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Regulation of immune cell signaling by activated protein C

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Abstract

Innate immune cells are an essential part of the host defense response, promoting inflammation through release of pro-inflammatory cytokines or formation of neutrophil extracellular traps. While these processes are important for defense against infectious agents or injury, aberrant activation potentiates pathologic inflammatory disease. Thus, understanding regulatory mechanisms that limit neutrophil extracellular traps formation and cytokine release is of therapeutic interest for targeting pathologic diseases. Activated protein C is an endogenous serine protease with anticoagulant activity as well as anti-inflammatory and cytoprotective functions, the latter of which are mediated through binding cell surface receptors and inducing intracellular signaling. In this review, we discuss certain leukocyte functions, namely neutrophil extracellular traps formation and cytokine release, and the inhibition of these processes by activated protein C.

Keywords

Neutrophils; activated protein C; APC; sepsis; Mac-1; EPCR; PAR1; PAR3

Introduction

Neutrophils, a type of innate immune cell, are among the first responders to sites of infection and injury, and they are indispensable for the initiation, amplification and resolution of inflammation [1–4]. Inflammation, for better or worse, is promoted by release of pro-inflammatory cytokines [5] and formation of DNA-rich neutrophil extracellular traps

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Authorship

L.D.H., J.H.G. and O.J.T.M. planned, researched and wrote the review. R.A.R. participated in writing and critical editing of the manuscript.

Disclosures

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(NETs) [6,7]. For the better, these processes are invaluable for successful host defense. For the worse, cytokine-driven inflammation and NETs have each been implicated in a multitude of acute and chronic inflammatory diseases [7–14]. Thus, new understandings of the regulatory mechanisms in NETs formation and cytokine release can provide insights into host defense and identify potential therapeutic targets when host defense mechanisms become pathologic.

The crosstalk between leukocytes, especially neutrophils, and coagulation serine proteases is a broad area that has been extensively reviewed [15–17]. Here, we focus on the crosstalk between leukocytes and the serine protease activated protein C (APC). Endogenous regulation of the coagulation cascade occurs, in part, through the protein C system in which the trypsin-like serine protease APC catalyzes inactivation of the activated coagulation factors Va and VIIIa to reduce thrombin generation [18,19]. APC is also well recognized to have cell signaling actions whereby it can exert a spectrum of cytoprotective effects [20–22]. The goal of this review is to present a summary of certain leukocyte functions, namely NETs formation and inflammation, and to discuss the regulatory role of APC and the protein C pathway on these leukocytic processes.

Leukocyte Cell Functions

Leukocytes are essential for successful host defense, but excessive activity and resultant inflammation contribute to serious infectious or autoinflammatory diseases [3,11,23–25]. Pathologic leukocyte activation also contributes to increased thrombotic risk [15,17,26]; however, the complex topic of immunothrombosis will not be addressed in this review. As part of the normal host defense response to pathogen-associated or damage-associated molecular patterns (PAMPs and DAMPs, respectively), pattern recognition receptors (PRRs) are activated to elicit rapid responses either against foreign pathogens or to endogenous host-derived signals [3,23,27,28]. The ability of leukocytes to sense and respond to their microenvironment is tightly regulated by a series of receptor-mediated oxidative and nonoxidative signaling pathways that control the assembly of intracellular proinflammatory complexes, degranulation, chemotaxis, cell death, and resolution [4,23,24,29–31].

In 2004, it was first described that neutrophils form NETs [6], and this process can cause suicidal NETosis, an alternative form of cell death. Suicidal NETosis involves a general pro-inflammatory mechanism whereby in response to various stimuli, intracellular signaling results in decondensation and extrusion of chromatin decorated with antimicrobial enzymes, including myeloperoxidase (MPO) and neutrophil elastase (NE) (Figure 1) [7,32–34]. Vital NETosis has also been reported whereby nuclear or mitochondrial DNA is extruded without cell membrane lysis, such that the resulting anucleate cytoplasm retains cellular functions [35–37]. Clearly, continued characterization of the stimuli and mechanisms driving vital and suicidal NETosis is required to resolve their respective roles in the host response. Limited, early formation of NETs is considered beneficial in select disease states like sepsis to promote pathogen degradation and clearance [35,38]. On the pathologic side, aberrant extracellular trap formation is associated with a multitude of chronic and acute diseases [39–43].

As one of their pro-inflammatory responses, leukocytes release the pro-inflammatory cytokines interleukin (IL)-1β and IL-18 [44]. These cytokines are generated from their pro-IL precursors by caspase-1, which itself is generated by activation of pro-caspase-1 by inflammasomes. Inflammasomes are intracellular aggregates of multiple proteins, most commonly driven by one protein, nucleotide-binding domain and leucine-rich repeat-containing protein 3 (NLRP3, also known as cyropyrin). Inflammasome signaling has been reviewed in detail elsewhere [14,45,46]. In general, canonical inflammasome assembly, activation and signaling require multiple signals and steps, including initiation of signaling by cell surface receptors, upregulation of transcription of select inflammasome components, multi-protein inflammasome assembly, and, finally, inflammasome activation resulting in activated caspase-1. The identification of activating missense mutations in cryopyrin results in a constitutively active protein and causes chronic autoinflammation in humans [47]. This discovery highlighted the importance of tight regulation of inflammasomes for healthy host defense. Aberrant activation of the inflammasome may be associated with, inter alia, multiple sclerosis, type 2 diabetes, and atherosclerosis (see Inflammation subsection below) [13,14,44].

The Protein C System

APC is a multi-functional plasma serine protease with potent anticoagulant activity as well as multiple cell signaling activities that are protective against diverse injuries [20–22] (Figure 2). For activation of the inactive zymogen protein C (PC), it binds to endothelial protein C receptor (EPCR) to form a complex, whereby it is cleaved by thrombomodulin-bound thrombin to generate the active protease, APC [18,48,49]. In its capacity as an anticoagulant, APC dampens thrombin generation through irreversible proteolytic inactivation of coagulation factors Va and VIIIa (Figure 2) [18,50,51]. To initiate intracellular signaling that can limit activation of inflammatory pathways and enable cell survival in the face of pro-apoptotic challenges, APC binds several receptors and can then activate the G-protein coupled receptors (GPCRs) protease activated receptor (PAR)1 and/or PAR3 (Figure 2). Intracellular mediators of APC-induced signaling include β-arrestin-2, PI(3)K/Akt and Rac1 [52–56]. Other key receptors that can bind APC and that may mediate APC's signaling include β1-3 integrins, especially Mac-1 [57–60], apolipoprotein E receptor 2 (ApoER2) [61,62], and Tie2 [63].

APC's multiple cytoprotective activities [20–22] have been demonstrated on a variety of cell types including but not limited to endothelial cells [55,64], monocytes [62,65–67], macrophages [65,68] and neutrophils [58,60,69–72]. The beneficial effects of APC on immune system cells are summarized in Table 1 [20,73,74]. Highly relevant for immune system cells is the recent discovery that APC inhibits development of the activated inflammasome [68] which is discussed in a separate inflammation subsection below.

APC and sepsis

Following the success of the Recombinant Human Protein C Worldwide Evaluation in Severe Sepsis (PROWESS) trial in 2001 for recombinant wt-APC for adult severe sepsis, wt-APC was approved for this indication [75]. The subsequent PROWESS-SHOCK trial was

completed in 2011 and failed to show benefit for wt-APC for severe adult sepsis [76], resulting in withdrawal of the drug from the market. Despite this, controversy remains, as meta-analysis and meta-regression of the effectiveness and safety of wt-APC for sepsis covering >40,000 patients concluded that real-life use of wt-APC was more in line with the PROWESS trial rather than the PROWESS-SHOCK trial [77,78]. Unfortunately, the rationale and design for the PROWESS trial and subsequent trials of APC for sepsis lacked a true understanding of APC's mechanisms of action in preclinical sepsis models and thus of its potential mechanisms of action in humans. Indeed, as of now, abundant evidence from preclinical models emphasizes the major role for APC's cell-signaling actions rather than its anticoagulant actions for reducing death in sepsis [73,79–84]. Cytoprotective-selective APC mutants reduce death in a murine pneumonia sepsis model due to APC's biased signaling via cleavage of PAR1 at Arg46 [85]. One such APC mutant, 3K3A-APC, is safe in humans when given as a high-dose bolus [86], and a recently completed clinical trial for treatment of ischemic stroke (RHAPSODY trial) showed that this APC mutant is safe in stroke patients (P. Lyden, unpublished data). Based on these observations and on a wealth of preclinical data, we believe that there is now a compelling rationale for development of cytoprotective-selective APC mutants such as 3K3A-APC for novel sepsis therapies that employ bolus dosing.

NETs

Sepsis

Severe sepsis is defined as a systemic inflammatory host response presenting with infection and acute organ dysfunction [16,27,87,88]. In the development of sepsis, inflammation is initiated in response to infection, activating the immune response and the blood coagulation cascade, the latter of which can exacerbate inflammation. Crosstalk between the immune response and the coagulation cascade results in the consumption of immune cells and coagulation factors, exhausting the immune and coagulation responses and increasing the risk for adverse bleeding events or thrombosis as well as death.

The formation of NETs in the pulmonary airways and liver microvasculature was initially observed using *in vivo* mouse models of sepsis, and NETs formation was determined to be partly beneficial for survival by capturing pathogens and promoting their clearance [35,38]. However, the observation that degradation of NETs by DNases reduced intravascular coagulation and reduced organ damage associated with sepsis and septicemia highlighted the delicate balance of the immune response between successful normal host defense and harmful pathological responses [38,89].

NETs and Activated Protein C (APC)

Genetic overexpression of APC in mice decreases neutrophil influx into lung tissue and bronchial lavage fluid during pneumococcal pneumonia [90]. The additional findings that APC cleaved extracellular histones in baboon [72] and mouse [70] models of sepsis raised the hypothesis that one or more of APC's cytoprotective or anti-inflammatory actions might include regulation of neutrophil function and NETs formation. As low dose infusions of recombinant wt-APC initially succeeded but later failed to provide an overall benefit in adult

severe sepsis trials (see above), studies of APC's cell signaling effects on neutrophils acquired greater relevance.

Receptors on the neutrophil surface that can bind APC and influence neutrophil migration include the canonical APC receptor EPCR [71] as well as the neutrophil β1-3 integrins [57,58] and VLA-3 ($\alpha_3\beta_1$; CD49c/CD29) [60]. Notably, APC inhibits induction of NETs by phorbol 12-myristate 13-acetate (PMA) or by autologous platelet secretome, and this effect of APC requires the neutrophil receptors EPCR, PAR3, and Mac-1 ($\alpha_M\beta_2$; CD11b/CD18) [69]. The signaling-selective APC mutant 3K3A-APC demonstrated a similar degree of inhibition of NETs generation as did wt-APC, further supporting the immuno-regulatory potential of cytoprotective-selective APC mutants. Evidence for APC's *in vivo* beneficial effects on neutrophils in sepsis came from a baboon model of *E. coli*-induced sepsis in which infusion of APC reduced levels of myeloperoxidase, a marker for neutrophil activation linked to NETs formation [69]. Currently, much remains to be clarified regarding receptor interactions and intracellular signaling pathways that mediate APC's anti-inflammatory activities involving neutrophils.

Inflammation and Inflammasomes

Inflammation is mediated by the release of cytokines and chemokines that help to drive innate immune cell responses and mobilization of the adaptive immune system. Many view IL-1β as the quintessential proinflammatory mediator in acute and chronic inflammation and one of the most powerful inducers of the innate immune response [5,91,92]. The inflammasome, a source for IL-1β, is a primary driver of inflammation and is best characterized in macrophages [9,25,45,46,93], but is present in other cell types, including epithelial cells [94] and neutrophils [95,96]. Neutralization of IL-1β by a monoclonal antibody in the Canakinumab for Atherosclerotic Disease (CANTOS) trial recently demonstrated that reduction of inflammation without concomitant change in lipid levels reduces the risk of atherothrombosis [13], and is strong evidence pointing to the broad reach of inflammation, especially a pathologic role for IL-1β in cardiovascular disease. Additional *in vivo* evidence shows that myocardial ischemia reperfusion injury involves the NLRP3 inflammasome. Recent studies showed that development of the inflammasome and injury was suppressed by APC treatment [68], raising the broad question of whether APC's inhibition of inflammasome development in many cell types partially explains APC's multiple effects in a large number of preclinical injury models [20]. Future studies of the regulation of inflammasomes in neutrophils and many other cell types will help broaden our understanding of inflammation in its multiple manifestations.

Concluding Remarks

The literature reviewed above highlights the crosstalk between leukocyte-driven inflammation and APC, which has notable therapeutic potential for its anti-inflammatory functions. Specific to neutrophils, APC initiates cytoprotective signaling through select cell surface receptors, including EPCR, Mac-1 and PAR3, which results in inhibition of NETs formation (Figure 3). Nonetheless, much remains to be learned concerning the mechanisms

that regulate inflammation and NETs formation, knowledge that will hopefully lead to methods limiting the progression of inflammatory pathologies.

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Abbreviations page

APC	Activated protein C
ApoER2	Apolipoprotein E receptor 2
BMDM	Bone marrow-derived macrophages
CAM	Camptothecin
CANTOS	Canakinumab for atherosclerotic disease
DAMPs	Damage-associated molecular patterns
EPCR	Endothelial protein C receptor
GPCR	G protein-coupled receptors
IL-1β & IL-18	Interleukin-1β & -18
INF-γ	Interferon-γ
LPS	Lipopolysaccharide
Mac-1	Macrophage Antigen-1; $\alpha_M\beta_2$; CD11b/CD18
MCP-1	Monocyte chemoattractant protein-1
MIP-1-α	Macrophage inhibitory protein 1-α
MPO	Myeloperoxidase
NE	Neutrophil elastase
NETs	Neutrophil extracellular traps
NLRP	Nucleotide-binding domain and leucine-rich repeat-containing proteins; NOD-like receptor protein
PAMPs	Pathogen-associated molecular patterns
PAR1,3	Protease activated receptors-1,3
PC	Protein C
PKC	Protein kinase C

PMA	Phorbol 12-myristate 13-acetate
PROWESS	Recombinant Human Protein C Worldwide Evaluation in Severe Sepsis
PRRs	Pattern recognition receptors
ROS	Reactive oxygen species
THP-1	Monocytic cell line
TNF-α	Tumor necrosis factor-α
U937	Monocytic cell line
VLA-3	Very Late Antigen-3; $\alpha_3\beta_1$; CD49c/CD29

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Summary sentence

This review focuses on select leukocytic functions and how they are regulated by the anti-inflammatory protease, activated protein C.

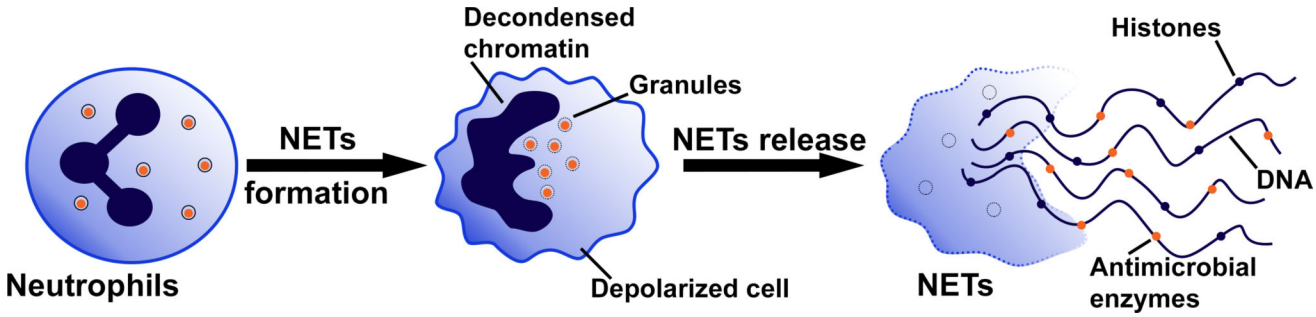

Figure 1. Neutrophil Extracellular Trap formation

In response to inflammatory stimuli, neutrophils are activated and can undergo neutrophil extracellular trap (NETs) formation whereby the cell depolarizes and chromatin comprised of histones and DNA is ejected from the cell. NETs are decorated with antimicrobial enzymes, including myeloperoxidase and neutrophil elastase. Figure adapted from [7,26].

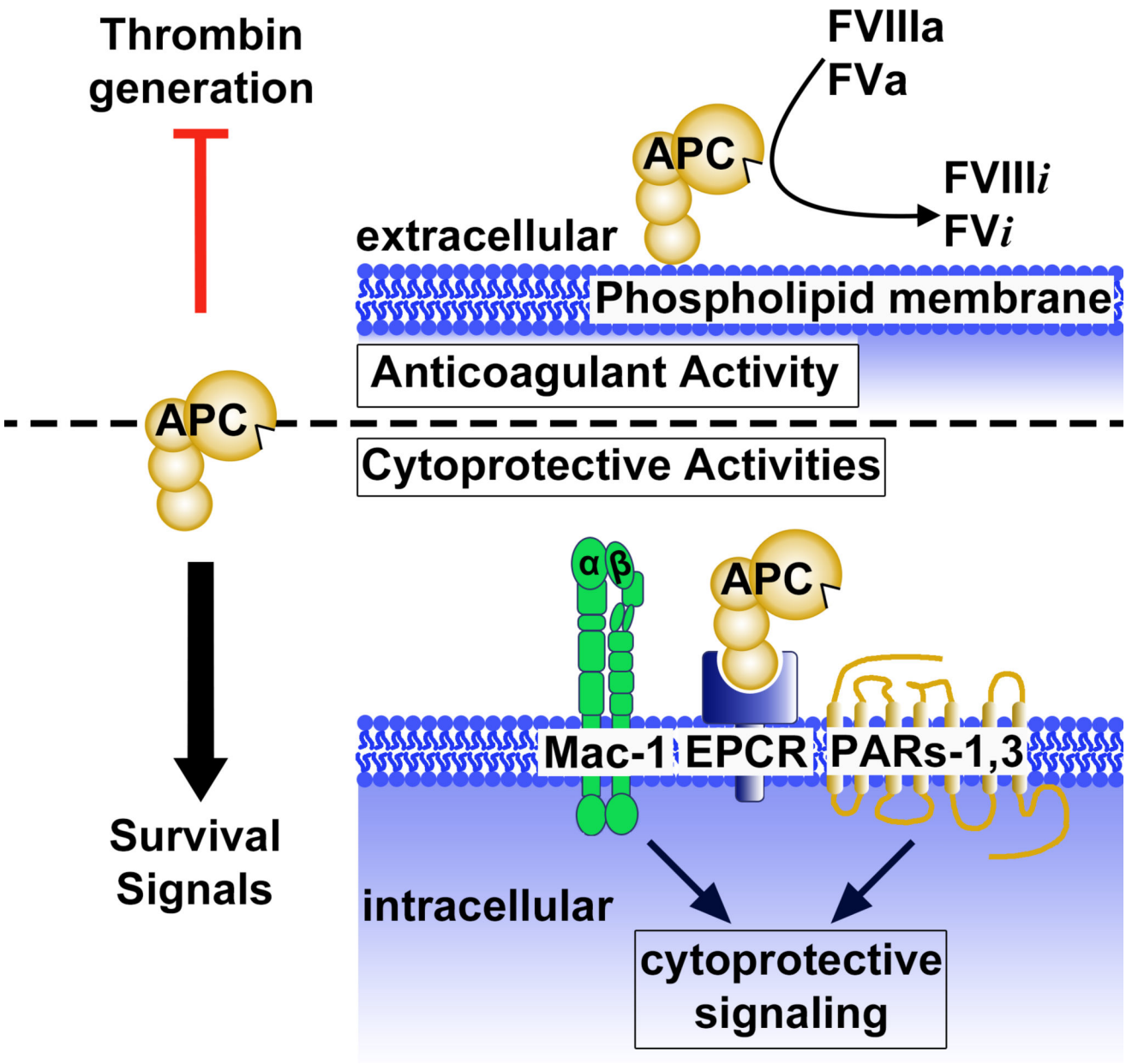

Figure 2. Anticoagulant and cytoprotective functions of activated protein C (APC)

APC has potent anticoagulant and cell survival effects. Thrombin generation is inhibited by APC on phospholipid membranes via proteolytic irreversible conversion of activated coagulation factors FVIIIa and FVa into their inactive forms, FVIIIi and FVi, respectively. APC also exerts diverse cytoprotective activities, including promotion of cell survival, by binding to select cell surface receptors, including Mac-1 (CD11b/CD18) and endothelial protein C receptor (EPCR), and by subsequent activation of protease activated receptors (PAR)1 and PAR3 to promote intracellular cytoprotective signaling. Figure adapted from [20].

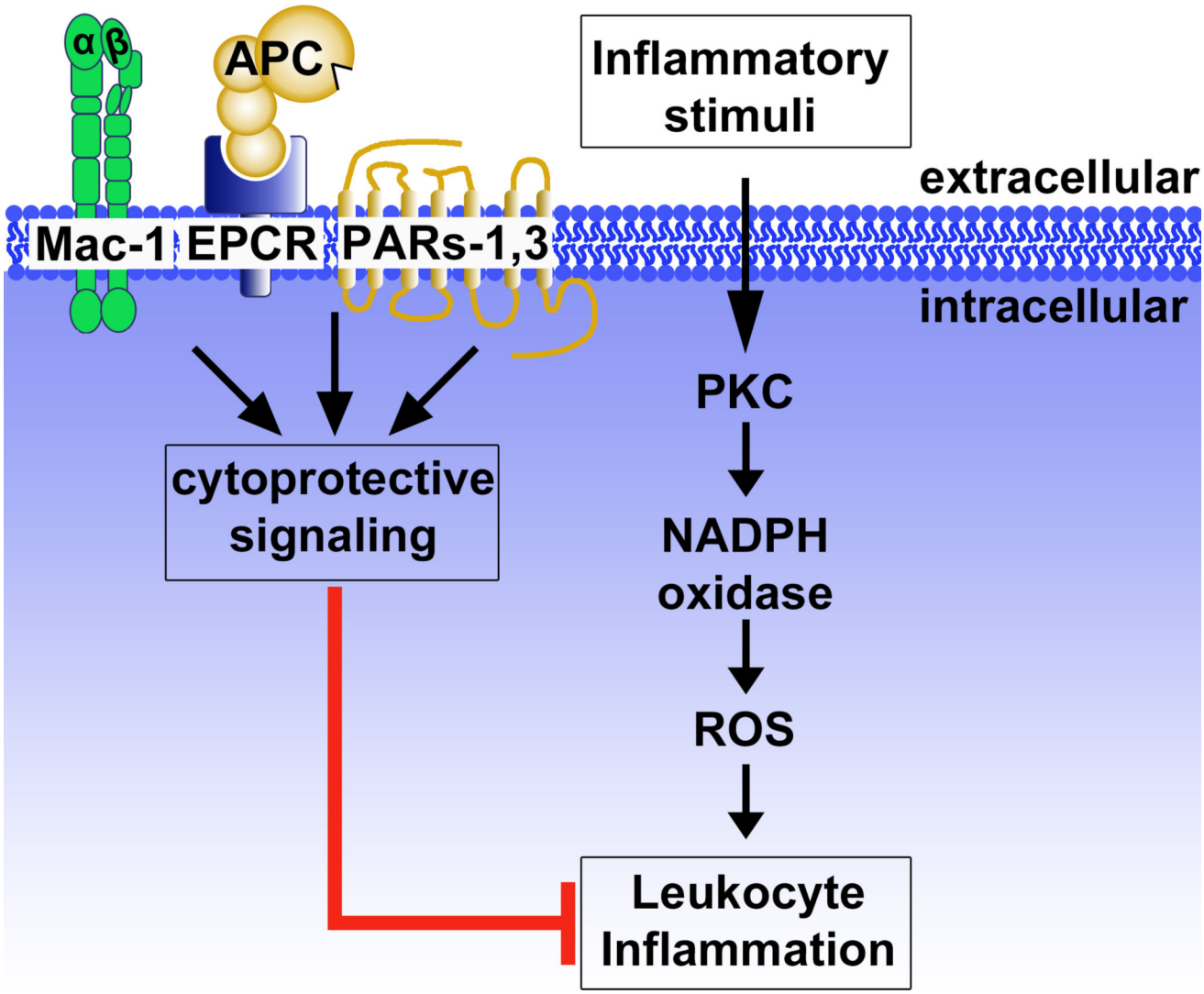

Figure 3. Activated protein C (APC) and leukocyte inflammation

Inflammatory signaling by leukocytes is driven in part via protein kinase C (PKC) signaling to generate reactive oxygen species (ROS) through NADPH oxidase, resulting in development of an activated inflammasome and/or formation of NETs. APC inhibits leukocyte inflammatory processes in a receptor-dependent manner, requiring Mac-1 (CD11b/CD18), endothelial protein C receptor (EPCR), PAR1 and PAR3, inducing intracellular cytoprotective signaling. Figure adapted from [69].

TABLE 1

Innate immune cells regulated by APC.

Cell type	Cell line	Receptors	Inhibitory effects	References
Monocytes	Primary [Human] (blood)	Unknown	Reduction in MIP-1-α release in septic patients and healthy volunteers	[65,66]
			Reduction in expression of CD14, CD11b and CD18 induced by INF-γ or PMA	
	THP-1 U937	EPCR, PAR1, ApoER2	Reduction in MIP-1-α, MCP-1, TNF-α release ± LPS	[62,65,67]
			CAM-induced apoptosis	
			Dab1-dependent signaling	
Macrophages	Primary (alveolar)	Unknown	Reduction in TNF-α release in response to LPS	[66,68]
	[Mouse] (BMDM)	PAR 1	Suppression of NLRP3 inflammasome activation	
Neutrophils	Primary (blood)	EPCR, integrins, PAR3	Reduction in chemotaxis	[58,60,69–72]
			No effect on spontaneous apoptosis	
			APC cleavage of extracellular histones (*in vitro* and *in vivo*)	
			Inhibition of NET s formation	

Footnote. Table Abbreviations. APC: Activated protein C; BMDM: bone marrow-derived macrophages; THP-1: monocytic cell line; U937: monocytic cell line; EPCR: endothelial protein C receptor; PARs-1,3: protease activated receptor-1/3; NLRP3: NOD-like receptor 3; ApoER2: Apolipoprotein E receptor 2; MIP-1-α: macrophage inhibitory protein 1-α; INF-γ: Interferon-γ; PMA: phorbol 12-myristate 13-acetate; MCP-1: monocyte chemoattractant protein-1; TNF-α: tumor necrosis factor-α; LPS: lipopolysaccharide; CAM: camptothecin.