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Sepsis, complement and the dysregulated inflammatory response

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Abstract

Sepsis in human beings is a major problem involving many individuals and with a high death rate. Except for a single drug (recombinant activated protein C) that has been approved for treatment of septic patients, supportive measures represent the main clinical approach. There are many models of experimental sepsis, mostly in rodents. A commonly used model is cecal ligation and puncture (CLP). In this model, robust activation of complement occurs together with up-regulation of C5a receptors (C5aR, C5L2) in a variety of different organs (lungs, kidneys, liver, heart). In septic human beings there is abundant evidence for complement activation. Interception of C5a or its receptors in the CLP model greatly improves survival in septic rodents. There is compelling evidence that CLP causes an intense pro-inflammatory state and that C5a interaction with its receptors can be linked to apoptosis of the lymphoid system and cells of the adrenal medulla, loss of innate immune functions of blood neutrophils, consumptive coagulopathy and cardiac dysfunction. These findings may have implications for therapeutic interventions in human beings with sepsis.

Keywords: sepsis • complement • C5a • C5a receptors • apoptosis

Introduction

Sepsis in human beings is a serious and often life-threatening situation that involves nearly 600,000 patients in North America, with a death rate approaching 50% [1–6]. Besides supportive therapy (fluid resuscitation, ventilatory support and related treatments) and specific therapy (broad spectrum antibiotics), there is only one FDA-approved drug: recombinant activated protein C (APC). APC causes modest improvements in survival but has a central drawback. It is an anti-coagulant that is used in the setting of sepsis, which per se is associated with intrinsic coagulant changes (consumptive coagulopathy). Accordingly, therapeutic use of APC carries with it potentially life-threatening complications of haemorrhage and is a reason for the strict limitations in its use in septic human beings.

Sepsis has been classified clinically as: sepsis, severe sepsis, septic shock and multiorgan failure. There are, as yet, no specific markers for sepsis, although plasma levels of interleukin (IL)-6 and procalcitonin tend to correlate with the intensity of sepsis, but these biomarkers are entirely non-specific biomarkers of sepsis. Their plasma presence occurs after trauma and a variety of other conditions. Clearly, we do not know enough about the fundamentals of sepsis to adequately understand what is happening. Why is sepsis occurring, and how can the progression to the more serious stages of sepsis be stopped or reversed? In this review we will focus chiefly on animal models of sepsis and what has been learned from animal studies, mostly involving rodents. We will also emphasize the role of the complement system in sepsis and the fact the experimental sepsis converts a regulated inflammatory response into a completely dysregulated inflammatory condition.

Models of sepsis

One of the early models of sepsis was vascular infusion of live bacteria such as *E. coli*. In subhuman primates (monkeys, baboons), vascular infusion of live *E. coli* results in complement

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activation, hypotension, consumptive coagulopathy, fever, leukocytosis followed by leucopoenia, and a variety of other signs and symptoms which are known to develop during experimental and human sepsis [7–10]. The model of vascular infusion of live bacteria has been criticized as having little relevance to human sepsis because of the massive number of bacteria injected. In view of recent PCR-based quantitation of bacterial DNA, this methodology has suggested a far greater presence of bacteria in blood compared to the standard method of colony forming units (CFUs) in blood. Accordingly, until the new methodology can be widely compared to other methods for *in vivo* bacterial quantitation and validated, it is premature to be definitive regarding the best method to quantitate the bacterial burden *in vivo*. Another model of sepsis is that of endotoxemia in which bacterial lipopolysaccharide (LPS) (often from *E. coli*) is injected intravenously or intraperitoneally, usually into rodents. It has recently been suggested that this model is not a good surrogate for human sepsis for a variety of reasons [11]. One of the more common models of sepsis is cecal ligation and puncture (CLP) in rodents, which induces polymicrobial sepsis (involving blood borne gram positive and gram negative bacteria). The intensity of CLP-induced sepsis is related to the area of cecum ligated, the number of punctures and the size of the needle used for puncture. Because about 50% of human beings develop sepsis associated with gram positive bacterial pneumonia [12, 13], there has been considerable controversy about whether CLP animal model of sepsis as well as the endotoxemic and infusion of live *E. coli* is relevant to what is occurring in septic human beings. Another problem in this regard is differences in rodent susceptibility compared to human susceptibility to bacteria such as Staphylococcus aureaus. This organisms is a major problem in bacterial pneumonia in human beings, but mice tend to be highly resistant to the same organisms. Such species differences in susceptibility imply that it may be difficult to extrapolate data from mice to human beings, and vice versa.

Evidence for complement activation in sepsis

There is considerable evidence in both animal sepsis and human sepsis that complement activation has occurred, as reflected by consumptive depletion of complement and the appearance in plasma of complement activation products (C3a, C4a, C5a anaphylatoxins) which are small peptides (circa 10 kD) with pro-inflammatory activities, especially in the case of C5a. These small peptides, collectively referred to anaphylatoxins, are present in low concentrations but often have intense pro-inflammatory activities. C5a concentrations rarely exceed 10–100 nM in plasma from septic human beings. C5a is highly reactive with C5a receptors (C5aR, C5L2) on phagocytic cells (macrophages, neutrophils [polymorphonuclear leucocytes – PMNs]) at very low concentrations (1–10nM), inducing chemotaxis, enzyme release and a respiratory burst (with H_2O_2 production), all of which are features of innate immune responses. When the levels of C5a are present locally as in soft tissue, lung, joint spaces and in limited in amounts, C5a tends to be protective, enhancing responses of resident phagocytic cells or phagocytes that migrate into the area in order to kill and clear bacteria. In the setting of sepsis, excessive production of C5a resulting in detectable levels (*e.g.* 100 nM) in plasma can lead to signalling paralysis in PMNs, preventing activation of mitogen activated protein kinases (MAPKs) that are required for chemotaxis, phagocytosis and the respiratory burst [14]. Details of how excessive levels of C5a are highly harmful are described below.

Sepsis as a dysfunctional inflammatory state

It has been known for some time that sepsis in human beings or animals exhibits evidence suggesting that regulation of the inflammatory response has been lost, resulting in high levels of pro-inflammatory mediators (IL-1β, tumour necrosis factor [TNF]-α, IL-6, IL-8, etc.) in plasma. This has been referred to as the 'systemic inflammatory response syndrome' (SIRS) [14]. The precise reasons for the high blood levels of these inflammatory mediators are not understood and the extent to which these mediators are responsible for multi-organ damage is still a matter of speculation. Over time during sepsis, there is often the presence of anti-inflammatory mediators (*e.g.* IL-10, IL-12, IL-13), originally considered to be a response to the earlier appearing pro-inflammatory mediators of SIRS. This situation was described by R. Bone to be a 'compensatory anti-inflammatory response syndrome' (CARS) [15]. Below, we will review evidence that sepsis causes major changes in phagocytic cells and endothelial cells, leading to a pro-inflammatory state that favours accumulation of PMNs in tissues which may perhaps be related to attendant tissue and organ damage during sepsis.

Protective effects of C5a generated in limited amounts

To put C5a into a biological perspective, if the amount of C5a generated locally (as at a site of bacterial infection) is limited ($\leq$10 nM), remaining in the physiological range, there will be several beneficial effects as described in Fig. 1. Interaction with C5aR will lead to 'priming' of PMNs and macrophages, such that their innate immune responses (respiratory burst, release of granule enzymes, etc.) to a second agonist such as LPS are considerably enhanced. C5a alone can also induce responses of phagocytic cells, such as chemotactic migration, limited enzyme release and translocation of cytosolic subunits of NADPH oxidase, which results in assembly of a fully active oxidase on the cell membrane, thereby allowing generation of $O^{\bullet}_2$ and H_2O_2 within the phagocytic vacuole and killing of ingested bacteria by HOCl, which is generated by the interaction of myeloperoxidase with its substrate, H_2O_2 C5a can also interact with endothelial cells, resulting in rapid expression of P-selectin on the cell surface, paving the way for intermittent adhesion (rolling) of PMNs on endothelial cells and the eventual transmigration of PMNs (Fig. 1). C5a has two other effects on endothelial cells: 1. Production of IL-8, the presence of

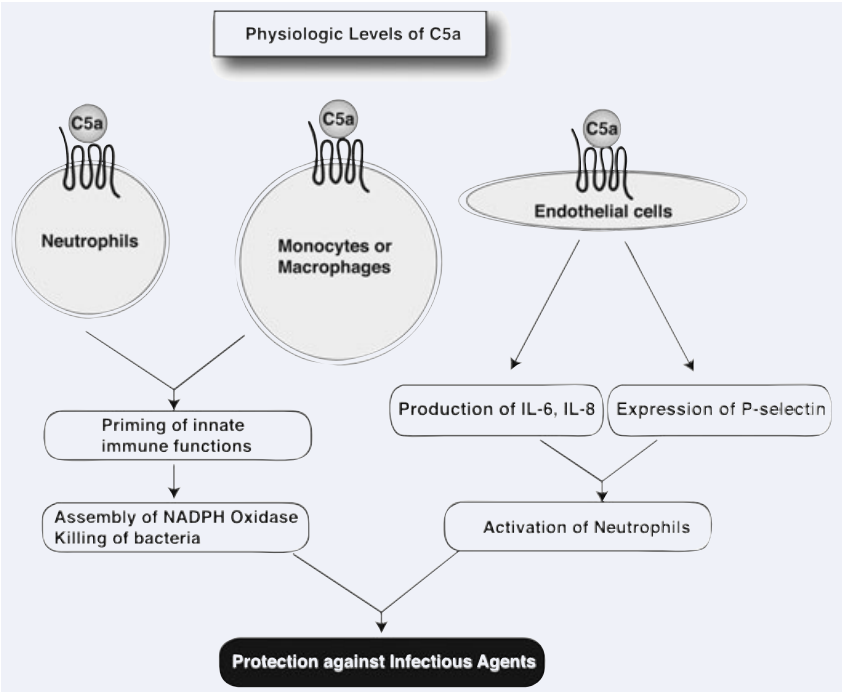

Fig. 1 Effects of physiological levels of C5a (circa 10nM) on neutrophils, monocytes/macrophages and endothelial cells. The responses of the various cell types result in enhanced innate immune responses to infectious agents.

which on endothelial cells can prime PMNs as they begin to adhere to the endothelial surface; 2. A potentially dangerous effect of C5a, induction of tissue factor expression on the endothelial cell surfaces, setting the stage for intravascular coagulation [16–18]. It should be pointed out that there is a controversy related to the ability of endothelial cells to express tissue factor *in vivo*, implying the need for caution in assumptions of the *in vivo* expression of the procoagulant molecule. Collectively, small, regulated amounts of C5a results in enhanced innate immune functions of phagocytic cells, while affecting endothelial cells in a way that initiates the acute inflammatory cascade. Such responses of endothelial cells to C5a may 'prime' adherent PMNs and assist in their adhesion to the endothelium and subsequent transmigration into a site of bacterial infection. Excessive amounts of C5a can lead to loss of innate immune functions of PMNs, which may lead to a catastrophic outcome (see below).

Sepsis-induced changes in signalling cascades

Sepsis triggers a cascade of alterations both in PMNs and endothelial cells which results in conversion of these cells to a pro-inflammatory state. In neutrophils, generation of limited amounts of C5a activates phosphokinase C and cytosolic phospholipase A_2 which ultimately leads to phosphorylation of cytosolic subunits of NADPH oxidase ($_{p47}^{phox}$, $p67^{phox}$, $p50^{phox}$) and oxidase assembly and activation in the context of the phagocyte cell membrane, resulting in H_2O_2 production and its conversion to toxic oxygen products (*e.g.* hypochlorous acid), which can kill bacteria. However, if excessive amounts of C5a are generated, this can result in paralysis of MAPK pathways, resulting in signalling paralysis and loss of bactericidal function of neutrophils (Fig. 2 and [14]). Limited amounts of C5a will also activate the PI3K and Akt signalling pathways (Fig. 2), which results in events that inhibit PMN apoptosis. The loss of PMN apoptosis *in vivo* is thought to result in sustained presence of PMNs in blood and in tissues, setting the stage for accentuated tissue injury [14]. Many different mediators (C5a, IL-1β, G-CSF [colony stimulating factor], etc.) have been shown to modify PMNs, such that they become resistant to apoptosis [19, 20]. In CLP-induced sepsis, *in vivo* blockade of C5a greatly reduces the development of resistance of blood PMNs to apoptosis [19]. In sepsis there is intense activation of coagulation pathways (Fig. 2). As indicated, C5a can induce expression of tissue factor on endothelial cells, triggering intravascular coagulation and consumptive coagulopathy. During sepsis blood PMNs become activated, with increased expression of β_2 integrins (CD11b/CD18), the process of which is C5a-dependent [21]. Sepsis also causes expression of β_1 integrin (as measured by cell surface content of CD29, the common β_1 integrin unit) on surfaces of blood PMNs [21]. This integrin is not ordinarily expressed on surfaces of blood PMNs. In addition, sepsis leads to expression of the chemokine receptor on PMNs, CCR-2, which reacts with MCP-1 (CCL-2), MCP-2 (CCL-4), MCP-3 (CCL-7) and MCP-4 (CCL-12) (Fig. 3, [22]). Up-regulation of β_1 and β_2 integrins (the latter of which binds to ICAM-1 and iC3b) as well as expression of CCR-2 implies that PMNs now have a 'gain of function', such that they can more robustly bind to ligands as well as being able to respond to ligands that they usually do not recognize. Collectively, blood PMNs and endothelial cells are converted to a pro-inflammatory state during CLP-induced sepsis,

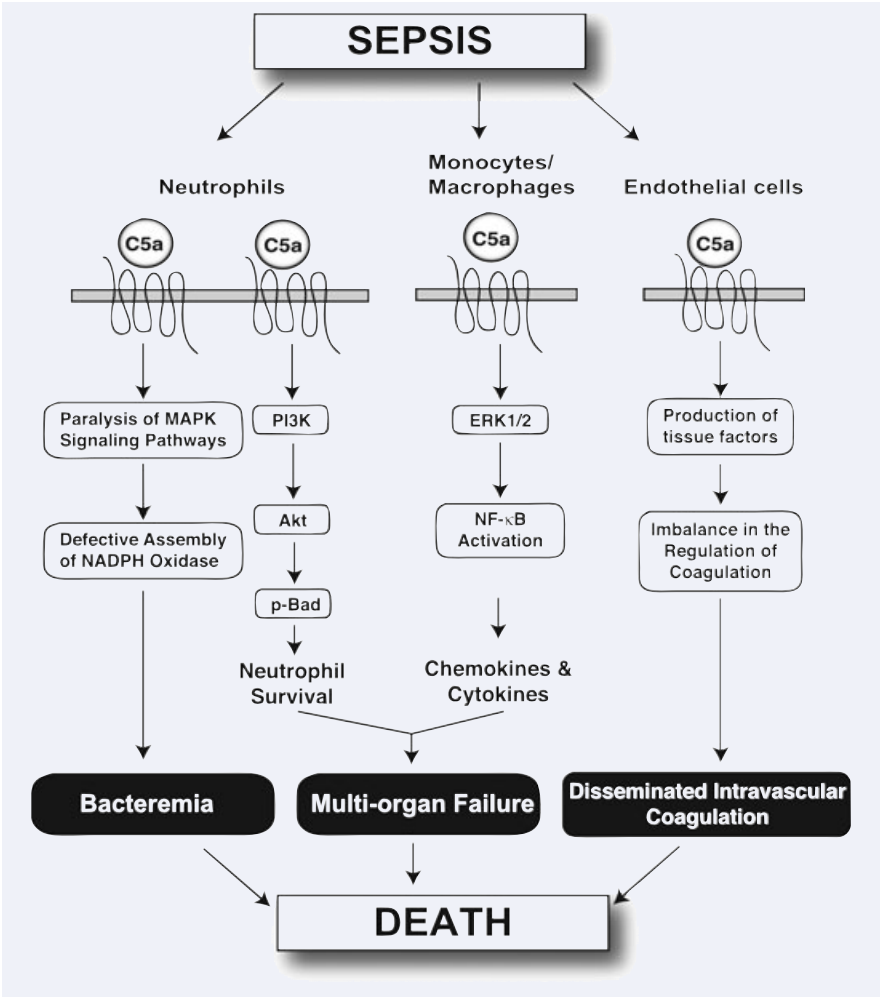

Fig. 2 Signalling pathway activation by C5a on neutrophils, monocytes/macrophages and endothelial cells, resulting in compromised innate immunity, resistance of neutrophils to apoptosis, a cytokine storm and disseminated intravascular coagulation.

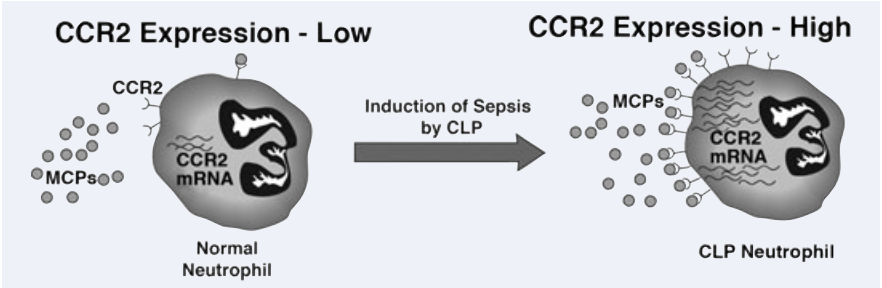

Fig. 3 Perturbations in blood neutrophils during sepsis, resulting in the appearance of high levels of CCR-2, which now allows cells to respond to MCP-1 (CCR-2) and related ligands. This represents a 'gain of function' for neutrophils.

which may put tissues at risk of injury due to an intensified inflammatory response.

Divergent effects on phagocytic cells during sepsis

During CLP-induced sepsis in rats, excessive production of C5a can lead to signalling paralysis of blood PMNs, resulting in loss of protective innate immune functions [14]. Table 1 summarizes studies in which blood PMNs, peripheral blood mononuclear cells (PBMCs) and alveolar macrophages were isolated and compared for functional responses before and after onset of sepsis (CLP). As has been shown in many different studies of sepsis in animals and in human beings, the innate immune responses of blood PMNs were defective. Chemotaxis (to C5a and to a chemically unrelated peptide, N-formyl Met Leu Phe) was defective, phagocytosis was

Table 1 Functional changes in phagocytic cells during sepsis*

Function	PMNs	PBMCs	Macrophages
Phagocytosis, chemotaxis, respiratory burst (H_2O_2 production)	Depressed	Unknown	Unknown
Cytokine, chemokine production (*in vitro*)	Depressed	Depressed	Enhanced
MAPK (ERK1/2) activation *in vitro*	Depressed	Unknown	Enhanced

*PMNs, neutrophils; PBMCs, peripheral blood mononuclear cells.

depressed and the respiratory burst (H_2O_2 production) was impaired [14]. The last abnormality was due to a C5a-induced defect in the ability of stimulated PMNs to translocate cytosolic subunits ($p47^{phox}$) of NADPH oxidase to the cell membrane where full assembly and activation of the oxidase takes place [14]. The effects on functional innate immune parameters in PBMCs and alveolar macrophages remain to be determined.

When cytokine and chemokine production was assessed *in vitro* following *in vitro* incubation of leucocytes with LPS, mediator production was suppressed in PMNs and PBMCs but enhanced in alveolar macrophages [23]. Finally, in a similar manner, LPS-induced activation (phosphorylation) of the MAPK, ERK1/2, was depressed in blood PMNs but enhanced in lung macrophages in the setting of CLP-induced sepsis. These data suggest, in general, that there is a loss in function involving blood PMNs and PBMCs and a gain in function in lung macrophages. Either lung macrophages cannot be rendered functionally defective by exposure to C5a, or the amounts of C5a to which lung macrophages are exposed after CLP is much lower than C5a levels in blood, resulting in priming of lung macrophages. It is not clear why there are such dramatic differences in functional responses of phagocytic cells based on their location (vascular *versus* extravascular) during sepsis.

Harmful effects of C5a and C5a receptors in experimental sepsis

As has been alluded to above, there are many harmful outcome related to interaction of C5a with its receptors during sepsis. As described above, considerable C5a is generated during sepsis and appears within the vascular compartment, reaching plasma concentrations as high as 100 nM [24]. C5a receptors on PMNs and in tissues are in a dynamic state after CLP. In general, mRNA and protein for C5aR are up-regulated in a variety of organs (lung, heart, kidney, liver) [25]. It appears that up-regulation of C5L2 also occurs, but not as robustly when compared to C5aR [25]. Figure 4 summarizes the results of C5a interacting with its receptors after CLP. As described above, C5a binding with C5aR on PMNs leads to MAPK signalling paralysis in these cells, resulting in loss of innate immune functions. The outcome is loss of protective functions of PMNs and attendant lethal bacteraemia. The up-regulation on blood PMN surfaces of CD18 indicates that these cells have been activated. Increased expression of IL-6, IL-8, etc. by endothelial cells implies that adherent PMNs will be activated after contact with IL-6 and IL-8. C5a interaction with tissue macrophages appears to be linked to development of the SIRS of sepsis, due to priming of these cells and the resulting surge of pro-inflammatory cytokines and chemokines in plasma and in tissues. To what extent the presence of these mediators can be directly linked to harmful events such as multi-organ damage and failure is currently a matter of much debate. The interaction of endothelial cells with C5a *via* interaction with C5aR results in a pro-inflammatory state that can be linked to chemokine expression and induction of tissue factor, the latter contributing to disseminated intravascular coagulation, consumptive coagulopathy and formation of intravascular thrombi [16–18].

Development of immunosuppression during sepsis is well documented and has been assumed to be the result of apoptosis of lymphoid cells (both T and B cells) and the ensuing development of the immunosuppression of sepsis [26, 27]. Our own studies have shown in CLP rats that the thymus undergoes massive apoptosis that is both C5a and C5aR dependent [28]. Within 3 hrs after CLP, thymocytes bind higher amounts of C5a (due to increased C5aR protein on surfaces of thymocytes) and can be shown to undergo apoptosis *in vitro* after the addition of C5a [28]. Apoptosis is associated with engagement of the intrinsic (mitochondrial) apoptotic pathway featuring activation of caspases 3, 6 and 9. When anti-C5a is given at the time of CLP, caspase activation and apoptosis in thymocytes are greatly reduced [28]. There is other evidence that the extrinsic pathway of apoptosis is also engaged after CLP in mice, as defined by the requirements for Fas and Fas ligand [29, 30].

We have recently shown that adrenal medullary cells also undergo apoptosis during sepsis [31]. This would imply that the major source of catecholamines (epinephrine and norepinephrine) is lost during sepsis, compromising the ability to sustain adequate blood pressure for tissue perfusion. During sepsis, it is well described that the ‘cardiomyopathy of sepsis’ develops, which refers to falling cardiac output, diminished blood pressure and declining vascular perfusion (Fig. 4). Apoptosis of adrenal medullary cells may lead to the loss of catecholamines, resulting in the inability to maintain vascular tone. In human beings with septic shock, restoration of blood pressure is accomplished by infusion of vasopressors (to increase blood pressure) or positive ionotropes (to increase cardiac output). There is evidence that the

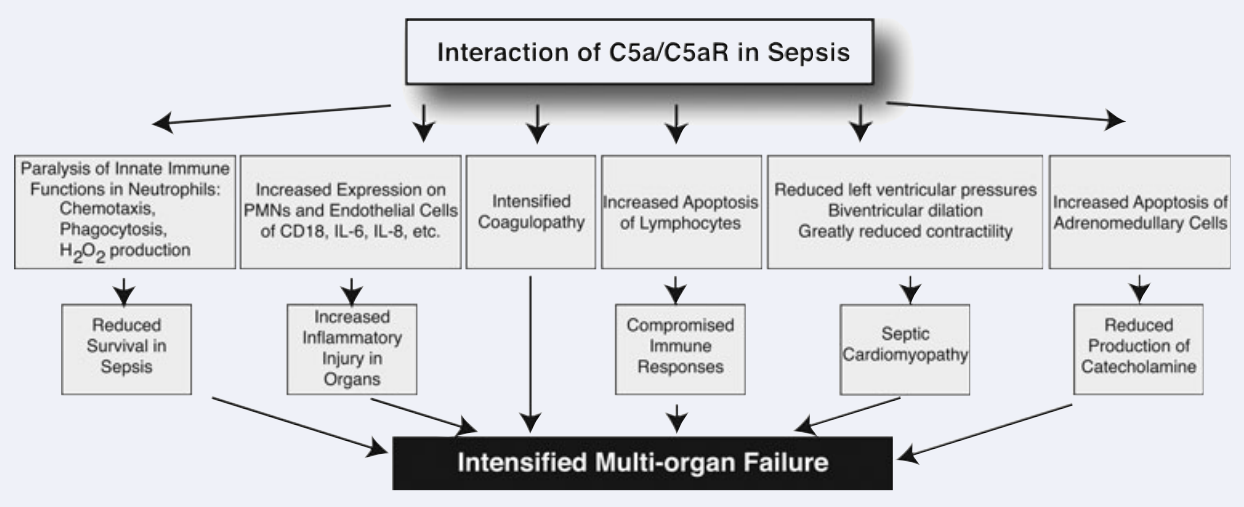

Fig. 4 Interaction of C5a with C5aR during sepsis, resulting in compromised innate immune functions of neutrophils, together with activation markers and increased expression of pro-inflammatory mediators, coagulopathy, apoptosis of lymphoid cells, cardiomyopathy and apoptosis of adrenal medullary cells.

loss of adrenal medullary cells after CLP is linked to participation to engagement of C5a receptors [31]. In addition to reductions in plasma catecholamines during experimental sepsis, there are also direct effects of C5a and C5aR on cardiomyocyte function. It has been shown that sepsis causes increased levels of mRNA and protein for C5aR on cardiomyocytes [32]. Furthermore, cardiomyocytes isolated from rat hearts after CLP show profound contractile dysfunction, involving reduced contractility (sarcomere shortening) and prolonged relaxation times [32]. If C5a is blocked at the beginning of CLP, contractility function of cardiomyocytes and left ventricular pressures are essentially restored to normal values. Furthermore, if sham or CLP cardiomyocytes are incubated *in vitro* with recombinant C5a, a profound contractility defect develops in these cells [32]. To what extent C5L2 may also be involved in the acquisition of these defects is unknown at the present time.

In the literature there is evidence, at least in the endotoxemia model in mice and in a model of burn injury, that cardiomyocyte production of IL-6, Il-1β or TNF-α may be linked to cardiac and cardiomyocyte dysfunction [33]. Accordingly, these mediators have been referred to 'cardiosuppressive cytokines'. Precisely how such observations might be related to 'septic cardiomyopathy' is unknown at the present time.

Future directions

As indicated above, in both experimental sepsis and in human beings with sepsis, there is compelling evidence for robust activation of complement. In sepsis following CLP in rodents, it appears that C5a engagement with its receptors leads to a series of events that: impair innate immune functions of blood PMNs; trigger apoptosis of lymphoid cells as well as adrenal medullary cells; initiate a generalized pro-inflammatory state; and lead to development of septic cardiomyopathy which impairs vascular perfusion. In rodents, the consequences of blockade of C5a, C5aR or C5L2 are highly protective and greatly improve survival after CLP suggesting that engagement of both C5aR and C5L2 lead to adverse outcome [34]. It is possible in human beings with sepsis that such blocking interventions might be protective, with C5a being the most obvious target using a humanized, neutralizing mAb to C5a that is unable to bind to C5 (which, when activated, generates C5a as well as the membrane attack complex (C5b-9), the latter having lytic activity for gram negative bacteria). Obviously, one broad concern is that a strategy aimed at intercepting C5a *in vivo* might potentially compromise innate immunity. However, one is talking about short term therapy (days). It might also be pointed out that in C5 deficient human beings the only clear problem is susceptibility to Neisserial infections. Critical issues include whether an intact IgG or an antibody fragment should be used in the clinical setting of sepsis. The problem with the antibody fragment is that its rapid clearance from blood would bring about the need to infuse very large amounts of antibody continuously over a 2–3 day period. Another critical issue is patient selection. There has been only one successful clinical trial in sepsis out of 40 or more failed clinical trials (see 'Introduction'). It is now clear that septic patients who are the most ill are the most likely to benefit from such therapy, implying that patient selection is absolutely key for the design of clinical trials. In several of the clinical trials, the 28-day lethality was around 30%, meaning that data from the majority of patients will likely dilute out data from a

small cohort of patients who would benefit from the special intervention. As of yet, there are no specific biomarkers of sepsis, only biomarkers that indicate a pro-inflammatory state that correlates with the severity of the clinical condition. Finally, it is clear that we do not understand enough about the pathophysiology of sepsis in order to intuitively move towards novel and more effective therapies.

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