [38]. Moreover, Tissue Factor can be induced by the membrane attack complex (C5b-9) [39]. Tissue factor itself activates the alternative pathway of complement, requiring properdin [40]. As expected, platelet-neutrophil aggregate formation, as part of a localized Shwartzman reaction, is optimal in the presence of properdin (Figure 1). The functions of complement and coagulation cascades are very closely interconnected [42-46].

In contrast to monocyte-platelet aggregates, the formation of neutrophil-platelet aggregates is sensitive to properdin activity [47], possibly reflecting a relative greater source of properdin, more oxidative stress in neutrophils compared to monocytes (respiratory burst in neutrophils), and greater procoagulant characteristics in neutrophil-platelet aggregates (NET formation, capture of Tissue Factor positive microparticles) [48]. Platelet-derived microparticles have been shown to sustain complement activation even in the presence of membrane bound complement regulators [49].

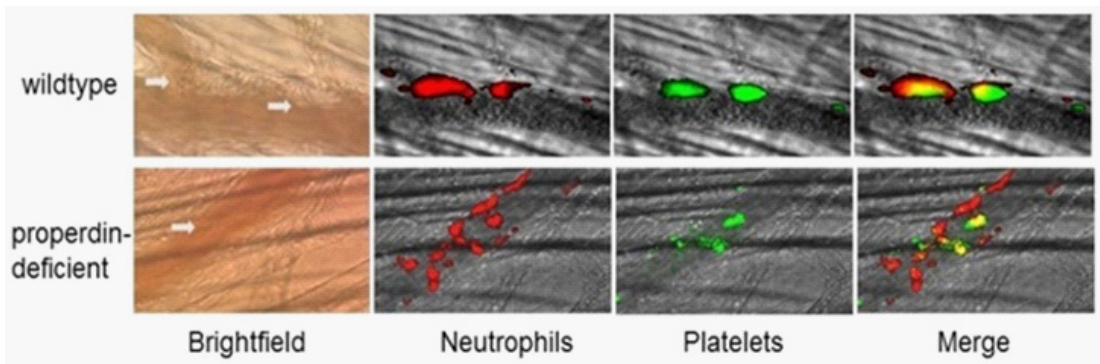


Figure 1: Intravital microscopy images of aggregates formed during a Shwartzman reaction in wildtype and properdin-deficient mice [41]; white arrows point to aggregates in brightfield (venules of exteriorized cremaster muscle are depicted horizontally and diagonally, respectively); granulocytes were labeled with Alexa (red) 555-RB6-8C5 F(ab')₂ fragments and platelets were labeled with FITC (green)-conjugated anti-CD41 antibody MWReg30.

MOLECULAR MECHANISMS OF AGGREGATE FORMATION IN SEPSIS

Intravital microscopy of the hepatic microcirculation has helped to delineate the sequence of cellular interactions: because LPS-stimulated platelets showed impaired adherence in liver sinusoids when neutrophils were depleted, contrary to LPS-stimulated neutrophils when platelets were depleted, it appeared likely that platelets aggregated with adherent neutrophils [50]. Similarly, another study, which compared C57Bl/6 mice and mice depleted of neutrophils after administration of the anti-granulocyte antibody RB6-8C5 in a model of localized Shwartzman reaction, documented lower adhesiveness of platelets in neutrophil-depleted animals [51]. A study investigating the relative contributions of TLR4 on endothelial cell and bone marrow cells to systemic LPS response demonstrated that the rolling flux of leukocytes in cremasteric venules of endothelial TLR4 mutant chimeric mice was significantly higher compared to that of intravascular TLR4 mutant chimeric mice [52]. Administration of TNF- α locally and of LPS systemically produces a localized phenotype of activated endothelial cells in postcapillary venules, which leads to adhesion of activated neutrophils, aggregation of activated platelets as well as increased microvessel permeability and erythrocyte leakage. The sequence of these interactions has been demonstrated in a study of rat mesentery microvessels perfused with autologous blood, after administration of TNF- α systemically and of platelet activating factor locally, using intravital microscopy [53]. Using high speed microfluorography in intravital imaging and blocking monoclonal antibodies, platelet glycoprotein Iba was identified as a significant receptor in the adherence of leukocytes on inflamed rat endothelium [54]. Platelet activating factor is released from adhering neutrophils and leads to platelet-neutrophil aggregates [55]. A reduction in expression of thrombomodulin by endothelial cells mediated by complement activation [34]

is another factor, which contributes to a procoagulant phenotype of the microvasculature. In a model of high dose intravenous LPS stimulation of C57Bl/6 mice, Jenne et al [56] observed (using spinning-disk confocal microscopy) recruitment of platelets to adhering neutrophils in liver and brain as well as to circulating neutrophils, forming platelet neutrophil aggregates.

TREATMENT OF SEPSIS

Early recognition and management of sepsis significantly decreases in-hospital mortality. Initial management involves administration of intravenous fluids and electrolytes, source control including antimicrobial drugs, continuous monitoring of clinical signs, and measures to maintain tissue oxygen delivery and organ function. Other measures to maintain normoglycemia, normothermia, thromboprophylaxis, avoid anaemia and coagulaopathy are needed (blood transfusion, insulin as glycaemic control, deep venous thrombosis prophylaxis such as heparin). Invasive monitoring, vasopressor or inotropic drugs and other organ support (renal, respiratory) are required in more severe cases. It is important to identify the source of infection in order to direct adjustment of treatment or surgical intervention. It should be noted that most current therapies are supportive rather than curative though several therapeutic agents with various targets in sepsis are under development [57].

CLINICAL TARGETS

Improved clinical guidelines have reduced mortality of patients from sepsis. Novel treatments, which are tested in trials, therefore target a relatively smaller margin to show convincing benefit, compared to a decade ago [58-60]. Among the pharmacotherapeutics relevant to this review are those which target coagulation, proinflammatory cytokines, complement/kininogen, and LPS (Table 1).

Table 1: A summary of candidate therapeutics investigated in past clinical trials.

Agents	Mechanism of action
MD2-TLR4-LPS antagonist	Blocks TLR4 signalling
sThrombomodulin	Co-factor of Thrombin, enhancing Protein C activation and fibrinolysis inhibitor activity
Activated Protein C	Inhibits coagulation factors of intrinsic and common pathway
Tissue factor pathway inhibitor	Reduces tissue factor expression and activation of extrinsic coagulation pathway
Antithrombin	Inactivates thrombin and inhibits coagulation factors of the intrinsic and common pathway of coagulation
Heparin	Activates antithrombin, inhibits platelet aggregation, histone neutralising effect
Platelet activating factor antagonist	Inhibits platelet aggregation and leukocyte activation
TNF inhibitors	Block TNF- α
C1 Inhibitor	Blocks classical pathway of complement activation and kallikrein-bradykinin pathway

CONCLUSION

Aggregates are formed during sepsis as part of the systemic inflammation leading to concerted activation of cells circulating in blood. A hyperdynamic phase of sepsis dislodges the marginal pool of neutrophils [61]. Inflammatory mediators mobilise cells from the bone marrow, while there is tissue resident expression of chemoattractive mediators. Importantly, virulence of pathogens and individual susceptibility to factors determining virulence may change over the duration of sepsis [62].

Aggregates may be formed *in vitro* by whole blood stimulation assays and *in vivo*, are found adhering to inflamed microvasculature. The purpose of homo- and heterotypic aggregates appears to be to concentrate antimicrobial activities of neutrophils and platelets [63] and as such fulfils a useful aim. It follows that hypercoagulation is the unwanted, serious side effect in the response of the immune system to sepsis. The dynamic changes in coagulation can be sensitively captured by thromboelastography [64] and may be a useful measurement to consider within the need to stratify patients with sepsis [65]. In novel treatment approaches it may be desirable not to disrupt aggregates, but rather to curtail coagulation. However, at present, coagulation activity is insensitively measured in routine static coagulation assays. By contrast, aggregate disruption may be beneficial in certain other (sterile) pathologies, such as acid induced acute lung injury [66], ischaemia-reperfusion injury [67] or haemodialysis [68].

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