New players in the sepsis-protective activated protein C pathway

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Recombinant activated protein C (aPC) improves the survival of patients with severe sepsis, but the precise molecular and cellular targets through which it mediates this effect remain incompletely understood. In this issue of the *JCI*, Kerschen et al. show that endothelial cell protein C receptor (EPCR) is specifically expressed by mouse CD8$^+$ dendritic cells and that these coordinators of host responses to systemic infection are required for aPC to provide protection against the lethality of sepsis. An additional study, by Cao and colleagues, recently published in the *JCI*, implicates the leukocyte integrin CD11b in the pathways by which aPC mediates antiinflammatory effects in the context of lethal sepsis in mice, suggesting a common thread of synergistic control of innate immune responses by life-saving aPC therapy.

Sepsis affects as many as 500,000 individuals in the United States each year, and in many of these cases it is lethal. Uncontrolled inflammation and coagulation are two hallmarks of severe sepsis. The latter is a result of increased coagulation mediated by tissue factor and impaired anticoagulation following cellular downregulation or depletion of thrombomodulin, endothelial cell protein C receptor (EPCR), and protein C (PC). The fact that sepsis-induced uncontrolled coagulation leads to microvascular thrombosis, which in turn can result in organ failure, provided the rationale for the clinical development of recombinant activated PC (aPC) as a therapy for sepsis. This approach markedly improved overall 28-day mortality in patients, specifically in the subgroup of patients with severe sepsis.

After the discovery that aPC could improve the survival of patients with severe sepsis, researchers sought to understand the molecular and cellular mechanisms underlying its effects. Initial observations that aPC attenuated inflammatory NF-κB signaling in endothelial and monocytic cells led to the hypothesis that the effects of aPC were not mediated simply by its ability to promote anticoagulation. Subsequent analysis identified an aPC signaling complex in which aPC binds to EPCR, facilitating the proteolytic cleavage of protease-activated receptor 1 (PAR1) on endothelial cells by aPC (1). Endothelial EPCR/aPC/PAR1 signaling typically counterbalances the detrimental effects of inflammation as well as those of thrombin/PAR1 signaling, which promote apoptosis, barrier disruption, and endothelial activation (2, 3). Although the paradoxical, opposing roles of PAR1 signaling are incompletely understood, several contributing factors have been identified, including the inability of EPCR/aPC-cleaved PAR1 to cross-activate PAR2 (3); coupling of EPCR/aPC signaling to sphingosine-1-phosphate receptor signaling (2); localization of EPCR/aPC signaling to caveolae, resulting in PAR1 desensitization (4); and ligand occupancy of EPCR, leading to EPCR/caveolin dissociation and a broad switch of PAR1 signaling specificity (5).

The development of mutants of aPC that were either permissive for signaling but had minimal anticoagulant activity (6) or potent anticoagulants with drastically reduced signaling function (7) provided the decisive tools to investigate whether aPC-mediated signaling, rather than its anticoagulant activity, was crucial for mortality reduction following aPC therapy in mouse models of sepsis, and this was demonstrated to be the case (6, 7). Furthermore, mice with very low levels of EPCR or deficiency of PAR1 did not benefit from aPC administration (6). However, these studies did not provide insight into relevant cellular targets for sepsis-protective aPC signaling (6, 7). Notably, the effects of aPC in other cell types can be independent of EPCR or PAR1 and may involve additional receptors. EPCR also supports PAR2 cleavage by aPC (1), aPC requires PAR3 for neuroprotection in stroke (8), and aPC utilizes the endocytic receptor LDL receptor–related protein 8 (LRP8; also known as ApoER2) to directly trigger disabled 1 (Dab1) and glycogen synthase kinase 3β phosphorylation in myeloid cells (9). Data published recently in the *JCI* by Cao et al. (10) and in this issue of the *JCI* by Kerschen et al. (11) elucidate new non-anticoagulant mechanisms by which aPC can protect against lethal sepsis in mice. Specifically, these reports indicate that aPC can control immune responses and are consistent with another recent article showing that aPC suppresses neutrophil migration (12).

DCs are required for lethality protection by therapeutic aPC

Kerschen et al. (11) provide an important advance in our understanding of the cellular targets of aPC therapy for severe sepsis and shed new light on recently identified alternative pathways for the non-anticoagulant activities of aPC. In a mouse model of lethal sepsis, administration of an aPC mutant permissive for signaling but with minimal anticoagulant activity indicated that EPCR and PAR1 in hematopoietic cells were required for mortality reduction by aPC. Consistent with previous studies (13), hematopoietic EPCR deficiency failed to increase the susceptibility of mice to the sepsis challenge, indicating that endogenous aPC does not appreciably support survival through this pathway and that therapeutic aPC given at a high dose may have unique pharmacological effects on immune cells.

While monocytes and macrophages express EPCR, Kerschen et al. (11) found that EPCR also marked selectively the CD8$^+$ subset of mouse DCs in the spleen. DCs are widely distributed as immature cells in the periphery, serving as antigen-sampling sentinels. Peripheral DCs mature and migrate to draining lymph nodes that contain a complex repertoire of migratory and resident DCs. The spleen of unchallenged mice

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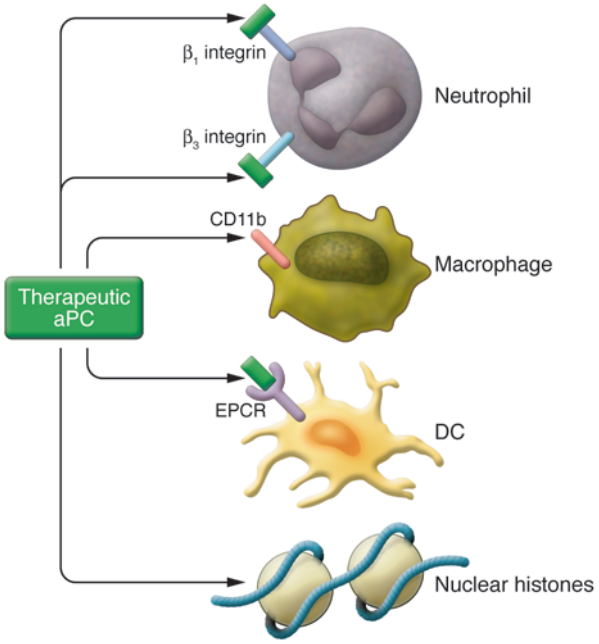

Figure 1
Targets for aPC that elicit its therapeutic effects in severe sepsis. The schematic illustrates newly identified targets for aPC in the innate immune system. Therapeutic aPC regulates neutrophil migration and extravasation by direct engagement of β_1 and β_3 integrins (12), suppresses macrophage activation dependent on integrin CD11b/CD18 (11), controls maturation and activation of $CD8^+$ DCs dependent on EPCR (11), and neutralizes late-stage inflammatory mediators by degrading nuclear histones from apoptotic cells (17).

predominantly harbors immature, lymphoid organ–resident conventional DCs, most of which are $CD8^-CD24^{lo}CD11b^{hi}$ and approximately 20% of which are $CD8^+CD205^+CD24^{hi}CD11b^{lo}$ DCs with a larger repertoire of endocytotic receptors (14). Kerschen et al. (11) capitalized on the relative homogeneity of steady-state spleen DC populations to isolate conventional DCs based on the common DC marker CD11c and negative selection to exclude the minor population of plasmacytoid DCs involved in antiviral immunity. Adoptive transfer of these purified DCs from either wild-type or EPCR-deficient mice into bone marrow chimeras, in which the EPCR-deficient hematopoietic compartment was unresponsive to aPC, clearly showed that DCs were sufficient and EPCR was required to restore the ability of aPC therapy to protect the mice from sepsis-induced death.

Spleen DCs are optimally positioned to sample the blood for systemic danger signals. $CD8^+$ DCs and their human counterparts (15) are highly efficient in presenting on MHC class I exogenous antigens derived from endosomal degradation, in particular following endocytotic uptake of apoptotic cells. $CD8^+$ DCs are therefore crucial regulators of both T cell activation and immune-regulatory, suppressive pathways in the context of cell death, which is a prominent feature in lymphoid organs in sepsis (16). It is intriguing that aPC also degrades released nuclear histones and thereby attenuates the inflammatory collateral damage of immune cell apoptosis (17), indicating cooperative effects of aPC therapy that may support the sepsis-protective roles of $CD8^+$ DCs.

Expression profiling of spleen $EPCR^+$ DCs isolated 3 hours after LPS challenge showed directly that a single dose of aPC given systemically briefly after intraperitoneal LPS suppressed the response of this DC population to LPS challenge (11). Evaluation of the same genes in a profile obtained 16 hours after LPS challenge in the absence of single-dose aPC therapy revealed that the early wave of LPS-induced DC activation had largely subsided at this time. However, the 16-hour transcriptional changes of the same gene set in DCs from LPS-challenged, aPC-treated mice showed marked similarities with that observed in DCs isolated at 3 hours from mice challenged in the absence of aPC, indicating that the applied single-dose aPC therapy delays or attenuates, rather than completely prevents, the activation or maturation of DCs. Kerschen et al. (11) also attempted to define the contributions of aPC signaling receptors by comparing expression profiles of the total $CD11c^+$ conventional DC populations purified from wild-type, EPCR-deficient, and PAR1-deficient mice 16 hours after LPS challenge. These data provided evidence for pharmacological effects of the aPC mutant that are EPCR- and to a lesser extent PAR1-independent, but no definitive connections could be extracted from this complex data set.

The data mining of the late-stage DC gene expression profiles generated by Kerschen et al. (11) is highly challenging, because TLR signaling not only induces the maturation of spleen-resident DCs, but also initiates considerable crosstalk between innate immune cells and dynamic changes in the local cytokine milieu. Resident DCs orchestrate the clustering and activation of neutrophils and IFN-γ–producing $CD11c^+NK1.1^+CD56^{bright}$ NK cells that co-isolate with $CD11c^+$ DCs. $CCR2^+Ly6C^+$ monocytes are recruited by resident DCs and differentiate within 24 hours into TNF-α– and iNOS-producing (TIP) DCs in a process that is dependent on NK cell–produced IFN-γ (18). Indeed, transcriptional profiling of $CD11c^+$ cells during the critical time window (approximately 12 hours after LPS challenge) for $CD11c^+NK1.1^+$ NK cell expansion in the spleen (12) provided initial evidence that aPC interrupted this inflammatory network that is coordinated by DC activation in the spleen. Marked reductions in the amount of the $CD11c^+NK1.1^+$ NK cell signature cytokine IFN-γ were further observed in both aPC-treated EPCR- and PAR1-deficient mice, indicating that aPC regulates the recruitment or subsequent activation of these $EPCR^-CD11c^+$ NK cells, at least in part, independently of the canonical aPC signaling receptors. Thus, although EPCR and PAR1 are required for aPC therapy–induced survival in these sepsis models, accessory signaling pathways must exist,

and these likely contribute to the overall efficacy of aPC in sepsis therapy.

Integrins are partners for aPC in innate immune signaling

Additional studies with aPC lacking the EPCR-interacting amino-terminal Gla domain have unraveled novel EPCR-independent signaling pathways involving integrins expressed by innate immune cells (10, 12). In a recent article published in the *JCI*, Cao et al. (10) showed that the suppression of LPS-induced inflammatory cytokine production by macrophage aPC/PAR1 signaling is independent of EPCR but requires the expression of the leukocyte integrin CD11b/CD18, which, similar to EPCR, was colocalized with PAR1 in lipid raft domains. Recapitulating findings in endothelial cells (2), Cao et al. found that macrophage aPC/PAR1 signaling coupled to sphingosine kinase 1 activation, leading to increased sphingosine-1-phosphate production (10). Furthermore, direct agonists of sphingosine-1-phosphate receptor 1 mimicked the ability of aPC to reduce production of the inflammatory cytokine IL-6. Cao and colleagues developed a coherent line of evidence that this pathway is relevant to the protective effects of aPC in mouse models of sepsis by demonstrating that aPC lacking the EPCR-interacting Gla domain suppresses IL-6 levels in vivo and improves survival when given in a highly critical time window 20 minutes after LPS, similar to the dosing scheme used by Kerschen et al. (11). In contrast, sphingosine-1-phosphate production in vivo was efficiently induced by treatment of mice prior to the LPS challenge. Therefore, in the absence of similar experiments with aPC lacking the Gla domain, the in vivo connection between sphingosine-1-phosphate signaling and reduced inflammation remains ambiguous.

Cao et al. (10) further showed that aPC did not improve survival of CD11b-deficient mice. Although it will be necessary to eventually prove the direct interaction between the two proteins in vivo (for example, with an aPC mutant devoid of CD11b binding), this genetic evidence implicates $CD11b^+$ innate immune cells in the response to life-saving aPC therapy. In monocytes/macrophages, aPC attenuates Wnt5A induction (19) and reduces inflammatory cytokine production without impairing phagocytotic bacterial clearance (20). Considering the role of CD11b in complement-dependent phagocytosis, cooperative interactions between CD11b and aPC may preserve the clearance of apoptotic cells by macrophages and possibly DCs. Additional more indirect synergistic effects may result from altered recruitment of $CD11b^+$ inflammatory monocytes and their differentiation into DCs and macrophages in secondary lymphoid organs (21).

Elphick et al. (12) provide another example of aPC interactions relevant to its protective effects in mouse models of sepsis. They found that aPC interacted with another class of integrins on innate immune cells and demonstrated that aPC binds directly to activated $\alpha_3\beta_1$, $\alpha_5\beta_1$, and $\alpha_V\beta_3$ integrins through a single RGD motif, preserved as a conservative QGD substitution in the mouse. The RGD site is cryptic in the protease domain of zymogen PC but exposed after activation, providing a mechanism by which therapeutic administration of aPC can produce effects that are distinct from the typical interactions of endogenous zymogen PC. aPC lacking the Gla domain was as effective as wild-type aPC at inhibiting neutrophil chemotaxis in vitro. In vivo, wild-type aPC, but not a site-specific integrin binding–deficient mutant, prevented neutrophil extravasation into the bronchoalveolar space, a finding that recapitulated experimental pharmacological effects of aPC in humans (22). While additional in vivo experiments with aPC lacking the Gla domain could have clarified the previously demonstrated role of EPCR in aPC effects on neutrophil migration, this study provided clear evidence that aPC targets neutrophil integrins other than CD11b.

Future perspective

While the results from a short-term administration of aPC during the first hours of a highly standardized sepsis challenge in rodents are not easily translated into the diverse syndrome of clinical sepsis for which aPC therapy is given as a continuous infusion over 72 hours, the studies of Cao et al. (10), Kerschen et al. (11), Elphick et al. (12), and Xu et al. (17) identify new mechanisms and several targets in the innate immune system that are indispensable for the life-saving effects of aPC. A picture emerges in which therapeutically administered aPC simultaneously modulates immune cell migration, activation, and maturation or directly neutralizes proinflammatory mediators that sustain the activation of the innate immune response in sepsis (Figure 1).

Although aPC is given in the clinic based on the principle of substitution therapy, the high-dose bolus injection of PC administered in experimental models and unfavorable binding interactions of PC with some of the identified targets (10) strongly suggest that aPC has unique pharmacological effects that go beyond the physiological regulatory roles of the anticoagulant pathway. Improving these sepsis-protective activities in variants of aPC will be essential to further validate these new pathways in animal models and to explore how to better administer aPC for life-saving therapy in the clinic. Variants with substantially reduced anticoagulant activity do not carry the liability of bleeding complications and may be given with higher and potentially intermittent dosing to interrupt the escalation of sepsis syndrome caused by neutrophil, macrophage, or DC activation, with the possible additional benefit of preventing the immune paralysis that frequently develops in survivors of severe sepsis. Such safer variants with improved alternative target specificity may also broaden the use of aPC in less severe sepsis and potentially other, more chronic immune disorders.

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Fitness and freezing: vector biology and human health

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Microbes transmitted to mammals by arthropods contend with many factors that could impede survival. To survive, host fitness with infection must outweigh costs. In this issue of the *JCI*, Neelakanta et al. demonstrate that ticks infected with *Anaplasma phagocytophilum* show enhanced fitness against freezing injury owing to induced expression of tick "antifreeze glycoprotein." This allows *A. phagocytophilum* to successfully propagate and survive to cause disease in nonnatural hosts, such as humans. How an intracellular microbe with a small genome subverts host cell function for survival provides insight into the control of some cellular function programs and underscores how vector biology can have an impact on human health.

Emergence and reemergence of infectious diseases is often attributed to many factors, most of which are influenced by human activities, for example, climate change, environmental change, changes in human demographics and behaviors, and the rise of global trade and travel (1). Perhaps not surprisingly, pathogens evolve or acquire genetic mechanisms that enhance fitness under circumstances of human intervention, such as increasing resistance to potent antimicrobial pharmacologic agents. Emerging infectious disease is most likely to be caused by zoonotic or vector-borne agents (1). This is readily understood for zoonotic and vector-borne RNA viruses (such as tick-borne encephalitis viruses, West Nile virus, and the viruses that cause yellow fever, dengue fever, and Crimean-Congo hemorrhagic fever), which undergo frequent genetic change and thus have repeated opportunities to improve fitness (1). However, the substantial evolution of fitness needed to emerge (or reemerge) as a significant health concern in humans is surprising in the context of vector transmission for organisms with less flexible genomes, few opportunities for genetic exchange, and the need to contend with protective mechanisms in at least two distinct hosts. Yet drug-resistant malaria, African and American trypanosomiasis, Lyme disease, and rickettsial infections such as Rocky Mountain spotted fever (RMSF) and human granulocytic anaplasmosis (HGA) have emerged or reemerged to increase in prevalence over the past few decades (2). While considerable investigation is being conducted for some vector-borne emerging diseases and pathogens, bacteria of the order Rickettsiales and their resulting human diseases are understudied, in part because they are obligate intracellular pathogens, which makes biological study difficult, and in part because it is extremely difficult to establish definitive diagnosis, which makes clinical study a challenge (3). Vector biologists who study arthropod-transmitted pathogens understand the role of the vector not only in transmission, but also in disease "ecology." In this issue of the *JCI*, Neelakanta et al. demonstrate a new paradigm as to how a tick-borne pathogen can manipulate its arthropod host to foster vector survival and indirectly, pathogen transmission, mammalian reservoir maintenance, and, inevitably, human disease (4).

Increasing rickettsial disease prevalence

While Lyme disease, which is caused by bacteria belonging to the genus *Borrelia*, is the most common vector-borne human infectious disease in the US (5), rickettsial infections are also increasingly being reported in North America, South America, and Europe, and serological studies show that rickettsial infections are common causes of febrile illness in regions where most fever is attributed to malaria or typhoid fever (5). Recognition of tick-borne rickettsial infections, such as RMSF (caused by *Rickettsia rickettsii*), African tick-bite fever (caused by *Rickettsia africae*), human monocytic ehrlichiosis (caused by *Ehrlichia chaffeensis*), and HGA (caused by *Anaplasma phagocytophilum*) has markedly expanded in recent years (3). The 4,727 confirmed and unconfirmed reports

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